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THIN-LAYER CHROMATOGRAPHY OF ENAMINO KETONES

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SUMMARY

The synthesis of sixteen enamino ketones was carried out by reaction of ethyl 3-oxobutanoate, ethyl 2-methyl-3-oxobutanoate, 2,4-pentanedione and 3-methyl-2,4-pentanedione with variously substituted aliphatic and aromatic amines. The studied compounds were separated by thin-layer chromatography and the R_F values determined on pre-coated silica gel plates and on commercially available ready-coated Silufol sheets using four solvent systems. The spots were detected by application of three different reagents, and in UV light. The dependence of the R_F values on the structure of the studied compounds is discussed A diagonal technique was used to control the purity of enamino ketones.

INTRODUCTION

Enamino ketones are important basic substances employed for the synthesis of more complex products, *e g*, medicaments, dyes, pesticides, etc, important intermediates in various organic syntheses[1], and they may also serve as protecting groups of primary and secondary amines during physiological processes The utilization of enamino ketones as intermediates and blocking groups in organic chemistry has been reviewed[2].

The first studies concentrated on the determination of the physical and spectral characteristics of enamino ketones[3]. Several authors studied the UV[4–7], IR[8–10] and NMR[11,12] spectra. In particular, the aim of most of these studies was the elucidation of the structure and the tautomeric forms of these compounds. However, to our knowledge, a detailed survey of the thin-layer chromatography (TLC) of enamino ketones has not been carried out. Therefore, we undertook to develop a method for identification and separation of enamino ketones, including those having similar R_F values. Sixteen derivatives of 2,4-pentanedione and ethyl 3-oxobutanoate were prepared, five of which had not been described previously. They were subjected to TLC on pre-coated silica gel plates and on commercially available ready-coated Silufol sheets in four different solvent systems. The possible decomposition of the com-

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pounds during the development of the chromatograms was studied by application of the diagonal technique.

EXPERIMENTAL

The UV spectra were measured on a Specord UV VIS spectrophotometer (Carl Zeiss, Jena, G. D. R.) in 1-cm silica cells. Melting points were determined on a Kofler block and were not corrected. The refractive indices were measured on a refractometer (Carl Zeiss) at 25°C.

Preparation of the compounds

3-Methyl-2,4-pentanedione was prepared as described[13]. The crude product was purified by distillation *in vacuo* (65–66°C, 19 Torr; in accord with ref. 14).

Ethyl 2-methyl-3-oxobutanoate. Ethyl 3-oxobutanoate was methylated with a solution of methyl iodide[15]. Fractional distillation gave a product of b.p. 75–76°C (15 Torr).

Ethyl 2-(n-butyl)-3-oxobutanoate. Ethyl 3-oxobutanoate was treated with butyl bromide in a medium of sodium methanolate. Fractional distillation gave a product of b.p. 93–96°C (8 Torr) in accord with ref. 16.

Enamino ketones I–VI. 2,4-Pentanedione or 3-methyl-2,4-pentanedione was treated[5,8,12,17] with a 25% solution of ammonia or with solutions of amines in the molar ratio 1:1 (Table I).

4-(1-Methylpropylamino)-3-penten-2-one (VII). In a flask, 9.75 g (0.1 mol) of 2,4-pentanedione were mixed with 7.4 g (0.1 mol) of 1-methylpropylamine and refluxed for 4 h. Then the reaction mixture was extracted four times each with 20 ml of benzene, the organic layers were combined and dried over anhydrous sodium sulphate and benzene was distilled off. Distillation *in vacuo* gave 15 g (96.5%) of compound VII (yellow liquid), b.p. 66–68°C (5 Torr). UV spectrum (methanol) 313.5 nm (log ε = 4.29); n_D^{25} = 1.5032. For $C_9H_{17}NO$ (MW 155.2) calculated: 69.68% C, 10.97% H, 9.03% N; found: 69.35% C, 10.64% H, 9.3% N.

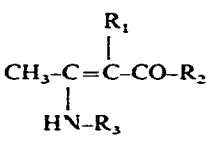
3-Methyl-4-(1-methylpropylamino)-3-penten-2-one (VIII). The above procedure was applied using 11.4 g (0.1 mol) of 3-methyl-2,4-pentanedione and 7.4 g (0.1 mol) of 1-methylpropylamine to give 14 g (83.0%) of a yellow liquid, b.p. 85–87°C (4 Torr). UV spectrum (methanol) 323.3 nm (log ε = 4.22); n_D^{25} = 1.4835. For $C_{10}H_{19}NO$ (MW 169.3) calculated: 71.01% C; 11.24% H, 8.28% N; found: 70.92% C, 11.32% H, 8.46% N.

Ethyl 3-amino-2-butenoate (IX). To 26 g (0.2 mol) of ethyl 3-oxobutanoate was added dry benzene (150 ml). The mixture was cooled to 4°C and a stream of dry NH_3 was bubbled through for 4 h. It was then left to stand for 24 h, followed by bubbling NH_3 for a further 4 h and finally standing for 24 h. Then the organic layer was separated, dried over anhydrous potassium carbonate and distilled *in vacuo* to give[6] 20 g (77.5%) of compound IX, b.p. 107–109°C (20 Torr).

Ethyl 3-amino-2-methyl-2-butenoate (X). Ethyl 2-methyl-3-oxobutanoate (29 g, 0.2 mol) was mixed with ethanol (50 ml) and gaseous ammonia was passed into the solution for 7 days. Ethanol was removed by distillation *in vacuo* and the residue distilled at 30 Torr to give 13 g (45.0%) of compound X, b.p. 75–76°C (30 Torr), m.p. 48–49°C in accord with ref. 17.

TABLE I

ENAMINO KETONES PREPARED BY REACTION OF DIKETONES AND KETO ESTERS WITH AMINES



Compound	R_1	R_2	R_3	*Yield (%)*	*Bp (°C/Torr)*		*Mp (°C)*	
					Found	*Literature*	*Found*	*Literature*
I	H	CH_3	H	86 0	–	–	39–42	39–41[18]
II	CH_3	CH_3	H	71 0	–	–	111–112	109–110[18]
III	H	CH_3	C_6H_5	72 5	75–76/30	74–75/30[17]	46–48	47–48[19]
IV	CH_3	CH_3	C_6H_5	72 3	122–125 5	not found	40–41	39[9]
V	H	CH_3	*n*-Butyl	84 0	92–94 2	94–95 4[20]	–	–
VI	CH_3	CH_3	*n*-Butyl	88 5	97–100/3	111–112/20[8]	–	–
VII	H	CH_3	1-Methyl-propyl	96 5	66–68/5	–	–	–
VIII	CH_3	CH_3	1-Methyl-propyl	93 0	85–87 4	–	–	–
IX	H	OCH_2CH_3	H	77 5	107–109 20	91–93 8[6]	–	–
X	CH_3	OCH_2CH_3	H	45 0	–	–	48–49	52[21]
XI	H	OCH_2CH_3	$COCH_3$	78 5	–	–	61–62	63[7]
XII	H	OCH_2CH_3	*n*-Butyl	92 0	88 3	not found	–	–
XIII	CH_3	OCH_2CH_3	*n*-Butyl	75 5	109–111 3	–	–	–
XIV	H	OCH_2CH_3	1-Methyl-propyl	89 2	78/3	not found	–	–
XV	CH_3	OCH_2CH_3	1-Methylpropyl	65 3	92–93/3	–	–	–
XVI	*n*-Butyl	OCH_2CH_3	*n*-Butyl	86 0	123–124 3	–	–	–

The compounds XI, XII and XIV were obtained by reaction of ethyl 3-oxobutanoate or ethyl 2-methyl-3-oxobutanoate with solutions of the appropriate amines[7] (Table I)

Ethyl 3-(n-butylamino)-2-methyl-2-butenoate (XIII). To 14.4 g (0 1 mol) of ethyl 2-methyl-3-oxobutanoate were added 7 4 g (0 1 mol) of *n*-butylamine and the mixture was refluxed for 4 h. Then the solution was extracted twice each with 20 ml of benzene, and the organic layers were combined, dried over anhydrous sodium sulphate and distilled *in vacuo* to give 15 g (75.5%) of a yellowish liquid, b p 109–111°C (3 Torr) UV spectrum (*n*-hexane) 298 5 nm (log ε = 4 26), n_D^{25} = 1.4745. For $C_{11}H_{21}NO_2$ (MW 199.3) calculated. 66 33% C, 10 55% H, 7 04% N; found 66.59% C, 10 42% H, 6 9% N

Ethyl 2-methyl-3-(1-methylpropylamino)-2-butenoate (XV) Ethyl 2-methyl-3-oxobutanoate was mixed with 1-methylpropylamine in 1.1 molar ratio to give 13 g (65 3%) of a yellow liquid, b p 92–93°C (3 Torr). UV spectrum (*n*-hexane) 301 2 nm (log ε = 4 23), n_D^{25} = 1.4770. For $C_{11}H_{21}NO_2$ (MW 199 3) calculated. 66 33% C, 10 55% H, 7 04% N; found· 66 12% C, 10 7% H, 6 94% N

Ethyl 2-(n-butyl)-3-(n-butylamino)-2-butenoate (XVI) was prepared in the same manner Distillation *in vacuo* yielded 20.7 g (86%) of a yellowish liquid, b p 123–124°C (3 Torr) UV spectrum (*n*-hexane) 300 nm (log ε = 4 20); n_D^{25} = 1 4687

TABLE II

R_F VALUES AND SPOT COLOURS OF THE ENAMINO KETONES I–XVI

Compound	Silica gel 60 F-254				Silufol				UV light		Iodine	Iodine–iodine reagent
	S1	S2	S3	S4	S1	S2	S3	S4	254 nm	366 nm		
I	0.14	0.18	0.23	0.14	0.08	0.09	0.19	0.13	Light gray	Violet	Light yellow	Brilliant yellow-white
II	0.23	0.37	0.44	0.26	0.18	0.33	0.42	0.19	Light gray	Light gray violet	White	White
III	0.41	0.61	0.59	0.55	0.44	0.48	0.62	0.72	Violet	Violet	Light yellow	Yellow orange
IV	0.51	0.63	0.64	0.59	0.46	0.53	0.66	0.78	Light gray	Light gray	Beige	White yellow white
V	0.33	0.55	0.49	0.45	0.37	0.35	0.42	0.61	Gray	Gray-violet	Yellow	Brilliant yellow-orange
VI	0.37	0.56	0.54	0.53	0.43	0.41	0.53	0.67	–	Gray-violet	White	White
VII	0.39	0.48	0.51	0.41	0.47	0.36	0.44	0.55	Dark violet	Violet	Light yellow	Yellowish orange-beige
VIII	0.43	0.57	0.55	0.50	0.52	0.46	0.53	0.65	Dark violet	Violet	Grayish beige	Yellow-white yellow
IX	0.47	0.45	0.62	0.57	0.53	0.37	0.52	0.53	Bluish gray	White gray	White	White
X	0.51	0.57	0.64	0.59	0.56	0.43	0.54	0.64	Light gray violet	Light gray-violet	White	White
XI	0.54	0.67	0.68	0.65	0.59	0.66	0.67	0.68	–	Violet	White-yellow	Deep yellow white
XII	0.35	0.56	0.58	0.64	0.46	0.49	0.49	0.65	–	Light gray-violet	White	White
XIII	0.43	0.62	0.65	0.70	0.51	0.50	0.59	0.72	–	Light gray-violet	White	White
XIV	0.31	0.51	0.61	0.58	0.40	0.44	0.51	0.57	–	Light gray-violet	White	White
XV	0.41	0.59	0.69	0.68	0.49	0.53	0.58	0.67	–	Light gray-violet	White	White
XVI	0.72	0.77	0.79	0.80	0.61	0.70	0.78	0.86	–	Violet	Light yellow	Yellow-deep yellow

For $C_{14}H_{27}NO_2$ (MW 241.4) calculated: 69.71% C, 11.21% H, 5.81% N; found: 70.02% C, 10.96% H, 5.66% N.

Thin-layer chromatography

Use was made of commercially available plates pre-coated with silica gel 60 F-254, 20 × 20 cm (E. Merck, Darmstadt, G.F.R.), and Silufol UV_{254} (aluminium sheets coated with silica gel bound with starch) 20 × 20 cm (Kavalier, Votice, Czechoslovakia).

The chromatograms were developed by application of the following solvent systems. S1 = chloroform; S2 = cyclohexane–ethyl acetate (3:2), S3 = benzene–ethyl acetate (8:2); S4 = benzene–cyclohexane–methanol (8:5:1:0.5). For the diagonal technique, the systems S1, S4, S5 = benzene–chloroform (1:1) and S6 = benzene–ethanol (18:2) were employed.

The compounds were dissolved in methanol to obtain a 0.5–0.8% solution, and aliquots of 1 *µl* were quickly applied to the plate which was then immediately placed into the developing chamber saturated with vapours of the mobile phase at 20°C. The spots were detected under UV light at 254 and 366 nm (minUVIS; Desaga, Heidelberg, G.F.R.). The spots were also exposed to vapours of iodine or visualized by spraying with the following reagents[22]. Iodine–iodide reagent: iodine (0.2 g) and potassium iodide (0.4 g) were dissolved in water (100 ml). After spraying, the compounds appear as white, yellow to orange spots on a beige-gray background. Potassium hexacyanoferrate(III) and ferric chloride: an aqueous solution of $K_3Fe(CN)_6$ (1%) and an aqueous solution of $FeCl_3$ (2%) were combined (1:1) shortly before use. After spraying the compounds gave deep green-blue spots on a light gray background. A more intense colour can be achieved by further spraying with 2 *M* HCl.

RESULTS AND DISCUSSION

The separation of enamino ketones was carried out by TLC on different adsorption layers using several solvent systems. The R_F values and the modes of detection are given in Table II (pre-coated plates with silica gel 60 F-254 and Silufol UV_{254}). Ready coated silica gel plates were used for the diagonal technique. It was found that the order of the R_F values of the analyzed compounds did not depend on the adsorbents.

The analyzed compounds comprised 2,4-pentanedione derivatives (substances I–VIII) and ethyl 3-oxobutanoate derivatives (compounds IX–XVI). Both groups included compounds having an unsubstituted amino group (I, II or IX, X), an alkylamino group (V–VIII or XII–XVI) and an arylamino or an acylamino group (III, IV or XI). The compounds III and IX served as references.

The compounds I–IV could be well separated in solvents S1 and S2 containing chloroform or ethyl acetate. Compounds V–VIII did not separate so readily. The best results were obtained in solvents S1 and S4, *i.e.*, in those containing larger volumes of chloroform or methanol. Compounds I–VIII could be well detected with iodine vapours, with the iodine–iodide reagent or with $K_3Fe(CN)_6$–$FeCl_3$.

The separation of compounds IX–XVI was also rather difficult. Satisfactory results for IX–XI could be achieved with solvents S2 and S3 containing ethyl acetate.

The compounds XII–XVI were well separated in solvents S2 and S4. The compounds of this group could easily be detected by exposure to iodine vapours and by spraying with the iodine–iodide reagent. The spots of compounds IX–XI gave, however, only a slightly blue colour with $K_3Fe(CN)_6$–$FeCl_3$; for the detection of compounds XII–XVI this reagent is unsuitable

The results showed that, in all the solvent systems, the R_F values of the 3-methyl-2,4-pentanedione and ethyl 2-methyl-3-oxobutanoate derivatives were higher than those of the corresponding unmethylated compounds with the same amino group. Compound XVI had the highest R_F value in all these solvents.

The R_F values of ethyl 3-oxobutanoate derivatives were, in all the solvents, higher than those of the corresponding derivatives of 2,4-pentanedione with the same amino group. The same was true in the case of their methyl derivatives except in solvent S1 where the R_F values of substances XIV and XV were lower than those of VII and VIII.

The dependence of the R_F values on the substituents of the amino group was not as unambiguous. Substances I and II had the lowest R_F values in all the solvents, the corresponding compounds IX and X, in the solvent systems S2 and S4, *i.e.*, in those without chloroform (for compound X, the results were, however, not convincing) Compounds III and IV of the 2,4-pentanedione group showed the highest R_F values in solvents S2, S3 and S4. In all the solvent systems, compound XI migrated more rapidly than the related compounds IX, XII and XIV. Interesting behaviours were shown by the compounds containing a *n*-butylamino or a 1-methylpropylamino group. Changes in the order of spots on the chromatogram occurred depending on the components of the solvent systems (Table II). These changes cannot satisfactorily be accounted for as yet.

The compounds XII–XV showed forward streaking in polar solvents containing alcohol (S4 and S6). To confirm our belief that the formation of the streaks is due to decomposition of the substances during development, we used the diagonal technique[23]. The sample was applied at the bottom left-hand corner of the plate and the plate was developed using the ascending technique with solvent S5. After drying, it was developed vertically with the same solvent. After detection with the iodine–iodide reagent, no streak formed and the spot was located on the diagonal of the chromatogram, indicating that no decomposition had occurred during development[23]. The experiment was repeated with solvent S1 with the same result. Then the development was carried out with solvents S4 and S6, and the chromatograms were again sprayed with the iodine–iodide reagent. Two spots whose R_F values were higher than that of the enamino ketone appeared in both cases, one on the diagonal of the chromatogram and one off-diagonally. Consequently, these spots arise from decomposition of the chromatographed compound during development. The other analyzed substances were also examined by this method, using solvents S4 and S5 In all cases, only one spot appeared which was situated in the diagonal of the chromatogram Thus, the diagonal method may be useful for the determination of the purity of the enamino ketones or of their degree of decomposition

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RAPID METHOD OF ESTIMATING VIABLE SPORES OF *ASPERGILLUS*

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SUMMARY

A rapid method of counting viable spores of spoilage fungi has been developed The method is based on the production of the enzyme pectinesterase during germination of the spores This enzyme hydrolyses the ester linkages of pectin to produce methanol which can be assayed by gas–liquid chromatography

A linear relationship can be established between the methanol produced and the spore concentration. The relationship is common to the six species of *Aspergillus* tested. Results obtained by this method in 19 h compare closely with plate counts of *Aspergillus* obtained at 48 h.

INTRODUCTION

Fungi of the genus *Aspergillus* are found widely in a variety of agricultural produce, where they are frequently present as dormant spores which may germinate under inadequate storage conditions and cause spoilage

Several types of spoilage may take place, the most important being the production of various toxins, especially the aflatoxins Non-toxigenic strains may also cause spoilage by production of heat leading to a loss of seed viability, alteration of flavour components and also by causing hydrolysis of triglycerides to free fatty acids. These are subsequently desaturated and subjected to autoxidation, thus leading to rancidity Considerable financial losses are caused by these fungi, especially in some developing countries where storage facilities and transport networks are inadequate and the ambient temperature and humidity are both high

Traditional methods for the estimation of fungi consist of plate counts, which may take 7 days or more depending upon the species[1] Such methods are of little value in a modern produce control laboratory, as toxigenic strains would have commenced toxin synthesis during this time period.

It is obvious that there is a great need for a rapid method of counting fungal spores and predicting whether or not they are viable

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Many species of the genus *Aspergillus* produce pectin degrading enzymes when grown in pectin-containing medium[2,3]. The enzyme pectinesterase (E C. 3.1.1.11) hydrolyses pectin to pectic acid and methanol. The enzyme may be measured by the titrimetric estimation of the carboxylic acid groups of pectic acid[4], or the methanol liberated may be measured either as formaldehyde[5] or by gas chromatography[6,7].

This work utilizes the activity of the pectinesterase found after 15 h germination to determine the number of viable dormant spores of six *Aspergillus* species known to cause spoilage.

MATERIALS AND METHODS

Organisms and media

The six species of *Aspergillus* used were obtained from the Commonwealth Mycological Institute (Ferry Lane, Kew, Great Britain) They were *A. flavus* 15959, 39178a, 86769, *A. niger* 31821, *A. nidulans* 16643 and *A. luchuensis* Inui 83356

Cultures were maintained by growth on slopes of the following medium; maltose, 38 g; neutralized soya peptone, 8 g; yeast extract, 2 5 g; malt extract, 2 g; Agar technical No. 3 (Oxoid), 20 g. These were dissolved in 1 l of distilled water and the pH adjusted to 5 4 before autoclaving Cultures were incubated at 30°C for 5 days and then stored at 4°C until required Subcultures were prepared at intervals of 4 weeks.

Organisms were grown on the following medium to obtain spores: KH_2PO_4, 2 0 g; KNO_3, 2 0 g; $CaCl_2$, 0 25 g; yeast extract, 5.0 g; glucose, 10.0 g; bactocasamino acids, 5.0 g; metals solution, 0.1 ml; distilled water, to 1 l. The medium was adjusted to pH 5 4 before autoclaving. The metals solution was made up as follows. $MgSO_4$, 0.5 g; $ZnSO_4$, 0.02 g; $FeSO_4$, 0 02 g; $MnSO_4$, 0 01 g; $CuSO_4$, 0.05 g; water, to 10 ml.

The medium for the assay of methanol was made up as follows: glucose, 4 g; pectin (citrus pectin, rapid set-type 104; Bulmer Ltd , Hereford, Great Britain), 1 g, soya peptone, 1 g; chloramphenicol, 100 mg; chlortetracycline, 100 mg; 1% phosphate buffer pH 7 0, to 100 ml.

Plate counts for viable spores were carried out on the following medium: maltose, 38 g; yeast extract, 2.5 g; mycological peptone, 8 g; malt extract, 2.0 g; agar, 20 g; distilled water, to 1 l. The medium was adjusted to pH 5.4 before autoclaving

Growth of spores

The sporulation medium (200 ml) was placed in Roux flasks, inoculated with a loopful of organisms from the slopes and incubated at 30°C for 3 weeks At the end of this period, a thick mycelial mat covered with spores was obtained. A spore suspension was obtained[8] and microscopic examination was carried out to ensure the absence of mycelial contamination.

The spores were stored as stock suspensions at 0°C until required, and to maintain a high level of viable spores fresh stock suspensions were prepared every 3 months.

Before use the spore suspensions underwent heat shock activation at 50°C for 25 min[9].

Production of methanol

Aliquots (100 ml) of the assay medium were placed in 500-ml Erlenmeyer

flasks. Fifty ml of spore inoculum were added and the flasks were incubated at 30°C on an orbital incubator at a speed of 150 rpm for 15 h. After this time, fungal germination and pectinesterase activity were stopped by placing the flasks in a water-bath at 80°C for 10 min Control flasks were inoculated with 50 ml of sterile distilled water.

The methanol produced was separated from the growth medium by dialysis across a Visking membrane using a modification of the dialysis cell method of Lee and Wiley[6] The dialysis cell consists of two poly(methyl methacrylate) half-cells separated by a Visking membrane. Each half-cell has an injection port and a cavity with a volume of 5 ml The contacting surfaces were smeared lightly with silicone grease and the front half of each cell was loaded with 3.0 ml of culture medium The rear half of each cell was loaded with an equal volume of distilled water and the injection ports were sealed The cells were then rotated on a revolving power unit at 70 rpm and 30°C for 4 h At the end of this time the concentration of methanol in the two halves of the cell is equal

Estimation of methanol

Methanol was estimated by gas–liquid chromatography (GLC) on a Pye 104 chromatograph using a hydrogen flame detector. The column (180 cm × 6 mm, I D was packed with Porapak Q (80–100 mesh, Waters Assoc) The column was operated isothermally at 110°C with the detector system at 140°C using nitrogen (flow-rate 20 ml/min) as carrier gas

A sample of 10 μl was injected and the methanol was determined from peak areas calculated by triangulation[10]. An equal volume of a standard solution of purified methanol was injected into the column before and after each test sample, and the amount of methanol in the test sample was then calculated Each run lasts approximately 5 min

Experimental procedure

The master spore suspension for each organism was randomly diluted 10–$7 \cdot 10^5$ spores per ml Each spore suspension was then mechanically shaken for 20 min to disperse clumps Aliquots of each sample were subjected to the following sequence

(a) A total spore count using a Helber chamber.

