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SINGLE-STAGE CLEAN-UP OF ANIMAL TISSUE EXTRACTS FOR ORGANOCHLORINE RESIDUE ANALYSIS

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SUMMARY

The use of column chromatography in the clean-up of hexane extracts of animal tissues for pesticide analysis is described, using dry, partially deactivated alumina and silica columns to remove fat and other unwanted materials. By limiting the fat loading of the columns, complete elution of organochlorine residues, free of substances interfering in gas chromatographic analysis, is achieved.

Two types of alumina, of different activities, and one type of silica have been tested and the order of elution of fourteen organochlorine residues determined. Alumina, activated at 800° and partially deactivated with 5% by weight of water, was found to be the most efficient. Silica columns were more effective in the separation of pesticides by differential elution.

INTRODUCTION

The methods most commonly used in pesticide analysis for the removal of fats and other interfering substances from solvent extracts of dairy products and animal tissues involve a liquid–liquid partition stage followed by a column chromatography stage. The first typically employs hexane and acetonitrile[1,2], or hexane and dimethylformamide (DMF)[3], and several successive extractions are made into the more polar solvent (acetonitrile or DMF). This solvent is then diluted with a large volume of water and the pesticides are transferred back to hexane, which is finally passed through an adsorbent column in the second stage to remove the final traces of fats and pigments. For the second stage, Florisil[2] or partially deactivated alumina[3] is used, and with Florisil it is possible to obtain a useful separation[4] of organochlorine residues into two groups by eluting with solvents of different polarity. Unfortunately the variability among batches of Florisil necessitates the testing of individual columns to determine the exact volumes of the two solvents required to produce the desired separation.

Single-stage clean-up of plant extracts can usually be achieved by column chromatography alone, but many animal tissue extracts contain too much lipid material for such clean-up to be sufficient for analysis by gas chromatography. The columns are normally prepared by slurrying Florisil or alumina into chromatographic tubes

with solvent, and the samples for clean-up are added to the solvent-wet columns. The efficiency of recovery of added residues from these columns is usually high.

In two-stage clean-up processes, a significant fraction of the total pesticide content of an extract is lost at the liquid–liquid partition stage, the percentage loss varying with the nature of the pesticide. Recovery from the column chromatography stage is usually complete, however, and the use of column chromatography alone would therefore offer an advantage in quantitative analysis. Furthermore, it is usual to obtain a relatively large final volume of extract of which only a few microlitres are taken for GLC analysis, the remainder being discarded if not required for other methods used in confirmation of identity. By concentrating the original extract to a suitable degree before clean-up, and obtaining a small volume of final solution, an appreciable saving of both adsorbents and solvents can be achieved. For this reason small columns have been developed, using fresh adsorbent for each sample. This also avoids the possibility of contamination between samples, which may occur if large columns are used repeatedly. The first part of this paper describes the use of dry alumina powder, in columns of small dimensions, which has been found to give results superior, both qualitatively and quantitatively, to the two-stage technique previously employed. In the second part, the alternative use of silica gel is discussed, this adsorbent being more useful than alumina in separating pesticides by differential elution.

EXPERIMENTAL

The following material was used: Glass columns, 45 cm long × 0.6 cm bore, drawn to a tip at the lower end, and plugged at the tip with glass wool, or solvent-washed cotton wool. Pipettes, graduated for content, 1 ml. Tubes, test or tapered centrifuge, graduated 10 × 0.1 ml, with glass stoppers. Flasks, graduated 1 ml, 10 ml and 20 ml, with glass stoppers. *n*-Hexane, 67–70°, redistilled and the 68–69° fraction collected. Tested to be free of residues detectable by electron capture GLC after evaporating 100 ml to 1 ml in a clean air-stream. Alumina powder, analytical grade aluminium hydroxide activated at 800° for 4 h, cooled in a desiccator, partially deactivated by shaking with 5% by weight of distilled water for 30 min and stored in a closed vessel. Silica gel, for chromatography, Merck No. 7754, 70–325 mesh (ASTM), dried at 120° for 2 h, cooled, and deactivated as for alumina.

