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by GERALD H. WAGMAN, *Manager, Antibiotics Research Department, and* MARVIN J. WEINSTEIN, *Director, Microbiology Research Division, Schering Corporation, Bloomfield, New Jersey, U.S.A.*

1973, about 256 pages. Dfl. 65.00 (about US$22.80) ISBN 0-444-41106-2

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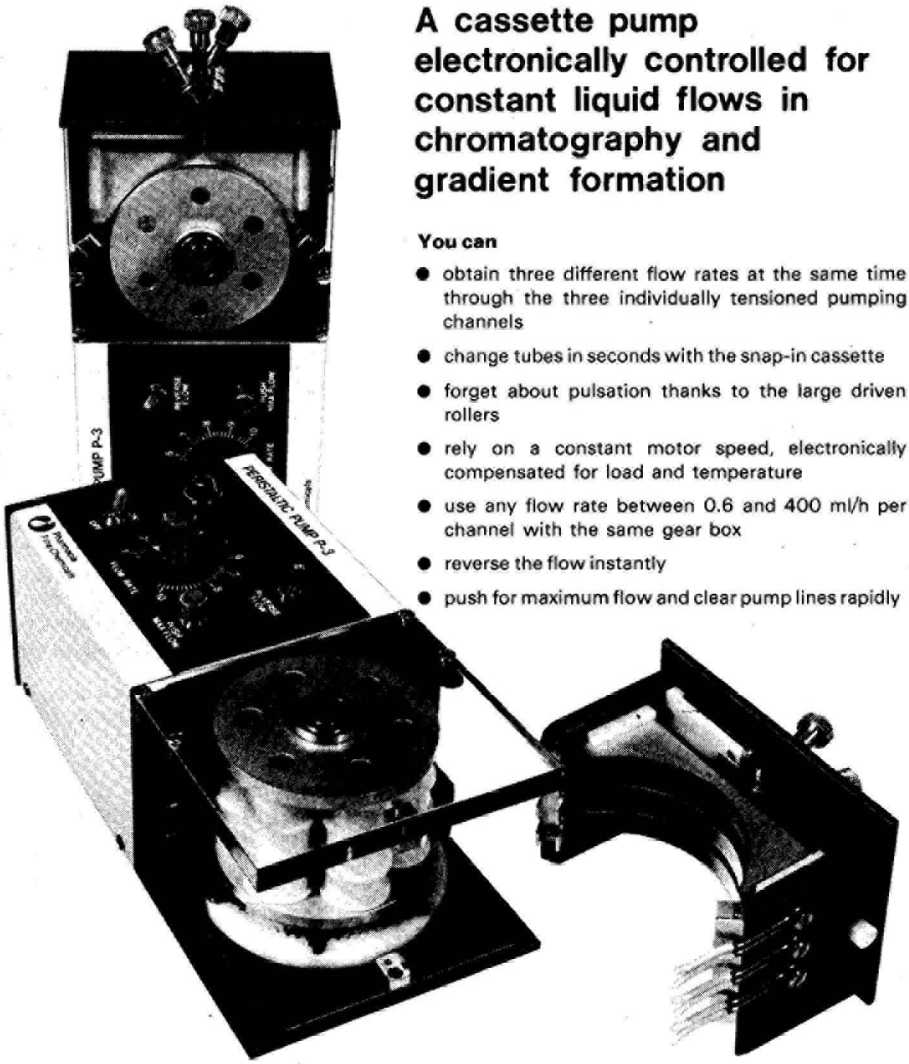

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CHROM. 7074

A CRYOGENIC GAS CHROMATOGRAPH

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(Received August 20th, 1973)

SUMMARY

A programmable, cryogenic gas chromatograph has been constructed in this laboratory for use in air pollution studies. Construction of the instrument is modular and utilizes many commercially available components and a few "scratch built" parts. The temperature control range is from −100° to +300° with five programming rates available. The instrument is also capable of dual column–dual detector operation.

Separations of laboratory prepared samples are presented to illustrate instrument versatility.

INTRODUCTION

There have been many reports of separations performed at subambient temperatures[1–7]. In 1963, Merritt and Walsh[8] described a simple, programmed, cryogenic gas chromatograph used in the separation of volatile odors from foodstuffs. In 1964, Merritt *et al.*[9] reported on improvements to their instrument, specifically in the program-rate control. Separations of mixtures of C_1 to C_4 olefins and paraffins, by a technique similar to the one used by Merritt and Walsh, have been described by Baumann *et al.*[10]. Isotopes have even been separated by programmed, cryogenic gas chromatography[11].

These achievements have led to the construction of the instrument described, with the intention of using it for air pollution studies. Cryogenic temperatures are achieved using liquified nitrogen and a solenoid valve activated by a temperature controller. The controller itself has been modified to perform both in the isothermal and programmed temperature mode.

EXPERIMENTAL OPERATION

At conditions above ambient, operation of the instrument is essentially the same as any other gas chromatograph. Temperature is controlled and maintained by a Harrell isothermal controller, Model TC-118-DL (Harrell Inc., East Norwalk, Conn., U.S.A.). In subambient operation, the Harrell unit is still the main controlling device. After setting the desired initial temperature, the controller activates a cryogenic solenoid valve (Asco 8262C22LT-2NC) admitting liquid nitrogen to

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the column compartment. The nitrogen is drawn from a demand regulated siphon tank (Welder's Supply, Philadelphia, Pa., U.S.A.). The demand regulator of the nitrogen flow is critical to the instrument's operation. Each time the solenoid valve is activated, the regulator allows liquid nitrogen to be drawn from the tank. When the solenoid closes, the regulator effectively shuts off the tank, prohibiting the liquid from building up extreme pressures within the piping. A safety release valve is employed since some liquid nitrogen can be trapped between the regulator and solenoid. The regulator is set for a pressure below the 22-p.s.i. safety release pressure.

Several types of cooling methods were employed and discarded before settling upon liquid nitrogen. Using liquid nitrogen provides an efficient and economical means of achieving cryogenic temperatures. One 150-l tank provides enough coolant for *ca.* five days of operation at $-35°$. As the temperature of the compartment begins to lower, the controller begins to cycle the solenoid valve on and off, yielding a time-proportional cooling rate, until set-point temperature is reached. This time-proportioned on–off action continues to maintain a constant temperature.

Sensing of the compartment temperature for the controller utilizes a multi-element, platinum resistor bridge. This circuit becomes one arm of the internal controller bridge. This arrangement tends to average out local deviations in the column compartment and provides an extremely accurate and reliable temperature reference.

As stated before, in both above and subambient operation, the Harrell unit is the main controlling unit. This unit has been altered to allow both isothermal and programmed temperature control. The set-point potentiometer of the Harrell unit has been replaced by a motor-driven potentiometer, Model 1106S (Valley Forge Instrument Co., Valley Forge, Pa., U.S.A.). The programmer is a temperature setting device for use with a suitable controller (*e.g.* Harrell unit) to control and linearly ramp temperature with time. Several fixed rates are available and a program may be changed, started or stopped at any time during operation. The rates available on our unit are 0.5, 1.0, 2.0, 3.0, 5.0 and 10.0 °/min.

The basic controlling action of the Harrell unit is a time-proportioning (on–off) output, in both heating and cooling modes, modified by an automatic reset function. This function tends to avoid overshoot due to high gain but still has the effect of bringing temperature back (automatically resetting) to the set-point. Deviations of 0.5° are easily sensed and compensated.

The combination of time-proportioning the power output to the solenoid and the regulated liquid nitrogen tank has resulted in excellent control of temperature in the cryogenic range. The overall control range is $-100°$ to $+300°$.

In actual use, the column compartment is allowed to equilibrate to the initial temperature and samples are then injected onto the column and retention times are recorded. At any time during operation a program may be started by simply choosing the desired program-rate and switching from isothermal mode to program mode.

CONSTRUCTION

The instrument is composed of three parts; an electronics module, an oven

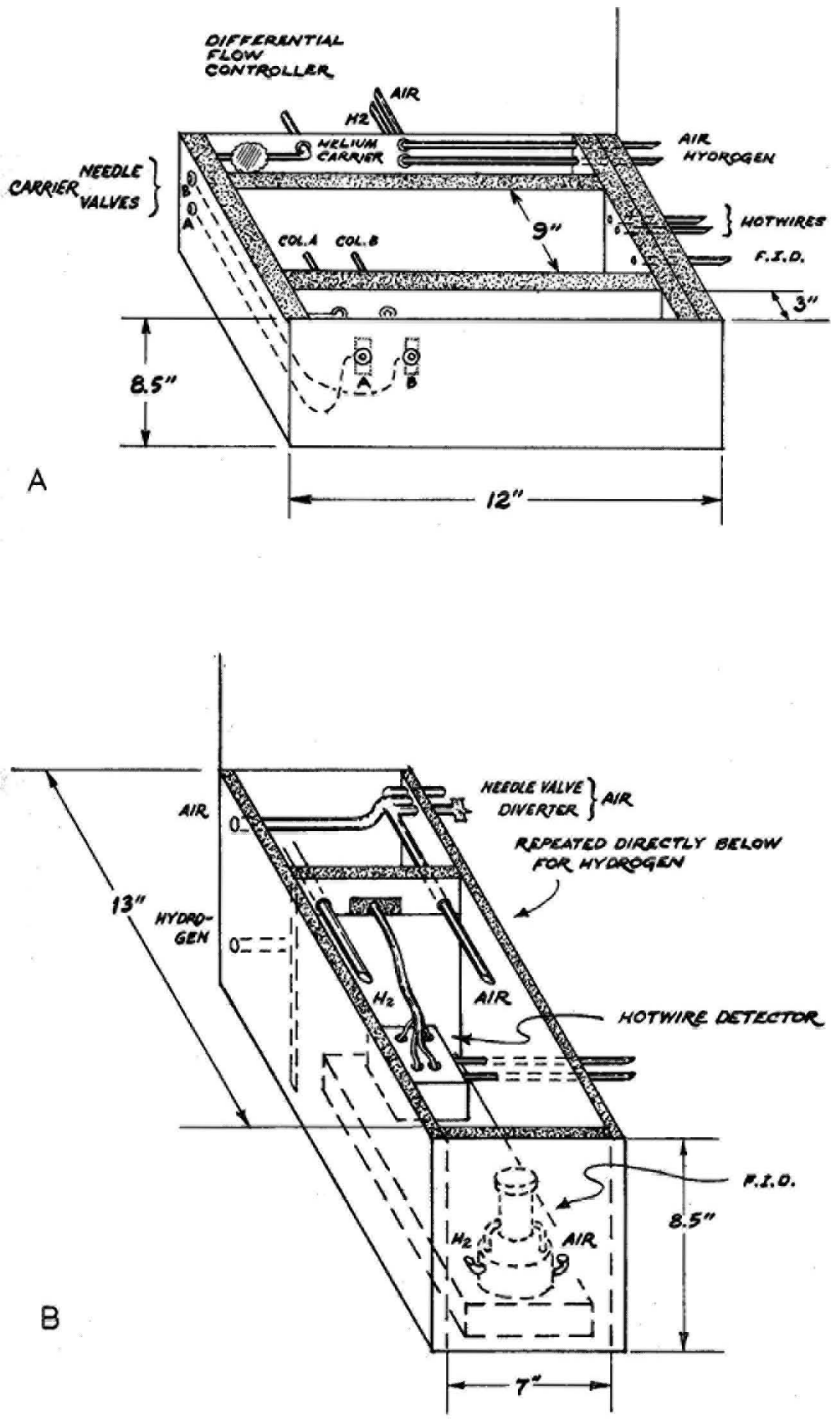

Fig. 1. Oven module. (A) Column compartment; (B) detector compartment.

module and a recorder module. The recorder module is an angle iron framework, housing the recorder and an ionization amplifier, Model 154-0449 (Perkin-Elmer, Norwalk, Conn., U.S.A.). The oven module contains the column compartment (Fig. 1A), detector compartment (Fig. 1B), various flow controls, cryogenic solenoid and fan. Exterior and interior dimensions of the oven module are shown in Figs. 1A and 1B. Both detector and column compartments are insulated with 1.5 in. thick Maronite with a 3-in. wall between the two compartments to insure the stability of the temperature in the detector compartment.

Two heating coils are located in the column compartment behind the fan shroud (Fig. 2). The fan itself is a squirrel cage type to aid in the rapid distribution of heat and coolant, permitting uniform and reproducible temperatures. Coolant is admitted into the compartment and is directed at the fan. The column compartment itself is large enough to accommodate two columns which can be used simultaneously.

Adjacent to the column compartment is the detector compartment, housing both a hot-wire detector (Gow-Mac Madison, N.J., U.S.A.) and a Perkin-Elmer ionization detector, Model 154-0407. Both detectors are in thermal contact with a heated aluminum block for temperature control.

Directly behind the column compartment are the flow controls. The flow controls are comprised of flow controllers, needle valves, flow diverters and associated hardware. The constant flow, differential controllers are essential to an instrument of this nature. Without them, flow-rates would vary erratically over the wide range of program temperatures.

The injection ports were designed by the authors and machined in the chemistry

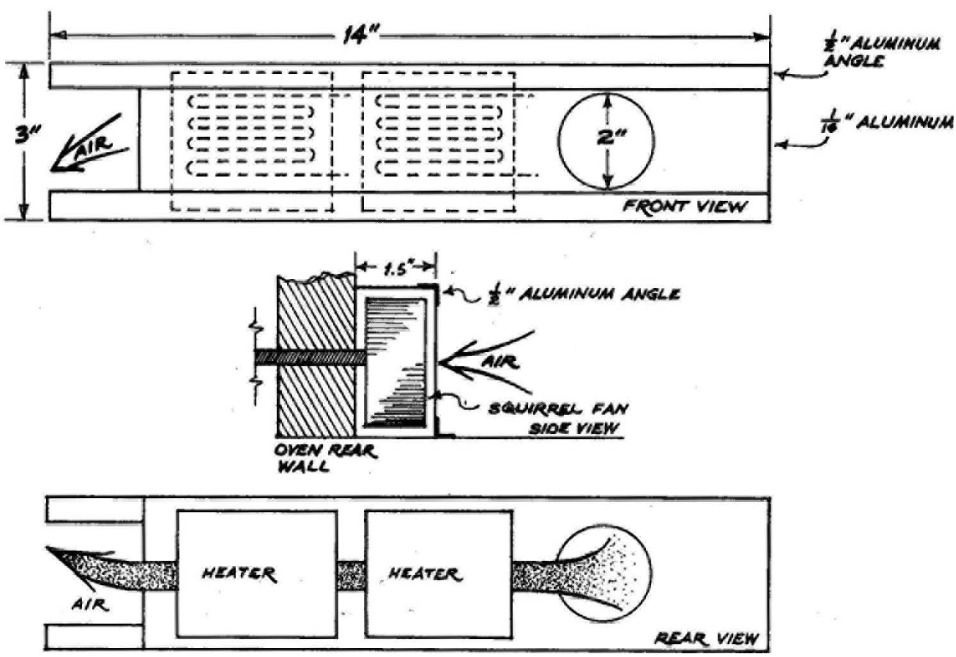

Fig. 2. Fan housing (shroud)

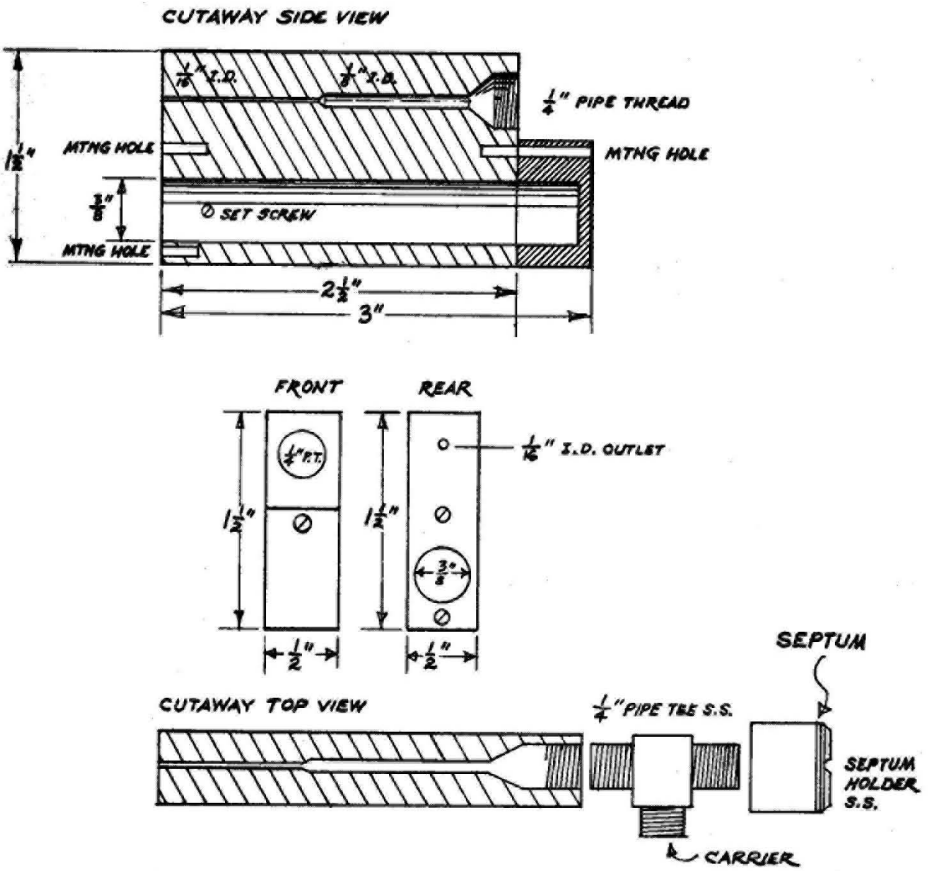

Fig. 3. Stainless-steel injection ports.

department shop. Each port (Fig. 3) is made in two parts for ease of maintenance. The result of this design is efficient heating with good thermal stability.

TABLE I

CONTROLS AND MONITORS FOR INSTRUMENT OPERATION

Controls	*Monitors*
Temperature programmer	Thermocouple selector
Temperature controller	High- and low-temperature pyrometer
Hot-wire power supply	Hot-wire current meter
Hot-wire current adjustment	Column heater pilot lamp
Recorder zero	Column coolant pilot lamp
Signal attenuator	Master power pilot lamp
Heating power to detector block	
Heating power to injection ports	
Master circuit breaker	
Master power switch	

Air and hydrogen, for the flame ionization detector (FID), are directed to the needle valves and flow diverters (Envirotech Corp. L-203-A, Mountain View, Calif., U.S.A.). These diverters act as two-way valves and allow flow-rate measurements without disturbing the FID unit.

Piping for both compartments is shown in Figs. 1A and 1B.

The electronics module contains all the necessary controls and monitors for instrument operation. These are listed in Table I and shown in Fig. 4. The electronics module itself is supported by an angle iron framework which straddles the oven module. Electrical connections employ terminal strips on both modules and multi-stranded cables terminated in cable plugs.

The instrument can be placed anywhere as long as the liquid nitrogen tank is within reasonable distance. All piping between the tank and the instrument is covered, first with styrofoam, then with glass wool and finally with asbestos tape.

RESULTS AND DISCUSSION

The separations presented were achieved on a 6 ft.×6 mm O.D. glass column packed with 33% DC-550 on 60–80 mesh Chromosorb W, AW, DMCS. The flow-rate, in all cases, was 33 cm^3/min. The hot-wire detector was employed. Current to the detector was 240 mA and an attenuation of eight was used. Initial temperature was −35°.

Two gas mixtures were prepared to illustrate the instrument operation.

Mixture I contains the inorganic gases CO_2, N_2O, H_2S, COS and SO_2. Fig. 5A is the chromatogram of mixture I run isothermally at −35°. This low temperature was necessary to separate the CO_2 and N_2O peaks; and even at this temperature they are not fully resolved. The effects of this temperature on the other components are increased retention times and severe band spreading; especially in the case of SO_2. Fig. 5B is a chromatogram of the same mixture utilizing a temperature program starting at −35°. After injection of the sample, temperature was maintained isothermally at −35° until the CO_2 and N_2O peaks were eluted.

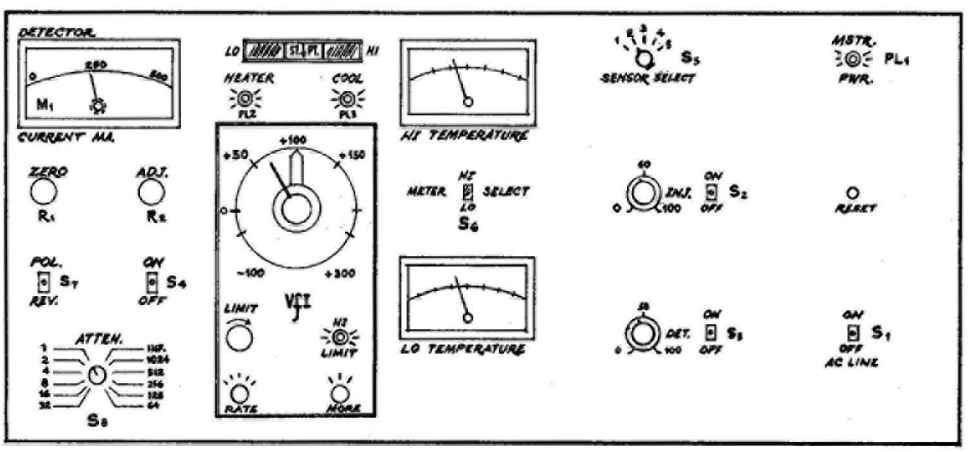

Fig. 4. Electronics module, front panel.

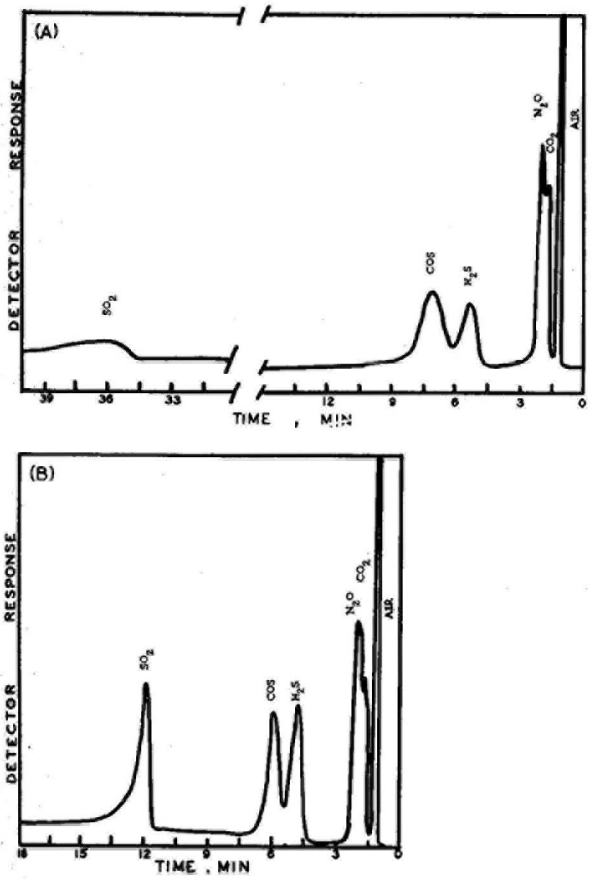

Fig. 5. Separation of some inorganic gases. (A) Isothermal mode, temperature −35°; (B) programmed mode, temperature 3 min at −35°, then programmed at a rate of 5°/min.

Programming was then initiated at a rate of 5°/min until the SO_2 peak was eluted. The result is that all the component peaks are sharpened and exhibit shorter retention times. Most dramatic is the change in the retention time for SO_2, which was roughly halved.

Fig. 6A in the isothermal chromatogram of mixture II, which contained the light hydrocarbons; ethane, propane, isobutane and butane. The isobutane and butane peaks exhibit severe band spreading. Fig. 6B is this same mixture under temperature programmed conditions. Again, all component peaks are sharpened with a corresponding decrease in retention time. Methane is not separated from the air peak under the conditions presented. The utilization of the FID will give us the methane content of the mixture.

CONCLUSIONS

The addition of cryogenic temperature control can increase the operating range of any gas chromatograph. Couple this extended range with temperature program-

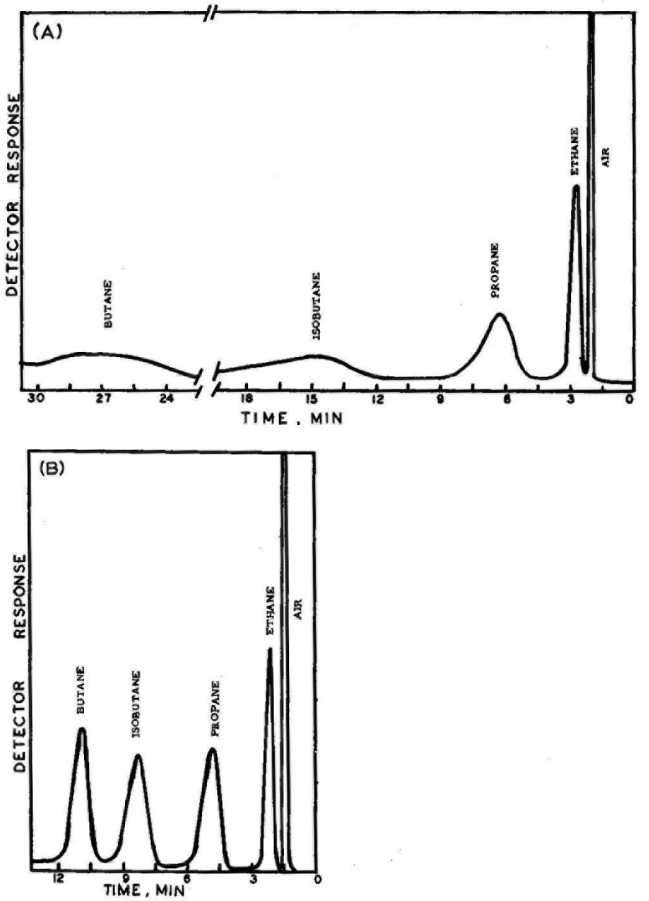

Fig. 6. Separation of some organic gases. (A) Isothermal mode, temperature −35°; (B) programmed mode, temperature −30°, then programmed at 5°/min.

ming and the operator now has another variable that he can manipulate to achieve a desired separation. Additionally, cryogenic operations allow the use of liquid phases that may have proved unsuitable at ambient temperatures and higher.

Cryogenic, programmable gas chromatography can provide researchers with an instrument of long range capabilities. Separations of atmospheric gases to everyday routine analyses could be performed simply by changing columns and temperature.

The separations already achieved on this instrument have led the authors to believe that it can be a powerful tool for use in air pollution studies. Efforts are now being directed toward this goal.

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Journal of Chromatography, 89 (1974) 11–15

CHROM. 7037

DETERMINATION OF CARBAMAZEPINE IN PLASMA

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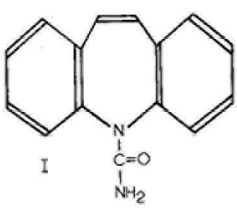
(Received September 6th, 1973)

SUMMARY

A description is given of a simple gas chromatographic method for assay of plasma levels of carbamazepine. This uses OV-1 as stationary phase and avoids high column temperatures. The calibration curve is linear and the results are reproducible. Plasma levels in patients under treatment with carbamazepine alone ranged from 1.1 μg/ml to 22.4 μg/ml.

INTRODUCTION

Carbamazepine (I) has found increasing application as an anti-convulsant drug but there have been few attempts to relate clinical actions and plasma levels. Most of the early methods for assay and some recent ones[1,2] use spectrophotometry.



I

The indirect measurement of carbamazepine by spectrophotometric measurement of 9-methylacridine produced by the acid hydrolysis of carbamazepine has been reported[2]. Recently, gas chromatographic methods for carbamazepine have been described[3–7]. These have various drawbacks such as tedious extractions, necessity to form derivatives and consequent cost or the use of high column operating temperatures. This paper describes a gas chromatographic method for the assay of plasma levels of carbamazepine which avoids these problems.

EXPERIMENTAL

Instrumental

In the method finally adopted, analyses are performed on a Varian Aerograph 1400 gas–liquid chromatograph equipped with an alkali flame ionization detector (AFID). The carbamazepine is extracted from plasma into an organic solvent and,

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after concentration, is subsequently chromatographed without derivatization on a 3% OV-1 column operated isothermally at 175°. The stationary phase, OV-1 (Varian Aerograph), is dissolved in chloroform and stirred with the appropriate amount of the support material Gas-Chrom Q (80–100 mesh, AW-HMDS, Applied Science Labs.). The solvent is evaporated overnight and the coated support packed into a 2 m × 1.5 mm I.D. borosilicate glass coil. The carrier gas is nitrogen at a flow-rate of 25 ml/min. Other flow-rates are 35 ml/min for hydrogen and 235 ml/min for air.

Extraction from plasma

The extraction method is summarized in Fig. 1.

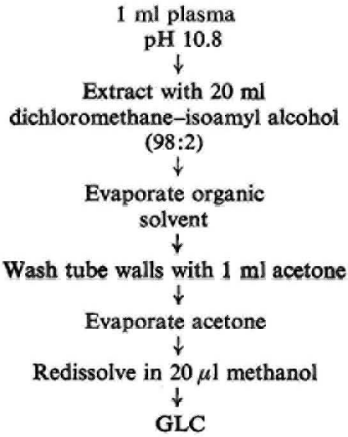
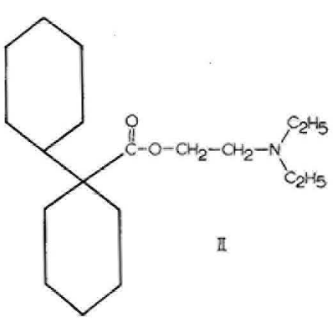


Fig. 1. Flow diagram of extraction procedure.

One millilitre of plasma is placed in a 40-ml glass centrifuge tube and the internal standard, dicyclomine (II), is added prior to extraction. The sample is made alkaline with one ml of bicarbonate buffer (pH 10.8), prepared by combining 87.9 ml of 0.1 *M* $NaCO_3$ and 12.1 ml of 0.1 *M* $NaHCO_3$, and extracted with 20 ml of dichloromethane–isoamyl alcohol (98:2) by shaking for 10 min. The aqueous layer is removed by aspiration. The organic layer is evaporated at 40° in a water-bath aided by a stream of clean compressed air. To avoid excessive dispersion of the dried deposit on the wall of the vessel, this is done in a 10-ml tapered centrifuge tube by repeated additions of 5-ml aliquots.

The deposit is washed from the walls of the tube with 1 ml of acetone and evap-



II

orated again at 60° in a water-bath using air as the evaporative aid, to give a concentrated deposit, which is redissolved in 20 μl of methanol. 1–2 μl of this solution are injected into the gas–liquid chromatograph.

Quantitation

Peak heights measured from the baseline proved to be as satisfactory as areas measured with a planimeter and are preferred for routine use. Known ratios of carbamazepine/dicyclomine are added to drug-free plasma and the resulting peak height ratios used to construct a calibration curve. Table I shows the typical ratios used. From this relationship the peak height ratios obtained from the chromatograms are converted directly to weight ratios.

TABLE I

CONSTRUCTION OF CALIBRATION CURVES

Tube No.	*Carbamazepine (μg/ml)*	*Dicyclomine (μg/ml)*
1*	0	20
2*	15	0
3	4	20
4	6	20
5	8	20
6	9	20
7	10	20
8	15	20

* Tubes 1 and 2 are used as retention time markers for dicyclomine and carbamazepine, respectively.

Preparation of drug stock solutions

Carbamazepine (Tegretol—Ciba-Geigy, Basel, Switzerland): 10 mg is dissolved in 50 ml of acetone.

Dicyclomine·HCl (Richardson-Merrell, Cincinnati, Ohio, U.S.A.): 50 mg is dissolved in 50 ml of water.

RESULTS

The best column performance was obtained with 3% OV-1 as the stationary phase (Fig. 2).

If more than 5 μg of carbamazepine is applied to a column packed with OV-1, two peaks emerge with markedly different retention times. The second peak is not seen if the column load is less than 5 μg. The reason for this has not been elucidated but since in the present method less than one tenth of the carbamazepine extracted from 1 ml of plasma is eventually injected, the mass injected is always much less than 5 μg and the second peak does not occur.

Internal standard is added to the plasma prior to extraction. This form of internal standardization has some advantages. It not only compensates for variations in column performance, injection volumes and for mechanical losses incurred during the extraction, but, provided that the internal standard chosen behaves in a similar

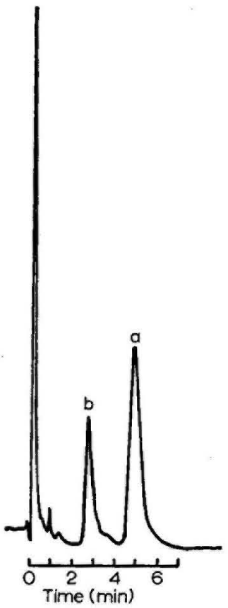


Fig. 2. Typical chromatogram trace of carbamazepine (a) and dicyclomine (b) on OV-1 (3%) at 175°.

fashion to the drug being assayed, it in part allows for variation in efficiency of the extraction procedure. With the present method, the average recovery from plasma of carbamazepine and dicyclomine relative to water blanks are 85.6% (S.E. 5.9%) and 78.0% (S.E. 5.1%), respectively. A typical calibration curve is shown in Fig. 3. It is linear over the range usually encountered in clinical samples.

Reproducibility of the method was estimated by analysis of variance of 23 duplicate estimations. The between injection standard deviation was 0.3 μg/ml and the between extraction standard deviation was 1.1 μg/ml.

Plasma levels of carbamazepine in patients being treated with it as the sole anti-convulsant ranged from 1.1 to 22.4 μg/ml.

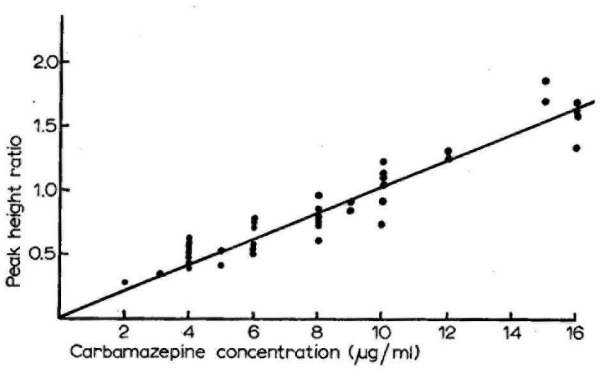


Fig. 3. Calibration curve relating peak height ratios (carbamazepine/dicyclomine) to carbamazepine concentration in the extracted plasma, derived from seven different extractions.

DISCUSSION

The present method utilizes the AFID with an $RbSO_4$ salt tip. Although its actual sensitivity is similar to that of the flame ionization detector (FID), *i.e.* of the order of 10^{-12} g, the AFID has the advantage of being 10,000 times less sensitive to hydrocarbons than is the FID[8] and in addition it can be tuned to specific heteroatoms, *e.g.* nitrogen, phosphorus or sulphur. The insensitivity to hydrocarbons produces very short solvent fronts which allows rapidly eluted peaks to be adequately detected. This increase in relative sensitivity of the AFID is utilized in the present method to permit adequate assay by the extraction of small volumes of plasma. Although 1 ml of plasma is routinely extracted in the method, successful assays can be accomplished using 0.5-ml samples.

Several recent methods for gas–liquid chromatographic analysis of carbamazepine[5,6] claim short analysis times. That of Meijer[4], using a QF-1/XE-60 column, has the considerable disadvantage of interference with the analysis by cholesterol. This was partially eliminated by a two-dimensional thin-layer chromatographic extraction step.

Although short retention times are reported by Larsen *et al.*[5] and Toseland *et al.*[6], using SE-52 and SP-1000, the high operating temperatures lead to rapid column breakdown. Another disadvantage is that the extraction is rather complex for routine application.

Kupferberg's method[7] involves a long extraction and derivatization, which increase both the time and cost of the assay. Another disadvantage is that phenobarbital and phenytoin interfere with the procedure and an extra extraction step is required to separate them from carbamazepine.

The plasma levels of carbamazepine obtained using the method described here are in agreement with the values found by Toseland *et al.*[6] (3–12 μg/ml), Larsen *et al.*[5] (4–11 μg/ml) and Kupferberg[7] (13.1 μg/ml). This method has been used for several months for routine assay to assist in the control of carbamazepine dosage.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance of Mrs. Ruth Meier and the secretarial assistance of Mrs. Suzy Quaife. This work was supported by grant No. 72/8836 from the National Health and Medical Research Council of Australia.

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CHROM. 7094

THE ASSAY OF PHENYLBUTAZONE IN HUMAN PLASMA BY A SPECIFIC AND SENSITIVE GAS–LIQUID CHROMATOGRAPHIC PROCEDURE

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(Received October 1st, 1973)

SUMMARY

A sensitive specific method is described for the determination of phenylbutazone in plasma. After simple solvent extraction from 1 ml of plasma the drug was estimated without interference from the known metabolites, oxyphenbutazone and the side-chain hydroxyphenylbutazone, by gas–liquid chromatography using a flame ionization detector. The procedure compared favourably with a less specific spectrophotometric method and was of sufficient sensitivity to determine plasma levels (<0.5 μg/ml) of the drug after single 200-mg doses.

