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EXTRACTIVE PENTAFLUOROBENZYLATION OF FORMIC, ACETIC, LEVULINIC, BENZOIC AND PHTHALIC ACIDS, STUDIED BY LIQUID CHROMATOGRAPHY AND DUAL-OVEN CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

Extractive pentafluorobenzylation of different short-chain carboxylic acids, including formic acid and a dicarboxylic acid, phthalic acid, was examined. Extractive alkylations were carried out in two different two-phase systems, *viz.*, dichloromethane–water and methyl isobutyl ketone–water, at a reaction temperature of 25°C. Quantitative yields were obtained within 50 min for both solvent systems, except for phthalic acid, which required an alkylation time exceeding 65 min in the dichloromethane–water system. Extractive pentafluorobenzylation of the monocarboxylic acids followed second-order rate kinetics.

The derivatives formed have good liquid and gas chromatographic properties. In order to utilize fully the electron-capture properties of the esters formed, a dual-oven capillary gas chromatographic separation procedure with electron-capture detection was developed.

INTRODUCTION

An important class of organic compounds with considerable biological interest and environmental and technical significance is short-chain carboxylic acids. These hydrophilic carboxylic acids are found in various aqueous solutions such as fermenter broth[1,2], plasma and serum[3–5], intravenous solutions[6], resulting from cleavage reactions of carbohydrates[7], sea water[8] and rain and fog samples[9].

The development of methods for the determination of low concentrations of short-chain carboxylic acids has gained considerable interest. The methods have generally followed two lines: either stable derivatives are prepared followed by

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chromatographic analysis[4–6,8–19], or direct analysis of the anion by ion chromatography is performed[7,20,21]. Moreover, the analysis of underivatized carboxylic acids by gas chromatography (GC) have been demonstrated[1–3]. Sensitive methods have been developed involving alkylation of the carboxylic acid with incorporation of a moiety with a good electron-capture response, *e.g.*, pentafluorobenzyl[4,8,14,15] and bromophenacyl[22]. The high sensitivity of the electron-capture detector to formic and acetic acid derivatives, especially the former, is generally obscured by excess of reagent or other matrix-associated compounds, however[4,14,15,22]. Further, halogenated solvents cannot be used with electron-capture detectors. Evaporation of, *e.g.*, dichloromethane followed by dissolution of the residue in *n*-hexane increases the possibility of losses of the derivatives and also prolongs the work-up procedure. However, the good electron-capture response and stability of pentafluorobenzyl derivatives make pentafluorobenzyl bromide a good candidate as an alkylation reagent for short-chain carboxylic acids.

Extractive alkylation, since its introduction as an analytical technique by Ehrsson[23], has been applied to a wide range of compounds. Gas chromatographic determinations of, *e.g.*, carboxylic acids[5,12,15,16,22,24], phenols[23] and theophylline[25] have been reported.

In this paper a study of the extractive alkylation of formic, acetic, levulinic, benzoic and phthalic acids with tetrahexylammonium as the counter ion and pentafluorobenzyl bromide as alkylation agent in two different solvent systems is described. The kinetics of the extractive alkylation were studied in dichloromethane–water and methyl isobutyl ketone–water two-phase systems. The effect of temperature on the reaction rate of formic acid was also investigated. Moreover, in order to circumvent the problems connected with excess of reagent and matrix-associated compounds and to be able to analyse the dichloromethane extract directly, although an electron-capture detector was used, dual-oven capillary GC separation procedures were also demonstrated. To exemplify the feasibility of the extractive alkylation method in combination with dual-oven capillary GC, a heat-sterilized dialysis solution was examined.

EXPERIMENTAL

Chemicals and reagents

Formic, acetic, acrylic and propanoic acid of analytical-reagent grade (Merck) and benzoic and phthalic acid of synthesis quality (Merck) were used. Levulinic acid and the internal standards fluoroacetic acid and *p*-fluorobenzoic acid were obtained at a purity of 98% (Sigma). Tetrahexylammonium hydrogen sulphate was obtained from Niel Clauson-Haas or Fluka (purum quality). The derivatization reagent was 2,3,4,5,6-pentafluorobenzyl bromide (Fluka).

