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# JOURNAL OF CHROMATOGRAPHY

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*(Continued overleaf)*

# DRUGS OF ABUSE

*by* LAWRENCE FISHBEIN, *National Center for Toxicological Research, Jefferson, AR, and University of Arkansas for Medical Sciences, Little Rock, AR, U.S.A.*

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The main objective of this volume is to provide the analytical and clinical chemist, as well as the forensic toxicologist, with a practical text which is also a literature reference source, of selected descriptive chromatographic procedures that stress the separation, detection and quantification of a spectrum of drugs of abuse, e.g. depressants, narcotics and analgesics, stimulants and hallucinogens, from biological, licit and illicit samples. The principal methods are thin-layer chromatography, gas-liquid chromatography, gas-liquid chromatography-mass spectrometry and high performance liquid chromatography. The procedures described are those which are primarily useful in therapeutic monitoring and pharmacokinetic studies, emergency, clinical, forensic and toxicological analyses and in monitoring in drug abuse screening programs.

Relevant information has been included, where available, regarding the synthesis or isolation from natural sources, the salient chemical, biological and physical properties, as well as the pharmacology, metabolism, pharmacokinetics, disposition and toxicity of the abused drugs. This information is important to investigators in diverse disciplines including biochemistry, clinical chemistry and medicine, pharmacology, toxicology, public health, and others such as clinicians and drug counselors who are all equally concerned with detecting, ameliorating, managing or eliminating the effects of this social and environmental hazard.

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# EVALUATION OF ANALYTICAL METHODS IN BIOLOGICAL SYSTEMS

## Part A: Analysis of Biogenic Amines

*edited by* GLEN B. BAKER *and* RONALD T. COUTTS, *Neurochemical Research Unit, Department of Psychiatry, and Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Canada*

TECHNIQUES AND INSTRUMENTATION IN ANALYTICAL CHEMISTRY, 14

This is the first volume of a new multi-volume work entitled "Evaluation of Analytical Methods in Biological Systems" that will evaluate the various analytical techniques and approaches that can be used in a particular bioanalytical field. This volume provides an overview of techniques for the analysis of catecholamines, 5-hydroxytryptamine (serotonin), 'trace amines' and histamine in biological sytems.

The authors describe the basic principles underlying the various techniques and discuss their advantages and disadvantages relative to other available methods, thus making this a valuable reference both for those experienced in analysis of biogenic amines who are considering using a new technique, and to those entering the field for the first time.



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# SOLVENT SELECTIVITY IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF ISOMERS OF POLYSTYRENE OLIGOMERS

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(Received February 23rd, 1983)

## SUMMARY

Isotactic, syndiotactic and atactic isomers of oligomers of low-molecular-weight polystyrene were successfully resolved using a highly efficient $C_{18}$ column and an acetonitrile–methylene chloride gradient. Structural assignments for some of the collected trimer peaks were made by gas chromatography–mass spectrometry and $^{13}C$ nuclear magnetic resonance analysis. After the initial separation, 27 different mobile-phase solvents, several reversed-phase bonded packings, and different column temperatures were tested in an effort to maximize the resolution of the stereoisomers. Peak separation ($P_i$) for each peak pair and chromatographic response function (CRF) for each chromatogram were calculated and compared. Only a few of the solvents tested produced stereoisomer separation. The sample solubility of polystyrene in the mobile-phase solvent appeared to be the best predictor for an optimum mobile phase. Only "weak" solvents, those solvents that could not easily dissolve polystyrene, gave good isomer separation when used as the mobile phase. Hansen's solubility model appears promising for optimizing the mobile-phase composition for separations of other solutes using reversed-phase high-performance liquid chromatography. A phenyl bonded phase, a $C_8$ bonded phase, and a synthesized perfluorinated bonded phase were compared to octadecylsilane. Only the $C_8$ column produced isomer separation. Decreasing the column temperature resulted in better resolution of the stereoisomers at the expense of longer retention times. Based on the high-performance liquid chromatographic data, it has been proposed that the mobile-phase affects both the conformations of the polystyrene stereoisomers and of the long-chain hydrocarbon bonded phases. The resolution of stereoisomers by capillary gas chromatography was found to be significantly less than that obtained by high-performance liquid chromatography with the $C_8$ and $C_{18}$ columns.

## INTRODUCTION

Polystyrene has long been used as a model for predicting the properties and

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behavior of other polymers. It is available in the form of well characterized samples having narrow dispersivity. Ready detectability of its oligomers by ultraviolet (UV) and refractive index (RI) measurements is also an advantage.

Many reports on the separation of polystyrene have been concerned with optimizing the fractionation of oligomers[1–6]. Some of these reports discuss "undesirable" peak splitting or misshapen peaks that have also been observed using supercritical fluid chromatography (SFC)[1], as well as normal-phase[2,3] and reversed-phase (RP) high-performance liquid chromatography (HPLC)[4]. This splitting of oligomer peaks has sometimes been ascribed to sampling difficulties and to effects other than adsorption or the separation of stereoisomers. To date, no reported attempt has been made to increase or optimize the resolution of these split peaks in RP-HPLC. In a recent investigation on the separation of stereoisomers of polystyrene by normal-phase HPLC, the separation was explained in terms of differences of repeat-unit rotational barriers and chain coiling between oligomer isomers, but an optimization scheme was not presented[3].

For several years, there has been increasing interest in the development of a general framework for predicting retention and selectivity in RP-HPLC. The most widely used method for predicting selectivity in liquid chromatography was proposed by Snyder and Kirkland[5]. Their solvent selectivity concept classifies the solvents according to relative proton-donating ($X_d$), proton-accepting ($X_e$), and dipole moment ($X_n$) properties. The contributions of each type of interaction are expressed as fractions of the total rather than on an absolute scale[7]. Not all solvents can be grouped in the triangle, *e.g.*, cyclohexane, carbon tetrachloride. Furthermore, this concept for predicting selectivity does not consider the properties of the solutes that are to be separated.

Several good methods for the prediction of solvent strength for HPLC have been developed which are based on experimental solubility data reported by Rohrschneider[21] and Snyder[5]. The solubility parameter concept has been used extensively in normal-phase HPLC; however, in the special case of reversed-phase systems, a more precise solvent strength parameter, $S'$, for several solvents have been experimentally determined and reported[5].

A similar approach has been used to predict the solubility of polymers[8]. In this case the absolute contributions of the molecular interactions rather than their relative values were employed. The cohesive energy, which is related to the solubility parameter, was divided into three types of interaction forces: hydrogen bonding ($\delta h$), dispersion ($\delta d$) and polarity ($\delta p$) forces. Hansen[9] developed a method for the indirect determination of $\delta h$, $\delta d$ and $\delta p$ for a number of solvents and polymers. A three-dimensional plot of the polymer and solvents of interest, with each force on a separate axis, was then used to predict solubility. As the distance between the plotted polymer and solvent increases, tendency towards dissolution decreases. This method does have the disadvantage that three-dimensional structures are necessary for a graphical representation of the interaction between polymers and solvents; thus, a two-dimensional method is preferred.

Bagley *et al.*[10] concluded that the effects of dispersion and polarity show close similarity, while the effect of hydrogen bonding is of a considerably different nature. Thus, they introduced the parameter $\delta v = \sqrt{(\delta d^2 + \delta p^2)}$. This led to a diagram in which $\delta v$ and $\delta h$ were plotted on two axes. At the present time, this $\delta v$–$\delta h$ diagram

appears to be the most efficient way to represent polymer–solvent interactions. From this plot a predicted circle could be drawn for each solute, *e.g.* a given polymer. Those solvents inside the circle are generally capable of dissolving the polymer while those outside the circle exhibit low or no solubility for the polymer. Since interactions between solute and solvent in the mobile phase are important in governing retention, it seemed worthwhile to examine the ability of this solubility model to predict solvent strength and selectivity in the RP-HPLC separation of isomers of polystyrene oligomers.

Most of the recent work on predicting retention and selectivity in RP-HPLC has focused on the behavior of model compounds containing different functionalities. This large diversity of the substituent groups provides insight into general optimization schemes[5,11]. An interesting alternative is to examine the chromatographic behavior of model compounds that possess very similar structures and properties, such as the separations of stereoisomers of polystyrene oligomers.

The first objective of this work was to examine the effect of different mobile-phase solvents in RP-HPLC on the separation of polystyrene stereoisomers. A second objective was to make structural assignments of some of the stereoisomers which could provide information that would permit an understanding of the basis of the separation. Finally, and most importantly, a theoretical basis for the isomer separations and a scheme for optimum mobile-phase selection for future isomer separations of other polymers and, perhaps, other types of compounds by RP-HPLC was desired.

## EXPERIMENTAL

### *Chemicals*

A total of 27 solvents obtained from various sources were used as received unless otherwise noted. Acetic acid, acetone, acetonitrile, carbon tetrachloride, chloroform, cyclohexane, diethyl ether, dimethylformamide, ethyl acetate, ethylene chloride, isopropanol, methanol, methylene chloride, nitromethane, and tetrahydrofuran were either Baker "photrex" or reagent grade (J. T. Baker, Phillipsburg, PA, U.S.A.). Tetrahydrofuran was distilled over 5:1 potassium–sodium alloy to remove water and butylated hydroxytoluene (BHT). *n*-Butanol, 1-chlorobutane, 2-cyanoethyl ether, 2-methoxyethanol, nitroethane and propylene carbonate were obtained from Aldrich (Metuchen, NJ, U.S.A.). Technical-grade 2-cyanoethyl ether and nitroethane were distilled to remove impurities. ACS-grade dimethyl sulfoxide and dioxane were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Dodecafluoroheptanol and tetrafluoropropanol were obtained from PCR (Gainesville, FL, U.S.A.). Absolute ethanol was purchased from U.S. Industrial Chemicals (New York, NY, U.S.A.). "House" distilled water was passed through a deionizing system and a Corning Mega-Pure 1 L still and then collected in glass bottles. All solvents were thoroughly degassed with helium before use.

Monodisperse 800 molecular weight (MW) polystyrene samples were obtained from Pressure Chemical (Pittsburgh, PA, U.S.A.). Monodisperse 666 MW polystyrene samples were acquired from Arro Labs. (Joliet, IL, U.S.A.). For UV detection, the 800 MW and 666 MW polystyrene were dissolved in methylene chloride–acetonitrile (1:19) at a concentration of 20 mg/ml. For RI detection, the 800 MW polystyrene was dissolved in 1:1 methylene chloride–acetonitrile at a concentration of 200 mg/ml.

For the capillary gas chromatography (GC) separation, the 666 MW polystyrene was dissolved in iso-octane at a concentration of 300 mg/ml. For the GC–mass spectrometric (MS) analysis, the two trimer fractions were dissolved in chloroform at a concentration of 300 mg/ml.

IBM $C_{18}$ and $C_8$ HPLC columns (IBM Instruments, Yalesville, CT, U.S.A.) (25 cm × 4.5 mm I.D.), having end-capped, spherical, 5-$\mu$m particles and 100-Å pore sizes, were used as received. Phenyl bonded-phase column packing having spherical, 7.5-$\mu$m particles and 100-Å pore sizes was obtained from Macherey, Nagel & Co. (Düren, G.F.R.). A glass GC column (6 m × 2 mm I.D.) coated with 3% OV-1 on 100–120 mesh Gas Chrom Q (Applied Science, State College, PA, U.S.A.) was used as received for GC–MS analysis. 1H,1H,2H-Perfluoro-1-decene (Columbia Organic Chemicals, Columbia, SC, U.S.A.) dimethyl monochlorosilane (Petrarch Systems, Bristol, PA, U.S.A.) and Licrospher SI 100, 5-$\mu$m particles (Batch No. YE605, Charge No. 8554867, E. Merck, Darmstadt, G.F.R.) were used for the preparation of the heptadecafluorodecyl-dimethylsilyl (RPF-10) bonded-phase packing. A fused-silica capillary GC column (15 cm × 0.32 mm I.D.) coated with DB-1 from J. and W. Scientific (Rancho Cordova, CA, U.S.A.) was also used as received.

*Apparatus*

A DuPont HPLC system consisting of a Model 8800 gradient controller, a Model 870 pump module, an oven compartment for the column, a manually operated Rheodyne Model 7125 injector having a 10-$\mu$l injection loop, and a Model 852001-901 UV variable-wavelength spectrophotometer was used to generate solvent gradients. An RI detector from Varian Associates (Palo Alto, CA, U.S.A.) together with an Altex Model 110A solvent metering pump (Altex, Berkeley, CA, U.S.A.) and an air-actuated six-port valve, Model ACV-6UHPa (Valco, Houston, TX, U.S.A.) having a 25-$\mu$l injection loop, were used to perform most of the isocratic separations. Chromatograms were recorded using a Linear Instruments Model 385 dual-pen chart recorded (Linear Instruments, Irvine, CA, U.S.A.) for UV detection and an Omniscribe Recorder (Houston Instruments, Houston, TX, U.S.A.) for RI detection.

The two trimer fractions were collected using a Model 270 fraction collector (ISCO, Lincoln, NE, U.S.A.). A Finnigan 4000 quadrupole GC–MS system (Finnigan, Sunnyvale, CA, U.S.A.) and a Nicolet $^{13}C$ nuclear magnetic resonance (NMR) system (Nicolet Instrument Group, Madison, WI, U.S.A.) were used to characterize the trimer fractions. A Hewlett-Packard 5880 GC with an on-column capillary injector was used to obtain the results for comparison with those obtained by HPLC.

*Procedures*

The RPF-10 bonded-phase packing was synthesized according to the procedure reported by Berendsen *et al.*[12,13]. The phenyl and heptadecafluorodecyl columns were constructed from precision-bore 316 stainless-steel tubing (25 cm × 4.5 mm I.D.; Alltech, Arlington Heights, IL, U.S.A.) that had been rinsed successively with 6 *M* nitric acid, distilled water, methanol and tetrahydrofuran. The tubing was then blown dry with nitrogen. The column was terminated using Swagelock® stainless-steel fittings (Crawford Fitting, Solon, OH, U.S.A.) and 0.5-$\mu$m frits from Alltech. The column was then packed at 6000 p.s.i. using a Micromeritics Model 705-P slurry packer (Micromeritics, Norcross, GA, U.S.A.) and a Varian Model 8500

pump equipped with a solvent programmer. The packing solvent for the phenyl and heptadecafluorodecyl bonded phases were chloroform–cyclohexane (2:1) and 100% carbon tetrachloride, respectively. After the packing pressure had been reached, the slurry was forced into the column until it was filled.

Before each successive run, all columns were prepared for sample injection by passing 20 column volumes of tetrahydrofuran or methylene chloride through before returning to the desired mobile-phase composition for the next injection. Fewer column volumes, *ca.* 10, of tetrahydrofuran or methylene chloride were later used and found to have no effect on the chromatograms.

Sample injection was performed in the following manner. Once the baseline had stabilized, the sample loop was filled and the sample injected. The mobile phase flow-rate was held at 1.0 ml/min for all separations and confirmed volumetrically by collecting the column effluent for an appropriate length of time. In solvent gradient studies, the gradient was initiated at the time of injection. Two gradients were run on the $C_{18}$ and $C_8$ columns. The first gradient went from 100% acetonitrile to acetonitrile–methylene chloride (50:50) in 30 min. The second gradient went from acetonitrile–water (80:20) to 100% acetonitrile in 30 min, was held at 100% acetonitrile for 10 min, and was then changed to acetonitrile–methylene chloride (50:50) in 30 min. The gradient performed on the phenyl column started at acetonitrile–water (70:30), was ramped to acetonitrile–water (90:10) in 30 min, and was held at this composition until the end of the run. The mobile-phase composition was held at acetonitrile–water (60:40) for the RPF-10 column.

Mixed-solvent studies using isocratic and gradient profiles were performed on the $C_{18}$ column in an effort to duplicate the Snyder selectivity properties of the gradient that started with acetonitrile and changed to acetonitrile–methylene chloride (50:50) in 30 min. The solvent combinations and proton-donating ($X_d$), proton-accepting ($X_e$) and dipole moment ($X_n$) properties are presented in Table I.

The rest of the separations were performed isocratically. After elution of the polystyrene pentamer, higher oligomers were quickly removed by elution using only strong solvent. For isocratic separations, water-miscible solvents were appropriately diluted in order to maintain the capacity factor, $k'$, of the two trimer peaks between 9 and 10. Since $k'$ is a measure of the solvent strength of the mobile phase, approximately equal $k'$ values will provide relatively constant mobile phase solvent strengths, which is necessary for selectivity comparisons. The extent of dilution was determined in the following way. The percentage of water necessary in the mobile phase was first approximated by using the reported values[5] for the solvent strength parameter, $S$, then further adjusted, based on the polymer retention data obtained, until the $k'$ values were within the desired range.

Fraction collection of the two trimer peaks was carried out using the $C_{18}$ column and a gradient from acetonitrile–water (80:20) to 100% acetonitrile in 20 min. After an appropriate number of injections (usually at least 50), the solvent was removed from the two collected fractions by a flash evaporator (Buchler, Fort Lee, NJ, U.S.A.) and the fractions were dried *in vacuo* over $PCl_5$. These fractions were then further purified by repeating the process until a single peak for each of the two trimer fractions was obtained (>99% purity).

Approximately 1 μl of each of these two trimer peaks (*ca.* 300 μg/ml) was injected into a GC–MS system using the following chromatographic conditions. The

TABLE I

ISOCRATIC AND GRADIENT SOLVENT COMBINATIONS AND THEIR SNYDER CONSTANTS

| | $X_e$ | $X_d$ | $X_n$ |
|---|---|---|---|
| UV detection —30-min gradient | | | |
| Chloroform | 0.25 | 0.41 | 0.33 |
| Tetrahydrofuran | 0.38 | 0.20 | 0.42 |
| Ethylene chloride | 0.30 | 0.21 | 0.49 |
| Initial chloroform–tetrahydrofuran–ethylene chloride (33:33:34) | 0.31 | 0.27 | 0.41 |
| Final chloroform–tetrahydrofuran–ethylene chloride (10:10:80) | 0.30 | 0.23 | 0.48 |
| RI detection —in five isocratic steps | | | |
| Nitroethane | 0.28 | 0.29 | 0.43 |
| Tetrahydrofuran | 0.38 | 0.20 | 0.42 |
| Ethylene chloride | 0.30 | 0.21 | 0.49 |
| Initial nitroethane–tetrahydrofuran (50:50) | 0.33 | 0.25 | 0.43 |
| Final nitroethane–tetrahydrofuran–ethylene chloride (25:25:50) | 0.31 | 0.23 | 0.46 |
| Original system | | | |
| Acetonitrile | 0.31 | 0.27 | 0.42 |
| Methylene chloride | 0.29 | 0.18 | 0.53 |
| Initial acetonitrile | 0.31 | 0.27 | 0.42 |
| Final acetonitrile–methylene chloride (50:50) | 0.30 | 0.23 | 0.48 |

injector and detector temperatures were maintained at 100°C and 300°C, respectively. The oven temperature was initially held at 100°C for 4 min, then changed to 400°C at 160°C/min. Electron impact (EI) (70 eV) spectra were obtained on each peak. $^{13}C$ Nuclear magnetic resonance (NMR) spectra were obtained from *ca.* 3 mg of the first trimer peak and 9 mg of the second trimer peak. Using $10^5$ scans on each sample, spectral regions of 14–45 ppm and 120–140 ppm were recorded.

The 666 MW polystyrene was separated by capillary GC. A 1-μl aliquot of a 300 μg/ml solution was injected. The column head pressure was maintained at 20 p.s.i.g. (helium), providing a linear flow-rate of 120 cm/sec. The injector and detector temperatures were 50°C and 300°C, respectively. The oven temperature was initially set at 50°C for 4 min, then changed at 8°C/min to a final temperature of 350°C.

*Calculations*

The capacity factor, $k'$, was calculated according to the following equation:

$$k' = \frac{t_R - t_0}{t_0}$$

where $t_R$ is the retention time of a particular component (in this case the trimer of polystyrene) and $t_0$ is the value for an unretained peak. Values of $t_0$ were obtained by injecting a small volume of solvent having a slightly weaker composition than the actual mobile phase. The RI detector was then used to monitor the first peak resulting from this injection (avoiding the total exclusion peak) to determine the $t_0$ value.

In order to compare the separation quality of the isocratic separations ob-

tained with each solvent, the peak separation of the isomers of each oligomer from trimer through pentamer was calculated for those solvents that gave isomer separations. Peak separation is defined as follows:

$$P_i = \frac{f}{g}$$

where $P_i$ is the peak separation of the $i$th pair of peaks in a system with $k$ total pairs of interest, $f$ is the depth of the valley below a straight line connecting two adjacent peak maxima and $g$ is the height of the straight line above the baseline of the valley[14,15]. The peak separation numbers were then used to calculate the chromatographic response function (CRF), which allows one to judge conveniently the overall separation quality or chromatographic performance of each set of conditions. In the CRF, overall resolution of all peaks is important:

$$\text{CRF} = \sum_{i=1}^{k} \ln P_i$$

The total number of peak pairs of interest in the isocratic separations was seven; *i.e.*, one for the trimer and three each for the tetramer and pentamer.

## RESULTS AND DISCUSSION

### *Structural assignments*

The first gradient, performed with acetonitrile–methylene chloride on a highly efficient octadecylsilane column, produced multiple peaks for each oligomer of the 800 MW polystyrene, as shown in Fig. 1A. The excellent resolution observed for the multiple peaks of polystyrene is the best reported thus far for RP-HPLC. Addition of water to the mobile phase greatly improved the separation but at the expense of longer retention times, as seen by comparison of Fig. 1A and B and by examination of the peak separation factors and CRF values in Table II. Since the second gradient gave almost baseline resolution for the two trimer peaks, these conditions were used for fraction collection.

Structural elucidations of the two presumed trimer peaks were performed by GC–MS and $^{13}C$ NMR. The GC–EI-MS of the two collected fractions (Fig. 2) produced the same fragmentation pattern and molecular ion (370.3 *m/e*). Therefore, both peaks collected were trimers having the same general structure $ArCH_2CH_2CH(Ar)CH_2CH_2(Ar)(CH_2)_4CH_3$, where $Ar = C_6H_5$ (Fig. 3). The *n*-butyl end-group of each oligomer arose from the polystyrene polymerization process in which *n*-butyllithium was added as an initiator[16,17]. Structures I and II and structures II and IV are enantiomeric pairs which are distinguishable by NMR.

The $^{13}C$ NMR spectra obtained on the two trimer peaks are presented in Fig. 4A and B, and they show clear differences, suggesting two different enantiomeric pairs. Based on work reported by Breitmaier and Bauer[18] concerning the $^{13}C$ NMR chemical shifts of aromatic carbons in atactic and isotactic polystyrene, the differences in the region *ca.* 128.2 ppm in the two spectra permit one to identify the first trimer peak as atactic (in this case syndiotactic) isomers (structures III and IV). The second

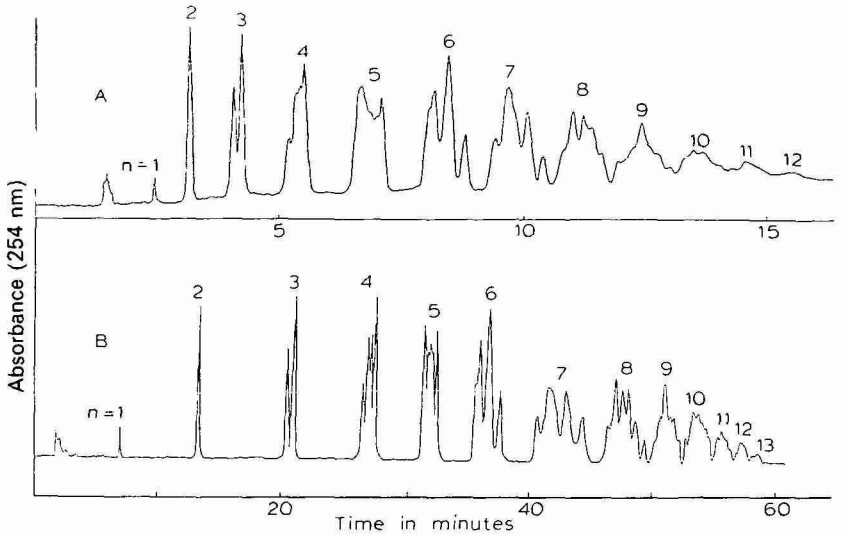



Fig. 1. Chromatograms of 800 MW polystyrene separation on a $C_{18}$ column with gradient (A), acetonitrile to acetonitrile–methylene chloride (50:50) in 30 min, and (B), acetonitrile–water (80:20, v/v) to acetonitrile in 30 min, held at acetonitrile for 10 min, then changed to acetonitrile–methylene chloride (50:50) in 30 min.

trimer peak is isotactic (structures I and II). On the basis of steric factors, the isotactic isomer should interact to a greater extent with the $C_{18}$ stationary phase than the syndiotactic or atactic isomers and thus be retained longer, an expectation consistent with the $^{13}C$ NMR data obtained.

*Solvent studies*

A few solvents that did not give isomer separation were selected and mixed together so that the Snyder selectivity properties (proton donating, proton accepting, and dipole moment) were approximately equivalent to those of the initial acetonitrile–methylene chloride gradient (see Table I). The severe limitations on solvent selection were primarily miscibility problems with solvents from different selectivity groups. These mixed-solvent attempts failed to produce any isomer separation in isocratic runs.

TABLE II

CALCULATED PEAK SEPARATIONS ($P_i$) AND CHROMATOGRAPHIC RESPONSE FUNCTIONS (CRF) FOR GRADIENTS PERFORMED ON A $C_{18}$ COLUMN

| *Gradient* | *Peak pair* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Trimer, 1–2* | *Tetramer* | | | *Pentamer* | | | *CRF* |
| | | *3–4* | *4–5* | *6–7* | *7–8* | *8–9* | *9–10* | |
| A | 0.88 | 0.61 | 0.07 | 0.16 | 0.14 | 0.01 | 0.21 | −13.3 |
| B | 0.91 | 0.67 | 0.22 | 0.61 | 0.27 | 0.07 | 0.54 | −6.7 |

* See gradient profiles in Fig. 1.

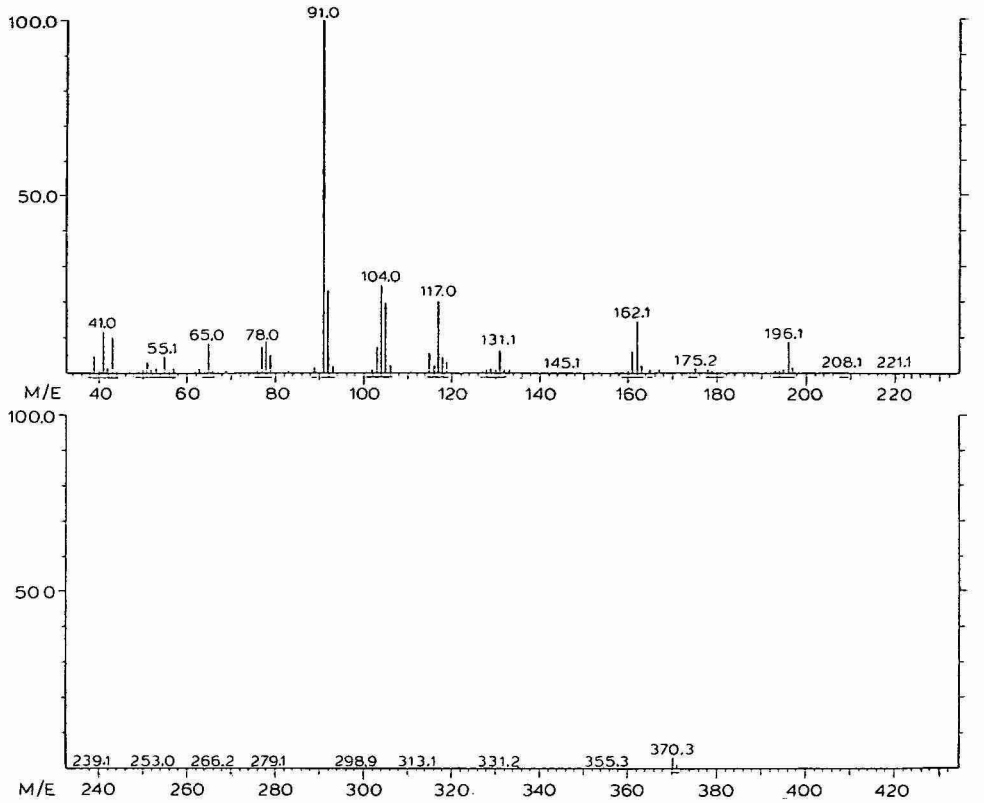
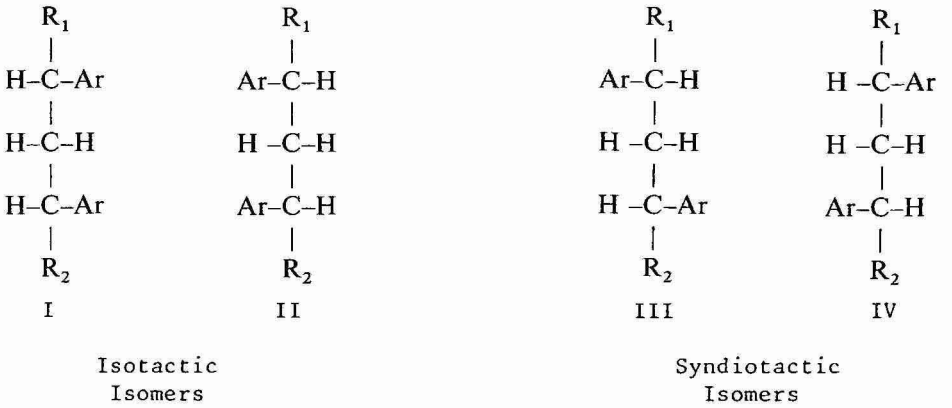



Fig. 2. Electron impact mass spectra of the two trimer peaks using gradient (B) of Fig. 1.




$Ar = C_6H_5$, $R_1 = ArCH_2CH_2$, $R_2 = (CH_2)_4CH_3$

Fig. 3. Four possible structural configurations for the trimer of polystyrene.

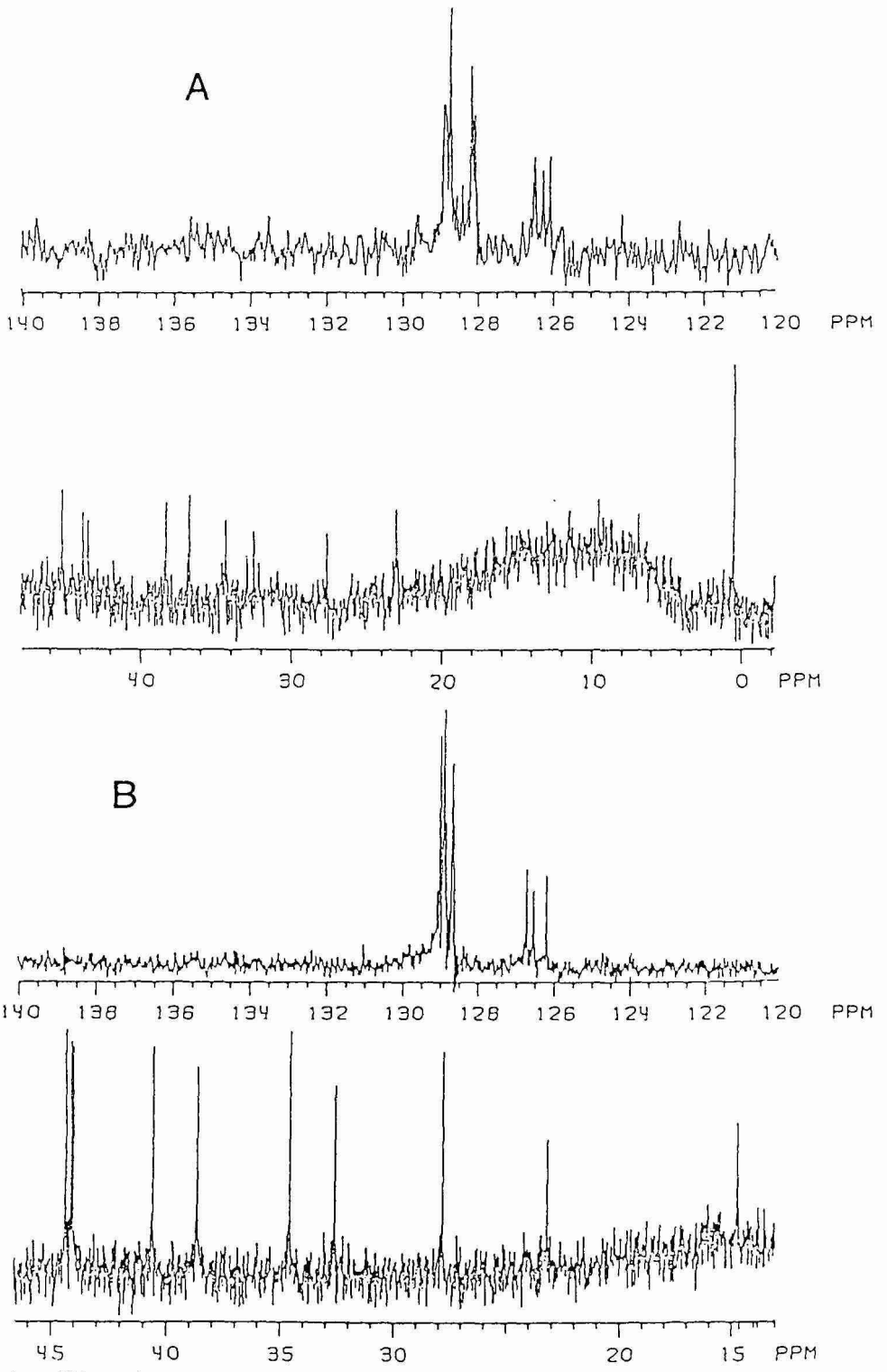



Fig. 4. $^{13}C$ NMR spectra of the two trimer peaks using gradient (B) of Fig. 1.

TABLE III

CALCULATED PEAK SEPARATIONS ($P_i$) AND CHROMATOGRAPHIC RESPONSE FUNCTIONS (CRF) FOR SOLVENTS PRODUCING ISOMER SEPARATION ON A $C_{18}$ COLUMN

| *Solvent composition* | *Peak separations ($P_i$)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Trimer, peak pair* | *Tetramer* | | | *Pentamer* | | | *CRF* |
| | *1–2* | *3–4* | *4–5* | *6–7* | *7–8* | *8–9* | *9–10* | |
| Propylene carbonate (100) | 1.00 | 0.81 | 0.16 | 0.96 | 0.49 | 0.28 | 1.00 | −4.05 |
| Nitromethane (100) | 0.95 | 0.82 | 0.34 | 0.79 | 0.72 | 0.15 | 0.83 | −4.00 |
| Dimethyl sulfoxide–water (90:10) | 0.92 | 0.71 | 0.34 | 0.38 | 0.33 | 0.19 | 0.54 | −6.63 |
| Acetonitrile–water (80:20) | 0.80 | 0.56 | 0.34 | 0.31 | 0.40 | 0.15 | 0.34 | −8.94 |
| 2-Cyanoethyl ether–water (80:20) | 0.70 | 0.54 | 0.14 | 0.25 | 0.35 | 0.03 | 0.42 | −9.74 |
| Ethanol–water (87:13)* | – | ( | 0.26 | ) | ( | 0.09 | ) | −∞ |
| Methanol–water (90:10)* | – | ( | 0.14 | ) | ( | 0.32 | ) | −∞ |

* For the methanol and ethanol mobile phases, peak separations were calculated on the basis of a single peak for trimer and only two peaks for tetramer and pentamer.

A number of mobile-phase solvents were evaluated for their ability to separate oligomer stereoisomers. Solvents were selected from each of Snyder's solvent selectivity groups. These separations were performed isocratically using an RI detector. In all, 27 solvents were investigated. Fig. 5 shows the solvents tested in their Snyder selectivity triangle and Table III lists the separation factors and the CRF values for those solvents that produced isomer fractionation. Partial or complete isomer separation was observed for seven solvents. From the CRF values, propylene carbonate and nitromethane appeared to give the best overall isomer separation, followed by dimethyl sulfoxide, acetonitrile, and 2-cyanoethyl ether, respectively. Methanol and ethanol each gave a slight hint of isomer fractionation in the form of a slight shoulder. Examples of chromatograms from solvents that produced complete (propylene carbonate), partial (methanol), and no (tetrahydrofuran) stereoisomer separation are shown in Fig. 6. The shapes of the resolved peaks suggested the same order of the isomers in different solvents.

It is evident from Fig. 5 that the Snyder solvent selectivity scheme, although good for a first approximation, does not accurately predict selectivity for this separation. Solvents from *different* groups gave isomer separations, yet solvents within the same group showed widely different selectivity for the isomers of the oligomers. Thus, one can not determine accurately which of the Snyder selectivity properties (proton accepting, proton donating or dipole moment) is important in the separation.

Sample solubility in a *pure* solvent appeared to be extremely important in achieving isomer separation, and it was a much better predictor for selecting those mobile phases that eventually provided isomer separations. This factor also appeared to be important in the separation of oligomers[2]. Using too strong a pure solvent as the mobile phase, *i.e.*, one that too readily dissolved the polystyrene, *even after adjusting solvent strength with water*, resulted in no isomer resolution. The strengths of solvents that gave good isomer separations were determined to be substantially lower than those reported in the literature[5] (see Table IV). Addition of water to the mobile

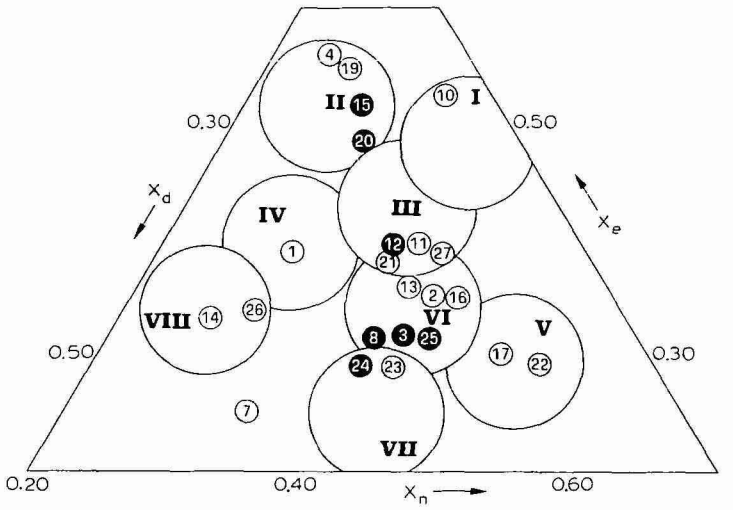



Fig. 5. Snyder selectivity triangle for the solvents tested. Solid numbers correspond to those solvents producing isomer separations. Clear numbers represent those solvents producing no isomer separations. The numbers correspond to: 1 = acetic acid; 2 = acetone; 3 = acetonitrile; 4 = *n*-butanol; 5 = carbon tetrachloride; 6 = 1-chlorobutane; 7 = chloroform; 8 = 2-cyanoethyl ether; 9 = cyclohexane; 10 = diethyl ether; 11 = dimethylformamide; 12 = dimethyl sulfoxide; 13 = dioxane; 14 = dodecafluoroheptanol; 15 = ethanol; 16 = ethyl acetate; 17 = ethylene chloride; 18 = hexane; 19 = isopropanol; 20 = methanol; 21 = 2-methoxyethanol; 22 = methylene chloride; 23 = nitroethane; 24 = nitromethane; 25 = propylene carbonate; 26 = tetrafluoropropanol; 27 = tetrahydrofuran. *Note:* Not all solvents tested could be plotted on this diagram: carbon tetrachloride, 1-chlorobutane, cyclohexane, hexane.

phase, however, did not appear to change the selectivity of the organic solvent, weak or strong, but only increased the retention time. For example in tetrahydrofuran, a 10% increase in the water component resulted in an increase in retention time by a factor of *ca.* 2.

A plot in Fig. 7 of Hansen's hydrogen bonding forces *vs.* combined dispersion

TABLE IV

PERCENTAGE OF WATER EXPECTED AND OBSERVED WITH VARIOUS SOLVENTS FOR EQUIVALENT SOLVENT STRENGTH

| *Solvent* | *Water expected (%)** | *Water necessary (%)*** | *Reported $S'$ value used* |
|---|---|---|---|
| Acetonitrile | – | 20 | 3.1 |
| Acetone | 22 | 63 | 3.4 |
| Dioxane | 23 | 65 | 3.5 |
| Ethanol | 23 | 13 | 3.6 |
| Isopropanol | 27 | 23 | 4.2 |
| Methanol | 19 | 10 | 3.0 |
| Tetrahydrofuran | 28 | 68 | 4.4 |

* Percentage of water expected for an equivalent solvent strength of an acetonitrile–water (80:20) mixture using reported $S'$ values[5].

** Water necessary in the mobile phase in order to make the capacity factor of the trimer of polystyrene equivalent in each chromatogram.

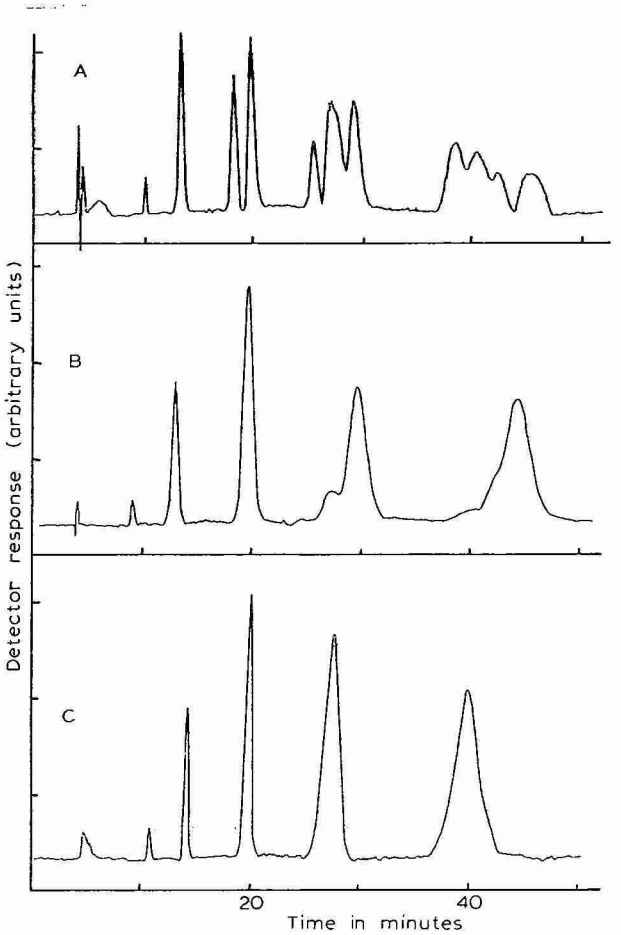



Fig. 6. Chromatograms of 800 MW polystyrene under isocratic conditions showing typical separations by solvents producing (A) good isomer resolution (propylene carbonate), partial isomer resolution (methanol–water, 90:10) and no isomer resolution (tetrahydrofuran–water, 60:40).

forces and polarity forces[9] was a much better model for predicting solvent strength and selectivity for this particular separation. All of the solvents tested that lay inside the solubility circle gave no diastereomer separation while all of the tested solvents that produced at least some hint of isomer separation lay outside the circle. Furthermore, solvents in a particular area of the graph or those with high dispersion and polarity contributions as well as medium hydrogen-bonding contributions gave excellent diastereomer separations. Dimethylformamide was the exception only because this model was not able to predict solubility or solvent strength in all cases. In other words, polystyrene was observed to be very soluble in dimethylformamide and, therefore, it was not expected to give diastereomer separation. This is still consistent with the idea that the diastereomer separation is controlled by the forces governing the solubility of polystyrene in the pure solvent, particularly dispersion and polarity forces.

Stationary-phase effects were not thoroughly tested, but results on the $C_8$,

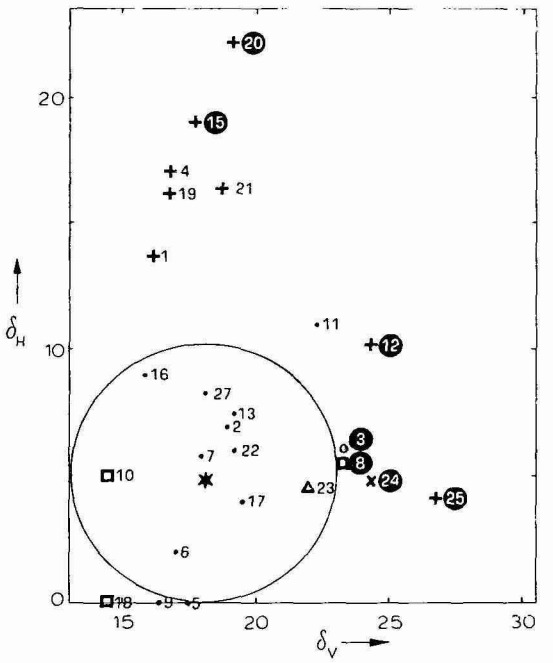



Fig. 7. Solubility of polystyrene in solvents tested using a $\delta v$ ($\delta p^2 + \delta d^2$ Hansen's dispersion and polarity forces) *vs.* $\delta h$ (Hansen's hydrogen bonding forces) diagram. The symbol, *, corresponds to polystyrene. The other symbols correspond to whether polystyrene is soluble (·), almost soluble (△), strongly swollen (□), slightly swollen (×), or has no effect (+) in the various solvents tested. See the caption of Fig. 5 to identify the solvents.

phenyl, and RPF-10 columns were used for comparison with those on the $C_{18}$ column. The $C_8$ column gave very similar diastereomer separations (Fig. 8A). However, the chromatograms obtained from the gradient performed on the phenyl and RPF-10 columns (Fig. 8B and C) showed no diastereoisomer separation and much shorter retention times compared with the $C_{18}$ column. The shorter retention times would be expected for the phenyl column because the surface area was smaller and also because, as a result of steric effects, the oligomers might not interact as well with the isopropylphenyl groups bonded on the silica surface.

The data do suggest that the $C_{18}$ and $C_8$ stationary phases have significantly better interactions than the other stationary phases with the polystyrene, particularly in certain solvents. Reasons for this are not clear; however, some indirect evidence[19,20] indicates that $C_{18}$, $C_8$ and phenyl bonded phases may have a "matted" or associated form in certain mobile phases and a "brush" or "bristle" form in others. Perhaps the $C_{18}$ is in a matted form with the solvents that gave isomer separation, thereby contributing to the increasing selectivity. The phenyl column may have also been in a matted form with these solvents, but it probably could not interact as well with the polystyrene due to steric effects or the smaller molecular volume of the isopropylphenyl-bonded phase. The fluorinated stationary phase may have been ineffective because it was not in a matted or coiled configuration with the solvents tested or because it did not interact as strongly with the polystyrene. Additional investiga-

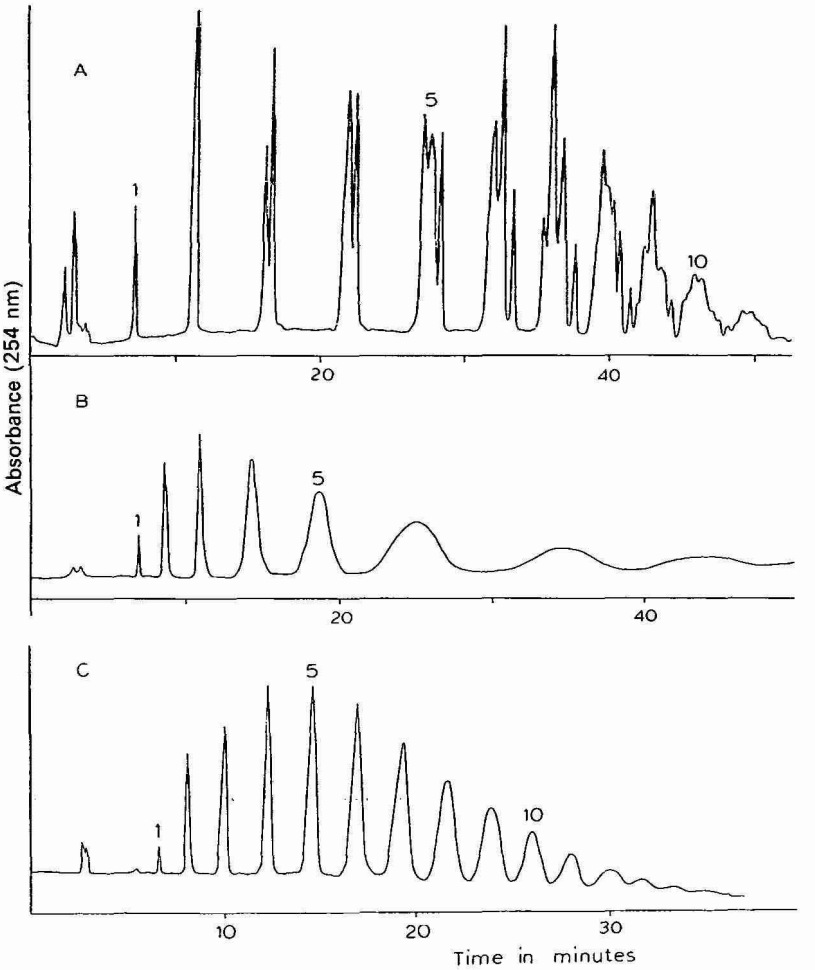



Fig. 8. Chromatogram of the 800 MW polystyrene separation on (A) a $C_8$ column using gradient (B) in Fig. 1, (B) a fluorinated bonded phase (RPF-10) column holding the mobile-phase composition at acetonitrile–water (60:40), and (C) a phenyl bonded-phase column using a gradient starting at acetonitrile–water (70:30), ramped to acetonitrile–water (90:10) in 30 min and held at this composition until the end of the run.

tions would be necessary to arrive at a better understanding of the results, but it is proposed that the solvents affect both the conformations of the polystyrene stereoisomers, resulting in differences in molecular volumes for each stereoisomer, and of the $C_{18}$ chains on the stationary phase. Furthermore, the bulky phenyl groups on the syndiotactic and atactic stereoisomers may affect the conformation of the long-chain hydrocarbon stationary phase. A proposed mechanism consistent with the results is that the hydrocarbon chain on the stationary phase lines up in a coiled fashion with the polystyrene chain when using "weak" solvents, *e.g.*, acetonitrile, nitromethane, etc., and as many bulky phenyl rings on the polystyrene as possible are pointing away from the $C_8$ or $C_{18}$ as shown in Fig. 9. Fraction collection and structural identification of the stereoisomers of the tetramer and pentamer could be used

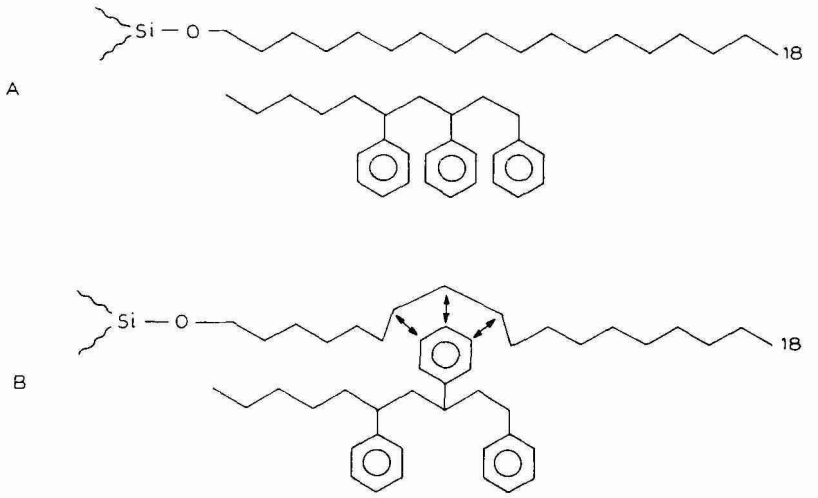



Fig. 9. Proposed mechanism for separations using the $C_{18}$ column: (A) represents the isotactic trimer interaction and (B) represents the syndiotactic trimer interaction with the $C_{18}$ chain and of the two pairs of the trimer diastereomers of polystyrene.

to test this idea; however, obtaining the necessary amounts of the *pure* species for $^{13}C$ NMR analysis would be rather difficult owing to the poor resolution between certain stereoisomers and the small amounts of material collected from each injection.

*Temperature effects*

Temperature effects on the resolutions of the isomers were tested using the $C_{18}$ column and 100% acetonitrile (isocratic conditions). Separations carried out at *ca.* 4°C (by cooling with ice), 25°C, and 40°C indicated that increasing the temperature resulted in decreasing the retention times at the expense of decreasing the isomer resolution. However, when the $k$ values were made nearly equal by addition of water to the mobile phase, the resolution was approximately the same at all three temperatures. Water added to the 4°C, 25°C and 40°C experiments was 0, 10 and 15%, respectively.

*Gas chromatographic separations*

The capillary GC work presented in Fig. 10 was performed for comparison with the RP-HPLC separations. Under the reported conditions, elution of oligomers through $n = 9$ was possible. There are clearly two trimer and two tetramer peaks. In general, resolution for GC was considerably less than in LC where at least four tetramer peaks were noted. Although difficult to discern, the higher oligomers also showed multiple peaks in the GC chromatogram, but they were not nearly as well resolved as in the HPLC separations.

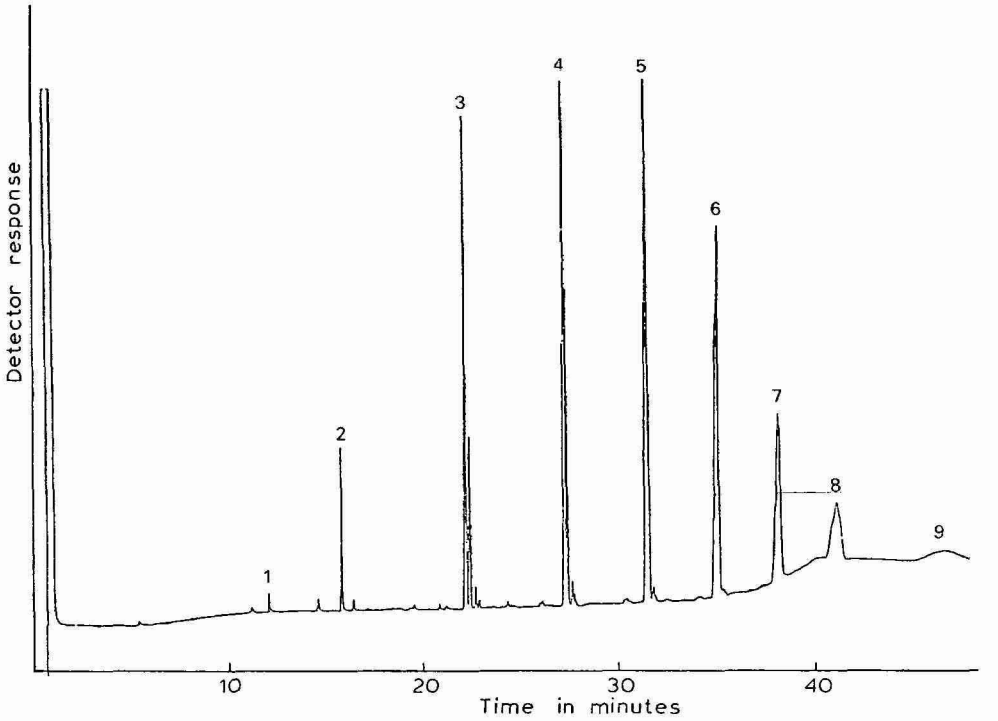



Fig. 10. Capillary GC chromatogram of 666 MW polystyrene separation using a DB-1 column. Column head pressure of $1.4 \cdot 10^5$ Pa (20 p.s.i.) provided a linear flow-rate of 120 cm/sec. Oven temperature was set at 50°C for 4 min, then ramped at 8°C/min to a final temperature of 350°C.

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# DIRECT LIQUID INTRODUCTION MICRO-LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY COUPLING

## OPTIMIZATION OF DROPLET DESOLVATION AND INSTRUMENTAL PARAMETERS FOR HIGH SENSITIVITY*

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SUMMARY

Direct coupling of microbore liquid chromatography to mass spectrometry via the Direct Liquid Introduction interface allows the mass spectrometer to be adapted to the eluent, rather than the other (usual) way that uses an external splitter to limit the liquid chromatograph eluent flow entering the mass spectrometer. We have examined a new design of desolvation chamber that achieves internal splitting with solute enrichment. This device, combined with the micro-bore column, permits utilization of the entire injected sample and therefore provides high-sensitivity liquid chromatography–mass spectrometry.

The greater ease and reproducibility in the tuning of the mass spectrometer, together with the stability of the chemical ionization conditions over a period of 1 day, permits quantitative measurements. Examples of the analysis of corticosteroids at the low-nanogram level in equine plasma and urine are presented.

## INTRODUCTION

As microbore liquid chromatography ($\mu$LC) columns (1 mm I.D.) become more readily available and more efficient, they may become more widely used to monitor low levels of drugs in biological fluids. Certain potent drugs have lower effective plasma levels and often the available sample volume is necessarily limited. The advantage of microbore columns over conventional 4.6 mm I.D. columns is that the samples can be concentrated to a suitable injection volume and, after separation,

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eventually enter the detector in a much more concentrated solution than after separation on a larger bore column.

Although the conventional liquid chromatographic (LC) detectors (UV, fluorescence and electrochemical) offer detection limits sufficient for the pharmacological studies of a wide range of drugs, they lack sufficient specificity. An extensive sample preparation before analysis is required to eliminate the interference of endogenous biological compounds. This often results in a low recovery of drug-related substances as very low levels of drugs are involved. Screening of biological fluids containing trace levels of unknown substances necessitates minimal sample preparation and a universal, though specific, detector.

The mass spectrometer coupled to a microbore column through a direct liquid introduction (DLI) interface[1] meets most of these requirements. In particular, it takes advantage of the transfer of the entire μLC column effluent to the mass spectrometer, thus promoting high sensitivity.

