Journal of Chromatography, 580 (1992) 257–278
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6350

Review

Methods for the analysis of persistent chlorinated hydrocarbons in tissues

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(First received December 23rd, 1991; revised manuscript received March 24th, 1992)

ABSTRACT

Chlorinated hydrocarbons bioaccumulate in tissues and may have severe health consequences. These compounds occur individually, in small groups or as complex mixtures; examples of each category include aldrin, hexachlorocyclohexane and the polychlorinated biphenyls. Tissue extraction and purification schemes have been established, although new approaches such as supercritical fluid extraction are promising. Analyses often require the resolving power of capillary gas chromatography, in combination with the sensitivity and selectivity of electron-capture detection, electrolytic conductivity detection and mass spectrometry. Difficulties arise in quantitating chlorinated hydrocarbons in tissues, due to the number of components present and the fact that individual constituents may be reduced or enhanced in concentration in tissues, compared with the original formulation. Congener specific analysis and computer-assisted identification techniques have been applied to the problem.

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0378-4347/92/$15.00

LIST OF ABBREVIATIONS

BHC	Hexachlorocyclohexane
DDD	1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethane
DDE	1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethene
DDT	1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane
ELCD	Electrolytic conductivity detector
ECD	Electron-capture detector
ECNCI	Electron-capture negative chemical ionization
EI	Electron ionization
GC	Gas chromatography
GPC	Gel permeation chromatography
HCB	Hexachlorobenzene
HPLC	High-performance liquid chromatography
I.D.	Inner diameter
MS	Mass spectrometry
MS–MS	Mass spectrometry–mass spectrometry
PCB	Polychlorinated biphenyl(s)
PCN	Polychlorinated naphthalene(s)
PCT	Polychlorinated terphenyl(s)
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SIM	Selected ion monitoring
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TLC	Thin-layer chromatography

1. INTRODUCTION

1.1. Compounds of interest

This review of analytical methods focuses on the persistent chlorinated hydrocarbons. These chemicals are characterized by considerable stability. Although chlorinated hydrocarbons are simple in elemental composition, by definition consisting of only carbon, hydrogen and chlorine, a variety of structural types have been synthesized. These range from simple aliphatic to elaborate cyclic polychlorinated structures. Some examples are provided in Table 1. The chlorinated dibenzo-*p*-dioxins and related compounds are outside the scope of this review, although many of the techniques discussed below are also applicable to these compounds.

The persistent chlorinated hydrocarbons are typically anthropogenic in nature. Most were produced intentionally, *e.g.* the pesticides [1]. However, some were unintentionally made, such as those generated from the chlorination of wastewaters and wood pulp bleaching [2,3].

TABLE 1

REPRESENTATIVE CHLORINATED HYDROCARBONS AND USES

Compound[a]	Uses
PCBs	Dielectric fluids, plasticizers, sealants, lubricants
PCTs	Plasticizers, hydraulic fluids, fire retardants, sealants, investment casting waxes, vapor suppressors, lubricants
PCNs	Dielectric fluids, flame retardants, fungicides
Chlorinated paraffins	Investment casting waxes, flame retardants, plasticizers
DDT, DDE, DDD	Insecticide and breakdown products
Toxaphene	Insecticide
BHC	Insecticide
Chlordane	Insecticide
Mirex	Insecticide
Aldrin	Insecticide
Hexachlorobenzene	Fungicide

[a] For abbreviations see List of abbreviations.

Volatile compounds, such as chloroform, although of toxicological interest due to, for example, occupational exposure, will not be covered here. Preparative techniques for the volatiles differ considerably from the environmentally persistent compounds discussed below.

1.2. Health and environmental considerations

Unfortunately, attendant with the utility and efficacy of chlorinated hydrocarbons have been a myriad of environmental and human health effects, including acute toxicity, in addition to impacts resulting from chronic exposure, such as carcinogenesis and reproductive failure [4–7]. Deleterious interactions have been observed in both humans and other species. Not only have individuals working directly with the chemicals been exposed, but essentially the entire biosphere has become contaminated through secondary contact via air, water and food. Direct evidence of this is provided in the form of analyses conducted on biota far removed from immediate sources, such as arctic and deepwater organisms [8–10].

Crucial to the widespread occurrence of chlorinated hydrocarbons, as mentioned above, is their stability. This is apparent in the difficulties encountered in destroying polychlorinated biphenyls (PCBs)) present in contaminated soils and other matrices. To date the process is expensive and requires very rigorous reaction conditions.

The properties of chlorinated hydrocarbons include low water and high lipid solubility [11,12]. Therefore, biological accumulation factors in tissues may be exceptionally high [13]. Organisms possess enzyme systems, *e.g.* the mixed-function oxygenases, which are capable of transforming many xenobiotic chemicals to more easily excreted compounds [14]. However, the chlorinated hydrocarbons are typically poor substrates for these enzymes [15]. Organisms, such as shellfish, crustaceans and finfish, are less efficient at transforming these xenobiotic chemicals than humans [16]. Therefore, these biota may act as sources of these compounds, through the food chain, to man. Certain bacteria have been reported to possess considerable capabilities for dechlorinating recalcitrant compounds, such as PCBs [17]. Their abilities may be utilized to help solve disposal problems.

On account of the bioaccumulative properties of chlorinated hydrocarbons, it is likely that these compounds will be found in any fatty tissue sample examined, even fetal material. Tissues containing high concentrations of lipids are typical matrices chosen for examination by analysts. Fluids, such as blood, are also valuable because of their ease of collection and function as conduits for inter-organ transport of organochlorines. Table 2 lists some human tissues recently used for monitoring levels of chlorinated hydrocarbons.

1.3. Analytical rationale

Chlorinated hydrocarbons were released to the environment as individual compounds, small groups of related compounds or complex formulations consisting of up to several hundred individual constituents. Some of these were and are still being transformed, in the environment, to

TABLE 2

HUMAN TISSUES RECENTLY USED TO MONITOR FOR THE PRESENCE OF CHLORINATED HYDROCARBONS

Tissue	Analyte	References
Serum	Pesticides	99, 133, 134
	PCB	135, 136
Blood	Pesticides	28, 56, 65
	PCB/PCT	46, 100, 142
Sebum	Pesticides	31, 65, 134
	PCB	134
Milk	Pesticides	37, 137, 138
	PCB	61, 139, 140
Adipose tissue	Pesticides	56, 99, 141
	PCB	29, 64, 99, 100

other compounds. This factor greatly complicates their accurate determination in tissues. On account of this complexity, the high cost of analyses and the fact that many of these chemicals exhibit similar behavior during extraction and subsequent chromatographic steps, researchers have developed multi-residue methods for their detection. To avoid descriptive redundancy this review will take this approach where feasible. Most methods have been devised for the pesticides and the PCB-like compounds, rather than the by-products. However, on account of their similar properties, methods are easily adapted for these related compounds. Individual chlorinated hydrocarbons will be discussed where necessary or for emphasis.

2. SAMPLING

2.1. Sample contamination considerations

The health effects of chlorinated compounds at trace levels require methods capable of measuring parts per million, billion and occasionally trillion concentrations. Therefore, extreme care is required to prevent inclusion of extraneous analyte during the sampling, storage and analysis of samples. This necessitates that the highest-quality reagents be used and that their purity be assured. Glassware must be scrupulously cleaned. Baking at temperatures in excess of 500°C, followed by multiple solvent rinses just prior to use are suggested for non-volumetric glassware. Samples should be stored separately from standards. Obviously, blanks should be analyzed with the samples.

2.2. Sampling rationale

Accurate identification and quantitation of any analyte first requires that a representative sample be collected and properly preserved prior to work-up. This cannot be overemphasized. Sample collection is matrix-specific. As a consequence, the analyst must be familiar with the properties of the analyte and the matrix. Care must be taken to examine the individual data objectives and design an appropriate sampling protocol to meet these. In some cases objective planning, sampling and analysis are conducted by different individuals and groups. Communication between these three functions is critical.

