Gas Chromatographic Method for the Determination of Organochlorine Pesticides in Human Milk

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Among several suitable analytical methods for the determination of organochlorine pesticides in food, including milk, the Environmental Protection Agency (EPA) recommends a method which is based upon extraction of the lipid soluble material with petroleum ether. The pesticide residues are separated from the lipid/petroleum ether solution by extraction with acetonitrile. Thereafter, the pesticide residues are re-extracted with petroleum ether by diluting the acetonitrile extract with an aqueous solution of 2% sodium chloride. The petroleum ether extract is concentrated and transferred to a Florisil column and petroleum ether/diethyl ether used as the eluating agent.

Even though this method can be used for the analysis of organochlorine pesticides such as DDE and DDT, SMYTH (1972) found that it was less suitable in connection with the quantitative determination of hexachlorobenzene (HCB), as the method gave a recovery rate of 20 - 30% HCB from fatty tissue to which 0.05 - 2 ppm HCB had been added. TAYLOR and KEENAN (1970) explained this low recovery rate as being due to the pronounced non-polar nature of HCB. A consequence of this is that HCB in a mixture of petroleum ether and acetonitrile dissolves selectively in petroleum ether. In order to avoid this problem, STIJVE (1971) and CRIST et al. (1975), among others, employed direct Florisil column clean-up, whilst ENOS et al. (1967) developed an analytical method in which acetonitrile was used to extract pesticides directly from the sample material. The acetonitrile solution was thereafter diluted with an aqueous 2% solution of sodium sulphate and the pesticides extracted with hexane. Clean-up was carried out with a micro Florisil column by eluating with 1% methanol in hexane. CRIST and MOSEMAN (1976) used the latter method to analyse fatty tissue and found a recovery rate of 60-65% HCB. They modified the method by using 20% acetone in acetonitrile as extraction solvent, the recovery of HCB then increasing to 88%.

TAYLOR and KEENAN (1970) and STIJVE (1972) have studied the stability of HCB in acid and alkali. They

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found that HCB is not affected by concentrated sulphuric acid, and is broken down only to a certain degree in a solution of sodium hydroxide in methanol.

A method which has been practised at the Department of Pharmacology and Toxicology for the analysis of organochlorine pesticides in human milk is described below. The method is relatively rapid and gives a recovery rate of over 90% HCB. Acetone and hexane are added to the human milk and the mixture disintegrated ultrasonically. The hexane phase is isolated and cleaned-up with acid and alkali according to The method of BJERK and SUNDBY (1970). HCB is detected in the acid cleaned-up hexane phase.

MATERIALS AND METHODS

Reagents.

a) Solvents: n-hexane and acetone redistilled in an all-glass apparatus and tested by GLC after 50 fold concentration.
b) Standards: p,p'-DDE, p,p'-DDT, p,p'-DDD supplied by Analytical Standards and HCB from Analabs Inc. dissolved in hexane.

Apparatus.

a) Gas chromatograph: Varian 1700 with elctron-capture detector tritium source. Columns: 150 cm glass columns with an internal diameter of 2 mm, packed with 10% QF-1 or 4% SF-96 on Chromosorb W, 100 - 120 mesh.
b) Ultrasonic disintegrator: Sonifier B-12, from Branson Sonic Power Co.
c) Centrifuge: Roto Silenta III from Hettich, with 80 ml centrifuge tubes.

Gas chromatography.

Identification and quantification of HCB in fatty tissue is reported to be problematical as α-hexachlorocyclohexane (α-BHC) has almost the same retention time in several GLC columns (EPA, 1974). A satisfactory separation of these compounds has been achieved by using a 150 cm glass column with an internal diameter of 2 mm, packed with 10% QF-1 or 4% SF-96 on Chromosorb W, 100 - 120 mesh. The operating conditions were as follows: Nitrogen 35 ml/minute, injector temperature 190 ^{o}C, column temperature 180 ^{o}C, and detector temperature 200 ^{o}C. Retention times for some pesticides are given in Table 1.

TABLE 1.

Relative retention times of some organochlorine pesticides in two columns packed with 10% QF-1 or 4% SF-96 on Chromosorb W, 100 - 120 mesh.

Compound	10% QF-1 RRT[a]	4% SF-96 RRT[a]
HCB	0.42	0.40
∝-BHC	0.60	0.35
Lindane	0.79	0.44

[a]Relative to aldrin.

In order to ensure the identification of HCB, the acid-treated hexane extract is derivatised according to The method of CRIST et al. (1975). The hexane phase is evaporated carefully and the residual material treated with pyridine and potassium hydroxide in isopropanol. The solution is heated for about 30 minutes on a boiling water bath and an aqueous solution of 2% sodium sulphate together with hexane is then added. CRIST et al. (1975) identified the derivatives as isopropoxypentachlorobenzene (IPB) and bis-isopropoxytetrachlorobenzene (BITB). A pure HCB standard is derivatised in an analogous manner. The derivatives are injected into the gas chromatograph and retention times compared. The retention times, relatively to aldrin, were 0.42, 0.70 and 1.03 for HCB, IPB and BITB, respectively, on the QF-1 column and 0.40, 0.63 and 0.93, respectively, on the SF-96 column.

