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C_{18} REVERSED-PHASE TRACE ENRICHMENT OF SHORT- AND LONG-CHAIN (C_2–C_8–C_{20}) FATTY ACIDS FROM DILUTE AQUEOUS SOLUTIONS AND SEA WATER

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SUMMARY

A method is presented for the quantitative determination of aliphatic carboxylic (fatty) acids in dilute aqueous solutions. C_{18} reversed-phase trace enrichment allows for recoveries ranging from 35% for acetic acid to 100% for heptanoic and higher acids. The influence of the flow-rate, the matrix and the pH of water on the efficiency of enrichment is established. To enhance detectability, acids are converted into pentafluorobenzyl esters by extractive alkylation. The optimum conditions of derivatization are studied for a wide spectrum of aliphatic acids. The derivatives are analysed by means of capillary column gas chromatography with electron-capture detection. The described method is employed for the determination of fatty acids in sea water. The separating power of a fused-silica capillary column permits the resolution of some 40 peaks which are identified both by correlation of retention times with actual standards and/or by gas chromatography–mass spectrometry.

INTRODUCTION

Fatty acids dissolved in sea water are used as indicators of the transformation and distribution of moderately labile compounds[1,2]. This requires processing of many samples in a relatively short, ship-board time, where they are routinely extracted from 5–20 dm^3 of water and analysed by gas chromatography after conversion into methyl esters, which exhibit better chromatographic properties than the free acids[1–8]. In this procedure a concentration factor of 1000–10,000 for relatively insoluble long-chain acids, combined with their relatively high concentration, allows utilization of a flame-ionization detector (FID)[1–3]. However, it usually fails to produce information on acids with fewer than ten carbon atoms[9] because of their very low concentrations in sea water and their unfavourable coefficients of distribution between water and organic phases[10]. Therefore a need arises for a method of simul-

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taneously determining long- and short-chained acids over a broad range of concentrations in sea water. Two problems must be resolved in this respect: (i) preconcentration of the acids from the water and (ii) their separation and quantification.

The amounts of acid needed for quantification can be dramatically decreased by conversion of the acids into halogenated derivatives and use of an electron-capture detector (ECD)[8], which is several orders of magnitude more sensitive than an FID. Among other methods, alkylation of acids in water[11], extractive alkylation[7,12,13], and crown-ether-catalysed alkylation[14,15] can be employed. Extractive alkylation is based on the extraction of ion pairs from water into an organic solvent which only slightly solvates the anions, and on subsequent derivatization with an alkylating agent dissolved in the solvent, in an S_N2-type reaction[7,13,16]. Alkylation with pentafluorobenzyl bromide (PFBBr) produces esters characterized by excellent gas chromatographic properties and sensitivity[7,12,13,17–20]. When these derivatives are used, preconcentration of acids by a factor of 1000 should provide enough material for quantification of acids in the picomole/dm^3 concentration range from 1 dm^3 of water[12].

Dimethyl ether has been used for extraction of short-chain acids from aqueous solution[21,22]; however, a high solvent-to-water ratio had to be used to ensure quantitative recovery. Thus this method cannot be used when large volumes of water are to be processed. Other techniques of preconcentration such as distillation and drying[10,11] and freeze-drying are also deemed impractical because of time limitations and the need for processing relatively large volumes of sea water. Adsorption on a solid matrix is commonly used for extraction of trace organics from water[23]. Macroreticular resins such as Amberlites® as well as strong and weak anion exchangers have been shown to have a high retention capacity towards acids[24,25]; however, low breakthrough volumes were observed in another study[26]. Reversed-phase trace enrichment provides another possibility which has not yet been tested for fatty acids, but which has been successfully applied to the isolation of chlorinated hydrocarbons[27], phenols[28], high-molecular-weight substances[29] and metal–organic complexes[30] from surface water. High concentration factors can be achieved by this technique because of the small amounts of solvent necessary for elution.

