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INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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JOURNAL OF CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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5TH SYMPOSIUM ON HANDLING OF ENVIRONMENTAL AND BIOLOGICAL SAMPLES IN CHROMATOGRAPHY

Baden-Baden (Germany), September 26–27, 1991

Guest Editor

K. ZECH

(Konstanz)

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Foreword

The 15th Symposium on "Handling of Environmental and Biological Samples in Chromatography" was held on September 26th and 27th, 1991, in Baden-Baden, Germany. It was the continuation of a series of very successful meetings initiated by the late Professor Roland Frei. The event was organized following in his footsteps, to bring together experts and practising analytical chemists for exchanges of both the state-of-the-art in this fast-growing discipline and first-hand experience in practical sample handling.

Every aspect of all of the modern sample handling methods was discussed, including more recent developments such as liquid membranes, electrophoresis, electrodialysis, supercritical fluid extraction and the on-line combination of liquid and gas chromatography. Special emphasis was placed on automation. Twenty-one keynote lectures and 40 posters (limited to this number) were presented.

Twenty-five companies took part in the instrument exhibition and also contributed to a Short Course on the same subject which preceded the Symposium.

Karl Zech as Chairman welcomed more than 150 participants to the Symposium; the Short Course, limited to 30 participants, was overbooked.

The Symposium was held under the auspices of the International Association of Environmental Analytical Chemistry. The organizers are especially grateful to Marianne Frei, Secretary of IAEAC, whose professional preparation ensured a successful and smooth-running meeting. The next Symposium will be held in Guildford, UK, on July 19–21, 1993, in cooperation with the Chromatographic Society and the University of Surrey.

K. Zech
G. J. de Jong
K.-P. Hupe

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Electrodialytic sample treatment coupled on-line with column liquid chromatography for the determination of basic and acidic compounds in environmental samples

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ABSTRACT

The use of electrodialytic sample treatment coupled on-line with column liquid chromatography for environmental samples is described. The influence of the ionic strength on analyte recovery and the linearity and repeatability of the method were studied using anthraquinone-1,8-disulphonic acid and paraquat as model compounds. The completely automated procedure allows 10–20-fold selective enrichment of the analytes from 0.5–1.0-ml samples within 20 min. A group of sulphonic acids and the basic compounds paraquat and diquat were determined in ground and surface water samples.

INTRODUCTION

Dialysis is a well known technique for the separation of compounds of different molecular size. Coupled to column liquid chromatography (LC), dialysis can be used for on-line sample treatment [1–5]. In that case low-molecular-weight analytes diffuse through a high-molecular-weight cut-off membrane from the sample (donor) solution to an acceptor solution. However, if efficient and/or complete mass transfer in dialysis is required, dilution of the sample is inevitable. Consequently, a precolumn is needed to concentrate the analytes again before introduction into the LC system.

Electrodialytic sample treatment (EDIST) coupled on-line with LC is an alternative method for the isolation and enrichment of charged compounds from complex aqueous samples. The principle has been described recently [6]. The separation of analyte and matrix constituents is based on differences in molecular size and in electric charge. Transfer of analytes from the sample (donor) solution to the acceptor phase is achieved by both diffusion and electromigration. A laboratory-made block was used which contains various spacers and membranes which create donor, acceptor and electrode compartments. The donor and acceptor compartments (channels) are separated by a high-molecular-weight

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cut-off membrane (separation membrane). The flux, J, of (low-molecular-weight) analytes through the separation membrane is given by the Nernst–Planck equation:

$$J = J_{diff} + J_{migr} = -D\left(\frac{dC}{dx}\right) - \left(\frac{DCzF}{RT}\right)\left(\frac{dV}{dx}\right) \quad (1)$$

where J_{diff} and J_{migr} are the fluxes due to diffusion and migration, respectively, D is the diffusion coefficient of the analyte, x the coordinate perpendicular to the membrane, C the analyte concentration, z the valency of the analyte, F the Faraday constant, R the gas constant, T the temperature and dV/dx the electric field strength across the membrane.

When a voltage is applied to such a system, charged analyte molecules start to migrate from the donor to the acceptor phase (J_{migr}). As the concentration of the analyte in the acceptor phase increases, diffusion of analyte into the donor phase will occur; that is, J_{migr} will be counteracted by J_{diff}. When equilibrium has been reached ($J_{diff} = -J_{migr}$), the analyte concentration in the acceptor phase has reached its maximum value. In a previous study, with ephedrine as a model compound, 200 μl of sample could be treated in 10 min with a flowing donor and a stagnant acceptor phase, the enrichment being about tenfold. It was also shown that selective enrichment from blood plasma samples can be obtained. However, owing to the high conductivity of plasma, the enrichment was lower than that obtained with purely aqueous samples.

In this study, EDIST was used for the determination of basic and acidic compounds in ground and surface waters. Such samples usually have a lower ionic strength than biological samples, which should facilitate analyte enrichment. Further with the present automated EDIST system, larger sample volumes can be pretreated in the same time. Anthraquinone-1,8-disulphonic acid and several other sulphonic acids, which are used in different production processes, *e.g.*, for dyes and detergents, are often determined in river water. The sulphonic acids are usually determined by means of an ion-pair LC separation with UV detection at 220–230 nm because fluorescence detection is possible for only a few of them. Sample clean-up and enrichment are achieved by laborious off-line solid-phase extraction using an ion-pair reagent [7]. At the low UV wavelengths used, however, many interferences still show up in the chromatograms. The herbicides paraquat and diquat, which have found extensive industrial and domestic application as weedkillers, are used to study the determination of basic compounds in environmental samples. For their reversed-phase LC determination, complicated and time-consuming off-line sample treatment methods [8] are usually required because solid-phase enrichment of these strongly ionic compounds is difficult, especially if analysis has to be carried out at low-ppb levels.

EXPERIMENTAL

Chemicals

Acetonitrile, methanol, diethylamine, phosphoric acid, sodium dihydrogenphosphate, disodium hydrogenphosphate, sodium chloride and phosphoric acid (85%) were purchased from J. T. Baker (Deventer, Netherlands). Hexanesulphonic acid was obtained from Eastman Kodak (Rochester, NY, USA) and tetrabutylammonium bromide from Aldrich Chemie (Steinheim, Germany). Anthraquinone-1,8-disulphonic acid was a gift from the Institute for Inland Water Management and Waste Water Treatment (RIZA, Lelystad, Netherlands), paraquat and diquat were from the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, Netherlands) and 1-amino-2-naphthol-4-sulphonic acid, *p*-toluenesulphonic acid and 4-nitrotoluene-2-sulphonic acid from Dr. W. Giger (Eidgenössische Anstalt für Wasserversorgung, Dübendorf, Switzerland). Paraquat solutions were prepared and stored in poly(vinyl chloride) vessels. Pooled Rhine water and pooled ground water samples were used without any further clean-up. The water sample from the river Rhine contaminated with anthraquinone-1,8-disulphonic acid was sampled by RIZA at Lobith on January 14th, 1991.

Chromatography

The LC system consisted of a Spectroflow 400 pump (Kratos, Ramsey, NJ, USA), a Spectroflow 757 UV detector and a 15 cm × 4.6 mm I.D. stainless-steel column packed with 5-μm RoSil C_{18} (Research Separation Labs., Eke, Belgium). The mobile phase for the paraquat and diquat analysis

consisted of 100 ml of acetonitrile, 900 ml of water, 3.64 g of hexanesulphonic acid, 10 ml of diethylamine and 16 ml of concentrated phosphoric acid, at a flow-rate of 0.7 ml/min. With the sulphonic acids, methanol–water (33:67, v/v) containing 1 m*M* tetrabutylammonium bromide and 0.02 *M* phosphate buffer (pH 6.5) was the LC eluent (flow-rate 0.8 ml/min). Peak areas and peak heights were measured with an HP 3396 A integrator (Hewlett-Packard, Waldbronn, Germany).

A Philips (Eindhoven, Netherlands) PW 9561 conductivity meter was used to measure the conductivity of the various sample solutions.

RESULTS AND DISCUSSION

Instrumental development

Electrodialysis block. The electrodialysis block consists of a set of spacers and membranes, held between two Perspex blocks which contain the electrode compartments (Fig. 1). Each compartment holds a coiled platinum wire electrode. Two extra spacers are used for mechanical support (not shown in Fig. 1). Ion-exchange membranes (Thomapor, Reichelt Chemie Technik, Heidelberg, Germany) are used to separate the acceptor and donor channels (equal volumes, each *ca.* 50 μl) from the electrode compartments. The donor and the acceptor phases are separated by a 10 000–15 000 molecular-weight cut-off membrane (Gilson, Villiers-le-Bel, France). The spacers of the EDIST block are made from PTFE sheets; PEEK (polyether ether ketone) tubing is used for the capillary connections.

The EDIST block is coupled on-line to an LC system by means of an automated six-port switching valve with a fixed injection loop. After electrodialytic treatment of the sample, the contents of the acceptor channel are transferred to the injection loop (volume 200 μl) by means of a syringe. This volume is acceptable, as band broadening is slightly reduced in the LC system by peak compression of the analytes in the usually aqueous injected zone on the top of the analytical column. Using a volume of 125 μl to transfer the contents of the acceptor channel (50 μl) to the injection loop, the highest recoveries (85%) were obtained. The same recoveries were obtained in experiments (data not shown here) where the EDIST block was replaced with a 50-μl loop, which means that the non-quantitative recovery is due to the incomplete transfer of the acceptor phase.

Compared with the EDIST block described in a previous paper [6], the new block has two major advantages. First, the *ca.* twofold larger volume of both the donor and acceptor channels of the new block allows the treatment of larger sample volumes. In order to obtain maximum recovery, a donor flow-rate of at best 25 μl/min could be used with the earlier block. In that event, the residence time of the analytes in the donor compartment (volume *ca.*

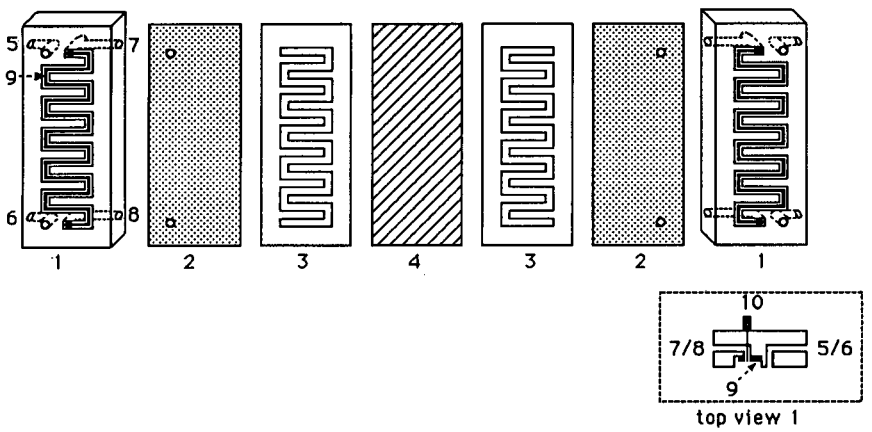

Fig. 1. Electrodialysis block. 1, Electrode vessel containing electrode compartment (meander); 2, ion-exchange membrane; 3, PTFE spacer with 50-μl flow channel; 4, separation membrane; 5, donor/acceptor outlet; 6, donor/acceptor inlet; 7, outlet electrode compartment; 8, inlet electrode compartment; 9, platinum electrode; 10, electrode connection.

20 μl) was *ca.* 1 min. For higher flow-rates the residence time was too short, which resulted in an incomplete recovery, *i.e.*, analyte was lost. In the present set-up, the volume of the donor compartment is *ca.* 50 μl and donor flow-rates of up to 50 μl/min can now be used. That is, the sample throughput is about twofold larger with the new block. Second, the electrode compartments of the present EDIST block are flushed continuously with an aqueous phosphate buffer at a flow-rate of *ca.* 1 ml/min. With the previous block only five or six electrodialysis experiments (10 min electrodialysis; 7.5 V) could be carried out without exhausting the buffer solution (0.1 *M* phosphate) [6]. With the present block, the continuous flushing of the electrode compartments with buffer solution prevents fouling and exhaustion of the buffer, that is, the number of experiments that can be performed without interruption is in principle limited only by the lifetime of the membranes.

With the basic drug ephedrine as a model compound, the influence of the electrode buffer concentration on analyte recovery was studied. Maximum recovery (*i.e.*, 85%) was obtained with 0.1–0.5 *M* phosphate buffer (pH 7). The use of 0.02 *M* phosphate buffer resulted in a decreased analyte recovery of only 30%. Probably the potential drop in the electrode compartments during electrodialysis was too high in the latter instance, causing a low electrical field strength in the acceptor and donor compartments and so a decreased J_{migr}. In all further work 0.1 *M* phosphate buffer solution (pH 7) was used to flush the electrode compartments.

EDIST set-up. The EDIST experiments were performed using a set-up similar to that described previously [6]. Fig. 2 shows the complete set-up. In this study, the EDIST procedure was fully automated by using an ASTED (Gilson) autosampler unit. Comparable results were obtained with two modified HP 1050 autosamplers (Hewlett-Packard), one for sample introduction and one for acceptor phase transfer.

In an EDIST run (coupled on-line to LC), several steps can be discerned (see Table I and Fig. 2). First, after loading the programme the donor pump (pump 1) flushes the EDIST unit with demineralized water and a reversed voltage is applied (reversed to the voltage applied during EDIST) in order to remove contaminants introduced during the previous run, from the separation membrane surface and the acceptor channel (PREP). Using this procedure, no clogging of the analytical system was observed during the lifetime of the membrane (*ca.* 200 analyses). After this flushing step, an automated syringe (syringe 1) injects sample into a sample loop which is attached to a six-port valve (sample valve) (LOAD).

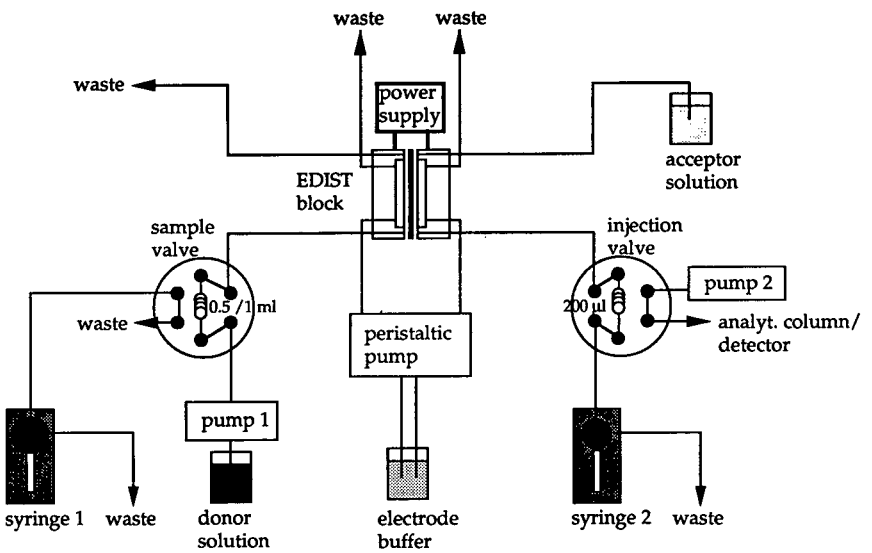

Fig. 2. Schematic set-up of the EDIST system with the sample valve and injection valve in the electrodialysis (ED) position.

TABLE I

TIME SCHEDULE FOR ELECTRODIALYTIC SAMPLE TREATMENT

Conditions: sample, 1.0 ml; donor flow-rate, 50 μl/min; acceptor, stagnant; electrodialysis time, 20 min.

Time (min)	Event	Step
0.0	Programme loading	
0.1	Pump 1 pumps donor solution; syringe 2 draws acceptor solution; reversed voltage on	PREP
3.0	Reversed voltage off; syringe 1 draws sample and injects sample in sample loop	LOAD
4.0	Sample valve switched; voltage applied	ED
24.0	Voltage switched off; acceptor phase drawn into injection loop	
24.1	Injection valve switched	ELUTE
25.0	End of run; start of next run	

Next, the sample valve is switched, the selected voltage is applied and the contents of the sample loop are flushed into the electrodialysis block by the donor pump (ED). After electrodialysis, a second automated syringe (syringe 2) transfers the contents of the acceptor channel to the injection loop which is attached to another six-port valve (injection valve). In the final step the injection loop is switched in-line between the LC pump (pump 2) and the analytical column and separation is performed (ELUTE). While one sample is being analysed, the next sample can be pretreated.

Determination of paraquat and diquat

If cationic compounds have to be determined, anion-exchange membranes are used to separate the electrode compartments from the acceptor/donor channels. If a voltage is applied only small anions will pass through the membrane, providing the electric current, while the cationic analytes will be retained in the acceptor phase.

During EDIST of a sample the analyte is enriched in the acceptor phase. This analyte enrichment can be expressed by means of the enrichment factor (*EF*) [6]:

$$EF = C_a/C_s \quad (2)$$

where C_a is the analyte concentration in the acceptor phase after (electro)dialysis and C_s is the initial analyte concentration in the sample (donor) solution. *EF* will be maximum when $J_{diff} = J_{migr}$ (see eqn. 1). Fig. 3 shows the dependence of *EF* for paraquat on the time of electrodialysis for two different donor flow-rates. The time of electrodialysis has been limited to 20 min because longer times would make the total time of analysis too long. As time increases more sample is introduced into the donor channel and more analyte will migrate to the acceptor channel. When a sample in 0.01 *M* phosphate buffer containing 0.01 *M* NaCl is used and the donor flow-rate is 50 μl/min, the equilibrium concentration in the acceptor solution is not reached within the time allotted. In other words, $|J_{diff}|$ still is smaller than $|J_{migr}|$. One way to increase *EF* is, of course, to increase the donor flow-rate. Fig. 3, however, shows that increasing the donor flow-rate from 50 to 100 μl/min does not cause a twofold increase in *EF* over the complete time interval. Probably the residence time of the sample in the donor channel is too short for complete analyte transfer from the donor to the acceptor phase. Small fluctuations in sample composition (ionic strength) may well cause irreproducible results under such conditions.

Since the electric field strength in the donor/acceptor solutions decreases with increasing ionic strength (the ionic strength in the electrode compartments being constant), the migration flux, J_{migr}, of the analyte from the donor phase to the acceptor phase and, therefore, the total flux, *J*, will be larger

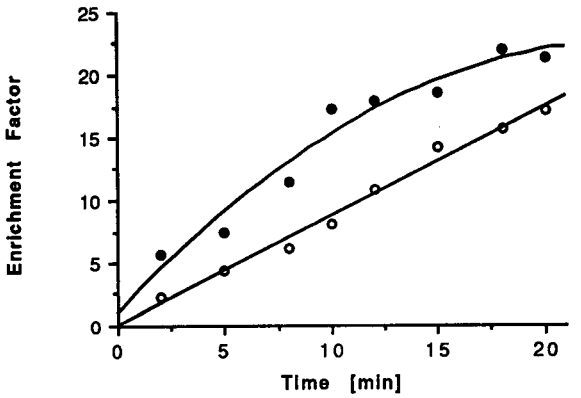

Fig. 3. Dependence of *EF* on the time of electrodialysis for a 1 ppm paraquat sample solution containing 0.01 *M* NaCl and 0.01 *M* phosphate buffer (pH 7). Donor flow-rate: (○) 50; (●) 100 μl/min. Voltage: 7.5 V. Each point represents the mean of two experiments.

TABLE II

DEPENDENCE OF RECOVERY OF PARAQUAT ON SAMPLE COMPOSITION

Sample (donor) flow-rate, 50 µl/min; $n = 2$; for further conditions, see text.

10 ppb paraquat solution in	Recovery (%)		Conductivity ($m\Omega^{-1}$)
	0.5 ml of solution	1.0 ml of solution	
0.01 *M* NaCl	57; 63	50; 70	1.1
0.02 *M* NaCl	56; 64	49; 71	2.3
0.05 *M* NaCl	50; 70	33; 47	5.2
Ground water	55; 65	48; 62	1.0

at low sample ionic strength. As the analyte is present, in most instances, in low concentration ($<10^{-6}$ *M*), the ionic strength will mainly be determined by the matrix constituents. The influence of the ionic strength on analyte recovery is shown in Table II. For solutions with low conductivity (0.01 *M* NaCl), which are comparable to the ground water samples, 60% recovery is obtained for both 0.5- and 1.0-ml sample solutions. The highest recovery found for samples containing paraquat is 60%. This is lower than the 85% recovery found with the model compound ephedrine and with anthraquinone-1,8-disulphonic acid. The low recovery is possibly caused by sorption of paraquat to capillaries or compartment walls. This will lead to an incomplete transfer of the analyte from the acceptor

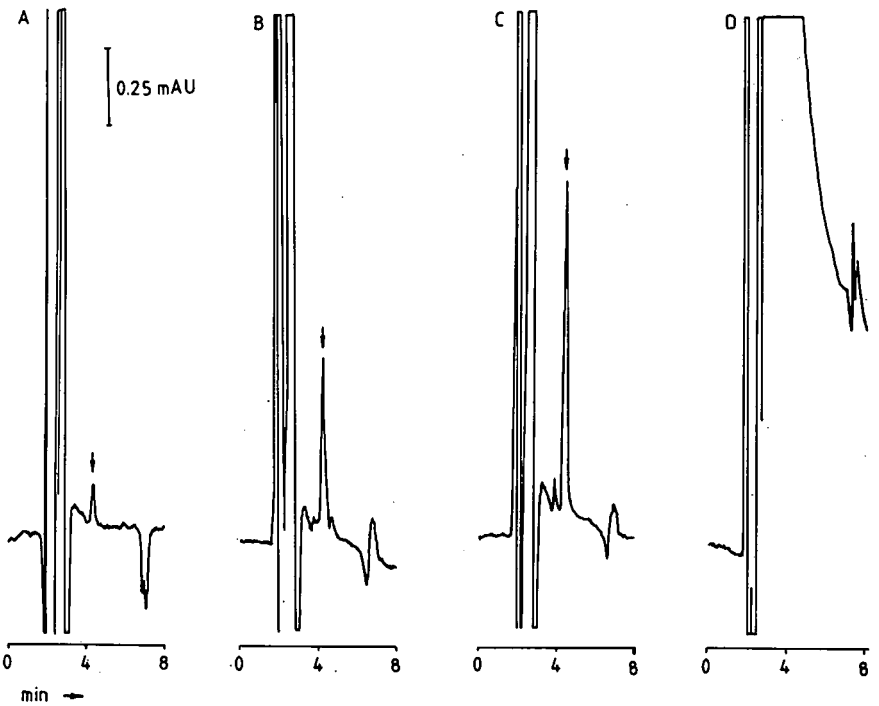

Fig. 4. LC–UV of ground water samples spiked with 10 ppb of paraquat. (A) 0.5-ml sample after dialysis (0 V); (B) 0.5-ml sample after EDIST (7.5 V); (C) 1.0-ml sample after EDIST (7.5 V); (D) direct injection of 200 µl of sample. For LC conditions, see text; UV detection at 254 nm. The arrow indicates the paraquat peak. Blank samples do not show a peak at the position of paraquat.

TABLE III

ANALYTICAL DATA FOR DETERMINATION OF PARAQUAT IN GROUND WATER

Parameter	Sample volume (ml)	
	0.5	1.0
Calibration plot (5–100 ppb)[a]	$y = 0.03 + 0.09x$	$y = -0.02 + 0.2x$
Regression coefficient (r^2)	0.996	0.992
R.S.D.[b] (%) ($n = 8$)	4	7
Detection limit (ppb) (S/N = 2)[c]	1	0.5

[a] y = Peak-height ratio; x = concentration.
[b] R.S.D. = Relative standard deviation.
[c] S/N = Signal-to-noise ratio.

channel to the injection loop. Increasing the ionic strength and, thus, the electric conductivity by a factor of two does not cause a decrease in recovery. At a fivefold higher ionic strength (0.05 *M* NaCl), however, a distinct loss is observed for sample volumes of 1.0 ml. At these high ionic strengths J_{migr} is smaller and an equilibrium between J_{diff} and J_{migr} is obtained, that is, plateau conditions are reached already for electrodialysis times of less than 20 min, *i.e.*, for sample volumes smaller than 1.0 ml. On prolonging the electrodialysis, no further enrichment of analyte will take place. For sample volumes of 0.5 ml the recovery is still 60%, indicating that after 10 min of electrodialysis plateau conditions obviously have not been reached.

LC–UV of spiked ground water samples with and without EDIST is shown in Fig. 4. Compared with the direct injection of ground water (Fig. 4D), the pretreatment by electrodialysis (Fig. 4B and C) shows its considerable advantage in terms of selectivity and enrichment. The enrichment which can be achieved by using EDIST is further demonstrated by comparing Fig. 4C and A, which shows a chromatogram obtained by dialysis (applied voltage 0 V; flowing donor, stagnant acceptor). Finally, it is interesting to compare the peak heights in Fig. 4B and C; obviously, 20-min EDIST of the paraquat-containing sample indeed yields twice the enrichment found after 10-min EDIST (5 ppb of paraquat; donor flow-rate 50 · μl/min, stagnant acceptor; 7.5 V).

Data on the linearity (concentration range 5–100 ppb) and the repeatability of EDIST are given in Table III for 10 min (0.5 ml of sample) and 20 min (1.0 ml of sample) of sample treatment. As is to be expected, the slope of the plot for 1.0 ml of sample is about twice as large as that for 0.5 ml of sample. With the present set-up the detection limit for paraquat is 0.5 ppb. With pretreatment methods for ground water samples involving the use of pre-

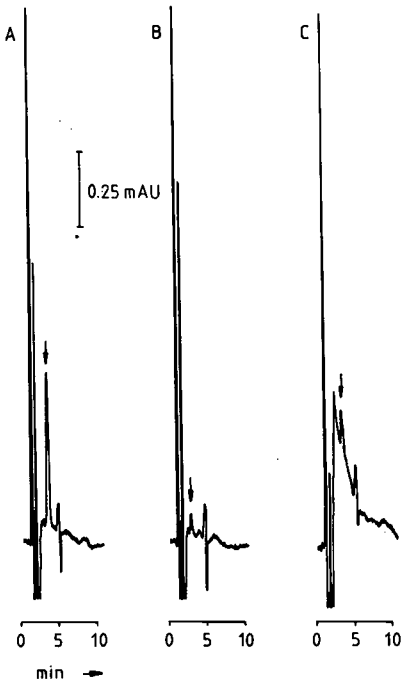

Fig. 5. LC–UV of ground water samples spiked with 10 ppb of diquat. (A) 1.0-ml sample after EDIST (7.5 V); (B) 0.5-ml sample after dialysis (0 V); (C) direct injection of 200 μl of sample. For LC conditions, see text; UV detection at 310 nm. The arrow indicates the diquat peak. Blank samples do not show a peak at the position of diquat.

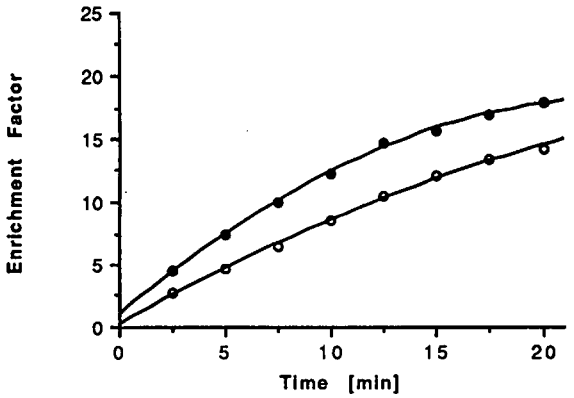

Fig. 6. Dependence of *EF* on the time of electrodialysis for a 1 ppm ADS sample solution containing 0.01 *M* NaCl and 0.01 *M* phosphate buffer (pH 7). Donor flow-rate: (○) 50; (●) 100 μl/min. Voltage: 7.5 V. Each point represents the mean of two experiments.

columns, irreproducible results are generally obtained even at 10–20-fold higher concentration levels [9]. In addition, these methods, which usually are off-line, are difficult to automate.

Fig. 5 shows the LC–UV determination of diquat in ground water using (A) EDIST, (B) conventional dialysis and (C) a direct injection of 200 μl of ground water. As the optimum detection wavelength for diquat is 310 nm, which is a more selective wavelength than the 254 nm used with paraquat, the direct-injection chromatogram shows less interferences. The selectivity gain is, however, still obvious and the enrichment due to EDIST is clearly demonstrated by comparing Fig. 5A and B.

Determination of sulphonic acids

For EDIST of acidic compounds, cation-exchange membranes have to be used to prevent migration of the anionic analytes into the electrode compartments. The dependence of *EF* for anthraquinone-1,8-disulphonic acid (ADS) on the time of electrodialysis for two donor flow-rates is shown in Fig. 6. It is evident, as with paraquat, that although *EF* increases, the recovery decreases at higher donor flow-rates. Table IV shows data on analyte recovery as a function of the ionic strength of the sample solution. Apart from the higher maximum recovery (85% *vs.* 60%; see above), the results are closely similar to those found with paraquat. Substantial losses start to occur for 0.05 *M* NaCl solutions and relatively large, *i.e.*, 1.0-ml, sample solutions, indicating that plateau conditions are reached.

Analytical data on the linearity (concentration range 5–100 ppb) and the repeatability of the determination of ADS in river Rhine water are shown in Table V. The detection limit of *ca.* 2 ppb is essentially the same as that reported using off-line solid-phase extraction [7]. The main advantage of EDIST is, of course, the automation potential.

LC–UV of spiked Rhine water samples is shown in Fig. 7. The accuracy of the method is demonstrated by the equal height of the ADS peaks obtained after 10-min EDIST of a 0.5-ml sample containing 20 ppb of ADS (Fig. 7A) and 20-min EDIST of a 1.0-ml sample containing 10 ppb of ADS (Fig. 7B). The number of interferences in the chromatogram is larger than with the paraquat samples. Probably negatively charged low-molecular-weight humic and fulvic acids, which are pres-

TABLE IV

DEPENDENCE OF RECOVERY OF ANTHRAQUINONE-1,8- DISULPHONIC ACID ON SAMPLE COMPOSITION

Sample (donor) flow-rate, 50 μl/min; $n = 2$; for further conditions, see text.

10 ppb ADS sample solution in	Recovery (%)		Conductivity ($m\Omega^{-1}$)
	0.5 ml of solution	1.0 ml of solution	
0.01 *M* NaCl	83; 87	82; 88	1.1
0.02 *M* NaCl	83; 87	79; 91	2.3
0.05 *M* NaCl	83; 87	50; 60	5.2
Rhine water	83; 87	81; 89	1.1

TABLE V

ANALYTICAL DATA FOR DETERMINATION OF ANTHRAQUINONE-1,8-DISULPHONIC ACID IN RIVER RHINE WATER

Parameter	Sample volume (ml)	
	0.5	1.0
Calibration plot (5–100 ppb)[a]	$y = -0.02 + 0.02x$	$y = 0.21 + 0.04x$
Regression coefficient (r^2)	0.997	0.996
R.S.D. (%) ($n = 8$)	5	3
Detection limit (ppb) (S/N = 2)	2.5	1.5

[a] y = Peak-height ratio; x = concentration.

ent in large amounts in river Rhine water, are transferred to the acceptor phase together with ADS. This explanation is supported by the fact that the interfering peaks in the chromatogram are larger when a 1.0-ml instead of a 0.5-ml sample is analysed (Fig. 7B *vs.* Fig. 7A). With the positively charged paraquat, the humic and fulvic acids of course migrate in the opposite direction and will not reach the acceptor phase. Nevertheless, comparison with the chromatogram from direct injection of a spiked Rhine water sample (Fig. 7D) adequately illustrates the potential of EDIST.

The determination of ADS in a real sample is shown in Fig. 7C. The sample was taken from the river Rhine at Lobith on January 14th, 1991. Analyses of a number of water samples from the same river (December 1990–January 1991) performed by the RIVM showed the presence of ADS in

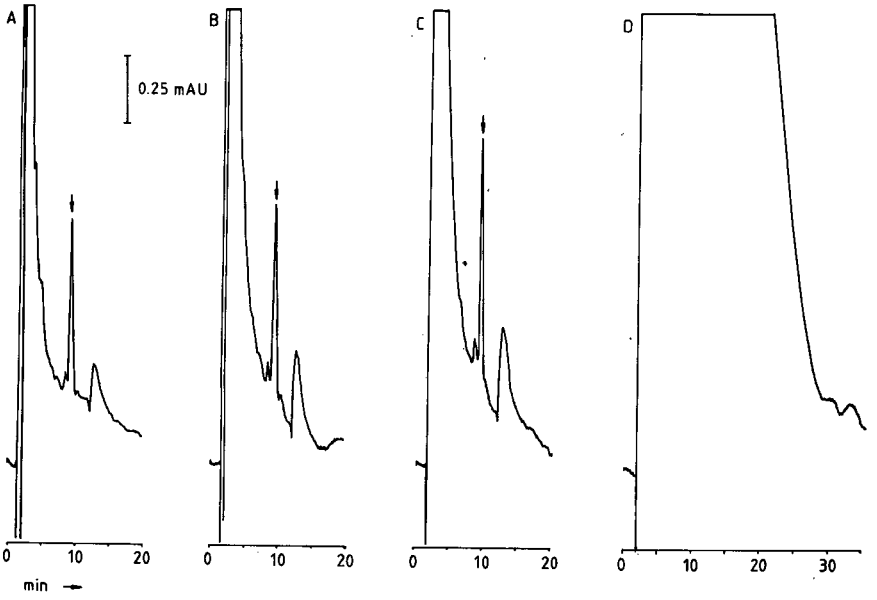

Fig. 7. LC–UV of ADS-spiked Rhine water samples after EDIST (7.5 V). (A) 0.5-ml sample spiked with 20 ppb; (B) 1.0-ml sample spiked with 10 ppb; (C) 1.0-ml sample taken at Lobith (January 14th, 1991). For comparison: (D) direct injection of 200 μl of Rhine water spiked with 10 ppb. For LC conditions, see text; UV detection at 254 nm. The arrow indicates the ADS peak. Blank samples do not show a peak at the position of ADS.

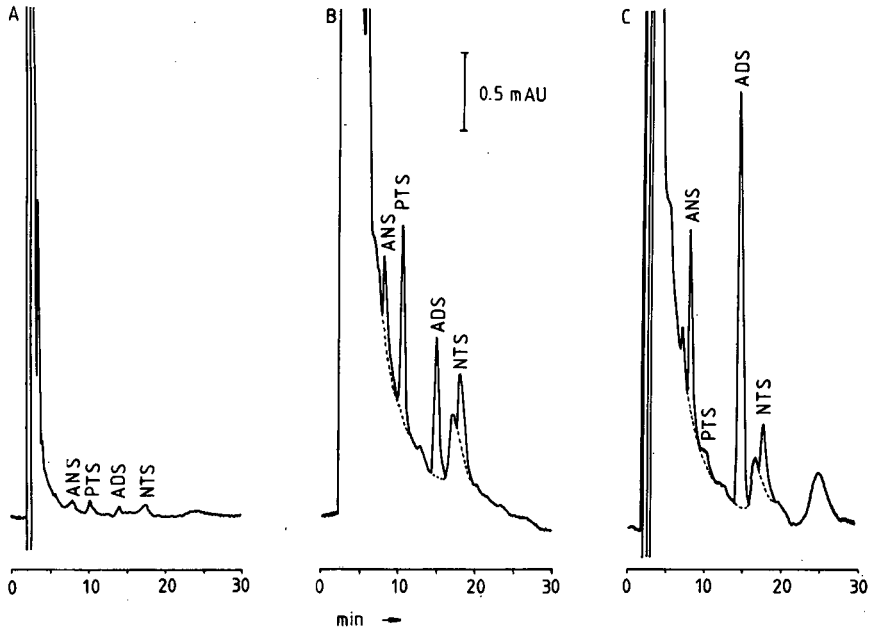

Fig. 8. LC–UV of Rhine water spiked with 50 ppb of a mixture of four sulphonic acids: 1-amino-2-naphthol-4-sulphonic acid (ANS), *p*-toluenesulphonic acid (PTS), anthraquinone-1,8-disulphonic acid (ADS) and 4-nitrotoluene-2-sulphonic acid (NTS). (A) Dialysis of 1.0-ml sample; (B) EDIST (7.5 V) of 1.0-ml sample, UV detection at 230 nm; (C) EDIST (7.5 V) of 1.0-ml sample, UV detection at 254 nm. Dashed lines, blank run. For LC conditions, see text.

the river water at that time, probably as the result of an accidental spill. The concentration levels determined using solid-phase extraction were comparable to that found by us in the sample shown in Fig. 7C (14 ppb).

As a further illustration of the potential and limitations of on-line EDIST and LC–UV, Fig. 8 shows the analysis of a mixture of four sulphonic acids. Compared with conventional dialysis (Fig. 8A), EDIST effects an enrichment of the negatively charged analytes (Fig. 8B); the gain in selectivity, however, is unsatisfactory. For two compounds, ADS and amino-2-naphthol-4-sulphonic acid, both the selectivity and sensitivity could be improved considerably by changing the detection wavelength from 230 to 254 nm (Fig. 8C).

CONCLUSIONS

EDIST is an effective sample treatment method for the clean-up and enrichment of anionic and cationic analytes from river and ground water samples prior to LC. The on-line coupling to LC is easy to automate, which makes the method suitable for routine analysis. EDIST can be used as a fast screening method at low concentration levels; it displays good linearity and repeatability. The method is especially suited for the determination of basic compounds in environmental samples. When determining acidic compounds, humic and fulvic acids appear to interfere to some extent. Further study is required in this area, using for instance other types of membranes, *e.g.*, with a lower molecular-weight cut-off.

The low ionic strength of environmental water samples allows enrichment factors of up to 20 to be obtained. With the present EDIST unit, sample volumes of *ca.* 1 ml can be treated within 20 min (Fig. 3). The on-line combination of EDIST with other sample treatment methods, such as solid-phase extraction, may well allow the treatment of larger sample volumes in order to obtain the low detection limits often required in trace analysis. Current research is devoted to this area, and also to the optimization of EDIST for the treatment of biological fluids, with which enrichment factors of up to 10 have already been realized.

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Optimization of supercritical fluid extraction of volatile constituents from a model plant matrix

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ABSTRACT

α-Cellulose has been used as a model plant matrix to investigate the conditions required to optimize the supercritical fluid extraction of typical plant constituents, limonene, caryophyllene, carvone, eugenol and santonin, using carbon dioxide as the extraction medium. The conditions required for the successful recovery of the analytes were monitored by gas–liquid chromatography. Timed recovery studies enabled differences in the rates of extraction to be determined to ensure that sufficiently long extraction runs were used. Subcritical and supercritical extractions over the ranges − 10 to 80°C and 50–250 bar were examined and 250 bar and 40°C were chosen as the optimum conditions. The effects of the addition of modifiers to the supercritical fluid were also examined. The work also demonstrated that increased selectivity for polar analytes such as lactones could be obtained by trapping the extract on a silica column coupled with selective elution.

INTRODUCTION

Supercritical fluid extraction (SFE) has been used for many years for the extraction of volatile components such as essential oils, aromas and flavours from plant materials on an industrial scale [1,2]. Recently, the application of this technique on an analytical scale has started to attract wide interest [3–5] and a range of examples, including lemon oils [6] and flavour and fragrance components from spices [7–9], have been reported. The supercritical fluid is depressurized to yield the extract, which is then usually analysed chromatographically. Off-line gas–liquid chromatography (GLC) is frequently employed. On-line capillary GLC, in which the fluid is depressurized within the column, can also be used [7–11] but in order to retain volatile constituents cryogenic focusing (less than −10°C) is usually needed [7,8,10]. Directly coupled SFE–supercritical fluid chromatographic (SFC) systems have also been reported [6,12–14]. A disadvantage of on-line systems is that the sample size may be limited and the chromatographic column can become contaminated by highly retained components. In addition, with inhomogeneous matrices the small protion analysed may not be representative of the bulk sample.

However, although the extract obtained by SFE often resembles that obtained by other techniques such as extraction with an organic solvent or steam distillation, many of the reported examples on an analytical scale have been qualitative rather than quantitative. Usually only one set of conditions are described and the effects on temperature and density of extraction on the selectivity of the extraction are not reported. However, Hawthorne and coworkers have reported quantitative recoveries of compounds from spiked rosemary samples [7] and have compared the extraction of basil by SFE and solvent extraction [8]. Sandra *et al.* [15] noted that it was easier to establish the optimum conditions for SFE in an off-line mode as the assay step was then independent of the extraction.

This study followed a similar approach and examined in detail the conditions required to give quantitative extraction of a range of test analytes present at known concentrations on a model cellu-

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lose matrix. The work also examined the conditions needed to obtain good recoveries of the analytes on depressurization of the supercritical fluid and the ability of the system to discriminate between different analytes. The work was designed to establish suitable conditions for the extraction of the volatile components from the herbal medicine feverfew, which contains a thermally labile sesquiterpene lactone, parthenolide, and a range of volatile terpenoids [16]. Initial studies of the complex extract obtained by SFE of dried feverfew were difficult to interpret and it was felt desirable to develop a simpler model system to examine the extraction conditions.

EXPERIMENTAL

Chemicals

Eugenol, santonin and α-cellulose were obtained from Sigma (Poole, UK), carvone and anhydrous sodium sulphate from BDH (Poole, UK), limonene and caryophyllene from Koch-Light (Colnbrook, UK), carbon dioxide of industrial grade (99.98%) from BOC (Middlesex, UK) and dichloromethane [high-performance liquid chromatographic (HPLC) grade] and hexane (HPLC grade) from FSA Laboratory Supplies (Loughborough, UK). Water was deionized and scrubbed.

Model plant material

A solution of limonene (60 mg), carvone (60 mg), eugenol (60 mg), caryophyllene (60 mg) and santonin (125 mg) in dichloromethane (50 ml) was added to α-cellulose (25 g). The solvent was allowed to evaporate at room temperature. The model plant material was then stored in a sealed container at −10°C.

SFC–SFE equipment

Supercritical extractions and separations were carried out using a Jasco Model 880 pump (Japan Spectroscopic, Tokyo, Japan), fitted with a pump head cooling jacket (−10 to −12°C) attached to a Haake KT2 cooling system, for the delivery of carbon dioxide, and a second Jasco Model 880 pump for the addition of modifier through a Gilson Model 811b dynamic mixer. A Jasco 1-ml extraction vessel was mounted in the sample loop position of a Rheodyne (Cotati, CA, USA) Model 7125 valve housed in a Jasco Model 815 oven. [For extractions at −10°C, a 1-m length of equilibration tubing and the extraction vessel were placed in a bath of acetone–ice (1:1)]. The eluent was monitored using a Jasco Model 820 absorbance detector at 220 nm. The pressure in the system was maintained using a Jasco Model 812 back-pressure regulator and the extracts were collected in a trap made from a 100-ml round-bottomed flask fitted with a side-arm and cooled in liquid nitrogen.

For the study using a silica trap, a short column (50 × 4.6 mm I.D.) dry packed with Spherisorb S5W (12 μm) (Phase Separations) was placed in the oven between the Rheodyne valve and the detector.

Analytical-scale extraction

A Jasco extraction vessel (1 ml) was packed with spiked cellulose samples (*ca.* 0.5 g) and exposed to various temperatures, pressures and flow-rates of sub- and supercritical carbon dioxide. The eluate from the extraction was trapped in a flask cooled in liquid nitrogen at −170°C. The solidified carbon dioxide was allowed to evaporate at −10°C in a refrigerator and the residual extract was dissolved in dichloromethane, containing safrole as an internal standard, and examined by GLC.

GLC analysis of extract

The GLC analyses were performed using a Carlo Erba Vega 6000 gas chromatograph. Samples (0.5 μl) were injected using a 10-μl syringe in the split injection mode (splitting ratio 20:1) on to a BP1 dimethylpolysiloxane fused-silica column (5 μm film thickness, 12 m × 0.33 mm I.D.) (Scientific Glass Engineering). The injection port was maintained at 180°C and the column oven was programmed from 60 to 300°C at 8°C min^{-1}, then held isothermal at 300°C for 8 min. The analytes were detected using a flame ionization detector and the results were recorded using a Perkin-Elmer Nelson 2600 data system on an Opus III computer. The concentrations of the analytes were calculated from calibration graphs prepared with standard solutions.

RESULTS AND DISCUSSION

Model plant matrix

In order to establish suitable conditions for the

quantitative extraction of terpenoids from plant material, it was decided to examine a model plant material of known composition so that the effects of the conditions on analytes of different structural types could be compared. Spiked matrices, such as polymer resins [17] and glass-wool [18], have been used previously to investigate the solubility of analytes in supercritical fluids. In this study, trial extractions indicated that 500-μm silanized solid glass beads were a poor model for the plant material and typical terpenes were rapidly washed off the surface even under mild extraction conditions. A microparticulate porous silica (Hypersil, 12 μm) matrix was also investigated but this proved too retentive and 5% methanol was needed to extract the simple oxygenated terpenes, whereas most of the components from feverfew were eluted with carbon dioxide at 45°C and 200 bar.

Powdered α-cellulose was found to be a more appropriate model as trial terpenes could be extracted using carbon dioxide at 45°C and 250 bar and it was selected for a more detailed study. As the cellulose is porous the analytes should be distributed throughout the body of the material and they should thus experience sorption and diffusion effects more typical of dried plant material.

A test mixture was prepared which was designed to be representative of the monoterpenes, sesquiterpenes, lactone and plant phenolics found in plant materials. The mixture contained limonene and caryophyllene as non-polar hydrocarbons, carvone as a polar monoterpene, eugenol as a less volatile plant phenolic and santonin to represent a sesquiterpene lactone. The test samples were spiked on to α-cellulose as a dichloromethane solution at similar levels to those of the essential oils in ferverfew. After air drying the treated cellulose, the resulting model plant material was stored in a sealed container at −10°C to prevent any loss of the analytes.

Trapping studies

Samples of this model plant material (0.5 g, which contained about 1 mg of each terpene and 2.5 mg of santonin) were used to assess the SFE recoveries using a number of different collection techniques. In the initial experiments with carbon dioxide at 40°C and 250 bar, a 15-ml tapered test-tube was placed under the back-pressure regulator to collect the sample. The collected material was dissolved in dichloromethane and the solution was examined using capillary GLC. When the yields of the test compounds were compared with a direct dichloromethane extraction of the model plant material the recoveries of all the compounds were poor (Table I). It appeared that as the carbon dioxide evaporated the test compounds were condensing as a mist in the gas phase, which was not collected but was swept out of the trap by the flow of gaseous carbon dioxide. This problem has also been noted by other workers [17]. Reducing the carbon dioxide flow-rate increased the recovery slightly but greatly extended the extraction time. Collection traps with

TABLE I

COMPARISON OF EFFICIENCIES OF DIFFERENT EXTRACTION AND COLLECTION TECHNIQUES FROM MODEL PLANT MATRIX

Method[a]	Recovery of analyte (%)					
	Limonene	Carvone	Eugenol	Caryophyllene	Santonin	Total
(a) CH_2Cl_2	46	88	81	95	98	82
(b) 15 ml tube	0	18	13	20	21	18
(c) Methanol	12	27	21	32	35	25
(d) −15°C	2	10	8	16	15	10
(e) −60°C	9	28	34	32	62	33
(f) −170°C	23	86	81	97	93	76

[a] (a) Extraction with dichloromethane overnight at room temperature; (b–f) extraction with carbon dioxide at 40°C and 250 bar; (b) collection in 15-ml tapered test-tube; (c) extract bubbled into methanol, crimped fine-bore HPLC tube; (d) 100-ml collection vessel cooled in methanol–ice (*ca.* −15°C); (e) 100-ml collection vessel cooled in acetone–dry-ice (*ca.* −60°C); (f) 100-ml collection vessel cooled in liquid nitrogen (*ca.* −170°C).

spiral flow paths or increased surface areas, originally designed for preparative GLC, were then tried but with little improvement in recovery rates.

Other workers, particularly Hawthorne and coworkers [7–9], have obtained good recoveries by bubbling the carbon dioxide at a low flow-rate through fused-silica tubing into a solvent, such as dichloromethane. When a similar technique using stainless-steel tubing and a methanol trapping solution was examined with the present samples, the recoveries were low (Table I). The tubing frequently became blocked and caused an erratic flow.

A series of extractions were then carried out using a 100-ml round-bottomed flask fitted with a vent arm as a trap. This was cooled to −15°C (methanol–ice), −60°C (acetone–dry ice) or −170°C (liquid nitrogen) and the extraction yields were compared (Table I). Under the last conditions the carbon dioxide was collected as a solid, which was then allowed to evaporate at −10°C. This method gave recoveries of the test compounds that were similar to those obtained by solvent extraction and was therefore adopted for the extraction study.

The collection vessel can be used for up to 1 h at 1 ml min^{-1} of carbon dioxide. For shorter extractions a smaller collection vessel, such as a test-tube with a side-arm, could be used. The flow of the carbon dioxide into the trap was important and with the 100-ml flask the optimum flow-rate was about 0.8 ml min^{-1}, which was used in all the subsequent studies. At lower flow-rates liquid oxygen condensed in the vessel and at higher flow-rates (1.5 ml min^{-1}) not all the extract was trapped.

In all the extraction studies, including solvent extraction with dichloromethane, the recovery of limonene was very low (0–46%, Table I). It was considered that this was due to the loss of this volatile monoterpene by evaporation during the preparation of the model plant material. However, in reporting the recoveries in the extraction studies it was assumed that the nominal concentration was present.

Effect of temperature and pressure on extraction

A series of extraction experiments were then carried out on the model plant material using carbon dioxide over a range of temperatures from −10 to 80°C to investigate the effect of changing pressure and temperature on the extraction efficiency. Each

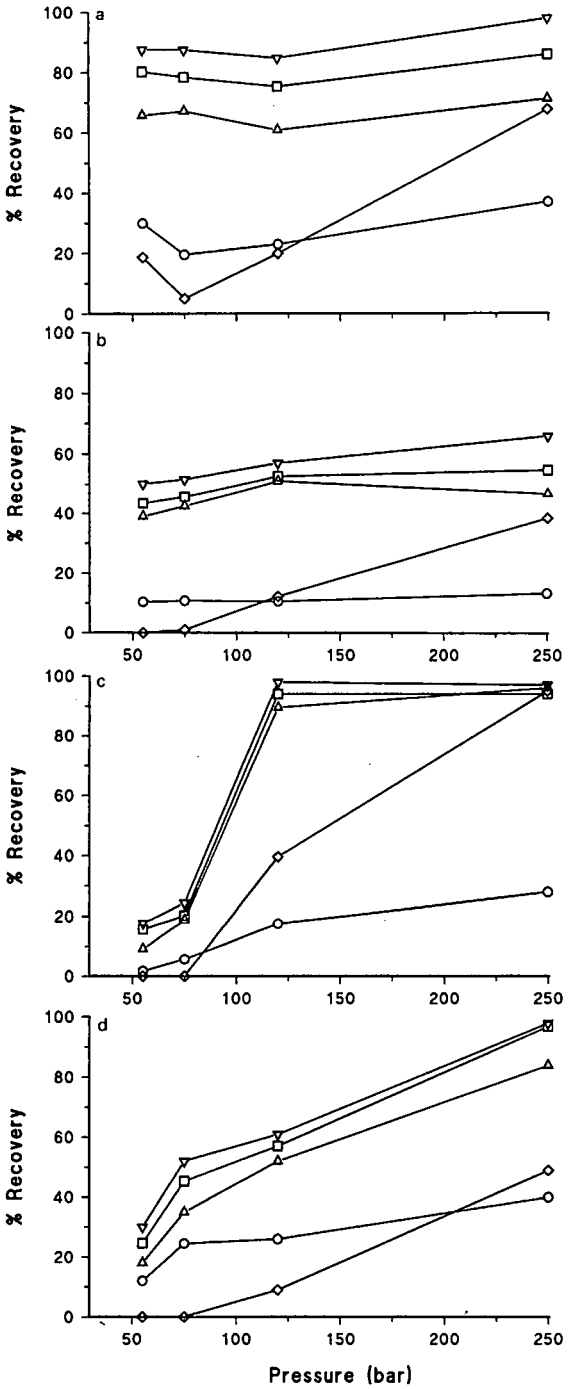

Fig. 1. Recovery yields of essential oils from a spiked cellulose matrix. Analytes: ○ = limonene; □ = carvone; △ = eugenol; ▽ = caryophyllene; ◇ = santonin. Extraction conditions: (a) −10°C, 0.8 ml min^{-1} CO_2; (b) 20°C, 0.8 ml min^{-1} CO_2; (c) 40°C, 0.8 ml min^{-1} CO_2; (d) 80°C, 0.8 ml min^{-1} CO_2.

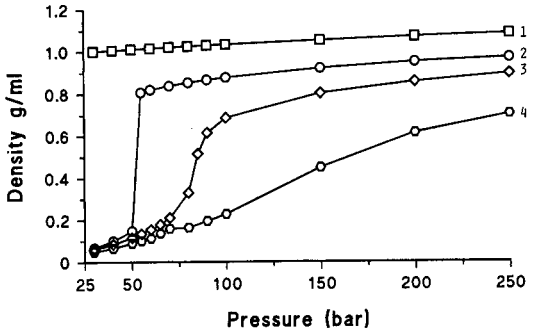

Fig. 2. Density profile of CO_2 (based on ref. 19). Temperature: 1 = −10°C; 2 = 20°C; 3 = 40°C; 4 = 80°C.

extract was monitored by UV spectrophotometry at 220 nm, which was carried out until the signal returned to the baseline (typically 20–40 min).

On extraction with subcritical liquid carbon dioxide at −10°C the yields of most of the terpene test compounds were independent of extraction pressure (Fig. 1a). This result was expected as the density of carbon dioxide varies very little with pressure at this temperature (Fig. 2, based on ref. 19). However, the yield of santonin increased markedly with increase in pressure.

On increasing the extraction temperature to 20°C, the yields of each compound were reduced (Fig. 1b), presumably because the density of the liquid carbon dioxide was lower at this temperature. It appeared that the reduced extraction strength of the carbon dioxide was not compensated for by the increase in the volatility of the terpenes.

When the extractions were carried out at 40°C a different extraction profile with pressure was obtained (Fig. 1c). At low pressures the yields of all the test compounds were low, reflecting the low carbon dioxide density (Fig. 2). The yields remained largely unchanged when the pressure was increased up to 70 bar, but then improved markedly with further increases in pressure and were nearly quantitative for carvone, eugenol and caryophyllene at 120 bar. A higher pressure of 250 bar was required to achieve a similar recovery for santonin. These changes in the yields of the lower molecular weight compounds closely mirrored the changes in the eluent density with pressure. However, at 120 and 250 bar the carbon dioxide density is lower than at 20°C so that the volatility of the analytes must now play a role in giving the higher extraction yields at the higher temperature. The lower viscosity of the supercritical carbon dioxide will also assist mass transfer of the analytes from the matrix. The increased extraction strength may also be due to the formation of solvent clusters which are considered to be prevalent near the critical point in the supercritical phase [20]. Thus, at 120 bar, the local solvent density about the solute may be higher than the bulk density [21].

On further increasing the temperature to 80°C, the extraction profile changed again (Fig. 1d). At 75 bar there was an small increase in the recovery yield compared with 40°C even though the corresponding density was smaller. This change can be related to significant increases in the vapour pressure of the analytes (except for santonin) [18]. At higher pressures the recovery was lower than at 40°C, which was attributed to a lower eluent density at the higher temperature (Fig. 2). Thus, at 75 bar the volatility of the analyte appears to dominate the extraction, but at 120 bar the eluent density is the more important factor. As the pressure (and hence eluent density) was increased further to 250 bar most of the test compounds, with the exception of santonin, were efficiently extracted. These competing effects have also been demonstrated for similar essential oils by Stahl and Gerard [18], who reported comparable solubility isotherms for essential oil components coated on silanized glass beads.

Rate of extraction

As the time required for a quantitative extraction may limit the sample throughout of an analytical extraction system, the extraction profiles of the different test compounds with time were examined. Under subcritical dense gas conditions (−10°C and 250 bar), the extraction of the less polar compounds was initially rapid but santonin was only slowly extracted and levelled off at less than 60% (Fig. 3a).

At a higher temperature but lower pressure (40°C and 55 bar) the extraction was again rapid for the less polar terpenes carvone and caryophyllene, but levelled off at a 55% yield (Fig. 3b). The recovery of eugenol appeared to be increasing with time but the signal from the spectroscopic monitor returned to the baseline after 30 min. Very little of the more polar lactone santonin was obtained. On raising the

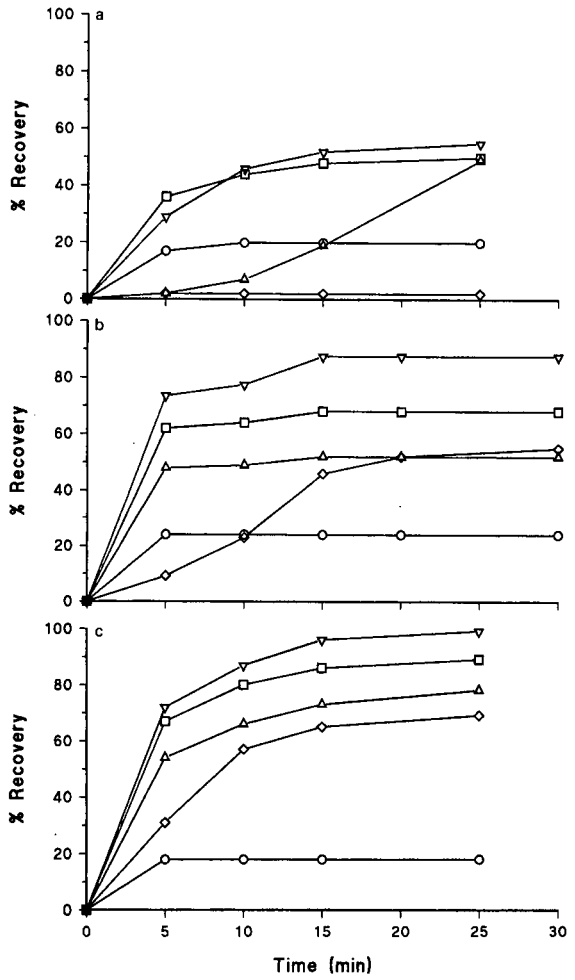

Fig. 3. Extraction profiles for the extraction of model plant matrix. Analytes as in Fig. 1. Extraction conditions: (a) 55 bar, 40°C, 0.8 ml min^{-1} CO_2; (b) 250 bar, −10°C, 0.8 ml min^{-1} CO_2; (c) 250 bar, 40°C, 0.8 ml min^{-1} CO_2.

pressure to 250 bar the rate and overall recovery of all the test compounds increased (Fig. 3c) and from the spectroscopic profile the extraction was effectively complete in less than 15 min. In each of these experiments the final results were similar to those of the single-step extraction shown earlier.

As all the test compounds were present at low concentrations in the model plant material, it was expected that the concentrations of the analytes in the supercritical extraction fluid should be below their solubility limits [18]. Sample solubility should not be the limiting factor in the extraction process. The extraction of the test compounds must therefore depend on their distribution between the carbon dioxide and sorptive sites on the sample matrix as well as diffusion from the matrix into the supercritical fluid.

Bartle *et al.* [22] proposed that in extractions two mass transfer steps may be present, an initial rapid surface extraction then a slower diffusion-limited step of extraction out of the matrix. However, insufficient data were available here to permit a similar analysis.

From these studies, 250 bar and 40°C were selected as suitable starting conditions for the study of the extraction of the essential oils from feverfew [23].

Modified extraction eluents

Because of the slow and sometimes incomplete extraction of santonin, the possible application of the addition of polar modifiers to the carbon dioxide extraction fluid was examined. Under mild conditions of 120 bar and 40°C, only 38% of santonin could be obtained from a spiked cellulose matrix with carbon dioxide alone. The recovery increased to 85% with the addition of 4% of acetonitrile to the carbon dioxide. If the carbon dioxide was bubbled through a water-filled trap to give a saturated solution the recovery of santonin was 92%. However, the addition of 4% of methanol or 4% of chloroform had no effect on the recovery. In each instance a second extraction at 250 bar and 40°C, using just carbon dioxide, confirmed that the santonin which had not had been extracted by the modified eluent had remained on the matrix and could be recovered by the more severe conditions.

It was surprising that methanol had little effect as it has been widely used to deactivate adsorption sites on stationary phases in SFC [24,25]. However, apart from acetonitrile, none of the organic modifiers could match the yield achieved using unmodified carbon dioxide at 250 bar.

Use of a silica "trap" to obtain selectivity

It is useful to be able to use differences in extraction conditions to achieve selective separations, as there can often simplify subsequent chromatographic separations. However, in this study stepwise changes in the extraction pressure or temper-

ature were insufficiently discriminating to resolve any of the present test compounds.

Differences in extraction rates caused by polarity differences can be enhanced by using a selective trap of a polar material to give a greater discriminating power than the original sample matrix. A short silica column was therefore placed in the oven between the extraction vessel and the detector. A sample of the model plant material was extracted under the optimum conditions of carbon dioxide at 250 bar at 40°C, which should extract all the components of the test mixture. The eluent flow was passed through the silica column and an on-line spectroscopic detector showed a series of four broad peaks, which were each collected and examined by GLC. These fractions contained, respectively, predominately limonene and caryophyllene, carvone and finally eugenol. No santonin was eluted from the column and it appeared to have been completely retained on the silica.

The extraction vessel was then switched out of the carbon dioxide flow and carbon dioxide containing 12% of methanol was passed directly through the silica trap. This yielded a santonin fraction free from the less polar components. However, the santonin was released only slowly from the silica column and to obtain a good recovery a higher than usual flow-rate of the eluent (2.5 ml min^{-1}) was needed. This fractionation technique has subsequently been used with feverfew to give a highly purified sesquiterpene lactone fraction containing the active principle parthenolide, free from the less polar terpenes [23].

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CHROMSYMP. 2525

New approaches to coupling flow-injection analysis and high-performance liquid chromatography

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ABSTRACT

An overview of the advantages gained in coupling a flow-injection manifold to a liquid chromatograph is presented. Improvements in the analytical features arising from this association and the peculiar pre- and postcolumn arrangements are discussed, as are the promising prospects of arrangements to be developed for avoiding the preliminary steps of the analytical process.

INTRODUCTION

Flow-injection analysis (FIA) [1,2] and high-performance liquid chromatography (HPLC) are the two most promising hydrodynamic techniques. The instrumentation used in both is remarkably similar; in fact, both use analogous liquid reservoirs, propulsion systems, injectors and continuous detection systems (whether optical, electroanalytical or otherwise). The greatest difference between the modules or units required for each lies in the presence of the separation column, which is essential to HPLC and dispensable in FIA. Other operational differences between these techniques are the working pressure, which is high in HPLC and low in FIA (this accounts for the high cost of HPLC compared with FIA systems) and the presence of a liquid–solid interface, which is always used in HPLC but only occasionaly in FIA [3]. However, the most significant difference perhaps lies in the main analytical goal of each technique, namely the discrimination and/or determination of several components in the same sample in HPLC, and the determination of one of a few analytes in many samples in FIA.

In relation to batch and segmented flow methods, FIA methods are more selective as a result of the kinetic nature of FIA measurements. On the other hand, FIA methods are less sensitive as a result of both the dilution or dispersion of the sample into the carrier stream and the lack of completion of the chemical derivatization reaction involved. Hence FIA manifolds are frequently coupled on-line to a separation or preconcentration system to improve the sensitivity of a given method when low analyte concentrations are to be determined. On the other hand, HPLC methods also require their sensitivity and selectivity to be improved in many instances. This can be accomplished by developing (bio)chemical derivatization reactions in pre- or postcolumn arrangements [4,5] and/or by coupling the HPLC system on-line to a non-chromatographic separation system (liquid–liquid extraction, ion-exchange, etc.) in a precolumn arrangement [6]. Both approaches can be implemented by using a flow-injection manifold which can be coupled on-line to the chromatograph in a pre- or postcolumn position, thus increasing the potential of the derivatization, preconcentration or separation steps. Coupling these two techniques therefore increases their individual potentials through a synergistic effect.

Coupled FIA–HPLC systems are usually intended to improve on such basic features of the analytical process as sensitivity, selectivity, precision, human participation, rapidity, cost, etc.

The features of a given FIA–HPLC system depend on whether the two techniques are coupled in a pre- or postcolumn arrangement. Thus, in precolumn couplings, the FI system is placed before the

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liquid chromatograph, which includes the detection module. Strictly, this is not an FI system proper as it lacks its own detection module. In postcolumn couplings, the chromatographic process takes place before the FIA step. In this instance, the detector is inserted into the FI manifold; hence, strictly again, the chromatograph is not used as an instrument as it has no detection system. Different arrangements offer specific advantages that warrant selection for specific applications.

In this paper we discuss the advantages of FIA–HPLC coupled systems as used in some recently developed methods using pre- and postcolumn arrangements. Unexplored possibilities which could be of interest for special applications are also commented on.

PRECOLUMN FIA–HPLC ARRANGEMENTS

The specific objectives of precolumn arrangements are automation of sample clean-up and/or preconcentration steps, automatic implementation of derivatization reactions, saving of reagents and direct introduction of troublesome samples (solid, viscous, heterogeneous) into FI systems.

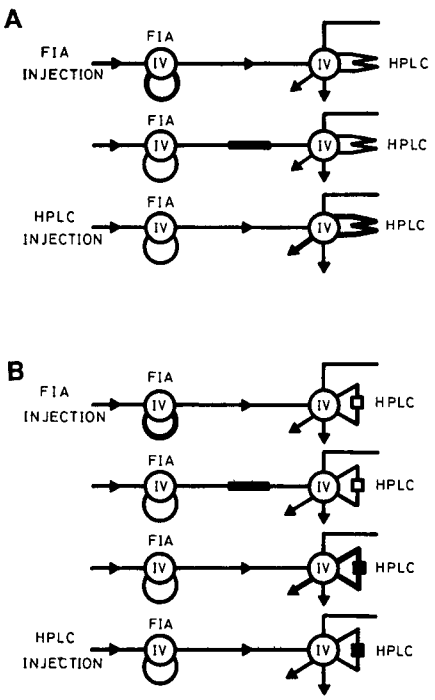

Fig. 1. Two different ways of synchronizing the functioning of the FI and HPLC systems: (A) by trapping the injected sample plug in the loop of the HPLC injection valve, and (B) by using a precolumn in the loop of the HPLC valve. IV = Injection valve.

Precise synchronization of the functioning of the FI manifold and the chromatograph is of paramount importance in precolumn arrangements, which can be accomplished in the two ways shown in Fig. 1. In mode A, the sample plug injected through the FI valve passes through the loop of the HPLC valve, which is then switched to introduce the plug into the column. The second mode involves retaining the analyte injected into the FI system in a precolumn placed in the loop of the high-pressure injection valve of the chromatograph. Large sample volumes can also be continuously aspirated.

There are several possible precolumn FIA–HPLC arrangements and those most frequently used are depicted in Fig. 2.

The merging-zones approach (Fig. 2A) can be very useful for saving expensive or scarce reagents as in chiral separations, which call for expensive derivatization reagents. The basic objective in this instance is to avoid the use of such reagents in the mobile phase. The FI manifold can include a solid reactor (Fig. 2B), whether enzymatic, redox, ion exchanger, etc., depending on the particular purpose. This kind of arrangement has rarely been used in precolumn FI arrangements. An example is the determination of zinc based on its activating effect on immobilized metal-free carboxypeptidase A. The FI valve is used to insert the analyte sequentially into a water stream in order to activate the enzyme first (immobilized on controlled-pore glass and filling a reactor placed between the FIA and the HPLC injection valves) and then the substrate (hippuryl-L-phenylanaline) in order to assess the enzyme activity as its decomposition product is determined spectrophotometrically at 228 nm after the chromatographic separation. The metal-free enzyme is regenerated by pumping 1,10-phenanthroline through the reactor between successively processed samples. This step is performed with the aid of a selecting valve placed before the injection valve which allows the water carrier to be replaced with the regenerating solution [7].

The on-line coupling of a non-chromatographic separation technique (dialysis, gas diffusion, liquid–liquid extraction, etc.) to an FI manifold (Fig. 2C) results in indirectly enhanced sensitivity and selec-

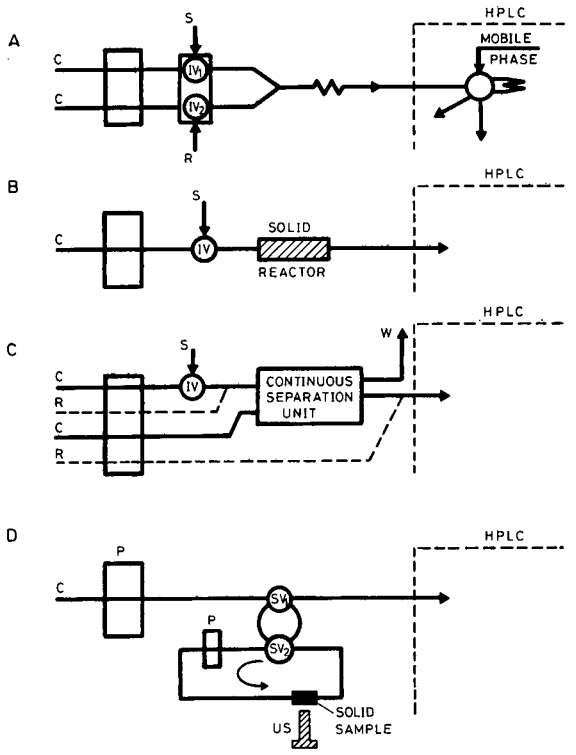

Fig. 2. Generic precolumn FIA–HPLC arrangements. (A) Merging-zones approach; (B) use of a manifold including a solid reactor; (C) on-line coupling of a non-chromatographic continuous separation technique to an FI configuration; (D) use of ultrasonic radiation on a solid sample placed in a special cell in the FI manifold. C = Carrier; R = reagent; S = sample; IV = injection valve; SV = selecting valve; US = ultrasonic probe; W = waste.

tivity and hence in substantial improvements in the subsequent chromatographic separation. An example is the coupling of a liquid–liquid extractor to a normal-phase chromatograph for the determination of caffeine. The sample is injected into an aqueous carrier and then segmented with methylene chloride. The separated organic phase is fed to the normal-phase chromatograph via a precolumn in order to preconcentrate the sample. This method has been successfully applied to real samples such as beverages and urine [8].

A special cell can be used to hold a solid sample for irradiation with ultrasound in the FI manifold (Fig. 2D) in order to enact a solid–liquid extraction process known as "lixiviation" or "leaching". This is a promising application of precolumn coupling aimed at automating the first step of the analytical process [9]. A manifold such as that shown in Fig. 2D has been used for the determination of boron in soils. An amount of 5 mg of sample is placed in a small reaction cell (1.5 mm × 1 mm I.D.). In the leaching step, 300 μl of 0.1 *M* HCl leaching carrier held in the closed circuit is passed through the sample for 5 min. As switching valves SV_1 and SV_2 are simultaneously actuated, the carrier drives the contents of this circuit to the FI manifold, where they are merged with a buffer–masking solution containing the reagent, namely azomethine H. A sharp peak is obtained as a result, the height of which is proportional to the concentration of boron in the solid sample. The results obtained are consistent with those provided by the conventional manual method, which is tedious and time consuming; about 2 h are necessary to perform the manual leaching step, which involves an ammonium acetate solution as leaching agent, whereas up to 30 samples per hour can be processed by the FI method [10].

POSTCOLUMN HPLC–FIA ARRANGEMENTS

Some HPLC workers consider any postcolumn derivatization system to be an FIA system, which is incorrect when the only valve included in the overall system is the high-pressure injection valve of the chromatograph. In fact, only those systems which include two valves should be considered to be true HPLC–FIA configurations. Examples are two of the applications recently developed by our group, namely the twofold use of an FI system and the coupling of a liquid chromatograph to an open–closed circuit. The chief objectives of these approaches were enhanced sensitivity, avoidance of the permanent use of a liquid chromatograph in routine analyses and implementation of continuous monitoring of reaction rates of separated analytes.

The use of the same FI configuration for two different purposes allows the implementation of new, promising methods aimed at avoiding permanent usage of HPLC for monitoring large numbers of samples. The principles behind this approach are illustrated in Fig. 3. Routine monitoring of the total analyte content (*e.g.*, toxic substances) is achieved with the FI manifold. A large number of samples are injected and, within a few hours, it is possible to

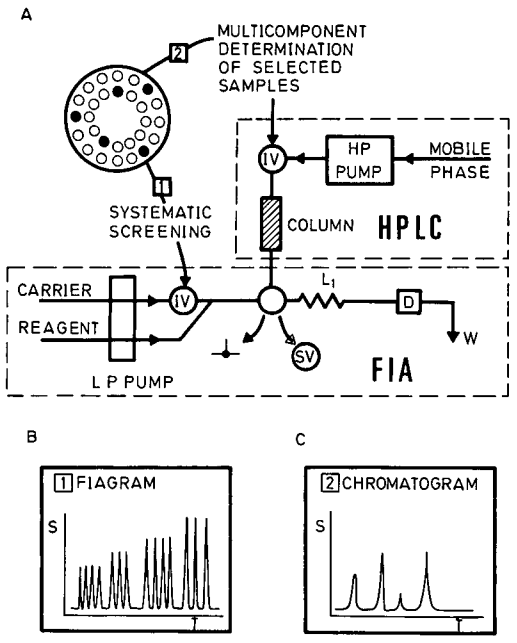

Fig. 3. (A) Twofold use of an FI manifold as a screening system and a continuous postcolumn reaction–detection system. (B) Recordings obtained in routine monitoring. (C) Chromatogram of the discriminated analytes in the selected samples. HP = High pressure; LP = low pressure; IV = injection valve; SV = selecting valve; L = reactor; D = detector; W = waste; S = instrumental signal; T = time.

select those with potentially high contents of toxic substances among 200–500 of them. When this overall determination is finished, only the selected samples are injected into the HPLC system, which uses the same FI configuration as a postcolumn reaction–detection system. In this way, the HPLC system is only used for a small number of samples, which is of practical relevance from an economic point of view. The recordings obtained in the routine monitoring (fiagrams) and in the discrimination of the analytes in the selected samples (chromatograms) are also shown in Fig. 3. This approach has been tested on several analytical problems.

One such problem is the determination of aflatoxins based on enhancing their fluorescence by means of a redox reaction with bromine. In the overall determination, all the samples are injected through the FI valve while the chromatograph remains at rest. Discrimination between the analytes in contaminated samples is accomplished by injecting the selected samples via the chromatograph, the FI system thus acting as a continuous postcolumn reaction–detection system. The results obtained by applying this procedure to foodstuffs (peanut, maize, etc.) show the usefulness of this HPLC–FIA approach [11].

One clinical use of this type of arrangement is the determination of bile acids in serum. Healthy individuals feature total bile acid levels below 1 mg/ml, so higher levels are indicative of liver complaints. The ratios between the concentrations of different bile acids allow specific diseases such as cirrhosis, jaundice and cholesterosis to be diagnosed. Thus, samples from a large hospital are first screened through the FI system in order to select those containing bile acid levels higher than 1–2 mg/l, and only those need be analysed by HPLC, by injecting them through the high-pressure injection valve of the chromatograph, which uses the same FI configuration as a postcolumn reaction detection system. The analytical indicator reaction employed for this purpose is the oxidation of the 3-hydroxy group in the acids by NAD^+, catalysed by 3-α-hydroxy-steroid-dehydrogenase which is used immobilized on controlled-pore glass. The NADH formed in this reaction is monitored fluorimetrically. In this instance, the switching valve (SV) allows the FI system to be isolated from the HPLC system. The former is thus a conventional flow-injection system furnished with a packed enzyme reactor located between the injection valve and the flow cell (L_1 in Fig. 3). In the discriminating determination, the selected samples are inserted through the HPLC valve. The switching valve allows the continuous introduction of the chromatographic eluate into the FI derivatization–detection system. In this way, it is possible to discriminate between bile acids in less than 12 min by careful optimization of the experimental conditions of the combined system. This approach has been successfully applied to serum samples from healthy and sick individuals with excellent results [12].

Coupling of a liquid chromatograph to an open–closed circuit is another example of the use of a true FI system coupled on-line to a liquid chromatograph in a postcolumn arrangement. The operational scheme of this combined system [13] is shown in Fig. 4. The switching valve (SV) allows the first-eluted analyte to be trapped in the circuit (2),

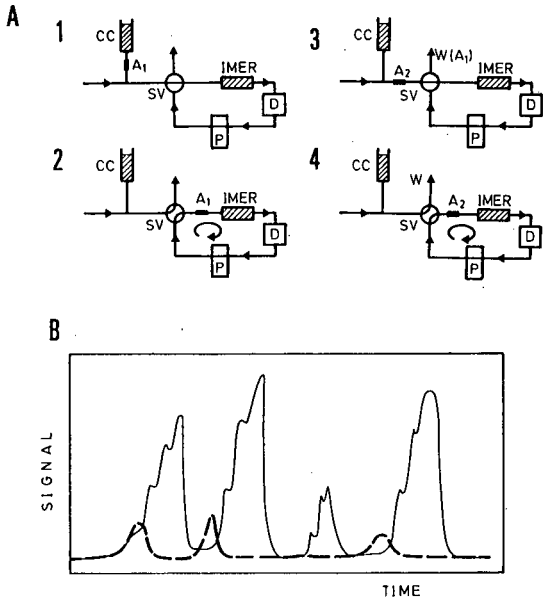

Fig. 4. (A) Operational scheme of a combined HPLC–open–closed FIA arrangement. (B) Normal chromatogram obtained with a conventional continuous postcolumn derivatizing system [valve SV is always kept in the open position (dashed line), and atypical multi-peak chromatogram obtained by using the open–closed postcolumn arrangement. CC = Chromatographic column; SV = selecting valve; P = low-pressure pump; A = analyte; D = detector; IMER = immobilized enzyme reactor; W = waste.

which includes a detector and an enzyme reactor if required. The iterative passage of the sample through the detector is repeated as many times as required; then, the valve is switched to flush the circuit (3) and introduce the next-eluted analyte, which is also trapped in the circuit (4), and so on. A multi-peak recording per analyte rather than a single HPLC peak is obtained (Fig. 4B), which can be compared with the ordinary chromatogram obtained under the same working conditions. Two practical conclusions can be drawn from these recordings: reaction-rate measurements on each analyte can be performed in a continuous fashion by using the distance between maxima or minima whether consecutive or not; and the sensitivity is clearly enhanced. Note that some species yield no peak in the ordinary chromatogram. Good results in this respect are only obtained if the delay time between successively eluted analytes is long enough (at least 1 min) and a microcomputer is used to synchronize the operation of the HPLC injection valve and the switching valve.

This association has been used for the individual determination of creatine isoenzyme activities based on three consecutive reactions. In a first step, the phosphate group in creatine is transferred to ADP in a reaction catalysed by the analyte enzyme. The other two steps involve auxiliary enzymes (hexokinase and glucose-6-phosphate dehydrogenase) immobilized on controlled-pore glass and make up the indicator reaction, which yields the monitored product, NADPH, the reduced form of the coenzyme, which is monitored spectrophotometrically at 340 nm. This methodology has been successfully applied to the determination of these isoenzymes in biological fluids with excellent results [14].

CONCLUSIONS

The development of automatic analytical procedures is one of the main objectives of today's analytical chemistry to respond to the increasing demands for chemical information by a society in frantic evolution [15]. Combined FIA–HPLC arrangements can be regarded as a way of solving real analytical problems in routine laboratories handling large numbers of samples and experiencing problems with conventional HPLC approaches, and interested in the reduction of human participation in analytical processes. Direct introduction of solid or heterogeneous samples, the twofold use of an FIA manifold as a screening system and a postcolumn reactor–detector are the most promising alternatives in this context. The use of postcolumn (bio)chemical FIA sensors based on integrated detection–reaction and/or separation in the flowcell is an interesting trend, as inferred from the first few attempts performed in our laboratories.

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CHROMSYMP. 2508

Coupled-column high-performance liquid chromatographic method for the determination of 1-hydroxypyrene in urine of subjects exposed to polycyclic aromatic hydrocarbons

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ABSTRACT

A coupled-column high-performance liquid chromatographic system for integrated, on-line sample processing and the determination of free and conjugated 1-hydroxypyrene in urine has been developed. The method is based on a "tailor-made" copper phthalocyanine-modified porous-glass precolumn packing material, which allows a direct and repeated injection of urine samples and a selective enrichment of trace amounts of particular components. The fully automated method has a low detection limit (0.01 pmol), a quantitative and matrix-independent recovery and a highly reliability, as shown by an interlaboratory comparison of methods.

INTRODUCTION

The urinary pyrene metabolite 1-hydroxypyrene is a suitable biological indicator for internal exposure to polycyclic aromatic hydrocarbons (PAHs) in several work environments [1]. In the urine of workers occupationally exposed to coal tar and derived products containing PAHs, increased levels of 1-hydroxypyrene, which is mainly excreted as its sulphate and glucuronide conjugate, can be found [1–3].

Until now, urinary 1-hydroxypyrene has been determined by a high-performance liquid chromatographic (HPLC) method developed by Jongeneelen and co-workers [4,5]. This procedure involves a manually performed liquid–solid phase extraction on a cartridge packed with a conventional C_{18} reversed-phase material. Subsequently, the pretreated sample is evaporated and redissolved before injection.

To handle the potentially large number of urine samples to be analysed in the biological monitoring of subjects exposed to PAHs we developed a fully automated coupled-column HPLC method for the urine and direct determination of 1-hydroxypyrene. The method is based on a "tailor-made" precolumn packing material which (1) shows a high selectivity for 1-hydroxypyrene, (2) enriches trace amounts of 1-hydroxypyrene, (3) eliminates the residual sample matrix and (4) allows the direct and repeated injection of native or enzymatically treated urine samples.

EXPERIMENTAL

Chemicals

1-Hydroxypyrene was obtained from Janssen Chimica (Beerse, Belgium). β-Glucuronidase–arylsulphatase solution (100 000 Fishman U/ml and 800 000 Roy U/ml) was from Boehringer (Mann-

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heim, Germany). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). HPLC-grade water was generated by double glass distillation. All other chemicals were of the highest purity obtainable.

Samples

Urine samples (20–25 ml) were collected in standard polythylene or polypropylene tubes and immediately frozen at −20°C until sample preparation. After slowly thawing the sample, 2 ml of the urine were placed in a glass vessel and ajusted to pH 5.0 with 1 and 0.1 *M* hydrochloric acid in 10-μl steps using a pH meter. The solution was then diluted with a sodium acetate buffer (0.1 *M*, pH 5.0) to a final volume of 4 ml and incubated for 3 h with 5 μl of 4β-glucuronidase–arylsulphatase at 37°C in a thermostated water-bath. The samples were centrifuged for 5 min at 2000 *g* and 2 ml of the supernatant were transferred into an autosampler glass vial. The enzymatically hydrolysed samples are stable for at least 12 h at 4°C and for at least 6 months at −20°C.

