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SHORT COMMUNICATIONS

In articles with more than one author, the name of the author to whom correspondence should be addressed is indicated in the article heading by a 6-pointed asterisk (*)

Gas Chromatography in Air Pollution Analysis

by **V.G. Berezkin,** *A.V. Topchiev Institute of Petrochemical Synthesis, Moscow, USSR and* **Y.S. Drugov,** *I.M. Gubkin Institute of Oil and Gas, Moscow, USSR*

Journal of Chromatography Library Volume 49

Air pollution determination is one of the most important fields of gas chromatography application in practice. This book provides a systematic description of the main stages of air pollution determination, ranging from sampling problems to the quantitative estimation of the acquired data.

Special attention is paid to the problem of gas, vapor, spray and solid particles extraction from air. The main methods of sampling procedure, namely, container utilization, cryogenic concentration, absorption, adsorption, chemisorption and filter usage, and successive impurities extraction are also handled. Sorption theory and the problems of sorption and desorption efficiency for hazardous impurities being extracted from traps with sorbents are discussed in detail. The practical utilization of different sorbents (silica, activated carbon, polymers etc.) to carry out sampling procedures for 200 main pollutants with known TLV (USSR and USA) is also considered.

This highly informative book, reflecting several insufficiently known techniques as well as the experience of both western and Soviet researchers, should be of interest to both beginners and skilled researchers.

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Optimization of the direct enantiomeric separation by high-performance liquid chromatography of the D-2 dopamine agonist N-0437 using response surface methodology and the multi-criteria decision-making method

DIRK T. WITTE*, JAN BOSMAN and ROKUS A. DE ZEEUW
Research Group Bioanalysis and Toxicology, University Centre for Pharmacy, A. Deusinglaan 2, 9713 AW Groningen (Netherlands)
and
JAN H. DE BOER and DURK A. DOORNBOS
Research Group Chemometrics, University Centre for Pharmacy, A. Deusinglaan 2, 9713 AW Groningen (Netherlands)
(First received March 4th, 1991; revised manuscript received May 3rd, 1991)

ABSTRACT

The applicability of a Chiralcel OD column for the separation of the enantiomers of the racemic dopamine agonist 2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin (N-0437) was studied. The resolution between the enantiomers was described by means of the response surface methodology and optimum chromatographic conditions were found using Smilde's multi-criteria decision-making technique. The variables studied were eluent composition, flow-rate and temperature. The effects of these variables on the retention time of the last-eluting enantiomer and the resolution between the enantiomers were examined. The optimum result was considered to be the highest resolution possible within a short retention time for the last-eluting enantiomer. It appeared that a decrease in temperature gave higher resolutions. With hexane–ethanol (95:5, v/v) as eluent at 10°C and a flow-rate of 0.5 ml/min, a resolution of 2.5 and a retention time of the last-eluting enantiomer of 23 min were obtained. Under these conditions calibration graphs for the (+)- and (−)-enantiomers were prepared using racemic N-0437.

INTRODUCTION

N-0437 (Fig. 1) is a potent racemic dopamine agonist. Evidence has been reported for differences in pharmacological behavior between the two possible enantiomers, (−)-, indicated as N-0923, and (+)-, indicated as N-0924 [1–4]. The agonistic D-2 dopaminergic activity was ascribed to N-0923. For pharmacological studies with N-0923 the enantiomeric purity had to be established. Until now, the enantiomers could only be separated by reversed-phase high-performance liquid chromatography (RP-HPLC) after precolumn derivatization with D-(+)-glucuronic acid [5]. This method, however, is very time consuming.

With the development of new chiral stationary phases other approaches became

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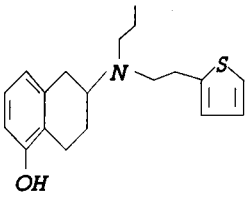

Fig. 1. Structure of N-0437.

available. The applicability of a Chiralcel OD column, containing chiral cellulose carbamates, for the separation of the N-0437 enantiomers was studied. This column was chosen because it already proved its applicability for drugs containing amines and hydroxyl groups [6]. Our preliminary studies with this column and the N-0437 enantiomers showed that an eluent consisting of hexane and ethanol gave better results than an eluent consisting of hexane and isopropanol with or without ethanol. Our first results with the column seemed promising, so optimization of the separation was carried out chemometrically, using response surface methodology (RSM) [7] and multi-criteria decision making (MCDM) [8]. Variables used during this optimization were the hexane concentration in the binary eluent (Hex), temperature (Temp) and flow-rate (Fl).

The effects of these variables on the chromatographic parameters, resolution (R_s) and retention time (t_R), were studied. The aim of this study was to have a high R_s between the enantiomers within the shortest retention time possible for the second-eluting enantiomer (t_{R2}). Chromatograms were obtained at different flow-rates, temperatures and eluent compositions. The settings of these variables were fixed in a factorial experimental design. In the starting design four different temperatures from 25 to 40°C were studied. From the data obtained with this design, an increase in R_s at lower temperatures became clear. However, with the temperatures used in this design no R_s values greater than 2 could be obtained. With these results in mind, lower temperatures were studied. The total design (Table I) therefore covered temperatures from 0 to 40°C.

Statistical polynomial models were used to describe the effects of the variables on R_s and t_{R2} within the factor space covered by the design. In theory the models to describe the effects of the variables on R_s and t_{R2} could contain linear and quadratic terms and maybe even terms of higher order. Effects caused by interactions between the variables should also be taken into account. In practice, in the model describing the effects of the variables on R_s, only significant linear effects of all variables were found. In the model describing the logarithm of t_{R2}, all variables showed linear effects but in addition Fl and Hex gave quadratic effects. With the models (Table II) and the application of MCDM, the pareto-optimum (PO) points were selected for the two responses R_s and t_{R2}. The MCDM methodology eliminates the necessity for making preliminary assumptions about the relative importance of objectives used in the optimization of HPLC separations. In this approach it is not necessary to preselect acceptable values of minimum resolution or maximum retention time. In MCDM the predicted values of R_s and t_{R2} were calculated from the models, using a small grid for each variable inside the whole factor space covered by the design. From the calculated

values of the R_s and t_{R2} the PO points were selected. A point is called a PO point if there exists no other point in the region which yields an improvement in one response without causing a degradation in another response. In the MCDM plot the PO points for R_s and t_{R2} are shown. In this plot the relationship between R_s and t_{R2} in the PO points is shown. Each PO point corresponds to a combination of variable settings.

Under conditions near one of the PO points thus found, calibration graphs for the two enantiomers were obtained using racemic N-0437. The influence of different concentrations N-0437 on R_s and t_R was investigated. Also, the molar absorptivities of the enantiomers at 225 nm were verified. Using the same conditions enantiomeric purity determinations of several batches N-0923 were made [9].

EXPERIMENTAL

Apparatus

The HPLC system utilized a (250 × 4.6 mm I.D.) Chiralcel OD column (Daicel Chemical Industries, Tokyo, Japan). For temperatures above 20°C the temperature of the column was regulated with a laboratory-made water jacket, thermostated with a Thermomix 1442 D (Braun, Rijswijk, Netherlands). For temperatures between 20 and 0°C a cooling device from HETO (Birkerod, Denmark) was used in combination with the thermostat. The solvent-delivery system was a Model 2150 HPLC pump (Pharmacia LKB, Uppsala, Sweden). Injections of 10 μl were made by a Model 710 A WISP (Waters Assoc., Milford, MA, USA) and detection was carried out with a Model 770 spectrophotometric detector at 225 nm (Spectra-Physics, Santa Clara, CA, USA). For integrating the chromatograms a C-R3A Chromatopac (Shimadzu, Kyoto, Japan) and for recording a BD 40 recorder (Kipp, Delft, Netherlands) were used. The models describing the influences of the variables Fl, Temp and Hex on R_s and t_{R2} were calculated by means of the SAS 6.04 package (SAS Institute, Cary, NC, USA).

Chemicals

The eluent contained hexane and ethanol, both of analytical-reagent grade, obtained from E. Merck (Darmstadt, Germany) and filtered through a 0.45-μm membrane filter (Schleicher & Schüll, Dassel, Germany). Mixtures were prepared by volume and degassed in a Sonicor (Farmingdale, NY, USA) ultrasonic bath before using the eluent. Racemic N-0437 · HCl and the enantiomers N-0923 · HCl and N-0924 · HCl were obtained from Whitby Research (Richmond, VA, USA). Stock solutions of N-0437 · HCl, N-0923 · HCl and N-0924 · HCl were prepared in ethanol and diluted to the desired concentrations with the eluent. Direct dissolution in the eluent was difficult because of solvation problems with the salts in the non-polar eluent.

HPLC conditions used for optimization

The eluent compositions tested were mixtures of hexane and ethanol, the temperature was varied from 0 to 40°C and the flow-rates were between 0.1 and 1 ml/min. The sample used during the optimization contained 390 μg/ml of N-0437. From this sample 10-μl injections were made. The HPLC conditions tested to establish the effect of the variables on the chromatographic parameters are summarized in Table I.

TABLE I

FLOW-RATES (ml/min) USED UNDER THE DIFFERENT HPLC CONDITIONS IN THE OPTIMIZATION EXPERIMENTS

Eluent, hexane–ethanol (v/v)	Temperature (°C)					
	0	10	25	30	35	40
80:20	0.25	0.50	0.10	0.50[a]	0.10	0.50
	0.50[a]		0.25[a]		0.25	
	0.60		0.50[b]		0.50	
	0.75		0.75[a]		0.75	
			1.00		1.00	
85:15	0.50	0.50	0.10	0.50	0.10	0.50
			0.25		0.25	
			0.50[a]		0.50[c]	
			0.75		0.75	
			1.00		1.00	
90:10	0.25[a]	0.50	0.25	0.50	0.25	0.50
	0.50[a]		0.50		0.50	
	0.75		0.75		0.75	
95:05	0.10	0.50[a]	0.50	0.50	0.50	0.50
	0.25[a]					
	0.50[a]					

[a] Indicates two runs under these conditions.
[b] Indicates eight runs under these conditions.
[c] Indicates three runs under these conditions.

If each set of conditions (four eluent compositions, five flow-rates, six temperatures) is tested once, $4 \times 5 \times 6 = 120$ runs are necessary. Information was obtained by carrying out 70 runs under 51 different conditions. To study the reproducibility of the measurements under some conditions several runs were carried out. From each chromatogram t_R and R_s were calculated.

Calibration graph for the enantiomers of N-0437

For the calibration graph, samples with concentrations of N-0437 in the range 500–0.25 μg/ml were prepared. From these samples 10 μl were injected in the HPLC system. The eluent was hexane–ethanol (95:5). The temperature was 10°C and the flow-rate 0.5 ml/min. The graph was measured twice with new eluent each time. This calibration graph was made with racemic N-0437. With racemic N-0437 the absorptivities of the enantiomers at 225 nm were compared. The peak areas, peak widths at half-height and t_R of the enantiomers were measured. Each enantiomer was identified by comparing the retention times in the racemic samples with samples of authentic N-0923 and N-0924.

RESULTS

Optimization

The models describing the effect of the variables on the chromatographic

TABLE II

MODELS DESCRIBING THE CHROMATOGRAPHIC PARAMETERS

$R_s = a + b\text{Hex} + c\text{Temp} + d\text{Fl}.$
$\text{Ln } t_{R2} = e + f\text{Hex} + g\text{Temp} + h\text{Fl} + k\text{Hex}^2 + m\text{Fl}^2.$

Regression parameter	S.D.	Regression parameter	S.D.
a 1.31	0.48	e 23.3	3.1
b 0.016	0.005	f −0.48	0.07
c −0.051	0.002	g −0.0050	0.0007
d −0.47	0.14	h −5.23	0.15
		k 0.0030	0.0004
		m 2.69	0.15

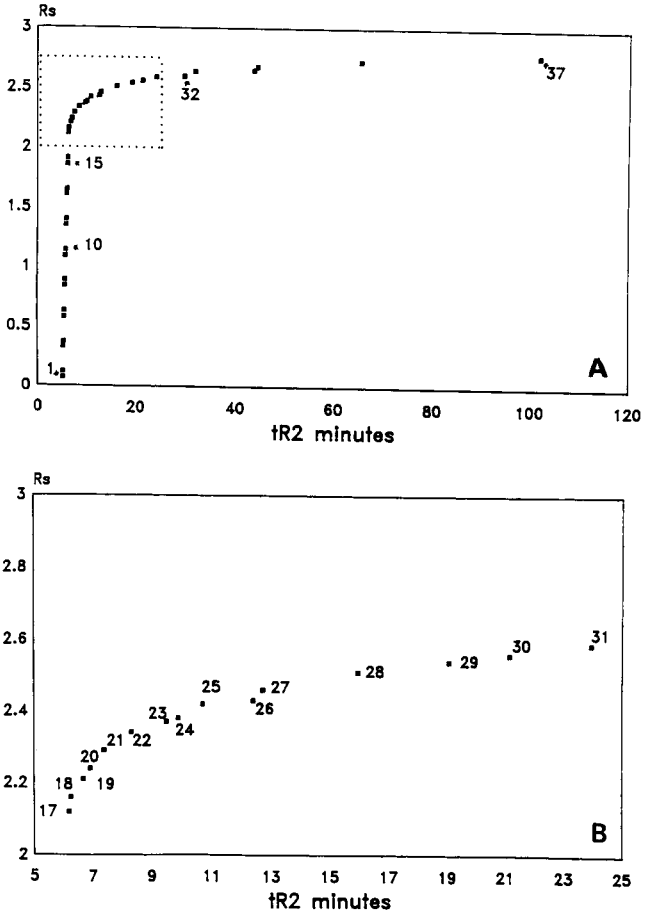

Fig. 2. (A) PO points for maximum resolution and minimum retention time of the last-eluting enantiomer. (B) Enlargement of the part in (A) in the dotted box, which contains the most relevant PO points.

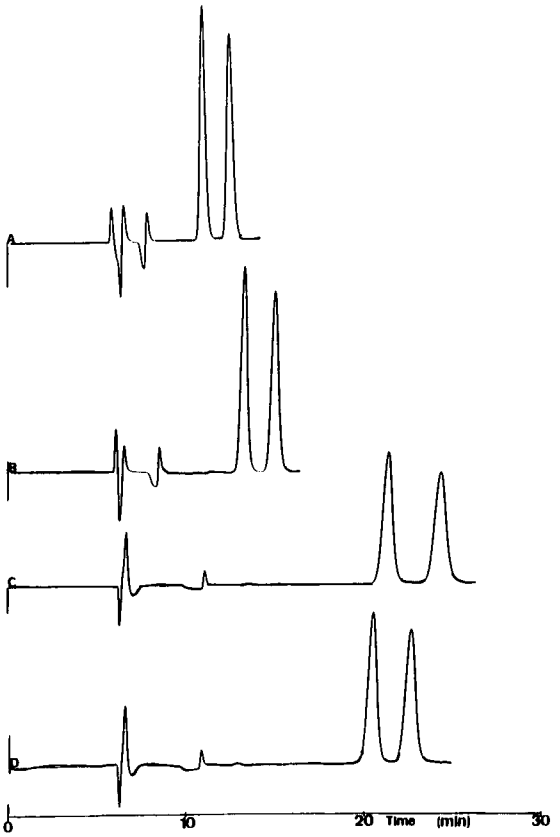

Fig. 3. Chromatograms of N-0437 (390 μg/ml) obtained under various conditions: (A) eluent, hexane–ethanol (85:15), temperature 0°C, flow-rate 0.5 ml/min, PO point 26; (B) eluent, hexane–ethanol (90:10), temperature 0°C, flow-rate 0.5 ml/min, PO point 28; (C) eluent, hexane–ethanol (95:5), temperature 0°C, flow-rate 0.5 ml/min. PO point 31; (D) eluent, hexane–ethanol (95:5), temperature 10°C, flow-rate 0.5 ml/min.

parameters are given in Table II. With aid of the models describing the effect of flow-rate, temperature and percentage of hexane in the eluent on R_s and t_R, the calculations used in the MCDM technique were made. The objectives were for R_s to be as high and t_{R2} as short as possible.

In Fig. 2A the MCDM plot is given and Fig. 2B shows an enlargement of that part of the MCDM plot containing the most relevant PO points. The variable settings corresponding to the PO points are given in Table III.

Calibration graph for N-0437

The calibration graphs were obtained at a temperature of 10°C instead of 0°C for the practical reason that 10°C is easier to control. The eluent was hexane–ethanol (95:5) and the flow-rate 0.5 ml/min. Under these conditions resolutions of 2.5 were obtainable in practice within 23 min. A chromatogram obtained under these

TABLE III

VARIABLE SETTINGS OF THE PO POINTS

PO point	Eluent (hexane, %)	Flow-rate (ml/min)	Temperature (°C)	R_s	t_{R2} (min)
1	80	1.0	40	0.07	5.03
2	80	0.9	40	0.12	5.09
3	80	1.0	35	0.33	5.16
4	80	0.9	35	0.37	5.22
5	80	1.0	30	0.58	5.29
6	80	0.9	30	0.63	5.35
7	80	1.0	25	0.84	5.42
8	80	0.9	25	0.89	5.49
9	80	1.0	20	1.09	5.56
10	80	0.9	20	1.14	5.63
11	80	1.0	15	1.35	5.71
12	80	0.9	15	1.40	5.78
13	80	1.0	10	1.61	5.85
14	80	0.9	10	1.65	5.92
15	80	1.0	5	1.86	6.00
16	80	0.9	5	1.91	6.08
17	80	1.0	0	2.12	6.16
18	80	0.9	0	2.16	6.23
19	80	0.8	0	2.21	6.65
20	85	0.9	0	2.24	6.90
21	85	0.8	0	2.29	7.37
22	85	0.7	0	2.34	8.31
23	90	0.8	0	2.37	9.50
24	85	0.6	0	2.38	9.88
25	90	0.7	0	2.42	10.70
26[a]	85	0.5	0	2.43	12.40
27	90	0.6	0	2.46	12.73
28[a]	90	0.5	0	2.51	15.97
29	95	0.6	0	2.54	19.07
30	90	0.4	0	2.56	21.15
31[a]	95	0.5	0	2.59	23.93
32	90	0.3	0	2.60	29.56
33	95	0.4	0	2.64	31.69
34	90	0.2	0	2.65	43.59
35	95	0.3	0	2.68	44.29
36	95	0.2	0	2.73	65.32
37	95	0.1	0	2.78	101.64

[a] Fig. 3 shows chromatograms obtained under these conditions.

conditions is shown in Fig. 3D. The curve for the total area [(+)- + (−)-] is given in Fig. 4. The absorbance ratio, N-023/N-0924, at 225 nm was found to be 1.02 with a relative standard deviation (R.S.D.) of 9% over the whole concentration range used. The calibration graph was measured twice. The correlation coefficient of the mean N-0437 curve was 0.9998. For this curve, the N-0923 and N-0924 peak areas were calculated. The retention times of the enantiomers were measured and the resolutions were calculated for each point on the calibration graph. The mean retention times were 20.40 min with an R.S.D. of 0.3% ($n = 20$) for N-0924 and 22.94 min with an R.S.D. of

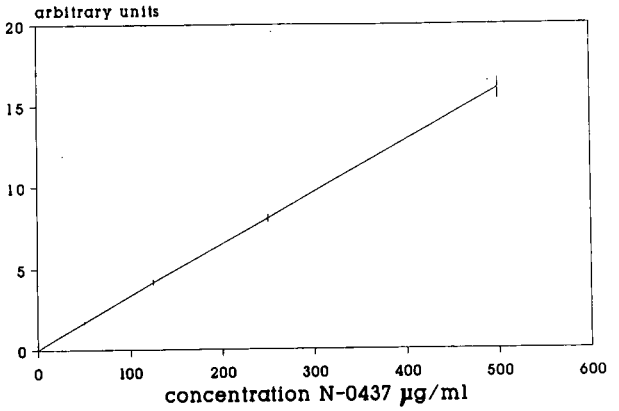

Fig. 4. Calibration graph for N-0437. Bars indicate the range.

0.3% ($n = 20$) for N-0923. The mean resolution between the enantiomers was 2.50 with an R.S.D. of 6.6% ($n = 20$). A sample of 25 µg/ml of N-0437 was injected eight times. The mean R_s and t_R values were calculated: $R_s = 2.57$ with an R.S.D. of 2.3% ($n = 8$); $t_{R1} = 20.9$ min with an R.S.D. of 0.4% ($n = 8$), t_R of the first-eluting enantiomer; and $t_{R2} = 23.2$ min with an R.S.D. of 0.6% ($n = 8$), t_R of the second-eluting enantiomer.

DISCUSSION

The models given in Table II give a good description of the effects that the variables have on the chromatographic parameters. The logarithmic transformation of t_{R2} is carried out because the error in the measurement of the retention time increases as the retention time increases. The assumption made in RSM is that the error in the response is constant over the whole range of the response [7]. R^2_{adj} for the model describing the resolution was 0.9104 and for the model describing the retention time of the last-eluting enantiomer R^2_{adj} was 0.9844. This $R^2_{adj} = 1$ if all the variance in the response is explained by the model. If the variance in the response cannot be explained by the model, this R^2_{adj} becomes smaller.

Table IV gives the differences between the predicted and observed values of the chromatographic parameters for five PO points and under the conditions used in the experiments for the calibration graphs. The effect of temperature on resolution was remarkable. Lowering the temperature gave a better resolution. This phenomenon may make the Chiralcel OD column more widely applicable than has been appreciated so far, if used at even lower temperatures. The manufacturer advises not to use temperatures below 0°C. Experiments at lower temperatures may be done in the future after consultation with the manufacturer. A temperature below 11°C was essential for the baseline separation of the N-0437 enantiomers. Lowering the flow-rate and increasing the proportion of hexane in the eluent also led to higher resolutions.

With the present objectives it is clear that there is not just one optimum set of conditions but that it depends on the relative importance of each objective which optimum is chosen. With these PO points, each representing a setting of the variables,

TABLE IV

RESULTS OF MEASUREMENTS UNDER PO CONDITIONS AND THE CONDITIONS USED FOR THE CALIBRATION GRAPHS

PO point[a]	Eluent (hexane, %)	Flow-rate (ml/min)	Temperature (°C)	Predicted		Observed	
				R_s	t_R (min)	R_s	t_R (min)
3	80	1.0	35	0.33	5.16	0.00	4.63
26[b]	85	0.5	0	2.43	12.40	2.51	12.50
28[b]	90	0.5	0	2.51	15.97	2.45	15.95
31[b]	95	0.5	0	2.59	23.93	2.72	23.50
37	95	0.1	0	2.78	101.64	2.78	104.76
Cal[b,c]	95	0.5	10	2.08	22.65	2.50	23.20

[a] The PO points correspond to those in Table III.
[b] Fig. 3 shows chromatograms obtained under these conditions.
[c] Conditions used for the preparation of the calibration graphs.

the balance between the chosen criteria is seen. It is easy to see in Fig. 2A what an improvement in resolution costs with respect to the retention time. An increase in resolution from 1.61 to 2.60 (PO points 13 to 32) is, according to the models, linked with an increase in retention time from 5.85 to 29.56 min. An increase in resolution is impossible without an increase in retention time along the curve formed by the PO points. It depends on the requirements of the user and on the availability of cooling instrumentation which variable settings are chosen. Determination of enantiomeric impurity, where a vast difference in concentration between the two enantiomers may occur, requires the highest resolution possible within workable limits. As the number of batches to be analysed is normally small, the retention time is of minor importance. In practice, a temperature of 10°C is more easily controllable than 0°C and 10°C was therefore selected for further experiments. The decrease in resolution caused by working at a 10°C instead of 0°C is to overcome by using a higher proportion of hexane in the eluent and working at a lower flow-rate. With higher temperature (> 10°C), the resolution decreases to an unacceptable value.

For quantitative work and enantiomeric impurity determinations, the variable settings used in this study appeared to be applicable. The reproducibility under these conditions was high and the calibration graph showed good linearity. Both enantiomers had the same absorption at 225 nm. The minimum amount that could be determined was 0.25 μg/ml of N-0437, which resulted in a minimum concentration of 0.125 μg/ml for each of the enantiomers. With an injection volume of 10 μl this corresponds to an absolute amount of 1.25 ng of each enantiomer. There was no effect of the concentration of N-0437 on the chromatographic parameters studied.

CONCLUSIONS

For the separation of N-0437 enantiomers the Chiralcel OD column is a good choice. At low temperatures (< 11°C) the separation between the N-0437 enantiomers is good, $R_s > 2.0$, under the conditions used. For the clean synthetic samples used in this study the normal-phase character of the column is not a disadvantage. However, if

one wishes to use this column for biological samples, it should be noted that the column cannot withstand aqueous solutions, so that extraction and removal of traces of water are required.

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Characterization of cellulase-based enzyme reactors for the high-performance liquid chromatographic determination of β-D-glucan oligosaccharides

P. C. MAES and L. J. NAGELS*
Laboratory of General and Organic Chemistry, Rijksuniversitair Centrum, Groenenborgerlaan 171, B-2020 Antwerp (Belgium)
C. DEWAELE
Bio-Rad/R.S.L., Begoniastraat 5, B-9731 Eke (Belgium)
and
F. C. ALDERWEIRELDT
Laboratory of General and Organic Chemistry, Rijksuniversitair Centrum, Groenenborgerlaan 171, B-2020 Antwerp (Belgium)
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ABSTRACT

Post-column enzyme reactors were used in series with an electrochemical detector for the selective high-performance liquid chromatographic (HPLC) determination of β-D-glucan oligo- and polysaccharides (degree of polymerization up to 30). Immobilized cellulase converted the eluting oligomers to β-D-glucose, which was oxidized by immobilized glucose oxidase. The production of hydrogen peroxide was measured with an electrochemical detector. The functioning of this system was verified for a whole range of glucosaccharides varying in both structure (positional isomers) and degree of polymerization. Fractional conversions and molar response factors were determined for all the compounds under study. Rate constants are discussed for the reactor system used applying a first-order kinetics model. Efficient HPLC separations were obtained for these oligo- and polysaccharides on a reversed-phase column using gradient elution. Detection limits were of the order of a few nanograms. The reactors were stable for several months.

INTRODUCTION

Interest in complex carbohydrates is growing very rapidly, especially in the field of biotechnology. Specific oligosaccharides have been reported as biologically active molecules in plant biochemistry (the so-called "oligosaccharins" [1]). Some of these compounds have a β-glucan structure [2]. β-Glucans are small polysaccharides consisting of only β-D-glucose units in the six-membered pyranose ring form, and all are linked from the glycosidic carbon 1 of the first glucose unit to carbon 2, 3, 4 or 6 of the next glucose unit. Complex structures with branched arrangements and mixed glycosidic linkages can occur. β-Glucan oligo- and polysaccharides also occur in food and beverage products. Their presence in biological samples is masked by the more abundant digestible α-glucans. In this laboratory, a method was needed for the

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quantitation of trace amounts of β-glucans in the presence of other types of oligo- and polysaccharides. A very selective and sensitive method was therefore required.

Enzyme reactors have been used successfully as post-column reactors in the high-performance liquid chromatographic (HPLC) determination of several biochemically interesting compounds [3] and in flow-injection analysis (FIA). In the latter field, selective enzymes are used for the measurement of mono- and disaccharides [4] and α-glucans [5,6]. For such measurements, oligomer hydrolysing reactors are used in combination with some system which detect the monosaccharides produced. Immobilized cellulase has been used in the bioengineering sciences for the large-scale saccharification of cellulose [7]. As far as is known, this enzyme complex has not yet been studied for analytical purposes. Little is known about its action on compounds other than β-(1→4)-linked glucans. This paper reports an investigation of the ability of cellulase-based post-column enzyme reactors for the selective and sensitive HPLC determination of β-glucan oligo- and polysaccharides in general.

As enzyme reactors require water-rich eluents, reversed-phase (RP) columns were chosen for the separation of the oligo- and polysaccharides. Separations of carbohydrates on RP columns have been described previously (see refs. 8 and 9 for recent reviews and refs. 10–12 especially for RP separations). Until now the practical use of RP columns for the separation of carbohydrates had limited success because no sensitive and gradient-compatible detectors were available for this purpose. Recently, the combination of novel column materials with pulsed electrochemical detection (PED) highly improved the existing HPLC separation and detection capabilities for these compounds [13]. However, the non-selectivity of PED detectors is disadvantageous when specific carbohydrate molecules have to be determined in the very complex carbohydrate mixtures that are characteristic of biological extracts. Also, the technique requires highly basic eluents, which restricts the choice of chromatographic conditions and the recovery of separated compounds. The wide possibilities of the combination RP columns and enzyme reactors with electrochemical detection (ED) in carbohydrate analysis are reported here.

EXPERIMENTAL

HPLC determination

For gradient elutions, an SP8700 solvent delivery system (Spectra-Physics, San Jose, CA, USA) was equipped with an extra 1-ml mixing chamber (Ultrograd gradient mixer, LKB, Bromma, Sweden) at the low pressure side to eliminate gradient disturbances. Samples were injected with a Valco EC10U injector (VICI, Houston, TX, USA). Analytical separations were performed on a RoSiL C_{18} column (Bio-Rad/RSL, Eke, Belgium), 150 × 4.6 mm I.D., 5 μm particle size. The detector consisted of a laboratory-made large-volume wall-jet electrochemical cell controlled by a three-electrode potentiostat (see ref. 14 for a detailed description). The working electrode was a 3 mm diameter platinum disc, operated at +700 mV *versus* the saturated calomel electrode. The chromatograms were recorded with an SP4100 computing integrator (Spectra-Physics). Enzyme reactors were placed between the analytical column and the electrochemical detector. The analytical column was omitted in FIA measurements. Since oxygen must be present for the regeneration of the flavin-adenine dinucleotide (FAD) co-enzyme (glucose oxidase), the eluents should not be thoroughly degassed.

Enzyme reactors

Glucose oxidase and cellulase were immobilized on aminopropyl-derivatized glass beads using glutaraldehyde as described by Weetall [15]. A 100-mg mass of aminopropyl glass, 50 nm pore size, 200–400 mesh (Sigma, St. Louis, MO, USA) was first activated in 3 ml of a 2.5% (v/v) solution of glutaraldehyde (grade 1, Sigma) in 0.1 *M* phosphate buffer (pH 7) for 90 min under vacuum. The derivatized glass beads were rinsed with water and added to a solution of either 5 mg of glucose oxidase [EC 1.1.3.4, from *Aspergillus niger*, 283 U mg^{-1} (Serva, Heidelberg, Germany)] or 50 mg of cellulase [EC 3.2.1.4, from *Penicillium funiculosum*, 5.9 U mg^{-1} (Sigma)] in 3 ml of 0.05 *M* acetate buffer (pH 5.0). Vacuum was applied for 15 min, after which the reaction proceeded for 3 h at 4°C with occasional shaking (vortex-mixing). After filtration, the glass beads with the immobilized enzyme were slurry-packed (10 MPa) into 2.1 mm I.D. stainless-steel columns of either 60 or 50 mm length. The reactors were stored at 4°C with buffer solution when not in use. All experiments with the reactors were run at ambient temperature at pH 5. This pH was found to be a good compromise for the optimum functioning of the enzyme reactors and the electrochemical detector.

Carbohydrates

Glucose (Merck, Darmstadt, Germany), cellobiose (Nutritional Chemical, Cleveland, OH, USA), laminaribiose (Sigma), gentiobiose (Sigma), cellulose (microcrystalline, Merck), curdlan (Serva) and laminarin (Sigma) were commercially available. Cellodextrins were obtained through the partial hydrolysis of cellulose by hydrochloric acid; 100 mg of cellulose were added to 2 ml of 37% hydrochloric acid (p.a., UCB, Leuven, Belgium). After 2 h of stirring, 6 ml of water were added. The acid was evaporated under reduced pressure and 6 ml of water were added to the residue. The remaining acid was neutralized with sodium carbonate. Insoluble material was removed by centrifugation at 10 000 *g* for 30 min.

Laminaridextrins were prepared from curdlan [16]. A 10-mg mass of curdlan was subjected to formolysis in 5 ml of 90% formic acid for 60 min at 100°C. The acid was removed under reduced pressure and 5 ml of a 0.1 *M* solution of trifluoroacetic acid were added to the residue. Hydrolysis occurred at 100°C for 60 min. The resulting hydrolysate was neutralized over Dowex MWA-1 (OH^- form, Serva) and centrifuged.

A 10-mg mass of laminarin was hydrolysed as described for curdlan, except that formolysis was omitted and the hydrolysis time was reduced to 30 min.

For the reduction of oligosaccharides with sodium borohydride (Sigma), 10-ml solutions of these hydrolysates were adjusted to pH 12 using concentrated sodium hydroxide, and 200 mg of sodium borohydride were added. After standing overnight at ambient temperature, the solutions were adjusted to pH 5 with acetic acid. These solutions were injected without further purification.

Semi-preparative chromatography

Cellodextrins (cellotriose to cellohexaose). A 10-g mass of cellulose was hydrolysed as described earlier and the volume was reduced to 40–50 ml. In a first chromatographic step, the higher oligomers of the hydrolysate were separated on a semi-preparative octylsilica column (RSiL C_8, 250 × 10 mm I.D., 10 μm particle size, Bio-Rad/RSL). Water was used as an eluent at a flow-rate of 5 ml min^{-1} (HP-1084A

liquid chromatograph, Hewlett-Packard, Avondale, PA, USA), with refractive index detection (R404, Waters, Milford, MA, USA). A first fraction contained cellobi-, -tri- and -tetraose. Cellopentaose and cellohexaose were collected in a second and a third fraction. Ninety 200-μl injections were performed. In a second step, the first fraction was injected on an analytical octadecylsilica column (RoSiL C_{18} column, 150 × 4.6 mm I.D., 5 μm particle size, Bio-Rad/RSL), eluted with water at 0.8 ml min^{-1}. Cellotri- and -tetraose were collected using fifty 200-μl injections. The amounts of collected oligomers ranged from 23 to 90 mg with a purity >90% for cellotriose and >95% for cellotetra-, -penta- and -hexaose.

Oligosaccharides from curdlan hydrolysates were separated by gradient preparative-scale HPLC on an octylsilica column. Injections (6 ml) containing 80 mg of hydrolysed curdlan were performed on a 250 × 10 mm I.D. RSiL C_8 column, 10 μm particle size (Bio-Rad/RSL). The column was eluted with a water (A)–acetonitrile (B) gradient (4 ml min^{-1}, from 0% B to 5.5% B in 55 min). The column eluent was split into a detection and a collection line using a flow splitter (Valco) and a metering valve (Hoke, Creskill, NY, USA) to obtain a split-ratio of 7 (collection line) over 1 (detection line). The detection line was mixed with a 0.05 *M* acetate buffer (pH 5). Detection was performed using the cellulase–glucose oxidase–ED system. Ten repetitive injections yielded 27 oligomers in amounts ranging from 22.6 mg [degree of polymerization (DP) 3] to 1.2 mg (DP30).

RESULTS AND DISCUSSION

Detection principle

β-Glucans, eluting from the chromatographic column, are hydrolysed to glucose monomers through the action of immobilized cellulase in a first enzyme reactor. The produced β-D-glucose is oxidized in a second enzyme reactor containing immobilized glucose oxidase. The oxidation of glucose is coupled to the reduction of oxygen to hydrogen peroxide. The hydrogen peroxide produced is detected electrochemically. (The glucose oxidase reactor has been studied for many FIA and HPLC applications [17].)

Commercial cellulase contains a mixture of enzymatic activities. Three major activities have been defined and can be found in all cellulase preparations [18]: *endo*-1,4-β-D-glucanase, *exo*-1,4-β-D-glucanase and β-D-glucosidase. Their action converts β-(1→4)-linked D-glucosaccharides (cellodextrins) to β-D-glucose. The cellulase under study (from *P. funiculosum*) is also known to act on β-(1→3)-D-(laminarioligomers), β-(1→6)-D-(gentiooligomers) and β-(1→2)-D-glucosidic bonds (sophorooligomers). Hydrolysis yields β-D-glucose (same configuration of the anomeric carbon) [19]. Michaelis constants (K) and relative velocities can be found for the hydrolysis of DP2 to DP6 cellodextrins only [20]. Information on the relative rates of hydrolysis of other than cellodextrin oligosaccharides by cellulase preparations is unfortunately very scarce. The following section therefore gives quantitative information on the relative rates of hydrolysis of different glucosaccharides obtained with immobilized cellulase reactors.

Quantitative evaluation of the enzyme reactors: theory

Under the conditions used in this work (low substrate concentrations) and in

analytical applications of enzyme reactors in general, first-order kinetics can be assumed (see also the discussion by Goldstein [21]) (see eqn. 1). This is particularly true under these conditions of strict detector linearity. For applications of enzyme reactors in fields where high productivities are required, this is not so, and other descriptions of reactor behaviour need to be found.

$$v = \frac{dS}{dt} = -K_{ps}^{(app)}S \tag{1}$$

Here v is the rate of substrate conversion in the enzyme reactor, S is the substrate concentration, t is time and $K_{ps}^{(app)}$ the apparent pseudo first-order rate constant, equal to $V_{max}/K_M^{(app)}$ (V_{max} is the maximum rate of substrate conversion and $K_M^{(app)}$ is the apparent Michaelis constant) [22]. In enzyme reactors, the rate of substrate conversion is affected by mass transport limitation phenomena. Therefore, the $K_{ps}^{(app)}$ values depend on the flow-rate, on the carrier particle characteristics (see Johansson *et al.* [22] for a detailed discussion) and on the reactor dimensions. This work was restricted to measuring $K_{ps}^{(app)}$ values at flow-rates of 1 ml min^{-1}, keeping the type of matrix particles and reactor dimensions constant.

Integration of eqn. 1 yields eqn. 2, which can be rearranged to eqn. 3:

$$\ln\left(\frac{S_{t=\tau}}{S_{t=0}}\right) = \ln(1 - X) = -K_{ps}^{(app)}\tau \tag{2}$$

$$X = 1 - e^{-K_{ps}^{(app)}\tau} \tag{3}$$

wherein $S_{t=\tau}$ is the amount of substrate at the outlet of the enzyme reactor and $S_{t=0}$ is the amount of substrate at the inlet of the enzyme reactor, τ and X are, respectively, the residence time of the substrate in the reactor and the fractional conversion of the substrate.

The effectiveness of an enzyme reactor can be expressed quantitatively using $K_{ps}^{(app)}$ values, fractional conversions (X), or $\tau_{1/2}$ values (the residence time required to convert 50% of the substrate). Still more practical than half-life values may be the use of a half-length $L_{1/2}$, *i.e.*, the reactor length required to convert 50% of the substrate: $L_{1/2} = \tau_{1/2}F^{-1}$ when no retention occurs during transit through the enzyme reactor (F being the linear velocity of the eluent in the reactor). As mentioned earlier, $K_{ps}^{(app)}$ and therefore also $\tau_{1/2}$ and $L_{1/2}$ are dependent on parameters which influence mass transport.

Characteristics of the cellulase reactor

Tables I and II show the fractional conversions (X) and the molar response factors (MRFs) obtained with the cellulase reactor for fifteen different oligomers. These measurements were performed by injecting known concentrations of carbohydrates in an FIA set-up (no column) consisting of a pump, an injector, the cellulase and glucose oxidase reactor and the electrochemical detector. Fractional conversions were defined as the ratio of the amount of β-D-glucose liberated by the enzyme reactor, divided by the maximum amount of β-D-glucose that could theoretically be liberated

TABLE I

MRFs AND FRACTIONAL CONVERSIONS (X) FOR CELLODEXTRINS [β-(1→4) GLUCO-OLIGOMERS] FOR DIFFERENT DP VALUES

Obtained with a cellulase reactor operated at a flow-rate of 1 ml min^{-1} using 0.05 M acetate buffer of pH 5 as an eluent. Reactor length × I.D. are given as column heading. The third series of values was obtained with sodium borohydride-reduced cellodextrins; the fourth series was obtained using 10% acetonitrile as the eluent.

DP	Column dimensions (mm)							
	50 × 2.1		100 × 2.1		50 × 2.1 (reduced)		50 × 2.1 (10% acetonitrile)	
	MRF	X (%)	MRF	X (%)	MRF	X (%)	MRF	X (%)
2	1.2	62	1.3	63	0.7	34	1.0	50
3	2.0	67	2.0	66	1.0	33	1.5	50
4	2.3	57	2.5	63	1.9	46	1.8	44
5	2.9	57	3.2	64	2.4	47	2.7	53
6	3.6	61	3.5	59	3.9	66	3.3	55

(100% hydrolysis). To determine the amount of glucose freed from the oligosaccharides by the cellulase reactor, known concentrations of β-glucans ($2 \cdot 10^{-10}$ mol) containing standard additions of glucose ($4 \cdot 10^{-10}$, $8 \cdot 10^{-10}$ and $16 \cdot 10^{-10}$ mol) were injected (a correction factor is applied to deal with the α/β anomerisation, as the glucose oxidase reactor only detects the β-anomer, which is present at 65% (25°C) for a glucose solution in equilibrium [23]). At least three standard additions were made for each data point and each sample was injected three times. The relative errors for these

TABLE II

MRFs AND FRACTIONAL CONVERSIONS (X) OBTAINED WITH A CELLULASE REACTOR AT DIFFERENT FLOW-RATES

Samples were β-(1→4) (cellobiose and cellopentaose), β-(1→6) (gentiobiose) and β-(1→3)-linked gluco-oligomers (laminaribiose and curdlan DP5–30). Column dimensions 50 × 2.1 mm.

Carbohydrate	Flow-rate (ml min^{-1})					
	0.5		1		4.5	
	MRF	X (%)	MRF	X (%)	MRF	X (%)
Cellobiose	0.57	28.5	0.54	27	0.38	19
Gentiobiose	0.64	32.2	0.79	40	0.60	30
Laminaribiose	0.44	22.0	0.42	21	1.4	68
Cellopentaose	3.57	71.4	3.6	72	2.6	52
Curdlan DP5	1.43	28.7	1.0	20	1.6	31
Curdlan DP10	1.61	16.1	1.1	11	0.96	9.6
Curdlan DP15	0.93	6.3	0.95	6.4	0.64	4.3
Curdlan DP20	0.99	4.9	1.0	5.2	0.45	2.2
Curdlan DP25	0.87	3.5	0.83	3.3	0.55	2.2
Curdlan DP30	0.82	2.7	0.55	1.8	0.57	1.9

data are up to 10% (linear regression error variance analysis). The MRFs are defined as the response (peak area, or number of Coulombs for an amperometric detector) provoked by the β-glucan, divided by the response provoked by an equimolar amount of β-D-glucose.

The fractional conversions are highest, around 60%, for the cellodextrins [β-(1→4)-glucans] (Table I). There is little variation in the fractional conversion within the series for degrees of polymerization from 2 to 6. This may indicate that for these substances, the reactor works at its maximum conversion capacity. This is in accordance with the fact that doubling the reactor length has no effect on the fractional conversion. Table I also shows that MRFs increase with increasing DP. The ratio MRF/(DP − 0.35) (0.35 is a correction factor, taking into account the α/β anomerisation of the end-glucose) is reasonably constant within each series of experiments. For the sodium borohydride reduced cellodextrins, this constancy is found in the ratio MRF/(DP − 1) as one of the glucose units is chemically transformed, and therefore not available for oxidation by glucose oxidase. After reduction pretreatment, the MRFs and fractional conversion are smaller, especially for molecules with low DP values. However, reducing the end-glucose unit of a β-glucan chain does not drastically alter the detection capability of the system for these compounds. This can be ascribed to the reaction route of the β-glucanases in which the terminal glucose units at the non-reducing end are hydrolysed first. The addition of 10% acetonitrile to the eluent also reduces the MRF and fractionation values, with 16 and 18%, respectively, as mean values for the five compounds.

Table II shows the MRF and fractionation values, measured for three disaccharides, cellopentaose and for six linear β-(1→3)-linked oligosaccharides at different flow-rates. It is clear from these data that the immobilized cellulase also catalyses the hydrolysis of β-(1→6)-linked compounds (gentiobiose) and β-(1→3)-linked oligomers from DP2 (laminaribiose) up to DP30. There is a tendency of decreasing MRFs and fractional conversions towards β-(1→3)-linked oligomers, as compard to the β-(1→4)-linked cellodextrins (Table I). These results suggest a lower activity of the immbilized enzyme complex towards other than β-(1→4)-linked glucans. However, the compounds are still easily detectable with the described system, as the MRFs are comparable to that of β-D-glucose. There are no significant differences between the results for flow-rates from 0.5 to 4.5 ml. This suggests that the enzyme reactor works at its maximum fractional conversion, *i.e.*, that the reactor length is much higher than $L_{1/2}$ (this is confirmed by results discussed for Table III later). For β-(1→3) oligomers, the MRFs decrease with increasing degrees of polymerization. This trend is shown in Fig. 1. There is no straightforward explanation as to why higher oligomers of this type have lower fractional conversions while the reactor works at its maximum efficiency. Substrate hydrolysis of 100% does not seem to be realizable, no matter how long the reactor is. A better understanding of the way in which the enzymes catalyse complex β-glucans is needed to improve the reactor efficiency, which leads to higher MRFs and lower detection limits. Little quantitative data are known for the MRFs for saccharides in PED applications. However, it is clear that the latter technique can give drastically reduced MRFs for higher oligomers, as Larew and Johnson [24] used enzyme reactors to improve the PED molar responses for such compounds.

Fig. 2 shows the fractional conversions as a function of the reactor length for

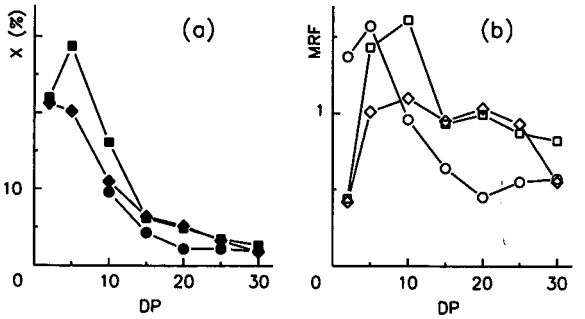

Fig. 1. (a) Dependence of fractional conversion (X) on DP value for β-(1→3)-linked glucooligomers of DP2, -5, -10, -15, -20, -25 and -30 at flow-rates of (■) 0.5 ml min^{-1}, (◆) 1 ml min^{-1} and (●) 4.5 ml min^{-1}, obtained with the immobilized cellulase reactor (50 × 2.1 mm I.D.). (b) Dependence of MRF on DP value for β-(1→3)-linked glucooligomers of DP2, -5, -10, -15, -20, -25 and -30 at flow-rates of (□) 0.5 ml min^{-1}, (◇) 1 ml min^{-1} and (○) 4.5 ml min^{-1} for the immobilized cellulase reactor (50 × 2.1 mm I.D.).