Dichloromethane (Merck) and methyl isobutyl ketone (Merck) of analytical-reagent grade were used without further purification. Milli-Q purified water was distilled after refluxing with potassium persulphate (1 g dm^{-3}) and phosphoric acid (1 cm^3 dm^{-3}) in an all-glass system. Phosphate buffers were prepared from sodium dihydrogenphosphate monohydrate (Merck) and disodium hydrogen phosphate dihydrate (Merck). Orthophosphoric acid (Merck) of Suprapur quality was used.

Preparation of standards

The acids (9 mmol) were derivatized with a mixture of 50 ml of acetone, 2.8 mmol of pentafluorobenzyl bromide and 6 mmol of potassium carbonate, the solution being refluxed for 6 h and then filtered[10]. The solvent was removed in a Rotavaporator and the residue was dissolved in 10 ml of *n*-hexane and washed with 50 ml of water. The hexane extract was then flushed through a silica gel column with *n*-hexane, which removed the remainder of the acid and the reagent. After evaporation, the esters were diluted with dichloromethane, checked by purity and identified by gas chromatography–mass spectrometry.

Pentafluorobenzyl esters of formic, acetic, acrylic, propanoic, levulinic, benzoic, phthalic, monofluoroacetic and *p*-fluorobenzoic acids were prepared.

Apparatus

The liquid chromatographic (LC) equipment used consisted of an LDC Minipump and an LDC UV-III detector. The column (25 cm × 4.6 mm I.D.) was packed with μBondapack C_{18} (mean particle diameter 10 μm) and HPLC-grade acetonitrile (Rathburn Chemicals) and Milli-Q filtered water were used as the mobile phase. Generally, the mobile phase was acetonitrile–0.001 *M* sulphuric acid (30:70) at a flow-rate of 1.0 ml min^{-1}. Peak-area integration was effected with a Spectra-Physics Minigrator.

The gas chromatographs used were connected in tandem by two different laboratory-constructed cold trap/reinjection interfaces. Either an interface based on Deans switching developed for multi-separation GC[26] or a valve-based interface was used. The valve-based interface was a slightly modified dynamic headspace equipment[27].

The first gas chromatograph in the tandem system was a Varian 1400 gas chromatograph modified with a laboratory-constructed glass-lined injector for capillary column GC. The first column, *i.e.*, the precolumn, was 10 m × 0.53 mm I.D. and contained CP-wax 57 (Chrompack) with a film thickness of 3.0 μm. The column temperature was programmed from 50 to 220°C at 10°C min^{-1}. The flame ionization detector temperature was 250°C and the injector temperature 200°C. The interface was maintained at 225°C. The second gas chromatograph was either a Perkin-Elmer Sigma 1 or a Carlo Erba Fractovap 4160. The Perkin-Elmer instrument was equipped with a CP-wax 52 fused-silica capillary column (25 m × 0.22 mm I.D.). The electron-capture detector temperature was 300°C. The column temperature was either maintained isothermally at 90°C or programmed from 90 to 240°C at 10°C min^{-1}. A schematic diagram of this set-up is shown in Fig. 1A.

The Carlo Erba gas chromatograph was equipped with a DB-5 fused-silica capillary column (30 m × 0.25 mm I.D.) with a film thickness of 0.25 μm (J & W Scientific). The column was temperature programmed from 70 to 235°C at 10°C min^{-1}. The electron-capture detector temperature was 260°C. A schematic diagram of this set-up is shown in Fig. 1B.

The integration of peaks generated on the second gas chromatograph was performed on a Nelson 6000 laboratory data system.

Fractions from the first column were transferred to the second column by intermediate cold trapping in order to effect solute band concentration (focusing).