For the interlaboratory method comparison, urine samples from workers of a creosote oil impregnation plant and from non-exposed control subjects were collected and frozen at −20°C at the University of Nijmegen (Netherlands). These samples were analysed in a double-blind study with Dr. Jongeneelen (Department of Toxicology, Faculty of Medicine, University of Nijmegen), who used a manual extraction procedure [4,5].

Calibration procedure

From a starting solution (approximately 200 μg/ml methanol), a standard stock solution of 1-hydroxypyrene in HPLC-grade methanol (approximately 10 nmol/ml) was prepared and the concentration photometrically confirmed at 242 nm using the extinction coefficient $\varepsilon = 56.64$ μmol cm^2 in methanol. The calibration standards with concentrations ranging from approximately 0.4 to 32 pmol per 100 μl were prepared by diluting the stock solution with an aqueous methanol solution methanol–doubly distilled water (50:50, v/v). The starting solution is stable for 1 month at −20°C, the stock solution for 1 week at −20°C, and the calibration standards for 2 days at 4°C.

The calibration standards were analysed in the coupled-column mode to obtain the calibration graph. Once the linearity of this curve was checked, a single-point calibration with the external standard was applied for day-to-day analysis. Peak areas were used for quantification.

Instrumentation

The HPLC system consisted of a Model L-6000 pump (P 1), a Model L-6200 gradient pump with a programmable microprocessor unit (P 2), a Model 655A-40 autosampler (optional) (AS), a Model ELV 7000 automatic switching valve (ASV), a Model F-1050 fluorescence detector (FD), a Model D-2050 integrator, a precolumn (copper phthalocyanine trisulphonic acid-modified porous glass, 30–60 μm, 5 × 4 mm I.D.) (PC) and an analytical column (LiChrospher RP-18, 5μm, 125 × 4 mm I.D.) (AC). The modular units of the HPLC system were obtained from Merck.

Chromatography

To perform a coupled-column switching technique, the precolumn and the analytical column were connected via the automatic six-port switching valve, the configurations and positions of which are shown in Fig. 1.

This instrumental set-up allows the use of the HPLC system in the coupled-column (precolumn–analytical column) as well as in the single-column (analytical column) mode. The microprocessor unit of the gradient pump controls the pump itself, the automatic switching valve and the integrator. The timetable with eluent compositions and the switching valve positions for a coupled-column analysis are given in Table I.

For the detection of 1-hydroxypyrene, its natural fluorescence is used (excitation 242 nm, emission 388 nm).

With an injection valve in front of the gradient pump and in the switching valve position LOAD, the instrumental set-up allows the independent use of the single-column (RP-18) HPLC system beside the coupled-column mode. In this mode standard samples can be injected directly onto the analytical column to control the recovery in the coupled-column mode.

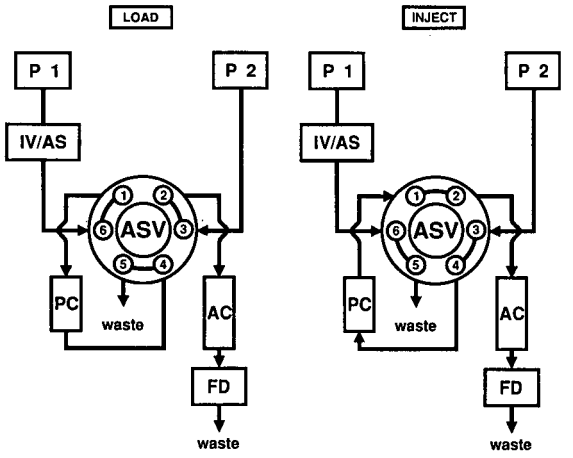

Fig. 1. Valve switching positions. P1 = Pump 1; P 2 = gradient pump; AS = autosampler (optional); IV = injection valve; PC = precolumn; AC = analytical column; FD = fluorescence detector.

TABLE I

TIMETABLE FOR THE COUPLED-COLUMN DETERMINATION OF 1-HYDROXYPYRENE

During the analysis cycle pump 1 operates at a flow-rate of 1 ml/min with eluent A. Eluent A, doubly distilled water–methanol (90:10 v/v); eluent B, HPLC-grade methanol; eluent C, doubly distilled water. Injection volume: 250 µl of the enzymatically treated urine sample, or 100 µl of a calibration standard. Fluorescence detection: excitation 242 nm, emission 388 nm.

Step	Time (min)	Eluent (%)		Flow-rate (ml/min)	Switching valve position (Fig. 1)
		B	C		
1	0	60	40	1.0	LOAD
2	15	60	40	1.0	INJECT
3	19	60	40	1.0	LOAD
4	33	85	15	1.0	LOAD
5	35	100	0	1.0	INJECT
6	44	100	0	1.0	LOAD
7	50	100	0	1.0	LOAD

RESULTS AND DISCUSSION

The most important part of the HPLC method introduced in this paper is the precolumn packing material, a porous glass support, that shows characteristic properties owing to its composition and modification of the chemical surface.

The hydrophilic surface (glyceryl coating) of the solid-phase material in combination with its size-exclusion properties allows a rapid and almost quantitative elution of the residual biological matrix. The selectivity for 1-hydroxypyrene is introduced by the immobilization of a copper phthalocyanine trisulphonic acid moiety at the surface of the solid phase (Fig. 2) [6].

This surface modification was chosen because water-insoluble salts of copper phthalocyanine tetrasulphonate and solid supports, on which copper trisulphonates are immobilized (Blue Cotton [7–9], Blue pearls [10]), selectivity adsorb compounds with a planar structure consisting of at least three fused rings from aqueous solutions. This selective adsorption is due to hydrophobic and steric interactions, which can easily be destroyed by organic solvents such as methanol. HPLC-integrated sample processing with the new precolumn packing material is thus performed by two different modes of liquid chromatography.

Adsorption chromatography results in a selective retention of the analyte, whereas size-exclusion chromatography allows a simultaneous and quantitative elution of the residual biological matrix into the waste. In the coupled-column mode, an analysis cycle is characterized by the following steps (Table I and Fig. 1).

Sample processing (step 1)

Sample application (standard solution, 100 µl; or enzymatically treated urine, 250 µl) is via the autosampler or an injection valve in valve position LOAD, followed by selective sample processing on

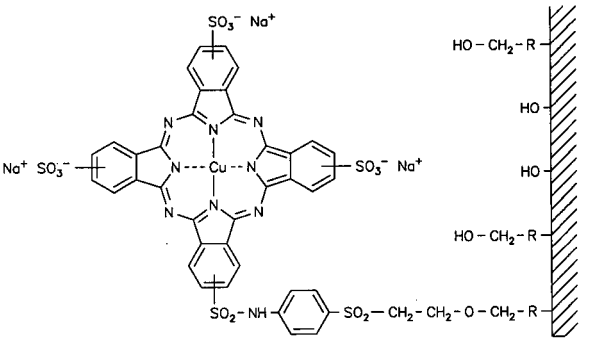

Fig. 2. Structure of the copper phthalocyanine trisulphonic acid derivative bound to the porous-glass support.

the precolumn with eluent A (water–methanol 90:10, v/v; pump 1). The analytical column is simultaneously equilibrated with methanol–water (60:40, v/v) via the gradient pump.

Transfer (steps 2–3)

This step involves automatic valve switching into position INJECT and automatic start of the integrator. The analyte is quantitatively eluted from the precolumn in a backflush mode by increasing the methanol content of the eluent (methanol–water, 60:40, v/v; gradient pump) and simultaneously transferred in a single, narrow elution band through valve positions 3–4–1–2 to the top of the series-connected analytical column.

Separation (steps 3–5)

The valve is switched into position LOAD and separation on the analytical column is carried out by a linear methanol gradient from 60 to 85% in 14 min and from 85 to 100% in 2 min delivered by the gradient pump. 1-Hydroxypyrene is detected and quantified by its natural fluorescence.

Reconditioning (steps 5–7)

In valve position INJECT the analytical column and precolumn are washed with methanol for 9 min to elute highly hydrophobic substances (gradient pump). During the last 6 min of the cycle, the valve is switched into position LOAD and the precolumn is reconditioned for the next analysis (pump 1), which can be started every 50 min. Fig. 3 shows chromatograms obtained by the coupled-column analysis of different urine samples. In addition to 1-hydroxypyrene, which is fully separated, only a few residual sample components appear in the chromatograms. It is interesting to note that no urine sample could be found, even when taken from infants, which contained no 1-hydroxypyrene.

To assess the accuracy of the system recovery (r) experiments were carried out. For the matrix-independent recovery, 100 μl of standard solutions (n =

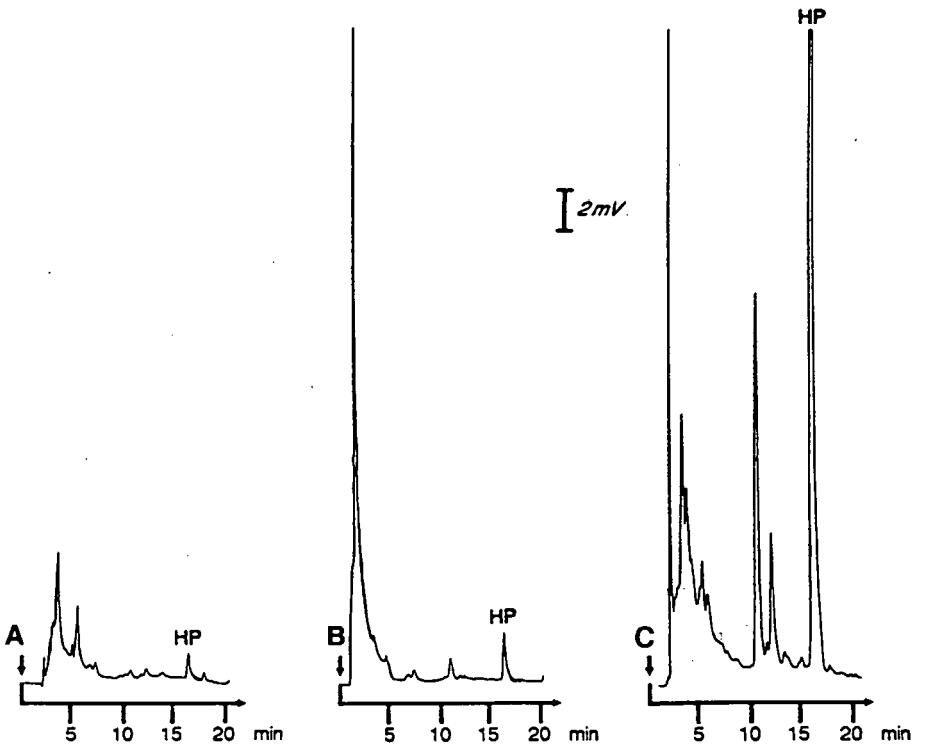

Fig. 3. Coupled-column analysis of 1-hydroxypyrene (HP). (A) Urine of a non-exposed subject after enzymatic hydrolysis (0.27 pmol ≡ 2.16 nmol/l). (B) and (C) Urine sample of an exposed worker of a creosote oil impregnation plant before (B) (0.50 pmol ≡ 4.0 nmol/l) and after (C) enzymatic hydrolysis (12.55 pmol ≡ 100.40 nmol/l). Injection volume 250 μl.

10) in the concentration range 0.4–32.02 pmol per 100 μl were analysed in the single-column mode and in the coupled-column mode (r = 101.99 ± 3.81%). For the matrix-dependent recovery, a pooled urine sample was enzymatically hydrolysed and the concentration of 1-hydroxypyrene was determined before the addition of a known amount of 1-hydroxypyrene. The spiked urine samples (injection volume 250 μl; n = 10) had concentrations ranging from 0.71 to 28.25 pmol per 250 μl (r = 99.79 ± 3.46%). It showed a quantitative recovery for the coupled-column mode, which is independent of the amount of 1-hydroxypyrene and the biological matrix injected.

To obtain data on the precision of the system, within-series an a between-day precision for the analysis of standards (coefficients of variation: $C.V._{series}$ = 2.49%, n = 15; $C.V._{day/day}$ = 1.83%, n = 7) and urine samples ($C.V._{series}$ = 2.57%, n = 15; $C.V._{day/day}$ = 2.87%, n = 7) were determined. For the determination of the between-day precision of the analysis of urine, including the enzymatic hydrolysis step (C.V. 4.91%), one urine sample from an exposed subject was aliquoted and frozen. The aliquots were enzymatically treated and analysed on 7 subsequent days. The results show that 1-hydroxypyrene can be analysed with high precision, even under routine conditions.

The linearity between peak area and the amount of 1-hydroxypyrene ranges from 0.2 to 35 pmol and covers the concentrations that are of interest in the biological monitoring of exposed subjects.

The detection limit was 0.01 pmol and thus even the very low concentrations which occur in urine of controls or subjects exposed to small amounts of PAHs can be quantified exactly. The specificity of the method relies on the detection of the native fluorescence and on the selective retention mechanism for the analyte on the copper phthalocyanine trisulphonic acid-substituted porous-glass support (only compounds with three or more fused aromatic rings are retained quantitatively).

The good reliability of the HPLC system could be confirmed by an interlaboratory comparison of methods [5]. The results of this double-blind study and its statistical treatment indicate that both methods yield equivalent results with respect to accuracy and precision (Table II).

The principal difference between the two methods is found in the respective sample processing steps. Compared with manually performed off-line method, the coupled-column, *i.e.* HPLC-integrated sample processing, offers many advantages. Time-consuming and error-prone manual working steps such as activation, rinsing and elution of the solid-phase extraction cartridge and evaporation and dissolution of the eluate are greatly reduced, resulting in an increased practicability and high reliability. Finally, the coupled-column system can handle more than 250 urine injections (approximately 32 ml) without any loss of chromatographic performance. Owing to the matrix-independent and quantitative recovery of the analyte, calibration is simplified and needs only an external standard.

TABLE II

RESULTS OF THE INTERLABORATORY COMPARISON OF METHODS

The samples were analysed with the off-line method [4] and the coupled-column method. Spearmans coefficient of rank correlation (r_S = 0.984) and the result for Student's t-test [t = 1.60; t(95%, 16) = 2.12] indicate that there is no significant difference between the two series of analytical results.

Sample No.	Concentration of 1-hydroxypyrene (nmol/l)	
	Off-line method	Coupled-column method
1	13.3	3.54
2	0.8	0.49
3	24.1	20.66
4	87.2	88.10
5	154.0	151.23
6	13.0	13.80
7	1.0	1.12
8	18.6	11.13
9	385.0	342.19
10	71.4	42.90
11	44.6	26.60
12	30.6	30.12
13	168.9	189.77
14	92.8	84.94
15	87.2	75.98
16	1.5	1.00
17	37.1	37.58

CONCLUSIONS

The chemical modification of a glyceryl-coated porous-glass support with a copper phthalocyanine

trisulphonic acid derivative and its integration into a conventional HPLC system via a precolumn and a column switching device has led to an automated coupled-column HPLC system for the determination of 1-hydroxypyrene in urine.

The practicability and high reliability make the system attractive for routine analysis in the biological monitoring of exposure to PAHs.

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Trace-level determination of polar phenolic compounds in aqueous samples by high-performance liquid chromatography and on-line preconcentration on porous graphitic carbon

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ABSTRACT

The use of porous graphitic carbon (PGC) was investigated for the trace enrichment and the on-line liquid chromatographic separation of polar phenolic compounds (phenol, di- and trihydroxybenzenes, aminophenols, etc.) from aqueous samples. Comparison between retentions obtained with PGC and with the copolymer-based sorbent PRP-1 showed similar variations of the capacity factors with the mobile phase composition, but an inverse retention order. The capacity factor of a very polar analyte, such as 1,3,5-trihydroxybenzene (phloroglucinol), is 1000 in pure water, whereas this analyte is not retained by C_{18}-silica and is poorly retained by PRP-1 ($k' = 3$ in water). A precolumn packed with PGC can be coupled to a PGC analytical column for simple separation in the reversed-phase mode. This methodology has been applied to the direct determination of pyrocatechol, resorcinol and phloroglucinol below the 0.1 μg/l level in a 50-ml sample.

INTRODUCTION

There is an increasing need for trace-level determinations of high-polarity compounds in aqueous environmental samples. On-line preconcentration on short precolumns prior to liquid chromatography has been shown to be useful in the trace determination of organic compounds in aqueous environmental or biological samples. As a first approach, liquid–solid extraction can be described as a simple chromatographic process with aqueous media as the mobile phase and the sorbent as the stationary phase; during the enrichment step, the analytes should be well retained by the sorbent and not eluted by water. Convenient sorbents are therefore reversed-phase materials, such as the widely used octadecyl-bonded silicas (C_{18}), some divinylbenzene–styrene copolymers and carbon-based sorbents. Trace enrichment of apolar compounds can be effected efficiently with C_{18}-silicas. Carbon-based materials and copolymer-based sorbents (*e.g.*, PRP-1 or PLRP-S) were found to have an enhanced affinity for medium-polarity compounds such as mono- and dichlorophenols [1–4].

Graphitized carbon black (GCB) has proved to be as an excellent gas chromatographic adsorbent in a wide variety of applications [5], but practical application in high-performance liquid chromatography (HPLC) is prevented by its poor mechanical properties [6]. GCB has also been employed in prepacked cartridges for off-line preconcentrations of medium-polarity organic compounds [7–9]. Colin *et al.* [10] strengthened GCB by deposition of pyrolytic carbon. The first attempts to pack precolumns with carbon materials for on-line preconcentration were made by Werkhoven-Goewie and co-workers [1–3] with these pyrocarbon sorbents. Although this material was never commercially available, their studies have shown that pyrocarbon sorbents are much better suited than C_{18}-silicas for preconcen-

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trating analytes containing polarizable substituents, such as nitro, phenyl or halogen. Nevertheless, similar results have been obtained by using PRP-1 sorbent for the preconcentration of these compounds [2].

Significant progress in the preparation of carbonaceous stationary phases for HPLC has been made in the last decade [11], and columns with high-efficiency porous graphitic carbon have become commercially available, but no development of their use for on-line preconcentration has been reported. It has been shown that PGC acts as a strong reversed-phase sorbent; it behaves like C_{18}-silica but requires eluents containing a lower proportion of water for equivalent retention. PGC also has unique separation qualities, such as selectivity for diastereoisomers and for closely similar geometric isomers [11–19]. Nevertheless, Colin and co-workers [12,13] reported that the retention order can be different from that obtained with C_{18}-silica for hydroxybenzenes and nitrobenzenes, indicating differences in the adsorption of polar solutes. In this work, PGC was investigated as a sorbent for preconcentration of these compounds from aqueous media and also as a stationary phase for HPLC. The retentions of phenol and di- and trihydroxybenzenes were studied with mobile phases having a high water content and compared with values obtained with C_{18}-silicas and PRP-1 copolymer.

EXPERIMENTAL

Apparatus

On-line percolation of water was performed with a Milton Roy pump (LDC, Riviera Beach, FL, USA). Precolumn elutions and analyses were carried out with a Varian (Palo Alto, CA, USA) Model 5060 liquid chromatograph equipped with a variable-wavelength UV 200 spectrophotometer and a Coulochem Model 5100 electrochemical detector (ESA, Bedford, MA, USA). Precolumns and analytical column switching were connected with two Rheodyne (Berkeley, CA, USA) valves. Quantitative measurements of peak areas were provided by a CR3A integrator–computer from Shimadzu (Kyoto, Japan).

Stationary phases and columns

A commercial column packed with Hypercarb porous graphitic carbon (100 × 4.6 mm I.D., 7 μm particle size) (Shandon, Runcorn, UK), a column laboratory-packed with PRP-1 copolymer (100 × 4.6 mm I.D., 10-μm particle size) (Hamilton, Reno, NV, USA) and a column laboratory-packed with LiChrosorb RP-18 (150 × 4.6 mm I.D., 5-μm particle size) (Merck, Darmstadt, Germany) were used. Stainless-steel precolumns (10 × 2 mm I.D.) from Chrompack (Middelburg, Netherland) and laboratory-made stainless-steel precolumns (22 × 4.6 mm I.D. or 27 × 4.6 mm I.D. were used for high-retention measurements in water-rich mobile phases and for on-line preconcentration. Precolumns were laboratory-packed using a thick slurry and a microspatula.

Chemicals

HPLC-grade acetonitrile was obtained from Rathburn (Walkerburn, UK) and methanol from Prolabo (Paris, France). LC-grade water was prepared by purifying demineralized water in a Milli-Q filtration system (Millipore, Bedford, MA, USA). Other chemicals were purchased from Prolabo, Merck or Fluka (Buchs, Switzerland). Stock solutions of selected solutes were prepared by weighing and dissolving them in methanol. LC-grade water samples were spiked with these solutions at the μg/l or ng/l level. The final standard solutions did not contain more than 0.5% of methanol.

Procedure

Retention volumes were measured for each mobile phase composition and reported values are the means of three measurements, made using either long columns for low retention volumes or shorter columns for longer retention volumes in water-rich eluents. Mobile phases were obtained by mixing methanol and a 0.05 *M* solution of perchloric acid–lithium perchlorate (pH 4) in different proportions. The flow-rate was 1 ml/min and the temperature was 25°C. The void volume was determined by injection of a 2 *M* solution of sodium nitrate for each mobile phase composition and capacity factors were calculated with this value for each mobile phase. Columns were equilibrated by percolating at least 100 ml of mobile phase before injection. Solutes were dissolved in the mobile phase at 25 ppm concentration and 20 μl were injected.

The on-line experimental set-up was as described

in ref. 4, for instance, the stainless-steel precolumn being placed in the sample-loop position of the six-port liquid switching valve. The water sample was adjusted to pH 6 with perchloric acid and percolated through the PGC precolumn. The precolumn was flushed with 2 ml of LC-grade water adjusted to pH 6 with perchloric acid and was then backflush-eluted with an analytical methanol–water or acetonitrile–water gradient.

Drinking water samples were analysed without any filtration.

RESULTS AND DISCUSSION

Comparison of retentions on PGC, PRP-1 and C_{18}-silica

These three stationary phases are non-polar materials used in reversed-phase chromatography. However, differences exist between these packings. With C_{18}-silicas, solute–stationary phase interactions are weak and non-selective whereas they play an important role with the two other sorbents. Fig. 1 shows the plots of the logarithm of the capacity factor ($\log k'$) against the methanol volume fraction (φ) in the water–methanol mobile phase. The addition of methanol to the mobile phase results in the same dependences for the three packings, showing a reversed-phase behaviour. The differences are in the retention values and retention order. It is clear that both PRP-1 and PGC are much more hydrophobic than C_{18}-silica, as shown by considering the retention of phenol. When comparing the plots obtained with C_{18}-silica (Fig. 1a) with those obtained with PRP-1 (Fig. 1b), we observe the same decreasing retention order with the solute polarity from phenol to phloroglucinol. Plots for phloroglucinol with C_{18}-silica have not been reported; this polar analyte is not retained by C_{18}-silica with methanol-rich mobile phases and has been proposed as an experimental probe for the determination of the void vol-

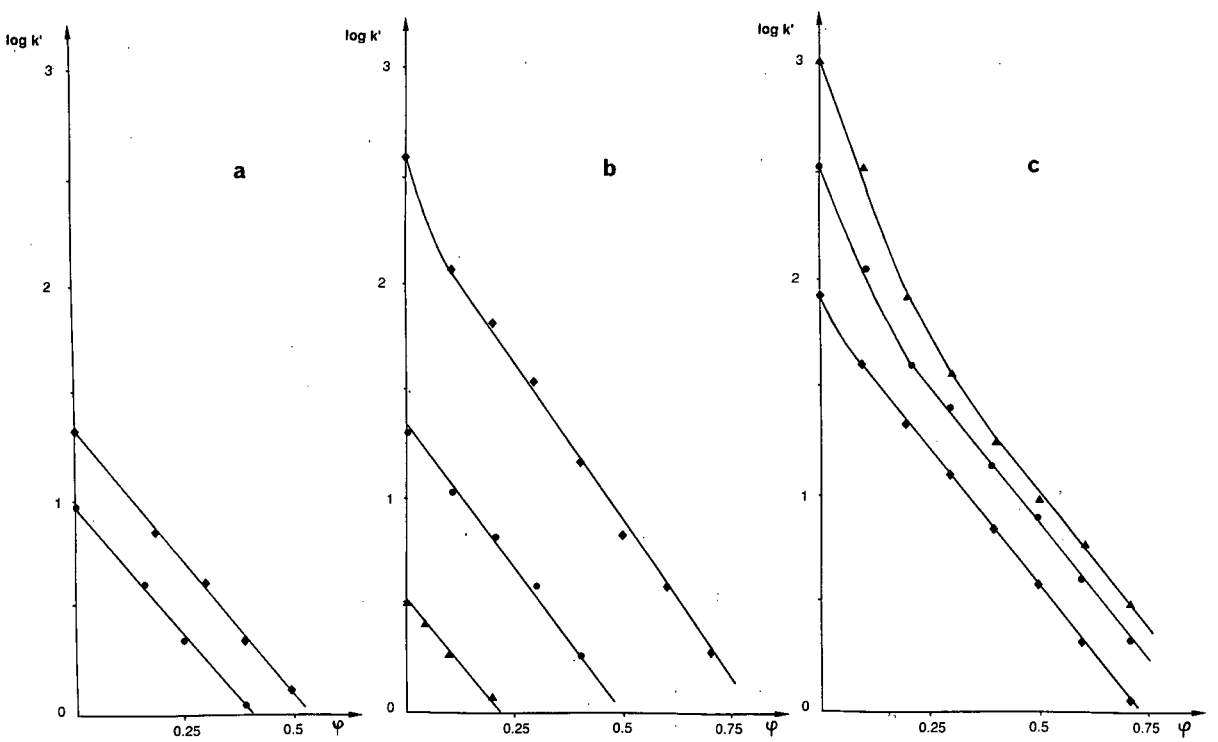

Fig. 1. Variations of capacity factors ($\log k'$) with the mobile phase composition obtained on (a) LiChrosorb RP-18, (b) PRP-1 and (c) PGC. Solutes: ◆ = phenol; ● = resorcinol; ▲ = phloroglucinol; φ is the volume fraction of methanol in the mobile phase [mixture of methanol and a 0.05 *M* solution of perchloric acid–lithium perchlorate (pH 4)]. Flow-rate, 1 ml/min; unretained compound, 2 *M* sodium nitrate.

ume of the column [20]. In pure water, it is very slightly retained (log k'_w = 0.5).

The capacity factors with PRP-1 are much higher than those with C_{18}-silica for the three solutes represented, k'_w of phenol being 15 times higher with PRP-1. The observed stronger retentions are explained by the π–π interactions between these aromatic compounds and the styrene–divinylbenzene matrix of the PRP-1 sorbent, which do not exist with the *n*-alkyl chains of C_{18}-silicas. When comparing plots obtained with PGC (Fig. 1c) with those obtained with C_{18}-silica and PRP-1, it can be observed that the retention order is reversed and increases from the less polar phenol to the more polar phloroglucinol. Phenol is four times more retained by PRP-1 than by carbon, indicating that interactions between phenol and PRP-1 are stronger than those between phenol and PGC. This result is in agreement with the results of Werkhoven-Goewie *et al.* [2], showing the superiority of PRP-1 over pyrocarbon-modified carbon black for preconcentrating monochlorophenol and dichlorophenols.

More surprising is the great difference in retention for phloroglucinol with k'_w values of 1050 with PGC, only 3 with PRP-1 and 0.3 with C_{18}-silica. This can only be explained by different retention mechanisms of polar solutes and by a strong interaction of hydroxy groups with polar species at the carbon surface. Further work is in progress to explain these interactions.

One can see easily the potential use of this sorbent for preconcentrating these non-volatile and very polar phenolic compounds directly from water samples. Chlorophenols are an important class of environmental pollutants and some of their degradation products by photolytic dehalogenation are less chlorinated and more hydroxylated phenols. One can expect in environmental waters that some of the degradation products of many pollutants will be hydroxylated.

Other hydroxylated phenolic compounds are now being tested and the first results are presented in Table I. Capacity factors measured in pure water are reported for PRP-1 and for PGC stationary phases. Retention volumes were calculated for a 2.2 cm × 0.46 cm I.D. precolumn in order to investigate the sample volume that can be handled for preconcentration because, to a first approximation, breakthrough volumes can be calculated from retention volumes in water [2–4]. Except for phenol, the solutes tested are much more retained by the PGC sorbent than by the PRP-1 sorbent. Their retention in water is sufficient for an on-line preconcentration and HPLC analysis. As indicated above, the capacity factor increases with increasing number of hydroxy substituents on the aromatic ring but, when examining capacity factors of di- and trihydroxybenzenes, 1,3- and 1,4-dihydroxybenzene give similar results (331 and 228) whereas 1,2-dihydroxybenzene has a capacity factor of 120, closer to that of phenol (81); the capacity factor of 1,2,3-trihydroxybenzene is between those of phenol and resorcinol or hydroquinone. When two hydroxy groups are substituted on vicinal carbons, only one seems to be taken into account for retention, indicating that the steric effects influence the adsorption of these phenolic hydroxy compounds.

Comparing now the variations of log k' obtained with the three sorbents with the composition of the water–methanol mixtures in Fig. 1, we can observe linear relationships between log k' and the methanol volume fraction for each sorbent, except for mobile phases containing more than 80% of water, where similar deviations to those described for C_{18}-

TABLE I

EXPERIMENTAL CAPACITY FACTORS OBTAINED IN WATER (k'_w) FOR PRP-1 AND PGC STATIONARY PHASES AND CORRESPONDING CALCULATED RETENTION VOLUMES (V_r) WITH A 2.2 × 0.46 cm I.D. PRECOLUMN

See Experimental for conditions.

Compound	PRP-1		PGC	
	k'_w	V_r (ml)	k'_w	V_r (ml)
Phenol	400	92	81	21
1,2-Dihydroxybenzene (pyrocatechol)	46	11	120	31
1,3-Dihydroxybenzene (resorcinol)	21	5	331	86
1,4-Dihydroxybenzene (hydroquinone)	7	1.8	288	75
1,2,3-Trihydroxybenzene (pyrogallol)	5.6	1.5	172	45
1,3,5-Trihydroxybenzene (phloroglucinol)	3	0.9	1050	273
4-Aminophenol	12	3.1	112	30
3-Aminobenzoic acid	37	9	151	40

silicas are obtained. For the same compound, the slopes of the linear plots of log k' *vs.* methanol volume fraction obtained with the three sorbents are very similar or identical, indicating that the changes in log k' observed on just decreasing the methanol content of the mobile phase are similar for the three stationary phases.

On-line preconcentration with PGC

The measurements of retention volumes in water indicated that it was possible to preconcentrate di- and trihydroxybenzenes by percolating water samples directly through the 2.2 cm long precolumn. As these polar analytes are strongly adsorbed on PGC, it was necessary to investigate their desorption for on-line transfer to an analytical column packed with the same sorbent. Fig. 2a shows the analytical separation when injecting 20-μl of a mixture of hydroxybenzenes directly into the Hypercarb column eluted by a methanol–water gradient. The first peak is broad and the resolution between the first two peaks is not very good, but we did not made further attempts to optimize the separation of all hydroxy derivatives, as our aim was to illustrate the potential of carbon for the on-line enrichment of these compounds. Nevertheless, it must be pointed out that the observed plate number decreases when using mobile phases having a high water content for the separation of more polar analytes, compared with the plate number obtained with less polar analytes eluted with mobile phases having a low water content. This is not peculiar to PGC and this variation can also be observed for some other reversed-phase materials.

Fig. 2b shows the chromatogram obtained after preconcentration of a 50-ml sample of LC-grade water spiked with 10 μg/l of each compound through the 2.2-cm long precolumn and on-line elution with the same analytical gradient as that used in Fig. 2a. Peak heights cannot be compared between the chromatograms in Fig. 2a and b because the amounts injected were not exactly known with direct injection, the point of interest being the comparison of peaks widths. The chromatograms are similar and we can just observe a small band broadening for the first two peaks. Generally band broadening comes from desorption of the solute from the precolumn to the analytical column and can be suppressed by compressing the relatively broad profile in the precolumn at the top of the analytical column by the choice of a proper mobile phase and by a backflush desorption. From a geometrical point of view, in order to avoid band broadening, it is necessary to use precolumn of small dimensions compared with those of the analytical column [21]. First using a 1 cm × 0.2 cm I.D. precolumn we studied the on-line preconcentration and elution of 2-chlo-

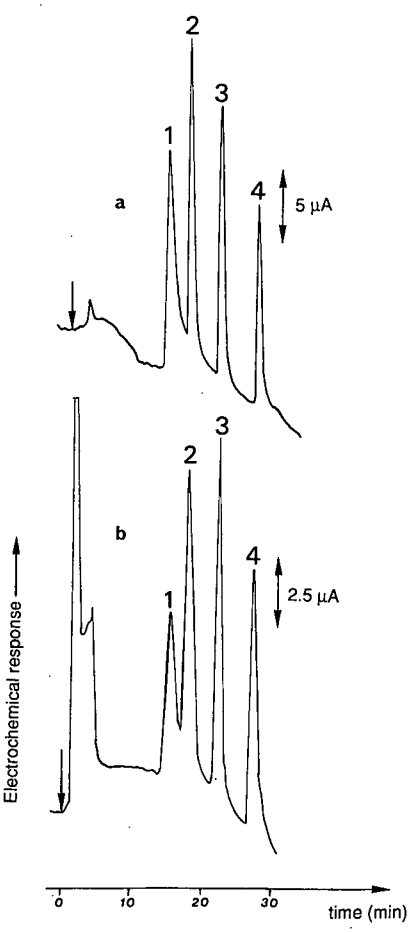

Fig. 2. (a) Direct 20-μl loop injection of hydroxybenzene mixture. (b) Chromatogram corresponding to the on-line elution of the PGC precolumn after preconcentration of a 50-ml LC-grade water sample spiked with 10 μg/l of each compound. Peaks: 1 = pyrocatechol; 2 = resorcinol; 3 = phloroglucinol; 4 = 2-chlorophenol. Analytical column, Hypercarb (10 cm × 0.46 cm I.D.) packed with 7-μm PGC; mobile phase; methanol gradient with a 0.05 *M* solution of perchloric acid–lithium perchlorate (pH 4) at a flow-rate of 1 ml/min; gradient 25 to 100% methanol from 0 to 40 min; electrochemical detection at 1.1 V (*vs.* Ag/AgCl); preconcentration at pH 6 through a 2.2 cm × 0.46 cm I.D. precolumn packed with 7-μm PGC at a flow-rate of 3 ml/min.

rophenol; no band broadening occurred under the experimental conditions selected. Nevertheless, possibly owing to the 7 μm particle size of PGC, the pressure in the precolumn increased rapidly, preventing its re-use. In previous on-line studies, 10-μm packings were selected in order to percolate water samples at a flow-rate of at least 5 ml/min. It was then decided to take a larger diameter of the precolumn, and a 2.2 cm × 0.46 I.D. precolumn was selected. This precolumn is too long for good coupling to the analytical column and this is certainly the reason for the observed band broadening in Fig. 2b. On another hand, this precolumn had the advantage of handling a larger sample volume, owing to stronger retentions of analytes, with an acceptable band broadening for trace determination as illustrated below. The same precolumn was reused more than 30 times with reproducible results.

Breakthrough volumes were not measured directly but calculated by comparing the chromatograms obtained after preconcentration of increasing sample volumes [22]. The peak areas were first measured on the chromatogram when preconcentrating a 10-ml volume spiked with 50 μg/l. A comparison with a direct injection of the same amount of analytes indicated that breakthrough did not occur with any of the analytes tested. The sample volume was then increased and the concentration decreased in order always to have the same amount preconcentrated. Recoveries were calculated as the ratio of the corresponding peak area to that obtained with the 10-ml samples and are reported in Table II. Breakthrough volumes (V_b) can be approximately calculated when the recoveries begin to decrease. V_b is less than 20 ml for pyrocatechol, between 20 and 50 ml for resorcinol and between 50 and 100 ml for phloroglucinol and 2-chlorophenol. These values are lower than the experimental retention volumes calculated in Table I (31, 86 and 273 ml for pyrocatechol, resorcinol and phloroglucinol, respectively). This can be easily explained by the fact that breakthrough curves can be spread over a large volume range. When determining experimental breakthrough curves on PRP-1 sorbent, we measured a breakthrough volume of 130 ml for simazine, whereas the retention volume was 207 ml and the end of the breakthrough curve 280 ml [22]. Another reason is that when several compounds are present together in the sample, they do not behave as if they were alone in LC-grade water, and we can expect lower V_b values also for this reason. This is why it is not necessary to measure V_b for each solute and it is better to calculate them by preconcentrating real samples spiked with the analytes of interest, following the method described above. It is important also to note that owing to the spreading of the breakthrough curves, the amount of analytes preconcentrated increases when the sample volume is increased above V_b, even if the corresponding recovery decreases. As an example, the peak height of phloroglucinol is higher for a 100-ml than for a 50-ml sample, even if breakthrough occurred between 50 and 100 ml.

Application to drinking water samples

A 50-ml sample of drinking water was spiked with 0.2 μg/l each of pyrocatechol, resorcinol and phloroglucinol and the preconcentration procedure was applied. The corresponding chromatogram is shown in Fig. 3. There is a large interfering peak during the first 30 min of the gradient, which appears also with the non-spiked water sample (blank run). We did not investigate any further clean-up but just modified the gradient shape in order to delay the elution of analytes after this interfering peak. The most important point here is that combination of preconcentration using PGC, on-line elution with water–acetonitrile and electrochemical detection allows a very simple determination of these very polar analytes with low detection limits, calculated to be at the 0.05 μg/l level under the selected experimental conditions. The large interfering peak shows also that PGC is a non-specific sorbent, as

TABLE II

PERCENTAGE RECOVERY OF COMPOUNDS DEPENDING ON THE SAMPLE VOLUME PERCOLATED THROUGH A 2.2 × 0.46 cm I.D. PRECOLUMN

See text for calculation; mean relative standard deviation < 3% ($n=3$). Each sample contained 0.5 μg of each compound and concentrations were 25, 10, 5 and 2 μg/l in the 20-, 50-, 100- and 250-ml samples, respectively.

Compound	Sample volume (ml)			
	20	50	100	250
Pyrocatechol	87	52	19	6.5
Resorcinol	98	66	34	13
Phloroglucinol	100	94	62	33
2-Chlorophenol	99	93	68	36

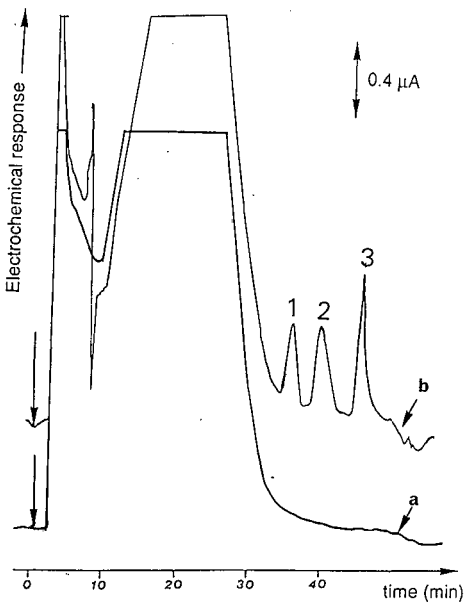

Fig. 3. Preconcentration and on-line analysis of 50 ml of drinking water (a) non-spiked and (b) spiked with 0.2 μg/l of each compound. Peaks: 1 = pyrocatechol; 2 = resorcinol; 3 = phloroglucinol. Analytical column Hypercarb (10 cm × 0.46 cm I.D.) packed with 7-μm PGC; mobile phase acetonitrile gradient with a 0.05 *M* solution of perchloric acid–lithium perchlorate (pH 4) at a flow-rate of 1 ml/min; gradient, 5% of acetonitrile from 0 to 18 min and to 25% of acetonitrile at 58 min; electrochemical detection at 0.85 V (*vs.* Ag/AgCl); preconcentration at pH 6 through a 2.7 cm × 0.46 cm I.D. precolumn packed with 7-μm PGC at a flow-rate of 3 ml/min.

are other reversed-phase materials. Nevertheless, as it retains analytes that are not retained at all by C_{18}-silica, it is possible to couple two precolumns on-line during the preconcentration step [23]. The first, packed with C_{18}-silica, should retain many apolar to moderately polar interfering compounds and not phenolic hydroxy compounds, which would then be recovered on the PGC precolumn.

CONCLUSIONS

The potential of PGC for both trace enrichment and on-line elution of very polar phenolic compounds has been demonstrated. This is an unexplored field of potential applications to some water-soluble, non-volatile and very polar solutes that are impossible to concentrate by the usual techniques. Applications to the handling of aqueous environmental samples are promising.

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Determination of divalent trace metals in natural waters by preconcentration on N,N,N′,N′-tetra(2-aminoethyl)ethylenediamine-silica followed by on-line ion chromatography

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ABSTRACT

A method for the on-line preconcentration and chromatography of trace metals, *e.g.*, Co, Ni, Cu, Zn, Cd and Pb, on N,N,N′,N′-tetra-(2-aminoethyl)ethylenediamine-bonded silica is described. The preconcentrated metals were desorbed with 0.13 *M* tartrate, which allows direct separation on a cation-exchange chromatographic column. The metals separated were detected by postcolumn reaction with 4-(2-pyridylazo)resorcinol and measuring the absorbances at 500 nm. Linear calibration graphs were obtained over the range $1 \cdot 10^{-8}$–$3 \cdot 10^{-6}$ *M*. The synthesis and characteristics of the chelated silica are described. The method was applied to the analysis of river and interstitial sediment waters.

INTRODUCTION

The use of ion chromatography (IC) for the determination of trace metals is attracting considerable attention because it allows multi-element analysis and, unlike anodic stripping voltammetric techniques, it is not limited to electroactive elements. However, the low level of trace metals and the presence of high levels of alkaline earth metals in natural waters hampers the direct determination of trace metals by IC. Consequently, a separation (to eliminate alkaline earth metals and interfering components present in the sample) and preconcentration step prior to IC analysis is necessary. For this purpose, chelating resins or silicas have frequently been used.

Natural waters contain organic ligands that complex with trace metals. Chelating resins or silica will retain only the labile metal fraction. Therefore, using this method and without sample pretreatment, only the free metal and moderately complexed ion concentration can be measured. However, it is very important to have a knowledge of these fractions as they are most toxic to microorganisms. Therefore, preconcentration of metals by chelating resins or silicas followed by IC separation may be used as a complementary method to spectroscopic methods for metal ion determination.

Metals preconcentrated on the chelating silicas may be determined either (directly) by on-line chromatography or (indirectly) by off-line chromatography. The former is preferable for several reasons, *e.g.*, contamination due to sample handling is minimized, and no dilution of the preconcentrated samples will be incurred, *i.e.*, high preconcentration factors would be achieved.

Unfortunately, on-line determination is not simple owing to the difficulty in finding an eluent that is suitable for both desorbing metals retained on the chelating silica (strong acid) and separation by IC (weak acid). To date only three methods have been proposed to overcome this problem [1–3], but each of them has shortcomings.

For instance, preconcentration of metals on 8-hydroxyquinolinol-bonded silica (8Q-silica) followed by desorption of retained metals with 0.1 *M* KCN

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allows the determination of only Cu and Ni [1]. Siriraks *et al.* [2] applied an iminodiacetate resin to the preconcentration of metals and showed that Mn, Fe(II), Co, Ni, Cu and Zn can be determined in complex matrices such as natural water and biological samples. However, their method is complicated and laborious owing to the use of two precolumns. Recently, we reported a similar method using ethylenediaminetriacetate-bonded silica (ED3A-silica) for preconcentration of metals [3]. The metals were desorbed in this instance with 0.1 *M* HNO_3 and the eluted metals were pumped into the analytical column, but before separation the acidic solution was neutralized with a tartrate buffer solution which was delivered using a second pump. Co, Ni, Cu, Zn, Cd and Pb ions were determined by this method. The necessity to use a second pump for the neutralization of the acid makes the method slightly delicate to use.

In this paper, the use of a novel silica, N,N,N′,N′-tetra(2-aminoethyl)ethylenediamine (PENTEN)-bonded silica, for on-line preconcentration of transition metal ions prior to determination by IC with UV–visible detection is described. This method avoids the use of a second pump. Owing to the high acidic constants of PENTEN, the metals retained on the silica are readily desorbed using weakly acidic solutions. This allows the same eluent to be used for metal desorption and separation on the analytical column. Hence the use of PENTEN-silica for the preconcentration of metal ions simplifies the high-performance liquid chromatographic (HPLC) instrumentation, *i.e.*, any type of HPLC instrument equipped with a preconcentration unit (made of plastic or titanium) can be use for performing the analysis and, most important, the delicate step of neutralizing the eluent [3] after desorption of metals from the precolumn is avoided.

EXPERIMENTAL

Apparatus

The chromatographic equipment (Fig. 1) consisted of a Knauer metal-free electric valve, a Knauer Model 64 titanium pump for delivering the eluent and a Dionex QIC preconcentration pump. A laboratory-made titanium precolumn (13 × 1.7 mm I.D.) was packed manually with PENTEN-silica. A Macherey–Nagel analytical column (30 cm × 4 mm I.D.) was packed with Nucleosil 10 SA silica. Plastic

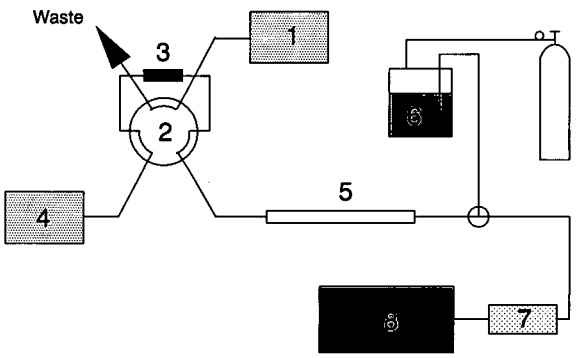

Fig. 1. Schematic diagram of the manifold used for metal analysis. 1 = Preconcentration pump; 2 = valve; 3 = precolumn; 4 = HPLC pump; 5 = analytical column; 6 = post-column reagent; 7 = reactor; 8 = detector.

tubing was used. The postcolumn reagent was pumped under nitrogen pressure (4.5 atm) and to ensure the completion of 4-(2-pyridylazo)resorcinol (PAR)–metal complexation reaction a PTFE coil (3 m × 0.5 mm I.D.) was used. A Hewlett-Packard Model 1040 A diode-array UV–visible detector was used for detection of metal complexes.

Reagents

Preconcentration. Unless stated otherwise, all chemicals were of analytical-reagent grade from Merck or Fluka. All solutions were made with freshly prepared doubly distilled water. Freshly prepared 0.1 *M* diethanolamine buffer (pH 8.5–9) was used for conditioning the precolumn. It was obtained by diluting a 1 *M* stock solution. The pH of the stock solution was adjusted with HNO_3 (Suprapur, Merck). A 1-ml volume of this 1 *M* buffer solution was also added to 20 ml of acidified samples prior to preconcentration.

PENTEN was synthesized as described by Dietrich *et al.* [4]. PENTEN-bonded silica was prepared using a modification of Kudryavtsev *et al.*'s [5] method of synthesis of tetraethylenepentamine-bonded silica. A 0.03-mol (6-g) amount of γ-chloropropyltrimethoxysilane (Petrarch Systems) were added to 10 g of silica (Polygosyl 100, 40–63 μm; Macherey–Nagel) in 45 ml of toluene. Water (40 μl) and 0.2 g of *p*-toluenesulphonic acid were added to the mixture (these act as catalysts), which was then refluxed for 4 h without stirring. The solid was filtered, washed successively with hot toluene, eth-

anol and diethyl ether and dried under vacuum at 120°C; elemental analysis, C 4.07, H. 1.08%; calculated loading capacity, 1130 μmol/g. A 2-g amount of chloropropyl-bonded silica was suspended in 10 ml of toluene, 0.004 mol (0.8 g) of bistrimethylsilylacetamide were added and the mixture was refluxed for 4 h. The solid was filtered, washed with hot toluene and diethyl ether and dried at 120°C under vacuum. Finally, to 12 ml of ethanol–water (50:50) containing 0.003 mol (2.1 g) of PENTEN · 6HBr (pH 9.5), 2 g of end-capped chloropropyl-bonded silica were added. The mixture was refluxed for 48 h (without stirring). The solid was filtered, washed successively with ethanol, water, 0.01 *M* HNO_3, water, ethanol and diethyl ether and dried at 120°C under vacuum; elemental analysis, C 6.71, H 1.59, N 1.94%; calculated loading capacity, 230 μmol/g.

Eluent. The eluent used was 0.13 *M* tartaric acid (pH 3) [6]. The pH was adjusted with NaOH. A few drops of pentachlorophenol solution (5 mg/l in 98% ethanol) were added to the tartrate solution to prevent bacterial growth.

Postcolumn reagent. A solution containing $5 \cdot 10^{-4}$ *M* PAR and $5 \cdot 10^{-5}$ *M* Zn-EDTA in 2 *M* ammonia solution (pH 11) was used as the postcolumn derivatizing reagent [6,7].

Procedure

A 20-ml (or 50-ml) volume of sample solution containing the metal ions [buffered with diethanolamine (pH 8.5–9) if required, for instance, with acidified solution] was pumped into the chelating silica precolumn (flow-rate 5 ml/min) where the metals were preconcentrated. The precolumn was rinsed with 5 ml of water and the metals retained were desorbed with 0.13 *M* tartaric acid (pH 3) delivered by means of an HPLC pump (flow-rate, 1.5 ml/min, pressure 120 atm). During the desorption step (total duration 1 min), the precolumn was linked to the analytical column. The metals, after separation on the analytical column, were derivatized with the PAR–Zn-EDTA solution and detected at 500 nm. While separation was proceeding, clean-up and conditioning of the precolumn were done by washing it with 5 ml of 0.5 *M* HNO_3 followed by 10 ml of 0.1 *M* diethanolamine buffer (pH 9) using the preconcentration pump. A new sample can be preconcentrated while the separation of the previous sample is in progress.

RESULTS AND DISCUSSION

Characteristics of PENTEN-silica

Conditions of preconcentration. As the p*K* values of PENTEN are high, preconcentration has to be done at high pH to minimize competition by metal-complexing ligands which are present in the samples. Moreover, preconcentration of metals must be carried out at pH 9 because hydroxide ions interfere with metal preconcentration. Therefore, the chelating silica precolumn was preconditioned with diethanolamine buffer. Once the column has been conditioned, silica itself acts as an autobuffer and samples to be preconcentrated need not be buffered unless their pH values are lower than 6.5–7.

In contrast, the ionic strength of the sample solution should be adjusted to 0.1 *M* by adding inert electrolytes such as potassium nitrate in instances where low-ionic-strength unbuffered samples are used. This is to minimize electrostatic repulsion between similarly charged ions (metals and ligands on the surface of the silica).

Breakthrough volumes. Using the above-mentioned conditions, the breakthrough volumes (V_b) for various metals were determined. The V_b values were found to be 120 and 250 ml for Pb, the least well retained metal, and Cu, the most strongly retained metal, respectively (concentration of metal ions = 10^{-6} *M*). After 50 preconcentration–elution cycles, a *ca.* 20% decrease in these V_b values was observed.

Effect of major ions on trace metal preconcentration. The results showed that the presence of 0.5 *M* of Na^+ and Cl^-, $5 \cdot 10^{-3}$ *M* of Ca^{2+} and $1 \cdot 10^{-3}$ *M* of SO_4^{3-} and CO_3^{2-} did not affect the preconcentration of metal ions (concentration of metal ions = $2 \cdot 10^{-7}$ *M*). A small fraction of Ca^{2+} (*ca* 1%) was found to be retained by PENTEN-silica.

Efficiency of PENTEN-silica. The stability constants of transition metals with PENTEN are high [8] (see Table I). However, neither the thermodynamic nor the kinetic reaction constants for these metals with PENTEN-silica are known. Metal retention depends on the latter, *i.e.*, if the metal complexation reaction is not fast, metals will not be retained. Therefore, in order to determine the metal retention characteristics of PENTEN-silica, preconcentration of metals were done in the presence of tartrate, nitrilotriacetate (NTA) and EDTA.

The results were compared with the retention

TABLE I

STABILITY CONSTANTS OF METALS WITH PENTEN [8]

Metal	$\log \beta_1$	Metal	$\log \beta_1$
Mn	9.37	Cu	22.44
Fe(II)	11.2	Zn	16.24
Co	15.75	Cd	16.15
Ni	19.3	Hg	29.59

characteristics of 8Q-silica and ED3A-silica reported previously [1,3]. Metal retentions were unaffected by the presence of up to 10^{-2} M tartrate concentrations for all three chelating silicas.

The influence of NTA and EDTA on the retention of Cu, Pb, Zn, Ni, Co and Cd ($2 \cdot 10^{-7}$ M in each of the metals) are shown in Fig. 2. NTA has protonation constants of 1.66, 2.95 and 10.28 [8]. Preconcentration of metals on PENTEN-silica was done at pH 8 whereas that on 8Q-silica and ED3A-silica was done at pH 5. In all three instances, the same protonated NTA species are present and the metal–NTA complexation strengths for a given metal at pH 5 and 8 are comparable. The results show that PENTEN-silica is best for the retention of Cu^{2+} owing to its high stability constant with Cu^{2+}, and also for Zn and Cd owing to the fact that their complex formation kinetics are fast. On the other hand, Ni and Co react slowly with PENTEN-silica and exhibit the same type of behaviour as observed with 8Q-silica. The striking feature of PENTEN-silica is that it shows a very poor affinity for Pb.

With EDTA, the protonation constant of 6.24 is different from that of NTA and in addition EDTA forms stronger complexes with metals than NTA. The results showed that all three chelating silicas exhibit similar behaviour towards the retention of all the target metals except Cu^{2+}, which is retained better on PENTEN-silica than the other two silicas.

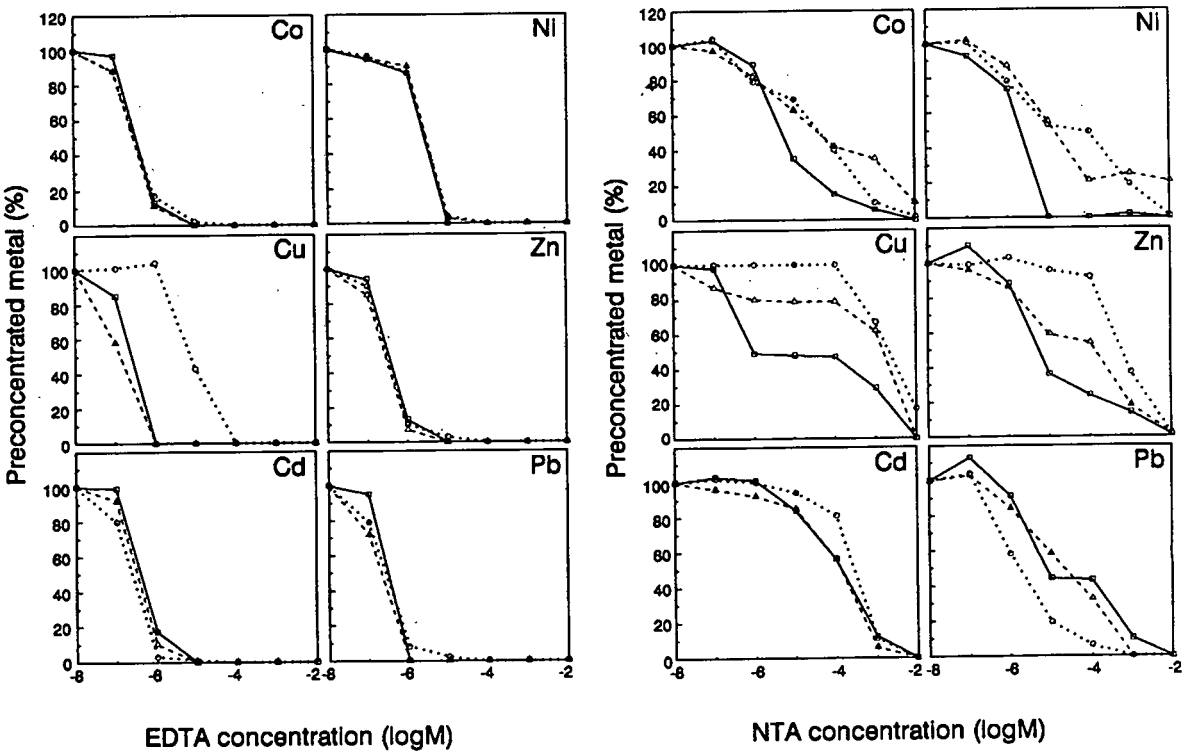

Fig. 2. Comparison between (○) PENTEN-, (△) 8Q- and (□) ED3A-silicas. Recoveries of metals with increasing concentrations of NTA and EDTA (20 ml of $2 \cdot 10^{-7}$ M metal samples).

Choice of buffer

The anions of the buffers may hamper the preconcentration of metals owing to problems due to precipitation or complexation. Several buffers were tested and the results showed that (1) phosphate buffer (pH 8.5–9) was not suitable as calcium ions which are present in high concentrations in natural aquatic systems form calcium phosphate precipitate; (2) ammonia is unsuitable owing to the formation of strong complexes with transition metals; (3) the low solubilities of boric acid and trishydroxymethylaminomethane (Tris) make these buffers unsuitable; and (4) diethanolamine, which is highly soluble in water and which is not a strong complexing agent, was found to be the best buffer for the preconcentration of metals. The only drawback to using this buffer is that Co(II) is oxidized to Co(III) in this medium in the presence of dissolved oxygen. In fact, an 80% decrease in the Co(II) signal was observed with Co(II) samples preconcentrated 30 min after their preparation. Co(II) therefore cannot be measured using this buffer when the sample itself is buffered (acidic sample, see above).

On-line chromatography

Metal separations were carried out using Yan and Schwedt's chromatographic separation procedure [6]. From the results in Fig. 3, it can be seen that a 2-ml sample containing 10^{-5} *M* Cu^{2+}, preconcentrated on PENTEN-silica, can be eluted in less than 1 min by using 1.5 ml of 0.1 *M* tartrate (pH 3). However, a 30-cm (instead of 20-cm) analytical column was used. Hence, in order to obtain acceptable retention times, a more concentrated tartrate solution (0.13 *M*) was used as the eluent.

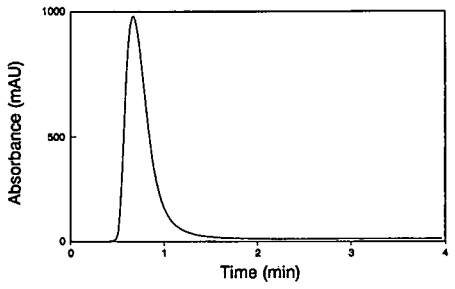

Fig. 3. Elution of preconcentrated copper (2 ml of 10^{-5} *M* Cu^{2+}) with 0.1 *M* tartaric acid (pH 3).

20-ml samples containing $2 \cdot 10^{-7}$ *M* of test metal ions were preconcentrated, separated and detected. The results obtained were compared with the direct injection method by injecting 20-μl samples containing $2 \cdot 10^{-4}$ *M* of the test metal ions. The peak areas of the preconcentrated samples were generally found to be smaller than in the injection method. The differences in peak areas between the two methods can be as much as 50%, depending on the metal and the conditions used. A possible reason for this discrepancy is that the metal ions present are not totally preconcentrated owing to hydrolysis problems. However, this does not affect the determination of metal ions because these differences in peak areas are reproducible and the peak areas are directly proportional to the metal ion concentration.

Precision and detection limits

The results obtained from five replicate measurements of 20-ml samples containing $2 \cdot 10^{-7}$ *M* of the various metal ions are given in Table II. The day-to day reproductibility is essentially dependent of the reproductibility of the preparation of the post-column reagent. Taking this into account, the day-to-day reproducibility is never more than 5% for each of the metals.

The detection limit for all the test metal ions was found to be $3 \cdot 10^{-9}$ *M* (three times the baseline height). Linear calibration graphs were obtained over the ranges $1 \cdot 10^{-8}$–$3 \cdot 10^{-6}$ *M* for Cu, Zn, Ni, Co and Cd and $1 \cdot 10^{-8}$–$3 \cdot 10^{-7}$ *M* for Pb. The narrow linear range for Pb is due to the use of a low Zn-EDTA concentration for postcolumn reaction (see ref. 3).

TABLE II

REPRODUCIBILITY OF THE METHOD

Relative standard deviations (R.S.D.) for five replicate measurements with 20-ml samples containing $2.5 \cdot 10^{-7}$ *M* target metal ions.

Metal	R.S.D. (%)	Metal	R.S.D. (%)
Cu	1.8	Ni	3.1
Pb	3.6	Co	1.8
Zn	2.5	Cd	1.8

Analytical applications

Determination of trace metals in river water. River Arve (Geneva, Switzerland) water samples were filtered through a Schleicher & Schüll 0.2-μm filter and buffered with diethanolamine buffer. A sample of 50 ml was spiked with 10^{-7} *M* metal ions, immediately preconcentrated on PENTEN-silica and separated as described above (Fig. 4C). The results showed that there was no matrix effect (Table III) except for the appearance of Ca, Mn and Mg at the end of the chromatogram. As can be seen from Table III, quantitative recoveries were obtained for all the target metals except Cu and Zn. Analogous results were observed in our earlier studies [3]. The lower recovery of Cu is due to the fact that a fraction of the total copper is present as an inert complex and cannot be determined by this method. For zinc, the high values for the blank make the results imprecise (Fig. 4A). The recoveries are defined as the difference between the metal found after spiking and the metal spiked (taking into account the metal already present in the sample and the blank).

A typical chromatogram of river Arve water sample preconcentrated and separated is shown in Fig. 4B. As can be seen, Cu, Zn and Ni can be determined without any problems. The concentration of free or labile lead is low and Cd is present at levels below the detection limit.

To test if the accuracy of the method is dependent on the preconcentrated volume, the measurement was repeated using a larger sample volume (100 ml). The results obtained with this sample were in good agreement with those obtained with the 50-ml sample volume (Table III).

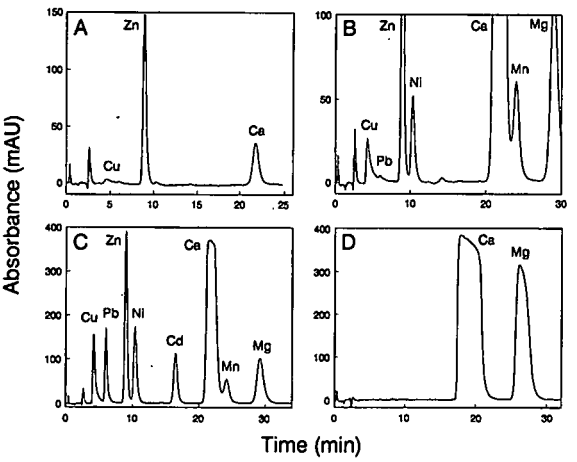

Fig. 4. Determination of trace metals in river Arve water. (A) Blank; (B) river water, 50-ml sample volume; (C) river water spiked with 10^{-7} *M* test metal ions, 50-ml sample volume; (D) same as (B) but preconcentrated on classical high-capacity cation-exchange silica.

The effectiveness of chelating silicas for preconcentration of metals was established by comparing the results obtained with a classical high-capacity cation-exchange silica (Nucleosil 10SA, 1 mequiv./g) (Fig. 4D). The cation-exchange silica is unsuitable for preconcentration of metals in samples containing high levels of alkaline earth metals (in this instance $1.3 \cdot 10^{-3}$ *M* for Ca^{2+} measured by atomic absorption spectrometry (AAS)).

Analysis of interstitial sediment water. Analysis of interstitial water by preconcentration of the sample on PENTEN-bonded silica followed by chromatog-

TABLE III

ANALYSIS OF RIVER ARVE WATER

Metal	Metal concentration found (*M*)				
	Blank	50-ml sample	100-ml sample	10^{-7} *M* spiked sample	Recovery (%)
Cu	$5.3 \cdot 10^{-9}$	$2.9 \cdot 10^{-8}$	$3.4 \cdot 10^{-8}$	$1.2 \cdot 10^{-7}$	−12
Pb	$1.2 \cdot 10^{-9}$	$1.7 \cdot 10^{-9}$	$1.8 \cdot 10^{-9}$	$9.9 \cdot 10^{-8}$	−2
Zn	$6.8 \cdot 10^{-8}$	$3.0 \cdot 10^{-8}$	$2.8 \cdot 10^{-8}$	$1.2 \cdot 10^{-7}$	−14
Ni	$1.6 \cdot 10^{-9}$	$4.7 \cdot 10^{-8}$	$4.7 \cdot 10^{-8}$	$1.5 \cdot 10^{-7}$	+3
Cd	0	0	$1.3 \cdot 10^{-9}$	$1.1 \cdot 10^{-7}$	+7

TABLE IV

ANALYSIS OF INTERSTITIAL SEDIMENT WATERS

Comparison between IC analysis (PENTEN- and ED3A-silicas, 20-ml samples) and AAS.

Metal	Metal concentration found (M)								
	Aire water			Nant d'Avril water			Nant de Châtillon water		
	PENTEN	ED3A	AAS	PENTEN	ED3A	AAS	PENTEN	ED3A	AAS
Cu	$2.0 \cdot 10^{-8}$	$1.9 \cdot 10^{-8}$	$3.0 \cdot 10^{-8}$	$1.9 \cdot 10^{-8}$	$3.5 \cdot 10^{-8}$	$5.2 \cdot 10^{-8}$	$8.6 \cdot 10^{-8}$	$8.7 \cdot 10^{-8}$	$1.9 \cdot 10^{-7}$
Pb	–	$4.5 \cdot 10^{-9}$	$4.3 \cdot 10^{-9}$	–	$7.3 \cdot 10^{-9}$	$6.5 \cdot 10^{-9}$	$3.3 \cdot 10^{-8}$	$2.9 \cdot 10^{-8}$	$3.8 \cdot 10^{-8}$
Zn	$2.2 \cdot 10^{-7}$	$1.8 \cdot 10^{-7}$	$2.8 \cdot 10^{-7}$	$2.4 \cdot 10^{-7}$	$2.5 \cdot 10^{-7}$	$3.8 \cdot 10^{-7}$	$2.1 \cdot 10^{-7}$	$2.4 \cdot 10^{-7}$	$3.1 \cdot 10^{-7}$
Ni	$9.5 \cdot 10^{-8}$	$9.4 \cdot 10^{-8}$	$1.2 \cdot 10^{-7}$	$8.2 \cdot 10^{-8}$	$9.1 \cdot 10^{-8}$	$1.1 \cdot 10^{-7}$	$4.4 \cdot 10^{-8}$	$5.7 \cdot 10^{-8}$	$4.4 \cdot 10^{-8}$
Co	$8.3 \cdot 10^{-9}$	$6.9 \cdot 10^{-9}$	$3.0 \cdot 10^{-8}$	–	$6.2 \cdot 10^{-9}$	–	–	$8.7 \cdot 10^{-9}$	–
Cd	–	–	$6.3 \cdot 10^{-10}$	–	–	$5.5 \cdot 10^{-10}$	–	–	$1.7 \cdot 10^{-9}$

raphy was compared with metal analysis by AAS (electrothermal for Cu, Pb, Ni, Co and Cd; flame for Zn) and preconcentration on ED3A-silica followed by IC [3]. Sediment samples from three polluted rivers in Geneva Canton (Switzerland), the Aire, Nant d'Avril and Nant de Châtillon, were collected. The samples were centrifuged and the supernatant interstitial water was filtered through a 0.2-μm filter and acidified to prevent adsorption problems during their storage. The results are given in Table IV.

It can be seen that results obtained by the two chromatographic methods are comparable except that cobalt can be determined reliably only with ED3A-silica. The oxidation of Co(II) to Co(III) by diethanolamine buffer used with the PENTEN-silica hampers the determination of Co.

AAS yields either similar or higher values than IC method, because IC measures only the free and moderately complexed metals whereas AAS measures the total concentration. Reliable results for cobalt could not be obtained by AAS in this concentration range.

CONCLUSIONS

The novel PENTEN-bonded silica allows the simple and efficient on-line preconcentration and separation of trace metals by IC. The method can be used with any standard HPLC equipment which includes an inert (plastic or titanium) preconcentration pump, switching valve, precolumn and tubing. It can be automated.

Detection limits lower than those reported here can be achieved using larger sample volumes, but at the expense of longer analysis times. Alternatively, the sensitivity of the method could be increased using a different detection system, *e.g.*, chemiluminescence [9].

The drawback of the proposed approach is that the determination of metals in biological samples containing high concentrations of phosphate or Fe(III) cannot be done owing to the precipitation of either metals as phosphates or Fe as hydroxides at pH 8.5–9.

It should be remembered that only the free and moderately labile metal fraction is measured by the use of chelating silicas. If the total concentration is required, the sample must be mineralized (*e.g.*, using UV radiation). Further, PENTEN is not a good ligand for lead, The results for the determination of lead are therefore not very useful. Finally, cobalt cannot be determined when the sample must be buffered (acidic sample).

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Application of an internal surface reversed-phase column for the automated determination of flucycloxuron residues

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ABSTRACT

A liquid chromatographic column-switching system for the automated determination of flucycloxuron, a benzoylphenylurea pesticide, in crop and environmental matrices is described. The system consists of an internal surface reversed-phase (ISRP) column, a phenyl-bonded precolumn and an analytical reversed-phase (RP) C_{18} column. Sample extracts are evaporated to dryness and dissolved in the mobile phase of the ISRP column. An aliquot of this solution is injected into the column-switching system. Clean-up, with regard to removal of large molecules, is performed on the ISRP column. The flucycloxuron fraction from the ISRP column is concentrated on the phenyl-bonded precolumn. Additional clean-up can be performed by washing the precolumn. Finally, the compound is desorbed from the precolumn and separation and determination of the *Z*- and *E*-isomers of flucycloxuron are performed with the analytical RP-C_{18} column using UV detection at 254 nm. The total analysis time required is 40 min. The reproducibility of the method obtained with the column-switching system, expressed as relative standard deviation, varies between 3.7 and 10% for apple, strawberry, citrus and soil samples for flucycloxuron levels between 0.04 and 0.33 mg/kg. The system showed no loss of analytical performance after more than 300 analyses.

INTRODUCTION

Problems encountered in residue analysis are often caused by non-selective extraction procedures. Apart from the compound of interest, many matrix constituents are co-extracted and they can disturb the chromatographic determination and/or decrease the lifetime of a separation column. Sample extracts are, therefore, often subjected to a special clean-up step. Size-exclusion chromatography (SEC) is a widely used clean-up technique to remove high-molecular-mass components that could interfere with liquid or gas chromatographic determination of analytes, such as pesticides and other organic compounds, in environmental matrices [1–5].

Flucycloxuron is a benzoylphenylurea which has acaricidal and insecticidal properties. It is an experimental pesticide, to be used for protection of fruit and vegetable crops against a range of insects and mite species [6]. Extraction of flucycloxuron from these fruits and vegetables is performed by macerating the tissue with a suitable organic solvent. Clean-up from matrix constituents is usually performed by liquid–liquid extraction or by adsorption chromatography using silica gel or Florisil.

Flucycloxuron is determined by high-performance liquid chromatography (HPLC) on a reversed-phase (RP) C_{18} column. The clean-up steps mentioned have the disadvantages that they are time consuming, they are not easy to automate and they always cause some loss of analyte, which unfavourably influences the reproducibility. We investigated on-line LC clean-up based on size-exclusion chromatography (SEC).

Bio-beads, an SEC packing material, can only be used for off-line clean-up procedures, as the mobile phase of this kind of column [gel permeation chromatography (GPC)] is not compatible with RP mobile phases [1,2]. Heart cutting is an alternative way to use a GPC column, with polystyrene–divinylbenzene (PS–DVB) as packing material and tetrahydrofuran as mobile phase coupled on-line with RP-HPLC. This method has been described for the analysis of malathion in tomatoes and lemonin in

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grapefruit peel [4,5]. Williams *et al.* [7] developed an interface in order to couple non-aqueous SEC on-line with RP-HPLC. A GPC column based on PS–DVB, with tetrahydrofuran as mobile phase, was used. The eluate from this column, containing the analyte, was mixed with water and transferred via a switching valve to an analytical RP-C_{18} column. This method was described for the determination of 2,6-di-*tert.*-butyl-4-methylphenol in snacks and of dibutyl phthalate in chocolate at levels above 0.5 mg/kg. From initial experiments, we concluded that the clean-up performance of a PS–DVB column was not sufficient for our purposes, as the greater part of the matrix constituents had molecular masses that did not differ sufficiently from the molecular mass of flucycloxuron to obtain an acceptable separation. As flucycloxuron is almost insoluble in water, we started our study with a gel filration chromatographic (GFC) column packed with a cross-linked methacrylate for clean-up purposes in flucycloxuron analysis. This material could be used with a mobile phase containing up to 20% of organic modifier but we concluded that, apart from size exclusion, adsorption also occurred to such an extent that it was impossible to obtain an acceptable chromatographic behaviour.

Our efforts were more sucessful with another type of SEC, using an internal surface reversed-phase (ISRP) column which was introduced by Pinkerton and co-workers [8,9] in 1985. ISRP is a new concept for LC packing materials for bioanalytical purposes. The ISRP material has the ability to exclude large molecules, such as proteins, from the pores with negligible adsorption of these molecules on the external surface. Low-molecular-mass compounds, such as drugs, are separated with good capacity, selectivity and efficiency. Several methods have been described for the direct injection of biological matrices, such as serum and plasma, for the determination of drugs [10–14], in which proteins did not interfere with the chromatographic analysis. A review on the use of ISRP material was published by Pinkerton [15].

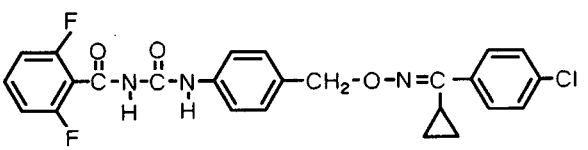
As fruit and vegetable extracts also contain relatively large molecules, we tried this type of packing as on-line LC clean-up material. We compared the results obtained with the ISRP column-switching system with the results obtained using standard methods, using liquid–liquid extraction and classical (low-pressure) column chromatography.

EXPERIMENTAL

Apparatus and materials

The chromatographic system consists of the following apparatus: a Model 231-401 diluter and programmable autosampler (Gilson, Villiers le Bel, France) fitted with a 100-μl loop; an HP1050 quaternary pump (Hewlett-Packard, Waldbronn, Germany), an HP1050 isocratic pump; three pneumatically operated Model 7010 valves (Rheodyne, Cotati, CA, USA); a Model 757 variable-wavelength UV detector (Applied Biosystems, Ramsey, NJ, USA); and an HP3396A integrating system (Hewlett-Packard). All three Rheodyne valves and two HP1050 pumps were controlled by the Gilson 231-401 autosampler. A Pinkerton ISRP GFF-S5-80 (5 μm) column (50 × 4.6 mm I.D.) was obtained from Regis (Morton Grove, TL, USA). A Zorbax ODS reversed-phase column (250 × 4.6 mm I.D.) was purchased from Chrompack (Middelburg, Netherlands). Precolumns filled with C_8 (10 μm), C_{18} (7 μm) and phenyl-bonded (5 μm) (all 30 × 4.6 mm I.D.) and PRP-1 (10 μm) (15 × 3.0 mm I.D.) materials were obtained from Brownlee Labs. (Santa Clara, CA, USA).

Acetonitrile and tetrahydrofuran used in preparing the mobile phases were of HPLC quality and were purchased from Baker (Deventer, Netherlands); ethanol was of pharmaceutical quality and distilled water was of HPLC quality. Solvents used for sample extraction (dichloromethane, acetonitrile and methanol) were of residue quality, obtained from Baker, or were purified by distillation. Light petroleum (b.p. 40–65°C) was obtained from Shell and purified by distillation. Florisil was obtained from Supelco (Bellefonte, PA, USA). Silica cartridges (500 mg) and alumina cartridges (neutral, 1000 mg) were obtained from Baker. An Ultra Turrax Model T 50 homogenizer (Janke & Kunkel, IKA Lab. Technology, Staufen, Germany) was used for grinding and extraction of samples. Fruit and soil samples were obtained from field trials. Flucycloxuron, (*E,Z*)-N-[({4-[({[(4-chlorophenyl)cyclopropylmethylene]amino}oxy)methyl]phenyl}amino)carbonyl]-2,6-difluorobenzamide, obtained from our own resources, has the following structure:



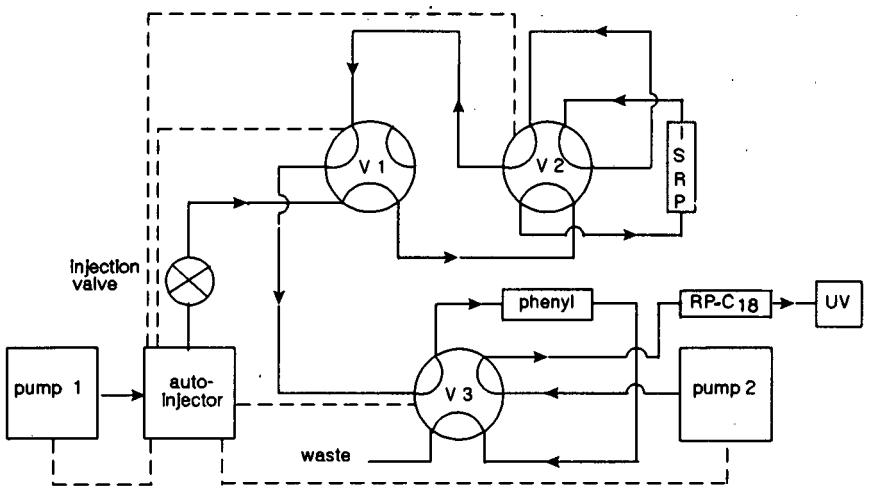


Fig. 1. Schematic representation of column-switching system.

Chromatographic system

The chromatographic system is presented in Fig. 1. Table I shows the schedule of the automated analysis; each valve position 1 corresponds to the position shown in Fig. 1.

In status A, the sample has been diluted with water in order to obtain a solution with the same composition as the ISRP mobile phase. Flucycloxuron cannot be stored in the mobile phase as the solubility during a longer period is insufficient. In

TABLE I

SCHEDULE OF THE AUTOMATED ANALYSIS OF SAMPLES FOR FLUCYCLOXURON USING ISRP AS ON-LINE CLEAN-UP PROCEDURE

Status	Time (min)	Pump 1					Pump 2	Description	Valve position		
		Flow-rate (ml/min)	Proportions (v/v)				Flow-rate (ml/min)		V_1	V_2	V_3
			Water	ACN[a]	THF[a]	EtOH[a]					
A	–	1.00	70	15	15	0	2.50	Sample dilution	1	1	1
B	0.00	1.00	70	15	15	0	0.00	Clean-up ISRP	1	1	2
B	7.00	1.00	70	15	15	0	0.00				
C	7.01	1.00	70	15	15	0	2.50	Preconcentration	1	1	1
C	12.99	1.00	70	15	15	0	2.50				
D	13.00	5.00	70	15	15	0	2.50	Wash precolumn	2	1	1
D	14.99	5.00	70	15	15	0	2.50	(clean-up)			
E	15.00	0.00	70	15	15	0	2.50	Desorption	1	1	2
E	17.00	0.00	70	15	15	0	2.50				
F	18.00	1.00	0	0	100	0	2.50	Back-flush ISRP	1	2	1
F	19.00	1.00	0	0	100	0	2.50				
F	20.00	0.00	70	15	15	0	2.50				
G	21.00	1.00	0	0	0	100	2.50	Wash precolumn	2	1	1
G	22.00	1.00	0	0	0	100	2.50				
G	23.00	0.00	70	15	15	0	2.50				
H	24.00	1.00	70	15	15	0	2.50	Ready for A	1	1	1

[a] ACN = Acetonitrile; THF = tetrahydrofuran; EtOH = ethanol.

status B, the ISRP eluate is directed to waste. In status C, the ISRP eluate is directed to the phenyl precolumn in order to preconcentrate flucycloxuron. In status D, an additional clean-up can be performed by washing the precolumn with the ISRP mobile phase. It is possible to increase the washing step up to a volume of *ca.* 35 ml (we determined the breakthrough volume to be 42 ml). After preconcentration and an additional clean-up step, flucycloxuron was eluted from the precolumn with acetonitrile–ethanol–water (50:15:35, pump 2) to the analytical column (status E). To prevent increasing back-pressure and decreasing ISRP performance, the ISRP column was back-flushed with tetrahydrofuran (status F). In status G, the precolumn was washed with ethanol and subsequently equilibrated with the mobile phase. The total procedure, including quantification by HPLC, is performed in 40 min.

Extraction procedures

Weighed amounts (50–100 g) of apple, strawberry, citrus and soil samples were extracted with 200–250 ml of dichloromethane (apple and strawberry), methanol (citrus) and acetonitrile (soil). For grinding, a top-drive mixer or Ultra Turrax homogenizer was used. For apple, strawberry and soil samples, sodium sulphate (50–100 g) was added to the samples to bind the water. After filtration, the solvent was removed by rotary evaporation. Clean-up from matrix constituents is performed, according to standard procedures, either by liquid–liquid partitioning (dichloromethane–water and acetonitrile–hexane) and adsorption chromatography on Florisil and alumina (citrus), by adsorption chromatography on silica (soil) or Florisil (apple and strawberry) only or by ISRP column switching. Final determination of the *Z*- and *E*-isomers of flucycloxuron is performed, in both instances, by chromatography on an RP-C_{18} column with acetonitrile–water–ethanol (50:35:15) as mobile phase and using UV detection at 254 nm.

RESULTS AND DISCUSSION

ISRP chromatography

Before studying the clean-up performance of an ISRP column, we optimized the chromatography of flucycloxuron on the ISRP column (5 μm) (50 × 4.6 mm I.D.). In the analysis of samples such as plasma and serum for drugs, the mobile phase may contain up to 20% (v/v) of any of four organic solvents: acetonitrile, isopropanol, methanol or tetrahydrofuran [16]. A combination of these solvents must not exceed the limit of 20% when blood serum or plasma samples are assayed. This upper level is applied, as serum albumin is denatured in mixtures containing over 20% of organic modifier. Denatured proteins cause clogging of the stationary phase. However, the ISRP stationary phase can withstand higher concentrations of organic solvents as Regis, the supplier of the ISRP columns, advises [11] flushing of the column with pure tetrahydrofuran in the case of increased back-pressure.

