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CONTENTS

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CHROMATOGRAPHY OF ENVIRONMENTAL HAZARDS

Volume III: Pesticides

by **LAWRENCE FISHBEIN,** Chemistry Division, National Center for Toxicological Research, Jefferson, Adjunct Professor of Chemistry, University of Arkansas, Little Rock, Arkansas, U.S.A.

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Pesticides represent a major area of increasing environmental concern both in terms of their potential ubiquity as well as toxicity. The main objective of this third volume in the series Chromatography of Environmental Hazards is to provide the analytical chemist with a practical text as well as a literature reference source of selected descriptive chromatographic procedures. Information is provided wherever possible concerning the basic equipment, operating parameters, sensitivity, and interferences encountered in the analysis of a particular pesticide and/or group of related contaminants.
The unique feature of the book is the blending of relevant information regarding the synthesis, areas of utility, degradation and metabolic fate of pesticide toxicants, thus presenting as thorough and cohesive a picture as possible of the specific environmental hazard.

CONTENTS: Introduction. DDT and metabolites. Cyclodienes: Dieldrin, Aldrin and Endrin. Chlordane, Heptachlor and Heptachlor epoxide. Miscellaneous organochlorine pesticides Perthane, Methoxychlor, Endosulfan and Toxaphene. Benzene hexachloride and hexachlorobenzene. Multiple organochlorine analyses. 2,4-D and its esters. Pentachlorophenol. Miscellaneous herbicides and acaricides (bipyridylium salts, Dinoseb, Trifluralin and Cycocel). Parathion, Methyl Parathion and Malathion. Bidrin, Azodrin, Diazinon, Dursban and Dasanit. Disyston, Dimethoate, Phorate. Multiple organophosphorus pesticide analysis. Carbamates. Ureas. Triazines. Pesticidal synergists. Index.

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DESIGN AND CHARACTERIZATION OF A COULOMETRIC DETECTOR WITH A GLASSY CARBON ELECTRODE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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(Received February 27th, 1976)

SUMMARY

The use of a glassy carbon electrode for detecting electrochemically oxidizable compounds with high current yield in high-performance liquid chromatography is described.

The various properties of the detector, such as sensitivity, dynamic behaviour, linear working range, detection limit and selectivity are discussed on the basis of theoretical considerations and experimental results. The detector has a detection limit in the picogram range with good dynamic behaviour.

The application of the detector to the rapid separation of biogenic aromatic acids is described and its applicability to the analysis of neuroleptics is discussed.

INTRODUCTION

Voltammetry and coulometry are valuable techniques in the quantitative analysis of electroactive compounds. However, when in a mixture the half-step potentials lie too close together, a separation process is necessary and in such instances liquid chromatography combined with electrochemical detection can be a convenient method.

The adaptation of a measuring system to a high-performance chromatographic separation process is possible only if the measurement system fulfils certain requirements with respect to dynamic behaviour, linear working range and detection limit. For electrochemical detection with the dropping mercury electrode, this adaptation has been shown to be possible[1,2]. However, in the anodic range the mercury electrode is of only limited use because of the anodic dissolution of the metal at potentials above 0.2–0.4 V *vs.* S.C.E. Moreover, the noise generated by the drop time variations influences the detection limits of these devices considerably. These limitations can be circumvented by using a solid electrode. Because of its chemical inertness and its

* Presented at Euroanalysis II, Budapest, August 24–29th, 1975.

larger potential range compared with metals such as gold or platinum[3] (from about —1.0 V to + 1.0 V *vs.* S.C.E.), glassy carbon was chosen as the electrode material.

The possible deactivation of the electrode surface, due to adsorption, can be a disadvantage of these solid electrodes. Knowledge of these effects and of the electrochemical behaviour above + 0.4 V *vs.* S.C.E. is limited compared with the numerous detailed studies that have been made with the dropping mercury electrode.

Detection in liquid chromatography by means of solid glassy carbon electrodes has been reported[4,5], using electrodes with small surface areas, but the detectors have not been characterized with respect to dynamic behaviour, linear range, sensitivity and noise. In this paper, the use of a glassy carbon electrode with a relatively large surface area is described.

It is necessary to define the concept of coulometric yield as used throughout this paper; it is defined as $i/\dot{m}nF$, where i = current (A), $\dot{m}$ = mass flow (mole/sec), n = number of electrons transferred per molecule and F = Faraday constant (C/mole). The term coulometric yield should not be confused with the term current efficiency which is used in electrochemistry.

A large surface area has the following advantages, all of which result from the fact that nearly all molecules can react at the electrode:

(a) an increase in the oxidation yield and therefore an improvement in the chromatographic signal will be obtained;

(b) small variations in the flow-rate will not affect the peak area, as the cell is acting effectively as a mass flow-sensitive device;

(c) the amount of compound can be calculated from the peak area directly if the number of electrons involved in the reaction is known;

(d) small variations in the temperature will have little effect; and

(e) partial deactivation of the electrode surface by adsorption of reaction products will have a relatively small influence.

APPARATUS

Construction

The detection cell, presented in Fig. 1, consists of two glassy carbon plates (V25, Carbone Lorraine, Paris, France) of dimensions 15 × 100 × 1.5 mm. The plates are polished with carborundum powder. In one plate the inlet tube (3-cm stainless-steel tube, I.D. 0.25 mm) and the outlet tube (3-cm silver tube, I.D. 1 mm) are cemented in electrically insulating epoxy resign (Araldite AV129). The necessary holes in the plates were made by ultrasonic drilling with carborundum powder. The cell volume is formed by a Teflon spacer between the plates, the thickness of the spacer being the height of the cell; Teflon sheets of 100 and 50 μm were used. The length of the duct is 80 mm and the width 7 mm. The plates are pressed together by means of two stainless-steel plates (thickness 10 mm) and six screws (M6). The glassy carbon plates are insulated from the steel by means of a Teflon sheet (thickness 1 mm). Electrical contact with the plates is made by means of a silver contact at the top of a Teflon screw.

Electrical part (Fig. 2)

A three-electrode system was used in order to minimize the influence of the

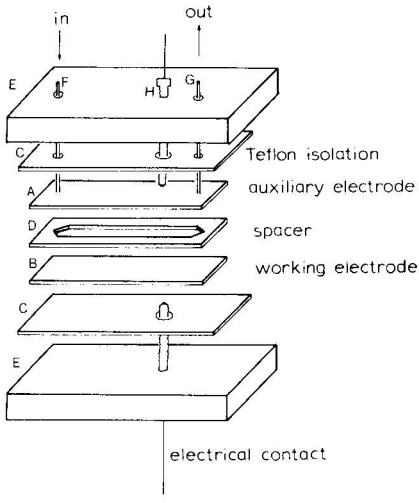

Fig. 1. Cell construction. A and B, glassy carbon plates constituting auxiliary and working electrode, respectively; C, Teflon isolation sheets; D, Teflon spacer; E, steel plates; F and G, inlet and outlet capillaries, respectively (the outlet tube is the reference electrode); not shown, six screws for pressing steel plates together.

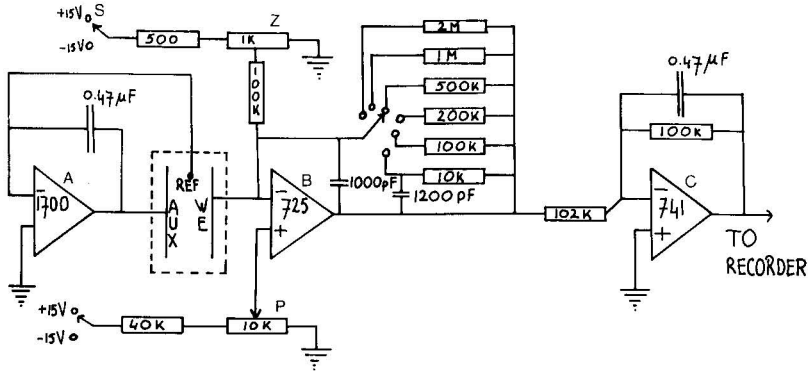

Fig. 2. Detector electronics. A, Amplifier (Philbrick 1700); B, Fairchild 725; C, Fairchild 741 (the current range can be chosen by switching the resistors at the second amplifier); P, potential control; Z, zero control, S, polarity of zero control.

resistance of the solution (*iR* drop) on the potential of the working electrode. Silver was used for the outlet tube because a reference electrode can be obtained with it. Two different electrode arrangements were used:

(a) both glassy carbon plates as the working electrode and downstream from the silver tube a stainless-steel tube (I.D. 2 mm) as the auxiliary electrode; and

(b) the upper plate as the auxiliary electrode and the opposite one as the working electrode.

The potential is controlled by a low-noise chopped operational amplifier (Philbrick 1700) and the current is amplified by a low-noise operational amplifier (Fairchild 725), followed by an active first-order filter (RC time 48 msec).

Chromatographic equipment

For testing the detector under chromatographic conditions, a pump (Labotron LDP 13A or Orlita DMP 1515), a stainless-steel column, a manometer with stainless-steel connecting tubes, a syringe injection device and an injection valve (Chromatronix HP SV 20) were used.

Chemicals

Potassium chloride, acetic acid, sodium acetate and L-ascorbic acid (pro analysi grade, E. Merck, Darmstadt, G.F.R.) and doubly distilled water were used. Amines and acids used as solutes were of different origin, most of them being of analytical-reagent grade.

THEORETICAL

General

When the eluent flows through the cell, electrochemically active compounds will react at the electrode surface and an electric current, proportional to the mass flow, will result at a constant flow-rate of the eluent. The liquid flows over the surface as a thin film of thickness about 0.1 mm and width 0.7 cm. As a result of the reaction at the electrode, transport by diffusion will start from the bulk towards the electrode surface. With the flow-rates encountered in chromatography and with dimensions of the cell used, the flow in the cell will be laminar[6]. Therefore, transport of electrochemically active material from the bulk towards the electrode surface can occur by molecular diffusion only.

In this paper, an expression is derived for the current as a function of the flow-rate and the surface area of the electrode. Mathematically, the problem has an analogy with heat conduction by flowing fluids. Graetz[7] was the first to describe the combination of heat conduction and laminar flow. Since then, several workers have dealt with this problem under different conditions and using different mathematical methods[8–10].

For the theoretical discussion the following assumptions are made:

(a) the cell is a rectangular duct in which an instantaneous reaction takes place at one wall of the cell;

(b) the flow is laminar;

(c) in the entrance rectangular cross-section the velocity is constant and the concentration is constant;

(d) all fluid properties are constant;

(e) diffusion in the direction of the flow is neglected; and

(f) mass transfer is two-dimensional, which means that the boundary effects at the vertical walls are neglected.

The following nomenclature is used:

b = width of the duct;
c_a = concentration of oxidizable compound a;
c_{a0} = concentration at $x = 0$;
$\langle c_{ax} \rangle$ = bulk concentration at x;
d = height of the duct;
D_a = diffusion coefficient of a in the eluent;

Gz = dimensionless Graetz number = $\frac{4d^2 v_x}{xD_a}$;

J = molar flux;

k_{ln} = convective mass transfer coefficient, based on the log mean concentration; log mean concentration = $\frac{\langle c_{ax}\rangle - c_{a0}}{\ln \frac{\langle c_{ax}\rangle}{c_{a0}}}$;

Sh = dimensionless Sherwood number = $\frac{2k_{ln}\, d}{D_a}$;

v_x = velocity in x-direction;
$\bar{v}_x$ = mean velocity in x-direction;
v_{x0} = velocity at $x = 0$;
v_y = velocity in y-direction;
x = coordinate in flow direction;
y = coordinate perpendicular to the plates.

By means of a mass balance in the xy plane, the following equation is obtained:

$$v_x \cdot \frac{\partial c_a}{\partial x} + v_y \cdot \frac{\partial c_a}{\partial y} = D_a \cdot \frac{\partial^2 c_a}{\partial y^2}$$

This equation must be solved for the following boundary conditions:

(a) $x = 0$: $-\frac{1}{2}d \leqslant y \leqslant +\frac{1}{2}d$; $v_x = v_{x0}$; $c_a = c_{a0}$

(b) $x > 0$: $y = +\frac{1}{2}d$; $v_y = v_x = 0$; $c_a = 0$

(c) $y = -\frac{1}{2}d$; $v_y = v_x = 0$; $\frac{\partial c_a}{\partial y} = 0$

The identical case for energy transport has been calculated numerically[9]. The dimensionless Nüsselt number has been given as a function of $1/Gz$; for mass transport, Sh is the analogous number. For the number of moles transferred per second to the electrode we have:

$$Jbx = \bar{v}_x db(c_{a0} - \langle c_{ax}\rangle) \tag{1}$$

The definition of the mass transfer coefficient gives[6]

$$J = k_{ln} \frac{(c_{a0} - \langle c_{ax}\rangle)}{\ln \frac{c_{a0}}{\langle c_{ax}\rangle}} \tag{2}$$

and the definition of the Sherwood number for this geometry is

$$Sh = \frac{k_{ln}\, 2d}{D_a} \tag{3}$$

From eqns. 1–3, we obtain

$$\ln \frac{c_{a0}}{\langle c_{ax}\rangle} = \frac{Sh\, D_a\, x}{2\bar{v}_x\, d^2} \tag{4}$$

As *Sh* is known to be a function of *Gz*, the number of moles transferred to the surface can be calculated from this formula. The result for the coulometric yield is

$$\frac{c_{a0} - \langle c_{ax} \rangle}{c_{a0}} = 1 - \exp\left(\frac{-Sh\, D_a\, x}{2\bar{v}_x\, d^2}\right) \tag{5}$$

Calculation of the coulometric yield under chromatographic conditions

Using the cell described under normal chromatographic conditions, the Graetz number will be less than 10. According to Stephan[9], *Sh* has then approached a constant value, which means that the mass transfer coefficient is no longer a function of the flow-rate. This value for *Sh* is 4.86.

Using eqn. 5, the yield can be calculated as a function of the electrode surface. This is illustrated in Fig. 3, and it can be seen that with reasonable cell dimensions it is possible to obtain nearly 100% coulometric yield (91%).

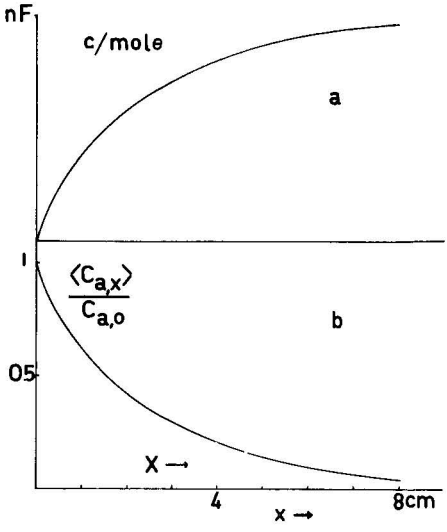

Fig. 3. Calculated percentage of reacted compound (a) and fraction of mean concentration from the beginning concentration (b) *versus* distance to the inlet point. Conditions: $\bar{v}_x = 0.8$ cm/sec; $D_a = 10^{-5}$ cm^2/sec; height of the spacer = 100 μm; width = 0.7 cm.

Prediction of dynamic behaviour

For 100% coulometric yield, each molecule will reach the electrode within the residence time in the cell. The dynamic behaviour of the cell is described by the response to a concentration impulse, *i.e.*, the current *versus* time curve after an amount of substance in a very small volume has entered the cell. This current *versus* time curve will be exactly the same as if the oxidation took place after diffusion from a stagnant fluid film, as the chance of molecules reaching the electrode is independent of the movement alongside the electrode caused by the flow. Diffusion from a stagnant fluid film has been described[11,12]. The bulk concentration as a function of time is given as

$$\frac{\langle c_a \rangle}{c_{a0}} = \frac{8}{\pi^2} \sum_{v=0}^{\infty} \frac{1}{(2v+1)^2} \exp\left\{ - \left[\frac{(2v+1)\pi}{2d}\right]^2 D_a\, t \right\}$$

The current caused by the impulse will be the derivative of this expression. In this way, a theoretical estimate of the peak broadening effect would be possible. However, comparison of theoretical with experimental values of the standard deviation, σ_d, caused by the detector is difficult, as the experimental value is based on measurement of the width at 0.6 maximal height of the peak. The same procedure applied to the theoretical curve would yield zero for σ_d, as the theoretical equation predicts an infinite value of the current for the moment that the substance enters the cell. A means of dealing with this problem is to consider the time interval in which a certain percentage of the substance reacts, and to compare it with the value expected for a gaussian curve. From Jost[11], it can be derived that when $\Delta t = 0.95\ 4d^2/\pi^2 D_a$, 68.5% of the substance has reacted. For a gaussian curve, the same percentage of the area is present between the boundaries $t_m - \sigma$ and $t_m + \sigma$, where t_m is the time of the maximum. When we equate 2σ to $\Delta t = 0.95\ 4d^2/\pi^2 D_a$, we obtain a rough estimate of σ, and accordingly we put

$$\sigma_d = \frac{0.95}{2} \cdot \frac{4d^2}{\pi^2 D_a}$$

These estimates are included in Table III.

Linearity

Although there may be other causes, non-linearity in electrochemical cells is caused mainly by the voltage drop in the electrolyte solution when a current flows. This so-called *iR* drop decreases the potential difference prevailing at the working electrode–solution interface. As a linear relationship between concentration and current can be expected only for a constant value of this potential difference, this *iR* drop must be kept as small as possible. There are several methods of accomplishing this requirement:

(i) Use of a solution of high conductivity (water with a high concentration of a conducting inert electrolyte). From both the chromatographic and electrochemical points of view, this procedure gives serious limitations.

(ii) Use of a cell of suitable geometric shape, in which the resistance between the working and reference electrodes is as small as possible. This procedure means a short distance and a wide cross-section between the electrodes.

(iii) Use of a three-electrode system. The two functions, conducting the current and serving as a reference point for the potential, normally performed by one electrode are separated in this instance, between a reference electrode and an auxiliary electrode which carries the current. A small reference electrode with high impedance can be used. These electrodes can be positioned very near to the working electrode (method ii).

A properly designed coulometric liquid chromatography detector should be capable of conducting fairly large currents, should have a small effective cell volume and should be compatible with different eluents. Therefore, a combination of methods ii and iii is the most suitable.

The relative positions of the working, reference and auxiliary electrode are important. The geometry should be such that the voltage drop that exists between the working and auxiliary electrodes as a result of the current is observed by the reference electrode to only a very small extent. This means that the current paths from the

auxiliary and reference electrodes to the working electrodes should have a common part of very small resistance.

Kissinger *et al.*[4] described an electrochemical detector with a three-electrode arrangement in which almost the whole of the voltage drop from the working to the auxiliary electrode is "seen" by the reference electrode. In this arrangement the eluent flows from the small cell through a high-resistance capillary into a larger vessel in which the reference and auxiliary electrodes are positioned. In this way, the essential advantage of a three-electrode system with respect to linearity is lost. The method used by Fleet and Little[5], where the auxiliary and reference electrodes are placed in separate outlets of the cell, is adequate. In the device described in this paper, the auxiliary electrode, having the same surface area as the working electrode, is placed directly in front of the latter. In this way, the total resistance is so small that large currents can be tolerated.

TESTING THE DETECTOR

During testing of the detector, the working electrode had a constant potential of + 0.75 V *vs.* Ag|AgCl|0.05 *M* Cl^-. The eluent was a solution 0.03 *M* in sodium acetate, 0.16 *M* in acetic acid (pH 4) and 0.05 *M* in potassium chloride. The silver tube was electrolytically covered with silver chloride. Together with the chloride added to the eluent, this arrangement results in a stable reference potential. L-Ascorbic acid was used as the test compound because it oxidises easily and no adsorption at the working electrode was observed. In order to reduce oxidation by air, the solvent for the samples was deaerated with nitrogen and cooled in ice before use. During the test, the temperature was held constant at 22°.

Static properties

Standing current. With eluent only there is a standing current due to impurities in the eluent. When using nylon connecting tubes only and hydrostatic syphoning, this current was 0.32 μA at the chosen voltage and using a spacer thickness of 100 μm.

Using stainless-steel tubes, a pump (Orlita DMP 1515), a manometer and a column (stainless steel), the standing current was 0.58 μA. The difference is probably due to metals dissolved from the tubes.

Sensitivity. The static mass flow sensitivity is defined as

$$S = \frac{i}{w\,c}$$

where S = sensitivity (A sec/mole), i = signal (A), w = flow-rate (l/sec) and c = concentration (mole/l). It was measured using a sampling valve with a large loop[2].

When the coulometric yield is 100%, the sensitivity (C/mole) can be calculated by the equation

$$S = nF$$

where n = number of electrons transferred per molecule and F = Faraday constant. The dependence of the coulometric yield on the flow-rate is shown in Fig. 4, in which

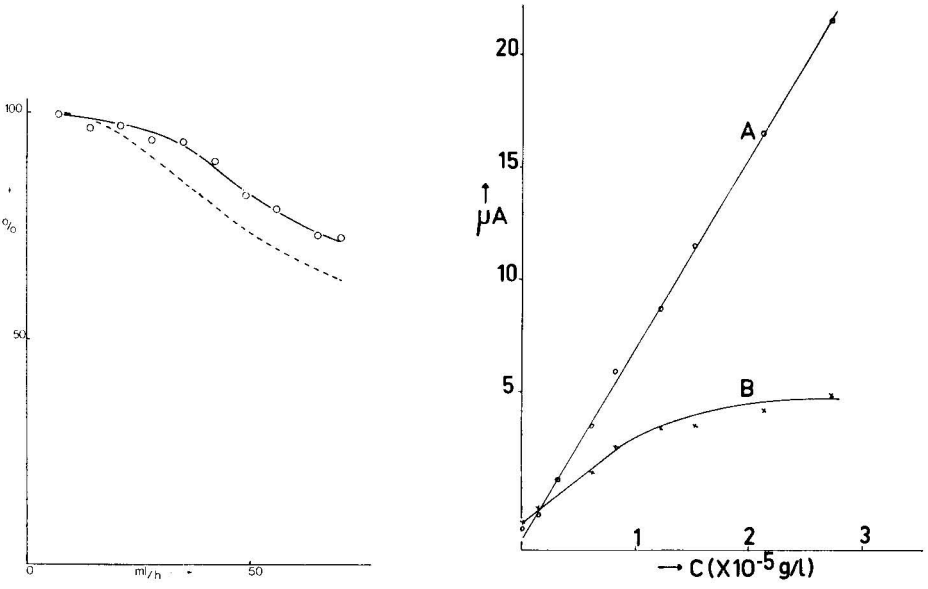

Fig. 4. Coulometric yield *versus* flow-rate; straight line, experimental; broken line, calculated. Conditions: voltage, + 0.90 V; spacer thickness, 50 μm; compound, ascorbic acid; concentration, $2.19 \cdot 10^{-4}$ mole/l.

Fig. 5. Linearity of the detector signal for two electrode arrangements: (A) working and auxiliary electrodes opposite each other; (B) glassy carbon plates as the working electrode and the auxiliary electrode downstream from the silver outlet. Conditions: Labotron pump; flow-rate, 20 ml/h; spacer thickness, 90 μm; voltage, + 0.75 V.

both experimental results and data calculated according to the theory described under Theoretical are plotted. The diffusion coefficient for ascorbic acid has been calculated to be $0.69 \cdot 10^{-5}$ cm^2/sec at 22° using Wilke's equation[13]. The coulometric yield of 100% corresponds to two electrons transferred per molecule, in agreement with the results in the literature[14]. As can be seen, the difference between the two lines is less than 10%. The concentration of the ascorbic acid solution was determined by titration with N-bromosuccinimide[15], which had been standardised with arsenic (III)[16].

Dependence of coulometric yield on spacer thickness. The coulometric yield, as can be calculated from eqn. 5, will increase when the term $Sh/4\bar{v}_x d^2$ increases. In Table I, the yield is given for various duct heights. From these data, it can be concluded that the coulometric yield is higher when using thinner spacers. Moreover, peak broadening will decrease under these circumstances. Spacers thinner than 50 μm should be avoided because of the limited flatness of the electrode material.

Linearity and linear dynamic range. The linearity was tested for the two different electrode arrangements described under *Electrical part*. The reason for using a downstream auxiliary electrode was to prevent reduction after the oxidation, which could occur for reversible reactions. However, as can be seen in Fig. 5, the arrangement of the working electrode opposite to the auxiliary electrode has a far greater linear range. The linear range is much larger than given in Fig. 5 (up to 10^{-3} A).

As most electrode reactions of interest for chromatographic applications are irreversible, the latter arrangement was chosen for all further experiments. The smaller

TABLE I

COULOMETRIC YIELD AT VARIOUS DUCT HEIGHTS

For D_a, a value of $0.69 \cdot 10^{-5}$ cm²/sec was taken, corresponding to the value calculated for ascorbic acid. Duct width, 0.7 cm; length, 7.6 cm; flow-rate, 20 ml/h.

Spacer height (μm)	*Coulometric yield (%) calculated*	*Measured*
50	96	97
75	88	—
107	75	64
200	57	—

linear range can be explained as a result of the electrode arrangement as described under *Linearity*.

