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DETERMINATION OF HEXACHLOROCYCLOHEXANE PESTICIDE RESIDUES IN WOOL FAT BY A COMBINED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC–GAS–LIQUID CHROMATOGRAPHIC METHOD

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SUMMARY

Beta- and gamma-hexachlorocyclohexane residues were determined in twelve wool fat samples by using a combined high-performance liquid chromatographic (HPLC)–gas–liquid chromatographic (GLC) method. After extraction and chromatographic clean-up on a silica-gel column, the sample was further purified by HPLC on a reversed-phase C-18 column with methanol as the mobile phase. The final determination was effected by GLC with a 1-mCi nickel-63 electron-capture detector. The analytical method was checked by addition of carbon-14-labelled lindane and measurement of the radioactivity in a liquid scintillation counter.

INTRODUCTION

The major components of wool fat (lanolin; *Adeps lanae*) are fatty esters and free fatty alcohols amounting to about 91 and 8%, respectively. In general, the fatty esters present are those which contain *n*-alkonic acids plus *iso*-alkonic, *anteiso*-alkonic and hydroxyalkonic types with chain lengths from C_{10} to C_{30}. The alcohol mixture is likewise complex, consisting of three groups, namely aliphatic alcohols (both straight- and branched-chain), triterpenols and sterols[1]. Pharmaceutical wool fat has a wide application in the pharmaceutical and cosmetic industries as a major ingredient of ointments, creams, etc.

Gamma-hexachlorocyclohexane (HCH), more commonly known as lindane, is widely used as an insecticide in agriculture; recently a high HCH content was found in the milk of cows that had been previously treated for a skin disease with an ointment containing wool fat[2]. The presence of organochlorine pesticides, especially HCH isomers, in wool fat has not received any great attention and no legal upper limit, as in the case of food products, has been fixed.

The purpose of this work was to determine residues of individual HCH isomers, especially alpha-, beta- and gamma-HCH, in wool fat samples. A combined high-performance liquid chromatographic (HPLC)–gas–liquid chromatographic (GLC) method, together with a clean-up and extraction procedure, suitable for the special requirements of wool fat, has been developed. The reliability of the procedure and

the recovery of HCH isomers have been checked by using carbon-14-labelled lindane and measuring the radioactivity in a liquid scintillation counter.

There have been a number of reports on the determination of pesticide residues in food, depot fat and blood and a GLC separation of HCH isomers on various columns and a column chromatographic separation of organochlorine residues prior to GLC analysis have been described[3–7]. A novel combination of HPLC and electron-capture detection (ECD) in the analysis of pesticides has also been reported[8].

EXPERIMENTAL

Chemicals

All of the solvents used, *viz.*, *n*-hexane, acetonitrile, light petroleum (b.p. 40–60°), toluene and methanol, were of reagent grade (Merck, Darmstadt, G.F.R.) and redistilled over Florisil. The Florisil (60–100 mesh) was previously dehydrated by heating for 6 h in a furnace at 600° and then cooled in an oven at 130° for 2 h. Dehydrated Florisil should be used on the day it is prepared.

Silica gel, 0.032–0.063 mm for column chromatography, was obtained from Woelm (Eschwege, G.F.R.). It was shaken vigorously with toluene in a stoppered erlenmeyer flask, filtered and dried for 6 h at 110° in a heating oven. Carbon-14-labelled lindane was obtained from Amersham Buchler (Braunschweig, G.F.R.).

Kinard scintillator was prepared by mixing intimately 800 ml of ethanol, 500 ml of dioxan, 500 ml of xylene, 14 g of PPO (2,5-diphenyloxazole), 0.75 g of POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene] (Packard, Hanau, G.F.R.) and 240 g of naphthalene and shaking well.