3. TISSUE PREPARATION

3.1. Handling, storage and pretreatment

Fortunately for the chromatographer some properties of the chlorinated hydrocarbons present advantages in terms of their analysis. The inherent stability of the chlorinated hydrocarbons means that concern for losses during sample storage are lessened, compared with other classes of analytes [18]. This stability is also beneficial during extraction, purification and subsequent chromatographic steps. Nonetheless, some handling losses are inevitable [19].

Tissue samples should be kept cold from time of collection, typically, they are frozen. Repeated thawing and freezing may rupture cells, due to the formation of ice crystals, and affect sample integrity, as well as subsequent extraction processes. Minimization of storage time is recommended, as some changes may occur in the sample over time. This precaution is as much to protect the tissue itself, as the analyte. Analytical results are generally reported on a concentration

basis, rather than as simply the total amount present. Therefore, changes in the mass of matrix due to desiccation or decomposition will result in an alteration of results. Reporting of data on a dry or lipid mass, rather than a wet or fresh mass basis, has been suggested as a means to compensate not only for these differences, but also for variables such as reproductive condition, nutritional state, sex or age [20,21]. Lipid content data may also be used to avoid overloading chromatographic purification steps. As information such as dry mass and lipid content are relatively easily determined, it is recommended that these data be collected on all samples so that results from other sources may be more easily compared.

The content of lipoidal material in tissues is typically operationally defined, *i.e.*, it is a function of the technique utilized. Methods range from simple gravimetric measurements of extractable material, obtained during the course of the chlorinated hydrocarbon analysis, to more specialized procedures with discrete chromatographic determinations of lipid components [22–24]. Schneider [25] illustrated the consequences of lipid composition determination by expressing the concentration of PCBs in muscle tissue, gonads and livers of cod as a function of several parameters. As shown in Fig. 1, large differences in concentrations between tissues were observed for data expressed on either a wet or dry mass basis. These differences decreased when the data was normalized to lipid content, and decreased yet again when only non-polar lipids were considered.

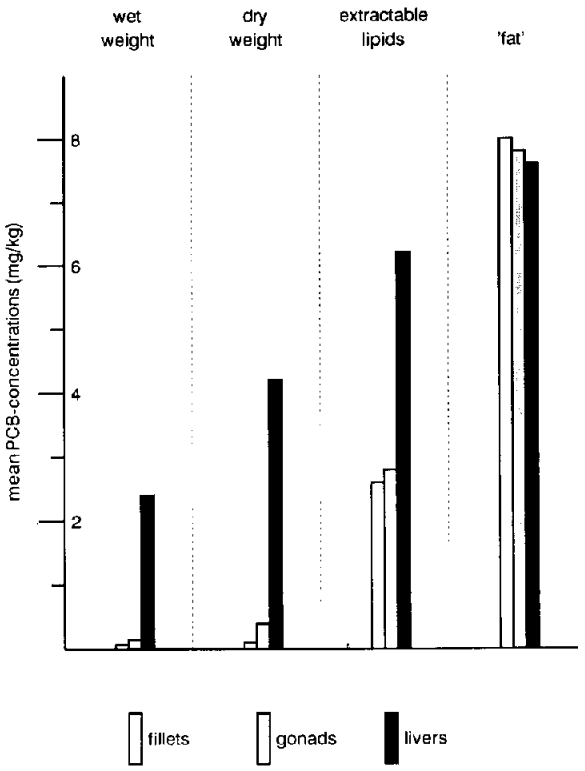

Fig. 1. Mean PCB concentrations in several different cod tissues, calculated with respect to wet mass, dry mass, total extractable lipids and non-polar lipids. This illustrates the importance of matrix in the reporting of results. Reprinted with permission from ref. 25.

3.2. Extraction

Prior to chromatographic analysis, chlorinated hydrocarbons must be separated from tissues. A number of extraction techniques have been published for biological matrices; these may begin with desiccated or wet samples. Methodology selection depends upon matrix type, analyte(s) of interest and availability of equipment. Ideally, tissues should be analyzed immediately after collection and with as little alteration as feasible. Sample handling results in potential analyte loss and contamination.

Standards should be added prior to extraction to assess the loss of compounds through the analysis process. Compounds added in this manner do not exactly mimic those present within tissues, since the latter may be associated with biological membranes and other components. Commonly used nomenclature designates these standards as "surrogates". These should correspond as closely as possible to the chemical properties of the chlorinated hydrocarbons sought. Recently, a number of isotopically labeled standards have become available for use in mass spectrometry (MS)-based detection schemes.

3.2.1. Desiccated tissue-based extraction methods

The intent of sample desiccation is to remove water and, therefore, allow greater solvent–ana-

lyte contact. In cases where only small amounts of moisture are present, such as wipe samples to determine organochlorines in skin lipids, simple passive air-drying may be sufficient. Tissue may also be dried by lyophilization or by the addition of a chemical desiccant [26,27]. As specific examples, Kanja *et al.* [28] ground subcutaneous fat with anhydrous magnesium sulphate and sea sand, prior to extraction of tissues for organochlorine pesticides; Focardi and Romei [29] freeze-dried samples before examining human tissues for PCBs. Lyophilization allows for the extraction of a greater amount of tissue at a time, provides a convenient mechanism for dry mass determination and eliminates the need to add drying agents to the sample. This latter material may introduce contaminants, if not pretreated. However, lyophilization equipment is expensive, contamination from pump oils is possible if preventative actions are not taken and loss of the more volatile compounds may occur.

Typically, non-polar solvents are employed for subsequent extraction of dry tissue. For example, Holden and Marsden [30] extracted desiccated tissues with hexane. Sasaki *et al.* [31] utilized a 1:1 mixture of acetone–hexane to separate organochlorine pesticides from skin lipids. Other solvents, such as dichloromethane, toluene and various combinations, have also been used [32,33]. Choice of solvent may determine not only analyte recovery, but also the quantity and identity of co-extractives.

Desiccated tissues may be subjected to continuous extraction, *e.g.* Soxhlet extraction [34], or packed into open chromatographic columns and eluted with solvent [35]. Alternatively, mechanical devices, such as ball mills and mixers, have been employed. Continuous extraction methods provide more sample–solvent contact time and typically operate at elevated temperatures. As a consequence, these may remove greater amounts of non-polar compounds than less rigorous extraction conditions [36]. Shortcomings include decomposition of extracted materials due to elevated temperatures, *e.g.* lipids and some analytes, and the requirement for delicate and expensive glassware.

Liquid matrices such as milk, blood or urine have generally been handled by liquid–liquid extraction techniques. However, recently, some researchers have examined the efficacy of adding or sorbing these liquids to solid phases such as silica gel, octadecyl-bonded silica and others [37–40]. As an example, Steinwandter [38] homogenized milk with silica gel. This material was then added to a column containing additional deactivated silica gel. On-line extraction and clean-up for organochlorine pesticides was achieved by elution with a 80:20 (v/v) mixture of petroleum ether and dichloromethane.

Several investigators have combined extraction and purification steps for solid tissues, either by placing the dried sample above a bed of adsorbent or mixing the two in open chromatographic columns [41–43]. Again, this affords a degree of sample purification, concurrent with the extraction step. Long *et al.* [39] used this technique, termed matrix solid-phase dispersion, to extract chlorinated pesticides from animal fat.

A number of commercially available, prepacked solid-phase extraction columns have also become popular. These columns contain a variety of stationary phases, in a number of sizes and with recommendations on candidate elution methods. Columns are available in plastics, as well as PTFE and glass configurations. The latter are recommended, to reduce interferences from extractables present in the plastic. These solid-phase extraction approaches have the advantage that minimal solvent volumes are required and a degree of additional analyte purification may be realized, compared to other techniques.

3.2.2. Wet tissue-based extraction methods

The simplest method, in theory, for separating chlorinated hydrocarbons from tissues involves partitioning between the wet or fresh sample and an organic solvent. This is more straightforward with matrices such as blood, urine and milk [44]. However, "solid" samples, such as adipose and liver, have been macerated or ultrasonically disrupted and subjected to liquid–liquid extraction as well. The matrix is typically homogenized with added water and an organic solvent, such as acetonitrile or methanol [44,45].

