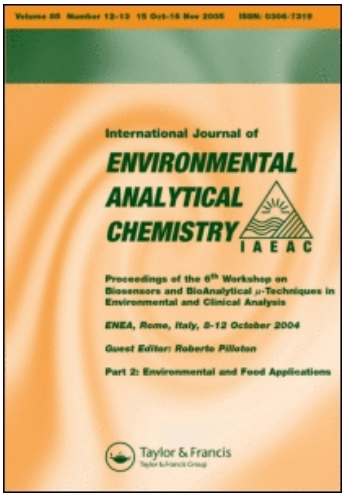

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Fingerprint Analyses of Plant and Animal Tissues with Respect to the Occurrence of Foreign Compounds

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Fingerprint Analyses of Plant and Animal Tissues with Respect to the Occurrence of Foreign Compounds

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Glass capillary chromatography can be used for the characterization of complex mixtures of plant and animal samples after clean-up by counter-current distribution. For good reproducibility of fingerprint patterns a special splitless injection system is used. This method prevents any loss at injection. In order to determine organochlorine substances also in trace amounts (a few pg) an electron capture detector (ECD) was used. Each step in the analysis procedure was checked with the aid of radioactively labelled substances, e.g. the polar compound pentachlorophenol and the non-polar compound hexachlorobenzene. With the aid of capillary chromatography-mass spectrometry substances in complex mixtures in a concentration of 10 to 100 ng/μl can be characterized.

Suitable glass capillaries for these investigations were prepared in this laboratory.

The results indicate that a standardization of the procedure is practicable.

INTRODUCTION

Comparative time-course studies are an essential aspect in assessing the history and trends of chemical residues in the environment. In a feasibility study within the US–German Environmental Specimen Bank Program, analytical sample description, residue determinations in organisms were performed, and data were obtained on the fate of polar and lipophilic organochlorine compounds upon long-term storage.

Residues reflect the true conditions at the time of sampling only when they are not subject to secondary alterations during the storage time. Therefore, using aldrin, as an example, the alterations were investigated that may occur with residues in samples of aldrin and its conversion products such as dieldrin, photodieldrin, hydrophilic metabolites, unextractable residues, in soil and plant samples during five to seven years

storage time at minus 18°C. The analyses showed that no significant changes or losses of aldrin-derived residues occur upon several years storage at low temperature.[1]

For unknown pollutants, however, it would be inappropriate to simply assume stability during storage in a bank. Therefore, a standardized analytical cross-characterization of environmental samples which immediately follows the sampling may provide a safe procedure. The characterization of the specimen with respect to the occurrence of unknown foreign compounds was therefore performed in a "fingerprint" type.

With the aim, analytical methodologies, especially for clean-up and detection, had to be screened. While clean-up by absorptive bubble analysis[2] did not give sufficient results (poor recovery), counter-current distribution which avoids decomposition of constituents, proved to be an effective and reproducible method. For some well defined chemicals the results can even be mathematically predicted. Using a feasible extraction, an adapted solvent system for counter-current distribution, a modified capillary injection system and capillary gaschromatographic–mass spectrometric combination for confirmation, we were able to characterize plant and animal samples by fingerprint patterns.

EXPERIMENTAL

For the investigation of plants, lettuce served as the matrix for the polar compound pentachlorophenol (PCP); for animal studies, we used several samples of rhesus monkey tissues. The monkeys had been dosed with hexachlorobenzene (HCB) for 15 months. The dose was equivalent to 1 ppm in the diet.[3] Furthermore, we analyzed a liver sample of a chimpanzee which contained residues of 13 compounds, for example, hexachlorobenzene, dieldrin, trichlorobiphenyl, etc. In this case, a single dose of a mixture of chemicals had been given at a level of 1 ppm bodyweight for each compound. The animals were sacrificed three days after dosing.[4]

Homogenization

Lettuce was homogenized with a regular kitchen blender. 50 g sub samples were deep freeze. The freeze-dried animal organs (stomach and liver of rhesus monkey and chimpanzee) were homogenized with the aid of a "Retsch"-grinder under continuous cooling with liquid nitrogen (−195°C). After the ^{14}C-hexachlorobenzene content was determined by combustion, two sub-samples of 5g were prepared for parallel study.