β-(1→3) oligomers. The experimental data were obtained using standard additions as described earlier (nine measurements for each data point). A decreased fractional conversion with increasing DP can be seen from this figure. Conversion rates, $K_{ps}^{(app)}$ (or half-length $L_{1/2}$), remain more or less constant for the different oligomers: the six lines shown in Fig. 2 reach a plateau value for approximately the same reactor lengths. The experimental data from Fig. 2 were used in a non-linear least-squares curve-fitting algorithm (the program was based on simplex iterations and run on a PC/AT 286 computer). The data were fitted in a function of the form $X = X_{max}[1 - \exp(-K_{ps}^{(app)}\tau)]$, which is theoretically predicted to describe the reactor behaviour (see eqn. 3) if first-order kinetics are used. Using the G-test [$G = \Sigma(X_{i,exp} - X_{i,cal})^2/\sigma_i^2$] of a χ^2 distribution, all the calculated plots described the experimental data at the 95% confidence level, except for DP30. These results suggest that the simple first-order kinetics model, although probably oversimplified, gives a fairly practical description of the reactor behaviour. From the theoretically fitted curves, $K_{ps}^{(app)}$, X_{max} and $L_{1/2}$ values were obtained (except for DP30) (Table III). It is clear that the $K_{ps}^{(app)}$ and $L_{1/2}$ values are reasonably independent of DP. The measured $L_{1/2}$ values confirm that

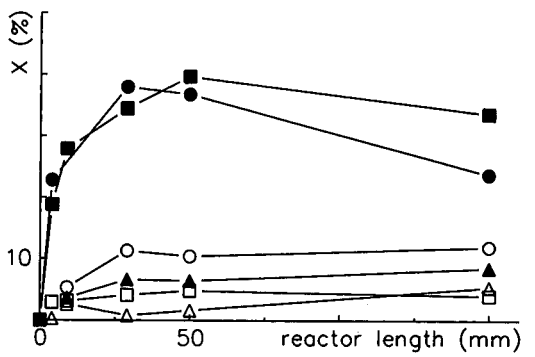

Fig. 2. Fractional conversion (X) of curdlan oligomers [β-(1→3) glucooligomers] as a function of reactor length (L) for an immobilized cellulase reactor (2.1 mm I.D.) at a flow-rate of 1 ml min^{-1}. (■) DP5; (●) DP10; (▲) DP15; (□) DP20; (○) DP25; and (△) DP30.

TABLE III

APPARENT PSEUDO FIRST-ORDER RATE CONSTANT, $K_{ps}^{(app)}$, MAXIMUM FRACTIONAL CONVERSION, X_{max}, AND HALF-LENGTH, $L_{1/2}$, OBTAINED FOR SOME CURDLAN OLIGOMERS WITH AN IMMOBILIZED CELLULASE REACTOR SYSTEM

Column, 2.1 mm I.D.; flow-rate, 1 ml min^{-1}. Curdlan oligomers [β-(1→3) glucooligomers] with known DP value. The values were calculated from the experimental data in Fig. 2 (simplex iterative curve fitting) (see text).

DP	$K_{ps}^{(app)}$ (s^{-1})	X_{max} (%)	$L_{1/2}$ (mm)
5	0.68	37	4.3
10	0.60	37	4.9
15	0.70	6.3	4.1
20	0.55	4.3	5.2
25	0.32	12	9.1
30	–	–	–

maximum reactor efficiencies were obtained, as the $L_{1/2}$ values are about ten times smaller than the reactor length.

The selectivity for β-glucans was evaluated by injecting a mixture which contained α-glucans (starch hydrolysate) at a concentration 50 times higher than the working range for β-glucans. Except for a large glucose peak, no major baseline disturbances could be detected at high sensitivity (10 nA). Some commercial glucose oxidase preparations were found to contain α-glucan hydrolysing enzymes. This causes the immobilized glucose oxidase reactor to behave as an α-glucan detector. Such contaminations were absent in the glucose oxidase preparation used in this study.

Characteristics of the glucose oxidase reactor

The quantitative determination of the fractional conversions as a function of reactor length (residence time) for the immobilized glucose oxidase reactor was rather troublesome. Similar problems were experienced in evaluating the hydrogen peroxide concentrations as those discussed by Tyrefors and Carlsson [25]. In Fig. 3 the integrator counts are plotted against the glucose oxidase reactor length (I.D. 2.1 mm) for a flow-rate of 1 ml min^{-1}. The same curve-fitting algorithm as described earlier was applied to the data in Fig. 3. The $K_{ps}^{(app)}$ value calculated from the resulting curve was 0.76 s^{-1}. From this value, $L_{1/2}$ was determined to be 3.8 mm and $\tau_{1/2}$ = 0.9 s. Therefore, an immobilized glucose oxidase reactor with an I.D. of 2.1 mm and a length of 60 mm has a fractional conversion >99.99%.

For both reactors, no major loss of activity could be detected for at least 3 months. The detection system was linear for glucose in the range 10^{-6} (1.8 ng injected) to $5 \cdot 10^{-3}$ *M* (9 μg injected). Linear calibration graphs were also obtained for cellodextrins of DP2 and DP6, and for laminaridextrins of DP2, -6 and -28 in the range $5 \cdot 10^{-6}$–$5 \cdot 10^{-4}$ *M*. Extra-column band broadening caused by the reactors was determined by a method described by Verzele and Dewaele [26]. A σ_{ec} value of 31 μl was obtained for a reactor of 50 mm length and 2.1 mm I.D.

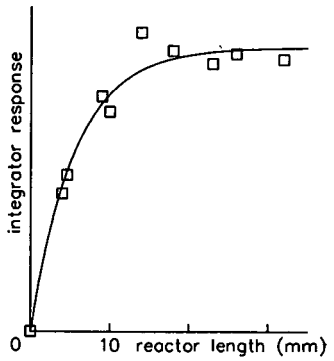

Fig. 3. Conversion of glucose (or production of hydrogen peroxide) with increasing reactor length by an immobilized glucose oxidase reactor (2.1 mm I.D., flow-rate 1 ml min^{-1}, 10^{-9} mol glucose injections).

Chromatographic separations

One of the problems in the RP chromatography of reducing carbohydrates is the doublet formation or band broadening due to α/β anomeric equilibria. Previous attempts to avoid this phenomenon by speeding up mutarotation were rather unsatisfactory [12]. Reduction of the terminal aldehyde group with sodium borohydride eliminates the anomerization problem, leading to less complicated chromatograms. Fig. 4a shows an isocratic mode separation of sodium borohydride-reduced cellodextrins [β-(1→4)-linked linear glucose chains] on an octadecylsilica column; Fig. 4b shows a gradient mode separation of non-reduced cellodextrins using

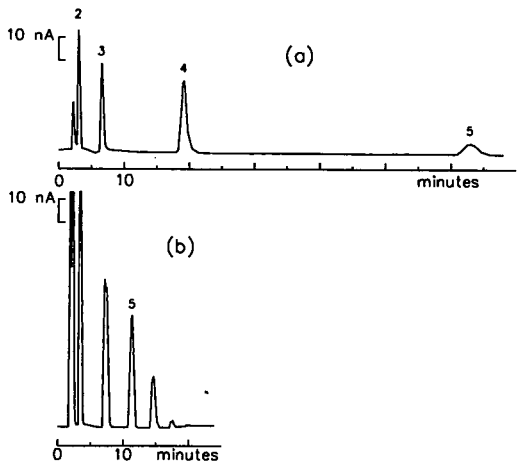

Fig. 4. (a) Chromatogram of sodium borohydride-reduced cellodextrins (reduced partial hydrolysate of cellulose) run under isocratic conditions using an immobilized cellulase (50 × 2.1 mm I.D.) and glucose oxidase (60 × 2.1 mm I.D.) reactor. Peak numbers refer to the DP value of the eluting compound. Chromatographic conditions: column, RP C_{18}; eluent, 0.05 *M* acetate buffer (pH 5); flow-rate, 1 ml min^{-1}; detector, platinum electrode at +700 mV *versus* saturated calomel electrode. (b) Chromatogram of cellodextrins (not reduced) using the immobilized enzyme reactors (cellulase and glucose oxidase) for detection run under gradient conditions. Eluent: A = 0.05 *M* acetate (pH 5); B = acetonitrile. Gradient programme from 0 to 5% B in 15 min; other conditions as in (a).

acetonitrile as the organic modifier. Peak broadening and splitting due to the separation of α/β anomers can be seen, but the overall separation efficiency is still sufficiently high. Laminaridextrins, obtained through the partial hydrolysis of curdlan [a mainly β-(1→3)-linked linear glucose chain], are also separated using the gradient system (Fig. 5). A simpler chromatographic pattern is obtained with sodium borohydride reduction pretreatment (Fig. 5b) than without this pretreatment (Fig. 5a). Sodium borohydride reduction is not strictly necessary for baseline separation of the oligosaccharides. More complex, branched oligosaccharides were obtained through the partial hydrolysis of laminarin. Water-soluble laminarin itself is a branched β-D-glucan containing about 30 glucopyranosyl units. The backbone is made up by (1→3) and (1→6) bonds while branching points consist of (1→6) bonds. The hydrolysate shows a complex chromatographic peak pattern (Fig. 6). Figs. 4–6 show that the immobilized cellulase can be used effectively for the detection of β-glucans after RP separations, for DP values as high as 30. The retention of the glucosaccharides increases with decreasing water solubility. Highly water-soluble carbohydrates such as the lower amylose [α-(1→4) linked] oligomers up to DP5 could not be retained on the C_{18} RP phases which were used in this study. A highly derivatized RP C_{18} phase, provided by Bio-Rad/RSL, could achieve this. Detection of the carbohydrates was very sensitive. For cellotetraose [capacity factor (k') ≈ 10] a detection limit was estimated to be 6 ng (10^{-11} mol injected, peak height about $10\sigma_{noise}$).

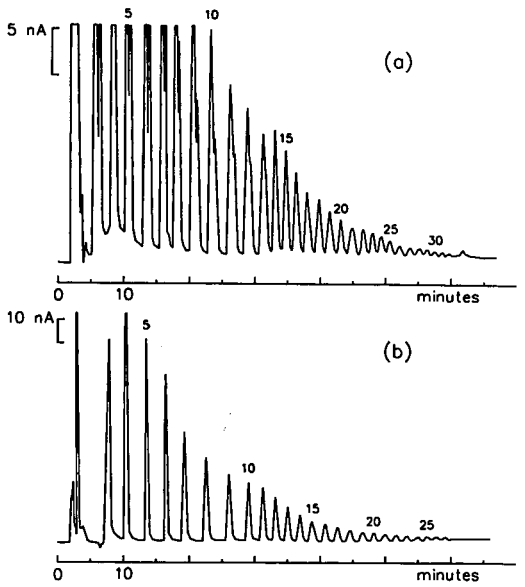

Fig. 5. (a) Chromatogram of partial hydrolysate of curdlan [laminaridextrins, linear β-(1→3)-linked glucooligomers], run under gradient conditions. Eluent: A = 0.05 *M* acetate (pH 5); B = acetonitrile. Gradient programme from 0 to 2.5% B in 3 min, 5% B at 12 min, 6.7% B at 30 min, 8.3% B at 60 min, 10% B at 65 min. Other conditions as in Fig. 4a. (b) Chromatogram of sodium borohydride-reduced partial hydrolysate of curdlan, run under gradient conditions. Eluent and gradient pogramme as in (a). Other conditions as in Fig. 4a.

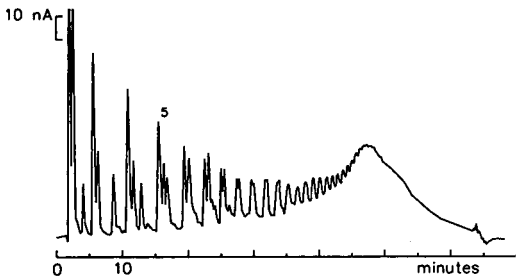

Fig. 6. Chromatogram of partial hydrolysate of laminarin (complex mixture of branched glucooligomers with mixed linkages, see text). Eluent: A = 0.05 *M* acetate (pH 5); B = acetonitrile. Gradient programme from 0 to 5% B in 20 min, 10% B at 60 min. Other conditions as in Fig. 4a.

Both the enzyme reactors and the ED system behaved well in water–acetonitrile gradient systems. There are indications that the large-volume wall-jet detector has better characteristics for gradient elution than thin-layer designs [27]. At high detector sensitivities (10 nA full scale), baseline problems were experienced during gradient elution when methanol was used as an organic modifier. Working electrode currents increased significantly with increasing methanol concentrations, resulting in a shifting baseline (probably due to the oxidation of methanol on the platinum electrode). Replacing methanol by acetonitrile solved this problem. It is known that enzymes can often exert their catalytic activity even in the presence of organic solvents, and that the stability of enzymes is also frequently enhanced after immobilization. No significant loss of activity of the reactor couple was observed for at least fifty gradient elution analyses comparable to that shown in Fig. 5.

CONCLUSIONS

In combination with gradient RP-HPLC and ED, immobilized cellulase reactors provide a powerful means for the selective and sensitive determination of β-glucans in complex carbohydrate mixtures. β-(1→4)-Linked glucosaccharides and various other structurally isomeric glucosaccharides up to at least DP30 are rapidly broken down by the immobilized enzyme complex. The reactors can be used in a gradient system, which highly improves and facilitates the separation of oligosaccharides on RP columns. The stability of the reactor is very good. The cellulase reactor can be improved to yield higher MRFs, especially for compounds other than β-(1→4)-linked glucans. Therefore, a search for enzymes with a higher hydrolytic activity towards such β-glucans and a better understanding of their mode of action is necessary. Future work in this laboratory will also be directed to the use of selective hydrolases in multiple enzyme reactor systems to obtain qualitative (structural) information on the chromatographically separated carbohydrates.

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Extraction of xanthine oxidase from milk by counter-current distribution in an aqueous two-phase system

AURORA ORTIN, MANUEL J. LÓPEZ-PÉREZ*, MARÍA T. MUIÑO BLANCO and JOSÉ A. CEBRIAN PEREZ

Departamento de Bioquimica, Biología Molecular y Celular, Facultad de Veterinaria, Miguel Servet 177, 50013 Zaragoza (Spain)

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ABSTRACT

By comparing the partitioning of total milk protein with that of pure xanthine oxidase, it was established that a two-phase system containing 7% (w/w) Dextran T 500 and 5% (w/w) polyethylene glycol 6000 in the range of 5–25 m*M* sodium phosphate is suitable for the extraction of the enzyme from milk. In this system, the bulk of milk protein partitioned in the upper phase, whereas the pure enzyme showed a preferential affinity for the lower phase. The enzyme appears also to be released from the membrane of the milk fat globules as a consequence of the phase partitioning itself, as the enzyme activity (undetectable in the boud form) increased when successive partitionings of milk were carried out. After 57 transfers, centrifugal counter-current distribution (CCCD) of milk in this two-phase system allowed the separation of the xanthine oxidase from the bulk of milk protein. The enzyme thus isolated was mostly resolved as an unique peak, identified densitometrically after sodium dodecyl sulphate polyacrylamide gel electrophoresis. The results obtained show that CCCD of milk in a two-phase system is an useful procedure to achieve the release and isolation of xanthine oxidase from milk.

INTRODUCTION

Xanthine oxidase (EC 1.2.3.2) is a complex enzyme abundant in the milk fat globule membrane, containing flavin adenine dinudeotide (FAD), molybdenum and iron–sulphur cofactors [1]. The purification of the enzyme from milk or butter requires a previous step involving the release of the enzyme from membrane material. Solvent extraction and protease or detergent treatment have been used for this purpose [2]. Diverse procedures have been employed for its isolation [3–6] and, more recently, an affinity chromatographic method using Sepharose 4B–folate [7] has been described.

The extraction of biological material using aqueous two-phase systems prepared from solutions of two water-soluble polymers is a powerful technique for the separation of macromolecules [8,9]. One of the systems most employed for the purification of proteins consists of dextran and polyethylene glycol (PEG) [10]. PEG is a water-soluble synthetic polymer with interesting properties. In addition to its use for the preparation of two-phase systems, it has applications as a precipitating or fusing

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agent [11]. The influence of PEG on the partitioning of membrane components between the external phase and the membrane has already been described [12], and this effect has been discussed as a smooth detergent-like behaviour [13].

The aim of this work was to apply the dextran–PEG two-phase system as a single procedure to achieve the purification of xanthine oxidase from whole cow milk. The results obtained show that the enzyme is both released and isolated from the bulk of the milk protein by counter-current distribution in a Dextran 500–PEG 6000 two-phase system.

EXPERIMENTAL

Chemicals

Dextran (relative molecular mass, M_r = 500 000) was obtained from Pharmacia and PEG 6000 (M_r 6000–7500) from Serva. Pure xanthine oxidase from buttermilk (X-4500) and all other chemicals were obtained from Sigma.

Milk

Fresh dairy milk was kept at 4°C (2–3 h). Any kind of physical treatment was completely avoided before phase partitioning.

Xanthine oxidase assay

Xanthine oxidase was assayed by the method of Avis *et al.* [14] in experiments in which commercial pure enzyme was employed. This method consisted in the spectrophotometric measurement at 290 nm of the uric acid formed in the enzymatic reaction. However, this method is inadequate for the determination of the enzyme in samples of milk with a high protein content. In these instances, the enzymatic activity was determined by measuring the hydrogen peroxide formed in the enzyme reaction. This was carried out by means of two coupled enzymatic reactions. The first involved catalase (E.C. 1.11.1.6) plus ethanol, producing acetaldehyde. The second coupled reaction was accomplished by aldehyde dehydrogenase (E.C. 1.2.1.5) plus NADP [15]. Reduction of NADP by acetaldehyde was then determined spectrophotometrically at 340 nm. The activity was linear during the first 4–5 min. When this coupled reaction was used (high milk protein content in batch experiments, see Fig. 3), different sample dilutions were performed, and the activity was determined in the range where the absorbance increase was proportional to the protein concentration.

Two-phase systems

The systems were prepared from stock aqueous solutions of the polymers, 40% (w/w) PEG and 20% (w/w) dextran, made as described previously [8]. The polymer solutions and stock solutions of 1.2 *M* phosphate buffer, 2 m*M* EDTA, milk and water were weighed and mixed to give the final concentrations described below. When batch experiments were carried out, 5.0-g two-phase systems containing 0.5 g of milk were prepared and mixed by 20 inversions. Phase separation was speeded up by centrifugation at 1000 *g* for 1 min. All operations were carried out at 0–4°C either in batch or in counter-current distribution experiments. When batch experiments were carried out, protein and/or xanthine oxidase were determined in the mixed two-phase system and in the upper phase after separation of the batch had occurred.

Centrifugal counter-current distribution

The counter-current distribution system used was constructed on the basis of that described by Akerlund [16]. With this device, the time for the separation of the two phases is shortened by centrifugation. The apparatus contains 60 chambers arranged in a circle, allowing transfer of the upper phases relative to the lower phases. To carry out centrifugal counter-current distribution (CCCD) experiments, 100 g of a two-phase system containing 7% (w/w) dextran, 5% (w/w) PEG, 0.2 m*M* EDTA and 10 m*M* sodium phosphate (pH 7.0) were prepared and mixed, and 1.55 ml of this two-phase system were loaded in chambers 3–59. A 8.0-g two-phase system was prepared with the same composition but also containing 3.5 g of milk, and 1.55 ml of this mixture were then loaded in chambers 0–2. The shaking and centrifugation time were 45 and 30 s, respectively, and 57 transfers were performed. After the run, the systems were transformed into homogeneous solutions by the addition of 1.55 ml of 50 m*M* sodium phosphate pH 7 in each cavity. The fractions were then collected and analysed. Protein was determined by the method of Bradford [17], by which no interference with the two-phase-forming polymers is produced. Results are presented as the percentage in every chamber of the total enzyme activity and protein determined after the run, as described previously [18].

Electrophoresis

Samples collected from each CCCD chamber were lyophilized and dissolved in 0.75 ml of 50 m*M* sodium phosphate (pH 7.5), then 0.4 ml was incubated with 50 μl of 30% mercaptoethanol and 50 μl of 45% sodium dodecyl sulphate (SDS) at 100°C for 5 min. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of 5 μl of this treated sample was carried out in a 10–15% PhastGel gradient (Pharmacia PhastSystem). Staining was carried out by the optimized silver method for SDS–PAGE gradient gel media (PhastGel TM) and densitometric tracing was performed on an LKB Ultroscan XL laser densitometer.

RESULTS

By comparing the partitioning of the total milk protein with that of xanthine oxidase, the usefulness of phase partitioning for enzyme purification could be evaluated. A number of parameters were first investigated to establish the optimum conditions for the extraction of the enzyme by phase partitioning. Fig. 1 shows that an increase in the concentration of dextran and PEG results in an enhanced preference of the bulk of milk protein for the lower, dextran-rich phase. The greatest extent of milk protein partitioning in the upper, PEG-rich phase was achieved in a two-phase system containing 7% dextran and 5% PEG in the range of 5–25 m*M* sodium phosphate. At higher polymer concentrations, the maximum partitioning of the milk protein in the upper phase occurred when the sodium phosphate concentration exceeded 25 m*M*.

The partitioning behaviour of pure xanthine oxidase (0.001% protein) in a 7:5 dextran–PEG two-phase system is shown in Fig. 2a. The maximum partitioning of the enzyme in the upper phase also occurred in the range 5–20 m*M* sodium phosphate, whereas higher salt concentrations promoted a stronger enzyme preference for the lower phase. It is remarkable that the percentage distribution in the upper phase of the bulk protein is much higher than that the enzyme. This effect made this poly-

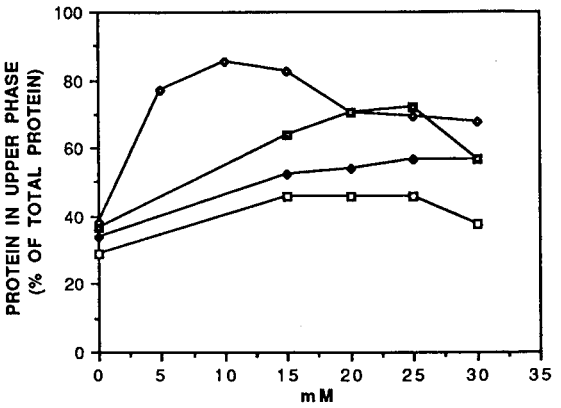

Fig. 1. Partitioning of milk protein as a function of sodium phosphate concentration at pH 7. The two-phase system contained (◇) 7 and 5, (◘) 7.5 and 5.5, (◆) 8 and 5.5 and (□) 8 and 6% dextran and PEG, respectively, and 0.2 m*M* EDTA.

mer concentration a promising variable for achieving the enzyme purification. In the presence of 10 m*M* sodium phosphate, an increasing concentration of potassium chloride promoted an enhanced affinity of the xanthine oxidase for the lower phase (Fig. 2b), and the partition behaviour was very similar between pH 5.5 and 8 (Fig. 2c). It is noteworthy that increasing amounts of enzyme in the two-phase system enhanced the partitioning in the lower phase (Fig. 2d).

However, when the xanthine oxidase activity was determined after partitioning of milk in a two-phase system of the composition established previously (Fig. 2b), an impaired behaviour of the enzyme was found. The enzyme activity was barely detected and the distribution of the scarce activity found was preferential for the upper phase. A study of the effect of successive extractions on the activity and partitioning of the xanthine oxidase from milk was therefore carried out. The results obtained after two further extractions of a first initial two-phase system are shown in Fig. 3. It can be observed how each re-extraction accounts for an increase in the xanthine oxidase activity. This increase was substantial in the upper phase, although it was also important in the lower phase after the second re-extraction. These results clearly indicated that an adequate extraction of the enzyme from milk by phase partitioning could only be achieved by a multi-step extraction process, such as the counter-current distribution can perform.

Fig. 4 shows the CCCD diagrams obtained from (a) pure xanthine oxidase and (b) milk. In both instances, the enzymatic activity was mostly located in the first ten chambers, whereas the protein bulk mostly appeared in the last 20 chambers (Fig. 4b) with another peak in the middle of the run.

The distribution of 1 molecule in a two-phase system is represented by the partition ratio, *G*. The *G* value is defined as the percentage of the molecule in the mobile part of the system (upper phase in counter-current distribution) divided by the percentage in the stationary part (lower phase). From the positions of the peaks in the CCCD diagram, the *G* value can be calculated using the approximate equation $G = i/$

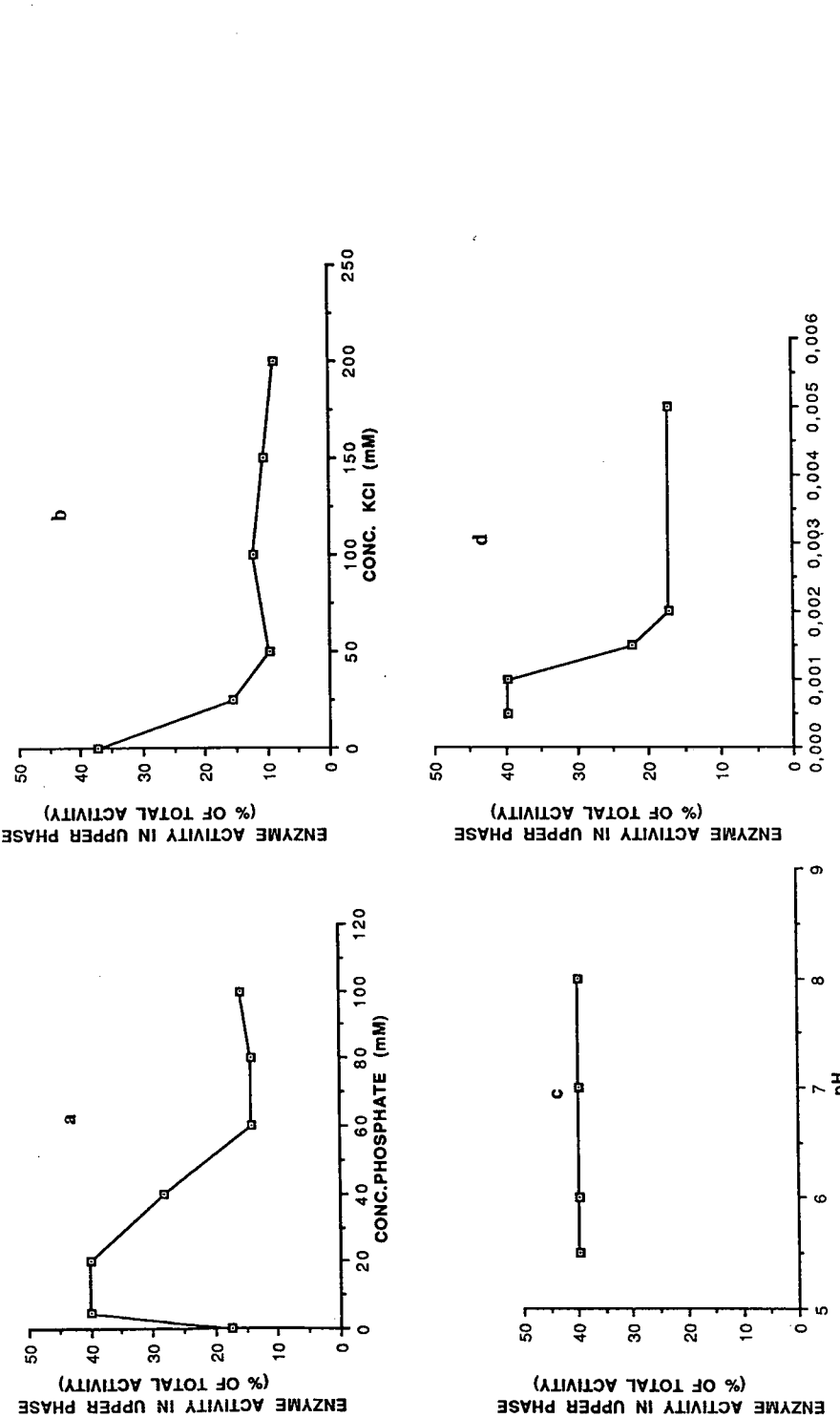

Fig. 2. Partitioning of pure xanthine oxidase as a function of (a) sodium phosphate concentration at pH 7 in a two-phase system containing 7% dextran, 5% PEG and 0.2 m*M* EDTA; (b) KCl concentration in the presence of 7% dextran, 5% PEG, 0.2 m*M* EDTA and 10 m*M* sodium phosphate (pH 7); (c) pH with a two-phase system containing 7% dextran, 5% PEG, 0.2 m*M* EDTA and 10 m*M* sodium phosphate; and (d) xanthine oxidase concentration, expressed as % (w/w), in a two-phase system with the same composition as (c) to pH 7.

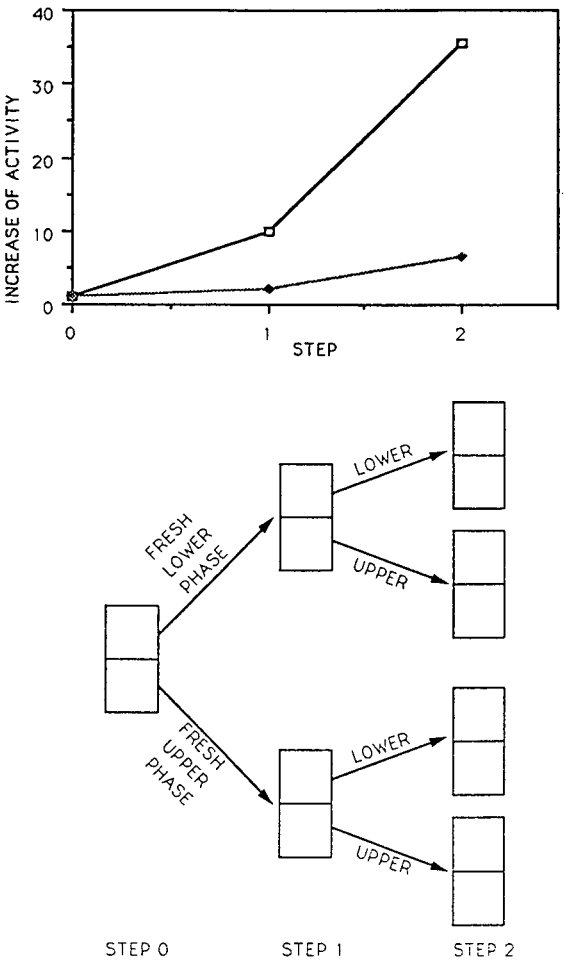

Fig. 3. Increase in the xanthine oxidase activity in the (□) upper and (◆) lower phases of successive phase partitionings of milk, in a two-phase system containing 7% dextran, 5% PEG, 0.2 m*M* EDTA and 10 m*M* sodium phosphate (pH 7). A diagram of the experimental procedure is shown underneath. The initial upper and lower phases of a first two-phase system (step 0) were re-extracted with fresh upper and lower phase, respectively, obtained from a new two-phase system of the same composition. Then, each of the four phases thus obtained (step 1) was newly re-extracted with the corresponding opposite fresh phase of a two-phase system of the same composition (step 2). The increase in the enzyme activity shown is then the ratio of the sum of the activities found in all the upper and lower phases obtained in each of step 1 or 2 from the initial upper or lower phase, divided by the activity found in the upper or lower phase of the initial step 0 two-phase system, respectively.

$(n-i)$, where i is the number of the chamber into which the enzyme peak has moved and n is the number of transfers [18]. The G values of xanthine oxidase and the bulk protein were calculated from the positions of the peaks in the CCCD diagram (Fig. 4). The protein peaks with G values of 1.33 and 6 correspond to the whey protein bulk (including β-lactoglobulin and α-lactalbumin) and caseins, respectively (data not shown).

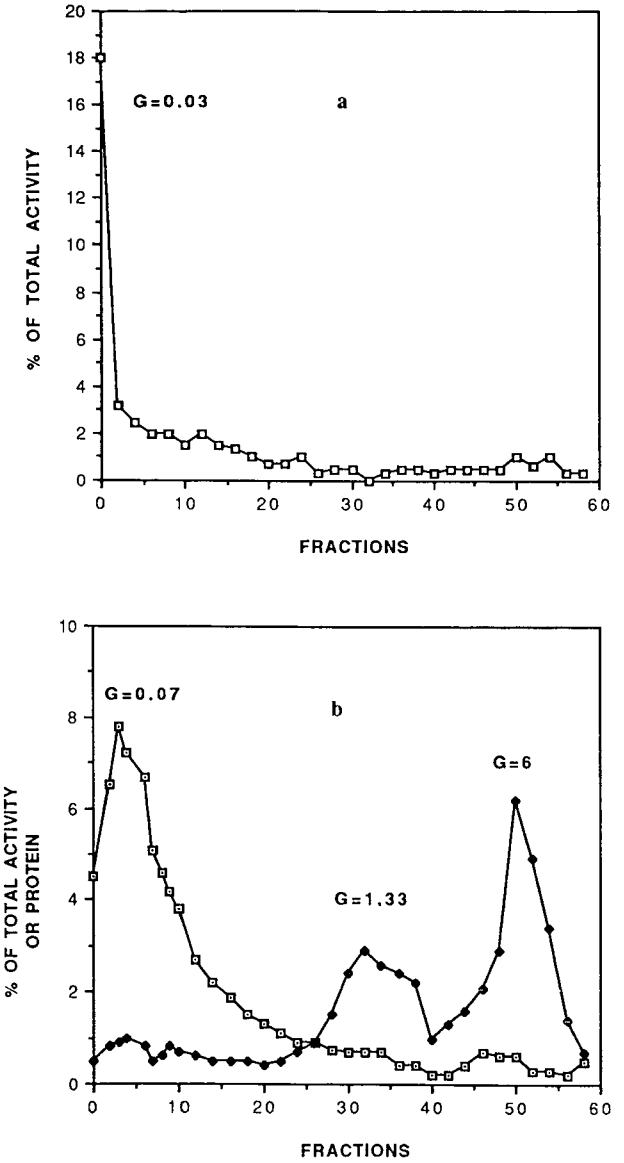

Fig. 4. CCCD diagrams of (a) pure xanthine oxidase (0.005% of protein in the loading chambers) and (b) milk. Results show the percentage in each chamber of the total (□) enzyme activity and (◆) protein obtained and determined after the run.

Protein extracted from milk and obtained from chambers 0–10 in CCCD (Fig. 4b) was resolved as an apparently unique electrophoretic band. This band can be easily made visible, but densitometric tracing was carried out to acheive a more precise and quantitative analysis of the gel obtained. The material thus detected was almost entirely located in a major peak, although some other very minor peaks ap-

Fig. 5. Densitometric traces of material obtained from chambers 0–10 after CCCD of (a) milk and (b) commercial xanthine oxidase; (c) trace for whole milk. KD = kilodalton.

peared in the upper part of the gel (Fig. 5a). Commercial pure xanthine oxidase used as a control showed a main densitometrically determined peak (Fig. 5b) of the same mobility as that of the milk xanthine oxidase obtained from CCCD chambers 0–10 (Fig. 5a). The other milk proteins showing a higher mobility than xanthine oxidase (Fig. 5c) were not detected in the first ten CCCD chambers.

As a precise determination of the enzyme activity cannot be performed in whole milk, the increase in the specific activity of the purified xanthine oxidase with respect to the milk xanthine oxidase cannot be determined. However, quantification of the densitometric tracing of the SDS-PAGE of the material extracted from milk and obtained from chambers 0–10 (Fig. 4b) shows that more than 80% of this material appeared in the major peak, which has the same electrophoretic mobility as the major component of the commercial pure enzyme (Fig. 5b). This percentage is *ca.* fifteen times higher than that of the corresponding peak (with the same electrophoretic mobility) obtained from whole milk.

DISCUSSION

The results obtained show that analytical CCCD of milk in a two-phase system containing 7% dextran, 5% PEG, 0.2 m*M* EDTA and 10 m*M* sodium phosphate (pH 7) is an adequate technique for extracting milk xanthine oxidase. The enzyme thus obtained appeared to be substantially free from other contaminant proteins detectable by SDS-PAGE under the experimental conditions used.

The high efficiency of this method for purifying xanthine oxidase from milk is due to two different effects. First, the enzyme is probably extracted from the membrane of the milk fat globules as a consequence of the phase partitioning itself. It is well known that xanthine oxidase bound to the fat globules shows very low activity, and that enzyme determination requires previous release of the enzyme [2]. Thus, when milk was partitioned in a single-batch two-phase system (Fig. 3; step 0), the enzyme activity was almost undetactable. However, successive extraction gave a substantial increase in the activity in the upper phase (Fig. 3; steps 1 and 2). As extractions proceed (Fig. 3; step 2), the enzyme appears to show a moderate increase in its affinity for the lower phase. These results can be interpreted as a partial and progressive release of the enzyme from the fat globules, with the consequent detection of the activity. As release is finally accomplished, the enzyme increases its partitioning in the lower phase, showing a very low G value in a multi-step extraction procedure (CCCD of milk; Fig. 3b). Such a releasing action of the two-phase system could be due to a combined effect of the mentioned ability of PEG for membrane extraction [11–13] together with the likely preference of the milk lipids for the upper PEG-rich phase in opposition to the preferential partitioning of the xanthine oxidase in the upper phase (Fig. 2).

In addition, the very different partition ratios of the milk bulk protein (Figs. 1 and 4b) and xanthine oxidase (Figs. 2 and 4a) is an important complementary advantage of this phase partition extraction of xanthine oxidase from milk. The great affinity of the xanthine oxidase for the lower phase is showed by its constant partition behaviour over wide ranges of pH (Fig. 2c) and temperature (4–25°C; data not shown), and even rises to 90% enzyme partitioning in the lower phase at a potassium chloride concentration higher than 25 m*M* (Fig. 2c). The slight difference in the G

values obtained for the pure enzyme and for the enzyme separated from milk (Fig. 4) could be due to the fact that the enzyme has first to be released from the fat globule membrane during the first transfers, inducing a lag effect in its preferential partitioning for the lower phase.

In the CCCD experiments using pure commercial xanthine oxidase, almost all the total initial activity (more than 90%) was found in the first ten chambers. However, this estimation cannot be made in the case of CCCD with whole milk, owing to the difficulty in detecting the activity in the sample, as stated above.

The G values obtained for the xanthine oxidase in the CCCD experiments (Fig. 4), both for the commercial pure form and for the enzyme separated from milk, were lower than those which could be expected from the percentage partitioning of the enzyme in the upper phase in the batch experiments (Figs. 1 and 2). This discrepancy could be explained by the different amount of enzyme present in each instance. In fact, we have found that increasing concentrations of xanthine oxidase in a two-phase system account for a higher partitioning of the enzyme in the lower phase (Fig. 2d).

Xanthine oxidase extracted from milk by CCCD appears as an almost unique band (Fig. 5b) in SDS-PAGE under the experimental conditions used here. This band showed the same electrophoretic mobility as the main component of the commercial enzyme (Fig. 5b).

The main protein components of milk are casein, α-lactalbunin and β-lactoglobulin [1,2]. Casein corresponds to the major peaks electrophoretically separated in the middle of the SDS-PAGE of milk, and α-lactalbunin and the two isoforms of β-lactoglobulin are the three major fastest components found at the end of the trace in Fig. 5c (data not shown). Therefore, the two minor peaks appearing in the xanthine oxidase extracted from milk by CCCD (Fig. 5a) do not correspond to any of these major milk proteins. It cannot be ruled out that they could represent some oligomeric forms of the enzyme.

These results clearly show that CCCD in a dextran–PEG two-phase system can be an advantageous single method for purifying xanthine oxidase from milk. Therefore, further studies on the use of different (cheaper) two-phase-forming polymers and on large-scale techniques could be justified in order to achieve the purification of the enzyme in the most convenient way for preparative and/or commercial purposes.

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CHROM. 23 450

Determination of a metabolite of the herbicide pyridate in drinking and groundwater using high-performance liquid chromatography with amperometric detection

ANTON PACHINGER*, ELISABETH EISNER, HELMUT BEGUTTER and HUBERT KLUS
Ökolab, Gesellschaft für Umweltanalytik, Hasnerstrasse 124a, 1160 Vienna (Austria)
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ABSTRACT

The main metabolite of the herbicide pyridate is 3-phenyl-4-hydroxy-6-chlorpyridazine (CL9673). A high-performance liquid chromatographic method with amperometric detection is described for determining CL9673 at residue levels in water samples. Sample preconcentration is performed by passage through a C_{18} extraction cartridge. A recovery study using tap water samples spiked with CL9673 at a concentration of 0.1 μg/l showed a recovery of 84.8% (coefficient of variation 6.2%). The method is suitable for the determination of CL9673 in drinking and groundwater.

INTRODUCTION

In the case of residue analysis of the herbicide pyridate {*O*[3-phenyl-6-chlorpyridazinyl-(4)]-S(*n*-octyl)-thiocarbonate}, which is used in the agricultural management of cereals, maize and rape, attention has primarily focused on the determination of its metabolite 3-phenyl-4-hydroxy-6-chlorpyridazine (CL9673, Fig. 1). CL9673 is physiologically active in plants and is the first metabolite of pyridate, which acts only as a carrier [1]. The occurrence of residues of pyridate and CL9673 are to be expected in treated plants and have previously been investigated in detail [1–5]. Leaching of CL9673 residues from topsoils may pollute groundwater. Until now, the determination of trace amounts of CL9673 in water samples has not been reported.

Analytical methods for the determination of pyridate residues use high-performance liquid chromatography (HPLC), gas chromatography (GC) and tandem mass spectrometry (MS–MS). Two-dimendional HPLC with UV detection has been applied to the determination of CL9673 in plant matrices [1,2]. Owing to its low volatility and low thermal stability, CL9673 cannot be determined by GC without derivatization. Pentafluorbenzylchloride has been used to form stable and volatile derivatives of CL9673 [3,4]. Jaklin *et al.* [5] have reported the use of MS–MS with direct probe insertion to determine CL9673 in crude plant extracts without derivatization. Buchberger *et al.* [6] have used HPLC with both UV and electrochemical detection to determine the phenolic metabolites bromoxynil and ioxynil of a herbicide formulation containing bromoxyniloctanoate, ioxyniloctanoate and pyridate. Al-

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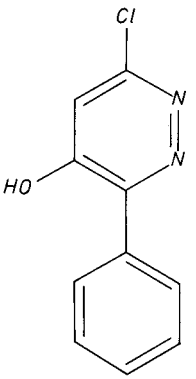


Fig. 1. Structure of 3-phenyl-4-hydroxy-6-chlorpyridazine (CL9673).

though this work focuses on sample preparation for the simultaneous determination of the metabolites of this herbicide formulation, it is not clear why CL9673 was excluded from these investigations.

Several recent papers have described the electrochemical behaviour of phenolic compounds [7–10]. The chemical structure of CL9673 encouraged these authors to investigate the application of amperometric detection for the determination of CL9673.

This paper describes a procedure for the determination of CL9673 in drinking and groundwater using solid-phase extraction and reversed-phase HPLC with amperometric detection.

EXPERIMENTAL

Apparatus

The liquid chromatograph used was a HP1090, Series M (Hewlett-Packard, Avondale, PA, USA) system fitted with a reversed-phase 5 μm ODS–Hypersil column (100 × 2 mm I.D.). Elution of the solutes was monitored with a programmable electrochemical detector (Model HP1049A, Hewlett-Packard) in amperometric mode. The detector was equipped with a glassy carbon working electrode and a solid state Ag/AgCl reference electrode. The working electrode was set at +1.25V.

Data integration was carried out using a series 300 HP 9000 computer (Hewlett-Packard).

Reagents

CL9673 was obtained from Chemie Linz (Linz, Austria). Working standard solutions of CL9673 of various concentrations were obtained by diluting a standard stock solution (1 mg/ml in methanol) with HPLC-grade water. The concentrations used were 0.4, 0.2, 0.04, 0.02 and 0.004 μg/ml. The working standard solutions were freshly prepared every 2 weeks.

Analytical-reagent grade acetic acid, acetone and sodium chloride were purchased from Merck (Darmstadt, Germany). HPLC-grade water and methanol used

for the preparation of the mobile phase were purchased from Promochem (Wesel, Germany) and J. T. Baker (Phillipsburg, NJ, USA), respectively. Bond-Elut C_{18} solid-phase extraction cartridges containing 1 g of sorbent were purchased from Analytichem International (Harbor City, CA, USA). The cartridges were activated by washing with methanol (5 ml) followed by water (5 ml) prior to use.

Millex-SLCR004NB 0.5-μm membrane filters were purchased from Millipore (Bedford, MA, USA).

Chromatographic conditions

Separations were effected under isocratic conditions at 40°C using a pre-mixed mobile phase of methanol–water (15:85) containing 3.5 m*M* sodium chloride and 0.3% acetic acid at a flow-rate of 0.5 ml/min. The mobile phase was continously degassed by purging with helium.

Sample preparation

Water samples (300 ml) were adjusted to pH 2 with 6 *M* hydrochloric acid. For dynamic solvatisation of the sorbent material, methanol (5 ml) was added and the sample applied to an activated Bond-Elut C_{18} cartridge under suction at a flow-rate of about 10 ml/min. The extraction column was allowed to dry prior to elution with acetone (6 ml). The eluate was concentrated to approximately 0.2 ml under a gentle stream of nitrogen. The sample was diluted with water to 0.5 ml. The exact volume was measured with a microlitre syringe and filtered through a 0.5-μm membrane filter. Sample aliquots (25 μl) were injected into the HPLC system.

RESULTS AND DISCUSSION

A chromatogram of Vienna tap water fortified with 0.1 μg/l CL9673 is shown in Fig. 2. CL9673 was eluted from the column with a retention time of 8.05 min. A sufficient separation of the peak from other constituents present in the water sample

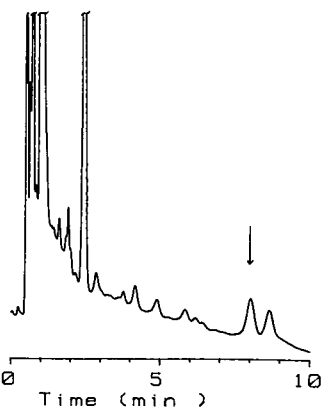
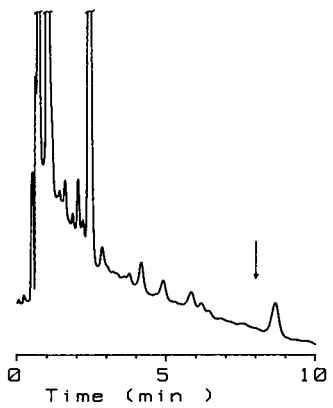


Fig. 2. HPLC chromatogram of Vienna tap water fortified with 0.1 μg/l CL9673. Arrow indicates retention time of CL9673.