The DLI interface sprays the LC effluent into droplets through a pinhole diaphragm[2]. One of the most effective ways of controlling the desolvation of the solutes from the droplets is to use a heated zone, usually called a *de*solvation *c*hamber (DSC), in which the droplets acquire a high speed before entering the chemical *i*onization (CI) source[3,4]. The commercial version of the DSC installed on our instrument was designed for flow-rates in the neighborhood of 10 μl/min. It did not allow operation of μLC at its optimum flow-rate (40 μl/min) without critical, delicate adjustment of the DLI interface tip position relative to the DSC.

This paper describes a new design of the DSC that allows tuning of the desolvation and the CI conditions with a wider margin of reproducible operation. In addition, this DSC provides a higher sensitivity by a higher effective use of the separated solutes.

## EXPERIMENTAL

### *Chemicals*

Methanol was distilled-in-glass grade (Burdick & Jackson, Muskegon, MI, U.S.A.) and water was Baker Analyzed HPLC Reagent (J. T. Baker, Phillipsburg, NJ, U.S.A.). The premixed solvents were filtered through a 0.2-μm pore size filter (Millipore, Bedford, MA, U.S.A.) to eliminate particulate matter, degassed in an ultrasonic bath and then continuously purged with helium.

The drug standards, dexamethasone, betamethasone, 6-methylprednisolone and hydrocortisone, were obtained from Steraloids (Wilton, NH, U.S.A.). A sample of 6-hydroxybetamethasone was kindly donated by Dr. R. Draper, Schering Corp.

### *Micro liquid chromatography*

The micro-high-performance liquid chromatographic system used consisted of a Waters M-6000A pump (Waters Assoc., Milford, MA, U.S.A.) controlled by a Waters M-660 solvent programmer modified for lower flow-rates[5], a 0.5-μm solvent line filter (Rheodyne, Cotati, CA, U.S.A.), a Rheodyne Model 7410 micro loop injector equipped with a 0.5-μl loop, a Whatman glass-lined μLC column (ODS-3, 7-μm particle size, 25 cm × 1 mm I.D., Whatman No. 4240-128, Clifton, NJ, U.S.A.) and a 1/16-in. low-dead-volume union equipped with a 0.5-μm stainless-steel frit

(Valco, No. ZUVF-0620.5, Houston, TX, U.S.A.) lined with two layers of 0.2-μm Millipore filter. Stainless-steel tubing with 1/16 in. O.D. and 0.1 mm (0.004 in.) I.D. connected the components of the system; before installing the short sections of tubing, their bores were opened with the electropolishing technique previously described[6].

*Modified DLI micro-liquid chromatography–mass spectrometry interface*

The μLC-mass spectrometry (MS) interface has been described previously[1]. In addition, three different designs of a DSC were successively used in this study: the standard HP DSC (Part No. 05985-20587, Hewlett-Packard, Palo Alto, CA, U.S.A.), a heated "extended" DSC and a heated "solvent stripping" DSC (*cf.* Fig. 3). The two last designs involve essentially the same hardware: a 304 stainless-steel rod (40 mm × 16 mm O.D.; 1.600 × 5/8 in.) machined to accomodate a cartridge heater (Hewlett-Packard cartridge, Part No. 05985-60171) in a separately machined stainless-steel holder, a probe guide and seal, and a droplet transfer line (Fig. 1).

The DSC was threaded to the source block in the usual manner. The DSC heater was supplied with current from the same computer-controlled supply as the source block heaters. In addition to these features, the "solvent-stripping" DSC includes a 0.5-mm gap between the tip of the DSC and the CI source aperture that is pumped with the high vacuum of the mass spectrometer through the threaded hole of the removed electron-controller-mask holding screw (6-32 machine screw). The aperture of the HP CI source plunger was also enlarged to 3.2 mm (1/8 in.) to maintain a constant section of the transfer line matching the I.D. of the DSC (see Fig. 1).

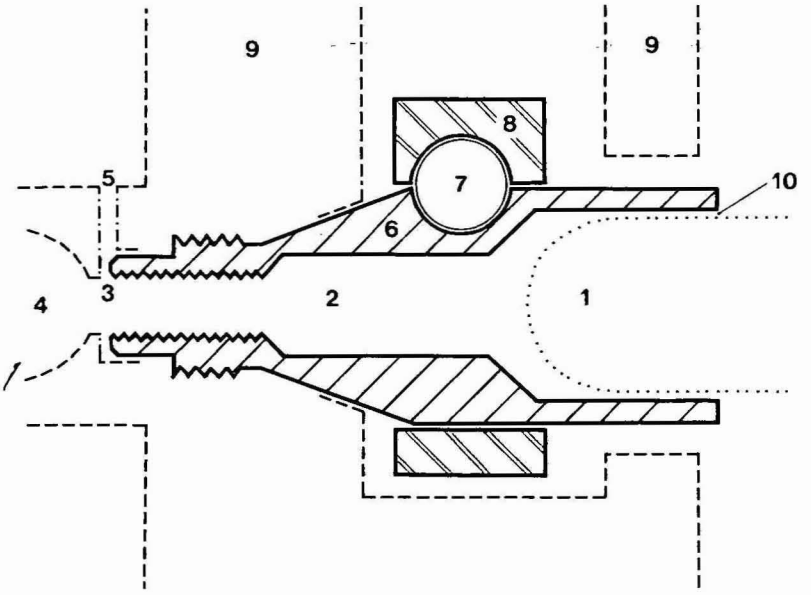



Fig. 1. Solvent-stripping desolvation chamber (longitudinal section). 1 = Tip of DLI LC–MS probe; 2 = heated droplet desolvation chamber and transfer line; 3 = solvent-stripping annular pumping gap (not present in extended DSC); 4 = CI ion source; 5 = solvent-stripping pumping line (screw hole through source body); 6 = desolvation chamber walls; 7 = heater cartridge; 8 = cartridge holder ring; 9 = source body and magnet; 10 = adjustable internal splitting gap.

The principle of operation for the different interfaces in the μLC–MS mode is described in the Results section.

*Mass spectrometry*

Mass spectra were recorded on a Hewlett-Packard Model 5985B gas chromatography (GC)–MS instrument equipped with option 04 for LC–MS operation. The liquid nitrogen-cooled cryopump was used in all experiments. Primary ionization of the solvent vapours was accomplished by a 140-eV beam of electrons from a heated rhenium wire. Improved pumping around the filament was obtained by replacing the standard electron shield by a stainless-steel mesh (mesh size 40, 50% transmission) of the same geometry.

The typical CI operating parameters of the mass spectrometer for the μLC–MS experiments were as follows: emission current, 200 μA; ion source temperature, 200°C; ion source pressure, 0.7 Torr (as measured at the CI GC–MS interface thermocouple); repeller, 8 V. The μLC eluent and CI reagent gas was methanol–water (65:35) and the detection was in the negative ion mode with a 2400 V electron multiplier voltage.

*Sample clean-up*

Urine and plasma samples were collected at 2-h intervals after intramuscular administration of the corticosteroids to standard-bred horses. Urine saturated with sodium sulphate and plasma were extracted at pH 4 with 1 volume of diethyl ether–dichloromethane–isopropanol (2:1:1). The organic phase was washed with 1 volume of 15% sodium sulphate in 2 *N* sodium hydroxide and the extract was dried under a stream of nitrogen. The residue was redissolved in ethyl acetate and applied onto an analytical silica gel thin-layer chromatography plate. The sample was developed to 5 cm with chloroform–ethyl acetate–light petroleum (b.p. 65–95°C) (5:3:2). The areas corresponding to corticosteroids and metabolites were located with 254-nm UV light, scraped, and eluted with ethyl acetate. The residual urine or plasma material was then analysed by μLC–MS.

## RESULTS

After development of a satisfactory μLC separation of a particular sample mixture utilizing a micro flow cell UV detector[1], at least two problems face the analyst using MS as a detection method: first, how to tune the mass spectrometer in order to ensure steady and sensitive levels of detection; secondly, how to document these conditions in order to reproduce them in later experiments.

*μLC solvent composition*

The solvent composition which gave a satisfactory separation of the corticosteroids of interest in this study (in particular hydrocortisone, betamethasone, dexamethasone and 6-methyl prednisolone) was methanol–water (65:35). Since the MS detection limits for these solutes were sufficient in the negative CI mode for our study, no attempt was made to modify the pH or the water–methanol solvent ratio. Furthermore, methanol–water at CI pressures forms mainly negative and positive methanol cluster ions. Other studies with methanol–water mixtures have shown that the

intensity of the protonated solvent ions is independent of the amount of water[7] and that the absolute sensitivity for a protonated solute remains constant over the entire solvent composition range[8].

*μLC solvent flow-rate*

The optimum chromatographic efficiency of the μLC column was observed at a flow-rate of 20 μl/min, but a compromise flow-rate of 40 μl/min was utilized to expedite the separations within 20 min. At this flow-rate the column head pressure was 900 p.s.i. (6.4 MPa).

Because the mass spectrometer diffusion pump above could not handle this flow-rate, a liquid nitrogen cryopump was used to complement the standard CI pumping system. The source envelope pressure was then $2.4 \cdot 10^{-4}$ Torr, which was one quarter of the maximum operating pressure. This was independent of the dimensions or supplier of a particular diaphragm.

*Ion source pressure*

Fig. 2 shows the typical source pressures that can be obtained by changing the distance between the DLI probe tip and each one of the three different DSCs (see Fig. 3). The source block and DSC temperatures were maintained constant at 200°C throughout this experiment. With the standard Hewlett-Packard DSC and the extended DSC, the same high pressure (2.2 Torr) was observed when the probe butted against the DSC, keeping the chamber tightly closed. When the probe was withdrawn however, the pressure dropped much more rapidly in the standard DSC, within the first millimeter from the DSC seat. The pressure was therefore much more difficult to regulate in this chamber than in the extended DSC. The proposed flow of droplets

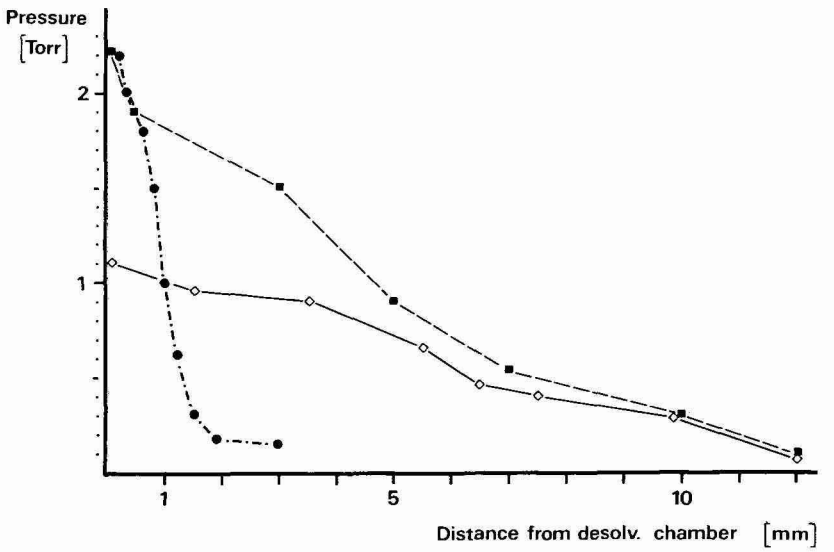



Fig. 2. Plots of ion source pressure *versus* distance between DLI probe tip and seat of desolvation chamber (DSC) for three different DSCs: ●– · · · –● = standard Hewlett-Packard DSC; ■ – – ■ = extended DSC; ◇——◇ = solvent-stripping DSC. Solvent methanol–water (65:35); source and DSC temperature, 200°C.

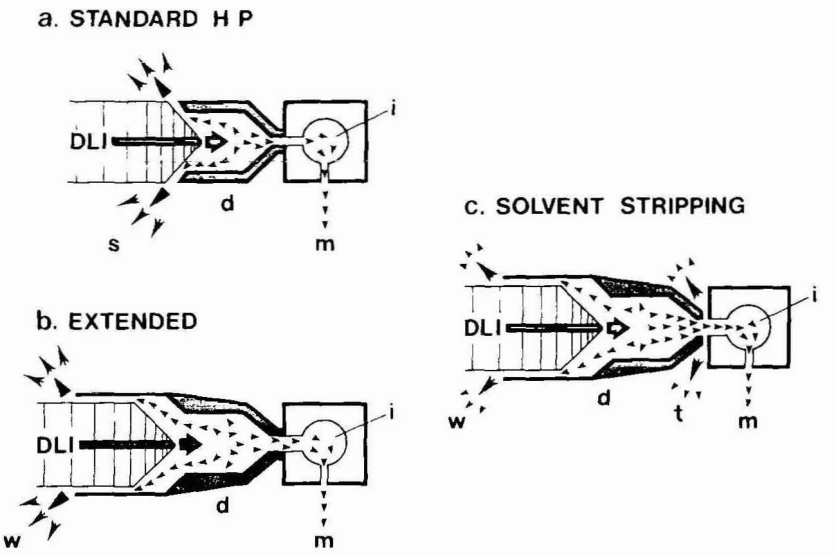



Fig. 3. Schematic diagram showing functioning of the three different DSCs. DLI = μLC probe producing a stream of droplets; d = desolvation chamber; i = ion source; m = ionized stream of solute and solvent molecules focused towards *mass* filter; s = flow pumped between DSC *seat* and μDLI probe tip; w = flow pumped through the gap between DSC *walls* and μDLI probe; t = flow pumped through the gap between DSC *tip* and ion source body.

and gases in these DSCs is schematically depicted in Fig. 3a and b. It consists basically in the splitting, outside of the DSC (*i.e.*, external to the ionization chamber), of the solvent *and solute* vapours that are in excess of the optimum CI working pressure. The differences between the standard and the extended DSC are that the latter features a more progressive splitter, a separate heater, a larger source inlet diameter and a possible minor extension of the jet length.

The third DSC design, schematically shown in Fig. 3c, may be called a "solvent-stripping desolvation chamber" and its basic pressure control is the same as that of the extended DSC. In addition, a gap of adjustable width pumped to a high vacuum is introduced between the DSC and the CI ion source. Thus, typically, pressures reached a maximum of 1.1 Torr with the probe tightly fitting the DSC, and the solvent vapours were removed from the *inside* of the DSC at a point where the droplets and gases have reached their highest temperature and velocity immediately before entering the ion source. Lower pressures could be obtained by an additional external splitting, controlled by the probe-to-DSC distance.

The source pressure provided useful information about the CI operating conditions but since its reading with a thermocouple gauge was not very precise and the optimal pressure varied with the different diaphragms, a pressure of 0.6–0.7 Torr at 200°C can only be given as an indication of average CI conditions. The tuning and recording of the CI conditions was actually carried out with the solvent cluster ions and the repetitive injection of a reference solute (see below).

*Temperature of source and DSC*

The effects of source and DSC temperature on the chromatographic peak

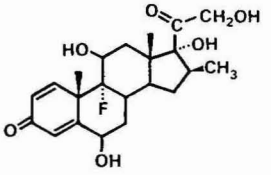
shapes and fragmentation were studied for the model compound 6-hydroxybetamethasone (6-OH-β-METH), the major urinary metabolite of betamethasone in horses[9]. The negative CI fragmentation of 6-OH-β-METH can be rationalized by comparison with the negative CI mass spectra of corticosteroids with similar structures[10]; the principal fragments are reported in Table I. Of particular interest is the ion of *m/z* 313, corresponding to the loss of neutral hydrogen fluoride from the *m/z* 333 ion.

These experiments were carried out with the source and DSC at the same temperature, between 150 and 300°C. Below 185°C, the peaks were hatched and tailing occurred, showing that too little heat was being transferred to the droplets in order to achieve an extensive desolvation of the solute. However, these low temperatures gave mild ionization conditions and a relationship was observed between fragment ion intensity and temperature: the loss of hydrogen fluoride becomes more extensive with higher temperatures. With the extended DSC, the *m/z* 313:*m/z* 333 ratio varied from 0.3 (at 150°C) to 1.1 (at 185°C). At DSC temperatures greater than 190°C, the peaks exhibited smooth shapes, showing that enough heat was being evenly transferred to the droplets during their drift along the DSC. In the extended DSC, higher temperatures produced greater fragmentation, the *m/z* 313 peak being 1.4 times more intense than the *m/z* 333 one at 200°C and 5 times more at 250°C. Although the fragmentation increased with temperature, the absolute intensity of the *m/z* 333 ion measured at the apex of the chromatographic peaks doubled from 150 to 200°C and decreased at higher temperatures.

The performance and sensitivity of the micro-DLI interface could be improved further by using the solvent-stripping DSC (Fig. 3c): at 200°C, the fragmentation of

TABLE I

FRAGMENT IONS DERIVED FROM 6-HYDROXYBETAMETHASONE UNDER NEGATIVE ION CI MASS SPECTROMETRIC CONDITIONS




6-Hydroxybetamethasone
Mol. wt. 408

CI reagent gas, methanol–water (65:35); 200°C; extended DSC. Solvent tuning ions ratios: *m/z* 63:81:95, 55:26:100% relative intensity.

| *Fragment(s) lost* | *m/z* | *Relative intensity (%)* |
|---|---|---|
| Hydrogen | 406 | 2 |
| Water | 390 | 10 |
| Water and a methyl radical | 375 | 25 |
| Water, a methyl radical and hydrogen | 373 | 46 |
| Ketene and hydrogen | 364 | 20 |
| Water, a methyl radical and HF | 355 | 36 |
| Methanol, water and a methyl radical | 343 | 52 |
| Ketene, water and a methyl radical | 333 | 88 |
| Ketene, water, a methyl radical and HF | 313 | 100 |

6-OH-β-METH was substantially reduced, with a ratio of *m/z* 313 to *m/z* 333 of 0.2, and the total ion current intensity of the smooth chromatographic peaks was doubled as compared with the extended DSC. The overall gain in sensitivity was eight-fold for the *m/z* 333 ion.

*Tuning for optimal CI conditions*

Most experimental procedures for optimizing jet length and source CI parameters use a continuous introduction of the tuning solution[8,11,12]. However, this method has the following limitations in the case of μLC–MS when it is used as a daily start-up procedure.

(1) The column and the connecting tubing of the entire chromatographic system must be filled with the tuning solution and then rinsed with pure solvent. It takes considerable time to return the column to its original condition after being "saturated" with the test solution.

(2) Continuous introduction of the test solution into the mass spectrometer unnecessarily contaminates the ion source.

(3) If the DLI probe is directly connected to the tuning solution pump during optimization, the jet must be interrupted to hook up the column and the injector. Furthermore, the diaphragm may clog after a column change.

(4) Column and injector switching with three-way valves would be effective with 4 mm I.D. columns, but the currently available hardware introduces an excessive dead volume when μLC columns are used.

(5) The maximum sensitivity does not mean that tuning is best for the stability and the shape of the chromatographic peaks. For example, Fig. 4 shows that the peak shape is better at a lower-than-maximum sensitivity.

The repetitive injection of a tuning solute is therefore the method of choice to determine the best jet conditions. Once these conditions have been established, the recording of the solvent cluster ion ratios gives a rapid solute-independent means of documenting the exact CI plasma conditions.

The intensity of solvent ions is thirty times larger than that of the solute ions. Unfortunately, the standard software package of the mass spectrometer (Hewlett-Packard, No. 05985-1004) does not allow simultaneous changes of scanning mass range and electron multiplier (EM) high voltage during an acquisition run. The solvent cluster ion ratio must be obtained with a low EM voltage, after interruption of continuous scanning of the mass range of the solute ions.

Typical solvent cluster ion patterns as a function of the source pressure are shown in Fig. 5. The ions selected as "solvent tuning ions" in the negative CI mode using methanol–water were those of *m/z* 63, 81 and 95. These ions correspond to $[(CH_3OH)_2 - H]^-$, $[(CH_3OH)_2 \cdot H_2O - H]^-$ and $[(CH_3OH)_3 - H]^-$, respectively, although we have no direct evidence for these compositions. As observed for positive CI solvent cluster ions[7,8], in negative CI the extent of solvent clustering and the relative intensity of the clusters depend strongly on the source pressure. As expected, the higher the pressure, the larger the degree of aggregation. However, ion quenching occurs above 1 Torr and decreases the intensity of the solute ions and the solvent cluster ions. A 2:1 intensity ratio of methanol trimer (*m/z* 95) to methanol dimer (*m/z* 63) was found to give the best conditions for obtaining a high intensity and a good chromatographic peak shape for the model solute 6-OH-β-METH. The intensity of

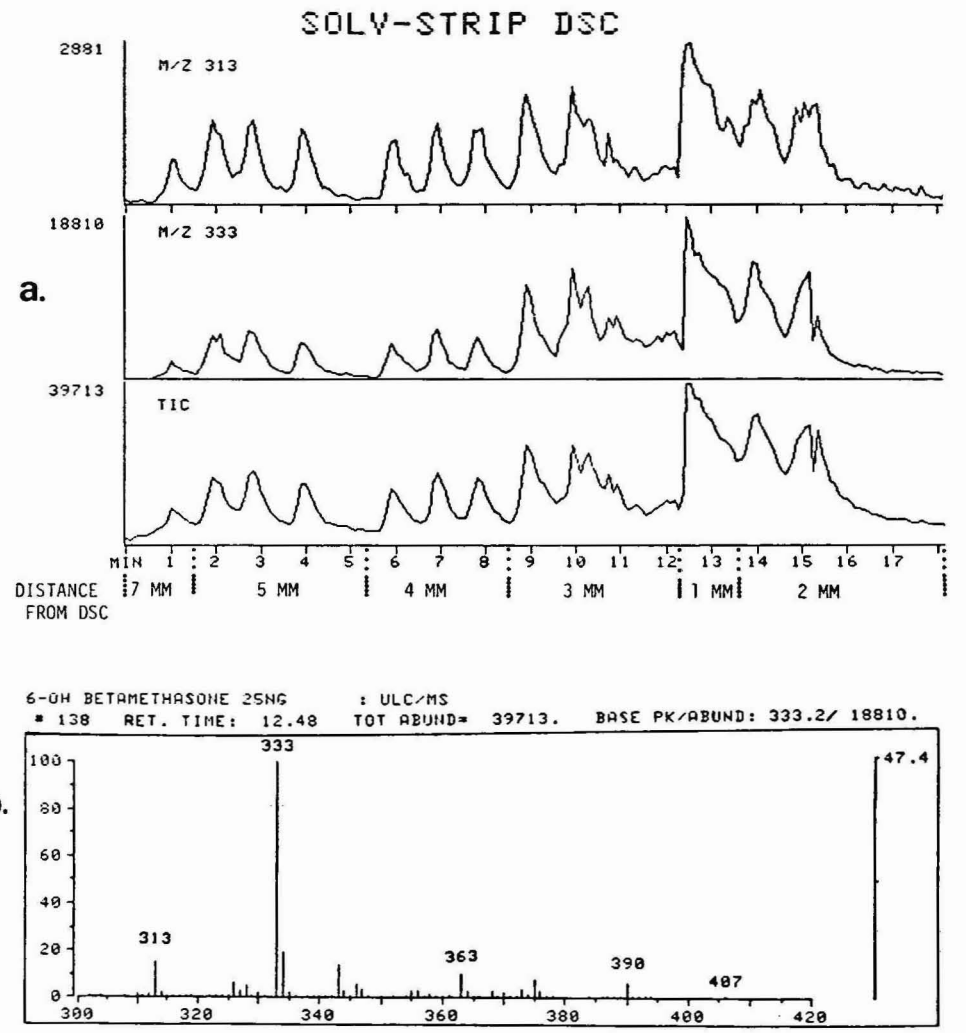



Fig. 4. $\mu$LC–MS separation of 6-hydroxybetamethasone (6-OH-$\beta$-METH) after repeated 25 ng injections; solvent-stripping DSC at 200°C (other standard parameters described in Experimental section). (a) Reconstructed ion chromatograms at different distances of DLI probe in DSC. (b) Mass spectrum recorded at the top of the largest peak ($t_R$ = 12.5 min); solvent tuning ion ratios: $m/z$ 63:81:95, 36:38:100% relative intensity.

the solvent cluster ions also depends strongly on the temperature of the DSC and ion source. Typical solvent tuning ion ratios at different temperatures of the solvent-stripping DSC and source are shown in Fig. 6. At 200°C, the trimer-to-dimer methanol cluster ion intensity ratio could be easily adjusted, using the variable splitter of the DSC, to stay at or slightly above the desired 2:1 ratio value.

With the extended DSC, the same procedure and a solvent tuning ion ratio of at least 1:1 at 200°C could be used. However, a 50% decrease of the relative intensity of the hydrated methanol dimer cluster ion ($m/z$ 81) was observed, as compared with the solvent-stripping DSC (see Fig. 5).

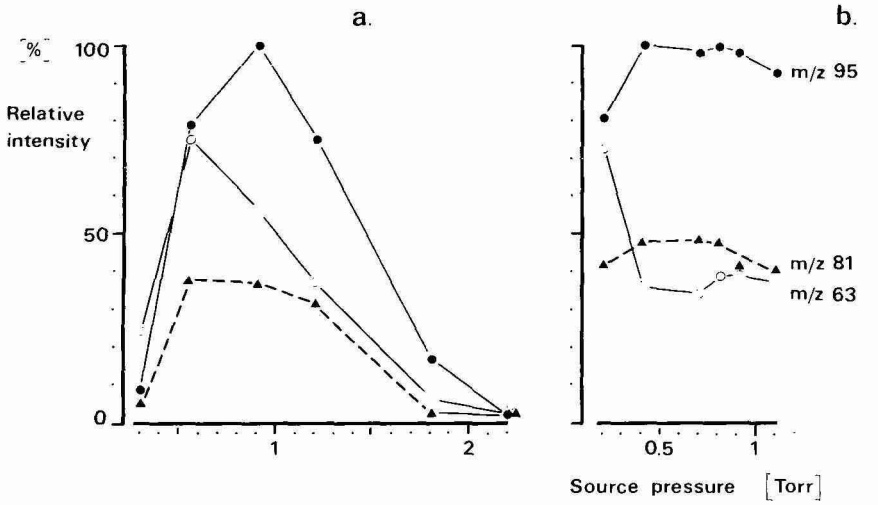



Fig. 5. Plot of the intensity of the three solvent cluster tuning ions *versus* source pressure, in negative CI at 200°C source and DSC temperature. (a) Extended DSC; (b) solvent-stripping DSC. Intensity normalized according to the most intense value in each DSC; solvent, methanol–water (65:35); flow-rate, 40 μl/min.

*High sensitivity detection*

An evaluation of the sensitivity provided by the solvent stripping DSC interface was made with the model compound 6-OH-β-METH. On-column 0.5-μl repetitive injections of 250 and 500 pg gave the chromatogram shown in Fig. 7. With full scan mode detection starting at *m/z* 240 to avoid continuous background ions originating from solvent impurities, the total ion current trace had a signal-to-noise ratio of 20. Similar values were observed for the reconstructed ion chromatograms at *m/z*

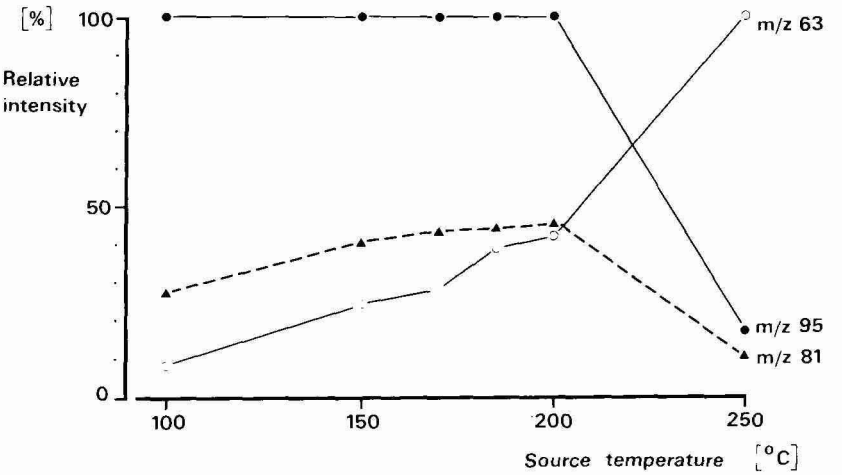



Fig. 6. Plot of the intensity of the three solvent cluster tuning ions *versus* source temperature, in negative CI at the pressure giving the highest intensity ratio of methanol trimer ion (*m/z* 95) to methanol dimer ion (*m/z* 63) with the solvent-stripping DSC. Intensity normalized according to the most intense ion in each measurement; solvent, methanol–water (65:35); flow-rate, 40 μl/min.

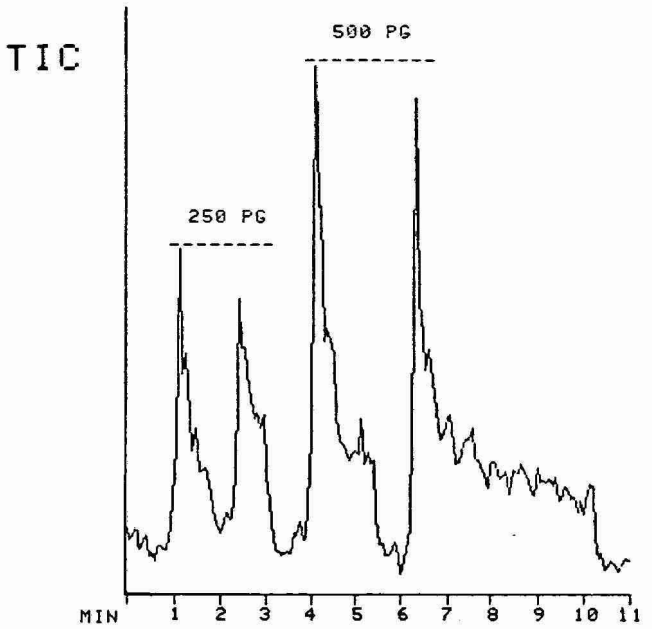



Fig. 7. Negative ion CI $\mu$LC–MS total ion current (TIC) profile for repeated injections of 250 and 500 pg of 6-OH-$\beta$-METH. The $\mu$LC–MS eluent/CI reagent gas was methanol–water (65:35) maintained at 40 $\mu$l/min through a $C_{18}$ 250 mm × 1 mm I.D. microbore column; solvent-stripping DSC; MS conditions described in Experimental section.

313 and 333. One could therefore expect that detection limits below 1 pg could be obtained in the selected ion monitoring mode.

*Analysis of biological samples*

Figs. 8 and 9 show the $\mu$LC–MS separation of equine urine extracts and Fig. 10 shows the analysis of crude extracts of equine plasma.

The urine for Fig. 8 was collected during the 6 h after administration of 6-methylprednisolone (6-Me-PRED). On the total ion current trace (Fig. 8b), no peak could be observed at the retention time expected for 6-Me-PRED. However, the mass spectrum recorded at this retention time (Fig. 8c) did reveal the presence of the drug, together with endogenous compounds. Fig. 8a, the reconstructed ion chromatogram for *m/z* 356, showed the peak corresponding to the loss of one molecule of water from the molecular ion of 6-Me-PRED to be present, albeit at a level of only *ca.* 10 ng in a biological matrix of 20 $\mu$g.

The urine for which data are shown in Fig. 9 was collected during the 6 h after administration of betamethasone ($\beta$-METH). In Fig. 9a, the resolution of the different overlapping polar compounds constituting the major peaks is seen to be better than in Fig. 8 because of the lower column load. This could be achieved because the ratio of the urinary level of $\beta$-METH to that of the biological matrix was more favourable than for 6-Me-PRED. The mass spectrum of the largest peak in Fig. 9a, (peak b) shown in Fig. 9b, and its retention time corresponded to those of 6-OH-$\beta$-METH. The unmetabolized drug, $\beta$-METH, could also be detected in this urine extract (peak c) and characterized, albeit at a much lower level (Fig. 9c).

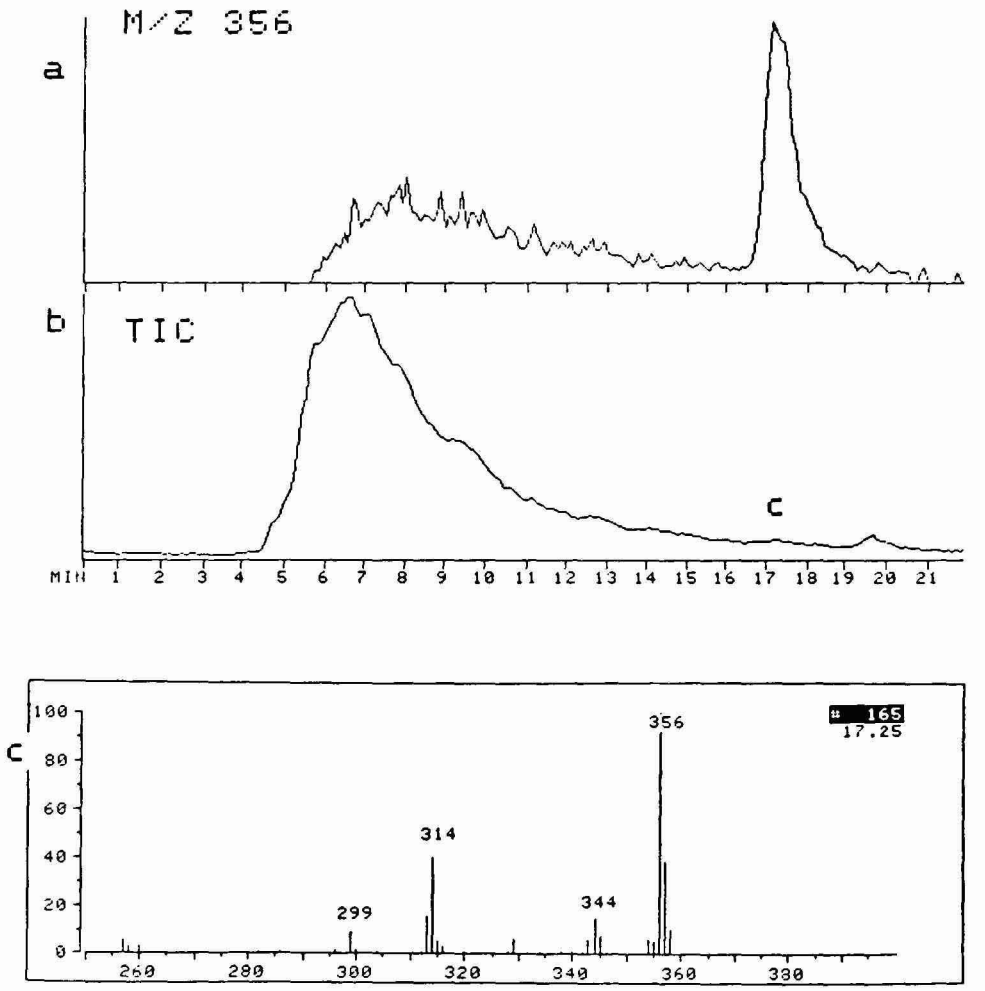



Fig. 8. Negative ion CI $\mu$LC-MS analysis of the extract of equine urine collected for 6 h post i.m. administration of 6-Me-PRED. Injection volume, 0.5 $\mu$l. $\mu$LC-MS conditions as in Fig. 7. (a) Reconstructed *m/z* 356 ion and (b) total ion current profiles; (c) negative CI mass spectrum of 6-Me-PRED, taken at the maximum of the *m/z* 356 peak in Fig. 8a. Solvent tuning ions ratios: *m/z* 63:81:95, 47:21:100% relative intensity.

For the same administration of $\beta$-METH to horses, the plasma levels were also measured. Fig. 10 shows an example of a chromatogram of a plasma extract obtained after $\mu$LC separation and selected ion monitoring (SIM) MS detection of $\beta$-METH, 6-OH-$\beta$-METH and hydrocortisone. Quantification of the compounds selectively detected in the samples was performed by comparing their peak heights with those of an external standard, a mixture of pure compounds injected before and after the samples. For the example shown in Fig. 10, 6 ng of $\beta$-METH and 0.6 ng of 6-OH-$\beta$-METH were determined in the plasma extract 1 h after administration, together with 21 ng of the endogenous corticosteroid hydrocortisone.

## DISCUSSION

An ideal detector for liquid chromatography has not yet been developed; however, MS is a highly sensitive detection method that fulfills most of the needs for a detector that is at the same time both universal and specific. Commercially available mass spectrometers that can be interfaced to LC were not specifically designed for this use; without modification of the mass spectrometer, a DLI interface has provided good results[13], but 99% of the LC flow had to be split while only 10 $\mu$l/min could be introduced into the mass spectrometer. To handle LC effluent flow-rates in excess of 10 $\mu$l/min, the conventional vacuum pumping system had to be modified by supplementing a cryopump[14,15] or a vacuum-lock system[16]. This improvement was still not sufficient for pumping the entire flow from a conventional 4.6 mm I.D.

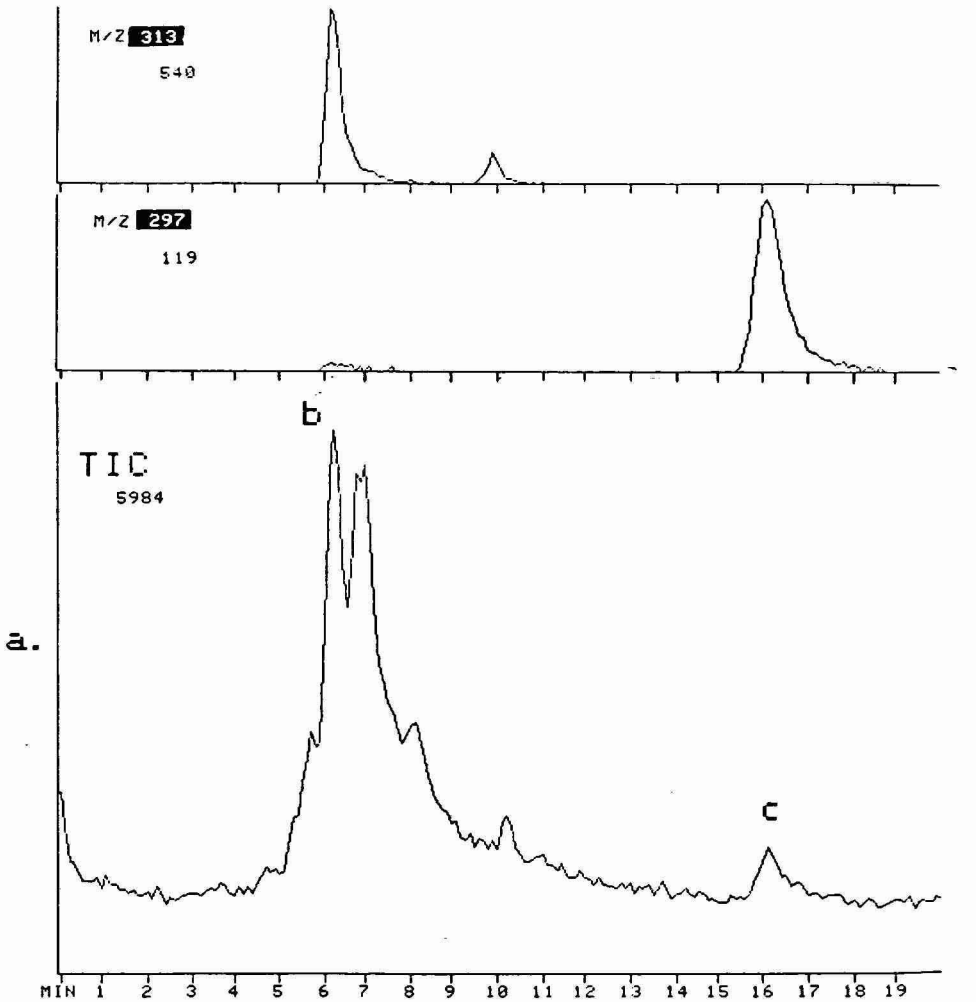



Fig. 9

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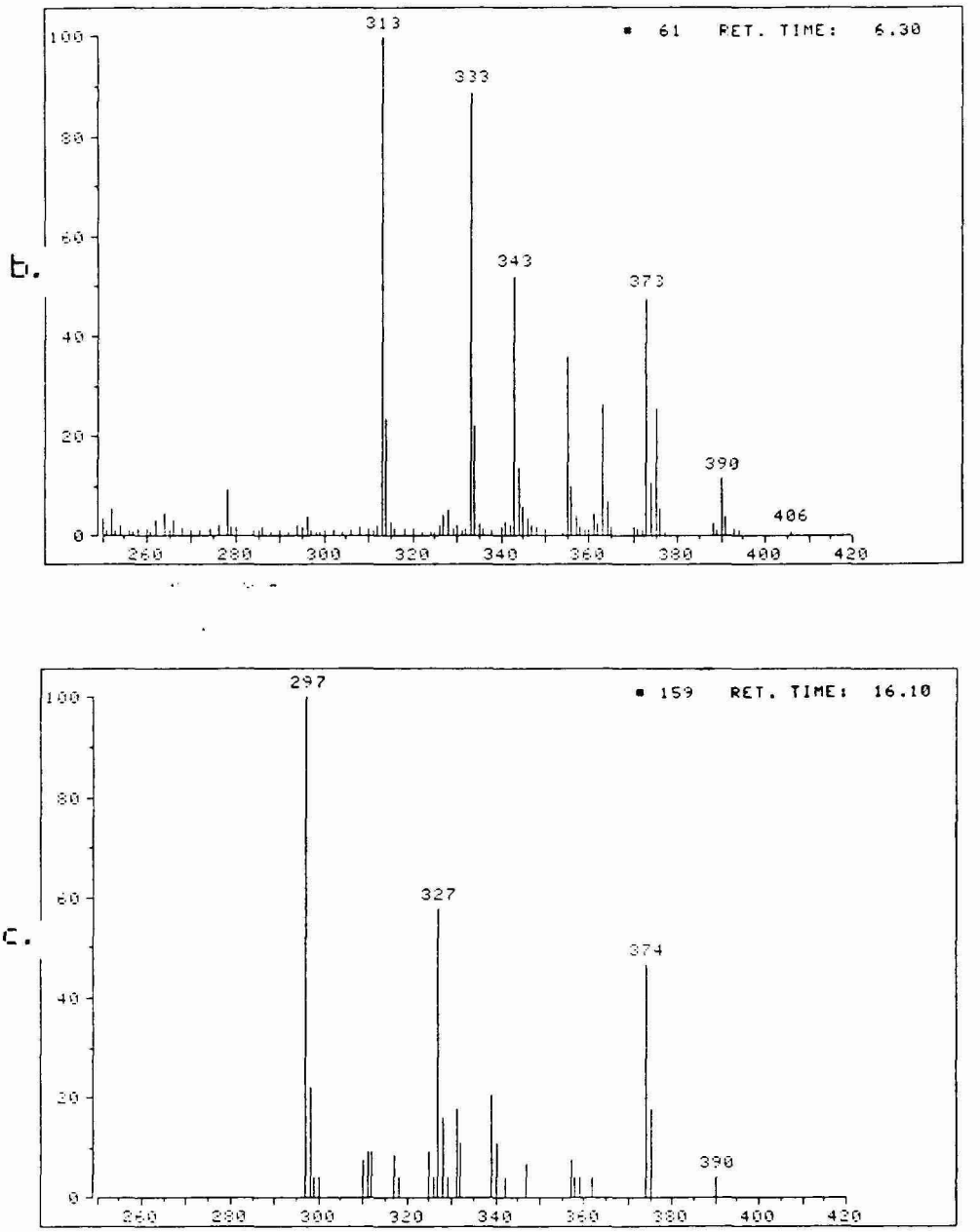


Fig. 9. Negative ion CI $\mu$LC–MS analysis of the extract of equine urine collected for 6 h post i.m. administration of $\beta$-METH. Injection volume, 0.5 $\mu$l; extended DSC; solvent tuning ion ratios as ion Fig. 8, other $\mu$LC–MS conditions as in Fig. 7. (a) Negative CI LC–MS total ionization chromatogram; (b) negative CI mass spectrum of 6-OH-$\beta$-METH (MW 408), obtained at 6.3 min at the apex of the most intense peak; (c) negative CI mass spectrum of $\beta$-METH (MW 392), obtained at 16.1 min.

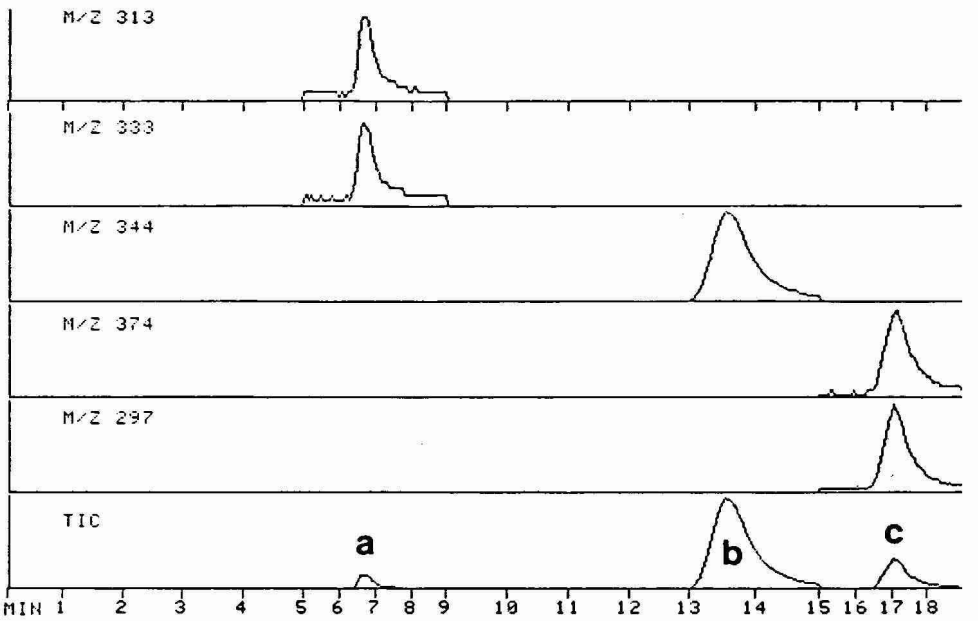


Fig. 10. SIM negative ion CI $\mu$LC–MS analysis of the extract of equine plasma collected 1 h post i.m. administration of $\beta$-METH. Injection volume, 5 $\mu$l; extended DSC; LC conditions as in Fig. 7. TIC = total selected ion current profile. The same dwell-time (100 msec) was used for each selected ion, but the frequency of data acquisition was two times slower in the 5–9-min and 15–19-min periods, where there was a pair of ions. Peaks: a = 6-OH-$\beta$-METH; b = hydrocortisone; c = $\beta$-METH.

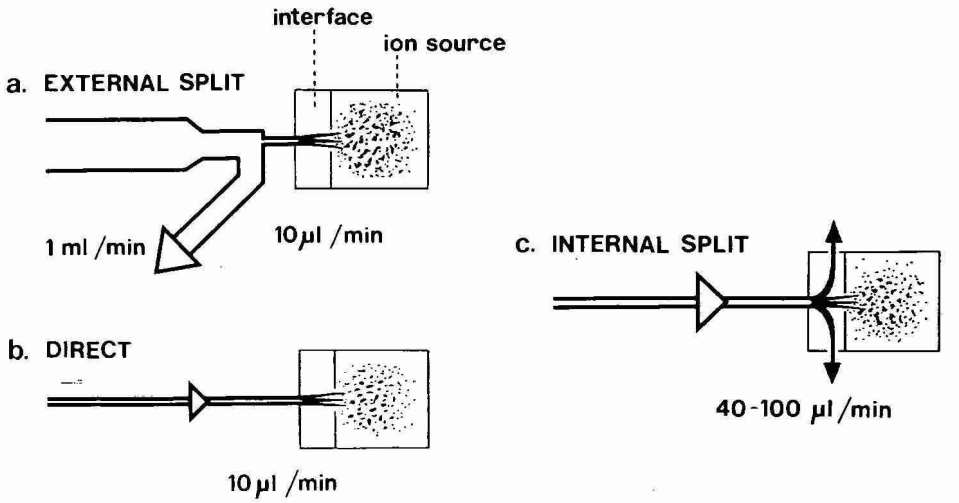


Fig. 11. Schematic diagram of different possible splitting strategies in DLI LC–MS, with typical values of LC flow-rates. (a) Splitting external to the mass spectrometer of a major portion of the LC flow from a conventional 4 mm I.D. LC column; (b) unsplit introduction of the entire flow from a $\mu$LC column; (c) introduction of the entire flow from a $\mu$LC column (up to 2 mm I.D.) and selective splitting (solute enrichment) internally to the mass spectrometer.

column. By allowing only *ca.* 5% of the eluent into the mass spectrometer through a splitter, DLI LC–MS interfacing could be achieved, but at the cost of a loss in sensitivity (Fig. 11a).

Interfacing μLC columns with a mass spectrometer has closed the gap between the solvent flow-rate which gives optimal LC separations and the flow-rate pumping capacity of the mass spectrometer with the transfer of the complete eluent flow into the spectrometer. This represented a radical change for the chromatographer: instead of having to adapt chromatography to the predominant needs of the mass spectrometer, he could choose the optimal μLC conditions and expect the flexibility from the mass spectrometer within its pumping range. The transport-type LC–MS interface allows one to take advantage of μLC by putting the entire effluent of a μLC column on the belt[17], but the full potential of the DLI-type interface has been limited by the high CI pressure generated by the LC solvent in the ion source. Unfortunately, the commercially available CI ion sources used to date with DLI have a fixed geometry; they generally can accept, without splitting, at the most 10 μl/min of LC solvent. This situation is illustrated in Fig. 11b. The major drawback of 1 mm I.D. microbore columns eluted at 10 μl/min is that elution times are extremely long; in addition their efficiency is hampered by diffusion at this low flow-rate.

An attempt to shorten retention times by using a higher flow-rate combined with splitting external to the mass spectrometer resulted in a decrease in sensitivity[18]. This is in essence similar to the situation shown in Fig. 11a, but with a more favorable splitting ratio.

Another approach for increasing the sensitivity is to use a heated desolvation chamber[3,4]. This device promotes uniform vaporization of the droplets with a range of solvents, although one can avoid the use of this accessory if the diaphragms have a pinhole size which ideally matches the LC solvent and flow-rate[13,18]. In the absence of a splitter, the desolvation chamber transmits the entire LC flow to the source. Subsequently the CI pressure limits the LC flow-rate and the sensitivity is, at best, the maximum that can be obtained from the unsplit direct coupling shown schematically in Fig. 11b.

To allow a higher throughput of solute to enter into the mass spectrometer, the ion-source side of the LC–MS instrument was modified. A variable gas conductance of the ion source was set by retracting the GC interface tip which seals the source; this reduced the extent of clustering by solvent tuning ions but did not increase the sensitivity for solutes. The pressure was also reduced, although a direct measure of the source pressure through the GC interface was not possible under these circumstances. More sophisticated modifications of source conductance would have required redesigning of the source, a task beyond the scope of this research.

The principle of the solution that has been shown in the present paper is illustrated in Fig. 11c. It consists of introducing the entire LC flow into the mass spectrometer (between 10 and 100 μl/min) and then using an interface designed to match the flow with the needs of the CI source. In this way all the adjustments occur within the mass spectrometer. Therefore, the parameters of the interface can be fully documented for the understanding of its operation and for reproducing its optimal conditions.

A practical way to deal with the excess of pressure and solvent reaching the ion source was to equip the interface with an adjustable splitter inside the mass

spectrometer; the standard Hewlett-Packard DSC with a narrow gap in front of the DLI probe (*cf.* Fig. 3a) answered the purpose, but the gap (*ca.* 0.8 mm) was very difficult to adjust reproducibly. The controlled leak of the "extended" DSC, adjustable over 10 mm (*cf.* Fig. 3b), solved this problem.

Another advantage gained with LC flow-rates of 40 $\mu$l/min and higher was that they permitted the use of diaphragms with relatively large pinholes, typically 4–6 $\mu$m I.D. (larger than the 2–3 $\mu$m pinholes used at 10 $\mu$l/min). These diaphragms have the advantage of being easier to manufacture in reproducible dimensions and are much less likely to be plugged by deposits.

The above solved two pertiment problems: first, how to have a LC–MS ion source with an adjustable CI pressure and secondly, how to improve the lifetime and the uniformity of the DLI diaphragms. However, the two splitting DLI interfaces described previously removed, with little discrimination, a large part of the solute vapors while little sensitivity was gained as compared with a directly coupled $\mu$LC interface.

This problem was solved by adding to the "extended" DSC a pumping slit that selectively removed the more volatile and diffusible vapors from the mist of droplets. This "solvent stripping" version of a DSC (*cf.* Fig. 3c) contributed to transfer of the less volatile droplets, containing the solutes, into the ion source. These have a high momentum and reach preferentially the source while the light, diffusible vapors (mostly solvent molecules) are pumped away by the solvent-stripping annular pumping gap (see Fig. 1). This allowed a higher flow of solute to reach the ion source and increased the sensitivity, but at the same time the solvent vapors were not in such excess so as to cause ion quenching; the water-to-methanol ratio in the solvent cluster ions also increased, showing the greater ease of the "solvent stripping" DSC to remove the more volatile molecules.

The final step in this development was to adjust the variable DSC for the highest sensitivity. In this simple "solvent stripping" DSC version, the area of the gap between the chamber and the ion source could be adjusted only when the DSC was threaded onto the source block, and not during routine high-vacuum operation of the mass spectrometer. Thus, only one control is needed, the distance between the DSC and the tip of the DLI, in order to determine the source pressure which gives the highest sensitivity.

When the LC–MS experiment is entirely new, the optimal temperature and pressure conditions can be determined by repeated injections of a solution of a model compound. As compared with a continuous infusion, this procedure provides optimum tuning because it checks the whole instrument, offers a lower contamination of the system and permits optimization of the sensitivity while maintaining the shape integrity of the chromatographic peaks. Source temperature and pressure are intimately interrelated, as both control the vaporization of the LC eluent. Vacuum alone is insufficient since it would freeze the solvent[2]; therefore, a minimum of heat must be transferred to the droplets.

A universal recipe for the selection of the optimum ion source temperature and pressure could not be devised, but general guidelines with adaptations depending on the thermal stability of the compounds analysed can be given as follows. First, rough values of 150°C and 0.7 Torr in negative CI or 0.3 Torr in positive CI are established. The temperature of the ion source and the DSC is increased to a value at which the

constancy of pressure and total ion current indicate that even vaporization conditions are achieved. Then, the source pressure is adjusted by moving the DLI probe to a position where the intensity of the repetitively injected solute is peaking and a good chromatographic peak shape is maintained. If the mass spectrum of the model compound used for tuning shows an acceptable intensity ratio of molecular ion to fragments, the temperature can be raised to produce less critical vaporization conditions in the DSC; conversely, for labile compounds, the temperature can be decreased to a value at which just enough heat can vaporize the droplets.

The results obtained with the solvent and solute ions have shown that changes of temperature have more dramatic effects than changes of pressure: temperature modifies mostly the ratio of the ions, while pressure has more influence on their overall intensities.

In the particular case of 17-hydroxy corticosteroids and our model compound 6-OH-$\beta$-METH, abundant $(M + 1)^+$ ions have been obtained in positive CI, as previously described for some of these compounds[14]. However, when compared with negative CI, positive CI has been less sensitive (see also ref. 10) and has given a higher background from the impurities of the solvent. Negative CI was therefore preferred for high-sensitivity measurements, but it had the drawback of not giving direct molecular weight information. Except for very weak $(M - 2)^-$ ions, 17-OH corticosteroids lose one molecule of water after electron capture to give a more stable $(M - 18)^-$ ion. Further losses of methanol, ketene or hydrogen fluoride are due in part to thermal degradation which could be prevented with the solvent-stripping DSC. Betamethasone, for example, yields a relative intensity of the $(M - H_2O)^-$ ion ($m/z$ 374) of 4% for vaporization from a heated-belt interface[10] and 41% for the solvent-stripping DSC. Another typical thermal fragmentation process, the loss of hydrogen fluoride, could be reduced in 6-OH-$\beta$-METH from 300% to 15% of the $m/z$ 333 peak by using the solvent-stripping DSC at a setting close to that which produces the minimum of energy required for the desolvation (see Fig. 4).

Once the optimal pressure and temperature conditions have been established, they must be documented so as to be readily reproducible later either with the same, or a different instrument. The *intensity ratios* of the solvent cluster ions give an accurate description of the CI conditions obtained in the source which is independent of any particular solute. For our methanol–water eluent, we observed that a 1:2 ratio of $m/z$ 63 to $m/z$ 95 ions, along with a 1:1 or slightly higher ratio of $m/z$ 63 to $m/z$ 81 ions, gave optimal CI-LC–MS conditions. Simultaneously, the $m/z$ 95 ion reached an absolute intensity maximum. These conditions could be obtained over a range of pressures with the solvent-stripping DSC at a temperature of 200°C (see Fig. 5b). For the solute, a higher sensitivity was obtained on the high pressure side of this range (*ca.* 0.8 Torr).

In later analyses of biological samples containing corticosteroids using the same HPLC conditions, the original optimal MS detection conditions were readily reproduced by tuning according to the solvent cluster ion ratios only. These ratios also gave between chromatographic runs an accurate diagnostic of the quality and straightness of the jet of droplets. Therefore, it is advised that the LC–MS contributions should include, along with the mass spectra, a plot of solvent cluster ions[19] or, better, a simple table of the intensity of characteristic solvent tuning ions. This would greatly help other researchers to reproduce the original conditions, without

problems occurring with instrument-to-instrument variations in temperature, pressure or rough data[20].

Comparison of our data with those obtained in DLI LC–MS with different solvents and compounds, particularly in positive CI, shows that the most sensitive MS conditions are strongly dependent on the thermal stability of the solutes analyzed. While one group, on the basis of small volatile solutes (*e.g.* benzoic acid), concluded that "optimization... is best achieved by adjusting the DLI probe for the maximum solvent cluster ion intensity"[8], another group showed with penicillins that reducing "the extent of clustering ... yields higher sensitivity at higher ion source temperature"[21]. This illustrates the need for using a relatively high pressure and low temperature for thermally labile molecules, in order to maintain a thin solvent "blanket" around the solute molecules when they enter the source[22]. On the other hand, thermally stable molecules can withstand contact with hot source walls and be entirely vaporized while still giving abundant molecular weight information.

