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OPTIMUM USE OF PAPER, THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY FOR THE IDENTIFICATION OF BASIC DRUGS

I. DETERMINATION OF EFFECTIVENESS FOR A SERIES OF CHROMATOGRAPHIC SYSTEMS

ANTHONY CHRISTOPHER MOFFAT, KENNETH WALLACE SMALLDON and COLIN BROWN

Home Office Central Research Establishment, Aldermaston, Berks. RG7 4PN (Great Britain)

(Received August 27th, 1973)

SUMMARY

A method is described for the determination of effectiveness for a series of chromatographic systems which uses the concept of discriminating power. The discriminating power for a series of chromatographic systems is defined as the probability that two drugs selected at random from a large population would be discriminated in at least one of the systems. The series of systems with the highest discriminating power is shown to produce the best overall separations for a large specified drug population and therefore leads to the identification of an unknown drug using a minimum number of systems. The conditions for maximum discriminating power are described and discussed.

INTRODUCTION

When analysing a drug sample, be it a pure compound, pharmaceutical or extract from a biological material, an analyst is faced with a bewildering selection of paper (PC), thin-layer (TLC) and gas–liquid chromatographic (GLC) systems from which to make his choice. It may be that the particular problem requires the separation of two or more known drugs, in which case the compilations of analytical data would be consulted[1–3] and an appropriate system or systems chosen. However, if the analyst is routinely engaged in the identification of basic drugs, the data compilations do not help him to choose the best system or combination of systems, since the data were generated independently by different workers and on different drug populations. As a result of the growing multiplicity of possibilities, each analyst tends to select the systems with which he is most familiar and for which he has some sort of data collection. As a result, there is a tendency for each laboratory to have a library of data for its own particular systems, often without being aware of the collections in other laboratories. It is therefore apparent that an urgent need exists to assess objectively the effectiveness of the systems which are currently in common use so that a comprehensive data collection is produced

for the most useful systems and thus a minimum amount of effort is involved in the routine identification of basic drugs. The effectiveness of new systems can be evaluated in a similar manner as they become available and then compared with existing systems.

In order to provide the maximum amount of information, a system used in a procedure for the identification of a large number of compounds should have a random distribution of values over the whole chromatogram. A further requirement is that the system should provide results which are as reproducible as possible. Ideally the reproducibility of each system should be determined by experiment. However, if the number of systems available is large, this approach may be too time consuming for practical use and an estimate of the reproducibility may have to be made.

When systems are used in combination, the order of elution of the compounds must vary from system to system or no more information may be obtained. Previous work by one of us[4] demonstrated that the correlation coefficients between a number of commonly used TLC systems cannot be regarded as negligible.

The retention values of compounds in single systems can be displayed as frequency histograms and those showing grossly non-random distributions can readily be observed. However, when system reproducibility and inter-system correlation are also considered clearly a mathematical model is required so that the optimum procedure can be selected for the identification of an unknown drug.

THEORY

Two compounds are regarded as having been separated or discriminated in a particular chromatographic system (i) if the difference between their retention values exceeds a certain critical value, which is termed the error factor (E_i). The error factor of the system can be determined practically so that virtually all the experimentally determined retention values of a particular drug would fall within the range $\pm E_i$ about the standard value.

The discriminating power of a single chromatographic system is therefore defined as the probability that two compounds selected at random would be discriminated in that system. Similarly the discriminating power of a series of systems is defined as the probability that two compounds selected at random would be separated in at least one of the systems. Compounds which are not discriminated are assumed to be chromatographically similar.

In a previous paper[5] a method was described and discussed for the calculation of discriminating power for any series of correlated attributes provided that the distribution of each attribute over the population is known and an estimate for each error factor can be made from experience. In this application the distributions are known to be non-Gaussian and therefore the appropriate theory must be used.

Suppose that the discriminating power is required for k systems in combination and that chromatographic values for N compounds have been recorded in each system. The total number of possible pairs is given by

$$NC_2 = \frac{N(N-1)}{2}$$

If the number of pairs which are similar in all k systems is M, then the probability of selecting a similar pair at random is $2M/[N(N-1)]$. The discriminating power (DP_k) for the k systems is thus given by

$$DP_k = 1 - \frac{2M}{N(N-1)}$$

The number of similar pairs, and then the discriminating power, is calculated by means of the computer search program given as an Appendix. The procedure used to calculate the discriminating power for k systems in series is termed a "kth order" computer search. The software in the Appendix allows searches up to 6th order to be made from any eight chromatographic systems. An example of the computer routine which calculates the 2nd order discriminating power for systems 3 and 7 is shown below.

```
INSERT NO. OF DATA ITEMS AND SYSTEMS
  100, 8
INSERT ORDER OF SEARCH AND SYSTEMS REQUIRED
  2, 3, 7
INSERT ERROR FACTORS TO BE USED IN THE SAME ORDER
  10, 10
DISCRIMINATING POWER=0.929
```

The ideal system would have a random or rectangular distribution of drugs over the whole chromatogram. A rectangular distribution function, $f(x)$, is of the form

$f(x) = 1$ when $0 \leqslant x \leqslant 1$

$f(x) = 0$ when $x < 0$ or $x > 1$

Suppose that two compounds are selected at random from this distribution and x for the first compound lies anywhere between E to $1-E$ (where E is expressed as a fraction of the range of possible chromatographic values). The probability that the two compounds are chromatographically similar is

$$\int_E^{1-E} 2E\,dx = 2E(1-2E)$$

If x for the first compound lies anywhere in the range 0 to E then the probability that the two compounds are similar is

$$\int_0^E (x+E)\,dx = \frac{3E^2}{2}$$

Similarly if x for the first compound lies in the range $(1-E)$ to 1 the probability is also $3E^2/2$.

Therefore, the overall probability that the two compounds are chromatographically similar is

$$2E(1-2E)+3E^2 = 2E-E^2$$

The maximum discriminating power that can be obtained with a chromatographic system is therefore given by

$$DP = 1 - 2E + E^2$$

This expression is true for all values of E and when E is small the E^2 term may be neglected.

As an aid to interpretation, correlation coefficients (r) may be determined for pairs of systems using the expression

$$r = \frac{\frac{1}{N}\Sigma(x-\bar{x})(y-\bar{y})}{\sigma_x \sigma_y}$$

where x and y are the chromatographic values in the two systems, $\bar{x}$ and $\bar{y}$ being their respective means, and σ_x and σ_y their respective standard deviations.

If k chromatographic systems are all uncorrelated then the combined discriminating power (DP_k) is given by

$$DP_k = 1 - \prod_{i=1}^{k} (1 - DP_i)$$

If, in addition, the distributions are all rectangular, then

$$DP_k = 1 - \prod_{i=1}^{k} (2E_i - E_i^2)$$

This represents the maximum possible discriminating power for any given error factors (E_i's) so that as all the E_i's$\rightarrow 0$, $DP \rightarrow 1$.

DISCUSSION

The measurement of the discriminating power for a series of chromatographic systems allows the effectiveness of the series to be expressed as a single value, the series with the highest discriminating power being the most effective. If only a small number of compounds have to be chromatographed, it may be that complete separation is possible ($DP = 1$), in which case further evaluation is unnecessary. However, if the number of compounds to be chromatographed is large, complete separation of all the possible pairs is no longer feasible and the aim becomes to maximise the discriminating power. In this case the conditions for maximum discriminating power are: (a) rectangular distributions of compounds over the chromatograms, (b) good system reproducibility, and (c) no correlation between systems.

The compounds for which chromatographic values are recorded can be determined from the nature of the particular problem and the work records of the laboratory. A statistically adequate number of samples which genuinely reflects the problem under consideration must be recorded before reliable values for discriminating power are obtained.

The number of systems available may preclude the experimental determination of all the error factors. In order to reduce the number of systems to manageable proportions the error factors could initially be estimated from experience and then the true inter-laboratory reproducibility could be determined for the small number of systems selected by this procedure.

Correlation coefficients can be calculated for non-Gaussian distributions, such as the distribution of drugs across a chromatogram, but their significance cannot be tested in the usual manner. However, values for correlation coefficients remain a useful aid to interpretation for a number of reasons. For example, if other factors are equal, the pair of systems showing the lowest correlation coefficient will provide the highest discriminating power for two systems in combination. When a large number of systems are considered, the amount of computation can often be reduced in the following ways: Those systems showing very low discriminating powers can be eliminated from further consideration, the discriminating power for series of uncorrelated systems can be calculated from the individual discriminating powers using the formula already described[5], and if two systems are highly correlated the system with the lower discriminating power can be eliminated.

If systems are selected so that the discriminating power is maximised, then the probability of discriminating a pair of compounds selected at random is also maximised. The same argument applies if any number of compounds are selected at random since N compounds can be represented by NC_2 pairs for which discrimination is required.

The discriminating power for a series of systems (DP_k), which has been calculated from the chromatographic values of N compounds, can be used to predict the average number of possibilities which would be retrieved during a search to identify an unknown compound. Since systems with high discriminating power have nearly rectangular distributions, the actual number of compounds retrieved does not vary much about the mean value. The method used to calculate the error factors ensures that there is only a very small chance that the true identity of the unknown compound will be excluded. Thus the true identity will be retrieved with certainty and further $(N-1)$ comparisons will be made, each with a probability $(1-DP_k)$ of providing a random match within the error factors. An estimate of the total number of compounds (T) retrieved during an average search is therefore given by

$$T=1+(N-1)(1-DP_k)$$

This expression shows that if the sequence of systems showing the maximum discriminating power (*i.e.*, $DP\rightarrow 1$) were used, then the unknown compound would be identified (*i.e.*, $T\rightarrow 1$) using the minimum number of systems. This argument naturally assumes that chromatographic values have been recorded for the unknown compound and this can only be achieved by carefully compiling a list of reference compounds from the work records of the laboratory.

The selection of systems purely on the basis of their discriminating power assumes that the effort involved in setting up each system is similar and that the time of analysis is not too important. Individual analysts would probably wish to apply their own particular constraints to results calculated in the manner described. For example, a clinical toxicologist would probably place a high priority on the speed

of analysis, whereas a pharmaceutical analyst would be less likely to do so. Under these circumstances final decisions would be made by balancing the discriminating powers against the particular constraints which apply.

The theory described here can be applied to any chromatographic problem which involves a large compound population in order to obtain the best overall separations and to identify an unknown compound with the minimum number of systems. In subsequent papers this theory has been applied to the PC, TLC, and GLC of basic drugs.

APPENDIX

Written in Fortran IV for a Hewlett-Packard Model 2100 computer.

```
ØØØ1 FTN
ØØØ2        PROGRAM DP
ØØØ3        DIMENSION IN (1ØØ,8), IERR(8), IPONT(6)
ØØØ4        WRITE(1,2)
ØØØ5 2      FORMAT("INSERT NO OF DATA ITEMS AND SYSTEMS")
ØØØ6        READ(1,*)N,ISYST
ØØØ7        DO 1Ø I=1,ISYST
ØØØ8        DO 2Ø J=1,N
ØØØ9        READ(5,*)IN(J,I)
ØØ1Ø 2Ø     CONTINUE
ØØ11        PAUSE
ØØ12 1Ø     CONTINUE
ØØ13 6      WRITE(1,3)
ØØ14 3      FORMAT("INSERT ORDER OF SEARCH AND SYSTEMS
            REQUIRED")
ØØ15        READ(1,*)IORD, IPONT
ØØ16        WRITE(1,4)
ØØ17 4      FORMAT("INSERT ERROR FACTORS TO BE USED IN THE
            SAME ORDER")
ØØ18        READ (1,*)IERR
ØØ19        A=Ø.Ø
ØØ2Ø        DO 5Ø I=1, (N-1)
ØØ21        DO 4Ø J=(I+1),N
ØØ22        DO 3Ø K=1,IORD
ØØ23        IF(IABS(IN(I,IPONT(K))-IN(J,IPONT(K))).LE.IERR(K))3Ø,4Ø
ØØ24 3Ø     CONTINUE
ØØ25        A=A+1.Ø
ØØ26 4Ø     CONTINUE
ØØ27 5Ø     CONTINUE
ØØ28        B=N
ØØ29        B=B*(B-1.Ø)/2.Ø
ØØ3Ø        DP=1.Ø-A/B
ØØ31        WRITE(1,5)DP
ØØ32 5      FORMAT("DISCRIMINATING POWER=",F5.3//)
```

```
ØØ33    GO TO 6
ØØ34    END
ØØ35    END$
```

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2 A. S. Curry, *Poison Detection in Human Organs*, Thomas, Springfield, Ill., 2nd ed., 1969.
3 I. Sunshine, *Handbook of Analytical Toxicology*, Chemical Rubber Co., Cleveland, Ohio, 1969.
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5 K. W. Smalldon, A. C. Moffat and J. B. Parker, *J. Forensic Sci. Soc.*, in press.

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OPTIMUM USE OF PAPER, THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY FOR THE IDENTIFICATION OF BASIC DRUGS

II. PAPER AND THIN-LAYER CHROMATOGRAPHY

ANTHONY CHRISTOPHER MOFFAT and KENNETH WALLACE SMALLDON
Home Office Central Research Establishment, Aldermaston, Berks. RG7 4PN (Great Britain)
(Received August 27th, 1973)

SUMMARY

The concept of discriminating power has been applied to the paper and thin-layer chromatography of 100 basic drugs in eight systems which are representative of the systems in current use. The design of the most effective series of chromatographic systems for the separation and identification of a large drug population is discussed in terms of the individual discriminating powers of the systems and inter-system correlation. It is shown that both the substrate and solvent may have to be changed to obtain significantly different systems.

INTRODUCTION

Paper (PC) and thin-layer (TLC) chromatographic methods have been extensively studied in order to develop screening tests for the detection, identification and quantitation of basic drugs (those drugs extracted from alkaline aqueous solutions by organic solvents) in formulations and biological materials. Many different systems have been advocated for use in general screening methods and for the separation of pharmacologically or chemically similar compounds. If a small group of known compounds is examined it may well prove feasible to separate all the members using one or more chromatographic systems. However, if the whole group of basic drugs is considered, complete separation and identification of each drug may be no longer feasible using a small number of systems.

It is important that the analyst should select the series of systems with the highest discriminating power for the PC and TLC of extracts containing basic drugs. The systems which have been reported for this purpose are too numerous to mention individually, but collections of chromatographic data have been produced. Fox[1] lists data for three PC systems, Curry[2] gives data for eight TLC systems and Sunshine[3] gives eleven PC and thirty-five TLC systems. In many cases the populations of drugs chromatographed in each system are so different that a comparison of the effectiveness of the systems is impossible and the choice of the best series of systems for general screening purposes is very difficult.

A method has been described which measures the effectiveness of chromatographic systems (discriminating power) in terms of the probability of separating

two bases selected at random from a specific drug population[4]. This concept is now applied to the analysis of basic drugs using PC and TLC.

EXPERIMENTAL

Choice of basic drugs

The records, from two forensic science laboratories in Great Britain, involving drugs (6000 items) were searched to obtain those basic drugs which had been received at least twice in any one year during the period 1970–1972. To this list was added a number of other commonly used drugs for which analytical data were available, *e.g.* caffeine and nicotine, to give a total of 100 drugs.

Choice of chromatographic systems

The PC, two reversed-phase PC and five TLC systems were selected as being representative of those which are, or have been, in common use in the field of drug analysis (Table I). Those drugs for which data were unavailable from the literature were chromatographed with the procedures used by the respective authors. Reference compounds were used to ensure compatibility of data. Drug spots were located by means of UV light, spraying with 1% iodine in methanol, acidified potassium iodoplatinate solution, or 1% potassium permanganate solution.

TABLE I

PC AND TLC SYSTEMS STUDIED

Type	*System No.*	*Plate or paper*	*Solvent*	*Reference*
TLC	1	Silica gel dipped or prepared with 0.1 *M* KOH	Cyclohexane–benzene–diethylamine (75:15:10)	5
	2	Silica gel dipped or prepared with 0.1 *M* KOH	Methanol	5
	3	Silica gel dipped or prepared with 0.1 *M* KOH	Acetone	5
	4	Silica gel dipped or prepared with 0.1 *M* $KHSO_4$	Methanol	5
	5	Silica gel dipped or prepared with 0.1 *M* $KHSO_4$	Ethanol (95%)	5
PC	6	Whatman No. 1 paper dipped in 5% sodium dihydrogen citrate	Butanol–water–citric acid (870:130:4.8 g)	6
	7	Whatman No. 1 paper dipped in 10% tributyrin in acetone	Acetate buffer, pH 4.58, run at 95°	7, 8
	8	Whatman No. 1 paper dipped in 10% tributyrin in acetone	Phosphate buffer, pH 7.4, run at 86°	9, 10

Treatment of results

The discriminating power[4] was calculated for each system using error factors of 0.05, 0.10, 0.15, and 0.20 in R_F value and then the discriminating power for each pair was calculated using an error factor of 0.10 for each system. Correlation coefficients were also determined for each pair of systems.

TABLE II

R_F (×100) VALUES FOR 100 BASIC DRUGS IN EIGHT SYSTEMS*

Drug	*System No.*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Acetophenazine	3	51	1	15	5	23	58**	0
Ametazole	19	20	47	38	17	8	95	87
Amethocaine	18	50	28	38	14	48	56	0
Amitriptyline	72	50	34	41	28	77	32	0
Amphetamine	34	28	33	59	53	51	85**	88**
Antazoline	8	11	6	61	38	74	85	52
Atropine	9	11	1	31	14	37	94	86**
Benzocaine	6	68	73	63	57	90	17**	16**
Benzphetamine	79	70	85	54	49	75	17	0
Bromodiphenhydramine	50	37	26	48	33	69	31	0
Buphenine	5	68	64	66	68	80	75	5
Butacaine	8	63	71	59	51	78	43	0
Butethamine	5	58	49	58	47	51	82	24
Caffeine	4	55	42	48	34	65	85**	74
Carbetapentane	63	37	30	44	18	76	73	8
Carbinoxamine	29	21	6	5	0	46	78	10
Chlorcyclizine	49	44	30	43	19	74	2	0
Chlordiazepoxide	0§	80§	40§	68**	59**	82	11**	8**
Chlorpheniramine	38	19	6	8	1	45	69**	13**
Chlorpromazine	57	44	37	44	26	68	20	2
Cinchonine	10	37	11	22	9	47	72	10
Clemizole	33	67	61	47	27	77	4	0
Cocaine	58	57	64	26	10	38	71**	12**
Codeine	7	28	6	24	10	16	89	22
Cyclizine	55	46	27	41	16	55	47	0
Cyclopentamine	52	10	2	52	40	64	73**	89**
Desipramine	26	18	3	62	45	65	70	6
Dextropropoxyphene	72	62	60	54	30	75	25	2**
Diamorphine	22	39	20	24	9	33	84	12
Diazepam	33	65	62	65	54	89	26	4**
Diethylpropion	76	69	78	44	27	58	52	0
Dimethoxanate	24	31	13	33	13	54	61	4
Diphenhydramine	52	37	26	45	25	62	70	0
Diphenylpyraline	42	25	11	44	23	46	50	4
Dipipanone	55§	55§	60§	17**	8**	85	16	0
Ephedrine	8	18	2	54	42	45	83**	85**
Ethoheptazine	59	29	11	39	19	52	84	17
Ethopropazine	68	62	82	40	25	72	14	5
Fluphenazine	6	60	25	15	6	36	13	7
Guanethidine	0	3	0	15	5	3	83	82
Hydroxyzine	8	59	39	56	25	67	28	0
Hyoscine	9	54	33	34	13	23	93	57
Imipramine	61	35	18	39	25	63	44	0
Iproniazid	4	64	34	34	22	75	83	79
Isocarboxazid	28	71	70	65	61	94	17	20
Isothipendyl	51	47	32	36	18	58	67**	5**
Levallorphan	31	60	53	63	47	73	74	6
Lignocaine	39	70	69	47	23	62	64	4
Lysergide	0§	70§	35§	49**	30**	47	55**	8**

(*Continued on p. 12*)

TABLE II (*continued*)

Drug	*System No.*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Meclozine	69	71	84	74	60	92	0	0
Mephentermine	50	15	3	52	39	62	82**	92**
Mepivacaine	37	62	57	43	30	62	79	12
Mepyramine	42	33	24	12	1	32	71	5
Methadone	76	37	43	51	25	74	59	0
Methapyrilene	47	36	27	14	2	32	71	5
Methaqualone	35§	75§	60§	69**	62**	94	6	4
Methotrimeprazine	56	56	65	46	23	65	13	4
Methylamphetamine	46	18	4	50	39	56	87**	89**
Methyl phenidate	55	56	35	54	45	63	76**	30**
Morphine	2	28	4	23	10	14	88	81
Naphazoline	5	8	1	43	30	51	81	76
Nialamide	0	55	9	35	23	78	78	77
Nicotine	53	52	29	8	2	7	99	39
Nicotinyl alcohol	3	60	40	30	5	16	89	81
Nikethamide	24	64	44	38	25	86	65	68
Nitrazepam	0§	75§	60§	64**	60**	92	63**	5**
Nortriptyline	35§	20§	10§	6**	6**	74	55	4
Orphenadrine	60	48	32	45	22	67	71	0
Papaverine	11	62	53	62	21	49	8	0
Perphenazine	6	57	20	12	4	23	19	0
Pethidine	55	48	21	49	24	0	83	3
Phenelzine	51	72	75	62	49	38	84**	95**
Phenindamine	55	53	35	41	25	63	37	0
Pheniramine	40	18	5	6	1	27	92	20
Phenmetrazine	24	44	11	49	37	49	83**	88**
Phenylpropanolamine	9	35	50	58	56	44	90**	94**
Phenyramidol	14	68	66	57	40	52	86	29
Pipamazine	0	60	32	48	27	50	36	3
Piperidolate	70	65	65	42	25	76	14	0
Piperocaine	63	45	42	47	29	68	79	5
Pramoxine	52	61	55	48	28	61	6	0
Procaine	5	52	47	39	18	31	89	27
Procyclidine	78	36	39	52	41	84	53	4
Promazine	50	36	25	39	20	58	33	2
Promethazine	46	47	37	45	23	65	23	2
Propiomazine	42	59	53	40	26	77	7	4
Prothipendyl	53	37	20	24	15	55	50	4
Pyrrobutamine	62	39	34	59	40	84	11	4
Quinine	5	47	11	37	14	46	71	11
Strychnine	13	17	3	17	7	30	81**	55**
Thenyldiamine	47	32	25	12	1	27	65	7
Thiopropazate	44	66	67	30	11	53	67	7
Thioridazine	52	45	31	41	27	76	67	5
Thonzylamine	41	38	27	29	12	52	73	5
Tranylcypromine	55	57	58	58	56	45	79	0
Trifluoperazine	45	49	19	10	2	34	6	3
Trimeprazine	64	55	62	44	22	70	29	94
Tripelennamine	50	35	27	12	3	35	74	57
Triprolidine	41	45	13	18	2	59	74	28
Yohimbine	9	68	61	55	11	54	64	7

* Data taken from original papers except where stated.
** This study.
§ Data recorded by Smalldon[11].

RESULTS AND DISCUSSION

The R_F values for the 100 basic drugs in the eight systems are given in Table II. Although there are many thousands of synthetic and natural basic drugs, the sample of 100 used in this study is representative of the population of interest since 98% of the items submitted to laboratories, in which a basic drug was found, contained one of those listed in Table II.

Table III gives the discriminating power *DP* for each system at a range of error factors. The inter-laboratory reproducibility, and hence the error factor *E*, should ideally be determined for each system. This was prohibited by the amount of work needed and therefore an estimate of the likely error factors was made from experience.

TABLE III

DISCRIMINATING POWERS FOR SINGLE CHROMATOGRAPHIC SYSTEMS AT VARIOUS ERROR FACTORS

Total number of possible pairs = 4950.

System No.	*Error factor*			
	0.05	*0.10*	*0.15*	*0.20*
1	0.839	0.730	0.645	0.562
2	0.837	0.688	0.564	0.439
3	0.863	0.749	0.659	0.569
4	0.813	0.657	0.525	0.410
5	0.808	0.672	0.535	0.412
6	0.862	0.742	0.620	0.508
7	0.867	0.753	0.650	0.567
8	0.661	0.549	0.492	0.442

Dhont *et al.*[12,13] found that the standard deviations (σ) for the determination of inter-laboratory TLC R_F values for single and multi-component systems are 0.12 and 0.06, respectively. Only three determinations carried out under essentially different conditions (tank saturation, activation, etc.) were used for the calculation of σ's and therefore they may be taken as upper limits of irreproducibility. When using two reference standards and the calculation of corrected R_F^c values[14] with the same systems, they found that the values of σ were reduced to 0.02 and 0.03, respectively. The inter-laboratory reproducibility of R_F values can therefore be dramatically improved by using R_F^c values and for the purposes of this paper we have estimated the error factors of all the systems to be 0.10.

It can be seen from Table III that if the value of *E* can be reduced for a given system, the discriminating power is greatly increased, *e.g.* System 4 has nearly twice the *DP* at an *E* of 0.05 than it has at an *E* of 0.20. It is therefore apparent that R_F^c rather than R_F values should be used in future identification procedures. However, assuming that all the systems have similar error factors, the order of systems in terms of discriminating power is virtually the same regardless of the actual value of *E*.

At our estimated error factor of 0.10 the maximum possible *DP* is 0.81 and the system with the highest discriminating power is System 7 ($DP_7 = 0.753$) followed by Systems 3, 6, and 1 (Table III). System 7 has a high *DP* because it has nearly a rectangular distribution of R_F values over the whole chromatogram, as shown in Fig. 1. This may be compared with Fig. 2, which shows that no less than 61% of all the R_F values in System 8 are 0.10 or lower, and this explains why it has the smallest discriminating power ($DP_8 = 0.549$). It is interesting to note that the best and the worst systems studied are both reversed-phase systems and only the mobile phase is different. The pH of the aqueous buffer is obviously critical and reproducible results are only obtained if the buffer solutions are made exactly to the correct pH.

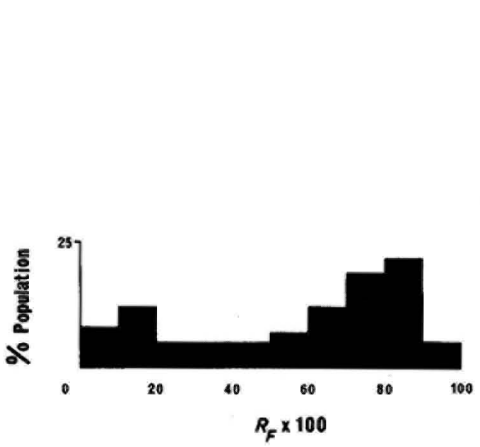

Fig. 1. Frequency distribution of R_F values of 100 basic drugs using System 7 (Whatman No. 1 paper dipped in 10% tributyrin in acetone/acetate buffer, pH 4.58, run at 95°).

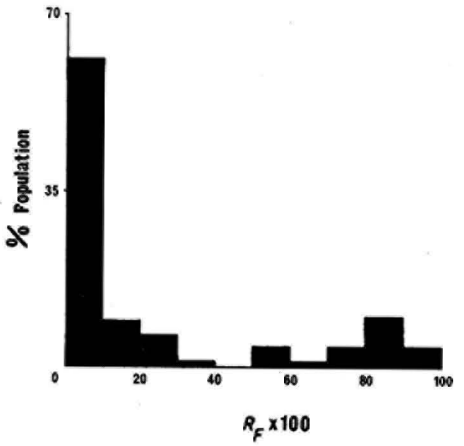

Fig. 2. Frequency distribution of R_F values of 100 basic drugs using System 8 (Whatman No. 1 paper dipped in 10% tributyrin in acetone/phosphate buffer, pH 7.4, run at 86°)..

When more than one chromatographic systems are used, the combined discriminating power obviously increases and the discriminating powers for the 28 possible pairs of systems (at an error factor of 0.10 in R_F value for each system) are given in Table IV. The pair with the largest discriminating power is System 3 and System 7 ($DP_{3,7} = 0.929$), followed by Systems 3 and 6, 6 and 7, and then 1 and 7. The reasons why these are the best combinations of systems may be seen by examining the factors necessary to produce a large *DP* of combined systems.

Firstly the two systems must themselves have large *DP*'s. System 8 has the lowest individual *DP* and consequently, in combination with any other system, although the *DP* increases from 0.549 to between 0.834 and 0.875, it does not approach the $DP_{3,7}$ of 0.929.

The second factor governing the size of the second-order *DP* is the correlation between systems. The correlation coefficients of each pair of systems are given in Table V. Systems 4 and 5 are both silica gel, with methanol and ethanol (95%), respectively, used as the mobile phase. The order of elution of the drugs on each system is virtually the same, as can be seen from the high correlation coefficient of 0.902 and the graphical plot shown in Fig. 3. Because the systems are highly cor-

TABLE IV

DISCRIMINATING POWERS FOR PAIRS OF CHROMATOGRAPHIC SYSTEMS WITH AN ERROR FACTOR OF 0.10 IN R_F FOR EACH SYSTEM

Total number of possible pairs = 4950.

System No.	*System No.*						
	2	*3*	*4*	*5*	*6*	*7*	*8*
1	0.903	0.917	0.898	0.902	0.917	0.925	0.875
2		0.866	0.875	0.876	0.912	0.921	0.844
3			0.900	0.899	0.928	0.929	0.871
4				0.763	0.871	0.915	0.839
5					0.878	0.916	0.834
6						0.927	0.865
7							0.873

TABLE V

CORRELATION COEFFICIENTS BETWEEN CHROMATOGRAPHIC SYSTEMS FOR 100 BASIC DRUGS

System No.	*System No.*						
	2	*3*	*4*	*5*	*6*	*7*	*8*
1	0.007	0.256	0.027	0.000	0.277	−0.288	−0.324
2		0.804	0.396	0.352	0.387	−0.462	−0.368
3			0.502	0.465	0.449	−0.434	−0.279
4				0.902	0.583	−0.226	0.000
5					0.596	−0.177	−0.068
6						−0.525	−0.285
7							0.544

related, the value of $DP_{4,5}$ is only 0.763 (Table IV) and even the combination of one of these systems with System 8 provides a higher *DP* ($DP_{4,8}=0.839$; $DP_{5,8}=0.834$).

The attainment of a large *DP* for systems used in combination is therefore achieved by large individual discriminating powers and low correlation between systems, although the best result usually involves a compromise between these two factors, *viz.* Systems 3 and 7, as shown in Fig. 4. The usefulness of the concept Discriminating Power is now apparent, since a single numerical value can be obtained which expresses the effectiveness of chromatographic systems whether used singly or in combination.

The series of three systems with the largest discriminating power is 3, 6, and 7 ($DP_{3,6,7}=0.979$) followed by Systems 1, 6, and 7 and then Systems 1, 3 and 7. The series of four systems with the largest discriminating power is 1, 3, 6, and 7 ($DP_{1,3,6,7}=0.993$). Thus, if a compound in Table II is to be identified using these four systems then, on average, 1.7 compounds will be found to be chromatographically similar[4].

Locating sprays can be used for the characterisation of drugs in chromatographic systems, but because the hue and colour intensity depend upon the freshness

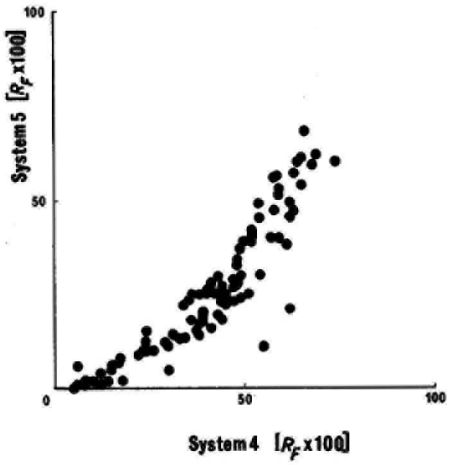

Fig. 3. Correlation of R_F values of 100 basic drugs using System 4 (silica gel with 0.1 *M* $KHSO_4$/methanol) and System 5 (silica gel with 0.1 *M* $KHSO_4$/ethanol, 95%). Correlation coefficient = 0.902.

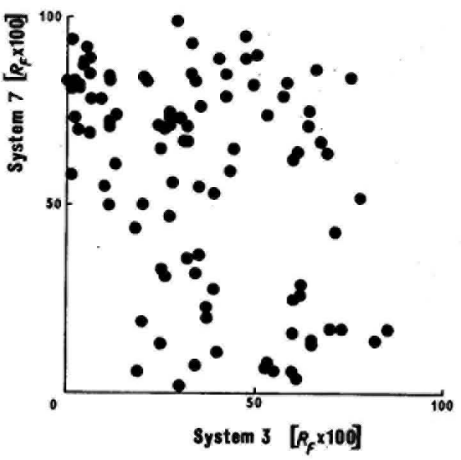

Fig. 4. Correlation of R_F values of 100 basic drugs using System 3 (silica gel with 0.1 *M* KOH/acetone) and System 7 (Whatman No. 1 paper dipped in 10% tributyrin in acetone/acetate buffer, pH 4.58, run at 95°). Correlation coefficient = −0.434.

of reagent, amount of drug in the spot, and presence of interfering substances, the results must be used with caution. The reactions of drugs with different spray reagents have been used as part of a systematic identification procedure[15]. However, most authors prefer to use spray reagents merely as locating agents when screening samples for the presence of basic drugs[3,16,17]. Therefore, although many spray reagents can be used to detect and locate any spots on the chromatogram, the searching system for identification should in future be based on the $R_F{}^c$ values and the colour reactions used only as a means of confirmation.

The eight systems used in this study are representative of those in common use and the correlation coefficients in Table V indicate how additional systems may be designed. Systems 2 to 5 use single solvents and, in general, are highly correlated showing that, although numerically different R_F values may be obtained by using a more polar solvent and the same substrate, the final order of elution of the compounds is similar. This is not surprising since there is a nearly rectilinear relationship between partition coefficients for a series of congeners in different solvent systems[18] and this has been shown to apply to chromatographic solvents[19]. Attempts to overcome this involve the use of a single mobile phase and a range of substrates, *viz.* cellulose, acetylated cellulose, aluminium oxide, and silica gel[20]. However, excluding the acetylated cellulose system, which showed poor separation, the remaining three pairs had correlation coefficients of between 0.79 and 0.81, which is not better than the results obtained in this study using the same substrate and different mobile phases (Table V).

The only system that shows a negligible correlation with all the other systems is System 1 (the mixed solvent system). The three components of the mobile phase obviously allow a completely different kind of separation to be obtained compared

to that achieved using a single solvent on the same substrate. Therefore, when creating a new system to be used in conjunction with existing systems, a combination of change of substrate and the change from single to mixed solvent or *vice versa* may be necessary to obtain a truly different system. When the system is designed, its *DP* can then be compared to those of the systems already in use to decide if it merits use in drug identification procedures.

The practical limitations of the systems may influence the choice of a system even if it has a high *DP*. For example, TLC is more sensitive and rapid than PC, and TLC may therefore be favoured by analysts to save time and sample. Also the best system was System 7, which must be run at elevated temperatures and has an objectionable smell because of the tributyrin and is therefore unpleasant to use. An analyst should therefore consider the practical constraints in conjunction with the relevant discriminating powers when choosing the most effective systems for the identification of basic drugs.

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OPTIMUM USE OF PAPER, THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY FOR THE IDENTIFICATION OF BASIC DRUGS

III. GAS–LIQUID CHROMATOGRAPHY

ANTHONY CHRISTOPHER MOFFAT, ANTHONY HOWARD STEAD and KENNETH WALLACE SMALLDON

Home Office Central Research Establishment, Aldermaston, Berks. RG7 4PN (Great Britain)

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SUMMARY

The concept of discriminating power has been applied to the gas–liquid chromatography of 62 basic drugs using eight stationary phases which show a wide range of polarity and are representative of those in current use. The SE-30 and OV-17 columns possessed the highest discriminating powers and SE-30 was the only phase which eluted all the drugs studied. More polar columns not only failed to elute many drugs altogether but the retention indices of those that were eluted also showed high correlation with all the other columns. A single low-polarity phase, such as SE-30 or OV-17, is recommended as the "preferred liquid phase" for the identification of basic drugs. If only low-molecular-weight drugs are considered, DEGS/KOH is recommended as the second "preferred liquid phase". The use of derivatisation for the identification of basic drugs by gas–liquid chromatography is briefly discussed.

INTRODUCTION

Many stationary phases have been, and still are, used in gas–liquid chromatography (GLC). In the years 1968–1969 Preston[1] found that approximately 300 different liquid phases were cited. This is not surprising considering that Applied Science Laboratories Inc. alone offer 245 stationary phases for sale[2]. The situation does not improve even when a small chemical class of compounds is examined—as many as 210 polysiloxanes have been used as phases at one time or another[3]. It might be expected that for the analyses of toxicological extracts only a few stationary phases would have been used. Unfortunately this is not the case, and Sunshine lists 172 stationary phases in his *Handbook of Analytical Toxicology*[4].

Two monographs[5,6] deal specifically with the gas chromatographic analyses of drugs, but do not discuss which systems are the most effective for their analyses. Some authors have developed systems for the analysis of basic drugs using more than one column[7–10] and standard analytical textbooks also quote retention data using several columns[4,11].

In spite of there being so many stationary phases available, only a handful have been extensively used for the analysis of drugs, with SE-30 being the most

popular[7–10,12] and also one of the first phases to be used[13]. The other popular non-polar phase that has been widely used is Apiezon L[7,14]. Of the polar phases, Carbowax 20M has been the most popular[7,8,11,15], with CDMS also being used by some workers[16–18]. To overcome the limitations of single stationary phases, mixtures have been used with varying degrees of success, *e.g.* SE-30–Carbowax 20M[19] and Hallcomid M-18–Carbowax 600[10]. Columns have even been prepared with different stationary phases in different parts of the column, *e.g.* ethylene glycol adipate in the middle with SE-30 at both ends[20].

To overcome the difficulty with the large number of phases in use it has been suggested that "preferred liquid phases"[1] be designated and that all future work should be carried out using them, and further that systems already in existence should be modified to incorporate these phases. The designated phases include some of those already in use in systems for the analysis of basic drugs, *viz.* SE-30, Apiezon L, polyethylene glycol 20M, and DEGS[21]. The above phases were examined, together with OV-17 and CDMS, which are also often used in toxicological analyses, thus obtaining a good range of column polarity, to determine which stationary phases are the most suitable for the identification of basic drugs.

EXPERIMENTAL

Materials

The stationary phases were obtained from the following sources: SE-30 (Silicone GE SE-30, GC grade) from Supelco (Bellefonte, Pa., U.S.A.); Apiezon L, OV-17, and Carbowax 20M from Perkin-Elmer (Beaconsfield, Bucks., Great Britain); CDMS (cyclohexane–dimethanol succinate, HI-EFF-8BP) from Applied Science Labs. (State College, Pa., U.S.A.), and DEGS (diethylene glycol succinate, LAC-3-R-728) from Cambridge Industries (Watertown, Mass., U.S.A.).

Choice of basic drugs

This was made on the same basis as before[22], except that only 62 drugs were chosen.

Preparation of samples

The bases were dissolved in ether or chloroform, or extracted from aqueous solutions of their salts after the solution had been made alkaline, to give final concentrations of approximately 1 μg/μl. Solutions of the compounds for the determination of McReynolds' constants[23] were made at a concentration of 10 μg/μl. Hydrocarbon standards were dissolved in hexane or toluene to give solutions of 1 μg/μl and 10 μg/μl.

Gas chromatography

Perkin-Elmer Model F11 and Pye Model 104 (Pye-Unicam, Cambridge, Great Britain) gas chromatographs were used with flame ionisation detectors and either 1- or 2-m columns. The stationary phases shown in Table I were coated on Chromosorb G (acid-washed, DMCS-treated, 80–100 mesh).

McReynolds' constants[23] were determined for each column with 10-μg

TABLE I
GLC SYSTEMS STUDIED

Column	*Stationary phase* *	*Column length (m)*	*Maximum operating temperature (°C)*
SE-30	2% SE-30	2	350
Apiezon L/KOH	2% Apiezon L +5% KOH	2	300
OV-17	5% OV-17	1	350
Carbowax 20M/KOH	1% Carbowax 20M +5% KOH	1	230
Carbowax 20M	1% Carbowax 20M	1	230
CDMS	1% CDMS	2	230
DEGS/KOH	1% DEGS +5% KOH	1	190
DEGS	1% DEGS	2	190

* On Chromosorb G (acid-washed, DMCS-treated, 80–100 mesh).

quantities of compounds, and 1-μg quantities of drugs and hydrocarbons were used for the determination of the retention indices of the drugs[24].

Treatment of results

The discriminating power[25] was calculated for each column using error factors of 10, 30, and 50 retention index units and then the discriminating power for each pair of columns was calculated using an error factor of 30 for SE-30, Apiezon L/KOH, and OV-17 and 50 for the remaining systems. Solely for the purpose of calculating the discriminating power, those drugs that failed to elute from a particular column were assigned a unique retention index. Correlation coefficients were also calculated for each pair of columns. Any drugs giving multiple peaks were excluded from the calculations.

RESULTS AND DISCUSSION

The columns examined are given in ascending order of polarity in Table II.

TABLE II
McREYNOLDS' CONSTANTS (ΔI) FOR THE GLC SYSTEMS STUDIED

Column	*Retention index of solvent*					ΔI*
	Benzene	*2-Pentanone*	*Butanol*	*Nitropropane*	*Pyridine*	
SE-30	650	680	690	710	740	249
Apiezon L/KOH	658	697	740	675	752	301
OV-17	740	796	755	896	887	853
Carbowax 20M/KOH	759	808	999	1006	945	1296
Carbowax 20M	819	872	1053	1042	1038	1603
CDMS	840	915	1006	1058	1072	1670
DEGS/KOH	1001	1038	1172	835	952	1777
DEGS	968	1021	1129	1197	1207	2301

* Calculated by summing the retention indices of the five solvents for each column and subtracting from it the sum of the retention indices of the same solvents on a squalane column ($\Sigma = 3221$).

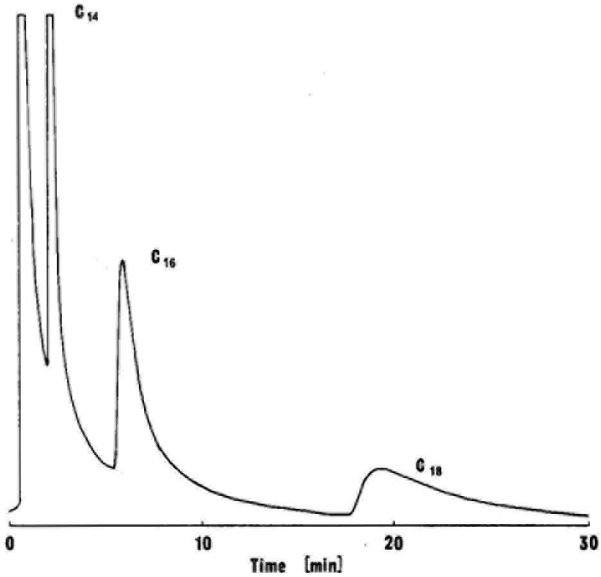

Fig. 1. Chromatogram of hydrocarbon standards (1-μg quantities) on a 2-m 1% DEGS column at 80 °.

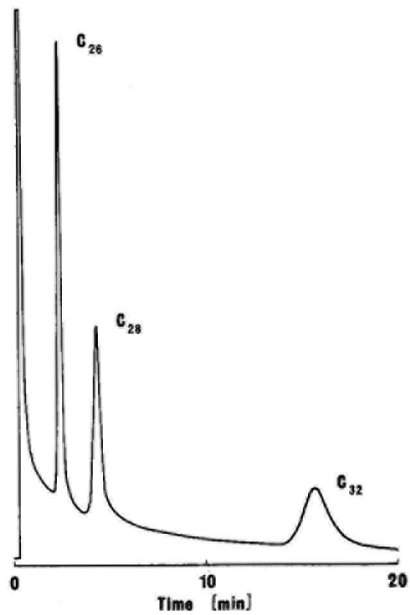

Fig. 2. Chromatogram of hydrocarbon standards (1-μg quantities) on a 2-m 1% DEGS column at 150 °.

They represent the maximum range in polarity of stationary phases suitable for the analysis of basic drugs and can be divided into three groups: the non-polar, SE-30 and Apiezon L; the semi-polar, OV-17 and Carbowax 20M; and the polar,

CDMS and DEGS. Although there are more polar phases than DEGS, it is the most polar phase that can be used up to a temperature of 190°.

Of the compounds eluted from the columns, most give symmetrical peaks indicating a minimum of adsorptive action, but a temperature of at least 100° is required for the polar columns before they act as good stationary phases. Even the hydrocarbon standards tail badly on the DEGS column at 80° (Fig. 1) and it is not until the temperature of the oven is above 120° that the column gives symmetrical peaks for the hydrocarbons (Fig. 2). This feature obviously limits the useful range of the polar columns, especially since they also tend to have lower maximum operating temperatures (Table I).

The retention indices of the 62 drugs studied on the eight columns are given in Table III, together with information regarding their stability during chromatography. Some drugs give small peaks with 1-μg quantities, but disproportionately

TABLE III

RETENTION INDICES OF 62 BASIC DRUGS IN EIGHT GLC SYSTEMS

— Indicates that no peak was seen between 1000 and 3500 retention index units.

Drug	*Stationary phase*							
	SE-30	*Apiezon L/KOH*	*OV-17*	*Carbowax 20M/KOH*	*Carbowax 20M*	*CDMS*	*DEGS/KOH*	*DEGS*
Amitriptyline	2200*	2216	2550*	2788	2924	—	—	—
Amphetamine	1110*	1136	1300*	1475	1587	1694	1507	1729
Amylocaine	1574	1579	1773	1915	2029	1985	1848	2183
Antazoline	2330*	2383	2820*	—	—	—	—	—
Atropine	2048**	2012	2310	2506	2658	2650	2527	3695§
Bromodiphenhydramine	2150*	2180	2465*	2792	2942	2842	2296	3268
Butacaine	2471	—	2826	—	3646	—	—	—
Caffeine	1810*	1980	2265*	—	2941	2873	2607	3587
Carbinoxamine	2052	2070	2398	2735	2903	—	2460	3270
Carbromal	1500*	1451	1790*	1735	1844	2158	2366§	2261
Chlorcyclizine	2215*	2324	2535*	2848	2965	—	—	3310
Chlordiazepoxide	2780*	3164	3140*	—	—	—	—	—
Chlorpheniramine	2000*	2021	2285*	2577	2725	2786	2126	3031
Chlorphentermine	1320*	1360	1530*	1710	1829	1931	1673	2113
Clemizole	2680	—	3135	—	—	—	—	—
Cocaine	2180*	—	2625*	—	—	—	—	3429
Codeine	2385*	—	2820*	—	3567	—	—	—
Colchicine	3340	—	—	—	—	—	—	—
Cyclizine	2010*	2036§	2350*	2516	2664	2652	2053	2972
Dextropropoxyphene	2180*	1793§,§§ 1855	2465*	2634	2818§§ 3194 3343 3701	—	2505	3052
Diamorphine	2615*	—	3050*	—	—	—	—	—
Diazepam	2407	—	2930	—	—	—	—	—
Diethylpropion	1480*	1487§	1705*	1845	1906	1915	1827	2145
Dihydrocodeinone	2411	—	2962	—	—	—	—	—

(*Continued on p. 24*)

TABLE III (*continued*)

Drug	*Stationary phase*							
	SE-30	*Apiezon L/KOH*	*OV-17*	*Carbowax 20M/KOH*	*Carbowax 20M*	*CDMS*	*DEGS/KOH*	*D*
Diphenhydramine	1855*	1877	2135*	2377	2513	2470	2047§	28
Dipipanone	2467	2466	2821	—	—	—	—	—
Ephedrine	1350*	1360	1590*	1965**	2082	2102	2284§	23
Ethoheptazine	1844	1827	2111	2328	2453	2425	2204	27
Levallorphan	2340	—	2747	—	3665	—	—	38
Lysergide	3445*	—	—	—	—	—	—	—
Meclozine	3050*	3147	3490*	—	—	—	—	—
Mephentermine	1240*	1255	1400*	1502	1602	1617	1478	17
Meprobamate	1790*	—	2185*	1941	1981	1803	—	21
Mepyramine	2204	2228§	2585	2970§	3134	—	—	—
Mescaline	1690*	1964§	2030*	2457§,§§ 2652	2638	—	2674	—
Methadone	2170*	2105	2445*	2609	2794	2811	2288	30
Methaqualone	2095	2182	2553	3050	3227	—	2746	36
Methyl-amphetamine	1170*	1182	1340*	1461	1590	1635	1400	16
Methyl phenidate	1780*	1786§	2060*	2260	2385	2301	2182	27
Morphine	2435*	—	2950*	—	—	—	—	—
Nicotine	1340*	1382§	1540*	1658	1814	1855	1676	20
Nikethamide	1500*	1474***	1840*	2142	2294	2220	2344	26
Nitrazepam	2674	—	—	—	—	—	—	—
Nortriptyline	2214	2275	2548	2899§	3062	—	—	36
Orphenadrine	1927	1921	2195	2423	2562	2527	2109	26
Papaverine	2806	—	—	—	—	—	—	—
Pethidine	1740*	1720	2000*	2144	2332	2256	2118	25
Phenacetin	1660*	1661	2035*	1996§	2107	1966	—	26
Phenazone	1830*	2237	2310*	2934	3082	—	3195	36
β-Phenethylamine	1120*	1110	1310*	1497	1622	1856	1435	18
Pheniramine	1802	1796§	2100	2339	2469	2538	1990	27
Phenmetrazine	1430*	1482§	1670*	1974§	2089	2058	1723	21
Phentermine	1130*	1170	1310*	1467	1566	1675	1493	17
Phenylephrine	2158	—	2603	—	—	—	—	—
Phenyl-propanolamine	1310*	1332§	1500*	2046	2231	2154	—	22
Procaine	1995*	—	2410*	—	3250	3016	—	—
Pyrrobutamine	2430*	2497	2830*	—	3270	—	—	—
Quinine	2755*	—	3300*	—	—	—	—	—
Strychnine	3040*	—	3760*	—	—	—	—	—
Thonzylamine	2045*	2183	2585*	2935	3095	—	2480	34
Tripelennamine	1960*	1999	2300*	2540	2682	2713	2109	30
Tryptamine	1710*	1712	2140*	—	—	—	2430	—

* Data from Kazyak and Permisohn[9].

** Major peak.

*** Nikethamide consistently gave a value of 1646 on another of our Apiezon L/KOH columns.

§ Result with 10-μg samples.

§§ Approximately equally sized peaks were obtained.

larger peaks with 10-μg quantities, indicating that some partial on-column decomposition takes place. Other drugs give minor peaks as well as a large major peak, and in these cases decomposition either in solution or in the injection port is indicated. Both dextropropoxyphene and mescaline give more than one major peak and therefore all the peaks are listed in Table III. In fact, these decompositions are so characteristic of these two compounds that they can provide a useful aid to identification.

For a column to be of the maximum use in an analysis, it should obviously elute all the drugs under consideration before its maximum operating temperature is reached. Only the SE-30 column achieves this, followed by the OV-17 column, which elutes 58 of the 62 drugs studied. The other columns show varying degrees of success, with the CDMS and DEGS/KOH columns eluting only about half of the drugs (30 and 33 drugs, respectively).

Several observations can be made from the distribution of the retention indices of the drugs on the columns used (Fig. 3). The more polar the column, the higher the retention index of the first drug to be eluted, *e.g.* the first drug to elute from the SE-30 column has a retention index of 1110, whereas the first to elute on the most polar column (DEGS) has a value of 1687. In practice the operating conditions of all the columns, *i.e.* percentage of stationary phase, length of column, etc., are always adjusted so that the first drug to be eluted from that column does so at the minimum operating temperature, thus allowing the maximum operating temperature range to be used for each column. However, because of the lower temperature maxima of the polar columns, their temperature range of operation is considerably less than that of the non-polar columns. Polar phases are therefore much less useful for the screening of extracts for basic drugs.

The number of compounds eluted by the KOH-treated columns is less than the number eluted from the non-treated columns. It has been common practice for many years to coat supports with KOH before the application of stationary phases for the analysis of basic drugs[26,27] in order to reduce the adsorptive effects of the supports. Although it does have this effect it also prevents the elution of any of the phenolic bases, *e.g.* morphine, by converting them to their non-volatile potassium phenates. Many alkaloids, especially those of high molecular weight, become thermolabile under alkaline conditions and do not elute from the KOH-treated columns (Apiezon L, Carbowax 20M and DEGS). This on-column decomposition occurs to a much lesser extent on the non-KOH-treated columns, as can be seen from Fig. 3, where the maximum retention index obtained on the Carbowax 20M column is 3700 but only 3100 on the KOH-treated column because of the non-elution of the higher molecular weight compounds.

The reproducibility of the retention indices on the columns generally decreases with increase in polarity. On individual columns operated at one temperature, the indices are always within ± 10 on the non-polar and slightly polar columns (SE-30, Apiezon L/KOH and OV-17) and within ± 25 on the more polar columns. However, when different columns using the same stationary phase are prepared, the reproducibility of the non-polar columns remains at ± 10, but the more polar columns, *e.g.* DEGS, give much more variance of retention indices. This lack of reproducibility is more noticeable at lower temperatures, *e.g.* a variation of 237 retention index units was obtained for β-phenethylamine on two DEGS/KOH

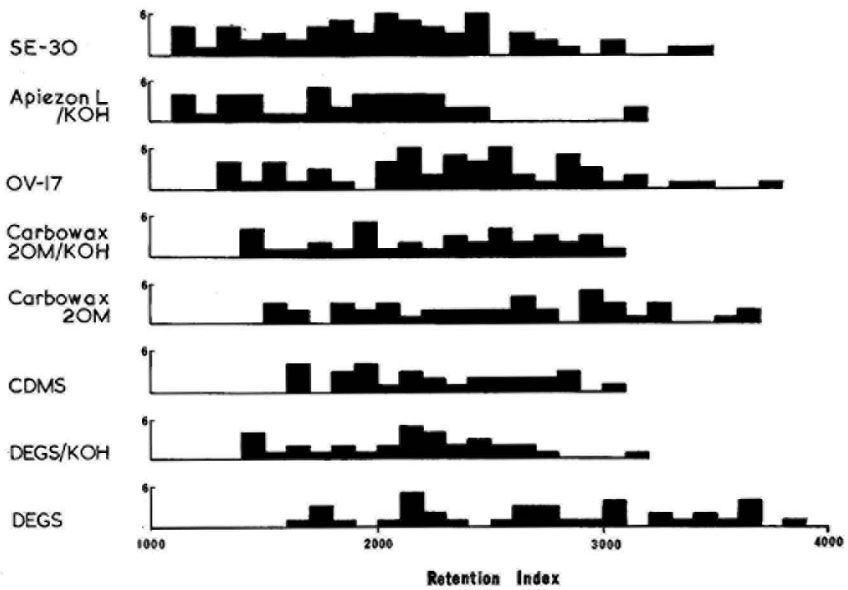

Fig. 3. Frequency distribution of retention indices of some basic drugs on GLC columns of different polarities.

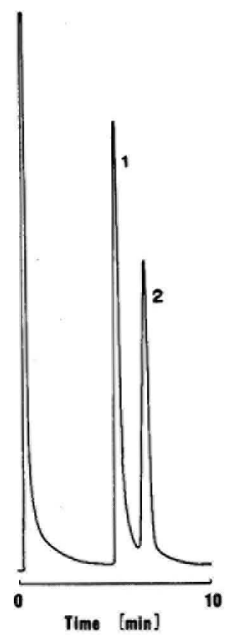

Fig. 4. Chromatogram of chlorphentermine (1) and diethylpropion (2) (retention indices 2113 and 2145, respectively) on a 2-m 1% DEGS column at 120°.

TABLE IV

DISCRIMINATING POWERS FOR SINGLE GLC SYSTEMS AT VARIOUS ERROR FACTORS

Total number of possible pairs = 1770.

Column	*Error factor (in retention index units)*		
	10	*30*	*50*
SE-30	0.987	0.967	0.945
Apiezon L/KOH	0.908	0.899	0.885
OV-17	0.983	0.971	0.953
Carbowax 20M/KOH	0.840	0.834	0.824
Carbowax 20M	0.911	0.898	0.890
CDMS	0.753	0.749	0.745
DEGS/KOH	0.766	0.760	0.756
DEGS	0.855	0.847	0.837

columns. However, most of the values can be reproduced within ± 50 provided that the column operating temperature is greater than 120°. Even when data are obtained using temperature programming (methylene units[28]), the differences between the retention indices and the methylene units $\times 100$ are never greater than ± 50.

When the data obtained in this study are compared with previously published data on the retention indices of drugs, very good agreement is found. The SE-30 results[9] are all within ± 36 and, provided that a linear regression equation is used with which to compare the data, variations of less than 26 are obtained for Apiezon L/KOH columns and less than 56 for Carbowax 20M/KOH columns, whether they are packed or support-coated open tubular columns[29].

From the above it is reasonable to assume that an appropriate error factor (E) for the SE-30, Apiezon L/KOH and OV-17 columns is 30, but that this is increased to 50 for the other columns. (Fig. 4 shows that on a single isothermal run on a polar column peaks that are separated by only 32 units can easily be distinguished and, provided all analyses are made at a single temperature, the error factor for polar columns may be reduced to 30.)

At the estimated error factors the column with the highest DP is OV-17 (DP=0.971), closely followed by SE-30 (DP=0.967). These values are very near to the maximum DP for a column of useful range, 1000–4000 retention index units, which is 0.980 at an error factor of 30 (ref. 25). The remaining columns have a much lower value for DP (Table IV). The variation in DP can be mainly attributed to the number of compounds that each column eluted. For example, the OV-17 and SE-30 columns eluted nearly all the drugs, whereas the CDMS column eluted only 30 and it therefore has the lowest value (DP=0.745). In the calculation of DP, drugs which did not elute were assigned a unique retention index so that they were all indistinguishable from each other but distinguishable from all those which did elute. Unlike the situation with PC and TLC[22], the DP does not change rapidly with variation in E. This arises principally because E is always small compared to the total range of possible retention indices. All the columns also have good

TABLE V

DISCRIMINATING POWERS FOR PAIRS OF GLC SYSTEMS

Error factor for SE-30, Apiezon L/KOH and OV-17 = 30 units; error factor for remaining columns = 50 units. Total number of possible pairs = 1770.

System	*System*						
	Apiezon L/KOH	*OV-17*	*Carbowax 20M/KOH*	*Carbowax 20M*	*CDMS*	*DEGS/KOH*	*DEGS*
SE-30	0.990	0.992	0.989	0.993	0.985	0.984	0.990
Apiezon L/KOH		0.990	0.919	0.951	0.928	0.911	0.937
OV-17			0.983	0.988	0.981	0.984	0.985
Carbowax 20M/KOH				0.897	0.862	0.866	0.874
Carbowax 20M					0.905	0.918	0.915
CDMS						0.828	0.863
DEGS/KOH							0.864

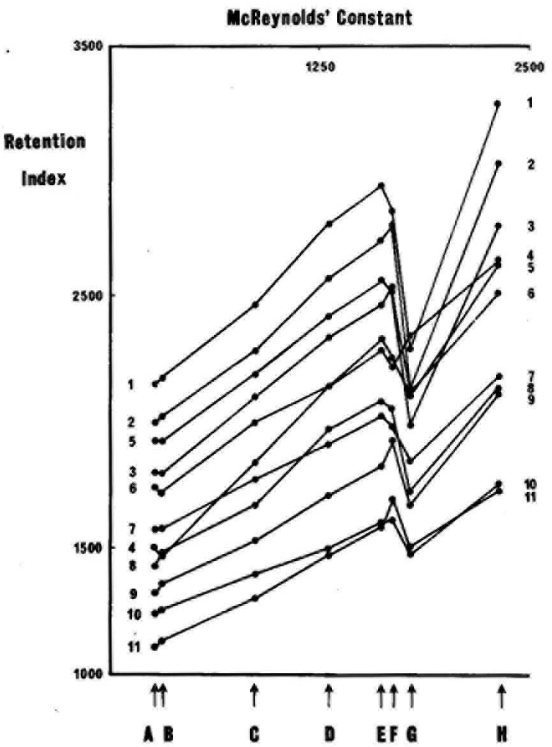

Fig. 5. Change of retention indices of some basic drugs with the polarity of the column. A = SE-30; B = Apiezon L/KOH; C = OV-17; D = Carbowax 20M/KOH; E = Carbowax 20M; F = CDMS; G = DEGS/KOH; H = DEGS; 1 = bromodiphenhydramine; 2 = chlorpheniramine; 3 = pheniramine; 4 = nikethamide; 5 = orphenadrine; 6 = pethidine; 7 = amylocaine; 8 = phenmetrazine; 9 = chlorphentermine; 10 = mephentermine; 11 = amphetamine.

frequency distributions for the peaks that are eluted —no column having more than six compounds within a band of 100 retention index units (Fig. 3).

When more than one chromatographic system is used, the combined discriminating power obviously increases and the *DP*'s for the 28 possible pairs of systems at the estimated error factors are given in Table V. The highest second order *DP*'s are associated with either the OV-17 or SE-30 systems, although they have only increased marginally from the first order *DP*'s. Nevertheless, many drug detection systems have been advocated which use both a polar and a non-polar column, such as SE-30 and Carbowax 20M/KOH[7,8,10,30]. However, as expected from the second order *DP* values, chromatographing drugs on several columns produces little extra information. Fig. 5 shows the results obtained using eleven representative

TABLE VI

CORRELATION COEFFICIENTS BETWEEN GLC SYSTEMS FOR 60 BASIC DRUGS

System	*System*						
	Apiezon L/KOH	*OV-17*	*Carbowax 20M/KOH*	*Carbowax 20M*	*CDMS*	*DEGS/KOH*	*DEGS*
SE-30	0.981	0.991	0.934	0.929	0.881	0.713	0.899
Apiezon L/KOH		0.982	0.972	0.968	0.939	0.796	0.941
OV-17			0.951	0.946	0.883	0.789	0.927
Carbowax 20M/KOH				0.999	0.964	0.849	0.963
Carbowax 20M					0.974	0.846	0.962
CDMS						0.817	0.926
DEGS/KOH							0.895

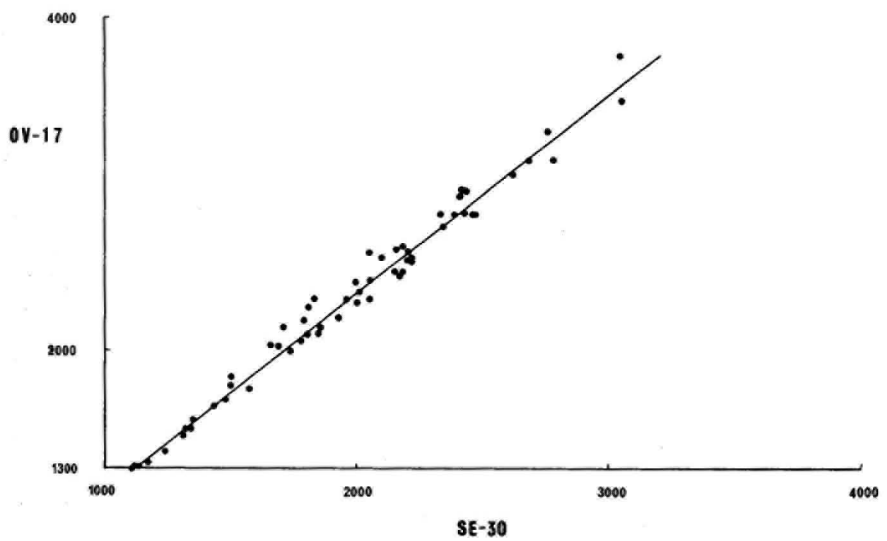

Fig. 6. Correlation of the retention indices of some basic drugs on SE-30 and OV-17 columns ($r=0.991$).

drugs on all the columns studied. The retention indices of the drugs increase as the polarity of the column increases, but the order of elution on the least polar column is different in only two cases from that on the most polar column. Thus, all the columns exhibit similar retention behaviour to the drugs. This clearly shows that the selection of "preferred liquid phases" for basic drugs purely on the basis of polarity differences would be unwise.

The correlation coefficients (r) for each pair of phases are shown in Table VI and all the pairs are seen to be highly correlated. This explains why the second order *DP*'s have only increased marginally from the values obtained from a single low-polarity column. The highest correlation coefficient occurs for the combination Carbowax 20M and Carbowax 20M/KOH (r=0.999), followed by the two silicone phases SE-30 and OV-17 (r=0.991). Fig. 6 shows the results for the latter combination graphically and from the high degree of correlation it is obvious that if one of the columns is used for the analysis of basic drugs, the use of the second column will give very little or no additional information. Excluding the DEGS/KOH data in Table VI, the correlation is seen to be highest between non-polar and slightly polar, and also between polar and slightly polar columns. As expected, the correlation is lowest, although still highly significant, between the non-polar and polar columns.

DEGS/KOH is a special case among the phases studied, the correlation with other phases (Table VI) being much lower than would be expected purely on the basis of polarity (Table II). It is likely that the KOH treatment of the support reacts with the polyester stationary phase to modify it in such a way as to make its partition properties somewhat different from those expected. A second DEGS/KOH column was prepared using a different source of DEGS in order to check that this phenomenon was not a property of one particular column. That it is a function merely of the polyester phase can be seen from the fact that although the KOH treatment of Carbowax 20M and DEGS drastically reduces both their

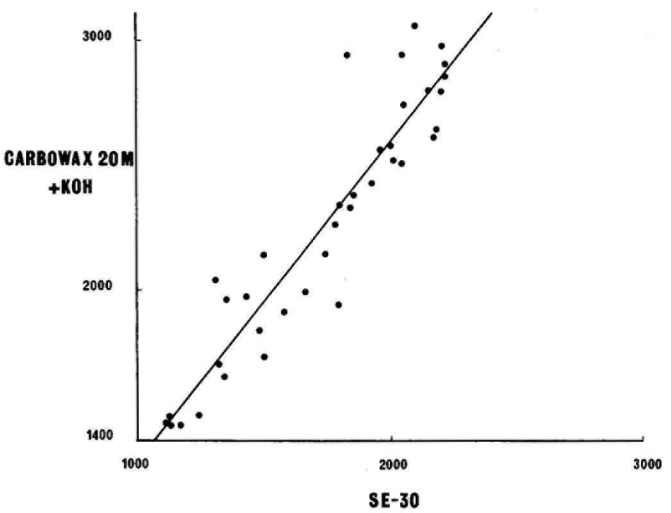

Fig. 7. Correlation of the retention indices of some basic drugs on SE-30 and Carbowax 20M/KOH columns (r=0.934).

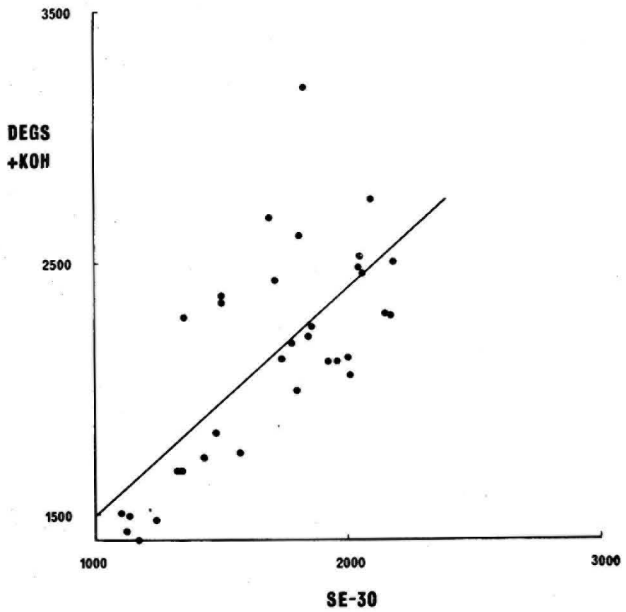

Fig. 8. Correlation of the retention indices of some basic drugs on SE-30 and DEGS/KOH columns ($r=0.713$).

polarities (from 1603 to 1296 and 2301 to 1777, respectively, as shown in Table II), the retention properties of the two Carbowax 20M columns are virtually identical ($r=0.999$), but DEGS and DEGS/KOH show distinct differences in their behaviour ($r=0.895$). The most popular columns used together, *viz.* SE-30 and Carbowax 20M/KOH (Fig. 7), are certainly not as good as the SE-30 and DEGS/KOH combination ($r=0.713$), as shown by a comparison of Fig. 8 with Figs. 6 and 7.