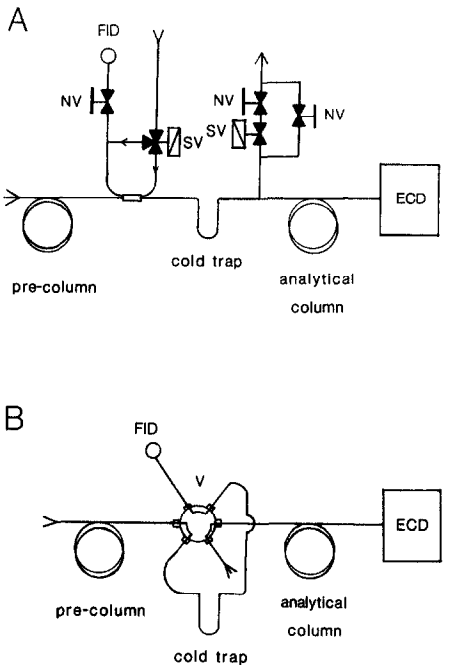

Fig. 1. Schematic diagram of capillary multi-separation configurations. NV = needle valve; SV = solenoid valve; FID = flame ionization detector; ECD = electron-capture detector; V = Valco six-port valve. (A) Capillary multi-separation technique based on flow switching according to Deans. (B) Capillary multi-separation technique based on flow switching with a rotary valve.

Kinetic methods

The acid (1.82 μmol) in 1.00 ml of phosphate buffer (pH 7.4, ionic strength = 0.1) and 1.00 ml of dichloromethane or methyl isobutyl ketone with pentafluorobenzyl bromide (138 μmol), tetrahexylammonium hydrogensulphate (10 μmol) and 0.100 ml of internal standard solution in dichloromethane or isobutyl methyl ketone were shaken in 10-ml tubes on an Evapo-mix (Buchler Instruments). One tube for each measuring interval was prepared. After an appropriate reaction time, the reaction was inhibited by addition of 5 ml 1.0 *M* phosphoric acid to the tube. After centrifugation and phase separation, the organic phase was diluted with 2.0 ml of acetonitrile. The organic phase was reduced to 1.0 ml by evaporation by blowing with nitrogen at room temperature. This was carried out in order to reduce most of the dichloromethane present in the sample, *i.e.*, to make the sample solution more compatible with the reversed-phase LC conditions used. The solution obtained was further diluted with 1.5 ml of acetonitrile and analysed by reversed-phase LC. A typical isocratic LC profile is shown in Fig. 2.

Peak areas obtained with the different measuring intervals were calibrated using the internal standard technique.

Extractive alkylation of dialysis solutions for GC analysis

Samples of the aqueous solution (0.5 ml) were shaken thoroughly with

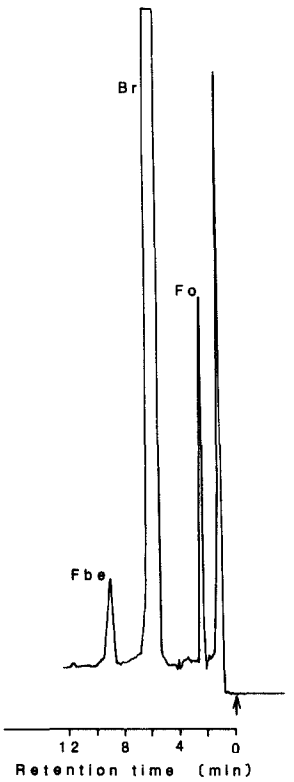

Fig. 2. Isocratic LC separation of a mixture of pentafluorobenzyl bromide (Br), pentafluorobenzyl ester of formic acid (Fo) and pentafluorobenzyl ester of monofluorobenzoic acid (Fbe). Flow-rate, 1.0 ml/min; mobile phase, acetonitrile–0.001 *M* sulphuric acid (30:70). UV detection at 254 nm. Full-scale recorder deflection corresponds to 0.1 absorbance units.