INTRODUCTION

Phenylbutazone is one of the most widely prescribed antiarthritic drugs and although blood level studies have been described[1–3], the currently available methods for determination of the drug in plasma are not ideal for pharmacokinetic studies in which single doses may be compared. Burns *et al.*[1], who initiated metabolic studies of phenylbutazone, extracted plasma samples containing the drug with *n*-heptane, back extracted into alkali and measured the amount by UV absorbance at 265 nm. This method suffers from a lack of sensitivity (>10 μg/ml) and specificity. An improved extraction procedure developed by Hermann[2] and later by Van Petten *et al.*[3] removed most of the 1-phenyl-2-*p*-hydroxyphenyl-3,5-dioxo-4-butylpyrazolidine (oxyphenbutazone) metabolite by means of buffers and use of a mixed 1,2-dichloroethane–heptane solvent. However, the background was still significant (5–10 μg/ml limit). A permanganate oxidation procedure first reported by Wallace[4] was adapted for small samples recently by Jänchen and Levy[5]. Although oxyphenbutazone does not interfere with this procedure and the background at 314 nm can be minimized to give sensitivity limits of 2 μg/ml, any 1,2-diphenyl-3,5-dioxo-4-(3-hydroxybutyl)-pyrazolidine (hydroxyphenylbutazone) present can not be differentiated from the parent drug[5]. The objective of this study was to increase the sensitivity of the UV procedure and to develop a new, specific gas–liquid chromatographic (GLC) procedure for the measurement of single dose levels of phenylbutazone in plasma.

MATERIALS AND METHODS

Chemicals and reagents

Phenylbutazone and oxyphenbutazone were obtained from Ciba-Geigy Canada

Ltd. and hydroxyphenylbutazone from Dr. A. Sallmann of Ciba-Geigy, Basel, Switzerland. Spectral grade *n*-heptane (Caledon Labs. Ltd., Georgetown, Ont., Canada) was used throughout the determinations.

Spectrophotometric procedure

The method of Janchen and Levy[5] was employed with the following modifications.

1-ml plasma samples were shaken with 1 ml of *N* HCl and 6 ml of *n*-heptane for 15 min instead of 30. 4 ml of the heptane layer were treated as in the literature procedure except that the final incubating time was changed from 3 to 1 h. The absorbance was read at 314 nm with 1-cm microcuvets (1 ml) on a Model 500 Unicam spectrophotometer.

GLC procedure

A Perkin-Elmer Model F 11 gas chromatograph equipped with a flame ionization detector and a Perkin-Elmer Model 56 recorder was employed. The chromatographic column consisted of glass tubing 3 ft. $\times \frac{1}{4}$ in. O.D. packed with 3% Apiezon L on 80–100 mesh Chromosorb W-HP supplied by Chromatographic Specialities, Brockville, Ont., Canada. The column was conditioned by injecting Silyl 8 (Pierce, Rockford, Ill., U.S.A.) and by maintaining the column at 300° for 18 h with low nitrogen flow.

The injection port and detector temperatures were 310 and 300°, respectively, and the column temperature was 230°. Compressed air and hydrogen flow-rates were adjusted to give maximum response. Nitrogen was used as the carrier gas at a flow-rate of 120 ml/min. (Retention time (t_R) 3.6 min for phenylbutazone and 6.0 min for diphenylphthalate.) The chart speed was 0.5 cm/min. The efficiency of the column was maintained by injecting 5 μl of Silyl 8 followed by 5 μl of Freon 113 (Pierce) each day before analysis.

Sample collection

Blood samples (10 ml) from two healthy human male subjects (170 and 200 lbs., respectively) were drawn into Vacutainers (Becton Dickinson Co., Mississagua, Ont., Canada) containing sodium citrate or heparin. The blood was centrifuged, plasma recovered and either analyzed immediately or stored at −15°.

Sample preparation

Add 1 ml of *N* HCl and 6 ml of *n*-heptane to 1 ml of heparinized plasma in a screw-cap centrifuge tube (15 ml). Secure the cap on the tube and mix the contents for 15 min on a rotorack before centrifugation for 5 min at $1200 \times g$. Transfer 2 ml of the heptane layer to another screw-cap tube (5 ml) and evaporate the solvent at 70° (water bath). Add 0.02 ml of ethyl acetate containing the external standard diphenylphthalate[6] (500 μg, *i.e.* 25 μg/μl) to the residue and mix on a vortex mixer. Inject 1-μl samples on to the gas chromatograph within 2 h.

Calibration curves and quantitation

Stock solutions were prepared by suspending phenylbutazone (20 mg) in distilled water (50 ml) and dissolving the drug by dropwise addition of 5 *N* sodium hydroxide. 40 ml of 0.2 *M* phosphate buffer (pH 7.4) were added and the pH of the

solution was adjusted to 7.4 with 5 *N* HCl. The final volume was made up to 100 ml with phosphate buffer (pH 7.4) to yield a solution of 200 μg/ml.

Standard calibration curves were established by adding phenylbutazone in the range of 2–32 μg/ml to freshly collected plasma and proceeding as described under *GLC procedure*. Quantitation was performed by plotting the peak height ratio of drug/external standard against concentration to obtain a standard curve linear in the concentration range 0.5–200 μg/ml with a slope 0.2105±0.012.

RESULTS AND DISCUSSION

Although Jänchen and Levy[5] suggested that the color development of phenylbutazone oxidation product required 3 h incubating at 65°, it was observed that the color was reproducible in 1 h. Spectral grade heptane was also required in the procedure and each new batch should be checked for interfering materials. The precision of the method is illustrated in Table I, the overall coefficient of variation in the range 2–32 μg/ml is 4.04%.

TABLE I

ESTIMATION OF PHENYLBUTAZONE ADDED TO PLASMA BY SPECTROPHOTOMETRIC METHOD

Phenylbutazone added (μg/ml)	*Mean absorbance n= 7*	*S.D.*	*C.V.*
2.0	0.033	0.001	3.03
4.0	0.062	0.004	6.45
8.0	0.117	0.004	3.41
16.0	0.233	0.011	4.72
32.0	0.428	0.011	2.57
			Mean 4.04

The extractability of phenylbutazone from acidified plasma into heptane was checked using and UV absorption spectrophotometer. The extinction coefficient of the UV absorption maximum of phenylbutazone in heptane at 265 nm was sufficient to determine down to 10 μg/ml of phenylbutazone from plasma. The extraction efficiency of heptane for partitioning phenylbutazone from plasma was determined by means of this UV procedure. The recovery of the drug from acidified plasma (pH 1–2) was better than 90%.

The response of the flame ionization detector to phenylbutazone was linear with concentration in the range 0.5–200 μg/ml. Watson *et al.*[6] had found diphenylphthalate a useful reference standard in a procedure for determination of pharmaceutical purity of phenylbutazone. The peak height ratio of the drug and external standard was used as the index of detector performance and overall efficiency of the analytical procedure. Thus the external standard curve from the solvent and the plasma-recovered external standard curve were constructed by plotting peak height response ratios of phenylbutazone/diphenylphthalate *versus* concentration of phenylbutazone solutions containing a constant amount of diphenylphthalate as the reference

TABLE II
RECOVERY OF PHENYLBUTAZONE FROM WATER DETERMINED BY GLC ASSAY

Phenylbutazone added (μg/ml)	*Phenylbutazone recovered (μg/ml)*	*Recovery (%)*
2.0	1.85	92.5
4.0	3.79	94.7
8.0	8.42	105.2
16.0	14.34	89.6
32.0	30.27	94.6
		Mean 95.32 ± 6.19

TABLE III
RECOVERY OF PHENYLBUTAZONE FROM PLASMA DETERMINED BY GLC ASSAY

Phenylbutazone added (μg/ml)	*Phenylbutazome recovered (μg/ml)*	*Recovery (%)*
2.0	1.80	90.0
4.0	3.79	94.7
8.0	7.54	94.2
16.0	14.62	91.4
32.0	30.98	96.8
		Mean 93.42 ± 2.9

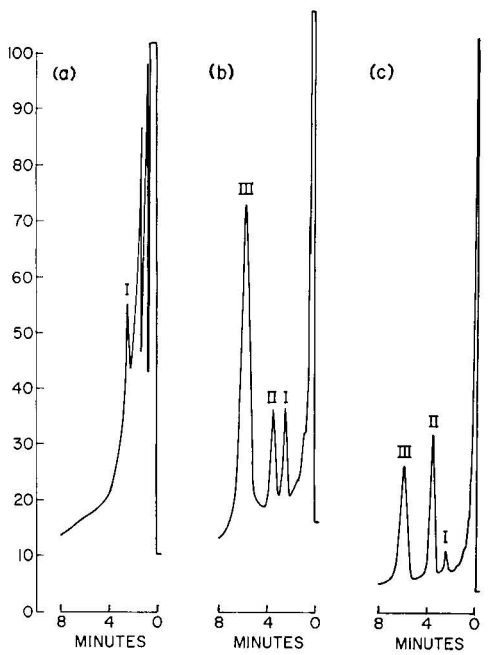

Fig. 1. Chromatograms of human plasma extracts. (a) Blank plasma, (b) control plasma to which 2.0 μg/ml of phenylbutazone and external standard are added, (c) plasma unknown from a human volunteer who received an oral dose of 200 mg of phenylbutazone. I= Peak from endogenous plasma material (t_R= 2.4 min); II= peak for phenylbutazone (t_R= 3.4 min); III= peak for external standard (t_R= 6.0 min).

standard per millilitre of ethylacetate. The overall recoveries of 2–32 μg of phenylbutazone from water and from plasma were of the order of 95.32±6.19 and 93.42± 2.9%, respectively (Tables II and III).

During the development of this method various solvents for injection were evaluated and it was found that ethyl acetate gave the cleanest separation free from interfering substances. Fig. 1a shows a chromatogram obtained by treating fresh plasma containing no drug as described under *GLC procedure* but omitting the external standard. The single extraneous peak with a t_R of 2.4 min was observed in chromatograms of all human plasma samples. A chromatogram observed when the method was applied to spiked plasma containing 2 μg/ml of phenylbutazone is shown in Fig. 1b where it is evident that the extraneous peak I does not interfere with the

TABLE IV

ESTIMATION OF PHENYLBUTAZONE ADDED TO PLASMA BY GLC

Phenylbutazone added (μg/ml)	*Mean peak height ratio drug/standard n= 4*	*S.D.*	*C.V.*
2.0	0.36	0.038	10.64
4.0	0.71	0.021	2.91
8.0	1.45	0.036	2.45
16.0	2.98	0.038	1.27
32.0	6.67	0.092	1.37
			Mean 3.73

$y = mx$
where $m = 0.2105 \pm 0.012$
$r^2 = 0.998$.

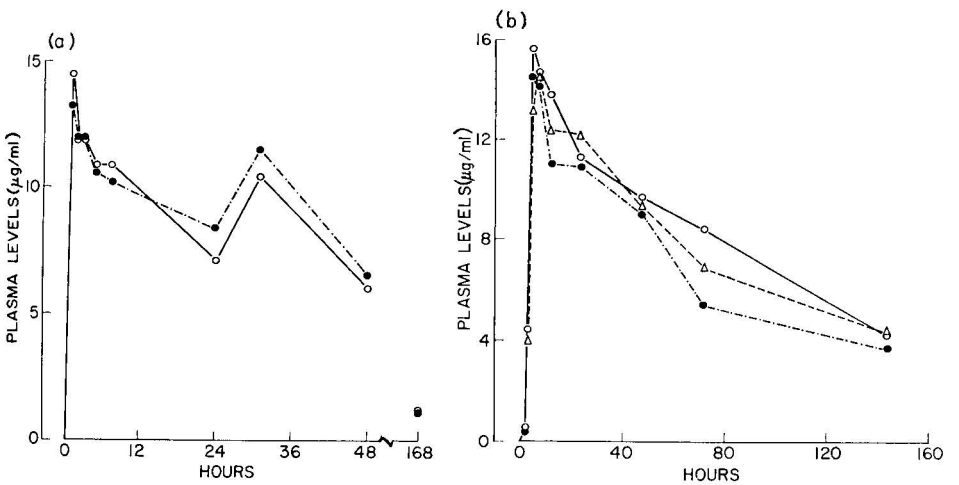

Fig. 2. Comparison of plasma levels determined by different analytical methods following single doses of phenylbutazone to volunteers. (a) 100-mg solution to subject I; (b) 200 mg (two 100-mg tablets) to subject II. ○—○, GLC; ●-·-·-●, UV; △---△, HSLC.

peaks from phenylbutazone (II, $t_R = 3.4$ min) or diphenylphthalate (III, $t_R = 6.0$ min). The known metabolites oxyphenbutazone and hydroxyphenylbutazone do not interfere in the procedure. Oxyphenbutazone does not come through the column and the hydroxyphenylbutazone gives a peak of poor response with a t_R of 8.5 min.

The accuracy and precision of the GLC determination are demonstrated in Table IV. Four aliquots each of five solutions of phenylbutazone ranging from 2 to 32 μg/ml in plasma were treated as described under *GLC procedure*. The overall coefficient of variation (C.V.) was 3.73%.

Application of the method to plasma level determinations is demonstrated in Fig. 2 along with comparisons with other methods. A 100-mg dose of phenylbutazone was given in solution to a healthy male volunteer (170 lbs.), plasma withdrawn at intervals over 48 h and assayed for phenylbutazone using both the UV method and the GLC procedure. The methods compare favourably (Fig. 2a) with an overall 6.0% difference. A second male volunteer was given a 200-mg (two 100-mg tablets) dose, plasma levels over 144 h being compared by the UV, GLC and a recent high-speed liquid chromatographic (HSLC) procedure[7]. The comparison is shown in Fig. 2b, some scatter being evident between the methods but with acceptable variation (GLC to UV, 12.5%; GLC to HSLC 8.9% overall). It should be noted that the UV procedure does not differentiate phenylbutazone from all metabolites which may interfere in some assays.

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ANALYSIS OF PHENYLBUTAZONE IN PLASMA BY HIGH-SPEED LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive, specific method for the determination of phenylbutazone in plasma in the presence of metabolites is described. The *n*-hexane extract of acidified plasma, redissolved in chloroform, is chromatographed on a SIL-X column using a mobile phase of 10% tetrahydrofuran in *n*-hexane on a high-speed liquid chromatograph fitted with ultraviolet absorbance detector. Quantitation of plasma samples containing less than 2 μg/ml of phenylbutazone is reported, using the 2,4-dinitrophenylhydrazone of benzaldehyde as an internal standard. Detection is limited to approximately 0.2 μg/ml. The retention times for the metabolites are such that they do not interfere in the procedure. For comparison, results determined by both electronic integration and peak-height measurements are quoted.

INTRODUCTION

The existing methods for the assay of phenylbutazone in blood or plasma, while adequate for therapeutic levels (40–100 μg/ml) are not ideal for biopharmaceutical studies in which single doses may be compared. The classical method of Burns *et al.*[1], in which the drug is extracted from acidified plasma by *n*-heptane, then back extracted into alkali to be measured by ultraviolet (UV) absorbance at 265 nm, suffers from a lack of sensitivity (>10 μg/ml) and specificity. Hermann[2] and later Van Petten *et al.*[3], by means of buffers and use of a mixed 1,2-dichloroethane–*n*-heptane solvent in the first extraction, removed most of the oxyphen butazone metabolite; however, background remained high (sensitivity 5 μg/ml in our hands). Wallace[4] reported a procedure based on permanganate oxidation which Jänchen and Levy[5] adapted for small samples. The UV background at 314 nm was minimized in this method with a sensitivity limit of approximately 2 μg/ml; but, although oxyphenbutazone does not interfere, any of the side-chain hydroxymetabolite (hydroxyphenylbutazone) present cannot be differentiated from the parent drug[5]. Thus, there is a requirement for more specific sensitive methods for measurement of phenylbutazone and its major metabolites. This report describes a high-speed liquid chromatographic (HSLC) analysis of phenylbutazone in the presence of these metabolites.

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EXPERIMENTAL

Materials

Complementary samples of phenylbutazone (Mount Royal Chemicals, Montreal, Canada), oxyphenbutazone (1-phenyl-2-*p*-hydroxyphenyl-3,5-dioxo-4-butylpyrazolidine) and hydroxyphenylbutazone [1,2-diphenyl-3,5-dioxo-4-(3-hydroxybutyl)pyrazolidine] (both from Ciba-Geigy, Montreal, Canada) were used as received. Tetrahydrofuran, freshly distilled and dried over Davison® Molecular sieves, Type 3A (both from Fisher Scientific, Montreal, Canada), and *n*-hexane (Caladon Labs., Georgetown, Canada) were used as solvents. The 2,4-dinitrophenylhydrazone of benzaldehyde, which was used as the internal standard, was prepared by the reported procedure[6], m.p. 234–236°; ref. 6, 237°.

Chromatographic procedure

Details of the modified constant-pressure high-speed liquid chromatograph (Varian Aerograph, Palo Alto, Calif., U.S.A., Model 4000) used in this study have been previously reported[7]. The chromatograph was equipped with a stop-flow injection port, a fixed-wavelength (254 nm) UV absorbance detector, attenuated to 0.04 absorbance units full scale (a.u.f.s.), and a digital integrator (Vidar, Mountain View, Calif., U.S.A., Model 6300).

The column (100 cm × 1.8 mm I.D.; 304 stainless steel) was dry-packed with SIL-X® (Perkin-Elmer, Norwalk, Conn., U.S.A.) adsorbent by a procedure similar to the one described by Kirkland[8], then coiled (radius 15 cm) and fitted horizontally into the water-bath of the instrument. Analyses were performed using a mobile phase of 0.002% glacial acetic acid and 10.0% tetrahydrofuran in *n*-hexane at a flow-rate of 60 ml/h (475 p.s.i.) and a temperature of 35°. The mobile phase was "degassed" by applying vacuum (*ca.* 100 mm) to the solvent reservoir for approximately 1 min before use. Extraction residues were redissolved in chloroform and 10-μl aliquots of this solution were injected directly on-column with a 25-μl syringe (Hamilton, Reno, Nev., U.S.A.) using a stop-flow injection technique.

Preparation of standard solutions

Phenylbutazone, accurately weighed, was dissolved in 95% ethanol (1 ml) in a 100-ml volumetric flask and brought to volume with phosphate buffer (pH 7.2). An aliquot of this solution was further diluted with water to produce a final solution of the desired concentration. Spiked plasma solutions (1.75–40 μg/ml) were prepared by transferring an aliquot (1.0 ml) of the aqueous phenylbutazone solution to a 5-ml volumetric flask. The flask was then brought to volume with citrated human plasma and the resulting solution mixed thoroughly.

General procedure

An aliquot (1.0 ml) of the plasma sample was transferred to a 10-ml screw-cap centrifuge tube, 1 *M* hydrochloric acid (1 ml) and a solution of the internal standard (1.5–5 μg/ml, accurately weighed) in *n*-hexane (6.0 ml) were added, and the tube tumbled on a Multi-Rotator® (Scientific Industries, Springfield, Mass., U.S.A.) for 15 min at 30 rpm. The tubes were then centrifuged for 2–3 min at 3000 rpm and the upper *n*-hexane fraction transferred with a pasteur pipette to a 15-ml conical screw-

capped centrifuge tube. The *n*-hexane was evaporated under nitrogen in a constant-temperature (60°) water-bath. The inner walls of the tubes were rinsed with chloroform (1 ml) which was also evaporated. The resulting residue was promptly redissolved in 50–200 μl of chloroform to give an anticipated phenylbutazone concentration of 30–200 ng/μl. Aliquots (10.0 μl) of this solution were chromatographed.

The amount of phenylbutazone in the sample was determined from the following expression:

$$W_p = \frac{R \cdot W_I}{m}$$

where

W_p = micrograms of phenylbutazone
R = response ratio of phenylbutazone to internal standard
W_I = weight of internal standard
m = slope of calibration curve (Fig. 2)

RESULTS AND DISCUSSION

Fig. 1b illustrates the chromatogram obtained from the analysis of a plasma sample containing 5.5 μg/ml of phenylbutazone using the system described above. This represents an on-column injection of 0.7 μg of internal standard and 1.1 μg of phenylbutazone. An analysis time of less than 7 min is achieved. No interfering compounds are extracted from plasma with *n*-hexane (Fig. 1a). Samples for this study were prepared from plasma collected from six different subjects. No extraneous compounds were encountered in any of these samples.

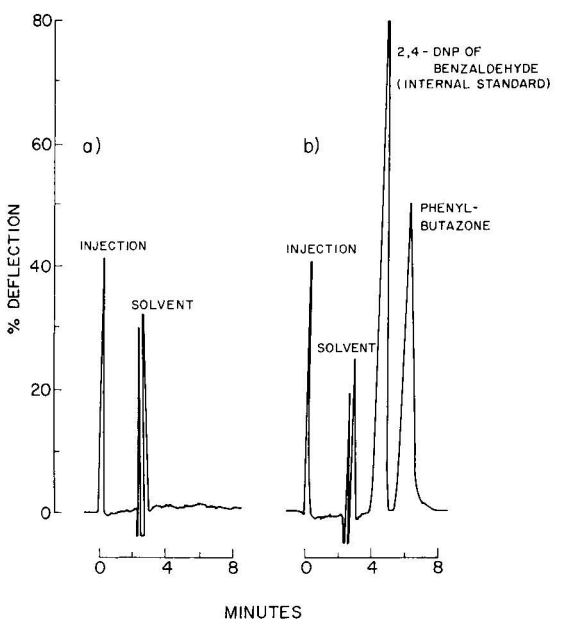

Fig. 1. High-speed liquid chromatogram of human plasma extract: (a) from blank plasma; (b) from plasma spiked with phenylbutazone (5.5 μg/ml).

Adsorption chromatography was chosen because it offers the advantage of rapid analyses and excellent column stability. A simple solvent system of *n*-hexane modified with 10% tetrahydrofuran was used as a mobile phase. The addition of a small amount (0.002%) of glacial acetic acid to the mobile phase produced a symmetrical phenylbutazone peak by reducing the slight tendency of this compound to tail. Operation at a column temperature of 35° reduced the column back pressure (475 p.s.i.) and modestly improved the efficiency of the column.

This chromatographic separation also eliminates any interference from either hydroxyphenylbutazone or oxyphenbutazone, two known metabolites of phenylbutazone[1], if either were present in the plasma. Under these chromatographic conditions retention times of 4.4, 5.8, 33.0, and 40.8 min are obtained for the internal standard, phenylbutazone, hydroxyphenylbutazone, and oxyphenbutazone, respectively. Neither hydroxyphenylbutazone nor oxyphenbutazone was detected in any of the biological samples that were analysed. This may be attributed to the low partition coefficient of these compounds in *n*-hexane and to the poor detector response partially caused by peak broadening of these highly retained compounds. Studies are presently in progress to develop analytical procedures for the analysis of oxyphenbutazone in biological samples.

Plasma samples were prepared by a modification of the partitioning procedure reported by McGilveray *et al.*[9]. Aliquots of plasma were treated with 1 *M* hydrochloric acid, to release the protein bound phenylbutazone, and partitioned for 15 min with *n*-hexane containing a known amount of internal standard. Previous procedures[1–3] used *n*-heptane or *n*-heptane modified with 2–3% of a polar solvent such as dichloromethane as an extraction solvent. *n*-Hexane was used in this procedure because the partition coefficient of phenylbutazone into *n*-hexane and *n*-heptane were comparable (*ca.* 98%), no interfering UV absorbing substances were extracted from the plasma with *n*-hexane, and the higher volatility of *n*-hexane simplified evaporation of the sample extracts.

Although refrigerated plasma samples containing phenylbutazone were found to be stable for several weeks, degradation of the evaporated extracts presented a problem initially. It was observed that degradation occurred when the residues were stored, even if refrigerated. Since this effect appeared to be random, it is suggested that traces of aqueous acid, which may have been carried over into some of the tubes, caused this degradation. This problem was eliminated by analyzing the samples within two to three hours following partitioning. Degradation, when present, is readily detected by the appearance in the spectrum of two, as yet unidentified, peaks with retention times of 7.1 and 11.6 min.

Chloroform was chosen as the solvent for redissolving the sample residues for injection since the internal standard and phenylbutazone are both readily soluble in this solvent, the injection of 10 μl of chloroform does not affect the resolution of the separation, and the vapor pressure of chloroform is high enough to prevent excessive loss due to evaporation of the sample between injections.

The output of the UV detector is linear in absorbance units; therefore, for compounds that obey the Lambert-Beer law, the area under the peak is proportional to concentration, within the linear range of the detector. As a result, it is possible to quantitate the phenylbutazone in plasma by electronic integration and peak-height measurements. Quantitation by peak-height measurement is particularly applicable

to HSLC since solute peaks tend to be symmetrical. This study (Table I), along with previous work in this laboratory[10], and results reported by Roos[11] indicate that peak-height data compare favourably with that obtained by electronic integration. This is particularly true for the measurement of peaks with relatively long retention times. The integrator in use in this laboratory provides a maximum slope sensitivity setting of 0.2 μV/msec. Since the peak base width increases with retention time, the slope of small peaks is often too low to trip the integrator. Therefore, a significant percentage of the peak area of small, broad peaks may not be integrated. In this study, the on-column injection of less than 200 ng of phenylbutazone gave poor results when the detector was attenuated to 0.04 a.u.f.s. Although it is possible to reduce the detector attenuation by a factor of 8, *i.e.* 0.005 a.u.f.s., at this attenuation problems caused by detector noise and baseline drift frequently produce spurious results. An attenuation setting of 0.04 a.u.f.s. was used routinely in this procedure since it provided adequate sensitivity for the levels of phenylbutazone to be analyzed and minimized any interference caused by detector noise or drift. In order to analyze samples containing as little as 2 μg/ml of phenylbutazone, plasma samples containing less than 6 μg/ml of drug were extracted with *n*-hexane containing 25% of the normal amount of internal standard. The resulting sample residues were re-dissolved in 50 μl of chloroform. This provided a final concentration of at least 200 ng per injection. Using this procedure it was possible to quantitate routinely plasma samples containing as little as 1.75 μg/ml (*i.e.*, 350 ng per injection) of phenylbutazone by electronic integration.

At a detector attenuation setting of 0.005 a.u.f.s. the injection of as little as 40 ng of phenylbutazone would produce a recorder response of approximately 10% full scale deflection. This would represent a minimum detectable amount of 0.2 μg/ml of phenylbutazone.

The 2,4-dinitrophenylhydrazone of benzaldehyde was used as an internal standard. It is added directly with the extracting solvent before partitioning the

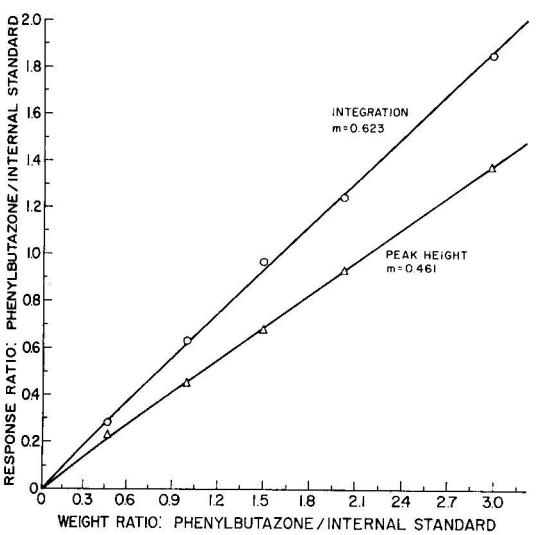

Fig. 2. Phenylbutazone/internal standard calibration curve.

samples. This reduces errors caused during transfer and evaporation procedures. The use of 2,4-dinitrophenylhydrazine derivatives as internal standards shows particular promise due to their ease of synthesis, stability, and intense UV absorbance at 254 nm. Judicious selection of the ketone or aldehyde reagent makes it possible to prepare internal standards with the required retention time for a particular separation. Fig. 2 represents the calibration curves obtained by plotting the response *vs.* concentration ratios of phenylbutazone/internal standard obtained from the analysis of spiked plasma samples. Data determined by integration and peak-height measurements are shown. Both plots are straight lines ($y=mx$) over the concentration range of 1.8 to 41.7 μg/ml. Mean slope values of $0.623 \pm 3.8\%$ (correlation coefficient, $r=1.0$) and $0.461 \pm 4.7\%$ ($r=1.0$) were obtained by integration and peak-height measurements, respectively. These calibration curves were checked frequently over the course of the study and the drift from the curve was always less than the mean slope error.

Table I lists the results obtained from the analysis of a number of spiked plasma samples. These data reflect the accuracy and precision of this method. Mean recovery values of $99.9 \pm 1.7\%$ and $99.3 \pm 3.1\%$ were obtained by integration and peak-height measurement, respectively.

TABLE I

ANALYSIS OF SPIKED PHENYLBUTAZONE-PLASMA SAMPLES

Results from the analysis of duplicate aliquots are shown.

Sample number	*Phenylbutazone concentration (μg/ml)*				
	Theoretical	*Found**	*Recovery (%)*	*Found***	*Recovery (%)*
1	38.86	39.60	101.9	39.91	102.7
		39.83	102.5	39.61	101.9
2	29.00	28.42	98.0	27.73	95.6
		27.97	96.4	27.52	94.9
3	19.33	19.27	99.7	19.18	99.2
		19.27	99.7	19.05	98.6
4	19.30	19.88	103.0	19.40	100.5
		19.82	102.7	20.39	105.7
		19.28	99.9	19.13	99.1
		18.98	98.1	18.57	96.2
		19.72	102.2	19.93	103.3
	Mean***		101.2		101.0
	Coefficient of variation		±2.1%		±3.7%
5	6.96	7.14	102.6	7.03	101.0
		6.68	96.1	6.64	95.4
6	6.18	6.15	99.5	6.08	98.4
		6.04	97.7	6.02	97.4
7	3.10	3.19	102.9	3.14	101.3
		2.96	95.6	3.02	97.4
	Mean§		99.9		99.3
	Coefficient of variation		±1.7%		±3.1%

* Determined by integration.

** Determined by peak-height measurement.

*** Mean of five aliquots of the same sample.

§ Mean of 17 analyses.

The plasma phenylbutazone profile of a human subject (male, 200 lbs.) who had been administered two 100-mg tablets of phenylbutazone (Butazolidin®; Ciba-Geigy, Montreal, Canada) is illustrated in Fig. 3. Blood was collected in heparinised 10-ml Vacutainers (Becton-Dickinson, Toronto, Canada), and the plasma, separated by centrifugation, was either analysed immediately or stored in a freezer (−15°).

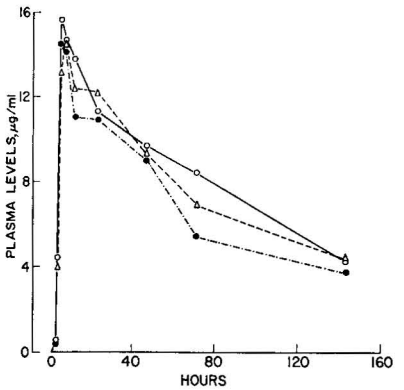

Fig. 3. Comparison of plasma levels by different analytical methods following single dose (two 100-mg tablets) of phenylbutazone. ○—○, GLC; ●–·–●, UV; △ – – –△, HSLC.

These results compare the plasma levels over 144 h determined by a UV procedure[5], a gas–liquid chromatographic (GLC) procedure[9] and the HSLC procedure described above. The overall difference between the HSLC and UV methods was 8.2% and that between the GLC and HSLC 8.9%. The UV determination would not distinguish between any side-chain hydroxymetabolite present and the parent drug and could contribute to the difference between levels estimated by this and the HSLC procedure. Two specific points —the 5 and 72 h levels— contribute greatly to the variations between the GLC and HSLC methods, no explanation being evident. Analysis of spiked plasma by the two chromatographic procedures gave greater precision (2% at 4 μg/ml level) and give greater confidence in the comparison.

This HSLC method is a sensitive procedure for the determination of phenylbutazone in plasma. It offers high specificity, since it differentiates the parent drug from the two known metabolites, and could form the basis of a method for metabolic studies.

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CHROM. 7072

ANALYTICAL PERFORMANCE OF THE PIEZOELECTRIC CRYSTAL DETECTOR

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SUMMARY

A piezoelectric crystal, which can be coated with various low-vapor-pressure materials, has been used as a detector in a gas chromatograph that can operate at room temperature using an air or nitrogen carrier gas. Performance data are presented on sensitivity, response and gas chromatographic peak parameters for a range of compounds from SO_2 to heavy hydrocarbons. The use of selective coatings to increase sensitivity is demonstrated.

INTRODUCTION

In the past, piezoelectric quartz crystals have been used successfully for many purposes, from frequency controllers to micro balances. Recently, a piezoelectric crystal has been effectively utilized in a gas chromatographic (GC) detector to provide a uniquely simple yet sensitive analytical instrument that can operate at room temperature and with an air or nitrogen carrier gas[1].

King[2] was the first to demonstrate that the principle of piezoelectric crystal oscillators could be applied in a GC detector. He coated quartz plates with the same partitioning liquids used in GC columns. The separated components were detected after leaving the column when they passed over the coated crystal and partitioned into the liquid coating, changing the natural resonance frequency of the oscillating quartz crystal. This frequency shift can be converted linearly to a voltage and displayed on a recorder to produce a chromatogram. In a more recent paper[3], King described many applications for the piezoelectric detector, including its ability to accomplish chromatography of high-boiling compounds at low temperatures. Both King[2] and Guilbault[4] have demonstrated that through the use of specific coatings the piezoelectric crystal becomes a sensitive and versatile detector. Guilbault and Lopez-Roman[5] have since shown its application to the detection of SO_2 in air. Janghorbani and Freund[6] have evaluated coated piezoelectric crystals as digital sensors and have suggested their use for continuous monitoring of gas streams.

We have recently been investigating the qualitative and quantitative performance of a chromatograph utilizing a piezoelectric detector (P/Z Chromatograph, Laboratory Data Control, Riviera Beach, Fla., U.S.A.), whose design is based on the work of Karasek and Gibbins[1]. It is the purpose of this paper to present data on the analytical performance of this detector and limits of detection for a number

of compounds as well as data on the use of specific coatings to selectively increase sensitivity. Response factors and analytical results for a range of compounds from SO_2 to mixtures of heavy hydrocarbons indicate the scope of its analytical capabilities.

EXPERIMENTAL

Instrument design

Fig. 1 is a simplified scheme of the P/Z chromatograph. The sample to be analyzed is injected through a conventional injection port system onto a packed GC column, where separation of the components is achieved. Because component separation is accomplished at room temperature, the columns used are short (20 in.) compared to columns of conventional lengths. Immediately after leaving the column the components pass through a stainless-steel detector block that houses a suitably coated 9,000 MHz piezoelectric crystal. The components are sensed as a frequency change, the variation of which is determined by heterodyning the detector signal against a similar reference oscillating circuit. The audio signal representing the difference between the reference and detector circuits is amplified, clipped and differentiated; the resulting pulses are then rectified and filtered. The DC voltage produced, which is proportional to the frequency shift, is compared to an offset or zeroing control and is amplified and displayed on a millivolt chart recorder.

Generally the crystal partitioning coating is the same as that used on the column in order that column and crystal bleed compensate each other. The crystal coating is obtained by depositing an appropriate volume of a solution of known weight

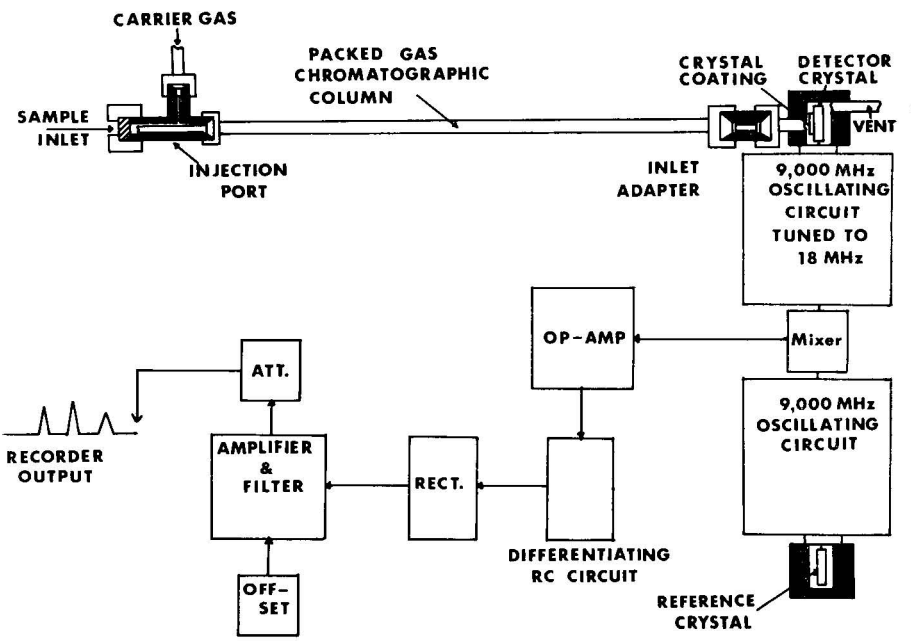

Fig. 1. Schematic diagram of P/Z chromatograph.

percent on the center of a clean crystal with a microsyringe. After an 8- to 10-h conditioning period at a temperature 25 to 100° below the maximum recommended temperature of the liquid support, this deposit forms a uniform layer over most of the electrode surface. The coated crystal detector is placed in the instrument where it is operated under a carrier flow of 20–40 ml/min until a stable baseline is achieved. This usually requires 1 to 2 h of operation. It has been observed that the prolonged heating of the crystal during the initial conditioning period does not adversely affect its performance but does reduce the time required for the detector to reach equilibrium stability.