Extraction of samples

Ethyl acetate was found to be the most suitable solvent for PCP and *n*-hexane for HCB extraction. The lettuce samples were treated with 1 ml conc. acetic acid, then mixed twice with 100 ml and twice with 50 ml ethyl acetate for several minutes with the aid of an Ultra-Turrax and decanted. Finally the homogenate was suction filtered with cleaned filter paper and cotton and washed with a few ml of the solvent. The recovery was 98% with a standard deviation of $\pm 1.9\%$.

The animal tissue extract samples were treated once with 100 ml solvent and twice with 50 ml solvent using an Ultra-Turrax. Decanting and filtering was done as with the lettuce. The recovery from stomach was 96% and from liver 95% with a standard deviation of $\pm 3.0\%$.

Clean-up

The clean-up was carried out in a counter-current distribution system which permits both upper and lower phases to be transferred simultaneously in opposite directions. The whole sample was inserted into the middle of the apparatus and fractionated in 50 steps. In several cases the fractions were also examined after 20 or 25 steps. The solvent systems[5-9] tested and used for distribution studies of PCP, are summarized in Table I.

In systems 1 and 2 PCP moved with the front of the lower phase and thus was separated from the main portion of materials contained in the plant. It could easily be obtained quantitatively (see discussion). To obtain PCP together with the plant components and to test the presence of these materials, the distribution coefficient of PCP was increased more and more through variation of the solvent system and by increasing the pH value. The distribution coefficient of HCB in stomach and liver extracts in the system *n*-hexane (upper phase)/methanol-water 1:9 (lower phase) is 57.

The investigation of the individual fractions as well as some combination (after concentration) was either by *liquid scintillation counting* or by *capillary chromatography*.

A dioxane-based solution was used as scintillation solvent for measurement of radioactivity. To obtain correct values also at a higher quench, the internal standard method was used. GC-determinations for PCP were done using a 75 m glass capillary, i.d. 0.25 mm, coated with a 3% OV 101. The coating of the HCl-etched surface with the stationary liquid was carried out by the dynamic method.[10-14] Injector temperature 250°C, detector (Ni-63ECD) temperature 275°C; nitrogen carrier gas 1.2 ml/min; temperature program 70 to 250°C at a rate of 3°C/min.

TABLE I

Solvent systems tested and used for distribution studies of PCP

Upper phase	Lower phase	Distribution coefficient of PCP
1. ligroin 9 ethylacetate 1	methanol 9 water 1	0
2. ligroin	methanol 9 water 1	0
3. ligroin 9 ethylacetate 1	methanol 9 water 1 (pH 1)	0.4
4. ligroin	methanol 9 water 1 (pH 1)	0.4
5. ligroin	phosphate- buffer pH 6.7	5.7
6. ligroin	phosphate- buffer pH 5.9	8.1
7. *n*-hexane	acetic acid 5%	21.7
8. *n*-hexane	acetic acid 10%	46.3

The detection limit for PCP-acetate was 0.02–0.03 ng (20–30 pg); the standard deviation for the determination of 0.1 ng was ±6%. Acetylation of the lettuce extract was carried out with acetic anhydride in an alkaline medium.[15-19] After extraction of the reaction mixture with *n*-hexane, the gaschromatographic analysis was done directly. Recovery after acetylation was 98%.

The *capillary-GC-conditions for the HCB* investigations:

1) length 71 m; i.d. 0.25 mm; coated with 1% OV 101; carrier 1.2 ml/min; temperature-program of 60°C to 220°C; rate 30°C/min.
2) length 58 m; i.d. 0.25 mm; 3% OV 101; 5 min 60°C then temperature-program from 60°C to 190°C; rate 4°C/min.