Derivative formation is often used as an aid to identification[6,7,10]. The fact that a derivative is formed with a particular reagent indicates the functional groups that an unknown drug may contain, and the retention indices of the derivatives provide additional data. Primary amines may be converted to Schiff bases by aldehydes or ketones, primary and secondary amines as well as phenols can be acylated by anhydrides, and alcohols and phenols can be converted to trimethylsilyl derivatives with trimethylsilylimidazole. Beckett *et al.*[7] chromatographed a number (n) of acetone Schiff bases and acetyl derivatives on both SE-30 and Carbowax 20M/KOH columns in order to identify basic drugs. On the SE-30 column the correlations between the log retention times of the base with the acetone and acetyl derivatives are 0.94 ($n=11$) and 0.98 ($n=27$), respectively, *i.e.* very little information is obtained by measuring the retention time of the product. The useful information gained is that the reaction has occurred. Chromatographing the acetone or acetyl derivatives on the Carbowax 20M/KOH column is the most useful where the correlations between the log retention times of the base and the two products are 0.49 ($n=8$) and 0.77 ($n=14$), respectively. These correlation coefficients cannot be directly compared with those in Table VI, because they are calculated in terms of log retention time instead of retention index. However, the correlation between the log retention

times on the SE-30 and Carbowax 20M/KOH columns is 0.75 (n=14) and this may be compared with the coefficient obtained from retention index data on the same columns, which is 0.934 (n=37).

Of the 62 basic drugs studied in this work, only twenty-nine would be expected to form acetyl derivatives and of these only nine are primary amines and could form Schiff bases. (The possibility of transesterification of some esters when treated with acid anhydrides or acid chlorides should not be ignored.) The formation of derivatives may therefore be used as an adjunct to the GLC analysis of basic drugs, but the retention characteristics of any reaction product are of somewhat limited value especially when the possibility of multiple derivative formation, depending on the reaction conditions used, is considered. It is of even less value when analysing large-molecular-weight drugs whose derivatives may well not elute.

It is therefore apparent that a low-polarity phase, such as SE-30 or OV-17, should be chosen as the "preferred liquid phase" for the GLC analysis of drugs. The more polar columns have much smaller *DP*'s because they fail to elute many compounds and have high error factors. The use of a second column in combination with the above would not be advocated because of the high degree of correlation characteristics which results in only a slight increase in *DP* except in the case of basic drugs having retention indices <2000 on the non-polar column, when a DEGS/KOH column could be recommended as a second "preferred liquid phase". There are insufficient data to recommend a "preferred liquid phase" for derivatisation of basic drugs. However, it is already apparent that high correlation with the retention index of the parent compound will occur using SE-30 and that only the derivatives of low-molecular-weight bases will elute on a less correlated column such as Carbowax 20M/KOH. In practice it may be preferable to use one GLC column only and then an alternative technique for further identification. For example, the combination of GLC and TLC systems might be more appropriate and this aspect will be pursued in future work.

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GAS CHROMATOGRAPHIC INVESTIGATION OF ORGANOMETALLIC COMPOUNDS AND THEIR CARBON ANALOGUES

III. A STUDY OF KOVÁTS RETENTION INDICES FOR TETRA-ALKOXYSILANES CONTAINING BRANCHED ALKOXY GROUPS

I.-B. PEETRE

Department of Technical Analytical Chemistry, Chemical Center, Lund (Sweden)

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SUMMARY

A method previously developed for the calculation of retention indices (Apiezon and XE-60 stationary phases) of mixed tetraalkoxysilanes with normal alkoxy groups was applied to some counterparts which also contained branched alkoxy groups (isopropoxy, isobutoxy and *sec.*-butoxy). It was found that the best agreement between experimental and calculated values was obtained for the Apiezon M retention indices, the average difference being about 3 index units. For the XE-60 retention indices, good agreement was obtained only for tetraalkoxysilanes that contain isobutoxy groups.

The linear relationship in the two-phase plot for retention indices of tetra-alkoxysilanes, belonging to the same structure group (same number of CH_3, CH_2, CH and C groups), previously established for purely normal compounds, was found also to be valid for the structurally more complicated tetraalkoxysilanes studied in this work.

INTRODUCTION

The two previous papers[1,2] in this series were devoted to a study of Kováts retention indices of tetraalkoxysilanes with normal alkoxy groups. Methods for calculating retention indices were developed, and various interrelations between retention indices, as well as relationships between retention indices and certain physical constants, were established. In this paper, the previous studies are expanded to include tetraalkoxysilanes with branched alkoxy groups. It was of special interest to investigate whether the calculation methods valid for mixed tetraalkoxysilanes with normal alkoxy groups could also be applied to the counterparts with branched alkoxy groups. Another aim was to examine if the linear relationship in the two-phase plot, previously found to exist for normal tetraalkoxysilanes belonging to the same structure group[1], could be extended to include the present compounds.

EXPERIMENTAL

Packed columns (2–4 m × 1/8 in. O.D.) containing Apiezon M or cyanosilicone XE-60 as stationary phase were used in conjunction with a Varian Model 1400 gas chromatograph with a flame ionization detector. These columns were the same as those used in Part I, and further details can also be found in that paper[1]. The retention indices were determined using the computer method developed by the present author[3].

All compounds investigated were prepared in this laboratory. The symmetrical tetraalkoxysilanes were prepared from silicon tetrachloride and alcohols, and the mixed tetraalkoxysilanes by alkoxyl interchange between symmetrical tetraalkoxysilanes or by alcoholysis of the latter. Gaseous hydrogen chloride was used as a catalyst in both instances. The difficulty in separating certain compound mixtures by gas chromatography caused some limitations on the material available for the study.

RESULTS AND DISCUSSION

Retention indices and ΔI values of symmetrical tetraalkoxysilanes with branched alkoxy groups

Table I gives the retention indices and ΔI values of tetraisopropoxy-, tetraisobutoxy- and tetra-*sec.*-butoxysilanes. For comparison, the data for the corresponding normal tetraalkoxysilanes, taken from ref. 1, are also given. The retention indices of the branched compounds are considerably lower than those of the normal compounds, which was to be expected because of their lower boiling points. The marked difference between the ΔI values of normal and branched compounds reflects the increased shielding of the polar Si–O and C–O bonds in the latter, which increases their activity on the polar XE-60 stationary phase.

TABLE I

RETENTION INDICES AND ΔI VALUES FOR SYMMETRICAL BRANCHED AND NORMAL TETRAALKOXYSILANES

Compound	I_{160}^{ApM}	I_{160}^{XE}	$\Delta I_{160} = I_{160}^{XE} - I_{160}^{ApM}$
(iso-PrO)$_4$Si	915	994	79
(*n*-PrO)$_4$Si	1163	1315	152
(iso-BuO)$_4$Si	1304	1410	106
(*sec.*-BuO)$_4$Si	1264	1337	73
(*n*-BuO)$_4$Si	1488	1630	142

Calculation of retention indices

An equation for calculating retention indices of mixed tetraalkoxysilanes on the basis of the symmetrical counterparts was given previously[1]:

$$I(\mathrm{RO})_4\mathrm{Si} = \Sigma I(\mathrm{RO})\mathrm{Si} + \Sigma(n \cdot d \cdot k)_{\mathrm{RO-RO}} \qquad (1)$$

where

RO = *n*-alkoxy;
I(RO)Si = group retention index, obtained from the retention index of a symmetrical tetraalkoxysilane by division by four;
n = so-called combination number, obtained by multiplying the numbers of the various alkoxy groups in a combination. A combination is formed between any unlike alkoxy groups bonded to the silicon atom. Thus, in the tetraalkoxysilane $(RO)_nSi(OR')_{4-n}$, RO and R'O form a combination with the combination number $n(4-n)$. In the tetraalkoxysilane $(RO)_nSi(OR')_m(OR'')_{4-n-m}$, there are three combinations with the combination numbers $n \cdot m$, $n(4-n-m)$ and $m(4-n-m)$ (see also ref. 1);
d = carbon number difference between alkoxy groups in a combination;
k = constant, dependent on the smallest alkoxy group in a combination.

The second term in eqn. 1 can be considered as a correction term that accounts for the deviation of the sum of the group retention indices in the mixed tetraalkoxysilane from additivity. In the calculation of retention indices of the mixed tetraalkoxysilanes in Table II, it has been assumed that the constant k for a combination containing a branched alkoxy group is the same as when the alkoxy group is normal. Accordingly, isopropoxy is treated as an *n*-propoxy group and isobutoxy and *sec.*-butoxy as an *n*-butoxy group. The k values used were taken from Table V in ref. 1.

The best agreement between experimental and calculated values was obtained for the Apiezon M retention indices, the mean deviation being about 3 index units. Thus, it appears that the method used gives an at least approximately correct value for the correction term for the type of compound studied. For 5 of the 28 compounds, the deviation is 6 index units or more. All of these compounds contain *sec.*-butoxy groups. This deviation is considerably greater than the error in the retention index measurement, and it is concluded that eqn. 1 is less suited for the calculation of Apiezon M retention indices of mixed tetraalkoxysilanes with *sec.*-butoxy groups, than for those with isopropoxy or isobutoxy groups.

This fact is even more apparent for the XE-60 retention indices, where the difference between experimental and calculated values for *sec.*-butoxy silanes is considerable. Good agreement is obtained in this case only for mixed tetraalkoxysilanes with isobutoxy groups. As the calculated values for *sec.*-butoxy silanes are always too high, it is obvious that a better agreement should be attained by using smaller $k \cdot d$ values for the combinations methoxy–*sec.*-butoxy and ethoxy–*sec.*-butoxy, respectively.

To summarize, the results obtained warrant the conclusion that a successful application of eqn. 1 using the k values valid for *n*-alkoxy groups is dependent both on the type of branched alkoxy group and on the polarity of the stationary phase. On a polar stationary phase such as XE-60, better agreement between experimental and calculated values can be expected when the branching takes place at the β-carbon atom, than when the alkoxy group is branched at the α-carbon atom. On a non-polar phase such as Apiezon M, not only the position of branching but also the size of the

TABLE II

EXPERIMENTAL AND CALCULATED RETENTION INDICES FOR MIXED TETRAALKOXYSILANES CONTAINING BRANCHED ALKOXY GROUPS

Compound	I_{160}^{ApM}			I_{160}^{XE}			ΔI_{160}	*Structure code*
	Exptl.	*Calcd.*	*Diff.*	*Exptl.*	*Calcd.*	*Diff.*		
$(MeO)_3SiOPr$-iso	743	741	−2					5–0–1–0
$(MeO)_2Si(OPr\text{-}iso)_2$	813	812	−1					6–0–2–0
$MeOSi(OPr\text{-}iso)_3$	869	870	+1					7–0–3–0
$(MeO)_3SiOBu$-iso	851	848	−3	1077	1076	− 1	226	5–1–1–0
$(MeO)_2Si(OBu\text{-}iso)_2$	1022	1018	−4	1206	1207	+ 1	184	6–2–2–0
$MeOSi(OBu\text{-}iso)_3$	1172	1171	−1	1314	1319	+ 5	142	7–3–3–0
$(MeO)_3SiOBu$-*sec.*	839	838	−1	1057	1057	0	218	5–1–1–0
$(MeO)_2Si(OBu\text{-}sec.)_2$	996	998	+2	1163	1171	+ 8	167	6–2–2–0
$MeOSi(OBu\text{-}sec.)_3$	1135	1141	+6	1252	1264	+12	117	7–3–3–0
$(EtO)_3SiOBu$-iso	974	973	−1	1131	1130	− 1	157	5–4–1–0
$(EtO)_2Si(OBu\text{-}iso)_2$	1091	1089	−2	1231	1229	− 2	140	6–4–2–0
$EtOSi(OBu\text{-}iso)_3$	1201	1200	−1	1324	1323	− 1	123	7–4–3–0
$(EtO)_3SiOBu$-*sec.*	959	963	+4	1107	1111	+ 4	148	5–4–1–0
$(EtO)_2Si(OBu\text{-}sec.)_2$	1063	1069	+6	1182	1193	+11	119	6–4–2–0
$EtOSi(OBu\text{-}sec.)_3$	1164	1170	+6	1260	1268	+ 8	96	7–4–3–0
$(PrO)_3SiOPr$-iso	1103	1101	−2	1232	1235	+ 3	129	5–6–1–0
$(PrO)_2Si(OPr\text{-}iso)_2$	1040	1039	−1	1149	1155	+ 6	109	6–4–2–0
$PrOSi(OPr\text{-}iso)_3$	977	977	0	1066	1074	+ 8	89	7–2–3–0
$(BuO)_3SiOBu$-iso	1438	1442	+4	1573	1575	+ 2	135	5–10–1–0
$(BuO)_2Si(OBu\text{-}iso)_2$	1393	1396	+3	1517	1520	+ 3	124	6–8–2–0
$BuOSi(OBu\text{-}iso)_3$	1345	1350	+5	1460	1465	+ 5	115	7–6–3–0
$(MeO)_2Si(OBu)OBu$-iso	1066	1064	−2	1264	1262	− 2	198	5–4–1–0
$MeOSi(OBu)_2OBu$-iso	1262	1263	+1	1428	1429	+ 1	166	5–7–1–0
$MeOSi(OBu)\,(OBu\text{-}iso)_2$	1216	1217	+1	1372	1374	+ 2	156	6–5–2–0
$(MeO)_2Si(OBu)OBu$-*sec.*	1054	1054	0	1242	1244	+ 2	188	5–4–1–0
$MeOSi(OBu)_2OBu$-*sec.*	1247	1253	+6	1402	1410	+ 8	155	5–7–1–0
$MeOSi(OBu)\,(OBu\text{-}sec.)_2$	1189	1197	+8	1324	1337	+13	135	6–5–2–0
$(MeO)_2Si(OPr\text{-}iso)OPe$	1056	1053	−3	1245	1245	0	189	5–4–1–0

group is of importance, as tetraalkoxysilanes that contain isopropoxy groups show better agreement than those that contain *sec.*-butoxy groups.

I_{160}^{ApM} *versus* I_{160}^{XE}

In the two-phase plot of I_{160}^{ApM} *versus* I_{160}^{XE} for normal tetraalkoxysilanes, two linear relationships were established[1]. One was between retention indices of members in homologous series and the other between retention indices of compounds that have the same structure code. The compounds available permit no linearity test for members in homologous series, but it is undoubtedly reasonable to assume that the relationship is linear to the same extent as for purely normal tetraalkoxysilanes.

In Table II, a structure code is given for each compound. This code is written *a–b–d–e*, where *a*, *b*, *d* and *e* are the number of CH_3, CH_2, CH and C groups, respectively. As it was found in the previous work that normal tetraalkoxysilanes with

the same structure code align themselves along straight lines, it was of interest to examine if the same rule was applicable to the structurally more complicated tetraalkoxysilanes studied in this work.

The retention indices of members of two structure groups with the codes 5–4–1–0 and 6–4–2–0 are plotted in Fig. 1, where the points are seen to lie on straight lines. This is also borne out by the correlation coefficients in Table III. The difficulties encountered in the gas chromatographic separation of various mixtures resulting from the synthesis of the tetraalkoxysilanes investigated have prevented the determination of retention indices for more than two compounds in each of the remaining four structure groups in Table III. However, the equations of the straight lines assumed to belong to these structure groups have nevertheless been calculated and are given in the table.

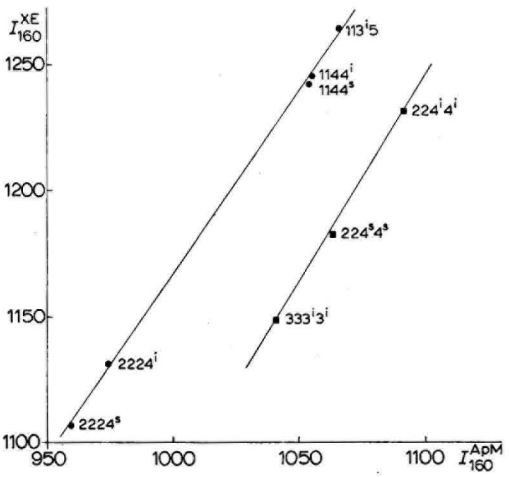

Fig. 1. Two-phase plot of I_{160}^{ApM} *versus* I_{160}^{XE}. Structure codes: ●, 5–4–1–0; ■, 6–4–2–0. The formula code denotes the alkoxy groups bonded to the silicon atom: 1 = methoxy, 2 = ethoxy, 3 = *n*-propoxy, 3^{i} = isopropoxy, 4^{s} = *sec.*-butoxy, etc.

TABLE III

CONSTANTS IN AND CORRELATION COEFFICIENTS OF THE LINEAR RELATIONSHIP $I_{160}^{XE} = k \cdot I_{160}^{ApM} + l$ FOR TETRAALKOXYSILANES WITH THE SAME STRUCTURE CODE *a–b–d–0*

Structure code	*Number of compounds*	*k*	*l*	*Correlation coefficient*
5–4–1–0	5	1.43	−264	0.9996
6–4–2–0	3	1.61	−530	0.9990
6–2–2–0	2	1.65	−484	
6–5–2–0	2	1.78	−790	
7–3–3–0	2	1.68	−650	
7–4–3–0	2	1.73	−753	

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CHROM. 7199

GAS CHROMATOGRAPHIC INVESTIGATION OF ORGANOMETALLIC COMPOUNDS AND THEIR CARBON ANALOGUES

IV. DETERMINATION, CALCULATION AND CORRELATION OF KOVÁTS RETENTION INDICES FOR TETRAALKYLSILANES

I.-B. PEETRE and B. E. F. SMITH

Department of Technical Analytical Chemistry, Chemical Center, Lund (Sweden)

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SUMMARY

Equations for the calculation of retention indices for mixed tetraalkylsilanes are given. The significance of the sign of the charge on the terminal methyl carbon atom of the alkyl groups bonded to silicon in determining the magnitude of the retention index and the ΔI value is discussed. Rules for the estimation of the temperature dependence of retention indices for tetraalkylsilanes are given. Linear relationships are shown to exist between retention indices for homologous series of mixed tetraalkylsilanes on the one hand and retention indices of symmetrical tetraalkylsilanes or of various tetraalkoxysilanes on the other. The two-phase plot of I_{160}^{ApL} *versus* I_{160}^{XE} for tetraalkylsilanes is shown to be completely different from that for tetraalkoxysilanes.

INTRODUCTION

The three previous papers[1–3] in this series dealt with Kováts retention indices for tetraalkoxysilanes. Methods for the calculation of retention indices were developed, and various interrelations between retention indices, as well as relationships between retention indices and certain physical constants, were established. In this paper, a similar study of tetraalkylsilanes, R_4Si, with normal alkyl groups is reported.

Tetraalkylsilanes belong to the types of organosilicon compounds most frequently investigated by gas chromatography. Thus, Semlyen and co-workers[4,5] measured the retentions of a considerable number of symmetrical and mixed tetraalkylsilanes relative to mesitylene on squalane, and Pollard *et al.*[6], in connection with redistribution studies on tetraalkylsilanes, determined retention volumes on silicone oil E-301. In similar work by Hailey and Nickless[7], specific retention volumes were given for some tetraalkylsilanes mainly containing branched alkyl groups. Garzó *et al.*[8] reported retention indices for several organosilicon compounds on Apiezon L, SE-30 and QF-1, including tetramethyl- and tetraethylsilane. Wurst and Churáček[9] measured a quantity called the silicon index (I_{Si}) for tetramethyl-, tetraethyl- and tetrapropylsilane. However, no data were given that permit the recalculation of the silicon index to Kováts retention index.

EXPERIMENTAL

Apparatus and columns

A Varian Model 1400 gas chromatograph with a flame ionization detector, and equipped with an auxiliary thermocouple and temperature gauge (Mettler TM 15), was used for the retention index measurements. The temperature could be read to $\pm 0.1°$ and was constant within this range during a measurement. Any significant deviation of the observed temperature from the measurement temperature in the tables was corrected for.

Perkin-Elmer capillary steel columns (50 m $\times$ 0.25 mm) containing Apiezon L and cyanosilicone XE-60, respectively, as stationary phases were used throughout. For the determination of accurate retention indices, the recently described computer method[10] was utilized.

Materials

Most of the compounds investigated were synthesized in this laboratory. The symmetrical tetraalkylsilanes were prepared from alkyl-lithium and silicon tetrachloride. The same method was used for preparing some of the mixed compounds, starting from appropriate alkyl chlorosilanes and alkyl-lithium. However, the majority of the mixed tetraalkylsilanes were obtained by alkyl interchange between symmetrical tetraalkylsilanes using aluminium chloride (1 mole-%) as catalyst[6]. Depending on the boiling points of starting material and products, the reaction mixture was heated either under reflux or in a sealed tube to 150–175° for 2 h. Under these conditions, the formation of undesired by-products was generally limited.

The syntheses by alkyl interchange were performed on a small scale. No separations of the compounds formed were made prior to the gas chromatographic investigation. The peaks in a chromatogram could generally be unequivocally assigned, as the various tetraalkylsilanes resulting from the systematic exchange of one alkyl group for another appeared in order of increasing molecular weight on both stationary phases. In some doubtful cases, this was proved by comparison with model compounds obtained by the alkyl-lithium–chlorosilane method.

RESULTS AND DISCUSSION

Retention indices and ΔI values of tetraalkylsilanes

Table I summarizes retention indices and ΔI values of tetraalkylsilanes with R = methyl to pentyl, and in Table II the corresponding group values are given. These values were obtained by division of the indices and ΔI values for the symmetrical tetraalkylsilanes, SiR_4, in Table I by four. In Table I, I_{160}^{ApL} values are generally given to one decimal place, while I_{160}^{XE} values are generally given as integers, which is in agreement with the general rule that the retentions of non-polar compounds can be determined more accurately on non-polar than on polar stationary phases. As the XE-60 column has a recommended maximum temperature of 150°, I_{160}^{XE} values were obtained by extrapolation from I^{XE} values determined at lower temperatures. The ΔI values for *n*-alkyl groups bonded to silicon are strikingly low, and only 1/15th to 1/5th of the corresponding ΔI values for *n*-alkoxy groups[1]. This is a consequence of the low polarity of the Si–C bond compared with the Si–O–C bond.

TABLE I

EXPERIMENTAL AND CALCULATED RETENTION INDICES AND ΔI VALUES FOR TETRAALKYLSILANES

Compound	I_{160}^{ApL}			I_{160}^{XE}			$\Delta I_{160} = I_{160}^{XE} - I_{160}^{ApL}$		
	*Exptl.**	*Calcd.***	*Diff.*	*Exptl.****	*Calcd.***	*Diff.*	*Exptl.*	*Calcd.*§*	*Diff.*
Me_4Si	420			437			17		
Et_4Si	922.2			948.1			25.9		
Pr_4Si	1192.9			1204.4			11.5		
Bu_4Si	1485.6			1508.7			23.1		
Pe_4Si	1820			1847			27		
Me_3SiEt	550	559	+ 9	569	578	+ 9	19	19	0
Me_2SiEt_2	676.9	688	+11	697	710	+13	20	22	+2
$MeSiEt_3$	800.1	810	+10	825	833	+ 8	25	23	−2
Me_3SiPr	634.0	638	+ 4	649	654	+ 5	15	16	+1
Me_2SiPr_2	838.8	841	+ 2	852	854	+ 2	13	13	0
$MeSiPr_3$	1026.0	1025	− 1	1037	1038	+ 1	11	13	+2
Me_3SiBu	722.7	724	+ 1	741	743	+ 2	18	19	+1
Me_2SiBu_2	1008.4	1003	− 5	1026	1023	− 3	18	20	+2
$MeSiBu_3$	1264.4	1257	− 7	1284	1279	− 5	20	22	+2
Me_3SiPe	814.0	820	+ 6	838	840	+ 2	24	20	−4
Me_2SiPe_2	1188.6	1187	− 2	1212	1209	− 3	23	22	−1
$MeSiPe_3$	1528.8	1520	− 9	1549	1545	− 4	20	25	+5
Et_3SiPr	994.1	996.5	+ 2.4	1020	1019	− 1	26	23	−3
Et_2SiPr_2	1064.4	1066.4	+ 2.0	1086	1085	− 1	22	19	−3
$EtSiPr_3$	1130.0	1131.8	+ 1.8	1145	1147	+ 2	15	15	0
Et_3SiBu	1076.3	1076.3	0.0	1103	1101	− 2	27	25	−2
Et_2SiBu_2	1222.1	1221.5	− 0.6	1247	1246	− 1	25	24	−1
$EtSiBu_3$	1358.7	1358.0	− 0.7	1382	1382	0	23	24	+1
Et_3SiPe	1164.0	1167	+ 3	1193	1193	0	29	26	−3
Et_2SiPe_2	1400.1	1397	− 3	1423	1424	+ 1	23	27	+4
$EtSiPe_3$	1618.0	1616	− 2	1640	1642	+ 2	22	26	+4

* The slope of the linear part of the *n*-alkane $\log t'_r$–carbon number plot at 160° was 0.226.

** Calculated using eqn. 1; the *k* values on both columns are 4.2 and 2.2 for MeO and EtO, respectively.

*** The slope of the linear part of the *n*-alkane $\log t'_r$–carbon number plot at 150° was 0.199.

§ Obtained by subtraction of I_{160}^{ApL} (calcd.) from I_{160}^{XE} (calcd.).

The increase in the group index per CH_2 group added is also given in Table II. The large increase for the first CH_2 group added is remarkable and it is concluded that the retention index of Me_4Si is abnormally low on both Apiezon L and XE-60. On the basis of refractometric studies, it has been proposed by Fajans[11] that in an alkyl chain bonded to silicon, the charge distribution is

$$\overset{-}{\underset{\alpha}{Si}}-\overset{+}{\underset{\beta}{CH_2}}-\overset{-}{\underset{\gamma}{CH_2}}-\overset{+}{\underset{\delta}{CH_2}}-\overset{-}{\underset{\varepsilon}{CH_2}}-\ldots$$

where the magnitude of the charge diminishes with increasing distance from the silicon atom. Comparison with the $\delta I(CH_2)$ values in Table II shows that the high β-CH_2 value is connected with a positive charge and the low γ-CH_2 value with a

TABLE II

GROUP RETENTION INDICES AND GROUP ΔI VALUES FOR *n*-ALKYL GROUPS BONDED TO SILICON IN SYMMETRICAL TETRAALKYLSILANES

R	$I_{160}^{ApL}(RSi)$	$\delta I(CH_2)$	$I_{160}^{XE}(RSi)$	$\delta I(CH_2)$	$\Delta I_{160}(RSi)$
Me	105.0		109.3		4.3
Et	230.6	125.6	237.0	127.7	6.5
Pr	298.2	67.6	301.1	64.1	2.9
Bu	371.4	73.2	377.2	76.1	5.8
Pe	455.0	83.6	461.8	84.6	6.8

negative charge. From refractometric investigations, in which a similar change in the group refraction of CH_2 was demonstrated, it was concluded that the influence of the silicon atom did not reach appreciably beyond the δ-CH_2 group[12]. Although tetraalkylsilanes with alkyl groups larger than pentyl have not been investigated, there is reason to assume that the ε-$\delta I(CH_2)$ value is close to the normal one, just as was found for the refraction of the ε-CH_2 group.

Thus, on the basis of the Fajans' theory, the low retention index for Me_4Si should be attributed to the negatively charged methyl carbon atoms, which cause the solubility of the molecule in the stationary phase to be lower than expected for normal behaviour. Similarly, the high retention index of Et_4Si should be connected with the now positively charged terminal methyl carbon atoms, causing the solubility to be higher than expected.

For the previously investigated tetraalkoxysilanes, a similar variation in the $\delta I(CH_2)$ values in the vicinity of the silicon atom existed (see Table IV in ref. 1). In this instance, the charge distribution is assumed to be[12]

$$\overset{-}{\text{Si}}\text{–}\overset{+}{\text{O}}\text{–}\underset{\alpha}{\overset{-}{\text{CH}_2}}\text{–}\underset{\beta}{\overset{+}{\text{CH}_2}}\text{–}\underset{\gamma}{\overset{-}{\text{CH}_2}}\text{–}\underset{\delta}{\overset{+}{\text{CH}_2}}\text{–}\underset{\varepsilon}{\overset{-}{\text{CH}_2}}\text{–}\ldots$$

The difference between I(EtOSi) and I(MeOSi) is strikingly small, which is mainly caused by the too high retention index for $(MeO)_4Si$. Accordingly, the positive charge on the methyl carbon atoms in $(MeO)_4Si$ renders the molecule more soluble in the stationary phase than expected for normal behaviour. Judging from the $\delta I(CH_2)$ values, the influence of the silicon (and oxygen) atom does not reach appreciably beyond the δ-CH_2 group in this instance either.

A comparison between the I(RSi) values and the corresponding I(ROSi) values (see Table III) shows a considerable variation in the differences with the size of the R group. On Apiezon L, I(ROSi) is about 60 index units higher than I(RSi) for R = methyl, which must be attributed to the opposite charges on the two methyl groups. For R = ethyl, I(ROSi) is about 18 index units lower than I(RSi), as a consequence of the now reversed charges of the terminal methyl groups. With increasing R, the group indices become more alike and are nearly identical for R = butyl and pentyl. Accordingly, we can conclude that on Apiezon L, for small R values, the charge of the terminal methyl group has a dominating influence on the retention, whereas for larger R groups, their size is the retention-determining factor, the charge in this instance

TABLE III

COMPARISON OF GROUP RETENTION INDICES FOR *n*-ALKYL AND *n*-ALKOXY GROUPS BONDED TO SILICON IN SYMMETRICAL TETRAALKYL- AND TETRA-ALKOXYSILANES

R	*Diff.* $[I_{160}^{ApL}(ROSi) - I_{160}^{ApL}(RSi)]$	*Diff.* $[I_{160}^{XE}(ROSi) - I_{160}^{XE}(RSi)]$
Me	+62	+118.0
Et	−17.7	+ 15.8
Pr	− 7.7	+ 22.7
Bu	− 0.6	+ 23.2
Pe	0	+ 23

being too low to be of any importance. For larger RO groups, the presence of the oxygen atom has only a minor influence on the retention of tetraalkoxysilanes on the non-polar stationary phase.

For the XE-60 phase, there is still a difference of about 20 index units between *I*(ROSi) and *I*(RSi) for R = butyl and pentyl. This difference can be considered as the contribution of the oxygen atom in the RO group to the retention on the polar phase.

The ΔI values are also markedly influenced by the charge of the terminal methyl groups. Thus, low ΔI values are connected with a negative charge: Me_4Si ($\Delta I = 17$), Pr_4Si ($\Delta I = 11.5$), Me_nSiPr_{4-n} ($\Delta I = 11$–15); and high ΔI values with a positive charge: Et_4Si ($\Delta I = 25.9$), Bu_4Si ($\Delta I = 23.1$), Et_nSiBu_{4-n} ($\Delta I = 23$–27).

Equation for the calculation of retention indices

Semlyen and Phillips[4] observed the non-additivity of group retentions in mixed tetraalkylsilanes and concluded that the more similar were the sizes of the alkyl groups in a mixed tetraalkylsilane, the smaller was the deviation from additivity. A rough correction method was based on this observation, but it does not permit accurate calculation of the retentions of mixed tetraalkylsilanes. In a previous paper[1], an equation was given for the calculation of retention indices of mixed tetra-alkoxysilanes from those of the symmetrical counterparts. If this equation is applied to mixed tetraalkylsilanes, it will become

$$I(R_4Si) = \Sigma I(RSi) + \Sigma(n \cdot d \cdot k)_{R-R} \qquad (1)$$

where

R = *n*-alkyl;

I(RSi) = group retention index (see Table II);

n = combination number (cf. refs. 1 and 3);

d = carbon number difference between alkyl groups in a combination;

k = constant, dependent on the smallest alkyl group in a combination.

When eqn. 1 was applied to the calculation of retention indices of mixed tetra-alkylsilanes, it appeared that there were certain limitations in its utilization. Thus, when the equation was applied to mixed tetraalkylsilanes containing methyl groups bonded to silicon, deviations between the experimental and calculated retention indices of up to 13 index units were obtained, which is considerably more than the

experimental error. When applied to mixed tetraalkylsilanes with ethyl as the smallest group, eqn. 1 gave values in much better agreement with the experimental values (see Table I). In the material investigated, no mixed tetraalkylsilanes with a group larger than ethyl as the smallest group are present. However, it is believed that eqn. 1 may be used for the calculation of retention indices of all types of mixed tetraalkylsilanes with good accuracy except for those which contain methyl groups. The results for the latter compounds are, as shown by Table I, only approximate, the deviation amounting to 1–13 index units. Another method of calculating retention indices of mixed tetraalkylsilanes with methyl groups is given later in this paper.

The reason for the different behaviour of mixed tetraalkylsilanes with and without methyl groups is associated with the different structures of the two types of tetraalkylsilanes. It was previously shown[1] that the correction term in the equation for the calculation of retention indices of mixed tetraalkoxysilanes (identical with the correction term in eqn. 1) could be obtained by summing a number of interaction increments arising from an assumed interaction between certain CH_3 and CH_2 groups. From this group interaction point of view, the CH_3CH_2Si group is equivalent to the CH_3OSi group, the $CH_3CH_2CH_2Si$ group to the CH_3CH_2OSi group, etc., while the CH_3Si group has no counterpart among the ROSi groups. It is thus understandable that eqn. 1, which is identical with the equation used for calculating the retention indices of mixed tetraalkoxysilanes, is not suitable for accurate calculations on mixed tetraalkylsilanes that contain methyl groups.

Temperature dependence of retention indices

The measured change in retention index with temperature for symmetrical and mixed tetraalkylsilanes is given in Table IV. In the previous investigations of tetraalkoxysilanes[1,2], their change in retention index with temperature was found to be always negative and to increase with molecular size. For the tetraalkylsilanes, the temperature increments are considerably lower and alternate between positive and negative values in an apparently erratic manner. However, there is also a tendency towards increasing negative values for larger molecules.

With the tetraalkoxysilanes, it was shown that approximate values of $10\,dI/dT$ for mixed compounds could be obtained by addition of group values, derived from the symmetrical counterparts by division by four. When the same method was applied to mixed tetraalkylsilanes, the mean deviation between experimental and calculated values of $10\,dI/dT$ for Apiezon L was found to be 0.35. Better agreement resulted when ($I - 100 \cdot$ carbon number) was plotted against $10\,dI/dT$. It appeared that the points were collected along three straight lines, one for symmetrical tetraalkylsilanes, one for mixed compounds containing methyl groups and one for mixed compounds without methyl groups. The calculated values in Table IV were obtained from the equations of these three straight lines (see Table V). The mean deviation between experimental and calculated values by this method is 0.17 for both columns.

The above relationship means that the sign and magnitude of the retention index temperature increment for a tetraalkylsilane is dependent on the difference between its retention index and the retention index of the *n*-alkane with the same number of carbon atoms. Thus, when the former index is smaller than the latter, *i.e.* the difference is negative, the temperature increment is always negative. When the difference is positive, the temperature increment is always positive for tetraalkylsilanes

TABLE IV

COMPARISON OF EXPERIMENTAL AND CALCULATED TEMPERATURE INCREMENTS OF RETENTION INDICES FOR TETRAALKYLSILANES

Compound	*10dI/dT(ApL)*			*10dI/dT(XE-60)*		
	Temp. range (°C)	*Exptl.*	*Calcd.*	*Temp. range (°C)*	*Exptl.*	*Calcd.*
Me_4Si	80–120	0	0.1	80–120	(0)*	
Et_4Si	100–180	+1.9	+1.9	100–150	+2.6	+2.6
Pr_4Si	120–180	−0.4	−0.4	120–150	+0.7	+0.7
Bu_4Si	140–180	−2.0	−2.2	120–150	−0.6	−0.6
Pe_4Si	160–180	−3.5	−3.3	150–160	(−1.5)*	
Me_3SiEt	100–140	0	+0.2	100–120	+0.7	+0.7
Me_2SiEt_2	100–140	+0.8	+0.7	100–120	+0.9	+1.1
$MeSiEt_3$	100–140	+1.2	+1.0	100–120	+2.0	+1.6
Me_3SiPr	120–160	0	−0.1	120–140	0.0	+0.4
Me_2SiPr_2	120–160	+0.3	+0.1	120–150	+0.5	+0.5
$MeSiPr_3$	120–160	+0.3	−0.1	120–150	+0.4	+0.3
Me_3SiBu	140–180	−0.5	−0.2	120–150	0.0	+0.3
Me_2SiBu_2	140–180	−0.4	−0.4	120–150	0.0	+0.1
$MeSiBu_3$	140–180	−0.9	−1.0	120–150	−0.1	−0.5
Me_2SiPe_2	160–180	−0.3	−0.7			
$MeSiPe_3$	160–180	−1.6	−1.6			
Et_3SiPr	140–180	+1.4	+1.5	120–150	+2.5	+2.4
Et_2SiPr_2	140–180	+1.3	+1.0	120–150	+2.1	+1.8
$EtSiPr_3$	140–180	+0.8	+0.5	120–150	+1.2	+1.1
Et_3SiBu	160–180	+1.1	+1.2	120–150	+1.7	+2.1
Et_2SiBu_2	160–180	+0.7	+0.4	120–150	+0.8	+1.1
$EtSiBu_3$	160–180	−0.5	−0.5	120–150	0.0	0.0
Et_3SiPe	160–180	+1.0	+1.1			
Et_2SiPe_2	160–180	−0.5	—0.1			
$EtSiPe_3$	160–180	−1.2	−1.3			

* Approximate values.

TABLE V

CONSTANTS IN AND CORRELATION COEFFICIENTS FOR THE LINEAR RELATIONSHIP $10dI/dT = k \cdot (I - 100 \times \text{CARBON NUMBER}) + l$ FOR TETRAALKYLSILANES COLLECTED IN THREE GROUPS

Group	*Apiezon L*			*XE-60*		
	k	*l*	*r*	*k*	*l*	*r*
R_4Si	0.017	−0.25	0.997	0.013	+0.63	1.00
Me_nSiR_{4-n}	0.015	−0.50	0.95	0.015	−0.28	0.90
Et_nSiR_{4-n}	0.015	−0.10	0.96	0.017	+0.27	0.96

without methyl groups, but positive or negative for tetraalkylsilanes that contain methyl groups. In the latter instance, the negative increment values are associated with smaller index differences up to about 30 index units. It can be concluded that the possibility of predicting reasonably accurate retention index temperature increments for tetraalkylsilanes not included in this study, on the basis of the equations given in Table V, seems to be rather good.

Relationship between retention indices and carbon numbers

Although available data are limited, it seems justifiable to state that for homologous series of tetraalkylsilanes there will exist an approximately linear relation between retention index and carbon number counted from the third or fourth member in a series.

Pollard *et al.*[6] reported a linear relationship between the logarithm of the retention volume and the carbon number for tetraalkylsilanes belonging to the series R_4Si–R_3SiR'–$R_2SiR'_2$–$RSiR'_3$–SiR'_4. However, this is true only for certain series. Thus, for the seven series Me_nSiR_{4-n} (R = ethyl to pentyl) and Et_nSiR_{4-n} (R = propyl to pentyl) studied in this work, the above statement is approximately correct only for the two series Me_nSiEt_{4-n} and Et_nSiPr_{4-n}. For the other five series, there will be an increased deviation from linearity with increase in the difference in size between the two alkyl groups in the tetraalkylsilane, as a consequence of the increased departure from additivity of the group retention indices as expressed by the correction term in eqn. 1. Similar relationships were previously found to exist for the corresponding series of tetraalkoxysilanes.

Retention indices of homologous series of mixed tetraalkylsilanes versus retention indices of the symmetrical counterparts

When the retention indices of homologous series of mixed tetraalkylsilanes were plotted against retention indices of the symmetrical counterparts, the points for the majority of the series were found to lie on very nearly straight lines. The largest deviation existed for the series Me_3SiR on Apiezon L (see Table VI). The reported correlation coefficient ($r = 0.9995$) means that when the retention indices for the members in the series are calculated from the equation

$$I(R_xSiR'_{4-x}) = p_x I(R'_4Si) + q_x \qquad (2)$$

TABLE VI

VALUES OF p_x AND q_x IN EQN. 2 AND OF THE CORRESPONDING CORRELATION COEFFICIENT r

R	x	*Apiezon L*			*XE-60*		
		p_x	q_x	r	p_x	q_x	r
Me	3	0.2945	281.1	0.9995	0.2992	287.2	0.9998
	2	0.5702	155.4	0.9997	0.5721	159.0	0.9998
	1	0.8115	55.2	0.99993	0.8055	64.6	0.99993
Et	3	0.2704	672.7	0.99991	0.2723	691.1	0.99993
	2	0.5330	429.9	0.99999	0.5281	448.7	0.99997
	1	0.7757	206.0	1.00000	0.7706	217.7	0.99999

(R = Me, $x = 3$), taking the values of p_x and q_x from Table VI and the values of $I^{ApL}_{160}(R'_4Si)$ from Table I, the standard deviation between calculated andexperimental values will become 2.9 index units. This value is greater than the error in the index measurement. However, for the majority of the series in Table VI, the standard deviation will be comparable with the error in the index measurement.

A relationship identical with that in eqn. 2 was recently reported for tetraalkoxysilanes[2]. In this case, an improved method for calculating retention indices for mixed tetraalkoxysilanes from the retention indices of the symmetrical counterparts could be developed from eqn. 2. However, in the present instance no further simplification of eqn. 2 is possible. Consequently, no simple relationship exists among either the p_x or the q_x values for the three series $R_xSiR'_{4-x}$ ($x = 1$–3), which was found to be the case for the corresponding tetraalkoxysilanes (see ref. 2).

Retention indices for homologous series of mixed tetraalkylsilanes versus retention indices for tetraalkoxysilanes

Several linear relationships exist between retention indices for homologous series of mixed tetraalkylsilanes on the one hand and retention indices for tetraalkoxysilanes on the other. Thus, the following relationship holds for mixed tetraalkylsilanes and the corresponding mixed tetraalkoxysilanes:

$$I(R_xSiR'_{4-x}) = k_1 I(RO)_xSi(OR')_{4-x} + l_1 \quad (3)$$

In this expression, R and x are the same throughout a series, while the R′ groups form a "homologous" suite (see full lines in Fig. 1). The good linearity of this relationship, especially for R = Me, is shown by the correlation coefficients in Table VII. The broken lines in Fig. 1 demonstrate that in addition to the above linear relationship there exists another, which concerns mixed tetraalkylsilanes, $R_xSiR'_{4-x}$ ($x = 0$–4), and the corresponding mixed tetraalkoxysilanes, where one group is exchanged for another in a regular manner. However, the linearity of the correlations for these series is not as good as for the former.

It was previously shown[1,2] that a linear relationship exists between the retention indices for homologous series of mixed tetraalkoxysilanes, $(RO)_xSi(OR')_{4-x}$, and the retention indices for their symmetrical counterparts, $(R'O)_4Si$. Accordingly, it follows from eqn. 3 that the following relationship will be valid:

$$I(R_xSiR'_{4-x}) = k_2 I(R'O)_4Si + l_2 \quad (4)$$

The values of k_2 and l_2 and of the correlation coefficient r for six series on two stationary phases are given in Table VIII.

The significance of a linear relationship between retention indices for two homologous series of compounds is as follows. There will exist a constant ratio between the increase in retention for corresponding compounds in the two series, as expressed by the change in retention index or its equivalent, the logarithm of the retention volume, when a CH_2 group is added to the compounds in question. This is partly a consequence of the linear relationship between retention index and carbon number for homologous series. However, the linear relationship between retention indices of homologous series, discussed in this section, is also valid for the first part of the series where the retention index is non-linear with the carbon number. For

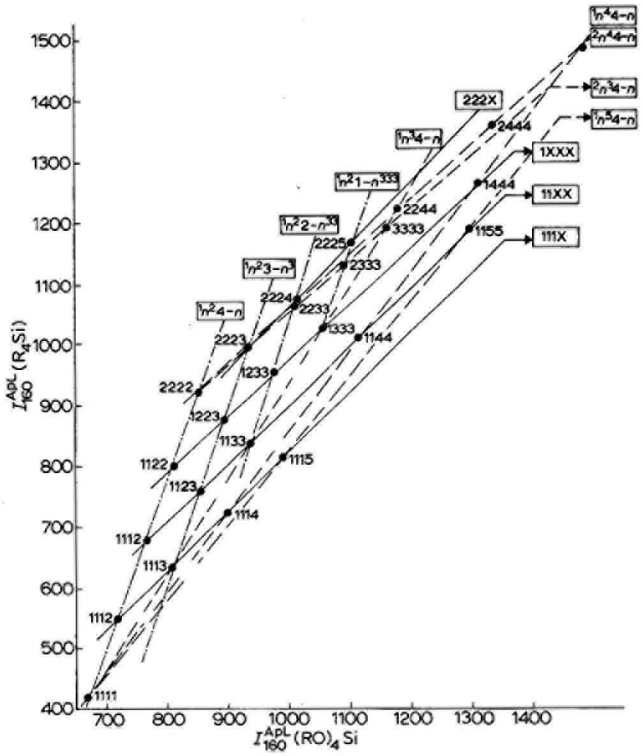

Fig. 1. I_{160}^{ApL} (R_4Si) *versus* I_{160}^{ApL} ($(RO)_4Si$). ———, "homologous" lines; – – – –, exchange of methyl (1) for propyl (3), butyl (4) and pentyl (5) or ethyl (2) for propyl (3) and butyl (4); –·–·–, exchange of methyl (1) for ethyl (2).

TABLE VII

VALUES OF k_1 AND l_1 IN EQN. 3 AND OF THE CORRESPONDING CORRELATION COEFFICIENT r

The retention indices for the tetraalkoxysilanes used in the calculation were taken from ref. 2.

R	x	*Apiezon L*			*XE-60*		
		k_1	l_1	r	k_1	l_1	r
Me	3	0.9605	−142.4	1.0000	1.0205	−395.0	0.99996
	2	0.9631	− 64.4	1.0000	1.0185	−294.0	0.99996
	1	0.9361	39.0	0.99998	0.9880	−159.7	0.9997
Et	3	0.9816	79.0	0.99998	1.0336	−100.9	0.9998
	2	0.9890	62.4	0.9997	1.0001	− 69.6	0.99999
	1	0.977	70.4	0.9997	1.0091	− 93.2	0.99999

compounds on this part of the curve, the linearity depends on the fact that the retention indices of the two compounds, *e.g.* Me_2SiEt_2 and $(MeO)_2Si(OEt)_2$, deviate equally from linearity in their respective retention index–carbon number plots.

TABLE VIII

VALUES OF k_2 AND I_2 IN EQN. 4 AND OF THE CORRESPONDING CORRELATION COEFFICIENT r

The retention indices for the tetraalkoxysilanes used in the calculation were taken from ref. 2.

R	x	*Apiezon L*			*XE-60*		
		k_2	I_2	r	k_2	I_2	r
Me	3	0.2730	317.3	0.99999	0.2906	274.4	0.99994
	2	0.5284	225.8	0.99998	0.5564	132.9	0.99994
	1	0.7517	115.6	0.9998	0.7825	29.5	0.99990
Et	3	0.2583	693.6	0.99999	0.2623	682.0	0.9998
	2	0.5106	468.9	0.9998	0.5225	409.5	1.00000
	1	0.7422	264.1	0.9998	0.7675	151.6	0.99999

TABLE IX

COMPARISON OF EXPERIMENTAL AND CALCULATED RETENTION INDICES FOR TRIFUNCTIONAL TETRAALKYLSILANES

Compound	*Exptl.*	*Calcd. (eqn. 1)*	*Diff.*	*Calcd. (eqn. 3)*	*Diff.*
$Me_2SiEtPr$	759	766	+7	758	−1
$MeSiEt_2Pr$	877	886	+9	876	−1
$MeSiEtPr_2$	953	957	+4	951	−2

Calculation of retention indices for mixed tetraalkylsilanes containing methyl groups

It was mentioned that eqn. 1 was less adequate for calculating retention indices for mixed tetraalkylsilanes containing methyl groups. On this account, we have investigated the usefulness of the linear relationships discussed in the previous section for making such calculations. It appeared that eqn. 3 was best suited for this purpose. In order to test the validity of eqn. 3, some trifunctional tetraalkylsilanes were prepared and their retention indices on Apiezon L determined. In Table IX, the experimental values are compared with those obtained using eqn. 1 and eqn. 3, respectively. Eqn. 3 is seen to be superior, and the agreement between experimental and calculated values is good. This proves that all mixed tetraalkylsilanes that contain one methyl group lie on the 1XXX line in Fig. 1, and all mixed tetraalkylsilanes that contain two methyl groups on the 11XX line.

For the mixed tetraalkylsilanes in Table I, a similar comparison between experimental and calculated retention indices shows that for compounds without methyl groups, eqns. 1 and 3 give comparable values, while for compounds that contain methyl groups, eqn. 3 is superior and should be used.

I_{160}^{ApL} *versus* I_{160}^{XE}

When I_{160}^{ApL} values for tetraalkylsilanes are plotted against the corresponding I_{160}^{XE} values, it appears that the points fall satisfactorily on a straight line of equation

$$I_{160}^{XE}(R_4Si) = 1.011 \cdot I_{160}^{ApL}(R_4Si) + 8.3 \qquad (5)$$

The correlation coefficient is 0.9997 when all available data are utilized, giving a standard deviation of ± 4.2 index units. The fact that the relationship between polar and non-polar retention indices for all of the tetraalkylsilanes investigated can be approximately expressed by the equation of a single straight line is in sharp contrast to the case for tetraalkoxysilanes, in which the retention indices for each homologous series of compounds fell on their own straight line (see Fig. 4 in ref. 1). The reason for this difference is the low polarity of the tetraalkylsilanes, as previously discussed in connection with the ΔI values.

In the case of the two-phase plot for tetraalkoxysilanes, two linear relationships existed. One was that mentioned above for homologous series and the other was for compounds belonging to the same structure group. A structure group is formed from compounds with the same number of $CH_3(a)$, $CH_2(b)$, $CH(d)$ and $C(e)$ groups and the code is written a–b–d–e. As only compounds with normal alkyl or alkoxy groups bonded to silicon are considered here the code will become a–b–0–0.

It was found that tetraalkyl- and tetraalkoxysilanes differ in the two-phase plot also for compounds that belong to the same structure group. In the latter case, there is a very good linear relationship between I_{160}^{ApL} and I_{160}^{XE} for all compounds within a structure group, while for the tetraalkylsilanes there is a considerable scatter in the points representing a structure group. However, closer inspection reveals that, in fact, linear relationships exist between the retention indices for certain compounds within a structure group.

This is demonstrated in Fig. 2 for the structure group 4–6–0–0. There are two parallel diagonal lines and three additional lines intersecting them, and the compounds 1117–1126–1225–2224* and 1135–1234–2233 are collected along the two diagonal lines. The tetraalkylsilanes in these two series are obtained from the first compound by the systematic transfer of a CH_2 group from an alkyl group larger than propyl to a methyl group. The compounds 1144–1135–1126, 1333–1234–1225 and 2233–2224 are gathered along the three intersecting lines. These series are formed from the first compound by transfer of a CH_2 group from a propyl group to another propyl or another, larger, alkyl group, or between alkyl groups larger than propyl. The significance of these linear relationships is that there exists a constant relationship between the change in I_{160}^{XE} and I_{160}^{ApL} for a CH_2 transfer in each of the series mentioned. The generality of these linear relationships between tetraalkylsilanes within a structure group cannot be decided on the basis of the available material, and more data are necessary in order to answer this question.

From a comparison between Fig. 2 in this paper and Fig. 4 in ref. 1, another difference between tetraalkyl- and tetraalkoxysilanes is apparent. In the structure group 4–6–0–0 for tetraalkoxysilanes, and all other structure groups investigated, the compounds are eluted according to decreasing formula code. In the structure group 4–6–0–0 for tetraalkylsilanes, the elution of compounds along the diagonal lines takes place according to increasing formula code and along the intersecting lines according to decreasing formula code. This means that in both of the latter cases the elution takes place according to symmetry with the important difference that along the diagonal lines the least symmetrical compounds are eluted first and along the intersecting lines the most symmetrical are eluted first.

* The figures in the formula code denote the *n*-alkyl groups bonded to the central silicon atom: 1 = methyl, 2 = ethyl, 3 = propyl, etc.

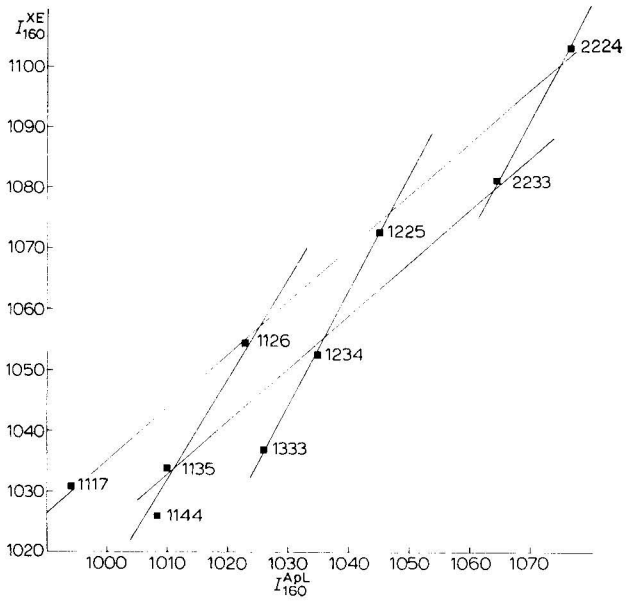

Fig. 2. Two-phase plot of I_{160}^{XE} *versus* I_{160}^{ApL} for tetraalkylsilanes belonging to the structure group 4-6-0-0.

The difference in elution behaviour of tetraalkyl- and tetraalkoxysilanes is perhaps best shown by comparing the elution order of the series of tetraalkylsilanes along the upper diagonal lines in Fig. 2 with that of the corresponding tetraalkoxysilanes along the 4-6-0-0 structure line in Fig. 4 in ref. 1. The two series of compounds, in order of elution on both Apiezon L and XE-60, are 1117–1126–1225–2224 and 2224–1225–1126–1117, *i.e.* the elution order is reversed for the tetraalkoxysilanes. Regarding the 4-6-0-0 tetraalkylsilane structure group as a whole, the elution picture is less clear. Thus, while the tetraalkoxysilanes within a structure group are eluted strictly according to decreasing formula code, the elution order of the tetraalkylsilanes is 1117–1144–1135–1126–1333–1234–1225–2233–2224 on the Apiezon L stationary phase and 1144–1117–1135–1333–1234–1126–1225–2233–2224 on the XE-60 stationary phase. This is a consequence of the variation of the $\delta I(CH_2)$ values (see Table II).

Boiling point versus retention index

Fig. 3 demonstrates the relationship between I_{160}^{ApL} and the boiling point for tetraalkylsilanes in the index range 400–1200. It appears that the curve in this range can be approximated by two linear relationships, one valid for tetraalkylsilanes containing two or three methyl groups bonded to silicon and the other for the remainder of the tetraalkylsilanes. The equation is

$$t(^{\circ}C) = k \cdot I_{160}^{ApL} + l \qquad (6)$$

In this expression, $k = 0.2792$, $l = -90.2$ for the first group of tetraalkylsilanes and $k = 0.2319$, $l = -58.9$ for the second group. It is possible to calculate the boiling

point from eqn. 6 to about 1°, *i.e.* with the same accuracy as that in the experimental boiling point determination[13].

For comparison, retention indices and boiling points for *n*-alkanes have also been plotted in Fig. 3. The curve for *n*-alkanes is seen to coincide with that for the second group of tetraalkylsilanes but not with that for the first group. This is another example of the divergent behaviour of tetraalkylsilanes that contain several methyl groups. The last tetraalkylsilane in Fig. 3, tetrabutylsilane ($I_{160}^{ApL}=1486$), lies far outside the *n*-alkane curve. Either the reported boiling point[13] for this compound is about 30° too low or the tetraalkylsilane curve makes a sharp turn for higher retention index values. The question of the appearance of the tetraalkylsilane curve outside of $I_{160}^{ApL}=1200$ cannot be decided on the basis of existing data, and a decision has to await the availability of further experimental results.

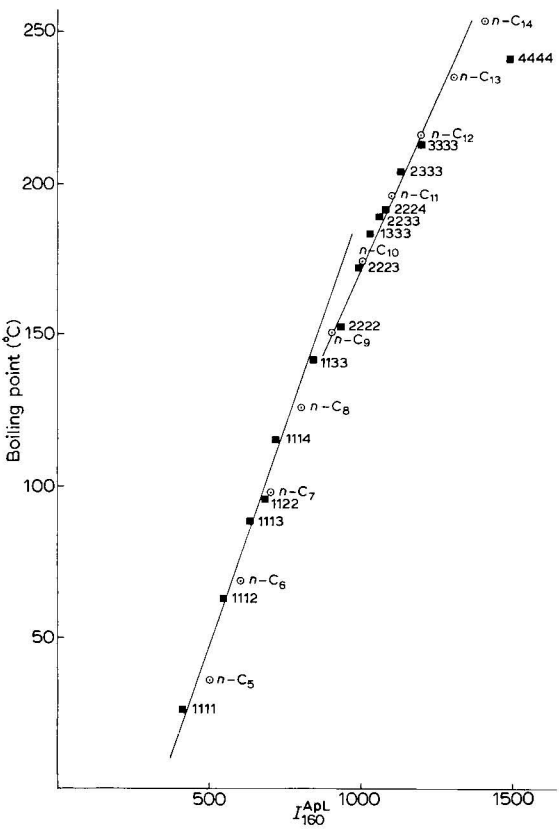

Fig. 3. Boiling point *versus* I_{160}^{ApL}: ■, tetraalkylsilanes; ⊙, *n*-alkanes.

Identification of tetraalkylsilanes

Tetraalkylsilanes can be recognized as a group and distinguished from most other types of organosilicon compounds by their small ΔI-values. However, the identification of a specific tetraalkylsilane by gas chromatography alone is by no means easy. As pointed out previously, the two-phase plot is less regular than for

tetraalkoxysilanes and there is considerable crowding of points for the various compounds, which makes the interpretation more difficult. If, however, the linear relationships found to exist within the structure groups investigated are found to be of general validity, it opens possibilities for a more reliable identification on the basis of the two-phase plot.

It was previously shown[1] that in the case of tetraalkoxysilanes, the ΔI values were useful for the distinction of different types of compounds belonging to this group. For tetraalkylsilanes, some use may also be derived from this physical quantity. Thus, a low ΔI value (<20) is an indication of the presence of only methyl and/or propyl groups in the molecule or at least three of these groups together with ethyl or butyl. On the other hand, high ΔI values (>25) indicate the absence of methyl and propyl in the molecule. However, certain exceptions to these rules exist. Earlier in this paper, some rules were given for the estimation of the temperature increments for the retention indices for tetraalkylsilanes. These data may also be of value in identification work.

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A SAMPLE TRAPPING AND REINJECTION TECHNIQUE FOR USE WITH GAS CHROMATOGRAPHY

EDWARD HOUGHTON

School of Chemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033 (Australia)

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SUMMARY

A technique for the collection and transfer of microgram or submicrogram quantities of gas chromatographic fractions is described. The technique has proved extremely useful for the examination of trapped components by combined carbon-skeleton gas chromatography–mass spectrometry.

INTRODUCTION

In the identification of trace components of volatile mixtures the chemist is often hampered by the limited amount of material available. Mass spectra can be obtained by combined gas chromatography–mass spectrometry (GC–MS), but with completely unknown samples positive identification based purely on mass spectral evidence can be difficult. The problem encountered in the isolation of sufficient pure material for examination by infrared and nuclear magnetic resonance spectroscopy often renders these techniques inapplicable as aids to identification. In such cases, valuable structural evidence can be obtained from retention time data and microchemical techniques such as carbon-skeleton gas chromatography[1,2] and on-line[3–5] or off-line[6] hydrogenation. For optimum performance, such techniques require the trapping and reinjection of microgram or submicrogram quantities of material without the use of solvents. Brownlee and Silverstein[7] have successfully trapped GC fractions in glass capillaries and reinjected them using a capillary breaker. Bierl *et al.*[8] have described a collection and transfer device using traps packed with coated support, but these authors reported a broadening of the trapped and reinjected peaks. Murray *et al.*[9] have developed a technique for reinvestigation of trapped components on capillary columns using an injector system built into the lid of a laboratory-built gas chromatograph. This technique has now been adapted for use with a conventional commercial gas chromatograph. It provides an efficient transfer of trapped components when used in conjunction with packed columns, and further has found useful application in the investigation of gas–liquid chromatographic fractions by hydrogenolysis and hydrogenation.

EXPERIMENTAL

Apparatus

The trap (Fig. 1A) was a thin-walled (0.42 mm) stainless-steel tube (102 × 3.2 mm

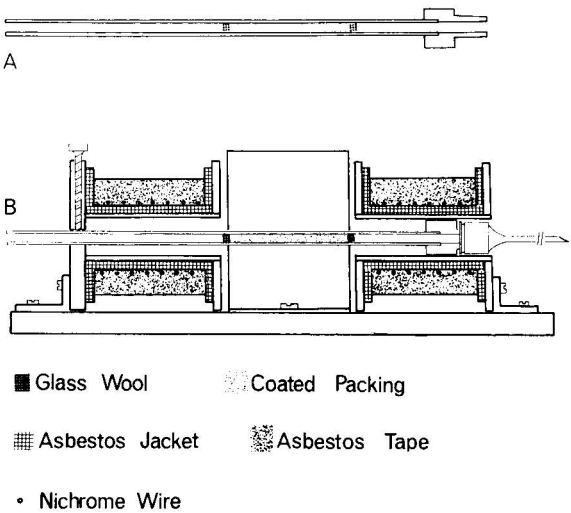

Fig. 1. (A) Stainless-steel collection tube; (B) cross section of the collection-tube heater with tube in position.

O.D.) with a male luer adaptor silver soldered to one end. A packing of glass wool or coated support was placed in the trap in the region which occupies the space between the two sections of the collection tube heater (Fig. 1B). The latter was constructed of brass. Each section consisted of two discs (31.75 mm diameter) attached to a tube (29 × 8 mm I.D.). An asbestos jacket was fitted to the two sections which were then wound with nichrome wire to yield a maximum temperature of 200° when used with a 32-V power supply. A layer of asbestos insulation tape was then applied and the two sections attached to a brass plate (114 × 38 × 4.8 mm) leaving a space in the centre (28 mm) to accommodate the hot and cold probes. A brass plate was fixed to the central section to support the two probes. The heater was mounted on a block of Syndanio board and attached to the gas chromatograph by means of two grooved right angle brackets (Fig. 2). The hot and cold probes were constructed according to Murray *et al.*[8].

Operation

Two gas chromatographs were used. Samples were collected from a Varian Aerograph 204 instrument fitted with a 5% SE-30, 1.5 m × 6.4 mm aluminium column. The collection tubes were attached to the exit port of the gas chromatograph using Swagelok fittings and Teflon ferrules and were cooled in the packed region with dry ice.

The injector device was attached to a Varian Aerograph 1740 instrument and the trapped samples reinjected onto a 1.5 m × 3.2 mm stainless-steel column packed with 3% SE-30 on 100–120 mesh Varaport. A miniature three port valve was installed to direct the carrier gas either to the GC column or to the front of the collection tube.

To reinject a collected sample, the collection tube is inserted partially into the heater from the rear and the syringe needle (19.05 mm, 26 gauge) attached.

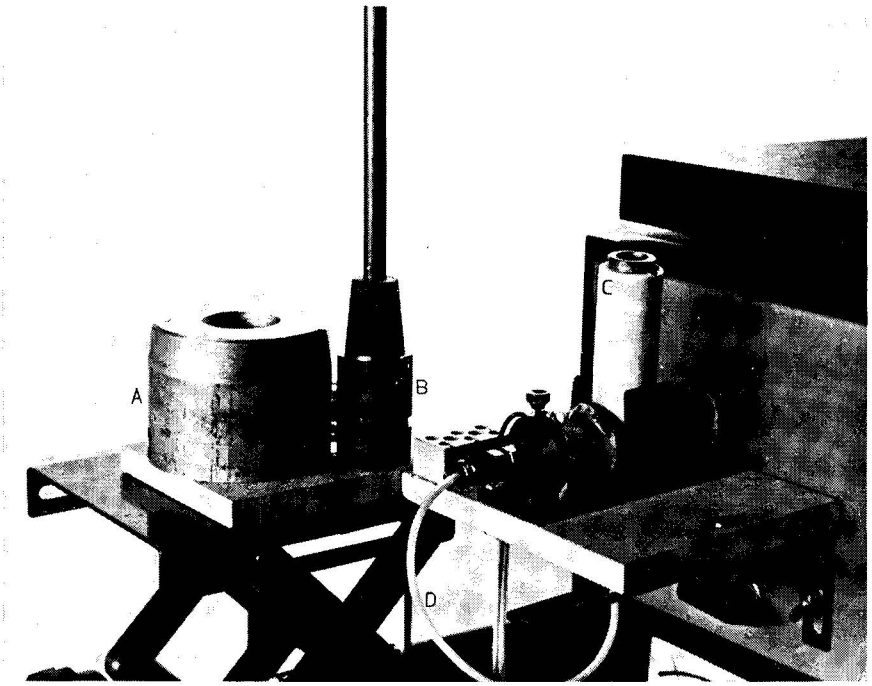

Fig. 2. Collection-tube heater attached to gas chromatograph. (A) Aluminium block heater for hot probe; (B) hot probe; (C) cold probe; (D) Teflon line for redirecting the carrier gas.

After insertion into the heater to the hilt of the needle the tube is held in position by tightening the thumb screw (Fig. 1B). The 3.2-mm Teflon line for the redirection of the carrier gas is attached to the front of the tube using a Swagelok union and Teflon ferrules. The packed section of the tube is now cooled by application of the cold probe, cooled with liquid nitrogen, and the carrier gas is diverted through the tube. The injection unit is pushed forward on the grooved brackets until the syringe needle penetrates the septum of the injection block. With the cold probe still in position, the low-voltage power supply to the two heating sections is switched on. When these reach a steady temperature, the sample is injected by removal of the cold probe and application of the hot probe pre-heated to a selected temperature in an aluminium block fitted with a cartridge heater. A tube cooled to $-110°$ attains a temperature of $150°$ in approximately 6–8 sec by application of the hot probe at $220°$.

RESULTS

Fig. 3a shows the chromatogram obtained for a 1-μg sample of *n*-tridecane in methylene chloride injected by the conventional method using a syringe. Fig. 3b shows the chromatogram of 1 μg of the same sample after collection on 30–40 mesh Chromosorb A coated with 10% SF-96 and reinjected by the method described above. There is no loss in resolution of the column and no tailing of the peak although the sample is retarded slightly. There is a slight loss in sample some of which is

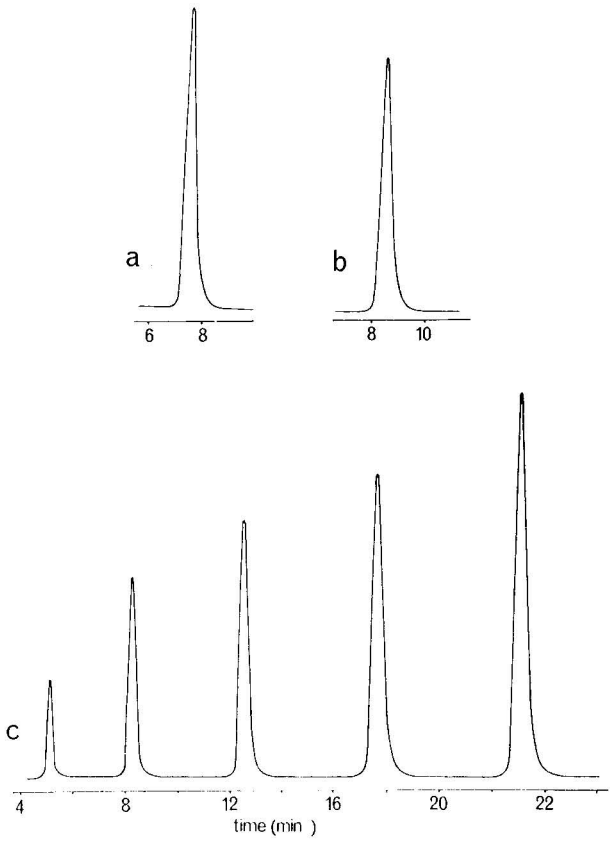

Fig. 3. (a) 1-μg sample of *n*-tridecane injected by conventional syringe method. Column, 1.55 m × 3.2 mm 3% SE-30; temperature, isothermal at 100°. (b) 1-μg sample of *n*-tridecane after trapping and reinjection. Column as in (a). (c) *n*-C_{11}–C_{15} hydrocarbons injected by injector device. Column as in (a); temperature programmed from 100° at 2°/min.

accounted for by the split ratio of the effluent splitter on the preparative instrument.

Fig. 3c shows the chromatogram for the series of *n*-alkanes C_{11}–C_{15} after trapping and reinjection. Again the peaks were as sharp as those obtained by conventional syringe injection under the same conditions. The technique thus provides an efficient method for the collection and reinjection of GC fractions on a second stationary phase or for investigation by combined GC–MS.