All columns used in the instrument for this work were 20 in. long and constructed of 1/8 in. O.D. stainless-steel tubing packed with 5% loadings of either Carbowax 400 or UCON LB550X on 100–120 mesh Chromosorb W, or silicone fluid OV-17 on 80–100 mesh Chromosorb P. All columns provided adequate separation with short retention times for the compounds chromatographed. A 4-μg coating on the detector crystal was used throughout the investigation.

Procedure

A mixture of *n*-acetates in equal proportions by weight was chromatographed at room temperature to demonstrate component separation and detector response to increasing molecular weight.

In order to establish the precision and accuracy with which the instrument can perform quantitative analysis, response factors for the components of two typical mixtures were calculated. The procedure involved preparing an accurate weight percent mixture of the components, making 0.5-μl injections and then calculating the individual peak areas with the aid of a Sargent Model SRG recorder in conjunction with a Model 240 disc integrator. The response factors were calculated relative to ethanol for the first mixture using an average of six runs. The same procedure was followed for a series of normal hydrocarbons. In this case the factors were calculated relative to *n*-hexane using an average of ten runs. Kováts retention indices for benzene and toluene were calculated.

The minimum detectable quantity for a large number of compounds was determined. A dilute solution of each compound was prepared in a suitable solvent. Injections of decreasing volume were made until the signal produced by the component of interest measured twice that of the ambient noise level. The minimum detectable volume was then converted to mass units.

Improved detector sensitivity through the use of specific coatings was illustrated by redetermining the minimum detectable quantity for *n*-nonane, *n*-decane, *n*-undecane, and *n*-dodecane using a rubber crystal coating. This coating was produced using commercial rubber cement (Carter's Clean Grip Rubber Cement, The Carter's Ink Company of Canada, Toronto, Canada).

Nitrogen samples containing low concentrations of SO_2 were also analyzed. Varying concentrations of SO_2 in N_2 were obtained by first filling a 1-ml Hamilton gas-tight syringe to capacity with anhydrous SO_2 and then evacuating it to the appropriate volume and refilling it to capacity with dry nitrogen. This procedure was repeated until the desired dilution was obtained. The gases were allowed to equilibrate between each dilution and before injection.

Reagents

The analytical standards used in the minimum detectable quantity study were obtained from kits Nos. 11A, 21A, 25A, 41A, and 62A (PolyScience Corp., Evanston, Ill., U.S.A.). All other compounds were reagent grade as specified by the American Chemical Society.

RESULTS AND DISCUSSION

A crystal coated with a liquid phase will respond when a critical mass is sorbed onto its surface. Fig. 2 illustrates the response of the piezoelectric detector to an equal weight percentage mixture of *n*-acetates. It can be observed that the response increases with increasing molecular weight, making the higher-boiling compounds at the end of the chromatogram more detectable. A plot of either log response *versus* boiling-point or log retention time *versus* boiling-point yields a straight line, indicating that the detector response is predictable. King[3] reports a detector response time in

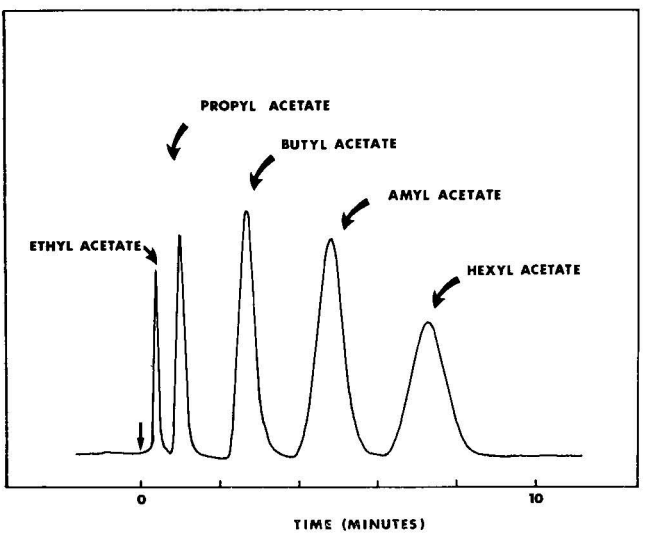

Fig. 2. Chromatogram of a mixture of *n*-acetates. Conditions: temperature, 25°; column: 1/8 × 20 in. O.D. stainless-steel column packed with 5% UCON LB550X on 100–120 mesh Chromosorb W; crystal coating, 4 μg UCON LB550X; sample size, 0.5 μl; carrier gas, air; flow-rate, 60 ml/min.

the 40-msec range. The rapid response time is reflected by the symmetry of the peaks and can be attributed to the small detector volume, minute mass of the crystal coating, and short component residence time in the detector. Because of the relatively low temperature operation, component separation can be achieved with the short 20-in. columns. Peak shapes are quite symmetrical with all the columns studied.

The following are some columns other than those already mentioned in this paper which have been used: 5% SF-96 on 80–100 mesh Chromosorb P, 5% Carbowax 20M on 100–120 mesh Chromosorb W, 5% SE-30 on 80–100 mesh Chromosorb P, 5% dinonyl phthalate on 100–120 mesh Chromosorb W, 5% Lexan on 100–120 mesh Chromosorb W, 5% Hallcomid M180L on 100–120 mesh Chro-

mosorb W, 3% UC W-98 on 80–100 mesh Chromosorb W, and 5% diethylene glycol adipate on 100–120 mesh Chromosorb W.

A prerequisite for quantitative GC analysis is that the detector response be linearly proportional to the quantity of components injected on the column. Table I displays data resulting from the quantitative study of a simple alcohol mixture. Determined values are accurate to within $\pm 0.1\%$. Fig. 3 shows a typical chromatogram displaying the elution of a series of normal alcohols at room temperature.

Table II presents response data for a similar quantitative study of a series of *n*-hydrocarbons. The error of the determination varies between 0.8 and 0.1%. The percent relative standard deviations quoted are those of the six individual runs relative to the average calculated weight percent.

TABLE I

QUANTITATIVE ANALYSIS OF A MIXTURE OF NORMAL ALCOHOLS

Sample	*Relative response*	*Known weight percent*	*Average calculated weight percent*	*Relative standard deviation* (%)
Ethanol	1.0	53.96	54.10	0.012
1-Propanol	2.26	32.48	32.35	0.005
Butanol	5.20	13.56	13.55	0.035

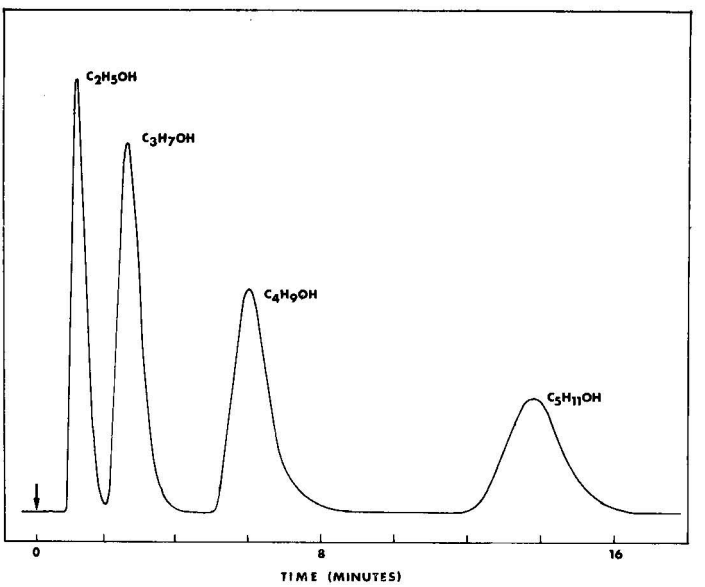

Fig. 3. Chromatogram of a mixture of *n*-alcohols. Conditions: temperature, 25°; column: 1/8×20 in. O.D. stainless-steel column packed with 5% Carbowax 400 on 100–120 mesh Chromosorb W; crystal coating, 4 μg Carbowax 400; sample size, 0.5 μl; carrier gas, air; flow-rate, 50 ml/min.

TABLE II

QUANTITATIVE ANALYSIS OF A MIXTURE OF HYDROCARBONS

Sample	*Relative response*	*Known weight percent*	*Average calculated weight percent*	*Relative standard deviation (%)*
n-Hexane	1.00	46.22	45.42	0.008
n-Heptane	2.60	10.41	10.92	0.037
n-Octane	7.09	27.62	28.10	0.007
n-Nonane	18.95	8.70	8.82	0.032
n-Decane	51.95	7.05	6.70	0.061

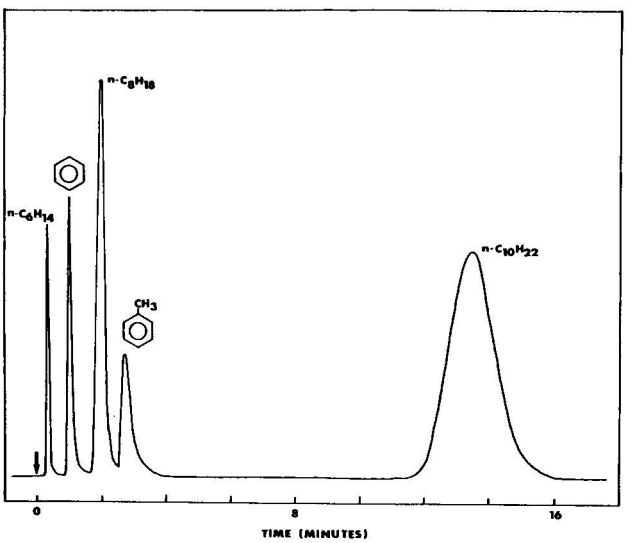

Fig. 4. Chromatogram of a mixture of normal and aromatic hydrocarbons. Conditions: temperature, 25°; column, 1/8 × 20 in. O.D. stainless-steel column packed with 5% OV-17 on 80–100 mesh Chromosorb P; crystal coating, 4 μg OV-17; sample size, 0.5 μl; carrier gas, air; flow-rate, 30 ml/min.

Fig. 4 shows a chromatogram of a mixture of normal and aromatic hydrocarbons. These compounds are all separated with a minimum resolution of 1.7. This permits the calculation of the Kováts retention index at room temperature for benzene and toluene.

Table III displays the results of the limits of detection study. It can be seen that in general the detection limit improves with increasing molecular weight. All compounds in this study were chromatographed at room temperature using a Carbowax 400 coating on the column packing and detector. This appears to be a very versatile coating and can be satisfactorily used for many compounds. Table IV illustrates the improvement in sensitivity which can be achieved by using coatings that are more specific to the compounds of interest. The four hydrocarbons were

TABLE III

LIMITS OF DETECTION OF THE PIEZOELECTRIC CRYSTAL DETECTOR FOR VARIOUS COMPOUNDS USING A CRYSTAL COATING OF CARBOWAX 400

Compound	*Molecular weight*	*Boiling-point (°C)*	*Minimum detectable quantity (g)*
n-Hexane	86.18	68.9	$2 \cdot 10^{-6}$
n-Heptane	100.21	98.4	$7 \cdot 10^{-7}$
n-Octane	114.23	125.6	$4 \cdot 10^{-7}$
n-Nonane	128.26	150.8	$4 \cdot 10^{-8}$
n-Decane	142.29	174.1	$3 \cdot 10^{-8}$
n-Undecane	156.32	195.9	$2 \cdot 10^{-8}$
n-Dodecane	170.34	216.3	$2 \cdot 10^{-8}$
Ethanol	46.07	78.5	$8 \cdot 10^{-6}$
Benzene	78.12	80.1	$4 \cdot 10^{-7}$
o-Xylene	106.17	144.4	$2 \cdot 10^{-7}$
Cumene	120.20	152.4	$3 \cdot 10^{-8}$
Mesitylene	120.20	164.7	$9 \cdot 10^{-8}$
p-Cymene	134.22	177.1	$9 \cdot 10^{-8}$
n-Hexylbenzene	162.28	277.0	$3 \cdot 10^{-8}$
2-Hexanone	100.16	128.0	$2 \cdot 10^{-7}$
2-Heptanone	114.19	151.5	$2 \cdot 10^{-7}$
2-Octanone	128.22	173.0	$2 \cdot 10^{-7}$
2-Nonanone	142.24	195.3	$2 \cdot 10^{-7}$
Ethyl acetate	88.12	77.1	$4 \cdot 10^{-7}$
Ethyl propanoate	102.13	99.1	$3 \cdot 10^{-7}$
Ethyl butanoate	116.16	124.0	$2 \cdot 10^{-7}$
Ethyl pentanoate	130.19	144.6	$2 \cdot 10^{-7}$
Ethyl hexanoate	144.22	168.0	$4 \cdot 10^{-8}$

TABLE IV

LIMITS OF DETECTION OF THE PIEZOELECTRIC CRYSTAL DETECTOR FOR SOME *n*-HYDROCARBONS USING A SPECIFIC CRYSTAL COATING PREPARED FROM RUBBER CEMENT

Compound	*Minimum detectable quantity (ng)*
n-Nonane	4
n-Decane	5
n-Undecane	2
n-Dodecane	4

chromatographed at room temperature with a Carbowax 400 column and a rubber crystal coating. This coating displays a larger component interaction specifically with hydrocarbons and consequently the sensitivity is increased by an order of magnitude.

One determination for which the piezoelectric detector is sensitive is the detection of SO_2 in air. Fig. 5 illustrates the linearity of response obtained when dif-

ferent concentrations of SO_2 by volume in nitrogen are chromatographed. The absolute limit of detection, corresponding to a signal-to-noise ratio of 2:1, is found to be in the range of 3–5 ppm using a 1-ml sample size.

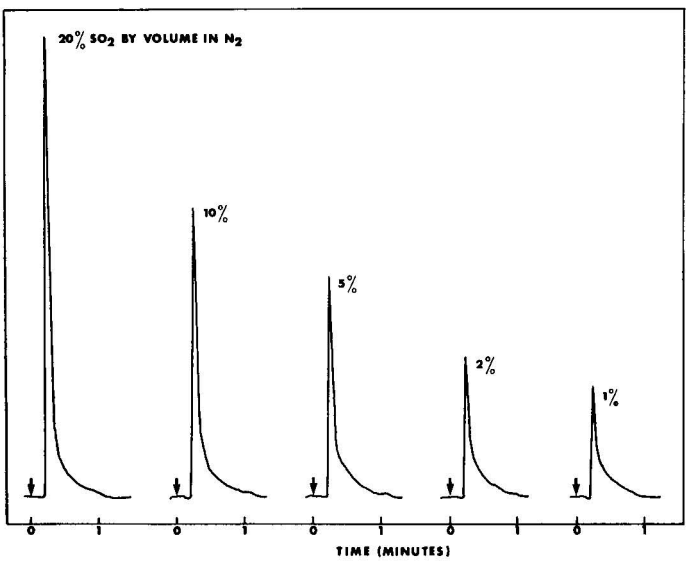

Fig. 5. Chromatograms of varying concentrations of SO_2 in nitrogen. Conditions: temperature, 25°; column, 1/8 × 20 in. O.D. stainless-steel column packed with 5% Carbowax 400 on 100–120 mesh Chromosorb W; crystal coating, 4 μg Carbowax 400; sample size, 1 ml; carrier gas, air; flow-rate, 60 ml/min.

CONCLUSIONS

From studies to date it appears that the scope of analytical capabilities of the piezoelectric detector are considerable. Based on its sensitive range of detection and high stability (noise < 0.05 Hz), the detector represents an effective means of detection of trace compounds. Perhaps its most exciting potential lies in the air pollution monitoring field. Further work is currently being done with specific coatings for light inorganic gases. It is expected that they will extend the useful range of this detector in qualitative and quantitative GC.

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CHROM. 7085

GRUNDLAGEN ZUR RATIONELLEN ANWENDUNG DER QUANTITATIVEN DÜNNSCHICHTCHROMATOGRAPHIE

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(Eingegangen am 27. August 1973)

SUMMARY

Basic considerations for a rational application of quantitative thin-layer chromatography

Using the Lambert–Beer law one obtains for transmission measurements for quantitative thin-layer chromatography calibration curves according to the function $y=ax$, if oversaturation of the spots to be measured is avoided. Once the optimal conditions for the starting spot have been attained, they can be easily reproduced with an automatic spot applicator. Up to nine samples can be applied simultaneously in a very short time. The diameter of the starting spots can be chosen. The calibration curve according to the Lambert–Beer law and the automatic applicator permit rational utilisation of quantitative thin-layer chromatography. The coefficient of variation for this method lies between 0.8 and 3.5%.

EINLEITUNG

Die Direktauswertung von Dünnschichtchromatogrammen ist ein Analysenverfahren, das wegen seines breiten Anwendungsbereichs immer grössere Bedeutung gewinnt. So lassen sich im Prinzip alle Verbindungen mit Eigenfluoreszenz oder Absorption im UV- und sichtbaren Spektralbereich quantitativ bestimmen. Hinzu kommen die Substanzen, bei denen die Fluoreszenz oder die Absorption durch eine chemische Reaktion auf der Dünnschichtplatte erreicht wird. Es ist deshalb sehr wichtig, die Voraussetzungen für eine rationelle Anwendung dieser Methode zu erreichen.

Eine wesentliche Aufgabe besteht darin, geeignete funktionelle Zusammenhänge zwischen der Konzentration und dem Messwert zu finden, die lineare Eichgeraden liefern. Am einfachsten liegen die Verhältnisse bei der Fluoreszenzmessung. Wie Seiler[1,2] gezeigt hat, ist die integrale Fluoreszenzintensität eines Flecks, unabhängig von dessen Form, in niedrigen Konzentrationen direkt der Substanzmenge proportional. Es kann in Reflexion oder in Transmission gemessen werden.

Wesentlich komplizierter liegen die Verhältnisse bei der quantitativen Analyse absorbierender Verbindungen. Als einer der ersten hat Jork[3] mit Hilfe der Kubelka–Munk-Funktion quantitative Bestimmungen in Remission durchgeführt. Da diese Methode recht aufwendig ist, hat man nach einfacheren empirischen Beziehungen gesucht. Eich *et al.*[4] verwenden für ihre Bestimmungen die Beziehung zwischen den

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Quadraten der Fläche unter der Absorptions–Ortskurve und den Substanzmengen der Flecke. Für einen begrenzten Konzentrationsbereich erhalten sie eine Gerade. Sie gehorcht der Funktion $y=ax+b$. Das bedeutet, die Eichgerade geht nicht durch den Nullpunkt. Treiber *et al.*[5] haben die Kubelka–Munk-Funktion mit dem Lambert–Beerschen Gesetz kombiniert, um durch eine simultane Remissions- und Transmissionsmessung die Platten quantitativ auszuwerten. Diese Methode ist jedoch mit grossem mathematischem Aufwand verbunden.

In dieser Arbeit wird gezeigt, wie es generell möglich ist, das Lambert–Beersche Gesetz für quantitative Bestimmungen anzuwenden. Die dabei erarbeiteten optimalen Bedingungen für den Startfleck können jederzeit mit Hilfe eines automatischen Auftragegerätes reproduziert werden.

EXPERIMENTELLES

Zur Chromatographie werden DC-Fertigplatten Kieselgel 60 F_{254} der Fa. Merck, Darmstadt, B.R.D., verwendet. Die Proben werden mit Hamilton Mikroliterspritzen (Hamilton, Reno, Nev., U.S.A.) bzw. mit einem automatischen Auftragegerät aufgetragen. Bei diesem Gerät handelt es sich um eine Eigenkonstruktion der Fa. Dr. Karl Thomae GmbH (Gebrauchsmusterschutz liegt vor).

Die quantitative Auswertung erfolgt mit dem Schoeffel Spektrodensitometer Modell SD 3000 (Schoeffel Instrument Corp., Westwood, N.J., U.S.A.) und einem Schreiber mit Integrator von Vitatron, Modell UR 400 (Vitatron, Milton, Mass., U.S.A.). Entwickelt werden die Platten in gesättigten N-Kammern.

Zur Überprüfung der Dosierungsgenauigkeit des Auftragegerätes wurde eine 0.1%ige wässrige Lösung des Lebensmittelfarbstoffes Gelborange verwendet. Die Lösungen wurden gesteuert auf Kieselgelfolien von Macherey, Nagel & Co. (Düren, B.R.D.) aufgetragen. Der Durchmesser der Startflecke betrug 4 mm. Anschliessend wurden die Flecke ausgeschnitten und mit 10 ml Wasser eluiert. In 1-cm Küvetten wurde bei 480 nm gegen Wasser die Absorption der jeweiligen Lösung bestimmt.

Den statistischen Aussagen liegen die folgenden Formeln zugrunde:

Standardabweichung, s: $$s = \sqrt{\frac{\Sigma(\bar{x}-x_i)^2}{n-1}}$$

Variationskoeffizient, V: $$V = \frac{s}{\bar{x}} \cdot 100$$

Vertrauensbereich des Mittelwertes, $\bar{x}_V$: $$\bar{x}_V = \bar{x} + t_{(n\cdot p)} \cdot s \cdot \frac{1}{\sqrt{n}}$$

Toleranzbereich des Einzelwertes, x_T: $$x_T = \bar{x} \pm t_{(n\cdot p)} \cdot s \cdot \sqrt{\left(1+\frac{1}{n}\right)}$$

ERGEBNISSE UND DISKUSSION

Eichgerade nach dem Lambert–Beerschen Gesetz

Das Lambert–Beersche Gesetz liefert eine lineare Beziehung zwischen der Extinktion E und der Konzentration C

$$E = \log \frac{I_0}{I} = \varepsilon \cdot d \cdot C = K \cdot C$$

Analog ist es möglich, dieses Gesetz in der quantitativen Dünnschichtchromatographie anzuwenden, wenn man in Transmission auswertet. Das Chromatogramm wird mit einem Lichtstrahl gegebener Intensität durchstrahlt. I_0 ist der Anteil der Lichtenergie, der an einer substanzfreien Stelle die Platte durchdringt. I ist die durch die absorbierende Substanz geschwächte Lichtintensität. Um auch im UV absorbierende Verbindungen bestimmen zu können, verwendet man DC-Platten mit Fluoreszenzindikator. Die Absorption der zu analysierenden Substanz bewirkt eine Fluoreszenzminderung. Gemessen wird hier

$$\log \frac{I_0}{I} = \log \frac{\text{Fluoreszenz}}{\text{verminderte Fluoreszenz}}$$

Indem man Probe- und Vergleichsbahn mit konstanter Geschwindigkeit an je einem Spalt vorbeibewegt, erhält man die Fläche der Extinktionskurve. Das Lambert–Beersche Gesetz liefert jetzt die lineare Beziehung zwischen der Fläche der Extinktionskurve (=Integratoreinheiten) und der Substanzmenge.

Erhält man jedoch eine Eichgerade[6–8] die der Funktion $y=ax+b$ gehorcht, dann bedeutet es einmal, dass man für exakte quantitative Aussagen mit der Probe eine Konzentrationsreihe von mindestens vier Werten auf die Platte auftragen muss, um den Gehalt mit Hilfe der daraus resultierenden Regressionsgeraden zu bestimmen. Zum anderen ergibt sich aus diesen Ergebnissen ein Widerspruch. Verlängert man die Eichgeraden, die oft nur für einen bestimmten Konzentrationsbereich angegeben sind, bis zum Schnittpunkt mit der y-Achse, dann erhält man nach der Funktions-

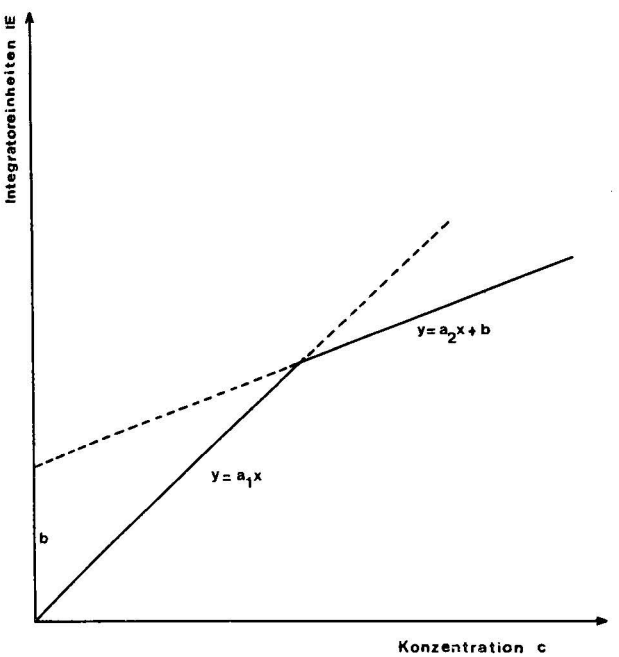

Fig. 1. Gegenüberstellung der beiden Eichgeraden: $y=a_1x$, die durch den Nullpunkt geht und $y=a_2x+b$, mit dem hypothetischen Wert für b.

gleichung $y=ax+b$ bzw. $IE=aC+b$ (IE=Integratoreinheiten) den Wert für b (Siehe Fig. 1). Es kann sich jedoch nur um einen hypothetischen Wert handeln, denn bei $C=0$ ist die Absorption auch 0. Demnach müsste es zwei Eichgeraden geben; die erste gehorcht der Funktion $y=a_1x$ und läuft von $C=0$ bis zum Schnittpunkt mit der zweiten Eichgeraden, die durch die Funktion $y=a_2x+b$ beschrieben wird. Verlängert man die erste Eichgerade über den Schnittpunkt hinaus, dann erhält man für die einzelnen Konzentrationen grössere Integratorwerte, da $a_1 > a_2$.

Dieser Widerspruch hebt sich auf, die beiden Eichgeraden fallen zusammen, wenn man die unterschiedlichen Konzentrationen nicht auf gleiche Flächen, sondern pro Flächeneinheit die gleichen Konzentrationen aufträgt. Das heisst, mit zunehmender Konzentration muss der Durchmesser der Startflecke steigen. Danach zeigt der Schnittpunkt der beiden Eichgeraden den Beginn einer Art Übersättigung an. Von hier ab wird nicht mehr die eigentliche Konzentration sondern nur noch die Fleckgrösse gemessen. Da der Fleck immer bis zu einem gewissen Grad diffundiert, steigt die Eichgerade nach der Funktion $y=ax+b$ mit zunehmender Konzentration trotz Übersättigung an. Aus dem gleichen Grund gibt es auch keinen scharfen Knick, sondern einen kontinuierlichen Übergang.

Es hat sich gezeigt, dass alle bis jetzt untersuchten Substanzen unter Berücksichtigung der gleichen Flächenbelastung für einen bestimmten Konzentrationsbereich eine Eichgerade nach der Funktion $y=ax$ liefern.

Die Begrenzung erfolgt durch die Spaltbreite der Auswertegeräte. Beim Schoeffel Spektrodensitometer beträgt sie 1 cm. Damit der entwickelte Fleck voll erfasst wird, muss sein Durchmesser kleiner als 1 cm sein.

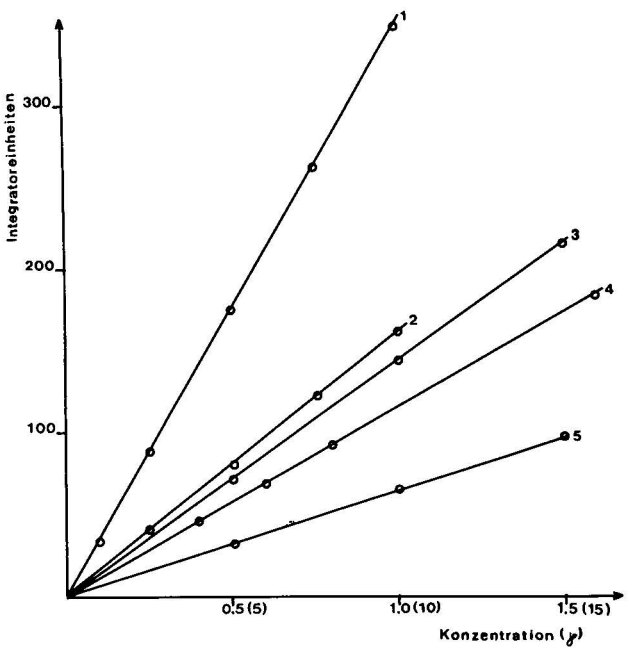

Fig. 2. Eichgeraden von Isopropylantipyrin (1), Novalgin (2), Bisolvon® (3), Prostigminbromid (4) und Codeinphosphat (5).

Tabelle I zeigt die Ergebnisse von fünf Substanzen, während in Fig. 2 die Eichgeraden graphisch dargestellt sind. Der grosse Vorteil dieser Eichgeraden besteht darin, dass eine Gehaltsbestimmung nicht mit Hilfe einer Regressionsgeraden sondern durch einen einzigen Vergleichswert durchgeführt werden kann.

Abhängigkeit der Integratorsignale vom R_F-Wert

Durch die Übersättigung lässt sich auch sehr einfach die in der Literatur[9] mehrfach beschriebene Abhängigkeit der Integratorsignale vom R_F-Wert erklären. Durch Diffusionsvorgänge während des Entwickelns kommt es zu einer Abnahme der Konzentration pro Flächeneinheit. Deshalb nimmt das Integratorsignal mit steigendem R_F-Wert zu und erreicht je nach Übersättigung des Startflecks und der Polarität des Fliessmittels ab einem R_F-Wert von *ca.* 0.3 seinen Maximalwert. Ab R_F 0.7 kann der Fleck so weit auseinanderdiffundiert sein, dass er beim Ausmessen nicht mehr ganz erfasst wird. Dadurch wird eine Abnahme vorgetäuscht. Wenn es gelingt, den Startfleck homogen und ohne Übersättigung aufzutragen, dann ist das Integratorsignal völlig unabhängig vom R_F-Wert.

Automatisches Auftragegerät

Für die quantitative Dünnschichtchromatographie sind die Konzentration pro Flächeneinheit, die Konzentrationsverteilung innerhalb des Flecks, die Fleckform und die Fleckgrösse sehr wichtige Faktoren. Die meisten dieser Kriterien werden entscheidend durch den Startfleck beeinflusst. Für eine rationelle Anwendung dieser Analysenmethode ist es sehr wichtig, dass einmal erarbeitete optimale Bedingungen

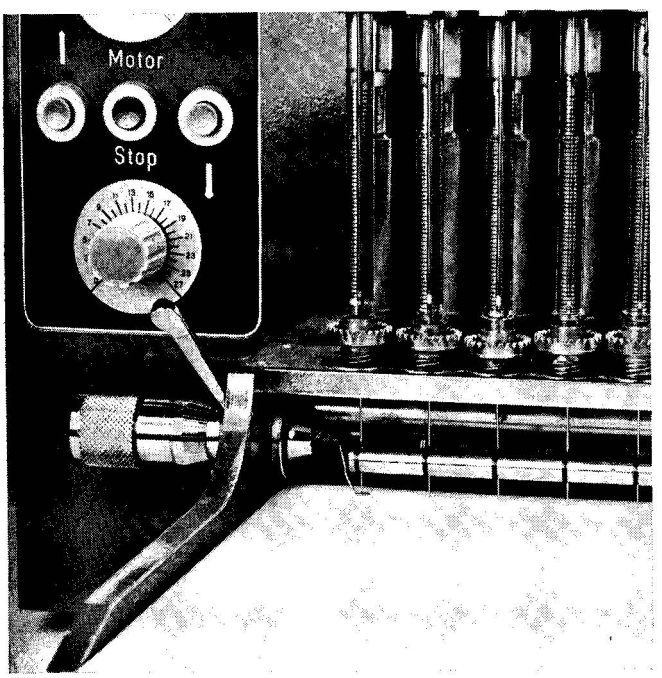

Fig. 3. Detailaufnahme des Auftragegerätes. Der Durchmesser des Startflecks ist durch zwei Elektroden festgelegt.

TABELLE I

INTEGRATORSIGNAL IN ABHÄNGIGKEIT VON DER SUBSTANZMENGE

Isopropylantipyrin ($\lambda_{max.}$ *270 nm*)		*Novalgin* ($\lambda_{max.}$ *270 nm*)		*Bisolvon®* * ($\lambda_{max.}$ *300 nm*)		*Codeinphosphat* ($\lambda_{max.}$ *270 nm*)		*Prostigminbromid* ($\lambda_{max.}$ *260 nm*)	
Substanzmenge (γ)	*Integratoreinheiten*	*Substanzmenge* (γ)	*Integratoreinheiten*	*Substanzmenge* (γ)	*Integratoreinheiten*	*Substanzmenge* (γ)	*Integratoreinheiten*	*Substanzmenge* (γ)	*Integratoreinheiten*
0.1	34	0.25	40	0.5	72	0.5	32	4.0	45
0.25	88	0.5	79	1.0	144	1.0	63	6.0	68
0.5	174	0.75	122	1.5	208	1.5	95	8.0	92
0.75	261	1.0	160	2.0	282	2.0	132	16	183
1.0	345	1.5	218	2.5	310	2.5	158	24	267
1.2	400					3.0	182	32	352
1.5	470					4.0	200		

* N-Cyclohexyl-N-methyl-(2-amino-3,5-dibrombenzyl)amin.

für eine Eichgerade oder eine Gehaltsbestimmung jederzeit reproduziert werden können. Dies ist möglich mit Hilfe eines automatisch arbeitenden Auftragegerätes.

Es arbeitet nach dem folgenden Prinzip: Eine bestimmte Menge der Probelösung wird aus einer Mikroliterspritze auf die Dünnschichtplatte aufgetragen. Dabei dient die Kanüle der Spritze als eine Elektrode. Mit Hilfe einer zweiten Elektrode kann jeder beliebige Durchmesser für den Startfleck festgelegt werden (siehe Fig. 3). Sobald der Auftragfleck die zweite Elektrode erreicht, fliesst ein Strom, der die Zugabe sofort stoppt. Ist der Fleck getrocknet, wird erneut Probelösung zugegeben. Dieser Vorgang wiederholt sich so lange, bis die gewünschte Menge aufgetragen ist.

Das verwendete Lösungsmittel muss gerade so polar sein, dass die Leitfähigkeit gewährleistet ist. Aber hierin liegt ein grosser Vorteil, denn es ermöglicht eine weitgehend homogene Substanzverteilung im Startfleck. Setzt man dagegen ein sehr polares Lösungsmittel ein, dann erhält man einen "Startring", während ein apolares Lösungsmittel einen fast punktförmigen Startfleck liefert. Beides sind Formen extremer Übersättigung und damit ungünstig.

Fig. 4 zeigt den Prototyp des Auftragegerätes. In wesentlich verbesserter Form und Aufmachung wird dieses Gerät durch die Fa. Camag (Muttenz, Schweiz) vertrieben.

Bei diesem Gerät sind neun Spritzen parallel zueinander angeordnet. Will man Proben auftragen, dann werden zunächst das gewünschte Volumen und der Durchmesser für den Startfleck eingestellt. Die Zugabegeschwindigkeit lässt sich durch regulierbares Heizen der DC-Platte von unten oder durch einen einstellbaren Luft- bzw. Stickstoffstrom von oben beeinflussen. Als Ergebnis erhält man automatisch in kürzester Zeit reproduzierbar neun identische Startflecke. Es können aber nicht nur

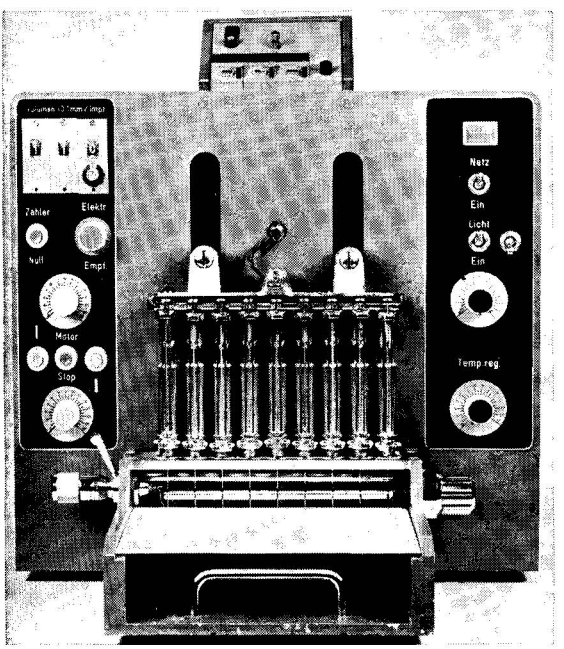

Fig. 4. Auftragegerät zur reproduzierbaren Applikation von neun Proben.

neun vergleichbare Proben, sondern auch Konzentrationsreihen äusserst rationell aufgetragen werden. Wie gezeigt wurde, kommt es ab einer bestimmten Konzentration pro Flächeneinheit zur Übersättigung. Daraus folgert umgekehrt, dass man bei einer Konzentrationsreihe alle Flecken so lange gleich gross auftragen kann, bis die Grenzkonzentration erreicht wird, denn eine "Verdünnung" beeinflusst das Flächenintegral nicht. Zur Aufstellung einer Eichgeraden trägt man steigende Konzentrationen in gleichen Volumina mit dem maximal möglichen Durchmesser auf (der Durchmesser des entwickelten Flecks muss kleiner als 1 cm sein) und stellt fest, bis zu welcher Konzentration die Eichgerade der Funktion $y=ax$ entspricht.