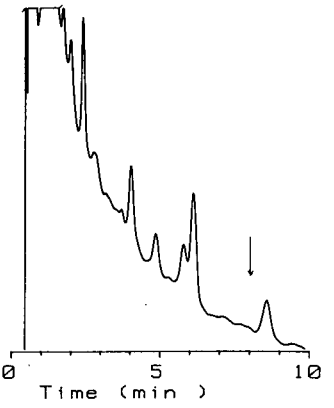


Fig. 3. HPLC chromatogram of Vienna tap water (blank). Arrow indicates retention time of CL9673.

Fig. 4. HPLC chromatogram of an untreated water sample (blank) from a well near Vienna. Arrow indicates retention time of CL9673.

was obtained. As an example, a chromatogram of Vienna tap water (blank) is shown in Fig. 3 and the chromatogram of an untreated groundwater sample (blank) obtained from a well near Vienna is presented in Fig. 4.

The effect of the applied potential on the peak area of CL9673 was determined by changing the potential from +1.0 V to +1.4 V. The relationship between the potential of the working electrode and the recorder response is shown in Fig. 5. An applied potential of +1.25 V was considered to be optimum as a lower potential resulted in a considerable loss of sensitivity and a higher potential resulted in unacceptable background noise. The detection limit was 0.1 ng per injected volume at a

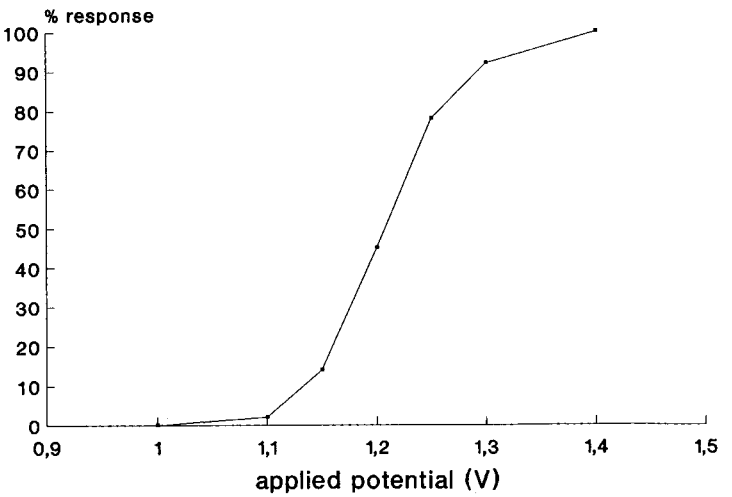


Fig. 5. Effect of applied potential on the response of CL9673.

signal-to-noise ratio of 5. The linearity of the detector response was obtained on the injection of 0.1–10 ng of CL9673 (coefficient of correlation 0.9992).

A recovery study using six tap water samples spiked with CL9673 at a level of 0.1 μg/l showed a recovery of 84.8% with a coefficient of variation of 6.2%. The results were calculated via peak areas using an external calibration graph.

From the results obtained it can be concluded that the proposed method is suitable for the determination of trace amounts of CL9673 in drinking and groundwater.

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CHROM. 23 479

Comparative analysis of thirty polychlorinated biphenyl congeners on two capillary columns of different polarity with non-linear multi-level calibration

EVA STORR-HANSEN

Ministry of Environment, National Environmental Research Institute, Division of Environmental Chemistry, Mørkhøj Bygade 26H, DK-2860 Søborg (Denmark)

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ABSTRACT

The elution patterns of polychlorinated biphenyls (PCBs) are different on capillary columns of different polarities. Congeners which elute as single peaks on one type of column may co-elute with other congeners on another type of column. This paper describes quantitative results from the analysis of calibration standards and various sample extracts from seals using a capillary dual-column gas chromatography system with electron-capture detection, where the two capillary columns are operated in parallel with a glass T-split. Non-linear multi-level calibration graphs and choice-of-fit functions are discussed with respect to the quantitative analysis of thirty PCB congeners. The differences in the results of the analyses on the two columns are evaluated, and an approach for preparing a single quantitative report from the two sets of analytical results is proposed.

INTRODUCTION

The analysis of specific polychlorinated biphenyl (PCB) congeners in environmental samples by capillary gas chromatography–electron-capture detection (GC–ECD) has been widely reported [1–4]. The precise identification and quantitation of specific PCB congeners is required as the toxicity of the congeners varies considerably [2]. The burden of the most toxic congeners, the non-*ortho* co-planar PCBs and their mono- and di-*ortho* derivatives, must be determined with the best precision possible in environmental samples.

The most common methods use a single capillary column for the separation, identification and quantitation of the PCB congeners. However, the complete separation of all congeners on a single capillary column has not yet been reported. Identification based solely on retention time (t_R) on a single column with ECD may still leave interfering compounds to be falsely identified as PCBs. Confirmation analysis of PCBs is frequently performed by analysing the sample on a second column of a different polarity [4–9]. Dual-column chromatography, where the two columns are installed in parallel in a GC oven, enables confirmation analysis to be routinely performed [5,8].

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Comparable analytical results are expected from the two columns when the analyte elutes as a single-component chromatographic peak on both columns. In this instance, either of the two columns may act as a confirmation column for the other. However, many PCBs do not elute in single-component peaks, and possible co-eluting PCBs have only been reported for the DB-5 column [1–3]. The problems of co-elution increase with the number of PCB congeners analysed [9], and the resolution of the PCB congeners must be verified for each of the two columns used. This applies in particular to less abundant congeners, and to congener clusters containing both major and minor analytes.

There are relatively few reports of quantitation procedures for dual-column chromatography [4,7–9]; the two analyses are usually performed separately [4,7,9]. The quantitation procedure reported by Erickson [4] used the average value if the results were within 20%. The lower value was taken when the results differed by more than 20%. Co-eluting or unresolved PCBs were summed.

Analytical systems in which the two columns are operated simultaneously allow the determination of interferences for each PCB congener and eliminate variability from repeated injections of the sample [5,8]. The determination of technical Aroclor mixtures (total PCBs) and organochlorine pesticides in various samples has been reported as an average of the results from the two columns, but the quantitation procedure was not discussed further [8].

The precision of the quantitation procedure depends on the mode of calibration. Many electron-capture detectors display non-linear response curves [6,10]. As PCB congeners in environmental samples show a wide range of concentrations, calibration is a possible source of error in the quantitative analysis of PCBs.

EXPERIMENTAL

Chemicals

PCB congeners were selected on the basis of toxicity and persistency [3,5,11] for a study of chlorinated organic micropollutants in marine mammals. In the following, the PCB congeners are referred to by their IUPAC numbers [4]; the molecular structures of the PCB congeners in the calibration mixture are listed in Table I. The PCB standards were purchased from Ultra Scientific, Promochem, Cambridge Isotope Laboratories and Community Bureau of Reference, EEC. The purity was greater than or equal to 99% and the PCBs were not purified further before use. PCB-15, -44, -136, -70 and -95 were generous gifts from Dr. L. G. Hansen (University of Illinois, Urbana-Champaign, IL, USA).

Pentachlorobenzene, hexachlorobenzene and *p,p'*-DDE were found in the PCB fraction from the clean-up of samples and were included in the calibration standard mixture. PCB-53 and PCB-155 were used as internal standards, as recommended by Wells *et al.* [12], as these compounds are not found in technical PCB formulations and are thus unlikely to occur in the environment. Octachloronaphthalene (OCN) was used as a late eluting internal standard. Any one of the three internal standards could be used for quantitation, but PCB-155 was usually preferred as it eluted in the middle of the PCB chromatogram. In total the mixture contained 36 compounds, of which three were internal standards and two were recovery surrogate standards (PCB-3 and PCB-198) [12].

TABLE I

PCB CONGENERS IN CALIBRATION STANDARD MIXTURE

Pentachlorobenzene, hexachlorobenzene, *p,p'*-DDE and octachloronaphthalene are included in the mixture, but not discussed further here. PCB-149 (2,2',3,4',5',6-H_xCB) was added in later preparations of the mixture. M_oCB = Monochlorobiphenyl; D_iCB = dichlorobiphenyl; T_rCB = trichlorobiphenyl; T_e = tetrachlorobiphenyl; P_eCB = pentachlorobiphenyl; H_xCB = hexachlorobiphenyl; H_pCB = heptachlorobiphenyl; O_cCB = octachlorobiphenyl.

Structure	IUPAC No.	1-*ortho*[a]	2-*ortho*[b]	ICES[c]	Other purpose
4-M_oCB	PCB-3				x[d]
4,4'-D_iCB	PCB-15				
2,4,4'-T_rCB	PCB-28			x	
2,4',5-T_rCB	PCB-31			x	
3,4,4'-T_rCB	PCB-37				x[e]
2,2',3',5-T_eCB	PCB-44				
2,2',5,5'-T_eCB	PCB-52			x	
2,2',5,6'-T_eCB	PCB-53				x[f]
2,3',4',5-T_eCB	PCB-70				
3,4,4',5-T_eCB	PCB-81				x[e]
2,2',3,5',6-P_eCB	PCB-95				
2,2',4,5,5'-P_eCB	PCB-101			x	
2,3,3',4,4'-P_eCB	PCB-105	x		x	
2,3,3',4',6-P_eCB	PCB-110				x[g]
2,3,4,4',5-P_eCB	PCB-114	x			
2,3',4,4',5-P_eCB	PCB-118	x		x	
2,2'3,3',4,4'-H_xCB	PCB-128		x		
2,2',3,3',6,6'-H_xCB	PCB-136				
2,2',3,4,4',5-H_xCB	PCB-137		x		
2,2',3,4,4',5'-H_xCB	PCB-138		x	x	
2,2',4,4',5,5'-H_xCB	PCB-153		x	x	
2,2',4,4',6,6'-H_xCB	PCB-155				x[f]
2,3,3',4,4',5-H_xCB	PCB-156	x		x	
2,3,3',4,4',5'-H_xCB	PCB-157	x			
2,3,3',4,4',6-H_xCB	PCB-158		x		
2,3,4,4',5,6-H_xCB	PCB-166		x		
2,3',4,4',5,5'-H_xCB	PCB-167	x			
2,3,3',4,4',5-H_xCB	PCB-170		x		
2,2',3,4,4',5,5'-H_pCB	PCB-180		x	x	
2,3,3',4,4',5,5'-H_pCB	PCB-189	x			
2,2',3,3'4,4',5,5'-O_cCB	PCB-194		x		
2,2',3,3',4,5,5',6-O_cCB	PCB-198				x[d]

[a] Mono-*ortho* co-planar PCBs [2].
[b] Di-*ortho* co-planar PCBs [2].
[c] Compounds recommended by ICES [6].
[d] Recovery surrogate standard.
[e] Non-*ortho* co-planar PCB, less toxic than PCB-77, -126, -169 [2].
[f] Internal standard. Octachloronaphthalene is third internal standard.
[g] Interfering compounds known from DB-5 (PCB-77 and PCB-110) [1,3].

A stock solution of the calibration mixture containing approximately 1000 ng/ml of each compound (PCB-3 = 43 000 ng/ml because of low ECD response) was prepared in iso-octane. Standard calibration mixtures were made by diluting the stock solution by volume.

Samples

A number of samples originating from seals in the Danish part of the Wadden Sea were analysed using the dual-column system.

Sample extraction and clean-up

The sample extraction and clean-up was a modified version of that of Jensen *et al.* [13]. A detailed description of the procedure and the method validation study is in preparation. The main features were as follows: 10 g of homogenized sample were blended three times with dichloromethane–methanol (2:1, v/v) in an Ultra-Turrax blender. The combined extract was shaken with acidified water in a separating funnel. The organic phase was filtered through anhydrous sodium sulphate and the solvent evaporated. The residue was redissolved in hexane and treated with sulphuric acid adsorbed on silica gel. The final clean-up of the extract was by chromatography on basic aluminium oxide, deactivated with 1% (w/w) water. Internal standards were added to the samples and the volume of the extract was adjusted to 1 ml. Extracts of blubber samples were analysed at dilutions 1:200 and 1:20, and extracts of other tissues at dilutions 1:20 and undiluted.

Most of the samples were submitted to chromatography on Carbopack C, which retained the non-*ortho*-substituted co-planar PCBs (PCB-37, -81, -77, -126 and -169). These congeners were eluted from Carbopack C with toluene and analysed by isotope dilution technique and combined GC and mass spectrometry (MS) for the precise determination of the much lower levels of these particular congeners.

Gas chromatographic analysis

Analysis was performed on a HP-5890A (II) gas chromatograph with an HP-7376 autosampler. The GC parameters were as follows: Column 1, J&W DB-5 (5% phenylmethylsilicone), 60 m × 0.25 mm I.D., 0.11 μm d_f; and column 2, J&W DB-1701 (14% cyanopropylphenyl), 60 m × 0.25 mm I.D., 0.15 μm d_f.

A splitless injection of 2 μl of sample was used, with a splitless time of 1 min and an injector temperature of 250°C. Detection was with two ^{63}Ni electron-capture detectors operated at 300°C. The carrier gas was helium, with a column head pressure of 170 kPa, corresponding to a linear flow-rate of approximately 25 cm/s at 150°C. The make-up gas was nitrogen at a flow-rate of 50 ml/min. The temperature was 90°C for 1 min, then 90 to 180°C at 25°C/min, 180°C for 2 min, then 180 to 220°C at 1.5°C/min, 220°C for 2 min, then 220 to 275°C at 3°C/min and finally 275°C for 10 min.

The columns were connected to the injector by 1 m of uncoated 530 μm pre-column and a glass T-piece. The collection and processing of data were performed by a Vectra QS/20 PC, with HP Chemstation software.

RESULTS AND DISCUSSION

Qualitative analysis

Chromatograms of the entire calibration mixture and retention times on the two columns have been given previously [5]. The identification of the congeners was on the basis of t_R, where the Chemstation software used the three internal standards to adjust the t_R values of the calibration table to t_R values of the sample [5,14]. A t_R tolerance of 1% was used for the internal standards and 0.2% for the analytes. The stability of the t_R values and injection volume have been described previously [5].

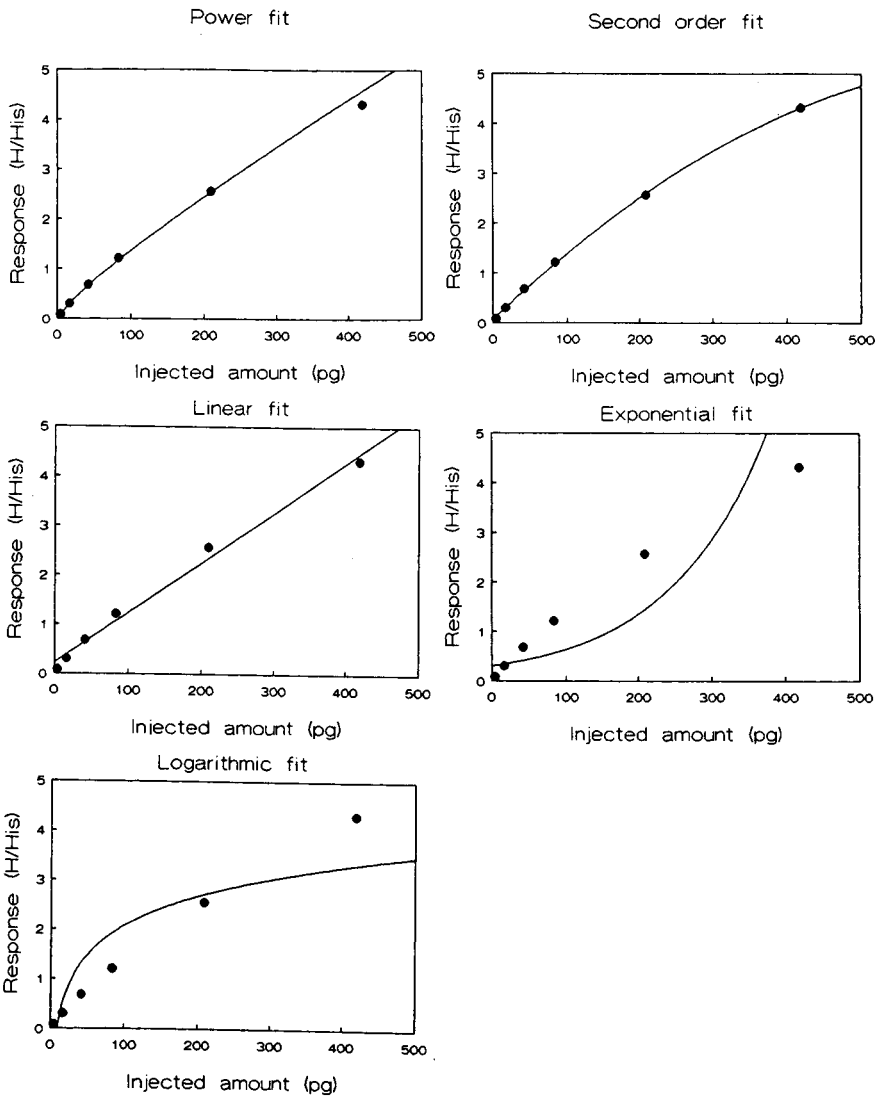

Fig. 1. Response curve fits for PCB-101 showing power, second-order, linear, exponential and logarithmic fits. The *X*-axis units are the injected amounts of PCB-101 in picograms and the *Y*-axis units are the peak heights of PCB-101 relative to the peak height of the internal standard PCB-155 (IS-2).

Quantitative analysis

The instrumental detection limit was calculated as $3SC_s$ (ng/ml), where S is the standard deviation of the response [peak heights relative to the peak height of the internal standard PCB-155 (H/H_{IS})] for three injections of standard calibration mixture with a concentration C_s = 5 ng/ml. The values ranged from 0.1 to 0.5 ng/ml injected. In samples, the detection limit could be higher, corresponding to the degree of dilution of the extracts. Dilutions were required as a result of the large amount of sample used, which was in turn required by the low levels of non-*ortho* co-planar PCBs present in the samples.

The response of both the electron-capture detectors in the system was non-linear and thus multi-level calibration was required for the simultaneous determination of the range of concentrations of individual PCB congeners present in marine mammal samples. The Chemstation software provided five possible mathematical functions to use as calibration graphs (linear, power, exponential, logarithmic and second-order fit) and three ways of handling the origin (as a data point, ignored or connected to the lowest data point by a straight line [14]). The diluted calibration standard solutions (5, 20, 50, 100, 250 and 500 ng/ml) were analysed by the dual-column system, and the various curve fits applied to the data for PCB-101 (Fig. 1). PCB-101 was representative of the remaining PCBs with respect to the appearance of the response graph. The exponential and logarithmic fits were not useful as fit functions. Among the remaining three fits, the power fit (ax^b) yielded values which were too low at the high level of PCB-101, whereas the second-order curve ($ax^2 + bx + c$) fitted the data well. The linear fit overestimated the middle-level concentrations and did not pass through the origin. The three types of fit were compared by calculating the relative error as a percentage:

$$\text{Relative error} = (X_{\text{calc}} - X_{\text{meas}})100/X_{\text{meas}} \quad (1)$$

where X_{calc} is the calculated response of the PCB congener with IUPAC number x, and X_{meas} is the actual measured response for the same congener. For a representative congener, PCB-101 (Fig. 2), the results indicated that the linear fit had a large relative fit error at low concentrations. The power fit and the second-order fit were compara-

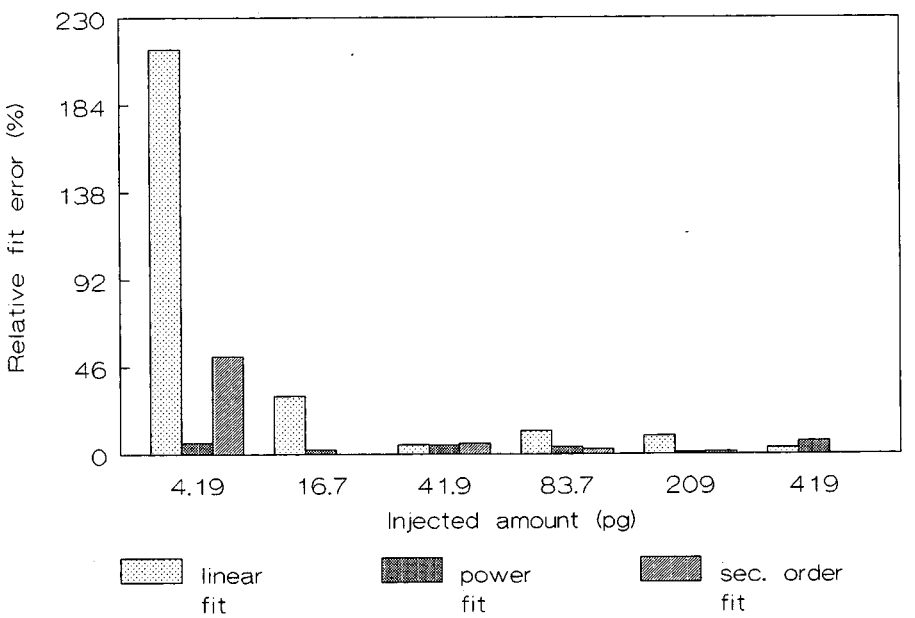

Fig. 2. Relative fit error (%) for the three best fits: power, second-order and linear fit in the concentration range 5–500 pg PCB-101 injected. Power fit is the best choice in the concentration range 5–250 ng/ml (250 pg injected onto each column). This corresponds to a calibrated range from the detection limit to 250 ng/ml.

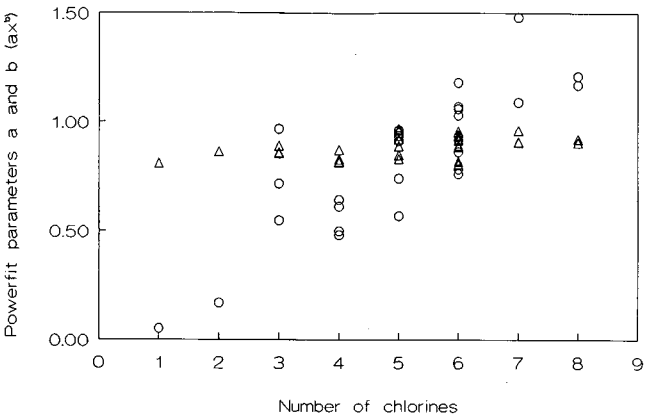

Fig. 3. Coefficient a (○) and power b (△) of the power fit function ax^b of each analysed PCB congener as a function of the number of chlorines. The value of a increases with degree of chlorination, whereas b is fairly uniform for different degrees of chlorination.

ble with respect to relative error, except near the detection limit where the power fit had the lowest relative error. Based on this, the calibrated range was narrowed down from detection limit up to 250 ng/ml, and the power fit method was chosen for all PCB congeners. As a result of the relatively large number of analytes, only four calibration levels were used, namely 5, 20, 100 and 250 ng/ml, because of the limitations of the software in data handling. As the power function passes the origin by definition, the origin was ignored as a data point.

For each PCB congener in the mixture, and for both columns, the best fit of the power fit function, based on the least-squares method, was then calculated during calibration. The fit was characterized by coefficient a and power b (ax^b) in the fit function, and a correlation coefficient r^2. The value of r^2 was in the range 0.998–1.000 for all PCB congeners ($n=30$) for both the DB-5 and DB-1701 columns. The fit parameters for each PCB congener on the DB-5 column were plotted *versus* the degree of chlorination (N_{Cl}) (Fig. 3). The power of the fit function (b) was seen to be fairly uniform for the different degrees of chlorination, whereas the coefficient (a) increased with degree of chlorination. This finding was consistent with literature reports of increasing response factors (RFs) with increasing numbers of chlorines [1,4] from linear or single level calibration.

The values for the coefficient (a) and the power (b) of the fit functions from the two columns were compared for all congeners by a two-tailed Student's t-test. A significant correlation ($p=0.05$) between both the a values from the two columns and between the b values from the two columns were found. This was taken to indicate that similar calibration functions apply on both columns and detectors.

If a larger calibration range was used, the second-order fit was preferable. The fit error near the detection limit was reduced by plotting a straight line from the lowest calibrated point to the origin.

The stability of the RFs was investigated to determine how often recalibration should be performed during larger numbers of analyses. An RF was calculated and

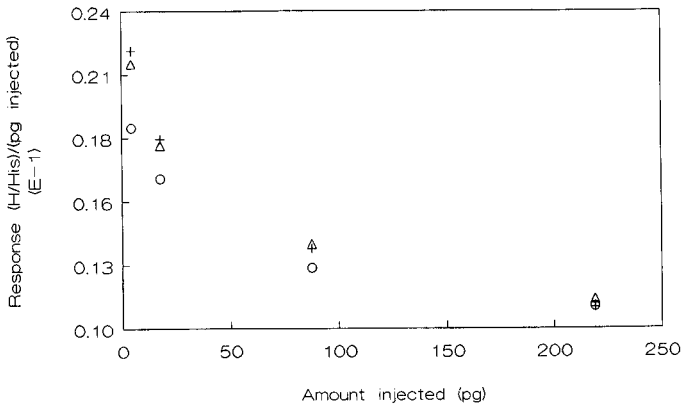

Fig. 4. RFs from three complete calibrations, performed approximately 10 days apart. + = June 26th, 1990, △ = July 7th, 1990 and ○ = July 19th, 1990. The RFs show a small decrease during the period. A linear range, defined as a range with a maximum of 10% variation in response factors, could only be defined for small concentration ranges (10% is approximately one division at the *Y*-axis).

compared for (1) six repeated injections of the same 50 ng/ml solution during 7 h of analysis and (2) a number of sample batch runs over several days.

$$\mathrm{RF} = H/(H_{\mathrm{IS}} \times \text{injected amount}) \tag{2}$$

From the repeated injections of the standard solution, the RF for PCB-101 had a relative standard deviation (R.S.D.) of 0.90%. This indicated a very stable detector response during daily analysis. It was therefore decided to recalibrate once every four samples, to adjust the retention times in the calibration table, rather than to adjust the RFs.

The long-term stability of the detector response in three batches of samples, analysed about 10 days apart (Fig. 4), indicated a change in the RFs during this period. It was concluded that a complete recalibration at all four levels was necessary prior to the analysis of a new batch of samples.

Comparison of quantitative results from DB-5 and DB-1701 columns

After the correction of any blank values, the difference between the Front and Rear signals (DB-5 and DB-1701 columns, respectively), $\Delta_{\mathrm{F/R}}$, was calculated as a percentage for each PCB congener analyte:

$$\Delta_{\mathrm{F/R}} = (X_{\mathrm{DB\text{-}5}} - X_{\mathrm{DB\text{-}1701}})100/(X_{\mathrm{DB\text{-}5}} + X_{\mathrm{DB\text{-}1701}}) \tag{3}$$

X_n is the analytical result from column n in ng/ml and $\Delta_{\mathrm{F/R}}$ is a percentage. When $\Delta_{\mathrm{F/R}}$ was positive, the DB-5 result was the larger value and *vice versa*, and the maximum and minimum values were ± 100%, respectively. The validity of $\Delta_{\mathrm{F/R}}$ as an evaluation parameter was tested on standard calibration mixtures and a number of samples of blubber and other tissues from seals.

Calibration standards

The difference in the quantitative results from the DB-5 and DB-1701 columns

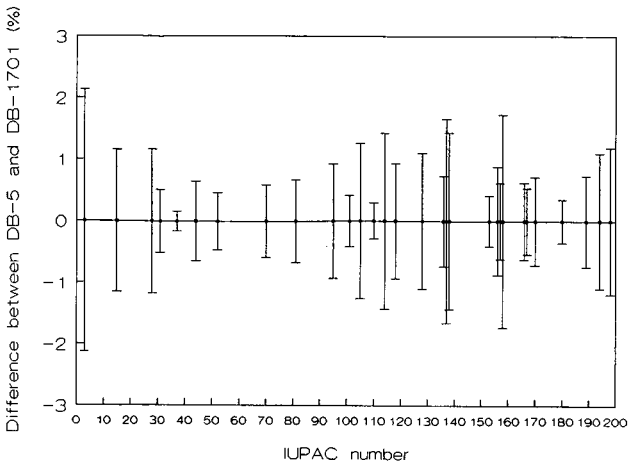

Fig. 5. Difference between analytical results from DB-5 and DB-1701 columns as a percentage ($\Delta_{F/R}$). Mean ± standard deviation of values from four calibration levels for each PCB congener. The two quantitative results are comparable and independent of calibration level.

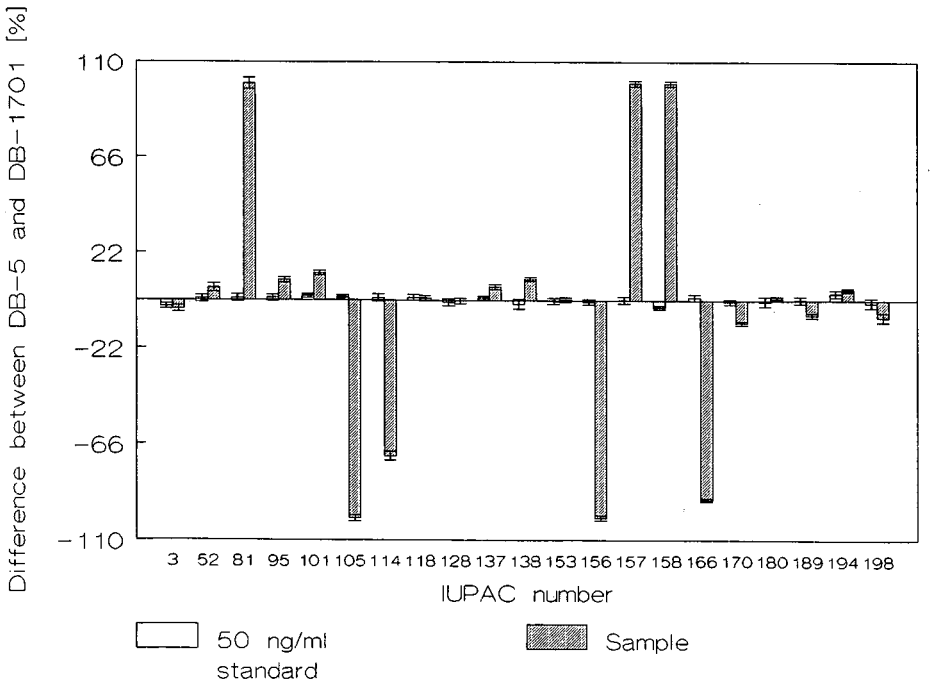

Fig. 6. Difference between analytical results from DB-5 and DB-1701 columns as a percentage ($\Delta_{F/R}$). Mean ± standard deviation of $\Delta_{F/R}$ values for repeated injections of a 50 ng/ml calibration standard compared to repeated injections of a seal blubber extract.

was investigated for the 5, 20, 100 and 250 ng/ml calibration standards. For each PCB congener, $\Delta_{F/R}$ was calculated for all levels, and subsequently the mean value of the four calibration levels was calculated. The results were all within the range ±2%, as shown in Fig. 5. This result indicates that the quantitative results from the analysis of calibration standards on the two columns were comparable and independent of the concentration level within the calibrated range for all the PCB congeners studied.

Comparison of standard mixture and blubber extract

The mean and standard deviation for six injections of a 50 ng/ml standard mixture was compared to six injections of a blubber extract (Fig. 6). The variance of the measurement of each congener, indicated by the error bar, was small. Significant differences were observed between the quantitative results for most PCB congeners in the sample and in the standard mixture. The mean values of some congeners in the standard mixture were not zero (PCB-3, -101, -137, -194), as a result of the fact that the calibration was not performed on the 50 ng/ml standard mixture, in contrast to the four calibration levels in Fig. 5.

Based on the maximum standard deviation of ±2% in the calibration standards in Fig. 5, and the observed non-zero mean of $\Delta_{F/R}$ in the uncalibrated standard

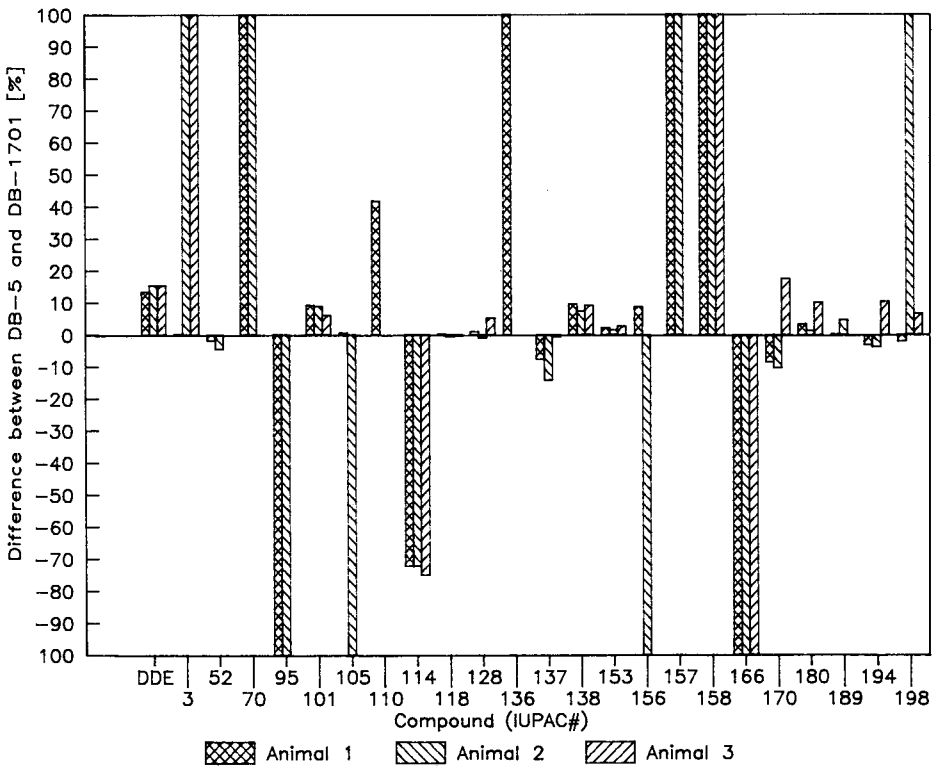

Fig. 7. Difference between analytical results from the DB-5 and DB-1701 columns as a percentage ($\Delta_{F/R}$) in extracts of blubber samples from three seals. PCB-101 is overestimated by about 10% by the DB-5 column in all three samples; PCB-138 is overestimated on the DB-5 column by about 10%.

mixtures in Fig. 6, a maximum value for $\Delta_{F/R}$ of ± 5% was chosen as an estimate for the insignificant difference between the two signals during sample analysis.

Analysis of animal tissues

Blubber samples from three animals and several tissues from one seal were analysed and the values of $\Delta_{F/R}$ in the sample extracts evaluated (Figs. 7 and 8). The blubber extracts were diluted 2–400 times. The tissue samples were analysed in sequences as given in the caption to Fig. 7.

In all samples, PCB-114 had a co-eluting compound on the DB-1701 column as reported previously [5]. PCB-101 was overestimated by the DB-5 column ($\Delta_{F/R}$ = 7–11%) in all samples. This was in accordance with earlier reports, which stated that PCB-90 is a possible co-eluting compound with PCB-101 on the DB-5 column [3,7,15], and that it constitutes about 10% of the peak at the t_R value of PCB-101 on the DB-5 column [16]. PCB-138 was overestimated on the DB-5 column ($\Delta_{F/R}$ = 3–11%). This was in agreement with the results reported by Larsen and Riego [7], in which PCB-163 (2,3,3′,4′,5,6,-H_xCB) was identified to constitute 10–30% of the peak at the t_R value of PCB-138 in various environmental and technical samples analysed on the DB-5 column. Williams and Lebel [9] also reported consistently higher values (about 10%) for PCB-138 on an SPB5 column relative to values found by a more polar SPB35 column. In all samples, *p,p′*-DDE had positive values of $\Delta_{F/R}$ (13–30%).

In the tissue samples, PCB-170 was overestimated by the DB-1701 column

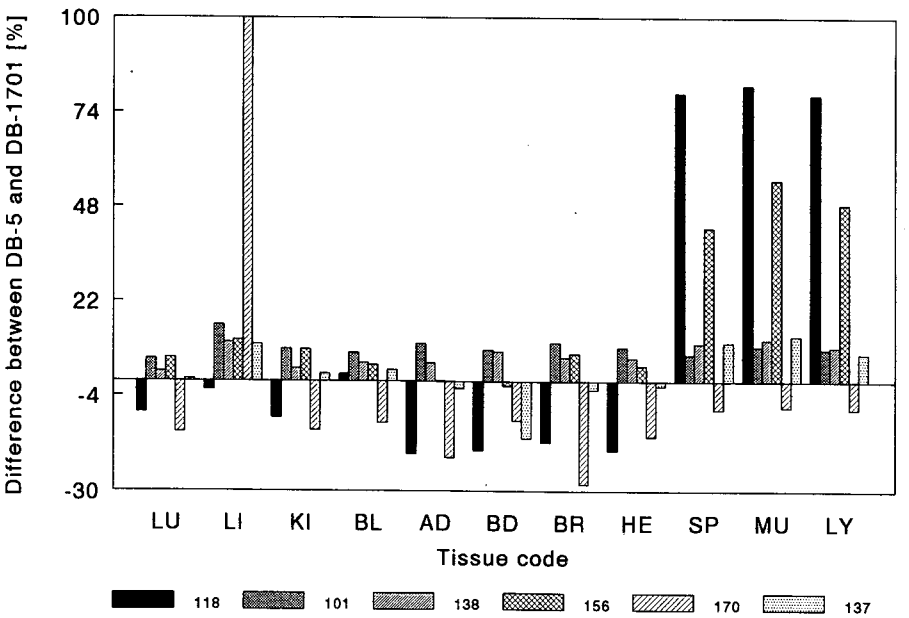

Fig. 8. Difference between analytical results from the DB-5 and DB-1701 columns as a percentage ($\Delta_{F/R}$) for selected PCB congeners, in extracts of various tissues from one seal. Capital letters = tissue code in figure, date of analysis of batches in parentheses: LIver/LUng/KIdney (May 18th, 1990), BLubber (July 6th, 1990), ADrenals/BlooD/BRain/HEart (July 19th, 1990) and SPleen/MUscle/LYmph nodes (July 26th, 1990). The value of $\Delta_{F/R}$ of PCB-118 follows the batch; a possible co-eluting compound on DB-5 was PCB-149 [3,15].

($\Delta_{F/R}$ = −7 to −28%). The value of $\Delta_{F/R}$ for PCB-156 in tissue samples was generally positive ($\Delta_{F/R}$ = −1 to 55%), in contrast to what was found for humane adipose samples analysed on SPB5 and SPB35 columns [9]. The $\Delta_{F/R}$ behaviour of PCB-118 in tissue samples followed the batch. The quantitation of this congener was extremely sensitive to separation from a close eluting congener, possibly PCB-149 (2,2′,3,4′,5′,6-H_xCB) on DB-5 [3,15]. The separation of PCB-118 and PCB-149 was later tested (Fig. 9) and was better on the DB-1701 column than on the DB-5 column; PCB-149 was not present in the original standard calibration mixture, but was included in later preparations of the standard mixture. The analysis of blubber extracts with the new standard preparation revealed PCB-149 to be present at similar levels to PCB-118. Fig. 8 illustrates that the ability to separate CB-149/118 on DB-5 is lost for the last batch of tissue samples (spleen/muscle/lymph nodes).

PCB-105 was not identified on the DB-5 column in some samples, possibly due to incomplete separation from PCB-153, which was the most abundant PCB congener in all analysed samples. PCB-132 (minor) could influence quantitation on both columns [17], but was not part of the analytical programme. The separation of PCB-132 and PCB-105 seemed most critical on the DB-5 column. PCB-157 co-eluted with PCB-180 (major congener) on the DB-1701 column, and was only detected on DB-5 in all samples analysed ($\Delta_{F/R}$ = 100%). PCB-158 co-eluted with the second most abundant PCB congener PCB-138 on the DB-1701 column, and was only detected on DB-5 ($\Delta_{F/R}$ = 100% in all samples). PCB-167 and PCB-128 were best separated on the DB-1701-column, but only just separated from the baseline. PCB-167 was not reported in any samples on the DB-5 column.

In some blubber extracts, analysed in dilution of 1:20, the internal standard PCB-155 had a co-eluting compound on the DB-1701 column, which rendered it unsuitable as an internal standard. OCN was used as an alternative internal standard for these samples. For the recovery of the standards PCB-198 and PCB-3, co-eluting compounds were observed on the DB-1701 column in one and two of the blubber samples, respectively. As these co-eluting compounds eluted close to and were comparable in intensity to the analytes, the software did not detect the analytes. No trend was observed for the remaining congeners.

Proposed criteria for handling of data from dual-column systems

The first step is to subtract blank values from the sample data. Data should only be reported within the calibrated range. Chromatograms, including calibration standards, should be examined in detail for each analysis to identify critical separations which may influence the identification or quantitation of the target compounds. Dilution factors calculated from the data should correspond to the actual dilution factors for major congeners identified in both dilutions.

In addition to these procedures the following set of criteria for the preparation of a single quantitative report from the dual-column system are proposed. The criteria are based on these presented analytical results in standard calibration mixtures and seal tissues, and a knowledge of the incomplete separation of PCB congener pairs from the literature [3,17,18].

(1) A congener, identified on only one of the two columns, is reported as not detected (n.d.).

(2) If $|\Delta_{F/R}|$ is less than 5%, the difference is regarded as insignificant, based on

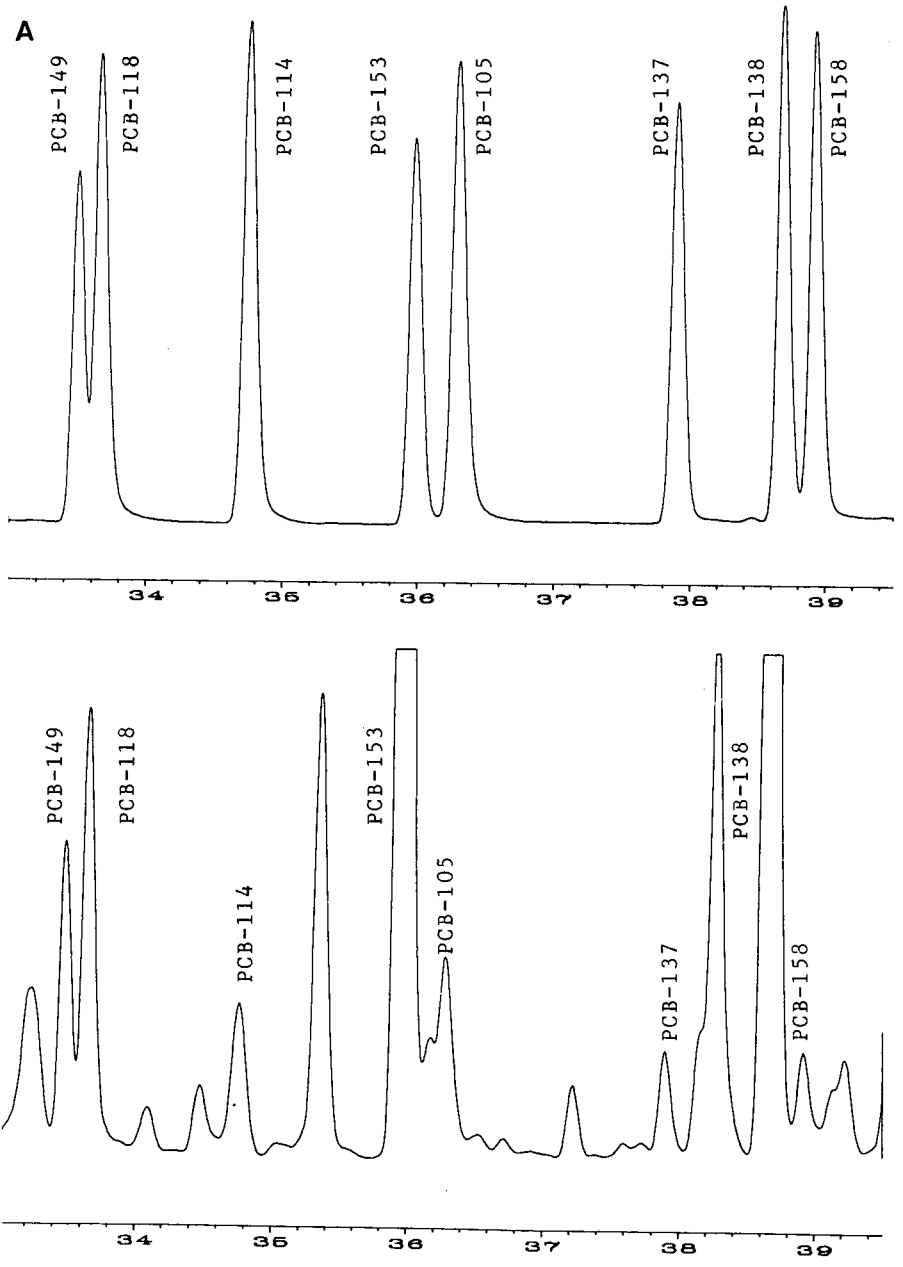

Fig. 9.

