Journal of Chromatography, 508 (1990) 159–178
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 302

Gas chromatographic analysis and gas chromatographic–mass spectrometric identification of components in the cyclohexane-extractable fraction from contaminated sediment samples

MIKAEL REMBERGER, PER-ÅKE HYNNING and ALASDAIR H. NEILSON*
Swedish Environmental Research Institute, Box 21060, S-100 31 Stockholm (Sweden)
(Received September 20th, 1989)

SUMMARY

A comprehensive investigation was carried out on the cyclohexane-soluble extracts from three sediment samples. Elemental analysis of the residue after careful removal of solvent showed that nitrogen was absent, sulphur accounted for 10–20% of the residue and the carbon/chlorine ratio was high. Gas chromatography and gas chromatography–mass spectrometry confirmed that a substantial fraction of the extracts consisted of non-chlorinated long-chain alkenoic and alkanoic acids, terpenes and plant steroids and that the free organochlorine compounds were dominated by chlorinated long-chain alkanoic acids and chlorinated resin acids, whereas chloroform, chemically bound phenolic compounds, and polychlorinated biphenyls made only a small contribution. The chemical structures of constituents of the extractable organic chlorine fraction and of the corresponding methanolic alkali extracts from whole sediments were completely different; the former did not have a major contribution from products originating in chlorinated guaiacyl-C_3 residues. Only *ca.* 8% of the organically bound chlorine could be accounted for in terms of known compounds which originated in bleachery effluents. Three hypotheses may be put forward for the nature of the unidentified components: they may not be constituents of bleachery effluents, they may not hitherto have been characterized in such effluents or they may be transformation products formed in the sediment phase. It was not possible in the light of current evidence to access which of these was correct. It was concluded that procedures used for characterization which rely heavily on gas chromatographic separation must be substantially improved to enable an acceptable level of indentification to be attained.

INTRODUCTION

A wide range of structurally diverse chlorinated compounds[1–5] is formed during the production of bleached pulp by conventional processes. Ultimately, a substantial

0021-9673/90/$03.50

fraction of this organically bound chlorine reaches the aquatic environment even after treatment. Considerable effort has therefore been directed to evaluating the impact of such discharge on the biota[6]. In addition, environmental monitoring for exposure to bleachery effluents has utilized sum parameters such as extractable organic chlorine (EOCl)[7,8] and analysis for specific compounds has been used in both field[9] and laboratory studies[10].

It is generally recognized that the persistence, the toxicity or the potential for bioconcentration of a compound may present a potential environmental hazard[11]. The bioconcentration potential is generally assessed from the octanol–water partition coefficient or some suitable surrogate, so that compounds extractable with cyclohexane and therefore components of the EOCl fraction are clearly lipophilic and putatively have a high bioconcentration potential. Substantial interest has therefore centred on the nature of the compounds contained in this cyclohexane-extractable fraction[12,13].

It has been shown, however, that at least as far as the sediment phase is concerned, the total amount of cyclohexane-EOCl significantly exceeds the sum of the concentrations of chloroguaiacols, chlorocatechols and chlorovanillins which can be extracted from sediments, even using relatively aggressive procedures[14]. On the basis of the results of a comparison of various extraction methods, we have postulated that a hitherto undetermined fraction of these compounds is bound to organic components of the sediment phase and is not chemically, and probably not biologically, accessible[14]. It is therefore important to identify the compounds responsible for this discrepancy.

As a prerequisite to achieving a realistic environmental hazard assessment of compounds originating in bleachery effluents and subsequently entering the environment, it is important to characterize the precise nature of the compounds in the EOCl fraction of both sediments and biota. Characterization of the organochlorine components of whole bleachery effluents have relied heavily, indeed almost exclusively, on application of gas chromatographic (GC) and gas chromatographic–mass spectrometric (GC–MS) procedures[1,2,15–17]. Clearly, then, these should be applied to the cyclohexane-EOCl fraction; this was the specific object of this investigation, which was directed at sediment samples from areas in the neighbourhood of the discharge of bleachery effluents. In this investigation, it was found that, in spite of considerable effort, only *ca.* 8% of the EOCl could be identified in terms of known compounds, and it is therefore suggested that attention be directed to analytical procedures that do not depend exclusively on the use of GC.

EXPERIMENTAL

Solvents and reagents

The solvents cyclohexane, benzene, methanol, acetonitrile, dichloromethane, 1,2-dichloroethane, carbon tetrachloride were obtained from LabScan (Stillorgan, Ireland), *tert.*-butyl methyl ether, pentane and hexane from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and diethyl ether from Fisons (Loughborough, U.K.).

Octadec-9-enoic acid, octadec-9,12-dienoic acid, hexadec-7-enoic acid, glyceryl trihexadecanoate, octadecanyl octadecanoate, cholesteryl octadecanoate, cholesterol, lupeol and betulin were obtained from Sigma (St. Louis, MO, U.S.A.), abietic acid and

dehydroabietic acid from K & K Labs. (Plainview, NY, U.S.A.), β-sitosterol from Serva (Heidelberg, F.R.G.), even-membered straight-chain alkanoic acids (C_{10-22}) from Alltech (Dearfield, IL, U.S.A.), tetrahydroabietic acid and pimaric acid from Hercules Powder (Wilmington, DE, U.S.A.), acetic anhydride from Fluka (Buchs, Switzerland), N-methyl-N-nitroso-*p*-toluenesulphonamide and boron tribromide–dimethyl sulphide complex from Janssen (Beerse, Belgium), hydrogen chloride in methanol from Tokyo Kasai (Tokyo, Japan) and methoxamine hydrochloride in pyridine and trimethylsilylimidazole (TMSI) from Pierce (Rockford, IL, U.S.A.). Silica gel 60 (70–230 mesh, aluminium oxide (70–230 mesh), acidic and basic, and sulphur (puriss. cryst.) were purchased from Merck (Darmstadt, F.R.G.). All other reagents were of analytical-reagent grade.

Synthesis of reference compounds

7,8-Dichlorohexadecanoic acid and 9,10-dichlorooctadecanoic acid were prepared by chlorination of hexadec-7-enoic acid and octadec-9-enoic acid with chlorine in dichloromethane at room temperature. Solvent was removed to give a white, low-melting waxy material whose methyl esters were >99% pure (GC–MS). No attempt was made to resolve the mixture of diastereoisomers. The mass spectra [electron impact (EI)] of the methyl esters had extremely weak parent ions (*m/z* 338 and 366, corresponding to $C_{17}H_{32}O_2Cl_2$ and $C_{19}H_{36}O_2Cl_2$), which gave rise to daughter ions with *m/z* 266 ($M^+ - 2$ HCl) and 294 ($M^+ - 2$ HCl).

9,10,12,13-Tetrachlorooctadecanoic acid was prepared in a similar way from octadec-9,12-dienoic acid by chlorination with a stoichiometric amount of chlorine in dichloromethane. Solvent was removed and the residue extracted several times with hexane to give a white powder which was free from compounds other than the desired product. The mass spectrum (EI) of the methyl ester had a weak parent ion (*m/z* 434, corresponding to $C_{19}H_{34}O_2Cl_4$), which gave rise to a daughter ion with *m/z* 326 ($M^+ - 3$ HCl).

9,10-Dibromooctadecanoic acid was prepared as for the corresponding chloro compound. The mass spectrum (EI) of the methyl ester had an extremely weak parent ion (*m/z* 454, corresponding to $C_{19}H_{36}O_2Br_2$), which gave rise to a daughter ion with *m/z* 295 ($M^+ - 2$ HBr).

