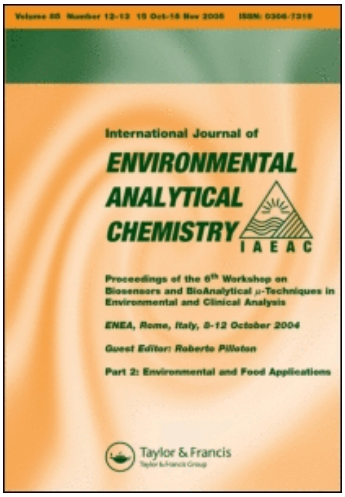

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Separation of Lipophilic Substances in Environmental Samples with Special Reference to Toxaphene

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A microcolumn of alumina, activated at 250°C over night and deactivated with 2% water in an oven at 150°C, has shown better separation characteristics than similar ones, deactivated at room temperature, for a great number of organochlorine compounds, particularly with respect to multicomponent toxaphene and PCB. The microcolumn is a simple disposable Pasteur pipette. Three fractions are collected: one with PCB and DDE, one with toxaphene components having similar lipophilicity to DDT and the third, toxaphene components similar to DDD in lipophilicity. Two such toxaphene fractions give a better GLC-pattern than only one fraction. DDD and DDT, which interfere with the analysis of toxaphene can be eliminated, after their GLC analysis, from the "Toxaphene fractions" by nitration followed by reduction of the nitro compounds formed to their corresponding amines. Performance of the column using environmental samples showed that it is a useful tool in routine pesticide residue analysis, especially when toxaphene is present.

KEY WORDS: Alumina microcolumn, organochlorine residue analysis, lipophilicity, GLC, pesticide analysis.

INTRODUCTION

In order to achieve reliable results in the gas liquid chromatographic analysis of anthropogenic substances in environmental samples, it is

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essential to employ various clean-up and separation techniques prior to the final GLC analysis. Because of their similarity in chemical and physical characteristics, PCB and the organochlorine pesticides, particularly the DDT family (DDT, DDE and DDD), chlordane and toxaphene, often interfere with one another in the gas chromatographic systems usually employed for the analysis of organochlorine compounds.

Most of the reported works in this direction employed standard fat clean-up procedure, including hexane/dimethylformamide, hexane/dimethylsulphoxide or hexane/acetonitrile solvent partition, followed by column chromatography. Other clean-up procedures include treatment of fat extracts with sulphuric acid and/or ethanolic potassium hydroxide.

A number of authors have reported the use of microcolumns with florisil,[1] silica gel[2-5] and aluminum oxide,[6] with a measure of success. The degree of efficiency or success of the various adsorption methods seem to reflect the different procedures for activation and/or deactivation of the adsorbents, and also the maximum load of fat extract that could be placed on the column without affecting the fractionation capability. In many of the investigations, large elution volumes were used, and in some cases the investigations were applicable only to standard solutions.[1,2] Very little has been reported on alumina microcolumns.

The aim of this work was to investigate the use of a simple alumina microcolumn for the efficient separation of PCB and the usual organochlorine pesticides, including toxaphene, and finally the elimination of the DDT complex from the toxaphene fractions by nitration. The polynitro compounds produced can even be reduced to the corresponding amines, which could be released, if necessary, by hydrochloric acid.

Thus the following criteria were established:

1) It should be possible to carry out the entire analysis from a fat amount as large as 1 g.

2) The column for fractionation should be a disposable Pasteur pipette.

3) Three fractions should be taken in such a way that DDE, DDT and DDD are totally separated from each other.

4) The total elution volume should not exceed 31 ml.

It was presumed that the success of the entire system would pave the way for simpler routine residue analyses of organochlorine compounds.