One also observes that the optimal pressures for negative and positive CI are not equal; in negative CI, an efficient electron capture ionization can be reached at 1 Torr pressure of reagent gas[23,24], while 0.05–0.3 Torr seems to be optimal in positive CI[8,20].

## CONCLUSIONS

In DLI LC–MS an optimum tuning of the mass spectrometer is critical for reaching high sensitivity, especially for heat sensitive molecules. With a standard DSC, this can be achieved either at the cost of analysis time by using a low $\mu$LC flow-rate (10 $\mu$l/min) or at the cost of sensitivity and reproducibility by using a splitting gap that is difficult to adjust. The data presented in this paper demonstrate that positioning of the DLI probe relative to the source for maintaining an optimum CI pressure was achieved easier with the extension feature of the new DSCs. This allowed operating $\mu$LC–MS from 10 to 100 $\mu$l/min without having to split the LC eluent before it enters the mass spectrometer.

Furthermore, the data demonstrate that after removing a part of the vaporized solvent, the new solvent-stripping DSC promotes a more efficient transfer and milder CI ionization conditions for the solutes introduced into the ion source as high momentum droplets. The detection limits were in the picogram range in the full scan mode after a chromatographic separation of corticosteroids, which showed the level of sensitivity that the combined qualities of this new DSC allows.

The data reported here also demonstrate that a complete, accurate and easy control of the LC–MS conditions over broad tuning ranges can be achieved with the new DSC owing to its physical properties. Changes in pressure have a lesser effect on the CI reagent gas composition (*i.e.* the CI source is better "buffered") and another advantage is that one can use DLI diaphragms with pinholes larger than those used at 10 $\mu$l/min for pre-mass spectrometer splitting DLI interfaces. Moreover, the simple tuning procedure outlined in this paper demonstrates that although the same solvent has to be used for both LC separation and CI reagent gas, the flexibility of the new DSC permits the adjustment of LC–MS parameters somewhat independently towards maximum sensitivity. After a first round of tuning with repeated injections of a tuning solution, the ability of re-setting the pressure and com-

position of the CI reagent gas according to the intensity *ratios* of low- and high-mass solvent cluster ions (instead of maximizing the intensity of one ion only) was one major factor which made it possible to easily reproduce daily the original analysis conditions.

Stability and ease of tuning the system, combined with a low maintenance (cleaning of diaphragm after 2 days and cleaning of the ion source after 2 months of daily use) demonstrate clearly that DLI μLC–MS can reach the reliability needed for routine use. The analysis data for complex mixtures of biological materials after a crude sample preparation presented in this paper are an indication of what the full analytical power of the DLI μLC–MS technique will provide for high sensitivity detection and structural information on mixtures of unknown compounds.

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# EFFECT OF THE SKELETAL STRUCTURE OF SORBENTS CONTAINING L-HYDROXYPROLINE GROUPS ON ENANTIOSELECTIVITY IN LIGAND-EXCHANGE CHROMATOGRAPHY OF AMINO ACID RACEMATES

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(First received December 1st, 1982; revised manuscript received March 24th, 1983)

## SUMMARY

Asymmetric sorbents containing L-hydroxyproline groups on modified polystyrene skeletons have been synthesized. The sorbents, saturated with copper ions, were used for ligand-exchange chromatography of amino acid racemates. The modification of the polymeric skeleton is shown to be a promising way of increasing the enantioselectivity in racemate chromatography.

## INTRODUCTION

Ligand-exchange chromatography is increasingly used for separating amino acid racemates. Polystyrene sorbents containing L-proline and L-hydroxyproline groups have successfully been used for enantiomeric analysis of amino acids[1], for obtaining tritium-labelled optically active amino acids[2,3] and for micropreparative separation of proline and leucine racemates[4,5]. However, these sorbents, are not highly enantioselective with respect to alanine, asparagine, methionine, lysine, ornithine, aspartic acid and glutamic acid, which makes it difficult to use them for separating the racemates of those amino acids.

Polystyrene sorbents containing various amino acid groups, including complex polydentate ones[6], have been synthesized in an attempt to raise the enantioselectivity of the chromatographic process, yet this has not so far yielded any sorbents that might be of practical value.

With a view to raising the efficiency of ligand-exchange chromatography, 5–10 $\mu$m sorbents containing proline groups were synthesized on silica gel[7] and on microporous polyacrylamide[8]. These sorbents are of some interest for the analytical separation of racemates; they have successfully been used for a rapid quantitative separation of some racemates, but mostly those that were also readily separated on polystyrene sorbents containing proline and hydroxyproline groups[3].

Compared to silica gel and polyacrylamide-based sorbents, the polystyrene sorbents are superior in capacity, strength and chemical stability, which makes them a promising candidate for the preparative separation of racemates. In the present study we looked at the possibility of raising their enantioselectivity by modifying the poly-

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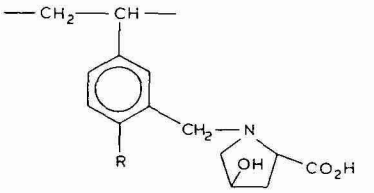
styrene skeleton by means of alkylation and acylation. Subsequent chloromethylation and amination led to the following sorbent structure:



1 R = $(CH_3)_3C$—
2 R =  (cyclohexyl radical)
3 R = $CH_3$—$(CH_2)_3$—
4 R = —$(CH_2)_2$—

The chromatographic properties of the modified polystyrene sorbents are compared to those of sorbents based on cross-linked unmodified polystyrene as well as polyacrylamide and polymethcrylate. The repeating units of these sorbents (5–8) can be represented as follows:

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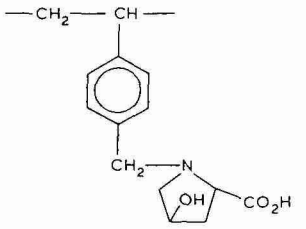

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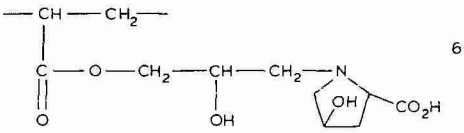

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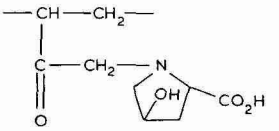

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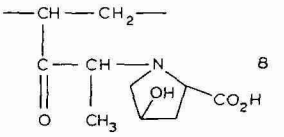


## MATERIALS AND METHODS

Cross-linked polystyrene Bio-Beads SX1 (200–400 mesh, Bio-Rad) was used as starting material.

*Synthesis of sorbent 1*

*Alkylation.* Bio-Beads SX1 (4.0 g, 38 mmol), 15.0 ml (140 mmol) of *tert.*-butyl chloride 23 ml of dichloroethane and 3 ml (26 mmol) of $SnCl_4$ were placed in a three-necked flask. The reaction mixture was periodically stirred at 20°C for 20 h. The polymer was filtered off, then washed with dichloroethane, acetone, a mixture of acetone and 1 *M* hydrochloric acid, acetone and diethyl ether. As a result, 5.9 g of yellow polymer were obtained.

*Chloromethylation.* Alkylated polystyrene skeleton (5.9 g) was placed in a three-necked flask. A cold (0°C) mixture comprising 23 ml of dichloroethane, 8.0 ml (105 mmol) of monochlorodimethyl ether and 1.0 ml (8.6 mmol) of $SnCl_4$ was added. The reaction solution was periodically stirred at 20°C for 10 h. The polymer was filtered off and washed with dichloroethane, acetone, a mixture of acetone and 1 *M* hydrochloric acid, acetone and sulphur ether. As a result, 7.3 g of yellow polymer containing 18.6% chloride were obtained.

*Amination.* Chloromethylated polymer (7.3 g) was aminated with methyl L-hydroxyprolinate hydrochloride in methanol–dioxane (1:4) in the presence of sodium bicarbonate and iodide as described earlier[9]. The capacity of the sorbent obtained is 2.4 mmol/g.

*Synthesis of sorbent 2*

*Alkylation.* Bio-Beads SX1 (3.0 g, 29 mmol), 15 ml of nitrobenzene, 3.0 ml (26 mmol) of $SnCl_4$ and 6.0 ml (52 mmol) of cyclohexyl chloride were placed in a three-necked flask. The reaction mixture was periodically stirred at 80°C for 10 h. The polymer was filtered off and washed on the filter, as for sorbent 1. As a result, 5.8 g of yellow polymer were obtained.

Chloromethylation and amination were carried out as for sorbent 1. The capacity of the sorbent is 2.6 mmol/g.

*Synthesis of sorbent 3*

Bio-Beads SX1 (3.0 g, 29 mmol), 20 ml of nitrobenzene, 6 ml (58 mmol) of butyl chloride, 2.5 ml (21 mmol) of $SnCl_4$ and 1.0 ml (7.1 mmol) of boron trifluoride etherate were placed in a steel autoclave. The reaction mixture was heated for 4 h at 90°C. The polymer was filtered off and washed on the filter. As a result, 4.4 g of yellow polymer were obtained.

Chloromethylation and amination were carried out as for sorbent 1. The capacity of the sorbent is 2.9 mmol/g.

*Synthesis of sorbent 4*

*Acylation.* Bio-Beads SX1 (3.0 g, 29 mol), 20 ml of nitrobenzene, 2.5 ml (21 mmol) of $SnCl_4$ and 8.0 g (46 mmol) of cyclohexapropyl chloride were placed in a three-necked flask. The reaction solution was kept at 40°C for 20 h. The polymer was filtered off and washed on the filter. As a result, 6.1 g of brown polymer were obtained.

Chloromethylation and amination were carried out as for sorbent 1. The capacity of the sorbent is 2.2 mmol/g.

Bio-Gel P-4 granules (−400 mesh, Serva) were used as starting material for synthesizing polyacrylamide-based sorbents.

*Synthesis of sorbent 5*

The synthesis of sorbent 5 containing L-hydroxyproline ligands fixed to a cross-linked polystyrene matrix was described earlier[9].

*Synthesis of sorbent 6*

The synthesis of sorbent 6 via the reaction of L-hydroxyproline with epoxy groups of poly(2,3-epoxypropyl methacrylate) was as described[10].

*Synthesis of sorbent 7*

Bio-Gel P-4 (10 g), 100 ml of a 5% aqueous solution of $Na_3PO_4$ and 10.2 ml (34 mmol) of formaldehyde solution were placed in a three-necked flask. The reaction mixture was heated to 60°C with continuous stirring and kept at this temperature for 1 h. L-Hydroxyproline (6.66 g, 50 mmol) was dissolved in 40 ml of 1 *M* sodium

hydroxide and added to the reaction mixture. The reaction mixture was then kept at 70°C for 30 min. The polymer was filtered off and washed on the filter with water and acetone. As a result, 14.9 g of polymer were obtained having a capacity of 2.5 mmol/g.

*Synthesis of sorbent 8*

Bio-Gel P-4 (4.0 g), 36 ml of water, 1.2 ml (27.5 mmol) of acetaldehyde and 4.0 g (22 mmol) of L-hydroxyproline methyl ether hydrochloride were placed in a three-necked flask. The solution was heated to 60°C over 1 h and kept at this temperature for 4 h. The polymer was washed with water on a filter and placed in 100 ml of 2 *M* ammonium hydroxide containing 0.1 *M* $[Cu(NH_3)_4]SO_4$. The reaction solution was periodically stirred at 20°C for 20 h. The polymer was washed on a filter with 0.1 *M* hydrochloric acid, water and acetone. As a result, 4.7 g of polymer were obtained having a capacity of 1.0 mmol/g.

*Chromatography of racemates*

The sorbents were saturated with copper ions to the required degree[1], suspended in 0.1 *M* ammonium hydroxide and placed in a 140 × 8 mm glass column. A 0.5-mg amount of L- and D-amino acids was introduced into the column in the form of a 1% solution. A UV detector was used at a wavelength of 250 nm.

## RESULTS AND DISCUSSION

The chromatographic elution order of amino acid isomers depends on the relative stability of the diastereomeric sorption complexes formed between the fixed ligand, $Cu^{2+}$ and mobile ligand. Enantioselectivity may be assessed from the viewpoints of the equilibrium distribution and chromatography.

Fig. 1 shows tentative structures for the more stable sorption complexes formed on sorbents containing L-hydroxyproline ligands during the chromatography of phenylalanine isomers. For polystyrene-based sorbent 5 the value of the free energy difference, $\delta\Delta G$, for is +2630 J/mol, sorption complex formation for glycidyl methacrylate-based sorbent 6 it is −1480 J/mol, for polyacrylamide-based sorbents 7 and 8 the values are −2010 and −240 J/mol.

With Sorbent 5, which contains a hydrophobic benzyl group, the sorption complex formed with D-phenylalanine is the more stable; but with sorbents 6–8 the sorption complex with the L isomer is the more stable. Sorbents 6–8 have the following feature in common: L-hydroxyproline is attached to them by hydrophilic groups which may be involved in coordinative interaction with an axially positioned copper ion.

The sign and magnitude of the enantioselective effect depend not only on the nature of the fixed ligand but largely on the nature of the group binding the ligand to the skeleton. The nature of the skeleton itself does not seem to be crucial. The enantioselective effects observed for sorbent 6 proved to be of about the same magnitude as for a silica gel-based sorbent[7], containing the same fixed ligand and binding group.

Hydrophobic interactions play an important part in the stabilization of sorption complexes. This is especially manifest when one compares ligand-exchange chro-

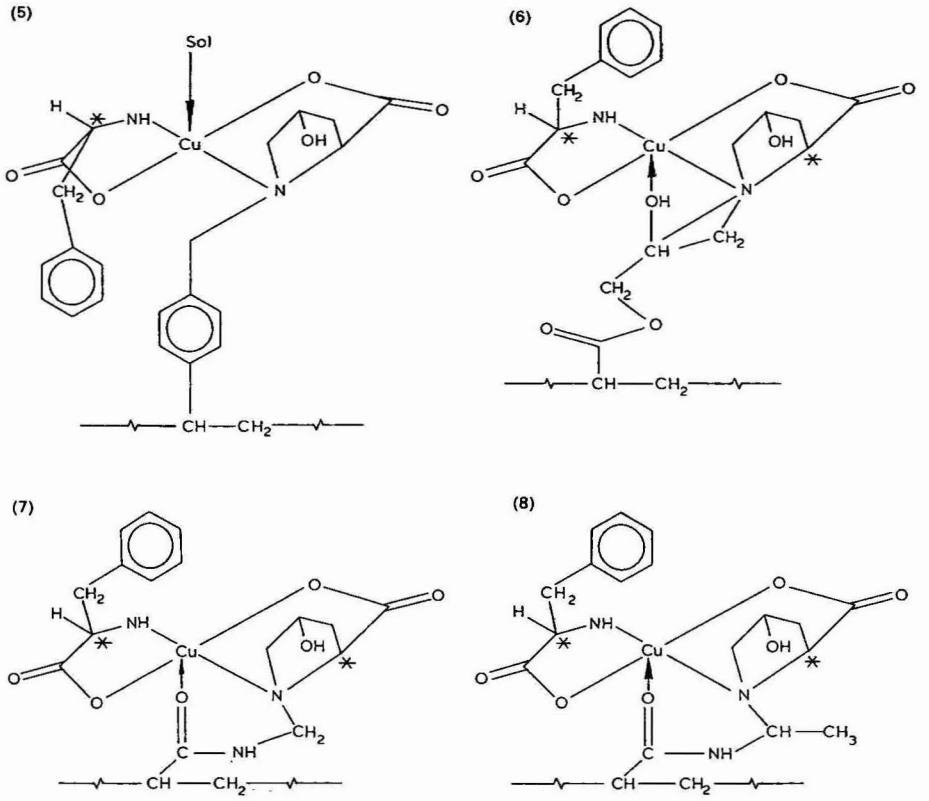



Fig. 1. Structures of more stable mixed-ligand sorption complexes formed by L- or D-phenylalanine on asymmetric sorbents (5-8) containing L-hydroxyproline. $k'_D/k'_L$ values; 5, 2.89; 6, 0.55; 7, 0.44; 8, 0.91.

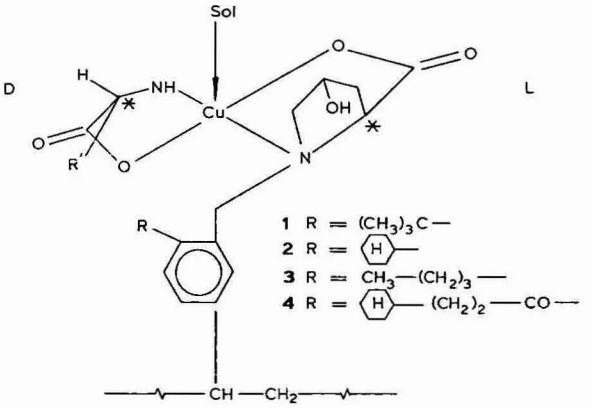



Fig. 2. Structure of mixed-ligand sorption complexes formed by D-amino acids on modified polystyrene sorbents (1–4) containing L-hydroxyproline.

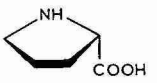
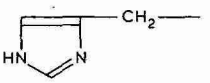
TABLE I

RETENTION PARAMETERS FOR AMINO ACID ENANTIOMERS ON THE POLYSTYRENE RESINS WITH L-HYDROXYPROLINE GROUPS AND SATURATED WITH $Cu^{2+}$

$k'$ = Capacity factor; $\delta\Delta G$ = enantioselectivity. Eluent: 0.1 *M* ammonium hydroxide (1–14); 1.0 *M* ammonium hydroxide (15, 16).

| *No.* | *Amino acid* | *R'* | *Sorbent* | $k'_L$ | $k'_D$ | $\delta\Delta G$ *J/mol)* |
|---|---|---|---|---|---|---|
| 1 | Ala | $CH_3$– | 1 | 5.5 | 7.4 | 740 |
| | | | 2 | 6.0 | 7.0 | 370 |
| | | | 3 | 7.8 | 8.4 | 300 |
| | | | 4 | 7.6 | 9.2 | 620 |
| | | | 5 | 5.8 | 6.0 | 150 |
| 2 | Abu | $CH_3CH_2$– | 1 | 8.0 | 12.9 | 1180 |
| | | | 2 | 7.5 | 10.5 | 830 |
| | | | 3 | 10.4 | 12.4 | 450 |
| | | | 4 | 8.0 | 12.0 | 1010 |
| | | | 5 | 6.5 | 8.0 | 490 |
| 3 | Val | $(CH_3)_2CH$– | 1 | 9.2 | 13.1 | 920 |
| | | | 2 | 9.0 | 17.5 | 1620 |
| | | | 3 | 9.2 | 12.0 | 650 |
| | | | 4 | 9.0 | 18.4 | 1620 |
| | | | 5 | 7.3 | 11.8 | 1180 |
| 4 | Leu | $(CH_3)_2CHCH_2$– | 1 | 45 | 50 | 260 |
| | | | 2 | 8.5 | 11 | 650 |
| | | | 3 | 20.8 | 30.0 | 900 |
| | | | 4 | 24.8 | 42.8 | 1890 |
| | | | 5 | 14.2 | 24.2 | 1320 |
| 5 | Ile | $CH_3CH_2CH(CH_3)$– | 1 | 32 | 45 | 830 |
| | | | 2 | 8 | 11 | 800 |
| | | | 3 | 15.2 | 26.4 | 1380 |
| | | | 4 | 26.0 | 62.0 | 2170 |
| | | | 5 | 11.1 | 20.9 | 1580 |
| 6 | Nva | $CH_3CH_2CH_2$– | 1 | 53 | – | – |
| | | | 2 | 8.2 | – | – |
| | | | 3 | 16.8 | – | – |
| | | | 4 | 14.4 | 23.2 | 1200 |
| | | | 5 | 11.2 | 19.9 | 1290 |
| 7 | Ser | HO–$CH_2$– | 1 | 4.4 | 4.7 | 170 |
| | | | 2 | 4.0 | 4.8 | 450 |
| | | | 3 | 5.3 | 5.7 | 170 |
| | | | 4 | 6.0 | 7.8 | 650 |
| | | | 5 | 3.5 | 4.5 | 630 |
| 8 | Thr | $CH_3CH(OH)$– | 1 | 15.0 | 16.5 | 240 |
| | | | 2 | 4.3 | 5.6 | 650 |
| | | | 3 | 6.0 | 6.2 | 130 |
| | | | 4 | 8.2 | 10.8 | 690 |
| | | | 5 | 3.5 | 5.3 | 1040 |

TABLE I *(continued)*

| *No.* | *Amino acid* | *R'* | *Sorbent* | $k'_L$ | $k'_D$ | $\delta\Delta G$ *(J/mol)* |
|---|---|---|---|---|---|---|
| 9 | Asn | $NH_2COCH_2$– | 1 | 7.0 | 7.5 | 170 |
| | | | 2 | 4.0 | 4.8 | 450 |
| | | | 3 | 1.4 | 4.0 | 1090 |
| | | | 4 | 6.8 | 8.4 | 470 |
| | | | 5 | 4.6 | 5.4 | 390 |
| 10 | Gln | $NH_2COCH_2CH_2$– | 1 | 7.2 | 9.0 | 550 |
| | | | 2 | 3.9 | 5.1 | 650 |
| | | | 3 | 2.0 | 2.4 | 450 |
| | | | 4 | 8.0 | 8.8 | 240 |
| | | | 5 | 2.5 | 3.7 | 1000 |
| 11 | Met | $CH_3$–S–$CH_2CH_2$– | 1 | 2.5 | 35 | 830 |
| | | | 2 | 9.0 | 10.0 | 240 |
| | | | 3 | 18.0 | 31.2 | 1360 |
| | | | 4 | 28.8 | 42.0 | 930 |
| | | | 5 | 11.7 | 14.3 | 460 |
| 12 | Tyr | HO–$C_6H_4$–$CH_2$– | 1 | 22 | 44 | 1600 |
| | | | 2 | 17.0 | 70.0 | 3500 |
| | | | 3 | 25 | 30 | 450 |
| | | | 4 | 14.4 | 32.0 | 1840 |
| | | | 5 | 9.0 | 19.9 | 2000 |
| 13 | Phe | $C_6H_5$–$CH_2$– | 1 | 112 | 141 | 650 |
| | | | 2 | 110 | 225 | 1780 |
| | | | 3 | 88 | 110 | 550 |
| | | | 4 | 82 | 278 | 3040 |
| | | | 5 | 34.0 | 98.0 | 2630 |
| 14 | Pro |  | 1 | 13.0 | 60 | 3800 |
| | | | 2 | 41.0 | 108 | 1530 |
| | | | 3 | 8.0 | 68 | 5100 |
| | | | 4 | 39 | 250 | 4620 |
| | | | 5 | 15.2 | 91.2 | 4450 |
| 15 | His |  | 1 | 40.0 | 9.0 | −3680 |
| | | | 2 | 66.0 | 19.0 | −3090 |
| | | | 3 | 35.0 | 11.3 | −2820 |
| | | | 4 | 43.5 | 10.6 | −3500 |
| | | | 5 | 27.3 | 7.8 | −3100 |
| 16 | Lys | $NH_2(CH_2)_3CH_2$– | 1 | 9.0 | 10.0 | 240 |
| | | | 2 | 21 | 23 | 240 |
| | | | 3 | 12.6 | 15.1 | 450 |
| | | | 4 | 12.3 | 16.0 | 650 |
| | | | 5 | 4.0 | 5.0 | 550 |

matography on ODS silica gels in the presence of bis(L-prolinato)copper[11] and its N-alkylhydroxyproline derivative[12], large enantioselective effects with different signs being observed. The more stable sorption complexes are those that favour interaction

between the hydrocarbon α-radical of the amino acid concerned and a hydrophobic ODS phase. It seems that the N-benzyl radical in sorbent 5 can serve as such a hydrophobic phase. The hydrophobic interaction must be most pronounced for bulky alkyl and aryl radicals of amino acids. A change in $\delta\Delta G$ does in fact occur in the series alanine, α-aminobutyric acid, valine and leucine. It would seem that the hydrophobic interactions in respect of D-alanine must be enhanced if additional hydrophobic groups are introduced at the *ortho* position of the N-benzyl group. Such an increase in enantioselectivity was indeed observed will sorbents 1–4 comparing to the resolving power of sorbent 5 which is based on the unsubstituted polystyrene matrix.

Fig. 2 shows suggested structures of the more stable sorption complexes formed on the sorbents synthesized. These complexes contain mobile ligands of the D configuration (for sorbents based on modified or unmodified polystyrene). Table I shows the chromatographic data for those sorbents.

In many cases the enantioselectivity of sorption is considerably higher for hydroxyproline sorbents on modified polystyrene skeletons than for sorbent 5 which has an unmodified skeleton. The value of $\delta\Delta G$ with respect to alanine and aminobutyric acid is especially different in the case of a skeleton containing a tertiary butyl group. The enantioselectivity is considerably increased in the chromatography of tyrosine on a sorbent containing an *n*-butyl group and in the chromatography of methionine on a sorbent containing a cyclohexyl group.

An increased enantioselectivity with respect to most amino acids was observed for sorbent 4 containing a cyclohexyl propionic acid residue. This radical allows interaction between the carbonyl oxygen and the copper ion at the axial position, which in turn must cause the cyclohexyl group to take up a position near the α-hydrocarbon radical of the amino acid in question.

For all the modified sorbents, the chromatographic elution order of the amino acid isomers is preserved, but the $\delta\Delta G$ values are somewhat lower for amino acids containing hydrophilic groups, such as serine, threonine and glutamine, as compared to values on the unmodified sorbent 5.

Thus, modification of the polymeric skeletons used for synthesizing asymmetric sorbents may increase the enantioselectivity in racemate chromatography, as is the case with amino acids containing hydrophobic α-radicals.

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# SEPARATION OF ORGANIC AND METAL IONS BY HIGH-VOLTAGE CAPILLARY ELECTROPHORESIS

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## SUMMARY

Electro-osmotic flows were measured in Pyrex glass capillary, fused-silica capillary and poly(fluoroethyl–propylene) tubings. The electro-osmotic flow exhibited a good linear relationship with electric current density. The factors which influence electro-osmosis and separations are discussed. Good separations of pyridinium salts, sulphonic acids and metal ions were demonstrated, eluting towards the negative or positive terminal at up to 16 kV in aqueous solution. Theoretical plate numbers for pyridinium salts were around $1.5 \cdot 10^5$–$2 \cdot 10^5$.

## INTRODUCTION

Zone electrophoresis is generally carried out in stabilizing media, such as paper, cellulose acetate or gels[1]. If non-viscous media, such as water, are used instead, the migration path of a solute might become completely straight because it has no need to pass through a matrix, such as cellulose fibres, and the dissipation of the heat generated by the application of a voltage is more rapid, allowing the possibility to input high voltages. Although several geometrical designs would be possible, two are more obvious.

The first involves the use of a horizontal electrophoresis tube (3 mm I.D.) rotating around its longitudinal axis to counteract convective disturbances. This device was proposed by Hjertén[2], and he applied it to the separation of, for example, metal ions and nucleotides. The second possibility is to use a cell of very narrow cross-section, that is, a capillary tubing[3–6]. Mikkers *et al.*[3] used a poly(tetrafluoroethylene) tubing of 0.2 mm I.D. and succeeded in a good and rapid separation of carboxylic acids with a potential gradient detector in conductive mode. Jorgenson and Lukas[4,5] used a very narrow-bore glass capillary of 75 $\mu$m I.D. and applied voltages up to 30 kV, and obtained good separations of fluorescent derivatives of amino acids with a fluorescence detector.

In the present paper we use Pyrex glass capillary, fused-silica capillary and poly(fluoroethylene–propylene) (FEP) tubings. The conditions for zone electrophoresis in capillary tubings with on-column UV detection are discussed and applications to the separation of pyridinium salts, sulphonic acids and metal ions are described.

0021-9673/83/$03.00 

## EXPERIMENTAL

Straight Pyrex glass capillary (30–120 cm × 50–200 μm I.D.) was drawn by a glass drawing machine (GDM-1; Shimadzu, Kyoto, Japan). Fused-silica capillary (Scientific Glass Engineering, North Melbourne, Australia) and FEP were kindly supplied by Shimadzu. These tubings were used as columns for zone electrophoresis without any surface modification. In some cases, fused-silica capillary was connected with Pyrex glass capillary by the aid of a small piece of poly(tetrafluoroethylene) tubing for sensitive detection or the electro-osmosis experiment. At the point of UV detection, the Pyrex glass capillary and FEP were used as "cells" without any modification, but in the case of fused-silica capillary the resin on it was flamed off before use. The column parts of these tubings were regarded as the parts from the inlet to detector. High-voltage d.c. power supplies (IP-2A, Shimadzu; V-5, Toyo Kagaku-sangyo, Osaka, Japan) were operated in both constant-voltage and constant-current modes and delivered up to 25 kV. Platinum–iridium tubing was used as electrode. The solvents were aqueous solutions of $Na_2HPO_4$, $KH_2PO_4$, phosphate buffer ($Na_2HPO_4$–$KH_2PO_4$) and acetic acid, with or without 0.5% ethylene glycol.

### *Procedure*

Solvent was degassed with helium gas or by the aid of an ultrasonic bath. Columns were filled with solvent under pressure or by dipping one end of the tube, which was kept 10 cm higher than the other end, in the solvent. After capillary had been filled, both ends were dipped in small beakers. Sample introduction was carried out by using electro-osmotic flow or by "downhill" flow in the same manner as used for filling a capillary with solvent[4–6]. The electric field was then applied.

## RESULTS AND DISCUSSION

The linear velocity, $u_{osm}$, of a liquid under the influence of an applied field, $E$, is derived[6–9] as follows

$$u_{osm} \approx keE/Z\,\eta\,(C^s)^{\frac{1}{2}} \tag{1}$$

where $k$, $e$, $\eta$, $Z$ and $C^s$ are a constant, the amount of charge per unit surface area of the capillary tubing, the viscosity of the liquid, the number of valence electrons and the concentration of the electrolyte in water, respectively. In a previous paper[6] we discussed the effects of the electrolyte concentration and electric current, $I$, on the electro-osmotic flow. In the present study, the effects of the electric current density, $i$, tubing materials and pH on electro-osmotic flow are considered.

### *Relationship between electric current density and electro-osmosis*

The relationship between applied voltage and electric current is, at first, linear and then becomes non-linear as shown in Fig. 1. This behaviour is supposed to be due to the increase in temperature in solution, the change in dielectric constant and/or a change in the kinetics of electrophoresis[7,10]. Also, there cannot be a linear relationship between electro-osmosis and applied voltage, $E$.

However, when the electric current, $I$, or current density, $i$, is used as the basic

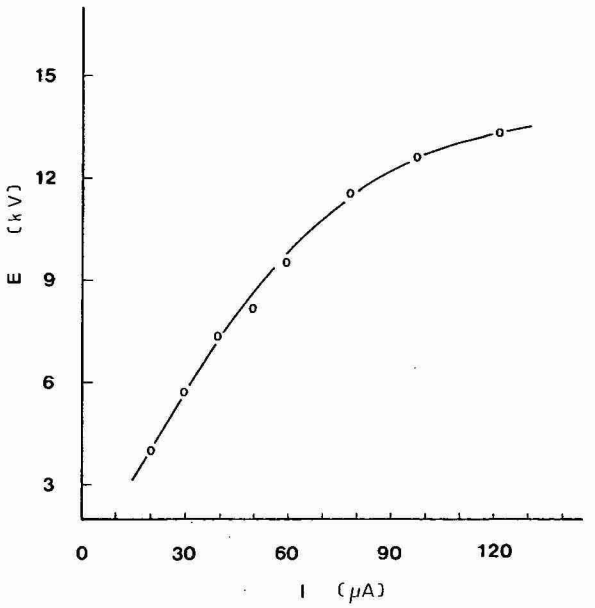


Fig. 1. Relationship between applied voltage, $E$, and electric current, $I$, under the conditions of zone electrophoresis. Capillary tubing: Pyrex glass capillary (422 mm × 85 μm I.D.) connected with fused-silica capillary (142 mm × 195 μm I.D.). Solvent: 0.02 $M$ phosphate buffer, pH 7, with 0.5% ethylene glycol.

parameter instead of $E$, a good linear relationship between electro-osmotic flow, $u_{osm}$, and $I$ or $i$ is obtained as shown in Fig. 2. The parameter $i$ is preferred to $I$, because the former includes the geometrical factor, namely, capillary radius, $r$. The parameter $i$ is not affected by the increasing temperature in solution. From these considerations the following equation can be derived

$$U_{osm} = fi = fI\,(\pi r^2)^{-1} \tag{2}$$

where $f$ is a constant calculated from Fig. 2 and is dependent on all parameters included in eqn. 1 except $E$, its dimensions being $cm^3\ A^{-1}\ sec^{-1}$. Current density is a more basic parameter than voltage in the study of electro-osmosis.

*Effects of capillary materials and pH on electro-osmosis*

The experimental values obtained by using Pyrex glass capillary columns of different I.D. can be plotted on the same lines, A and B in Fig. 2. The difference between the slopes of these lines is readily explained by the difference in concentration of each electrolyte in eqn. 1 (ref. 6). As the Pyrex glass capillaries were home-made, their surface conditions were quite similar in spite of the difference in their I.D. But this is not true for the fused-silica capillaries and FEPs, C–E and F, respectively, in Fig. 2. In these cases, each tubing has its own relationship between $u_{osm}$ and $i$, even though for the same solution. This is due to the different manufacturing processes of fused-silica capillaries and FEPs which lead to different surface conditions with each column of different I.D.

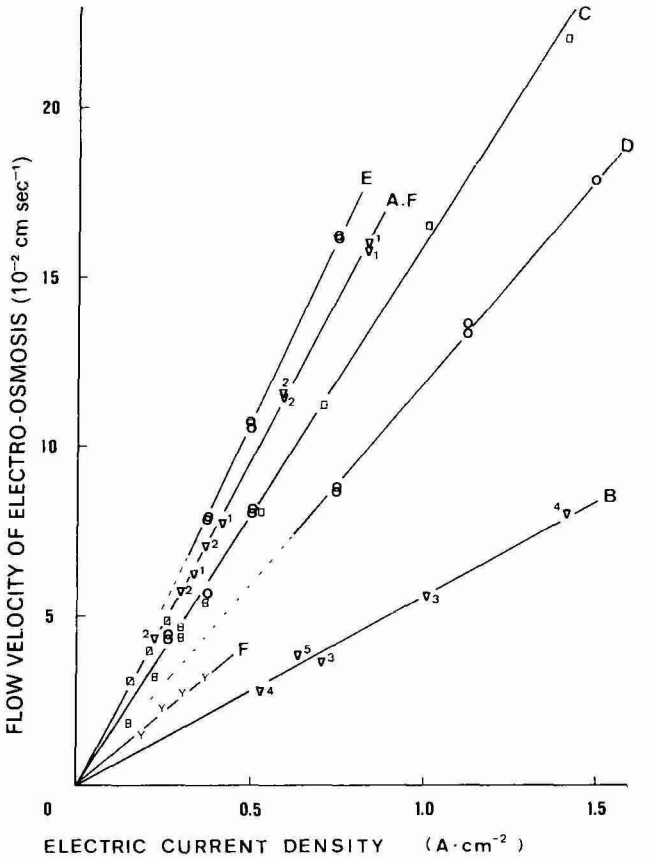


Fig. 2. The relationship between electro-osmotic flow and electric current density. A and B, Pyrex glass capillaries (▽) of 88 (1), 208 (2), 60 (3), 85 (4) and 200 μm I.D. (5); C–E, fused-silica capillaries (○) with 195, 92 and 72 μm, I.D., respectively. F, FEP with 323 (Y) and 500 μm I.D. (▨); C also shows (□ and ⊟) combined tubings of Pyrex glass and fused-silica capillary, 85 and 195 μm I.D. (□) and 208 and 195 μm I.D. (⊟), respectively. The solution was 0.02 *M* phosphate buffer, pH 7, with 0.5% ethylene glycol, except in the case of B, where it was 0.05 *M* $Na_2HPO_4$. Solutes were benzene or pyridine.

Another interesting result in Fig. 2 is the $u_{osm}$–$i$ relationship for the combined Pyrex glass and fused-silica capillary tubings. These tubings were connected face to face with the aid of a small piece of poly(tetrafluoroethylene) tubing, 5 × 0.2 mm I.D. The relationship obtained is the same as that given by the fused silica capillary of 195 μm I.D., *i.e.*, C in Fig. 2. So it is clear that the total electro-osmotic flow in such a combination is controlled by the tubing which gives the lower electro-osmotic flow.

Electro-osmotic flow is slightly dependent on pH in the range shown in Fig. 3.

*Elution time at different current densities and direction of electro-osmotic flow*

Elution times, $T_{e,1}$ and $T_{e,2}$, for a given solute at different current densities, $i_1$

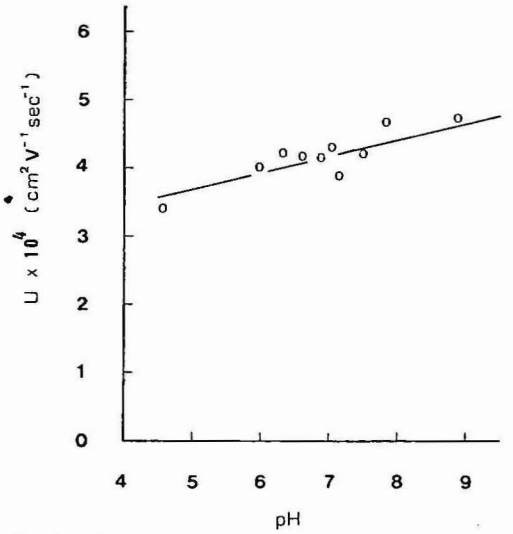


Fig. 3. Effect of pH on electro-osmotic flow. Pyrex glass tubing: 132 $\mu$m I.D., length 40 cm from inlet to detector and 10 cm from detector to electrode. Solute: benzene. Solvent: 0.05 *M* phosphate buffer, pH 7. Constant-voltage mode; 5000 V.

and $i_2$, in the same solvent are easily calculated from

$$\frac{T_{e,1}}{i_1} = \frac{T_{e,2}}{i_2} \tag{3}$$

where subscripts 1 and 2 correspond to the different experimental conditions. For example, with a Pyrex glass capillary (60 cm × 200 $\mu$m I.D.), N-methyl-2,4-dimethylpyridinium iodide was eluted at 17 min and 200 $\mu$A (case 1); using a Pyrex glass capillary of 60 cm × 60 $\mu$m I.D. it was eluted at 5.2 min and 60 $\mu$A (case 2). The solvent in each case was 0.05 *M* aqueous $Na_2HPO_4$, pH 8.9. The value of $T_{e,2}$ calculated by using eqn. 3 and the values of $i_1$, $i_2$ and $T_{e,1}$ is 5.1 min, in good agreement with the experimental value of 5.2 min.

When the present method is applied to separations, we should be careful to estimate values of the electrophoretic mobilities, $u_{mob}$, of the components of the sample and the electro-osmotic flow velocity, $u_{osm}$, in the capillary tubing, and also to determine their flow directions. In our case, $u_{osm}$ was always in the direction towards the negative terminal in the aqueous solution used. However, Terada *et al.*[11] recently reported that the direction was towards the positive terminal when the solution contained cetyltrimethylammonium bromide. In that case the surface active agent was adsorbed on the inner wall, which thus was positively charged. In our case, the surface was always negatively charged under the applied electric field.

*Effect of sample size on theoretical plate number, N*

The relationship between theoretical plate number, *N*, and the injected amount of pyridine using a 72 $\mu$m I.D. fused-silica capillary is shown in Fig. 4. Smaller injected amounts give higher theoretical plate numbers. As the limit of detection of the sample in this experiment was around 1 ng, it was not clear by how much we should reduce the sample size in order to reach the region in which *N* was independent of the sample amount. In other words, if we used a detector with higher sensitivity

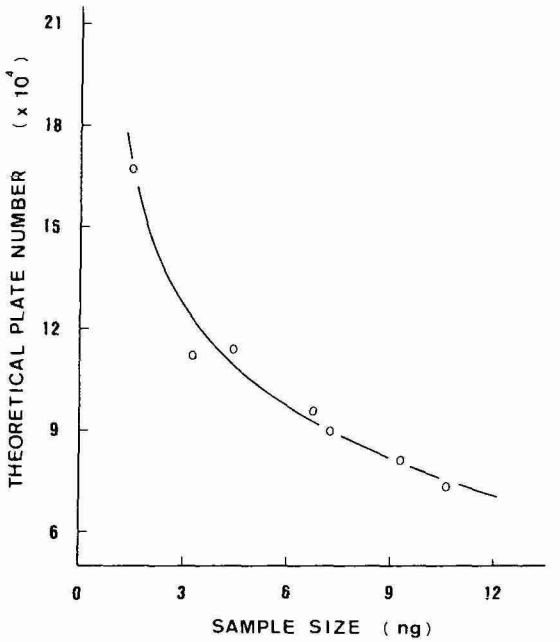


Fig. 4. Relationship between theoretical plate number and sample size. Fused-silica capillary; 72 μm I.D. × 780 mm, of which 610 mm was used as column. Solvent: 0.02 *M* phosphate buffer, pH 7, with 0.5% ethylene glycol. Sample: pyridine. Constant-voltage mode: 12.6 kV.

than the present one, a higher plate number should be obtained. In capillary electrophoresis, $N$ is strongly dependent on the sample size. Therefore, one has to consider carefully the relationships which include the sample size; such as $N$ and $u_{osm}$ or $u_{osm} + u_{mob}$.

*Separations of cations and anions*

As the direction of $u_{osm}$ is always towards the negative terminal under the present experimental conditions, cations move rapidly towards the negative terminal with the apparent velocity, $u_{app}$;

$$u_{app} = u_{osm} + u_{mob} \tag{4}$$

Therefore, the separation of cations could be performed in a very short time.

However, in the case of anions, such as those of sulphonic acids, these flow towards the positive terminal and against the electro-osmotic flow. So the apparent flow velocity for anions is:

$$u_{app} = u_{osm} - u_{mob} \tag{5}$$

Here the positive sign of $u_{app}$ means that the sample moves towards the negative terminal. Therefore the separation of anions usually requires longer times compared to the separation of cations.

Typical examples of separations are shown in Fig. 5–8. The $u_{app}$ for adenosine 5'-monophosphate, shown in Fig. 7, is positive, but the $u_{app}$ values for sulphonic

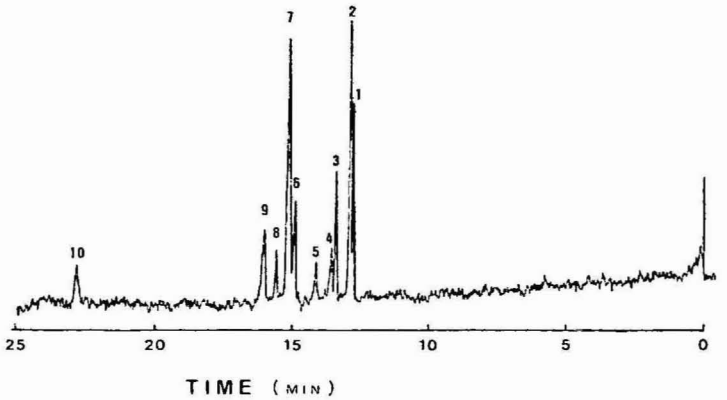


Fig. 5. Separation of pyridinium salts. Pyrex glass capillary: 85 $\mu$m I.D. × 105 cm, of which 90 cm was used as column. Solvent: 0.05 $M$ $Na_2HPO_4$. Constant-current mode: 80 $\mu$A (*ca.* 14.2 kV). Each solute shows $1.5 \cdot 10^5$–$2 \cdot 10^5$ theoretical plates. Solutes: 1 = N-methyl-4-methyl-; 2 = N-methyl-3-methyl- or N-methyl-2-methyl-; 3 = N-methyl-2,4-dimethyl- or N-methyl-2,3-dimethyl-pyridinium salt; 4 = N-methyl-2-aminopyrimidinium salt; 5 = N-benzyl-; 6 = N-benzyl-4-cyano-; 7 = N-2,4-dinitrophenylpyridinium salt; 8 = unknown; 9 = 1,2,3-trimethyl-3,5-bis(ethoxycarboxyl)pyridinium salt; 10 = pyridine.

acids are negative (Fig. 8). Therefore, if we want to elute the sample at a given terminal, negative or positive, it is necessary to control the velocity of electro-osmosis. First, as $u_{osm}$ is strongly dependent on the electrolyte concentration, we should select the concentration of the electrolyte. Secondly, we should choose the material of the capillary tubing. Thirdly, we should choose an additive to modify the surface of the capillary.

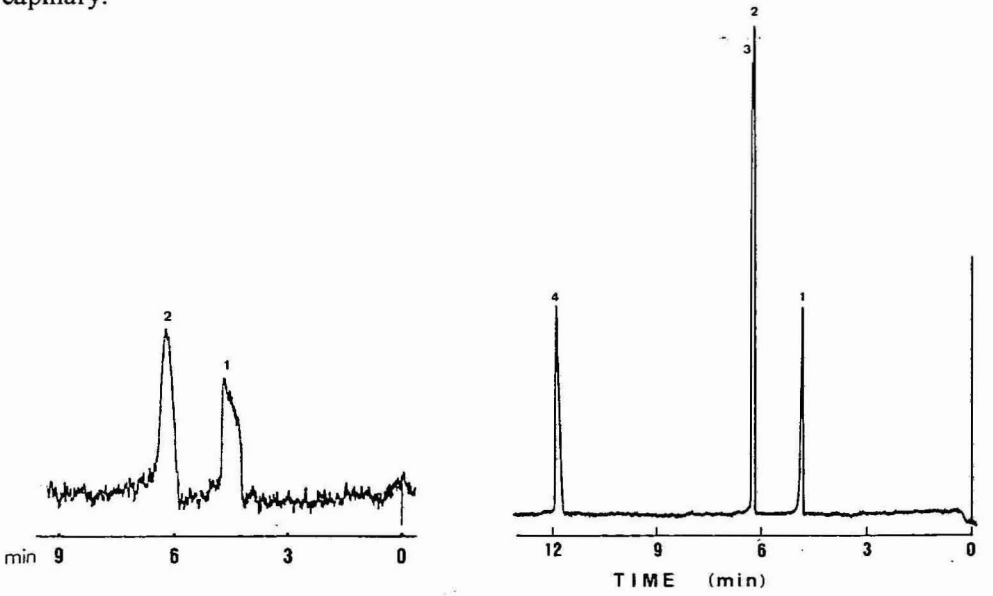


Fig. 6. Separation of cupric (1) and ferric (2) ions. Column: Pyrex glass tubing, 80 $\mu$m I.D. × 36 cm. Solvent: 0.05 $M$ acetic acid. Constant-current mode: 10 $\mu$A (*ca.* 8.6 kV).

Fig. 7. Separation of a mixture including a cation and an anion. Column: fused-silica capillary, 610 cm × 72 $\mu$m I.D. Solvent: 0.02 $M$ phosphate buffer, pH 7, with 0.05% ethylene glycol. Constant-current mode: 30 $\mu$A (*ca.* 16.6 kV). Solutes: 1 = pyridoxamine; 2 = pyridine; 3 = unknown; 4 = adenosine 5′-monophosphate.

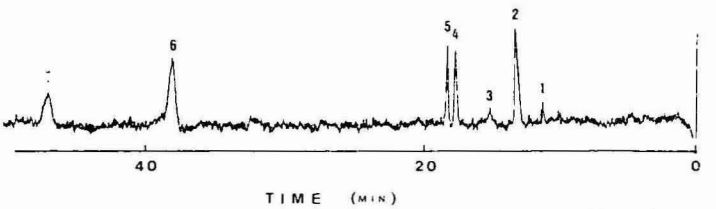


Fig. 8. Separation of sulphonic acids. Column: Pyrex glass capillary, 60 cm × 130 μm I.D. Solvent: 0.05 *M* $KH_2PO_4$. Constant-current mode: 200 μA (*ca.* 11 kV). Solutes: 1 = unknown from reagent 2; 2 = naphthalene-1,3,6-trisulphonic acid; 3 = unknown from reagent 2; 4 = 2,6-naphthalenedisulphonic acid; 5 = 3-(4-sulphophenylazo)-4,5-dihydroxynaphthalene-2,7-disulphonic acid; 6 = *m*-nitrobenzenesulphonic acid; 7 = 2-naphthalenesulphonic acid (all sodium salts).

Pyridine (10 in Fig. 5), which was neutral under the conditions of Fig. 5, was eluted only by the electro-osmotic flow. Peak 1 in Fig. 5 flowed to the negative terminal with an apparent velocity of which the mobility and electro-osmotic flow comprised 40 to 60%, respectively. Plate numbers for solutes in Fig. 5 are around $1.5 \cdot 10^5$–$2 \cdot 10^5$. Although metal cations in Fig. 6 were eluted within 8 min, it is better to use another detector because they have no strong absorbance at 254 nm. If we had used a detector with a high sensitivity for metal ions, we might have obtained a better electropherogram. Work along these lines is now proceeding in our laboratory.

Concerning the separation of metal ions, Hjérten[2] achieved a good separation between $Bi^{3+}$ and $Cu^{2+}$ within 3 min using his alternative method. As the solutes were detected by *in situ* UV scanning of the tube, their migration distance from the original spot was small compared to those in the present method.

From peaks 4 and 2 in Fig. 7, $-u_{mob}$ of adenosine 5′-monophosphate was about one-half of $u_{osm}$. Naphthalenedi- or -trisulphonic acids were eluted within 20 min owing to their high mobilities. The biomedical application of the present method to nucleotides in blood or liver will be discussed elsewhere[12].

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# IMPROVED RADIOACTIVITY DETECTOR FOR FUSED-SILICA CAPILLARY COLUMNS*

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## SUMMARY

$^{14}C$-labeled and unlabeled organic compounds eluting from fused-silica capillary columns were detected by the parallel combination of a radioactivity detector (RAD) and a flame ionization detector (FID). The RAD consists of a small-volume counter tube and ancillary electronics. It is constructed from readily available parts. For maximum signal-to-noise ratio, the RAD is operated at 70% counting efficiency. The resulting background is less than 5 cpm. The instrumentation delivers high chromatographic efficiency and eliminates combustion artifacts. Radioactive peaks are 12 sec wide, while the detection limit is *ca.* 440 cpm (0.2 nCi) for peaks with $k' \leqslant 5$. No degradation in column performance is apparent in the FID trace.

## INTRODUCTION

Separations of $^{3}H$- and $^{14}C$-labeled materials by gas chromatography (GC), and their detection in the gas stream, are well documented in the literature. An excellent review of this topic appeared in this journal[1]. The continuous monitoring of $^{14}C$-labeled and unlabeled compounds is typically performed after splitting the eluted sample. One fraction of the sample goes to a flame ionization detector (FID) while the remaining fraction is counted. Frequently, the counting is done after conversion of the compounds to $^{14}CO_2$ (refs. 2–4). This conversion facilitates transfer of the radiolabel to the radioactivity detector (RAD) and permits room-temperature operations of the RAD. In addition, it eliminates possible compound-related quench problems.

The RAD can be a flow-through detector of a variety of types: the $^{14}CO_2$ can be trapped in a liquid stream which contains a scintillator, and then counted, or it can be trapped in aqueous NaOH and counted by flowing the liquid stream through a cell packed with a solid scintillator[5]. Alternatively, the $^{14}CO_2$ can be counted as a gas by means of a solid scintillator[6,7] or a proportional counter[2–4,8].

The use of flow-through detectors requires a compromise between sensitivity

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and resolution. Sensitivity is dependent on the counting time, and therefore depends on the residence time of the material in the counter. A long residence time increases sensitivity but may impair resolution. For packed-column GC, a long residence time (*e.g.*, 60 sec) may not significantly affect resolution. This is not true for capillary columns where the peak-width may be only a few seconds.

Instrumentation to perform FID and RAD detection on effluents from capillary columns has been reported[6,8,9]. Gross *et al.*[6] used a microfurnace to convert the radiolabel to $^{14}CO_2$ to minimize band spreading prior to radioactivity detection. A commercial flow monitor, equipped with an anthracene cell (total volume *ca.* 7 ml), was used as the detector. The flow-rate through the detector was adjusted to 12–14 ml/min with a suction pump. They reported a limit of detection of 7 Bq (420 dpm or 0.2 nCi). We estimated the minimum peak-width of the radioactive peaks shown at *ca.* 30–60 sec.

Ernst *et al.*[8] adapted a commercial gas flow proportional counter (with a 20-ml volume) for use with capillary columns. Make-up gas was added to adjust the gas flow through the counter to 60 ml/min. They estimated their detection limit at about 100 dpm (0.05 nCi). However, based on their experimental conditions, the minimum peak-width of the radioactive peaks is likely to exceed 30 sec.

A short residence time in the flow-through RAD is necessary to match better the resolution available by the use of capillary columns. However, a short time requires a high signal-to-noise ratio to achieve an acceptable detection limit.

Gas flow-through proportional counters have the potential for higher signal-to-noise ratios than approaches based on the use of solid or liquid scintillators. For example, an anti-coincidence gas counter having *ca.* 95% efficiency for $^{14}C$ and a background of *ca.* 1 cpm has been reported[10]. In addition, the counter tube and ancillary instrumentation can be simple and reliable. For these reasons, a gas flow-through proportional counter was chosen for our design.

In this report we describe the instrumentation developed for parallel detection of carbon-containing and $^{14}C$-labeled compounds separated by capillary GC. A small-volume counter, a simple and inexpensive electronics package and the elimination of combustion artifacts combine to produce low detection limits while maintaining the narrow bandwidths associated with capillary columns.

## EXPERIMENTAL

### *Gas chromatograph*

A Perkin-Elmer 3920 gas chromatograph, equipped with a quartz injector/trap[11], was modified to accept the RAD. A diagram of the instrumentation is shown in Fig. 1. Two modifications were performed: first, a splitter was installed in the detector manifold section of the gas chromatograph. Second, access to the splitter from the right side of the unit was provided by drilling a 1-in. hole through the side panel and a 0.5-in. hole through the manifold wall. A Vycor combustion tube was brought into the manifold through these holes. The larger hole holds an Inconel tube used to house the combustion tube.

The splitter and the inlet of the combustion tube are shown in Fig. 2. The necessary assemblies were made from stainless-steel (SS) Swagelok fittings: 1/2-in. to 1/16-in. reducing union and two 1/16-in. tees. The union and one of the tees were

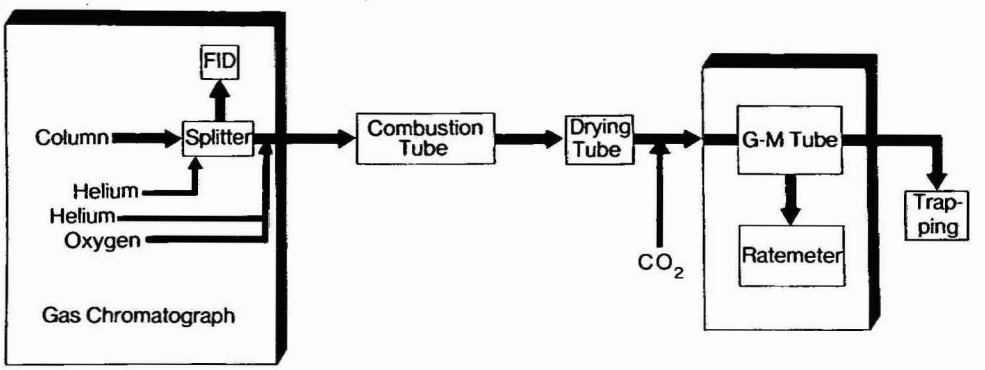



Fig. 1. Diagram of FID/RAD for detection of $^{14}C$-labeled compounds. G-M = Geiger-Müller gas flow-through proportional counter.

drilled to accept 1/16-in. O.D. SS tubes which were silver-soldered in place.

These tubes are used to add make-up gases. The split ratio was adjusted by varying the length of 0.2-mm I.D. fused-silica lines connecting the modified tee to the detectors. Two 15-in. lengths were used to produce a *ca.* 50/50 split. Vespel ferrules were used to obtain gas-tight connections. A make-up gas (I, Fig. 2), He at 20 ml/min, was added to the modified tee to keep the residence time of a peak in the splitter to less than 0.1 sec.

*Combustion tube*

A 60-cm long Vycor tube (6 mm O.D., 4 mm I.D.) was loosely packed with a mixture of ground quartz and CuO wire (0.4–0.5 mm diameter): 6–8 g of mixture occupied a section of tube *ca.* 23 cm long. Quartz wool was used to keep the bed in

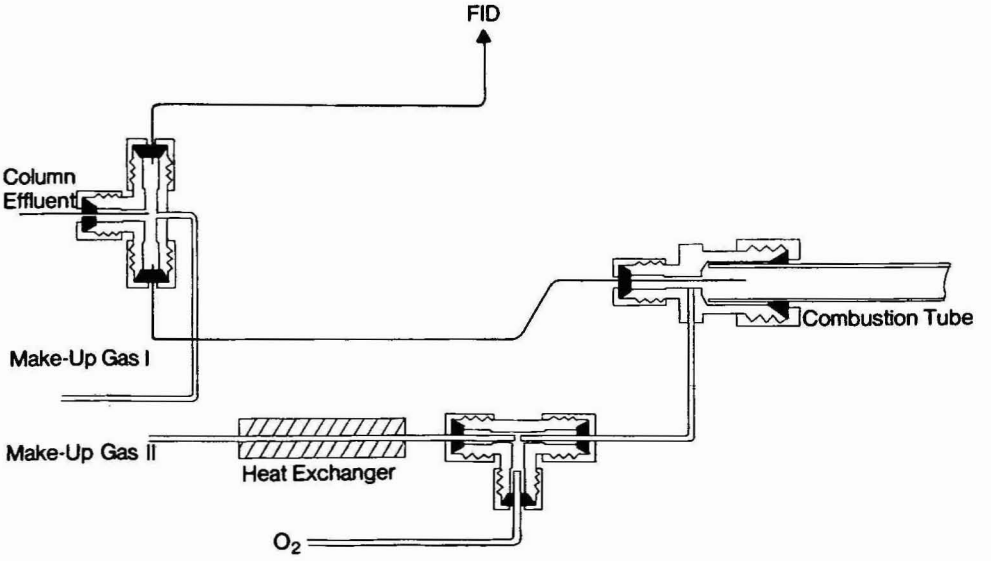



Fig. 2. Diagram of splitter and combustion tube inlet. All components shown are installed in the detector manifold of the gas chromatograph.

place and quartz rod inserts (3 mm O.D.) were used to occupy the empty ends of the tube.