The synthesis of 12-chloro-, 14-chloro- and 12,14-dichlorodehydroabietic acid has been described[18], but in attempting to repeat this work we encountered two serious problems: inclusion of the free-radical scavenger 2,3-dichloro-5,6-dicyanobenzoquinone brought about ring dehydrogenations[19] to products that were difficult to remove, and separation of the two monochloro isomers through formation of the amides was unreliable in our hands. The following modifications were therefore adopted. Only the $FeCl_3$–SiO_2 catalyst was used; the monochloro isomers were prepared by chlorination in dichloromethane at 0°C with slightly over one molar equivalent of chlorine, and the products were separated by semi-preparative high-performance liquid chromatography (HPLC). A Nucleosil C_{18} column (250 × 10 mm I.D., particle size 5 μm) (Jones Chromatography, U.K.) was used at a flow-rate of 4 ml min^{-1}, and the products were detected by their absorption at 280 nm. Two mobile phases were prepared by mixing 6% (v/v) acetic acid with acetonitrile in the ratios 30:70 (system A) and 40:60 (system B). Purification of the chlorinated resin acids was carried out first with system A and further purification of the product with system B.

The mass spectra of the methyl esters had *m/z* 348, corresponding to $C_{21}H_{29}O_2Cl$. The dichloro compound was prepared using excess of chlorine in dichloromethane at room temperature for 2 h and the product was purified by semi-preparative HPLC using system A. The mass spectrum of the methyl ester had *m/z* 382, corresponding to $C_{21}H_{28}O_2Cl_2$.

The procedure for the synthesis of 5,6-dichloro-β-sitosterol O-benzoate was first evaluated using the more readily available cholesterol, which was benzoylated with benzoyl chloride in pyridine[20]. After the reaction was complete, potassium carbonate solution (8 ml, 0.8 *M*) was added and the mixture shaken for 5 min. The precipitate was filtered and washed with hydrochloric acid and water. The dried product was crystallized from *tert.*-butyl methyl ether, chlorinated with chlorine at 0°C in carbon tetrachloride and the product purified first by semi-preparative HPLC using a Nucleosil C_{18} column with acetonitrile as the mobile phase and the detector set at 232 nm, and then finally recrystallized from acetonitrile. The direct probe mass spctrum had *m/z* 560, corresponding to $C_{34}H_{50}O_2Cl_2$. Pure β-sitosterol was benzoylated in the same way, and the EI mass spectrum, which lacked a parent ion (typical of Δ^5-steroids) had a principal peak at *m/z* 105 ($C_6H_5CO^+$) which showed that benzoylation had occurred. The O-benzoate was chlorinated at 0°C in carbon tetrachloride, the solvent removed and the product purified by semi-preparative HPLC on a Nucleosil C_{18} column with 100% methanol (6 ml/min) as the mobile phase and with the detector set at 232 nm. The direct-probe mass spectrum had a parent peak at *m/z* 588, corresponding to $C_{36}H_{54}O_2Cl_2$.

Source of sediments

All the samples were recovered from within 2 km of bleachery discharges, samples A and B from the Gulf of Bothnia, Sweden, and sample C from Oslo Fjord, Norway. Determinations of dry weight and ignition loss were carried out by procedures already described[15].

Extraction procedures for EOCl

These were carried out by established procedures[7]. The following experiment was carried out to exclude the possibility of interference from inorganic chloride. Cyclohexane was spiked with octadecanyl octadecanoate, glyceryl trihexadecanoate, dehydroabietic acid (all at three concentrations, 25, 75 and 225 μg/ml) and cholesteryl octadecanoate (5, 15 and 45 μg/ml), and chloride was added to the water–propan-2-ol phase to give a concentration of 0.7%. The extraction with cyclohexane was then carried out by the standard procedure used for sediment samples and the organic phase analysed for EOCl.

Elemental analysis

The extracts were evaporated to dryness *in vacuo* under a stream of nitrogen and dried in a vacuum desiccator over phosphorus pentoxide. These residues were analysed by ICI Agrochemicals (Jealotts Hill, U.K.).

Chemical treatment of samples

Analyses of polychlorinated biphenyls (PCBs) were carried out with extract residues prepared as above for elemental analysis. The residues were dissolved in

a small volume of *n*-hexane, chromatographed on silica gel deactivated with 5% (w/w) water, the hexane eluates collected, elemental sulphur removed[21], the extracts shaken repeatedly with concentrated sulphuric acid, washed with water and finally chromatographed on a column of acidic alumina overlayered with basic alumina. The hexane eluate was discarded and the PCBs were eluted with benzene, the extracts concentrated and used for analysis.

The following chemical modifications were carried out with *ca.* 2 mg of residue:

(i) The sample was treated with sulphuric acid (85%, w/w) overnight at room temperature, diluted with water and extracted twice with *tert.*-butyl methyl ether–hexane (1:1), the extracts dried (sodium sulphate), solvent removed and the residues derivatized as below.

(ii) Alkali treatment was carried out with 1 *M* aqueous methanolic (1:1) potassium hydroxide overnight at room temperature, the sample diluted with water, acidified, extracted and derivatized as below.

(iii) Treatment with $BBr_3 \cdot (Me_2S)$ (100 mg) was carried out in 1,2-dichloroethane for 1 h at 80°C in a closed tube. Excess of reagent was destroyed by adding water and the products were extracted with *tert.*-butyl methyl ether–hexane and derivatized as below.

(iv) The residue was dissolved in *tert.*-butyl methyl ether, anhydrous sodium methoxide added to give a final concentration of 0.2 *M* and the mixture was shaken vigorously for 1 min, acidified with dilute hydrochloric acid and the organic phase was used directly for analysis. Except for simple lipid esters, it was necessary to use *tert.*-butyl methyl ether as solvent for the transesterification.

(v) The residue was treated with 3 *M* aqueous potassium hydroxide and $CuSO_4$ $5H_2O$ (300 mg) at 100°C for 1 h. The sample was acidified, extracted and analysed for chlorophenolic compounds as described below.

(vi) The residue was dissolved in propan-2-ol, hydrobromic acid (0.1 ml, 48%) was added and the mixture was heated for 1 h at 100°C, diluted, extracted with *tert.*-butyl methyl ether–hexane and esterified as below.

Preparation of derivatives of standards and of EOCl extracts for GC and GC–MS analysis

Acetylation of phenolic compounds was carried out by standard procedures[14]. Free alkanoic acids were methylated with diazomethane or methanolic hydrogen chloride and resin acids with diazomethane[22], and non-chlorinated sterols and pentacyclic triterpenes were trimethylsilylated[23]. For quantification in EOCl extracts, these samples were used directly; for identification, however, they were first purified. The derivatized samples were evaporated to dryness, dissolved in hexane and purified by chromatography on a column of silica gel; the hexane eluate was discarded and the desired compounds eluted with benzene. Benzoylation of EOCl extracts was carried out as described for the synthesis of β-sitosterol benzoate except that after destruction of excess of reagent, the product was extracted with hexane (2 × 1.5 ml) and the extracts were washed with hydrochloric acid and dried (sodium sulphate). The extracts were chromatographed on silica gel and the product was eluted with benzene. Solvent was removed, and the product rechromatographed twice by semi-preparative HPLC as described for the synthesis of dichloro-β-sitosterol benzoate.

Gas chromatography–mass spectrometry

GC–MS analysis was carried out as described[24] with the modifications that the injector temperature was 260°C and the temperature programming was continued to 300°C and held for 20 min. For direct-probe analysis, the following temperature programme was used: 50°C (30 s isothermal), increased at 150°C/min to 450°C, which was maintained for 3 min.