(b) A plate count for viable spores using the medium described earlier

(c) A dry weight determination of the spores in each test sample

(d) Portions (50 ml) of the spore suspension were then inoculated aseptically into 100 ml of the methanol assay medkum and shaken at 30°C for 15 h

(e) An aliquot of (d) was taken and the dry weight of the growing mycelium after 15 h incubation was determined.

(f) An aliquot of (d) was taken and the methanol was determined after 15 h incubation. Methanol production could then be described as μg/ml per mg dry weight of mycelium

RESULTS

The production of methanol as a function of the size of spore inoculum for each strain is shown in Figs 1–6.

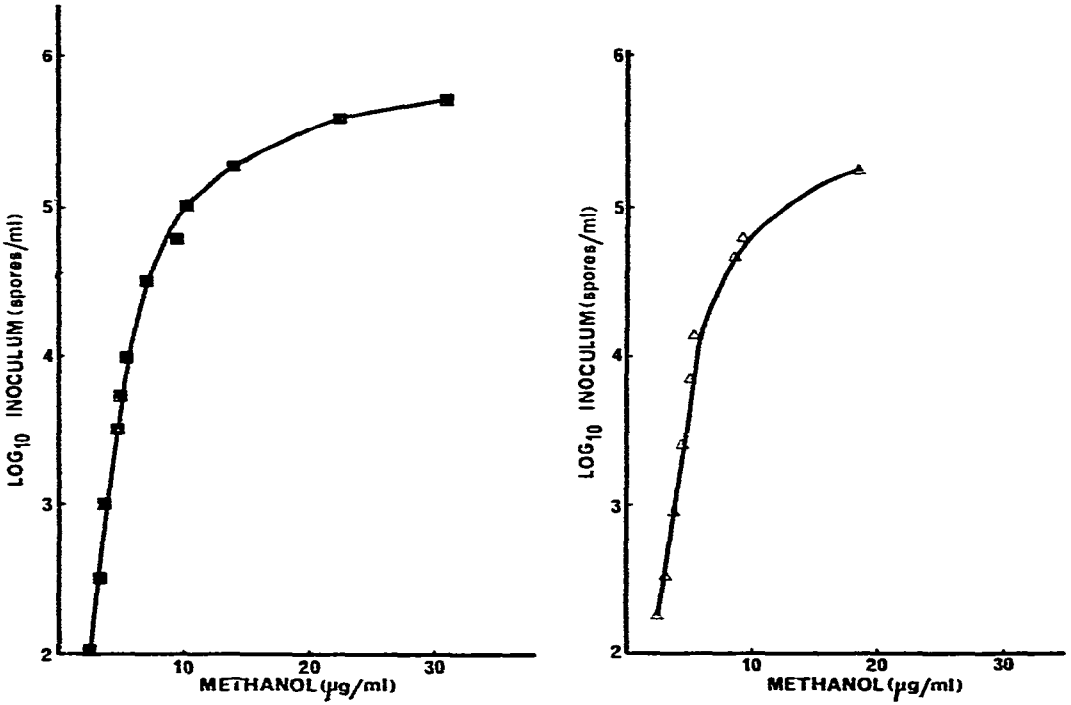

Fig. 1. The influence of inoculum size on methanol production for *A niger*. See text for experimental procedure.

Fig. 2. The influence of inoculum size on methanol production for *A flavus* 15959 See text for experimental procedure.

The results show that an increasing spore concentration liberates an increasing amount of methanol into the medium, reaching an upper limit when the spore concentration is infinitely large. As no methanol is produced when sterile water is used as inoculum, then the curve will pass through both axes.

The results shown in Figs. 1–6 can therefore be described by the equation[11]

$$y = Ce^{-\frac{B}{x}} \tag{1}$$

where $y = \log_{10}$ of the initial spore number per ml, x = amount of methanol released at the end of the incubation period (μg/ml) and C, e and B are all constants.

Eqn. 1 may be transformed to

$$\log_{10} y = \log_{10} C - \frac{B}{x} \tag{2}$$

and a plot of $\log_{10} y$ against $1/x$ should give a straight line. When this was done using the results shown in Figs. 1–6, straight line plots were obtained whose slopes and intercepts are shown in Table I. The six lines were almost parallel, the slopes having a

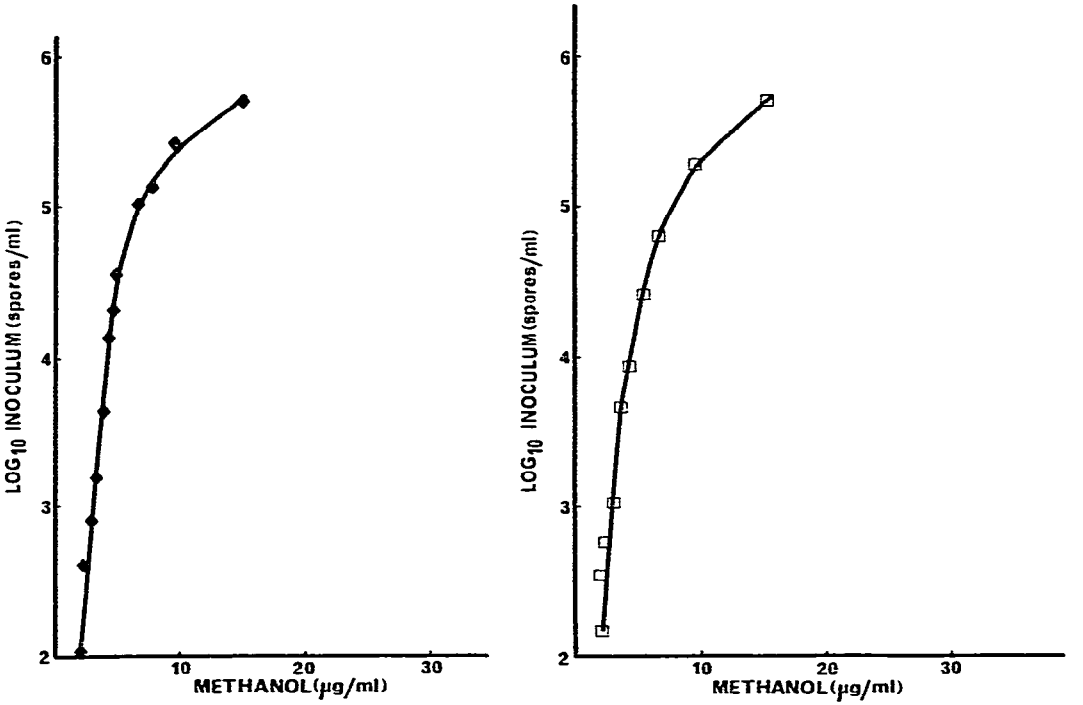

Fig. 3 The influence of inoculum size on methanol production for *A flavus* 86769 See text for experimental procedure

Fig 4 The influence of inoculum size on methanol production for *A flavus* 39178a See text for experimental procedure

standard deviation of $-1.2576 \pm 0\,063$. The intercepts were not identical and correspond to spore numbers ranging from $4\,7 \times 10^4$ to $7\,2 \times 10^5$

It was thought that the methanol production by various strains might be a more consistent property if it were related to some character indicating the germination rate of the spores, for example, protein content or mycelial dry weight at 15 h (the period of incubation).

This was carried out measuring methanol production per mg of dry weight of mycelium at 15 h incubation The results for individual species are shown in Table II using both $\log_{10}$ dry weight of spore material and $\log_{10}$ $\log_{10}$ spore numbers *versus* the reciprocal of methanol production.

The results were then pooled and plotted as a single line (Table II and Fig 7) for each set of data.

Once the level of methanol produced has been established these relationships may be used to determine spore numbers in an inoculum. The results obtained (Table III) are in good agreement with those obtained by standard surface plate methods which took 48 h.

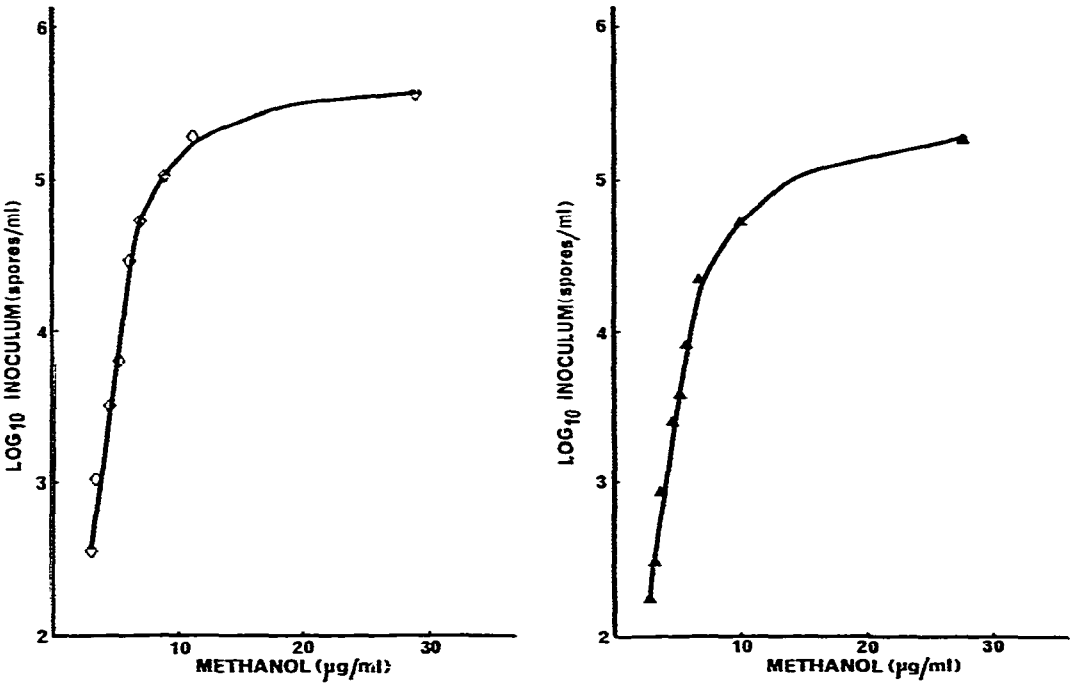

Fig. 5. The influence of inoculum size on methanol production for *A. nidulans* See text for experimental procedure.

Fig. 6 The influence of inoculum size on methanol production for *A. luchuensis* Inui See text for experimental procedure

DISCUSSION

The presence of spoilage fungi in grain and cereals represents a major problem for industries producing or using these commodities. Due to the toxic nature of some fungal metabolites, rapid analysis for the purpose of quality control is obviously highly desirable

One method for the rapid estimation of fungi is the determination of

TABLE I

COMPARISON OF THE RESULTS SHOWN IN FIGS 1–6 PLOTTED AS LOG_{10} LOG_{10} SPORE NUMBERS AGAINST THE RECIPROCAL OF METHANOL PRODUCTION USING EQN 2

Organism	*Slope*	*Intercept*	*Correlation coefficient*	*Standard deviation*
A. flavus 39178a	−1.228	0 883	−0 946	0 023
A. niger	−1 328	0 842	−0 952	0 021
A. flavus 15959	−1.305	0 851	−0 921	0 026
A. luchuensis Inui	−1.75	0 816	−0 914	0 016
A. flavus 86769	−1.249	0 913	−0.942	0 02
A. nidulans	−1.160	0 853	−0 937	0 011

TABLE II

COMPARISON OF THE RESULTS OF PLOTTING LOG_{10} LOG_{10} SPORE NUMBER OR LOG MYCELIAL DRY WEIGHT AGAINST THE RECIPROCAL OF METHANOL PRODUCTION

Organism	*log_{10} dry weight vs reciprocal methanol*			*log_{10} log_{10} (spore number per ml) vs reciprocal methanol*		
	Slope	*Intercept*	*Correlation coefficient*	*Slope*	*Intercept*	*Correlation coefficient*
A flavus 39178a	−6 62	1 034	−0 993	−0 741	0 701	−0 975
A niger	−6 64	1 098	−0 999	−0 725	0 706	−0 968
A flavus 15959	−6 41	1 076	−0 995	−0 721	0 704	−0 976
A luchuensis Inui	−6 55	1 094	−0 997	−0 712	0 704	−0 984
A flavus 86769	−6 82	1 127	−0 986	−0 733	0 703	−0 979
A nidulans	−7 06	1 106	−0 987	−0 745	0 710	−0 973
All six Organisms	−6 727	1 078	−0 988	−0.739	0 706	−0 963

chitin[12–15]. This method is rapid, requiring 4–5 h, but it suffers from several serious drawbacks High levels of contamination are required to produce sufficient chitin for analysis, and the method does not distinguish viable from non-viable spores. A further problem is that the chitin found in insects which frequently infest stored products would interfere with the use of chitin as an index of fungal contamination

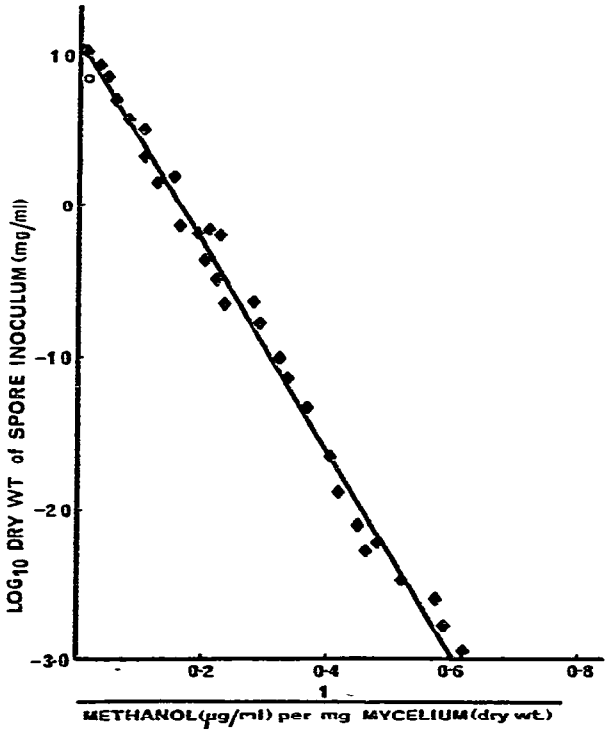

Fig. 7 Calibration curve obtained using data from all six species. The calibration curve is based on eqn 2.

TABLE III

COMPARISON OF SPORE NUMBERS IN SPORE SUSPENSIONS BETWEEN SURFACE PLATE METHOD AND USING DRY WEIGHT OF SPORES AND LOG_{10} LOG_{10} SPORE NUMBERS

Two suspensions of each of the six organisms were prepared by random dilutions. In addition two suspensions were prepared by mixing spores of all six organisms at random. When using the dry weight method a calibration curve of dry weight and viable spore number is necessary.

Organism		*Spore numbers per ml (mean ± standard deviation)*		
		Surface plate method	*Use of spore dry weight*	*Use of log_{10} log_{10} spore numbers*
A. flavus	1	950 ± 350	1123 ± 372	1386 ± 544
39178a	2	1100 ± 270	1002 ± 274	1295 ± 308
A. niger	1	190 ± 47	205 ± 53	312 ± 104
	2	4800 ± 420	5041 ± 480	4606 ± 608
A. flavus	1	11000 ± 2700	10217 ± 2729	9711 ± 3063
15959	2	600 ± 130	689 ± 160	874 ± 187
A. luchuensis	1	8600 ± 1100	9182 ± 1504	12248 ± 4338
Inui	2	38000 ± 6400	36815 ± 5775	42253 ± 6280
A. flavus	1	180 ± 100	270 ± 99	264 ± 119
86769	2	77000 ± 13800	91115 ± 8851	105056 ± 24630
A. nidulans	1	840 ± 152	915 ± 186	1243 ± 331
	2	570000 ± 56000	562500 ± 62250	510700 ± 46175
Mixture of all	1	47000 ± 11600	57915 ± 12777	64103 ± 12193
6 organisms	2	990 ± 320	1195 ± 308	1632 ± 546
Time		48 h	19 h	19 h

The method described in this paper, although not as rapid as the chitin method, has the advantage of distinguishing viable from non-viable spores, and as it measures dormant viable spores capable of germination it is also predictive, *i e.*, it can give an indication of possible sources of contamination in the future. A further advantage is that it is linear over a wide concentration range of spores, thus removing the need for numerous dilutions.

The results (Table II) show that there is a good correlation (−0.988) between inoculum size (in mg dry weight) and methanol production when all the results are pooled. Although the correlation is not so good for spore numbers (−0.963, Table II), it is more convenient as it gives a direct count of viable spores without the need for a calibration curve. It also allows the estimation of lower numbers of spores than the dry weight method due to the difficulties in obtaining accurate dry weight measurements when only a small number of spores are involved.

An examination of Table III shows that the results obtained by this method are in good agreement with those obtained by traditional plate counts

A useful point to make is that as the results for all six species of *Aspergillus* are so similar, it is therefore not necessary to identify the organism to species level when counting. A count may be made on a worst possible prediction basis, *i.e.*, the assumption can be made that all species are toxigenic and the material can be accepted or rejected on the basis of the spore count alone.

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SEPARATION AND IDENTIFICATION OF SYNTHETIC ORGANIC PIGMENTS IN ARTISTS' PAINTS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A thin-layer chromatographic method on silica gel is described for the separation and identification of synthetic organic pigments in artists' oil and acrylic paints Six solvent systems are proposed, and it is established that, except in the case of paints containing phthalocyanine pigments, the presence of the binding medium has no effect on the separations and R_F values obtained For the removal of binding medium from phthalocyanine paints, a multiple extraction with suitable solvents is suggested

INTRODUCTION

Knowledge of the composition of paints is of great importance for the solution of the growing problem of the conservation and restoration of paintings, as well as for the recognition of their authenticity and dating. The identification of paints is also indispensible for the control of their purity. Finally, a knowledge of paint composition is important in view of the fact that often there is a difference between the details supplied on the container and the actual chemical composition, not all paint manufacturers provide catalogues of the chemical compositions of their paints

Different methods have previously been applied for the analysis of synthetic pigments used in artists' paints, *e g*, optical and chemical microscopy, infrared spectrometry, reflexion spectrophotometry, etc Recently, a spectrophotometric method was used for the identification of pigments which were initially separated by dissolving them in suitable solvent systems[1] However, in all these methods, difficulties arise when chemically similar compounds are to be identified; the task is made even more difficult by the fact that samples are not pure, *i e*, homogeneous, in the chemical sense. Therefore, it seems that chromatographic methods of analysis which allow a simultaneous separation and identification of these pigments offer an appropriate solution. Such methods may also be regarded as complementary to those mentioned above.

So far several authors have used thin-layer chromatography (TLC) for the analysis of synthetic organic pigments. Most attention was paid to the analysis of azo pigments which are often encountered Thus, Mc Clure *et al.*[2] separated pigments of

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the following groups: acetoacetarylide, 2-naphthol, 2-hydroxy-3-naphtharylide, dye derivatives, diazo pigments, phthalocyanine and perylene, respectively. Gašparič[3] carried out the separation of azo pigments belonging to the acetoacetarylide, 2-naphthol, pyrazolone, 2-hydroxy-3-naphtharylide groups, as well as lake azo dyes; in addition to TLC, he also applied paper chromatography. Baier[4] investigated the TLC of azo and diazo pigments, including sparingly soluble azo pigments and polycyclic pigments. The potential of TLC for the separation of chemically similar pigments whose spectra exhibit only slight differences has also been pointed out by Billmayer and Kumar[1].

All these investigations were carried out with pure pigments; to our knowledge, no attempt has been made to analyze chromatographically pigments present in artists' paints. In this study we have investigated the possibility of application of TLC to the rapid and efficient separation and identification of synthetic organic pigments most often present in artists' oil and acrylic paints.

EXPERIMENTAL

For TLC separations thin-layers of silica gel H (Type 60; E. Merck, Darmstadt, G.F.R.) were used. Chromatographic plates (20 × 20 cm and 13 × 20 cm) were coated with layers of 300 μm thickness; a mixture of 35 g of silica gel and 85 ml of distilled water was used, which was sufficient for coating 5 or 8 plates, respectively. The coated plates were dried in the air overnight. Pure pigment solutions were chromatographed in parallel with solutions of samples taken from paint tubes. These solutions (2.5–3 mg per 3 ml of the solvent), prepared by heating, were applied to the plate by means of a capillary tube. Spots were dried in a stream of warm air. The development was carried out in commercial tanks for 20-cm plates for 1–1.5 h at 20 ± 2°C. Only in the case of systems 17 and 18, the development time was 3.5 h.

Reagent grade solvents were used for dissolving the samples and for preparation of the solvent systems. Multi-component solvent systems were thoroughly shaken in a separatory funnel before use, and where two layers were obtained the upper layer was applied for chromatography (solvent systems 14 and 20).

The removal of the binding medium from acrylics containing phthalocyanine pigments was effected by multiple extractions with acetone; before extraction, the paints were dried in the air for 24 h. For the removal of the binding medium from oil paints, successive extractions were made with the following solvents: petroleum ether, benzene, ether and acetone. The residue after extraction was dried in a drying oven at 110°C. In the case of other paints it was not necessary to remove the medium, since it was found that this does not affect the separation and R_F values obtained.

All the investigated pigments are in use in the firm Talens (The Netherlands), as were the oil and acrylic paints which belonged to the Rembrandt series.

RESULTS AND DISCUSSION

A list of the pigments investigated is presented in Table I.