dichloromethane (1 ml) at 30°C for 3–4 h. The organic phase contained the derivatizing reagent, pentafluorobenzyl bromide (25 μl ml^{-1}) and tetrahexylammonium hydrogensulphate (11.7 mg). The dialysis solution was phoshate-buffered (pH = 7.4) prior to the extractive alkylation. After an appropriate reaction time, the organic phase was washed with excess of 1 *M* phosphoric acid, the aqueous layer removed and the dichloromethane solution diluted with 5 ml of *n*-hexane. After centrifugation the organic phase was directly subjected to multi-separation capillary GC. Alternatively, the dichloromethane extract was evaporated to dryness and the residue dissolved in 200 μl of *n*-hexane.

RESULTS AND DISCUSSION

Determination of rate constants

The extractive alkylation can be described as a two-step reaction, which is illustrated by the following equations:

$$Q^+ + A^- \rightleftharpoons QA_{org} \quad (1)$$
(ion-pair extraction)

$$QA_{org} + PFBBr_{org} \rightarrow PFBA_{org} + QBr_{org} \quad (2)$$
(alkylation)

where Q^+ represents the quaternary ammonium ion, A^- the anion of the acid, QA_{org} the ion pair in the organic phase, PFBBr pentafluorobenzyl bromide and PFBA the benzyl ester of the acid. The evaluation of the rate constants follows the principles given by Tivert and Gustavii[28]. The kinetic model used for two-phase alkylation is given by the equation

$$\ln ([PFBA]_\infty - [PFBA]_t) = \ln C_{A,0} - k'_{A,obs}t \quad (3)$$

where $k'_{A,obs}$ is the observed pseudo-first-order rate constant when the alkylation reagent and the quaternary ammonium ion are present in excess. A straight line with a slope equal to $-k'_{A,obs}$ is obtained on plotting $\ln([PFBA]_\infty - [PFBA]_t)$ *versus* t. The observed pseudo-first-order rate constants obtained for the two different alkylation solvent systems are given in Table I.

The extractive alkylations were followed for more than five half-lives. For the two different two-phase systems complete alkylation (*i.e.*, >99% yield) is obtained within 50 min, except for phthalic acid in the dichloromethane–water system (>65 min). The alkylation proceeds throughout more rapidly in the methyl isobutyl ketone–water system than in the dichloromethane–water system. It is not clear why this is so, but it may be attributed to the solution properties of methyl isobutyl ketone. Dipolar aprotic solvents, such as ketones, are known to solvate cations, whereas anions are poorly solvated[29]. As a result, many reactions can be considerably more rapid in dipolar aprotic solvents when the reaction involves anions.

TABLE I

OBSERVED PSEUDO-FIRST-ORDER RATE CONSTANTS FOR TWO-PHASE ALKYLATION

Alkylation reagent, 0.138 *M* pentafluorobenzyl bromide; $C_A = 1.82 \cdot 10^{-3} M$ at pH 7.4; $C_Q = 0.01$ *M*; the alkylations were carried out at 25°C.

Acid	$k'_{A,obs}$ (min^{-1})	
	Dichloromethane–water	*Methyl isobutyl ketone–water*
Formic	0.097	0.123
Acetic	0.106	0.137
Levulinic	0.123	0.148
Benzoic	0.127	0.143
Phthalic*	0.070	0.101

* Apparent value; see discussion on dicarboxylic acids.