The tissue extracts used in the investigations were obtained by Soxhlet extraction, for 30 min, of 5 g aliquots of animal tissue (previously ground to a dry powder with anhydrous AR grade crystalline sodium sulphate) using approximately 100 ml of hexane. The extracts were subsequently made up to 100 ml, and 50 ml evaporated to dryness in a tared dish to determine the extractable fat. The remaining 50 ml were evaporated and made up to smaller volumes, from 1 to 25 ml depending on the fat content, before clean-up.

ALUMINA COLUMNS

Alumina was prepared by activating aluminium hydroxide at 800° for 4 h and partially de-activating with 5% by weight of distilled water. Initially 2 g of the de-activated alumina was slurried into the columns with hexane, and the solvent drained

to the surface of the alumina, but these "wet" columns were found to be ineffective in retaining a high proportion of the lipid in fatty extracts. It is believed that the initial diffusion of the sample (1 ml applied to 2 g of alumina) into the interstitial hexane reduces the ability of the alumina to adsorb the lipid material. The use of columns prepared without slurrying ("dry" columns) enables the sample to be applied directly to the alumina surface and adsorbed into the upper 2–3 mm. These columns were found to be much more effective in retaining fat and pigments. The "channelling" effect, which may occur when dry columns of large dimensions are used, in which the solvent passes through the bed in an uneven manner (usually on one side only), does not occur with the narrow-bore tubing used for the method now described.

For extracts of fairly low fat content a single alumina column is often sufficient for satisfactory clean-up but for extracts of high fat content two identical columns are used serially. The first removes the greater proportion of the unwanted fat, and the eluate from this column is concentrated and transferred quantitatively to the second column. Each column is prepared by placing 2.0 ± 0.1 g of dry alumina powder (activated at 800°, partially deactivated with 5% by weight of water) in a 45 cm long × 0.6 cm bore chromatographic tube having a glass wool plug at its lower end. 1.0 ml of a hexane extract of animal tissue, containing up to 100 mg of extracted fat per millilitre, is placed on the alumina surface by means of a 1-ml pipette calibrated for content. (Pipettes calibrated for delivery of 1 ml of water are liable to give a drainage error when used for hexane solutions.) The extract is drained down one side of the glass column, and washed into the alumina by refilling the pipette with 1 ml of hexane and draining down the same side of the column. (A slight incline of the column from the vertical position is advantageous.) With a suitable receiver below the column a further 10 ml of hexane is added above the alumina, and additional volumes added as required for elution. The eluate is evaporated to 1 ml or slightly less in a stream of cold dry, filtered air and transferred by means of the 1-ml pipette to the surface of the second alumina column. One millilitre of hexane is added to the first receiver, removed by the same 1-ml pipette, and used to rinse the first millilitre into the second column. The elution from the second column is then made with ten or more millilitres of hexane and the eluate adjusted to a suitable volume by evaporation. Glass-stoppered test-tubes calibrated to 10 ml have been found very suitable, the final volume after evaporating being adjusted to 5 ml or 10 ml as required. When necessary, a reduction of the volume to 1 ml can be made in tapered graduated centrifuge tubes, but these have been found to be insufficiently accurate for the most precise work.

Results

Table I shows the typical order in which a number of organochlorine pesticides and metabolites were eluted from a single alumina column of the type described above. The eluates were collected in 1-ml fractions. With such a small total volume it is not convenient to separate the pesticides into two or more fractions, although subdivisions at 5 ml might be of value in confirming the identity of certain pesticides such as dieldrin and β-BHC, which would be recovered in the second fraction. (Silica columns, described in the second part of this paper, are more effective for this purpose.) It was found that an eluate of about 15 ml was necessary in order to elute β-BHC and DCBP (*p,p'*-dichlorobenzophenone) from the column.

TABLE I

PERCENTAGE RESIDUES IN 1-ml ELUATE FRACTIONS FROM A 2-g COLUMN OF ALUMINA (800° ACTIVATION, 5% H_2O)

Abbreviations: *p,p'*-DCBP = *p,p'*-dichlorobenzophenone (= DBP); *p,p'*-MDE = 1-chloro-2,2-bis(*p*-chlorophenyl)ethylene (= DDMU); *p,p'*-DDE = 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; *p,p'*-TDE = 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (= DDD); *p,p'*-DDT = 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane; *o,p'*-DDT = 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.