Saponification procedures have also been used. For example, Watanabe *et al.*, [46] examining PCBs and polychlorinated terphenyls (PCTs) in adipose, milk, blood, fetal skin, liver and fat, refluxed the tissue with 2 *M* ethanolic potassium hydroxide. The saponification process cleaves ester linkages present in many lipids, rendering them more water-soluble. Often, however, serious emulsions result. Treatment by centrifugation, filtration, freezing or salting out are then required. Saponification may, however, free chlorinated hydrocarbons bound to endogenous material. This material may not be easily extractable by less rigorous procedures [36]. Evaluation of the procedure for the analytes of concern should be conducted, as alterations in some organochlorines under these harsh conditions have been reported [47].

Saponified tissues and extracts are often filtered or centrifuged to remove insoluble material. Chlorinated analytes may then be obtained by extraction with a non-polar solvent, such as hexane or petroleum ether, in a separatory funnel or other suitable vessel [44]. Filtrates and extraction solvent may be mixed by a number of processes. These include automated or manual shaking and mixing by sonication, vortex mixer or blender. Water washing steps, often accompanied by the addition of salt to reduce emulsions, are sometimes used to further purify the extracts.

3.2.3. Supercritical fluid extraction

Supercritical fluid extraction (SFE) is a relatively new technique which holds considerable promise for the analysis of chlorinated hydrocarbons [48]. Toxicologically innocuous solvents, such as carbon dioxide, may be used to extract organochlorines from biological matrices in relatively short periods of time. Direct coupling of SFE to chromatographic techniques has been accomplished as well [49]. Alterations in temperature and pressure may be utilized during SFE to optimize the desired separation. Other parameters affecting recovery are extraction time, sample moisture and sample size. Polar modifiers may be used to alter selectivity of the supercritical fluid [50]. Sample preparation typically involves mixing the tissue with a sorbent or drying agent, such as sodium sulfate. This material is then loaded into pressurized cells for extraction. Nam *et al.* [51] examined the SFE of chlorinated pesticides and PCBs from biological tissues. Extractions were conducted with supercritical carbon dioxide at 40–60°C and 100–150 bars. Reported recoveries were similar to those obtained by Soxhlet extraction, in less than one half of the time.

Current disadvantages of the technique involve the capital expense of the instrumentation and the relatively small sample cells commercially available. However, potential time savings and lower solvent-associated costs, for both purchase and disposal, may compensate for the capital investment. Multi-port extraction instrumentation is now becoming available. This advance, in combination with the inherent speed of the technique, may compensate for the small cells available. As with other extraction techniques, many evaluations conducted to date have only compared recoveries of laboratory-added standards from samples. Additional comparisons of analyte concentrations measured in replicate field samples by competitive methods should also be made.

3.3. Extract clean-up

A number of techniques have been employed to remove co-extracted interferences from tissue samples. Unfortunately, some endogenous materials, such as lipids, possess polarities similar to the chlorinated hydrocarbons. As a consequence, separation of these prior to final chromatographic analysis is often difficult. Extraction techniques which result in lower levels of co-extractives should be considered by the analyst when evaluating potential methodologies for their specific application.

3.3.1. Caustic chemical treatment

One widely used technique for the removal of lipids is the addition of caustics to the extract. Concentrated acids, bases or peroxides may be used directly. Alternatively, these may be added to a solid support, packed into a chromatogra-

phy column and eluted [52–55]. Sasaki *et al.* [56] treated blood extracts with concentrated sulfuric acid to remove interferences. Solid sodium bicarbonate was then added, the sample dried and the residue redissolved in hexane. DiMuccio *et al.* [57] added concentrated sulfuric acid to a column packed with macroporous diatomaceous earth and eluted extracts of fatty tissues with light petroleum. Caustic treatment often is followed by an additional step, such as column chromatography or water washes, to remove the reagent and the degraded by-products. Care should be exercised to ensure that the desired analytes are not altered by this step, *e.g.* aldrin and the related family of pesticides which are often analyzed with the chlorinated hydrocarbons [58].

3.3.2. Liquid–liquid partitioning

One of the first widely used methods for the purification of extracts was back-partitioning of the target analytes. Specifically, chlorinated hydrocarbons, initially extracted from tissue with a non-polar solvent, are partitioned into a more polar solvent, leaving the bulk of the extracted lipids behind [59]. Kuwabara *et al.* [60] examined the recovery of organochlorine pesticides from spiked animal fat. The lipoidal material, dissolved in hexane, was partitioned with acetonitrile by sequential batch extractions in a separatory funnel. The contents of the funnel were allowed to stand and the acetonitrile layer drained. This layer was then diluted with 2% aqueous sodium chloride and re-extracted with hexane. The hexane was washed with water and the extract dried with anhydrous sodium sulfate. Some difficulties in back-extracting very non-polar compounds, such as the PCBs, have been observed with these methods. In many instances, column-based technologies have replaced liquid–liquid partitioning for the removal of non-polar interferences due to their speed, reproducibility and lower solvent requirements [61].

3.3.3. Open column chromatography

Open or gravity column chromatography is an integral feature of many tissue preparation procedures [45,62,63]. Typically, a column is charged with a solid sorbent, and the sample is added to the head of the column in a weak solvent and eluted with sequentially stronger solvents. Before use, sorbents are normally purified by extraction or prior elution. The material is often heated and then partially deactivated with a known percentage of water to optimize separations. Columns may be topped with anhydrous sodium sulfate to prevent additional deactivation, due to residual moisture in the sample. Periodic recalibration of elution volumes is suggested due to differences in activity between batches. Care should also be exercised to avoid overloading columns with excess lipid.

Florisil has been one of the most popular adsorbents [59]. Williams and LeBel [64] eluted Florisil, deactivated with 2% water, with 5% dichloromethane in cyclohexane for the purification of PCBs in human adipose tissue. Wariishi and Nishiyama [65] used this sorbent to purify blood and sebum for chlordane analysis. Kuwabara *et al.* [60] examined the utility of Florisil for the clean-up of pesticides from animal fat. Silica gel has also been widely used. Sasaki *et al.* [56] used 15 g of deactivated silica gel and eluted with 100 ml of methylene chloride–petroleum ether (1:4) to purify organochlorine pesticides in fat. Kveseth and Brevik [63] examined the effect of silica gel water content on the separation of PCBs and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethene (DDE). Duarte-Davidson *et al.* [61] used 3 g of 5% deactivated alumina, eluted with 20 ml of hexane to clean up PCBs in human milk. Prapamontol and Stevenson [66] used 500 mg of C_{18} solid-phase extraction columns to clean up pesticides extracted from milk. Combinations of sorbents have also been employed. Schecter *et al.* [67] used a column containing both Florisil and silica gel to purify organochlorine residues present in extracts of a variety of human tissues.

In addition to removing lipids and other interferences, open column chromatography has been used to perform organochlorine group separations [45,59,60]. As an example, Price *et al.* [41] separated pesticides and PCBs on a 5.0-g silica gel column. The column was initially rinsed with 3 ml of hexane, which was discarded. This was

followed by 15- and 20-ml aliquots of hexane which eluted hexachlorobenzene (HCB) and Mirex, and the PCBs and DDE, respectively. Approximately 20 ml of benzene were then added and collected to obtain chlordane, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), toxaphene, hexachlorocyclohexane (BHC) and the remainder of the DDE.

Recently, considerable interest has arisen in separating planar and non-planar PCBs, due to their differing toxicities [68]. Activated carbon columns have been used for this purpose [3,69,70]. Williams and LeBel [64] added 250 mg of an activated carbon–celite (1:19) mixture to a column. The material was packed between thin layers of anhydrous sodium sulfate and glass wool. An extract of adipose tissue, pretreated with Florisil as described above, was added to the column in 10 ml of hexane. The column was eluted with an additional 20 ml of dichloromethane and the eluate discarded. The column was then inverted and the coplanar PCBs eluted with 4 ml of toluene.