Optimization of the chromatography of flucycloxuron resulted in the mobile phase composition tetrahydrofuran–acetonitrile–water (15:15:70). The flow-rate of the mobile phase was 1.00 ml/min and the column temperature was 35°C. In this case, the mobile phase contains over 20% of organic modifier. However, this caused no problems as fruit and vegetable samples contain few proteins. As mentioned by Pinkerton and Koeplinger [17], the diol–Gly–Phe–Phe-bonded phase favours the retention of aromatic compounds and separates species primarily by a reversed-phase mechanism. We concluded that the retention behaviour of flucycloxuron did not fully correspond with that on reversed-phase material. When tetrahydrofuran was replaced with acetonitrile, the capacity factor did not increase as in reversed-phase chromatography, but remained almost constant. As flucycloxuron is a neutral component, this difference cannot be caused by the carboxylic group of the ISRP material, which is a weak cation exchanger.

ISRP clean-up

We prepared samples as described under *Extraction procedures*. Sample extracts were dissolved in the ISRP mobile phase and 100 μl of these solutions were, after filtration, injected directly on to the ISRP column. The outlet of the column was connected to a UV detector set at 254 nm. The chromatograms of apple, strawberry and soil samples showed that clean-up effects obtained with the ISRP column were good. The *Z*- and *E*-isomers of flucycloxuron were well separated from the bulk of the matrix constituents. After the injection of several samples,

we observed an increased back-pressure and decreased performance. This problem was overcome by back-flushing the ISRP column with tetrahydrofuran after each sample injection.

Preconcentration and HPLC analysis

The fraction in which flucycloxuron elutes from the ISRP column is too large to be transferred directly to the analytical RP-C_{18} column. A precolumn was used to preconcentrate the ISRP fraction in which flucycloxuron elutes and to perform an additional clean-up step. The choice of precolumns depended on the following criteria: the precolumn should not have any adverse effect on the chromatography of flucycloxuron on the RP-C_{18} column and the breakthrough volume should exceed 6 ml, as the ISRP fraction had a volume of 6 ml.

The breakthrough volumes were determined for precolumns (30 × 4.6 mm I.D.) packed with C_8 (10 μm), C_{18} (7 μm), phenyl-bonded (5 μm) and PRP-1 (10 μm) (15 × 3.0 mm I.D.) materials. The breakthrough volumes were determined by injecting 100 μl of a flucycloxuron solution (composition of ISRP mobile phase) containing *ca.* 1 μg/ml on to the precolumn. The precolumn was washed with a variable volume of the ISRP mobile phase, after which on-line desorption was carried out to the analytical RP-C_{18} column using acetonitrile–water–ethanol (50:35:15) as the mobile phase. The breakthrough volumes were between 30 and 50 ml and were sufficient in all instances. The phenyl-bonded (5 μm) precolumn (30 × 4.6 mm I.D.) with a breakthrough volume of 42 ml was selected for further experiments, as this column has the smallest particles and would contribute least to peak broadening on the analytical column.

Analytical data

Calibration solutions were used to study the repeatability, linearity and memory effects (alternate injections of solutions containing 50 μg/ml flucycloxuron and blank solutions) of the total system. Repeatability was studied by tenfold injection of three calibration solutions at levels of 1, 5 and 10 μg/ml [sum of *Z*- (30%) and *E*- (70%) isomers]. Relative standard deviations (R.S.D.) determined for the *Z*- and *E*-isomers ranged between 9.9% (for the lowest *Z*-isomer level, 0.3 μg/ml) and 3.4%. The average R.S.D. was 5%. Linearity was determined by a twelve-point calibration graph at levels between 0.3 and 10 μg/ml (sum of *Z*- and *E*-isomers). The correlation coefficient was 0.9993 for both isomers. The memory effect was less than 0.3%.

Applications

Extracts of blank and treated samples were prepared according to the procedures outlined under *Extraction procedures*. Extracts were prepared to perform analysis in quintuplicate, according to both the standard and ISRP methods. In this way, we compared the clean-up performance obtained with the column-switching system with that of the standard method. Typical chromatograms, obtained from strawberry and citrus samples, are presented for both methods in Figs. 2 and 3.

Fig. 2a and b show the chromatograms of analyses of a strawberry blank and a strawberry sample (treated with flucycloxuron) analysed with the standard method and Fig. 2c and d show the chromatograms of the same samples analysed with the column-switching system. Fig. 2a shows a component that interferes with flucycloxuron determination at lower levels. It was difficult to remove this component using traditional clean-up procedures. However, using the ISRP system, this component was completely eliminated. We have therefore obtained a better clean-up procedure, without performing any off-line clean-up step, within a reduced analysis time.

Fig. 3a and b show the chromatograms of the analysis of a citrus blank and a citrus sample (treated with flucycloxuron), respectively, according to the standard method, and Fig. 3c and d show the chromatograms of the same samples analysed according to the ISRP method. As the extracts of citrus samples contained such a large amount of sample constituents that interfered with the HPLC determination of flucycloxuron, it was necessary to perform an extended clean-up procedure. Using the ISRP method, the off-line clean-up could be limited to one extra liquid–liquid extraction step. This resulted in a sharply reduced analysis time (1.25 h instead of 2.75 h/sample, calculated from a series of eight samples) in comparison with the standard method with the same clean-up effect. During optimization of the automated clean-up procedure, we observed that additional washing of the phenyl-bonded precolumn with a volume of 10 ml of the

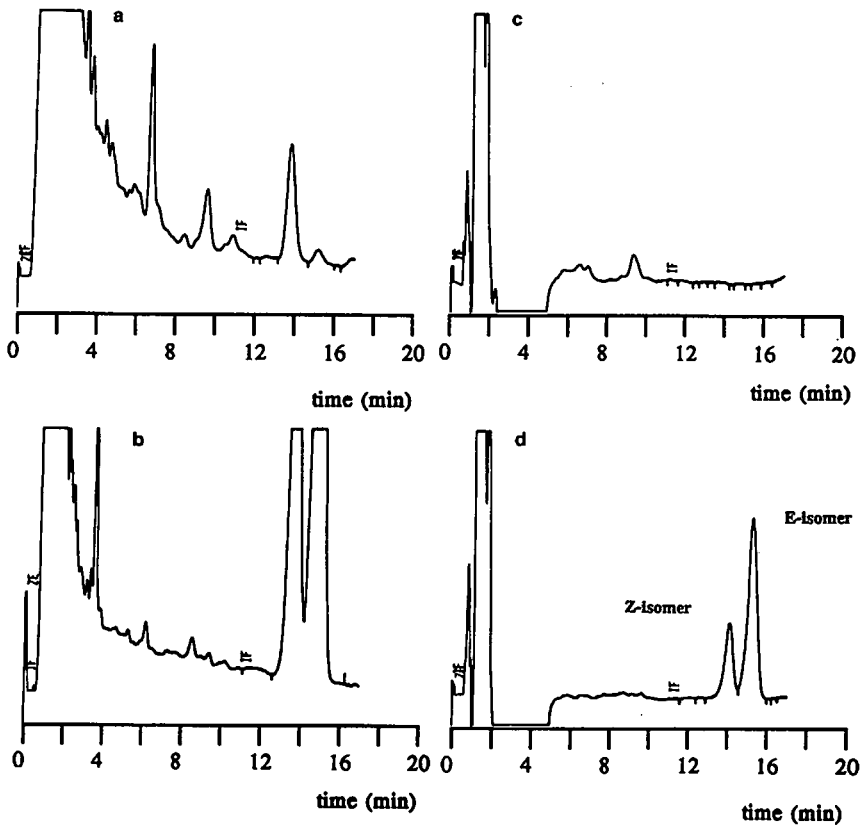


Fig. 2. Determination of flucycloxuron in strawberry samples. Amounts of 100 g of (a) blank sample and (b) a treated sample [*ca.* 0.5 mg/kg (*Z*- + *E*-isomers)] were analysed according to the standard method. An aliquot of the extract (100 ml from 250 ml) was taken and the solvent was evaporated. The residue was dissolved in a mixture of 3 ml of dichloromethane and 25 ml of light petroleum (b.p. 40–65°C) and submitted to Florisil clean-up; the final volume was 3.00 ml. A 100-μl volume was injected on to an RP-C_{18} column with acetonitrile–ethanol–water (50:15:35) as mobile phase. The same samples were analysed according to the ISRP method (c and d): 100 g of sample were extracted, the solvent was removed by evaporation and the residue was dissolved in 2.00 ml of acetonitrile–tetrahydrofuran (1:1). This solution was placed in the Gilson autosampler, where 300 μl were mixed with 700 μl of water. A 100-μl volume of this acetonitrile–tetrahydrofuran–water (15:15:70) mixture were injected into the column-switching system.

ISRP mobile phase resulted in a better clean-up performance. Two components with retention times almost equal to that of flucycloxuron were removed by this additional wash step of the phenyl-bonded precolumn.

The results of flucycloxuron analyses in several matrices are summarized in Table II. All analysis were performed in quintuplicate. For all samples, the amount of flucycloxuron residue determined by using the ISRP method was equal to or greater than that determined by the standard method. The R.S.D.s calculated from the five indvidual results of the ISRP method were satisfactory compared with the results of the standard method.

CONCLUSIONS

The ISRP material has excellent clean-up properties in residue analysis of flucycloxuron in crop and soil matrices. The combination of SEC and RP chromatography, which is characteristic for the ISRP material, has shown much better clean-up properties from matrix constituents than customary SEC techniques.

The column-switching system consists of an ISRP column, a phenyl-bonded precolumn and an RP-C_{18} analytical column. The clean-up is performed on the ISRP column. The fraction in which flucycloxuron elutes from the ISRP column is preconcen-

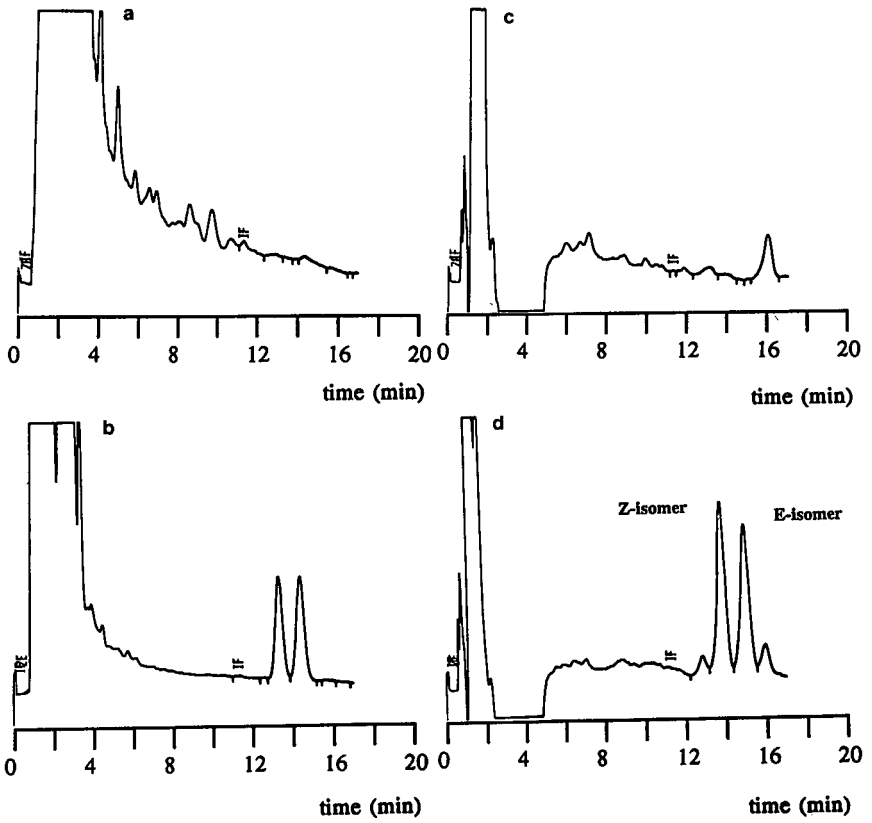


Fig. 3. Determination of flucycloxuron in citrus samples. Amounts of 100 g of (a) blank sample and (b) a treated sample [*ca.* 0.1 mg/kg (*Z*- + *E*-isomers)] were analysed according to the standard method. After evaporation of the methanol, the extract contained only water. An extra 150 ml of water were added and the flucycloxuron was extracted with three 50-ml portions of dichloromethane. The dichloromethane of the combined extract was evaporated and the residue was dissolved in 100 ml of hexane. The flucycloxuron was extracted with three 50-ml portions of acetonitrile. After evaporation of the acetonitrile, the residue was dissolved in a mixture of 3 ml of dichloromethane and 25 ml of light petroleum (b.p. 40–65°C) and submitted to Florisil and alumina chromatography. Finally, the residue was dissolved in 3.00 ml of the mobile phase and an aliquot of 100 μl was injected on to an RP-C_{18} column with acetonitrile–ethanol–water (50:35:15) as mobile phase. The same samples were analysed according to the ISRP method (c and d): 100 g of sample were extracted, the extract was reduced to contain only water by evaporation and then extracted with hexane. The residue was dissolved in a 2.00 ml of acetonitrile–tetrahydrofuran (1:1). This solution was placed in the Gilson autosampler, where 300 μl were mixed with 700 μl of water. A 100-μl volume of this acetonitrile–tetrahydrofuran–water (15:15:70) mixture was injected into the column-switching system.

TABLE II

COMPARISON OF STANDARD METHOD AND ISRP METHOD FOR RESIDUE ANALYSIS OF FLUCYCLOXURON

Type of sample	Standard method				ISRP method			
	Z-Isomer		*E*-Isomer		*Z*-Isomer		*E*-Isomer	
	Average amount (mg/kg)	R.S.D. (%)	Average amount (mg/kg)	R.S.D. (%)	Average amount (mg/kg)	R.S.D. (%)	Average amount (mg/kg)	R.S.D. (%)
Clay soil	0.058	2.3	0.133	1.0	0.059	6.6	0.133	6.1
Apple	0.143	7.6	0.070	7.8	0.145	10.0	0.071	9.0
Strawberry	0.173	5.7	0.328	6.5	0.203	4.4	0.334	3.7
Citrus	0.058	20.3	0.050	15.8	0.068	9.1	0.057	9.8

trated on the phenyl-bonded precolumn. If desired, additional clean-up can be performed by washing the precolumn. After desorption from the precolumn, separation and determination of flucycloxuron are performed on an RP-C_{18} column with acetonitrile–ethanol–water (50:15:35) as mobile phase. The column-switching system is suitable for performing automated sample clean-up of apple, strawberry, citrus and soil samples for residue analysis of flucycloxuron. The total chromatographic analysis time is 40 min. The total analysis time is less than 1.5 h per sample, for all sample types tested, based on a series of eight samples.

Over 300 samples were analysed without a noticeably decreased performance of the ISRP column. Compared with standard methods, using off-line clean-up procedures, such as liquid–liquid extraction and classical column chromatography, the present method has the advantages of reduced loss of analyte (higher recovery), higher selectivity and considerable reduction in analysis time. The column-switching system may also be applicable for the determination of micro-organic pollutants in matrices such as soil. Another application could be automated off-line (perhaps even on-line) clean-up in GC analysis.

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High-performance affinity chromatography of DNA

II☆. Porosity effects

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ABSTRACT

A DNA–silica, $(dT)_{18}$–silica, was prepared and used in a study of the chromatography of the oligonucleotide, $(dA)_{18}$, based upon base pairing. It was shown that hybridization efficiency did not depend upon flow-rates up to 2 ml/min for the small columns (22 × 2 mm) used. As increasing amounts of $(dA)_{18}$ were loaded onto the columns, the columns were found to saturate at a well defined capacity that was always less than the amount that theoretically could have been bound. Maximum capacity was achieved whenever the loading temperature was at least 20–25°C below the temperature at which the loaded oligonucleotide would elute. The effects of porosity on both coupling efficiency and capacity were measured and suggest that pore sizes in the 300–500 Å range are most appropriate for this form of chromatography.

INTRODUCTION

Previously [1], we synthesized a DNA–silica, $(dT)_{18}$–silica and showed it was able to resolve oligonucleotides differing in length by a single nucleotide. In other experiments, a $(dT)_{50}$–silica was synthesized and used to fractionate polyadenylated messenger RNA [poly(A) mRNA] from *Saccharomyces* by poly(A) tail length [2]. In either case, 300 Å pore silica was used and even small columns (*e.g.*, 30 × 4.6 mm) were capable of binding adequate amounts (*e.g.*, 2.2 units of mRNA, *ca.* 88 μg) of polynucleotides. However, we noticed during these experiments that actual column capacity was always less than the theoretical capacity predicted assuming that all DNA coupled to the silica was capable of hybridization [3]. Here, we further investigate this phenomenon. While we did not learn the cause of the diminished capacity, the experiments do give a great deal of information about how to make and use these columns.

The basis for chromatographic separations on DNA–silica is hybridization of the column attached strand with injected, single stranded DNA or RNA. For this affinity chromatography, the amount of $(dT)_{18}$ linked to the column is the maximum theoretical capacity of the column for binding the same length complementary strand; *i.e.*, $(dA)_{18}$. The fact that this theoretical capacity is much larger than the determined capacity for oligoadenylate binding could be explained if: (1) the flow-rate or other conditions of chromatography were inappropriate for maximal binding, (2) the pore size used was too small to allow the applied samples to interact with DNA inside the pores, or (3) the spacer length between the silica surface and the attached DNA chain was too short to allow productive interactions with applied samples.

The last of these can probably be excluded. From the silica surface to the 5′-phosphate on $(dT)_{18}$–silica is the following chain backbone [1]:–Si–C–C–C–

☆ For Part I, see ref. 1.

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N–C–C–C–C–N–C–C–P, a 13-atom spacer with a fully extended length of more than 15 Å. Furthermore, oligoadenines can hybridize anywhere along the length of the $(dT)_{18}$ chain. A $(dT)_{18}$ chain would have a fully extended length of nearly 130 Å and even in a double helix, would extend nearly 61 Å. Thus, an aligoadenine could base pair anywhere in a region extending from near the silica surface to about 150 Å distant. Given that the spacer chain length is probably adequate, the other possible explanations for diminished capacity were investigated.

METHODS

Oligonucleotide synthesis and end labeling

$(dA)_{18}$ was synthesized by the standard phosphoramidite chemistry using the departmental DNA synthesis facility and deblocked on the last cycle. 5′-Aminoethyl-$(dT)_{18}$ [amino-$(dT)_{18}$] was synthesized in a similar manner except that a last cycle utilizing the AminoLink reagent (Applied Biosystems) was included. $(dT)_{18}$ was 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase using standard procedures [4].

Chromatography

The chromatograph was a Gilson Model 9000 binary gradient chromatograph outfitted with a Jasco variable-wavelength UV detector (260 nm, unless otherwise specified), a Gilson 231/401 autosampler, and a Gilson 203 fraction collector. Data collection and programming of the chromatograph were with an Uniq 386sx computer and the Gilson 714 software. Chromatography was conducted at room temperature unless otherwise stated. When the temperature was varied from room temperature, the column and a preheating coil of 1/16-in. stainless-steel tubing (150 cm × 0.010 in. I.D.) was submerged in a water bath at the desired column temperature. Buffer A was 5 m*M* Na_2HPO_4, 0.99 *M* NaCl pH 7.0; buffer B was 5 m*M* Na_2HPO_4 pH 7.0; TE500 buffer was 10 m*M* Tris, 0.1 m*M* EDTA, 500 m*M* NaCl, pH 7.5.

Column preparation

All columns were packed from an isopropanol slurry (100 mg silica per ml, 2 ml/min constant flowrate) using a specially made reservoir and Alltech's direct-connect guard column (22 × 2 mm) as the column hardware. The silica was Macrosphere-WCX 7-μm beads supplied by Alltech (Deerfield, IL, USA) in various pore sizes. The starting material for Macrosphere-WCX was in all cases silicas in the appropriate pore size from Macherey-Nagel derivatized in the same way using the same proprietary process [5]. The columns were activated to the N-hydroxysuccinimidyl ester (NHS) as previously described [6]. Amino-$(dT)_{18}$ was then coupled by recirculation as described by Goss *et al.* [1].

Determination of $(dT)_{18}$ coupling

Unless otherwise stated, the amount of amino-$(dT)_{18}$ coupled was determined by the difference in the absorption (260 nm) units added and the units recovered in column washes. All fractions were titrated to pH 2 to minimize NHS absorption as described by Goss *et al.* [1]. Absorption units were then converted to nmol using a molar absorptivity of 8400 M^{-1} per (dT) base in an oligonucleotide [7]. This gives a conversion factor of 1 unit = 6.61 nmol for $(dT)_{18}$.

For one experiment (Table I), the amount of $(dT)_{18}$ coupled to the silica was also measured using an assay for the inorganic phosphate released when the DNA is oxidatively hydrolyzed. After $(dT)_{18}$ coupling by recirculation as described above, the silica was removed from the 22 × 2 mm columns, washed with water and then acetone and dried in a 110°C oven. The silica was then weighed and the DNA was hydrolyzed to release the inorganic phosphate using a modification of the procedure of Ames and Dubin [8]. Briefly, 200 μl of 10% Mg $(NO_3)_2$ in 95% aqueous ethanol was added to each sample of DNA–silica (30–33 mg). This was then gently heated to evaporate the liquid. The samples were then strongly heated over a Bunsen burner until the brown fumes had disappeared and the bottom of the Pyrex test tube glowed a dull red. After cooling, 0.3 ml of 1 *M* HCl was added and the samples were heated in a boiling water bath for 15 min with the test tubes capped by glass marbles. A 0.7-ml volume of 0.005 *M* H_2SO_4 was then added and 0.1-, 0.2- and 0.4-ml portions were removed and assayed for inorganic phosphate using a modification of the Malachite green method [9]. Briefly, each sample was adjusted to 0.5 ml with 0.005 *M* H_2SO_4 and 2 ml of a freshly prepared solution containing

0.0135% Malachite green, 0.84% ammonium molybdate, 0.1 *M* H_2SO_4 and 0.04% Tween 20 were added. After 5 min, absorption at 660 nm was determined for each fraction. Phosphate standards prepared from K_2HPO_4 were included in each assay. The amount of inorganic phosphate released from the silica is then divided by 18 [the number of phosphates in $(dT)_{18}$] to obtain the $(dT)_{18}$ content of the silica.

Determination of capacity by temperature-dependent elution (Table III and Fig. 4)

Columns containing $(dT)_{18}$–silica of each pore size tested were equilibrated at 5°C in a RTE-210 refrigerated, circulating water bath (Neslab Instruments, Newington, NH, USA), and an excess [over the $(dT)_{18}$ content of the column] of $(dA)_{18}$ was injected. Refrigeration was then discontinued and the water bath set temperature was changed to 60°C to elute the column. Throughout, the mobile phase was buffer A, the flow-rate was 0.2 ml/min, and 4-min fractions were collected. The water bath used consistently warms at approximately 1°C/min under these conditions and the $(dA)_{18}$ consistently elutes near 45 min. Thus, the column is always washed with over 100 column volumes before retained $(dA)_{18}$ elutes. Fractions containing eluted $(dA)_{18}$ were pooled and quantified by absorption at 260 nm using a molar absorptivity of 15 200 M^{-1} cm^{-1} per (dA) base [7]. This yields a conversion factor for $(dA)_{18}$ of 1 unit = 3.66 nmol.

RESULTS AND DISCUSSION

DNA–silica columns were prepared by recirculating 5'-aminoethyl-$(dT)_{18}$ over a prepacked column containing an activated, NHS-ester silica. One advantage to this procedure is that prepacked columns available commercially can be used for coupling which require no column packing expertise. From the difference in the amount of $(dT)_{18}$ added to and recovered after coupling, the amount of $(dT)_{18}$ coupled to silica can be calculated [1]. This method of estimating coupling (by difference) may overestimate the amount coupled and this could explain the lower-than-expected capacity found for such columns. Thus, the amount of $(dT)_{18}$ coupled was also determined by second independent procedure in which the DNA-silica is hydrolyzed and the inorganic phosphate released from the DNA is measured. The results are shown in Table I.

TABLE I

COMPARISON OF TWO TECHNIQUES FOR DETERMINING $(dT)_{18}$ COUPLING

Pore size (Å)	Added (nmol)[a]	Amount coupled	
		Absorption difference (nmol)[a]	Phosphate assay (nmol)[b]
100	27.5	21.0	29.5
	25.2	24.0	25.2
300	30.4	23.9	29.3
	16.2	12.7	15.3
500	16.1	12.4	10.8
	15.9	11.3	14.1

[a] nmol $(dT)_{18}$ determined by difference in DNA absorption as described by Goss *et al.* [1].

[b] nmol $(dT)_{18}$ determined by phosphate assay of the $Mg(NO_3)_2$ hydrolyzed silica assuming 18 equivalents of phosphate per $(dT)_{18}$ equivalent.

The results in Table I clearly show that both methods give similar estimates for the amount of $(dT)_{18}$ coupling regardless of the porosity of the silica used. The method based upon absorption difference gives somewhat lower but comparable numbers to those obtained upon hydrolysis and phosphate assay. The agreement between the two methods suggests that either accurately measures $(dT)_{18}$ coupling. Since both methods give a reliable estimate of $(dT)_{18}$ coupling and the method based upon absorption difference is non-destructive to the DNA–silica, this method was used for all further studies.

Since the amount of $(dT)_{18}$ coupled is accurately measured, we next investigated whether low capacity may have resulted from insufficient time for DNA hybridization during chromatography. The rate of hybrid formation between two stands of DNA is a function of the length and complexity of the hybridizing sequences, the concentration of each strand, and the temperature [7]. Since the kinetics of hybrid formation for DNA immobilized to nitrocellulose is similar to that of DNA in solution [10], the rate of hybrid formation inside high-performance liquid chromatography (HPLC) columns also probably follows similar kinetics. When one

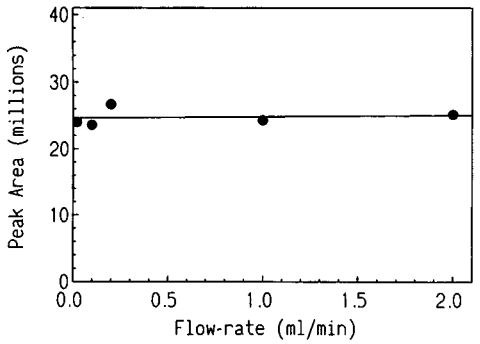

Fig. 1. Column capacity is independent of flow-rate. A 300 Å $(dT)_{18}$ column [containing 35 nmol $(dT)_{18}$ coupled] was loaded with 4 μl containing 0.47 nmol of $(dA)_{18}$ at flow-rates of 0.02, 0.1, 0.2, 1.0 and 2.0 ml/min in buffer A. The column was washed with 0.4 ml of buffer A (5 m*M* Na_2HPO_4, 0.99 *M* NaCl, pH 7). Then the flow-rate was changed to 0.2 ml/min and washing was continued for an additional 3 min. The column was then eluted with buffer B (5 m*M* Na_2HPO_4, pH 7) at 0.2 ml/min. Peak areas are reported in integration units of 10 μV ··milliminute.

strand of a potential DNA duplex is in excess, the rate of hybrid formation is pseudo-first order and the rate constant, k, may be calculated as:

$$k = 3 \cdot 10^5 \, L^{1/2}/N \, (M^{-1} \, s^{-1})$$

where L is the length of the strands and N is the hybrid complexity in base pairs [7]. For $(dT)_{18}$ hybridizing with $(dA)_{18}$, L and N are both 18 and $k = 7 \cdot 10^4 \, M^{-1} \, s^{-1}$.

For this experiment, the column-attached DNA is usually in excess and allows for rapid hybridization as shown by the experiment in Fig. 1. When flow-rate during sample injection was varied over a two order of magnitude range (0.02 to 2 ml/min), the amount of DNA retained by hybridization to the column and subsequently eluted did not change significantly. The small variations found are probably due more to inaccuracies in injecting small samples (4 μl) and in integrating the peaks obtained than to any real effect of flow-rate. The column used in this experiment contains 35 nmol of $(dT)_{18}$ in a column volume of 69 μl or about 0.51 m*M* $(dT)_{18}$. From the equation above, hybridization should occur with a half-time of about 20 ms. Even at 2 ml/min, the highest flow-rate used in Fig. 1, the transit time through the column would be about 2 s or about 100 half-times. At 2 ml/min, backpressures in excess of 150 bar were encountered, thus flow-rates much more than twice this would not be possible with these silica supports and this column size. Thus, even at the highest flow-rate possible for these columns, the amounts of DNA coupled to silica give such rapid hybrid formation that injected DNA is retained regardless of flow-rate. Thus, the lower-than-theoretical capacities under study are not due to slow hybridization under less than optimal conditions.

The lower than expected capacities could also arise if only some fraction of applied DNA hybridizes because of poor mass transfer during chromatography. This is apparently also not the case as the experiment in Fig. 2 shows. As the amount of $(dA)_{18}$ injected onto a column is increased, the amount bound increases up to the point at which the column capacity is reached. The column capacity found in this experiment is the same as that found by simply loading a large excess of $(dA)_{18}$ sample, washing the column, and measuring the amount which elutes. Thus, sample binding by the column is a saturable phenomenon, saturating at a well-defined capacity.

Fig. 2 also demonstrates a strategy that can be used to increase recovery of precious DNA samples: the greatest recovery of injected DNA occurs at sample loads which are only a fraction off the column's actual capacity. The three smallest DNA samples injected in Fig. 2 (leftmost data points,

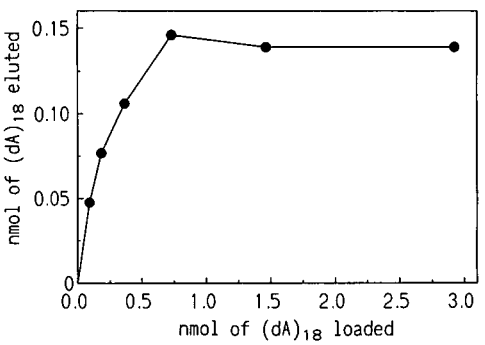

Fig. 2. Column capacity determined with increasing sample load. A 100 Å $(dT)_{18}$ column in buffer A and 30°C was loaded with different amounts of $(dA)_{18}$ (0.092, 0.18, 0.37, 0.73, 1.46 and 2.93 nmol) in 4 μl buffer A and washed for 10 min. Each load was eluted by abruptly changing the mobile phase to water. The flow-rate was 0.5 ml/min and 1.5-min fractions were collected. The $(dA)_{18}$ eluted was determined by absorption of pooled fractions.

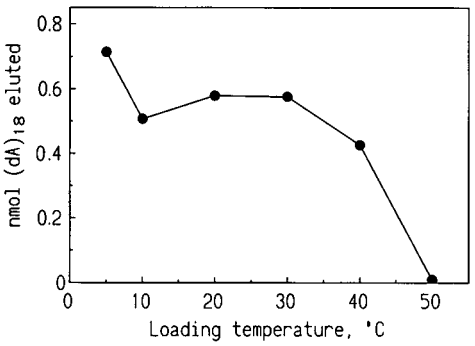

Fig. 3. Effect of loading temperature on column capacity. A 100 Å $(dT)_{18}$ column (containing 11.8 nmol $(dT)_{18}$ and listed on line 3 of Table III) equilibrated in TE500 buffer (10 m*M* Tris, 0.1 m*M* EDTA, 500 m*M* NaCl, pH 7.5) in a water bath at the various temperatures shown was loaded with 0.99 nmol of 5′ end labeled $(dA)_{18}$ (23.1 cpm/pmol) in 10 μl of TE500. The column was then washed for 15 min under the loading conditions and eluted by transferring the column to a 65°C water bath. The flow-rate was 0.5 ml/min and 1-min fractions were collected. The fractions were counted for Cerenkov radiation without scintillation fluid.

0.09, 0.18, 0.37 nmol $(dA)_{18}$) demonstrate this trend. As injected DNA was increased the proportion of it bound by the column decreased (52, 42 and 29%, respectively). This trend has not been rigorously explored but in some experiments where the sample load was approximately 1% of column capacity, 90% of the injected DNA was bound by and eluted from the column (data not shown).

In other experiments (data not shown), 5′ end labeled DNA was either injected as described in Fig. 2 onto a complementary DNA–silica column or recirculated through the column for 1 h. In either case, the amount of labeled DNA retained by the column and subsequently eluted was the same. Thus, recirculation does not improve sample retention and is thus unnecessary.

The effect of loading temperature on column capacity was also investigated as shown in Fig. 3. For this experiment, an amount of $(dA)_{18}$ approximately equal to the column's capacity was injected and the column was washed while maintaining the temperatures shown in the figure. The column was then eluted by abruptly changing the column temperature to 65°C. Under the conditions used, the temperature for elution (T_e) of $(dA)_{18}$ has been measured [1] to be 52°C. While it is clear that loading a sample onto a column at temperatures too close (*i.e.*, within about 25°C) to T_e adversely affects capacity and should be avoided, capacity is reasonably constant from 5–30°C, a fairly wide range of loading temperature.

TABLE II

THE EFFECT OF PORE SIZE ON THE EFFICIENCY OF $(dT)_{18}$ COUPLING

Pore size (Å)[a]	Area (m^2/g)[a]	Added (nmol)[b]	Coupled (nmol)[b]	Coupled (%)
50	450	56.2	18.9	34
		19.2	8.3	43
100	350	72.7	8.9	12
		75.3	11.8	16
300	100	41.9	34.8	83
		66.1	57.3	87
500	35	66.1	47.8	72
1000	25	109.7	40.3	37
4000	10	77.1	35.0	45

[a] Values supplied by the manufacturer.
[b] nmol $(dT)_{18}$ determined by absorption, 260 nm.

The above experiments were carried out on two pore sizes (*i.e.*, 100 and 300 Å). Other pore sizes were also investigated. The effect of pore size on the efficiency of $(dT)_{18}$ coupling was investigated; the results are shown in Table II. Silicas of all porosities tested coupled $(dT)_{18}$ effectively. The most efficient coupling was found for silicas with pores in the 300–500 Å range but the dependence of coupling efficiency on porosity is clearly complex. The lower

TABLE III

EFFECT OF PORE SIZE ON COLUMN CAPACITY FOR $(dA)_{18}$ BINDING

Pore size (Å)	Coupled $(dT)_{18}$ (nmol)	$(dA)_{18}$ Eluted (nmol)	Ratio (%)
50	18.9	0.57	3.1
100	8.9	0.40	4.5
	11.8	0.61	5.2
300	34.8	0.90	2.6
500	47.8	1.18	2.5
1000	40.3	0.57	1.4
4000	35.0	0.01	0.0

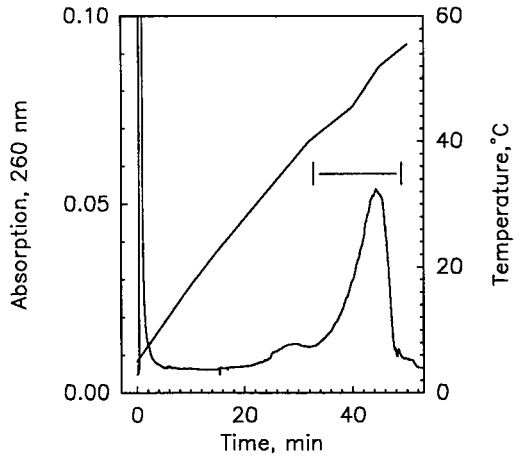

Fig. 4. Chromatography by temperature-dependent ellution. A 100 Å $(dT)_{18}$ column (11.8 nmol coupled) was equilibrated at 5°C in buffer A. A 30-μl volume of buffer A containing 11.6 nmol of $(dA)_{18}$ was then loaded. The temperature gradient was produced as described in the Methods section using a water bath. The temperature of the water bath was measured every 4 min during the separation with a mercury thermometer. The bar shows the position of the fractions pooled to determine column capacity.

amounts coupled to the largest pore sizes are probably due to the lower surface area of these silicas. The lower coupling to the smaller pore sizes may result from restricted access of $(dT)_{18}$ because of its large size. The highest coupling efficiencies for the intermediate pore sizes probably results from a trade-off of adequate pore size with adequate surface area.

The various pore size columns were also tested for the amount of $(dA)_{18}$ they would retain. The results are shown in Table III. For each pore size, an excess of $(dA)_{18}$ [*i.e.*, an amount greater than the $(dT)_{18}$ content of the column] was loaded onto each column at 5°C in 1 *M* Na^+ (*i.e.*, buffer A), and eluted by raising temperature to 60°C. Before elution, over 100 column volumes of buffer A passes through the column (see Fig. 4) ensuring that any non-specifically retained DNA is washed away before the eluted DNA is measured. The data in Table III show that under these conditions never is more than about 5% of the theoretical capacity of the columns actually realized in practice. In other experiments (data not shown), column capacity was also measured using different loading temperatures and eluting by lowering salt concentration; the results are in qualitative agreement with those shown. Perhaps the most remarkable feature of these data is that while $(dT)_{18}$ apparently couples well to 4000 Å pore silica (Table II), the coupled DNA does not retain injected $(dA)_{18}$ (Table III). The reason for this phenomenon is not known.

Considering the data in both Tables II and III, it can be seen that 300 and 500 Å pore silicas give both high coupling efficiencies and relatively high capacities and would be best suited for chromatography of oligonucleotides. Since 300 Å pore columns have also been used to separate mRNA [2], this pore size would also be appropriate for kilobase size polynucleotides.

Fig. 4 shows a typical chromatogram obtained with the temperature-dependent elution protocol used to measure capacities in Table III. It shows that injected DNA not bound by the column appear as a sharp peak; in less than 3 min following injection, the absorption at 260 nm falls to baseline values and remains there until about 25 min into the separation. Thus, diminished capacity is not due to DNA slowly bleeding off the column during the extensive washing procedure used. After about 25 min, the water bath has reached 33°C and some DNA begins to elute in a broad hump extending to about 32 min. This hump contains 12% of the total eluted DNA and was not included in the pooled fractions or used to calculate the capacities in Table III. Capacity was calculated from the major peak containing 88% of the total eluted DNA which reaches a maximum at 44 min when the temperature was 51°C. This melting temperature agrees closely with the 52°C elution temperature previously reported for $(dT)_{18}$:$(dA)_{18}$ hybrids [1]. What the broad hump represents was not investigated other than to demonstrate that it is not a baseline artifact and occurs to about 12% of the total area in all the chromatograms used for Table III. It is likely that this represents oligomers of dA shorter than 18 in length present in the synthetic $(dA)_{18}$ sample since average efficiency of this DNA synthesis was 99% and thus only 83% ($= 0.99^{18}$) of the total synthesized should be an 18-mer and this agrees reasonably well with the 88% peak area for the major peak. Thus, the early eluting broad hump probably represents short oligomers which should elute at lower temperatures as observed. Fig. 4 demon-

strates that low capacity does not arise from slow bleeding of DNA from the column but rather suggests that only a fraction (about 5%, Table III) of the $(dT)_{18}$ coupled to silica ever binds injected DNA at all and that this binding is, for the most part, a normal hybridization between 18-mers. Thus, while capacity is lower than expected, coupled DNA which does not participate in the affinity chromatography appears to be inert and does not interfere with the separations obtained.

The experiments show that high flow-rates can be used with DNA–silica columns since hybridization is quite rapid (Fig. 1). The columns become saturated by high sample loads and bind only a well defined capacity (Fig. 2). The capacity of a column is also relatively unaffected by loading temperatures well below T_c (Fig. 3). However, the capacity of the column is a function of the porosity of the silica support, and the amount of DNA coupled to the silica (Table III). While all pore size silicas couple DNA efficiently (Table II), porosities of 300 and 500 Å give the best combination of coupling efficiency (Table II) and column capacity (Table III). However, even a column optimized for porosity will typically bind less than 5% of the amount it should be capable of binding (Table III). We did not discover the cause for this diminished capacity, but a lower than expected capacity has also been observed for other kinds of affinity chromatography. For example, melittin forms a 1:1 complex with calmodulin [11] but melittin–silica containing 1.4 mg of melittin (0.49 μmol) bound only 1.6 mg (0.096 μmol) or about 20% of the theoretical capacity [12]. In other cases, actual and theoretical capacity were found to be nearly identical [13].

Hybridization was rapid at the levels of $(dT)_{18}$ coupled in the experiments here. The highest flow-rates permissible with these columns allowed 100 half-times for hybrid formation (Fig. 1). Since hybridization would be *ca.* 97% complete in only 5 half-times, the amount of DNA coupled could be decreased about 20-fold and still allow rapid chromatography. However, the results in Fig. 2 show that when sample load is well below column capacity, a larger fraction of the sample is retained by the column. When sample load is only a small fraction of capacity, nearly quantitative recovery would be expected. Thus, column capacity should be kept high to improve recovery and detection of injected samples; an aditional consequence of this high capacity will be that chromatography can be performed rapidly. Highest capcity was found at temperatures less than about 25°C below T_c (Fig. 3). It is interesting to note that in using DNA probes for complementary sequences, the most rapid hybrid formation occurs at 25°C below the hybrid melting temperature [7]. In previous studies, we have also found a reasonably good agreement between the temperature and salt dependence of DNA–silica elution and what would be predicted from other studies of DNA hybridization [1]. Thus, chromatography follows similar rules to those already discovered in other DNA hybridization experiments and thus is a very predictable form of chromatography. This is not surprising since all DNA hybridization, whether in an HPLC column or not, depends ultimately upon the chemistry of the specific base pairing in double-stranded DNA.

The observation that no more than about 5% of theoretical capacity could be obtained with DNA–silica would be important if adequate capacities could not be obtained and this is clearly not the case. This is not a significant limitation since the capacities measured are already quite large for many practical experiments. For example, DNA–silica columns have been used to fractionate poly (A) mRNA, from yeast [2]. For these uses, very little of the mass of a mRNA sample is actually polyadenylate and so fairly large capacities are obtained. For example, for a 2-kilobase mRNA with a poly(A) tail 50 long (such as is typical in *Saccharomyces*), only about 2.5% of the length is actually poly(A). For such an mRNA, a column which binds 0.7 nmol of $(dA)_{18}$ (such as was encountered in Fig. 3) should bind about 8 units of mRNA (about 400 μg). Since we obtain capacities over 0.7 nmol $(dA)_{18}$ routinely on some pore sizes in 23 × 2 mm columns (Table III) containing only 33 mg of silica, capacities are already quite high and larger columns or coupling larger amounts of $(dT)_{18}$ should allow columns of adequate capacity for most experiments.

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Purification of DNA-derived deoxynucleotides from leukocytes involving nuclease elution of an ion-exchange column

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ABSTRACT

A method has been developed in which the DNA of leukocytes (as the buffy coat from blood) is isolated in the form of its constituent deoxynucleotides. The steps in this method are as follows: (1) lyse the leukocytes with sodium dodecyl sulfate (SDS) and enzymatically digest the proteins and RNA, (2) remove the SDS on a non-polar adsorbent (Bio-Beads SM-4) and then trap the DNA on a quaternary amine silica cartridge, (3) wash the column with 1 *M* NaCl–buffer, (4) digest the DNA on the column with staphylococcal nuclease and (5) elute the digested DNA with 0.5 *M* NaCl–buffer and digest it further with bovine spleen phosphodiesterase II to deoxynucleotide-3′-monophosphates. From a 40-μl sample of butty coat was obtained 126 ± 14 μg (two experiments, eight sample total) of deoxynucleotides. Reversed-phase high-performance liquid chromatography, which removed the added enzymes, showed only peaks for deoxynucleotides. For comparison, the amount of deoxynucleotides obtained from the leukocytes by an automated phenol extraction procedure was 101 ± 5.4 μg (one experiment in triplicate).

INTRODUCTION

Several methods are available for extracting DNA from biological samples. Typically these methods start with cell lysis followed by the addition of proteases and ribonucleases to digest the corresponding components of the sample. The DNA can then be isolated by a variety of techniques including aqueous–organic partitioning [1,2], ion-exchange chromatography [3,4] size-exclusion chromatography [5,6], ethanol precipitation [7,8] or centrifugation [9]. Aside from practical considerations, a given method for isolating DNA may be selected depending on the nature of the DNA (*e.g.* chromosomal or plasmid), how pure the DNA needs to be, and the degree to which the DNA must be isolated in an intact *versus* fragmented form.

One purpose for isolating DNA from a biological sample is for the determination of DNA adducts. The latter refers to the chemical damage, typically covalent, that DNA can undergo upon exposure to toxic chemical or physical conditions. Good correlations have generally been observed between the ability of genotoxic agents to cause cancer or mutations, and their capacity to form DNA adducts [10]

We are interested in the determination of DNA adducts by capillary electrophoresis and also mass spectrometry. For some of these studies it is appropriate to isolate the DNA from a biological sample as deoxynucleotides. Here we report a procedure for this purpose which is applied to a sample of leukocytes (buffy coat) obtained from human blood. In one of the steps, DNA bound to an anion exchanger is digestively eluted with a nuclease.

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MATERIALS AND METHODS

Chemicals and reagents

Leukocyte-enriched blood (Leukopack) was kindly provided by Children's Hospital, Harvard Medical School (Boston, MA, USA). Wild-type staphylococcal nuclease (E.C. 3.1.4.7) was isolated in our laboratory by a previously described procedure [11]. An engineered strain of *Escherichia coli* carrying the expression plasmid PFOG405 was utilized. The bacterium was kindly provided by David Shortle (Johns Hopkins University, Baltimore, MD, USA). Highly polymerized calf thymus DNA type I, ribonuclease A type III-A, proteinase-K type XI, sodium dodecyl sulfate (SDS), and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma (St. Louis, MO, USA). Bovine spleen phosphodiesterase type II (E.C. 3.1.4.18) was from Worthington (Freehold, NJ, USA). Bio-Beads SM-4 were from Bio-Rad (Rockville Center, NY, USA). Bakerbond quaternary amine silica (40 μm, Bulk Prep LC Packing 7043-00, "QAE-silica") was from J. T. Baker (Phillipsburg, NJ, USA).

High-performance liquid chromatography (HPLC)

The HPLC system comprised a Series 4 LC pump from Perkin-Elmer (Norwalk, CT, USA), an Ultra-Sphere-5 ODS 150 × 4.6 mm column (Beckman, San Ramon, CA, USA), a 9060 Polychrom diode array detector (Varian, Walnut Creek, CA, USA), and a Cl-10B Integrator (LDC Milton Roy, Bloomfield, CT, USA). Solvent A was 0.1 *M* potassium dihydrogenphosphate (pH 4.6 inherently), and solvent B was acetonitrile. The deoxynucleoside-3′-monophosphates from the enzymatic digests were separated using a gradient of 0 to 6% B in 9 min followed by 6–35% B in 21 min at a flow-rate of 1 ml/min. Absorbance units full scale was 0.1 and the detection wavelength was 260 or 215 nm.

DNA extraction–digestion procedure

Buffy coat. Leukocyte-enriched blood, obtained at Children's Hospital from a Baxter-Fenwal (Deerfield, IL) CS-3000 plateletpheresis apparatus, was centrifuged at 200 *g* for 30 min at 4°C. The plasma was aspirated and the buffy coat was aliquoted using a Pasteur pipette into plastic tubes and stored at −20°C until further use.

Buffy coat (40-μl samples, two experiments: n = 2 and 6 samples) was thawed and added to 0.5 ml of 10 m*M* Tris–HCl buffer, pH 7.4, 1% SDS, 0.1 m*M* EDTA (TE buffer) in a borosilicate glass tube. To test recovery, a second set of buffy coat samples were run in parallel which were each spiked with 56 μg of calf thymus DNA in 40 μl of water. After each tube was sonicated (water bath sonicator) for 5 min at room temperature, 12 μl of ribonuclease A (10 μg/μl in water) was added. The tube was incubated at 37°C for 1 h in a shaking water bath. The addition of ribonuclease A and 1 h incubation was repeated. Proteinase K (15 μl of 2.5 μg/μl in water) was added and a similar incubation was done for 0.5 h. This proteolytic step was repeated twice more.

Bio-Beads SM-4 (0.5 g) were dry-packed into a Supelclean solid-phase extraction tube (3 ml size, 6.4 × 0.9 cm, catalogue No. 5-7024) and washed under gravity flow with approximately 3 ml each (fill column to the top) of water and then TE buffer. The residual solvent was forced out with a brief stream of nitrogen, and the bottom of the column was capped.

The entire sample was added to the extraction tube (for SDS removal). After the top was capped, the tube was placed on a rocking plate for 10 min at room temperature. The tube was drained (both caps off) and eluted with 3 × 0.5 ml of TE buffer, followed by a brief nitrogen flow through the column at the end. The SDS-free eluents were combined, giving a 2-ml sample which was placed in a boiling water bath for 30 min and then in an ice bath for 15 min, yielding a single-stranded DNA solution.

QAE-Silica (0.5 g) was dry-packed into an extraction tube and washed as above (including intermittent nitrogen flows) with approximately 3 ml each of hexane, methanol, water and TE buffer, followed at the end by nitrogen. The single-stranded DNA sample was applied and rocked in this tube for 2–18 h at room temperature (equivalent results were obtained throughout this period). The QAE-silica was drained and washed with 12 ml of 10 m*M* Tris–HCl, pH 8.8, 1 *M* NaCl, followed by 6 ml of the same buffer containing 0.5 *M* NaCl. Residual buffer was forced out with a brief stream of nitrogen.

To the bottom-capped tube was added 1 ml of 10 m*M* Tris–HCl, pH 8.8, 0.5 *M* NaCl, 10 m*M* $CaCl_2$, and 25 μl of a 1 μg/μl solution of staphylococcal

nuclease in water. After incubating on a rocking plate for 2 h at room temperature, the tube was drained and eluted with 3 × 1 ml of 10 m*M* Tris–HCl, pH 8.8, 0.5 *M* NaCl, followed by nitrogen. The A_{260} values of the fractions were recorded. The pH was adjusted to 5.8 by the addition of 200 μl of 1 *M* sodium acetate, pH 5.8. Each fraction was treated with 20 μl (0.2 Units) of bovine spleen phosphodiesterase II, followed by a 1-h incubation at 37°C in a shaking water bath. A 30-μl aliquot was then subjected to HPLC.

Automated DNA extraction. We are grateful to James Swenberg and Vernon Walker, previously at the Chemical Industry Institute of Toxicology (CIIT), for performing DNA extractions (for comparison purposes) on three 40-μl samples of buffy coat that we provided. They used a Model 340A nucleic acid extractor (Applied Biosystems, Foster City, CA, USA) for this purpose.

RESULTS AND DISCUSSION

Procedure

Our method for the isolation of nucleotide-3′-monophosphates from leukocytes is summarized in Fig. 1. The leuckocytes are first lysed in 1% SDS and the proteins and RNA are enzymatically digested. The SDS is then removed by hydrophobic adsorption onto Bio-Beads SM-4, a polystyrene–divinylbenzene packing.

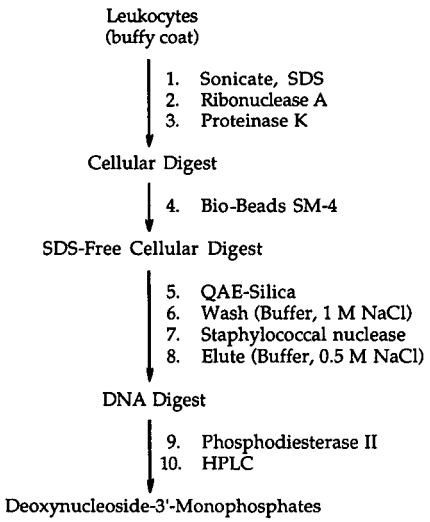

Fig. 1. Scheme for isolating DNA-derived deoxynucleotides from leukocytes.

The SDS-free solution containing the DNA is applied to a cartridge column packed with quaternary amine-silica (QAE-silica). Based on preliminary experiments with calf thymus DNA (data not shown), better recoveries are obtained when the DNA is heat denatured prior to adsorption on this anion exchanger. Contaminants are removed by washing with buffer containing 1 *M* NaCl. The DNA, due to its high molecular weight and polyanionic structure, does not elute from the anion exchanger under these conditions.

In order to prepare the column for the next step, it is washed with a lower-ionic-strength buffer (0.5 *M* NaCl) also containing a small amount of calcium chloride (calcium is essential for nuclease activity). The DNA is then digested on the column with staphylococcal nuclease, giving a mixture of 3′-deoxynucleotides and small oligonucleotides [12]. This low-molecular-weight material readily elutes from the QAE-silica with buffer containing 0.5 *M* NaCl. Prior to HPLC, the final step, any oligonucleotides remaining in the eluate are digested with phosphodiesterase II [12].

Application to buffy coat leukocytes

When deoxynucleotide-3′-monophosphates are isolated from a 40-μl aliquot of buffy coat leukocytes by the above method, the final sample (3.0 ml) gives 1.24 ± 0.13 A_{260} units/ml (mean ± S.D., data from two runs, total of 8 samples). One absorbance unit of DNA at 260 nm corresponds to approximately 50 μg of DNA per ml [13]. We determined that nuclease digestion causes one A_{260} unit/ml of heated DNA to increase up to 1.47 A_{260} units/ml (hyperchromic shift). Relying on this latter value, 126 ± 14 μg of nucleotides are obtained per sample of buffy coat. The A_{260}/A_{280} for the sample is 2.30. For comparison, a sample of calf thymus DNA was similarly digested, also giving an A_{260}/A_{280} of 2.30. Completion of DNA digestion is evidenced by the absence of significant HPLC dinucleotide peaks as we have demonstrated before [12], and equivalent results in this respect are obtained for calf thymus DNA digested directly or via the new procedure.

To estimate recovery, we spiked each of six buffy coat aliquots with 56 μg of calf-thymus DNA. The spiked samples give 183 ± 13 μg of nucleotides

(1.80 ± 0.14 A_{260} units/ml). This indicates a 100% recovery of the added calf thymus DNA.

For comparison, we extracted DNA from identical samples of buffy coat by an automated phenol extraction procedure (Applied Biosystems nucleic acid extractor). After comparable enzymatic digestion of the isolated DNA in 1.0 ml of buffer, the yield is 2.98 ± 0.16 A_{260} units/ml (n = 3) or 101 ± 5.4 μg of deoxynucleotides. This is 20% lower than the apparent quantitative yield of 126 μg (see above) by our method. The automated procedure has been reported by the manufacturer to give 37 ± 3 μg of DNA per ml of blood starting from 6 ml of blood [14]. If one assumes that the blood which was extracted contained 45 μg/ml of DNA, a typical value [2], then the yield of 37 μg corresponds to an 83% recovery. This is consistent with our observation that the automated method appears to recover 80% of the DNA from the buffy coat samples. The value of A_{260}/A_{280} for the deoxynucleotides obtained after the phenol extraction procedure (followed by the exhaustive enzymatic digestion to deoxynucleotides) is 2.31.

Subjecting the deoxynucleotides from leukocytes to reversed phase HPLC gives the chromatograms shown in Fig. 2a (detection at 260 nm) and Fig. 2b (215 nm). The four major deoxynucleotides elute, as seen, in the following order: deoxycytidine-, deoxyguanosine-, thymidine- and deoxyadenosine-3′-monophosphate. The identity of the peaks was confirmed as before [12]. The early-eluting salt peaks are also present in a method blank in which the initial sample is 40 μl of water instead of buffy coat (data not shown). The two minor peaks which elute after deoxyadenosine-3′-monophosphate are apparently trace amounts of residual dinucleotides. The latter have been investigated in more detail in a related study [12]. Enzymatic digestion of the DNA obtained by the automated phenol extraction procedure gives identical chromatograms (data not shown). Ribonucleotides are absent: their retention times are approximately one-half those of the corresponding deoxyribonucleotides by reversed-phase HPLC (*e.g.* ref. 15).

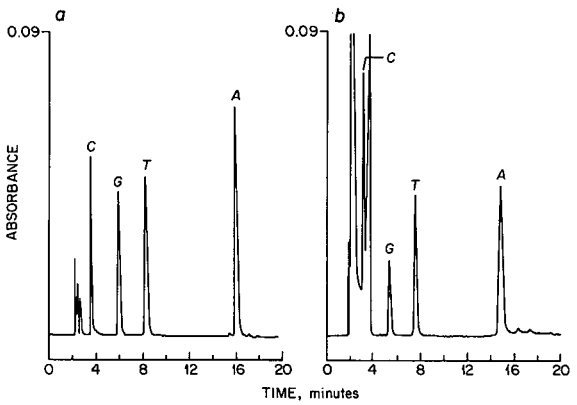

Fig. 2. Reversed-phase HPLC chromatograms of deoxynucleoside-3′-monophosphates obtained from leukocytes. A 30-μl aliquot from the final 3.0-ml sample was injected. Based on calibration with external standards, approximately 2 nmol of each of the four major deoxynucleotides were injected. The HPLC solvent is a gradient of acetonitrile in 0.1 *M* potassium phosphate, pH 4.5: 0–6% acetonitrile in 9 min followed by 6–35% acetonitrile in 21 min. The wavelength of detection is (a) 260 nm or (b) 215 nm.

CONCLUSIONS

We have established a convenient, low-cost, high-yield method for extracting DNA-derived deoxynucleotides from leukocytes. An interesting step in the procedure is the digestive elution of the DNA from a quaternary amine silica packing. Potentially the method will be useful for the isolation of trace amounts of DNA adducts from leukocytes, and from other cells as well.

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Purification of recombinant human interferon-β by immobilized antisense peptides

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ABSTRACT

Synthetic antisense peptides encoded in the antisense strands of DNA corresponding to the 1–14, 42–54 and 103–115 fragments of the human interferon-β sequence were applied in the purification of recombinant human interferon-β from a mammalian cell culture. The protein fragments were selected on the basis of their computer-predicted exposure on the surface of the protein. The antisense peptides were synthetized by the solid-phase method directly on the resin used as the stationary phase in affinity chromatography. All the tested antisense peptides showed a selective affinity for human interferon-β, permitting a ten-fold purification of the protein.

INTRODUCTION

Several studies have been already reported on the affinity between peptides encoded by the antisense strands of DNA, named antisense (AS) peptides, and those coded by the corresponding sense strands. These include peptides complementary to adrenocorticotropic hormone [1,2], ribonuclease S peptide [3], γ-endorphin [1], substance P [4], lutein izing hormone-releasing hormone (LH–RH) [5], anglotensin II [6], interleukin-2 [7], fibronectin [8], insulin [9] and glycoprotein GPIIa [10]. As the interaction between peptides and the corresponding AS-peptides is selective, their use in affinity technology has been proposed. AS-peptides immobilized on chromatographic supports have been employed to isolate native polypeptides, such as the Arg^8-vasopressin–bovine neurophisin II biosynthetic precursor [11], recombinant c-raf protein [12] and the Arg^8-vasopressin–receptor complex [13].

Human interferon-β (hIFN-β) is a glycoprotein of 166 amino acid residues, secreted by human primary fibroblasts after induction with virus or double-stranded RNA. Alternatively, hIFN-β can be obtained by recombinant bacterial or mammalian cell cultures [14–16]. The interest in this protein is connected with its therapeutic value in viral diseases and against certain types of tumours such as brain tumours and malignant melanomas [17,18]. The purification of hIFN-β from various sources has been attempted by a variety of methods over many years and some procedures have been proposed that yield a homogeneous protein [19–23].

To evaluate the potential of AS peptides in affinity chromatography, we applied this approach in the purification of hIFN-β from a recombinant Chinese hamster overay cell line (CHO-rhIFN-β). Fragments 1–14, 42–54 and 103–115 of the hIFN-β sequence were chosen on the basis of their computer-predicted exposure on the surface of the protein [24]. More precisely, the sequences 42–54 and 103–115 were selected for their highly hydrophilic character deduced from the hydropathic profile [25]. Sequence 1–14 was selected because in the Chou–Fasman-predicted secondary structure [26] it contains a β-turn between two β-sheet strands, which is frequently found in exposed regions of proteins [24].

The sequences of the AS-IFN peptides, AS-IFN 1–14, AS-IFN 42–54 and AS-IFN 103–115, shown

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hIFN-β 1-14

H-Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser-Asn-OH

AS-IFN 1-14

H-Ile-Ala-Ala-Ser-Leu-Gln-Glu-Ser-Lys-Gln-Val-Val-Ala-His-OH

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hIFN-β 42-54

H-Glu-Glu-Ile-Lys-Gln-Leu-Gln-Gln-Phe-Gln-Lys-Glu-Asp-OH

AS-IFN 42-54

H-Val-Leu-Leu-Leu-Glu-Leu-Leu-Gln-Leu-Leu-Asp-Leu-Leu-OH

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hIFN-β 103-115

H-Glu-Glu-Lys-Leu-Glu-Lys-Glu-Asp-Phe-Tyr-Arg-Gly-Lys-OH

AS-IFN 103-115

H-Phe-Ser-Pro-Gly-Glu-Ile-Phe-Phe-Leu-Gln-Phe-Phe-Phe-OH

Fig. 1. Amino acid sequences of the hIFNβ fragments 1–14, 42–54 and 103–115 and of the corresponding AS-IFN peptides.

in Fig. 1 were specified by DNA complementary to the DNA coding the corresponding hIFN-β fragments. The nucleotide sequences of the gene encoding hIFN-β was obtained from the λEMBL4 library of the partial Sau3A fragment of the human lymphoblastoid cell line GM 1416.

The AS-IFN peptides were synthesized by the solid-phase method on a resin, which was used directly as the stationary phase without detaching the peptides, thus avoiding expensive and time-consuming procedures for peptide isolation and immobilization [11–13].

The results achieved in purification with the AS-IFN peptides were evaluated and compared with those obtained with a monoclonal antibody anti-hIFN-β column. An attempt was made to demonstrate the selectivity of the interaction between AS-IFN peptides and CHO-rhIFN-β.

EXPERIMENTAL

Materials

CHO-rhIFN-β produced in recombinant CHO cell culture at a concentration of 0.5 μg/ml with a specific activity of $1.2 \cdot 10^5$ I.U./mg [27] was purified 50-fold by controlled-pore glass (CPG) chromatography [28] and extensively dialysed against 0.125 *M* NaCl–0.1 *M* sodium phosphate buffer (pH 7) [29].

The final solution contained CHO-rhIFN-β with a specific activity of $6 \cdot 10^6$ I.U./mg with a concentration of $3 \cdot 10^6$ I.U./ml, corresponding to 10 μg/ml of IFN-β.

The chromatographic supports containing the immobilized AS-peptides AS-IFN 1–14, AS-IFN 42–54 and AS–IFN 103–115, called R-AS 1–14, R-AS 42–54 and R-AS 103–115, respectively, were prepared on a Biolynx 4175 peptide manual synthesizer, using the standard continuous-flow 9-fluorenylmethoxycarbonyl–polyamide strategy [30] and commercial Ultrosyn resin with 0.1 mequiv./g of sarcosine (Pharmacia–LKB). The resin was activated with 1,6-diaminohexane (Aldrich) prior to use. Details of the synthesis have been described elsewhere [31].

The AS-IFN peptide resins were swollen in dimethylformamide (DMF), then equilibrated with solutions of DMF–water (80:20, 50:50 and 20:80, v/v) and finally conditioned with 0.125 *M* NaCl–0.1 *M* sodium phosphate buffer (pH 7).

Monoclonal antibodies against hIFN-β (mAb-IFN) were purchased from CellTech and purified on Protein A–Sepharose 4 FF (Pharmacia).

Citric acid, diammonium sulphate, disodium hydrogenphosphate, sodium chloride, DMF and triethylamine (TEA) were obtained from Carlo Erba. Acetonitrile (ACN, HPLC grade) and trifluoroacetic acid (TFA) were supplied by Merck. Water was purified with a Milli-Q system (Millipore).

Aqueous solutions were filtered through a 0.45-μm cellulose acetate filter and organic solutions through a 0.5-μm PTFE filter. All the eluents were degassed by purging with helium prior to use.

Apparatus

All the chromatographic experiments were carried out on a Pharmacia apparatus consisting of a Model P-500 pump, a Model MV-7 injection valve equipped with a Super Loop (10 ml), a Model UV-1 monitor, a Model Rec-1 recorder and a Model Frac-100 fraction collector. The eluents were connected to the pump through a Model MV-8 eight-way valve. Samples were introduced into the Super Loop by a Model P-1 peristaltic pump. All the components were controlled by a Model LCC-500 controller.

Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a BakerBond Wide-Pore octadecyl column (100 × 4.5 mm I.D., 5-μm particle size) (Baker) and using an HPLC system assembled from two Model 114M pumps, a Model 420 System Organizer, a Model 163 variable-wavelength detector (Beckman) and a Model 7700 Professional computer for data acquisition and reporting (Perkin-Elmer).

Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on Phast System apparatus on precast gels (Phast Gel gradient 10–15, Pharmacia). The electrophoretic gels were evaluated on a Model Ultro-Scan XL laser densitometer (LKB).

Amino acid analyses were performed on an Alpha-Plus amino acid analyser (LKB).

Methods

The IFN-β antiviral activity was measured by the method of cytophathic effect inibition [32]. Protein mass was determined by the Lowry method [33]. mAb-IFN were immobilized on cyanogen bromide-activated Sepharose 4B, following the procedure described by Pharmacia.

The stationary phases R-AS 1–14, R-AS 42–54, R-AS 103–115, Ultrobsyn, DEAE-Sepharose FF and mAb-IFN Sepharose 4B were packed in 100 × 5 mm I.D. columns and conditioned with 0.125 *M* sodium chloride–0.1 *M* sodium phosphate buffer (pH 7.0). All the columns were run under similar chromatographic conditions. In a typical experiment, after having loaded 30 ml of the CHO-rhIFN-β solution, the columns were washed with ten column volumes of 0.125 *M* sodium chloride–0.1 *M* sodium phosphate buffer (pH 7.0), then the adsorbed products were eluted with ten column volumes of 0.1 *M* citric acid (pH 2.0). In the run with human serum albumin (HSA), 3 ml of a 0.1 mg/ml solution were loaded.

In the cation-exchange experiment a prepacked Mono-Q 50 × 5 mm I.D. column (Pharmacia) was used under the above conditions. In all the chromatographic runs the linear velocity was 0.6 cm/min.

Preparative purification of CHO-rhIFN-β by RP-HPLC was carried out at a flow-rate of 1 ml/min on the above RP column. A 30-ml volume of CHO-rhIFN-β solution was loaded on the column, previously conditioned with 30 m*M* TEA–TFA (pH 7.0), then the column was washed with 30 ml of 30

m*M* TEA–TFA in ACN–water (52:48) at pH 7.0 and finally it was eluted with 0.1% TFA in ACN–water (52:48).

RESULTS

The RP-HPLC and SDS-PAGE profiles of the crude solution of CHO-rhIFN-β are shown in Fig. 2. The proportion of CHO-rhIFN-β in the total protein content was about 8%, calculated from the SDS-PAGE profile.

The amino acid analysis of the AS-peptide resins R-AS 1–14, R-AS 42–54 and R-AS 103–115, reported in Table I, gave a purity of the synthesized peptide of *ca.* 90%.

As it has been reported that hydropathic complementary might provide the driving forces in the recognition between sense and antisense peptides [12, 34–36], in Fig. 3 the hydrophatic profiles of AS-IFN peptides are compared with the native hIFN-β sequences.

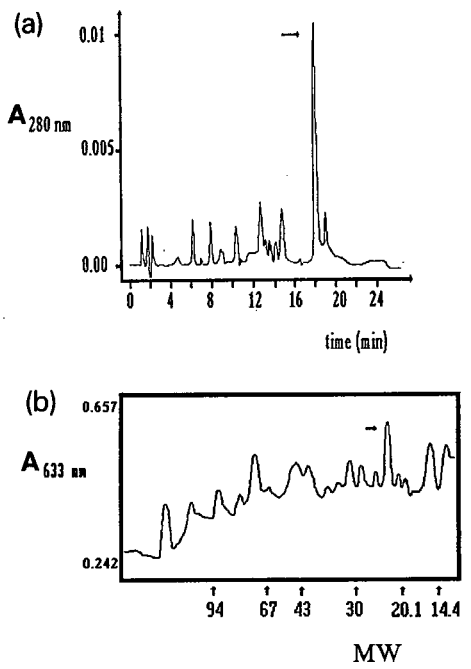

Fig. 2. Chromatographic profiles of crude CHO-rhIFN-β solution. (a) RP-HPLC analysis. Column, 100 × 4.6 mm I.D. Baker-Bond Wide-Pore ODS. Eluents: (A) 0.1% TFA in ACN–water (5:95); (B) 0.1% TFA in ACN–water (95:5). Linear gradient of B from 20% to 55% in 15 min and then 55% for 3 min. Flow-rate, 1 ml/min. Sample, 20 μml of the ten-fold concentrated solution. (b) SDS-PAGE gel scan. Sample, 1 μl of the ten-fold concentrated solution. CHO-rhIFN-β peaks indicated by arrows; MW = molecular weight in kilodalton.

TABLE I

AMINO ACID ANALYSIS OF THE AS-IFN STATIONARY PHASES

Hydrolysis was carried out with 6 *M* HCl at 110°C for 18 h. Theoretical values in parentheses.

R-AS 1–14	R-AS 42–54	R-AS 103–115
His 0.94 (1)	Leu 8.30 (9)	Phe 5.70 (6)
Ala 3.00 (3)	Asp 1.00 (1)	Gix 2.13 (2)
Val 0.86 (1)	Glx 2.21 (2)	Leu 1.00 (1)
Glx 3.35 (3)	Val 0.90 (1)	Ile 0.97 (1)
Lys 1.11 (1)		Gly 9.90 (1)
Ser 1.83 (2)		Pro 0.91 (1)
Leu 0.95 (1)		Ser 0.94 (1)
Ile 0.90 (1)		

The AS-IFN stationary phases gave similar chromatographic behaviours and an elution profile of a representative experiment for the purification of CHO-rhIFN-β on the AS-IFN columns is reported in Fig. 4. As can be seen, almost all the protein material was eluted from the column during the loading of the sample. The amount of CHO-rhIFN-β loaded was chosen to exceed the saturation level of the AS-IFN stationary phases, thus making possible the calculation of the binding capacities. The adsorbed CHO-rhIFN-β was eluted from the column with 0.1 *M* citric acid and no further material was eluted with 1 *M* citric acid.

The chromatographic yields and the CHO-rhIFN-β purity obtained in these runs are summarized in Table II, and in Fig. 5 the scans of the SDS-PAGE gels of the peaks collected at pH 2 are reported. With the stationary phase R-AS 42–54, an increase in the sodium chloride concentration in the wash step to 0.5 *M* slightly reduced the binding capacity to $1.1 \cdot 10^7$ I.U./ml with a specific activity of $6.7 \cdot 10^7$ I.U./mg.

When the AS-IFN columns were replaced with a DEAE-Sepharose FF or Mono-Q column, and tested under the same chromatographic conditions, no adsorption of CHO-rhIFN-β was detected. The same result was obtained when the AS-IFN columns were replaced with the unfunctionalized Ultrosyn column. In addition, no adsorption was

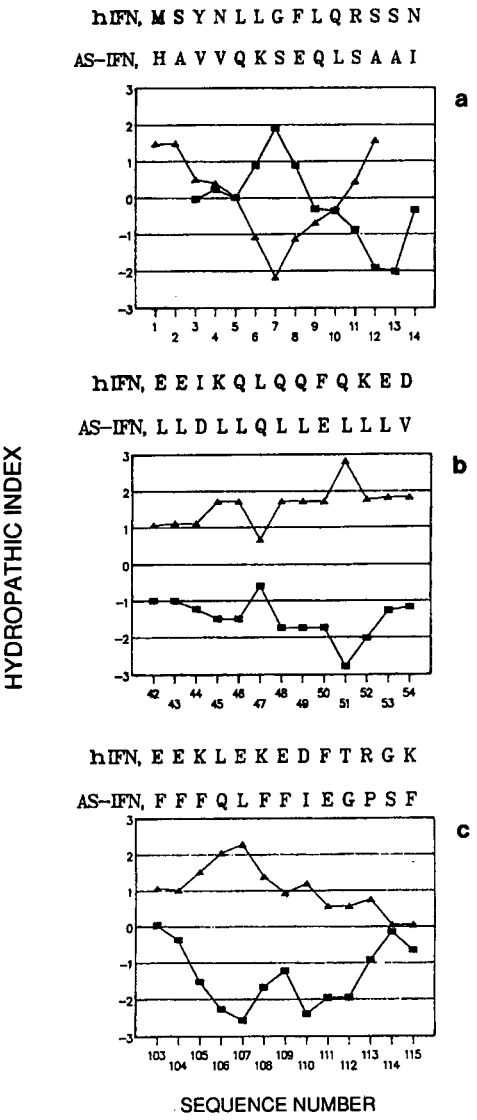

Fig. 3. Comparison of the hydrophatic profiles of (■) hIFN fragments with those of the (▲) AS-IFN peptides. (a) hIFN-β 1–14 and AS-IFN 1–14. (b) hIFN-β 42–54 and AS-IFN 42–54. (c) hIFN-β 103–115 and AS-IFN 103–115. The best complementarity is obtained with the AS-IFN peptides laid down in opposite alignment to the hIFN-β sequences. The hydrophatic indices used are those proposed by Kyte and Doolittle [37].

found when HSA was loaded on the R-AS 42–54 column.

In RP-HPLC, CHO-rhIFN-β was completely retained at pH 7.0 with 30 m*M* TEA–TFA in any ACN gradient, whereas it was eluted with 0.1% TFA at pH 2 in an ACN gradient (see Fig. 2).

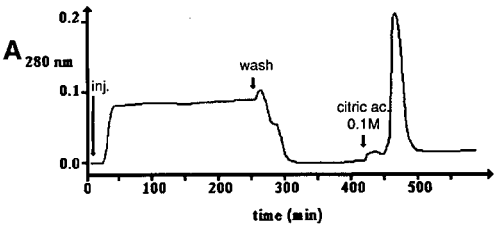

Fig. 4. Elution profile of the preparative purification of CHO-rhIFN-β obtained with R-AS 42–54. Similar elution profiles were obtained with the other AS-IFN stationary phases.

Fig. 6 shows the profile of the preparative purification of CHO-rhIFN-β on the RP column. In the inset the gel scan of the collected material at pH 2 is shown.

An outline of the purification of CHO-rhIFN-β obtained with the mAb-IFN column is reported in Fig. 7; the inset shows the gel scan of the peak collected at pH 2. Purified CHO-rhIFN-β was collected with a specific activity of $1 \cdot 10^8$ I.U./mg and a purity of 82%. The binding capacity was $3.4 \cdot 10^7$ I.U./ml.

A comparison of the RP-HPLC analysis of the purified material obtained with the R-AS 42–54 stationary phase and mAb-IFN Sepharose 4B stationary phase is reported in Fig. 8.

DISCUSSION

The tenfold purification of biologically active CHO-rhIFN-β achieved with each of the AS-IFN stationary phases, R-AS 1–14, R-AS 42–54 and R-AS 103–115, confirms the selective recognition of sense peptides by AS peptides and their useful application in affinity chromatography.

In an attempt to recognize which mechanism is mainly responsible for the interaction of AS-IFN

TABLE II

AS-IFN CHROMATOGRAPHY

Stationary phase	Binding capacity (I.U./ml)	Specific activity (I.U./ml)	IFN-β purity (%)
R-AS 1–14	$9.2 \cdot 10^6$	$6.6 \cdot 10^7$	84.3
R-AS 42–54	$1.4 \cdot 10^7$	$6.4 \cdot 10^7$	86.8
R-AS 103–115	$5.2 \cdot 10^6$	$4.5 \cdot 10^7$	83.2

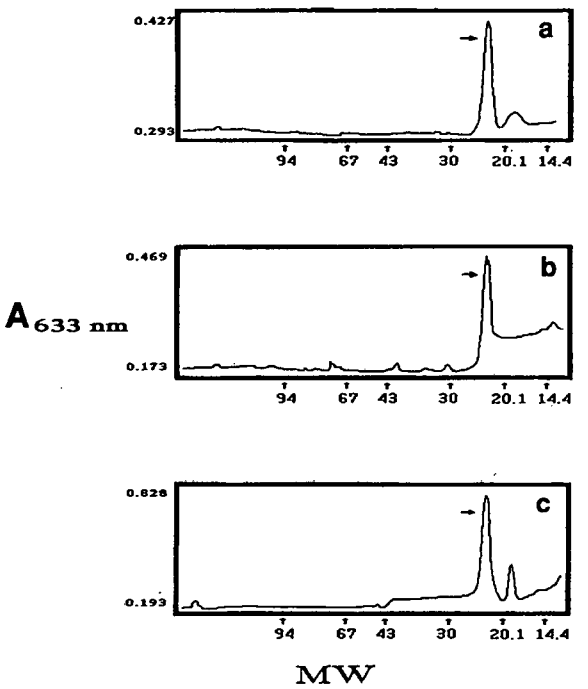

Fig. 5. Gel scans of the peaks collected at pH 2 in AS-IFN chromatography. (a) AS-IFN 1–14; (b) AS-IFN 42–54; (c) AS-IFN 103–115. CHO-rhIFN-β peaks indicated by arrows; MW = molecular weight in kilodalton.

peptides with CHO-rhIFN-β, coulombic attraction, dipolar and/or hydrogen bonding interactions and hydrophobic interaction were evaluated.

The chromatographic behaviour of CHO-rhIFN-β on the AS-IFN columns cannot be explained by a predominant ion-exchange interaction,

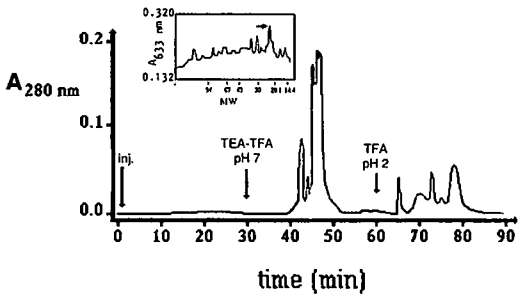

Fig. 6. RP-HPLC profile of the crude CHO-rhIFN-β purification. Inset: gel scan of the peak collected at pH 2 (CHO-rhIFN-β peak indicated by arrow; MW = molecular weight in kilodalton).

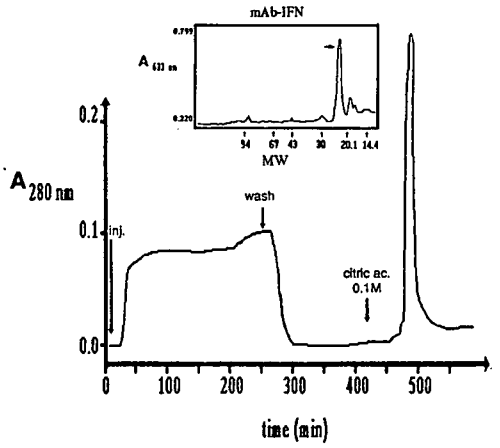

Fig. 7. Profile of the crude CHO-rhIFN-β purification on mAb-IFN column. Inset: gel scan of the peak collected at pH 2 (CHO-rhIFN-β peak indicated by arrow; MW = molecular weight in kilodalton).

as an increase in the ionic strength of the eluents only slightly reduced the binding capacity without affecting the purity of the collected material, and on replacement of the AS-IFN columns with cation- or anion-exchange columns not detectable adsorption

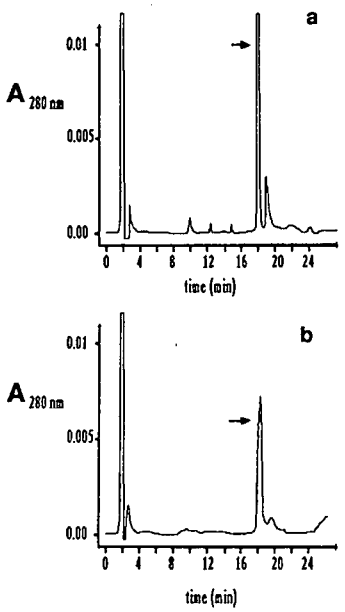

Fig. 8. RP-HPLC analysis of purified CHO-rhIFN-β (peaks indicated by arrows) obtained with (a) R-AS 42–54 and (b) mAb-IFN Sepharose 4B. Conditions as in Fig. 2.

of CHO-rhIFN-β was observed. The purification of CHO-rhIFN-β cannot be related to any non-specific peptide–protein interaction because, for example, HSA was not adsorbed when loaded on R-AS 42–54 stationary phase. Dipolar and/or hydrogen bonding interactions can also be excluded as the main mechanism accounting for the selective adsorption of CHO-rhIFN-β on the AS-IFN stationary phases. Indeed, all the proteins present in the CHO-rhIFN-β solution came from a CPG purification, where they were eluted at pH 2, after being retained at neutral pH. In this kind of chromatographic technique the dipolar and hydrogen bonding interactions have been recognized as making the main contribution to rhIFN-β adsorption [38]. Therefore, the same type of interaction that produced a CHO-rhIFN-β with a purity of 8% in the CPG chromatography cannot justify alone the selectivity obtained in the AS-IFN chromatography.

A hydrophobic interaction could be hypothesized considering the CHO-rhIFN-β behaviour in RP-HPLC at pH 7.0 and 2.0. However, the roughly similar chromatographic performances obtained with the AS-IFN peptides in contrast to their different hydrophobic characters, whose values obtained by summing the hydrophatic contributions of each amino acid are AS-IFN 1–14 +2.9, AS-IFN 42–54 +27.6 and AS-IFN 103–115 + 15.3 and the poor selectivity obtained with the RP purification, also precludes any predominant role of this kind of interaction.

Finally, considering that non-specific adsorptions with the solid-phase synthesis matrix are also absent, we can conclude that only a multi-modal and sequence-dependent binding mechanism is responsible for the interaction of CHO-rhIFN-β with the AS-IFN peptides. This conclusion is supported by the similar chromatographic behaviours of CHO-rhIFN-β with the AS-IFN peptides and mAb-IFN, even though the latter instance a higher binding capacity was achieved.

It is worth noting that the main impurities, still present after the As-IFN chromatography, have molecular weights lower than that of CHO-rhIFN-β and similar products are also present after the mAb-IFN column, thus suggesting possible IFN-β fragments and confirming the specificity of the interaction of the AS-IFN peptides with hIFN-β sequences.

The good hydropathic complementary of the AS-IFN peptides and the corresponding native hIFN-β fragments does not exclude that this could be the reason of the interaction, leaving the possibility of improving the purification performance by optimizing their hydrophatic complementary.

Further, the results demonstrate that it is possible to localize portions of proteins that could interact with properly designed AS-peptides, following the computer-assisted approach previously applied in the search for immunodominant sequences. We believe that the possibility of using as a stationary phase the peptides attached to the solid support used in the synthesis makes the AS-peptides approach more feasible, as it permits rapid screenings of many sequences. Nevertheless, once proper peptides and sequences have been recognized, it would be advisable to use less expensive and more suitable chromatographic supports especially when large-scale purification is needed.

Finally, it could be significant for the explanation of the specific interaction that the best chromatographic performance was obtained with AS-IFN 42–54, *i.e.*, a peptide that should interact with the hIFN-β region where the active site is assumed to be located [39].