Gas chromatography

Analysis of chlorophenolic compounds was carried out as described previously[14]. For the analysis of chloroform, samples were extracted with pentane and the following temperature programme was used: 35°C (6 min isothermal), increased at 10°C/min to 265°C. Attention is drawn to serious interference in the analysis of chloroform resulting from compounds released from PTFE-coated screw-cap liners, the use of which must therefore be avoided.

Analysis of PCBs was carried out using splitless injection with a Varian 3700 gas chromatograph fitted with an electron-capture detector, and the following temperature programme: 50°C isothermal for 1 min, increased at 4°C/min to 295°C, which was held for 5 min. Quantification was carried out using authentic standards (IUPAC Nos. 28, 31, 40, 52, 53, 101, 105, 138, 153, 180 and 209).

Analysis of elemental sulphur was carried out by GC with electron-capture detection (ECD) using the same temperature programme as for chlorophenolic compounds described above.

For the analysis of all other compounds, splitless injection was used with a flame ionization detection (FID) system and a Hewlett-Packard Model 5880 gas chromatograph using the following temperature programme: 50°C (2 min isothermal), increased at 15°C/min to 215°C, then at 8°C/min to 300°C, which was maintained for 30 min. The injector temperature was 250°C and the detector temperature 275°C. A DB-5 fused-silica capillary column (30 m × 0.25 mm I.D., film thickness 0.25 μm) (J & W Scientific, Folsom, CA, U.S.A.) was used with a column head pressure of 70 kPa. Quantification was carried out as follows: surrogate standards [dibromooctadecanoic acid for the carboxylic and resin acids and cholesterol for the sterols (100 μg of each)] were added to the extracts (1.0 ml) and the extracts were evaporated to dryness under nitrogen, esterified with diazomethane, again evaporated to dryness and silylated with TMSI. Hexane–*tert.*-butyl methyl ether (1:1) (1.0 ml) containing naphthalene (50 μg/ml) as internal standard was added and the organic phase analysed as above. Quantification was made using authentic standard compounds.

RESULTS AND DISCUSSION

General characteristics of the EOCl extract

In order to avoid confusion, it is important at the outset to appreciate that the term EOCl (extractable organic chlorine) is, by itself, an imprecise specification. The values obtained, together with the nature of the substances extracted, clearly depend critically on the choice of solvent. Cyclohexane was originally selected not primarily on account of its effectiveness as a solvent, but because it does not dissolve appreciable amounts of water. In this way, interference from inorganic chloride during neutron activation analysis of organic chlorine is minimized[25]. In the following discussion, we

TABLE I

LOSSES ON DRYING AND ON IGNITION OF DRIED SEDIMENT SAMPLES

Loss	*Sample*		
	A	*B*	*C*
Loss on drying (%)	85	67	52
Loss on ignition (%)	15	6	6

use the term EOCl exclusively for the fraction obtained by extraction of a sediment with propan-2-ol followed by back-extraction with cyclohexane. However, as the extracts contained high concentrations of non-chlorinated surface-active compounds, it was imperative to eliminate conclusively the possibility that the EOCl analyses were compromised by an artefact resulting from inadvertently introduced inorganic chloride. The experiments using the three "model" substances clearly showed that there was no interference from inorganic chloride.

The gross characteristics of the sediments are given in Table I and the elemental analysis of the residues after evaporation of the solvent showed that they contained no nitrogen and a high percentage of sulphur (Table II). GC and GC–MS confirmed the sulphur analysis, and showed that it was in the form of elemental sulphur. Organic nitrogen and sulphur compounds therefore made only a negligible contribution to the organic components of the extracts.

Quantification of organochlorine compounds

There were two main types of chlorinated compounds whose GC relative retention times and response factors are given in Table III.

The first consisted of a group of carboxylic acids. The concentrations obtained by treatment with diazomethane are designated "free"; treatment with sodium methoxide, however, which brings about transesterification of lipid esters[26], resulted in the release of a further contribution from chloroalkanoic acids, designated "bound", although not from the chlorinated resin acids (Table IV). In a separate experiment, it was shown that the treatment with methoxide did not result in any destruction of the chloroalkanoic acids, which were sensitive to treatment with aqueous methanolic alkali. The latter reagent did not release concentrations of the chlorinated resin acids exceeding those designated "free", so that it was assumed that these resin acids occurred only in the free form. The quantification of these compounds

TABLE II

MEAN DUPLICATE ELEMENTAL ANALYSES FOR RESIDUES AFTER EVAPORATION OF EOCl EXTRACTS

Sample	*C (%)*	*H (%)*	*Cl (%)*	*S (%)*
A	65.3	9.5	1.87	13.5
B	71.8	9.9	1.83	11.0
C	67.0	9.5	0.99	11.4

TABLE III

RETENTION TIMES AND RESPONSE FACTORS OF CHLORINATED CARBOXYLIC ACID METHYL ESTERS RELATIVE TO THAT OF 9,10-DIBROMOOCTADECANOIC ACID METHYL ESTER

Methyl ester	*Relative retention time*	*Relative response factor*
7,8-Dichlorohexadecanoic	0.782	0.74
9,10-Dichlorooctadecanoic	0.931	0.89
9,10,12,13-Tetrachlorooctadecanoic	1.064	0.90
12-Chlorodehydroabietic	0.956	0.93
14-Chlorodehydroabietic	0.972	1.44
12,14-Dichlorodehydroabietic	1.040	1.27

presented a technical problem as all of them have only low GC electron-capture responses and quantification was carried out using an FID system. Quantification of the chlorinated resin acids was carried out by both GC with FID and GC–MS procedures, and the results were in excellent agreement.

The second group of compounds, consisting of chlorophenolic compounds in the free form, constituted only a small fraction of the extracts, although this was increased by chemical modification; these components are therefore designated "bound" (Table V). There was also a minor contribution from chloroform, although the precise value depends critically on the details of the extraction procedure, and in particular the temperatures used during working up.

The chlorinated dehydroabietic acids and chlorinated octadecanoic acids, which clearly accounted for the greater fraction of the identified organochlorine compounds (Table VI), even though their total concentrations represented only a small fraction

TABLE IV

CONCENTRATIONS (mg/kg ORGANIC C) OF MAJOR ORGANOCHLORINE COMPONENTS OF EOCl; THE VALUES IN PARENTHESIS ARE GIVEN IN mg Cl/kg ORGANIC C

Compound	*Locality*		
	A	*B*	*C*
9,10-Dichlorooctadecanoic acid:			
Free	148 (28)	102 (20)	44 (8.3)
Bound	154 (30)	66 (13)	19 (3.6)
9,10,12,13-Tetrachlorooctadecanoic acid:			
Free	13 (10)	51 (14)	49 (16)
Bound	<[a]	44 (14)	19 (6)
12-Chlorodehydroabietic acid	28 (6.4)	32 (15)	31 (5)
14-Chlorodehydroabietic acid	70 (9)	66 (8)	66 (7)
12,14-Dichlorodehydroabietic acid	350 (90)	181 (31)	35 (8.8)
Chloroform	1.5 (1.3)	1.8 (1.6)	0.6 (0.33)
EOCl	(2180)	(2260)	(780)

[a] < = Lower than the limit of quantification.

TABLE V

CONCENTRATIONS (mg/kg ORGANIC C) OF CHLOROPHENOLIC COMPOUNDS RELEASED FROM EXTRACTS OF SAMPLE A AFTER CHEMICAL MODIFICATION

Modification	*Component*[a]					
	3,4,5-CG	*3,4,5-CC*	*TCG*	*TCC*	*2,4,5-CP*	*Penta-CP*
Untreated	0.64	0.13	0.39	1.78	<[b]	<
KOH	1.78	0.06	0.64	0.32	<	0.13
BBr_3 $(Me)_2S$	<	1.93	1.28	1.93	4.49	1.93

[a] CG = Chloroguaiacol; CC = chlorocatechol; CP = chlorophenol.
[b] < = Lower than the limit of quantification.

of the organically bound chlorine, are an environmentally important group of compounds because even the non-chlorinated derivatives of dehydroabietic acids are toxic[27–29], and both groups of compounds were apparently persistent in the sediment phase.