Clean-up procedure

A 5-g amount of wool fat was weighed in a separating funnel and dissolved in 50 ml of *n*-hexane by gently rotating the funnel. A 25-ml volume of acetonitrile was then added and the contents were shaken for 15 min and allowed to stand for the separation of the two phases. The acetonitrile layer was transferred from the separating funnel into a round-bottomed flask and the *n*-hexane phase was shaken for a further three 15-min periods, each with 25 ml of acetonitrile. The combined acetonitrile extract was condensed in a rotary evaporator to a few millilitres and the final extract was dried under a stream of nitrogen. The residue was taken up in 15 ml of light petroleum (b.p. 40–60°) and set aside. A 10-g amount of silica gel (Woelm), 0.032–0.063 mm, was shaken with light petroleum (b.p. 40–60°), the slurry was transferred into a glass column (20 cm × 1.5 cm I.D.) up to a height of 16 cm and the light petroleum was allowed to drain without pressure. The light petroleum fraction containing the sample was brought into the packed column and the solvent was allowed to pass through slowly. The column was washed with a further 10 ml of light petroleum (b.p. 40–60°) and the eluate was rejected. Most of the fat content remained at the top of the column and was visible as a yellow zone. HCH isomers were then eluted slowly with about 60 ml of toluene at the rate of 40 drops/min. The toluene eluate was collected in a ground-glass jointed round-bottomed flask and condensed in a rotary evaporator to a few millilitres, which were removed with a stream of nitrogen. The residue was taken up in 1 ml of toluene and was set aside for further purification by HPLC.

HPLC purification

A Perkin-Elmer 1220 liquid chromatograph with a fixed-wavelength (254 nm) detector and a 1-mV Varian Aerograph Model 20 recorder were used. A Merck LiChrosorb reversed-phase column, packed with C-18 phase chemically bonded silica gel, mean particle size 10 μm, was used for separation. The mobile phase was pure methanol, the flow-rate being 1 ml/min. A 10-μl volume of the toluene fraction was injected into the liquid chromatograph and, as soon as the recorder pen moved upwards, the mobile phase coming from the detector outlet was collected for 75 sec in a 2-ml reagent glass. Finally, the eluate was removed gently with a stream of nitrogen and the residue was taken up, according to the amount of HCH present, in 0.1–1 ml of toluene. This solution was used for GLC determination with ECD. Each sample was injected twice into the liquid chromatograph. Standard solutions of different concentrations of beta- and gamma-HCH were also injected into the liquid chromatograph and collected in the same manner. The main contents of HCH isomers were in this fraction. Two further fractions, one from just after the injection up to the upward movement of the recorder pen and the second from just after the collection of the main fraction for a further 60 sec, were also collected. They contained no or only trace amounts of HCH isomers.

GLC determination of beta- and gamma-HCH in wool fat samples

Aliquots of 1 μl were withdrawn with a Hamilton micro-syringe and injected five or six times directly into the gas chromatograph. There were two main eluted fractions from each wool fat sample (from two separate 10-μl portions injected into the liquid chromatograph) for GLC analysis, in addition to two other subsidiary fractions from each run (six fractions in all). The average of the results for the two main fractions as peak areas were taken for calculation. Standard solutions of beta- and gamma-HCH, which were submitted to the same HPLC purification process as the samples, in order to compensate for any losses during the separation, were analysed by GLC in the same manner.

The GLC analysis was carried out on a Perkin-Elmer Model F22 gas chromatograph equipped with a 1-mCi nickel-63 ECD (wrapped in 0.5-μm thick nickel foil) in the pulse mode and a Hewlett-Packard Model 3380 recorder–integrator–calculator. The instrument contained a 2 m $\times$ 4 mm I.D. coiled glass column, packed with 10% DC 200 methylsilicone oil, which was coated on Chromosorb W (acid washed, 60–80 mesh) support. Argon–5% methane was used as the carrier gas at a flow-rate of 70 ml/min and the column temperature was 220° (isothermal). The amount of beta- and gamma-HCH in the samples was calculated from corresponding standards.

Checking of analytical procedure by addition of carbon-14-labelled lindane to a wool fat sample

A 5-g amount of wool fat was dissolved in 50 ml of *n*-hexane, 5 μg each of inactive beta- and gamma-HCH plus a known amount of carbon-14-labelled lindane were added and the solution was extracted four times with 25-ml portions of acetonitrile. The further clean-up and purification steps were as described above for the sample. Volumes of 0.5 ml were withdrawn from each fraction of acetonitrile (four fractions), *n*-hexane (four fractions), combined acetonitrile fraction after distillation, light petroleum (b.p. 40–60°) (two fractions, before and after elution through a chromato-

graphic column), toluene (two fractions, after elution through a chromatographic column and after distillation), three fractions in the HPLC procedure, and transferred into separate scintillation tubes. A 10-ml volume of Kinard scintillator was added to each tube, the tubes were shaken well, inserted in an ICN-Tracerlab liquid scintillation counter and the radioactivity of carbon-14 weak beta-emitters was measured three times for each sample for a 1-min period. The counts per minute, after subtracting the blank counts, were multiplied by the corresponding factors and the results were compared with that of the original activity added (counts/minute) at the beginning of the experiment. About 99.0% of the added activity was recovered, indicating that the loss of HCH in the analytical procedure was minimal.