MS when used in conjunction with carbon-skeleton GC can provide a means of rapid analysis of the hydrogenolysis products. The device described above proved extremely useful in the examination of trapped components by combined carbon-skeleton GC–MS, a decided advantage being that microgram samples could be handled and injected without the use of solvents. Hydrogen was used as carrier gas and the catalyst, 500 mg of neutral 1% palladium on Chromosorb W (ref. 2), was contained in an injection port liner (a 140×6.4 mm O.D. stainless-steel tube). The hydrogenolysis products were investigated using a 1.5 m×3.2 mm stainless-steel column packed with 5% SE-30 on 80–100 mesh Chromosorb W. The gas

TABLE I
ANALYSIS OF HYDROGENOLYSIS PRODUCTS BY GC–MS
Catalyst, 1% Pd–Chromosorb W; temperature, 250 °.

Compound	*Product*
Citral	2,6-Dimethylheptane
n-Decanal	Nonane
2-Methylcyclopentanone	No reaction
2-Methyl-5-ethylcyclopentanone	No reaction

chromatograph was connected to an AEI MS12 mass spectrometer via a single stage Llewellyn separator. Samples (10–50 μg) were trapped in collection tubes packed with glass wool and injected onto the catalyst as described above. Some preliminary results are summarised in Table I.

The two aldehydes citral and *n*-decanal were reduced almost quantitatively to the hydrocarbons 2,6-dimethylheptane and *n*-nonane whereas the two cyclopentanones passed over the catalyst unchanged.

The device has also been used successfully for the hydrogenation of olefins using the injection port hydrogenator described by Beroza and Sarmiento[4], the products were again analysed by combined GC–MS.

CONCLUSION

The injector device provides an efficient method for the trapping and injection of samples into a conventional gas chromatograph. It provides a means of handling and transferring microgram quantities of material without the use of solvent, which is an advantage in the investigation of compounds by carbon-skeleton GC. It offers a simple and effective means for the trapping of a minor component of a complex mixture for investigation by micro-chemical techniques and has been used successfully in this manner in this laboratory for the investigation of insect volatiles by combined carbon-skeleton GC–MS[10].

ACKNOWLEDGEMENTS

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CHROM. 7176

QUANTITATIVE DETERMINATION OF SULFUR COMPOUNDS IN THE GAS PHASE OF CIGARETTE SMOKE

A. D. HORTON and M. R. GUERIN

Oak Ridge National Laboratory, Oak Ridge, Tenn. 37830 (U.S.A.)

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SUMMARY

Flame photometric detection gas chromatography affords a reliable approach for the determination of sulfur compounds in smokes. Reliable quantitative data can only be generated if the analytical system allows a minimum of time to elapse between smoke generation and the onset of analyses. Contact with stainless-steel surfaces must be minimized and the chromatographic column must be conditioned to avoid irreversible adsorption. Typical cigarettes deliver approximately 85 μg of H_2S, 35 μg of COS, 2 μg of CS_2, and 3 μg of SO_2 when smoked under standard conditions. The gas phases of smokes contain at least 28 sulfur components. Quantitative distribution of these components is highly sensitive to sampling methodologies.

INTRODUCTION

Applications of flame photometric detection gas chromatography (GC) to the study of sulfur components in the gas phase of cigarette smoke have suggested that this component of smoke is significantly more complicated than had been assumed. Groenen and Van Gemert[1] found up to 37 sulfur-containing species in the gas phases of various cigarette smokes. Guerin[2] reported the presence of at least eleven sulfur-containing components and found that previously held estimates of the relative quantities of H_2S, COS, and SO_2 appeared to be in error. Absolute quantitative deliveries of the sulfur gases and organosulfur components were not reported in either work. Quantitative studies, while demonstrating the potential utility of the method, enjoyed only limited success.

A system is described here which allows the generation of reliable quantitative results for sulfur constituents of cigarette smokes. Results are reported for the H_2S, COS, SO_2, and CS_2 deliveries of typical cigarettes. Applications of the system for a further study of the sulfur components of smokes are discussed briefly.

EXPERIMENTAL

A Model MT 220 gas chromatograph (Tracor, Austin, Texas, U.S.A.) equipped with a flame photometric detector (FPD) and a 6 ft. $\times$ 1/8 in. O.D. fluoroelastomer (FEP) column packed with Tracor Special Silica was used for the deter-

mination of COS, H_2S, CS_2, and SO_2. The column was operated at room temperature (24 °C) with a nitrogen carrier gas flow-rate of 15 ml/min. Inlet and detector temperatures were 75 °C and 125 °C, respectively. An 18 ft. × 1/4 in. O.D. glass column of 20% FFAP (Varian, Walnut Creek, Calif., U.S.A.) on 60–80 mesh Chromosorb W AW-DMCS was used for surveys of higher-boiling sulfur components. Chromatographic conditions were as follows: column program, isothermal at 24 °C for 6 min then 4 °C/min to 225 °C; nitrogen carrier gas flow-rate, 60 ml/min; inlet and detector temperatures, 225 °C. A sample volume of 0.14 ml of gas phase was injected for the determination of the low-boiling sulfur components and a volume of 3.5 ml was injected for high-boiling components. For both columns, FPD gases were: hydrogen 180 ml/min, air 40 ml/min, and oxygen 20 ml/min.

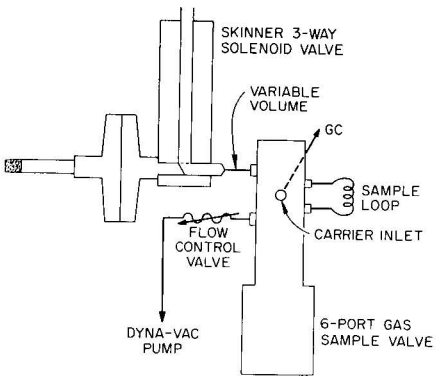

Fig. 1. Single-port smoking machine.

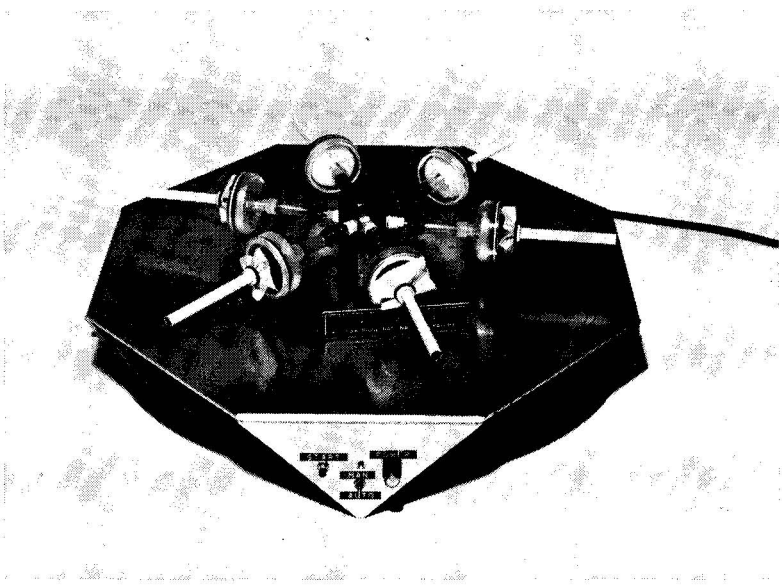

Fig. 2. Six-port smoking machine-"Hexapuffer".

Two smoke generation-sampling systems were used in this work. A single-port machine (Fig. 1), consisting of a filter assembly, a Skinner solenoid valve, a Varian 6-port gas sampling valve, a Dyna-vac vacuum pump, and an electro-mechanical cam timer necessary to achieve standard puff parameters, was used for preliminary quantitative studies. All cigarettes smoked on the single-port machine were conditioned and weight-selected as follows: Cigarettes were conditioned at 60 ± 2% RH and 75 ± 2 °F. Cigarettes that were free from obvious defects, *e.g.* pin holes, frayed wrappers and/or loss of filler, were weight-selected from a batch of 100–200 cigarettes by discarding those differing by more than ±20 mg from the average and setting aside as samples those within ±20 mg of the average weight of the batch. Resistance-to-draw (*RTD*) of the weight-selected cigarettes was determined and only those that matched within ±5% of the average *RTD* were used.

A modified version of the six-port puff averaging system developed by Norman *et al.*[3] (Fig. 2) was used for generating the quantitative data reported here. Modifications include the use of Teflon flow lines, direct vacuum puffing rather than evacuated manifold puffing, and the incorporation of manual operation to allow smoking cigarettes of large puff number. A detailed description of the system is in preparation.

All cigarettes smoked on the Hexapuffer were conditioned for at least 48 h at 60% RH and 75 °F before smoking. Cigarettes were randomly selected from experimental products on hand or single packs of commercial products purchased locally. Cigarettes were inserted through a latex dental dam attached to standard Cambridge filter assemblies[4] and lit using an electric cigar lighter.

Standards of carbonyl sulfide and of hydrogen sulfide, both approximately 500 ppm in nitrogen, were prepared by Matheson Gas Products (East Rutherford, N.J., U.S.A.). Standard sulfur dioxide, approximately 700 ppm in nitrogen, was prepared locally and standardized iodometrically. Carbon disulfide standards were prepared in *n*-hexane using reagent-grade chemicals. Sulfur compounds used for co-chromatographic identification of peaks were obtained primarily from Polyscience Corporation (Kit No. 71B). Gaseous standards for quantitative calibration were introduced either as received or by dynamic dilution with nitrogen.

RESULTS AND DISCUSSION

Flame photometric detection provides a convenient method for visualizing sulfur-containing components in smokes. The sensitivity is such as to allow detecting trace components and selectivity is such as to allow detecting sulfur components in the presence of great excesses of other smoke constituents. Determination of the absolute quantities of these compounds delivered in smokes is complicated by their generally high reactivity. The problem is particularly severe for hydrogen sulfide and for sulfur dioxide.

Table I illustrates the loss of hydrogen sulfide resulting from the use of stainless-steel tubing in the smoking machine and in the transfer line. Considering a total residence time of no more than 4 sec and that the sample is swept to the instrument in a stream of inert gas, the extent of the loss, 60–70%, is surprisingly high. Losses as a result of stainless-steel tubing are apparently due to a process

TABLE I

EFFECT OF STAINLESS STEEL ON HYDROGEN SULFIDE DELIVERIES

Product	*H_2S (μg/cigarette)**		*Per cent loss*
	Stainless-steel tubing	*Teflon tubing*	
Kentucky Reference-1Rl	27	90	70
Commercial, 85-mm non filtered	32	79	59
Commercial, 85-mm charcoal filter	7	27	74

* Single-port smoking device

TABLE II

EFFECT OF CONSTRUCTION MATERIALS AND AGING ON THE GAS PHASE SULFUR COMPOSITION

Peak No.	*Per cent increase* in peak height*		
	Teflon loop Age, 30 sec	*Stainless-steel loop Age, 0 sec*	*Stainless-steel loop Age, 30 sec*
1	–**	–	–
2	–	–	–
3–5	See Tables I and III		
6	–	–	–
7	–	–	–
8	–	–	100
9	–	–	–
10	50	0	50
11	0	0	300
12	0	0	–
13	50	0	50
14	0	0	200
15	50	0	50
16	50	50	200
17	10	0	50
18	–	–	–
19	–	–	–
20	–	–	–
21	–	–	–
22	60	200	300
23	50	0	70
24	10	0	200
25	50	0	50
26	40	60	220
27	40	60	300
28	10	20	200

* Increase relative to Teflon loop, age 0 sec.

** < 10% increase.

more complicated than simple reaction with or adsorption by the metal surface. Losses due to adsorption alone would tend to cancel since standard and sample are routed identically. The presence of the other gas phase constituents may accelerate losses of hydrogen sulfide. Data in Table II, illustrating a substantial increase in higher-molecular-weight sulfur components in the presence of stainless steel further suggests an involvement by other smoke constituents. The metal surfaces may both irreversibly adsorb the reactive sulfur gases and catalyze reaction with other gas phase constituents. Considering that the increase in organosulfur constituents illustrated in Table II results from replacing only the Teflon sampling loop with stainless steel and that contact times are in the order of 4 sec or less, catalysis may be a major factor.

Aging is also a major factor in changing the composition of the sulfur component of smokes. Significant increases in higher-molecular-weight sulfur compounds are observed (Table II) when the sample is held for 30 sec in a Teflon loop before injection into the chromatograph. Lower-molecular-weight components, particularly hydrogen sulfide, exhibit a simultaneous decrease in concentration (Table III). The finding that the sulfur dioxide concentration does not change is unexpected. A possible explanation is that sulfur dioxide is formed as efficiently as it is consumed. No substantive data are available for this view, however.

TABLE III

DECREASE IN SULFUR DELIVERY WITH AGING

Age 0 = 1.00.

Age (*sec*)	*Relative quantity*			
	COS	H_2S	CS_2	SO_2
10	0.93	0.75	0.98	1.00
30	0.87	0.68	0.79	1.00
60	0.65	0.31	0.75	1.00

A final difficulty associated with the generation of quantitative data concerns the GC process itself. Hydrogen sulfide and sulfur dioxide in particular, are irreversibly lost to the column packing. Chromatographic packings suggested for the analyses of sulfur gases at high concentrations inevitably fail in applications to trace quantities. Of the packings evaluated for quantitative analyses, Porapak Q was found the most adsorptive while Deactigel, Porapak P, and Tracor Special Silica were found, respectively, superior. No packing evaluated to date was found completely free of adsorptive properties. The difficulty was overcome in this work by "conditioning" the packing through repetitive injections of H_2S and, when SO_2 is to be analyzed, SO_2 at the beginning of the day. Fifteen 5-ml injections of a gas standard with the average of the last three injections measured as a calibration point are sufficient to condition the column. No further losses occur with continuous use. Fig. 3 illustrates the chromatographic response observed on injection of gas phase on to the Tracor Special Silica Column.

Table IV summarizes the sulfur delivery of typical commercial and experimental cigarettes as determined by use of the Tracor Special Silica column and the puff averaging smoking device. Using randomly selected cigarettes, *i.e.* not selected by weight or pressure drop, allows the analyses to be carried out with a coefficient of variation of approximately $\pm 5\%$ when three machine loads are used. Results obtained with the puff averaging system are in excellent agreement with those obtained with the single-port machine (compare Tables I and IV). As many as three determinations can be carried out without changing Cambridge filters, indicating little effect on the analysis by accumulated tar.

TABLE IV

QUANTITIES OF COS, H_2S, CS_2 AND SO_2 DELIVERED BY COMMERCIAL AND EXPERIMENTAL SMOKING PRODUCTS

Product	*Amount of sulfur compound (μg/cigarette)*			
	COS	*H_2S*	*CS_2*	*SO_2*
Kentucky Reference-1R1	41	87	1.8	2.6
Commercial, 85-mm non-filtered	30	81	1.0	3.7
Commercial, 85-mm cellulose acetate-filtered	30	89	2.0	3.3
Commercial, 85-mm charcoal*-filtered	27	25	0.6	2.9
Experimental (A), 85-mm non-filtered, control	22	55	1.2	4.0
Experimental (A), 85-mm non-filtered, low-ammonium sulfamate	24	65	2.0	6.3
Experimental (A), 85-mm non-filtered, high-ammonium sulfamate	38	102	2.6	8.8
Commercial little cigar, 85-mm filtered	37	112	1.6	7.0
Marihuana, 85-mm non-filtered	29	80	2.0	2.2

* Cellulose acetate–charcoal–cellulose acetate.

The hydrogen sulfide delivery is seen to be approximately twice that reported by Terrell and Schmeltz[5] and by Mattina[6]. Carbonyl sulfide deliveries are found to be 30 to 50% that of hydrogen sulfide rather than five to twenty times that of hydrogen sulfide as reported. Sulfur dioxide is shown to be present to the extent of a few per cent of hydrogen sulfide rather than in equal quantities. Carbon disulfide levels are in good agreement with those reported by Phillipe and Hobbs[7]. Since aging and metal surfaces should not have been serious problems in these studies, differences are likely a result of the use of non-specific analytical techniques or reactions after trapping.

As further evidence of the reliability of the method, product modifications expected to affect sulfur delivery were found to do so. Cigarettes containing ammonium sulfamate produced almost twice the H_2S and SO_2 deliveries when compared to the same blend without the additive. Charcoal filters produce decreased H_2S deliveries and bisulfite used in tobacco processing is reflected in increased sulfur delivery by the resulting cigarettes.

As illustrated in Fig. 4, the gas phases of smokes contain a large number of other sulfur compounds. Approximately 28 peaks are observed using an FFAP column. Use of 1,2,3,4-tetrakis(2-cyanoethoxy)butane, an analog of 1,2,3-tris-

(2-cyanoethoxy)propane, the liquid phase employed by Groenen and Van Gemert[1], proved less successful than FFAP, yielding only 12 peaks. The larger number of components observed by Groenen and Van Gemert[1] may be a result of a difference in cigarettes or, as is likely from observations discussed above, artifacts of the sampling method.

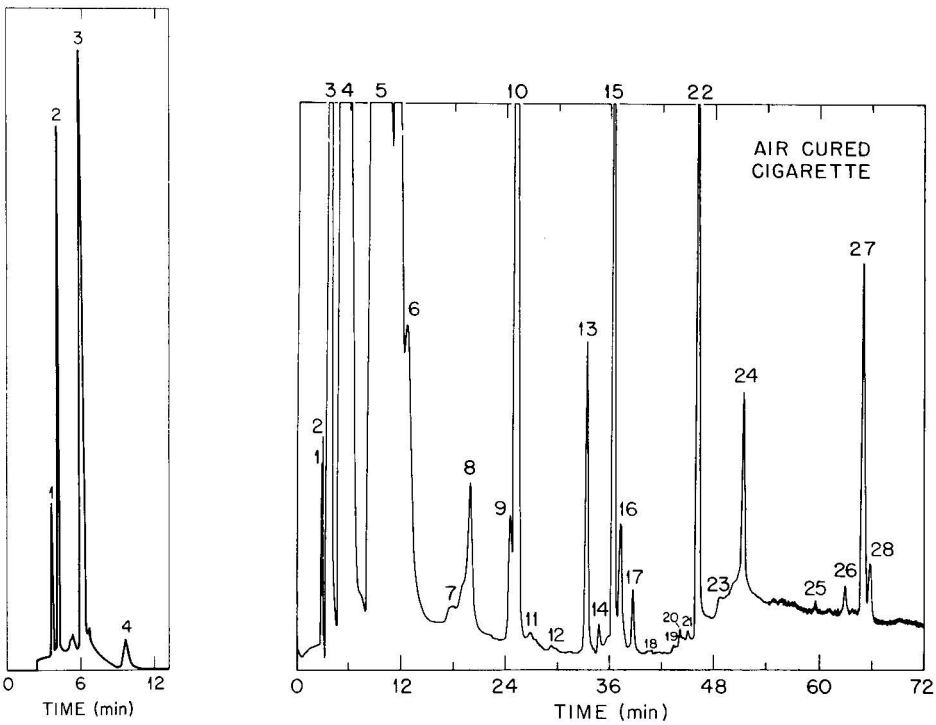

Fig. 3. Gas chromatogram of major sulfur compounds in the gas phase of cigarette smoke. Column, Tracor Special Silica. Nitrogen carrier gas flow-rate, 15 ml/min; temperature, 24 °C; sample size, 0.4 ml. 1 = COS (attenuation × 10^6); 2 = H_2S (attenuation × 10^6); 3 = CS_2 (attenuation × 10^4); 4 = SO_2 (attenuation × 10^4).

Fig. 4. Gas chromatographic profile of sulfur components in the gas phase of cigarette smoke. Column, Varian FFAP, 20% on 60–80 mesh Chromosorb WAW-DMCS. Nitrogen carrier gas flow-rate, 60 ml/min; temperature, 24 °C for 6 min, increase 4 °/min to 225 °C; sample size, 3.5 ml. Attenuation first 54 min, × 10^4; last 18 min, × 10^3. 3 = COS; 4 = dimethyl sulfide; 5 = H_2S; 6 = CS_2; 10 = dimethyl disulfide; 13 = thiophene; 15 = diethyl sulfide.

Although it is apparent that the sulfur component of smoke is much more complex than had been assumed, additional study is needed before the quantitative distribution of higher-molecular-weight components is confidently known. In the best of the systems used in this study, the Skinner valves and the gas sampling valves of both machines allow some contact with metal surfaces. Since some components (Table II) are seen to increase when a stainless-steel loop replaces the Teflon loop —the rest of the system remains Teflon— even this very brief opportunity for contact on passage through the valves may provide artifactual results.

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DETERMINATION OF 2,3,7,8-TETRACHLORODIBENZO-1,4-DIOXIN AT PARTS PER BILLION* LEVELS IN TECHNICAL-GRADE 2,4,5-TRICHLOROPHENOXYACETIC ACID, IN 2,4,5-T ALKYL ESTER AND 2,4,5-T AMINE SALT HERBICIDE FORMULATIONS BY QUADRUPOLE MASS FRAGMENTOGRAPHY

HANS-RUDOLF BUSER and HANS-PAUL BOSSHARDT

Swiss Federal Research Station for Arboriculture, Viticulture and Horticulture, CH-8820 Waedenswil (Switzerland)

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SUMMARY

A fast, highly selective gas chromatographic method was developed for the determination of 2,3,7,8-tetrachlorodibenzo-1,4-dioxin (TCDD) in technical 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and also in 2,4,5-T alkyl ester and amine salt herbicide formulations using quadrupole mass fragmentography. The method is faster, more sensitive and has a higher specificity than previously reported methods using conventional gas chromatography. It requires minimal or no sample clean-up. The content of TCDD found in batches of 2,4,5-T produced from 1967 to 1973 by one manufacturer showed a sharp decrease after 1970 from 500 to 50 ppb.*

INTRODUCTION

Polychlorinated dibenzo-1,4-dioxins, especially 2,3,7,8-tetrachlorodibenzo-1,4-dioxin (TCDD), are extremely toxic[1,2], teratogenic[3] and mutagenic[4] compounds. Owing to their stability in biological systems[5,6] and their potential for accumulation through food chains[7], they present a threat to man, animals and the environment. In order to prevent environmental contamination with TCDD and thus to prevent irreparable biological damage, the further use of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and other chemically related pesticides possibly contaminated with TCDD must therefore depend on the purity of the active ingredient. The Swiss Federal Research Station at Waedenswil, as the authority for the licencing of agricultural pesticides in Switzerland, established a specification for a maximum content of 50 ppb* of TCDD in 2,4,5-T. Similar specifications for TCDD in 2,4,5-T have been adopted in other countries. There has been a considerable international effort in the development of methods[1,6,8–11] to enable manufacturers and government laboratories to control the purity of these products. As a contribution, this paper describes a simple method that permits the rapid and accurate determination of

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

TCDD at the required parts per billion level in technical-grade 2,4,5-T and its formulations. Mass spectrometry is widely used for pesticide analysis[12]. But mass fragmentography so far has been used mainly for the analysis of drugs and their metabolites in biological systems[13–15]. We assume that this technique will also gain more and more acceptance in the fields of pesticide residue analysis and formulation control.

EXPERIMENTAL

Reagents

Methanol, diethyl ether and ethyl acetate were obtained in puriss quality and light petroleum (b.p. 40–65 °) and lithium hydroxide in purum quality from Fluka (Buchs, Switzerland). Aluminium oxide (basic, cationotropic) from Woelm (Eschwege, G.F.R.) was used as received.

Reference compound

2,3,7,8-Tetrachlorodibenzo-1,4-dioxin (TCDD) was obtained from Stickstoffwerke Linz (Linz, Austria) and its purity was checked by gas chromatographic (GC), mass spectrometric (MS) and micro-elemental analysis. It was found to be suitable for use as a standard without further purification. Standard solutions of TCDD in ethyl acetate were prepared at concentrations of 1 and 10 μg/ml. The safety precautions to be taken when handling this highly toxic compound have been stressed elsewhere[11].

Instrumentation

A Finnigan Model 1015D quadrupole gas chromatograph–mass spectrometer was used to obtain conventional mass spectra on the reference compound. A GC column was interfaced with the mass spectrometer via a venting system and a glass jet separator. The glass column (1.5 m$\times$2 mm I.D.) was packed with 3% silicone OV-225 on Chromosorb W AW-DMCS. The column had been previously treated with silylation reagent. The helium carrier gas flow-rate was adjusted to 25 ml/min. The injection port temperature was 250 °, the column temperature 230 ° and the separator temperature 250 °. Mass spectra were recorded at 70 eV. The ion energy used was approximately 10 V and the electron multiplier voltage 3 kV. A scan time of 2 sec was found to be suitable for an *m/e* range of 35–550.

For mass fragmentography, the instrument was operated in a similar fashion. With the instrument set at unit resolution, the ion intensities at *m/e* 320 for TCDD were monitored (single ion detection). This adjustment was made using the precision mass meter of the instrument. The signals were recorded on a regular 10-mV recorder using an additional 0.16-Hz filter.

A second, similarly treated glass column with an auxiliary T-port at approximately one third of the column length from the inlet was used for back-flushing possible late-eluting, interfering substances. This column was packed in the first portion (50 cm) with 3% Carbowax 20M on Chromosorb W AW-DMCS, and in the second portion (100 cm) with 3% silicone OV-225. The column was installed in the gas chromatograph with helium carrier gas lines connected to both the inlet port and the auxiliary port. Additionally, venting lines were mounted for the inlet port and

auxiliary port. The carrier gas and venting lines could be individually switched on and off by toggle valves outside the chromatograph. Operation of this column at 240° was done with constant pressure (2.0 atm) rather than constant flow-rate in order to achieve efficient back-flushing. The total retention time for TCDD on both this composite column and on the first conventional column was between 5 and 6 min.

Sample preparation

A sample preparation based on the work of Edmunds *et al.*[11] was used. Twenty grams of sample were dissolved or suspended in 150 ml methanol and 25 ml of 5 *N* lithium hydroxide solution were added. The mixture was refluxed for 20 min and then cooled to room temperature, and 500 ml of water and 200 ml of light petroleum were added and the mixture was vigorously shaken. After separation of the phases, the organic phase was dried over anhydrous sodium sulphate. A portion of about 100 ml was concentrated under vacuum on a rotary evaporator (maximum temperature 50°) to about 10 ml. An aliquot was usually taken because some formulations formed emulsions that were difficult to separate.

The concentrate was transferred to an alumina column (45 ml of basic aluminium oxide in a 50 cm × 20 mm I.D. column). The column was eluted with 100 ml of light petroleum, 50 ml of 5% diethyl ether and then by 100 ml of 25% diethyl ether in light petroleum. These eluates were discarded. TCDD was eluted with 150 ml of diethyl ether and the solution was concentrated to approximately 10 ml. After transfer to a suitable receiver, it was further concentrated to about 0.2 ml with a gentle stream of nitrogen. Care was taken never to allow the sample to be evaporated completely to dryness. The samples, made up to 1.0 ml with ethyl acetate, were now ready for GC–MS analysis.

A shortened procedure was used for technical 2,4,5-T or 2,4,5-trichlorophenol by omitting the refluxing and the clean-up steps. To a 20-g sample, dissolved in 150 ml of methanol, 500 ml of water and 25 ml of 5 N lithium hydroxide solution were added. After addition of 200 ml of light petroleum and vigorous shaking, the organic phase was separated, dried and an aliquot concentrated on the rotary evaporator. Again, final concentration was carried out with a gentle stream of nitrogen. The samples, made up to volume (1.0 ml) with ethyl acetate, were then analyzed by GC–MS.

Samples that contained TCDD at levels above 100 ppb were re-analyzed by mass fragmentography after being methylated with diazomethane according to the micro-method described by Schlenk and Gellerman[16]. Methylation was carried out in 10% methanol–diethyl ether solution.

Recovery

Recovery experiments were carried out by adding differing amounts of TCDD, ranging from 0.1 to 10 μg, to the hydrolysis reagents, to technical 2,4,5-T, 2,4,5-trichlorophenol or to formulations. These fortification levels correspond to 5–500 ppb for technical materials and 50–5000 ppb for 10% 2,4,5-T formulations.

Determination

For quadrupole mass fragmentography, the mass spectrometer was used to

monitor at unit resolution the molecular ion for TCDD at *m/e* 320. The precision mass meter was adjusted by slowly evaporating a few nanograms of TCDD from the direct insertion probe.

A standard curve was prepared by injecting differing amounts of TCDD (0.5–100 ng) on the GC column and plotting peak heights *versus* amount injected. Quantitation of TCDD in samples was then achieved by comparing their peak heights with the standard curve.

In order to allow injection of up to 50 μl of solution, a venting period of 1 min was used to prevent overloading the MS vacuum system with solvent vapour. Samples prepared by the shortened procedure were analyzed using the dual-column system. An initial venting period of 1 min was used and back-flushing initiated after 4 min, which prevented early- and late-eluting components from entering and contaminating the separator and ion source. This timing period was previously determined by a few standard injections so as to ensure that none of the TCDD is lost through venting and back-flushing.

Confirmation of the identity of TCDD in samples with a suspected content higher than 100 ppb was carried out by MS. A larger aliquot, corresponding to approximately 100 ng of TCDD, was injected and complete mass spectra were recorded. Five mass spectra taken at 15-sec intervals bracketting the retention time of TCDD were sufficient. The spectra were evaluated by comparison of the mass peaks in the region *m/e* 200–500 of scans obtained before, during and after elution of suspected TCDD. The ion intensities of *m/e* 320, 322 and 324 (M^+, four chlorine atoms) and 257 and 259 (M^+—COCl, three chlorine atoms) were compared with that of a TCDD standard. In addition, the spectra were checked for the absence of chlorine containing higher molecular ions that could possibly form fragments at these *m/e* values.

In addition, samples containing large amounts of TCDD were reassessed by mass fragmentography after methylation in order to observe any reduction of the TCDD content caused by this treatment.

RESULTS AND DISCUSSION

The MS data for TCDD indicate intense molecular ions with the characteristic clustering due to chlorine isotopes at *m/e* 320, 322 and 324. The only other ions of some intensity in the higher mass range are at 257 and 259 (M^+–COCl). Quadrupole mass fragmentography was carried out by single ion detection of the ion at *m/e* 320. This ion was used rather than the more intense ion at *m/e* 322 because of possible interference of PCBs. In addition, column bleeding from silicone OV-225 showed a minor signal at *m/e* 322.

The response of the Finnigan 1015D quadrupole mass spectrometer proved to be sufficiently stable over a suitable period of time to allow the use of an external standard for quantitation with acceptable precision. The peak height of the response was found to be linear in the range 0.5–100 ng of TCDD injected. A detection limit of better than 0.5 ng could easily be achieved without any attempt to maximize the response. This corresponds to a level of approximately 1 ppb based on a 20-g sample of technical material. This detection limit was found to be sufficient for

the present work. For the samples investigated, a single GC peak was usually obtained.

Back-flushing and venting the dual-column system was achieved by switching the carrier gas stream and vent by toggle valves outside the heated zones. Such a system has been described by Deans[17] as early as 1965 and more recently by Warner *et al.*[18]. The system allowed the back-flushing of unwanted late-eluting components and prevented them from entering the second, final GC column. With the same system, early-eluting components are vented and do not contaminate the separator and ion source. It is of importance in pesticide analysis that the components of interest are in contact only with glass and not with any metallic parts such as valves. Only components with retention times in a narrow range are determined. Of course, this system is applicable only when a few components are of interest. The use of a first column to cut out unwanted components results in an efficient clean-up and simplifies sample preparations prior to GC analysis. Such systems are easily built, easy to operate and suitable for routine application and automation. In combination with single ion detection, this leads to a high specificity of detection. We are sure that such techniques will gain an increasingly wider application in pesticide analysis.

Recovery experiments for TCDD added to the reagents and carried through all the steps of the procedure ranged from 40% at the 0.1 μg level to 80–100% at the 1–10 μg level. Addition of similar amounts of TCDD to 2,4,5-T and 2,4,5-trichlorophenol led to recoveries of 80–100% at levels corresponding to 50–500 ppb. Recoveries in both 2,4,5-T ester and amine salt formulations were lower (60–80%).

Some results for the content of TCDD in 2,4,5-T are reported in Table I. The samples listed represent the production of a German manufacturer over a period of 6 years. We were told after this investigation that the sample with code 12.8.70 and a content of *ca.* 2000 ppb TCDD was not of German but of American origin.

TABLE I

TCDD CONTENT IN SAMPLES OF 2,4,5-T OBTAINED FROM A GERMAN MANUFACTURER

Sample code	*TCDD content (ppb)*	
	Complete procedure	*Simplified procedure*
7.8.67	300	240
17.7.70	390, 490	520
12.8.70	1840	1950
12.11.70	420	580, 640
12.12.70	50	60
14.9.71	80	60, 70
22.9.72	n.a.*	40
18.9.73	n.a.*	80
Dowicide 2 (2,4,5-trichlorophenol)	2	<3

* Not analysed.

The first column in Table I lists the results obtained according to the original procedure, while the second column lists the results obtained by the shortened procedure.

The results indicate acceptable agreement for duplicate samples and also for the two procedures. The results show that the content of TCDD in technical 2,4,5-T for this manufacturer ranged from 300 to 600 ppb prior to 1971. Since then, the TCDD content decreased to 40–80 ppb, which is about compatible with the 50 ppb specification valid in Switzerland. As a comparison, a single result for a sample of 2,4,5-trichlorophenol (Dowicide 2) is included, which shows a content of TCDD of about 2 ppb.

In addition, a comparison of the results listed in Table I indicates that refluxing samples with methanolic lithium hydroxide does not increase the TCDD content. The simplified method results in a very fast procedure for the analysis of TCDD in technical 2,4,5-T and 2,4,5-trichlorophenol and is suitable for routine analysis in quality control.

Table II lists some typical results obtained by analyzing 2,4,5-T formulations taken from commercial sources in Switzerland in June, 1973. These formulations were analyzed using the complete procedure with refluxing and alumina column clean-up. The results indicate TCDD contents of 20–70 ppb relative to 2,4,5-T. Again, the results are acceptable with our specification.

TCDD in samples with a suspected content above 100 ppb could be confirmed in all instances by MS. Confirmation of TCDD by complete MS analysis on these samples was preferred to multiple ion detection on three or four prominent ions. Scanning the whole mass range from *m/e* 35 to 550 in fixed intervals before, during and after elution of the suspected TCDD allowed the confirmation and identification of TCDD in all those samples, although no GC peak could usually be observed on the total ion monitor, because of a multitude of other components that were eluted from the column at about the same retention time as TCDD. These components

TABLE II

TCDD CONTENT IN 2,4,5-T FORMULATIONS TAKEN FROM COMMERCIAL SOURCES IN SWITZERLAND IN JUNE, 1973

Results are given in ppb relative to 2,4,5-T.

Formulation	*Sample*	*TCDD content*
Ester	Manufacturer I (15% 2,4,5-T)	50
	Manufacturer II (45% 2,4,5-T)	20
Amine salt	Manufacturer III (12% 2,4,5-T)	10, 20
	Manufacturer IV (9% 2,4,5-T)	50, 70
	Manufacturer V (9% 2,4,5-T)	20, 50

could be identified by MS as polychlorinated biphenyls, dibenzofurans and diphenyl oxides.

The presence of polychlorohydroxydiphenyl ethers as possible precursors of TCDD could not be substantiated. This was observed by Rappe and Nilsson[19] for octachlorodibenzo-1,4-dioxin. Our finding is based on the fact that with the samples examined in this study, no significant reduction of the apparent TCDD content occurred after methylation of the neutral part.

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CHROM. 7187

DETERMINATION OF TOTAL HYDROCARBONS IN SEA WATER AT THE MICROGRAM LEVEL WITH A FLOW CALORIMETER

ÁDÁM ZSOLNAY*

Institut für Meereskunde, Kiel (G.F.R.)

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SUMMARY

One litre of water is extracted with 10 ml of 1,2,2-trichlorotrifluoroethane and the extract is then concentrated to 100 μl. A 50-μl volume is injected into a high-performance liquid chromatographic system with a flow calorimeter that measures the absorbance of hydrocarbons on porous glass beads. A short silica gel column in the system removes non-hydrocarbon material. A chromatogram is obtained within 3 min and only one peak has to be evaluated. The minimum detectable amount is 4.0 ± 3.4 μg/l. These values can be improved by extracting larger volumes. The equipment used is fairly inexpensive and can readily be taken to sea.

INTRODUCTION

In order to make a meaningful study of the distribution of hydrocarbons in an aquatic environment under normal conditions, one is often faced with the problem of making a large number of determinations on a relatively small sample volume (under 5 l) containing compounds that are present in only low concentrations[1]. It was therefore desirable to develop a method that was capable of fulfilling these requirements. In addition, it was hoped to make the method sufficiently simple to be used at sea, to decrease the time between sampling and measuring and thereby to help reduce the complications brought about by storage.

Infrared detectors have been used for measuring quantitatively the hydrocarbon content in water[2–4]. However, they usually have a lower sensitivity of only 0.05 mg (ref. 3), and great care must be taken to separate the hydrocarbons from other non-polar compounds, as the detector measures all the C–H bonds present regardless of any other functional groups that might possibly be attached to the molecule.

Sensitive methods are available for the measurement of aromatic hydrocarbons based on their absorption of ultraviolet (UV) light[5] or on measuring their fluorescence in the UV region after having been excited[6–8]. These measurements, however, do not show the amount of non-aromatic hydrocarbons present, which quantitatively are far more abundant in natural waters[1,9]. Another disadvantage

* Present address: Biostation, St. George's West, Bermuda.

of these optical methods is that the response per unit weight varies from compound to compound, making it necessary to report all experimental values with respect to a more or less arbitrary standard.

Gas–liquid chromatography (GLC) has often been used in the analysis of mineral oil present in polluted water[2,4,10] and gives a high resolution of the individual hydrocarbon compounds. However, in water not directly concerned with oil pollution, it is difficult to obtain quantitative values, as the small amount of the hydrocarbon material present would produce individual peaks that are too weak to measure accurately. Also, the extracted samples have to be purified by liquid chromatography in order to separate the hydrocarbons from other compounds, before they can be analyzed by means of GLC. Another difficulty is that a single chromatogram takes about a half an hour to run and requires some time to evaluate and may involve sophisticated electronic equipment such as integrators.

In this laboratory, a method has been used[11] that consists in purifying the extract on a thin-layer chromatographic (TLC) plate, scratching off the part containing the hydrocarbons, eluting them, and then determining their amounts in a CHN analyzer. This method, however, was found to be too cumbersome to be used for rapid determinations and contained too many steps, each of which were prone to contamination.

EXPERIMENTAL

The layout of the high-performance liquid chromatographic (HPLC) system used is shown in Fig. 1. All of the major parts were obtained from Varian Aerograph (Walnut Creek, Calif., U.S.A.). The solvent delivery system is a pressurized solvent container made of stainless steel. A small helium bomb provides the pressure, which forces the solvent through the system. The tubing is also made of stainless steel with an O.D. of 1/8 in. and an I.D. of 1.8 mm. The injector is of the septum type with a silicone rubber/PTFE laminated septum. The flow calorimeter is an adsorption detector and is placed in a well insulated water-bath containing about 12 l of water.

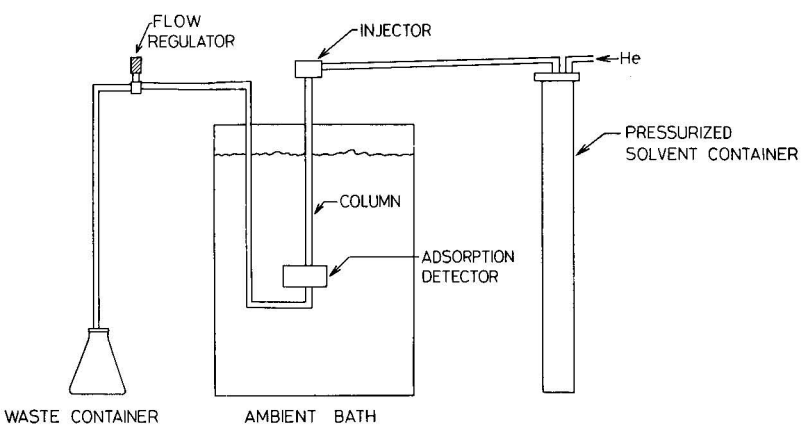
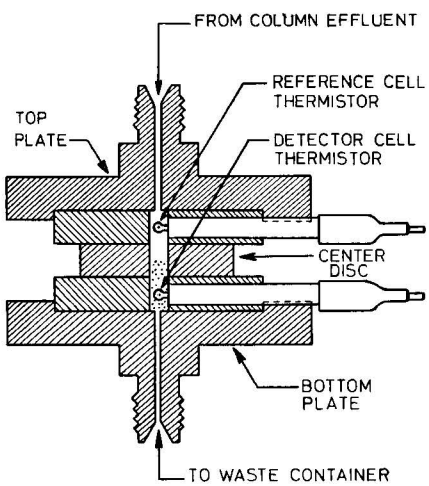


Fig. 1. Layout of the HPLC system. The tubing has an O.D. of 1/8 in. and the ambient bath a volume of about 12 l.

The detector is shown in detail in Fig. 2, and it consists of two thermistors placed within the solvent stream. The upper thermistor is embedded within a non-adsorbing material or is not embedded at all, while the lower thermistor is embedded in an adsorbing material. Any compounds transported by the solvent through the detector that are capable of adsorbing on this material do so and produce a heat of adsorption, which in turn changes the resistance in the lower thermistor. An electrical imbalance occurs and is registered on a 0–1 mV potentiometric strip-chart recorder. Under ordinary conditions, desorption of the compound from the adsorbent follows, resulting in a loss of heat around the lower thermistor, and a negative peak results after the positive peak (Fig. 3).

In order to test the characteristics of the detector and to investigate various solvent–adsorbent combinations, a column 5 cm long with an I.D. of 1.8 mm was filled with non-porous glass and attached between the detector and the injector. It was found that the detector was very sensitive to changes in the flow-rate, and a flow regulator was therefore built into the system. The detector was also sensitive to changes in pressure and the injection of a sample often resulted in a pressure front, which produced a sharp deflection on the recorder. This was avoided by placing the flow regulator after the detector, so that a pressure is maintained on the entire system and the effects resulting from the additional pressure due to an injection are proportionally not as great.

The solvent used has to be non-polar with a low boiling point, as the samples are obtained by extracting the hydrocarbons, which are non-polar, from the water. These extracts must then be concentrated rapidly with the lowest possible loss of the hydrocarbon material. At first, *n*-hexane was used as the solvent with *n*-tetracosane as the material to be determined. The following materials were tried as



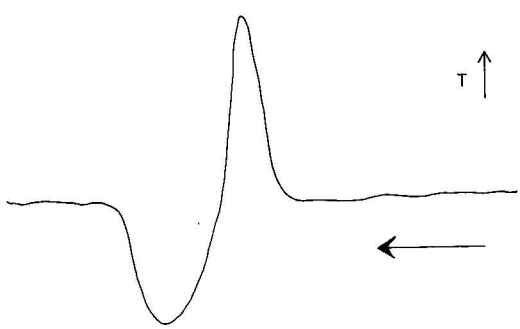


Fig. 2. Diagram of the flow calorimeter used. It is an adsorption detector, with the detector cell thermistor embedded in a material that is capable of adsorbing hydrocarbons.

Fig. 3. Peak obtained from the flow calorimeter with an *n*-hexane–graphite system. First a heat of adsorption occurs, which is then followed by desorption, resulting in a negative peak after the positive peak.

adsorbents: porous glass beads, Amberlite XAD-2, silica gel, cellulose powder and cast iron[12], but all of these produced an adsorption that was too weak to be measured satisfactorily. Graphite resulted in a very strong heat of adsorption with the larger hydrocarbons dissolved in *n*-hexane and was used in two studies in this laboratory[13,14]. The use of graphite, however, had two serious limitations: the response per unit weight varied from hydrocarbon to hydrocarbon in a non-linear manner and, in addition, the active sites on the graphite became irreversibly blocked after several extracts had been injected, which resulted in a loss of sensitivity with use, and the adsorbent had to be replaced fairly often.

In order to avoid these problems, the solvent was changed from a low-boiling hydrocarbon to a halogenated hydrocarbon. Any of the non-polar halogen compounds would have been suitable, but as a low boiling point was desired, 1,2,2-trichlorotrifluoroethane (TCF) was chosen. This compound also has the additional advantage that it is transparent to UV light at 254 nm, as measurements on the aromatic hydrocarbons are also made in this laboratory[1,5]. Of the various adsorbents that had been tried before, porous glass beads with a pore size between 3.0 and 4.5 nm were found to be the most suitable. The TCF–glass beads system has a sensitivity that is only about one-tenth of that of the *n*-hexane–graphite system for the longer-chain hydrocarbons, but it has the advantage that the response per

TABLE I

RESPONSE PER UNIT WEIGHT RELATIVE TO NONADECANE

Compound	*No. of carbon atoms in compound*	*Response (%)*	
		5-cm column with non-porous glass beads	*5-cm column with 10% deactivated silica gel*
Nonadecane	19	100	100
Squalane	30	92	93
Octacosane	28	78	76
Tetracosane	24	– *	83
Docosane	22	94	93
Eicosane	20	–	91
Pristane	19	–	90
Octadecane	18	–	107
Heptadecane	17	–	97
Hexadecane	16	101	103
Pentadecane	15	–	97
Undecane	11	93	91
Hexane	6	–	110
β-Carotene	40	–	0
Squalene	30	81	32
1-Docosene	22	–	81
1-Nonadecene	19	82	79
1-Heptadecene	17	79	75
Naphthalene	10	45	29
Stearic acid *n*-decyl ester	28	15	1
Methyl palmitate	17	20	0

* A dash means not tested.

unit weight for the various saturated hydrocarbons is the same regardless of the degree of branching or chain length (Table I), and the adsorbed material is readily desorbed again, causing no blocking of the active sites on the glass beads. The adsorption curve in this system, however, is different in that at first a decrease in temperature is registered, followed by an increase (Fig. 4). Evidently the hydrocarbons displace solvent molecules from the surface of the glass beads, and this desorption is registered by the detector. This desorption also occurred when halogenated hydrocarbons other than TCF, such as carbon tetrachloride or tetrachloroethane, were used as the solvent. This desorption peak was found to be linearly proportional to the amount of hydrocarbons injected.

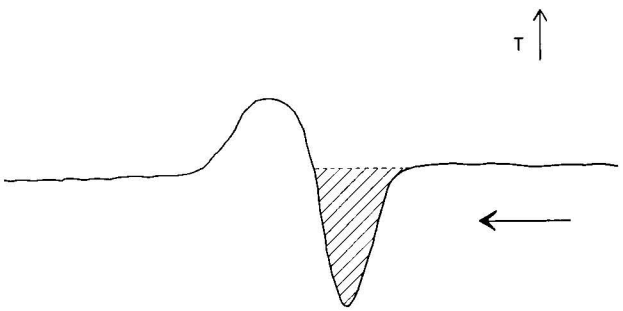


Fig. 4. Peak obtained from the flow calorimeter with a halogenated hydrocarbon–porous glass bead system. A desorption occurs before the adsorption. The area indicated with hatched lines was proportional to the amount injected on the column.

It is important to make certain that only the hydrocarbon material is measured and not other compounds. It can be seen from Table I that the glass beads in the detector react preferentially with non-aromatic hydrocarbons. In addition, the non-polar solvent that is used to extract the sea water will not extract more polar compounds, such as waxes or fatty acid esters, as well as the non-polar hydrocarbons. However, in order to be certain that the non-hydrocarbon compounds, which are far more abundant in nature, are also not measured, the column is filled with silica gel (0.063–0.200 mm grain size) that has been activated overnight at 110° and then partially deactivated with 10% (w/w) of distilled water. The area of the curve that is measured is indicated in Fig. 4 with hatched lines, and the response per unit weight relative to *n*-nonadecane of various compounds is shown in Table I. *n*-Nonadecane was chosen as the standard because it has an "intermediate" size among the hydrocarbons that are most likely to be found in a natural environment. It can be seen from Table I that the silica gel prevents both fatty acid esters and waxes from having an influence on the hydrocarbon measurements. The saturated hydrocarbons have responses per unit weight that range from 76 to 110% of that of *n*-nonadecane. In general, there is a tendency for the response to decrease with increasing chain length, but branching, judging from squalane and pristane, has no effect. Unsaturation, however, does decrease the response per unit weight. This decrease is not too pronounced with the mono-unsaturated compounds, heptadecene, nonadecene and docosene, but squalene, with six double bonds, has a response that is only 32% that of *n*-nonadecane, and carotene shows

no response at all. This means that this method will tend to underestimate the hydrocarbon content when there is a large proportion of unsaturated compounds present. If one assumes that 50% of the hydrocarbons present are unsaturated and that the unsaturated hydrocarbons have, on average, a response per unit weight that is 50% of that of *n*-nonadecane, the value determined will be 25% too low. This is naturally only a very rough estimate, but even under these extreme conditions, one can see that the underestimate is still at a tolerable level, when one considers the complexity of the problem of measuring hydrocarbons in the aquatic environment.

PROCEDURE

The sample consists of 1 litre of water, which must be taken with a sampling device that is scrupulously free from hydrocarbon material. When it is possible to take larger samples, it is better to do so. The sample is shaken for 5 min with 10 ml of Uvasol-grade TCF (E. Merck, Darmstadt, G.F.R.) and then allowed to stand for 10 min so as to permit the solvent to settle. It is then removed from the bottom of the shaking container with a pipette and transferred to a small test-tube, which is placed under vacuum in a desiccator. The desiccator is partially filled with water in order to take advantage of the relatively high specific heat of the liquid and by this means to prevent the temperature in the desiccator from becoming too low, which would retard the concentrating rate too much. This concentration step is the most time-consuming, but the extracts from a large number of samples can be placed in the desiccator at the same time, thus making this step considerably more efficient when several samples are being processed at the same time. When the extract in the test-tube has been concentrated to about 1 ml, the extract is transferred by means of an Eppendorfer pipette into a concentration tube, which is simply a small test-tube that has part of a sealed pipette with 10-μl gradiations fused on it so that the amount of extract that remains after concentration can be read with an accuracy of 5 μl. The extract is concentrated to 100 μl by means of a stream of nitrogen gas. The nitrogen used was the purest commercially available and was further purified by the use of a 1-nm molecular sieve. All glassware was pre-cleaned with dichromate–sulphuric acid in the laboratory and then wrapped in solvent-cleaned aluminium foil until required for use. The Eppendorfer pipette tips were pre-cleaned with solvent in the laboratory and disposed of after use.

The flow-rate of the solvent in the HPLC system is 5.2 ml/h and 50 μl of the concentrated extract is injected. As the standard, about 25 μg of *n*-nonadecane in a 50-μl volume are injected before the first sample and after every fifth sample. The peak on the recorder is complete after about 3 min, and 4 min after each injection, the next injection can be made. Every time the standard is injected, a blank consisting of 50 μl of solvent is also injected. Sometimes a peak results from the blank, presumably due to a pressure front, and the area of this peak is then subtracted from the peak areas of the standard and of the samples. The peak area of the sample is then divided by the peak area of the standard and multiplied by the weight of *n*-nonadecane that was injected in order to give the amount of hydrocarbons that were present in 500 ml of sample. The smallest amount of *n*-nonadecane that could readily be detected in a 50-μl injection was 2.0 μg. Several sub-samples from the

same water sample were extracted, in order to determine the standard deviation of the method, which was found to be ± 1.7 μg. Because the method, as used here, gives only the value for 500 ml, this value must be multiplied by 2, which means that the smallest amount of hydrocarbons that can be detected in 1 litre is 4.0 ± 3.4 μg. When larger amounts of sea water are extracted or when a larger percentage of the concentrated sample extract is injected into the system, these values can be considerably improved.

It is desirable to know to what extent the TCF extracts the hydrocarbons from the water, but there is no simple way of spiking a sample and maintaining natural conditions at the same time. In another study[5], it was determined that $87 \pm 8\%$ of the phenanthrene introduced into a sea water sample was recovered. Presumably the recovery of the more hydrophobic paraffins should be even better.

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CHROM. 7171

PREPARATION OF ADSORBENTS FOR BIOSPECIFIC AFFINITY CHROMATOGRAPHY

I. ATTACHMENT OF GROUP-CONTAINING LIGANDS TO INSOLUBLE POLYMERS BY MEANS OF BIFUNCTIONAL OXIRANES

LARS SUNDBERG and JERKER PORATH

Institute of Biochemistry, University of Uppsala, Uppsala (Sweden)

(Received November 9th, 1973)

SUMMARY

A new method for preparing adsorbents for biospecific affinity chromatography is described. Bisoxiranes (*e.g.* 1,4-butanediol diglycidyl ether) have been used for the introduction of reactive oxirane groups into agarose, and for simultaneous stabilization of the gel by cross-linking. Optimal conditions for the activation and subsequent coupling of proteins, peptides and aliphatic and aromatic amines have been evaluated.

Fractionation of different forms of trypsin on soya bean trypsin inhibitor agarose is described in order to illustrate the use of oxirane–agarose in biospecific affinity chromatography.

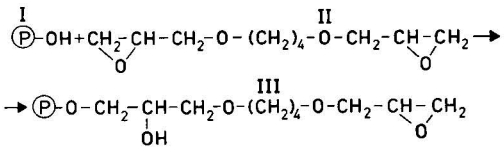
INTRODUCTION

The synthesis of adsorbents for biospecific affinity chromatography requires suitable matrices and effective coupling methods for attachment of ligands. These aspects have recently been discussed by Porath and Kristiansen[1]. Agarose fulfils most demands on a carrier for adsorptive groups or ligands, and its properties can be further improved by cross-linking with agents such as diglycidyl ethers, epihalohydrins[2,3] and divinyl sulphone[4]. A variety of coupling methods are also available but only a few of them satisfy demands such as the formation of stable bonds between ligand and matrix, high yield and no introduction of non-specific centres such as charged groups, etc.[5–7].

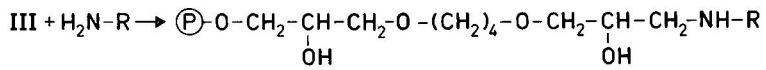
This paper describes the use of bisoxiranes for the introduction of reactive oxirane groups into agarose and for stabilization of the gel by simultaneous cross-linking. The use of bisoxiranes such as 1,4- or 1,3-butanediol diglycidyl ether consequently fulfils the dual function of bridging the gel strands as well as allowing attachment of adsorptive groups or molecules to the matrix via long hydrophilic or amphiphilic spacers[8–10]. The method has been used successfully for the immobilization of proteins, peptides and amino acids, and some of these applications have been described earlier[11,12]. A comprehensive description of the optimal conditions

for activation and coupling is included. To illustrate the use in biospecific affinity chromatography, we have chosen the isolation and fractionation of different forms of trypsins on an inhibitor agarose, because this system lends itself particularly well as a model for comparison with other methods of preparing biospecific adsorbents and immobilized enzymes. The following parameters were studied during activation: pH, reaction time, temperature, solvent and concentration of bisoxirane. Coupling was studied as a function of pH, reaction time and temperature.

For 1,4-butanediol diglycidyl ether, the activation of the polymer matrix Ⓟ–OH takes place in the following manner:



and the amino group containing compounds $R–NH_2$ are coupled to III according to the reaction:



Agarose (Sepharose 2B, 4B and 6B) was obtained from Pharmacia (Uppsala, Sweden) and 1,3- and 1,4-butanediol diglycidyl ether from EGA Chemie (Steinheim, G.F.R.). Glycyl-L-leucine, L-arginine, L-lysine, L-glycine, *p*-aminobenzamidine hydrochloride, soya bean trypsin inhibitor (STI), twice-crystallized trypsin, *p*-tosyl-L-arginine methyl ester (TAME), benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and sulphanilamide were all purchased from Sigma (St. Louis, Mo., U.S.A.).

MATERIALS AND METHODS

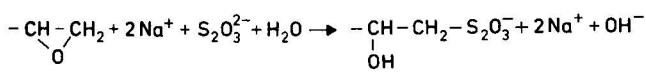
Determination of the amount of bound substance

The coupled products were analyzed with respect to amino acid[13], nitrogen (Kjeldahl method) and sulphur[14]. Prior to the analyses, the gels were washed thoroughly with water and acetone on a glass filter-funnel and freeze-dried. The water content in the dried gels was determined using a moisture analyzer. Reported values for bound substances were corrected for the residual water in the gel. The amounts of proteins coupled to the gels were determined by amino acid analysis in all experiments. Gels containing fixed amino acids and other amines were analyzed with respect to the nitrogen content. Gels with coupled sulphanilamide were analyzed for both nitrogen and sulphur.

Determination of oxirane groups

We have used the method suggested by Axén[15] involving a reaction between

the oxirane ring and sodium thiosulphate in order to determine the amount of oxirane in the solution and in the gel. The reaction is as follows:



The release of OH^- was followed by titration with 0.1 *M* hydrochloric acid in a pH-stat (pH meter 51, Autoburett ABU 12, Radiometer, titrator 11).

Oxirane in solution was determined as follows. The oxirane-containing solution (50 μl) was added to 1.5 ml of 1.3 *M* sodium thiosulphate solution and pH was kept constant by additions of hydrochloric acid until the reaction was complete. The amount of oxirane present in the solution was then calculated from the amount of hydrochloric acid needed in order to maintain neutrality.

Oxirane groups in the gel were determined in the following way. Wet agarose gel (0.5 g) was added to 1.5 ml of 1.3 *M* sodium thiosulphate solution of pH 7.0 and the oxirane content of the gel was determined by titration with hydrochloric acid. The agarose gel was suction-dried under vacuum on a glass filter-funnel for 5 min and weighed. In our experiments, the samples had a dry weight of approximately 27 mg.

Activation and cross-linking

One gram of suction-dried agarose (Sepharose 6B) was washed on a glass filter-funnel with water and then mixed with 1 ml of diglycidyl ether and 1 ml of 0.6 *M* sodium hydroxide solution containing 2 mg of sodium borohydride per millilitre. The suspension was mixed by rotation for 8 h at 25° and the reaction stopped by washing the gel on a glass filter-funnel with large volumes of water (500 ml).

Coupling

The coupling for 1 g of suction-dried oxirane–agarose was accomplished by dissolving the desired reactant in 2 ml of a buffer adjusted to a predetermined pH. Thus proteins were coupled in the pH range 8.5–10 at a temperature of 25° and a reaction time of 15–48 h. For amino acids, amines, carbohydrates and other more stable substances, the corresponding reaction conditions were pH 9–11, temperature 25–75° and reaction time 4–15 h. An increased coupling yield was obtained at higher pH and temperature. However, the decreased yield at the low pH and temperature necessary for coupling proteins can be partially compensated for by prolonging the reaction time.

RESULTS

Conditions for introduction of oxirane groups in agarose gel

Optimum pH. Samples of 0.5 g of suction-dried Sepharose 6B were mixed in a 25-ml round-bottomed flask with 0.5 ml of diglycidyl ether and 0.5 ml of sodium hydroxide solution of varying molarity containing 2 mg of sodium boro-

hydride per millilitre. The flask was rotated at a slow speed at 25° for 8 h. Washing of the oxirane–agarose was performed on 25-ml plastic filter-funnels with 10-ml portions of water in order to remove excess of reagent. The last portion of the washings was found to contain no oxirane. In Fig. 1, the amount of oxirane expressed in μmoles per gram of dry gel is plotted against concentration of added hydroxide ions. The maximum activity, expressed as oxirane concentration in the gel, was found at a final concentration in the reaction mixture of 0.2 *M* sodium hydroxide, which corresponds to a concentration of 0.6 *M* of the sodium hydroxide solution added.

Optimum temperature. Samples of 0.5 g of suction-dried Sepharose 6B were mixed with 0.5 ml of diglycidyl ether and 0.5 ml of 0.6 *M* sodium hydroxide solution (containing 2 mg sodium borohydride per millilitre) at 4°. The samples were then brought to different temperatures and were slowly stirred for 8 h. Fig. 2 shows the curve for the amount of free oxirane groups in μmoles introduced per gram of dry gel as a function of temperature.

Optimum reaction time. Samples of 0.5 g of suction-dried Sepharose 6B were mixed with 0.5 ml of diglycidyl ether and 0.5 ml of 0.6 *M* sodium hydroxide solution (containing 2 mg sodium borohydride per millilitre) and rotated at 25° for different lengths of time. Fig. 3A shows the number of μmoles of oxirane groups introduced as a function of reaction time in hours while Fig. 3B displays the results from a set of experiments under the same conditions in which the amount of oxirane groups in the gel and in the solution during activation was determined. A sample of 50 μl of supernatant was withdrawn, titrated and the total amount of oxirane in the supernatant was calculated. A blank experiment was carried out in order to determine the oxirane stability in the solution in the absence of gel. In a third

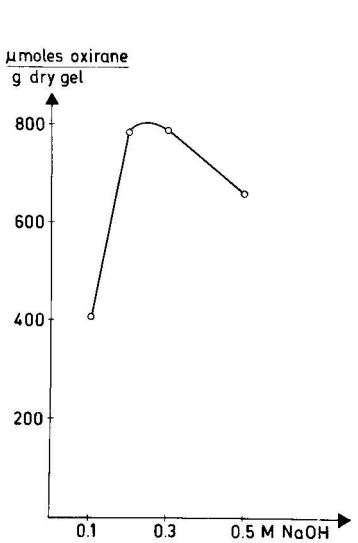


Fig. 1. Oxirane content in the gel as a function of the alkalinity of the reaction mixture.

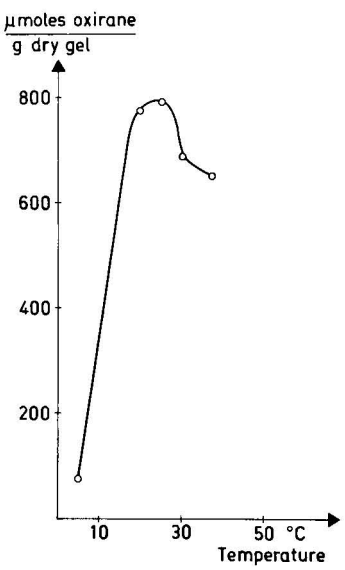


Fig. 2. Oxirane content in the gel as a function of the temperature during the reaction.

experiment, pre-cross-linked Sepharose 6B (ref. 3) was used in order to determine the degree of cross-linking under normal activation conditions.

Optimization of diglycidyl ether concentration. Samples of 0.5 g of suction dried Sepharose 6B were mixed with 0.5 ml of 0.6 *M* sodium hydroxide solution (containing

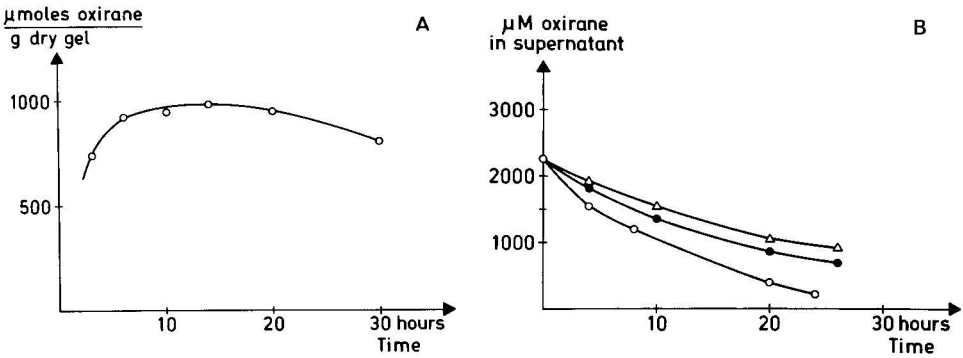


Fig. 3. (A) Oxirane content in the gel as a function of reaction time. (B) Oxirane content in the supernatant as a function of reaction time. ○—○, Oxirane in the supernatant during reaction with Sepharose 6B; ●—●, oxirane in the supernatant during reaction with epichlorohydrin cross-linked desulphated Sepharose 6B (ECD-Sepharose 6B); △—△, stability of diglycidyl ether under the conditions used for preparation of oxiran-Sepharose expressed as remaining oxirane as a function of reaction time.

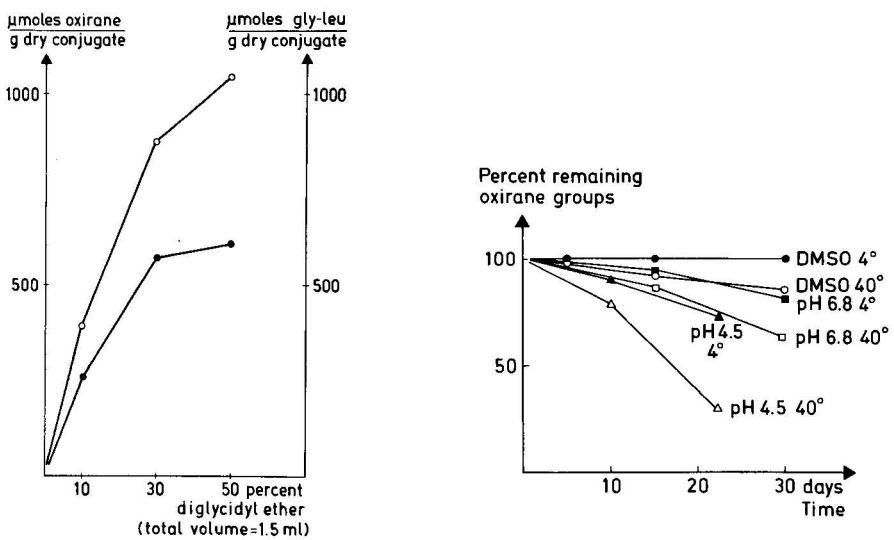


Fig. 4. Oxirane content and amount of coupled dipeptide (glycyl-L-leucine) in the gel as a function of concentration of diglycidyl ether in the reaction mixture. ○—○, Oxirane-Sepharose 6B; ●—●, amount of coupled glycyl-L-leucine in µmoles per gram of dry conjugate.

Fig. 5. Stability of oxirane–Sepharose 6B expressed as a percentage of remaining oxirane groups in the gel as a function of temperature and solvent. ○—○, Dimethyl sulphoxide, 40 °; ●—●, dimethyl sulphoxide, 4 °; △—△, 0.1 *M* sodium acetate, pH 4.5, 40 °; ▲—▲, 0.1 *M* sodium acetate, pH 4.5, 4 °; □—□, 1*M* sodium chloride, pH 6.8, 40 °; ■—■, 1 *M* sodium chloride, pH 6.8, 4 °.

2 mg sodium borohydride per millilitre) and varying concentrations of diglycidyl ether and rotated for 8 h at 25°. Fig. 4 shows the amount of oxirane coupled to the gel (μmoles per gram of dry gel) as a function of percentage of oxirane in the reaction mixture. It also shows the amount of bound glycyl-L-leucine in milligrams per gram of dry conjugate as a function of oxirane concentration in the reaction mixture. The dipeptide coupling was performed with 100 mg of glycyl-L-leucine in 1 ml of 0.5 *M* sodium carbonate buffer, pH 10, mixed with 0.5 g of suction-dried oxirane–Sepharose 6B at 50° for 15 h.

Stability of oxirane–agarose

Samples of suction-dried Sepharose 6B were incubated with a variety of solutions at 4°, 23° and 40° for different periods of time. Fig. 5 shows the stability of oxirane–agarose in distilled water (1 *M* in sodium chloride), dimethyl sulphoxide and 0.1 *M* acetate buffer, pH 4.5 (1 *M* in sodium chloride).

Conditions for coupling

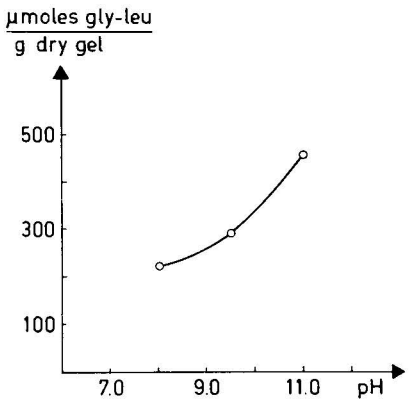
In all coupling experiments, the following activation procedure was used: 0.5 g of suction-dried Sepharose 6B was mixed with 0.5 ml of diglycidyl ether and 0.5 ml of 0.6 *M* sodium hydroxide solution (containing 2 mg of sodium borohydride per millilitre) in a round-bottomed flask and rotated for 8 h at 25°.

Coupling of dipeptide at different pH values. Glycyl-L-leucine (100 mg) was dissolved in 1 ml of buffer solutions at different pH values (0.1 *M* sodium phosphate buffer for pH 6.0 and 8.0, 0.3 *M* sodium carbonate buffer between pH 8.5 and 11) and mixed with 0.5 g of suction-dried oxirane gel. In Fig. 6 the amount of chemically bound glycyl-L-leucine (μmoles per gram of dry gel) is plotted as a function of pH. After the coupling step, the gel was washed systematically with the following solutions: 0.5 *M* sodium carbonate buffer, pH 9.5, 1 *M* in sodium chloride (100 ml); distilled water (50 ml); and 0.05 *M* glycine–hydrochloric acid buffer, pH 3.0, 1 *M* in sodium chloride (100 ml).