Fraction No.	*α-BHC*	*β-BHC*	*γ-BHC*	*Hepta-chlor*	*Hepta-chlor epoxide*	*Aldrin*	*Diel-drin*	*Endrin*	*p,p'-DCBP*	*p,p'-MDE*	*p,p'-DDE*	*p,p'-TDE*	*p,p'-DDT*	*o,p'-DDT*
1				47		69				48	52		24	39
2	18			44		25				49	40	11	71	53
3	69		4	5		4				2	5	70	5	5
4	7		43	3	17	2		3		1	2	16		2
5	3		41	1	58			36			1	2		1
6	2		11		15		41	46				1		
7	1		1		6		48	15						
8					3		9							
9					1		2		4					
10		7							21					
11		25							36					
12		31							25					
13		26							11					
14		9							3					
15		2												
16														
17														
18														

If β-BHC and p,p'-DCBP are of no interest, or known not to be present, a total eluate of 11–12 ml is sufficient to ensure the elution of the other twelve residues. The percentages of the various residues eluted in successive 1-ml fractions will be rather variable, but several tests on the procedure using the same combination of organochlorines have shown that the order of elution is as shown in Table I, within ± 1 ml. Slight variations between individual columns may be expected, due to differences in internal diameter, and in the weight of alumina used.

The use of alumina activated at 500° and partially de-activated with 6% distilled water was considered as a possible alternative where separation of the residues into two groups is desirable. This adsorbent required 15 ml of hexane for the elution of approximately 95% of most residues (Table II) but endrin and heptachlor epoxide required a further 15 ml. Dieldrin, β-BHC, and DCBP were not eluted with hexane, or with 5% diethyl ether in hexane. The use of a hexane–diethyl ether solvent mixture with a higher proportion of ether is liable to elute a significant proportion of fat from tissue samples. In view of the incomplete recovery of some residues from this column, its use was not further investigated.

A series of six replicate pairs of alumina columns was tested by eluting 1 ml aliquots of a mixture of standard pesticides in 25 mg corn oil dissolved in hexane through each pair, according to the method described (using 800° alumina). The final extracts, adjusted to 10 ml, were analysed on two GLC columns and peak heights compared with those from a 1-ml aliquot of the standard mixture diluted to 10 ml. The GLC columns were 5 ft. long and 1/8 in. O.D., containing (a) a 10% coating of DC-200 silicone oil on 80–100 mesh Chromosorb W (AW/DMCS) and (b) 5% DC-200 plus 7.5% QF-1 fluorosilicone on the same support. The chromatograph used was an Aerograph Model 205-2B, fitted with electron capture detectors. The mean and standard deviations of the pesticide concentrations, expressed as a percentage of the values for the unprocessed standard solution, are given in Table III. The standard deviations of replicate injections of the standard solution were in the range of ± 1.2 to ± 1.6%, and the recoveries of the pesticides from the columns were found to be complete.

Using the dry alumina columns, extracts of seal blubber, which are difficult to clean up even with the normal two-stage procedure (involving also an appreciable loss of pesticide), gave excellent GLC chromatograms in which the return to base line following the solvent peak was rapid. Recoveries of a number of organochlorine pesticides added to 250 g of penguin fat (selected for its comparative freedom from pesticides) were quantitative, and similar to those obtained with corn oil. Comparison of GLC chromatograms obtained by the single-stage and two-stage techniques (the latter involving hexane–DMF partition[3], followed by a slurried alumina column) has confirmed the improved clean-up of the single-stage method.

For samples which contain only a small proportion of fat, resulting in the transfer of not more than about 10 mg of fat to the first column, it is usually sufficient to use this column alone for clean-up. The second column is necessary only for higher fat loadings.

Table IV shows the percentage recoveries of eight organochlorine pesticides added to two animal tissue extracts, of pike liver (*Esox lucius*) and penguin fat (*Aptenodytes forsteri*). Recoveries, within the limits of error of the determinations, were 100%, after allowing for the residues originally present. By comparison, re-

TABLE II

PERCENTAGE RESIDUES IN 1 ml ELUATE FRACTIONS FROM A 2-g COLUMN OF ALUMINA (500° ACTIVATION, 6% H_2O)

For chemical identities of residues, see Table I.