RESULTS AND DISCUSSION

Results for twelve wool fat samples containing various amounts of beta- and gamma-HCH are given in Table I and a gas chromatogram of one sample is shown in Fig. 1. Although only gamma-HCH is used exclusively as an insecticide, the presence of beta-HCH shows that in addition to lindane, technical HCH, which contains large amounts of beta-HCH, has also been applied in agriculture. Alpha-HCH was present in most samples in trace amounts (*ca.* 0.1 ppm) and was not quantitated further.

TABLE I

DETERMINATION OF BETA- AND GAMMA-HCH IN WOOL FAT SAMPLES

Sample No.	*Beta-HCH (ppm)*	*Gamma-HCH (ppm)*
1	1.68	0.05
2	0.65	0.76
3	Not detected	Not detected
4	1.1	0.85
5	6.56	1.37
6	16.5	2.54
7	0.25	Not detected
8	1.7	1.2
9	7.78	0.05
10	2.60	0.05
11	1.40	4.2
12	0.5	0.1

On account of the very high fat content of wool fat, an *n*-hexane–acetonitrile system was used for the separation of HCH isomers. This mode of extraction is often used for the extraction of organochlorine and organophosphorus pesticides from samples with high fat contents, such as butter, margarine and cooking oil[9–11]. It could be demonstrated by the addition of carbon-14-labelled lindane to the sample that the extraction of HCH was quantitative after the sample solution had been shaken three times with 25-ml portions of acetonitrile. In the procedure applied, *n*-hexane solution is shaken four times with acetonitrile, and no activity was found in either the fourth acetonitrile fraction or the remaining *n*-hexane phase. The combined acetonitrile fraction still contained, in addition to HCH isomers, a considerable

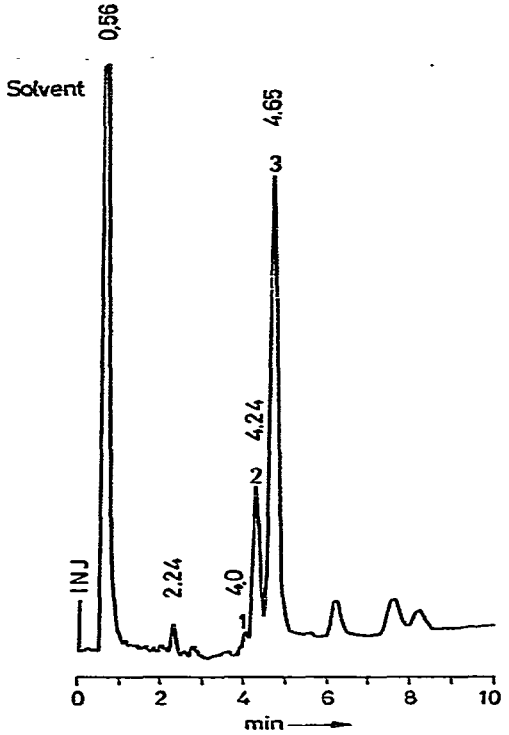

Fig. 1. GLC determination of HCH isomers in wool fat after extraction, clean-up and HPLC purification. Column, 2 m × 4 mm I.D. coiled glass, packed with 10% DC 200 methylsilicone oil on Chromosorb W AW (60–80 mesh); column temperature, 220° (isothermal); injector temperature, 280°; detector temperature, 300°; carrier gas, argon–5% methane, flow-rate 70 ml/min. Peaks: 1 = alpha-HCH; 2 = beta-HCH; 3 = gamma-HCH.

amount of fatty esters and alcohols which had to be removed through a column chromatographic clean-up.