CONCLUSIONS

Immunoaffinity chromatography is a very versatile method in protein purification. However, in industrial applications the use of this technique is hampered by the cost of antibodies and by the need to validate the process, where the problems come from the biological sources of antibodies and by their leakage into purified products. Therefore, affinity techniques with synthetic ligands which exhibit selectivity and a wide range of applications similar to immunoaffinity chromatography are desirable.

In this work, it has been demonstrated that AS peptides can be usefully applied as ligands in the purification of a recombinant protein with performances similar to those obtained with a monoclonal antibody column. The use of AS-peptide columns in tandem could improve the purification performance, whereas the proteic sequences of the impurities contain only one biding site among those which chould interact with the chosen AS-peptide station-

ary phases. The possibility of deducing the correct AS peptide sequences only on the basis of the sequence of proteins to be purified makes this approach general and permits a wide application to a number of known proteins.

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Use of basic alumina in fractionation of fossil fuels

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ABSTRACT

The use and efficiency of extrographic fractionation are reported for a number of fossil fuels and their processing products. The influence of basic alumina, which is a constituent of the column packing, on the fractionation selectivity is discussed. This fractionation is capable of an efficient separation of non-polar hydrocarbons, pyrrolic-type nitrogen compounds, and phenolic compounds. The hydrocarbon fraction also includes highly condensed polyaromatics. In the chromatographic fractionation these polyaromatics are eluted along with heteroatom-rich compounds. The extrographic fractionation has been found to be independent of column loading on going from a sample-to-sorbent ratio of 1:15 to 1:8 (w/w). However, it is not possible to separate aromatic hydrocarbons according to the ring number, and therefore the extrography is a suitable method for the fractionation of coal-derived liquids containing large amounts of heteroatom compounds of different polarity.

INTRODUCTION

The elucidation of the compound class composition of fossil fuels and their processing products is a prerequisite for process control work and for obtaining valuable fundamental information on cracking, hydrotreating and refining processes. A substantial amount of work has been done on developing appropriate fractionation procedures, which meet most of the generally desired capabilities, *e.g.*, separation of saturated compounds, mono-, di- and polyaromatics, basic and neutral nitrogen compounds, sulphur compounds and phenols. Unfortunately, no simple procedure exists, and a complex separation scheme, which involves a number of separation steps, is commonly needed for a detailed fractionation [1–13].

Basic alumina has not often been used for the fractionation of petroleum- and coal-derived liquids. Nevertheless, it possesses the potential for an excellent separation of nitrogen compounds [7,14–17]. As opposed to neutral alumina [5,18,19], its basic properties are responsible for the strong retention of compounds with hydrogen-donating capability on a chromatographic column [7,15]. On the other hand, the retention of basic nitrogen compounds may be lowered, and some of these elute together with aromatic hydrocarbons [7,14]. The spreading of basic nitrogen compounds over several fractions has been overcome by using a combination of basic alumina and silica gel in the fractionation column [15,16]. The basic compounds can thus be eluted together with phenolic compounds, allowing fractions of nitrogen-free aromatics and pyrrolic-type compounds to be obtained. Further, some highly condensed polyaromatics can be present in the nitrogen fraction as significant contaminants because of the strong retention of highly condensed polyaromatics on basic alumina [20]. On the other hand, a clean cut is obtained between the fractions of nitrogen and phenolic compounds [21,22].

However, as a result of an electron donor–accept-

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or interaction between the sorbent and solute, the extent of irreversible adsorption of the more polar and acidic compounds on the sorbent surface is higher for basic than for neutral alumina. It is almost impossible to release dihydric phenols and related compounds from the fractionation column [7]. Hence basic alumina fractionation is a valuable method particularly for the separation of nitrogen compounds from middle distillates [14,17]. Nonetheless, it has also been used for the extensive fractionation of coal liquids from a solvent-refined coal process [23–27].

The results presented in this paper are based on extrographic fractionation with the combined use of silica gel and basic alumina in the column [15]. This fractionation has been utilized in our laboratory for 3 years. A number of materials have been fractionated and analysed by spectroscopic methods to give compositional and average structural characteristics of the samples. The aim of this work was to evaluate the separation efficiency of the silica gel–basic alumina-based extropgraphic method relative to the material fractionated.

EXPERIMENTAL

Materials studied

The materials fractionated in our laboratory by using the extrographic technique [15] have so far involved the following coal-derived liquids: a distillation residue from the hydroliquefaction of bituminous coal, a coal-tar pitch, a laboratory pyrolysis tar from the carbonization of bituminous coal (samples F and S), commercial phenols from gasification of tar and a chloroform extract of a subbituminous coal. Other fractionated liquids were shale oil and maltenes derived from petroleum vacuum residue. The fractionation and analysis of some of these material have been described elsewhere [15,16,28].

Sorbents

Silica gel 60 (Merck) (particle size 0.063–0.200 mm) was activated in a vacuum oven for 16 h at 130°C. Its activity was then adjusted by addition of 4% (w/w) of water.

Neutral and basic alumina 90 (Merck) (particle size 0.063–0.200 mm, activity I) were reactivated for 8 h at 400°C.

Fractionation procedures

All extrographic fractionations were performed as described [15]. Briefly, a column (450 mm × 13.5 mm I.D.) was dry packed successively with silica gel (75 mm), a sample precoated on silica gel in a ratio of 1:15 (w/w) (300 mm) and basic alumina (75 mm). Two hydrocarbon fractions and a pyrrolic-type compounds fraction were eluted through basic alumina with *n*-hexane, toluene and chloroform. Polar fractions were eluted by reversing the solvent flow through the silica gel filter section with chloroform–diethyl ether (9:1, v/v) and tetrahydrofuran–methanol (17:3, v/v).

Additionally, the compound-class composition of petroleum-derived maltenes was determined according to the chromatographic method of Sawatzky *et al.* [4]. The purity of the individual hydrocarbon classes was carefully controlled by UV spectrophotometry through the determination of the molar absorptivities and by ^{1}H NMR spectroscopy [29]. The elution solvents of Sawatzky *et al.* [4], including chloroform to yield the pyrrolic nitrogen fraction, were also applied in the extrographic fractionation of petroleum maltenes in addition to the solvents mentioned above. The separation procedure on neutral alumina with the elution order of Sawatzky *et al.* [4] was also used for the fractionation of the aromatic extrographic fractions of the pyrolysis tars.

Analytical methods

Infrared spectra were acquired on a Bruker IFS 88 Fourier transform IR or a Specord M80 dispersive IR spectrometer at a resolution of 2 or 4 cm^{-1}. Solutions in dichlormethane were measured at a concentration of 30 or 50 mg/ml in a 0.6-mm sodium chloride cell.

Average molecular weights were measured on a Knauer vapour-pressure osmometer in benzene or tetrahydrofuran at 32 and 45°C, respectively. Four measurements were made at finite concentrations and data were extrapolated to infinite dilution.

Nitrogen contents in samples together with carbon and hydrogen percentages were determined with a Perkin-Elmer Model 240 C elemental analyser.

RESULTS AND DISCUSSION

Separation efficiency

Details on the extrographic fractionation have been published recently [15,16]. One of the advantages of this procedure lies in obtaining a nitrogen-free aromatic fraction and a highly concentrated pyrrolic-type compounds fraction. Infrared spectra of these two fractions in the spectral region 3700–3300 cm^{-1} for a number of fractions are shown in Fig. 1.

Except for petroleum maltenes, the aromatic fractions exhibited only negligible or no absorption around 3460 cm^{-1}, which is characteristic of N–H bond vibrations. This was also revealed by the determination of the nitrogen percentage, which is presented in Table I as being below 0.2% (w/w), but the elemental analyses gave values from 0 to 0.15% (w/w). The pyrrolic-type compound fractions showed a significant absorption in the infrared spectra due to N–H bond vibrations around 3460 cm^{-1} (Fig. 1). Also, the nitrogen percentages in Table I are correspondingly high. The best concentrate of nitrogen compounds was obtained for shale oil,

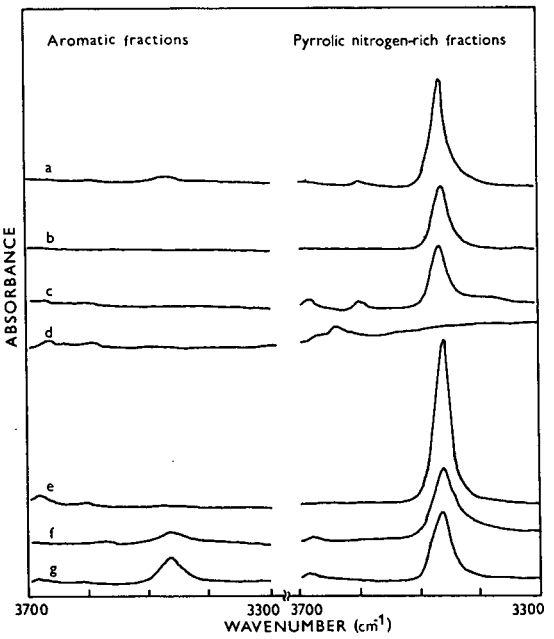

Fig. 1. Infrared spectra of aromatic and pyrrolic nitrogen-rich fractions. a = Liquefaction product; b = coal tar pitch; c = commercial phenols; d = coal extract; e = shale oil; f and g = petroleum maltenes.

TABLE I

YIELD AND NITROGEN PERCENTAGE OF AROMATIC HYDROCARBON AND PYRROLIC-TYPE COMPOUND FRACTIONS IN THE STUDIED SAMPLES

Sample	Aromatic fraction		Pyrrolic-type compounds fraction	
	Yield (%, w/w)	N (%, w/w)	Yield (%, w/w)	N (%, w/w)
Liquefaction product: distillation residue[a]	24.1	<0.2	2.5	2.0
Coal tar pitch: toluene extract	59.2	<0.2	3.2	1.3
Pyrolysis tar: maltenes[b]	39.5–52.9	<0.2	–	–
Gasification tar: commercial phenols[a]	9.1	<0.2	1.0	1.9
Sub-bituminous coal: chloroform extract	9.9	<0.2	4.7	0.2
shale oil[a]	14.4	<0.2	1.9	4.2
Petroleum bottom: maltenes	59.2	0.2	3.5	1.0
	17.0[c]	0.3	4.3	1.0

[a] From ref. 15.
[b] From ref. 28.
[c] Polyaromatics developed with benzene by separation with the elution order of Sawatzky *et al.* [4].

which is generally known to be rich in nitrogen compounds, including amines. These can also contribute to a high peak due to N–H vibrations in infrared spectra. The clean cut between pyrrolic-type and phenolic compounds is documented by the absence of a hydroxyl absorption band around 3600 cm^{-1} in the infrared spectra (Fig. 1).

Negligible amounts of nitrogen compounds were found in the pyrrolic-type compounds fraction of the coal extract. This is probably due to the low nitrogen percentage (0.3%, w/w) and/or the low abundance of purely non-basic nitrogen compounds in the chloroform-extractable material of coal. However, owing to the high oxygen content in the coal extract (10.5%, w/w), the existing nitrogen bridges between aromatic rings may be accompanied by oxygen functionalities within the molecule. This changes the chromatographic behaviour of such molecules by the superposition of moderately polar entities. Instead of the nitrogen compounds, a high abundance of carbonyl compounds (ketones, esters) was present in the "nitrogen compound fraction". The relevant regions of the infrared spectrum are shown in Fig. 2. The possibility of eluting the carbonyl species in this fraction has also been proposed in earlier work [15].

The separation of pyrrolic-type compounds from aromatics in the petroleum maltenes was not as successful as for coal-derived liquids and shale oil (Fig. 1). The nitrogen content of the total aromatics slightly exceeded 0.2% (w/w) and in the polyaromatic fraction obtained with an elution order according to Sawatzky *et al.* [4] was even 0.3% (w/w) (Table I). Taking into account the average molecular weights of aromatics (790) and pyrrolic-type compounds (910), the nitrogen compound content in these fractions can be as high as 15% (w/w). On the other hand, about one third of the nitrogen fraction is constituted by non-nitrogen compounds, probably polyaromatics [20]. This is probably also the case for the pyrrolic-type compounds fraction of coal-tar pitch, which exhibited a low nitrogen content of only 1.5% (w/w) (Table I), although by size-exclusion chromatography on Sephadex LH-20 a nitrogen fraction of coal-tar pitch with a nitrogen content as high as 5.5% (w/w) was obtained [30].

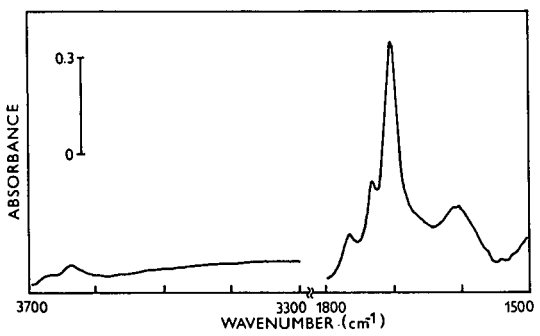

Fig. 2. Relevant part of the infrared spectrum for chloroform-extractable material of coal (measured in dichlormethane at a concentration of 50 mg/ml).

Both of these materials are rich in polyaromatics and the content of polar compounds is low. These aspects and possible steric shielding of nitrogen sites in the case of petroleum maltenes can lead to a poorer separation efficiency of these materials in comparison with the other fractionated liquids.

Effect of column loading

To assess the separation efficiency at higher column loadings, shale oil was fractionated at a sample-to-sorbent ratio ranging from 1:15 to 1:8 (w/w). The corresponding infrared spectra of aromatic and pyrrolic-type compound fractions are shown in Fig. 3, and integrals of the relevant spectral band together with the yield of the fractions are presented in Table II. These data lead to the following conclusions.

The percentage of hydrocarbon compounds is independent of the sample-to-sorbent ratio. The in-

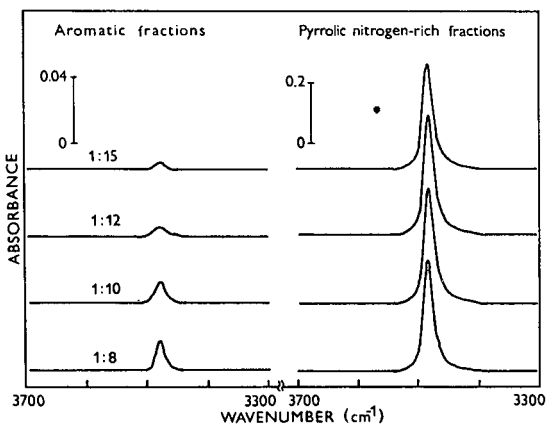

Fig. 3. Infrared spectra for aromatic and pyrrolic-nitrogen rich fractions of shale oil at different sample-to-sorbent ratios from 1:8 to 1:15 (w/w) (measured in dichlormethane at a concentration of 30 mg/ml).

TABLE II

EFFECT OF COLUMN LOADING ON THE YIELD OF FRACTIONS AND ABUNDANCE OF N–H BONDS FOR FRACTIONATION OF SHALE OIL

Elution solvent	Sample-to-sorbent ratio (w/w)							
	1:15		1:12		1:10		1:8	
	Yield (%, w/w)	INT_{3460} (A cm^{-1})[a]	Yield (%, w/w)	INT_{3460} (A cm^{-1})	Yield (%, w/w)	INT_{3460} (A cm^{-1})	Yield (%, w/w)	INT_{3460} (A cm^{-1})
Hexane	41.6	–	42.2	–	43.2	–	43.8	–
Toluene	16.5	0.1	16.6	0.2	15.4	0.3	15.0	0.3
Chloroform	1.1	9.1	1.2	10.2	1.4	9.5	1.6	9.2
Tetrahydrofuran–methanol (17:3, v/v)	25.6	–	26.5	–	25.0	–	25.1	–

[a] Integral of N–H bond vibrations; see Fig. 3. A = Absorbance.

crease in the yield of fraction 1 developed with hexane is at the expense of fraction 2 developed with toluene.

The abundance of nitrogen compounds in aromatics (fraction 2) has a slightly increasing tendency with increasing column loading, but it can still be considered to be negligible.

There is an increase in the amount of the pyrrolic-type compounds fraction developed with chloroform (fraction 3) with increasing column loading. However, the abundance of nitrogen compounds in these fractions is almost constant, and consequently some of the nitrogen compounds seem to remain unrecovered in the corresponding fraction at lower loadings. Of course, quantitative elution of these compounds at higher loadings is also in question.

No phenolic compounds were eluted even at the highest sample-to-sorbent ratio in either aromatic or pyrrolic-type compound fractions. Nonetheless, the changes in the analytical results described are small, and higher loadings can succesfully be used for the fractionation of coal-derived liquids with satisfactory efficiency.

Separation of aromatic hydrocarbon classes

For materials rich in aromatics, there is often a need to know the distribution of aromatics in the individual aromatic hydrocarbon classes. To evaluate the possibility of the utilization of the extrographic technique for this purpose, the petroleum maltenes were fractionated by using three methods: (1) the chromatographic technique on dual silica gel–neutral alumina columns according to Sawatzky *et al.* [4] with careful control of the eluting material by UV and ^1H NMR spectroscopy [29]; this procedure provides the fractionation of aromatics according to ring number, at least into mono-, di- and polyaromatic fractions; (2) the extrographic technique with elution solvents as for the above method; chloroform was ordered after benzene to yield a pyrrolic nitrogen-rich fraction; (3) the extrographic technique with the originally proposed elution solvents [15].

Table III gives the yields of fractions produced by the respective methods. It can be clearly seen that the extrographic technique produced more material in the individual hydrocarbon fractions, especially in the first fraction of saturates. Therefore, the extrographic technique cannot be used to fractionate an aromatics-rich material according to ring number. Also, the cummulative yield of fractions up to benzene–toluene elution and the total material recovered are higher for the extrographic than for the chromatographic technique (Table III). These findings may be due to a larger retention of the fractionated material on alumina in chromatography. A column loading of about 1% (w/w) relative to sorbents used is commonly utilized, as opposed to extrography with a column loading more than five times higher. The short section of basic alumina in the extrographic column is overloaded and, therefore, incapable of retaining more condensed poly-

TABLE III

FRACTIONATION OF PETROLEUM MALTENES BY DIFFERENT METHODS

Elution solvent	A[a]		B[a]		C[a]	
	Yield (%, w/w)	Cumulative yield (%, w/w)	Yield (%, w/w)	Cumulative yield (%, w/w)	Yield (%, w/w)	Cumulative yield (%, w/w)
Hexane	8.8	8.8	19.1	19.1	19.4	19.4
Hexane–benzene (19:1, v/v)	8.0	16.8	10.6	29.7	–	–
Hexane–benzene (17:3, v/v)	8.5	25.3	10.3	40.0	–	–
Benzene or toluene	22.2[b]	47.5	17.0[b]	57.0	38.8[c]	59.2
Chloroform	–	–	4.3	61.3	3.5	62.7
Chloroform–diethyl ether (9:1, v/v)	–	–	–	–	22.1	84.8
Methanol–benzene–diethyl ether (3:1:1, v/v/v)	43.6	91.1	36.6	97.9	–	–
Tetrahydrofuran–methanol (17:3, v/v)	–	–	–	–	12.9	97.7

[a] A = Chromatography according to Sawatzky *et al.* [4]; B = extrography with the elution solvents of Sawatzky *et al.* [4] and chloroform; C = extrography with the solvents from ref. 15.
[b] Benzene-eluted fraction.
[c] Toluene-eluted fraction.

aromatics in benzene/toluene elution.

The elution of more condensed polyaromatics in the toluene fraction by extrography was revealed by using chromatographic fractionation of this extrographic aromatic fraction. The aromatic fraction obtained by extrographic fractionation of pyrolysis tars [28] contained neither nitrogen nor phenolic compounds. Results of the chromatographic fractionations on neutral alumina are given in Table IV. In both instances the entire aromatic fractions contained a material which remained on the neutral alumina after the toluene elution, although it had passed through the basic alumina in the extrographic column. Even a stronger elution solvent, toluene–methanol, did not flush all the material from alumina, as it remained slightly coloured after fractionation at the entrance to the column. However, the irreversibly adsorbed material did not account for such a percentage as may result from the total yields in Table IV. A high proportion of the losses was probably due to evaporation during the fraction treatment.

Hence it can be deduced that the extrographic procedure [15] is capable of separating the more condensed aromatics (more than approximately four rings) from pyrrolic-type nitrogen and phenolic compounds. This is probably not the case with

TABLE IV

CHROMATOGRAPHIC FRACTIONATION OF EXTROGRAPHIC AROMATIC FRACTIONS OF PYROLYSIS TARS F AND S ON NEUTRAL ALUMINA

Elution solvent	Yield of aromatics F (%, w/w)	Yield of aromatics S (%, w/w)
Hexane	14.5	11.6
Hexane–toluene (19:1, v/v)	27.9	17.2
Hexane–toluene (17:3, v/v)	11.1	15.6
Toluene	15.4	25.2
Toluene–methanol (17:3, v/v)	24.9	28.1
Total	93.8	97.7

chromatographic fractionation on alumina [5,7,17–19]. In this fractionation polyaromatic hydrocarbons must contribute to a significant extent to the yields of heteroatom compound fractions. The probable overlapping portion of polyaromatic hydrocarbons is represented in Table IV as the fraction eluted with toluene–methanol. This contamination of the nitrogen fraction with polyaromatics has already been described for both neutral and basic alumina [20,31].

CONCLUSIONS

Based on the compilation of fractionation results of many fossil fuel processing products and almost 10 years of experience with extrographic fractionation, the following conclusions can be drawn. Extrographic fractionation with a basic alumina section in a column [15] is capable of providing fractions of hydrocarbons including more condensed polyaromatics, pyrrolic-type nitrogen compounds, and phenolic compounds. A clean cut can be obtained between the respective fractions. Separation into the individual hydrocarbon compound classes, *i.e.*, saturated compounds and mono-, di- and polyaromatics, cannot be achieved by a simple extrographic procedure. A column loading in the sample-to-sorbent ratio range from 1:15 to 1:8 (w/w) does not have a significant effect on the fractionation efficiency. The extrographic method [15] is especially suitable for the fractionation of coal-derived liquids with a large content of heteroatom compounds with different polarities.

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Efficiency of gas extraction in headspace analysis

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ABSTRACT

A study of the variation of volatile concentrations in static and dynamic versions of headspace analysis was carried out to compare the efficiencies of single- and multistep gas extraction procedures with a stationary liquid or gas phase, and with two moving phases.

INTRODUCTION

Gas chromatographic headspace analysis (GCHSA) is based on gas extraction procedures performed under static and dynamic conditions [1]. The efficiency is a major characteristic of the extraction and is determined by the amount of analyte stripped by a fixed volume of the extracting phase. It is affected by various parameters of the heterogeneous system such as the partition coefficient, temperature, pressure and phase volume ratio. The influence of these parameters on the principal metrological characteristics of GCHSA has been extensively studied [1–3]. The actual conditions under which the extraction and analytical process are performed can also noticeably affect the efficiency and performance of the system.

This paper reports a comparison of static and dynamic versions of GCHSA used analytically and characterized by different relationships.

SINGLE-STEP CONTINUOUS GAS EXTRACTION

The efficiencies of single-step static gas extraction (the simplest version of GCHSA) and continuous gas extraction (CGE) underlying the widely adopted dynamic GCHSA technique will be compared first. It can be shown that, other conditions being equal, CGE is always more efficient than the discontinuous extraction carried out under static conditions. The so-called extraction coefficient (X) can be introduced

$$X = V_G C_G/(V_L C_L) = V_G/(KV_L) \quad (1)$$

where $K = C_L/C_G$ is the partition coefficient representing the concentration ratio of a volatile in the liquid (L) and gas (G) phases and V is the volume of gas or liquid. According to the above definition of efficiency (E_j), for the static conditions (j = s) we have [4]

$$E_s = C_L^0/[K(1 + X)] \quad (2)$$

whereas for CGE (j = d) this equation can be written as

$$E_d = C_L^0[1 - \exp(-X)]/(KX) \quad (3)$$

where C_L^0 is the analyte concentration in solution before extraction. The ratio of these quantities representing the relative coefficient of efficiency (h) is always greater than unity

$$h = E_d/E_s = (1 + X)[1 - \exp(-X)]/X \geqslant 1 \quad (3a)$$

which follows from the well known inequality $\exp(-X) < 1/(1 + X)$. The highest gain in the dynamic process is reached, however, under optimum or close to optimum conditions. An analysis of eqn. 3a shows that the function $h(x)$ is described by a curve with a maximum (Fig. 1) at $x = 1.79$, allowing the calculation of the most efficient extraction of a volatile analyte from the solution under study. For

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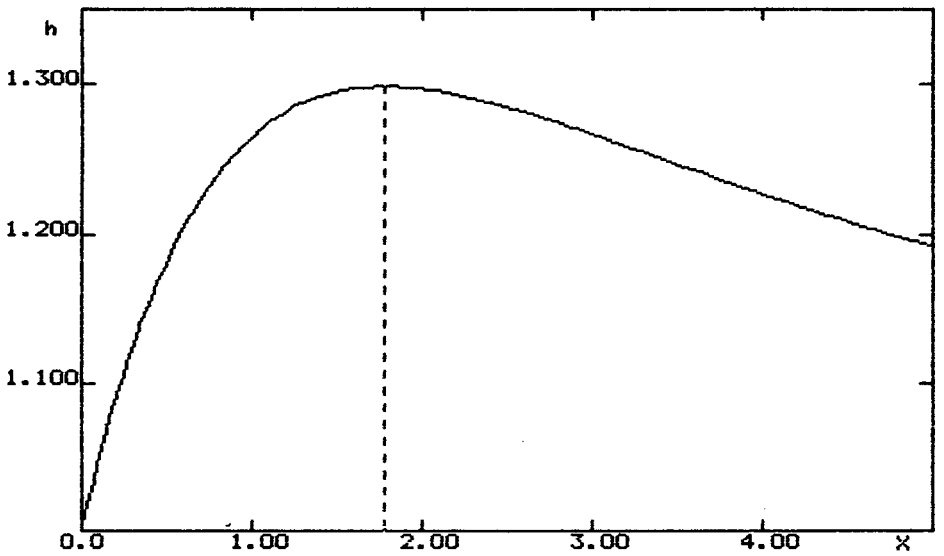

Fig. 1. Relative coefficient of efficiency (h) as a function of the extraction coefficient (X).

such a value of h, and other conditions being equal, the same volume of the stripping gas under dynamic conditions extracts 30% more of the volatile from the solution than the single extraction under static conditions.

It is well known that if the volume of the stripping agent is divided into q portions, then the total amount of the extracted analyte under static conditions will increase compared with a single-step use of this volume. For $q \to \infty$ the fractions of the extracted analyte under static and dynamic conditions will be equal.

Dynamic GCHSA is also preferable from the standpoint of the volume of the stripping gas required to extract a given fraction of the analyte. Indeed, for 95% extraction under dynamic conditions 7 times, and for 99% extraction 22 times, less gas is used than is needed in the single-step procedure under static conditions. However, for small fractions of extracted analyte dynamic GCHSA practically loses its advantages over the static version. It should also be pointed out that despite the higher efficiency of CGE, its application is useful only when used together with headspace cryogenic or adsorption trapping, or for the characterization of compounds with large partition coefficients (K). For small K values, although the total stripping gas volume decreases very little, the analytical techniques become more complicated and the accuracy is poorer because of the difficulties involved in maintaining a constant K value under dynamic conditions.

Expressing the efficiency in the form of a dimensionless quantity (Z), the fraction of extracted analyte [4]

$$Z = (C_L^0 - C_L)/C_L^0 \quad (4)$$

It can be seen that for the cases considered here this quantity will depend only on the extraction coefficient X.

MULTISTEP CGE

The efficiency of CGE can be increased by using a multistep procedure [4] which consists in dividing the analyte solution into N portions and passing the gas stream sequentially through them. A version of multistep CGE using a clean carrier gas was studied by Marinichev and Vitenberg [4]. Consider now a more general case of this process, when through a solution with an initial (mass/volume) concentration of the volatile C_L^0 a gas is passed containing the vapour of the same compound at a concentration $C_G^{\#}$. After the exchange of the analyte between the solution of volume V_L and the carrier gas stream of volume V_G (resulting in an enrichment or depletion

of the solution in the volatile analyte) its initial concentration in the solution will change to C_L^I, related to C_L^0 by

$$C_L^0 = C_L^I + m_G^I/V_L \quad (5)$$

Here $m_G^I = V_G(C_G^{-I} - C_G^{\#})$ is the mass of the volatile exchanged between the condensed and gas phases, C^{-I} is the mean concentration in the volume V_G. Taking into account the fact that in CGE the analyte distribution reaches equilibrium in the limit of the volume V_G being brought in contact with the solution in infinitely small amounts, eqn. 5 can be rewritten in the form of a differential equation

$$dC_L^I/dx = -C_L^I + KC_G^{\#} \quad (6)$$

with the solution

$$C_L^I = C_L^0 \exp(-X) + KC_G^{\#}[1 - \exp(-X)] \quad (7)$$

In the second step, the gas volume V_G after the interaction in the first step is again brought into contact with another amount of the solution with the same initial concentration C_L. In place of eqn. 5, the equation can now be written as

$$(V_G/V_L)C_G^{-I} + C_L^0 = C_L^{II} + (V_G/V_L)C_G^{-II} \quad (8)$$

where C_L^{II} is the volatile concentration after the completion of extraction from the second portion of the solution, and $V_G C_G^{-II}$ is the mass of the analyte extracted in contact with this portion. The solution of the corresponding differential equation, which is similar to eqn. 7, can be written as

$$C_L^{II} = KC_G^{\#} + (C_L^0 - KC_G^{\#})(1 + X)\exp(-X) \quad (9)$$

If a sequence of N vials is made up with solutions of the same initial concentration C_L^0, and these are passed consecutively through a stream of the stripping gas with the initial analyte concentration $C_G^{\#}$, the concentration in the solution at the Nth stage will be described, as shown by Marinichev and Vitenberg [4], by the equation

$$C_L^N = KC_G^{\#} + (C_L^0 - KC_G^{\#})\exp(-X)\sum_{s=0}^{N-1}(X^s/s!) \quad (10)$$

The fraction of the extracted analyte in the case of N-step CGE (Z_{NL}) can be calculated by the relation

$$Z_{NL} = (V_L C_L^0 - V_L C_L^I)/NV_L C_L^0 + \ldots + (V_L C_L^0 - V_L C_L^N)/NV_L C_L^0 \quad (11)$$

It is assumed here that the volumes of the solutions involved in each step of the CGE are the same and equal to V_L/N. Bearing this in mind and substituting eqn. 10 into eqn. 11 gives

$$Z_{NL} = (1 - KC_G^{\#}/C_L^0)\{1 - \exp(-NX) \cdot [1 + \sum_{s=1}^{N-1}(NX)^s(1 - s/N)/s!]\} \quad (12)$$

The coefficient of relative efficiency of MHE is defined by the relation $h_{NL} = Z_{NL}/Z_s$; from eqn. 2 the following equation for Z_s can be derived

$$Z_s = (1 - KC_G^{\#}/C_L^0)X/(1 + X) \quad (13)$$

Using these equations

$$h_{NL} = (1 + 1/X)\{1 - \exp(-NX) \cdot [1 + \sum_{s=1}^{N-1}(NX)^s(1 - s/N)/s!]\} \quad (14)$$

Eqn. 14 describes the efficiency ratio for the N-step CGE and the static method —in other words, the relative efficiency.

The calculations made using eqn. 12 for the case $C_G^{\#} = 0$ and illustrated by Fig. 2 show that the efficiency of CGE increases with an increasing number of vials N containing the solution of the same total volume V_L. It is seen to grow noticeably up to N values of five or six. A further increase in N results only in an insignificant increase of Z_{NL}, and this is what places a limit on the required number of vials (steps) N.

An essential asset of multistep CGE lies in the possibility of stabilizing the volatile concentration in the gas stream at the outlet of the last vial (Fig. 3). An increase in the number of vials substantially increases the part of the curve with the almost constant analyte concentration in the last vial. This property can be used to advantage in producing gas flows with a given and practically constant trace concentration of a volatile.

CGE WITH STATIONARY GAS PHASE

The dynamic versions described in the preceding section have a common feature, namely, the liquid phase (or any other condensed phase) remains stationary while the gas phase is mobile. In practice, however, a reverse dynamic version used in characterization of gases dissolved in liquids is sometimes used [5,6]. This method is based on the outgassing of a liquid during filtration through a column filled

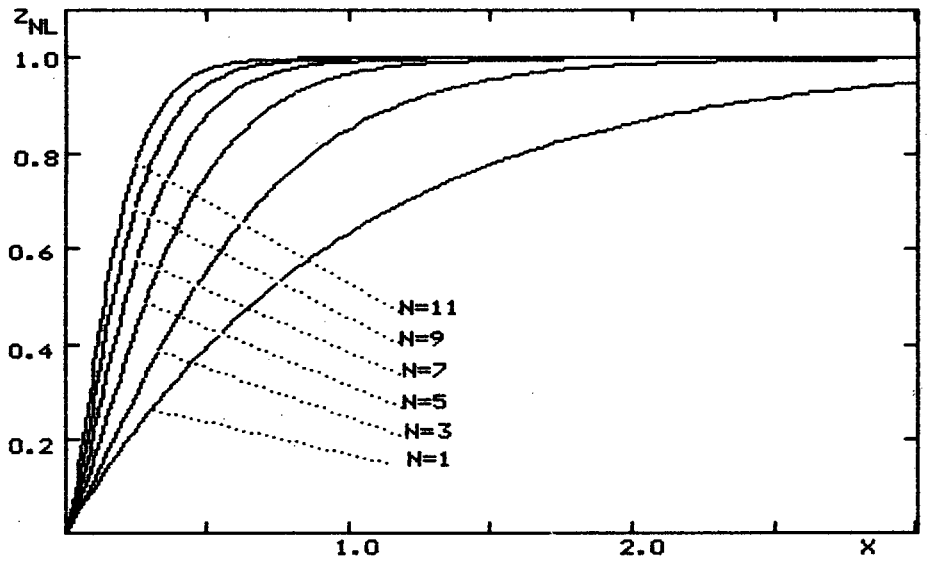

Fig. 2. Fraction of extracted analyte for N-step CGE (Z_{NL}) as a function of the extraction coefficient (X) for N = 1, 3, 5, 7, 9 and 11.

with a porous polymer which has a free gas volume acting as a "fixed" extracting gas. This method is actually a variant of the chromatographic technique, referred to as "liquid–gas distribution chromatography" [6], which is a reverse analogue of the frontal concentration of volatiles present in gases [7]. The calculations in these two methods are based on the relations conventionally used in chromatography and assuming that the retention volume is proportional to the volume of the stationary phase.

Volatiles can be extracted by a stationary gas phase from a moving solution not only in the chromatographic regime but also, for instance, by passing a stream of liquid under a gas bubble or by

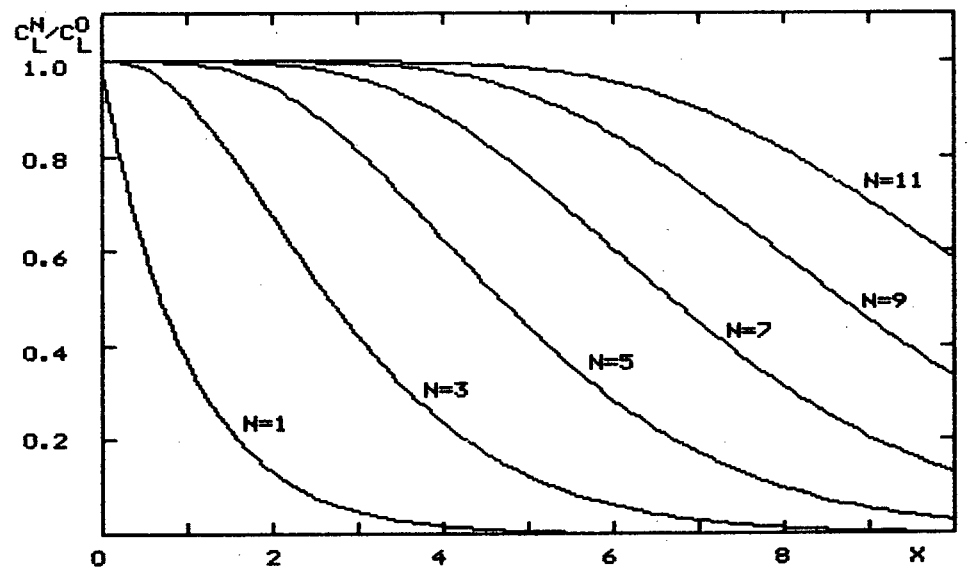

Fig. 3. Fraction of analyte in the liquid phase of the last vial for N-step CGE (N = 1, 3, 5, 7, 9 and 11).

spraying the liquid in a vial containing a volume of gas. This process is a reverse variant of the equilibrium absorption concentration proposed in the early 1970s and is used to trap volatiles present in a gas by volatile or non-volatile liquids [8]. The single-step variant of the gas extraction of volatiles from a moving liquid flow by a stationary gas phase is described by the equation [9]

$$C_G = C_L^{\#}[1 - \exp(-1/X)]/K$$

which is similar to the relations characterizing the absorption equilibrium concentration of volatiles present in a gas [10]. The multistep modification with a stationary extracting gas has not yet been considered and can be described by equations similar to those derived for CGE with a stationary liquid. If the gas phase is divided into N cells (portions) and $C_G^0 = 0$, while the liquid phase moves and comes into contact consecutively with each cell, with $C_L^{\#} > 0$ before extraction, then after replacing X and K in eqns. 10 and 12–14 by the corresponding reciprocal quantities, as well as G by L, and L by G

$$C_G^N = C_L^{\#}/K + (C_G^0 - C_L^{\#}/K)\exp(-1/X)\sum_{s=0}^{N-1}(X^{-s}/s!) \quad (15)$$

$$Z_{NG} = (1 - C_L^{\#}/KC_G^0)\{1 - \exp(-1/X)[1 + \sum_{s=1}^{N-1}(X/N)^{-s}(1 - s/N)/s!]\} \quad (16)$$

$$Z_s = (1 - C_L^{\#}/KC_G^0)/(1 + X) \quad (17)$$

$$h_{NG} = (1 + X)\{1 - \exp(-N/X)\cdot[1 + \sum_{s=1}^{N-1}(X/N)^{-s}(1 - s/N)/s!]\} \quad (18)$$

The relative efficiencies $h_{NL}(X)$ and $h_{NG}(X)$ calculated for several values of N and X are listed in Table I. It is readily seen that for $X > 1$ the highest efficiency is provided by the modification with a stationary liquid, and for $X < 1$ by the method using a stationary stripping gas. As the number of steps N increases for X = constant = 1, both quantities, h_{NL} and h_{NG}, grow monotonically and approach one another. In the particular case of $X = 1$ the gas extraction efficiencies are equal irrespective of the state of aggregation of the stationary phase.

CGE WITH MOVING PHASES

In analytical practice this process is used in the characterization of organic volatiles (simple and halogenated hydrocarbons) present in water under counter-current conditions [12–14] and when analysing gases dissolved in water under the co-current regime [15]. The variation of the volatile concentration in the liquid phase is described by the relation [10]

$$C_L(t) = C_L^{\#}[(1 - a/b) + (a/b)\exp(-bt)] \quad (19)$$

where $a = w_G/(Kv_1)$, $b = a + w_L/v_1$ and t is time, w_L, w_G are, respectively, the flow-rates of the liquid and gas phases and v_1 is the volume of liquid from which the analyte is extracted—in other words, the volume of the liquid in the vial. Using eqn. 19 the following relation is obtained for the fraction of the extracted analyte

$$Z = X\{1 - \exp[-R(1 + X)]\}/(1 + X) \quad (20)$$

Here $R = V_L/v_1$ is the volume ratio of the liquid passed through the vial to that present in it, or, which is the same, the number of "fillings" of the volume v_1 during the extraction process. If $Y = RX$, then eqn. 20 takes the form

$$Z = Y\{1 - \exp[-(Y + R)]\}/(Y + R) \quad (21)$$

The parameter Y represents the extraction coefficient reduced to the volume v_1, in contrast to X which relates to the total volume V. In the limit, as $R \to 0$, eqn. 21 degenerates to the relation for Z for single-step CGE.

The relative efficiency (h) for moving phases, as follows from eqns. 13 and 20, is described by

$$h = 1 - \exp[-R(1 + X)] \quad (22)$$

which shows that the efficiency of this modification

TABLE I

RELATIVE EXTRACTION EFFICIENCIES h_{NL}/h_{NG} FOR VARIOUS VALUES OF N AND X

N	X				
	0.5	1	1.5	2	3
1	1.18/1.30	1.26/1.26	1.29/1.22	1.30/1.18	1.28/1.15
3	1.41/1.46	1.55/1.55	1.53/1.48	1.46/1.41	1.33/1.30
5	1.46/1.49	1.65/1.65	1.59/1.56	1.49/1.46	1.33/1.32
10	1.49/1.50	1.75/1.75	1.64/1.63	1.50/1.49	1.33/1.33

of CGE is always lower than that of the static method for the same liquid and gas volumes, the two quantities closely approaching one another for a sufficiently small volume v_1 (*i.e.* when it can be assumed that $R \gg 0$). CGE with moving phases may be preferable when the purpose is to extract the largest possible amount of analyte.

CONCLUSIONS

The method chosen for gas extraction substantially affects the efficiency. This increases when dynamic rather than static modifications are used, and multi- in place of single-step head-space analysis techniques.

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Optimized method for the determination of 1,2-diacyl-*sn*-glycero-3-phosphocholine and 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine molecular species by enzymatic hydrolysis and gas chromatography

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ABSTRACT

A method was developed for the analysis of molecular species of 1,2-diacyl-*sn*-3-glycerophosphocholine and 1,2-diacyl-*sn*-3-glycerol-phosphoethanolamine from natural sources. The method involves enzymatic hydrolysis of the *sn*-3 position of the glycerophospholipids, acetylation of the newly liberated hydroxyl group and high-temperature gas chromatography of the 1,2-diacyl-3-acetyl-*sn*-glycerols. Both the enzymatic hydrolysis and the gas chromatographic separation were optimized with chemometric methods.

INTRODUCTION

Glycerophospholipis, *i.e.* phospholipids (PLs), are found as major constituents in cell membranes in both the plant and animal kingdoms. They play an important role in biological systems, not only as barriers in cells but also as the site of many reactions that are vital to life [1]. The conformation of the phospholipid molecules, with a polar head group and two fatty acyl chains, provides them with powerful emulsifying capabilities. This fact has made phospholipids interesting commercial products. Some phospholipid mixtures, called lecithins [2], are frequently used as emulsifying agents in the food, cosmetic and pharmaceutical industries. The main sources of vegetable lecithin are soybean, rapeseed and sunflower seed. The predominant polar lipid classes of lecithins are usually 1,2-diacyl-*sn*-3-glycerophosphocholine (PC) and 1,2-diacyl-*sn*-3-glycerophosphoethanolamine (PE) [2].

The fatty acyl composition of glycerophospholipid species has important physicochemical and bioavailability implications. Therefore, the analysis of molecular species with the glycerol backbone intact provides valuable structural and metabolic information.

The analysis of highly polar lipid molecules presents problems in chromatographic systems such as high-performance liquid chromatography, (HPLC) and gas–liquid chromatography (GLC) owing to adsorption and thermal instability. It is therefore desirable to reduce the polarity of the molecules, either by entirely removing the head group or by utilizing some suitable derivative [3–7]. Monoacetyldiacylglycerol (MADG) derivatives of diacylglycerols (of phospholipid origin) have been successfully used in high-temperature GLC analysis [4]. There exist two general methods for the conversion of phospholipids to MADGs: (1) acetolysis of phospholipids with acetic acid anhydride and acetic acid at approximately 150°C [4] and (2) enzymatic hydrolysis to remove the phosphoryl moiety, followed by acetylation at 70°C [4].

Diacylglycerols (DGs) isomerize under the influence of acidic, basic or polar solvents [4,8]. Thus, the DG residues of phospholipids have to be deriv-

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atized promptly into stable MADGs in order to avoid intramolecular acyl migration. Some reports have indicated that this type of isomerization may be promoted by the acetolysis procedure [9]. However, it has been shown that intermolecular acyl migration does not occur [8]. The removal of the phosphoryl group at the *sn*-3 position with a specific enzyme and the subsequent acetylation can in this respect be considered a much less violent procedure.

Complete removal of the polar head group in glycerophospholipids can be accomplished by enzymatic hydrolysis with phospholipase C [7,10–12], a well known but time-consuming reaction. As long as the fatty acyl chains are of sufficient length to possess a hydrophobic region which can bind to the enzyme [13], PC is a suitable substrate for phospholipase C (from *Bacillus cereus*). Soybean PC fulfils this requirement since the fatty acids mainly contain sixteen to eighteen carbon chains. The phospholipase C from *Bacillus cereus* has also been used for the hydrolysis of PE and 1,2-diacyl-*sn*-3-glycerophosphoinositol (PI) [4,7].

The aim of this investigation was therefore to optimize the performance of two well known and existing methods, enzymatic hydrolysis and high-temperature gas chromatography (GC) of MADGs, for the analysis of PC and PE in natural lipid materials.

EXPERIMENTAL

Study design

This study consisted of two separate steps: (1) enzymatic hydrolysis of PLs to 1,2-diacyl-*sn*-glycerols and (2) GLC analysis of the hydrolysis products after their conversion to 1,2-diacyl-3-acetyl-*sn*-glycerols, *i.e.* MADGs. Steps 1 and 2 were separately optimized by the combined use of factorial design and response surface modelling [14,15].

Experimental design

In order to obtain optimal conditions for the enzymatic hydrolysis and subsequent GLC analysis, factorial design was used [14,15]. The design and experimental domains are shown in tables I and II. For the enzymatic hydrolysis (Table I) the effects of the amount of enzyme, amount of substrate (phospholipid) and reaction temperature were explored in a reduced two-level design (2^{3-1}) [14,15]. Two additional points of measurement (experiments) were collected, one for calculating interactions and the centrepoint of the design to check for non-linearities. This was done in order to produce optimal conditions for the complete transformation of PC and PE into their diacylglycerol residues in as short a time as possible. Five minutes were considered a practical goal for this reaction.

A full factorial, with centrepoint, using the temperature programming rate, linear velocity of carrier gas and sample concentrations as design variables (Table II), was used to optimize the GLC analysis. This was done using a mixture of 1,2-diacyl-3-acetyl-*sn*-glycerol standards in equal amounts. In order to ensure stable GLC analysis conditions, a new design was laid out using the conditions from the best run from the previous design as the centrepoint (see Fig. 2). Through response surface modelling (see below), the influence of sample concentration was found to be negligible within

TABLE I

ENZYMATIC VARIABLES AND RATIOS OF UNREACTED PHOSPHOLIPID TO REACTION PRODUCT

	Design			Enzyme concentration (Units)[a]	Substrate concentration (mg/ml)	Temperature (°C)	Phospholipid/ diglyceride (%)
	A	B	C				
1	−	−	+	1	2	37	0.431
2	+	−	−	5	2	22	0.0
3	−	+	−	1	10	22	0.375
4	+	+	+	5	10	37	0.246
5	Centre point			2.5	5	30	0.756
6				2.5	5	22	0.542

[a] One Unit will liberate 1.0 μmol of organic phosphorus from PC per minute at pH 7.3 at 37°C (manufacturer's definition).

TABLE II

CHROMATOGRAPHIC VARIABLES AND CRS VALUES

Run[a]	Design			Temperature rate (°C/min)	Linear velocity[b] (cm/s)	Sample concentration (mg/ml)	CRS value
	A	B	C				
1	−	−	−	2	21.4	0.05	3.42
2	+	−	−	6	24.4	0.05	4.48
3	−	+	−	2	47.6	0.05	3.39
4	+	+	−	6	47.6	0.05	3.22
5	−	−	+	2	21.7	0.25	4.62
6	+	−	+	6	24.4	0.25	4.24
7	−	+	+	2	48.6	0.25	4.97
8	+	+	+	6	47.6	0.25	3.18

[a] Executed in random order.
[b] Actual values.

the explored interval. Therefore only the temperature programming rate and linear velocity of carrier gas were used as design factors in this set of four runs.

Evaluation of experimental data

The ratio of unreacted phospholipid to liberated diacylglycerol (PL/DG) was used as a response (dependent variable) for the enzymatic hydrolysis. In order to obtain a quantitative and objective response from the chromatograms a multivariate ranking function was used, the chromatographic resolution statistic (CRS) [16]

$$\mathrm{CRS} = \{\sum[(R_i - R_{\mathrm{opt}})^2/R_i(R_i - R_{\mathrm{min}})^2] + \\ + \sum (R_i)^2/a\bar{R})^2\}\ (T_f/n)$$

where R_i = resolution element for the ith peak pair, R_{min} = chosen value of minimum resolution, a = total number of resolution elements, T_f = retention time of final peak, n = total number of peaks, $\bar{R}^2$ = square of mean resolution. The optimal chromatographic conditions are found at the minimum of the CRS function. The optimal peak resolution value (R_{opt}) was set to 1.5 in this study.

The above-described experimental responses were fitted to polynomial functions in the experimental variables (Tables I and II). Linear models were found to be sufficient to describe the data. These models were used to generate response surface plots, which were used graphically to locate optimal conditions within the explored domains. All calculations were done in Statgraphics (STSC; Rockville, MD, USA).

Materials

The solvents used in this study were all of pro analysi quality and were purchased from Merck (Darmstadt, Germany). Phospholipase C (No. P-7147), from *B. cereus*, was purchased from Sigma (St. Louis, MO, USA) and suspended in a phosphate buffer (pH 7), composed of 50 cm^3 of 0.1 mol/dm^3 potassium dihydrogenphosphate and 29.1 cm^3 of 0.1 mol/dm^3 sodium hydroxide. The stock enzyme suspension was diluted to 25 U/ml of buffer and stored at 5°C until used. Natural PC and PE from soybean lecithin were obtained through Lipid Teknik (Stockholm, Sweden). All 1,2-diacylglycerol standards were purchased from Larodan (Malmö, Sweden). Phospholipid standards [1,2-di-14:0 PC, 1,2-di-16:0 PC, 1,2-di-18:0 PC, 1,2-di-18:2 PC, 1,2-di-20:0 PC (Larodan) and 1-16:0, 2-18:1 PC (Sigma)] were used to generate stereospecifically intact MADGs, with the optimized method described herein, for a standard GLC run.

Enzymatic hydrolysis

The experimental domain for the enzymatic hydrolysis was chosen on the basis of previous experience in our laboratory. According to standard practice in experimental design (Table I), the experiments were executed in random order, utilizing the following general procedure. A 1-ml volume of substrate suspension (in all experiments PC and PE

were suspended in the above-described phosphate buffer, and it was found to be important to minimize the size of aggregates, especially when preparing the PE suspension) (concentration according to design) was transferred to a test tube equipped with a PTFE-lined screw cap. The test tube was placed in a ultrasonic water bath and held at a temperature given in the design for 5 min for temperature equilibration. Either 40 or 200 μl of the stock enzyme suspension were added to the test tube. While maintaining control of the temperature, the ultrasonic function was activated for 5 min. The hydrolysis was discontinued by the addition of 2 ml of diethyl ether–isooctane (20:5, v/v), and heating the sealed tube in a thermoblock at 140°C for 5 min. After the subsequent addition of 3 ml of water, the test tube was shaken vigorously for 30 s and centrifuged at 3000 *g* for 5 min. The organic layer was recovered and the aqueous phase was extracted twice in the same manner with 1 ml of iso-octane. The three organic phases were combined and washed once with 1 ml of deionized water.

Evaluation of reaction performance was done by thin-layer chromatography (TLC) and scanning densitometry. The organic phase containing the diacylglycerols was evaporated to dryness in a stream of nitrogen. The residue was dissolved in 200 μl of chloroform–methanol (1:1, v/v), of which 1 μl was drawn and subsequently spotted on a HPTLC plate (see Fig. 1). The remaining diacylglycerols were again evaporated to dryness under nitrogen and immediatly derivatized into 1,2-diacyl-3-acetyl-*sn*-glycerols.

Thin-layer-chromatography

TLC evaluation was conducted according to an optimized method [17] on prewashed silica HPTLC plates (Merck). Sample application was conducted with a PS 01 TLC spotter (Desaga, Heidelberg, Germany); the solvent system was chloroform–methanol–1-butanol–ethyl acetate–ammonia (25%)–Ca^{2+} (aq., 0.25%, w/v) (80.2:44.1:4.9:5:5:6, v/v). Densitometric evaluation of the HPTLC plates was performed with the Desaga CD 60 scanning densitometer, utilizing the spot optimization programme.

Derivatization

In order to preserve structural integrity and to render the hydrolysis products amenable to analysis by GC, the hydrolysis products, *i.e.* the 1,2-diacyl-*sn*-glycerols, were acetylated by a modified method [4]. The diacylglycerols were dissolved in 2 ml of a freshly prepared solution consisting of pyridine–acetic acid anhydride (1:5, v/v), and sealed in a test tube at 70°C for 30 min. The reaction was stopped by the addition of 2 ml of isooctane and 3 ml of deionized water, after which the test tube was shaken vigorously for 30 s and centrifuged at 3000 *g* for 5 min. The organic layer, containing the acetylated diacylglycerols, was recovered and the water phase was extracted once again with 1 ml of isooctane. The combined organic phases were evaporated in a stream of nitrogen. The residue was dissolved in isooctane (2.0 ml) and injected into the gas chromatograph.

Gas–liquid chromatography

GLC of the acetylated diacylglycerols was conducted on a moderately polar fused-silica capillary column, TAP (65% phenyl–35% methyl polysiloxane), 25 m × 0.25 mm I.D. (phase thickness: 0.10

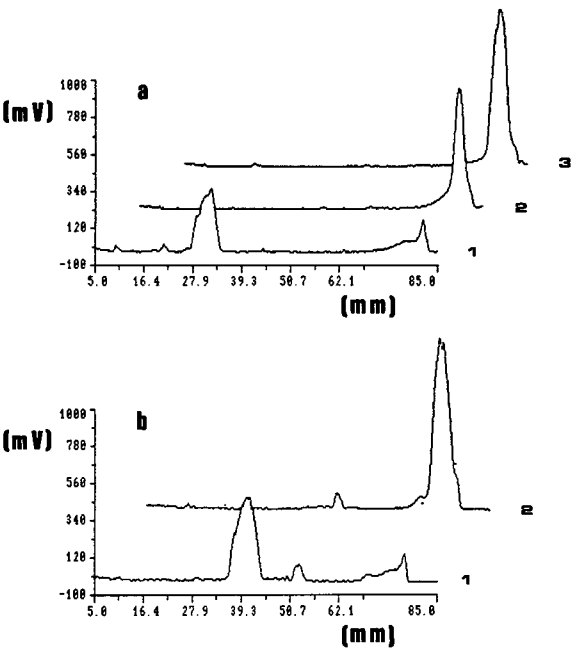

Fig. 1. (a) Densitograms of (1) unreacted PC, (2) reaction product and (3) dioleoylglycerol. (b) Densitograms of (1) unreacted PE and (2) reaction product.

μm), purchased from Chrompack (Middelburg, Netherlands). This column was installed in a Varian 3500 gas chromatograph, equipped with a temperature-programmable on-column injector and a flame ionization detector (FID) (Walnut Creek, CA, USA). A retention gap was installed between the injector and the analytical column. The detector signals were recorded by the PC Integration Pack (Kontron, Milan, Italy). The standard mixture of MADGs was composed of Ac-di-14:0, Ac-di-15:0, Ac-di-18:0, Ac-di-18:1, Ac-di-18:2 and Ac-di-18:3 in equal amounts, dissolved in isooctane. The profile of the oven temperature programme was as follows: from 100°C (hold 1 min) to 335°C at 50°C/min, then from 335°C (hold 1 min) to 360°C at 2 (or 6) °C/min, and hold at the final temperature for 5 min. The temperature programme of the injector was 100°C (hold 1 min) to 360°C at 200°C/min and hold at the final temperature for 2 min. The detector temperature was held at 360°C throughout this investigation.

The fatty acid composition (as methyl esters) of

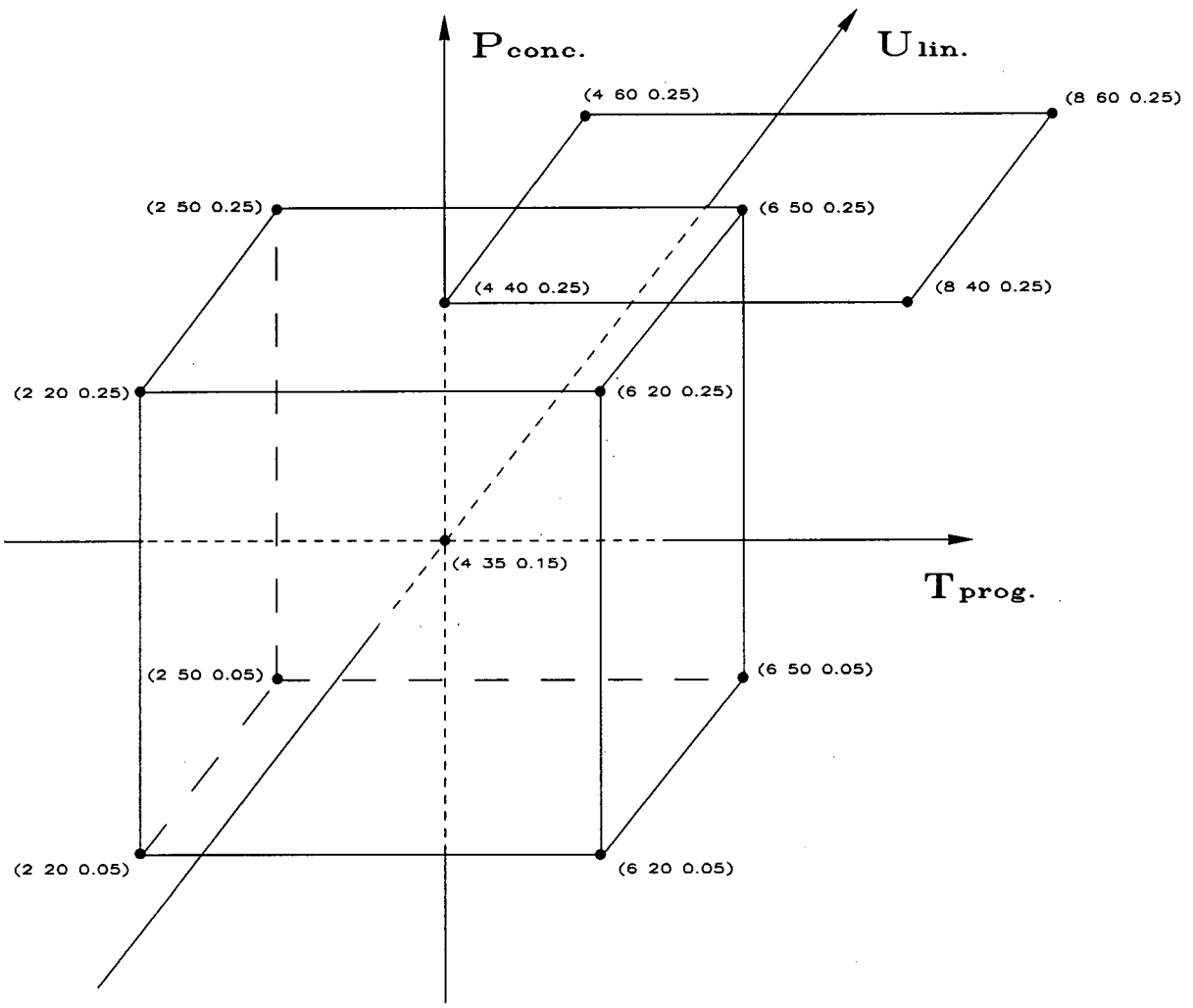

Fig. 2. Two-level, three-factor factorial design (2^3) with centre point, for the gas chromatographic separation of MADGs. Optimal conditions: Temperature programme rate, 6°C/min; linear velocity, 50 cm/s; sample concentration, 0.25 mg/ml. New design with the found optimal conditions as centre point.

PC and PE was determined according to an optimized method which has been described elsewhere [18]. However, a DB-WAX (30 m × 0.25 mm I.D.) column, purchased from J&W (Folsom, CA, USA), was used in this study. Information about the fatty acid composition is essential for the interpretation of peaks representing molecular species in the high-temperature GLC analysis.

RESULTS AND DISCUSSION

The optimum conditions for the enzymatic hydrolysis of PC were located in the experimental domain (Table I) by response surface modelling. Evaluation of reaction performance was done by TLC in combination with scanning densitometry (Fig. 1a). Optimum conditions obtained for PC were also

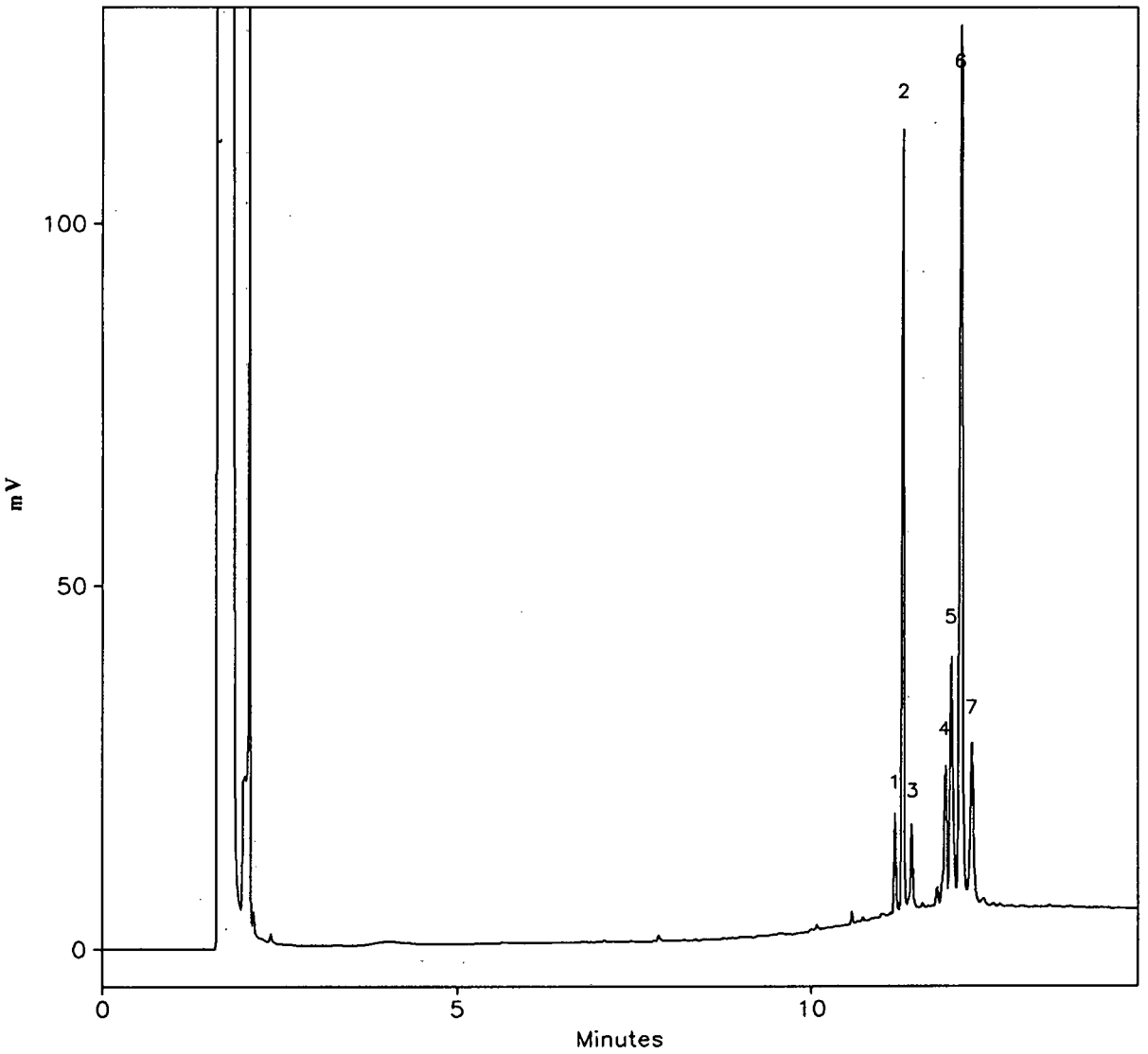

Fig. 3. Gas chromatogram of MADGs of PC origin. Optimized separation for conditions see text. Peaks: 3 = 16:0,18:2; 6 = 18:2,18:2; 7 = 18:2,18:3.

found to be suitable for the hydrolysis of PE, as illustrated in Fig. 1b. The response surfaces (not shown) of the data show a minimum (the optimum) at 22°C and 5 U in the experimental domain investigated. The response surfaces of reaction temperature *vs.* substrate and enzyme concentration *vs.* substrate concentration (not shown) all confirm that the optimal conditions for the enzymatic hydrolysis are at 22°C, 2 mg/ml and 5 U. The hydrolysis of both PC and PE went to 100% completion, with no side-products, after 5 min at ambient temperature.

The response surfaces (not shown) of the GLC experimental data showed that optimal conditions for the GLC analysis were 6°C/min temperature programming, a linear velocity of carrier gas of 50 cm/s (actual value: 47.6 cm/s) and a sample concentration of 0.25 mg/ml.

In order to validate the method, a new design was laid out with optimum conditions as the centre

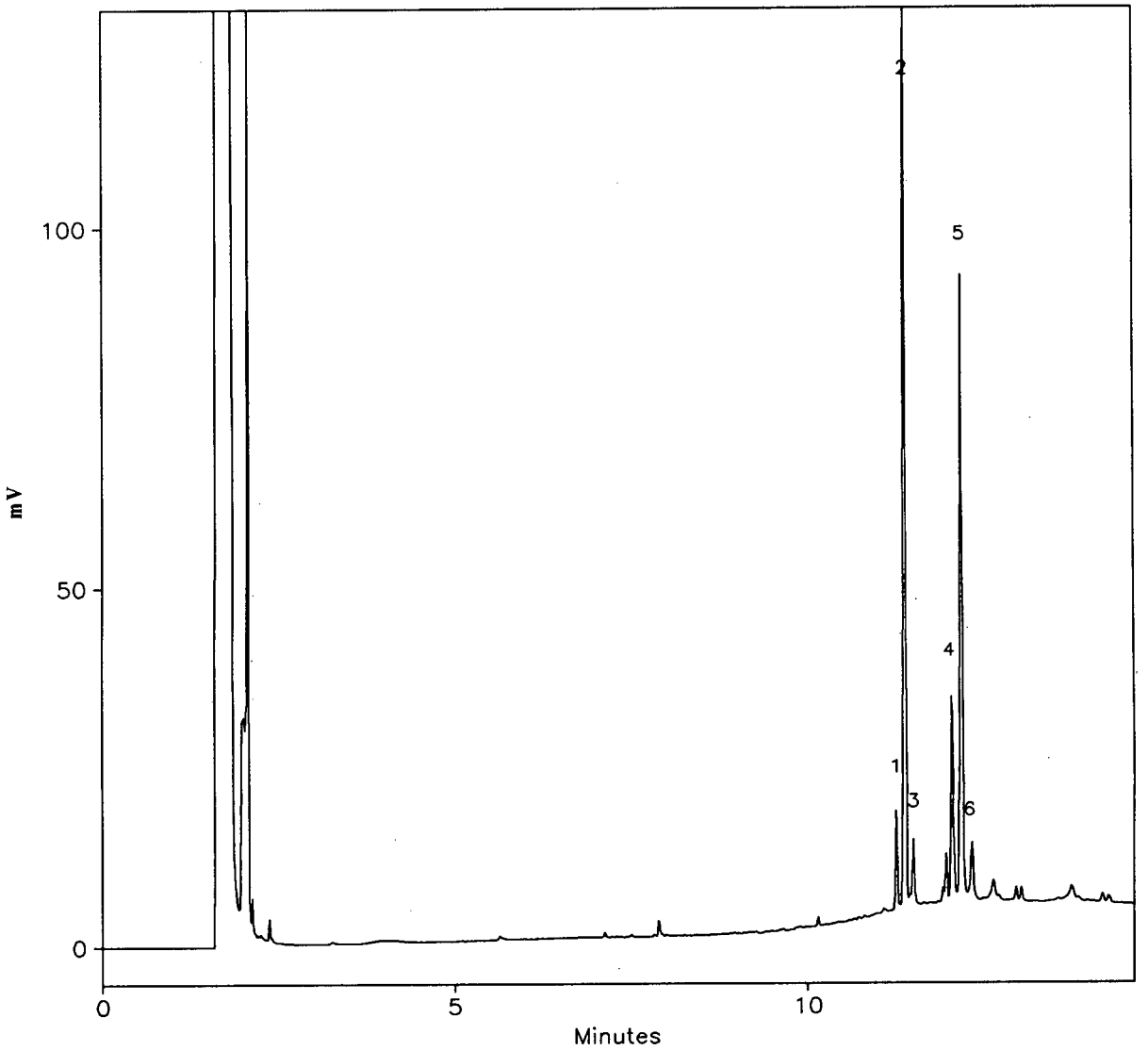

Fig. 4. Gas chromatogram of MADGs of PE origin. Optimized separation. For conditions see text. Peaks: 3 = 16:0,18:2; 5 = 18:2,18:2; 6 = 18:2,18:3.

TABLE III

FATTY ACID COMPOSITION (AS METHYL ESTERS) OF PC AND PE

Fatty acid	Composition (mean ± S.D.) (weight-%)	
	PC	PE
14:0	0.1 ± 0.00	Trace[a]
15:0	Trace	Trace
16:0	14.8 ± 0.14	18.8 ± 0.05
17:0	Trace	Trace
18:0	3.2 ± 0.01	0.8 ± 0.01
20:0	Trace	Trace
22:0	Trace	
24:0	Trace	
16:1(*n*−7)	0.1 ± 0.01	0.1 ± 0.03
17:1[b]	Trace	Trace
18:1(*n*−9)	7.5 ± 0.04	6.2 ± 0.11
18:1(*n*−7)	1.6 ± 0.04	0.6 ± 0.11
20:1(*n*−9)	Trace	
20:1(*n*−11)	Trace	
18:2(*n*−6)	62.6 ± 0.42	64.8 ± 0.30
18:3(*n*−3)	7.6 ± 0.06	7.8 ± 0.04
Unidentified[c]	2.5 ± 0.30	0.9 ± 0.13
Total	100.0	100.0

[a] <0.10%.
[b] Position not determined.
[c] Including minors.

point (Fig. 2). By perturbing the system in such a manner, the stability of the method, measured as the variation in the CRS value, could be deduced. This also served to confirm that the optimal conditions had in fact been located. Naturally, different optimal chromatographic conditions might result by increasing the optimal peak resolution term (R_{opt}) in the CRS function. The resolution obtained for molecular species of natural mixtures of PC and PE can be seen in Figs. 3 and 4.

The overall fatty acid compositions of PC and PE are given in Table III. Since there are fewer fatty acids in PE than there are in PC (4 *vs.* 6; >1.0%), the number of molecular species (as MADGs) should be lower in PE. This is illustrated in Figs. 3 and 4. The fatty acid composition of the underivatized material determines the number of peaks in MADG analysis. Consequently, minor peaks in Figs. 3 and 4 represent unidentified molecular species containing fatty acids (>1.0%) and species comprised of a combination of one of these acids and one of the four or six (PE or PC) major acids. The chromatogram of the system blank (without substrate) shows no peaks at all, and chromatograms of individual stereospecifically intact DG residues, transformed into their respective MADGs, show no extraneous peaks other then those expected from their initally somewhat poor purity level.

TABLE IV

METHOD REPRODUCIBILITY; PC FROM SOYBEAN

Peak No.	GLC separation[a]		Overall[b]		
	Area (%)	S.D. (%)	Area (%)	S.D. (%)	R.S.D. (%)[c]
1	3.2	0.03	3.1	0.18	5.8
2	24.6	0.22	24.9	0.66	2.6
3	3.1	0.09	2.7	0.43	15.9
4	7.0	0.36	7.0	0.37	5.3
5	11.1	0.55	11.2	0.55	4.9
6	37.8	0.24	37.7	0.34	0.9
7	8.3	0.14	8.3	0.17	2.1
Minors[d]	4.9	0.40	5.1	0.48	
Total	100.0		100.0		

[a] Average of six gas chromatographic runs of the same batch of MADGs.
[b] Average of six gas chromatographic runs of six individually prepared batches of MADGs, *i.e.* including the enzymatic hydrolysis step.
[c] Relative standard deviation. Overall method reproducibility = 5.4%.
[d] < 1.0%.

Further, the presence of only six peaks in the chromatogram of the stereospecifically intact standard mixture of MADGs confirms that interacyl migration does not occur with the described derivatization procedure (Fig. 5).

As a measure of the reproducibility of the method, six batches of 1,2-diacyl-*sn*-glycerol (of soybean PC origin) were prepared and subsequently acetylated and submitted to the optimized GLC analysis. The standard deviation of the overall method can be seen in table IV. These figures are not dramatically higher than those obtained with the GLC separation alone. The overall mean relative standard deviation (peaks 1–7) of the method was 5.4%.

Our goal in this work was to develop an optimized method for the GLC analysis of molecular species of naturally occurring phospholipids. To this end, experimental design and response surface modelling were used. The two main steps in this work, the hydrolysis of PLs and the GLC analysis,

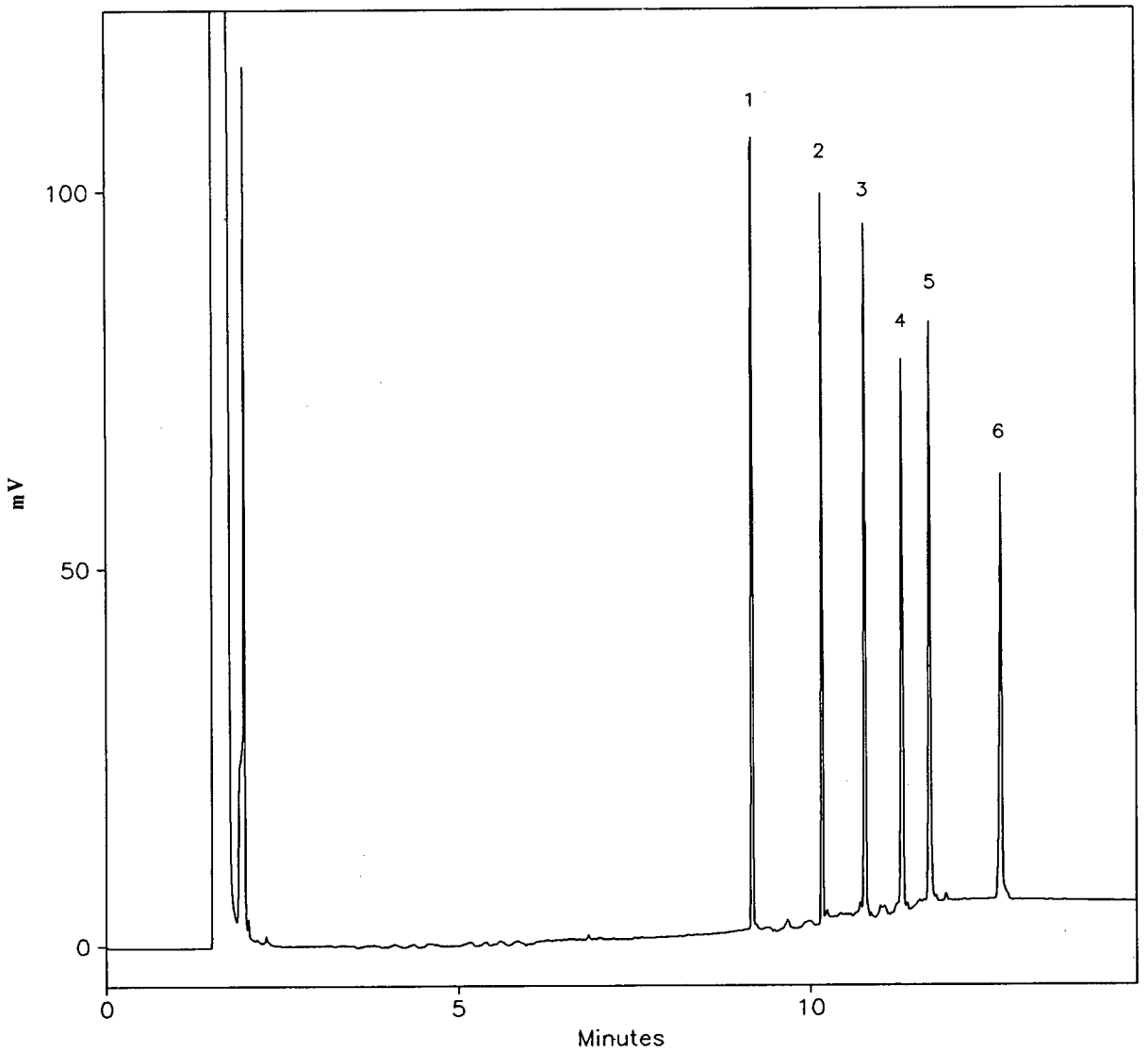

Fig. 5. Gas chromatogram of stereospecifically intact MADGs of PC standards. Optimized separation. For conditions see text. Peaks: 1 = Ac-1,2-di-14:0; 2 = Ac-1,2-di-16:0; 3 = Ac-1-16:0,2-18:1; 4 = Ac-1,2-di-18:0; 5 = Ac-1,2-di-18:2; 6 = Ac-1,2-di-20:0.

were optimized in terms of reaction performance and chromatographic separation, respectively.

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CHROM. 24 101

Capillary column gas chromatography–ammonia and deuterated ammonia chemical ionization mass spectrometry of sulfur vesicants

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ABSTRACT

Capillary column gas chromatography–ammonia and deuterated ammonia chemical ionization mass spectrometry was found to be a highly specific technique for the detection and identification of three long-chain sulfur vesicants, 2-chloroethyl (2-chloroethoxy)ethyl sulfide, sesquimustard and bis[(2-chloroethylthio)ethyl]ether. All three vesicants exhibited significant $(M + NX_4)^+$ (where X = H or ^2H) pseudo-molecular ions and structurally significant chemical ionization fragmentation ions during capillary column gas chromatographic–ammonia chemical ionization mass spectrometric analysis. This method was utilized during analysis of contaminated painted panels circulated during the 3rd round robin verification exercise (1991). Chemical ionization data obtained during this exercise complemented the electron impact data obtained for sesquimustard and bis[(2-chloroethylthio)ethyl]ether and the specificity of the technique enabled the confirmation of 2-chloroethyl (2-chloroethoxy)ethyl sulfide, a compound masked by hydrocarbons in the painted panel extracts.

INTRODUCTION

The possible ratification of a United Nations Chemical Weapons Convention has prompted many nations to consider the development of appropriate analytical techniques for chemical warfare agent detection and confirmation. Capillary column gas chromatography (GC) with flame ionization detection (FID) may be used for the routine screening of samples for the presence of mustard and other sulfur vesicants [1,2]. However, it is generally agreed that confirmation of the chemical warfare agents or their degradation products requires identification by mass spectrometry (MS).

Electron impact (EI), the traditional MS method of ionization, has gained wide acceptance for the verification of sulfur vesicants, as the EI mass spectra of numerous chemical warfare agents, their decomposition products and related compounds have been published [3–9]. EI mass spectra generally provide excellent structural information, but the presence of little or no molecular ion information for longer chain sulfur vesicants often hinders the identification of these compounds. Isobutane chemical ionization (CI) MS, a milder ionization technique, has been used to provide molecular ion information for these sulfur vesicants and related compounds [7]. However, isobutane lacks the specificity of ammonia and in hydrocarbon contaminated samples difficulties may occur in the detection of trace levels of sulfur vesicant contamination.

The efficacy of ammonia CI-MS [10] has been demonstrated for the detection of organophosphorus chemical warfare agents, their decomposition products and related impurities [11–15], including those contaminated with high levels of hydrocarbons [16]. Ammonia CI-MS has been used for long chain and cyclic mustard degradation products [8], but has not been previously utilized for long chain sulfur vesicants. The primary objective of this study was the evaluation of capillary column GC–ammonia and deuterated ammonia CI-MS for the detec-

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tion and confirmation of long-chain sulfur vesicants controlled under the proposed United Nations Chemical Weapons Convention. The sulfur vesicants studied exhibited significant $(M + NX_4)^+$ (where X = H or ^2H) pseudo-molecular ions and structurally significant CI fragmentation ions during capillary column GC–ammonia CI-MS analysis. The specificity of this technique was recently demonstrated during the analysis of contaminated painted panels circulated to fourteen United Nations Conference on Disarmament Technical Group national laboratories during the 3rd round robin verification exercise.

EXPERIMENTAL

Standards and sample handling

2-Chloroethyl (2-chloroethoxy)ethyl sulfide, sesquimustard [bis(2-chloroethylthio)ethane] and bis[(2-chloroethylthio)ethyl]ether were provided by the Defence Research Establishment Suffield Organic Chemistry Laboratory. Distilled-in-glass hexane was purchased from BDH (Edmonton, Canada). Anhydrous-grade ammonia (99.99% ; Liquid Carbonic) and deuterated ammonia (99% ; MDS Isotopes) were used during all CI-MS analyses.

Painted metal panels (2 cm × 4 cm with 60 μm paint), provided by Prins Maurits Laboratory TNO (Netherlands) as part of the 3rd round robin verification exercise, were placed in the bottom of a 100-ml glass beaker containing 10 ml of hexane. The panel was extracted by ultrasonic vibration for 5 min and the hexane removed and concentrated by nitrogen blowdown to 1 ml prior to analysis. All sample extracts and standards were stored in 1.8-ml PTFE-lined screw-capped vials at 4°C prior to GC analysis.