The linear dynamic range is the ratio between the upper linearity limit and the noise of the standing current, and gives the useful working range of the detector. Its value is given in Table II. The very large dynamic range is noteworthy, especially when compared with those for other electrochemical detectors used in high-performance liquid chromatography[1-4].

Noise and detection limit. The method for measurement of noise has been described previously[1,2]. The integrated noise of the standing current was calculated for integration times corresponding to chromatographic peaks. The noise observed is present only when connecting the glassy carbon working electrode. Two explanations of the noise can be given.

(a) As the electrode material is porous, its complete wetting by the eluent takes place only after a very long time. Electrodes in use are therefore incompletely wetted. Changes in the area covered by the eluent will generate electric currents. If a new pore is filled with eluent, a spike will result; indeed, the noise observed seems to be composed of current spikes. A remarkable improvement was achieved by conditioning the working electrode in hot paraffin wax. After cooling, the solid wax was removed from the upper surface by wiping with tetrachloroethane. The improvement can be explained from the fact that part of the cooled wax stayed in the pores and a flat electrode surface was obtained.

(b) Voltage noise of the amplifier, which regulates the potential difference between the working electrode and the reference electrode at the desired value, will generate voltage fluctuations at the working electrode–solution interface. As this interface behaves like a large capacitor, this results in current fluctuations. The situation can easily be simulated. The result was that, with the amplifier used, a noise level of about 10^{-10} A can be expected from this source, assuming a capacity of 200 μF/cm² across the interface[3]. This is of the same order of magnitude as the observed noise and therefore this effect may give a significant contribution.

The standard deviations of the noise are represented in Table II.

When the amount of sample is limited, for instance in clinical and biochemical analysis, it is more relevant to give the detection limit as an absolute amount. The detection limit, expressed in moles, can be calculated using the equation

$$q_{det} = 3\sigma_{det} V_{peak}$$

TABLE II

STATIC NOISE OF THE DETECTOR AND LINEAR DYNAMIC RANGE

Conditions: spacer thickness, 100 μm; potential of working electrode, + 0.75 V *vs.* Ag|AgCl|0.05 *M* Cl^-; temperature, 22°; the eluent was syphoned hydrostatically and nylon connecting tubes were used.

Integration time (sec)	*Standard deviation, integral (A sec × 10^{-9})*		*Standard deviation, current (A × 10^{-9})*		*Corresponding concentration (mole/l × 10^{-9})*		*Linear dynamic range*
	*a**	*b***	*a**	*b***	*a**	*b***	
1	0.27	0.48	0.27	0.48	0.40	0.32	$> 10^{-6}$
3	0.48	1.2	0.16	0.40	0.28	0.27	
10	0.90	2.4	0.09	0.24	0.15	0.16	
30	2.2	7	0.07	0.23	0.12	0.16	
100	10.5	22.5	0.10	0.23	0.17	0.16	
300	145	110	0.48	0.37	0.82	0.26	

* Flow-rate 13.5 ml/h.
** Flow-rate 53.5 ml/h.

where q_{det} = lowest detectable amount of component present in the sample, σ_{det} = the concentration corresponding to the standard deviation of the noise and V_{peak} = peak volume. In modern liquid chromatography, the peak volume is often of the order of 50 μl. Using the value for σ_{det} from Table II, q_{det} is found to be $5 \cdot 10^{-14}$ mole.

Dynamic properties

Peak broadening. A good detector must follow a chromatographic signal sufficiently rapidly, in other words, peak broadening caused by the detector must be small compared with that of the column. In order to measure the peak broadening of the detector, 0.5 μl of a $3.4 \cdot 10^{-3}$ *M* solution of ascorbic acid was injected by means of a precise syringe and a sampling device[17]. The peak broadening, as given in Table III, was determined by measuring half the width of the peak at six tenths of the maximal height. Calculated values, according to the method described under *Prediction of dynamic behaviour*, are also given. It can be seen that these values agree with the ex-

TABLE III

CONTRIBUTION TO PEAK WIDTH FOR ASCORBIC ACID EXPRESSED AS THE STANDARD DEVIATION, MEASURED AS THE HALF-WIDTH AT SIX TENTHS OF THE MAXIMAL HEIGHT OF THE PEAK.

The calculated values correspond to 100% coulometric yield.

Flow-rate (ml/h)	*Duct height (μm)*	*Peak broadening*		
		Experimental		*Calculated (sec)*
		sec	*μl*	
40	50	0.65 ± 0.03	7.2	0.7
20	50	0.64 ± 0.4	3.5	0.7
10	50	0.7 ± 0.8	1.9	0.7
40	100	0.87 ± 0.02	9.7	2.8
20	100	1.44 ± 0.02	7.9	2.8
10	100	2.80 ± 0.08	7.7	2.8

perimental results provided that 100% coulometric yield is approached, *i.e.*, with a spacer thickness of 50 μm and for a spacer thickness of 100 μm at the lowest flow-rate. For the diffusion coefficient, a value of $0.69 \cdot 10^{-5}$ cm^2/sec was taken.

SELECTIVITY

When changing the potential of the working electrode, the sensitivity for some compounds can be changed, depending on their half-step potentials. When the separation of two peaks is bad, the peak of the compound with the higher half-step potential can be made to disappear by lowering the potential. The amount of both compounds can then be calculated from the two peaks areas. Information on the peaks can be obtained by varying the potential.

APPLICATION TO CHEMICAL ANALYSIS

At present, there is increasing interest in the analysis of endogenous metabolites and drugs and their metabolites in body fluids. As many of these compounds are oxidizable, liquid chromatography with electrochemical detection is of interest because of its low detection limit and its selectivity. Detection by means of anodic electrochemical oxidation has been applied by Kissinger *et al.*[4] and Fleet and Little[5] for the detection of ascorbic acid, uric acid and some catecholamines.

Some psychopharmaceuticals that are oxidizable at + 0.9 V are as follows: *neuroleptics:* chlorpromazine, thioridazine, periciazine, thiethylperazine, thioproperazine, perphenazine, fluphenazine, chlorprothixene, clopenthixol, flupenthixol and thiothixene; *antidepressants:* amitriptyline, nortriptyline, imipramine, desipramine and doxopine. The detection of the so-called "long-acting depot" neuroleptics such as the decanoic esters of fluphenazine, perphenazine, clopenthixol and flupenthixol is of special interest.

Reversed-phase chromatography using coulometric detection might be a useful method for analysis in blood serum. Fig. 6 shows a peak of 0.5 ng of fluphenazine, separated from the solvent peak by a column filled with modified silica[18].

The results of the separation of some biogenic amines are given in Fig. 7. Fig. 7a gives the result for UV detection; the phase system will be described elsewhere[18]. The coulometric detector was placed in series with the UV detector. Fig. 7b gives the result when the applied voltage is 0.75 V; only peaks 2 and 5 are detected. Fig. 7c gives the result when the applied potential is + 0.6 V. When the UV detector is placed in series after the coulometric detector, the peak belonging to 5-aminosalicylic acid appears to be much higher. This interesting increase in sensitivity is illustrated in Fig. 8, which should be compared with Fig. 7a.

CONCLUSION

The results show that the use of a large electrode surface area has a number of advantages, among which the most important are: the higher sensitivity and the smaller influence of electrode contamination on the sensitivity. The detection limit lies in the picogram range and the electrode arrangement results in a linear dynamic range of 10^6.

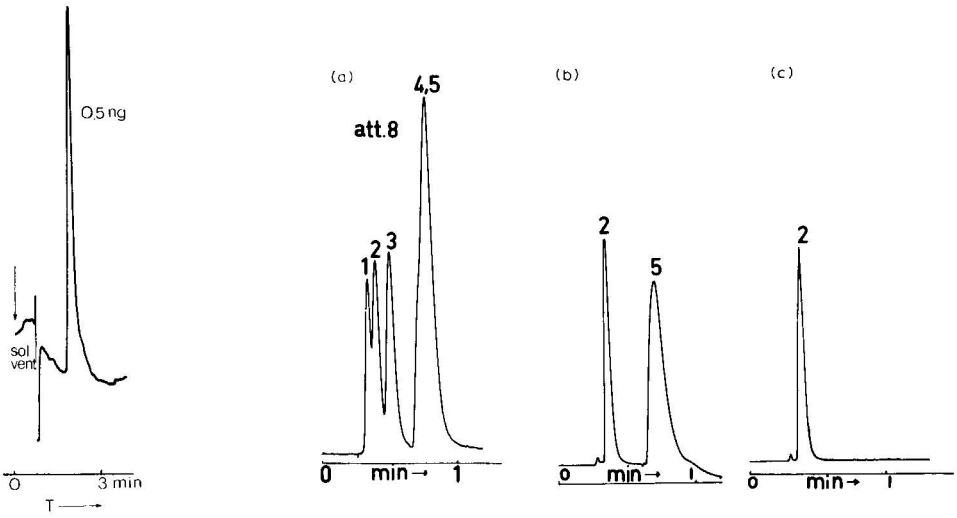

Fig. 6. Peak from 0.5 ng of fluphenazine. Conditions: voltage, 0.9 V; column, 3 cm stainless-steel tube, I.D. 2.8 mm, filled with modified silica (particle size 4–7 μm); eluent, 60% methanol, 40% water, 0.05 *M* phosphate buffer, pH 6.4; flow-rate, 3.5 μl/sec; pressure, 20 bar.

Fig. 7. Separation of biogenic amines. (a) Rapid separation of biogenic aromatics: 1 = 3-amino-toluenesulphonic acid; 2 = 5-aminosalicylic acid; 3 = 3-hydroxymandelic acid; 4 = 3,5-dihydroxybenzoic acid; 5 = 4-methylaminobenzoic acid. Conditions: UV detection at 265 nm; column, 10-cm stainless-steel tube, I.D. 2.8 mm, filled with modified silica (particle size 4–7 μm); eluent, 0.05 *M* perchloric acid, 0.05 *M* potassium chloride, 1.7% butanol in water; flow-rate, 28 μl/sec; pressure, 220 bar. (b) Coulometric detection at 0.75 V; peaks 2 and 5 are detected. (c) Coulometric detection at 0.60 V; only peak 2 is detected.

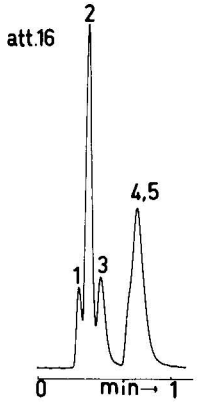

Fig. 8. UV detection after reaction in the electrochemical cell (potential + 0.75 V). The reaction products of peak 2 have a greater UV absorption at the chosen wavelength compared with Fig. 7a.

Other eluents having a lower electrical conductivity can be used. More compounds will be detectable with, *e.g.*, acetonitrile as the mobile phase because of its wider anodic range[19]. The possibility of using eluents of low conductivity permits a wider choice of chromatographic phase systems.

For 100% coulometric yield, the apparatus can also be used for the determination of the number of electrons transferred per molecule.

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GAS CHROMATOGRAPHIC SEPARATION OF SOME SULPHUR COMPOUNDS ON GLASS CAPILLARY COLUMNS USING FLAME PHOTOMETRIC DETECTION

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SUMMARY

Due to the adsorptive properties of some sulphur compounds, gas chromatographic analysis of samples containing sulphur compounds requires columns proven to be inactive towards small amounts of certain test substances. Moreover, for reliable sulphur analyses using flame photometric detectors the columns should possess high resolution properties, since large amounts of hydrocarbons are known to quench the emission of small quantities of sulphur compounds when eluted simultaneously.

The purpose of this investigation is to exemplify the above statements with selective sulphur detection, and using glass capillary columns deactivated by a thin layer of non-extractable Carbowax 20M coated with SF-96. As a practical application, the gas phase of fresh tobacco smoke has been analyzed.

INTRODUCTION

The flame photometric detector (FPD), presented in 1962 by Dräger and Dräger[1] and further developed for use in gas chromatography (GC) by Brody and Chaney[2], has been extensively used for the selective detection of volatile sulphur and phosphorus compounds. Several applications have been reported, such as the analysis of atmospheric sulphur compounds[3–8], of sulphur- and phosphorus-containing pesticides[9–11], of urinary volatile sulphur compounds[12] and of sulphur gases in soil atmospheres[13,14]. The GC separation has in most cases been performed on packed columns; the use of capillary columns seems to be rather uncommon. This might be due in part to the difficulty in preparing a capillary column which is sufficiently deactivated for the proper separation of sulphur compounds. Many sulphur compounds are extremely sensitive to adsorption and catalysis, and this is especially noticeable when they occur in low concentrations[15]. For example, if the "adsorption capacity" of the system is 20 pg for a given substance, adsorption will be very serious if the sample amount is 50 pg but hardly noticeable in the chromatogram if the amount of sample is 50 ng. Thus, an inert column system is a requisite for reliable analysis when utilizing the low limit of detection which is possible for sulphur with the FPD.

The low hydrocarbon response of the FPD is sometimes considered to be an advantage in the respect that separation would become less important[16]. However, large amounts of organic compounds are known to have a quenching effect on the sulphur response when eluted simultaneously[11,17–20]. For reliable quantitation, the sulphur compounds must therefore be separated from the major hydrocarbon peaks; in several cases, high-resolution capillary columns are required for such separations.

The use of deactivated Pyrex capillary columns for the separation of sulphur compounds is demonstrated in this paper. The detector employed was constructed at this laboratory.

EXPERIMENTAL

Pyrex glass capillaries were drawn on a vertically operating Schimadzu glass-drawing machine, and etched with HF produced by the thermal decomposition of chlorotrifluoroethyl methyl ether[21]. They were then deactivated with a thin non-extractable layer of Carbowax 20M as described earlier[22] and coated dynamically[23] at a rate of 20 mm/sec with a solution of 10% (v/v) SF-96 methylsilicone in freshly distilled toluene.

The analyses were carried out on a home-made all-glass GC system; thus the only metal surface in contact with the sample was the needle of the injection syringe. The flame emission due to sulphur passed an interference filter (AGA Sweden) having a transparency maximum at 395 nm and a band width of 5 nm at half maximum, before being measured on a photomultiplier tube (EMI 9524B) operated at 1150 V The photomultiplier tube was enclosed in a glass cooler in which circulated acetone cooled by ice to 4°. The temperature in the flame section of the detector was 110°, and the temperature of the injection block was 120°.

Research cigarettes (University of Kentucky) were equilibrated at 65% relative humidity at 25° and smoked through a Cambridge filter by means of a 50-ml Hamilton glass syringe[24]. Puffs of 35 ml were drawn during 2 sec and at 1-min intervals. From the third puff, 5 ml of smoke was withdrawn by means of a gas-tight 10-ml syringe and injected in the gas chromatograph, the oven of which was cooled to −70° by spraying with liquid nitrogen. The oven temperature was then programmed to 190°.

RESULTS AND DISCUSSION

In the detector modification used, the column effluent was mixed with hydrogen and led into the burner jet. The flame was thus similar to that of a conventional flame ionization detector (FID), Fig. 1. Such an arrangement was found to give the highest signal to noise ratio[25] and no solvent flame-out effects were experienced[25]. For 1-butanethiol, the optimum response was achieved with a hydrogen gas flow-rate of 190 ml/min and an air flow-rate of 120 ml/min. However, the response was found to vary somewhat between sulphur compounds, probably due to the different efficiency of the formation of S_2 species[26,27]. The flame was also supplied with nitrogen from the column, at a flow-rate which was relatively small, 0.9 ml/min, when capillary columns were used for the separation. Nitrogen is considered to be of great importance, acting as a third body for the formation of S_2 species[28–30]. Consequently the flame might be deficient in nitrogen. However, the addition of up to 10 ml/min of purge nitrogen

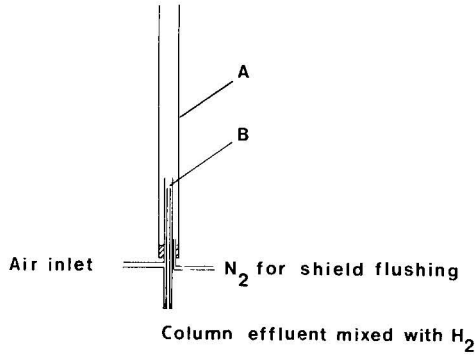

Fig. 1. Burner used in the flame photometric detector. A = Pyrex glass sheath and B = quartz tube jet (0.8 mm I.D.).

to the flame had no effect on the peak height for 1-butanethiol, and at higher purge rates the response was slightly lowered, probably due to cooling of the flame. It is assumed that nitrogen for the reaction is supplied by the air outside the flame. A further possibility is the action of the sheath wall as a third body in the formation of S_2 species[30,31].

The presence of organic compounds in the flame is found to decrease the sulphur response of the detector. This might be due to inactivation of excited S_2 species by collision with hydrocarbons and/or their decomposition products, as proposed by Sugiyama *et al.*[20]. The quenching effect of *n*-hexane on the response of 1-propanethiol was examined. The lowest amount of hydrocarbon that caused a detectable reduction of the sulphur response was 150 ng (Fig. 2). The reduction of sulphur response as a consequence of the presence of the hydrocarbon was essentially the same for 250 pg and 10 ng of 1-propanethiol. This was also observed by Sugiyama *et al.*[20] for benzo[*b*]-thiophene, but Perry and Carter[19] found an increased quenching effect of 2-methyl-1-propanol when the amount of 2-methyl-2-propanethiol was lowered. The importance of efficient separations for the detection of small amounts of thiophene in benzene is demonstrated in Fig. 3.

In some chromatographic systems, hydrocarbons cause a negative signal on the FPD. This is considered[32,33] to be due to a background of sulphur compounds being continuously eluted from the column, thus raising the baseline. However, the baseline

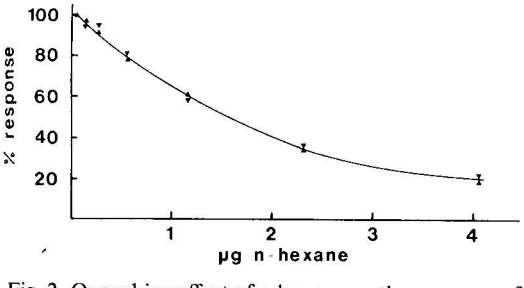

Fig. 2. Quenching effect of *n*-hexane on the response of 1-propanethiol. ▼ = 10 ng of 1-propanethiol; ▲ = 250 pg of 1-propanethiol. Pyrex glass capillary column (20 m × 0.25 mm I.D.), treated with Carbowax 20M and coated with SF-96.

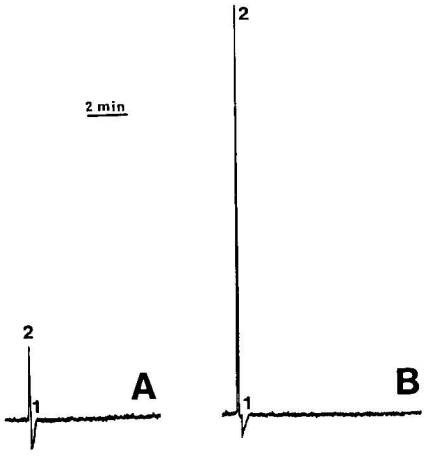

Fig. 3. Effect of separation power on the FPD response. Pyrex glass capillary column (90 m × 0.25 mm I.D.), treated with Carbowax 20M and coated with SF-96. 1 = Benzene; 2 = thiopene (100 pg). A = Poor separation at column temperature 110°; B = good separation at 28°.

is stable when programming the temperature up to 200°. This sulphur background response is quenched when a major hydrocarbon component is eluted, thus resulting in a negative peak. In the system used in this investigation a negative peak for *n*-hexane was first observed with a sample amount of 250 ng. Consequently slight quenching of sulphur compounds might take place at concentrations lower than those giving a response on the FPD. The detection limit for sulphur is *ca.* 40 pg, and this low detection limit might be explained by the enhancement in peak heights caused by the continuous background of sulphur eluting from the column[32,34]. The width and form of the negative hydrocarbon peaks depends to a considerable extent on the condition of the glass sheath of the detector. An adsorptive sheath leads to broad tailing hydrocarbon peaks. This peak-broadening effect was reduced when the sheath tube was continuously flushed with nitrogen at a flow-rate of 110 ml/min. In addition, the flushing should reduce the effect on the flame of sulphur and phosphorus compounds adsorbed on the sheath. However, silanization of the sheath caused a broadening of the hydrocarbon peaks, and this seems to be due to increased adsorption of hydrocarbons.

Using conventional flame photometry, Dagnall *et al.*[35] found that acetonitrile and pyridine produced intense CN emission at 389 nm. Moreover, the interference of CN bands has been reported[36] in the analysis of the pesticides Methidathion and Diazinon when measured at 383 nm. In my system, no positive response was observed for acetonitrile, pyridine or α-picoline, which otherwise would interfere in the analysis of the tobacco smoke.

Since the smallest detectable amount is limited by the noise level, it is essential to reduce the noise when attempting to obtain a low detection limit. The overall noise consists of several components: the flame, the interference filter, the photomultiplier and the electronics. The flame noise has been found to be dependent on the burner design[25,33] and the gas flow-rates. However, a great deal of the noise originates from the photomultiplier tube, and is reduced when the tube is cooled[5,37–39] (Fig. 4). In addition the dark current is lowered and stabilized[40]. The individual photomultiplier

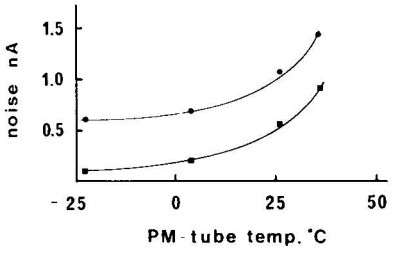

Fig. 4. The effect of photomultiplier (PM) temperature on the detector noise. ● = Flame on; ■ = flame extinguished.

tubes are found to differ somewhat. Thus it is important to optimize the tube voltage when attempting to achieve a high signal to noise ratio. The noise from a detector connected to a packed column might be reduced with the aid of an RC-filter[5,41], thus increasing the time constant of the detector. However, this method is quite unsuitable when capillary columns are used, since the narrow peaks eluted from these require a detection system showing a low time constant if a true peak representation is to be obtained. The highest frequency noise is suppressed due to the time constant of the amplifier and recorder.

Capillary columns have to some extent been used for the separation of sulphur compounds. Capillaries of materials such as stainless steel[42,43], nickel[12,39,44] and glass[45] have been used. These materials are considered to be active to sulphur compounds and some kind of deactivation is necessary. In most cases, this deactivation has been obtained by means of thick layers of polar stationary phases, and a final on-column deactivation was achieved by the injection of large amounts of a suitable sulphur compound. However, such deactivation does not seem to be sufficient, and in the results presented some of the sulphur compounds occurring in low concentrations formed broad and tailing peaks. It also appears that the polar phases have been chosen primarily for deactivation purposes, and that the chromatographic properties have been somewhat neglected. PTFE seems to be the most inert material[5–7]; however, its low critical surface tension does not allow coating of capillaries with the stationary phases used. It is possible to etch PTFE, *e.g.*, with sodium–naphthalene–tetrahydrofuran[46], thus improving its wettability. However, at this laboratory it was found that the inert properties were lost with such treatment.

Aue *et al.*[47] discovered that when Chromosorb W coated with Carbowax 20M was heat-treated to 280° and then exhaustively extracted, a thin non-extractable layer of phase remained. The support was found to possess a high degree of deactivation after this treatment. A further advantage is that the deactivated support can be coated with different stationary phases, *e.g.*, Carbowax or less polar phases; thus it is possible to adapt the chromatographic properties to the actual separation problem. This deactivation method has been applied in our laboratory to the deactivation of glass capillary columns[22], and in this investigation such deactivated columns are used for the separation of sulphur compounds. As column material, Pyrex glass was chosen due to its slightly acidic properties which are favourable for the slightly acidic sulphur compounds. The silicone oil SF-96 was found to be a suitable stationary phase for these analyses, since it gives highly efficient columns when using my coating methods[23].

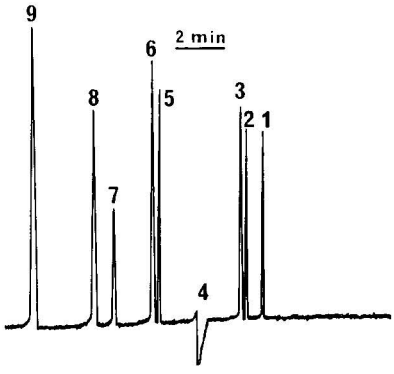

Fig. 5. Gas chromatogram (FPD) of a test mixture. Column as in Fig. 3. Column temperature, 28°. Sample amount, 75–300 pg. Peaks: 1 = methanethiol; 2 = dimethyl sulphide; 3 = carbondisulphide; 4 = *n*-hexane; 5 = 2-butanethiol; 6 = thiophene; 7 = diethyl sulphide; 8 = 1-butanethiol and 9 = dimethyl disulphide.