Investigations of the solubility of these pigments in a large number of organic solvents have shown that some dissolve in almost all the solvents applied (methanol, cyclohexane, chloroform, acetone), whereas others are either sparingly soluble or

TABLE I

LIST OF INVESTIGATED PIGMENTS

Generic name	*Constitution number*	*Pigment type*
Pigment Yellow 1 (PY 1)	11680	Acetoacetarylide
Pigment Yellow 3 (PY 3)	11710	Acetoacetarylide
Pigment Yellow 97 (PY 97)	11767	Acetoacetarylide
Pigment Yellow 100 (PY 100)	19140 1	Metal azo salt
Pigment Yellow 110 (PY 110)		Azamethine pigment (isoindolinone)
Pigment Orange 1 (PO 1)	11725	Acetoacetarylide
Pigment Orange 5 (PO 5)	12075	2-Naphthol
Pigment Orange 43 (PO 43)	71105	Vat Pigment (perinone)
Pigment Red 7 (PR 7)	12420	2-Hydroxy-3-naphtharylide
Pigment Red 12 (PR 12)	12385	2-Hydroxy-3-naphtharylide
Pigment Red 83 (PR 83)	58000 1	"Vat Pigment" (anthraquinone)
Pigment Red 112 (PR 112)	12370	2-Hydroxy-3-naphtharylide
Pigment Red 122 (PR 122)	73915	Quinacridone
Pigment Brown 25 (PBr 25)	12510	Benzimidazolone
Pigment Violet 19 (PV 19)	46500	Quinacridone
Pigment Violet 23 (PV 23)	51319	Dioxazine
Pigment Green 8 (PG 8)	10006	Miscellaneous metal complex pigments
Pigment Green 7 (PG 7)	74260	Phthalocyanine
Pigment Blue 15 (PB 15)	74160	Phthalocyanine

insoluble in the most of the solvents used; however, the last group was found to dissolve in dimethyl sulphoxide, concentrated sulphuric acid and ethanol–sodium hydroxide (9 1), respectively Accordingly, the pigments were classified into two groups (a) monoazoacetarylide pigments, monoazo-2-naphthols and monoazo-2-hydroxy-3-naphtharylides, (b) metal azo salts, isoindolinones, perinones, quinacridones, monoazobenzimidazolones, anthraquinones, dioxazines, metal complexes and phthalocyanines Dimethyl sulphoxide was found to be suitable for the most of pigments examined, only PV 23, PO 43 and PR 122 were disolved in sulphuric acid

For the chromatographic separation of the pigments more than 150 two- and multi-component solvent systems have been examined; those which were found to afford the best results are listed in Table II

The chromatographic results for the first group of pigments are shown in Table III. As is seen, the best separations were achieved with solvent system 14 However, better separations of red pigments, which exhibited very similar R_F values, were afforded by solvent system 4

Chromatographic separation of the second group of pigments presented a complex problem on account of their poor solubility, the pigments either remained at the starting points, or gave diffuse or tailing chromatographic zones On the basis of investigations carried out with more than 100 solvent systems it has been found that those listed in Table IV allow the identification of some pigments in spite of their similar R_F values, since different colour zones are obtained For example, the separation of PG 8 from PY 100 and PR 83 can be effected by the use of the solvent system 16, whereas pigments PV 19 and PG 8 which exhibit only slightly different R_F values (only 0 03 R_F units) can be identified on the basis of differently coloured parts of

TABLE II

SOLVENT SYSTEMS USED FOR THE SEPARATION OF PIGMENTS BY TLC ON SILICA GEL H

No.	*Composition*	*Proportion (v/v/v)*
1	Pyridine–xylene–acetone	60:40:20
2	Chloroform–toluene–benzene	40:40:40
3	Toluene–pyridine–ethyl acetate	70:30:30
4	Benzene–chloroform–cyclohexane	25:50:25
5	Ethyl acetate–pyridine–toluene–water	30:30:60:10
6	Benzene–cyclohexane–pyridine–water	50:20:20:10
7	Benzene–cyclohexane–pyridine	50:30:20
8	Toluene–acetic acid	70:30
9	Pyridine–toluene	50:50
10	Ethyl acetate–pyridine–acetone	40:40:40
11	Nitrobenzene–xylene	50:50
12	Nitrobenzene–toluene–dimethylformamide	40:40:40
13	Benzene–cyclohexane–chloroform–50% acetic acid	50:20:10:20
14	Benzene–cyclohexane–chloroform–50% acetic acid	60:20:10:10
15	Benzene–cyclohexane–chloroform–50% acetic acid	60:20:15:15
16	Dimethylformamide–dimethylsulphoxide–pyridine–benzene	16:24:40:40
17	Ethanolamine–dimethyl sulphoxide–benzene	20:60:20
18	Ethanolamine–dimethyl sulphoxide–benzene	15:30:55
19	Nitrobenzene–dimethyl sulphoxide–pyridine	25:25:50
20	Formic acid–1,2-dichlorobenzene	80:20

TABLE III

R_F VALUES OBTAINED FOR THE TLC SEPARATION OF THE FIRST GROUP OF PIGMENTS ON SILICA GEL

Pigments are dissolved in pyridine except where otherwise indicated

Pigment	$R_F \times 100$														
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
PY 1	78	34	83	36*	62	83	91	83	50	–	–	–	28*	46*	29*
PY 3	81	43	89	46*	75	90	96	88	66	–	–	–	51*	58*	46*
PY 97	74	1	54	20*	51	65	62	60	18	–	–	–	24*	21*	15*
PY 100	0	0	0	0*	0	0	0	0	0	–	–	–	0*	0*	0*
PY 110	0	0	0	0*	0	0	0	0	0	–	–	–	0*	0*	0*
PO 1	80	–	–	33*	–	–	–	73	78	88	–	–	27*	27*	18*
PO 5	76	–	–	34*	–	–	–	76	72	82	–	–	26*	31*	24*
PO 43	0	–	–	0*	–	–	–	0	0	0	–	–	0*	0*	0*
PR 7	–	–	–	44*	–	–	–	–	–	–	70	94	33*	42*	32*
PR 12	–	–	–	31*	–	–	–	–	–	–	49	85	26*	35*	16*
PR 112	–	–	–	39*	–	–	–	–	–	–	55	89	24*	36*	21*
PR 83	–	–	–	0*	–	–	–	–	–	–	0	81	0*	0*	0*
PR 122	–	–	–	0*	–	–	–	–	–	–	0	0	0*	0*	0*

* Dissolved in dimethyl sulphoxide.

TABLE IV

R_F VALUES OBTAINED FOR THE TLC SEPARATION OF THE SECOND GROUP OF PIGMENTS ON SILICA GEL.

Pigments are dissolved in dimethyl sulphoxide unless otherwise indicated

Pigment	$R_F \times 100$				
	16	*17*	*18*	*19*	*20*
PBr 25	0–100**	79	0–100**	96(tail)	0
PV 19	97	73–99**	96	93	0
PV 23*	88	98	99	81	92
PG 8	94	97	97	86	89
PY 100	84	100	98	85	65–99**
PY 110	0–100**	76	68–93**	0–100**	0
PG 7	0	0	12	0	0
PB 15	0	0	0	0	0
PR 83	98	99	97	98	99
PR 122*	1	1	2	2	7
PO 43*	2	0	7	3	65

* Dissolved in concentrated sulphuric acid

** In the case of diffuse zones the R_F values of both ends of the spots are given

zones which do not overlap Solvent system 17 is the only one which allows separation of PBr 25 from PY 110. As is seen, PG 7 can migrate from the starting point only upon the application of solvent system 18 Finally, the separation of PO 43 and PR 122, as well as of PV 19 from PR 83, can be achieved by use of solvent system 20 The results obtained show that a given pigment can be chromatographed by the application of several different solvent systems, the actual choice depending on the composition of the sample analyzed

On the basis of these results we attempted to apply this method to the separation and identification of pigments present in about 30 oil and acrylic paints The results of the chromatographic separations are shown in Table V It may be concluded that the analysis of these oil and acrylic paints can be performed with the use of the six solvent systems 4, 14, 16, 17, 18 and 20 Good agreements between the R_F values of pure pigment samples and those obtained for samples taken from paint tubes were achieved It follows that the binding medium has no effect on the separations and R_F values obtained, which makes possible analysis of paint samples without prior removal of this medium. However, in the case of paints containing phthalocyanines the medium has to be removed since it was found to interfere with the chromatography. The identification of the pigments was carried out both on the basis of their R_F values and on the colours of the corresponding spots From the results obtained it can be seen that artists' paints often contain mixtures of pigments, since manufacturers mix different pigments in order to attain various colour tones.

We concluded that the chromatographic method described is very suitable for rapid analysis of synthetic organic pigments in artists' oil and acrylic paints Its advantage lies in its accessibility to small laboratories which are not equipped with expensive and complicated apparatus

TABLE V

CHROMATOGRAPHIC RESULTS FOR SYNTHETIC ORGANIC PIGMENTS IN ARTISTS' OIL AND ACRYLIC PAINTS

Sample number	*Sample name*	*Pigment identified*	*Solvent system*
Oil paints			
1	Talens yellow	PY 1	14
2	Talens yellow lemon	PY 3	14
3	Talens yellow deep	PY 1 + PO 43	14, 20
4	Stil de grain jaune	PY 100	16
5	Dutch vermilion ex.	PO 5	14
6	Madder lake light	PR 83	16
7	Madder lake deep	PR 83	16
8	Rose madder	PR 83	16
9	Rose madder lt (alız)	PR 83	16
10	Talens red purple	PR 12 + PR 112	4
11	Talens red deep	PO 5 + PR 112	14, 4
12	Brownish madder	PBr 25 + PR 83	17
13	Rembrandt rose	PV 19	16
14	Rose madder antique	PV 19	16
15	Asphaltum extra	PY 100 + PG 8 + PR 83	16
16	Stil de grain brun	PV 100 + PG 8 + PR 83	16
17	Permanent violet	PV 23	16
18	Rembrandt blue	PB 15	18
19	Cinnabar green light*	PY 3	14
20	Permanent green light*	PY 3	14
21	Sap green	PY 110 + PG 8	17
22	Rembrandt green	PG 7	18
Acrylic paints			
1	Lemon yellow	PY 3	14
2	Talens yellow light	PY 1 + PY 3	14
3	Talens orange	PO 1	14
4	Talens yellow deep	PY 1 + PO 43	14, 20
5	Carmine	PR 12 + PV 19	4 16
6	Permanent red light	PO 5 + PR 112	14, 4
7	Permanent red deep	PR 7	4
8	Talens rose	PV 19	16
9	Permanent violet	PV 23	16
10	Rembrandt blue	PB 15	18
11	Rembrandt green	PG 7	18

* The green colour of the pigment originates from the inorganic pigment

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Note

Precautions in preparing whisker-walled open tubular columns

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Previous reports demonstrated that the poor wettability of the smooth glass surface complicated the coating of open tubular columns. Roughening techniques, such as the whisker growth method were developed to alleviate the problems associated with coating polar liquid phases. Studies by Schieke *et al.*[1–3], using scanning electron micrographic techniques, revealed the nature of the whisker surface of the glass open tubular columns after treatment with 2-chloro-1,1,2-trifluoroethyl methyl ether. The conditions of etching ether concentration and treatment temperature were further studied by Sandra and Verzele[4] for preparation of this microcrystalline silica growth. Although capillary column technology has advanced considerably with the development of fused-silica columns, the whisker glass surface remains advantageous for coating polar liquid phases. In addition, this approach has utility for gas–solid chromatography applications[5]. When used for such applications, it was found necessary to make adjustment to the methods cited to optimize column characteristics. This report describes such modifications as well as the precautions that should be taken during the preparation of whisker-wall-coated open tubular (WWCOT) columns.

EXPERIMENTAL

Materials

All solvents were distilled in glass and purchased from Burdick and Jackson Labs. (Muskegan, MI, U.S.A.). 2-Chloro-1,1,2-trifluoroethylmethyl ether (etching ether) was purchased from PCR Research Chemicals (Gainesville, FL, U.S.A.). Corning Pyrex 7740 glass tubing, 10 mm O.D. × 2.5 mm I.D. and 8 mm O.D. × 4 mm I.D., which was purchased from A. H. Thomas (Philadelphia, PA, U.S.A.), was drawn into capillary tubing 1 mm O.D. × 0.25 mm I.D. and 1 mm O.D. × 0.5 mm

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I D., respectively, using a Model GDM-1 glass drawing machine manufactured by Shimadzu Scientific Instruments (Columbia, MD, U S A) A Packard Model 7400 gas chromatograph and nitrogen carrier gas (99.999" ,) were employed

RESULTS AND DISCUSSION

The whisker-growth technique originally attempted was that as outlined by Schieke *et al.*[1] In our laboratory, the resulting whisker growth was non-uniform

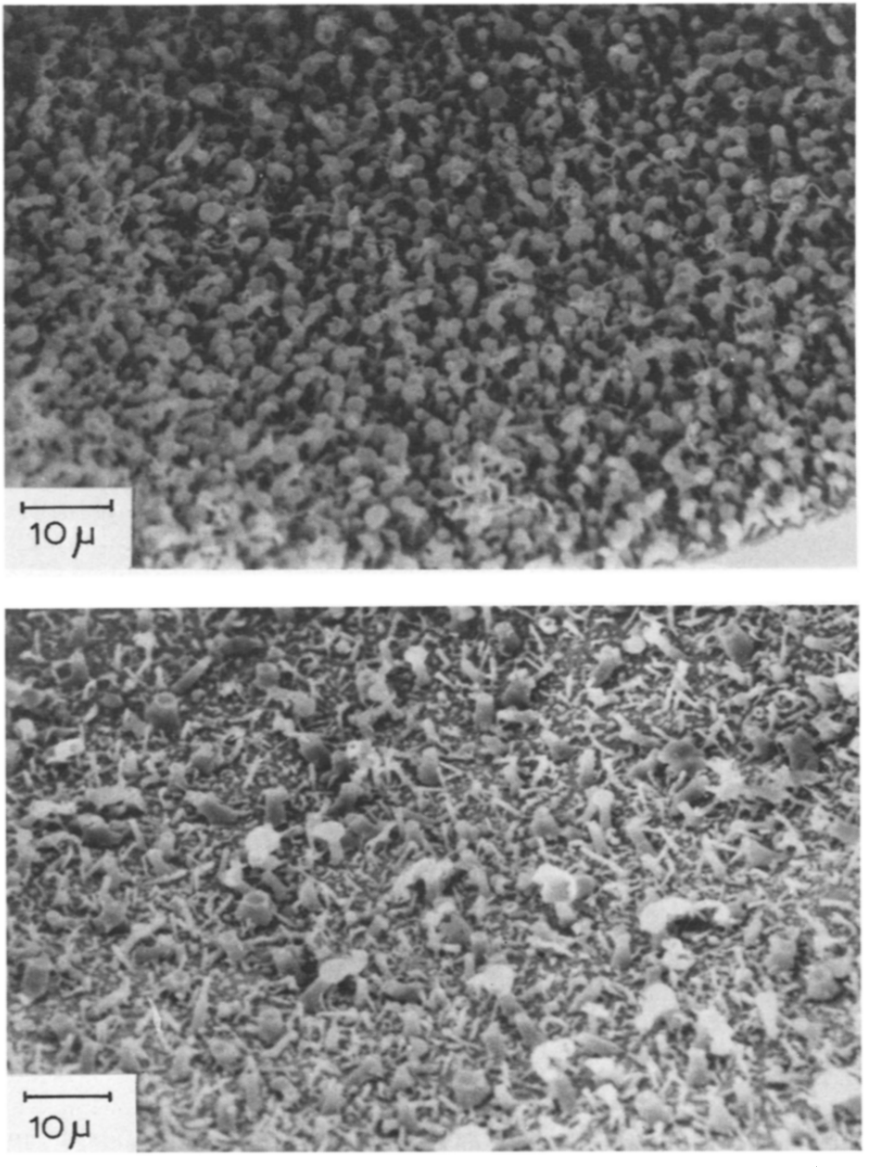

Fig 1 Scanning electron micrographs of the whisker surface resulting from the static vacuum etchin method (heated to 400 C, held there for 1 h and left to cool overnight) a = septum end b = vacuum end

throughout the column As illustrated in the scanning electron micrographs (Fig 1) the whisker density at the end where the etching ether was injected (septum end) was more dense than at the opposite end (vacuum side) Obviously the etching ether was not distributed evenly throughout the column The spherical caps on top of the whiskers obtained in this procedure were also reported by Schieke *et al*[1] These spheres which were removed after washing the column with ethanol were thought to result from the relatively short hold of 1 h at 400 C Another attempt to obtain a

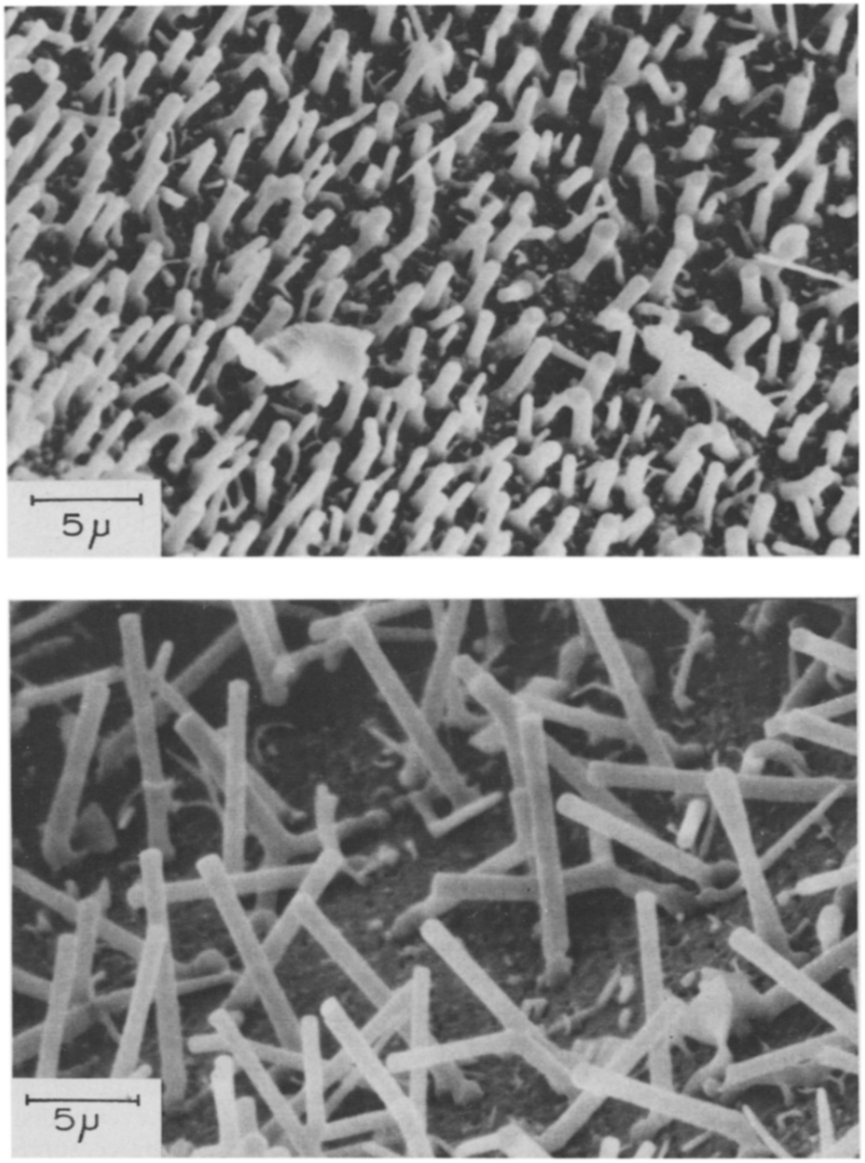

Fig 2 Scanning electron micrographs of the whisker surface resulting from the static vacuum etching method (temperature programmed to 400 C and held there for 12 h) a = septum end b = vacuum end

uniform whisker growth using their approach was made employing the modification described by Sandra and Verzele[4], namely temperature programming the column at 1 5 min to 400 C. The final temperature was maintained for 12 h as suggested by both groups. Although Sandra and Verzele[4] stated that the etching ether is more evenly distributed, scanning electron micrographs of the resulting surface (Fig. 2) still indicate that the whisker density was greater at the septum end. The whiskers at the vacuum end were approximately twice as tall as those at the septum end. It is noteworthy that the spherical caps were not present with this treatment.

In view of the lack of uniformity of whisker growth, the following modifications were made the vacuum remained attached to the glass capillary column while the etching ether was injected through the septum end in such a manner that bubbles were interspersed between small quantities of the etching ether. This end was microtorch sealed and evacuation was continued to distribute evenly the liquid dynamically throughout the column The end of the column, which was attached to the vacuum system was in contact with dry ice to prevent loss of the etching ether through vaporization This vacuum end was then microtorch sealed and the column was temperature treated for 12 h according to the previous procedure Scanning electron micrographs of the septum and vacuum ends of a 51-m glass capillary column treated in this fashion are presented in Fig. 3. As indicated, the whisker growth was of equal density and height at both ends of the column Unfortunately, the results from treating a 102-m glass capillary column with this procedure produced non-uniform whisker growth in a fashion opposite to that experienced previously (Fig 4) This phenomenon was rationalized to occur because the dynamic coating of the etching ether under vacuum proceeded so slowly that the concentration of ether originally present at the septum end continually moved toward the vacuum end. The method of dynamic coating the capillary with the etching ether was modified using nitrogen pressure as described by Clarke[6] Appropriate precautions were included to insure that the required amount of etching ether[4] remained in the column for proper and reproducible whisker formation The procedure used was as follows: the capillary tubing was washed with one column volume of methanol in each direction. Dry nitrogen, which was used at 50 p.s i. to push the methanol through the column, was allowed to pass through the column overnight. This column was then installed on a glass tube support in the gas chromatograph and connected to dry nitrogen at 50 p s i. The column oven temperature was programmed from 25 to 350°C at 5°/min with a final hold at 350 C for 12 h. One end of this capillary was then connected using heat-shrinkable Teflon® tubing to a 2 mm O D. end of a tapered 1/4 in O D standard glass tube. The appropriate amount of etching ether (0 3 mm^3/cm^2; 2.46 μl/m for a 0 25 mm I D. capillary or 4.9 μl m for an 0 5 mm I D. capillary), as determined optimal by Sandra and Verzele[4] to form whiskers 4–5 μm long, was inserted into this 1 4 in glass tube. This tube was secured with a Swagelok connection to a dry nitrogen supply at 50 p.s.i. The capillary was dynamically coated in this manner at constant linear velocity until the ether plug was exhausted within the last 5–10 coils of the column. Both ends of the capillary were attached to vacuum and dry ice was placed at both ends to restrict removal of the ether vapor. With the etching ether evenly distributed after 10–15 min, both ends of the capillary were sealed with a microtorch flame and the capillary was placed in the gas chromatograph. The column oven temperature was programmed from 25 to 400°C at 1.5°/min with a hold at 400°C for

12 h Under these conditions a unfirom whisker density was obtained throughout the entire column After cooling, the ends of the capillary were broken in a hood to release the generated hydrogen fluoride gas The column was then connected to dry nitrogen at 50 p s i in the gas chromatograph and the oven temperature was programmed from 25 to 200 C at 2°/min with a hold at 200°C for 6 h Upon cooling, the nitrogen was replaced with oxygen at 50 p s i and the column temperature pro-

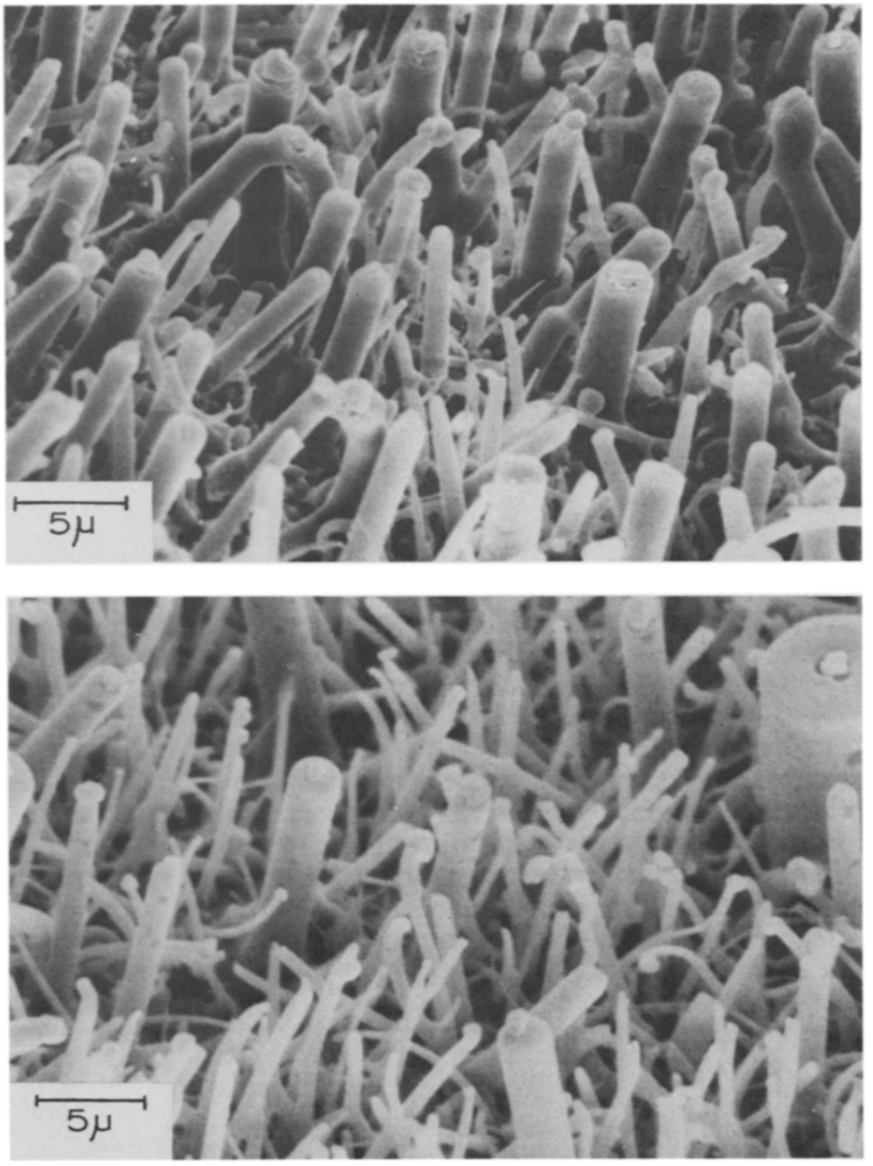

Fig 3 Scanning electron micrographs of the whisker surface resulting from the dynamic vacuum etching of a 51-m column (temperature programmed to 400 C and held there for 12 h) a = septum end b = vacuum end