After the determination of the distribution curve, the fractions 1–5, 6–10 and 11–15 were combined, treated with anhydrous Na_2SO_4, concentrated and chromatographed. The detection limit was 2 pg.

The proper introduction of complex mixtures onto capillary columns is quite problematic,[20-27] however, this method is very efficient for the separation of such mixtures. To obtain good reproducibility, a specific splitless injection system was employed in view of the fact that the great sensitivity of electron capture detector accentuates the slightest fluctuations. The modification introduced here by using graphite-ferrule, in

contrast to Schulte and Acker,[22] facilitates easier changing of the capillary column as it excludes temperature fluctuations and bleeding of the ferrule.

Integrator: Hewlett Packard, 3385 A, automatic system, chart speed 0.5 cm/min; zero 10; attn. 2^1; aux sgnl A; slope sensitivity 0.1; after 6 min area reject 0. GC–MS-conditions: Glass capillary length 24 m; i.d. 0.25 mm; coated with 3% SE 30; injector temperature 235°C; accessory temperature 200°C; temperature program 4 min 50°C then from 50°C to 240°C; rate 4°C/min; carrier gas 0.5 ml He/min.

Quadrupole-mass-spectrometer Finnigan: Beam current 0.32: ion energy 6.9; electron energy 55.1; collector 33.0; emission 39.0; extractor 19.6; lens 149.1; mass range from 100 to 282 and from 100 to 400 m/e.

RESULTS AND DISCUSSION

The internal standards PCP and HCB were easily extracted with a high yield of over 90%. The results for the extraction of stomach mucosa and liver were determined twice, once by radioactivity measurement of the extract and once by burning which gave the difference in radioactivity of the sample before and after extraction. The recovery after evaporation and counter-current distribution was unusually high.

The distribution curve for PCP for the various systems is shown in Figure 1. The reproducibility of the counter-current distribution is shown in Figure 2 for PCP in system 8 (see Table I) and Figure 3 for HCB.

Capillary chromatography using ECD is a more exact method for the control of reproducibility of the distribution. Figure 4 shows the results of two parallel investigations of two extracts from one lettuce sample. The graphs demonstrate the good reproducibility also throughout the whole procedure.

A variation of the method is provided by derivatizations. Following acetylation[18,19] of a respective fraction, e.g. the internal standard pentachlorophenol could be determined readily because of the quantitative derivatization. Figure 5 shows this determination of PCP, in more dilute solutions, in a range of 0.01 to 0.1 ppb.

Figures 6 and 7 show fingerprints of stomach and liver extract of rhesus monkey after counter-current distribution and demonstrate also the possibility to obtain good results with chlorinated hydrocarbons. One can easily see the presence of hexachlorobenzene, which dominates this fingerprint pattern. Pentachlorobenzene was identified, as one of two major metabolites[3], and so, this compound is also detectable in the fingerprint patterns.

Figure 8 shows chromatograms of two parallel investigations of a liver extract of a chimpanzee after separation from lipids. Table II summarize