Moats[12] compared a number of adsorbents for the clean-up of butter fats and found that Florisil, deactivated with 5% of water, was suitable for the elution of pesticides such as dieldrin. Leoni[13] used silica gel micro-columns for the separation of a number of pesticides. Holden and Marsden[14] found a partially deactivated silica gel column to be more effective in the separation of organochlorine pesticides by differential elution from animal tissue extracts. Of the numerous adsorbents that have been tested, such as aluminium oxide, various silica gels, cellulose and magnesia, silica gel (Woelm), 0.032–0.063 mm, wetted with light petroleum, retained most satisfactorily a substantial proportion of fat on the top of the column. Partial deactivation of the silica gel with water was not necessary in this instance. HCH isomers were retained on the silica gel column and the light petroleum fraction was free of them after passage through the column. This was confirmed by radioactivity measurements and by concentrating the light petroleum eluate to about 1 ml and injecting a 1-μl volume into the gas chromatograph. No positive signal was obtained in either instance.

Great care should be taken in removing the solvents from the various extracts.

They should not be evaporated to dryness, in order to avoid losses of HCH, which are generally in the range 20–30%. The recovery is greatly affected by the manner in which the extracts are condensed. Distillation of the solvent on a rotary evaporator to a volume of about 2–3 ml and removal of the remainder by passing a gentle stream of nitrogen gave quantitative and reproducible results. No activity was found in the distilled portion of the solvents, indicating the absence of any losses of HCH isomers.

All three HCH isomers (alpha-, beta- and gamma-) were eluted from the chromatographic column quantitatively with about 60 ml of toluene. This was checked by adding a known amount of each to the sample, eluting with toluene and subsequently carrying out a GLC determination and also by radioactivity measurements. At this stage, either a direct GLC determination could be carried out by simply diluting the toluene fraction to a suitable volume, or the sample could be purified further by HPLC. When peaks from other substances almost overlap those of HCH isomers on the gas chromatogram or the detector is overloaded by giving large signals, an HPLC purification is essential. In this work, all twelve samples were analysed after HPLC purification.

HCH isomers have almost no UV absorption and therefore could not be detected directly in HPLC. In a number of experiments in which HCH was added to the samples, it was found that they were eluted almost with the toluene solvent front. At 254 nm toluene shows a high UV absorption and, as soon as it passed through the detector cell, the recorder pen moved upwards. The mobile phase coming from the detector was then collected immediately for 75 sec and this fraction contained about 93% of HCH isomers. The fractions collected before and after this fraction contained 1.3 and 4.7% of gamma-HCH, respectively. These results were obtained by radioactivity measurements. All of the standard solutions of beta- and gamma-HCH were submitted to this HPLC separation step and the results for the wool fat samples were quantified only on the basis of these standards.

The sensitivity of HPLC could be further increased by applying larger injection volumes. Thus, samples with a very low HCH content could also be analysed by injecting 50–100 μl of the solution. This led occasionally to a large increase in the column pressure owing to the fat particles collected in the upper section of the column. In this instance, *n*-hexane and methanol were passed through the column for several hours until the column pressure was again in the range 300–400 p.s.i. In combined HPLC–GLC, the high sensitivity and specificity of the latter for organochlorine compounds is combined with the large resolving and separating power of the former. This technique has already been applied by Willmott and Dolphin[8] for the analysis of pesticides, with a direct combination of an ECD to the HPLC apparatus. Because of difficulties with the apparatus and operating problems, an indirect combination was chosen here that was simple but still retained all of the advantages of the ECD.

Efforts to apply a thin-layer chromatographic (TLC) method to the separation of HCH isomers using TLC plates coated with various adsorbents and solvents of diverse polarities did not give the desired results. Apart from the losses that occurred on scraping off the adsorbent, treating it with a solvent, filtration etc., there was always some fat present in the samples owing to an inadequate separation on the TLC plates. This ultimately interfered with the HCH peaks in the gas chromatogram and quantitative work was not possible.

Of the three GLC columns tested (10% DC 200 on Chromosorb W AW, 3% OV-17 on Varaport 30 and 3% SE-30 on Chromosorb G), the first column gave the optimal separation of alpha-, beta- and gamma-HCH. Other chlorinated pesticides such as heptachlor, methoxychlor, aldrin and dieldrin were also separated and did not interfere with the HCH peaks (Fig. 2). Only dieldrin was present in some wool fat samples and was not quantitated. Other liquid phases mentioned in literature for the separation of HCH isomers are OV-61[3], QF-1 + 2,2-dimethylpropane-1,3-diol succinate[4], NPGS + XF-1112[5] and XE-60[6]. On these columns, the sequence of elution is reported to be alpha-, gamma- and beta-HCH. However, beta-HCH was eluted between alpha- and gamma-HCH on the column used here (Fig. 2).