Moreover, the use of a non-polar phase minimizes the retention times and thus some of the possibilities for adsorption and catalysis. However, newly prepared columns showed a slight adsorption of sulphur compounds, and an additional deactivation was necessary. About ten injections of 50 ng of a sulphur compound, having a suitable boiling point, usually resulted in a deactivation that allowed reproducible peak heights. A chromatogram of a test mixture of methanethiol, dimethyl sulphide, carbon disulphide, 2-butanethiol, thiophene, diethyl sulphide, 1-butanethiol and dimethyl disulphide is shown in Fig. 5.

Using flame photometric detection, sulphur compounds in the gas phase of tobacco smoke have been analyzed on packed columns[48–51] and on nickel capillary

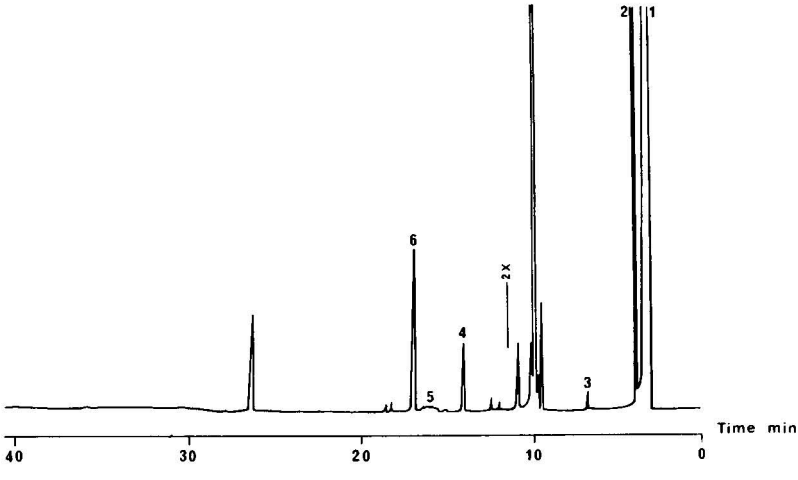

Fig. 6. Gas chromatogram (FPD) of the gas phase of tobacco smoke. Column as in Fig. 3. Splitting ratio, 1:145. Carrier gas, nitrogen. Initial temperature on injection, −70°; programmed to −5° at 25°/min and to 190° at 5°/min. Peaks: 1 = hydrogen sulphide; 2 = carbonyl sulphide; 3 = sulphur dioxide: 4 = thiophene; 5 = unknown and 6 = dimethyl disulphide.

columns[39,44]. A reduction in sulphur response due to the quenching effect of organic compounds is certainly a possibility when packed columns are used for the separation. Due to the large number of components in the tobacco smoke, the risk of artefact formation is unusually high. This has been demonstrated by Horton and Guerin[48] for the analysis of sulphur compounds in tobacco smoke. They found drastic losses of H_2S when the smoke was in contact with stainless steel for 4 sec. When aging the smoke for 30 sec in a stainless-steel loop, a substantial increase in higher-molecular-weight sulphur components was observed, probably formed by the reaction of low-molecular-weight sulphur compounds, *e.g.*, H_2S and other constituents of the smoke. These observations indicate the need for inert chromatographic systems as well as sampling methods. Trapping the smoke on an adsorbent such as Tenax GC[39,44] might introduce artefacts and losses: the use of an unsuitable transfer line from the smoking device to the chromatograph might have the same effect. The injection of fresh tobacco smoke by means of a glass syringe is assumed to minimize the formation of artefacts[24,52–55]. It is also likely that artefacts are formed in the injection part of the chromatograph. Thus it is essential to keep the injection temperature as low as possible and to minimize the sample residence time in the injection system.

A typical gas chromatogram (FPD) of a relatively low amount of fresh tobacco smoke is shown in Fig. 6 and that of a somewhat larger sample is shown in Fig. 7. In the case of compounds present in low concentrations, especially those disposed to adsorption, the peak form might be improved if the sample amount is increased, as can be seen when comparing peak 5 in Figs. 6 and 7; the large peaks, however, show slight tailing in Fig. 7, which is probably due to overloading of the column. The relation between the amounts of the reactive substances H_2S, COS and SO_2, respectively, agrees with the results achieved by Horton and Guerin[48] when special precautions are taken in order to avoid artefacts; our deactivation thus seems to be equivalent. Moreover, when using nickel capillary columns, Bertsch[39] found a large number of higher-molecular-weight sulphur compounds in high concentrations. Our results, however, show the presence of only a few high-intensity peaks and a considerable

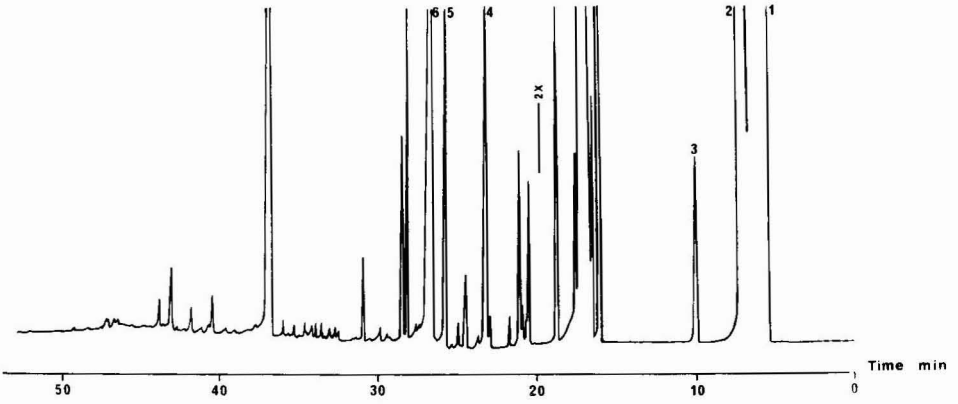

Fig. 7. Gas chromatogram (FPD) of the gas phase of tobacco smoke. Column as in Fig. 3. Splitting ratio, 1:7. Initial temperature on injection, −70°; programmed to 195° at 5°/min. Peaks: 1 = hydrogen sulphide; 2 = carbonyl sulphide; 3 = sulphur dioxide; 4 = thiophene; 5 = unknown and 6 = dimethyl disulphide.

number of sulphur compounds occurring in low concentrations; these findings also seem to agree with the results obtained by Horton and Guerin[48] for the analysis of sulphur compounds in the gas phase of tobacco smoke.

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CHROM. 9270

THE USE OF TENAX FOR THE EXTRACTION OF PESTICIDES AND POLYCHLORINATED BIPHENYLS FROM WATER

II. TESTS WITH ARTIFICIALLY POLLUTED AND NATURAL WATERS

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SUMMARY

The application of Tenax® to the extraction of organic micro-pollutants such as pesticides and polychlorobiphenyls from waters has been studied, and it has been observed that also in the presence of other pollutants (oil, surface-active substances, etc.) the results obtained with an absorption column of Tenax–Celite are equivalent to those obtained with the continous liquid–liquid extraction technique. For natural waters that contain solids in suspension that adsorb pesticides, it may be necessary to filter the water before extraction with Tenax and then to extract the suspended solids separately. Analyses of river and estuarine sea waters, filtered before extraction, showed the effectiveness of Tenax, and the extracts obtained for the pesticides analysis prove to be much less contaminated by interfering substances than the corresponding extracts obtained by the liquid–liquid technique.

INTRODUCTION

Surface waters can be polluted by many organic substances, most of which are present in small or trace amounts. Among such substances, pesticides are of particular importance owing to their high toxicity towards aquatic animals and the possibility that they may not be completely eliminated by water-treatment processes and may therefore reach man. Recently, tolerance limits for some organochlorine pesticides in drinking water have been proposed[1] at the 0.1 ppb (μg/l) level; therefore, in order to effect analytical controls it is necessary to develop suitable techniques for the concentration of the pollutants. Favourable results have been obtained for the concentration of many organic substances[2] and recently also for some pesticides[3–5] using macromolecular resins of the Amberlite XAD-2 type; progress has also been made in liquid–liquid extraction techniques[6].

In a previous investigation, we showed the possibility of using Tenax (a porous polymer; trade mark registered by Enka N.V.; developed by AKZO Research Labs.,

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Arnhem, The Netherlands) for the extraction of organic micro-pollutants such as pesticides and aromatic polycyclic hydrocarbons[7] from waters. The recoveries of these substances from unpolluted waters (mineral and drinking waters) when added at the level of 1 ppb averaged 90%. The use of Tenax, compared with the other techniques mentioned, has the advantages of requiring no preliminary treatment (activation or purification) and of permitting the utilization of automated equipment for field extraction of the pollutants.

The physical characteristics of Tenax were examined by Sakodynskii *et al.*[8] and recent applications have included the analysis of atmospheric pollutants[9,10] and the volatile components of wines[11]. On the other hand, as noted by Beyermann and Eckrich[12], the capacity of polymers to extract organic micro-pollutants from waters can be adversely affected by the presence in the waters of larger amounts of other pollutants. For instance, in the particular case of a hydrophobic polymer such as Tenax, mineral oils could "deactivate" the adsorbents and result in low recoveries of the micro-pollutants.

This paper describes tests carried out in order to study these phenomena and to ascertain the possibilities of the use of Tenax. Initially, mineral and drinking waters were enriched with pesticides and then artificially polluted with various products (surfactants, mineral oils, emulsifying agents) and, on the basis of the results obtained for the recoveries of some pesticides, the absorption column was partially modified. Using the modified absorption column and the liquid–liquid extraction technique[13–15], extraction tests were carried out with natural waters (sweet surface and coastal sea waters). The results of these tests showed the fields in which extraction with Tenax can be applied.

EXPERIMENTAL

The equipment used for the extraction experiments is illustrated in Fig. 1 and consists of the following components. A glass container (1) of 20 l capacity was used. The silicone rubber hose (3) (type MCO/3, Cellai, Milan, Italy), in order to eliminate eventual interfering substances, was extracted before use by immersion in light petroleum for 48 h, changing the solvent after the first 24 h. Watson-Marlow Type MHRE/88 peristaltic pumps (4) with the canals and variable speed (Watson-Marlow Ltd., Marlow, Buckinghamshire, Great Britain) were used. The flow-rate of water through successive absorption columns (5) was regulated at about 3 l/h using two canals of the pump (the first incoming and the second, inverted outgoing). Therefore, with one of these pumps it is possible to carry out five extractions simultaneously. The glass absorption column (5), dimensions 48 cm × 1 cm I.D., was fitted with a porous septum. During preliminary tests, the column was filled exclusively with 1.5 g of Tenax, while during standard tests it was filled from the bottom with 1.5 g of Tenax (60–80 mesh), a layer (about 2 cm high) of glass pellets of diameter about 3 mm; a layer (about 10 cm high) of a mixture of 1–1.5 g of Celite 545 (Johns-Manville, Denver, Colo., U.S.A.) and plugs of silanized glass-wool (Applied Science Labs., State College, Pa., U.S.A.); and a layer of glass pellets (2 cm high).

The extraction and recovery tests were carried out with waters to which pesticides and other pollutants had been added, and with natural waters. In the recovery

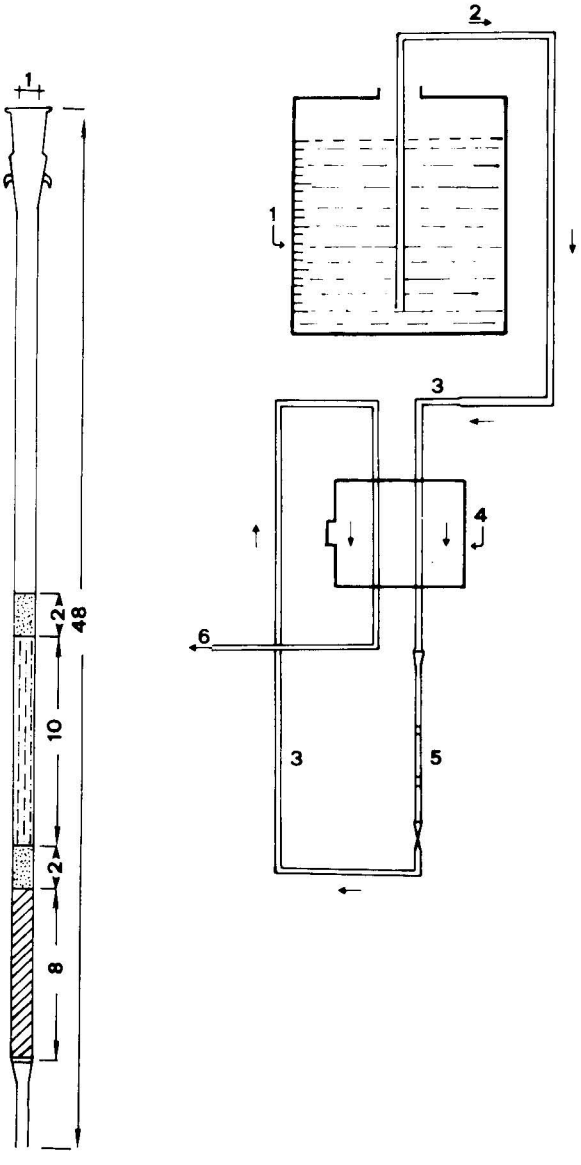

Fig. 1. Right: Equipment used for the extraction of micro-pollutants. 1, Glass container; 2, glass tubes; 3, silicone rubber hose; 4, peristaltic pump; 5, glass absorption column; 6, extracted water discharge. Left: adsorption column (dimensions in cm). From bottom to top: Tenax, glass pellets, mixture of Celite and plugs of silanized glass-wool, glass pellets.

tests, standard mixtures of pesticides prepared in acetone were added to 8–15 l of water at a level of 100 μl of standard solution per liter of water, so that the concentration of the various pesticides was 1 ppb (1 μg/l).

The various water samples were then passed through the peristaltic pump into

the absorption column at a flow-rate of about 3 l/h. When the absorption was completed, the pesticides were eluted with three 10-ml volumes of diethyl ether, in such a way that the solvent also passed through the section of hose through which the water reached the column. Finally, the diethyl ether was dried over anhydrous sodium sulphate. The water container was washed with light petroleum in order to remove the pesticides adsorbed by the glass walls, and the washing solution, after concentration, was added to the eluate obtained from the absorption column.

For recovery tests carried out with mineral waters enriched with pesticides and other known pollutants, the mixed diethyl ether and light petroleum extract was evaporated just to dryness, the residue dissolved in *n*-hexane and the solution analyzed directly by gas chromatography with an electron-capture or phosphorus detector. However, for the analysis of naturally polluted waters, the mixed diethyl ether and light petroleum extract was evaporated, the residue dissolved in light petroleum and the solution partially purified by partitioning with acetonitrile saturated with light petroleum[15,16]. The acetonitrile partitioning is not needed for samples that are not highly polluted or for sea waters.

The resulting solution was evaporated just to dryness, the residue dissolved in 1 ml of *n*-hexane and pesticides and polychlorobiphenyls were separated into four fractions by deactivated silica-gel microcolumn chromatography[15] (silica gel Type Grace 950, 60–200 mesh). The deactivation of the absorbent was effected by adding 7–10% of water, instead of the 5% used previously[15], in order to compensate for the increased activity observed in recent years in many batches of the product. For the standardization of the adsorbent, a yellow dye, *p*-methoxyazobenzene, was also used as suggested by Claeys and Inman[17]. The various eluates from the silica gel were then analyzed by gas chromatography[18].

All solvents and reagents used were of "for pesticide residue analysis" grade (Carlo Erba, Milan, Italy), except for the light petroleum (b.p. 40–60°, BDH, Poole, Great Britain) which was distilled before use. All necessary precautions for analyses of this type were taken and a few blank trials were carried out.

It is noteworthy that the recoveries of hexachlorobenzene during the acetonitrile partitioning were not greater than 75%, and therefore for the calculations a correction factor was used. In order to evaluate the effectiveness of extraction from natural waters with the Tenax–Celite column, the samples were also extracted simultaneously by the liquid–liquid technique using the equipment suggested by Kahn and Wayman[13], consisting of three consecutive chambers; light petroleum was used as the solvent in the first and second chambers and benzene in the third. Before the extraction by the liquid–liquid method, the water was acidified to pH 1–3 with hydrochloric acid.

RESULTS

Table I indicates the tests effected, adding pesticides and other pollutants to a mineral water (pH 6.8 with a constant residue of about 0.4 g/l, consisting mainly of calcium salts and containing no carbon dioxide) that had been shown to be pesticide free prior to the analyses. These recovery trials were carried out with the pesticides most frequently found in Italian surface waters, usually also polluted by organophosphorus pesticides[19–21].

TABLE I

SUMMARY OF TRIALS ON THE TENAX EXTRACTION OF PESTICIDES FROM WATERS ARTIFICIALLY POLLUTED WITH PESTICIDES (1 ppb) AND OTHERS SUBSTANCES

Trial No.	*Volume of extracted water (l)*	*Pollutant*	*Adsorption column*	*Pesticides examined*	
				Organo-chlorine	*Organo-phosphorus*
1	10	0.1 ppm of Arkopal N-100	Tenax	+	+
2	10	0.1 ppm of alkylbenzene-sulphonate (Na salt)		+	+
3	10	3% NaCl (marinesalt)		+	+
4	10	0.1 ppm of alkylbenzene-sulphonate + 1 ppm of mineral oil		+	+
5	10	1 ppm of mineral oil + 2 ppm of Tween 80		—	+
6	8				+
7a	14		Tenax–Celite	—	+
7b	10	1 ppm of mineral oil + 4 ppm of Tween 80		+	+
7c	10			+	+
7d	10			+	+
7e	10			—	+

Table II shows the results obtained. In the absorption with Tenax alone, the first three trials gave satisfactory results, while in the presence of mineral oil (trials 4,5 and 6) a considerable proportion of the organophosphorus pesticides (particularly malathion and methylparathion) was not adsorbed and was recovered in the filtered water. Test 7 (7a–7e) showed that this drawback can be overcome by placing, ahead of the layer of Tenax in the absorption column, a layer of Celite 545 which, in order to prevent blocking of the column, is mixed with silanized glass-wool plugs (Fig. 1).

A number of analyses of surface and estuarine sea waters were carried out by this modified Tenax column and simultaneously by the liquid–liquid extraction technique. To some of the samples taken, standard mixtures of pesticides were also added, each at the level of 1 ppb, *i.e.*, in concentration from 13 to 500 times higher than that usually found in the waters analyzed. One recovery trial also specifically concerned polychlorobiphenyls. The scheme of these tests is shown in Table III and the results obtained are given in Tables IV and V.

Table IV (trials 8, 9 and 11) shows that the two extraction methods, when applied to surface waters that were not filtered before extraction, yielded very similar results for many pesticides, with the exception of compounds of the DDT series, for which discordant results were frequently obtained (see the differences between trials 8a and b and 11a and b). Similarly, as shown in Table V (trial 11c), when the standard mixture of pesticides was added to a nonfiltered surface water, *i.e.*, containing suspended solids, the recoveries of active substances such as DDT and malathion were unsatisfactory. It is known that some pesticides that are present in waters are partially adsorbed by suspended or sedimentable solids[22]; therefore, such adsorbed

TABLE II

PERCENTAGE RECOVERY OF PESTICIDES ADDED AT THE 1 ppb LEVEL FROM MINERAL OR DRINKING WATERS ARTIFICIALLY CONTAMINATED IN THE LABORATORY WITH OIL, SURFACTANTS, ETC.

Pesticides and trials	*Adsorption with Tenax alone*							*Adsorption with Tenax–Celite*								
	1	*2*	*3*	*4*	*5*	*6**	*6***	*7a*	*7b**	*7b***	*7c**	*7c***	*7d**	*7d***	*7e**	*7e***
Hexachlorobenzene	93.5	82.5	94.7	102.9	—***	—	—	—	—	—	—	—	88.8–85.9	Abs.§	—	—
Dieldrin	85.9	95.6	98.9	89.4	—	—	—	—	92.0	Abs.	100.6	Abs.	87.2–80.0	Abs.	—	—
Heptachlor	82.2	79.7	85.2	96.0	—	—	—	—	—	—	—	—	—	—	—	—
o,p'-DDE	73.2	83.9	77.7	101.3	—	—	—	—	—	—	—	—	—	—	—	—
o,p'-DDT	75.6	83.2	82.9	111.8	—	—	—	—	77.8	Abs.	87.0	Abs.	86.5	Abs.	—	—
p,p'-DDT	75.9	89.2	83.9	106.9	—	—	—	—	93.4	Abs.	95.4	Abs.	88.1	Abs.	—	—
γ-BHC	—	—	—	87.7	—	—	—	—	—	—	—	—	85.8	Abs.	—	—
β-BHC	—	—	—	—	—	—	—	—	—	—	—	—	86.3–79.3	Abs.	—	—
Ronnel	93.4	85.5	98.9	82.9	—	—	—	—	—	—	—	—	—	—	—	—
Dursban	—	—	—	—	98.1	88.6	Abs.	89.6	—	—	—	—	—	—	88.7	Abs.
Malathion	—	—	—	—	12.0	20.1	64.9	78.0	50.0	33.3	32.8	38.1	32.6	30.7	82.2	20.0
Parathion	96.5	87.2	96.6	51.2	66.3	74.5	13.3	85.0	—	—	—	—	—	—	98.7	Abs.
Methylparathion	76.6	68.4	87.5	—	27.3	39.9	43.0	81.0	—	—	59.1	27.2	71.4	23.2	91.9	Abs.
Trithion	—	—	—	—	—	—	—	—	—	—	—	—	—	—	94.2	Abs.
Ethion	—	—	—	—	—	—	—	—	—	—	—	—	—	—	90.5	Abs.

* Percentage recovery of the column, hose and container.

** Percentage recovery in the filtered water on the Tenax column.

*** Not determined.

§ Abs. = absent.

TABLE III

SUMMARY OF TRIALS OF TENAX AND LIQUID–LIQUID EXTRACTION OF PESTICIDES FROM NATURAL POLLUTED WATER

Trial No.	*Sample*	*Trials effected*
8	Tiber river water (30 l)	8a: 15 l, Tenax–Celite extraction
		8b: 15 l, liquid–liquid extraction
9	Sea-coast water (20 l)	9a: 10 l, Tenax–Celite extraction
		9b: 10 l, liquid–liquid extraction
10	Sea-coast water (30 l)	10a: 10 l, Tenax–Celite extraction
		10b: 10 l, liquid–liquid extraction
		10c: 10 l + 1.6 ppb standard of PCB* and Tenax–Celite extraction
11	Tiber river water (30 l)	11a: 10 l, Tenax–Celite extraction
		11b: 10 l, liquid–liquid extraction
		11c: 10 l + 1 ppb standard of pesticides and Tenax–Celite extraction
12	Tiber river water (30 l) filtered before extraction	12a: 10 l, Tenax–Celite extraction
		12b: 10 l, liquid–liquid extraction
		12c: 10 l + 1 ppb standard of pesticides and Tenax–Celite extraction

* PCB made by Caffaro (Milan), Italy, added as standard mixture of Fenclor 42, 54 and 60 equivalent to 1.6 ppb of decachlorobiphenyl in total.

TABLE IV

DETECTION OF PESTICIDES IN SURFACE WATER SAMPLES EXTRACTED BY ADSORBTION ON TENAX–CELITE (SERIES a) AND BY THE LIQUID–LIQUID TECHNIQUE (SERIES b)

Results expressed in ppt (ng/l).

Pesticide identified	*Trial No.*							
	*8a**	*8b**	*9a**	*9b**	*11a**	*11b**	*12a***	*12b***
Hexachlorobenzene	5.4	5.8	3.3	3.8	2.6	—	8.1	6.6
Dieldrin	5.8	6.4	—	—	15.6	8.2	14.6	14.6
Heptachlor	1.5	1.7	—	—	0.9	0.9	7.1	2.7
p, p'-DDE	2.2	3.5	—	—	2.8	2.5	10.6	5.2
o, p'-DDT	5.0	12.2	—	—	13.0	12.3	24.8	19.8
p, p'-DDT	8.8	18.0	9.0	15.7	32.2	24.7	37.2	35.5
α-BHC	—	—	—	—	5.2	4.0	—	—
β-BHC	37.2	43.9	6.0	5.7	75.8	77.5	58.4	67.5
γ-BHC	9.7	13.6	—	—	19.4	17.3	10.5	15.5
Ronnel	18.9	21.7	—	—	2.1	2.1	—	—
Dursban	27.5	29.4	—	—	41.2	43.3	—	—
Diazinon	37.4	37.4	—	—	—	—	19.7	22.3
Malathion	26.5	31.7	—	—	27.0	31.0	27.3	27.3
Parathion	—	—	—	—	37.5	39.3	—	—
Methylparathion	32.6	38.2	—	—	21.0	26.0	—	—
Total (ppt)	218.6	263.5	18.3	25.2	296.3	289.1	218.3	217.0

* Waters not filtered before extraction.

** Waters filtered through paper before extraction.

TABLE V

RECOVERY TESTS ON EXTRACTION WITH TENAX-CELITE OF PESTICIDES AND POLYCHLOROBIPHENYLS ADDED TO SURFACE WATERS AT LEVELS OF 1.0 AND 1.6 ppb, RESPECTIVELY.

All results for corresponding non-treated samples are corrected. Results given are percentage recoveries.