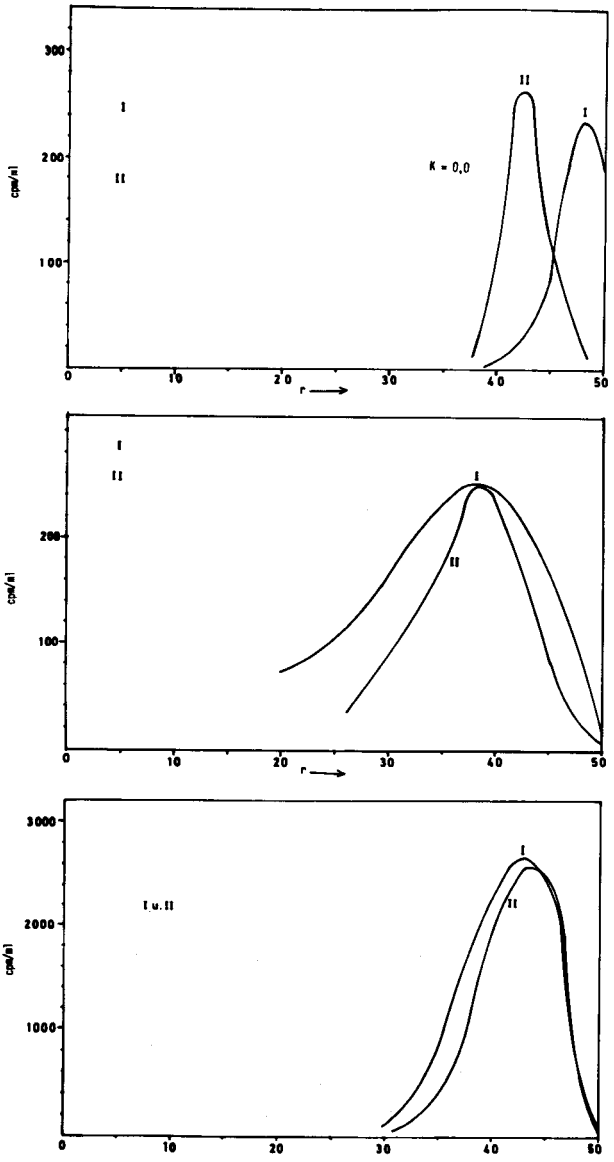

FIGURE 1 Distribution curves of PCP (see Table I).

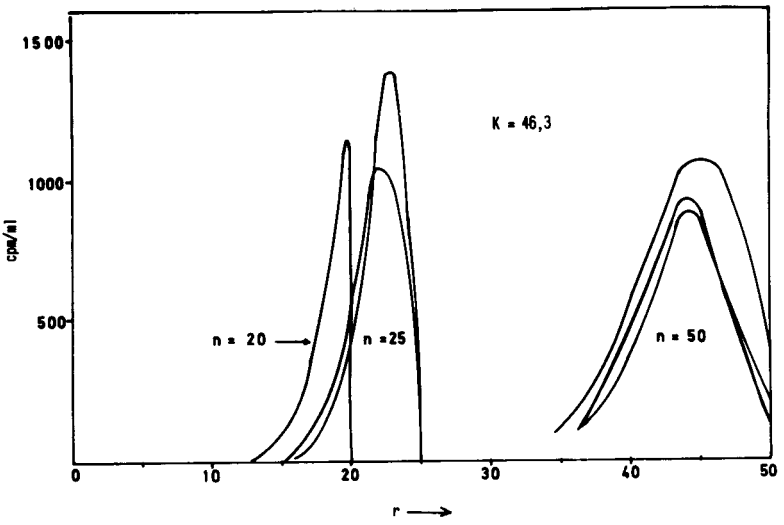

FIGURE 2 Reproducibility of the counter-current distribution of PCP in system *n*-hexane acetic acid 10%.

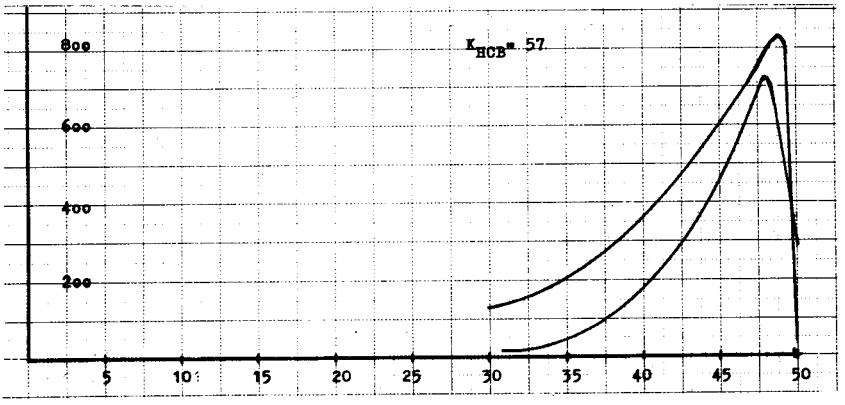

FIGURE 3 Reproducibility of the counter-current distribution of HCB in a system *n*-hexane/methanol-water 1:9.