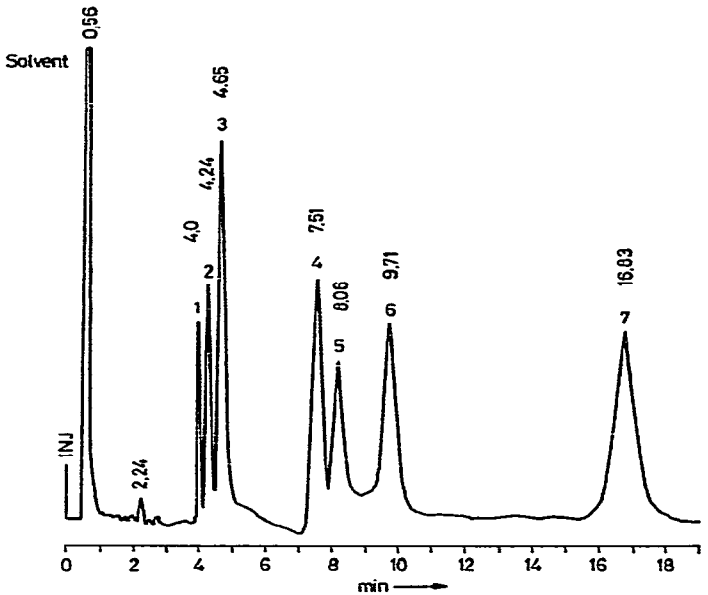

Fig. 2. Separation of some pesticides on a 10% DC 200 methylsilicone oil on Chromosorb W AW (60–80 mesh) column. GLC conditions as in Fig. 1. Peaks: 1 = alpha-HCH; 2 = beta-HCH; 3 = gamma-HCH; 4 = heptachlor; 5 = methoxychlor; 6 = aldrin; 7 = dieldrin.

As the amounts of HCH isomers in the various wool fat samples differed considerably, it could not be assumed that the isomers were in the linear range of detection in the solutions injected into the GLC column. The results in Table I therefore do not refer to a single reference solution, but to a series of standards of various concentrations, which were prepared so as to cover the range of the sample peaks.

The recovery of HCH isomers during all of the extraction and clean-up operations was also tested by using a wool fat sample that was originally free of these pesticides. Known amounts of beta- and gamma-HCH (5 μg of each) were added to it and the sample was treated as described above. After the GLC determination it was found that 98% of gamma-HCH and 95% of beta-HCH were recovered. The measurements with carbon-14-labelled lindane also confirmed that almost no loss of activity occurred during these operations. HPLC purification resulted in a 6% loss, which was compensated for by using corresponding standards.

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REFERENCES

1 E. W. Clark, Westbrook Lanolin, Bradford, Great Britain, personal communication.
2 Personal communication.
3 A. di Muccio, L. Boniforti and R. Monacelli, *J. Chromatogr.*, 71 (1972) 340.
4 G. Czegledi-Janko, *Analyst (London)*, 48 (1973) 863.
5 G. Czegledi-Janko, *Chromatographia*, 6 (1973) 89.
6 A. Szokolay, J. Uhnák, M. Sackmauerová and A. Maďarič, *J. Chromatogr.*, 106 (1975) 401.
7 D. E. Wells and S. J. Johnstone, *J. Chromatogr.*, 140 (1977) 17.
8 F. W. Willmott and R. J. Dolphin, *J. Chromatogr. Sci.*, 12 (1974) 695.
9 L. R. Jones and J. A. Riddick, *Anal. Chem.*, 24 (1952) 569.
10 P. A. Mills, *J. Ass. Offic. Agr. Chem.*, 42 (1959) 734.
11 Deutsche Forschungsgemeinschaft S. 5–20, *Methodensammlung zur Rückstandsanalytik von Pflanzenschutzmitteln*, Verlag Chemie, Weinheim, 1972.
12 W. A. Moats, *J. Ass. Offic. Anal. Chem.*, 46 (1963) 172.
13 V. Leoni, *J. Chromatogr.*, 62 (1971) 63.
14 A. V. Holden and K. Marsden, *J. Chromatogr.*, 44 (1969) 481.