The combustion tube was housed within a 45 × 2.5 cm O.D. Inconel tube and was placed inside a combustion furnace (Model 55035; Lindberg, Watertown, WI, U.S.A.) kept at 800°C. The placement of the assemblies is such that the head of the combustion bed is 10 cm from the end of the furnace nearest to the gas chromatograph. The furnace was located next to the wall of the gas chromatograph.

Make-up gas (II, Fig. 2), helium at 49 ml/min, was added to the combustion tube via the modified reducing union. A trickle of oxygen, at *ca.* 1 ml/min, was added to the second make-up gas via the tee placed in the detector manifold. The effluent of the combustion tube was passed through a drying tube containing $Mg(ClO_4)_2$, and was mixed with quench gas ($CO_2$, 6 ml/min) prior to entering the counter tube.

*Split ratio measurements*

The FID jet tip was removed and replaced with a SS tube silver-soldered to the base of a FID jet. The split ratio was calculated from either measured flow-rates or from radioactivity collected at the FID and RAD. The $^{14}CO_2$ was collected with an ethanolamine-based cocktail. Liquid scintillation counting (LSC) was done by a Packard Tri-Carb Scintillation spectrometer (Model 2001; Packard, Downers Grove, IL, U.S.A.). The cocktail collection efficiency for $CO_2$ and intact organic compounds was 100%, while the combined scintillation/counting efficiency of the cocktail and counter was 59%.

Some split ratio measurements, and all of the experiments designed to quantitatively measure contamination, were performed without a column. In these experiments, transfers of materials from the injector/trap to the splitter were made via a 0.3 mm I.D. PTFE line.

*Contamination measurements*

For these experiments a 10-ml sample of $^{14}CH_2Br_2$ in air, containing *ca.* 150 μg and *ca.* 48 nCi, was trapped in the injector/trap. After transfer to the detectors via the PTFE line, the radioactivity was collected for 5 min. The split ratio was calculated from these data. Residual radioactivity was calculated from the radioactivity collected for an additional 5 min.

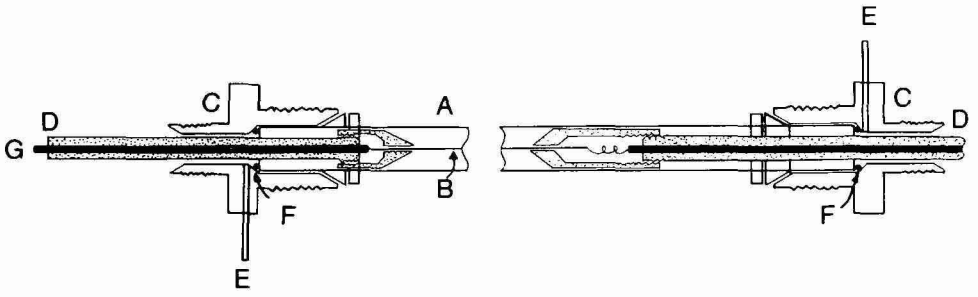



Fig. 3. Diagram of counter tube. A = 12.5-mm O.D. SS tube; B = tungsten wire; C = nylon reducing union; D = Delrin holders; E = 1/16-in. O.D. SS tube; F = O-ring spacer; G = brass rod.

*Radioactivity detector (RAD)*

The RAD consists of a counter tube and associated electronics. The counter tube is a 10 cm × 12.5 mm O.D. × 11 mm I.D. SS tube. The interior was polished to a smooth finish. The tube was polarized with a −2.2 kV regulated supply (No. 224; Keithley, Cleveland, OH, U.S.A.). The anode is a 5 cm × 0.1 mm O.D. tungsten wire. The anode is centered in the tube by Delrin holders placed in modified Nylon 1/2-in. to 1/4-jn. reducing unions. These unions were drilled to accept a 1/16-in. O.D. tube. A tube was fixed to each of the unions with epoxy glue. A schematic diagram is shown in Fig. 3. The assembly is housed in a 1-in. thick lead shield.

Current pulses occurring at the anode are converted to a voltage by an operational amplifier (gain = $2 \cdot 10^{-8}$ A/V), stretched in time (time constant, $\tau$ = 0.5 msec), discriminated with respect to amplitude and time, and counted. The counter module (TIL 307; Texas Instruments, Dallas, TX, U.S.A.) serves also as a display and latch. The contents of the latches are updated every 6 sec and displayed on a recorder via a 12-bit digital-to-analogue converter. A diagram is shown in Fig. 4.

*Calculations*

The displayed counts (RAD counts) are related to the radioactivity injected by the equation

$$\text{RAD (counts)} = A \times S \times \varphi \times E \times (V/F)$$

where $A$ = radioactivity injected (dpm), $S$ = split ratio, $\varphi$ = fraction of sample

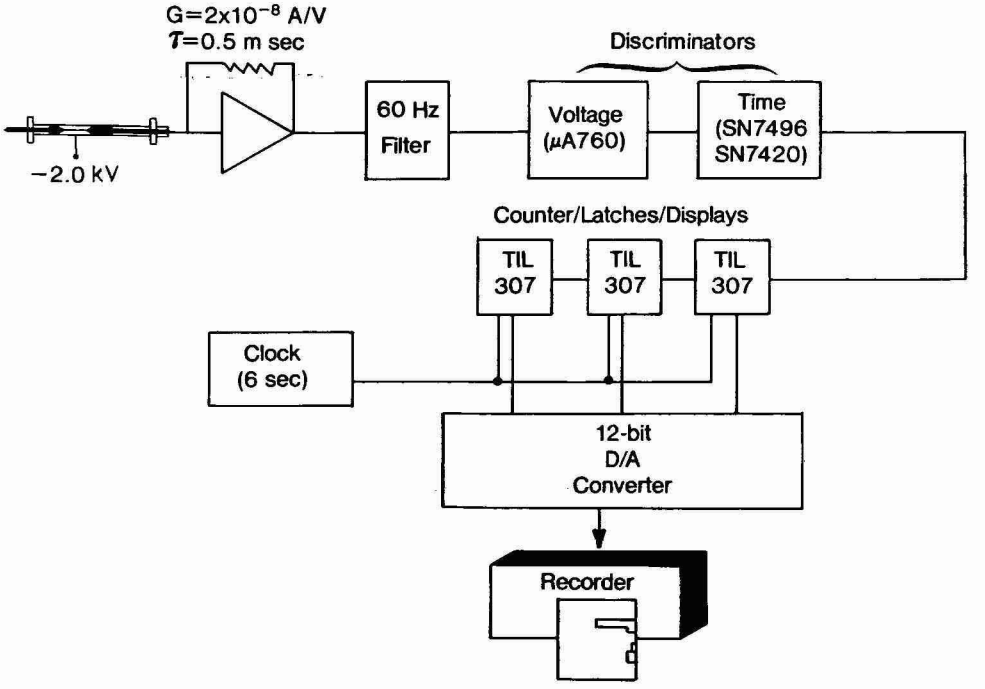



Fig. 4. Diagram of counter electronics. G = Gain; D/A = digital-to-analogue.

recovered from the system, $E$ = counter efficiency and ($V/F$) = residence time in the counter ($V$ is the effective counter volume in mL, $F$ is the gas-flow rate in ml/min). For most of the work presented here, $S = 0.46$, $E = 0.70$ and $V/F = 0.070$ min. Under these conditions the equation reduces to:

$$\text{RAD (counts)} = A \times \varphi \times (0.023)$$

$\varphi$ was calculated by dividing the radioactivity collected from the RAD effluent by the product of $A$, $S$ and the cocktail trapping/counting efficiency.

*Racioactive standards*

*n*-Decane, *n*-$^{14}C_{10}$ (1-$^{14}$C, 7.5 mCi/mmol), and *n*-decanol, *n*-$^{14}C_{10}OH$ (1-$^{14}$C, 7.5 mCi/mmol), were purchased from ICN (Irvine, CA, U.S.A.). Naphthalene (1(4,5,8)-$^{14}$C, 5 mCi/mmol) was purchased from Amersham (Amersham, Great Britain). Working standards were prepared by dilution in pentane. Dibromo [$^{14}$C]methane was purchased from California Bionuclear Corp. (Sun Valley, CA, U.S.A.) and was diluted with the cold materialm to an activity of 0.054 mCi/mmol.

## RESULTS AND DISCUSSION

Several experimental variables affect the split ratio. To eliminate column-oven temperature as a variable, the splitter was placed in the thermostated detector manifold of the gas chromatograph. However, the split is a sensitive function of the backpressure generated downstream from the splitter.

The effect of downstream pressure changes on the split ratio is minimized by reducing the backpressure generated by the components placed after the fused-silica lines: the FID, the combustion tube and the counter tube. Of these, only the combustion tube presents a problem. The other components generate less than 0.5 p.s.i. at the flow-rates used.

When the combustion tube was packed with CuO particles, 0.1–0.3 mm diameter, and in the absence of $O_2$, the backpressure exceeded 13 p.s.i. with the bed at 800°C. Dilution of the packing with ground quartz and the use of CuO wire, 0.3–0.5 mm long, produced a more porous bed with lower backpressure. A 3:1 mixture of CuO wire and quartz particles, 0.5–1 mm mean diameter, generated 3.4 p.s.i. of backpressure at 800°C. However, after three injections of $^{14}CH_2Br_2$, corresponding to the combustion of a total of *ca.* 210 $\mu$g of compound, the backpressure increased to 6.4 p.s.i. The split ratio (RAD/FID) changed from 57/43 to less than 50/50, as shown in Fig. 5I, curve C. Furthermore, the radioactivity remaining in the combustion tube, after the elution of the main body of radioactivity, increased with mass or number of injections made. This is shown in Fig. 5II, curve C.

The changes in backpressure and the residual radioactivity in the combustion tube are a consequence of the surface exhaustion of the CuO bed. As the surface changes, the Cu-containing particles fuse together, thereby decreasing the permeability of the packing and increasing the adsorptive capacity of the bed. Because of the high temperature, the adsorbed compounds eventually decompose or diffuse to oxygen-containing bed sites. The result is a slow release of radioactivity from the combustion tube. Practical consequences of this behavior are poor peak shape and

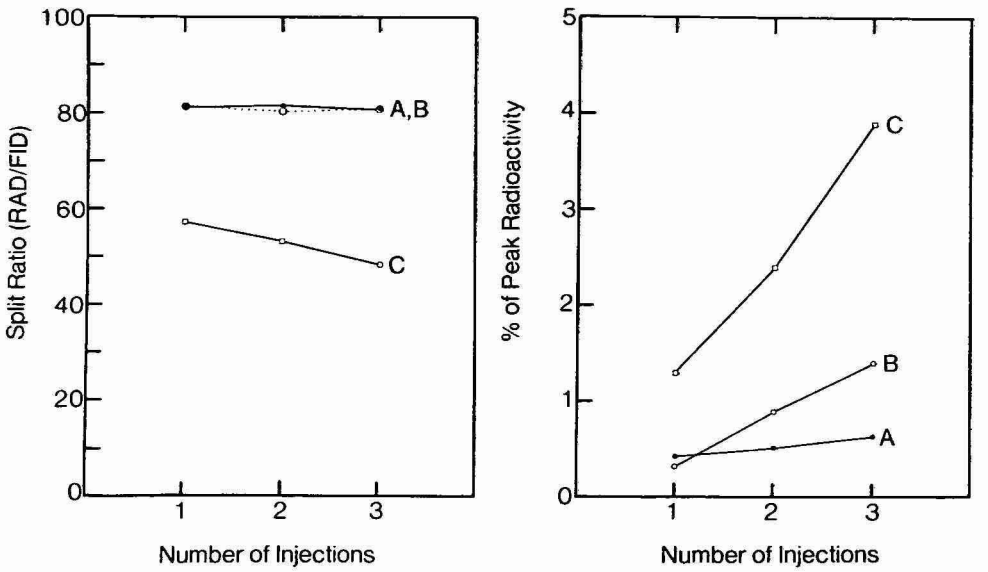


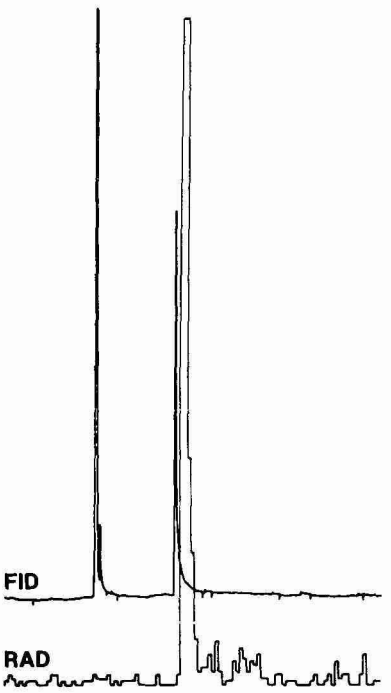

Fig. 5. I, Variation of split ratio after injection of $^{14}CH_2Br_2$; II, radioactivity remaining in the combustion tube after injection of $^{14}CH_2Br_2$. Bed composition: A, CuO: quartz mixture 5:1 (w/w) + a trickle of $O_2$; B, CuO: quartz mixture 5:1 (w/w) with no added $O_2$; C, CuO: quartz mixture 3:1 (w/w).

poor recovery of radioactivity. This is illustrated in Fig. 6, where only 61 % of the expected radioactivity is found under the peak. Note the increased background after the peak and the lack of corresponding changes in the FID trace. Increasing the CuO wire length increases bed permeability and decreases the retention of radioactivity in the combustion tube. A 80:20, RAD/FID ratio was selected to illustrate these two points. Curve B, Fig. 5I and II, corresponds to a 5:1 CuO: quartz bed packed with 1–3 mm CuO wire and 0.3–0.5 mm quartz particles. With this packing the backpressure is less than 2 p.s.i. and the split ratio remains constant (5I). The radioactivity remaining in the combustion tube is *ca.* 1.5% after three injections, as shown in Fig. 5II, curve B. Further decreases in residual radioactivity were achieved by the continuous introduction of a trickle of oxygen into the combustion tube, as shown in Fig. 5II, curve A.

While the introduction of $O_2$ into the combustion tube results in a stable bed that requires no repacking, it changes the operating parameters of the counter tube. This is apparent in Fig. 7. A broad efficiency maximum is observed in the absence of $O_2$ (7A) but a continuous increase in efficiency with polarizing voltage is seen when $O_2$ is present (7B).

The broad maximum, rather than the expected Geiger-Müller plateau, is a consequence of the d.c. approach, the gain and the time constant we have chosen to process the current pulses. As the internal gain of the tube increases with polarizing voltage, the magnitude of the current pulses also increases. These pulses are very large in the Geiger-Müller region, and, because of their magnitude, they produce short-term saturation of the amplifier at the chosen gain. The saturation and long time constant combine to produce pulse pile-up and an apparent decrease in efficiency.

Because $O_2$ is a more efficient quencher than $CO_2$, its use requires a higher tube voltage to achieve the same counting efficiency. However, there is no apparent



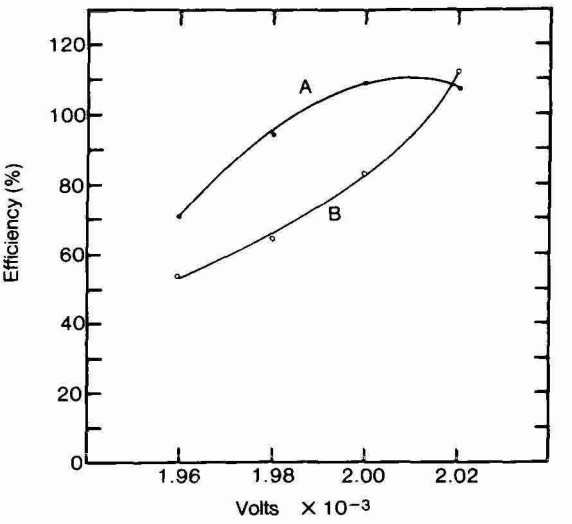




Fig. 6. Chromatogram of *n*-decanol. Combustion without added oxygen. Isothermal run: 140°C, SE-54, 30 m × 0.32 mm J&W fused-silica column. Mass (activity) injected = 96 ng (4.6 nCi). Injection volume = 5.8 µl. FID sensitivity = 5 pA full scale. Split: 80/20 (RAD/FID). Recovery of radiolabel under the peak: 61 %

Fig. 7. Counter-tube efficiency as a function of gas composition. A, He 59 ml/min, $CO_2$ 6 ml/min; B, He 59 ml/min, $CO_2$ 6 ml/min, $O_2$ 1 ml/min.

Geiger-Müller region. This is inconsequential to the operation of the RAD because we operate the counter tube in the proportional region where efficiency is dependent on applied voltage. This mode of operation does not represent a serious drawback when highly regulated supplies (0.01 %) are used and when the current measurement approach (*i.e.*, a d.c. measurement with the anode at virtual ground) ensures a constant field gradient across the tube. Therefore, to operate the tube in the presence of $O_2$, it is only necessary to use a more negative potential to achieve the desired efficiency. For maximum signal-to-noise ratio, the tube is operated at 70 % efficiency. The resulting background is 3–5 cpm.

The chromatographic performance of the system is illustrated in Fig. 8. No increase in background radioactivity is seen after the elution of *n*-$^{14}C_{10}$. Because this compound elutes shortly after the solvent (capacity factor, $k' < 1$) the background radioactivity after the $C_{10}$ peak would have shown an increase if there were surface exhaustion of the combustion bed by the solvent. Since the mass present in the solvent peak is likely to exceed that of any other compound in the chromatogram, we expect a constant, low radioactivity background under most separation conditions.

The number of theoretical plates, $N$, calculated from the FID trace of the *n*-$C_{12}$ hydrocarbon (isothermal run, $k' \approx 3$), is *ca.* $10^5$. For peaks with $k'$ between 3

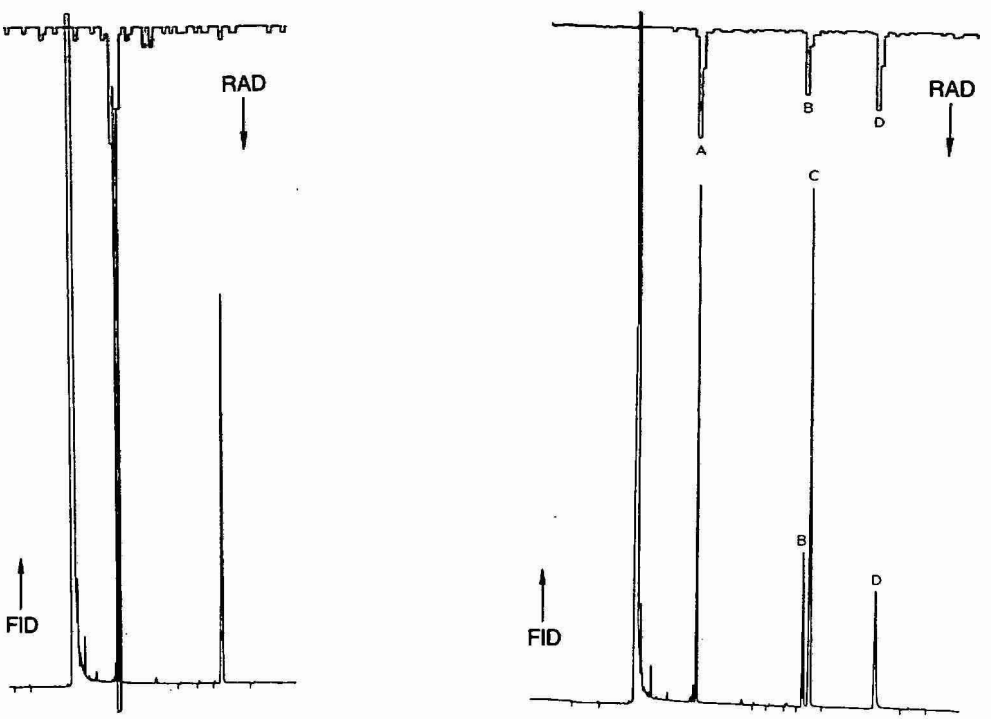



Fig. 8. Chromatogram of $n$-$^{14}C_{10}$ and $n$-$C_{12}$ hydrocarbons in pentane. Temperature-programmed run: 110°C isothermal hold 4 min, 4°C/min to 120°C and hold. Durabond 5 J&W, 30 m × 0.32 mm I.D. Absolute recovery of radiolabel under the peak: 80%. RAD efficiency: 68%. Sensitivity: 100 counts full scale. RAD/FID split ratio: 46/54. Mass injected: *ca.* 70 ng per compound.

Fig. 9. Chromatogram of $^{14}C$-labeled standard materials and cold $n$-$C_{12}$. Mass (radioactivity) injected: A, $n$-$^{14}C_{10}$, 63 ng (2.4 nCi); B, $^{14}C$-Naphthalene, 25 ng (0.9 nCi); C, $n$-$C_{12}$, 110 ng; D, $n$-$^{14}C_{10}OH$, 38 ng (1.5 nCi).

TABLE I

ANALYSIS OF RADIOLABELED STANDARDS

Mass (radioactivity) injected: $n$-$^{14}C_{10}$, 50–80 ng (2.6–4.2 nCi); $^{14}C$-naphthalene, 20–30 ng (0.8–1.2 nCi); $n$-$^{14}C_{10}OH$, 30–50 ng (1.4–2.4 nCi).

| | *Nominal distribution (%)* | | *Experimental distribution (%)* | | |
|---|---|---|---|---|---|
| | *Radioactivity* | *Mass* | *FID area* | *$^{14}CO_2$ liquid scintillation* | *RAD counts* |
| $n$-$^{14}C_{10}$ | 54 | 50 | 52 ± 1 | 50 ± 1 | 47 ± 2 |
| Naphthalene | 14 | 20 | 21 ± 1 | 18 ± 1 | 19 ± 5 |
| $n$-$^{14}C_{10}OH$ | 30 | 30 | 27 ± 1 | 32 ± 1 | 35 ± 3 |

TABLE II

FID RESPONSE FACTOR DETERMINATION BY "COLD" AND RADIOLABELED STANDARDS

| *No. of measurements* | *Mass (ng) range tested* | *Compound* | *Apparent response factor (Coul/g compound)* | *Absolute recovery (%)* | *Corrected response factor (Coul/g compound)* |
|---|---|---|---|---|---|
| 5 | 36-90 | $n$-$C_{12}$ | 0.014 ± 0.001 | | 0.018 |
| 6 | 38–94 | $n$-$^{14}C_{10}$ | 0.014 ± 0.001 | 80 ± 4 | 0.018 |

and 5, the chromatographic efficiency calculated from the RAD trace is *ca.* $2 \cdot 10^4$ theoretical plates.

While the high chromatographic efficiency measured with hydrocarbons is an indicator of splitter performance, a more severe test is posed by the quantitative, temperature-programmed analysis of a mixture containing polar compounds. A chromatogram is shown in Fig. 9. The results are summarized in Table I.

The distribution of radioactivity recovered as $^{14}CO_2$ from the RAD agrees, withinin experimental error, with the distribution calculated from the RAD counts. The larger relative error observed in the RAD distribution is the result of the

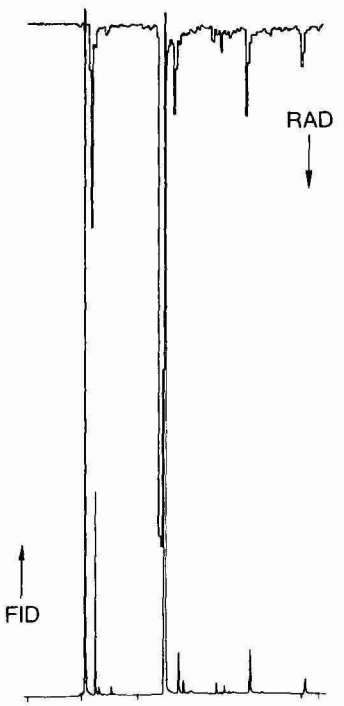



Fig. 10. Chromatogram of underivatized $^{14}$C-diethylenetriamine. Conditions: 140°C isothermal hold 2 min, 8°C/min to 190°C and hold. Carbowax J&W, specially deactivated for amines, 30 m × 0.25 mm I.D. RAD/FID split: 46/54. RAD sensitivity = 1000 counts full scale. Mass (radioactivity) injected = 2 μg (500 nCi).

TABLE III

RADIOCHEMICAL DATA OF DIETHYLENETRIAMINE (DETA) SAMPLE

| | |
|---|---|
| *Specfic activity determination* | |
| FID area | $(5.77 \pm 0.08) \cdot 10^6$ |
| Mass DETA eluted (g)* | $1.15 \cdot 10^{-6}$ |
| $^{14}CO_2$ activity (cpm) | $(1.68 \pm 0.1) \cdot 10^5$ |
| Radioactivity eluted (nCi)** | 280 |
| Calculated DETA specific activity (mCi/mmol) | 25 |
| Bulk specific activity reported (mCi/mmol) | 20 |

*Distribution and radiopurity of DETA sample by three detection methods*

| | *FID (area %)* | *LSC (cpm %)* | *RAD (counts %)* |
|---|---|---|---|
| Ethylenediamine | 7.1 ± 0.3 | 6.3 ± 0.4 | 7.3 ± 1.1 |
| DETA | 85.5 ± 0.1 | 82.7 ± 1.9 | 82.6 ± 2.3 |
| Unknown I | 2.4 ± 0.3 | 4.3 ± 1.5 | 3.8 ± 0.4 |
| II | 3.4 ± 0.2 | 4.4 ± 0.5 | 3.5 ± 1.2 |
| III | 1.6 ± 0.1 | 2.4 ± 0.7 | 2.8 ± 0.6 |

* Calculated from sensitivity of FID in C/g carbon, carbon content of DETA and split ratio.
** Calculated from scintillation/counting efficiency and split ratio.

short residence time and low activity of the samples. Less than 50 RAD counts were recorded for the naphthalene peak.

The distribution calculated from FID areas agrees with the nominal mass distribution calculated from the radioactivity and the specific activity of the standards. This agreement is due to the similar FID response factors and transfer efficiency (recovery) of the compounds studied.

Knowledge of the transfer efficiency of a compound is most important when performing parts-per-billion ($1/10^9$) analyses on gaseous samples. This is true because it is nearly impossible to prepare accurate gaseous standards at those levels and the internal-standard technique is not generally applicable. The use of a radiolabeled model compound, if not the analyte itself, allows accurate calibration of the FID detector. Once this is done, the FID is available to perform ultra-trace analyses. An illustration of this application is shown in Table II, where the apparent FID response measured by injecting known masses of $n$-$^{14}C_{10}$ is corrected by the transfer efficiency of the compound. As expected, the uncorrected values measured for the $n$-$C_{10}$ and $n$-$C_{12}$ hydrocarbons agree since they should have the same transfer efficiency. However, only 80% of the compound is reaching the detectors. Therefore, for accuracy, the results can now be corrected to account for the transfer efficiency of the system.

Finally, the ability to perform accurate mass and radioactivity measurements can be used in establishing radiopurity and specific activity. This point and the high resolution possible with the equipment are illustrated in Fig. 10 and Table III.

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# SIMULTANEOUS DETERMINATION OF SMALL AMOUNTS OF HYDROCHLORIC AND HYDROBROMIC ACIDS BY DERIVATIZATION WITH ETHYLENE OXIDE AND GAS CHROMATOGRAPHY

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## SUMMARY

A method has been developed to determine quantitatively micromole amounts of hydrochloric and hydrobromic acids by derivatizing the acids with ethylene oxide and determining the 2-haloethanols by gas chromatography. The advantages of the method are that chloride and bromide are determined simultaneously and that only small amounts of samples and reagents are required. Hydrobromic acid can be analyzed over the concentration range from $1 \cdot 10^{-1}$ to $5 \cdot 10^{-4}$ *M* and hydrochloric acid from $1 \cdot 10^{-1}$ to $2 \cdot 10^{-4}$ *M*. Derivatizations can be performed on 0.5 ml of solution. At $5 \cdot 10^{-4}$ *M*, this volume contains 0.25 μmole of halide. The relative precision of the method is 3–10% at the 95% confidence limit.

The method is applicable to a number of analytical problems and provides a means of measuring the amounts of hydrochloric and hydrobromic acids evolved during the combustion of flame-retarded polymers in a Schöniger flask. Samples weighing 10–200 mg and containing *ca.* 1–20% (w/w) total halogens have been analyzed.

## INTRODUCTION

Small amounts of HCl, HBr, and HI can be determined by treating the acids with a 1,2-olefin epoxide and quantifying the corresponding haloalcohols by gas chromatography (GC) as first reported by Russel[1]. Despite the indication that the method would be useful for simultaneous determination of the halides, subsequent published work has pertained only to the analysis of HCl: Petruj *et al.*[2] determined small amounts of HCl in chlorinated organic solvents by direct reaction of the solvents with gaseous ethylene oxide and GC determination of the 2-chloroethanol. Vierkorn-Rudolph and co-workers[3–5] have undertaken detailed studies of the reaction of HCl with a number of epoxides, with the goal of obtaining sufficient sensitivity to measure HCl in samples of air from the upper troposphere. This paper reports the development of a method for the simultaneous, quantitative analysis of HCl and HBr as their haloethanols.

In addition to the fact that the halides are simultaneously determined, the

primary advantages of the method are its sensitivity and the requirement of only small amounts of samples and reagents. About 0.5 ml of solution is required for derivatization, and quantitative results can be obtained to a lower limit of *ca.* $5 \cdot 10^{-4}$ *M*. Semiquantitative results can be obtained to *ca.* $10^{-5}$ *M*.

The method is generally applicable to analyses of solutions in which halide ions are present as their hydrohalic acids and was developed specifically in order to satisfy the requirements for measuring the amounts of HCl and HBr released in small-scale laboratory tests of flame-retarded materials containing both chlorinated and brominated compounds*. For these tests, such as combustion in a Schöniger flask, gases evolved during combustion or pyrolysis of milligram amounts of material are collected in a small volume of an aqueous absorbent which is subsequently analyzed. It is therefore necessary to determine bromide and chloride simultaneously at concentrations of *ca.* $10^{-2}$–$10^{-4}$ *M* in sample volumes of *ca.* 10 ml. Also, since materials being studied include formulations based on poly(vinyl chloride), chlorinated polyethylene, and polyethylene, which can release large and widely varying amounts of HCl and/or $CO_2$ when burned, it is necessary that the determination of $Br^-$ be unaffected by large excesses of $Cl^-$, and that all methods be insensitive to excess $CO_2$ and to variations in pH.

Because of these requirements, a number of other analytical methods were not applicable. For example, neither classical gravimetric and titrimetric methods nor ion-selective electrodes[6]** have sufficient sensitivity and selectivity under the conditions described above. Direct GC determination of small amounts of HCl and HBr is not feasible for several reasons: the poor sensitivity of thermal conductivity detectors; the need for special detector filaments and for materials not corroded by HCl and HBr; and the tendency of HCl and HBr to produce tailing, asymmetric peaks from which it is difficult to obtain quantitative data. Other indirect GC methods employing derivatization of halide ions and flame-ionization and electron-capture detectors are not suitable because they use potentially hazardous organo-mercury compounds[7], need relatively large and concentrated samples[8], require non-aqueous solvents[9], or are appropriate for bromide and iodide, but not for chloride[10].

Although the requirements listed previously can also be satisfied by ion chromatography[11,12], this relatively new and still-evolving variant of ion-exchange chromatography employs columns of specially modified and sometimes proprietary resins and requires the use of extensively modified high-performance liquid chromatographs or dedicated ion chromatographs. For applications not requiring the greater sensitivity obtainable with ion chromatography and for laboratories not performing large numbers of routine analyses for ions, the indirect GC method can offer advantages in terms of simplicity, economy, and availability of materials and instrumentation.

The remainder of this paper will present procedures for analyses in aqueous solutions. Results from analyses of two flame-retarded materials will be presented to illustrate applications of the method.

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* It is assumed that the sole source of halide in the absorbent is the HCl and HBr evolved by combustion, since no inorganic halides are present in the formulations being studied.

** Although ion-selective electrodes have been used to determine $Br^-$ and $Cl^-$ in coal samples combusted in an oxygen bomb, *ca.* 1 g of material was needed and it could be inferred from the author's discussion that the method lacked the sensitivity for use with milligram samples, such as combusted in a Schöniger flask.

## EXPERIMENTAL

### *Derivatization*

*n*-Pentanol, as an internal standard, and $HNO_3$, as a catalyst, are added to solutions just prior to derivatization. A 1.0-ml aliquot of a solution which is 0.006 *M* in *n*-pentanol and 0.94 *M* in nitric acid is added to 5.0 ml of the sample. A 0.5-ml aliquot of this solution is placed in a 1-ml micro-reaction vial (Pierce) with a magnetic stirrer and is sealed with a PTFE-lined silicone septum and screw cap.

Ethylene oxide from a lecture bottle is introduced into the vial through a 22-gauge stainless-steel needle and is bubbled into the solution for 5 min. Another 22-gauge needle is inserted into the headspace of the vial for 5 sec per minute to release the pressure. Sealed vials are heated in a constant temperature bath at 45°C and the solutions stirred for 1 h. Venting needles are then inserted into the headspace, and the solutions are stirred at room temperature overnight.

### *Gas chromatography*

Samples are analyzed on a 6 ft. × 1/8 in. I.D. stainless steel column packed with 10% Carbowax K20M on 80–100 mesh Chromosorb W AW DMCS. The gas chromatograph (Perkin-Elmer 990) is equipped with dual columns, dual flame ionization detectors, and an electrometer operated in the differential mode for column compensation. Helium is the carrier gas at 30 ml/min. Samples (0.1 μl) are injected into a glass-lined injector port at 200°C. The column oven is held at 120°C for 8 min, heated to 175°C at 24°C/min and held at 175°C for 25 min. The manifold is at 250°C. Graphical and numerical data are obtained on a Hewlett-Packard 3390A recording integrator.

### *Quantitation*

A series of eight standard solutions of reagent grade HCl and HBr are prepared at concentrations from 0.0005 *M* to 0.1 *M*. The standard solutions are derivatized and then chromatographed according to the procedures outlined above. For each acid at each concentration, a ratio is calculated from the areas of the haloethanol and *n*-pentanol peaks. These ratios are plotted *versus* concentration of acid to construct calibration curves.

### *Sample preparation for combustion studies*

Weighed samples (from 10 to 200 mg, depending on the composition of the samples) are enclosed in ashless paper wrappers (Whatman No. 42). A 1000-ml Schöniger flask (A. H. Thomas) is flushed with oxygen for 3 min, charged with 10.0 ml of distilled water and flushed with oxygen for an additional minute. The samples are burned in accordance with the manufacturer's instructions and the flasks are stored unopened at room temperature overnight. The absorbents are filtered through a 0.45 μm filter (Millex-HA, Millipore) and stored in glass vials with polyethylene-lined screw caps. Aliquots of the solutions are derivatized and chromatographed as outlined above.

## RESULTS

Chromatograms for standard solutions of derivatized HCl and HBr are shown in Fig. 1a and b. The peaks for excess ethylene oxide, *n*-pentanol, 2-chloro- and 2-bromoethanol are eluted isothermally at 120°C. The larger peaks eluting at longer retention times are ethylene glycol and higher-molecular-weight glycols formed from excess ethylene oxide in acidic aqueous solution.

The identification of the chloro- and bromoethanol peaks at 3.1 and 5.9 min was confirmed by GC–mass spectrometry (MS) analyses[13] of the derivatized solutions of HCl and HBr and of reagent-grade 2-chloroethanol and 2-bromoethanol (Aldrich). No other compounds were detected co-eluting with the haloethanols in any of the solutions. However, chromatograms of the standard haloethanols showed several additional peaks, comprising *ca.* 2% of the total area, which interfered with the quantitation of the *n*-pentanol standard. Therefore, calibration curves were constructed from derivatized solutions of HCl and HBr rather than from primary standards.

Fig. 1c is the chromatogram of a blank from a derivatized solution of 0.001 *M* pentanol and 0.16 *M* nitric acid. There is a small peak with the same retention time as

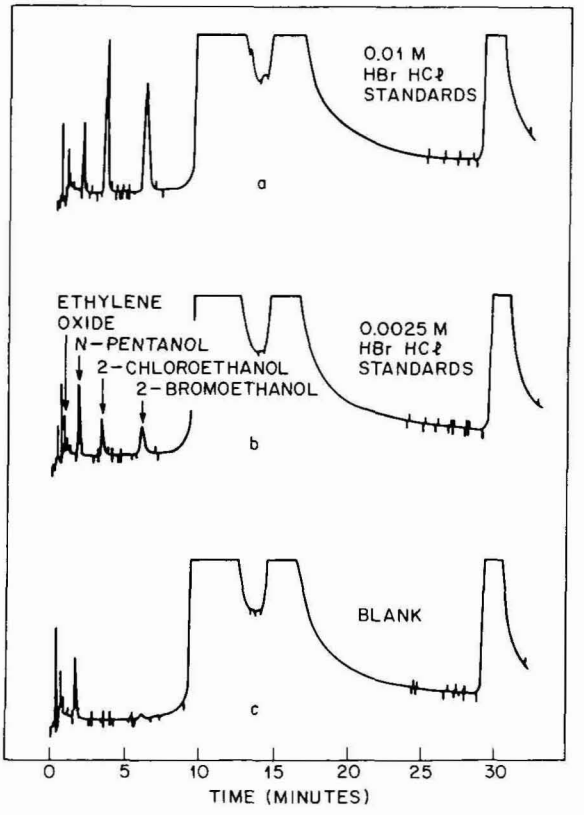


Fig. 1. Chromatograms of derivatized HBr and HCl standards and a blank.

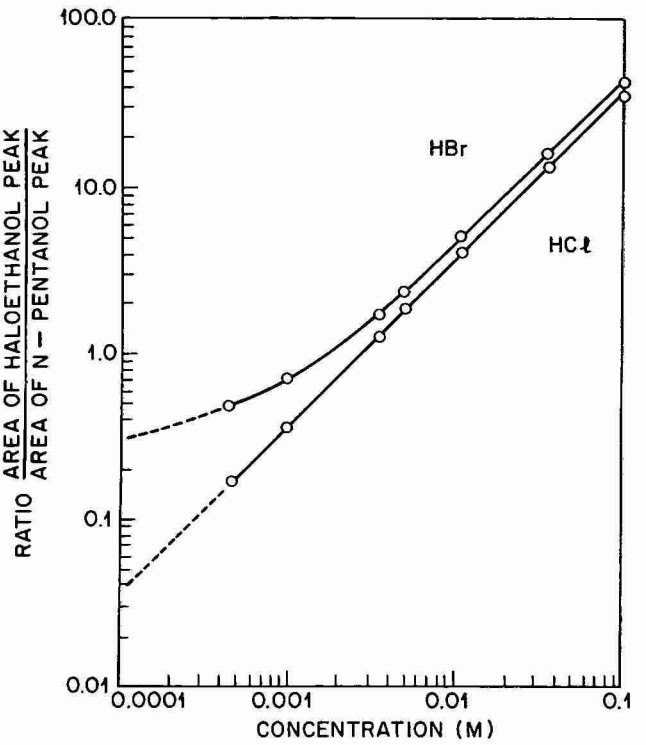


Fig. 2. Calibration curves for derivatized HBr and HCl standards.

bromoethanol and and an average area corresponding to about $1.2 \cdot 10^{-4}$ *M* HBr. Efforts to identify this peak by GC–MS have been unsuccessful.

Calibration curves of HCl and HBr are shown in Fig. 2. The curve for HBr is not linear at low concentrations due to the contribution of the small peak in the blank. However, the working calibration curve extends to at least $5 \cdot 10^{-4}$ *M*. The calibration curve for HCl is linear to at least $2 \cdot 10^{-4}$ *M*.

The most significant source of error in the determination appears to be the derivatization reaction. For eight determinations of one derivatized standard solution, the average R.S.D. for both HCl and HBr was 3%. However, for determinations of seven different derivatizations of this one standard solution, the R.S.D. was 10%. On the average, the R.S.D. is 10–12% at 0.001 *M* and 3–8% at 0.01 *M*.

Fig. 3a and b are chromatograms of two flame-retarded materials and are presented to illustrate an application of the method. The chromatograms are comparable to those of the standards. There are no additional peaks to interfere in the analysis. Quantitative data are summarized in Table I. Results have been converted from determined concentrations of haloacids to equivalent weight-percent halides in the original samples.

Sample A, a thermosetting molding compound, consisting of *ca.* 20% epoxy novolac resin and 80% silica filler, had been previously cured, crushed, and passed through a 60-mesh screen. Neutron activation analyses (NAA) had shown 0.925 ± 0.012% Br and 256 ± 27 ppm Cl. Three samples of the powdered material were

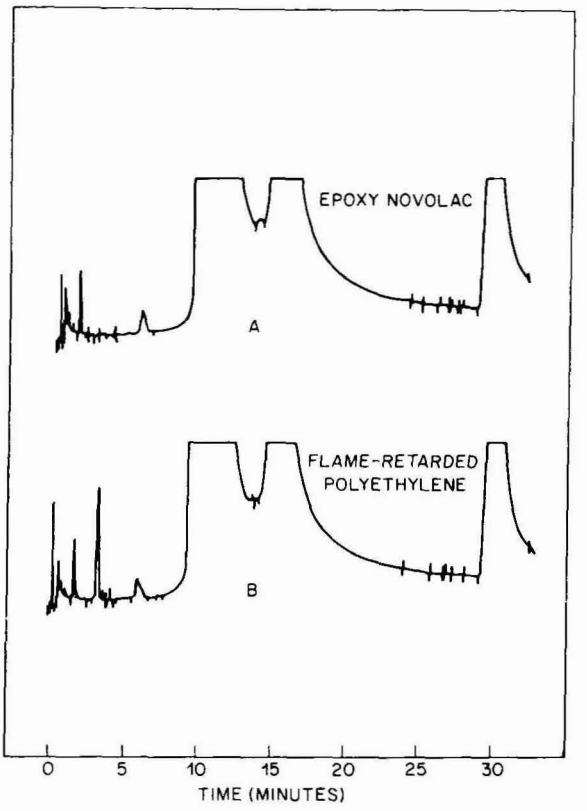


Fig. 3. Chromatograms of derivatized absorbents from burned polymers.

burned in Schöniger flasks and analyzed. The determined concentration of HBr in the absorbent corresponds to the release of 1.00 ± 0.17% (w/w) Br from the samples. At the 95% confidence level, this value is not significantly different than the 0.925% Br determined in the unburned material by NAA. Small peaks observed for chloroethanol provided semiquantitative results; 0.06 ± 0.03% Cl was determined. This is the same order of magnitude as the 0.026% found by NAA.

Sample B, a flame-retarded polyethylene had been compounded to contain 8.9% Br from decabromodiphenoxyethane and 13.6% Cl from chlorinated polyethylene. Samples B and B′ had the same nominal composition but had been processed under different conditions. B′ was darker and less flammable than B. Results for analysis of five samples of B and two samples of B′ are shown in Table I, rows I and III. Because only two samples of B′ were analyzed, results for the two of the five samples of B which were burned at the same time are shown separately in row II.

There were no significant differences in the amounts of HCl released from B and B′. Differences in the yields of HBr were significant at 90% confidence limits when two samples of each material (rows II and III) were compared and were significant at 95% when the larger sampling of B (row I) was considered. Thus, the described analytical method has made it possible to show that differences in the

TABLE I

EVOLDED $Br^-$ AND $Cl^-$ FROM COMBUSTION STUDIES

| *Sample* | *Br (%, w/w)* | | | *Cl (%, w/w)* | | | *R.S.D. (%)* | | *n** |
|---|---|---|---|---|---|---|---|---|---|
| | *Nominal* | *Determined* | | *Nominal* | *Determined* | | *Br* | *Cl* | |
| | | *Av.* | *S.D.* | | *Av.* | *S.D.* | | | |
| A. Molding compound | 0.93 ± 0.01 | 1.00 | 0.17 | 0.026 ± 0.003 | 0.06 | 0.03 | 16.5 | 50 | 3 |
| B. FR-Polyethylene | 8.9 | | | 13.6 | | | | | |
| I. Light = B | | 7.60 | 0.94 | | 13.74 | 0.44 | 12.3 | 3.2 | 5 |
| II. Light = B | | 8.35 | 0.35 | | 13.75 | 0.50 | 4.2 | 3.6 | 2** |
| III. Dark = B′ | | 9.45 | 0.21 | | 14.85 | 1.02 | 2.2 | 8.1 | 2 |

* $n$ = number of determinations, each of which is the average of data from two chromatograms from the same solution.

** These are two of the five determinations listed above.

processing conditions, as indicated by the darkening of the polymer, have influenced the amount of Br incorporated into and/or released from this material.

## DISCUSSION

Although the results presented in this report have emphasized analyses of aqueous absorbents of combustion products, the described method is also suitable for other applications. Samples of tetrabutylammonium chloride from various suppliers have been tested for contamination with bromide, and HBr has been detected in carbon tetrachloride[14]. This latter analysis extends the work of Petruj[2], who determined HCl in organic solvents. For non-aqueous solutions, the preparation of standard solutions is more difficult, but chromatograms are shorter because high-molecular-weight glycols are not formed as side products of the derivatization.

Four aspects of the experimental procedure require brief comments:

(1) We have found that addition of 0.16 *M* nitric acid and heating and stirring of the reaction mixture are critical for obtaining adequate sensitivity and reproducibility. Before these steps were included, quantitative results varied with the total elapsed time between derivatization and analysis and with the pH of the solution. Both modifications to the procedure increase the rate of ring-opening of the epoxide, thereby (a) ensuring the completeness of the reaction between the haloacids and epoxide and (b) promoting the consumption of excess ethylene oxide by its reaction with water to form glycol. Bächmann *et al.*[5] observed similar results in studies of the reaction of HCl with epibromohydrin. Also, removal of unreacted ethylene oxide is important because it bubbles into the sampling syringe and prevents accurate measurement and transfer of the sample.

(2) The stability of derivatized solutions was evaluated for a month. The haloethanol–pentanol ratios increased gradually and the areas of the glycol peaks at long retention times became significantly larger. In view of this evidence of slow, secondary reactions among residual ethylene oxide, pentanol, haloethanols, and glycols, all quantitative data are obtained within two days of derivatization.

TABLE II

MOLAR CONCENTRATIONS IN STANDARD SOLUTIONS

| *Solution No.* | *Nominal* | | | *Treatment* | *Determined* | |
|---|---|---|---|---|---|---|
| | *HCl* | *HBr* | $Br_2$ | | *HCl* | *HBr* |
| 1 | 0.005 | 0.005 | – | None | 0.0043 | 0.0047 |
| | | | | Extraction | 0.0043 | 0.0045 |
| 2 | 0.005 | 0.005 | 0.005 | None | 0.0048 | 0.0072 |
| | | | | Extraction | 0.0048 | 0.0048 |
| 3 | 0.005 | 0.005 | 0.001 | None | 0.0048 | 0.0055 |
| | | | | Extraction | 0.0047 | 0.0049 |
| 4 | 0.005 | 0.005 | 0.02 | None | 0.0048 | 0.0072 |
| | | | | Extraction | 0.0048 | 0.0048 |

(3) Among a large number of compounds evaluated for internal standards, only *n*-pentanol was both soluble in water and separable from the haloethanols under reasonable chromatographic conditions. it was assumed that halogenation of a primary alcohol in dilute acidic solution at room temperature would be minimal[15]. Therefore, in our initial work with standard solutions and with flame retardants containing low levels of chlorine and bromine, *n*-pentanol and nitric acid were added to the absorbent before combustion. However, when neat samples of a flame retardant (decabromodiphenoxyethane) containing 77% bromine were burned, the area of the *n*-pentanol peak was significantly smaller than usual, suggesting a possible reaction between *n*-pentanol and HBr. By using distilled water as the absorbent and adding the *n*-pentanol and nitric acid just prior to derivatization, the area of the *n*-pentanol peak has been restored to reproducible values which show no evidence of significant reactions between sample and standard.

(4) There is evidence[16], that $Br_2$ as well as HBr can be released during the combustion of brominated organic materials in a Schöniger flask. Therefore, a series of standard solutions, summarized in Table II, were studied to determine the effect, if any, of $Br_2$ on the quantitation of chloride and bromide. A small and non-quantitative enhancement in the yield of bromoethanol was observed when $Br_2$ was added to standard solutions. However, following a two-fold extraction of $Br_2$ with a 2:1 excess of carbon tetrachloride, quantitative results were obtained for HBr. Alternatively, $Br_2$ can be reduced with hydrazine before derivatization in order to determine total bromine as bromide[16].

## CONCLUSIONS

A method has been developed to determine simultaneously and quantitatively micromole amounts of HCl and HBr by treating the haloacids with ethylene oxide and determining the corresponding 2-haloethanols by GC. Advantages of the method are that chloride and bromide are determined simultaneously and that only small quantities of samples and reagents are required. HBr can be determined at concentrations as low as $5 \cdot 10^{-4}$ *M* and HCl at $2 \cdot 10^{-4}$ *M*. Derivatizations require 0.5 ml of solution. At $5 \cdot 10^{-4}$ *M*, this corresponds to 0.25 μmole of halide. The relative preci-

sion of the method is 3–10%, depending on concentration. The method is applicable to a number of analytical problems and, in particular, provides a means of measuring a small amount of HCl and HBr released in small-scale tests of flame-retarded polymers containing chlorinated and brominated compounds. Samples weighing 10–200 mg and containing 1–20% Cl and/or Br have been analyzed.

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# ANALYSIS OF AMPHOTERIC SURFACTANTS BY LIQUID CHROMATOGRAPHY WITH POST-COLUMN DETECTION

## I. MONO- AND DIALANINE TYPE SURFACTANTS

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SUMMARY

β-Alanine type amphoteric surfactants were analysed by high-performance liquid chromatography with a novel post-column detection. The separation was performed on a reversed-phase column (Develosil ODS-3, 3 μm) with acetonitrile as an organic modifier in the eluent. The chromatographic behaviour of the surfactants (mono- and dialanines) was examined, and the specificity and sensitivity of post-column detection was demonstrated. The post-column detection system was based on a specific reaction with the amine structures of the surfactants. By this method, information on both the alkyl chain distribution and the degree of N-substitution could be obtained. The proposed method enables a direct and convenient determination of β-alanine type amphoteric surfactants in shampoos.

## INTRODUCTION

The most important amphoteric surfactants have been generally classified according to types: alanines; glycines; imidazolines and betaine derivatives. These have been extensively applied to toiletry and household products such as shampoos, hair rinses and fabric softeners.

However little has been reported on their analysis. Takano *et al*[1] reported on the analysis of betaine type amphoteric surfactants by reaction gas chromatography. Dmitrieva *et al*[2] determined amphoteric surfactants by potentiometric titration. Sanders *et al*[3] and Keough *et al*[4] reported on the filed desorption mass spectrometry of carboxybetaine amphoteric surfactants. These analytical methods need pure mixtures solely of amphoteric surfactants and/or cumbersome sample preparation for the elimination of interferences. Therefore little information is available on the methods of analysis when the surfactants are present in a complicated matrix.

For reversed-phase high-performance liquid chromatographic (HPLC) analysis of amphoteric surfactants, Parris and co-workers[5,6] and Nakamura and Morikawa[7] used a differential refractive index detector and a UV absorption detector (at 210 nm) respectively. These detection systems were neither specific nor sensitive to the amphoteric surfactants.

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We report a new HPLC method for convenient and direct determination of amphoteric surfactants in commercial products by using novel post-column reaction systems. The post-column detection is based on the specific reaction with amine structures (primary, secondary and tertiary) of the surfactants. The amphoteric surfactants from an analytical column are first converted into the corresponding N-chloramines with hypochlorite ; the N-chloramines are then treated react with iodide to form triiodide which can be monitored at 355 nm.

This report is concerned with structurally confirmed mono- and dialanine type amphoteric surfactants. Their chromatographic behaviour and the specificity and sensitivity of the post-column reaction system were examined.

## EXPERIMENTAL

### *Apparatus*

A schematic diagram of the liquid chromatograph is shown in Fig. 1. Sample solutions were injected via a Hitachi sample injector. An Hitachi 635 pump was used to force eluent through the analytical column at a flow-rate of 1.0 ml $min^{-1}$. Acid-resistant pumps (Nihon Seimitsu NMP-2u) were used for post-column reagents each at a flow-rate of 0.4 ml $min^{-1}$. Develosil ODS-3 (3 $\mu$m ; Nomura Kagaku, Japan) was slurry packed in a stainless-steel column (150 × 4.6 mm I.D.). The post-column reaction coils were made of PTFE tubing (5 m × 0.5 mm I.D.). The effluent was monitored at 355 nm with a variable-wavelength detector (Hitachi 635-T, range 0.32) equipped with a flow cell (inner volume 8 $\mu$l, path length 10 mm) in combination with a multirange recorder (Hitachi Model 056) and a data processor (Shimadzu Chromatopac E1A).

### *Reagents*

$\beta$-Alanine type amphoteric surfactants, sodium laurylaminoethylcarboxylate (R = $C_{12}H_{25}$), sodium alkylaminoethylcarboxylate (R = $C_8H_{17}$–$C_{18}H_{37}$), disodium laurylaminodi(ethylcarboxylate) (R = $C_{12}H_{25}$) and disodium alkylaminodi(ethylcarboxylate) (R = $C_8H_{17}$–$C_{18}H_{37}$), were made in our laboratories by a minor variant

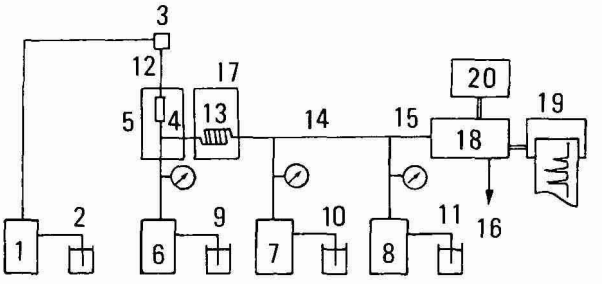



Fig. 1. Schematic diagram of the liquid chromatograph. 1 = pump ; 2 = eluent ; 3 = sample injector ; 4 = analytical column ; 5, 17 = water-bath ; 6–8 = acid-resistant pumps ; 9 = hypochlorite reagent ; 10 = nitrite reagent; 11 = iodide reagent ; 12 = 0.25 mm I.D. × 0.5 m stainless tubing ; 13 = 5 m × 0.5 mm I.D. PTFE reaction coil; (14/15) = 0.5 m × 0.5 mm I.D. PTFE tubing; 16 = 5 m × 0.5 mm I.D. PTFE suppressor tubing ; 18 = UV detector ; 19 = recorder ; 20 = data processor.

of the literature method[8]. In the reactions the molar ratio of ethyl acrylate to amine was 1.2:1 for monoalanines and 2.1:1 for dialanines:

$$\underset{\text{I}}{\text{R–NH}_2} + \text{CH}_2\text{CHCO}_2\text{C}_2\text{H}_5 \xrightarrow[\text{C}_2\text{H}_5\text{OH saponification}]{\text{Heat}\quad \text{NaOH/H}_2\text{O}} \underset{\text{II, monoalanine}}{\text{R–NHC}_2\text{H}_4\text{CO}_2\text{Na}} + \underset{\text{III, dialanine}}{\text{R–N(C}_2\text{H}_4\text{CO}_2\text{Na)}_2}$$

Sample solutions for HPLC were prepared by dissolving weighed samples in deionized water or in methanol. For HPLC analysis, the eluent was 0.2 *M* sodium perchlorate containing 60% acetonitrile (buffered at pH 2.5 by phosphoric acid). The post-column reagents were as follows : hypochlorite reagent, 0.25 *M* phosphate buffer (pH 8.0) containing 1% sodium hypochlorite; nitrite reagent, 0.5% sodium nitrite aqueous solution; iodide reagent, 0.5% potassium iodide aqueous solution.

## RESULTS AND DISCUSSION

### *Post-column detection system*

The mechanism of the proposed post-column reaction as follows :

Compounds N-chloramines

$$\underset{\text{I}}{\text{RNH}_2} \xrightarrow{\text{NaOCl}} \text{RNCl}_2 \xrightarrow{\text{KI}} 2\text{I}_3^-$$

$$\underset{\text{II}}{\text{RNHC}_2\text{H}_4\text{CO}_2\text{Na}} \rightarrow \text{RN(Cl)C}_2\text{H}_4\text{CO}_2\text{Na} \rightarrow \text{I}_3^-$$

$$\underset{\text{III}}{\text{RN(C}_2\text{H}_4\text{CO}_2\text{Na)}_2} \rightarrow \overset{+}{\text{RN(Cl)}}(\text{C}_2\text{H}_4\text{CO}_2\text{Na})_2 \rightarrow \text{I}_3^-$$

$$\underset{\text{(excess)}}{\text{NaOCl}} + \text{NaNO}_2 \rightarrow \text{NaCl} + \text{NaNO}_3.$$

The post-column detection system consisted of three reagents as described previously[9]. The active ingredients I–III of the mono- and dialanine amphoteric surfactants were first converted into the corresponding N-chloramines with hypochlorite. Then the excess of hypochlorite was selectively destroyed with nitrite, and the N-chloramines were treated with iodide to form triiodide which can be monitored at 355 nm.

In this system, 1 mole of compound I, II and III could be converted into 2 moles, 1 mole and 1 mole of triiodide, respectively provided that the overall reaction proceeds stoichiometrically. Therefore information on the degree of N-substitution is obtained by optimizing the conditions of post-column reaction.