In this study a procedure for the determination of short- and long-chain acids is described, which involves in essence preconcentrations, derivatization and gas chromatographic separation. Preconcentration is based on the C_{18} reversed-phase trace-enrichment technique. After enrichment, acids are converted into their pentafluorobenzyl esters and analysed by capillary gas chromatography with an ECD. The method has been successfully used for the quantification of both short- and long-chain acids in sea water.

MATERIALS AND METHODS

Reagents and materials

Organic-free water was obtained by refluxing water obtained from a Milli Q® system (Millipore, U.S.A.) with potassium persulphate (1 g/dm^3) and phosphoric acid (1 cm^3/dm^3) for 1 h and then distilling it in an all-glass system. Methanol and methylene chloride were pesticide grade. Hydrochloric acid, ammonium hydroxide and tetrabutylammonium hydrogen sulphate (TBA-HSO_4) were analytical-reagent grade. PFBBr was obtained from Pierce (U.S.A.). Fatty acids, both straight-chain

and branched, which were used to prepare the standards, were obtained from Analabs (U.S.A.). Phosphate buffer (pH 7.4) as well as borax buffer (pH 9.2) were prepared from prepacked buffer salts obtained from Fisher (U.S.A.). 10-Undecenoic acid and 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethylene (*o,p*-DDE), obtained from Analabs, were used as internal standards. C_{18} reversed-phase prepacked cartridges were obtained from Waters (U.S.A.). In order to minimize contamination of samples, all-glass syringes, Teflon® tubing, reaction vials equipped with Teflon-lined caps, and Teflon-covered magnetic stirring bars were used. All reagents and materials were tested for blanks. Ammonium hydroxide had to be further purified by isothermic distillation. C_{18} cartridges were cleaned by passing methylene chloride (20 cm^3), methanol (70 cm^3), 0.1 *M* ammonium hydroxide (2 cm^3) and water (2 cm^3) through them before use.

C_{18} reversed-phase trace enrichment

Water samples (1–2 dm^3) of different pH in the range 1–7 were spiked with 25.0 pmoles to 25.0 nmoles of the following acids: acetic, propanoic, 2-methylpropanoic, butanoic, 2-methylbutanoic, pentanoic, 4-methylpentanoic, hexanoic, 4-methylhexanoic, heptanoic, 6-methylheptanoic and octanoic; they were subsequently passed through the C_{18} cartridges at a flow-rate of 5–30 cm^3/min. The cartridges were then washed with 1 cm^3 of 0.01 *M* hydrochloric acid. Sorbed acids were eluted directly into reaction vials with 2 cm^3 of 0.1 *M* ammonium hydroxide in water–methanol (50:50) followed by 1 cm^3 of methanol. Acetone and methanol were also tested as eluting agents. After stripping the ammonia and methanol in a gentle stream of nitrogen, the acids remaining in the water (1 cm^3) were derivatized.

Extractive alkylation with PFBBr

Two buffers (borax and phosphate) were tried, and the concentrations of PFBBr, as well as the time, temperature and solvent:water ratio, were varied in order to find the optimal conditions for simultaneous derivatization of the wide spectrum of acids. The following procedure, which is a variation of the procedure used by Fogelqvist *et al.*[12], was chosen as the most suitable.

To the aqueous solution produced in the trace-enrichment step, 0.1 cm^3 of 0.2 *M* TBA-HSO_4 in water was added followed by 0.1 cm^3 of borax buffer (pH 9.2). After mixing, 0.5 cm^3 of 0.20 % (w/w) PFBBr solution in methylene chloride containing 1.2 nmoles (378 ng) of *o,p*-DDE and 1.0 nmoles of 10-undecenoic acid was added, a stirring bar was dropped in and the vial was tightly closed with a cap. The derivatization was carried out in a constant-temperature heating block (105°C for 3 h). During this time the reaction mixture was vigorously agitated with the stirring bar. The aqueous and organic phases were separated thoroughly by centrifuging the vial at 1000 *g*. The organic layer, which was covered by a layer of water, was taken into a syringe without removal of the water layer.