3.3.4. Preparative gel permeation chromatography

Low-pressure size-exclusion or gel permeation chromatography (GPC) is experiencing increased usage for the separation of organochlorines from large-molecular-volume lipids and other interferences [58,71]. Rather than working on a polarity basis, as the adsorption columns mentioned above, GPC operates on a size-exclusion principle. Larger molecules are excluded from some or all of the pores of the stationary phase, typically consisting of synthetic beads of controlled pore size. As a consequence, the larger molecules, *e.g.* lipids, emerge first from the column and may be discarded. The analyst simply increases or decreases the volume of eluate retained to include the desired compounds. Thus, the technique is very amenable to multi-residue analyses.

GPC has the advantage that chemical interactions between the analyte and the stationary phase are minimal, as compared to adsorption chromatography. This reduces the possibility of irreversible binding and analyte decomposition, although this is typically not a problem with the stable chlorinated hydrocarbons.

As adsorption is not a major factor, solvent gradient elution is not generally required. Mobile phases are typically pumped through the GPC column at low pressures. Commonly used solvents include dichloromethane, cyclohexane, acetone, toluene or mixtures thereof. Large capacity, automated models, some of which include solvent evaporation capabilities, are commercially available.

Common column packing materials have included Biobeads, consisting of a styrene–divinylbenzene copolymer, and Sephadex, a cross-linked dextran. Sasaki *et al.* [31] used a 21 cm × 1.0 cm bed of Biobeads S-X2, eluted with a 1:1 mixture of dichloromethane–hexane to separate chlorinated pesticides from skin lipids. Hale *et al.* [72] compared the abilities of S-X8 and S-X3 to separate PCTs from lipoidal material. The latter resin, in concert with a 1:1 solvent regime of cyclohexane–dichloromethane, was reported to provide superior separation.

3.3.5. High-performance liquid and thin-layer chromatography

High-performance liquid chromatography (HPLC) has found some utility in the area of extract purification, although most researchers rely on less sophisticated equipment. Typically, the higher resolution capabilities of HPLC have not been required for the class separations desired, although use has been made of this technique for the complex PCBs and PCTs. Both size exclusion- and polarity-based techniques have been utilized [54,73]. As an example, Krahn *et al.* [74] injected 500-μl tissue extracts onto a preparatory 100-Å size-exclusion column and eluted chlorinated hydrocarbons with methylene chloride at 5 ml/min. PCBs and pesticides were obtained within 15 min. A silica-alumina open column was used, prior to HPLC, to remove some lipoidal material. Recently, Haglund *et al.* [75] applied electron–donor–acceptor HPLC to separate planar from non-planar PCBs using a single solvent. Some usage of HPLC for the determination of pesticides has also been made [76]. However, gas chromatography (GC), as desribed below, has been much more popular due to the excellent GC

properties of chlorinated hydrocarbons, the technique's superior resolving capabilities and the sensitivity of GC detectors to halogenated compounds.

Thin-layer chromatography (TLC) has found limited utility in the purification of tissue extracts for chlorinated hydrocarbons. However, some researchers have used it to separate classes of PCBs and PCTs, prior to GC analysis [73,77,78].

4. FINAL SEPARATION AND IDENTIFICATION

4.1. Capillary-column gas chromatography

Capillary GC is the separation method of choice for the analysis of chlorinated hydrocarbons because of its potentially high chromatographic resolution. The replacement of fragile glass with flexible fused-silica columns has added to its popularity.

The application of this technique to PCBs was reviewed by Pellizzari *et al.* [79] in 1985. Many of the basics of that review remain applicable, although refinements in column technology have continued. As mentioned previously, chlorinated hydrocarbons are non-polar and exhibit excellent chromatographic characteristics on commercially available capillary columns. The main consideration is whether adequate separation of the individual components of a complex analyte is achieved.

A detailed discussion of chromatographic theory is beyond the scope of this review, several texts are available on this subject [80–82]. Briefly, however, to achieve resolution of the individual components of complex chlorinated hydrocarbon mixtures, a number of factors need to be considered. These items include column length and internal diameter (I.D.), carrier gas, phase type and film thickness.

One of the most common stationary phases used in the analysis of organochlorines is cross-linked 5% phenyl methylsilicone (*e.g.* DB-5, SE-54) [32,83,84], but other phases such as 50% phenyl methyl silicone (DB-17) [64] and 50% trifluoropropyl methyl silicone (DB-210) [85] have been employed to enhance specific separations or to fulfil a second-phase requirement for identification purposes. Storr-Hansen [86] used a dual-column system and a T-split, installed at the injector, for the separation of PCBs, DB-5 and DB-1701 columns were linked in parallel and the behavior of the congeners on the columns used for confirmatory purposes. Highest resolution is obtained on long, narrow-I.D. columns with thin liquid phase films, thus maximizing the number of theoretical plates. For example, a 60 m × 0.15 mm I.D. column with a 0.20 μm film thickness was used by De Boer and Dao [87] to separate PCB congeners in fish extracts. Alternatively, some researchers have chosen to connect shorter columns in series [88].

Carrier gas selection also affects separation capabilities. Hydrogen is preferred, however, helium is often used due to the former's explosive hazard. Nitrogen is seldom utilized on account of the increased analysis times required with this gas. Column oven temperature programs may also be manipulated to obtain the needed resolution. Mullin *et al.* [89] used a temperature program of 1°C/min, over a 140-min run, to separate PCB congeners on a 50 m × 0.2 mm I.D. SE-54 column with hydrogen carrier gas.

On account of the low concentrations of chlorinated hydrocarbons encountered in tissues, splitless injection techniques are generally used. On-column injection is growing in popularity, as this limits sample discrimination in the injector [80–82].

Judicious selection of detection system may reduce reliance on optimization of chromatographic resolution, as using the best available technology may be expensive in terms of time, equipment, carrier gases and column needs. High-resolution systems are vulnerable to column overload problems, particularly for biological samples. Despite improved sample preparation procedures, tissue extracts often contain significant amounts of interfering material.

Another approach to achieving difficult separations has been the use of multi-dimensional GC or heart cutting. This technique involves transferring the unresolved compounds onto a second column, containing an alternative liquid phase,

on which the necessary resolution can be achieved. Schultz *et al.* [90] used a Siemens Sichromat-2 dual-oven gas chromatograph equipped with a 25-m SE-54 and a 30-m OV-210 capillary column to provide a complete separation of all components in several common Aroclor and Clophen formulations. Both columns were 0.32 mm I.D. and had a film thickness of 0.25 μm. Fractions were selected during the run from the former and transferred to the latter column, via a valveless pneumatic system, to attain complete separation of the mixture components.

4.2. Packed-column gas chromatography and other techniques

A variety of other techniques are available to the chromatographer to meet their particular needs. These include packed columns, wide-bore capillary columns and supercritical fluid chromatography (SFC).

Prior to the emergence of capillary GC, the majority of studies examining chlorinated hydrocarbons in tissues were conducted on packed columns. This technique is still used effectively in cases where the resolution of capillary GC is not required. Situations include those in which a limited number of specific compounds are targeted [43,45,91], or sophisticated separations are employed to purify and separate the compounds of interest into specific subfractions [41]. The difference in resolution obtainable with packed *versus* capillary columns for chlorinated hydrocarbons has been demonstrated (Fig. 2). Packed columns are easily made in the laboratory with a multitude of stationary phases and supports. However, this potential diversity is typically not required for non-polar analytes, such as the chlorinated hydrocarbons.

Specific applications of packed columns for the analysis of chlorinated hydrocarbons are available in the literature. For example, Noren and Sjovall [37] analyzed pesticides and PCBs on 2 m × 2 mm I.D. glass columns, packed with a mixture of 3% silicone GE SF-96 and 6% DC QF-1 on Chromosorb W HP. Wariishi and Nishiyama [65] examined chlordane obtained from blood and sebum on a 2% OV-1 column. Putnam *et al.* [92] chromatographed PCTs with a 3% OV-210 phase on a Gas Chrom Q support.