Pesticides + additives	*Trial*		
	*10c**	*11c**	*12c***
PCB	100.2		
Hexachlorobenzene (HCB)		84.8	83.9
o, p'-DDT		38.0	81.8
p, p'-DDT		47.0	93.1
β-BHC		70.0	73.0
γ-BHC		84.9	92.0
Dieldrin		75.4	92.7
Methylparathion		82.3	101.0
Malathion		25.0	93.3

* Water not filtered before addition of standards and extraction.

** Water filtered through paper before addition of standards and extraction.

amounts cannot be extracted by filtration procedures on polymers while, at least to a great extent, they can be extracted by procedures in which the water is extracted with solvents. Trials 12a and b (Table IV) show that when the two extraction techniques are applied to filtered water, almost identical results were obtained, and trial 12c (Table V) shows that with Tenax extraction the recoveries of pesticides added to a filtered surface water before extraction were satisfactory.

During the tests it was observed that amounts of up to 30% of some pesticides can be adsorbed by the walls of the hose and the container, so that at the end of the extraction it is essential to wash these surfaces with solvents.

We consider that the tests described above have defined the fields in which Tenax extraction can be applied.

CONCLUSIONS

The extraction of pesticides from waters by absorption on Tenax, yields results equivalent to those obtained by the liquid–liquid procedure when applied to mineral, drinking and surface waters that completely or almost completely lack solid matter in suspension. For waters that contain suspended solids that can adsorb some pesticides in considerable amounts, the results of the two methods are equivalent only if the water has previously been filtered. In these instances, therefore, the analysis will involve filtered water as well as the residue of filtration.

Compared with liquid–liquid extraction, the main advantages of Tenax are the considerable amount of time saved, the possible automation of the process and that gas chromatographic analysis shows the "extracts" obtained with Tenax to be less contaminated by interfering substances. Another advantage of Tenax is that the product can be used "as received", without preliminary treatment.

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CHROM. 9164

DETERMINATION OF NALOXONE AND NALTREXONE AS PERFLUOROALKYL ESTER DERIVATIVES BY ELECTRON-CAPTURE GAS–LIQUID CHROMATOGRAPHY

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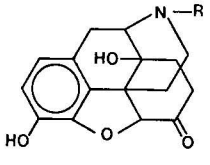
SUMMARY

An electron-capture gas chromatographic method is described for the determination of naloxone and naltrexone as the perfluoroalkyl esters. Each compound serves as internal standard for determination of the other. The method permits quantitation of 2–100 ng of either compound. Conditions for derivatization with heptafluorobutyric anhydride (HFBA), pentafluoropropionic anhydride (PFPA), and trifluoroacetic anhydride (TFAA) have been investigated. When catalyzed with pyridine, derivatization with HFBA and PFPA at 70° gives naloxone and naltrexone triesters. Evidence for triester formation was obtained from gas chromatography–methane chemical ionization mass spectrometry and infrared spectral analysis. It was found that both the HFB and PFP triesters are suitable for quantitation of the narcotic antagonists, the HFB derivatives having greater stability than the PFP derivatives. The TFA derivatives are substantially less stable.

INTRODUCTION

Naloxone (Ia) and naltrexone (Ib) are potent, rapidly acting narcotic antagonists currently undergoing extensive clinical trials for prevention of narcotic addiction in man[1–3].



Ia: R = $CH_2CH{=}CH_2$

Ib: R = CH_2 

They are active at low plasma concentrations, typically in the ng/ml range[4], and are extensively metabolized[5–12]. Thus, analytical methodology of high sensitivity

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and specificity is required to study the mechanism of action as well as the absorption, distribution, and elimination of these compounds.

Verebely *et al.*[13] have recently reported a gas chromatographic assay for naltrexone and its metabolite, β-naltrexol in human urine which is based upon flame ionization detection (FID) of the silylated derivatives. They reported that the absolute sensitivity of detection of these compounds is 10–20 ng/ml. On examining the plasma concentration–time profiles following intravenous administration of naltrexone in dogs and monkeys, it was found that the data is best fit to a two-compartment open model[14]. Plasma levels in the terminal phase of the profiles are in the range 2–20 ng/ml following a 0.72 mg/kg dose to a dog and in the range 10–45 ng/ml following a 1-mg/kg dose to a monkey. These concentrations are in the region of the absolute sensitivity of the FID method. Accordingly, a method having greater sensitivity is required for a pharmacokinetic study utilizing plasma samples.

This report describes conditions for submicrogram scale derivatization of naloxone and naltrexone with perfluoroalkyl anhydrides and for determination of the resulting derivatives by electron-capture gas–liquid chromatography (GLC–ECD).

EXPERIMENTAL

Reagents

Naloxone hydrochloride (Ia-HCl) and naltrexone hydrochloride (Ib-HCl) were obtained from the National Institute on Drug Abuse (Rockville, Md., U.S.A.) and were converted to free bases. Purity was established by thin-layer chromatography (TLC) and by GLC–ECD of the heptafluorobutyryl derivatives.

Trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA), heptafluorobutyric anhydride (HFBA), and heptafluorobutyryl imidazole (HFBI) were obtained from Pierce (Rockford, Ill., U.S.A.) and were used without further purification. They were carefully protected from moisture and were stored under refrigeration. Pyridine of Sequanal® quality was also obtained from Pierce and was stored under nitrogen at −5°.

Methanol and benzene of "Nanograde" purity were purchased from Mallinckrodt (St. Louis, Mo., U.S.A.). All other chemicals were reagent grade or better and were used as purchased.

Solutions containing 1% pyridine in benzene for use as a catalyst were prepared fresh daily.

Glassware

All glassware was soaked overnight in sulfuric acid–nitric acid (4:1). After thorough rinsing in distilled water, it was siliconized by immersion for one min in a 1% solution of Siliclad® (Clay Adams, Parsippany, N.J., U.S.A.) in water. Siliclad solutions were adjusted to pH 8–9 by the addition of concentrated ammonium hydroxide and were prepared fresh. Glassware was immediately rinsed with 50% methanol in water, and then dried at 105°.

Stock solutions

Stock solutions of Ia and Ib in methanol (0.01 mg/ml) were prepared. No evidence of decomposition was observed after storage of the solutions for six months at −5°.

Submicrogram derivatization of naloxone and naltrexone

Samples containing submicrogram quantities of naloxone and naltrexone in 15 × 125-mm glass culture tubes (Pyrex® No. 9826) were prepared from stock solutions of the compounds in methanol. Solvent was carefully evaporated under a stream of nitrogen. To each tube were added 25 μl of anhydride and 50 μl of 1% pyridine in benzene. Contents were thoroughly mixed and the tubes were tightly sealed with Teflon®-lined screw caps. Tubes were then placed in an oil bath (pre-heated to 70°) to a depth of about 50 mm so that the upper parts of the tubes acted as reflux condensers. After 2 h the tubes were placed in an ice bath.

Excess derivatizing agent was removed before chromatography by adding 5 ml of a saturated aqueous solution of sodium borate to the cooled reaction mixture. Tubes were immediately rotated on a Labquake® mixer for 3.0 min and then centrifuged for 4.0 min to separate the layers. When a series of samples was derivatized, excess agent was removed from each sample just before it was chromatographed in order to minimize hydrolysis of derivatives. Aliquots (1–3 μl) of the upper (organic) layers were immediately withdrawn into a 10 μl syringe and injected on to the GC column.

Electron-capture gas–liquid chromatography

Analyses were made using a Hewlett-Packard 5700A gas chromatograph equipped with a ^{63}Ni electron-capture detector (pulsed, variable-frequency type). Chromatographic columns were 183 cm × 2 mm I.D., coiled, borosilicate glass treated with trimethylchlorosilane (5% solution in toluene). With the aid of gentle vacuum, columns were packed with either 3% OV-1 or 3% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). Packed columns were conditioned at 325° for 4 h with no carrier gas flow, and then at 275° for 16 h with a carrier gas flow-rate of about 10 ml/min. Columns conditioned in this manner were ready for use without further treatment.

The carrier gas was argon–methane (95:5) at a flow-rate of 40 ml/min. For separations on OV-1 columns, the oven was programmed from 190° to 270° at 4°/min. Separations on OV-17 columns were carried out isothermally at 205°. The injection port and detector temperatures were 250°.

Gas chromatography–chemical ionization mass spectrometry

Low resolution mass spectra were obtained at Battelle Columbus Laboratories (Columbus, Ohio, U.S.A.) on a computerized GLC–mass spectrometry (MS) system consisting of a Varian 1740 gas chromatograph coupled to a Finnigan quadrupole mass spectrometer. Naloxone and naltrexone derivatives were chromatographed on a 183 cm × 2 mm I.D., glass column packed with 3% SE-30 on 100–120 mesh Gas-Chrom Q. The column oven was programmed from an initial temperature of 190° to 270° at 4°/min. The injection port and detector temperatures were 280° and 290°. The carrier gas, methane (flow-rate, 20 ml/min), also served as reactant gas in the ionization chamber.

Derivatives were prepared by reacting 1 μg of naloxone or naltrexone with 50 μl of HFBA and 100 μl of 1% pyridine in benzene at 70° for 20 min. Excess reagent was removed by washing the reaction mixtures with saturated sodium borate solution as described earlier.

Plots of total ion current *versus* scan number were obtained and compared with chromatograms obtained by GLC–ECD on 3% OV-1. Mass spectra of major peaks were recorded.

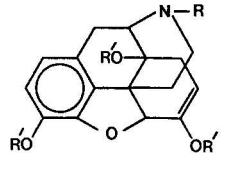
Infrared analysis

Infrared (IR) spectra of derivatives in benzene solution were obtained on a Perkin-Elmer 257 grating IR spectrometer using 0.1-mm sodium chloride cells.

Derivatives for IR analysis were prepared by heating 10 mg of naloxone with 50 μl of HFBA and (a) 500 μl of benzene or (b) 500 μl of 1% pyridine in benzene at 70°. Reaction mixtures were sampled periodically by removing 50-μl aliquots. After decomposition of excess HFBA and dilution with benzene, the aliquots were examined by GLC–ECD on the OV-17 column. When chromatograms indicated the presence of one derivative (either IIa or IIIa), the reaction mixtures were washed with sodium borate solution and centrifuged to separate the layers. The supernatant organic layers were removed and dried with anhydrous sodium sulfate before their IR spectra were obtained.

RESULTS AND DISCUSSION

Reaction of submicrogram quantities of naloxone and naltrexone with HFBA in the presence of pyridine results in derivatization of the hydroxyl groups at C-3 and C-14 as well as the carbonyl group at C-6 to yield the tri-substituted heptafluorobutyryl derivatives, IIa and IIb. Structures of IIa and IIb were confirmed by GLC–MS and IR spectroscopy.



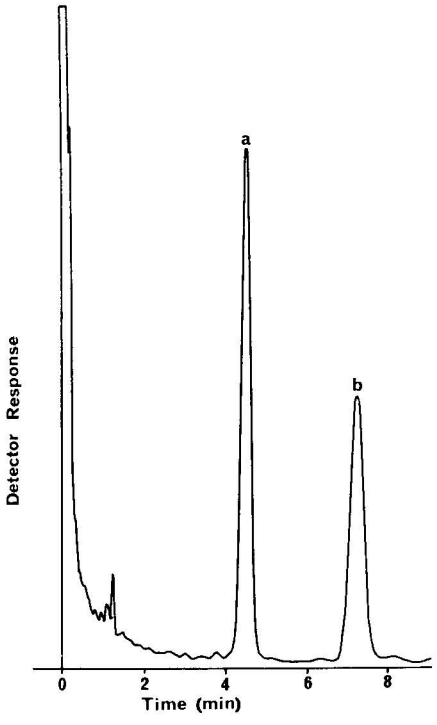
IIa: R = $CH_2CH{=}CH_2$

IIb: R = CH_2– 

R′ = $\overset{O}{\overset{\|}{C}}CF_2CF_2CF_3$

The derivatives appear as single, symmetrical peaks by GLC–ECD (Fig. 1) and are suitable for quantitation. Minimum detectable quantities correspond to about 40 pg of Ia and 70 pg of Ib injected on-column.

For quantitative studies, naloxone and naltrexone were used as internal standards for each other. Because of the differences in the ECD responses to IIa and IIb, 100 ng of naltrexone was used for naloxone (10–100 ng) determinations and 60 ng of naloxone was used for naltrexone (10–100 ng) determinations. Standard curves were obtained by plotting peak height ratios against the amounts of naloxone or naltrexone added to standard samples. Fig. 2 shows a typical standard curve for the determination of 10–100 ng of naloxone with 100 ng of naltrexone added as internal standard. All standard curves were linear with zero intercepts and were very reproducible. Precision was about 1.0% relative standard deviation at 100 ng of naloxone ($n = 8$) and 8.5% at 10 ng ($n = 5$). High ECD responses of derivatives and high precision of peak height ratios permit accurate quantitation of 2–100 ng of either naloxone or naltrexone.



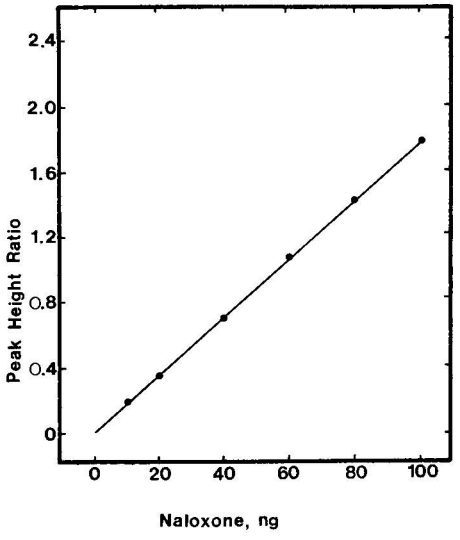


Fig. 1. Representative chromatogram of heptafluorobutyrate derivatives of (a) naloxone (100 ng) and (b) naltrexone (100 ng). Derivatization with HFBA catalyzed with pyridine. Column, glass (6 ft. × 2 mm), packed with 3% OV-17 on Gas-Chrom Q; column temperature, 205°; carrier gas, 5% methane in argon at a flow-rate of 40 ml/min.

Fig. 2. Calibration plot of 10–100 ng of naloxone assayed as its 3,6,14-HFB derivative. Internal standard, naltrexone-3,6,14-HFB.

Reaction conditions for submicrogram derivatization of naloxone and naltrexone to the corresponding tri-substituted heptafluorobutyryl derivatives, IIa and IIb, were investigated. The rate of formation of IIa was determined under the conditions described in Experimental with the exception that 9-bromophenanthrene was used as the internal standard instead of naltrexone. Reaction mixtures were heated at 70° for 3, 6, 9, 12, 15, 30, 60 and 120 min and then were quenched by decomposing the HFBA with sodium borate solution. The plot of peak height ratios (IIa/9-bromophenanthrene) against derivatization time (Fig. 3) indicates that derivatization is complete in about 1 h. The peak height ratio observed after heating for 16 h was identical with the ratio observed after heating for 1 h.

When pyridine was omitted from the HFBA derivatizations of naloxone and naltrexone, the major derivatives which were obtained exhibited longer retention times and lower ECD responses (Tables I and II) than the corresponding tri-substituted derivatives, IIa and IIb. Small quantities of IIa and IIb were also detected by GLC–ECD thus suggesting that derivatization is incomplete without the addition of pyridine.

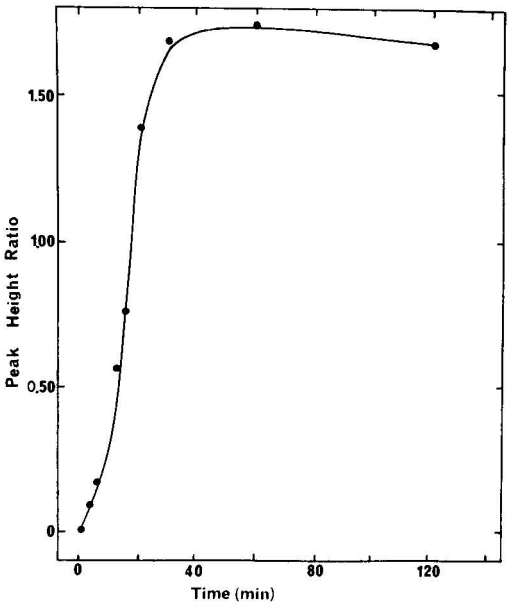


Fig. 3. Rate of formation of naloxone-3,6,14-HFB from 100 ng of naloxone and 25 μl HFBA in 50 μl benzene catalyzed by 1% pyridine at 70°.

A preliminary GLC–MS study indicated that the derivatives (IIIa and IIIb) result from esterification of the hydroxyl groups at C-3 and C-14.

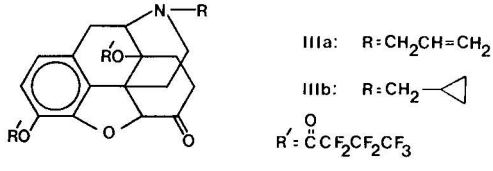


Fig. 4 shows a methane chemical ionization mass chromatogram of a mixture of naloxone and naltrexone derivatized with HFBA using pyridine catalyst and con-

TABLE I

RETENTION TIMES OF NALOXONE AND NALTREXONE DERIVATIVES RELATIVE TO 9-BROMOPHENANTHRENE

Separations were carried out on 3% OV-17 on Gas-Chrom Q at 205° with carrier gas flowing at 40 ml/min.

Compound	*Relative retention time*					
	Derivatization with pyridine			*Derivatization without pyridine*		
	TFAA	*PFPA*	*HFBA*	*TFAA*	*PFPA*	*HFBA*
Naloxone	0.898	0.658	0.680	0.898	1.45	1.46
Naltrexone	1.55	1.12	1.15	1.55	2.49	2.46

TABLE II

PEAK HEIGHTS AND PEAK AREAS OF NALOXONE AND NALTREXONE DERIVATIVES RELATIVE TO 9-BROMOPHENANTHRENE

Separations were carried out on 3% OV-17 on Gas-Chrom Q at 205° with carrier gas flowing at 40 ml/min.

Compound	*Peak height (peak area)*					
	Derivatization with pyridine			*Derivatization without pyridine*		
	TFAA	*PFPA*	*HFBA*	*TFAA*	*PFPA*	*HFBA*
Naloxone	2.89 (2.69)	2.59 (2.29)	2.81 (2.37)	2.89 (2.69)	1.42 (2.02)	1.03 (1.60)
Naltrexone	1.35 (1.95)	1.56 (1.87)	1.64 (2.03)	1.35 (1.95)	0.575 (1.43)	0.487 (1.14)

ditions so that partial derivatization of the molecules occurs. Mass spectra were obtained at the centroids of each of the peaks and background spectra were subtracted. The peaks in these spectra are listed in Table III. Peak 1 was found to correspond to naloxone-3,6,14-HFB. The only prominent ions in the mass spectrum have masses of 215, 197 and 169. These ions correspond to $[CF_3CF_2CF_2COOH \cdot H]^+$, $[CF_3CF_2CF_2CO]^+$, and $[CF_3CF_2CF_2]^+$, respectively. Since neither the molecular ion nor any fragments containing naloxone were observed, the structure could not be established from the MS data. However, confirmation of the structure was obtained by IR analysis. The mass spectrum obtained at peak 2 corresponds to naloxone-3,14-HFB. The spectrum shows a protonated molecular ion, $[M \cdot H]^+$, at 720 and significant fragment ions at 534, 506, 310, 215, 197, and 169. The ions at 534 and 506 result from loss of $CF_3CF_2CF_2COOH$ from $[M \cdot C_2H_5]^+$ and $[M \cdot H]^+$, respectively. The ion at 310 is due to loss of $[CF_3CF_2CF_2CO]^+$ from the ion at 506 to give a neutral molecule with a mass of 309. Protonation of this molecule gives an ion of mass 310. The ions at 215, 197, and 169 result from fragmentations similar to those observed with the naloxone triester. The mass spectrum of naltrexone-3,6,14-HFB was obtained at the centroid of peak 3. The spectrum exhibits ions of masses 732, 716, 520, 215, and 197. Although no molecular ion is observed, an ion of mass 716 corresponding to fragmentation of $CF_3CF_2CF_2COOH$ from the protonated molecular ion is observed thereby suggesting the identity of the compound. The ion of mass 520 results from loss of $[CF_3CF_2CF_2CO]^+$ from the ion of mass 716 to give a neutral molecule of mass 519. Protonation of this molecule results in an ion of mass 520. The ion of mass 732 is presently unexplained. The spectrum obtained at peak 4 shows that the peak corresponds to a mixture of two compounds, naloxone-3-HFB and naltrexone-3,14-HFB. The only ion which could be positively attributed to naloxone-3-HFB is the protonated molecular ion of mass 524. Ions in the spectrum having masses 734, 548, 520, 324, 215, and 169 result from the fragmentation of naltrexone-3,14-HFB. The protonated molecular ion at mass 734 confirms the identity of the compound. The ions of mass 548 and 520 originate from fragmentation of $CF_3CF_2CF_2COOH$ from $[M \cdot C_2H_5]^+$ and $[M \cdot H]^+$, respectively. Peak 5 is due to underivatized naloxone. The spectrum is characterized by a protonated molecular ion of mass 328. Peak 6 in the mass chroma-

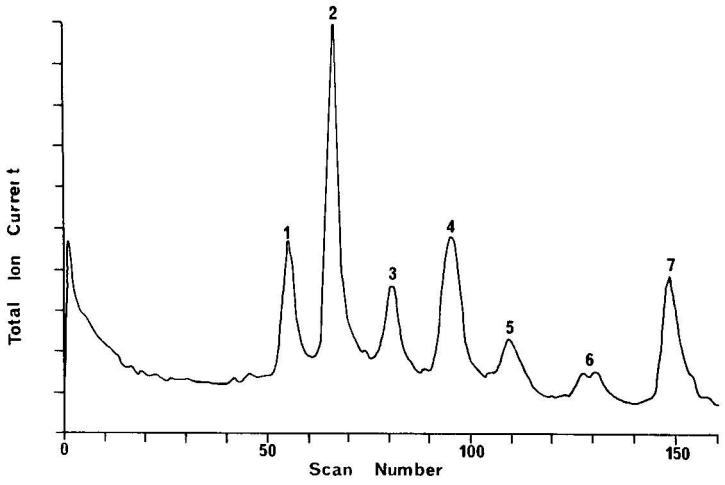


Fig. 4. Mass chromatogram of HFBA derivatives of naloxone and naltrexone: 1 = naloxone-3,6,14-HFB, 2 = naloxone-3,14-HFB, 3 = naltrexone-3,6,14-HFB, 4 = naltrexone-3,14-HFB and naloxone-3-HFB, 5 = naloxone, 6 = naltrexone-3-HFB, and 7 = naltrexone.

togram is due to naltrexone-3-HFB. The only significant ions in the mass spectrum of the monoester are found at masses 566, 538, and 342. Ions of masses 566 and 538 correspond to $[M \cdot C_2H_5]^+$ and $[M \cdot H]^+$, respectively, whereas the ion of mass 342 results from loss of $[CF_3CF_2CF_2CO]^+$ from the protonated molecular ion followed by protonation of the neutral molecule. Because the phenolic esters fragment with loss of $[RCO]^+$ whereas alcohols fragment with loss of $[RCOOH]$[15], the spectrum is attributed to naltrexone-3-HFB. Peak 7 is due to underivatized naltrexone. The spectrum exhibits ions of masses 370 and 342 corresponding to $[M \cdot C_2H_5]^+$ and $[M \cdot H]^+$, respectively.

Evidence that enol ester formation has not occurred at C-6 in the diesters IIIa and IIIb was obtained by comparison of the IR spectra of the diesters and triesters.

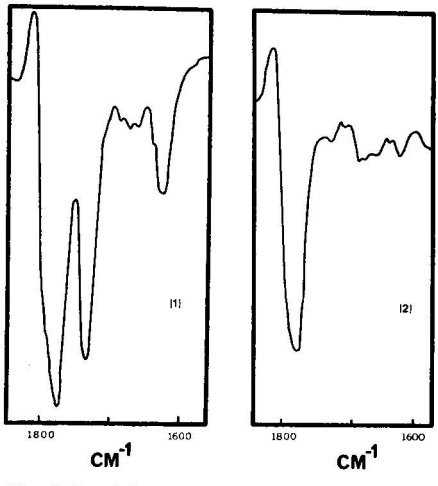


Fig. 5. Partial IR spectra of (1) naloxone-3,14-HFB (IIIa) and (2) naloxone-3,6,14-HFB (IIa).