In order to establish the efficiency of the derivatization, standard solutions of acetic, propanoic, butanoic, pentanoic, hexanoic, heptanoic, octanoic, nonanoic, decanoic, undecanoic, dodecanoic, tetradecanoic, hexadecanoic and oxtadecanoic acids in methanol were used. To 1 cm^3 of water were added 1.0 cm^3 of these standards containing 20.0 pmoles to 2.0 nmoles of individual acids, 0.1 cm^3 of the TBA-HSO_4 solution and 0.1 cm^3 of borax buffer. Methanol was stripped off in a stream of

nitrogen and the remaining aqueous solution (1 cm^3) was treated as described previously for derivatization of the trace-enrichment concentrates.

Gas chromatography

The organic extracts (2.0 μl) were injected into a Hewlett-Packard 5730A gas chromatograph equipped with a ^{63}Ni electron-capture detector and a SE-30 (30 m × 0.25 mm) fused-silica capillary column obtained from J&W Scientific (U.S.A.). The temperature was initially held at 120°C for 2 min, then increased at 4°C/min to 270°C and finally held at 270°C for 16 min. Helium was used as the carrier gas (1.0 cm^3/min) and argon–methane (95:5) as the make-up gas (39 cm^3/min). The instrument was used in the splitless mode, and detector signals were integrated with a Hewlett-Packard 3390A reporting integrator.

Quantification

In the range tested, there was a linear relationship between the amounts of acids used and the amounts of esters obtained. Calibration curves were prepared for each ester and all quantifications were based on these curves.

Gas chromatography–mass spectrometry

A Finnigan 4000 gas chromatograph–mass spectrometer in the electron-impact mode (70 eV energy) with an INCOS data system was utilized. The organic extracts (2.0 μl) were injected at 250°C onto an SP 2100 (25 m × 0.2 mm) fused-silica capillary column. The temperature was initially held at 50°C for 2 min and then increased at 4°C/min to 250°C where it was held for 16 min. The mass range was scanned from m/z 45 to 500 every 2 sec.

RESULTS AND DISCUSSION

Reversed-phase trace-enrichment step

Data presented in Fig. 1 suggest that the pH of the water passed through the

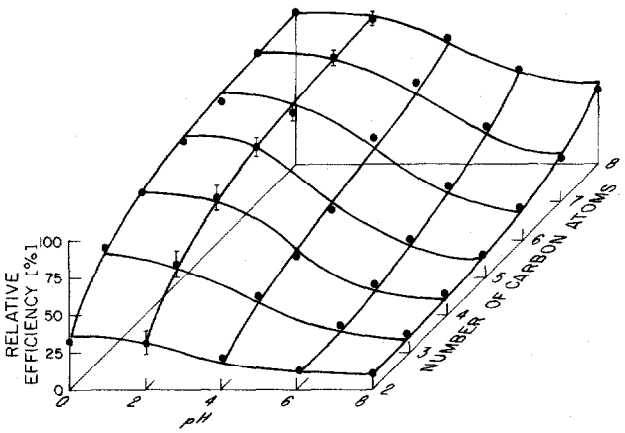

Fig. 1. Relation between the effectiveness of the C_{18} reversed-phase trace enrichment of short-chain carboxylic acids and the pH of the aqueous solution. (Error bars indicate one standard deviation above and below the mean as determined from six experiments.)

cartridges strongly influences the efficiency of sorption. Undoubtedly this is connected, through the degree of dissociation, to the polarity of the molecules subjected to sorption. Reversed-phase trace enrichment is especially efficient in the case of sorption of non-polar substances[28,29,31,32]. In the case of solute ionization, sorption occurs as long as the pH of the solution is at least two units below the pK_a value[28,32]. The curves presented in Fig. 1 show that the influence exerted by the pH of water upon the efficiency of sorption depends on the length of the aliphatic chain. At pH 2 the efficiency of sorption increases from 35% for acetic acid to 80% for butanoic acid and to 100% for heptanoic and octanoic acids. This proves that the polarity of the undissociated molecules influences the efficiency to a high degree.