(*Continued on p. 388*)

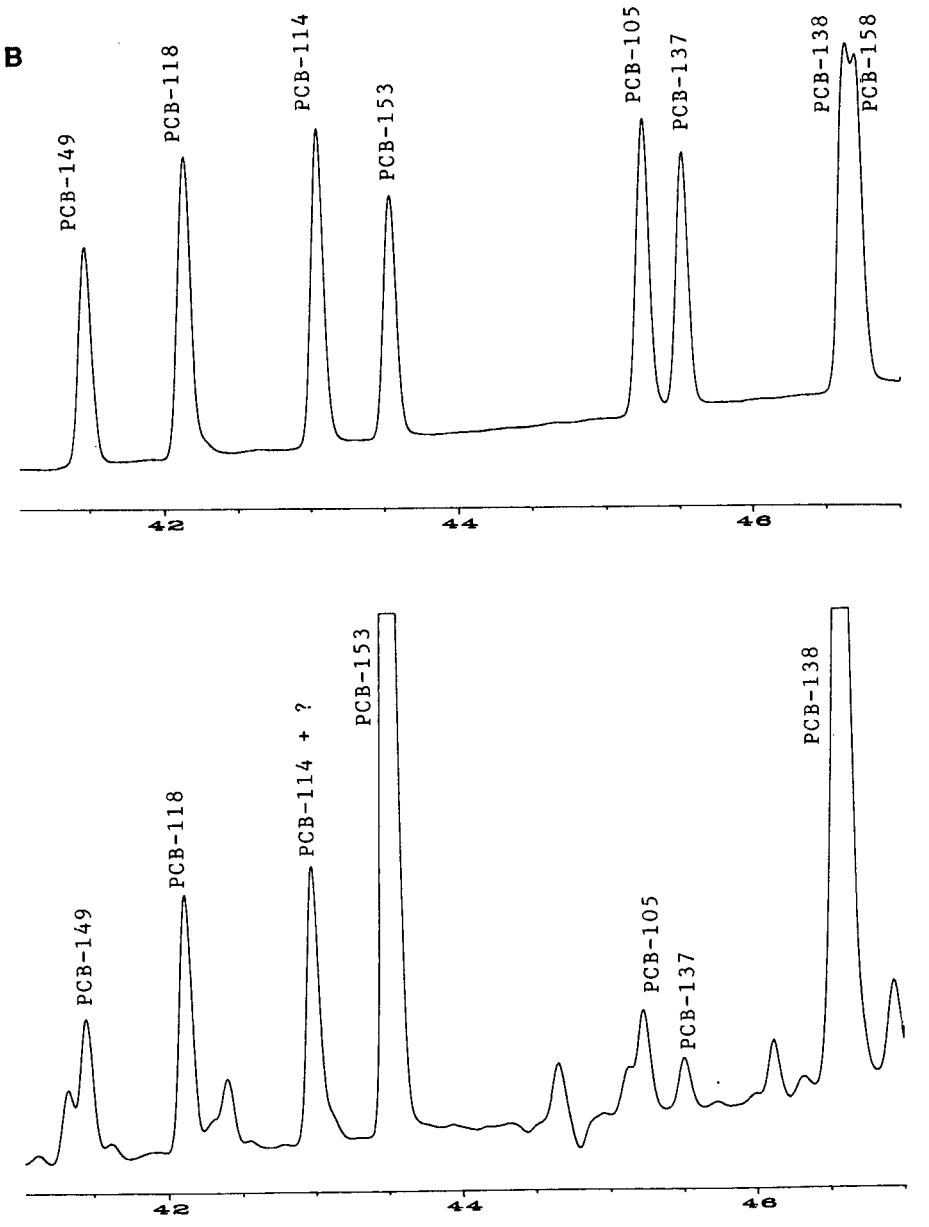
B
PCB-149
PCB-118
PCB-114
PCB-153
PCB-105
PCB-137
PCB-138
PCB-158
42
44
46
PCB-149
PCB-118
PCB-114 + ?
PCB-153
PCB-105
PCB-137
PCB-138
42
44
46

Fig. 9.

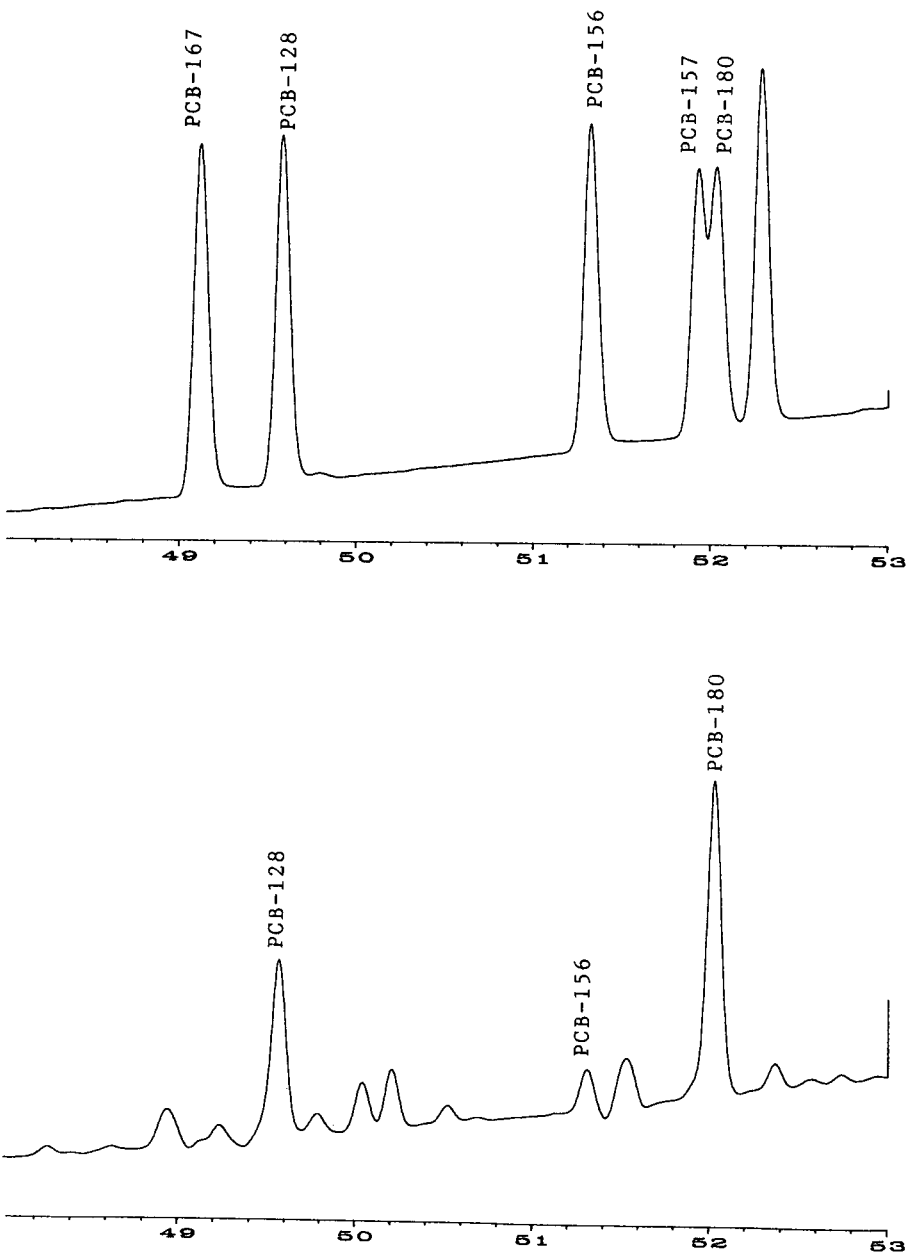

Fig. 9. (A) Critical chromatographic separations of PCB-118 and PCB-149, PCB-153 and PCB-105 and PCB-138 and PCB-158 in a standard mixture (upper panel) and a seal blubber extract (lower panel) on the DB-5 column. (B) Critical chromatographic separations of PCB-118 and PCB-149, PCB-153 and PCB-105, PCB-138 and PCB-158 and PCB-157 and PCB-180 in the same standard mixture (upper panel) and a seal blubber extract (lower panel) as in (A), analysed on the DB-1701 column.

the variation from the calibration standards as discussed earlier. Either of the two results may be used. Erickson [4] proposed the use of the average value.

(3) If the $|\Delta_{F/R}|$ value is larger than 5%, the lowest quantitative result is accepted, based on the assumption that the higher value was caused by an impurity or co-eluting congener. This is consistent with the procedure discussed by Erickson [4]. However, matrix interferences should be considered, for instance, in the analysis of waste oils, which may lower the ECD response of the PCBs.

Excepted from these criteria are the following congeners:

PCB-105 is quantitated on the DB-1701 column, and the result is accepted even if n.d. is reported from the DB-5 column. The close eluting congeners on DB-5 are PCB-153 (major) and PCB-132 (minor). Confirmation analysis may be performed by GC–MS.

PCB-157 is quantitated on the DB-5 column, and the result is accepted even if n.d. is reported from the DB-1701 column. The close eluting congener on the DB-5 column is PCB-180 (major). Confirmation analysis may be performed by GC–MS.

PCB-158 is quantitated on the DB-5 column, and the result is accepted even if n.d. is reported from the DB-1701 column. The close eluting congener on DB-5 is PCB-138 (major). Confirmation may be performed on another polarity column; confirmation by GC–MS not possible as both PCB-138 and PCB-158 are hexachlorinated.

PCB-167 and PCB-128 should both be reported from the DB-1701 column, even if one (PCB-167, minor) is reported as n.d. on the DB-5 column. Confirmation may be performed on another polarity column. Confirmation by GC–MS not possible as both PCB-167 and PCB-128 are hexachlorinated.

If a congener is reported from only one column, the result should be followed by the statement "unconfirmed".

Recommendations for the analysis of marine mammal samples

The separation of PCB-153 and PCB-105 and the possible co-eluting PCB-132 was critical in all samples. The separation of PCB-118 and PCB-149 was also critical in all the presented samples, and PCB-149 was found at levels comparable to PCB-118. There is currently considerable interest in the determination of both PCB-105 and PCB-118, partly based on toxicity, and partly as both congeners are included in the set of congeners recommended by the ICES for marine monitoring [6,10].

It is recommended to include PCB-132 and PCB-149 in the standard calibration mixture as column performance controls, even is these compounds are not analytes in the analytical programme. This use of column performance controls is generally recommended for high-resolution congener-specific PCB analysis.

CONCLUSIONS

The comparative quantitative analysis of thirty PCB congeners on two capillary columns of different polarity was performed. The columns were connected to the injector via a glass T-split, and accordingly the variation due to replicate injections of samples was eliminated. This dual-column system offers a convenient route to improve the quality of PCB analysis by GC–ECD.

Non-linear multi-level calibration was required for the determination of specific PCB congeners in marine mammal samples using this system. Power fit (ax^b) was the best fit for the calibrated range from detection limit up to 250 ng/ml, whereas the second-order fit (ax^2+bx+c) was a better choice for larger calibrated ranges.

The dual-column analysis of blubber and other tissues from seals revealed that PCB-101 and PCB-138 were overestimated by about 10% on the DB-5 column relative to the DB-1701 column. This finding is consistent with earlier reported work, and the DB-1701 column is thus preferred as a quantitative column for these congeners.

The difference in analytical results from the two columns were evaluated as a percentage, $\Delta_{F/R}$. $\Delta_{F/R}$ was useful in the evaluation of the very large amount of data from the dual-column system. A quantitation procedure based on the value of $\Delta_{F/R}$ is proposed. In general, the smallest analytical result was accepted, with exceptions for PCB-105, -158, -157, -128 and -167. These PCB congeners could be identified or quantitated on only one of the two columns, and accordingly other techniques are necessary to confirm the identity for these PCB congeners. A 5% difference between the DB-5 and DB-1701 quantitative results is considered insignificant, based on the overall measurement uncertainty in this automated capillary GC–ECD system.

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Methods for the analysis of thiodiglycol sulphoxide, a metabolite of sulphur mustard, in urine using gas chromatography–mass spectrometry

ROBIN M. BLACK* and ROBERT W. READ
Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire SP4 0JQ (UK)
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ABSTRACT

Two methods have been developed for the analysis of thiodiglycol sulphoxide, a metabolite of sulphur mustard, in urine. The first method recovers thiodiglycol sulphoxide from urine by extraction from a solid absorbent tube and clean up on Florisil. In the second method thiodiglycol sulphoxide is reduced to thiodiglycol with acidic titanium trichloride prior to extraction. This method detects thiodiglycol, thiodiglycol sulphoxide, and their acid-labile esters, as the single analyte thiodiglycol. In both cases the recovered analytes were converted to the bis(pentafluorobenzoyl) derivative of thiodiglycol and detected by gas chromatography–mass spectrometry using negative ion chemical ionisation. The limits of detection were 1 ng per 0.5-ml sample of urine. Urine from five normal human subjects showed low background levels of thiodiglycol sulphoxide in the range 2–8 ng/ml. However, a sixth subject was found to be excreting levels of thiodiglycol sulphoxide as high as 36 ng/ml. The first method has been used in toxicokinetic studies of sulphur mustard and the second method is intended to be used for the retrospective confirmation of mustard poisoning in casualties of chemical warfare.

INTRODUCTION

The use of sulphur mustard, 1,1′-thiobis(2-chloroethane), in the Iraq–Iran conflict [1–3], plus the negotiations towards a verifiable chemical weapons treaty, have lead to renewed interest in analytical methods for the retrospective confirmation of sulphur mustard poisoning [4–9]. There is currently no unambiguous forensic method to support a medical diagnosis of mustard poisoning in casualties believed to have been exposed to the agent. The detection of unchanged sulphur mustard in body fluids, such as blood or urine, is unlikely because of its extensive metabolism, rapid hydrolysis and general reactivity with nucleophiles present in proteins and nucleic acids. It has been assumed that an important excretion product following systemic absorption of sulphur mustard is its simple hydrolysis product thiodiglycol (2,2′-thiobis-ethanol). Early metabolism studies [10,11] suggested that excreted thiodiglycol, plus unidentified acid-labile conjugates which release it on treatment of urine with hydrochloric acid, may account for around 15% of an injected dose of sulphur mustard in the rat. Methods for the analysis of thiodiglycol in urine have been reported by Wils and co-workers [7,8] and by Black and Read [9]. The method of Black and

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Read, which is more specific for thiodiglycol, was used to determine the quantitative elimination of thiodiglycol in rat urine following cutaneous administration of sulphur mustard [12]. Somewhat surprisingly, it was found that excreted thiodiglycol accounted for $<0.3\%$ of an applied cutaneous dose of sulphur mustard, and, even after treatment of urine with hydrochloric acid, the amount of thiodiglycol detected in urine over the first nine days following application of the agent accounted for only 1 to 1.5% of the applied dose. This amount was around an order of magnitude lower than had been expected from the reports of early metabolism studies [10,11]. More recent metabolism studies at this establishment [13] have shown that thiodiglycol itself is not the major excretion product derived from the hydrolysis of sulphur mustard. Metabolic oxidation on sulphur converts it to its sulphoxide (2,2′-sulphinylbisethanol) which has been consistently observed as a urinary metabolite of sulphur mustard in the rat [13]. Thiodiglycol sulphoxide was also shown to be the major urinary metabolite following intravenous administration of thiodiglycol in the rat [13].

In this present paper we report two analytical methods for the recovery of this very polar, water-soluble metabolite from urine and its quantitative determination. The first method is specific for thiodiglycol sulphoxide and recovers it from urine unchanged. We have applied this method to the quantitative measurement of the urinary excretion of thiodiglycol sulphoxide in the rat following administration of sulphur mustard. However, the method is not ideal for the purpose of retrospective identification of mustard poisoning because it requires the extraction of urine from a solid absorbent using relatively large volumes of polar solvent, a procedure which also recovers large amounts of extraneous materials. This results in faster column degradation and increased chemical noise in the selected ion current chromatograms (see Results and Discussion). The recovery of thiodiglycol on the other hand is easier and gives much cleaner selected ion current chromatograms [9]. We therefore sought an additional method in which the sulphoxide is reduced to thiodiglycol prior to extraction, and which would allow the simultaneous determination of both excretion products as the single analyte thiodiglycol. The second method reported below employs acidic titanium trichloride solution to reduce thiodiglycol sulphoxide to thiodiglycol. This method therefore also detects thiodiglycol, acid labile esters of thiodiglycol and its sulphoxide, and any other metabolites of sulphur mustard which might convert to thiodiglycol under these conditions. It is intended to be used for the retrospective identification of sulphur mustard poisoning rather than for the specific analysis of thiodiglycol sulphoxide. Both methods involve the conversion of the recovered analyte to the bis-pentafluorobenzoyl derivative of thiodiglycol and detection using gas chromatography (GC) combined with negative ion chemical ionisation mass spectrometry (MS), as reported previously by us for the analysis of thiodiglycol [9].

EXPERIMENTAL

Materials

Thiodiglycol (99%) was purchased from Aldrich (Gillingham, UK). [1,1,1′,1′-$^2H_4$]thiodiglycol was prepared by reacting sodium sulphide with ethyl bromoacetate to give diethyl thiodiglycollate, followed by reduction with lithium alumin-

ium deuteride [14]. Thiodiglycol sulphoxide, m.p. 113–4°C, was obtained by oxidation of thiodiglycol with 30% hydrogen peroxide in water; [1,1,1′,1′-$^2H_4$]thiodiglycol sulphoxide was prepared similarly from [$^2H_4$]thiodiglycol [14]. Standard solutions were made up in methanol at concentrations of 0.1–100 μg/ml.

Titanium trichloride (15% solution in 20–30% hydrochloric acid) was purchased from Aldrich. Pentafluorobenzoyl chloride (puriss) was purchased from Fluka (Glossop, UK) and pyridine (Regis derivatisation grade) from Phase Separations (Deeside, Clwyd, UK). Fisons (Loughborough, UK) Distol grade solvents were used, with the exception of toluene which was Aldrich HPLC grade.

Florisil Sep-Pak cartridges were purchased from Fisons and were conditioned with ethyl acetate before use. Chem Elut tubes (Analytichem International, Harbor City, CA, USA) were washed with methanol (3 × 5 ml) and dried in a vacuum oven at 60°C prior to use. All glassware was pretreated with Aquasil siliconising fluid (Pierce & Warriner, Chester, UK).

Samples of human urine were collected from laboratory volunteers. Samples of rat urine were collected from male Porton strain rats in the Biology Division, CDE.

Extraction and clean-up

Method A. Urine (0.5 ml), to which [1,1,1′,1′-$^2H_4$]thiodiglycol sulphoxide (50 ng) (or 25 ng) was added as internal standard, was absorbed onto a 3-ml Chem Elut tube. Some less polar extraneous materials were eluted with ethyl acetate–methanol 100:2 (3 × 5 ml) (from which thiodiglycol can be recovered if required) and the eluate discarded. The sulphoxide was then extracted by elution with ethyl acetate–methanol 100:7 (5 × 5 ml) into a 50-ml round-bottomed flask. After concentration of the eluate to dryness at 40°C using a rotary evaporator, the residue was loaded onto a Florisil Sep-Pak cartridge in acetone (2 × 2 ml). The cartridge was washed with chloroform–methanol (100:20) (2 × 5 ml), which was discarded, and then eluted with chloroform–methanol (50:50) (2 × 4 ml) into a 25-ml round-bottomed flask. The eluate was concentrated to dryness at 40°C on a rotary evaporator, the residue transferred to a 1-ml vial with methanol (2 × 0.5 ml) and the solution finally concentrated to dryness under a stream of nitrogen at 60°C prior to derivatisation.

Method B. To urine (0.5 ml) in a micro-centrifuge tube was added titanium trichloride solution (0.5 ml) and the mixture incubated at 40°C overnight (16 h) (or 75°C for 1 h). [1,1,1′,1′-$^2H_4$]thiodiglycol (50 ng) was then added as internal standard and the mixture transferred to a 3-ml Chem Elut tube connected in series to a Florisil Sep-Pak cartridge. The tube plus cartridge were eluted with ethyl acetate (5 × 5 ml) into a 50-ml round-bottomed flask. The solution was concentrated to *ca.* 1 ml at 30°C on a rotary evaporator, transferred to a 1-ml vial with methanol (2 × 0.5 ml) and concentrated to dryness under nitrogen at 40°C.

Derivatisation

To the dried residue was added pyridine (80 μl method A or 50 μl method B) and pentafluorobenzoyl chloride (20 μl method A or 10 μl method B). The mixture was vortexed and then stood at ambient temperature for 2 min. The solution was made up to 500 μl with toluene, vortexed and centrifuged. This procedure converts both thiodiglycol sulphoxide and thiodiglycol to the bis-(pentafluorobenzoyl) derivative of thiodiglycol.

GC–MS analysis

Analyses were performed using a Finnigan 4600 quadrupole GC–MS system with only minor modifications to the instrumental conditions reported previously [9]. The gas chromatograph was fitted with a 25 m × 0.22 mm OV-1701 bonded phase column (Thames Chromatography, Maidenhead, UK) or a 25 m × 0.22 mm BP 10 column (SGE, Milton Keynes, UK), film thickness 0.25 μm; carrier gas helium at 15 p.s.i. The oven was held at 90°C for 0.5 min then programmed from 90 to 230°C at 20°C/min, from 230 to 260°C at 3°C/min and finally held at 260°C for 1 min. Splitless injections (0.5 μl) were made with toluene needle flush; split delay 0.5 min; 2 mm I.D., injector liner; injector temperature 250°C. The transfer line and interface were at 260°C. The mass spectrometer was operated in the selected ion monitoring mode using negative ion chemical ionisation with methane as reagent gas; source pressure 0.8 Torr; source temperature 120°C; electron energy 150 eV; emission current 0.3 mA; electron multiplier 1300 V. Molecular ions *m/z* 510 and 514 (internal standard) were monitored, dwell time 0.2 s, total scan time 0.5 s. For additional confirmation the isotope ion at *m/z* 511 could also be monitored with a relative intensity (peak area) of 10–15% compared to *m/z* 510. The retention time for thiodiglycol bis-(pentafluorobenzoate) was *ca.* 12 min.

Quantitation was performed by comparing the computer integrated peak area for the ion *m/z* 510 at the appropriate retention time for thiodiglycol bis-(pentafluorobenzoate) with that for the analogous peak for *m/z* 514 derived from the tetradeuterated internal standard. Calibration curves were established for thiodiglycol sulphoxide and thiodiglycol standards, and for samples of human and rat urine spiked with thiodiglycol sulphoxide at concentrations of 0, 2, 5, 10, 25, 50, 75, 100, 150 and 200 ng/ml. These calibration curves were superimposable on curves constructed from standards after allowing for any background levels of the sulphoxide present in urine (see below) and the molecular weight difference between the sulphoxide and thiodiglycol. Quantitation for method A was therefore performed against a calibration curve constructed form thiodiglycol sulphoxide standards and for method B against a calibration curve constructed from thiodiglycol standards. For method B, spiking experiments were also performed using thiodiglycol.

RESULTS AND DISCUSSION

Recovery and clean-up

Method A. Thiodiglycol sulphoxide is considerably more polar than thiodiglycol, as is evident from its strong retention on normal-phase silica gel [15]. Conversely, it is not retained from aqueous solution by non-polar reversed phases such as C_{18}, nor by polymeric matrices such as the various XAD resins. Two possible alternative approaches to recovery were, (i) removal of anionic and cationic materials from urine using ion-exchange resins followed by concentration of the remaining solution to dryness, or, (ii) extraction with a polar solvent after absorption of urine onto a solid phase, as was used previously by us for the recovery of thiodiglycol [9] and other water soluble materials such as nivalenol [16], followed by clean-up on normal-phase chromatographic material. Attempts at recovery by concentration of deionised urine gave poor and very variable recoveries, even with the use of silanised glassware. Extraction from a solid absorbent was therefore adopted as the most fruitful approach.

Preliminary experiments indicated that the use of ethyl acetate as extracting solvent, as used for thiodiglycol [9], gave no recovery of the sulphoxide. The polarity of the eluting solvent for Chem Elut tubes can be increased up to a maximum of ethyl acetate-methanol (10:1) before breakthrough of the aqueous phase occurs, but the problem with highly polar elution is that as the polarity increases so does the amount of extraneous material extracted. A compromise therefore has to be made between recovery of the analyte and clean-up. Experiments indicated that for a solution of the sulphoxide in water (50 ng/ml) absorbed onto a 3-ml Chem Elut tube, 4 or 5 × 5 ml elutions with ethyl acetate–methanol (10:1) were required for near quantitative extraction. However, not surprisingly, these elution conditions when applied to urine recovered large amounts of extraneous materials, which may cause problems in the derivatisation stage and introduce significant chemical background into the selected ion current traces. Experiments using gradually decreasing amounts of methanol in the eluting solvent indicated that ethyl acetate–methanol (100:7) was the least polar mixture which gave more than 75% recovery. This extract when applied to urine then required additional clean-up. Silica and Florisil cartridges were compared for retention of the sulphoxide. Florisil was more retentive than silica and was selected to allow some separation of the sulphoxide from extraneous materials. The sulphoxide was not eluted from Florisil with chloroform–methanol (100:20), allowing some of the extraneous material to be removed. The sulphoxide was then recovered by elution with chloroform-methanol (50:50). Using this procedure recoveries of thiodiglycol sulphoxide, from 5 replicate samples of urine spiked at levels of 50 ng/ml, were 52, 52, 44, 47 and 50% respectively, average recovery 49% (coefficient of variation of 6.8%).

Although thiodiglycol is essentially excluded by this procedure, spiking experiments indicated that small amounts (*ca.* 7% from a 0.5-ml aliquot spiked with 50 ng) were recovered in the final eluate. This presumably arises from a small amount of thiodiglycol being bound more strongly to a few very polar sites in the Chem Elut tube and Florisil cartridge, which was eluted only with the more polar eluting solvent. However, since thiodiglycol is normally present in much lower amounts than the sulphoxide in urine from rats treated with mustard, the small amount recovered using this procedure was considered insignificant for the purpose of determining elimination profiles of the sulphoxide.

Method B. There are few reagents which will efficiently reduce sulphoxides to sulphides in aqueous solution. One possible reagent is titanium trichloride, as was used by Nishimura *et al.* [17] to reduce dimethyl sulphoxide to dimethyl sulphide prior to analysis using GC–MS. Preliminary experiments with thiodiglycol sulphoxide indicated that reduction with titanium trichloride in hydrochloric acid proceeded in high yield and was tolerant to a wide variation in reaction conditions. Reduction to thiodiglycol occurred in essentially quantitative yield (>98%) in less than 1 h at 75°C or within 16 h at 40°C. Reduction at 75°C had the slight disadvantage of some leakage of the acidic solution around the caps of the plastic micro-centrifuge tubes. For our purposes an overnight (16 h) reduction at 40°C was convenient and was adopted as the standard procedure. Optimum yields were obtained using an equal volume of reagent to 0.5 ml of urine. Since the reagent is highly acidic (pH < 1) it should also hydrolyse any acid-labile esters of thiodiglycol, or of the sulphoxide, which may be present in urine. This was supported by the analysis of urine from rats which had been treated with sulphur mustard. Treatment of 0–24 h urine with acidic titanium trichlo-

ride released amounts of thiodiglycol which were up to 1.3 to 1.4 times greater than the total amounts of thiodiglycol and the sulphoxide determined separately after treatment of urine with hydrochloric acid. These experiments in fact suggested that there may be other metabolites which convert to thiodiglycol under these conditions; further details will be reported elsewhere. The titanium trichloride reagent did not affect the extraction of thiodiglycol from Chem Elut tubes or clean up on Florisil, although extraction on Chem Elut tubes was noticeably slower due to clogging of the top of the tube or the frit, presumeably with colloidal titanium salts.

Recoveries determined for replicate 0.5-ml samples of urine spiked with 50 ng of deuterated thiodiglycol sulphoxide were 68, 67, 61, 69 and 70% respectively, average recovery 67%, coefficient of variation 5.3%. These were similar to the recoveries (60–80%) determined previously for thiodiglycol [9].

Derivatisation

As reported previously [9] the derivatisation procedure results in reduction of the sulphoxide to the bis-(pentafluorobenzoyl) derivative of thiodiglycol. The conversion proceeds in essentially the same yield as from thiodiglycol itself. Reductions of sulphoxides to sulphides by electrophilic reagents have been reported previously and proceed by initial O-acylation of the sulphoxide group to form a sulphonium species which then eliminates an acyloxy moiety [18]. Due to the greater amount of extraneous material in the residue using method A, a larger amount of derivatising agent was required to ensure reliable derivatisation.

Quantitation

Method A. The procedure gave a linear calibration over the range 10–200 ng/ml for spiked urine with a correlation coefficient of 0.9991, slope 0.252. Slight curvature was observed at concentrations below 10 ng/ml; quantitation in this lower range was performed using calibration points at 0, 2, 5 and 10 ng/ml. Calibration curves for pure standards and for spiked urine were superimposable after adjusting for any low background levels of thiodiglycol sulphoxide present in the urine (see below). The limit of detection for urine was estimated as 2 ng/ml (1 ng per 0.5-ml sample) based on a signal-to-noise ratio of 3:1. However, as discussed below, background levels in normal control urine were usually higher than this. Fig. 1 shows the selected ion current trace for a normal sample of human urine containing a small amount of the sulphoxide, quantitated at 4 ng/ml. Fig. 2. shows selected ion current traces for the same urine spiked with the sulphoxide at 50 ng/ml. These selected ion current trace show considerably greater chemical noise than was observed for the analysis of thiodiglycol [9], reflecting the greater amounts of extraneous materials recovered from urine by the polar extracting/eluting solvents required to recover the sulphoxide. Urine sample volumes were accordingly limited to 0.5 ml to minimise any interference of extraneous materials in the derivatisation procedure. The method showed good precision. Six replicate determinations for a sample of urine, containing a background level of 7 ng/ml and spiked with 50 ng/ml of the sulphoxide, gave values of 58, 58, 58, 56, 62 and 60 ng/ml, average 59 ng/ml, with a coefficient of variation of 3.5% (standard deviation, $\sigma_{n-1} = 2.07$).

Method B. A calibration curve constructed from urine spiked with thiodiglycol sulphoxide was linear over the range 10–200 ng/ml with slight curvature in the 0–10

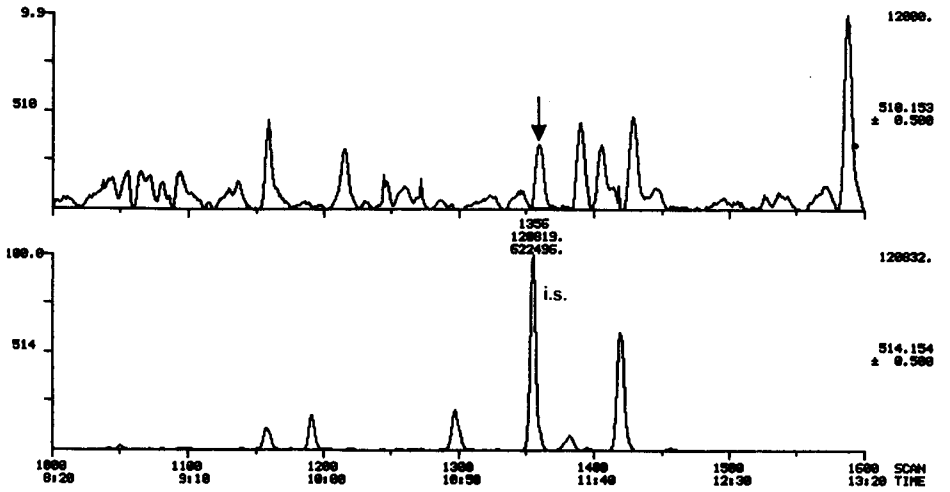

Fig. 1. Selected ion current chromatogram (method A) showing the presence of low levels (4 ng/ml) of thiodiglycol sulphoxide in normal human urine monitoring *m/z* 510 (upper) and the response to the internal standard (50 ng/ml) monitoring *m/z* 514 (lower). Time in min:s.

ng/ml range. Quantitation in this lower range was performed using calibration points at 0, 2, 5 and 10 ng/ml. Calibration points constructed from thiodiglycol standards lay on the same curve, shown in Fig. 3, after adjusting for any background level of the sulphoxide present in the urine and the molecular weight difference between thiodiglycol and the sulphoxide. Quantitation of unknowns was performed against a calibration curve constructed from thiodiglycol standards. The limit of detection was

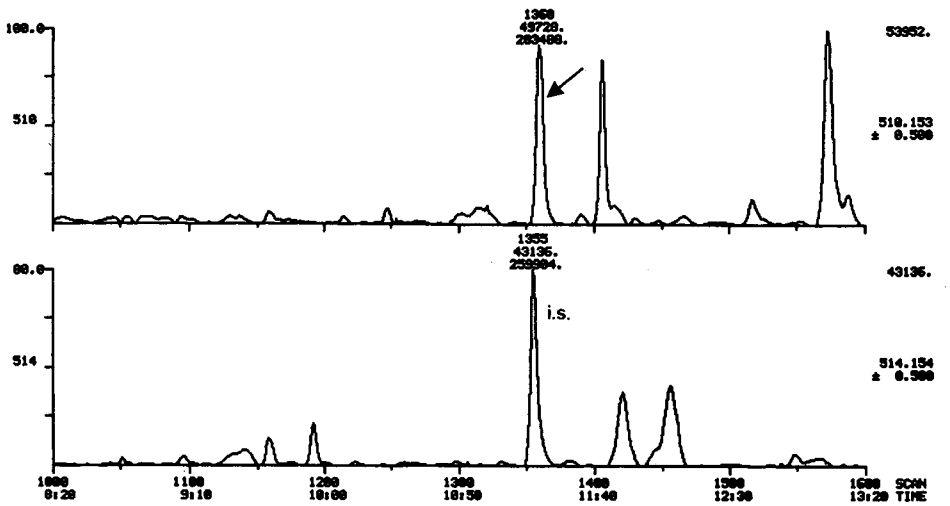

Fig. 2. Selected ion current chromatograms (method A) showing the detection of thiodiglycol sulphoxide (50 ng/ml), spiked into human urine, monitoring *m/z* 510 (upper) and the response to the internal standard (50 ng/ml) monitoring *m/z* 514 (lower). Time in min:s.

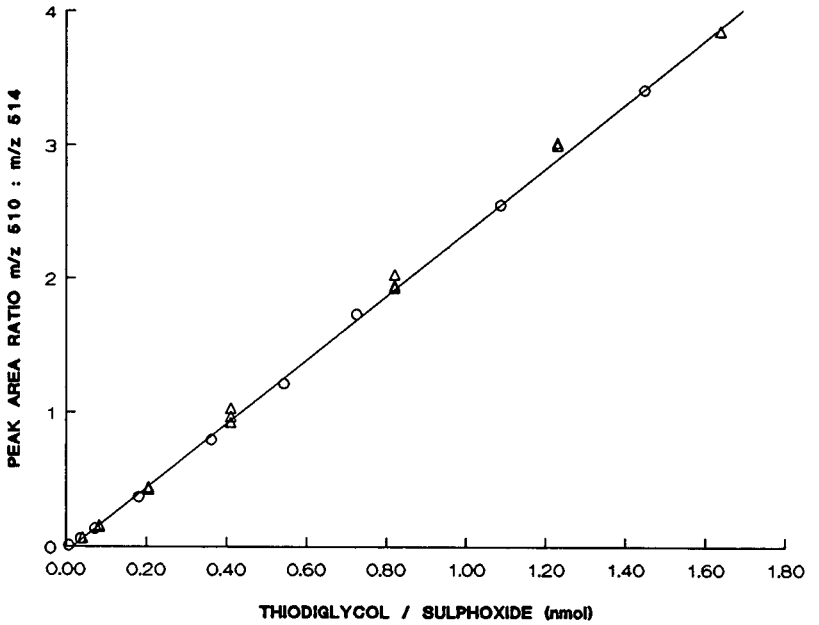

Fig. 3. Calibration curve for thiodiglycol sulphoxide in urine (adjusted for background level) using method B, and thiodiglycol standard. ○ = Thiodiglycol sulphoxide; △ = thiodiglycol.

1 ng per 0.5-ml sample. However, consistent with our observations with thiodiglycol sulphoxide using method A, normal control urine (rat and human), after reduction with titanium trichloride, contained a small background level of thiodiglycol, normal-

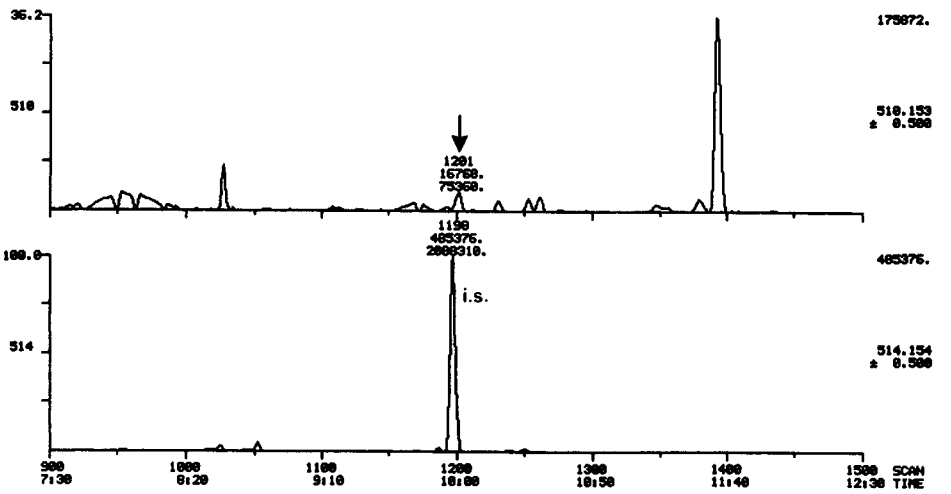

Fig. 4. Selected ion current chromatograms (method B) showing the presence of low levels (7 ng/ml) of analyte in normal human urine monitoring *m/z* 510 (upper) and the response to the internal standard (100 ng/ml) monitoring *m/z* 514 (lower). Time in min:s.

ly in the range 2–10 ng/ml (see further discussion below). Fig. 4 shows selected ion current chromatograms from a control sample of human urine with a background level of analyte determined as 7 ng/ml. Fig. 5 shows the same sample spiked with 20 ng/ml of thiodiglycol sulphoxide. Additional experiments indicated that the reducing agent did not interfere with the analysis of free thiodiglycol. The precision of the method was good. Six replicate determinations performed on rat urine, containing a background level of 6 ng/ml of thiodiglycol sulphoxide and spiked with 100 ng/ml of sulphoxide ($\equiv$93.7 ng/ml of thiodiglycol), gave values of 90, 92, 98, 98, 96 and 90 ng/ml, average 94 ng/ml, with a coefficient of variation of 4.0% (σ_{n-1} = 3.8). Because the method is intended to be used for the combined detection of thiodiglycol, thiodiglycol sulphoxide and their acid-labile esters, as evidence of mustard poisoning, [$^2H_4$]thiodiglycol was employed as internal standard and added after treatment with titanium trichloride. Experiments indicated no significant difference when [$^2H_4$]thiodiglycol sulphoxide was added as internal standard before treatment with titanium trichloride.

As referred to above, control samples of human urine contained a small background level of analyte using both of the methods described. Levels in urine from five different subjects were in the range 2–8 ng/ml, one subject being sampled on eight different occasions. Negative control samples of distilled water run prior to these samples were negative, thereby eliminating any possibility that the background levels reflected contamination of laboratory equipment. However, a sixth subject was found to be excreting levels of analyte determined as 32 and 24 ng/ml respectively using method B, when sampled on two different occasions. Analysis of the first of these samples separately for thiodiglycol [9] and for the sulphoxide indicated that this background level was due almost entirely to the sulphoxide. Levels of thiodiglycol

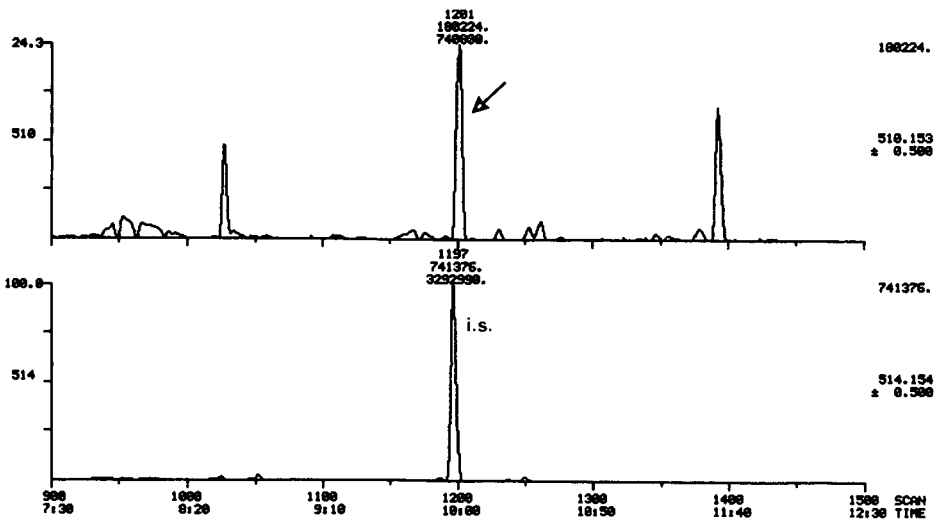

Fig. 5. Selected ion current chromatograms (method B) showing the detection of thiodiglycol sulphoxide (20 ng/ml), spiked into human urine, monitoring *m/z* 510 (upper) and the response to the internal standard (100 ng/ml) monitoring *m/z* 514 (lower). Time in min:s.

and the sulphoxide were determined as <1 ng/ml and 36 ng/ml (= 32 ng/ml thiodiglycol), respectively. The detection of this background using both of the methods reported in this paper lends additional support to its identification as the sulphoxide rather than an unknown interferent. The chances of an interferent being present which has similar GC–MS characteristics to thiodiglycol after derivatisation, and which behaves like the sulphoxide before and after reduction, must be extremely remote. As we have discussed previously [9], the concentration of the ion current in the molecular ion with minimal fragmentation gives the analytical method great sensitivity but does present problems where additional confirmation of identity is required. The isotopic ion at *m/z* 511 can be monitored and shown to be within the ratio 10–15% (peak area) of the parent ion. We have also employed GC columns with a different selectivity to lend additional support to the identification [9]. Low levels of the sulphoxide (<2–10 ng/ml) were also found in control rat urine.

In our previous work [9] we reported that normal urinary levels of thiodiglycol were <1 ng/ml but that levels up to *ca.* 16 ng/ml were found in blood. These earlier results with urine contrasted with those of Wils and co-workers [7,8] who, using a method less specific for thiodiglycol, found background levels in control subjects mostly in the range equivalent to 1–10 ng/ml of thiodiglycol but in one case as high as 55 ng/ml [7] and in another 21 ng/ml [8]. Our findings with background levels of the sulphoxide are more consistent with those reported [7,8] for thiodiglycol. The procedure used by Wils and co-workers [7,8] converts thiodiglycol back to mustard by heating with concentrated hydrochloric acid. We thought that thiodiglycol sulphoxide might be reduced to thiodiglycol via a sulphonium species under these conditions, since reductions of sulphoxides with hydrochloric acid have been reported previously [18]. However, refluxing a solution of the sulphoxide with concentrated hydrochloric

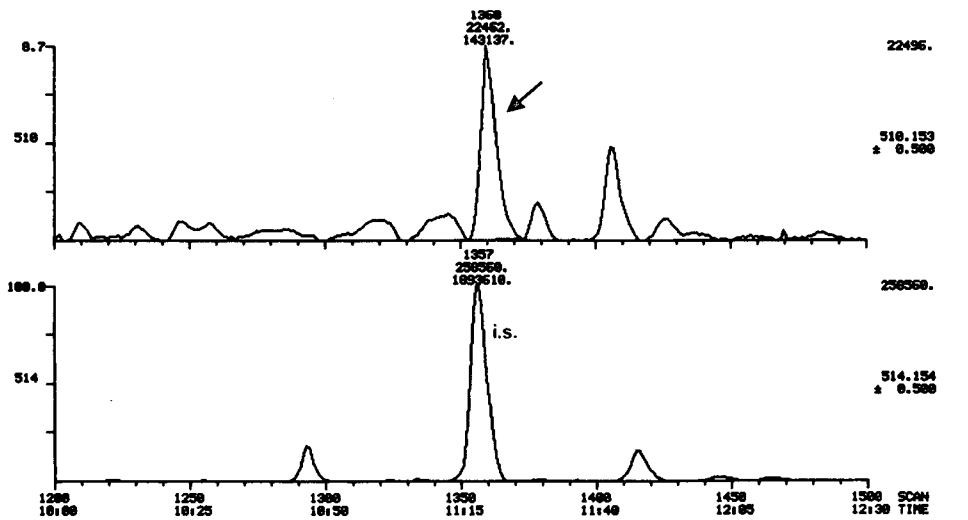

Fig. 6. Selected ion current chromatograms (method A) showing the detection of thiodiglycol sulphoxide (16 ng/ml) in rat urine, collected 8 days after a cutaneous application of 2 μmol of sulphur mustard, monitoring *m/z* 510 (upper) and the response to the internal standard (100 ng/ml) monitoring *m/z* 514 (lower). Time in min:s.

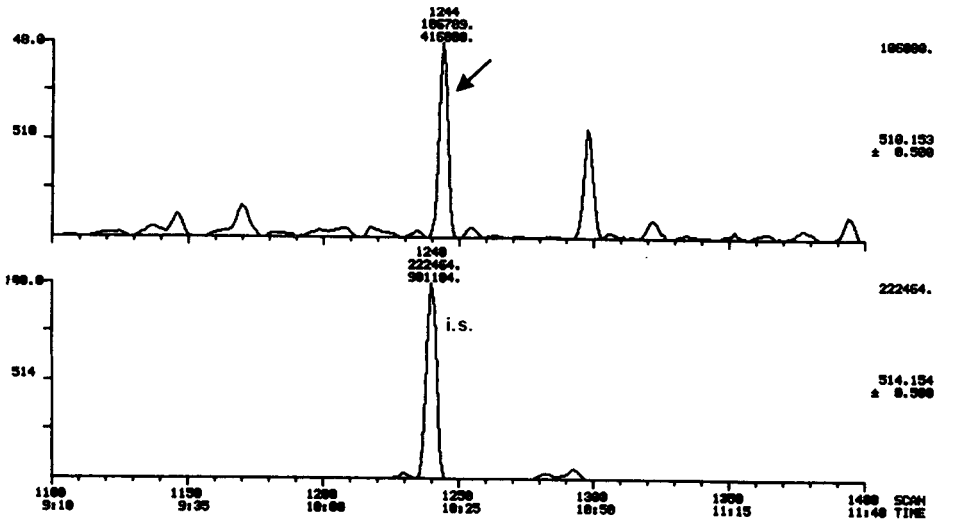

Fig. 7. Selected ion current chromatograms (method B) showing the detection of thiodiglycol (60 ng/ml) in rat urine after reduction with titanium trichloride, collected 8 days after a cutaneous application of 2 μmol of sulphur mustard, monitoring *m/z* 510 (upper) and the response to the internal standard (100 ng/ml) monitoring *m/z* (lower). Time in min:s.

acid for 1 h, and neutralisation with sodium hydroxide, did not produce any thiodiglycol.

The source of this low background level is unknown, but sulphur–rich foods in the diet is one possibility. A compound which was identified by Reichstein and Goldschmidt [19,20] in 1936 as thiodiglycol sulphoxide, on the basis of elemental analysis, melting point and comparison with a synthetic sample, was isolated from the saponified lipid fraction of the adrenal glands (1000 kg) of cattle. The source of the compound was unknown but the authors postulated that the sulphoxide was associated with lipids by esterification in the same way as glycerol. This explanation seems unlikely on the basis of known naturally occurring lipids, but there appears to be no evidence to refute it.