In addition, careful attention was directed to the possible presence of any compound that had been identified in bleachery effluents, or that was suspected of having a global distribution. Although a range of chlorinated thiophenes have been identified in bleachery effluents[5], and tetrachlorothiophene was the dominant thiophene in an effluent from the same factory in the neighbourhood of which the sediment sample was collected, none of these compounds could be identified in the EOCl fraction in spite of their ready extractability with cyclohexane. Chlorinated quinones, which have been putatively identified in bleachery effluents[30] and which might be environmental chemical modification products of chlorinated catechols, were also absent. An examination was also made for the presence of PCBs and other pesticides, but the contribution of all of these was extremely low. The identity of the PCB congeners was carried out by selected ion monitoring mass spectrometry of the parent ions taking into account the characteristic chlorine isotope patterns, but the only congener which occurred in sufficient concentration for conclusive identification was 2,3,2′,3′-tetrachlorobiphenyl (IUPAC No. 40), which occurred in samples B and C at concentrations of 2.9 and 0.1 mg/kg organic C.

TABLE VI

SUMMARY OF CONCENTRATIONS (mg Cl/kg ORGANIC C) OF IDENTIFIED COMPONENTS IN THE EOCl FRACTION IN SAMPLE A

Component	*Concentration*	*Component*	*Concentration*
Total chlorinated resin acids	105	Chloroform	1.3
Total chlorinated fatty acids	68	PCBs	0.4
Total phenolic compounds	9.5	EOCl	2180

"Free" and "bound" residues

Since chemical treatment of the extracts released additional amounts of chloroalkanoic acids (Table IV) and chlorophenolic compounds (Table V), a significant fraction of these compounds has been designated as "bound", and we have hypothesized that these compounds were covalently linked to organic components either in the sediment phase or to those originating in the effluents. We suggest that the chloroalkanoic acids existed as lipids, and emphasize that care must be taken in the choice of reagent for the release of "bound" residues. For example, the chlorinated alkanoic acids were rapidly destroyed by treatment with aqueous methanolic alkali which was used for release of the chlorophenolic compounds, but were stable to anhydrous sodium methoxide. The chlorocatechols were clearly demethylation products of the initially released chloroguaiacols, so that the latter group of substances were quantitatively the more significant. All these observations are consistent with the previously established fact that whereas chlorocatechols can be recovered from sediment samples by direct solvent extraction, the chloroguaiacols are apparently "bound" in some hitherto unknown manner so that they are effectively extractable only by more aggressive procedures[14].

These apparently "bound" residues were clearly extractable with cyclohexane, in contrast to the "bound" chlorophenolic compounds in whole sediments which were not effectively removed with water-immiscible solvents[14]. Whereas the essential nature of this covalent binding of the phenolic components in the cyclohexane extracts might have been similar to that in the bulk sediment, significant differences have now clearly emerged. Treatment of whole sediment samples with alkaline copper sulphate[31] effectively released amounts of chlorovanillins comparable to those of the phenolic compounds after treatment with methanolic alkali (Table VII). In addition, these values do not take into account the presence of substantial concentrations of the de-O-methylated vanillins whose presence was conclusively demonstrated by mass spectrometric examination. By contrast, application of similar procedures to the EOCl produced only low concentrations of chlorovanillins. We interpret the results from whole sediments as involving oxidative degradation of high-molecular-weight chlorinated lignin with initial fission into guaiacyl-C_3 entities, and subsequent loss of the side-chain with formation of the vanillins. These entities were presumably part of the polymeric chlorolignin structure, and these results are consistent with the fact that

TABLE VII

CONCENTRATIONS (mg/kg ORGANIC C) OF CHLOROPHENOLIC COMPOUNDS RELEASED FROM WHOLE SEDIMENT SAMPLE A AFTER CHEMICAL MODIFICATION

Modification	*Component*[a]			
	6-CV	*5,6-DCV*	*3,4,5-CG*	*TCG*
Untreated	<[b]	<	3	2
Methanolic KOH	13	15	77	61
Cu^{2+}–KOH	59	32	79	57

[a] CV = chlorovanillin; DCV = dichlorovanillin; CG = chloroguaiacol; TCG = tetrachloroguaiacol.

[b] < = Lower than the limit of quantification.

substantial amounts of unchlorinated vanillins were also released, presumably from lignin itself. Comparable polymerized entities were apparently of relatively minor significance in the EOCl fractions. In agreement with this is the fact that oxidation of EOCl residues with $KMnO_4$ and KIO_4, which has been used successfully for chlorolignin[32], yielded negligible amounts of chlorinated aromatic fragments.

Mass spectrometric identification of EOCl components

In a matrix as complex as that encountered in heavily contaminated sediment samples, identification of organic components clearly cannot be based solely on comparison of GC retention times with those of reference compounds[33]. All of these components were therefore conclusively identified by EI-MS comparison with reference compounds (Figs. 1 and 2). To facilitate identification, and eliminate as far as possible interfering compounds, all samples were purified by chromatography on silica gel before GC–MS analysis. A serious technical difficulty emerged because, under EI conditions, the chlorinated alkanoic acid esters had extremely weak parent ions, and readily formed daughter ions lacking chlorine atoms[34]; this considerably exacerbated their conclusive identification in complex mixtures of organic compounds and indeed the presence of chlorine in the compounds could readily have been overlooked. These results emphasize the necessity for access to pure samples of reference compounds. Although these chlorinated alkanoic acids and resin acids have been identified in bleachery effluents[22,35], we are not aware of their recovery from environmental samples prior to this study.

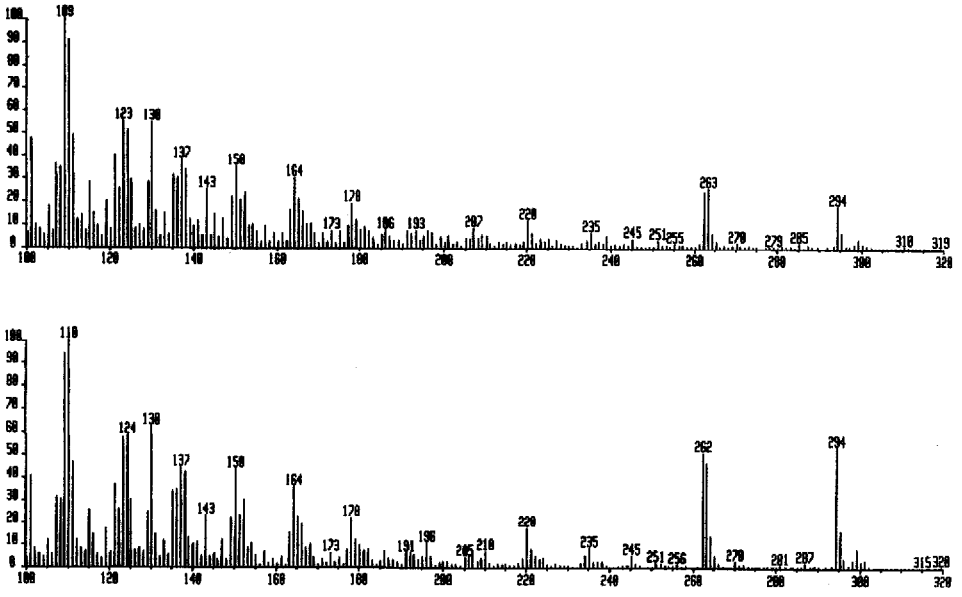

Fig. 1. Comparison of EI mass spectra of derivatives of compounds isolated from EOCl extracts (top) with those of authentic reference compounds (bottom): 9,10-dichlorooctadecanoic acid methyl ester.