Für die Anwendung dieses Gerätes spielt die Frage nach seiner Dosierungsgenauigkeit eine entscheidende Rolle. Dazu wurden verschiedene Volumina einer farbigen Lösung auf Kieselgelfolien aufgetragen. Die erhaltenen Startflecke wurden ausgeschnitten. Der Farbstoff wurde eluiert und seine Konzentration photometrisch bestimmt. In Tabelle II sind die Ergebnisse zusammengefasst.

Um in etwa vergleichbare Extinktionen zu erhalten, wurde eine Stammlösung verschieden verdünnt. Mit Erhöhung der Probenmenge wird der Dosierungsfehler erwartungsgemäss kleiner. Aber selbst im ungünstigsten Fall bei 5 μl liegt der Toleranzbereich des Einzelwertes unter 1%.

Um das angewandte Verfahren selbst abzusichern, wurden die Extinktionen von drei Farblösungen bestimmt, deren Konzentrationen sich um je 5% unterschieden. Tabelle III zeigt die Ergebnisse, während in Fig. 5 die graphische Darstellung erfolgt.

TABELLE II

ÜBERPRÜFUNG DER DOSIERGENAUIGKEIT DES AUTOMATISCHEN AUFTRAGEGERÄTES

	Zugegebene Menge (μ)		
	5	*20*	*40*
Gemessene Extinktionen für die Spritzen[1-9]	0.636	0.725	0.740
	0.635	0.725	0.743
	0.633	0.728	0.742
	0.635	0.723	0.743
	0.636	0.729	0.742
	0.635	0.725	0.741
	0.632	0.722	0.742
	0.631	0.723	0.743
	0.631	0.722	0.743
Mittelwert	0.634	0.725	0.742
Standardabweichung	0.0021	0.0022	0.0011
Vertrauensbereich des Mittelwertes	0.0016	0.0017	0.0008
In % vom Mittelwert	0.249	0.228	0.109
Toleranzbereich des Einzelwertes	0.0049	0.0052	0.0025
In % vom Mittelwert	0.787	0.721	0.346

TABELLE III

ÜBERPRÜFUNG DER ANGEWANDTEN METHODE

Es wurden jeweils 40 μl von drei verschieden konzentrierten Lösungen zugegeben.

Spritzen	*Konzentration* (%)	*Extinktionen*	*Mittelwert*
1–3	90	0.750, 0.749, 0.748	0.749
4–6	95	0.790, 0.790, 0.790	0.790
7–9	100	0.831, 0.837, 0.833	0.834

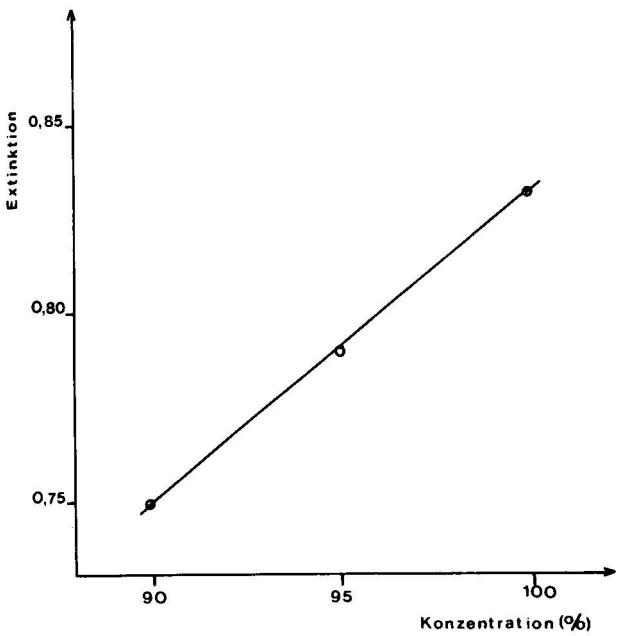

Fig. 5. Überprüfung des Analyseverfahrens, nach dem die Dosierungsgenauigkeit des Auftragegerätes bestimmt wurde.

Einfluss vorgekühlter Platten auf die quantitative Auswertung

In vielen Fällen hat es sich für die quantitative Auswertung als günstig erwiesen, die DC-Platte vor der Entwicklung zu kühlen. Bringt man die vorgekühlte Platte (10 min bei 4°) in die gesättigte Normalkammer, dann schlagen sich sofort im Gasraum befindliche Fliessmittelmoleküle auf der kalten Schicht nieder. Da sich die Platte schnell auf Zimmertemperatur erwärmt, führt diese Vorbeladung maximal bis zur sorptiven Sättigung der Schicht. Für die quantitative Auswertung ist dabei sehr wichtig, dass man durch eine derartige Behandlung der Platte in vielen Fällen wesentlich homogener verteilte Flecken erhält und eine Schwanzbildung weitgehend unterbindet. Diese Verbesserung wurde in den verschiedensten Fliessmittelsystemen beobachtet. Die Hauptursache liegt wohl in einer gleichmässigen Vorbeladung bzw. Desaktivierung der Schicht.

Nach der Standardisierung der Auftragsbedingungen hat man durch diese Vorbeladung die Möglichkeit, die Entwicklung der Platten weitgehend reproduzierbar zu gestalten.

Statistische Beurteilung des Analysenverfahrens

Unter Berücksichtigung der beschriebenen Bedingungen liegt der Variationskoeffizient für diese Analysenmethode zwischen 0.8 und 3.5%.

DANK

Den Herren J. Ackelbein, H.-J. Dentler und E. Schwortschik sei an dieser Stelle für viele wertvolle Beiträge zu dieser Arbeit gedankt.

ZUSAMMENFASSUNG

Mit Hilfe des Lambert–Beerschen Gesetzes erhält man für Transmissionsmessungen in der quantitativen Dünnschichtchromatographie Eichgeraden nach der Funktion $y=ax$, wenn man eine Übersättigung der zu messenden Flecke vermeidet. Einmal ermittelte optimale Bedingungen für den Startfleck können mit einem automatisch arbeitenden Auftragsgerät jederzeit reproduziert werden. Es ist möglich, bis zu neun Proben parallel in kurzer Zeit aufzubringen. Für den Startfleck kann man jeden beliebigen Durchmesser vorwählen. Die Eichgeraden nach Lambert–Beer und das Auftragegerät erlauben eine rationelle Anwendung der quantitativen Dünnschichtchromatographie. Der Variationskoeffizient für diese Methode liegt dabei zwischen 0.8 und 3.5%.

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CHARGE TRANSFER THIN-LAYER CHROMATOGRAPHY AND MULTIPLE SITE COMPLEXATION

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SUMMARY

The effect of impregnating silica gel with different types of acceptors on the separation of different aromatic hydrocarbons by thin-layer chromatography has been investigated. Benzoquinone and substituted benzoquinones were studied in detail, and it was found that benzoquinone was most sensitive to moderate differences in the structure of most aromatic hydrocarbons.

Benzoquinone-impregnated silica gel thin-layer chromatographic R_F values were measured for a number of aromatic hydrocarbons, and the "binding constant" of Harvey and Halonen calculated for these hydrocarbons. The question of multiple site complexation between the aromatic hydrocarbon and either the silica gel surface or the benzoquinone was studied extensively. The R_F values for the biphenyl–terphenyl–quaterphenyl series appears to give some evidence for some type of multiple site binding. A novel method of locating spots on impregnated silica gel adsorbent has also been devised. It involves spraying with a dilute naphthacene solution; the yellow fluorescence of naphthacene is quenched everywhere except on the spots.

INTRODUCTION

Electron donor–acceptor complexes, or pi complexes, have been used extensively in various types[1] of chromatography. The complexes are considered to be formed from a pi acceptor, or pi acid, and a pi donor, or pi base. A requirement for a good acceptor in chromatography is that there must be more than one strong electronegative or electron-withdrawing group attached to the carbon atoms of the pi bond. The main requirements of donor molecules for chromatographic use are that steric hindrance and electron-withdrawing groups should be minimal.

Morris[2] and Barrett *et al.*[3] independently introduced the impregnation of the adsorbent into adsorption chromatography with the thought that a chemical could react selectively with a particular constituent or a functional group that might be present in the sample. Morris and Barrett *et al.* impregnated the adsorbent with silver(I) ions to effect the separation of *cis* and *trans* isomers of olefins.

Harvey and Halonen[4] and Berg and Lam[14], formalized the term "charge

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transfer chromatography" in applying the formation of pi complexation to thin-layer chromatographic (TLC) separations. They predicted that the migration of aromatic hydrocarbons over impregnated silica gel adsorbent could be selectively retarded to a degree depending on the strength of the charge transfer (CT) complex formed. They did in fact observe that when a CT complex is formed, migration of the donor is usually retarded on TLC plates. If the donor molecule does not migrate, it is assumed that the CT complex it forms is so stable that the solvent interactions are too weak to facilitate migration. None of these workers made any attempt to interpret or study situations where the R_F values increase. We believe that increasing the number of acceptor molecules on the silica gel plate reduces the total number of available hydroxyl sites per square area. Therefore in the case where the donor molecule will interact more with the hydroxyl sites than with the acceptor, the adsorption will be decreased and the R_F values will be increased.

To evaluate the effect of impregnation of adsorbents, Harvey and Halonen[4] proposed the so-called binding constant B, defined as:

$$B = \frac{R_F - R'_F}{R_F} \times 100$$

where R_F is the value obtained on non-impregnated silica gel and R'_F is the value obtained on impregnated silica gel for the same donor molecule. The binding constant should reflect the strength of the charge transfer complex formed by a donor on a particular adsorbent impregnated with a particular acceptor. A comparison of the binding constants of a series of compounds should reveal relative strengths with which the molecules bond to the surface at one or more sites.

The value of the binding constant can be positive, negative, or zero. A positive value for the binding constant represents the formation of CT complexes. Large positive values signify strong CT complexes, which will be reflected by small R_F values on impregnated plates. A value of zero for a binding constant means that the migration is unaffected by the presence of the impregnator in the plate. Negative values for the binding constant signify that the migration rate is increased for the impregnated plate as compared to non-impregnated plates. Presumably, the acceptor is blocking enough Si–OH sites to reduce the hydrogen bonding of the donor to these sites; the complex formed by the acceptor is too weak to compensate for this binding.

It is thought that the aromatic hydrocarbons are adsorbed to the silica gel surface by orienting their pi electrons to the surface silanol groups. Hydrogen bonds then can form between the silanol groups and the pi electron cloud, thus forming a weak CT complex. Snyder[5] has stated that the amount of adsorption does not depend directly on the energy of the highest filled π orbitals of the donor molecules as would be expected.

Snyder[5,6] has made several contributions to the understanding of TLC separations on silica gel which are useful for understanding separations on impregnated silica gel. He has proposed[6] that the "reactive" hydroxyl sites are the preferred sites for complexation of the aromatic hydrocarbon, since the approach of the hydrocarbon can break the hydrogen bond between the two hydroxyl groups at each site, allowing for several hydroxyl groups to bond to different orbitals of the same molecule. This would have to involve some multisite bonding. He has also

formulated[5] a method for calculating the effective molecular areas by defining benzene as having a base value of six units, which equals 51 Å^2. The molecular areas for related unsaturated hydrocarbons of the formula C_cH_h is calculated by using the formula:

$$\text{Molecular area } (\text{Å}^2) = 6 + 0.8(h-6) + 0.25(c-h) \quad (1)$$

Also, the planarity of the aromatic hydrocarbon governs the degree of adsorption. Planar molecules tend to be adsorbed more strongly while non-planar molecules are less adsorbed. This can be rationalized by considering that for adsorption the pi cloud of the donor molecule must achieve a specific orientational distance. Planar molecules can approach bonding sites easier while donors that have some degree of steric hindrance theoretically cannot orientate their entire pi electron cloud to the adsorbent.

EXPERIMENTAL

Reagents

All aromatic hydrocarbons used were purified by sublimation, except for bibenzyl, which was recrystallized. Their melting points were verified by checking against values tabulated in ref. 7. The stock solutions used in spotting the thin-layer plates were made up as 0.02 *M* solutions in benzene except: naphthalene 0.2 *M*; biphenyl, 0.2 *M*, and napthacene 0.002 *M*.

Of the pi acceptors used, *p*-benzoquinone was sublimed; bromanil and chloranil were recrystallized from chloroform, and 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) was recrystallized from ethylene dichloride. The benzene used in the eluent was an ACS-grade solvent; the heptane was solvent grade.

The adsorbent used was either MC and B plain silica gel G (TLC, Stahl) or silica gel treated with an inert fluorescent material that fluoresces green under short-wave UV light (254 nm). A slurry was made with benzene–95% ethanol (92:8) mixed in the ratio of 40 g silica gel to 100 ml solvent. Ethanol was mixed with benzene to improve the solubility of the acceptor when making impregnated plates, and also to increase the binding of the adsorbent on the glass. A slurry made with pure benzene was found to bind very poorly. When impregnated plates were prepared, 5.40×10^{-3} moles of the acceptor were dissolved in 100 ml of the solvent with stirring and added to 40 g of powdered silica gel.

Apparatus and preparation of TLC plates

The glass plates (20 cm × 10 cm) were cleaned by washing with soap and water and then soaking in chromic acid cleaning solution. After a minimum of 4-h soaking, the plates were rinsed with distilled water and dried at 110°. Before the plates were processed, they were wiped with 95% ethanol to remove any grease contaminants.

The Camag applicator (Mutter-Hofstetter design made for 20 cm × 10 cm plates) was used to spread the slurry (Camag, Muttenz, Switzerland). The screw settings on the front exit plate of the applicator were adjusted to 250 μ for uniform adsorbent thickness. This adjustment was checked with a 250-μ spacer before each series of plates were made.

The prepared plates were stored on 20 cm×20 cm aluminum trays which could be stacked one on top of another. It was found that 14 to 18 plates could be made from the slurry of 100 ml of solvent and 40 g of silica gel G.

The plates were then covered with aluminum foil and stored at room temperature (about 26°) overnight. Any plates that would be used after a 24-h time period were stored in a desiccator. There was no activation of any plates because the *p*-benzoquinone impregnant will decompose at elevated temperatures.

Spot detection was made by: (1) use of an UV view box (Chromato-View; Ultra-violet Products, Inc., San Gabriel, Calif., U.S.A.) capable of producing both long-wave (365 nm) and short-wave (254 nm) mercury lines, or (2) by spraying the plate with a solution of naphthacene (0.05 *M* in benzene) or a 0.002 *M* solution of perylene in benzene. The sprayer used was a Chromatosprayer with Jet-pak made by Scientific Manufacturing Industries, Emeryville, Calif., U.S.A.

Experimental procedures

The spotting and development for both impregnated and non-impregnated plates was the same. The solutions of aromatic hydrocarbons were applied with micropipets, using a spotting guide. The volume of liquid applied to the spot was determined by the intensity of fluorescence emission of the aromatic hydrocarbon. For very strong emitters the spot was 2 μl, while for very weakly fluorescent aromatics the load was increased to 25 μl. In the case of naphthalene and biphenyl, new stock solutions were made up at 0.2 *M* in benzene and the liquid load was reduced to 5 μl.

Before development, the development tank was lined with filter paper on three sides to ensure tank saturation, and filled with eluent to a depth of about 10 mm. Spotted TLC plates were carefully placed in the tank and allowed to elute until the solvent front had moved exactly 10 cm from the start line.

Spot detection was performed on dried plates in the UV view box. Most aromatic hydrocarbons are fluorescent and can be detected on non-fluorescent silica gel. The relative fluorescence intensities of the hydrocarbons used in this study, using excitation by the 254-nm line of the mercury source, range from zero for azulene, to medium for naphthalene, and to very high for chrysene and anthracene. The corresponding relative intensities using excitation by the 365-nm mercury line range from very low for naphthalene, biphenyl, azulene, triphenylene and *p*-terphenyl, to medium for phenanthrene, pyrene and chrysene, and to high for anthracene and its derivatives. This means that both the exciting wavelength and the detection limit vary with different hydrocarbons. The short-wave mercury line can be used to excite all of the hydrocarbons except azulene, while the long-wave mercury line is only effective for phenanthrene, anthracene, chrysene, and pyrene.

Detection on non-impregnated plates did not present any problems once the liquid load was determined. The plates impregnated with *p*-benzoquinone did present problems. These impregnated plates have a light yellow-brown color which hindered detection of many of the aromatics which fluoresce violet against the background of the non-impregnated silica gel G. Therefore, the problem of detection of the aromatics was two-fold on the impregnated thin-layer plates: first, the problem of the light yellow-brown background, and secondly, the charge transfer interactions.

To overcome these problems, the *p*-benzoquinone-impregnated plate was sprayed with a 0.05 *M* naphthacene solution when the aromatic hydrocarbons did not fluoresce. After spraying with naphthacene (or perylene), the dull yellow background of the plate disappeared after 5 min, and only the spots containing the aromatics fluoresced yellow under UV radiation. (The yellow fluorescence of naphthacene in these spots was not quenched by the benzoquinone because the latter was complexed by the aromatic hydrocarbon in the spot.)

R_F values were measured as usual, but to insure reliable values, each R_F value was measured fifty times on both impregnated and non-impregnated plates. The maximum standard deviation was 1×10^{-2}.

RESULTS AND CONCLUSIONS

Evaluation of acceptors

The state of the acceptor in an impregnated silica gel layer is difficult to describe. It is not in a crystalline state since the acceptor molecules are not close enough together, yet it is not in solution. Since the impregnated silica is a homogeneous mixture, and since it is assumed that the mobile phase does not appreciably disturb the molecular arrangement of the acceptor and silica, it is probably more realistic to consider the acceptor as in a semi-crystalline state.

The acceptor will be bonded weakly to the silica gel since it blocks some "free" hydroxyl sites. It is likely that there will be some hydrogen bonding between the hydroxyl sites and the acceptor carbonyl groups. The extent of this depends on the molecular structure of the acceptor.

It has been shown[8] that *p*-benzoquinone molecules are planar and in crystals are arranged in rows at almost right angles to one another, with the minimum inter-oxygen distance being about 3.6 Å and the minimum intermolecular carbonyl carbon–oxygen distance being about 3.4 Å. There appears to be little intermolecular hydrogen bonding.

Both chloranil and bromanil have been shown[9] to be non-planar in their crystalline form. The carbon atoms are in one plane, but the oxygen and halogen atoms protrude alternately above and below the plane of the ring. This is not the conventional "chair" form, as the carbon atoms are coplanar.

It does not appear that the crystal structure of DDQ has been determined. However, 2,5-dichloro-*p*-benzoquinone, 2,5-dibromo-*p*-benzoquinone, and 2,5-bromochloro-*p*-benzoquinone have all been shown[10] to be planar, yet 2,3-dichloro-*p*-benzoquinone[10] and chloranil[9] have been shown to be non-planar. This would indicate that the adjacent chlorine atoms are responsible for non-planarity, and it can logically be assumed that DDQ will also be non-planar. The nitrile groups will not sterically interfere with each other or with the carbonyl oxygens, so it can be assumed that they will not contribute to the non-planarity of the molecule.

A number of different benzoquinone-type acceptors were evaluated with regard to their structural effects on TLC separations. Some representative R_F values are listed in Table I. Unsubstituted *p*-benzoquinone itself proved to be of prime importance because it was so sensitive to moderate structural changes. It at first appeared to have no effect on the R_F value of biphenyl, yet it lowered the

TABLE I

EFFECT OF ACCEPTOR STRUCTURE ON R_F VALUES ON IMPREGNATED SILICA GEL

Solvent: benzene–heptane (1:4).

Acceptor	*R_F value*		
	Biphenyl	*Naphthalene*	*Anthracene*
None	0.35	0.40	0.33
Benzoquinone	0.35	0.34	0.27
Choranil	0.37	0.40	0.32
Bromanil	0.36	0.38	0.33
DDQ	0.32	0.36	0.24
Trinitrobenzene*	0.31	0.27	0.24

* Calculated for our conditions using B values of Harvey and Halonen[4].

R_F value of naphthalene, which also has two benzene rings. It also lowered the R_F value of anthracene.

Two tetrahalo-substituted benzoquinones were also evaluated, *viz.* 2,3,5,6-tetrachlorobenzoquinone (chloranil) and 2,3,5,6-tetrabromobenzoquinone (bromanil). In both of these, the steric hindrance of the halo substituents was too pronounced to provide much of a difference in R_F values. The minimum possible interplanar distance for chloranil complexes is 3.65 Å and that for bromanil complexes is 3.80 Å. The extent of complexation is small because these distances are less than normally found in pi complexes.

DDQ has two chlorine atoms on one side of the molecule and comparatively small nitrile groups on the other. Since only one side of the molecule might block the approach of a hydrocarbon, one can expect DDQ to be able to complex better with a hydrocarbon than either chloranil or bromanil. As can be seen in Table I, DDQ retards hydrocarbons more than any of the other acceptors. It appears that its high pi acidity relative to benzoquinone is more than enough to overcome its greater steric hindrance (compared to benzoquinone). In spite of this, we decided to investigate benzoquinone in detail because it was more sensitive to moderate structural differences among various aromatic hydrocarbons.

Study of benzoquinone-impregnated TLC plates

Table II shows the donor molecules studied. Each R_F value reported is the mean value of a population of fifty spots. The maximum standard deviation was 1×10^{-2} R_F units. All impregnation R_F values were with the silica gel impregnated with *p*-benzoquinone. Effective molecular areas (Å^2) were calculated according to Snyder's[5] method. Binding constants were calculated according to Harvey and Halonen[4].

In evaluating the R_F values of the non-impregnated silica gel, it must be remembered that these values depend on the degree to which the aromatics are adsorbed on the silica gel. Therefore, it is important to consider the planarity of the donors concerned and their effective molecular areas.

TABLE II

R_F VALUES OF AROMATIC HYDROCARBONS

Solvent: benzene–heptane (1:4).

Aromatic hydrocarbon (Area in Å²)	*R_F value*		*B*
	Plain silica gel	*Impregnated silica gel*	
C_6H_5-R-C_6H_5 series			
Biphenyl (83)	0.39	0.44	−13
Diphenylmethane (94*)	0.36	0.36	0
Bibenzyl (100**)	0.40	0.42	− 5
Long ring series			
9-Methylanthracene (94)	0.47	0.32	32
9,10-Dimethylanthracene (100)	0.44	0.32	27
Naphthalene (69)	0.45	0.42	7
Anthracene (87)	0.36	0.32	11
Naphthacene (105)	0.00	0.00	0
Phenyl substitution series			
Triphenylmethane (159)	0.27	0.25	7
trans-1,2-Diphenylethene (114)	0.34	0.32	6
Triphenylethene (167)	0.29	0.28	3
Tetraphenylethene (218)	0.20	0.23	−15
1,4-Diphenyl-1,3-butadiene (122)	0.30	0.34	−13
1,6-Diphenylhexatriene (132)	0.26	0.26	0

* Diphenylmethane is not planar and models indicate its area is less than that of two benzene rings; this area is an estimate using eqn. (1).

** Bibenzyl is almost planar and models indicate its area is almost the same as two benzene rings laid end to end.

In considering the planarity of the donor molecules, it is reasonable to assume that the more planar the molecule, the more easily it will be adsorbed on the surface of the silica gel at the "free" hydroxyl sites. As stated earlier, the "free" hydroxyl sites are slightly mobile, which will make them more available to bond to slightly planar donors and to at least a small extent to non-planar donors. In the case of non-planar molecules, the hydroxyl sites may not always be able to extend themselves to the degree needed for adsorption, which will result in high R_F values and very little adsorption.

The effective molecular area of the donor molecule will indicate an approximate maximum number of "free" hydroxyl sites available for adsorption. Utilizing Snyder's[5] calculated value of four to five hydroxyl sites per 100 Å² and the effective molecular area of the donor, a general evaluation of the R_F value can be made. The larger the effective molecular area representing the donor molecule, the greater the possibility for adsorption to take place.

In the first series of biphenyl, diphenylmethane, and bibenzyl in Table II, there seems to be no effect in increasing the carbon linkage. Actually, the increase in the effective molecular area is not large enough to increase the maximum number of "free" hydroxyl sites available to the donor molecule. Complicating the situation is the fact that neither biphenyl nor diphenylmethane are completely planar. The

two rings of biphenyl are twisted[11] about 23° and models indicate that the two rings of diphenylmethane are not coplanar either. It is possible that at any given time one hydroxyl site can bond strongly to the benzene ring that is oriented completely parallel to the silica gel surface, and that a second hydroxyl site then bonds weakly to the other, non-planar benzene ring. (In any given 100 Å^2 covered by one of these molecules, there are four to five hydroxyl sites available for such bonding.)

When silica gel is impregnated by the amount of benzoquinone used in our experiments, about half of the surface is covered by benzoquinone. The negative B values in Table II therefore reflect an interaction with benzoquinone that is weaker than that with the silica gel hydroxyl sites. There is no increase in aromaticity in this case because the carbon linkage which has been added is a saturated linkage. The strength of the pi complex formed does not increase because there is no increase in the conjugation of this series.

In the remaining two series (Table II), there is a definite increase in the degree of adsorption. This additional increase can be attributed to both the increase in the number of available sites and the increase in aromaticity. The increase in aromaticity is the more important factor. With the expansion of the pi electron cloud, there are additional site(s) at which a pi complex may form. Therefore, with the additional pi complexation, and with the increase in the number of available hydroxyl sites, it is reasonable to assume that the degree of adsorption should increase.

In the large ring series of naphthalene–anthracene–naphthacene, the R_F values for impregnated silica gel decrease while the binding constants do show some increase. There is a slight increase in adsorption of the unsubstituted aromatics on impregnated silica gel. (In the case of naphthacene there is no migration on either the non-impregnated plate or the impregnated plate under the solvent conditions stated in the experimental section.) In contrast, there is a large increase in the adsorption of 9-methyl- and 9,10-dimethylanthracene on impregnated silica gel (large B values). Since it can be calculated that there are two to three hydroxyl sites and one benzoquinone per 100 Å^2, it is obvious that the methyl-substituted anthracenes are complexing with benzoquinone far more strongly than anthracene itself.

Our R_F values are in general much lower than those reported by Harvey and Halonen[4] for naphthalene, anthracene, and 9-methylanthracene on silica gel alone. We believe this reflects the difference in the activation used: they activated their plates by heating at 60° or 100° whereas our plates were allowed to air dry at room temperature. Our B values for benzoquinone are also much lower than their[4] B values for trinitrobenzene. This reflects the fact that trinitrobenzene forms stronger pi complexes than benzoquinone.

At a quick glance, the phenyl substitution series does not seem to exhibit any consistent trend. It is true that one of the main differences in the compounds of this series is the length of carbon linkage between the phenyl rings. But, with a closer examination of the series, it will be recognized that this series is composed of two different types of compounds. The first type is compounds in which only one phenyl ring can be parallel to the surface of the silica gel. Both triphenylmethane and tetraphenylethene fall into this category. Diphenylmethane could be considered

to fall into this class as well. All of these compounds are sterically oriented so that only one phenyl ring is able to be completely parallel to the silica surface. It would be expected that these compounds would complex very weakly with benzoquinone on the surface of the silica gel. However, they can complex moderately well with the hydroxyl sites on the silica gel because this type of interaction probably does not require a completely parallel orientation. Thus these compounds have low R_F values but small or negative B values.

In contrast to these compounds, triphenylethene, diphenylbutadiene, and diphenylhexatriene can orient themselves so that at least two phenyl rings are parallel to the silica gel surface. Triphenylethene ought to complex more with benzoquinone than tetraphenylethene and indeed its B value is larger. Diphenylbutadiene and diphenylhexatriene also ought to complex with benzoquinone strongly, but for some reason their B values are not positive. It may be that both compounds strongly prefer to complex with the hydroxyl sites for some electronic reason.

The possibility of multiple site complexation

Although they studied rather large ring systems, Harvey and Halonen[4] did not consider the possibility that aromatic hydrocarbons might interact at more than one bonding site on either plain silica gel or impregnated silica gel. Snyder[12] does picture adsorption of bibenzyl on two SiOH sites on plain silica gel; he also implies that naphthalene (two fused rings) is not large enough to overlap two SiOH sites. It occurred to us that the polyphenyl series might provide a useful test of whether multiple site complexation was occurring (Table III).

In referring to the molecular models of this series, it will become apparent that this series consists of molecules which are not quite planar. The two benzene rings of biphenyl, the first member of the series, are not quite coplanar. One ring is twisted an approximate angle[11] of 23° to the other phenyl ring, and this spatial orientation will decrease the effective molecular area of the molecule. (Snyder's

TABLE III

BEHAVIOR OF POLYPHENYL HYDROCARBONS

Solvent: benzene–heptane (1:4); impregnation with benzoquinone.

Polyphenyl (Area in Å²) *	*No. of SiOH sites per area* **	*R_F Value* ***		*B*
		Plain silica gel	*Impregnated silica gel*	
Fluorene (85) ("rigid biphenyl")	3 + to 4	0.34	0.30	12
Biphenyl (83)	3 + to 4	0.39	0.44	−13
p-Terphenyl (114)	4 + to 6	0.28	0.27	4
p-Quaterphenyl (155)	6 to 8	0.25	0.17	32

* This area is relative to a base value of 51 Å² for benzene.

** This is based on an estimate of four to five sites per 100 Å².

*** Each R_F value is the mean of fifty measurements, with a maximum standard deviation of ±0.01 R_F units.

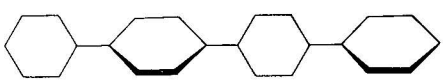
effective molecular area calculation does not take into consideration the spatial geometry of the molecule but only the empirical formula.) This may reduce the interaction of biphenyl with the hydroxyl sites because of the lower probability of a given hydroxyl site interacting with the non-planar phenyl ring. Similar problems are involved with the interaction of *p*-terphenyl and *p*-quaterphenyl with the SiOH sites; these will be discussed later.

Plain silica gel. The first question is whether there are multiple site interactions between any of the polyphenyls and plain silica gel. One notes that the R_F value of biphenyl is lower than that of naphthalene (Table II), which cannot undergo multiple site bonding[12] and which is a stronger pi donor in general. Fluorene, whose structure approaches that of "rigid biphenyl", has an R_F value about 10% lower than that of biphenyl (Table III). In contrast, it is 4.5 times stronger a donor towards tetracyanoethylene[13], which complexes only one ring in solution (equivalent to a one-site interaction on a TLC plate). It is thus possible that one ring of biphenyl is interacting strongly with a hydroxyl site, and the other (twisted) ring is interacting weakly with a second hydroxyl site. It can be postulated that the reason fluorene does not interact more strongly with the silica gel than it does relative to biphenyl is that it is only interacting more strongly than biphenyl with the second hydroxyl site.

The multiple site hypothesis also accommodates the R_F values of *p*-terphenyl and *p*-quaterphenyl on plain silica gel, if the most favorable orientations of the twisted rings are assumed to predominate. In the most favorable orientation of terphenyl, it can orient its two outer rings in a plane parallel to the silica gel surface, while the center, twisted, ring will be at an approximate angle of at least 23° to the surface:

(parallel) (twisted) (parallel)

(This orientation allows for greater interaction with the silica gel surface than the situation where only one ring can have a parallel orientation.) In this orientation, it is possible for *p*-terphenyl to interact strongly with two hydroxyl sites and weakly with one hydroxyl site. If this occurs, it would rationalize the R_F of *p*-terphenyl being lower than either that of fluorene or biphenyl. This again contrasts with the fact that fluorene is 1.5 times a stronger donor towards tetracyanoethylene than is *p*-terphenyl[13]. Tetracyanoethylene complexes over one ring of any of these three donors, and this implies that more than one ring per donor must be interacting with the silica gel. In either of the most favorable orientations of *p*-quaterphenyl, there will always be two rings oriented in a plane parallel to the silica gel surface and two twisted rings. One of these is:



(parallel) (twisted) (parallel) (twisted)

In this orientation, it is possible for *p*-quaterphenyl to interact strongly with two hydroxyl sites and weakly with two hydroxyl sites. Its R_F value is thus not much lower than that of *p*-terphenyl because it is interacting via only one more twisted ring.

Although the multiple site concept is only a hypothesis at this point, it does appear to accommodate the R_F measurements on plain silica gel. The hypothesis will now be tested with the data on silica gel impregnated with *p*-benzoquinone.

Impregnated silica gel. It should be recalled that about half of the surface of the impregnated silica gel is covered by *p*-benzoquinone molecules; this also probably blocks half of the available hydroxyl sites listed in Table III. If fluorene and biphenyl are again compared, it appears that fluorene is interacting more strongly with benzoquinone than with the hydroxyl sites, whereas biphenyl is not. It is likely that only one benzoquinone complexes with fluorene; it is possible that a hydroxyl site can complex with fluorene at the same time, but it is not necessary since fluorene complexes so strongly with acceptors like benzoquinone. In the case of biphenyl, the negative B value indicates that there is little if any interaction between the *p*-benzoquinone and this aromatic hydrocarbon. It appears that this molecule interacts more strongly with two hydroxyl sites than with one molecule of impregnant. Therefore, because of the decrease in the number of "free" hydroxyl sites per 100 Å^2, the R_F value shows an increase as compared to that on a non-impregnated plate.

The results (Table III) for *p*-terphenyl and *p*-quaterphenyl at first glance do not appear to conform to the multiple site hypothesis. However, there is only one benzoquinone molecule per 100 Å^2. This limits *p*-terphenyl to interacting with one benzoquinone at a time whereas *p*-quaterphenyl may interact with two benzoquinones at a time. Thus *p*-terphenyl may interact simultaneously with one benzoquinone and one hydroxyl site whereas *p*-quaterphenyl may simultaneously interact with two benzoquinones. It would be difficult to rationalize the dramatic 30% decrease from 0.25 to 0.17 R_F units for *p*-quaterphenyl without involving some sort of multiple site hypothesis. The small decrease in the R_F value for *p*-terphenyl may be rationalized by recalling that it does not bond strongly in solution to "one-ring" acceptors like tetracyanoethylene[13]. It is only about 2.5 times as strong a donor towards tetracyanoethylene as is biphenyl so that a small decrease in R_F value on impregnated silica gel is not unreasonable.

At this point in time, the multiple site hypothesis appears to be reasonable in that it rationalizes R_F trends in the biphenyl–terphenyl–quaterphenyl series. The hypothesis needs further testing but would appear to be useful in predicting TLC separations.

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PROGRAMS FOR USE WITH THE AUTOMATIC AMINO ACID ANALYSER TO IDENTIFY, COMPUTE AND CORRELATE AMINO ACID CONCENTRATIONS IN BIOLOGICAL SAMPLES

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SUMMARY

A series of computer programs is presented that enable chromatograms obtained from different machines run under a variety of analytical conditions to be processed. Many chromatograms may be examined per run to establish the identity of the amino acids and determine their concentrations. Using tissue analysis as an example, those chromatograms obtained from the same specimen but passed through different columns are combined to provide a profile for that tissue. Tissues are further grouped into sets of experiments which are jointly compared, and from the results, questions are formulated to aid in the elucidation of the biochemical changes occurring in tissues under various conditions.

INTRODUCTION

The rate of acquisition of data from such machines as automatic amino acid analysers can at times prove to be an embarrassment unless adequate facilities are available to process this information. Partial solutions to this imbalance in operations are available which, though an improvement, have not eliminated the race with the machine that incompletely resolved data promotes.

The integration of the chromatogram curve is one of those problems that has attracted some interest and for this a solution is to be found using an electronic integrator or a computer program[1,2]. Association of the peaks in the chromatogram to amino acids is another requirement and this has been accomplished to a limited extent in the more modern analysers. Additionally, computer programs have been published[3,4] that compute the concentrations of those amino acids recognized. Present techniques are, however, insufficient for a variety of reasons. Some of these outstanding problems are when unknown or unexpected peaks occur, when a series of samples is run over a period of time during which the decay of the ninhydrin reagent becomes a significant factor and when the results from more than one chromatogram must be combined to determine the concentrations of amino acids present in a tissue. Furthermore, existing methods present the biochemist with a vast number of experimental findings measured in terms of amino acid concentrations. This and similar information often requires statistical analysis. To inhibit this growth of un-

digested information and to reunite the researcher with the significance of his experiments, a set of computer programs that embrace something of the total experimental environment in which amino acids are involved has been written.

