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DETERMINATION OF PENTACHLOROPHENOL AND RELATED COMPOUNDS IN ANIMAL MATERIALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY

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SUMMARY

The rapid determination of pentachlorophenol and tetrachlorophenols in animal materials by high-performance liquid chromatography (HPLC) on porous silica is described. Non-fatty substrates are digested in alkali and the chlorophenols extracted as ion pairs. Fatty materials are extracted with ethyl acetate–hexane after acidification. Extracts are cleaned up on "Sep-Pak" silica or Florisil cartridges. Penta- and tetrachloroanisoles are recovered by the extraction procedures and can be determined by gas chromatography if required.

Mean recoveries of the chlorophenols were 73–108% at fortification levels of 0.1–10 mg/kg. A concentration of 0.1 mg/kg can readily be determined and the method can be adapted to reach about 1 μg/kg. Identities can be confirmed by ion-pair HPLC on a reversed-phase column.

INTRODUCTION

A rapid method of analysis for pentachlorophenol (PCP) in animal tissues, serum and eggs with a limit of determination of 0.1 mg/kg or lower was needed; one which would also allow the determination of tetrachlorophenols (TCPs) was desirable. Low levels of PCP in animal tissues have usually been determined by gas chromatography (GC) with electron-capture detection after conversion to alkyl[1–6] or acetyl[7–9] derivatives. A method which did not rely on derivatisation was sought for speed and convenience and to avoid possible losses. Although free PCP has been determined by GC on polar columns[10,11], high-performance liquid chromatography (HPLC) seemed likely to be more suitable for the analysis of a range of animal materials. HPLC has been used to determine PCP in water[12–15] and timber[16]. Its application to the analysis of fish, shrimps and oysters has also been briefly reported, but only at rather high PCP levels (5–15 mg/kg)[6]. PCP has been determined in formulations by ion-pair HPLC[17].

Published HPLC methods have employed adsorption, reversed-phase or ion-exchange columns. Previous work at this laboratory[18] had shown the advantages of

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exclusion chromatography for separating compounds of relatively high molecular weight from retained co-extractives, and it was found in the course of this work that PCP could also be eluted with methanol from porous glass or silica before co-extractives, although the mechanism of separation was not clear.

In the method to be described, PCP and the 2,3,4,6- and 2,3,5,6-TCPs were determined in animal materials by HPLC on porous silica. Muscle, liver, serum and egg whites were dissolved in alkali and extracted as ion pairs. This procedure was unsuccessful for the analysis of egg yolks and fat: satisfactory results were obtained by extracting the chlorophenols from acid solution with ethyl acetate–hexane (15:85, v/v) before chromatography. Both variants are rapid and the eluates from the column can be used to confirm identity without additional clean-up. Confirmation by ion-pair HPLC on a reversed-phase column is described.

Since penta- and tetrachlorophenols are known to be metabolically converted to the corresponding anisoles which can cause taint in poultry and eggs (see ref. 19, where other references are given), analysis for chloroanisoles and chlorophenols in the same samples may sometimes be required. The possibility of determining chloroanisoles in the chlorophenol extracts was therefore briefly examined. Recoveries, as determined by GC, were somewhat below those of the chlorophenols but would suffice for most practical purposes.

EXPERIMENTAL

Materials and apparatus

Technical pentachlorophenol (Koch-Light, Colnbrook, Great Britain) was recrystallized repeatedly from ethanol–water mixtures containing decolorizing carbon. The three tetrachlorophenols, pentachloroanisole and trichlorophenols used in exploratory work were from Field Instruments (Surbiton, Great Britain) and were used as received. Tetrachloroanisoles were prepared in small quantities by methylation of the tetrachlorophenols, using a modification of the method of Roper and Ma[20]. Methanol was HPLC grade (Fisons, Loughborough, Great Britain). Other solvents were "AnalaR" or of similar quality.

Tetrabutylammonium hydroxide (TBAH) was obtained as a 40% aqueous solution from the Aldrich (Gillingham, Great Britain) and solid tetrabutylammonium phosphate from Magnus (Sandbach, Great Britain).