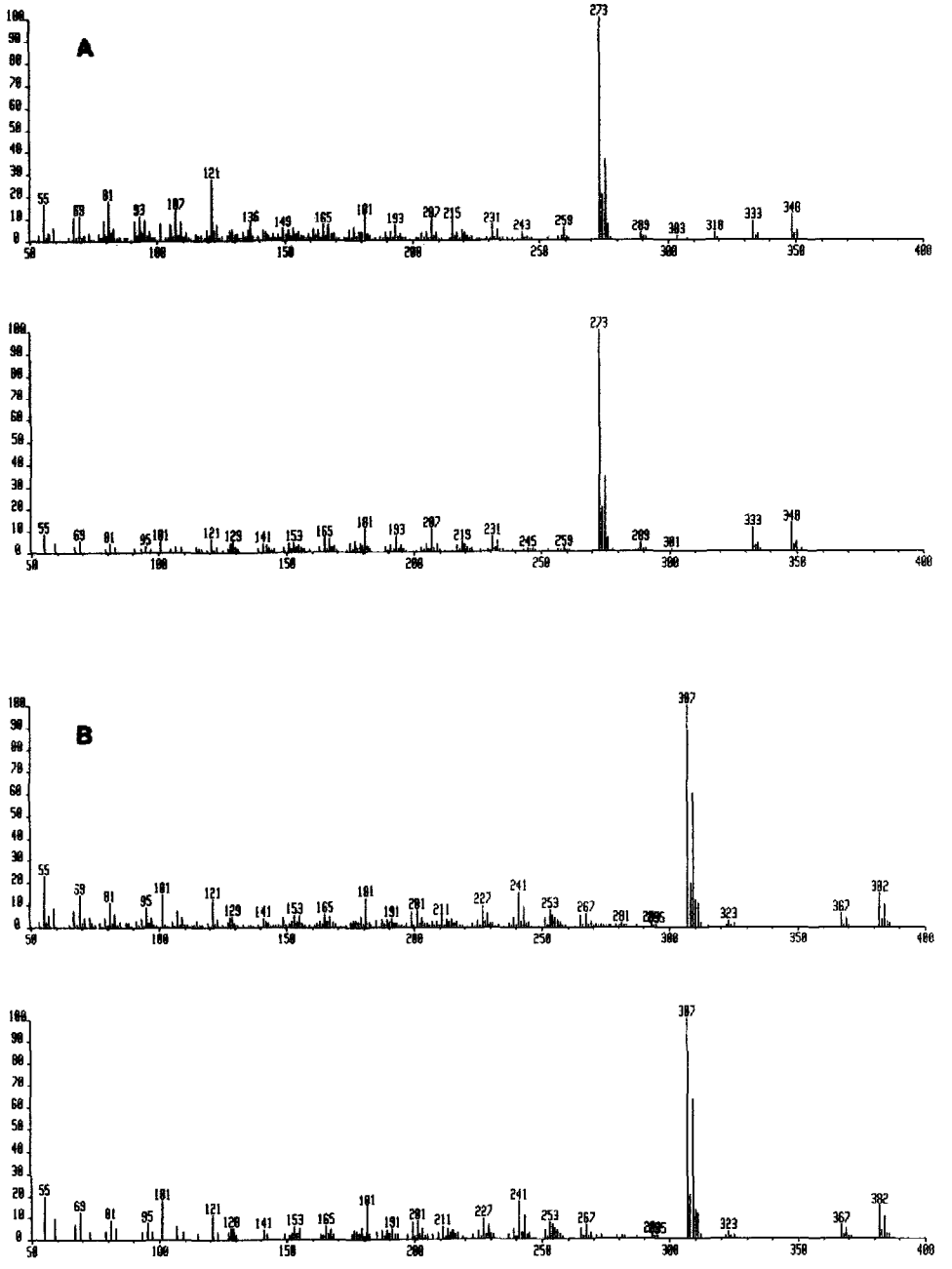

Fig. 2. Comparison of EI mass spectra of derivatives of compounds isolated from EOCl extracts (top) with those of authentic reference compounds (bottom): (A) 14-chlorodehydroabietic acid methyl ester; (B) 12,14-dichlorodehydroabietic acid methyl ester.

Origin and nature of the organochlorine compounds in the EOCl fraction

All the compounds identified above, with the exception of the PCBs, have been reported in bleachery effluents so that there can be little doubt as to their origin. It should be appreciated, however, that our samples were collected in the close proximity of the discharge of such effluents, so that a point discharge source could be clearly identified. Although less than 10% of the organically bound chlorine compounds in the EOCl fractions have been identified in terms of specific compounds, we feel that the comprehensive nature of this investigation, which employed procedures effectively used for analysis of bleachery effluents[1,2,15–17], reasonably excludes any major contribution from other compounds hitherto identified in such effluents. Three hypotheses concerning the origin of the unidentified compounds may be put forward: they do not originate in bleachery discharge and have a hitherto unknown origin, they have not yet been identified in bleachery effluents or they are transformation products of the original compounds contained in the effluents. In the light of current knowledge, it is impossible to decide between these alternatives.

Problems in identifying the components of the EOCl fraction in biota have been encountered by other workers[12,13] so that this may be assumed to be a general issue. It is important to realize, however, that the EOCl in biota from remote locations may be entirely different; possibly the major components are neutral, free organochlorine compounds transported via the atmosphere[36,37]. The possible confusion resulting from the uncritical use of sum parameters is therefore again emphasized.

The data presented clearly show that the use of sum parameters such as EOCl in environmental hazard assessment of bleachery effluents is unsatisfactory since such a substantial fraction remains unidentified in spite of intensive efforts. Among the more critical issues are (i) it is impossible to determine unambiguously the source of the contamination and (ii) it is not realistic to attempt an environmental impact assessment when the cardinal issues of persistence, bioavailability and potential toxicity cannot be addressed. The use of parameters such as adsorbable organic halogen (AOX) is fraught with even greater uncertainty, since a distinction between chloro- and bromo-organics clearly cannot be made.

Application of alternative analytical procedures

A compound was recovered from extracts whose benzoate after purification by HPLC had a mass spectrum identical with that of the benzoate of 5ξ,6ξ-dichloro-β-sitosterol (Fig. 3). However, as it co-chromatographed with another substance, identifiable by its mass spectrum as dihydro-β-sitosterol benzoate, quantification was not possible. Because, however, it was identified in sub-samples from the same sediment on two separate occasions, we feel confident of its occurrence and identification. This presented an interesting example of a compound whose existence had not hitherto been reported in bleachery effluents although its presence is eminently reasonable by analogy with the occurrence of the chlorinated long-chain alkanoic acids and that cannot be analysed by GC–MS owing to apparent disintegration on injection or during GC. This appears to be a general problem with chlorinated steroids (and probably also chlorinated triterpenoids) as GC analysis of the related 5ξ,6ξ-dichlorocholesterol acetate was unsuccessful even using on-column injection. Application of HPLC to the purification of the derivatized (benzoylated) extracts before direct probe mass analysis was clearly successful, so that further exploitation of this