COMPUTER PROGRAMS

Dividing the system into sections, the first deals with the identification of amino acids contained within the chromatogram and the computation of their concentrations. The second is concerned with the grouping of chromatograms derived from different samples of the same tissue. The third program groups similar tissues subject to like treatments into experimental sets and thence performs statistical comparisons between experiments. The fourth program examines the differences between these experiments and allows formulation and solution of pertinent questions.

The programs have been designed so that they may be used together or as individual modules. When used as a set the amount of data is reduced at each stage with a corresponding increase in the information derived. Thus, many experiments using different types of machines or even other information can be combined at different stages within the information flow. The programs, though complex in character, are simple to run, each providing a full description of the tasks performed together with any errors or inconsistencies that are found.

A chromatogram is here defined as a set of pairs of numbers, the retention time and the integrated optical density time function (referred to as counts). The column through which the liquid carrying the amino acid mixture is passed is referred to as a physical column. Since the system caters to samples in which the identity and number of amino acids may be only partially known and as these amino acids are to be identified with respect to standards, the concept of a higher order standard or a theoretical standard is introduced. This has three functions, primarily it acts as a consistency check on the standard being run and secondly it allows the representation of the column in the program to be tuned to a particular amino acid eluted at a time when more than one amino acid may be eluted. The third use for this concept is to afford a mechanism that accounts for phenomena peculiar to a particular run, for example, time dilation which is discussed later. To each such column is associated a set of times corresponding to all the peaks that this type of column expects whether they be generated by amino acids or other substances. It is this set of values that is the theoretical standard, though its establishment may well be by experiment. Currently the program contains seven such columns. It is immaterial how the columns are distributed between machines. There may be seven different machines with identical columns or one machine with seven different columns and so forth. Since the first program can accept chromatographic data from several sources using a variety of columns, each unit of information, which itself may be a set, is a member of a set. Comprehension of the first program may be enhanced by thinking of each set as a singleton.

Normally amino acid analyses are run sequentially, that is, a standard is passed through a particular column followed by a series of samples and finally a re-run of the original standard. This series of runs is referred to as a chromatographic partial set. The set becomes complete when all the amino acids in the samples can be identified, which may require the inclusion of additional supplemental standards. The program

can conveniently accommodate 100 chromatograms per run, each of which may contain up to 30 amino acids. There is no restriction on the number of complete sets that can be constructed from these 100 chromatograms. The only restriction (for age correction) is that a sequential enumeration of the sample chromatograms (allowing gaps) is required. Since many different complete sets can be accepted, there is no restriction in the order in which the chromatograms are presented to the computer. Furthermore, the same physical column can be used with many such sets. A collection of concentration sets, each member of which contains acceptable amino acid names, together with the concentrations of these amino acids is also required.

Idiosyncratic behavior, as well as intentional manipulation, of the automatic analyser is accommodated by allowing for correction of such things as baseline shift, time drift and count changes. These are applied on a piece-wise basis by specifying the chromatogram, the type of correction and the time at which it is to be applied. The phenomenon of time dilation, that is stretching of the time axis of the chromatogram, induced by different concentrations of amino acids, can be accounted for either by the range that an amino acid is expected to be eluted or by functionally changing the theoretical standard. This function will generally be the addition of a constant to each time after a particular time (that is, after the elution of a particular amino acid) or by the addition of an element that itself increases linearly with time. Although a complete history is kept of the manipulations applied to each chromatogram, it is preferable to adjust the theoretical standard rather than each individual chromatogram. It is then the interpretation that is placed, by the investigator, on the retention times which is changed and not the retention times of the samples themselves.

Trimming or filtering is afforded to standards, this normally consists of removal of the tail and spurious peaks from the chromatogram. Such filtering is not extended to samples for fear of systematically excluding amino acids present in small quantities.

EXPERIMENTAL AND RESULTS

The capabilities of the system are best described by an example and except for omitting the numerous error messages, all the manipulations extended to a particular tissue will now be described in terms of chromatogram 339. This and similar samples were passed through a 'long column' (denoted by DC1ANA3) using a sodium buffer. Chromatogram 339 contained six peaks not present in the standard. A series of partial standards was therefore sequentially analysed to determine the identities of these unknown amino acids. The chromatograms used for this identification are shown in Fig. 1 as numbers 337, 350, 464, 697 and 933. Whether these additional compounds are run as individual chromatograms or together is immaterial to the program. Indeed, these numbers indicate that they were run well after the sample. These chromatograms together with any between numbers 338 and 361 constitute a complete chromatographic set. Fig. 1 displays this information, the prime or non-augmented standards are shown as a set of retention times and counts without the amino acid names. This is because, at this point in the processing, the retention times and their corresponding names have not been confirmed by the theoretical standard. The list of concentrations for all the amino acids contained

```
FOR STANDARD TYPE DC1ANA3D THE FOLLOWING CHROMATOGRAMS HAVE BEEN USED
STANDARDS    336   362   337   350   464   697   933
SAMPLES  338 TO  361
NOISE FILTERED CHROMATOGRAM NUMBER  336
            TIME      COUNT

              22      410578
              28      223490
              32      357837
              40      414170
              48      414462
              51      437341
              54      432823
              63       69808
              69      140078
              74      410041
              77      410583
              83      428992
              89      414527
              93      439414
              97      426397
             109      414728
             116      410992
             123      421432
             129      486552
             149      361258
             190      271830
NOISE FILTERED CHROMATOGRAM NUMBER  362
              22      398414
              28      215398
              32      355955
              40      407718
              48      400360
              51      427981
              54      425288
              63       70316
              69      188950
              74      397798
              77      404814
              83      420149
              89      402990
              93      427240
              97      412808
             109      395570
             116      390155
             123      394051
             129      464292
             149      362470
             187      397942
```

Fig. 1. A complete chromatographic set together with the prime standards. At this stage each standard is unresolved and is represented by a set of pairs of retention times and counts.

within the composite standard (that is, prime standards plus additional standards) were found in concentration set D, hence the designation 'STANDARD TYPE DC1ANA3D'.

The pre- and post-chromatograms were first filtered and then compared for consistency. They must both have the same number of pairs and corresponding retention times and these must not differ by more than a fixed amount (3 min). The additional standards are then filtered and should they contain retention times not present in the main standard, these times and counts are included to form a composite standard (Fig. 2). The names of the amino acids contained in concentration set D were compared with the theoretical standard established for column DC1ANA3 and theoretical retention times were associated with these names (Fig. 3*). In the

* In Figs. 3–11 and in the text, the following non-standard abbreviations are used: CITRU= citrulline; $CYSO_3H$=cysteic acid; CYSTAT=cystathionine; ETAM=ethanolamine; GABA= γ-aminobutyric acid; GAM=glucosamine; G-P-E=glycerophosphoryl ethanolamine; HCAR= homocarnosine; O-P-E=phosphoethanolamine; TAU=taurine.

```
NOISE FILTERED CHROMATOGRAM NUMBER  337
          126       264869
          149        91865
RETENTION TIME 126 INSERTED INTO CHROMATOGRAM  336
NOISE FILTERED CHROMATOGRAM NUMBER  350
           59       201309
          149        38191
RETENTION TIME  59 INSERTED INTO CHROMATOGRAM  336
NOISE FILTERED CHROMATOGRAM NUMBER  464
          112       215836
          150        51560
          195       372079
RETENTION TIME 112 INSERTED INTO CHROMATOGRAM  336
NOISE FILTERED CHROMATOGRAM NUMBER  697
          106       232551
          152       189427
          196       190142
RETENTION TIME 106 INSERTED INTO CHROMATOGRAM  336
RETENTION TIME 152 INSERTED INTO CHROMATOGRAM  336
NOISE FILTERED CHROMATOGRAM NUMBER  933
           25        26277
RETENTION TIME  25 INSERTED INTO CHROMATOGRAM  336
```

```
THE FOLLOWING ARE THE REPORTED CONSTITUENTS FOR          THIS STANDARD
        AMINO ACID          CONCENTRATION
          CYSO3H                100.120
          O-P-E                 101.400
          TAU                   101.000
          ASP                   100.000
          THR                   100.000
          SER                   100.000
          GLU                   100.000
          PRO                   100.000
           -CYS                 100.000
          GLY                   100.000
          ALA                   100.000
          VAL                   100.000
          MET                   100.000
          ILE                   100.000
          LEU                   100.000
          TYR                   100.000
          PHE                   100.000
          HIS                   100.000
          LYS                   100.000
          NH3                   100.000
          ARG                   100.000
          CITRU                  50.120
          GAM                    52.440
          ORN                    59.030
          GABA                   50.000
          G-P-E                  46.300
```

Fig. 2. Additional standards. Counts of those times not occurring in the prime standard are merged with the prime standard.

Fig. 3. Concentration set D.

theoretical standard, the times 82, 83, and 84 min correspond to buffer change one, cysteic acid, and valine (not illustrated). This means that at around 83 min a change of buffer may be expected and either valine or cysteic acid can be expected. This column is tuned at this stage to recognize valine as the appropriate amino acid for this series by including a concentration for valine but excluding one for cysteic acid in the concentration set D (Fig. 3). The effect of this on the sample will be seen later. These theoretical times are now matched using closest neighbor, unique position, and bounded difference criteria with the retention times of the pre-composite standard, thereby producing a resolution between the names of the amino acids and the times at which this standard has established that they are eluted. It is seen that valine is expected at 83 min (Fig. 4). The retention times of the sample chromatogram are

RESOLUTION BETWEEN REPORTED CONSTITUENTS AND RETENTION TIME WITH ABSOLUTE STANDARD

AMINO ACID	TIME	COUNTS
CYSO3H	22	410578
G-P-E	25	26277
O-P-E	28	223490
TAU	32	357837
ASP	40	414170
THR	48	414462
SER	51	437341
GLU	54	432823
CITRU	59	201309
PRO	63	69808
-CYS	69	140078
GLY	74	410041
ALA	77	410583
VAL	83	428992
MET	89	414527
ILE	93	439414
LEU	97	426397
GABA	106	232551
TYR	109	414728
GAM	112	215836
PHE	116	410992
HIS	123	421432
ORN	126	264869
LYS	129	486552
NH3	149	361258
NH3	152	189427
ARG	190	271830

Fig. 4. Resolution between theoretical and first standard. This ensures that the standard run is acceptable.

in turn matched against the first or composite pre-standard (again using the closest neighbor, unique occupancy, and bounded difference criteria). If any two of these three comparisons yield the same name it is assumed that this name identifies that amino acid at this time. In sample 339 at 81 and 83 min, both standards recognize these as valine yet the theoretical standard recognizes these as buffer change one and cysteic acid (Fig. 5). These are alternate possibilities. But from other criteria it is known that the first valine is actually brought about by a buffer change while the second is the true valine. The mechanism of accepting the second valine over the

SAMPLE CHROMATOGRAM 339 FOUND

COMPARE 1ST STD	COMPARE LST STD	COMPARE ABS STD	RETENTION TIME	COUNT	CONCENTRATION
CYSO3H	CYSO3H	CYSO3H	23	8034	1.97
G-P-E	G-P-E	G-P-E	25	13881	24.46
O-P-E	O-P-E	O-P-E	28	164079	74.76
TAU	TAU	TAU	32	1153311	325.72
ASP	ASP	ASP	40	80419	19.45
THR	THR	THR	48	59311	14.37
SER	SER	SER	50	77386	17.74
GLU	GLU	GLU	53	115024	26.63
CITRU	CITRU	CITRU	59	2014	0.50
PRO	PRO	PRO	62	54150	77.50
GLY	GLY	GLY	74	1008981	246.92
ALA	ALA	ALA	77	264031	64.41
VAL	VAL	BCH1	81	13490	3.15
VAL	VAL	CYSTAT	83	19797	4.63
MET	MET	MET	89	3430	0.83
LEU	LEU	LEU	99	3893	0.92
		BCH2	102	5557	0.56
GABA	GABA	BCH3	104	7127	1.53
HIS	HIS	HIS	123	7170	1.71
ORN	ORN	ORN	126	4405	0.98
LYS	LYS	LYS	129	15567	3.22
NH3	NH3	ETAM	149	146657	40.58

Fig. 5. Sample chromatogram 339. The amino acids contained in this sample are recognized by comparison with the first, last and theoretical standard and their concentrations computed. BCH= buffer change.

first is deferred to the description of the next program. A similar situation occurs with GABA and buffer change three.

Using the enumeration number of the chromatogram as the independent variable, an interpolation between the count of the pre- and post-standard is made for each identified amino acid and from this the concentration of the amino acid is determined. It is assumed that all chromatograms belonging to a particular series take about the same length of time to process and that the ninhydrin reagent ages uniformly throughout this time (and any other factor such as packing occurs uniformly with use). A linear interpolation is then both valid and results in a significant correction. The example shows differences between the pre- and post-standards and whatever interpretation is placed on these differences, the correction applied tends to give greater weight to the standard that was run nearest in time to the particular sample. Since the additional standards are only represented once, no interpolation is applied to the amino acids they represent. This comment also applies to those situations in which only one prime standard is run.

All the chromatograms in the sample set, namely chromatograms 338 to 361, are similarly processed and the program continues with subsequent complete chromatographic sets. Together with the printout, an additional data set, which may reside on cards, tape, or private file, is made of all successfully processed chromatograms. This stipulates the column used for a chromatogram, the amino acids contained therein together with their computed concentrations. This, in combination with other such data form part of the input for the next program.

The amino acids contained in a tissue sample normally require more than one type of column for their resolution. Occasionally, a sample is passed twice through the same column using different dilutions. Additionally, a particular amino acid may be imperfectly resolved by one type of column and acceptably resolved by another. The program, whose description follows, has been designed to accommodate these various analytical techniques. This program accepts, together with output from the previous program, that information which allows the assemblage of component chromatograms and quantities such as tissue dilution factors, cell weight or volume and so forth to build a tissue profile.

Using tissue 229T as an example, this tissue was divided into two parts, the first of which was passed through a sodium buffered column (chromatogram 339, the subject of the previous example) and the other was passed through a basic physiological column (chromatogram 514). From the set of chromatograms that the program may access these two were found. The program contains certain manipulative features for both changing amino acid concentrations or selecting from a multiplicity of the same amino acid a particular one. In chromatogram 339, as illustrated in Fig. 6 compared with Fig. 5, the concentration for GABA has been set to zero. The two values for valine remain in their original order. If the first value had been desired their order would have been reversed. This is achieved by specifying the chromatogram number, the amino acid name and either a cardinal number or a concentration, the amino acid referred to with that cardinality is then used (here this could be the first valine) or the new concentration is applied (here the concentration of GABA is set to zero).

The program contains a matrix (the acceptance matrix), the rows of which are amino acid names and the columns represent the types of chromatographic

```
TISSUE 229T CONSISTS OF THE FOLLOWING CHROMATOGRAMS
CHROMATOGRAM  339 FOUND

CYSO3H          1.97
G-P-E          24.46
O-P-E          74.76
TAU           325.72
ASP            19.45
THR            14.37
SER            17.74
GLU            26.63
CITRU           0.50
PRO            77.50
GLY           246.92
ALA            64.41
VAL             3.15
VAL             4.63
MET             0.83
LEU             0.92
GABA            0.00
HIS             1.71
ORN             0.98
LYS             3.22
NH3            40.58
THE ABOVE HAVE BEEN RUN THROUGH COLUMN TYPE DC1ANA3
CHROMATOGRAM  514 FOUND

PHE             0.13
ETAM            0.75
NH3            35.56
LYS             3.14
HIS             0.93
TRP           272.22
HCAR            0.00
THE ABOVE HAVE BEEN RUN THROUGH COLUMN TYPE DC2ABPH
    THR,SER,GLU, WILL BE ACCEPTED FROM THE DC1ANA3  COLUMN
```

Fig. 6. Component chromatograms for tissue 229T. These chromatograms were obtained using different portions of the same tissue.

columns. If the amino acid is deemed resolved by a particular physical column then a one is present in the appropriate position of the matrix and if not, a zero. Thus application of the information contained within this matrix to the component chromatograms enables them to be combined. For example, lysine is resolved by both columns DC1ANA3 and DC2ABPH in an acceptable manner. The content of lysine within the tissue is then computed by taking the weighted mean of the concentrations obtained from both chromatograms, where the weights reflect any sample dilution. Unusual methods of analysis may be incorporated into the program by

SUMMARY FOR TISSUE 229T			μMOLE/ML	PERCENT	CONCENTRATION OF ELEMENTS		
	DC1ANA3	DC2ABPH	PACKED CELLS	CONC	N	S	P
CYSO3H	1.97	0.00	0.0985	0.17	0.0082	0.0187	0.0000
G-P-E	24.46	0.00	1.2230	2.07	0.0793	0.0000	0.1754
TAU	325.72	0.00	16.2860	27.62	1.8226	4.1720	0.0000
O-P-E	74.76	0.00	3.7380	6.34	0.3710	0.0000	0.8205
ASP	19.45	0.00	0.9725	1.65	0.1023	0.0000	0.0000
THR	14.37	0.00	0.7185	1.22	0.0845	0.0000	0.0000
SER	17.74	0.00	0.8870	1.50	0.1182	0.0000	0.0000
GLU	26.63	0.00	1.3315	2.26	0.1268	0.0000	0.0000
CITRU	0.50	0.00	0.0250	0.04	0.0060	0.0000	0.0000
PRO	77.50	0.00	3.8750	6.57	0.4714	0.0000	0.0000
GLY	246.92	0.00	12.3460	20.94	2.3040	0.0000	0.0000
ALA	64.41	0.00	3.2205	5.46	0.5063	0.0000	0.0000
VAL	4.63	0.00	0.2315	0.39	0.0277	0.0000	0.0000
MET	0.83	0.00	0.0415	0.07	0.0039	0.0089	0.0000
LEU	0.92	0.00	0.0460	0.08	0.0049	0.0000	0.0000
PHE	0.00	0.13	0.0065	0.01	0.0006	0.0000	0.0000
ORN	0.98	0.00	0.0490	0.08	0.0104	0.0000	0.0000
ETAM	0.00	0.75	0.0375	0.06	0.0086	0.0000	0.0000
LYS	3.22	3.14	0.1590	0.27	0.0305	0.0000	0.0000
HIS	1.71	0.93	0.0660	0.11	0.0179	0.0000	0.0000
TRP	0.00	272.22	13.6110	23.08	1.8671	0.0000	0.0000
			TOTAL CONCENTRATION OF ELEMENTS		7.9722	4.1996	0.9958

Fig. 7. Profile for tissue 229T.

manipulation of the acceptance matrix. For example, threonine, serine and glutamic acid are normally resolved using a lithium buffered column but for technical reasons they were, in this example, resolved by the sodium column. This is noted in Fig. 6.

Fig. 7 illustrates the profile for tissue 229T. In addition to the contribution that each amino acid makes to this tissue the amount of the inorganic elements nitrogen, sulphur and phosphorus that are contained within each amino acid together with their totals that the tissue contains is computed. The units of measure depend upon the units in specifying the concentrations of the standard and the tissue constants. Several hundred chromatograms containing several thousand concentrations can be processed in each run of this program. There is no limitation on the number of tissues from which these chromatograms were derived, each such tissue can contain up to five component chromatograms. The names of the tissues, together with their amino acid contents are passed to the next program.

Experiments usually consist of like treatments using many samples of similar tissues. By naming an experiment and specifying the member tissues of that experiment, a summary for the experiment is obtained. Fig. 8 shows experiment C5 TRY. In this experiment, tissue 229T, the subject of the last example, is a member. That which was previously computed for the individual tissues is now presented for the experiment. In addition, the total nitrogen content is treated as a pseudo amino acid.

With the computation of many such sets of experiments, various cross-comparisons can be made. In Fig. 9, the pair-wise contrast between experiment C5 CONT and C5 TRY is presented. Using the t statistic, a single asterisk denotes significance at the 95% level whilst the double asterisk the 99% level. It will be seen in this example that leucine is significantly lower in the C5 TRY experiment than in the C5 CONT experiment. By convention, the first experiment is called the control. Introduction of pseudo amino acids at the tissue level allows any numerically describable property of the tissue (for example, the nitrogen content) to be included in an independent way

FOR SET C5 TRY THE FOLLOWING TISSUES HAVE BEEN USED

225 T
227 T
229 T
237 T

A ACID	NUM	MEAN	S.D.	PERCENT	N	S	P
CYSO3H	4	0.1244	0.0486	0.23	0.0103	0.0236	0.0000
G-P-E	4	1.0879	0.1255	2.00	0.0705	0.0000	0.1560
TAU	4	16.3198	4.2785	29.94	1.8264	4.1807	0.0000
O-P-E	4	3.4112	0.7488	6.26	0.3386	0.0000	0.7487
ASP	4	1.0037	0.1604	1.84	0.1056	0.0000	0.0000
THR	4	0.8086	0.2193	1.48	0.0951	0.0000	0.0000
SER	4	0.8939	0.2187	1.64	0.1191	0.0000	0.0000
GLU	4	1.4467	0.3895	2.65	0.1377	0.0000	0.0000
CITRU	3	0.0397	0.0307	0.07	0.0095	0.0000	0.0000
PRO	3	3.9292	1.0913	7.21	0.4780	0.0000	0.0000
GLY	4	13.2011	4.5948	24.22	2.4636	0.0000	0.0000
ALA	4	3.0920	0.7140	5.67	0.4861	0.0000	0.0000
VAL	4	0.2287	0.0477	0.42	0.0273	0.0000	0.0000
MET	3	0.0345	0.0061	0.06	0.0032	0.0074	0.0000
ILE	1	0.0215	0.0000	0.04	0.0023	0.0000	0.0000
LEU	4	0.0309	0.0272	0.06	0.0033	0.0000	0.0000
TYR	3	0.0782	0.0347	0.14	0.0060	0.0000	0.0000
PHE	4	0.0642	0.0547	0.12	0.0054	0.0000	0.0000
ORN	4	0.0960	0.0880	0.18	0.0203	0.0000	0.0000
ETAM	1	0.0375	0.0000	0.07	0.0086	0.0000	0.0000
LYS	4	0.3346	0.3492	0.61	0.0641	0.0000	0.0000
HIS	4	0.1592	0.1594	0.29	0.0431	0.0000	0.0000
TRP	4	8.0570	5.7267	14.78	1.1053	0.0000	0.0000
N TOT	4	7.2974	1.4507				

Fig. 8. Profile for experiment set C5 TRY.

THE CONTROL C5 CONT IS NOW BEING COMPARED WITH C5 TRY

A ACID	MEAN	MEAN	DF	T
CYSO3H	0.1421	0.1244	9	0.629
G-P-E	0.7672	1.0879	9	-3.500**
TAU	17.8680	16.3198	9	0.763
O-P-E	4.4857	3.4112	9	1.922
UREA	25.2402	0.0000	9	0.000
ASP	1.0712	1.0037	9	0.517
THR	1.4049	0.8086	9	4.247**
SER	1.1460	0.8939	9	1.551
GLU	1.6641	1.4467	9	0.418
CITRU	0.0000	0.0397	9	0.000
PRO	4.4152	3.9292	8	0.756
GLY	14.4500	13.2011	9	0.580
ALA	5.7531	3.0920	9	4.461**
VAL	0.1064	0.2287	9	-5.001**
MET	0.1251	0.0345	8	10.053**
ILE	0.0159	0.0215	6	-0.546
LEU	0.0693	0.0309	9	2.381*
TYR	0.1307	0.0782	8	1.519
PHE	0.0918	0.0642	9	0.774
ORN	0.0000	0.0960	9	0.000
ETAM	0.0000	0.0375	9	0.000
LYS	0.9354	0.3346	9	4.159**
HIS	0.3751	0.1592	9	2.688*
TRP	0.0000	8.0570	9	0.000
ARG	0.0472	0.0000	9	0.000
N TOT	19.2417	7.2974	9	4.479**

Fig. 9. Result of comparisons between experiments C5 CONT and C5 TRY.

while cross-comparing experiments. At the conclusion of all the requested comparisons only those amino acids (including pseudo amino acids) for which significant differences were found are passed to the last program in the set. These form the data base from which specific questions can be answered. The data base in the example was derived from several hundred tissues involving some 25,000 amino acids.

The last program is in essence a retrieval system. On the presentation of certain key words the information these words imply is obtained. Fig. 10 examines the information that is obtained when the key word 'SETC' followed by the experiment name 'C5 CONT' is provided. Here a table of all pair-wise comparisons using C5 CONT as the control set is exhibited. Leucine in C5 TRY is represented. Care must be exercised in interpreting the table, for though leucine is significantly different

KEYWORD "SETC" HAS BEEN SPECIFIED

USING C5 CONT AS THE CONTROL SET THE FOLLOWING MULTICOMPARISON TABLE HAS BEEN GENERATED

	C5 HIS	C5 GLN	C5 TRY	B5 CONT	C5 ARG	C5 GAMGLN	C7 CONT
CYSO3H						+	
G-P-E			+			+	
TAU	-	-				-	
O-P-E	-	-					-
UREA		-				-	
ASP	-	+				+	
THR	-	-	-		-	-	-
SER		-		+		-	-
GLU		+				+	
PRO	-	-				-	-
GLY	-	-		+		-	
ALA	-		-	-		-	-
VAL		+	+			+	
MET		-	-				
ILE		+					
LEU			-		-		
TYR				-			
LYS	-	-	-			-	-
HIS	+	-	-			-	
ARG					+		
N TOT			-				

Fig. 10. Multicomparison table showing all comparisons that were made with experiment C5 CONT. '+' indicating significant elevation of the amino acids over the control, '—' the converse.

```
                    KEYWORD "ACID" HAS BEEN SPECIFIED

AMINO ACID LEU    SHOWS SIGNIFICANT DIFFERENCES BETWEEN THE FOLLOWING COMPARISONS
          S CONT BR IS HIGHER THAN S ILE  BR
          S CONT BR IS HIGHER THAN S LEU BR
          B6 GAM    IS HIGHER THAN B7 CONT
          B7 GLN    IS HIGHER THAN B7 CONT
          S LEU CL  IS HIGHER THAN S CONT CL
          B5 TRY    IS HIGHER THAN C5 TRP
          S VAL CL  IS HIGHER THAN S CONT CL
          S CONT FL IS HIGHER THAN S PHE FL
          C5 CONT   IS HIGHER THAN C5 ARG
          B5 GLN    IS HIGHER THAN B5 CONT
          B7 GLN 30 IS HIGHER THAN C7 GLN 30
          S ARG CL  IS HIGHER THAN S CONT CL
          B7 GAMGLN IS HIGHER THAN B7 CONT
          B5 GLN 30 IS HIGHER THAN C5 GLN 30
          C5 CONT   IS HIGHER THAN C5 TRP
          B6 GAM    IS HIGHER THAN B5 CONT
          B5 GAMGLN IS HIGHER THAN B5 CONT
          B7 CONT   IS HIGHER THAN B7 ARG
          B5 TRP    IS HIGHER THAN B5 CONT
          B6 GAM    IS HIGHER THAN C6 GAM
          S CONT BR IS HIGHER THAN S MET BR
          B7 GLN    IS HIGHER THAN C7 GLN
          S MET CL  IS HIGHER THAN S CONT CL
          B7 GLN 30 IS HIGHER THAN B7 CONT
```

Fig. 11. A retrieval of all those cross-comparisons between experiments for which leucine was significantly different.

in both C5 TRY and C5 ARG, these are independent evaluations with respect to C5 CONT with very definite probability levels, and may in concert reflect a different probability level. In those situations where an overall probability level is required, Dunnett's test[5] is to be preferred over the separate t tests. Similar key words are defined for samples and combinations of samples and controls. Fig. 11 illustrates the use of the key word 'ACID'. The amino acid leucine is specified and it is seen that all experimental groups in which the level of leucine was significantly different are displayed.

In addition to these elementary questions, more complex logical constructs can be built. Such an interrogation scheme gains in power as the information base develops.

All programs were written in the PL/1 language. Most of the input information is presented to the program in free form strings with character delimiters and separators and is relatively simple to construct. The author invites those who would like to try the system either for complex or simple problems to contact him.

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Note

One-step analysis of a mixture of permanent gases and light hydrocarbons by gas chromatography

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Mixtures of permanent gases and light hydrocarbons are usually encountered in petrochemical systems that involve cracking, steam reforming, partial combustion, etc. The complete analysis of such mixtures by gas chromatography with a single sample injection still remains a problem.

Various methods involving the use of combinations of multiple columns, multiple detectors and multiple sample injections have been reported by Sevenster[1], Hobbs[2], Takamiya and Sukenaga[3], Trowell[4], Archer[5], Lo-Chang[6] and others, and these methods are usually very complicated. Cross[7] described a polymer column which separated hydrogen, carbon monoxide, methane, carbon dioxide, acetylene and ethane. Gornak and Komarov[8] reported that natural glauconite was an excellent adsorbent for the separation and analysis of mixtures of hydrogen, air, carbon dioxide and C_1–C_{12} hydrocarbons using helium as the carrier gas. A mixture of hydrogen, oxygen, nitrogen, carbon monoxide, carbon dioxide and C_1–C_4 hydrocarbons was separated[9] by using a single column (Poropak Q), a single sample injection and a single detector system with temperature programming from $-65°$ to $+200°$, and with helium as the carrier gas.

This paper describes the development of an analytical technique for mixtures of permanent gases and light hydrocarbons with a single sample injection using hydrogen as the carrier gas and without the use of any sub-ambient temperature programming. This technique is somewhat similar to those reported by Madison[10] and Cvejanovich[11], but is simpler and less complicated.

EXPERIMENTAL AND RESULTS

A Perkin-Elmer (Model 900) chromatograph was modified as shown in the flow diagram in Fig. 1. Two standard Poropak Q columns were fitted inside the programmed oven of the dual column chromatograph; the outlet of one of the columns was divided into two lines through a gas sampling valve (Pye 104), one passing through a molecular sieve 5A column maintained at 55° in a box with a hot-air blower and the other passing through a needle valve in order to keep the pressure drop through two lines the same. Both of these lines were then connected through a T-piece to the thermal conductivity detector.

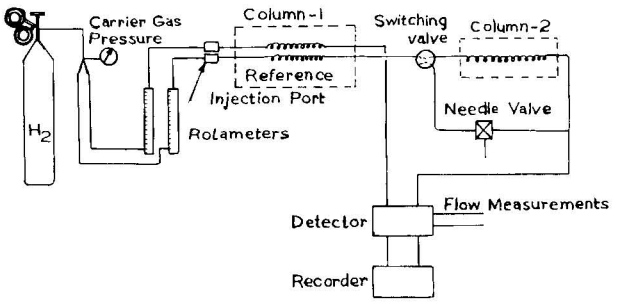

Fig. 1. Flow diagram of chromatographic system.

Initially, the Poropak and the molecular sieve columns were arranged in series, and nitrogen, oxygen, methane and carbon monoxide were allowed to leave the first column (50°) and enter the second. Then the molecular sieve was by-passed and carbon dioxide and hydrocarbons were allowed to leave the first column with temperature programming and pass into the detector. Subsequently, the effluent from the first column was again allowed to pass through the molecular sieve so as to separate nitrogen, oxygen, methane and carbon monoxide. The time required for a complete analysis was about 30 min.

Fig. 2 shows the recorder response for the analysis of a mixture of nitrogen,

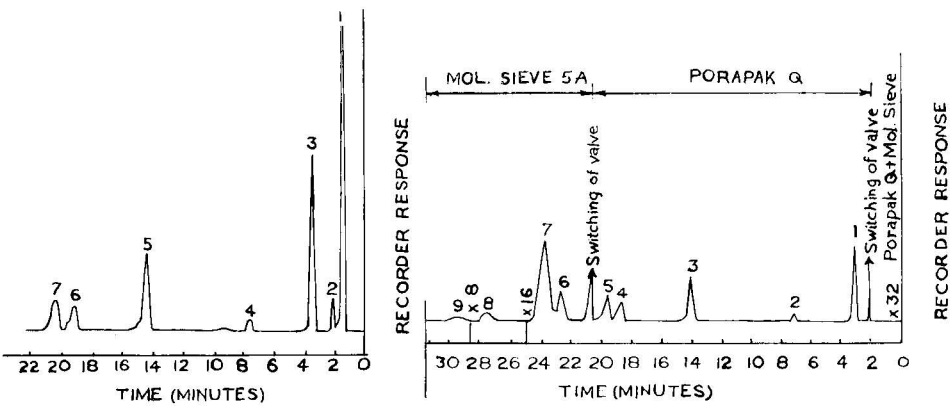

Fig. 2. Chromatography of a mixture of nitrogen, oxygen, methane, carbon monoxide and LPG using the Porapak Q column alone. Column: 6 ft. × 1/4 in. O.D. stainless steel. Column packing: Poropak Q. Carrier gas: hydrogen, flow-rate 40 ml/min. Temperature programme: initial by 50° for 3 min, increasing at 8°/min to 150°. Detector: thermal conductivity, temperature 150°; bridge current 100 mA. Peaks: 1= air; 2= methane + carbon monoxide; 3= carbon dioxide; 4= ethane; 5= propane; 6= isobutane; 7= *n*-butane.

Fig. 3. Chromatography of the same mixture as in Fig. 2 using the present technique. Column 1: 6 ft. × 1/4 in. O.D. stainless steel, Porapak Q. Column 2: 10 ft. × 1/4 in. O.D. copper, 30–60 mesh molecular sieve 5A. Carrier gas: hydrogen, flow-rate 40 ml/min. Temperature programme: column 1, initially 50° for 3 min, increasing at 8°/min to 150°; column 2, isothermal (55°). Detector: thermal conductivity, temperature 150°; bridge current 100 mA. Peaks: 1= carbon dioxide; 2= ethane; 3= propane; 4= isobutane; 5= *n*-butane; 6= oxygen; 7= nitrogen; 8= methane; 9= carbon monoxide.

oxygen, methane, carbon monoxide and liquefied petroleum gas (LPG) (containing ethane, propane, *n*-butane and isobutane) using the Poropak Q column alone. Fig. 3 shows the analysis of the same mixture using the present technique.

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CHROM. 7092

Note

Quantitative gas chromatographic determination of the major alkaloids in gum opium

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(Received September 18th, 1973)

A rapid gas chromatographic method for the simultaneous and quantitative determination of codeine, morphine, thebaine, papaverine and narcotine has been developed. These alkaloids are completely separated on a column packed with a 50:50 mixture of OV-17 and SE-30. The standard deviation of the method for the individual alkaloids is between 0.05 and 0.18%.

MATERIALS AND METHODS

Materials

OV-17 phenylmethyl silicone (50% phenyl) on 80–100 mesh Varaport 30 from Varian Aerograph, Palo Alto, Calif., U.S.A. SE-30 silicone gum rubber on Chromosorb W (AW, DMCS) from Perkin-Elmer, Norwalk, N.J., U.S.A. Resmethrin [(±)*cis,trans*-(5-benzyl-3-furyl)-methyl 2,2-dimethyl-3-(2-methylpropenyl)-cyclopropane-carboxylate] from S. B. Penick & Co. Didecyl phthalate from Eastman-Kodak, Rochester, N.Y., U.S.A. Analytical standards (codeine, morphine, thebaine, papaverine and narcotine) purified samples from S. B. Penick & Co.

Extraction of alkaloids from gum opium

A sample of opium was cooled for several hours at -10° and then powdered with a mortar and pestle. 1 g of the powdered gum opium was placed in an erlenmeyer flask containing 30 ml of water, 1.0 g of isoascorbic acid and 50 mg of sodium hydrosulfite. The flask was placed on a reciprocating shaker for 2 h and the contents were then filtered through a small (1/8 in.) bed of filter aid. After washing the filter cake with 3×5 ml of a 0.25 *M* aqueous solution of isoascorbic acid, the insoluble resins were scraped off the filter aid and returned to the erlenmeyer flask. This material was extracted two additional times using the above procedure. To the combined filtrates and washes were added 10 g of sodium chloride and 50 ml of a chloroform–isopropyl alcohol mixture (85:15). The mixture was heated to 50° and the pH adjusted to 8.8 with 10% ammonium hydroxide. The layers were separated and the aqueous phase re-extracted with 3×40 ml of chloroform–isopropyl alcohol (85:15). The combined organic extracts were washed once with 10 ml of water. This water phase was re-extracted with 10 ml of chloroform–isopropyl alcohol (85:15). The

combined organic phase was evaporated *in vacuo* and the residue prepared for gas chromatography as described below.

Preparation of sample

The above residue was quantitatively transferred, with the aid of a methanol–chloroform (25:75) solvent mixture to a 25-ml volumetric flask containing 50 mg of didecyl phthalate and 25 mg of resmethrin (internal standards). The mixture was warmed slightly to effect solution, cooled to room temperature and diluted to 25 m with the above solvent mixture.

Preparation of standard solution and determination of response factor

The following materials were accurately weighed into a 25-ml volumetric flask: codeine (35 mg), morphine (125 mg), thebaine (60 mg), papaverine (50 mg), narcotine (70 mg), resmethrin (12 mg) and didecyl phthalate (25 mg). The mixture was dissolved in and diluted to 25 ml with a methanol–chloroform (25:75) mixture. The response factor for each of the alkaloids was obtained in the usual manner.