One advantage of packed-column GC is its greater sample capacity. A compromise between resolution, speed of analysis and capacity may be realized by the use of wide-bore capillary columns for specific compounds. Many packed-column GC instruments and techniques are directly amenable to this technology. Hopper [93] analyzed a variety of chlorinated pesticides present in lipid extracts on both a packed column (183 cm × 0.2 cm I.D., 3% OV-101 on 80–100 mesh Supelcoport) and a fused-silica capillary column (30 m × 0.53 mm I.D., 1.5 μm film thickness, DB-1). Comparable results were obtained with both columns, although a faster analysis was possible with the capillary column on account of its greater number of theoretical plates.

SFC has been applied by Onuska *et al.* [94] to the analysis of PCBs and PCTs. Carbon dioxide was used as the supercritical fluid. The authors rated the SFC resolution similar to that obtainable by packed-column GC. Their sensitivity with the UV detector employed was inferior to that obtainable with an electron-capture detector (ECD), although the former detector has the advantage that it is non-destructive. SFC also holds promise for the analysis of low-volatility compounds which are not amenable to GC [95]. SFE, as discussed previously, has been gaining acceptance as an extraction technique. The possibility for a direct link between the extraction unit and chromatograph with this method may, in certain instances, provide a valuable and rapid analytical technique [49].

4.3. Gas chromatographic detectors

4.3.1. Electron-capture detector

The ECD has been the most commonly used detector for the determination of chlorinated hydrocarbons, with numerous references to its credit [61,67,79,91,96,97]. Some specific applications have included pesticides in milk [66], blood [98] and skin lipids [31], PCBs in spleen and testis [67], adipose [99], milk [37] and blood [100] and PCTs

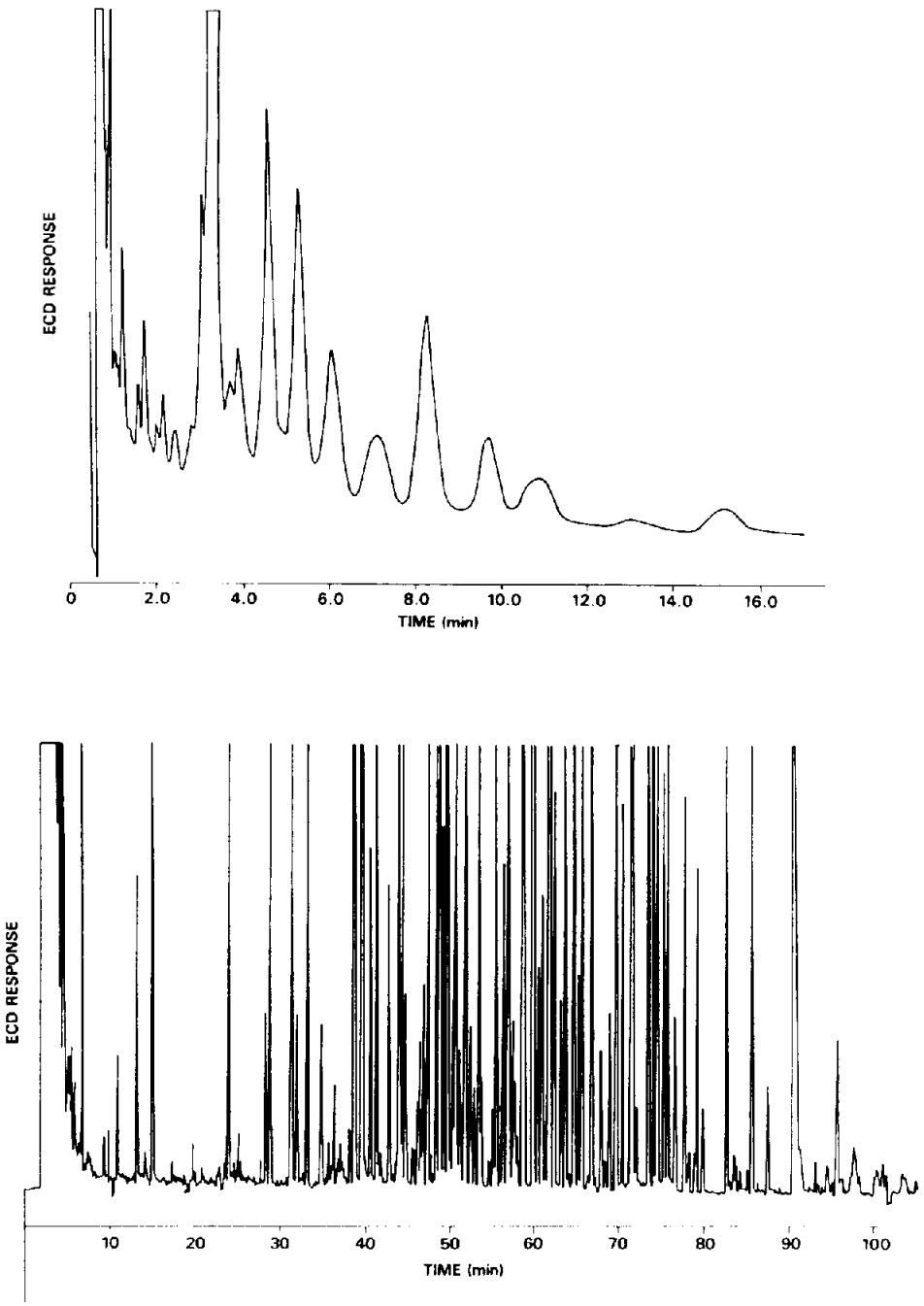

Fig. 2. Comparison of chromatographic resolution obtained on a packed (top) *versus* a capillary column (bottom) for a human adipose tissue extract. Detection was accomplished with an ECD. Reprinted with permission from ref. 79.

in fat, milk, blood and fetal tissues [46]. This popularity is attributable to its sensitivity, low cost, simplicity of operation and ease of maintenance. Descriptions of its operating principles are available elsewhere [79,80,101].

Although the ECD exhibits heightened sensitivity to chlorinated compounds, some response to non-halogenated compounds with electron-capturing characteristics, such as those containing oxygen and sulfur, is observed [80]. Also, be-

cause ECD response is specific to the structural geometry of the analyte, as well as to the level of chlorination, individual response factors are typically needed for each compound analyzed [88,89]. This can become very difficult to manage for complex mixtures, such as the PCTs, where several thousand congeners are possible.

4.3.2. Electrolytic conductivity detector

The electrolytic conductivity detector (ELCD), or Hall detector, is an alternative to ECD for the analysis of chlorinated hydrocarbons in tissues. Detection of these compounds is based on the reduction of the component chlorine atoms to HCl by hydrogen, in the presence of a nickel catalyst at elevated temperature. Temperatures as high as 960°C are recommended for complete reduction. The gases produced in the reactor are then mixed with an electrolyte, such as *n*-propanol, altering its conductivity. The change in conductivity is reported by the associated electronics as a voltage. Additional details are available elsewhere [102].

The ELCD is less sensitive than the ECD. Analysis of actual environmental samples indicates approximately an order of magnitude difference in sensitivity between the two detectors, which is in reasonable agreement with instrument manufacturers [103]. Early ELCDs suffered from reliability problems. Newer designs still require greater maintenance than the ECD, but are much improved. Detector-specific expendables include reaction tubes, electrolyte and resins.

The ELCD has the advantage of being more selective than the ECD. This can considerably reduce the time and costs associated with the sample purification steps required prior to GC analysis [45,103]. Fig. 3A and B show ELCD chromatograms of Aroclor 1254 and 1260. For comparison of specificity, ELCD- and ECD-based chromatograms of a raw, unfractionated water sample, contaminated with these Aroclors, are presented (Fig. 3C and D). Finally, an ECD chromatogram of this same water extract, after Florisil clean-up, is provided (Fig. 3E).