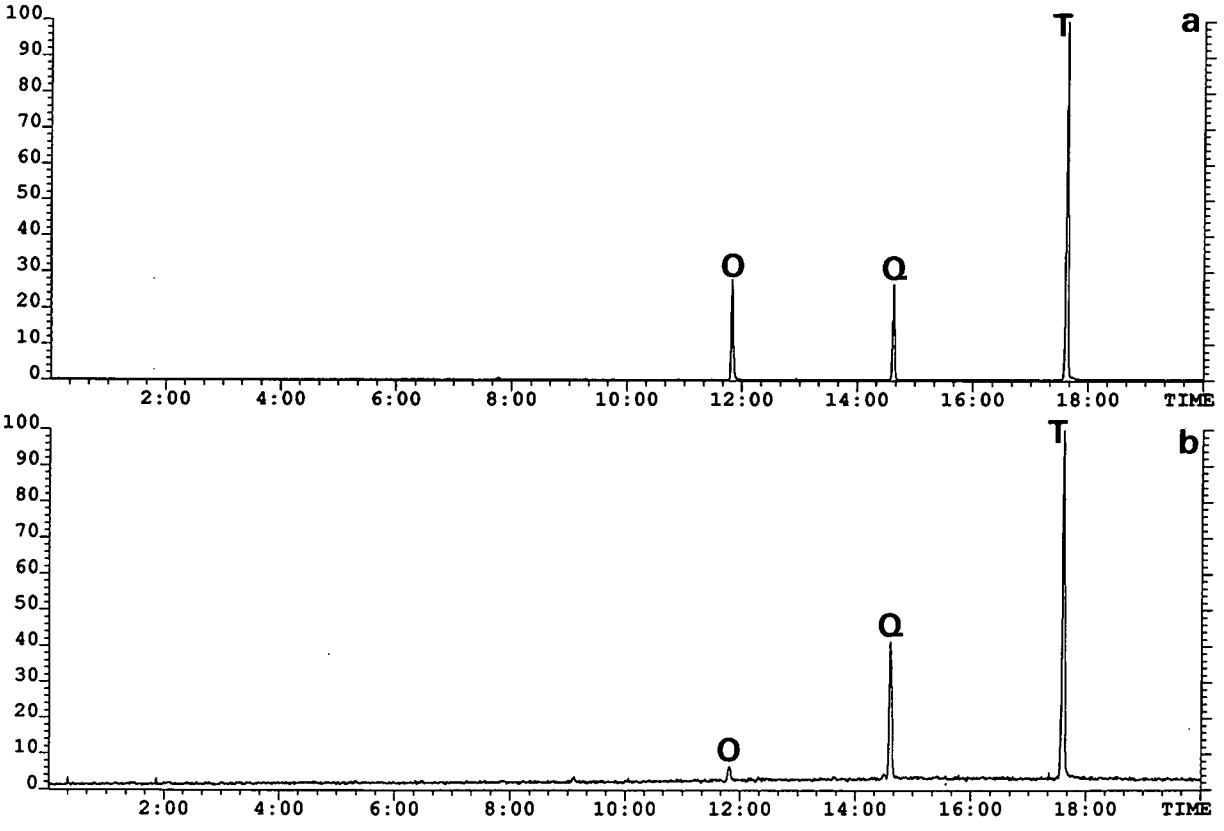

Fig. 1. Capillary column GC–MS (ammonia CI) chromatograms (200 to 300 u) of (a) a standard containing 2-chloroethyl (2-chloroethoxy)ethyl sulfide (O), sesquimustard (Q) and bis[(2-chloroethylthio)ethyl]ether (T) and (b) a hexane extract of round robin painted panel. Time in min; *y*-axis: relative intensity in %.

Instrumental

Capillary column GC–MS analyses were performed with an Autospec-Q hybrid tandem mass spectrometer (VG Analytical, Wythenshawe, UK) interfaced to a Hewlett-Packard 5890 gas chromatograph under the following chromatographic conditions. All injections were on-column [1] at 40°C onto a 15 m × 0.32 mm ID J&W DB-1701 (0.25 μm) capillary column with a 40°C (2 min) → 10°C/min → 280°C (5 min) temperature program. EI-MS operating conditions were as follows: accelerating voltage, 8 kV; emission, 0.2 mA; electron energy, 70 eV; source temperature, 200°C and source pressure, $2 \cdot 10^{-6}$ Torr. CI-MS operating conditions were as follows: accelerating voltage, 8 kV; emission, 0.3 mA; electron energy, 50 eV; source temperature, 120°C and source pressure (near source), $8 \cdot 10^{-5}$ Torr. The ratio of NH_4^+: $NH_3^{+\cdot}$ was approximately 15:1 in the VG Autopsec EI/CI source. Full scanning EI and CI data were collected over the 400 to 40 u mass range at 0.5 s/decade.

RESULTS AND DISCUSSION

Fig. 1 illustrates capillary GC–ammonia CI-MS chromatograms for a standard containing three long chain sulfur vesicants, 2-chloroethyl (2-chloroethoxy)ethyl sulfide, sesquimustard and bis[(2-chloroethylthio)ethyl]ether and the hexane extract of a round robin painted panel contaminated with an envelope of hydrocarbons and the same sulfur vesicants. The envelope of hydrocarbons in the hexane extract of the painted panels did not hinder the EI-MS detection and confirmation of sesquimustard and bis[(2-chloroethylthio)ethyl]ether (Fig. 2).

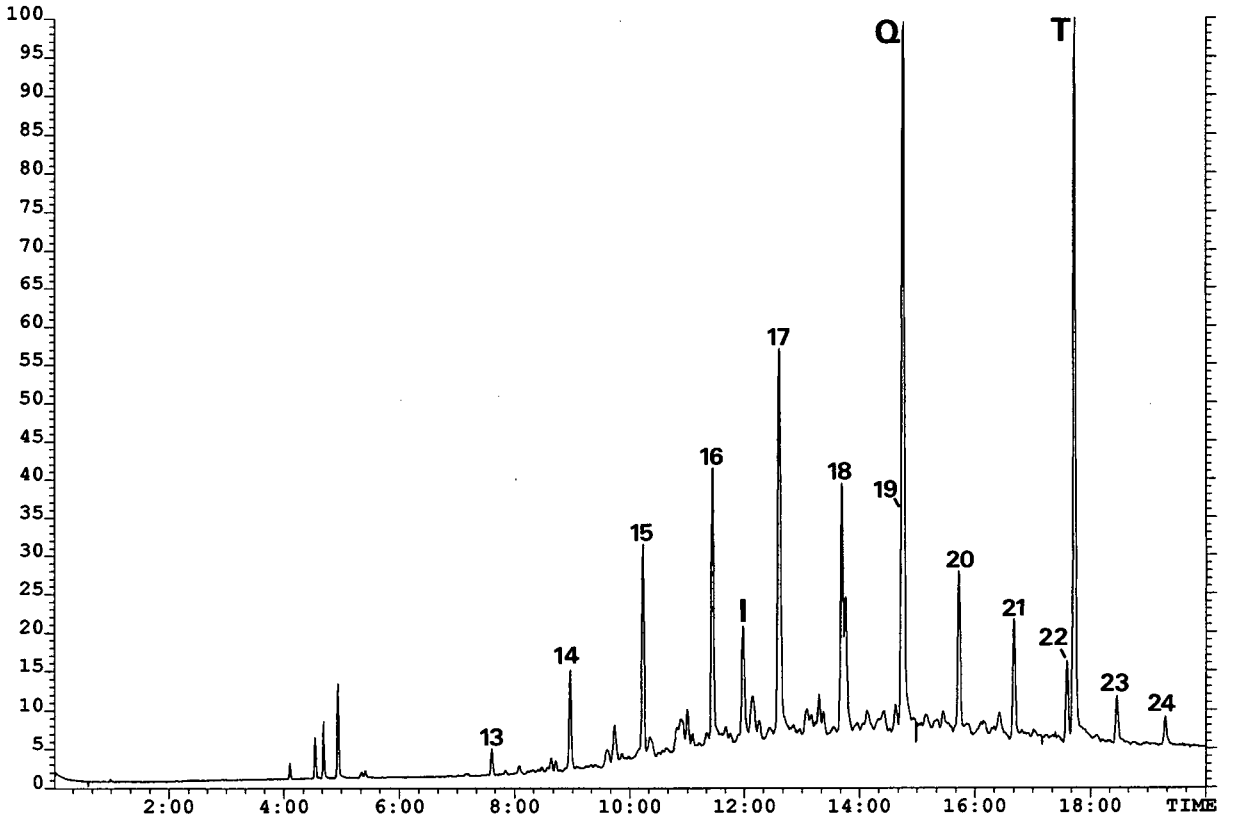

Fig. 2. Capillary column GC–MS (EI) chromatogram (40 to 400 u) of a hexane extract of round robin painted panel containing 2-chloroethyl (2-chloroethoxy)ethyl sulfide [masked by interference (I)], sesquimustard (Q) and bis[(2-chloroethylthio)ethyl]ether (T). The numbered components indicate the carbon number of *n*-alkanes. Scales as in Fig. 1.

However, the presence of co-eluting hydrocarbon(s) hindered the identification of 2-chloroethyl (2-chloroethoxy)ethyl sulfide, present at 4 ng level in the extract chromatogram (Fig. 1b). EI-MS identification was tentative at best. However under ammonia CI-MS the co-eluting hydrocarbon(s) were not ionized and this compound was detected and confirmed along with the other two higher-level sulfur vesicants in the painted panel extract. A full scanning ammonia CI-MS detection limit of about 1 ng was estimated based on the detection of 2-chloroethyl (2-chloroethoxy)ethyl sulfide.

The ammonia CI mass spectra of the compounds found in the painted panel hexane extract (Fig. 3) were characterized by the presence of intense $(M + NH_4)^+$ pseudo-molecular ions and ammonia CI fragmentation ions due to $(M + H)^+$, $(M + NH_4 - 34)^+$, $(M + NH_4 - NH_4Cl)^+$, $[C_2H_4SC_2H_4Cl]^+$ and $(M + NH_4 - C_2H_4S - 34)^+$. Most of the ions, listed in Table I were readily interpreted with the exception of those involving loss of 34. It was apparent from the isotopic abundance data that the loss involved chlorine, but there was uncertainty as to the source of the additional hydrogen. Deuterated ammonia CI-MS was performed on the standards in an attempt to ascertain whether the ammonia CI gas was involved in the gain of hydrogen by these ions. Fig. 4 illustrates the deuterated ammonia CI mass spectra for 2-chloroethyl (2-chloroethoxy)ethyl sulfide, sesquimustard and bis-[(2-chloroethylthio)ethyl]ether. Gains in mass (5 u) by the deuterated ammonia CI fragmentation ions confirmed that the ions in question were due to $(M + NX_4 - Cl + X)^+$ and $(M + NX_4 - Cl - C_2H_4S + X)^+$ (where X = H or ^2H). The other deuterated ammonia CI ions correlated well with

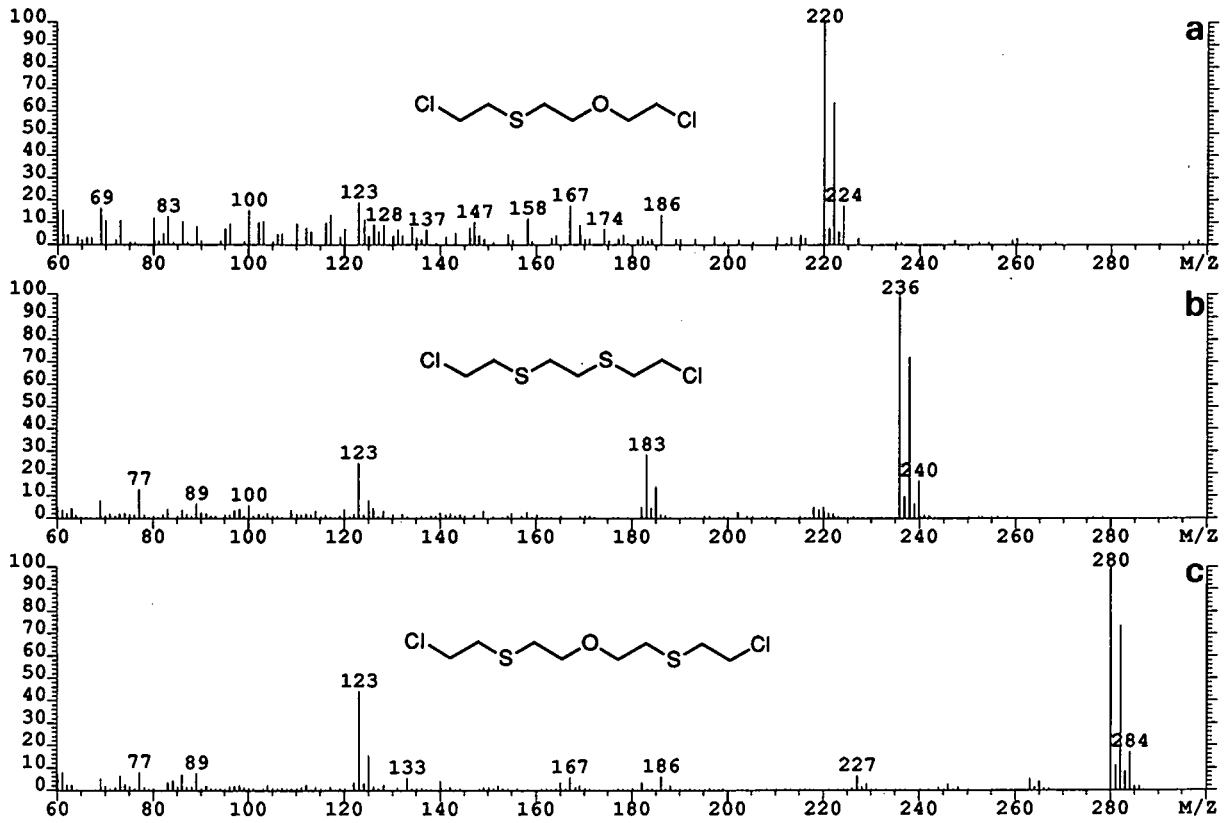

Fig. 3. Ammonia chemical ionization mass spectra of (a) 4 ng of 2-chloroethyl (2-chloroethoxy)ethyl sulfide, (b) 35 ng of sesquimustard and (c) 50 ng of bis[(2-chloroethylthio)ethyl]ether found in the hexane extract of the round robin painted panel (refer to Fig. 1b). *y*-axis: relative intensity in %.

TABLE I

NX_3 (X = H OR ^2H) CHEMICAL IONIZATION MASS SPECTROMETRIC DATA FOR 2-CHLOROETHYL (2-CHLOROETHOXY)ETHYL SULFIDE (O), SESQUIMUSTARD (Q) AND BIS[(2-CHLOROETHYLTHIO)ETHYL]ETHER (T)

Ion structure	*m/z* (% Relative intensity)					
	X = H			X = ^2H		
	O	Q	T	O	Q	T
$(M + NX_4)^+$	220 (100)	236 (100)	280 (100)	224 (100)	240 (100)	284 (100)
$(M + X)^+$	203 (1.4)	219 (3.5)	263 (4.8)	204 (1.6)	220 (5.8)	264 (3.9)
$(M + NX_4 - Cl + X)^+$	186 (6.4)	202 (3.0)	246 (3.0)	191 (3.1)	207 (2.2)	251 (2.1)
$(M + NX_4 - NX_4Cl)^+$	167 (17)	183 (28)	227 (6.1)	167 (11)	183 (13)	227 (5.3)
$(M + NX_4 - Cl - C_2H_4S + X)^+$	–	–	186 (4.0)	–	–	191 (4.5)
$[C_2H_4SC_2H_4Cl]^+$	123 (16)	123 (35)	123 (40)	123 (20)	123 (27)	123 (30)

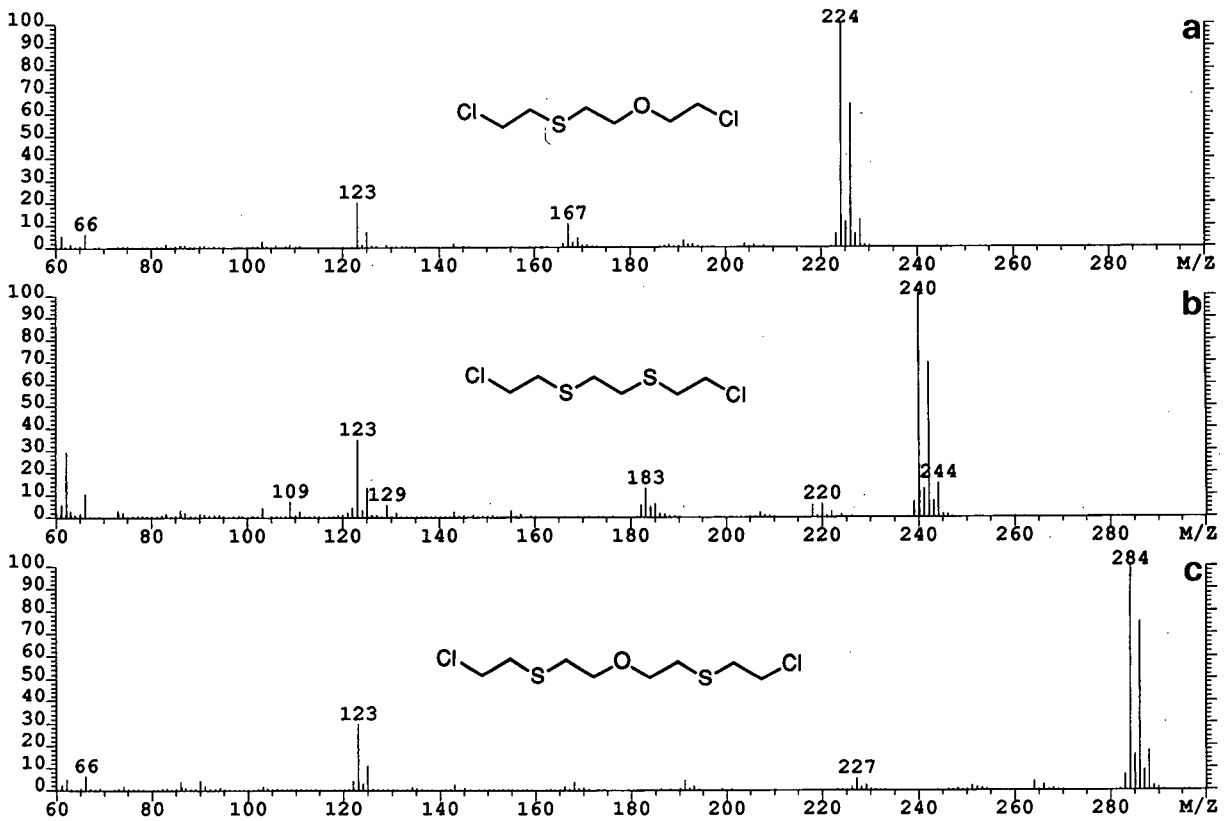

Fig. 4. Deuterated ammonia chemical ionization mass spectra of (a) 2-chloroethyl (2-chloroethoxy)ethyl sulfide, (b) sesquimustard and (c) bis[(2-chloroethylthio)ethyl]ether. *y*-axis: relative intensity in %.

the ammonia CI data and Table I compares the deuterated ammonia and ammonia CI ions observed under similar source conditions.

CONCLUSIONS

Capillary column GC–ammonia and deuterated ammonia CI-MS was found to be a highly specific technique for the detection and identification of three long-chain sulfur vesicants, 2-chloroethyl (2-chloroethoxy)ethyl sulfide, sesquimustard and bis-[(2-chloroethylthio)ethyl]ether. All three vesicants exhibited significant $(M + NX_4)^+$ (where X = H or ^2H) pseudo-molecular ions and structurally significant CI fragmentation ions during capillary column GC–ammonia CI-MS analysis. Deuterated ammonia CI data were acquired for all three compounds to confirm the identity of two unusual CI fragmentation ions.

This new approach to vesicant analysis was demonstrated during analysis of contaminated painted panels circulated during the 3rd round robin verification exercise (1991). CI data obtained during this exercise complemented the EI data obtained for sesquimustard and bis[(2-chloroethylthio)ethyl]ether and the specificity of the technique enabled the confirmation of 2-chloroethyl (2-chloroethoxy) ethyl sulfide, a compound masked by the presence of hydrocarbons during EI-MS analysis of the painted panel extracts. The CI data provided are sufficient for the detection and confirmation of three long-chain sulfur vesicants controlled under the proposed United Nations Chemical Weapons Convention and application of this highly specific technique appears to be likely during future analyses.

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CHROM. 24 080

Supercritical fluid extraction of chemical warfare agent simulants from soil

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ABSTRACT

Chemical warfare agent simulants are efficiently recovered from 2-ppm spikes in 1 g of Rocky Mountain Arsenal Standard Soil using methanol–carbon dioxide (5:95) at 300 atm for 2 min at 60°C. Recoveries (n = 3) were 79 ± 23% for dimethylmethylphosphonate, 93 ± 14% for 2-chloroethylethyl sulfide, 92 ± 13% for diisopropylfluorophosphate and 95 ± 17% for diisopropylmethylphosphonate. Recoveries are higher than, but less precise than those achieved from a 5-min ultrasonic micro-scale extraction using methanol. Much less laboratory waste is generated than the current standard organic solvent extraction method (33 g of soil shaken with 100 ml of chloroform).

INTRODUCTION

Current standard methods [1] for extracting agents bis(2-chloroethyl)sulfide (HD) and O-isopropylmethylphosphonofluoridate (GB) or O-ethyl-S-2-(diisopropylaminoethyl)methylphosphononothioate (VX) from soil involve shaking 33 g samples with 100 ml of chloroform. Although the method is simple and straightforward, the mass of toxic waste is considerable, and the sensitivity of the final analytical methods is limited by the solid:liquid ratio if the solvent is not concentrated.

Supercritical fluid extraction (SFE) appears to offer a good alternate to solvent extraction in this application. Carbon dioxide and nitrous oxide have been used [2–8] in both off- and on-line interfacing with gas chromatography (GC) to achieve highly efficient recoveries and sensitive analyses of many low polarity organic compounds from soil, sediment, diesel exhaust and air particulates, using small masses of sample and short extraction times. Recoveries for many solutes have been superior to those achieved using soxhlet or ultrasonic solvent extraction [2,7]. Binary supercritical fluids [3,4,6] can be used to improve extraction recoveries from sorptive matrices. This paper reports the successful recovery and analysis of chemical warfare agent simulants from soil using SFE followed by off-line GC.

EXPERIMENTAL

Equipment

SFE was performed using two commercially available devices. A Suprex SFC/200A supercritical fluid chromatograph was used for most of the work. The column was replaced with a Brownlee high-performance liquid chromatography (HPLC) guard column or a Keystone Scientific SFE vessel (nominal volume of *ca.* 0.5 ml) holding *ca.* 1 g of soil. The UV detector was replaced with a high pressure shut-off valve and a *ca.* 30 cm length of 25 μm I.D. fused-silica tubing from SGE was attached to the valve outlet for a restrictor. The experiments with 10 g of soil were performed using an ISCO System 1200, a *ca.* 30 cm length of 50 μm I.D. fused-silica tubing, and a 5-ml extraction cell.

Off-line gas chromatographic analysis of the simulants was conducted on two instruments. The initial work was done on a Perkin-Elmer Model 3920

using a 30 m × 0.53 mm I.D. × 1.5 μm film thickness DB-5 fused-silica capillary column with a 7 ml/min flow-rate of helium. The column oven was temperature programmed from 70°C (after a 4 min isothermal hold) to 150°C at 8°C/min with the injector and flame ionization detector held at *ca.* 120°C and 200°C, respectively. A 3-μl volume was injected slowly using the solvent flush technique, and quantitation was performed by the method of internal standards using a Maxima chromatography data system on an IBM XT personal computer. Later work used a Varian Model 3400 gas chromatograph equipped with the same column and a 1:1 split of the column effluent to flame photometric (P mode) and electron-capture detectors. The column oven temperature program was 70°C (2 min isothermal hold) to 130°C at 4°C/min. The injector was held at 200°C and the detectors were maintained at 220°C. A 1-μl injection was made using the solvent flush technique, and quantitation was conducted by the method of internal standards using either a Maxima chromatography data system or a Model 4400 Varian integrator.

Reagents

The simulants were purchased from the indicated vendors and were used as received: chloroethylethyl sulfide (CES), diisopropylfluorophosphate (DIFP), triethyl phosphate (TEP), and diethylaminoethanethiol hydrochloride (DEAT·HCl) all were from Aldrich (Milwaukee, WI, USA), and the dimethylmethyl phosphonate (DMMP) and diisopropylmethyl phosphonate (DIMP) were from Alfa (Danvers, MA, USA). The free base of DEAT was prepared by dissolving DEAT·HCl in water at pH 11, extracting with diethyl ether, and evaporating the solvent. Solvents were Burdick & Jackson distilled in glass grade from American Scientific Labs. (Atlanta, GA, USA). The SFC-grade carbon dioxide and methanol–carbon dioxide (5:95) were obtained from Scott Specialty Gases (Plumsteadville, PA, USA).

Caution: The DIFP simulant is highly toxic and is an acetylcholinesterase inhibitor. The CES can cause burns.

SFE

The SFE procedure consisted of weighing *ca.* 1 g of Rocky Mountain Arsenal Standard Soil into an extraction cell, and injecting a known volume of simulant spiking solution into the soil *ca.* 2 cm from the inlet end, assembling the cell and letting the sample set for 15 min at room temperature. The cell was installed in the apparatus and allowed to warm up to operating temperature. The inlet valve was opened to admit supercritical fluid to the cell, and the outlet valve was then opened to begin the collection of the extract. In early work, the fused-silica restrictor tubing was dipped into a vial containing 3 ml of methanol and TEP internal standard, while later, the volume of methanol was 2 ml and the TEP was added after the SFE was completed. The extractions were conducted at 60°C and 300 atm (unless otherwise listed). The vial containing the collecting solution was placed in a beaker of water at room temperature to prevent ice formation when extractions were carried out longer than 5 min.

RESULTS AND DISCUSSION

This study used simulants which have structural features similar to the actual agents but which lack the very high toxicity of the latter. DMMP was the simulant for agent VX, DIFP and DIMP modelled agent GB, and CES was used in place of HD. In addition, DEAT·HCl, a byproduct from VX manufacture, also was tested. All of the simulants can be separated and determined in a single GC run, as shown by the bottom chromatogram in Fig. 1. Low ppm solution concentrations can be determined using the flame ionization detector, and sub-ppm concentrations with the combination of flame photometric (P-mode) and electron-capture detectors.

It was found that pressures around 300 atm are needed to extract low-ppm concentrations of agent simulants from 1-g samples of soil. As shown in Table I, straight supercritical carbon dioxide at 60°C and 300 atm can easily extract the CES from soil in 5 min, but it is not able to efficiently extract the phosphonates and fluorophosphate even at higher extraction pressures or longer extraction times. Experiments in which supercritical carbon dioxide was bubbled through methanol spiked with the simulants showed that the latter were not volatilized from the collection solution by the decompressing supercritical fluid. DIMP has been recovered from water [8] in unknown yield using supercritical carbon dioxide. For soil, a 5% methanol

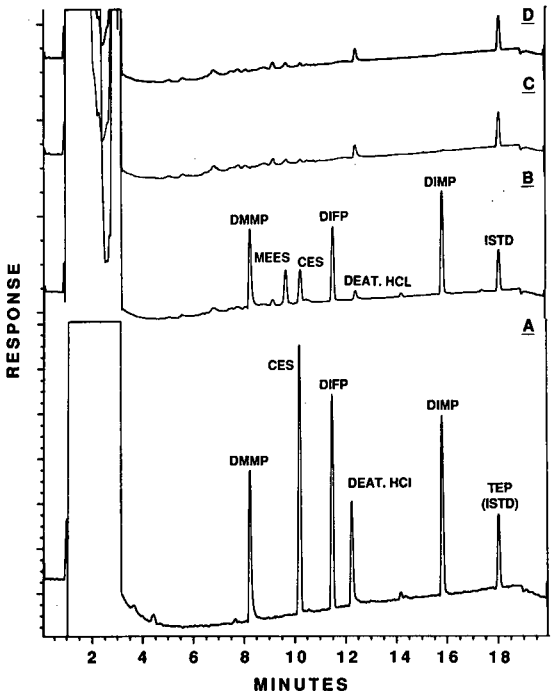

Fig. 1. Gas chromatographic analysis (flame ionization detection) of (A) simulant standard and (B) first 10 min SFE of spiked soil, (C) second 10 min SFE of soil, and (D) third 10 min SFE of soil. (Acronyms are defined in the Experimental section, and SFE conditions are listed in footnote *b* of Table II).

modifier is necessary in the carbon dioxide to recover all the simulants, and at 300 atm and 60°C, good recoveries are achieved in a 5 min SFE. Even low-polarity compounds such as five-ring polycyclic aromatic hydrocarbons and tetrachlorodibenzodioxin require methanol modifiers in their SFE from sorptive matrices [3,4,7].

The SFE recoveries are slightly higher but less reproducible than those achieved using a single ultrasonic solvent extraction (5 min) with methanol. The latter is probably better controlled at this stage of technology development, and non-uniformity of SFE flow may contribute [2] to the variability of the former. These SFE recoveries of simulants also are much higher but less precise than those reported [9] for sequential ultrasonic extractions with hexane and methylene chloride of triethylphosphate and agents GB, HD and GD (soman) spiked at 5 to 50 ppm in soil.

As shown in Fig. 1, a single SFE is sufficient for the simulants. In contrast, the agent manufacturing byproduct DEAT·HCl was recovered in low and irreproducible yield (typically 10% per extract fraction). This may be due to a low solubility of the compound in the supercritical fluid and/or strong sorption of the compound by the soil. Reaction of the amine with carbon dioxide to form a urea derivative also is possible [8], but is not consistent with the observed extraction behavior. Fig. 1 shows that while the simulants are extracted in the first 10 min fraction, DEAT·HCl continues to slowly extract in subsequent fractions. The free amine did not extract at all, suggesting that sorptive interactions with acidic sites on the soil may be the limiting factor. Nitrous oxide has been used successfully [8] to extract basic amines from soil, and may be useful

TABLE I

SFE RECOVERIES OF CHEMICAL WARFARE AGENT SIMULANTS FROM ROCKY MOUNTAIN ARSENAL SOIL

Fluid	Extraction[a]		Spike (ppm)	Replicates	Recovery (%), average ± S.D.			
	Pressure (atm)	Time (min)			DMMP	CES	DIFP	DIMP
CO_2	300	5	2	2	12	95	17	43
CO_2	300	12	2	1	9	90	ND[c]	15
CO_2	350	12	2	1	4	93	59	7
CO_2–CH_3OH	300	5	2	3	79±23	93±14	92±13	98±25
CO_2–CH_3OH	300	5	12	3	73± 9	ND	86±10	95±17
CH_3OH[b]	–	5	2	3	80± 3.5	85±15	63± 4.2	87± 6.1

[a] SFE at 60°C using 25 μm I.D. restrictor and conditions shown. A 1-g amount of soil was extracted, and supercritical fluid was decompressed in 2 ml of methanol. Analysis by GC–flame photometric detection/electron-capture detection.

[b] Ultrasonic extraction of 1 g spiked soil with 2 ml of methanol for 5 min.

[c] ND = Not determined.

here. The DEAT·HCl also was not recovered (3%) from ultrasonic extraction using methanol.

In early SFE development work two GC peaks were recovered for the CES, as shown in Fig. 1B. Relative retention data [10] would suggest 1,4-dithiolane as a candidate for the earlier-eluting peak, and this product has been identified [11] as a degradation product of pure CES. GC–mass spectrometry suggested that the new, earlier-eluting peak shown in Fig. 1 is a methyl ether derivative, possibly methoxyethylethylsulfide (MEES). Major *m/z* observed in the mass spectrum of the new peak were 120 (apparent M), 75, 58 and 45, *versus* 120 (M), 92, 61 and 46 in the spectrum for 1,4-dithiolane. The first step in the decomposition of CES has been postulated [11] as the formation of a reactive ethylene sulfonium ion via an S_N1 mechanism. MEES presumably could be formed by a nucleophilic attack on the ion by methanol (solvent for standards). The new product was observed in methylene chloride solvent extractions of the soil, but not when carbon dioxide–methanol was bubbled through a spiked solution of simulants in methanol (see Table II). It was not observed in the work reported in Table I where fresh spiking solutions were used. The controlling factors in its production have not yet been identified, but could include a catalytic effect from soil surfaces.

In earlier work with 25 μm I.D. fused-silica restrictor tubing, problems were encountered with tubing breakage at the point where the tubing dipped into the methanol collection solution. This was thought to result from ice crystal formation in the tubing. A small, *ca.* 10-mg layer of calcium chloride or sodium sulfate was placed in the downstream end of the extraction cell to trap water extracted from the soil. Tubing breakage was not solved, and, as listed in Table II, the recoveries of the phosphonates were reduced. However, the CES recovery was improved. With sodium sulfate, the production of the MEES also was increased such that the sum of the MEES and CES recoveries accounted for the CES spike. Sheathing the last 10 cm of the 25 μm I.D. tubing with 325 μm I.D. fused-silica tubing minimizes breakage. However, breakage has not been as much of a problem with the larger-bore 60 μm I.D. fused-silica tubing, and extraction recoveries appear to be equivalent.

Extraction of soil masses larger than 1 g is feasible. Experiments with 10 g masses of soil spiked at 2 ppm each simulant and extracted in the ISCO apparatus for 20 min yielded good recoveries: DMMP 70%, CES 81%, DIFP 103% and DIMP 110%. None of the simulants were detected in second and third 20-min SFE fractions. The lower recoveries of DMMP and CES could have resulted from purging out of the collecting solution with the greater flowrate of the 50 μm I.D. restrictor. Purging from the

TABLE II

EFFECTS OF RESTRICTOR AND DRYING AGENTS ON SFE OF SIMULANTS

Variable[b]	Replicates	Recovery[a] (%) average ± S.D.				
		DMMP	MEES[c]	CES	DIFP	DIMP
25 μm I.D. restrictor	3	96 ± 3.2	24 ± 11	28 ± 11	71 ± 19	97 ± 11
60 μm I.D. restrictor	1	103	29	27	75	93
$CaCl_2$[d]	2	5	27	40	94	14
Na_2SO_4[d]	1	51	40	60	101	57
Spiked CH_3OH[e]	1	98	0	96	92	103

[a] Recoveries from spiked Rocky Mountain Arsenal Standard Soil: DMMP = 32 ppm, CES = 10.6 ppm, DIFP = 10.7, DIMP = 16.2 ppm. Analysis by GC–flame ionization detection.

[b] SFE conditions: methanol–CO_2 (5:95) at 300 atm, 60°C, 10 min extraction of 1 g soil spiked as noted in footnote (*a*), and supercritical fluid decompressed in 3 ml of methanol.

[c] Methoxyethylethylsulfide recovery from spiked CES.

[d] 25 μm I.D. restrictor used, *ca.* 10 mg of drying agent packed in bottom of SFE cell.

[e] Supercritical CO_2–methanol bubbled through 3 ml of methanol spiked with simulants at concentrations equivalent to 100% recovery from soil.

collection solution was not tested with the larger bore restrictor tubing, but the flow-rate was obviously much greater than the *ca.* 200 ml/min gas flow-rate with the 25 μm restrictor. It is quite likely that detection limits may be lowered by an order of magnitude using the larger mass of soil.

CONCLUSIONS

The results of experiments with simulants suggest that SFE holds considerable promise for providing a rapid and efficient means of recovering chemical warfare agents from soil with less laboratory waste than current methods.

ACKNOWLEDGEMENTS

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Capillary zone electrophoresis of linear and branched oligosaccharides☆

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ABSTRACT

The electrophoretic behavior of derivatized linear and branched oligosaccharides from various sources was examined in capillary zone electrophoresis with polyether-coated fused-silica capillaries. Two UV-absorbing (also fluorescent) derivatizing agents (2-aminopyridine and 6-aminoquinoline) were utilized for the electrophoresis and sensitive detection of neutral oligosaccharides, *e.g.*, N-acetylchitooligosaccharides, high-mannose glycans and xyloglucan oligosaccharides. The oligosaccharides labelled with 6-aminoquinoline yielded eight times higher signal than those tagged with 2-aminopyridine. Plots of logarithmic electrophoretic mobilities of labelled N-acetylchitooligosaccharides with 6-aminoquinoline or 2-aminopyridine *versus* the number of sugar residues in the homologous series yielded straight lines in the size range studied, the slopes of which were independent of the tagging functions. The slopes of these lines are referred to as the N-acetylglucosaminyl group mobility decrement. The oligosaccharides were better resolved in the presence of tetrabutylammonium bromide in the running electrolyte. Furthermore, the electrophoretic mobilities of branched oligosaccharides were indexed with respect to linear homooligosaccharides, an approach that may prove valuable in correlating and predicting the mobilities of complex oligosaccharides.

INTRODUCTION

The separation of carbohydrates by electrophoresis has often required the *in situ* conversion of these compounds into charged species. Early investigations on carbohydrate electrophoresis described anionic complexes with sodium borate [1], sulphonated benzene boronic acid [2], sodium germanate [3], sodium stannate [1] or sodium tungstate [4]. Cationic complexes of carbohydrates with lead acetate [5], or cations of the alkali and alkaline earth metals [5] were also described for the electrophoresis of neutral carbohydrates. Furthermore, because of the ionization of the hydroxyl groups of the sugars at high pH, sodium hydroxide was also useful for the electrophoresis of neutral carbohydrates [6]. These buffer systems are expected to find their way to the capillary zone electrophoresis of sugars. In fact, recently capillary zone electrophoresis (CZE) systems based on borate complexes for mono- and oligosaccharides have been described [7,8].

Another difficulty in the analysis of sugars is the lack of chromophores in their structures. This can be overcome by precolumn derivatization [7–11] or by indirect detection [12]. However, a precolumn derivatization that supplies both the charge and the chromophore for the electrophoresis and the sensitive detection, respectively, is preferred.

In recent reports from our laboratory, we have demonstrated the potential of CZE in the separation of 2-aminopyridyl derivatives of maltooligosaccharides [9] and complex-type glycans cleaved from glycoproteins [10]. In the present article, the scope of applications of CZE was extended to the separation and determination of the homologous series of N-acetylchitooligosaccharides, the branched xylo-

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glucan oligosaccharides from cotton cell walls, and the high-mannose glycans of ribonuclease B. The electrophoretic mobility of the 2-aminopyridyl derivatives of xyloglucan fragments, was indexed to that of the homologous series, which permitted the evaluation of the contribution of sugar residues and degree of branching to the electrophoretic mobility of the derivatized xyloglucan oligosaccharides. In addition, the potential of 6-aminoquinoline as a new tagging agent that provides both the charge and the center for detection was investigated.

EXPERIMENTAL

Instruments

The instrument for capillary electrophoresis was assembled in our laboratory from commercially available components. It comprised two high-voltage power supplies of positive and negative polarity from Glassman High Voltage (Whitehouse Station, NJ, USA) and a Linear (Reno, NV, USA) Model 200 UV–VIS variable-wavelength detector equipped with a cell for on-column capillary detection. The detection wavelength was set at 240 nm for sensing the derivatized oligosaccharides. The electropherograms were recorded with a Shimadzu computing integrator (Columbia, MD, USA) equipped with a floppy disk drive and a cathode-ray tube monitor.

The absorption spectra of the derivatizing agents, *i.e.*, 2-aminopyridine and 6-aminoquinoline were performed on a UV–VIS spectrophotometer (Model UV-160, Shimadzu) by scanning from 200 to 310 nm.

Capillary columns

Fused-silica capillary columns of 50 μm I.D. × 365 μm O.D. were obtained from Polymicro Technology (Phoenix, AZ, USA). All capillaries used in this study were coated in the laboratory with an interlocked polyether coating according to previously described procedures [13]. The running electrolyte was renewed after each run. To ensure reproducible separations the capillary column was flushed successively with fresh buffer, water, methanol, water and again running buffer. The capillary was allowed to equilibrate for 10 min before each injection.

Reagents and materials

Xyloglucan oligosaccharides were obtained from cotton cell walls as described earlier [14]. They were supplied as 2-aminopyridyl derivatives by Dr. A. Mort from the Department of Biochemistry. N-Acetylchitooligosaccharide standards having a degree of polymerization (DP) from 2 to 6 were from Seikagaku Kogyo (Tokyo, Japan). High-mannose oligostandard, $(GlcNAc)_2$-Man_5, was purchased from Dionex (Sunnyvale, CA, USA). Ribonuclease B (RNase B), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, N-acetylglucosamine (GlcNAc), 2-aminopyridine (2-AP), tris(hydroxymethyl)aminomethane (Tris) and Brij 35 were obtained from Sigma (St. Louis, MO, USA). Peptide-N-glycosidase F (PNGase F) from *Flavobacterium meningosepticum* was obtained from Boehringer Mannheim (Indianapolis, IN, USA). Mercaptoethanol, sodium cyanoborohydride, 6-aminoquinoline (6-AQ), trifluoroacetic acid (TFA) and tetrabutylammonium bromide (Bu_4N^+) were from Aldrich (Milwaukee, WI, USA). Reagent-grade sodium phosphate monobasic and dibasic, phosphoric acid, hydrochloric acid, acetic acid, sodium hydroxide, calcium chloride, ethylenediaminetetraacetic acid disodium salt (EDTA), and HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was used to prepare the running electrolyte. All solutions were filtered with 0.2-μm UniPrep syringeless filters obtained from Genex (Gaithersburg, MD, USA) to avoid column plugging.

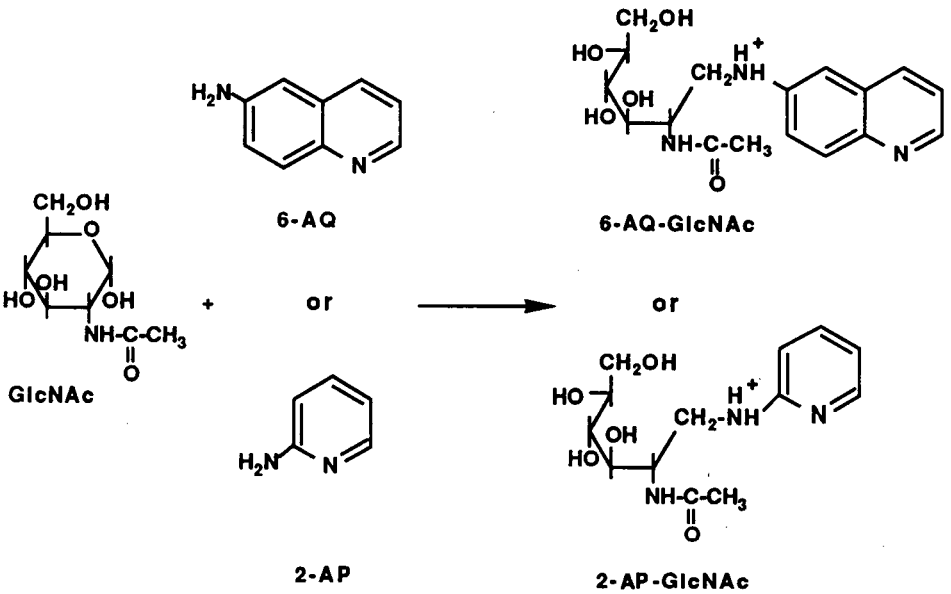
Cleavage of high-mannose glycans

Bovine RNase B was first digested with TPCK-treated trypsin using a 10 m*M* Tris buffer containing 100 m*M* ammonium acetate and 0.1 m*M* calcium chloride, pH 8.3, at a trypsin substrate ratio of 1:100 and a temperature of 37°C [15]. Trypsin was added again after 2 h and the digestion was stopped after a total of 4 h by addition of phosphoric acid. Thereafter, the whole digest was desalted by passing it on a Bakerbond wide-pore octadecyl-silica column (250 × 4.6 mm) equilibrated with water at 0.05% (v/v) TFA. The bound materials were eluted stepwise with acetonitrile–water (80:20, v/v) at 0.05% TFA (v/v). The pooled fraction was evaporated to dryness using a SpeedVac concentrator (Savant,

Farmingdale, NY, USA). The dried materials were then dissolved in 20 m*M* phosphate buffer containing 2 m*M* EDTA, 1% (v/v) mercaptoethanol and 0.1% (w/v) Brij 35, pH 7.5. To this solution 3 units of peptide-N-glycosidase F were added, and the incubation was maintained at 37°C for 24 h [16]. Thereafter, the mixture was evaporated to dryness with a Savant SpeedVac concentrator. The dried material containing the cleaved oligosaccharides, the peptide fragments, and other reagents employed in the incubation step was used as is without a sample clean up prior to the derivatization of its oligosaccharide components.

Derivatization of oligosaccharides

Commercially available N-acetylchitooligosaccharides were tagged with 2-AP or 6-AQ whereas the oligosaccharides cleaved from bovine ribonuclease B were derivatized with 2-AP, at their reducing termini via reductive amination according to the following equations:



where N-acetylglucosamine is taken as a typical example. To that end a 0.26 *M* aqueous solution of 2-AP or 6-AQ was titrated to pH 5.8 with glacial acetic acid. Thereafter, sodium cyanoborohydride was added at a concentration of 20 mg/ml just prior to the addition of the carbohydrate. The reaction mixtures were incubated overnight at 70°C. Following, the reaction mixtures containing the derivatized oligosaccharides were evaporated to dryness. The dried materials thus obtained were dissolved in water and then applied to capillary electrophoresis without any sample clean up from excess derivatizing agent and other components of the reaction mixture.

TABLE I

LOGARITHMIC N-ACETYLGLUCOSAMINYL GROUP MOBILITY DECREMENT, δ_{GlcNAc}, AS ESTIMATED FROM THE SLOPE OF THE LOGARITHMIC ELECTROPHORETIC MOBILITY *VERSUS* THE NUMBER OF GlcNAc RESIDUES IN THE 2-AP-GlcNAc$_n$ HOMOLOGOUS SERIES

Electrolytes: 0.1 *M* phosphate solution, pH 5.0, at various tetrabutylammonium bromide concentrations.

$[Bu_4N^+]$ (m*M*)	δ_{GlcNAc}	Correlation coefficient
0	−0.082	0.981
10.0	−0.105	0.990
30.0	−0.109	0.989
50.0	−0.122	0.990

RESULTS AND DISCUSSION

The electrophoretic behavior of derivatized linear

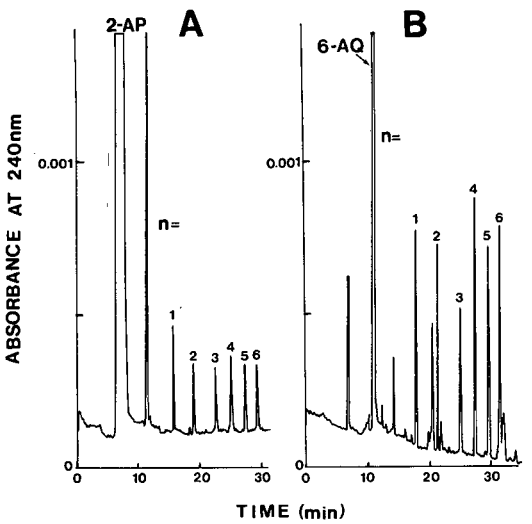


Fig. 1. Electropherograms of 2-aminopyridyl (A) and 6-aminoquinolyl (B) derivatives of N-acetylchitooligosaccharides. Capillary, fused-silica tube with polyether interlocked coating on the inner walls, 50 cm (to the detection point), 80 cm total length × 50 μm I.D.; electrolyte, 0.1 *M* phosphate solution containing 50 m*M* tetrabutylammonium bromide, pH 5.0; running voltage, 18 kV. 2-AP = 2-Aminopyridine; 6-AQ = 6-aminoquinoline.

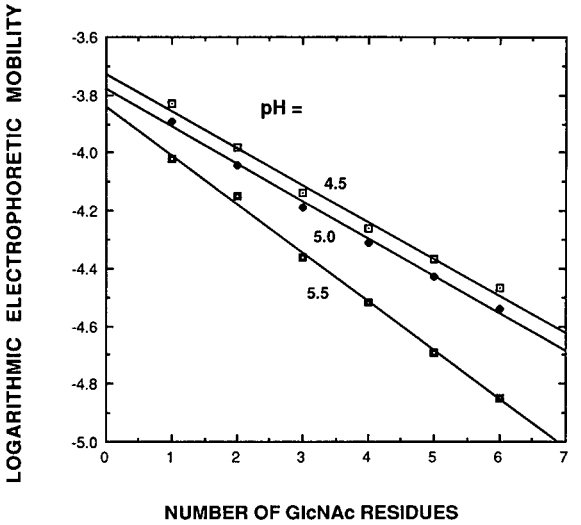


Fig. 2. Plots of logarithmic electrophoretic mobility *versus* the number of GlcNAc residues in the 2-AP-GlcNAc$_n$ homologous series at various pH. Electrolytes, 0.1 *M* sodium phosphate solutions containing 50 m*M* tetrabutylammonium bromide. Other conditions as in Fig. 1.

and branched oligosaccharides from various sources was examined using fused-silica capillaries with hydrophilic coating. The results obtained with 2-aminopyridyl derivatives were compared to those of 6-aminoquinolyl derivatives. A mobility indexing system for branched oligosaccharides was established with repect to linear homooligosaccharides.

CZE of derivatized N-acetylchitooligosaccharides

N-Acetylglucosaminyl group mobility decrement. Fig. 1A and B illustrates the high resolution separation of 2-aminopyridyl and 6-aminoquinolyl derivatives of N-acetylchitooligosaccharides, respectively. It was brought about by adding tetrabutylammonium bromide (Bu_4N^+) to the running electrolyte, a medium that we have reported earlier for the separation of maltooligosaccharides [9] and complex glycans cleaved from glycoproteins [10]. To further characterize the effect of the organic salt on the separation of derivatized oligosaccharides, the 2-aminopyridyl derivatives of N-acetylchitooligosaccharides (2-AP-GlcNAc$_n$) were electrophoresed over a wide range of pH and Bu_4N^+ concentrations.

Fig. 2 illustrates typical plots of the logarithmic eletrophoretic mobility, log μ, of 2-AP-GlcNAc$_n$ *versus* the number of N-acetylglucosamine residues (GlcNAc) in the homologous series at different pH. These plots were linear in the size range studied. It has to be noted that the derivatized oligosaccharides carry the same net positive charge, and therefore the charge to size ratio decreases with the number of GlcNAc residues in the homologous series. As can be seen in Fig. 2, the electrophoretic mobility decreased with the pH as the derivatized oligosaccharides become less positively charged. As a result, the slope of the straight lines, which are referred to as the "N-acetylglucosaminyl group mobility decrement", δ_{GlcNAc}, increased in absolute value with increasing the pH in the range studied. However, the resolution between the homologs decreased as the pH increased due to an increase in the electroosmotic flow of the medium with increasing pH.

As expected, the 6-aminoquinolyl derivatives of N-acetylchitooligosaccharides (6-AQ-GlcNAc$_n$) exhibited behavior similar to 2-AP-GlcNAc$_n$. Plots of log μ *vs.* the number of GlcNAc residues of the homologous series labelled wth 6-AQ, were also straight lines, and their slopes were identical to those

obtained with 2-AP-GlcNAc$_n$ under otherwise identical conditions. This is to say that the δ_{GlcNAc} is the same regardless of the tagging functions. Note that both derivatizing agents have similar characteristic charges.

To study the effect of Bu_4N^+ on the electrophoretic behavior of derivatized N-acetylchitooligosaccharides, 2-AP-GlcNAc$_n$ were analyzed in the presence of various amounts of the organic salt in 0.1 *M* phosphate solutions, pH 5.0. Upon adding the salt to the running electrolyte, the migration time of the homologous series increased monotonically with increasing the salt concentration in the range studied. The plots of log μ *versus* the number of GlcNAc residues at different concentrations of Bu_4N^+ in the running electrolyte were also linear. Their slopes represent δ_{GlcNAc}, and are summarized in Table I. The increase in the values of δ_{GlcNAc} with increasing Bu_4N^+ concentration reflects an increase in the resolution between the homologous series. It can be seen in Table I that the addition of only 10 m*M* of the organic salt to the running electrolyte was enough to yield an absolute increase of about 28% in the value of δ_{GlcNAc}, while 50 m*M* Bu_4N^+ exhibited only 48% increase in the absolute value of δ_{GlcNAc}.

To better assess the effect of the organic salt, the electroosmotic flow was measured at each salt concentration using methanol as the inert tracer. Typical results obtained with N-acetylchitohexaose are depicted in Fig. 3 by plots of the overall and electrophoretic mobilities of this sugar as well as the electroosmotic flow *versus* the concentration of the organic salt in the running electrolyte. As shown in Fig. 3, the decrease in the net mobility of the various homologues could not be only accounted for by the slight decrease in the electroosmotic flow at higher salt concentration, but due to the continuous decrease in the electrophoretic mobilities of the 2-AP-GlcNAc$_n$ with increasing the ionic strength of the running buffer [17–19].

Comparison of the derivatizing agents. Although the derivatization with 2-AP is quite reproducible and allows the electrophoresis of carbohydrates by CZE, the 2-aminopyridyl derivatives of mono- and oligosaccharides exhibited limited detection sensitivity in the UV. In search for a more sensitive tagging agent, the performance of 6-AQ, a UV-absorbing and fluorescent tag, was investigated in this study and compared to that of 2-AP using an

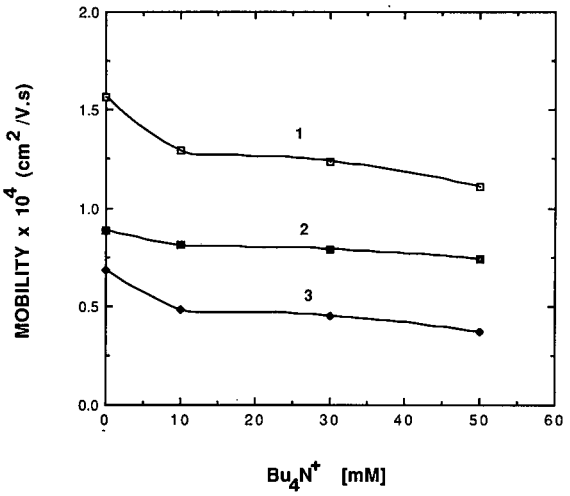


Fig. 3. Plots of overall and electrophoretic mobilities of the 2-aminopyridyl derivative of N-acetylchitohexaose as well as electroosmotic flow at various concentrations of tetrabutylammonium bromide in the running electrolyte, pH 5.0. Curves: 1 = overall; 2 = electroosmotic; 3 = electrophoretic. Other conditions as in Fig. 1.

N-acetylchitooligosaccharides mixture ranging in size from N-acetylglucosamine to N-acetylchitohexaose as model sugar substrates.

Returning to Fig. 1A and B, the 6-aminoquinolyl derivatives of the oligosaccharides (6-AQ-GlcNAc$_n$) exhibited slighty higher migration times than that of 2-AP-GlcNAc$_n$. This is attributed to the lower pK_a value of 6-AQ (pK_a = 5.64) as compared to 2-AP (pK_a = 6.71). Also, 6-AQ has a slightly higher molecular size. As can be noted from Fig. 1A and B, the homologues taggad with 6-AQ showed almost 2 to 3 times higher detection signal than those labelled with 2-AP as estimated from peak height and area calculations from six consecutive runs.

To further characterize the derivatization reactions under investigation, spectral analysis was carried out on both tagging agents and their sugar derivatives. The absorption spectrum of 2-AP (see Experimental) revealed two maxima at 229 and 290 nm with the most intense being at 229 nm, while the 6-AQ spectrum showed an absorption band with a maximum at 242 nm. Due to the difficulty of obtaining the derivatized oligosaccharides in a highly pure form, their absorbance were measured from the electropherograms obtained from various elec-

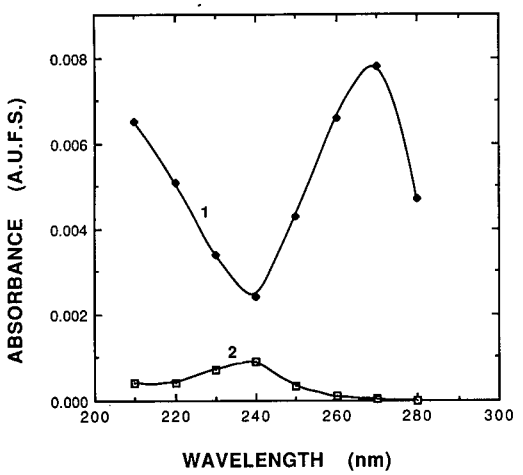


Fig. 4. Plots of absorbance of 6-AQ-GlcNAc$_3$ (1) and 2-AP-GlcNAc$_3$ (2) *versus* the detection wavelength. Conditions are as in Fig. 1, except electrolyte was 0.1 *M* sodium phosphate solution at pH 5.0.

trophoretic runs. Typical results obtained with 2-AP-GlcNAc$_3$ and 6-AQ-GlcNAc$_3$ are depicted in Fig. 4 by plots of the absorbance at maximum peak heights as a function of the detection wavelength in the range 210 to 280 nm in an increment of 10 nm. The λ_{max} for both the 2-aminopyridyl and 6-aminoquinolyl derivatives shifted toward higher wavelengths than the pure derivatizing agents. The λ_{max} of 2-AP-GlcNAc$_n$ is around 240 nm and that of 6-AQ-GlcNAc$_n$ is at *ca.* 270 nm. As can be seen in Fig. 4, under the same electrophoretic conditions and in the wavelength range investigated, the 6-aminoquinolyl derivatives exhibited higher absorbance. When compared at their λ_{max}, 6-AQ-GlcNAc$_n$ yielded a detection signal 8 times higher than that of the sugar tagged with 2-AP. The derivatization procedure described here may prove useful for fluorescence detection. In fact, 6-AQ possess ideal fluorescence properties [20] in the sense that its excitation wavelength (355 nm) is far removed from its emission wavelength (550 nm) and is therefore expected to give low detection limits with fluorescence detector.

CZE of 2-AP-xyloglucan oligosaccharides

Mapping. Xyloglucans are branched oligosaccharides having a backbone identical to that of cellulose [*i.e.*, the (1 → 4) β-linked D-glucan] with various side chains comprising xylose, galactosyl-xylose, fucosyl-galactosyl-xylose and seldom arabinosyl-xylose. When digested with cellulase, the backbone of xyloglucans are cleaved after any glucosyl residue with no side chain linked to it. Therefore, the fragments obtained are reflective of xyloglucan branching.

The 2-aminopyridyl derivatives of the xyloglucan fragments (2-AP-XG) obtained by cellulase digestion were first analyzed by reversed-phase chromatography as reported earlier [14]. The collected fractions the structures of which were assessed tentatively by liquid secondary-ion mass spectrometry and previously proven structures were then analyzed by CZE using the electrophoretic system described for the homologous series. Fig. 5 is a typical electropherogram of a mixture reconstituted by mixing aliquots from selected reversed-phase chromatography fractions. It was performed on a

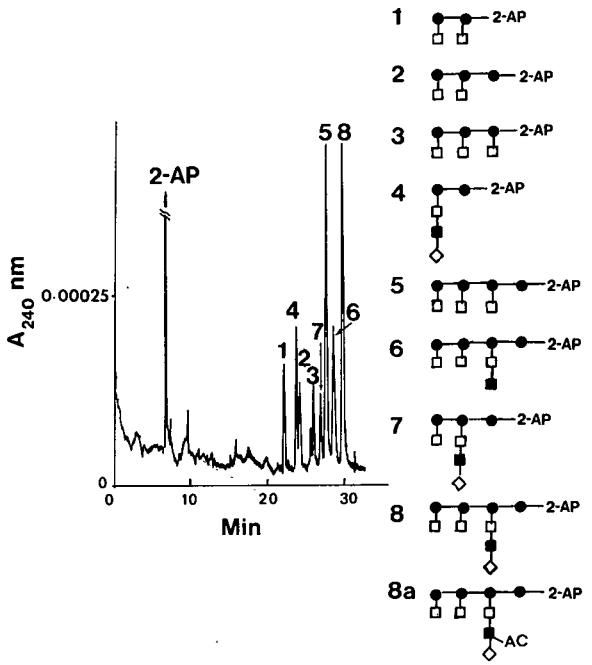


Fig. 5. Capillary zone electrophoresis mapping of the 2-aminopyridyl derivatives of xyloglucan oligosaccharides reconstituted by mixing aliquots from fractions collected during various chromatographic runs. Capillary, fused-silica tube with polyether interlocked coating on the inner walls, 50 cm (to the detection point), 80 cm total length × 50 μm I.D.; electrolyte, 0.1 M sodium phosphate solution containing 50 m*M* tetrabutylammonium bromide, pH 4.75; running voltage, 20 kV. Symbols: ● = glucose; □ = xylose; ■ = galactose; ◇ = fucose; AC = acetyl group.

fused-silica capillary with a polyether interlocked coating using 0.1 *M* phosphate containing 50 m*M* Bu_4N^+ at pH 4.75. As expected the elution pattern in CZE was different from that in reversed-phase chromatography. The peak numbering on the electropherogram (see Fig. 5) reflects the elution order obtained in reversed-phase chromatography. In CZE the elution order was mainly governed by the number of sugar residues and the degree of branching, whereas in reversed-phase chromatography the elution order was mainly influenced by the size of the oligosaccharide and the hydrophobic character of the sugar residues. For instance, fragment 4 which is smaller in size than fragment 3 was more retarded on the reversed-phase chromatography column. This may be attributed to the presence of fucosyl residue in structure 3, which is more hydrophobic than any other sugar residues in the molecule. The same reasoning can explain the elution order for fragments 6 and 7. In CZE, due to the fact that all 2-AP-XG fragments possess the same charge they migrated in the order of increasing size as the charge to mass ratio decreased. However, for the same number of residues but with slight differences in molecular weight the less branched oligosaccharides eluted earlier than the more branched one. In fact, structure 4, which has a slightly higher molecular weight than structure 2 eluted first. This may be explained by the fact that structure 2 is doubly branched as opposed to fragment 4 which is singly branched. The same behavior was observed for fragments 7 (doubly branched) and 5 (triply branched). Thus, as the extent of oligosaccharide branching increased, the electrophoretic mobility decreased.

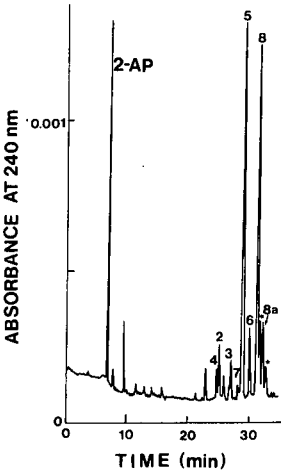


Fig. 6. Capillary zone electrophoresis mapping of 2-aminopyridyl derivatives of acetylated xyloglucan oligosaccharides from cotton cell walls. For fragment structures, see Fig. 5. Conditions as in Fig. 5.

Fig. 6 illustrates the high separation efficiency of acetylated xyloglucan oligosaccharides generated by a slightly different method [14] than that used to generate the fragments used in Fig. 5. The average plate count per meter was about 225 000 as calculated from the two major fragments 5 and 8. Both structures 8 and 8a are nonasaccharides with the same extent of branching. The only difference is that fragment 8a has an acetyl substitution at the galactosyl residue that is not present in structure 8. This once more shows the high resolving power of CZE in recognizing small differences between the various xyloglucan fragments. The peaks labelled with asterisks may be attributed to other oligosaccharides present in the mixture.

Mobility indices. To interpret the electrophoretic behavior of the various 2-AP-XG and to quantitatively describe the effects of the various sugar residues on the electrophoretic mobility, we have attempted the indexing of the electrophoretic mobilities of the branched xyloglucan oligosaccharides with respect to the linear 2-AP-$GlcNAc_n$. In this regard, the mobility indices, M.I., of the 2-AP-XG fragments were calculated using the following equation:

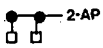
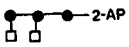
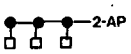
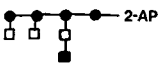
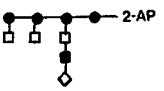
$$\text{M.I.} = 100n + 100\left(\frac{\log \mu_s - \log \mu_{n+1}}{\log \mu_n - \log \mu_{n+1}}\right)$$

where μ_s is the electrophoretic mobility of the 2-AP-XG solute, μ_n and μ_{n+1} are the electrophoretic mobilities for the two homologues with n and $n + 1$ repetitive units which eluted before and after the xyloglucan fragment, respectively. This indexing is similar to that of Kováts retention indices for gas–liquid chromatography [21]. Table II compiles the values of mobility indices of selected 2-AP-XG fragments with respect to the homologous series, 2-AP-$GlcNAc_n$. In this table the mobility indices, M.I. were averaged over the pH range 4.5 to 5.25 and for Bu_4N^+ concentrations from 0 to 50 m*M*. The difference in mobility indices between an adjacent pair of 2-AP-XG fragments, ΔM.I., is referred

TABLE II

MOBILITY INDICES (M.I.) AND GROUP MOBILITY INDEX DECREMENTS (ΔM.I.) OF VARIOUS 2-AP-XG FRAGMENTS WITH RESPECT TO 2-AP-$GlcNAc_n$

See text for calculations. Group I, average values obtained with 0.1 *M* sodium phosphate solutions, pH 5.0, at 0, 10, 30 or 50 m*M* tetrabutylammonium bromide; group II, average values obtained with 0.1 *M* sodium phosphate solutions containing 50 m*M* tetrabutylammonium bromide at pH 4.50, 4.75, 5.00 or 5.25; group III, overall average. Symbols: ● = glucose; □ = xylose; ■ = galactose; ◇ = fucose.

Xyloglucan	Group					
	I		II		III	
	M.I.	ΔM.I.	M.I.	ΔM.I.	M.I.	ΔM.I.
	267.5 ± 5.6	51.5 ± 4.0	277.0 ± 7.8	53.5 ± 4.0	272.3 ± 4.8	52.5 ± 2.8
	319.0 ± 9.0	57.1 ± 1.2	330.5 ± 10.9	53.7 ± 3.0	324.8 ± 7.1	55.4 ± 1.6
	376.1 ± 9.0	53.3 ± 4.7	384.2 ± 10.0	54.6 ± 5.2	380.2 ± 6.7	54.0 ± 3.5
	429.4 ± 14.0	37.4 ± 4.1	438.8 ± 16.0	39.5 ± 5.0	434.1 ± 10.6	38.4 ± 3.2
	466.8 ± 18.0	37.9 ± 4.0	478.3 ± 20.0	36.8 ± 5.6	472.5 ± 13.5	37.4 ± 3.4
	504.7 ± 20.0		515.1 ± 20.0		509.8 ± 14.1	

to as the group mobility index decrement contributed by a sugar residue added to a parent oligosaccharide molecule. This value was practically the same over the pH range or salt concentrations studied for a given pair of derivatized xyloglucan fragments. Based on these findings, the M.I.s were averaged as indicated in group III in Table II. As can be seen, the xyloglucan fragment 1 eluted between the homologues of DP 2 and 3, respectively. Fragment 2, which has an additional glucosyl residue in the backbone chain of the oligosaccharide, exhibited a mobility index decrement of 52 and eluted between the homologues of DP 3 and 4, respectively. The presence of a xylosyl residue in structure 3 showed a similar change in the mobility index decrement. Furthermore, the addition of a glucosyl unit to the linear core chain imparted an identical increment in the migration time and xyloglucan fragment 5 eluted between the homologues of DP 4 and 5. It is therefore wise to say that a glucosyl residue in the backbone of the xyloglucan behaves as one half a GlcNAc residue in terms of its contribution to the eletrophoretic mobility of the 2-AP-XG. The same can be stated about adding a xylosyl residue at the glucose loci. However, the addition of a galactosyl residue to an already branched xylosyl residue exhibited less retardation (almost 70%; see mobility index decrements in Table II) than the addition of a glucosyl or xylosyl unit to the backbone of the xyloglucan oligosaccharide. The same observation can be made about adding a fucosyl residue to a branched galactosyl residue. Thus, as the molecule becomes more branched, the addition of a sugar residue does impart a slightly less decrease in its mobility. Such indexing may prove valuable in correlating and predicting the mobilities of oligosaccharides.

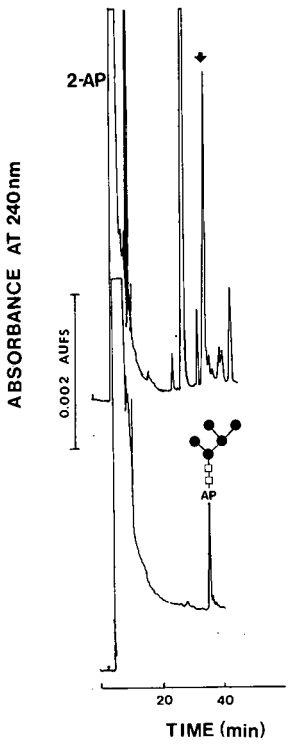


Fig. 7. Capillary zone electrophoresis mapping of 2-aminopyridyl derivatives of high mannose oligosaccharides cleaved from bovine ribonuclease B (top electropherogram), and of 2-AP-$(GlcNAc)_2$-Man_5 standard (lower electropherogram). Symbols: □ = GlcNAc; ● = mannose. Conditions as in Fig. 1.

CZE mapping of oligosaccharide chains from ribonuclease B

To further illustrate the resolving power of the electrophoretic system under investigation, high-mannose glycans were analyzed by CZE. In this regard, oligosaccharides from bovine RNase B were cleaved from the tryptic digest of the protein using peptide-N-glycosidase F, an endoglycosidase that cleaves all types of N-linked oligosaccharide chains between the asparagine and the carbohydrate units. The CZE mapping of the 2-aminopyridyl derivatives of these oligosaccharides is portrayed in Fig. 7. It was performed on a capillary with a polyether interlocked coating using 0.1 *M* phosphate solution, pH 5.0, containing 50 m*M* tetrabutylammonium bromide as the running electrolyte. As can be seen in Fig. 7, the peak labelled with an arrow was identified as $(GlcNAc)_2$-Man_5 using an oligosaccharide standard. In fact, ribonuclease B is known as a source of high mannose oligosaccharides with only one glycosylation site. This results in heterogeneous populations of structurally related oligosaccharides which renders RNase B, as most other glycoproteins, highly microheterogeneous. This may expain the presence of several peaks in the CZE map besides that of $(GlcNAc)_2$-Man_5 oligosaccharide which, according to Liang *et al.* [22], is the most predominant carbohydrate moiety of bovine ribonuclease B.

ACKNOWLEDGEMENTS

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Capillary gel electrophoresis of single-stranded DNA fragments with UV detection*

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ABSTRACT

Capillary gel electrophoresis has proven to be a powerful tool in biomedical research. We report our investigation of some of the critical parameters affecting separations of single-stranded DNA fragments as monitored by ultraviolet (UV) absorbance detection. Although not as sensitive as laser-induced fluorescence (LIF), UV absorbance detection allows one to calculate quite accurately, and inexpensively, the molarity of each separated DNA fragment and, moreover, the signal "fading" effect normally observed with LIF detection can be, in many cases, substituted for fluorescence to detect the many different single-stranded DNAs, as well as for detection of sequencing reactions.

INTRODUCTION

Capillary gel electrophoresis (CGE), a new analytical and micropreparative technique, is being rapidly developed [1–6]. Included among the advantages of CGE are ultraresolution, extremely high efficiency, rapid separation time, easy quantitation and amenability to automation. Many interesting applications in the biomedical field are just now emerging. One of the newly attractive applications of CGE is separation and identification of DNA sequencing mixtures, where the fragments have one common 5′-endpoint and differ in successive length by a single nucleotide. Because of the very small sample band volumes (a few to tens of nl) and extremely low molar concentrations of separated DNA bands, a highly sensitive detection scheme is required. Laser-induced fluorescence (LIF) detection is, thus far, one of the most sensitive methods [7–13], that is applicable to molecules having at least one fluorescent label attached. However, the exploration of alternative detection schemes in the separation and identification of DNA sequencing samples is also interesting. We herein demonstrate that CGE coupled downstream with UV absorbance detection is likewise a very sensitive, accurate, and relatively inexpensive scheme for the separation and detection of single-stranded DNAs consisting of homopolymeric deoxyadenosine, deoxythymidine and dideoxythymidine terminated sequencing reaction derived from the single-stranded becteriophage, M13.

EXPERIMENTAL

Polyacrylamide gel-filled capillaries were prepared according to the procedure described by Karger and co-workers [1,2]. Fused-silica capillary (Polymicro

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0021-9673/92/$05.00

Technologies, Phoenix, AZ, USA), 150 μm I.D. × 360 μm O.D., with a detection window at 30 to 60 cm from the inlet were used in most of the experiments. A 30-kV direct current power supply (Model MJ30N400; Glassman, Whitehouse Station, NJ, USA) was used to generate the electrical field across the capillary gel column. A UV absorbance detector (Model 757; Applied Biosystems, Ramsey, NJ, USA) with a capillary holder was used as an on-column detector, generally set at 260 nm. The UV absorbance output was monitored with a dual-channel chart recorder (Model BD41; Kipp & Zonen, Netherlands) and also acquired and stored on an IBM PC/AT computer using an analog/digital interface (Model 970; Nelson Analytical, Cupertino, CA, USA).

Tris(hydroxymethyl)aminomethane (Tris), boric acid, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), ammonium persulphate (APS) and urea were purchased from Fluka (Buchs, Switzerland). N,N,N′,N′-Tetramethylenediamine (TEMED) was supplied by Merck (Darmstadt, Germany). Methacryloxypropyltrimethoxysilane was supplied by Sigma (St. Louis, MO, USA). Premixed acrylamide–N,N′-methylenebisacrylamide (29:1) was purchased from Schwarz/Mann Biotech (Cleveland, OH, USA).

Polydeoxyadenylic acids [poly-d(A)] were purchased from Boehringer Mannheim (Indianapolis, IN, USA). The sample containing the oligodeoxythymidilic acids poly-d(T) 20–160 was synthesized at Genentech (South San Francisco, CA, USA). Electrophoresis buffer solutions were filtered through a Supelco (Gland, CH, USA) nylon 66 filter unit with a pore size of 0.25 μm before use.

Single-stranded M13 mp18 (Pharmacia, Piscataway, NJ, USA) was annealed with a universal sequencing primer (−40; New England Biolabs, Beverly, MA, USA), extended with deoxynucleotides (dNTPs) using T7 polymerase (US Biochemicals Corp., Cleveland, OH, USA) or Bst polymerase (Bio-Rad, Richmond, CA, USA) and terminated with dideoxythymidine. The molar ratio of dTTP to dideoxythymidine (ddTTP) was generally 20:1. Reactions were terminated by addition of one-tenth volume of 0.5 *M* EDTA, followed by phenol–chloroform extraction, ethanol precipitation, and lyophilization. Samples were resuspended in deionized formamide, 2 m*M* EDTA, and heated to >85°C for 3–5 min immediately prior to column application.

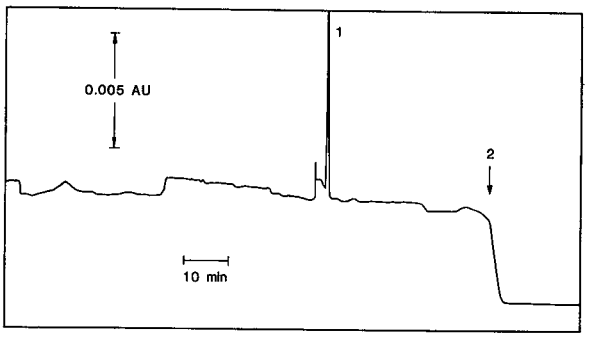

Fig. 1. A typical background UV absorbance trace recorded during pre-electrophoresis of a fresh gel-filled column: 1 = unidentified peak; 2 = sharp drop in UV absorbance. Capillary is 150 μm I.D.; 75 cm total length, 50 cm to detector. Gel composition is 4%T, 3.3%C. Electrolyte: TBE, 7 *M* urea; running voltage: 6 kV; UV absorbance at 260 nm.

RESULTS AND DISCUSSION

Pre-electrophoresis of gel-filled column

All columns were pre-run electrophoretically before any injection of DNA samples. Two phenomena were observed during pre-electrophoresis. First, at constant voltage the current steadily decreased until after a period of time (about 2 h under our experimental conditions), it reached a stable level. Secondly, the baseline UV absorbance is initially stable and then decreases sharply and finally stabilizes at a new lower level. These observations suggest that a mixture of ions from the polymerization, at least one of which is UV absorbing (acrylate?) are being eluted from the column. These small ionic substances occur along the entire length of a fresh gel-filled column. During pre-electrophoresis, they migrate toward the anode. With continuous depletion, the conductivity of the entire gel-filled column decreases resulting in the decrease of the current. When the plug of ions has finally migrated past the detector, a sharp drop in UV absorbance is observed. Fig. 1 gives a typical UV absorbance trace of a pre-run. For the gel-filled capillary with 150 μm I.D., 4%T and 3.3%C[a], 50 cm to detector at electrical field strength of 80 V/cm, the mean (n = 4)

[a] C = g N,N′-methylenebisacrylamide (Bis) /%T; T = (g acrylamide + g Bis)/100 ml solution.

migration rate was $1.0 \cdot 10^{-4}$ cm^2/V s. The mean (n = 4) background UV absorbance change at 260 nm was 4.5 mAU for the capillary with 150 μm I.D. (calibrated light path is 120 μm) or equivalent to 0.38 AU for 1 cm light path. Due to this large change in background of UV absorbance, the pre-run procedure is necessary.

Concentrating the sample during introduction in CGE

In CGE, the dimensions of the electrophoretic channel are very small, and it is difficult to introduce sample into the inlet of the capillary gel by a mechanical method. Instead, the electrokinetic injection method is usually employed; the sample ions are migrated into the column under the influence of an electrical field. A sample concentrating phenomenon occurs during electrokinetic injection when DNA fragments are prepared in electrolytes with low ionic strength. Fig. 2 demonstrates this "concentrating" effect during electrokinetic injection. A poly-d(T) sample is prepared in either deionized water or in 90 m*M* Tris–borate, 2.5 m*M* EDTA (TBE) running buffer, and then electrokinetically loaded at 15 kV for 5 s. In Table I, the UV absorbance of five consecutive peaks in the calibrated light path of the capillary is used to quantitate the extent of the concentration. It shows about 22 times more of the sample is injected when DNA is prepared in water than when it is prepared in 90 m*M* running buffer. This phenomenon can be explained as follows. At a constant injection voltage, the current passing the gel column is determined predominantly by the huge resistance of the gel column (*ca.* 500 MΩ in our experiments). Anions in sample solution have to migrate into the inlet of the gel column to maintain the current level. When the DNA sample is prepared in running electrolyte, there are relatively many borate anions in solution. During electrokinetical injection, the amount of DNA molecules that get into the column depends on the mole ratio and mobility ratio of DNA anions to all other anions. Since the DNA is of very low concentration compared to 90 m*M* borate, only a small portion of DNA can get into column during injection. When the DNA sample is prepared in pure water, the amount of other anions is much lower (depends on the desalting process used for the DNA sample). Therefore a much higher portion of DNA can get into the column. The advantage of low ionic strength load solution is that it can reduce the difference between sample volume and sample injected volume. In other words, it improves the detection since the concentration in the sample band is increased.

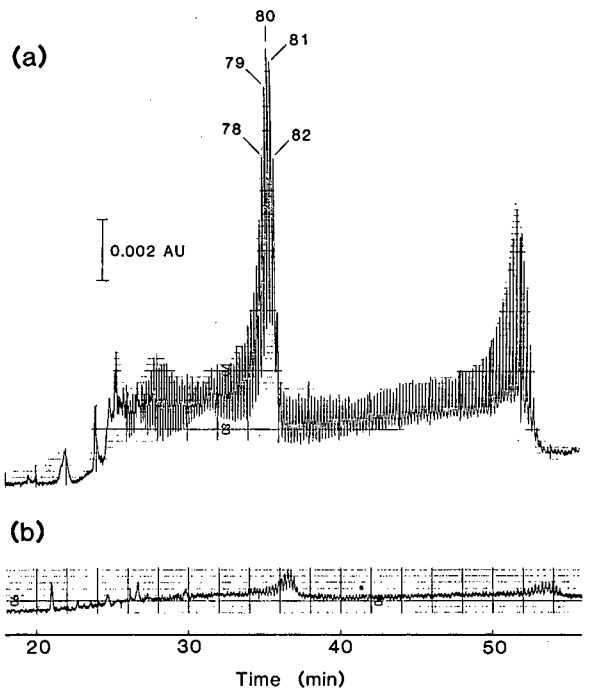

Fig. 2. Comparison of amount of sample injected between sample prepared (a) in water and (b) in running electrolyte; 200 V/cm (32 μA), Sample: synthetic poly-d(T) 20–160. Injection conditions: 15 kV, 5 s, 1 μl sample volume. Other conditions as in Fig. 1.

TABLE I

RELATIVE PEAK HEIGHT RATIOS FOR SAMPLES PREPARED IN WATER AND IN RUNNING BUFFER

Peak order	Absorbance (mAU)		Ratio
	in water	in buffer	
78	5.3	0.25	21.2
79	6.6	0.31	21.3
80	7.5	0.36	21.2
81	7.3	0.34	21.5
82	5.7	0.25	22.8
Mean			21.6

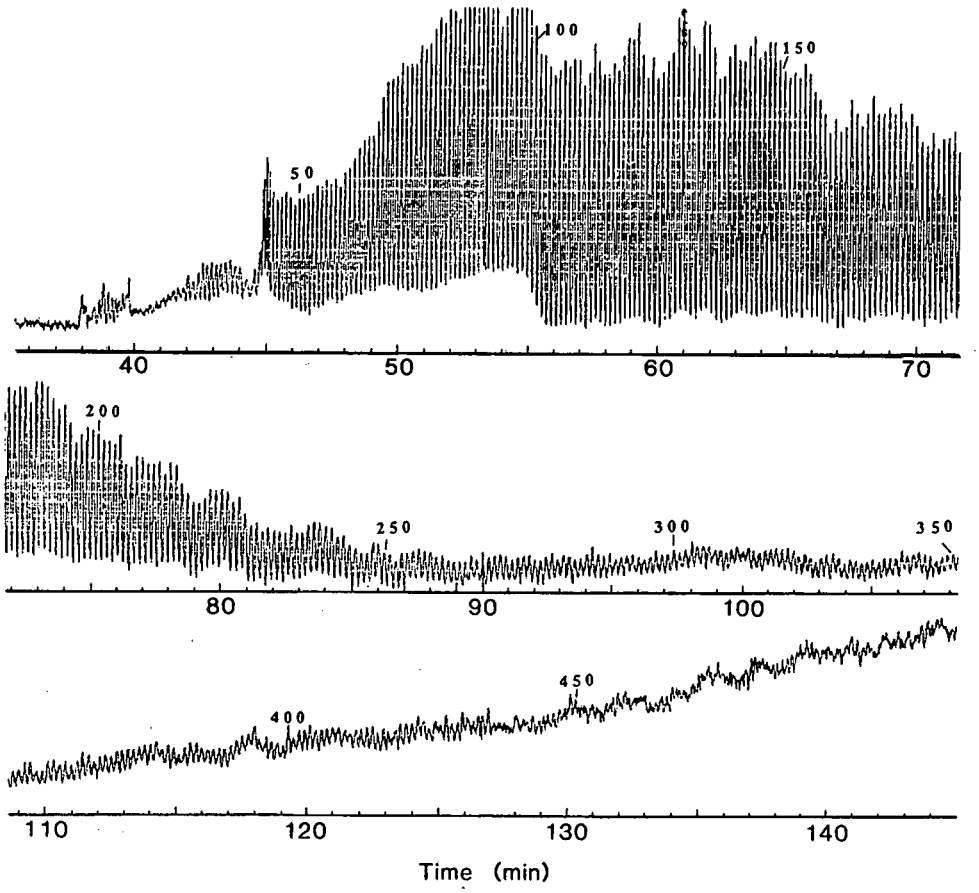

Fig. 3. Electropherogram of poly-d(A) sample. Capillary is 100 cm total length, 70 cm to detector. Gel composition is 3%T, 3.3%C; injection conditions: 6 kV, 3 s, 3 μl sample volume. Running voltage: 15 kV. Other conditions as in Fig. 1.