### *Effects of organic modifier in the eluent*

In this method, detection is influenced by both the type and the content of organic modifier in the eluent. Acetonitrile was more advantageous than methanol as a modifier for the post-column detection. In the case of methanol, detection could not

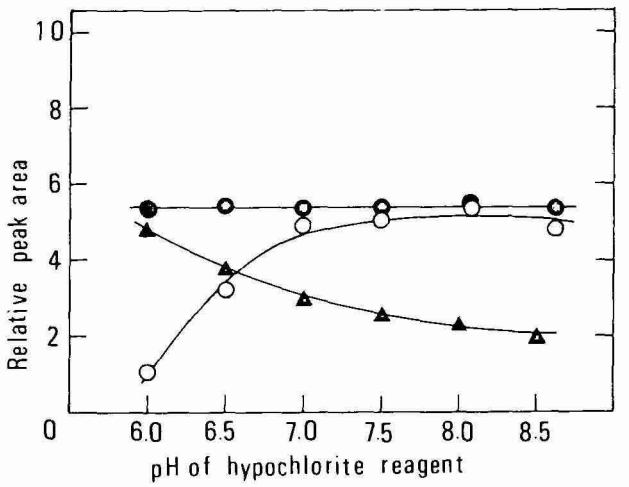



Fig. 2. Effects of pH of hypochlorite reagent on the peak areas: ▲, compound I (R = $C_{12}H_{25}$); ●, II (R = $C_{12}H_{25}$); ○, III (R = $C_{12}H_{25}$). The reaction temperature was fixed at 60°C.

be performed at all when its content was higher than 10% owing to the high blank absorbance caused by oxidation of methanol with hypochlorite. When acetonitrile was used the blank absorbance was low and the detection could be performed with up to 70% of acetonitrile in the eluent. Therefore acetonitrile was selected as the modifier.

*Effects of the pH of the hypochlorite reagent*

The effects of varying the pH of the hypochlorite reagent in the range pH 6.0–8.5, on the post-column reaction were tested. The eluent used was that given in *Reagents*.

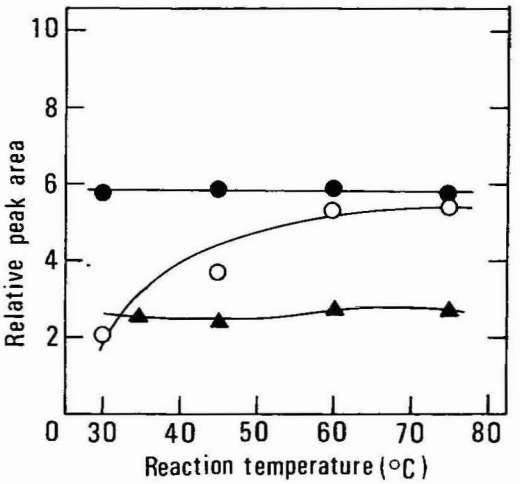
Fig. 2 shows that the sensitivity varied with the pH of the hypochlorite reagent in different ways depending on the type of amine. For primary amines (I), the sensitivity decreased with increasing pH, for monoalanines (II), the sensitivities were fairly constant over the range examined, and the sensitivities of dialanines (III) increased with increasing pH. In practice, primary amines (I) were not found in the commercially available β-alanine amphoteric surfactants. Thus, the optimum pH of the hypochlorite reagent was found to be 8.0.

*Effects of reaction temperature*

Fig. 3 shows the effects of reaction temperature on sensitivity. With increase of reaction temperature, the sensitivity behaved in a similar manner to that in Fig. 2, except for primary amines(I). Above 70°C, a stable baseline could not be achieved due to the formation of air bubbles. Thus a temperature of 60°C is recommended.

On the basis of these results, it is assumed that the rate of reaction and the stabilities of the N-chloramines differ for each type of amine structure. Primary and secondary amines (I, II) would react with hypochlorite reagent faster than tertiary amines (III), and the N-chloramines derived from primary amines would be more unstable than those of the other amines at higher pH and at higher reaction temperature.

In our preliminary experiments, the post-column detection was applied to all sorts of amines, and it was possible to determine the structures of unknown amines



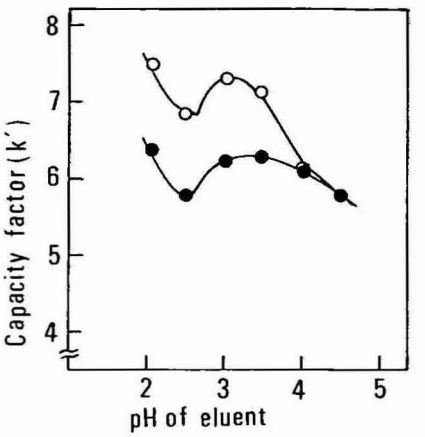




Fig. 3. Effects of reaction temperature on the peak areas. Compounds as in Fig. 2. The pH of the hypochlorite reagent was fixed at 8.0.

Fig. 4. Effects of eluent pH on $k'$. Compounds as in Fig. 2. Eluent : 0.2 *M* sodium perchlorate containing 60% acetonitrile (pH adjusted by phosphoric acid).

(such as primary, secondary and tertiary amines) by observing both the pH and temperature profiles of the compounds in the post-column detection.

*Chromatographic separation of alanine type amphoteric surfactants*

In the proposed method, β-alanine type amphoteric surfactants were separated by reversed-phase HPLC according to both the carbon number of the original fatty amines and the degree of N-substitution.

Nakamura and Morikawa reported the chromatographic behaviour of some amphoteric surfactants using methanol as an origanic modifier in the eluent. In the proposed post-column detection, methanol could not be used as the organic modifier, therefore we studied the chromatographic separation of β-alanine type amphoteric surfactants using acetonitrile as an organic modifer.

HPLC separation of ionic surfactants is usually achieved in the presence of inorganic salts[7,10]. Here, sodium perchlorate was chosen as the inorganic salt in the eluent. High concentrations of inorganic salts in the eluent lead to irregular baselines in the post-column detection. We found that the concentration of inorganic salt could be minimized while retaining the ability to separate ionic surfactants, 0.2 *M* sodium perchlorate was chosen.

Fig. 4 shows the effect of eluent pH on the capacity factor $k'$. Large changes in $k'$ were not observed, but the peaks of alanines broadened and tailed with increasing pH. Thus, the eluent pH chosen was 2.5. The elution order was dialanine, monoalanine and fatty amine for the same alkyl chain.

The effect of column temperature on the separation was small. With increase of column temperature, the $k'$ values decreased but the elution order of compounds I–III did not change. Therefore a column temperature of 40°C, was employed.

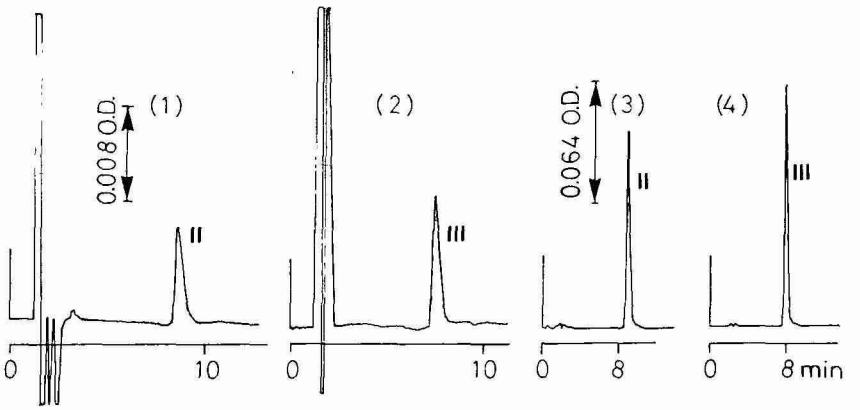



Fig. 5. Comparison of the relative responses to mono- and dialanines with UV and/or post-column detection. Compounds ($R = C_{12}H_{25}$) : 1, II, 40 $\mu$g (143.4 nmol), UV at 210 nm, 2, III, 34.92 $\mu$g (93.6 nmol), UV at 210 nm ; 3, II, 1 $\mu$g (3.58 nmol), post-column detection ; 4, III, 1.74 $\mu$g (4.66 nmol), post-column detection.

*Sensitivity and selectivity*

The sensitivity of the proposed method was examined by use of standard pure materials (mono- and dialanines : $R = C_{12}H_{25}$), see Fig. 5. Considering the stability of the baseline in the post-column detection, the practical range of the detector was fixed as 0.32 absorbance units full scale (a.u.f.s.), corresponding to about 3 $\mu$g of each alanine.

The relative molar response to mono-/dialanine ($R = C_{12}H_{25}$) was 105.1/100 in the post-column detection. Therefore the molar responses to mono- and dialanines were almost identical. This result shows that the degree of N-substitution can be calculated in the proposed method.

The detection limits of the method were about 80 times higher for a $C_{12}$ monoalanine and about 30 times higher for a $C_{12}$ dialanine compared with those achieved by UV detection (at 210 nm, 0.08 a.u.f.s.). Furthermore, the post-column detection has a higher selectivity compared to UV detection.

*Determination of alkyl chain distribution and degree of N-substitution*

Representative chromatograms obtained with the proposed method are shown in Figs. 6 and 7. The compounds were assigned by use of standard samples. From the results, both the alkyl chain distribution and the degree of N-substitution of the $\beta$-alanines could be obtained, provided that almost identical molar responses were yielded regardless of the length of the alkyl chain.

The results for the alkyl chain distribution obtained in the proposed method are compared with those from gas chromatographic analyses of the original fatty amines in Table I. The agreement between the two sets of results is good and the average molecular weight of the original fatty amines can be calculated conveniently in the proposed method.

In practice, the original fatty amines were not found in commercially available $\beta$-alanine amphoteric surfactants. Thus, the degree of N-substitution can be calculated from the peak area ratio of mono-/dialanine in the chromatogram, using the relative molar response(105.1/100) of the standard pure materials, as indicated previously.

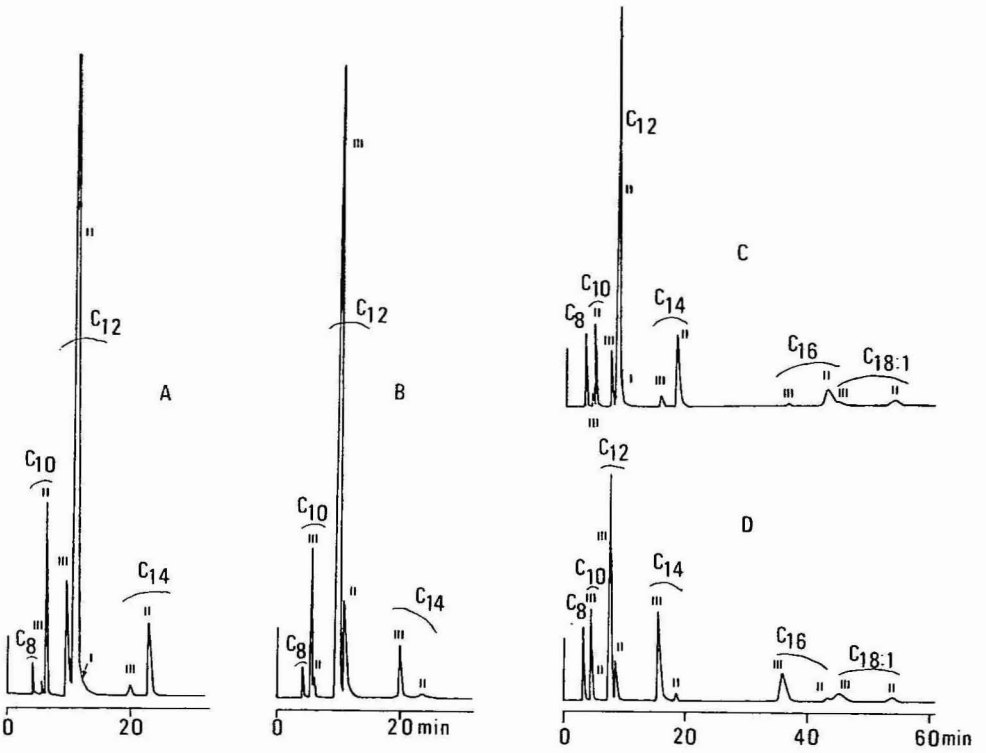



Fig. 6. HPLC of sodium laurylaminoethylcarboxylate (A) and disodium laurylaminodi(ethylcarobxylate) (B). The alkyl-chain distribution and the assignments of the compounds are indicated on the peaks. The surfactants were commercially available ones.

Fig. 7. HPLC of sodium alkylaminoethylcarboxylate(C) and disodium alkylaminodi(ethylcarboxylate)(D). Notation as in Fig. 6. The surfactants were commercially available ones.

TABLE I

COMPARISON OF THE PROPOSED HPLC METHOD WITH GAS CHROMATOGRAPHIC (GC) ANALYSES OF THE ALKYL CHAIN DISTRIBUTION

Sample : Surfactant C in Fig. 7.

| *Alkyl chain of original fatty amine* | *HPLC method (mol %)** | *GC method* | |
|---|---|---|---|
| | | *(wt. %)*** | *(mol %)**** |
| $C_8$ | 7.00 | 4.54 | 6.39 |
| $C_{10}$ | 8.85 | 7.34 | 8.66 |
| $C_{12}$ | 59.85 | 60.21 | 61.06 |
| $C_{14}$ | 16.40 | 18.24 | 16.22 |
| $C_{16}$ | 7.90 | 9.68 | 7.67 |

* Calculated by summing the peak areas (%) of compounds II and III, assuming that identical molar responses were yielded regardless of the length of the alkyl chain.

** GC-flame ionization detection response of acetylated original fatty amine.

*** Calculated from wt. %.

TABLE II

DETERMINATION OF MONO- AND DIALANINES IN A STANDARD SHAMPOO

1 = Sodium alkylaminoethylcarboxylate(R = $C_8H_{17}$–$C_{18}H_{37}$) :
2 = disodium alkylaminodi(carboxylate) (R = $C_8H_{17}$ $C_{18}H_{37}$) ;
3 = sodium laurylaminoethylcarboxylate.

| *Compound* | *Alanine type amphoterics* | | | | |
|---|---|---|---|---|---|
| | *Added(%)* | *Found (%)* | *n* | *C.V. (%)* | *Recovery(%)* |
| 1 | 5.00 | 4.84* | 4 | 2.4 | 96.8 |
| 2 | 5.00 | 4.87* | 4 | 2.1 | 97.4 |
| 3 | 2.50 | 2.49 | 4 | 1.3 | 99.6 |

* Calculated by summing the peak areas of $C_8$–$C_{18}$ alkyl chains.

However, the separation between the mono- and dialanines of $C_8$ and $C_{10}$ was not achieved. Therefore, the degree of N-substitution was calculated from the peak area ratio of $C_{12}$ alanines, assuming that almost identical responses were yielded regardless of the alkyl chain length.

Thus the alkyl chain distribution and the reaction molar ratio of ethyl acrylate to fatty amines can be estimated from the chromatogram. The information obtained from the post-column detection were not affected by interfering substances even when the surfactants were formulated in shampoos.

*Commercial products analysis*

In the proposed method, the calibration curve of peak areas *vs.* concentrations was completely linear for each alkyl chain of the alanines ($C_8$–$C_{18}$) and passed through the origin. For example, the curves for both mono- and dialanines of $C_{12}$ were linear in the range of 0.3–12 $\mu$g. Thus, the content of alanines in commercial products can be determined by summing the peak areas due to each alkyl chain. The precision and accuracy of the method were tested by adding known amounts of mono- and dialanines to a standard shampoo. Table II demonstrates that the proposed method can be applied to commercial products analysis.

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# FLUOROMETRIC DETERMINATION OF SECONDARY AMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POST-COLUMN DERIVATIZATION

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## SUMMARY

To obtain suitable conditions for the simultaneous determination of primary and secondary amines by high-performance liquid chromatography with post-column derivatization, the conversion of secondary amines into primary amines with sodium hypochlorite was reinvestigated by flow injection analysis. While NaOCl was required primarily for the conversion of secondary amines into primary amines, it also decreased the detectability of the latter with *o*-phthalaldehyde-2-mercaptoethanol reagent, depending on the reaction temperature and time. The conditions established for the post-column derivatization were applied to the determination of amino acids including L-proline, L-4-hydroxyproline and N-methyl-amino acids, catecholamines and their 3-O-methyl derivatives.

## INTRODUCTION

Various methods using high-performance liquid chromatography (HPLC) with fluorescence detection have been developed for the determination of secondary amines in biological materials. For this purpose, pre-column derivatization procedures with Dns chloride[1], 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole[2] or 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole[3] and a post-column procedure with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole[4] have been devised. These reagents derivatize both primary and secondary amines to fluorescent compounds. Alternatively, *o*-phthalaldehyde–2-mercapthoethanol (OPA–ME) reagent which is selective to primary amines has been used in post-column derivatization after the conversion of secondary amines into primary amines with sodium hypochlorite (NaOCl)[5,6].

This NaOCl–OPA method has now almost replaced the conventional ninhydrin method for the determination of amino acids. However, there is a problem in that the sensitivities of primary amino acids are much lower compared with the case in the absence of NaOCl. These reduced sensitivities are supposed to be due to side reactions caused by the excess of NaOCl, such as the transformation of primary amino acids to corresponding N-chlorinated derivatives[7]. Böhlen and Mellet[5] reported a

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method in which NaOCl was added to the column eluate only when proline was eluted (switching flow method). Continuous addition of NaOCl resulted in a marked reduction in the sensitivities of primary amino acids, *i.e.*, to less than 10%. In contrast, Ishida *et al.*[8] recently developed the "non-switching flow method" in which NaOCl is always added to the column eluate. Contrary to the results of Böhlen and Mellet[5], they claimed that the sensitivity of the method was so high that a few picomoles of primary amino acids could be determined.

In a previous study[7] we succeeded in suppressing the effects of the excess of NaOCl by the addition of 2,2'-thiodiethanol (TDE) to NaOCl-treated secondary amines and established manual procedures for the fluorometric determination of secondary amines. As an extension of that work, we have reinvestigated the NaOCl–OPA fluorogenic reactions of typical secondary amines together with analogous primary amines by flow injection analysis (FIA) to find the optimum conditions for the simultaneous determination of primary and secondary amines. These conditions were then applied to the HPLC post-column derivatization of some biogenic primary and secondary amines.

## EXPERIMENTAL

### *Materials*

Packed columns of Partisil-10 SCX (25 cm × 4.6 mm I.D., 10 μm; strong cation exchanger bonded to silica gel) and TSK LS-410 (30 cm × 4.0 mm I.D., 5 μm; ODS-bonded silica gel) were purchased from Whatman (Clifton, NJ, U.S.A.) and Toyo Soda (Tokyo, Japan) respectively. OPA (Funaphthal) was purchased from Funakoshi (Tokyo, Japan), DL-metanephrine hydrochloride, DL-normetanephrine hydrochloride and N-methyl-L-leucine from Sigma (St. Louis, MO, U.S.A.), L-DOPA, dopamine hydrochloride, γ-aminobutyric acid (GABA), L-proline, L-4-hydroxyproline, DL-alanine and Brij-35 from Nakarai (Kyoto, Japan) and L-aspartic acid and L-lysine hydrochloride from Ajinomoto (Tokyo, Japan). Ethanol, antiformin (0.7 *M* NaOCl solution when assayed by iodometry) and reagents for preparing buffers were obtained from Kanto (Tokyo, Japan). L-Epinephrine bitartrate, L-norepinephrine bitartrate, methylamine hydrochloride and other chemicals were purchased from Tokyo Kasei Kogyo (Tokyo, Japan).

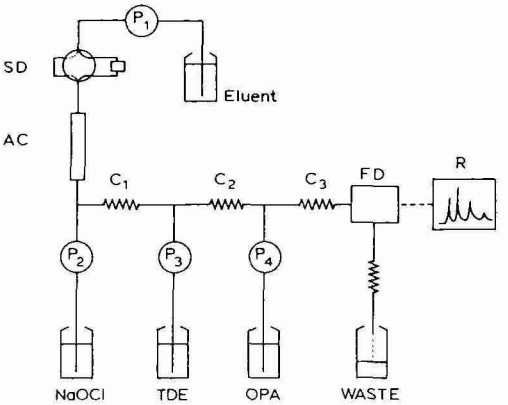


Fig. 1. Flow diagram of the present NaOCl–TDE–OPA post-column derivatization method. SD = Sampling device; FD = fluorescence detector; R = recorder; $P_1$–$P_4$ = pumps; $C_1$–$C_3$ = reaction coils.

*Preparation of solutions*

Ten millimolar solutions of amines were prepared with 20 m*M* citrate buffer (pH 3). The buffer was used to dilute the solutions. The NaOCl solution was prepared by diluting antiformin with buffers of various pH values. The TDE solution was prepared by dissolving TDE in phosphate buffer (pH 8) or in 5.7% acetic acid.

The OPA–ME reagent was prepared by a modification of the method of Benson and Hare[9]: 1 ml of OPA solution (80 mg/ml in ethanol) and 0.2 ml of ME were mixed with 100 ml of 1 *M* borate buffer (pH 10.2).

*Apparatus for HPLC of secondary amines*

Fig. 1 shows the flow diagram of the present method. All tubings and coils were made of PTFE (0.5 mm I.D.). The eluent was delivered through a Mini-micro pump (Type KHD-26; Kyowa Seimitsu, Tokyo, Japan) to the analytical column (AC). The column temperature was ambient. The eluate was mixed in a three-way "tee" with the NaOCl solution delivered at a flow-rate of 0.55 ml/min through a Mini-micro pump (Type KSD-16; Kyowa Seimitsu). The mixture was incubated in the coil $C_1$ and mixed in the second three-way "tee" with TDE solution delivered at a flow-rate of 0.53 ml/min through a Mini-micro pump (Type KHD-16; Kyowa Seimitsu). The outlet of the second "tee" was connected through the coil $C_2$ to a third three-way "tee" and mixed with the OPA reagent delivered at a flow-rate of 0.60 ml/min through a Mini-micro pump (Type KSU-45H; Kyowa Seimitsu). Unless otherwise stated, the length of coils was 10 m and the reaction temperature was ambient, except

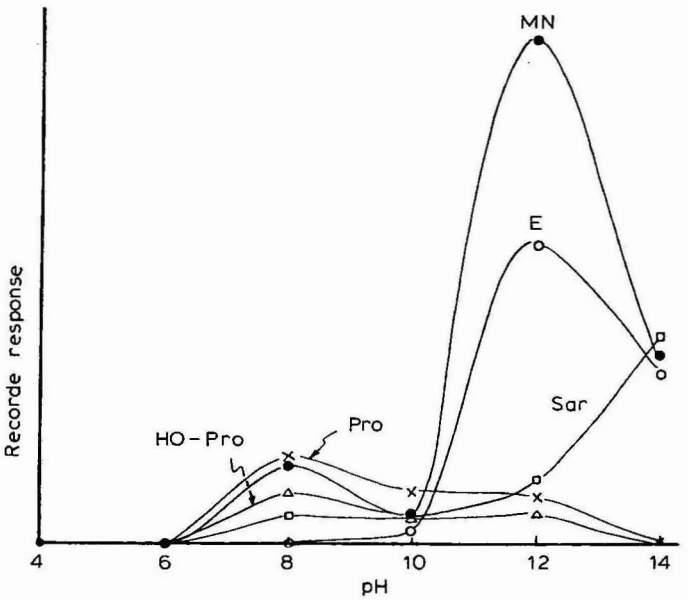


Fig. 2. Influence of pH on the conversion reaction. FIA was carried out as described in Experimental without delivering the TDE solution. The flow-rate of the carrier was 0.99 ml/min. NaOCl solutions (0.7 m*M*) were prepared as follows: 0.1 *M* citrate buffer (pH 4 and 6); 0.1 *M* phosphate buffer (pH 8 and 12); 0.1 *M* borate buffer (pH 10) and 1 *M* NaOH (pH 14). Injected amount of amines; 10 nmol. Abbreviations: Pro = proline; HO-Pro = 4-hydroxyproline; E = epinephrine; MN = metanephrine; Sar = sarcosine.

that the coil $C_1$ was set at 40°C. The outlet of the coil $C_3$ was introduced to a 14-$\mu$l quartz flow cell in a fluorescence detector (Type FLD-1; Shimadzu Seisakusho, Kyoto, Japan). It was equipped with a coated low-pressure mercury lamp which emits light of wavelength 300–400 nm (emission maximum at 360 nm) and a secondary filter (EM-3) which cuts off light of wavelength shorter than 405 nm. The fluorescence intensities were recorded with a recorder (Model EPR-100A; Toa Electronics, Tokyo, Japan).

*Optimization of post-column derivatization by FIA*

All the experiments on the optimization of the post-column reactions were conducted by FIA. For these investigations the post-column derivatization system described above was modified by removing the analytical column and delivering distilled water as the carrier.

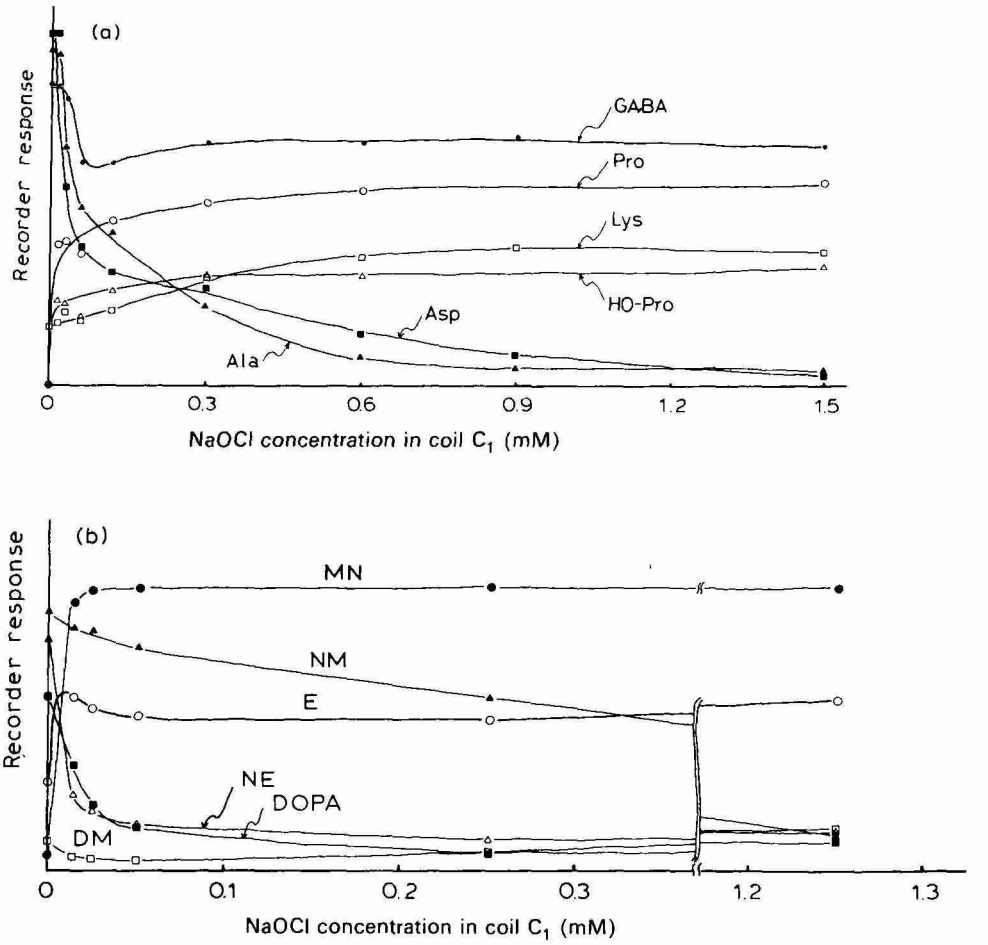


Fig. 3.

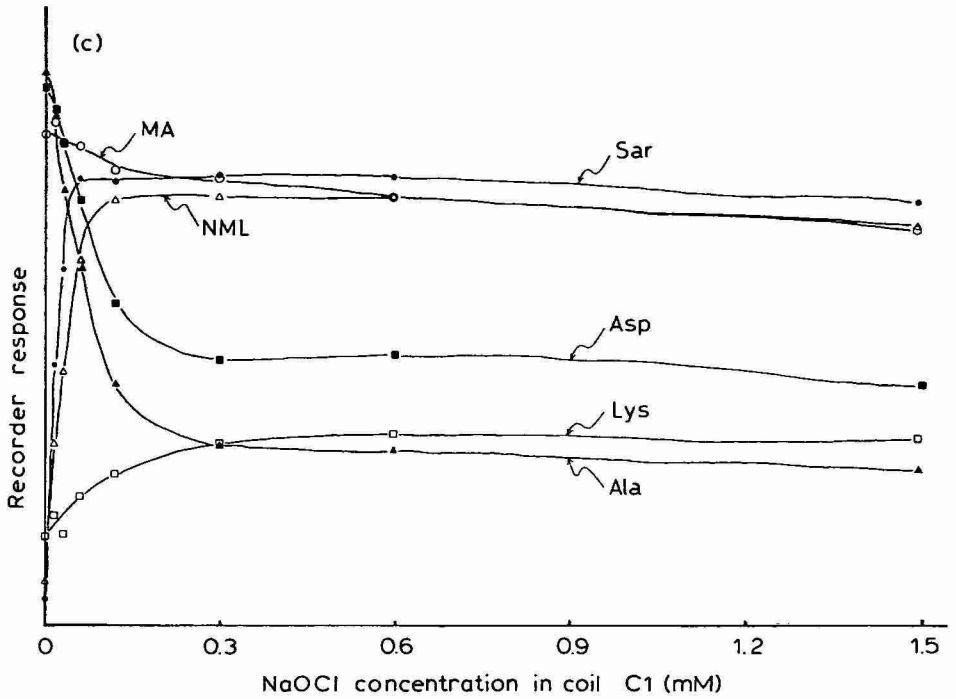


Fig. 3. Influence of NaOCl concentration on the conversion reaction. FIA was carried out as described in Experimental. The carrier flow-rates were 0.70 ml/min for group I(a) and group III(c), and 0.99 ml/min for group II(b). The NaOCl solution was prepared with 0.1 *M* phosphate buffer (pH 8) for group I, with 0.1 *M* phosphate buffer (pH 12) for group II and with 1 *M* NaOH for group III. For group II, 200 m*M* TDE solution was prepared with 0.5 *M* phosphate buffer (pH 8). Injected amounts of amines; 0.5 nmol (groups I and III) and 1.0 nmol (group I). Abbreviations: Asp = aspartic acid; Ala = alanine; GABA = γ-aminobutyric acid; Lys = lysine; NE = norepinephrine; NM = normetanephrine; DM = dopamine; NML = N-methylleucine; MA = methylamine.

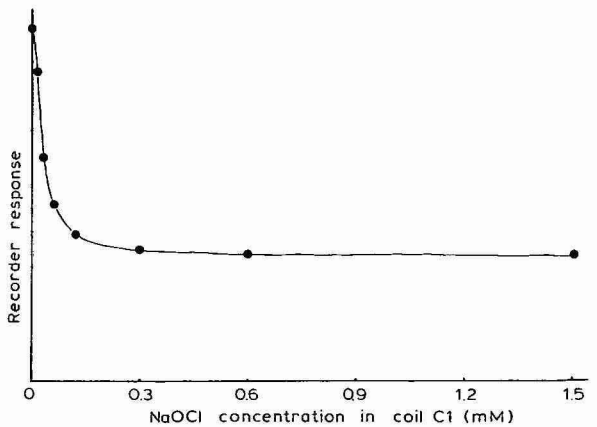


Fig. 4. Influence of NaOCl concentration on the fluorescence intensity of lysine with the addition of Brij-35 to the OPA–ME reagent at the concentration of 1.0 g/l. The conditions were the same as those for group III in Fig. 3. Injected amount of lysine: 0.3 nmol.

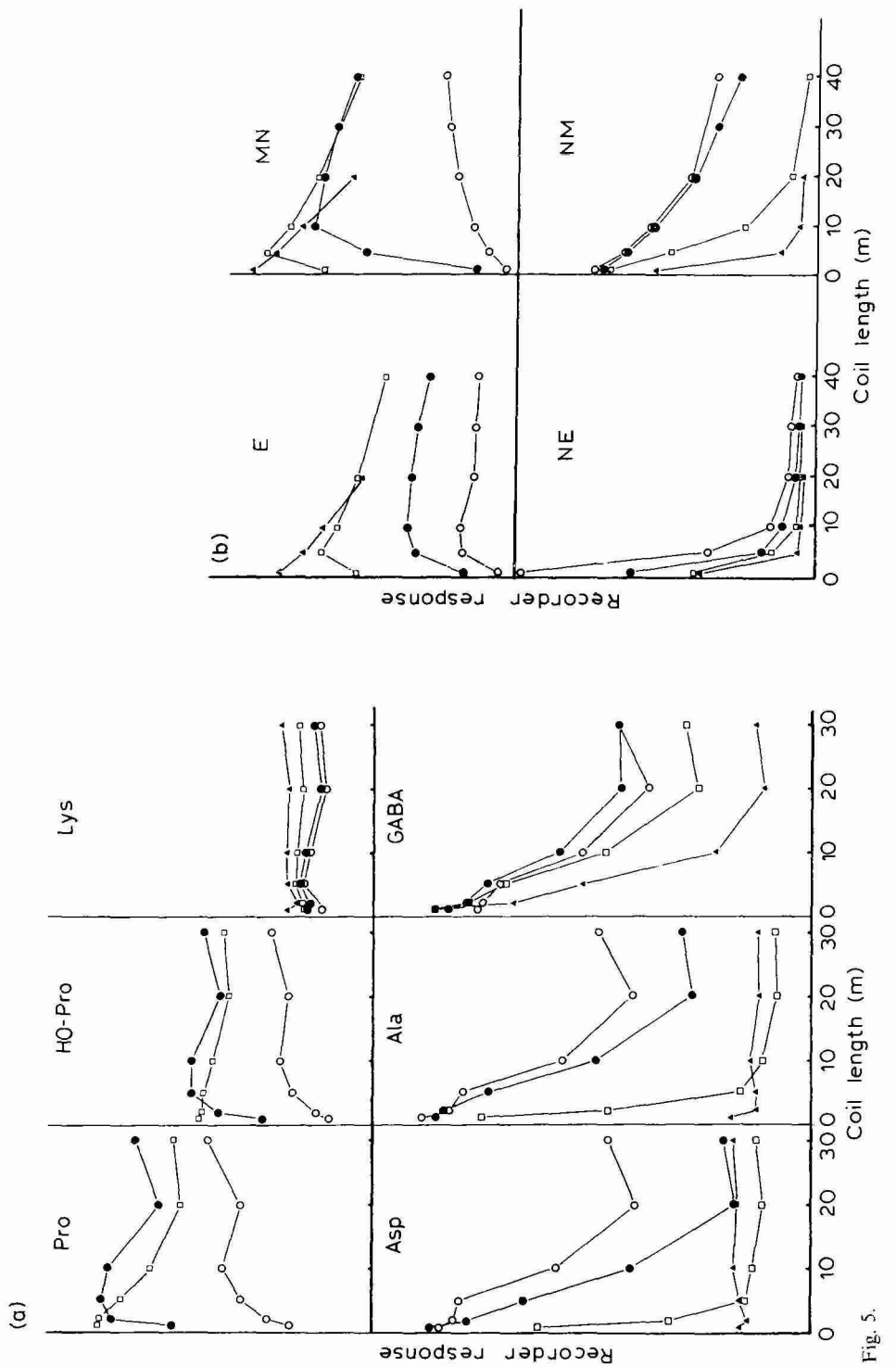
(a)
Pro
HO-Pro
Lys
Asp
Ala
GABA
Recorder response
0 10 20 30
Coil length (m)
(b)
E
NE
MN
NM
Recorder response
0 10 20 30 40
Coil length (m)

Fig. 5.

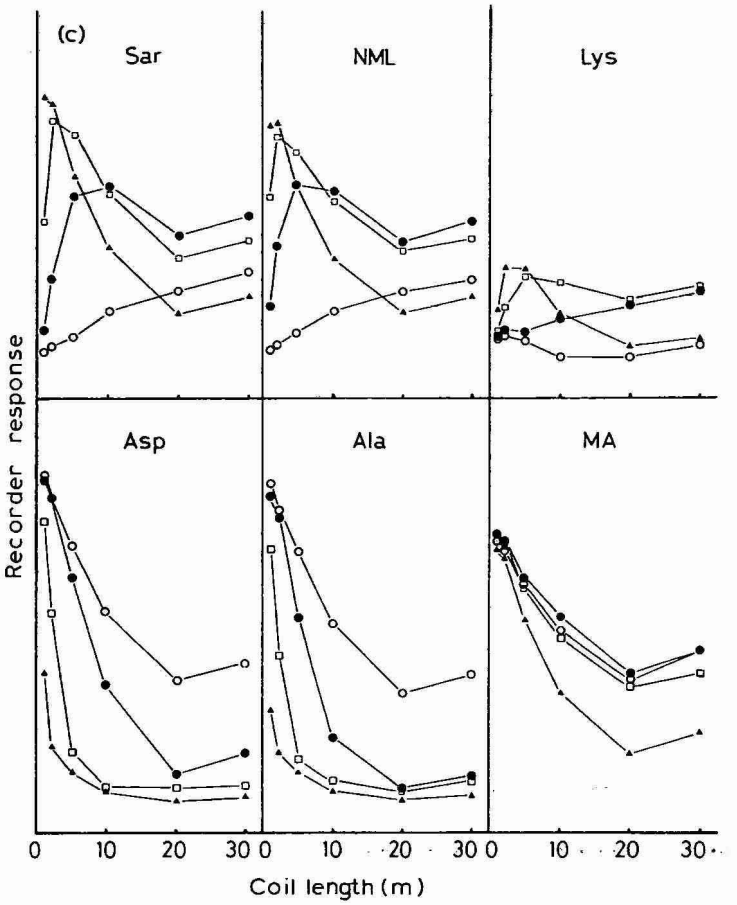


Fig. 5. Influence of reaction temperature and length of coil $C_1$ on the conversion reaction. The reaction temperature was changed from 20°C to 80°C and the coil length from 1 m to 40 m. Other conditions were the same as those of Fig. 3 except for the NaOCl concentration: a, 0.07 m*M* for group I; b, 0.14 m*M* for group II and c, 0.35 m*M* for group III. Injected amounts of amines: 0.5 nmol for groups I and III except prolines (2.5 nmol), and 1.0 nmol for group II. ○, 20°C; ●, 40°C; □, 60°C; ▲, 80°C.

## RESULTS AND DISCUSSION

### *Optimization of the conversion reaction*

*pH.* The influence of pH on the conversion reaction with NaOCl was investigated using FIA without delivering the TDE solution. Solutions of NaOCl were prepared with buffers of various pH values. As shown in Fig. 2, the optimum pH for the conversion reaction was about 8 for prolines, about 12 for epinephrine and metanephrine and about 14 for sarcosine. Therefore, in the following investigations, the model secondary amines were divided into three groups, *i.e.*, group I (prolines), group II (epinephrine and metanephrine) and group III (N-methyl amino acids) and reacted with NaOCl at their respective optimum pH values. Within each group, related primary amines were treated in the same manner. Based on our previous finding[7], the reaction with TDE was carried out at about pH 8.

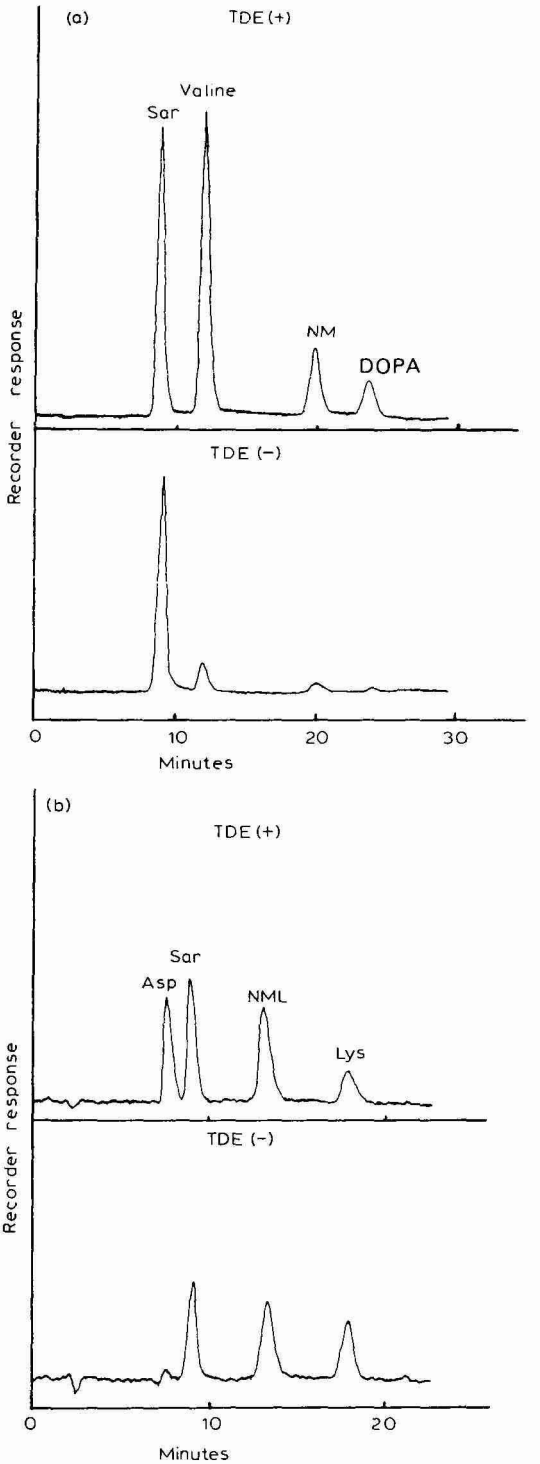


Fig. 6. Detection of amines with or without the addition of TDE. a, Method II was used except that reaction temperature was set at 60°C; injected amount of each amine was 4 nmol. b, Method III was used; injected amount of each amine was 0.2 nmol except lysine (0.3 nmol).

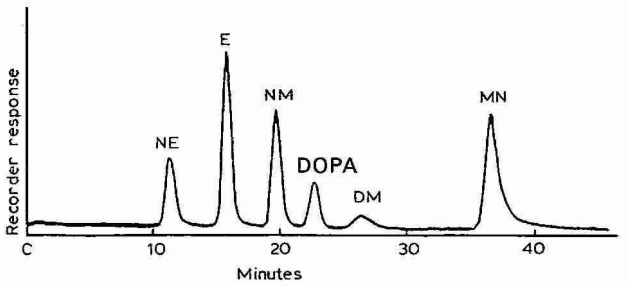


Fig. 7. Separation of catecholamines and their 3-O-methyl derivatives. The conditions were as in Fig. 6a. Injected amount of each amine: 2.7 nmol.

*NaOCl concentration.* The influence of NaOCl concentration on the conversion reaction was next investigated by FIA. As shown in Fig. 3a (pH 8), the fluorescence intensities of prolines and GABA reached plateaus at NaOCl concentrations over 0.3 m*M* in coil $C_1$, while those of aspartic acid and alanine decreased rapidly with increasing NaOCl concentration. Unlike other α-amino acids, lysine showed a similar profile to those of prolines and GABA. When the conversion reaction was performed at pH 12 (Fig. 3b), epinephrine and metanephrine fluoresced intensely even with higher concentrations of NaOCl, while the fluorescence intensities of norepinephrine, normetanephrine, DOPA and dopamine decreased with increasing NaOCl concentration. As shown in Fig. 3c (pH 14), the fluorescence intensities of sarcosine and N-methylleucine reached plateaus at >0.2 m*M* NaOCl. Similar to the case in Fig. 3a, the fluorescence intensities of aspartic acid and alanine decreased rapidly and that of lysine increased gradually with increasing NaOCl concentration, while that of meth-

TABLE I

CONDITIONS FOR HPLC WITH NaOCl–TDE–OPA POST-COLUMN DERIVATIZATION

| | *Method I* | *Method II* | *Method III* |
|---|---|---|---|
| Column | Partisil-10 SCX | TSK LS-410 | Partisil-10 SCX |
| Eluent | 0.1 *M* citrate buffer (pH 3) | phosphate buffer (pH 3)* | 0.1 *M* citrate buffer (pH 3) |
| Flow-rate | 0.70 ml/min | 0.48 ml/min | 0.70 ml/min |
| Length of coil $C_1$ | 3.0 m | 1.0 m | 2.0 m |
| Temperature | 40°C | 80°C | 60°C |
| NaOCl concentration in coil $C_1$ | 0.03 m*M* | 0.07 m*M* | 0.15 m*M* |
| Solvent for NaOCl solution | 0.5 *M* phosphate buffer (pH 8) | 0.1 *M* phosphate buffer (pH 12) | 1 *M* NaOH |
| TDE concentration in coil $C_2$ | 6.0 m*M* | 68 m*M* | 6.0 m*M* |
| Solvent for TDE solution | 0.5 *M* phosphate buffer (pH 8) | 0.5 *M* phosphate buffer (pH 8) | 5.7% acetic acid |

* Prepared by titrating 50 m*M* $NaH_2PO_4$ with $H_3PO_4$.

TABLE II

DETERMINATION RANGES OF THE PRESENT METHOD

| *Compound* | *Determination range (nmol)* | | |
|---|---|---|---|
| | *Method I* | *Method II* | *Method III* |
| Proline | 0.1–2.0 | | |
| Hydroxyproline | 0.1–2.0 | | |
| Sarcosine | | | 0.02–2.0 |
| N-Methylleucine | | | 0.02–2.0 |
| Aspartic acid | 0.02–10.0 | | 0.02–10.0 |
| Lysine | 0.02–0.2 | | 0.075–15.0 |
| γ-Aminobutyric acid | 0.02–5.0 | | |
| Epinephrine | | 0.5–10.0 | |
| Metanephrine | | 0.5–10.0 | |

ylamine was hardly affected. The unexpected result obtained with lysine is thought to be due to the quenching property of the intramolecular interaction between the two isoindole fluorophores derived from the two amino groups of lysine[10]. The apparent increase in the fluorescence intensity induced by lysine with increasing NaOCl concentration is due to the decrease in this interaction caused by the loss of the α-amino group sensitive to NaOCl, but not obviously due to the increase in the number of primary amino groups. In fact, when a surfactant Brij-35 was added to the OPA–ME reagent to suppress the interaction, the fluorescence intensity obtained from lysine decreased with increasing NaOCl concentration (Fig. 4), showing a similar profile to those of other primary aminon acids. On the basis of the above results, the NaOCl concentration in coil $C_1$ was set at 0.03 m*M* for group I, 0.07 m*M* for group II and 0.15 m*M* for group III.

*Reaction temperature and time.* Fig. 5 shows the dependence of the conversion reaction on the reaction temperature and the length of coil $C_1$. The fluorescence intensities of secondary amines increased with increasing reaction temperature. However, at elevated temperatures, they decreased as the length of coil $C_1$ increased. The

TABLE III

REPRODUCIBILITIES OF THE PRESENT METHOD

| *Compound* | *Coefficient of variation (%)* | |
|---|---|---|
| | *Method I (n = 10)* | *Method III (n = 8)* |
| Proline | 2.7 (1.0 nmol*) | |
| Hydroxyproline | 2.2 (1.0 nmol) | |
| Sarcosine | | 2.5 (0.2 nmol) |
| N-Methylleucine | | 1.8 (0.2 nmol) |
| Aspartic acid | 2.3 (0.1 nmol) | 6.3 (0.2 nmol) |
| Lysine | | 6.6 (0.3 nmol) |
| γ-Aminobutyric acid | 1.7 (0.1 nmol) | |

* Injected amount of the compound is given in parentheses.

fluorescence intensities of primary amines except lysine decreased rapidly as the reaction temperature or time increased. The adopted conditions for the NaOCl–TDE–OPA post-column derivatization are summarized in Table I. Methods I, II and III were for the determination of groups I, II and III respectively.

*Application to the HPLC of biogenic amines*

The present NaOCl–TDE–OPA post-column derivatization was applied to the HPLC of some standard compounds. Fig. 6 shows the chromatograms (upper) obtained with the present method and the corresponding chromatograms (lower) obtained by delivering the solvent in place of the TDE solution. Comparison of the two sets of chromatograms revealed that TDE suppressed the effects of the excess of NaOCl except in the case of lysine. Fig. 7 shows a chromatogram of catecholamines and their 3-O-methyl derivatives obtained by Method II. Tables II and III show the determination ranges and reproducibilities, obtained by a peak height method, of the present method.

We were not able to obtain any condition under which all secondary amines were sensitively detected. This is thought to be due mainly to differences either in the optimum conditions for the conversion reaction of secondary amines or in the stability to NaOCl of primary amines converted from secondary amines. However, as can be seen from the profile of fluorescence intensity and NaOCl concentration (Fig. 3), the primary amine compounds produced from the secondary amines tested seem to be relatively stable to NaOCl. This suggests that the differences in the optimum conditions for the conversion reaction dominate the overall determination of secondary amines. The identification of the reaction products of the conversion reaction is now in progress.

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CHROM. 15,867

# QUANTITATIVE ANALYSIS OF TWO-DIMENSIONAL ELECTROPHEROGRAMS WITH A TELEVISION CAMERA–MICROCOMPUTER SYSTEM

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## SUMMARY

A system to quantitate dye-stained proteins on two-dimensional polyacrylamide gels employing a television camera for data acquisition and a microcomputer for data analysis is described. All the equipment comprising the system and most of the software are commercially available. Therefore, minimal knowledge of computer software is needed to construct the system. The system is handy, low cost and useful for practical quantitation of protein spots, although it is semi-quantitative compared with the previously available systems. The system is especially suited for the analysis of micro two-dimensional gels since high resolution up to 30 $\mu$m is obtained.

## INTRODUCTION

Previously we have reported a two-dimensional electrophoretic technique in which no denaturing agent is used[1], and demonstrated its applicability to clinical purposes[2,3]. A microscale-multisample version of the technique[4] enabled us to analyze eight to sixteen samples simultaneously and it facilitated comparative studies of multiple two-dimensional patterns[5]. During these comparative studies, we tried to analyze the changes in quantity of several specific proteins. Previously, we used a thin-layer chromatographic scanner, which is a flat bed $x$–$y$ scanner, for the quantitation of a specifically increased serum protein in rat to which carbon tetrachloride had been administered[3].

However, for the quantitation of individual protein spots on a complex two-dimensional pattern, the scanner should be combined with a computer system for data analysis. Several reports have dealt with sophisticated systems for quantitation of individual proteins on two-dimensional gels and for comparisons of two-dimensional patterns, combining a flat-bed scanner or a drum scanner with a computer system[6–9], but these systems are costly and not handy for laboratory use.

In this report we describe a system for quantitation of two-dimensional electropherograms, which employs a television (TV) camera and a digitizer for data acquisition, a mini-floppy disk unit for data storage and a microcomputer for data analysis. The system is especially useful for quantitating proteins on micro two-dimensional electropherograms.

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## MATERIALS AND METHODS

### *Two-dimensional electrophoresis*

Two-dimensional electrophoresis of normal human serum proteins in the absence of denaturing agents using macro slab gels[10] or micro slab gels[4] was performed as described previously, except that the macro slab gel was 160 mm wide, 120 mm long and 3 mm thick. Micro two-dimensional electrophoresis of bovine brain soluble proteins was performed as described previously[11]. The macro slab gels were stained overnight in 0.025% Coomassie Brilliant Blue R-250–7% acetic acid–50% methanol and destained in 7% acetic acid. In the case of the micro slab gels, gels were stained in the above staining solution for 1 h and destained in 7% acetic acid. The micro slab gel was 38 mm wide, 40 mm long and 1 mm thick. Silver staining of micro slab gels was performed as described by Oakley *et al.*[12].

### *Instruments*

An illustration of the TV camera–microcomputer system is shown in Fig. 1. The whole system is composed of commercial equipment. A stained two-dimensional gel was sandwiched between two glass plates, placed on a fluorescent light-box (Yodobashi Camera, Tokyo, Japan) and a black and white TV camera (NEMCO Model CN-120; Pax Electronica Japan, Tokyo, Japan) was set above the gel using a camera stand. The TV camera is equipped with a Cosmicar lens ($f$ = 16 mm) and a Toshiba Y-2 filter (Tokyo Shibaura Electric, Tokyo, Japan). The magnification rate of the camera was adjusted by changing the length of extension tubes (Cosmicar extension tube kit; Asahi Precisin, Tokyo, Japan) attached to the lens. For example, by attaching a 15-mm extension tube, gel sections (8 × 6 mm) could be projected on the full screen of a TV display. The video signal is digitized by a digitizer (Computer Eye Model 4000; Pax Electronica Japan) which has an interface for an NEC PC-8801 microcomputer (Nippon Electric Co., Tokyo, Japan). The digitizer divides the video image into 256 × 256 dots and each dot has four levels of gray scale. The digitizer

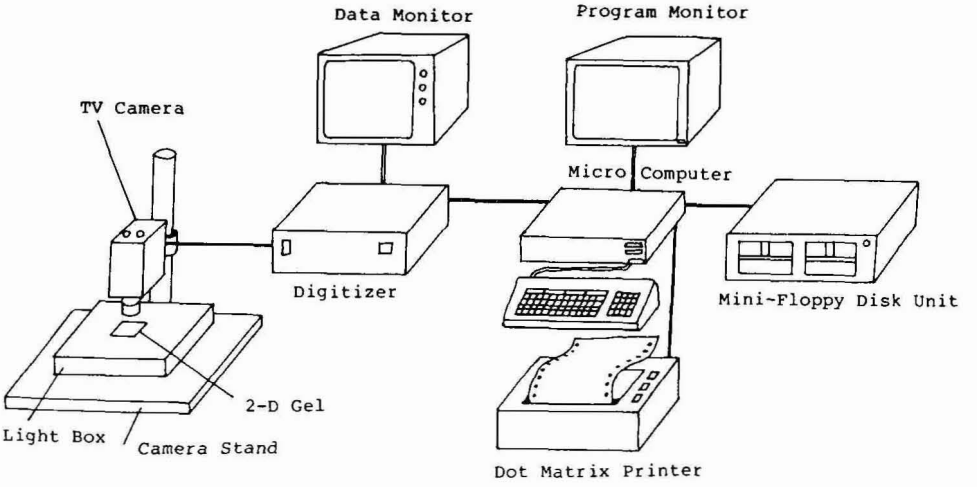


Fig. 1. The TV camera–microcomputer system.

is equipped with two variable resistors and these are adjusted when the gray levels are converted into dye density. The digitized image is acquired in the digitizer 1/60 sec after triggering a switch, and monitored by a TV display (NEC PC-8041 12-in. green display, Nippon Electric Co.). The digitized image can be stored in a 5-in. magnetic disk (143 kbytes of memory) by a floppy disk unit (NEC PC-8031 mini-floppy disk unit, Nippon Electric Co.). The floppy disk unit has two drives, one of which is used to set a 5-in. disk which stores the microcomputer programs to control the system and the other sets a disk for storage of the digitized images. One 5-in. disk can store seven digitized images. The programs to control the system were selected by monitoring a TV display (NEC PC-8851 14-in. monochrome display, Nippon Electric Co.). A dot-matrix printer (EPSON MP-82; Shishu Seiki, Shiojiri, Japan) was used for hard-copy of the digitized images.

*Computer programs*

The microcomputer programs, commercially available as a software package from Pax Electronica Japan, were used with some modifications. The software is written in BASIC language for the operator–computer interactions and in machine language for the computer–peripheral machine interactions. Our modifications were restricted to the BASIC programs. The software package is composed of the following programs: (1) "cecp", a program to select the subroutines described below; (2) "save", which reads the digitized image data in the digitizer and stores them in a magnetic disk file; (3) "load", which reads the image data in a magnetic disk and stores them in the memory of the digitizers; (4) "data match", which compares the digitized image in the digitizer with any of the images stored in a magnetic disk and displays the unmatched dot positions on the TV display; (5) "demo", which allows an operator to select any rectangular area of a digitized image using a cursor; [The number of dots in the selected area, in four gray levels ("white", "light gray", "dark gray" and "black"), is counted.] (6) "package II", which prints out the digitized image in the digitizer on a dot-matrix printer.

We added the following functions to the program "demo"; (a) after dot counting, the integrated density of the spot was calculated and printed out by the procedure described in the next paragraph: (2) after calculating integrated density, the selected area is distinguished from the remaining area by inversing the gray levels.