EXPERIMENTAL

Materials and reagents

a) Aluminum oxide 90 active, acidic for column chromatography (activity I) 0.063–0.200 mm (70–230 mesh ASTM), MERCK, Cat. No. 1078.

b) Solvents: All solvents were distilled-in-glass quality suitable for pesticide residue analysis.

c) Eluting mixtures: Pentane, pentane/toluene mixture (90:10), pentane/toluene/diethyl ether mixture (45:5:50) and pentane/diethyl ether mixture (90:10).

d) Analytical standards: Hexachlorobenzene (HCB); hexachlorocyclohexane isomers (alpha, beta and gamma-BHC); *p,p'* and *o,p'* isomers of DDE, DDD and DDT; aldrin; dieldrin; endrin; *cis* and *trans* isomers of chlordene, chlordane and *trans*-nonachlor; heptachlor; heptachlor epoxide; oxychlordane; polychlorinated biphenyls (PCB); and toxaphene standards were kindly supplied by Professor C. A. Wachtmeister at the Wallenberg Laboratory, University of Stockholm, the National Swedish Environment Protection Board, and the United States Environmental Protection Agency.

e) Chromatographic columns: Disposable Pasteur pipettes (24 cm × 5.8 mm i.d.).

f) Apparatus: Gas chromatograph Varian Model 3700, equipped with an electron capture detector (^{63}Ni) and a cross-linked fused silica capillary column (25 m × 0.32 mm i.d.) coated with SE 54 (1% vinyl, 5% phenyl methyl silicone).

Operating conditions: Detector temperature 320°C; injection temperature 220°C; oven programme—initial temperature 80°C, programme 15°C/min to 200°C, then 3°/min to 260°C: carrier gas—nitrogen at 10 psi constant pressure (3–4 ml/min); splitless injection.

Aluminum oxide preparation

Aluminum oxide was activated by placing the desired quantity in an open conical flask and heated overnight (16–17 hours) at 250°C in an oven. After heating the flask was covered with aluminium foil and allowed to cool to room temperature. Deactivated alumina was prepared by weighing 4.9 g of the activated alumina in an ampoule. One-hundred mg distilled water was added using a 100 μl Hamilton syringe, and the ampoule sealed and weighed. For 5% water deactivation, 4.75 g alumina and 250 mg H_2O were weighed in the ampoule.

The ampoule was then placed in an oven at 150°C for one hour, after which it was immediately removed and rotated gently for at least two hours. It was then reweighed and if the weights before and after the deactivation were different, the ampoule was discarded.

Sample extraction

Extraction of organic tissues was carried out as described by Jensen *et al.*[6]

Clean-up of fat extract

The clean-up of the fat extracts was done using acetonitrile.

Instead of employing the usual liquid–liquid extraction methods[7,8] by using two immiscible solvents, which could be very long and tedious, the method by Jensen and Jansson[9] was employed. The fat itself served as the lipophilic phase and acetonitrile as the polar phase.

One gram fat extract was rotated five minutes with each of two 10 ml portions of acetonitrile followed by centrifugation. The acetonitrile extracts were removed and combined. Two ml pentane plus 2 ml concentrated sulphuric acid were added to the fat remaining after acetonitrile extraction, rotated five minutes, then centrifuged. The pentane phase was added to the combined acetonitrile extract, and the solvents removed in a rotary evaporator at 50°C or under a gentle stream of nitrogen on a 70°C water bath, and the fat amount determined.

The fat extracted with the acetonitrile (about 10%) was dissolved in a suitable volume of pentane for column chromatography. If only

20 mg (or less) of the fat extract was to be used for analysis, the separation column (containing 2% water) below could be employed directly, but if a larger fat portion was to be used, the pre-column (5% water) should first be run. If the epoxides are not of interest, the extract can be treated with sulphuric acid and the residue run directly on the separation column.