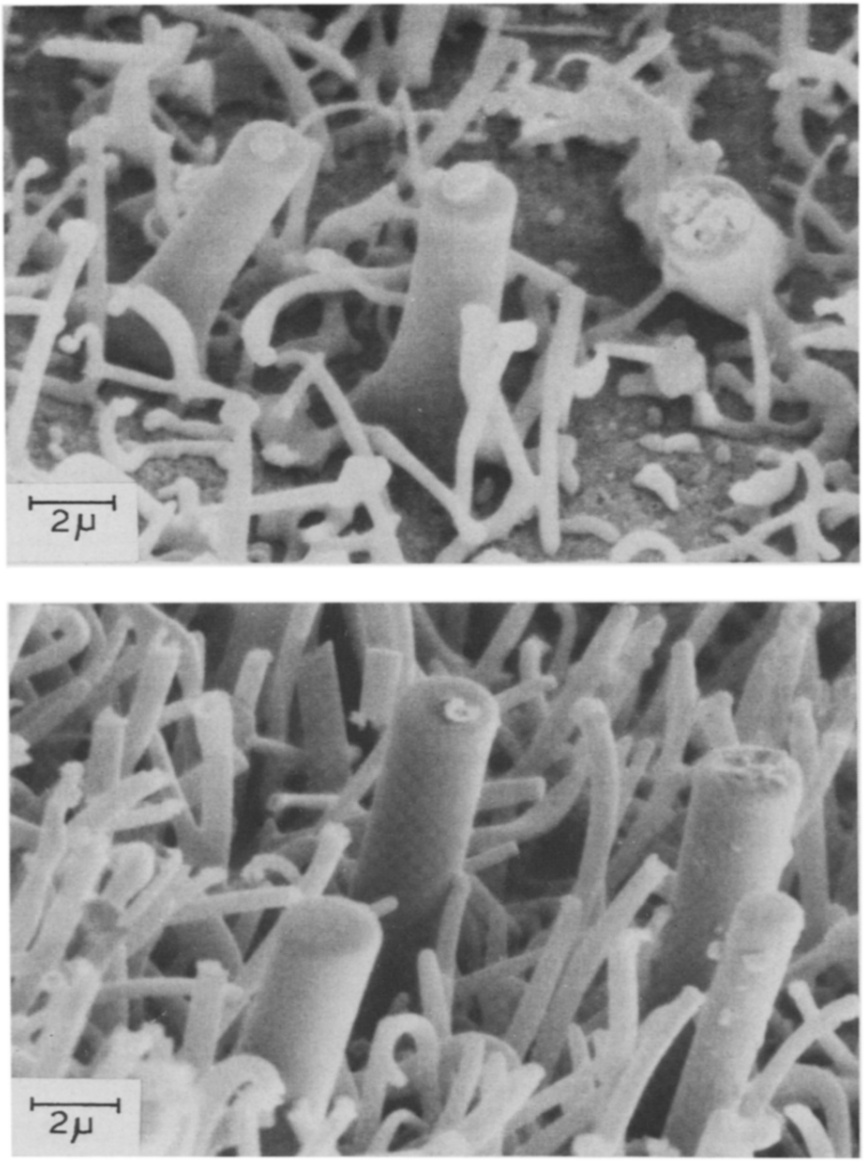

Fig 4 Scanning electron micrographs of the whisker surface resulting from the dynamic vacuum etching of a 102-m column (temperature programmed to 400°C and held there for 12 h) a = septum end, b = vacuum end

grammed from 25 to 450°C at 1°/min with a hold at 450°C for 12 h to remove the residual carbon deposit. After cooling, the whisker capillary column was microtorch sealed for storage if not immediately used

The means of supporting the capillary during the whisker-growing process was found to be very important to obtain uniform whisker growth in each coil of the column. Originally, a wire was used to suspend the capillary (horizontal coil axis) in

the gas chromatograph oven A slight irregular frosted look was observed where the capillary was touching the hanger wire This area became a bare spot after acid leaching with 20% hydrochloric acid A photomicrograph of this bare spot compared to that of an unexposed area for an 0 5 mm I D glass capillary column is presented in Fig 5 A large stainless steel support covering one quarter of the coil diameter was found to cause irregular whisker growth where the capillary touched this surface This was indicated by small bare blotches (after acid leaching) rather than a continuous bare area, as seen in the photomicrograph of an 0 25 mm I D glass capillary column in Fig 6 compared to a photomicrograph of an unexposed area Since the gas chromatograph oven was constructed of stainless steel, asbestos sheets were used to line as much of the oven as possible to eliminate its influence on the whisker growth process A glass tube support created bare spots at times where the capillary column touched the glass tubing Also, when the coils of the capillary column were too closely packed together during the whisker growth process, bare spots along the sides of the coils were observed A support method which did not create bare spots and provided undisturbed whisker formation employed a wire wrapped with asbestos tape upon which the coils of the column were evenly spaced

The vacuum method of introducing the etching ether into the glass capillary is suitable for preparing columns of less than 50 m in length However, the dynamic procedure taking care to distribute evenly an appropriate and reproducible concentration and of etching ether, provides uniform whisker growth in columns in excess of 100 m in length The bare spots in the whiskered columns were only visible as heavily frosted areas until the acid-leaching step of the deactivation method was found to remove the apparently unattached whiskers from the glass surface Taking care to line the oven walls and capillary support with a non-flaking asbestos material and

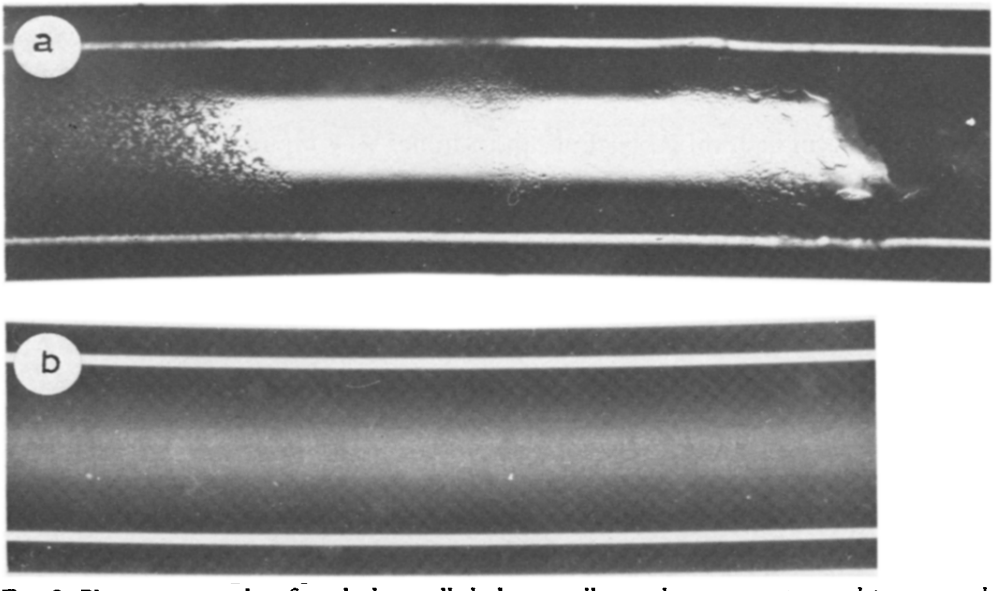

Fig 5 Photomicrographs of a whisker-walled glass capillary column a = exposed to a wire b = an unexposed area

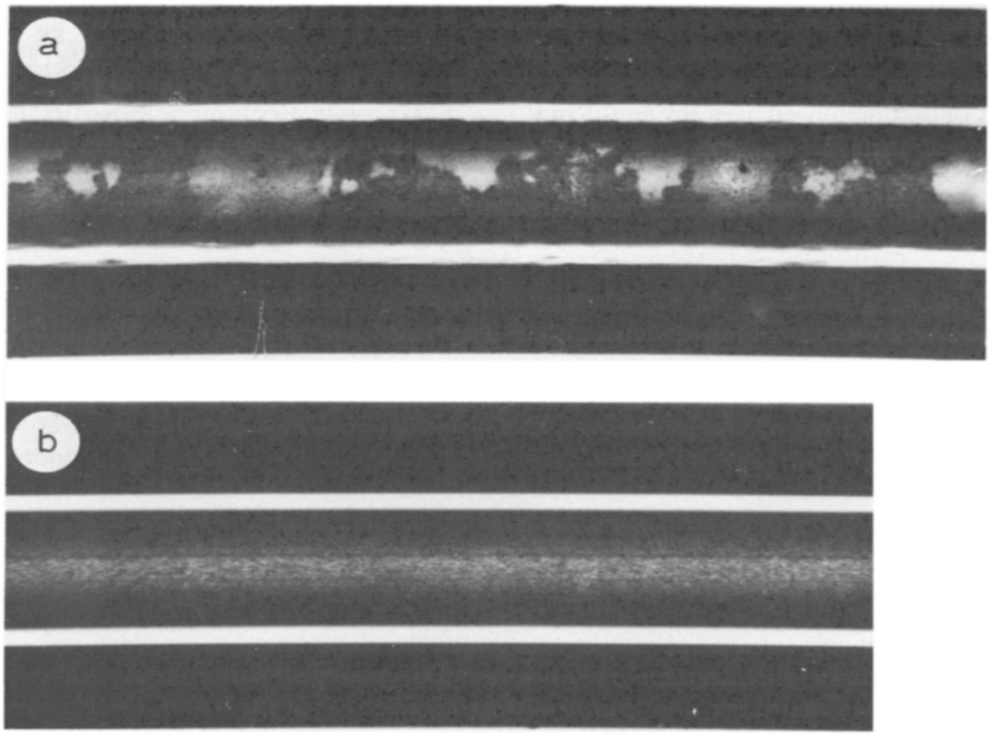

Fig. 6 Photomicrographs of a whisker walled glass capillary column. a = exposed to a sheet of stainless steel; b = an unexposed area

keeping the coils evenly spaced during the whisker-growth step eliminated this problem.

ACKNOWLEDGEMENTS

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Note

Deactivation of whisker-walled open tubular columns with octamethylcyclotetrasiloxane

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The increasing awareness of the glass surface chemistry of open tubular columns has led to improvements in deactivation methods involving acid leaching techniques followed by silanization. Reports[1,2] have indicated that acid leaching removes metal ions from the glass surface, an observation that was confirmed by Auger spectroscopy[3]. Back-migration of the metal ions from the bulk glass to the surface was observed at high temperature[4,5] and a leaching depth of 5–8 μm was suggested to prevent this phenomenon[5]. Various silanization methods at moderate temperatures were investigated for deactivation of the glass surface[6–11], however, silanization only succeeded as a viable deactivation technique when high temperatures (300–400°C) were used[12,13]. Subsequent studies which incorporated these modifications, as well as new silanization reagents[4,14–16], demonstrated marked improvement in deactivation. With the advent of the fused silica column, Stark *et al.*[17] demonstrated that octamethylcyclotetrasiloxane provided thermostability to 350°C for coating apolar liquid phases on such a surface. This report describes a method to deactivate whisker-walled open tubular columns with this reagent.

EXPERIMENTAL

The whisker-walled open tubular columns were prepared as reported elsewhere[18]. Octamethylcyclotetrasiloxane (OMCTS) was purchased from Ohio Valley Specialty Chemical (Marietta, OH, U.S.A.). Concentrated hydrochloric acid was purchased from Fisher Scientific (King of Prussia, PA, U.S.A.), and a 20% solution was prepared in Milli-Q® filtered distilled water.

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RESULTS AND DISCUSSION

The persilylation method of the Grobs[13,14], employing hexamethyldisilazane (HMDS), was studied for the deactivation of whisker-walled capillaries. However, the resulting columns, although reasonably good when evaluated with a polarity test mixture, deteriorated rather quickly (≈2 weeks), indicating loss of deactivation, even when the columns were sealed under nitrogen. With whisker glass capillary columns, a prohibitive amount of ammonia was liberated as a by-product of the HMDS reaction in comparison to a 30% column volume produced with smooth glass capillary columns. This observation was thought to result from the increased surface area (*ca* 10 times) of the whisker-walled column reacting with more HMDS than observed with a smooth-walled column of similar dimension. This excess ammonia was postulated to cause the poor deactivation stability since, as mentioned by Grob[14], excess ammonia continually exposes more of the silica glass structure.

The Grob technique[13,14] of acid leaching and dehydration combined with the octamethylcyclotetrasiloxane reagent introduced by Stark *et al*[17] for deactivating fused silica capillary columns produced well deactivated whisker glass capillary columns.

The whisker-walled glass capillary was leached with acid according to the following procedure: both ends of the capillary were straightened to approximately 30 cm in a Bunsen burner flame. The column was filled to a volume of *ca* 92% capacity with a 20% hydrochloric acid solution using dry nitrogen. The ends of the capillary were microtorch sealed and the column placed in a beaker in a vacuum oven where the temperature was gradually raised to 180°C and the oven evacuated to 60 Torr. After 16 h, the acid solution was washed out of the capillary with one column volume of water followed by a one-column volume water wash in the opposite direction. The capillary was positioned in the gas chromatograph with the straight ends protruding outside above the oven. The capillary surface, regardless of column length, was dehydrated at 150°C for 20 min with the column ends open to the atmosphere. Vacuum (120 Torr) was then applied to both ends of the capillary through a glass T-piece connected to a water aspirator for a time dictated by the column length as described by Grob[14]. As soon as the oven temperature was lowered, both ends of the capillary were immediately sealed under vacuum. One end of the cooled dehydrated capillary was broken and immediately immersed in octamethylcyclotetrasiloxane. After a sufficient amount of this solution was drawn into the column, this end of the column was attached to an SGE glass septum connection and dynamically coated at a velocity of 2 cm/sec with pressure regulated dry nitrogen. Immediately after coating, both ends of the capillary were attached to vacuum (120 Torr) for a specified time[14]. The ends were microtorch sealed and the column was placed in the gas chromatograph. The oven temperature was programmed from 250°C to 400°C at 10°/min and held at 400°C for 12 h. One end of the column was broken under toluene and sequentially washed once in each direction with one-third column volume of toluene, methanol and diethyl ether. Dry nitrogen was employed to push the solvent through the column. The dry column was subsequently connected to the injection port of a gas chromatograph and subjected to temperature program treatment from 40°C to 250°C at 6°/min with a final hold of 1 h at 250°C. A helium carrier gas pressure of 10 p.s.i. was maintained during this period. At this point, the deactivated

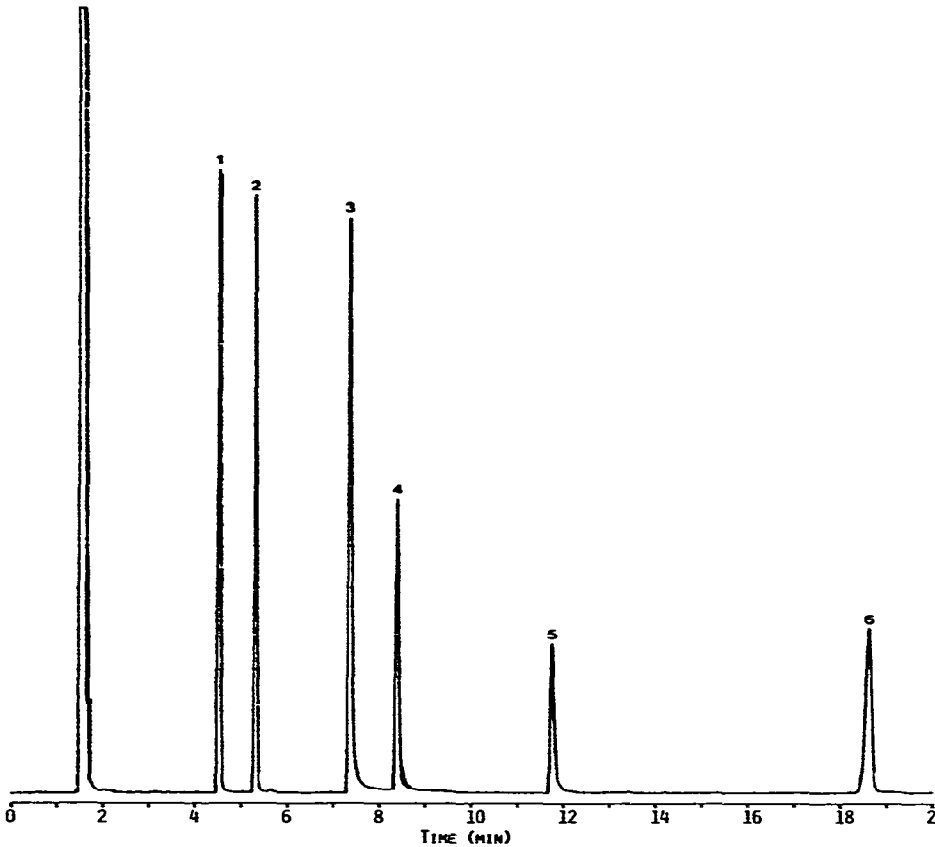

Fig 1 Chromatogram of a polarity test mixture on an octamethylcyclotetrasiloxane deactivated whisker-walled glass capillary column (25 m × 0 25 mm I D , d_f = 0 2 μm), static coated with SE-30 Linear velocity 28.5 cm/sec Λ = 3378 plates per m Helium carrier gas at 20 p s i k = 11 5 Temperatures detector, 245°C, injector, 250°C, column, 83°C Split ratio 100 1 Attenuation 32 × 1 Sample polarity test mixture in hexane Peaks 1 = 2-octanone, 2 = *n*-decane, 3 = 1-octanol, 4 = 2 6-dimethylphenol, 5 = 2,4-dimethylaniline, 6 = *n*-dodecane

column was sealed under nitrogen for storage or directly coated with a liquid phase

A 25 m × 0 25 mm I.D. whisker walled glass capillary column deactivated in this manner was static-coated with SE-30 at a film thickness of 0 2 μm A chromatogram (Fig 1) of a test mixture, which was separated on this column, illustrates the results obtained using this deactivation technique Whisker-walled capillaries deactivated with this procedure have provided thermal stable columns coated with OV-17 (≥270°C), OV-210 (250°C) and Carbowax 20M (220°C) Using this technique on an OV-210 coated column was found useful in the separation of biologically important prostaglandins[19]. The dramatic difference in this separation compared to that obtained with an SE-30 column resulted from the inherent variation in the selectivity offered by OV-210. This deactivation procedure allows the preparation of stable columns coated with moderately polar and polar liquid phases Such stable polar columns provide the increased selectivity necessary for difficult separations, as illustrated by the prostaglandin studies

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Note

New injector design for splitless capillary column gas chromatography

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Recent advances in capillary column technology has made capillary column gas chromatography more attractive than ever, although more or less serious imperfections of the sample introduction techniques may restrict its full utilization Various methods have been developed to circumvent inadequate injector designs Split injection[1,2], pre-column sampling[3] and the so-called falling-needle technique[4] are examples of methods that are designed to reduce or eliminate the problem with the solvent The so-called solvent front effect technique[5–8] takes advantage of the presence of the solvent, which in fact aids in utilizing the high resolution of the capillary column This latter technique is employed mainly in connection with splitless injection and enables injection of microliters of sample The relationship between the boiling point of the solvent and the initial column temperature is essential in this technique A quite thorough evaluation of various parameters of the technique has been performed by Yang *et al.*[9] using a Varian splitless injector with septum and injector purge They conclude, for example, that the initial column temperature should be 15–30°C below the boiling point of the solvent; that sample sizes between 0 1 and 10 μl can be injected; that the rate of sample injection should be about 1 μl/sec, that the period of time in which the syringe needle is resident in the injector should not be less that 20 sec and that the injector purge delay time should not be less than 40 sec

This paper presents an injector design that eliminates most of the drawbacks of the conventional sample introduction systems with their rudiments from the era of packed column gas chromatography and allows an uncomplicated introduction of a sample.

EXPERIMENTAL

Instrumentation

In a Varian 3700 gas chromatograph, equipped with flame ionization detectors and a make-up gas device, was a Varian splitless injector (with septum purge but no injector purge) installed. The dual-pen recorder was a Varian 9176 with one channel adjustable span.

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RESULTS AND DISCUSSION

The Varian splitless injector

This commercially available injector consists of a metal body in which a glass-lined stainless-steel tubing is inserted. The carrier gas enters through the intermediate space, which is enclosed by metal surfaces (Fig. 1). The capillary column is connected to the glass-lined insert. A part of the carrier gas flow is purging the septum to prohibit the septum bleed to enter the column and to avoid memory effects. The purge flow is controlled by a fix restriction.

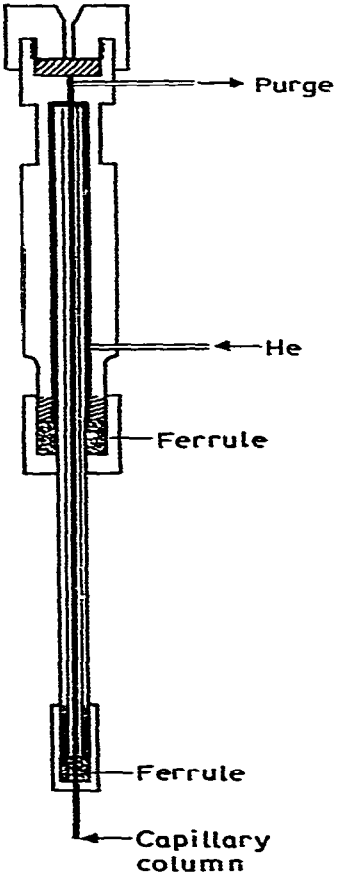

Fig. 1. The Varian splitless injector for capillary column gas chromatography Shaded areas indicate the volumes that are accessable to the carrier gas and vaporized sample

Extracellular fluids (from biopsies and cell cultures), which had been electrophoretically extracted and fractionated[10,11], were analyzed regarding their contents of low-molecular-weight organic compounds. A very limited fraction of acidic metabolites, derivatized in N,N-dimethylformamide with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA, Regis), was analyzed by capillary column gas chromatography employing the Varian splitless injector. Unacceptable chromatograms, similar to that in Fig. 2 (upper panel), were obtained.