*Settings of density levels and integration of density*

A density standard tablet (Fuji Density Step Tablet; Fuji Photo Film, Tokyo, Japan), which is a photo-film representing stepwise thirteen levels of diffuse visual density, was used to convert gray levels into density. The tablet was put on the light-box and a digitized image of the tablet was shown on the TV display. By adjusting the two variable resistors of the digitizer, we set the four gray levels, white, light gray, dark gray and black, to the following diffuse visual density; 0.04, 0.28, 0.67 and 1.32, respectively. Since white corresponded to gel background, the density value of 0.04 was subtracted from the density value of each gray level. Then the integrated density of a spot was calculated by multiplying the dot counts in each gray level by the converted density and summing the products

$$\text{I.D.} = 0.24L + 0.63D + 1.28B$$

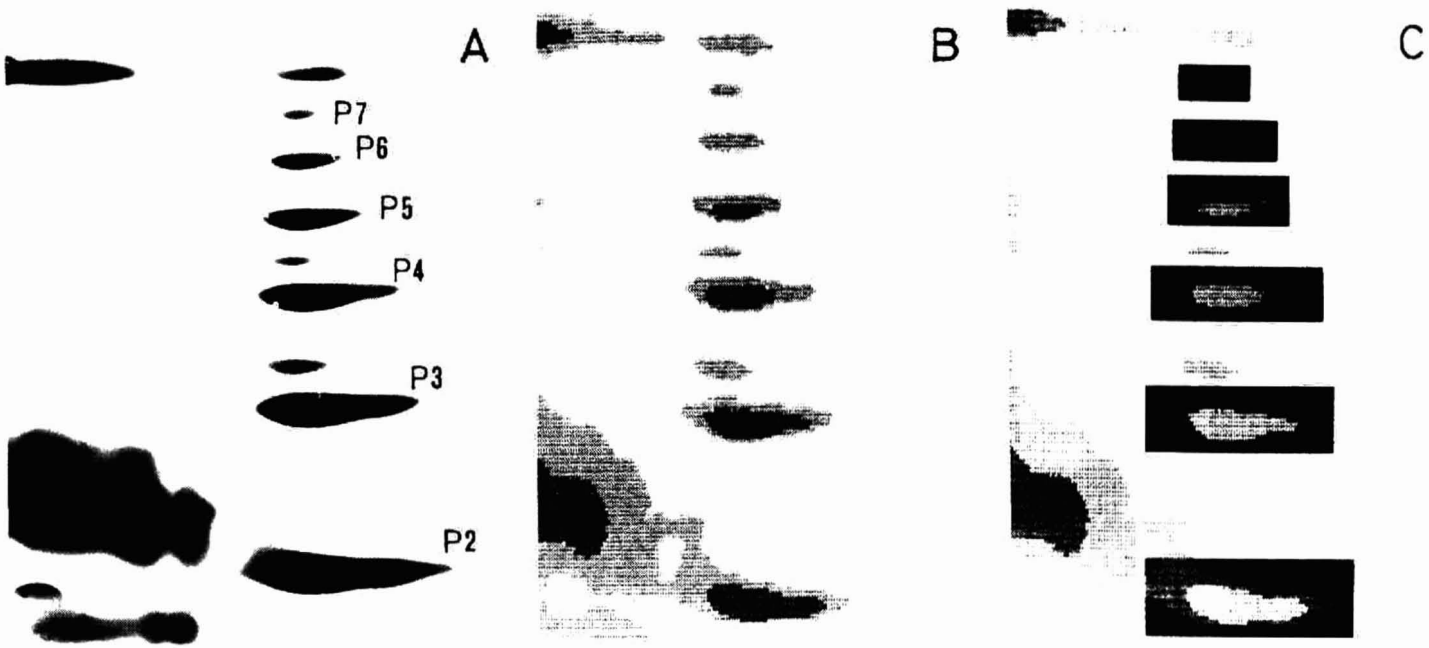


Fig. 2. The process of quantitating stained spots on two-dimensional (2-D) electropherograms. A, A section of a macro slab gel (160 × 120 × 3) pattern of a haptoglobin Type 2-1 serum. Haptoglobin polymer series, P2 P7, are indicated. B, A gray scale counter map of the gel section shown in A in 200-$\mu$m resolution. The print-out image is expanded 1.3-fold in the vertical axis. C, The gray scale contour map shown in B, after calculation of integrated density. By running the program "demo", a rectangular area which contained a spot of haptoglobin 2-1 polymer series was selected by keyboard control, the integrated density was calculated and the gray levels within the area were inverted.

where I.D. represents integrated density, $L$, $D$ and $B$ the number of dots in levels light gray, dark gray and black, respectively.

*Comparison with a flat-bed scanner*

A flat-bed $x$–$y$ scanner, Shimadzu dual-wavelength thin-layer chromatographic (TLC) scanner CS-910 (Shimadzu, Tokyo, Japan), was used to compare the results of quantitation. The sample wavelength was set at 580 nm and the reference wavelength was 750 nm. The densitometer was operated in "zigzag scanning mode" and the protein amounts were quantitated by measuring the step height of the integrating signal.

*Albumin quantity standard*

In order to examine the sensitivity of the TV camera system, an albumin quantity standard slab gel was prepared. Varying amounts of bovine serum albumin (crystallized four times; Sigma, St. Louis, MO, U.S.A.) ranging from 0.05 to 3.2 $\mu$g were subjected to polyacrylamide SDS gradient gel electrophoresis. Micro polyacrylamide slab gels containing 1% SDS were prepared as described previously[11]. Electrophoresis was run in 0.05 $M$ Tris–0.38 $M$ glycine containing 0.1% SDS at 5 mA for 100 min.

## RESULTS

*Resolution*

As the first step of quantification, we determined the degree of magnification of the image of a two-dimensional gel by selecting the lens focal length of the TV camera. Since the digitizer divides the video image into 256 × 256 dots, the resolution of the system depends on the degree of magnification. We routinely attached a 1.0-mm extension tube to the lens of the TV camera for the analysis of 50 × 40 mm sections of macro two-dimensional gels (160 × 120 × 3 mm), which corresponded to 200 × 160 $\mu$m resolution. In case of micro two-dimensional gels (38 × 40 × 1 mm), a 10-mm extension tube was attached and 12 × 9 mm sections were analyzed, which corresponded to 47 × 35 $\mu$m resolution.

*Performance of the software*

A haptoglobin Type 2-1 serum was subjected to two-dimensional polyacrylamide gel electrophoresis using a macro (160 × 120 × 3 mm) slab gel and a 50 × 40 mm gel section (Fig. 2A) which showed haptoglobin polymer series was analyzed by the TV camera system. The image data were acquired 1/60 sec after triggering a switch of the digitizer and the digitized image was displayed on the monitor TV as a gray scale contour map (Fig. 2B) without the aid of software. A spot to be quantitated was selected by keyboard control, the "integrated density" of the spot was calculated, and the gray levels in the selected area were inverted (Fig. 2C) by program "demo". The digitized image on the TV display could be copied on the printer by the program "package II". The time needed for the area selection and calculation for one spot was 10–20 sec, for hard-copy of one image it was 4 min. Routinely, we stored the image data in magnetic disks by use of the program "save" and then read the data from the disk by the program "load" when we wanted to quantitate spots of interest. The time needed to save or load one digitized image was about 20 sec.

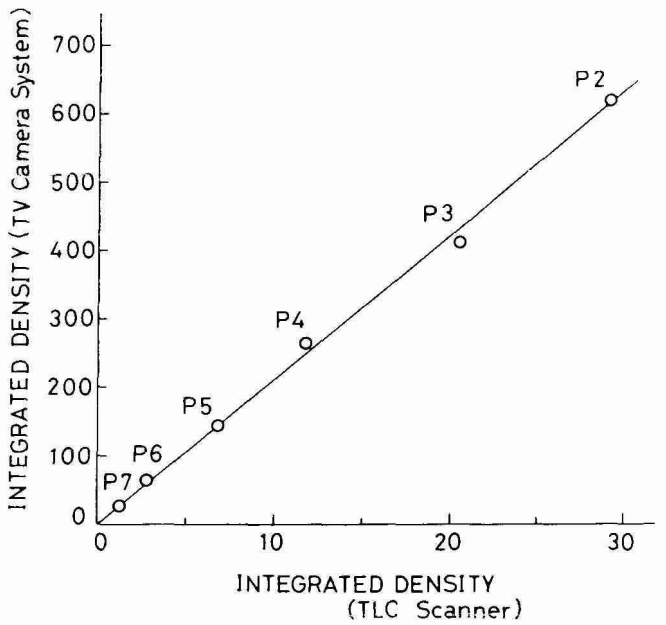


Fig. 3. Comparison of the values of integrated density calculated by the TV camera-microcomputer system with those obtained by an $x$–$y$ scanner.

*Quantitation of haptoglobin polymer series*

The integrated density of each haptoglobin spot in Fig. 2A was calculated by the program "demo". A TLC scanner was also employed to measure the relative quantity of the haptoglobin spots. The values of integrated density calculated by the TV camera system were plotted against those obtained by the TLC scanner. As shown in Fig. 3, the two sets of results were in good agreement.

*Application to micro two-dimensional gels*

One of the advantages of the TV camera system is that high resolution is obtained simply by magnifying the video image. Fig. 4 shows a 12 × 9 mm section of a micro two-dimensional gel pattern of bovine brain soluble proteins, a print-out image at 47 × 35 $\mu$m resolution. The minimum spot size in the pattern was 500 $\mu$m in the direction of isoelectric focusing and 200 $\mu$m in the direction of gradient gel electrophoresis. Thus, the resolution of 47 × 35 $\mu$m was required for the analysis of spots on micro two-dimensional gels. The time needed to acquire image data is 1/60 sec, irrespective of the resolution. However, in the case of mechanical scanners, much longer time is necessary to obtain high resolution. Since the TV camera system can analyze stained gels directly, the time of data acquisition must be compared with that of flat-bed $x$–$y$ scanners, which scan very slowly (at least 30 min being needed to scan a 40 × 38 mm slab gel in 50-$\mu$m steps).

*Sensitivity of the system*

As shown in Fig. 3, the integrated density obtained by the TV camera system correlated very well with that obtained by a densitometer equipped with an $x$–$y$

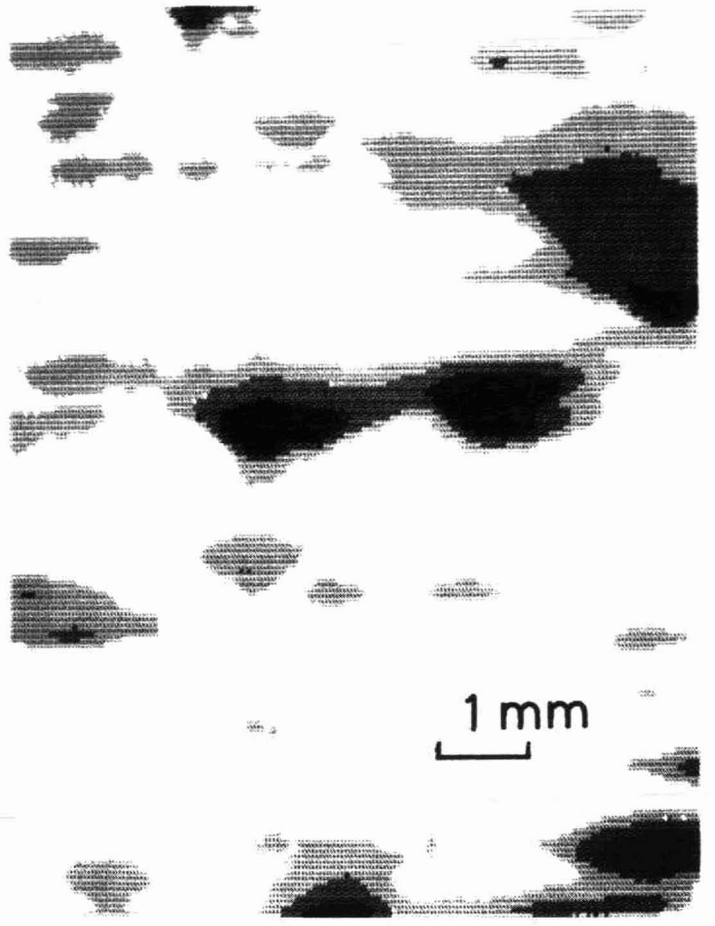


Fig. 4. A gray scale contour map of a gel section of a micro two-dimensional electropherogram, a print-out image. Bovine brain soluble proteins (20 $\mu$g) were subjected to micro two-dimensional gel electrophoresis, silver stained and analyzed by the TV camera system in 47 × 35 $\mu$m resolution.

mechanical scanner. Next we examined the sensitivity of the TV camera system. Various amounts of bovine serum albumin, ranging from 0.05 to 3.2 $\mu$g, were applied on the slots of a polyacrylamide gradient micro slab gel (40 × 38 × 1 mm), electrophoresed and the gel was stained with Coomassie Blue R-250. The gel was set on the light-box and the image data were acquired at 47 × 35 $\mu$m resolution. As shown in Fig. 5, the system could detect as little as 0.05 $\mu$g albumin. Since the albumin samples were only subjected to one-dimensional electrophoresis, the size of the 0.05-$\mu$g spot was about five times larger than the minimal spot obtained by two-dimensional electrophoresis, 0.1 mm$^2$. Therefore, the sensitivity of the system for two-dimensionally separated and Coomassie Blue-stained spots must be about 0.01 $\mu$g. The value of the integrated density was almost linearly correlated with the amount of albumin applied, up to 1.6 $\mu$g albumin.

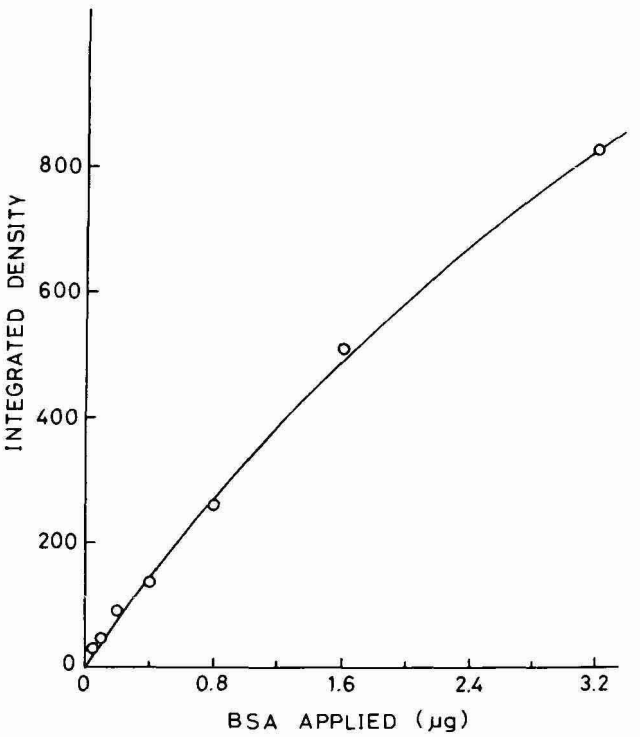


Fig. 5. Integrated density of albumin quantity standard spots. Various amounts of bovine serum albumin, ranging from 0.05 to 3.2 μg, were subjected to micro gradient gel electrophoresis, the gel was stained and the albumin spots were quantitated in 47 × 35 μm resolution.

## DISCUSSION

Several reports have dealt with the quantitation of proteins on two-dimensional electrophorograms[6–9], each of which employed a mechanical scanner and a high-performance computer. These approaches are orthodox and should permit not only accurate quantitation of proteins but automated comparisons of two electropherograms. However, the quantitation systems are costly and not handy for laboratory use.

The advantages of the present system are as follows: (1) direct analysis of stained gels (not possible with a drum scanner); (2) rapid data acquisition (flat-bed scanners take time to scan especially when high resolution is required); (3) construction from commercially available equipment and software (although we slightly modified the commercial software, this is easy since the software is written in BASIC for the operator–computer interactions); (4) low cost, the whole system including all the hardware shown in Fig. 1 and the software being purchased for about $ 4000.

The sensitivity of the system was 0.05 μg protein per 0.5 $mm^2$ for Coomassie Blue-stained albumin spots on polyacrylamide micro slab gels. The minimum detectable quantity of proteins on two-dimensional electropherograms should depend on the minimum spot size on the slab gels (the resolution of the two-dimensional

electrophoretic technique) and the staining technique employed. The minimum spot size on macro two-dimensional gels (160 × 120 × 3 mm) was 2 $mm^2$ and that on micro gels (38 × 40 × 1 mm) was 0.1 $mm^2$. Therefore, the sensitivity of the system for the Coomassie Blue-stained proteins on macro and micro two-dimensional gels can be calculated to be 0.2 and 0.01 $\mu$g, respectively. Since our silver staining technique is at least twenty times more sensitive than Coomassie Blue staining, this system will have a sensitivity of 0.5 ng for proteins on silver-stained micro two-dimensional gels.

The values of integrated density were reproducible within 10% when a spot showed more than 100 units of integrated density per 600 dots of spot area, corresponding to 0.4 $\mu$g protein per $mm^2$. This level of reproducibility will be sufficient for most of biological studies, since the staining techniques have inherent limitations.

Recently, Mariash *et al.*[13] reported a system for quantitating two-dimensional radioautofluorograms employing a TV camera and a digitizer for data acquisition. However, their approach is quite different from ours. They aimed to attain an accuracy comparable with that of the mechanical scanner–high performance computer systems. They employed 64 levels of gray scale, scanned a whole 18 × 10 cm radiofluorogram at a relatively low resolution of 440 $\mu$m taking almost 1 h and calculated the quantitity of all the protein spots on the gel. In contract, we wanted to quantitate several spots of interest on multiple micro two-dimensional gels, for example, for comparisons of samples of different origin or to monitor quantities of substances after various sample treatments. Therefore, four gray levels were employed to digitize the video image, which facilitated easy programming and rapid calculation of integrated density. As shown in Figs. 3 and 5, the present system is useful in practical quantitation of stained spots on two-dimensional electrophorograms. Quantitative analysis of various changes in plasma proteins of experimental animals is now in progress.

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# DETECTION OF β,β′-DICHLOROETHYL SULPHIDE ON THIN-LAYER CHROMATOGRAMS

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## SUMMARY

Thin-layer chromatography (TLC) has been employed to detect and quantify very low concentrations of β,β′-dichloroethyl sulphide (sulphur mustard, HD), on the basis of visualization of spots and $R_F$ values. Various reagents and reactions were examined with respect to their sensitivity and selectivity. In particular, chemically similar compounds like thiodiglycol or β,β′-dichloroethyl oxide as well as a variety of organophosphorus esters (pesticides) and halogen-containing organic compounds were subjected to the same analytical procedures so as to check the selectivity of the TLC methods developed for HD. The best separations were usually obtained by developing silica chromatoplates in dichloromethane–*n*-hexane (1:1) solvent. The most satisfactory evidence for the presence of HD is based on a reaction with iodoplatinate $[PtI_6]^{2-}$ and the subsequent application of starch solution (ultimate sensitivity ' 0.1 μg/μl). Somewhat less sensitive (0.5 and 1 μg, respectively), but quite selective, is a reaction utilizing Michler's ketone in the presence of mercury bromide, which gives rise to intense purple spots on a yellow background, and a photochemical reaction using silver nitrate. Other sensitive and selective reactions are those with triphenylmethane dyes in conjunction with $AgNO_3$ and with selenic acid.

## INTRODUCTION

In contrast to sophisticated analytical techniques, surprisingly little effort has so far been made to determine trace quantities of sulphur mustard (HD) by means of thin-layer chromatography (TLC), despite the sensitivity and simplicity of this technique[1–3]. Well established analytical procedures using colorimetric methods are based on the so-called "DB 3" reaction in solution in which HD reacts with 4-(*p*-nitrobenzyl)pyridine to form an intense blue dye[4]. In order to detect rapidly contamination of water, soil and vegetation with mustard outside the laboratory there is need for a much simpler but nevertheless reliable and sensitive method of detecting HD. TLC seems to fulfil most of these requirements if the sensitivity, selectivity and possible interferences of similar compounds are known and certain precautions are taken. This led us to conduct a systematic study of the versatility and utility of TLC for identification and determination of HD.

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## EXPERIMENTAL

### *Thin-layer chromatography*

Pure freshly distilled HD was dissolved in reagent-grade dichloromethane and diluted to give standard solutions of 100, 10, 1 and 0.1 $\mu g/\mu l$. For the TLC experiments, commercial glass plates coated with 0.25 mm silica gel (Fertigplatte Type 60 $F_{254}$, E. Merck) were used. A 1-$\mu l$ volume of standard HD solution was carefully applied to a chromatoplate with a micropipette to form a very small sample starting spot. Thereafter the plates were immersed vertically in glass development chambers maintained at 22°C and containing ' 100 ml of reagent-grade dichloromethane and *n*-hexane (1:1). This solvent is known to yield rather high $R_F$ values for mustards but only very low $R_F$ values for organophosphorus compounds (pesticides, etc.). Uniform saturation of the chamber atmosphere was achieved in the usual manner by lining the chamber with filter-paper. After development, the chromatoplates were first dried in air to remove most of the solvent. Thereafter they were uniformly sprayed with appropriate reagents (see below) using a spray gun applicator (Desaga, Heidelberg, G.F.R.). If necessary, the plates were heated in an oven to a defined temperature for a certain period of time, or exposed to UV light (mercury line at 253.7 nm) in order to accelerate photochemical reactions.

### *Chemicals and procedures*

Five different reactions were studied in detail (see below), requiring the following reagents.

*Detection via* $[PtI_6]^{2-}$–starch solution. (a) One millilitre of an aqueous solution of 5% hexaiodoplatinate (potassium salt) was added to 3.5 ml sodium iodide solution (' 1 *M*) (prepared immediately before use) and diluted in distilled water to a total of 60 ml. (b) One gram of soluble starch was moistened and stirred and then dissolved in 100 ml boiling water (prepared shortly before use). (c) Reagent grade glacial acetic acid.

The spray reagent comprised 1 ml of reagent a plus 3 ml of b plus 2 ml of c plus 1 ml distilled water and was prepared immediately before use. The developed chromatoplate was uniformly sprayed with this reagent. The presence of HD is indicated by blue spots on a white or pink background. The intensity of the spots fades after a while. Detection limit: $< 0.1$ $\mu g$ HD.

*Detection with Michler's ketone and mercury salts.* (a) A 0.1% solution of Michler's ketone [4,4'-bis(dimethylamino)benzophenone] in toluene (p.a.). (b) A 5% solution of mercury dichloride or bromide ($HgCl_2$, $HgBr_2$) in absolute ethanol.

The spray reagent comprised equal parts of reagents a and b, thoroughly mixed. The developed chromatoplate was uniformly sprayed with the reagent and then heated to 80°C. HD is indicated by intense purple spots on a yellowish background. The detection limit depends strongly on the concentration of the mercury salt solution, and is lowest for the conditions given above. With the standard chromatoplates described above the detection limit is approximately 0.5 $\mu g$ HD. If high-performance TLC chromatoplates are used the detection limit can be lowered to 0.01 $\mu g$.

*Detection with silver nitrate using a photochemical reaction.* The spray reagent comprised a 0.1 *M* aqueous solution of silver nitrate, prepared immediately before

use, stored in the dark. The developed chromatoplate was uniformly sprayed with the reagent, then placed in an oven for *ca.* 5 min at 80°C before exposure to UV light (mercury line at 253.7 nm) for 3 min. HD is indicated by gray or purple spots. Detection limit: 1 μg HD.

*Detection with triphenylmethane dyes and silver nitrate.* (a) Thymolphthalein (0.17 g) was dissolved in 20 ml absolute ethanol to which 2 ml of 5 *M* sodium hydroxide solution were added. Instead of thymolphthalein, 0.17 g α-naphtholphthalein, 0.13 g phenolphthalein or 0.15 g alizarine-S (sodium sulphonate salt of dihydroxyanthraquinone), respectively, can be used. (b) A 0.1 *M* aqueous solution of silver nitrate, prepared shortly before use. (c) 25% Sulphuric acid or acetic acid.

The developed chromatoplate was uniformly sprayed with reagent a. A dark blue colour is obtained with thymolphthalein and α-naphtholphthalein, a purple colour with phenolphthalein or alizarine-S. Thereafter reagent b was carefully sprayed; a thin homogeneous coating is crucial. A heat treatment in an oven at 120 or 140°C then follows, for about 10 min until dark brown spots appear. Excessive heat will result in a brown background, thus preventing visualization of the spots. After this treatment solution c was sprayed which helps to lighten the background to a yellowish finish. The detection limit is around 1 μg HD.

*Detection with selenic acid.* One gram of selenic acid ($H_2SeO_3$) was dissolved in 25 ml sulphuric acid (25%). The developed chromatoplate was homogeneously sprayed with this solution. Subsequent heating in an oven at 120°C for about 10 min (too long a heat treatment is deleterious) produced orange or light-brown spots on a white background if HD was present. The detection limit is approximately 1 μg HD. A photometric evaluation (by shining light through the transparent plate) is recommended since it increases the detection limit by a factor of 2. The spots are very stable and do not change in intensity even weeks after the reaction.

## RESULTS

The experiments were first performed in order to test the applicability of various analytical reactions (so far mostly carried out in solution) to TLC. Secondly, the sensitivity was determined and the physicochemical parameters (concentration, composition of mixtures, temperature, exposure, etc.) were varied systematically so as to improve the suitability and detection limit for HD. Thirdly, the selectivity of a given reaction was checked by directly comparing HD with various chemically related compounds and some other harmful substances such as organophosphorus esters or irritants.

Some of the results of our investigations concerning the first two of these categories have already been touched upon in that only those reactions listed gave satisfactory chromatograms. Also, the best analytical procedures were also briefly reported. Here, we concentrate on the third category, namely, the selectivity of a given reaction and possible interferences in mixtures. Experiments were performed in which HD was allowed to migrate together with a variety of other chemical compounds in similar concentrations, under otherwise identical conditions (same developing solvent, heat treatment, etc.). Basically three different groups of compounds were investigated:

(i) Chemically related species: "oxygen mustard" β,β'-dichloroethyl ether,

TABLE I

SUMMARY OF THE TLC RESULTS OF THE DETERMINATION OF HD

| *Reaction* | *Temperature (°C)* | *Detection limit (μg)* | *Interferences* | | *Reproducibility* |
|---|---|---|---|---|---|
| | | | *Irritants related components* | *Pesticides* | |
| DB 3 (NPB reagent) | 110 | 1 | + | ? | Very good |
| Iodoplatinate | 25 | 0.1 | – | Few | Very good |
| Michler's ketone + mercury salts | 80 | 0.5 | + | + | Very good |
| Silver nitrate + UV light | 80 | 1 | – | – | Very good |
| Triphenylmethane dyes + $AgNO_3$ | 120–140 | 1 | + | + | Poor |
| Selenium dioxide | 120 | 1 | – | Few | Very good |

$(ClCH_2CH_2)_2O$; thiodiglycol; $\beta,\beta'$-dihydroxyethyl sulphide, $(OHCH_2CH_2)_2S$; "nitrogen mustard", $(ClCH_2CH_2)_3N$; 1,4-dithiane;

(*ii*) Irritants and organic compounds containing active halogen atoms: "CN", chloroacetophenone, $CH_2Cl\text{-}CO\text{-}C_6H_5$; "CS", *o*-chlorobenzalmalondinitrile, $ClC_6H_4CHC(CN)_2$; benzoyl chloride, $C_6H_5COCl$; benzyl bromide, $C_6H_5CH_2Br$; bromoacetic acid methyl ester, $CH_2BrCOOCH_3$.

(iii) Pesticides (organophosphorus esters, thioesters, etc.) (trivial names given only): dimethoat, $(CH_3O)_2PSSCH_2CONHCH_3$; parathion, $(C_2H_5O)_2PSOC_6H_4NO_2$; malathion, $(CH_3O)_2PS\text{–}SCH(COOC_2H_5)\text{–}(CH_2COOC_2H_5)$; ethion, $(C_2H_5O)_2PS\text{–}SCH_2S\text{–}SP(OC_2H_5)_2$; dimefox, $[(CH_3)_2N]_2POF$; DDVP (Dichlorvos), $((CH_3O)_2POOCH = CCl_2)$; metasystox, (demeton-*o*-methyl), $(CH_3O)_2PO\text{–}S\text{–}CH_2\text{-}CH_2SCH_2CH_3$; chlorfenvinfos, $(C_2H_5O)_2POOC = CHClC_6H_3Cl_2$.

In the chromatographic experiments, 10 μg HD were directly compared to 100 μg each of the compounds listed above. The developing solvent was again dichloromethane–*n*-hexane (1:1). The chromatoplates were then subjected to the various analytical procedures described. The results will be reported in the same order.

*Selectivity of the iodoplatinate–starch reaction*

*Chemically related species, organic halides, irritants.* Only HD forms intense blue spots; CN and CS are visible as white spots, but their $R_F$ values are much lower than that of HD. Thiodiglycol does not perturb the confirmation of HD. Nitrogen mustard gives no response.

*Pesticides.* Of all the compounds studied only metasystox slightly perturbs the HD detection in that it gives rise to weak blue spots (a group of two spots with different $R_F$ values) the first of which has a similar $R_F$ to that of HD. In order unequivocally to determine HD a second reaction has to be performed, *e.g.*, the photochemical reaction with silver nitrate which is negative with metasystox.

*Selectivity of the reaction using Michler's ketone and mercury salts*

*Chemically related compounds.* Only HD forms an intense purple spot, whereas oxygen mustard gives no response. Thiodiglycol produces a white spot with very low $R_F$ value and 1,4-dithiane gives a white spot with a lower $R_F$ value than that of HD.

Nitrogen mustard likewise gives a positive reaction with Michler's ketone, its $R_F$ value and detection limit being similar to those of HD. As will be shown later, HD can be distinguished from nitrogen mustard by means of the reaction with iodoplatinate–starch or with $SeO_2$ which gives a negative result in the presence of nitrogen mustard.

*Active organic halides, irritants.* CN produces a very weak dark spot, whereas CS shows an orange spot whose intensity increases if $HgBr_2$ is used instead of $HgCl_2$. Compounds like "Clark I", diphenylarsine chloride, $(C_6H_5)_2AsCl$, or "Pfiffikus", phenylarsine dichloride, all show negative reactions. Benzoylchloride reacts immediately with Michler's ketone (without any mercury salt) to give a sea-green precipitate; 1 μg gives a clearly visible spot. It is worth mentioning that aromatic halides substituted in the side chain like xylyl bromide, $CH_3C_6H_4CH_2Br$, or benzyl bromide, $C_6H_5CH_2Br$, give the same reaction as does HD, *viz.*, a purple spot. Here, only the different $R_F$ values would render a distinction from HD.

*Pesticides.* Chlorfenvinphos, dimefox and metasystox give negative reactions. Dimethoat gives rise to a group of three separate spots, a pink one with very low $R_F$ and two green ones with appreciable $R_F$ values. Parathion exhibits similar behaviour; besides the pink spot only one weak pink-yellow spot was observed. Blue spots are obtained with dichlorvos and malathion, and the latter pesticide produces an additional green starting spot. Ethion gives rise to a very weak greenish spot. So all the pesticides studied essentially do not perturb the HD determination with Michler's ketone.

*Selectivity of the photochemical reaction with $AgNO_3$*

*Chemically related species.* Nitrogen mustard gives exactly the same reaction as HD, *i.e.*, a dark purple spot on a white background. Also, the detection limit is similar, namely, 1 μg. Thiodiglycol and oxygen mustard as well as 1,4-dithiane do not show any reaction at all and therefore do not perturb the HD detection.

*Irritants and active organic halides.* Only CN gives a (washed-out) light spot which shows intense purple fluorescence if irradiated with UV light; its $R_F$ value is about half that of HD. CS gives a negative reaction as does benzyl bromide. Benzoyl chloride, however, gives a brownish spot which can easily be distinguished from the respective HD features.

*Pesticides.* Immediately after spraying a yellow starting spot appears with dimethoat, a weak yellow spot with parathion, $R_F$ value approximately half that of HD, and an intense yellow spot is obtained with ethion ($R_F$ ‘2/3 that of HD). After irradiation with UV light for 5 min some alteration of the features occurs in that the parathion and ethion spots become very intense. A weak yellow spot appears with malathion. All the spots, however, do not interfere with the HD spot as far as $R_F$ value and colour are concerned. The other pesticides showed no responses.

In summary, the photochemical reaction with silver nitrate, despite its simplicity, is a quite selective and reasonably sensitive method to detect mustards having activated chlorine atoms in the β-position.

*Selectivity of the triphenylmethane dye–silver nitrate reaction (thymolphthalein, in particular)*

*Chemically related compounds.* In contrast to the very intense dark brown spot

obtained with HD, thiodiglycol only shows a brown starting spot and oxygen mustard gives no reaction. 1,4-Dithiane and nitrogen mustard were not studied in this series.

*Irritants and active organic halides.* Both CN and CS show fairly intense orange and yellow-brown spots, respectively, with slightly different $R_F$ values (about 1/2 to 2/3 that of HD). The CS spot sometimes has a characteristic yellow halo around it. Benzoyl chloride exhibits a quite intense whitish or pink spot with about the same $R_F$ value as that of CN and CS.

*Pesticides.* Many pesticides give rise to brown or orange spots similar to HD, particularly parathion, malathion, ethion, dimefox, metasystox, dichlorvos and chlorfenvinfos. Dimefox produces an intense brown spot ($R_F \approx 60\%$ that of HD) and a much weaker greyish spot with appreciably higher $R_F$ value ($\approx 80\%$ that of HD) and may therefore render difficult a distinction from HD. All other pesticide spots occur at markedly lower $R_F$ values than that of HD, so a separation should be possible.

However, the thymolphthalein–silver nitrate method, although quite sensitive, cannot be regarded as a very specific reaction for HD. Moreover, it is a rather crucial reaction (also true for the other triphenylmethane dyes investigated) as far as exposure to the spray reagents and the thermal treatment is concerned, and thus cannot be recommended for routine determinations of sulphur mustards, despite the fact that it gives good results in solution[5].

*Selectivity of the selenium dioxide reaction*

*Chemically related compounds.* Oxygen mustard does not show up at all, and thiodiglycol only produces an intense orange starting spot. Interestingly, nitrogen mustard does not react with $SeO_2$. Therefore, this reaction can be used to distinguish between sulphur mustard and nitrogen mustard. 1,4-Dithiane has not been investigated with respect to $SeO_2$.

*Irritants and active organic halides.* Here, the excellent selectivity of the HD detection with $SeO_2$ becomes quite apparent. CN, CS (which produces a brown starting spot), benzoyl chloride end benzyl bromide do not give a positive reaction.

*Pesticides.* Although somewhat less selective (parathion, malathion and ethion all give pronounced yellowish spots; $R_F = 40\%$ that of HD for parathion, about 10% for malathion and 60% for ethion; dimethoat and metasystox only give brown starting spots), a clear separation from HD is easily achieved by choosing an appropriate developing solvent.

## DISCUSSION

The aim of this section is not only to review briefly and discuss analytical reactions that have been used before to detect and identify sulphur mustard (particularly with respect to TLC), but also to correlate the sensitivity and selectivity of the various reactions with the actual chemistry that occurs when HD interacts with the respective reagents.

A fairly detailed study on the TLC determination of HD was recently reported by Sass and Stutz[3] who used the NBP reagent [4-(*p*-nitrobenzyl)pyridine], a method also known as the "DB 3" reaction[6]. Although reasonably sensitive ($\approx 1$ μg HD), this method exhibits only poor selectivity since all alkylating agents will give positive

results, and an unequivocal identification of HD is only possible if the $R_F$ values are carefully considered. However, as these authors results showed, this reaction is about a factor of 5 more sensitive than that using *o*-dianisidine and cupric acetate.

To our knowledge the iodoplatinate reaction has not been employed for TLC before, although its suitability for HD determination has long been known (Tschugajeff reaction)[7]. Although the details of the reaction pattern are quite complex, an essential step is certainly the formation of elemenal iodine which is most sensitively indicated by the well known reaction with starch. Iodine is at least partially released from the hexaiodoplatinate anion as the HD molecule becomes a ligand. Of course, all reagents that lead to the formation of elemental iodine will perturb this method. However, as our results demonstrate, there are very few such compounds amongst the chemically similar species formed in the course of HD hydrolysis. Surprisingly, most of the other materials studied (irritants, pesticides) are inactive, which makes this method rather selective. A serious disadvantage, however, is that the reagents are expensive and, more importantly, not stable and have to be prepared immediately before use. Furthermore, the persistence of the TLC spots is poor.

The other quite promising method to identify HD is based on a reaction with Michler's ketone. This has only been reported before as a drop reaction[8]; its applicability to TLC is one of the key results of this work. It is superior to the DB 3 method, and it competes very well with the iodoplatinate or the thymolphthalein method as far as sensitivity and selectivity are concerned. Excellent results are obtained if high-performance TLC chromatoplates are used, about 10 ng HD being detectable. Again, the underlying chemistry must be very complex. Supposedly, the cyclic dialkylsulphonium ion (into which HD is easily transformed) attacks the base N atom(s) of the dimethylamino groups of Michler's ketone, thereby forming a sort of diphenylmethane dye molecule. Addition of mercury may be essential in order to achieve an auxochromic effect.

Much clearer is the chemistry of the photochemical reaction using silver ions. This method has also not been applied to TLC before, although it is sensitive and extremely simple, and a similar reagent has been employed for the detection of some pesticides[9]. Since it essentially takes place in aqueous solution, the β-activated chlorine atom(s) can react with $Ag^+$ to form a precipitate of silver chloride, which then suffers photochemical decomposition into chlorine and metallic silver. Accordingly, this reaction should also be given by all those organic halides (except fluorides) that have a high dissociation constant, yielding the respective halogen atom(s). Surprisingly, however, few perturbations arise from the other compounds investigated, the most important being the reaction with nitrogen mustard which proceeds in exactly the same fashion as with HD.

As far as the triphenylmethane dye–silver nitrate reaction is concerned, again quite complex chemical processes are involved. In previous work concerning the detection of HD in solution, the formation of at least five different coloured species was established[10]. The yellow or red colour of the product species (which is believed to consist of an alkylated triphenylmethane dye molecule) very likely arises from an extended system of conjugated double bonds in which, for some (as yet unknown) reasons, the HD molecule has a strong auxochromic effect. However, despite its utility in solution analytical chemistry, its complexity and poor reproducibility make this dye reaction unsuitable for routine TLC determination of HD.

On the other hand, this is not true for the detection of HD via selenium dioxide, wich is the other important result of this work. In the monograph of Stahl[11] the reaction is listed among others for the detection of active organic halides. It had not been employed for the determination of HD. Chemically, the nucleophilic central S atom of the HD molecule can be oxidized to sulphoxide compounds. Apparently, selenium dioxide is a very selective oxidizing agent which is in turn reduced to elementary selenium with its characteristic red or orange colour.

## CONCLUSIONS

We have demonstrated that several analytical methods, previously confined to solution or drop reaction methods, can be favourably employed in TLC. Specifically, reactions involving hexaiodoplatinate, Michler's ketone, silver nitrate, triphenylmethane dyes and selenium dioxide have been investigated as regards their sensitivity and selectivity. Except for the triphenylmethane dye, all the reactions listed gave good analytical results and point to the suitability and versatility of thin-layer chromatography. A summary of our results is given in Table I.

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## Note

# Thin-layer chromatographic separation and spectra of oxygenated benzo[*a*]pyrene derivatives in dichloromethane

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Benzo[*a*]pyrene (B*a*P), a polycyclic aromatic hydrocarbon found in our environment, induces tumors in experimental animals and is implicated in human cancer. B*a*P requires metabolic activation for carcinogenicity and produces oxygenated derivatives[1]. We describe a thin-layer chromatographic (TLC) method as a substitute for expensive high-performance liquid chromatography[7] and time-consuming paper chromatography methods[2,3]. This TLC method separates B*a*P-7,8-dihydrodiol, the precursor of B*a*P-7,8-dihydrodiol 9,10-epoxide (BPDE), which is the ultimate carcinogen of B*a*P[4], from other oxygenated B*a*P derivatives. We also discuss then use of dichloromethane, which extracts smaller amounts of interfering substances from TLC plates and thereby facilitates mass spectrometric examination of the metabolites.

B*a*P (Aldrich, Milwaukee, WI, U.S.A.) was crystallized[5,6]. Oxygenated B*a*P derivatives (through Dr. David G. Longfellow, NCI Chemical Repository of the IIT Research Institute, Chicago, IL, U.S.A.) were used as received.

Milligram amounts of the substances were dissolved in 10 ml of dichloromethane. B*a*P-*cis*- and *trans*-4,5-dihydrodiols required 30 ml of warm solvent, while B*a*P-7,*trans*-8,9-triol called for 100 ml. These solutions were then diluted (Table I) and the UV spectra were recorded between 410 and 250 nm with a Cary 15 UV–VIS spectrophotometer.

Silica gel plates (UV-254, Brinkman Instruments, Westbury, NY, U.S.A.) were used in an ascending direction in Shandon tanks. The synthetic samples were applied either as spots (singles) or as 3-cm wide streaks (mixtures). They were dried (nitrogen) and developed in the respective solvents for at least 1 h (Table II). The fluorescence of wet plates and the $R_F$ values of the substances were noted under both 360-nm and 254-nm UV lamp. B*a*P-4,5-dihydrodiols do not fluoresce under 360 nm, but rather appear as dark spots under a 254-nm UV-lamp. When the same plate was run in more than one solvent it was dried in a vacuum oven at room temperature for 15 min and then developed in the next solvent.

For recovery, UV absorbance of *trans*-B*a*P-4,5-dihydrodiol (highly polar) was recorded. The solution was concentrated, applied to a 1-cm area, dried, scraped off, extracted with dichloromethane and concentrated. The spectrum was then recorded to determine recovery.

For mass spectral determination, an AEI MS-9 instrument with a direct inlet probe at 220°C was used.

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TABLE I

UV SPECTRA OF OXYGENATED BENZO[*a*]PYRENE DERIVATIVES

| *Compound (μg/ml dichloromethane with which the spectra were taken)* | *Wavelength (nm) (absorbance)* |
|---|---|
| Benzo[*a*]pyrene (B*a*P) (1.36) | 280 (0.11), 287 (0.189), 293 (0.12), 299 (0.26), 310 (0.005), 350 (0.06), 357 (0.05), 367 (0.118), 376 (0.065), 380 (0.1), 387 (0.14), 400 (0.009), 404 (0.02). 11.14 μg/ml at 385 nm. |
| *Phenols* | |
| 1-OH-B*a*P (1.19) | 265 (0.175), 269 (0.19), 282 (0.105), 289 (0.158), 294 (0.128), 300 (0.2), 320 (0.015), 350 (0.03), 378 (0.11), 384 (0.1), 388 (0.11), 398 (0.119), 410 (0.035) 10 μg/ml at 398 nm. |
| 2-OH-B*a*P (1.28) | 274 (0.191), 283 (0.16), 289 (0.178), 299 (0.122), 305 (0.138), 325 (0.021), 353 (0.068), 360 (0.061), 371 (0.111), 379 (0.078), 383 (0.09), 391 (0.108), 405 (0.02), 410 (0.03) 11.85 μg/ml at 391 nm. |
| 3-OH-B*a*P (1.0) | 265 (0.13), 270 (0.149), 278 (0.059), 280 (0.062), 293 (0.105), 300 (0.078), 307 (0.145), 320 (0.008), 363 (0.065), 370 (0.05), 380 (0.1), 390 (0.041), 397 (0.07), 410 (0.021) 14.28 μg/ml at 397 nm. |
| 4-OH-B*a*P (1.05) | 269 (0.295), 280 (0.138), 290 (0.152), 295 (0.142), 302 (0.18), 310 (0.038), 336 (0.035), 345 (0.04), 355 (0.08), 360 (0.08), 373 (0.118), 380 (0.1), 387 (0.105), 395 (0.102), 405 (0.05), 410 (0.055) 10.29 μg/ml at 395 nm. |
| 5-OH-B*a*P (1.0) | 267 (0.195), 275 (0.12), 277 (0.125), 284 (0.105), 291 (0.165), 296 (0.125), 304 (0.185), 315 (0.02), 355 (0.058), 367 (0.07), 376 (0.091), 390 (0.078), 396 (0.088), 403 (0.04), 407 (0.092) 11.36 μg/ml at 396 nm. |
| 6-OH-B*a*P (2.36) | 268 (0.295), 276 (0.181), 282 (0.208), 285 (0.199), 293 (0.318), 297 (0.24), 305 (0.41), 315 (0.048), 370 (0.12), 378 (0.138), 388 (0.185), 403 (0.155), 410 (0.171) 12.75 μg/ml at 388 nm. |
| 7-OH-B*a*P (2.0) | 263 (0.422), 269 (0.51), 285 (0.258), 293 (0.43), 297 (0.331), 304 (0.632), 320 (0.04), 358 (0.155), 364 (0.14), 376 (0.288), 387 (0.172), 398 (0.299), 410 (0.07) 6.68 μg/ml at 398 nm. |
| 8-OH-B*a*P (1.57) | 160 (0.372), 265 (0.368), 270 (0.388), 274 (0.386), 278 (0.412), 288 (0.308), 291 (0.321), 303 (0.268), 306 (0.271), 331 (0.045), 336 (0.051), 342 (0.049), 354 (0.129), 359 (0.112), 372 (0.22), 378 (0.141), 391 (0.22), 403 (0.02) 7.13 μg/ml at 391 nm. |
| 9-OH-B*a*P (1.7) | 263 (0.33), 270 (0.422), 276 (0.315), 288 (0.438), 298 (0.239), 304 (0.305), 315 (0.028), 361 (0.172), 367 (0.129), 379 (0.279), 388 (0.1), 393 (0.149), 405 (0.03) 6.09 μg/ml at 379 nm. |
| 10-OH-B*a*P (2.2) | 266 (0.285), 277 (0.175), 280 (0.179), 285 (0.161), 292 (0.295), 297 (0.23), 304 (0.46), 315 (0.045), 335 (0.014), 360 (0.096), 367 (0.088), 379 (0.179), 388 (0.11), 400 (0.181), 405 (0.1) 12.29 μg/ml at 379 nm. |

TABLE I (*continued*)

| *Compound (μg/ml dichloromethane with which the spectra were taken)* | *Wavelength (nm) (absorbance)* |
|---|---|
| 11-OH-BaP (2.3) | 260 (0.385), 269 (0.61), 282 (0.23), 292 (0.32), 297 (0.286), 303 (0.355), 315 (0.05), 330 (0.021), 364 (0.16), 368 (0.138), 377 (0.223), 380 (0.219), 383 (0.242), 388 (0.148), 393 (0.2), 405 (0.045)<br>9.50 μg/ml at 383 nm. |
| 12-OH-BaP (2.82) | 262 (0.351), 269 (0.399), 275 (0.335), 289 (0.462), 293 (0.461), 298 (0.528), 310 (0.1), 320 (0.032), 360 (0.132), 365 (0.13), 377 (0.258), 387 (0.178), 399 (0.265), 405 (0.135)<br>10.93 μg/ml at 377 nm. |
| *Diones* | |
| BaP-4,5-dione (2.73) | 260 (0.451), 267 (0.658), 271 (0.662), 275 (0.8), 286 (0.208), 295 (0.048), 305 (0.05), 312 (0.072), 315 (0.078), 320 (0.096), 327 (0.125), 331 (0.119), 337 (0.14), 348 (0.108), 352 (0.1), 355 (0.05)<br>19.5 μg/ml at 337 nm. |
| BaP-1,6-dione (13.6) | 250 (0.891), 253 (0.93), 257 (0.19), 260 (0.93), 287 (0.192), 290 (0.191), 295 (0.15), 302 (0.162), 317 (0.091), 410 (0.455)<br>83.95 μg/ml at 302 nm. |
| BaP-6,12-dione (3.6) | 275 (0.188), 282 (0.249), 286 (0.241), 292 (0.281), 298 (0.19), 304 (0.25), 316 (0.045), 352 (0.13), 360 (0.119), 369 (0.159), 385 (0.059)<br>22.64 μg/ml at 369 nm. |
| BaP-3,6-dione (3.68) | 250 (0.26), 255 (0.24), 263 (0.211), 266 (0.215), 286 (0.089), 292 (0.091), 298 (0.05), 304 (0.059), 309 (0.058), 317 (0.082), 324 (0.072), 342 (0.105), 370 (0.069)<br>35.04 μg/ml at 345 nm. |
| BaP-7,8-dione (3.27) | 320 (0.222), 338 (0.43), 344 (0.402), 352 (0.515), 365 (0.145)<br>6.34 μg/ml at 352 nm. |
| BaP-11,12-dione (3.48) | 258 (0.378), 276 (0.81), 294 (0.1), 308 (0.071), 350 (0.16), 385 (0.038)<br>21.75 μg/ml at 350 nm. |
| *Dihydrodiols and triol* | |
| *cis*-BaP-4,5-dihydrodiol (1.83) | 260 (0.241), 261 (0.39), 268 (0.38), 275 (0.615), 285 (0.09), 297 (0.061), 300 (0.071), 306 (0.053), 313 (0.071), 320 (0.042), 326 (0.069), 335 (0.01)<br>26.52 μg/ml at 326 nm. |
| *trans*-9,10- (3.12) | 260 (0.155), 272 (0.34), 275 (0.348), 282 (0.455), 287 (0.371),288 (0.378), 297 (0.152), 302 (0.212), 313 (0.058), 318 (0.075), 323 (0.061), 332 (0.151), 338 (0.07), 348 (0.282), 355 (0.035)<br>11.06 μg/ml at 348 nm. |
| *trans*-4,5- (2.79) | 260 (0.455), 262 (0.48), 264 (0.47), 271 (0.741), 290 (0.076), 296 (0.091), 303 (0.079), 308 (0.092), 315 (0.051), 322 (0.09), 330 (0.012)<br>31 μg/ml at 322 nm. |
| *cis*-7,8- (3.71) | 260 (0.465), 269 (0.118), 270 (0.113), 277 (0.111), 284 (0.241), 289 (0.161), 296 (0.325), 309 (0.031), 335 (0.181), 340 (0.165), 351 (0.43), 359 (0.266), 368 (0.577), 380 (0.085), 390 (0.022), 395 (0.035), 400 (0.018)<br>5.37 μg/ml at 368 nm. |
| 7,8,9-Tetrahydrotriol (*ca.* 4) | 265 (0.099), 269 (0.195), 274 (0.1), 281 (0.36), 288 (0.019), 310 (0.035), 316 (0.08), 321 (0.065), 331 (0.21), 337 (0.099), 347 (0.32)<br>Approx. 12.5 μg/ml at 347 nm. |

TABLE II

$R_F$ VALUES

| *Compound* | *Solvent 1: hexane benzene (3:1)* | *Solvent 2: dichloro-methane* | *Solvent 3: dichloromethane–benzene (97:3)* | *Solvent 4: ethyl acetate* | *Solvent 5: dichloromethane ethanol (9:1)* | *Solvent 6: methanol THF (19:1)* |
|---|---|---|---|---|---|---|
| B*a*P | 0.5, 0.52 | 0.87 | 0.88 | | | |
| 1-OH-B*a*P | 0, 0 | 0.34, 0.36, 0.44 | 0.54 | | | |
| 2-OH | 0 | 0.33 | | | | |
| 3-OH | 0, 0 | 0.36, 0.36 | 0.32, 0.44, 0.44, 0.53 | 0.76, 0.76 | | |
| 4-OH | 0 | 0.49 | | | | |
| 5-OH | 0 | 0.40 | | | | |
| 6-OH | 0 | 0.51, 0.54 | 0.68 | | | |
| 7-OH | 0, 0 | 0.39 | 0.36, 0.49, 0.50 | 0.77 | | |
| 8-OH | 0 | 0.25 | | | | |
| 9-OH | 0, 0 | 0.27 | 0.24, 0.34, 0.36 | 0.76 | | |
| 10-OH | 0 | 0.54 | | | | |
| 11-OH | 0 | 0.54 | | | | |
| 12-OH | 0 | 0.40 | | | | |
| *cis*-4,5-Diol | 0, 0 | 0 | 0, 0.01, 0.02 | 0.61, 0.69, 0.72, 0.73 | 0.77, 0.82 | 0.82 |
| *trans*-9,10-Diol | 0, 0 | 0, 0 | 0.006, 0.007, 0.01 | 0.42, 0.44, 0.49, 0.51 | 0.57, 0.63 | 0.83 |
| 7,8,9-Triol | 0 | 0 | | 0.08 | 0.36 | 0.80 |
| *trans*-4,5-Diol | 0 | 0, 0 | 0.015 | 0.71, 0.73 | 0.71, 0.73 | 0.82 |
| *cis*-7,8-Diol | 0, 0 | 0, 0 | 0, 0.015, 0.01, 0.02 | 0.48, 0.51, 0.53, 0.57 | 0.66, 0.72 | 0.80 |
| B*a*P-4,5-quinone | 0 | 0.23 | | 0.85 | | |
| B*a*P-1,6-quinone | 0, 0 | 0.06, 0.12 | 0.06, 0.06, 0.08, 0.09 | 0.70, 0.77, 0.78 | | |
| B*a*P-6,12-quinone | 0, 0 | 0.10, 0.24 | 0.12, 0.14, 0.15, 0.16 | 0.72, 0.83, 0.88 | | |
| B*a*P-3,6-quinone | 0, 0 | 0.04, 0.10 | 0.04, 0.04, 0.05, 0.06 | 0.65, 0.76 | | |
| B*a*P-7,8-quinone | 0 | 0.20 | | 0.87 | | |
| B*a*P-11,12-quinone | 0 | 0.32 | | 0.87 | | |

Table II shows that a sequence of dichloromethane-based solvents separates B*a*P, B*a*P-phenols, the quinones and the dihydrodiols. In solvent 1 (hexane–benzene, 3:1), B*a*P has an $R_F$ of 0.5. The B*a*P-phenols, quinones and the dihydrodiols have zero $R_F$. For large-scale metabolite mixtures, solvent 1 is suitable to remove unchanged B*a*P on silica gel columns. In solvent 2 (dichloromethane) B*a*P is at the solvent front with $R_F$ 0.87, the blue fluorescent phenols have $R_F$ values of 0.25–0.54 and the colored quinone —yellow (4,5-, 1,6-, 6-12-), red (3,6-), violet (7,8-) or pink (11,12-)— have $R_F$ values smaller than the phenols. The dihydrodiols have zero $R_F$ in dichloromethane.

A mixture of 1-OH-, 3-OH-, and 6-OH-B*a*P, B*a*P-1,6-, 3,6- and 6,12-diones, and BP-*trans*-4,5-, -*trans*-9,10-, and -*cis*-7,8-dihydrodiol was run in dichloromethane. 1-OH- and 3-OH-B*a*P did not separate ($R_F$ 0.33). 6-OH-BP separated out at $R_F$ 0.54, and the three quinones formed two spots: (1) red, $R_F$ 0.05 and (2) yellow, $R_F$ 0.15. When this plate was developed in dichloromethane–benzene (97:3), all three quinones separated out. The $R_F$ values were as follows: quinones, 1,6-, 0.09; -3,6-, 0.06; -6,12-, 0.16. 1-OH- and 3-OH-B*a*P still remained mixed. The dihydrodiol moved very little (Table II). In a mixture of 3-OH-, 7-OH- and 9-OH-B*a*P, the quinones and the dihydrodiols and the phenols separated well in dichloromethane–benzene (97:3). $R_F$ values: 3-OH-, 0.44; 7-OH-, 0.52; and 9-OH-, 0.33. Quinone $R_F$ values were as mentioned earlier.

The dihydrodiols were best separated with ethyl acetate. There was further resolution, including separation of *cis*- and *trans*-4,5-dihydrodiols in dichloromethane–ethanol (9:1). In the latter solvent, even the triol moved. B*a*P-7,8-dihydrodiol, the precursor of BPDE, separated out from the other dihydrodiols. $R_F$ values: ethyl acetate, *cis*-4,5, and *trans*-4,5-dihydrodiol as a mixture, 0.72; *trans*-9,10-, 0.51, and *cis*-7,8-, 0.57. Values for the same plate after dichloromethane–ethanol (9:1) were *cis*-4,5-, 0.78; *trans*-4,5-, 0.71; *trans*-9,10-, 0.57, and *cis*-7,8-, 0.66. In methanol–tetrahydrofuran (solvent 6) the diols and the triol had large $R_F$ values. We examined the separation of a mixture (as if from a metabolic experiment) of 185 μg B*a*P with 1.8 μg each of 3-OH-, 7-OH-, 9-OH-, 1,6-, 3,6-, and 6,12-diones and *cis*-4,5-, *trans*-9,10-, and *cis*-7,8-dihydrodiols. In dichloromethane–benzene (97:3), the phenols were a mixture at $R_F$ 0.53, the colored quinones at 0.37, the bluish dihydrodiols at 0.06, and B*a*P at $R_F$ 0.86. The phenols, quinones and the dihydrodiols were extracted with ethyl acetate, dichloromethane and methanol. The solutions were concentrated and separated, as described above. A sequence of solvents 3, 4 and 5 (Table II) separates a mixture of oxygenated B*a*P derivatives.

We recovered 51% B*a*P-*trans*-4,5-dihydrodiol with dichloromethane extraction. Either B*a*P or 3-OH-B*a*P, after a chromatographic run, was extracted with dichloromethane and the mass spectrum determined without interference: B*a*P ($C_{20}H_{12}$, 252.0931) and 3-OH-B*a*P ($C_{20}H_{12}O$, 268.0892). In Table I, UV spectra (maxima–minima) are given to allow reconstruction of the spectra. The last line indicates μg/ml (factor) that produces an absorbance of 1 at the wavelength mentioned. Concentration (total μg) = ml × absorbance × factor.

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## Note

### Amino acids and peptides

### CLXXXI*. Separation of diastereoisomers of oxytocin analogues

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A number of examples of reversed-phase chromatographic separations of diastereoisomeric peptides have been reported[1–5]. The separation of all oxytocin isomers formed by a change in configuration of any one amino acid may be achieved[5].

In this study, the separation of diastereoisomers was investigated in the synthesis of analogues containing an unnatural amino acid in position 2, starting with the racemic derivatives of phenylalanine, and it was examined whether diastereoisomers of oxytocin analogues modified in other ways (in position 1 and in the disulphide bridge) may also be separated.

#### EXPERIMENTAL

The oxytocin analogues were synthetized as described previously[6,7]. Chromatography was carried out on a 25 × 0.4 cm I.D. column packed with Separon SI-C-18 (Laboratory Apparatus, Prague, Czechoslovakia), using an SP-8700 liquid chromatograph equipped with an SP-8400 continuously variable wavelength UV detector and an SP-4100 integrator (all from Spectra-Physics, Santa Clara, CA, U.S.A.). Mixtures of methanol or tetrahydrofuran with aqueous buffers were used as mobile phases.

#### RESULTS AND DISCUSSION

A number of oxytocin analogues, I–XX (Table I) containing an amino acid of L or D configuration in position 2, were prepared. The synthesis of some compounds I–X employed optically inactive aromatic amino acids as starting materials, and in order to obtain pure compounds reversed-phase liquid column chromatography was used. For the determination of which enantiomer of phenylalanine derivative is present in an analogue, a comparison was made with a substance prepared from optically pure amino acid, and enzymatic incubation of the hydrolysate with L-amino acid oxidase was also carried out.

* For Part CLXXX see ref. 6.