Column preparation

2.5 g of the deactivated alumina was dry-packed in a simple disposable Pasteur pipette, using a glass wool plug at the bottom. The column packing was added in small increments followed by gentle tapping. Proper care should be taken to avoid air-bubbles during subsequent elution. Wet packing was also employed in the following way. 2.5 g of the deactivated alumina was weighed into a conical test tube, and a slurry made with 4 ml pentane. The slurry was transferred by pipette, to a disposable Pasteur pipette lightly plugged with a small piece of clean glass wool. To ensure uniform packing, it was best to close the tip of the Pasteur pipette using the forefinger and filling the column approximately one-fourth with pentane. Then the first portion of the alumina slurry was added and thereafter the forefinger removed, allowing the solvent to run through the column into a beaker. Addition of the slurry continued and the pipette lightly tapped with a pencil so that the alumina settled. Tapping of the column continued after the last addition of slurry until the alumina had stopped settling in the column. At this point the pentane was allowed to drain from the column until it reached the top of the column packing.

A solvent reservoir for the column was constructed by attaching a glass tube (150 mm × 12 mm i.d.), threaded at one end, using a perforated screw cap and teflon-lined rubber washer (Figure 1). To minimize evaporation of solvent in the reservoir, a cork pierced with a syringe needle was used to cap the reservoir. The pre-determined volume of solvent was placed in the reservoir and allowed to elute through the column. The flow of solvent stopped on its own as soon as the tail end reached the top of the column. Thus up to ten columns could be run simultaneously.

The fat residue (up to 200 mg) from the acetonitrile extraction procedure above was dissolved in 200 μl pentane and added to the

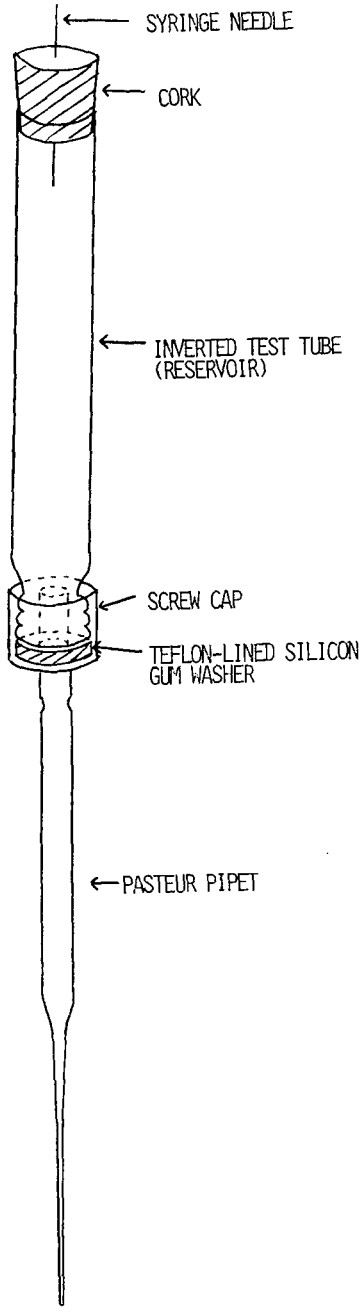

FIGURE 1 A simple micro-column of Pasteur pipette connected to a reservoir of glass tube.

top of the pre-column, followed by two 200 μl rinses of the tube containing the fat residue. The column was then eluted with 9.5 ml pentane/diethyl ether mixture (90:10). The eluate, containing the organochlorine compounds of interest, was evaporated under a gentle stream of nitrogen in a 40°C water bath and the fat amount determined.

The residue (less than 20 mg) from the acetonitrile extraction or pre-column or from a sulphuric acid clean-up was dissolved in 100 μl pentane and transferred to the top of the column, followed by two 100 μl rinses of the tube containing the residue. The following three fractions were collected:

Fraction 1—13 ml pentane (14.5 ml in wet-packed columns)
Fraction 2— 8.0 ml pentane/toluene mixture (90:10)
Fraction 3— 8.0 ml pentane/toluene/ether mixture (45:5:50).