Quantification using ELCD is more straightforward than for the ECD, because the former

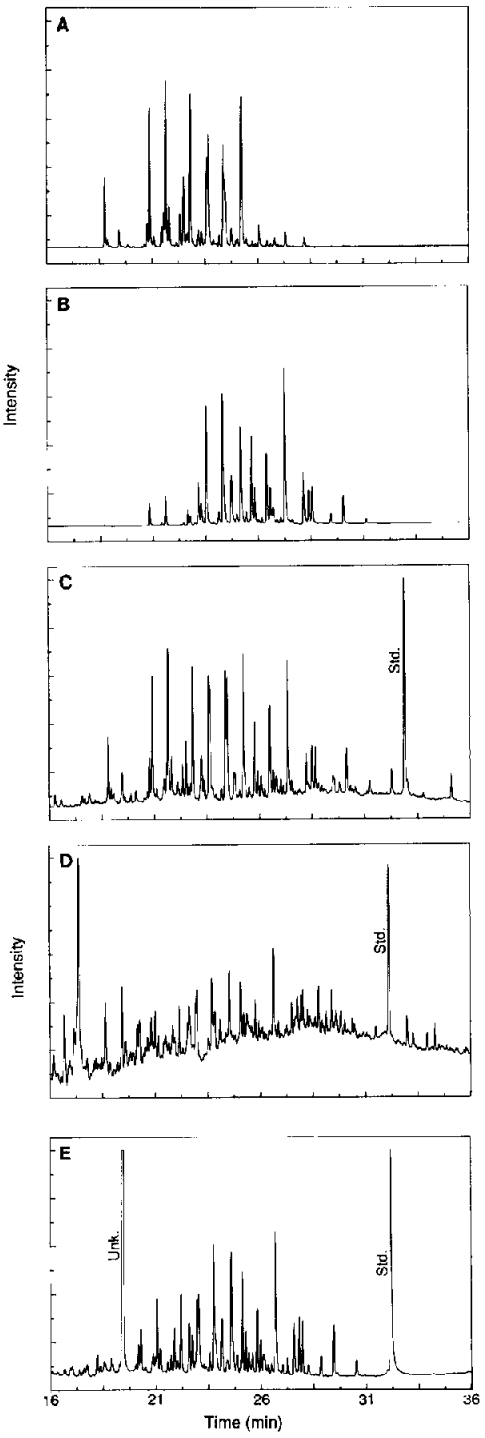

Fig. 3. ELCD chromatograms of Aroclor 1254 (A) and 1260 (B) standards. ELCD (C) and ECD (D) chromatograms of an unfractionated water sample containing these Aroclors are presented for comparison of relative detector specificities. Finally, an ECD chromatogram (E) of this same water extract, after Florisil clean-up, is provided.

responds only to the molar chlorine content of the analyte, irrespective of the substitution pattern of the chlorines. Thus, only 10 response factors, rather than 209, are required for the PCBs [104], *i.e.* one for each level of biphenyl chlorination. Complete configurational identification of individual compounds are not required, if the number of chlorine atoms for a given structure can be established [105]. This advantage may be critical for complex mixtures where complete sets of standards are not available, or are impractical to employ because of the sheer number required. As an example, for PCT analysis, knowledge of chlorine content of chromatographic peaks in a sample may be obtained by MS. Instrument response factors can then be determined with a set of fourteen standards, corresponding to the various levels of chlorination of the terphenyl molecule.

Both the ELCD and ECD, discussed previously, are halogen-selective but exhibit, to differing degrees, some response to non-halogenated compounds. Identification and quantification of analytes with these detectors are dependent on the resolution that can be achieved by the GC column. Identifications from these GC detectors must inevitably be tentative. A higher degree of confidence may be attached to identifications, depending on the analytes, knowledge of the sample itself (*e.g.* type and source) and the purification steps employed. Dual-column analysis using dissimilar phases may also be used, although this too becomes tenuous when the analyst is dealing with environmentally altered, complex mixtures.

The ELCD holds significant promise for use in biomedical applications. However, to date, most researchers have used the more stable and sensitive ECD.

4.3.3. Mass spectrometry

MS provides a level of confidence in the identification of pollutants in tissues that cannot be obtained by techniques such as ECD and ELCD [97]. Both full-scanning and selected ion monitoring (SIM) modes have enjoyed considerable use in the environmental field for a number of years [79].

Recently, a proliferation of MS techniques has occurred, many of which have been applied to environmental samples. Methods have included new ionization techniques such as negative chemical ionization with various reagent and moderator gases, high-resolution SIM, and mass spectrometry–mass spectrometry (MS–MS) including collisionally activated dissociation. A major driving force behind the application of new MS techniques in environmental analysis has been the need for improved methods for the determination of the highly toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

Electron ionization (EI) is the most commonly applied form of ionization due to its availability, universal applicability, reproducibility, the structural information obtained, the presence of reference libraries and inter-laboratory transferability. Muir *et al.* [106] used full-scanning EI-MS for identification of PCB, DDT and its metabolites, chlordane and toxaphene in adipose tissue from white beaked dolphins and pilot whales. Mass chromatograms formed from summing the intensities of the major ions of the analytes proved to be a very effective way of deconvoluting this complex set of analytes. One drawback that does limit the use of EI is that in the full-scanning mode it is often not a sensitive or selective enough technique to obtain spectra at the concentrations at which organochlorines are typically encountered in tissues. For this reason, SIM is frequently used [79].

Negative chemical ionization is a term that has been applied to various ionization processes that generate anions. These include ion–molecule reactions, such as addition of O^- [107] and are, therefore, analogous to those occurring in positive chemical ionization. In the case of organohalogens an alternative process involving the capture of an electron to form an anion [108] is the most commonly used ionization procedure. This anion may remain as the molecular ion or dissociate, often by progressive losses of chlorine atoms, to form fragment ions. The latter process is often referred to as dissociative electron capture. The electron-capture ionization process is frequently used for the analysis of organochlo-

rine pollutants. This ionization mode is called by a variety of names including negative chemical ionization, electron-capture negative chemical ionization (ECNCI), electron-capture negative ionization and high-pressure electron-capture ionization. For simplicity, ECNCI is the term used in this review.

ECNCI has been used in many environmental studies because of its inherent sensitivity and selectivity [103,107,109,110]. It is, however, a process that is somewhat structure-specific, with sensitivity being both a function of the number of chlorine atoms present and the structural geometry of the molecule. Compounds ionized most effectively in ECNCI, *i.e.* producing intense molecular ions or high-mass-fragment ions as opposed to generating predominately Cl^- ions, are those which are highly chlorinated (greater than four chlorine atoms per molecule). In addition the chlorine atoms should be attached to aromatic structures [111]. For example, better sensitivity is expected for the higher chlorinated PCB than for DDT.

The choice of full scanning *versus* SIM is often dependent on whether a general screening for organochlorine pollutants is desired or whether highest sensitivity is needed. In going from full scanning to SIM, some identification information is lost in exchange for sensitivity. This can be counteracted to a degree by monitoring several ions, which in combination with chromatographic retention can be highly specific. SIM has been applied to the analysis of chlorinated hydrocarbons in tissues by a number of researchers [37,61,84,112,113]. Dearth and Hites [84] showed very effective use of ECNCI combined with SIM for the quantification of components of chlordane in rat adipose tissue. Detection limits (signal-to-noise ratio $>10:1$) were approximately 100 fg injected.

Fig. 4 provides an example of the use of SIM as a selective method to obtain information on the presence of specific compounds in a complex mixture. Fig. 4A depicts a total ion chromatogram from an ECNCI-MS analysis of an environmental extract. Apparent are a significant quantity of PCBs, which obscure the presence of PCTs. SIM was then conducted on this same extract. Ions m/z 331 and 297, corresponding to trichloro- and dichloroterphenyls, respectively, were monitored in the extract (Fig. 4B and D). For comparison, ion profiles of an Aroclor 5432 standard, monitored at these same m/z, are also provided (Fig. 4C and E). This demonstrates the selectivity obtainable with SIM.

^{13}C-Labeled internal standards have proved particularly useful for attendant quantitation purposes with MS techniques [64,114]. The benefits of such standards include the precise match attainable between the analyte and the quantification standard. For instance, Williams and LeBel [64] used ^{13}C-labeled coplanar PCBs (IUPAC Nos. 77, 126 and 169) to quantify environmentally accumulated ^{12}C coplanar congeners in human adipose tissue samples.