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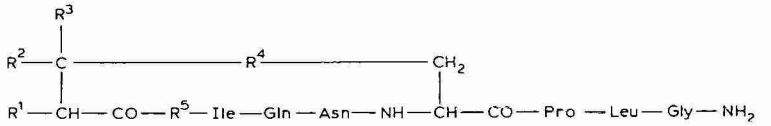
TABLE I

STRUCTURES OF OXYTOCIN ANALOGUES




Et = Ethyl; Me = methyl.

| *Compound* | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ |
|---|---|---|---|---|---|
| I | $NH_2$ | H | H | S–S | L-Phe(Et) |
| II | $NH_2$ | H | H | S–S | D-Phe(Et) |
| III | H | H | H | S–S | L-Phe(Et) |
| IV | H | H | H | S–S | D-Phe(Et) |
| V | $NH_2$ | $CH_3$ | $CH_3$ | S–S | L-Phe(Et) |
| VI | $NH_2$ | $CH_3$ | $CH_3$ | S–S | D-Phe(Et) |
| VII | H | H | H | S–$CH_2$ | L-Phe(Et) |
| VIII | H | H | H | S–$CH_2$ | D-Phe(Et) |
| IX | H | H | H | S–$CH_2$ | L-Phe(Me) |
| X | H | H | H | S–$CH_2$ | D-Phe(Me) |
| XI | H | H | H | S–$CH_2$ | L-Phe |
| XII | H | H | H | S–$CH_2$ | D-Phe |
| XIII | H | H | H | S–$CH_2$ | L-Phe(Cl) |
| XIV | H | H | H | S–$CH_2$ | D-Phe(Cl) |
| XV | H | H | H | S–$CH_2$ | L-Tyr(Et) |
| XVI | H | H | H | S–$CH_2$ | D-Tyr(Et) |
| XVII | H | H | H | S–$CH_2$ | L-Tyr |
| XVIII | H | H | H | S–$CH_2$ | D-Tyr |
| XIX | H | H | H | $CH_2$–S | L-Tyr |
| XX | H | H | H | $CH_2$–S | D-Tyr |

As is evident from Table II, the diastereoisomers were separated in all cases. Almost in all instances the substance containing a D-amino acid was eluted later, indicating its higher hydrophobic interaction with the stationary phase. The analogues containing tyrosine in position 2 are the sole exception. In this case the analogue with a D-tyrosine was eluted earlier when methanol was used as the organic component of the mobile phase, but with respect to the L-tyrosine-containing compound its separation coefficient is very low and only little affected by the composition and the pH value of the buffer used. (For XVII and XVIII the value of $\alpha$ is between 0.93 and 0.96 for buffers of pH 2–8.1.) However, if tetrahydrofuran is used instead of methanol the separation factor is 1.2, while the D-tyrosine-containing compound XVIII is eluted later, in agreement with the chromatographic behaviour[5] of (2-D-tyrosine)oxytocin. Tetrahydrofuran also increased the selectivity of the separation in other cases; for example, compounds IX and X have $\alpha = 1.59$ (21% tetrahydrofuran in the mobile phase) in contrast to $\alpha = 1.39$ obtained when methanol was used.

In the case of analogues VII–XX we also investigated the chromatographic behaviour of the corresponding sulphoxides. The separation factor of the sulphoxides of analogues containing a D- and L-amino acid in position 2 is in most instances not much different from the corresponding value for the pair of analogues in sulphide form, and the order of elution of the D and L diastereoisomers is also unchanged (see

TABLE II

RETENTION DATA OF DIASTEREOISOMERIC PEPTIDES

| *Compound* | *Amino acid in position 2 ($R^5$)* | $k'$ | $\alpha$* | $k'_{so}$** | $\alpha_{so}$** | *Mobile phase*** | |
|---|---|---|---|---|---|---|---|
| I | L-Phe(Et) | 2.09 | 1.74 | | | A | 7:3 |
| II | D-Phe(Et) | 3.65 | | | | | |
| III | L-Phe(Et) | 9.25 | 1.61 | | | B | 13:7 |
| IV | D-Phe(Et) | 14.87 | | | | | |
| V | L-Phe(Et) | 3.05 | 1.96 | | | A | 7:3 |
| VI | D-Phe(Et) | 5.89 | | | | | |
| VII | L-Phe(Et) | 5.18 | 1.44 | 4.32 | 1.40 | B | 7:3 |
| VIII | D-Phe(Et) | 7.48 | | 6.04 | | | |
| IX | L-Phe(Me) | 4.75 | 1.39 | 3.84 | 1.35 | B | 3:2 |
| X | D-Phe(Me) | 6.60 | | 5.18 | | | |
| XI | L-Phe | 3.90 | 1.41 | 3.12 | 1.38 | B | 3:2 |
| XII | D-Phe | 5.51 | | 4.30 | | | |
| XIII | L-Phe(Cl) | 4.45 | 1.39 | 3.67 | 1.42 | B | 13:7 |
| XIV | D-Phe(Cl) | 6.18 | | 5.22 | | | |
| XV | L-Tyr(Et) | 7.03 | 1.30 | 5.48 | 1.15 | B | 11:9 |
| XVI | D-Tyr(Et) | 9.14 | | 6.28 | | | |
| XVII | L-Tyr | 10.44 | 0.95 | 7.56 | 0.63 | B | 2:3 |
| XVIII | D-Tyr | 9.92 | | 4.77 | | | |
| XIX | L-Tyr | 17.8 | 0.98 | 11.4 | 1.02 | B | 2:3 |
| XX | D-Tyr | 17.4 | | 11.6 and 8.25 | or 0.73 | | |

* Ratio of $k'$ for D-amino acid-containing analogue/$k'$ for L-amino acid-containing analogue.
** Value for the corresponding sulphoxide.
*** A, Methanol–0.1 *M* ammonium acetate pH 7; B, methanol–0.05% trifluoroacetic acid.

Table II). However, in compounds XVII and XVIII containing tyrosine the value of the separation factor changed distinctly ($\alpha = 0.63$) on oxidation, while the analogue containing D-tyrosine is eluted earlier. Use was made of this fact in the preparation of analogue XVIII from which even a trace of contamination with compound XVII had to be excluded. The sulphoxide was prepared by oxidation with sodium periodate and purified by liquid chromatography. After reduction with hydrogen bromide and acetone[8,9] and rechromatography, pure analogue XVIII was obtained. The entire reaction sequence, including lyophilization of the product, could be carried out in 1 day. For compounds XIX and XX a similar procedure was employed, even though the situation is complicated here by the fact that the sulphoxide of XX is eluted in two peaks (*R* and *S* configuration of the sulphur atom of the sulphoxide) even when an acidic buffer is used (in contrast to other sulphoxides of the analogues of the carba-1 series*), and that only the more rapidly eluted peak ($\alpha = 0.73$) is utilizable, which decreases the yield of the whole procedure.

* Carba-substitution means replacement of the sulphur atom in the disulfide group by a methylene group. Here, we distinguish carba-1 of carba-6 series: carba-1, if cysteine in sequence position 1 was changed for methylene group (analogues XIX and XX); carba-6 if this replacement was done in position 6 (analogues VI–XVIII).

On the basis of the oxidation rate of individual carba-analogues, attempts had previously been made to infer a better or poorer accessibility of the oxidant to the sulphur atom of the carba-bridge[10,11]. In the case of carba-6 analogues, no interaction of the aromatic residue with the sulphur atom should take place on the basis of earlier findings, and so the rate of oxidation of analogues with a different phenylalanine configuration should be the same. This assumption was checked by following the course of the oxidation of analogues VII and VIII with sodium periodate, and the oxidation rate in each case was found to be the same within the experimental errors.

The possibilities of preparing oxytocin analogues by starting with a racemate of the amino acid in position 2, containing various modifications of the molecule (deamination in position 1, introduction of penicillamine into this position, carba-1 and carba-6 modification of the bridge) have been examined. It was shown than some difficult separations of the carba-analogues can be circumvented by preparing easily reducible sulphoxides.

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## Note

# Separation by high-performance liquid chromatography of two types of subunit from horse spleen ferritin

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Understanding the mechanism by which ferritin stores and mobilizes iron depends upon a thorough understanding of the chemical nature and structural organization of the protein subunits of ferritin. Currently, a major problem which must be solved before the ultimate questions about structure/function can be fully answered is the problem of how many chemically distinct subunits may exist in the 24 equivalent positions[1] of the ferritin quaternary structure. It is generally accepted that ferritins from different tissues can be distinguished electrophoretically. In addition, isoelectric focusing has shown heterogeneity within, as well as among, tissue ferritins and suggests that many tissue ferritins contain common "isoferritins" (reviewed in ref. 2). Based on their observation by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) that two different-sized subunits, designated "H" and "L", existed in different ratios in ferritins from various tissues, Drysdale and coworkers[3,4] suggested that multiple isoferritins represented hybrid molecules composed of different proportions of the two subunit types. The ultimate test of this model will be the separation of two or more different types of ferritin subunit in sufficient amount and purity to allow sequence determination of each.

The recent advances made in the use of high-performance liquid chromatography (HPLC) for protein separation[5] prompted this investigation which represents the first use of HPLC for separating the different types of ferritin subunit. The procedure described affords complete purification of each ferritin subunit type in amounts sufficient for subsequent sequence analysis.

## EXPERIMENTAL

Horse spleen ferritin was obtained from three sources: a twice-crystallized preparation from Miles Labs. (lot 63A), lot No. 1089305 from Boehringer-Mannheim, and a sample prepared in our laboratory according to the procedure of May and Fish[6] from fresh horse spleens.

Ferritin subunits were routinely prepared by performing simultaneous iron reduction and subunit dissociation via treatment of the ferritin with acetic acid–thioglycolic acid and subsequent gel chromatographic exchange into 0.01 *M* glycine, pH 2.85, or 0.1% trifluoroacetic acid (Collawn and Fish, unpublished results). Subunits prepared in this manner behaved by analytical ultracentrifugation and circular di-

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chroism in a similar fashion to subunits prepared from apoferritin by the method of Harrison and Gregory[7].

HPLC was performed on a Varian 5060 liquid chromatograph. A $C_{18}$ reversed-phase column of 300-Å pore size (SynChropak RP-P, 25 cm × 4.1 mm I.D., SynChrom, Linden, IN, U.S.A.) was employed for the protein separation. The column was maintained at a constant temperature of 30°C, and elution was conducted at a solvent flow-rate of 1 ml/min. The solvent systems employed were: solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile. Although a number of different solvent programs resolved the two subunit species, the following program was used for routine preparation of the two subunits: injection of the sample at 100% A, a linear gradient from 0% B to 75% B over 25 min, and an isocratic elution at 75% B for 3 min.

SDS-PAGE was performed on 1.5 mm × 12 cm × 18 cm gels of 15% polyacrylamide in a Bio-Rad apparatus. The discontinuous buffer system of Laemmli[8] was employed. Isoelectric focusing was on pH 3–10 slab gels according to the modifictions of Ames and Nikaido[9] and Lavoie *et al.*[10] for the O'Farrell procedure[11].

## RESULTS

As illustrated by the elution profile shown in Fig. 1, profile a, two chromatographic species were observed when subunits from commercial horse spleen ferritin were subjected to reversed-phase HPLC. When each of the chromatographic species was re-chromatographed using the same solvent-gradient program, it eluted as a single peak at the same binary solvent composition as in its initial elution (*cf.* Fig. 1, profiles b and c). Estimates of the relative proportions of each of the chromato-

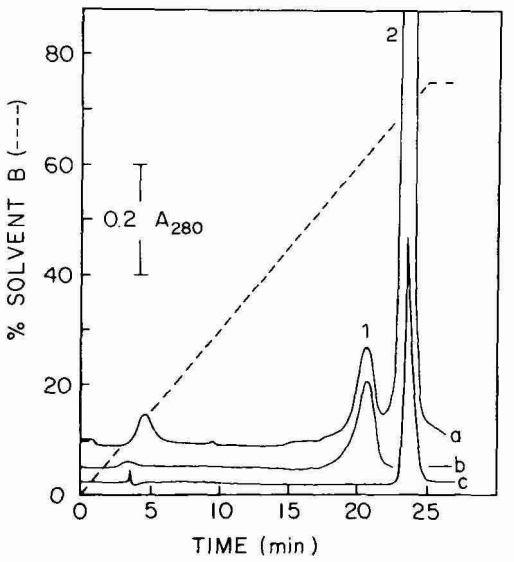


Fig. 1. Reversed-phase HPLC of horse spleen ferritin subunits. Profile a, horse spleen ferritin subunits. Profile b, re-chromatography of peak 1. Profile c, re-chromatography of peak 2. ( ) solvent gradient expressed as %B.

graphic species and of the recovery of protein from each column run indicated that 0.08 mg of protein from peak 1 and 0.69 mg from peak 2 were recovered from each milligram of protein applied to the column. This amounted to a 77% overall recovery of protein from each run. A number of ancillary experiments indicated that most of the protein which remained adsorbed to the $C_{18}$ column was species 2; this protein could be cleared from the column by a wash cycle in which the column was repeatedly washed with gradients of 0–75% solvent B. The same two chromatographic species were obtained when a second source of commercial ferritin (purified by a different procedure) was used. Similarly, subunits from horse spleen ferritin which was prepared from fresh tissue without crystallization[6] yielded the same two HPLC species in the same proportions as observed for the commercial ferritins.

The same two chromatographic species were obtained when horse spleen ferritin was dissociated and the iron reduced by treatment of ferritin in 6 *M* guanidinium chloride plus 1% thioglycolic acid, pH 2.85, before subjection to HPLC. Likewise, subunits which were reduced and carboxymethylated in 6 *M* GdmCl, either prior to HPLC separation or after subjection to gel chromatography in 6 *M* GdmCl, eluted

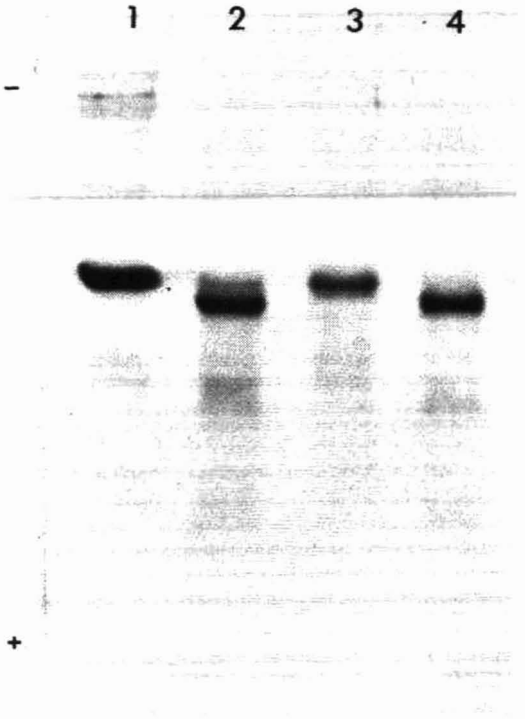


Fig. 2. SDS-PAGE electrophoresis of ferritin subunits separated by HPLC. Samples: 1 = porcine spleen ferritin subunits[6] included for reference; 2 = horse spleen ferritin subunits before chromatography; 3 = HPLC peak 1; 4 = HPLC peak 2.

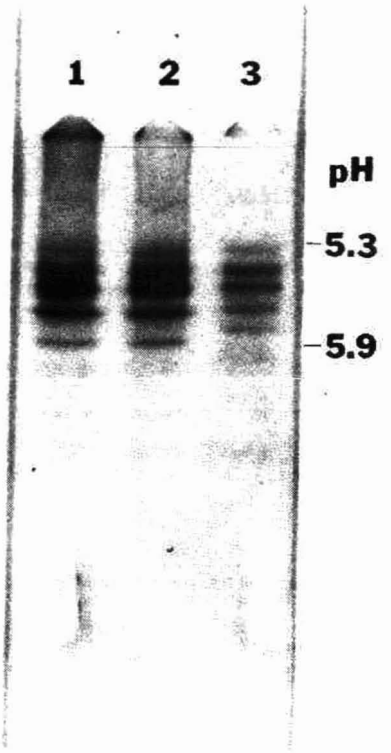


Fig. 3. Isoelectric focusing of ferritin subunits separated by HPLC. Samples: 1 = HPLC peak 2; 2 = horse spleen ferritin subunits before chromatography; 3 = HPLC peak 1.

at their characteristic binary solvent composition upon HPLC. When 2-propanol was employed instead of acetonitrile as the less polar solvent, the same two polypeptides (as identified by SDS-PAGE) were obtained in approximately the same ratio. The preceding three observations argue against one of the two HPLC species being an artifact of the subunit preparation or the elution procedure.

Fig. 2 demonstrates that the two chromatographic species could be differentiated by SDS-PAGE. All other things being equal, the relative electrophoretic mobilities of the two polypeptides suggest that species 1 is slightly larger than species 2 by a molecular weight difference of *ca.* 2000. This was substantiated by gel chromatography of the two polypeptides on Sephacryl S-200 in the presence of 6 *M* GdmCl. By labeling one of the polypeptides with iodo[$^3$H]acetate, the reduced, carboxymethylated derivatives of both species could be compared simultaneously. When so compared, species 1 eluted slightly ahead of species 2. The elution difference corresponded to an apparent molecular weight difference of *ca.* 2000. Since gel chromatography in random-coil-producing solvents is generally less fraught with anomalies than is SDS-PAGE[12], this observation adds considerable credence to the postulated size difference between the two polypeptides first observed by SDS-PAGE[13].

As illustrated in Fig. 3, differences could also be seen between the isoelectric focusing (IEF) patterns of the two polypeptides. Consistent with previous reports for unfractionated horse spleen ferritin subunits[13,14], we observed multiple bands which

TABLE I

AMINO ACID COMPOSITIONS OF HPLC FRACTIONS*

| *Amino acid* | *HPLC No. 1* | *"H" subunit*** | *HPLC No. 2* | *"L" subunit*** | *Sequence**** |
|---|---|---|---|---|---|
| Asx | 27 | 19 | 18 | 16 | 18 |
| Thr | 7 | 7 | 6 | 5 | 6 |
| Ser | 10 | 9 | 10 | 8 | 10 |
| Glx | 29 | 23 | 23 | 22 | 26 |
| Pro | 4 | – | 2 | – | 2 |
| Gly | 10 | 12 | 12 | 9 | 11 |
| Ala | 14 | 15 | 16 | 14 | 15 |
| Val | 6 | 8 | 9 | 7 | 8 |
| Met | 3 | 3 | 3 | 3 | 3 |
| Ile | 6 | 6 | 4 | 3 | 4 |
| Leu | 22 | 20 | 28 | 25 | 28 |
| Tyr | 6 | 6 | 6 | 6 | 6 |
| Phe | 8 | 7 | 8 | 7 | 8 |
| Lys | 13 | 11 | 9 | 9 | 9 |
| His | 9 | 6 | 6 | 4 | 6 |
| Arg | 7 | 9 | 12 | 9 | 11 |

* Trp and ½Cys were not quantitated.
** Ref. 13.
*** Ref. 5.

focused between pH 5.0 and pH 5.9. Though each species from the HPLC separation exhibited multiple IEF components, those components of HPLC species 1 as a whole appeared slightly more acidic than those of HPLC species 2. Multiple components were also observed for horse ferritin "H" and "L" subunits[13].

Finally, Table I presents the amino acid compositions of HPLC species 1 and 2. Also included for comparison are the amino acid compositions of "H" and "L" subunits from horse ferritin[13] as well as the composition of horse spleen ferritin based on its amino acid sequence[15]. Two points are worth mentioning in regard to a comparison of these data. First, with the exception of Asx, Leu, Pro, and the basic amino acids, the compositions of HPLC species 1 and 2 are indistinguishable. Second, the amino acid composition of HPLC species 1 more closely resembles that of "H" subunit while the amino acid composition of HPLC species 2 is more like those published for "L" subunit and the amino acid sequence for horse spleen ferritin subunit.

## DISCUSSION

The major point to be defended in this report is whether the minor component isolated by HPLC is truly an apoferritin subunit and not just a minor contaminant which co-purifies with ferritin. Though the evidence presented herein cannot resolve this question unequivocally, it and a substantial amount of information from other laboratories strongly support our contention that this polypeptide is indeed a ferritin subunit. First, HPLC-species 1 is found in horse spleen ferritin preparations which have been purified in three different ways. These include separations by size, charge, and repeated crystallization. It is highly unlikely that the same protein would survive so many different types of discriminatory procedures always to appear in the same ratio with the major apoferritin subunit species. Second, the chemical and physical properties of HPLC species 1 are quite similar to those of the major component,

HPLC species 2, which suggests that the two are closely related. Third, there is, of course, evidence from other laboratories that horse spleen ferritin has two types of subunit which can be differentiated by SDS-PAGE[13]. Additionally, earlier investigations led by Drysdale's and Listowsky's laboratories have shown that the ratios of the two peptide species vary from one type of tissue to the next[10,13]. In fact, ferritin "H" and "L" subunit types have been observed in various ratios from different tissues for a variety of animal species (reviewed in ref. 2). In this regard, we have recently observed that porcine ferritin subunits can also be separated into more than one chemically distinct species by reversed-phase HPLC[16].

If one accepts the premise that both HPLC fractions are indeed ferritin subunits, it then becomes tempting to speculate that HPLC species 1 corresponds to the "H" subunit and HPLC species 2 corresponds to the "L" subunit. This correlation is based on their relative behaviors on SDS-PAGE (Fig. 2), on their behaviors during gel chromatography in 6 *M* GdmCl, and on their amino acid compositions (Table I).

To our knowledge, the results presented herein represent the first report of the application of reversed-phase HPLC to separate different forms of apoferritin subunits in analytical or preparative amounts. The only other preparative means presented to date has been the use of electrochromatography by Otsuka and Listowsky[17] to separate limited quantities of "H" and "L" subunits. Certainly, reversed-phase HPLC represents an important tool for the study of ferritin structure and function. It offers the investigator the ability to separate completely milligram amounts of (at least) two different forms of ferritin subunits so that the actual chemical differences between the subunits species may then be elucidated.

ACKNOWLEDGEMENTS

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## Note

# Quantitative high-performance liquid chromatographic analysis of the bitter quassinoid compounds from *Simaruba glauca* seeds

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(Received March 18th, 1983)

For several years, the "Centre d'Informatique Appliquée au Développement et à l'Agriculture Tropicale" (CIDAT, Tervuren, Belgium) has helped the Republic of Burundi to combat desertification by transplanting the tree *Simaruba glauca* in some regions of the country. The seeds of *S. glauca* are also very rich in edible fat, up to 60% (w/w)[1,2].

This edible fat is used for cooking in tropical countries[1,3]. The cake from oil extraction is rich in proteins[1,2] but cannot be used for cattle feed unless the toxic and bitter compounds are removed. The most efficient and least expensive method of removal is extraction with hot water[2], improved by use of 0.01 *N* acetic acid for the third extraction step[3].

The major compounds extracted from the seeds of *S. glauca* are glaucarubine[3–5] and glaucarubinone, which is derived from glaucarubine by oxidation (Fig. 1)[6].

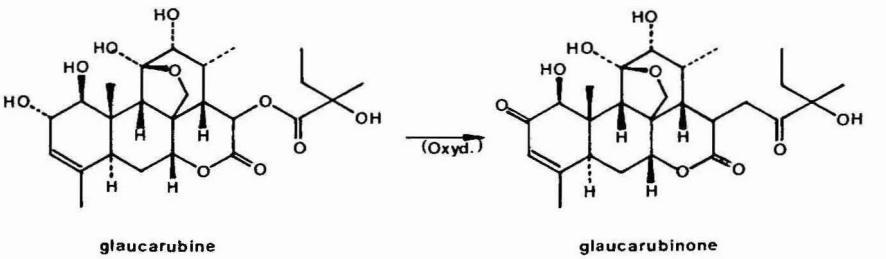


Fig. 1. Oxidation of glaucarubine. Oxidation with $MnO_2$ gives glaucarubinone in 50% yield[7].

As well as edible fat[7], the seeds contain amoebicide molecules[8] that are used in tropical countries. Glaucarubinone has also been claimed to exhibit antileukaemic activity[9].

In this work, an analytical method to detect traces of the toxic and bitter quassinoids was developed. The most efficient analysis technique was shown to be an aqueous extraction followed by reversed-phase high-performance liquid chromatography for the quantitation of these natural products.

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## EXPERIMENTAL

### *Products*

The seeds of *S. glauca* were imported from Burundi. Other sources of Simarubaceae were obtained from Costa Rica, Belize and Senegal. The bitter compounds, glaucarubine and glaucarubinone, were extracted and crystallized until they were chromatographically pure. The ultraviolet, infrared and mass spectral characteristics of these compounds were found to be identical with those given in the literature[3–6].

Pure methanol was purchased from SBA Chimie (Belgium) and filtered on a 0.45-$\mu$m Millipore filter before use. Water was distilled twice and then filtered on a Norganic system from Millipore.

### *Instrumentation*

The modular high-performance liquid chromatographic system used was equipped with a 6000 A pump, a U6K injector and a R401 refractometer, all from Waters Assoc.

The column used was a R-Sil $C_{18}$ HL/D stainless-steel column (250 × 4.7 mm I.D., particle size 10 $\mu$m) modified following Verzele and Dewaele[10] and purchased from Altech Europe. The results are recorded on a HP 3388 A terminal from Hewlett-Packard.

### *Calibration*

Fig. 2 shows the calibration curves obtained by diluting 50 mg of each compound in 20 ml of methanol (2.5 $\mu$g/$\mu$l), and adding successively 80, 60, 50, 40, 30, 20 and 10 $\mu$l of these alcoholic solutions to 10 $\mu$l of internal standard (phenylacetone). The mobile phase was added to obtain a final volume of 100 $\mu$l in each case.

## RESULTS AND DISCUSSION

The mobile phase was selected in order to obtain good selectivity in a relatively short time. The best isocratic separation was obtained with water–methanol (52.5:47.5) as the mobile phase. Other compositions were tested but gave inferior results. The flow-rate was 0.7 ml/min. A plot of capacity factors *versus* the composition of the mobile phase is shown in Fig. 3, which reveals that glaucarubine and glaucarubinone both have a very high capacity factor in water and a very low one in methanol. Aqueous extraction enabled us to use this fact to develop an analytical method based on preconcentration $C_{18}$ cartridges.

In practice, the aqueous fractions obtained from the extraction steps were filtered and cooled to 20°C. Then an aliquot was filtered on a preconditioned $C_{18}$ cartridge and washed twice with water. The bitter compounds were eluted from the cartridge with methanol, which can either be used directly or concentrated to the volume necessary for preparation of the injection volume. The recovery of both compounds was quantitative. The detection limit was 0.3 $\mu$g.

This time-saving method has the following advantages: a lower detection limit (a ratio of 100 was observed), better protection of the column, and a small amount of seeds required even when the content of bitter compounds is low.

Fig. 4 shows a typical chromatogram of a hot-water extraction of *S. glauca*

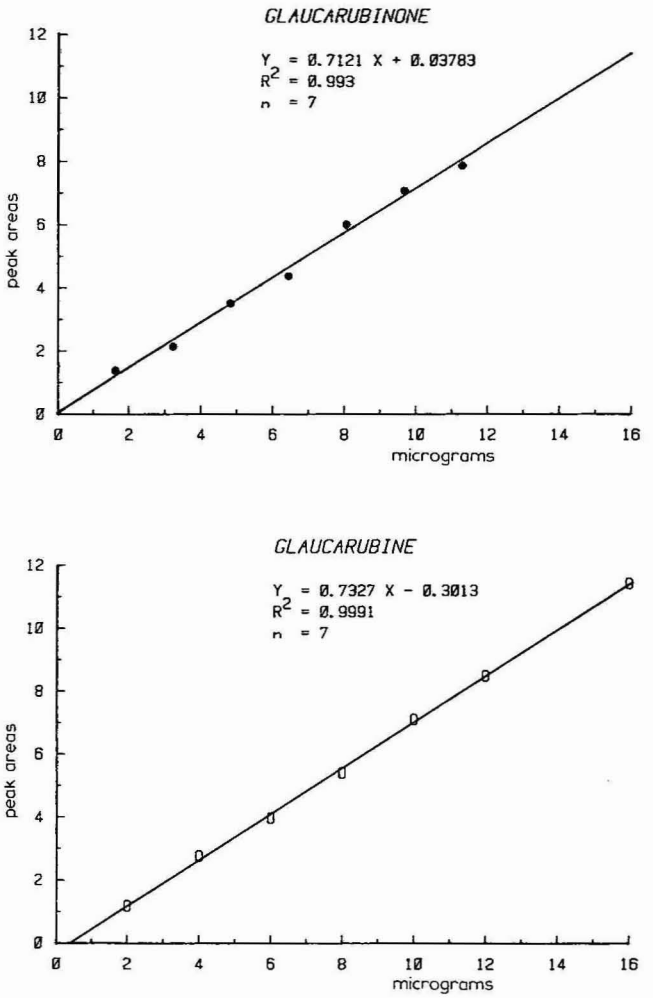


Fig. 2. Calibration curves used for quantitative determination of the concentration of the glaucarubine (○) and glaucarubinone (●) in crude water extracts. The peak areas are linearly correlated with the amount of injected quassinoid.

seeds with water–methanol (52.5:47.5) as mobile phase. In order to quantify the concentration of the quassinoid derivatives, calibration curves were made and, as shown in Fig. 2, very good linear correlations were found between the peak areas and the amount of the bitter compounds injected. The standard deviation was 0.15. Table I lists the amounts extracts with each successive extraction.

The preconcentration $C_{18}$ cartridges were used to analyse the glauca content of some crops received from Belize, Costa Rica, Senegal and Burundi. The results are shown in Table II, which reveals that the concentrations of glaucarubine and glaucarubinone vary with the origin of the crop. The sample from Senegal was a crop from *Hannoa undulata*, and besides the very low content of glaucarubine and glaucarubinone, it is rich in undulatone and ailanthinone as described by Wani[11]. The un-

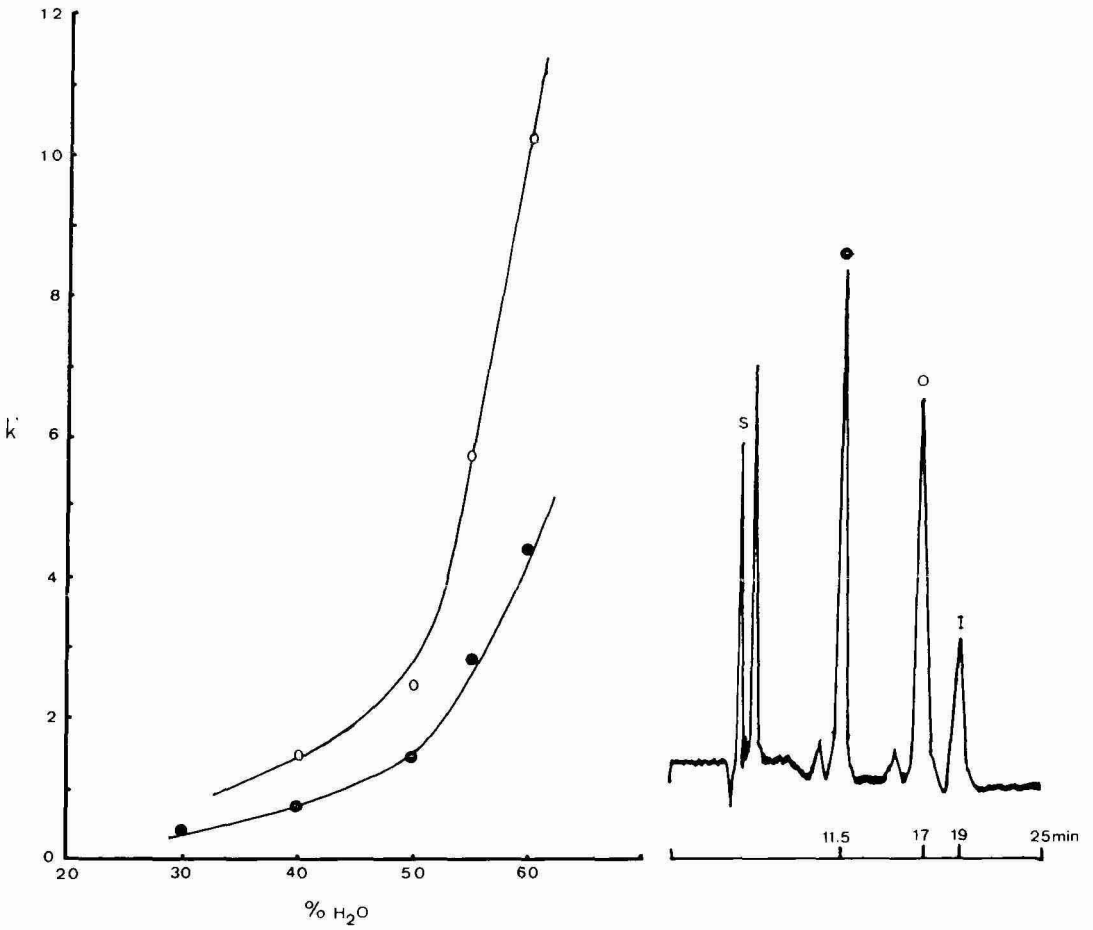


Fig. 3. Capacity factors ($k'$) of glaucarubine (○) and glaucarubinone (●) as a function of the amount of water in the mobile phase.

Fig. 4. Typical separation of the hot-water extraction of the *S. glauca* seeds using water–methanol (52.5–47.5) as mobile phase. Injection of 10 $\mu$l of filtered crude water extracts. Flow-rate, 0.7 ml/min. Peaks: S = solvent; ● = glaucarubinone; ○ = glaucarubine; I = internal standard (phenylacetone).

TABLE I

GLAUCA CONTENTS OF *SIMARUBA GLAUCA* SEEDS FROM BURUNDI

The values, which are averages from three determinations, are given following the successive extractions. The amounts, calculated from the calibration curves shown in Fig. 2, are reported in grams per 100 g dry cakes from oil extraction.

| *Compound* | *Amount extracted (g/100 g)* | | | |
|---|---|---|---|---|
| | *Step 1* | *Step 2* | *Step 3* | *Total* |
| Glaucarubinone | 0.932 | 0.177 | 0.01 | 1.119 |
| Glaucarubine | 1.112 | 0.305 | 0.005 | 1.422 |

TABLE II

AMOUNTS OF GLAUCARUBINE AND GLAUCARUBINONE IN SIMARUBACEAE FROM VARIOUS SOURCES

The amounts of glaucarubine (A) and glaucarubinone (B) given are those found in crude water extracts following the method based on the preconcentration with $C_{18}$ cartridges. Results, reported in g/100 g dry cakes from oil extraction, are given following the successive extractions.

| *Sample* | *Origin* | *Quassinoid* | *Amount extracted (g/100 g)* | | |
|---|---|---|---|---|---|
| | | | *Step 1* | *Step 2* | *Step 3* |
| SCR 1 | Costa Rica | A | 0.2 | – | – |
| | | B | 0.63 | 0.039 | $257.5 \cdot 10^{6}$ |
| SCR 2 | Costa Rica | A | 1.081 | 0.037 | $76.3 \cdot 10^{-6}$ |
| | | B | 0.988 | 0.021 | $46.3 \cdot 10^{-6}$ |
| SCR 4 | Costa Rica | A | 0.974 | – | – |
| | | B | 0.884 | – | – |
| HS | Senegal | A | 0.126 | – | – |
| | | B | 0.055 | – | – |
| B5 | Belize | A | 1.596 | 0.407 | 0.041 |
| | | B | 1.45 | 0.34 | 0.03 |

known compounds found in the sample SCR 4 from Costa Rica are under investigation and will be reported later.

The analytical method described can identify and quantify the toxic and bitter compounds present in the seeds before their use as a protein source for cattle feed. The analysis and the quantification of these compounds become easier and the detection limit is increased by at least two orders of magnitude by the use of preconcentration $C_{18}$ cartridges.

ACKNOWLEDGEMENTS

We thank Mr. Ergo[12] (CIDAT), Mr. Severin and Mr. Lognay (Faculté Agronomique de Gembloux) for providing us with seeds and cakes from oil extraction.

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## Note

# Determination of sucrose, glucose and fructose in plant tissue by high-performance liquid chromatography

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Increased interest in use of crop biomass for energy has created additional demand for a rapid method to analyse for the common sugars in crop plants, mainly sucrose, glucose and fructose. The method should include capabilities to qualitate and quantify for the sugars and involve a minimum of sample preparation due to large numbers of samples that are often produced from a experiment.

Use of high-performance liquid chromatography (HPLC) is gaining wide acceptance for carbohydrate studies. Development and subsequent use of the silica column[1–7], with various modifications, has greatly increased the ability to detect many of the carbohydrates and their derivatives. Silica columns have also been particularly applicable to resolution of sugars such as the disaccharides. Insufficient resolution of glucose from fructose for quantification is sometimes experienced, however[1]. The cation-exchange resin columns[2,8–13] have further enhanced HPLC analysis of various sugar mediums. Care must be exercised to protect the column[5] with either precolumns or sample pretreatment when large numbers of samples containing contaminants are to be analysed.

This paper describes a rapid procedure to extract, and quantify for sucrose, glucose and fructose in oven-dried ground plant tissue using a cation-exchange resin column ($Ca^{2+}$) and protective precolumns.

## EXPERIMENTAL*

The HPLC equipment used in this study was a Beckman system (Beckman Instruments, Berkeley, CA, U.S.A.) consisting of a Model 421 microprocessor, Model 110A pump, Model 210 sample injector, Model 156 refractive index (RI) detector set at attenuation × 16, and a Scientific Systems (State College, PA, U.S.A.) CH 20-C column heater. Output was recorded on a Hewlett-Packard (HP; Avondale, PA, U.S.A.) 3390 A reporting integrator equipped with an input/output board. An electrical contact fitted on the injector was connected through the microprocessor to

* Product and company names are included for the benefit of the reader and not to imply endorsement or preferential treatment of the product by the Texas Agricultural Experiment Station, College Station, TX, U.S.A.

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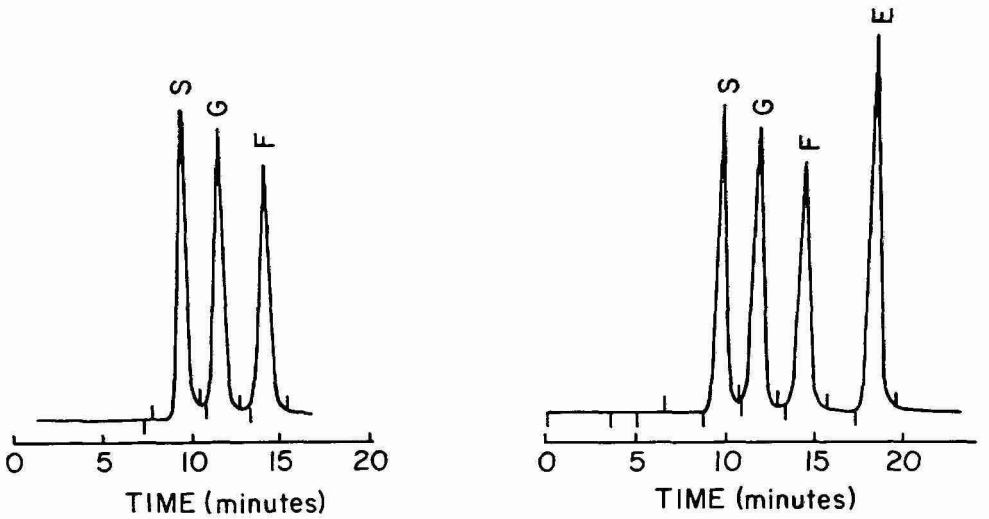


Fig. 1. Example of chromatogram for standard solution of 0.125% sucrose (S), glucose (G), and fructose (F) prepared in water and used for some tissue analyses. Attenuation for RI detector, × 16. Attenuation and threshold for integrator, ×4 and 3, respectively; chart speed, 0.3 cm/min; peak width, 0.16; area reject, 5. Flow-rate, 0.6 ml/min. Eluent, water. Column temperature, 85°C. Sample size, 20 μl. Column, Aminex HPX-87. Precolumns, Aminex HPX-85H, A-25.

Fig. 2. Chromatogram of standard solution of 0.5% sucrose; glucose; fructose and 2% ethanol (E). Attenuation, × 6; threshold, 4 for integrator. See Fig. 1 for other details.

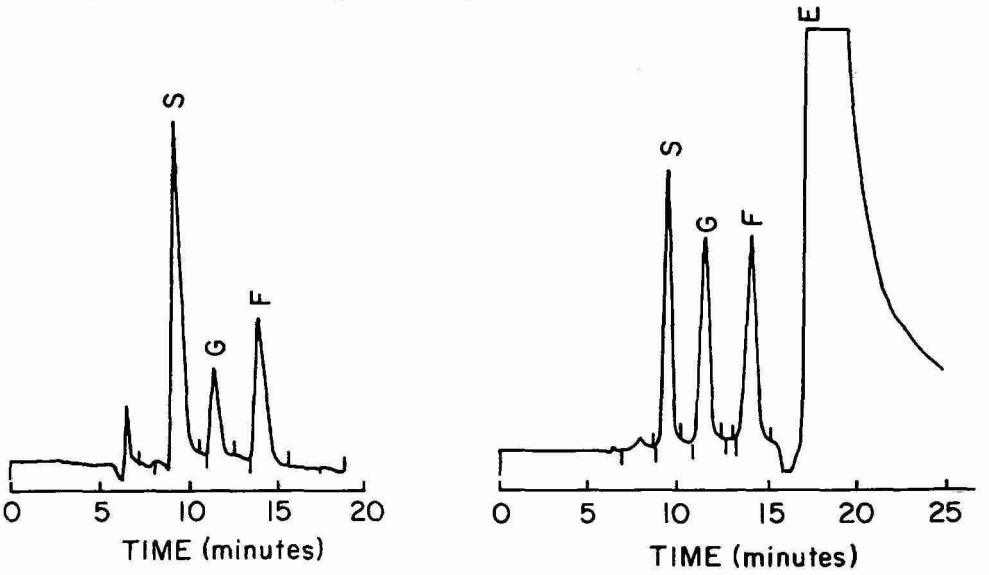


Fig. 3. Typical chromatogram showing quantitative amounts of sucrose, glucose and fructose in low-sugar-type cultivary (37) extracted with warm water (1 g/100 ml). Attenuation, × 2; threshold, 2 for integrator. See Fig. 1 for other details.

Fig. 4. Typical chromatogram for low-sugar-type cultivar (37) with sugars (Table I) added to plant sample before extraction in boiling 95% ethanol (1 g/100 ml). Attenuation, × 3; threshold, 3. See Fig. 1 for other details.

the HP integrator so that the microprocessor and integrator were both started upon injection of the sample. A precolumn arrangement consisted of an in-line series of two precolumn holders (Micro-Guard; Bio-Rad Labs., Richmond, CA, U.S.A.) containing a cation- and anion-exclusion cartridge (Aminex HPX-85H, A-25; Bio-Rad Labs.) in respective sequence and connected to the injector. The precolumns were followed by an HPX-87 carbohydrate column (Bio-Rad Labs., 300 × 7.8 mm I.D.) placed in the column heater. The column heater was maintained at 85°C and degassed water was used as the eluent at a flow-rate of 0.6 ml/min.

Culms from two sorghum cultivars, ATx378 × RTx7000 (31) and ATx623 × R74CS5388 (65), were used which contained relatively low and moderate concentrations of sugar, respectively. A 1-g amount of oven-dried tissue, ground to pass a 1-mm screen, was refluxed for 30 min with boiling 95% ethanol in 50-ml centrifuge tubes with stoppers and fitted capillaries[14]. They were centrifuged after each extraction and the supernatant saved. The process was repeated three times and the collected supernatant was brought to 100-ml volume. Duplicate sets of the plant material were also extracted in deionized, distilled water using the same method with exclusion of the capillaries. The bath for water extraction was set at 60°C and samples extracted for 5 min with periodic stirring. A minor trace of the sugars could only be detected in ethanol extracts and none in water subsequent to four extractions of tissue for the HPLC conditions described. These procedures allow for extraction of a minimum of 32 samples within 4–6-h period.

Three variations of sample preparation were used: (1) sample extracted and pooled extracts brought to 100 ml with extracting solution, (2) sample extracted, measured amount of standard sugar solution (sucrose, glucose, fructose) added to the pooled extractions and resulting solution brought to 100 ml, and (3) prior to extractions, measured amount of standard sugar solution added to oven-dried material and then pooled extracts brought to 100 ml. Subsamples from the extractions

TABLE I

CONCENTRATION OF SUGARS IN CULMS OF TWO SORGHUM CULTIVARS AND PERCENT RECOVERY OF ADDED SUGARS

Refer to legends of Fig. 1, 3 and 4 and Experimental section for conditions. Values are the means ± S.E. of 4 replications and duplicated chromatograms.

| *Cultivar* | *Method of extraction* | *Sucrose* | | | |
|---|---|---|---|---|---|
| | | *Initial concn. (mg/g)* | *Added (mg/ 100 ml)* | *Recovery (%)** | |
| | | | | *Var. 2* | *Var. 3* |
| Low-sugar type | Water | 34.06 ± 0.54 | 12.50 | 103.02 ± 1.41 | 101.44 ± 1.11 |
| | Ethanol | 29.30 ± 0.26 | 12.50 | 99.30 ± 0.89 | 100.60 ± 2.86 |
| Moderate-sugar type | Water | 149.61 ± 0.62 | 12.50 | 99.12 ± 0.76 | 99.47 ± 0.70 |
| | Ethanol | 149.08 ± 0.38 | 12.50 | 100.40 ± 1.21 | 101.98 ± 0.09 |

* Var. 2: standard sugar solution added to extract and brought to volume; Var. 3: standard sugar solution added to oven-dried, ground plant material, extracted and then brought to volume.

were filtered through a 5-$\mu$m filter prior to chromatography. A 20-$\mu$l injector loop was used to insure precise sample size. All samples were replicated four times and the HPLC runs were duplicated. A silica-based column (Bio-Sil Amino 5S, Bio-Rad Labs.) was initially used to check the plant extractions to ascertain that the sucrose peak was pure and did not contain maltose.

The external standard method (ESTD) of quantification was used for the integrator. Standards for the determinations were prepared from reagent grade sugars. Attenuation and threshold values were varied on the integrator to accommodate the different levels of saccharides. The integrator was programmed so that results were recorded (mg sugar/g oven-dried material) immediately after completing resolution of the sugars for water extracts and upon elution of ethanol for the ethanol extracts.

## RESULTS AND DISCUSSION

Values shown in Table I indicate that HPLC can be effectively used to quantify for various mono- and disaccharides in dried, ground plant tissue. The method of extraction is critical because water will remove some of the starch, particularly amylose. Extraction with boiling ethanol has the advantage of separating starch from the sugars of interest. An advantage in use of water is reduction of elution time. Generally, samples extracted in water were eluted and the column equilibrated in approximately 17 min whereas samples extracted in ethanol required approximately 40 min.

A typical chromatogram for the standards is shown in Fig. 1. Standards were rerun and the integrator recalibrated at least twice before analysing and quantifying for the tissue samples. The chromatogram shown in Fig. 2 illustrates this method may also be used to evaluate fermentation broth and potential ethanol production. Values for the extracted oven-dried material without sugar additions and percent

| *Glucose* | | | | *Fructose* | | | |
|---|---|---|---|---|---|---|---|
| *Initial concn. (mg g)* | *Added (mg/ 100 ml)* | *Recovery (%)** | | *Initial concn. (mg/g)* | *Added (mg/ 100 ml)* | *Recovery (%)** | |
| | | *Var. 2* | *Var. 3* | | | *Var. 2* | *Var. 3* |
| 10.13 ± 0.54 | 31.25 | 101.36 ± 0.26 | 101.94 ± 4.53 | 18.83 ± 0.42 | 31.25 | 102.14 ± 3.84 | 98.54 ± 2.45 |
| 8.32 ± 0.29 | 31.25 | 102.50 ± 5.80 | 92.00 ± 6.14 | 15.66 ± 0.55 | 31.25 | 98.70 ± 4.64 | 102.08 ± 3.46 |
| 19.07 ± 0.18 | 31.25 | 102.99 ± 1.63 | 101.36 ± 3.73 | 26.84 ± 0.23 | 31.25 | 95.19 ± 1.66 | 98.16 ± 1.07 |
| 17.32 ± 0.31 | 31.25 | 103.05 ± 1.90 | 98.68 ± 2.53 | 26.26 ± 0.46 | 31.25 | 95.40 ± 2.46 | 94.25 ± 2.73 |

recovery for both methods of sugar addition are given in Table I. An example of a chromatogram obtained for extraction of the material with water is shown in Fig. 3 and with ethanol in Fig. 4. Sample size for extraction is critical and must be initially determined from experience or by trial. Since 50-ml centrifuge tubes were used for extraction, weights exceeding 1 g of oven-dried material resulted in failure to extract all the sugars from the samples. Weights less than 1 g could have been used for the cultivar with the moderate sugar concentration, but this quantity was necessary to obtain sufficient glucose and fructose concentrations from the low sugar type. An attenuation value of 2 for the integrator with the respective setting of × 16 for the RI detector was near the minimum value that could be used and obtain desirable peaks for quantification.

Minimal sample preparation is required when using this method. In fact, samples may be extracted concurrently while the HPLC analysis is in progress for previous extractions. By use of the cation and anion precolumn cartridges, a range of 300–350 samples were injected before the cartridges required replacement. After injection of several thousand samples, the column appears to be functioning satisfactorily and shows no significant signs of deterioration. This indicates the value of precolumns in preserving longevity of the column and is probably due to removal of contaminants, particularly the salts, that may be present in significant quantities within plant tissue.

This procedure appears to be applicable for accurate analysis of certain types of oven-dried ground plant material that vary over a wide range of sugar concentrations. Since the integrator prints the quantitative values after completion of tracing the chromatogram, results can be obtained immediately. Minimal sample preparation, rapid quantitative analysis and a high degree of repeatability support this HPLC technique as a promising method for analysis of sugars in oven-dried plant tissues.

ACKNOWLEDGEMENT

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## Note

# Urea–polyacrylamide gel electrophoresis of alginic acid

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(Received April 12th, 1983)

Alginic acid is a major component of brown algal cell walls. It is a linear polyanion, a co-polymer of β-D-mannuronate and α-L-guluronate, arranged in regions of homopolymeric blocks separated by regions approaching an alternating structure[1]. Homopolymeric alginate blocks are chemically produced from high-molecular-weight alginic acid by mild acid hydrolysis and selective precipitation in acid solutions[2]. Isolated blocks have been used as substrates in characterizing alginolytic enzymes[3], as well as for investigating solution behavior and physical structure of alginate[4,5].

A rapid method of analysis would be desirable to follow purification of alginate blocks and to determine their degradation by bacterial enzymes. Since algal alginates have a uniform charge to mass ratio, electrophoretic systems using polyacrylamide gels[6], agarose gels[7], and free-boundary methods[8] have been utilized. However, due to heterogeneity of chain length, analysis of alginate composition by electrophoresis has generally been less successful than other methods, such as nuclear magnetic resonance[4] or circular dichroism[5].

This report describes a urea–polyacrylamide gel electrophoresis (urea–PAGE) system to analyze acid degradation of high-molecular-weight alginate, to rapidly separate short alginate blocks, and to provide a technique that is rapid and sensitive for determining the specificity of enzymic degradation of alginate.

## EXPERIMENTAL

### *Alginate*

High-molecular-weight alginate from brown algae (*Laminaria hyperborea* and *Macrocystis porifera*) was obtained from Kelco (San Diego, U.S.A.). Alginate samples were converted to sodium salts, precipitated by addition of ethanol to 70% (v/v), and lyophilized. In all cases, alginate concentrations were given as the mass of sodium alginate per volume of buffer. Homopolymeric blocks of poly-D-mannuronate (M-blocks) and poly-L-guluronate (G-blocks) were obtained from high-molecular-weight alginate after mild heterogenous acid hydrolysis and selective acid precipitation[2]. The blocks had an average degree of polymerization of 24 and were approximately 97% M-blocks or G-blocks as determined by $^1H$ nuclear magnetic resonance[4] and circular dichroism[5]. Purified alginate blocks were used as electrophoretic standards or substrates for bacterial enzymes as previously described[3].

*Electrophoresis*

Separation of alginate blocks was performed using urea–PAGE. The stacking gel contained 6% (w/v) acrylamide, 6 *M* urea, and 50 m*M* Tris, pH 7. The separating gel contained 8 to 21% acrylamide, 4.5 to 6 *M* urea, and 50 m*M* Tris, pH 8.7. Where appropriate, separating gel composition is given in the text and figure legends. Both gels contained 1 m*M* calcium chloride, N,N′-methylene bis-acrylamide as 1% of the total acrylamide concentration, and were polymerized by addition of ammonium persulphate and N,N,N′,N′-tetramethylethylenediamine to a final concentration of 0.07%. The vertical slab gels (1.5 mm thick and 140 mm in length) were polymerized between glass plates enclosed by 6.4 mm thick plexiglass casting units and were run immediately at room temperature with a constant potential of 130 V. The upper-tray buffer was 400 m*M* glycine, 50 m*M* Tris (pH 8.7), while the lower-tray buffer was 50 m*M* Tris (pH 8.9) and 1 m*M* calcium chloride. The samples (0.5 to 30 μg alginate) were dissolved in 4 *M* urea, 10 m*M* ethyleneglycol-bis(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA), and 50 m*M* Tris (pH 7.5), with 0.001% bromphenol blue and 0.005% xylene cyanole as tracking dyes. Electrophoresis continued for about 2 h or until bromphenol blue reached the end of the gel. Gels were stained for alginate by immersion for 15 min or longer in aqueous 0.1% toluidine blue O (TBO) and were destained in distilled water[7].

## RESULTS

*Acid hydrolysis of alginic acid*

High-molecular-weight alginate from *L. hyperborea* was electrophoresed before and after partial acid hydrolysis to determine the electrophoretic behavior of alginate and the relationship between acid-insoluble hydrolysis products and chemically purified M-blocks and G-blocks. Alginate had low mobility prior to hydrolysis, since the majority of the stained material remained at or very near the origin (Fig. 1, lane D). The low electrophoretic mobility was due to the effect of calcium on the high-molecular-weight alginate, since when calcium was omitted from the gel, all of the high-molecular-weight alginate entered the stacking gel and formed a smear (results not shown). The effect of short, partial acid hydrolysis on alginate mobility was striking. After 2 h of hydrolysis (lane C), alginate disappeared from the origin (even with calcium in the gel), became highly mobile, and separated into three distinct staining bands near the electrophoretic front and one faint band midway through the gel. The mobility of the lead band was identical to M-blocks (lane B), with the following bands corresponding to G-blocks (lane A). More extensive acid degradation (up to 8 h, lane E) did not change this pattern.

*Calcium effect on block separation*

Urea–PAGE clearly separated M-blocks from G-blocks, only if a discontinuous gel system containing calcium was employed. When the separating gel was omitted (in effect a 140-mm stacking gel), both blocks co-migrated with bromphenol blue at the electrophoretic front. Even with a discontinuous gel, when calcium was omitted from the gels, all alginate blocks co-migrated with bromphenol blue. When a separating gel containing 8% acrylamide, 6 *M* urea, and 1 m*M* calcium was utilized, the majority of G-blocks (Fig. 1, lane A) migrated with xylene cyanole behind M-

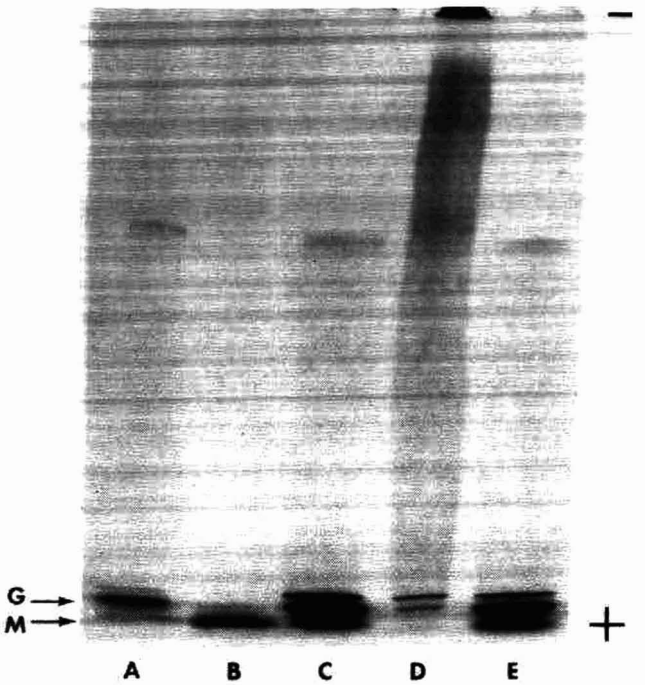


Fig. 1. Urea–PAGE (6 *M* urea, 8% acrylamide) of alginate. High-molecular-weight alginate from *L. hyperborea* was electrophoresed before (lane D), after 2 h (lane C), and after 8 h (lane E) of partial acid hydrolysis. For comparison, chemically purified G-blocks (lane A) and M-blocks (lane B) were also electrophoresed. Lanes A and B contained 5 μg alginate, while lanes C, D, and E contained 20 μg alginate. The location of the main bands of M-block and G-blocks near the electrophoretic front are indicated by arrows.

blocks (lane B), which migrated with bromphenol blue at the electrophoretic front. The separation of G-blocks from M-blocks by the addition of calcium was consistent with the results from free-boundary electrophoresis[8].

*Enzymic degradation of alginate blocks*

Urea–PAGE also proved to be a sensitive technique to visualize enzymatic degradation of alginate blocks. Two alginate lyase enzymes (one specific for M-blocks, another specific for G-blocks) were isolated from marine bacteria[3]. Purified homopolymeric alginate blocks were incubated with the enzymes, then electrophoresed (Fig. 2). The poly-D-mannuronate lyase degraded M-blocks (lane E) but not G-blocks (lane C), while the poly-L-guluronate lyase degraded G-blocks (lane D) but not M-blocks (lane F). Urea–PAGE allowed detection of alginolytic activity with concentrations of blocks as low as 0.05 to 0.1 μg per lane, due to the tight banding of alginate blocks and the sensitivity of the TBO stain. In contrast, determinations by colorimetric or UV absorbance measurements would have required considerably more substrate, a minimum of 50 to 100 μg, and viscometric analysis would have failed entirely, due to the inherently low viscosity of the blocks.