Within the range studied, neither the flow-rate of water through the cartridge nor the concentrations of the acids affected the efficiency of sorption, as can be seen from the results presented in Table I. Both factors, however, play important roles in the analysis of natural waters since the low concentrations of some acids require the processing of considerable amounts of water in order to obtain measurable amounts.

TABLE I

THE INFLUENCE OF THE FLOW-RATE, THE CONCENTRATION AND AMOUNTS OF ACIDS, AND THE MATRIX ON THE EFFICIENCY OF THE C_{18} REVERSED-PHASE TRACE ENRICHMENT OF THE SHORT-CHAIN CARBOXYLIC ACIDS FROM WATER (pH 1.7)

Solution	*Flow-rate* (cm^3/min)	*Concentration* (pM)	*Volume* (dm^3)	*Recovery of acids with the given number of carbon atoms* (%)*			
				2	*4*	*6*	*8*
Distilled water	5	50	2	34	79	95	104
Distilled water	30	50	2	36	72	96	99
Distilled water	10	500	2	32	78	93	101
Sea water**	10	500	2	35	77	94	102

* As the percentage of amounts of acids added.

** Fatty-acid-free sea water was obtained by passing sea water (salinity, $S = 3.17\%$) through Amberlite XAD-2 resin and charcoal.

The time needed for the preconcentration step can be shortened when a high flow-rate is used. This is especially important for substances subject to biochemical degradation, when a prolonged time of analysis may affect the accuracy. Moreover, it is well known that the concentrations of different acids in the same sample of water may cover a wide range[1,2]. It has been shown, for instance, that the concentration of hexadecanoic acid can be several orders of magnitude higher than that of pentadecanoic acid[3]. Since the efficiency of trace enrichment does not depend on concentration and the cartridges have a high capacity towards carboxylic acids, they may be used for the preconcentration of acids from natural waters. Table I shows that the salts present in sea water do not influence the efficiency. According to Horváth and Melander[32], the matrix can affect the capacity of a reversed-phase column to some degree, which might be crucial if the system worked close to the limit. As natural waters differ in inorganic matrices, this lack of influence permits the wide use of this technique for the preconcentration of fatty acids from both fresh and sea water.

TABLE II

EFFICIENCY OF TRACE ENRICHMENT OF STRAIGHT AND BRANCHED SHORT-CHAIN FATTY ACIDS FROM DISTILLED WATER SPIKED WITH 2.0 nmoles OF ACIDS

Straight-chain	*Efficiency*		*Branched acid*	*Efficiency*	
	Average (%)	*S.D. (n=6)*		*Average (%)*	*S.D. (n=6)*
Acetic	34	9			
Propanoic	58	8			
Butanoic	76	5	Isobutanoic	80	6
Pentanoic	89	5	2-Methylbutanoic	93	5
Hexanoic	95	4	4-Methylpentanoic	96	3
Heptanoic	102	3	4-Methylhexanoic	101	4
Octanoic	99	5	6-Methylheptanoic	103	4
			2-Ethylhexanoic	100	3

In Table II the efficiency of sorption of a series of straight-chain and branched acids from aqueous solutions is given. Higher values for branched acids may be accounted for by their more hydrophobic properties and lower solubility compared to straight-chain ones. Complete recoveries of heptanoic and octanoic acids suggest that long-chain acids should be characterized by complete recoveries as well. However, it has been observed in experiments with water spiked with a methanol solution of these acids (20–200 pmoles of individual acids) that the recoveries are much lower. A thorough investigation proved that the "missing" amounts of acids were adsorbed on the glass walls and Teflon tubing. No acids were found in water passed through the cartridges. No irreversible sorption on the reversed-phase occurred, as the sum of the acids recovered from the cartridges and adsorbed in the system equalled the sum of the amounts applied.