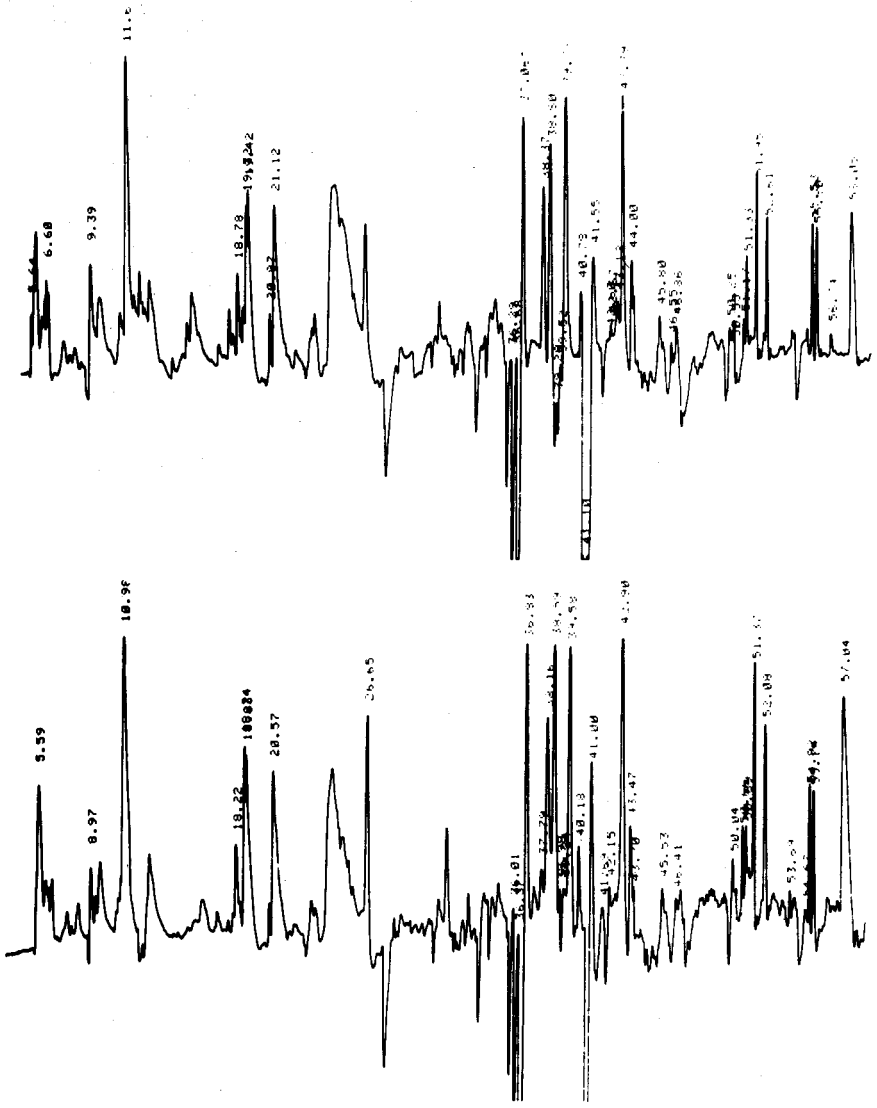

FIGURE 4 Fingerprint analysis of two parallel investigations of lettuce extracts.