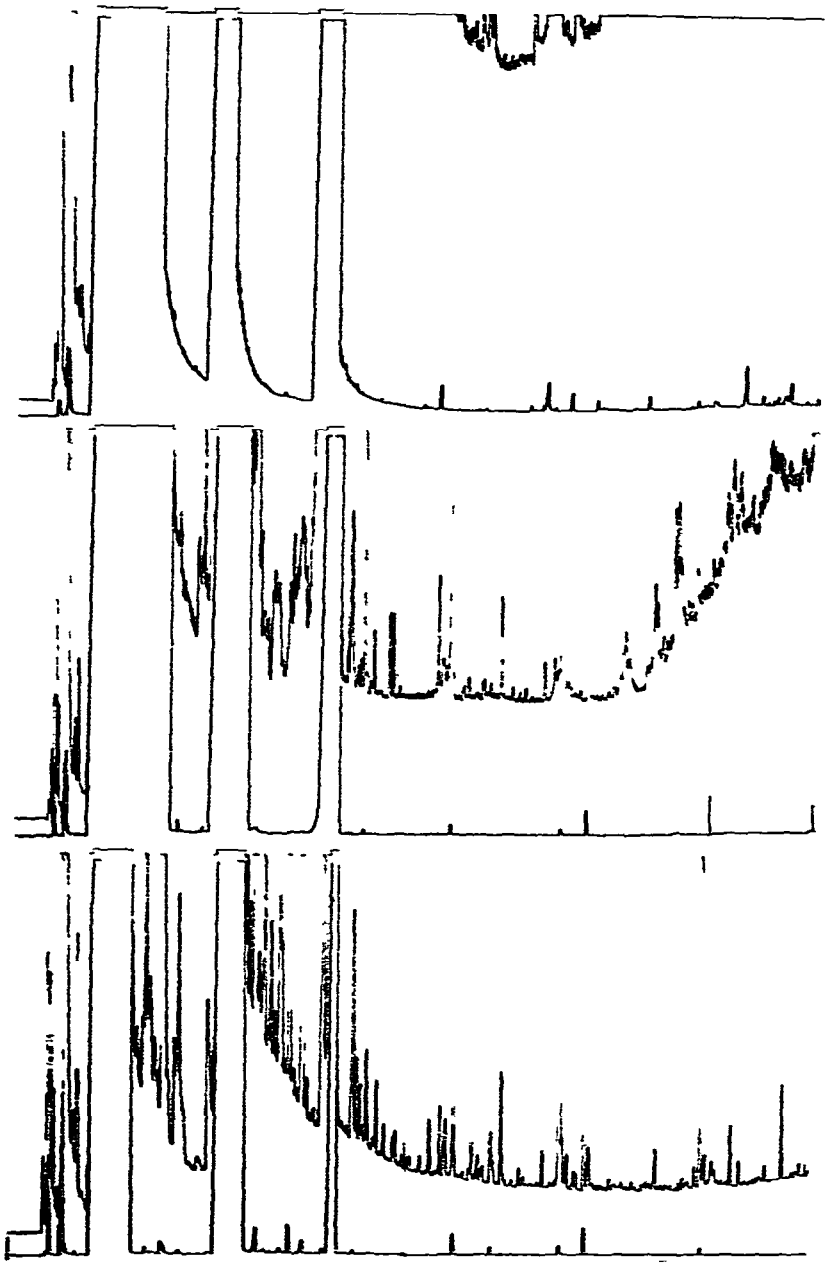

Fig. 2. Splitless injections (2 μl) of the same MTBSTFA-derivatized sample dissolved in dimethylformamide and chromatographed on a 30 m × 0.25 mm Durabond DB-5 column (J & W Scientific), but with different injectors. The oven temperature was 100°C during the initial 8 min and then increased at 4°/min to 300°C. The injector temperature was 220°C and the detector temperature was 310°C. The velocity of the helium carrier gas was about 20 cm/sec. The chromatograms were recorded at a span ratio of 1:50. Upper panel: injection with the Varian splitless injector, middle panel: injection with the modified Varian injector; lower panel: injection with the novel, pre-evacuated injector.

Tests with pure and non-polar solvents, such as isooctane, showed that a decent solvent front could not be obtained at high attenuation (*e g*, $2 \cdot 10^{-11}$ A). The insert was replaced twice with new, cleaned and silanized ones and various column temperatures, sample volumes, rates of injection and periods of time in which the syringe needle resided in the injector were tested without any acceptable result. In most cases a second and even a third small shoulder appeared, which together with the wide and tailing main solvent front strongly indicated problems with dead volumes and back flush.

The shaded areas in Fig 1 show the volumes, which are filled with carrier gas and during an injection will contain the vaporized sample. From the viewpoint of dead volumes the space between the insert and the injector body is excessive and in particular the volume between the gas inlet and the ferrule. During the vaporization process part of the sample will expand into this space and be exposed to a large, hot metal surface and with the well known risk of decomposition of sensitive organic compounds. Another remarkable dead volume is between the extended inner tubing of the insert, the nut and the ferrule for the connection of the capillary column.

The modified Varian splitless injector

The following modifications were made to reduce the dead volumes to improve the performance of the injector. The purge outlet was replaced by the carrier gas inlet and the original inlet was plugged. The inner diameter of the inlet tubing was reduced to diminish the risk of back flush. The space between the insert and the injector body was eliminated by sealing the top of the insert to the inner top of the injector body with a graphite disc. A hole was made in the center of the disc for the passage of the syringe needle. The dead space in the nut for the column attachment was filled with graphite by wrapping a small piece of graphite band around the protruding part of the insert. The dead volume was now reduced to the volume between the septum and the entrance of the column and which is enclosed by glass walls except for a short distance in the injector body between the septum and the insert.

The result of this modification is shown in Fig. 2, middle panel. Tests with non-polar solvents showed a greatly improved shape of the solvent front; total elimination of the third shoulder and an almost eliminated second one. The wide shape of these latter shoulders indicated bleeding from volumes not flushed by the carrier gas. This was substantiated by removing the graphite filling in the lower nut which caused the last eluted shoulder to reappear.

The rate of injection and the time the syringe needle was kept in the injector had no longer any influence upon the appearance of the chromatogram. Thus, it is most likely that the syringe needle has to be kept in the injector for a period of time to reduce the tendency of back flush into both the dead volume between the insert and the injector body and the inlet tubing. The slow rate of injection avoids a fast buildup of pressure, which increases the back flush. Even though the performance of this modified version of the Varian injector could be regarded as substantially improved, it was still unsatisfactory.

The pre-evacuated injector

The new design of an injector for splitless injection, as shown in Fig. 3, allows the introduction of a sample into a closed and evacuated volume. The vaporization

process is not hampered by an initial pressure; the sample is not diluted and spread out in volume and time by the carrier gas flow during the vaporization and the risk of back flush is reduced.

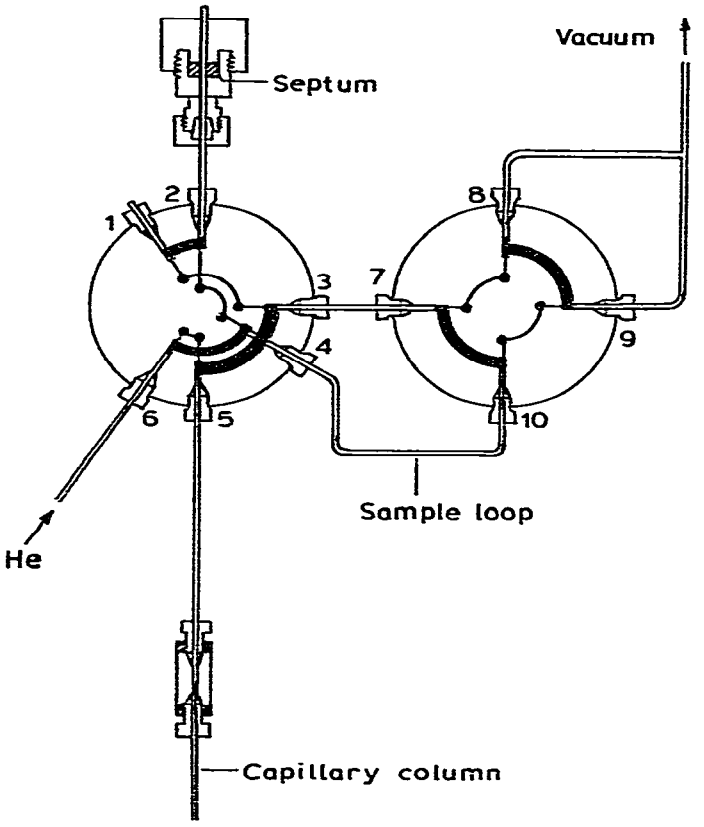

Fig. 3 The design of the novel pre-evacuated injector (For details, see text)

The injector was constructed by means of two zero-dead-volume, high-temperature valves (Valco Instruments Co.) and glass-lined stainless-steel tubing (Scientific Glass Eng). The valves were mounted on an L-shaped bar as close to each other as the nuts at the ports 3 and 7 (Fig 3) allow Two holders (aluminum) for two Varian injector heaters (85 W each and coupled in parallel) and a temperature probe were mounted on the L-shaped bar, which was fastened to the frame of the gas chromatograph at the location of the original injector. The entire construction (valves, sample loop and heaters) was insulated with insulation material.

The length of the glass-lined tubing (0.4 mm I.D.), which serves as transfer line from the injector to the capillary column, was made as short as possible by mounting the zero-dead-volume connector (1/16 in.) close to the ceiling of the oven. The septum holder was made of a reducing union (1/4 to 1/16 in.) and the bottom of which had been flattened. In the center of the septum nut (made of aluminum) a glass-lined tubing (0 3 mm I D) was soldered to guide the syringe needle through the septum and to hit the 0.3-mm hole of the glass-lined tubing, which is connected to port 2 of the

six-port valve. The length of this tubing was adapted to the length of the syringe needle so that, when fully inserted, the tip of the needle is 1–2 mm from the core of the valve rotor. Port 1 has a short and plugged glass-lined tubing (0.3 mm I.D.). The glass-lined tubing (0.7 mm I.D.) connecting port 4 with port 10, the sample loop, was arbitrarily cut to a length of 10 cm. Thus, the injector volume is about 40 μl. A single stage, oil vacuum pump (ultimate vacuum of 10 Torr) was used to evacuate the injector through ports 8 and 9.

The injector is operated in three sequential modes: evacuation, sample loading and injection. During the evacuation ports number 1 and 3; 2 and 4; 5 and 6; 7 and 8; 9 and 10 are connected (indicating by thin lines in Fig 3). The injector system is evacuated all the way from the septum while the column is supplied with carrier gas. The injector will be ready for the loading of the sample by switching the four-port valve to the position, which connects ports 7 with 10 and 8 with 9 (thick lines in Fig. 3). The vaporized sample is injected, without removing the syringe needle, by switching the six-port valve to the position where ports 1 and 2; 3 and 5; 4 and 6 are connected (thick lines in Fig. 3). The carrier gas is now diverted so it will push the volatilized sample out of the injector and onto the column. A negligible small fraction of the sample will be trapped and lost in the volumes at the ports 1 and 2 as can be seen in Fig. 3. The syringe needle is left inserted during the sample loading and injection to diminish the dead volume and reduce the sample loss. Depending upon the velocity of the carrier gas the sample is transferred to the column in 10–20 sec. Usually after 30–60 sec, the six-port valve is reversed first followed by the four-port valve. The injector will then be evacuated, while the chromatographic process continues uninterrupted.

The performance of this injector is exemplified in Fig. 2, lower panel. Besides the noticeable differences in sharpness of the solvent and the two reagent peaks, a steady baseline is achieved. The peaks with longer retention times, particularly in the upper panel, are mainly ghost peaks with a characteristic non-reproducibility. There are fewer of those in the middle panel, and none in the lower panel.

The internal volume of the injector was arbitrarily chosen (see above) and has not been evaluated regarding a likely optimal ratio between its volume and the injected sample volume, but has been found satisfactory for sample volumes up to 0.5 μl. Larger volumes (up to 2 μl) have been injected with good reproducibility but with loss of linearity. A wider range of linearity should be achievable by increasing the volume of the sample loop. The loss of linearity is most likely due to leakages developed at the high pressure created by the injection of too large a sample volume. Incomplete volatilization due to excessive pressure cannot explain non-linearity as the entire sample should be vaporized as soon as the pressure is released by opening the valve to the column.

Even though the two valves are quite costly as well as the pump, it falls short of the price tag of most commercially available injectors. The performance of this new injector surpasses at least the commercial one used in this laboratory. The pre-evacuated and closed sample loop ensures efficient volatilization of the appropriate sample volume at reasonable temperature without a dynamic dilution by the carrier gas. The excellent reproducibility may be attributed partly to a clean injector (exposure to continuous vacuum) with no hot metal surfaces. Additional factors such as hardly any exposure of the septum; no unnecessary and unflushed dead volumes and most likely very little back flush make this injector reliable and easy to operate.

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Note

Gas chromatography of aromatic boronic acids: on-column derivatization

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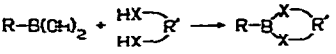
Substituted benzeneboronic acids are of considerable importance as reactive ligands for affinity chromatography[1] and as derivatizing reagents for use in gas chromatography (GC) with electron-capture detection[2]. Since simple boronic acids, such as butaneboronic acid, are well known as derivatizing agents for bifunctional organic compounds[3], it is reasonable to expect simple bifunctional compounds to be useful derivatizing reagents for the more complex boronic acids. A general scheme for the reaction is given (Scheme 1). Other workers[4] have briefly reported the derivatization of electronegative aromatic boronic acids with pinacol (2,3-dihydroxy-2,3-dimethylbutane). For the purpose of analysis by GC–mass spectrometry (MS), we preferred the use of propane-1,3-diol or 1,2-dihydroxybenzene as derivatizing agent for substituted benzeneboronic acids[5] Both of these methods[4,5] involved derivatization prior to GC analysis. This note describes a simple on-column derivatization of boronic acids with propane-1,3-diol. The new procedure has a number of advantages over the conventional one. For example, it is shown that volatile components in a mixture of boronic acids can be examined separately by injection of the underivatized mixture, followed later by an injection of the derivatizing reagent for subsequent characterization of the boronic acid(s) present



X = O, NH
R' = $(CH_2)_{2 \text{ or } 3}$ $(C(CH_3)_2)_2$ ∝
Scheme 1.

A third method for analysis of boronic acids by GC has been reported[6]. It entails dehydration and trimerization of boronic acids to boroxines (trioxatriborans), a reaction which can also be brought about in the gas chromatograph if the injector port is maintained at an elevated temperature[4,6]. This older procedure has not been used because many substituted benzeneboronic acids after cyclization afford more than one GC peak[4] and because boroxines of substituted benzeneboronic acids have very long retention times[5]. Also, the method is not suitable for mixtures since condensation between different boronic acid components would lead to complex boroxines.

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EXPERIMENTAL

Materials

The boronic acids described herein were synthesised in this department in respect of its work on reactive ligands for affinity chromatography[1] Their synthesis will be described elsewhere The exception to this is 2-dimethylaminomethylbenzeneboronic acid which was kindly supplied by Professor G Wüiff Propane-1,3-diol was obtained from Fisons Scientific. All solvents used were redistilled before use

The propane-1,3-diol reagent comprised 50 mmol of the diol in 100 ml pyridine–ethyl acetate (1.1)

Gas chromatography

A Pye-Unicam 104 series gas chromatograph with flame-ionization detector was used The glass column (5 ft × 4 mm I D) was packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh The oven temperatures used were between 140 and 210°C (see below) and the injector temperature was set to be as near as possible to that of the oven Argon (40 ml/min) was used as carrier gas

Gas chromatography–mass spectrometry

The instrument used was a VG Micromass 7070F coupled to a Pye-Unicam 204 gas chromatograph and a Finnigan Incos data system The GC column and conditions were as above except that helium was used as carrier gas The GC–MS interface was a single-stage, all-glass jet separator, maintained at 250°C Ion source conditions accelerating voltage, 4 kV, electron beam energy, 70 eV; emission current, 200 μA; ion source temperature, 200–250°C.

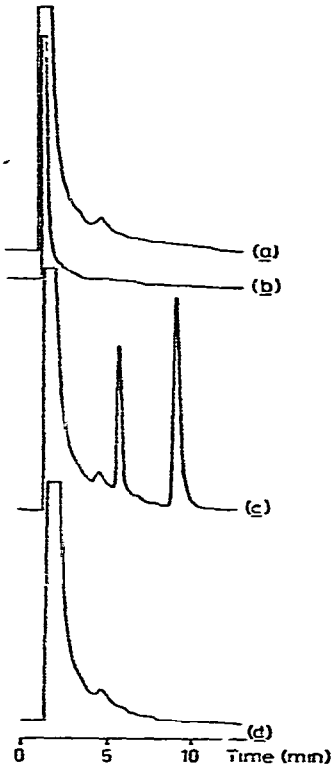
Methods

The aromatic boronic acid sample was dissolved in a suitable solvent (usually ethyl acetate or methanol) and an aliquot (ng–μg range) injected onto the GC column. After elution of the solvent, the propane-1,3-diol reagent (2 μl) was injected to derivatize and hence elute the boronic acid(s) The time between the two injections was varied between *ca* 2 and 30 min (see below)

Conventional "bench" preparation of cyclic boronate esters uses the same derivatizing agent and has been described elsewhere[5]

RESULTS AND DISCUSSION

A typical analysis is shown in Fig. 1 for a mixture of benzene- and 4-methylbenzeneboronic acids. The first trace is that obtained for the propane-1,3-diol reagent only (*i e* a blank) and the second, showing no peaks other than solvent, for the underivatized boronic acid mixture After 10 min, the derivatizing agent was injected and trace c was obtained Two well-shaped peaks were observed for the two cyclic boronate derivatives, the small peak eluting soon after the solvent also being present in the blank (an impurity in the derivatizing reagent) The identity of the eluting species in all cases cited here was checked by combined GC–MS[5]. To check that derivatization was complete, a further injection of the diol reagent was performed and this showed no cyclic boronate ester peaks (Fig 1d) In fact, even when the column



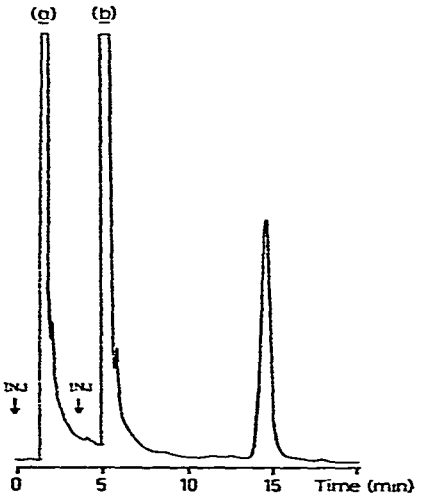


Fig. 1. GC analysis of a mixture of benzene- and 4-methylbenzeneboronic acid. (a) Propane-1,3-diol in ethyl acetate–pyridine only, (b) underivatized mixture of boronic acids in ethyl acetate, (c) injection of propane-1,3-diol in ethyl acetate–pyridine to effect on-column derivatization, and (d) repeat injection of propane-1,3-diol in ethyl acetate–pyridine. All injections were consecutive with a 10-min delay between each. GC conditions: 3% OV-17 at 140°C; injector temperature, 150°C.

Fig. 2. GC analysis of 4-methyl-3-nitrobenzeneboronic acid. Injection points (INJ), of underivatized acid in methanol (a) followed by propane-1,3-diol derivatizing reagent (b), are shown GC conditions. 3% OV-17; column and injector temperature, 200°C.

was overloaded with a boronic acid, on-column derivatization was complete and no memory effects were observed.

Isolated boronic acids occur as mixtures of the acid and the trimeric cyclic anhydride (boroxine). Also, it has been reported that benzeneboronic acid undergoes conversion to triphenylboroxine in the injection port of a gas chromatograph[4,6]. Therefore, it seems likely that the material adsorbed at the top of the column prior to derivatization is a mixture of the boronic acid itself and its boroxine, the composition depending on the temperature of the injector heater and the chemical structure of the acid. Since the diol derivatizing agent reacts with both species to give the same cyclic boronate ester, this situation does not interfere with the analysis. The temperatures of the injection port and a packed OV-17 column required to form triphenylboroxine from benzeneboronic acid and then to elute it are 270 and 200°C respectively[6], whereas the temperatures of the injector and oven in the present study were 150 and 140°C respectively, reflecting the greater volatility of propane-1,3-diol benzenebo-

ronate. If the boronic acid itself or the boroxine derived from it were to spread or elute slowly through the column before on-column derivatization were effected, broad peak shapes would be obtained with retention times significantly less than those of the authentic cyclic boronate derivatives. Since this problem would be most severe with the most volatile boronic acid of the aromatic family, a comparison was made of the GC behaviour of pre-formed propane-1,3-diol benzeneboronate[5] and the same derivative formed on-column. Under conditions giving a retention time of 5.7 min for the pre-formed derivative, the retention time in the on-column experiment was 5 5 min. Whilst this slight reduction in retention time was observed (relative retention time 0 965), there was no observable band-spreading with both peaks having widths at half-height of 17.7 sec

The potential problem of efficient trapping on the top of the column was examined also for 4-methyl-3-nitrobenzeneboronic acid With the inlet port and column at 200°C, the retention time of the cyclic ester derivative was measured to be 10.1 min when the reagent solution was injected 6 min after the boronic acid, 9.7 min when the delay between injections was 12 min, and 9.5 min with a delay as long as 30 min The peak width at half-height was 33 sec in each case with no significant tailing Again, some movement of the boronic acid and/or its boroxine through the column is indicated but no loss of resolution occurs. In this short study, GC conditions were not optimized. In particular, lowering the temperature of the injector was not investigated but would be expected to reduce or eliminate the variation in retention time Reproducibility would be enhanced by injecting the derivatizing agent a set time after the boronic acid injection.

For conventional formation of cyclic boronate esters, the range of suitable solvents is limited to ethyl acetate, dimethylformamide, pyridine, tetrahydrofuran

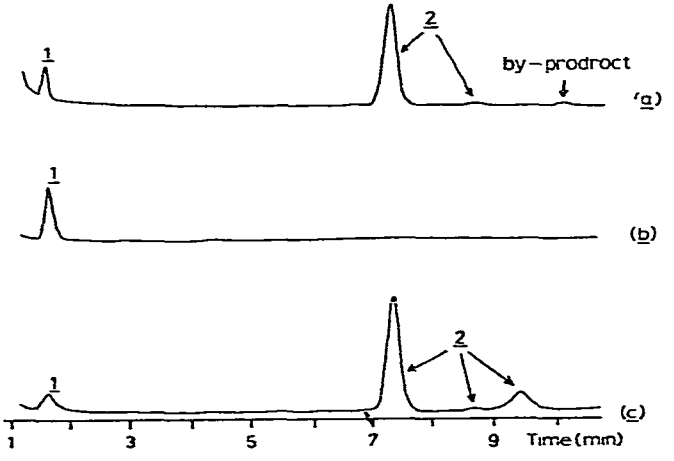
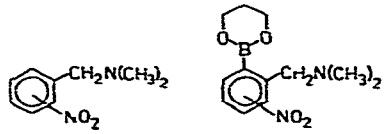


Fig. 3 Analysis by GC–MS of the mixture resulting from nitration of 2-dimethylaminomethylbenzeneboronic acid using conventional derivatization (a) and on-column derivatization (b) injection of underivatized mixture and (c) injection of derivatizing agent The inlet to the ion source was opened in each case immediately after elution of the solvent Note that all three isomeric products (compounds 2) were observed only with the on-column procedure (*cf.* traces a and c) and that compound 1 was present prior to derivatization (trace b) GC conditions· 3% OV-17 at 180°C for 2 min, then temperature increased to 220°C at 8°/min, injector temperature, 250°C (a) or 175°C (b, c)

and chloroform[3]. Some of the more polar benzeneboronic acids are hardly, or not at all, soluble in these solvents and so could not be derivatized. However, the restriction on solvents is removed by use of the on-column reaction and even alcohols can be used. To illustrate this, on-column derivatization of a sample of 4-methyl-3-nitrobenzeneboronic acid in methanol is shown in Fig. 2 with a 3.3 min delay between injection of the methanolic solution and the derivatizing agent The peak width at half-height (38 sec) was increased slightly over that obtained (33 sec) when using ethyl acetate as solvent.