Extraction of fat.

Ten g human milk is poured into a 80 ml centrifuge tube and 15 ml acetone and 20 ml hexane added. Homogenization is carried out using ultrasonic disintegration for about 9 minutes. Thereafter the sample is centrifuged at 3000 rpm for 5 minutes. The sample is frozen at -70 °C in a bath of ethanol and dry ice, causing precipitation in the hexane layer. The hexane layer is cleaned by centrifuging the sample in the cold state at 3000 rpm for 10 seconds. The hexane layer is removed with a pipette immediately after centrifugation is completed and transferred to a 50 ml measuring cylinder.

Ten ml hexane and 5 ml acetone are added to the remainder of the centrifugate and the extraction process is repeated.

The pipetted hexane layers are collected in the

measuring cylinder which is put over a water bath at 40 °C, and the hexane phase is evaporated to about 2 ml in a gentle stream of dry air. The rest of the hexane is transferred to a graduated centrifuge tube, which has been weighed beforehand. The measuring cylinder is rinsed 3 times with 2 ml hexane. The centrifuge tube is put over a water bath at 40 °C and the hexane phase carefully evaporated. The tube is weighed and the fat percentage calculated.

Cleanup.

The sample is cleaned up using acid and base, according to The method of BJERK and SUNDBY (1970). The method is briefly as follows. The fat phase in the graduated centrifuge tube is dissolved in hexane, such that the total volume is 1.0 ml. Two samples of 0.4 ml are transferred to a test tube and the hexane phase carefully evaporated.

To one of the test tubes are added 1.5 ml conc. sulphuric acid and 1.0 ml hexane. After mixing, it is stoppered and allowed to stand for one hour at 20 °C. The sample is centrifuged and frozen. Five µl of the hexane layer are injected into the gas chromatograph.

To the other test tube is added 1.5 ml 10% potassium hydroxide in methanol. After mixing, the sample is stoppered and put on a water bath at 40 °C and allowed to stand overnight. Three ml of 2% sodium chloride solution and 1.0 ml hexane are then added to the sample. Five µl of the hexane layer are injected into the gas chromatograph.

RESULTS AND DISCUSSION

Fat in milk is present as globules with an average diameter of 3 to 4 microns. The interior of the globule consists essentially of triglycerides, whilst the outer membrane contains phospholipids, vitamin A and other components. Milk fat is extracted by adding hexane to the milk sample. If the extraction is to be effective, the outer membrane must be burst so that the triglyceride fraction can come into contact with the hexane phase. The membrane is burst by addition of acetone to the milk sample and by homogenization, using an ultrasonic disintegrator.

The reproducibility of the method was tested on two different milk samples. The results, which are given in Table 2, suggest that the same percentage of organochlorine pesticides is extracted from each human milk sample.

TABLE 2

Repeated quantitative determination of HCB and DDE in two different samples of human milk.

Sample	Parallel	Concentration in ppb HCB	DDE
1.	a	10.7	31
	b	9.1	32
	c	9.7	31
	d	9.0	29
	Range	10.7-9.0	32-29
2.	a	4.1	46
	b	3.7	52
	c	4.1	46
	Range	4.1-3.7	52-46

To determine if any residues of organochlorine pesticides remain after the milk had been extracted, the milk was re-extracted. This extract contained about 2% of the total amount of organochlorine pesticides and fat.

In the recovery investigations, organochlorine pesticides were added to the human milk sample and shaken thoroughly for 5 minutes. The concentration of the added standard was of the same order as the residue levels of organochlorine pesticides which have previously been demonstrated in human milk (Table 2). The recovery percentages were determined by subtraction of the peak heights on the chromatograms of samples with and without the pesticide standards. Results are given in Table 3.

The lower limits of detectability for organochlorine pesticides in 10 g human milk using this method are about 0.1 ppb HCB and about 2 ppb DDE, DDD, and DDT.

TABLE 3

Recovery of organochlorine pesticides added to human milk in % of added standards [a].

Analysis	HCB	DDE	DDD	DDT
1	90	158	89	88
2	89	127	85	90
3	90	146	86	90
4	85	100	81	71
5	109	97	88	75
6	109	124	98	84
7	94	95	87	89
8	102	96	114	88
9	118	101	80	117
10	100	92	90	102
Mean	98.6	113.6	89.8	89.4
Standard deviation	10.8	23.7	9.9	12.9

[a]Organochlorine pesticides added to human milk: 5 ppb HCB, 10 ppb DDE, 25 ppb DDD and 40 ppb DDT.

The results show that this method of analysis is reliable for concentrations of organochlorine pesticides in human milk down to the ppb level. The method requires care but is not laborious. It can be carried out using simple glass apparatus and small amounts of chemicals, and should therefore be suitable for long series of analysis.

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