These fractions were concentrated to suitable volumes for gas chromatographic determination. It should be noted that strict control over operating conditions of the micro-column system is important to ensure reproducible results. Every new batch of alumina should be tested after deactivation, using BHC mixture (alpha, beta and gamma), *p,p'*-DDE, *p,p'*-DDT and *p,p'*-DDD as test compounds. These compounds should be distinctly separated into each of the three fractions as shown in Table I. Initial heating of new batches of alumina at 800°C for four hours prior to subsequent use greatly reduces batch-to-batch variation. The columns should stand in a cool draught-free environment without direct exposure to heat or light.

Removal of DDT and DDD

If the GLC chromatograms of fractions 2 and 3 indicated the presence of DDT and DDD, the DDT and DDD were eliminated prior to toxaphene determination by nitration, followed by reduction of the nitro compounds formed to amines.

Nitration

Fractions 2 and 3 were evaporated carefully just to dryness under a stream of nitrogen. To each of them was added 2 ml 38% by volume

TABLE I

Separation of organochlorine compounds on 2% water deactivated alumina column

Substance	Percent substance eluted		
	Fraction 1	Fraction 2	Fraction 3
PCB	100		
Toxaphene	5	60	35
HCB	100		
alpha-BHC		100	
beta-BHC			100
gamma-BHC			100
*p,p'*DDE	100		
o,p'-DDE	100		
p,p'-DDD			100
o,p'-DDD		40	60
p,p'-DDT		100	
o,p'-DDT	100		
cis-chlordene	100		
trans-chlordene	100		
cis-chlordane		100	
trans-chlordane		100	
cis-nonachlor			100
trans-nonachlor		100	
Heptachlor	100		
Heptachlor epoxide			100
Oxychlordane		85	15
Aldrin	100		
Dieldrin			100
Endrin			100
Methoxychlor			100
Mirex	100		

concentrated nitric acid in concentrated sulphuric acid. This was rotated 1 hour in a 45°C oven. After cooling with tap water, 4 ml distilled water and 3 ml pentane were added and shaken for two minutes. After centrifugation, the aqueous phase was removed and a fresh 2 ml portion of water added and shaken. Again after centrifugation the aqueous phase was discarded. The organic phase was then ready for GLC injection, but the interference which resulted from the nitro derivatives formed made it necessary to reduce these nitro compounds to the corresponding amines.

Two ml absolute ethanol was added to the organic phase from the preceeding step, and the pentane evaporated under a gentle nitrogen stream in a 40°C water bath until between 1.5–2 ml ethanol remained. One hundred μl concentrated hydrochloric acid and 50 mg ferrum reductum were added, and the mixture shaken for three minutes. Then 2 ml distilled water and 2 ml pentane were added. The mixtures was again shaken for three minutes. After centrifugation, the aqueous phase was removed, and the organic phase shaken with 2 ml concentrated sulphuric acid. After phase separation and concentration to a suitable volume, the organic phase was ready for GLC analysis.

RESULTS AND DISCUSSION

Table I shows the separation of different organochlorine compounds on micro alumina columns (2% H_2O). With the exception of toxaphene, oxychlordane and *o,p'*-DDD, all the organochlorine compounds tested are cleanly separated into each of the three fractions. Fraction 1 contained about 5% of the total toxaphene (composed of relatively few components). Fractions 2 and 3 contained 60% and 35% respectively. Figure 2 shows the chromatograms of both the technical toxaphene and the different fractions. In most of the known environmental samples, however, where both PCB and toxaphene are found, the ratio of PCB to toxaphene is so large that the front-running toxaphene in fraction 1 will not constitute any threat to the accurate determination of the PCBs. In cod liver oil, the amount of DDT, DDD and dieldrin were significantly larger than the amount of toxaphene in fractions 2 and 3. It was therefore easy to determine the levels of these pesticides in the presence of toxaphene. Treatment of fraction 3 with H_2SO_4 (conc.), and nitration, followed by reduction, of both fractions 2 and 3, eliminated DDT, DDD and dieldrin. After the necessary concentration the level of the toxaphenes in the fractions could be determined.