Fraction No.	*α-BHC*	*β-BHC*	*γ-BHC*	*Hepta-chlor*	*Hepta-chlor epoxide*	*Aldrin*	*Diel-drin*	*Endrin*	*p,p'-DCBP*	*p,p'-MDE*	*p,p'-DDE*	*p,p'-TDE*	*p,p'-DDT*	*o,p'-DDT*
1						4								
2				12		59				6	18			3
3				59		27				47	57		3	48
4				20		6				31	17		32	37
5				6		3				10	5		43	9
6				2		1				3	2		17	2
7	4			1						2	1		4	1
8	20									1			1	
9	35	Not eluted					Not eluted		Not eluted			5		
10	25		4									17		
11	12		11									28		
12	4		24									24		
13			26									14		
14			18									7		
15			10									4		
16			5					1				1		
17			2		2			3						
18					3			5						
19					3			6						
20					4			8						
21–30					88			77						

TABLE III

PERCENTAGE RECOVERY OF ADDED PESTICIDES FROM CORN OIL

Corn oil spiked with eight organochlorine pesticides, and 25 mg cleaned up on six pairs of 2-g alumina columns, 800°/5 % H_2O type.

Pesticide	*Mean % recovery from corn oil*	*S.D.*
α-BHC	101.5	±1.3
γ-BHC	99.4	±1.7
Heptachlor	100.5	±2.2
Heptachlor epoxide	101.2	±2.7
Aldrin	100.8	±1.9
Dieldrin	101.0	±1.9
p,p'-DDE	101.0	±3.1
o,p'-DDT	101.9	±2.5

TABLE IV

PERCENTAGE RECOVERY OF ADDED PESTICIDES FROM ANIMAL TISSUE

S.D. = ± 5 %.

Pesticide	*% recovery from pike liver*	*% recovery from penguin fat*
α-BHC	97	100
γ-BHC	103	103
Heptachlor	97	106
Heptachlor epoxide	102	108
Aldrin	97	104
Dieldrin	105	108
p,p'-DDE	108	105
o,p'-DDT	104	109

coveries from spiked extracts of the same tissues cleaned up by the hexane–DMF process were low. The chromatograms for the penguin fat were unusable, the partition stage in the clean-up being difficult to achieve owing to emulsification. In the case of pike liver spiked with dieldrin, recoveries were 90% by the DMF process and 105 % by the alumina column. Cod liver (*Gadus morrhua*) extracts similarly spiked gave 31 % recovery by the DMF process and 103% by the alumina column. With some samples additional peaks have been detected on the chromatograms. These are possibly due to substances which, in the liquid–liquid partition process, remain in the discarded hexane extract or are removed by the aqueous wash, but their identity has so far not been established. The polychlorinated biphenyls, which are often found in wildlife samples, are eluted from the columns in the first 2–3 ml of hexane.

The single-stage method has proved very successful with a variety of samples, including seal blubber, fish liver and muscle, shellfish tissues and vegetable oil. A few types of sample may prove to be more difficult to clean up, however, and limitation of the column loading to 100 mg of extractable lipids is advised. If polar solvents are used in the initial extraction of tissue samples, the organochlorine residues must be transferred to hexane solution before using the alumina columns described. The

columns have not been tested with samples containing organophosphorus or other pesticides, and the technique should be fully examined before use in the analysis of such compounds.

An improved version of the elution column has now been developed in place of that used in the investigation. This has a 15-cm section of 0.7 cm bore at the lower end, and is joined at the upper end to a section of 2.5 cm bore, 8 cm long. It is thus possible to apply the 1 ml aliquots of sample directly to the upper surface of the alumina, and to add 20 ml of hexane for elution if necessary to the upper reservoir. The elution time for these modified columns is longer, owing to the smaller head of pressure available.

The advantages of the twin alumina column technique over the liquid–liquid partition method are (a) small quantities of solvent and adsorbent reduce operative costs, (b) alumina as an adsorbent is less variable than Florisil, (c) recoveries of the fourteen organochlorine residues tested are complete, in contrast to the experience with solvent partition, (d) results are more reproducible, (e) clean-up of fats is more effective, (f) samples can be processed more rapidly and with less laboratory space, (g) some residues lost in solvent partition are recovered by the new technique, and (h) the emulsion formation often experienced with liquid partition of fats is avoided.