The migration behavior of single-stranded DNA

A difference between slab gel and CGE is that in CGE with on-column detection, all of the components injected into the column migrate the same distance before they are detected and recorded. In a slab gel autoradiography, during the same time period, the small fragments migrate further than the large ones. Thus, the pattern of migration rate of peaks in these two methods may not be same. One of the advantages of CGE with on-column detection is that it provides faster and more accurate electropherograms. Fig. 3 is a typical electropherogram of poly-d(A) sample. In order to determine the order of peaks, a slab gel purified known poly-d(A) was spiked as an internal DNA length marker. Since this marker is well defined, it is helpful in determining the numbers of bases for each peak. The migration time for the individual peaks is plotted in Fig. 4a and shows the relationship between poly-d(A) base number and migration time. It is almost a perfect linear relationship. Based on migration time, detection distance and electrical field strength, the migration rate can also be calculated. Fig. 4b shows the relationship between poly-d(A) base number and the migration rate. The curve fits well with a second-order polynomial relationship ($r = 0.998$).

A very interesting problem on the separation of single-stranded DNA is how to achieve good resolution between the nth fragment and the $(n + 1)$th fragment of a DNA polymer. Since poly-d(A) and poly-d(T) have no severe secondary structure problem, we thought it might be a useful tool for

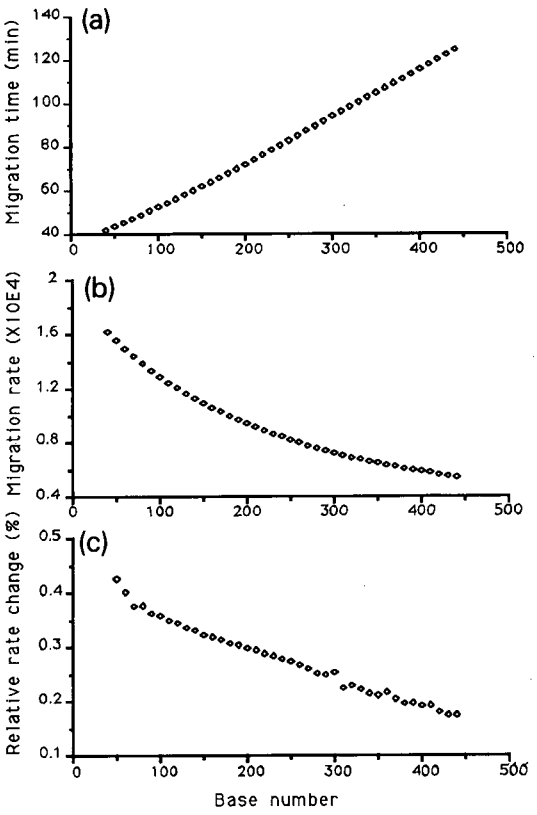

Fig. 4. (a) Plot of migration time *vs.* base number. (b) Plot of migration rate (in cm^2/V s) *vs.* base number. (c) Plot of relative migration rate change (R_c) *vs.* base number.

evaluating the capillary gel column. The most challenging problem is to separate high-molecular-weight single-stranded DNA. Fig. 4c shows the relationship between relative migration rate change and DNA base number. The relative migration rate change, R_c, is calculated using the equation:

$$R_c = \frac{\mu_{10n} - \mu_{10(n+1)}}{\frac{1}{2}(\mu_{10(n+1)} + \mu_{10n})}$$

Here, μ is migration rate of DNA fragments and n is an integer between 4 and 44. It is cleat, that with an increase in DNA size, the relative migration rate change is decreased. Since the resolution of the DNA depends upon the relative migration rate change, the resolution for large DNA molecules become increasingly difficult.

UV absorbance detection of M13 sequencing products terminating in ddT

CGE with UV detection can be used to separate DNA sequencing samples. Since the molar concentration of DNA fragments in sequencing reactions is very small, it is difficult to obtain a good signal to noise ratio for the DNA fragments. To address this problem, several measures were taken. Firstly, a more "concentrated" sequencing reaction was prepared. Secondly, a thorough desalting of the sample in 70% ethanol was performed to enhance the injection process. Thirdly, an injection using higher current for an increased period of time was attempted to ensure improved transfer of the DNA sample into the gel-filled column. Finally, a larger capillary (150 μm) was utilized to obtain longer optical path. Fig. 5 shows an electropherogram of a typical M13 T track. It is remarkable that using CGE with UV absorbance detection, peak heights do not decrease as much with increasing molecular weight as they do with LIF detection. In other words, the signal "fading" phenomenon is not significant in UV absorbance detection. In UV absorbance detection, with an increase in the length of the DNA molecule, the molar absorbance is also increased. This compensates to some degree the decrease of molar concentration with long DNA fragments. Since UV absorbance detection gives concentration information, this method can be employed to calculate the molar concentration of individual DNA fragments in each band. Table II shows the calculated DNA concentration and mass quantity of different DNA fragments in some selected bands. The data in the table give an estimation of the amount of DNA

TABLE II

CALCULATED MOLAR CONCENTRATION AND MASS QUANTITY OF THE SINGLE-FRAGMENT BANDS OF M13 T TRACK SEQUENCING PRODUCT

Base number	DNA concentration (n*M*)	Mass of DNA (fmol)
102	5.1	0.29
149	5.2	0.29
252	2.7	0.15
311	1.2	0.07
344	1.7	0.10
399	1.5	0.08

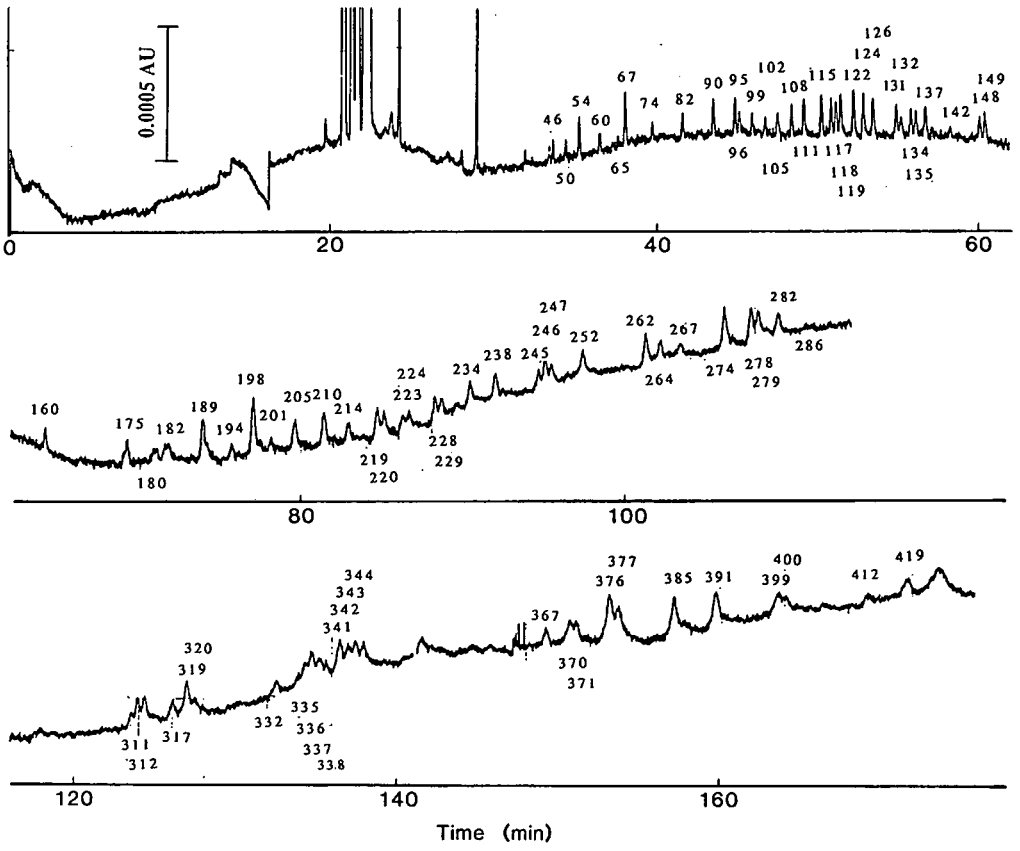

Fig. 5. Electropherogram of M13 T track sequencing product. Capillary is 85 cm total length, 50 cm to detector. Injection conditions: 12 kV, 10 s, 4 μl sample volume. Running voltage: 12 kV. Running temperature: 60°C. Other conditions as in Fig. 1.

sequencing sample injected under our experiment conditions. The mass quantity of the DNA fragments in each band ranges from 0.08 fmol of T399 mer to 0.44 fmol of T149 mer. The low detection limits obtained are quite impressive showing that, using a commercially available UV detector, it is possible to detect single-stranded DNA fragments at the sub-femtomole level.

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Isoelectric focusing field-flow fractionation

III. Investigation of the influence of different experimental parameters on focusing of cytochrome *c* in the trapezoidal cross-section channel

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ABSTRACT

In addition to the electric field and pH gradient used in isoelectric focusing, a recently introduced technique, isoelectric focusing (or electrical hyperlayer) field-flow fractionation, employs the flow of the liquid carrier through a thin separation channel as a third factor affecting separation. Focusing of cytochrome *c* (CYTC) in a trapezoidal cross-section channel of 0.875 ml volume and 25 cm length was investigated as a function of the injection procedure, relaxation time, flow-rate of the carrier ampholyte solution and applied electric power. The influence of different initial conditions was also investigated by computer simulation. Both computed and experimental data showed an important contribution of the injection procedure and relaxation time on the retention and shape of the CYTC zone. It follows from these data that the sample should be injected as a narrow zone into the centre of the stream rather than homogeneously together with the carrier solution. For the described experimental set-up it could be demonstrated that the time necessary for zone formation should be at least 15 min and that relaxation times in excess to 20 min do not influence the final shape of the CYTC zone. It could further be shown experimentally that the sample must be injected under an applied electric field, that the relaxation time should be about 10 min, that the elution flow-rate should not be larger than 100 μl/min, that focusing becomes more efficient with increasing electric fields and that, for a given assembly and specified flow conditions, there is an electric power window only within which proper operation is possible.

INTRODUCTION

Isoelectric focusing field-flow fractionation (IEF_4) is a technique for the separation of ampholytes using the combination of an electric field, a pH gradient and a flow velocity profile whose shape is determined by the geometry of the separation channel [1–3]. Amphoteric solutes are transported via isoelectric focusing to the equilibrium positions, where these compounds possess no net overall charge, and narrow focused solute zones with nearly Gaussian concentration distributions are formed. Provided that solutes exhibit different isoelectric points, they focus in different positions across the separation channel. Unequal flow velocities (Fig. 1A) cause differential migration of focused solutes along the channel, *i.e.*, their longitudinal separation.

IEF_4 was experimentally introduced by Chmelík *et al.* [2] in the trapezoidal cross-section channel and

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by Thormann *et al.* [3] in the rectangular cross-section channel. The latter group named this technique electrical hyperlayer field flow fractionation following the terminology of Giddings [4]. The formation of the pH gradient [5] and IEF_4 of a low-molecular-mass compound (methyl red) in the trapezoidal cross-section channel [6] have also been studied. This work was concerned with IEF_4 of a high-molecular-mass amphoteric compound, cytochrome *c* (CYTC), in the trapezoidal cross-section channel. The influence of different experimental parameters, including sample injection, relaxation, elution flow-rate and power, on the retention and zone width of CYTC is reported. The focusing process for two sample injection procedures was investigated both experimentally and by computer simulation using a mathematical model developed for electrophoretic processes [7,8].

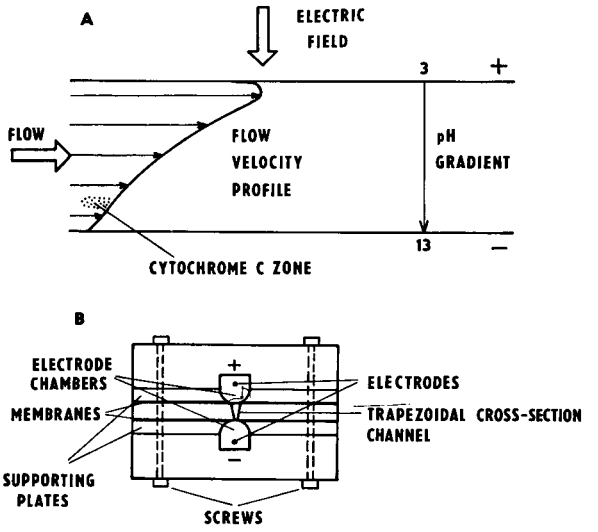

Fig. 1. Schematic representation of (A) IEF_4 in the trapezoidal cross-section channel and (B) cross-section of the IEF_4 channel assembly.

EXPERIMENTAL

Chemicals

All chemicals were of analytical-reagent grade. Cytochrome *c* from horse heart (M_r = 12 384, p*I* = 9.3) was obtained from Sigma (St. Louis, MO, USA) and Ampholine (pH 3.5–10) from Pharmacia–LKB (Bromma, Sweden).

Instrumentation and experimental conditions

The scheme of the experimental IEF_4 arrangement with the trapezoidal cross-section channel has been published elsewhere [6]. A schematic representation of the flow velocity profile and a cross-sectional view of the IEF_4 cell assembly are shown in Fig. 1A and B, respectively. The length of the channel was 25 cm, the height was 0.5 cm and the lengths of the two opposite walls of the trapezoid were 0.45 and 0.95 mm (volume 0.875 ml). Two additional Perspex plates were used for the mounting of PLGC ultrafiltration membranes (Millipore, Bedford, MA, USA) and to avoid changes in the channel flow conditions. For trouble-free operation the channel was run with the electrode compartments sideways, *i.e.*, turned 90°C with respect to Fig. 1B. A two-channel peristaltic pump (Vario Perpex, H. J. Guldener, Zürich, Switzerland) was used to pump 0.1 *M* acetic acid and 0.1 *M* sodium hydroxide solution through the electrode chambers, each at a flow-rate of 250 μl/min. A Model 2150 high-performance liquid chromatographic (HPLC) pump (LKB, Bromma, Sweden) was employed to pump 0.5% (w/v) carrier ampholyte solution through the IEF_4 channel at a flow-rate in the range 10–200 μl/min. A Model 355 syringe pump (Sage Instruments, Cambridge, MA, USA) was used to introduce the sample, $5 \cdot 10^{-5}$ *M* CYTC in 0.5% Ampholine solution.

Employing a four-port valve with a 5-μl sample loop, two ways of sample introduction were studied (Fig. 2). In one, sample was slowly admitted through a capillary situated 2 cm downstream from the carrier inlet (Fig. 2B). In the other experimental arrangement (Fig. 2A), the sample was introduced through the carrier stream. In this instance the four-port valve was located between the HPLC pump and the carrier inlet capillary. In all instances the carrier flow-rate during injection was 10 μl/min. Eluting zones were monitored with a Model 2158 Uvicord SD photometric detector (LKB) at 405 or 280 nm and a Model 2210 recorder (LKB). A Model 2297 Macrodrive 5 power supply (LKB) was used to apply up to 20 V (the maximum current was *ca.* 150 mA). In most instances the total electric power was limited to 1 W to avoid deleterious electrohydrodynamic effects [9].

Concentrations of ampholyte, sample and electrode solutions were chosen on the basis of previous

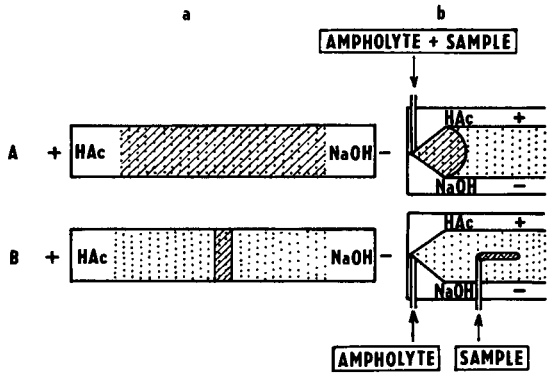

Fig. 2. Comparison of two sample introduction schemes for IEF_4. (A) Homogeneous sample distribution after injection of the sample together with the ampholyte solution through the carrier inlet. (B) Pulse sample distribution after injection of the sample through the capillary located 2 cm downstream of the carrier inlet. Initial distribution of compounds used for computer simulation and experiments are shown in (a) and (b), respectively. The dotted areas show the distribution of carrier ampholytes and the hatched areas depict the distribution of CYTC.

measurements [5,6]. Unless stated otherwise, the sample (5 μl of $5 \cdot 10^{-5}$ *M* CYTC) was injected over a period of 4 min with power applied and a carrier flow-rate of 10 μl/min. Relaxation, *i.e.*, the formation of a focused zone under this reduced flow-rate, occurred either within the flowing stream or, for relaxation times longer than 10 min, under stop-flow conditions. The zones were then eluted from the channel at a flow-rate of 40, 100 or 200 μl/min. The carrier ampholyte and sample solutions were degassed by vacuum and filtered through 0.2-μm Nalgene (25-mm diameter) disposable syringe filters (Nalge, Rochester, NY, USA).

Computer simulation

For simulation, the PC-adapted software package of the transient electrophoretic model developed by Bier *et al.* [7] was employed. This model is one-dimensional and isothermal and assumes the absence of fluid flows. For this reason, simulation does not describe separation in IEF_4 but focusing at zero or very low flow-rates without elution. Briefly, the model is capable of treating biprotic ampholytes, weak and strong monovalent acids and bases, peptides and proteins. Component fluxes result from electromigration and diffusion. It predicts the concentration distribution of individual components, pH and conductivity profiles as a function of time. The input required includes pK and mobility values of each component, the length of the separation space and its segmentation, the electrophoresis time, the current density, the initial distribution of each component and a table of net charge *versus* pH and also the diffusion coefficient for each protein. The permeabilities of the ends of the separation space are also specified. Simulations were performed on a Mandax AT 286 personal computer.

Ten biprotic carrier ampholytes were used to establish a pH gradient between acetic acid and sodium hydroxide. The pI values uniformly span the range 3–12 (ΔpI = 1). For each ampholyte, ΔpK was 2, the ionic mobility was $3 \cdot 10^{-8}$ m^2/V · s and the initial concentration was $2 \cdot 10^{-3}$ *M*. The concentrations of acetic acid (pK = 4.76) and sodium hydroxide solution were 0.1 *M* and the mobilities of acetate and sodium were $4.12 \cdot 10^{-8}$ and $5.19 \cdot 10^{-8}$ m^2/V · s, respectively. The diffusion coefficient for CYTC was taken as $1.33 \cdot 10^{-6}$ cm^2/s [10] and the net charge *vs.* pH table was constructed from a titration curve according to Theorell and Akesson [11] and under consideration of the charge of the iron ion (for details see ref. 12). All simulations were performed with a 1.5-cm separation space divided into 200 segments of equal length and with a constant current density of 10 A/m^2. The lengths of the focusing space and electrode compartments were 1 and 0.25 cm, respectively.

RESULTS AND DISCUSSION

An IEF_4 experiment consists of three important processes: sample injection, relaxation and elution. In order to compare the influences of different experimental conditions on retention, all fractograms are expressed in units of elution volume and not time. Comparison of fractograms of CYTC without and with an electric field applied and monitored at two detection wavelengths [280 nm (dashed lines) and 405 nm (solid lines)] is shown in Fig. 3. There is a clear difference between elution volumes of unretained (graphs 1 and 1′) and retained protein (graphs 2 and 2′). The observed difference agrees well with the theoretically expected situation because CYTC is focused in the narrower part of the channel where elution is slower (see Fig. 1A). It is

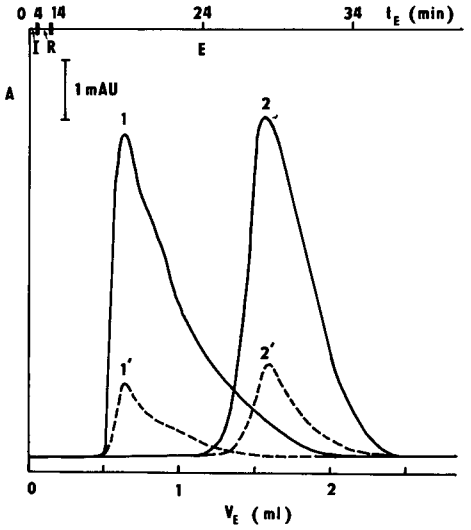

Fig. 3. Comparison of fractograms of CYTC without (1 and 1') and with (2 and 2') electric field applied and monitored at 280 nm (1' and 2') and 405 nm (1 and 2). Conditions: sample, 5 μl of $5 \cdot 10^{-5}$ *M* CYTC; carrier ampholyte solution, 0.5% (w/v) Ampholine (pH 3.5–10); electrode solutions, 0.1 *M* acetic acid (anode) and 0.1 *M* sodium hydroxide (cathode); applied voltage, 10 V; maximum current, 100 mA; relaxation time, 10 min; elution flow-rate, 100 μl/min. The time axis with the three stages, sample injection (I), relaxation (R) and elution (E), is shown at the top.

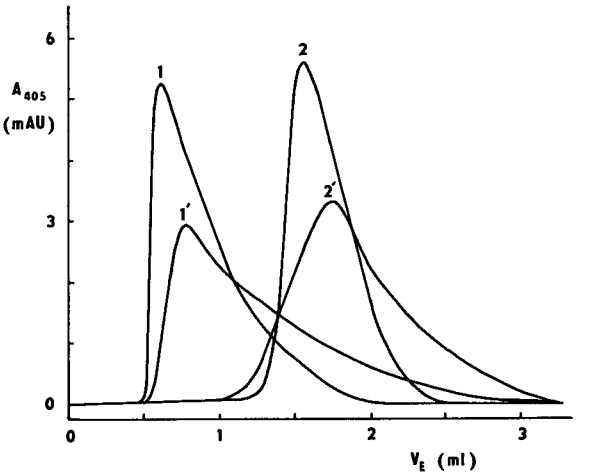

Fig. 4. Influence of different ways of sample injection on the shape of CYTC zone without (1 and 1') and with (2 and 2') electric field applied. Graphs 1 and 2 were obtained with pulse sample injection into the centre of the channel (Fig. 2B). Graphs 1' and 2' resulted from homogeneous sample injection according to Fig. 2A. Other conditions as in Fig. 3.

also evident that the detected signal is higher at 405 nm (curves 1 and 2) and, therefore, all other measurements were made at this wavelength.

Two experimental arrangements for sample injection were studied. In the first (Fig. 2A), the sample is introduced into the channel together with the ampholyte solution through the inlet capillary and its initial distribution in the inlet part of the channel is homogeneous. In the other (Fig. 2B), the sample is introduced through a capillary located 2 cm downstream from the carrier inlet. The sample is introduced as a narrow zone into the centre of the channel. The schemes of the initial distributions of components used for computer simulation and the experimental arrangements for both ways of sample injection are shown in panels a and b, respectively. As is depicted in Fig. 4, much narrower protein zones were found experimentally for the pulse sample injection both for unretained (graph 1) and retained (graph 2) CYTC compared with homogeneous sample injection (graphs 1' and 2'). In all instances the sample was focused for 10 min and then eluted at 100 μl/min.

Computer simulation data obtained with the input parameters listed under Experimental are presented in Figs. 5–7. Distributions of electrolytes and carrier ampholytes after 0, 10, 20 and 100 min of current application are shown in Fig. 5. At the beginning (A) the ampholytes are uniformly distributed throughout the focusing space, *i.e.*, the space between 0.25 and 1.25 cm of the column which is demarcated by the membranes (M). Comparison of the concentration profiles of the first two time points (A and B) reveals the progress of focusing of the ampholytes within 10 min of current flow, as well as a significant penetration of the sodium hydroxide (right-hand side) and acetic acid (left-hand side) into the focusing space. Further, some of the very acidic and very basic ampholytes are beginning to enter the electrode compartments. This is even more pronounced after 20 and 100 min of current flow (C and D, respectively), time points at which full separation of the ampholytes is predicted. These data clearly show that a relatively rapid separation phase is followed by a stabilizing phase which is characterized by a slow adjustement of the edge components (see refs. 13–15 for details).

The formation of the pH gradient (Fig. 6A) is predicted to be faster than the conductivity distribution (Fig. 6B). This is best seen by comparing the

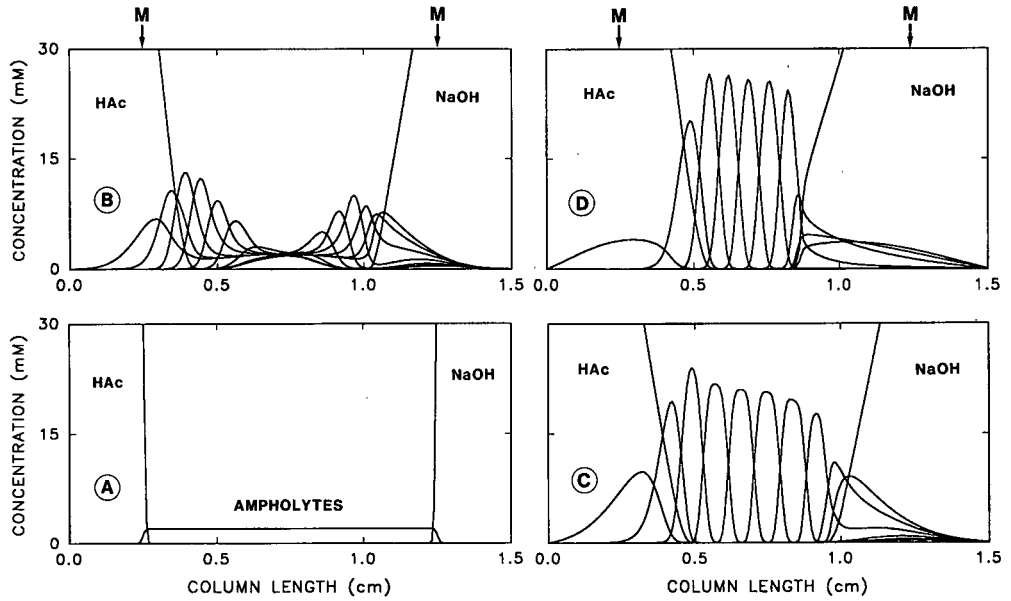

Fig. 5. Computer-simulated distribution of carrier ampholytes, anolyte (acetic acid, HAc) and catholyte (NaOH) after (A) 0, (B) 10, (C) 20 and (D) 100 min of current flow. The anode is to the left. M marks the locations of the mebranes which define the focusing space between 0.25 and 1.25 cm column length.

15-min profiles. In prolonged runs small changes can be seen especially in the neighbourhood of electrolyte chambers where the slow adjustments referred to above take place [13–15]. For the purpose of this study the most interesting results are shown in Fig. 7A and B. It is evident that focusing of a protein zone is predicted to be faster in the case of pulse sampling in the centre of the column (Fig. 7B)

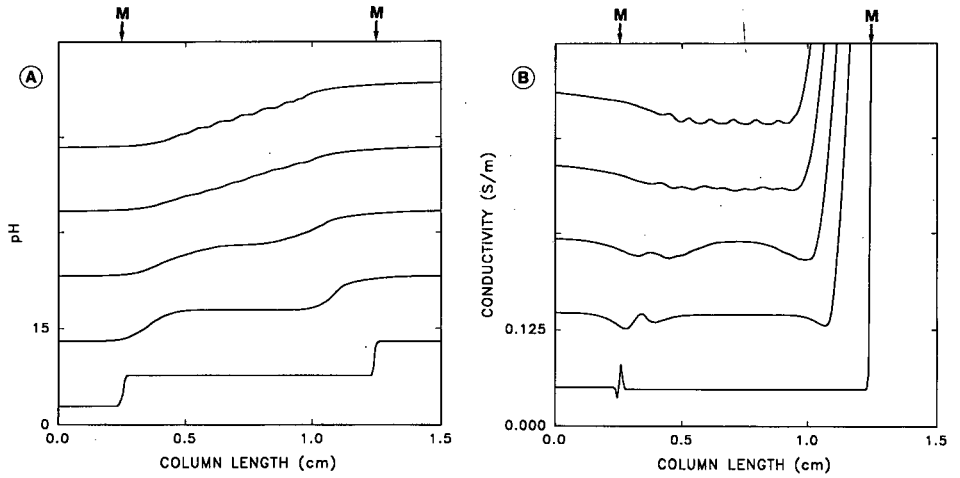

Fig. 6. Computer simulation of (A) pH gradient formation and (B) corresponding conductivity profiles after 0, 5, 10, 15 and 20 min of current flow (from bottom to top). Each successive time point is offset from the previous one by a constant amount for presentation purposes. Other conditions as in Fig. 5.

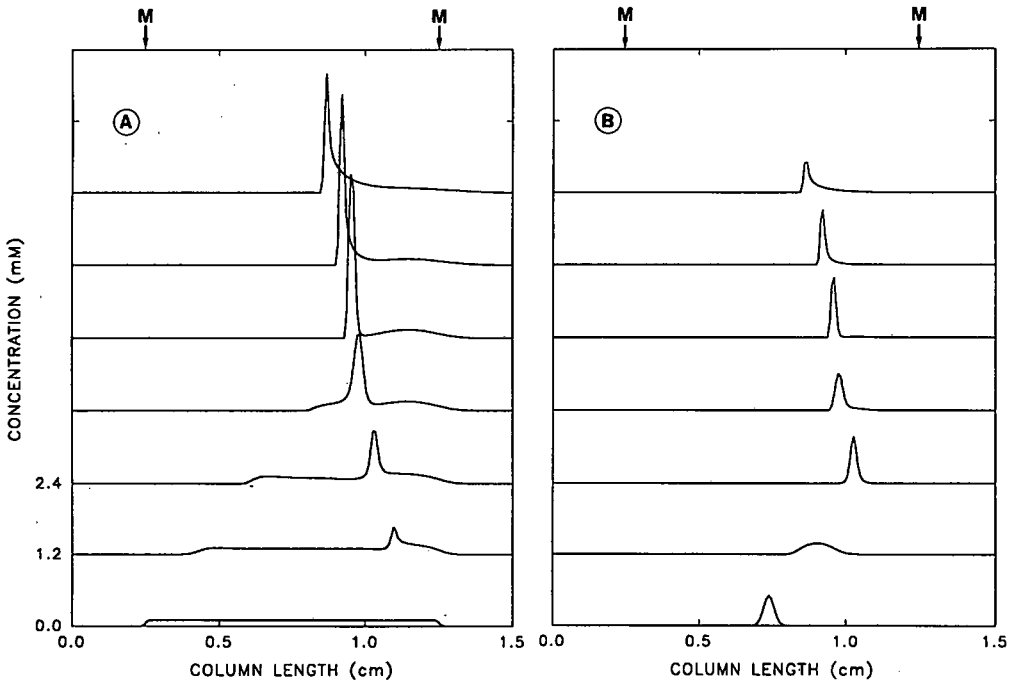

Fig. 7. Computer simulation of CYTC zone formation with (A) homogeneous sample injection (0.1 m*M*) and (B) pulse sample injection (0.5 m*M*). The initial protein distribution (bottom profiles) and offset time points after 5, 10, 15, 20, 50 and 100 min of current application are shown. Other conditions as in Fig. 5.

compared with an initial homogeneous distribution (Fig. 7A). Moreover, the transient protein zone is narrower in the former instance. Therefore, CYTC should disperse less in IEF_4 with pulse sample injection, which is exactly what is seen experimentally (Fig. 4). Another interesting finding of computer simulation is the fact that no complete steady-state distribution of CYTC is reached during 100 min of current flow. CYTC is focusing in a region where slow zone adjustements occur. However, because of the eluting nature of IEF_4, the changes between 20 and 100 min are irrelevant. There are three important conclusions for IEF_4. First, pulse sample injection (Fig. 2B) is much more efficient than homogeneous sample injection (Fig. 2A). Therefore, all remaining experiments were done with the pulse sample injection. Second, the total run time (sum of injection, relaxation and elution time intervals, see Fig. 3) should be at least 15 min, and third, focusing time >20 min only marginally influence the CYTC zone width and thus have almost no influence on the spreading of this zone during elution.

The importance of application of the electric field during sample injection is shown with the fractograms presented in Fig. 8. After sample injection the zones were immediately (without any relaxation) eluted at a flow-rate of 100 μl/min and an applied voltage of 10 V. Graph 1 shows the fractogram of CYTC injected without an applied voltage. It is evident that this broad fractogram consists of frontal and rear parts corresponding to non-retained and retained protein, respectively. With application of the electric field during sample injection only the rear part (retained) was observed (graph 2). Sample injection in the presence of the electric field resulted in a much narrower zone compared with the case of sample injection in the absence of electric field. All remaining experiments were therefore done with sample injection under an applied voltage.

The deleterious effect of the absence of the electric field during sample injection was so evident in the above experiment because no relaxation time was used after sample injection. The sample was immediately eluted at a relatively high flow-rate of 100 μl/min. The influence of relaxation time on the

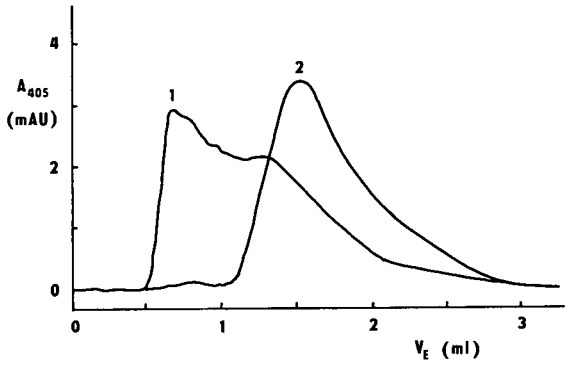

Fig. 8. Comparison of CYTC fractograms recorded without (1) and with (2) applied voltage of 10 V during sample injection. Other conditions as in Fig. 3.

elution volume of the peak maximum (V_E) and on the peak width (W_E) is shown in Fig. 9. Because of the asymmetric sample peak shape, the peak width was measured at one third of its height. It is necessary to take into account that the total focusing time during IEF_4 is the sum of the time periods of sample injection (in all instances presented this time is 4 min), relaxation (this time is variable) and elution (this time is related to the flow-rate used). It is evident from Fig. 9 that the elution volumes of the peak maxima (V_E) first increase and the peak widths decrease with higher relaxation time. The equilibrium values are reached for a relaxation time of about 10 min. This finding is in agreement with the computer simulation data.

The influence of the elution flow-rate on the shape of fractograms without and with applied voltage is shown in Fig. 10. It can be seen that the elution volumes of the peak maxima are barely influenced by different flow-rates but the peak widths increase and peak heights decrease with increasing flow-rates. The dependences of the elution times of the peak maxima and the peak widths on applied voltage and current are shown in Figs. 11A and B, respectively. The elution volumes of the peak maxima (V_E) were found to increase and the peak widths (W_E) to decrease before reaching equilibrium at a power level of about 1 W (20 V in A and 100 mA in B). Therefore, under the conditions employed and with a power <1 W, complete focusing cannot be reached until the end of the column and transient stages are monitored. For a power exceeding 1.5 W (data not shown) the peaks became broader, indicating the occurrence of appreciable convective disturbances probably caused by Joule heating and

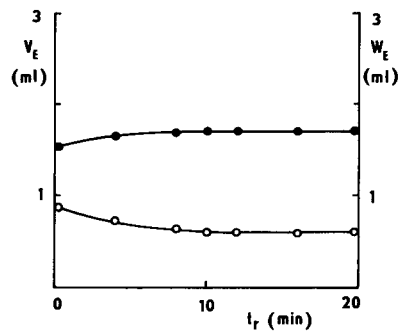

Fig. 9. Influence of relaxation time t_r, on the elution volume of the CYTC zone maximum, V_E (●), and the peak width at one third of its height, W_E (○). Other experimental conditions as in Fig. 3.

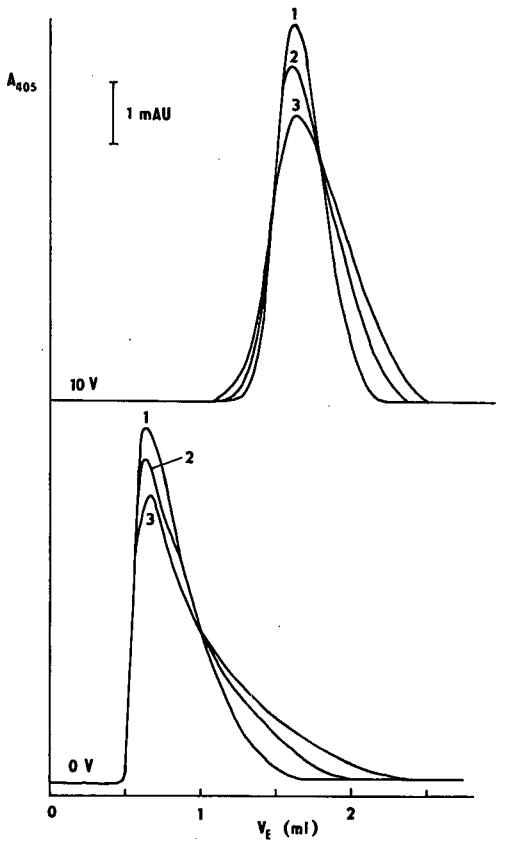

Fig. 10. Influence of the elution flow-rate on the shape of CYTC zone without (bottom) and with (top) applied power of about 1 W. Elution rates: 1 = 40; 2 = 100; 3 = 200 μl/min. Other conditions as in Fig. 3.

electrohydrodynamics. Thus, a given channel assembly with specied flow conditions should only be operated within a distinct electric power window.

CONCLUSIONS

IEF_4 of CYTC in a trapezoidal cross-sectional channel was investigated as a function of different experimental parameters, including sample injection, relaxation, elution flow-rate and applied electric power. Under the chosen conditions (pH gradient and polarity with respect to trapezoidal channel), CYTC focused in the narrower section of the channel where elution velocities are lower than in the wider part. Location of the CYTC zone is shown schematically in Fig. 1A. The observed elution volume (and of course retention time) of the retained CYTC zone was indeed found to be higher than that in absence of the electric field. Both computer simulation and experimental data showed that pulse sample injection is more efficient than homogeneous sample injection. For the described instrumental set-up it follows from the data presented that the time necessary for zone formation should be at least 15 min and that an increase in the relaxation time to >20 min does not influence the final shape of the CYTC zone. It could further be shown experimentally that the sample must be injected under an applied electric field, that the relaxation time should be about 10 min, that the elution flow-rate should not be larger than 100 μl/min and that focusing becomes more efficient with increasing electric field. The data also reveal that, under given flow conditions, the IEF_4 process should only be executed between 1 and 1.5 W, *i.e.*, within a specific power window. Investigations on the separation of proteins is the subject of another paper [12].

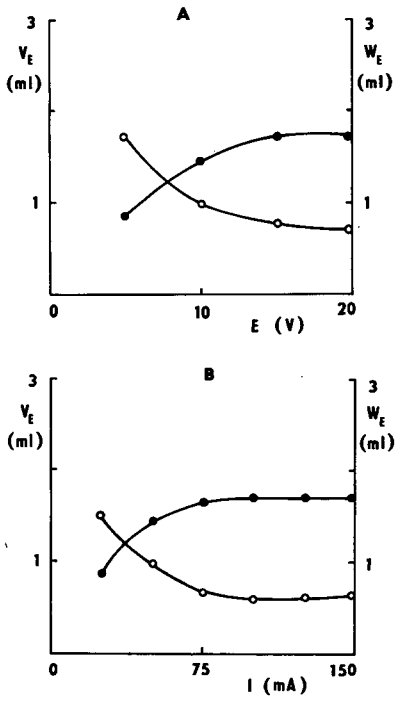

Fig. 11. Influence of applied electric power with (A) varying voltage (under a constant current of 50 mA) and (B) current (under a constant voltage of 10 V) on V_E (●) and W_E (○) of the CYTC zone. Other conditions as in Fig. 3.

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Isoelectric focusing field-flow fractionation

IV. Investigations on protein separations in the trapezoidal cross-section channel

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ABSTRACT

The first separation of three proteins (horse spleen ferritin, equine myoglobin and horse heart cytochrome *c*) by isoelectric focusing field-flow fractionation in a trapezoidal cross-section channel of 0.875 ml volume and 25 cm length is reported. Separation and elution are shown to proceed within about 1 h at a power application of about 1 W. The separation of the three proteins is demonstrated to be dependent on applied electric power, carrier ampholyte concentration and the concentrations of anolyte and catholyte. It follows from these data that the resolution is improved with increasing carrier ampholyte concentration and/or decreasing concentrations of the electrode solutions. The experimentally observed effects are in agreement with predictions made by computer simulation.

INTRODUCTION

Isoelectric focusing (IEF), particularly implemented in gels, has become an important method for the high-resolution separation and analysis of amphoteric compounds [1]. Recently, research was focused on free fluid IEF methods in order to extend the use of IEF to particles and cells which cannot pass through gels, for preparative IEF purposes in continuous-flow and recyling systems and to protein analysis in flowing streams which permit the use of detectors designed for liquid chromatography or capillary electrophoresis [2–4]. Separation in IEF is carried out in a pH gradient which is established between the anode and cathode. Amphoteric compounds migrate under the influence of the electric field until they concentrate at their isoelectric positions where the net charge (and therefore the migration) is zero. IEF is an equilibrium technique with a dynamic equilibrium between diffusion and other dispersing factors and the electrical focusing forces.

In addition to the electric field and pH gradient used in IEF, isoelectric focusing field-flow fractionation (IEF_4) employs the flow of the liquid carrier through a thin separation channel as a third factor affecting separation. The flow is perpendicular to the electric field and the flow velocity profile is determined by the geometry of the separation channel [5]. Amphoteric solutes are transported via isoelectric focusing to the equilibrium positions, where these compounds possess no net overall charge, and narrow focused solute zones with nearly Gaussian concentration distributions are formed. Provided that solutes exhibit different isoelectric points, they focus in different positions across the separation

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channel. Unequal flow velocities cause differential migration of focused solutes along the channel, *i.e.*, their longitudinal separation. Owing to the dimensions of the channels high electric field strengths can be applied with small voltages, thus keeping Joule heating at a low level. IEF_4 is an elution technique, its instrumental set-up being similar to that of high-performance liquid chromatography (HPLC) [5,6].

IEF_4 was experimentally introduced by Chmelík *et al.* [6] in the trapezoidal cross-section channel and by Thormann *et al.* [7] in the rectangular cross-section channel. The latter group named this technique electrical hyperlayer field flow fractionation following the terminology of Giddings [8]. So far, the formation of the pH gradient in a thin channel [9] and IEF_4 of a low-molecular-mass [10] and a high-molecular-mass compound [11] in the trapezoidal cross-section channel have been carefully studied. It was found that the pH gradient formation was sufficiently fast and reproducible for IEF_4. This work was devoted to the separation of three proteins under different experimental conditions and by computer simulation.

EXPERIMENTAL

Chemicals

All chemicals were of analytical-reagent grade. Cytochrome *c* from horse heart (CYTC, molecular mass M_r = 12 384, pI = 9.3) was obtained from Sigma (St. Louis, MO, USA) and ferretin from horse spleen (FER, M_r = 450 000, pI = 4.2–4.5) and equine myoglobin from skeletal muscle (MYO, M_r = 17 800, pI = 6.8–7.0) from Serva (Heidelberg, Germany). Ampholine (pH 3.5–10) was obtained from Pharmacia–LKB (Bromma, Sweden).

Instrumentation and experimental conditions

The scheme of the experimental IEF_4 arrangement has been described in detail elsewhere [6,7] and the experimental conditions used were selected on the basis of previous measurements [10,11]. The length of the trapezoidal cross-section channel was 25 cm, the height was 0.5 cm and the lengths of the two opposite walls of the trapezoid were 0.45 and 0.95 mm (volume 0.875 ml). PLGC ultrafiltration membranes (Millipore, Bedford, MA, USA) separated the focusing channel from the electrode compartments. Proteins were dissolved in carrier ampholyte solution and introduced with a four-port valve (featuring a 5-μl sample loop) through a capillary inlet situated 2 cm downstream from the carrier ampholyte inlet. Sample injection occurred over a period of 4 min using a Model 355 syringe pump (Sage Instruments, Cambridge, MA, USA). A Model 2150 HPLC pump (LKB, Bromma, Sweden) was employed to pump the carrier ampholyte solution at a pump rate of 10 μl/min during sampling and the subsequent 10-min relaxation period. The flow-rate was increased to 40 μl/min during elution. Eluting zones were monitored with a Model 2158 Uvicord SD photometric detector (LKB) at 405 nm and a Model 2210 Recorder (LKB). A Model 2297 Macrodrive 5 power supply (LKB) was used to apply up to 10 V (maximum current 100 mA). The electric field was applied during the entire experiment, including sample injection. A two-channel peristaltic pump (Vario Perpex, H. J. Guldener, Zürich, Switzerland) was used to pump solutions of acetic acid and sodium hydroxide through the anodic and cathodic electrode chambers respectively (pump rate 250 μl/min each). The carrier ampholyte and sample solutions were degassed by vacuum and filtered through 0.2-μm Nalgene (25-mm diameter) disposable syringe filters (Nalge, Rochester, NY).

Computer simulation

As described elsewhere [11], the PC-adapted software package of the transient electrophoretic model developed by Mosher *et al.* [12] was employed to predict the IEF behaviour of the proteins. This model is one-dimensional and isothermal and assumes the absence of fluid flows, hence it does not describe separation in IEF_4 but focusing at zero or very low flow-rates without elution. Ten biprotic carrier ampholytes were used to establish a pH gradient between acetic acid and sodium hydroxide. The pI values uniformly span the range 3–12 (ΔpI = 1). For each ampholyte, ΔpK was 2 and the ionic mobility was $3 \cdot 10^{-8}$ m^2/V · s. The pK and mobility value for acetic acid were $4.76 \cdot 10^{-8}$ and $4.12 \cdot 10^{-8}$ m^2/V · s, respectively, and the mobility of the sodium ion was $5.19 \cdot 10^{-8}$ m^2/V · s. The diffusion coefficients for CYTC and MYO were taken as $1.33 \cdot 10^{-6}$ [13] and $1.027 \cdot 10^{-6}$ cm^2/s [14], respectively. The diffusion coefficient for FER was taken to be equal to that of CYTC. The net charge *vs.* pH tables

employed are summarized in Table I. All simulations were performed with 67 grid points/cm and with a constant current density of 10 A/m^2. The three proteins were sampled as a Guassian peak (0.5 m*M* peak concentration) in the centre of the focusing column. The lengths of focusing space and electrode compartments were 1 and 0.25 cm, respectively [11].

TABLE I

pH DEPENDENCE OF THE IONIZATION OF PROTEINS

Ionization data were adopted with consideration of the charge of the iron ion from refs. 15 and 16 for CYTC and MYO, respectively. The data used for FER are hypothetical.

pH	Net charge		
	CYTC	MYO	FER
2.0	26.9		25
2.4			20
2.5	24.6		
3.0	21.3	24	
3.1			10
3.4			6.0
3.5	17.4	20	
3.9			3.0
4.0	14.7	16	
4.4			0
4.5	12.7	12	
4.8			−3.0
5.0	11.6	8.0	
5.4			−10
5.5	10.5	6.6	
6.0	9.9	4.6	
6.4		2.4	
6.5	9.7		
6.8		0	
7.0	9.4		
7.1			−14
7.2		−2.4	
7.5	9.1		
7.8		−4.6	
8.0	8.8		
8.5	8.6	−6.0	
8.9			−18
9.0	7.7	−8.0	−22
9.1			−26
9.2			−34
9.5	6.1	−12	
10.0	3.3	−16	
10.5	−0.1		
11.0	−5.3		

RESULTS AND DISCUSSION

The experimental conditions for the successful performance of an IEF_4 experiment were reported elsewhere [11]. It was found that (i) the sample has to be injected under applied electric power into the centre of a slowly flowing stream (10 μl/min), (ii) the relaxation time, *i.e.*, the time period necessary for formation of a focused zone, should be of the order of 10 min with no or minimum flow only and (iii) the efficiency decreases with increasing flow-rate of the carrier ampholyte solution.

For the characterization of individual compounds, the three proteins were first studied separately. The fractograms of FER, MYO and CYCT obtained at different applied voltages are depicted in Fig. 1. The IEF_4 procedure consists of three phases, sample injection, relaxation and elution. Typically each phase is executed at a different carrier flow-rate. Therefore, in order to be able to compare the influences of different experimental conditions on retention, all fractograms are expressed in units of elution volume (V_E) and not time. For all three proteins, there is a clear difference between the elution volume of unretained (bottom graphs) and retained protein (centre and top graphs). It was further found that with application of power FER has the lowest and CYTC the highest elution volume, with MYO eluting at volumes between those for the other two proteins. This observation agrees well with theory because with the configuration employed CYTC is expected to focus in the narrower part of the channel where elution is slow, MYO somewhere in the centre and FER towards the wider part where elution is fast (Fig. 2). The highest power level applied was 1 W, a value which was previously found to be safe for proper operation [11].

Fractograms depicting the separation of FER, MYO and CYTC at different power levels are presented in Fig. 3. Not surprisingly, there is no separation without application of the electric force field, as can be seen in the bottom graph. Application of a constant 5 V shows partial separation (centre graph) and almost complete resolution is obtained with 10 V (top graph). The last run was executed at a power level of about 1 W. This represents the first IEF_4 experiment showing the separation of three proteins and hence demonstrates the feasibility of this separation technique.

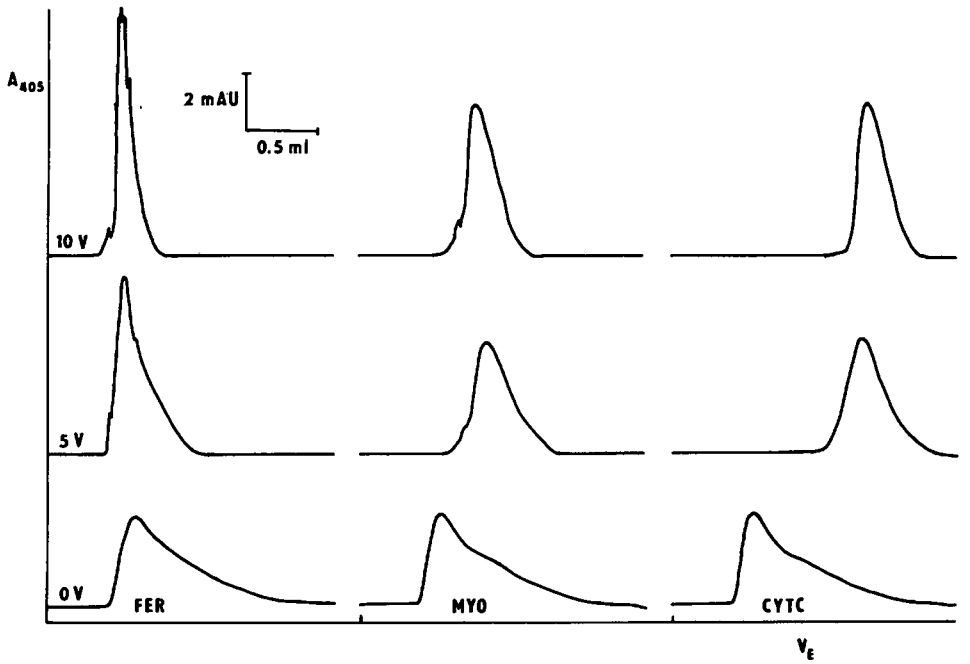

Fig. 1. Comparison of fractograms of FER (left hand panel), MYO (centre panel) and CYTC (right-hand panel) obtained without applied power (bottom graphs), with a constant 5 V (centre graphs) and a constant 10 V (top graphs). A 5-μl volume of sample containing either FER (3 μM), MYO (50 μM) or CYTC (50 μM) was injected in each instance. Carrier flow-rates under sample injection (4 min), relaxation (10 min) and elution were 10, 10 and 40 μl/min, respectively. The carrier ampholyte concentration was 2% (w/v) and the concentrations of anolyte and catholyte in the electrode compartments were 100 mM. The maximum current was 100 mA. The fractograms were monitored at 405 nm and are expressed as function of the elution volume, V_E. Origin of each fractogram indicated on V_E axis.

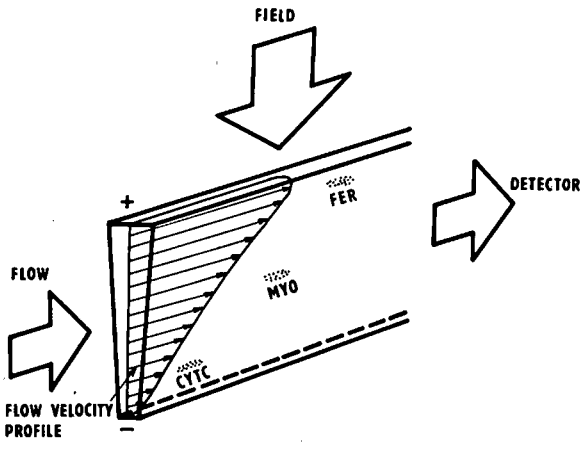

Fig. 2. Schematic representation of IEF_4 of CYTC, MYO and FER in the trapezoidal cross-section channel with the cathode on the narrower and the anode on the wider part of the channel.

The influence of the carrier ampholyte concentration on the separation of the three model proteins is depicted in Fig. 4. It is seen that the resolution of proteins is incomplete at a low (0.5%) Ampholine concentration (a) and significantly improved with 2% (c) compared with 1% (b) or 0.5% (a) Ampholine.

The influence of the buffer concentrations in the electrode chambers on resolution is presented in Fig. 5. Comparison of the fractograms obtained with (a) 100 and (b) 50 mM concentrations of anolyte and catholyte reveals that the proteins are better separated at the lower concentrations of the electrode solutions. The Ampholine concentration in that case was 2%. These findings are in good agreement with previous results which demonstrate that the pH gradient becomes shallower with either

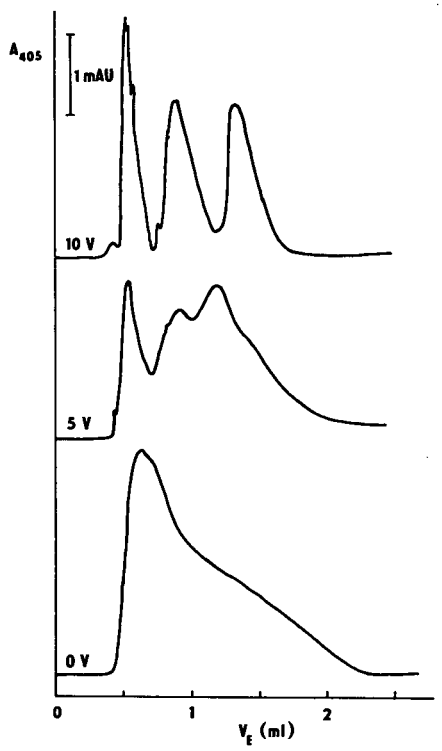

Fig. 3. Comparison of fractograms of a three-component protein mixture with FER (1 μM), MYO (17 μM) and CYTC (17 μM) at 0, 5 and 10 V. Other conditions as in Fig. 1.

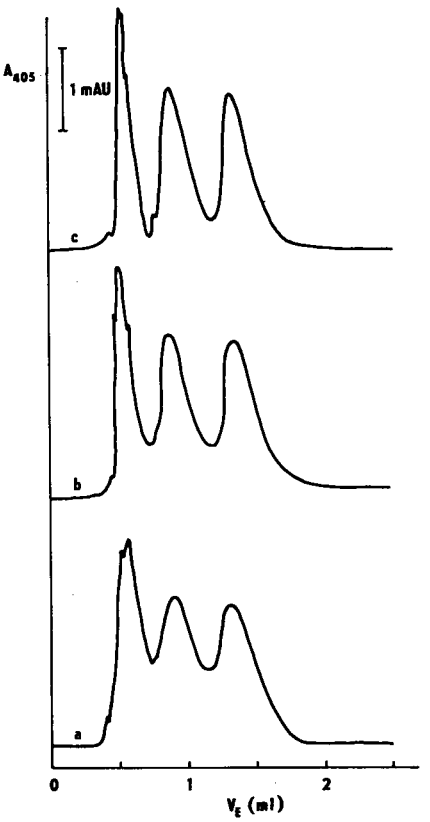

Fig. 4. Influence of carrier ampholyte concentration on protein separation with (a) 0.5, (b) 1 and (c) 2% (w/v) Ampholine. The applied voltage was 10 V. The sample solution was the same as in Fig. 3 and other conditions were as in Fig. 1.

an increasing concentration of carrier ampholytes and/or a decreasing concentration of electrolytes in the electrode chambers [9]. Generally, of course, protein separation is favoured in shallower pH gradients, which is exactly what is seen in the IEF_4 experiments.

Computer simulation was employed to confirm these basic dependences on protein separations. The computer-predicted dynamics of the three proteins with 2 mM concentrations of ampholytes are depicted in Fig. 6. The data in A and B were obtained with 100 and 50 mM electrode solutions, respectively. Note that the concentration profiles of ampholytes, catholyte and anolyte are not depicted, because they are very similar to those presented in ref. 11. With pulse sampling in the centre of the column, rapid protein separation is predicted (within 5 min) followed by focusing of the three proteins at characteristic locations. No significant change in the protein distributions is noted between 20 and 25 min of current flow, indicating that the separation phase was terminated between 15 and 20 min [17–19].

More interesting for the purposes of this paper are the pH gradients produced and the positions of the foci in relation to the initial ampholyte concentrations and the concentrations of the electrode solutions. The pH gradients after 25 min of current application for the two cases in Fig. 6 are shown in Fig. 7. Comparison of the two profiles reveals that a shallower gradient is predicted with 50 than with 100 mM electrode solutions and that the difference on the basic (cathodic) side is larger than that on the acidic side. This explains the fact that the protein foci are further apart in Fig. 6B compared with Fig. 6A, with the position of focused CYTC showing the largest effect on electrode solution concentration. Similar, but less pronounced, shifts are predicted with increasing carrier ampholyte concentrations

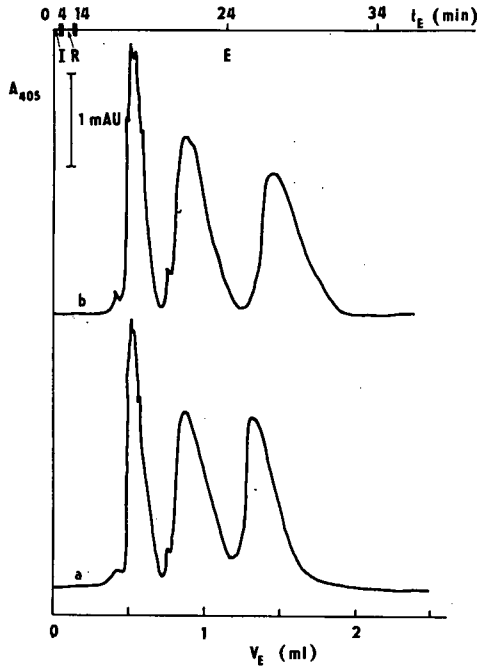

Fig. 5. Influence of anolyte and catholyte concentration on protein separation with (a) 100 and (b) 50 m*M* concentrations of acetic acid and sodium hydroxide, respectively. The applied voltage was a constant 10 V. The sample solution was the same as in Fig. 3 and other conditions were as in Fig. 1. The time axis with the three stages, sample injection (I), relaxation (R) and elution (E), is shown on the top.

(data not shown). Hence the computer simulation data are in agreement with the experimental observations. These data demonstrate the applicability of the electrophoresis model without incorporation of fluids flow to predict basic focusing behaviour in IEF_4, and the establishment of proper flow conditions in the employed trapezoidal cross-section IEF_4 channel.

CONCLUSIONS

The data presented on the separation of proteins demonstrate the validity of the IEF_4 separation principle in a trapezoidal cross-section channel. The time period of an IEF_4 experiment in the channel employed is about 1 h and the applied electric power required is about 1 W. Separation is shown to be dependent on applied electric power, concentration of carrier ampholytes and concentration of electrode solutions. These effects are in agreement with predictions made by computer simulation. The feasibility of IEF_4 has been demonstrated, but not its resolving power or limit, because the investigated proteins are characterized by large differences in p*I* values. For a complete elucidation of the potential

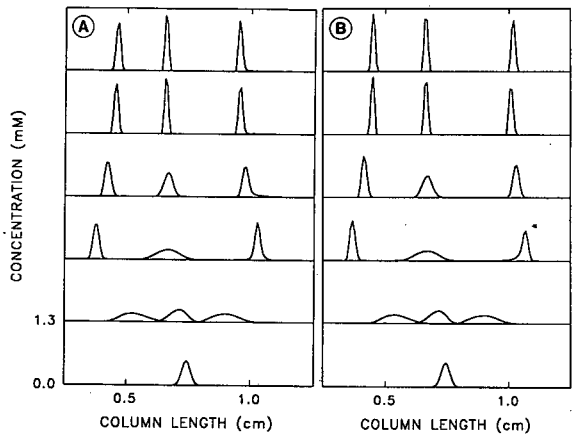

Fig. 6. Computer-simulated distribution of the three proteins after 0, 5, 10, 15, 20 and 25 min (from bottom to top) of current flow within the focusing space (from 0.25 to 1.25 cm column length) having (A) 100 and (B) 50 m*M* acetic acid and sodium hydroxide as anolyte and catholyte, respectively. The initial concentration of each carrier ampholyte was 2 m*M*. The anode is to the left. The distributions of the carrier ampholytes and the components from the electrode solutions are not shown. Each successive time point is offset from the previous one by a constant amount for presentation purposes. The left-hand, centre and right-hand foci are formed by FER, MYO and CYTC, respectively.

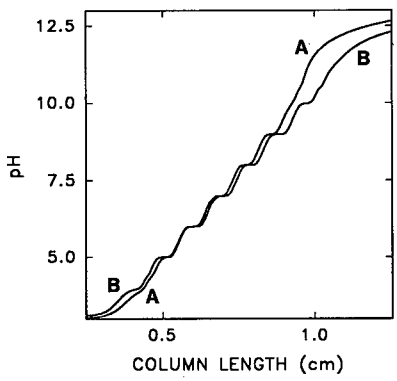

Fig. 7. Predicted pH gradients for the 25-min time points in Fig. 6 with electrode solution concentrations of (A) 100 and (B) 50 m*M*.

of IEF_4, further studies with other proteins and with channels of different lengths and cross-sectional areas will have to be executed. Further, comparative studies between IEF_4 and other IEF techniques in flowing streams are in progress and will be reported in due course.

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Short Communication

Suitable chiral packing material for the high-performance liquid chromatographic separation of derivatives of 1′-hydroxyeugenol

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ABSTRACT

Plants of the genus *Coreopsis* contain rare phenylpropanoids of the 1′-hydroxyeugenol (Eu) type. To investigate the biological activities of these compounds, 1′-hydroxyeugenol isobutyrate (Eu1) and its derivatives were synthesized. The aim was to separate racemic Eu1 by high-performance liquid chromatography into its enantiomers on a preparative scale, in order subsequently to synthesize the equivalent esters. To control the stereoselectivity of esterification of Eu1, analytical systems investigated for Eu1–Eu5 can be utilized. The best results were obtained on microcrystalline cellulose triacetate (CTA), Pirkle Convent phenylglycine and Chiracel OK columns. Racemic Eu1 could be separated on a preparative scale on CTA.

INTRODUCTION

The genus *Coreopsis* accumulates phenylpropanoids with unusual structures and rare occurrence, and derivatives of 1′-hydroxyeugenol (Eu) (Fig. 1) are of great interest regarding their biological activities [1–5]. Eu2–Eu4 are naturally occurring compounds in the roots of *Coreopsis* species, whereas Eu1 and Eu5 are totally absent in the genus *Coreopsis*. A screening programme for the biological activities of Eu1–Eu5 required large amounts of these compounds. On this account, the racemates of Eu1–Eu5 were synthesized. In addition to Eu2–Eu4, the racemates of Eu1 and Eu5 were also integrated into the screening programme to obtain information about a supposed stucture–activity relationship.

It is well known that many naturally occurring or synthesized optically active compounds with one asymmetric carbon atom produce full biological activities only when they have a high enantiomeric purity. For this reason it was considered useful to test also the optically pure enantiomers of Eu1–Eu5,

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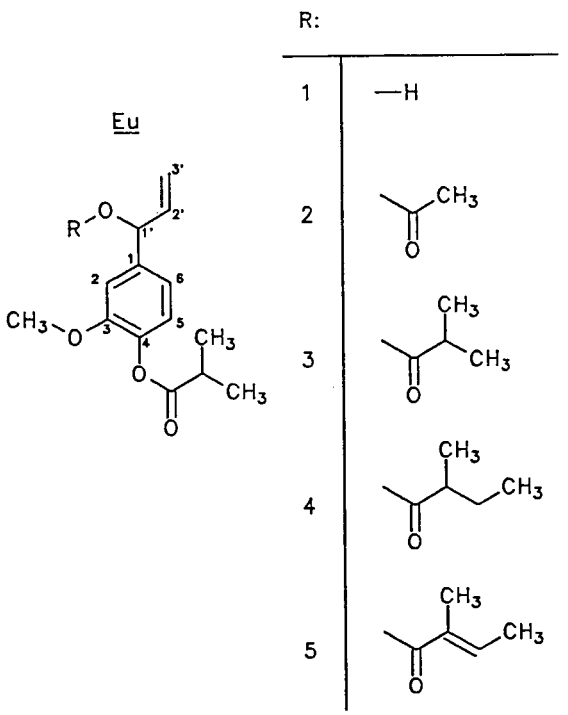

Fig. 1. Derivatives of 1′-hydroxyeugenol (Eu). Eu1 = 1′-hydroxyeugenol isobutyrate; Eu2 = 1′-acetoxyeugenol isobutyrate; Eu3 = 1′-isobutyryloxyeugenol isobutyrate; Eu4 = 1′-isovaleroyloxyeugenol isobutyrate; Eu5 = 1′-tigloxyeugenol isobutyrate.

depending on the results with racemic compounds.

Separation of enantiomers by liquid chromatography on chiral stationary phases has become a practical and useful method for obtaining pure optical isomers. In this paper, we report high-performance liquid chromatographic (HPLC) separations of racemic Eu1 into pure optical enantiomers on a preparative scale and analytical separations of Eu1–Eu5 on different chiral stationary phases.

EXPERIMENTAL

Chiral stationary phases and eluents

Microcrystalline cellulose triacetate (CTA). The column contained CTA prepared by heterogeneous acetylation (250 × 6 mm I.D.) (Macherey–Nagel, Düren, Germany). The injection volume was 20 μl (25 mg of substance/ml of eluent).

For Eu1, Eu4 and Eu5 the eluent was *n*-hexane–isopropanol (90:10, v/v) at flow-rates of 1.5, 0.5 and 1 ml/min, respectively. For Eu2 the eluent was ethanol at a flow-rate of 1 ml/min. Eu3 was not tested.

Pirkle convent phenylglycine. The column (250 × 10 mm I.D.) contained as stationary phase (*R*)-(−)-N-3,5-dinitrobenzoylphenylglycine bonded on aminopropyl-functionalized silica gel (Regis, Austin, USA); N = 57 800 theoretical plates. The injection volume was 20 μl (25 mg of substance/ml of eluent) and the flow-rate was 1 ml/min.

For Eu1 and Eu2 the eluent was *n*-hexane–isopropanol (90:10, v/v) and for Eu3, Eu4 and Eu5 *n*-hexane–isopropanol (98:2, v/v).

Chiracel OK. The column (250 × 4 mm I.D.) contained as stationary phase cellulose cinnamate on silica gel [6,7] (Daicel Chemical Industries, Deventer, Netherlands); N = 17 500 theoretical plates. The injection volume was 20 μl (1 mg of substance/ml of eluent).

The eluent was *n*-hexane–isopropanol (98:2, v/v), at a flow-rate of 1.0 ml/min for Eu2 and 0.5 ml/min for Eu1 and Eu3–Eu5.

Preparative separation of Eu1

A CTA column (250 × 20 mm I.D.) (Macherey–Nagel) was used. The injection volume was 250 μl (80 mg of Eu1/ml of eluent). The eluent was *n*-hexane–isopropanol (90:10, v/v) at a flow-rate of 7 ml/min. The resolution factors were $k'_1 = 6$, $\alpha = 1.33$ and $R_s = 0.4$ (for definitions, see Table I).

A 456-mg amount of racemic 1′-hydroxyeugenol isobutyrate was dissolved in the eluent, of which 20 mg were always loaded on to the column. The weighed fractions showed 164 mg of enantiomerically pure (−)-Eu1 and 266 mg of (+)-Eu1.

Equipment

Analytical chromatography was performed using a Kontron (Munich, Germany) Model 420 pump, a Perkin-Elmer (Überlingen, Germany) Model 241 polarimeter and an Erma Optical Works UV detector (wavelength 279 nm) (Besta, Heidelberg, Germany). The effluent from the column was guided through the polarimeter and then through the UV detector.

For preparative chromatography a Latek (Heidelberg, Germany) P700 pump and a Milton-Roy (Hasselroth, Germany) Spectro Monitor 3100 UV detector (flow-rate 7 ml/min, wavelength 279 nm) were used.

Synthesis of Eu1–Eu5

Grignard reaction of vanillin isobutyrate (obtainable through esterification of vanillin with isobutyryl chloride) with vinylmagnesium bromide gave racemic Eu1 [3]. Esterification of Eu1 with acetyl chloride or isobutyryl chloride yielded Eu2 and Eu3, respectively.

Eu4 and Eu5 were prepared using a method for the direct esterification of carboxylic acids described by Neises and Stegliz [8].

RESULTS AND DISCUSSION

Analytical separation of enantiomers

Microcrystalline cellulose triacetate. CTA, developed by Hesse and Hagel [9,10], is a useful stationary phase and has been widely employed for the separation of aromatic compounds [11,12]. First we examined the extent of separation using ethanol as the eluent and changing the flow. Using a flow-rate of only 0.1 ml/min we observed a partial separation of Eu1. This was insufficient for transfer to a preparative system, so we used more lipophilic eluents in place of ethanol. Finally, using *n*-hexane–isopropanol (90:10), Eu1 was completely separated into its enantiomers ($k'_1 = 3.43$; $\alpha = 1.42$; $R_s = 0.83$) (Table I). The polarimetric results show that the first peak represents the laevorotatory enantiomer of Eu1 and the second peak the dextrorotatory enantiomer.

All efforts to separate racemic Eu2–Eu5 into the enantiomers using CTA failed.

Pirkle phenylglycine column. This chiral stationary phase, described by Pirkle and co-workers [13,14], showed an ability to separate the enantiomers of Eu1 using *n*-hexane–isopropanol (90:10) as eluent. The enantiomer eluted first was (+)-Eu1. It was also possible to separate Eu3–Eu5 when the eluent was changed to *n*-hexane–isopropanol (98:2) (Fig. 2). Resolution of Eu2 on this chiral stationary phase was impossible (Table I). In contrast to Eu1, the laevorotatory enantiomers of Eu3–Eu5 left the column faster than the dextrorotatory enantiomers.

Chiracel OK column. With a Chiracel OK column and *n*-hexane–isopropanol (98:2) as eluent (flow-rate 1 ml/min), racemic Eu2 was successfully separated into its antipodes (Table I). The separations of te enantiomers of Eu3 and Eu4 were even more effective when the flow-rate was halved. In all

TABLE I

RESULTS OF CHROMATOGRAPHIC RESOLUTION OF Eu1–Eu5 ON DIFFERENT COLUMNS

k'_1 (capacity factor for less retained enantiomer) = (retention time − dead time)/dead time; α (separation factor) = (capacity factor for more retained enantiomer)/k'_1; R_s (resolution factor) = 2(distance of the two peak positions)/(sum of band widths of the two peaks); for separation conditions, see Experimental. k'_1: +/− indicates sign of optical rotation of first eluting enantiomer.

Column	k'_1	α'	R_s	Substance
CTA	3.43(−)	1.42	0.83	Eu1
Pirkle	2.00(+)	1.25	1.00	
Chiracel OK	0.88	1.00	−	
CTA	2.33(−)	1.00	−[a]	Eu2
Pirkle	1.29(+)	1.00	−[a]	
Chiracel OK	5.63(+)	1.19	1.06	
Pirkle	2.29(−)	1.12	1.14	Eu3
Chiracel OK	1.69(+)	1.78	1.75	
CTA	0.85(−)	1.00	−[a]	Eu4
Pirkle	1.96(−)	1.09	1.00	
Chiracel OK	2.25(+)	1.17	0.67	
CTA	1.09	1.00	−	Eu5
Pirkle	3.14(−)	1.08	1.00	
Chiracel OK	4.87	1.00	−	

[a] No separation was observed with the UV detector, although partial separation was obtained with the polarimeter detector.

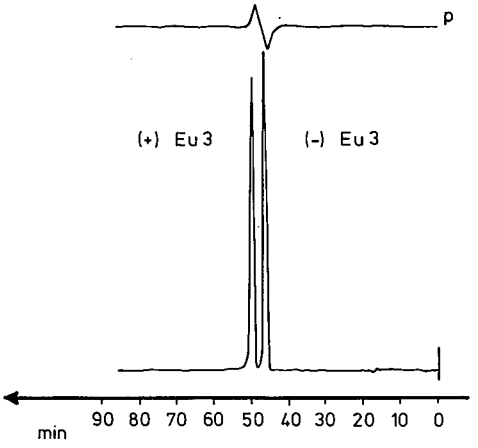

Fig. 2. Analytical separation of Eu3 using the Pirkle convent phenylglycine column (250 × 10 mm I.D.) with *n*-hexane–isopropanol (98:2) as eluent at a flow-rate of 1 ml/min. UV detection at 279 nm. Concentration, 25 mg/ml; injection volume, 20 µl. Polarimeter: Hg lamp emitting at 302 nm (p = polarimeter curve).

instances the dextrorotatory enantiomer was eluted first. Separation of Eu1 and Eu5 was impossible under these conditions.

The results of the analytical resolution of Eu1–Eu5 on the different chiral stationary phases are summarized in Table I. All racemic compounds were separable chromatographically on one or other of the three columns. The selection of the chiral stationary phases was made empirically, because it is very difficult to determine the structure requirements for a chiral stationary phase based on the structural parameters of the enantiomers. The best results were obtained on the Pirkle phenylglycine column. This column showd a remarkable chiral discrimination against Eu1 and Eu3–Eu5, whereas the CTA column resolved only Eu1 with high efficiency. The latter is unexpected as CTA has been widely employed for the separation of aromatic compounds [12,15,16].

It is also noteworthy that on the Pirkle column the enantiomers of Eu1 were eluted in reversed order compared with the enantiomers of Eu3–Eu5. This indicates that esterification of the alcoholic OH group on the side-chain of Eu1 has the consequence that the chiral discrimination of Eu3–Eu5 on the Pirkle phenylglycine column changed with respect to Eu1.

Separation of Eu1 on CTA on a preparative scale

The next objective was to separate the synthetic racemate of Eu1 into its antipodes on a preparative scale.The optimization strategy for preparative chromatography in the form of overload experiments was applied to the direct HPLC separation of the enantiomers. Considering the time–performance factor, we chose a system that allowed only a partial separation. The analysis of the fractions to ascertain the points of intersection and to determine the enantiomeric purity was achieved with the Pirkle phenylglycine column. The chromatography resulted in a high optical purity of (−)-Eu1 and an enantiomeric excess of (+)-Eu1 of 99%. The mean fractions containing the enantiomers in the ratio 1:1 were separated again.

CONCLUSIONS

With microcrystalline CTA as a chiral stationary phase and *n*-hexane–isopropanol (90:10, v/v) as eluent, racemic Eu1 was successfully separated into its antipodes by HPLC on both an analytical and a preparative scale. It seems to be possible to separate pure (+)-Eu1 and (−)-Eu1 in large amounts using this technique.

Based on the results of the biological tests, the equivalent esters of (+)- and (−)-Eu1 will now be synthesized, and the optical purity of the esterification products will be controlled with these analytical systems investigated for Eu1–Eu5.

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CHROM. 24 131

Short Communication

Monitoring the effluents of the trichloroacetic acid process by high-performance liquid chromatography☆

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ABSTRACT

A simple and rapid high-performance liquid chromatographic method for the simultaneous determination of small amounts of nitric acid and trichloroacetic acid in process effluents was developed. Acidic components of the effluents were separated on a reversed-phase C_{18} column using 0.15 *M* ammonium sulphate as mobile phase and determined quantitatively by UV absorption at 210 nm. The detection limits for nitric acid and trichloroacetic acid were 1.4 and 10 μg/l, respectively.

INTRODUCTION

Trichloroacetic acid is an important intermediate in the chemical industry and is also used as a herbicide against couch grass and wild oats [1]. It is manufactured [2–4] in large amounts either by oxidation of chloral or by chlorination of acetic acid. Our laboratory has studied the production of trichloroacetic acid by the oxidation of chloral using nitric acid. Effluents obtained during this process contain nitric acid and trichloroacetic acid in considerable amounts. Their recovery would be helpful in improving the yield of trichloroacetic acid and for recycling the nitric acid through the process. Rapid and reliable analytical methods are needed not only for monitoring the reactions but also for disposing of the effluents safely.

Titrimetric, potentiometric and polarographic techniques [5,6] have been employed extensively to determine trichloroacetic acid in water, but these methods are tedious and time consuming. Spectrophotometric methods [7–9] have been found to be unattractive owing to a lack of specificity. Gas–liquid chromatographic methods [10–12] have limitations because carboxylic acids dimerize through hydrogen bonding in the vapour state. Ion-exchange chromatography [13], size-exclusion chromatography [14] and high-performance liquid chromatography (HPLC) [15] have been attempted but were unsuccessful.

In this paper, we describe a simple and rapid HPLC method for the separation and determination of small amounts of nitric, acetic and trichloroacetic acids in process effluents using a ChromSphere C_{18} column and an eluent containing 0.15 *M* ammonium sulphate at ambient temperature.

EXPERIMENTAL

Materials and reagents

All reagents were of analytical-reagent grade unless stated otherwise and glass-distilled water was

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used throughout. Nitric acid, acetic acid, chloral and trichloroacetic acid were obtained from Spectrochem (Bombay, India) and ammonium sulphate from BDH (Poole, UK).

Apparatus

A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) with a 20-μl loop injector having a high-pressure six-way valve was used. A Model SPD-6AV variable-wavelength UV–VIS spectrophotometric detector (Shimadzu) was connected after the column. A ChromSphere C_{18} (10 μm) column (250 mm × 4.6 mm I.D.) (Chrompak, Middelburg, Netherlands) was used for separation. The chromatograms and the integrated data were recorded with a Chromatopac-CR3A processing system.

Chromatographic conditions

The mobile phase used was 0.15 *M* aqueous ammonium sulphate. Samples were dissolved in the mobile phase. Analyses were carried out under isocratic conditions at a flow-rate of 1 ml/min and a chart speed of 5 mm/min at room temperature (27°C). Chromatograms were recorded at a wavelength of 210 nm.

Analytical procedure

Standard mixtures containing known amounts (10–20 mg) of nitric acid and trichloroacetic acid were prepared by dissolution in 25 ml of the mobile phase. A 10-μl volume of each mixture was injected and chromatographed under the above conditions and the response factors were calculated. Process effluents were analysed under identical conditions.

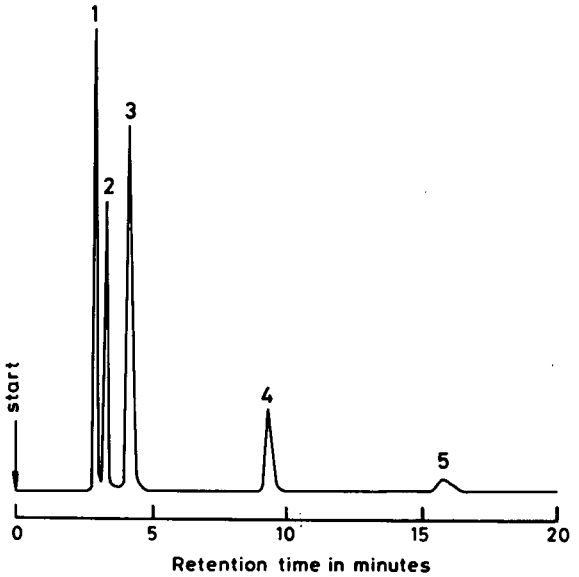

Fig. 1. HPLC profile of a synthetic mixture (10 μg) containing 1 = nitric acid, 2 = acetic acid, 3 = dichloroacetic acid, 4 = trichloroacetic acid and 5 = chloral. Chromatographic conditions: column, ChromSphere, 10 μm (250 mm × 4.6 mm I.D.); mobile phase, 0.15 *M* aqueous ammonium sulphate; flow-rate, 1 ml/min; UV detection at 210 nm.

The amounts of nitric and trichlaoroacetic acid were calculated from the peak areas.

RESULTS AND DISCUSSION

HPLC separation of nitric, acetic and trichloroacetic acids is shown in Fig. 1. The peaks were identified by injecting the individual compounds. The retention data and the λ_{max} values are given in Table I. It can be seen that trichloroacetic acid is well

TABLE I

RETENTION DATA

Compound	Retention time (min)	Relative retention time	λ_{max} (nm)
Nitric acid	2.78	1.00	210
Acetic acid	3.20	1.15	207
Dichloroacetic acid	4.15	1.49	205
Trichloroacetic acid	9.78	3.52	205
Chloral	16.90	6.08	208

TABLE II

RESPONSE FACTORS OF NITRIC ACID AND TRICHLOROACETIC ACID

Sample	Area under the peak[a]	R.S.D.[b] (%) (n = 5)
Nitric acid	8353	1.72
Trichloroacetic acid	1074	2.58

[a] For 1 ml of sample.
[b] Relative standard deviation.

TABLE III

ANALYTICAL DATA FOR STANDARD MIXTURES

Sample No.	Nitric acid (%)				Trichloroacetic acid (%)			
	Taken	Found[a]	Error	S.D.	Taken	Found[a]	Error	S.D.
1	0.63	0.62	−1.59	3.22	0.55	0.56	+1.82	3.57
2	0.97	0.99	+2.06	2.50	1.08	1.11	+2.77	1.80
3	1.54	1.52	−1.30	1.97	1.92	1.89	−1.04	2.12
4	2.76	2.81	+1.81	2.76	2.50	2.45	−1.99	2.04

[a] Average of three determinations.

separated not only from nitric acid but also from acetic acid. Further, nitric acid elutes at 2.78 min, which is close to the void volume of the column. Therefore, inorganic impurities such as hydrochloric acid and sodium nitrate may interfere in the analysis as they also elute in the same region. However, their interference can be avoided by adjusting the concentration of the mobile phase and increasing the elution time of nitric acid. The response factors for all the compounds were determined and are given in Table II. When the UV detector was set at 0.001 a.u.f.s. the limits of detection for nitric acid and trichloroacetic acid were 1.4 and 10 μg/l, respectively, with a signal-to-noise ratio of 4.0.