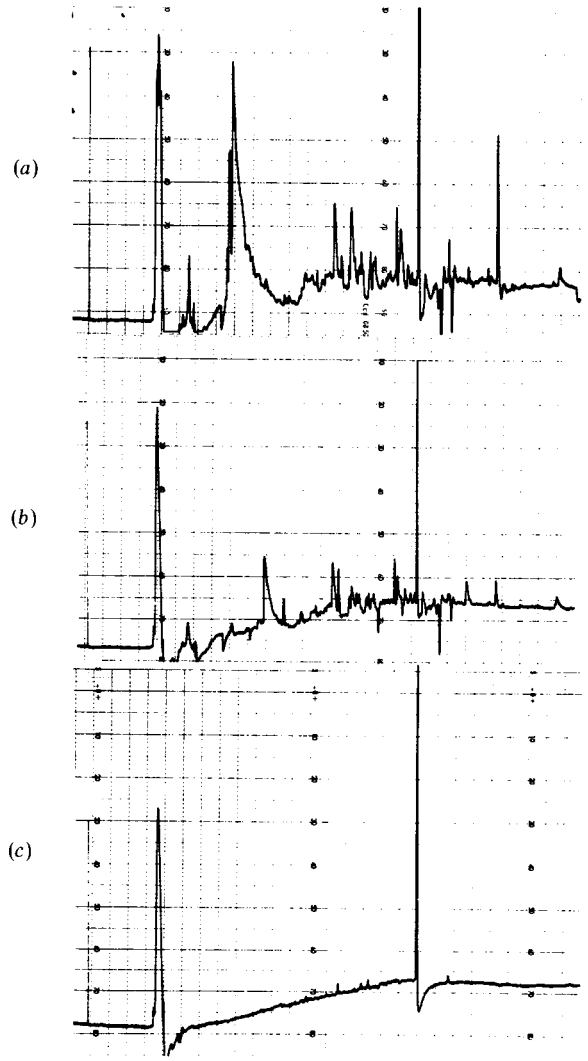

FIGURE 5 Determination of PCP in lettuce after acetylation; *a* and *b* are two different extracts, *c* is the chromatogram of a standard solution about 40 pg.

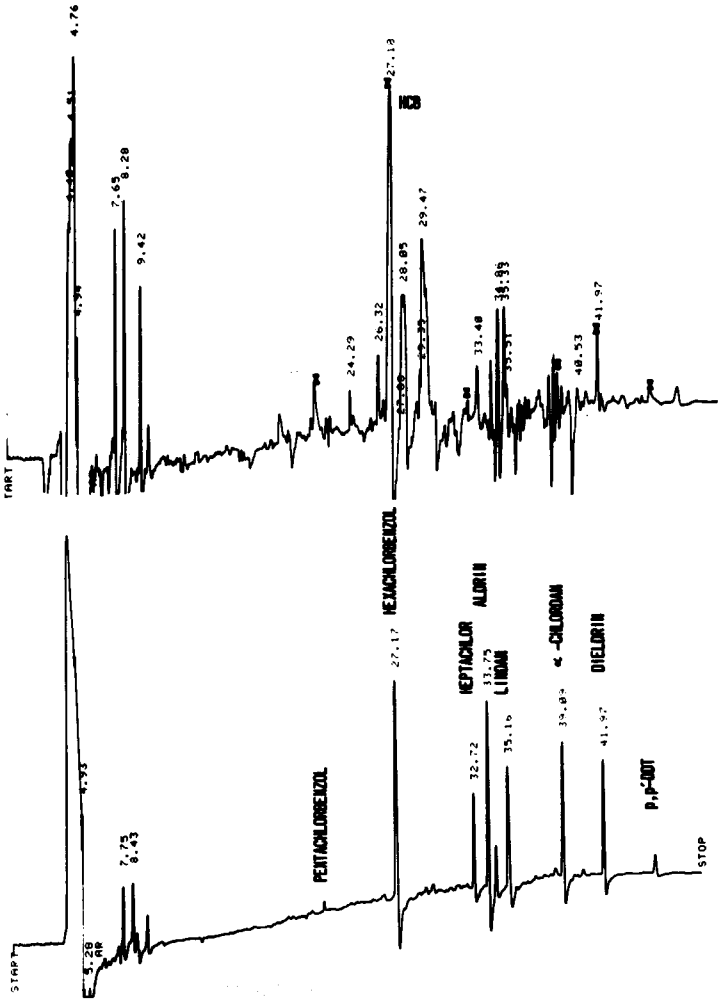

FIGURE 6 (*a*) Fingerprint pattern of stomach extract; (*b*) Standard mixture of chlorinated hydrocarbons.