High-resolution MS also has been used for the determination of chlorinated compounds in tissues [114,115]. This technique provides specificity such that, for an interference to occur, the interfering peak must have an m/z value within a few millimass units of the analyte of interest. Working with adipose tissue from atlantic dolphin, Kuehl *et al.* [115] were able to use multiple mass spectrometric techniques to provide information on different classes of compounds. High-resolution EI-SIM was used for chlorinated dibenzo-*p*-dioxins, dibenzofurans and polybrominated compounds. Low-resolution EI-SIM and GC–ECD were applied to PCB analysis, while full-scanning low-resolution EI and ECNCI (moderator gas = argon) were effective in general screening procedures for non-target compounds.

Another MS technique that is maturing is MS–MS. In this technique a second level of separation is achieved within the mass spectrometer itself. This reduces the resolution necessary prior to the introduction of a sample to the ion source. Occasionally, GC is not required at all and a sample can be introduced directly into the mass spectrometer [116]. The applications of this technique in environmental analysis have primarily been to remove PCB interferences when analyzing for the polychlorinated dibenzo-*p*-dioxins and related

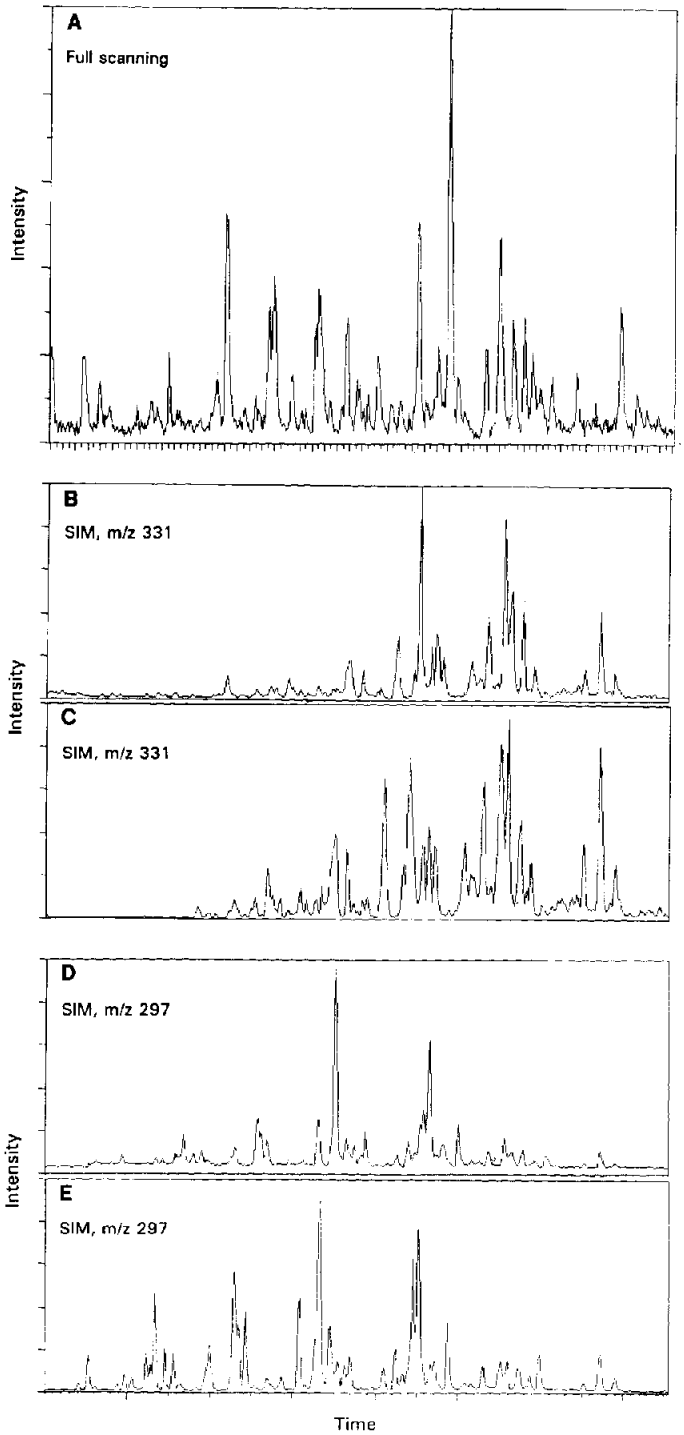

Fig. 4. Total ion chromatogram from an ECNCI-MS analysis of a sample extract (A) showing that PCBs obscure the presence of PCTs. SIM was then conducted on this same extract, and ions *m/z* 331 (B) and 297 (D), corresponding to trichloro- and dichloroterphenyls, respectively, were monitored. For comparison, ion profiles of an Aroclor 5432 standard, monitored at these same *m/z*, are also provided (C and E), demonstrating the selectivity obtainable with SIM.

compounds [117,118]. However, the use of oxygen-enhanced negative chemical ionization MS–MS in structural analysis of individual PCB congeners [119] suggests future applications in congener-specific analyses and the identification of components of other complex mixtures.

4.4. Perchlorination

Perchlorination has been used by some investigators to simplify the complexity of multi-component chlorinated hydrocarbon formulations. In the case of PCBs, this leads to the production of a single peak, *i.e.* decachlorobiphenyl. For PCTs, three peaks result, representing the fully chlorinated *o*-, *m*- and *p*-terphenyls. Reaction of the chlorinated hydrocarbons, *e.g.* PCBs and PCTs, with antimony pentachloride at elevated temperature has been the most popular technique [73,120,121]. Although this simplifies the chromatography, considerable information concerning the source, disposition and potential effects of the compounds present may be lost [122]. Dechlorination of compounds has also been employed for the analysis of organochlorines [123]. In eliminating the chlorines, one also loses the potential to use the chlorine-selective ECD and ELCD, in addition to the individual component information.

4.5. Individual congener analysis

Advancements in chromatographic techniques have resulted in greatly improved separations of the components present in complex chlorinated hydrocarbon mixtures. For example, Mullin *et al.* [89] have reported the separation of all 209 PCB congeners using multi-dimensional GC. This influx of chromatographic information requires advancements in data acquisition, manipulation and interpretation.

Chlorinated hydrocarbons in tissues and other matrices have been previously compared and quantitated relative to parent standards. For single-component organochlorines this is straightforward. However, difficulties surface for the multi-component analytes [122]. For these a number of approaches are possible. The simplest is to sum the concentrations of all compounds within a window encompassing the chromatographic extremes of the formulation of concern [124]. This approach requires that no additional detector-responding compounds are present in the window selected. This is a test rarely met in tissues without considerable prefractionation. Alternatively, smaller windows may be selected which exclude interfering compounds. Increasing the specificity of the detector, *e.g.* using SIM-MS or ELCD, may minimize these problems. A common practice has been to select several dominant peaks in the sample [67,100]. However, changes in the chromatographic profile of the mixture, *i.e.* the relative concentrations of the analyte constituents compared to standards, are typical for tissues. As a consequence, varying degrees of error will result if samples are quantitated against parent formulations. As mentioned previously, biotic and abiotic processes are effective at selectively degrading individual constituents and thus altering the original chromatographic profiles.

Identifying and quantifying each individual congener of a complex chlorinated hydrocarbon mixture separately and then summing all or the majority of the corresponding peaks is a difficult task. However, more accurate estimates of total concentration are obtained [125]. Eganhouse and Gosset [126] examined the relative errors associated with a variety of PCB quantitation schemes using ECD. They reported large discrepancies in the determination of a number of individual components, due to variations in response factors. Quantitation as total PCBs was found to be the most accurate, while determination on an Aroclor basis was reported to be relatively inaccurate on account of incomplete chromatographic resolution and overlapping of Aroclor mixtures.

Individual congener data may also provide valuable information on the potential toxicity and source of the contaminant [96,100]. Automated procedures have been developed to handle the quantitation task. For example, Slivon *et al.* [127] used nine PCB standards, representing various levels of biphenyl chlorination, to calibrate a

GC–MS system. Dedicated software was then utilized to sum the quantified peaks to produce a final concentration. Utilization of levels of chlorination within a homologous series to quantify pollutants has also been conducted with ELCD [104,105].