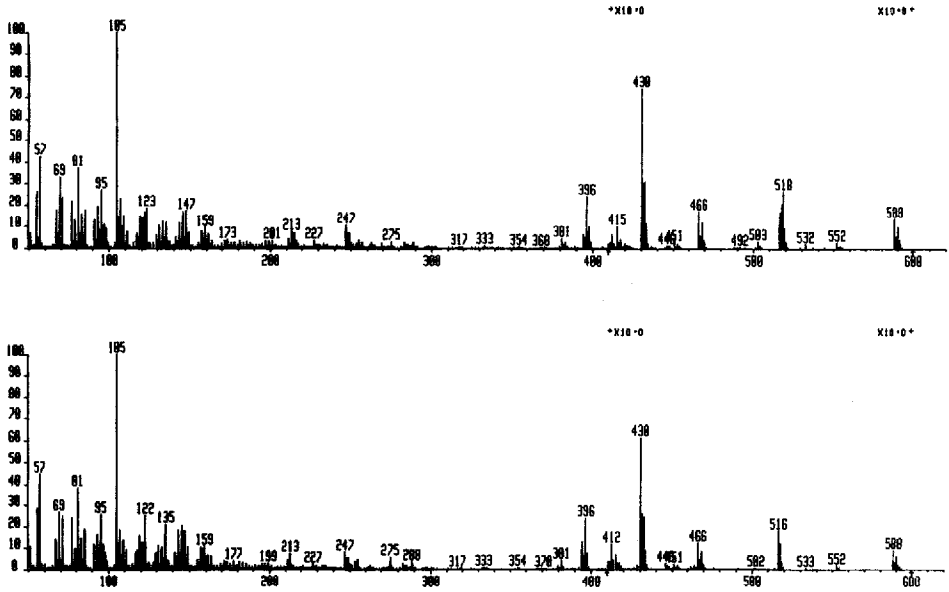

Fig. 3. Comparison of EI mass spectra of derivatives of compounds isolated from EOCl extracts (top) with those of authentic reference compounds (bottom): 5ξ,6ξ-dichloro-β-sitosterol benzoate.

technique opens new horizons for the characterization of hitherto unidentified compounds in EOCl extracts.

Non-chlorinated organic compounds

The preceding discussion has considered only organochlorine compounds. The elemental analyses (Table II) clearly illustrated, however, the high C–Cl ratio which could be rationalized on the basis either of a low degree of chlorination of the components or the presence of unchlorinated analogues. The latter conclusion finds support in the results of mass spectrometric examination of the derivatized extracts, which showed a range of unchlorinated aliphatic long-chain alkenoic and alkanoic acids, β-sitosterol and its reduction product and a range of terpenes including a few pentacyclic triterpenes (Table VIII, Figs. 4–6). The GC relative retention times and response factors of these compounds are given in Table IX.

As with the chlorinated analogues, only the alkanoic acids occurred in the form of lipid esters. These non-chlorinated compounds were found at concentrations often ten times greater than those of the chlorinated analogues, and their recovery suggests a substantial persistence. Many of the terpenes and sterols were sensitive to chemical modification by the procedures adopted for the chlorophenolic compounds so that these methods cannot be employed to assess their existence as "bound" residues. Some of the compounds such as the terpenes are toxic to aquatic organisms[27–29], and as it may plausibly be assumed that all compounds in this fraction have a high bioconcentration potential, attention should probably be directed to all these compounds, and also to the organochlorine compounds discussed above. As the relative fraction of identified organochlorine compounds in the EOCl extracts and of

TABLE VIII

CONCENTRATIONS (mg/kg ORGANIC C) OF PRINCIPAL NON-CHLORINATED COMPOUNDS IN EOCl EXTRACTS WITH CONCENTRATION OF LIPID-BOUND FATTY ACIDS IN PARENTHESES

Compound	*A*	*B*	*C*
Fatty acids:			
C_{14}	160 (135)	58 (102)	49 (141)
C_{16}	353 (263)	459 (379)	234 (127)
C_{18}	109 (45)	262 (102)	58 (24)
C_{20}	1136 (128)	1283 (235)	653 (205)
C_{16}:$\Delta 7$	340 (295)	58 (262)	49 (141)
C_{18}:$\Delta 9$	230 (116)	182 (226)	175 (73)
Terpenes and sterols:			
Abietic acid	539	488	385
Dehydroabietic acid	3126	5051	1946
Pimaric acid	565	1108	63
Tetrahydroabietic acid	725	488	385
β-Sitosterol	2277	2693	1513
Dihydro-β-sitosterol	841	1179	439
Lupeol	<[a]	924	29
Betulin	380	7278	<
Methylcycloartenol	165	718	344

[a] < = Lower than the limit of quantification.

TABLE IX

RETENTION TIMES OF NON-CHLORINATED CARBOXYLIC ACID METHYL ESTERS AND TRIMETHYLSILYL ETHERS OF STEROLS AND TRITERPENES RELATIVE TO THAT OF 9,10-DIBROMOOCTADECANOIC ACID METHYL ESTER

Compound	*Relative retention time*	*Relative response factor*
Methyl esters:		
Dodecanoic	0.536	0.81
Tetradecanoic	0.612	0.99
Hexadecenoic	0.682	0.98
Hexadecanoic	0.689	1.05
Octadeca-8,10-dienoic	0.759	1.03
Octadecenoic	0.762	1.07
Octadecanoic	0.770	1.23
9,10-Epoxyoctadecanoic	0.777	1.16
Eicosanoic	0.854	1.63
Docosanoic	0.937	1.47
Pimaric	0.760	–[a]
Tetrahydroabietic	0.872	–[a]
Dehydroabietic	0.876	1.34
Abietic	0.897	–[a]
O-Trimethylsilyl ethers:		
Cholesterol	1.178	1.42
β-Sitosterol	1.287	1.06
5,6-Dihydro-β-sitosterol	1.297	–[b]
Lupeol	1.332	1.36
Betulin	1.419	0.96

[a] Response factors assumed to be the same as for dehydroabietic acid.
[b] Response factor assumed to the same as for β-sitosterol.