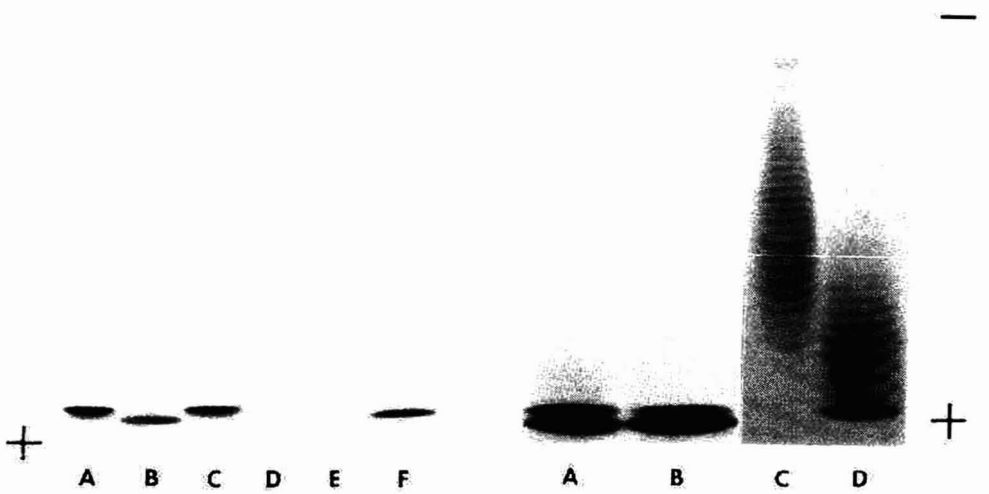


Fig. 2. Urea PAGE (6 *M* urea, 8% acrylamide) of alginate blocks after incubation with alginate lyases. All lanes contained 10 μg alginate, and all enzyme incubations were for 120 min. Undegraded G-blocks (lane A) and M-blocks (lane B) were electrophoresed for comparison. G-blocks were incubated with poly-D-mannuronate lyase (lane C) and with poly-L-guluronate lyase (lane D). M-blocks were incubated with poly-D-mannuronate lyase (lane E) and poly-L-guluronate lyase (lane F).

Fig. 3. Urea PAGE (4.5 *M* urea, 21% acrylamide) of purified alginate blocks. G-blocks (lane A) and M-blocks (lane B) were electrophoresed in a gel which contained no calcium. G-blocks (lane C) and M-blocks (lane D) were electrophoresed in a gel containing 1 m*M* calcium.

*Alginate block size heterogeneity*

Although urea-PAGE with low acrylamide concentrations (8%) and calcium in the gels successfully separated G-blocks from M-blocks, increasing the acrylamide concentration revealed size heterogeneity within the samples and indicated the important role of calcium in the separations. The effects occurred gradually as the acrylamide concentration was increased from 8 to 21% in steps of 2 to 3%. The results of urea PAGE using 21% acrylamide gels with 4.5 *M* urea are presented in Fig. 3. In the absence of calcium (lanes A and B) both G-blocks and M-blocks migrated near the electrophoretic front; however, trailing behind the main bands of G-blocks were a series of evenly spaced, faint bands. When 1 m*M* calcium was present, G-blocks (lane C) were separated into a series of evenly spaced bands, well behind the electrophoretic front. M-blocks (lane D) migrated at the electrophoretic front with 8 to 10 trailing bands overlapping the G-blocks. According to the migration of DNA restriction fragments (HaeIII digests of φX-174 DNA, not shown), urea PAGE separated polyanions on the basis of size.

## DISCUSSION

The electrophoretic patterns of G-blocks and M-blocks can be attributed to both the gel composition and to the physical behavior of the blocks. Although the average polymer size of both blocks was similar, calcium–urea–PAGE was able to separate the blocks. The presence of sharp, separate, highly mobile bands (G-blocks and M-blocks) indicated that in gels with low (less than 10%) acrylamide concentrations block mobility was limited by ion effects near the electrophoretic front, since the gel pore-size was too large to cause sieving of the blocks. An increase in acrylamide concentration decreased the pore-size of the gel, and in the presence of calcium, G-blocks but not M-blocks separated completely into discrete bands well behind the electrophoretic front. Although calcium may have affected the structure of the acrylamide gel, the results from other studies suggest that the effect was probably directly on alginate. For instance, calcium-induced retardation of G-blocks but not M-blocks was reported[8] using free-boundary electrophoresis under conditions which would not have affected the stationary phase. Although the presence of urea may have precluded calcium-induced gel formation, ion pairing due to transient binding of calcium may have reduced the net charge sufficiently to cause retardation[9]. Hence, the tight bands of G-blocks were probably discrete size classes and not inter- or intra-molecularly cross-linked molecules.

Several reservations must be presented with respect to separations of alginates by urea–PAGE. High-molecular-weight poly-D-mannuronate (greater than 95% D-mannuronate residues) migrated as a smear behind G-blocks with some overlap. Therefore, high-molecular-weight alginates should be slightly depolymerized prior to electrophoresis. In addition, electrophoresis of bacterial alginates, with varying degrees of O-acetylation, may require de-acetylation to preserve a constant charge-to-mass ratio.

Urea–PAGE, with calcium present, can clearly and rapidly separate small quantities of alginate blocks in a large number of samples, typically 10 to 30 samples per gel. Unlike any other electrophoretic method yet reported, urea–PAGE allows one to make direct comparisons of enzymic or acid degradation of alginate. No other method has demonstrated separation of discrete populations of blocks on the basis of size, indicating that urea–PAGE may be valuable as a means of obtaining fine structure analysis of alginate during synthesis or degradation. Overall, it appears that urea–PAGE requires less time and smaller amounts of material to yield information not attainable by other techniques.

## ACKNOWLEDGEMENTS

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## Letter to the Editor

# Correlation between the retention of cardiac glycosides in reversed-phase high-performance liquid chromatography with a diphenylsilyl stationary phase, their structure and biological activity

Sir,

The recent investigation by Davydov *et al.*[1] on the correlation between the retention of cardiac glycosides in reversed-phase high-performance liquid chromatography (RP-HPLC), their structure and biological activity is indeed an important one in pharmaceutical chemistry.

However, some questions can be raised as to the presentation of the data. The authors represented the biological activity of the cardiac glycosides, LD, in units of mg/kg, although LD is usually evaluated in the units of moles/kg and/or any other units including numbers of molecules, especially as the molecular weight of the compounds ranges widely in this instance. In addition, the relationship between LD and the retention volume, $V_m$, on the hydrophobic silica gel surface containing diphenylsilyl groups is represented in Fig. 12 of the paper as LD *versus* $V_m$. But such relationships are generally expressed as plots of log LD *versus* log $V_m$.

In Fig. 1 I have replotted the authors' data, using the units of moles/kg instead of mg/kg for LD. A good linear relationship is observed between log LD and log $V_m$ of cardiac glycosides except G-strophanthin. It seems that the most important contribution to this relationship is the hydrophobic properties of glycosides, as was stated[1].

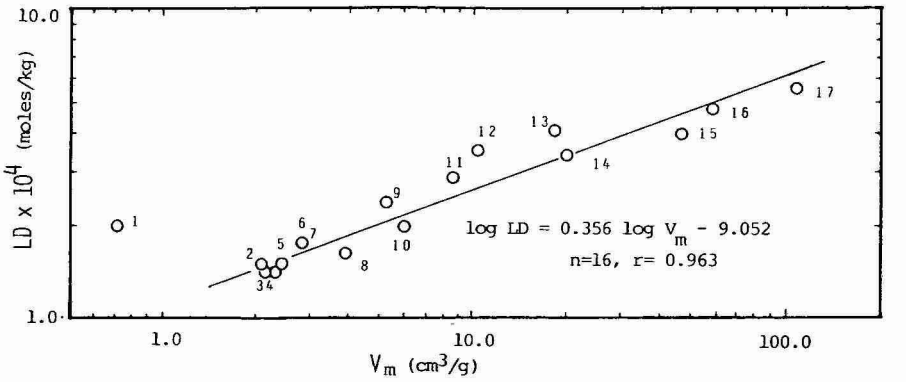


Fig. 1. Correlation between the biological activity, LD, of cardiac glycosides and their retention volumes, $V_m$, on the hydrophobic silica gel surface with water–ethanol as eluent. Glycosides: 1 = G-strophantin; 2 = corelborin-$\pi$; 3 = K-strophanthoside; 4 = convallatoxin; 5 = olitoriside; 6 = K-strophantin-$\beta$; 7 = desglucocheirotoxin; 8 = erysimin; 9 = desacetyl-lanatoside C; 10 = cymarin; 11 = lanatoside C; 12 = digoxin; 13 = lanatoside B; 14 = oleandrin; 15 = lanatoside A; 16 = digitoxin; 17 = acetyldigitoxin.

0021-9673/83/$03.00 

In conclusion, by use of the alternative method of presentation described here, the data of Davydov *et al.*[1] clearly indicate that chromatographic retention in RP-HPLC can be employed as a descriptor of the biological activity of cardiac glycosides.

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KIYOKATSU JINNO

1 V. Ya. Davydov, M. Elizalde Gonzalez and A. V. Kiselev, *J. Chromatogr.*, 248 (1982) 49.

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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*, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.
4 R.H. Doremus, B.W. Roberts and D. Turnbull (Editors), *Growth and Preparation of Crystals, Proc. Int. Conf. Crystal Growth, Coopertown, NY, August 27–29, 1958*, Wiley, New York, 1958.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The JOURNAL OF CHROMATOGRAPHY; JOURNAL OF CHROMATOGRAPHY, BIOMEDICAL APPLICATIONS and CHROMATOGRAPHIC REVIEWS should be cited as *J. Chromatogr.*

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Vol. 264, No. 3

journal of chromatography news section

## NEW BOOKS

**Sample preparation for gas chromatographic analysis,** by W.G. Jennings and A. Rapp, Hüthig, Heidelberg, 1983, 104 pp., price DM 46.00, ISBN 3-7785-0858-X.

**GC/LC, instruments, derivatives in identifying pollutants and unknowns,** by R.C. Crippen, Pergamon, Oxford, 1983, 353 pp., price £41.50, US$ 75.00, ISBN 0-08-027185-5.

**The analysis of gases by chromatography,** by C.J. Cowper and A.J. DeRose, Pergamon, Oxford, 1983, 159 pp., price £14.00, US$ 25.00, ISBN 0-08-024027-5.

**Laborpraxis, Band 4, Analytische Methoden,** Birkhäuser, Basel, Boston, Stuttgart, 1983, 208 pp., price SFr. 28.00, DM 32.00, ISBN 3-7643-1395-1.

**Methods of biochemical analysis,** edited by D. Glick, Wiley, Chichester, New York, **Vol. 29,** 1983, *ca.* 448 pp., price *ca.* US$ 65.00, £41.50, ISBN 0-471-86283-5.

**TrAC – Trends in Analytical Chemistry: Reference edition 1981–1982,** edited by P.T. Shepherd, Elsevier, Amsterdam, Oxford, New York, 1983, XVI + 398 pp., price US$ 89.25 (U.S.A. and Canada), Dfl. 210.00 (rest of world), ISBN 0-444-42193-3.

**Wilson and Wilson's Comprehensive analytical chemistry, Vol. XII, Thermal analysis, Part C,** by V. Balek and J. Tölgyessy, Elsevier, Amsterdam, Oxford, New York, 1983, *ca.* 270 pp., price US$ 80.75 (U.S.A. and Canada), Dfl. 190.00 (rest of world), subscription price US$ 72.25 (U.S.A. and Canada), Dfl. 170.00 (rest of world), ISBN 0-444-99659-1.

**Environmental radioanalysis,** by H.A. Das, A. Faanhof and H.A. van der Sloot, Elsevier, Amsterdam, Oxford, New York, 1983, VI + 298 pp., price US$ 83.00 ( U.S.A. and Canada), Dfl. 195.00 (rest of world), ISBN 0-444-42188-2.

**Analytical chemistry of molybdenum,** by G.A. Parker, Springer, Berlin, Heidelberg, New York, 1983, *ca.* 200 pp., DM 98.00, *ca.* US$ 42.30, ISBN 3-540-12235-4.

**Immobilized cells and organelles,** edited by B. Mattiasson, CRC Press, Boca Raton, FL, **Vol. I,** 1983, 152 pp., price US$ 53.00 (U.S.A.), US$ 61.00 (rest of world).

**Immobilized cells and organelles,** edited by B. Mattiasson, CRC Press, Boca Raton, FL, **Vol. II,** 1983, 168 pp., price US$ 53.00 (U.S.A.), US$ 61.00 (rest of world).

## MEETINGS

FACSS X – THE FEDERATION OF ANALYTICAL CHEMISTRY AND SPECTROSCOPY SOCIETIES 10th ANNUAL MEETING, PHILADELPHIA, PA, U.S.A., SEPTEMBER 25–30, 1983

In 1983, the FACSS meeting will be celebrating its 10th anniversary. Over the past decade, FACSS has consistently been a forum for the presentation of research results of the highest quality. At the 1983 meeting, papers will be presented in the following categories: atomic spectroscopy, infrared and Raman spectroscopy, NMR spectroscopy, mass spectroscopy, chromatography (gas, liquid,

ion, etc.), electrochemistry, thermal analysis, particle/surface analysis, data processing/computerization/laboratory automation, biological/clinical/forensic analysis, environmental analysis, and new analytical techniques. Within these categories, symposia on special topics are also being prepared.

Workshops and short courses. As in previous years, workshops and short courses will be offered prior to, during, and after the conference. Details of these will be available soon.

Equipment exhibits. Centrally located at the meeting will be an exhibition of scientific equipment and services. This exhibition can afford an excellent opportunity for suppliers and users of scientific equipment to meet and discuss mutual interests. For exhibit information contact: Peter Keliher, Exhibits Director, P.O. Box 96, Collegeville, PA 19426, U.S.A.

Preliminary programme. If you wish to receive a copy of the preliminary program please write to: FACSS X Program Chairman, John O. Lephardt, Philip Morris Research Center, P.O. Box 26583, Richmond, VA 23216, U.S.A.

## WORKSHOP ON LOW DISPERSION LIQUID CHROMATOGRAPHY, AMSTERDAM, THE NETHERLANDS, JANUARY 19–20, 1984

"Microbore", "miniature" and "high speed" are words that are appearing more and more frequently in references to high-performance liquid chromatography. The trend that they reflect is a reduction in volume of all the components in a chromatographic system. The critical implication is that the dispersive contributions of all instrument factors external to the column have to be kept to a minimum. Therefore these techniques are grouped under the name: low dispersion liquid chromatography (LDLC). There are obvious benefits to be gained by applying LDLC: savings in stationary phase and solvent, reduced analysis time, improved absolute detection limit, enhanced separation efficiency, easier compatibility with hyphenated systems, etc.

The forthcoming LDLC workshop will address this topic in detail, with special emphasis on miniaturized columns and particle dimensions, high speed separations, systems for the generation of low flows, small volume injectors, miniaturization of system elements, detectors with small detection volumes and fast response and measurements of dispersion effects.

The workshop will be held at the Free University, in Amsterdam. The workshop language will be English (no simultaneous translation will be offered). The proceedings will be published by Elsevier Science Publishers B.V. as a special issue of the *Journal of Chromatography*, after the usual refereeing procedure. Besides regular lectures, instrument demonstrations and applications will be offered and discussed. A short course on "Low Dispersion LC" will be held at the Free University on January 17 and 18. The deadline for registration is December 15, 1983.

Further information may be obtained from: LDLC Workshop Office, Dept. of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

## BIOCHEMISCHE ANALYTIK 84 – INTERNATIONAL CONFERENCE ON BIOCHEMICAL AND INSTRUMENTAL ANALYSIS, MUNICH, G.F.R., APRIL 10–13, 1984

The 9th Conference on Biochemical Analysis will be held in 1984 at the Münchener Messegelände. The breadth of the scientific themes will be considered in 16 half-day symposia. The themes selected by the Scientific Committee may be supplemented by poster demonstrations. Besides the provision of information during the course of the scientific programme, more time than is usual will be allowed for discussion in individual scientific meetings. The conference will be broadened in the direction of the technical development and field of application by the "Analytica Forum München", at which novelties of industrial development by the technical exhibition will be presented.

Further information may be obtained from: Dr. Rosmarie Vogel, General Secretary, Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universität München, Nussbaumstrasse 20, D-8000 München 2, G.F.R. Tel: (089) 153032; telex 5 216 018 bird d.

## CALENDAR OF FORTHCOMING MEETINGS

| | |
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| July 18–22, 1983<br>Plymouth, NH, U.S.A. | **Gordon Research Conference on Ion Exchange**<br>Contact: Alexander M. Cruickshank, Director, Gordon Research Conferences, Pastore Chemical Laboratory, University of Rhode Island, Kingston, RI 02881, U.S.A. Tel: (41) 783-4011 or 783-3372. (Further details and short program published in Vol. 261, No. 1.) |
| Aug. 13–19, 1983<br>Vancouver, BC,<br>Canada | **Canadian Society of Forensic Science Annual Conference**<br>Contact: Mrs. Joanne Cottingham, Executive Secretary, Canadian Society of Forensic Science, Suite 303, 171 Nepean Street, Ottawa, Ontario K2P 0B4, Canada. Tel.: (613) 235-7112. |
| Aug. 15–19, 1983<br>New London, NH, U.S.A. | **Gordon Research Conference on Separation and Purification**<br>Contact: Alexander M. Cruickshank, Director, Gordon Research Conferences, Pastore Chemical Laboratory, University of Rhode Island, Kingston, RI 02881, U.S.A. Tel.: (401) 783-4011 or 783-3372. |
| Aug. 15–19, 1983<br>New Hampton, NH, U.S.A. | **Gordon Research Conference on Analytical Chemistry**<br>Contact: Alexander M. Cruickshank, Director, Gordon Research Conferences, Pastore Chemical Laboratory, University of Rhode Island, Kingston, RI 02881, U.S.A. Tel.: (401) 783-4011 or 783-3372. |
| Aug. 28–Sept. 2, 1983<br>Amsterdam,<br>The Netherlands | **9th International Symposium on Microchemical Techniques**<br>Contact: Symposium Secretariat, c/o Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel: (020) 552 3459. |
| Aug. 29–Sept. 2, 1983<br>Bratislava, Czechoslovakia | **4th Danube Symposium on Chromatography and 7th International Symposium "Advances and Application of Chromatography in Industry"**<br>Contact: Professor J. Garaj, Department of Analytical Chemistry, Faculty of Chemical Technology, Jánska 1, 81237 Bratislava, Czechoslovakia. (Further details published in Vol. 235, No. 1.) |
| Sept. 5–9, 1983<br>Bucharest, Romania | **MACRO '83: 29th IUPAC International Symposium on Macromolecules**<br>Contact: IUPAC MACRO '83, Calea Plevnei 139, R-77131 Bucharest, Romania. |
| Sept. 5–9, 1983<br>Rome, Italy | **1st Italo-Hungarian Symposium on Spectrochemistry: Environmental Protection and Spectrochemistry**<br>Contact: S. Caroli, Laboratorio di Tossicologia Applicata, Istituto Superiore di Sanità, Viale Regina Elena, 299, I-00161 Rome, Italy. |
| Sept. 21–29, 1983<br>Amsterdam,<br>The Netherlands | **het instrument**<br>Contact: het instrument, Birkstraat 108, Postbus 152, 3760 AD Soest, The Netherlands. Tel. (02155) 18204. |
| Sept. 22–23, 1983<br>Cambridge, Great Britain | **Symposium: "Columns in High-Performance Liquid Chromatography"**<br>Contact: Mrs. Annet Pullen, Hewlett-Packard Ltd., Analytical Instrumentation, Nine Mile Ride, Easthampstead, Wokingham, Berks. RG11 3LL, Great Britain. Tel.: (03446) 3100, ext. 3465. |

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| Sept. 25–30, 1983<br>Philadelphia, PA, U.S.A. | **10th Annual Meeting of the Federation of Analytical and Spectroscopy Societies**<br>Contact: FACSS X Program Chairman, John O. Lephardt, Philip Morris Research Center, P.O. Box 26583, Richmond, VA 23261, U.S.A. |
| Sept. 28–30, 1983<br>Würzburg, G.F.R. | **International Workshop on Analysis of Volatiles: New Methods and Their Applications**<br>Contact: Prof. Dr. P. Schreier, University of Würzburg, Food Chemistry, Am Hubland, D-8700 Würzburg, G.F.R. |
| Sept. 29–30, 1983<br>Schliersee, G.F.R. | **Symposium "Chiralität und Aktivität"**<br>Contact: Gesellschaft Deutscher Chemiker, Geschäftsstelle, Postfach 90 04 40, D-6000 Frankfurt am Main 90, G.F.R. Tel.: 0611/7917-366. |
| Oct. 3–4, 1983<br>Bad Nauheim, G.F.R. | **Anwender-Kolloquium über die Gaschromatographische Dampfraumanalyse**<br>Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, D-6000 Frankfurt am Main 90, G.F.R. Tel.: 0611/ 7917-366. |
| Oct. 3–6, 1983<br>Amsterdam, The Netherlands | **20th Anniversary – International Symposium on Advances in Chromatography**<br>Contact: Professor A. Zlatkis, Department of Chemistry, University of Houston, Houston, TX 77004, U.S.A. (Further details published in Vol. 256, No. 1.) |
| Oct. 10–12, 1983<br>Tarrytown, NY, U.S.A. | **Capillary Chromatography – 2nd International Symposium**<br>Contact: Professor A. Zlatkis, Department of Chemistry, University of Houston, Houston, TX 77004, U.S.A. (Further details published in Vol. 261, No. 3.) |
| Oct. 12–14, 1983<br>London, Great Britain | **Analyticon 83**<br>Contact: Mr. G.C. Young, SIMA, Leicester House, 8 Leicester Street, London WC2H 7BN, Great Britain. |
| Oct. 17–21, 1983<br>Neubrandenburg, G.D.R. | **Analytiktreffen 1983: Fortschritte in der Gas- und Flüssigkeits-Chromatographie**<br>Contact: Dr. sc. W. Engewald, Karl-Marx-Universität Leipzig, Sektion Chemie, Leibigstrasse 18, DDR-7010 Leipzig, G.D.R. |
| Oct. 18–19, 1983<br>Saarbrücken, G.F.R. | **"Dünnschichtchromatographie – Säulenflüssigkeitschromatographie: Partner oder Konkurrenten?"**<br>Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, D-6000 Frankfurt am Main 90, G.F.R. Tel.: (0611) 7917-366. |
| Nov. 10–16, 1983<br>Düsseldorf, G.F.R. | **9th International Congress and Exhibition for Instrumentation and Automation (INTERKAMA 83)**<br>Contact: INTERKAMA 83, Düsseldorfer Messegesellschaft mbH, NOWEA, Postfach 32 02 03, D-4000 Düsseldorf 30, G.F.R. |
| Nov. 14–16, 1983<br>Monte Carlo, Monaco | **3rd International Symposium on HPLC of Proteins, Peptides and Polynucleotides**<br>Contact: Shirley E. Schlessinger, 400 East Randolph, Chicago, IL 60601, U.S.A. Tel: (312) 527-2011. (Further details published in Vol. 275, No. 1.) |

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| Nov. 16–18, 1983<br>New York, NY, U.S.A. | **22nd Eastern Analytical Symposium**<br>Contact: Norman Gardner, 73 Ethel Street, Metuchen, NJ 08840, U.S.A. Tel.: (201) 548-7377. |
| Nov. 24–25, 1983<br>Lausanne, Switzerland | **Workshop on Handling of Environmental and Biological Samples in Chromatography**<br>Contact: Prof. R.W. Frei, The Free University of Amsterdam, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 261, No. 3.) |
| Dec. 7–10, 1983<br>Singapore, Singapore | **Chem Asia '83 Conference**<br>Contact: Singapore Exhibition Services, Ltd., 601 Cathay Building, Singapore 0922, Singapore. |
| Jan. 19–20, 1984<br>Amsterdam, The Netherlands | **Workshop on Low Dispersion Liquid Chromatography**<br>Contact: LDLC Workshop Office, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. |
| March 5–9, 1984<br>Atlantic City, NJ, U.S.A. | **35th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy**<br>Contact: Linda Briggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A. |
| April 8–13, 1984<br>St. Louis, MO, U.S.A. | **187th National Meeting of the American Chemical Society**<br>Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A. |
| April 10–13, 1984<br>Munich, G.F.R. | **9th Conference on Biochemical Analysis (BIOCHEMISCHE ANALYTIK 84) & ANALYTICA 84 Exhibition**<br>Contact: Secretary General, Dr. Rosmarie Vogel, Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universität München, Nussbaumstrasse 20, D-8000 München 2, G.F.R. Tel.: (089) 15 30 32; Telex: 5 216 018 bird d. |
| April 16–19, 1984<br>New York, NY, U.S.A. | **20th International Symposium on Chromatography**<br>Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. |
| May 15–18, 1984<br>Ghent, Belgium | **5th International Symposium on Mass Spectrometry in Life Sciences**<br>Contact: Prof. Dr. A. De Leenheer, Symposium Chairman, Laboratoria voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel.: (091) 21.89.51. |
| May 20–26, 1984<br>New York, NY, U.S.A. | **8th International Symposium on Column Liquid Chromatography**<br>Contact: Professor Cs. Horváth, Mason Laboratory, Yale University, P.O. Box 2159, Yale Station, New Haven, CT 06520, U.S.A. (Further details published in Vol. 256, No. 1.) |
| Aug. 26–31, 1984<br>Philadelphia, PA, U.S.A. | **188th National Meeting of the American Chemical Society**<br>Contact: A.T. Winstead, American Chemical Society, 1155 16th Street, NW, Washington, DC 20036, U.S.A. |

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| Sept. 2–6, 1984<br>Hradec Králové,<br>Czechoslovakia | **4th International Symposium on Isotachophoresis – ITP 84**<br>Contact: ITP 84, Dr. Z. Prusik, C.Sc., Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Flemingovo nám. 2, CS-166 10 Praha 6, Czechoslovakia. (Further details published in Vol. 256, No. 1.) |
| Sept. 23–28, 1984<br>Philadelphia, PA, U.S.A. | **11th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies**<br>Contact: R.F. Hirsch, Division of Analytical Chemistry, American Chemical Society, 304 Beach Wood, Orange, NJ 07050, U.S.A. |
| Oct. 1–5, 1984<br>Nürnberg, G.F.R. | **15th International Symposium on Chromatography**<br>Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), G.F.R. |
| Oct. 8–10, 1984<br>Tarrytown, NY, U.S.A. | **3rd International Symposium on Capillary Chromatography**<br>Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. |
| Oct. 24–26, 1984<br>Montreux,<br>Switzerland | ***Third Workshop on LC–MS and MS–MS***<br>Contact: Professor Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. |
| Nov. 22–24, 1984<br>Barcelona,<br>Spain | **3rd International Congress on Analytical Techniques in Environmental Chemistry**<br>Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; telex: 50458 FOIMB-E. |
| Nov. 22–24, 1984<br>Barcelona,<br>Spain | **14th Annual *Symposium on* Analytical Chemistry of Pollutants**<br>Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; telex: 50458 FOIMB-E. |
| Feb. 25–March 1, 1985<br>New Orleans, LA, U.S.A. | **36th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy**<br>Contact: Linda Biggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A. |
| April 28–May 3, 1985<br>Miami Beach, FL, U.S.A. | **189th National Meeting of the American Chemical Society**<br>Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A. |
| July 1–6, 1985<br>Edinburgh, Scotland,<br>Great Britain | **9th International Symposium on Column Liquid Chromatography**<br>Contact: J.H. Knox, Department of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, Scotland, Great Britain. |
| Sept. 8–13, 1985<br>Chicago, IL, U.S.A. | **190th National Meeting of the American Chemical Society**<br>Contact: Meeting Department American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A. |

## SHORT REPORT

### 34th PITTSBURGH CONFERENCE AND EXPOSITION

The Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy continued its record of successful meetings with the 34th Pittsburgh Conference and Exposition held in Atlantic City, NJ, U.S.A., the week of March 7–12, 1983. Total attendance showed a 9.3% increase over the previous year. Although statistics alone cannot measure the impact of this international meeting, comparative figures can document its increase in stature in the scientific community.

| | 1983 | 1982 |
|---|---|---|
| Conferees | 14,720 | 12,387 |
| Exhibitors | 7,008 | 7,497 |
| Total Registration | 21,728 | 19,884 |
| Exhibiting Companies | 579 | 560 |
| Booth Spaces | 1,475 | 1,373 |
| Seminar Rooms | 34 | 35 |
| Technical Papers | 948 | 849 |

President Dr. Richard Obrycki was very pleased with the success of the 1983 Pittsburgh Conference and Exposition and said, "It was a huge success. Both exhibition and conferees were well satisfied. Exhibitors felt that they made good contacts, many more than usual asking for quotes on instrumentation, and conferees commenting on the excellent papers which were given, as well as the Exposition." He also said, "There appears to be an upturn in business and we are moving forward."

The 35th Conference and Exposition will be held in Atlantic City, NJ, U.S.A., the week of March 5–10, 1984. The Technical Sessions will be Monday through Friday, the Exposition will be open Monday through Thursday only, and there will be a Short Course on Friday and Saturday, March 9 and 10.

## SYMPOSIUM PROGRAM

### 20th ANNIVERSARY – INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY

The above symposium will be held on Oct. 3–6, 1983, at the Hotel Okura in Amsterdam, The Netherlands. A total of 102 papers will be presented at the symposium, representing contributions from 22 countries. A special feature of this meeting will be an exposition of the latest instrumentation and books.

Registration should be made in advance. The programs, registration forms and hotel reservation forms can be obtained from:

Professor Albert Zlatkis
Chemistry Department
University of Houston
4800 Calhoun
Houston, TX 77004
U.S.A.
Tel.: (713) 749-2623

The detailed program of the symposium is given below.

## MONDAY, OCTOBER 3

### Monday Morning

*L.S. Ettre, presiding*

9:00 Welcome to the Symposium
Presentation of Tswett Chromatography Award

### CONTEMPORARY CHROMATOGRAPHY

*L.S. Ettre, presiding*

9:20 E. Hellum, A.K. Thorsrud and P. Helland (Institute of Clinical Biochemistry, Rikshospitalet, Oslo, Norway) and F.W. Karasek (University of Waterloo, Waterloo, Ontario, Canada) – A new test system using human cells and a high resolution separation technique for evaluation of potentially hazardous substances.

9:40 G. Schomburg, F. Weeke and H. Husmann (Max-Planck-Institut für Kohlenforschung and Max-Planck-Institut für Strahlenchemie, Mülheim-Ruhr, G.F.R.) – Trace analysis with MDGC (multi-dimensional gas chromatography) using capillary columns.

10:00 Y. Ghaemi, B. Kaur and J.H. Knox (University of Edinburgh, Edinburgh, Scotland, U.K.) – Porous glassy carbon: chromatographic performance in relation to conditions of preparation.

10:20 R.P.W. Scott, E. Katz and K. Ogan (Perkin-Elmer Corp., Norwalk, CT, U.S.A.) – Column design in liquid chromatography.

10:40 Intermission

*A.T. James, presiding*

11:00 M.Y.B. Othman, J.H. Purnell, P. Wainwright and P.S. Williams (University College of Swansea, Swansea, Wales, U.K.) – Total optimisation of the separation of $C_1$–$C_2$ chlorohydrocarbons by gas–liquid chromatography.

11:20 V. Pretorius and E.R. Rohwer (Institute for Chromatography, University of Pretoria, Pretoria, South Africa) – Manufacture and evaluation of nickel silicon carbide capillary columns for gas–liquid chromatography.

11:40 R. Leshem, S. Lam and A. Karmen (Albert Einstein College of Medicine, Bronx, New York, U.S.A.) – Change of chromatographic properties of GLC columns with addition of organic vapor to carrier gas.

12:00 E. Bayer and K. Albert (Institut für Organische Chemie, Universität Tübingen, Tübingen, G.F.R.) – Characterization of chemically modified silica gels by $^{29}Si$ and $^{13}Ci$ cross-polarization and magic angle spinning nuclear magnetic resonance.

### Monday Afternoon

### CHROMATOGRAPHY – GENERAL

*E.sz. Kováts, presiding*

2:00 G. Guiochon, M.S. Gonnord, H. Colin and A. Siouffi (Ecole Polytechnique, Palaiseau, France) – Bidimensional column chromatography.

2:20 D.R. Deans (I.C.I. Petrochemicals Division, Wilton, Middlesborough, Cleveland, U.K.) – A new gas sampling device for gas chromatography.

2:40 S.-C. Chang and E. Gil-Av (Weizmann Institute of Science, Rehovot, Israel, and Chung-Shan Institute of Science and Technology, Taiwan, Republic of China) – Extension of the scope of GC separation of enantiomers on chiral phases. Resolution of α-halo carboxylic acids.

3:00 G. Alexander and I. Hazai (Hungarian Academy of Science, Budapest, Hungary) – Analysis of hard coal extracts by chromatographic methods.

3:20 F. Mangani and F. Bruner (Istituto di Scienze Chimiche, Università di Urbino, Urbino, Italy) – Chromatographic properties and analytical applications of a low-surface-area graphitized carbon black.

3:40 Intermission

*G. Guiochon, presiding*

4:00 F. Lancas, H. Karam and H. McNair (Virginia Polytechnic Institute and State University, Blacksburg, VA, U.S.A.) – Analysis of high ash coal-derived liquids by high resolution chromatographic techniques.

4:20 G. Holzer and A.J. Vella (Colorado School of Mines, Golden, CO, U.S.A.) – Gas chromatography–mass spectrometry study of monoalkylglycerol ethers.

4:40 J.R. Chretien, K. Szymoniak and J.E. Dubois (Institut de Topologie et de Dynamique des Systèmes, Université Paris VII, Paris, France) – EURECAS bibliographic data base in chromatography: the power of DARC software for structure and substructure search.

5:00 T.G. Andronikashvili, L.Ya. Laperashvili, V.G. Berezkin and N.A. Nadiradze (Institute of Physical and Organic Chemistry, Academy of Sciences of the Georgian SSR, Tbilisi, U.S.S.R.) – Chromatographic separation of hydrocarbon gases on zeolites with the use of carbon dioxide as carrier gas.

## TUESDAY, OCTOBER 4

### Tuesday Morning

### GAS CHROMATOGRAPHY – CAPILLARY COLUMNS

*J.E. Lovelock, presiding*

9:00 J. Curvers, T. Chapman, F. Maris, C. Cramers, C. Schutjes and J. Rijks (Eindhoven University of Technology, Eindhoven, The Netherlands) – Increased speed of quantitative steroid analysis by capillary GC and GC–MS; effects of sample pretreatment, sample introduction and column efficiency.

9:20 R.C. Kong and M.L. Lee (Brigham Young University, Provo, UT, U.S.A.) – Preparation of small diameter capillary columns for gas and supercritical fluid chromatography.

9:40 D.C. Williams, J.R. Whitaker and W. Jennings (Department of Food Science and Technology, University of California, Davis, CA, U.S.A.) – Detection of nicotine residues in workplace environments.

10:00 P.G. Simmonds (International Science Consultants, Ringwood, Hants., U.K.) – Purge without trap for the determination of trace halocarbons in water.

10:20 S.R. Lipsky and W.J. McMurray (Yale University School of Medicine, New Haven, CT, U.S.A.) – Recent advances in the development of fused-silica glass capillary columns coated with a special variety of cross-linked non-polar and highly polar films.

10:40 Intermission

*D.H. Desty, presiding*

11:00 J. Buijten, L. Blomberg, K. Markides and T. Wännman (Department of Analytical Chemistry, University of Stockholm, Stockholm, Sweden) – Cross-linked methyltolyl and methylphenylsilicones as stationary phases for fused-silica capillary gas chromatography.

11:20 P. Sandra (Laboratory of Organic Chemistry, University of Ghent, Ghent, Belgium) – On-line selective sample introduction in capillary gas chromatography.

11:40 C.P.M. Schutjes, E.A. Vermeer, R.W. Bally and C.A. Cramers (Eindhoven University of Technology, Eindhoven, The Netherlands) – Fast gas chromatography on 50-μm I.D. capillary columns: a sensitive deactivation test; combination with electron-capture detection.

12:00 C.P.M. Schutjes, P.A. Leclerq, J.A. Rijks and C.A. Cramers (Eindhoven University of Technology, Eindhoven, The Netherlands) and C. Vidal-Madjar and G. Guiochon (Ecole Polytechnique, Laboratoire de Chimie Analytique Physique, Palaiseau, France) – A model describing the role of the pressure gradient on efficiency and speed of analysis in capillary gas chromatography.

Tuesday Afternoon

CAPILLARY COLUMNS – INSTRUMENTATION AND APPLICATIONS

*M. Verzele, presiding*

2:00 L.S. Ettre (Chromatography Division, The Perkin-Elmer Corp., Norwalk, CT, U.S.A.) and B. Kolb (Bodenseewerk Perkin-Elmer & Co. GmbH, Überlingen, G.F.R.) – Quantitative headspace gas chromatography with capillary columns.

2:20 S. Trestianu, F. Munari and C. Saravalle (Carlo Erba Strumentazione, Milan, Italy) – Peak splitting phenomena in capillary gas chromatography.

2:40 W.A. König (Institut für Organische Chemie und Biochemie der Universität Hamburg, Hamburg, G.F.R.) – Unusual constituents in peptide antibiotics: the application of capillary gas chromatography in the analysis of structure and configuration.

3:00 J. Curvers, Th. Noy, C. Cramers and J. Rijks (Eindhoven University of Technology, Eindhoven, The Netherlands) – Possibilities and limitations of dynamic head-space sampling as a preconcentration technique for trace analysis of organics by capillary gas chromatography.

3:20 D.W. Later and B.W. Wright (Pacific Northwest Laboratory, Richland, WA, U.S.A.) – Capillary gas chromatographic methods for the selective separation of biologically active amino polycyclic aromatic hydrocarbon isomers in coal-derived materials.

3:40 Intermission

*E. Gil-Av, presiding*

4:00 S.H.G. Andersson and J. Sjövall (Department of Physiological Chemistry, Karolinska Institutet, Stockholm, Sweden) – Enol *tert.*-butyldimethylsilyl ethers for gas chromatographic–mass spectrometric analysis of steroids in tissue extracts.

4:20 F.I. Onuska and K. Terry (National Water Research Institute, Burlington, Ontario, Canada) – Requirements for instrumental modifications and application of narrow-bore WCOT columns in environmental analyses.

4:40 F. Shunbo, P. Literathy, F. Abdilly and M. Al-Ali (Kuwait Institute for Scientific Research, Safat, Kuwait) – Assessment of chlorinated hydrocarbon pollution in Kuwait's marine environment.

5:00 P. de Voogt and U.A.Th. Brinkman (Free University, Amsterdam, The Netherlands) – Clean up and quantification of PCBs in waste oils.

5:20 A. Zhu and G.-Y. Xu (Institute of Chemistry, Academia Sinica, Beijing, People's Republic of China) – Derivatization at the liquid–solid interface: application to the trace analysis of aqueous samples.

WEDNESDAY, OCTOBER 5

Wednesday Morning

GC–MS AND GC GENERAL

*E.C. Horning, presiding*

9:00 R.W. Walker, L.R. Mandel, L. DeLisi, R.J. Wyatt and W.J.A. VandenHeuvel (Merck Sharp & Dohme Research Labs., Rahway, NJ, U.S.A., National Institute of Mental Health, Bethesda, MD, U.S.A., and St. Elizabeth's Hospital, Washington, DC, U.S.A.) – Capillary column GLC–selected ion monitoring assay for [$^{13}$C, $^{15}$N]N-methyltryptamine in human urine.

9:20 C.J.W. Brooks, G.M. Barrett and W.J. Cole (University of Glasgow, Glasgow, Scotland, U.K.) – Studies of the selective derivatisation of methyl hyocholate and related steroidal ring B diols by GC–MS.

9:40 E. Gelpí, R. Freixa and J. Roselló (Instituto de Química Bio-Orgánica C.S.I.C., Barcelona, Spain) and M. Rigaud and J.C. Breton (Faculty of Medicine, Limoges, France) – Contributions of high-performance chromatographic techniques to the study of the toxic oil syndrome.

10:00 H. Miyazaki, M. Ishibashi, K. Yamashita and I. Ohguchi (Pharmaceutical Division, Nippon Kayaku Co., Tokyo, Japan) – Microdetermination of prostaglandins and thromboxane $B_2$ by negative-ion chemical ionization mass spectrometry.

10:20 H.M. Liebich, A. Pickert and B. Tetschner (Medizinische Universitäts-Klinik, Tübingen, G.F.R.) – GC–MS analysis of abnormal organic acids in serum of patients with chronic renal insufficiency.

10:40 M. Galli Kienle, G. Galli, G. Cighetti, E. Bosisio and R. Paoletti (University of Milan, Milan, Italy) – Evaluation of enzyme activities by gas chromatography–mass spectrometry; HMGCoA reductase and cholesterol 7α-hydroxylase.

11:00 Intermission

*H.J. Purnell, presiding*

11:20 J.C. Giddings, J.M. Davis and M.R. Schure (University of Utah, Salt Lake City, UT, U.S.A.) – Applications of the statistical model of component overlap in chromatography.

11:40 C.F. Poole, S.C. Dhanesar and F. Pacholec (Wayne State University, Detroit, MI, U.S.A.) – Organic molten salt and perfluorocarbon phases for gas chromatography.

12:00 V. Schurig (Institut für Organische Chemie, Universität Tübingen, Tübingen, G.F.R.) – Utilization of glass and fused-silica open-tubular columns for complexation gas chromatography.

12:20 C.L. Guillemin (Rhone-Poulenc Recherches, Aubervilliers, France) – Steam gas–solid chromatography: a flexible technique of separation.

## Wednesday Afternoon

## LIQUID CHROMATOGRAPHY – GENERAL

*D.W. Grant, presiding*

2:00 M. Verzele, L. Use and M. Van Kerrebroeck (Laboratory of Organic Chemistry, State University of Ghent, Ghent, Belgium) – On the problem of the internal standard in high-performance liquid chromatography.

2:20 S. Lam and A. Karmen (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) –Stereoselective D- and L-amino acid analysis by HPLC.

2:40 J.J. Donkerbroek, C. Gooijer, N.H. Velthorst and R.W. Frei (General and Analytical Chemistry, Free University, Amsterdam, The Netherlands) – Phosphorescence quenching as a new detection technique in liquid chromatography.

3:00 U.A.Th. Brinkman, A. de Kok and R.W. Frei (Department of Analytical Chemistry, Free University, Amsterdam, The Netherlands) – New approaches to the chromatographic analysis of phenylurea herbicides and the corresponding anilines.

3:20 H. Hatano, K. Sumizu and H. Yamamoto, Kyoto University and Kyoto Blood Research Institute, Kyoto, Japan) – New packings, polyvinylalcoholic porous gel, for high-performance gel filtration of organic and biochemical compounds.

3:40 Intermission

*E. Grushka, presiding*

4:00 P. Assenza and P.R. Brown (University of Rhode Island, Kingston, RI, U.S.A.) - Characterization of purines and pyrimidines by post-column ionization reactions and ultraviolet and fluorescence detection.

4:20 M. Apfel, A.J. Repta and L.A. Sternson (The University of Kansas, Lawrence, KS, U.S.A.) – Design and evaluation of a theophylline-modified bonded stationary phase for HPLC.

4:40 D. Fazio, B. Crowther and A. Hartwick (Rutgers University, New Brunswick, NJ, U.S.A.) – Analysis of acid hydrolysis products of bonded stationary phases used in HPLC.

5:00 J.P. Langenberg and U.R. Tjaden (Department of Analytical Chemistry and Pharmaceutical Analysis, State University of Leiden, Leiden, The Netherlands) – Determination of K-vitamins in human plasma using RP-HPLC with fluorometric reaction detection.

## WEDNESDAY, OCTOBER 5

### Wednesday Morning
### Esperance Room

## CHROMATOGRAPHY – GENERAL

*D.R. Deans, presiding*

9:00 D.W. Grant (Walton, Chesterfield, Derbyshire, U.K.) – Use of HPLC and spectrofluorescence in the analysis of nitrogen heterocycles.

9:20 J.H. Haken and M.A. Rohanna (University of New South Wales, Kensington, N.S.W., Australia) – Chromatographic analyses after chemical degradation of polyester resins in liquid and cured laminate forms.

9:40 E.R. Adlard and S.N. Davenport (Shell Research Ltd., Thornton Research Centre, Chester, U.K.) – Photoionization detectors for gas chromatography.

10:00 P.H. Degen, A. Schweizer and A. Sioufi (Ciba-Geigy Ltd., Basle, Switzerland, and Laboratoires Ciba-Geigy SA, Rueil Malmaison Cedex, France) – Determination of pirprofen in biological material by gas–liquid chromatography with nitrogen-specific and electron-capture detection.

10:20 A. Bidlingmeyer and F.V. Warren (Waters Assoc., Milford, MA, U.S.A.) – Practical considerations when using UV-visualization liquid chromatography.

10:40 Intermission

*B.S. Middleditch, presiding*

11:00 A. de Zeeuw, J. Wijsbeek, J.P. Franke and M. Bogusz (State University, Groningen, The Netherlands) – Mechanisms in the concentration dependent retention of drugs in capillary gas chromatography with splitless injection.

11:20 J. Churáček, A. Horna, K. Komárek and O. Dufka (Institute of Analytical Chemistry, University of Chemical Technology, Pardubice, Czechoslovakia) – Glass capillary GC–MS of halogenated esters.

11:40 E. Grell, E. Lewitzki, S. Dehal, I. Oberbäumer, F. Raschdorf and W.J. Richter (Max-Planck-Institut für Biophysik, Frankfurt, G.F.R., and Ciba-Geigy AG, Zentrale Forschung, Basel, Switzerland) – Chromatographic isolation and characterization of streptogramicin antibiotics.

12:00 F.F. Andrawes (American Cyanamid Co., Stamford, CT, U.S.A.) – Trace water analysis by gas chromatography and helium ionization detection.

12:20 W.D. Spall (Los Alamos National Laboratory, Los Alamos, NM, U.S.A.) and S.R. Lipsky (Yale University School of Medicine, New Haven, CT, U.S.A.) – A chromatographic system for the separation, identification and assessment of potential biological activity of individual compounds in complex organic mixtures.

Wednesday Afternoon
Esperance Room

## THIN-LAYER CHROMATOGRAPHY

*A. Tishbee, presiding*

2:00 R.E. Tecklenberg, E.K. Johnson, M.J. Wenning, B.L. Maidak and D. Nurok (Indiana University–Purdue University at Indianapolis, Indianapolis, IN, U.S.A.) – Optimization of continuous development thin-layer chromatography.

2:20 R. Segura, X. Navarro and J. Prat (Autonomous University of Barcelona, Bellaterra, Spain) – Separation and sub-fractionation of neutral lipids by a monocomponent solvent system.

2:40 H.J. Schneider and G.R. Taylor (NASA, Johnson Space Center, Houston, TX, U.S.A.), R.P. Landry and D.G. Winkler (Northrup Services, Johnson Space Center, Houston, TX, U.S.A.) and R. Segura (Universidad Autonoma de Barcelona, Bellaterra, Spain) – Analyses of plasma lipids and glucose, collected during space flight, by HPLC and imaging enhancement.

3:00 A. Aszalos (Food and Drug Administration, Washington, DC, U.S.A.) – Comparison of thin-layer chromatographic and high-pressure liquid chromatographic mobility of peptide-type antibiotics.

3:20 W. Ritter (Dept. of Pharmacokinetics Bayer AG., Wuppertal, G.F.R.) – Determination of nafazatrom in body fluids by quantitative HPTLC with post-chromatographic derivatization.

3:40 Intermission

4:00 V. Justová, Z. Wildtová and V. Pacovský (3rd Internal Clinic, Prague, Czechoslovakia) – Determination of 1,25-dihydroxyvitamin $D_3$ in plasma using HPTLC.

4:20 H.T. Butler and C.F. Poole (Wayne State University, Detroit, MI, U.S.A.) – Quantitative HPTLC analysis of polycyclic aromatic hydrocarbons in environmental samples.

## THURSDAY, OCTOBER 6

Thursday Morning

## LIQUID CHROMATOGRAPHY – GENERAL

*P.R. Brown, presiding*

9:00 C. Guttel and E. Grushka (The Hebrew University, Jerusalem, Israel) – Regelation separation on pseudoelastic polymers.

9:20 J.R. Benson (Interaction Chemicals, Inc., Los Altos, CA, U.S.A.) – Ion chromatography using a single polymeric column.

9:40 V.V. Berry (326 Reservoir Rd., Boston, MA, U.S.A.) – Universal liquid chromatography methods 4: 190 nm full gradients with gases to adjust eluent UV absorbance and pH.

10:00 R.S. Brazell and R.W. Holmberg (Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.) – Application of HPLC–flow injection analysis for the determination of polyphosphoric acids in phosphorous smokes.

10:20 I. Jane and R.J. Flanagan (Metropolitan Police Forensic Science Laboratory, London, U.K., and Poisons Unit, Guy's Hospital, London, U.K.) – Analysis of basic drugs by HPLC on silica columns using non-aqueous ionic eluents – mechanism of retention.

10:40 Intermission

*W.J.A. VandenHeuvel, presiding*

11:00 A. Liberti, P. Ciccioli and R. Tappa (Istituto Inquinamento Atmosferico C.N.R., Monterotondo Stazione, Rome, Italy) – Narrow-bore HPLC columns packed with graphitized carbon black.

11:20 F. Kreuzig (Biochemie GmbH, Kundl/Tirol, Austria) – High-speed liquid chromatography with conventional instruments for the determination of Cyclosporin A, B, C and D in fermentation broths.

11:40 E.R. White and D.N. Laufer (SmithKline and French Laboratories, Philadelphia, PA, U.S.A.) – Reversed-phase high-performance liquid chromatography of antibiotics on microbore columns.

12:00 T. Hanai and J. Hubert (Université de Montreal, Montreal, Quebec, Canada) – Retention *vs.* Van der Waal's volume and $\pi$ energy in liquid chromatography.

Thursday Afternoon

## LIQUID CHROMATOGRAPHY – GENERAL

*C.J.W. Brooks, presiding*

2:00 H.D. Meyer, W. Vogt and K. Jacob (Institut für Klinische Chemie, Universität München, Munich, G.F.R.) – Improved separation and detection of free porphyrins by high-performance liquid chromatography.

2:20 H. Lingeman, A. Hulshoff, B. Loriaux, F.B.J.M. Offerman, J. Renema and W.J.M. Underberg (Department of Analytical Pharmacy, State University of Utrecht, Utrecht, The Netherlands) – A rapid, sensitive and specific derivatization method for the fluorimetric detection of carboxylic acids prior to reversed-phase HPLC separation.

2:40 S.A. Hauffe and J.P. Dubois (Ciba-Geigy Ltd., Pharma Research & Development, Basle, Switzerland) – Determination of cadralazine in human plasma and urine by high-performance liquid chromatography.

3:00 P. Roch, N. Davant and M. Lassegues (Centre de Morphologie Expérimentale de CNRS and Département de Biologie du Développement de l'Université de Bordeaux, Talence, France) – Isolation of agglutinins from lysins in earthworm coelomic fluid by gel filtration then chromatofocusing.

3:20 A. Niederwieser, W. Staudenmann and E. Wetzel (Department of Pediatrics, University of Zurich, Zurich, Switzerland) – HPLC with column switching for analysis of pterins and biogenic amine metabolites.

3:40 Intermission

*V. Berry, presiding*

4:00 J. Wagner, C. Danzin, M. Palfreyman and S. Huot-Olivier (Centre de Recherche Merrell International, Strasbourg, France) – High-performance liquid chromatographic analysis of S-adenosylmethionine and its metabolites in rat tissues: interrelationship with changes in catechol levels following treatments with L-Dopa.

4:20 P. Padovani, G. Bianchetti, J. Fraisse-Andre, P.L. Morselli and J.P. Thenot (Department of Clinical Research, LERS-Synthelabo, Meudon la Foret, France) – Determination of progabide and its major metabolites by HPLC and negative-ion mass spectrometry.

4:40 P. Moree-Testa and Y. Saint-Jalm (S.E.I.T.A. Département Biologie-Chimie, Paris, France) – Qualitative and quantitative study of imidazole derivatives in cigarette smoke.

5:00 R.V. Golovnya and D.N. Grygoryeva (Institute of Organo-Element Compounds of USSR Academy of Science, Moscow, U.S.S.R.) – Application of energy equivalent to $\Delta I$ for identification of organic compounds.

THURSDAY, OCTOBER 6

Thursday Morning
Esperance Room

## CHROMATOGRAPHY – GENERAL

*E.R. Adlard, presiding*

9:00 N. Ikekawa (Tokyo Institute of Technology, Tokyo, Japan) – Analysis of natural brassino steroids by gas chromatography and gas chromatography–mass spectrometry.

9:20 M.A. Spain and B.S. Middleditch (University of Houston, Houston, TX, U.S.A.) and D. Bafus and E. Galen (Northrop Services, NASA, Houston, TX, U.S.A.) – Generation of toxic substances in enclosed recirculating atmospheres.

9:40 Th. Kuster, A. Niederwieser and A. Matasovič (Department of Pediatrics, University of Zurich, Zurich, Switzerland) – Application of GC–MS for the study of biopterin metabolism in man.

10:00 H.-J. Stan and B. Steinbach (Institut für Lebensmittelchemie, Technische Universität Berlin, Berlin, G.F.R.) – Automated development of temperature programs for optimum separation of complex mixtures on capillary columns by means of a computer.

10:20 J. Sevcik (Analytical Laboratory Systems, Deidesheim, G.F.R.) – Application of microwave desorber to analysis of volatiles.

10:40 Intermission

*C.F. Poole, presiding*

11:00 A.B. Attygalle and E.D. Morgan (University of Keele, Staffordshire, U.K.) – Chemical reactions with nanogram quantities of compounds collected from GC effluent.

11:20 K. Jacob and W. Vogt (Institut für Klinische Chemie, Universität München, Munich, G.F.R.) – Sensitive mass fragmentographic determination of acidic catecholamine metabolites in human body fluids.

11:40 G. Schomburg, U. Häusig and E. Ziegler (Max-Planck-Institut für Kohlenforschung, Mülheim-Ruhr, G.F.R.) – The application of "COLACHROM" a new COmmand LAnguage for CHROMatography, data processing within the Mülheim computer system.

12:00 T.K. Korpela and J.-P. Himanen (Department of Biochemistry, University of Turku, Turku, Finland) – Determination of equilibrium constants with gel chromatography binding of small molecules into cyclodextrins.

12:20 N.A. Katsanos and G. Karaiskakis (University of Patras, Patras, Greece) – Measurement of rate coefficients by the reversed-flow gas chromatography technique.

12:40 B.V. Ioffe (Leningrad State University, Leningrad, U.S.S.R.) – New applications of gas chromatographic headspace analysis.

1:00 K.I. Sakodynskii (Institute of Physical Chemistry, Moscow, U.S.S.R.) – Investigation of polyfunctional polymer sorbents.

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# MASS SPECTROMETRY ADVANCES 1982

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**Plenary Lectures:** Unimolecular and photo-induced ion fragmentation *(J. H. Beynon)*. Analysis of solids and surfaces by mass spectrometry *(B. Blanchard)*. New instrumentation in mass spectrometry *(C. Brunnée)*. Isotopic analyses of inorganic and organic substances by mass spectrometry *(K. Heumann)*. New ionization techniques in mass spectrometry *(D. F. Hunt)*. Fundamental aspects of electron impact ionization *(T. Märk)*. Aspects and prospects of structure elucidation of organic compounds by mass spectrometry today *(J. Seibl)*. Twenty **Keynote Lectures** reviewed different aspects of the field. **Contributed Papers** were divided over the following sections: Instrumentation. Combination of Techniques (GC/MA, LC/MA, MS/MS). Theory of Gaseous Ions. Ionization Processes. Neutral Impact Particle and Ion Impact (FAB and SIMS). Pyrolysis and Laser Ionization. Physical Chemistry of Ion Decay. Ion Molecule Reactions. Negative Ions. High temperature Mass Spectrometry. Structure Elucidation and Fragmentation Mechanisms. General Chemical Applications. Biochemical and Medical Applications. Applications in Environmental and Food Chemistry. Isotope Ratios, Geological and Nuclear Applications. **Author Index. Subject Index.**

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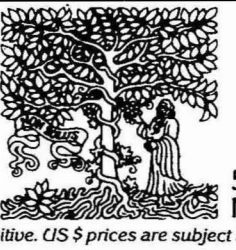

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# ATOMIC ABSORPTION SPECTROMETRY

*edited by*
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**Techniques and Instrumentation in Analytical Chemistry Vol. 5**

Atomic absorption spectroscopy is now established as one of the most useful tools for analysing trace metals in samples which may be taken into solution. It has wide applicability, is inexpensive and can be used with confidence by a wide range of analysts. The rapid growth and advancement of electrothermal atomisation methods and their subsequent automation has consolidated the technique's position by extending the dynamic analytical range down to concentration levels that other techniques cannot reach.

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## PUBLICATION SCHEDULE FOR 1983

*Journal of Chromatography* (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

| MONTH | D 1982 | J | F | M | A | M | J | J | A | |
|---|---|---|---|---|---|---|---|---|---|---|
| Journal of Chromatography | 252<br>253/1<br>253/2 | 254<br>255<br>256/1 | 256/2<br>256/3<br>257/1 | 257/2<br>258<br>259/1 | 259/2<br>259/3<br>260/1 | 260/2<br>261/1<br>261/2 | 261/3<br>262<br>263 | 264/1<br>264/2<br>264/3<br>265/1 | 265/2<br>266 | The publication schedule for further issues will be published later. |
| Chromatographic Reviews | | | | | 271/1 | | 271/2 | | 271/3 | |
| Biomedical Applications | | 272/1 | 272/2 | 273/1 | 273/2 | 274 | 275/1 | 275/2 | 276/1 | |

## INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 244, No. 2, pp. 401–404. A free reprint can be obtained by application to the publisher.)

**Types of Contributions.** The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

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