Coupling of dipeptide at different temperatures. Glycyl-L-leucine (100 mg) was dissolved in 1 ml of 0.5 *M* sodium carbonate buffer, pH 11, and mixed with 0.5 g of suction-dried oxirane–Sepharose 6B at 4°. The temperature was adjusted and the reaction mixture carefully stirred for 8 h. The results are shown in Fig. 7A. In another set of experiments, the contact time for the reaction between oxirane–agarose and glycyl-L-leucine was studied at 23° and 40°; the conditions were the same as above and the results are shown in Fig. 7B. The effect of concentration was studied and the conditions used were as follows: 0.5 g of suction-dried oxirane–Sepharose 6B was mixed with varying amounts of glycyl-L-leucine dissolved in 1 ml of 0.5 *M* sodium carbonate buffer, pH 11, under rotation at 50° for 15 h. Fig. 8 shows the amount of coupled dipeptide in μmoles per gram of dry gel as a function of the concentration of glycyl-L-leucine.

Coupling of proteins. Soya bean trypsin inhibitor (200 mg) was dissolved in 20 ml of 0.5 *M* sodium carbonate buffer, pH 9.5, and carefully stirred with 10 g of suction-dried oxirane–Sepharose 6B for 24 h at 25° (activation: 10 g of suction-dried agarose was mixed with 5 ml of diglycidyl ether and 5 ml of 0.6 *M* sodium hydroxide solution containing 2 mg of sodium borohydride per millilitre) for 8 h at 25°). The inhibitor–gel was then washed with the following solutions in order to remove

unbound protein: sodium carbonate buffer, pH 9.5, 1 *M* in sodium chloride (250 ml); distilled water (100 ml); 0.1 *M* glycine, pH 3.0, 1 *M* in sodium chloride (250 ml); and finally 0.05 *M* Tris–hydrochloric acid buffer, pH 7.8, 0.5 *M* in sodium chloride and 0.02 *M* in Ca^{2+} (250 ml).



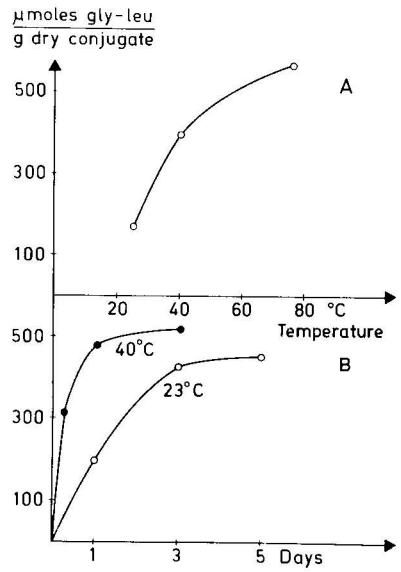
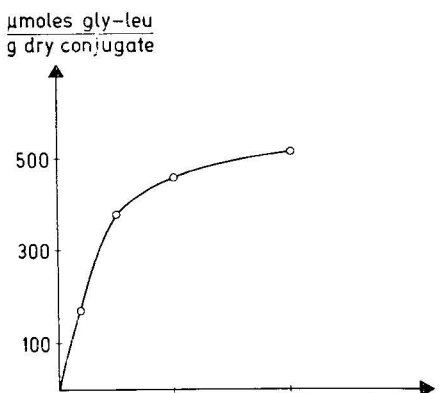


Fig. 6. Amount of coupled glycyl-L-leucine (expressed as μmoles of dipeptide per gram of dry conjugate) as a function of the pH in the reaction mixture.

Fig. 7. Amount of coupled glycyl-L-leucine (expressed as μmoles of dipeptide per gram of dry conjugate) as a function of reaction temperature.



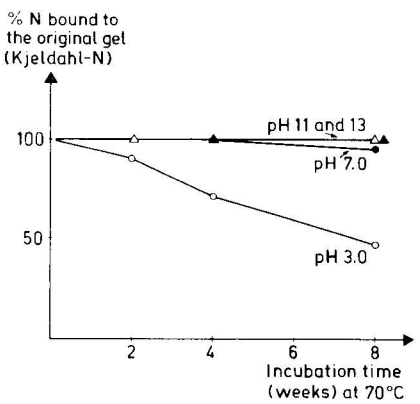


Fig. 8. Amount of coupled glycyl-L-leucine (expressed as μmoles of dipeptide per gram of dry conjugate) as a function of peptide concentration in the reaction mixture.

Fig. 9. Stability of coupled glycine expressed as a percentage of remaining nitrogen in the gel as a function of pH and incubation time at 70 °. ○—○, 1 *M* sodium acetate buffer of pH 3.0; ●—●, 0.5 *M* sodium phosphate buffer of pH 7.0; △—△, 1 *M* sodium carbonate buffer of pH 11; ▲—▲ 0.1 *M* sodium hydroxide solution (pH 13).

Deactivation of remaining oxirane groups after coupling. Immobilization of proteins results in a gel that still contains oxirane groups capable of further coupling. These groups can be blocked by treatment of the gel with a solution of, for example, 2 *M* glycine or ethanolamine, preferably at a pH above 8.5, at 23° for 24 h. Such deactivation blocks oxirane groups that are sterically available for coupling. Titration with sodium thiosulphate solution shows that some groups remain, presumably within sites that do not interfere in the chromatographic experiments.

Stability of coupled glycine

The stability of glycine coupled to Sepharose 6B (450 mg per gram of conjugate) was studied by incubation of the mixture at various pH values (1 *M* sodium acetate buffer, pH 7.0, 1 *M* sodium carbonate buffer, pH 11, and 0.1 *M* sodium hydroxide solution, pH 13) at 70° for 8 weeks. Samples were withdrawn after different incubation times, and the amounts of nitrogen in the gel and in solution were determined.

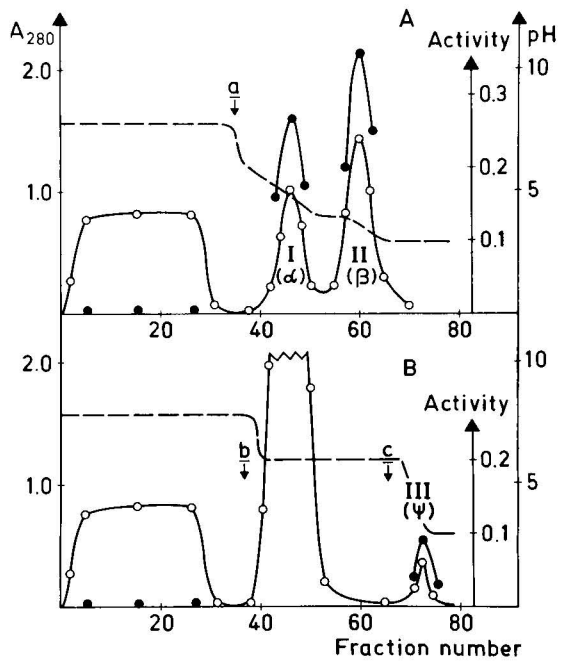


Fig. 10. (A) Chromatography of bovine trypsin on an STI–Sepharose 6B column (10 × 1.4 cm) previously equilibrated with 0.05 *M* Tris–hydrochloric acid buffer, pH 7.8 (0.5 *M* in sodium chloride, 0.02 *M* in Ca^{2+}). Amount of trypsin: 400 mg. Flow-rate: 50 ml/h. Fraction volume: 9 ml. At a, the pH gradient started (– – –). The absorption at 280 nm (○—○) and the trypsin activity (ΔE_{405}/min, ●—●) of eluted fractions were measured. The N-terminal amino acid residues of fractions I and II indicated the composition of mainly α- and β-trypsin (see Table I). (B) The same conditions as in A. At b, specific desorption was performed with citrate–phosphate buffer, pH 6.0, containing 50 mg of benzamidine per millilitre, and at c elution with 0.05 *M* glycine buffer, pH 3.0 (0.5 *M* in sodium chloride) was started. The eluted trypsin–benzamidine complex could be split and separated by chromatography on Sephadex G-25 previously equilibrated with 10^{-3} *M* hydrochloric acid. N-Terminal amino acid analyses of fraction III indicated the presence of ψ-trypsin (see Table I).

In Fig. 9, the stability is expressed as percentage of original nitrogen remaining in the gel after the incubation.

Demonstration of bioaffinity chromatography employing adsorbents produced by bisoxirane coupling

Soya bean trypsin inhibitor agarose obtained as described in the section *Coupling of proteins* was used to isolate and separate different active trypsins from commercial preparations. Adsorption was performed at pH 7.8 (0.05 *M* Tris–hydrochloric acid, 0.5 *M* in sodium chloride) and the adsorbed material displaced either by using a non-linear pH gradient (LKB 1130 Ultrograd gradient mixer operating on the following buffers: citrate–phosphate, pH 6.0 (0.05 *M* dipotassium hydrogen orthophosphate adjusted with 1 *M* citric acid), 0.5 *M* in sodium chloride; and citrate–phosphate, pH 3.0 (0.05 *M* dipotassium hydrogen orthophosphate adjusted with 1 *M* citric acid), 0.5 *M* in sodium chloride); or by consecutive displacement with citrate–phosphate buffer, pH 6.0, 0.05 *M* in phosphate and 0.5 *M* in sodium chloride containing 50 mg of benzamidine per millilitre and with 0.05 *M* glycine–hydrochloric acid, pH 3.0, 0.5 *M* in sodium chloride. Enzyme activities were determined with BAPNA as substrate[16], measuring the change in absorption at 405 nm. The amino acid terminal sequence was determined according to the method of Edman[17] and Iwanaga *et al.*[18]. As the three active trypsins, α, β and ψ, differ in their N-terminal residues, this property can be used for identification[19]. In Table I, literature values of N-terminal residues for the α-, β- and ψ-trypsins are compared with those actually found in bioaffinity chromatography of commercial trypsin (shown in Fig. 10).

TABLE I

N-TERMINAL SEQUENCE OF THE MATERIAL IN THE CHROMATOGRAPHIC FRACTIONS I, II AND III (SEE FIGS. 10A AND B)

β-Trypsin is a single polypeptide chain, whereas α-trypsin consists of two and ψ-trypsin of three polypeptide chains[19, 22].

Fraction	*N-terminal amino acid residues*
I	Ile, Ser (traces of Asn)
II	Ile
III	Ile, Ser, Asn
α-Trypsin[19, 22]	Ile, Ser
β-Trypsin[19, 22]	Ile
ψ-Trypsin[19, 22]	Ile, Ser, Asn

DISCUSSION

Treatment of hydroxyl-containing polymers with bifunctional oxiranes (*e.g.* 1,4-butanediol diglycidyl ether) in alkaline medium has proven to be an effective means of preparing adsorbents for biospecific affinity chromatography. Attempts to find the optimal conditions for activation are shown in Figs. 1–5. Fig. 1 shows

that a final concentration of 0.2 *M* sodium hydroxide in the reaction mixture is sufficient to give the maximal introduction of oxirane groups in the gel. At higher concentrations of sodium hydroxide, hydrolysis and cross-linking will produce gels with fewer free groups available for coupling. The most suitable temperature for activation is found to be about 25° (Fig. 2). Higher temperatures can be used if the reaction time is shortened. Fig. 3 shows a rather broad optimum for the reaction time; 6–10 h gives a maximum yield under the experimental conditions used.

The parameters involved in the activation and coupling are not mutually independent. The determination of exact optimum conditions of introduction of oxirane groups into the gel and for coupling would require a much larger series of experiments than those described in this paper. From a practical point of view, such an extentive study is not necessary because the approximate optimal conditions obtained simply by superimposing the results from the separate series of experiments are likely to approach the exact optimal conditions very closely.

In order to determine the degree of cross-linking and the amount of oxirane remaining in solution, experiments were performed which gave the results shown in Fig. 3A. During activation, it is necessary to work with an excess of diglycidyl ether owing to the side-reaction leading to formation of cross-links. The difference between the hydrolysis curves obtained in the absence and presence of gel is a measure of the total amount of oxirane reacted with gel, *i.e.* both involved in cross-links and available for titration. From Fig. 3, it is obvious that only a small part of the diglycidyl ether used is involved in the production of active gel-linked oxirane groups, whereas the major portion of reacting material has produced cross-links. Further evidence for this conclusion was provided by the experiment in which pre-cross-linked agarose was treated with diglycidyl ether under the same conditions. Here, less oxirane should be consumed because most of the reactive hydroxyl groups in the gel should already be involved in cross-links. The results in Fig. 3A show this to be so. The amount of available oxirane can be seen to decrease progressively when activation is allowed to continue for periods longer than 10 h, under which conditions the oxirane group is presumably hydrolyzed. The number of available oxirane groups is also a function of the initial concentration of diglycidyl ether. Fig. 4 shows that an increase in concentration gives a considerable increase in oxirane groups in the gel. For practical use, a concentration of 30% of diglycidyl ether in the reaction mixture is sufficient to give good results. One of the advantages of this method is that the oxirane gel can be stored in the wet state at 4° in 1 *M* sodium chloride solution for more than 4 weeks with less than 10% reduction in the oxirane content.

Both low- and high-molecular-weight substances have been coupled to oxirane agarose. Table II summarizes the results obtained and gives the amount of coupled substance under optimal conditions. Glycyl-L-leucine was used as a model substance in the attempts to determine optimal conditions for coupling. Figs. 6–8 show the influence of pH, temperature, time and reagent concentration on this coupling reaction. The best results were obtained at high pH (11) and a temperature of about 50°. At high temperatures, a shorter contact time can be used, but for coupling of proteins, milder conditions have to be used to retain biological activity. Table II shows the results obtained in the coupling of soya bean trypsin inhibitor, STI, to Sepharose 6B (see Methods). The amount of coupled inhibitor per gram

of dry polymer, 7 μmoles, corresponds to about 75% of that obtained with the cyanogen bromide method under similar conditions.

The STI–agarose has been used as an adsorbent for the isolation of proteolytic enzymes from crude pancreatic extracts and for separation of active and inactive trypsins in commercial preparations[11] (see refs. 20–22). From these experiments, it is clear that 1 mole of trypsin binds to 1 mole of coupled inhibitor.

Different forms of active trypsins can also be separated by bioaffinity chromatography on bisoxirane-coupled STI–agarose. Absorbed trypsin can be eluted by pH gradient elution, which completely resolved α-, β- and ψ-trypsin (Fig. 10A). The separation of ψ-trypsin from its analogues can be carried out by specific desorption with benzamidine, a strong inhibitor for α- and β-trypsin[19]. ψ-Trypsin is not eluted under these conditions, but can be subsequently desorbed by changing the pH of the eluent (Fig. 10B).

These experiments illustrate how the bisoxirane method can be used to produce effective adsorbents for bioaffinity chromatography in which non-specific adsorption is to be avoided and high selectivity is required.

In addition to active groups for coupling and cross-linking of agarose, this method provides a spacer or arm, which is of great importance in biospecific adsorption when the ligand is small and the affinity constant low[23–27]. In a study of these effects, we have used sulphanilamide coupled to agarose (Table II) to adsorb and isolate carbonic anhydrase directly from human erythrocytes (to be published). Sulphanilamide coupled to cross-linked dextran (Sephadex) with cyanogen bromide has also been used for the purification of carbonic anhydrase[28]. The corresponding agarose adsorbent possessed a very low affinity, presumably owing to more severe steric hindrance by the matrix. Adsorbents formed from oxirane agarose, however, showed a high capacity for carbonic anhydrase. The results indicate that the spacer is necessary in this case for obtaining a good adsorbent.

TABLE II

COVALENT BINDING OF SUBSTANCES CONTAINING AMINO GROUPS TO SEPHAROSE 6B

The oxirane–gel was prepared as described under *Activation and cross-linking*. Soya bean trypsin inhibitor (STI) was coupled at 25 °, pH 10.0, for 24 h, and the other substances listed at 75 °, pH 11.0, for 18 h.

Substance	*Amount of substance added to 5 g of wet polymer (mg)*	*Amount of coupled substances*	
		μmoles/g of dry polymer	*% of added substance*
p-Amino benzamidine	100	450	21
Sulphanilamide	100	430	20
Sulphanilamide	500	650	6
Glycyl-L-lysine	100	380	25
Lysine	100	428	21
Soya bean trypsin inhibitor	100	5.8	38

ACKNOWLEDGEMENTS

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CHROM. 7190

FRACTIONATION OF RNA ON A METAL ION EQUILIBRATED CATION EXCHANGER

I. CHROMATOGRAPHIC PROFILES OF RNA ON AN AMBERLITE IR-120 (Al^{3+}) COLUMN

V. SHANKAR and P. N. JOSHI

Department of Chemistry, University of Poona, Poona 411 007 (India)

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SUMMARY

Amberlite IR-120, a polystyrene sulphonate type of cation exchanger, equilibrated with Al^{3+} ions, has been employed for the fractionation of RNA. This adsorbent affords a quantitative and reproducible recovery of RNA into six fractions.

INTRODUCTION

Although several chromatographic procedures exist for the resolution of RNAs and DNAs[1–6], they do not provide adequate resolution of a complex mixture of functionally different RNAs necessary for the precise study of their metabolic role. In addition, they are not free from operational limitations such as clogging of the column, slow flow-rate leading to irreversible adsorption and consequent loss of biological activity, denaturation, low recovery, etc. In view of the above considerations, alternative methods of fractionation are necessary.

EXPERIMENTAL

Ribonucleic acid

The sodium salt of RNA was isolated from the liver of buffalo (Mammalia, Ruminantia), the chief milking animal of India, essentially by the procedure of Sevag *et al.*[7]. The purity of the isolated RNA was characterized by the usual methods[8–12], and was found to be 80% pure, containing about 10% protein residue and no DNA contamination. Hyperchromic studies showed a 40% increase in the UV absorbance at 260 nm, indicating that the isolated RNA is a native RNA.

Preparation of IR-120 (Al^{3+})column

A 5-g amount of dry regenerated Amberlite IR-120 (Na^+) resin was allowed to swell in water for about 4–5 h and the slurry obtained was packed in a clean Pyrex glass column (45 × 1 cm). It was then equilibrated with aluminium ions by passing 50 ml of 0.2 *M* aluminium chloride solution through the column at a

flow-rate of 10–15 ml/h. This amount was found to be adequate for equilibration with respect to Al^{3+} ions, as judged from preliminary experiments. Finally, the pH of the column was adjusted to 4.0 by passing a sufficient amount of acetate buffer (pH 4.0, ionic strength, $\mu = 0.05$). The washing with buffer removes all of the loosely retained Al^{3+} ions. The amount of Al^{3+} in the influent, effluent and buffer washings were determined by Vogel's method[13], and it was observed that the resin retained 39.2 mg of Al^{3+} per gram of resin under the conditions used.

Typical chromatographic profiles of RNA

A homogeneous solution of buffalo liver RNA, isolated by the method of Sevag *et al.*[7] and characterized for its purity and nativeness, was prepared in acetate buffer (pH 4.0, $\mu = 0.05$) and was applied to a column containing 5 g of IR-120 (Al^{3+}), previously equilibrated with the above buffer. The RNA solution was allowed to percolate through the column at the rate of 12–15 ml/h, and this slow flow-rate permitted satisfactory equilibrium conditions to be attained. The effluent was collected and the column was washed with four bed volumes of the above buffer in order to remove any loosely retained RNA species.

The adsorbed RNA was then eluted by the continuous passage of 0.05 *M* ammonium acetate solution. The fractions, each of 10 ml, were collected and assayed for their RNA content by the thymol–iron (III) chloride–hydrochloric acid reaction[9].

The percentage of total RNA adsorbed and eluted is given in Table I. Fig. 1 shows typical chromatographic profiles of RNA on the IR-120 (Al^{3+}) column, the total optical density at 590 nm being plotted against the test-tube numbers of the fractions eluted.

TABLE I

TYPICAL CHROMATOGRAPHIC PROFILES OF BUFFALO LIVER RNA ON AN IR-120 (Al^{3+}) COLUMN

Percentage retention	*Percentage elution by 0.05 M ammonium acetate*						*Total elution* (F_1–F_6)	*Total recovery* (%)
	F_1	F_2	F_3	F_4	F_5	F_6		
100	32.18	19.33	15.04	19.67	1.97	4.63	92.82	100

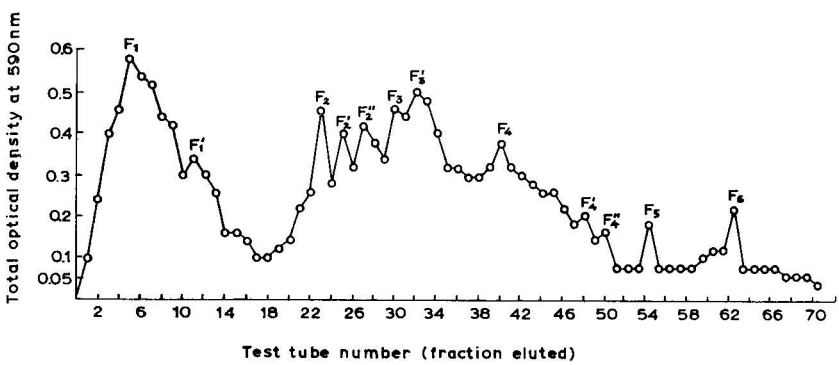

Fig. 1. Typical chromatographic elution profiles of buffalo liver RNA (method of Sevag *et al.*[7]) on an IR-120 (Al^{3+}) column.

Re-chromatography

Re-chromatography of a given fraction or a peak corresponding to a given fraction is an important aspect of any chromatographic technique. Reproducible chromatographic behaviour can be regarded as a sound criterion of the homogeneity of the material eluted in a specific fraction.

As fraction one (F_1) was the largest and most distinct fraction, it was chosen for re-chromatographic studies. It was extensively dialyzed against physiological saline at 4° in order to remove ammonium acetate completely, then re-chromatographed on a fresh IR-120 (Al^{3+}) column, as described above. The fraction was completely adsorbed and was eluted in a single peak and at the same position on the chromatogram (Fig. 2).

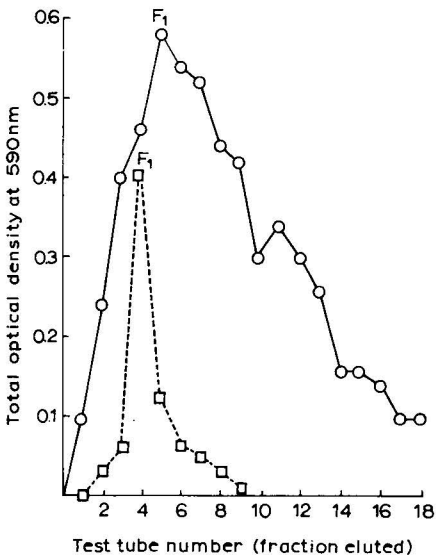

Fig. 2. Re-chromatography of fraction F_1 on an IR-120 (Al^{3+}) column: ○—○, chromatography; □- - -□, re-chromatography.

Base composition

Different RNAs may differ in their base composition and/or the sequence of bases in the chain. In order to ascertain whether the IR-120 (Al^{3+}) column fractionates RNAs according to their composition, the base composition of three of the major isolated fractions was therefore studied.

RNA fractions were hydrolyzed by incubating them with 0.3 *M* potassium hydroxide solution at 37° for 16 h [14]. The resulting nucleotides were further subjected to hydrolysis with perchloric acid in order to liberate the bases.

From this digest, adenine was determined by the method of Woodhouse[15] and guanine by the Folin phenol reaction according to Hitchings[16]. After the determination of purine bases, they were quantitatively removed by precipitation as silver salts[17] and the pyrimidines, *viz.*, cytosine and uracil, were determined according to the method of Soodak *et al.*[18]. From this mixture, cytosine was then removed

by adsorption on Decalso cation exchanger[17] and uracil was determined as mentioned above[18]. The results are given in Table II.

TABLE II

BASE COMPOSITION OF RNA FRACTIONS ON AN IR-120 (Al^{3+}) COLUMN

Concentrations of bases are expressed as moles/100 moles of phosphorus.

Type of RNA	*Base contents*						
	A	*G*	*C*	*U*	$\frac{A}{U}$	$\frac{G}{C}$	$\frac{A+U}{G+C}$
Unfractionated	12.00	40.26	39.57	9.45	1.42	1.02	0.26
Fraction 1 (F_1)	11.80	40.40	39.72	8.48	1.39	1.02	0.25
Fraction 2 (F_2)	11.80	40.00	39.00	8.40	1.41	1.03	0.26
Fraction 3 (F_3)	19.50	35.30	37.89	7.35	2.65	0.93	0.37

RESULTS AND DISCUSSION

The failure of the IR-120 (Na^+) column to adsorb RNA[19] and the large increase in retention on the IR-120 (Al^{3+}) column shows that interaction with Al^{3+} ions on the IR-120 (Al^{3+}) column is the basis of adsorption. Dissociation of the Al^{3+}–RNA complex and its subsequent removal could be the basis for resolution–elution.

There are likely to be reversal types of linkages that bind RNA molecules to the IR-120 (Al^{3+}) column. In order that specific RNA molecules might progress down the column, these multiple linkages must be dissociated or disrupted simultaneously. Reagents such as EDTA, citrate and fluoride elute RNA from the IR-120 (Al^{3+}) column by their intrinsic ability to form a complex with Al^{3+} ions and thus displace RNA. With such reagents, most of the RNA is eluted in a single fraction, making resolution impossible. In such instances, the elution characteristics are less dependent on the concentration of the eluting agent than on its chemical nature. A changing gradient of these eluting agents will not, therefore, be of much help in such instances, when a basic technique has had to be established in order to study broadly the heterogeneity of RNA. In view of these considerations, a simple system consisting of only one eluting agent was employed, which resulted in complete elution.

The ability of ammonium acetate solution of low concentration (0.05 *M*) to effect elution, in spite of its not being a chelating agent, suggests that the binding between RNA and Al^{3+} ions is weak. The fact that 0.05 *M* ammonium acetate solution is able to effect complete elution together with fractionation indicates that the mechanism which governs this column chromatographic behaviour is probably a distribution between two phases.

Quantitative elution of a fraction in a single peak after re-chromatography not only shows that the eluted fraction represents one group of RNA, but also suggests that the fractionation procedure is reproducible.

Base composition studies indicated that the unfractionated buffalo liver RNA and the fractions (F_1–F_3) obtained after passage through the IR-120 (Al^{3+}) column have similar base compositions, and that they are guanine–cytosine rich.

The results suggest that the fractionation of RNA on an IR-120 (Al^{3+}) column is likely to be independent of base composition, and similar observations were reported by Mahler *et al.*[20] and Miura and Suzuki[21].

CONCLUSIONS

As the present studies were aimed mainly at understanding the heterogeneity of RNA and studying broadly its chromatographic behaviour, sub-fractionation or finer resolution was not attempted. It can be stated that the present fractionation procedure does not appear to be of a conventional ion-exchange chromatographic type because, in addition to the resin, Al^{3+} ions are also involved in the adsorption.

The present investigation has shown that the use of the IR-120 (Al^{3+}) column is a promising technique for the fractionation of RNA. Further, the IR-120 (Al^{3+}) resin is reasonably stable at elevated temperatures, over a wide range of pH and at high salt concentrations, and the technique offers a simple, reproducible and inexpensive system for the fractionation of nucleic acids. In addition, certain factors such as the amount of adsorbent, concentration of the adsorbate, marginal variation in flow-rate and ageing of RNA, do not have a significant effect on the chromatographic behaviour of RNA on the IR-120 (Al^{3+}) column.

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CHROM. 7179

QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF ATP AND THE PRODUCTS OF ITS DEGRADATION IN MEAT TISSUE

G. A. NORMAN, M. J. FOLLETT and D. A. HECTOR

J. Sainsbury Ltd., Stamford House, Stamford Street, London, S.E.1 (Great Britain)

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SUMMARY

A method has been developed for the quantitative analysis of adenosine triphosphate and the products of its degradation in *post mortem* meat tissue, by means of thin-layer chromatography and fluorescent quenching techniques.

INTRODUCTION

In recent years it has become increasingly apparent that the products of ATP* degradation in *post mortem* meat tissue merit closer investigation by the food scientist since it has been suggested that these breakdown products contribute to the flavour (Doty *et al.*[1], Jones[2], Disney *et al.*[3]) eating quality (Howard *et al.*[4]), and water-holding properties (Hamm[5,6] and Lawrie[7]) of meat.

Until recently, it has been difficult to quantify ATP and all its degradation products to Hypox in meat tissue within a single analysis. Hurlbert *et al.*[8] developed a method by which nucleotides could be separated and quantitatively determined using ion-exchange techniques. Jones[9] subsequently modified and extended this procedure for the analysis of nucleotides, nucleosides, and purines in fish muscle, but the method was time-consuming and required two separate ion-exchange procedures. Bendall and Davey[10] also devised a method based on ion exchange but this suffered from the same faults and was rather limited in application.

Enzymatic analysis has also been used to measure ATP and some of the isolated intermediates derived from it (Bergmeyer[11]), but these methods are not favoured because the experimental procedures involved are often complicated and because the cost of dealing with large numbers of samples is high.

Recently Potthast and Hamm[12] developed a new procedure based on thin-layer chromatography (TLC) in which ATP and its intermediates were separated on silica gel coated plates impregnated with a fluorescent material. This method has the advantage that all of the intermediates from one meat extract can be separated and quantitatively measured on one TLC plate during a single analysis.

Initial trials carried out by the present authors with the TLC method showed that although good separations were achieved with standard solutions, practical difficulties arose when meat extracts were assayed. Investigation has since shown,

* The following abbreviations have been used throughout this paper: ATP, ADP, AMP = adenosine tri-, di- and monophosphate; IMP = inosine monophosphate; Ino = inosine; Hypox = hypoxanthine; PCA = perchloric acid.

however, that difficulties and disadvantages in the procedure of Potthast and Hamm can be obviated by introducing the appropriate modifications detailed and discussed in this paper. Incorporating these modifications has produced a fast and accurate method for separating and quantitatively measuring ATP and the products of its degradation in meat tissue.

EXPERIMENTAL

Materials

All biochemical substrates were obtained from Boehringer (London, Great Britain). TLC plates were 20×10 cm silica gel F_{254} type, supplied by Anderman (London, Great Britain).

Organic solvents were purchased from either E. Merck (Darmstadt, G.F.R.) or British Drug Houses (Poole, Dorset, Great Britain).

The instrument used for spectrofluorometric measurements was the Vitatron TLD 100 universal densitometer supplied by Fisons (Loughborough, Great Britain). All other chemicals were purchased from British Drug Houses and were of AnalaR grade.

Constriction pipettes (25 μl) and chromatographic tanks (22 cm×7 cm×22 cm depth) were purchased from Shandon (London, Great Britain).

Extraction of meat tissue

The procedure for obtaining a deproteinised meat extract relies upon the action of 5% (v/v) PCA to denature any protein material and at the same time to extract all of the soluble components of the meat tissue[13]. The meat sample is stored and later powdered in liquid nitrogen, and approximately 1 g macerated in 8 ml of ice-cold 5% PCA. A further 8 ml of PCA are added to the macerate and the whole is allowed to stand for 20 min before it is filtered through a Whatman No. 42 filter paper. 10 ml of the supernatant is adjusted to pH 3.0–4.0 with 5 *M* potassium carbonate, using thymol blue as indicator. (At the correct pH the indicator colour change is from red to yellow.)

It is essential to bring the extracts to this pH since when the chromatogram is developed it has been observed that (i) in unneutralised extracts the purine derivatives move rapidly with the leading solvent front with little or no separation and (ii) with a pH greater than 7.0 the derivatives remain aggregated just above the base line.

'Neutralised' extracts are stored at 0° for at least 30 min before use, to allow the precipitate of potassium chlorate to settle out. The clear supernatant thus obtained is used directly for the TLC separation.

Stock standard solutions

Stock standard solutions (10 μmoles/16 ml; this adequately covers the observed range of concentrations of each compound present in 1 g of meat tissue) are prepared by dissolving the individual compounds in glass-distilled water and adjusting the pH to around 4.0 (PCA or K_2CO_3 as appropriate) before making up to 100 ml.

Allowance should be made for the percentage purity and water of hydration of the commercially prepared substrates.

All compounds readily dissolve in distilled water, with the exception of Hypox, which requires alkaline conditions (K_2CO_3) for solvation, followed by acidification to pH 4.0 with PCA prior to making up to the final volume. These stock solutions are diluted to give a further series of standard solutions with concentrations equivalent to 8, 6, 4, 2, and 1 μmoles/16 ml.

Spot application

Extracts and standard solutions are applied to the chromatographic plates using 25-μl constriction pipettes. It is important that the spot diameter is kept to between 8–9 mm by applying the 25 μl as ten individual drops, ensuring that each spot is dry before a further application is made to the plate.

Formulation of solvent system

Although standard solutions of ATP and its intermediates gave adequate separations with the multi-component solvent system of Potthast and Hamm[12], poorer separations were obtained with meat extracts. It was therefore necessary to modify the solvent system. A most satisfactory separation was obtained with the system isobutyl alcohol–amyl alcohol–ethoxyethanol–ammonia–glass-distilled water (15:10:30:15:25).

Plate development

Plates are developed in a tank containing 100 ml of the solvent system. Well defined separations are achieved if: (1) The solvent system is 'matured' for at least 48 h before use; (2) The solvent system is added to the tank 1 h before introduction of the plates; (3) The tank is placed in a constant environment to minimise the effects of draughts, sunlight and uneven heating; (4) When more than one plate is being developed, the silica gel layers are placed facing one another. This eliminates the increased rise of the solvent at the plate edges resulting in a concave-shaped solvent front at the end of the run.

Principle of measurement

Quantitative measurement of the separated compounds relies upon fluorescent quenching, which measures the loss in intensity of fluorescent light emitted from an activated surface by light of a shorter wavelength. Fluorescence in this instance is produced by fluorescein-impregnated silica gel which emits green light ($\lambda_{max.}$ = 526 nm) when exposed to ultraviolet (UV) light at a wavelength of 254 nm.

ATP and its intermediates present in the silica gel layer appear blue due to the absorption characteristics of these purine-based compounds in UV light[14]. The degree to which the emitted green light is masked by the blue is a function of the concentration of the compound on the plate.

Instrumentation

The Vitatron TLD 100 is specifically designed for the quantitative measurement of compounds separated as spots on TLC plates and where the distribution of the compound is not necessarily homogeneous within the spot.

The separated spot, which may not be homogeneous, and which need not have a particular shape either, can be considered to be composed of many smaller spots which may be regarded as homogeneous. The quantity of light reflected from each of the smaller spots is measured individually, all values are integrated, and the averaged value passed continuously to the recorder.

As the sample on the plate is scanned horizontally by the incident light beam, a curve is produced on the recorder chart the area of which may be related directly with the amount of material on the plate. The recorder is fitted with an integrator and the area is determined as the number of integrator units under the curve.

Spot alignment and spot density measurement

The TLC plate is secured such that the incident light beam passes through the centre of each of the separated compounds. Initial marking of the TLC plate at the top and the bottom of each separation run facilitates this alignment.

The light beam of the densitometer is positioned onto a blank area of the plate which has been developed by solvent. The light shutter is then moved into a position which blocks the transmitted light from the plate to the measuring unit, and the recorder is set to full-scale deflection (0% transmission). The light shutter is now opened and the recorder pen set to give zero deflection, *i.e.* 100% transmission. Each plate is calibrated in this way prior to quantitative measurement of the spots.

The loss of green fluorescent light, due to the presence of the blue spots, is recorded as a series of peaks, the areas of which are measured in arbitrary integrator units.

RESULTS

Separation

The following $R_F \times 100$ values were obtained for ATP and each intermediate: ATP, 26; ADP, 35; AMP, 44; IMP, 52; Ino, 65; Hypox, 72.

Calibration curves

Table I shows the mean areas and standard deviations (S.D.), expressed in integrator units, recorded for standard solutions of ATP and its degradation products. Coefficients of variation are also shown in the table.

It can be seen from Table I that the mean values for ATP and AMP were similar as were those for IMP, Ino, and Hypox. Accordingly, composite curves were produced and these are shown in Fig. 1, together with the curve for ADP.

Recovery trials

Volumes of the stock ATP solution were added to neutralised 5% perchloric acid extracts of beef muscle to produce a series of solutions with added ATP concentrations equivalent to 10.0, 5.0, 2.5, 1.3, and 0.6 μmoles/16 ml (or 1 g of meat). An extract blank was also included in the series. 25-μl aliquots of each solution were applied to the TLC plates as six individual spots and these were developed and quantitatively measured. ATP concentrations were calculated using the composite calibration curve for ATP/AMP. Mean recoveries of added ATP, standard deviations and coefficients of variation for the different concentrations are shown in Table II.

TABLE I

PEAK AREAS RECORDED FOR STANDARD SOLUTIONS OF ATP AND RELATED COMPOUNDS

Intermediate	*Number of plates measured*		*Concentration (μmoles/16 ml)*					
			1	*2*	*4*	*6*	*8*	*10*
ATP	10	Mean	21.3	40.3	59.4	76.6	95.8	107.9
		S.D.	2.9	3.9	2.2	3.9	1.5	4.8
		Coefficient of variation, %	14	10	4	5	2	4
ADP	13	Mean	17.4	27.5	40.1	51.2	59.9	67.3
		S.D.	1.7	1.7	3.0	5.0	4.5	4.7
		Coefficient of variation, %	10	6	7	10	7	7
AMP	10	Mean	20.2	38.2	62.0	81.4	94.0	104.8
		S.D.	3.4	3.1	1.8	2.1	4.9	3.8
		Coefficient of variation, %	17	8	3	3	5	4
IMP	12	Mean	10.5	21.1	38.9	56.4	68.0	78.3
		S.D.	2.0	1.9	2.4	3.4	5.2	5.0
		Coefficient of variation, %	18	9	6	6	8	6
Ino	10	Mean	12.8	23.0	40.5	54.2	64.4	74.4
		S.D.	1.4	2.6	3.4	4.7	6.9	6.4
		Coefficient of variation, %	12	12	8	9	11	9
Hypox	8	Mean	10.5	21.8	39.3	51.6	63.5	72.1
		S.D.	1.6	1.4	2.5	1.8	2.5	1.4
		Coefficient of variation, %	15	6	6	3	4	2

TABLE II

RECOVERIES OF STANDARD SOLUTIONS OF ATP ADDED TO MEAT EXTRACTS

Concentration of added ATP (μmoles/16 ml)	*N*	*Observed recovery (μmoles/ml)*		
		Mean	*S.D.*	*Coefficient of variation (%)*
10.0	6	9.9	0.4	4
5.0	4	4.8	0.2	5
2.5	6	2.6	0.3	11
1.3	6	1.5	0.2	16
0.6	6	0.8	0.1	16

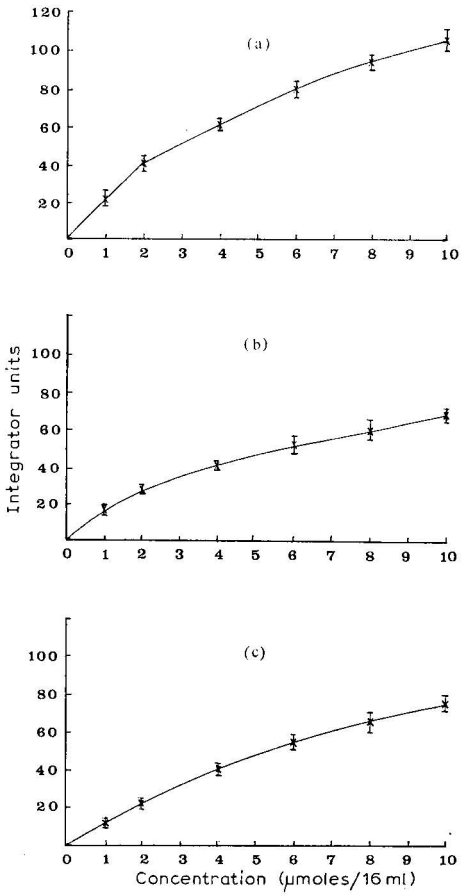

Fig. 1. Relationship between peak area (expressed in integrator counts) and concentration of (a) ATP and AMP, (b) ADP, and (c) IMP, Ino, and Hypox.

DISCUSSION

The original TLC method[12] for separating ATP and its degradation products was found to be satisfactory when using standard solutions but not sufficiently precise with beef extracts to allow accurate quantitative measurement. The streaking effect often observed between the spots is effectively eliminated by adjusting the pH of the extract prior to spotting onto the TLC plate. Furthermore, a more consistent spacing between spots has been achieved by altering the solvent system. These modifications, coupled with the moving spot method of density measurement, enables ATP and all of its degradation products to be quantitatively determined by a single assay procedure. Also, because of the small spot size after development, as many as nine different extracts can now be separated on a single plate.

It was anticipated that the calibration curves for all the compounds would be similar, or more probably that they would bear a direct relationship to one another in proportion to their individual absorption characteristics at a wavelength of

254 nm (ref. 14). However, it is clear from the values presented in Table I that this was not entirely the case. Values for ATP and AMP were similar and could be easily combined as a single curve (Fig. 1a), but the unexpected deviation of ADP (Fig. 1b) (which has identical absorption characteristics to ATP and AMP in UV light) was not easy to explain. The reduced values may result from a supersaturation effect caused by concentration of the nucleotide within a narrower spot area.

Similarities observed in the calibration curves for IMP, Ino and Hypox can be directly related to their corresponding absorption characteristics in UV light[14].

As reported previously[12], none of the calibration curves were found to be linear throughout the range of concentrations studied. In the composite curve for IMP, Ino and Hypox (Fig. 1c) the relationship of concentration to peak area was curvi-linear, whereas in the ATP/AMP composite and ADP calibration curves, straight-line correlations between peak area and concentration were noted up to 2 μmoles/16 ml and above this concentration a less definable pattern.

Although some of the standard deviations presented on the calibration curves may appear to be at first sight excessively high, it may be noted that deviations from the mean value rarely represent concentrations greater than 0.5 μmoles/16 ml or 1 g of meat.

The mean percentage recovery of ATP added to the meat extracts was 107% (S.D. 2%, coefficient of variation 17%). This inordinate level was possibly due to the high observed recovery at lower concentrations of added ATP.

This method has been successfully applied to the determination of ATP and its degradation products in a number of beef hindquarter muscles and the results of this work will be published elsewhere in the near future.

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CHROM. 7169

CONTAMINATION PROBLEMS WITH ^{63}Ni ELECTRON CAPTURE DETECTORS

CLAUDE GOSSELIN, GÉRARD B. MARTIN and ARMAND BOUDREAU

Département des Vivres, Faculté des Sciences de l'Agriculture et de l'Alimentation, Université Laval, Quebec G1K 7P4 (Canada)

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SUMMARY

A study was performed on two ^{63}Ni electron capture detectors operating in d.c. mode. Several parameters have been examined. It appeared that the temperature difference between the detector and the column is important. Scavenger gas temperature and detector isolation are not less important. Furthermore the detector polarization switch must always be off when no chromatogram is being run. These modifications help to keep the detector sensitivity much longer, with a consequent reduced number of washings.

INTRODUCTION

In gas chromatography, the electron capture detectors (ECDs) using tritium foil, commonly used in the analyses of organochlorine residues, are of the greatest sensitivity. However, they must be used at relatively low temperature which causes serious problems of contamination. Different ways of overcoming these problems, as already mentioned[1], have been used: (1) The pulse mode; (2) the use of ^{63}Ni as a radioactive source allowing use of higher temperature, and electron capture detection using a rare gas discharge to produce electrons.

The first method has its limitations, since it needs argon and methane, both relatively expensive gases. Moreover, these gases cannot be used when an ECD is coupled in series with a flame ionization or a flame photometric detector.

The use of ^{63}Ni, although allowing much higher temperatures, is also faced with some problems. In view of the low activity of ^{63}Ni, it has been necessary to substitute the plane-parallel geometry of Lovelock and Lipsky[2] for the cylindric configuration of the argon detector. This increases the area of the radioactive cell and consequently makes the detector as sensitive or even more sensitive than the tritium detector (Fig. 1). To decrease further the degree of contamination of this ^{63}Ni detector, we have tried a few modifications that we wish to report here.

MATERIALS AND APPARATUS

Our experiments were run on a Microtek 220 equipped with two ^{63}Ni (10 mCi) ECDs in a d.c. mode. Three types of columns were used: (A) Dexsil 300-GC (15%) on Gas-Chrom Q (80–100 mesh); (B) SE-30 (4%) plus QF-1 (6%) on Chromosorb W

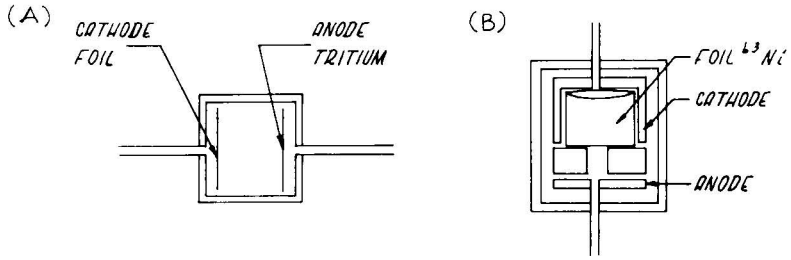

Fig. 1. Geometrical types of electron capture detectors. (A) Parallel-plane configuration; (B) cylindric configuration (*cf.* Microtek operation manual[3]).

(80–100 mesh); (C) diethylene glycol succinate (DEGS) (2%) plus phosphoric acid (0.5%) on Chromosorb W (80–100 mesh). This latter column has been recently proposed by Uyeta *et al.*[4]. The size of the columns was 6×0.25 in. I.D.. The oven temperature was set at 265° for column A and 185° for columns B and C, while the detector was kept at 295° for column A and at 285° for columns B and C. An attenuation of $16 \cdot 10^2$ was used throughout. Solutions of organochlorine standards were chromatographed as well as water and food extracts.

METHODS

Problems of operation

The two ECDs operating in d.c. mode were used to analyse pesticides in several food products. Under the conditions prevailing before the below-mentioned modifications, it was necessary to run frequent clean-ups, *i.e.* once every two, three or four weeks.

The need for a clean-up was indicated by the following symptoms: (a) after the detector has been switched on at the beginning of the day, a baseline is obtained which first shows a rapid decrease of sensitivity followed by a gradual return to a plateau (Fig. 2). That reduction of sensitivity was directly related to the degree of contamination; (b) when injecting a sample or a standard, the chromatogram illustrated in Fig. 3 was obtained. The sensitivity showed an immediate increase but decreased in the same way as in the case of symptom a.

Moreover, in order to maintain good sensitivity with increasing number of analyses, we had to adjust the polarizing voltage up to a maximum level, after which the sensitivity decreases, indicating the necessity for a clean-up.

Using a Dexsil column, stable up to 450–500°, at a low bleeding temperature (265°) has not reduced the necessity of frequent clean-ups. In the meantime, we have noted that one detector, even if heavily contaminated, could clean itself when kept at its operating temperature for three months without polarization, purge or carrier gas.

Modifications made to the instrument

As a first corrective measure, we have replaced the Dexsil 300-GC by an already known column, 4% SE-30–6% QF-1, and used a lower temperature: 185°.

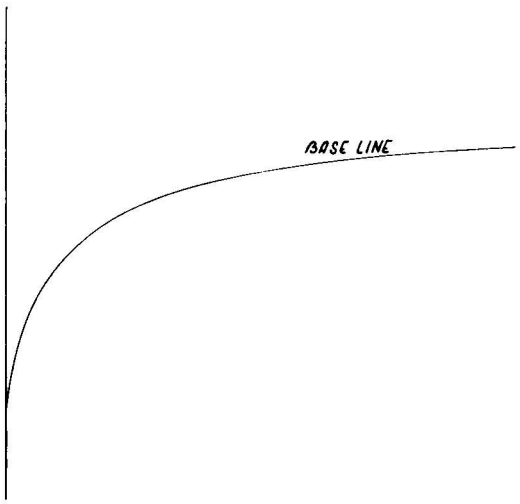

Fig. 2. Drifting of the baseline following polarization of a contaminated ECD.

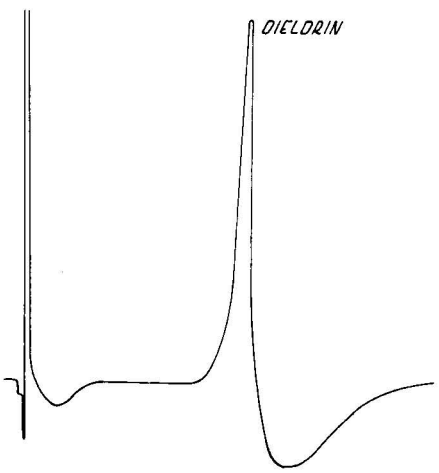

Fig. 3. Sample peak obtained by a contaminated ECD.

Even if the QF-1 phase has a non-negligible bleeding at that temperature compared to Dexsil, the frequency of decontamination was reduced by a factor of two or three.

The second detector was used with the column of 2% DEGS–0.5% phosphoric acid at 185°. Due to the considerable bleeding, the sensitivity of that detector was lowered somewhat, but there was no comtamination, even after several months of use. It then follows that the temperature difference between the column and the ECD must be larger than the one used with the flame ionization detector in order to avoid the problems of contamination.

As a second means of improving the instrument, we introduced some insulating material between the detector and the supporting plate which also serves as the heating unit of the inlets. This plate was normally at 50 or 60° below the temperature

of the detector when used with the second and third columns. By the addition of the insulating material, the temperature of the detector was markedly increased.

As a further step we added two more spirals to the gas line around the ECD in order to increase the temperature of the scavenger gas.

As a last measure, we stopped the polarization of the ECD during the nights, week-ends and when possible between analyses.

RESULTS

During a period of one year, we cleaned the ECD connected to the DEGS column only once, due to an accidental contamination. The second ECD was used for seven months with the afore-mentioned modifications. About 400 samples went through this detector during that time without major signs of contamination (Fig. 4).

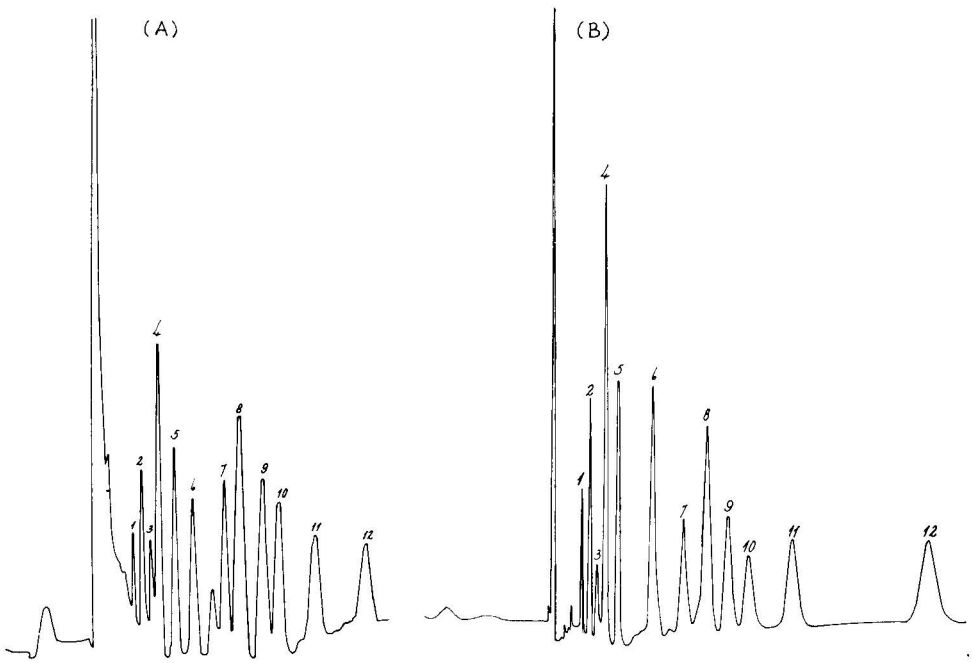

Fig. 4. Degree of contamination as obtained with unmodified (A; after 75 samples) and modified (B; after 400 samples) instrument. Pesticides were eluted in the order indicated. 1 = α-BHC; 2 = γ-BHC; 3 = β-BHC; 4 = Heptachlor; 5 = Aldrin; 6 = Heptachlor epoxide; 7 = DDE; 8 = Dieldrin; 9 = *o,p*-DDT; 10 = TDE; 11 = *p,p*-DDT; 12 = Methoxychlor.

DISCUSSION

The contamination of an ECD first leads to an adsorption of materials on electrodes and to a contact potential set up on the cathode[1,5].

Beta particles, when striking the contaminated cathode, cause charges to be set up. These charges are not neutralized by low-energy electrons which flow towards the anode. Subsequently, these adsorbed positive charges, which are in

contact with the negatively charged electrode, cause a fall in the polarizing potential as well as in the resulting current. A sample, negatively charged by the capture of electrons, or an electron coming from the ionization when polarization is off, can easily neutralize these adsorbed positive ions and leave the possibility for a normal electric field to develop. This would partly explain the increased sensitivity, soon after polarization has been switched on, and soon after electron absorbing materials have passed through the ECD (symptoms a and b). Thereafter ionization of adsorbed layers would gradually appear, setting up the contact potential.

However, the anode of our ECD does not build up a contact potential, due to an appropriate device. Indeed, a narrow window, about one centimeter long, prevents nearly all β-particles from reaching the anode and thus ionizing adsorbed materials. Moreover, electrons formed by ionization, being of too weak energy, cannot set up this contact potential on the contamination layers.

Furthermore, another phenomenon comes to strengthen the latter: that is the space charge effect. As suggested by the slow decontamination reported, it seems that there is, in the detector, a very slow and continuous evaporation of adsorbed materials as well as an ionization of these substances. Because these oily substances have poor electron affinity, they have little chance of capturing electrons and when ionized, they migrate slowly towards the cathode as a positively charged cloud. This phenomenon has been called space charge effect[5]. These space charge effects can even be enhanced by an uneven distribution of temperature within the detector. When an electron absorbing substance goes through the detector, there is formation of negative ions. These particles then neutralize positive ions and diminish the space charge effect of the cathode. Soon after the electron adsorbing substance has gone out, the space charge effect is diminished as well as the contact potential. The electric field and ionic current are increased, but they decrease rapidly due to the set up of the contact potential and of the space charge effect, both originating from the contaminating layers and vapors. Furthermore a scavenger gas, if kept at a too low temperature when reaching the ECD, will cause a condensation of contaminating vapors, bringing all sorts of troubles.

Finally, we must be conscious that even polarization enhances contamination. In fact, the cathode attracts clouds of positive ions and this suggests leaving the polarizing voltage off as often as possible between analyses. This will allow contaminating vapors to be evacuated by the gas flow. This corresponds to the non-polarization which improves the pulse mode operation.

Even if ^{63}Ni ECD is less susceptible than ^{3}H ECD to contamination, problems still remain when used on direct current, so that a few clean-ups have to be run every year, the frequency depending on the utilization of the instrument. If more clean-ups are necessary the following points have to be examined:

(1) The difference between column and detector temperature.

(2) The presence of a temperature gradient in the detector, due to a poor insulation.

(3) The temperature of the scavenger gas; if this is too low, it can be improved by increasing the number of spirals in the gas line on the detector wall.

(4) The feasibility of stopping the polarization during nights, week-ends and between analyses.

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CHROM. 7189

ANALYTICAL STUDIES OF PYRETHRIN FORMULATIONS BY GAS CHROMATOGRAPHY

III. ANALYTICAL RESULTS ON INSECTICIDALLY ACTIVE COMPONENTS OF PYRETHRINS FROM VARIOUS WORLD SOURCES*

YOSHIHIKO KAWANO, KARL H. YANAGIHARA and ARTHUR BEVENUE

University of Hawaii, Honolulu, Hawaii 96822 (U.S.A.)

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SUMMARY

Gas chromatographic techniques were applied to studies of the insecticidally active esters of pyrethrum. Pyrethrin extracts from various world sources were compared. The greater potential importance of more definitive and precise results acquired by gas chromatography compared with results obtained by older classical procedures is illustrated and it is more evident when comparisons are made of the "true" pyrethrin I and pyrethrin II ester fractions of the extracts. Results of this more precise nature should be of increased benefit to the pesticide formulator and to the insect toxicologist.

INTRODUCTION

Pyrethrum (*Chrysanthemum cinerariaefolium* Vis), native to temperate and boreal regions[1], is grown commercially primarily in Kenya, Tanzania, Ecuador and, to a lesser extent, in India and Japan as a source of the insecticidally active pyrethrin ingredients. The increased application of gas chromatographic (GC) techniques to studies of pyrethrum extracts has created renewed interest in research activities with the active components of the extracts, such as studies on the relative toxicity of the pyrethrin I and pyrethrin II fractions to the housefly (*Musca domestica*) and other insects and also the application of modified techniques for the measurement of the relative amounts of these two components in the extracts.

Published reports have been somewhat contradictory in so far as pyrethrin I has been considered to be more toxic than pyrethrin II (and *vice versa*) to the housefly[2–9] and perhaps to other insects. It has been suggested that this anomalous observation may be attributed to poorer storage stability characteristics of pyrethrin II and it has been shown that insecticidal activity is diminished when pyrethrins are exposed to sunlight and air[10].

To date, six insecticidally active esters have been identified in pyrethrin extracts[11–15]: pyrethrin I (Py_I), jasmolin I (J_I), cinerin I (C_I), pyrethrin II (Py_{II}),

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jasmolin II (J_{II}) and cinerin II (C_{II}). Prior to the advent of GC, accepted methods of analysis for pyrethrins defined two insecticidally active fractions, Py_I and Py_{II}, in which each fraction contained Py_I, J_I, C_I, and Py_{II}, J_{II}, and C_{II}, respectively[16,17]. In this paper, the 3-component Py_I group and the 3-component Py_{II} group will be referred to as Py_1 and Py_2, respectively. Results previously reported by other workers were based on $Py_1:Py_2$ ratios and not on the true component pyrethrin I:pyrethrin II ratios[18]. This paper presents results on the six components of pyrethrin extracts obtained from various world sources utilizing flame ionization GC techniques with variable operational conditions of isothermal, temperature-programmed and matrix-programmed modes, plus mass spectral data obtained by a combined GC–mass spectrometric technique. Results are presented on the "true" $Py_I:Py_{II}$ ratio values and these results are compared with the mixed component $Py_1:Py_2$ results obtained by previously discussed methods of analysis.

MATERIALS AND METHODS

Gas chromatograph

An F & M Model 810 instrument with a flame ionization detector was used. Three sets of operating conditions were employed, as follows.

(1) Isothermal (at various temperatures, to establish the best conditions), with oven temperatures of 160°, 170°, 180° and 190°, injector temperatures of 180°, 190° and 200° and detector temperatures of 180°, 190° and 210° and a nitrogen flow-rate of 30 or 45 ml/min, a hydrogen flow-rate of 25 ml/min and an air flow-rate of 210 ml/min.

(2) Temperature-programmed, with the oven temperature starting at 155°, and increasing at the rate of 4°/min up to 210° and maintained at that temperature.

(3) Matrix-programmed. The initial oven temperature was 155° and as soon as the solvent peak appeared, the temperature was maintained for 6 min 2 sec (timed with a stop-watch). The temperature was then raised to 175° at the rate of 20°/min. When the Py_I peak reached the maximum height on the recorder chart, the temperature was maintained at 175° for 2.25 min; the temperature was then raised to 205° at the rate of 20°/min and maintained at this temperature until the Py_{II} peak and other subsequent peaks appeared on the recorder chart. The other operating conditions for the gas chromatograph were an injector port temperature of 190° and a detector temperature of 210°.

Gas chromatograph–mass spectrometer

A Finnigan Model 3000 peak identifier was used with a sensitivity of 10^{-6} A/V, electron multiplier high voltage −2.00 kV and electron energy −69.5 V; a mass spectrogram was taken at the apex of each peak. The column was 18 in. × 2 mm I.D., 2.5% XE-60 on Chromosorb W, 60–100 mesh, the helium flow-rate was 20 ml/min and the system was matrix-programmed as described above.

Gas chromatographic column

Thirty different column material preparations were examined in an effort to find a column with optimum conditions for the separation of the six pyrethrin esters on the gas chromatograph (Table I). A column mixture which satisfied these

TABLE I

GC COLUMN MATERIALS USED IN STUDY OF SIX PYRETHRIN ESTERS

Stationary phase	*Support* *	*Column*			
		Type **	*Dimensions*		*Usefulness* ***
			Length (ft.)	*O.D. (in.)*	
Carbowax 20M, 3%	Chromosorb W, 60–80 mesh	G	4	1/4	U
DC 200, 5%	Chromosorb W, 60–80 mesh	G	4	1/4	U
FFAP, 5%	Chromosorb W, 60–80 mesh	G	4	1/4	U
Neopentyl glycol succinate, 1%	Chromosorb W, 60–80 mesh	G	4	1/4	U
Neopentyl glycol succinate, 3%	Chromosorb W, 60–80 mesh	G	4	1/4	U
Neopentyl glycol succinate, 1.5%	Supelcoport, 80–100 mesh	G	4	1/4	U
OV-17, 3%	Chromosorb W, 60–80 mesh	G	4	1/4	U
OV-22, 2%	Chromosorb W, 60–80 mesh	G	4	1/4	U
OV-25, 2%	Chromosorb W, 60–80 mesh	G	4	1/4	U
OV-101, 2%	Chromosorb W, 60–80 mesh	G	4	1/4	U
OV-210, 2%	Chromosorb W, 60–80 mesh	G	4	1/4	U
OV-225, 2%	Chromosorb W, 60–80 mesh	G	2	1/4	U
OV-225, 5%	Chromosorb W, 60–80 mesh	G	4	1/4	U
QF-1, 5%	Anakrom SD, 70–80 mesh	G	4	1/4	U
QF-1, 5%	Chromosorb W, HP, 80–100 mesh	G	4	1/4	U
SE-30, 3%	Chromosorb W, 60–80 mesh	G	2	1/4	U
SE-30, 3%	Chromosorb W, 60–80 mesh	G	4	1/4	U
SE-30, 5%	Chromosorb W, 60–80 mesh	G	2	1/4	U
SE-30, 5%	Chromosorb W, 60–80 mesh	G	4	1/4	U
SE-52, 3%	Chromosorb W, 60–80 mesh	G	4	1/4	U
SF-96, 2%	Chromosorb W, 60–80 mesh	G	4	1/4	U
XE-60, 2%	Chromosorb W, 60–80 mesh	SS	3	1/8	U
XE-60, 2%	Chromosorb W, 60–80 mesh	G	4	1/4	U
XE-60, 2%	Chromosorb W, 60–80 mesh	SS	5	1/8	U
XE-60, 3%	Chromosorb W, 60–80 mesh	SS	3	1/8	U
XE-60, 3%	Chromosorb W, 60–80 mesh	G	4	1/4	U
XE-60, 3%	Chromosorb W, 60–80 mesh	SS	5	1/8	U
XE-60, 3%	Chromosorb W, HP, 80–100 mesh	G	4	1/4	U
XE-60, 2.5%§	Chromosorb W, 60–100 mesh	G	2	1/4	S
XF-1105, 2%	Chromosorb W, 60–80 mesh	G	4	1/4	U

* All supports are AW-DMCS.

** G = glass; SS = stainless steel.

*** S = satisfactory; U = unsatisfactory.

§ A mixture of equal parts of 2% XE-60 on Chromosorb W, 60–80 mesh, and 3% XE-60 on Chromosorb W, 80–100 mesh.

conditions consisted of 2.5% XE-60 on Chromosorb W AW-DMCS, 60–100 mesh, packed in a 2 ft. × 1/4 in. I.D. glass column. This preferred column material was prepared by mixing equal parts of (1) 2% XE-60 on Chromosorb W AW-DMCS, 60–80 mesh, and (2) 3% XE-60 on Chromosorb W AW-DMCS, 80–100 mesh. Matrix-programmed conditions for the gas chromatograph gave the most satisfactory resolution of the six major pyrethrin esters.

Pyrethrin extracts

Refined and crude pyrethrin extracts from Ecuador, Kenya and Tanzania were supplied by Mr. Dean Kassera, McLaughlin Gormley King Co., Minneapolis, Minn., U.S.A. Extracts from areas of Japan were supplied by Mr. Takenosuke Takano, Institute for Japanese Pyrethrum Research, Kyoto, Japan. Extracts from the area of Srinagar, Kashmir, India, were supplied by Dr. S. Prasad, Council of Scientific and Industrial Research, Jammu-Tawi, India. World Standard extracts for the years 1970 and 1972 were supplied by Dr. S. W. Head, the Pyrethrum Bureau, Nakura, Kenya.

Preparation of sample extracts and World Standard solutions for analysis

A 0.4-g sample of the World Standard (21.3% purity) was weighed into a 10-ml calibrated flask and made up to volume with redistilled carbon disulphide. The standard solution was stored in a brown bottle. Samples of the pyrethrin extracts (about 100 mg per 10 ml as pyrethrins) were weighed into 10-ml calibrated flasks and made up to volume with redistilled carbon disulphide. Then 2–3-μl aliquots of the solutions were applied to the gas chromatograph for analysis. The crude extracts were passed through Florisil columns prior to analysis, as previously described[19].

TABLE II

Py_1:Py_2 AND Py_I:Py_{II} RATIOS OF PYRETHRINS FROM VARIOUS WORLD SOURCES

Pyrethrin extract	Py_1:Py_2* (*AOAC*)**	Py_I:Py_{II}* (*GC method****)
World Standard, 1970	1.12	1.63
World Standard, 1970 (restandardized 2 years later)	1.13	1.40
World Standard, 1972	1.13	1.63
Ecuador, crude	1.54	2.01
Ecuador, crude, cleaned up§	—	2.18
Ecudor, refined	1.53	2.05
India, crude	—	2.10
Japan, Fumakira	0.77	1.22
Japan, Nagaoka	1.08	1.58
Japan, Dainihon Jyochukiku	1.72	0.00
Kenya, crude	0.96	1.49
Kenya, crude, cleaned up§	—	1.46
Kenya, refined	0.94	1.36
Tanzania, crude	1.01	1.42
Tanzania, crude, cleaned up§	—	1.53
Tanzania, refined	1.08	1.47

* $Py_1:Py_2=(Py_I+J_I+C_I)/(Py_{II}+J_{II}+C_{II})$.

** The values are based on the results obtained by the method of the Association of Official Agricultural Chemists (AOAC), U.S.A.[20], and were supplied by the donors of the samples.

*** The values are based on data obtained from a gas chromatograph containing a 2 ft. × 1/4 in. glass column packed with 2.5% XE-60 on Chromosorb W, 60–100 mesh (see text). Each value is based on the average of 3 or more determinations.

§ Florisil column clean-up.

Quantitative measurement of pyrethrin esters

The areas of the pyrethrin ester peaks recorded on the gas chromatograph chart were determined with a planimeter. The World Standard peaks were used as the frame of reference; 20 mg of the World Standard was arbitrarily selected and all other sample peaks were recalculated to this sample size for ready comparison. Py_I:Py_{II} ratios were determined and are presented in Table II.

RESULTS AND DISCUSSION

As previously discussed elsewhere[19,21], the classical AOAC analytical procedure for the determination of "pyrethrins"[20] involves the hydrolysis of the samples and subsequent measurement of the chrysanthemum monocarboxylic and dicarboxylic acids. Thus, the pyrethrin 1 value would encompass the total value for pyrethrin I, jasmolin I and cinerin I; similarly, a single value would be obtained for the pyrethrin 2 fraction. Such values would also include any other potentially hydrolyzable esters, thereby inflating the true value; hydrolyzed degraded esters would also be measured. Obviously, such results could possibly present difficulties

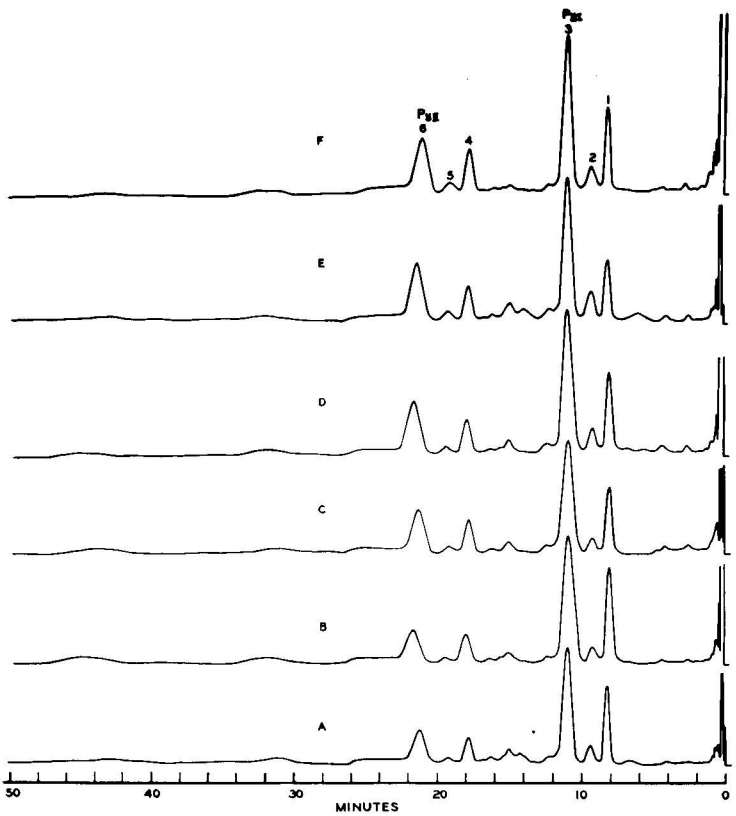


Fig. 1. GC curves of pyrethrin extracts. (A) Ecuador crude; (B) Ecuador refined; (C) Tanzania crude; (D) Tanzania refined; (E) Kenya refined; (F) World Standard, 1970. Peaks are (1) cinerin I; (2) jasmolin I; (3) pyrethrin I; (4) cinerin II; (5) jasmolin II; (6) pyrethrin II.