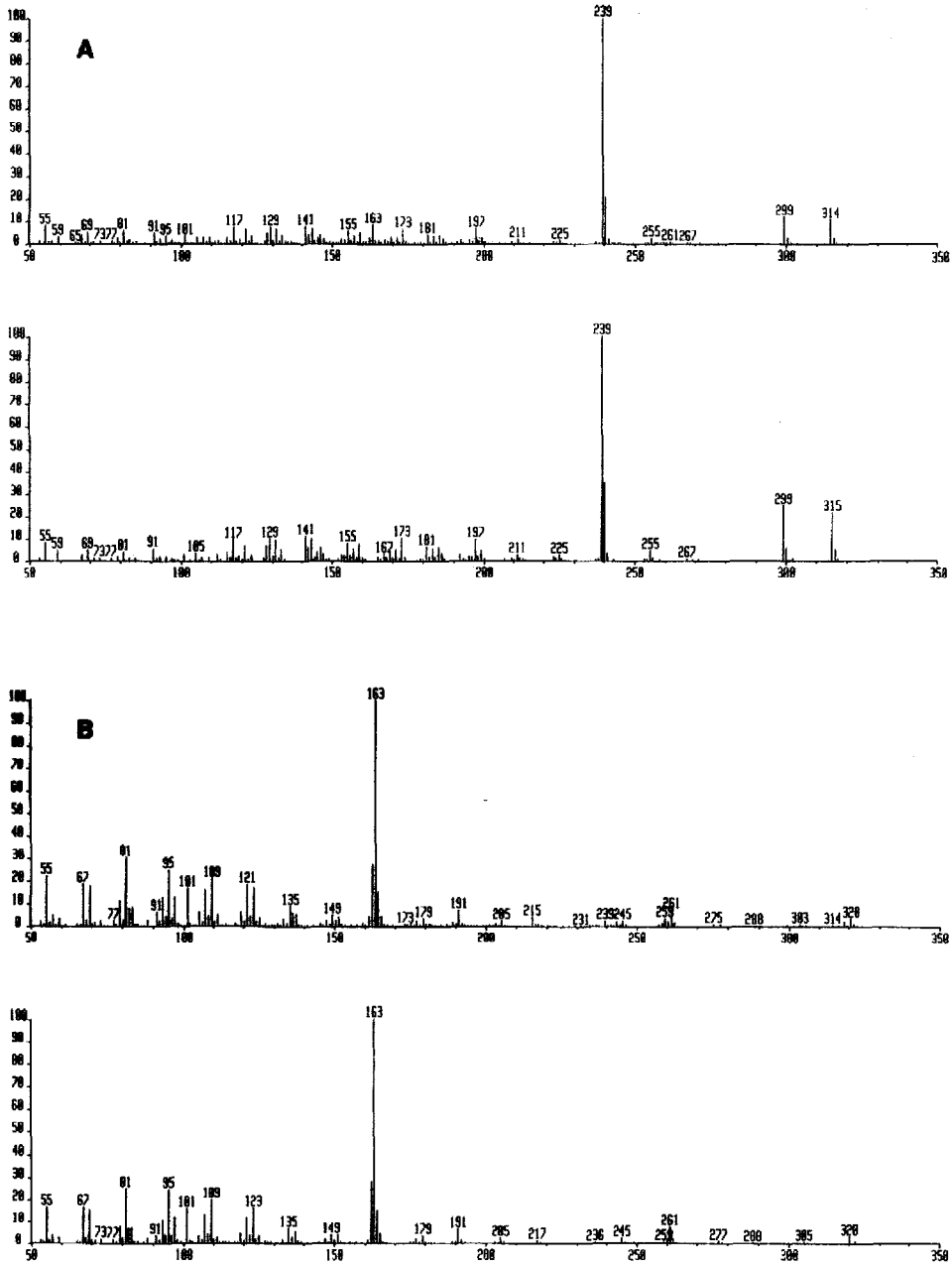

Fig. 4. Comparison of EI mass spectra of derivatives of compounds isolated from EOCl extracts (top) with those of authentic reference compounds (bottom): (A) dehydroabietic acid methyl ester; (B) tetrahydroabietic acid methyl ester.

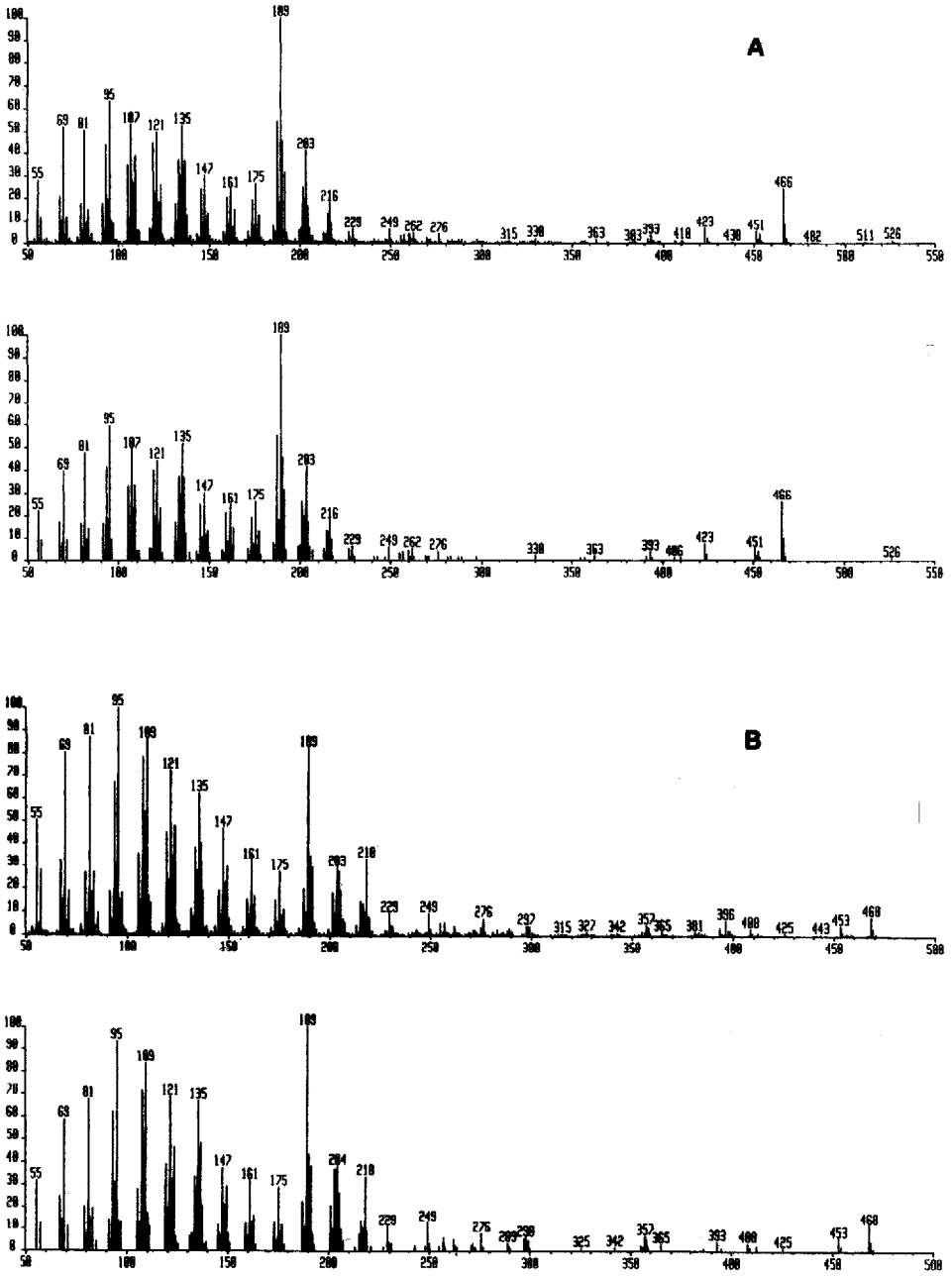

Fig. 5. Comparison of EI mass spectra of derivatives of compounds isolated from EOCl extracts (top) with those of authentic reference compounds (bottom): (A) betulin O-acetate; (B) lupeol O-acetate.

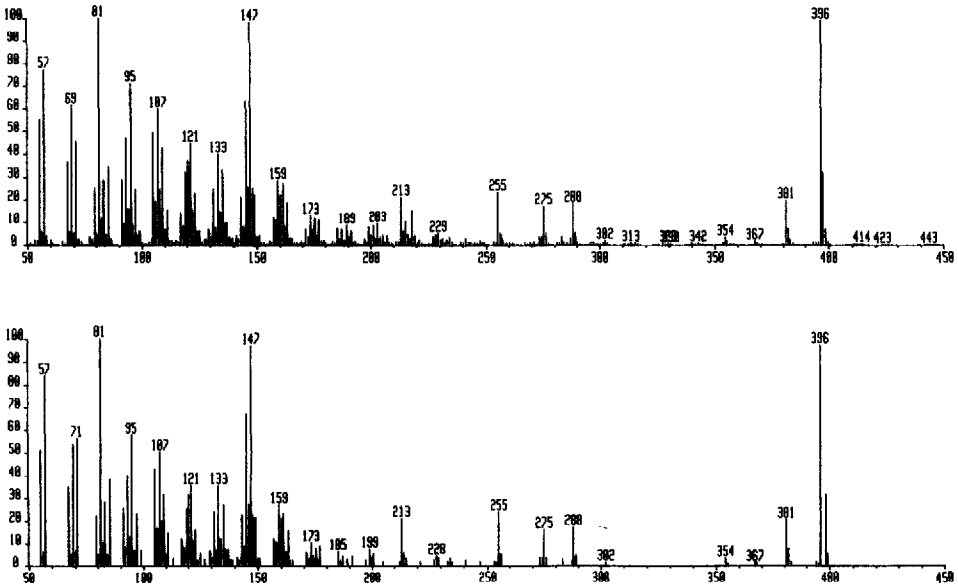

Fig. 6. Comparison of EI mass spectra of derivatives of compounds isolated from EOCl extracts (top) with those of authentic reference compounds (bottom): β-sitosterol O-acetate.

identified non-chlorinated compounds in the total organic fraction of the sediments was comparable (*ca.* 10%), we hypothesize that the unidentified components belong to classes of compound structurally distinct from those identified in this study. This emphasizes our conclusion from examination of the organochlorine compounds that analytical procedures based exclusively on application of GC methodologies are inadequate for analysis and identification of these constituents.