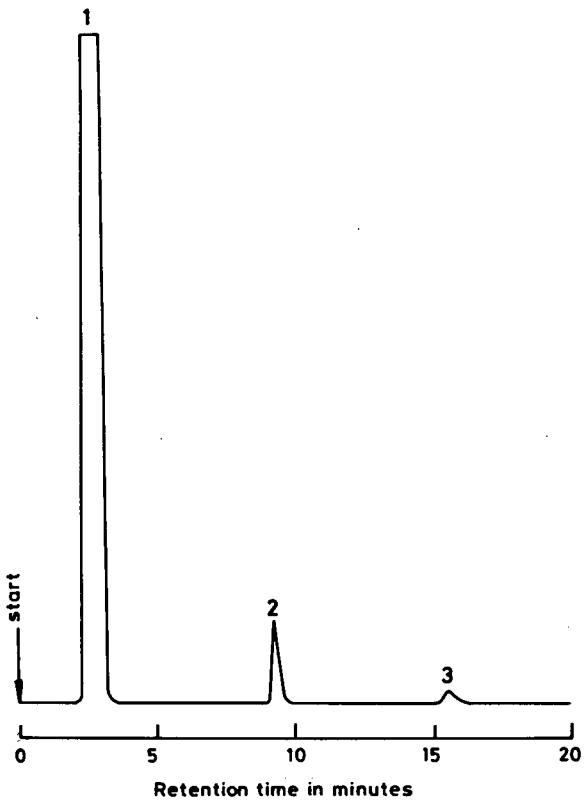

Fig. 2. Typical chromatogram of effluent of trichloroacetic acid process. Peaks: 1 = nitric acid; 2 = trichloroacetic acid; 3 = chloral. Chromatographic conditions as in Fig. 1.

Standard mixtures containing different amounts of nitric acid and trichloroacetic acid were prepared and analysed by HPLC. The results are given in Table III. The measured amounts of nitric acid and trichloroacetic acid agreed with the actual values to within 0.06% and 2.77%, respectively.

Process effluents were collected during the course of oxidation of chloral with nitric acid and analysed by the proposed method. A typical chromatogram of an effluent is shown in Fig. 2. The unreacted chloral does not interfere, as it elutes at 16.90 min. The results are given in Table IV and show that the method is simple, rapid and convenient for monitoring effluents of the trichloroacetic acid process.

TABLE IV

ANALYTICAL DATA FOR PROCESS EFFLUENTS

Sample No.	Nitric acid (%)[a]	Trichloroacetic acid (%)[a]
1	1.60	3.52
2	1.45	1.49
3	2.10	4.50
4	1.19	0.41

[a] Average of three determinations.

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CHROM. 24 130

Short Communication

High-performance liquid chromatographic separation of chiral metallocenic ketones and alcohols

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ABSTRACT

The enantiomers of chiral (arene)tricarbonylchromium ketones and alcohols were separated by high-performance liquid chromatography with a Chiralcel OD column. The absolute configuration of the ketones was assigned on the basis of the sign of optical rotation determined with an on-line detector.

INTRODUCTION

High-performance liquid chromatography (HPLC) has long been used for the preparative and analytical separation of organometallic compounds. However, in only a few instances has HPLC been applied to the determination of enantiomeric excesses of organometallic derivatives [1].

Recently, the enantiomers of (η^6-benzene)tricarbonylchromium alcohols, methyl esters and acetates and (η^5-cyclopentadienyl)tricarbonylmanganese and ferrocene compounds were separated by HPLC with a Chiralcel OD column [2]. In contrast, this column did not resolve the related aldehydes.

As chiral (arene)tricarbonylchromium ketones are useful starting materials for asymmetric synthesis [3], we have verified if the Chiralcel OD column is suitable for determining their optical purity and that of the corresponding alcohols obtained by sodium tetrahydroborate reduction.

EXPERIMENTAL

HPLC was conducted on a 10-μm BakerBond Chiralcel OD column (250 × 4.6 mm I.D.), using a Jasco Model 880-PU pump, a Rheodyne Model 7125 injector with a 20-μl sample loop, a Jasco 870-UV detector set at 315 nm and a Hewlett-Packard HP-3394A integrator. The order of elution of (arene)tricarbonylchromium ketones was determined employing a Jasco DIP-370 on-line optical rotation detector equipped with a flow cell (5 cm path length) and a solid-state laser beam (670 nm). The system was operated at room temperature at a flow-rate of 0.5 ml/min with 1.5–10% (v/v) ethanol in hexane as the mobile phase.

The $Cr(CO)_3$ complexes **1–6** were prepared by

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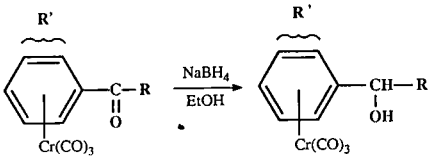
complexation of the parent ketones with $Cr(CO)_6$ [4]. They were reduced to the corresponding alcohols **7–12** with sodium tetrahydroborate using ethanol as the solvent under nitrogen in the dark. After reaction for 10 min the solvent was evaporated and the residue was diluted with water and extracted with diethyl ether.



1;7a,7b	$R = CH_3$	$R' = o\text{-}CH_3$
2;8a,8b	$R = CH_3$	$R' = o\text{-}OCH_3$
3;9a,9b	$R = CH_3$	$R' = m\text{-}CH_3$
4;10a,10b	$R = CH_3$	$R' = m\text{-}OCH_3$
5;11a,11b	$R = C_7H_{15}$	$R' = o\text{-}OCH_3$
6;12a,12b	$R = C_7H_{15}$	$R' = m\text{-}CH_3$

Letters **a** and **b** refer to the two diastereoisomers differentiated by ^1H NMR spectroscopy on a Bruker AC 300 instrument.

RESULTS AND DISCUSSION

The racemic $Cr(CO)_3$ complexes **1–6** and the corresponding alcohols **7–12** were analysed using the Chiralcel OD column.

The results obtained with the $Cr(CO)_3$ ketones are summarized in Table I. The compounds were completely resolved (R_s values ranging from 1.01 to 2.22), with the exception of **1**. The order of elution of the enantiomers was determined with an on-line optical rotation detector. For *ortho*-substituted ketones the (−)-enantiomer was eluted first and the opposite was found for the *meta*-substituted compounds.

The absolute configuration of *ortho*- and *meta*-substituted complexes **1–6** was assigned on the basis of the model proposed by Solladié-Cavallo [3] for (η^6-arene)tricarbonylchromium ketones. In this model the σ plane of the ring is divided into octants of different size and the signs of optical rotation of the ketones are correlated with the absolute configuration at C-1. It was found that the 1*R*-enantiomers were eluted before the 1*S*-enantiomers (Table I).

The same regularities in the elution order had been already found by Yamazaki *et al.* [2] for organometallic alcohols, acetates and esters; they attributed the planar chirality as "the predominant factor in controlling the binding mode with the substituents of the adsorbent".

With the alcohols **7–12**, which were obtained as diasteromeric mixtures, HPLC was carried out after separation of the diastereoisomers by flash chromatography on silica gel 60 (230–400 mesh, Merck) with mixtures of dichloromethane and diethyl ether as the mobile phase. Table II shows that, with the exception of one diastereoisomer of **10**, the resolution of the enantiomers was good to excellent. Therefore, HPLC with Chiralcel OD is very effec-

TABLE I

RESOLUTION OF CHIRAL (ARENE)CARBONYLCHROMIUM KETONES ON CHIRALCEL OD

Compound	Ethanol in hexane (%)	Elution order	k'^a	α^b	R_s^c
1	1.5–10		7.56–2.87	1	–
2	6.5	(−)-(*R*); (+)-(*S*)	5.78; 6.22	1.08	1.01
3	6.5	(+)-(*R*); (−)-(*S*)	3.13; 3.67	1.17	2.06
4	5	(+)-(*R*); (−)-(*S*)	3.90; 4.61	1.18	1.93
5	8	(−)-(*R*); (+)-(*S*)	2.35; 2.94	1.25	2.22
6	8	(+)-(*R*); (−)-(*S*)	1.67; 2.00	1.20	1.25

[a] Capacity factor for the resolved enantiomers. For **1**, where there was no enantiomeric resolution, k' values refer to peak retention as a function of eluent composition.
[b] Separation factor.
[c] Resolution.

TABLE II

RESOLUTION OF CHIRAL (ARENE)CARBONYLCHROMIUM ALCOHOLS ON CHIRALCEL OD

Compound	Ethanol in hexane (%)	k'	α	R_s
7a	3.3	5.20; 7.10	1.36	4.20
7b	8.3	8.60; 14.41	1.67	6.51
8a	8.5	3.23; 3.52	1.09	0.82
8b	8.5	5.24; 5.90	1.12	1.75
9a	1.5	12.25; 13.50	1.10	1.18
9b	1.5	16.25; 18.25	1.12	1.52
10a	3.5	7.38	1	–
10b	3.5	7.85; 9.67	1.23	4.50
11a	3	0.91; 2.01	2.21	2.03
11b	3	13.70; 14.18	1.04	0.71
12a	2	2.28; 2.46	1.08	0.91
12b	2	2.86; 3.07	1.07	0.63

tive for the separation of racemic chromium-complexed ketones and alcohols and is also simpler than other methods such as ^{1}H NMR spectroscopy in the presence of chiral-shift reagents [3].

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CHROM. 24 144

Short Communication

Plant growth regulators G_1, G_2, G_3

Synthesis, extraction and determination of leaf content in *Eucalyptus grandis*

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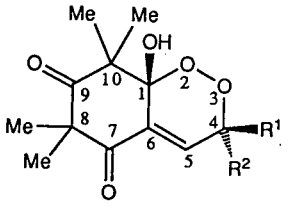
ABSTRACT

Endogenous concentrations of plant growth regulators G_1, G_2, G_3 from leaves of French-planted *Eucalyptus grandis* were determined. The leaves were extracted with methanol and the resulting extracts were purified by means of silica gel column chromatography and reversed-phase medium-pressure liquid chromatography. Improved synthesis, purification and physico-chemical characterization of the G factors, for use as external standards, were developed.

INTRODUCTION

Three closely related growth regulators designated as G substances occur in the leaves of *Eucalyptus grandis* and some other myrtaceous plants [1]. For some years there has been growing interest, especially in Australia [2,3], concerning these compounds. It has been suggested that in addition to auxin-like activity, the G factors affect stomatal conductance and photosynthesis [4], reduce water loss [5] and are probably involved in the frost resistance of *E. grandis* by controlling the active electron transport properties of membranes [6].

The G cyclic peroxides have been either isolated from *E. grandis* [2,7] or synthesized from precursor syncarpic acid [8] and their structures determined by X-ray analysis [9].



G	R_1	R_2
C_1	C_2H_5	CH_3
G_2	CH_3	C_2H_5
G_3	CH_3	CH_3

Me = CH_3

Endogenous concentrations of G factors have been determined for Australian *E. grandis*. The leaf content of G varies from 18.6 μg/g for juvenile trees to 7500 μg/g fresh weight for adult trees [6]. Although phytogeographical differences between the two hemispheres may affect the G content in *E. grandis*, to our knowledge no such data exist concerning the French-planted Eucalyptus. In connection with our current work on this topic [10,11], this paper reports on the research, extraction and isola-

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tion of the G substance from *E. grandis* and its research in *E. gunii* species.

EXPERIMENTAL

Plant material

Leaves from 4-month-old *E. grandis* seedlings were kindly provided by Afocel (Toulouse, France). For *E. gunii*, leaves were collected from two different clones of 4-month-old seedlings, one frost-resistant and the other frost-sensitive. All samples were grown in soil in a glasshouse.

Instrumentation

Medium-pressure liquid chromatography was performed on an Axxial apparatus (Axxial Modulo Prep, Martigues, France). Analytical chromatography was performed on a Waters Model 600 E apparatus equipped with a Rheodyne injector (20 μl) and a Waters Model 990 UV Specroflow detector. The detection wavelength was set at 240 nm. The column used was Nova-Pak silica (150 mm × 4.6 mm I.D.; 4-μm particle size) purchased from Waters. The eluent used was isooctane–dichloromethane (35:65) at a flow-rate of 1 ml/min.

NMR spectra were recorded in deuterochloroform on a Bruker AC 200 apparatus. IR and UV spectra were recorded on a Perkin-Elmer Model 883 and a Lambda 7 spectrophotometer, respectively. Melting points were measured on a Kofler bank.

Chemicals

The G_1, G_2, G_3 compounds used as external standards were chemically synthesized from syncarpic acid, which was obtained by a procedure developed in this laboratory [10]. Although the synthesis of G_1 and G_2 compounds has been described differently to that of G_3 [8], we obtained them under identical experimental conditions. Syncarpic acid (1 g, 5.5 mmol) in ethanol (40 ml) was added dropwise to a solution of the appropriate aldehyde in excess (110 mmol) [2-methylbutyraldehyde (9.4 g) for G_1 and G_2 and 2-methylpropionaldehyde (7.9 g) for G_3] in 10 ml of ethanol containing a catalytic amount of piperidine (0.2 ml). The mixture was allowed to stand for 15 min then solvent and excess of aldehyde were removed under reduced pressure.

G_3 was purified on a silica gel column (40 g, 70–200 μm Amicon, 500 mm × 20 mm I.D., eluent dichloromethane) to give 1.1. g (yield 75%) of isolated product, m.p. 170°C (lit. [8] m.p. 170–171°C); the purity was also checked by thin-layer chromatography (TLC) on a silica gel plate (R_F = 0.37, eluent dichloromethane).

A crude mixture of the two diastereoisomers G_1 and G_2 (75 mg) was purified by medium-pressure liquid chromatography on a silica gel column (20 g, 6–35 μm Amicon, 500 mm × 20 mm I.D.). The detection wavelength was set at 257 nm and the eluent used was dichloromethane–light petroleum (b.p. 45–60°C)–ethyl acetate (75:20:5) [TLC: R_F (G_1) = 0.35, R_F (G_2) = 0.29]. Intermediate fractions were repurified. The purity of the samples was greater than 97% from HPLC results. G_1 and G_2 were identified by their melting points: G_1, m.p. 100°C (lit. [8] m.p. 99–100°C); G_2, m.p. 127°C (lit. [8] m.p. 127–128°C). They were obtained in 46% (710 mg) and 31% (480 mg) total yield, respectively.

The IR spectra of G_1, G_2 and G_3 were recorded as 0.05 *M* solutions in chloroform [ν (cm^{-1}) ± 1]: G_1, 3578 (OH), 1724 (C_9=O), 1691 (C_7=O), 1636 (C=C); G_2, 3576 (OH), 1726 (C_9=O), 1691 (C_7=O), 1636 (C=C); G_3, 3570 (OH), 1725 (C_9=O), 1690 (C_7=O), 1638 (C=C).

The ^{1}H NMR spectra at 200 MHz and ^{13}C NMR spectra at 50.32 MHz were recorded in C^2HCl_3, with the following results [δ (ppm)]. G_1: ^{1}H NMR, 0.94 (t, J = 7.5 Hz, 3H), 1.03 (s, 3H), 1.30 (s, 3H), 1.34 (s, 3H), 1.35 (s, 3H), 1.44 (s, 3H), 1.71 (q, J = 7.5 Hz, 2H), 4.70 (s, 1H), 7.15 (s, 1H); ^{13}C NMR, 210.7 (C-9), 198.2 (C-7), 141.4 (C-5), 132.4 (C-6), 97.4 (C-1), 82.0 (C-4), 54.9 (C-8), 51.7 (C-10), 30.4 (CH_2), 26.6, 24.0, 21.6, 20.9, 15.2, 7.6 (6 × CH_3). G_2: ^{1}H NMR, 1.01 (dd, J = 7.5 Hz, 3H), 1.03 (s, 3H), 1.28 (s, 3H), 1.30 (s, 3H), 1.35 (s, 3H), 1.36 (s, 3H), 1.78 (m, J_{AB} = 13.5 Hz; J_{AX} = J_{BX} = 7.5 Hz, 2H), 3.70 (s, 1H), 7.20 (s, 1H); ^{13}C NMR, 210.7, 198.3, 143.0, 131.8, 97.3, 81.8, 55.0, 51.6, 29.3, 26.6, 24.1, 20.9, 20.1, 15.1, 7.7 (6 × CH_3). G_3: ^{1}H NMR, 1.00 (s, 3H), 1.08 (s, 3H), 1.25 (s, 6H), 1.28 (s, 3H), 1.43 (s, 3H), 4.09 (s, 1H), 7.09 (s, 1H); ^{13}C NMR, 210.8, 198.5, 143.2, 131.2, 97.3, 79.4, 54.9, 51.7, 26.6, 24.1, 23.9, 23.6, 20.9, 15.2 (6 × CH_3).

The UV spectra were recorded in ethanol G_{1-3} [λ_{max} (nm); ε ($l\,mol^{-1}\,cm^{-1}$)]: G_1, 238, 6950; G_2, 239, 6950; G_3, 237, 6900.

Extraction and purification procedure

The extraction procedure was identical with that applied previously but the purification steps were significantly different [1,7]. Leaves of *E. grandis* (30 g) were pulverized with a pestle and mortar, then placed in methanol (750 ml) and stirred at room temperature for 15 h. The mixture was filtered through a Büchner funnel with a filter-paper and the filtrate was evaporated to dryness. The residue was then dissolved in water (100 ml) and the solution extracted with light petroleum (200 ml). The organic phase was dried over magnesium sulphate and evaporated to dryness. The residue (330 mg) was first separated by silica gel column chromatography [eluent cyclohexane (600 ml) then diethyl ether (200 ml)]. The G substances detected were collected along with other chlorophilic product and the solvent was evaporated (200 mg). Rapid filtration over reversed-phase silica [Amicon C_{18}, 90–130 μm, 20 g, eluent water–methanol (20:80), 150 ml] left 130 mg of product, which was purified by medium-pressure liquid chromatography (RSil C_{18}, 15–35 μm, 25 g, purchased from RSL Alltech, France) using water–methanol (25:75) as eluent (flow-rate 6 ml/min).

We thus obtained in two fractions (volume 25 ml each) the G substances along with a residual impurity that absorbs at the detection wavelength utilized for G_1–G_3 mixture analysis; it was eliminated by filtration of the mixture (50 mg) through a Sep-Pak silica cartridge [eluent ethyl acetate–dichloromethane–light petroleum (4:46:50), 4 ml]. The final purified extract contained principally G_1, G_2 and G_3 (Fig. 1) and was used for quantitative analysis.

The same purification procedure as described above was applied to two other samples of *E. grandis* leaves and was found to be reproducible.

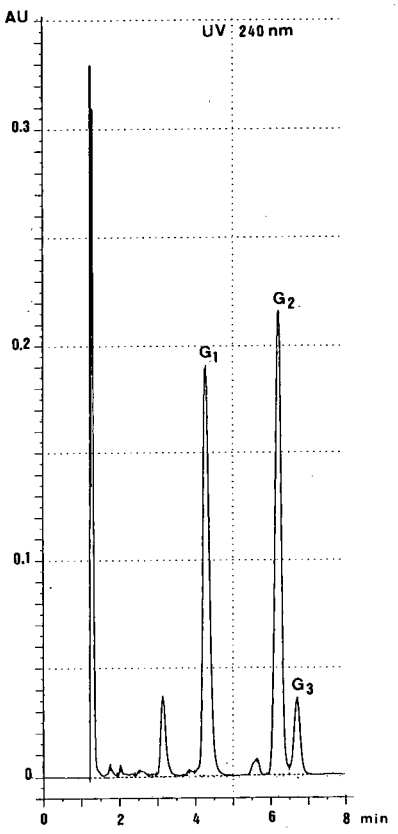

Fig. 1. HPLC of the final purified extract issued from leaves of *E. grandis*. Column, Nova-Pak (4 μm) (15 cm × 4.6 mm I.D.); detection, absorption at 240 nm; mobile phase, isooctane–dichloromethane (35:65); flow-rate, 1 ml/min; temperature, 20°C; injection, 20 μl of *ca.* 2.4 mg of purified extract in 10 ml of dichloromethane. Values on the ordinate are absorbance.

RESULTS AND DISCUSSION

The HPLC separation of an artificial mixture of G_1, G_2, G_3 was performed. Under the conditions described above, the capacity factors (k') obtained were $G_1 = 1.65$, $G_2 = 2.9$ and $G_3 = 3.2$.

Determination of each compound was possible using a G_1–G_3 mixture as external standards. Calibration was performed by plotting known concentrations of mixtures of G compounds *versus* peak area. The proportions of G were chosen in the range estimated from the purified extract ($[G_1]$, $[G_2] = 0.4 \cdot 10^{-4}$–$2.2 \cdot 10^{-4}$ M, $[G_3] = 0.7 \cdot 10^{-5}$ M). The results obtained for four concentration values included in these ranges showed very good linearity (correlation coefficient $r > 0.998$).

Quantitative analysis of a sample of the purified extract was then undertaken. For a 30-mg extract the following average values for three assays of G content were obtained: $G_1 = 7.7 \pm 0.15$, $G_2 = 8.6 \pm 0.12$ and $G_3 = 1.4 \pm 0.03$ mg. Thus the total leaf content of G substances in the *E. grandis* examined was calculated to be 600 μg/g fresh weight.

For a second sample of 60 mg of an extract, obtained before HPLC purfication using RSil, from 17 g of fresh leaves and checked by analytical chromatography, the $(G_1 + G_2)/G_3$ peak-area ratio

was found to be in the same range as that obtained with the preceding extract (9.1 *versus* 10.3), indicating a relative deviation of 12%.

Leaves of two *E. gunii* species were also treated following the same extraction and purification procedure, but we did not detect any G substances under the analytical conditions applied. Nevertheless, we observed major differences in the chromatograms of the two purified extracts of the *E. gunii* samples. Compounds derived from G substances may occur in these species through metabolic transformations so that it will be interesting to extract and identify the substances contained in the purified *E. gunni* extracts.

CONCLUSION

This improved synthesis, purification and characterization of G_1 and G_3 will enable us to examine their chemical behaviour. We have also developed a preparative method for the extraction of G compounds from the leaves of *E. grandis* and measured their content. This method is more convenient and consumes less solvent than previously reported methods.

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Short Communication

Determination of the herbicide diclofop in human urine

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ABSTRACT

A simple and sensitive method for the gas chromatographic determination of diclofop residues in human urine is described. Recoveries of diclofop, as its methyl ester, from fortified urine were greater than 85% at 100, 50, 10 and 1 $\mu g\ kg^{-1}$, and were similar with and without the inclusion of a hydrolytic step in the analytical method. However, a hydrolytic step was necessary for analysis of 24-h urine samples collected from a male applicator following a single exposure to diclofop-methyl during application to wheat using a tractor-pulled sprayer. Diclofop residues determined with hydrolysis were approximately double those without hydrolysis, suggesting that a significant portion of diclofop was excreted in the conjugated form.

INTRODUCTION

Applicators are exposed to pesticides both dermally and by inhalation [1,2] resulting in absorption of these chemicals into the body both through the skin [3] and the respiratory tract [4]. When excreted in the urine, the cumulative urinary excretion of pesticides provides an indirect estimate of the amount of pesticide which entered the body.

Conjugation of acidic herbicides, such as (2,4-dichlorophenoxy)acetic acid (2,4-D) [5], (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T) [6] and (±)-2-[4-(2,4-dichlorophenoxy)phenoxy]propanoic acid (diclofop) [7], is now a well-established metabolic pathway in plants and has also been recognized in animals. For example, conjugates of 2,4-D and 2,4,5-T have been found in the urine of pigs [8] and humans [9], and rats [10], respectively. Thus, in order to accurately determine cumulative urinary excretion of acidic herbicides, such conjugates must be cleaved by the analytical method prior to quantitation. Previous studies involving the urinary excretion of acidic herbicides have included a hydrolytic step to cleave possible herbicide conjugates [11–13].

Several acidic herbicides, such as 2,4-D [11,14,15], (4-chloro-2-methylphenoxy)acetic acid (MCPA) [15], 2,4,5-T [16], (±)-2-(2,4-dichlorophenoxy)propanoic acid (dichlorprop) [15], (±)-2-(4-chloro-2-methylphenoxy)propionic acid (mecoprop) [15] and 3,6-dichloro-2-methoxybenzoic acid (dicamba) [14], are known to be excreted in the urine of exposed applicators. Diclofop (Fig. 1) is an acidic herbicide which is extensively applied as its methyl ester (diclofop-methyl) for the post-emergent control of wild oats (*Avena fatua* L.) and other grassy weeds in a variety of crops. However, there are no published data available regarding human urinary excretion of diclofop following exposure. The objectives of the

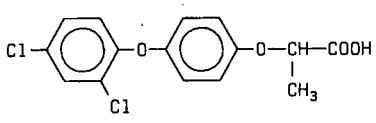

Fig. 1. Structural formula for diclofop.

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present study were (i) to develop, for use in a subsequent applicator exposure study, a sensitive analytical method for the determination of diclofop residues in human urine which included a hydrolytic step to cleave possible conjugates and (ii) to determine whether diclofop conjugates were present in urine collected from an exposed applicator by analysis with and without hydrolysis.

EXPERIMENTAL

Materials

All solvents were distilled-in-glass grade (Caledon Laboratories). The Florisil (Fisher Scientific) was heated at 600°C for 48 h and then deactivated by the addition of 5% (w/w) water. The sodium sulphate was heated at 600°C for 24 h. Deionized water was obtained using the Nanopure II cartridge system (Barnstead). The boron trifluoride–methanol reagent was 14% (w/w) BF_3 (BDH). Analytical standards of diclofop and diclofop-methyl were obtained from Hoechst Canada (Regina, Canada).

Urine collection

Two volunteers, who were not involved in any spray operations, provided urine samples for method development. Later, they also participated as controls by alternately providing 24-h urine samples every second day throughout the 12-days experimental period during the spring spraying season, and also for a 10-days period after the spraying season ended to ascertain background levels.

In order to test the effectiveness of the developed method with and without hydrolysis, composite 24-h urine samples were collected from an exposed applicator beginning 1 day before spraying and then continuously until 10 days after spraying. The samples were collected such that the first void of the day was included as part of the previous day's sample.

All urine voids for each 24-h period were collected in disposable polyethylene-lined, 2.5-l urine specimen storage containers (Fisher Scientific, Cat. No. 14-375-119). The containers were collected and dated each day, and then stored at −10°C until extraction.

Field procedures

The farmer applicator (body weight 109 kg) who provided "exposed" urine samples followed his normal mixing and spraying procedures and did not wear protective gloves or a respirator. Diclofop-methyl [624 g acid equivalent (a.e.) ha^{-1}], tank mixed with bromoxynil (3,5-dibromo-4-hydroxybenzonitrile, 247 g phenol equivalent ha^{-1}), was applied in 110 l water ha^{-1} to 36.4 ha of wheat at the 3- to 4-leaf stage using a tractor-pulled sprayer. The sprayer, equipped with a 910-l tank, a 13-m boom and 28 Tee-jet 6502 nozzles, was operated at a pressure of 275 kPa using a boom height of 51 cm above the crop canopy and a ground speed of 9 km h^{-1}. The tractor used to pull the sprayer was equipped with a closed cab which provided filtered air (dust filter only). The spraying operation lasted 550 min, during which the sprayer tank was filled four times.

Non-hydrolytic extraction

Urine (100 ml), contained in a 250-ml separatory funnel, was acidified to pH 1–2 by the addition of 3 *M* H_2SO_4 and extracted twice with 100-ml portions of methylene chloride. Each methylene chloride extract was passed through 25 ml of anhydrous sodium sulphate (contained in a 9-cm diameter long-stemmed funnel on top of a glass wool plug) into a 250-ml round-bottom flask, followed finally by a 25-ml methylene chloride wash of the sodium sulphate. The combined extracts were taken just to dryness using a rotary evaporator and the extract residue transferred with three 1.5-ml portions of methanol to a 150 mm × 18 mm I.D. test tube.

Boron trifluoride–methanol methylation

Boron trifluoride reagent (4.5 ml) was added to the test tube and the tightly stoppered test tube was heated in a dry block heater at 70°C for 1.5 h. The tube was then cooled by emmersing in an ice–water bath. Saturated NaCl solution (10 ml) and hexane (10 ml) were added and the stoppered tube shaken vigorously for 1 min. The organic layer was transferred by using a disposable Pasteur pipette to a test tube containing 2 g of anhydrous sodium sulphate. The extraction of the aqueous solution was repeated with a further 10 ml of hexane. The combined hexane extracts were decanted from the drying agent into a 40-ml centrifuge tube together with a 5-ml hexane rinse of the sodium sulphate. The hexane solution was evaporated to approximately 1 ml

using a stream of nitrogen gas and the concentrated extract subjected to Florisil column cleanup.

Florisil column cleanup

Florisil (4 ml) was added to a 200 mm × 10 mm I.D. column containing 10 ml of hexane, and topped with 1 cm of anhydrous sodium sulphate. The hexane was drained to the surface of the sodium sulphate and the methylated extract was transferred to the column, including two 1.5-ml hexane rinses of the centrifuge tube. The column was then eluted with 60 ml of 0.5% acetone in hexane solution, the last 48 ml of which were concentrated to approximately 0.5 ml using a stream of nitrogen gas and then taken to volume (1 ml) with hexane.

Hydrolytic extraction

Sodium hydroxide solution (10 ml, 10 *M*) was added to 100 ml of urine contained in a 250-ml flat-bottom flask and the contents gently refluxed (6-unit Vari-heat extraction rack, GCA Corp.) for 4 h. After cooling, the condenser was rinsed with water and then 3 *M* H_2SO_4 (25 ml) was used to acidify the solution (pH 1–2). The acidified urine sample was then extracted with methylene chloride and subsequent sample workup carried out as described above.

Gas chromatographic system

An Hewlett-Packard Model 5890A gas chromatograph, equipped with a ^{63}Ni electron-capture detector and an on-column injector, was used with the Model 7673A autosampler set to inject 2 μl, and the Model 5895A data station. A Hewlett-Packard 30 m × 0.53 mm I.D. HP-1 (film thickness, 0.88 μm) fused-silica column was used with the following temperature program: 70°C for 1 min, then 10°C min^{-1} until 270°C, and hold for 1 min. Using a carrier gas (helium) flow-rate of 5 ml min^{-1} and detector make-up gas (nitrogen) flow-rate of 70 ml min^{-1}, diclofop methyl ester had a retention time of 12.82 min.

Fortification

Urine (250 ml), collected for method development and contained in the polyethylene-lined urine speci-

TABLE I

RECOVERIES OF DICLOFOP FROM FORTIFIED URINE SAMPLES COLLECTED FROM NON-EXPOSED VOLUNTEERS USING HYDROLYTIC AND NON-HYDROLYTIC PROCEDURES

Fortification level ($\mu g\ l^{-1}$)	Sample		Diclofop recovery (%)	
	Volunteer	Date	With hydrolysis	Without hydrolysis
100	2	June 16	88.3	80.4
	2	July 10	117.8	–
	2	July 14	82.4	–
	1	July 20	78.9	77.4
50	2	June 12	77.4	86.8
	1	July 12	83.3	–
	1	July 16	74.2	78.4
	1	July 20	80.6	–
10	1	June 6	89.1	–
	2	June 16	90.2	–
	2	July 10	–	95.3
	1	July 12	81.1	–
	1	July 16	85.1	97.8
1	1	July 10	90.3	–
	1	July 12	84.7	–
	2	July 14	130.0	90.6
	1	July 20	92.9	99.5
Mean ± standard deviation			89.1 ± 14.7	88.3 ± 8.9

men containers, was fortified at 1, 10, 50 and 100 μg kg^{-1} by the addition of diclofop acid (0.25, 2.5, 12.5 and 25 μg, respectively) in 2.5 ml of methanol. The fortified samples were stored at $-10°C$ for a minimum of 24 h prior to extraction. Two replicate samples were analyzed at each fortification level using the non-hydrolytic extraction, whereas four at each level were analyzed using the hydrolytic procedure.

RESULTS AND DISCUSSION

In plants, conjugation of acidic herbicides containing the carboxyl moiety generally involves the formation of an amido linkage by reaction with amino acids/proteins and/or an ester linkage by reaction with sugars, and either type of conjugate could also be expected in exposed animals. Both linkages are susceptible to acid or base hydrolysis and thus, in previous studies [11–13], a hydrolytic step has been part of the analytical method to ensure more accurate quantitation of the parent herbicide.

In the present study, ten of the 24-h urine samples collected from the two "non-applicator" volunteers were analyzed. Half of the samples analysed were collected (June 6–16, 1988) during the normal period of herbicide application on the Canadian prairies with the remainder being collected (July 10–20) following the normal spring spraying season. Background interferences at the retention time for diclofop-methyl for these samples were 0.1 ± 0.1 μg kg^{-1} for both the hydrolytic and non-hydrolyitc methods, and were not significantly greater for urine samples collected during the normal period of herbicide application.

Observed background interferences permitted a limit of quantification of 1.0 μg kg^{-1}. Similar recoveries of diclofop from fortified urine were obtained for both the hydrolytic and non-hydrolytic methods (Table I), and these exceeded 85% (Fig. 2). However, when urine from the exposed applicator was subjected to alkaline hydrolysis (1 *M* NaOH), the concentration of diclofop detected was generally double that from non-hydrolyzed samples (Table II). This indicates that approximately half of the diclofop present in the urine of the exposed applicator was in a conjugated form that would only have been detected after hydrolysis. Consequently, if the hydrolysis had been ommitted, the absorbed dose or amount of diclofop excreted in the urine would have been underestimated by about half. The hydrolytic method had the additional advantage that emulsion problems associated with the methylene chloride extraction were essentially eliminated.

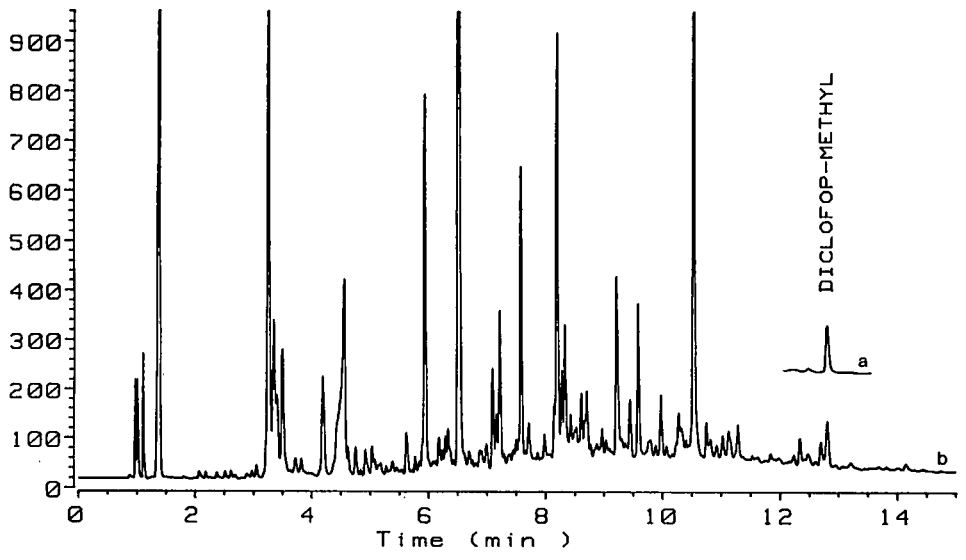

Fig. 2. Gas chromatographic analysis of diclofop residues in urine. Chromatogram a: diclofop-methyl standard (0.2 ng) equivalent to 1 μg kg^{-1}; chromatogram b: recovery (92.9%) of diclofop from urine (volunteer 1; July 20) fortified at 1 μg kg^{-1}.

TABLE II

DICLOFOP CONCENTRATIONS ($\mu g\ l^{-1}$ ACID EQUIVALENT) DETERMINED WITH AND WITHOUT HYDROLYSIS OF URINE SAMPLES COLLECTED FROM THE EXPOSED APPLICATOR

Urine sample	Date	24-h void volume (ml)	Diclofop concentration ($\mu g\ l^{-1}$) in the urine[a]		Total amount (μg) of diclofop excreted in the urine per day	
			With hydrolysis	Without hydrolysis	With hydrolysis	Without hydrolysis
Pre-spraying	June 3	–	–	0.04	–	–
Day of spraying	3	491	0.6	0.4	0.3	0.2
Day 1	4	746	5.7	3.1	4.3	2.3
Day 2	5	948	9.9	5.4	9.4	5.1
Day 3	6	1148	5.5	3.0	6.3	3.4
Day 4	7	1402	7.5	2.7	10.5	3.8
Day 5	8	1260	6.6	2.7	8.3	3.4
Day 6	9	1608	4.4	2.2	7.1	3.5
Day 7	10	961	5.7	4.0	5.5	2.9
Day 8	11	1622	3.1	1.8	5.0	2.9
Day 9	12	893	3.9	2.7	3.5	2.4
Day 10	13	1258	3.9	2.0	4.9	2.5
Cumulated amount					65.1	33.3

[a] Residues are uncorrected for recoveries.

The urinary residue data (Table II) describe an excretion pattern in which the concentration of diclofop in the urine reached a maximum 2 to 4 days after exposure and then continually decreased through to day 10. Similar urinary excretion patterns following dermal exposure have been reported for other acidic herbicides. For example, maximum urinary concentrations have been detected at 1 to 2 days following applicator exposure to MCPA [17] and at 3 days for 2,4-D [18]. However, in contrast to the present study where the urinary concentration of diclofop remained well above background at 10 days after exposure, concentrations of MCPA [17] and 2,4-D [18] returned to background levels 5 and 8 days, respectively, after exposure.

The total amount of diclofop applied by the farmer in the present study was 22.7 kg (a.e.). Cumulative urinary excretion of diclofop (a.e.) following this exposure was 65 μg 10 days after exposure, however, since diclofop residues in the urine remained substantially above background levels even at 10 days after the single exposure (Table II), this represents an underestimation.

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Short Communication

Collaborative study of the determination of cloxacillin by column liquid chromatography

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ABSTRACT

A previously published column liquid chromatographic method proposed for the analysis of cloxacillin preparations was subjected to an interlaboratory collaborative study. The method is rigorously defined in terms of performance requirements, yet allows a degree of flexibility to the individual analyst. Eight participating laboratories submitted results for the analysis of three samples in duplicate. The data from one laboratory were rejected because they failed to meet the prescribed performance criteria. Estimates for the repeatability and reproducibility of the method, expressed as relative standard deviations of the results of the analysis of cloxacillin preparations, were found to be less than 0.65% and 1.33%, respectively.

INTRODUCTION

A more specific and reliable method is needed for the assay of cloxacillin preparations because the current official procedures lack specificity [1,2]. This paper reports the results and evaluation of a collaborative study to validate a column liquid chromatographic (LC) method for the determination of the potency of bulk cloxacillin and cloxacillin capsules and injections. The protocol of analysis was basically that described previously [3], except that it was revised for this study by allowing the individual analyst a degree of flexibility while rigorously defining the performance criteria of the method to maintain control.

The control of the method is maintained by specifically defined minimum performance criteria or a system suitability test. The flexibility of the method lies in the discretion given to the analyst to select the specific analytical system (*i.e.*, instrument, injector, detector and column, etc.). The analyst is encouraged to use individual judgement in adjusting the operating conditions to meet those criteria.

EXPERIMENTAL

Collaborative study

Each participating laboratory was provided with the protocol of analysis and duplicate samples of cloxacillin sodium bulk drug, cloxacillin capsules (China Biological and Chemical Laboratories, Taiwan) and cloxacillin injections (Bristol Industries, Taiwan). These samples were to be measured against a reference cloxacillin sodium sample with a potency of 899.7 μg/mg. Analysts were requested to submit all data from duplicate injections for each sample and to report their calculated assay results. They were also asked to describe specific operating parameters of the instrument system used.

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Instrumentation

Each laboratory was asked to use routine LC equipment. This instrument must be equipped with a 254-nm UV detector and a recording device. In order to obtain a wider diversity of systems, analysts were encouraged to use their own columns. However, only microparticulate reversed-phase packing materials that exhibit some degree of polarity, such as hydrocarbon-bonded silicas, were used.

Reagents

Analytical-reagent grade dimethyl phthalate was purchased from E. Merck (Darmstadt, Germany). Reference material cloxacillin sodium was an NLFD house standard (National Laboratories of Foods and Drugs, Taiwan). Methanol was of LC grade. Glacial acetic acid and acetonitrile were of analytical-reagent grade. Triply distilled water with a resistivity greater than 15 MΩ was used.

Mobile phase

The mobile phase was methanol–4% acetic acid (60:40, v/v). The mobile phase was filtered (0.45-μm Millipore filter) and degassed by ultrasonication prior to use. The mobile phase may be sparged with helium through a 2-μm metal filter for the duration of the analysis.

Internal standard solution

The internal standard, dimethyl phthalate (3 g), was dissolved in 100 ml of acetonitrile–water (1:1).

Standard solution

To an accurately weighed amount of cloxacillin sodium standard, equivalent to 50 mg potency of cloxacillin, was added 0.5 ml of internal standard solution and the volume was made up to 50.0 ml with distilled water.

Sample solution

All solutions of cloxacillin samples were prepared in a manner identical with that of reference material.

Conditions for determination

A constant operating temperature (15–30°C) was maintained. The eluent flow-rate, which was not to exceed 2.0 ml/min, was adjusted to give peaks of satisfactory retention and configuration. The detector sensitivity was adjusted to produce peak heights of 40–90% full-scale deflection, with a chart speed of 0.5 mm/min.

System suitability test

The column was equilibrated with mobile phase. A minimum of three injections of cloxacillin standard solution were chromatographed. The relative standard deviation for the ratio of peak responses should be $\leqslant 2.0\%$. Injection volume for all solutions to be analysed was 10 μl.

Assay and calculations

Identical volumes of carefully measured standard and sample solutions were injected sequentially into the chromatograph. The peak response was normalized to the internal standard and compared with that of the reference material to give the cloxacillin content as follows: $(P_u C_s I_s)/(P_s C_u I_u) \cdot 899.7$ = cloxacillin potency (μg/mg), where P = peak response of cloxacillin, C = concentration of solution, I = peak resposne of internal standard, u = analyte sample and s = reference material. Calculations and data reductions may be performed manually or with a data processing system. Duplicate injections were run for each preparation.

RESULTS AND DISCUSSION

Table I shows the diversity of instrument systems used by the collaborators. The adoption of suitability tests can obviate many problems arising from deficiencies in most analytical instrument systems because they demonstrate whether a particular system can perform satisfactorily.

Most of the collaborators were able to meet the system suitability requirements of the method. However, the data from one laboratory were rejected because they failed to meet the prescribed performance criteria. The times required for the collaborators to complete the analysis of the samples in the study varied from one to several days.

The Dixon test for outliers, when applied to laboratory averages for each sample, showed only one outlier overall. The highest result for capsules, that of laboratory 5, was flagged as an outlier. The data for capsules from laboratory 5 were omitted.

The statistical terms used are those given by the Association of Official Analytical Chemists [4] and/or commonly used by statisticians. Results of the analysis of the samples, together with means and

TABLE I

INSTRUMENT SYSTEMS USED IN COLLABORATIVE STUDY OF LIQUID CHROMATOGRAPHIC METHOD FOR CLOXACILLIN

Laboratory	Instrument	Detector	Injector	Mode[a]	Column[b]	Length × I.D. (cm × mm)
1	Waters 6000A	W-440	U6K	A	μBondapak C_{18}	30 × 3.9
2	Waters 6000A	W-450	U6K	M	Partisil ODS	25 × 4.6
3	Waters 600E	W-481	712WISP	A	μBondapak C_{18}	30 × 3.9
4	Waters M45	W-441	U6K	M	Chemcosorb ODS	15 × 4.6
5	Tosoh CCPD	Linear 204	Rheodyne	M	Nucleosil C_{18}	25 × 4.6
6	Hitachi	L-4000	Rheodyne	M	μBondapak C_{18}	30 × 3.9
7	Waters 600E	W-484	715WISP	M	Nucleosil C_{18}	30 × 3.9

[a] M = Manual; A = automatic.
[b] From manufacturer.

relative standard deviation (R.S.D.), are given in Table II. In addition to the mean, a measure of the precision was also calculated for (a) the within-laboratory standard deviation or repeatability (S_r), (b) the between-laboratories standard deviation or reproducibility (S_R), (c) repeatability relative standard deviation (R.S.D.$_r$) and (d) reproducibility relative standard deviation (R.S.D.$_R$). The R.S.D.$_r$ values were 0.28% for the bulk drug, 0.42% for capsules and 0.65% for injection and the R.S.D.$_R$ values were 0.73% for bulk drug, 1.33% for capsules and 1.01% for injection (Table II).

Collaborators' comments

Most collaborators commented favourably on the method. Collaborator 7 found that the capsule preparation was more easily dissolved in methanol than as specified in distilled water. The prescribed sample dissolution in distilled water was found to turn the solution turbid, which might affect the detector responses. To study this aspect, several concentrations of cloxacillin capsules were prepared in two sets by dissolution in distilled water and methanol. The results obtained using the two sets of solution were not significantly different. Collabo-

TABLE II

COLLABORATIVE RESULTS FOR CLOXACILLIN BULK DRUG AND DOSAGE FORMS

Collaborator	Bulk drug[a] (%)	Capsules[a] (%)	Injection[a] (%)
1	98.9, 98.8	96.8, 97.2	104.2, 104.3
2	97.8, 98.0	96.2, 96.3	106.2, 105,7
3	98.9, 98.5	96.5, 96.3	103.7, 103.6
4	98.1, 98.3	95.3, 94.1	105.2, 103.6
5	97.9, 97.7	104.8[b], 105.0[b]	103.2, 103.2
6	98.9, 99.8	96.6, 96.6	103.9, 105.8
7	97.4, 97.4	93.7, 94.2	105.1, 105.3
Mean	98.3	95.8	104.5
S_r	0.28	0.40	0.68
S_R	0.71	1.26	1.04
R.S.D.$_r$ (%)	0.28	0.42	0.65
R.S.D.$_R$ (%)	0.73	1.33	1.01

[a] Compared with reference substance.
[b] Outlier by Dixon's test.

rator 4 considered that the method was superior with respect to specificity to the official US Code of Federal Regulations microbiological and iodometric methods.

CONCLUSIONS

The collaborative study of the reversed-phase column LC method for the determination of cloxacillin in bulk, capsule and injection preparations showed good reproducibility. The method is now under consideration by the Chinese Pharmacopoeia.

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Short Communication

Cholesteryl acetate as a stationary phase for the gas chromatography of some volatile oil constituents

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(First received January 3rd, 1992; revised manuscript received March 6th, 1992)

ABSTRACT

Cholesteryl acetate provides a useful low-polarity stationary phase in packed columns for the gas chromatography of some volatile oil constituents like terpene hydrocarbons, certain terpenoids and some aromatics. With a high mobile phase flow-rate, it is best used above its melting point as a normal liquid (115°C and more) although it has a narrow mesomeric temperature range below this as a chiral nematic liquid crystal. It can be used to resolve racemic linalol, but not carvone.

INTRODUCTION

We have previously reported on the gas chromatographic behaviour of some volatile oil constituents when chromatographed on packed columns of three nematic liquid crystals [1–4]. These stationary phases were all multi-aromatic linear ether molecules, and showed appropriate affinity for the volatile oil linear aromatics whilst melted to the nematic state, and at temperatures both below this (supercooled or "unmelted") and above it when a normal isotropic liquid {for azoxydiphenetole (ADP) only [3]}. They were not well suited to studying most oxygenated terpenoids.

It seemed useful now to examine a differently shaped liquid crystal to see how general was the previously observed behaviour. Cholesteryl esters are interesting as being bulky rigid polycyclic non-aromatic molecules of terpenoid character with an unsaturated ring, also having a C_8 flexible terpene hydrocarbon branched side chain. Their liquid crystal condition however, is more complex than the simple roughly parallel oriented molecular nematic state of those previously used. Cholesteryl esters form chiral nematic (cholesteric) liquid crystals, with the molecules again arranged parallel, but here in layers, with each layer oriented at an angle to those adjacent to it. These liquid crystals therefore offer solutes a stratified three-dimensional terpenoid rather than an aromatic linear molecule to retain them, suggesting they may have special value for terpenoid gas chromatography.

Barrall *et al.* [5], who first used cholesteryl esters, observed gas chromatographic slope changes (plotting log retention time against column temperature) at 88°C and 105–113°C for the acetate with some *n*-alkanes, benzene and alkylbenzenes. They considered these indicated, under gas chromatographic conditions, the melting point of the cholesteryl acetate and its transition temperature (range, depending on the solute) to a normal liquid. Differential thermal analysis [6] had confirmed the melting point as 88.3°C but given a higher transition point of 118.6°C, the widest mesomeric range of the three

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aliphatic esters they used. A slightly lower transition was indicated by an earlier optical study of a cooling melt as 116.5°C [7], but this is still above the range of Barrall *et al.* They considered this discrepancy was due to the influence of the gas chromatographic support, as first suggested by Dewar and Schroeder [8], yet claimed "the gas chromatographic method appears to be an excellent and general determination for liquid crystal transition temperatures", noting "the elution times increase sharply in the transition to the isotropic liquid". Cholesteryl esters were also used independently as stationary phases in 1966 by the originator of liquid crystals for gas chromatography, Kelker, and Winterscheidt [9]. However, the results were confused because they mixed the bulky benzoate with a linear aromatic liquid crystal. Benzoates were later used by Kirk and Shaw [10] at their melting points, resolving α- and β-androstanes and -enes, but failing with racemic mixtures. They noted "the cholesteric liquid (phase) shows a strong tendency to become supercooled". Subsequently, other cholesteryl esters have been used in capillaries in their supercooled condition for olefinics using the cinnamate [11] and butyrate [12]. Thus after initial use, the acetate, which offers minimal size non-aromatic ester component, has been neglected for twenty-five years.

EXPERIMENTAL

Apparatus

A Pye Unicam GCD gas chromatograph fitted with flame ionisation detector and a wide range amplifier were used with a Hewlett-Packard 3390A recorder/integrator. Oven temperatures were observed with a Technoterm 7300 probe.

Two glass columns were used, 1.5 m × 2 mm I.D., packed with 10% cholesteryl acetate (Sigma) on Chromosorb W AW, 80–100 mesh. The weighed materials were mixed in ethanol and taken to dryness in a rotary evaporator for packing the column. No preheating was used before initial results were obtained, but subsequent behaviour of the columns depended on their history of heating. A high nitrogen mobile phase flow-rate was required, about 40 ml/min, and the detector thus needed a higher flow-rate of hydrogen than usual.

A Reichert hot stage microscope with polarisers was used to observe transition temperatures.

Materials and methods

Sources of some solutes used were as before [3,4] plus citronellal from Sigma, linalyl acetate (TCI, Tokyo), (−)-menthol (Plaimar) used in strong alcohol solution, and pulegone as the main peak from pennyroyal oil (D.G.F., Granada, Spain).

RESULTS AND DISCUSSION

On the hot stage, after an initial melting and cooling, the slowly heated cholesteryl acetate melted at about 112°C to give a birefringent fluid with the typical loop texture of a chiral nematic liquid crystal. These loops disappeared at 116°C when a normal liquid formed. This cholesteryl acetate thus had a short 4°C mesomeric range, and only observations at 115°C could be claimed to be truly made on a liquid crystal. However, previous studies [2–4] gave anticipation of successful use outside this limited range.

Linalol was taken as the relative retention standard, as before [1–4], but caused problems by being resolved more or less into two peaks on the cholesteryl acetate. Under optimum conditions of full resolution (around 125°C) these had almost identical peak areas, as would be expected from a racemic mixture. Comparison with lavender oil indicated that the slower of the two peaks was (−)-linalol, and this was used to calculate relative retention times. No other substance with optical isomers gave double peaks, not even a mixture of (+)-and (−)-carvones.

Gas chromatographic results are presented in Figs. 1 and 2, and show relative retention times against (−)-linalol.

As was found for the multi-aromatic liquid crystals [2,3] cholesteryl acetate exhibits a naive behaviour if it is not taken above a particular temperature, in this case 105°C. Previously, the naive response seen was that relative retention times were less than those obtained on the supercooled liquid crystal at the same temperature. Such naive lower values were only observed now with menthol (Fig. 2), whilst four other solutes (terpenoids included) showed an extension of the melted plot which was not greater than the naive state. On the naive column, (−)-linalol retention time was just less than 0.5 min at 105°C, rising to about 0.9 min at 110°C, and then about 2.9 min at 115°C, with the same

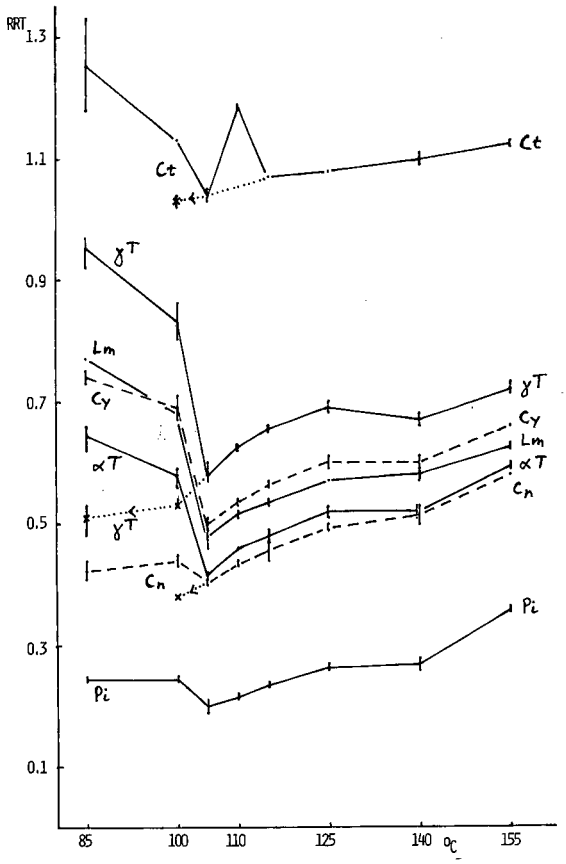

Fig. 1. Plots (connected points) at various temperatures of relative retention times (RRT) against (−)-linalol (1.00) for some volatile oil constituents on a packed column of 10% cholesteryl acatate. Cn = dashes connecting cineole; Ct = citronellal; Cy = dashes connecting *p*-cymene; Lm = limonene; Pi = α-pinene; αT = α-terpinene; γT = γ-terpinene. Dotted lines show supercooled results. Vertical bars depict range of results used for average value, and points show perfect confirmation of first observation.

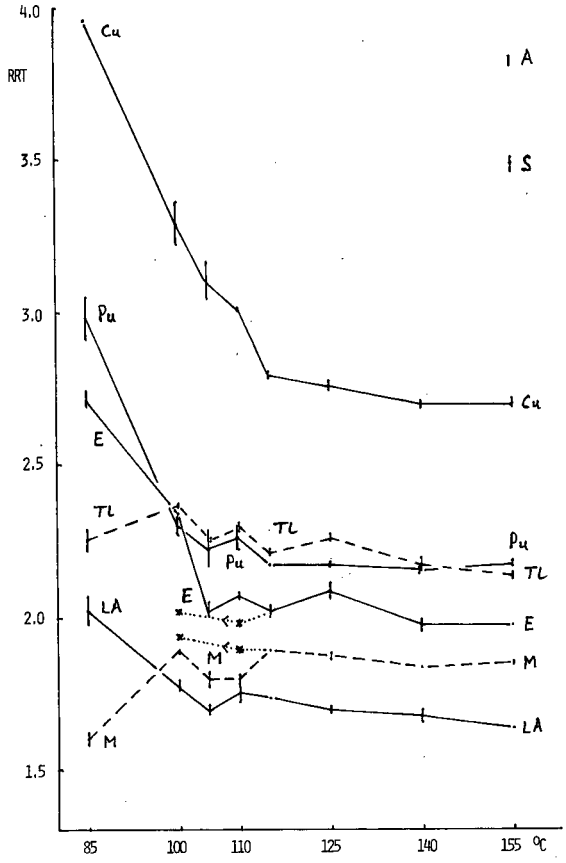

Fig. 2. Plots (connected points) at various temperatures of relative retention times (RRT) against (−)-linalol (1.00) for some volatile oil constituents on a packed column of 10% cholesteryl acetate. Cu = cuminal; E = estragole; LA = linalyl acetate; M = dashes connecting menthol; Pu = pulegone; Tl = dashes connecting α-terpineol. Dotted lines show supercooled results. A = anethole and S = safrole results at 155°C only. Vertical bars depict range of results used for average value, and points show perfect confirmation of first observation.

nitrogen flowrate. After heating to 125°C (linalol about 2.0 min retention) and cooling, values at 105°C were about 3.6 min.

Safrole preceded anethole in elution sequence on cholesteryl acetate, confirming the value of this "test" for liquid crystal phases [3], and this was observed both on a naive column at 100°C (before melting) and at 140°C and 155°C when the phase would be a normal isotropic liquid. At 155°C, the elution sequence of six standard probe solutes previously used [3] was estragole-α-terpineol-cuminal-safrole-anethole-thymol (last), which resembled melted ADP and mature unmelted bis(methoxybenzylidineanil-bi-toluidine) [$(MBT)_2$] apart from transposing their safrole-cuminal sequence. The polysiloxane polyaromatic liquid crystal "MPMS", used in a capillary below its melting point, also yielded safrole after cuminal, [3] like cholesteryl acetate, to which it otherwise seems unrelated.

Volatile oil constituents with short retention times such as the terpinenes, etc., show a rapid fall in relative retention time from 100°C to 105°C (Fig. 1), suggesting a gas chromatographic phase transition of the cholesteryl acetate then, agreeing with

Barrall *et al.* who observed a "break" in their plots [5]. Further heating does not suggest any obvious further transition with these solutes. The 100–105°C change is echoed by the plots for citronellal and estragole (Fig. 2), but these solutes show another fall from 110 to 115°C, agreeing with the microscopical observation of transitions for cholesteryl acetate. Other solutes studied show an angularity in their plots at 115°C, further supporting this, as does a sharp increase in actual retention times. Melted cholesteryl acetate can be used reliably for chromatography, with fairly high gas flow-rate, from 115°C upwards, and gives only slightly changing relative retention times in this its isotropic normal liquid condition. It is probably unwise to use it at lower temperatures, so it is not being used as a true liquid crystal.

Some solute positional shifts in sequence of their retention times were observed here, as previously [2,3] when these were thought to be indicators of changes in condition of liquid crystals. Between 95–100°C on the naive column these are *p*-cymene–limonene, menthol–linalyl acetate and α-terpineol–estragole–pulegone; and they provide some support for the literature melting point of about 90°C for cholesteryl acetate. α-Terpineol–pulegone provide a novel two shifts. The lower temperature one at about 100°C is the consequence of the distinctive initial upward slope seen for the two alcohol plots (menthol and α-terpineol). The higher temperature shift at about 143°C may be caused by partial decomposition of the liquid crystal, as some plots, eg. terpene hydrocarbons, rise from 140°C {as on decomposing $(MBCA)_2$ from 180°C [3]}.

There is hardly any difference, other than quantitative, between the plots of the aromatic cuminal and the terpenoid pulegone, both being smoothly continuous, apart from the angularity at 110°C. Similarly, plots for the aromatic estragole, and α-terpineol, linalyl acetate and citronellal are modified by the inflexions peaking at 110°C. Although linalyl acetate is an ester, like the liquid crystal used, it gave a plot no different to some other solutes tested.

The polarity of the cholesteryl acetate phase was evaluated as before [13], both methods being applied when it was a normal liquid. At 120°C, 2-octyne emerged last after *n*-butanol and pyridine, indicating a low-polar phase. Average retention indices observed were butanol 742, pyridine 831 and octyne 884. This was confirmed at 160°C when the indices for the three probe solutes were 1067 for cineole, 1165 for linalol and 1289 for estragole; their average differed from that of the phenylmethylpolysiloxane base by −59, this "P rating" being similar to $(MBT)_2$ and approaching that of a fully methyl polysiloxane. Such low polarity suggests that cholesteryl acetate is suited to the gas chromatography of terpene hydrocarbons and similar substances, as it is. The "standard" sequence [4] of α-pinene, α-phellandrene–limonene–γ-terpinene was seen whether melted or unmelted, with cineole about level with α-phellandrene (well ahead on naive phase), *p*-cymene just behind limonene (ahead if naive at 85°C), and α-terpinene just behind α-phellandrene. This is like the sequence on melted ADP and $(MBT)_2$, except that the aromatic *p*-cymene is not the last to emerge, reflecting the absence of aromatic structure from cholesteryl acetate. It should be used above its melting point, and with a high gas flowrate could be used to assay the constituents of oils of citronella, *Eucalyptus citriodora*, lavender, coriander, peppermint, basil, tarragon, pennyroyal, cumin and perhaps others, as a supplement to conventional phases, although it resolves no chiral solute pairs other than linalol.

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Short Communication

Effect of column material on sorption isotherms obtained by inverse gas chromatography

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ABSTRACT

The dependence of sucrose sorption isotherm data obtained by inverse gas chromatography on column material was studied. Columns made of polyamide glass, copper and stainless steel were used. The results obtained indicate that the interaction between water vapour and the column wall cannot be ignored when products with very low equilibrium moisture content are considered.

INTRODUCTION

Most of the current methods for determination of water sorption isotherms rely upon the measurements of the changes in weight of samples equilibrated in atmospheres with different relative humidities, as described by Labuza [1]. A different procedure that is particularly suitable for the study of the lower region of water activity and for products with very low equilibrium moisture contents uses inverse gas chromatography (IGC), and has been extensively used by Smith *et al.* [2].

With IGC the sorbed solute is injected into the carrier gas stream and its linear transport is retarded owing to interaction with the product under study, which constitutes the stationary phase. Kiselev and Yashin [3] derived a method for calculating sorption isotherms, based on Glueckauf's [4] theory, using the chromatographic data obtained. The development of this theory results in the following equations, which relate chromatograph operating parameters and peak data to the sorption isotherm

$$a = \frac{m_a I_{ads}}{m I_{pic}} \quad (1)$$

where a = uptake of sorbed water (g/g stationary phase), m_a = mass of water injected (g), m = mass of stationary phase (g) and I_{ads}/I_{pic} = ratio of the areas (A + B)/B calculated from the chromatogram (Fig. 1), and

$$p = \frac{m_a h R T}{I_{pic} W} \quad (2)$$

where p = partial pressure (atm), h = peak height (detector units), R = gas constant (82.0567 cm^3

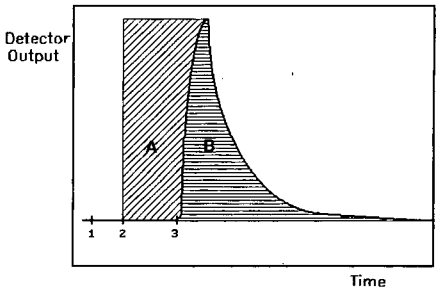

Fig. 1. Typical gas chromatogram obtained by IGC. 1 = Point of injection; 2 = point of emergence of unadsorbed peak (air); 3 = point of emergence of probe peak (water). I_{pic} = Area B; I_{ads} = area A + B.

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atm mol^{-1} K^{-1}), T = absolute temperature (K), W = flow-rate of carrier gas (cm^3/min) and I_{pic} = area B in Fig. 1 (detector units min).

IGC provides a rapid and reliable method for the determination of sorption isotherms, but some attention is required to the choice of the material for the chromatographic column, particularly with very low equilibrium moisture contents.

EXPERIMENTAL

A Perkin Elmer SIGMA 3B dual-column gas chromatograph with a thermal conductivity detector was used. Helium (2 ppm water, 6 ppm maximum impurity) at a flow-rate of 60 cm^3/min was used as the carrier gas.

The study as conducted at 45°C with sucrose as the stationary phase. A 50–63 mesh fraction of sucrose (0.03% moisture content, 0.007% ash and 0.002% inverted sugars, dry weight basis) obtained from Refinarias de Açucar Reunidas (Porto, Portugal) was packed into 5 ft. × 0.25 in. I.D. columns. Columns made from polyamide, glass, copper and stainless steel were used. Distilled water was injected in amounts ranging from 0.2 to 2 μl. In order to remove most of the moisture contained in the sucrose, the columns were purged for 24 h with dry helium before use. Injector and detector temperatures were kept at 150°C.

Detector data were acquired by an XT-type microcomputer using a custom-built amplifier and a 12-bit dual-slope integrator analogue-to digital converter from Digital Design & Development (UK). Data collection and calculations were made by an existing computer program, CROMADATA, developed by Cardoso and Sereno [5].

RESULTS AND DISCUSSION

The operating conditions were similar to the ones described by Smith *et al.* [2]. Water sorption isotherms calculated using eqns. 1 and 2 are plotted in Fig. 2. Each data point represents the mean value of three determinations with the same amount of injected water.

The set of data obtained with each column was fitted to the GAB (Guggenheim [6], Anderson [7] and De Boer [8]) equation using a non-linear regression program written by Sereno and Medeiros [9].

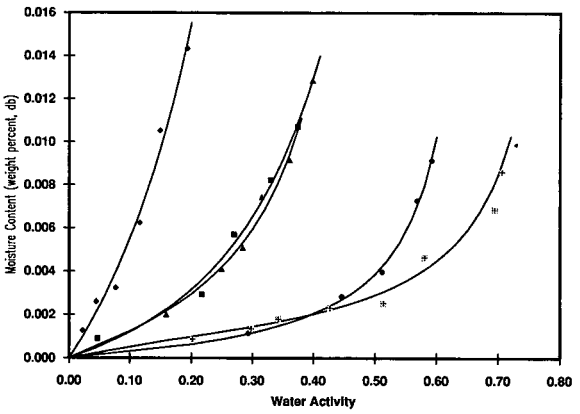

Fig. 2. Sorption isotherms for sucrose by IGC with different columns: polyamide (◆), glass (▲), copper (■), stainless steel (this work) (●) and stainless steel (Smith *et al.* [2]) (+). The solid lines represent the result obtained by fitting the data to the GAB equation.

The dependence of the results on column material is clear.

The results obtained by Smith *et al.* [2], using a stainless-steel column are also plotted in Fig. 2. The observed deviations are throught to be caused by different characteristics or ash contents of the sucrose crystals as implied in the works of Bienenstock and Powers [10], Rodgers and Lewis [11] and Bagster [12].

In conclusion, while IGC remains a fast and reliable technique for the experimental determination of water sorption isotherms of particulate or ground solids, the results of this research indicate that the interaction between water vapour and the column wall cannot be ignored when products with very low equilibrium moisture content are considered.

ACKNOWLEDGEMENTS

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Short Communication

Thermal desorption–gas chromatography for the determination of benzene, aniline, nitrobenzene and chlorobenzene in workplace air

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ABSTRACT

Sampling on Tenax TA of different mesh sizes followed by thermal desorption and gas chromatography was evaluated as a simple method for the determination of benzene, aniline, nitrobenzene and chlorobenzene in the workplace air. An alternative sampling technique in place of pump sampling was developed. Quantitative recoveries were obtained in the mass range 0.04–10 μg. It was found that air humidity had no effect on recovery. The charged tubes can be stored at room temperature for 5 days with no change in recovery. The particle size of Tenax TA has no significant effect on adsorption and desorption.

INTRODUCTION

The demand for accurate, reliable and sensitive techniques for the monitoring of organic trace pollutants in the workplace atmosphere has increased tremendously. Chromatography is a widely used technique which allows the identification and the determination of organic compounds in sub-nanogram amounts. Because of the relatively low concentrations of organic contaminants in the workplace air, most chromatographic methods for monitoring these contaminants requires a preconcentration step before the actual analysis. Traditional preconcentration of organic vapour used in most NIOSH procedures [1] utilizes charcoal or silica gel as the adsorbent, followed by solvent desorption and chromatographic analysis. A newer alternative technique uses a porous polymer adsorbent and thermal desorption into a gas chromatograph [2–5], which offers the best sensitivity with respect to solvent extraction as the whole sample is injected [6,7]. Hence this technique has gained wide acceptance in a variety of applications, including environmental trace analysis [8–14], industrial hygiene [15], stack sampling [16] and personal monitoring [17].

Among the various organic environmental pollutants, benzene, aniline, nitrobenzene and chlorobenzene are particularly poisonous with acute and chronic effects. The threshold limit values (TLVs) for aniline, nitrobenzene, benzene and chlorobenzene are 10, 5, 30 and 350 mg/m^3, respectively, for an

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8-h exposure [18]. The inhalation and absorption through the skin of nitrobenzene and aniline lead to cynosis with the formation of methaemoglobin [19]. Benzene is a known carcinogen causing aplastic anaemia, leukaemia and other types of cancer [20].

In a recent report, aniline in air was adsorbed on silica and desorbed with methanol for determination by HPLC with a desorption efficiency of 96% [21]. Nitrobenzene was detected in workplace air by adsorption on charcoal and Tenax GC with desorption efficiencies of 91% with toluene and 100% with diethyl ether [22]. Regarding the determination of benzene in the workplace air using solid sorbent adsorption and thermal desorption, Brown [23] has carried out extensive work and reviewed the subject. However, to our knowledge, no work has been reported so far on the thermal desorption of aniline and nitrobenzene.

The aim of this work was to determine trace levels of benzene, aniline, nitrobenzene and chlorobenzene in the workplace air in plants involving the processes of nitration of benzene to nitrobenzene and the reduction of nitrobenzene to aniline. Chlorobenzene occurs owing to the chlorination of benzene being carried out in an adjacent plant. The development of an alternative sampling technique in place of the conventional pump sampling method is also reported.

EXPERIMENTAL

Benzene, chlorobenzene, aniline, nitrobenzene and methanol were of analytical-reagent grade from Fluka (Buchs, Switzerland). Tenax TA of different mesh sizes (20–35, 35–60 and 60–80) was purchased from Alltech (Deerfield, IL, USA) and from Chrompack (Middelburg, Netherlands).

A Perkin-Elmer gas chromatograph equipped with an automatic thermal desorption system (ATD-50) was used. The ATD-50 was coupled via a heated transfer capillary to the GC column. GC analysis was carried out with flame ionization detection detector using a GP-100 printer–plotter. Stainless-steel sample tubes (Perkin-Elmer) of 89 mm × 5 mm I.D. with stainless-steel wire gauges on both ends to hold the adsorbent and having metal sealing caps on both sides for storage were used.

Solid adsorbent

Commonly used adsorbents are the Chromosorb Century series [13,15], the Porapak series [2,4], Amberlite XE [24] and Tenax GC, the most popular being Tenax GC (a polymer of 2,6-diphenyl-*p*-phenylene oxide), which has excellent properties for trapping the pollutants and of thermal stability [25–27].

Although Tenax GC, after proper pretreatment, has been shown to be useful as a trapping material, some problems still exist. Often significant amounts of aromatic hydrocarbons are observed in blank chromatogram of this material. However, by changing the process conditions during the preparation of Tenax, a new type of material, Tenax TA, was obtained. Tenax TA shows a substantial reduction in the contribution of impurities to the blank values, especially with regard to aromatic hydrocarbons. Hence, the adsorption characteristics of three different particle sizes of Tenax TA were evaluated and its utility in workplace air monitoring of benzene, chlorobenzene, aniline and nitrobenzene was studied.

Determination of retention volumes and safe sampling volumes

Glass chromatographic columns (2 m × 2 mm I.D.) were packed with known weights of Tenax TA of 20–35, 35–60 and 60–80 mesh sizes. After packing, each column was conditioned by passing pure nitrogen through for 45 min at room temperature, then heating at a rate of 2°C/min to 300°C and maintaining this temperature for 16 h. Standard solutions of benzene, chlorobenzene, aniline and nitrobenzene were injected at various temperatures (150–275°C) with a nitrogen flow-rate of 25 ml/min.

Standardization of the method by GC

Standard solutions. Stock standard solutions (1%) of benzene, chlorobenzene, aniline and nitrobenzene were prepared in methanol. Working standard solutions to cover the range of interest (0.04–10.0 μg/μl) were prepared by serial dilution of the stock standard solution with methanol.

Calibration. A 1-μl volume of each standard solution was injected on to the GC column under the conditions specified below.

GC conditions. The following conditions were used: column, stainless steel (2.25 m × 2 mm I.D.);

column packing, Tenax TA (60–80 mesh); injection port temperature, 250°C; flame ionization detector temperature, 275°C; oven temperature 185°C for 1 min, increased at 30°C/min to 240°C, held for 6 min; carrier gas (nitrogen) flow-rate, 30 ml/min; and chart speed, 5 mm/min.

The values of area under the curve are the means of five independent observations for each concentration of the standard sample of interest.

Thermal desorption recovery

To determine the thermal desorption efficiency of Tenax TA adsorbent of three different mesh sizes, 0.3 g of material was placed in sample tubes and conditioned under a flow of nitrogen (20 ml/min) at 350°C overnight. The conditioned sample tubes were fitted into GC injection port maintained at 250°C with a nitrogen flow-rate of 15 ml/min. A set of five tubes for each mesh size of Tenax TA were spiked with 1 μl of each standard solution at room temperature. The spiked tubes were disconnected after 2 min and thermally desorbed under the following optimum desorption conditions: desorption temperature, 250°C; desorption time, 10 min; transfer line temperature, 150°C; cold trap low, −30°C; cold trap high, 300°C; and cold trap adsorbent, Tenax TA (60–80 mesh).

The samples were analysed under GC conditions given above.

Workplace air sample collection

Generally, workplace air sampling on a solid sorbent has been carried out by drawing the air with a constant flow-rate pump. The high capital expenditure for large numbers of such pumps limits the number of sampling locations and the sampling frequency in the workplace. Further, repeated pump failure and maintenance forced us to develop an alternative air sampling technique which is economical and as accurate as pump sampling. In this sampling technique, water is displaced from an air-tight glass bottle (10 l) by siphoning to obtain an accurate and constant flow of air into the bottle through the sample tube. To keep the back-pressure constant to achieve a constant flow, each sample tube was filled with an identical amount (0.3 g) of Tenax TA adsorbent of three mesh sizes. To maintain a constant flow of water withdrawal from the

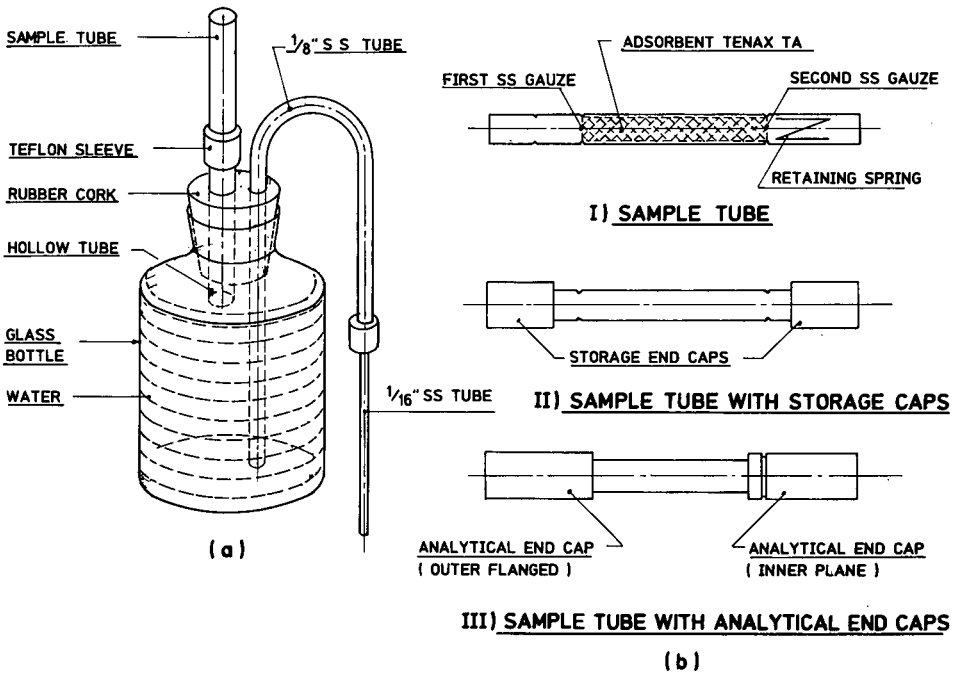

Fig. 1. (a) Diagram of sampler; (b) diagrams of sample tube. I = Sample tube; II = sample tube with storage caps; III = sample tube with analytical end-caps. SS = Stainless steel.

bottle siphoning, the water was siphoned through 1/8- and 1/16-in. stainless-steel tubing connected to each other. The variation in the length of each piece of tubing gave different constant flow-rates over a period of time with an accuracy of $\pm 2\%$, which is comparable to that with pump sampling. It was ensured that the water taken in the bottle was free from particles. The sampling system and sample tube with different end-caps are shown in Fig. 1.

The sample tubes containing 0.3 g of Tenax TA of three mesh sizes, conditioned as mentioned earlier, were used for sampling at each location. When selecting the four locations, emphasis was placed on the sources of potential exposure, such as sample taps, vents, manholes and drains, where the operator may be exposed to the process chemicals. During sampling, care was taken to keep each sampling set as close as possible to each other at every location so as to have the same concentration over three sampling tubes. The amounts of sample collected ranged from 4 to 8 l at rates of 25–30 ml/min. Tubes were sealed immediately for analysis.

RESULTS AND DISCUSSION

In order to establish the retention volume and safe sampling volume, standard solutions of benzene, chlorobenzene, aniline and nitrobenzene were injected at various temperatures (150–275°C) with a nitrogen flow-rate of 25 ml/min. The retention volumes of each analyte at different temperatures were recorded. The logarithm of specific retention volume was plotted against the reciprocal of the absolute column temperature, which gave a linear relationship. The retention volumes at 20 and 25°C were obtained by extrapolation [24] (Table I). Half of the retention volume was taken as the safe sampling volume, which is defined as the volume of air containing a particular vapour contaminant that may be sampled under a variety of circumstances without significant breakthrough [28].

The calibration graph for each analyte was obtained by plotting average peak area against known concentrations of the compounds in the range 0.04–10.00 $\mu g/\mu l$. A typical chromatogram of a mixture is shown in Fig. 2. The results for the concentration of each compound injected and measured are presented in Table II, together with standard deviation for each measurement. The calibration graphs were linear; the equations of the regression lines and regression coefficients for benzene, chlorobenzene, aniline and nitrobenzene are presented in Table III.

Recovery studies on the thermal desorption were carried out using different mesh sizes of Tenax TA for various concentrations of organic compounds. In general, no differences in recoveries were found on the different mesh sizes of Tenax TA, except for the recoveries of aniline, which were marginally better on Tenax TA (20–35 mesh). The results given in the Table IV indicate nearly quantitative recoveries of all the analytes at all levels of spiking in sample tubes. As the recoveries of the analytes were found to be independent of the mesh size of Tenax TA, only the data for 60–80-mesh Tenax TA are presented in Table IV. A typical chromatogram of thermal desorption is shown in Fig. 3. It was consistently observed that the blank values in ther-

TABLE I

ADSORPTIVE PROPERTIES OF TENAX TA

Compound	Retention volume (l/g)[a]			
	Tenax TA (60–80 mesh)	Tenax TA (35–60 mesh)	Tenax TA (20–35 mesh)	Tenax GC (60–80 mesh)
Benzene	38 (57)	22 (31)	13 (19)	36 (54)
Chlorobenzene	270 (400)	200 (314)	160 (221)	181 (284)
Aniline	1408 (2208)	1635 (2697)	943 (1480)	1212 (1900)
Nitrobenzene	1270 (7331)	6002 (9897)	2565 (4230)	3463 (5710)

[a] Values at 25°C; values in parentheses are retention volumes at 20°C.

TABLE II

STANDARDIZATION OF GC METHOD

Each value is average of five independent measurements.

Standard No.	Concentration (μg/ml)	Benzene	Chlorobenzene	Aniline	Nitrobenzene
1	Taken	0.040	0.040	0.040	0.040
	Found	0.040	0.041	0.042	0.040
	S.D.	0.001	0.003	0.003	0.003
2	Taken	0.200	0.203	0.202	0.201
	Found	0.201	0.206	0.189	0.199
	S.D.	0.007	0.012	0.009	0.007
3	Taken	0.400	0.407	0.404	0.403
	Found	0.403	0.409	0.388	0.405
	S.D.	0.017	0.022	0.018	0.015
4	Taken	1.200	1.220	1.212	1.208
	Found	1.220	1.234	1.223	1.232
	S.D.	0.029	0.034	0.035	0.037
5	Taken	2.000	2.033	2.020	2.013
	Found	1.923	1.950	1.945	1.933
	S.D.	0.046	0.042	0.045	0.041
6	Taken	2.800	2.846	2.828	2.818
	Found	2.957	2.879	2.894	2.855
	S.D.	0.074	0.073	0.073	0.073
7	Taken	3.600	3.659	3.636	3.623
	Found	3.701	3.670	3.788	3.723
	S.D.	0.027	0.029	0.036	0.035
8	Taken	4.400	4.473	4.444	4.429
	Found	4.410	4.475	4.551	4.461
	S.D.	0.073	0.079	0.127	0.118
9	Taken	5.200	5.286	5.252	5.239
	Found	5.202	5.276	5.383	5.258
	S.D.	0.132	0.130	0.085	0.088
10	Taken	6.000	6.099	6.060	6.039
	Found	6.010	6.096	6.205	6.052
	S.D.	0.115	0.125	0.148	0.140
11	Taken	8.000	8.132	8.080	8.052
	Found	7.875	8.031	8.149	7.955
	S.D.	0.312	0.311	0.252	0.252
12	Taken	10.000	10.165	10.100	10.065
	Found	9.763	10.040	10.048	9.899
	S.D.	0.274	0.30	0.252	0.296

TABLE III

LINEARITY OF ANALYTICAL PROCEDURE

Compound	Regression equation[a]
Benzene	$y = 36.739x + 2.049$; $r = 0.999$
Chlorobenzene	$y = 25.142x + 0.936$; $r = 0.999$
Aniline	$y = 28.036x + 0.779$; $r = 0.999$
Nitrobenzene	$y = 22.274x + 1.002$; $r = 0.999$

[a] y = Peak area; x = concentration; r = regression coefficient.

mal desorption on Tenax TA were low (10–20 ng) compared with the values obtained on Tenax GC (40–60 ng).