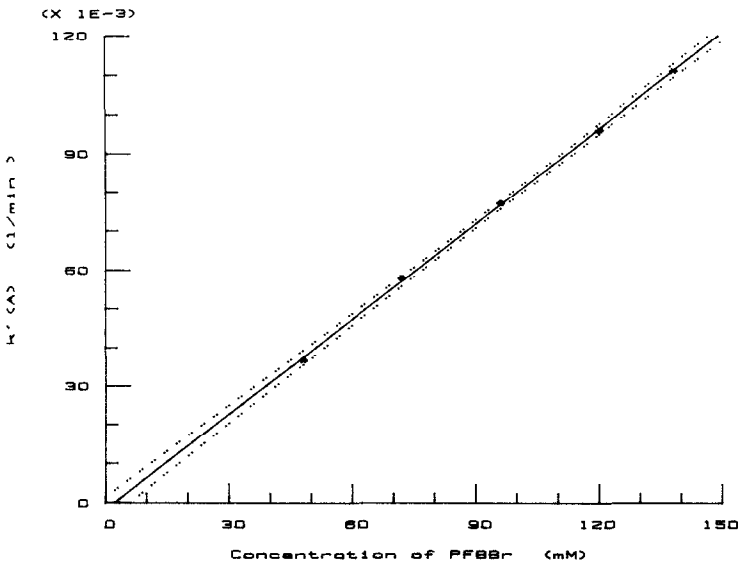

Fig. 3. Extractive alkylation of formic acid with pentafluorobenzyl bromide. Pseudo-first-order rate constant at different concentrations. The dotted lines indicate the confidence limits at the 95% level.

When the concentration of the alkylation reagent PFBBr was increased, $k'_{A,obs}$ also increased. According to Tivert and Gustavii[28], $k'_{A,obs}$ is related to the second-order rate constant k_A and the concentration of PFBBr by

$$k'_{A,obs} = k_A C_{PFBBr} \quad (4)$$

A plot of $k'_{A,obs}$ *vs.* C_{PFBBr} should give a straight line passing through the origin. The slope of the line gives the second-order rate constants. The second-order rate constant for formic acid was 0.81 l mol^{-1} min^{-1} at a reaction temperature of 35°C (see Fig. 3). This result indicates that the reactions follow second-order reaction kinetics and, together with the results in Table I, that the extractive benzylation is complete and that no major side-reactions occur.

The corresponding second-order rate constants for the other acids could be calculated using eqn. 4 and the information given in Table I. For example, the second-order rate constant for acetic acid in the dichloromethane–water system is 0.77 l mol^{-1} min^{-1} at 25°C. This value is considerably higher than the published second-rate order constant (0.018 l mol^{-1} min^{-1}) of acetic acid in a corresponding two-phase alkylation reaction but with tetrabutylammonium as counter ion and phenacyl bromide as alkylation agent[30]. This confirms that for hydrophilic acids a higher degree of extraction is obtained with larger counter ions such as tetrahexylammonium[25]. Further, the comparison gives additional support to the assumption that a highly lipophilic counter ion is needed for extractive alkylation of formic and acetic acid in order to achieve quantitative alkylation and sufficiently short analysis times. It has been reported[31] that an increase in the lipophilic character of the counter ion increases the degradation of pentafluorobenzyl bromide. This was considered to be

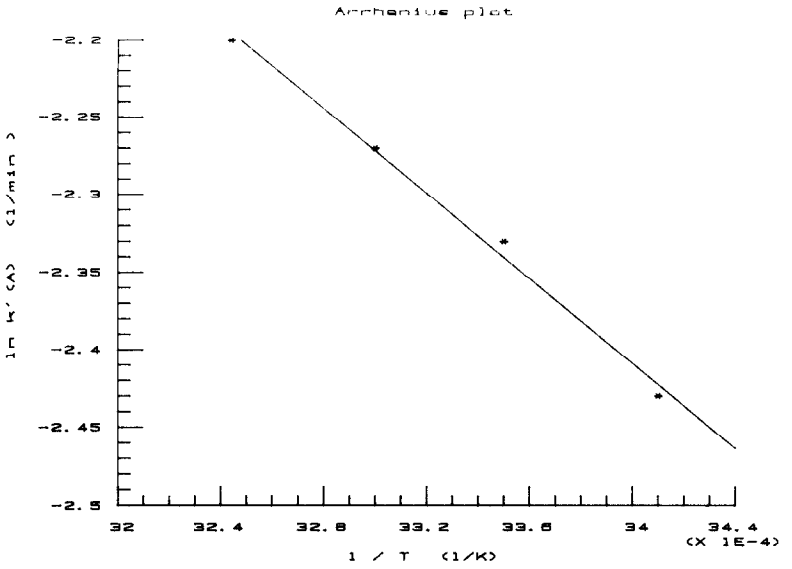

Fig. 4. Extractive alkylation of formic acid with pentafluorobenzyl bromide at different reaction temperatures.