SILICA COLUMNS

Columns of powdered silica have been reported[5] as being relatively inefficient for clean-up purposes, although KADOUM[6–8] has described a technique using small columns, in the solvent-wet form, for clean-up and separation of a number of pesticide residues. He found it necessary to use *n*-hexane and benzene in different proportions to elute substances of differing polarities, but thereby achieved a useful confirmatory technique. For samples of unknown pesticide content, however, such as wildlife specimens, this procedure could result in an inconveniently large number of extracts per sample for analysis, and a compromise would normally be accepted, by which several residues would be eluted with the most polar solvent mixture.

In the present investigation the use of silica columns containing 2 g of 70–325 mesh (ASTM) silica pre-wetted with hexane was found to result in a significant elution of fatty materials when a more polar solvent was applied to elute those pesticides not extracted by hexane. Columns identical to those previously described for alumina, but using 2 g of prepared silica in the dry form, were therefore tested. Some unidentified substances were found to be eluted from silica activated at 120° and partially deactivated by shaking with 5% (by weight) of distilled water, two of these substances being similar in retention time to α-BHC and γ-BHC when analysed by GLC using a DC-200 silicone column. Unlike α- and γ-BHC, however, they were eluted by hexane, whereas the two BHC isomers required 10% diethyl ether in hexane for elution.

The contaminants were removed by pre-washing the silica, before activation, in a Buchner funnel with hot distilled water, followed by a wash with diethyl ether, drying off by drawing air through the silica, and activating in an oven at 120° for 2 h. Partial deactivation by shaking for 30 min with 5% (by weight) of distilled water then produced the desired activity. Drying the silica at 300° was found to result in

a lower final activity, which was less suitable for the separation of organochlorine residues to be described.

The grade of silica found most effective for the purpose was Silica Gel No. 7754 (Merck), which is stated to have a mesh range of 70–325 (ASTM). Silica of this type, from which material passing a 200-mesh sieve had been removed, and silica from another supplier (Hopkin and Williams) stated to have an approximate mesh range of 100–200, were also found suitable for producing the separation between solvents described below, but the more rapid rate of elution of *p,p'*-DDT with these two types precluded the possibility of separating *p,p'*-DDT from other pesticides. The unsieved Merck silica was therefore finally selected for routine use, although columns of this material take longer for elution than the alumina columns previously described. Shorter columns, using less than 2 g of silica, are faster but give a less effective separation.

Results

The typical order of elution of fourteen organochlorine pesticides and related substances is shown in Table V. Two solvents are necessary for the elution, the first being *n*-hexane (10 ml) and the second 10% diethyl ether in hexane (10 ml). If the first 10 ml of hexane is followed by a further 10 ml of the same solvent, none of the substances in the second group (extracted by 10% ether in hexane) are eluted. Thus an excess of hexane is not critical, and it may be found convenient to collect 12–13 ml of eluate and reduce the final volume to 10 ml or 5 ml by evaporation. The polychlorinated biphenyls are eluted in the first 4 ml of hexane, and hexachlorobenzene, which is sometimes difficult to distinguish from α-BHC in GLC analysis, is eluted in the first 3 ml of hexane.

The fourteen substances listed in Table V are approximately equally divided between the two solvent fractions, but for wildlife samples in which the DDT group of substances and dieldrin, together with the PCBs, are often the only contaminants, the separation enables the PCBs, *p,p'*-DDE, *p,p'*-DDT and *o,p'*-DDT to be removed in the hexane, and *p,p'*-TDE and dieldrin in the 10% ether–hexane. Furthermore, it is possible (using the Merck grade of silica) to elute with 5 ml of hexane, thus removing the PCBs and *p,p'*-DDE, and following this with 10 ml of 10% ether–hexane, which elutes the *p,p'*-DDT with the *p,p'*-TDE and dieldrin. Any *o,p'*-DDT present will be split between the two fractions, but this is often an insignificant contaminant. Analysis by a single GLC column of the DC-200 or SE-30 silicone type is then sufficient for a complete analysis of both eluates, as the interference of PCBs with *p,p'*-TDE and *p,p'*-DDT is avoided. The interference of PCBs with *p,p'*-DDE is usually small.