To elute acids from the cartridges 0.1 *M* ammonium hydroxide solution in methanol (50:50) was used. The results presented in Table III show that other eluents gave lower recoveries of acids. These results are somewhat surprising since, in previous studies, plain solvents such as methanol[29,30] and acetone[28] managed to elute organic solutes sorbed from acidified water. However, larger volumes of solvents had

TABLE III

RECOVERIES OF ACIDS FROM C_{18} CARTRIDGES WITH VARIOUS ELUENTS

Eluent	*Recovery of acids with the given number of carbon atoms* as percentages of amounts used for spiking*						
	2	*3*	*4*	*5*	*6*	*7*	*8*
Acetone	18	21	25	28	20	27	25
Methanol	20	23	22	20	27	26	25
Methanol–water (50:50)	07	15	14	18	20	12	13
1 *M* Ammonium hydroxide in methanol–water (50:50)	34	58	76	89	96	102	99

* Distilled water spiked with 2.0 nmoles of each acid was used in these experiments.

to be used, undoubtedly, to wash out traces of inorganic acid left in the cartridges. In the procedure described in this paper ammonium hydroxide not only neutralizes the acid remaining after passing the acidified water, but causes dissociation of organic acids as well. This decreases the affinity of the acids towards the reversed-phase[32], enabling more efficient elution, and also prevents losses of volatile acids in the subsequent stripping of methanol in a stream of nitrogen.

Analysis of sea water

A typical gas chromatogram of pentafluorobenzyl esters of acids isolated from sea water by the C_{18} reversed-phase trace enrichment is shown in Fig. 2. Identifications were based on retention times and/or mass spectra. A number of peaks were not identified but, considering the similarity in the mass spectra, it can be safely assumed that most of them belong to esters of unsaturated and branched acids. These acids are present in sea water in great variety[1–3,32,33]. The concentrations of the identified acids are given in Table IV. As the efficiencies of preconcentration, the yields of derivatization and the detector responses of 10-undecenoic acid and undecanoic acid were the same, it was assumed that the recoveries of other unsaturated acids were similar to those characteristic of saturated fatty acids with the same number of carbon atoms.

The scatter of the results, described by the correlation coefficients, is considerable, especially for the lower acids. Because it is not easy to see a direct reason for this, the experiment was repeated after spiking water with branched acids. The con-