1 2

Two further advantages of the on-column procedure became clear during a study by GC–MS of a mixture resulting from nitration of 2-dimethylaminomethylbenzeneboronic acid. Conventional derivatization followed by GC–MS analysis afforded two major peaks corresponding to isomers of compounds 1 and 2, and two minor peaks assigned to a second isomer of compound 2 and a by-product due to further reaction of compound 2 with the diol (Fig. 3a). First, we required to know if compound 1 was formed during derivatization or was present in the nitration product. Injection of the underivatized mixture showed that compound 1 was already present (or formed on the column) as seen in Fig. 3b. This illustrates that volatile components can be characterized separately. Subsequent injection of the derivatizing agent afforded the trace shown in Fig 3c The two isomers of compound 2 were readily observed, the by-product was not detected, and a third isomer of 2 (not observed in the pre-formed boronate mixture) was seen to elute at the end of the analysis. We propose that this last cyclic boronate ester, when formed during conventional derivatization, reacts further to give, in part or whole, the observed by-product. During on-column derivatization, contact between substrates and reagents is transitory, thereby drastically reducing the chances of further reaction occurring. The fact that compound 1 is also present after on-column derivatization indicates that it is formed during derivatization as well as being present in the original mixture and/or being a thermal product of the boronic acids (see above).

CONCLUSION

The method of on-column derivatization reported herein has been employed successfully with several aromatic boronic acids and mixtures thereof. It is now used routinely in this laboratory. The advantages of the procedure are: (1) speed and simplicity of analysis, (2) use of any solvent commensurate with gas chromatography, including those unsuitable for the "bench reaction", (3) separate characterization of volatile compounds in mixtures of boronic acids is possible since long delays between injection of sample and derivatizing agent are tolerated without loss of chromatographic efficiency, and (4) difficulties with cyclic boronates that are prone to further

reaction upon conventional derivatization are removed The disadvantages are that (1) some solvent tailing is inevitable with pyridine and propane-1,3-diol in the reagent mixture and (2) slight variation in retention times is observed. Standardization of the procedure, improvements to the derivatizing reagent solution and optimization of GC conditions are expected to reduce or eliminate disadvantages

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Note

Gas chromatography of alditol acetates on a high-polarity bonded-phase vitreous-silica column

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Monosaccharides are often analysed by gas chromatography of their alditol acetates on polar phases. Recently, glass capillary columns coated with the polar cyanoalkyl silicone phases, OV-275[1] and Silar 10C[2,3] have been used to separate alditol acetates. However, these glass columns are difficult to handle because of their lack of flexibility and poor mechanical strength. This problem can be overcome by using flexible vitreous-silica columns which are easy to install in gas chromatographs and interfaces with mass spectrometers. Vitreous-silica also has lower levels of metal oxides and other impurities that may adsorb reactive compounds or cause catalytic breakdown of the chromatographic phase. Non-polar phases, such as SP-2100, can be coated onto silica columns, but give relatively poor resolution of alditol acetates[4]. Polar phases give good resolution, but are unstable when coated onto the non-polar surfaces of vitreous-silica columns. This instability can be overcome by cross-linking within the phase to give a bonded or immobilized phase[5,6]. In this paper we report the chromatography of alditol acetates on a high-polarity wall-coated open-tubular (WCOT) column, BP-75, produced by bonding the polar phase, OV-275, on vitreous-silica.

EXPERIMENTAL

Materials

Sugars were obtained from the following sources: L-arabinose, BDH, Poole, Great Britain; 2-deoxy-D-ribose, Koch-Light, Colnbrook, Great Britain; D-mannose, Pfanstiel Labs., Waukegan, IL, U.S.A.; D-allose, cellobiose, 2-deoxy-D-glucose, L-fucose, D-galactose, D-glucose, L-rhamnose, D-ribose and D-xylose, Sigma, St. Louis, MO, U.S.A. Myo-inositol and erythritol were from BDH. Laminaritetrose was prepared by the method of Clarke and Stone[7].

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Preparation of alditol acetates

Alditol acetates were prepared from monosaccharides by the method of Blakeney *et al.*[3]. Permethylated, peracetylated alditols were prepared as described by Jansson *et al.*[8].

Gas chromatography

Alditol acetates were separated on a 6 m × 0 2 mm I D , BP-75, vitreous-silica, WCOT column (S G E., Melbourne, Australia) in a Hewlett-Packard 5710A chromatograph equipped with a flame-ionization detector and a S.G E. Unijector capillary injection system, used in the split mode (split ratio 4 1) The end of the column was located at the point within the heated section of the injection splitter at which equivalent splitting of each component was achieved High-purity hydrogen was used as the carrier gas at a flow-rate of 1 3 ml/min. Alditol acetates were separated using a temperature program of 170°C for 4 min followed by a 4°C/min rise to 230°C. Permethylated, peracetylated alditol acetates were separated using a temperature program of 150°C for 4 min followed by a 2°C/min rise to 230°C The injection port and detector were heated to 250°C and 300°C, respectively. Peak areas were recorded using a Hewlett-Packard Model 3380A reporting integrator

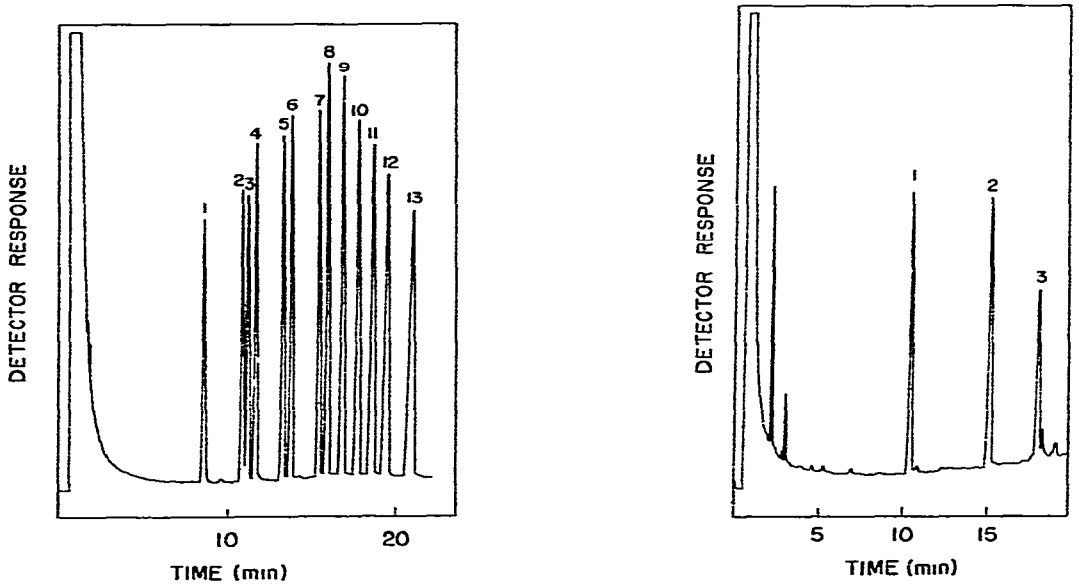

Fig. 1 Separation of 13 alditol acetates on a 6-m B-75 vitreous-silica column Temperature program 170°C for 4 min, then 4°C/min to 230°C Peaks 1 = erythritol, 2 = deoxyribitol, 3 = rhamnitol, 4 = fucitol, 5 = ribitol; 6 = arabinitol, 7 = xylitol, 8 = deoxyglucitol; 9 = allitol, 10 = mannitol, 11 = galactitol; 12 = glucitol, 13 = inositol 2 μl of dichloromethane containing the alditol acetates derived from 3 μg of each monosaccharide was injected

Fig. 2. Separation of permethylated alditol acetates on a 6-m BP-75 vitreous-silica column Temperature program· 150°C for 4 min, then 2°C/min to 230°C Peaks· 1 = 2,3,4,6-tetramethyl-1,5-diacetylglucitol, 2 = 2,4,6-trimethyl-1,3,5-triacetylglucitol, 3 = 2,3,6-trimethyl-1,4,5-triacetylglucitol

RESULTS AND DISCUSSION

The separation of 13 alditol acetates on a BP-75 vitreous-silica WCOT column is shown in Fig. 1. The resolution is comparable with that obtained on a Silar 10C glass support-coated open-tubular (SCOT) column (28 m × 0.5 mm I.D.)[3]. All components were separated in less than 20 min, however, faster separations are possible. For example, by using a temperature program starting at 140°C and rising at 16°C/min to 250°C all 13 alditols, except deoxyribitol and rhamnitol, were separated in less than 9 min. Routine separation of simple mixtures with widely different relative retention times may be completed in even shorter times. Permethylated alditol acetates were also well resolved on this column (Fig. 2).

The flexibility of the vitreous-silica column allowed easy manipulation of the column and the bonded-phase has several advantages[5,6]. Bonded phases have good thermal stability which increases the life of the column and the low phase bleed reduces background in mass spectrometry. Contaminants can be removed by washing with solvents without effecting the chromatographic properties of the column.

The combination of the high resolving power of a polar phase with the practical advantages of a bonded phase on vitreous-silica suggests that this column should find wide application in the analysis of complex mixtures of monosaccharides and in methylation analysis.

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Note

High-performance liquid chromatographic analysis of short chain carboxylic acids as *p*-bromophenacyl esters: identification and separation of a decay product

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It has been shown previously[1] that short chain carboxylic acids in aqueous systems can be determined by extraction with diethyl ether, esterification with *p*-bromophenacyl bromide[2] and analysis by reversed-phase high-performance liquid chromatography (HPLC). However, one problem encountered was the formation of a "decay" product from *p*-bromophenacyl bromide in the presence of chloride ion[1]. This non-reactive product co-eluted with the propanoate ester under the chromatographic conditions used As many aqueous samples —for example marine waters— contain significant amounts of chloride, this "decay" product could be mistaken for propanoate Thus a method was described which largely overcame this problem However, as *p*-bromophenacyl bromide itself appears to contain a small amount of this component, ultimately the best solution is to find chromatographic conditions — reported here— which allow separation of propanoate and the decay component In addition, the latter has been characterized from its mass spectrum, so that its suspected origin could be confirmed

EXPERIMENTAL

Reagents

Glassware, double distilled water and chemicals were prepared as before[1]

HPLC apparatus

The HPLC system consisted of 6000A and M45 pumps coupled to a M660 solvent programmer (all Waters Assoc.), a Rheodyne 7125 loop injector (20 μl) and μBondapak C_{18} column (30 cm × 3.9 mm I D , Waters Assoc.). Column effluent passed through an 8-μl flow-cell in a Cecil Instruments CE 2112 UV variable-wavelength spectrophotometric detector set at 254 nm, connected to a Bryans 28000 chart recorder. The mobile phase was acetonitrile and water, total flow-rate 2 ml min^{-1} Gradient and isocratic conditions are given below

Mass spectra

Mass spectra of *p*-bromophenacyl bromide and the decay product were obtained via a probe inlet on a Kratos MS 25 double focussing mass spectrometer.

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ionizing voltage 70 eV; filament emission current 100 μA; source temperature 200°C. Spectra were collected on a Kratos DS 55 data system.

Procedure

(a) Esterification of standard acids. Briefly, carboxylic acids (acetic, propanoic and butanoic) were converted into K^+ salts by addition of aqueous potassium hydrogen carbonate. Esterification occurred with addition of excess *p*-bromophenacyl bromide/18-crown-6 (ratio *ca.* 10:1) in acetonitrile and heating (80°C, 20 min)[2].

(b) Formation of the "decay" product. Hydrochloric acid (1 *M*) and potassium hydrogen carbonate (0.1 *M*) were added in equal mole amounts to form potassium chloride. *p*-Bromophenacyl bromide/18-crown-6 were added so that no bromide remained after heating. The decay product was the only organic component detected when the sample was analysed by HPLC. It was purified by thin-layer chromatography on silica gel G (eluent: hexane–diethyl ether, 7:3), before analysis by mass spectrometry.

(c) HPLC separation of propanoate ester and decay product. Aliquots of esteri-

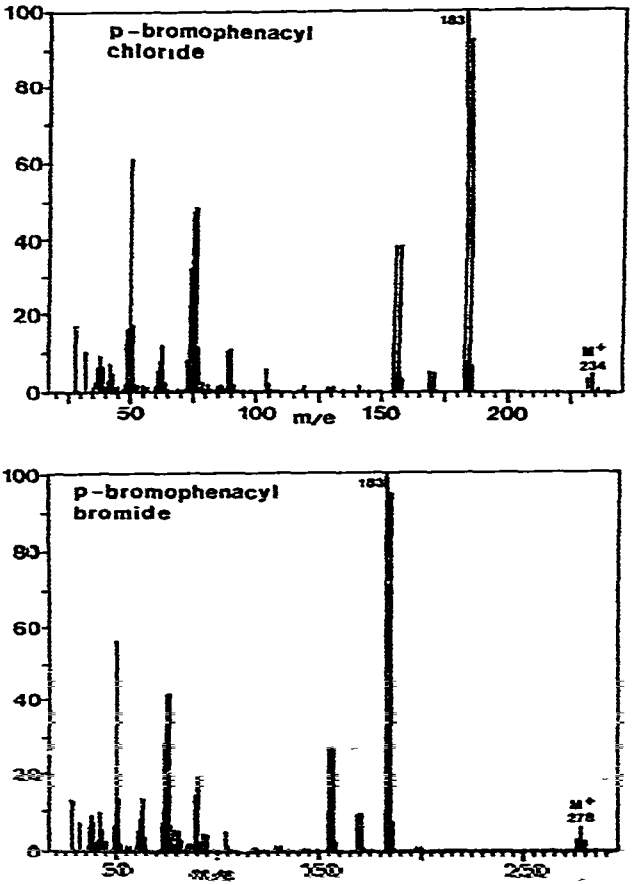

Fig. 1. Electron impact mass spectra of *p*-bromophenacyl chloride and *p*-bromophenacyl bromide.

fied acids formed in *a* and the product from *b* were combined to form one sample, which was used for separation of the two co-eluting components

RESULTS AND DISCUSSION

It was suggested previously that the decay product, formed from *p*-bromophenacyl bromide in the presence of chloride ion, might be *p*-bromophenacyl chloride This is indeed the case, as the mass spectrum in Fig 1 shows (the mass spectrum of the bromide is included for comparison). This is important for three reasons: (i) environmental samples which contain short chain carboxylic acids often contain significant amounts of chloride as well. Extraction techniques which do not exclude chloride will result in formation of *p*-bromophenacyl chloride at the derivatization stage (ii) *p*-Bromophenacyl chloride is non-reactive for esterification purposes and appears to

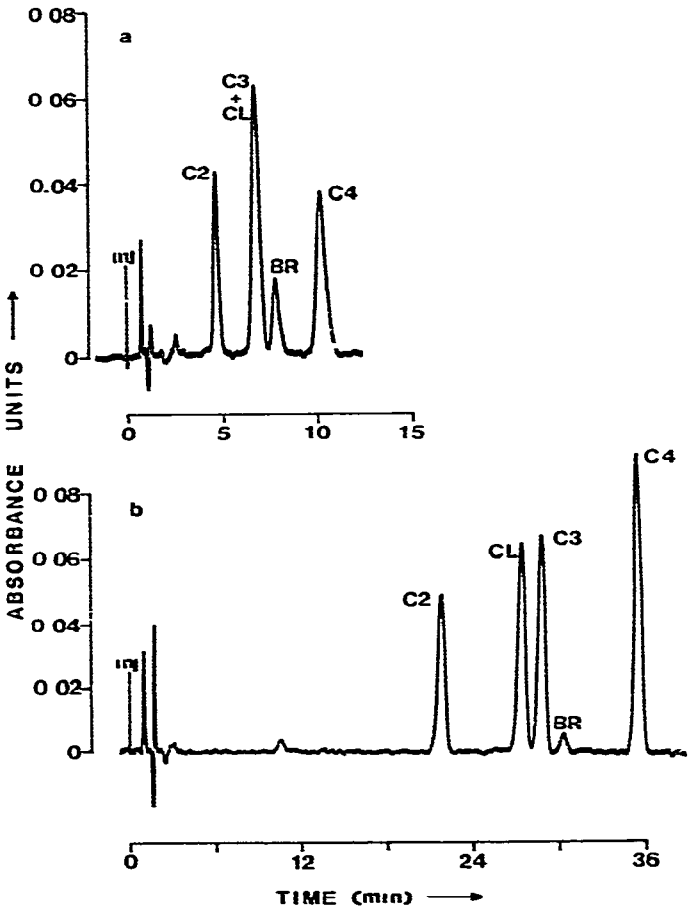

Fig. 2. HPLC separations of standard acids (C_2–C_4) (*p*-bromophenacyl esters) and *p*-bromophenacyl chloride Conditions 20-μl loop injection on μBondapak C_{18} column, flow-rate 2 ml min^{-1}, UV detection 254 nm (a) Acetonitrile–water (50 50) isocratic elution (b) Acetonitrile (20–52%) and water, linear gradient elution in 40 min C2 = acetate; C3 = propanoate, C4 = butanoate, CL = chloride, BR = bromide (starting material)

form more readily than the acid esters (iii) The chloride derivative and propanoate ester co-elute on reversed phase HPLC under isocratic conditions used previously (acetonitrile–water, 50:50) (Fig. 2a) As it is difficult to prevent completely formation of the chloride derivative, separation of the co-eluting components was attempted. This can be done under isocratic conditions, and baseline separation was achieved using 35% acetonitrile However, this leads to significant peak broadening and very long retention times for butanoate and higher acid esters (>60 min). Instead, a linear gradient programme of 20–52% acetonitrile in 40 min was found to be the best set of conditions (Fig. 2b). Each component is eluted as a sharp peak, and propanoate and the chloride are separated to baseline. Retention times are still longer than under the initial isocratic conditions, but this is felt to be a worthwhile sacrific since propanoate can be assigned with greater confidence.

ACKNOWLEDGEMENTS

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Note

High-performance liquid chromatographic determination of hydroxyproline after derivatization with 4-dimethylaminoazobenzene-4′-sulphonyl chloride

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Human fibroblast cultures can provide a good means for studying collagen metabolism and its alterations in numerous diseases Various methods have been reported for specifically measuring collagen in fibroblast cultures and in other biological samples, based on the presence of hydroxyproline (Hyp) which is characteristic of the collagen molecule[1]. Some of these methods are based on spectrophotometric determination of Hyp after hydrolysis, oxidation and extraction of the sample[2,3] Other procedures utilize the incorporation of [^{3}H]- or [^{14}C]-Hyp after a hydrolysis and a long series of oxidations and extractions[4–6], or measure the soluble ^{14}C-labelled proline and Hyp obtained by collagenase digestion of the sample[7,8].

Unfortunately, these procedures are often tedious and time-consuming and most of them utilize radioactive materials

The present paper reports a simple and very rapid method for Hyp assay in fibroblast cultures, using high-performance liquid chromatographic (HPLC) separation and determination of the 4-dimethylaminoazobenzene-4′-sulphonyl (dabsyl) derivatives of amino acids in the hydrolyzed samples.

MATERIALS AND METHODS

Apparatus

The HPLC system (Perkin-Elmer) consisted of a Model Series 3 solvent-delivery system, a Rheodyne 7120 injection valve with a 10-μl injection loop, a Model LC 100 column oven and a Model LC-55 B variable wavelength UV detector operat-

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ing at 486 nm. A reversed-phase RP-8 column (Brownlee; 25 cm × 4.6 mm I.D., particle size 10 μm) was used. All chromatograms were recorded on a Perkin-Elmer Model 56 recorder. The mobile phases were. A, acetonitrile, 1 66 m*M* acetic acid and 6 99 m*M* orthophosphoric acid, pH 3.5; B, 10 m*M* sodium acetate, 1.66 m*M* acetic acid, pH 3 00 adjusted with orthophosphoric acid[9]. Other conditions were: flow-rate, 1.5 ml/min; pressure, 3.5–4 MPa; column temperature, 40°C. Solvent program. T_1 = 10 min (30–34% solvent A), gradient curve 1; T_2 = 10 min (34–60% solvent A), gradient curve 0 3 (Fig. 1).

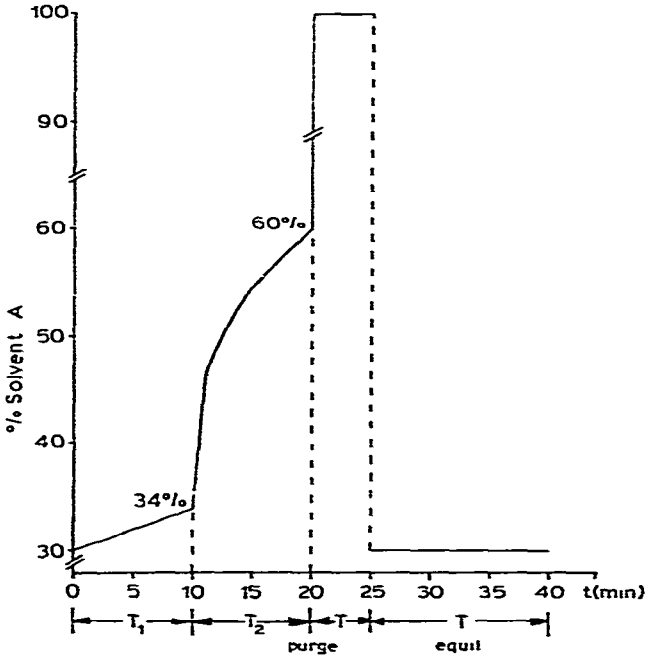

Fig. 1 Solvent program used for the column elution. Solvent program T_1 curve 1, T_2 curve 0 3

Reagents

L-4-[^{3}H(G)]Hydroxyproline ([^{3}H]Hyp), specific activity 5 4 Ci/mmol, was purchased from New England Nuclear (Boston, MA, U S A.). Dabsyl chloride, 3 3 mg/ml in acetone, was obtained from Pierce (Rockford, IL, U S A). N-Methyltaurine (99% purity according to a liquid chromatographic assay), kindly supplied by Professor Adembri, was used as an internal standard. Acetonitrile LiChrosolv, highly purified Hyp and other standard amino acids were obtained from E Merck (Darmstadt, G.F.R.). Solvents and other chemicals were of AR grade.

Procedure

Fibroblast cultures. Human fibroblasts, obtained from normal donor skin explants, were cultured as previously described[10]. Supernatants and cell monolayers of early confluent fibroblast cultures were used as samples for the HPLC procedure.

Fibroblasts (2 · 10^6 cells per flask) were cultured for 72 h at 37°C (pH 7.4) to reach visual confluency with and without ascorbic acid (100 μg/ml) in the medium. Then

supernatants were collected; cell monolayers were solubilized with a 0 5 *M* NaOH solution, washed three times with warm Hank's balanced salt solution and collected Supernatants and solubilized cell monolayers were then hydrolyzed (3 *N* HCl, overnight at 130°C) The hydrolyzates were neutralized at pH 8 9 with 4 *M* K_2CO_3

Dabsylation The dabsylation reaction was performed as described previously[11]. The reaction mixture consisted of 20 μl of the neutralized sample hydrolyzates, 20 μl of 0 1 *M* Na_2CO_3–$NaHCO_3$ buffer solution (pH 8 9), containing 6 2 μmol/ml of internal standard and 20 μl of dabsyl chloride solution The reaction was allowed to proceed at 70°C for 6 min with constant shaking A 10-μl volume of the reaction mixture was injected into the chromatograph

Calibration curve. Standard solutions of Hyp, ranging from 0 19 μmol/ml to 3 3 μmol/ml, were processed as described above The calibration curve was obtained by calculating the ratio between the peak heights of Hyp and the internal standard and plotting these ratios against the concentrations This curve was used to convert peak height ratios of unknown samples into Hyp concentration

RESULTS

Separation of dabsyl-Hyp

Fig 2 shows a typical chromatogram of biological samples (supernatants + cell monolayers from fibroblast cultures) processed by the method described It can be seen that a good separation of dabsyl chloride, N-methyltaurine and Hyp was obtained The retention times of the internal standard and Hyp were 8 and 15 2 min respectively Dabsylated standard Hyp gave a single peak

The Hyp peak was identified on the basis of its absolute and relative retention time and by adding known amounts of standard Hyp to the samples. By the same procedure it was possible to identify the peaks immediately before and after Hyp as shown in Fig 2

In order to better identify the Hyp peak [^{3}H]Hyp was also added to the samples at various concentrations and processed by the method described Throughout this procedure a linear correlation (r = 0 997) was obtained between the single amounts of [^{3}H]Hyp added to the samples and the radioactivity found in the fractions corresponding to the Hyp peak collected from the column

Furthermore, no radioactivity was recovered in the fractions collected before and after the one corresponding to the Hyp peak This confirmed that Hyp gave a single derivative under the analysis conditions used.