Instrument and operating conditions

A Perkin-Elmer Model 881 gas chromatograph equipped with hydrogen flame ionization detector, Leeds and Northrup Speedomax W recorder and Hewlett-Packard Digital Integrator 3373B was employed.

A glass spiral chromatographic column (Perkin-Elmer 008-1285) 6 ft. in length with an internal diameter of 0.08 in. was used. The column was packed with a 50:50 mixture of 3% OV-17 on 80–100 mesh Varaport 30 and 5% SE-30 on 80–100 mesh Chromosorb W (AW, DMCS).

The operating conditions were: injector temperature, 310°; oven temperature,

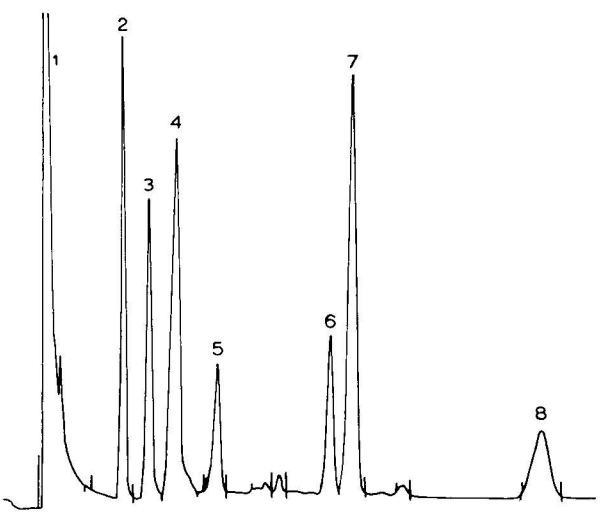

Fig. 1. GLC analysis of opium alkaloids. 1 = Solvent; 2 = resmethrin (internal standard) (t_R = 2.72 min); 3 = codeine (t_R = 3.51 min); 4 = morphine (t_R = 4.25 min); 5 = thebaine (t_R = 7.63 min); 6 = papaverine (t_R = 9.15 min); 7 = didecyl phthalate (internal standard) (t_R = 9.80 min); 8 = narcotine (t_R = 15.65 min).

TABLE I

PERCENTAGE ALKALOIDS IN GUM OPIUM

Values were calculated using resmethrin (*A*) or didecyl phthalate (*B*) as internal standard.

Deter-	*Codeine*		*Morphine*		*Thebaine*		*Papaverine*		*Narcotine*	
mination	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
1	3.50	3.46	9.79	9.89	2.21	2.20	2.27	2.27	5.93	5.67
2	3.54	3.55	9.40	9.66	2.51	2.13	2.22	2.26	5.69	5.85
3	3.52	3.57	9.39	9.77	2.29	2.35	2.18	2.24	5.75	5.59
4	3.60	3.55	9.28	9.37	2.37	2.15	2.24	2.24	5.49	5.61
5	3.54	3.64	9.77	9.56	2.57	2.19	2.16	2.25	5.77	5.93
6	3.63	3.47	9.26	9.59	2.32	2.33	2.37	2.30	5.76	5.50
7	3.50	3.46	9.24	9.74	2.49	2.28	2.30	2.28	5.67	5.91
8	3.34	3.49	9.56	9.39	2.35	2.27	2.11	2.22	5.77	5.93
9	3.53	3.54	9.54	9.45	2.31	2.16	2.27	2.28	5.76	5.59
10	3.54	3.56	9.45	9.86	2.26	2.11	2.22	2.25	5.53	5.72
11	3.55	3.58	9.31	9.88	2.26	2.30	2.23	2.26	6.07	5.66
12	3.56	3.46	9.31	9.76	2.30	2.21	2.22	2.26	6.10	5.64
Average	3.53	3.53	9.47	9.64	2.35	2.22	2.23	2.26	5.77	5.72
S.D.	0.05	0.05	0.17	0.18	0.09	0.07	0.05	0.02	0.16	0.13

programmed at 250° for 5 min followed by increasing the temperature to 280° at a rate of 48°/min; detector temperature, 270°; flow-rate of carrier gas (helium), 30 ml/min; hydrogen pressure, 10 p.s.i.g.; air pressure, 40 p.s.i.g.

DISCUSSION

Several gas–liquid chromatographic (GLC) methods have been developed for opium alkaloids[1–5]. Similar GLC procedures are in use for biological fluids, toxicology, and illicit drug screening[6–10]. Most of the procedures use silylated or acetylated derivatives for the opium alkaloids. We have found that these alkaloids can be chromatographed without derivatization on a mixed column of SE-30 and OV-17.

Fig. 1 depicts a typical chromatogram. Both resmethrin and didecyl phthalate have been found to be excellent internal standards. The reproducibility of the GLC analysis is illustrated in Table I. Values in columns A and B were calculated using resmethrin and didecyl phthalate, respectively, as the internal standards.

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Note

Gas chromatographic determination of ethambutol

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Ethambutol is an oral chemotherapeutic drug effective against the genus *Mycobacterium*, specifically, *Mycobacterium tuberculosis*. Several gas chromatographic methods for separating ethambutol and other antitubercular drugs have been previously reported[1,2]. However, these methods are not entirely satisfactory because a complicated derivatization process must be utilized along with a programmed temperature system. The method developed in this laboratory makes use of a single-step derivatization, isothermal temperature, and a multipurpose column. There is no interference from other antitubercular drugs that may be used in conjunction with ethambutol.

EXPERIMENTAL

Reagents

N-Trimethylsilylimidazole (TSIM), N,O-bis(trimethylsilyl)acetamide (BSA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), and hexamethyldisilazane (HMDS) (Pierce Chemical Company, Rockford, Ill. 61105, U.S.A.) were used to prepare the trimethylsilyl derivatives. Ethambutol (Myambutol®) was supplied by Lederle Laboratories, Pearl River, N.Y. 10965, U.S.A. Pyrazinamide, cycloserine, ethionamide, pyridoxine, and isoniazid, other antitubercular drugs often used in conjunction with ethambutol, were obtained from the Veterans Administration Hospital Pharmacy, Shreveport, La. 71130, U.S.A. Stock solutions containing 1 mg/ml of each drug as well as a mixture of the compounds were prepared. These stock drug solutions were refrigerated when not in use and were stable for several weeks.

Procedure

Preliminary attempts at silylation were performed at room temperature in 1-ml reaction vials fitted with PTFE-lined caps using the undiluted TMS reagents. One-tenth milliliter of each standard solution was pipetted into a 1-ml conical reaction vial and the solvent removed by evaporation in a temperature-regulated heating block. The residue was then dissolved in 0.1 ml of the various undiluted silylating reagents. One microliter of each drug derivative was injected into the gas chromatograph at 0 min, 1, 2, 3 and 4 h, and showed that only TSIM derivatized ethambutol

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immediately and completely. The other silylating agents required mild heating at 50° to complete the derivatization of ethambutol. Since TSIM reacts only with hydroxyl groups[3,4], only ethambutol and pyridoxine were derivatized because the others did not contain reactable functional groups (Fig. 1). A 1:5 dilution of TSIM in spectrophotometric-grade chloroform also derivatized ethambutol and did not affect retention time or peak symmetry but did reduce the solvent peak. The dilution also minimizes column damage.

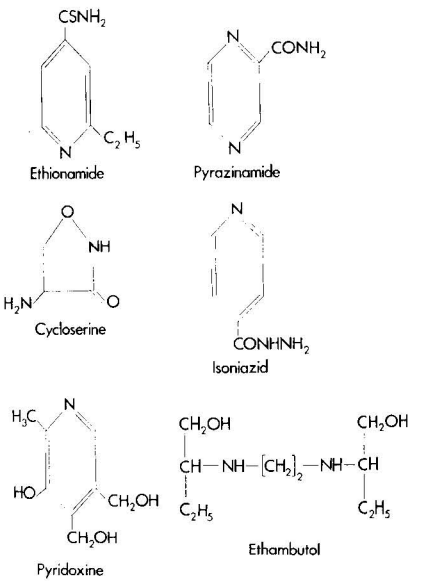

Fig. 1. Six antitubercular drugs investigated.

Gas chromatography

A Beckman GC-65 gas chromatograph (Beckman, Fullerton, Calif., U.S.A.) equipped with a flame ionization detector was used in conjunction with a Leeds and Northrup Speedomax XL 600 Series recorder (Leeds and Northrup, North Wales, Pa., U.S.A.) equipped with a disc integrator and automatic printer.

Column

A silanized glass column, 1.5 m×6 mm O.D.×2 mm I.D., packed with 3% OV-17 on Chromosorb W-HP, 100–120 mesh, was used. The column was conditioned overnight at 275° with a helium flow-rate of 20 ml/min.

Temperatures

The injection port was set at 200°, the column temperature at 150°, and the detector temperature at 200°.

Flow-rates

The flow-rates utilized were: helium, 90 ml/min; hydrogen, 60 ml/min; air, 300 ml/min.

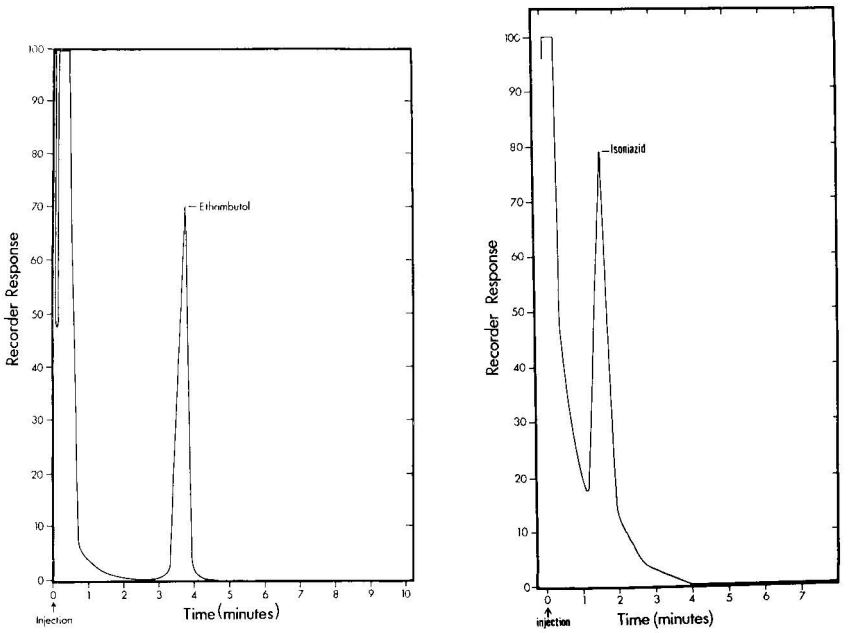

Fig. 2. Gas chromatogram of ethambutol TSIM derivative.

Fig. 3. Gas chromatogram of isoniazid TSIM derivative.

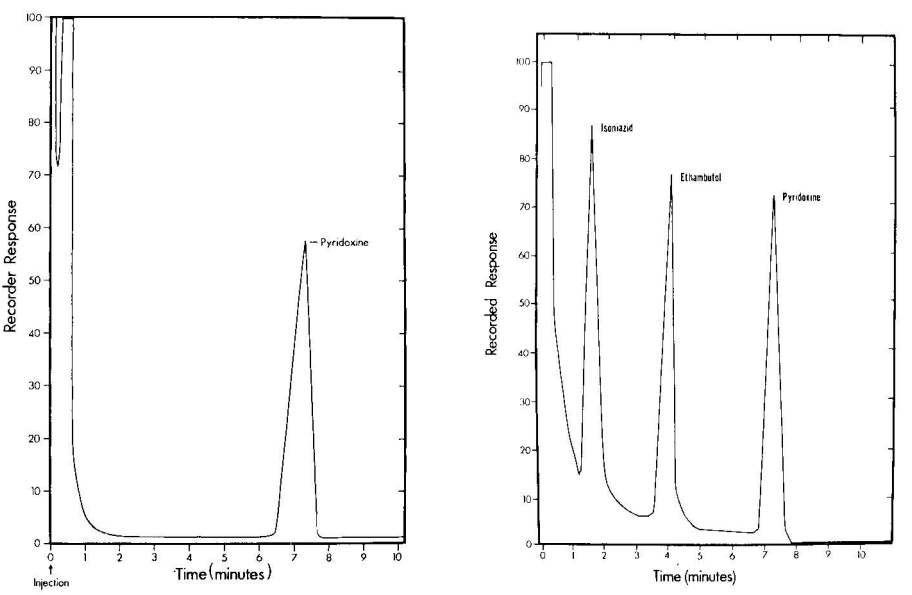

Fig. 4. Gas chromatogram of pyridoxine TSIM derivative.

Fig. 5. Gas chromatogram of a mixture containing TSIM derivatives of ethambutol, isoniazid, and pyridoxine.

RESULTS AND DISCUSSION

Under the chromatographic conditions described above, ethambutol has a retention time of 4 min (Fig. 2), isoniazid 1.8 min (Fig. 3), and pyridoxine 7.2 min (Fig. 4). The other antitubercular drugs are not eluted under the above conditions. Fig. 5 illustrates a chromatogram of a mixture of each of the drugs studied.

Several column packings were tested which could have multiple utility. A mixture of 1% OV-1 and 1% OV-17 on Chromosorb G-DMCS, a column we have found useful for chromatographing a large variety of drugs, eluted the derivatized sample but at the expense of much tailing. Thirty percent Carbowax 20M on Chromosorb W-HP, an excellent alcohol column[5], was decomposed by the silylation reagent. Neither decomposition or adsorption was encountered when 3% OV-17 on Chromosorb W-HP was used as the column packing. This column will also separate cannabinols, barbiturates, narcotics, and other drugs.

BSA, BSTFA, TMCS and HMDS were useful as derivatizing agents for ethambutol, but reacted only after mild heating or some other catalytic reaction. When TMCS was used as a catalyst in the HMDS reaction, a NH_4Cl precipitate was formed. Although the precipitate will not interfere with the chromatogram, mechanical problems will arise when small samples, such as 20 μl, are used. The sample cannot be centrifuged or withdrawn due to the excess NH_4Cl. Also, the precipitate solidifies very rapidly in the syringe. BSA, BSTFA, TMCS and HMDS are also extremely sensitive to moisture, decomposing upon contact with water. The usual precautionary measures necessary with other silylating agents with reference to water are not necessary with TSIM.

As a result of this study, a simple, rapid gas chromatographic method for determining ethambutol utilizing an isothermal temperature system and a multipurpose column has been developed.

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Note

Sodium chloride in buffers for amino acid analysis

Application to the analysis of lysine in maize samples

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(First received August 6th, 1973; revised manuscript received October 24th, 1973)

Although manual photometric methods of analysis for lysine or tryptophan[1] are possibly most suitable for screening the large number of samples associated with maize-breeding programs, the accuracy of an automatic amino acid analyser is invaluable for a more subtle distinction between selected samples of high lysine maize. Nevertheless, as the number of such selected samples can be large it is essential that the accuracy of the automatic analyser be coupled with a high speed of operation. In a previous communication[2] a method was described employing L-2,4-diaminobutyric acid (DAB) as an internal standard to minimise errors and using an overlapping system of elution from two short columns, each operated with the conventional short column buffer, to reduce the time of analysis. By this means a sample could be analysed in 40 min.

A further reduction in the time of analysis has now been achieved by the inclusion of sodium chloride in the elution buffer. As reported by Moore and Stein[3], a decrease in the elution time can be effected by an increase in either the pH or the ionic strength of the buffer. An increase in pH, however, is attended by a relatively poorer separation between lysine and the internal standard, DAB, and between histidine and ammonia. Similarly, if the ionic strength is increased by increasing the buffer concentration, lysine and DAB tend to be eluted together (*e.g.* Fig. 1a). However, we have observed that, in general, substitution of part of the sodium citrate by sodium chloride or addition of extra sodium chloride causes the DAB to be eluted later relative to lysine. Consequently, if the buffer concentration is kept constant and the ionic strength is increased by adding sodium chloride, a decrease in the elution time is effected without a concomitant deterioration in the resolution between lysine and DAB (*e.g.* Fig. 1b). Addition of sodium chloride to the conventional pH 5.28 buffer is attended by a decrease in pH which causes histidine to be eluted together with ammonia. For the analysis of lysine this is no disadvantage but should a separation between histidine and ammonia be required, this can be effected simply by increasing the pH slightly.

The buffer system described above has an advantage in common with the conventional short-column buffer[3] in that a single buffer can be used both for lysine analysis and for complete basic amino acid analysis. However, a buffer suitable specifically for lysine analysis can be prepared by further increasing the proportion

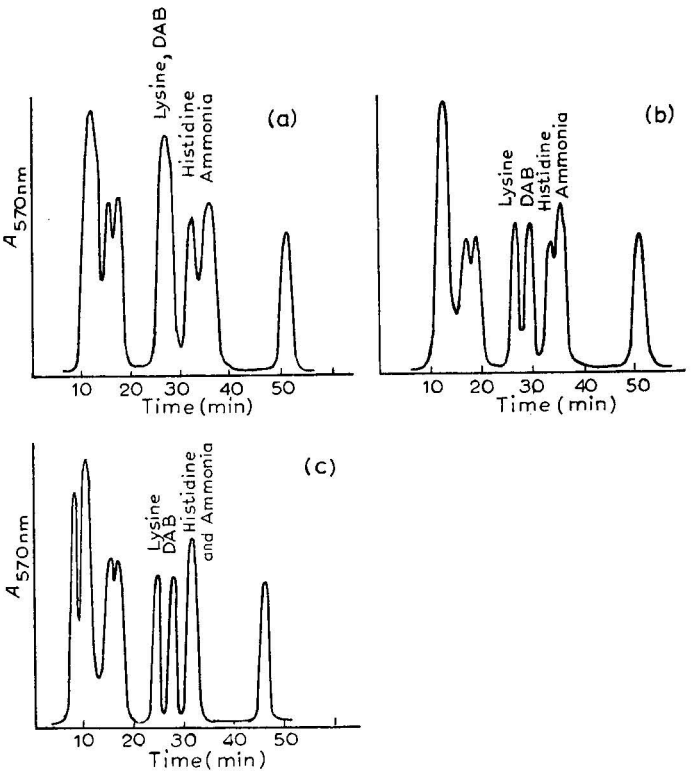

Fig. 1. The effect of substituting sodium chloride for sodium citrate on the elution of basic amino acids in buffers of elevated ionic strength. Buffers, (a) 0.7 *N* sodium citrate, pH 4.95; (b) 0.35 *N* sodium citrate–0.35 *M* sodium chloride, pH 4.95; (c) 0.1 *N* sodium citrate–0.6 *M* NaCl, pH 4.95; temperature, 58°; resin, 10.5 × 0.9 cm, Beckman type PA-35.

of sodium chloride, at the expense of sodium citrate, thus further improving the separation between lysine and DAB and by simultaneously lowering the pH to retard histidine. Lowering of the pH causes histidine to be co-eluted with ammonia but eliminates the overlap between DAB and histidine which results from the increased concentration of sodium chloride. For the routine analysis of lysine in maize samples we have used a buffer of pH 4.95 containing 0.1 *N* sodium citrate and 0.6 *M* sodium chloride, in addition to the usual proportions of detergent and preservative. Using this buffer system and a bed of PA-35 resin (Beckman, Palo Alto, Calif., U.S.A.; 10.5 × 0.9 cm), lysine and DAB are eluted at approximately 25 min and 28 min, respectively, while arginine is eluted at 47 min (Fig. 1c). In a two-column overlapping system[2] using this buffer, it is therefore possible to analyse a lysine sample, with internal standard, every 20 min. Twenty or more samples can thus be analysed per 8-h day.

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Breeding Section of the Natal Region of the Department of Agricultural Technical Services, headed by Mr. H. O. Gevers.

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CHROM. 7114

Note

A novel method for the separation and identification of bile acids and phospholipids of bile on thin-layer chromatograms

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(Received August 27th, 1973)

Cholesterol gallstone disease, so common in the western world, has considerable medical and economic importance. Under normal physiological conditions, cholesterol is held in solution in the bile. If the solubility of cholesterol in bile is changed, cholesterol crystals will precipitate and lead to the formation of gallstones. The solubility of cholesterol in bile depends on three major lipid components of bile: conjugated bile salts, phospholipids and cholesterol[1]. Knowledge of bile lipid composition is important in our understanding of the mechanism of gallstone formation.

Paper chromatography[2] and silicic acid column chromatography[3] have been used for the analysis of the composition of bile lipids. However, the latter technique has been found less satisfactory for routine bile analysis or to experiment on small animals because of the limited quantity of the bile samples. Although thin-layer chromatographic separation of bile lipids[4] can be performed using three successive solvent systems, it takes as long as 8 h and does not delineate the free bile acids one from the other. Since bile contains a variety of compounds, different plates and different solvent systems must be used for the separation of each class of compounds. Various solvent systems have been proposed to achieve the separation of bile acids and their conjugates[5,6] on thin-layer chromatograms. Our method successfully accomplishes the separation of several classes of bile lipids on a single plate which makes a direct comparison possible between different bile lipid components such as cholesterol, free and conjugated bile acids, lecithin and lysolecithin. The solvent system and the spray reagent used in our technique, not previously described, are satisfactory for a routine check of bile lipid composition and may be used for the separation of individual bile acids, both free and conjugated, in biological fluids on the same plate.

MATERIALS AND METHODS

Chemicals

Cholesterol, deoxycholic acid, chenodeoxycholic acid, taurodeoxycholic acid, glyco- and taurochenodeoxycholic acid, glycocholic acid, lysolecithin (all from Sigma, St. Louis, Mo., U.S.A.) cholic acid and lithocholic acid (Applied Science Labs., State College, Pa., U.S.A.), lecithin and taurocholic acid (Nutritional Bio-

chemicals, Cleveland, Ohio, U.S.A.), glycodeoxycholic acid (Calbiochem., San Diego, Calif., U.S.A.), isopropyl alcohol, acetic acid, sulphuric acid (Baker Analyzed Reagent, J. T. Baker, Phillipsburg, N.J., U.S.A.), ammonium molybdate, ethyl acetate, isooctane (Mallinckrodt, St. Louis, Mo., U.S.A.) chloroform, methanol (Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.) were used.

Bile samples were collected by puncture of the gallbladder during operation and when the gallbladder was removed, the total amount of bile was taken, frozen and stored at $-16°$.

Preparation of spray reagent

The spray reagent was the same as used for phospholipid detection[7]. A piece of copper wire is placed in a solution of 0.5 g of ammonium molybdate in 2 ml of distilled water. The mixture is chilled and 2 ml of concentrated sulphuric acid are added; the deep blue solution is then shaken. This reaction mixture is kept for 2 h at room temperature with occasional shaking. A 80-ml volume of distilled water is then added and shaken; the color changes from deep blue to light brown, the copper wire is then removed and 6.4 ml of concentrated sulphuric acid are added and mixed; the resulting solution remains light brown. This reagent can be stored for a week or more in the refrigerator.

Experimental

The solutions to be tested were applied on 20×20 cm pre-coated thin-layer plate silica gel F_{254} of 0.25 mm thickness (E. Merck, Darmstadt, G.F.R. distributed by Brinkmann, Westbury, N.Y., U.S.A.). The plate was marked at 6 cm and 13 cm from the starting line. About 10-μg samples of each of free and conjugated bile acids and phospholipids were applied as spots of 4 mm diameter at 1.5 cm above the bottom edge of the plate. Volumes of 0.5–1.0 μl of the bile samples were applied directly to the plate using a Hamilton microliter syringe. When samples are applied, the plate must not be dried with hot air as some of the bile acids might be adsorbed to the silica gel and might not move in the chromatographic system. The plate was then placed in a commercial chromatographic chamber (Gelman, Ann Arbor, Mich., U.S.A.) which was previously saturated with the solvent system isooctane–isopropyl alcohol–ethyl acetate–acetic acid (40:20:10:10, v/v) at room temperature. After the center of the solvent front reached the 13-cm mark, the plate was taken out of the chamber and dried with cold air for 2–3 min by means of a hair-drier. The plate is next placed in the second chamber and developed with the solvent system chloroform–methanol–water (65:35:4, v/v) up to the 6-cm mark and taken out of the chamber.

The plate was then kept at 70–80° in an oven for 1–2 min, taken out of the oven and sprayed with the spraying reagent. The plate was then heated in the oven at 70–80° for 5–7 min and the color of cholesterol and phospholipids noticed and the area marked. The plate is allowed to heat again for another 5–7 min and the color of bile acids noted.

RESULTS AND DISCUSSION

Bile samples from gallstone patients and pure bile acids, conjugated and

TABLE I

THE R_F VALUES OF BILE ACIDS, PHOSPHOLIPIDS AND CHOLESTEROL AND THE COLOR PRODUCED BY THE NEW SPRAY REAGENT

The R_F values are represented as percent of the distance of solvent front from the origin and the R_{st} values as percent of the distance of cholesterol from the origin.

Substance	R_F	R_{st}	*Color*
Lysolecithin	2.6	3	Blue
Lecithin	7.2	8.2	Blue
Taurocholic acid	14	15.6	Greyish green
Taurochenodeoxycholic acid	20	23	Purple
Taurodeoxycholic acid	21	24	Greyish green
Glycocholic acid	34	36.6	Greyish green
Glycochenodeoxycholic acid	55	63	Purple
Glycodeoxycholic acid	56	64	Greyish green
Cholic acid	71	80	Greenish yellow
Chenodeoxycholic acid	78	89	Purple
Deoxycholic acid	81	92	Yellow
Lithocholic acid	85	96	Light purple
Cholesterol	88	100	Pink, turns greenish grey

unconjugated, cholesterol, lysolecithin and lecithin were examined by this method. The R_F values and the color produced by each component are shown in Table I. The R_F values presented are means of at least five runs and they possibly apply to the particular batches of plates employed and hence are not an absolute value but would be expected to vary from one individual to another. It is suggested each investigator will want to prepare his own color standard and the R_F values.

The solvent front forms a concave shape at the end of the run and a solvent demixing occurs at the center of the plate showing disproportionate R_F values. Hence a mixture of known compounds was spotted between every fourth sample. The important feature of this new spray reagent is that, whatever might be the difference of R_F values, the compounds tested can be identified by their characteristic colors which are very distinct amongst each other. The blue phospholipid color and the pink cholesterol color appear within 5–7 min of heating in the oven, other bile acid colors appeared after that. All the colors stand out clearly in an almost white background. Various spray reagents have been used[8–11] in the past for the detection of bile acids on thin-layer chromatograms; the present reagent is a unique and useful addition.

The solvent systems previously[12,13] used for the separation of bile acids, conjugated and unconjugated, separate either the free bile acids or the conjugated bile acids but not all on the same plate, nor do they separate chenodeoxycholic acid from deoxycholic acid. The solvent systems that we have developed completely separate the conjugated bile acids from free bile acids leaving each component distinct and separate in the same plate, *e.g.* chenodeoxycholic acid is separated from deoxycholic acid. The dihydroxybile acid–taurine conjugates, taurodeoxycholic and taurochenodeoxycholic acid, have the same mobility, as do the dihydroxybile acid–glycine conjugates, glycodeoxycholic acid and glycochenodeoxycholic acid, but the

presence of both chenodeoxy- and deoxycholic acid conjugates in the same sample can be recognized by a mixed color of the spot *i.e.* greenish purple.

The main advantage of this technique for the separation of bile is its accomplishment on a single plate using only a small amount of 0.5 μl of bile, and the rapid completion of the operation within 4 h. Part of the bile pigment migrated with the solvent, while the rest stayed on the starting point, and did not interfere with the color development and identification of the bile salts. Thus this micro-technique can be used for routine bile analysis and permits the direct study of different components of bile important in gallstone formation. Furthermore, this method does not involve preliminary extraction and separation of each class of bile lipids, which is a part of the usual bile lipids analysis.

Thirty samples of gallbladder bile from gallstone patients were examined by this technique. Glycine- and taurine-conjugated cholic and chenodeoxycholic acid, lecithin and cholesterol were present in all of the samples tested. Lysolecithin and glycodeoxycholic acid were found in ten samples. No taurine-conjugated deoxycholic acid was found on the chromatogram by this method. Free bile acids are also absent in the bile which supports previous observations[14,15]. The presence of lecithin and lysolecithin in bile has been reported earlier[16]. The absence or presence of a trace amount of glycodeoxycholic acid in the bile of patients with gallstones may be the result of interrupting the enterohepatic circulation, as deoxycholic acid is formed by bacterial action on cholic acid during the enterohepatic circulation[17–19]. Deoxycholic acid was also found to be absent in patients with acute hepatitis, biliary dyskinesia and portal cirrhosis[15]. The explanation for the absence of taurine conjugation of deoxycholic acid in gallstone patients is obscure.

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Note

Two-dimensional TLC of lipophilic compounds

Characterization of a mixed stationary phase permitting both adsorption TLC and reversed-partition TLC on one plate

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Appropriate choice of solvents for two-dimensional thin-layer chromatography (TLC) of polar substances can allow unique separations in each dimension on a single stationary phase by varying the relative contributions of adsorption, partition, pH, and ionophilic character. No such flexibility exists for non-polar substances, which separate on an essentially unidimensional polarity basis with a hydrophilic stationary phase, or on a reversed-partition basis with a hydrophobic stationary phase.

Previous two-dimensional TLC of non-polar lipids has involved adding $AgNO_3$, paraffin, or silicone oil to change the stationary phase characteristics before the second elution[1–4]. Now a method is reported of achieving adsorption or partition TLC in successive developments on the same chromatoplate without changing stationary phase composition.

MATERIALS AND METHODS

Silica gels G, H, and "H (silanized)" were obtained from E. Merck (Darmstadt, G.F.R.). Calcium sulfate dihydrate (AR) (Mallinckrodt, St. Louis, Mo., U.S.A.) was converted to the hemihydrate by heating at 110 °. Standards from commercial sources were purified by crystallization or conventional TLC. All solvents were distilled before use. Dimethyldichlorosilane (DMCS) was obtained from Applied Science Labs., State College, Pa., U.S.A.

Silica gel H was silanized by slurrying in anhydrous toluene, and adding DMCS in 1-ml portions with stirring until further addition did not produce ebullition (caution: HCl gas evolved.) If the required excess of DMCS is present, addition of a few g of fresh silica gel H will produce rapid bubbling. The slurry was allowed to stand overnight at room temperature, loosely covered. The preparation was completed by vacuum-assisted filtration in a büchner funnel, rinsing with 3 volumes of anhydrous benzene, followed by 3 volumes of anhydrous methanol. The büchner funnel was loosely covered, and vacuum-assist maintained for 4 h. Drying was completed at 70° in a tray[5]. The product was designated silica gel "HS".

The mixed sorbent, termed silica gel "G + HS", was prepared by carefully mixing the following weight percentages: 50% silica gel G, 43% silica gel HS, and 7% $CaSO_4 \cdot \frac{1}{2}H_2O$. No significant differences were found upon substitution of silica gel "H" for "G", or commercial silica gel "H (silanized)" for "HS". Optimum layer integrity was obtained with $>10\%$ $CaSO_4 \cdot \frac{1}{2}H_2O$ (including the amount found in silica gel G). Layers were spread on 20×20 cm glass plates as a 70% aqueous ethanol slurry. Plates were dried overnight at room temperature. Activation, when desired, was accomplished at 70° overnight. Non-activated plates were found suitable for our purposes, and were used throughout. TLC was accomplished by the ascending technique in a vapor-saturated tank. If solvents were carefully removed from the plate between developments, the R_F values in the second dimension were equivalent to those obtained by unidimensional TLC in that solvent. Visualization of spots was accomplished with iodine vapor, or by spraying with 50% aqueous H_2SO_4 and heating at 120°. For preparative purposes, the still slightly damp plate was scraped and the scrapings eluted with ethyl acetate.

RESULTS

The solvent systems used were as follows: (I) hexane–ethyl acetate (95:5), (II) hexane–ethyl acetate (90:10), (III) hexane–ethyl acetate (80:20), (IV) methanol–water (70:30), (V) *p*-dioxane–water (75:25), and (VI) *p*-dioxane–acetic acid–water (50:30:20).

Table I shows R_F values for various standards in several different solvent systems. The major features of interest are the normal, silica gel G-type separations found with non-polar solvents (I, II, III), and the reversed-phase partition separations found with polar solvents (IV, V, VI). Trials in our laboratory have shown that methanol or acetonitrile-based solvents containing water are not suitable for reversed-partition TLC of non-polar high-molecular-weight lipids like monohydroxysteroids, as these tend to remain at the origin because of low solubility in the solvent system. This characteristic can be exploited to separate lower terpenes from steroids, as shown by solvent system IV. Otherwise, dioxane or tetrahydrofuran-based solvents are more suitable for steroid reversed-partition TLC. Even with these solvents, heavy loading (10 μg/spot) with high-molecular-weight lipids must be rigorously avoided.

The R_F values in Table I were obtained by unidimensional TLC, but the R_F values in each direction for two-dimensional TLC are quite comparable. Division of the plate into one- and two-dimensional areas, as shown in Fig. 1, has proved very useful, as standards may be run on the same plate and R_F values directly compared.

DISCUSSION

A practical method for two-dimensional TLC of non-polar lipids has been described. This method was developed for a specific application in our research, but it is a method which suggests general utility in separations of non-polar lipids, a group of compounds for which there has heretofore been no simple easily-reproducible method of two-dimensional TLC.

TABLE I

R_F VALUES FOR DIFFERENT COMPOUNDS USING NON-ACTIVATED SILICA GEL G+HS PLATES.

Elution distance was approximately 14 cm.

Compound	*Solvent systems*					
	I	*II*	*III*	*IV*	*V*	*VI*
Geraniol	0.14	0.24	0.48	0.63	0.72	0.60
Nerol	0.15	–	0.56	0.65	0.71	–
Linalool	–	–	0.81	0.46	0.71	–
Myrcene	–	–	1.0	0.64	0.42	–
α-Terpineol	–	–	0.64	0.65	0.76	–
Terpinolene	–	–	1.0	0.65	0.78	–
Eugenol	0.15	–	0.78	0.72	0.77	0.73
trans,trans-Farnesol	–	0.26	0.51	0.46	0.60	0.52
cis,trans-Farnesol	–	0.33	0.60	0.46	0.60	0.52
Pristane	1.0	1.0	1.0	–	–	–
Phytol	–	–	0.72	–	0.46	0.27
Cholesterol	0.11	0.22	0.59	0	0.45	0.22
5α-Cholestanol	0.11	–	0.57	0	0.40	0.17
Lanosterol	0.20	0.35	0.79	0	0.45	0.22
24,25-Dihydrolanosterol	0.20	–	0.79	0	0.40	0.17
Stigmasterol	0.10	–	0.51	0	0.39	–
β-Sitosterol	0.10	–	0.51	0	0.39	0.20
Cycloartenol	0.21	–	0.82	0	0.39	0.19
24-Methylenecycloartanol	0.21	–	0.82	0	0.39	0.20
Cholesteryl acetate	0.74	–	0.98	0	–	–
Lanosteryl acetate	0.77	–	0.99	0	–	–
Squalene	0.98	0.99	1.0	0	0.21	0.07
Squalane	1.0	1.0	1.0	0	–	–
2,3-Oxidosqualene	0.71	0.92	–	0	0.32	–

The principle of the method is that the two types of silica gel in the stationary phase act independently of each other. In polar solvents adsorption to silica gel G does not occur appreciably, so the major effect is reversed-phase partition with the hydrophobic silanized silica gel. Conversely, in non-polar solvents the major effect is adsorption to silica gel G. An intermediate polarity solvent, like ethyl acetate or ether, permits elution of most non-polar lipids to near R_F 1.0.

The method has the following advantages: (1) difficult-to-reproduce spraying or dipping operations are eliminated; (2) no paraffin or silicone oil contaminates non-polar lipids, so derivatization and/or subsequent TLC are rendered unnecessary; and (3) because the layer composition is uniform, stable, and reproducible, R_F values are stable and standards are easily correlated.

Further improvements may include: use of long-chain alkyl groups in silanization to improve partition properties for reversed-phase TLC[6]; addition of glass fiber or asbestos to improve layer durability; and argentation TLC by the normal method.