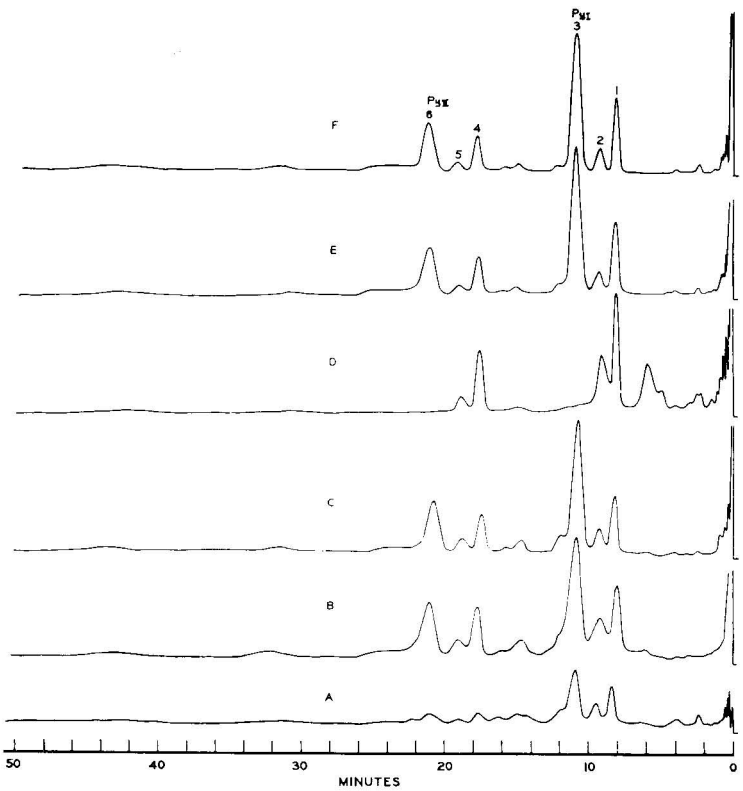
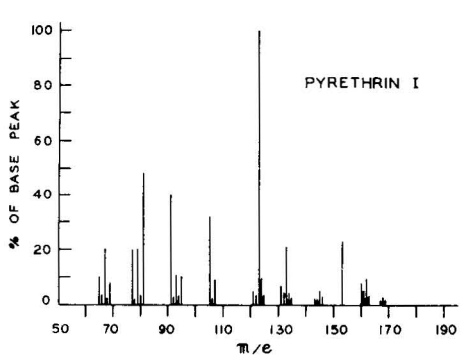
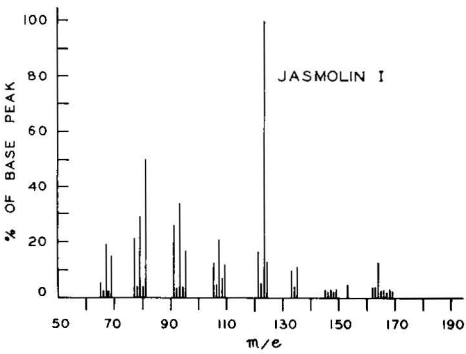


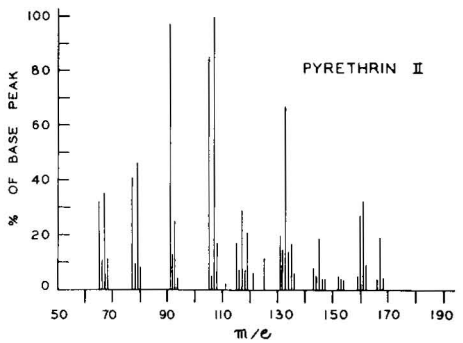
Fig. 2. GC curves of pyrethrin extracts. (A) India crude; (B) Fumakira, Japan, refined; (C) Nagaoka, Japan, refined; (D) Dainihon, Japan, refined; (E) World Standard, 1972; (F) World Standard, 1970. Peaks are (1) cinerin I; (2) jasmolin I; (3) pyrethrin I; (4) cinerin II; (5) jasmolin II; (6) pyrethrin II.

in any attempt to relate the insecticidally active ingredients of the pyrethrin extract to its true toxicity capabilities.

The results given in Figs. 1 and 2 and Table II, and verified by the mass spectral data shown in Figs. 3, 4 and 5[22,23] and Table III, illustrate that GC analyses of pyrethrin mixtures, under the conditions prescribed above, will provide the analyst and the insect toxicologist with information useful for a more precise evaluation of the insecticidal properties of a given pyrethrin extract. For example, the AOAC procedure for the Japanese samples (Table II) produced positive values for the pyrethrin I and 2 components. However, the GC procedure showed that one of the Japanese samples did not contain any measurable amounts of pyrethrin I and pyrethrin II (see Fig. 2D); subsequent investigation revealed that the sample was 10 years old, which (storage conditions unknown) suggested that complete degradation of the two components had occurred over this extended period of time. Again referring to Table II, the Py_I: Py_{II} ratios for the Equador and Indian samples were the same, yet a comparison of the gas chromatograms (Fig. 1A and Fig. 2A) indicated that on an equivalent weight basis the Ecuador extracts were superior in quality.







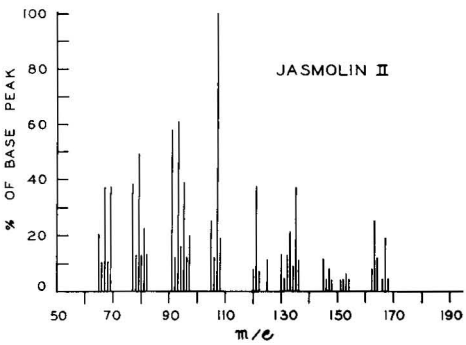
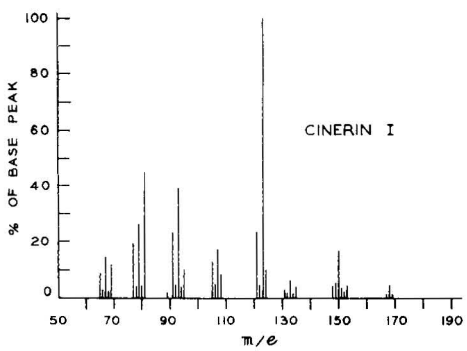


Fig. 3. Mass spectra of pyrethrins I and II.

Fig. 4. Mass spectra of jasmolins I and II.



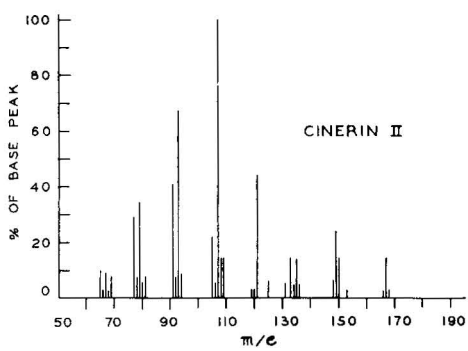


Fig. 5. Mass spectra of cinerins I and II.

Results obtained by the GC procedure are considerably more informative than those obtained by the AOAC procedure. For example, with the exception of the erratic results obtained in the Japanese samples, the African extracts contained approximately equal amounts of the Py_1 and Py_2 component mixtures and the Ecuador extracts contained about 1.5 times as much Py_1 components compared with their Py_2 content (Table II). However, when comparing the amounts of the two predominant insecticidally active components, Py_I and Py_{II}, obtained by GC, the amount of true Py_I was about 1.5 times the amount of Py_{II}. The exceptions were the Ecuador and Indian samples, which contained twice as much Py_I when compared tc their Py_{II} content.

The mass spectral data (Table III) indicate that the fragmentation patterns

TABLE III

INTENSITIES OF FRAGMENT IONS IN THE MASS SPECTRA OF THE PYRETHRIN I AND PYRETHRIN II ESTERS

Values are expressed as a percentage of the base peak.

m/e	*Py*$_I$	*J*$_I$	*C*$_I$	*Py*$_{II}$	*J*$_{II}$	*C*$_{II}$
168	3.0	3.3	2.8			
167				18.4	18.4	13.7
164		13.0			11.4	
163		3.5			24.6	
162	10.0	4.0		8.6	8.0	
161	3.5			32.0		
160	7.7			27.0		
153	2.3	3.5	2.2	3.4	6.0	1.7
150			17.0			13.7
149			3.9			23.0
148			3.2			6.0
147					8.0	
145	5.0	2.6		15.3	10.5	
143	2.0			8.6		
136				6.1	10.5	4.0
135	1.5	11.0			37.0	13.3
133	21.0	9.6	4.5	67.0	21.0	14.0
131			2.1			4.4
125	2.7			10.4	10.5	5.6
123	100.0	100.0	100.0			
121		16.5	22.6	6.1	37.7	44.0
117				29.0		
111				1.0		
108	4.8	7.8	7.3	17.0		13.7
107	8.6	20.8	16.7	100.0	100.0	100.0
105	31.5	13.0	12.2	85.0		21.0
94	2.3			3.0	15.0	8.0
93		33.5	38.4		61.0	67.0
91	40.0	26.0	23.0	97.0	58.0	40.7
81	48.0	50.0	42.7		22.0	
80				7.4	12.2	4.8
79		28.7	24.4		49.0	34.0
77		20.4	19.0		38.0	29.0
67	20.0	18.7	14.0	35.0	37.0	9.0
66	4.0			10.0		3.0

of the six pyrethrin esters are similar to those previously reported by King and Paisley[22] and Pattenden *et al.*[23]. The relative intensities of the major fragments are variable, especially for the pyrethrin II esters. No molecular ions were observed for any of the esters and no fragment ions were observed above *m/e* 168 for pyrethrin I esters and *m/e* 167 for pyrethrin II esters. The pyrethrin 1 esters (Py_I, J_I and C_I) had base peaks at *m/e* 123 and similar fragment ions at *m/e* 168, similar to previously published results[22,23]. The pyrethrin 2 esters (Py_{II}, J_{II} and C_{II}) had base peaks at *m/e* 107, in contrast to an *m/e* 133 peak for pyrethrin II, *m/e* 163 for jasmolin II, and *m/e* 149 for cinerin II reported earlier[22,23]. However, the fragment ions *m/e* 133, 163 and 149 had greater intensities for the pyrethrin II esters than for the pyrethrin I esters. All of the pyrethrin II esters revealed clusters of three ions separated by one mass unit; *m/e* 160, 161 and 162 for pyrethrin II; *m/e* 162, 163 and 164 for jasmolin II; and *m/e* 148, 149 and 150 for cinerin II. Similar results were reported by Pattenden *et al.*[23]. These clusters were also evident in mass spectra of the pyrethrin I esters but were of lower intensity for the respective clusters.

The differences in intensities of the fragmentations observed with the Finnigan peak identifier may be attributed to the higher temperatures applied to the pyrethrin 1 and 2 mixtures. The temperature of the gas chromatograph column was first set at 175° and maintained at this temperature until the three esters of the pyrethrin I group were eluted; then the temperature was raised to 205° to effect the elution of the three esters of the pyrethrin II group. The eluate from the GC column was then passed through a heated gas chromatograph–mass spectrometer interface set at 225° and finally through a heated transfer line at a temperature of 250°. The electron voltage was set at 69.5 V with no tangible differences in fragment ion intensity within a range from 30 to 100 V[24].

The acquisition of analytical data on pyrethrum as discussed in this paper should prove informative and helpful in the selection of a pyrethrin mixture for subsequent insecticidal use and it should aid the insect toxicologist in his efforts to interpret the toxic effects of pyrethrin mixtures on insect life.

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ION-EXCHANGE CHROMATOGRAPHIC SEPARATION AND FLUOROMETRIC DETECTION OF URINARY POLYAMINES

HANS VEENING*, W. WILSON PITT, Jr. and GUY JONES, Jr.

*Oak Ridge National Laboratory***, *Oak Ridge, Tenn. 37830 (U.S.A.)*

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SUMMARY

An ion-exchange chromatographic procedure has been developed for the separation and fluorometric detection of the urinary polyamines 1,3-diaminopropane, putrescine, spermidine, cadaverine, and spermine. The polyamines were separated on a 15×0.45 cm cation-exchange column (70°) employing a combined pH–salt gradient. The separation conditions were designed to enable utilization of the sensitive reagent fluorescamine as a means of monitoring these amines in physiologic fluids. This non-fluorescent reagent forms a fluorophor with most primary amines. Experiments were carried out to determine the effects of several variables such as pH, reagent concentration, and flow-rate. The method was successfully applied to both normal and pathologic samples of human urine.

INTRODUCTION

Various methods have been proposed for the early diagnosis of malignancy in humans. Those which have received increased attention during the last few years are based on the determination of polyamines in physiologic fluids. Recent experiments suggest that patients with active cancer may have elevated levels of these compounds in their urine[1–7].

The polyamines generally include the following compounds: 1,3-diaminopropane, $H_2N(CH_2)_3NH_2$; putrescine, $H_2N(CH_2)_4NH_2$; cadaverine, $H_2N(CH_2)_5NH_2$; spermidine, $H_2N(CH_2)_3NH(CH_2)_4NH_2$; and spermine, $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$. The separation and/or determination of polyamines in various biological samples has been accomplished by several techniques including fluorometric assay[8,9], enzymic assay[10], gas chromatography[11,12], ion-exchange chromatography[13–16], and thin-layer chromatography[17]. In the ion-exchange procedures, the ninhydrin detection system is generally used, although quantitative data for reactions of the polyamines with this reagent have not been reported.

The purposes of this investigation were to develop an ion-exchange technique for separating polyamines and a means of detecting these compounds fluorometrically

* N.I.H. Special Research Fellow, 1972–1973. Permanent address: Department of Chemistry, Bucknell University, Lewisburg, Pa. 17837, U.S.A.

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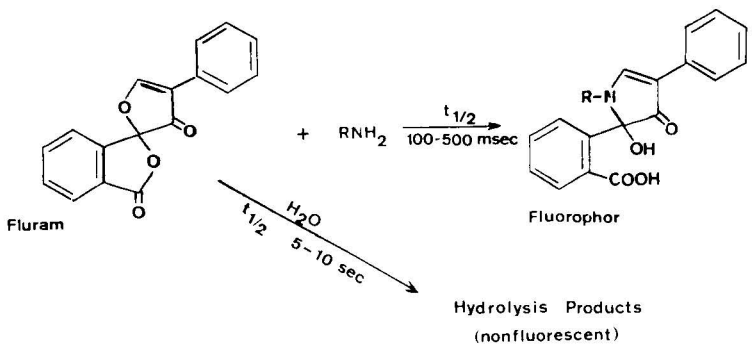
by utilizing the fluorophor that is formed when primary amines react with 4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′-dione (fluorescamine or Fluram™). Fluorescamine (which itself is non-fluorescent) has been reported to form a highly fluorescent product with primary amines[18], and has been demonstrated to be two orders of magnitude more sensitive for the detection of picomole quantities of amino acids than the standard ninhydrin procedure[19]. A manual fluorometric assay for nanogram quantities of proteins utilizing fluorescamine has also been reported[20]. This reagent reacts directly with primary amines in aqueous medium (pH 9–10) at room temperature with a half-life of a fraction of a second. Excess reagent is destroyed by water, with a half-life of several seconds, to form non-fluorescent hydrolysis products[18]. The reactions are shown below.



MATERIAL AND METHODS

Chemicals

Putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride, and spermine tetrahydrochloride were obtained from Sigma (St. Louis, Mo., U.S.A.). 1,3-Diaminopropane dihydrochloride was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Spectro-grade acetone was obtained from Eastman (Rochester, N.Y., U.S.A.). Fluorescamine (Hoffmann-La Roche) was purchased from Aminco (Silver Spring, Md., U.S.A.). Cross-linked sulfonated styrene and divinylbenzene copolymer Aminex A-5 cation-exchange resin were obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.).

Apparatus for manual procedures

Several experiments were carried out by manual procedures utilizing an Aminco-Bowman spectrophotofluorometer and a Sargent-Welch Model DR pH meter. A rotary evaporator was used to evaporate hydrolyzed urine samples to dryness.

Chromatographic system

Fig. 1 is a schematic flow diagram of the ion-exchange chromatograph which was constructed. Three minipumps (Milton-Roy) served to pump the column eluent, the buffering agent (0.1 *M* H_3BO_3) and the fluorescamine solution through the system. In the fluorescamine pump the rubber plunger seals were replaced by Rulon O-rings (Milton-Roy) in order to counteract the destructive effect of acetone. The

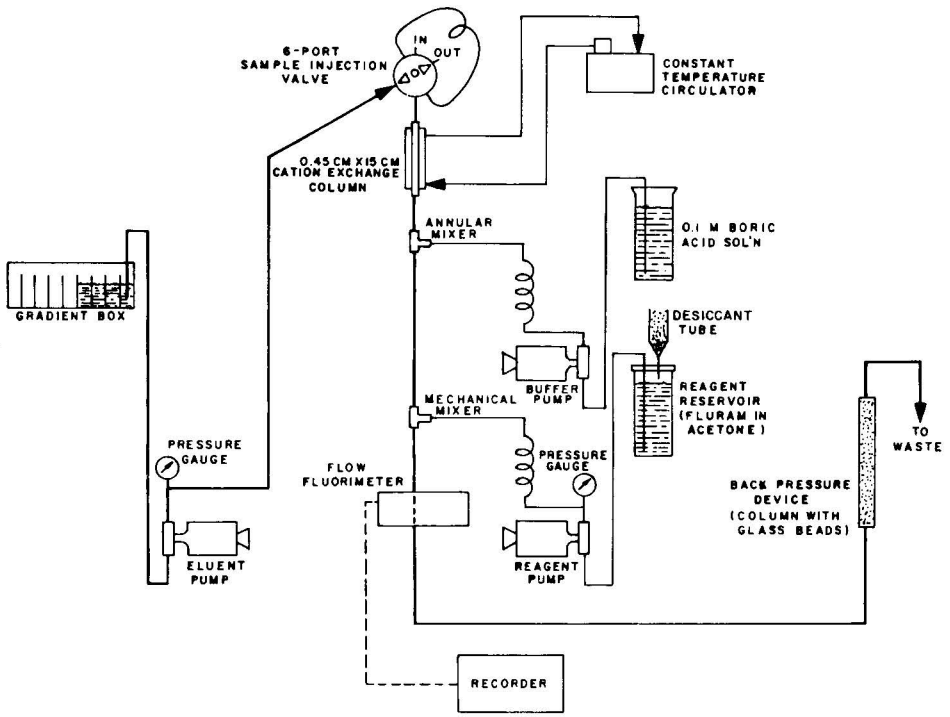


Fig. 1. Ion-exchange chromatograph for the separation and fluorescence detection of polyamines.

first four chambers of a nine-chamber gradient box supplied the eluent, which was pumped through a six-port sample injection valve supplied with a 0.450-ml sample loop. The design of this valve has been described previously[21]. A jacketed, nickel column, 15×0.45 cm I.D., was used in this investigation. Nickel was used to minimize the corrosive effects of sodium chloride solutions. The column was slurry-packed[22] with Aminex A-5 resin (13- to 20-μm particles), which was supported by a 1/4- to 1/16-in. reducer union containing a 0.5-μ stainless-steel frit. The column was operated at 70° utilizing a Haacke constant-temperature circulator.

The column effluent was first mixed with a stream of 0.1 *M* H_3BO_3 using an annular mixer. Details of the design and operation of this mixer have been reported previously[23]. The buffered stream was then fed to a T-mixer supplied with a small magnetic stirring bar, where the fluorescamine reagent stream was introduced.

A Laboratory Data Control fluoroMonitor was used to detect fluorescent components eluting from the column. The excitation lamp was a low-pressure, hot-cathode mercury lamp with a phosphor coating that emits near-UV energy peaking around 360 nm. Visible light was blocked by a Corning 760 primary filter; visible light emitted from the flow cell passed through a sharp cutoff UV blocking filter and impinged on the photosensitive elements of a dual photocell. The fluoro-Monitor was equipped with a 2-mm-I.D. quartz flow cell with a volume of *ca.* 20 μl. The signal from the detector was fed to a 10-mV Leeds & Northrup Speedomax pen recorder which operated at a chart speed of 2 in./h.

The chromatographic system was provided with a back pressure device at

a point beyond the detector in order to prevent the formation of bubbles when acetone was mixed with the aqueous stream at the T-mixer. This device consisted of a 45×0.45 cm stainless-steel column which was packed with Type 500–5005 finely divided glass beads obtained from the Reflective Products Division of the 3M Co. (St. Paul, Minn., U.S.A.). A back pressure of 40–50 p.s.i. was sufficient to prevent bubble formation.

Operation of the chromatograph

The polyamines were separated on Aminex A-5 cation-exchange resin by using a combined pH–salt gradient. Prior to each run, the column was equilibrated with 0.03 *M* phosphate buffer (pH 9.2). The gradient was prepared by placing the following solutions in each of the first four chambers of the nine-chamber Phoenix Varigrad gradient box: Chamber 1, 25 g of 0.05 *M* NaCl, pH 11.20; Chamber 2, 25 g of 0.10 *M* NaCl, pH 11.80; Chambers 3 and 4, 25 g per chamber of 0.20 *M* NaCl, pH 12.00. The pH of each solution was adjusted by using a pH meter and adding a sufficient quantity of dilute sodium hydroxide to each salt solution. The column was operated at a flow-rate of 36 ml/h and a column inlet pressure of 150 p.s.i.

In order to form the fluorescamine derivatives of the polyamines at the optimum pH for fluorescence measurement, it was found necessary to readjust the pH of the column effluent by mixing it with 0.1 *M* boric acid solution. The boric acid was pumped into the annular mixer at a flow-rate of 5 ml/h.

Fluorescamine reagent solution was prepared in the reagent reservoir by dissolving 150 mg of the reagent in 1 liter of anhydrous spectro-grade acetone. The reagent was pumped into the mechanical T-mixer at a flow-rate of 16 ml/h.

Standard solutions of the polyamines were prepared by weighing appropriate quantities of the amine salts, dissolving the salts in triply distilled water and diluting to volume. Samples were introduced into the column by means of the sample injection valve. Before the sample was loaded, the 0.450-ml sample loop was flushed with distilled water and emptied; then about 1.5 ml of sample was passed through the loop to ensure that the sample was not diluted by residual water.

Urine samples

A number of "normal" and pathologic urine samples were analyzed for polyamines; these samples were stored at −60° prior to sample preparation. It was found necessary to hydrolyze each urine with HCl prior to analysis in order to release the polyamines from the conjugates which they form[2–4] with other components in urine. This was accomplished by refluxing 10 ml of urine for 10 h with sufficient HCl to make the final mixture 6 *M* in HCl. The hydrolyzed urine was then evaporated to dryness in a rotary evaporator at a temperature of 60° and a pressure of 1 mm in order to remove the excess HCl and thus prevent its adverse retention effects on the resin. Finally, the dried residue was diluted to a known volume with triply distilled water and filtered. The hydrolyzed urine samples were stored at −25° until they were ready for chromatographic analysis.

RESULTS AND DISCUSSION

Fluorescence properties of fluorescamine derivatives of the polyamines

A number of experiments was performed to determine the optimum conditions

for chromatographic operation. Activation and fluorescence spectra were recorded at a pH of 10.5 for each of the fluorescamine derivatives of the polyamines. In all cases, the excitation and emission maxima were 395 nm and 475 nm, respectively.

It has been reported[18–20] that the relative fluorescence intensity of the primary amine derivatives of fluorescamine is strongly dependent on pH. This parameter was investigated for the polyamines. A series of solutions was prepared in which the concentration of polyamine was kept constant at 1 μg/ml and the total amount of fluorescamine added (as an acetone solution) was such that the final concentration was 100 μg/ml. The pH of each solution was adjusted by use of phosphate buffers. A sufficient number of solutions was prepared to provide data for the pH range 8.0 to 12.0. The fluorescence intensity of each sample was measured with an Aminco-Bowman spectrophotofluorometer using an excitation wavelength of 395 nm and an emission wavelength of 475 nm. The data, illustrated in Fig. 2, show that the fluorescence intensities for spermine and spermidine are considerably less than for the other three compounds. The pH for obtaining maximum fluorescence from putrescine, cadaverine, and 1,3-diaminopropane derivatives ranges from 9 to 10, whereas the optimum pH for spermine and spermidine is 10.5 in each case. Using these data, it was determined that the column effluent should be buffered to a pH of about 9 to 10 prior to fluorometric detection.

The effect of excess reagent on the fluorescence intensity of the polyamine fluorophors was also studied. A series of solutions was prepared in which the poly-

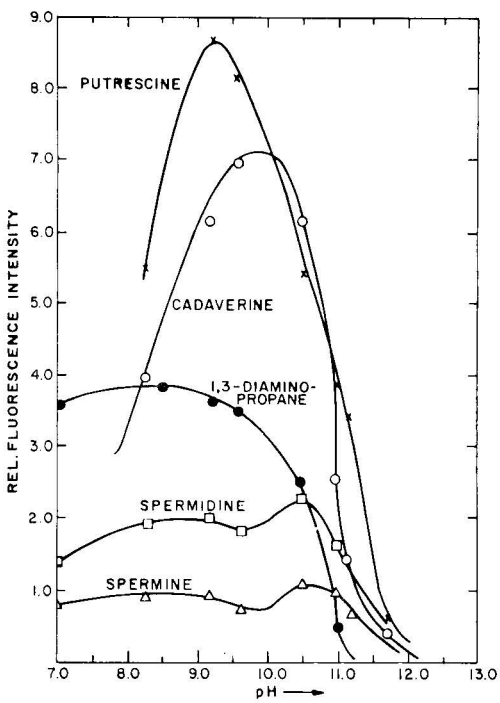


Fig. 2. Effect of pH on relative fluorescence intensity. Excitation wavelength, 395 nm; emission wavelength, 475 nm. Concentration of polyamines, 1 μg/ml (each); concentration of Fluram, 100 μg/ml.

amine concentration and the pH were kept constant at 1.0 μg/ml and 10.5, respectively, while the fluorescamine to polyamine weight ratio was varied. The results, shown in Fig. 3, showed that the curves of putrescine and cadaverine coincided. Maximum fluorescence intensities, in all cases, occurred at rather high fluorescamine to polyamine weight ratios (400 or 500:1). Several chromatographic runs were carried out using the fluorescamine reagent at a concentration of 400 μg/ml; however, the gain in sensitivity was not sufficient to justify the high reagent cost. Accordingly, the fluorescamine reagent concentration utilized in the chromatographic system was 150 μg/ml.

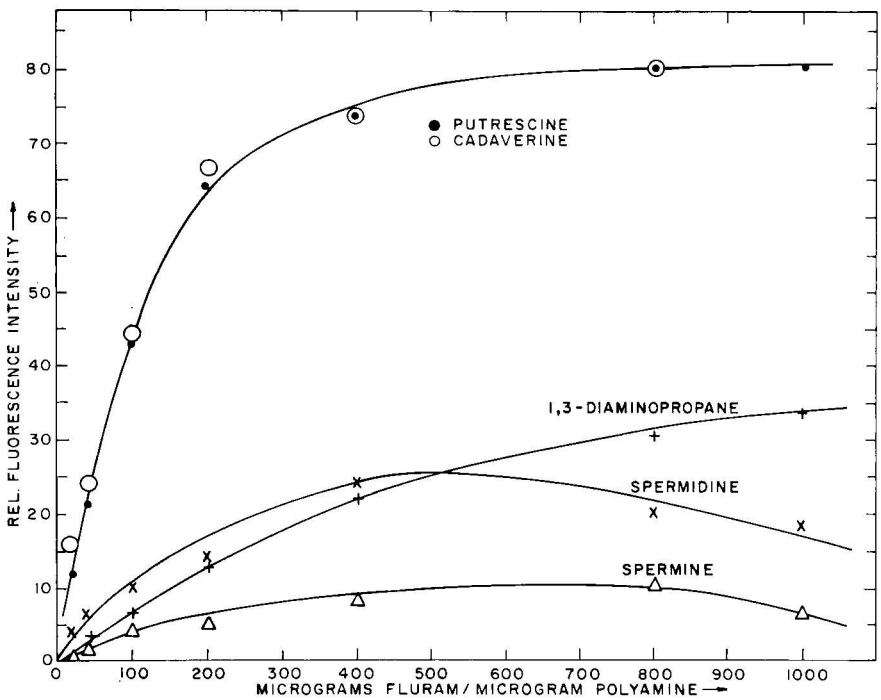
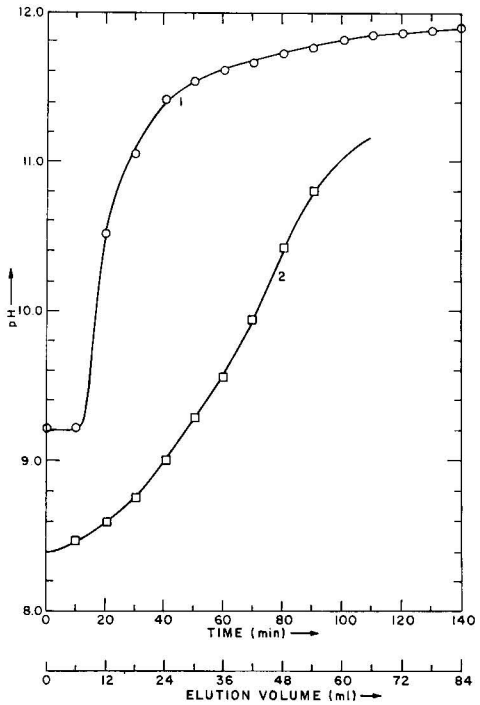


Fig. 3. Effect of fluorescamine concentration on the fluorescence intensity of several polyamines. Excitation wavelength, 395 nm; emission wavelength, 475 nm. pH = 10.5.

pH gradient and buffer stream

The pH gradient used to separate the polyamines was determined experimentally by monitoring aliquots of eluent at the column outlet. The resulting curve (curve 1) is shown in Fig. 4. Since the polyamines have retention volumes ranging from 30 to 57 ml, it is apparent from the data in Fig. 2 that the pH of the column effluent is above that necessary to achieve maximum fluorescence for the polyamine fluorophors. To lower the pH, and thus improve the sensitivity of the procedure, the column effluent was buffered with 0.1 *M* boric acid. At a boric acid flow-rate of 5.0 ml/h, the pH of the column effluent was lowered sufficiently that maximum fluorescence intensity for each component could be achieved. The pH of the buffered column effluent is shown in Fig. 4 (curve 2).



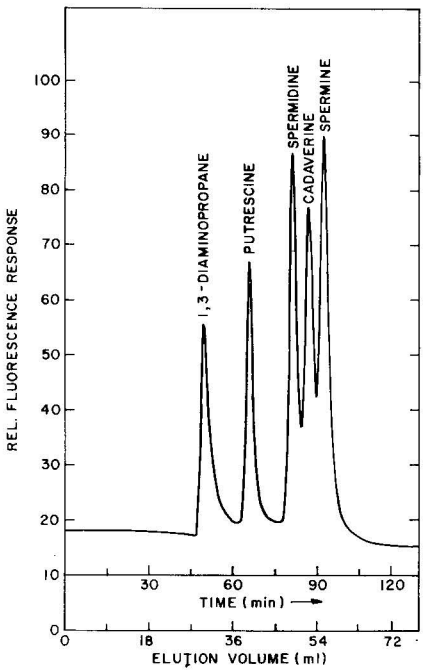


Fig. 4. pH gradient used for the separation of polyamines (curve 1) and pH of column effluent after buffering with 0.1 *M* boric acid (curve 2).

Fig. 5. Chromatogram of a standard mixture of 12.0 μg 1,3-diaminopropane, 3.6 μg putrescine, 15.0 μg spermidine, 7.0 μg cadaverine, and 35.0 μg spermine. (Fluorometer operated at lowest gain, × 64.)

Chromatographic separations and quantitative response

A typical separation of a standard mixture containing known amounts of the five polyamines is shown in Fig. 5. In this particular experiment, the boric acid stream was not employed. The resolution is satisfactory except for spermidine and cadaverine; however, quantitative measurements are possible for these two polyamines. The entire analysis can be completed in less than 2 h under the conditions described above. When the column was operated isocratically at a pH of 9.2 for 90 min after injection, and the gradient was started at this point, the elution of the polyamines was essentially unchanged, as shown for the four-component separation in Fig. 6. Such results indicate that the compounds are strongly retained on the resin at the lower pH until the gradient is begun. This procedure is useful because it automatically provides early elution of the less basic components in the front end of the chromatogram, prior to measuring the desired constituents.

The fluorescamine detection method is reasonably sensitive for 1,3-diaminopropane, cadaverine, and putrescine. The sensitivity for these compounds was greatly increased by the use of the boric acid stream to lower the pH of the column effluent, as shown in Fig. 7. The fluorescence response of each compound was measured with and without the boric acid flow. In all cases, peak heights were plotted.

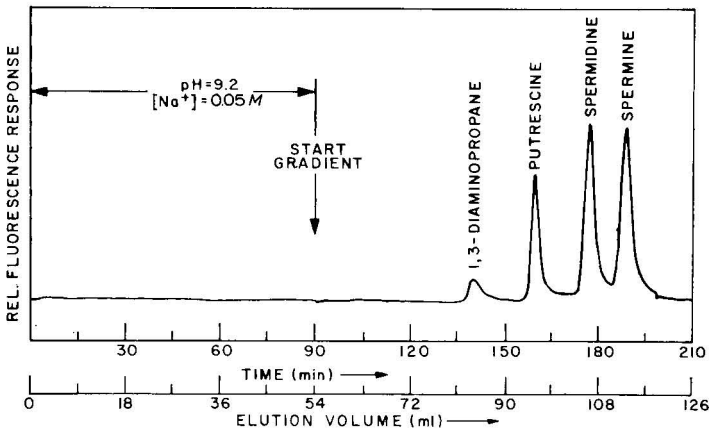


Fig. 6. Separation of a standard mixture of four polyamines using a delayed gradient.

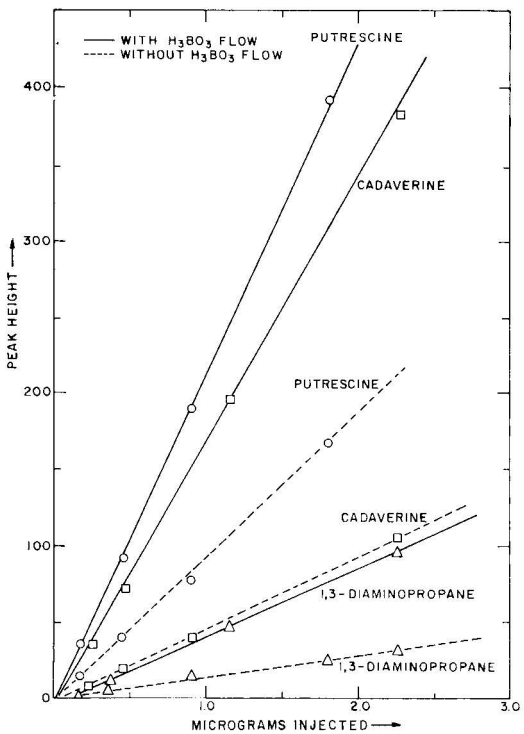


Fig. 7. Response plots for putrescine, cadaverine, and 1,3-diaminopropane with and without the effect of stream buffering with boric acid.

Decreasing the pH of the column effluent increases the response approximately twofold for each of the compounds. The minimum detectable quantity for each of these compounds was found to be 100 ng for 1,3-diaminopropane and cadaverine and 50 ng for putrescine. The sensitivity for spermidine and spermine is considerably less; the minimum detectable quantities were 1.0 and 3.0 μg, respectively. In the ninhydrin detection procedure, the limit of detection has been reported to be 0.5 μg for each polyamine[24].

Several urine samples were analyzed for polyamines. Typical examples of samples from a "normal" subject and from a cancer patient are shown in Fig. 8. Fig. 8a represents the chromatogram of a hydrolyzed, pooled urine sample obtained from eight normal males. Peaks 1, 3, 4, and 5 appear in the elution positions for 1,3-diaminopropane, putrescine, spermidine, and cadaverine, respectively. A smaller unknown peak (No. 2) also appears. The chromatogram in Fig. 8b represents a hydrolyzed urine sample from a cancer patient. Peaks 6 and 7 represent components located in the normal elution positions for putrescine and spermidine. Based on the previously established calibrations, the normal sample contains 0.7 μg/ml putrescine, 1.5 μg/ml spermidine, and a trace of cadaverine. The chromatogram in Fig. 8b shows the presence of more than 2.0 μg/ml putrescine (peak 6), and 4.0 μg/ml spermidine (peak 7). The major peak, eluting early in the chromatogram, contains the amino acids and other less basic components, leaving a reasonably clear region for the elution of the polyamines.

Slight changes in the elution position for standards were found after the

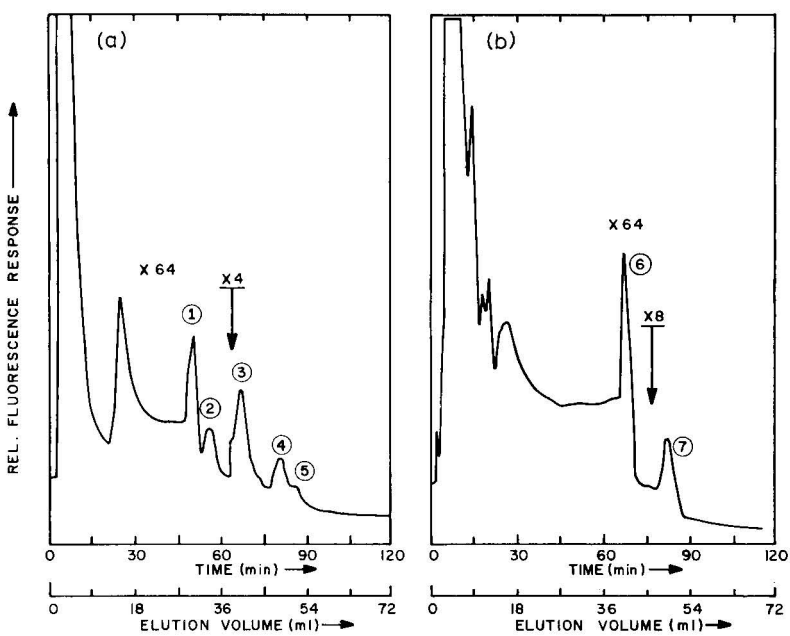


Fig. 8. (a) Chromatogram for 0.450 ml of a hydrolyzed, normal urine sample. The sample was diluted by a factor of 0.67. (b) Chromatogram for 0.450 ml of a hydrolyzed urine sample from a cancer patient. The sample was concentrated by a factor of 2.

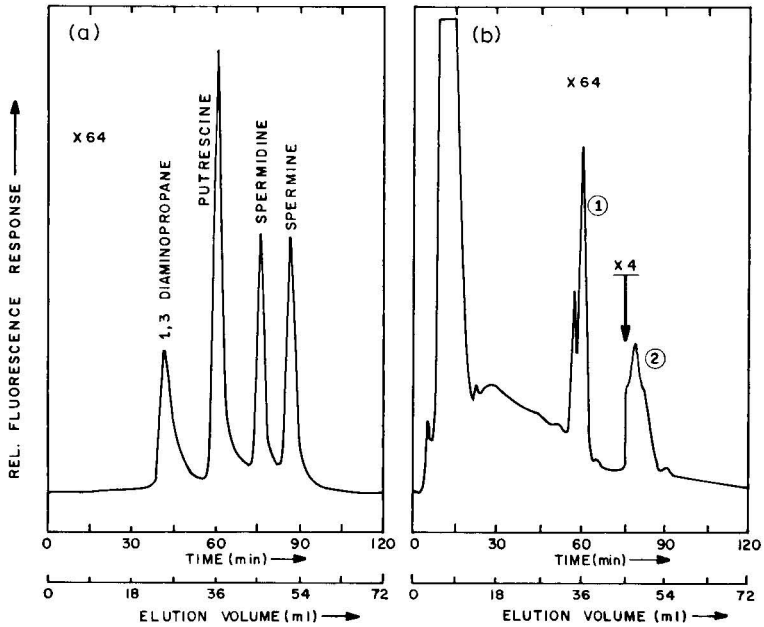


Fig. 9. (a) Chromatogram for a standard mixture of 1,3-diaminopropane, putrescine, spermidine, and spermine. (b) Chromatogram for 0.450 ml of a hydrolyzed urine sample from a cancer patient.

column had been repacked. This is illustrated in Figs. 5 and 9a. Fig. 9 compares the chromatogram for a standard mixture of 1,3-diaminopropane, putrescine, spermidine, and spermine (a) to that of a urine sample from a cancer patient (b). Putrescine (peak 1) and a smaller amount of cadaverine (peak 2) are present in somewhat elevated amounts.

CONCLUSION

The separation procedure described for the polyamines is rapid and gives satisfactory results. The sensitivity of fluorescamine toward 1,3-diaminopropane, putrescine, and cadaverine is acceptable for use in the analysis of physiologic fluids; however, the sensitivity for spermidine and spermine is not as good as had been anticipated from the earlier results obtained for amino acids[18,19]. It is interesting to note that the latter two compounds are the only ones with secondary amine groups (spermidine has one, spermine has two). Despite the presence of the two terminal primary amine groups in each of these molecules, the secondary amine groups apparently interfere with the fluorescamine reaction, or else they have a quenching effect on the activation of fluorescence in the fluorescamine derivatives of the polyamines.

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MIKROQUANTITATIVE BESTIMMUNG VON ALIPHATISCHEN AMINEN MIT 7-CHLOR-4-NITROBENZO-2-OXA-1,3-DIAZOL

H.-J. KLIMISCH und L. STADLER

Forschungsinstitut der Cigarettenindustrie e.V., D 2 Hamburg 54 (B.R.D.)*

(Eingegangen am 4. Oktober 1973)

SUMMARY

Microquantitative determination of aliphatic amines with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole

Fluorometric determination of microanalytical quantities of aliphatic amines with 7-chloro-4-nitrobenzo-2-oxa-1-3-diazole is described. Reaction conditions in a one-phase system, clean-up methods and column or thin-layer chromatographic separation procedures are investigated in order to know the potential of the reagent. For quantitative investigation, separation on polyamide sheets with subsequent *in situ* measurement by means of a Zeiss chromatogram spectrophotometer is particularly suitable. A linear calibration curve is obtained for quantities between 15 and 150 ng per spot. The high stability of the reaction products and the efficient reproducibility of the procedure justify recommendation of the reagent for the microanalytical determination of amines.

EINLEITUNG

Zur Bestimmung von Mikromengen primärer und sekundärer Amine hat das Dansylchlorid in letzter Zeit einige Bedeutung erlangt[1]. Umfassende Untersuchungen haben aber auch Probleme dieser Methode deutlich gemacht. Eigenfluoreszenz des Dansylchlorids sowie der durch Hydrolyse gebildeten Sulfonsäure machen sich störend bemerkbar. Ausserdem kann es beim Arbeiten mit Reagenzüberschuss zu Seitenreaktionen des Dansylchlorids kommen. Dabei täuschen Reaktionsprodukte wie z.B. Dansyl-dimethylamin und Dansyl-methylamin ein Vorkommen dieser Amine vor[1]. Hinzu kommt, dass Dansylchlorid nicht aminspezifisch ist, sondern auch mit aliphatischen und aromatischen Alkoholen fluoreszierende Derivate bildet. Das kürzlich eingeführte 7-Chlor-4-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl)[2] zeigte beim Nachweis von Sulfonamiden[3] und zentralstimulierenden Aminen[4] bemerkenswerte Vorteile. Es fluoresziert selbst nicht, seine Umsetzungsprodukte liessen sich gut chromatographieren und zeigten Vorteile in der NMR- und Massenspektrometrie[5]. Ausserdem ist das Reagenz spezifisch für den Nachweis primärer und sekundärer Amine als Dansylchlorid, da es mit Phenolen, Thiolen und Anilinen keine fluoreszierenden Produkte bildet[6]. Es erschien daher interessant, sein Reaktionsverhalten gegenüber Aminen sowie die Eigenschaften dieser Aminderivate näher zu untersuchen.

* Direktor: Prof. Dr. W. Dontenwill.

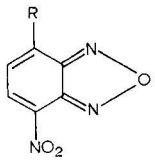
MATERIAL UND METHODEN

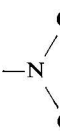
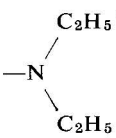
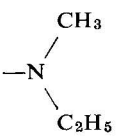
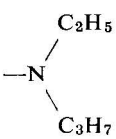
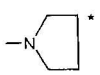
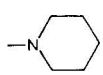
Reagenzien

NBD-Cl (Merck, Darmstadt, B.R.D.) wurde aus Methanol–Wasser umkristallisiert und anschliessend sublimiert. Es enthielt dann keine fluoreszierenden Verunreinigungen mehr. Schmelzpunkt: 96–96.5°.

TABELLE I

NBD-AMINE



R	*Schmelzpunkt* (°C)	% *Gehalt***					
		C		*H*		*N*	
		a	*b*	*a*	*b*	*a*	*b*
	217–218	46.15	46.18	3.85	3.81	26.90	26.26
 *	134–135	–	–	–	–	–	–
	171–172	48.60	48.80	4.55	4.58	25.20	24.99
	85–86	52.85	53.01	5.60	5.65	22.40	22.13
 *	211–212	–	–	–	–	–	–
	166–167	53.22	53.40	4.84	4.94	22.58	22.29

* Diese Derivate wurden uns dankenswerterweise von Dr. G. Eisenbrand, Deutsches Krebsforschungszentrum Heidelberg, zur Verfügung gestellt.

** a = Theoretisch; b = gefunden.

Alle Amine wurden frisch destilliert, ebenso alle Lösungsmittel, die ausserdem noch über Säulen (Al_2O_3, sauer, W-200; Woelm, Eschwege, B.R.D.) gereinigt wurden.

Synthese der NBD-Amine

Die in Tabelle I aufgeführten Derivate des NBD-Cl wurden analog einer von Reisch *et al.*[5] veröffentlichten Vorschrift synthetisiert. Sie wurden dünnschichtchromatographisch (DC), massenspektrometrisch und durch Elementaranalysen auf Reinheit und Identität geprüft.

Analytische Reaktionsbedingungen

25–500 μl einer methanolischen Aminlösung (1–20 μg Amin) wurden in einem 1 bis 3 ml-Messkolben mit dem vier- bis achtfachen Äquivalent einer 0.05% methanolischen NBD-Cl-Lösung und 50–100 μl einer 0.1 *M* wässrigen $NaHCO_3$-Lösung versetzt. Der Messkolben wurde sofort verschlossen und 1.5 Std. bei 55° im Wasserbad erwärmt. Nach dem Abkühlen wurde bis zur Marke aufgefüllt.

Reinigungsmethoden

(i) Die Reaktionslösung wurde am Rotationsverdampfer unter Wasserstrahlvakuum eingeengt, das Konzentrat in 10 ml Wasser aufgenommen und dreimal mit dem gleichen Volumen Essigsäureäthylester oder Methylenchlorid extrahiert. Nach dem Trocknen über Natriumsulfat kann die Lösung des NBD-Amins entweder konzentriert oder gleich in Küvetten UV- oder fluoreszenz-spektroskopisch vermessen werden.

(ii) Die auf die Hälfte eingeengte Reaktionslösung wurde auf eine Kieselgelsäule (1.25×60 cm, Kieselgel 60, d_p=10–40 μm; Merck) aufgetragen und mit Cyclohexan–Essigester (1:1) eluiert. Nach einem nicht fluoreszierenden Vorlauf (NBD-Cl) wurde die fluoreszierende Fraktion des NBD-Amins aufgefangen. Die Elution war beendet, wenn kein fluoreszierendes Eluat mehr von der Säule tropfte. Man kann die Säule auch vorher mit NBD-Cl und dem zu messenden NBD-Amin eintesten und dann die entsprechenden Fraktionen gewinnen.

UV-spektroskopische Bestimmung

Die Extinktion der gereinigten NBD-Amin-Lösung wurde bei der Wellenlänge maximaler Absorption (Tabelle II) mit einem Zeiss-Spektralphotometer PMQ II (Zeiss, Oberkochem, B.R.D.) in einer 1 cm-Küvette vermessen und die quantitativen Werte aus einer Eichkurve bestimmt.

Fluoreszenz-spektroskopische Bestimmung

Die Fluoreszenzintensität der gereinigten NBD-Amin-Lösung wurde am Zeiss-Fluorometer mit zwei Monochromatoren bei einer Anregungswellenlänge von 464 nm gemessen. Die Fluoreszenz Messwellenlängen sind in Tabelle II angegeben. Der Anregungsspalt war 1.4 mm, der Messspalt 0.3 mm.

Dünnschichtchromatographische Trennung

An Kieselgelplatten. 5 μl der ungereinigten Reaktionslösung wurden mit einem Desaga Auftragegerät (Desaga, Heidelberg, B.R.D.) auf Kieselgel 60, 20×20 cm

TABELLE II

MESSWELLENLÄNGEN DER NBD-AMINE IN ESSIGSÄUREÄTHYLESTER

Substanz	*UV-Maximum (nm)*	*Fluoreszenz-maximum (nm)**
NBD-Dimethylamin	464–466	522
NBD-Diäthylamin	475	527
NBD-Methyläthylamin	470	524–525
NBD-Äthylpropylamin	472	526–527
NBD-Pyrrolidin	476	523
NBD-Piperidin	473	531

* Anregung: 464 nm.

TABELLE III

R_F-WERTE DER NBD-AMINE AUF KIESELGELPLATTEN UND POLYAMIDFOLIEN

Kieselgel —Fliessmittel: I. Dimension, Cyclohexan–Essigsäureäthylester (1:1); II. Dimension, Petroläther–Chloroform–Äther–Eisessig (33:33:33:1). Polyamid-11 —Fliessmittel: Heptan–Essigsäureäthylester–*n*-Butanol (8:1:1).

NBD-Amin	*R_F-Werte*		
	Kieselgel		*Polyamid*
	I. Dimension	*II. Dimension*	
NBD-Dimethylamin	0.15	0.28	0.31
NBD-Methyläthylamin	0.23	0.38	0.44
NBD-Pyrrolidin	0.23	0.41	0.37
NBD-Diäthylamin	0.30	0.49	0.60
NBD-Piperidin	0.37	0.55	0.57
NBD-Äthylpropylamin	0.41	0.60	0.71

Platten (Merck) aufgetragen und in gesättigten N-Kammern entwickelt. Die Platte wurde zweimal mit Fliessmittel I in einer Dimension und zweimal mit Fliessmittel II unter 90° in dieser Dimension entwickelt. (Siehe Tabelle III.)

An Polyamid-11 Folien. Die Auftragung erfolgte wie oben beschrieben auf Polyamid-11 Folien (Macherey, Nagel & Co., Düren, B.R.D.). Die Folien wurden dreimal mit dem Fliessmittel Heptan–Essigsäureäthylester–*n*-Butanol (8:1:1) entwickelt. (Siehe Tabelle III.)

In situ fluorimetrische Messung auf Polyamid-11 Folien

Eine direkte *in situ* Vermessung der auf Polyamid-11 getrennten NBD-Amine wurde mit dem Chromatogramm-Spektralphotometer (Zeiss) in Fluoreszenzanordnung P-M vorgenommen. Eine Quecksilberdampflampe mit einem Zeiss M-436 Filter diente als Anregungsquelle. Bei einer Spaltbreite von 0.3 mm wurde der Emissionsmonochromator auf die jeweilige Wellenlänge maximaler Fluoreszenz eingestellt (NBD-Dimethylamin = 530 nm, NBD-Piperidin = 537 nm). Die quantitative Auswertung erfolgte über einen integrierenden Schreiber Vitatron UR 403 (Fig. 1).

Als Vergleichsstandard wurde das entsprechende NBD-Amin mitchromatographiert und vermessen. Eichkurven wurden mit verschiedenen Konzentrationen der chromatographierten NBD-Amine erstellt.

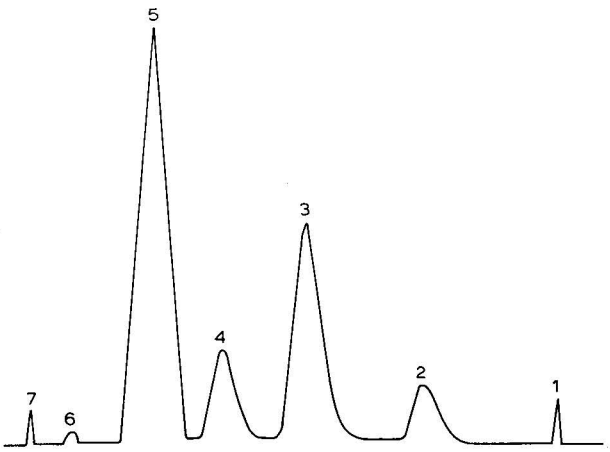


Fig. 1. Schreiberbild einer *in situ* analytischen Vermessung eines auf Polyamid-11 Folie getrennten derivatisierten Amingemisches. 1 = Peak des Auftragspunktes; 2 = NBD-Dimethylamin; 3 = NBD-Methyläthylamin; 4 = NBD-Piperidin; 5 = NBD-Äthylpropylamin; 6 = NBD-Cl; 7 = Peak der Fliessmittelfront.

ERGEBNISSE UND DISKUSSION

Ein ideales Reagenz zur Analyse von Aminen sollte nach einer Zusammenstellung von Seiler[1] folgende Bedingungen weitmöglichst erfüllen:

(1) Schnelle quantitative Reaktionen unter milden Bedingungen in Wasser oder Wasser enthaltenden Reaktionsmedien.

(2) Spezifische Reaktion mit primären oder sekundären Aminogruppen ohne Seitenreaktionen.

(3) Der Reagenzüberschuss soll sich einfach von den Reaktionsprodukten trennen lassen.

(4) Hohe Nachweisempfindlichkeit der Reaktionsprodukte.

(5) Günstige chromatographische Trenneigenschaften der Reaktionsprodukte.

Zwei weitere Eigenschaften erscheinen uns ausserdem wichtig:

(6) Günstige Stabilität der Reaktionsprodukte bei UV Bestrahlung in Lösung oder auf DC Platten.

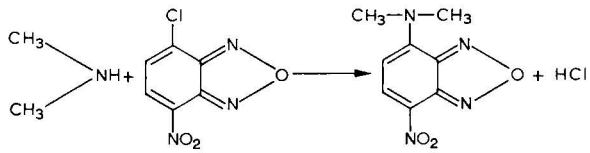
(7) Einheitliche Eigenschaften bei der Aufnahme von Massenspektren zur Identifizierung der Reaktionsprodukte.

Keines der bisher angewandten Aminreagenzien[1] erfüllt alle Bedingungen. Besonders eine Reaktion in wässrigen Medien, eine hohe Amin-Spezifität und Stabilität sind gleichzeitig bisher nicht zu erreichen gewesen.

Reaktionsbedingungen

Eingehende Untersuchungen mit NBD-Cl zeigten deutlich einige Vorteile dieses Reagenzes.

Da die Derivatisierung in rein wässrigen Medien nur sehr langsam verläuft, führten Lawrence und Frei[6] diese Reaktion in einem Zweiphasengemisch Wasser–Methylisobutylketon durch.



Wir konnten diese Methode gut reproduzieren. Allerdings zeigten DC Untersuchungen der Reaktionslösung mehr Nebenprodukte als sie bei der Verwendung von Methanol in einem einphasischen Lösungsmittelgemisch auftraten. Die niedrige Reaktionstemperatur von 55° sowie die leichte Verdampfbarkeit des Lösungsmittels erwiesen sich ebenso als vorteilhaft. Bei einer Konzentration von etwa 0.025% NBD-Cl in der methanolisch wässrigen Reaktionslösung war nach 1.5-stündigem Erwärmen auf 55° die Reaktion quantitativ verlaufen. (Siehe Tabelle IV.) Zur Verkürzung der Reaktionszeiten kann die NBD-Cl Konzentration erhöht werden. Bei der beschriebenen Mikrobestimmung der Amine bis zu einer Konzentration von 1 μg/ml erwies sich die geringe Reagenzkonzentration als günstig, wenn anschliessend ohne Reinigungsschritte eine direkte *in situ* Analyse der Derivate erfolgte.

TABELLE IV

DURCHSCHNITTLICHE AUSBEUTE AN NBD-R BEI VERSCHIEDENEN AMINKONZENTRATIONEN AUS FÜNF REAKTIONSANSÄTZEN

Amin-konzentration (μg/ml)	*Ausbeute* (%)	
	$R=N(CH_3)_2$	$R=$ 
20	95.5	96.2
15	94.7	96.3
10	97.1	94.4
5	94.8	92
1	95.8	86.8

Chromatographie und quantitative Bestimmung

Die quantitative Bestimmung der NBD-Amine kann colorimetrisch oder fluorimetrisch in Lösungsmitteln oder direkt als *in situ* Bestimmung auf DC-Platten durchgeführt werden.

Zur UV-spektroskopischen Bestimmung (Tabelle II) muss dazu der Reagenzüberschuss vollständig entfernt werden. Das geschieht am besten mit einer Kieselgelsäule. Da zwischen dem NBD-Cl und seinen Aminderivaten ein grosser Polaritätsunterschied besteht, ist die Trennung einfach. NBD-Cl kann im Vorlauf abgetrennt werden. Die Nachweisgrenze für das NBD-Dimethylamin bei der Verwendung von 1 cm-Küvetten liegt bei 0.3 μg/ml, entsprechend $1.5 \cdot 10^{-9}$ Mol Dimethylamin pro ml.

Bei einer fluorimetrischen Bestimmung lässt sich die Empfindlichkeit noch steigern. 10 ng/ml NBD-Dimethylamin lassen sich noch quantitativ auswerten. Da die Fluoreszenzanregung bei 464 nm erfolgt, ist die Verwendung von Quarzküvetten und UV-Anregungslicht nicht erforderlich (Tabelle II).

Es erwies sich auch hierbei als vorteilhaft, einen grossen Reagenzüberschuss vor der Vermessung zu entfernen, da sonst eine Verlagerung der Messwellenlängen eintrat. Da das NBD-Cl selbst nicht fluoresziert, genügt es, den grössten Teil des Reagenzüberschusses durch eine flüssig–flüssig-Verteilung in Wasser–Methylenchlorid oder Essigester zu entfernen.

Gemische von mehreren Aminderivaten müssen vor ihrer quantitativen Bestimmung aufgetrennt werden. Damit verbunden ist eine gleichzeitige Entfernung des Reagenzüberschusses. Die an einer Kieselgelplatte (Tabelle III) getrennten NBD-Amine lassen sich abkratzen und nach erfolgter Elution vermessen.

Für eine direkte fluorimetrische *in situ* Bestimmung mit einem Dünnschichtscanner haben sich Polyamid-11 Folien bewährt (Fig. 1). Im Vergleich zu Kieselgelplatten ist die Trennkraft des Systems grösser. Ausserdem ist die Fluoreszenzintensität gleicher Mengen auf den dünnen Polyamidfolien erheblich höher.

Für eine quantitative Bestimmung wurden bekannte Mengen der zu bestimmenden NBD-Amine als Standard mitchromatographiert. Jeder Fleck wurde fünfmal ausgemessen und die erhaltenen Integratorwerte gemittelt. Unter den angegebenen Messbedingungen erhielten wir zwischen 15 und 150 ng NBD-Dimethylamin pro Fleck eine lineare Eichkurve. Bei einer weniger empfindlichen Einstellung des Scanners lassen sich auch Werte bis 500 ng pro Fleck[6] vermessen.

Stabilität

Die an Polyamid-11 Folien chromatographierten NBD-Amine waren unter den Lagerungsbedingungen Zimmertemperatur (21°) und Luftfeuchtigkeit (72% rel.) stabil. Nach vier Wochen zeigten sie noch die gleiche Fluoreszenzintensität. Bei UV Bestrahlung der Flecken mit einer Quecksilberdampflampe mit Monochromatorfilter M 436 sank die Fluoreszenzintensität nach 10 min um 3%, nach 20 min um 10%. Damit tritt auf Kieselgelplatten[6] eine doppelt so schnelle Fluoreszenzabnahme als auf den Polyamidfolien ein.

Gemessen an den beschriebenen Anforderungen an ein ideales Aminreagenz werden viele der gestellten Bedingungen von NBD-Cl erfüllt. Besonders hervorzuheben sind dabei die grosse Stabilität des Reagenzes und der Umsetzungsprodukte gegenüber dem Einfluss von Wasser. Dies ermöglicht eine Umsetzung in wässrigen Reaktionsmedien bei sehr geringen Aminkonzentrationen unter milden Reaktionsbedingungen.

Die Spezifität des Reagenzes zur Bestimmung primärer und sekundärer aliphatischer Amine ist bei einer fluorimetrischen Auswertung gegeben, da Reagenz und Umsetzungsprodukte mit anderen funktionellen Gruppen nicht fluoreszieren.

Über die Vorteile in der Massenspektrometrie soll an anderer Stelle berichtet werden.

ZUSAMMENFASSUNG

Die fluorimetrische Bestimmung mikroanalytischer Mengen aliphatischer Amine mit 7-Chlor-4-nitrobenzo-2-oxa-1,3-diazol wird beschrieben. Reaktionsbe-

dingungen in einem Einphasensystem, Reinigungsmethoden und säulen- bzw. dünnschichtchromatographische Trennverfahren wurden untersucht, um die Anwendungsmöglichkeiten des Reagenzes kennenzulernen. Zur quantitativen Bestimmung eignet sich besonders eine Trennung an Polyamidfolien mit anschliessender *in situ* Vermessung mit einem Zeiss-Chromatogramm-Spektralphotometer. Zwischen 15 und 150 ng pro Fleck erhält man eine lineare Eichkurve. Die grosse Stabilität der Reaktionsprodukte und eine gute Reproduzierbarkeit des Verfahrens empfehlen das Reagenz zur mikroanalytischen Aminbestimmung.

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CHROM. 7197

COMPORTEMENT DES ÉTHERS

I. ÉTABLISSEMENT D'UNE ÉQUATION DE TYPE "TAFT" À PARTIR DE GRANDEURS DE RÉTENTION DES ÉTHERS SATURÉS

ROLAND FELLOUS, LOUISETTE LIZZANI-CUVELIER, ROBERT LUFT* et JEAN-PIERRE RABINE

Laboratoire de Chimie Organique, Institut Polytechnique Méditerranéen, Université de Nice, 28, Av. de Valrose, 06034-Nice Cédex (France)

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SUMMARY

Behaviour of ethers. I. Working out a "Taft"-like equation founded on retention data of saturated ethers

Variations in retention data of a series of saturated aliphatic ethers have been studied with a view to express them in a Taft-like equation. The linear relationships pointed out show the influence of the ramifications located on the atom neighbouring the oxygen. The numerical values of ρ^* are determined for six stationary phases. They express the selectivity of the stationary phases towards the ethers and they can be considered as an evaluation of the polarity of these substances.

INTRODUCTION

Depuis longtemps on a cherché à établir des relations linéaires entre les propriétés constitutives des substances et leur temps de rétention. Dans ce domaine, la littérature est trop abondante pour être citée intégralement; un examen détaillé de ces approches est dû à Lenfant *et al.*[1]. D'autres auteurs[2–6] tentent d'exprimer la variation de ces grandeurs de rétention par une somme d'interactions entre la substance injectée (soluté) et la phase stationnaire (solvant).

Dans ce qui suit, nous avons tenté d'évaluer l'influence des substituants sur les temps de rétention d'une série d'éthers aliphatiques. Pour accéder à la participation de l'environnement sur la rétention, nous avons pensé faire appel aux constantes d'effet polaire, σ^*, et stérique, E_s, de Taft qui caractérisent le groupe variable, R.

Une telle approche a été réalisée dans le cas des phénols substitués[7] ou des aziridines[8]. Ces auteurs suggèrent que les valeurs de ρ^* qu'ils ont déterminées pourraient traduire une mesure de la sélectivité des colonnes vis à vis de ces deux familles de composés.

* Auteur auquel les demandes de tirés à part doivent être envoyées.

PARTIE EXPÉRIMENTALE

Sauf pour ceux que l'on trouve dans le commerce, les éthers ROMe et ROEt utilisés dans notre étude ont été préparés par la méthode classique de Williamson. Nous avons réduit au minimum les risques de réaction d'élimination par le choix du dérivé halogéné. Aucune des substances utilisées dans nos essais ne dépasse un taux de 0.5% d'impuretés et leurs constantes physiques sont en bon accord avec les valeurs de la littérature.

Les mesures de grandeurs de rétention ont été réalisées à l'aide d'un appareil Varian-Aerograph, série 1200, équipé d'un détecteur à ionisation de flamme et couplé à un enregistreur Phillips à plusieurs vitesses de déroulement de papier.

La constance des débits d'azote et d'hydrogène est vérifiée régulièrement grace à un rotamètre Brooks.

Pour des chaînes alkyles allant de méthyle à octyle, nous avons obtenu le plus grand nombre de pics symétriques en respectant les conditions suivantes: température de l'injecteur, 200°; du four, 85°; du détecteur 230°; débit d'azote 20 ml/min et d'hydrogène 20 ml/min. Les colonnes sélectionnées ont une longueur de 10 pieds et sont remplies de support Chromosorb W (60–80 mesh) lavé à l'acide, contenant 10% de phase stationnaire.

Pour chaque produit nous avons effectué 5 à 8 déterminations des distances de rétention, par injection d'environ 0.02 μl. La répétabilité de la valeur log t'_R est comprise entre 0.003 et 0.006 unités logarithmiques, sauf pour le premier terme MeOEt, soit une erreur moyenne de 1 à 1.5%.

Le calcul de répétabilité a été effectué selon la méthode dite de "propagation des erreurs" adaptée à la chromatographie par Goedert et Guiochon[9].

Pour notre étude, nous avons sélectionné le temps de rétention réduit relatif $t'_R=(d_s-d_0)/(d_R-d_0)$ où d_0, d_R, d_s sont les distances de rétention du gaz vecteur, du corps de référence R et de la substance S à analyser. La détermination du temps de passage du gaz vecteur est faite à partir du méthane. En effet, la différence de déplacement du méthane et du gaz vecteur est si faible qu'elle peut être négligée[10].

De plus, pour effectuer les mesures dans des conditions prêtant le moins à des à coups, nous avons réalisé chaque série de mesures d'affilée, car on peut alors raisonnablement admettre que les facteurs expérimentaux n'évoluent ainsi que de façon très peu sensible.

L'étude entre les temps de rétention et les facteurs structuraux est basée sur les méthodes de régression effectué en fonction du nombre de paramètres, soit à l'aide d'une machine Programma Olivetti P 101, soit IBM 1130.

RÉSULTATS

Nous avons sélectionné[11] les deux séries d'éthers MeOR et EtOR et avons déterminé les temps de rétention relatifs réduits sur six phases stationnaires recouvrant une gamme de polarités[2] assez large.

Les résultats concernant ces méthoxyalcanes et éthoxyalcanes sont rassemblés dans les Tableaux I et II.