TABLE III

METHANE CHEMICAL IONIZATION MASS SPECTRAL DATA OF THE MAJOR CHROMATOGRAPHIC PEAKS (FIG. 4) ON PARTIAL DERIVATIZATION OF NALOXONE AND NALTREXONE WITH HFBA

Peak	*m/e*	*Assignment**	*M Fragment*
1	215	$RCOOH \cdot H^+$	
	197	$RCOOH \cdot H^+ - H_2O$	
	169	R^+	
2	720	$M \cdot H^+$	M = Naloxone-3,14-HFB
	534	$M \cdot C_2H_5^+ - RCOOH$	
	506	$M \cdot H^+ - RCOOH$	
	310	$M \cdot H^+ - RCOOH - RCO^+ + H^+$	
	215	$RCOOH \cdot H^+$	
	197	$RCOOH \cdot H^+ - H_2O (= RCO^+)$	
	169	R^+	
3	732	Unknown	M = Naltrexone-3,6,14-HFB
	716	$M \cdot H^+ - RCOOH$	
	520	$M \cdot H^+ - RCOOH - RCO^+ + H^+$	
	215	$RCOOH \cdot H^+$	
	197	$RCOOH \cdot H^+ - H_2O (= RCO^+)$	
4	734	$M \cdot H^+$	M = Naltrexone-3,14-HFB
	548	$M \cdot C_2H_5^+ - RCOOH$	M′ = Naloxone-3-HFB
	524	$M' \cdot H^+$	
	520	$M \cdot H^+ - RCOOH$	
	324	$M \cdot H^+ - RCOOH - RCO^+ + H^+$	
	215	$RCOOH \cdot H^+$	
	169	R^+	
5	328	$M \cdot H^+$	M = Naloxone
6	566	$M \cdot C_2H_5^+$	M = Naltrexone-3-HFB
	538	$M \cdot H^+$	
	342	$M \cdot H^+ - RCO^+ + H^+$	
7	370	$M \cdot C_2H_5^+$	M = Naltrexone
	342	$M \cdot H^+$	

* $R = CF_2CF_2CF_3$.

The partial IR spectra (Fig. 5) contrast the carbonyl absorption regions of naloxone derivatives, IIa and IIIa. It is noted that IIIa exhibits strong ketone absorption at 1736 cm^{-1} whereas IIa lacks this absorption thereby illustrating the role of pyridine in catalyzing enol ester formation.

IIIa and IIIb have longer retention times and give lower ECD responses than the corresponding trisubstituted derivatives (IIa and IIb). Furthermore, it was difficult to prepare IIIa and IIIb without also forming small quantities of IIa and IIb. Thus, IIa and IIb prepared by pyridine catalyzed derivatization with HFBA were considered more suitable for quantitative studies.

The amount of pyridine required to catalyze the formation of IIa and IIb was determined by varying the concentration of pyridine from 0.1 to 5% in benzene. Derivatization was incomplete when 0.1% pyridine in benzene was used. With 5% pyridine in benzene, derivatization was rapid and complete but high ECD backgrounds resulted. Satisfactory chromatograms were obtained with 1% pyridine in benzene.

It was also found that derivatization with 25 μl of HFBA in 50 μl of benzene at 70° for 2 h resulted in the formation of IIa and IIb without the use of pyridine. This procedure, however, resulted in high ECD backgrounds and was therefore not pursued.

Naloxone and naltrexone were also derivatized with TFAA and PFPA both with and without pyridine as a catalyst. The relative retention times and relative response factors of the derivatives are summarized and compared with the HFBA derivatives in Tables I and II. Derivatization with HFBA and PFPA in the presence of pyridine yielded the triesters and in the absence of pyridine primarily the diesters. Reaction with TFAA in the presence or absence of pyridine resulted in the formation of the same derivative for each compound. Furthermore, it is noted that all of the derivatives exhibit comparable electron-capture responses.

Because perfluoroalkyl anhydrides are strong electron absorbers, they must be removed from samples before GLC–ECD or high backgrounds will result. Most investigators remove excess anhydride by evaporation under nitrogen. However, in the procedure described here, evaporation is precluded by the presence of pyridine which reacts with anhydrides to form non-volatile residues which swamp the electron-capture detector. Satisfactory cleanup with minimal decomposition of derivatives was accomplished by washing the reaction mixtures with sodium borate solution.

Since perfluoroalkyl esters are hydrolyzed under mild conditions, the stabilities of derivatives after decomposition of excess anhydride with sodium borate solution were investigated. It was found that naltrexone derivatives decompose more rapidly than the corresponding naloxone derivatives. As a consequence, peak height ratios change with time (Fig. 6). Specifically, IIb decomposed at 23° with a half-life of about 520 min compared to 784 min for IIa. Furthermore, HFB-derivatives are more stable than PFP-derivatives which are in turn much more stable than TFA-derivatives. This observation is consistent with the report of Änggård and Sedvall[16] on the comparative stabilities of several perfluoroalkyl esters of catecholamines. Thus, the rate of change of peak height ratios is slowest for HFB derivatives, faster for PFP derivatives, and fastest for TFA derivatives. Further study of the TFA derivatives was not

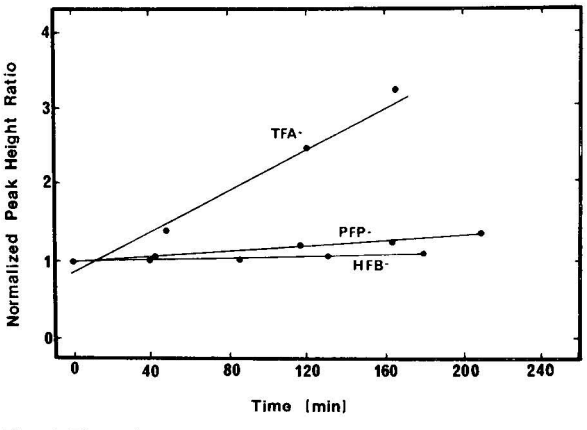


Fig. 6. Plot of normalized peak height ratios against time for naloxone/naltrexone derivatives prepared under pyridine-catalyzed conditions described in the text.

undertaken because of the substantially greater instability of these esters. Because of the time dependence of peak height ratios, excess anhydride was decomposed from each sample just before it was chromatographed. The procedure was carefully timed and samples were not allowed to stand after the anhydride had been decomposed but were chromatographed immediately. These precautions were necessary to obtain linear and reproducible calibration curves for quantitative studies.

It is postulated that the differences in stabilities of naloxone and naltrexone derivatives of the same type (*e.g.* IIa and IIb) are due to differences in basicities of the tertiary amine groups. Initially, it was assumed that the enol esters would be the least stable of the ester groups. However, the distance between the enol ester and the tertiary amine group is so great that differences in the basicities of the amine groups should have no effect on the relative stabilities of the enol esters. Furthermore, the peaks corresponding to the products of enol ester hydrolysis (IIIa and IIIb) do not increase with time as the parent compounds decompose. No hydrolysis products were detected by GLC–ECD. Therefore, it is postulated that the first step in the hydrolytic decomposition of the derivatives involves cleavage of the C-14 ester group as a result of intramolecular base catalysis (Fig. 7). Thus, the rate of hydrolysis increases as the basicity of the tertiary amine increases. Since naltrexone ($pK_a = 8.38$) is more basic than naloxone ($pK_a = 7.94$)[17], naltrexone derivatives are expected to hydrolyze more rapidly. This interpretation is in accord with the observed results. The products of hydrolysis are C-3,6-diesters possessing polar groups at C-14 and having greatly increased retention times.

Because narcotic antagonists such as naloxone and naltrexone are often administered with narcotic agonists, HFB derivatives of morphine, oxymorphone,

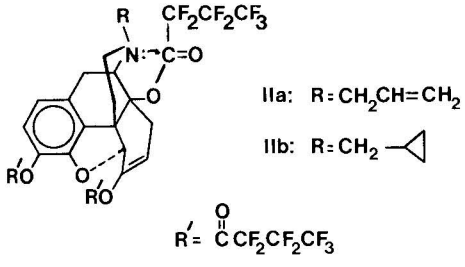


Fig. 7. 3,6,14-HFB esters of naloxone (IIa) and naltrexone (IIb).

TABLE IV

RETENTION TIMES OF DERIVATIVES RELATIVE TO RETENTION TIME OF 9-BROMOPHENANTHRENE

Derivatization catalyzed by pyridine. Separations were carried out on 3% OV-17 on Gas-Chrom Q at 205° with carrier gas flow at 40 ml/min.

Compound	*Relative retention time*		
	TFAA	*PFPA*	*HFBA*
Cyclazocine	—	0.368	0.387
Pentazocine	—	0.429	0.458
Oxymorphone	0.563	0.430	0.441
Morphine	—	1.10	1.13

cyclazocine, and pentazocine were prepared. Relative retention times of derivatives are shown in Table IV. It is noted that all derivatives except the morphine derivative are well resolved from the naloxone and naltrexone derivatives on OV-17.

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QUANTITATIVE DETERMINATION OF HEXOSAMINES IN GLYCOPROTEIN BY ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

An ion-exchange chromatographic method for the quantitative determination of glucosamine and galactosamine in glycoproteins is described. The hexosamines are completely separated from interfering peptides of the acid hydrolysate using a small column of cation exchanger in a manner described earlier by Boas. Chromatographic separation of the hexosamine fraction and the determination of glucosamine and galactosamine are accomplished on an amino acid analyzer with ninhydrin.

INTRODUCTION

During a study of the hexosamine content of κ-casein and potato glycoprotein in this laboratory, a method was required for quantitatively determining the hexosamines. While glucosamine and galactosamine are readily determined in the presence of each other by cation-exchange chromatography, this is limited to their presence unbound or in small peptides. The partial hydrolysis of glycoproteins, a condition for minimum loss of amino sugars, yields peptides with elution volumes in the chromatographic separation which overlap those of the hexosamines. This does not permit hexosamine determination by amino group reagents. Lee *et al.*[1] employed a neocuproine reducing sugar reagent with the column effluent to avoid interference by amino compounds. On the other hand, Fanger and Smyth[2] prefractionated the partial hydrolysate on an anion exchanger, eluting the amino sugars and lysine with a volatile buffer of N-ethylmorpholine. The hexosamines were finally determined with ninhydrin on a short amino acid analyzer column.

In this laboratory, limited success was experienced with the neocuproine method[1] because of some continuing interference by peptides, presumably by those containing hydroxy amino acids. An alternative method is reported here which utilizes a prefractionation of the hydrolysate as described by Boas[3], followed by chromatographic separation of the hexosamine fraction and determination with ninhydrin.

EXPERIMENTAL*

Columns and resins

Prefractionating columns consisted of 5–5.5 ml of Amberlite IR-120 (ref. 2) (200–400 mesh) resin packed in 140 × 9 mm I.D. nonjacketed glass columns. The resin was poured in segments. The columns were readied and regenerated after each sample according to the procedure of Boas[3]. Analytical chromatography was carried out in a water-jacketed 290 × 9 mm I.D. glass column packed in segments with Amberlite IR-120 resin to a height of 265 mm. Crushed resin, 25–30 μm particles, was sized by the Hamilton procedure[4].

Hexosamines and amino acids

D-Glucosamine hydrochloride and glycyl-L-leucyl-L-tyrosine were purchased from Mann Labs. (New York, N.Y., U.S.A.) and D-galactosamine hydrochloride from Pfanstiehl Labs. (Waukegan, Ill., U.S.A.). D-Mannosamine hydrochloride, L-serine, L-threonine, and L-tyrosine were obtained from Calbiochem (Los Angeles, Calif., U.S.A.).

Proteins

β-Lactoglobulin A and κ-casein were prepared by the Dairy Laboratory of the Eastern Regional Research Center. Sperm whale myoglobin was purchased from Schwarz/Mann (Orangeburg, N.Y., U.S.A.) and the iron removed by conventional procedure. A potato protein preparation was made in this laboratory from the expressed tuber juice by ammonium sulfate precipitation and dialysis in the usual manner.

Analyzer system

The modules and arrangement comprising an amino acid analyzer were used. A Milton Roy piston pump delivered the eluting buffer and another the ninhydrin reagent. When neocuproine was the developing reagent, the modules included a Technicon proportioning pump in lieu of the second piston pump and a 95° reaction bath assembled and employed in the manner described by Lee *et al.*[1]. The colored ninhydrin reaction products were measured with two Technicon colorimeters, using a 570-nm and a 450-nm filter with 15-mm flow cells. The 450-nm colorimeter was employed in the neocuproine assembly. The results were plotted on a 12-in. multiple-point recorder.

Buffers and reagents

Sodium citrate buffer, 0.35 *N*, was prepared from Baker's reagent grade salt adjusted to pH 5.28 $\pm$ 0.02 (ref. 5). The preservative was 0.1 ml octanoic acid per liter of buffer. Ninhydrin reagent, procured from Pierce (Rockford, Ill., U.S.A.), was prepared according to the procedure of Spackman *et al.*[5]. Neocuproine hydrochloride was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and the reagent was prepared as described for procedure II of the publication of Lee *et al.*[1].

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Hydrolysis of protein

Proteins and glycoproteins (2.6–10 mg) were hydrolyzed with 3–6 ml of 4 *N* HCl for 4 h in a 100° water-bath in evacuated sealed tubes. Hydrolysates were taken to dryness at 40° with a rotary evaporator until free of mineral acid.

Prefractionation

The residues from hydrolysis were dissolved in approximately 2 ml distilled water and passed through the small Amberlite IR-120 (H^+) prefractionating column. The column was washed with 15 ml distilled water (removing unabsorbed sugars, acidic amino acids, and peptides), and eluted with 9.5–18 ml 2 *N* HCl. The column was further eluted with 9–10 ml 4 *N* HCl. When studying the ion-exchange retention of hydrolytic fragments of β-lactoglobulin A, the column was water washed and further eluted with 4 *N* NH_4OH. All eluates were concentrated separately on the rotary evaporator at 40° to dryness until free of the eluting agent.

Analysis

Each of the residues was dissolved in distilled water and applied to the analyzer column either in its entirety or as a volumetric aliquot. With the ninhydrin method, samples in volumes as high as 0.350 ml were examined on the analytical column. The samples were washed into the column with three 0.020-ml aliquots of the eluting buffer. Elution of the column was carried out at 55° and at a flow-rate of 60.0 ml/h with 0.35 *N*, pH 5.28 ± 0.02 citrate buffer. Ninhydrin reagent was pumped at the rate of 20.9 ml/h with the second piston pump. Ninhydrin flow was initiated 15 min after the start of column elution. When the reagent was neocuproine, the module arrangement and flow-rates described under procedure II of Lee *et al.*[1] were employed. Peak areas were determined as with automatic amino acid analyzers, whereby the area is determined from half the peak height multiplied by the peak width.

RESULTS AND DISCUSSION

With the column length employed in this study, glucosamine and galactosamine were satisfactorily separated on the analyzer with maximum peak heights at 62.5 and 70 min, respectively. Separations carried out with stepwise lengthened analyzer columns indicated that a resin column of 300 mm would permit a complete return to the baseline between the two hexosamine peaks. This, however, was not essential to the quantitation.

Recovery determinations were made on standard binary solutions of the hexosamines at two levels differing by a factor of ten (Table I). No hexosamine was found in the water wash from the prefractionating column. After elution of the column with 9.5 ml of 2 *N* HCl, a small amount of residual hexosamines could still be recovered with further elution using 4 *N* HCl. However, 16 ml of 2 *N* HCl fully eluted the tenfold hexosamine sample. Since the sample not pretreated showed a ± deviation from 100% recovery, the 90–96% recovery totals of those that had undergone column pretreatment indicate that some losses had probably occurred in the manipulations. Greater care was taken in subsequent operations.

Both β-lactoglobulin A and sperm whale apomyoglobin are proteins devoid of carbohydrates and amino sugars. These two proteins were utilized to study the

TABLE I

DETERMINATION OF HEXOSAMINES IN STANDARD BINARY SOLUTIONS

The concentrations of Experiments 1 and 2 are tenfold dilutions of Experiment 3. Experiment 3 represents a mixture containing 0.1172 μM galactosamine and 0.0580 μM glucosamine in 100 μl of 0.01 M HCl.

Experiment	*Pretreatment*	*% Recovered*	
		Glucosamine	*Galactosamine*
1	None	94.2	103.7
2	IR-120; 9.5 ml 2 N HCl	92.8	87.5
2 wash	9 ml 4 N HCl	3.1	3.8
	Σ 2 + 2 wash	95.9	91.3
3	IR-120; 16 ml 2 N HCl	92	89.6
3 wash	10 ml 4 N HCl	0	0
	Σ 3 + 3 wash	92	89.6

effects of the prefractionating column on the removal of peptides with elution volumes overlapping those of glucosamine and galactosamine. The results of elution of a mild acid hydrolysate of β-lactoglobulin A using 2 N HCl followed by 4 N HCl and ultimately stripping the column with 4 N NH_4OH are shown in Fig. 1. Without pre-

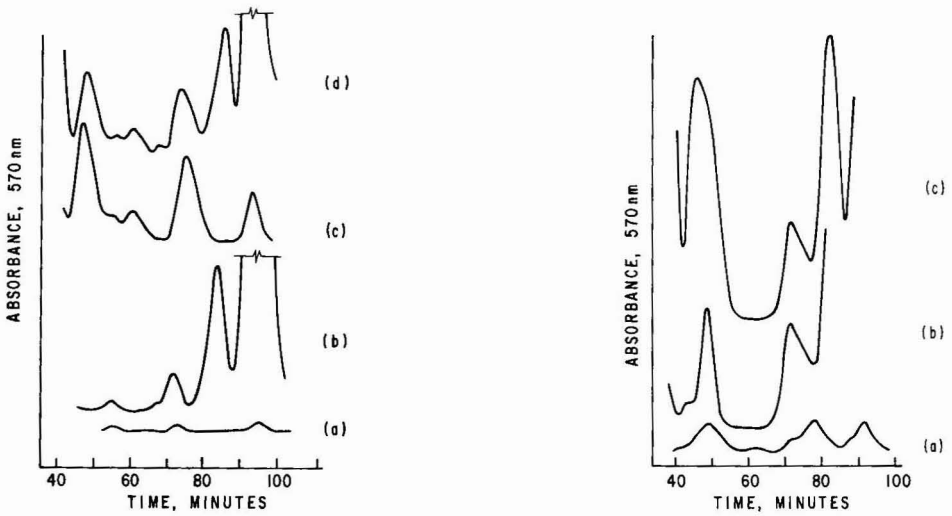

Fig. 1. Chromatography of a partial hydrolysate (4 N HCl, 4 h, 100°) of β-lactoglobulin A on a 265 × 9 mm I.D. Amberlite IR-120 analytical column, 55°, pH 5.28, 0.35 N sodium citrate buffer. Ninhydrin reagent. (a) Partial hydrolysate eluted from the prefractionating IR-120 column with 2 N HCl. (b) Fraction from the continued elution of the prefractionating column (a) with 4 N HCl. (c) Fraction from the continued elution of (b) with 4 N NH_4OH. (d) Partial hydrolysate chromatographed directly on the analytical column.

Fig. 2. Chromatography of a partial hydrolysate (4 N HCl, 4 h, 100°) of sperm whale apomyoglobin on a 265 × 9 mm I.D. Amberlite IR-120 analytical column, 55°, pH 5.28, 0.35 N sodium citrate buffer. Ninhydrin reagent. (a) Partial hydrolysate eluted from the prefractionating IR-120 column with 2 N HCl. (b) Fraction from the continued elution of the prefractionating column (a) with 4 N HCl. (c) Partial hydrolysate chromatographed directly on the analytical column.

treatment of the hydrolysate before analytical chromatography, ninhydrin-positive material is especially evident partially overlapping the galactosamine region (70 min). Only a very small portion of this peak is eluted with 2 *N* HCl, while 4 *N* acid removes an additional amount plus some interfering material in the glucosamine region of the chromatogram. Further amounts of interfering peptides are obtained from the pre-fractionating column with NH_4OH. Sperm whale apomyoglobin yielded a similar elution pattern (Fig. 2).

When the two hexosamines were added to the β-lactoglobulin A hydrolysate and pretreated on the IR-120 column, the 2 *N* HCl eluate yielded a good separation pattern and constant baseline. A 100% recovery of both amino sugars was obtained with 15 ml of 2 *N* acid (Table II). Similar results were obtained with the hexosamines added to sperm whale apomyoglobin with a 98% recovery of both compounds using 18 ml of 2 *N* acid (Fig. 3; Table III). The material eluted between 64 and 80 min with 4 *N* HCl, partially overlapping the galactosamine position, represents peptide material indigenous to the myoglobin hydrolysate.

The glycoprotein, κ-casein, was examined for its hexosamine content using the neocuproine procedure as described by Lee *et al.*[1]. With no pretreatment of the hydrolysate, a steeply changing baseline was observed in the hexosamine region of the chromatogram (Fig. 4), interfering with an accurate determination of their peak areas. It also raised doubts as to the complete composition of the peaks regarded as

TABLE II

DETERMINATION OF HEXOSAMINES ADDED TO β-LACTOGLOBULIN A

Experiments 1 and 2 represent 3 mg partially hydrolyzed β-lactoglobulin A to which was added 0.1172 μ*M* galactosamine and 0.0580 μ*M* glucosamine to give a total volume of 1.03 ml.

Experiment	*Pretreatment*	*% Recovered*	
		Glucosamine	*Galactosamine*
1	IR-120; 12 ml 2 *N* HCl	89.2	86.0
1 wash	10 ml 4 *N* HCl	11.5	14.0
	Σ 1 + 1 wash	100.7	100
2	IR-120; 15 ml 2 *N* HCl	100	101.7
2 wash	10 ml 4 *N* HCl	0	trace
	Σ 2 + 2 wash	100	101.7

TABLE III

DETERMINATION OF HEXOSAMINES ADDED TO MYOGLOBIN

Experiments 1 and 2 represent 4 mg partially hydrolyzed sperm whale apomyoglobin to which was added 0.1172 μ*M* galactosamine and 0.0580 μ*M* glucosamine to give a total volume of 0.430 ml.

Experiment	*Pretreatment*	*% Recovered*	
		Glucosamine	*Galactosamine*
1	IR-120; 16 ml 2 *N* HCl	86.5	90.7
1 wash	11 ml 4 *N* HCl	6.1	< 1
	Σ 1 + 1 wash	92.6	91
2	IR-120; 18 ml 2 *N* HCl	98.1	97.6
2 wash	10 ml 4 *N* HCl	0	0
	Σ 2 + 2 wash	98.1	97.6

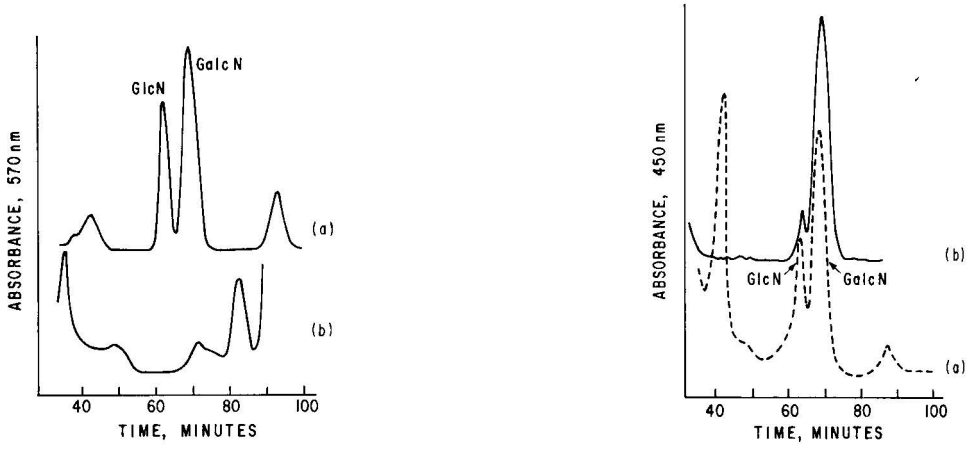

Fig. 3. Chromatography of glucosamine and galactosamine added to a partial hydrolysate (4 *N* HCl, 4 h, 100°) of sperm whale apomyoglobin on a 265 × 9 mm I.D. Amberlite IR-120 analytical column, 55°, pH 5.28, 0.35 *N* sodium citrate buffer. Ninhydrin reagent. (a) Fraction eluted from the prefractionating IR-120 column with 2 *N* HCl. (b) Fraction from the continued elution of the prefractionating column with 4 *N* HCl.

Fig. 4. Chromatography of a partial hydrolysate (4 *N* HCl, 4 h, 100°) of κ-casein on 265 × 9 mm I.D. Amberlite IR-120 analytical column, 55°, pH 5.28, 0.35 *N* sodium citrate buffer. Neocuproine reagent. (a) Direct chromatography of the partial hydrolysate on the analytical column. (b) Partial hydrolysate eluted from the prefractionating IR-120 column with 2 *N* HCl.

hexosamines. An improved separation was obtained when the hydrolysate was pretreated with the Boas-type column and the 2 *N* HCl eluate examined on the analytical column (Fig. 4). Hexosamine values for both κ-casein and a potato protein preparation obtained with the neocuproine reagent were in excellent agreement with those obtained with the ninhydrin reagent following the same column pretreatment and chromatography (Table IV).

TABLE IV

COMPARISON OF NEOCUPROINE AND NINHYDRIN REAGENTS IN DETERMINING GLYCOPROTEIN HEXOSAMINE

	*Neocuproine**		*Ninhydrin**	
	% Glucosamine	*% Galactosamine*	*% Glucosamine*	*% Galactosamine*
Potato protein	0.228	—	0.249	—
κ-Casein	0.044	0.579	0.053	0.533

* Mean value of two determinations.