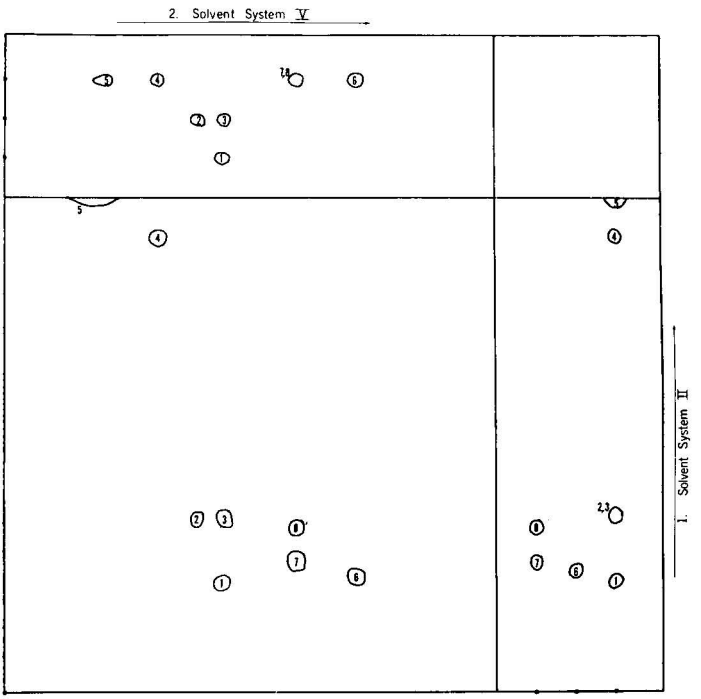

Fig. 1. Two-dimensional TLC of a synthetic mixture, with standards run in unidimensional sections of the plate in each dimension. A non-activated silica gel G+HS plate was developed to 14 cm in each dimension. Development times were 45 min in the first dimension and 2 h in the second. Compounds tested were: (1) cholesterol; (2) dihydrolanosterol; (3) lanosterol; (4) oxidosqualene; (5) squalene; (6) geraniol; (7) *trans,trans*-farnesol; (8) *cis,trans*-farnesol.

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Note

High-voltage electrophoresis of choline and its esters

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Numerous methods have been described[1,2] for the identification and determination of choline in biological materials. Assay procedures are commonly based on precipitation by non-specific reagents (*e.g.* ammonium reineckate, phosphotungstic acid, cadmium chloride) followed by gravimetric, spectrophotometric or titrimetric determination. Other methods include colorimetric measurement after a complexing reaction.

More specific identifications employ chromatographic procedures. Those based on paper[3] and thin-layer[4] techniques are simple but time consuming, whilst gas chromatography requires either conversion to stable, volatile compounds[5] or pyrolysis[6]. This communication describes a more rapid method for the separation and identification of choline salts and some of its more important esters by high-voltage electrophoresis under a range of pH conditions. The method may be applied to quantitative determinations in biological extracts and pharmaceutical preparations.

EXPERIMENTAL

The Camag HVE system (Camag, Muttenz, Switzerland) was employed, separations being carried out on 40 cm × 20 cm strips of Whatman No. 3MM filter paper.

Qualitative identification

Paper strips were soaked in electrolyte and blotted in order to remove excess solution before application of 5 μl of 1% (w/v) solutions of choline salts and esters in distilled water. Conditions for the separations are shown in Table I. After drying at 110° for 5 min pherograms were sprayed with Dragendorff's reagent and R_M values calculated with respect to choline chloride.

Quantitative separations

Ten microlitres of solutions containing from 5 to 100 μg of standard were applied by means of Drummond Microcap pipettes (Drummond, Broomall, Pa., U.S.A.) to separate origins on paper strips impregnated with 8% formic acid solution (pH 1.8). Electrophoresis was carried out for 20 min at 2,500 V and pherograms were dried before spraying with Dragendorff's reagent. The outlines of the spots produced were carefully traced onto 1-cm graph paper and spot areas calculated.

TABLE I

MIGRATION VALUES, RELATIVE TO CHOLINE CHLORIDE, AND EXPERIMENTAL CONDITIONS

Electrolytes:

I= pH 1.8, formic acid 8% (v/v); 2,500 V, 20 min.
II= pH 3.1, ammonia (0.88) 20 ml, formic acid (90%) 50 ml and water to 2.5 l; 1,300 V, 30 min.
III= pH 4.2, 0.2 *M* potassium hydrogen phthalate 50 ml, 0.2 *N* sodium hydroxide 3.7 ml and water to 200 ml; 2,500 V, 25 min.
IV= pH 5.1, 1×10^{-5} *N* hydrochloric acid; 3,500 V, 15 min.
V= pH 5.8, ammonium chloride 0.05 g/l; 3,500 V, 15 min.
VI= pH 6.5, pyridine 50 ml, acetic acid 2 ml and water to 1 l; 3,500 V, 15 min.
VII= pH 7.2, collidine 4.7 ml, acetic acid 1.3 ml and water to 500 ml; 3,500 V, 15 min.
VIII= pH 10.4, 0.2 *M* sodium hydroxide 43.9 ml; 0.2 *M* boric acid 50 ml, 0.2 *M* potassium chloride 50 ml and water to 200 ml; 2,000 V, 30 min.

Compound	*Migration value*								*Colour with Dragendorff's reagent*
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	
Choline chloride	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	orange-red
Acetylcholine chloride	0.85	0.84	0.87	0.86	0.89	0.92	0.91	0.91	orange
Propionylcholine chloride	0.79	0.79	0.83	0.90	0.88	0.86	0.89	0.89	orange-yellow
Butyrylcholine chloride	0.74	0.70	0.80	0.82	0.81	0.81	0.82	0.81	orange-yellow
Methacholine	0.79	0.78	0.85	0.91	0.86	0.81	0.87	0.89	orange
Acetyl-β-methyl choline	0.81	0.82	0.87	0.81	0.87	0.86	0.86	0.86	orange
Choline dihydrogen citrate	1.04	1.04	1.04	1.00	1.04	1.04	1.03	1.05	orange-red
Choline theophyllinate	1.03	1.02	1.02	1.00	1.02	1.03	1.02	1.02	orange-red
Carbachol	0.84	0.88	0.91	0.87	0.92	0.89	0.89	0.92	orange-red

Calibration graphs were established for each pherogram by plotting the logarithm of the weight of choline salt or ester against the spot area.

Sample preparation was carried out by extraction and dilution with water to give a final concentration of choline salt or ester in the range 0.5–7.5 μg/μl.

RESULTS AND DISCUSSION

Qualitative separations

Migration values for choline salts and esters are given in Table I. The most satisfactory separation of choline salts from the esters occurred under low pH conditions, optimum resolution being obtained using 8% formic acid as electrolyte. It was found possible to resolve the acetyl, propionyl and butyryl esters and methacholine from each other but not carbachol, which exhibited similar migration values to acetylcholine. As the pH of the electrolyte was increased the R_M values tended to converge. The technique was also found valuable for rapid detection of the occurrence of decomposition products of choline esters during storage, both choline and acetylcholine being easily detected.

Quantitative determinations

Correlation coefficients were calculated for each pherogram and found to be close to unity in all cases (minimum value 0.97). A linear relationship was established between the limits 10 to 75 μg of choline salt or ester per spot, the minimum detection limit being 5 μg. This method is less sensitive than previously attained by paper chromatography[3]. Calibration graphs constructed for successive pherograms, although linear, were displaced and showed slightly differing slopes. As separations were carried out under identical conditions, these slight differences were attributed to variations in impregnation of the papers with electrolyte. Thus, it was found necessary to run sample and standard solutions on each paper strip and to construct calibration graphs for each pherogram.

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Note

Polyacrylamide gel electrophoresis of alginic acid

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Polyacrylamide gel electrophoresis has been used extensively in the study of proteins[1] but much less in work on polysaccharides. It is an unsatisfactory method for separating classes of acid mucopolysaccharides because the sieving effect of the medium does not allow separation purely on the basis of charge density. However, in the polyuronide alginic acid the ratio of carboxyl groups to sugar residues is constant and movement on gels should be related closely to the molecular weight.

Polyacrylamide gel electrophoresis has been developed in this laboratory to provide a rapid and convenient method for assessing the "quality" of alginic acid samples.

METHODS AND MATERIALS

Various commercial alginate samples were investigated. Before study they were dialysed against 0.4% NaCl containing 0.4% EDTA, pH 8.5, then against distilled water and finally lyophilised. Samples of other polysaccharides were used without purification.

Most chemicals were obtained from BDH, Poole, Great Britain. Acrylamide and N,N′-methylene bisacrylamide were recrystallised as described by Shuster[2], Alcian Blue 8GX (now known as 8G, ref. 3) was from George T. Gurr, London S.W. 9, Great Britain.

The polyacrylamide gel for the routine study of alginate samples consisted of 6% (w/v) total monomer with N,N′-methylene bisacrylamide constituting 2% of the monomer. Gels were prepared in uniform bore glass tubes, 5 mm I.D. Monomers were dissolved in 0.25 M Tris–HCl, pH 8.3 containing 0.05% of the accelerator TEMED (N,N,N′,N′-tetramethylethylenediamine) and sufficient ammonium persulphate was added to give a final concentration of 0.08%. Aliquots (1.0 ml) were transferred from the bulk to the tubes using an automatic pipette. At 20° polymerisation was complete 20 min after the addition of the ammonium persulphate.

A discontinuous buffer system, based on the strategy of Williams and Reisfeld[4] was used. The sample gel was 6% (w/v) acrylamide polymerised without cross-linker in 0.02 *M* MOPS (3-(N-morpholino)propanesulphonic acid)–NaOH, pH 7.3. Alginate samples (20–60 μg in water) were mixed with the viscous sample gel (100 μl) *in situ* in the electrophoresis tube and 5 μl of 0.01% bromophenol blue was added as a

marker. The running buffer (0.1 *M* glycine–Tris, pH 9.0) was layered on top of the sample gel.

Electrophoresis proceeded for 90 min at 20° under 100 V (approx. 4 mA per tube). Gels were removed from the glass tubes by rimming with a syringe needle lubricated with water, and stained overnight in 0.08% Alcian Blue in 7% (v/v) acetic acid. Excess Alcian Blue was removed electrophoretically.

Alcian Blue is a permanent stain and gels may be stored indefinitely in the light in 7% acetic acid without appreciable fading. Records of gels were made either photographically or by scanning in a Joyce, Loebl & Co. Chromoscan densitometer.

RESULTS AND DISCUSSION

Electrophoresis of an alginic acid sample on gels of different pore sizes (Fig. 1) produced a broad band of slow-moving material and a sharp band of anodic material that coincided with the electrophoretic front, indicated in unstained gels by a band of marker dye. The maximum spread of stained material occurred in the gels of largest pore size but, since 4% and 5% gels with 2% cross-linker are soft and difficult to handle, 6% gels with 2% cross-linker were chosen as standard gels for further studies.

Fractionation of an alginate sample on Sephadex G-200 followed by gel electrophoresis confirms that the anodic band is due to material of low molecular weight (Fig. 2). The sample used here is an extreme case, being very polydisperse; a sample of sodium alginate supplied by BDH is much less disperse.

The method has been used for enzyme studies. Fig. 3 indicates the effect of incubating alginate with an alginate lyase partially purified from a marine *Pseudomonad*. This indication of enzyme action can be achieved rapidly without the use of viscometers or chemical methods for detecting breakdown products.

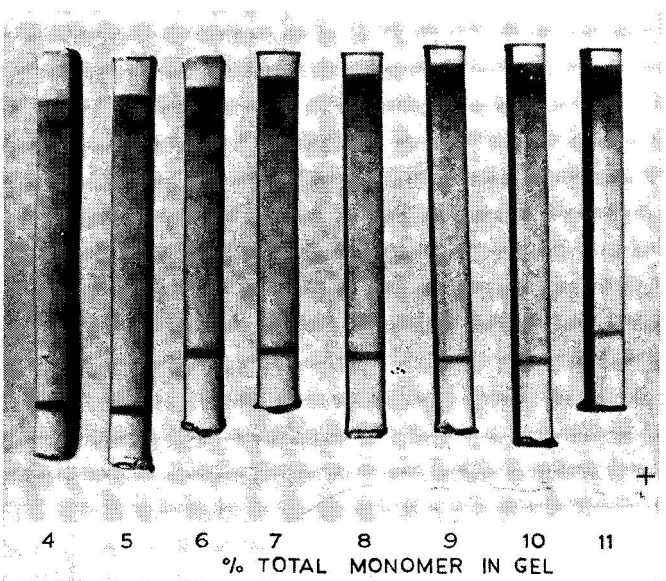

Fig. 1. Electrophoresis of sodium alginate (20 μg) with acrylamide contents from 6 to 11%. Each gel contained N,N′-methylene bisacrylamide as 2% of the total monomer.

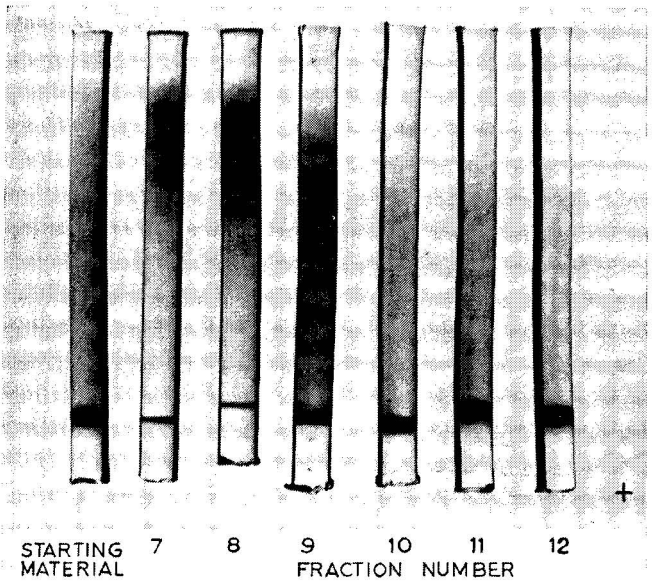

Fig. 2. Electrophoresis of sodium alginate fractionated by gel permeation on Sephadex G-200 using 6% gels.

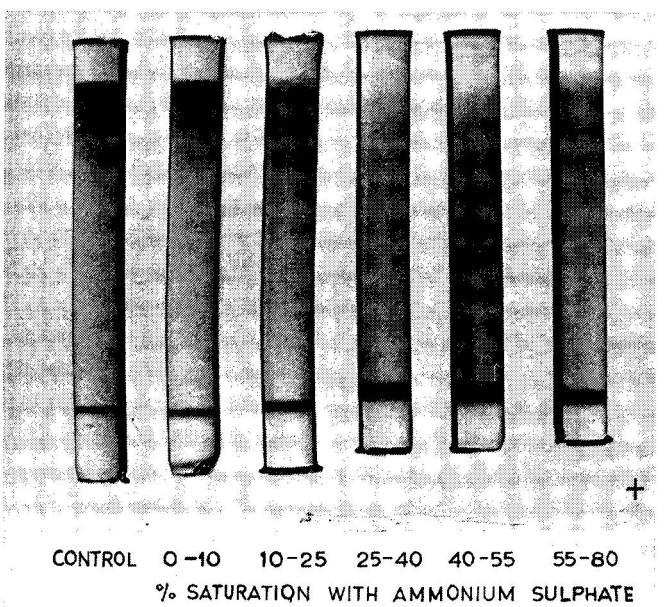

Fig. 3. Electrophoresis of alginate samples after incubation with ammonium sulphate fractions of protein from a marine bacterium.

Although the method was developed with the aim of investigating alginic acid preparations it is equally applicable to other acidic polysaccharides such as pectins and possibly carrageenans if it is known that the charge density is constant throughout the sample. The method can be used semi-quantitatively when coupled with densitometry but since the dye used, Alcian Blue 8GX, is probably a mixture of four geometrical isomers[3] the dye–alginate complex may not be stoichiometric.

The molecular weights of alginates could be determined using this method if samples of accurately-known molecular weight were available. The first fraction from Sephadex G-200 (Fig. 2) is totally excluded and will have a molecular weight in the range 400,000 to 800,000; the last fraction will have a molecular weight of around 20,000. A wider range of molecular weights can be studied using smaller pore gels in addition to those used here. Such gels would be of use in studying food-grade pectins.

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Note

An evaluation of the gas chromatographic estimation of trace quantities of hexachlorophane

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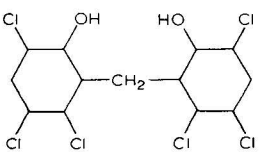
(First received June 13th, 1973; revised manuscript received August 29th, 1973)

Although the effectiveness of hexachlorophane (2,2'-methylenebis(3,4,6-trichlorophenol); HCP) as an antimicrobial agent is well defined[1], recent events[2,3] have led to speculation regarding its continued general use [1,4–7] and the introduction of new regulations[8]. At the present time, however, HCP continues to be widely used under controlled conditions, notably in hospitals[9]. Following recent toxicity reports[1,10–13] it was considered to be desirable that we should monitor the blood levels arising from the topical application of this drug to patients participating in clinical trials. The preliminary clinical results have been reported elsewhere[14], and the object of this present report is to discuss the problems which became evident during the gas chromatographic estimations.



HCP may be assayed colorimetrically[15] or spectrophotometrically[16], but gas–liquid chromatography (GLC) is undoubtedly the method of choice in the absence of more sophisticated and costly instrumentation. Although it may be chromatographed directly[17,18], the presence of two phenolic groups on this aromatic molecule renders the preparation of some volatile derivative necessary[19] if the problems of long retention times and unsymmetrical peaks are to be avoided. Porcaro[17] has overcome these problems by the use of very short columns (8 to 12 in.) and a high carrier gas flow-rate in the detection of amounts of HCP greater than 5 μg.

The formation of trimethylsilyl (TMS) derivatives[19–21] has been advocated, as have acetylation[22] and methylation[10]. This latter technique was not considered further since the methods applied to HCP have involved the use of diazomethane,

which can be hazardous[23], although it has recently been used by Ferry and McQueen[24] in conjunction with a comparatively short GLC column where it yielded improved peak symmetry over free HCP. The acetylation procedure described by Browning *et al.*[22] was selected as being the most appropriate for this study, and is based upon the partition of whole blood (3.0 ml) with ethyl acetate (10.0 ml), followed by concentration of the extract and its subsequent reaction with a mixture of equal volumes of acetic anhydride and pyridine (0.1 ml).

An instrument fitted with a flame ionisation detector (FID) may be employed in the estimation of HCP in pharmaceutical preparations[19], but for monitoring the trace therapeutic blood levels[2,10] arising from the topical application of this drug, an electron capture detector (ECD) is necessary. Gudzinowicz[25] has evaluated the ^{3}H ECD for the detection of HCP, and this detector was also used by Browning *et al.*[22]; the use of the helium discharge ECD has been reported by Porcaro and Shubiak[20]. The ^{63}Ni ECD used in this present study has been shown to give an erratic response following the direct injection of HCP[20] thus rendering the preparation of some volatile derivative necessary in conjunction with this detector.

EXPERIMENTAL AND RESULTS

A Pye Series 104 gas chromatograph (Pye-Unicam, Cambridge, Great Britain) was fitted with a 1.0 m × 4 mm I.D. glass column, packed with 3% Silicone OV-17 on Gas-Chrom Q, 80–100 mesh[26] (Phase Separations Ltd., Queensferry, Great Britain) which was maintained at 265°. The ECD, which comprised 7.5 mC of ^{63}Ni, was heated to 285° and was used in the pulsed mode at 50 μsec pulse space. The carrier gas was high-purity (oxygen-free) nitrogen at a flow-rate of 50 ml/min, and an ECD purge of 10 ml/min. Injections were made into a heated zone above the column at a temperature of *ca.* 300°.

In a typical chromatogram (Fig. 1a) one major peak is observed which correlates with the amount of acetylated HCP (AHCP) injected. Quantitative data were based upon the peak area function:

Peak height × peak width at half its height

The measurements were taken from a tangent drawn across the baseline of this major peak. The calibration graph of this function *versus* concentration of HCP (Fig. 2) gave a comparatively narrow linear concentration range, as would be expected from an ECD of this type. This problem may be overcome by the preparation of a series of calibration graphs at various attenuation settings corresponding to several concentration ranges.

Having established the linear range at each setting, the injection of a standard alongside each sample (corresponding to a concentration within the linear range of the appropriate calibration graph) presents a more practicable technique than the evaluation of a calibration graph together with each series of samples. This latter approach would also introduce problems which may arise from the lack of long term stability and reproducibility of the ECD.

No internal marker is used in this method[22], and it is therefore necessary to establish the reproducibility between successive replicate injections from the syringe. During the preparation of the calibration graph, it was observed that the reproducibility between the peak area functions (defined earlier) arising from duplicate injec-

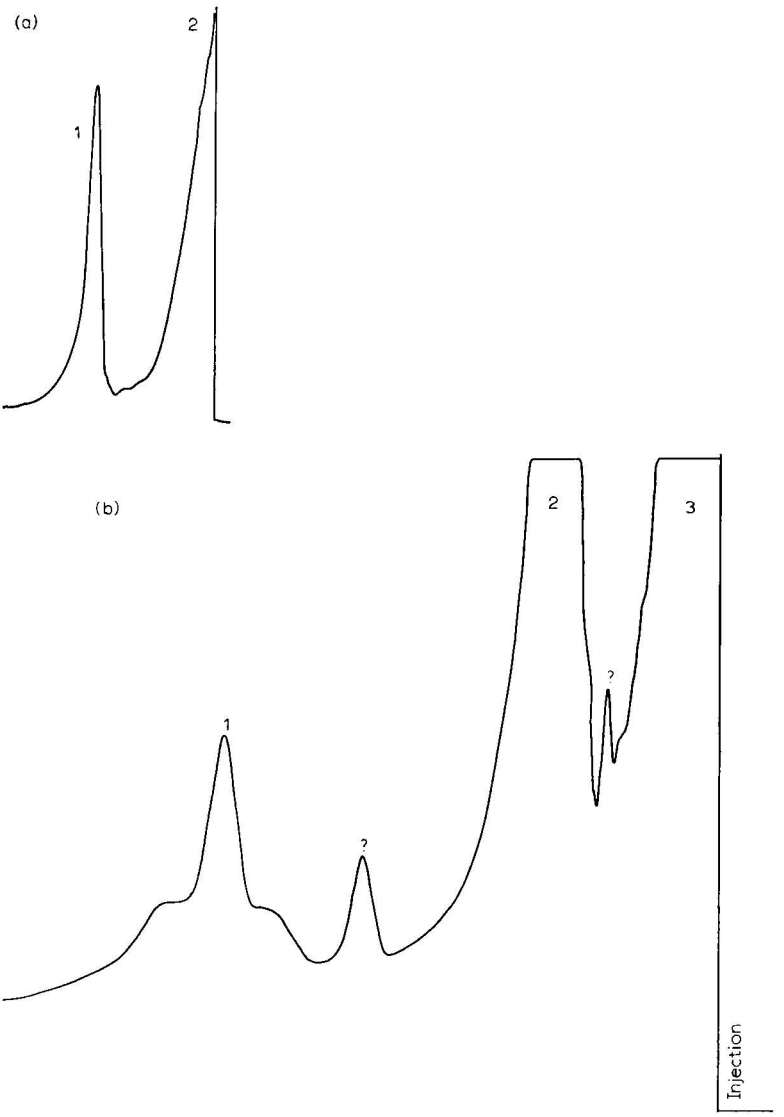


Fig. 1. Chromatogram of 3 μl acetylated hexachlorophane in ethyl acetate. (a) Original concentration, 100 ng/ml. 1= Hexachlorophane; 2= ethyl acetate. (b) Original concentration, 250 ng/ml. 1= Hexachlorophane (secondary peaks); 2= hexachlorophane (major peak); 3= ethyl acetate.

tions of the same solution of AHCP tended to decrease at higher concentrations with the linearity of the graph.

In view of the limitations of the ECD response, care was taken to ensure that quantitative data were based upon chromatograms in which the peak from the sample and standard alike emerged at a similar height on the solvent tail.

The preliminary results demonstrated the need for a comprehensive evaluation of the acetylation conditions[22]. Aliquots (5.0 ml) of a solution of HCP in ethyl acetate

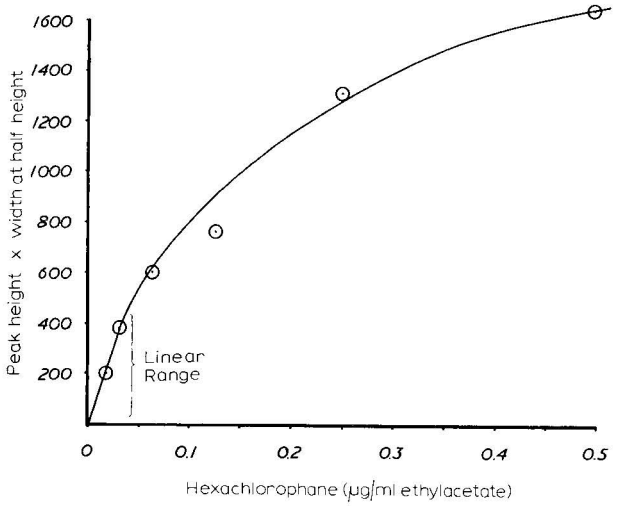


Fig. 2. Calibration graph (0–500 ng/ml).

at a concentration of 0.1 μg/ml were evaporated to *ca.* 0.5 ml and were then acetylated for 0, 5, 10, 15, 20, 25, and 30 min at 60°. Further aliquots (5.0 ml) were evaporated to dryness and volumes of 0.1, 0.2, 0.5 and 1.0 ml, respectively, and each of these was then acetylated for 10 min at 60°.

All the solutions were subsequently evaporated to dryness in a stream of nitrogen, and were reconstituted with ethyl acetate (100 μl) prior to injection into the chromatograph. The results, which are summarised in Tables I and II, respectively, indicate that complete acetylation is achieved very rapidly and is not dependent upon the concentration of HCP at that stage within the working range of the method.

Browning *et al.*[22] evaporated the solution to a volume of 1.1 ml after acetylation, but it was found more convenient to evaporate the solution to dryness in a conical test tube, care being taken to wash the AHCP into the base of the tube with ethyl acetate. The extracts could then be stored in a refrigerator under these conditions for several days without loss of AHCP. Reconstitution was achieved by washing the sides of the tube with 100 μl of ethyl acetate from a microlitre syringe and leaving to stand for *ca.* 5 min at room temperature. The tube was also warmed in the hand to assist the dissolution of the AHCP.

TABLE I

THE EFFECT OF THE ACETYLATION TIME UPON THE RECOVERY OF HEXACHLOROPHANE

	Time (min)				
	0	*5*	*15*	*20*	*30*
Peak function (mm^2)*	728	672	654	692	647

* Defined in the text.

TABLE II

THE EFFECT OF CONCENTRATION AT THE ACETYLATION STAGE UPON THE RECOVERY OF HEXACHLOROPHANE

	Volume of ethyl acetate extract at acetylation (ml)			
	0	*0.1*	*0.5*	*1.0*
Peak function (mm^2) *	750	725	726	726

* Defined in the text.

This modification to the technique, with an effective eleven-fold increase in the concentration of AHCP at the GLC stage, permits the detection of a concentration of 100 pg of HCP in 1.0 ml of ethyl acetate, corresponding to a blood level of 330 pg/ml assuming complete extraction from the blood. A chromatogram of AHCP at this level is illustrated in Fig. 3. This limit of detection represents a considerable improvement upon that of 0.05 μg/ml reported in the original paper[22], and is somewhat lower than that obtained by Porcaro and Shubiak [20] and Curley *et al.*[10]. It may be extended by the use of the lower attenuation settings, which do, however, result in excessive tailing of the solvent peak which may mask the AHCP peak.

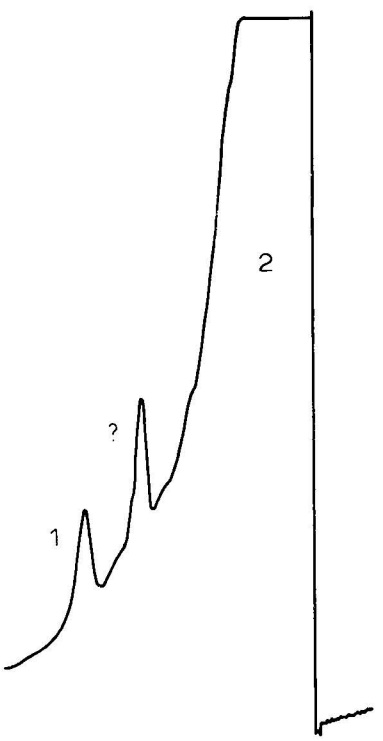


Fig. 3. Chromatogram of 3 μl acetylated hexachlorophane in ethyl acetate. Original concentration, 100 pg/ml. 1 = Hexachlorophane; 2 = ethyl acetate.

In addition to the major AHCP peak, a number of secondary peaks was also observed. They had considerably longer retention times than the major peak, but appeared to be a function of the amount of AHCP injected. These peaks become evident when a more concentrated extract is injected at a comparatively low attenuation setting, as shown in Fig. 1b. The slight leading of the major AHCP peak may be attributed to decomposition in the heated injection zone, rather than the elution of some component with a retention time similar to that of AHCP since the injection of pure solvent did not reveal any 'ghosting' of this peak[27].

Some unidentified peaks appear in all chromatograms, and it is evident that the artefacts on the solvent peak arise from the acetylation mixture. The peak which is eluted just before the major AHCP peak does not originate from the solvent, since it does not give a constant response when several aliquots of pure solvent are injected, and it would not be expected to arise from the column packing material, which was conditioned at 300° before use. Impurities in the carrier gas supply line or plasticisers from the septum[28] cannot be discounted, particularly in conjunction with an ECD, which is far more susceptible to such effects than is the FID. This peak is present in all the chromatograms to a greater or lesser extent, whereas that which is eluted with a retention time slightly longer than that of AHCP is not always observed. One possible origin of these two peaks is, however, the adsorption and retention of a trace of AHCP on the column, and its subsequent elution with each injection of solvent.

This hypothesis is supported by the pattern of anomalous relationships between the peak area function and concentration which were obtained during a preliminary calibration. An explanation may, however, be postulated in terms of the uptake of AHCP by active binding sites on the column system, which may be subsequently eluted with a correspondingly shorter retention time upon the injection of pure solvent. An injection of AHCP would then be partially absorbed onto the regenerated binding sites, resulting in an anomalously low value for that peak area function. The negligible size of the two peaks during the early stages of the work (as in Fig. 1a) and their increase in area as the work progressed (Figs. 1b and 3) lend further support to this explanation.

It should be stressed that no unidentified peaks coincide with the elution of AHCP, since injections of pure solvent yield only the two peaks which have already been discussed. The effect of these peaks upon the quantitative data will be minimal, provided standard reference solutions are injected alongside each sample.

The 'on-column' retention of free HCP has been observed by Browning *et al.*[22], but no such problems were reported from AHCP. The choice of Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.), which is one of the most inert supports currently available[29] would be expected to minimise any absorption problems. The precision of the method as a whole was subjected to a comprehensive evaluation by the original authors[22] and has not been investigated further.

It is essential that attention should be directed towards the potential problems associated with this assay, and their resolution, if valid results are to be obtained.

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Harrogate for the loan of the ECD equipment, and to Vick International for the supply of hexachlorophane. Thanks are also due to Messrs. K. S. P. Andrews and P. J. Eveleigh, and Miss J. C. Lewis for useful discussions, and to Miss E. Walker for the preparation of the original manuscript.

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CHROM. 7095

Note

Indomethacin estimation in plasma and serum by electron capture gas chromatography

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Investigations involving estimation of indomethacin concentrations in biological fluids have formerly utilized spectrofluorimetric or radioisotope techniques. Metabolites and co-extractives may interfere with estimations utilizing these methods.

The method described below utilizes electron capture gas chromatography of a derivative of indomethacin. Its freedom from interference has enabled it to be successfully applied to a study of the interaction of indomethacin and aspirin[1].

EXPERIMENTAL

One milliliter of plasma or serum was placed in a 40-ml glass-stoppered centrifuge tube. Two milliliter of Sorensen's buffer, 0.1 *M*, pH 5.0, and 10 ml of 10% amyl alcohol in hexane were added and the tubes shaken for 15 min on a vortex mixer. After centrifuging to separate the phases, 9 ml of the organic layer were transferred to a graduated tube. The extract was concentrated to approximately 0.5 ml on a 52° water-bath under a gentle stream of dry air. Approximately 0.3–0.5 ml of a hexane solution of diazoethane (prepared from N-ethyl-N′-nitro-N-nitrosoguanidine) was added, the samples were mixed on a vortex mixer and after a few minutes the excess diazoethane was removed and the solution concentrated under an air stream on the water-bath as above. After dilution to an appropriate volume with hexane, the sample was analysed by gas–liquid chromatography. The indomethacin was quantitated by comparison of the peak heights for samples and standards. Stock standard solutions were prepared in ethanol and stored under refrigeration. Dilute standards were prepared daily from these stock solutions. As the ethylated derivatives may not be stable when exposed to sunlight, it is considered advisable to protect extracts and dilute standard solutions as much as possible.

The gas chromatograph used was a Varian Model 2100 one (Varian, Palo Alto, Calif., U.S.A.) fitted with tritium electron capture detectors. The Pyrex column was U-shaped, 1 m × 2.5 mm I.D. packed with 2% OV-1 on 100–120 mesh Chromosorb W AW-DMCS, prepared by evaporation–fluidisation and conditioned for 48 h at 225°. Operating conditions were: oven temperature, 190°; injector port temperature, 225°; detector block temperature, 275 ; nitrogen carrier gas flow-rate 60 ml/min. The retention time of the ethylated derivative was 9 min.

RESULTS AND DISCUSSION

The recoveries of known amounts of indomethacin (0.1–5 μg/ml) added to serum are shown in Table. I The increase obtained by using two extractions instead of a single one was not considered sufficient to warrant the additional labour. The use of 10% amyl alcohol in hexane was found to be particularly important when extracting samples which had been stored deep-frozen for more than a few days. For example, a mixture of 1.5% amyl alcohol in hexane gave recoveries up to 98% when the extraction was carried out immediately after addition of the indomethacin to

TABLE I

RECOVERIES OF INDOMETHACIN

% amyl alcohol in hexane	*Number of extractions*	*Recovery (% ± S.D.)*	*Number of determinations*
5	1	71 ± 3	10
10	1	90 ± 3	22
5	2	92 ± 4	36
10	2	96 ± 3	24

plasma. When the samples were stored deep-frozen and subsequently analysed, the recovery decreased with time. After three weeks the recovery had fallen to 10%. Increasing the amyl alcohol concentration to 10% resulted in complete recovery of the indomethacin even after six weeks storage. The practice of analysing the samples as soon as possible after they are obtained is considered advisable as the effect of prolonged storage on indomethacin levels in experimental or clinical samples is not known. Other methods have used either 3% or 5% amyl alcohol in heptane. Hexane was used in this method as it is more readily removed during evaporation on the water-bath than heptane. It offers the additional advantage that amyl alcohol and hexane form an azeotropic mixture and by repeated addition of hexane the amyl alcohol can be completely removed permitting application of cleanup procedures or other derivative techniques if required.

The ethylation of indomethacin was essentially instantaneous and considerably improved the chromatographic response of indomethacin (Fig. 1), which was linear over the range of 0.1–3.0 ng injected (Fig. 2). The ethyl derivative was used as it permits estimation of indomethacin in the presence of one of its principal metabolites, desmethylindomethacin, which can be extracted by the above procedure. Under the above operating conditions the peaks corresponding to the ethylated derivatives of indomethacin and desmethylindomethacin are not completely separated. The separation is sufficient, however (Fig. 1) to ensure that a peak height for desmethylindomethacin of up to 10% of that of indomethacin does not significantly interfere. If the amount of desmethylindomethacin did appear to interfere with the estimation of indomethacin, the column length and conditions could be altered accordingly. In studying the interaction of indomethacin and salicylate the levels of desmethylindomethacin in the blood of volunteers was below the limits of detection.

Some other reagents were examined for the preparation of derivatives but they did not offer any advantages over diazoethane. Methylation of indomethacin with diazomethane produced a derivative with a slightly greater response and shorter

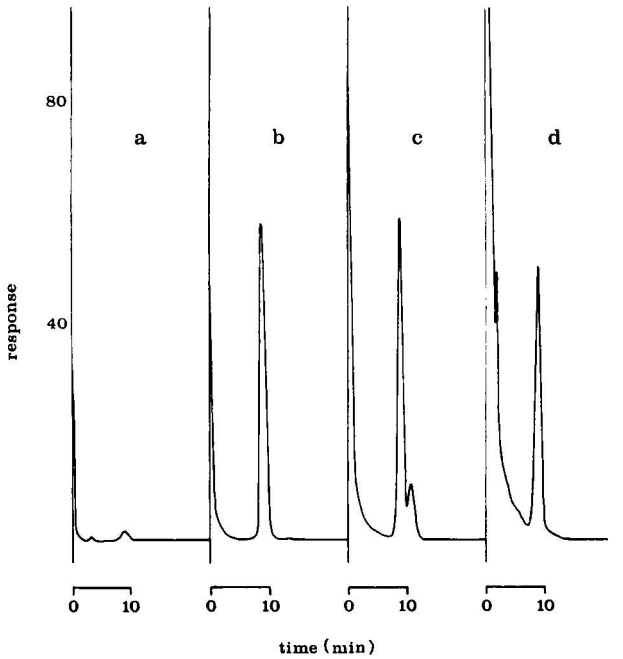

Fig. 1. Chromatograms of indomethacin. (a) 20 ng non-derivatised; (b) 2.5 ng ethylated indomethacin; (c) 2.5 ng ethylated indomethacin+0.25 ng ethylated desmethylindomethacin; (d) extract of human serum estimated to contain 0.5 μg/ml indomethacin.