due to an enhanced extraction of hydroxy ions into the organic phase, with a subsequent increase in the hydrolysis rate of the reagent. Those studies were carried out at pH 10 or above, whereas in the present study alkylation was performed at pH 7.4 with a phosphate buffer. This restricts the extraction of hydroxy ions and explains why no apparent degradation of the reagent is observed.

Phthalic acid has two reactive sites, generating a dibenzylated ester. Dialkylation of nonanedioic acid[30] has been proposed to proceed through the extraction into dichloromethane as Q_2A and a monoalkylated intermediate, followed by a reaction forming the dialkylated end product. The steps are governed by the pseudo-first-order rate constants k_1' and k_2', respectively. By establishing suitable equations and by plotting the concentration of dialkylated acid *versus* time the constants can be calculated by non-linear curve fitting[30]. However, as the reaction of phthalic acid proceeded approximately according to an observed pseudo-first-order rate, no attempt was made in this work to deconvolute the underlying rate constants.

In order to study the effect of temperature on alkylation, the reaction rate of formic acid in the range 20–35°C was studied. An increase in the reaction temperature from 20 to 35°C increased the pseudo-first-order rate constant from 0.088 to 0.111 min^{-1} (see Fig. 4). The Arrhenius activation energy for formic acid was 11.4 kJ mol^{-1}.

The pentafluorobenzyl esters formed on extractive alkylation have good UV absorption properties and are therefore suitable for LC. However, in order to utilize fully the excellent electron-capture properties of the pentafluorobenzyl derivatives, electron-capture detection (ECD) should be used. The problems associated with attaining good sensitivity when using ECD include chemical nosie due, *e.g.*, to halogenated solvents and the presence of interfering compounds. Owing to decrease in the ECD response when subjected to an excess of, *e.g.*, dichloromethane combined

with overlapping of the formic and acetic acid ester peaks by the pentafluorobenzyl bromide peak, the excess of the alkylation reagent and halogenated solvents must be removed prior the final analysis step. In order to overcome the problems due to the alkylation reagent, Chauhan and Darbre[4] used a column coated with PPSeb stationary phase and a low excess ratio (*e.g.*, 15) of pentafluorobenzyl bromide to the acid. For *p*-bromophenacyl esters, Kawamura and Kaplan[9] separated the alkylation reagent from the esters on a silica gel LC column. This procedure involved two evaporation steps. However, purification on silica gel columns is of limited value because, as pointed out by Davis[14], some decomposition of the pentafluorobenzyl esters occurs.

Brötell *et al.*[32] used two-dimensional GC and heart cutting in order to remove the excess of reagent and to shorten the analysis time in the determination of an amino alcohol in serum after trifluoroacetylation. In this work a similar approach was also utilized.

For removal of the alkylation reagent we developed a capillary precolumn–capillary column system based on multi-dimensional GC technology[26]. By use of the capillary precolumn technique, crude dichloromethane extracts can be analysed without distortion of the ECD performance. A typical example of the GC technique is given in Fig. 5.