Some experiments were performed using a Perkin-Elmer LC85-Autocontrol variable wavelength detector (equipped with a 2.4-μl flow-cell) which could record and analyze spectra of the single peak eluted from the column The UV spectrum of the fraction corresponding to the Hyp peak overlapped (absorbance maximum at 194 5 nm) that of standard dabsylated Hyp injected into the column under the same experimental conditions.

Further evidence that no other dabsyl-positive compounds might interfere with Hyp was obtained by using a thin-layer chromatographic (TLC) procedure The fraction eluted from the column between 15 and 20 min after injection of the sample (corresponding to the last five peaks including Hyp in Fig. 2) was separated on silica gel G plates (Merck, 20 × 20 cm) by two-dimensional TLC according to Lin and

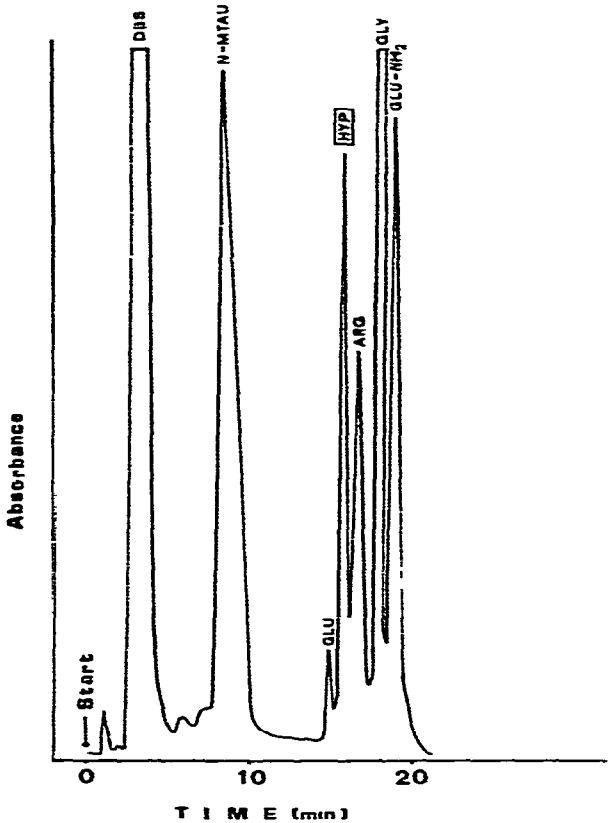

Fig. 2. Representative chromatogram of dabsyl derivative of the biological sample DBS = Dabsyl chloride; N-MTAU = N-methyltaurine, GLU = glutamic acid, HYP = hydroxyproline, ARG = arginine; GLY = glycine; GLU-NH_2 = glutamine.

Chang's method[11]. With this technique (Fig. 3), it was possible to demonstrate that: (1) only five dabsyl-positive substances were present in the fraction collected from the column and (2) these compounds corresponded to the five amino acids shown in Fig. 2. The resulting TLC red spot corresponding to Hyp was submitted to gas chromatography–mass spectrometry which confirmed the purity of this fraction

Analytical variables

Calibration curve and sensitivity. A linear calibration curve was obtained ($y = 0.2794113x - 0.0205341$; $r = 0.995$). The minimum detectable concentration of Hyp in our standard samples was 458 pmoles.

Recovery. The percentage recovery was determined by measuring the levels of Hyp in a biological sample to which known amounts of the standard amino acid had been added. The resulting yield was 93 $\pm$ 1.52% (mean value $\pm$ S.E.; $n = 9$).

Precision. Increasing amounts of Hyp were added to the same sample and processed by the same method. The results, expressed as the coefficient of variation (CV), are shown in Table I.

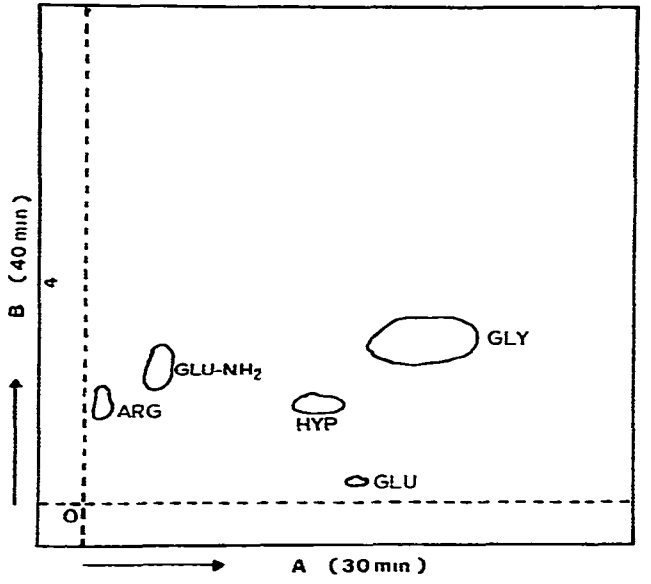

Fig. 3 Two-dimensional TLC of dabsyl amino acids The fraction eluted from the HPLC column (see text) was concentrated to dryness under nitrogen, redissolved in 0 5 ml of 0 1 *M* Na_2CO_3–$NaHCO_3$ buffer and then applied at the origin of the silica gel plate (cut into 10 × 10 cm strips) Solvents[11] A benzene–pyridine–acetic acid (80 20 5 v/v/v), B, toluene–2-chloroethanol–25% aqueous ammonia (100 80 6 7 v/v/v) The resulting five red spots were identified by the same method as the dabsyl derivatives of standard amino acids Abbreviations for amino acids as in Fig 2

Hyp concentration in the fibroblast cultures In order to verify whether the HPLC method was able to measure variations in the imino acid concentration in the cultures, Hyp was assayed in fibroblasts cultured with and without ascorbic acid in the medium[12–14]. The experiments were performed in duplicate during a period of 3 weeks A statistically significant decrease in total Hyp content (from 0 804 ± 0 007 to 0 434 ± 0.008 μmol/ml; $p < 0\ 001$; $n = 5$) was observed when cells were cultured in ascorbic acid-free medium.

TABLE I

PRECISION OF THE HYP HPLC DETERMINATION

A, Data obtained by processing the sample various times during a single day, B day-to-day precision, evaluated during a period of 6 weeks n = Number of assays

Hyp concentration (μmol/ml)	*CV (%)*	
	A (n = 10)	*B (n = 15)*
0 29	5 2	6 2
0 58	1 4	4 1
1 17	4 5	4 1
4 70	4 7	6 1

DISCUSSION

The aim of the present study was to produce a rapid and practical method for determining the concentration of Hyp in fibroblast cultures and other biological samples by HPLC with dabsyl derivatization

The specificity of the method was based on: (1) absolute and relative retention times of the amino acid: (2) collection of standard [^{3}H]Hyp in the fractions corresponding to the Hyp peak; (3) enrichment of biological samples by standard preparation of Hyp.

The sensitivity was higher than the method reported by Prockop and Udenfriend[2], who utilized a spectrophotometric assay. It was not possible to compare our results with those obtained by other groups[4–7] because they used radioisotopic assays and expressed values as dpm per mg of protein.

The yield was almost the same as those reported by Prockop and Udenfriend[2] and Peterkofsky and Diegelmann[7]. On the other hand, it was higher than those achieved in other procedures[4–6].

The present method has the advantage of not requiring an extraction step. In fact, after hydrolysis of samples, only 6 min are needed for dabsyl derivatization and a further 20 min for chromatographic separation Furthermore, it has a higher sensitivity and the same yield as the previously considered procedures Only a small volume (20 μl) is required for the analysis and, what is more, use of radioactive materials is avoided.

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Note

Facile, semi-preparative, high-performance liquid chromatographic separation of synthetic peptides using ammonium bicarbonate buffers

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An important goal of our high-performance liquid chromatography (HPLC) studies[1,2] has been the development of a simple procedure for the purification of synthetic peptides prepared by the solid phase method[3] Frequently a small scale synthesis will yield 25–100 mg of crude peptide which is contaminated by aberrant peptides produced by side-reactions. A recent study[4,5] demonstrated that the Radial-Pak C_{18} columns that are used in the Waters Radial Compression Module are particularly suitable for the separation of peptides[5] The purpose of this report is to demonstrate that this separation system can be used for surprisingly large sample loadings (typically 25 mg) with a volatile mobile phase (containing ammonium bicarbonate) that allows the rapid isolation of the purified peptide by lyophilisation

EXPERIMENTAL

Apparatus

A Waters Assoc (Milford, MA, U S A) HPLC gradient system was used for the separations (see Refs 1 and 2 for details) Sample injections were made using a Microliter 802 syringe or a 10-ml Gastight 1010 W syringe (Hamilton, Reno, NV, U S A.) The Radial-Pak CN and C_{18} cartridges (8 mm I D) were also purchased from Waters Assoc. For optimal column life the column was protected with a guard column and an in-line prefilter (Waters Assoc.)

UV spectra were determined using a Shimadzu MPS-5000 instrument

Chemicals and separation conditions

The solvents and chemicals used in this study are identical to those described in a previous paper[5] The peptide solutions were prepared as described previously[2], except that all solutions contained 6 *M* urea or 3 *M* guanidine–HCl to prevent sample aggregation.

The ammonium bicarbonate buffer (0 1 *M*, pH 7.7) was prepared freshly each day, and was used as the initial solvent (A) in the gradient separations The second solvent (B) consisted of isopropanol–acetonitrile–solvent A (30·30 40) (Figs 1 and 2) or isopropanol–solvent A (80 20) (Fig. 3). A linear gradient from 0 to 100% B over 60 min was used throughout this study. The separation was achieved at ambient

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temperatures and a flow-rate of 1 ml/min was used. The eluted peptides were detected by UV absorbance (generally at 280 nm).

RESULTS

Fig. 1. shows the purification of 60 μg of the synthetic peptide Leu–Glu–Ser–Phe–Lys–Val–Ser–Trp–Leu–Ser–Ala–Leu–Glu–Glu–Tyr–Thr–Lys–Ala (I) using the chromatographic system described in the Experimental Section Although this peptide had been previously purified by ion-exchange chromatography, the sample still contained two deletion peptides which were readily removed by HPLC. The large O.D.

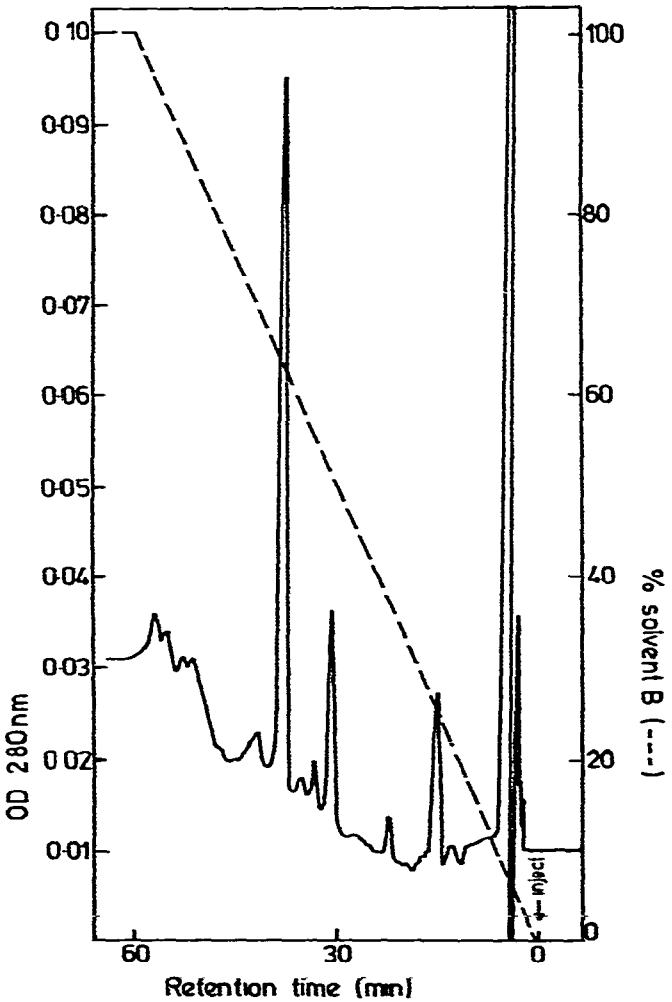

Fig. 1. The purification of 60 μg of the synthetic peptide Leu–Glu–Ser–Phe–Lys–Val–Ser–Trp–Leu–Ser–Ala–Leu–Glu–Glu–Tyr–Thr–Lys–Ala on a Radial-Pak CN column was achieved with a linear gradient from 0.1 *M* ammonium bicarbonate to isopropanol–acetonitrile–0 1 *M* ammonium bicarbonate (3.3.4) at a flow-rate of 1.0 ml/min.

peak at the start of the gradient was due to guanidine–HCl which was added to the sample to prevent aggregation

Fig. 2A shows the purification of 350 μg of the synthetic peptide Leu–Glu–Ser–Phe–Leu–Lys–Ser–Trp–Leu–Ser–Ala–Leu–Glu–Gln–Ala–Leu–Lys–Ala using the conditions described in Fig 1. Prior to the HPLC separation, this peptide had been partially purified by gel filtration and ion-exchange chromatography The sample was loaded in 5 ml of 6 *M* urea as three approximately equal volumes through the U6K sample injector. The large peak was collected (see bar in Fig 2A) and re-chromatographed as shown in Fig. 2B. Before rechromatography the trapped peak (2 ml) was diluted to 6 ml with 0.1 *M* ammonium bicarbonate and loaded as three approximately equal volumes using identical conditions as those in Fig 2A

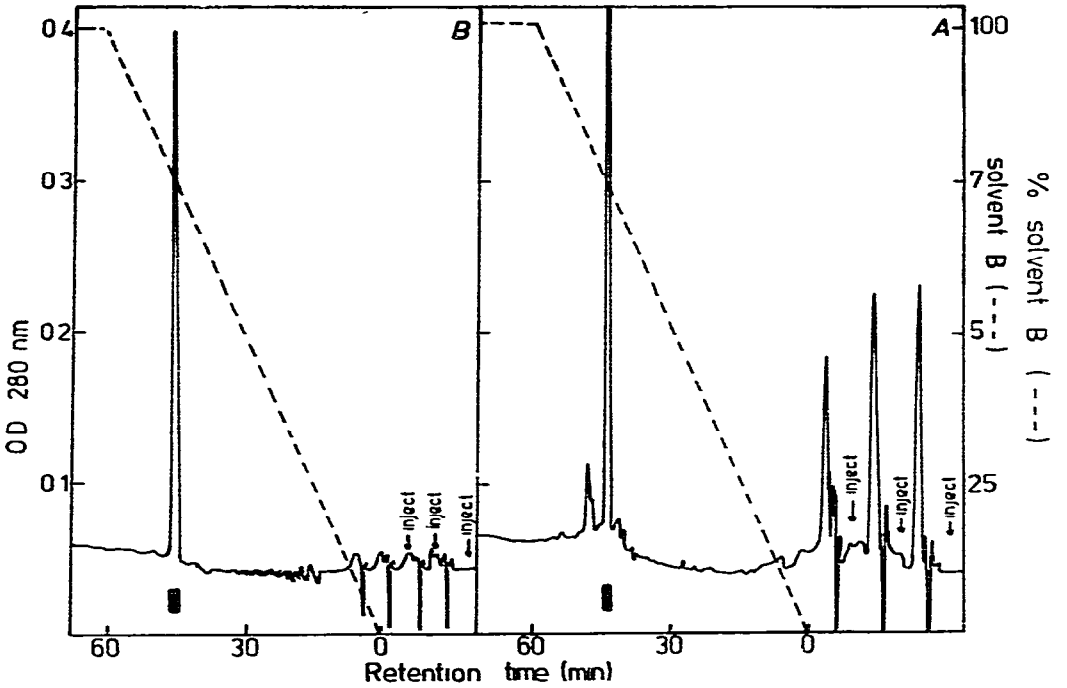

Fig 2. The purification of 350 μg of the peptide Leu–Glu–Ser–Phe–Leu–Lys–Ser–Trp–Leu–Ser–Ala–Leu–Glu–Gln–Ala–Leu–Lys–Ala was accomplished using the conditions described in the legend in Fig 1. The sample was loaded in three injections from a solution in 5 ml of 6 *M* urea Part A shows the elution profile for the crude peptide mixture. The area defined by the solid bar was pooled (2 ml), diluted to 6 ml with 0.1 *M* ammonium bicarbonate, and re-chromatographed using the same conditions as in Part A

Fig. 3A shows the separation of another synthetic peptide Val–Ser–Ser–Leu–Leu–Ser–Ser–Leu–Lys–Glu–Tyr–Trp(CHO)–Ser–Ser–Leu–Lys–Glu–Ser–Phe–Ser (2 5 mg) on the Radial-Pak C_{18} column The separation was achieved with a linear gradient of 0.1 *M* ammonium bicarbonate to 0 1 *M* ammonium bicarbonate–isopropanol (1.4). The separation shown in Fig. 3B was achieved under identical conditions as part A, except that the sample loading was only 0.37 mg and 0.1 *M* ammonium formate (pH 7.5) replaced the 0 1 *M* ammonium bicarbonate in both solvent A and B.

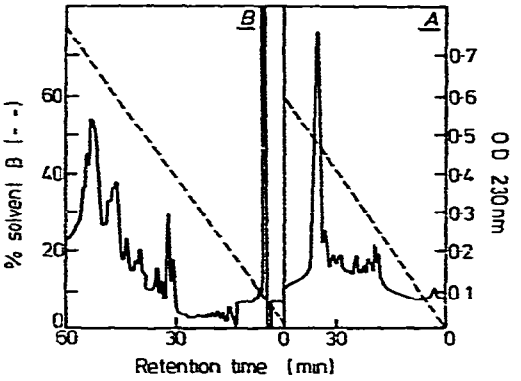

Fig. 3 The purification of 2.5 mg of the peptide Val–Ser–Ser–Leu–Leu–Ser–Ser–Leu–Lys–Glu–Tyr–Trp(CHO)–Ser–Ser–Leu–Lys–Glu–Ser–Phe–Ser on a Radial-Pak C_{18} column was achieved in part A with a linear gradient from 0.1 *M* ammonium bicarbonate to isopropanol–0.1 *M* ammonium bicarbonate (4:1). The absorbance was monitored at 300 nm with 2.0 a.u.f.s. Run B was achieved using identical conditions except that ammonium formate was used instead of ammonium bicarbonate. In this analysis the absorbance was monitored at 230 nm with 0.2 a.u.f.s. and 0.370 mg of peptide was loaded. A guard column filled with Porasil B C_{18} was used in both chromatograms.

DISCUSSION

We[4] and others[6] have reported the use of ammonium bicarbonate as a suitable mobile phase for the Radial-Pak C_{18} flexible-walled columns. The high apparent pH of this mobile phase (7.7–8) precludes its use with siliconaceous supports packed in inflexible columns, due to the generation of column voids caused by dissolution of the silica. The radial compression used with the flexible-walled columns circumvents this problem as any voids that may be generated are removed under compression. Provided the column is washed with water and then isopropanol each evening, we have found that an extended life-time of at least 6 months can be achieved with Radial-Pak C_{18} or CN columns and the mobile phases used in this study. As a further precaution we have recently introduced the use of a guard column filled with Porasil B C_{18} packing material. The elution profiles shown in Fig. 3 were achieved with the guard column in line with the prefilter. We have shown[7] that the guard column does not degrade the separation, but does allow for a significant increase in column lifetime due both to removal of contaminants from the sample and mobile phase and to dissolution of silica in the guard column (thus partially presaturating the mobile phase with silica).

The use of ammonium bicarbonate, with its excellent volatility, allows the semi-preparative separation of synthetic peptides as the separated material can be simply isolated by freeze-drying. The organic solvent can be also removed at this stage, provided the sample is diluted with water to allow freezing of the sample. Alternatively the organic solvent can be removed by reduced pressure evaporation before the freeze-drying step. The perfluoroalkanoic acids, which also have excellent volatility and can be used in peptide separations[8], are not suitable for use with the Radial-Pak C_{18} or CN columns due to the limited stabilities of these packing materials at low pH values[9]. Amine salts, which can be adjusted to suitable pH values, are not sufficiently volatile to allow facile preparative separations[10].

The only significant problem with ammonium bicarbonate buffers is the possibility of bubble formation with the use of either high concentrations of ammonium bicarbonate (0.5 *M*) or organic solvent (above 80%) Therefore, this mobile phase may not be suitable for the chromatography of extremely basic peptides which require high salt concentrations to suppress silanophilic interactions We are presently investigating[11] suitable volatile buffers for use in this circumstance

Figs. 1–3 show that a range of synthetic peptides can be separated with high efficiency by the ammonium bicarbonate system The eluted peptides were identified by amino acid analysis of an acid hydrolysate (see Table I for a representative example) Also it should be noted that an increase in sample load (0 06 to 0 35 to 2 5 mg in Figs. 1–3, respectively) was achieved with no loss of separation efficiency. Current studies with this system show that sample loads as high as 25 mg can be used without significant loss of separation efficiency This result, when combined with the excellent volatility of the mobile phase, demonstrates the suitability of this system for the semi-preparative separation of synthetic peptides

TABLE I

REPRESENTATIVE RECOVERIES AND AMINO ACID COMPOSITIONAL DATA* OBTAINED IN THE SEPARATION OF THE SYNTHETIC PEPTIDES

Amino acid	*Expected*	*Obtained*
Ser**	3	3 0
Glu + Gln	3	3 1
Ala	3	2 9
Leu	5	5 0
Phe	1	1 0
Lys	2	2 0
Trp***	1	1 0
Recovery	87%***	

* Expressed as number of residues per molecule
** Corrected for destruction of serine during acid hydrolyses
*** Obtained by UV spectra determination (λ_{max} = 280 nm, ε_{280} = 5200 l mol^{-1} cm^{-1})

One final feature that is crucial for preparative separations is the ability to achieve high recoveries of the purified material. Recoveries were determined either by amino acid analysis or UV spectra determination (using the characteristic tryptophan absorption) In all cases the recovery of the purified material was in excess of 85% and a typical example is shown in Table I.

Much of the selectivity that is achieved in reversed-phase HPLC of peptides has been attributed to ion-pairing[2,12] and silanophilic interactions[5]. As is shown by a comparison of the elution profiles shown in Fig. 3A and B ammonium bicarbonate allows excellent peak shapes which may, in some cases, be superior to those obtained with other buffers In Fig 3B, although the sample loading was significantly lower than in part A (0 37 *vs* 2 5 mg), the use of ammonium formate gave broader peak shapes and lower recoveries than did the use of ammonium bicarbonate (see part A) The success of ammonium bicarbonate as a mobile phase additive can be related to

the presence of ammonium ions which will suppress unwanted silanol-solute interactions, while bicarbonate may have similar ion-pair effects to that of phosphate[2].

In conclusion this report demonstrates that ammonium bicarbonate solutions when combined with suitable organic modifiers allows the facile semi-preparative HPLC separation of synthetic peptides. This system gives excellent peak shapes in a separation, as well as yielding purified peptides with excellent recoveries after a simple freeze-drying step.