Recovery

When known amounts of PCB components, HCB, DDT complex and BHC were eluted either alone or in 20 mg fat through the

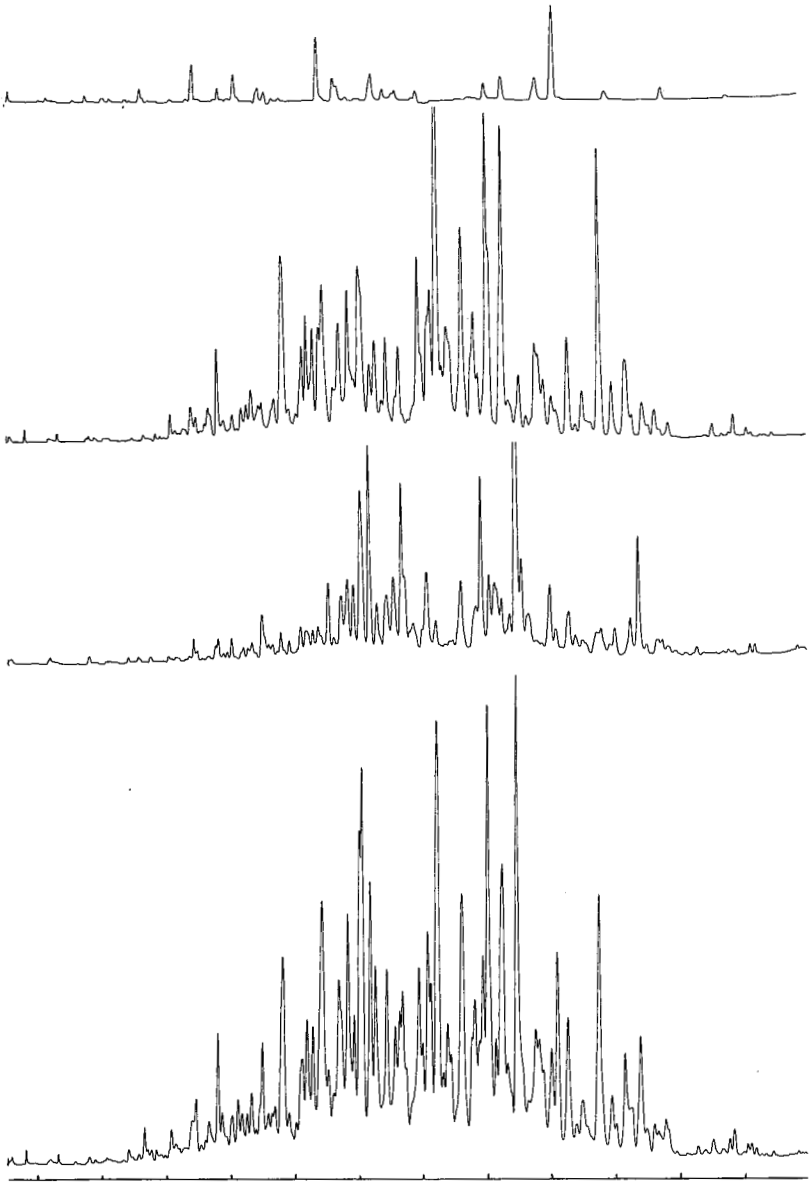

FIGURE 2 Technical toxaphene and the three fractions obtained after separation of this product on an alumina micro-column, chromatographed on a 25 m × 0.32 mm (i.d.) SE 54 cross-linked fused silica capillary column.
Temperature programme: 80°C–15°/min – 200°C–3°/min–260°C.

column, the percentage recoveries ranged from 96–102%. These recoveries were based on a number of fractionation trials, and precision for replicate runs was better than 2% in all the cases.