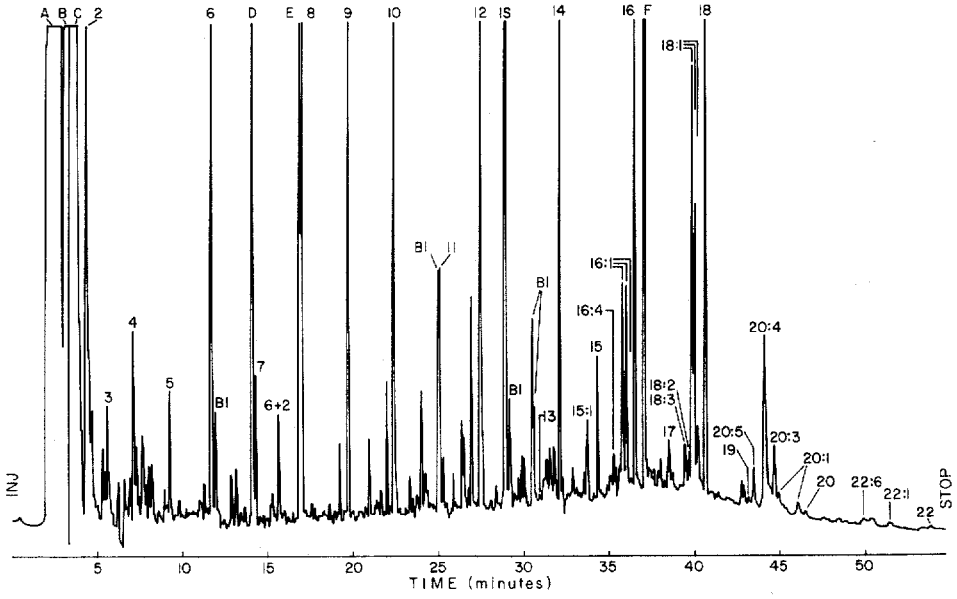

Fig. 2. Typical gas chromatogram of pentafluorobenzyl esters of acids isolated from filtered (0.8 μm glass-fibre filter) sea water by the C_{18} reversed-phase trace enrichment. Fatty acid peaks are described by the ratio of the number of carbon atoms in the chain to the number of double bonds. Peaks: A = solvent; B = pentafluorobenzyl chloride; C = pentafluorobenzyl bromide; D = pentafluorobenzyl ether; E = tributylamine; F = pentafluorobenzyl phthalate; IS = internal standard; Bl = unidentified peaks present in blanks.

TABLE IV

CONCENTRATIONS OF FATTY ACIDS IN FILTERED (0.8 μm GLASS-FIBRE FILTER) SEA WATER OBTAINED BY C_{18} REVERSED-PHASE TRACE ENRICHMENT, EXTRACTIVE PENTAFLUOROBENZYLATION AND FUSED-SILICA CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

Acid	*Mean concentration (n = 5) pM ± S.D.*			
	*Method 1**	*S.D.*	*Method 2***	*S.D.*
Acetic	89	28		
Propanoic	8.4	3.7		
Butanoic	24	7	39	4
Pentanoic	19	5	31	4
Hexanoic	380	68	395	42
Heptanoic	23	7	27	5
2-Ethylhexanoic	28	6	32	4
Octanoic	640	63	730	48
Nonanoic	720	58		
Decanoic	1250	94		
Undecanoic	220	46		
Dodecanoic	4250	280		
Tridecanoic	45	8		
Tetradecanoic	5100	240		
Pentadecanoic	350	46		
Hexadecenoic***	2300	320		
Hexadecanoic	7400	380		
Heptadecanoic	190	38		
Octadecenoic***	1550	220		
Octadecanoic	3700	170		

* Calculated on the basis of recoveries of straight-chain acids listed in Table III.

** Calculated on the basis of recoveries of branched acids used as internal standards, multiplied by the ratios of straight-to-branched acid efficiencies listed in Table III.

*** Sum of three isomers.

centrations were recalculated using the branched acids as internal standards. The precision was better for the straight-chain acids, but the overall recoveries of branched acids were again scattered.

One reason for this effect may involve the extraction of substances other than fatty acids. These substances may modify both the trace-enrichment and/or conversion steps. It has been shown that the reversed-phase trace-enrichment technique isolates up to 40% of the total organic substances dissolved in sea water[29]. Fatty acids comprise only 6–8% of the isolated organic load.

Sensitivity in relation to sea water

The sensitivity of the method in relation to sea water can be defined as the lowest determinable concentration of an acid. Considering that the ECD minimum detectable amount was equal to 0.01 pmole of a pentafluorebenzyl ester and that only 2.0 μl of organic extract out of 500 μl was injected, the amount of an ester in the extract has to equal 2.5 pmole, assuming 100% efficiency. Further concentration of the organic extract to 50 μl might decrease that value by ten-fold. However, the removal of excessive amounts of PFBBr and $TBA^{(+)}$ might be necessary[7]. It must be

remembered however, that the combined efficiencies of both the trace-enrichment step and the derivatization are much less than 100%. The efficiency for the short-chain acids is decreased by the trace-enrichment step, whereas the efficiency for the long-chain acids is reduced by incomplete conversion into esters.

The amount of esters in the extracts depend on the initial concentrations of the acids and the amount of water passed through the cartridge. For practical purposes the volume of water should not exceed 1.0 dm^3. A combination of both factors shows that the described method allows the quantitative determination of the concentrations of fatty acids in water, provided that they are present at concentrations higher than 5 p*M* (assuming an overal efficiency of 50%). By comparison, conventional methods of quantifying fatty acids in sea water are characterised by sensitivities in the range 1–10 nM and still require the extraction of 10–20 dm^3 of water [2,3,9]

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