The use of two silica columns in succession (as previously described for alumina) for cleaning up 1 ml aliquots of fat extracts, has been found fairly effective provided the fat content is not excessive, but the preferred technique is to use one alumina column (eluted with 20 ml of hexane) followed by a silica column using the two-solvent method to separate the pesticides into groups as described above. At least 100 mg of fat can be removed by this combination.

The efficiency of elution of pesticides from a silica column was examined by applying known weights of pesticide in 1 ml of hexane to the column, and eluting with 10 ml of hexane, followed by 10 ml of 10% ether in hexane, the results being compared on GLC analysis with those obtained from the original solution. Within the

TABLE V

PERCENTAGE RESIDUES IN 1-ml ELUATE FRACTIONS FROM A 2-g COLUMN OF SILICA GEL (120° ACTIVATION, 5% H_2O)

For chemical identities of residues, see Table I. Solvents: Fractions 1–10, hexane. Fractions 11–20, 10% ether in hexane.

Fraction No.	*α-BHC*	*β-BHC*	*γ-BHC*	*Hepta-chlor*	*Hepta-chlor epoxide*	*Aldrin*	*Diel-drin*	*Endrin*	*p,p'-DCBP*	*p,p'-MDE*	*p,p'-DDE*	*p,p'-TDE*	*p,p'-DDT*	*o,p' DDT*
1														
2						6								
3				17		69					22			
4				48		25				66	64			4
5				31						31	14			41
6				4						3			10	43
7													43	8
8													37	4
9													10	
10														
11														
12														
13														
14														
15								96				2		
16								4	64			6		
17	100	51	10		97		8		36			90		
18		41	62		3		63					2		
19		6	28				29							
20		2												

limits of the analytical errors, recoveries of all pesticides examined were found to be complete.

The efficiency of recovery from fat extracts, spiked with a number of pesticides and cleaned up on an alumina column followed by a silica column, has also been examined. In this instance the first 5-ml eluate of hexane was followed by a 10-ml elution with 10% ether in hexane, the eluates being analysed separately, and the concentrations of residues found being corrected for the presence of any similar residues in the original sample. As with the earlier tests of alumina columns, recovery of the added pesticides was complete.

The two-column alumina/silica gel method has been found very effective in cleaning-up hexane extracts of samples of seal blubber containing a number of PCB residues as well as *p,p'*-DDE, *p,p'*-TDE, *p,p'*-DDT and dieldrin. One-millilitre aliquots of the extracts, containing approximately 100 mg of extractable fat, are eluted on an alumina column with 20 ml of hexane, and the eluate is evaporated to 1 ml with a stream of air. This volume is then transferred quantitatively to the silica column as previously described. Hexane is used to elute the PCBs and *p,p'*-DDE, the first 5 ml being collected, and the remaining residues are eluted with 10 ml of 10% ether in hexane, which is subsequently reduced to 5 ml by evaporation.

The technique described has so far been employed only for organochlorine residues, and should be tested with standard solutions before the clean-up of extracts containing organophosphorus or other residues is attempted. It is also probable that the efficiency of clean-up will vary with the type of lipid material being removed. While it has been found possible to remove more than 200 mg of some types of extractable lipid, certain fish oils tend to elute more easily with hexane, and the column loading may have to be reduced below 100 mg. Alternatively an additional alumina column may be used. As the efficiency of recovery of the organochlorine residues is approximately 100%, the passage of the sample through a succession of columns does not result in any loss of these residues. The separation afforded by the silica columns has revealed the presence of residues of unknown origin in wildlife samples, these being in the 10% ether–hexane eluate, and thus distinguishable from PCBs. They have not so far been identified as any of the commonly used pesticides.

CONCLUSIONS

The twin-column chromatographic clean-up method described has several advantages over the more commonly employed liquid–liquid partition method. In particular, it gives 100% recovery of organochlorine compounds with a minimum use of solvents, and is more rapid and reproducible. Where pre-GLC sub-division of residues is not required, the twin alumina column version is preferred, but by replacing the second alumina column with a silica column a useful separation of PCB compounds from many organochlorine pesticides is obtained.

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