The applicability of the two methods to the detection of excretion products derived from sulphur mustard is shown in Figs. 6 and 7. Fig. 6 shows the detection of 16 ng/ml of thiodiglycol sulphoxide in rat urine, eight days after a cutaneous application of 2 μmol of sulphur mustard. Fig. 7 shows the detection of 60 ng/ml of thiodiglycol in rat urine, after treatment of urine with titanium trichloride using method B, also eight days after a cutaneous application of 2 μmol of sulphur mustard.

CONCLUSIONS

Sensitive methods have been developed for the detection and quantitation of thiodiglycol sulphoxide in urine. The limits of detection were 2 ng/ml of urine. The methods were validated by the analysis of spiked human urine samples and by the analysis of urine from rats which had been exposed to sulphur mustard. For the purpose of retrospective confirmation of mustard poisoning the second method is

preferred, which detects thiodiglycol sulphoxide, thiodiglycol, and their acid-labile esters, as the single analyte thiodiglycol. A complicating factor is the presence of low levels of analyte in samples of normal human urine. These were in the range 2–8 ng/ml in five subjects but were as high as 36 ng/ml in one subject.

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Analysis of 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane, metabolites of sulphur mustard, in urine using gas chromatography–mass spectrometry

ROBIN M. BLACK*, RAYMOND J. CLARKE and ROBERT W. READ
Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire SP4 0JQ (UK)
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ABSTRACT

A method has been developed for the detection of 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane, which have been identified as urinary metabolites of sulphur mustard in the rat. The two metabolites were reduced to the single analyte 1,1′-sulphonylbis[2-(methylthio)ethane] by treatment of urine with acidic titanium trichloride. 1,1′-Sulphonylbis[2-(methylthio)ethane] was readily extracted from urine by passing through a C_8 reversed-phase extraction column, or by solvent extraction from a solid absorbent tube, and detected by gas chromatography–mass spectrometry using ammonia positive ion chemical ionisation. The limit of detection was 2 ng/ml for 1-ml samples of urine. There were no background levels of analyte in human or rat urine. If man metabolises sulphur mustard by a similar pathway, the detection of these metabolites should constitute firm evidence of an exposure to sulphur mustard.

INTRODUCTION

The recent proliferation and use of chemical weapons, particularly in the Middle East, plus the negotiations towards a verifiable chemical weapons treaty, have lead to increased attention being given to the retrospective confirmation of specific agent poisoning in victims of chemical weapons. Of particular concern is sulphur mustard, following its use in the Iraq–Iran conflict [1–3]. At present there is no unambiguous means of confirming a medical diagnosis of mustard poisoning in casualties who have been externally decontaminated from any traces of agent. Greatest attention has been given to the detection of excretion products derived from the hydrolysis of sulphur mustard as possible indicators of mustard poisoning. Methods for the detection of thiodiglycol, the simple hydrolysis product of sulphur mustard, in urine, have been reported by Wils and co-workers [4,5] and by Black and Read [6]. Black and Read [7] have also reported methods for the detection of thiodiglycol sulphoxide, which recent metabolism studies [8] have shown to be the major urinary excretion product derived from the hydrolysis of sulphur mustard. However, a com-

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plicating factor in the detection of these hydrolysis products is the presence of low background levels of analyte in normal urine which, although usually in the range 1–10 ng/ml, were as high as 36 ng/ml [7] and 55 ng/ml [4] in extreme cases. We therefore sought an alternative metabolite whose detection in urine could be accepted as unambiguous evidence of mustard poisoning, in addition to the detection of products derived from hydrolysis.

Nine urinary metabolites of sulphur mustard have been identified in recent studies in these laboratories [8]. Several of these are conjugates of mustard with N-acetylcysteine, most of which have poor mass spectrometric and/or gas chromatographic properties, mainly due to thermal instability. More promising from the viewpoint of analysis were two closely related products assumed to be derived from the further action of the enzyme β-lyase on cysteine conjugates. These metabolites are 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] (**1**) and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane (**2**).

$O_2S(CH_2CH_2SOCH_3)_2$ $CH_3SCH_2CH_2SO_2CH_2CH_2SOCH_3$

1 **2**

We sought an analytical method which would detect both of these metabolites in urine down to levels of 5 ng/ml or lower. Compounds containing sulphoxide groups often possess poor gas chromatographic properties, giving rise to badly tailing peaks. An additional problem with these particular compounds was the possibility of thermal elimination of the CH_3SOH moiety to give an olefinic species $-SO_2CH=CH_2$, a process which is observed in the mass spectra of these compounds. The high polarity of the sulphoxide group also makes isolation from aqueous matrices more difficult. We therefore sought to reduce the sulphoxide groups to sulphide, which would increase both lipophilicity and thermal stability, and enable both metabolites to be detected as the common easily extractable analyte 1,1′-sulphonylbis[2-(methylthio)ethane] (**3**).

$O_2S(CH_2CH_2SCH_3)_2$

3

We had already adopted a similar approach for the simultaneous detection of thiodiglycol and its sulphoxide, by reducing thiodiglycol sulphoxide to thiodiglycol using acidic titanium trichloride [7]. This reagent had been previously employed by Nishimura *et al.* [9] to reduce dimethyl sulphoxide to dimethyl sulphide. In this present paper we report a method, employing gas chromatography–mass spectrometry (GC–MS), which enables both of the β-lyase metabolites (**1** and **2**) to be detected in urine as the single analyte 1,1′-sulphonylbis[2-(methylthio)ethane], after reduction with acidic titanium trichloride.

EXPERIMENTAL

Materials

1,1′-Sulphonylbis[2-(methylthio)ethane] (**3**) was synthesised from 1,1′-sulphonylbis(2-chloroethane) and methanethiol. 1,1′-Sulphonylbis[2-(methylsulphinyl)ethane] (**1**) and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane (**2**) were prepared by oxidation of **3** with sodium periodate. 1,1′-Sulphonylbis[2-(trideuteromethylsulphinyl)ethane] was synthesised by an analogous procedure using [$^2H_3$]methanethiol. Full experimental details will be reported elsewhere [10]. Standard solutions were made up in methanol at concentrations of 0.1–10 μg/ml.

Titanium trichloride (15% solution in 20–30% hydrochloric acid) was purchased from Aldrich (Gillingham, UK). Fisons (Loughborough, UK) Distol-grade solvents were used. Chem Elut tubes and Bond Elut columns (Analytichem International, Harbor City, CA, USA) were purchased from Jones Chromatography (Hengoed, Mid Glamorgan, UK). Chem Elut tubes were washed with methanol (3 × 5 ml) and dried in a vacuum oven at 60°C prior to use. All glassware was pretreated with Aquasil siliconising fluid (Pierce & Warriner, Chester, UK).

Samples of human urine were collected from volunteer laboratory workers. Samples of rat urine were collected and supplied by Biology Division, CDE. They were stored at −20°C prior to analysis.

Preliminary experiments

Solutions of metabolites **1** or **2** (10 μg) in water (1 ml) were treated with titanium trichloride solution (0.5 ml) at 75°C for 15 min or at 40°C overnight [7]. The cooled solution was absorbed onto a 5-ml Chem Elut tube, extracted with dichloromethane (2 × 8 ml), and analysed by full scanning GC–MS. Recoveries were estimated by comparison with a standard solution of 1.1′-sulphonylbis[2-(methylthio)ethane].

Extraction and clean-up

Method A. To urine (1 ml) was added 1,1′-sulphonylbis[2-(trideuteromethylsulphinyl)ethane] (50 ng) as internal standard. Titanium trichloride solution (0.4 ml) was then added and the sample incubated at 40°C overnight (16 h). The sample was transferred to a 3-ml (500 mg) C_8 Bond Elut column (previously conditioned with 2 × 2 ml methanol and 2 × 2 ml water) mounted on a Vac Elut vacuum manifold (Jones Chromatography), and the solution allowed to seep through the sorbent bed under gravity; a light vacuum (120–250 mmHg) was then applied to remove unretained material. Partial clean-up was performed by washing the column with water (2 × 0.5 ml) followed by methanol–water (40:60) (2 × 0.5 ml) under gentle suction (*ca.* 50 mmHg), and the column then sucked dry under maximum vacuum (water pump) for 5 min. The analyte was eluted with acetone (2 × 0.5 ml) using gentle suction (*ca.* 50 mmHg), the eluate was transferred to a 1-ml vial and concentrated to dryness under a stream of nitrogen or using a centrifugal evaporator.

Method B. To urine (1 ml) was added 1,1′-sulphonylbis[2-(trideuteromethylsulphinyl)ethane] (50 ng) and titanium trichloride solution (0.4 ml) and the sample incubated at 40°C overnight (16 h). The sample was then transferred to a 3-ml Chem Elut tube connected in series to a 3 ml (500 mg) silica Bond Elut column (previously

conditioned with 2 × 2 ml dichloromethane–*n*-hexane, 40:60) mounted on a Vac Elut vacuum manifold. The analyte was extracted from the Chem Elut tube with dichloromethane–*n*-hexane (40:60) (2 × 5 ml) and the eluate passed directly through the silica column using gentle suction (*ca.* 50 mmHg). The Chem Elut tube was removed and the retained analyte eluted from the silica column with acetone (3 × 0.5 ml) using gentle suction (*ca.* 50 mmHg). The eluate was transferred to a 1-ml vial and concentrated to dryness under a stream of nitrogen or using a centrifugal evaporator.

The residues were dissolved in toluene (100 μl) before GC–MS analysis.

GC–MS analysis

Preliminary experiments were performed using a VG7070EQ double focussing magnetic sector mass spectrometer, coupled to a HP 5890 gas chromatograph. The analytical method was developed using a Finnigan 4600 GC–MS system. The gas chromatograph was fitted with a 25 m × 0.22 mm I.D. BP10 silicone bonded phase column (SGE, Milton Keynes, UK), film thickness 0.25 μm, inserted directly into the mass spectrometer ion source. Helium at 20 p.s.i. was used as carrier gas. Splitless injections (1 μl) were made, with a 4 mm injector insert, split delay 0.5 min, septum purge 2 ml/min; injector temperature 260°C. The GC oven temperature was held at 90°C for 0.5 min, programmed from 90 to 180°C at 20°C/min, from 180 to 240°C at 10°C/min and finally held at 240°C for 2 min. The GC–MS interface oven and transfer line were held at 260°C. The mass spectrometer was operated in the selected ion monitoring mode using positive ion chemical ionisation with ammonia as reagent gas; source pressure 0.6 Torr; source temperature 120°C. The ion source was operated at 120 eV with an emission current of 0.3 mA.

The retention time of 1,1′-sulphonylbis[2-(methylthio)ethane] was *ca.* 10 min 20 s and that for the internal standard 10 min 17 s. For detection and quantitation, the ion at *m/z* 232 $[M+NH_4]^+$ was monitored for 1,1′-sulphonylbis[2-(methylthio)ethane], and *m/z* 238 for the reduced hexadeuterated internal standard; the ion at *m/z* 184 was monitored for additional confirmation at levels above *ca.* 20 ng/ml. Dwell time was 0.08 s, total scan time 0.3 s.

Quantitation was performed by comparing the computer integrated peak area for the ion *m/z* 232 at the appropriate retention time with that for the reduced hexadeuterated internal standard monitoring *m/z* 238. Calibration curves were established for samples of human and rat urine spiked with 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] (**1**) at levels of 0, 2, 5, 50, 100, 250 and 500 ng/ml.

RESULTS AND DISCUSSION

Preliminary experiments

Preliminary experiments indicated that both of the metabolites **1** and **2** were unstable to GC; neither compound eluted from non-polar silicone bonded phase columns. In contrast, the bis-methylthio reduction product **3** possessed good GC properties, giving a narrow well shaped peak under the conditions employed. The reduction of the metabolites **1** and **2** to the bis-methylthio product **3**, using titanium trichloride in hydrochloric acid, occurred in >95% yield at a temperature of 75°C for 15 min, or alternatively at 40°C overnight (16 h). At the higher temperature some

leakage of the acidic reagent around the caps of the plastic micro-centrifuge tubes tended to occur and for convenience the lower temperature was adopted, as was used for the reduction of thiodiglycol sulphoxide to thiodiglycol [7].

Isolation and clean-up

Two methods were developed for the isolation of **3**. The first method is intended to be used for the analysis of urine specifically for these β-lyase products. The second method was designed so that it could be integrated into our procedure for determining the hydrolysis products thiodiglycol sulphoxide and thiodiglycol [7] using a single sample of urine. The procedure employed for the analysis of the combined hydrolysis products also involves an initial reduction with acidic titanium trichloride.

Preliminary extraction experiments indicated that liquid–liquid extraction of **3** from aqeuous solution with dichloromethane was inefficient (*ca.* 30% recovery from 1 ml of water using 3 × 2 ml portions of dichloromethane). Superior recoveries were achieved either by retention of the analyte on reversed-phase silica cartridges, or by extraction with dichloromethane after absorption of the urine onto a solid absorbent tube.

Method A. Various reversed-phase silica extraction columns retained **3** from aqueous solution. Recovery experiments from urine spiked with **1**, after titanium trichloride treatment and using acetone as eluent, indicated recovery to decrease in the order $C_8 > C_{18} > Ph > CH > CN$, the more polar phases CH and CN giving poor recoveries. C_8 Bond Elut columns were selected for further method development. Elution experiments with 2 × 0.5-ml volumes containing increasing amounts (10, 20, 30% etc.) of methanol in water indicated 40% methanol to be the maximum concentration which did not elute the analyte, and this solvent was therefore used for partial removal of more polar extraneous materials from the column. Elution of the analyte could be achieved with methanol, acetone, acetonitrile or dichloromethane; acetone gave the most consistent recoveries. Six replicate recoveries from urine, spiked with 50 ng/ml of **1**, ranged from 82 to 94%, mean value 87.7%, with a coefficient of variation of 4.5%.

Method B. For the simultaneous determination of thiodiglycol and its sulphoxide, urine is treated with acidic titanium trichloride under conditions similar to those described above and is then absorbed onto a Chem Elut solid absorbent tube prior to extraction. Since the reduced β-lyase product **3** is considerably more lipophilic than thiodiglycol, selective extraction using a relatively non-polar solvent should be possible prior to the extraction of thiodiglycol with ethyl acetate. Dichloromethane extraction of the Chem Elut tube gave virtually quantitative recoveries of **3**. The polarity of the solvent was then gradually decreased by substituting increasing amounts of *n*-hexane; the optimum concentration which efficiently recovered **3** (*ca.* 90%), with minimal (*ca.* 10%) extraction of thiodiglycol, was hexane–dichloromethane (60:40). The eluate was partially cleaned up by retention of the analyte on a silica Bond Elut column which was then eluted with acetone. Six replicate recoveries from urine, spiked with 50 ng/ml of **1**, were in the range 83–103%, mean value 95.3%, with a coefficient of variation of 7.6%.

GC–MS analysis

Electron impact (EI) ionisation, methane chemical ionisation (CI) and ammo-

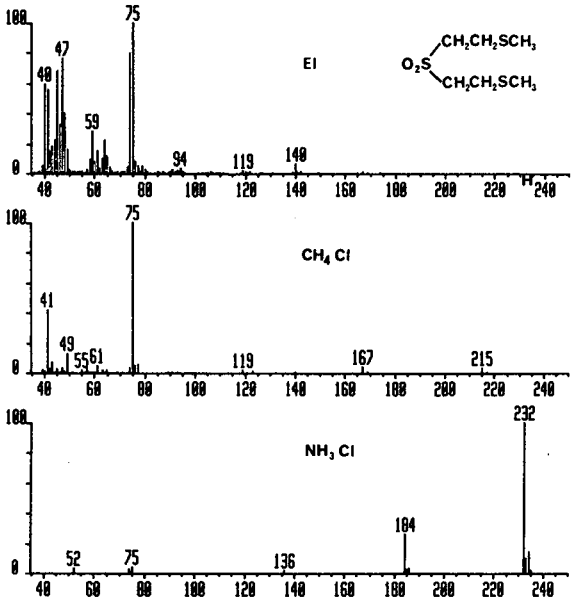

Fig. 1. EI, methane CI and ammonia CI mass spectra of 1,1′-sulphonylbis-[2-(methylthio)ethane].

nia CI positive ion mass spectra of **3** are shown in Fig. 1. Only ammonia CI gave a high intensity (100%) quasimolecular ion and was therefore adopted as the method of choice. Some variation in the ammonia CI mass spectra was observed when using the magnetic sector instrument, particularly as the source became dirty; ions at *m/z* 136, 138 and 140 became more intense and the quasimolecular ion weaker, especially with small amounts injected. Our experience with several metabolites of sulphur mustard has been that their mass spectra are sensitive to temperature and matrix effects, presumably due to thermal decomposition reactions. Such variability was minimal when using GC–quadrupole MS, which can operate at much lower source temperatures. The $[M+NH_4]^+$ ion at *m/z* 232 was monitored for quantitation and the fragment ion at *m/z* 184, formed by loss of CH_3SH, for confirmation at levels above *ca.* 20 ng/ml. Signal-to-noise ratios for this ion were poor at levels of analyte below 20 ng/ml. Additional sulphur isotope containing ions at *m/z* 234 and 186 could also be monitored if required for additional confirmation. The ion at *m/z* 136 was unsuitable for detection due to the heavy background present in urine at this mass.

Quantitation

Method A gave a linear calibration over the range 10–500 ng ml for metabolite **1** spiked into human urine, with a correlation coefficient 0.999, slope 0.0246. slight curvature was observed below 10 ng/ml; quantitation in this lower range was performed against calibration points at 0, 2, 5 and 10 ng/ml. Normal blank human urine gave a clean background at the retention time of interest as shown in Fig. 2. Ten samples of urine, from seven subjects, were analysed and all found to be clear at the retention time for the analyte **3**. The limit of detection and quantitation, monitoring

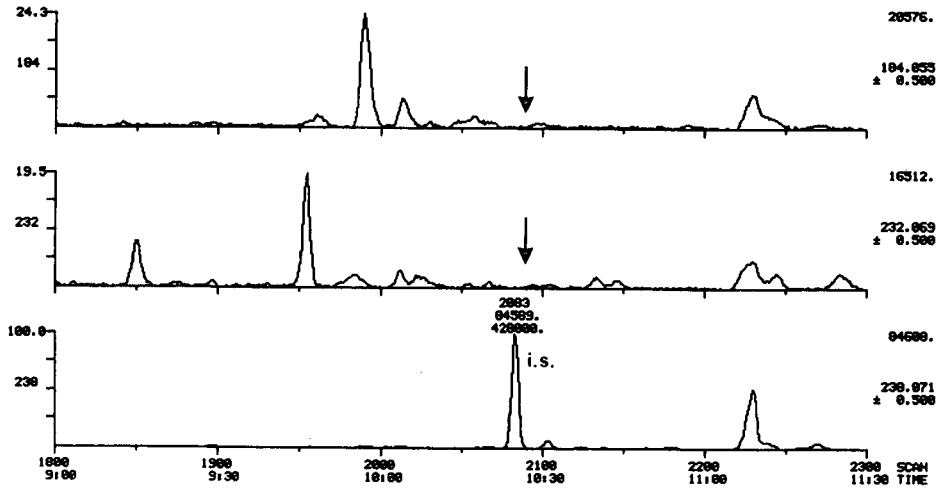

Fig. 2. Selected ion current chromatograms (method A) showing the absence of analyte in human urine monitoring *m/z* 184 (upper) and *m/z* 232 (middle), and the response to the internal standard (50 ng/ml) monitoring *m/z* 238 (lower). Time in min:s.

the quasimolecular ion and based on a signal-to-noise ratio of 3:1, was 2 ng/ml. The signal-to-noise ratio was lower for the ion at *m/z* 184 and monitoring this ion was useful only at levels above *ca.* 20 ng/ml. Fig. 3 shows selected ion current chromatograms from a sample of human urine spiked with **1** at a concentration of 50 ng/ml. The precision of the method was good; six replicate analyses of urine spiked at 50 ng/ml gave values of 48–52 ng/ml (Table I), mean value 49.8 ng/ml, with a coefficient

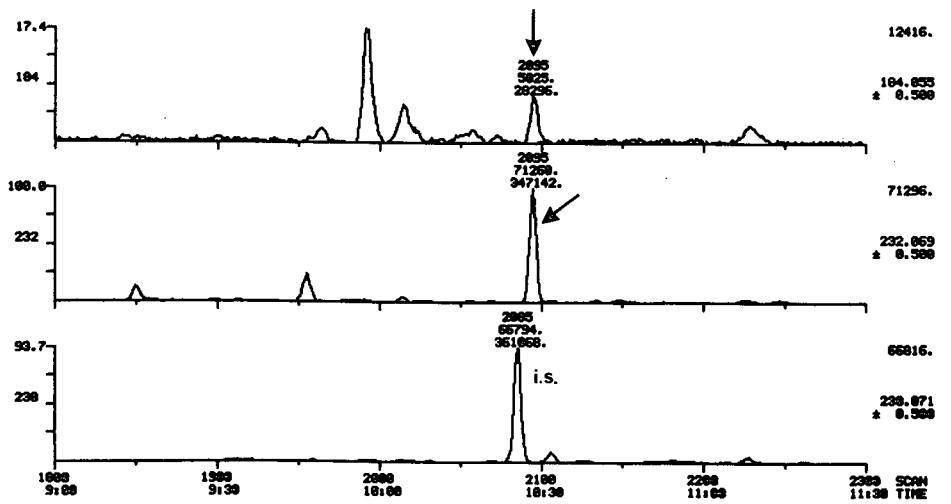

Fig. 3. Selected ion current chromatogram (method A) showing the detection of 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] (50 ng/ml), spiked into human urine, monitoring *m/z* 184 (upper) and *m/z* 232 (middle), and the response to the internal standard (50 ng/ml) monitoring *m/z* 238 (lower). Time in min:s.

TABLE I

PRECISION DATA FOR HUMAN URINE SPIKED WITH 50 ng/ml OF **1**

	Recovery (ng/ml)	
	Method A	Method B
	48	51
	51	49
	52	50
	50	49
	49	51
	49	52
Mean	49.8	50.3
Coefficient of variation (%)	2.95	2.4
σ_{n-1}[a]	1.47	1.21

[a] σ_{n-1} = Standard deviation.

of variation of 2.95%. Rat urine gave a linear calibration over the range 5–500 ng/ml. Six replicate analyses of a sample of rat urine, collected 5 days after cutaneous dosing with 2 μmol of sulphur mustard, gave values of 28–32 ng/ml, mean value 30 ng/ml, with a coefficient of variation of 0.9%. No analyte was detected in control samples of urine from 4 rats. There were however many more extraneous peaks in the selected ion current chromatograms derived from rat urine. Fig. 4 shows selected ion current chromatograms from a sample of rat urine, collected 5 days after a cutaneous exposure to mustard (2 μmol), found to contain β-lyase metabolites $\equiv$ 32 ng/ml of **1**.

Method B gave a linear calibration for human urine spiked with **1** over the range 10–500 ng/ml, with slight curvature below 10 ng/ml. The limit of detection and

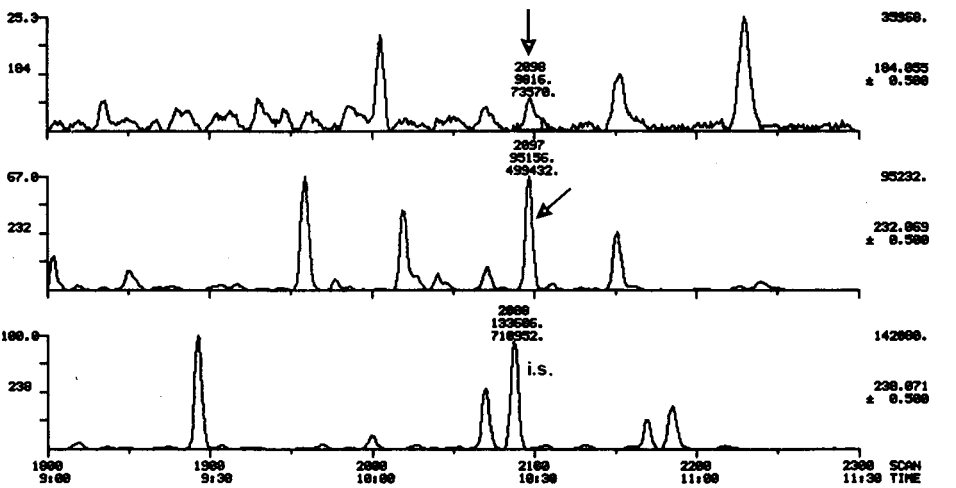

Fig. 4. Selected ion current chromatograms (method A) showing the detection of β-lyase metabolites [$\equiv$ 32 ng/ml of **1**] in rat urine, 4 days after a cutaneous application of sulphur mustard (318 μg), monitoring m/z 184 (upper) and m/z 232 (middle), and the response to the internal standard (50 ng/ml) monitoring m/z 238 (lower). Time in min:s.

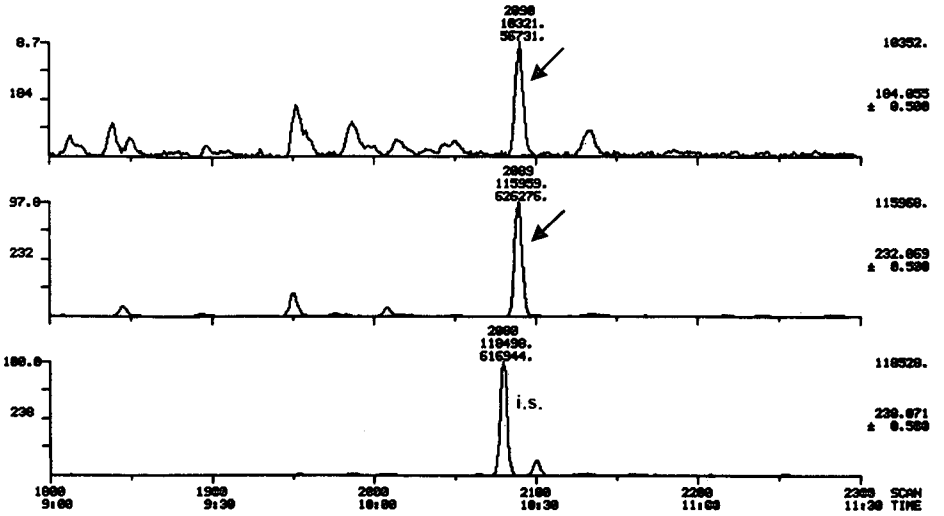

Fig. 5. Selected ion current chromatograms (method B) showing the detection of 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] (50 ng/ml), spiked into human urine, monitoring *m/z* 184 (upper) and *m/z* 232 (middle), and the response to the internal standard (50 ng/ml) monitoring *m/z* 238 (lower). Time in min:s.

quantitation monitoring *m/z* 234 was 2 ng/ml. Again precision was good; six replicate analyses of urine spiked with **1** at 50 ng/ml gave values of 49–52 ng/ml (Table I), mean value 50.3 ng/ml, with a coefficient of variation of 2.4%. A selected ion current chromatogram showing the detection of 50 ng/ml of **1** in spiked human urine is shown in Fig. 5. As with method A, control samples of urine from seven subjects were all negative for the analyte. Rat urine gave a linear calibration over the range 2–500 ng/ml, and again control samples from 5 rats were negative.

Both methods provide sensitive analytical procedures for the detection of β-lyase-derived metabolites of sulphur mustard, down to levels of 2 ng/ml in urine. Method A gave marginally cleaner traces from blank urine samples although there was little difference around the retention time of interest. In contrast to the detection of the hydrolysis products, thiodiglycol and its sulphoxide, there was no indication of any background levels of analyte in normal human or rat urine. Method A is preferred for the analysis of samples from casualties, provided sufficient urine is available to allow several different assays to be performed. Method B has been employed for the quantitative measurement of the urinary excretion of **1** and **2** plus the combined hydrolysis products, thiodiglycol and its sulphoxide, following cutaneous administration of sulphur mustard to rats. The results of these studies will be reported in a separate paper [11].

The applicability of the general method to the retrospective identification of mustard poisoning will, of course, depend on whether man also metabolises sulphur mustard by the same pathway, and on the elimination profile. The latter is currently being determined in rats but the question of metabolism in humans will only be answered if suitable samples become available in the event of a chemical attack.

CONCLUSIONS

A sensitive and specific method has been developed for the detection of two urinary metabolites of sulphur mustard, which are assumed to be derived from the action of β-lyase on cysteine conjugates. The metabolites were detected as the single analyte 1,1′-sulphonylbis-[2-(methylthio)ethane] after reduction with titanium trichloride. In contrast to the detection of hydrolysis products of sulphur mustard, there was no indication of any background levels of analyte in human or rat urine. If man metabolises sulphur mustard by the same pathway, then the detection of these metabolites should provide firm evidence of exposure to mustard.

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Comparative study of chromatography on thin layers impregnated with organic stationary phases

Chromatographic separation of nitrophenols

J. GASPARIČ*
Faculty of Pharmacy, Charles University, CS-501 65 Hradec Králové (Czechoslovakia)
and
J. SKUTIL
East Bohemian Chemical Works, CS-532 17 Pardubice-Semtín (Czechoslovakia)
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ABSTRACT

The chromatographic behaviour of nitrophenols on thin layers of silica gel and cellulose was compared, both without impregnation and impregnated with non-aqueous polar stationary phases (formamide, dimethylformamide) and less polar stationary phases (liquid paraffin, octan-1-ol, 1-bromonaphthalene). Cellulose is preferred when using formamide or dimethylformamide if a pure partition process is required. For each particular analyte a certain amount of the stationary phase is always required to suppress the adsorption activity of silica gel. Separation by reversed-phase thin-layer chromatography is strongly affected by the type of stationary phase (the possibility of forming charge-transfer complexes with 1-bromonaphthalene) and its support (the acidic properties of silica gel and its adsorption activity), the mobile phase (content of organic modifier, pH, presence of salts) and the properties of the solutes (polarity, ionizability).

INTRODUCTION

Much work was carried out in the 1960s in which thin layers impregnated with an organic stationary phase were used for analytical separations (for reviews, see Grassini-Strazza *et al.* [1] and Gasparič [2]). These layers were later largely replaced in analytical work by chemically bonded phases [3]. However, reversed-phase thin-layer chromatography (RP-TLC) became a simple method of obtaining quantitative information about the hydrophobic character of biologically active compounds in the study of quantitative structure activity relationships (QSAR) [4], and impregnated layers continued to be routinely, and often mechanically, used by medicinal chemists without considering the possible complications that could affect the partition mechanism [5].

These studies were based on the determination of the R_M values of the tested compounds on thin layers impregnated with a less polar stationary phase (such as

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octan-1-ol, liquid paraffin or silicone oil). Aqueous methanol or acetone were used as mobile phases. Buffer solutions were sometimes used instead of pure water. Straight-phase chromatography on layers impregnated with a non-aqueous polar stationary phase (dimethylformamide) were also used for this purpose [6]. It has been shown that the conditions of a pure partition mechanism required for these measurements are often strongly affected by the properties of the support used (silica gel, cellulose, Kieselguhr), the stationary and mobile phases and the properties of the solute [2]. The interaction of polar solutes with active sites on the support can sometimes be stronger than with the non-polar stationary phases; this is observed with sulphonamides and sulphonated azo dyes [7]. These two groups of compounds migrate in an identical manner on silica gel and cellulose layers either without impregnation or impregnated with octan-1-ol.

This work investigated the behaviour of relatively polar model compounds and considered the problems encountered with ionisable compounds in RP-TLC on impregnated layers.

EXPERIMENTAL

Chemicals

The nitrophenols used in this study have been described previously [8,9]. All liquids used as stationary phases and the mobile phases components were of analytical-reagent grade. Britton–Robinson buffer solutions of pH 3, 8 and 10 were prepared according to Sýkora and Zátka [10].

Thin-layer chromatography

All experiments were carried out on Silufol (silica gel) and Lucefol (cellulose) ready-made 15 × 15 cm sheets (Kavalier, Votice, Czechoslovakia). Experiments were also carried out with Kieselgel 60 "Fertigplatten" (E. Merck, Darmstadt, Germany) for comparison. The layers were impregnated by the dipping technique. The following solutions were used for impregnation: 1–40% solutions of dimethylformamide in ethanol, 1–20% solutions of formamide in ethanol, a 5% solution of 1-bromonaphthalene in chloroform, a 10% solution of liquid paraffin in *n*-hexane and 10% solutions of octan-1-ol or dodecan-1-ol in ethanol. After immersing the layers into the solutions, the sheets were dried vertically by standing on a pack of filter paper (the edge with the starting line at the bottom) for 10 min so that any excess impregnating solution was removed and the auxiliary solvent evaporated. Nitrophenols were applied in 5–10 μg amounts dissolved in ethanol or benzene. The development was carried out in glass tanks. The mobile phases used in the RP systems were always saturated with the stationary phase. Detection was carried out by exposing the developed and dried chromatogram to ammonia vapour for several seconds (until the appearance of a yellow colour).

The R_F values obtained on layers impregnated with formamide and the literature pK_A values of the nitrophenols are given in Table I. The behaviour of nitrophenols in RP systems was represented as "profiles" to enable a fast optical evaluation.

TABLE I

R_F AND pK VALUES OF NITROPHENOLS

Mobile phase: benzene–acetic acid (95:5).

No.	Phenol	pK_A [a]	R_F		
			TL_1 [b]	TL_2 [b]	TL_3 [b]
1	2-Nitrophenol	7.17	0.83	0.92	0.99
2	3-Nitrophenol	8.28	0.21	0.14	0.20
3	4-Nitrophenol	7.15	0.17	0.11	0.14
4	2,3-Dinitrophenol	4.96	0.24	0.10	0.14
5	2,4-Dinitrophenol	4.07	0.58	0.44	0.63
6	2,5-Dinitrophenol	5.2	0.68	0.70	0.92
7	2,6-Dinitrophenol	3.7	0.22	0.13	0.14
8	3,4-Dinitrophenol	5.42	0.11	0.09	0.10
9	3,5-Dinitrophenol	6.7	0.17	0.11	0.18
10	2,4,6-Trinitrophenol	0.38	0.33	0.04	0.00
11	2,4-Dinitro-6-methylphenol	4.70	0.76	0.85	0.97
12	2,4-Dinitro-3-methylphenol	4.0	0.20	0.11	0.18
13	2,4-Dinitro-6-*tert*.butylphenol	5.2	0.94	0.99	0.99
14	2,4-Dinitro-6-fluorophenol	2.40	0.54	0.10	0.09
15	2,4-Dinitro-6-chlorophenol	2.10	0.72	0.09	0.10
16	2,4-Dinitro-6-bromophenol	2.11	0.76	0.11	0.10
17	2,4-Dinitro-6-iodophenol	2.3	0.83	0.12	0.10
18	2,4-Dinitro-5,6-dimethylphenol	5.1	0.80	0.96	0.99
19	2,4-Dinitro-3,6-dimethylphenol	3.4	0.40	0.41	0.70
20	2,4-Dinitro-3,5-dimethylphenol	4.95	0.42	0.35	0.40
21	4-Nitro-2,6-dichlorophenol	3.1	0.31	0.13	0.30s
22	4-Nitro-2-chloro-6-bromophenol	3.1	0.35	0.14	0.25s
23	4-Nitro-2,6-dibromophenol	3.0	0.42	0.17	0.22
24	4-Nitro-2,6-diiodophenol	3.0	0.58	0.33	0.42s
25	2-Nitro-4,6-dichlorophenol	3.5	0.96	0.99	0.99
26	2-Nitro-4-chloro-6-bromophenol	4.8	0.97	0.99	0.99
27	2-Nitro-4,6-dibromophenol	5.4	0.97	0.99	0.99
28	2-Nitro-4,6-diiodophenol	2.4	0.98	0.99	0.99
29	2,4,6-Trinitro-3-methylphenol	–	0.35	0.06	0.02
30	2,4,6-Trinitro-3,5-dimethylphenol	–	0.41	0.09	0.10

[a] From Sergeant and Dempsey [11].

[b] TL_1 = Silica gel; TL_2 = silica gel impregnated with a 20% solution of formamide; TL_3 = cellulose impregnated with a 20% solution of formamide. s = Elongated spot.

RESULTS AND DISCUSSION

Straight-phase partition TLC with formamide as the stationary phase

This type of TLC using non-aqueous polar stationary phases was performed on layers of silica gel and cellulose impregnated with formamide. The impregnation was carried out with a 20% solution and established the conditions for the partition mechanism. Similar separations were obtained on both layers when benzene–acetic acid (90:5, v/v) was used as the mobile phase. The presence of acetic acid positively influenced the form of the spots of higher-acidity nitrophenols (tendency to tailing).

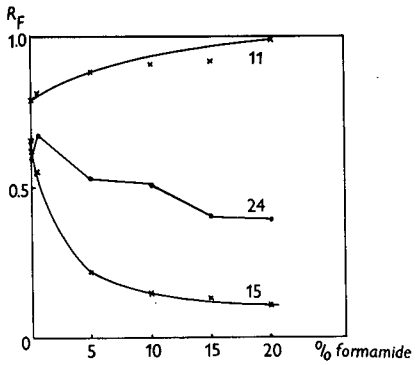

Fig. 1. Influence of degree of impregnation of silica gel with formamide on the mobility of nitrophenols. Mobile phase: benzene–acetic acid (95:5). See Table I for compound names.

The R_F values on silica gel were almost all lower than those on the cellulose layers. The same phenomenon had been observed previously for the phenoxyalkanoic acids and ascribed to adsorption [12]. Blanks on untreated layers using the same mobile phase showed that a very similar retention and separation of nitrophenols takes place on silica gel, whereas practically no interaction was observed with cellulose.

A certain amount of formamide is required in the layer to suppress the adsorption activity of the silica gel [13]. Therefore the response of individual nitrophenols to the gradual conversion of the adsorption mechanism into a partition mechanism was tested using chromatograms impregnated with 1–20% solutions of formamide. The results are summarized in Fig. 1. The R_F values of 2,4-dinitro-6-methylphenol increased due to deactivation of the silica gel, whereas a considerable decrease was seen in the R_F value of 2,4-dinitro-6-chlorophenol as a result of the increasing concentration of the stationary liquid. This must be taken into account if conditions for a pure partition system are to be established.

For comparison, 2-methoxyanthraquinone was chromatographed [14] on cellu-

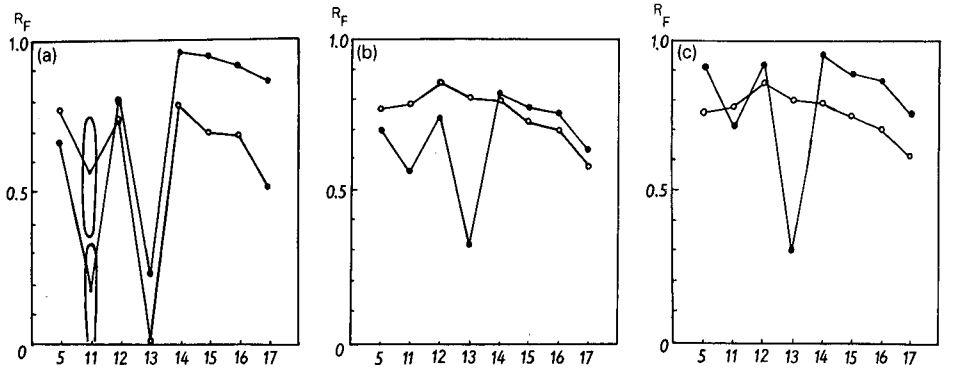

Fig. 2. Chromatograms on untreated layers of cellulose (○) or silica gel (●) using aqueous buffer solutions as mobile phases. (a) pH 3; (b) pH 8; (c) pH 10. See Table I for compound names.

lose and silica gel layers, both untreated and impregnated with 1–40% dimethylformamide using *n*-hexane as the mobile phase. The retention on untreated silica gel was strong (R_F 0.0) whereas on cellulose it was weak (R_F 0.70). A decrease of the R_F value was observed with increasing dimethylformamide content on cellulose owing to the gradual build-up of the partition system. On silica gel the R_F value increased under the same conditions as a result of successive deactivation of the sorbent and conversion to the partition system. At the highest degree of impregnation the R_F values were practically identical on both layers (0.25).

RP partition chromatography

Three types of less polar stationary phases were used to establish the conditions for RP chromatography: liquid paraffin, 1-bromonaphthalene and octan-1-ol (or dodecan-1-ol). Blank experiments were run for each mobile phase using untreated layers of both silica gel and cellulose to detect any interaction of the nitrophenols with the sorbent. Chromatograms using untreated layers and aqueous buffer solutions of pH 3, 8 and 10 as mobile phases gave results showing a strong interaction of some nitrophenols (especially of 6-alkyl-2,4-dinitrophenols) with silica gel over the whole pH range studied (see Fig. 2a–c). The sequence of spots for 2,4-dinitrophenol, its 6-methyl and 6-*tert.*-butyl derivatives as well as of the 6-fluoro, 6-chloro, 6-bromo and 6-iodo derivatives corresponds with that expected in RP chromatography. This is in agreement with the definition of RP chromatography of Bij *et al.* [15] according to which the use of silica gel layers with a neat aqueous or water-rich hydro-organic mobile phase also falls into the category of RP chromatography.

The interaction with cellulose at pH 3 is practically as strong as with silica gel; it is, however, only slight at higher pH values at which nitrophenols are supposed to be dissociated. This can be explained by the fact that only undissociated molecules are retained. The different behaviour on silica gel and cellulose can also be explained by the lower actual pH value on the silica gel layers. It was observed [16,17] that when using a buffer solution as the mobile phase the pH difference at the origin and near the mobile phase front can be two pH units as a result of sorption of the cations onto the Si–OH sites during the flow through the layer.

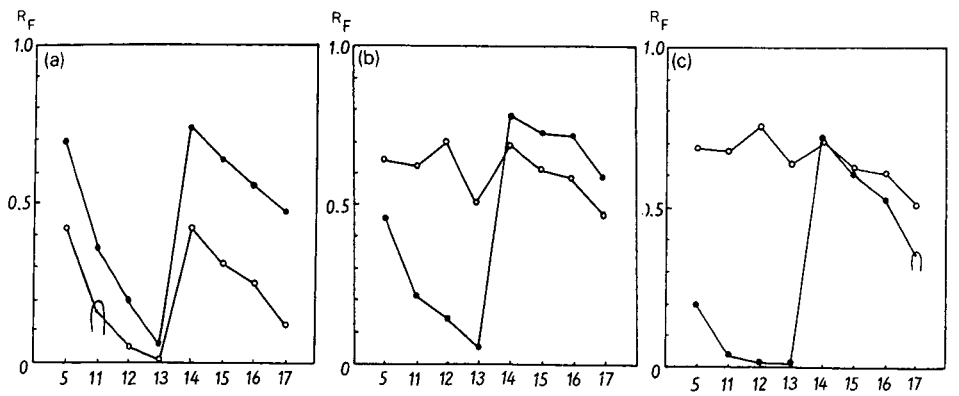

Fig. 3. Chromatograms on layers of cellulose (○) and silica gel (●) impregnated with octan-1-ol using aqueous buffer solutions as mobile phases. (a) pH 3; (b) pH 8; (c) pH 10. See Table I for compound names.

Impregnation with octan-1-ol brings about an increased retention on the silica gel layers which is practically identical at all three pH values studied. The retention on cellulose at pH 3 is stronger than that on the silica gel layers, but is very low at pH 8 and 10 (Fig. 3a–c).

Further experiments were carried out to compare liquid paraffin, 1-bromonaphthalene and octan-1-ol under the same conditions to determine whether the

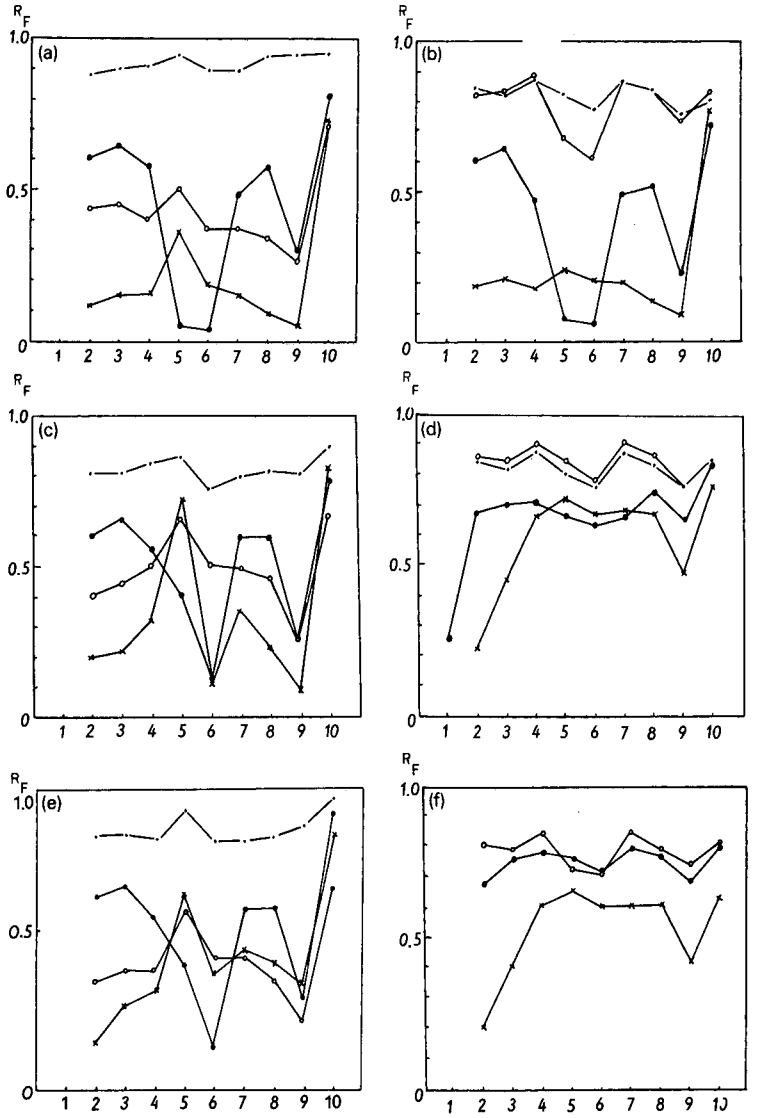

Fig. 4. Chromatograms on (---) untreated layers and impregnated layers with liquid paraffin (○), 1-bromonaphthalene (●) or octan-1-ol (x). Layers a, c, e = silica gel; b, d, f = cellulose. Mobile phases: a, b = buffer pH 3–ethanol (2:1); c, d = buffer pH 8–ethanol (2:1); e, f = buffer pH 10–ethanol (2:1). See Table I for compound names.

results can be influenced by the nature of the stationary phase. Ethanol was added to the mobile phase as an organic modifier to obtain reasonable R_F values. Mixtures (2:1) of the individual aqueous solutions with ethanol were always used saturated with the corresponding stationary phase. The addition of the organic modifier moved the spots on the untreated layers (blanks) to higher values. No significant difference between silica gel and cellulose was observed. The presence of the organic modifier suppressed the adsorption.