Nous avons observé dans tous les cas une relation linéaire du type

$$\log t'_{R(\mathrm{ROEt})} = a \log t'_{R(\mathrm{ROMe})} + b \qquad (1)$$

TABLEAU I

TEMPS DE RÉTENTION RELATIFS DES ÉTHOXYALCANES

No.	*ROEt*	*Log t'_R*					
		Apiezon L	*SE-30*	*Ucon-Polar*	*Carbowax 20M*	*XF-1150*	*DEGS*
1	Me	0.000	0.000	0.000	0.000	0.000	0.000
2	Et	0.205	0.166	0.109	0.106	0.062	0.006
3	*n*-Pr	0.495	0.445	0.403	0.366	0.260	0.200
4	*i*-Pr	0.378	0.358	0.281	0.243	0.153	0.033
5	*n*-Bu	0.836	0.773	0.732	0.636	0.517	0.442
6	*i*-Bu	0.684	0.631	0.550	0.447	0.340	0.198
7	*s*-Bu	0.690	0.635	0.566	0.470	0.370	0.236
8	*t*-Bu	0.564	0.536	0.450	0.362	0.167	0.115
9	*n*-Pent	1.173	1.086	1.041	0.903	0.786	0.681
10	*i*-Pent	1.043	0.976	0.912	0.780	0.677	0.661
11	*s*-Pent	0.996	0.941	0.847	0.716	0.605	0.451
12	*s,i*-Pent	0.915	0.878	0.747	0.618	0.521	0.329
13	Me-2 Bu	1.034	0.976	0.877	0.760	0.621	0.475
14	*t*-Pent	0.949	0.884	0.785	0.660	0.546	0.397
15	*néo*-Pent	0.775	0.731	0.568	0.431	0.304	0.078
16	*n*-Hex	1.501	1.399	1.332	1.169	1.001	0.915
17	*n*-Hept	1.835	1.679	1.607	1.427	1.241	1.140
18	*n*-Oct	2.161	1.955	1.903	1.700	1.529	1.364

TABLEAU II

TEMPS DE RÉTENTION RELATIFS DES MÉTHOXYALCANES

No.	*ROMe*	*Log t'_R*					
		Apiezon L	*SE-30*	*Ucon-Polar*	*Carbowax 20M*	*XF-1150*	*DEGS*
2	Et	0.000	0.000	0.000	0.000	0.000	0.000
3	*n*-Pr	0.301	0.291	0.213	0.261	0.190	0.121
4	*i*-Pr	0.213	0.130	0.141	0.138	0.107	−0.024
5	*n*-Bu	0.656	0.541	0.563	0.544	0.457	0.348
6	*i*-Bu	0.492	0.417	0.387	0.332	0.276	0.124
7	*s*-Bu	0.533	0.445	0.445	0.398	0.353	0.206
8	*t*-Bu	0.472	0.350	0.377	0.385	0.240	0.168
9	*n*-Pent	0.998	0.872	0.887	0.813	0.702	0.606
10	*i*-Pent	0.871	0.768	0.758	0.720	0.583	0.470
11	*s*-Pent	0.854	0.754	0.734	0.665	0.565	0.445
12	*s,i*-Pent	0.782	0.695	0.647	0.577	0.474	0.317
13	Me-2 Bu	0.860	0.763	0.739	0.674	0.546	0.425
14	*t*-Pent	0.842	0.742	0.729	0.663	0.574	0.463
15	*néo*-Pent	0.590	0.531	0.445	0.352	0.248	0.046
16	*n*-Hex	1.306	1.186	1.178	1.084	0.932	0.837
17	*n*-Hept	1.631	1.493	1.472	1.345	1.180	1.054
18	*n*-Oct	1.954	1.782	1.770	1.601	1.440	1.293

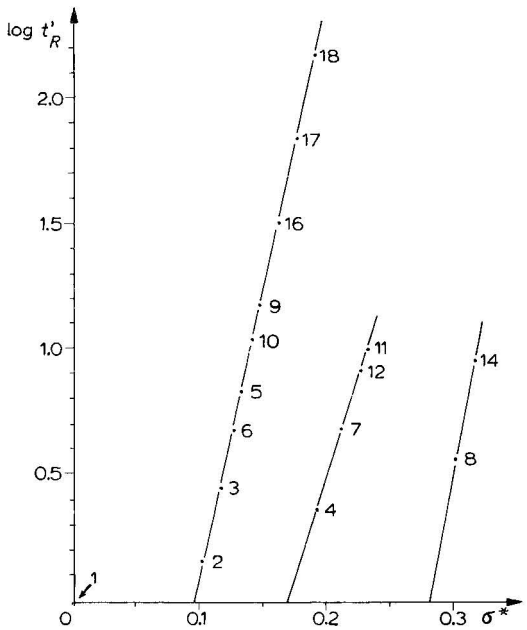

Fig. 1. Vérification de la relation de Taft dans le cas des éthoxyalcanes. Les nombres correspondent aux numéros donnés dans les Tableaux I et II.

caractérisée par des paramètres liés à la nature de la phase stationnaire et rassemblés dans le Tableau III.

TABLEAU III

VARIATION DES COEFFICIENTS DE L'ÉQN. 1 AVEC LA NATURE DE LA PHASE STATIONNAIRE

r = Coefficient de corrélation; ψ = test d'Exner[12]; p = nombre de substances utilisées pour la corrélation.

Phases stationnaires	*a*	*b*	*r*	ψ	*p*
Apiezon L	1.016	0.157	0.998	0.07	17
SE-30	0.998	0.194	0.999	0.06	17
Ucon-Polar	1.003	0.129	0.997	0.08	17
Carbowax 20M	0.999	0.073	0.996	0.10	17
XF-1150	1.034	0.031	0.995	0.11	17
DEGS	1.043	0.031	0.989	0.16	17

Les résultats du Tableau III indiquent clairement l'analogie de comportement des deux séries vis à vis d'une même phase stationnaire. La pente a des différentes droites obtenues est dans tous les cas sensiblement égale à 1. Pratiquement, cela implique que le groupe variable R intervient de façon analogue dans les deux séries. De plus, en première approximation, l'ordonnée à l'origine b caractérise l'incrément de passage d'une série à l'autre pour une phase stationnaire donnée. Pour l'obtention

de la meilleure séparation possible des homologues, il faut choisir la phase stationnaire pour laquelle le terme b est le plus élevé.

INTERPRÉTATION DES RÉSULTATS

La relation 1 que nous venons d'examiner traduit des "corrélations homogènes"[13] qui interviennent lors de la comparaison du comportement de deux populations face à un même phénomène physico-chimique (en l'occurence phase stationnaire identique).

Parallèlement à ces relations, il est possible de définir des "corrélations mixtes", établies à partir de deux séries de valeurs expérimentales concernant une même famille (p.ex. ROEt) et relatives à deux comportements distincts (phases stationnaires différentes).

L'étude de telles corrélations nous permettra d'apprécier le degré de polarité des phases. Enfin, nous rendrons compte des relations paramétriques entre un comportement et un "paramètre d'action"[13]. Un tel paramètre peut correspondre par exemple aux facteurs structuraux σ^* et E_s de Taft ou être la résultante de plusieurs paramètres liés à la juxtaposition de différents phénomènes d'interaction.

Bien entendu, les interprétations établies pour la série ROEt demeurent valables pour la série ROMe.

Relations mixtes

La variation des temps de rétention des éthoxyalcanes vis à vis des différentes phases stationnaires peut permettre d'apprécier le degré de polarité des phases stationnaires.

Chovin et Lebbe[14] ont proposé un système de classification à partir de phases stationnaires de référence; Bonastre et Grenier[15], faisant intervenir la volatilité α de deux alcanes homologues, montrent par contre qu'il n'est pas nécessaire de retenir une référence pour effectuer un tel classement.

Les travaux de Casteignau[16] sur les oxétanes font apparaître que les méthodes des deux groupes de chercheurs conduisent à un même classement.

Pour notre part, nous avons essayé d'établir une échelle de polarité à l'aide de la relation (2)

$$\log t'_{R(\phi_1)} = a \log t'_{R(\phi_2)} \tag{2}$$

où ϕ_1 et ϕ_2 représentent deux phases stationnaires quelconques.

Nous obtenons un ensemble de droites dont la pente a peut donc exprimer la polarité relative de ϕ_1. La classification ainsi obtenue est parallèle à celles établies par Chovin et Lebbe et Bonastre et Grenier.

Les résultats sont rassemblés dans le Tableau IV dans le cas où la phase de référence ϕ_2 est Apiezon L.

La valeur a de la pente ainsi déterminée est inversement proportionnelle à la polarité des colonnes.

Relations paramétriques

En opérant dans des conditions isothermes à l'intérieur d'une série de sub-

TABLEAU IV

CLASSIFICATION DES PHASES STATIONNAIRES SELON LEUR POLARITÉ RELATIVE

Phases stationnaires	*a*	*r*	*ψ*	*p*
Apiezon L	1.000	–	–	–
SE-30	0.915	0.999	0.04	18
Ucon-Polar	0.907	0.998	0.06	18
Carbowax 20M	0.809	0.995	0.10	18
XF-1150	0.745	0.988	0.16	18
DEGS	0.706	0.964	0.29	18

stances et compte-tenu des approximations classiques, l'établissement de relations linéaires extrathermodynamiques de type Hammett[7] ou Taft devient licite.

Dans le cas où le paramètre d'action est constitué par les effets polaires seuls, les graphes obtenus pour les différentes phases stationnaires se caractérisent par quatre séries de points qui se différencient entre-elles par le nombre de substituants portés par l'atome voisin de l'oxygène. Cette distribution selon quatre ensembles distincts (Fig. 1) suggère une forte influence du nombre d'atomes d'hydrogène de l'atome en α de l'oxygène sur la variation des grandeurs de rétention, à côté de l'effet polaire des substituants. Une telle participation a été envisagée par Kreevoy et Taft[17] dans l'hydrolyse acide des acétals.

Aussi avons-nous tenté d'établir une relation de la forme

$$\log t'_R = \rho^* \sigma^* + h(n-3) \tag{3}$$

tenant compte de ce phénomène où σ^* (lit. 18) représente le facteur d'effet polaire des substituants et n le nombre d'atomes d'hydrogène de l'atome en α de l'oxygène.

Ce type de relation permet d'évaluer d'une manière globale l'influence des substituants sur les grandeurs de rétention.

La relation 3 établie à partir des mesures effectuées sur six phases stationnaires conduit dans tous les cas à un plan contenant l'ensemble des structures étudiées (Tableau V).

TABLEAU V

VARIATION DES COEFFICIENTS DE L'ÉQN. 3 AVEC LA NATURE DE LA PHASE STATIONNAIRE

Phases stationnaires	ρ^*	*h*	*r*	*ψ*	*p*
Apiezon L	−21.168	1.909	0.995	0.11	16
SE-30	−19.396	1.740	0.995	0.11	16
Ucon-Polar	−19.135	1.744	0.994	0.12	16
Carbowax 20M	−17.000	1.564	0.992	0.14	16
XF-1150	−15.578	1.459	0.989	0.16	16
DEGS	−14.682	1.410	0.985	0.19	16

Les résultats sont satisfaisants, compte-tenu de la valeur du test ψ et de celle du coefficient de corrélation r. De plus, on observe que ces résultats sont meilleurs pour les phases apolaires que pour les phases polaires.

La constante ρ^* caractérise dans ce cas la sensibilité de la phase stationnaire à l'effet polaire.

Si en outre on fait intervenir l'effet stérique, la relation 3 devient

$$\log t'_R = \rho^*\sigma^* + \delta E_s + h(n-3) \tag{4}$$

On n'observe qu'une légère modification des valeurs des paramètres ρ^* et h. Les résultats obtenus sont groupés dans le Tableau VI.

TABLEAU VI

VARIATION DES COEFFICIENTS DE L'ÉQN. 4 AVEC LA NATURE DE LA PHASE STATIONNAIRE

Phases stationnaires	ρ^*	δ	h	r	ψ	p
Apiezon L	−21.238	0.148	1.829	0.996	0.11	11
SE-30	−19.333	0.120	1.665	0.995	0.12	11
Ucon-Polar	−19.209	0.130	1.673	0.996	0.12	11
Carbowax 20M	−17.100	0.166	1.476	0.995	0.12	11
XF-1150	−15.799	0.187	1.392	0.992	0.16	11
DEGS	−14.807	0.208	1.311	0.993	0.15	11

Pour une phase stationnaire donnée, la comparaison des résultats des Tableaux V et VI montre que la prise en considération de l'effet stérique entraîne une légère amélioration de la qualité des corrélations, surtout pour les phases polaires telles que XF-1150 et DEGS.

Une relation à trois paramètres telle que 4 permet d'évaluer le rôle de l'effet stérique sur le temps de rétention (i) en le corrigeant de l'influence de la substitution sur l'atome en α de l'oxygène et (ii) en le considérant comme la résultante $E_{s\,\mathrm{CPV}} = \delta E_s + h(n-3)$.

Les pentes ρ^* obtenues par les relations 3 et/ou 4 pour les six phases stationnaires sélectionnées varient dans le même sens que la polarité relative a précédemment définie par la relation 2. La phase stationnaire Apiezon L se révèle être la plus sensible aux effets polaires.

Si nous portons ρ^* en fonction de a, nous obtenons une relation linéaire du type

$$\rho^*_{\phi_i} = \lambda a + \beta \tag{5}$$

Selon que l'on utilise les valeurs ρ^* du Tableau V ou du Tableau VI les valeurs de λ obtenues à l'aide de la relation 5 divergent légèrement (Tableau VII).

L'existence d'une relation linéaire telle que 5 montre qu'il devient possible, en utilisant les valeurs de $\rho^*\phi_i$, d'aborder une classification de la polarité des phases stationnaires vis à vis du soluté utilisé.

TABLEAU VII

COEFFICIENTS DE L'ÉQN. 5

ρ^* *d'après*	λ	$\Delta\lambda$ *moyen*	β	p	r	ψ
Tableau V	−22.10	0.18	0.90	6	0.999	0.02
Tableau VI	−21.58	0.24	0.37	6	0.999	0.03

Dans un mémoire ultérieur nous montrerons que cette approche de la polarité des colonnes n'est pas limitée aux éther-oxydes, mais qu'elle présente un caractère général. Elle est applicable quelle que soit la fonction chimique de la population examinée.

RÉSUMÉ

L'étude de la variation des grandeurs de rétention d'une série d'éthers aliphatiques saturés est abordée sous l'angle de l'équation de Taft. Les relations linéaires mises en évidence font intervenir les ramifications dont l'atome en α de l'oxygène est le siège. Les valeurs de ρ^*, déterminées pour six phases stationnaires, sont assimilées à une mesure de la sélectivité vis à vis des éthers et peuvent être considérées comme caractéristiques de la polarité de ces substances.

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CHROM. 7167

GAS CHROMATOGRAPHIC ANALYSIS OF AMINES SEPARATED AS URETHANE DERIVATIVES

TORBJÖRN GEJVALL

Radiobiology Department, Wallenberg Laboratory, Stockholm University, Lilla Frescati, S-10405 Stockholm 50 (Sweden)

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SUMMARY

A simple method for the separation of low molecular weight amines as urethanes is described. Diethyl pyrocarbonate is used under restricted pH conditions in the reaction. Analysis of the derivatives was carried out on four columns using gas chromatography. The times of elution and substance-specific correction factors were determined.

INTRODUCTION

Diethyl pyrocarbonate is used in various biochemical and microbiological applications and has so been used for many years in our laboratory. The reaction with amines is well known and produces a carbethoxy derivative (a urethane):

$$R\text{–}NH_2 + C_2H_5O\text{–}CO\text{–}O\text{–}CO\text{–}OC_2H_5 \rightarrow R\text{–}NH\text{–}CO\text{–}OC_2H_5 + CO_2 + C_2H_5OH$$

This type of derivative has been made and analysed by Boehm and Mehta[1] and Pauli and Genth[2], but gas chromatography has been used for only a few amino products[3,4]. Special interest has been shown in herbicides. Spengler and Hamroll[5] investigated urethanes with a ring structure.

In this work low-molecular-weight amines are detected as urethanes.

MATERIALS

Compounds were obtained as follows: diethyl pyrocarbonate from Schuchardt, München, G.F.R.; ammonium chloride, propylamine, methylamine hydrochloride, 2-methyl-propylamine, 3-methyl-butylamine hydrochloride, aniline and methylaniline from E. Merck, Darmstadt, G.F.R.; ethylamine hydrochloride from Hopkin & Williams, Chadwick Heath, Great Britain; and butylamine from Eastman-Kodak, Rochester, N.Y., U.S.A. The pentylamine and 2-methylbutylamine were prepared by decarboxylation of norleucine and isoleucine[6]. Distillation into HCl was used to purify the amines for the gas chromatographic studies.

EXPERIMENTAL

Preparation of samples

The analysis was made on 0.05–0.7 mg amine in 5 ml aqueous solution treated with 10 mg diethyl pyrocarbonate for 30–40 minutes at room temperature. The pH was adjusted with NaOH to 9.5.

The excess of diethyl pyrocarbonate and the duration of treatment were enough for the complete carbethoxylation of all available amino groups (*cf.* the reaction kinetics of diethyl pyrocarbonate[7,8]). Mixtures of stock solutions were used to determine the times of elution and specific correction factors. The substance-specific correction factors, f_i, were calculated according to Kaiser[9] by

$$f_i = \frac{\text{wt. per cent}_i \times \text{area standard}}{\text{wt. per cent standard} \times \text{area}_i}$$

where wt. per cent$_i$ = wt. per cent of the substance i in the calibration mixture and area$_i$ = peak area for i.

The butylamine derivative was used as the standard.

Analysis

A Perkin-Elmer F11 gas chromatograph having a flame ionisation detector (FID) and all-glass column system was used in the experiments. The conditions for analysis are given in Table I. Injections were made with a 5-μl Scientific Glass (London, Great Britain) syringe. A 10-ml vial with a membrane was used for the reaction of each mixture. Before analysis the pressure was lowered with a needle through the membrane of the vial. *n*-Decane was added for comparison of elution times. Quantitative measurements were made on a Carbowax column. Aniline derivatives had to be separated on the other columns. Apiezon N and SE-30 columns were used in this part of the work. Peak areas were measured with a planimeter.

TABLE I

GAS CHROMATOGRAPHIC CONDITIONS

	Column			
	SE-30	*Apiezon N*	*Carbowax 1540*	*XF-1125*
Stationary phase	20% SE-30 + 1% KOH	10% Apiezon N	8% Carbowax 1540	1% XF-112
Support	Gas Chrom. Q (100–120 mesh)	Chromosorb G (60–80 mesh)	Chromosorb W (80–100 mesh)	Chromosor (60–80 m
Column dimensions	1.10 m × 2.0 mm I.D.	1.20 m × 2.0 mm I.D.	1.5 m × 3.0 mm I.D.	2.2 m × 2.0 I.D.
Gas (N_2) flow-rate	37 ml/min	60 ml/min	75 ml/min	20 ml/min
Sensitivity	× 100	× 100	× 100	× 100
Amount injected	1 μl	1 μl	2 μl	1 μl
Recorder speed	40 mm/min	40 mm/min	40 mm/min	40 mm/min

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of some aliphatic amines separated in the form of carbethoxy derivatives on an 8% Carbowax 1540 column. The derivative from ammonia (urethane) is eluted after butylamine. Under the conditions used on this polar column the aniline derivative could not be found.

A high percentage (20%) of SE-30 stationary phase resulted in a suitable separation of the urethanes (Fig. 2). The performance of this column was even better than that of the Apiezon N and XF-1125 columns used in the experiments.

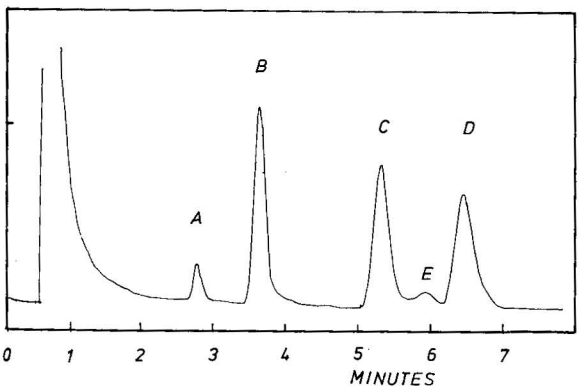

Fig. 1. Gas chromatographic separation of the urethane derivatives of methylamine (A), propylamine (B), butylamine (C), ammonia (E) and 3-methylbutylamine (D). An 8% Carbowax 1540 column was used for the analysis.

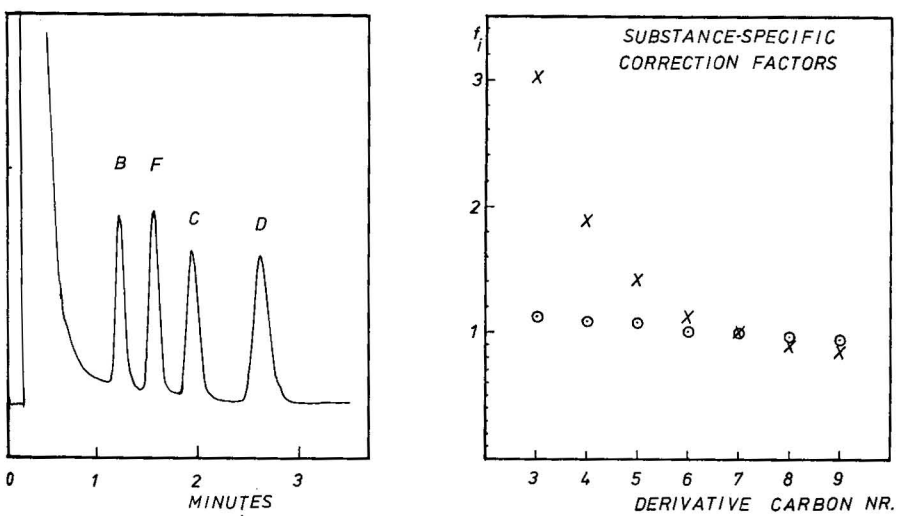

Fig. 2. Separation of the urethane derivatives of propylamine (B), 2-methylpropylamine (F), butylamine (C) and 3-methylbutylamine (D) on a 20% SE-30 column.

Fig. 3. ×, Substance-specific correction factors, f_i, as wt.% urethane produced; ⊙, f_i values as wt.% of the amine; both are plotted against the corresponding derivative carbon number.

TABLE II

ELUTION TIMES (min) OF AMINE DERIVATIVES

Compound	*Urethane derivative*	*Column*			
		SE-30	*Apiezon N*	*Carbowax 1540*	*XF-1125*
NH_3	urethane	0.725	0.70	5.78	–
CH_3NH_2	methylurethane	0.789	0.70	2.81	–
$C_2H_5NH_2$	ethylurethane	1.17	0.70	2.80	–
$C_3H_7NH_2$	propylurethane	1.55	1.30	3.68	1.16
$C_4H_9NH_2$	2-methylpropylurethane	1.98	1.65	3.89	1.24
$C_4H_9NH_2$	butylurethane	2.46	2.23	5.34	1.41
$C_5H_{11}NH_2$	3-methylbutylurethane	3.25	3.08	6.57	1.61
$C_5H_{11}NH_2$	2-methylbutylurethane	3.94	3.03	6.00	1.21
$C_5H_{11}NH_2$	pentylurethane	4.42	3.84	8.04	2.68
$C_{10}H_{22}$*		1.59	1.42	0.654	1.16

* *n*-Decane (reference)

TABLE III

ELUTION TIME (min) FOR ANILINE DERIVATIVES

Amine	*Column and temperature*		
	SE-30, 175°	*Apiezon N, 175°*	*XF-1125, 140°*
Methylaniline	2.30	2.30	2.66
Aniline	2.85	3.60	5.07

Samples injected on the XF-1125 material had tailing peaks so that some low-molecular-weight derivatives could not be observed. In Table II the times of elution at 140° on the four columns are given. For two separations, the cyclic amines, a higher temperature was needed. Table III lists the elution times for anilines.

Quantitative measurements

The derivatives have been correlated to the standard peak in two ways. Firstly, the original amount of amine was used for determination of the factors and, secondly, calculations were made for the urethanes. Fig. 3 includes both of these values.

A linear function between the carbon number and the f_i values from amines was found. One $-CH_2-$ unit alters the substance-specific correction value by 0.03 for this group. The f_i values in the urethane group are large for the low molecular weight derivatives. This is clearly due to the content of oxygen and nitrogen when analysed on an FID system.

Treatment of special samples

By use of a pH between 8.5 and 10.5 samples can be separated according to the method given. Unknown material might need extra diethyl pyrocarbonate to

ensure that all the nucleophiles present react. A modification of the system to use more reagent might require the use of ethanol to give a homogeneous solution. The urethanes formed are stable for several weeks at 5° in aqueous media. If long-term storage is necessary, a suggestion would be to neutralize the sample after reaction.

In order to relate the correction factors to other systems *n*-butanol was used as standard ($f=1.00$). A value of 3.85 ± 0.05 was obtained for urethane.

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CHROM. 7195

SELECTIVE GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC METHODS FOR THE QUANTITATION OF NORMETANEPHRINE, METANEPHRINE AND VANILLYLMANDELIC ACID

N. NARASIMHACHARI

Thudichum Psychiatric Research Laboratory, Galesburg State Research Hospital, Galesburg, Ill. 61401 (U.S.A.)

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SUMMARY

The partial derivatization of normetanephrine, metanephrine and vanillylmandelic acid (VMA) with alcoholic hydrochloric acid yields β- or α-O-ethyl ethers suitable for selective quantitation by gas chromatography–mass spectrometry (GC–MS). The isomeric 3-methoxy-4-hydroxy and 4-methoxy-3-hydroxy derivatives can be identified and quantitated in biological samples by this method. The GC–MS characteristics of the partial O-ethyl ethers of normetanephrine isothiocyanate, metanephrine and VMA ethyl ester are described.

INTRODUCTION

Normetanephrine (NM), 3-methoxy-4-hydroxyphenylglycol (MHPG) and 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid, VMA) are key metabolites in catecholamine metabolism. Several methods involving column, ion-exchange, paper or thin-layer chromatography (TLC) followed by colorimetry or fluorimetry have been used to determine the levels of these compounds in body fluids and tissues[1] (for earlier publications on methodology, see the bibliography in ref. 1). Gas chromatography (GC) of the trimethylsilyl (TMS) ethers or of the trifluoro- or heptafluorobutyryl derivatives of the methyl esters has also been used for their quantitation[2,3]. Although the *p*-methylation of catecholamines by catechol-O-methyltransferase (COMT) has been established[4], no attempt has been made to separate or to take into account such isomeric compounds.

In a recent study, we showed that the ethyl or methyl esters of homovanillic acid (HVA) and isohomovanillic acid (iso-HVA) could be separated on an OV-225 GC column[5]. We also reported the identification of these isomers by gas chromatography–mass spectrometry (GC–MS) as well as their ratios in urine and cerebrospinal fluid[6]. It was pointed out that the complete derivatization of the phenolic hydroxyl groups as TMS ethers renders the isomers inseparable.

A method for the partial derivatization of the β-hydroxy compounds to ethyl esters (alcoholic) so that the isomeric 3- and 4-methoxy compounds can be separated and quantitated is now reported.

EXPERIMENTAL

Materials and methods

NM, metanephrine, MHPG, VMA and *p*-methoxymandelic acid were obtained from commercial sources. Freshly distilled reagent-grade carbon disulphide was used in the preparation of isothiocyanate (NCS) derivatives[7]. A 3 *N* solution of ethanolic hydrochloric acid was prepared by passing hydrogen chloride gas through absolute ethanol.

Two millilitres of ethanolic hydrochloric acid and 3 mg of the compound were heated in a stoppered tube for 2 h at 90–100°. The solution was then evaporated to dryness under vacuum with more ethanol added for complete dehydration. The residues of the ethyl esters were dissolved in ethyl acetate to give a solution containing 1 mg/ml. The residues of NM and metanephrine were shaken for 15 min with 10 ml of ethyl acetate and 1 ml of 10% ammonia solution. After centrifugation, the aqueous layer was discarded and the ethyl acetate extract was dried over sodium sulphate and evaporated to dryness under vacuum. The residue of NM was redissolved in ethyl acetate and shaken for 30 min with 0.5 ml of carbon disulphide to form the NCS derivative.

Gas–liquid chromatography

The acid esters, the NCS derivatives and the β-O-ethyl ether of metanephrine were run on a 6-ft. column of 3% OV-225 on a Varian Model 2740 gas chromatograph at 190° (isothermal). Helium was used as the carrier gas at a constant flow-rate of 30 ml/min.

Gas chromatography–mass spectrometry

The mass spectra were recorded on a Varian CH 7 mass spectrometer interfaced with a Varian Model 2740 gas chromatograph and a Watson–Biemann separator. The GC conditions were as described above. The temperatures of the separator and the ion source were maintained at 280° and the ionization potential was 70 eV.

RESULTS

Under the conditions of the reaction with ethanolic hydrochloric acid, the β-hydroxyl group is quantitatively converted into the O-ethyl derivative. In a series of experiments to determine the optimal conditions for the reaction, it was found that a 3 *N* solution of hydrogen chloride gas in absolute ethanol gave the best results when the sample was heated in a stoppered tube at 90–100°. When the reaction with VMA was monitored by TLC and GLC, it was found that VMA ethyl ester and VMA ethyl ester-β-O-ethyl ether were distinctly separated and that underivatized β-hydroxy compound was present at levels of 5–10% of the mixture. Similar results were obtained with *p*-methoxymandelic acid. However, with NM and metanephrine, the reaction was quantitative.

The GC data for the various compounds are presented in Table I.

TABLE I

GC RESULTS FOR SOME CATECHOLAMINE METABOLITES

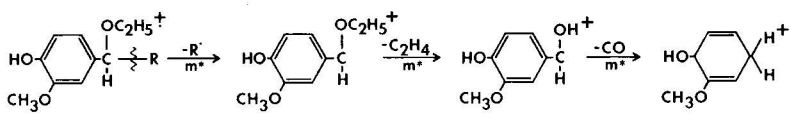
Column: 6 ft., 3% OV-225, 190° (isothermal), helium flow-rate 30 ml/min.

Metabolite	*Retention time (min)*
4-Methoxymandelic acid ethyl ester-α-O-ethyl ether	3.66
4-Methoxymandelic acid ethyl ester	4.65
VMA-ethyl ester-α-O-ethyl ether	9.62
VMA-ethyl ester	13.7
Metanephrine-β-O-ethyl ether	4.88
Normetanephrine-NCS-β-O-ethyl ether*	3.8

* Column temperature 200°.

Mass spectral data

The mass spectra of all the derivatives agreed with those required for the α- or β-O-ethyl ethers. In all instances the molecular ion was noticeable and the base peak due to the β-fission in the NCS derivatives[7] was at *m/e* $(M-72)^+$, at *m/e* $(M-73)^+$ for the acid esters and $(M-44)^+$ for metanephrine-β-O-ethyl ether. Thus NM-NCS-β-O-ethyl ether, metanephrine-β-O-ethyl ether and VMA-ethyl ester-α-O-ethyl ether had the base peak at *m/e* 181 and the other fragment ions derived from these were identical in all instances. The fragmentation is represented schematically below. The fragmentation mechanisms are confirmed by metastable transitions.



m/e **181** *m/e* **153** *m/e* **125**

The structures of the β-O-ethyl ethers were further confirmed by the preparation of O-TMS ethers, where mono-TMS derivatives were obtained. The mass spectra of the derivatives agreed with the required structures. As a typical example, the mass spectra of VMA-ethyl ester-β-O-ethyl ether and of the free ester, which is a minor component in the reaction, are given in Fig. 1. The mass spectral data of all the compounds studied are presented in Table II.

Under these experimental conditions of derivatization, MHPG loses water and forms an epoxide. The mass spectrum of the epoxide is shown in Fig. 2. The molecular ion at *m/e* 166 and the base peak at *m/e* 137 by loss of ·CHO are the characteristic features of the spectrum.

DISCUSSION

This method for the partial derivatization of the β-hydroxyl group of NM and metanephrine and the α-hydroxyl group of VMA renders the compounds volatile enough for GC analysis. The free phenolic hydroxyl group permits the

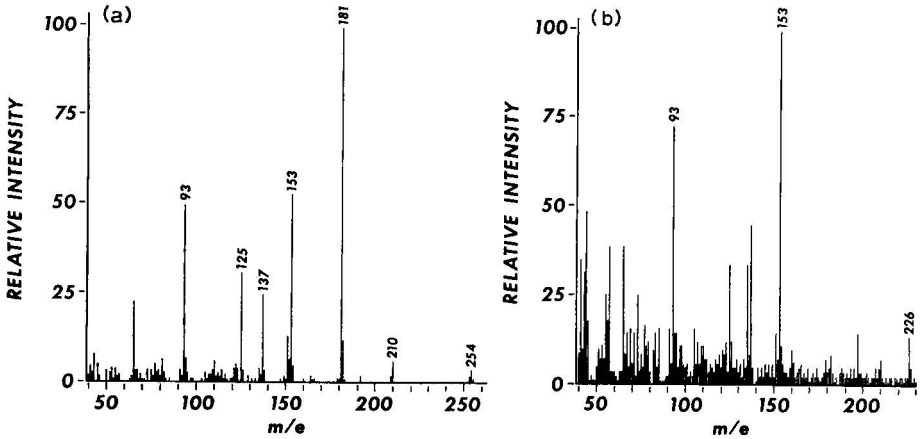


Fig. 1. Mass spectra of (a) VMA ethyl ester-β-O-ethyl ether and (b) VMA ethyl ester.

TABLE II

MASS SPECTRAL DATA FOR SOME CATECHOLAMINE METABOLITES

Metabolite	*m/e* (%)
VMA ethyl ester-α-O-ethyl ether	254 (7.5), 181 (100), 153 (42), 136 (6), 125 (18), 93 (36)
VMA ethyl ester-α-O-ethyl-4-O-TMS ether	326 (9), 311 (8), 253 (100), 225 (14), 197 (6), 73 (34)
Normetanephrine-NCS-β-O-ethyl ether	253 (13), 181 (100), 153 (60), 137 (14), 125 (40), 110 (10), 93 (87)
Metanephrine-β-O-ethyl ether	225 (5), 181 (100), 153 (52), 137 (9), 125 (22), 93 (31)
4-Methoxymandelic acid ethyl ester-α-O-ethyl ether	238 (1.2), 193 (3.5), 165 (100), 137 (82), 109 (30), 94 (13)
MHPG with HCl	166 (25), 137 (100), 122 (14), 94 (16), 77 (22)

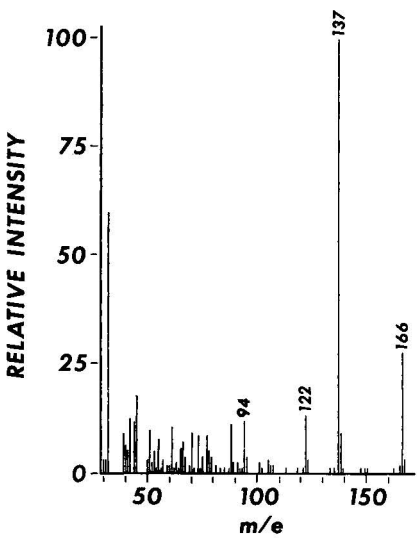


Fig. 2. Mass spectrum of the product formed from 3-methoxy-4-hydroxyphenylglycol (MHPG) with ethanolic hydrochloric acid.

separation of the isomeric 3-methoxy-4-hydroxy and 4-methoxy-3-hydroxy compounds on GC for quantitation by GC–MS.

Even if the isomeric compounds are obtained by standard techniques of liquid chromatography, ion-exchange column chromatography or TLC, when the isomers are not separated, they can subsequently be derivatized by this method and identified if they should occur in biological systems.

As overlapping of other peaks has been reported to occur with the use of the TMS derivatives of the methyl ethers[8], we reinvestigated by the present method the levels of VMA in urine samples of Parkinson patients receiving L-DOPA. When the levels were low and other peaks interfered, we performed preliminary preparative TLC. The ester fraction from the urine and standard VMA-ethyl ester-β-O-ethyl ester were spotted on a silica gel G thin-layer plate which was developed with chloroform–acetic acid (100:2). The reference standard was rendered visible by spraying with diazotized *o*-tolidine and the sample fraction corresponding to the reference standard was eluted with ethanol–ethyl acetate (1:1). This fraction was then quantitated by GC on an OV-225 column and also by monitoring the base peak at *m/e* 181 during GC–MS. The homogeneity of the peak was confirmed by its mass spectrum. The results obtained by the two methods agreed closely, and were further confirmed when the recovery was checked by using the same procedure with adequate controls with added standards. The values obtained by this specific method were lower than those in the literature[8]. Details of these results will be published elsewhere.

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CHROM. 7273

DETERMINATION OF PROSTAGLANDIN $F_{1\alpha}$ AND $F_{2\alpha}$ BY GAS–LIQUID CHROMATOGRAPHY

MAMORU SUGIURA and KAZUYUKI HIRANO

Tokyo College of Pharmacy, Ueno-sakuragi, Taito-ku, Tokyo 110 (Japan)

(Received August 29th, 1973)

SUMMARY

A quantitative gas–liquid chromatographic method has been developed for the determination of prostaglandin $F_{1\alpha}$ and $F_{2\alpha}$ ($PGF_{1\alpha}$ and $PGF_{2\alpha}$). The method involves the conversion of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ into their trimethylsilyl methyl esters or heptafluorobutyryl methyl esters and their chromatography using 3% OV-1 on Gas-Chrom Z; their structures were established by combined gas chromatography–mass spectrometry.

The method can be applied to human semen plasmas with prostaglandin $F_{2\beta}$ ($PGF_{2\beta}$) as an internal standard.

INTRODUCTION

Prostaglandins (PGs) were first detected as a result of their biological activities[1,2], and such activities have frequently been employed as the basis for their measurement. Spectrophotometric analyses are not sufficiently sensitive for the determination of the very small amounts that exist in tissues[3,4], and an enzymatic procedure for measuring PGs[5–7] also lacks specificity and has limited sensitivity of detection when applied to biological samples. Radioimmunoassays for PGs[8–11] have the capacity to measure picogram amounts, but have incomplete specificity owing to immunological cross-reactions.

Gas–liquid chromatography (GLC) has the potential to provide simultaneous separations according to the extent of unsaturation of PGs. GLC can be employed with several PGF derivatives[12–17], but it is difficult to apply these techniques to biological samples. Recently, combined gas chromatography–mass spectrometry (GC–MS) measurements[18–20] have made it possible to identify and measure nanogram amounts of individual PGs. However, most laboratories probably cannot afford to purchase the necessary equipment.

In this paper, we describe our studies on the extraction of PGF from biological samples and the use of GLC to determine $PGF_{1\alpha}$ and $PGF_{2\alpha}$. This technique has been applied to assay $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in human semen plasmas.

EXPERIMENTAL

Reagents

Methanol, ethanol, *n*-hexane, ethyl acetate and ether were distilled before use. Other solvents and reagents were purchased commercially and used as received.

Apparatus and conditions

A Jeol, Model JGC-20KPF, gas chromatograph equipped with a hydrogen flame ionization detector (HFID), and a Jeol, Model JGC-20KE, gas chromatograph equipped with an electron capture detector (ECD) were used. The 3 m × 2 mm I.D. and 1 m × 2 mm I.D. glass columns were packed with 3% OV-1 on Gas-Chrom Z (80–100 mesh).

Gas chromatography–mass spectrometry (GC–MS). The mass spectra were measured with a Hitachi Model RMU-6E mass spectrometer. The operating conditions were: chamber voltage, 70 eV; ion source temperature, 220 °; total emission, 80 μA; current, 70 μA. All the samples were introduced into the ionization chamber through a Hitachi K-53 gas chromatograph. A 2.0-m glass column packed with 3% OV-1 on Gas-Chrom Z (80–100 mesh) was used and the temperature was maintained at 210 °.

Scintillation counter. A Beckmann Model LS 100 scintillation counter was used, and ^{3}H-labelled PGF was counted in a dioxan scintillator.

Standard procedure

Method A (preparation of $PGF_{1\alpha}$–TMS–Me and $PGF_{2\alpha}$-TMS–Me derivatives). PGF was methylated with diazomethane in diethyl ether–methanol (9:1), allowed to stand for 10 min and then evaporated under a stream of nitrogen at room temperature. To the dried residue of PG–Me ester, 50 μl of dimethyl sulphoxide–ethyl acetate (1:5) or 50 μl of triethylamine–ethyl acetate (1:5) and 50 μl of bistrimethylsilyl trifluoroacetoamide (BSTFA) were addded. After 30 min, the excess of the reagents was evaporated under a stream of nitrogen at room temperature, and the dried residue was dissolved in a small volume of ethyl acetate and injected into the gas chromatograph (HFID).

Method B (preparation of $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives). Methylation of PGF was carried out according to Method A and 50 μl of ethyl acetate and 50 μl of heptafluorobutyryl imidazole (HFBI) were added to the dried residue. After 10 min, the reaction mixture was evaporated to dryness under a stream of nitrogen at room temperature. The dried residue was extracted with three 1-ml volumes of *n*-hexane, and the combined *n*-hexane extracts were concentrated and injected into the gas chromatograph (ECD).

Extraction of total PGs

The extraction of PGs from samples is shown in Fig. 1.

The sample was homogenized with 10 volumes of Bloor solution (ethanol–diethyl ether, 3:1) in a glass homogenizer, the homogenate was allowed to stand for 30 min and then filtered. The filtrate was evaporated to dryness under reduced pressure at 40 °, and 5 ml of carbon tetrachloride and 10 ml of water were added to the residue. The mixture was shaken vigorously and centrifuged at 3,000 rpm

Sample: Bloor solution (1:10)
↓
Filtrate — Mixed and allowed to stand for 30 min, filtered and washed with Bloor solution
↓
Upper phase — Evaporated to dryness under reduced pressure at 40°, dissolved in 5 ml of CCl_4, 10 ml of water added and shaken vigorously, then centrifuged at 3,000 rpm for 10 min
↓
Upper phase — Adjusted at pH 3 with 1 *N* HCl, 10 ml of ethyl acetate added and shaken vigorously, then centrifuged at 3,000 rpm (800 *g*) for 10 min
↓
Residue — 200 μl of 1 *N* ammonia solution and 2 ml of methanol added, then evaporated to dryness under reduced pressure at 40° (Total PGs)

Fig. 1. Procedure for extraction of PGs from samples.

(800 *g*) for 10 min, then the upper layer (aqueous layer) was adjusted to pH 3 with 1 *N* hydrochloric acid and 10 ml of ethyl acetate were added. The mixture was shaken vigorously and centrifuged at 3,000 rpm (800 *g*) for 10 min and then 200 μl of 1 *N* ammonia solution was added to the upper layer (ethyl acetate layer) and the mixture was evaporated to dryness under reduced pressure at 40°.

RESULTS AND DISCUSSION

Preparation of $PGF_{1\alpha}$–TMS–Me and $PGF_{2\alpha}$–TMS–Me derivatives

The optimum reaction conditions for the standard procedure as described under Experimental was established from preliminary experiments. Various trimethylsilylating reagents and reactive solvent systems were examined, and it was found that the PGF–TMS derivatives were most easily produced by using BSTFA as the trimethylsilylating reagent in the above mixed solvent systems. The effects of temperature and time on the production of PGF–TMS–Me derivatives were examined relative to standard conditions of 30 min and room temperature. The $PGF_{2\beta}$–TMS–Me derivative was used as the internal standard and a GLC sepa-

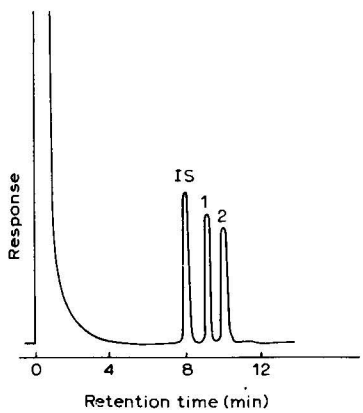

Fig. 2. Gas chromatogram of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ using $PGF_{2\beta}$ as the internal standard. Conditions: 3% OV-1; 3-m glass column; 230° (240°); HFID; nitrogen carrier gas, flow-rate 50 ml/min. IS, internal standard, $PGF_{2\beta}$–TMS–Me; (1) $PGF_{2\alpha}$–TMS–Me; (2) $PGF_{1\alpha}$–TMS–Me.

ration was achieved successfully by using a glass column, as shown in Fig. 2; the calibration curves passed through the origin for $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in the range 1–10 μg.

Structures of the $PGF_{1\alpha}$–TMS–Me and $PGF_{2\alpha}$–TMS–Me derivatives

The structures of the $PGF_{1\alpha}$–TMS–Me and $PGF_{2\alpha}$–TMS–Me derivatives were established by GC–MS and the spectral data are shown in Figs. 3 and 4.

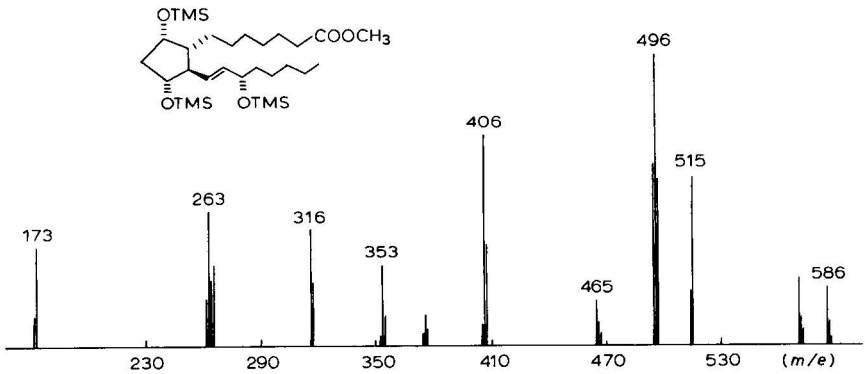

Fig. 3. Mass spectral data obtained on gas chromatographic effluent at a retention time corresponding to $PGF_{1\alpha}$–TMS–Me derivative. GC–MS conditions: mass range, 750; chamber voltage, 70 eV; chamber temperature, 230 °; column, 3% OV-1, 2 m; 210 °; helium carrier gas, flow-rate 50 ml/min.

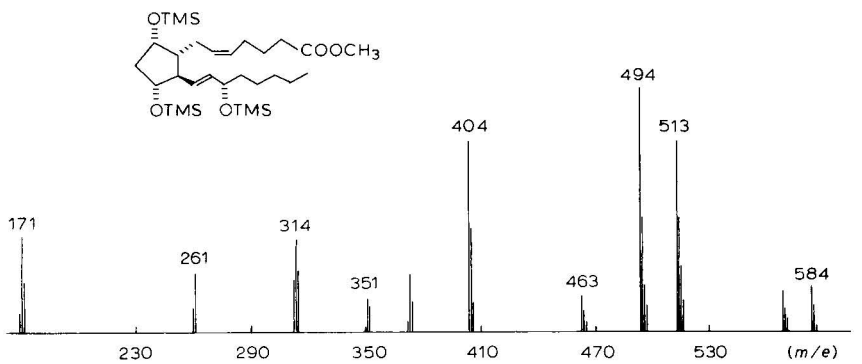

Fig. 4. Mass spectral data obtained on gas chromatographic effluent at a retention time corresponding to $PGF_{2\alpha}$–TMS–Me derivative. GC–MS conditions: mass range, 750; chamber voltage, 70 eV; chamber temperature, 230 °; column, 3% OV-1, 2 m; 210 °; helium carrier gas, flow-rate 50 ml/min.

In Fig. 3, the ion at *m/e* 586 indicates the molecular weight and *m/e* 496 corresponds to the loss of one TMS group, *m/e* 406 corresponds to the loss of two TMS groups and the peak at *m/e* 316 corresponds to the loss of three TMS groups from the $PGF_{1\alpha}$–TMS–Me derivative. In Fig. 4, the ion at *m/e* 584 indicates the molecular weight and that at *m/e* 494 corresponds to the loss of one TMS group, the peak at *m/e* 404 corresponds to the loss of two TMS groups and the peak at *m/e* 314 corresponds to the loss of three TMS groups from the $PGF_{2\alpha}$–TMS–Me

derivative. It is concluded that three hydroxy groups in both $PGF_{1\alpha}$ and $PGF_{2\alpha}$ are substituted with three TMS groups.

Preparation of $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives

The optimum reaction conditions for the preparation of $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives were as described under *Standard procedure*. PGs are unstable towards acids and bases and, as reagent for the formation of PGF–HFB–Me derivatives, it seems that HFBI is more desirable than heptafluorobutyric anhydride (HFBA), because heptafluorobyturic acid is produced from HFBA in the course of the preparation of PGF–HFB–Me derivatives. In fact, when HFBA is used, the PGF–HFB–Me derivatives give two peaks on the gas chromatogram, according to Levitt *et al.*[15], but when HFBI is used, PGF–HFB–Me derivatives give a single peak and can be separated successfully, as shown in Fig. 5.

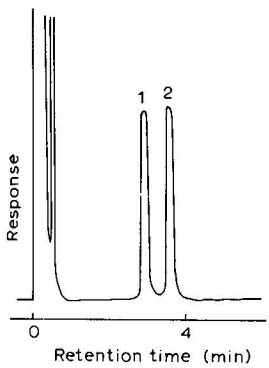

Fig. 5. Gas chromatogram of (1) $PGF_{1\alpha}$–HFB–Me and (2) $PGF_{2\alpha}$–HFB–Me derivatives. Conditions: 3% OV-1; 1-m glass column; 190° (260°); ECD (^{63}Ni); nitrogen carrier gas, flow-rate 60 ml/min.

The thermal decomposition of $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives was not observed during GLC under the experimental conditions used. The PGF–HFB–Me derivatives have suitable properties for the ECD gas chromatograph: they are volatile, adequately separated, have sufficient electron-capturing properties to be detectable at a low level and have adequate stability when stored in *n*-hexane at 0°. Calibration curves passed through the origin when $PGF_{2\beta}$–HFB–Me was used as the internal standard.

Structures of the $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives

The structures of the $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives were established by GC–MS and the spectral data are shown in Figs. 6 and 7.

In Fig. 6, a molecular peak at *m/e* 958 does not appear, the peak at *m/e* 744 corresponds to the loss of one HFB group, the peak at *m/e* 530 corresponds to the loss of two HFB groups and the peak at *m/e* 316 corresponds to the loss of three HFB groups from the $PGF_{1\alpha}$–HFB–Me derivative. In Fig. 7, a molecular peak at *m/e* 956 does not appear, the peak at *m/e* 742 corresponds to the loss of one HFB group, the peak at *m/e* 528 corresponds to the loss of two HFB groups and

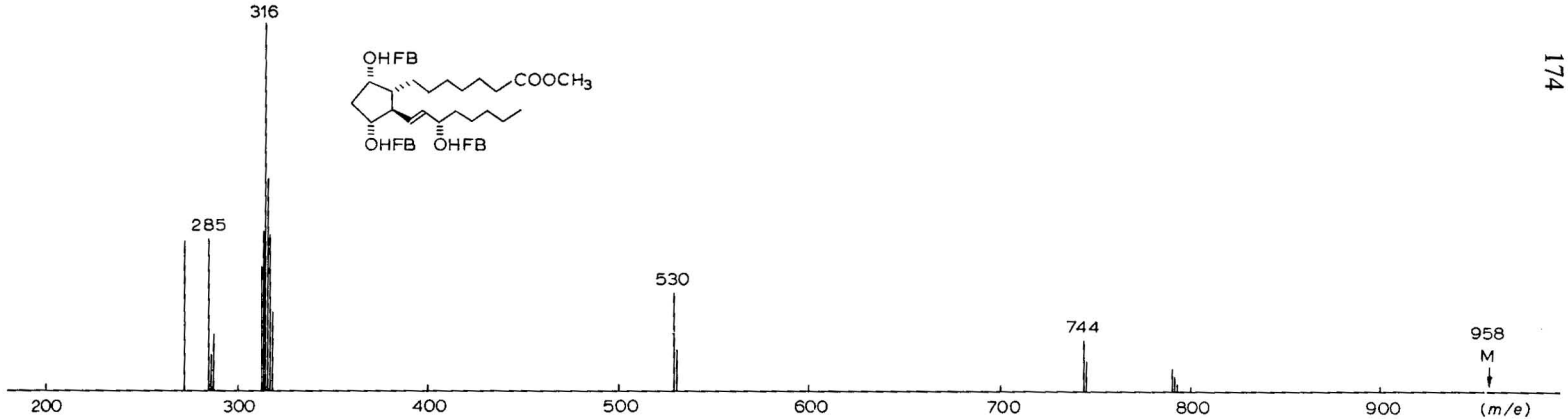

Fig. 6. Mass spectral data obtained on gas chromatographic effluent at a retention time corresponding to $PGF_{1\alpha}$–HFB–Me derivative. GC–MS conditions: mass range, 1,000; chamber voltage, 70 eV; chamber temperature, 230°; column, 3% OV-1, 2 m; 210°; helium carrier gas, flow-rate 50 ml/min.

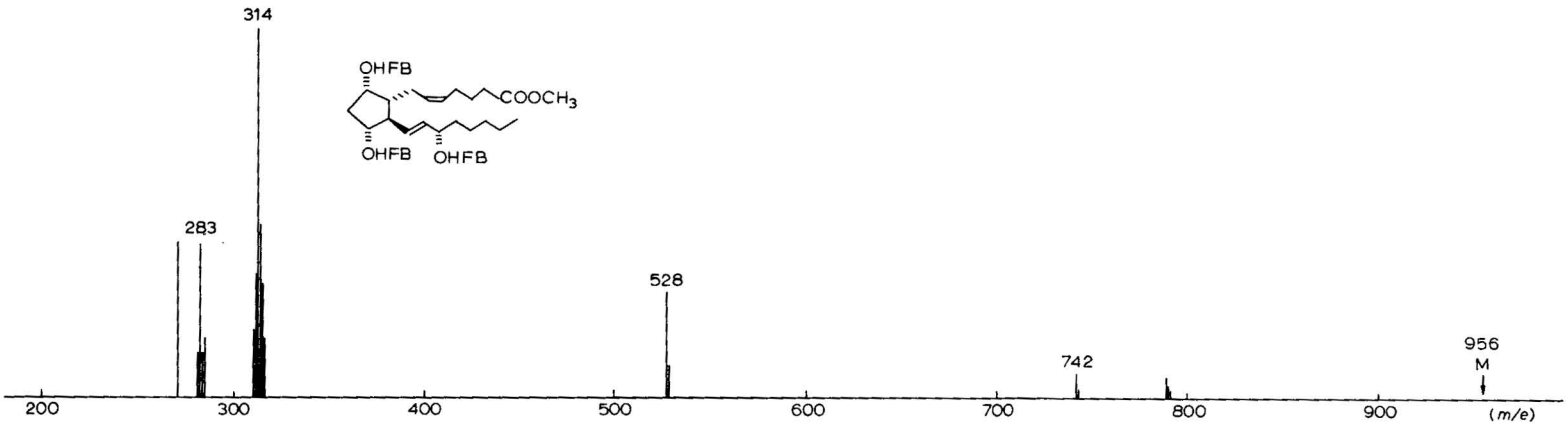

Fig. 7. Mass spectral data obtained on gas chromatographic effluent at a retention time corresponding to $PGF_{2\alpha}$–HFB–Me derivative. GC–MS conditions as in Fig. 6.

the peak at *m/e* 314 corresponds to the loss of three HFB groups from the $PGF_{2\alpha}$–HFB–Me derivative. From these results, it seems that three hydroxy groups in both $PGF_{1\alpha}$ and $PGF_{2\alpha}$ are substituted with three HFB groups.

Extraction of PGs

Recently, Inagawa *et al.* reported on the determination of PGs by means of radioimmunoassay[11], using Folch solution (methanol–chloroform, 1:2) for the extraction of all the lipids from the samples. In the gas chromatographic determination by ECD, the impurity peaks based on chloroform as the solvent appeared on the gas chromatograms and therefore, in this work, we used Bloor solution (ethanol–diethyl ether, 3:1) instead of Folch solution in the first step of the extraction of all the lipids from the samples and established the optimum procedure for the extraction of samples for GLC determination as shown in Fig. 1. The addition of 1 *N* ammonia solution to the ethyl acetate layer prevents the decomposition of PGF during evaporation at 40°, which would have occurred because PGs are unstable towards acids. The transfer of PGF from the carbon tetrachloride layer into the aqueous layer is specific for all lipids and, in this step, the lipids other than PGs can be removed.

Table I shows the recovery of 5 μg and 5 ng of PGF_2 according to Fig. 1 by use of tritium-labelled $PGF_{2\alpha}$, and the mean recoveries of $PGF_{2\alpha}$ were 88.7% and 84.3%, respectively. $PGF_{1\alpha}$ exhibits the same behaviour as $PGF_{2\alpha}$ in the extraction procedure.

TABLE I

RECOVERY OF PGs IN EXTRACTION PROCEDURE

No.	*PG*	*Amount*	*Recovery* (%)
1	$PGF_{2\alpha}$*	5 μg	92.8
2			85.5
3			87.8
		average	88.7
4		5 ng	86.2
5			82.2
6			84.6
		average	84.3

* $PGF_{2\alpha}$ included ^3H-labelled $PGF_{2\alpha}$ (200 pg). Counted in a dioxan scintillator with a Beckman Model LS 100 scintillation counter.

Determination of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in human semen plasma

$PGF_{1\alpha}$ and $PGF_{2\alpha}$ in human semen plasmas were assayed by using method B. A chromatogram obtained from human semen plasma is illustrated in Fig. 8 using $PGF_{2\beta}$ as the internal standard and the results are listed in Table II.

The amounts of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ determined in human semen plasmas were mid-way between the results so far reported by radioimmunoassay and GLC.

We are planning to apply this technique to the GLC determination of other PGs.

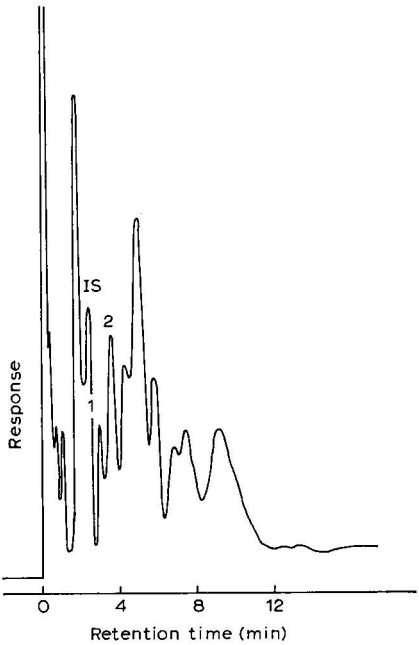

Fig. 8. Gas chromatogram of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in human semen plasma using $PGF_{2\beta}$ as the internal standard. Conditions: 3% OV-1; 1-m glass column; 190° (260°); ECD (^{63}Ni); nitrogen carrier gas, flow-rate 60 ml/min. IS, internal standard; (1) $PGF_{2\alpha}$–HFB–Me; (2) $PGF_{1\alpha}$–HFB–Me.

TABLE II

DETERMINATION OF PGF IN HUMAN SEMEN PLASMA

Sample No.	*Age (years)*	*$PGF_{1\alpha}$ ($\mu g/ml$)*	*$PGF_{2\alpha}$ ($\mu g/ml$)*
1	20	0.87	1.60
2	25	1.16	1.44
3	23	0.76	0.46

ACKNOWLEDGEMENTS

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CHROM. 7162

Note

A new solvent system for the thin-layer chromatographic separation of the Dansyl derivatives of some biogenic amines

GRAHAM C. BOFFEY and GLENN M. MARTIN

Clinical Pathology Service, Royal Inland Hospital, Kamloops, British Columbia (Canada)

(Received October 9th, 1973)

During our investigations of pharmacologically active compounds present in tick tissues, a methodology of high sensitivity and resolution was required for the separation of biogenic amines. The procedure was to accomplish these criteria without significant interference from amino acids.

Procedures are available for the separation of biogenic amines utilizing various chromatographic systems including paper, thin-layer and gas–liquid (GLC) chromatography. Seiler and Wiechmann[1–3], Seiler[4] and Creveling *et al.*[5] have described the derivative formation of primary and secondary amines with 5-dimethylamino-1-naphthalenesulfonyl chloride (Dansyl-Cl) to produce intensely fluorescent compounds which may subsequently be separated by chromatography on silica gel thin layers using semi-polar solvent systems. The utilization of the reaction of Dansyl-Cl with the amino groups of amino acids and peptides in procedures for the separation of these derivatives was described by Gray and Hartley[6] and subsequently by Boulton and Bush[7] and others[2,8]. The chromatographic resolution of the Dansyl derivatives of amine-containing biological materials is impaired by the diversity of derivatives produced during the reaction of Dansyl-Cl with constituents of biological extracts. Reaction of Dansyl-Cl with hydroxyl groups of phenolic compounds[1] and some alcohols[4] has been reported. Similar difficulties may also be anticipated using the more recently reported reaction[9] of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) with amino groups to produce fluorescent derivatives of amines since reaction also occurs with other electron donating groups but produces derivatives of substantially relatively weaker fluorescence.

The use of GLC to separate and identify biogenic amines involves certain fundamental difficulties such as the low volatility of the free amines and their basicity which produce tailing, long retention times and peak asymmetry. Reactions to produce trimethylsilyl[10,11], dinitrophenyl[12], trifluoracetyl[13], perfluoropropionyl[14] and isothiocyanate[15] derivatives of amines derived from biological samples have been proposed in order to reduce some of the problems of GLC separation. These approaches have also been of limited value even in conjunction with various stationary and liquid phases. As a result of the inherent problems of the above procedures it was decided to reinvestigate the feasability of developing a procedure for biogenic amine separation utilizing the proven Dansyl-Cl reaction as the basis of the method.

MATERIALS AND METHODS

Dansyl-Cl was recrystallized twice from hexane and stored desiccated at room temperature until required. Analytical grade reagents were used throughout the study except when otherwise specified. Reference amines were used as obtained from the supplier without further purification. The Dansyl derivatives of the reactive amino compound (0.01 to 1.00 μM) were prepared from the biological sample by a modification of the method of Creveling *et al.*[5] and after lipid removal with benzene as described the Dansyl derivatives of the amine were extracted into 3 ml of ethyl acetate–cyclohexane (1:9, v/v) by mechanical shaking for 2 min. The mixture was centrifuged at $1500 \times g$ for 10 min, the ethyl acetate–cyclohexane extract was removed and the solvent was evaporated at room temperature under a stream of dry nitrogen. The residue contained predominantly the Dansyl derivatives of the amines which were redissolved in 50 μl of ethyl acetate. The solution of the amine derivative was applied to a 20×20 cm silica gel coated thin-layer plate (E. Merck, Darmstadt, G.F.R. Cat. No. 5763 distributed by Brinkmann Instruments (Canada) Ltd.) of layer thickness 0.25 mm. Dansyl derivatives of standard biogenic amines (0.01 to 1.00 μM) were prepared as described and also applied to the chromatoplate.

The chromatogram was developed in the carbon tetrachloride–ethylene glycol monomethyl ether (85:15, v/v) solvent system until the solvent front had ascended

TABLE I

R_F VALUES FOR DANSYL DERIVATIVES OF SOME BIOGENIC AMINES

Solvent system: carbon tetrachloride–ethylene glycol monomethyl ether (85:15).

Substance	$R_F \times 100$	*S.D.* $\times$ *100* (*n* = *10*)	*Coefficient of variation*
Agmatine	36	4.5	12.7
Ammonia	22	1.5	6.7
D-Amphetamine	47	1.9	4.0
Cadaverine	36	4.2	11.5
Diethylamine	56	1.2	2.1
Histamine	7	2.1	30.0
5-Hydroxytryptamine	14	3.1	22.0
Metanephrine	27	1.7	6.3
Methylamine	34	2.2	6.3
Normetanephrine	20	2.1	10.6
Octopamine	15	1.5	9.8
Phenylethylamine	46	1.6	3.5
Phenylpropanolamine	33	5.1	15.4
Piperidine	60	2.4	4.1
Putrescine	35	3.5	10.1
Spermidine	33	5.2	15.8
Spermine	35	6.1	7.4
Synephrine	22	1.4	6.3
Triethylamine	55	1.5	2.7
Tryptamine	31	2.6	8.5
Tyramine	24	1.8	7.5
Dansyl-Cl	65	2.5	3.8

15 cm from the origin during approximately 100 min at 20°. The chromatogram was blown completely free of solvent to remove any residual carbon tetrachloride which had a marked quenching effect on the fluorescence of the Dansyl derivatives. The location of the separated derivatives was determined by inspection under long wavelength (366 nm) ultraviolet light.

RESULTS

Table I shows the mean R_F values for a number of amine derivatives using this solvent system and the reproducibility for a series of ten separate runs.

DISCUSSION

The procedure described resulted in an effective preliminary separation of amino acids and amines as their Dansyl derivatives as was demonstrated by tracer studies using the Dansyl derivatives of ^{14}C-labelled glycine and putrescine. These studies indicated that the derivative of putrescine was preferentially extracted into the ethyl acetate–cyclohexane solvent with an efficiency of at least 94% in comparison with the glycine derivative over a concentration range of 1.00 to 0.01 μM. The chromatographic system described produced reproducible, high-resolution separations of several biologically important primary and secondary amines. The sensitivity of the procedure was greater than 0.01 μM.

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Note

A new fluorescence method for the detection of hexosamines and their separation by means of thin-layer chromatography

Y. VLADOVSKA-YUKHNOVSKA, CH. P. IVANOV and M. MALGRAND*

Department of Organic Chemistry, Higher Institute of Chemical Technology, Sofia 56 (Bulgaria)

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A number of methods have been developed for the determination of amino sugars: the method of Elson and Morgan[1,2] and its modifications[3–5] for measuring their total content, the method of Dische *et al.*[6], the ninhydrin method[7] and gas chromatographic[8] and ion-exchange chromatographic methods[9]. Kelleher *et al.*[10] proposed a new method for the quantitative determination of glucosamine only by means of column chromatography and radio isotope dilution. More recent spectrophotometric and colorimetric methods, based on colour reactions with various reagents, such as trinitrobenzene-1-sulphonic acid[11], *p*-nitrobenzenediazonium salts in alkaline medium[12], *p*-nitrobenzaldehyde[13] and N-methylbenzothiazolone hydrazone[14] have also been described. Methods based on the 2,4-dinitrophenyl derivatives of amino sugars and their separation by means of paper (PC)[15] and thin-layer[16] chromatography (TLC) are also known.

The separation of the individual amino sugars is most often achieved by PC (ref. 17) and TLC (e.g., ref. 18), detection being effected with different reagents. Amounts of 10–100 μg of each individual sugar are needed for these methods. Moczar *et al.*[19] suggested a more sensitive method (1.5 μg).

The most sensitive methods involve fluorescence techniques. Galoyan *et al.*[20] described a sensitive method ($3–5 \times 10^{-9}$ mole) for the detection of amino sugars as 5-dimethyl aminonaphthalene-1-sulfonyl (Dns) derivatives and for their separation by means of TLC. Cho Tun *et al.*[21] described an automatic spectrofluorimetric determination of the formaldehyde released by periodate oxidation of amino sugars as 3,5-diacetyl-1,4-dinitro-2,6-dimethylpyridine. Maeda *et al.*[22] proposed a highly sensitive method for the determination of amino sugars by using pyridoxal and zinc(II) ions. The last two methods, however, do not provide the possibility of determining individual sugars, but only their total content.

In previous papers, we showed that Dis-chloride (diphenylindenone sulphonyl-chloride or 2-*p*-chlorosulphophenyl-3-phenylindenone) reacts with amines[23] and amino acids[23–25] by forming the corresponding Dis derivatives and that under the action of sodium ethylate, the latter are transformed into strongly fluorescent derivatives of diphenylisobenzofuran[26]. This reaction offered the opportunity of determining amino acids in amounts of 10^{-12}–10^{-13} mole. On this basis, a highly sensitive method

* Present address: Faculté des Sciences, Université de Constantine, Algérie.

for the detection of the N-terminal groups in proteins and peptides was developed[26], and we have attempted to establish whether we could also detect small amounts of hexosamines using Dis-chloride, and also to find conditions for the separation of Dis derivatives of hexosamines from each other and from the Dis derivatives of amino acids.

EXPERIMENTAL

α-D-Glucosamine hydrochloride (Koch-Light, Colnbrook, Great Britain) and D-galactosamine hydrochloride (Loba-Chemie, Vienna, Austria) were used.

Preparation of Dis derivatives of glucosamine and galactosamine

The procedure described[26] for the preparation of the Dis derivatives of amino acids was used with certain modifications: 40 μl of an acetone solution of Dis-chloride (1 mg/ml) was added to 2×10^{-11} mole of glucosamine or galactosamine dissolved in 20 μl of a 0.1 *M* solution of sodium hydrogen carbonate. After leaving the mixture for about 2.5 h at room temperature, the samples were evaporated to dryness in a vacuum desiccator, and the dry residue was dissolved in acetone and applied to the chromatographic plate.

Chromatography

The separation of the Dis derivatives was achieved by TLC using silica gel G (E. Merck, Darmstadt, G.F.R.) as the carrier. Before each chromatographic run, the plates were activated by heating them for 20 min at 105°. The following solvent systems were used:

(A) *n*-butanol–toluene–25% ammonia (80:10:10)[28]

(B) chloroform–ethyl acetate–acetic acid (50:66:2.5)[27]

(C) chloroform–ethyl acetate–methanol–propionic acid (70:40:22.5:0.5)

(D) toluene–ethylene chlorohydrin–25% ammonia (30:50:20)

After the chromatography, the plates were dried at 105° for 5 min in order to remove trace amounts of the solvents, cooled to room temperature and sodium ethylate solution (5 g of Na per 100 ml of 96% ethanol) was poured over them. The plates were immediately observed under UV light (365 nm), and the spots of the Dis derivatives of the hexosamines, as well as those of Dis-acid, Dis-amide and Dis-chloride, showed yellow-green fluorescence.