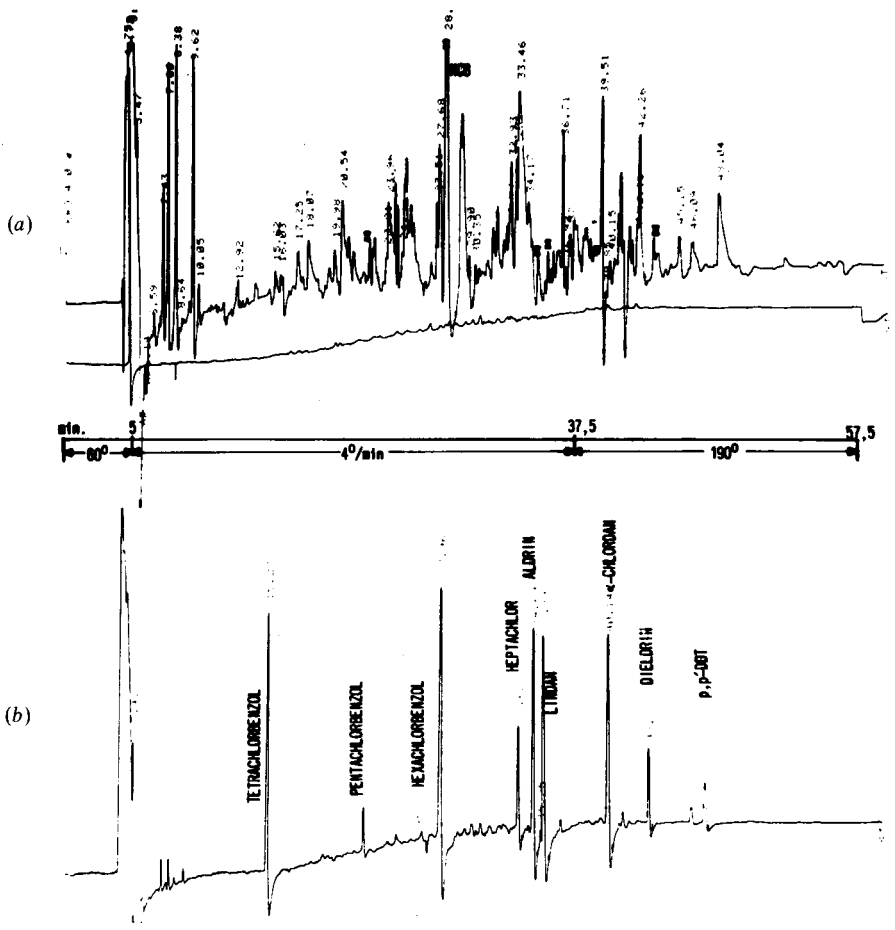

FIGURE 7 (*a*) Fingerprint pattern of liver extract; (*b*) Standard mixture of chlorinated hydrocarbons.