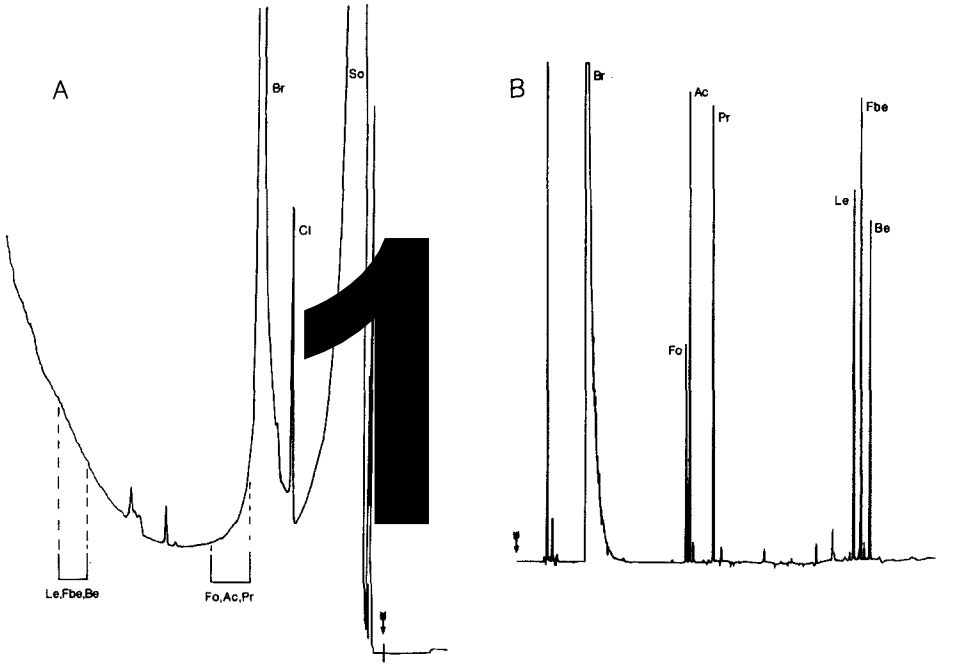

Fig. 5. Chromatograms generated by extractive pentafluorobenzylation of a standard saline solution. So = dichloromethane; Cl = pentafluorobenzyl chloride; Br = pentafluorobenzyl bromide, in chromatogram A also decomposition products of the counter ion; Fo = formic acid; Ac = acetic acid; Pr = propanoic acid; Le = levulinic acid; Be = benzoic acid; Fbe = monofluorobenzoic acid. (A) Chromatogram generated by injection of crude dichloromethane extract with flame ionization detection. Stationary phase, CP-wax 57. (B) Chromatogram obtained by intermediate cryogenic trapping of the indicated fractions from chromatogram A. Detection with electron-capture detector. Stationary phase, CP-wax 52.

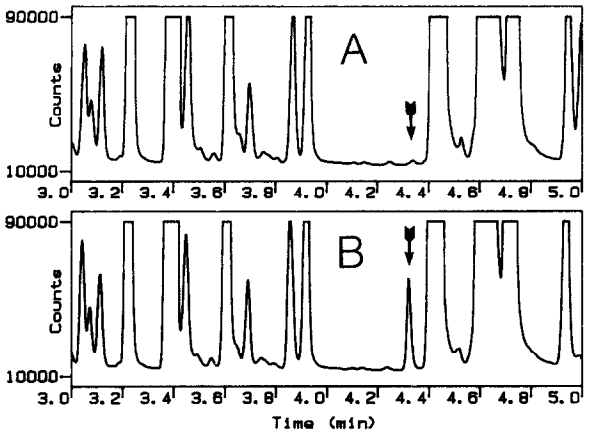

Fig. 6. Part of the chromatograms generated by reinjection on the non-polar DB-5 column of a 60 s wide fraction, with intermediate cryogenic trapping, from the polar CP-wax 57 pre-column. (A) Reinjected fraction from extractive alkylation of dialysis solutions. (B) Reinjected fraction from extractive alkylation of dialysis solutions fortified with acrylic acid (260 ng ml^{-1}).

Apart from nearly complete removal of dichloromethane and the derivatization reagent, the first GC set up, with a polar precolumn and a polar analytical column (see Experimental) is characterized by the resolution of pentafluorobenzyl bromide and the pentafluorobenzyl ester of formic acid. Similar resolutions were not obtained on the less polar column used in the second separation stage. Owing to the cross-linking of the Carbowax stationary phase, low column bleeding is obtained in the working temperature range, with a subsequent low distortion of the ECD performance.