RESULTS AND DISCUSSION

The solvent systems used for the separation of the corresponding Dns derivatives[20], as well as those for the separation of Dis amino acids[27], were tested for the separation of the Dis derivatives of glucosamine and galactosamine.

The solvent systems recommended for use with Dns derivatives in the preliminary application of buffers to the plates proved to be unsuitable. A relatively good separation was achieved by using systems C and D with preliminary impregnation of the plates with borate buffer (pH 8.6) and a single development in one direction (Table I).

A considerably improved and simplified separation, without preliminary

TABLE I

R_F VALUES OF Dis DERIVATIVES OF α-D-GLUCOSAMINE (GLA) AND D-GALACTOSAMINE (GAA) IN THE SOLVENT SYSTEMS USED

Carrier: silica gel G (0.25 mm layer thickness).

Dis derivative	*Solvent system*			
	A	*B*	*C*	*D**
GLA	0.61	0.45	0.45	0.27
GAA	0.53	0.29	0.28	0.20
OH	0.23	0.00	0.14	0.55
NH_2	0.86	0.86	0.90	0.92
Cl	0.90	0.97	0.96	0.00

* with borate buffer, pH 8.6.

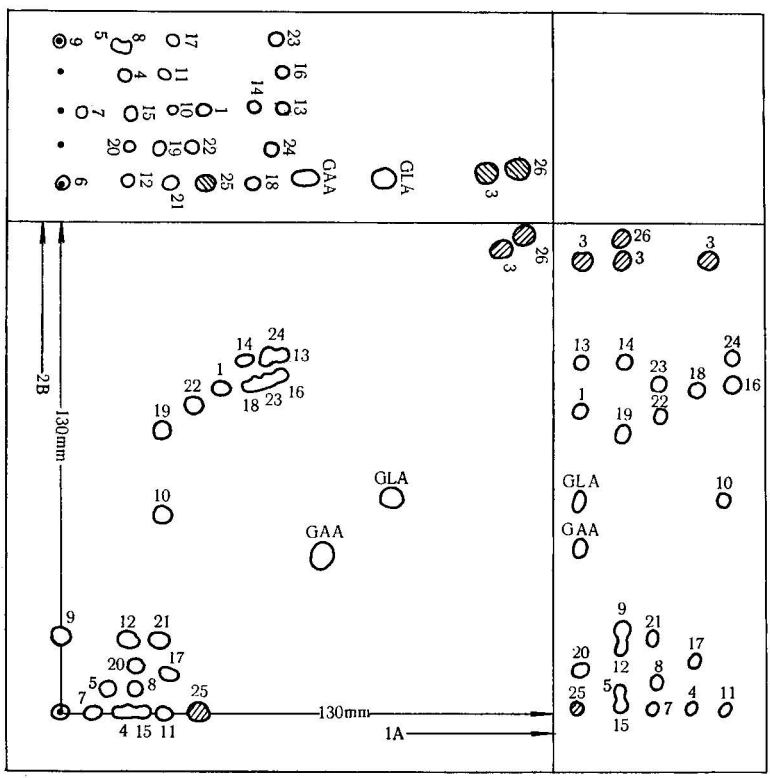

Fig. 1. Two-dimensional separation of the Dis derivatives of α-D-glucosamine (GLA), D-galactosamine (GAA) and common amino acids. (1) Dis-α-alanine; (3) Dis-amide; (4) Dis-arginine; (5) Dis-asparagine; (6) Dis-aspartic acid; (7) Dis-cysteic acid; (8) Dis-glutamine; (9) Dis-glutamic acid; (10) Dis-glycine; (11) di-Dis-histidine; (12) Dis-hydroxyproline; (13) Dis-isoleucine; (14) Dis-leucine; (15) ε-Dis-lysine; (16) di-Dis-lysine; (17) Dis-methionine-sulphone; (18) Dis-phenylalanine; (19) Dis-proline; (20) Dis-serine; (21) Dis-threonine; (22) Dis-tryptophan; (23) di-Dis-tyrosine; (24) Dis-valine; (25) Dis-OH; (26) Dis-Cl. Carrier: silica gel G (0.5 mm layer thickness). First run: *n*-butanol–toluene–25% ammonia (80:10:10) (A), developed once. Second run: chloroform–ethyl acetate–acetic acid (50:66:2.5) (B), developed once. Dimensions of plates: 200 × 200 mm.

impregnation of the plate, was achieved by the use of systems A and B with a single development (Table I). A reliable separation not only of the Dis derivatives of glucosamine and galactosamine from each other, but also of the Dis derivatives of the amino acids contained in the proteins, was obtained by using two-dimensional chromatograms in systems A and B (Fig. 1). Moreover, under these conditions, almost all Dis derivatives of amino acids were separated from each other. Only Dis-isoleucine and Dis-valine, Dis-arginine and ε-Dis-lysine, and also Dis-phenylalanine, di-Dis-tyrosine and di-Dis-lysine, remained unseparated. All these Dis derivatives of amino acids, however, can be separated from each other by using the solvent systems proposed by us[27].

The proposed fluorescence method makes possible the detection of amino sugars in amounts as low as 2×10^{-11} mole, which means that amounts of hexosamines 100 times lower than those detected by the method of Galoyan *et al.*[20] can be determined.

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CHROM. 7194

Note

A simple device for the collection of gas chromatographic effluents from small-bore packed columns*

A. A. CASSELMAN and R. A. B. BANNARD

Defence Research Establishment, Defence Research Board, Ottawa (Canada)

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In the past, we found it necessary to recover eluted compounds following the gas chromatographic (GC) analysis or purification of mixtures. This work included the preparative GC separation of isomers[1–5], to provide material for derivative formation and structural proof, collection and identification of compounds resulting from sample decomposition during chromatography[6] or recovery of fractions for additional instrumental examination[7,8] (IR, NMR, etc.). Many of these collections were carried out using the commercially available apparatus reported earlier[1–5]. Recently, however, the device shown in Fig. 1 has been designed and employed. The results obtained and some of the parameters that affect recovery efficiency are reported and discussed.

EXPERIMENTAL

Gas chromatography

An F & M Scientific Research Chromatograph, Model 5750 (Hewlett-Packard, Avondale, Pa., U.S.A.) equipped with a thermal conductivity detector (TCD; bridge current 150 mA) and coupled to an electronic integrator (Hewlett-Packard, Model 3370B) was used for all the GC analyses. A stainless-steel column (2 ft. × 1/8 in. O.D.) packed with 3.8 % OV-17 on Chromosorb W, AW and DMCS-treated, 80–100 mesh, was employed. The carrier gas (helium) flow-rate was set at 25 ml/min. The injection port and column temperatures were kept at 210° and 130°, respectively, while the TCD temperature was varied as shown in Tables I and II. The sample size ranged from 1 to 5 μl during collection experiments and was standardized at 1 μl for quantitative analysis.

Sample collection and quantitative analysis

One-microlitre aliquots of each of the four compounds methyl Cellosolve, Cellosolve, *n*-butyl Cellosolve and *n*-butyl carbitol were injected separately at the different TCD temperatures shown in Table I. The collection device illustrated in Fig. 1 was connected to the exit port of the gas chromatograph for 1 min prior to sample elution. Separate recoveries of each of the above compounds were made

* Issued as DREO Tech. Note No. 74-2.

TABLE I

RECOVERY EFFICIENCY OF GAS CHROMATOGRAPHIC EFFLUENTS AT DIFFERENT DETECTOR TEMPERATURES

Compound	*B.p.* (°C)	*Retention time (min)* *	*Recovery (%)*											
			Detector temperature (°C)											
			135				*170*				*230*			
			Wire out		*Wire in*		*Wire out*		*Wire in*		*Wire out*		*Wire in*	
			*RT***	*Cooled****	*RT*	*Cooled*	*RT*	*Cooled*	*RT*	*Cooled*	*RT*	*Cooled*	*RT*	*Cooled*
Methyl Cellosolve	124.3	0.2	86.5	97.9	87.0	99.3	82.3	98.9	90.0	100.9	52.2	62.2	51.5	69.9
Cellosolve	135.1	0.3	90.5	94.4	92.2	96.2	85.4	97.8	89.6	99.0	53.9	65.1	53.8	63.9
n-Butyl Cellosolve	170.6	0.4	–	–	–	–	97.0	97.4	94.5	100.1	92.2	99.2	95.5	102.0
n-Butyl carbitol	231.2	1.7	–	–	–	–	–	–	–	–	97.3	98.2	99.9	99.5

* Retention time of carbitol solvent = 1.2 min.
** RT = Collection device at ambient temperature.
*** Cooled = collection device cooled with liquid nitrogen vapour (−90 °).

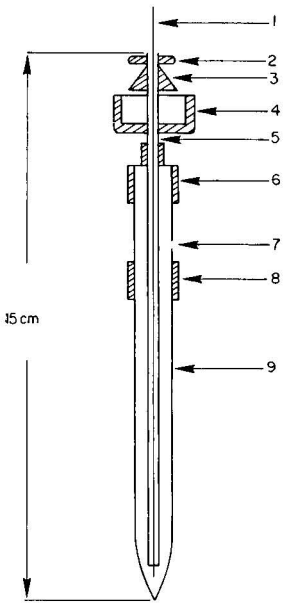

Fig. 1. Collection device assembly. 1 = Stainless-steel insert wire, diameter 0.018 in.; 2 = silicone rubber O-ring, 0.125 in. I.D.; 3 = Swagelok front ferrule, 1/8 in.; 4 = Swagelok nut, 1/8 in.; 5 = coagulation capillary tube, 6 in.; 6 = self-closing rubber stopper, sleeve type; 7 = pressure release vent; 8 = 1-cm length of rubber tubing, 1/4 in. I.D., 1/16 in. wall; 9 = sealed Pasteur pipette.

with the collector in four different modes: (1) at ambient temperature; (2) cooled with nitrogen vapour (−90°) (ref. 9); (3) at ambient temperature with wire insert; and (4) cooled with liquid nitrogen vapour and with wire insert. After peak elution, the collector was disconnected from the chromatograph and 50 μl of carbitol was added through the top end of the capillary tube. The piece of rubber tubing was slid over the vent and the entire assembly was centrifuged at 1500 rpm (g value, 1720) for 1 min. Approximately 1.5 in. of the tip containing the carbitol effluent solution was broken from the receiver. After thorough mixing of this solution, 1-μl samples were injected into the chromatograph for quantitative analysis.

Control samples, representing 100% recovery, were prepared by adding 1 μl of the appropriate Cellosolve or carbitol directly to the capillary tube of a collection device and treating it in the same manner as a recovered sample. All samples and controls were collected in duplicate, after which they were also analysed by duplicate injections. The recovery efficiency was determined by comparing the peak areas (provided by the electronic integrator) from a given sample with those of the corresponding control. The results are shown in Table I.

The above procedure was repeated for samples and controls of 2, 5 and 3×5 μl with the collector at ambient temperature without the wire insert. The results are shown in Table II.

TABLE II

RECOVERY EFFICIENCY OF GAS CHROMATOGRAPHIC EFFLUENTS FOR DIFFERENT SIZED SAMPLES

Collection device at ambient temperature.

Compound	*B.p.* (°C)	*Retention time (min)* *	*Recovery (%)*											
			Detector temperature (°C)											
			135				*170*				*230*			
			Sample size (μl)				*Sample size (μl)*				*Sample size (μl)*			
			*1***	*2*	*5*	*3×5*	*1***	*2*	*5*	*3×5*	*1***	*2*	*5*	*3×5*
Methyl Cellosolve	124.3	0.2	86.5	97.1	96.4	88.6	82.3	86.8	93.0	86.9	52.2	79.6	84.7	77.5
Cellosolve	135.1	0.3	90.5	100.2	94.9	87.8	85.4	85.3	92.3	93.6	53.9	80.3	89.8	82.2
n-Butyl Cellosolve	170.6	0.4	–	–	–	–	97.0	97.4	96.2	92.5	92.2	95.1	95.0	90.9
n-Butyl carbitol	231.2	1.7	–	–	–	–	–	–	–	–	97.3	100.0	99.7	99.2

* Retention time of carbitol solvent = 1.2 min.

** Values taken from Table I.

DISCUSSION

A large number and variety of collectors have been described in the literature, ranging from simple capillary tubes to elaborate coils and electronic precipitators. Among these have been devices with some similarity to the one shown in Fig. 1, that is, with a small-bore tube extending from the gas chromatograph exit port into a larger outer glass tube[10–12]. This design forces the sample-bearing carrier gas to travel a longer path before reaching the atmosphere, which in turn increases the efficiency of collection.

The collector shown in Fig. 1 is judged to possess several advantages. It is built from cheap materials that are readily available in most laboratories, virtually no skills, such as glass blowing, are required to assemble it and consequently the device can be made quickly by the user. The outer glass tube allows for repeated collections of a given GC peak or large injections, when greater amounts of sample are required such as for molecular structure investigations via derivative formation. Samples larger than 3 μl tend to be lost by blow-out when simple capillary tubes are used. The design lends itself to centrifugation and, because of its low cost and ease of construction, there need be no hesitation in sealing a sample in the tip for ampoule-type storage. For short-term storage, the unit is sealed by positioning the piece of rubber tubing over the pressure release vent and removing the capillary tube from the self-closing stopper. Connection to the Model 5750 gas chromatograph is simple, as the exit port of this instrument is equipped wth a 1/8-in. Swagelok fitting, while other instruments could be readily adapted.

The four compounds listed in Table I (methyl Cellosolve, Cellosolve, *n*-butyl Cellosolve and *n*-butyl carbitol) were chosen as test materials because they afforded a temperature range ($>100°$) from a moderate boiling point (124°) to a rather high boiling point (231°), which made it possible to examine the collection efficiency for a range of compounds at different detector temperatures. In addition, compounds boiling above 175° have long been noted for aerosol formation during collection[13], a feature which tends to make recovery more difficult.

There was a small decrease in the recovery of methyl Cellosolve and Cellosolve (Table I, collector at ambient temperature) when the detector temperature was increased from 135° to 170°. A similar loss was noted for *n*-butyl Cellosolve when the detector was set at 230°. In these instances, cooling (−90°) overcame the minor losses. Recovery of both methyl Cellosolve and Cellosolve, however, decreased markedly at the higher detector temperature. The advisability of employing low detector temperatures during the collection of low-boiling effluents has been mentioned previously[14] and is demonstrated here. The detector temperature (as in Tables I and II) must be at least equal to or greater than the boiling point of the compound in order to eliminate possible condensation within the instrument. The results in Tables I and II suggest that the detector can be operated at approximately 50° above the boiling point of the collected compound without serious loss in recovery efficiency. Progressively lower recoveries should be expected if larger differentials between effluent boiling point and detector temperature become necessary.

Warming the capillary tube by pre-flushing (1 min) with carrier gas prior to sample collection serves a dual purpose. The air is removed, thereby protecting

oxygen-sensitive or hygroscopic samples from degradation or contamination and a gradual temperature gradient is developed to decrease aerosol formation. The wire insert was introduced in an effort to lengthen this temperature gradient, but the results in Table I indicate that the effect is small both at ambient temperature and at $-90°$, except for the collection of methyl Cellosolve at detector temperatures of 170° and 230°.

A means of producing a temperature gradient with a metal tube cover for capillary collectors has been reported[15]. This report, however, did not include results obtained when the metal cover was not used. Therefore, comparison of the effectiveness of a metal insert *versus* a metal cover, relative to a straight capillary cannot be made.

In order to simulate the experimental conditions that exist during the collection of a single fraction present in a group of closely eluting peaks, both methyl Cellosolve and Cellosolve were recovered (TCD temperature 170°) by connecting the collection device to the gas chromatograph just prior to peak elution. This procedure eliminated the formation of a temperature gradient and the collection efficiency was reduced by 10–15%.

Table II illustrates the effect of increasing the sample size. In almost all instances the 2- and 5-μl samples provided better collection efficiency than 1-μl and 3 × 5-μl samples. The reason for this behaviour was not established although there may be a regular initial loss. This would represent more on a percentage basis for the 1-μl samples and result in a lower recovery efficiency. Lower recovery efficiency for the 3 × 5-μl than for the 5-μl sample is probably due to the evaporation of previously recovered material during the collection of subsequent samples.

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Note

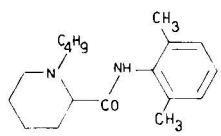
Quantitative micro-determination of 2,6-pipecoloxylidide by gas–liquid chromatography

R. BOUCHE and R. MINETTI

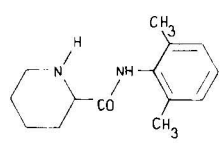
Laboratory for Pharmaceutical Analysis, Medicinal Chemistry Department, Catholic University of Louvain, Van Evenstraat 4, B-3000 Leuven (Belgium)

(Received October 24th, 1973)

Recently, 2,6-pipecoloxylidide (PPX) was detected by gas–liquid chromatography (GLC)[1] in blood and urine samples of patients anaesthetized with bupivacaine (Marcaine®). However, the use of a flame ionization detector limited the determination of this metabolite to a level of about 50 ng. With the aim of lowering the detection limit to the sub-nanogram level, an attempt was made to prepare derivatives with high electron affinity, suitable for electron capture detection. Acylation with heptafluorobutyric anhydride (HFBA), as described by various workers[2,3], was found to be inapplicable, as inconsistent and irreproducible results were observed throughout all the experiments.



Bupivacaine



PPX

The purpose of this paper is to explain this unusual behaviour and to propose a modification of the general method which allows the quantitative micro-determination of PPX.

A primary acylation product was synthesized on a preparative scale and its structure elucidated by means of elemental and spectroscopic analyses. It could not be quantitatively determined by GLC. However, when treated with an aliphatic alcohol it affords a new acylated compound, which was found to be suitable for GLC determination. The structure of this second product was also elucidated.

EXPERIMENTAL*

Preparation and analysis of acylation products

Primary acylation. A 1-ml volume of heptafluorobutyric anhydride (Macherey, Nagel & Co., Düren, G.F.R.) was added to a solution of 50 mg of 2,6-pipecoloxylidide (AB Bofors, Mölndal, Sweden) in 1 ml of ethyl acetate. The mixture was heated in a water-bath at 70° for 30 min, cooled, washed with saturated sodium

* Elemental analysis performed by Janssen Pharmaceutica, Beerse, Belgium.

hydrogen carbonate solution in order to eliminate the excess of reagent, then extracted with diethyl ether. The ethereal solution was washed with water, dried over sodium sulphate and evaporated to dryness. The residue was purified by preparative thin-layer chromatography on silica gel with a solvent mixture of diethyl ether and *n*-hexane (1:3). An oil was obtained ($R_f = 0.52$), which crystallized very slowly. The composition based on the formula $C_{22}H_{18}F_{14}N_2O_3$ was: C 42.32% (experimental 42.09%); H 2.91% (experimental 3.25%); F 42.60% (experimental 43.14%).

Alcoholysis of the primary acylation product. A 1-ml volume of methanol was added to 50 mg of the purified primary acylation product and the mixture evaporated to dryness at 50° to yield white crystals (corrected m.p. 178°). The composition based on the formula $C_{18}H_{19}F_7N_2O_2$ was: C 50.47% (experimental 48.84%); H 4.47% (experimental 4.40%); F 31.05% (experimental 29.31%). The same compound was obtained by treating the primary product with ethanol, *n*-propanol and *n*-butanol.

Infrared and mass spectrometry

A Perkin-Elmer 237 grating infrared (IR) spectrometer was used and mulls of isolated material were prepared with Nujol. An AEI-MS12 mass spectrometer was used with a ionization energy of 70 eV and a chamber temperature of 100°.

GLC determination

Equipment. The apparatus used for this work was a Hewlett-Packard Model 5750 gas chromatograph equiped with an electron capture detector (^{63}Ni) and a 1-m spiral glass column (3.5 mm I.D.). The column was packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, Pa., U.S.A.). The detector was maintained at 300° while the column temperature was 200° and the injection port temperature 250°. The pressure and flow-rate of nitrogen carrier gas were 2.5 kg/cm^2 and 35 ml/min, respectively.

Analytical procedure. The extraction of urine and blood samples was carried out by the method described by Reynolds[1]. The residues were treated with a solution of 0.2 ml of HFBA in 1 ml of ethyl acetate in a water-bath at 70° for 30 min. The reaction mixture was cooled and treated with 2 ml of water saturated with sodium hydrogen carbonate. The aqueous solution, which had a pH between 7.5 and 8.0, was extracted three times with 5 ml of peroxide-free diethyl ether, and the organic phases were combined, washed with 3 ml of water, dried by freezing the water in a cooling bath of solid carbon dioxide and acetone, followed by centrifugation. The ethereal solution was then poured into a ground-glass stoppered Pyrex conical tube, treated with 0.5 ml of methanol and evaporated to dryness at a temperature not exceeding 40°. The final residue was dissolved in a benzene solution of 800 ng/ml of the internal standard (heptafluorobutyrylbenzoctamine). The injected amounts of acylated PPX ranged from 0.5 to 3 ng, corresponding to 0.26 to 1.56 ng of PPX. The calibration curve for the determination was constructed by plotting the ratio of peak heights of PPX and internal standard against the weight ratio of these two compounds, whereby good linearity was observed. The accuracy of the determination was ±3% for amounts of PPX ranging from 0.5

to 1.5 ng and ±5% for 0.25 ng of PPX. Careful siliconization of the tubes reduced the adsorption losses to a considerable extent.

RESULTS AND DISCUSSION

The IR spectrum of the primary product shows CO vibrational absorption bands at 1725 and 1680 cm^{-1}. The NH deformation band that appears at 1540 cm^{-1} in PPX is missing. This observation suggests an acyclic imide structure, CO–NR–CO[4,5]. Moreover, the elemental analysis and the mass spectrum indicate the presence of two heptafluorobutyryl groups in one molecule (Fig. 1). The ions at *m/e* 308 and 280 correspond to the acylated pipecolyl moiety and its product of decarbonylation, whereas the peak at *m/e* 344 is thought to arise as a result of the breaking of the bond between the imide group and the heterocyclic ring. Those results provide strong evidence of the structure of a diacylated PPX, as shown in Fig. 1.

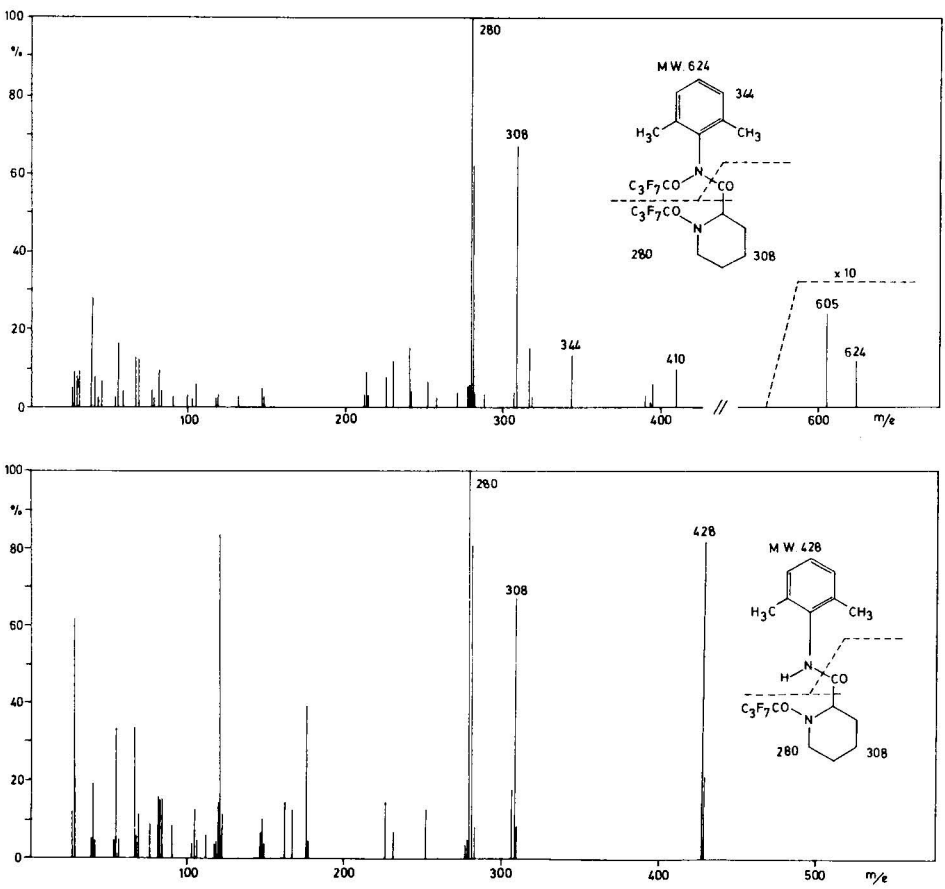


Fig. 1. Mass spectrum of di- and monoacylated products of PPX.

The diacylated PPX, when treated with an alcohol, undergoes rapid and quantitative solvolysis to yield the monoacylated PPX. Its IR spectrum does not show the imide stretching at 1725 cm^{-1} but shows an absorption band at 1530 cm^{-1}, which has been assigned to the NH bending of a secondary amide structure. The elemental analysis and the mass spectrum agree with the presence of a single heptafluorobutyryl group. The ion-fragments at *m/e* 308 and 280 are present but the peak at *m/e* 344 is missing. The product is thus a monoacylated PPX with the structure shown in Fig. 1.

In order to interpret the unusual one-step acylation of the very dissimilar amine and amide groups of PPX, the reaction conditions should be considered. Owing to the fact that HFBA was used without any base catalyst, the presence of a small amount of free acid is inevitable. The amine function of PPX is partially transformed into its conjugated acid, with the result of a decrease in its reactivity towards the anhydride. On the other hand, the trace amount of acid is likely to increase the reactivity of the anhydride towards the amide function, as observed previously[6], so that both the amine and amide are acylated simultaneously. The diacylated PPX contains an imide structure, which is liable to react with an alcohol to yield one amide molecule and one ester molecule.

CONCLUSION

The transformation of PPX into a derivative with high electron affinity by means of HFBA is a useful method for increasing the selectivity and the sensitivity of the chemical determination of PPX by GLC to the sub-nanogram level. However, in order to obtain successful results, the preparation of the derivative requires the treatment of the primary acylation product with an alcohol. This procedure permits the determination of PPX and possibly other aminoacylamine drugs and metabolites in small samples of biological fluid, such as foetal blood or the urine of new-borns.

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Note

Furan derivatives

LII. The gas chromatography of α,β-unsaturated sulphones of the 5-nitrofuran series

E. KOMANOVÁ, A. JURÁŠEK and J. KOVÁČ

Department of Organic Chemistry, Faculty of Chemical Technology, Slovak Technical University, Jánska 1, Bratislava (Czechoslovakia)

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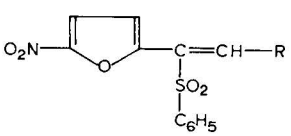
In a previous paper[1], we described a method for the preparation of saturated sulphones of the 5-nitrofuran series with the active methylene group localized between the 5-nitrofuran residue and the SO_2–R group, where R = alkyl, aryl or a heterocyclic ring. As we found, these sulphones undergo a condensation reaction with carbonyl compounds and thus make possible the direct synthesis of 1,1,2-trisubstituted derivatives of ethylene —α,β-unsaturated sulphones of the 5-nitrofuran series. By the condensation of 5-nitrofurfurylphenylsulphone with aldehydes of the benzene series we prepared the corresponding 1-(5-nitro-2-furyl)-1-phenylsulphonyl-2(X-phenyl)ethylenes (I)[2], and with 5-X-2-furaldehydes the 1-(5-nitro-2-furyl)-1-phenylsulphonyl-2-(5-X-2-furyl)ethylenes (II)[3].

The question of the geometric isomers and various conformers in these non-coplanar systems, together with the mechanism of their formation, is discussed elsewhere[2–4].

These 1,1,2-trisubstituted derivatives of ethylene (A) were previously unknown and are interesting from the point of view of both biological activity and physico-chemistry, so that chromatographic studies were thought to be useful.



(A)

R = aldehyde residue

The main purpose of this work was to investigate how to distinguish analytically α,β-unsaturated sulphones of the 5-nitrofuran series. We also studied the effect of substituents on the retention times.

EXPERIMENTAL

Preparation of compounds[1–4]

The starting 5-nitrofurfurylphenylsulphone was prepared by the reaction of 5-nitrofurfuryl nitrate with sodium benzenesulphinate.

Procedure A (derivatives I–XIV). Piperidine (0.5 ml) and ammonium acetate (3.0 g; 0.04 mole) were added to a mixture of 5-nitrofurfurylphenylsulphone (5.4 g; 0.02 mole) and the corresponding aldehyde (0.02 mole) in acetic acid (50 ml), the mixture was refluxed for 5 h, poured on to ice (150 g) and the precipitate was filtered and dried. The crude product was extracted with chloroform and the extract was boiled with charcoal, filtered and concentrated to a small volume. The addition of diethyl ether or light petroleum precipitated the product, which was contaminated with a small amount of starting sulphone. The product was purified by chromatography on an alumina column (16×2 cm, Brockmann activity II), usually using *n*-hexane–ethyl acetate (2:3) as eluent.

Procedure B (derivative XV). A mixture of 5-nitrofurfurylphenylsulphone (2.7 g; 0.01 mole), 5-nitro-2-furaldehyde (2.8 g; 0.02 mole) and acetic anhydride (25 ml) was refluxed for 4 h, cooled in a salt–ice mixture, and the precipitate (unreacted starting sulphone) was filtered off. The filtrate was treated with hydrochloric acid (20 ml; 1:1), the mixture was stirred for 1 h and then poured on to crushed ice (100 g). The precipitate was filtered, washed with diethyl ether and purified chromatographically as described in procedure A.

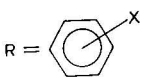
Gas chromatography

The instrument used was a Hewlett-Packard 7620 A research gas chromatograph with a dual hydrogen flame-ionisation detector. A 183 × 0.2 cm steel column packed with Diatoport (80–100 mesh) and coated with 10% UCW 98 silicone gum was used. The flow-rate of the carrier gas (nitrogen) was 20 ml/min. The injector port and detector temperatures were 250° and 270°, respectively, while the column temperature was programmed between 150 and 300° at 8° min.

The retention indices (*I*) were calculated graphically or from the equation given

TABLE I

RETENTION TIMES (t_R) AND RETENTION INDICES (*I*) RELATIVE TO *n*-PARAFFINS OF 1-(5-NITRO-2-FURYL)-1-PHENYLSULPHONYL-2-(X-PHENYL)ETHYLENE

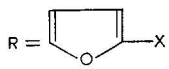


No.	*X*	*m.p.* (°C)	t_R (*min*)	*I*
I	H	136–138	20.18	2418
II	3-Cl	117–120	21.15	2492
III	4-Cl	132–134	22.01	2555
IV	3,4-Cl_2	176–178	24.13	2700
V	4-I	154–157	14.63	1917
VI	4-NO_2	157–158	17.35	2213
VII	4-CN	154–156	12.77	1727
VIII	4-OCH_3	171–172	9.79	1451

by Kováts[5]. For the graphical determination of retention indices, we used the group of paraffins from C_6 to C_{28}, which were chromatographed on UCW 98 under the same conditions.

TABLE II

RETENTION TIMES (t_R) AND RETENTION INDICES (I) RELATIVE TO *n*-PARAFFINS OF 1-(5-NITRO-2-FURYL)-1-PHENYLSULPHONYL-2-(5-X-FURYL)ETHYLENE



No.	*X*	*m.p.* (°C)	t_R (*min*)	*I*
IX	H	115–116	19.23	2351
X	CH_3	113–115	19.74	2386
XI	S-CH_3	124–125	23.20	2638
XII	Br	115–117	20.48	2441
XIII	I	131–132	23.14	2634
XIV	$COOCH_3$	140–142	7.96	1301
XV	NO_2	165–167	14.47	1900

RESULTS

The results in Tables I and II confirm that gas chromatography is a suitable method for the determination of 1-(5-nitro-2-furyl)-1-phenylsulphonyl-2-(X-phenyl)-ethylene and 1-(5-nitro-2-furyl)-1-phenylsulphonyl-2-(5-X-furyl)ethylene. They also show that electron-accepting substituents decrease the value of t_R while substituents with an electron-donor effect shift t_R to higher values.

From this work, it follows that one can apply the method of determination of retention indices described earlier[5,6] to the study of α,β-unsaturated sulphones of the 5-nitrofuran series.

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Note

Preparation and use of surface-modified adsorbents in clean-up techniques for pesticide residue analysis

P. G. BALAYANNIS

State Drug Control Laboratories (K.E.E.F.), Department for Pesticides, Athens (Greece)

(Received October 1st, 1973)

One of the main problems in residue analysis is the clean-up of the first crude extract. The numerous other plant substances that are extracted simultaneously can, in many instances, mask the presence of pesticide residues. Even with more specific analytical techniques such as gas–liquid chromatography (GLC) with electron capture detection for organochlorine compounds, clean-up procedures are necessary in order to obtain reliable results. The nature of the co-extracted plant materials depends on the extraction solvent; in most instances this is a non-polar organic solvent, so the impurities are generally lipophilic.

Adsorption column chromatography and thin-layer chromatography are often used for the clean-up of such extracts. These techniques generally result in separations by compound type and give very good results when the contaminants and the pesticides differ considerably in their polarity. In most instances in residue analysis, however, these differences are minor, and hence overlapping of spots or peaks in the developed chromatograms occurs.

In order to overcome the above disadvantage, the procedure described by Abel *et al.*[1] and adopted by Stewart and Perry[2] was examined and modified. It was considered that, if the active adsorptive sites of a porous material are replaced with aliphatic chains of different lengths, the resolving properties of the material will differentiate between lipophilic compounds in the extract. It would be expected that the modified material will separate the different compounds in the extract not adsorptively, but according to their partition coefficient between the aliphatic chains attached to the surface of the porous particles (stationary phase) and the eluent (mobile phase).

EXPERIMENTAL

Materials

Aluminium oxide: Camag MFC (Hopkin & Williams, Chadwick Heath, Great Britain) was dried by heating for 11 h at 550°. Silica gel: For Chromatographic Adsorption (BDH, Poole, Great Britain) and MFC (Hopkin & Williams) were dried by heating for 11 h at 480°.

Preparation of modified adsorbents

The dry powders were poured into a solution of 11.7 g of octadecyltrichloro-

silane (Aldrich, Milwaukee, Wisc., U.S.A.) in about 500 ml of sodium-dried light petroleum (boiling range 60–80°). The suspension was shaken for 5 h and the light petroleum was then removed by filtration. The powder was dried and placed in a round-bottomed flask. The flask was fitted to a rotary evaporator (Fig. 1) modified so that hot air saturated with water vapour was continuously passed through the powder while the powder was agitated. Fumes of hydrochloric acid were evolved for at least 24 h. The air stream was stopped when no further hydrochloric acid was detected (wet litmus paper) in the exit stream of air.

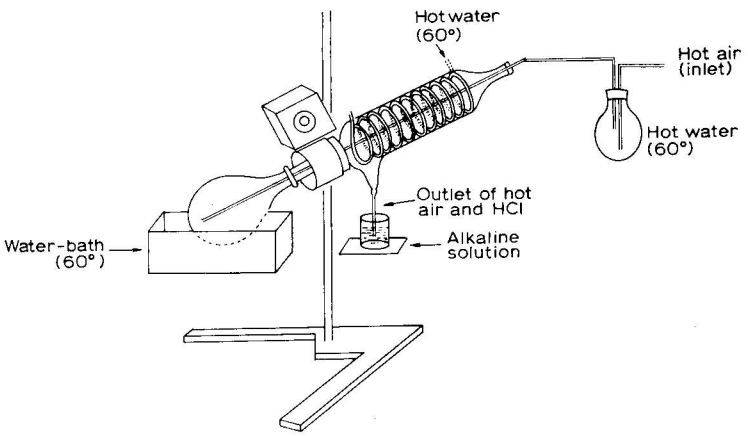

Fig. 1. Modified rotary evaporator used for the dehydrochlorination of the adsorptive material.

The resultant material was dried for 12 h at 60° and 0.1 mmHg. The powder, yellowish in colour, was washed successively with redistilled *n*-hexane, acetone and absolute ethanol and dried. The final fractions of each of the washing solvents were examined by GLC using both electron capture and flame ionization detectors. No peaks other than those due to the pure solvents were observed.

The same procedure was followed for the preparation of powders modified with dimethyldichlorosilane (11.8 g).

Use of modified adsorbents

A 2.5-g amount of the adsorbent was packed in a chromatographic column (15 cm × 0.5 cm I.D.). A 15-cm long extension was fitted on each column and the adsorbent washed with 20 ml of 5% acetone in *n*-hexane (one drop per second) followed by 20 ml of *n*-hexane. Then 2 ml of an *n*-hexane extract from tomatoes to which DDT, DDE and γ-BHC had been added were introduced into the column. The column was eluted with 10 ml of *n*-hexane, and the eluate was collected and concentrated to 1 ml on a water-bath (60°), employing a steam of dry air for evaporation. A 1-μl volume of the concentrate was examined by GLC.

RESULTS AND DISCUSSION

The results obtained from modified alumina or coarse silica gel columns were not very satisfactory. Those obtained from fine-grade silica gel (Hopkin & Williams) were much better.

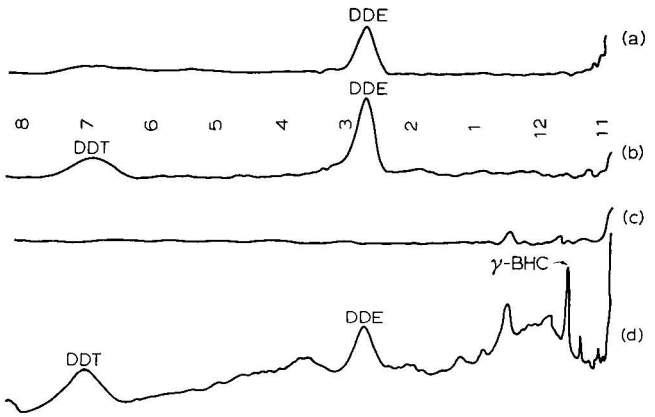

Fig. 2. Gas–liquid chromatograms obtained on the first 10 ml of *n*-hexane eluate from columns containing the modified adsorptive material. Traces: (a) silica gel modified with octadecyltrichlorosilane; (b) silica gel modified with dimethyldichlorosilane; (c) silica gel untreated; (d) *n*-hexane extract with no clean-up.

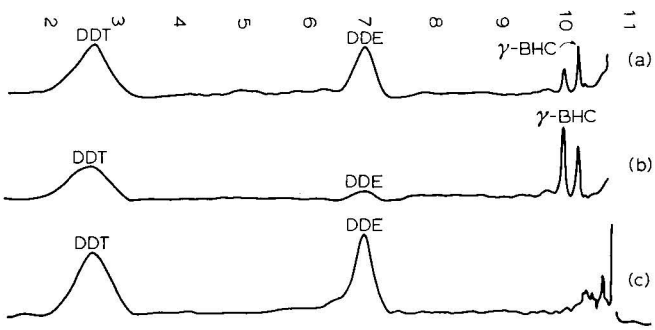

Fig. 3. Gas–liquid chromatograms obtained on the second 10 ml of *n*-hexane eluate from columns containing the modified adsorptive material. Traces: (a) silica gel modified with octadecyltrichlorosilane; (b) silica gel modified with dimethyldichlorosilane; (c) silica gel untreated.

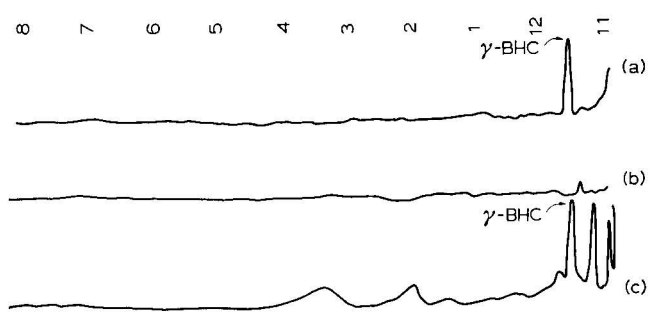

Fig. 4. Gas–liquid chromatograms obtained on the first 10 ml of acetone–*n*-hexane eluate from columns containing the modified adsorptive material. Traces: (a) silica gel modified with octadecyltrichlorosilane; (b) silica gel modified with dimethyldichlorosilane; (c) silica gel untreated.

Figs. 2, 3 and 4 show the chromatograms obtained with such columns. In each case, trace (a) is from silica gel modified with octadecyltrichlorosilane; (b) from silica gel modified with dimethyldichlorosilane; (c) untreated; and (d) with the *n*-hexane extract without any clean-up. It is obvious from Fig. 2 that in order to obtain easily identifiable peaks, it is necessary that the first extract be cleaned up (compare traced with traces a, b and c), and that the untreated silica gel (trace c), with its strong adsorptive sites, retards the elution of the interfering plant compounds (trace d) in addition to the γ-BHC, DDE and DDT when *n*-hexane is used as the eluent. The first millilitre of eluate shows a clear baseline in trace c. However, with modified adsorbents, the results are different (compare trace c with traces b and a).

In trace b, from the adsorbent modified with dimethyldichlorosilane, it seems that DDE is more soluble in the *n*-hexane than in methyl groups attached to the powders. For this reason, DDE is eluted quantitatively and in the first 4 ml of the *n*-hexane eluate. DDT is retained more strongly and only a trace amount is eluted in the first 4 ml of eluate.

In trace a, the result is the same except that DDT does not appear in the chromatogram, probably because of its strong lipophilic properties.

Fig. 3 shows the nature of the next 10 ml of *n*-hexane eluate. The untreated column (trace c) permits both DDE and DDT to be eluted but γ-BHC is retained completely. The dimethyldichlorosilane-modified column permits γ-BHC and DDT to be eluted, plus an unknown compound (first peak) and a trace amount of DDE. However, the octadecyltrichlorosilane-modified column (trace a) permits the remainder of the DDE to be eluted together with the DDT (quantitatively) and a small amount of γ-BHC. The unknown compound (first peak) is also present with the same area as in trace b.

Fig. 4 shows more obviously the selective and differential function of the three columns. The eluent in this instance was 10 ml of a 5% solution of acetone in *n*-hexane. The untreated column results in quantitative elution of the γ-BHC (trace c), but at the same time this peak is accompanied by three unknown peaks due to plant compounds. The dimethyldichlorosilane-treated column (trace b) gives trace amounts of the unknown compound (first peak), while the octadecyltrichlorosilane-treated column (trace a) gives quantitatively a very clear peak of γ-BHC. The next 10 ml of eluate from both the columns with the modified silica gel and the untreated column, containing the pigments of the plant material, are, as a consequence, coloured without the presence of any of the added compounds (γ-BHC, DDE and DDT).

The results obtained are difficult to explain on the basis of the affinities of the pesticides between the compounds used to modify the adsorptive material and the eluent. Nevertheless, it is clear that the compounds are separated not on the basis of their polarity but according to their affinity towards the modified material (compare the results in Fig. 4, traces a and b).

Much more work must be done in order to attain a better understanding of the mechanism of function of these modified columns. Nevertheless, it is obvious that the above technique may have potential in modifying the resolution of certain adsorptive materials. It may be more useful in TLC techniques. The main advantage is the number of parameters that can be changed in order to improve the resolution: the untreated adsorptive material; the number of methyl groups in the aliphatic chain;

and the combination of different eluents. We hope in the future to be able to present more convincing results for both GLC and TLC.

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Note

Vapour pre-adsorption thin-layer chromatography

Preliminary experiments

J. H. DHONT

Central Institute for Nutrition and Food Research TNO, Zeist (The Netherlands)

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The role of the vapour phase in thin-layer chromatography (TLC) has already been discussed in several papers. Geiss *et al.*[1] pre-adsorbed benzene and water vapour on to layers of alumina and silica gel. The influence of chamber saturation on the separation was studied by De Zeeuw and Feitsma[2], De Zeeuw[3], Takeuchi *et al.*[4], and other scientists. De Zeeuw developed a chamber for programmed vapour adsorption[5] which showed remarkable separation possibilities[6,7]. Other chambers for pre-adsorption were described by Takeshita[8] as well as by Suzuki *et al.*[9].

None of the chambers, however, is very suitable for studying the influence of vapour composition on the chromatographic behaviour of organic compounds, since during handling the thin layer is exposed to the moisture present in the laboratory atmosphere.

In this paper an experimental set-up is described which allows TLC plates to reach equilibrium with well defined vapour phase without coming into contact with the atmospheric moisture.

EXPERIMENTAL

Apparatus

The apparatus used is shown in Fig. 1. A is the chromatographic chamber, which consists of a glass tube of 50 cm I.D. The chamber can be filled with the developing solvent from reservoir B by opening stopcock J. Washing bottles C and D are partly filled with the liquid phase of the impregnating vapour (water, methanol, acetonitrile or nitromethane). A controlled stream of dry nitrogen is bubbled through the washing bottles with stopcocks F and G open. In this way chamber A is filled and washed with dry vapour. Stopcock G is connected to an adsorption tube filled with active carbon.

Before the experiment the TLC plate (20 cm × 5 cm) is heated for 2 h at 150°. After activation, the plate is immediately transferred from the oven into chamber A by removing stopper K, which is replaced as soon as the plate is in its proper position. The plate is then allowed to cool during a predetermined time in the stream of vapour-loaded nitrogen.

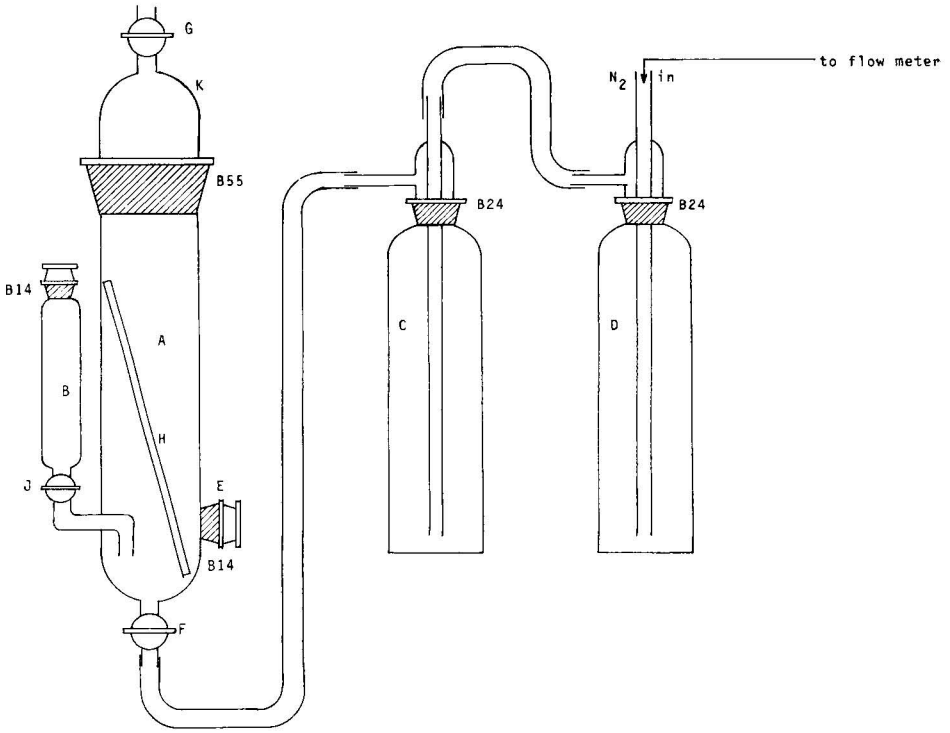

Fig. 1. Experimental set-up for vapour phase impregnation. A = Development chamber; B = solvent reservoir; C, D = washing bottles; E = ground glass joint for spotting purposes; F, G, J, = stopcocks; H = chromatoplate; K = ground glass stopper.

For spotting, stopcock G is closed and by removing ground glass joint E, the plate can be spotted while the nitrogen escapes through E, ensuring that no atmospheric moisture penetrates into chamber A during the spotting procedure.

By simultaneously replacing stopper E and opening stopcock G, the solvent can be run into chamber A with stopcock J open. The silicon tubing, connecting A with the washing bottles, is disconnected at stopcock F.

The experiments were carried out at ambient temperature ($24.5 \pm 1.0\,^{\circ}$). After development, the plate was removed from the chamber and inspected under UV light or sprayed with a suitable reagent.

Preparation of chromatoplates

Glass plates 20×5 cm, were coated with silica gel GF_{254} (E. Merck, Darmstadt, G.F.R). using standard Desaga or Camag equipment. After drying for at least 3 h under laboratory conditions, lines were drawn through the thin layer parallel with the long edges of the plate, about 0.5 cm apart. The starting-point was marked at 2.5 cm from the bottom edge of the plate. A front line was drawn at 11.5 cm above the starting-point.

Impregnating vapours

Nitrogen was bubbled through washing bottles C and D (Fig. 1), both filled with water, methanol, acetonitrile or nitromethane.

Mobile phase

Reagent-grade hexane was used as the mobile phase.

Test compounds

Solutions of the following compounds were used: (1) azobenzene; (2) diphenylamine; (3) dimethyl yellow; (4) methyl anthranilate; (5) *p*-aminoazobenzene.

Detection

Compound Nos. 1, 3 and 5 are coloured and, therefore, visible in daylight; No. 2 could be located by inspection under UV light of 254 nm; No. 4 was detected by its strong fluorescence in filtered UV light.

RESULTS AND DISCUSSION

When the plates were allowed to cool in a stream of vapour-saturated nitrogen for different periods of time, complete equilibrium was obtained for the non-aqueous vapours (Figs. 2–4). Equilibrium was thought to be reached as soon as the R_F values became constant. However, the system containing water-loaded nitrogen was an exception. In the atmosphere of nitrogen saturated with water vapour, it was impossible to reach equilibrium, even after 18 h (Fig. 5). The thin layer of silica gel then became very brittle and finally dropped off from the glass support. Better results were obtained when the nitrogen was bubbled through water saturated with NaCl. Even when exposing the plate to a lower water-vapour pressure, it took an unexpectedly long time to reach equilibrium, *i.e.* 5 h.

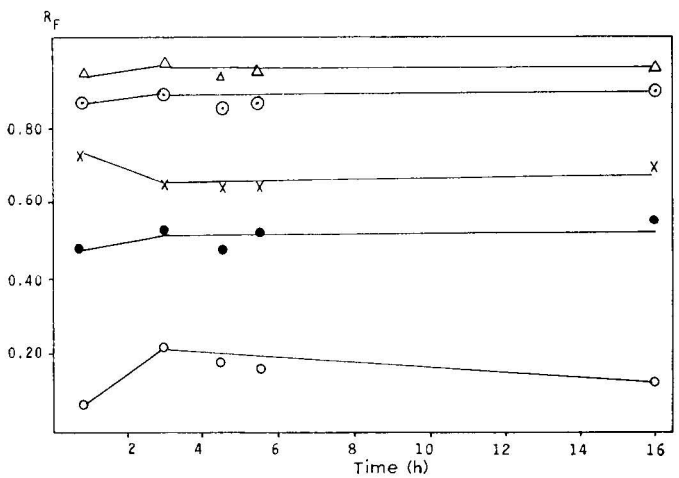

Fig. 2. Relationship between equilibration time and R_F value for the system silica gel/methanol/hexane. △, Azobenzene; ×, diphenylamine; ⊙, dimethyl yellow; ●, methyl anthranilate; ○, *p*-aminoazobenzene.

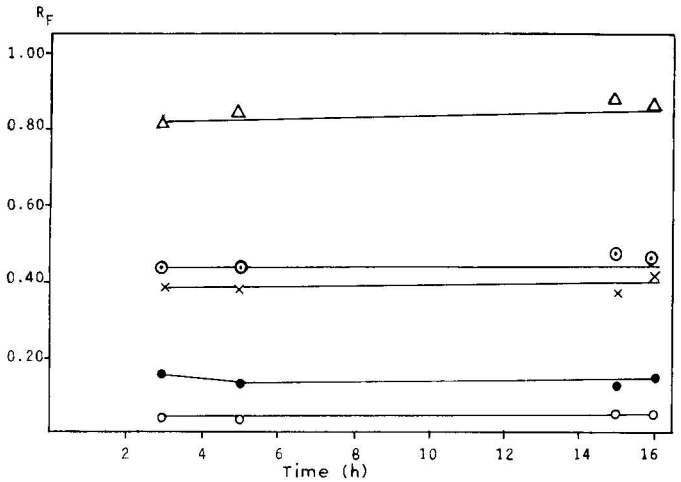

Fig. 3. Relationship between equilibration time and R_F value for the system silica gel/nitromethane/hexane. For explanation of symbols, see the legend to Fig. 2.

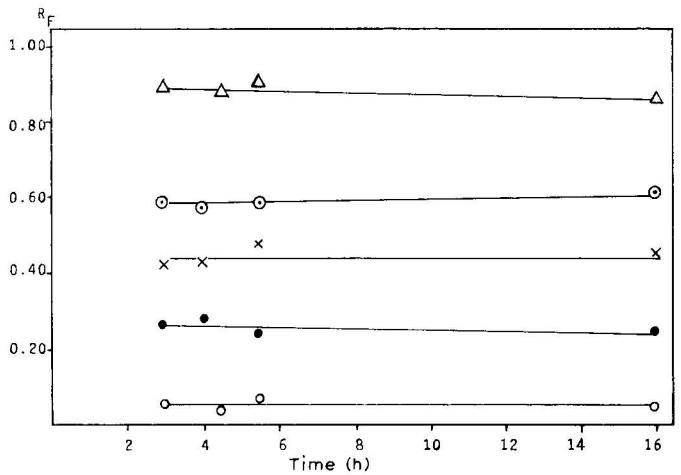

Fig. 4. Relationship between equilibration time and R_F value for the system silica gel/acetonitrile/hexane. For explanation of symbols, see the legend to Fig. 2.

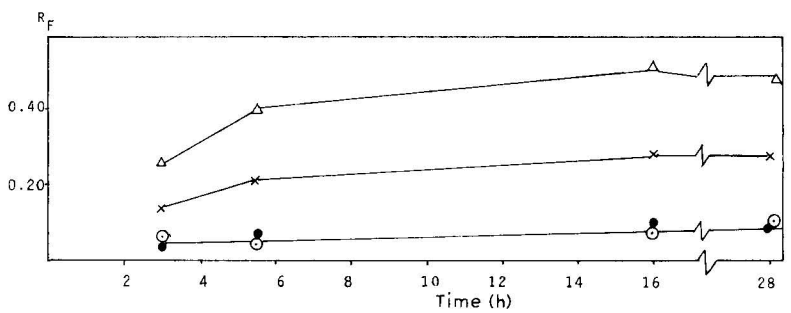

Fig. 5. Relationship between equilibration time and R_F value for the system silica gel/water/hexane. For explanation of symbols, see the legend to Fig. 2.

Contrary to the experiments with water, vapour equilibrium for the other vapours was reached in a much shorter time, *i.e.* 3 h. Suzuki *et al.*[9] exposed their plates for 5 to 50 min to a vapour-saturated nitrogen stream in a similar arrangement to the one described, except that the plates in Suzuki *et al.*'s arrangement could not be protected against atmospheric moisture. After 50 min no equilibrium was reached in their experiments, as can be seen from the graphs published in their paper.

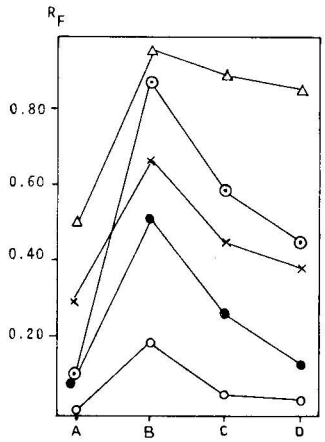

Fig. 6. Chromatographic spectra of the compounds studied using (A) water, (B) methanol, (C) acetonitrile, or (D) nitromethane as the impregnating vapour. For explanation of symbols, see the legend to Fig. 2.

In some experiments washing bottles C and D were filled with concentrated sulphuric acid. The plates could then be spotted and developed in their fully activated state. On these plates R_F values were zero, except the R_F value for azobenzene, which was about 0.15. It was thus shown that differences in R_F value obtained with different vapours are due only to adsorption of vapour on to the silica gel surface. The influence of adsorbed vapour on the R_F value is shown in Fig. 6.

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Note

Xerox copying, a simple method for recording chromatograms

R. FELICI, E. FRANCO and M. CRISTALLI
Laboratorio di Cromatografia del C.N.R., Via Romagnosi 18/A. Rome (Italy)
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During work on the kinetics of some cobalt(III) complexes in this laboratory, the need arose to make a permanent record of numerous thin-layer chromatograms. Photographic methods have been advocated for this purpose and commercial apparatus is available from several sources, but we did not possess the required equipment and even if it had been available the costs involved per chromatogram would still have been considerable. We were therefore rather pleased to find that our Xerox copier (Xerox Model 422) could be used to give excellent reproductions, especially as previous attempts with other Xerox machines gave poor results.

We have compared various copying machines in order to select the optimum conditions and feel that our results may be of interest to other workers.

The chromatograms to be recorded were silica gel thin layers on glass plates that had been sprayed with ammonium sulphide to yield dark brown spots of cobalt sulphide on a white background. It must be emphasized that while these brown spots were copied very well, more intense blue spots, *e.g.* those of proteins coloured with bromophenol blue, were reproduced very poorly. It is know that Xerox machines, which involve reflection of blue/green light, will not reproduce blue and green colours satisfactorily.

The Xerox Models 7000 and 3600 copiers, in addition to giving rather weak copies, were ruled out because they have curved windows. Several thin layers were copied on two different copying machines for direct comparison. These results are shown in Fig. 1. and show clearly that the Xerox Model 4000, the DEDEM copier and the Gevafax 50 (Agfa) copier all gave weaker copies than the Xerox 422 model used by us.

We then tried to establish whether the copy had a lower sensitivity than the original thin layer and chromatographed a series of dilutions of one of our mixtures on one thin layer. The result is shown in Fig. 2a. On the original, the last dilution is a hardly visible spot while on the copy it is not visible. There is therefore a decrease in the sensitivity but several workers in our laboratory failed to see a difference between the copy and the thin layer.

Finally, we found that the intensity could be improved by re-copying the first Xerox copy, as shown in Fig. 2b and c. The background, however, increases simultaneously, so that while there may be some advantage in making a first re-copy, there is certainly none in a second.

Although the Xerox Model 422 has several positions for higher and lower intensity, these do not seem to alter the result as far as the copying of chromatograms is concerned.

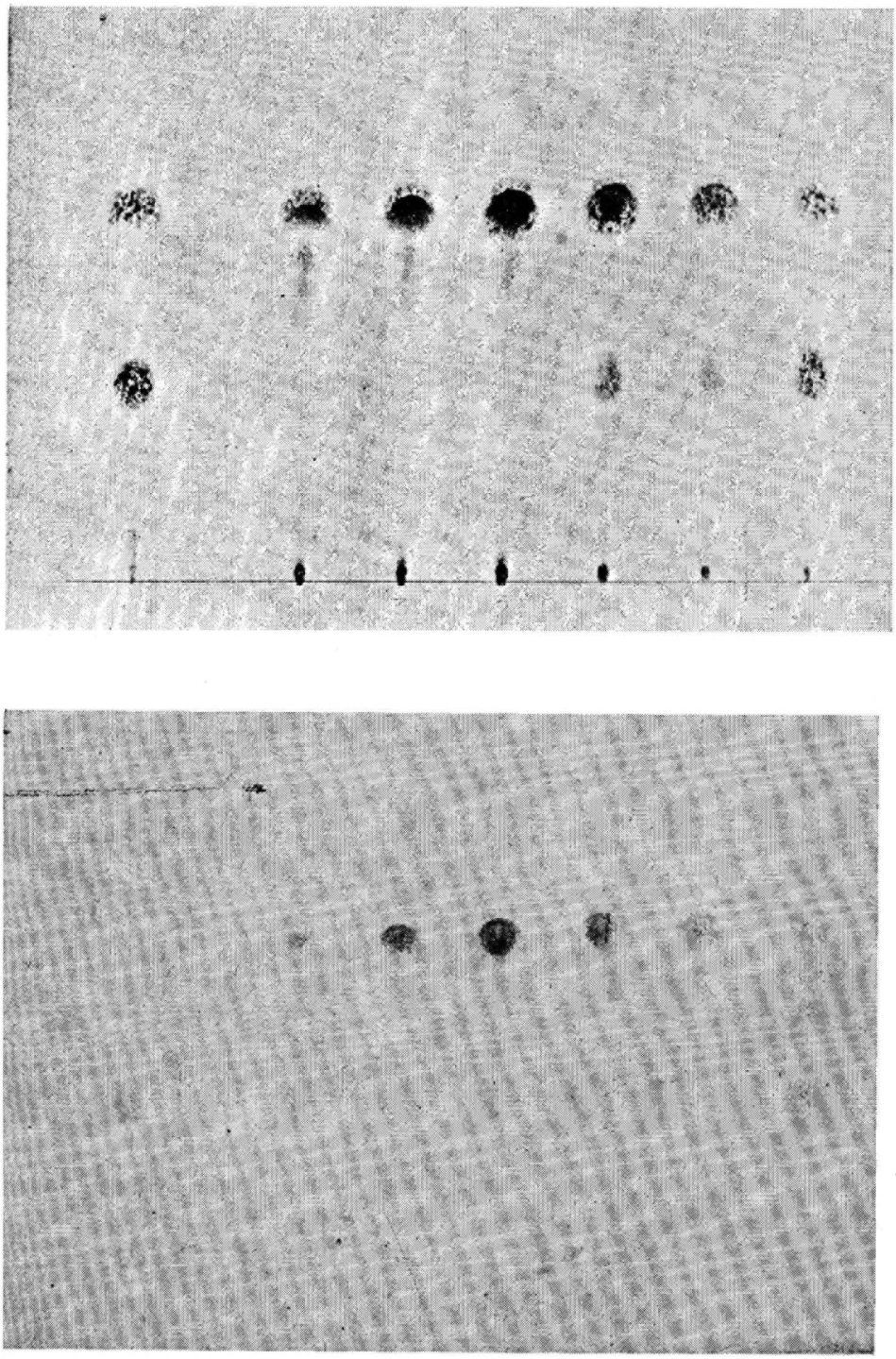

Fig. 1a.

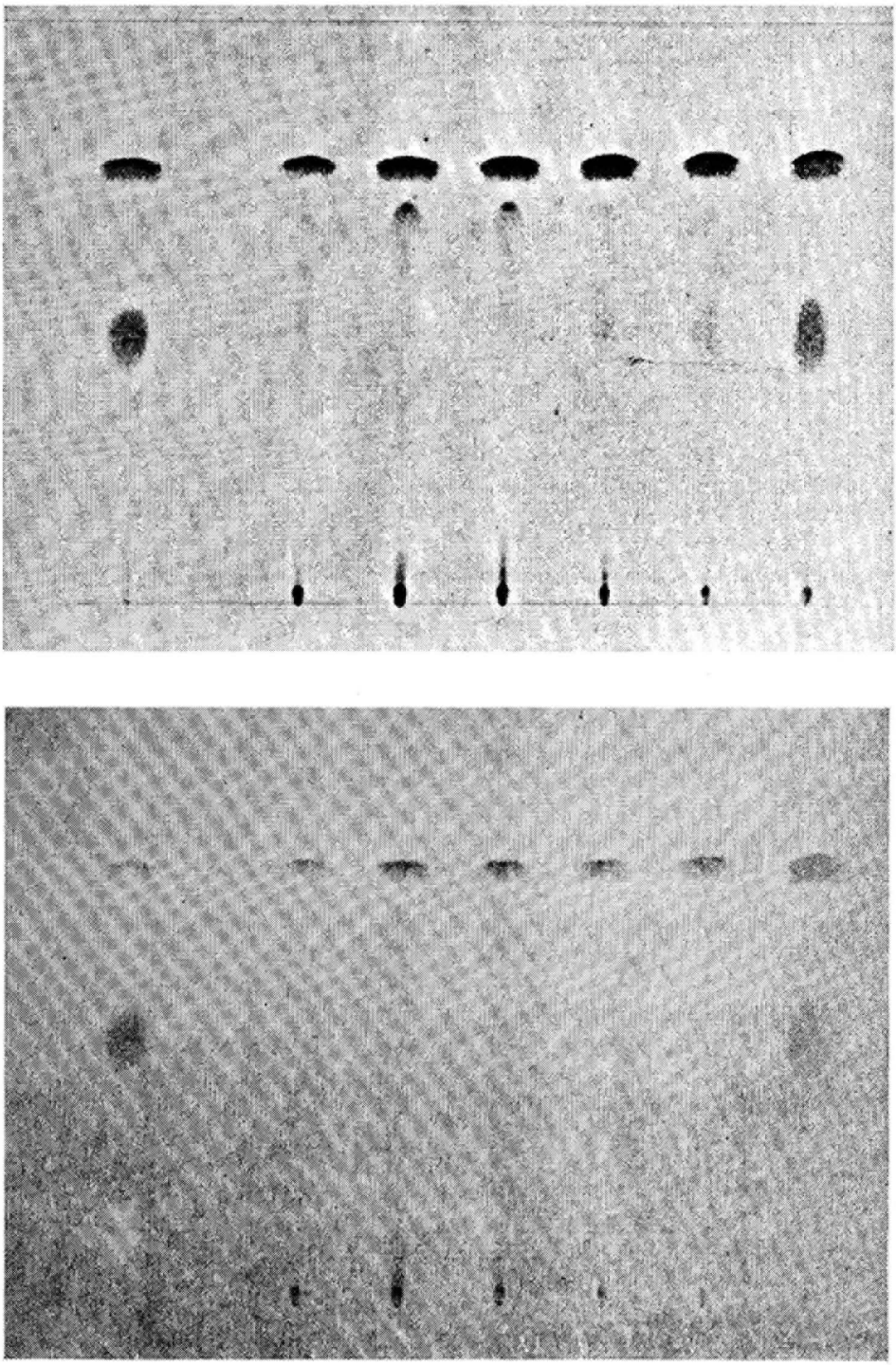

Fig. 1b.

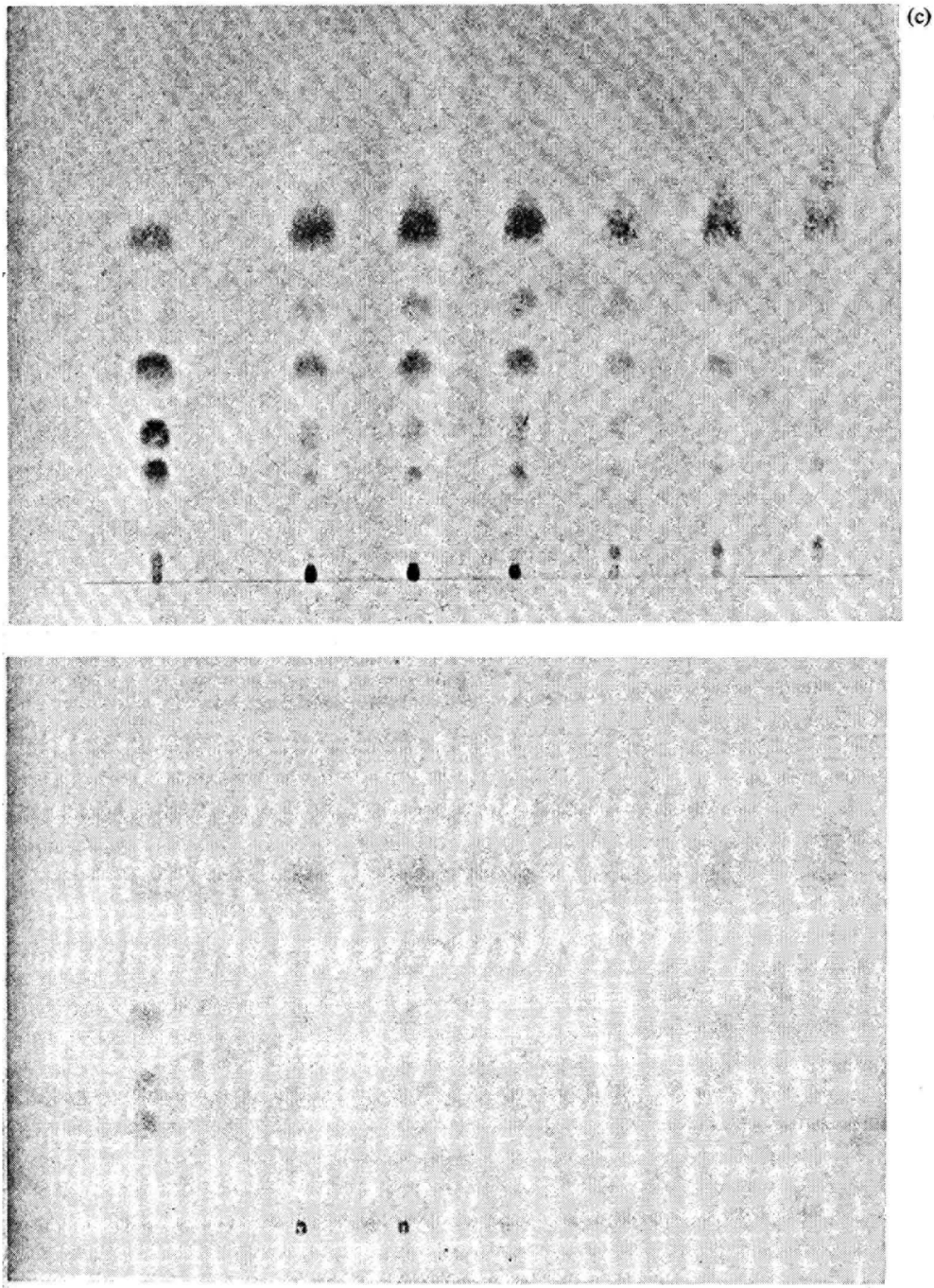

Fig. 1. (a) The same chromatogram reproduced on a Xerox 422 (above) and a Gevafax 50 copier (below). (b) Comparison of another chromatogram copied on a Xerox 422 (above) and a DEDEM copier (below). (c) Comparison of the Xerox 422 (above) and Xerox 4000 (below) copiers on another chromatogram.