Comparison of the results in Table II for standardization of the GC method and in Table IV for recovery studies shows good agreement, indicating that the adsorption of substances from the dry nitrogen calibration mixtures and subsequent thermal desorption were complete under the conditions

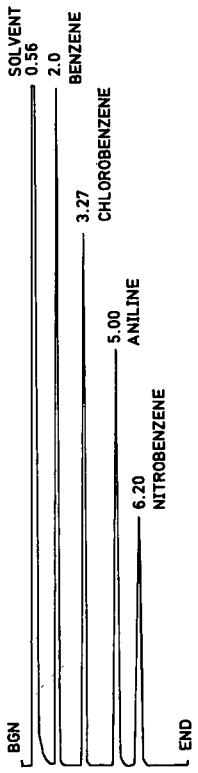

Fig. 2. Typical chromatogram showing the separation of benzene, chlorobenzene, aniline and nitrobenzene. BGN = Start; numbers at peaks indicate retention times in min.

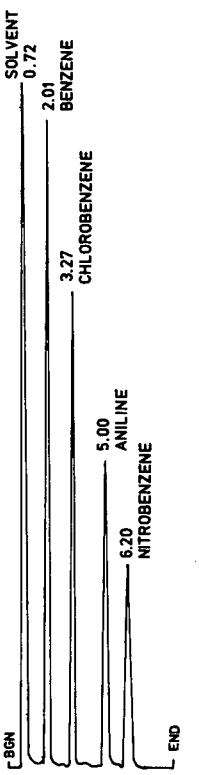

Fig. 3. Typical chromatogram for the thermal desorption recovery of benzene, chlorobenzene, aniline and nitrobenzene.

TABLE IV

THERMAL DESORPTION RECOVERY ON TENAX TA (60–80 MESH)

Standard No.	Recovery (μg)[a]			
	Benzene	Chlorobenzene	Aniline	Nitrobenzene
1	0.041 ± 0.006 (102)	0.056 ± 0.010 (121)	0.051 ± 0.006 (122)	0.041 ± 0.01 (93)
2	0.235 ± 0.008 (117)	0.237 ± 0.017 (115)	0.199 ± 0.033 (105)	0.121 ± 0.045 (105)
3	0.411 ± 0.009 (102)	0.414 ± 0.007 (101)	0.354 ± 0.015 (91)	0.394 ± 0.015 (98)
4	1.226 ± 0.018 (100)	1.30 ± 0.013 (105)	1.137 ± 0.039 (93)	1.182 ± 0.039 (96)
5	2.033 ± 0.041 (106)	2.107 ± 0.013 (108)	1.839 ± 0.046 (94)	1.926 ± 0.048 (100)
6	2.853 ± 0.024 (100)	2.953 ± 0.016 (102)	2.695 ± 0.032 (93)	2.746 ± 0.049 (96)
7	3.644 ± 0.030 (98)	3.825 ± 0.028 (104)	3.538 ± 0.045 (93)	3.698 ± 0.035 (99)
8	4.425 ± 0.262 (100)	4.755 ± 0.156 (106)	4.406 ± 0.147 (97)	4.502 ± 0.109 (102)
9	5.237 ± 0.028 (101)	5.545 ± 0.041 (105)	5.227 ± 0.105 (97)	5.248 ± 0.117 (100)
10	6.047 ± 0.052 (101)	6.409 ± 0.033 (105)	6.122 ± 0.052 (99)	6.168 ± 0.072 (102)
11	7.907 ± 0.063 (100)	8.433 ± 0.059 (105)	8.002 ± 0.083 (98)	8.034 ± 0.099 (101)
12	9.867 ± 0.091 (101)	10.640 ± 0.100 (106)	10.243 ± 0.109 (102)	10.054 ± 0.129 (102)

[a] Each value is average of five independent measurements ± standard deviation. The values in parentheses are percentage recoveries.

TABLE V

RESULTS OF ANALYSIS OF WORKPLACE AIR SAMPLES

Sample No.	Mesh size	Concentration (mg/m^3)			
		Benzene	Chlorobenzene	Aniline	Nitrobenzene
1	20–35	0.020	0.005	0.401	0.050
	35–60	0.020	0.004	0.417	0.070
	60–80	0.019	0.005	0.394	0.069
2	20–35	0.014	0.006	0.385	0.116
	35–60	0.014	0.006	0.339	0.098
	60–80	0.014	0.007	0.244	0.083
3	20–35	0.012	0.013	0.792	0.098
	35–60	0.013	0.014	0.754	0.092
	60–80	0.011	0.012	0.668	0.085
4	20–35	0.017	0.021	0.259	0.065
	35–60	0.030	0.037	0.280	0.134
	60–80	0.021	0.016	0.174	0.046
5	20–35	0.077	0.074	0.448	0.101
	35–60	0.081	0.067	0.355	0.077
	60–80	0.076	0.073	0.334	0.087
6	20–35	0.018	0.010	0.289	0.072
	35–60	0.019	0.011	0.268	0.074
	60–80	0.019	0.012	0.256	0.069
7	20–35	0.030	0.022	0.557	0.064
	35–60	0.032	0.025	0.546	0.063
	60–80	0.030	0.022	0.575	0.067
8	20–35	0.041	0.021	0.070	0.118
	35–60	0.041	0.020	0.062	0.129
	60–80	0.040	0.022	0.067	0.126
9	20–35	0.027	0.053	0.117	0.099
	35–60	0.025	0.032	0.099	0.086
	60–80	0.024	0.021	0.078	0.078
10	20–35	0.022	0.022	0.072	0.084
	35–60	0.038	0.025	0.078	0.106
	60–80	0.027	0.028	0.083	0.118

specified. In order to check for potential losses during field sampling, charged tubes were exposed to a stream of air with various relative humidities, generated by volumetric mixing of air with water-saturated air. The charged samples tubes were then purged with air at various relative humidities of air over different periods of time. The recoveries were almost complete within the relative standard deviation of the whole method (1–4%), indicating that relative humidity has no effect on sampling over Tenax TA. Similar observations were reported earlier [28,29].

The results of workplace air monitoring of organic pollutants using the proposed sampling technique are shown in Table V. The data obtained at each location on three different mesh sizes of Tenax TA are in reasonable agreement with each other. In conclusion, this work has clearly demonstrated the applicability of Tenax TA for the monitoring of airborne benzene, aniline, nitrobenzene and chlorobenzene in the workplace environment in conjunction with thermal desorption and gas chromatography.

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Short Communication

Reactor for prechromatographic fusion reactions

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ABSTRACT

An improved design of a reactor for alkaline fusion as a preliminary to chromatographic analysis is described. The reactor allows the use of a significantly reduced sample size, minimizes leakages and facilitates the removal of the reaction products. The use of the reactor is demonstrated by the analysis of several polyester samples exhibiting increased hydrolytic stability.

INTRODUCTION

The prechromatographic degradation of many materials including some polymers using hydrolytic cleavage was developed by Siggia and co-workers [1–4] and was described as fusion reaction gas chromatography. The technique employed a device constructed from an obsolete furnace pyrolyser attached to the injection port of a gas chromatograph. The apparatus was a modification of a furnace pyrolyser [5] formerly marketed by Perkin-Elmer (Norwalk, CT, USA) (pyrolysis accessory 154-0825). For volatile degradation products a cold trap was inserted between the reactor and the chromatograph. This was immersed in liquid nitrogen during the reaction and then heated, with the contents being swept directly into the chromatograph.

Fusion reactions and chromatographic detection as pioneered by Siggia and co-workers was extended by Glading and Haken [6] with semi-microfusion conducted externally to the gas chromatograph with heating for 0.5 h at 360°C. Subsequently the procedure with the associated work-up and derivatization has been applied to many condensation polymers possessing considerable hydrolytic stability, including polyamides [7], polyamides [8], polyurethanes [9] and polyesters [10], the polyesters having also been cross-linked using amine–formaldehyde resins [11].

The advantages of separate microfusion far outweigh those of *in situ* reaction and include the following: water necessary for the reaction remains in the reaction environment rather than tending to be preferentially swept from the reactor; the ability to conduct multipe fusions without restricting the use of the gas chromatograph or, more important, of restricting the examination to samples amenable to gas chromatography; the examination of materials normally retained in the reactor as potassium soaps or low-volatility compounds after appropriate chemical work-up and/or derivatization; the elimination of the presence of low-volatility compounds which gradually bleed or decompose into the system; the use of a diversity of chromatographic procedures, *i.e.*, gas chromatography–mass spectrometry, high-performance liquid chromatography and size-exclusion chromatography; the use of diethyl

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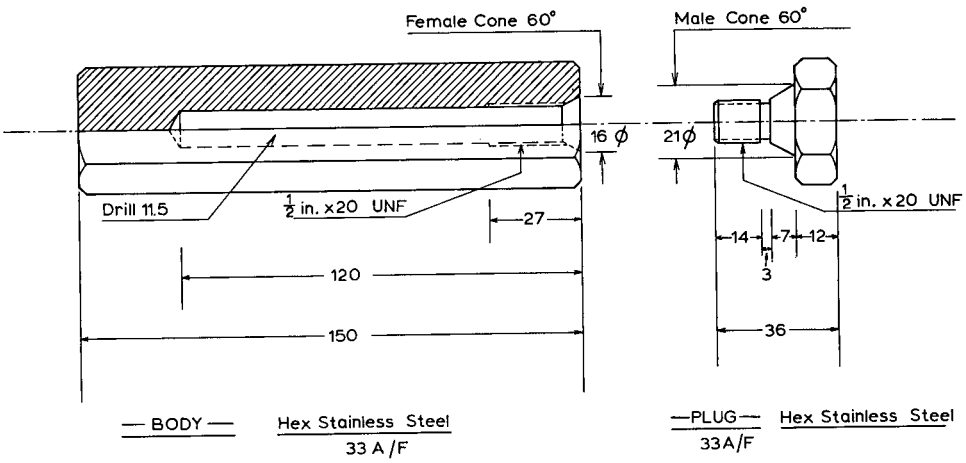

Fig. 1. Plan of stainless-steel pressure tube reactor. All dimensions in millimetres. ∅ = Diameter.

ether cleavage in addition to hydrolysis to be carried out singly or simultaneously; and the analysis of the complete molecule rather than solely volatile cleavage products to be conducted.

The reactions were first conducted in glass pressure tubes [6] but some dissolution of silica occurred and with the determination of silica from siloxanes as an organic derivative the procedure involved interferences. The metal reactor first used consisted of a stainless-steel screw-capped pressure tube as shown in Fig. 1. The cavity in which the reaction was conducted was 120 mm × 16 mm I.D. [12]. Limitations of the reactor included some difficulties in opening and sealing and preventing leakage during reaction. Owing to the relatively large volume of the reactor (24.0 cm^3), a relatively large sample, *i.e.*, 200 mg, was used to minimize losses associated with the difficulties of removing the reactants from the reactor.

A smaller (shorter) version of the reactor shown in Fig. 1 was constructed but difficulties in preventing leakage during the reaction were greatly accentuated owing to the higher internal pressure. The diameter of the reactor was 3 mm with the total length of the cavity being 35 mm. This reactor was also unsatisfactory as it was virtually impossible to remove the melt and the finer thread of the sealing plug often could not be removed from the body.

The quantitative nature of analytical fusion reactions was demonstrated by Siggia and co-workers in work with sulphonic acids [13], simple esters and polymeric esters [1], amides, urea and nitrile compounds [14] and polysiloxanes [3]. The initial work from this laboratory employing work-up of the potassium salts considered the quantitative examination of nylons [5] and subsequently quantitative studies of silicone polyesters [15] and cross-linked polyesters [16] were reported and the quantitative nature of the reaction was reviewed [17].

While the quantitative nature of the fusion reactions has been demonstrated, it has been shown [17] that the extraction steps in the work-up and derivatization steps introduce errors which may be as high as several percent. Recent studies [18] have eliminated or reduced the extraction steps and the losses have been reduced.

This paper reports the development of an alternative reactor which eliminates the problems previously experienced. Use of the reactor with a considerably reduced sample size, *i.e.*, 20 mg, is demonstrated with the degradation of two reactive polyesters and polyethylene terephthalate.

EXPERIMENTAL

Reactor

The stainless-steel reactor developed is shown in Fig. 2 and consists of two parts, namely the reactor shell and the flange. The reactor shell is a square stainless-steel block containing a cylindrical cavity

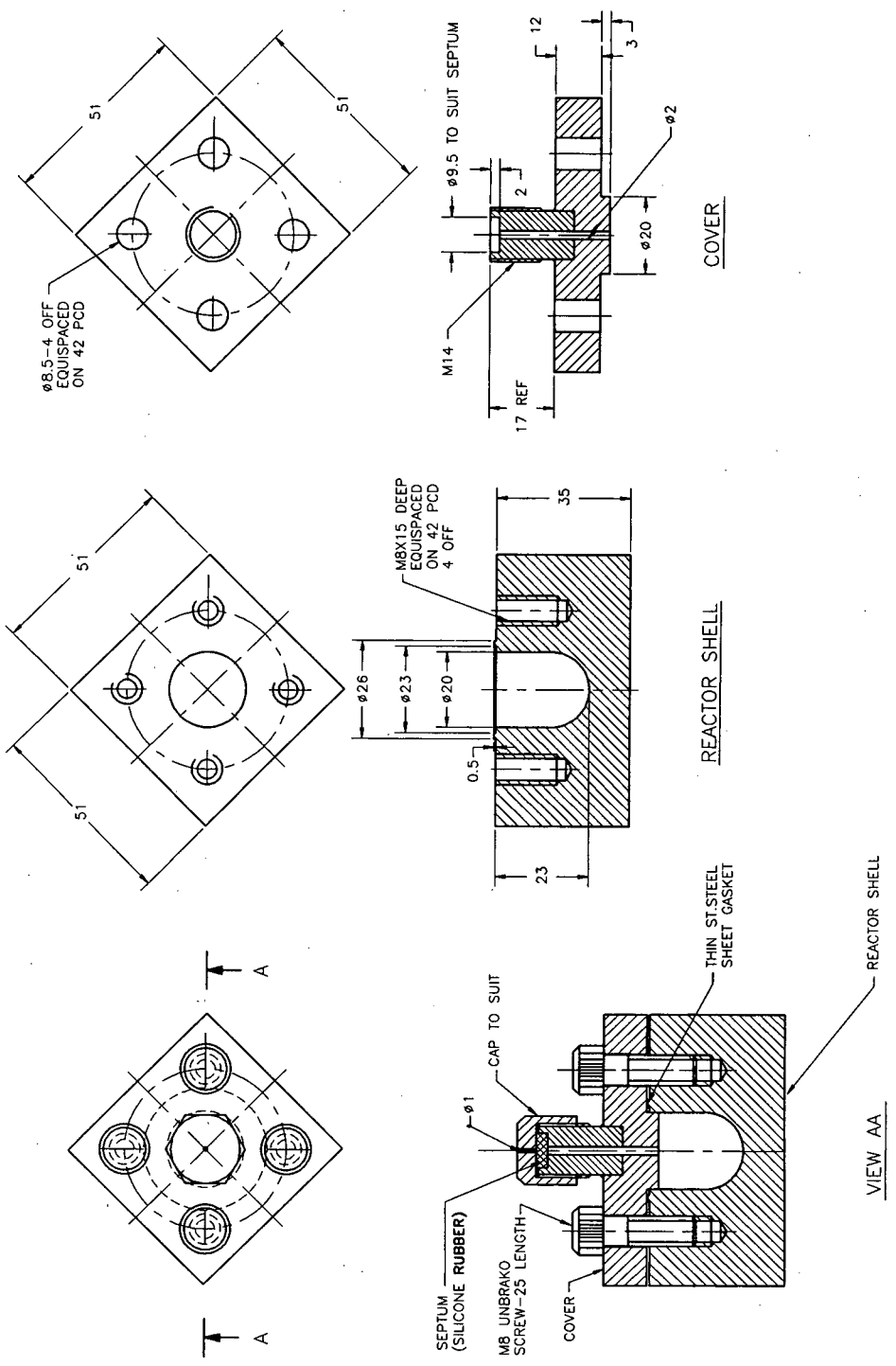

Fig. 2. Plan of improved stainless-steel reactor. All dimensions in millimetres.

23 mm deep and 20 mm in diameter. The flange has an orifice (1 mm diameter) in the centre which allows entry to the cavity and combines with the main reactor cavity of the shell. The orifice is sealed with a cap fitted with a heat-resistant silicone-rubber septum. A thin stainless-steel gasket seals the cover and the reactor shell. The cover is fitted to the reactor shell by four screws.

Fusion procedure

The polyester sample (20 mg) was mixed with the fusion reagent (0.2 g) consisting of a prefused mixture of potassium hydroxide and sodium acetate (5%) and heated in the reaction vessel for 1 h at 250°C. After cooling, the contents were transferred to a beaker and dissolved in water. The polyols were then extracted with chloroform and concentrated using rotary evaporation. Acetic anhydride (2.5 ml) and 1-methylimidazole (0.5 ml) were added to the concentrated polyol extract and refluxed for 1 h. After refluxing, the contents were transferred into a separating funnel and extracted with two 20-ml portions of chloroform and washed with two 20-ml portions of water. The chloroform layer was then dried using anhydrous magnesium sulphate and concentrated to 5–6 ml in a rotary evaporator and used in the analysis.

The aqueous layer containing the resin acid components was transferred into a beaker and a dilute hydrochloric acid solution was added until the pH was 1–2. Water was then evaporated and the solid acids were dried in an oven at 105°C for 30 min. Boron trifluoride–methanol reagent (5 ml) was then added and refluxed for 1 h. Methyl esters of acid components were extracted with two 20-ml portions of toluene and then washed with two 20-ml portions of water. The toluene layer was dried with anhydrous magnesium sulphate and concentrated in a rotary evaporator to 5–6 ml and used for gas chromatographic analysis.

Gas chromatography

For gas chromatography a Perkin-Elmer Model 8410 instrument fitted with flame ionization detectors was used. The column was aluminium (12 ft. × ¼ in. O.D.) packed with 10% Silar IOCP on Chromosorb W AW DMCS. The carrier gas (helium) pressure was 20 p.s.i. (120 kPa). The polyol acetates were separated isothermally at 180°C whereas for the carboxylate diesters the column temperature was programmed from 170 to 250°C at 8°C/min.

Samples

The samples were (1) a white crystalline solid sample of polyester based on phthalic anhydride, maleic anhydride and neopentyl glycol, (2) similar to (1) but based on propylene glycol and (3) commercially available polyethylene terephthalate film.

RESULTS AND DISCUSSION

The reactor shown in Fig. 2, owing to its broad and shallow reaction cavity, was found to facilitate handling of the sample and prevent loss of the reaction products, thus allowing smaller samples to be used. Samples of 20 mg are conveniently handled, as shown by the results reported here. Whereas this is a tenfold reduction in sample size, the actual amount of reaction product necessary for gas chromatographic examination is less than 1 μl of solution and is thus orders of magnitudes greater than available. For gas chromatography–mass spectrometry the sample requirement is much lower, it having been indicated that with modern instrumentation the identity of compounds in admixtures may be determined at about the 10^{-10} g ml^{-1} level [19]. Leclercq *et al.* [20] have shown that identifiable mass spectra are produced with 40–60 pg of the sample. Pyrolysis gas chromatographic–mass spectrometric studies of polyacrylic esters [21] have used 800-ng (0.8-μg) samples to produce a range of components and spectra capable of interpretation for nearly 30 compounds. Similarly, forensic studies have involved samples as small as 0.5 μg in case work [22]. With the present work further significant reductions in sample size are expected by reduction of losses experienced in the work-up stages. Earlier studies [17] have shown that the hydrolytic reactions are essentially quantitative and losses and errors of at least 1–2% per extraction step are experienced.

The smaller volume of the reactor cavity, *i.e.*, 7.2 cm^3, generates higher pressure during reaction, increasing the retention rate with a decrease in the reaction period necessary. The overall design prevents leakages and the four-screw system of attachment facilitates opening and closing of the reactor. The flange with the orifice allows the analysis of

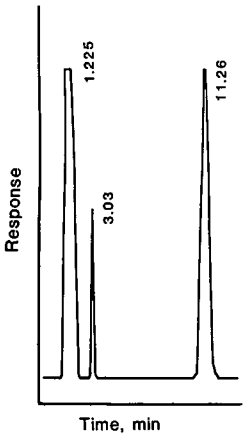

Fig. 3. Chromatogram showing peaks of solvent, diesters of maleic acid (3.03 min) and *o*-phthalic anhydride (11.26 min).

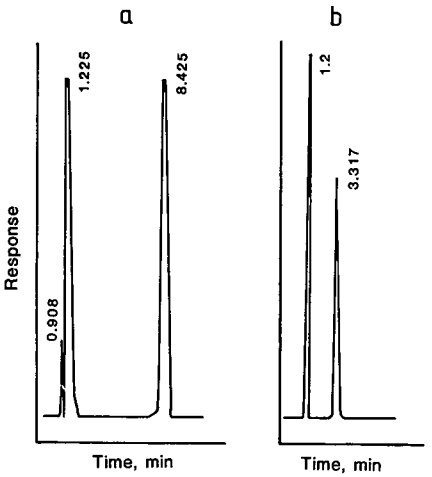

Fig. 5. Chromatograms showing solvent and (a) dimethyl terephthalate (8.425 min) and (b) ethylene glycol diacetate (3.317 min) from polyethylene terephthalate.

gaseous cleavage products and serves the same function as the septum incorporated into the sealing plug of the reactor shown in Fig. 1.

The separations of the acidic components of samples 1 and 2 are shown in Fig. 3 and the chromatograms of neopentyl glycol diacetate and propylene glycol diacetate from these two samples are shown in Fig. 4a and b, respectively.

The cleavage products of polyethylene terephthalate, a polyester of substantial hydrolytic stability, are shown in Fig. 5 for (a) dimethyl terephthalate and (b) ethylene glycol diacetate.

With fusion reactions the occurrence of leakages is normally extensive; basically the reactor is sealed or the volatile reaction products are essentially lost. In such a case, a high yield of non-volatile products is produced on working up and derivatizing the potassium salts whereas an almost negligible amount of volatile reaction products results. With leakages caustic residues and staining are evident on the external body of the reactor. This feature is clearly demonstrated by heating the charged reactor in an unsealed tinplate or zinc-lined can where on leakage attack and staining of the can surface are dramatic. The possibility of leakages is in any case minimized as standard analytical practice requires that multiple analyses (*i.e.*, fusions) be conducted.

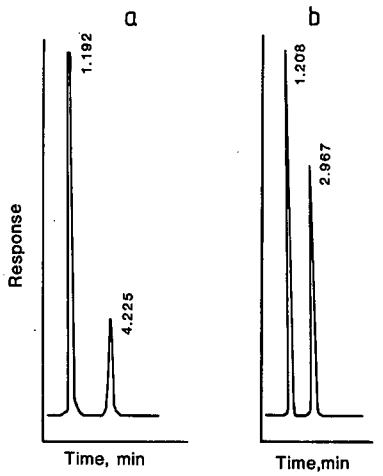

Fig. 4. Chromatograms showing solvent and diacetates of (a) neopentyl glycol (4.225 min) and (b) propylene glycol (2.967 min) from reactive polyesters.

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Short Communication

Permanganate-impregnated packed capillary columns for group separation of triacylglycerols using supercritical media as mobile phases

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ABSTRACT

Capillary columns packed with a silica-based anion exchanger were treated *in situ* with a solution of potassium permanganate. These columns showed highly reproducible retention times when used under supercritical conditions. Column temperatures up to 140°C could be applied. This type of column was successfully used for the group separation of triacylglycerols from vegetable oils according to their degree of unsaturation.

INTRODUCTION

Transition metal complexes provide high selectivities when used as stationary phases in chromatography [1], and a wide variety of metal acceptors have been shown to form complexes with olefins [2]. The selectivity obtained with Ag^+ was found to be particularly useful, and silver-containing stationary phases are now being extensively used. Christie [3] showed that very stable argentation columns for liquid chromatography were obtained when a silica-based cation modifier was used as a support for the silver ions. We have recently shown that capillary columns packed with a silver-impregnated cation exchanger are remarkably stable under supercritical fluid chromatographic (SFC) conditions [4,5]. These results encouraged us to evaluate the interactions in SFC with olefins of some other transition metal complexes.

Metal atoms of oxides are coordinated to silanol surface groups [6] and silica-carrying metal oxides has been used as catalysts. Metals such as Cr [7], Mn [8] and V [9] have been used. In this work, the separation of triacylglycerols on permanganate-impregnated columns in SFC was investigated. In the early days of gas chromatography (GC), manganese stearate was tried as a stationary phase for the separation of amines [10]. In addition, chelates containing manganese (II) have been used in GC [17]. Manganese is a weak acceptor [11]; however, the aim of this work was separation into groups rather than separation of molecular species of triacylglycerols.

EXPERIMENTAL

The chromatographic system consisted of a Lee Scientific 600 Series SFC system and an Isco μLC-10 variable-wavelength absorbance detector. Detection was performed at 210 nm on a short

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length of 250-μm I.D. fused-silica tubing according to the method of Fields *et al.* [12]. The width of the slit on the detection capillary was about 1 mm. Fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) of 11 μm I.D. was used as a restrictor in lengths of 20–25 mm.

Columns were prepared from fused-silica capillary tubing, 290 or 250 mm × 0.25 mm I.D. and 0.43 mm O.D. (Polymicro Technologies). The columns were packed with Nucleosil 5 SB (Macherey–Nagel, Düren, Germany) or Supersphere Si 60, 4 μm (Merck, Darmstadt, Germany). Slurry packing was excecuted as described previously [4]. After packing, the columns were washed with methanol, 400 μl of distilled water, 400 μl of 0.2 *M* potassium permanganate solution and 400 μl of distilled water. Finally, the columns were dried by flushing with carbon dioxide at 115°C and 275 atm.

The mobile phase consisted of carbon dioxide–acetonitrile–isopropanol, different contents of modifier being used for the different column types and the content of isopropanol being *ca.* 10% of that of acetonitrile. SFC-grade carbon dioxide (Scott Specialty Gases, Plumsteadville, PA, USA) was used. The mobile phase mixture was prepared in the SFC pump as described previously [13]. The mobile phase velocity was 3.5 mm/s.

Chromatographically purified olive oil, corn oil, soyabean oil and linseed oil were purchased from Larodan Fine Chemicals (Malmö, Sweden) and borage oil was obtained from LipidTeknik (Stockholm, Sweden). The solutes were dissolved in high-performance liquid chromatographic (HPLC)-grade pentane at concentrations of 30 mg/ml. Injection was performed with a splitting ratio of 1:1 and a timed split of 0.2 s.

RESULTS AND DISCUSSION

Excellent reproducibility of retention times was observed under the applied conditions. Further, the columns could be used at temperatures up to 140°C without deterioration of the performance.

The separation of some different vegetable oils is demonstrated in Figs. 1–5. As shown in Figs. 1–4, good group separation of the triacylglycerols according to the number of unsaturations was obtained. Fig. 5 shows the separation of a highly com-

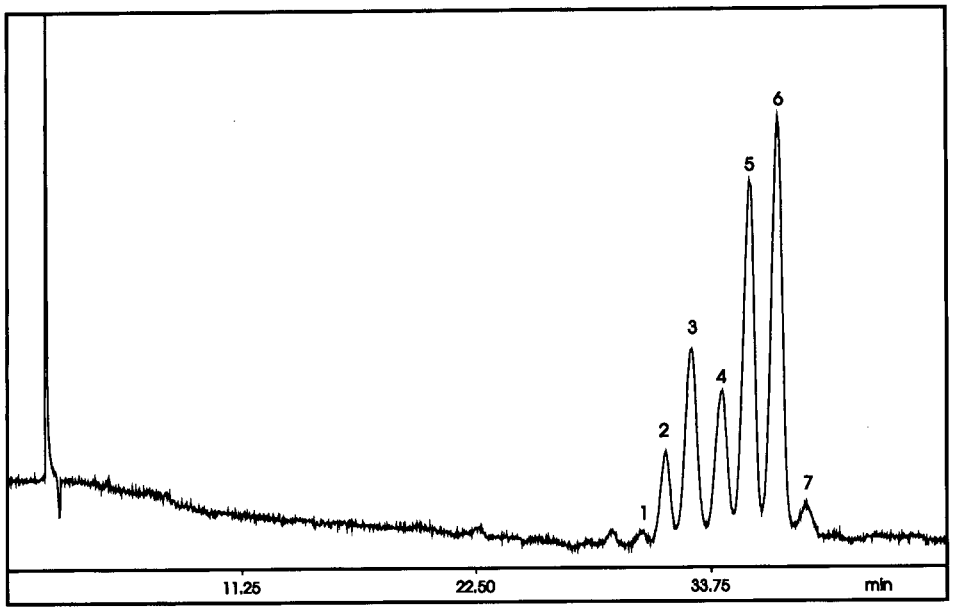

Fig. 1. Supercritical fluid chromatogram of corn oil. Capillary column (330 mm × 0.25 mm I.D.) packed with 5-μm anion exchanger and modified *in situ* with $KMnO_4$. Injection at 100°C and 275 atm; after 2 min, programmed at −1°C/min to 75°C and 0.5 atm/min to 288 atm. Mobile phase: carbon dioxide–acetonitrile–isopropanol (97.3:2.4:0.3, mol%). UV detection at 210 nm. Peak numbers refer to the number of double bonds.

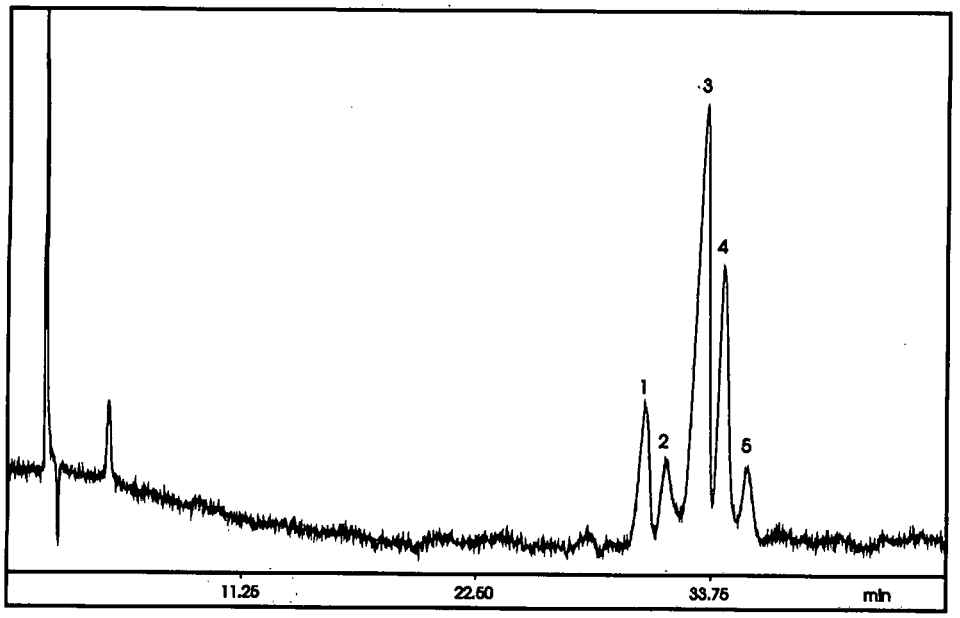

Fig. 2. Supercritical fluid chromatogram of olive oil. Column, conditions and peaks as in Fig. 1.

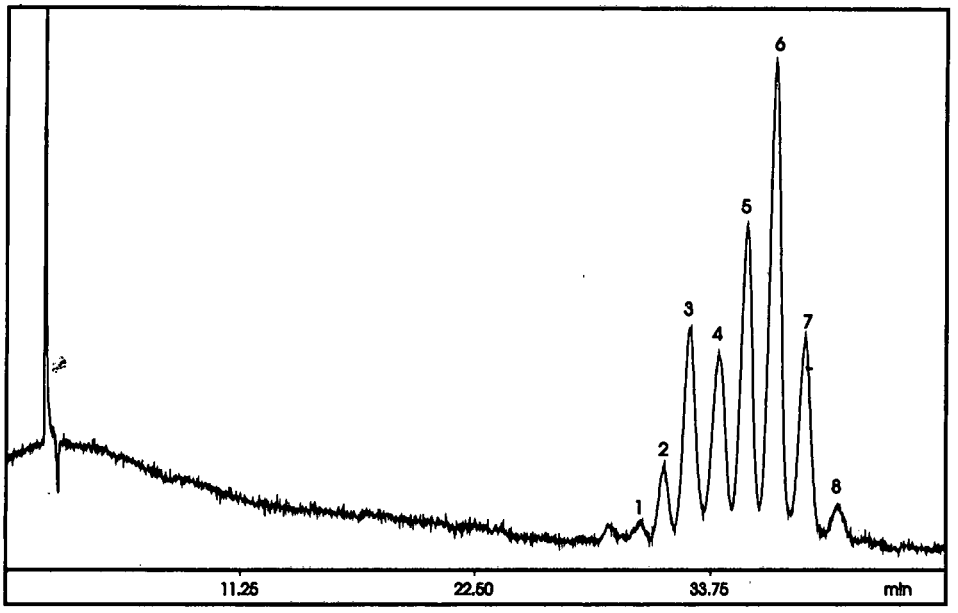

Fig. 3. Supercritical fluid chromatogram of soyabean oil. Column, conditions and peaks as in Fig. 1.

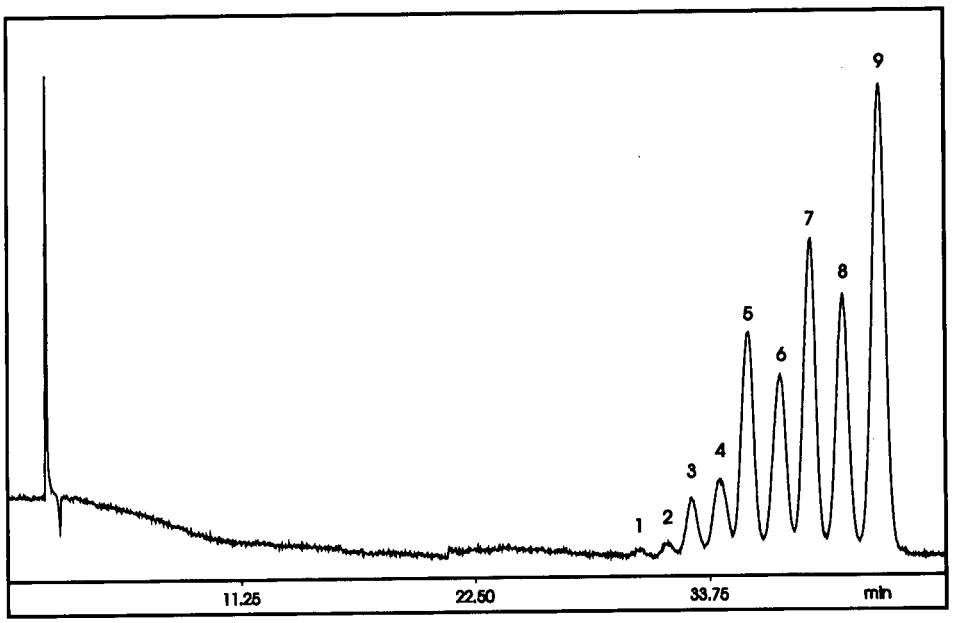

Fig. 4. Supercritical fluid chromatogram of linseed oil. Column, conditions and peaks as in Fig. 1.

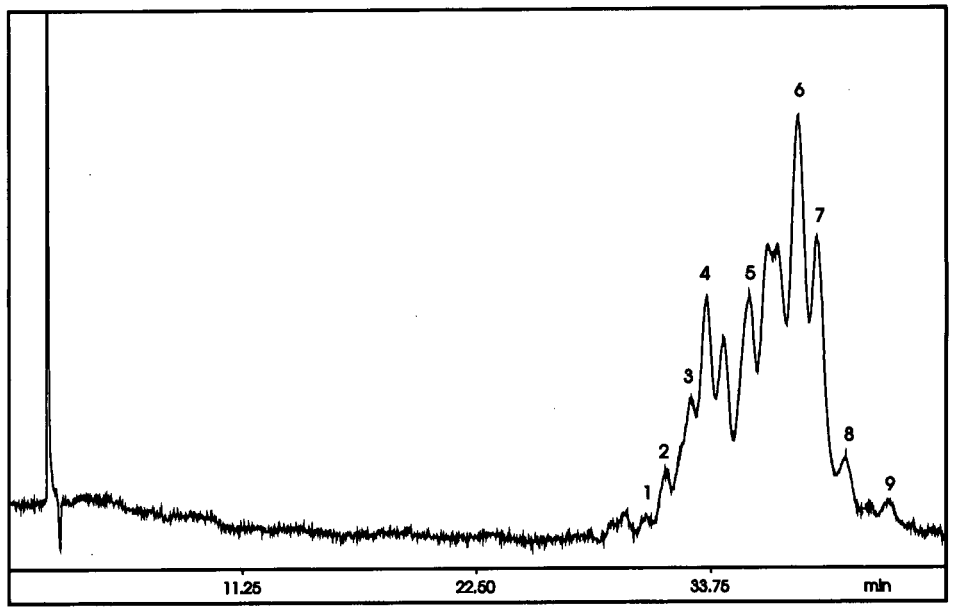

Fig. 5. Supercritical fluid chromatogram of borage oil. Column, conditions and peaks as in Fig. 1.

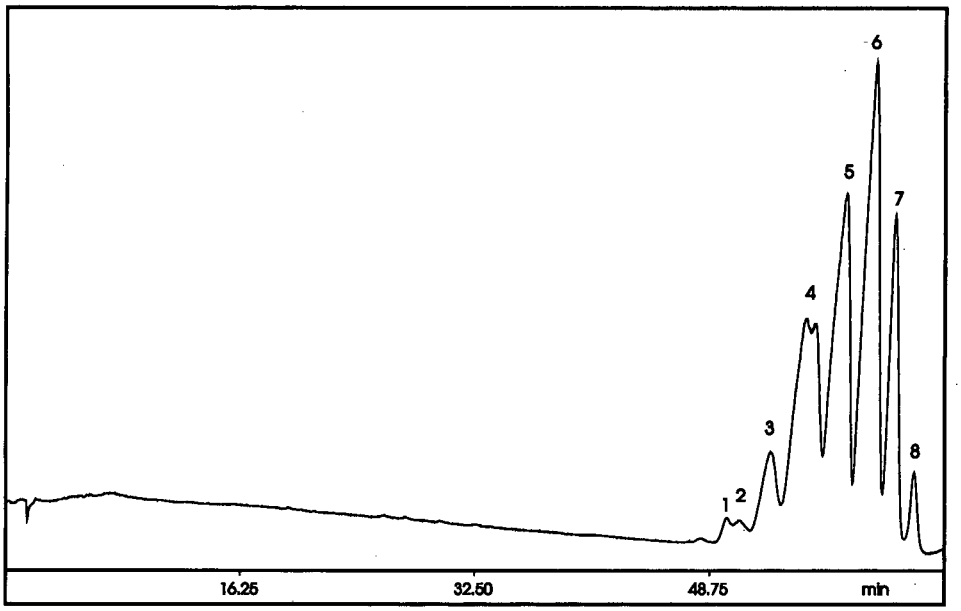

Fig. 6. Supercritical fluid chromatogram of soyabean oil. Capillary column (250 mm × 0.25 mm I.D.) packed with 4-μm silica. Injection at 150°C and 240 atm; after 2 min, programmed at −2°C/min to 70°C and 2 atm/min to 320 atm. Mobile phase: carbon dioxide–acetonitrile–isopropanol (98:1.8:0.2, mol%). UV detection at 210 nm. Peak numbers refer to number of double bonds.

plex oil [14], where some overlap between the different groups was inevitable. A chromatogram of soyabean oil obtained on a capillary packed with silica is shown in Fig. 6. The separation is relatively poor, but it is much more complete than previously reported separations on silica by HPLC [15].

In order to achieve optimum elution of triacylglycerols on permanganate-treated columns, it was necessary to add 2.8% of acetonitrile to the mobile phase. For the elution of the same samples on Ag^+-impregnated columns, 6.5% of acetonitrile was required [5]. The difference depends on two factors. First, the acetonitrile is needed for partial deactivation of the Ag^+ moieties, and second, the acetonitrile serves to deactivate residual surface silanol groups. Triacylglycerols are, however, relatively insensitive towards residual silanol groups, thus giving the poor separation shown in Fig. 6. Fatty acid methyl esters (FAME) are much more sensitive in this respect, and the silanol-deactivating effect of permanganate treatment was recently demonstrated for such compounds [16]. Finally, when using open-tubular columns, triacylglycerols are readily eluted with carbon dioxide alone, the modifier thus not being of crucial importance for the solubility in the mobile phase in this instance.

It has been pointed out that the chromatographic activity of the transition metal complexes cannot be ascribed solely to the central metal atom [1]. The ligands may also play a role through steric factors or inductive factors and, further, they stabilize the oxidation state of the metal. Columns packed with ion exchanger in addition to silica became light brown on permanganate treatment. Obviously, the anion-exchange moieties were degraded by the permanganate. It is not known in which form the manganese occurs on the silica surface, but the presence of manganese was detected by means of atomic absorption spectrometry.

ACKNOWLEDGEMENT

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Short Communication

Supercritical fluid chromatographic analysis of polyprenols in *Ginkgo biloba* L

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ABSTRACT

A supercritical fluid chromatographic (SFC) procedure for the quantitation of three major polyprenols present in the leaves of *Ginkgo biloba* was developed. In contrast to previously reported high-performance liquid chromatographic (HPLC) methods, the SFC method does not require extensive pre-purification for polyprenol analysis. The SFC analytical procedure described shows a very broad range of linearity and detects many known polyprenol isoprenologs with baseline separation. Dodecaprenol was used as the internal standard. The coefficient of variation of the method was 5.8% for the quantitation of C_{85}, C_{90} and C_{95} polyprenols. The SFC assay results showed that the content of polyprenols in ginkgo leaves were higher than the previously published values. In addition, the chromatogram of the highly concentrated leaf extract revealed the presence of an isoprenolog (C_{120}) not previously detected by HPLC methods.

INTRODUCTION

Several types of polyprenols have been found in the plant kingdom. Most of the polyprenols isolated from the angiosperms have the ω-t_3-c_n-OH structure. However, polyprenols from many gymnosperms are known to have the ω-t_2-c_n-OH structure (see Fig. 1) [1] and are therefore similar to the structure of the animal dolichols. Dolichols function in the transfer of the sugar moieties during the process of protein glycosylation [2]. Numerous recent studies have shown that dilochols are elevated in the urine of alcoholics suggesting that urinary dilochols could serve as an important laboratory marker for alcoholism [3].

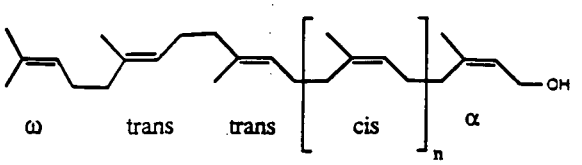

Fig. 1. Structure of Ginkgo polyprenols (di-*trans*,poly-*cis*-prenols), $n = 10$–18. Mixtures of polyprenols have different numbers of isoprene units. For example, if $n = 11$, it represents pentadecaprenol and if $n = 12$, it represents hexadecaprenol.

The precise physiological role of polyprenols in plants is not fully understood and it has been suggested that they may have physiological roles similar to that of dilochols in animals [4]. The ginkgo

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leaves have a very high content of polyprenols that have a chemical structure slightly different from that of animal dolichols. Therefore, they could provide an abundant source of an intermediate that could be used in the synthesis of dolichols [5,6].

In 1983, Ibata *et al.* [7] isolated and characterized polyprenols from the leaves of *Ginkgo biloba*. Using high-performance liquid chromatographic (HPLC) separation, field-desorption mass spectroscopy (FD-MS), ^{1}H NMR spectroscopy and/or ^{13}C NMR spectroscopy [1,7], they were able to show that the ginkgo polyprenol mixture contained compounds which had 14 to 22 isoprene units with 17, 18 and 19 isoprene-containing compounds being predominant. Numerous other studies using very laborious and elaborate purification schemes with HPLC separation have characterized and quantitated polyprenols in plant, and dolichols in animal samples [3,8–11].

Our studies describe the use of a very simple purification protocol followed by chromatographic separation using supercritical fluid chromatography (SFC) to quantitate the various polyprenols present in the leaves of *G. biloba*.

EXPERIMENTAL

Instrumentation and chromatographic conditions

A Series 600 capillary supercritical fluid chromatograph (Lee Scientific, Salt Lake City, UT, USA) was used for all analyses. The SFC system was equipped with a flame ionization detector and Valco injection system with a 200-nl injection loop operated in a time-split mode. All analyses were performed with the supercritical fluid carbon dioxide as the mobile phase (Scott Specialty Gases, PA, USA). For the separation of polyprenols, a chemically bonded SB-Phenyl-50 capillary column (10 m × 50 mm I.D., film thickness 0.25 mm) purchased from Lee Scientific was used. The injection time used was 0.2 s and the detector temperature was maintained at 325°C. Separation of the polyprenols was achieved by pressure gradient in which the initial pressure of 200 atm was increased at a rate of 20 atm/min to a final pressure of 400 atm. The oven temperature was maintained at 100°C. All of the data were processed by a Dionex AC1600 SFC–gas chromatographic system controller in an IBM PS/2 Model 60-071 computer.

Standards and identification

Dodecaprenol (C_{60}) from *Rhus typhina* was used as the internal standard and it was provided by Drs. E. Swiezewska and T. Chojnacki, Polish Institute of Physics and Chemistry (Warsaw, Poland). A polyprenol mixture from *G. biloba* was provided by Dr. Takigawa, Kuraray (Okayama, Japan). Polyprenol standards (C_{85}, C_{90}, C_{95}) were purchased from Sigma (St. Louis, MO, USA) and all reagents used were of analytical-reagent grade. Individual standards curves for C_{85}, C_{90} and C_{95} polyprenols were prepared in hexane at concentrations of 0.5, 1.0, 1.5 or 2.0 mg/ml for each compound with 0.5 mg of dodecaprenol (C_{60}) as internal standard. The ratio of the area under each polyprenol peak and the dodecaprenol peak was plotted against the individual polyprenol concentration. Standard curves were generated each day that samples were analyzed.

Plant materials

Ginkgo leaves were collected and pooled from three trees located on the campus of the University of Minnesota (Minneapolis, MN, USA) during the following seasons: spring (May), summer (August), fall (early October) and the late fall (late October) of 1990. The leaves were oven-dried at 60°C and pulverized. Ginkgo leaves were also collected from 3-month-old seedlings germinated and grown in a green house (College of Pharmacy, University of Minnesota).

Extraction and purification

Pulverized leaves (about 200 mg) spiked with 500 μg of the internal standard dodecaprenol were extracted for 1 h in *n*-hexane with occasional stirring. The resulting *n*-hexane extract was washed three times with 90% aqueous methanol and then vigorously stirred with potassium carbonate and methanol for 12 h at room temperature. After a further washing once with water and twice with saturated sodium chloride solution, the resulting *n*-hexane solution was passed through sodium sulfate. The eluted *n*-hexane solution was evaporated under nitrogen. The residue was reconstituted with 1 ml of *n*-hexane prior to separation of the polyprenols by SFC.

Quantitation and reproducibility of the method

Two ginkgo leaf samples collected for each sea-

son were extracted, purified and subjected to separation by SFC. The concentration of each polyprenol (C_{85}, C_{90} and C_{95}, respectively) was calculated from the standard curves generated, and the content of each polyprenol (mg/g of dried leaves) was calculated. The reproducibility of the SFC analysis method was validated by analyzing ten different samples of a batch of leaves that were harvested on October 25th, 1990. Polyprenol contents were calculated as described and the relative standard deviation (R.S.D.) for each polyprenol was determined.

RESULTS AND DISCUSSION

The use of SFC enabled us to rapidly separate the different polyprenols that were present in the leaves of the ginkgo (see Fig. 2). The extraction and saponification techniques we used were similar to those were described by Takigawa *et al.* [1] with the exception that we omitted the purification steps with adsorbent carbon treatment and column chromatography. Even though we were using a semi-purified mixture, the use of SFC enabled us to obtain baseline separation between different poly-

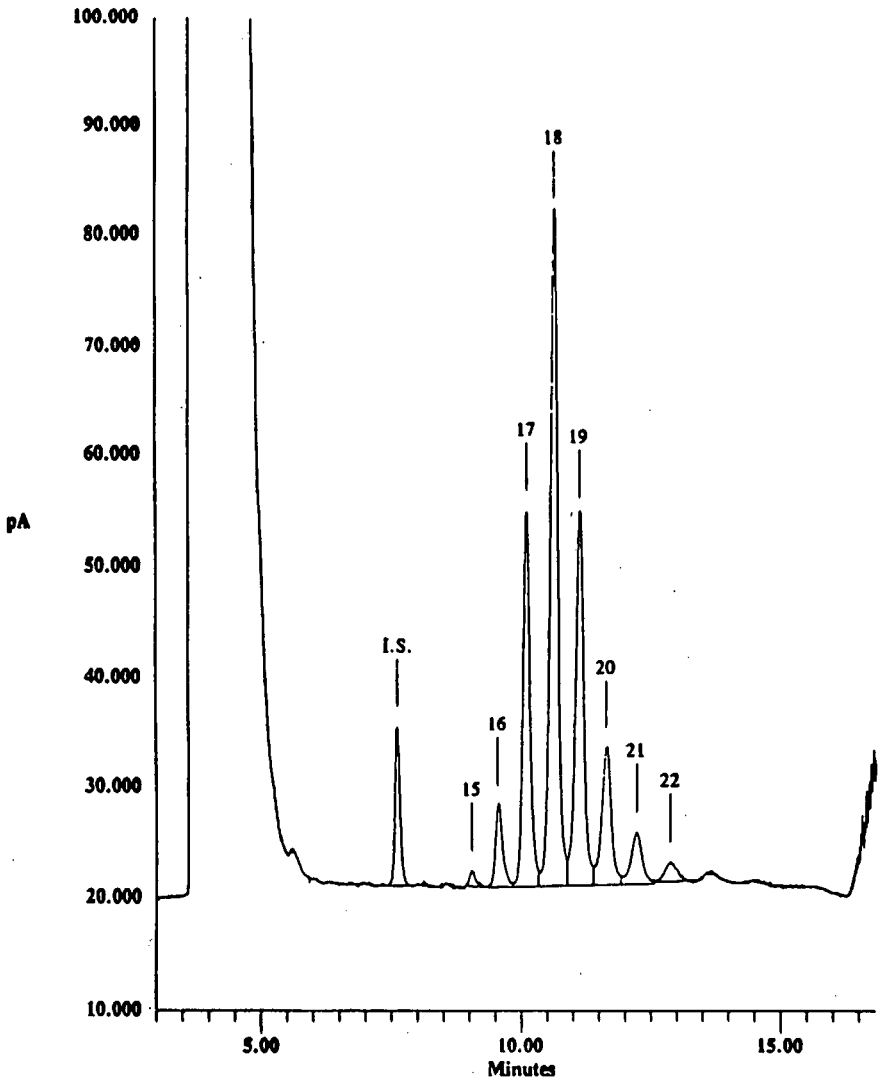

Fig. 2. SFC separation of polyprenol extract from ginkgo leaves. Numbers above the peaks represent isoprene units. Sample: leaf collected late October; 263 mg of dried leaves were extracted, saponified and reconstituted with 1 ml of *n*-hexane. Injection volume, 0.1 μl. I.S. = Internal standard.

prenols (see Fig. 2). Dodecaprenol (C_{60}, containing 12 isoprene units) from *R. typhina* was selected as the internal standard because this compound is not present in ginkgo but it has similar physicochemical properties to the other polyprenols that are present in the ginkgo. The use of this internal standard enabled the correction of recovery of polyprenols during the extraction process.

This present study shows for the first time the application of SFC to the separation and quantitation of polyprenols in plant tissues. A number of other investigators have used HPLC techniques to quantitate polyprenols in plant tissues [1,7,10]. Their techniques involve the use of very elaborate and laborious purification steps prior to HPLC. The use of SFC eliminates the need for extreme prepurification steps.

Fig. 2 shows the presence of polyprenols containing 15 isoprene units to 22 isoprene units to be present in the ginkgo leaf extract. As previously reported [7] and confirmed by our SFC techniques, polyprenols containing 17, 18 and 19 isoprene units

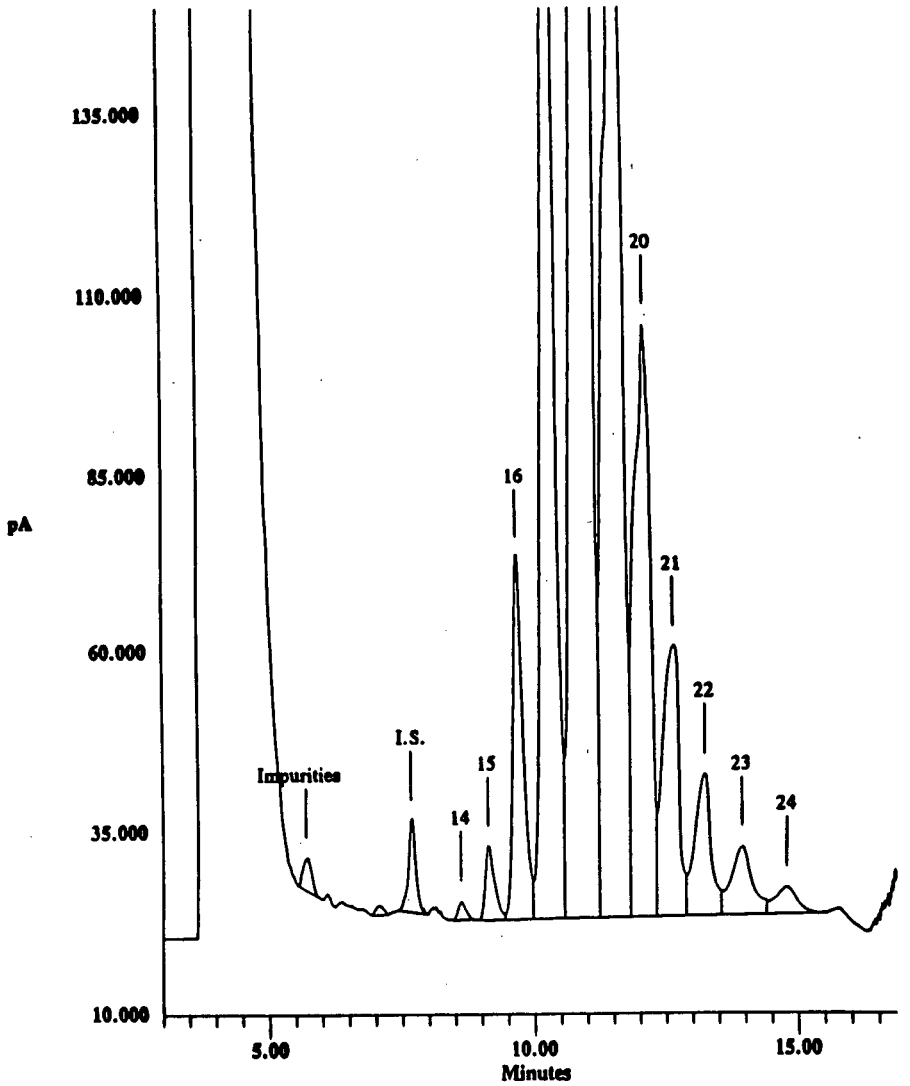

Fig. 3. SFC separation of concentrated ginkgo polyprenol extract. Numbers above the peaks represent isoprene units. Sample: leaf collected late October; 2.99 g of dried leaves were extracted, saponified and reconstituted with 1 ml of *n*-hexane. Injection volume, 0.1 μl.

were the predominant species present and they represented about 80% of the total ginkgo polyprenols.

In Fig. 3 shows that when we injected a highly concentrated extract from the leaves, we were able to show the presence of an additional peak with retention time of 14.8 min that appears to have 24 isoprene units (C_{120}). Such a polyprenol has not been previously described to be present in the ginkgo. Further characterization of this compound is currently being carried out to verify that it is indeed a C_{120}-containing polyprenol.

The calibration curves obtained for the three major polyprenols C_{85}, C_{90} and C_{95} show a wide range of linearity (see Fig. 4), up to 2 mg/ml for each compound. The reproducibility in quantitating ten different samples from the same batch of pulverized leaves was excellent (see Table I). The R.S.D. for the amounts of each of three compounds was less than 10%, and the value of the sum of total polyprenol content of these three species in the ten samples extracted had an R.S.D. of less than 6%. The recovery of the ginkgo polyprenols based on the recovery of our internal standard (500 μg) was 85.5 $\pm$ 13.7% ($n=9$).

The content of the three major polyprenols in ginkgo leaves analyzed by SFC and expressed in mg/g dry weight of the leaves was approximately

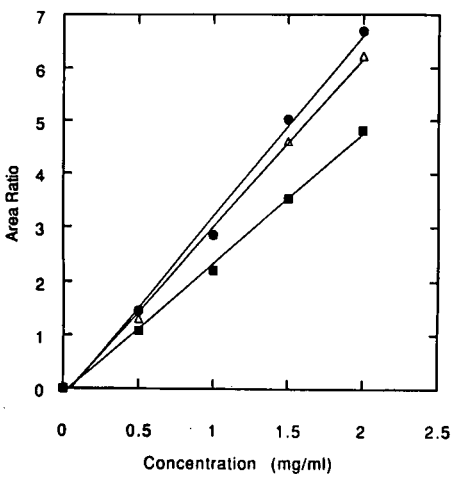

Fig. 4. Standard curves for three major polyprenols. The concentration coefficients of the regression lines of C_{85} polyprenol (△), C_{90} polyprenol (■) and C_{95} polyprenol (●) were 0.9989, 0.9990 and 0.9969, respectively.

TABLE I

REPRODUCIBILITY OF POLYPRENOL ANALYSIS BY SFC

Ten different samples from the same batch of pulverized leaves were subjected to the extraction and saponification procedure. Each of the polyprenols C_{85}, C_{90} and C_{95} was quantitated.

Polyprenol	Content (mean ± S.D.) (mg/g dry weight)	R.S.D. (%)
C_{85}	4.37 ± 0.296	6.78
C_{90}	10.58 ± 0.742	7.01
C_{95}	4.85 ± 0.405	8.35
Sum	19.57 ± 1.146	5.86

TABLE II

SEASONAL VARIATION OF POLYPRENOLS IN GINKGO LEAF

Season	Age of leaf (weeks)	Content (mg/g dry weight)	
		Present study	Ibata *et al.* [7][a]
Spring	6	1.5	1.0
Summer	20	10.2	8.0
Fall	26	16.9	14.0
Late fall	28	19.6	16.0

[a] Values calculated from Fig. 5 of ref. 7.

20% higher than the previously reported values [7]. This yield discrepancy might be resulted from either the quantitation methods used or geographical origin and/or environmental difference of the leaf samples. Our data also show seasonal variation in ginkgo polyprenol content (see Table II) and is in agreement with the findings of Ibata *et al.* [7].

The present study shows that SFC is an excellent alternative to HPLC for the analysis and quantitation of plant polyprenols and possibly of dolichols in animal tissues.

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We thank Dr. T. Takigawa, Kuraray (Okayama, Japan) for his generous gift of polyprenol standard mixture from *G. biloba*. We also thank Drs. T. Chojnacki and E. Swiezewska for their kindest courtesy of dodecaprenol standard. This work was in part supported by University of Minnesota Graduate School Grants-in-Aid to H. H.

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Short Communication

Determination of ephedrine alkaloids by capillary electrophoresis

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ABSTRACT

A simple and rapid method for the simultaneous determination of six ephedrine alkaloids (ephedrine, pseudoephedrine, norephedrine, norpseudoephedrine, methylephedrine and methylpseudoephedrine) in *Ephedrae herba* by capillary electrophoresis was developed. A buffer solution that contained 0.005 *M* barium hydroxide and 0.02 *M* isoleucine and adjusted to pH 10.0 with ammonia solution was found to be the most suitable electrolyte for this separation. The contents of the six alkaloids in the crude drug of *Ephedrae herba* could be easily determined.

INTRODUCTION

Ephedrae herba (Ma-Huang) is a commonly used Chinese herbal drug intended for diaphoretic purposes, and is known to contain (−)-ephedrine (E), (+)-pseudoephedrine (PE), (−)-methylephedrine (ME), (+)-methylpseudoephedrine (MPE), (−)-norephedrine (NE) and (+)-norpseudoephedrine (NPE) as its major bioactive components [1–3].

Several methods have been reported for the determination of some of these six alkaloids, including a copper complex method [4], thin-layer chromatography [5,6], gas–liquid chromatography [7] and ^{13}C NMR spectrometry [8] for E and PE and high-performance liquid chromatography (HPLC) [9–11] for three, four or five alkaloids. Recently, two methods for the simultaneous determination of all these six ephedrine alkaloids have been developed, one by HPLC [12] and the other by isotachophoresis [13]. However, the former requires tedious pretreatment of *Ephedrae herba* extracts before analysis and the latter is not able to separate ME and NPE well in *Ephedrae herba*.

We describe here the development of a simple, rapid and simultaneous method for determining these six alkaloids in crude and processed samples of *Ephedrae herba* by capillary electrophoresis.

EXPERIMENTAL

Reagents and materials

Ephedrine hydrochloride, pseudoephedrine hydrochloride, methylephedrine, methylpseudoephedrine and norephedrine hydrochloride were purchased from Aldrich (Milwaukee, WI, USA) and norpseudoephedrine hydrochloride, isoleucine and benzyltriethylammonium chloride from Merck (Darmstadt, Germany). Barium hydroxide and ammonia solution were of extra-pure grade. *Ephedrae herba* was purchased from the Chinese herbal market in Taipei (Taiwan).

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Preparation of Ephedrae herba extracts

A 1.0-g sample of pulverized *Ephedrae herba* was extracted with 50% ethanol (15 ml) by stirring at room temperature for 30 min, then centrifuged at 1500 *g* for 10 min. Extraction was repeated three times. The extracts were combined and filtered through a No. 1 filter-paper. After adding a 5-ml aliquot of internal standard solution (1 mg of benzyltriethylammonium chloride in 1 ml of water), the *Ephedrae herba* extract was diluted to 50 ml with 50% ethanol. This solution was passed through a 0.45-μm filter and about 1.7 nl (10-s hydrostatic sampling) of the filtrate was injected into the capillary electrophoresis system directly.

Apparatus and conditions

All analyses were carried out on a Waters Quanta 4000 capillary electrophoresis system equipped with a UV detector set at 185 nm and a 60 cm × 75 μm I.D. capillary. The running conditions were as follows: sampling time, 10 s hydrostatic; running time, 10 min; applied voltage, 28 kV; and temperature, 25.0–25.5°C. The electrolyte was a buffer solution consisting of 0.02 *M* isoleucine and 0.005 *M* barium hydroxide, adjusted to pH 10.0 with ammonium solution. The electrolyte was filtered through a 0.45-μm filter before use.

RESULTS AND DISCUSSION

Analytical conditions

We tried to apply HPLC to the constituents of *Ephedrae herba* and found that by connecting μBondapak C_{18} and phenyl columns (both 15 cm × 3.9 mm I.D.) in series, and eluting with ammonium buffer solution (consisting of 0.003 *M* di-*n*-butylamine, 0.002 *M* ammonium dihydrogenphosphate and 0.003 *M* ammonium chloride, adjusted to pH 2.8 with phosphoric acid), we were able to separate the six ephedrine alkaloids well. However, these conditions cannot be applied directly to water or ethanol–water extracts of the herb, owing to interferences from other constituents in the extract.

Following Zhang *et al.*'s pretreatment method [12], we used 0.5 *M* sulphuric acid for preliminary extraction, and neutralized the extract with 6 *M* sodium hydroxide solution, then extracted with diethyl ether and, after condensation, dissolved the extract in methanol to prepare a test solution that yielded clearly uninterfered peaks. However, this method cannot be used for analyses of herbs containing smaller amounts of constituents, because the NE signal can still be subjected to interference from methanol. Therefore, following Kasahara and Hikino's method [13], we adopted capillary electrophoresis for the analysis, using barium ion as the leading ion and β-alanine as the counter ion, which under a voltage of 28 kV was capable of separating the six alkaloids except for partial overlap of ME and NPE. On using other amino acids in association with barium hydroxide, we found that only 0.03 *M* isoleucine with 0.01 *M* barium hydroxide as electrolyte could completely separate all six peaks. When histidine, valine, glutamine, methionine, leucine, proline, alanine, threonine and lysine were used, ME and NPE completely or partially overlapped, and with the use of arginine and phenylalanine no absorption signal was obtained because of their own high absorbances. Also, in order to overcome the inconveniently low solubility of barium hydroxide, we replaced it with ammonia solution. As a result, the running time was shortened to 3 min and the absorption signals were enhanced, but ME and NPE could not be separated. Finally, we found that we could separate the six peaks completely and also shorten the retention time and enhance the peak intensity by mixing barium hydroxide and ammonia solution in an appropriate ratio.

An electrolyte containing 0.005 *M* barium hydroxide and 0.02 *M* isoleucine adjusted to pH 10.0 with ammonia solution was found to produce the best resolution. Fig. 1 is an electropherogram showing the separation of the six authentic ephedrine alkaloids with retention times of 4.1 min for the internal standard, 5.3 min for MPE, 6.1 min for PE, 6.7 min for E, 7.8 min for ME, 8.0 min for NPE and 8.7 min for NE. The separation of all the constituents can be completed within 10 min. As the ethanol–water extract of *Ephedrae herba* was injected directly and analysed, the results were as good as those obtained with pure chemical samples without interference for each peak and the analysis could be completed within 10 min, as shown in Fig. 2.

Calibration graphs for ephedrine alkaloids

Calibration graphs (peak-area ratio, y, *vs.* concentration, x, in mg/ml) were constructed in the range of 0.02–0.40 mg/ml for E and PE and 0.001–0.040 mg/

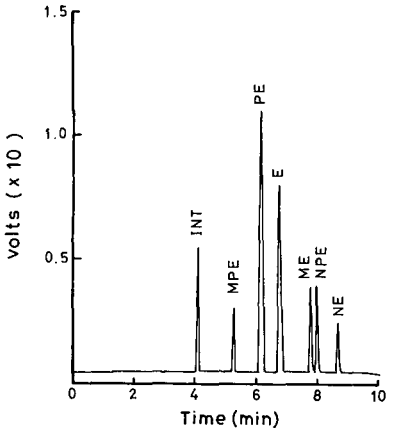

Fig. 1. Capillary electropherogram of a mixture of ephedrine alkaloids. INT = Internal standard (benzyltriethylammmonium chloride); MPE = methylpseudoephedrine; PE = pseudoephedrine; E = ephedrine; ME = methylephedrine; NPE = norpseudoephedrine; NE = norephedrine.

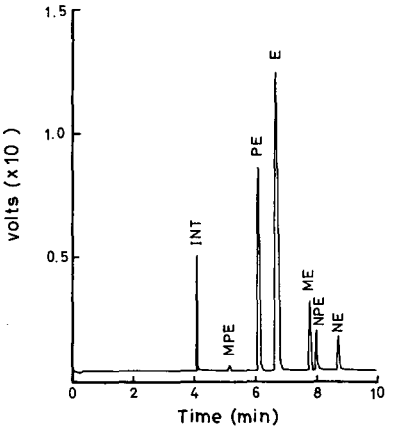

Fig. 2. Capillary electropherogram of the extract of an *Ephedrae herba* sample. Abbreviations as in Fig. 1.

ml for the other four alkaloids. They were linear with a good correlation coefficient of 0.999.

Determination of ephedrine alkaloids in Ephedrae herba

When the test solution of *Ephedrae herba* extract was analysed by capillary electrophoresis under the selected conditions, the graph shown in Fig. 2 was obtained. The calculated contents of the individual ephedrine alkaloids in the *Ephedrae herba* sample ($\pm$S.D.; $n = 3$) were MPE, <0.005; PE, 0.423 ± 0.019; E, 0.965 ± 0.017; ME, 0.133 ± 0.009; NPE, 0.057 ± 0.001; and NE, 0.059 ± 0.006%. Suitable amounts of the six ephedrine alkaloids were added to a sample of *Ephedrae herba* of known alkaloidal content and the mixture was extracted and analysed using the proposed procedure. The recoveries of the alkaloids were 95.5–102.9% with relative standard deviations of 2.2–4.0%.

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Book Review

Chromatography and isolation of insect hormones and pheromones, edited by A. R. McCaffery and I. D. Wilson, Plenum, New York, 1990, XIV + 376 pp., price US$ 89.50, ISBN 0-306-43707-4.

The book *Chromatography and isolation of insect hormones and pheromones*, edited by McCaffery and Wilson, is a welcome addition to the growing number of references in this area. Perhaps it is for the first time that insect hormones and pheromones are treated in one volume. The book is a collection of papers presented at an international symposium held in March 1989, and is divided into four main sections.

The juvenile hormone (JH) section contains four articles on JH and one on anti-JH. The JH papers rely on previously published methodologies: the *in vitro* radiochemical assay of Tobe and Pratt and/or the gas chromatographic (GC)–mass spectrometric (MS) quantification protocol developed by Bergot and co-workers. The articles include valuable information on thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) of these compounds.

The eight articles in the ecdysteroid section provide very useful information on recent developments in methodology for separation and purification of naturally occurring ecdysteroids from both insect and plant sources. Whether they occur as free ecdysteroids, as various conjugates, or esters, one can find tools described in this section to deal with fractionation of mixtures that are often quite intractable. The breadth of the subject matter of all the chapters, taken together, provides useful information for the novice as well as experienced researcher in ecdysteroid chemistry. In addition to quite thorough coverage of advances in HPLC applications and TLC technique, some rather new and novel methods such as supercritical fluid chromatography, plasmaspray HPLC–MS analysis, overpressure TLC, and the potential of immobilized phenylboronic acid separations are treated in this section.

The section on peptides deals primarily with isolation of neuropeptides from locusts, cockroaches, and crickets. While there is some overlap in technical information, particularly in reports from De Loof's and Holman's groups, readers will admire the productivity that is possible with a bioassay as robust as the cockroach hindgut and a skilled application of modern chromatographic techniques. Chromatographers should take note, especially of Holman's use of C_1 reversed-phase LC and Hayes' exploitation of the adsorptive properties of a size-exclusion column in totally aqueous media to complement the more widely used C_8 and C_{18} reversed-phase methods. Also introduced are methods for studying biosynthesis of neuropeptides with [^{3}H]amino acids and characterization of new neuropeptides by screening cDNA expression libraries with antisera; unfortunately, these methods have not been well served, in that positive outcomes are not demonstrated. Nevertheless, the success of the last eight years or so in characterization of peptide structures is much in evidence here. It is ironic, perhaps, that this success reveals the gap in our knowledge of, for instance, the significance of the numbers of closely related myotropic peptides in a single insect. A challenge in sorting out the physiological aspects of insect neuropeptides remains.

The section on pheromones presents a combination of old and new tricks of the trade. The value of gas chromatography combined with electroantennography in the identification of insect semiochemicals is nicely demonstrated in several of the chapters. Similarly GC–MS in the hands of an experienced operator has proved very helpful in confirming pheromone identifications. HPLC–MS is particularly useful where stability of compounds is at stake.

There is an interesting chapter on how a comput-

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erized system can be used for pattern recognition between gas chromatograms. The compound and subject indexes are thorough. References are current (at the time of publication) and give complete citation. Overall, the book will be a useful reference volume.

Beltsville, MD (USA) Ashok K. Raina

Journal of Chromatography, 600 (1992) 375
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 157

Book Review

Advances in electrophoresis, Vol. 4, edited by A. Chrambach, M. J. Dunn and B. J. Radola, VCH, Weinheim, 1991, XI + 413 pp., priceDM 268.00. ISBN 3-527-28212-2.

This volume (the fourth in a series that started in 1987) is divided into seven chapters: Pulsed field gel electrophoresis (by R. M. Gemmill), Detection and quantification of DNA in electrophoresis gels and blots (by D. M. Gersten and E. J. Zapolski), High sensitivity sequence analysis of proteins separated by polyacrylamide gel electrophoresis (by R. Aebersold), Temperature-gradient gel electrophoresis (by D. Riesner, K. Henco and G. Steger), Determination of size, isomeric nature and charge of enzymes by pore gradient gel electrophoresis (by G. M. Rothe), Application of two-dimensional electrophoresis to mutation studies (by C. S. Giometti and J. Taylor) and New aspects of electrophoresis of membrane proteins: light scattering and polyacrylamide gel electrophoresis in sodium dodecyl sulphate analogues (by T. Takagi).

The volume is written by some of the world authorities in the respective fields, and as such it represents a well balanced and up-to-date view of each topic. However, I cannot help having a feeling of *déjà vu*, as these reviews seem to be appearing simultaneously in several books and chapters in different fields, to the point that one suspects recycling. Perhaps, it is also a natural evolution law, a kind of darwinian survival of the "fittest".

In some instances, I would have liked to see some streamlinining and greater precision of terms. Thus, in the chapter by Rothe (in general very well written), which appears to be an update of an extensive chapter already written in M. J. Dunn's book, *Analytical Gel Electrophoresis of Proteins* (Wright, Bristol, 1986), I think that Figs. 4 and 6 (both giving a linear course of a porosity gradient) could certainly have been omitted. Also, the pictures of the various electrophoretic chambers and gradient mixers are so old and well known (and seen in so many books) that they do not appear to belong to a series of "advances", which preferably should be restricted to the latest progress in each topic. I was also puzzled by the persistence in using the same terms and units for electrophoretic velocity (v, in mm s^{-1}) and apparent free electrophoretic mobility (μ, also expressed in mm s^{-1}). A moment later, the free electrophoretic mobility U (this time in m^2 s^{-1} V^{-1}) appears and this is a strange camouflage to me; for as long as I can remember, μ and U have been used interchangeably for expressing mobilities, and as such they should both be in m^2 s^{-1} V^{-1}. Nobody has ever distinguished between these two terms, and the fact of attributing the same units (mm s^{-1}) to such different things as velocity and mobility only generates great confusion. Additional confusion also stems from the fact that the units used are sometimes mm s^{-1}, sometimes m s^{-1} and sometimes the good old unit of μm s^{-1}.

This last unit (μm s^{-1}) is the one that biochemists introduced long ago and it made sense, until one day it was decided that proteins should migrate down the length of a polyacrylamide gel at the fantastic speed of m s^{-1}, when not even Speedy Gonzales or a space rocket could get close to such a speed (in such a medium!).

Aside from these minor flaws, the book can certainly be recommended to both protein chemists and molecular biologists, as there is a good blend on DNA and protein electrophoresis.

Milan (Italy) P. G. Righetti

0021-9673/92/$05.00

Journal of Chromatography, 600 (1992) 376–377
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 167

Book Review

Advances in chromatography, Vol. 32, edited by J. C. Giddings, E. Grushka and P. R. Brown, Marcel Dekker, New York, 1991, 296 pp., price US$ 125.00 (USA and Canada), US$ 143.75 (rest of the world), ISBN 0-8247-8563-0.

This is another in the long-standing series of *Advances in Chromatography* published by Marcel Dekker for the past few decades. The current Volume 32 contains six chapters in different areas of chromatography, all written by established experts and published authorities in each area. One of these chapters deal with gas chromatography (GC), one is an overview of chromatography in forensic science, and the remaining four chapters deal with different aspects of high-performance liquid chromatography (HPLC). Two of these six chapters deal with forensic analytical chemistry, one for the HPLC of explosive materials and the other, as above, an overview of all chromatographic approaches in forensic science. The chapter (6) on HPLC of explosive materials by J. B. F. Lloyd is a very comprehensive and thorough overview of everything that has ever been reported using HPLC for all types of explosives, detection available, sample preparation required, HPLC stationary and mobile phases applicable, detection limits realizable, confirmation approaches, and so forth. Hubball's chapter (5) on the use of chromatography in forensic science is again an overview of all chromatographic methods applicable to a variety of forensic samples. Thus, he discusses analysis of commonly abused drugs, arson analysis, explosives analysis, and then miscellaneous applications. There is some degree of overlap between Chapters 5 and 6 in that they both deal with explosives analysis by HPLC, the former much less than the latter.

Chapter 3 (K. Robards, V. R. Kelly and E. Patsalides) details the use of GC for the analysis of dissolved gases in water, in terms of water sampling methods, recovery and GC sample introduction procedures, quantification, and finally specific applications/examples. It is a very good chapter for anyone interested or needing to analyze and quantitate for the commonly dissolved gases in various types of water samples. Chapter 4 (V. P. Pchelkin and A. G. Vereschchagin) deals with the thin-layer chromatography and HPLC analysis for lipids, hydrolysis products of lipids, diacylglycerol derivatives of lipids, and other lipid classes. It is a very good overview of how to analyze for these general classes of lipids, often difficult to detect by most common HPLC or thin-layer chromatography detectors. Chapter 2 by M. Dong of Perkin-Elmer deals with tryptic mapping by reversed-phase HPLC approaches, and was a very interesting and educational overview and survey of the various approaches now in use for tryptic map analysis. This discusses the various reversed-phase columns in use, HPLC conditions for separating peptide maps, and then mobile phase choices and optimization of operating conditions. However, there is no real discussion of identification or specific detection for peptides, such as interfacing with mass spectrometry, amino acid sequencing of individual peptides, computer software for reconstructing the original, parent protein that led to a given peptide map, and other computerized approaches and aids for using the peptide maps to reconstruct the purity and identity of the parent protein. What is discussed very nicely has to do just with HPLC conditions, fast HPLC, microbore HPLC, and so forth, but nothing about how that information is then used to come up with information about the structure and/or purity of the parent protein. Perhaps these areas have nothing to do with *Advances in Chromatography*?

Finally, Chapter 1 by C.-K. Lim deals with one of the newer stationary phases for HPLC, namely porous graphitic carbon, and its uses for biomedical applications. This is a very nice overview of where this particular packing material has come from, how

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it has been developed and then applied for inorganic and organic ions and neutrals, and how it can be successfully used for drugs and bioorganics in biofluids, sample preparation requirements, and so forth. The basic structure of the porous graphitic carbon surface morphology is discussed, separation mechanisms for inorganic ions and neutral organics are elucidated, and interactions with biofluid components and the surface are described. Finally, specific applications from the literature related to biomedical applications are described in detail.

In summary, this is a typical collection of non-interrelated and non-overlapping chapters and reviews of very different areas of chromatography. There is, as usual, no connecting theme evident, other than that each chapter uses some form of chromatography. The chapters are written on a reasonable level of sophistication, there is no over-emphasis on applications or sample analysis, and there are adequately detailed mechanisms and theoretical treatments presented in each chapter. The reference lists are extensive, up-to-date, non-selective, non-biased, and very useful for anyone interested in going back to the primary literature. I am not convinced that I would buy the book for my private library as opposed to making xerox copies of individual chapters that are of immediate interest, illegal as that may sound. Clearly the book belongs in any scientific library at a university or private company involved in chromatography, where several individuals in an organization would be interested in specific chapters. However, it seems less likely that a private scientist would have need for all of the chapters in this Volume 32 nor need his own individual copy of the book. However, this could be said about any volume in the series. One is always hesitant to make a book purchase, when but one or two chapters will be of use of interest/read. This may be an inherent problem with all *Advances in Chromatography* volumes? The current example is no better or worse than its predecessors.

Boston, MA (USA) Ira S. Krull

Journal of Chromatography, 600 (1992) 378
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 199

Book Review

Stationary phases in gas chromatography (*Journal of Chromatography Library*, Vol. 48), by H. Rotzsche, Elsevier, Amsterdam, 1991, XIV + 409 pp., price US$ 166.50, Dfl. 325.00, ISBN 0-444-98733-9.

The old cliché that the column is the heart of the gas chromatograph is well accepted with the most significant part of the column being generally the stationary phase with often unwanted contributions due to the support and the column material. This volume from the worker who developed the cyanoalkyl polysiloxane phases about a decade before they were available on the Western market, is most timely and collates an extensive amount of data. Few works have appeared that have dealt with stationary phases generally, reviews that have appeared tending to be restricted to a particular group or type of materials.

The volume consists of a single page introductory and eight subsequent chapters. The first of these considers the basic concepts of gas chromatography and includes a number of important applicable relationships with the treatment of necessity being rather mathematical. This reviewer has on a number of occasions noted that specialised chromatographic works have contained a vast amount of largely irrelevant general material which has been more elegantly presented in modern texts and it is pleasing to see that the present work does not follow this pattern.

Chapters 3 and 4 are both rather compact. Chapter 3 reviews the important aspects of the chromatographic column and includes both packed and the various types of capillary columns while Chapter 4 details the individual intermolecular forces and classifications which combine these interactions.

The individual types of solid stationary phases are described in Chapter 5 where valuable tabulations of available commercial materials are shown. Chemically bonded stationary phases are the subject of Chapter 6 and absorbents for bonding, commercially available types and bonding reactions are described. Chapter 7 entitled The solid support includes the major types available and particularly the treatment of diatomaceous earth support.

Liquid stationary phases (Chapter 8) is the major contribution. While all of the major groups of phases are included, the polysiloxanes as the most widely used phases, form a large part of the chapter. The chapter includes extensive tabulations showing comparisons of available products. The literature contains few references after 1987 and in an extremely rapidly developing field, particularly with polysiloxanes, where new phases appear almost weekly, the treatments on chiral and mesogenic phases will soon be dated. About 20 pages are devoted to chemically bonded stationary phases and a comparable treatment of the potentially more important immobilised stationary phases rather than mention at the end of individual sections might have been more appropriate.

Chapter 9 includes general recommendations for selection of stationary phases, lists of suggested phase types for various separations and attempts at rationalization that have appeared over several decades. A bibliography of more than 1000 works is included together with author, general subject and stationary phase indices.

The author has been able to produce a fine work which includes the development of stationary phases through to existing modern techniques. The work is highly recommended as a reference work for the bookshelves of all concerned with gas chromatography.

Kensington (Australia) J. K. Haken

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Author Index

Errata

J. Chromatogr., 593 (1992) 41–46

Page 44, Fig. 3, amend legend: Peaks: 1 = 9-methylanthracene, 2 = CRA, 3 = retinol.

J. Chromatogr., 596 (1992) 285–289

Page 289, Table III, first value under Burley: 26 should read 26.6.

PUBLICATION SCHEDULE FOR 1992

Journal of Chromatography and *Journal of Chromatography, Biomedical Applications*

MONTH	O 1991–F 1992	M	A	M	J	
Journal of Chromatography	Vols. 585–593	594/1 + 2 595/1 + 2	596/1 596/2 597/1 + 2	598/1 598/2 599/1 + 2 600/1 600/2	602/1 + 2 603/1 + 2 604/1	The publication schedule for further issues will be published later.
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Biomedical Applications	Vols. 573 and 574	575/1 575/2	576/1	576/2 577/1	577/2	

[a] Cumulative Indexes will be Vol. 601, to appear early 1993.

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