ACKNOWLEDGEMENTS

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Note

Analytical and semi-preparative high-performance liquid chromatography of oligosaccharides obtained by hydrazinolysis of hen ovomucoid

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Currently, monosaccharides and oligosaccharides are satisfactorily separated by paper, ion-exchange, thin-layer and gel filtration chromatography, however the methods are time-consuming High-performance liquid chromatography (HPLC) using a column of porous microparticles derivatized with amino groups allows a rapid separation of sugars[1–5] Since 1980, the HPLC on bonded primary amine packings of sugars derived from glycoprotein glycans have been described fractionation of oligosaccharides and glycopeptides excreted in the urine of patients with lysosomial diseases[6]; separations of oligosaccharides obtained by digestion with endoglycosidase H[7] or released by β-elimination of O-glycosylprotein[8 9], oligosaccharides derived from dolichol-linked oligosaccharide intermediates[10] Finally, clear separations of sialoglycopeptides or oligosaccharides have been obtained in less than 1 h by HPLC on a Micropak AX-10 ion-exchange column[11]

The present report shows that bonded primary amine packings are effective in the rapid separation of the mixture of neutral glycans liberated by hydrazinolysis of a hen ovomucoid neutral glycopeptide

MATERIALS AND METHODS

Glycoproteins, glycopeptides and oligosaccharides

Ovomucoid was prepared according to Fredericq and Deutsch[12] The asialoglycopeptide β was isolated after pronase hydrolysis of ovomucoid according to Monsigny *et al.*[13]. Oligosaccharides were released from asialoglycopeptide β by hydrazinolysis as previously described[14]. The resulting oligosaccharides were N-reacetylated according to Reading *et al* [15] and reduced with $NaBH_4$

Liquid chromatography on primary amine bonded silica

Analysis were carried out with a Spectra Physics Model 700 liquid chromatograph, equipped with an UV 8400 variable wavelength detector connected to a 4100 computing integrator.

HPLC was performed on a 5-μm Amino AS-5A column (0.4 × 25 cm, Chromatem 33; Touzart et Matignon). A 1-mg amount of oligosaccharides dissolved in 10

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μl of distilled water was injected into the column; for preparative chromatography, 3.5 mg of oligosaccharides dissolved in 20 μl of acetonitrile–water (50·50) were injected. The column was equilibrated with the initial solvent (acetonitrile–water, 65:35). After the injection, a linear gradient to acetonitrile–water (60:40) was applied for 30 min followed by isocratic conditions during 30 min and then a linear gradient to acetonitrile–water (50.50) for 30 min. The flow-rate was 1 ml/min. The oligosaccharides were detected at 200 nm under the following conditions: sensitivity of detec-

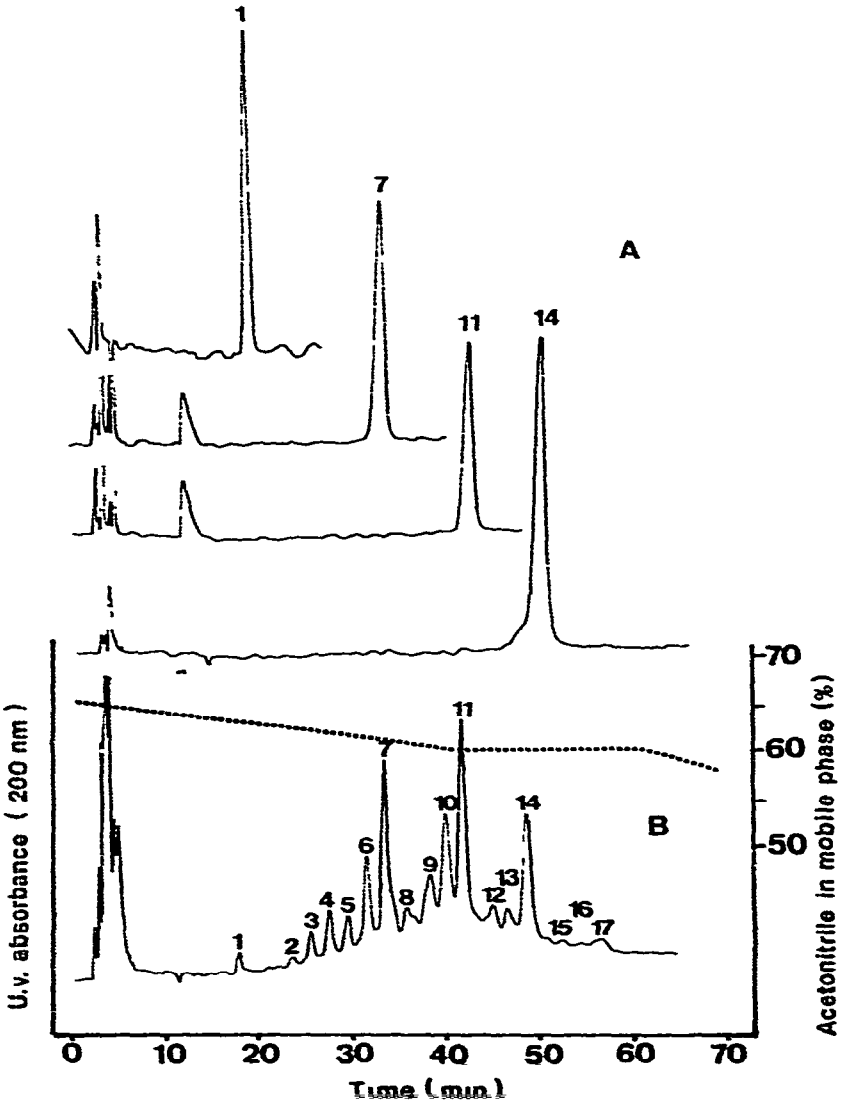

Fig. 1. Analysis (A) of oligosaccharides 1, 7, 11 and 14 obtained by semi-preparative chromatography (B) of oligosaccharides from the hydrazinolysis of hen ovomucoid neutral glycopeptide *β* on 5-μm Amino AS-5A (Chromatem 33, Touzart et Matignon) For chromatographic conditions, see Material and Methods.

tor, 0.35; integrator attenuation, 4, for analytical chromatography; sensitivity of detector, 0.32, integrator attenuation, 50, for semi-preparative chromatography.

Molar composition of oligosaccharides

The molar composition of oligosaccharides was determined by gas–liquid chromatography (GLC) of trifluoroacetylated methylglycosides according to Zanetta *et al.*[16].

Thin-layer chromatography (TLC) of oligosaccharides

TLC of oligosaccharides was performed on silica gel plates (pre-coated silica gel 60, Merck) using ethanol–*n*-butanol–pyridine–acetic acid–water (100.10 10.3 30 v/v/v/v/v)[17] during 7 h. Oligosaccharides were revealed with an orcinol–sulphuric acid reagent[18].

RESULTS AND DISCUSSION

The separation of a mixture of reduced oligosaccharides obtained by hydrazinolysis from hen ovomucoid is shown in Fig. 1. The effective separation of seventeen fractions was obtained within 90 min on an Amino AS-5A column (Fig. 1B) and semi-preparative chromatography on the same column allows one to obtain pure fractions 1, 7, 11 and 14 (Fig. 1A). The results of the semi-preparative chromatography of 4.2 mg of ovomucoid-derived oligosaccharides are given in Table I.

TABLE I

WEIGHTS OF FRACTIONS OBTAINED BY SEMI-PREPARATIVE CHROMATOGRAPHY OF 4.2 mg OF OLIGOSACCHARIDES LIBERATED BY HYDRAZINOLYSIS FROM OVOMUCOID NEUTRAL GLYCOPEPTIDE β

Fraction number	*Weight (μg)*
F-0	312.4
F-1	44
F-2	36.3
F-3	95.7
F-4	169.4
F-5	73.7
F-6	265
F-7	510.4
F-8	146.3
F-9	283.8
F-10	400.4
F-11	644.6
F-12	186
F-13	242
F-14	284
F-15	48.4
F-16	36.3
F-17	83.7
Recovered	3862

The use of a 0.4 × 25 cm column filled with silica gel modified by organic amines provides quantitative recovery (92%) of the oligosaccharides without loss of resolution. Each fraction was analysed by TLC (Fig. 2) and the carbohydrate composition was determined by GLC (Table II). Four fractions (1, 7, 11 and 14) are homogeneous on the basis of HPLC and TLC and of the monosaccharide molar composition. The latter is characterized by the absence of the presence in low amounts of galactose and by the relative high content of N-acetylglucosamine. Of interest is the ratio (N-acetylglucosamine + N-acetylglucosaminitol)/mannose, *i.e.*, (GlcNAc + GlcNAc-ol)/Man, the value of which is related to the number of N-acetylglucosamine branches on the mannose residues. The limiting values are 1.35 and 2.7 in the case of classical biantennary structures such as human serum transferrin[19] and of pentaantennary structures such as turtle-dove ovomucoid[20], respectively.

CONCLUSIONS

HPLC on bonded primary amine packings gives excellent fractionation of a

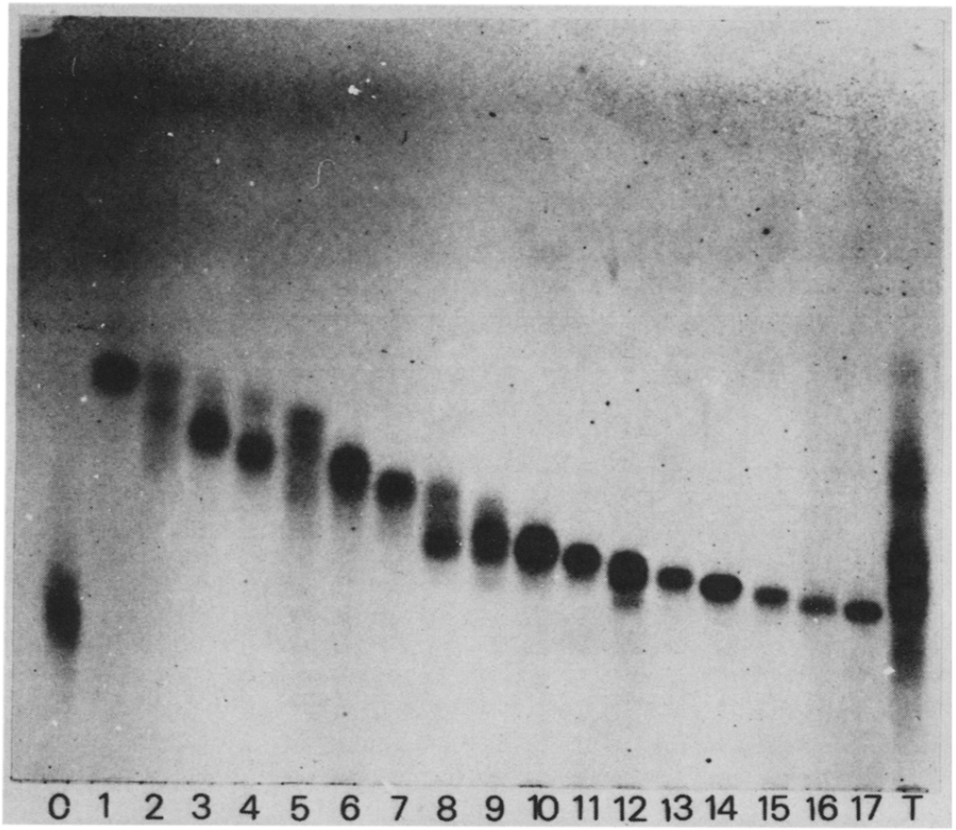

Fig. 2. TLC on silica gel plates (Merck) of seventeen fractions obtained by semi-preparative chromatography of oligosaccharides from the hydrazinolysis of hen ovomucoid neutral glycopeptide. Solvent: ethanol–*n*-butanol–pyridine–acetic acid–water (100:10:10:3:30 v/v/v/v/v). Development time: 7 h. Oligosaccharides revealed with orcinol–sulphuric acid reagent.

TABLE II

CARBOHYDRATE COMPOSITION OF FRACTIONS OBTAINED BY SEMI-PREPARATIVE CHROMATOGRAPHY OF OLIGOSACCHARIDES LIBERATED BY HYDRAZINOLYSIS OF HEN OVOMUCOID NEUTRAL GLYCOPEPTIDES β

Fractions	*Molar ratio**				*GlcNAc + GlcNAc-ol* / *Man*
	Gal	*Man*	*GlcNAc*	*GlcNAc-ol*	
1	0	3	1 3	0 94	0 74
2	0	3	2 94	0 81	1 25
3	0	3	3 4	0 50	1 3
4	0	3	3 63	0 66	1 43
5	0	3	3 4	0 2	1 2
6	0	3	3 9	0 60	1 5
7	0	3	4 64	0 94	1 86
8	0 44	3	5 02	0 61	1 87
9	0 44	3	5 38	0 65	2 01
10	0 59	3	4 63	0 64	1 75
11	0 19	3	6 8	1 01	2.6
12	0 72	3	5 70	0 57	2.09
13	1 03	3	5 99	0 30	2 09
14	1 07	3	6 8	1 07	2 62
15	1 52	3	6 9	0 71	2 53
16	1 5	3	6 21	0 57	2 26
17	1 85	3	6 8	0 84	2 54
Native glyco-peptide β	0 6	3		6	2

* The ratio of mannose (Man) was taken as 3 Gal = Galactose, GlcNAc = N-acetylglucosamine, GlcNAc-ol = N-acetylglucosaminitol

new class of glycans constituting a complex mixture of about twenty reduced neutral oligosaccharides liberated by hydrazinolysis from hen ovomucoid. These glycans are in general N-acetylglucosamine-rich and highly branched Our results illustrate again the usefulness of HPLC as a powerful tool to investigate the various compounds encountered in the field of glycoproteins. glycans of the "N-acetyl-lactosaminic type"[7 11], of the "oligomannosidic type"[7], of the "mucin type"[8 9] and lipid intermediate-derived oligosaccharides[10], dolichyl pyrophosphoryl oligosaccharides[21] and peracetylated mono- and oligosaccharides[22]

The results obtained demonstrate the high microheterogeneity of hen ovomucoid, which was not revealed by our first investigations[23] The primary structure of the numerous glycans isolated is now under investigation in order to understand the significance of their amazing microheterogeneity better and define the characteristics of the new class of glycans

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Note

Nachweis von LSD in Körperflüssigkeiten mit Hochleistungsflüssigkeitschromatographie

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(Eingegangen am 13 Juli 1982)

Labors, die sich mit dem Nachweis von Suchtmitteln befassen, sind immer wieder damit konfrontiert, eine Einnahme von LSD nachweisen zu mussen. Die Schwierigkeit dieses Nachweises liegt darin, dass aufgrund der geringen Dosierung von LSD nur niedrige Spiegel im Blut, Serum oder Urin entstehen Es konnen deshalb nur empfindliche Nachweismethoden verwendet werden, wie z B Hochleistungsflüssigkeitschromatographie (HPLC) mit Fluoreszensdetektion[1,2] oder Radioimmunoassay[2,3]

Die vorliegende Arbeit beschreibt den Nachweis von LSD in Korperflussigkeiten mit HPLC und Fluoreszensdetektion. Um die bei forensischen Fällen notwendige Sicherheit der Befunde zu erhalten, wurde die Absicherung durchgefuhrt mit zwei verscheidenen Laufmitteln und mit Saulenschaltung

EXPERIMENTELLES

Hochleistungsflüssigkeitschromatographie

Gerat Hewlett-Packard Hochleistungsflussigkeitschromatograph 1084 B mit automatischem Probengeber HP 79842 A und Spektralfluorimeter SFM 23 der Fa Kontron. Anregung bei 325 nm Emission bei 430 nm.

"Reversed-Phase"-Chromatographie, Säule Merck Hibar Fertigstahlsaule EC 250-4 mit C_8 Reversed-Phase Material LiChrosorb (7 μm), Fluss 1.5 ml/min, Ofentemperatur 60°C, Laufmittel A Methanol–Wasser mit 3 g KH_2PO_4/l, eingestellt mit H_3PO_4 auf pH 3 (50.50), Laufmittel B Methanol–Wasser mit 1 % $(NH_4)_2CO_3$ (60:40)

Saulenschaltung Laufmittel A, Saule 1· C_8 Reversed-Phase wie beschrieben, Saule 2· Merck Hibar Fertigstahlsaule EC 125-4 mit Kieselgel Merck LiChrosorb Si 60 (5 μm), Zeitprogramm· Zunachst Saule 1, nach 3 2 min Umschaltung auf Saule 2.

Probenmaterial

Es handelte sich um Blut und Urin von Personen, bei denen der Verdacht bestand, dass LSD eingenommen worden war. Wenn es moglich war, wurde aus dem Blut Serum gewonnen, ansonsten wurde das Blut direkt eingesetzt

Blut, Serum oder Urin (1–3 ml) wurden mit Boratpuffer pH 9 5 (5 g $Na_2B_4O_7 \cdot 10\ H_2O$ auf 1 l) auf 20 ml aufgefüllt und auf eine Extraktionssaule Ex-

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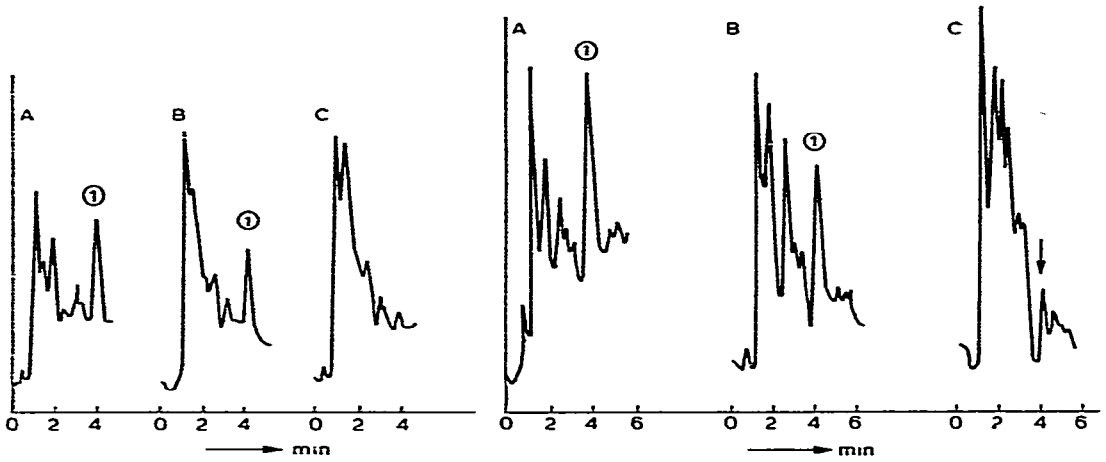

Fig. 1. Chromatogramme von (A) Serum mit Zusatz von 2 ng/ml LSD (1), (B) positive Serumprobe mit 1 4 ng/ml LSD (1), und (C) negative Serumprobe Säule, C_8 Reversed-Phase; Laufmittel, B

Fig. 2. Chromatogramme von Serumproben wie in Fig 1 Säule, C_8 Reversed-Phase; Laufmittel A 1 = LSD. C vorgetäuschtes LSD (Pfeil).

trelut® gegeben[4]. Die Säule wurde mit Dichlormethan–Isopropanol (85·15) eluiert, die organische Phase eingedampft und der Rückstand mit 200 μl Methanol aufgenommen. Von dieser Lösung wurden 10 μl eingespritzt.

Die quantitativen Bestimmungen im Blut oder Serum wurde über externe Standardisierung durchgeführt. Hierfür wurde eine Eichlösung von 2 ng pro ml LSD in Rinderblut oder Rinderserum hergestellt, die jeweils analog aufgearbeitet wurde.

ERGEBNISSE

Die Erfassungsgrenze für LSD betrug *ca.* 0 5 ng/ml. Die Wiederfindungsrate war 60–70%, die Standardabweichung 6.5% (n = 5). Die Bestimmung war von 0.5–20 ng/ml linear.

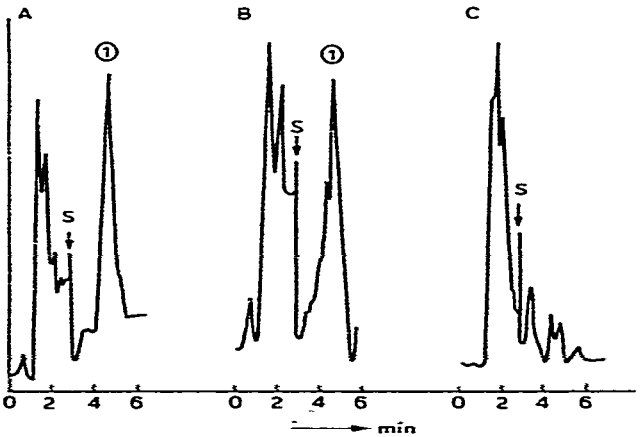

Fig. 3 Chromatogramme von Serumproben wie in Fig. 1. Säule, C_8 Reversed-Phase, nach 3 2 min Kieselgelsäule (S = Schaltkontakt); Laufmittel, A. 1 = LSD.

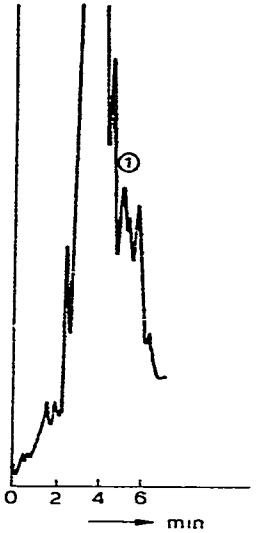

Fig 4 Chromatogramm einer Urinprobe Säule C_8 Reversed-Phase, Laufmittel A. 1 = LSD (0 5 ng/ml)

Fig. 1 zeigt eine positive und negative Serumprobe mit dem alkalischen Laufmittel B; Fig. 2 dieselben Proben mit dem sauren Laufmittel A und Fig 3 dieselben Proben nach Saulenschaltung Fig. 4 zeigt eine positive Urinprobe mit Laufmittel A.

Die Spiegelwerte von Serumproben, die wir gefunden haben, sind in Tabelle I angegeben.

TABELLE I

LSD-SPIEGEL (ng/ml)

	Fall						
	A	*B*	*C*	*D*	*E*	*F*	*G*
Serum	5 2	1 6	15 1	1 4	1 6	7	
Urin						5	0 5

DISKUSSION

Nach Einnahme von 160 μg LSD werden nach *ca.* 1 h Serum-Spiegel von 2–8 ng/ml erreicht[5] Die von uns gefundenen Werte liegen mit einer Ausnahme in diesem Bereich, wobei nähere Einzelheiten zu der Vorgeschichte nicht bekannt waren. Auch Twitchet *et al* [2] finden Werte in diesem Bereich.

Die Absicherung des Befundes wird erreicht durch Verwendung eines sauren und eines alkalischen Laufmittels, sowie durch Verwendung zweier Säulen unterschiedlicher Polarität, eine Reversed-Phase-Säule und einer Kieselgelsäule, die hintereinander geschaltet sind

Wie Fig 2 zeigt, ist eine derartige Absicherung notwendig, da manchmal geringe Konzentrationen von LSD vorgetauscht werden konnen Es wurde deshalb

auch als Erfassungsgrenze 0 5 ng/ml festgelegt, obwohl der Detektor noch eine Verstärkung um den Faktor 10 zuliesse.

Die Bestimmung ist ebenfalls im Urin möglich; wie Fig. 4 zeigt. Wenn möglich ziehen wir eine Bestimmung im Serum vor, da hier weniger Störungen auftreten.

Iso-LSD, Lysergsäureamid, Lysergsäure und Ergotamin, die dasselbe Fluoreszensverhalten haben, stören die Bestimmung nicht.

DANK

Der Fa. Sandoz (Nürnberg, B.R.D./Basel, Schweiz) danken wir für die Überlassung von Reinsubstanz.

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