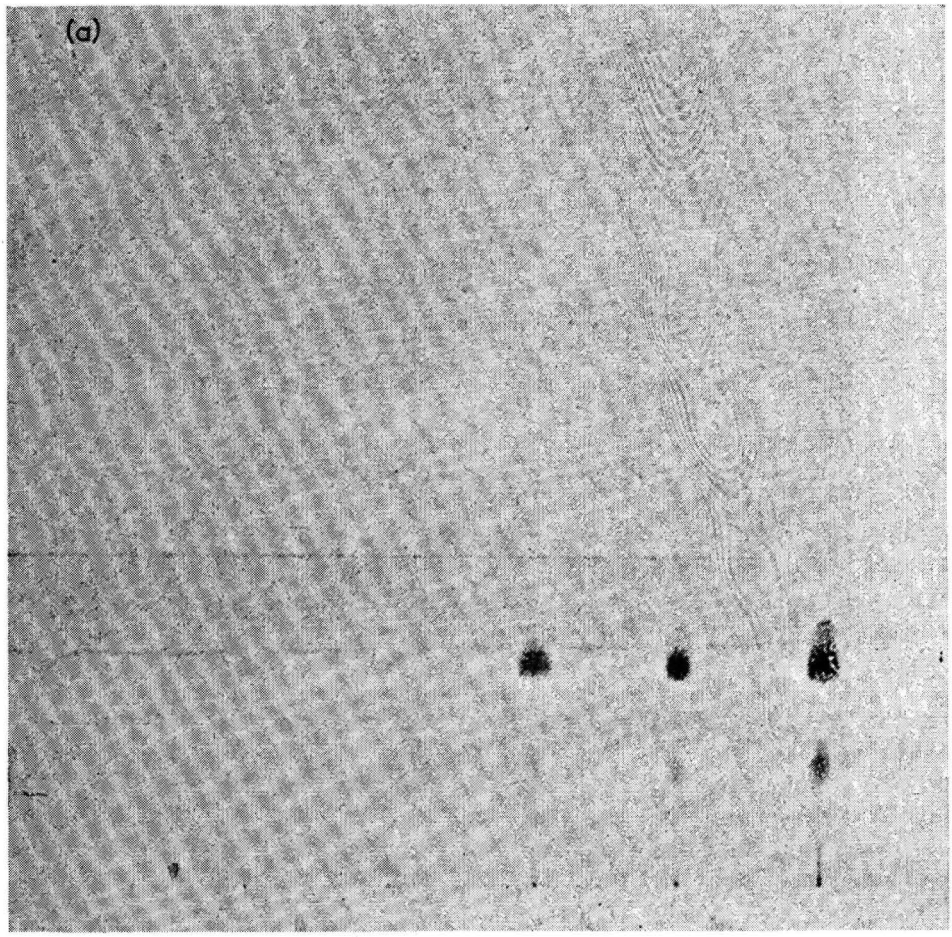

Fig. 2a.

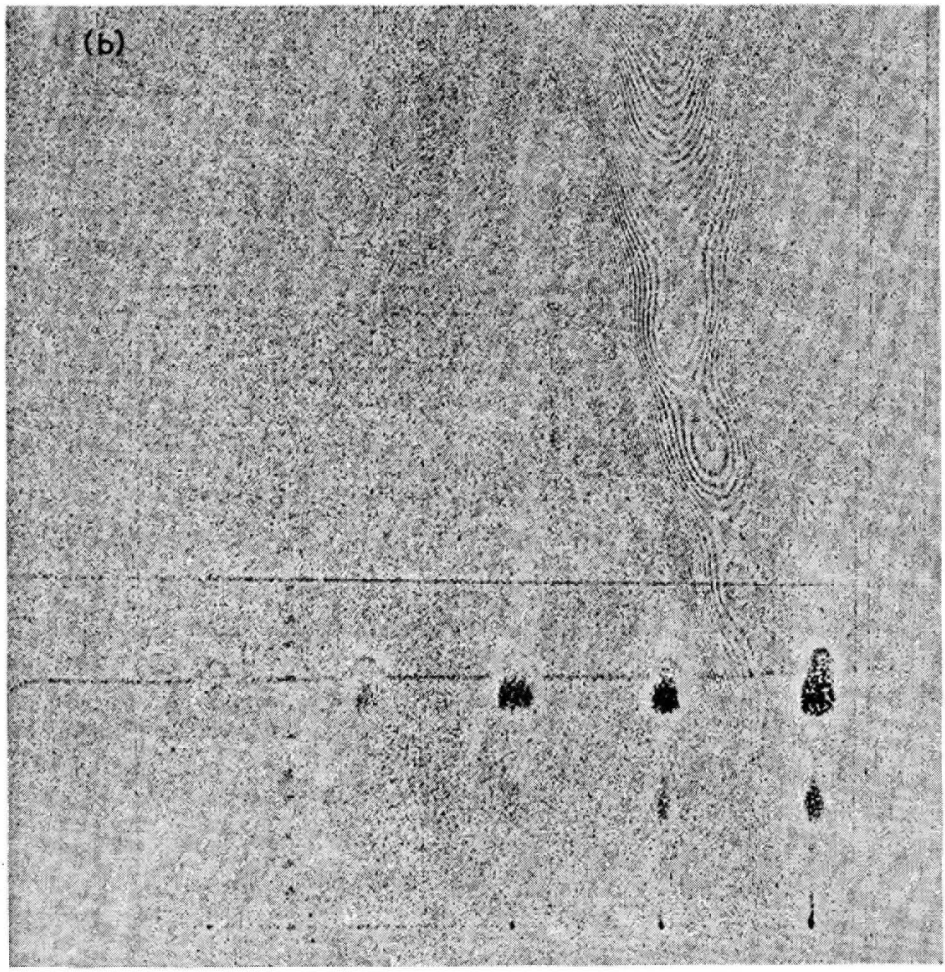

Fig. 2b.

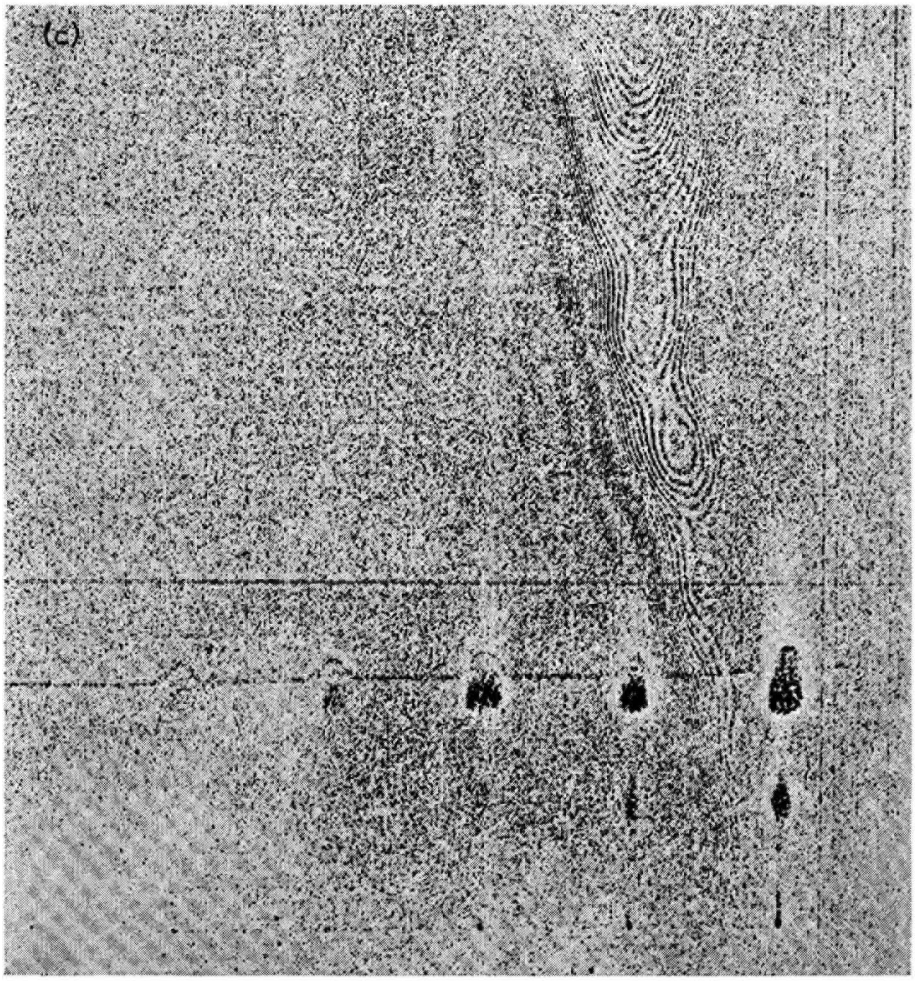

Fig. 2. (a) A chromatogram of various dilutions of the same solution. A faint spot is still visible for a concentration of $5 \cdot 10^{-5} M$. (b) Xerox copy of (a). (c) Xerox copy of (b).

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Note

Redox flat-bed techniques

A study of papers loaded with amalgams

A. MESSINA

Laboratorio di Cromatografia del C.N.R., Via Romagnosi 18/A, Rome (Italy)

(Received November 16th, 1973)

Filter-paper has been known as a support for sensitive precipitation and colour reactions for a long time and has been used extensively in the field of spot tests.

The obvious advantages of filter-paper or flat beds for partition chromatography and electrophoresis have been pointed out repeatedly. Other chromatographic processes, such as adsorption with loaded papers and ion exchange, have also been adapted to flat-bed techniques. Oxidation–reduction reactions in a flat-bed arrangement have so far been limited to a few experiments with papers loaded with redox resins[1]. On the other hand, redox column techniques have been used extensively in analytical chemistry, for example the Jones reductor. We therefore felt that it would be interesting to study the possibilities of flat-bed redox reactions using papers loaded with metal amalgams. Such papers are readily prepared, if the amalgam has the consistency of a soft solid, by rubbing the amalgam on pieces of filter-paper.

EXPERIMENTAL

Amalgams of zinc, lead and cadmium were prepared by heating the required amounts of the metal and mercury in a crucible with occasional stirring. Silver amalgam was best prepared by grinding silver powder with mercury in a mortar. The amount of metal giving an amalgam that can be rubbed on paper was established by trial and error, and the proportions used are given below in each instance.

Whatman No. 1 strips (usually 4×20 cm) were washed with 1 *N* hydrochloric acid and water and dried, and then loaded by rubbing the amalgam of the right consistency over both sides (using rubber gloves). It takes little experience to prepare very uniformly loaded strips. The first 2 cm were usually left free from amalgam (where the paper dips into the solvent) and zones of various lengths were made along the strips.

The metal ions to be studied were solutions of *ca.* 0.01 *M* in 0.5 *N* hydrochloric acid, and 1–2 μl were applied just below the amalgam zone and developed at room temperature by the ascending technique in glass jars 25 cm high and 11 cm wide. The solvent was usually allowed to move 16–17 cm and the metal ions, which had moved out of the amalgam into the upper, uncovered part of the paper strip, were detected with the usual spot tests such as rubeanic acid for copper(II), ammoniacal silver nitrate solution for manganese, α-nitroso-β-naphthol for cobalt, potassium hexa-

cyanoferrate(II) and hexacyanoferrate(III) for iron(III) and iron(II), respectively, tin(II) chloride for palladium, and potassium iodide for bismuth(III).

RESULTS

Zinc amalgam

Amalgams containing 18.2% and 28% of zinc were prepared and zones from 0.5 to 8 cm in length loaded on to paper strips.

The following metals did not leave the amalgam zone and were presumably reduced: bismuth(III), copper(II), lead(II), cadmium(II) and antimony(III). Nickel(II) and cobalt(II) were still detected after passing the amalgam but the spots observed were much weaker, indicating partial reduction in the amalgam.

Iron(III) was completely reduced if the amalgam zone was 5 cm or longer, while 1 cm of amalgam allowed both iron(II) and iron(III) to leave the amalgam zone. In 0.5 cm of amalgam, only iron(III) was washed out, *i.e.*, the reduction had not even started.

Manganese(II) was not reduced at all and could therefore be separated readily from the completely reduced metals above.

Lead amalgam

An amalgam containing 43% of lead proved suitable, and the papers were loaded with an amalgam zone 8 cm long. Copper(II) and bismuth(III) were completely retained on such amalgam zones, while nickel(II), cobalt(II), cadmium(II) and manganese(II) travelled out of the zones unreduced.

Cadmium amalgam

The amalgam contained 14.8% of cadmium. Papers with zones of 7 and 8 cm of this amalgam were prepared and were found to reduce bismuth(III), copper(II) and lead(II) but not nickel(II), cobalt(II) and manganese(II). For the complete conversion of iron(III) into iron(II), the amalgam zone had to be 20 cm long, otherwise iron(III) could still be detected.

Silver amalgam

The amalgam contained 16% or 17.7% of silver. Zones 8 cm and 20 cm long did not reduce bismuth(III), but both palladium(II) and gold(III) were completely reduced. Copper(II) was only partially reduced while platinum(IV) was not completely reduced in 8-cm zones but was reduced in a 20-cm zone. Very dilute solutions of platinum(IV) could be reduced in shorter zones. Platinum(II) was also tried and was reduced analogously to platinum(IV).

DISCUSSION

The reduction observed on amalgam papers is that expected from the electromotive series, except for cobalt and nickel, which are reduced rather slowly, a phenomenon that is also observed in polarography as well as in static experiments[2].

Iron(III) can be reduced to iron metal on amalgam paper, but this seems not to be the case in a Jones reductor.

The use of amalgam papers may be of interest when one requires to remove a large number of metals from one other metal, and from these experiments it seems to be relatively simple to plan suitable conditions for most individual cases.

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Note

Simultaneous gas chromatographic separation of volatile organic sulphur compounds and C_1–C_4 hydrocarbons

F. RAULIN and G. TOUPANCE

Université Paris Val de Marne, Laboratoire d'Energétique Biochimique, 94000-Créteil (France)

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In the course of investigations on the formation of volatile biochemical precursor compounds in methane–hydrogen sulphide atmospheres[1], it appeared necessary to carry out an extensive gas chromatographic separation of volatile C_1–C_4 hydrocarbons and organic sulphur-containing compounds.

Several types of supports and liquid phases have already been used for the gas chromatographic separation of organic sulphur compounds, especially for the homologous series of aliphatic thiols and sulphides. These studies were reviewed recently[2,3]. Some of the previously reported analyses include the results of only a few low-molecular-weight sulphur compounds[4–9]. In addition, the C_1–C_4 hydrocarbons may interfere in the separation[7], and even when there is no interference between volatile organic sulphur compounds and C_1–C_4 hydrocarbons, the separation of the hydrocarbons is poor[10,11].

This paper describes the simultaneous separation of volatile sulphur compounds and C_1–C_4 hydrocarbons on a Durapak OPN–Porasil C column, with an exhaustive calibration of this column for the C_1–C_4 alkanethiols and the C_1–C_4 alkyl sulphides. The relative molar responses to the flame ionization detector of the tested sulphur compounds relative to ethanethiol are presented.

EXPERIMENTAL

Apparatus

The chromatograph was a Varian Aerograph Model 1520 equipped with flame ionization detectors and a linear temperature programmer. An Autolab Model 6300 digital integrator was used for determining the peak areas.

Reagents

All the hydrocarbons were purchased from l'Air Liquide S.A. (Le Plessis-Robinson, France), 1,3-propanedithiol from Aldrich (Milwaukee, Wisc., U.S.A.), 1,2-ethanedithiol from J. T. Baker (Deventer, The Netherlands), diethyl disulphide from K & K Labs. (Plainview, N.Y., U.S.A.) and thiophene from Prolabo (Paris, France).

Methanethiol, 1-propanethiol, 2-propanethiol, 2-methyl-2-propanethiol, 1-butanethiol, 2-butanethiol, 1,2-propanedithiol, 1,4-butanedithiol, methyl ethyl sulphide, 1,2-epithiopropane (propylene sulphide) and tetrahydrothiophene were Fluka (Buchs, Switzerland) products.

2-Propene-1-thiol, 2-methyl-1-propanethiol, 2-methyl-2-propene-1-thiol, methyl isopropyl sulphide, methyl *n*-propyl sulphide, thiirane (methylene sulphide) and thiacyclobutane (trimethylene sulphide) were purchased from Pfaltz and Bauer (Flushing, N.Y., U.S.A.), and ethanethiol, dimethyl sulphide and diethyl sulphide from Schuchardt (Munich, G.F.R.).

Chromatography

The chromatographic column was a 2 m long, 1.5 mm I.D. PTFE tube packed with Durapak OPN–Porasil C, 80–100 mesh (Waters Ass., Framingham, Mass., U.S.A.). The Durapak packing is impregnated with the liquid phase (OPN, *i.e.* β,β'-oxidipropionitrile), chemically bonded to the siliceous support (Porasil C).

The temperature programme was: isothermal at 20° for 6 min, then increased at 2°/min to a final temperature of 130°, held for 10 min; the temperature of the injector was 200° and that of the detector (FID) was 210°C. The flow-rates employed were: carrier gas (nitrogen), 25 ml/min; hydrogen, 30 ml/min; and air, 300 ml/min.

RESULTS AND DISCUSSION

Fig. 1a shows a chromatogram for a sample containing C_1–C_4 hydrocarbons. A good separation of these hydrocarbons is obtained within 5 min, during the 20° isothermal step of the temperature programme. Nevertheless, ethane and ethylene, and isobutene and *trans*-butene-2, are co-eluted.

Fig. 1b represents the gas chromatographic analysis of virtually all of the volatile organic sulphur compounds that contain 1–4 carbon atoms. They are all eluted after the isothermal step of the temperature programme, so their separation does not interfere in the separation of the C_1–C_4 hydrocarbons. All of the alkanethiols and alkyl sulphides and most of the unsaturated thiols and sulphur heterocyclics containing 1–4 carbon atoms were present in the sample. In addition, some sulphur compounds with higher boiling points, including dithiols and disulphides, were also injected for comparison and extensive calibration of the column. There is no separation between ethyl methyl sulphide and 2-butanethiol, and 2-methyl-1-propanethiol and 2-methyl-2-propene-1-thiol, but all of the other sulphur compounds can be distinguished.

A quantitative study of the gas–liquid chromatography of these compounds was performed by determining the relative molar response (*RMR*) of each sulphur compound with respect to ethanethiol. The *RMR* values are given in Table I for each sulphur compound, according to structural groups present; boiling points and retention temperatures are also presented.

Theoretically, this list of volatile organic C_1–C_4 sulphur compounds is not exhaustive. From the behaviour observed on the Durapak column of the different homologous series of sulphur compounds examined, it is possible to determine approximately the retention temperatures of the missing compounds. Hence the calibration of the column can be considered to be exhaustive for sulphur compounds with retention temperatures up to 80°.

Classical qualitative analysis in gas chromatography is based on the comparison between retention times of known and unknown compounds. However, even with simultaneous injection of standards with the sample to be analyzed,

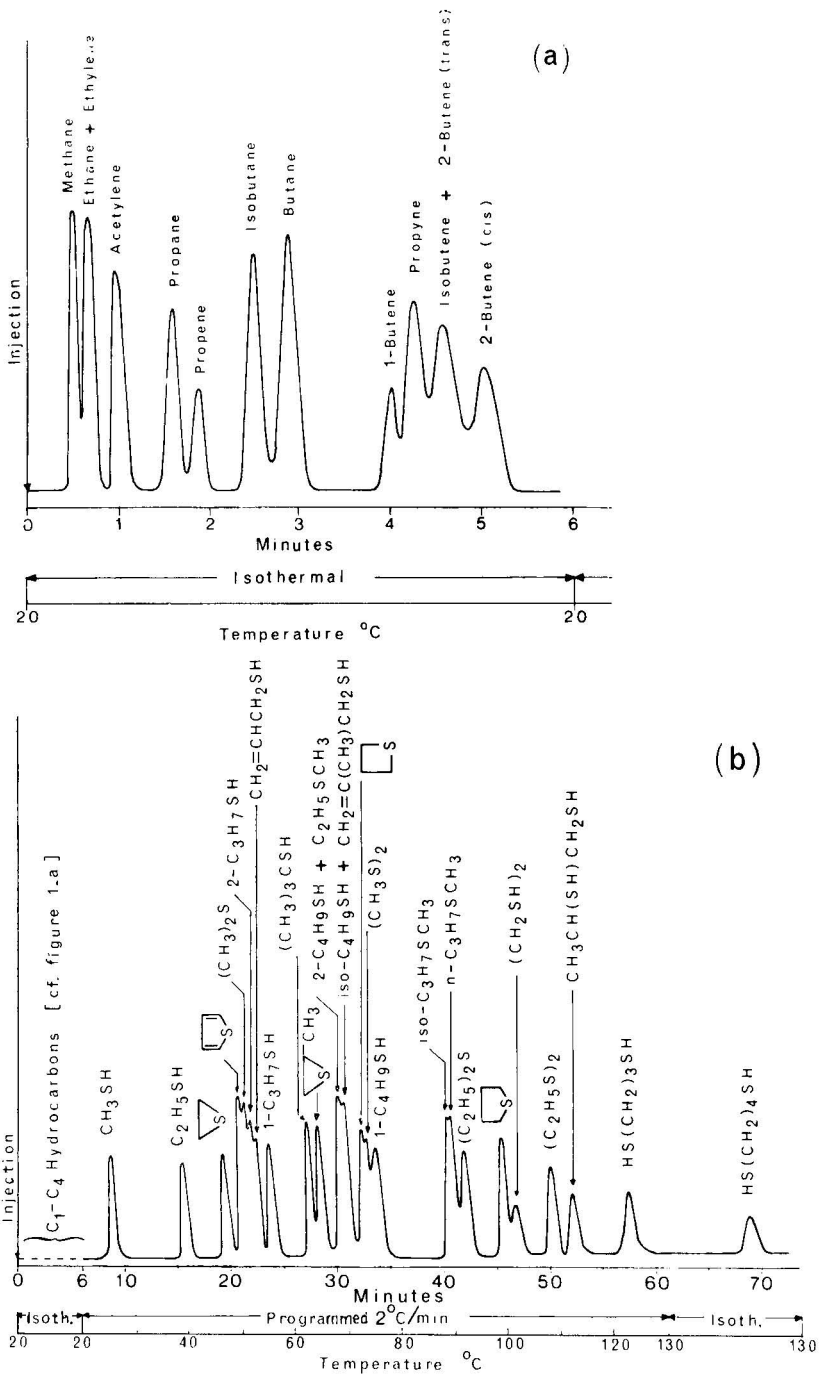

Fig. 1. (a) GLC of a C_1–C_4 hydrocarbons mixture. (b) GLC of 26 sulphur compounds. Amount injected: about 5 μg of each compound. Column: Durapak OPN–Porasil C, 80–100 mesh, 2 m × 1.5 mm I.D., PTFE. Conditions: injector, 200°; detector, 210°; initial temperature, 20°, 6 min initial hold, then 2°/min to 130°; carrier gas, nitrogen at a flow-rate of 25 ml/min. Sensitivity: $5 \cdot 10^{-9}$ A full-scale.

TABLE I

RELATIVE MOLAR RESPONSES, BOILING POINTS AND RETENTION TEMPERATURES OF C_1–C_4 SULPHUR COMPOUNDS

Sulphur compound	*RMR*, sulphur compound/EtSH*	*B.p. (°C)*	*Retention temperature (°C)*
Alkanethiols			
Methanethiol	0.56	6	25.3
Ethanethiol	1.00	33–36	39.0
2-Propanethiol	1.59	57–60	51.7
1-Propanethiol	1.55	66–68	55.3
2-Methyl-2 propanethiol	2.07	62–67	62.7
2-Butanethiol	1.93	85	68.3
2-Methyl-1-propanethiol	1.80	88	69.7
1-Butanethiol	1.84	96–98	75.7
Alkenethiols			
2-Propene-1-thiol	0.67	67–68	53.0
2-Methyl-2-propene-1-thiol	0.46**	90–95	69.7
Dithiols			
1,2-Ethanedithiol	0.86	146	102
1,2-Propanedithiol	1.08	150–160	112
1,3-Propanedithiol	1.48	173	123
1,4-Butanedithiol	1.12**	195–196	130
Sulphides			
Dimethyl sulphide	1.00	37	50.7
Methyl ethyl sulphide	1.40	66	68.3
Methyl isopropyl sulphide	1.70	85	88.7
Methyl *n*-propyl sulphide	2.07	95–96	89.3
Diethyl sulphide	2.21	92	92.0
Disulphides			
Dimethyl disulphide	0.91	116–118	74
Diethyl disulphide	1.80	153	108
Heterocyclics			
Thiirane	0.99	55–56	46.7
1,2-Epithiopropane	1.52	72–75	64.7
Thiophene	2.39	84	49.3
Thiacyclobutane	1.58	94	72.7
Tetrahydrothiophene	2.17	119	99.0

* Average of three independent analyses; ethanethiol as reference.
** Non-quantitative elution.

there is the risk of incorrect assignment of the identity of peaks, owing to coincidence of the retention values of several compounds at a particular position on the chromatogram. Nevertheless, if the peaks to be identified are located in the area where exhaustive gas chromatographic calibration has been carried out, the identification can be achieved with certainty.

The exhaustive calibration of the Durapak OPN–Porasil C column for retention temperatures up to 80° has been used successfully in our laboratory for qualitative and quantitative analyses of gaseous samples containing C_1–C_4 hydrocarbons and volatile organic sulphur compounds[1].

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We thank Dr. S. Roumazeilles of the S.N.P.A. Research Centre, Pau, France, for helpful technical data.

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CHROM. 7245

Note

Fluorimetrische Bestimmung von Nitrosaminen nach säurekatalysierter Denitrosierung und Derivatisierung mit 7-Chlor-4-nitrobenzo-2-oxa-1,3-diazol

H.-J. KLIMISCH und L. STADLER

Forschungsinstitut der Cigarettenindustrie e.V., D 2 Hamburg 54 (B.R.D.)*

(Eingegangen am 4. Oktober 1973)

Die Methodik der Spurenanalytik flüchtiger N-Nitrosoverbindungen ist in letzter Zeit vielseitig untersucht worden. In Kombination mit Anreicherungs- und clean-up-Verfahren war die Ausarbeitung spezifischer und empfindlicher Nachweismethoden von Interesse. Der direkte und damit spezifische gaschromatographisch–massenspektrometrische Nachweis der Nitrosamine ist meist nur bei höheren Nitrosaminkonzentrationen und einem sehr intensiven clean-up möglich. Andere Bestimmungsmethoden basieren ebenfalls auf dem Erhalt der N–N-Bindung. Sowohl die Reduktion der Nitrosamine zu Hydrazinen als auch die Oxidation zu Nitraminen führen unter einheitlichen Reaktionsbedingungen bei Nitrosamingemischen zu unterschiedlichen und teilweise niedrigen Ausbeuten. Ein gewisser Verlust an Spezifität stellt die gut reproduzierbare säurekatalysierte Denitrosierung[1] mit anschliessendem Nachweis der Amine dar. Sie führt dann zu sicheren Aussagen, wenn eine sorgfältige Abtrennung der freien Amine vorgeschaltet und eine Contaminierung mit Aminen vermieden wird. Bisher ist der Nachweis der Amine vorzugsweise durch Derivatisierung zu halogenhaltigen Amiden mit anschliessender gaschromatographischer Bestimmung mit dem "electron capture detector" vorgenommen worden[2–5]. Eine weitere Derivatisierung mit anschliessender fluorimetrischer Bestimmung der Amide wurden mit Dansylchlorid ausgeführt[2].

In einer eingehenden Untersuchung haben wir die Vorteile des 7-Chlor-4-nitrobenzo-2-oxa-1,3-diazols (NBD-Cl) für den mikroanalytischen Nachweis von Aminen[6] beschrieben. In der folgenden Arbeit beschreiben wir die Derivatisierung der Amine mit NBD-Cl nach der säurekatalysierten Denitrosierung der Nitrosamine.

MATERIAL UND METHODEN

Reagenzien

NBD-Cl (Merck, Darmstadt, B.R.D.) wurde aus Methanol–Wasser umkristallisiert und anschliessend sublimiert. Es enthielt dann keine fluoreszierenden Verunreinigungen mehr. Schmelzpunkt: 96–96.5°. Alle Lösungsmittel wurden frisch destilliert und über Säulen (Al_2O_3, sauer, W-200; Woelm, Eschwege, B.R.D.) gereinigt. Die Nitrosamine N-Nitrosodimethylamin und 1-Nitrosopiperidin (Schuchardt, München, B.R.D.) wurden frisch destilliert. Zur Denitrosierung wird eine Lösung von 3% HBr in Eisessig, Suprapur (Merck) hergestellt.

* Direktor: Prof. Dr. W. Dontenwill.

Denitrosierung und Derivatisierung mit NBD-Cl

125 µl einer Lösung der Nitrosamine in Methylenchlorid (entsprechend 8.22 µg N-Nitrosodimethylamin bzw. 6.7 µg 1-Nitrosopiperidin) wurden zusammen mit 500 µl 3% HBr in Eisessig in einem 1-ml Messkolben gemischt. Der fest verschlossene Messkolben wurde im Wasserbad bei 50° 30 min erwärmt. Nach dem Abkühlen wurde zur Trockne eingeengt, der Rückstand in 125 µl Methanol aufgenommen, mit 125 µl einer 0.05% NBD-Cl-Lösung in Methanol und 50 µl einer 0.1 *M* wässrigen $NaHCO_3$ Lösung versetzt, fest verschlossen und 1.5 Std. im Wasserbad bei 55° erwärmt. Nach dem Abkühlen wird bis zur Marke aufgefüllt.

Chromatographie auf Polyamid-11 Folien und in situ fluorimetrische Vermessung

5 µl der Reaktionslösung wurden mit einem Desaga Auftragegerät (Desaga, Heidelberg, B.R.D.) aufgetragen und in einer gesättigten N-Kammer dreimal entwickelt. Fliessmittel: Heptan–Essigsäureäthylester–*n*-Butanol (8:1:1).

Die direkte *in situ* Vermessung der getrennten NBD-Amine erfolgte mit dem Chromatogramm-Spektralphotometer (Zeiss, Oberkochen, B.R.D.) in Fluoreszenzanordnung P-M. Eine Quecksilberdampflampe mit einem Zeiss 436 Filter diente als Anregungsquelle. Bei einer Spaltbreite von 0.3 mm wurde der Emissionsmonochromator für NBD-Dimethylamin auf 530 nm, für NBD-Piperidin auf 537 nm eingestellt. Die quantitative Auswertung erfolgte über einen integrierenden Schreiber Vitatron UR 403. Als Vergleichsstandard wurden bekannte Mengen des entsprechenden NBD-Amins mitchromatographiert. Jeder Fleck wurde fünfmal ausgemessen.

ERGEBNISSE UND DISKUSSION

Bei der Denitrosierung der Nitrosamine mit HBr mussten wir gegenüber den Arbeitsbedingungen von Eisenbrand und Preussmann[1] die Reaktionszeit von 3 auf 15 min verlängern, da die Ausbeuten an Amin bei der kürzeren Reaktionszeit um *ca.* 10% niedriger lagen. Die anschliessende Umsetzung der Amine mit NBD-Cl erfolgte am günstigsten nach Abdestillieren des Eisessigs. Bei einer Neutralisation des Eisessigs mit anschliessender Derivatisierung beeinträchtigen die hohe Salzkonzentration der Lösung oder die erforderliche Verdünnung mit Wasser den Nachweis so geringer Aminmengen. Wie bereits beschrieben[6], erhielten wir bei der *in situ* Vermessung auf Polyamid-11 Folien Eichkurven, die bei der gegebenen Geräteeinstellung von 15–150 ng/Fleck linear waren. Bei einer Konzentration von 5 µg Nitrosamin pro ml erzielten wir bei zehn Bestimmungen folgende "recoveries": N-Nitrosodimethylamin 95.2% und 1-Nitrosopiperidin 89.2%. Dabei lag die Standardabweichung der Bestimmung von N-Nitrosodimethylamin bei 0.58, der Variationskoeffizient betrug 2.6% bei einer range von 93–98.1%.

Im Vergleich zu einer Derivatisierung mit Dansylchlorid ergeben sich wichtige Arbeitserleichterungen. Bei der dünnschichtchromatographischen Entwicklung der Dansylamide muss unter Lichtschutz in Kühlräumen gearbeitet werden. Ausserdem verblasst die Fluoreszenz der Flecken sehr rasch, da die Dansylamide insbesonders auf Kieselgelplatten empfindlich gegenüber hydrolytischen Einflüssen sind[2]. NBD-Amine sind dagegen sehr stabil[6], sodass diese Vorsichtsmassnahmen entfallen. Ausserdem treten keinerlei Störungen durch Eigenfluoreszenz des Reagenzes bzw. durch die bekannten Seitenreaktionen des Dansylchlorids[6] auf.

Die Nachweisempfindlichkeit bei einer *in situ* dünnschichtchromatographischen Vermessung liegt bei beiden Aminderivaten in der gleichen Grössenordnung von 10^{-12} Mol Amin pro Chromatogramm. Dabei lassen sich nach Anreicherungs- und clean-up-Schritten Nitrosaminkonzentrationen von 1 μg/ml mit einer Wiederfindungsrate von 90–95% quantitativ bestimmen.

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CHROM. 7251

Note

Thin-layer chromatography of methylthiohydantoin amino acids

P. RABIN and A. DARBRE

Department of Biochemistry, King's College, Strand, London WC2R 2LS (Great Britain)

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Edman[1] introduced the method of sequencing of peptides or proteins in which the 3-phenyl-2-thiohydantoin (PTH) of the amino acid at the N-terminal end was obtained by stepwise degradation. This method was automated[2,3], with identification of the PTH amino acid using thin-layer chromatography (TLC) or electrophoresis on silica gel plates. TLC of PTH amino acids on polyamide-coated glass plates was used with an iodine–sodium azide spray reagent[4] and on polyamide-coated plastic sheets with a fluorescent indicator[5] for their detection. PTH amino acids could also be identified by gas–liquid chromatography (GLC)[6–8]. PTH arginine did not give a peak on GLC.

A sequencing method was described in which the 3-methyl-2-thiohydantoin (MTH) derived from the amino acid at the N-terminal end of the peptide was identified by GLC[8–10]. However, MTH arginine required prior treatment with cyclohexanedione followed by trimethylsilylation for GLC analysis[10].

Using manual methods for the sequencing of peptides[11], we used GLC for the identification of MTH amino acids. Because arginine could not be identified, we required an independent method, similar to that used for PTH amino acids, which were analyzed by two methods, *viz.* GLC in parallel with TLC[12]. It was stated[9] that TLC was a standard technique for identification of PTH and MTH amino acids (for reviews, see refs. 13 and 14). Stepanov and Lapuk[15] described four solvent systems for the one-dimensional TLC separation of many, but not all of the MTH amino acids on silica gel glass-coated plates. We describe here a separation achieved in less than 1 h using two-dimensional TLC on polyamide pre-coated plates.

EXPERIMENTAL

Materials

Reagent-grade toluene and glacial acetic acid were obtained from Fisons (Loughborough, Great Britain) and *n*-heptane and amino acids from BDH (Poole, Great Britain). Polygram polyamide-6/UV_{254} pre-coated (0.1 mm) plastic sheets (20×20 cm) and a Desaga UVIS lamp with emission at 254 nm were purchased from Camlab (Cambridge, Great Britain). The crystalline MTH amino acid derivatives were prepared from D,L-amino acids using the methods published[16–18]. MTH serine could not be crystallized and was prepared freshly from D,L-serine by adaption of the method of Peterson *et al.*[11].

Method

Two-dimensional ascending chromatography was carried out on a pre-coated polyamide plate (10×10 cm) cut from the larger sheets purchased. The coating incorporated a fluorescent indicator. The plates were sectioned as shown in Fig. 1 by removing 3-mm strips of coating at right angles to each other 8 cm from the starting edges to stop the solvent flow. The origins for the unknown sample and the standard MTH amino acids were 1 cm from the edges of their respective plates. Glycine was chromatographed as the marker with both solvents in the outer zones of each plate. It was better to restrict the spot at the origin to about 2 mm diameter to give a good separation, as reported previously[5]. Usually 0.5 to 1.0 μl solution of MTH amino acids in pyridine was spotted on to the plate with a disposable micropipette. A stream of cold air or nitrogen was used to evaporate the solvent. Chromatography was carried out in a small glass tank with glass lid.

The plate was developed with Solvent 1, toluene–*n*-heptane–glacial acetic acid (60:30:20), for approximately 15 min and dried with cold air from a hair-drier. After turning through 90°, the plate was developed with Solvent 2, 35% acetic acid. This required about 30 min. After hot air drying (quicker) the plate was viewed under UV irradiation at 254 nm. The MTH amino acids were seen as purple spots on a green background and their positions noted by circling with a pin. A clear plastic template showing the positions of all the derivatives was used to aid identification. This was lowered on to the unknown plate and its position adjusted to coincide with that of the MTH glycine.

TABLE I

$R_F \times 100$ VALUES FOR 19 MTH AMINO ACID DERIVATIVES IDENTIFIED BY TLC ON POLYAMIDE-COATED PLASTIC PLATES

MTH amino acid	*$R_F \times 100$ value*	
	Solvent 1	*Solvent 2*
Alanine	67	70
Arginine	02	92
Asparagine	26	82
Aspartic acid	19	70
Cysteine	45	45
Glutamic acid	33	70
Glycine	54	74
Histidine	08	93
Isoleucine	89	43
Leucine	86	43
Lysine	22	60
Methionine	74	52
Phenylalanine	80	35
Proline	90	63
Serine	55	61
Threonine	57	45
Tryptophan	42	21
Tyrosine	18	43
Valine	82	57

RESULTS AND DISCUSSION

Many different solvent systems were tried. Solvent 1 was a modification of Solvent III and Solvent 2 was identical to Solvent V described by Kulbe[4]. Improved separations were not obtained by pre-saturating the atmosphere as claimed by Summers *et al.*[5].

Table I shows the $R_F \times 100$ values for 19 MTH amino acids. These are the average values for six replicates. The spots were easily identified and reproducibility was good. Fig. 1 gives the schematic representation of a chromatogram with 19 MTH amino acids. The limit of sensitivity was 0.05 to 0.1 nmoles for each derivative[5]. Solvent 2 affected the appearance of the plate under UV light. Approximately one third of the lower end of the plate after development with Solvent 2 was very dark but only MTH tryptophan was found in this zone. All the derivatives were separated except for MTH isoleucine and MTH leucine, but these could be easily distinguished by GLC. We could not distinguish between MTH glutamic acid and MTH glutamine with any TLC solvent system. The amide may have been hydrolyzed to its corresponding acid during its preparation. It should be noted that TLC separations of PTH amino acids on polyamide-coated plates with the same solvent systems as published by Kulbe (Fig. 1b)[4] and by Summers *et al.* (Fig. 1)[5], disagree markedly over the relative positions of PTH glutamic acid and PTH glutamine, although they agree in most other respects.

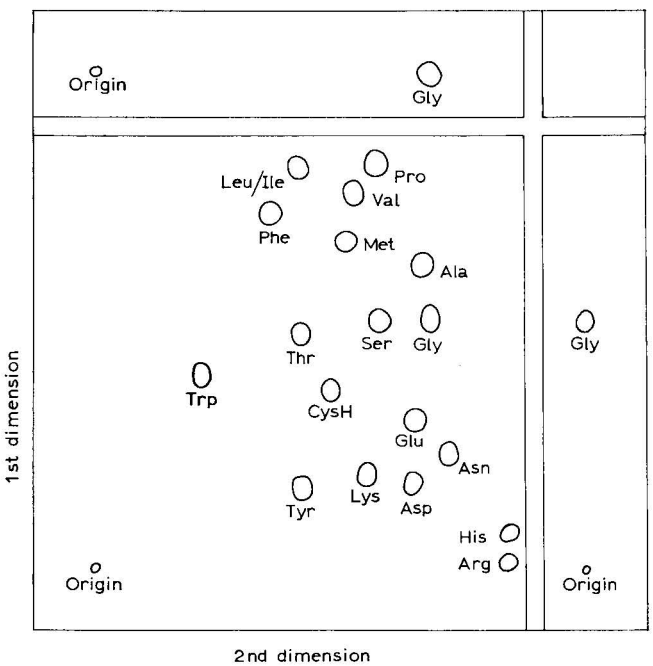

Fig. 1. Schematic representation of two-dimensional TLC chromatography of 19 MTH amino acids. For the first dimension Solvent 1 was used, for the second Solvent 2.

ACKNOWLEDGEMENTS

We thank Mrs. M. Rangarajan for preparing the MTH amino acids and Professor H. R. V. Arnstein for his support.

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CHROM. 7180

Note

Thin-layer chromatography of methyl N-trimethyl-γ-aminobutyrate chloride and related compounds

K. OHYA and N. TSUNAKAWA

Laboratory of Pharmaceutical Formulation, Research Institute, Daiichi Seiyaku Co., Ltd., Narihira 5-6-9, Sumida-ku, Tokyo (Japan)

(Received September 10th, 1973)

So far, only a few reports[1–4] on the application of thin-layer chromatography to aliphatic quaternary ammonium compounds have been published.

In this work, methyl N-trimethyl-γ-aminobutyrate chloride (MTB), which is a drug with excellent cholinergic action[5,6], has been separated from its structurally related compounds by a TLC method.

MATERIALS

The MTB used was the pharmaceutical material synthesized in this Institute and other related compounds shown in Table I were obtained by the following methods.

The acidic form (TB) was prepared by the hydrolysis of MTB in 1 *N* hydrochloric acid, and the esters (ETB, PTB and BTB) were prepared by the transesterification of MTB with the appropriate anhydrous alcohols contained in about 0.1 *M* equivalent hydrochloric gas. All of these reactions were carried out at 70° for 3 h.

METHODS

The TLC plates were prepared as follows. A slurry of 30 g of Kieselgel G (E. Merck, Darmstadt, G.F.R.) in 60 ml of water was spread on 10×20 cm glass plates to a thickness of 0.25 mm with a Camag (Muttenz, Switzerland) automatic applicator. The plates were dried at 120° for 1 h and stored in a desiccator.

A 1% aqueous solution of each compound was prepared and 1 μl of the solution (corresponding to 10 μg of each compound) was spotted at 2.5 cm from the edge of the plate. The plate was developed at about 25° with the solvent system ethyl acetate–formic acid–water (10:2:2) until the solvent front had travelled about 12 cm. About 40 min were usually required for the development.

The plate was then dried and spread with Dragendorff's reagent, and each of the compounds appeared as an orange spot.

TABLE I

THIN-LAYER CHROMATOGRAPHY OF MTB AND RELATED COMPOUNDS

General formula of MTB compounds: $[(CH_3)_3N^+-CH_2-CH_2-CH_2-COO-R]Cl^-$.
Plate: Kieselgel G, 0.25 mm layer, 120° for 1 h.
Developing solvent: ethyl acetate–formic acid–water (10:2:2).

Compound	*R*	R_F value*
Methyl N-trimethyl-γ-aminobutyrate chloride (MTB)	CH_3	0.21
N-trimethyl-γ-aminobutyrate chloride (TB)	H	0.17
Ethyl N-trimethyl-γ-aminobutyrate chloride (ETB)	C_2H_5	0.28
n-Propyl N-trimethyl-γ-aminobutyrate chloride (PTB)	C_3H_7	0.37
n-Butyl N-trimethyl-γ-aminobutyrate chloride (BTB)	C_4H_9	0.42

* Mean R_F values of five experimental results.

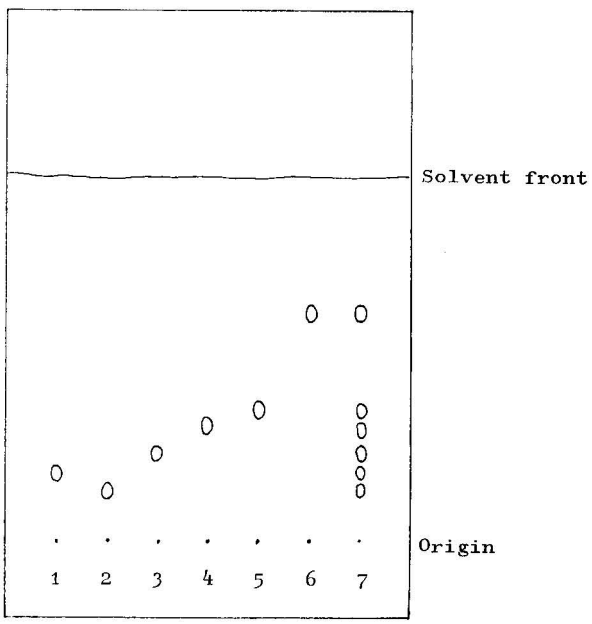

Fig. 1. Thin-layer chromatogram of MTB and related compounds. Plate: Kieselgel G, 0.25 mm layer, 120° for 1 h. Solvent system: ethyl acetate–formic acid–water (10:2:2). 1 = MTB; 2 = TB; 3 = ETB; 4 = PTB; 5 = BTB; 6 = crystal violet; 7 = mixture.

RESULTS AND DISCUSSION

The R_F values and a typical chromatogram are shown in Table I and Fig. 1.

The detection limit was found to be *ca.* 1 μg for each compound. The only adsorbent used in our work was Kieselgel G, but heating of the TLC plates at various temperatures (105–150°) was also examined. Several developing solvents, such as acetone–36% hydrochloric acid (10:1)[1], ethyl acetate–formic acid–water[4]

TABLE II

R_{st} VALUES OBTAINED USING CRYSTAL VIOLET AS THE STANDARD MATERIAL

Compound	*R_F value**		*Rst value**	
	Mean	*Coefficient of variation* (%)	*Mean*	*Coefficient of variation* (%)
MTB	0.21	10.0	28	7.1
TB	0.17	10.4	23	7.5
ETB	0.28	8.1	37	6.2
PTB	0.37	7.0	49	3.9
BTB	0.42	6.0	56	3.7
Crystal violet	0.75	3.5	100	–

* Mean values of five experimental results.

in various proportions, *n*-butanol–acetic acid–water (60:20:20)[7], phenol–water (75:25)[7], benzene–ethanol (5:1)[8] and methanol[9], were also examined.

Heating the plates hardly affected the chromatograms, but the best separation was achieved with a plate heated at 120° for 1 h. On the other hand, the various developing solvents had a much greater effect, and sharp spots free from tailing were found only in the solvent system ethyl acetate–formic acid–water. Increasing the proportion of formic acid gave higher R_F values for the each compound, but did not improve the separation. The most favourable result was achieved with ethyl acetate–formic acid–water in the proportions 10:2:2.

The R_F values obtained in this TLC system showed some variations (Table II), but the separations were adequate for the identification of each compound.

The R_{st} values[10] were applied using crystal violet as the standard material, and the results were better in terms of reproducibility, as shown in Table II.

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CHROM. 7181

Note

Determination and micro-preparative separation of chlorocholine chloride by paper chromatography

S. KOUDELA and V. CIELESZKY

Institute of Nutrition, Department of Toxicological Chemistry, Gyáli ut 3/a, Budapest 1097 (Hungary)

(Received September 6th, 1973)

The specificity of detection of chlorocholine chloride (CCC) on chromatograms or its determination by spectrophotometry with dipicrylamine[1–3] or iodine[4,5] depends on the efficiency of the separation from choline (CC) and other positive compounds by use of paper or thin-layer chromatography (*e.g.* refs. 6–8), ion-exchange resins[2,3,9], alumina columns[1–3] or iodine extraction[10,11].

In our experiments, which were conducted in order to obtain more effective separations by using various combinations of solvent systems on buffered and salt-impregnated papers, it was found that the optimum separation of CCC from CC could be achieved on Whatman No. 1 paper impregnated with 3% sodium chloride solution by development with the supernatant of the solvent mixture isoamyl alcohol–*n*-propanol–3% sodium chloride solution (1:2:3) (Fig. 1). By decreasing the sodium chloride concentration, the CCC and CC spots became more satisfactorily resolved, but also became more diffuse.

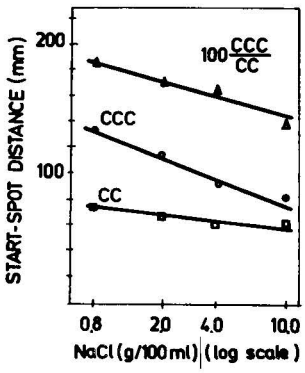
EXPERIMENTAL

Extraction and clean-up

The sample of the material to be tested (usually 50–100 g) was extracted with methanol and, after adding of water to the extract, the methanol was evaporated and the aqueous solution was purified by shaking with light petroleum (b.p. 40–70°). The aqueous solution was evaporated in a vacuum, the dry residue was redissolved by adding of 1 ml of water, 1 ml of methanol and 12 ml of acetone, and the solution obtained was transferred to an alumina column and eluted with acetone containing 25% of methanol[1,2]. From an acidic aluminium oxide (E. Merck, Darmstadt, G.F.R.) column, 20 cm long and 1 cm I.D., a total of 70 ml of eluate could be collected. Finally, the dry residue of the eluate was taken up in 0.2 ml of methanol.

Paper chromatography

A Whatman No. 1 paper sheet was immersed in 3% sodium chloride solution and dried at room temperature. An aliquot of the methanolic solution equivalent to one fifth of the sample extract and appropriate aliquots of stock CCC solutions were applied to the starting line. Development by the descending technique required 14–16 h. The air-dried paper was sprayed with potassium iodoplatinate solution



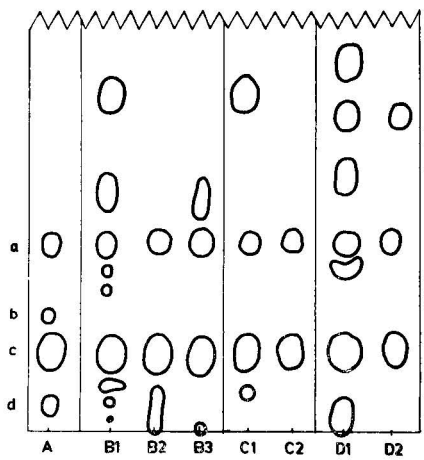


Fig. 1. Migration distance of CCC (○—○) and CC (□—□) spots, and the 100·CCC/CC distance ratio (△—△), plotted against the logarithm of the concentration of the impregnating sodium chloride solution.

Fig. 2. Paper chromatograms of some choline derivative standards (A) and of wheat seed (B), wheat flour (C) and grape (D) extracts from CCC-treated plants. Standards: (a) CCC; (b) acetylcholine; (c) CC; (d) betain. Clean-up: (1) Dowex 50 W-X8; (2) acidic alumina column; (3) extraction with iodine solution.

(1 g of platinum (IV) chloride and 10 g of potassium iodide were separately dissolved in water and the combined solutions were made up to 1 l with water). CCC and other detectable substances appeared as dark blue spots, and the chromatogram allowed a satisfactory semi-quantitative determination of the compound.

The efficiency of this separation technique was checked on some plant extracts, using for the clean-up an ion-exchange resin, aluminium oxide or extraction with iodine–potassium iodide solution (Fig. 2).

Micro-preparative separation

For the more sensitive and accurate spectrophotometric determination of CCC, paper chromatography can be used as a micro-preparative technique. The remaining four fifths of the methanolic extract were applied to the paper in two spots and developed as previously described. After location, the CCC spots (a piece of developed paper of a similar size served as a blank) were cut out and shaken with 10 ml of a 0.01% solution of dipicrylamine in dichloromethane[1] and the extracts were shaken with 5 ml of 0.03 *N* sodium hydroxide solution. The alkaline phases were discarded. The extracts were filtered through anhydrous sodium sulphate and finally evaporated to 5 ml. Spectrophotometric readings were made in 1-cm cells at 415 nm and corrected for the blank.

RESULTS

The procedure described permits the complete separation of CCC from CC even when the latter is present in a 1000-fold excess. Both substances can be easily

detected in the 2–500 μg range for CCC and 10–1000 μg range for CC. With a sample size of 50 g, the detection limit is 0.2 ppm.

The recovery of CCC in preparative separations, involving regeneration from the paper and conversion into the dipicrylamine complex, is about 90%. The sensitivity of the spectrophotometric method is 1 μg per millilitre of solution, the measuring range being 5–50 μg. The detection limit for a 50-g sample is 0.05 ppm.

ACKNOWLEDGEMENT

The authors wish to thank Mrs. Irene Leder for technical assistance.

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Book Reviews

CHROM. 7275

Determination of sequences in RNA, by G. G. Brownlee, North-Holland, Amsterdam and Elsevier, New York, 1972, II+265 pp., price Dfl. 30.00. (This book is also part of T. S. Work and E. Work (Editors), *Laboratory techniques in biochemistry and molecular biology*, Vol. 3, North-Holland, Amsterdam and Elsevier, New York.)

In 1965 F. Sanger, G. G. Brownlee and B. G. Barrell (*J. Mol. Biol.*, 13 (1965) 373) described a novel method for sequencing of RNA. This method entails biological labeling of RNA with [^{32}P]orthophosphate to high specific activity, isolation and degradation of RNA to oligonucleotides, and "finger-printing" by high-voltage electrophoresis. This approach, which was soon recognized as a major breakthrough in nucleic acid methodology, has been an extremely useful tool in sequence studies ever since its introduction, its main limitation being the fact that, unfortunately, a great number of interesting RNAs (*e.g.*, human and other mammalian RNAs *in vivo*) cannot be biologically labeled to the high specific activity required for sequencing.

The book by Brownlee, one of the initiators of this valuable method, provides a detailed survey of methods for sequence analysis of RNA. Emphasis is placed upon the radioactive methods, only about 35 pages out of a total of 265 pages being devoted to sequencing of non-radioactive RNA. The book presents a most complete and accurate account of the experimental details of the sequencing techniques and contains a large amount of useful information presented in a concise style. The center of the book (about 90 pages) contains a fascinating description of these techniques as applied by Brownlee and Sanger to the elucidation of the structure of 5S RNA of *E. coli*. Only a few errors were noticed, for example in the bibliography section. The statement (pp. 226–227) regarding film detection of tritium is no longer correct; there are now quite sensitive techniques for doing this. This book will certainly be welcomed by everyone interested in the structure of nucleic acids and other biological macromolecules. I regard it as a major contribution in the fields of analytical biochemistry and separation methods.

Houston, Texas (U.S.A.) K. RANDERATH

CHROM. 7270

Das Dithizon und seine Anwendung in der Mikro- und Spurenanalyse, by G. Iwantscheff, Verlag Chemie, Weinheim/Bergstr., 2nd ed., 1972, XVI+330 pp., 41 figs., 18 tables, price DM 118.00.

The second edition of Iwantscheff's well known book on the application of *di*phenyl*thio*carba*zone* (=dithizone) in micro- and trace analysis cites some 2000 references, whereas a mere 500 were included in the first edition published in 1957. In order to limit the size of his treatise, the author has revised and shortened the introductory chapter on theoretical aspects and also that on the use of dithizone in qualitative analysis. The description of the analytical techniques has remained essentially unchanged. The survey of the use of dithizone in quantitative analysis, which constitutes nearly half of the total text, includes many recent data on, for example, the elimination of interfering ions, the reaction of dithizone with organometallic compounds and analytical applications. The increasing attention that is presently being devoted to the use of dithizone in titrimetry, chromatography, polarography and atomic-absorption spectroscopy has led to a considerable increase in the length of the pertinent chapters.

The book contains 41 figures, 18 tables, a comprehensive list of references, an index of authors and an adequate subject index. It is a valuable handbook for analytical chemists and its use is recommended. Unfortunately, the price places it in the category of library reference works.

Amsterdam (The Netherlands) U. A. Th. BRINKMAN

CHROM. 7248

A programmed introduction to gas–liquid chromatography, by J. B. Pattison, Heyden & Son, London, 2nd ed., 1973, XV+303 pp., price £ 2.90, $ 8.00, DM 23.80.

This book is virtually a straight re-issue of the first edition —previously reviewed in the *Journal of Chromatography*, 46 (1970) 331— and only a few minor alterations have been made to the text, such as now telling the reader not to connect his column to the detector while conditioning it.

Some anomalous points still exist in the text, such as the 25% phenyl-substituted silicone MS 550 being described, and illustrated, as a non-polar methyl silicone oil. Benzene is also described as non-polar in comparison with paraffins, because its carbon atoms are "not combined with a strongly electro-negative element".

No attempt appears to have been made to update the text and, for instance, the coverage of support materials is still rather sketchy, being limited to obtaining mesh cuts within 60 to 100 mesh of Kieselguhr, crushed firebrick or Celite. These are described as suitable because they are "inert and porous".

Fortunately, however, the coverage of supports is not typical of the depth with which other topics in gas chromatography are treated and generally a good balance in breadth and depth of information is achieved.

Since 1970, the first edition has been read several times by staff coming into gas chromatography work, and the book has been very effective with their background training.

Abingdon (Great Britain) D. T. COKER

CHROM. 7249

High pressure liquid chromatography (Biochemical and biomedical applications), by Phillis R. Brown, Academic Press, New York, London, 1973, XII + 202 pp., 161 figs., 26 tables, price $ 11.50.

The term "high pressure" refers to the advanced column technology that is the characteristic feature of modern liquid column chromatography. The present state of liquid column chromatography is described in six chapters. The introduction (15 pages) is followed by a discussion of instrumentation (35 pages), experimental methods (36 pages), identification of peaks (25 pages), quantitation (16 pages) and applications (57 pages). A bibliography covering the literature up to 1972 (215 references), an author index and a subject index are included.

The book gives an elementary introduction to the techniques of modern liquid column chromatography and their application, and practical aspects are stressed. Theoretical aspects are discussed in a qualitative manner, and only a few very simple equations are given in an attempt to describe the chromatographic process. The author has relied to a great extent on figures, more than 130 chromatograms being shown, and the operating conditions relating to the chromatograms are given in detail. The chosen examples of applications give a good up-to-date survey of the field of application of liquid column chromatography. Special attention is paid to nucleic acid components, the particular field of research of the author.

As the book is elementary and oriented towards practical aspects, it can be recommended as a useful first reading for all beginners in the field of modern liquid column chromatography. It is well printed and good value at the price stated.

Amsterdam (The Netherlands) J. F. K. HUBER

Journal of Chromatography, 90 (1974) B71–B109

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Gas Chromatography

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31. PLASTICS AND THEIR INTERMEDIATES

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1709 Chakrabarti, P. B. and Gupta, S. K.: (Complete paper electrophoretic separation of a mixture of iron(III), aluminum(III), nickel(II), cobalt(II), and molybdenum(VI), using pyridine–acetic acid buffer). *Vijnana Parishad Anusandhan Patrika*, 15 (1972) 177–180; *C.A.*, 78 (1973) 131658d —paper.

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1717 Glew, R. H. and Lee, R. E.: Composition of the membranous deposits occurring in Gaucher's disease. *Arch. Biochem. Biophys.*, 156 (1973) 626–639 —SDS-polyacrylamide gel.

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1719 Kimes, B. W. and Morris, D. R.: Cations and ribosome structure. II. Effects on the 50S subunit of substituting polyamines for magnesium ions. *Biochemistry*, 12 (1973) 442–449 —polyacrylamide gel.

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journal of
chromatography news section

APPARATUS

N-415

SINGLE COLUMN GAS CHROMATOGRAPH

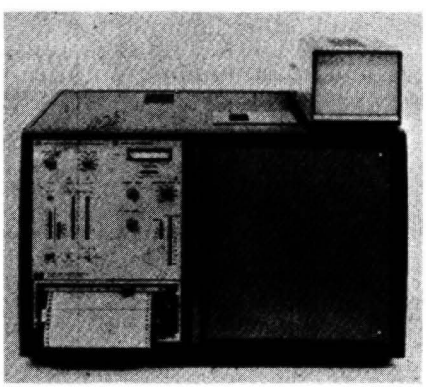
Hewlett-Packard introduces a high performance, single column, single detector gas chromatograph, the Series 5720A, available with either flame ionization or thermal conductivity detectors. They are suitable for both quality control and research laboratories and are capable of analyzing a wide variety of samples. GC accessories and GC data processing systems can be applied in connection with the 5720A Series.

Solid state temperature control is provided via independent temperature controllers for column oven and detector and an optional controller for use with heated injection ports or accessories. All employ a proportional feedback control circuit and are directly calibrated in °C. There is a digital isothermal controller or linear programmer for the column oven, and both units feature repeatable digital settings in 1° steps from 80° to 399°. The programmer has five repeatable digital settings for linear program rates to 12°/min.

The flame ionization detector is an improved grounded jet design that passes the entire column effluent through the collector electrode. A one-piece jet body that is easy to remove simplifies cleaning.

The electrometer can be ranged even during a run without creating transients and without changing the detector response factor. Dynamic range is $> 10^6$ on each of the three upper range settings (10, 100 and 1,000). In addition, range-to-range accuracy has been improved with the elimination of high-megohm resistors.

The thermal conductivity detector has replaceable filament cartridges. The tungsten–rhenium filaments are passivated at the factory to reduce oxidation problems. The TC detector control provides continuous adjustment of filament current which is readily observed on the 0–400 mA multifunction meter.



N-423

HP JOURNAL

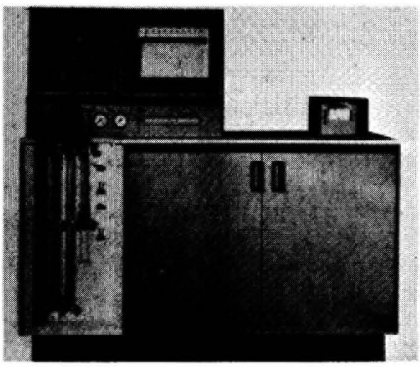
Hewlett-Packard announce in their December 1973 issue of "HP Journal" a portable strip-chart recorder (Model 7155A), a test desk cable fault locator (Model 4913A) and the use of switching regulators in power supplies.

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-416

LIQUID CHROMATOGRAPH

Beckman has introduced the liquid column chromatograph Multichrom B. It is mainly designed for amino acid analysis. By adding some accessories to the basic model it can also be used for the analysis of peptides, nucleotides and nucleosides as well as other compounds absorbing in the UV range.



The standard model includes a photometer for the wavelengths of 570 and 440 nm capable of measuring in % transmission as well as in five different selectable absorbance ranges (2 A–0.1 A). The Multichrom B also comprises a recorder with variable chart speeds and printing frequencies.

Accessories include an automatic sample injector with cooling box, an internal integrator, a peptide and stream divider accessory, preparative columns, a nucleotide accessory, a gradient pump, a battery supply, an operation control system, and computer systems for automatic evaluation of chromatograms.

NEW BOOKS

Die Chemische Industrie und ihre Helfer 1973–1974, Edition Selka, Industrieschau-Verlagsgesellschaft mbH, Darmstadt, 1973, 584 pp., price DM 30.00.

Collected accounts of transition metal chemistry, Vol. 1, by F. Basolo, J.F. Bunnett and J. Halpern (Editors), American Chemical Society, Washington, D.C., 1973, v + 250 pp., price S3,95.

Coordination chemistry –experimental methods, by I.T. Millar and D.W. Allen (Editors of the English translation), Butterworth & Co., London, 1973, 372 pp., price £10.00.

Drug analysis by chromatography and microscopy – a practical supplement to pharmacopoeias (translation from the German edition), by E. Stahl, AnnArbor Science Publishers, Ann Arbor, Mich., 1973, x + 238 pp., price $22.50.

Identification of organic compounds with the aid of gas chromatography, by R.C. Crippen, McGraw-Hill, New York, N.Y., 1973, x + 331 pp., price $19.50.

Ion exchange and solvent extraction, Vol. 4, by J.A. Marinsky and Y. Marcus (Editors), Marcel Dekker, New York, 1973, xii + 265 pp., price $19.75.

Methodicum Chimicum, Band 1, Deutsche Ausgabe, Teil 1 und Teil 2, by F. Korte (Editor-in-chief), Georg Thieme Verlag, Stuttgart, 1973, 1342 pp., price DM 496.00, subscribers DM 421.60.

Modern aspects of inorganic chemistry, by H.J. Emeléus and A.G. Sharpe, Routledge & Kegan Paul Ltd., London, 4th ed., 1973, xv + 678 pp., price £5.25.

Prostaglandins – isolation and synthesis, by J.C. Colbert, Noyes Data Corp., Park Ridge, N.J., 1973, viii + 280 pp., price $36.00.

Quantitative thin layer chromatography, by J.C. Touchstone (Editor), Wiley-Interscience, New York, 1973, xiv + 330 pp., price £7.50.

Lipid Analysis, by W.W. Christie, Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig, 1973, xiv + 338 pp., price £6.00.

PUBLICATION SCHEDULE FOR 1974

Journal of Chromatography (incorporating *Chromatographic Reviews*)

ONTH	J	F	M	A	M	J	J	A	S	O	N	D
URNAL	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
EVIEWS *			98/1						98/2		98/3	

* Volume 98 will consist of *Chromatographic Reviews*. The issues comprising this volume will ot be published consecutively, but will appear at various times in the course of the year.

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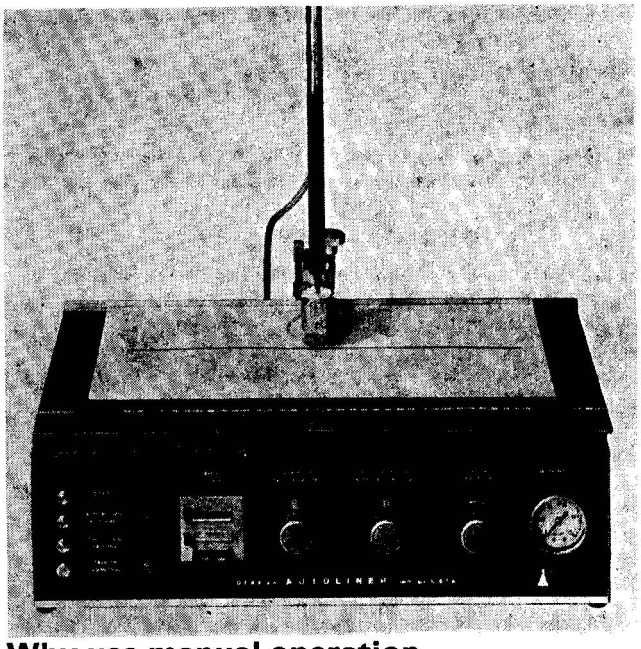

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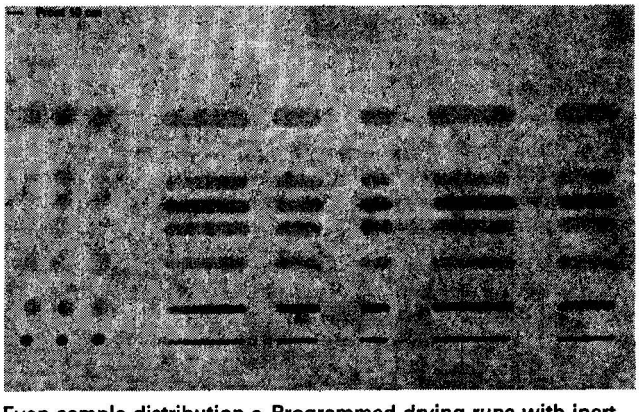

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