The use of 18 ml of 2 *N* HCl has proven to be most conducive to complete removal of the two hexosamines from prefractionating columns reported in this study. As much as 15 mg of caseins (1% hexosamine)[6] and as little as 1 mg of purified milk fat globule membrane protein (6% hexosamine)[7] have been partially hydrolyzed and successfully pretreated for hexosamine recovery from these columns.

Column effluents of serine, threonine, tyrosine, and glycylleucyltyrosine were found to produce peaks on the analyzer with the neocuproine reagent, although none had elution volumes interfering with the determination of glucosamine and galactosamine. However, it would not be incongruous to expect other peptides containing hydroxyamino acids to respond similarly with neocuproine and cause some interference in the determination of the hexosamines.

In this analytical system, mannosamine and galactosamine were found to have identical elution volumes. However, mannosamine has rarely been encountered as a constituent of glycoproteins.

During the course of this study, four commercially purchased samples of D-galactosamine regarded as chromatographically pure by their suppliers were found to be contaminated with glucosamine. These samples had been prepared from natural sources and the impurity varied from 5.0–16.5%. The synthetic preparation of galactosamine utilized in this study was found to be devoid of glucosamine.

Treatment of acid hydrolysates of glycoproteins was recommended by Boas[3] for improvement of the Elson–Morgan method for determining hexosamine. Although Boas had designed the modification in order to eliminate sugar–amine interactions responsible for abnormally high color values, this study finds it valuable in the ion-exchange chromatographic method for hexosamines. It improves the results obtained with neocuproine and permits the use of amino group reagents such as ninhydrin. Thereby, the final analytical step can be carried out directly on an amino acid analyzer.

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Note

A simplified numerical method for correction of polydispersities from gel permeation chromatography

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The problem of instrumental spreading in gel permeation chromatography has been considered in detail in recent years (*e.g.*, ref. 1).

The mathematical expression relating the experimental chromatogram f(v), the true chromatogram w(y) and the function g(v,y) describing the instrumental spreading can be represented by the following equation[2]:

$$f(v) = \int_{-\infty}^{\infty} g(v,y)\, w(y)\, dy \tag{1}$$

where v and y both represent elution volume.

In this discussion we shall assume that g(v,y) is a simple Gaussian distribution:

$$g(v,y) = \frac{h}{\sqrt{\pi}} \exp\left[-h^2 (v - y)^2\right] \tag{2}$$

where h is a parameter describing the width of the spreading. The calibration of instrumental spreading is reduced to the determination of the parameter h in eqn. 2, which can be accomplished by means of the reversed-flow technique[3].

In the theory of integral equations, eqn. 1 is called a "Fredholm equation of the first kind," and its solution can be obtained by expanding the functions that appear in it into Fourier series[4].

It is known that a system of Hermite polynomials, $H_n(t)$, is complete and orthonormal with $\exp(-t^2)$ in $(-\infty, \infty)$. From the physical sense of the function w(y), it follows that

$$\int_{-\infty}^{\infty} y \exp(-y^2)\, w^2(y)\, dy < \infty \tag{3}$$

Therefore, the Fourier series of the function w(y)

$$w(y) = \sum_{n=0}^{\infty} w_n H_n(y) \qquad y \in (-\infty,\infty) \tag{4}$$

where

$$w_n = \frac{1}{2^n n! \sqrt{\pi}} \int_{-\infty}^{\infty} w(y) \exp(-y^2) H_n(y)\, dy \tag{5}$$

converges to it in $L_2(-\infty, \infty)$.

Substituting eqns. 2 and 4 into eqn. 1, we obtain

$$f(v) = \sum_{n=0}^{\infty} w_n \frac{h}{\sqrt{\pi}} \int_{-\infty}^{\infty} \exp[-h^2 (v - y)^2] H_n(y)\, dy \tag{6}$$

Let

$$b_n(v) = \int_{-\infty}^{\infty} \exp[-h^2 (v - y)^2] H_n(y)\, dy \tag{7}$$

then eqn. 6 can be rewritten in the form

$$f(v) = \frac{h}{\sqrt{\pi}} \sum_{n=0}^{\infty} w_n b_n(v) \tag{8}$$

It can be shown that the solution of eqn. 1 is unique[5]. The function $f(v)$ is given in tabular form (experimental points) $\{f(v_i), (v_i); i = 0,1,2,3,\ldots, N\}$.

For any point, it is possible to calculate $b_n(v_i)$.

In numerical calculations, the infinite series in eqns. 4 and 8 are replaced by finite partial sums. Thus, eqn. 8 can be rewritten as follows:

$$f(v_i) = \frac{h}{\sqrt{\pi}} \sum_{n=0}^{N} w_n b_n(v_i); \qquad i = 0, 1, 2, \ldots, N \tag{9}$$

If $\det|b_n(v_i)| \neq 0$, then it is possible to determine the coefficients w_n by solving eqn. 9 and consequently the function $w(y)$, given by eqn. 4.

To test the method proposed in this paper, we have performed numerical calculations. There remains the pure mathematical problem of proving how far the inversion used affects the obtained form of $w(y)$.

For this purpose, we accepted the function[6]

$$w(y) = \frac{0.325}{\sqrt{\pi}} \{0.6 \exp[-(0.325)^2 (y - 25)^2] + 0.4 \exp[-(0.325)^2 (y - 31)^2] \tag{10}$$

with the same parameters as in Tung's paper[6]. In the Gaussian function $g(v,y)$, we accepted the constant value of $h = 0.4$. Using these functions, we evaluated numerically the function $f(v)$. Next, we chose 25 "theoretical" values for $f(v_i)$, $i = 0,1,2, \ldots, 24$. Using these values, we reproduced the function $w(y)$ according to the method described above.

Fig. 1 shows the results of our calculations. The solid line represents the function $w(y)$ from eqn. 10 and the dashed line the reproduced function $w(y)$. It can be concluded that the numerical method used reproduced the form of the function $w(y)$ well.

The method of calculation is very simple and fast, and has been reduced to the following operations:

(1) computation of the $(N + 1)$ integrals (eqn. 7);

(2) solution of the linear system (eqn. 9);

(3) calculation of the sum in eqn. 4.

It should be stressed that in the above calculations all of the integrals from eqn. 7 can be fully evaluated analytically.

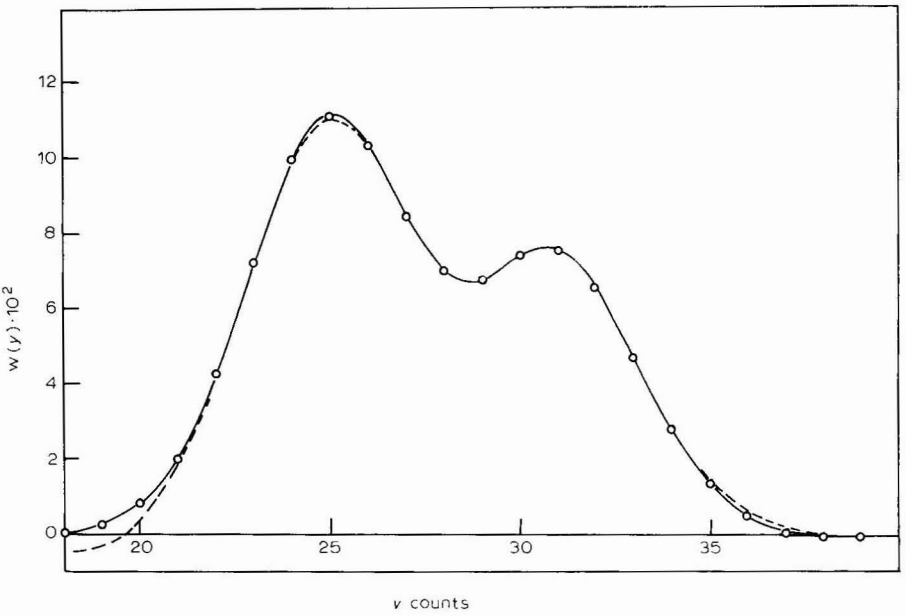

Fig. 1. Relationship between assumed chromatogram w(y) (solid line) and reproduced chromatogram (dashed line).

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Note

A simple combination of R_F value and melting-point determination for the identification of barbiturates

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Thin-layer chromatography is used extensively for drug screening either for drugs of abuse or to detect cases of poisoning.

Chromatographers are well aware that one single R_F value cannot be considered conclusive evidence for the presence or absence of a substance and the use of a wide range of confirmatory techniques, such as gas chromatography of the substance eluted from the spots, infrared or ultraviolet spectra, or even gas chromatography–mass spectrometry, has been proposed.

The following interesting method was recently suggested by Berezkin *et al.*[1]. A compound to be identified is partitioned between two immiscible solvents and both solutions are injected into a gas chromatograph. By using the retention time and the ratio of the two peaks (which indicates the partition coefficient between the two solvents), it is possible to identify it or distinguish it from others.

Thus the identification of a compound does not present problems if suitable instrumental techniques (and technicians) are available. It does, however, present a problem in a small hospital laboratory or similar unit that is not equipped with modern instruments.

A simple technique is reported here, which has been developed as a possible aid in the identification of barbiturates in urine and which seems to be of general application.

It is based on the determination of the melting point of the substance after separating it on a thin layer. This procedure was proposed earlier by Martinek[2], who eluted the substance from the adsorbent and recrystallized it under a microscope, thus employing microchemical techniques and skills that cannot be expected to be within the competence of a laboratory technician. The method described below is simpler in that it is based on a simple sublimation procedure[3,4]. The only remarkable feature of this technique is that it can be effected with the small amounts (several micrograms) that are available in spots on thin layers.

EXPERIMENTAL

Materials

The following pre-coated thin-layer plates were employed: silica gel 60 F_{254} (E. Merck, Darmstadt, G.F.R.), and Polygram Cell 300 DEAE, Polygram Cell 400

UV_{254} and Polygram Ionex-25 SB-Ac, which were obtained from Macherey, Nagel & Co. (Düren, G.F.R.). Desaga development jars, 26 × 11 × 20 cm, were used. The following reagents were prepared: 0.05% aqueous potassium permanganate; 0.2% diphenylcarbazone in 95% ethanol and 2% mercury(II) chloride in 95% ethanol, equal volumes of which were mixed before use.

All chromatograms were developed with the solvent chloroform–isopropanol–25% ammonia (45:45:10).

The various barbiturates were pharmaceutical products as obtained from the manufactures, but in some instances were extracted from tablets. Their origin and proprietary names are listed in Table I.

TABLE I

R_F VALUES FOR AND SYNONYMS OF A RANGE OF COMMONLY USED BARBITURATES (R_F VALUES ARE RELATIVE TO PENTOBARBITAL = 1)

Solvent: chloroform–isopropanol–25% ammonia (45:45:10).

Barbituric acid	*Commercial name*	*Proprietary name*	*Pre-coated silica gel G 60 F_{254}*	*Polygram Cell 300 DEAE*	*Polygram Cell 400 UV_{254}*	*Polygram Ionex-25 SB-Ac*
5-Phenyl-5-ethyl	Phenobarbital	Luminal	0.53	0.35	0.69	0
5,5-Diethyl	Barbital	Veronal	0.73	0.52	0.70	0.3
5,5-Diallyl	Diallylbarbital	Dial	0.76	0.62	0.77	0.3
5-(1-Cyclohexenyl)-5-ethyl	Cyclobarbital	Phanodorm	0.80	0.69	0.90	0.54
5-(1-Cycloheptenyl)-5-ethyl	Heptabarbital	Medomin	0.88	0.75	0.95	0.70
5-Allyl-5-isobutyl	Itobarbital	Sandoptal	0.91	0.80	0.93	0.70
5-Ethyl-5-(3-methylbutyl)	Amobarbital	Amytal	0.96	0.92	1	1
5-Allyl-5-(1-methylbutyl)	Secobarbital	Seconal	1	1	1	1
5-Ethyl-5-(2-pentyl)	Pentobarbital	Nembutal	1	1	1	1
5-Methyl-5-(1-cyclohexenyl)-N-methyl	Hexobarbital	Evipan	1.03	1.03	1	1.20

Chromatographic development

The chromatography chambers were lined with filter paper and 100 ml of the solvent mixture placed in a chamber, which was closed and allowed to equilibrate for 1 h. All chromatograms were developed at a room temperature of 22–25°.

With the Cell 300 DEAE and Cell 400 UV_{254} layers the samples to be chromatographed have to be placed behind the liquid front, that is, the thin layer is allowed to develop up to a distance of 3.5–4 and 1.5 cm, respectively, and only then are the samples applied to it. All four thin layers are then allowed to develop in the same jar simultaneously for 1 h and 15 min. The R_F values obtained are shown in Table I. We used the following five standard mixtures of barbiturates in solution in diethyl ether at a concentration of 1 mg/ml for each barbiturate: phenobarbital–itobarbital, barbital–cyclobarbital–amobarbital, diallybarbital–secobarbital, cyclobarbital–pentobarbital and heptabarbital–hexobarbital.

The volumes applied to the thin layers were as follows; for the silica gel layer, 10 μl; Cell 300 DEAE layer, 2.5 μl; and Cell 400 and Ionex-25 SB-Ac layers, 5 μl. The samples to be analysed were placed on the layer as follows: one (containing about the same amount of barbiturates as the standards) to each layer except on the silica gel which four spots were placed side by side, two of 10 μl and two of 40 μl (in the order 10, 40, 40 and 10 μl).

Preparation of spiked urine samples

Urine samples (50 ml) were spiked with 0.5 ml of an aqueous solution containing 0.5 mg of a barbiturate (if necessary 0.1 *N* sodium hydroxide was used to dissolve the barbiturates that are insoluble in water). The spiked urine samples were then extracted according to the method of Berry and Grove[5]. The urine was acidified to pH 1–2 with 1 *N* sulphuric acid and extracted with an equal volume of chloroform by shaking the mixture for several minutes in a Vortex shaker or for 10 min by hand. After centrifugation, the chloroform layer was filtered through a porous glass filter G 4 and evaporated to dryness under vacuum. The residue was then dissolved in 0.4 ml of diethyl ether.

Detection of spots after development

The thin layers, while still moist with the developing solvent, are viewed under an ultraviolet lamp at 254 nm and the spots marked with a pencil. As the eluent contains ammonia the spots are visible even on the DEAE-cellulose thin layers, although rather weakly, and on the Ionex layer (without a fluorescence indicator). The chromatograms are then allowed to dry in an oven at 60° until all the ammonia has volatilised.

The Cell 400, Ionex and Cell 300 DEAE layers are then sprayed with the $HgCl_2$–diphenylcarbazone reagent. The barbiturates appear as rather weak rose-coloured spots on a violet background, which become a bright rose colour on an almost white background on heating the layers in an oven at 80° for about 10 min or exposing them to sunlight. The chromatograms on the silica gel layer are divided into zone A containing a 10-μl spot, zone B the two 40-μl spots and zone C the other 10-μl spot. Zones A and C are sprayed with $HgCl_2$–diphenylcarbazone and permanganate reagents, respectively, as described by Lehmann and Karamustafaoglu[6]. While all barbiturates react with the former reagent, only those with double bonds give spots with the permanganate reagent (*i.e.* diallylbarbital, cyclobarbital, heptabarbital, itobarbital, secobarbital and hexobarbital).

Determination of melting points after chromatography

After development, there are two chromatograms for 40 μl of sample on the silica gel layer (in zone B, in which the spots visible under UV light were also outlined). Some of these spots also yielded positive reactions with the $HgCl_2$–diphenylcarbazone reagent on the adjacent 10-μl samples. These zones corresponding to positive reactions are scraped off from one of the chromatograms (the other being kept as a reserve) and the silica gel containing the spot is inserted into a micro-column, 2.5 mm I.D. and about 40 mm long, drawn out to a fine tip and with a small wad of cotton wool (previously washed with chloroform) at its lower end as described by Martinek[2]. The substance is eluted from the silica gel with chloroform (diethyl ether was also

TABLE II

MELTING POINTS DETERMINED FOR BARBITURATES

Barbiturate	*Melting point (°)*	
Phenobarbital	174	(by sublimation the low-melting form is obtained)
Barbital	176	(by sublimation the low-melting form is obtained. Transition to the higher-melting form (183°) is sometimes observed during heating)
Diallylbarbital	173	
Cyclobarbital	166–173	
Heptabarbital	174	
Itobarbital	138–139	(Merck Index)
Amobarbital	157	
Pentobarbital	129	
Hexobarbital	146	
Secobarbital	–*	

* The melting point of secobarbital (98–100°) was not obtained because no crystals suitable for a melting-point determination were formed. Secobarbital was therefore identified by applying the reagent of Wagenaar, as described by Davis[7], to the residue on the slide; oily droplets were formed, which subsequently became rosette-shaped crystal agglomerates.

tried but was less satisfactory) and the eluate evaporated to dryness on a microscope slide. The slide is then placed on a Kofler bank and a cover slip (supported on both sides by two other cover slips) placed over the residue so as to collect the sublimate that is formed, the cover slip being held a fraction of a millimetre above the residue. By slowly applying heat one can see whether a sublimate forms. If so, the cover slip is placed in a Kofler melting-point microscope and the melting point of the sublimate recorded.

RESULTS

We carried out melting-point determinations as described above with all the pure samples and with two spiked urine samples (Table II). For the pure samples we used the five mixtures of barbiturates described under Experimental and placed sufficient solution on the silica gel plates to give 20 μg of each barbiturate, which seems to be the smallest amount from which one can still obtain sufficient sublimate to enable its melting point to be determined.

The two spiked samples of urine contained: (a) a mixture of barbital, cyclobarbital and amobarbital and (b) secobarbital. The extraction of the acidified urine with chloroform was, of course, found to be only partial and consequently there is always much less material available than with the corresponding standard solution. It was for this reason that 40-μl samples were used instead of the 20 μl that suffice for the standards.

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CHROM. 9259

Note

Liquid chromatography and thin-layer chromatography of some substituted ureas

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A great deal of attention has been paid to the chromatographic behavior of urea-type compounds[1–4]. The main reasons for this interest are that these compounds constitute a widespread group of agricultural chemicals, they are of biochemical interest, and the group of compounds consists of a great number of structurally closely related compounds for which it is difficult to use classical analytical procedures for identification and quantitative assay. Traditional wet methods very often incur interferences; gas chromatography is amenable but subjects these compounds to possible decomposition. High-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC) techniques offer a rapid means of analysis for a large spectrum of substituted urea compounds and require a minimum of sample preparation.

EXPERIMENTAL

A Waters Model ALC 202/401 liquid chromatograph with an M6000 high-pressure pump, a U6K 2-ml loop injector, and a UV detector operating at a wavelength of 254 nm were used. A Hewlett-Packard Model 7130A recorder operated at 0.25 in/min and a Varian Model 620L 18K computer for integration were also employed. Chromatographic separation was effected using a stainless-steel column 1.3 m × 2 mm I.D. packed with octadecyltrichlorosilane (C_{18}) on Bondapak (37–50 μm grain size). Materials were obtained from Waters Assoc. (Framingham, Mass., U.S.A.). The mobile phase eluent was initially adjusted to 20% water and 80% acetonitrile, the polarity of which was adjusted by gradient elution for a 20-min period with a concave No. 7 curve to 90% water and 10% acetonitrile. A No. 7 curve is common to a Waters Model ALC 202/401 liquid chromatograph gradient elution curve selector. However, separation can be achieved with adequate success by eluting linearly for 20 min from 0–100% with water–acetonitrile (20:80). The column pressure was 2000 p.s.i. and the flow-rate 3 ml/min. The linear velocity was 2.8 cm/sec. A constant volume of 3 μl was injected by the stop-flow technique. The acetonitrile was Burdick and Jackson (Muskegon, Mich., U.S.A.) distilled-in-glass grade. Standards were obtained from CIBA-GEIGY (McIntosh, Ala., U.S.A.). Verification of purity was conducted by proton magnetic resonance and mass spectroscopy.

Thin-layer chromatography (TLC) was performed with silica gel G, neutral

(50% Merck silica gel–50% Bio-Rad Bio-Sil A) on 200 × 200 mm glass plates coated to a thickness of 200 μm and activated at 100° for 1 h. Solutions of chloroform–nitromethane (80:20) or benzene–chloroform–ethyl acetate (40:40:20) were used ás a mobile phase. Solvents were Fisher reagent grade (Atlanta, Ga., U.S.A.). After elution the plates were air dried and then exposed to chlorine in a saturated chamber for 30 sec. The plates were then air dried for 2 min and sprayed with potassium iodide–starch solution.

RESULTS AND DISCUSSION

Fig. 1 shows the separation achieved with Bondapak/C_{18} on a typical sample. Resolution is similar to that from gas–liquid chromatograms. The linearity response

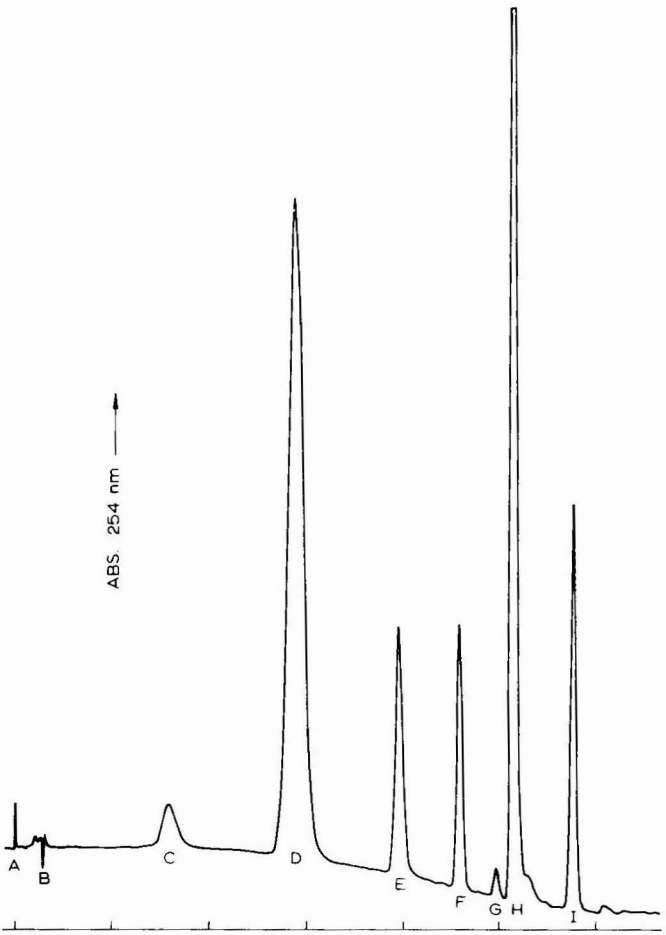

Fig. 1. Resolution of selected ureas. A = injection point; B = solvent front; C = 1,1-dimethyl-3-(1,1,1-trifluoro-*o*-tolyl)urea; D = 1,1-dimethyl-3-(1,1,1-trifluoro-*m*-tolyl)urea; E = 1,1-dimethyl-5-(1,1,1-trifluoro-*m*-tolyl)urea; F = 1,3-bis(1,1,1-trifluoro-*o*-tolyl)urea; G = impurity related to H; H = 1,3-bis(1,1,1-trifluoro-*m*-tolyl)urea; I = dimethylphthalate internal standard. Concentration adjusted to 8 mg/ml for sample components and 2 mg/ml internal standard. See text for experimental conditions. R_t/sec: C = 390; D = 690; E = 960; F = 1110; H = 1245; I = 1395.

of the UV detector to increasing concentrations is seen to be quite good. Substitution of the water–acetonitrile mobile phase by water–ethyl acetate–isopropanol (40:10:50) did not yield equivalent resolution. A water–methanol mobile phase did not allow separation of the bis compounds. Increasing the concentration of water tended to cause earlier elution of the compounds and loss of resolution. Increasing the acetonitrile concentration caused a corresponding increase in retention time for all compounds investigated.

Under the conditions specified, the number of theoretical plates was 2000. The detection sensitivity limits for compounds investigated were 50–300 ng by UV detection at 254 nm. Because the maximum absorbances of the individual ureas assayed are not always coincident with this wavelength, it is probable that an increase in sensitivity would occur by using a variable-wavelength spectrophotometer.

Dimethylphthalate was found to be useful as an internal standard. Areas determined by the Varian Model 620L 18K computer had a coefficient of variance of 0.51%. Response factor reproducibility was 0.82% relative standard deviation of the mean at 2 σ. Analyses of mixtures of varying concentrations produced an accuracy of 1.0% relative standard deviation of the mean at 2 σ. In Table I results are tabulated for various substituted ureas considered in this study.

TABLE I

LIQUID CHROMATOGRAPHY DATA COMPILATION

Results presented are for typical sample mixtures.

*Urea**	*Mixture 1*		*Mixture 2*		*Mixture 3*			*Retention time (sec)*	
	Present (%)	*Found (%)*	*Present (%)*	*Found (%)*	*Present (%)*	*Found (%)*		*Mixture 3(1)*	*Mixture 3(2)*
						1	*2***		
C	5.0	5.0	10.1	10.0	30.7	30.8	30.8	391	388
D	85.1	85.4	20.5	20.3	5.5	5.5	5.5	689	691
E	3.0	2.9	30.2	30.2	15.4	15.2	15.1	960	965
F	6.0	6.0	30.2	30.3	7.2	7.2	7.3	1110	1107
H	0.3	0.3	9.0	9.1	41.2	41.1	41.1	1246	1247

* For designations, see the legend to Fig. 1.