Relative retention indices, using marker compounds, have proven useful in identifying and subsequently quantifying components of complex mixtures in tissues. Fischer and Ballschmiter [113] used two PCBs as retention markers in conjunction with a mass-selective detector. Edstrom [104] employed four chlorinated compounds as markers and an ELCD. Mullin *et al.* [89] related retention to that of octachloronaphthalene using an ECD, while Zell and co-workers [96,128] used *n*-alkanes with flame ionization detection, as well as *n*-alkyltrichloroacetates with ECD.

For complex mixtures, such as the PCB, it may be informative in certain circumstances to relate individual constituent data back to, for example, an Aroclor basis. This may prove valuable for comparison with other data or for source identification. Principal component and multiple linear regression analyses of results have been used for this purpose [110,129,130].

One approach to expressing the results of analyses has been driven by a greater understanding of the toxicological properties of chlorinated compounds. A major thrust in recent PCB research has aimed at determining the concentrations of coplanar congeners in tissues. This concern is derived from their toxicological similarity to TCDD [7,64,131]. Recent papers have reported concentrations of the coplanar PCBs, while others have expressed these data in terms of various toxic equivalents [32,61,64,86]. Typically, tissue concentrations of the coplanar PCBs are low compared to the other congeners [32,64]. These low concentrations may not, however, negate the importance of these congeners in overall considerations of toxicity.

Several groups have suggested a standardized set of PCBs be reported. Some discussion in the literature concerning the criteria for selection has occurred [132]. Although it seems logical that toxicological concerns should be primary, knowledge in this area is rapidly expanding. Complete resolution of complex chlorinated hydrocarbon mixtures are only now being reported. Separations of some complex chlorinated hydrocarbon mixtures may not be feasible with current technology. Taking into consideration the effort and expense involved in analysis, the potential health consequences at stake and the evolution of our understanding of the effects and environmental behavior of organochlorines, extreme care should be exercised in choosing data sets for reporting and exclusion.

5. CONCLUSIONS

A number of techniques have been developed for the analysis of chlorinated hydrocarbons in tissues. These typically involve extraction and purification, prior to final chromatographic determination. Recent research has aimed at reducing the steps required to obtain a purified sample. This holds the potential to reduce analysis time and cost. In addition, reductions in solvent usage, attendant worker exposure and waste disposal may be realized. Less sample handling may also mean less opportunity for sample contamination and analyte loss.

It is recommended that incidental data concerning tissue wet mass, dry mass and lipid content be collected during sample work-up. This will permit greater comparison between studies and aid in interpretation of results. Additional work is needed to compare methods using homogenates of real tissues, containing burdens of organochlorines, as opposed to matrices to which standards have been added in the laboratory just prior to analysis.

Current capillary GC techniques appear adequate to determine tissue burdens of simple chlorinated hydrocarbons. A number of halogen-selective and -sensitive detectors, *e.g.* ECD, ELCD and MS, in various modes, are available. Each has advantages and disadvantages.

Fig. 5 shows a flow chart of analytical procedures for the determination of chlorinated hydrocarbons. It illustrates many of the choices that can be made in this process and the variety of

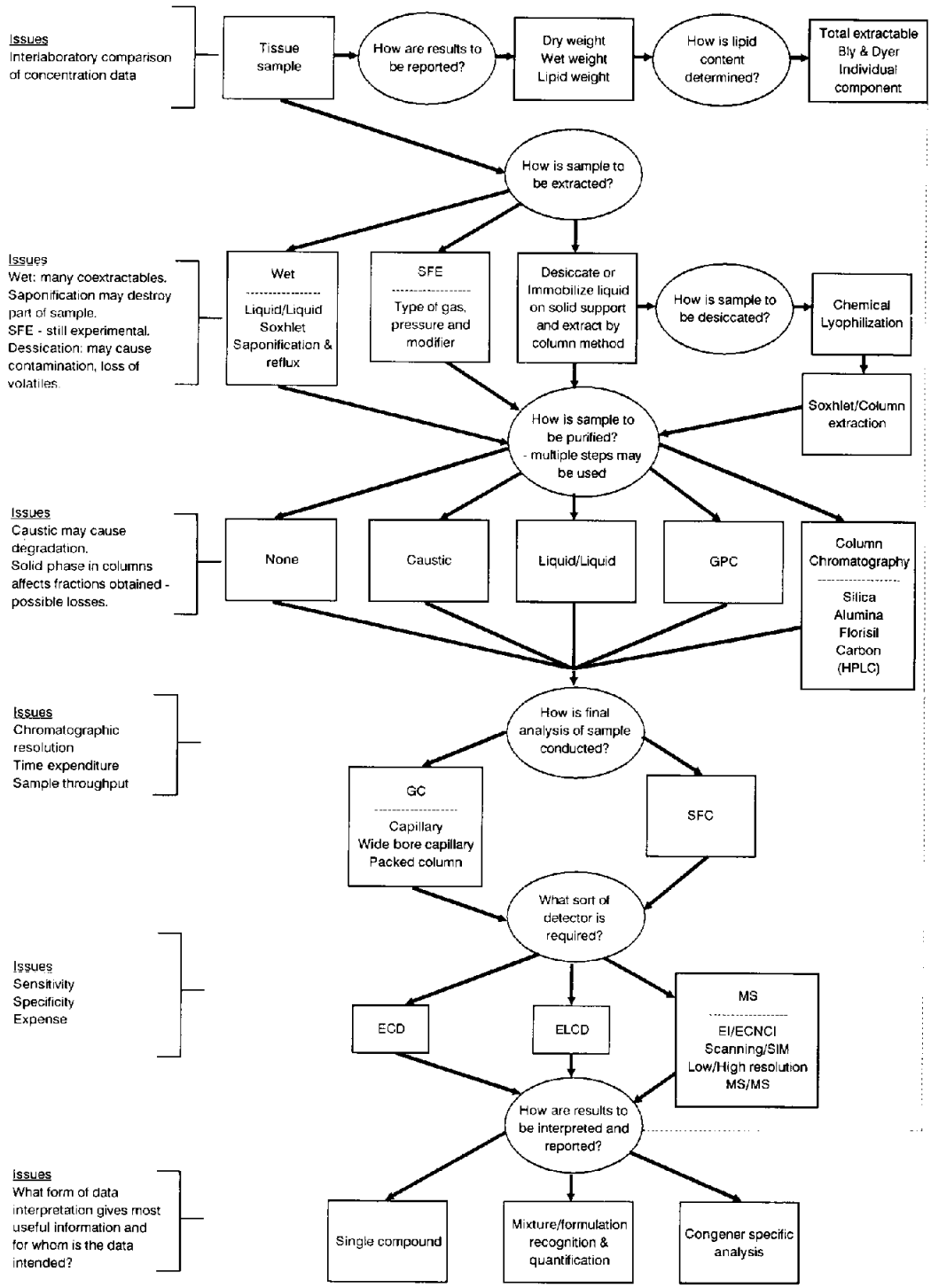

Fig. 5. Flow chart of analytical procedures for the determination of chlorinated hydrocarbons in tissues. It illustrates the choices required during the analysis and the issues and consequences which the analyst must consider.

issues and consequences of which the analyst must be aware.

Many chlorinated hydrocarbons consist of homologous series of related compounds, individual constituents of which may be altered to varying extents by biotic and abiotic processes. These processes may occur prior to uptake by the organism, within the organism and during sample handling and analysis. On account of these factors, and the different physical and biological (particularly toxicological) behavior of these components, detailed analysis of individual constituents is indicated.

Sophisticated, computer-assisted identification, quantitation and data manipulation techniques are currently being developed to assist the analyst in coping with the volume of data produced by high-resolution methods. As our knowledge of the effects of chlorinated hydrocarbons is rapidly increasing, care should be taken in regards to what analytical information is reported and what is discarded.

6. ACKNOWLEDGEMENTS

The authors wish to thank Ellen Harvey for her assistance in identifying and obtaining reference materials. This is Contribution No. 1733 from the Virginia Institute of Marine Science.

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