Results obtained for individual supports, stationary liquids and particular pH values of the mobile phases differ significantly (Fig. 4a–f). Thus, retention on layers impregnated with liquid paraffin is only observed on silica gel at pH 3, 8 and 10. On cellulose layers retention is observed only at pH 3; at pH 8 and 10 it is practically zero. The results on silica gel layers impregnated with octan-1-ol (dodecan-1-ol gave practically the same results) are similar to those of liquid paraffin. On cellulose layers there is a similar retention only at pH 3; there is practically no retention at pH 8 and 10 as seen with liquid paraffin.

The impregnation of silica gel layers with 1-bromoanphthalene and the mobile phase with pH 3 buffer gives a strong retention and the sequence of nitrophenols significantly differs from that obtained with other stationary phases. This indicates that another mechanism must be involved. This can be explained by charge-transfer complex formation, which is a known interaction between polycyclic hydrocarbons and nitro compounds often used in chromatography [18–20]. The change of mobile phase pH from 3 to 8 and 10 does not significantly change the behaviour of nitrophenols, with the exception of 2,4-dinitrophenol. The results for cellulose layers and the pH 3 mobile phase are practically identical with those obtained with silica gel; there is, however, decreased retention and separation efficiency at pH 8 and 10, similar to that seen with octan-1-ol and liquid paraffin as stationary phases. The application of 80% acetic acid as the mobile phase [8] results in analogous retention and separation on both silica gel and cellulose layers (see Fig. 5a and b).

A TLC system using impregnated layers is very complicated. Cellulose should be preferred when using formamide or dimethylformamide as the stationary phase if a pure partition process is required. For each particular analyte a certain amount of

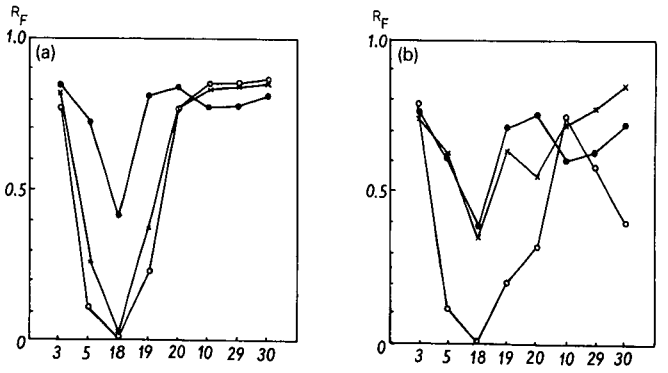

Fig. 5. Chromatograms on layers of (a) silica gel and (b) cellulose impregnated with 1-bromonaphthalene. Mobile phases: ● = 80% acetic acid; ○ = buffer pH 3–ethanol (2:1); x = buffer pH 8–ethanol (2:1). See Table I for compound names.

stationary phase is required to suppress the adsorption activity of the silica gel. RP-TLC results are strongly affected by the type of stationary phase (*e.g.* the possibility of charge-transfer complex formation with 1-bromonaphthalene) and its support (the acid properties of silica gel and its adsorption activity), the mobile phase (content of organic modifier, pH, presence of salts) and the properties of the solutes (polarity, ionizability). Mutual interactions between these constituents of the RP-TLC system can considerably affect the results.

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Isotachophoresis of proteins in uncoated open-tubular fused-silica capillaries with a simple approach for column conditioning

PETR GEBAUER[a] and WOLFGANG THORMANN*
Department of Clinical Pharmacology, University of Berne, Murtenstrasse 35, CH-3010 Berne (Switzerland)
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ABSTRACT

The isotachophoretic determination of proteins in uncoated open-tubular fused-silica capillaries of 50 and 75 μm I.C. with on-column multi-wavelength detection is reported. Small amounts of hydroxypropylmethylcellulose added to the leader provide an efficient method of dynamic column conditioning which allows the high-resolution isotachophoretic determination of most proteins to be performed in the presence of an electro-osmotic flow. Different approaches for cationic and anionic analyses are discussed and illustrated with selected examples.

INTRODUCTION

For more than 20 years, most isotachophoretic (ITP) analyses were performed in narrow-bore plastic tubes of 200–500 μm I.D. or separation channels of rectangular cross-section and with minimized electro-osmosis [1,2]. Modern capillary-type electrophoretic analysers feature fused-silica capillaries of 25–100 μm I.D. which can exhibit strong electro-osmotic flows. Work by Smith and co-workers [3–5], Hjertén and co-workers [6,7], Thormann and co-workers [8–11] and Beckers *et al.* [12] has shown that anionic and cationic ITP analyses can be performed in both untreated and coated open-tubular fused-silica capillaries, *i.e.* in the presence of and with minimized electro-osmosis, respectively. With these columns, most of the solutes investigated so far encompass low-molecular-mass compounds which show minimal interaction with the column walls.

In the ITP analysis of proteins, zones of high concentration (typically of the order of 10–20 mg/ml [8]) are formed. The proteins are vulnerable to interactions with the column walls. Not surprisingly, experiments with proteins in untreated fused-silica capillaries could not be reproduced, whereas much improved data were ob-

[a] Permanent address: Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, CS-611 42 Brno, Czechoslovakia.

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tained in capillaries coated with linear polyacrylamide [6–8] or with a bonded and cross-linked film of DB-17 [4,5]. The best capillary ITP results for proteins were obtained with PTFE capillaries [13]. This paper reports a simple approach for capillary conditioning which allows cationic and anionic analyses of proteins to be performed in untreated open-tubular fused-silica capillaries. ITP analysis with multi-wavelength detection is shown to represent a useful tool for the characterization of protein samples.

EXPERIMENTAL

Chemicals

All chemicals used were of research grade purity. Ribonuclease A (RNase) from bovine pancreas, carbonic anhydrase (CA) from bovine erythrocytes, γ-amino-*n*-butyric acid (GABA), 2-amino-2-methyl-1,3-propanediol (ammediol), β-alanine and hydroxypropylmethylcellulose (HPMC, No. 7509) were from Sigma (St. Louis, MO, USA). Ovalbumin (OVA) from chicken egg (5× crystallized, Lot No. 11840/D8), lysozyme (LYSO) from chicken egg white and conalbumin (CAL) were from Serva (Heidelberg, Germany). Bovine serum albumin (BSA) was purchased from Fluka (Buchs, Switzerland) and creatinine (CREAT), potassium acetate, formic acid and acetic acid were from Merck (Darmstadt, Germany). Ampholine (pH 3.5–10) was obtained from Pharmacia-LKB (Bromma, Sweden).

Electrolyte systems and sample preparation

For all the experiments presented here two electrolyte systems were used, a cationic system consisting of 0.01 *M* potassium acetate and acetic acid (pH_L 4.75) as the leader and 0.01 *M* acetic acid as the terminator, and an anionic system composed of 10 m*M* formic acid titrated with ammediol to pH_L 9.1 (leader) and 10 m*M* β-alanine–ammediol of pH 9.5 (terminator). In most instances 0.3% HPMC was added to the leaders. Sample components were dissolved in the leader without the addition of HPMC. Protein concentrations of 10–30 mg/ml were used.

Instrumentation and running conditions

The laboratory-instrument used in this work has been described previously [10]. It featured either a 50 or 75 μm I.D. fused-silica capillary of about 90 cm length (Polymicro Technologies, Phoenix, AZ, USA), together with a fast-scanning multi-wavelength detector (Model UVIS 206 PHD) and a capillary detector cell No. 9550-0155 (both from Linear Instruments, Reno, NV, USA). The effective separation distance was about 70 cm. Two 50-ml plastic bottles served as electrode vessels and a vacuum pump (Model VacTorr 150, CGA/Precision Scientific, Chicago, IL, USA) was used to rinse the capillary. Current was applied at a constant voltage (20 kV) with a Model HCN 14-20000 power supply (FUG Elektronik, Rosenheim, Germany). Sample application occurred manually via gravity through lifting the capillary end, dipped into the sample vial, some 34 cm for a specified time interval. Multi-wavelength data were read, evaluated and stored using a Mandax AT 286 computer system and running the 206 detector software package version 2.0 (Linear Instruments) with windows 286 version 2.1 (Microsoft, Redmont, WA, USA). Throughout this work the 206 detector was employed in the high-speed polychrome mode by scanning from

195 to 320 nm at 5-nm intervals (26 wavelengths). New capillaries were first rinsed with 1 *M* sodium hydroxide (20 min) and then 0.1 *M* sodium hydroxide (10 min). Before each run the capillary was cleaned with 0.1 *M* sodium hydroxide (10 min) followed by buffer insertion for at least 10 min.

RESULTS AND DISCUSSION

In the cationic mode, the cathode was on the detector side and the entire capillary was filled with leader prior to sample analysis. In this configuration both electro-osmotic and electrophoretic sample displacement occurred towards the detector. Experiments without the addition of a polymer to the solution were performed first. It was interesting to find that with the 50 μm I.D. capillary none of the six tested proteins could be monitored within 30 min after the application of a constant voltage of 20 kV. This is in contrast to the behaviour of low-molecular-mass compounds, which form sharp ITP zones and could be detected within a few minutes [9]. With a 75 μm I.D. capillary, the situation was improved. Some proteins, such as LYSO and CA, did reach the detector, but the sample zones were poorly defined and the elution times were unexpectedly high compared to low-molecular-mass constituents [10]; others, such as CAL and OVA, could not be detected within 40 min of current application. It is presumed that the proteins are adsorbed onto the capillary walls, thereby forming a coating which changes the surface charge and thus the electro-osmotic flow (reduction or even reversal of the net sample flux).

The addition of HPMC to the leader was found to allow the formation and migration of well developed cationic ITP zones of many proteins. The cationic ITP behavior of CA in the 75-μm capillary is shown in Fig. 1. Panel A depicts the three-

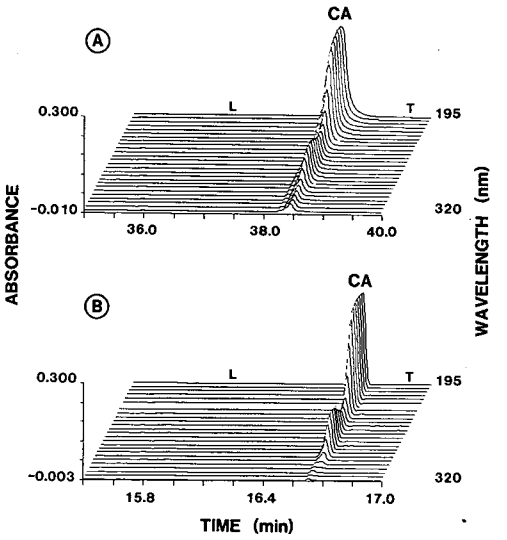

Fig. 1. Three-dimensional isotachopherograms of the cationic analysis of CA in a 75 μm I.D. fused-silica capillary (A) without and (B) with HPMC in the leader. Sample application occurred for 20 and 60 s, respectively, with about 10 mg/ml CA dissolved in the leader. The initial and final currents (at detection time) were 10 and 3, and 11 and 4 μA, respectively. L and T refer to leader and terminator, respectively.

dimensional data plot of CA without the addition of HPMC. A rather broad zone without sharp edges eluting after about 38 min of current application was monitored. With 0.3% HPMC in the leader, this zone is much better defined and elution occurred within 15–18 min (panel B), a time interval which is almost twice a high than that observed with low-molecular-mass compounds an in the absence of HPMC [10]. As the current densities are equal in the two approaches, HPMC obviously lowers the electro-osmotic flow. This effect was previously noted for 220 μm I.D. capillaries [14].

Fig. 2 shows three-dimensional electropherograms of a blank (panel A), of a model mixture composed of LYSO, CAL and OVA with the spacers CREAT and GABA (panel B) and of a commercial OVA sample which was spiked with two low-molecular-mass spacers (panel C). All these experiments were performed with 0.3% HPMC in the leader and sufficient sample was introduced to allow the formation of plateau zones in panels B and C. Sharp boundaries are formed which are marked by absorbance changes. Thus distinction between the zones is simple, even for the blank where small amounts of impurities mark the leader–terminator transition (panel A). With the model mixture (panel B), conditions for the ITP analysis of the three proteins were elucidated. LYSO and OVA were found to establish proper ITP zones, the lengths of which linearly increased with the addition of more sample.

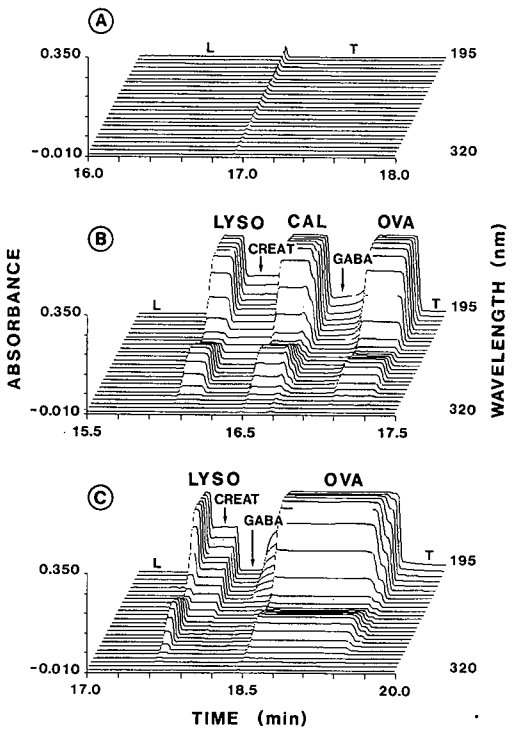

Fig. 2. Three-dimensional isotachopherograms for cationic analysis of (A) blank, (B) LYSO, CREAT, CAL, GABA and OVA and (C) OVA spiked with CREAT and GABA in a 75 μm I.D. capillary. The leader of all three experiments contained HPMC. The initial and final currents (at detection time) for the blank run were 12 and 2 μA, respectively.

CAL, however, behaved differently. Despite the presence of HPMC a loss of part of the protein was observed. This is particularly seen with the data shown in panel C. The commercial OVA product used in this study contained two major proteinaceous impurities, LYSO and CAL [15]. ITP analysis in a fused-silica capillary (as presented here) revealed the presence of LYSO only, whereas the same analysis in a PTFE capillary showed both proteins (date not shown; similar data are given in Caslavska *et al.* [15]).

With spacer compounds the purity of a protein can be assessed. As is shown in panel C of Fig. 2, the addition of a couple of well characterized, low-molecular-mass spacers can separate proteins by forming their own ITP zones. With sample detection over a range of wavelengths in one experiment, both protein and spacer zones are fully characterized. The identification of compounds via comparison of spectra, as is shown for OVA, LYSO and CREAT in Fig. 3, is possible. The presented spectra represent time slices extracted from the data of Fig. 2B. The described technique can also be used for the purity control or fractionation of proteins in the presence of a large number of spacer molecules, such as the carrier ampholytes manufactured for isoelectric focusing [13]. The three-dimensional electropherogram of RNase depicted in panel A of Fig. 4 shows that this protein forms an ITP zone with substantial impurites at its rear edge. These impurities are removed from RNase through the addition of carrier ampholytes to the sample (panel C). This analysis was only successful in the presence of HPMC and compared well with data obtained in a PTFE capillary (data not shown).

In the anionic mode protein ITP is predominantly executed at high pH. With the leader in the electrode vessel on the detector side, as well as initially throughout the entire capillary, and the anode on the detector side, no results are provided within 30 min of power application. Therefore the reversed buffer configuration reported previously for low-molecular-mass substances [9] was used. Initially, the capillary and the electrode vessel near the detector contained the terminator, whereas the leader was in the electrode compartment on the sample inlet side. The cathode was on the detector side. In this configuration electromigration and electro-osmosis occur in the opposite direction and the electro-osmotic flux has to be larger than that of electromigration (ITP) for the sample to reach the detector. Single-wavelength data for BSA obtained in a 50 μm I.D. capillary with no HPMC and in a 75 μm I.D. capillary with HPMC are depicted in Fig. 5A and B, respectively. In both instances BSA formed a concentration plateau and eluted rapidly as a result of strong electro-osmotic flows.

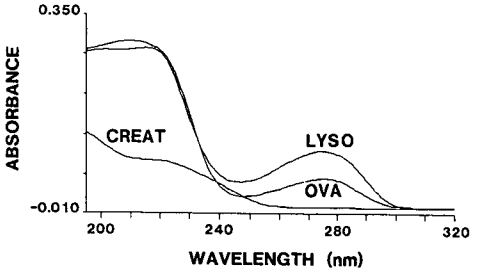

Fig. 3. Time slices of OVA, LYSO and CREAT extracted from the plateau zones of Fig. 2B.

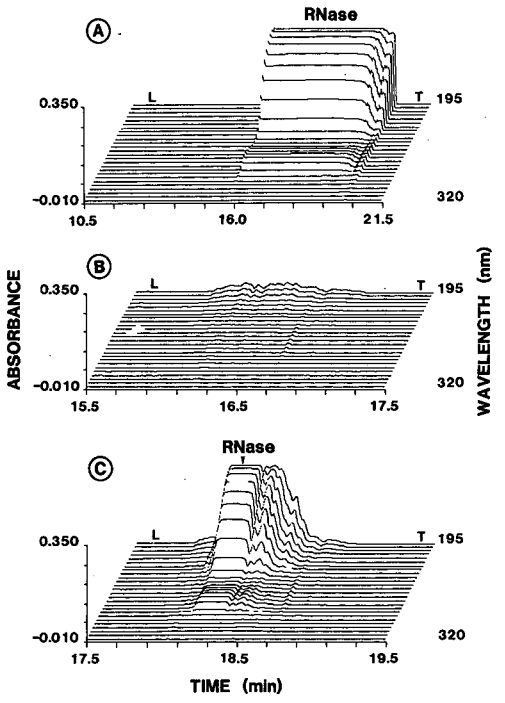

Fig. 4. Cationic isotachopherograms (75 μm I.D. capillary) in the presence of HPMC after (A) the injection of RNase (90 s) (B) a 1% (w/v) solution of pH 3.5–10 ampholine (30 s) and (C) RNase and ampholine (30 s each).

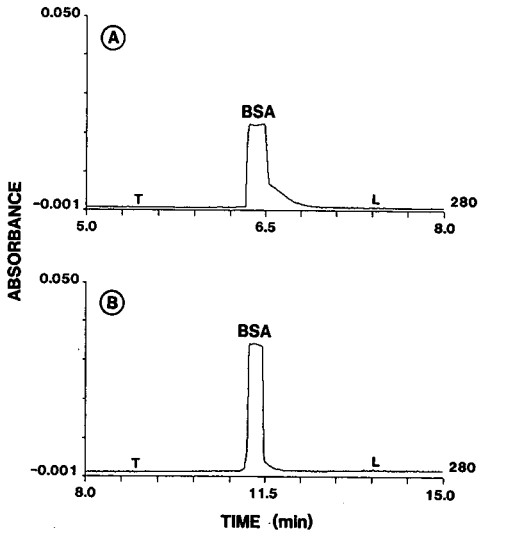

Fig. 5. Anionic ITP of BSA in (A) a 50 μm I.D. capillary without addition of HPMC and (B) in a 75 μm I.D. capillary with HPMC. The initial and final currents were 1 and 4, and 1 and 8 μA, respectively. Only pherograms obtained at 280 nm are displayed.

The difference in magnitude of absorbance is attributed to the difference in I.D. of the capillaries used. Comparison of zone shape reveals that the addition of the polymer does not drastically change the ITP behaviour of BSA, but prevents the formation of the tailing shown in panel A. Note that the rear ITP sample boundary (which passes the detector first) exhibits no tailing. This indicates that the observed dispersion is due to the net zone displacement towards the detector. Clearly, as a result of charge repulsion, anionic protein zones exhibit less interactions with the negatively charged fused-silica surface than the cationic proteins discussed earlier.

CONCLUSIONS

HPMC is believed to form a dynamic coating which drastically reduces the interaction between proteins and the walls as well as the electro-osmotic flow. The polymer is added to the leader in both cationic and anionic analyses so that the coating is renewed for each run. It provides a simple conditioning effect which allows the ITP analysis of many proteins (at the nanogram level) in plain open-tubular fused-silica capillaries of small I.D., *i.e.* in presence of an electro-osmotic flow. Fast-scanning polychrome detection is an interesting approach for the characterization (purity control) of protein zones.

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Short Communication

Separation of methylated and non-methylated cyanoginosin-LR homologues of the cyanobacterium *Microcystis aeruginosa* strain PCC 7806 by reversed-phase medium-pressure liquid chromatography

JOSEF CREMER*, HERMANN MEYER and KLAUS HENNING
Institute of Microbiology, Federal Armed Forces Medical Academy, Neuherbergstrasse 11, D-8000 Munich 45 (Germany)
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ABSTRACT

Cyanoginosins produced by *Microcystis aeruginosa* sp. are toxic cyclic heptapeptides with molecular weights of about 1000 dalton. *Microcystis aeruginosa* strain PCC 7806 proved to synthesize a methylated and non-methylated cyanoginosin-LR. A reversed-phase medium-pressure liquid chromatographic method was developed to separate the two cyanoginosin homologues on a preparative scale. The influence of chromatographic conditions on the separation such as solvent system, gradient parameters, flow-rate and temperature was investigated systematically. After optimization, up to 1.5 mg of the two compounds could be separated completely within 40 min.

INTRODUCTION

The formation of toxic cyanobacterial water-blooms in freshwater ecosystems and in drinking-water supplies is a well documented problem [1–4]. The most important organism in this context is the species *Microcystis aeruginosa*, which can produce highly stable hepatotoxins, termed cyanoginosins. The cyanoginosins are cyclic peptides with molecular weights of about 1000 dalton. Regularly these peptides are composed of five constant amino acids (*i.e.*, D-Glu, β-methyl-Asp, D-Ala, N-methyldehydro-Ala and ADDA, an atypical β-amino acid which is a 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid) and two strain-dependent variable L-amino acids [5]. Recently it became obvious that the hitherto constant part of the molecule can also exhibit some structural variations that influence toxicity [6,7].

The object of our investigations, *M. aeruginosa* strain PCC 7806, proved to synthesize cyanoginosin-LR (L = L-Leu, R = L-Arg) in two forms. Amino acid analysis confirmed that their difference consists only in methylation or non-methyla-

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tion of the D-Asp residue [8]. For toxicological and immunological studies we tried to separate these two homologues by preparative reversed-phase medium-pressure liquid chromatography (MPLC; maximum pressure = 40 bar). Results of an optimization procedure for the reversed-phase MPLC are presented, establishing that this method is a valuable supplement to separation techniques for small amphiphilic peptides.

EXPERIMENTAL

Cyanobacteria

M. Aeruginosa strain PCC 7806 was grown in continuous cultures supplied with BG-11 medium [9] and illuminated with 2000-lux fluorescent light.

Toxin isolation

Algal dry mass was obtained from the cultures by centrifugation (3000 *g*, 30 min) and lyophilization. After suspension in doubly distilled water, the cyanobacterial cells (5 g dry mass per 500 ml) were extracted by exposure to ultrasonics and centrifugation at 30 000 *g* for 30 min. For the solid-phase extraction, the supernatant was applied to a column (7.5 × 1.6 cm I.D.) containing 10 g of C_{18} silica gel (Baker Bond C_{18}; J. T. Baker, Gross-Gerau, Germany). Hydrophilic components were washed out with 100 ml of water. After elution with methanol, the toxic fraction was evaporated to dryness and the residue was dissolved in 200 ml of methanol–water (30:70, v/v). Precipitated lipids were removed by centrifugation (30 000 *g*, 30 min) and filtration (0.22 μm). At a flow-rate of 2 ml/min, the toxin solution was then pumped through a column (4.8 × 1.6 cm I.D.) containing 5 g of quaternary methylamine anion-exchange resin (QMA; Waters–Millipore, Eschborn, Germany) activated with 0.1 *M* ammonium hydrogencarbonate [in methanol–water (30:70, v/v)]. The loaded column was washed with 100 ml of methanol–water (30:70, v/v) and subsequently the cyanoginosin was eluted with 100 ml 0.02 *M* ammonium hydrogencarbonate [in methanol–water (30:70, v/v)] [10]. After drying, the toxin was dissolved in methanol–water (30:70, v/v) and photometrically adjusted to a concentration of 3 mg/ml with the aid of a standard calibration graph (cyanoginosin-LR; Medor, Herrsching, Germany).

Preparative reversed-phase MPLC

The separation studies of the two cyanoginosin homologues were performed on a fast protein liquid chromatography (FPLC) system (Pharmacia-LKB, Freiburg, Germany) of the following configuration: LCC-500 plus gradient controller connected with FPLC Manager software, two P-500 Model pumps, MV-7 motor injection valve, 500-μl sample loop, VWM 2141 variable-wavelength detector and FRAC-100 fraction collector. As stationary phase a HR 16/10 PepRPC column (10 × 1.6 cm I.D.), packed with 15-μm silica particles with covalently bonded C_2/C_{18}, average pore size 100 Å (Pharmacia–LKB), was used. Solvents for the gradient formation (solvents A and B) were different mixtures of acetonitrile (LiChrosolv; Merck, Darmstadt, Germany) and water purified with a Milli-Q system (Millipore, Eschborn, Germany). Depending on the experiment, trifluoroacetic acid (TFA) or pentafluoropropionic acid (PFPA) (Merck) were added to solvents A and B as ion-pairing agents.

TABLE I

INFLUENCE OF GRADIENT START, GRADIENT SLOPE AND FLOW-RATE ON THE MEAN RETENTION TIME (t_R) AND THE RESOLUTION (R_s)

Conditions	Gradient[a]		Flow-rate (ml/min)											
	Start (%)	Slope (%/min)	8		4		2		5		3		1	
			R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)
(a)	32	0.32	0.88	19	1.10	28	0.83	41						
	32	0.16	0.93	24	1.15	37	1.20	56						
	32	0.08	0.96	28	1.14	47	1.26	74						
(b)	40	0.00							0.84	11	0.97	19	1.15	55
	36	0.00							1.07	21	1.14	35		
	32	0.00							1.05	60	1.99	99		
(c)	36	0.10							1.06	20	1.08	29	1.12	69
	35	0.10							1.00	22	1.08	34		
	34	0.10							1.13	26	1.20	39		

[a] The percentage values refer to the acetonitrile concentration.

TABLE II

INFLUENCE OF TEMPERATURE ON RETENTION TIME AND RESOLUTION

Conditions: gradient start, 34% acetonitrile; slope, 0.1%/min; flow-rate, 3 ml/min.

Temperature (°C)	Retention time (min)		Peak width at half peak height (min)	R_s
	t_{R1}	t_{R2}		
24.8	36.2	39.4	1.42	1.18
13.3	35.6	39.4	1.40	1.42
9.3	35.0	39.2	1.42	1.51
4.3	34.2	38.6	1.42	1.57

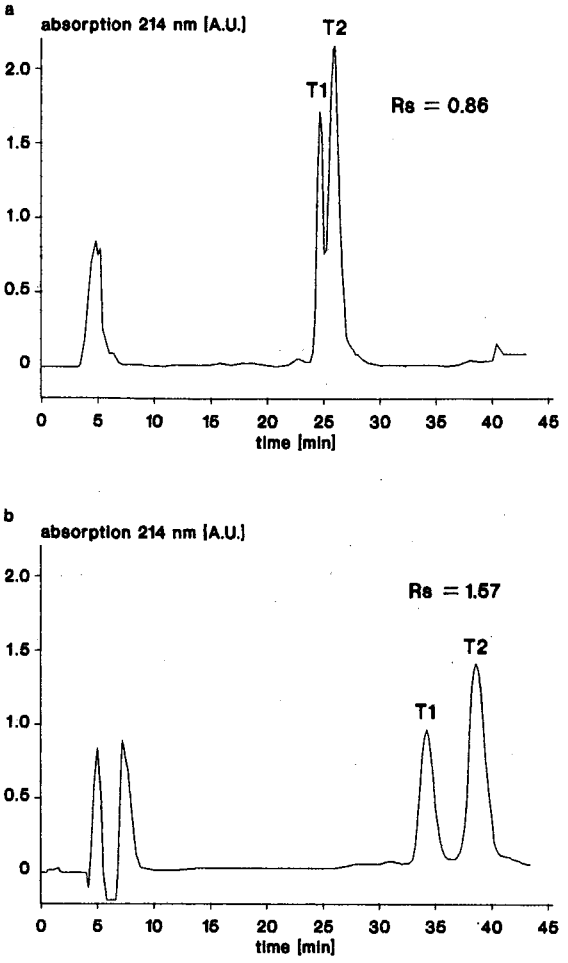

Fig. 1. Separation of the non-methylated (T1) and methylated (T2) cyanoginosin-LR homologues on an HR 16/10 PepRPC MPLC column. Sample, 1.5 mg of peptide mixture in 500 μl of methanol–water (30:70, v/v). (a) Starting conditions: gradient start, 30% acetonitrile; slope, 0.33%/min; flow-rate, 4 ml/min; temperature, 20°C; solvent A, Milli-Q-purified water containing 0.1% of TFA; solvent B, acetonitrile containing 0.1% of TFA. (b) Optimized method: gradient start, 34% acetonitrile; slope, 0.10%/min; flow-rate, 3 ml/min; temperature, 4.3°C, solvent A, acetonitrile–Milli-Q-purified water (24:76, v/v) containing 0.3% of PFPA; solvent B, acetonitrile–Milli-Q-purified water (50:50, v/v) containing 0.3% of PFPA.

RESULTS AND DISCUSSION

Apart from non-UV-absorbing contaminants, a *ca.* 90% pure cyanoginosin preparation was isolated by QMA-anion exchange chromatography. C_{18} thin-layer chromatography clearly demonstrated that the cyanoginosin fraction isolated from *M. aeruginosa* PCC 7806 is made up of two very similar compounds (data not shown). With the aim of isolating these two compounds by preparative reversed-

phase MPLC methods, the chromatographic conditions were investigated systematically and the success was verified by calculation of the resolution (R_s) [11].

In the first step, the concentration of the organic phase in which the two compounds were eluted from the column was determined in the range between 30 and 40% acetonitrile (Fig. 1a). Simply by changing the ion-pairing agent from 0.1% TFA to 0.3% PFPA, the resolution was increased successively from 0.86 to 1.11. This was mainly due to an extended retention time and increased selectivity (data not shown).

The influence of the gradient parameters and the flow-rates on the separation is presented in Table I. The resolution was increased to 1.26 by decreasing the flow-rate and running very shallow acetonitrile gradients [Table I (a)]. Isocratic runs in this range delivered the highest resolution (R_s = 1.99) but in a very long time [99 min, Table I (b)]. In consideration of these data, the influence of different gradient start points and flow-rates at a constant low gradient slope of 0.1%/min on the separation was investigated; as a result, a resolution of 1.20 could be achieved within 40 min [Table I (c)].

The decisive step for the optimization of the separation conditions was the variation of the temperature (Table II). By decreasing the temperature from 24.8 to 4.3°C, the R_s value increased from 1.18 to 1.57. This effect is caused by shortening the retention of the first peak without influencing the peak width, thus strongly increasing the selectivity. A typical chromatogram recorded under these optimized conditions is presented in Fig. 1b. The R_s value of 1.57 indicates that the two compounds are baseline separated according to the mathematical definition of resolution [11].

The results show that reversed-phase MPLC is a powerful method for the preparative separation of cyanoginosin homologues. The excellent performance of the preparative MPLC column, which exhibits a high loading capacity, a high selectivity but a low flow resistance, permits the complete separation of up to 1.5 mg of the peptides, which only differ in one methyl group, within 40 min. This may be an important result for further toxicological and immunological studies.

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Short Communication

High-performance liquid chromatographic separation and determination of small amounts of 2-naphthaleneacetic acid in 1-naphthaleneacetic acid[a]

SAJID HUSAIN*, P. NAGESWARA SARMA, N. S. SWAMY, S. N. ALVI and R. NAGESWARA RAO

Analytical Chemistry Division, Indian Institute of Chemical Technology, Hyderabad - 500 007 (India)

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ABSTRACT

A simple and rapid high-performance liquid chromatographic method was developed for the separation and determination of small amounts of 2-naphthaleneacetic acid in 1-naphthaleneacetic acid. The separation was achieved on a reversed-phased μBondapak CN column using 0.15 *M* ammonium sulphate–2-propanol (97.5:2.5, v/v) as the eluent. Reaction mixtures collected during the course of condensation of naphthalene with monochloroacetic acid were analysed by the proposed method and the yields of 1-naphthaleneacetic acid were monitored. The limit of detection of 2-naphthaleneacetic acid in a large excess of 1-naphthaleneacetic acid was $3 \cdot 10^{-9}$ g.

INTRODUCTION

1-Naphthaleneacetic acid (1-NAA) is an important plant growth regulator used not only to prevent the preharvest drop of apples but also as a fruit thinning agent [1,2]. It is manufactured generally by the alkylation [3,4] of naphthalene with acetic anhydride or by the condensation of naphthalene with monochloroacetic acid (MCA). Depending on the conditions, a mixture of 1-NAA, 2-NAA and di-, tri- and tetraacetic acid is obtained. High-purity 1-NAA is often required in vegetable cultivation. It is applied along with a recommended concentration of 2-NAA in controlling grape tendril atrophy [5]. Therefore a simple method is needed for the determination of 1-NAA and 2-NAA in mixtures.

No single method for the determination of 2-NAA in 1-NAA appears to have been reported. However, several methods [6,7] for the determination of 1-NAA in

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apples, grapes and oranges are available. Recently, Bertrand *et al.* [8] separated naphthaleneacetic acids by derivatizing them with cyanoethyldimethylsilane followed by gas chromatography–mass spectrometry. Ion-pair reversed-phased thin-layer chromatography and liquid chromatography (RP-LC) are used extensively [9,10] for the separation of naphthaleneacetic and sulphonic acids. Ion-pairing reagents such as tetrabutylammonium dihydrogenphosphate and cetyltrimethylammonium bromide are added to the mobile phase containing acetonitrile or methanol during RP-LC. However, using these reagents it is observed [11] that the peaks are often not only split but also show irreproducible shapes. Jandera and co-workers [12–14] have successfully overcome this problem by using solutions of inorganic salts as mobile phases for the separation of naphthalenesulphonic acids. They also compared the retention behaviours of various naphthalenesulphonic acids and concluded that the separation using inorganic salts is superior to that in systems containing ion-pair reagents [15]. Several workers [16–18] have also recommended the use of eluents containing inorganic salts for the separation of aromatic sulphonic and carboxylic acids.

In this paper, we describe a simple and rapid high-performance liquid chromatographic (HPLC) method for the separation and determination of 1-NAA and 2-NAA in standard and reaction mixtures using a μBondapak CN 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. Glass-distilled water, 2-propanol (Spectrochem, Bombay, India), 1-NAA and 2-NAA (Fluka, Buchs, Switzerland), ammonium sulphate (BDH, Poole, UK) and sodium 3-nitrobenzenesulphonate (3-NBSS) (Aldrich, Milwaukee, WI, USA) were used.

Apparatus

A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) with a 20-μl loop injector having a high-pressure six-way valve was used with a Model SPD-6AV variable-wavelength UV–VIS spectrophotometric detector (Shimadzu). A μBondapak CN (10 μm) column (300 mm × 3.9 mm I.D.) (Waters Assoc., Milford, MA, USA) was used for separation. The chromatograms and the integrated data were recorded by a Chromatopac C-R3A processing system.

Chromatographic conditions

The mobile phase was 0.15 *M* ammonium sulphate–2-propanol (97.5:2.5, v/v). Samples were dissolved in the mobile phase. The analysis was carried out under isocratic conditions at a flow-rate of 1 ml/min and a chart speed of 2.5 mm/min at room temperature (27°C). Chromatograms were recorded 223 nm.

Analytical procedure

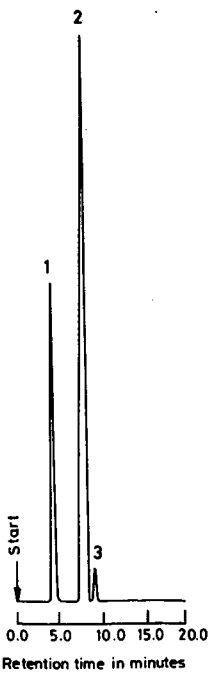
Standard mixtures containing 5 mg of internal standard, 27–30 mg of 1-NAA and 0.3–3.0 mg of 2-NAA were prepared by dissolving known amounts of the compounds in 25 ml of the mobile phase. A 5-μl volume of each standard mixture was injected and chromatographed under the above conditions. From the peak areas, the

response factors of 1-NAA and 2-NAA with respect to the internal standard were calculated.

Standard and reaction mixtures were analysed under identical conditions. The reaction mixture (25 mg) together with the internal standard (10 mg) was dissolved in 25 ml of the mobile phase and chromatographed. The percentage of 1-NAA and 2-NAA were calculated from the peak areas.

RESULTS AND DISCUSSION

The HPLC separation of 1-NAA, 2-NAA and 3-NBSS is shown in Fig. 1. The peaks were identified by injecting the individual compounds. It can be seen that 1-NAA is well resolved not only from its positional isomer 2-NAA but also from 3-NBSS used as an internal standard. The conditions for HPLC separation were optimized using three different stationary phases, *viz.*, μBondapak C_{18}, C_8 and CN, and eluents containing acetonitrile, methanol and 2-propanol. The μBondapak CN cyano-bonded reversed-phase column with 0.15 *M* ammonium sulphate–2-propanol (97.5:2.5, v/v) was selected over other systems not only because of the better separation obtained between 1-NAA and 2-NAA but also because the peak shapes were undistorted and reproducible.



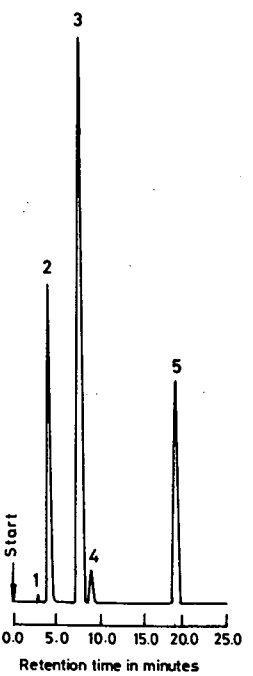


Fig. 1. Chromatogram of a typical mixture containing 1-NAA (1.2 μg), 2-NAA (0.1 μg) and 3-NBSS (6.1 μg). Peaks: 1 = 3-NBSS; 2 = 1-NAA; 3 = 2-NAA.

Fig. 2. Chromatogram of a reaction mixture (10 μg) together with internal standard (6.1 μg). Peaks: 1 = MCA; 2 = internal standard; 3 = 1-NAA; 4 = 2-NAA; 5 = naphthalene.

TABLE I

RETENTION DATA

Compound	Retention time (min)	k'	α	λ_{max} (nm)
1-Naphthalenecarboxylic acid	5.90	0.84	2.36	211
2-Naphthalenecarboxylic acid	9.54	1.98		222
1-Naphthaleneacetic acid	7.65	1.39	1.30	222
2-Naphthaleneacetic acid	9.00	1.81		223
1-Naphthalenesulphonic acid	7.90	1.47	1.53	225
2-Naphthalenesulphonic acid	10.39	2.25		226
1-Amino-naphthalene	12.09	2.78	1.12	236
2-Amino-naphthalene	13.19	3.12		235
Naphthalene 1-acetate	14.46	3.52	1.06	219
Naphthalene 2-acetate	14.92	3.72		219
1-Naphthalenol	22.35	5.98	1.05	219
2-Naphthalenol	23.26	6.27		221
Naphthalene	18.44	4.76	–	222
Sodium 3-nitrobenzene-sulphonate	4.02	0.26		260

A series of 1- and 2-substituted naphthalenes such as 1- and 2-naphthalenecarboxylic acid were also subjected to the HPLC separation under the same conditions. The wavelengths of maximum absorption and the retention data for all the compounds are given in Table I. A wavelength of 223 nm, where the compounds under investigation absorb UV radiation, was selected for detection. When the UV detector was set at 0.001 a.u.f.s., the limit of detection for 2-NAA in a large excess of 1-NAA was found to be $3 \cdot 10^{-9}$ g with a signal-to-noise ratio of 4.0. The response factors for 1-NAA and 2-NAA in the concentration ranges 90–100% and 1–10%, respectively, were determined and are given in Table II. They were found to remain constant throughout out these ranges.

Standard mixtures containing different amounts of 1-NAA and 2-NAA were prepared and analysed by HPLC. The results are given in Table III. The accuracy of the method was determined by the standard addition technique. Subsequent additions of 1-NAA and 2-NAA were accurately reflected in their peak heights. The measured amounts of 1-NAA and 2-NAA agreed well with the actual values, to within 1.59% and 2.34%, respectively. Linear regression analysis of the data with

TABLE II

RESPONSE FACTORS OF 1-NAA AND 2-NAA

Compound	Response factor[a] (mean, n = 10)	S.D.	R.S.D.[b] (%)
1-NAA	0.0865	0.0017	1.9653
2-NAA	0.0503	0.0014	2.7834

[a] With respect to 3-NBSS used as internal standard.

[b] Relative standard deviation.

TABLE III

ANALYTICAL DATA FOR STANDARD MIXTURES

Sample No.	1-NAA (%)		Error (%)	2-NAA (%)		Error (%)
	Taken	Found[a]		Taken	Found[a]	
1	90.18	90.64	+ 0.51	9.82	9.59	− 2.34
2	91.53	92.41	+ 0.96	8.47	8.63	+ 1.89
3	94.79	93.28	− 1.59	5.21	5.12	− 1.73
4	97.25	96.49	− 0.78	2.75	2.80	+ 1.82
5	98.97	99.32	+ 0.45	1.03	1.05	+ 1.94

[a] Average of three determinations.

x = percentage of the component taken and y = percentage of the component found yielded the equations $y = 0.9884x + 0.9387$ and $y = 0.9869x + 0.5361$ with correlation coefficients of 0.9359 and 0.9980 for 1-NAA and 2-NAA, respectively.

Reaction mixtures were collected during the course of condensation of naphthalene with MCA and analysed by the proposed method. A typical chromatogram of the reaction mixture is shown in Fig. 2. The unreacted naphthalene and MCA do not interfere as they elute at 18.44 and 3.26 min, respectively. These results show that the procedure is suitable not only for determining trace amounts of 2-NAA in 1-NAA but also for the separation and determination of several 1- and 2-substituted naphthalenes. The method is simple, rapid and convenient for the quality control naphthaleneacetic acids.

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Short Communication

Reversed-phase separations of high-molecular-weight polystyrenes in a dichloromethane–acetonitrile solvent system

ROSS ANDREW SHALLIKER* and PETER EDWIN KAVANAGH
Chemical and Analytical Sciences, Deakin University, Waurn Ponds, Victoria 3217 (Australia)
and
IAN MAXWELL RUSSELL
CSIRO, Division of Wool Technology, Belmont, Victoria 3216 (Australia)
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ABSTRACT

High-resolution separation of polystyrenes ranging from monomer and oligomers to the high-molecular-weight polymers (> 900 000 dalton) has been achieved in a single gradient separation on a variety of small-pore-size reversed-phase columns. Separation of the high-molecular-weight polymers in the acetonitrile–dichloromethane solvent system appeared to be based on retentive interactions with the stationary phase, despite the fact that the polymers were nominally too large to enter the pores of the small pore size column.

INTRODUCTION

In a previous publication [1] we examined the retention behaviour of polystyrenes in a methanol–dichloromethane solvent system on C_{18} reversed-phase columns of various pore sizes. Elution of the polystyrenes generally obeyed the principles of adsorption chromatography except when the polymer was excluded from the pores, at which point the elution was complicated by additional mechanisms which resulted in the polymer eluting at the polymer solubility composition according to the precipitation redissolution process, or prior to the polymer solubility composition by a process we have previously described as pre-elution. The process of pre-elution contributed to band broadening and the formation of multiple peaks of the higher-molecular-weight monodisperse polystyrenes on a 120-Å column and a 300-Å column. These effects resulted in virtually zero selectivity and limited the separation of molecular weights. In this methanol–dichloromethane solvent system, elution of the polystyrenes was also dependent upon the mass load and the flow-rate pre-elution being dominant at low mass loads and high flow-rates.

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In the present study we report the separations of polystyrenes of various molecular weights in a solvent system of acetonitrile and dichloromethane. By selection of gradient shape it is possible to achieve single-column separation of polystyrenes from monomer through to molecular weights of 10^6.

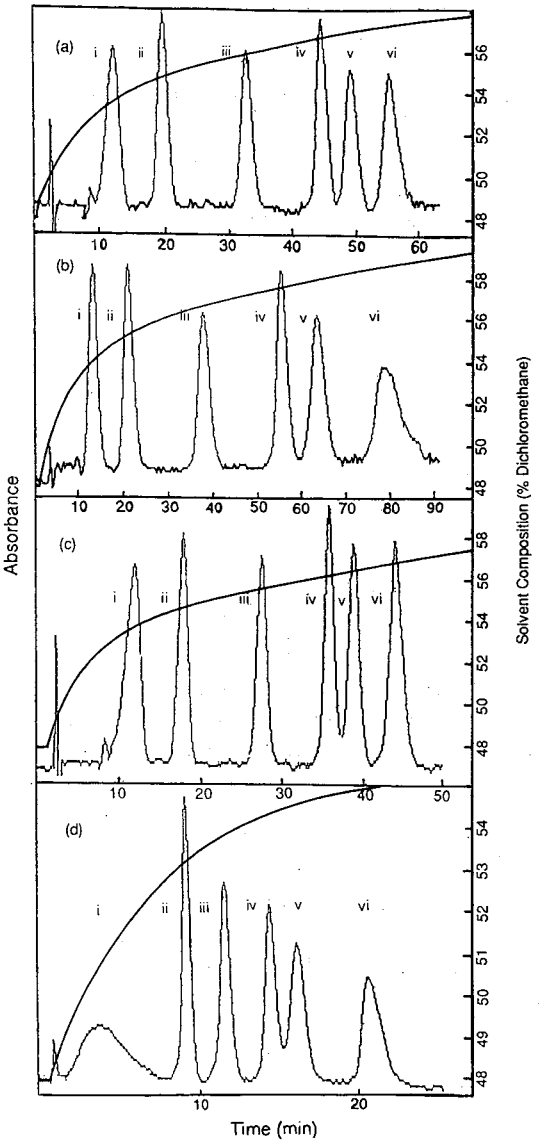

Fig. 1. Separation of polystyrenes on various columns. (a) μBondapak, (b) Serva, (c) Hibar 25 cm, (d) Hibar 12.5 cm. Molecular weights: i = 17 500; ii = 50 000; iii = 110 000; iv = 200 000; v = 410 000; vi = 929 000 dalton. Elution conditions: dichloromethane–acetonitrile (48:52); dichloromethane–acetonitrile (64:36); gradient run time 5 h on curve 3; flow-rate 1 ml/min.