** Reanalysis of mixture 3 with different sample weights.

Normal principles of adsorption chromatography were applied to effect a TLC separation procedure using activated silica gel. The time for elution varied from 30–40 min at ambient temperatures. Fig. 2 shows typical TLC separations with the corresponding eluents used. Concentrations were less than 5%. Samples should be double-spotted in order to give the necessary concentration to evaluate sample quantification at the normal standard range of: 1.0% (w:v), 2.5% (w:v), and 5% (w:v). The standard deviation of the analysis representing the relative standard deviation of the mean at 2 σ was found to be approximately 25%. Spots were visually estimated by comparing to known standards. Good sensitivities were obtained for the substituted ureas considered in this study. Spots appeared as purple spots on a light gray background. It was found necessary to prepare fresh solutions prior to analysis and to allow the solutions to equilibrate for 30 min.

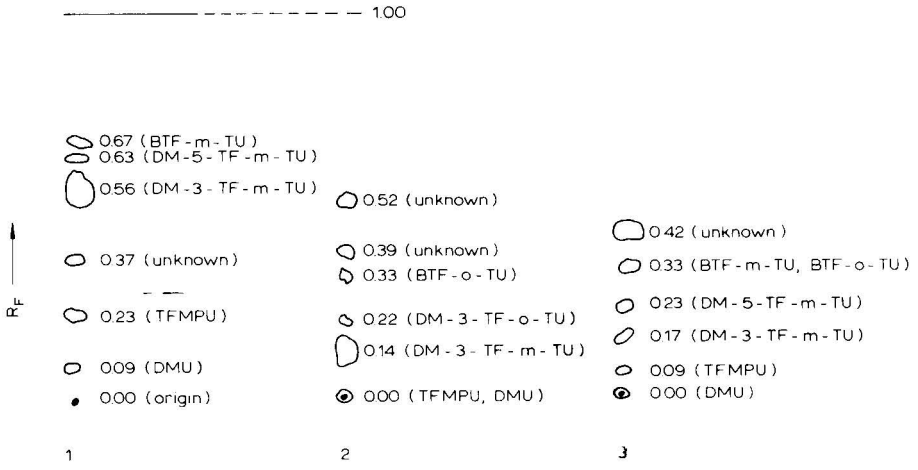

Fig. 2. TLC separation of selected ureas. 1 = chloroform–nitromethane–methanol (80:20:5); 2 = benzene–chloroform–ethyl acetate (40:40:20); 3 = chloroform–nitromethane (80:20). DMU = Dimethyl urea; TFMPU = trifluoromethylphenylurea; DM-3-TF-*m*-TU = dimethyl-3-trifluoro-*m*-tolyl urea, etc.; BTF-*m*-TU = bis-trifluoro-*m*-tolyl urea, etc.

It should be noted that no one TLC system described will separate all of the compounds studied. Combinations of two systems were generally used to assay mixtures. Slight variations in eluent component ratios may be necessary for certain applications, depending on the original sample matrix.

CONCLUSIONS

The present paper offers a TLC and HPLC method for the possible quantification of a very wide range of substituted ureas. With the modern pumping systems, flow gradients, and mixture gradients available many similar substituted urea compounds can be separated. Separation of the complex isomeric system investigated in this work suggests the above statement. Combination of the liquid chromatography method with the TLC procedure provides a most powerful analytical methodology for investigating highly substituted isomeric urea compounds.

ACKNOWLEDGEMENTS

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Note

Microdetermination of *o*-phenylphenol in citrus fruits by gas–liquid chromatography

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Biphenyl (BP), *o*-phenylphenol (OPP) and thiabendazole (TB) are used as preservatives for citrus fruits (or as fungicides) but only BP is allowed legally as a food additive in Japan.

Imported fruits sometimes contain OPP or TB, and about 10 ppm of OPP has been found in imported citrus fruits, grapefruits and lemons. OPP can be determined by gas–liquid chromatography (GLC)[1–4], thin-layer chromatography (TLC)[5,6], and high-performance liquid chromatography (HPLC)[7]. A flame-ionization detector (FID) is usually used in GLC but Hahn and Their[3] used an electron-capture detector (ECD) after brominating OPP. An FID is adequate for the determination of OPP at the level used as preservatives, but residual or impregnated OPP in fruits needs a more sensitive detector such as an ECD.

Phenols and benzoyl chloride react well in an aqueous medium and, in order to prepare phenol derivatives that would be detected with greater sensitivity by the ECD, PFB-Cl was reacted with OPP and optimal conditions were established.

EXPERIMENTAL

Reagents

Pentafluorobenzoyl chloride was obtained from Aldrich, (Milwaukee, Wisc., U.S.A.). All solvents and reagents were of analytical grade and were used as received. The extraction solvent contained heptachlor epoxide as the internal standard in *n*-heptane, accurately weighed to contain 1.6 μg/ml. The dialysis solution was prepared by dissolving 33 g of anhydrous sodium acetate and 200 g of sodium chloride in distilled water and making the volume up to 1000 ml.

Procedure

Weigh accurately 20 g of homogenized sample, add 50 ml of dialysis solution, add 80-ml of *n*-hexane and mix in an electric blender. After centrifugation, remove the hexane layer, add a further 80 ml of *n*-hexane extract the aqueous layer, combine the hexane layers and concentrate to 5 ml with a K-D concentrator. Extract the organic layer with two 5-ml portions of 0.1 *N* sodium hydroxide solution, combine the extracts, neutralize it with 1 *N* hydrochloric acid (2 ml) and add sodium hydrogen

carbonate powder so that the final concentration becomes approximately 1%. Then add 10 μl of PFB-Cl, shake vigorously for 30 sec and allow to stand for 10 min at room temperature. Add exactly 5 ml of the extraction solution and shake vigorously for 1 min. The extract is subjected to GLC after dehydration.

Gas–liquid chromatography

A Shimadzu GC-4CM gas chromatograph equipped with an electron-capture detector was fitted with a U-shaped glass column (2 m × 3 mm O.D.), packed with 5% DEGS on Gas-Chrom Q, GC grade, pre-conditioned at 240° for 20 h. The temperatures used were: column, 190° (isothermal); injection and detector, 210°. The gas (nitrogen) flow-rate was 30 ml/min.

RESULTS AND DISCUSSION

Phenol benzoate is formed by the Schotten–Baumann reaction[8] from phenol and benzoyl chloride in the presence of sodium hydroxide. In the reaction of OPP and PFB-Cl, the pH conditions of the reaction media were examined. Sodium hydroxide, sodium carbonate and sodium hydrogen carbonate were used and the reactions were carried out between pH 8 and 14. The results are shown in Fig. 1. The reaction rate reached a maximum at pH 12 when sodium hydroxide was used, but the yield was inadequate (32%). When sodium carbonate was used, the yield of the product was 20% at pH 9, and increased to 97% at pH 10. However, at pH 11 the yield was lower. The reaction proceeded very well in sodium hydrogen carbonate solution and the reaction was therefore performed in a 1% solution.

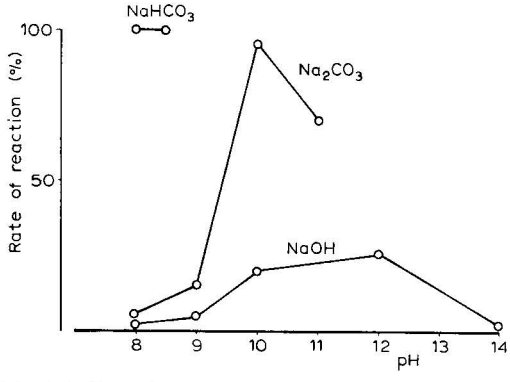

Fig. 1. Effect of pH on rate of reaction.

The yields of the product at various temperatures were compared; when the temperature exceeded about 50° the yield decreased, and good yields were obtained at room temperature and below. The reaction time was also varied at 5, 10, 20 and 30 min, and it was found that the reaction proceeded to completion within 5 min and the yield had not altered at the end of the 30-min period. The time of reaction was therefore set at 10 min.

The amount of PFB-Cl required for reaction with OPP was examined. To 100

and 1 μg of OPP, various amounts of PFB-Cl were added separately (2, 5 and 10 μl). The results are shown in Fig. 2. No undesirable effect was observed when excess of PFB-Cl was used, and 2 μl of PFB-Cl were found to be sufficient for use with both 1 and 100 μg of OPP. The permitted limit of OPP within the E.E.C. is 12 ppm, so that 10 μl of PFB-Cl is considered to be sufficient for most samples.

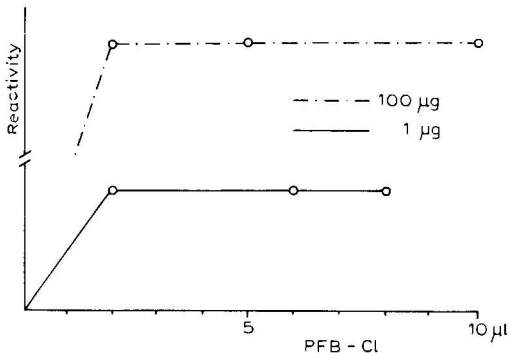

Fig. 2. Effect of amount of PFB-Cl on reactivity.

Solvents for the extraction of the reaction product were examined, and it was found that cyclohexane, *n*-hexane, benzene, *n*-heptane and ethyl acetate all had the same extraction efficiency. No disturbance to the gas chromatogram was found with any of these solvents, but a non-polar solvent was preferable because certain essential oils in fruits are not extracted. *n*-Heptane was used as it separates from water rapidly, and it is not miscible with water; shaking for 1 min was sufficient for extraction.

Unstable reaction products may affect on the accuracy of the assay, so that the stability of the products was examined. No change in the reaction products was observed when they were examined after 5, 15, 30 and 60 min and 10 h.

The reaction product was assumed to be the benzoate formed between OPP and PFB-Cl, but it is possible that the benzoate might have been decomposed during the GLC process, so the identity of the product was therefore confirmed by GC–MS. The mass spectrum is shown in Fig. 3. The molecular ion occurs at *m/e* 364 and the fragment of OPP ($C_{12}H_9O$–) is *m/e* 169 (M — 195) which corresponds to the loss of *m/e* 195 (C_6F_5CO–), indicating that the reaction product is *o*-phenylphenol pentafluorobenzoylester.

DEGS, QF-1, OV-17 and SE-30 were compared as the GLC column packing material. It was found that DEGS shown good separation from contaminating substances, while QF-1, OV-17 and SE-30 gave overlapped peak of OPP and interfering substances. DEGS was therefore selected.

The sensitivity of detection was extremely good as an ECD was used for the detection of OPP, and picogram amounts of OPP could be detected. A calibration graph for the determination of OPP was constructed with five points (from 0.1 to 1 ppm), and for the determination of the residual level the detector sensitivity was set back to the normal condition and five points (from 0.01 to 0.1 ppm) were used for the calibration graph (in this instance, the extraction solvent was diluted 8-fold with *n*-

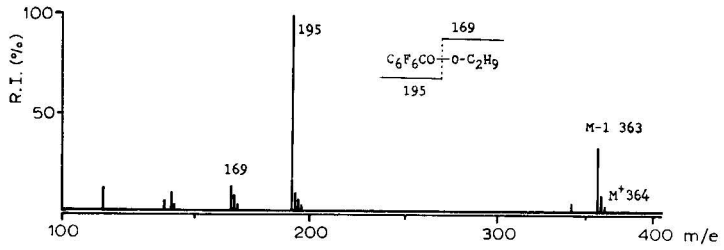

Fig. 3. GC–MS spectrum of reaction product.

heptane). The linear ranges of the calibration graph were from 0.1 to 1.2 ppm in the former and from 0.01 to 0.12 ppm in the latter instance. The results are shown in Fig. 4.

Quantitative determinations by GLC were made by using an internal standard. (heptachlor epoxide) and comparing relative peak heights. The internal standard is excluded from the sample during alkaline extraction, even if it is present in sample.

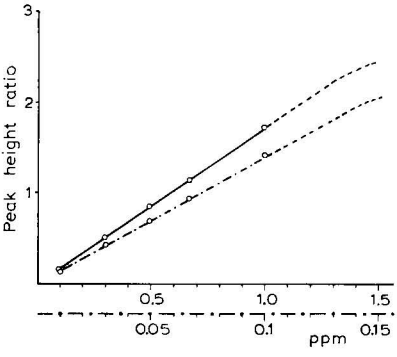

Fig. 4. Calibration graph for *o*-phenylphenol.

If the amount of hydrochloric acid exceeds that required for the neutralization of the reaction medium in the reaction of OPP and PFB-Cl, the subsequent reaction does not proceed well, so that precise neutralization is required.

The recovery of the sample was examined. Tonogai and co-workers [9,10] used ethyl acetate as the extraction solvent when BP and TB were determined simultaneously with good recovery. In our work *n*-hexane was used in order to exclude interfering substances such as certain essential oils, and good results were obtained, as shown in Table I.

TABLE I

RECOVERY OF OPP ADDED TO LEMON FRUITS

Results are mean values of five experiments.

Amount added (*μg*)	*Amount found* (*μg*)	*Recovery* (%)
0	2.12	—
1.0	3.02	90.0
5.0	6.88	95.2
10	11.9	97.8

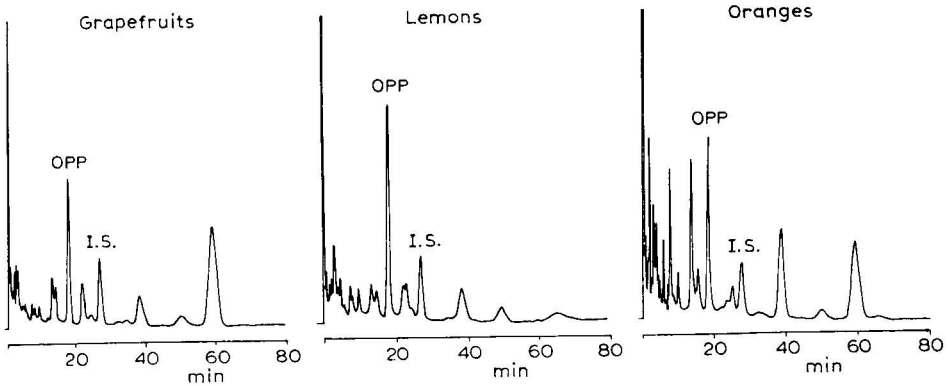

Fig. 5. Gas chromatograms of reaction products of extracts from citrus fruits. I.S. = internal standard.

OPP in lemon, orange and grapefruit was determined, and no interfering materials were found. Much more contaminating materials are present in orange than in lemon and grapefruit. The gas chromatograms are shown in Fig. 5.

It can be concluded from the results that the micro-determination of OPP in citrus fruits can be achieved by forming a derivative with PFB-Cl and using a sensitive ECD, to the extent of as low as 0.005 ppm OPP in solution.

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Note

Long-chain acetates as internal standards in the gas–liquid chromatography of volatile fatty acids

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Volatile fatty acids (VFA) are responsible for the flavours of many food products. The ordinary gas–liquid chromatographic (GLC) method of VFA analysis could be improved by using internal standards and the purpose of this work was to examine the use of some acetates for this purpose.

EXPERIMENTAL

C_2–C_7 volatile fatty acids and C_2–C_{12} alcohols were obtained from Polyscience Corp. and dichloroacetic acid (AnalaR grade from POCH) was used as a 10% (w/v) solution in distilled water[1]. Acetates of C_6–C_{12} alcohols were synthesized in our laboratory from the above alcohols and acetyl chloride (AnalaR grade, POCH).

Five microlitres of VFA solution in 10% dichloroacetic acid[1] containing 0.1 nl of each acid and acetate were injected onto the chromatographic column.

A Beckman GCM gas chromatograph was used, equipped with a dual column system, flame-ionization detectors and a Beckman recorder. The chromatographic conditions were: stainless-steel columns, 1.8 m × 4 mm I.D., filled with 20% EGA on Chromosorb W HP, 100–120 mesh; nitrogen flow-rate, 53 ml/min (1.8 atm); hydrogen, 1.5 atm; air, 2 atm; recorder chart speed, 0.2 in./min; column temperature, constant at 140°, 150° or 180° or programmed from 130° to 210° at the rate of 10°/min; injection block temperature, 250°; detector temperature, 300°.

RESULTS AND DISCUSSION

A typical chromatogram of VFA and long-chain acetates is shown in Fig. 1. The order of the various VFA on the chromatogram, depending on the carbon number, is typical for the conditions of analysis[2,3].

Of the internal standards studied, the most promising were heptyl acetate, which appears between acetic and propionic acids, and decyl acetate, which appears just after valeric acid. Hexyl acetate appears on the solvent peak, octyl acetate in the position of isobutyric acid, nonyl acetate could interfere with the two possible isomers of valeric acid (2-methylbutyric and 3-methylbutyric acid), while undecyl and dodecyl

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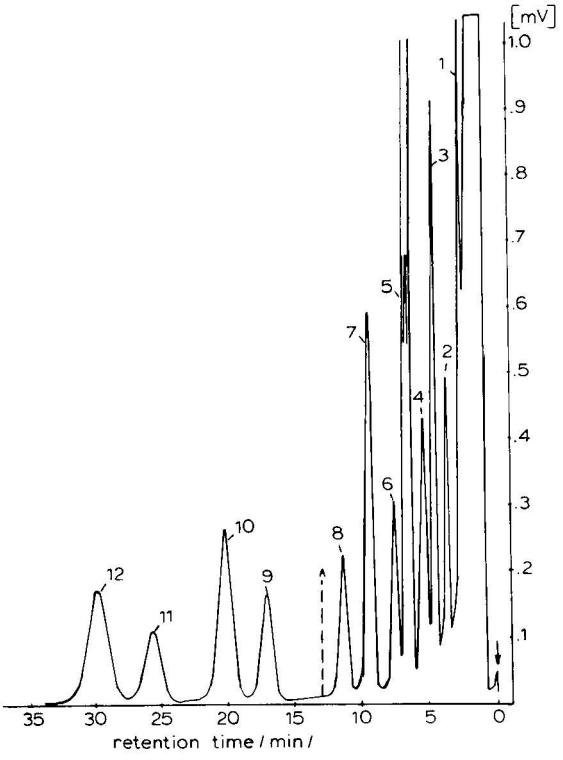

Fig. 1. Typical chromatogram of volatile fatty acids and some long-chain acetates. Constant column temperature, 150°. Peaks: 1 = hexyl acetate; 2 = acetic acid; 3 = heptyl acetate; 4 = propionic acid; 5 = octyl acetate; 6 = butyric acid; 7 = nonyl acetate; 8 = valeric acid; A = decyl acetate (position calculated from Fig. 3); 9 = caproic acid; 10 = undecyl acetate; 11 = enanthic acid; 12 = dodecyl acetate. The amount of each compound injected into the chromatographic column was 0.1 nl in 5 μl of 10% dichloroacetic acid solution.

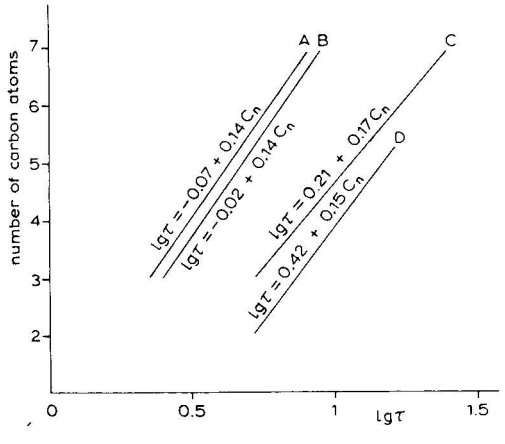

Fig. 2. Correlations between the logarithm of retention time and number of carbon atoms for different column temperatures. A, programmed temperature from 130° to 210° at the rate of 10°/min; B, isothermal at 160°; C, 150°; D, 140°.

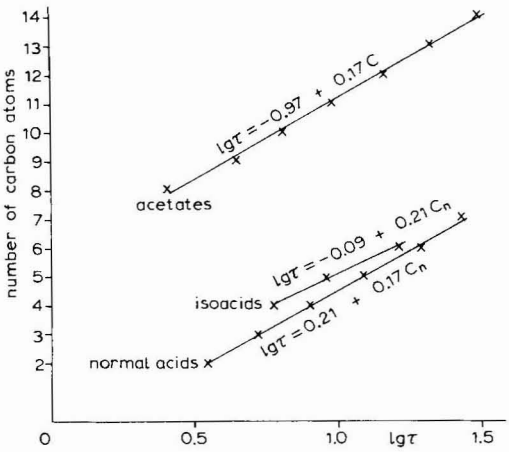

Fig. 3. Correlations between the logarithm of retention time and number of carbon atoms for acetates, iso-acids and normal volatile fatty acids for a constant column temperature of 150°.

acetates appear too late, so that these compounds cannot be good internal standards.

Long-chain normal alcohols have the same retention times as normal fatty acids with a carbon chain shorter by four carbon atoms; hence *n*-octanol appears in the place of butyric acid, *n*-nonanol in place of valeric acid and *n*-decanol in place of caproic acid.

Relationships between the number of carbon atoms in the molecules of fatty acids and the ogarithm of retention time for different conditions of analysis are shown in Fig. 2. Very similar results were obtained for a programmed temperature from 130° to 210° at the rate of 10°/min and for a constant column temperature of 160°, but in both instances the separation of some isomers was not satisfactory. We therefore chose for use in subsequent analyses a constant column temperature of 150°, which gave good separations and a time of analysis that was not too long. For these conditions there were linear relationships between the logarithm of the retention time and number of carbon atoms for normal acids, iso-acids and acetates of long-chain alcohols (Fig. 3). The lines for acetates and acids are exactly parallel. These relationships enable one to identify unknown compounds and to choose a suitable internal standard for each group of acids or acetates to be determined quantitatively.

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Journal of Chromatography, 125 (1976) 447–450

CHROM. 9290

Note

Simple thin-layer chromatographic identification method for erythromycin stearate

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(Received March 5th, 1976)

The broad-spectrum antibiotic erythromycin is formulated as salts (stearate, gluceptate, lactobionate) and esters (propionate, ethyl succinate, ethyl carbonate) as well as the free base and an ester–salt combination (estolate). Their structures are illustrated in Fig. 1.

The methods for analysis of these products in the British Pharmacopoeia[1] and Code of Federal Regulations[2] are microbiological with prior hydrolysis required in the case of the esters. In all cases, the results are calculated and expressed in terms of equivalence to erythromycin base and give no indication of the form of erythromycin assayed. This is determined by identity methods: colour tests in the British Pharmacopoeia for the base, salts, and esters as well as paper chromatography for the estolate, and infrared spectroscopy in the Code of Federal Regulations (for U.S.P. and N.F. products) for all erythromycin bulk drug materials.

In our search for more specific and less time-consuming identity methods, we reported a thin-layer chromatographic (TLC) method[3] which differentiated erythromycin base from erythromycin estolate and erythromycin ethyl succinate. The system, however, could not separate erythromycin base from the stearate, gluceptate or lactobionate salts, all of which chromatographed as the free base, nor could the stearate portion be detected.

We wish to report here a new TLC method that allows the detection of both the stearic acid and erythromycin portions of the erythromycin stearate molecule and thus differentiates this salt from other erythromycin derivatives. The method is simple, rapid, and applicable to both bulk drug and formulations.

EXPERIMENTAL

TLC plates

Commercially available pre-coated silica gel 60 (Merck) plates (20 × 20 cm, 0.25 mm thickness) were employed. The plates were activated for 30 min in the oven at 130° prior to use.

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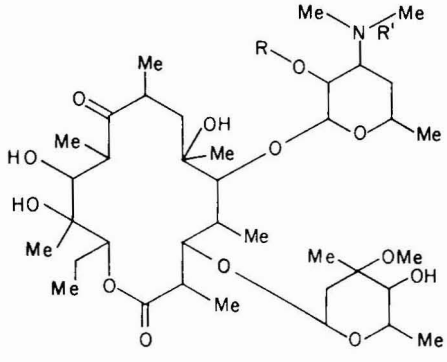

	R	R'
Erythromycin	H	
Propionyl erythromycin	CH_3CH_2CO	
Erythromycin estolate	CH_3CH_2CO	$C_{12}H_{25}OSO_3H$
Erythromycin stearate	H	$C_{17}H_{35}COOH$
Erythromycin lactobionate	H	$C_{11}H_{21}O_{10}COOH$
Erythromycin gluceptate	H	$C_6H_{13}O_6COOH$
Erythromycin ethyl succinate	$CH_3CH_2OOCCH_2CH_2CO$	
Erythromycin ethyl carbonate	CH_3CH_2OOC	

Fig. 1. Structural formulae of erythromycin derivatives. Me = CH_3.

Spray reagent[4]

Potassium dichromate (5.0 g) was dissolved in 40% sulfuric acid (100 ml).