Experiments with time of heating showed that Al_2O_3 activated at 250°C for only 2 hours and deactivated in the oven as described under "Experimental", gave slightly different elution volumes for the different tested compounds (Fraction 1: 0–11 ml, 2: 11–18 ml, 3: 18–25 ml). In this system the α-BHC appeared in both fractions 2 and 3 and reproducibility was relatively poor. When the alumina (activated at 250°C overnight) was deactivated at room temperature, the elution volumes were much lower than those from columns deactivated at 150°C (1: 0–9 ml, 2: 9–15 ml and 3: 15–21 ml) and the separation of components was also much poorer. Table II shows the differences in separation between the column deactivated at 150°C and that deactivated at room temperature. In the latter system DDT,

TABLE II

Effect of deactivation at 150°C in relation to the conventional room temperature deactivation for the separation of some organochlorine compounds on alumina microcolumn (2% H_2O)

	Percentage chlorinated compounds eluted					
	Deactivation at 150°C			Deactivation at room temperature		
Chlorinated compounds	0–13 ml	13–21 ml	21–29 ml	0–9 ml	9–15 ml	15–21 ml
HCB	100			100		
Aldrin	100			100		
p,p'-DDE	100			100		
PCB	100			100		
Toxaphene	5	60	35	15	60	25
p,p'-DDT		100		20	80	
α-BHC		100			100	
Chlordanes (*cis*- and *trans*-)		100			100	
β-BHC			100			100
γ-BHC			100		40	60
p,p'-DDD			100		35	65
Dieldrin			100			100

DDD and γ-BHC were not separated into distinct fractions. It would therefore appear that deactivation in the oven (150°C) should be preferred since it gave better separation and reproducibility than deactivation at room temperature.

Another important aspect of the use of support activated at 150°C was that up to 20 mg fat residue (equivalent to approximately 200 mg of original fat extract) could be run on the separation column (2% H_2O) without showing any inconsistency in the elution pattern.

The environmental significance of the three fractions eluted through the column, particularly with regard to the toxaphene components, might be related to the degree of toxicity of the various toxaphene components in these fractions. The toxaphene components are difficult to separate into distinct fractions and therefore only one fraction is generally taken. This gives rise to a very poor GLC-resolution. However, by taking more fractions the GLC-pattern can be improved. In addition this might also be of value in the future if biological parameters prove that there are differences in the persistence, accumulation and toxicological properties of toxaphene dependent on the lipophilicity of the various components. Thus in a preliminary study as to the toxicity of the three fractions, where the test animals were banana flies and fish, it was found that the most lipophilic fraction (fraction 1) posed by far the highest toxicity to flies whereas the second fraction (fraction 2) was the only one toxic to fish.[10]

Because of the narrowness of the columns the flow of solvents stops immediately when the tail end of a solvent flow reaches the top of the column. Thus it is not necessary to watch the fraction collection but only to add the pre-estimated volume of solvent to the column and remove the fraction when appropriate. One person can therefore easily run up to ten columns at the same time.

The method under study has been employed in the determination of organochlorine residues from numerous samples of biological origin from the arctic zone and the Baltic, and from different extracts of cod liver oil. In most of the samples no serious problem was encountered during the separation processes. The GLC chromatograms showed a good separation of the various compounds in accordance with the worked-out elution pattern. Details of the results are being presented on a separate publication.

CONCLUSION

This study shows that an alumina microcolumn employed with the described elution system is well suited for separating the DDT complex, multicomponent PCB, toxaphene and chlordane, HCB, dieldrin and the BHCs into three major fractions suitable for GLC analysis. DDE can be quantified in the presence of PCBs provided that the concentrations of the latter are not excessive. Toxaphene can be determined accurately only after the elimination of DDT, DDD and dieldrin in the appropriate fractions.

The microcolumns are easy to prepare, rapid to use, and require significantly small amounts of alumina and small volumes of elution solvents. Because of the small size of the columns, a large number of samples can be run simultaneously on separate columns.

Investigations on the application of the method to some environmental samples have indicated the versatility of the column.

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