EXPERMENTAL

Equipment

All chromatographic experiments were performed using two M6000A pumps, a 660 solvent programmer and U6K injector (Waters Assoc., Milford, MA, USA). The detector used was variable-wavelength UV–VIS 200 set at 262 nm (Activon Scientific Products, Thornleigh, Australia). Data acquisition and analysis was done with a laboratory-built system. The columns used were a μBondapak C_{18}, 30 cm × 3.9 mm I.D., nominal pore size 120 Å, carbon load 10% (Waters Assoc), a Serva 300 Å C_{18}, 5 μm particle size, 25 cm × 4.6 mm I.D., nominal pore size 300 Å, carbon load 11.6% (Serva, Heidelberg, Germany), and two Hibar C_{18} cartridge columns, of 25 cm and 12.5 cm column length, 4.0 mm I.D., 10 μm particle size, nominal pore size 60 Å (Merck). Column temperature was maintained at 25.0°C in a thermostatted water-jacket. The solvent composition at the peak maxima was calculated using the equation representing the gradient profile [2] and these values corresponded to the values obtained from gradient profiles.

Chemicals and reagents

Acetonitrile and dichloromethane (HPCL grade) were obtained from Mallinckrodt Australia. The monodisperse polystyrene standards used were molecular weights 3600, 110 000, 200 000, 410 000 and 929 000 dalton (Waters Assoc.), and 17 500 and 50 000 dalton (Polysciences, Warrington, PA, USA).

RESULTS AND DISCUSSION

Gradient elution of high-molecular-weight polystyrenes on small-pore-size columns in a methanol–dichloromethane solvent system generally produced peaks which had severe tailing or formed into multiple peaks [1]. In contrast, similiar experiments in a dichloromethane–acetonitrile solvent system produced gaussian like peaks. The initial separations were obtained on the same columns which were used for the previous work [1], and Hibar cartridge columns gave similiar results. Non-linear gradients gave best separations, beginning with an initial mobile phase composition of dichloromethane–acetonitrile (48:52) followed by convex gradient elution (NO. 3 on the Waters 660 solvent programmer) to a final mobile phase composition of dichloromethane–acetonitrile (64:36) using a gradient run time of 5 h. Elution of the highest-molecular-weight polystyrene occurred well within this time. Fig. 1 illustrates the separations on the various columns. It is obvious that these separations compare very favourably to size-exclusion chromatography [3–6] and field-flow fractionation [7,8]. Separation of broad-range polystyrenes also compared favourably with size-exclusion methods.

The optimal flow-rate for separations was found to be within range of 1–2 ml/min, although this may be an effect of the gradient function. High flow-rates (5 ml/min) had little affect on the resolution and elution of the highest-molecular-weight polystyrene was obtained within 16 min (Fig. 2).

The mobile phase elution composition φ_e, of all the polystyrenes was greater than that of the polymer solubility composition, φ_s (Table I). This is in strong contrast with the methanol–dichloromethane solvent system used in the previous paper

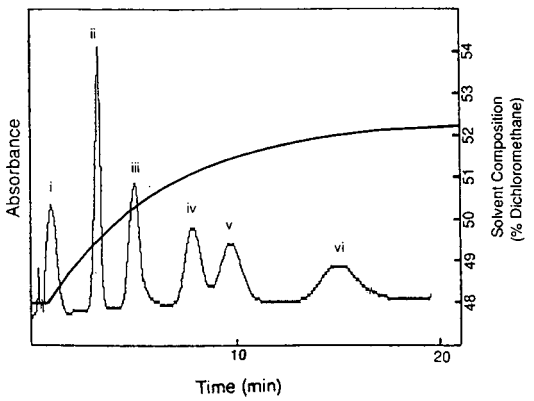

Fig. 2. Separation of polystyrenes on a Hibar 12.5 cm columns at a flow-rate of 5.0 ml/min. Elution order and conditions as in Fig. 1.

[1]. With the current solvent system it appears that polymer-stationary phase interactions were likely to be occurring even for the highest molecular weights. As the higher-molecular-weight polystyrenes would be excluded from the pores in all four columns in a good solvent such as dichloromethane, such interactions would only be expected if (a) the surface area outside the pores was sufficient for adsorption interactions to retain the polymer, (b) the polymer underwent solvent induced configurational changes, or (c) dissentanglement of polymer chains allowed retention by "snaking" into the pores. The latter mechanism was proposed in detail by Larman *et al.* [9] and was refered to as the "softball" model. Lochmüller and McGranaghan [10] have demonstrated that adsorption of the higher-molecular-weight polystyrenes is possible on small-pore-size columns as illustrated by the isocratic elution of these polystyrenes.

TABLE I

ELUTION COMPOSITION, φ_e (% DICHLOROMETHANE), OF THE POLYSTERENES ON THE μBONDAPAK, SERVA AND HIBAR 25 cm COLUMNS

φ_i (initial mobile phase composition): dichloromethane–acetonitrile (48:52); φ_f (final mobile phase composition): dichloromethane–acetonitrile (64:36); gradient curve 3, run time = 5 h; flow-rate = 1.0 ml/min. Polymer solubility composition φ_s, is also tabled.

Molecular weight	φ_e (%)			φ_s (%)
	μBondapak	Serva	Hibar	
929 000	56.98	58.13	56.17	53.0
410 000	56.62	57.40	55.83	51.0
200 000	56.31	56.96	55.63	48.5
110 000	55.38	55.71	54.90	46.5
50 000	53.88	53.97	53.71	39.5
17 500	52.43	52.47	52.45	28.5

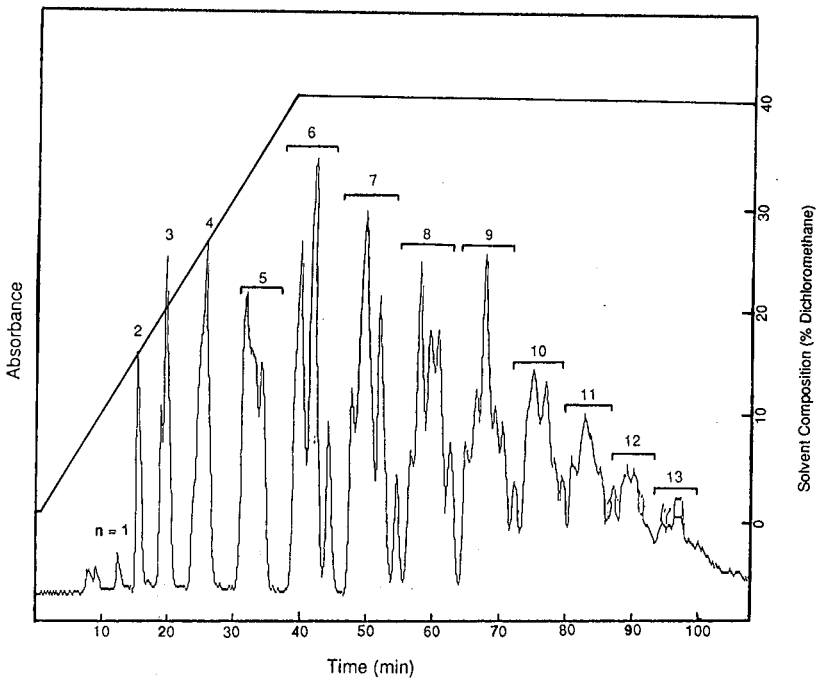

Fig. 3. Separation of oligomers of polystyrene molecular weight 800 on a Hibar 25 cm column. φ_i : 100% acetonitrile, φ_f : dichloromethane–acetonitrile (40:60); linear gradient at 1%/min; flow-rate 1 ml/min.

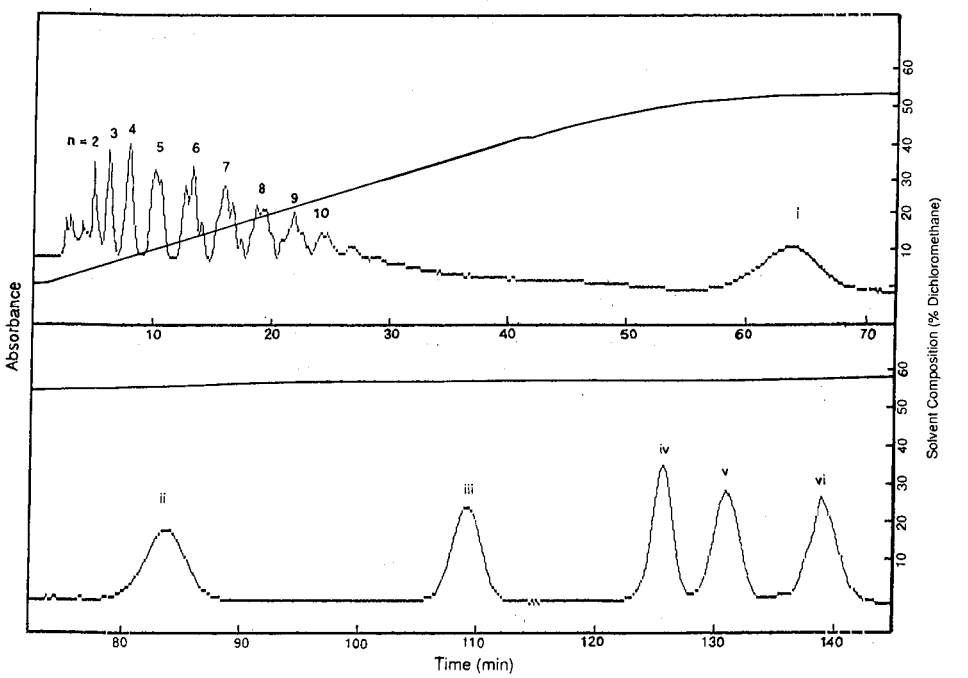

Fig. 4. Separation of polystyrene oligomers through to molecular weight 929 000. φ_i : 100% acetonitrile running to dichloromethane–acetonitrile (40:60) linear gradient at 1%/min followed by a 1-min hold then a convex gradient (curve 3) to a final mobile phase composition of dichloromethane–acetonitrile (66:34) in 6 h. Flow-rate was 1.0 ml/min. Sample concentration was 4 mg/ml for the oligomer 800 and 1 mg/ml for the molecular weights greater than 17 500. Injection volume 8 μl. Elution order as described in Fig. 1.

Studies showing the oligomeric separations of polystyrenes in various solvent systems were reported in detail by Lewis *et al.* [11]; oligomeric stereoisomers were shown to be separated in an acetonitrile–dichloromethane solvent system but not in a methanol solvent system. Similiar stereoisomer separations have also been achieved in the present study (Fig. 3). The stereoisomers were verified by mass spectroscopic examination of collected fractions. We were then able to combine the separation of the higher-molecular-weight polystyrenes with oligomeric separations in the same analysis. The final separation ranged from styrene monomer up to the 929 000-dalton polystyrene (Fig. 4). The gradient shape is somewhat complicated, although it would still be adaptable to any computer controlled system on a routine basis.

In the dichloromethane–acetonitrile solvent system the mass load of the polymer was not important. No change in the retention behaviour was observed for sample loads from 0.5 to 50 μg, in complete contrast to results obtained in the dichloromethane–methanol solvent system. This indicates that solvation effects within the polymer are playing an influential role in the dichloromethane–methanol system yet they do not affect the results to the same degree in the current dichloromethane–acetonitrile system.

As expected, injection in large volumes (greater than 40 μl of strong solvent, dichloromethane) gave poor results. Peaks size decreased as the injection volume increased. This was probably due to polymer eluting within the solvent front as described by Glöckner [12] and by Lochmüller and McGranaghan [10]. Studies to determine the mechanism of retention are in progress and will be published at a later date.

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Short Communication

Direct coupling of high-speed counter-current chromatography to thin-layer chromatography

Application to the separation of asiaticoside and madecassoside from *Centella asiatica*

B. DIALLO, R. VANHAELEN-FASTRÉ and M. VANHAELEN*
Laboratory of Pharmacognosy and Bromatology, Free University of Brussels, B205-4 Bd. du Triomphe, 1050 Brussels (Belgium)
(First received April 18th, 1991; revised manuscript received May 31st, 1991)

ABSTRACT

The direct coupling of high-speed counter-current chromatography to thin-layer chromatography is proposed as an efficient method for the on-line monitoring of the column effluent. It requires a simple adaptation of the fittings of a Linomat C device without modification of the software. An application to the separation of asiaticoside and madecassoside from a crude extract of *Centella asiatica* is presented.

INTRODUCTION

High-speed counter-current chromatography (HSCCC) has become a widely used method for the isolation of natural products [1]. Commercially available instruments are now very efficient, but simple and effective methods for the on-line monitoring of the column effluent are still lacking. For this purpose, UV, Fourier transform IR, thermospray mass spectrometry and evaporative laser-light scattering detection have been described, but all these methods have disadvantages which limit their application [2,3]; moreover, although in some instances they can provide structural information about the eluted compounds, they do not allow the control of the purity of the eluted fraction. Thin-layer chromatography (TLC) is a very attractive method for this last purpose as the solvent composition used in HSCCC is not a restricting factor and as it allows the combination of many chromatographic adsorbents to specific detection methods, including *in situ* UV spectrometry; however, the manual application of the column effluent on the plates is laborious, especially as the carry-over of the stationary phase, which frequently occurs in HSCCC, leads to

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the decantation of the solvent phases in the collected fractions. The combination of high-performance liquid chromatography (HPLC) with TLC has recently been reported using a sample spray-on apparatus [4]. We report here an adaptation of this device for the coupling of preparative HSCCC to TLC. The method of detection was applied to the separation of asiaticoside and madecassoside, the two main saponins from *Centella asiatica*; purified extracts of this medicinal plant are used to accelerate cicatrizing and grafting.

EXPERIMENTAL

Apparatus

HSCCC was performed using an Ito multi-layer coil separator–extractor [5] (PC. Inc., Potomac, MD, USA) equipped with a 66 m × 2.6 mm I.D. column (column capacity 350 ml). An LDC Milton Roy (Riviera Beach, FL, USA) minipump was used to pump the solvents through the column. The rotational speed was 800 rpm. A manual sample injection valve (Lobar Column Accessories, Merck, Darmstadt, Germany) equipped with a 10-ml loop was used to introduce the sample into the column. A flow splitter was inserted between the restrictor connected at the outlet of the column and the fraction collector. It was made up with a PEEK (polymer compatible with all solvents) three-ports fitting (Alltech, Laarne, Belgium); the original 0.30-mm throughholes of this tee were enlarged to 1.5 mm. Teflon tubing (30 mm × 0.25 mm I.D. × 1.6 mm O.D.) was used for the connection of the splitter to the syringe of a Linomat C device (Camag, Muttenz, Switzerland). Fractions of the eluent were collected using an LKB Ultrorac 7000. The flow diagram of the complete system is presented in Fig. 1.

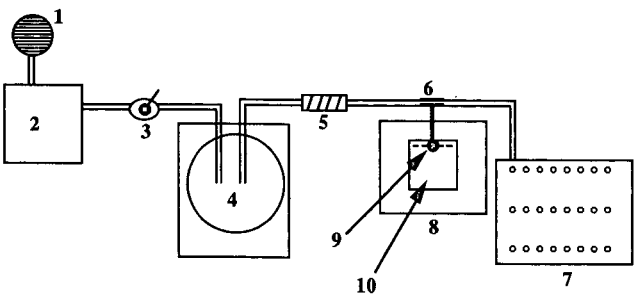

Fig. 1. Top view of the on-line coupling of high-speed counter-current chromatography with thin-layer chromatography. 1 = Mobile phase reservoir; 2 = pump; 3 = injector; 4 = counter-current chromatograph; 5 = restrictor; 6 = flow splitter; 7 = fraction collector; 8 = Linomat C device; 9 = syringe; 10 = chromatoplate.

Separation procedure

A two-phase solvent system was prepared by equilibrating chloroform–methanol–2-butanol–water (7:6:3:4, v/v) at room temperature. After separation, the two phases were degassed in an ultrasonic bath. The upper phase, used as the stationary phase, was pumped into the column at 6 ml/min. After sample injection and the start

of column rotation, the lower phase, used as the mobile phase, was pumped "head to tail" into the column at 4 ml/min.The separation was performed at room temperature and 80 fractions of 12 ml were collected. For the spraying of the effluent onto the silica gel plate, the settings of the Linomat C were as follows: band width, 5 mm; space, 7 mm; rate at which the effluent was aspirated by the syringe, 75 μl/min; peak window, 0.5 min; retention times, 1 min for the first fraction to be collected with an increment of 3 min for each following fraction; the run-time key was pressed when the mobile phase was coming out of the column (fraction 9 ± 1); gas (nitrogen) pressure, 4 bar.

Preparation of sample

Dried mother liquors obtained industrially after crystallization of asiaticoside extracted from the aerial parts of *Centella asiatica* L. were obtained from Syntex (Puteaux, France)). A 400-mg amount of this powder was dissolved in 10 ml of a 1:1 (v/v) mixture of the two phases of the solvent system and the solution was filtered before loading into the column.

Fractionation monitoring

Precoated silica gel $60F_{254}$ plates (10 × 20 cm) (Merck) were developed in an unsaturated tank (22 × 22 × 10 cm) with ethyl acetate–methanol–water (8:2:1, v/v/v) and detection was achieved by spraying a 3% methanolic solution of sulphuric acid and heating at 100°C for 5 min.

RESULTS AND DISCUSSION

The connection of a Camag device recently marketed for the on-line TLC detection in HPLC was modified in order to allow the coupling of HSCCC to TLC. A commercially available three-port fitting was used as a splitter; the through-holes were enlarged to avoid any back-pressure in the HSCCC column. The inside diameter of the tube connecting the splitter to the syringe of the Linomat C device was chosen to allow a suitable aspiration of a homogeneous sample of the effluent by the syringe and to reduce to a minimum the dead volume of the connection. In the proposed application, this dead volume (1.5 μl) represented only 4% of the sprayed volume (37.5 μl) and was negligible. It must be kept in mind that the dead volume could become inconvenient for some applications which require a mobile phase containing large amounts of water and, thus, low sprayed volumes to avoid excessive diffusion of the solution on the adsorbent; this band broadening could also arise when too low concentrations of the sample require high volumes to be sprayed for the subsequent monitoring of the fractionation.

In order to study the potential of the system, the separation of a mixture of triterpenic saponins from an extract of *Centella asiatica* was attempted. A very efficient solvent system was developed on the basis of the partition coefficients of the saponins between the two phases of several solvent systems. Despite the minor structural difference between asiaticoside and madecassoside, the saponins were separated without overlapping of the peaks. The retention of the stationary phase was 63%. Fig. 2 shows the separation obtained with the proposed direct coupling; in order to present a good illustration of the results on the same chromatoplate, the experimental

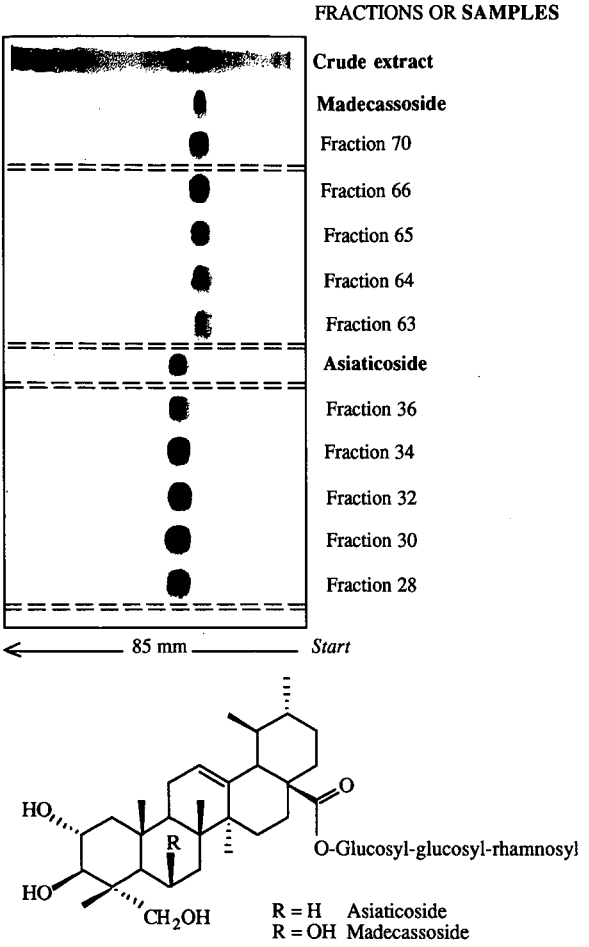

Fig. 2. HSCCC separation of madecassoside and asiaticoside from an extract of *Centella asiatica*. Experimental conditions: column, 66 m × 2.6 mm I.D.; sample size, 400 mg; solvent system, chloroform–methanol–2-butanol–water (7:6:3:4, v/v); mobile phase, lower non-aqueous phase; flow-rate, 4 ml/min; rotation speed, 800 rpm; detection, on-line effluent spraying on silica gel plate followed by TLC [solvent, ethyl acetate–methanol–water (8:2:1, v/v/v); detection, 3% methanolic solution of sulphuric acid and heating at 110°C for 5 min].

conditions related to the effluent spraying were modified in order to insert the bands corresponding to the standards and the starting sample and to reduce the track number. In usual experimental conditions, the apparatus software allows the running of the same effluent spraying program for several other chromatoplates.

The combination of the present method of detection with other detection systems such as on-line UV absorptiometry could improve the automation of the system. However, our results indicate that simple on-line TLC detection in HSCCC is a powerful and inexpensive method which avoid the tedious manual spotting of the numerous eluted fractions and which affords useful information about both peak purity and separation achievement.

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Short Communication

Capillary gas chromatographic analysis of oxo, oxo-hydroxy and unsaturated bile acid glycine conjugates

TAKASHI IIDA* and TAMAAKI TAMARU
College of Engineering, Nihon University, Koriyama, Fukushima-ken 963 (Japan)
FREDERIC C. CHANG
Department of Chemistry, Harvey Mudd College, Claremont, CA 91711 (USA)
and
JUNICHI GOTO and TOSHIO NAMBARA
Pharmaceutical Institute, Tohoku University, Aobayama, Sendai, Miyagi-ken 980 (Japan)
(First received April 2nd, 1991; revised manuscript received June 10th, 1991)

ABSTRACT

A method for the direct gas chromatographic analysis without prior hydrolysis has been developed for a series of glycine-conjugated bile acids having oxo or multiple functional groups, including oxo and hydroxyl groups and double bonds. The glycine conjugates without and with the hydroxyl groups were derivatized to their ethyl or methyl esters and ethyl ester-trimethylsilyl or methyl ester-dimethylethylsilyl ethers, respectively, and chromatographed on an aluminum-clad, flexible, fused-silica capillary column coated with a thin film (0.1 μm) of chemically bonded and cross-linked methyl polysiloxane. The change of methylene unit value exerted by derivatization and glycination for each compound is discussed.

INTRODUCTION

In previous papers [1,2], we have reported a simultaneous gas chromatographic (GC) analysis of the unconjugates and glycine conjugates of a series of hydroxylated bile acid stereoisomers as their ethyl ester-trimethylsilyl (Et-TMS) and methyl ester-dimethylethylsilyl (Me-DMES) ether derivatives on an aluminum-clad, flexible, fused-silica capillary column coated with a thin film (0.1 μm) of chemically bonded with non-selective methyl polysiloxane. A simple method for the micro-scale (< 1 mg) preparation of glycine-conjugated bile acid esters as authentic specimens from the corresponding unconjugates was also developed.

Since glycine-conjugated bile acids containing oxo or multiple functional groups, including oxo and hydroxyl groups and double bonds, are also potential metabolites [3], we are extending our GC approach to include these compounds. Thus the availability of a variety of oxo, oxo-hydroxy and unsaturated bile acids having

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one to four substituents at positions C-3, C-4, C-6, C-7 and/or C-12 has prompted us to compare the GC behavior of the glycine conjugates and the corresponding unconjugates reported previously [4].

EXPERIMENTAL

Samples and reagents

Almost all of the oxo, oxo-hydroxy and unsaturated bile acids related to 5α- and 5β-cholanoic acids, which differ from one another in the number, position and configuration of substituents in the molecules, were taken from our laboratory collections.

The silylating reagents, hexamethyldisilazane and trimethylchlorosilane in anhydrous pyridine (TMS-HT) and dimethylethylsilylimidazole (DMESI), were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), glycine methyl ester hydrochloride and glycine ethyl ester hydrochloride were obtained from Wako Pure Chemical Industries (Osaka, Japan). All solvents used were of analytical-reagent grade.

GC instrumentation and column

A Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector and data-processing system (Shimadzu Chromatopac C-R6A) was used. It was fitted with an aluminum-clad, flexible, fused-silica capillary column (25 m × 0.25 mm I.D.) with a thin film (0.1 μm) of bonded and cross-linked methyl polysiloxane (equivalent to OV-101) and operated under the following conditions: carrier gas (helium) flow-rate, 1.5 ml/min; auxiliary gas flow-rate, 40 ml/min; splitting ratio, 1:50; injection temperature, 330°C; and column temperature, 300°C (isothermal). The column, HiCap CBPM1, was purchased from Shimadzu (Kyoto, Japan).

Derivatization procedures

The Et-TMS and Me-DMES ether derivatives of glycine-conjugated bile acids as authentic specimens were prepared in two steps from their corresponding unconjugates by a combination of glycination followed by silylation [2]. Initially, oxo, oxo-hydroxy and unsaturated C_{24} bile acid samples were converted into their glycine conjugate ethyl (Et) or methyl (Me) esters by treatment with EEDQ, glycine ethyl (or methyl) ester hydrochloride and triethylamine in acetonitrile. Each of the glycine conjugate esters containing hydroxyl groups was then converted to the Et-TMS and Me-DMES ether derivatives using TMS-HT and DMESI as silylating reagents, respectively. An aliquot of the derivatized sample solutions was injected into the GC system together with an internal standard.

RESULTS AND DISCUSSION

Table I shows the retention data of 65 glycine-conjugated bile acids observed for the two classes of derivatization products on a HiCap CBPM1 column. Retention data were expressed as the relative retention time (RRT) and methylene unit (MU) values; RRT values were expressed relative to an appropriate derivative of glycodeoxycholic acid (GDCA: Et-TMS, 7.12 min; Me-DMES, 8.95 min) and MU values were

TABLE I

RRT AND MU VALUES OF THE Et-TMS AND Me-DMES ETHER DERIVATIVES OF OXO, OXO-HYDROXY AND UNSATURATED BILE ACID GLYCINE CONJUGATES[a]

Position and configuration of substituents	Et-TMS		Me-DMES		$\Delta[U_m]_{M-E}$
	RRT	MU	RRT	MU	
Monooxo[b]					
3-Oxo	1.05	39.12	0.69	38.50	−0.62
3-Oxo (5α)	1.14	39.56	0.76	39.00	−0.56
6-Oxo	0.94	38.62	0.63	38.02	−0.60
6-Oxo (5α)	1.06	39.17	0.71	38.60	−0.57
7-Oxo	0.89	38.33	0.59	37.73	−0.60
7-Oxo (5α)	1.01	38.99	0.68	38.41	−0.58
12-Oxo	0.84	38.09	0.56	37.49	−0.60
12-Oxo (5α)	0.93	38.58	0.62	37.98	−0.60
Dioxo[b]					
3,6-Dioxo	1.76	41.68	1.17	41.15	−0.53
3,6-Dioxo (5α)	1.76	41.68	1.17	41.15	−0.53
3,7-Dioxo	1.44	40.73	0.96	40.14	−0.59
3,7-Dioxo (5α)	1.66	41.42	1.10	40.86	−0.56
3,12-Dioxo	1.44	40.73	0.96	40.14	−0.59
3,12-Dioxo (5α)	1.62	41.29	1.07	40.72	−0.57
7,12-Dioxo	1.16	39.68	0.78	39.15	−0.53
7,12-Dioxo (5α)	1.37	40.47	0.91	39.90	−0.57
Trioxo[b]					
3,7,12-Trioxo	1.84	41.91	1.22	41.34	−0.57
3,7,12-Trioxo (5α)	2.17	42.73	1.45	42.18	−0.55
Monooxo-monohydroxy					
3-Oxo-6α-OH	1.39	40.57	1.17	41.15	0.58
3-Oxo-6β-OH	1.28	40.17	1.06	40.66	0.49
3-Oxo-7α-OH	1.28	40.17	1.04	40.56	0.39
3-Oxo-7α-OH (5α)	1.17	39.72	0.97	40.22	0.50
3-Oxo-7β-OH	1.75	41.70	1.43	42.09	0.39
3-Oxo-7β-OH (5α)	1.53	41.03	1.28	41.61	0.58
3-Oxo-12α-OH	1.16	39.68	0.92	39.97	0.29
3-Oxo-12α-OH (5α)	1.25	40.04	1.00	40.41	0.37
3-Oxo-12β-OH (5α)	1.25	40.04	1.00	40.41	0.37
6-Oxo-3β-OH (5α)	1.85	41.95	1.61	42.69	0.74
7-Oxo-3α-OH	1.38	40.53	1.15	41.08	0.55
12-Oxo-3α-OH	1.34	40.37	1.11	40.91	0.54
12-Oxo-3β-OH	1.34	40.37	1.15	41.08	0.71
12-Oxo-7α-OH (5α)	1.00	38.96	0.82	39.40	0.44
12-Oxo-7β-OH	1.15	39.62	0.96	40.14	0.52
12-Oxo-7β-OH (5α)	1.28	40.17	1.06	40.66	0.49
Monooxo-dihydroxy					
3-Oxo-7α,12α-$(OH)_2$	1.34	40.37	1.37	41.90	1.53
3-Oxo-7α,12α-$(OH)_2$(5α)	1.22	39.89	1.25	41.47	1.58
6-Oxo-3α,7β-$(OH)_2$ (5α)	1.46	40.80	1.48	42.30	1.50
7-Oxo-3α,6α-$(OH)_2$	1.38	40.53	1.43	42.09	1.56
7-Oxo-3α,12α-$(OH)_2$	1.49	40.89	1.47	42.27	1.38
12-Oxo-3α,7α-$(OH)_2$	1.57	41.15	1.59	42.61	1.46

(*Continued on p. 454*)

TABLE I (*continued*)

Position and configuration of substituents	Et-TMS		Me-DMES		$\Delta[U_m]_{M-E}$
	RRT	MU	RRT	MU	
12-Oxo-3α,7α-$(OH)_2$ (5α)	1.54	41.05	1.61	42.69	1.64
12-Oxo-3α,7β-$(OH)_2$	1.57	41.15	1.59	42.61	1.46
12-Oxo-3α,7β-$(OH)_2$ (5α)	1.61	41.28	1.66	42.85	1.57
12-Oxo-3β,7α-$(OH)_2$	1.39	40.55	1.42	42.06	1.51
12-Oxo-3β,7α-$(OH)_2$ (5α)	1.54	41.05	1.61	42.69	1.64
12-Oxo-3β,7β-$(OH)_2$	1.57	41.15	1.65	42.79	1.64
12-Oxo-3β,7β-$(OH)_2$ (5α)	2.00	42.32	2.08	43.96	1.64
Monooxo-trihydroxy					
6-Oxo-3α,7β,12α-$(OH)_3$ (5α)	1.39	40.56	1.69	42.92	2.36
Dioxo-monohydroxy					
7,12-Dioxo-3α-OH	1.82	41.85	1.53	42.45	0.60
7,12-Dioxo-3β-OH	1.77	41.72	1.53	42.45	0.73
Unsaturated					
3-Oxo-Δ^4 [b]	1.38	40.53	0.93	40.05	−0.48
3-Oxo-7α-OH-Δ^4	1.41	40.64	1.15	41.08	0.44
3-Oxo-12α,OH-Δ^4	1.45	40.77	1.15	41.08	0.31
3-Oxo-7α,12α-$(OH)_2$-Δ^4	1.53	41.03	1.50	42.35	1.32
12-Oxo-3α-OH-$\Delta^{9(11)}$	1.33	40.33	1.12	40.91	0.58
3β-OH-Δ^5	1.16	39.68	0.98	40.34	0.66
3α-OH-Δ^6	0.87	38.28	0.74	38.85	0.57
3α-OH-Δ^7	0.93	38.62	0.79	39.17	0.55
3α-OH-$\Delta^{8(14)}$	1.00	38.96	0.83	39.46	0.50
3α-OH-$\Delta^{9(11)}$	0.85	38.15	0.72	38.71	0.56
3α-OH-Δ^{11}	0.91	38.48	0.77	39.08	0.60
7α,12α-$(OH)_2$-Δ^3	0.80	37.83	0.82	39.40	1.57
3α,12α-$(OH)_2$-Δ^6	0.99	38.88	0.99	40.35	1.47
3α,12α-$(OH)_2$-Δ^7	0.95	38.70	0.94	40.11	1.41
3α,12α-$(OH)_2$-$\Delta^{8(14)}$	0.91	38.48	0.91	39.90	1.42

[a] RRT values were expressed relative to the Et-TMS or Me-DMES ether derivatives of GDCA. The designation 5α in parentheses refers to "*allo*" (*trans* 5α-H) compounds.

[b] RRT and MU values of these compounds correspond to the Et or Me ester derivatives of the glycine conjugates.

determined relative to *n*-alkane. The $\Delta[U_m]_{M-E}$ values [5], which are defined as the differences in the MU values between analogous Me-DMES and Et-TMS ethers, are also listed in the table.

Under the derivatization conditions, the GC peaks arising from bile acids containing hydroxyl groups are assigned to the corresponding glycine-conjugated Et-TMS or Me-DMES ethers, whereas those from oxo bile acids, which are not susceptible to silylation, correspond to the glycine-conjugated Et or Me esters. However, both the ester and ether derivatives afforded sharp and symmetrical peaks on this column, with brief analysis times (within 13 min for the Et esters and Et-TMS ethers, and within 17 min for the Me esters and Me-DMES ethers). Fig. 1 illustrates the distinct separation of a mixture of oxo and oxo-hydroxy bile acid glycine conjugate isomers.

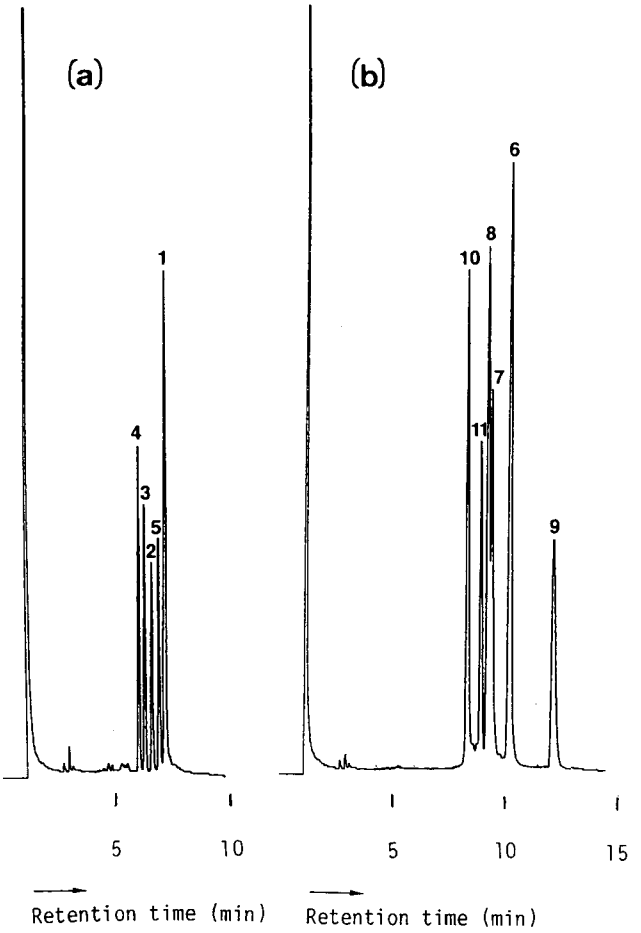

Fig. 1. Capillary GC of a mixture of (a) oxo and (b) oxo-hydroxy 5β-bile acid glycine conjugate isomers as their Et ester and Me-DMES ether derivatives. Peak identification and position of substituents: 1 = 3-oxo; 2 = 6-oxo; 3 = 7-oxo; 4 = 12-oxo; 5 = Et-TMS ether of GDCA; 6 = 3-oxo-6α-hydroxy; 7 = 3-oxo-6β-hydroxy; 8 = 3-oxo-7α-hydroxy; 9 = 3-oxo-7β-hydroxy; 10 = 3-oxo-12α-hydroxy; and 11 = Me-DMES ether of GDCA.

As shown in Fig. 2, plots of the MU values of glycine conjugate Et-TMS ethers (or Et estes) *versus* those of the corresponding Me-DMES ethers (or Me esters) afforded three regression lines, a, b and c, with a similar slope of 1 and good linearity depending upon the number of hydroxyl groups in the molecules. Line a (Fig. 2), expressed as $y = 1.03x + 0.38$ ($r = 0.998$, $n = 17$), consists of all of the mono-, di- and trioxo compounds without a hydroxyl group. The straight line reflects the fact that these compounds show nearly consistent negative $\Delta[U_m]_{M-E}$ values (*ca.* −0.57). On the other hand, regression line b, expressed as $y = 1.03x - 0.67$ ($r = 0.994$, $n = 28$), was obtained from the data for compounds with a hydroxyl group (*e.g.* mono-oxo-monohydroxy and dioxo-monohydroxy), indicating that these compounds have consistent postive $\Delta[U_m]_{M-E}$ values of *ca.* 0.50. Further, compounds possessing two

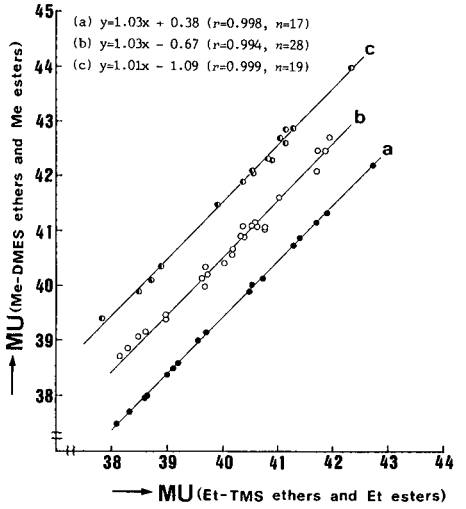

Fig. 2. Relationship between the MU values of Me-DMES and Et-TMS ether (or Me and Et ester) derivatives of glycine-conjugated bile acids without (a) or with one (b) or two (c) hydroxyl groups.

hydroxyl groups (*e.g.* monooxo-dihydroxy) belong to the remaining regression line c, defined as $y = 1.01x - 1.09$ ($r = 0.999$, $n = 19$), implying that the retention times of the Me-DMES ethers are much longer (*ca.* 1.53 in the $\Delta[U_m]_{M-E}$ values) than those of the corresponding Et-TMS ethers. These significant correlations are, therefore, useful for characterizing each of the three types of compounds.

As expected, glycine-conjugated bile acids are eluted much more slowly than the corresponding unconjugates, and the MU values observed for the glycine conjugate Me esters and Me-DMES ethers are in the range 37–44 (*cf.* 30–38 for the unconjugates [4]); 38–43 for the Et esters and Et-TMS ethers. For the purpose of com-

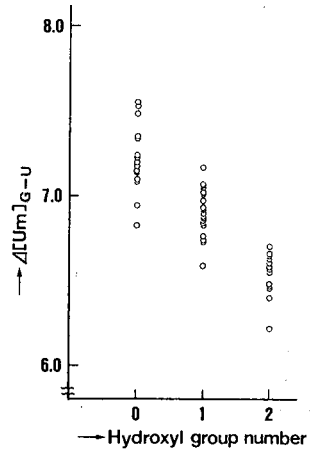

Fig. 3. Relationship of hydroxyl group number to $\Delta[U_m]_{G-U}$ value of oxo and oxo-hydroxy bile acids as their Me-DMES ether (or Me ester) derivatives.

parison, the differences in the MU values between the Me-DMES ethers (or Me esters) of analogous glycine-conjugated and unconjugated bile acids (determined on a HiCap CBPM1 column) [4], which are defined as $\Delta[U_m]_{G-U}$ values, were calculated. The result is expressed graphically in Fig. 3. In analogy with hydroxylated bile acid glycine conjugates reported previously [2], the $\Delta[U_m]_{G-U}$ values were found to depend on the number of hydroxyl substituents on the steroid nucleus, and the average values obtained were as follows: 7.3 for nineteen oxo (S.D. = 0.201), 6.9 for nineteen monooxo-monohydroxy and dioxo-monohydroxy (S.D. = 0.142) and 6.5 for fourteen monooxo-dihydroxy (S.D. = 0.128) compounds, as well as their unsaturated analogues.

The above generalization suggests that the elution order of each group of oxo and oxo-hydroxy bile acid glycine conjugate isomers is essentially identical and corresponds well with the order observed for the corresponding unconjugates [4] on this column. In fact, the positional isomers of the oxo bile acid glycine conjugates in both the 5α and 5β series are well separated as their Et and Me esters, emerging from the column in the order 12- < 7- < 6- < 3-ketones, 7,12- < 3,12- < 3,7- < 3,6-diketones, and then 3,7,12-triketones, precisely corresponding to the order found for their unconjugate esters [4]. In addition, the mono-, di- and triketones in the 5β series move faster than the corresponding ketones in the 5α series with the exception of the C-5 epimeric 3,6-diketones. Similar behavior was also observed for each of the two series of the monooxo-monohydroxy and monooxo-dihydroxy isomers.

The relative mobilities of individual analogues of the two derivatives were also in a similar order. However, some of recalcitrant pairs could be separated successfully by changing the derivatization from Et to Me esters or Et-TMS to Me-DMES ethers (or *vice versa*). For example, while epimeric pairs in the 5β series, 12-oxo-3β-hydroxy *versus* 7-oxo-3α-hydroxy and 7,12-dioxo-3α-hydroxy *versus* 7,12-dioxo-3β-hydroxy, completely overlap as the Me-DMES ethers, the two pairs are well resolved as the Et-TMS ethers. The reverse was true for the pairs 3-oxo-6β-hydroxy *versus* 3-oxo-7α-hydroxy and 12-oxo-3α-hydroxy *versus* 12-oxo-3β-hydroxy.

For the positional isomers of unsaturated 3α-hydroxy bile acid glycine conjugates, the following order of increasing retention was observed: $\Delta^{9(11)} < \Delta^{6} < \Delta^{11} < \Delta^{7} < \Delta^{8(14)}$. Interestingly, this elution order differs from that found for the analogous unsaturated 3α,12α-dihydroxy compounds: $\Delta^{8(14)} < \Delta^{7} < \Delta^{6}$.

The retention data reported here provide an insight into structural elucidation of these biologically important glycine-conjugated bile acids, and the method depends on the ability to measure simultaneously unconjugated and glycine-conjugated ketonic bile acids in biological fluids in a single profile without prior separation and hydrolysis.

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Short Communication

Capillary supercritical fluid chromatography of primary aliphatic amines using carbon dioxide and nitrous oxide as mobile phases

MAY BRITT BAASTOE and ELSA LUNDANES*
Department of Chemistry, University of Oslo, P.O. Box 1033, Blindern, 0315 Oslo 3 (Norway)
(First received April 16th, 1991; revised manuscript received June 7th, 1991)

ABSTRACT

The chromatographic behaviour of primary aliphatic amines on different capillary columns (OV-1, SB-phenyl-5 and SB-biphenyl-30) is described. The best performance was obtained using the OV-1 column; however, this column showed some peak tailing and losses probably due to adsorption effects. The primary amines heptylamine, undecylamine, tetradecylamine and stearylamine with $K_b \approx 4 \cdot 10^{-4}$ were eluted from the columns using both carbon dioxide and nitrous oxide as mobile phase, and no difference in chromatographic performance was observed using either mobile phase. The use of flame ionization detection using nitrous oxide as mobile phase is discussed.

INTRODUCTION

In supercritical fluid chromatography (SFC), carbon dioxide is by far the most commonly used mobile phase. Carbon dioxide has been utilized as the mobile phase for a wide variety of compounds but has been shown to be less suitable for polar compounds. It has been claimed that carbon dioxide cannot be used as a mobile phase for the elution of primary amines because of the reaction of carbon dioxide with basic compounds like amines [1,2]. A similar conclusion has been drawn from the results of extractions with supercritical carbon dioxide and nitrous oxide [3]. Some primary amines have, however, been successfully chromatographed using nitrous oxide [4] and sulphur hexafluoride [2] as eluents. Aromatic amines and tertiary aliphatic amines have been reported to be readily eluted from packed columns and capillary columns using neat carbon dioxide [5,6]. The rather low polarity of supercritical carbon dioxide limits its use as a mobile phase for more polar compounds. Nitrous oxide has a permanent dipole moment and is therefore expected to be a better solvent for these compounds. The results from a group separation of crude oil indicated that nitrous oxide was a stronger eluent than carbon dioxide for aromatics

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and polar compounds [7]. However, it has also been shown that supercritical carbon dioxide and nitrous oxide have nearly identical solvation properties and elute most solutes in the same range of densities [8,9].

The aim of this study was to investigate to what extent the rather non-polar fluids nitrous oxide and carbon dioxide can be used for the chromatography of polar compounds, especially primary and secondary amines, on capillary columns. When both carbon dioxide and nitrous oxide are used as the mobile phase, flame ionization detection (FID) can be utilized, even though it is known that nitrous oxide gives a high background signal with this type of detection [3,7,10,11]. It was found that aliphatic amines, also primary aliphatic amines, could be eluted from capillary columns with neat carbon dioxide as well as with nitrous oxide as the mobile phase. The chromatographic behaviour of primary aliphatic amines on different stationary phases is reported, and the possibility of using capillary SFC for the analysis of these compounds is discussed.

EXPERIMENTAL

Equipment

The SFC system consisted of a Model 602 supercritical fluid chromatograph from Lee Scientific (Salt Lake City, UT, USA). This instrument was equipped with a Valco C14W injector with a 200-nl loop; the injector could be operated with timed split injection. In addition, a 500 μm I.D. dynamic splitter [12] was installed inside the oven. Injections were performed at or slightly above ambient temperature, while dynamic splitting occurred under supercritical conditions. The columns were installed into the dynamic splitter at a position 2–3 mm below the injector rotor. The restrictor utilized was a 50-μm frit restrictor (Lee Scientific), while the dynamic split restrictor was either an integral restrictor [13] made from 50 μm I.D. fused silica or a linear restrictor made from 10 μm I.D. fused silica. The split restrictor was heated by an extra heating unit (copper block) with temperature control (Eurotherm, Worthing, UK). The heating unit was installed outside the oven (lower left side). The flame ionization detector was operated at 325°C, and normal flow-rates of hydrogen, air and make-up gas (nitrogen) were utilized. Peak-area determinations were accomplished using Multichrom software (VG Lab. System) on a Micro PDP 11/73 (Digital).