CONCLUSIONS

The major organic fraction of the cyclohexane-extractable fraction from sediment samples consisted of non-chlorinated compounds, including long-chain alkenoic and alkanoic acids, terpenes and some plant steroids. The free organochlorine compounds which could be identified by GC–MS were dominated by chlorinated long-chain alkanoic acids and chlorinated resin acids; chloroform and PCB isomers comprised a minor fraction. Free chlorinated phenolic compounds made a minor contribution, although such compounds were released in greater amounts after chemical modification.

In spite of intensive efforts, not more than *ca.* 8% of the EOCl fraction has been identified in terms of known compounds; with the exception of the PCBs, all were established constituents of bleachery effluents. It is concluded, therefore, that sum parameters such as EOCl do not provide an acceptable base for environmental hazard assessments of bleachery effluents and that, in addition, attention should be directed not only to chlorinated phenolic compounds but also to apparently persistent chlorinated and non-chlorinated compounds such as resin acids, steroids and long-chain alkenoic and alkanoic acids.

Application of chemical degradative procedures to the EOCl fraction and to whole sediments established that the major constituents of the two were significantly different; the unidentified EOCl components were probably not structurally related to chlorinated C_3-guaiacyl residues.

It is concluded that current procedures based on GC and GC–MS analysis are inadequate for revealing the identity of the major organochlorine constituent(s) of EOCl, and are unlikely to increase significantly the level of identification of the organochlorine components. It is suggested that attention be directed instead to HPLC methods and direct-probe mass spectrometric analysis.

ACKNOWLEDGEMENTS

We thank K. Martinsen and G. Carlberg, Senter for Industriforskning, Oslo, Norway, for the EOCl analyses, and D. J. Austin and M. L. Jamieson, ICI Agrochemicals, Jealotts Hill, U.K., for the elemental analyses. Funding for purchase of the mass spectrometer was provided by the Knut and Alice Wallenberg Foundation, and partial financial support was received from the Swedish Forest Industry Water and Air Pollution Research Foundation within the project "Environment 90, Bleaching Effluents".

REFERENCES

1 K. P. Kringstad and K. Lindström, *Environ. Sci. Technol.*, 18 (1984) 236A.
2 K. Lindström and F. Österberg, *Environ. Sci. Technol.*, 20 (1986) 133.
3 L. M. Strömberg, F. de Sousa, P. Ljungqvist, B. McKague and K. P. Kringstad, *Environ. Sci. Technol.*, 21 (1988) 754.
4 A. B. McKague, F. de Sousa, L. M. Strömberg and K. P. Kringstad, *Holzforschung*, 41 (1988) 191.
5 G. Carlberg, S. Johnsen, L. H. Landmark, B.-E. Bengtsson, B. Bergström, J. Skramstad and H. Storflor, *Water Sci. Technol.*, 20(2) (1988) 37.
6 A. Södergren, B.-E. Bengtsson, P. Johnsson, S. Lagergren, Å. Larsson, M. Olsson and L. Renberg, *Water Sci. Technol.*, 20(1) (1988) 49.
7 K. Martinsen, A. Kringstad and G. E. Carlberg, *Water Sci. Technol.*, 20(2) (1988) 13.
8 L. Håkansson, P. Johnsson, P. Johnson and K. Martinsen, *Water Sci. Technol.*, 20(2) (1988) 25.
9 T.-M. Xie, K. Abrahamsson, E. Fogelqvist and B. Josefsson, *Environ. Sci. Technol.*, 20 (1986) 457.
10 F. Österberg, A. Kierkegaard, L. Renberg and B.-E. Bengtsson, *Chemosphere*, 17 (1988) 1117.
11 L. Landner (Editor), *Chemicals in the Aquatic Environment*, Springer, Berlin, 1989.
12 C. Wesen, *Water Sci. Technol.*, 20(2) (1988) 185.
13 J. Hemming and K.-J. Lehtinen, *Nordic Pulp Paper Res. J.*, 4 (1988) 185.
14 M. Remberger, P.-Å. Hynning and A. H. Neilson, *Environ. Toxicol. Chem.*, 7 (1988) 795.
15 K. Lindström and J. J. Nordin, *J. Chromatogr.*, 128 (1976) 13.
16 G. Eklund, B. Josefsson and A. Björseth, *J. Chromatogr.*, 150 (1978) 161.
17 J. Knuutinen, *J. Chromatogr.*, 248 (1982) 289.
18 J. P. Kutney and E. Dimitriadis, *Helv. Chim. Acta*, 65 (1982) 1351.
19 E. A. Braude, L. M. Jackman, R. P. Linstead and G. Lowe, *J. Chem. Soc.*, (1960) 3123.
20 F. A. Fitzpatrick and S. Siggia, *Anal. Chem.*, 45 (1973) 2310.
21 S. Jensen, L. Renberg and L. Reutergårdh, *Anal. Chem.*, 49 (1977) 316.
22 B. Holmbom, *Pap. Puu*, 62 (1980) 523.
23 C. G. W. Brooks, E. C. Horning and G. S. Young, *Lipids*, 3 (1968) 391.
24 P.-Å. Hynning, M. Remberger and A. H. Neilson, *J. Chromatogr.*, 467 (1989) 99.
25 J. Gether, G. N. Lunde and E. Steinnes, *Anal. Chim. Acta*, 108 (1979) 137.
26 H. T. Badings and C. de Jong, *J. Chromatogr.*, 249 (1983) 493.
27 J. J. Tana, *Water Sci. Technol.*, 20(2) (1988) 77.
28 L. Matsoff and M. Nikinmaa, *Ecotoxicol. Environ. Saf.*, 14 (1987) 157.

29 B. Holmbom and K.-J. Lehtinen, *Pap. Puu*, 62 (1980) 673.
30 B. S. Das, S. G. Reid, J. L. Betts and K. Patrick, *J. Fish. Res. Board Can.*, 26 (1969) 3055.
31 S. Steinberg, M. E. Venkatesan and E. R. Kaplan, *J. Chromatogr.*, 298 (1984) 427.
32 M. Erickson and C. W. Dence, *Sven. Papperstidn.*, 10 (1976) 316.
33 A. H. Neilson, in L. Landner (Editor), *Chemicals in the Aquatic Environment*, Springer, Berlin, 1989, p. 74.
34 J. L. LaBrosse and R. J. Anderegg, *J. Chromatogr.*, 314 (1984) 83.
35 J. M. Leach and A. N. Thakore, *J. Fish. Res. Board Can.*, 32 (1975) 1249.
36 D. L. Swackhamer, B. D. McVeety and R. A. Hites, *Environ. Sci. Technol.*, 22 (1988) 664.
37 D. J. Gregor and W. D. Gummer, *Environ. Sci. Technol.*, 23 (1989) 561.