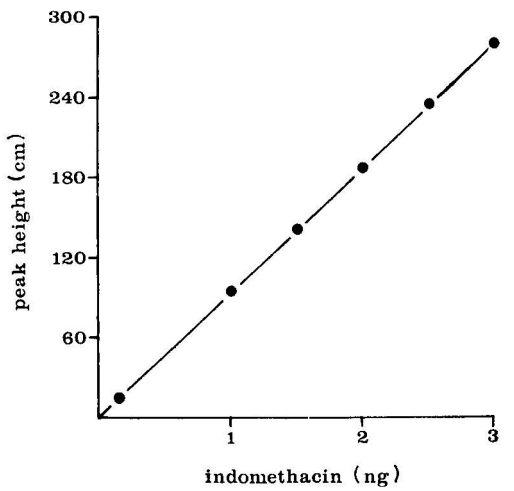

Fig. 2. Detector response of indomethacin.

retention time than the ethylated derivative but also converted desmethylindomethacin to indomethacin. N,O-Bis (trimethylsilyl)acetamide gave derivatives which could be distinguished by gas chromatography and BF_3–methanol formed a derivative with indomethacin only.

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CHROM. 7118

Note

A chromatographic comparison of the constituents of nutmeg and mace (*Myristica fragrans* Houtt.) with those of marihuana and hashish (*Cannabis Sativa* L.)*

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In recent years, there has been a marked increase in the illicit use of cannabis products, such as marihuana and hashish, necessitating the development of simple accurate "field tests" for such products. A rapid, sensitive and reasonably specific test of this type has been described by De Faubert Maunder[1] whereby products seized by law enforcement agents and suspected of containing cannabis products can readily be provisionally identified. The test involves a simple extraction of the suspected material with light petroleum (in practice lighter fluid is used). The extract, applied to a filter paper, is treated with a solution of Fast Blue salt B (FBB) after drying. A positive result is indicated by the rapid development of a red to violet colour[1]. Of approximately two hundred plant materials examined by this author[1] only two were found to give strongly false positives to this test, *i.e.* nutmeg and mace, both products of the tree *Myristica fragrans* Houtt. Of these two spices the colour produced by nutmeg is more intense than that produced by mace. Other interfering plant materials include agrimony which also gives a false positive reaction, although the colour produced is of a much paler hue than that obtained with cannabis[2]. Henna also gives a false positive result but only if an excessively large sample is tested[2].

A search for the psychoactive constituents of these spices, which have a long history of abuse (*i.e.* "non-culinary" use) and which have been used as substitutes for cannabis (*cf.* review by Forrest and Heacock[3]) lead us to make a chromatographic comparison of light petroleum extracts of marihuana and hashish with those of nutmeg and mace. Suitable extracts of these materials were subjected to thin-layer chromatography (TLC) in various solvent systems; the developed plates sprayed with the FBB reagent and the nature of the resulting coloured spots which developed, compared for identification purposes.

EXPERIMENTAL

Materials

Freshly ground nutmeg (or mace) (5 g) was shaken with 50 ml of light petroleum

* Issued as NRCC No. 13697.

(b.p. 30–60°) for 5 min. The resulting suspension was filtered and stored at 4° overnight and any solid that precipitated out was removed by filtration. The filtrate was concentrated to a total volume of *ca.* 10 ml.

Reference samples of Δ^9-tetrahydrocannabinol, Δ^8-tetrahydrocannabinol, cannabidiol, mixed hashish resin reference standard and standardized *Cannabis sativa* L. were kindly supplied for the purposes of this investigation by the Health Protection Branch, Health and Welfare, Canada. The mixed hashish resin reference standard is the purified phenolic fraction isolated from hashish and contained approximately 35% cannabidiol, 39% Δ^9-tetrahydrocannabinol, 18% cannabinol and other cannabinoids which have not been quantitated. The standardized *Cannabis sativa* L. was finely ground and extracted with light petroleum (b.p. 30–60°), in a similar manner to that used for the spices nutmeg and mace. The extract was evaporated to dryness and the residue redissolved in chloroform to give a similar extract concentration to that which had been used above for the nutmeg and mace.

Chromatography

Thin-layer chromatography. Commercially available silica gel F_{254} (Brinkmann, Westbury, N.Y., U.S.A.) pre-coated thin-layer plates (thickness 0.25 mm) were used. The plates were developed in the solvent systems listed below. Approximately 20 μg of the various spice and plant material extracts were applied to the plates in each case.

Chromogenic reagent. A 0.1% solution of FBB (Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A.) in 70% ethanol was freshly prepared prior to use.

Solvent systems used. (I) light petroleum (60–80°)–ethyl acetate (5:1); (II) light petroleum (60–80°)–ethyl acetate–ether (90:5:5); (III) light petroleum (60–80°)–diethyl ether (4:1); (IV) *n*-hexane–ether–acetic acid (87:12:1); (V) *n*-hexane–acetone–diisopropyl ether (10:1:1). The light petroleum (b.p. 60–80°) used in solvent systems I–III was AnalaR grade and was obtained from BDH, Poole, Great Britain.

RESULTS AND DISCUSSION

The colours obtained from the light petroleum extracts of nutmeg, mace, marihuana (*i.e. Cannabis sativa* plant material as supplied) and hashish resin, after samples of these extracts had been spotted onto paper, cellulose layers or silica gel layers and sprayed with the FBB reagent were all red to red-violet in nature and were virtually indistinguishable to the naked eye. However, it can readily be seen from Figs. 1–3 that comparison of the TLC behaviour of the extracts of nutmeg (A), mace (B), marihuana (C) and hashish (D) readily distinguishes the substances in question. The chromatograms were obtained using the developing solvents listed above and with triple development in each case. In view of the use of a multiple development technique no R_F values are reported in this paper. Numerous solvent systems have been described in the literature for the TLC of cannabis products (*cf.* refs. 4–6). The first four solvents described above have been previously reported in the literature for this purpose, whilst as far as the authors are aware the final, useful solvent V, has not been used before for the separation

of cannabinoids. Whilst the results of using only the first three solvent systems are given in this paper, those obtained with the other two are basically similar in nature.

The De Faubert Maunder field test procedure for cannabis derivatives elutes essentially the "fat-soluble" phenols, such as the tetrahydrocannabinols from the plant material. Such compounds, which will couple with the stabilised diazonium salt FBB (tetra-azotised di-*o*-anisidine), produce the typical red colour given by a phenol. A brief extraction of ground nutmeg and mace with light petroleum also readily extracted some "fat-soluble" phenols, however this solvent also extracts a vast quantity of unwanted fatty material in both cases. The trimyristin, which makes up about 80% of the total weight of the nutmeg, does however readily precipitate out of solution on cooling and can be removed by filtration, prior to carrying out the TLC.

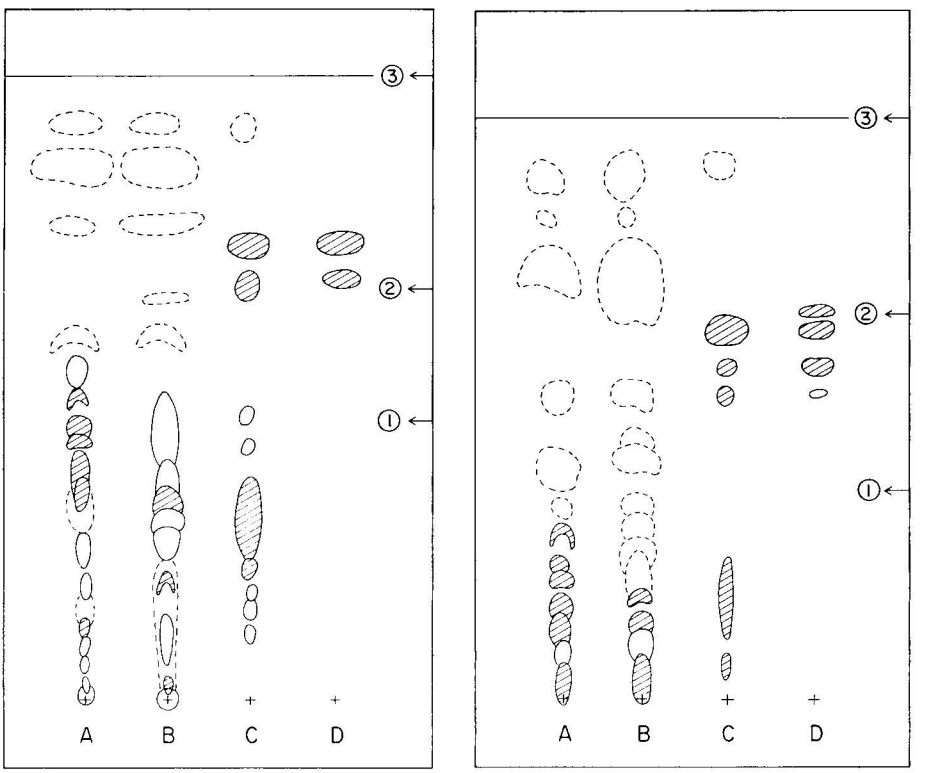

Fig. 1. TLC on silica gel F_{254} of light petroleum extracts of: (A) nutmeg; (B) mace; (C) marihuana and (D) hashish. Solvent I, multiple development was used, the solvent being allowed to ascend to position 1 in the first instance; the plate was then removed from the tank, dried and re-run to position 2; this procedure was repeated and the final development was allowed to proceed to position 3. Visualising reagent: FBB (freshly prepared); spots indicated by dotted lines did not give red colours with FBB and were located either by observing the developed plate in UV light or by dull yellow-brown colours given with FBB; spots indicated by solid line gave weak red colours with FBB and spots indicated by solid line with cross-hatching gave a strongly positive reaction with FBB.

Fig. 2. As for Fig. 1, except that solvent II was used.

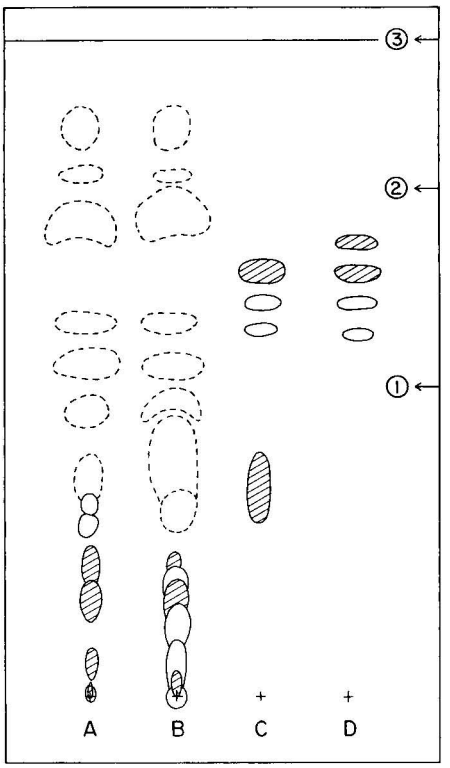

Fig. 3. As for Fig. 1, except that solvent III was used.

It is interesting to speculate on the chemical structures of the FBB positive compounds in nutmeg and mace. The presence of some relatively simple phenolic phenylpropanoids, such as eugenol, isoeugenol and methoxyeugenol has been known for some time (*cf.* ref. 3). However these products do not give red colours with the FBB reagent. This is not too surprising, since they do not have positions *para* to the phenolic $-OH$ group available for the *para*-coupling reaction with the diazonium salt to occur. Recently, a number of dimeric phenylpropanoids has been isolated from nutmeg and mace[8–12]. However, it is doubtful if these products are responsible for the production of the red colours since in the vast majoritity of cases they do not have the structural requirements that would be expected to give a strongly positive test with the diazo reagent.

In all the solvent systems utilized, the cannabinoids can readily be distinguished from the nutmeg and mace phenolics (see Figs. 1–3), which cannot be demonstrated by the preliminary field test. The cannabidiol, cannabinol and tetrahydrocannabinol have high R_F values in the systems used, whilst the cannabinoids having an acid function in the molecule tend to migrate more slowly[4]. These studies have shown that with the chromatographic systems used for the FBB positive substances extractable from nutmeg and mace with cold light petroleum tend to be in the lower half of the chromatogram in the cannabinoid acid region.

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Book Review

Techniques of Lipidology: isolation, analysis and identification of lipids, by M. Kates, North-Holland Publishing Co., Amsterdam, London, 1972, 610 pp., price Dfl. 36.00.

This well-produced pocket-monograph collects the basic knowledge and experimental procedures necessary for the study on the chemistry and metabolism of lipids. The contents of the book are divided into seven chapters. The first chapter contains the definition and classification of lipids. In the second part solvents, reference compounds, glassware and some analytical instruments available for lipid analysis are briefly described. Various general and analytical procedures are discussed in detail in the additional two parts. The most commonly used and effective fractionating methods are summarized in the fifth chapter. The subsequent chapter deals with the application of radioisotopic methods to lipid chemistry. The final section supplies much information on the identification of individual lipids and lipid moieties. From the chromatographic point of view, the last three chapters are of the greatest value. All fundamental column, paper, thin-layer, and gas–liquid chromatographic techniques suitable for the qualitative and quantitative determination of lipid classes are described and discussed here. The methods can serve as a starting-point for everyone who wants to inform himself about the present-day possibilities of lipid chromatography.

The author includes in the book his personal experiences resulting from many years of fruitful research. All the methods and procedures that he has proved to be most reliable and useful are presented in their most recently modified form.

The reviewer feels that the detailed presentation of the techniques and procedures concentrated in this monograph will be certainly wellcomed by everyone interested in lipidology.

Prague (Czechoslovakia) Č. MICHALEC

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Paper Chromatography

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1031 Hatch, M. D. and Mau, S.-L.: Activity, location and role of aspartate aminotransferase and alanine aminotransferase isoenzymes in leaves with C_4 pathway photosynthesis. *Arch. Biochem. Biophys.*, 156 (1973) 195–206 —polyacrylamide gel.

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20m. Enzymes: ligases

1048 Rao, D., Beyreuther, K. and Jaenicke, L.: A comparative study of pig and sheep brain glutamine synthetases: Tryptic peptides and thiol groups. *Eur. J. Biochem.*, 35 (1973) 582–592 —cellulose.

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1055 Reijnders, L., Sloof, P. and Borst, P.: The molecular weights of the mitochondrial-ribosomal RNAs of *Saccharomyces carlsbergensis*. *Eur. J. Biochem.*, 35 (1973) 266–269 —polyacrylamide gel.

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1064 Hamelin, R., Larsen, Ch. J. and Tavitian, A.: Effects of actinomycin D, toyocamycin and cycloheximide on the synthesis of low-molecular-weight nuclear RNAs in HeLa cells. *Eur. J. Biochem.*, 35 (1973) 350–356 – polyacrylamide gel, agarose gel.

1065 Lagner, H. J., Teufel, U., Siegert, M. and Frommhold, M.: (Chemical and microbiological detection of antibiotics). *Fleischwirtschaft*, 53 (1973) 243–246, 249; *C.A.*, 78 (1973) 134569m.

1066 Ochab, S.: Application of electrophoresis for the separation of tetracycline and polyene antibiotics. *Dissert. Pharm. Pharmacol.*, 24 (1972) 205–208; *Anal. Abstr.*, 24 (1973) – Whatman No. 4 paper.

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33. INORGANIC SUBSTANCES

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35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

35c. Complex mixtures and non-identified compounds

1071 Wigle, D. T.: Purification of a messenger-specific initiation factor from ascites-cell supernatant. *Eur. J. Biochem.*, 35 (1973) 11–17 – SDS-polyacrylamide gel.

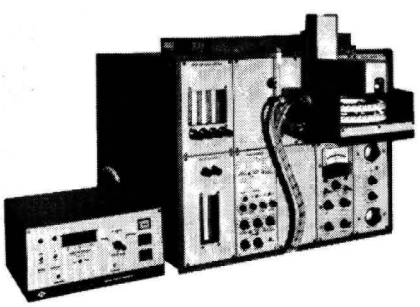
journal of
chromatography news section

APPARATUS

N-401

AUTOMATIC SAMPLING SYSTEM FOR GC

Tracor announces the Model 222 and 550 gas chromatographs with automatic sampling capabilities. The auto sampler, using a special Pressure-Lok syringe affords better than ± 1% reproducibility and infinite sample size adjustment within four ranges 0–2.0 μl, 0–10.0 μl, 0–25.0 μl, and 0–50 μl. In both vertical 222 GC Model and horizontal 550 GC



Model the syringe carriage moves in a single plane, eliminating the potential problem area of a complex tilting mechanism. Pre-flushing of the entire syringe volume with 70–150 μl of sample eliminates residual carry-over. Of the latest electronic design, the sampling system can be operated automatically, manually, or triggered via a remote source. Three output signals are available for control of auxiliary equipment, *i.e.*, GC temp. program initiation, recorder or integrator start/stop, etc.

N-388

HP JOURNAL

The November, 1973 issue of the Hewlett-Packard Journal describes 4 new instruments: a selfcontained hand-held digital multimeter, a portable high-resolution counter for low-frequency measurements, a high-speed pattern generator and an error detector, the models 970A, 5307A, 3760A and 3761A respectively, the latter two intended for testing digital systems.

N-386

CATALOG OF LC EQUIPMENT

Chromatronix has published a 32-page catalog covering the full range of equipment for liquid chromatography. The catalog includes equipment for HPLC as well as classical column chromatography. Equipment is available in the form of complete chromatographs and as individual parts ready for lab assembly.

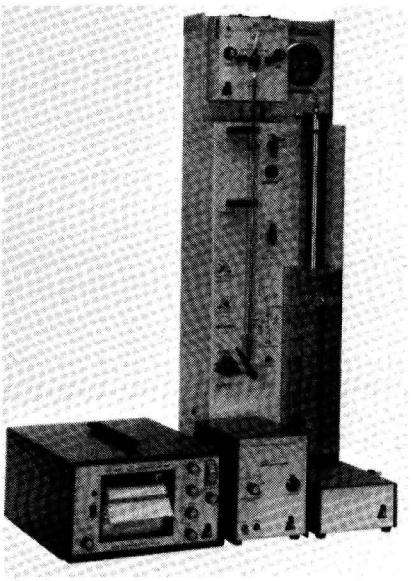
The line for high-pressure LC includes a 7000-p.s.i. gradient elution chromatograph capable of analytical and preparative work, a 3000-p.s.i. analytical chromatograph, and individual parts such as pumps, gradient programmers, detectors, sample injectors, stainless-steel columns, valves and fittings.

Equipment for classical column chromatography consists of stainless steel columns, injectors, detectors, pumps, fittings and valves in which only glass, Teflon and Kel-F contact the liquid stream. This equipment is useful for biochemical and other applications where metal contact must be avoided. Maximum pressure limit is 500 p.s.i.

N-387

HPLC MODEL 1440 FROM ISCO

The ISCO Model 1440 chromatograph features a constant flow, pulseless, 2000 p.s.i. solvent pump for isocratic or gradient elution. Rapid refill and washout valves provide fast solvent changing. A six-port injection valve



accomplishes rapid and reproducible sample introduction. Columns are stainless steel with non-clogging fittings and no dead volume. The dual beam photometer has 8 full scale absorbance ranges from 0.01 A to 2.0 A, low noise, micro flow cells, and 13 operating wavelengths.

N-399

ANALYTICAL ADVANCES

The Hewlett-Packard Journal "Analytical Advances" November, 1973 contains information on single-column gas chromatographs, Models 5700. Operating characteristics of Series 5700 FID with different carrier gases are described.

The effectiveness of pulsation damping in Model 1010 Liquid chromatograph is discussed.

The Model 5930 mass spectrometer is presented as a GC detector for polynuclear aromatic hydrocarbons.

N-402

QUICKFIT NON-PRECISION BORE LC COLUMNS

The columns are designed to meet the demand for simple low-cost equipment suitable for conventional chromatographic processes (gel permeation, de-salting, etc.), and for instructional purposes in educational establishments. The columns are fixed bed units manufactured from high quality borosilicate glass, with screw-threaded 'Delrin' ends fitted with PTFE liners so that when assembled they present an all-glass or PTFE liquid path to the eluent.

The bore sizes are 10, 20 and 30 mm, and in each of these sizes four different column lengths can be supplied: 150, 300, 450 and 900 mm. Input to the columns can be by simple gravity feed via a funnel assembly or direct coupling to a solvent reservoir (*e.g.* Marriott bottle or separating funnel), or by peristaltic pump via a pump adapter. The sample may be introduced by stopping the eluent flow and removing the column end. For more sophisticated on-line sample introduction the columns have been designed to accommodate either the sample injection head or rotary valve from the Jobling High Performance LC equipment and full inter unit PTFE accessories are available.

N-400

ELECTRON CAPTURE G.C. DETECTOR

Offering linearity over a range greater than 1–20,000, quantitation from low picogram to nanogram levels of chlorinated compounds is possible with the Ni^{63} electron capture detector from Tracor Instr. The integral, panel mounted, pulse width adjustment allows the analyst to optimize linearity for his particular compound of interest.

Operable with either Ar/5% CH_4 or N_2 carrier gas, Tracor's linear electron capture detector affords a significant increase in the linear range of electron capture GC.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-403

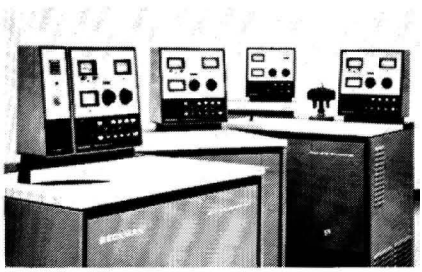
L5 PREPARATIVE ULTRACENTRIFUGE

Beckman Instr. introduces four models in the L5 series which offer speeds from 40,000 to 75,000 rpm. The new units combine the key features of the L2-65/75B and L3-40/05 preparative ultracentrifuges with advanced operating and control innovations to achieve improved operating performance.

Remarkable characteristics are direct-current, high-torque drives for rapid rotor acceleration and deceleration, variable acceleration and deceleration rates and electronic controls which maintain rotor speeds to within 1% of selected rpm. The new units also have automatic optical overspeed protection systems.

Three of the instruments have IR radiometric temperature control systems which keep rotor temperatures constant to within 0.5° on the L5-75 and to within 1° on the L5-65 and L5-50. The L5-40 has a manual temperature control.

The L5-75 and L5-65 are equipped with recirculating oil systems.



N-417

SYSTEM FOR CONTINUOUSLY VARIABLE WAVELENGTH UV DETECTION

Variscan™, a UV detection system for high performance liquid chromatography, is described in a brochure published by Varian. Variscan represents an analytical technique which combines liquid chromatography and UV spectrophotometry to provide multi-wavelength detection in HPLC applications. As it allows continuously variation of wavelengths between 210 and 780 nm the user can tune for maximum sample response and/or minimum solvent interference and can scan absorption spectra of separated components in stop-flow mode.

Equipped with micro-volume sample and reference cells it is specifically designed for HPLC.

The 8-page brochure details significant performance and design parameters in addition to presenting sample spectra of representative compounds.

N-418

SPECTROPHOTOMETRIC DETECTOR FOR LC

A 4-page bulletin from Schoeffel Instrum. describes in detail their UV–VIS (200–360 nm) liquid chromatography detector, the Model SF 770, suitable for high and low pressure. The noise level is better than 5×10^{-4} A.U. at 280 nm, the stability better than 5×10^{-4} A.U. per h at 280 nm. Flow cells of 8 µl volume and 10 mm pathlength are used.

N-419

GAS PURIFICATION FOR GC

A gas purifier to protect GC columns and ECD's is available from Supelco. It has an increased capacity and in typical use can be operated for over a year without the need for changing the purification system.

N-420

PIPETTES

A leaflet describing the MLA range of precision pipetting systems is available from Shandon Southern Instrum. Illustrations show tip seating, dispensing and disposal, each as a one-hand operation. Each pipette is made from aluminium and stainless steel with a 0.4% repeatability over the entire capacity range of 10–1,000 µl.

N-422

MONOCHROMATORS

A brochure from Schoeffel Instrum. gives information on different types of quartz prism and grating monochromators, accessories and systems.

N-421

AUTOMATIC BATCHWISE SAMPLE LOADING FOR SPECTROPHOTOMETERS

Varian Techtron introduces two automatic sampling accessories for use with the series 635 UV–VIS spectrophotometers. Using a combination of a vacuum pump intake dipper and a flow cell to transfer the sample, the units eliminate the need for cells to be manually filled and emptied. The result is greatly increased sample throughput, permitting automatic collection of analytical data.

The Sample transfer accessory draws a sample into a flow cell, holds it for measurement, and empties the cell. The "fill" and "empty" times can be varied between one and ninety-nine seconds with the read time fixed at 10 sec. The Auto-50 sample changer combines this "single shot" vacuum sample transfer accessory with an automatic fifty-position rotating sample tray, providing automatic measurement of up to 50 samples without operator attention.

CHEMICALS

N-425

GLASS PERMEATION CHROMATOGRAPHY MANUAL

A manual from Electro-Nucleonics describes the application of glass permeation chromatography using rigid, porous silica column packings. Contents include pore size selection, packing procedures, column technique, and elimination of surface adsorption. Methods apply to both aqueous and organic solvent systems, including high pressure applications.

N-424

CHROMATOGRAPHY LIPIDS

The Supelco periodical "Chromatography Lipids" Vol. VI, No. 7 (1973) supplies details on the use of Carbopack graphitized carbon supports for analysis of polar compounds, Dexsil 400 GC carborane siloxane stationary phase stable to 400°, Chromosil silicagel for sulfur gas analysis and the SP-216-PS packing for the separation of methyl esters of dibasic acids.

PROCEDURES

N-412

CARLO ERBA JOURNAL

The 2nd 1973 issue of "Short Notes", a Carlo Erba Journal describes several applications of the CLA 2500 colorimetric laboratory analyzer in biochemical analyses.

N-427

GC OF AMINES

Amine analysis by gas chromatography at the ppm level is possible with a new series of packings available from Supelco. The severe tailing and adsorption common to the methylamines has been drastically reduced, permitting analysis of dilute aqueous solutions of these troublesome compounds. A review of amine analysis by gas chromatography is provided in Technical Bulletin 737.

N-426

AMYLASE PROCEDURE

Pierce announces the Amylase Rapid Stat™ procedure which does not require centrifugation or deproteinization. The very low blank gives considerable mathematical advantage in accuracy. The ready-prepared liquid substrate is stable, the test color is stable and only a small sample (0.1 ml) is required to render good accuracy for both normal and pathological specimens. Data indicates linearity to 500 Somogyi units, enzyme activity is linear with time and results parallel those derived with Somogyi and Henry-Chiamori procedures.

CALENDAR OF FORTHCOMING MEETINGS

March 4–8, 1974
Cleveland, Ohio, U.S.A.

25th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy

Contact:
Robert W. Baudoux, Exhibition Chairman, U.S. Steel Corp. Research Lab., M.S. 57, Monroeville, Pa. 15146, U.S.A.
(Further details published in Vol. 82, No. 2)

March 5, 1974
Cleveland, Ohio, U.S.A.

Symposium of the American Society for Testing and Materials on Computerised Laboratory Systems

Contact:
J.B. Wheeler, ASTM, 1916 Race St., Philadelphia, Pa. 19103, U.S.A.

March 14–15, 1974
Oak Ridge, Tenn., U.S.A.

6th Annual Symposium on Advanced Analytical Concepts for the Clinical Laboratory

Contact: C.D. Scott, Biochemical Technology Section, Oak Ridge National Laboratory, Oak Ridge, Tenn. 37830, U.S.A.

March 19–21, 1974
Frankfurt (M), G.F.R.

35th International Technicon Symposium

March 20–22, 1974
Königstein/Ts., G.F.R.

Diskussionstagung der Deutschen Bunsengesellschaft für Physikalische Chemie: Physikalisch–chemische Aspekte flüssiger Kristalle.

March 31–April 5, 1974
Los Angeles, Calif., U.S.A.

167th ACS National Meeting

Contact:
Mr. A.T. Winstead, ACS, 1155-16th, N.W., Washington, D.C. 20036, U.S.A.

April 1–4, 1974
Mittenwald, G.F.R.

EUCHEM Conference Organic Electro Chemistry

Contact:
Dr. W. Fritsche, Gesellschaft Deutscher Chemiker, D-6000 Frankfurt (M), Postfach 90 04 40 (G.F.R.)

April 8–9, 1974
Tübingen, G.F.R;

Symposium of the Working group "Chromatography" of the "Analytic chemistry" division

Contact:
GDCh-Geschäftsstelle, D-6000 Frankfurt (M), Postfach 90 04 40, G.F.R.

April 9–11, 1974
Athens, Ga., U.S.A.

Identification and Transformation of Aquatic Pollutants Symposium

Contact:
Dr. W. Garrison, Southern Environmental Lab., EPA, Athens, Ga. 30601, U.S.A.

April 22–26, 1974
Munich, G.F.R.

Biochemical Analysis, joint meeting of Deutsche Gesellschaft für Klinische Chemie, Gesellschaft Deutscher Chemiker and Gesellschaft für Biologische Chemie

Contact:
GDCh-Geschäftsstelle, D-6000 Frankfurt (M), Postfach 90 04 40, G.F.R.
(Further details published in Vol. 80, No. 1)

May 1–4, 1974
Brugge, Belgium

22nd Annual Colloquium on Protides of the Biological Fluids

Contact:
22nd Colloquium on Protides of the Biol. Fluids, Simon Stevin Instituut, Jerusalemstraat 34, B-8000 Brugge, Belgium

May 28–31, 1974
At the Balaton Lake, Hungary

3rd Symposium on Ion Exchange

Contact:
The Hungarian Chemical Society, Organizing Committee, 3rd Symposium on Ion-Exchange, H-1368 Budapest, P.O. Box 240, Hungary
(Further details published in Vol. 84, No. 2)

June 10–12, 1974
Lindau (Lake Constance), G.F.R.

Meeting in analytical chemistry

Contact:
GDCh-Geschäftsstelle, D-6000 Frankfurt (M), Postfach 90 04 40, G.F.R.

June 12–14, 1974
Ft. Collins, Colo., U.S.A;

27th Annual Summer Symposium on Automatic Processes in Analytical Chemistry Instrumentation

Contact:
R.A. Hagstrom, Olin Mathieson Chemical Corp., 275 Winchester Ave., New Haven, Conn. 06504, U.S.A.

June 17–19, 1974
Basel, Dwitzerland

4th Annual Symposium on Recent Advances of the Analytical Chemistry of Pollutants

Contact:
Dr. D.M. Hercules, Chemistry Dept., University of Georgia, Athens, Ga. 30601, U.S.A.

June 24–26, 1974
Milan, Italy

2nd International Symposium on Mass Spectrometry in Biochemistry and Medicine

Contact:
Dr. A. Frigerio, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy.
(Further details published in Vol. 88, No. 2)

July 16–20, 1974
London, England

Centenary Celebration, Society for Analytical Chemistry

Contact:
SAS, 9/10 Saville Row, London, W1X 1AF, England

August 26–31, 1974 Warsaw, Poland	**IVth Polish Conference on Analytical Chemistry** Contact: The Secretary of the Organizing Committee, Dr. hab. R. Dybczynski, Institute of Nuclear Research, ul. Dorodna 16, 03-195 Warsaw, Poland (Further details published in Vol. 82, No. 20
September 9–October 14, 1974, Basel, Switzerland	**Internationale Fachmesse für Laboratoriums- und Verfahrens-technik, Messtechnik und Automatik in der Chemie (ILMAC 74)**
September 9–12, 1974 Prague, Czechoslovakia	**Conference on Analytical Chemistry of the Environment (Interan)** Contact: House of Technology SVTS, 01180-Žilina-Hliny, Czechoslovakia. (Further details published in Vol. 88, No. 2)
September 30–October 4, 1974 Barcelona, Spain	**10th International Symposium on Chromatography** Contact: Director of the G.A.M.S., 10, rue du Delta, 75009 – Paris, France (Further details published in Vol. 81, No. 1)
October 27–November 1, 1974 Buenos Aires, Argentina	**8th Panamerican Congress on Endocrinology** Contact: Dr. C. Bergadá, Octavo Congreso Panamericana de Endocrinologia, Cas. Correo No. 2593 C. Central, Buenos Aires, Argentina
November 4–7, 1974 Houston, Texas, U.S.A.	**Ninth International Symposium on Advances in chromatography** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A. (Further details published in Vol. 88, No. 2)
March 3–7, 1975 Cleveland, Ohio, U.S.A.	**26th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy**
November 18–22, 1974 Atlantic City, N.J., U.S.A.	**Federation of Analytical Chemistry and Spectroscopy Societies, 1st Annual Meeting** Contact: J.G. Grasselli, Standard Oil Co. (Ohio), 4440 Warrensville Ctr. Rd., Cleveland, Ohio 44128, U.S.A.
June 1975 Knoxville, Tenn., U.S.A;	**28th Annual Summer Symposium on New Horizons in Analytical Spectroscopy** Contact: J. Winefordner, Dept. of Chemistry, University of Florida, Gainesville, Fla. 32601, U.S.A.

October 6–10, 1975
Indianapolis, Ind., U.S.A.

Federation of Analytical Chemistry and Spectroscopy Societies, 2nd Annual Meeting

Contact:
J.G. Grasselli, Standard Oil Co. (Ohio), 4440 Warrensville Ctr. Rd., Cleveland, Ohio 44128, U.S.A.

PUBLICATION SCHEDULE FOR 1974

Journal of Chromatography (incorporating *Chromatographic Reviews*)

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
JOURNAL	88/1 88/2	89/1 89/2	90/1	90/2 91	92/1 92/2	93/1 93/2	94 95/1	95/2 96/1	96/2	97/1 97/2	99	100/1 100/2
REVIEWS *			98/1						98/2		98/3	

* Volume 98 will consist of *Chromatographic Reviews*. The issues comprising this volume will not be published consecutively, but will appear at various times in the course of the year.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. (a) Original research work not previously published in a generally accessible language in other periodicals (Full-length papers). (b) Review articles. (c) Short communications and Notes. (d) Book reviews; News; Announcements. (e) Bibliography of Gas Chromatography, Column Chromatography, Paper Chromatography, Thin-Layer Chromatography and Electrophoretic Techniques. (f) Chromatographic Data.

Submission of Papers. Two copies of manuscripts in English, French or German should be sent to: The Editor of the Journal of Chromatography, *c/o* Elsevier Scientific Publishing Company, P.O. Box 330, Amsterdam, The Netherlands. For *Review articles*, an outline of the proposed article should first be forwarded to the Editor for preliminary discussion prior to preparation.

Manuscripts. The manuscript should be typed with double spacing on pages of uniform size and should be accompanied by a separate title page. The name and the complete address of the author to whom proofs are to be sent should be given on this page. Authors of papers in French or German are requested to supply an English translation of the title. A short running title of not more than 50 letters (including spaces between the words) is also required for Full-length papers and Review articles. All illustrations, photographs, tables, etc., should be on separate sheets.

Heading. The title of the paper should be concise and informative. The title should be followed by the authors' full names, academic or professional affiliations, and addresses.

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References. References should be numbered in the order in which they are cited in the text and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places using superscript numerals. In the reference list, periodicals[1], books[2], and multi-author books[3] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 R. D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins*, Vol. 5, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.*

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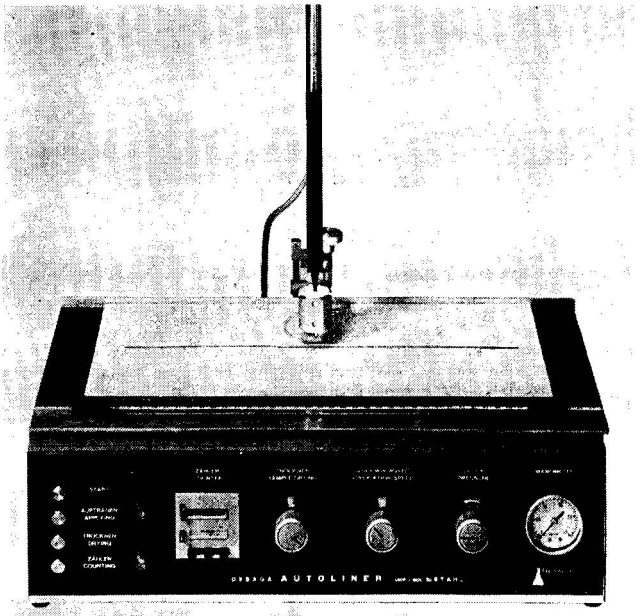

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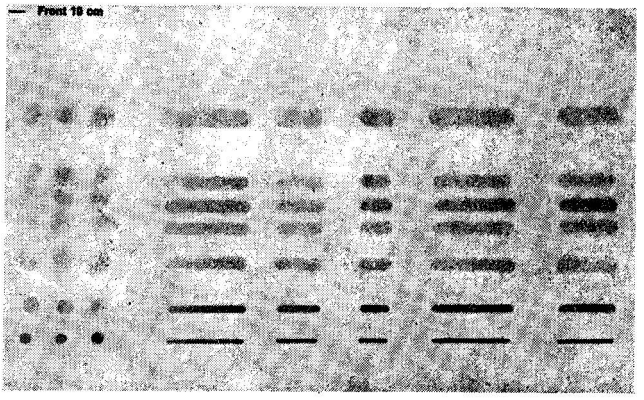

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