Solutions for spotting

Standard solutions. A solution (50 mg/ml) of each of the erythromycin compounds (Table I) was prepared in chloroform or, in the case of erythromycin base, gluceptate and lactobionate, in cloroform–methanol (2:1). Stearic acid (Applied Research Labs., Rochester, New York, U.S.A.) was dissolved in chloroform (20 mg/ml).

Sample solutions. In the case of tablets, one tablet (equivalent to 250 mg erythromycin activity) was ground using a mortar and pestle, the powder was then transferred into a 15-ml stoppered glass centrifuge tube, and a 5.0-ml portion of chloroform was added. Vigorous shaking for several minutes and centrifugation at 300 rpm for 5 min gave a clear supernatant solution for spotting on the TLC plate.

In the case of oral suspensions, a 5.0-ml portion of the well shaken liquid formulation (equivalent to 250 mg erythromycin activity) was transferred into a 125-ml separatory funnel and diluted with 10.0 ml of distilled water. A 5.0-ml portion of chloroform was added, and the organic layer separated for spotting on a TLC plate.

TABLE I

R_F VALUES OF ERYTHROMYCINS AND STEARIC ACID ON SILICA GEL 60

Solvent system: Chloroform–methanol–acetic acid (90:10:1).

Compound	*R_F value**
Erythromycin	0.05
Erythromycin stearate**	0.04, 0.68
Stearic acid	0.69
Erythromycin ethyl succinate	0.10
Erythromycin estolate	0.10
Erythromycin gluceptate	0.04
Erythromycin lactobionate	0.04

* Average of five plates.

** Two spots, *viz.* erythromycin free base and stearic acid.

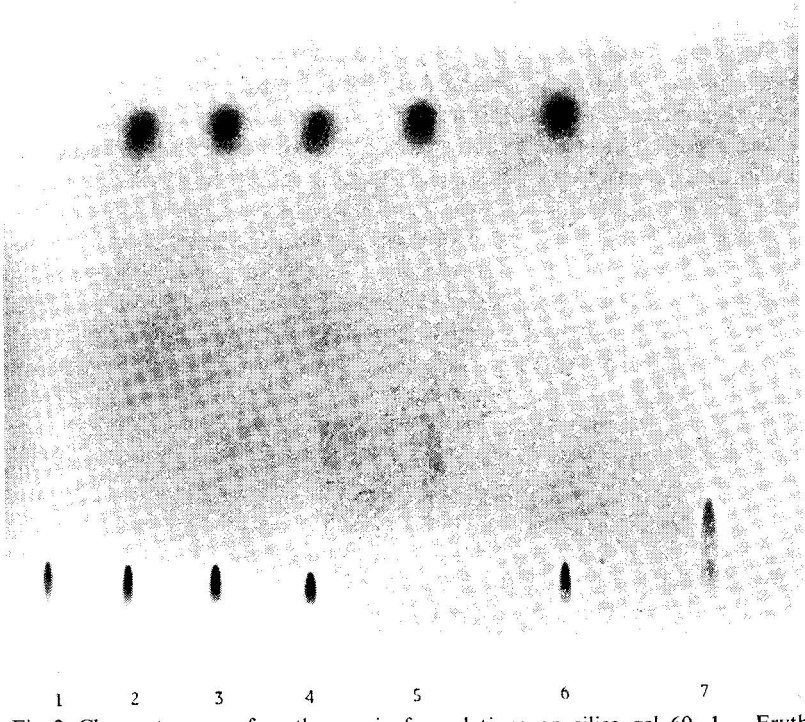

Fig. 2. Chromatogram of erythromycin formulations on silica gel 60. 1 = Erythromycin tablets, U.S.P.; 2 = erythromycin stearate tablets, B.P.; 3 = erythromycin stearate tablets, U.S.P.; 4 = erythromycin stearate reference standard, U.S.P.; 5 = stearic acid standard; 6 = erythromycin stearate oral suspension, manufacturer's standard; 7 = erythromycin estolate oral suspension, N.F.

Chromatographic procedure

Sample solutions (1 μl) representing 50 μg (20 μg for stearic acid) were applied to the plate by means of micropipettes and the plates were inserted into a filter paper-lined chromatographic chamber which had been saturated with solvent vapour for 1 h prior to use. The solvent system employed was chloroform–methanol–acetic acid

(90:10:1). The plates were developed to a height of 15 cm (approx. 75 min), then removed from the chamber, dried at room temperature, then uniformly sprayed with the spray reagent and heated for 1 h at 150°.

RESULTS AND DISCUSSION

Chromatography of erythromycin stearate in our solvent system produces two spots on a TLC plate after spraying with chromic acid–sulphuric acid reagent and charring, one due to stearic acid and the other to erythromycin moiety. Acetic acid, being a stronger acid than stearic acid, displaces the stearate anion, which is then protonated to release free fatty acid. The corresponding R_F values for various erythromycin derivatives and stearic acid are listed in Table I.

In Fig. 2, a typical chromatogram obtained with five formulations and erythromycin stearate and stearic acid standards is shown. The formulations included erythromycin tablets, erythromycin stearate tablets (both B.P. and U.S.P.), erythromycin estolate liquid, and erythromycin stearate liquid. As can be seen from Fig. 2, pharmaceutical excipients do not interfere with the method, both the stearate and erythromycin portions of the molecule being well resolved from each other.

The use of the chromic acid–sulphuric acid spray reagent and a prolonged heating period were required in order to detect the fatty acid portion of the drug. Although less than 50-μg quantities could be detected on the plate, 50 μg amounts gave well defined, reproducible spots.

The use of this proposed TLC identification method enables fast differentiation of erythromycin stearate from erythromycin base and other erythromycin derivatives —ethyl succinate, estolate, gluceptate and lactobionate.

REFERENCES

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2 *Code of Federal Regulations*, U.S. Government Printing Office, Washington, 1975, Title 21, Part 452.
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4 J. Bartetti, *Ann. Chem. (Rome)*, 44 (1954) 495.

Journal of Chromatography, 125 (1976) 451–452

CHROM. 9589

Book Review

Instrumental liquid chromatography. A practical manual on high-performance liquid chromatographic methods, (Journal of Chromatography Library, Vol. 5), by N. A. Parris, Elsevier, Amsterdam, Oxford, New York, 1976, X + 329 pp., price Dfl. 100.000, US$ 38.50, ISBN 0-444-41427-4.

For the analytical chemist who applies or has potential applications for high-performance liquid chromatography, this is probably the most useful, practical book dealing with this popular and rapidly expanding technique that has been published so far. The author has collected and collated the considerable experiences he has acquired in an applications laboratory of a company who market a range of liquid chromatographs and materials. From his first-hand knowledge of customer's problems he has been able to compile a laboratory handbook containing many practical hints that are not to be found in the more academic treatises.

The book is divided into four main sections of which the first is entitled "Fundamentals and instrumentation". After a brief introductory chapter, the second states the basic definitions and equations and illustrates, by means of examples, how the results of theory aid the practising chromatographer. Theoretical aspects of liquid chromatography are adequately discussed elsewhere and their omission from this particular book is understandable. What is disturbing, however, is the author's reason for this omission, *i.e.*, that many scientists are so busy getting results that they have no time to try and understand what they are doing and consequently need to rely on commercial suppliers. This is not only at variance with the scientists' naturally enquiring mind but also makes sweeping assumptions on the academic status of each and every supplier. The remainder of the first section includes an important chapter on column technology and two chapters on instrumentation. These are superbly authorative accounts but concentrate on commercially-attractive equipment at the expense of, say, specialised detectors and new innovations.

The second section discusses factors influencing selectivity and contains some excellent material. Its five chapters describe the properties of the mobile and stationary phases and contain guidelines for choosing the best chromatographic systems for attacking a particular problem. The chapter on steric exclusion chromatography is less strong. The principles governing column selection are not described well and whilst the molecular-weight distribution of polymers is discussed, the author never gets around to informing the inexperienced how to actually perform this determination.

The third section of the book describes the techniques of qualitative and quantitative analysis and discusses procedures for trace analysis and preparative-scale separations. The last section lists references to applications published during the last five years. Perhaps a reference to the numerous potential applications discussed in Volume 3 of the Journal of Chromatography Library would also have been appropriate.

The book on the whole is very readable and has obviously been written with much enthusiasm. The occasional sentence, such as the first one on p. 29, might leave the reader rather breathless. Most analysts will benefit by having a copy of this book alongside their chromatographs—whether a commercial or a home-assembled instrument.

Abingdon (Great Britain) R. AMOS

Journal of Chromatography, 125 (1976) 453

CHROM. 9404

Book Review

New developments in separation methods, edited by E. Grushka, Marcel Dekker, New York, Basel, 1976, VIII + 246 pp., price Sfr. 85.00, ISBN 0-8247-6411-0.

These eleven papers are from an April 1974 meeting of the American Chemical Society, and were originally published in volumes 9 and 10 of *Separation Science*. About half of the book is taken up by two papers on ultracentrifugation by Adams *et al.* and by Hsu. The Adams paper deals mainly with the use of ultracentrifugation to obtain molecular weight distribution data via the equilibrium technique, and to study molecular self-association. An extensive theoretical treatment is provided, along with comparisons of experimental results with gel permeation chromatographic measurements. The Hsu paper examines the various zonal centrifugal techniques for preparative bioseparations.

A second two papers by Grieves *et al.* and Somasundaran review various foam fractionation methods, with emphasis on anion separations from aqueous solutions by cationic surfactants. Little that is new or challenging appears here.

A paper on isolelectric focusing by Catsimpoolas includes a good review of principles along with a discussion of a new wrinkle introduced by the author: the TRANSIF technique in which both kinetics and equilibria of isoelectric focusing are monitored during separation. This provides additional parameters to characterize separated species.

The remaining six papers are less impressive, for different reasons. Two papers on the selection and sequencing of separation systems, and potential-controlled adsorption on chemically modified graphite are so fragmentary as to be almost meaningless. Another paper on plasma chromatography concludes mainly that the technique is useless for analyzing mixtures. Three remaining papers on phenol separation from waste water via liquid membranes, clathrates for analytical separations, and electrokinetic cell separations are rather brief examinations of techniques that have been well described elsewhere, and which add little as reviews, new applications or critical assessments.

In summary, I have great difficulty in seeing a valid rationale for producing the present book. It will hardly appeal to individual scientists for their own libraries, because the unifying theme and general interest of the collected articles is minimal, and because only one or two articles provide any in depth treatment of their subject. Libraries will probably already subscribe to *Separation Science*, and will not want to duplicate what is already contained there. My own impression is that the publisher found it to be inexpensive to reprint what was already set in type, and hoped that the addition of a binding would result in a salable product.

Tarrytown, N.Y. (U.S.A.) L. R. SNYDER

Journal of Chromatography, 125 (1976) 454

CHROM. 9480

Book Review

Separation methods in chemical analysis, by James M. Miller, Wiley, Chichester, New York, 1975, X + 309 pp., price £ 9.00, ISBN 0-471-60490-9.

This book is intended to provide a unified approach to the major analytical separation methods and to present a basic theory for separation science. The first six chapters deal with the general theoretical aspects of the subject and include discussion of classification, thermodynamics, kinetics, separation forces and separation methods based on chemical reactions. Chapters 8 and 9 complete the theoretical section with a discussion of zone broadening. The remainder of the book including Chapter 7 and Chapters 10–17 briefly discusses the following separation methods: distillation, liquid–liquid extraction, chromatography (general), gas chromatography, paper and thin-layer chromatography, zone electrophoresis and differential dialyses.

The book has been written essentially for undergraduate students and as such contains all the relevant subject matter. The need for brevity has, however, meant that a prior understanding of elementary thermodynamics and equilibrium concepts is a prerequisite to a full understanding of the theoretical discussions. The chapters concerning the individual separation methods are also brief but are supplemented by an up-to-date bibliography which should provide a useful list of source material to those readers meeting a particular topic for the first time. The author's choice of symbols and nomenclature may, as he points out, lead to some confusion when compared with other texts. However, such changes are necessary and desirable if uniformity is to be achieved between the various fields of study.

The book is well produced with few typographical errors, and the subjects are discussed without bias. The book is up to date and although brief provides an extremely useful undergraduate text in this important field of analytical chemistry.

Loughborough (Great Britain) L. A. GIFFORD

Vol. 125, No. 2

journal of
chromatography news section

APPARATUS

N-910

MASS SPECTROMETER DATA SYSTEM

A new data system for Hewlett-Packard mass spectrometers offers users an expanded software capability, a new 19,000-spectra chemical library and a semiconductor-memory HP 21MX mini-computer. The new, more-compact HP 5934A dual-disc system controls scan operations of HP mass spectrometers, stores spectral data on discs, then displays and tabulates these data at operator command.

N-916

PRECISION MICRODENSITOMETER

Gaertner Scientific Company presents a new microdensitometer which measures the position and optical density of any point on a film or plate to 0.001 mm, over a range of 255 mm x 100 mm. Optical density range is 0–4.0 density units. Several versions of the basic instrument are available. A motorised version, which operates automatically, is capable of recording at every 2 μm (or more) of carriage position. Other versions are available for manual operation and for greater ranges.

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

N-913

SOLVENT RECOVERY APPARATUS

The new line of solvent recovery apparatus from Kontes includes several convenient and compact laboratory-scale stills of various designs to meet a variety of needs. Output from the stills is residue-free and suitable for all analytical and preparative techniques.

N-917

NEW GAS CHROMATOGRAPH

The Dani 3400 is a routine-dedicated gas chromatograph with interchangeable analytical modules dedicated to a specific detector such as FID, ECD, AFD, FPD and HWD, and to specific applications such as pesticides or pollution. Features include digital programming, modular electronics and a hardware design that simplifies operation and maintenance.

N-914

TOTAL HYDROCARBON/METHANE MONITOR

The new Dani THMM 230 is capable of performing continuous and simultaneous determinations of total hydrocarbons with and without methane and of methane alone. The main features of the instrument are a dual hydrogen flame detector, parallel sample lines with one hydrocarbon-selective catalytic filter and a differentiating amplifier. An auto-calibration system compensates for zero and span variations against the standard.

PROCEDURES

N-912

NEW PHOTOGRAPHY PUBLICATIONS

The second edition of "Close-Up Photography and Photomacrography" is now available from Eastman Kodak Co. The 198-page hardbound book features more than 350 black-and-white and colour photographs to illustrate discussions on theory and practice that can be useful to photographers and students who use close-up photography and photomacrography in various scientific disciplines.

Also published is a new Kodak data book, "High-Speed Photography", for those using the technique for the first time. The 60-page book explains how high-speed motion pictures are made and also describes the necessary equipment (including black-and-white and colour films), and includes discussions of various types of cameras, their operating principles, and useful techniques. There are also basic explanations of lenses, lighting and film characteristics.

Kodak publication No. P-92 is the newly revised and enlarged data chart on the characteristics of Kodak black-and-white instrumentation films. The chart contains information of 17 Kodak films. In addition to sections defining Estar base, RAR films, Linagraph Shellburst films and various high-speed films, the chart includes tables listing colour sensitivity, applications, sizes, etc.

N-920

AUDIO-VISUAL CHROMATOGRAPHY

Beckman's "New Dimensions" range of audio-visual technical training programmes include presentations on basic aspects of gas chromatography, principles and practice of thin-layer chromatography, basic electrophoresis, and electrophoretic techniques utilizing the Microzone^{R} system. A revised 24-page brochure "Audio-Visual Guide" describes the expanded series of programmes. Other subjects covered include analysis of peptides/amino acids, enzymology, nuclear instrumental techniques, spectroscopy (IR and UV/visible), spectral interpretation, etc.

N-909

PROTEIN SEQUENCING BOOK

"Solid Phase Methods in Protein Sequence Analysis", recently published by Pierce, is the proceedings of the First International Conference on Solid Phase Sequencing (May 1975) and covers all advances and applications since the 1971 publication by Laursen on automated solid phase sequencing. The chemistry of solid-phase Edman degradation, sequencing techniques and applications, PTH analysis, and instrumentation are discussed.

NEW BOOKS

Spectrophotometric determination of elements, by Z. Marczenko, Ellis Horwood, Chichester, 1976, xi + 643 pp., price £ 19.50, US$ 42.90, ISBN 0-470-56865-8.

Environmental effects on molecular structure and properties, (Proceedings of the 8th symposium on quantum chemistry and biochemistry, Jerusalem, April 7–11, 1975), edited by B. Pullman, Reidel, Dordrecht, Boston, Mass., 1976, 588 pp., price Dfl. 195.00, US$ 75.00, ISBN 90-277-0604-2.

Mass spectrometry of steroids, by Z.V. Zaretskii, Halsted (Wiley), New York, Chichester, and Israel Univ. Press, Jerusalem, 1976, xi + 182 pp., price £ 9.90, US$ 19.80, ISBN 0-7065-1453-X.

Analysis of air pollutants, by P.O. Warner, Wiley-Interscience, New York, London, 1976, xi + 329 pp., price £ 11.00, US$ 22.00, ISBN 0-471-92107-6.

Recent contributions to geochemistry and analytical chemistry, edited by A.I. Tugarinov, Halsted (Wiley), New York, Israel Program for Scientific Translations, Jerusalem, 1975, viii + 695 pp., price £ 26.40, US$ 52.80, ISBN 0-470-89228-5.

Organic chemistry, by T.W.G. Solomons, Wiley, New York, London, 1976, xxiii + 1056 pp., price £ 12.00, US$ 24.00, ISBN 0-471-81220-X.

Spectral and chemical characterization of organic compounds; A laboratory handbook, by W.J. Criddle and G.P. Ellis, Wiley, New York, London, 1976, viii + 103 pp., price £ 4.60, US$ 10.15, ISBN 0-471-18767-4.

Structural studies of macromolecules by spectroscopic methods, edited by J.K. Ivin, Wiley-Interscience, New York, London, 1976, 339 pp., price £ 14.50, US$ 31.90, ISBN 0-471-43120-6.

Treatise on analytical chemistry, Part I, Theory and practice, Vol. II, edited by I.M. Kolthoff and P.J. Elving, Wiley-Interscience, New York, London, 1975, xxiii + 698 pp., price US$ 43.85, £ 22.00, ISBN 0-471-49967-6.

The quality control of medicines, (Proceedings of the 35th International Congress of Pharmaceutical Sciences, Dublin, 1975), edited by P.B. Deasy and R.F. Timoney, Elsevier, Amsterdam, New York, 1976, *ca.* 390 pp., price Dfl. 100.00, US$ 38.50, ISBN 0-444-41454-1.

Assay of drugs and other trace compounds in biological fluids, (Methodological Developments in Biochemistry, Vol. 5), edited by E. Reid, North-Holland, Amsterdam, New York, 1976, x + 254 pp., price Dfl. 65.00, US$ 24.95, ISBN 0-7204-0584-X.

Thermometric titrations, by J. Barthel and R. Wachter, Wiley-Interscience, New York, London, 1975, xi + 209 pp., price £ 12.25, US$ 24.55, ISBN 0-471-05448-8.

Organic functional group analysis by gas chromatography, by T.S. Ma and A.S. Ladas, Academic Press, London, New York, San Francisco, 1976, x + 173 pp., price £ 6.80, US$ 14.75, ISBN 0-12-462850-8.

CRC Standard Mathematical Tables, edited by W.H. Beyer, CRC Press, Cleveland, Ohio, 24th ed., 1976, 565 pp., price US$ 9.95, ISBN 0-87819-623-4.

Practical high performance liquid chromatography, edited by C.F. Simpson, Heyden & Son (in association with the Continuing Education Committee of The Chemical Society), London, 1976, xi + 315 pp., price £ 9.30, US$ 18.50, DM 59.50, ISBN 0-85501-089-4.

Quadrupole mass spectrometry and its applications, edited by P.H. Dawson, Elsevier, Amsterdam, New York, 1976, *ca.* 230 pp., price Dfl. 129.00, US$ 49.75, ISBN 0-444-41345-6.

Advances in mass spectrometry in biochemistry and medicine, Vol. 1, (Proceedings of the 2nd International Symposium, Milan, June, 1974), edited by A. Frigerio and N. Castagnoli, Spectrum Publications/Halsted (Wiley), New York, 1976, xxi + 586 pp., price £ 27.20, US$ 46.40, ISBN 0-470-28121-9.

Carbohydrate chemistry, Vol. 8, edited by J.S. Brimacombe, Chemical Society, London, 1976, xii + 485 pp., price £ 24.00, US$ 66.00, ISBN 0-85186-072-9.

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Advances in infra-red and Raman spectroscopy, Vol. 2, edited by R.J.H. Clark and R.E. Hester, Heyden & Son, London, 1976, ISBN 0-85501-182-3.

Ion selective electrodes, by P. Bailey, Heyden & Son, London, 1976, ISBN 0-85501-223-4.

Spectra and chemical interactions (Structure and Bonding, Vol. 26), edited by J.D. Dunitz, Springer, Berlin, New York, 1976, iv + 144 pp., price DM 58.00, US$ 23.80, ISBN 3-540-07591-7.

Handbook of biochemistry and molecular biology, Vol. 2, Nucleic acids, edited by G.D. Fasman, CRC Press, Cleveland, Ohio, and Blackwell, Oxford, 3rd ed., 1976, 293 pp., price £ 36.40, ISBN 0-87819-506-8.

Biosynthesis, Vol. 4, edited by J.D. Bu'lock, Chemical Society, London, 1976, ix + 274 pp., price £ 16.00, ISBN 0-85186-533-X.

Methods in zone electrophoresis, by J.R. Sargent and S.G. George, BDH Chemicals, Poole, 3rd ed., 1975, price £ 3.50.

Chemistry and biochemistry of plant pigments, edited by T.W. Goodwin, Academic Press, New York, London, 2nd ed., 1976; Vol. 1: xvi + 870 pp., price US$ 65.75, £ 26.50, ISBN 0-12-289901-6; Vol. 2: xiii + 373 pp., price US$ 29.75, £ 12.00, ISBN 0-12-289902-4.

CALENDAR OF FORTHCOMING MEETINGS

November 1–4, 1976
Houston, Texas, U.S.A.

Chromatography '76. 11th International Symposium on Advances in Chromatography

Contact:
Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A. (Further details published in Vol. 117, No. 1)

February 28–March 4, 1977
Cleveland, Ohio, U.S.A.

28th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy

Contact:
John E. Graham, Program Chairman, Koppers Company, Inc., 440 College Park Drive, Monroeville, Pa. 15146, U.S.A.

April 26–28, 1977
Bratislava, Czechoslovakia

5th International Symposium. Improvements and Application of Chromatography in the Chemical Industry

Contact:
Ing. J. Remeň, Analytická sekcia ČS VTS, pri. n. p. Slovnaft, 82300 Bratislava, Czechoslovakia

May 22–27, 1977
Davos, Switzerland

International Symposium on Microchemical Techniques 1977

Contact:
Dr. W. Merz, BASF A.G. Untersuchungslaboratorium, WHU, D-6700 Ludwigshafen, G.F.R. (Further details published in Vol. 117, No. 1)

July 17–22, 1977
Birmingham, Great Britain

4th SAC Conference

Contact:
Dr. A. Townshend or Dr. A.M.G. Macdonald, Chemistry Department, The University, P.O. Box 363, Birmingham B15 2TT, Great Britain.

September 27–30, 1977
Salzburg, Austria

3rd International Symposium on Column Liquid Chromatography

Contact:
Professor Dr. J.F.K. Huber, Analytisches Institut der Universität Wien, Währinger Strasse 38, A-1090 Vienna, Austria

PUBLICATION SCHEDULE FOR 1976

Journal of Chromatography (incorporating *Chromatographic Reviews*)

MONTH	D 1975	J	F	M	A	M	J	J	A	S	O	N	D
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* Volume 127 will consist of *Chromatographic Reviews*. The issues comprising this volume will not be published consecutively, but will appear at various times in the course of the year.

GENERAL INFORMATION

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

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

Submission of Papers. Three copies of manuscripts in English, French or German should be sent to: Editorial office of the Journal of Chromatography, P.O. Box 681, Amsterdam, The Netherlands. For *Review articles*, an outline of the proposed article should first be forwarded to the Editorial office for preliminary discussion prior to preparation.

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

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

Summary. Full-length papers and Review articles should have a summary of 50–100 words. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes will be published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Particular attention should be paid to the size of the lettering to ensure that it does not become unreadable after reduction. Sharp, glossy photographs are required to obtain good halftones. Each illustration should have a legend, all the *legends* being typed together on a *separate sheet*. Coloured illustrations are reproduced at the author's expense.

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

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

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

Proofs. Two sets of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

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Publication. The *Journal of Chromatography* (including *Chromatographic Reviews*) appears fortnightly and has 14 volumes in 1976. The subscription price for 1976 [Vols. 115–128 and Supplementary Vol. 5 (Bibliography of Paper and Thin-Layer Chromatography 1970–1973)] is Dfl. 1470.00 plus Dfl. 180.00 (postage) (total US$ 660.00). Subscribers in the U.S.A., Canada and Japan receive their copies by air mail. Additional charges for air mail to other countries are available on request. Back volumes of the *Journal of Chromatography* (Vols. 1 through 114) are available at Dfl. 100.00 plus postage. Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge.

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