Columns

The following columns were utilized: a 10 m × 50 μm I.D. (0.2 μm film thickness) OV-1 (dimethyl silicone) from Rescom (Kortrijk, Belgium), a 5 m × 50 μm I.D. (0.25 μm film thickness) SB-phenyl-5 (5% phenyl, 95% methyl polysiloxane) and a 10 m × 50 μm I.D. (0.25 μm film thickness) SB-biphenyl-30 (30% biphenyl, 70% methyl polysiloxane) from Lee Scientific.

Materials

Carbon dioxide (grade 5.0) was obtained from Aga Norgas (Oslo, Norway), while nitrous oxide (SFC grade) was obtained from Scott Specialty Gases (Plumstedville, PA, USA).

The standards were obtained from different commercial sources and were dissolved at a concentration of 1 mg/ml unless otherwise stated. The solvents used were

high-performance liquid chromatography grade chloroform from Rathburn (Walkerburn, UK) and carbon disulphide (analyzed reagent) from J. T. Baker (Deventer, Netherlands).

RESULTS AND DISCUSSION

Nitrous oxide was the mobile phase of choice since it was expected to give better solvation of polar compounds than carbon dioxide and since it has been argued that primary amines react with carbon dioxide. The elution of the primary aliphatic amines was examined on three different stationary phases. The main conclusion was that the best results with respect to peak shape were obtained with the methyl column. However, the peak shape of a primary amine was inferior to that of a tertiary amine and a normal alkane, as shown in Fig. 1A.

It has been observed [14] that primary aliphatic amines can be eluted from a glass capillary column using neat carbon dioxide as the mobile phase. Therefore the

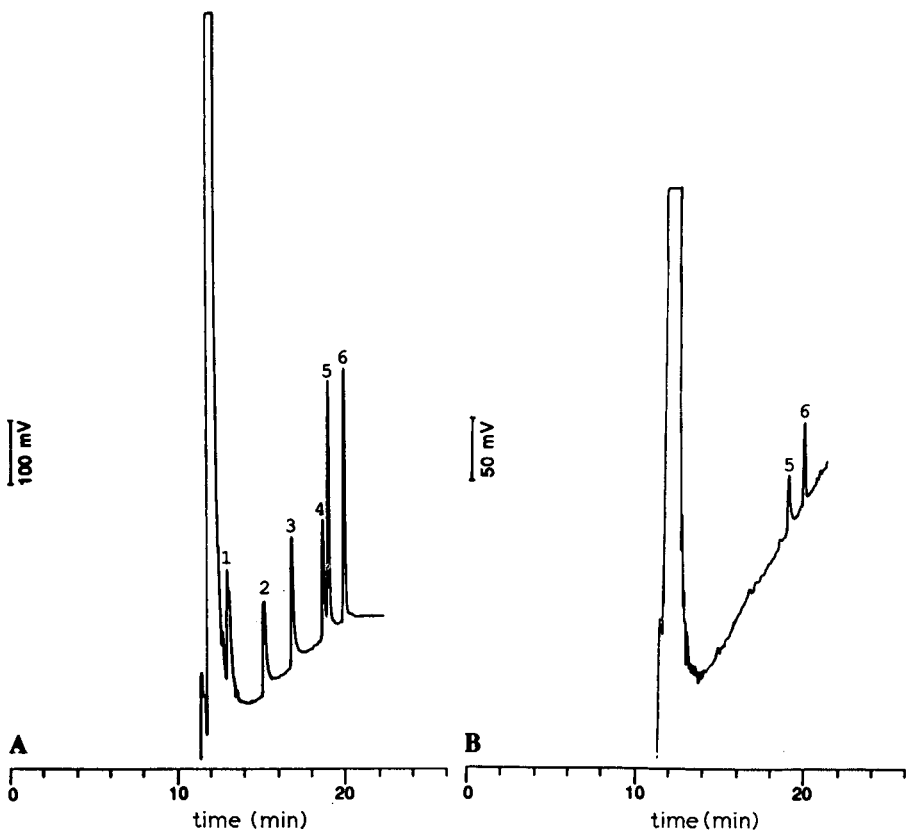

Fig. 1. Separation of heptylamine (1), undecylamine (2), tetradecylamine (3), stearylamine (4), trioctylamine (5) and NP-25 (6) on a OV-1 methyl column using nitrous oxide as the mobile phase at a column temperature of 70°C and using the following pressure programme: 90 bar for 10 min followed by 90 to 210 bar at 10 bar/min. About 15 ng (A) and 2.5 ng (B) of each component were injected onto the column.

same mixture as above was chromatographed with carbon dioxide as the mobile phase. The resulting chromatogram (Fig. 2A) resembled the results obtained with nitrous oxide. The amount injected was the same in both cases, and was about 15 ng of each compound (on column). It has been argued [15] that the low recovery of primary aliphatic amines on capillary columns using carbon dioxide as the mobile phase is due to the reaction with carbon dioxide. The peak areas of undecylamine and tretradecylamine were compared with the peak area of the normal alkane NP-25 in both nitrous oxide and carbon dioxide. The same amount of each compound was injected, however the peak areas of the two primary amines were both only about 70% of the peak area of NP-25. Even though we expect a lower FID response of the nitrogen-containing amines compared with a normal alkane [16], the whole difference cannot be explained by this. Therefore there must be some loss of the primary amines in the chromatographic system. Interestingly the same ratio was found in both carbon dioxide and nitrous oxide. We therefore concluded that the lower recovery was not caused by any reaction with carbon dioxide, but rather may be caused by losses to the column or possibly the injector. When a 1:10 diluted solution of the same mixture was chromatographed at the same conditions, the peak heights for the primary amines became very low compared with those of the tertiary amine and NP-25 (Fig. 2B). The

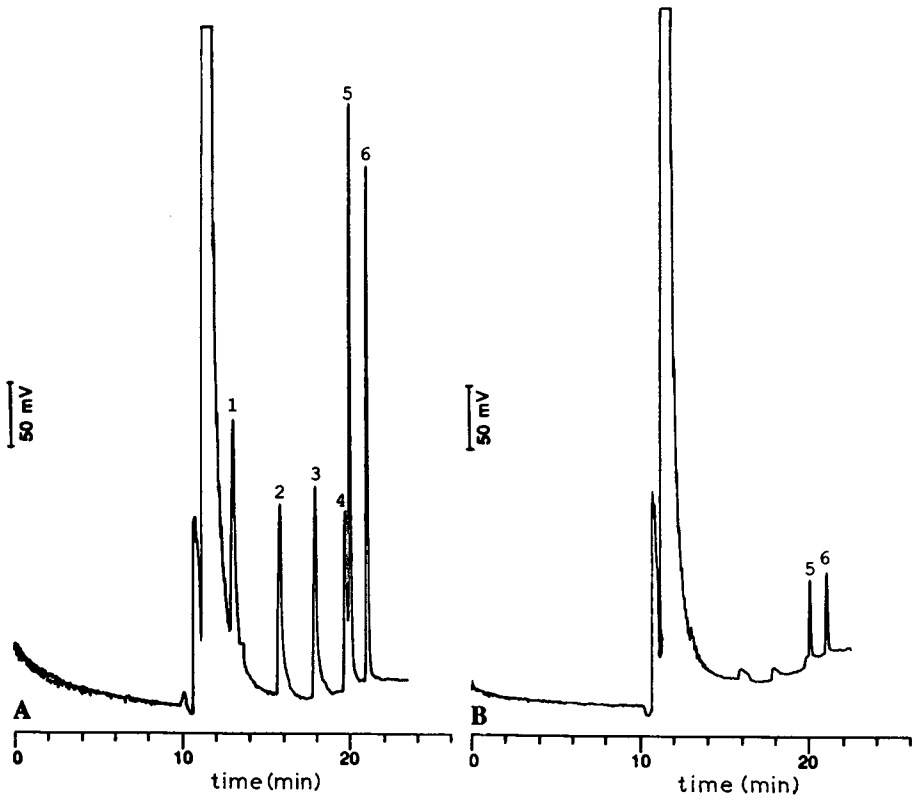

Fig. 2. Separation of heptylamine (1), undecylamine (2), tetradecylamine (3), stearylamine (4), trioctylamine (5) and NP-25 (6) on a OV-1 methyl column using carbon dioxide as mobile phase. Conditions and labeling as in Fig. 1.

same result was obtained with nitrous oxide as the mobile phase (Fig. 1B). Unfortunately the high background level with nitrous oxide prevented easy integration of the peaks at the low concentration (Fig. 1B), but from the inspection of the chromatograms it seems likely that the same reduction in peak height/area was found with both mobile phases. The high background level with nitrous oxide as the mobile phase is due to nitrous oxide itself [11]. The background level became smaller when the amount of hydrogen to the flame ionization detector was reduced, however the signals for the injected compounds also decreased, giving no overall gain in sensitivity. The mass sensitivity of the detector with nitrous oxide as the mobile phase was checked by determining the peak areas of a series of *n*-alkanes using gradient elution and comparing the results with a similar gradient using carbon dioxide as mobile phase. Identical relative areas confirmed the mass sensitivity of the flame ionization detector using gradients of nitrous oxide as the mobile phase. The detection limit with nitrous oxide was about ten times higher than with carbon dioxide at the mobile phase flow-rates used in this study.

Since the peak heights of the primary amines relative to the peak heights of NP-25 became smaller when the injected amount became smaller, we anticipate that this is caused by adsorption effects, most probably to active sites in the column. That this adsorption effect is most probably caused by the column and not by the injector or the mobile phase is also indicated by the fact that different peak shapes were observed on the different columns examined. Both the methyl column and the biphenyl column were new, while the phenyl column had been used for some time before this study was started. Better peak shapes were also obtained on the methyl column than on the phenyl column for a mixture of chlorinated phenols. Therefore adsorption onto active sites in the column is the most likely reason for the peak tailing and loss of primary amines. However, reaction of small amines with carbon dioxide cannot be ruled out. Pyrrole ($K_b \sim 10^{-9}$) was eluted with very short retention time on the methyl column with carbon dioxide as the mobile phase and could be detected when carbon disulphide was used as solvent instead of chloroform. However, pyrrolidine ($K_b \sim 10^{-3}$) could not be detected. This compound has been reported to react with carbon dioxide [17], and also reacts rather violently with carbon disulphide. The primary amine butylamine could not be detected when injected on the methyl column. This may be due to the short retention time but is most probably due to reaction with carbon dioxide, since this compound also reacts with carbon disulphide (at a concentration of 25 mg/ml). Thus we cannot rule out the possiblity that some primary amines react with carbon dioxide. It has been argued that the reaction between carbon dioxide and primary and secondary amines requires a carbon dioxide:amine ratio of less than 1:2 [18]. However, in chromatography the carbon dioxide:amine ratio is usually not favourable for carbamate formation. Owing to the somewhat unpredictable behaviour of the different amines, authentic reference materials should always be utilized when using this method. For successful chromatography it is also important that the free amines are injected. After desalting, the primary amine phenylpropanolamine was eluted from the phenyl column, as a rather broad peak, using either mobile phase, while this compound could not be detected when the salt form of the amine was used for injection. The same was observed for the secondary amine propranolol. The peak shape of the free base of propranolol was quite reasonable using either mobile phase on this column. These compounds are relatively polar compounds and solubility problems in the mobile phases can also be expected.

Unfortunately, the methyl column, which initially showed the best performance for the separation of primary amines, rather quickly (after more than 50 injections of amines and other compounds) deteriorated with respect to the chromatographic behaviour of the primary amines. The peaks became smaller, with more tailing, relative to the chromatograms shown in Figs. 1 and 2, even at the level of 15 ng injected on the column. At the same time no difference in column efficiency was detected for normal paraffins and chlorinated phenols. Thus, the possible explanation may be an increase in accessible active sites in the column either because of a small but immeasurable stripping of the stationary phase or because of absorption of compounds onto the stationary phase. It has also been observed by others [2] that the column lifetime may be important for the successful chromatography of amines. Recently, Gyllenhaal and Vessman [19] examined the chromatographic behaviour of some aliphatic amines using carbon dioxide and nitrous oxide as mobile phases. They concluded that a 5% phenyl methyl siloxane column was superior to a 25% cyanomethyl siloxane and a Carbowax 20M column for these compounds because of its greater inertness. However, poor and varying symmetry was found for the primary aliphatic amine octylamine on this column, which had a film thickness of 0.5 μm.

The conclusion from this work is that primary aliphatic amines such as heptylamine and larger (with $K_b \sim 4 \cdot 10^{-4}$) can be chromatographed with carbon dioxide as well as nitrous oxide as the mobile phase on rather non-polar capillary columns. However, peak tailing and losses, probably due to adsorption onto active sites in the columns, indicate that this system is not well suited for quantitative analysis of small amounts of amines. A better deactivated column and/or a thicker film could perhaps provide a better chromatographic performance.

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Author Index

Errata

J. Chromatogr., 539 (1991) 501–505.

Page 505: the authors would like to add the following "Acknowledgements" section: "The success of the work was critically dependent on an expression system which we received from Drs. Curtis L. Patton, Charles E. Egwuagu and James F. Young of the Yale University School of Medicine, New Haven, CT, USA."

J. Chromatogr., 554 (1991) 61–71.

Page 63, *Mobile phases*, line 4, "acetonitrile (ACN) (39%)" should read "acetonitrile (ACN) (30%)".

Journal of Chromatography

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- Analysis of compounds arising during food preparation and storage

Potential authors of reviews should contact Zdenek Deyl (Institute of Physiology, Czechoslovak Academy of Sciences, Vídeňská 1083, CS 14220 Prague, Czechoslovakia; tel.: +42-2-531267; fax: +42-2-4712253) prior to any submission. Research papers should be submitted to the above address as well.

The deadline for receipt of submissions is **January 31, 1992.** Manuscripts submitted after this deadline can still be published in the Journal, but then there is no guarantee that an accepted article will appear in the special, thematic issue. **Three** copies of the manuscript should be submitted to Zdenek Deyl. All manuscripts will be reviewed and acceptance will be based on the usual criteria for publishing in the *Journal of Chromatography.*

Instructions to Authors

JOURNAL OF CHROMATOGRAPHY

JOURNAL OF CHROMATOGRAPHY, BIOMEDICAL APPLICATIONS

JOURNAL OF CHROMATOGRAPHY, SYMPOSIUM VOLUMES

Elsevier Science Publishers
Desk-Editorial Office Journal of Chromatography:
Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands
P.O. Box 330, 1000 AH Amsterdam, The Netherlands
Tel. 31-20-5862793
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General

The JOURNAL OF CHROMATOGRAPHY publishes papers on all aspects of **chromatography, electrophoresis** and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications.

BIOMEDICAL APPLICATIONS. This section, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis or alterations during medical treatment; screening and profiling of body fluids or tissues with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; drug level monitoring and pharmacokinetic studies; clinical toxicology; analytical studies in occupational medicine.

SYMPOSIUM VOLUMES. This publication, which is under separate editorship, publishes proceedings of symposia on chromatography, electrophoresis and related methods.

Types of contributions

The following types of papers are published in the JOURNAL OF CHROMATOGRAPHY and the section on BIOMEDICAL APPLICATIONS: Regular research papers (full-length papers), Review articles and Short Communications. Short Communications are usually descriptions of short investigations, or they can report minor technical improvements of previously published procedures; they reflect the same quality of research as full-length papers, but should preferably not exceed six printed pages. Review articles are invited or proposed in writing to the Editors. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation.

Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere.

Upon acceptance of an article by the journal, the author(s) will be asked to transfer the copyright of the article to the publisher. This transfer will ensure the widest possible dissemination of information.

Submission of papers

Manuscripts (***four*** copies are required), in English, should be submitted to:

for the JOURNAL OF CHROMATOGRAPHY:

Editorial office
JOURNAL OF CHROMATOGRAPHY
P.O. Box 681
1000 AR Amsterdam, The Netherlands

for the BIOMEDICAL APPLICATIONS section:

Dr. K. Macek
The Editor of
JOURNAL OF CHROMATOGRAPHY,
BIOMEDICAL APPLICATIONS
P.O. Box 681
1000 AR Amsterdam, The Netherlands

Manuscripts for the SYMPOSIUM VOLUMES section (*three* copies are required) should be submitted during the symposium concerned. After the symposium, correspondence should be sent by *regular airmail (NOT by registered, special delivery of private mail services)* to the Editor handling the corresponding proceedings, i.e., either:

Dr. E. Heftmann
Editor of
JOURNAL OF CHROMATOGRAPHY,
SYMPOSIUM VOLUMES
P.O. Box 928
ORINDA, CA 94563-0818, U.S.A.

or Dr. Z. Deyl
Editor of
JOURNAL OF CHROMATOGRAPHY,
SYMPOSIUM VOLUMES
Institute of Physiology
Czechoslovak Academy of Sciences
Videnská 1083
14220 PRAGUE 4-Krc
Czechoslovakia

Every paper must be accompanied by a letter from the senior author, stating that he/she is submitting the paper for publication in the *Journal of Chromatography*.

Manuscripts

Manuscripts should be typed in ***double spacing*** on consecutively numbered paper of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. As a rule, papers should be divided into sections, headed by a caption (*e.g.* Abstract, Introduction, Experimental, Results, Discussion). All illustrations, photographs, tables, etc. should be on separate sheets. **Four** copies of the complete manuscript should be submitted.

Title

The title of the paper should be concise and informative. Since titles ares widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are not to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

Introduction

Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Abstract

All articles should have an abstract of 50–100 words which clearly and briefly indicates what is new, different and significant.

Illustrations

The figures should be submitted in a form suitable for reproduction, either drawn in Indian ink on drawing or tracing paper, or as sharp glossy prints. Axes of a graph should be clearly labelled. Please note that any lettering should also be in a form suitable for reproduction. Lettering (which should be kept to a minimum) and spacing on axes of graphs should be such that numbers, etc., remain legible after reduction. One original and three photocopies are required. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. The size of the figures should preferably not exceed the size of the text pages. Standard symbols should be used in line drawings, the following are available to the printers and can also be used in the legends:

○ ● □ ■ △ ▲ ◇ ◆

Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using Arabic numerals and the approximate position of the illustration should be indicated in the margin of the manuscript. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*.

If structures are given in the text, the original drawings should be provided.

Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed.

The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

Tables

The tables should be typed (in double spacing) on separate pages, and numbered in Roman numerals according to their sequence in the text. A brief descriptive heading should be given above each table. Below the heading the experimental conditions should be described. The layout of the tables should be given serious thought, so that the reader can grasp quickly the significance of the results.

Nomenclature, symbols and abbreviations

Widely accepted symbols, abbreviations and units (SI) should be used. If there is any doubt about a particular symbol or abbreviation, the full expression followed by the abbreviation should be given the first time it appears in the text. Abbreviations used in tables and figures should be explained in the legends. In general, the recommendations of the International Union of Pure and Applied Cemistry (I.U.P.A.C.) should be followed.

References

References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places in square brackets. In the reference list, periodicals [1], books [2], multi-author books [3], and proceedings [4] should be cited in accordance with the following examples:

1 D. P. Ndiomu and C. F. Simpson, *Anal. Chim. Acta*, 213 (1988) 237.
2 T. Paryjczak, *Gas Chromatography in Adsorption and Catalysis*, Wiley, Chichester, 1986.
3 M. Saito, T. Hondo and Y. Yamauchi, in R. M. Smith (Editor), *Supercritical Fluid Chromatography*, Royal Society of Chemistry, London, 1988, Ch. 8, p. 203.
4 F. I. Onushka and K. A. Terry, in P. Sandra, G. Redant and F. David (Editors), *Proceedings of the 10th International Symposium on Capillary Chromatography, Riva del Garda, May 1989*, Hüthig, Heidelberg, 1989, p. 415.

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

Dispatch

Before dispatch of the manuscript please check that the envelope contains four copies of the paper complete with references, legends and figures. One of the sets of figures must be the originals suitable for direct reproduction. Please also ensure that permission to publish has been obtained from your institute.

Proofs

One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. We shall be obliged to make a charge for all "extra corrections" at a rate in accordance with their cost to us.

To ensure fastest possible publication, proofs are sent to authors by *airmail* and must be returned to the publisher also by *airmail*. If this is not done, the article will be passed for publication with house correction only. Proofs may also be returned by FAX; the FAX number of *Journal of Chromatography* is: 31 (The Netherlands) -20 (Amsterdam) -5862304.

Reprints

Fifty reprints of Full-length papers and Short Communications will be supplied free of charge. Additional reprints can be ordered by the authors. The order form containing price quotations will be sent to the authors together with the proofs of their article.

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Subscription orders should be sent to Elsevier Science Publishers B.V., P.O. Box 211, 1000 AE Amsterdam, The Netherlands. The JOURNAL OF CHROMATOGRAPHY and the BIOMEDICAL APPLICATIONS section can be subscribed to separately. Cumulative Author and Subject Indexes can also be ordered separately. Write to the Marketing Manager, Chemistry, at the above address for more details.

Vol. 558, No. 2

journal of chromatography news section

ANNOUNCEMENTS OF MEETINGS

EUCHEM CONFERENCE ON CAPILLARY ELECTROSEPARATIONS, STORLIEN, SWEDEN, DECEMBER 9–11, 1991

EUCHEM (European Chemistry) was created in 1964 by the chairmen of the various National Committees for Chemistry in West European countries, with the purpose of establishing a series of high level conferences as a Western European counterpart to the Gordon Conferences in USA. Regarding the themes chosen for the conferences a particular emphasis has been put on newly evolving fields of chemistry and on areas where there is a strong industrial need for better scientific knowledge.

Modern electroseparations have developed into highly flexible systems and consequently can be used for all kinds of solutes from macromolecules to small inorganic compounds. Purely electrophoretic capillary techniques are mainly used for large molecules and include zone electrophoresis, isotachophoresis, isoelectric focusing and gel electrophoresis. Electroosmotic techniques are generally applied for smaller molecules and include micellar electrokinetic chromatography and capillary electrochromatography.

The different techniques are still undergoing intensive development and improvements are required mainly regarding theory, injection methods, capillary coating and detection techniques.

The purpose of the conference is to highlight the performance of the different techniques and to critically examine their possibilities and limitations in order to optimize future research activities and to identify suitable application areas for the different separation principles.

The programme will contain short lectures and poster sessions with accepted registered contributions. Sessions for discussions of current topics will also be an essential part of the programme. Each afternoon, several hours will be free for informal discussions, and typical activities for Nordic winter holidays.

For further details, contact: Dr. Agneta Sjögren, The Swedish National Committee for Chemistry, Wallingatan 26 B, S-111 24 Stockholm, Sweden. Tel.: (+46-8) 115260; Fax: (+46-8) 106678.

HPCE '92, 4th INTERNATIONAL SYMPOSIUM ON HIGH-PERFORMANCE CAPILLARY ELECTROPHORESIS, AMSTERDAM, NETHERLANDS, FEBRUARY 9–13, 1992

The program will include invited and contributed lectures. As in the previous symposia, posters will play a major role in the scientific program, and ample time will be set aside to view them and to speak to their authors. Sufficient space has also been allocated for the poster area so that all posters will remain up during the entire symposium. Lecture and poster topics will include: zone electropho-

resis; isoelectric focusing; isotachophoresis; micellar separations; CE–mass spectrometry; gel columns; detector design; instrumentation; analytical and micropreparative applications for pharmaceuticals, peptides, proteins, carbohydrates, oligonucleotides, sub-cellular structures, and whole cells.

Papers presented at the symposium will be eligible for publication in a special volume of the *Journal of Chromatography*. Authors wishing to contribute to this symposium volume will be required to turn in their manuscripts at the time of the Symposium.

Abstracts forms and a copy of the second circular which will contain the preliminary program and information about registration, travel arrangements, and hotel accomodations may be obtained by contacting the Symposium Manager: Shirley E. Schlessinger, HPCE '92, Suite 1015, 400 East Randolph Drive, Chicago, IL 60601, USA. Tel.: (312) 527 2011.

43rd PITTSBURGH CONFERENCE AND EXPOSITION ON ANALYTICAL CHEMISTRY AND APPLIED SPECTROSCOPY, NEW ORLEANS, LA, USA, MARCH 9–13, 1992

The technical program of PITTCON '92 will be a blend of presentations on analytical technology, materials characterization, environmental monitoring, laboratory management, biochemical and clinical applications, and related fields as well as topics of general interest.

The broad technical scope is an integral part of PITTCON '92. A plenary session, invited symposia, contributed papers and short courses will be presented.

Original, unpublished papers may be contributed in all areas of analytical chemistry, spectroscopy and related scientific areas.

PITTCON '92 will feature several technical sessions in which awards will be presented to distinguished scientists: Pittsburgh Analytical Chemistry Award; Keene P. Dimick Award; James L. Waters Symposium; The Williams-Wright Industrial Spectroscopist Award; Pittsburgh Spectroscopy Award; Charles N. Reilley Award; and Dal Nogare Award.

In addition, the exposition of modern laboratory instrumentation, equipment, supplies and services will showcase the latest developments of suppliers from around the world. In 1991, 970 companies from around the world exhibited their products in 2763 booths and 64 seminar rooms.

For further details contact: Mrs. Alma Johnson, Program Secretary, The Pittsburgh Conference, 300 Penn Center Blvd., Suite 332, Pittsburgh, PA 15235-5503, USA.

ANATECH '92, 3rd INTERNATIONAL SYMPOSIUM ON ANALYTICAL TECHNIQUES FOR INDUSTRIAL PROCESS CONTROL, ATLANTA, GA, USA, APRIL 6–8, 1992

Greater productivity and efficiency along with increased attention to quality are the goals of industrial manufacturing plants. Understanding of process parameters and appropriate control will increase profitability. One critical means of developing the necessary understanding and criteria for control can be obtained by continuous process monitoring.

Some of the techniques currently in use in product environments and contributing to process optimization and control are spectroscopy, chromatography, automated wet chemical analysis, advanced computing, etc, and new emerging techniques.

Like its European predecessors, this first symposium of ANATECH in the USA will focus on the latest developments in process analytical chemistry for an interdisciplinary audience. The participants will be encouraged to take an active part in a conference environment which promotes the development of long term interactions.

Participants wishing to present a paper may indicate their intention of submitting one or more abstracts so that instructions can be sent in time. Papers presented at the conference will be reviewed for publication in a special edition of *Process Control and Quality*. The deadline for abstracts is 1 November 1991.

An exhibition will be arranged in conjunction with the conference; potential exhibitors are invited.

For more information contact: ANATECH '92, Infoscience Services Inc., 3000 Dundee Road, Suite 313, Northbrook, IL 60062, USA. Tel: (708) 291-9161; Fax: (708) 291-0097.

ISPAC-5, 5th INTERNATIONAL SYMPOSIUM ON POLYMER ANALYSIS AND CHARACTERIZATION, INUYAMA-CITY, JAPAN, JUNE 2–4, 1992

This three-day symposium will consist of contributed poster sessions, invited lectures, and round-table discussions, and information exchange on recent advances in approaches and techniques on polymer analysis and characterization.

Topics will include: chromatography and other separation methods, UV and IR spectrometry, NMR, mass spectrometry, light scattering, viscometry, solution properties of polymers, as well as general contributions on polymer analysis and characterization. Contributions dealing with other techniques or special classes of polymers will also be included.

Contributed papers will be accepted for poster pressentations in any of the mentioned or related areas of the topics.

For further details contact: Dr. Sadao Mori, Faculty of Engineering, Mie University, Tsu, Mie 514, Japan. Fax: (592) 31-2252.

HPLC '92, 16th INTERNATIONAL SYMPOSIUM ON COLUMN LIQUID CHROMATOGRAPHY, BALTIMORE, MD, USA, JUNE 14–19, 1992

The scientific program will include presentations on the latest developments in HPLC and related techniques. Various sessions will address advances in separation methods, mechanisms, selective and sensitive detection, sample preparation, automation, derivatization, preparative LC, biopolymer separation, chiral recognition, capillary and conventional electrophoresis, supercritical-fluid chromatography and field-flow fractionation. A special emphasis for HPLC '92 will be on bioanalytical chemistry, including the separation and detection of biopolymers, pharmaceuticals, biorganics, and related biologically active materials.

The program will include general and invited lectures on fundamentals and applications in HPLC, poster papers and informal discussion sessions.

An exhibition of the latest instrumentation will be organized. The organizing committee welcomes your contributions of recent research results.

For further information contact: Ms Shirley E. Schlessinger, Symposium Manager, HPLC '92, 400 E. Randolph Drive, Suite 1015, Chicago, IL 60601, USA. Tel.: (312) 527-2011.

19th INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY, AIX-EN-PROVENCE, FRANCE, SEPTEMBER 13–18, 1992

The symposium will be organized by the Groupe pour l'Avancement des Sciences Analytiques (G.A.M.S.) in association with the Chromatographic Society (UK) and the Arbeitskreis Chromatographie der Fachgruppe Analytische Chemie der G.D.C. (FRG).

The scientific program will include all aspects (fundamentals, instrumentation, applications) of the various chromatographic techniques (liquid chromatography, supercritical fluid chromatography, gas chromatography, planar chromatography) and of related separation techniques (field-flow fractionation, capillary electrophoresis) with special emphasis on new developments and novel or rapidly expanding areas.

The dead-line for submission of Abstracts is December 1, 1991.

For further details contact: G.A.M.S., 88 Boulevard Malesherbes, 75008 Paris, France. Tel.: (1) 45639304; Fax: (1) 49530434.

ITP '92, 8th INTERNATIONAL SYMPOSIUM ON CAPILLARY ELECTROPHORESIS AND ISOTACHOPHORESIS, ROME, ITALY, OCTOBER 6–9, 1992

ITP '92 will deal with all aspects of capillary electrophoretic techniques: zone electrophoresis, isotachophoresis, isoelectric focusing and micellar electrokinetic chromatography.

The symposium will include invited lectures, oral communications and poster sessions. The contributions will be focused on the recent advances both in the theory and in the methodological and technical development of the techniques mentioned above. Special sections will be focused on applications in the areas of DNA, oligonucleotides, proteins, peptides, organic and inorganic small ions, drugs, optical isomers, etc.

The proceedings of the symposium (full papers and short notes) will be published as a special issue of the *Journal of Chromatography*.

An exhibition of commercial instrumentation and accessories for the related techniques will be given by the manufacturers during the symposium.

For newcomers to the field who are interested, a short course preceding the meeting will be held on October 6, which will present the necessary background for the symposium.

The registration fee is 450 000 Italian Lire and will include entrance to all sections and exhibitions, book of abstracts, refreshments and symposium dinner. The registration fee for the short course (which is limited to 50 participants) is 50 000 Italian Lire.

For further details contact: Dr. Salvatore Fanall, Istituto di Cromatografia, C.N.R., Area della Ricerca di Roma, P.O. Box 10, 00016 Monterotondo Scalo, Roma, Italy. Tel.: (+39 6) 9005328/9005836; Fax: (+39 6) 9005849; Telex: 624809 CNR ML 1.

9th MONTREUX SYMPOSIUM ON LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY (LC–MS; SFC–MS; CZE–MS; MS–MS), MONTREUX, SWITZERLAND, NOVEMBER 4–6, 1992

The symposium on LC–MS, SFC–MS, CZE–MS, and MS–MS dedicated to the late Roland W. Frei, will deal with all areas of this topic including technical developments with on-line aspects, theoretical considerations and applications of the techniques in environmental, clinical and pharmaceutical analysis and other fields. Subtopics will be introduced by plenary lectures and invited research lectures followed by brief research presentations and posters. A substantial part of the workshop will be devoted to panel and group discussions on the state-of-the-art of LC–MS, SFC–MS, CZE–MS and MS–MS.

The symposium will be preceeded by a 2-day short course on LC–MS, SFC–MS and CZE–MS, November 2–3, 1992.

An exhibition will take place during the five days. Those interested in giving a research lecture or a poster presentation should send an abstract of no more than 200 words to the chairman by March 1, 1992 (Prof. J. v.d. Greef, Center for Bio-Pharmaceutical Sciences, P.O. Box 9502, 2300 RA Leiden, Netherlands. Tel.: +31-3404-44400, Fax: +31-3404-57224.)

For further details and preliminary registration contact: M. Frei-Häusler, Postfach 46, CH-4123 Allschwil 2, Switzerland. Tel.: (+41 61) 632789; Fax: (+41 61) 4820805.

SHORT COURSE

CHROMATOGRAPHIC FUNDAMENTALS COURSE, KENT, OH, USA, OCTOBER 28–NOVEMBER 1, 1991

Kent State University's Chemistry Department will present a course, Fundamentals of Chromatographic Analysis, during October 28–November 1 for the ninth year.

The purpose of the course is to provide a coherent overview of chemical separations via chromatographic methods. It will be unique from most other short courses in that material will be included

on gas, liquid and thin-layer methods. The use of the three techniques as complementary rather than competing processes will be stressed. The course will be a blend of fundamental information on theory and instrumentation with emphasis placed on the latest developments and trends. Additional periods will provide time to discuss practical problems related to HPLC, GC, GC–MS, TLC and CE instrumentation.

Dr. Roger K. Gilpin, Chairman of Chemistry at Kent State University, and Dr. Neil D. Danielson, Professor at Miami University, will be the principle lecturers. Spectra-Physics personnel will discuss instrumentation in the laboratory and demonstrate various laboratory methods.

Information on this course can be obtained from: Carl J. Knauss, Chemistry Department, Kent State University, Kent, OH 44242, USA. Tel.: (216) 672-2327.

Announcements are included free of charge. Information on planned events should be sent well in advance (preferably 6 months or more) to: Journal of Chromatography, News Section, P.O. Box 330, 1000 AH Amsterdam, The Netherlands, Fax: (31) 20-5862845.

CALENDAR OF FORTHCOMING EVENTS

Oct. 15–16, 1991
Frederick, MD, USA

Frederick Conference on Capillary Electrophoresis
Contact: Margaret L. Fanning, Conference Coordinator, PRI, NCI-FRDC, P.O. Box B, Frederick, MD 21702-1201, USA. Tel.: (301) 846-1089; Fax: (301) 846-5886. (Further details published in Vol. 543, No. 2.)

Oct. 20–23, 1991
Washington, DC, USA

11th International Symposium on High-Performance Liquid Chromatography of Proteins, Peptides and Polynucleotides
Contact: Janet E. Cunningham, Barr Enterprises, P.O. Box 279, Walkersville MD 21793, USA. Tel.: (301) 898-3772; Fax: (301) 898-5596. (Further details published in Vol. 513.)

Oct. 20–24, 1991
Knoxville, TN, USA

7th Symposium on Separation Science and Technology for Energy Applications
Contact: Dr. J.S. Watson, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6223, USA. Tel.: (615) 574-6795 or 574-4934. (Further details published in Vol. 540.)

Oct. 21–24, 1991
San Miniato (Pisa), Italy

Colloquium Chemiometricum Mediterraneum
Contact: Dr. L. Lampugnani, Ist. Chimica Analitica Strumentale – C.N.R., Via Risorgimento 35, I-56100 Pisa, Italy. Tel.: (+39 50) 501224; Fax: (+39 50) 587260.

Nov. 11–15, 1991
Somerset, NJ, USA

30th Annual Eastern Analytical Symposium and Exposition
Contact: Program Committee Eastern Analytical Symposium, P.O. Box 633, Montchanin, DE 19710-0633, USA. Tel.: (302) 453-0785; Fax: (302) 738-5275. (Further details published in Vol. 543, No. 2.)

Dec. 9–11, 1991
Storlién, Sweden

* **EUCHEM Conference on Capillary Electroseparations**
Contact: Dr. Agneta Sjögren, The Swedish National Committee for Chemistry, Wallingatan 26B, S-111 24 Stockholm, Sweden. Tel.: (+46 8) 115260; Fax: (+46 8) 106678.

Jan. 6–11, 1992
San Diego, CA, USA

1992 Winter Conference on Plasma Spectrochemistry
Contact: Dr. R. Barnes, c/o ICP Information Newsletter, Department of Chemistry, CRC Towers, University of Massachusetts, Amherst, MA 01003-0035, USA. Tel.: (413) 545-2294; Fax: (413) 545-4490; Bitnet: RBARNES@UMASS. (Further details published in Vol. 543, No. 2.)

Feb. 9–13, 1992
Amsterdam, Netherlands

* **HPCE '92, 4th International Symposium on High Performance Capillary Electrophoresis**
Contact: Shirley E. Schlessinger, HPCE '92, 400 East Randolph Drive, Suite 1015, Chicago, IL 60601, USA. Tel.: (312) 527 2011

Feb. 17–18, 1992
Antwerp, Belgium

* **Hands-on Workshops on Supercritical Fluid Extraction and Supercritical Fluid Chromatography; on Liquid Chromatography–Gas Chromatography; and on Hyphenated Techniques in Capillary Gas Chromatography: Mass Spectrometery, Fourier Transform Infrared Spectroscopy, Atomic Emission Detection**
Contact: Dr. R. Smits, p.a. BASF Antwerpen N.V., Central Laboratory, Scheldelaan, B-2040 Antwerp, Belgium.

Feb. 18–21, 1992
Antwerp, Belgium

2nd International Symposium on Hyphenated Techniques in Chromatography
Contact: Dr. R. Smits, p.a. BASF Antwerpen N.V., Scheldelaan, B-2040 Antwerp, Belgium. Tel.: (32) 5682831; Fax: (323) 5683355; Telex: 31047 basant b. (Further details published in Vol. 508, No. 2.)

March 9–13, 1992
New Orleans, LA, USA

* **43rd Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy**
Contact: Mrs. Alma Johnson, Program Secretary, The Pittsburgh Conference, 300 Penn Center Boulevard, Suite 332, Pittsburgh, PA 15235-5503, USA.

April 6–8, 1992
Atlanta, GA, USA

* **ANATECH '92, 3rd International Symposium on Analytical Techniques for Industrial Process Control**
Contact: ANATECH '92, Infosciences Inc., 3000 Dundee Road, Suite 313, Northbrook, IL 60062, USA. Tel.: (708) 291-9161; Fax: (708) 291-0097.

April 6–8, 1992
Nancy, France

PREP-92, 9th International Symposium on Preparative and Industrial Chromatography
Contact: PREP-92 Secretary, E.N.S.I.C.–L.P.C.I., 1 rue Grandville, B.P. 451, F-54001 Nancy Cedex, France. Tel.: (33) 83300276; Fax: (33) 83350811. (Further details published in Vol. 543, No. 2.)

May 5–8, 1992
Liège, Belgium

4th International Symposium on Drug Analysis
Contact: Dr. J. Crommen, Drug Analysis '92-Liège, University of Liège, Institute of Pharmacy, rue Fusch 5, B-4000 Liège, Belgium. Tel.: (+3241) 237002; Fax: (+3241) 221855.

May 5–8, 1992
Munich, Germany

* **13th International Conference on Biochemical Analysis**
Contact: U. Arnold, Nymphenburger Strasse 70, D-8000 Munich 2, Germany. Tel.: (89) 1234500; Fax: (89) 183258.

May 12–14, 1992
La Grand Motte, France

4th European Meeting of Groupe Français de Bio-Chromatographie
Contact: Groupe Français de Bio-Chromatographie, Unité d'Immuno Allergie, Institut Pasteur, 28 rue de Docteur Roux, 75724 Paris Cedex 15, France. Tel.: (1) 45688000, ext. 7143; Fax: (1) 43069835; Telex: 250609 F. (Further details published in Vol. 540.)

May 17–22, 1992
Kyoto, Japan

4th International Conference on Fundamentals of Adsorption
Contact: Prof. M. Suzuki, Conference Chairman, Institute of Industrial Science, University of Tokyo, 7-22-1 Roppongi, Minatoku, Tokyo 106, Japan. (Further details published in Vol. 508, No. 2.)

June 2–4, 1992
Inuyama City, Japan

* **ISPAC-5, 5th International Symposium on Polymer Analysis and Characterization**
Contact: Dr. Sadao Mori, Faculty of Engineering, Mie University, Tsu, Mie 514, Japan. Fax: (592) 31-2252.

June 9–12, 1992
Dortmund, Germany

22nd Roland W. Frei Memorial Symposium on Environmental Analytical Chemistry and Workshop on Detection in Environmental Analysis
Contact: Symposium Office IAEAC, M. Frei-Häusler, P.O. Box 46, CH-4123 Allschwil 2, Switzerland. Tel.: (+41 61) 632789; Fax: (+41 61) 4820805. (Further details published in Vol. 543, No. 2)

June 14–19, 1992
Baltimore, MD, USA

* **HPLC '92, 16th International Conference on Column Liquid Chromatography**
Contact: HPLC '92, Ms. Shirley E. Schlessinger, 400 E. Randolph Drive, Suite 1015, Chicago, IL 60601, USA. Tel.: (312) 527-2011.

Aug. 24–27, 1992
Jena, Germany

COMPANA '92, 5th Conference on Computer Applications in Analytical Chemistry
Contact: COMPANA '92, Friedrich Schiller University Jena, Institute of Inorganic and Analytical Chemistry, Steiger 3, Haus 3, O-6900 Jena, Germany. Tel.: (82) 25467 or (82) 25029.

Aug. 31–Sept. 3, 1992
Cincinnati, OH, USA

* **106th Annual International Meeting and Exposition of the Association of Official Analytical Chemists**
Contact: Margaret Ridgell, AOAC, 2200 Wilson Boulevard, Suite 400, Arlington, VA 22201-3301, USA. Tel.: (703) 522-3032; Fax: (703) 522-5468.

Sept. 5–11, 1993 Edinburgh, U.K.	**EUROANALYSIS VIII, 8th European Conference on Analytical Chemistry** Contact: Miss P.E. Hutchinson, Analytical Division, The Royal Society of Chemistry, Burlington House,Piccadilly, London W1V 0BN, U.K. Tel.: (071) 4378656; Fax: (071) 7341227; Telex: 268001.
Sept. 13–18, 1992 Aix-en-Provence, France	* **19th International Symposium on Chromatography** Contact: G.A.M.S., 88 Boulevard Malesherbes, 75008 Paris, France. Tel.: (1) 45639304; Fax: (1) 49530434.
Oct. 6–9, 1992 Rome, Italy	* **ITP '92, 8th International Symposium on Capillary Electrophoresis and Isotachophoresis** Contact: Dr. Salvatore Fanali, Instituto di Cromatografica, C.N.R., Area della Ricerca di Roma, P.O. Box 10, 00016 Monterontondo Scalo, Rome, Italy. Tel.: (+39 6) 9005328; Fax: (+39 6) 9005849; Telex: 624809.
Nov. 4–6, 1992 Montreux, Switzerland	* **9th Montreux Symposium on Liquid Chromatography–Mass Spectrometry (LC–MS, SFC–MS, CZE–MS, MS–MS)** Contact: Marianne Frei, IAEAC Secretariat, P.O. Box 46, CH-4123 Allschwil, Switzerland. Tel.: (+41 61) 632789; Fax: (+41 61) 4820805.
May 9–13, 1993 Hamburg, Germany	* **HPLC '93, 17th International Symposium on Column Liquid Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abt. Tagungen. P.O. Box 900440, D-6000 Frankfurt 90, Germany.
June 20–24, 1994 Bournemouth, UK	* **20th International Symposium on Chromatography** Contact: Executive Secretary, The Chromatographic Society, Nottingham Polytechnic, Burton Street, Nottingham, NG1 4BU, UK. Tel.: (0602) 500596; Fax: (0602) 500614.

* Indicates new or amended entry

PUBLICATION SCHEDULE FOR 1991

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	D 1990–M 1991	J	J	A	S	O	N	D
Journal of Chromatography	Vols. 535–545/1	545/2 546/1 + 2 547/1 + 2	548/1 + 2 549/1 + 2 550/1 + 2	552/1 + 2 553/1 + 2 554/1 + 2 555/1 + 2	556/1 + 2 557/1 + 2 558/1	558/2 559/1 + 2		
Cumulative Indexes, Vols. 501–550				551/1 + 2				
Bibliography Section	560/1	560/2			561/1			561/2
Biomedical Applications	Vols. 562–566	567/1	567/2 568/1	568/2	569/1 + 2 570/1	570/2	571/1 + 2	572/1 + 2

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 558, pp. 469–472. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Review articles and Short Communications. Short Communications are usually descriptions of short investigations, or they can report minor technical improvements of previously published procedures; they reflect the same quality of research as Full-length papers, but should preferably not exceed six printed pages. For Review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he/she is submitting the paper for publication in the *Journal of Chromatography*.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Abstract, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

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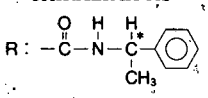

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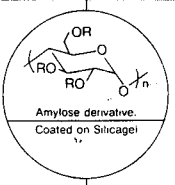

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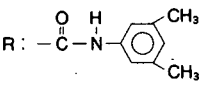
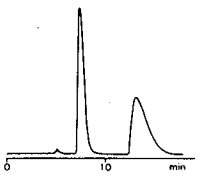
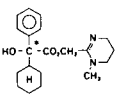


4-Acetoxy-2-azetidine

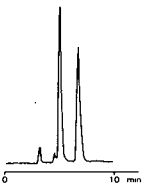
Eluent : Hexane/Ethanol = 8/2
Flow rate : 1.0ml/min
Temperature : r.t.
Detection : UV254nm



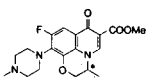
Oxyphencyclimine



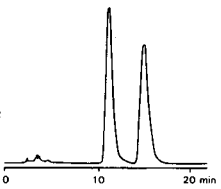
Eluent : Hexane/2-Propanol = 9/1
Flow rate : 1.0ml/min
Temperature : r.t.
Detection : UV254nm



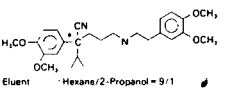
Ofloxacine methyl ester



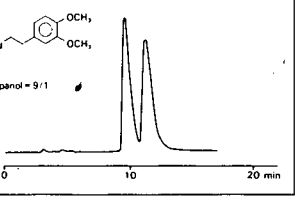
Eluent : Hexane/EtOH = 8/2
Flow rate : 1.2ml/min
Temperature : 40°C
Detection : UV254nm



Verapamil



Eluent : Hexane/2-Propanol = 9/1
Flow rate : 1.0ml/min
Temperature : r.t.
Detection : UV254nm



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