The use of multi-separation procedures utilizing columns of different selectivity has been found to be of a great advantage in the analysis of complex mixtures[27]. In the analysis of dialysis solutions, the increased separation power achieved by using two columns with different selectivities was needed in order to separate the pentafluorobenzyl esters from other matrix-associated compounds with an ECD response. The dialysis solutions contained glucose at up to 44 mg ml^{-1}, sodium lactate at *ca.* 4.5 mg ml^{-1} and sodium chloride at 5.8 mg ml^{-1}. Prior to use, these solutions were heat sterilized. Apart from thermal degradation of the dialysis solution, impurities can migrate from the PVC bag containing the solutions. Lactate and chloride ion are extracted as ion pairs into the organic phase and react with the reagent. Therefore, in order to avoid depletion of the pentafluorobenzyl bromide, the reagent concentration must be in excess of the sum of the molar concentrations of the lactate and chloride. Further, to allow the extractive alkylation to proceed at a sufficient rate, the concentration of the counter ion has to be high. This implies that the molar concentration of the reagent and the counter ion are far above the concentration of the acids under study. For example, the excess ratio of the reagent to some minor acids is $> 10^5$ (w/w) and the the counter ion also $> 10^5$ (w/w). Hence, even very low concentrations of impurities, *e.g.*, formic acid, in the extractive alkylation reaction chemicals could severely affect the analysis results for the dialysis solutions. It was found that it was not possible to determine formic and acetic acid in the low ng ml^{-1} range without further purification of the reagent and counter ion. Especially the latter has to be purified if the

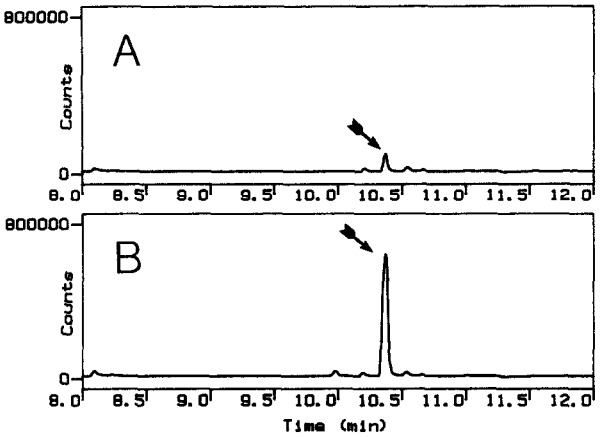

Fig. 7. Part of the chromatograms generated by reinjection on the non-polar DB-5 column of a *ca.* 60 s wide fraction, with intermediate cryogenic trapping, from the polar CP-wax 57 pre-column. (A) Reinjected fraction from extractive alkylation of a dialysis solution. (B) Reinjected fraction from extractive alkylation of a dialysis solution fortified with benzoic acid (284 ng ml^{-1}).

proposed method is to be used for the determination of formic and acetic acid in dialysis solutions. However, acrylic acid could be determined in the low ng ml^{-1} range by the proposed method (see Fig. 6), although the results are strongly dependent on the high resolution properties of multi-dimensional GC. The fraction that contains the ester of acrylic acid is close to the fractions containing the bulk of the reagents and degradation compounds of the counter ion. The situation improves considerably when fractions that are further away from the bulk of the reaction chemicals on the precolumn are analysed on the second column. As an example, the analysis of the pentafluorobenzyl ester of benzoic acid is shown in Fig. 7. These chromatograms show that the detection of short-chain carboxylic acids ($>C_2$) in the low ng ml^{-1} range is possible even in very complex solutions by direct analysis of extractive pentafluorobenzylation extracts by dual-oven capillary GC with ECD.

This work has demonstrated that extractive pentafluorobenzylation of the studied acids in combination with dual-oven capillary GC may be a feasible method for the trace analysis of complex samples. However, in order to validate the method for quantitative analysis, a more complete study of analytical parameters such as impurities in reagents, reproducibility and detection limits has to be carried out.

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