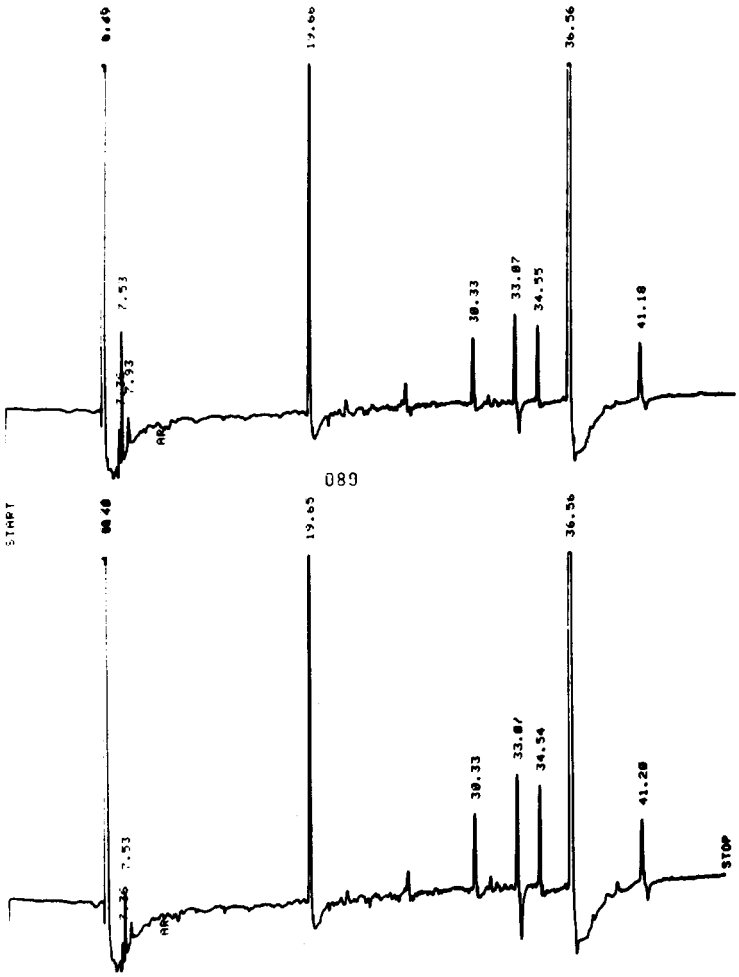

FIGURE 8 Parallel investigation of liver extract of chimpanzee.

TABLE II

Parallel investigation of liver of chimpanzee*

Rotation time (min)		Area (%)	
19.65	19.66	13.5	14.8
30.33	30.33	2.4	2.1
33.07	33.07	3.3	2.4
34.54	34.55	2.8	2.3
36.56	36.56	75.8	76.5
41.20	41.18	1.9	1.6

*Cf. Fig. 8.

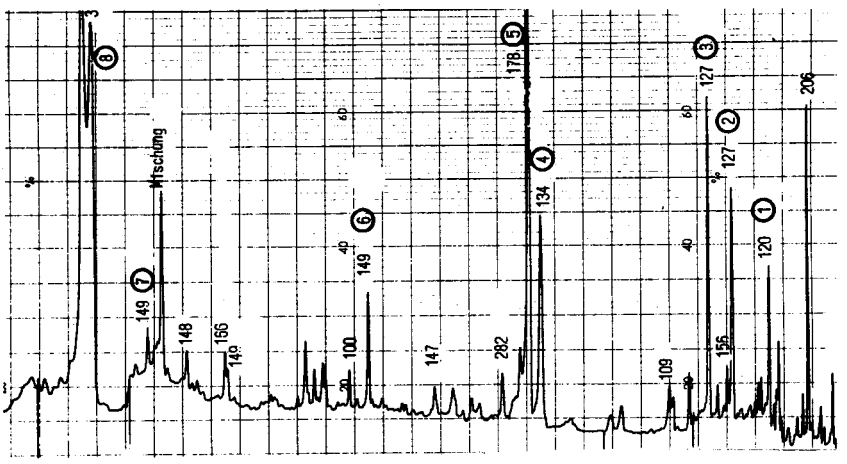

FIGURE 9 Combination GC–MS chromatogram.

the excellent corresponding values of retention times and integrations. By optimum programming of the integrator for several selected peaks, a satisfactory automatic integration can also be obtained for these chromatograms.

In order to characterize the total fingerprint region, and thus also unknown substances, the glass capillary chromatograph was combined with a mass spectrometer.

The results of the investigation using this combination are shown in Figure 9. The plain numbers at the various peaks give the molecular weight. The circled numbers are the peaks for which mass spectra were taken. Since the main object of this investigation was to test the method, complete analysis of the mass spectra was not carried out.

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