"Sep-Pak" silica and Florisil cartridges (disposable packed PTFE cartridges through which solvent can be forced with a syringe) were obtained from Waters Assoc. (Northwhich, Great Britain). They were prepared for use by washing successively with 10 ml each of methanol–chloroform (1:9, v/v), chloroform and diethyl ether.

Samples were homogenised in a sealed-unit model mixer-emulsifier (Silverson, Chesham, Great Britain).

The liquid chromatograph was as previously described[21], except that a Tracor Model 970A UV detector was used and samples were introduced through a 20-μl loop by means of a rotary valve (Valco 10-port switching valve; Chrompack, London, Great Britain). A Pye Model 204 gas chromatograph (Pye-Unicam, Cambridge, Great Britain) was used.

Analytical columns were stainless steel, internally polished, 250 × 5 mm I.D.

(Magnus). They were slurry-packed with "Hypersil" porous spherical silica (5-μm spheres with 10-nm pores; Shandon Southern Products, Runcorn, Great Britain) at a pressure of 175 kg/cm^2 (2500 p.s.i.; resolution was found to be impaired if columns were packed at substantially higher pressures). Reversed-phase columns for confirmation of identity, of the same tubing but 100 mm long, were packed with a C_{22} bonded stationary phase (Magnusil 8H C_{22}; Magnus). Analytical columns were tested for satisfactory resolution of PCP, 2,3,5,6-TCP and 2,3,4,6-TCP before use.

Extraction

Serum. The sample (1 ml) was mixed with sodium hydroxide (0.1 *M*, 10 ml). TBAH solution (0.2 ml) was added and the mixture extracted with diethyl ether (2 × 10 ml, 1 × 5 ml). The extract was dried with anhydrous sodium sulphate, filtered, concentrated under a stream of nitrogen to a low volume and diluted to 5.0 ml with ether.

Liver, muscle, egg white. The sample (5 g) was heated under reflux with a mixture of anhydrous sodium sulphate (5 g) and sodium hydroxide (0.1 *M*, 100 ml) for 1 h. Liver and muscle were finely chopped to aid disintegration. After cooling, TBAH (0.8 ml) was added and the mixture extracted with ether (2 × 100 ml, 1 × 50 ml). The extract was dried, filtered and concentrated, initially in a rotary evaporator and finally under nitrogen. The volume was adjusted to 5.0 ml with ether.

Egg yolk, fat. The sample (2 g) was homogenized with hydrochloric acid (5 *M*, 0.5 ml) and the mixture extracted twice with ethyl acetate–hexane (15:85, v/v, 25 ml). The homogenates were separated by centrifugation for 2 min at 900 *g* and the combined organic layers dried with anhydrous sodium sulphate. The extract was taken just to dryness under nitrogen and the residue dissolved in ether (5.0 ml).

Clean-up

All extracts were cleaned up on "Sep-pak" cartridges, Florisil being used for extracts of egg yolk and fat and silica for other substrates. The preparation of the two types of cartridge was identical and the same eluants were used.

An aliquot (1 ml) of the ethereal concentrate was injected onto a prepared "Sep-pak" cartridge, which was eluted with ether (2 × 2 ml). This eluate contained any chloroanisoles originally present in the sample and was retained if these were to be determined. The chlorophenols were eluted with methanol–chloroform (1:9, v/v, 4 ml), the solvent was evaporated with nitrogen at 35°C and the residue dissolved in methanol (0.1–1.0 ml) for HPLC.

Determination

Duplicate aliquots (20 μl) of the methanolic solutions were injected onto the analytical column. The column was eluted with methanol at a flow-rate of 1 ml/min and chlorophenols determined by comparison of the peak heights from samples with those from standard solutions.

If analysis for chloroanisoles was required, ethereal eluates from "Sep-pak" cartridges were taken just to dryness and the residue dissolved in methanol (0.1 ml). Duplicate aliquots (0.5 μl) were injected into the gas chromatograph. The column was XE-60 (3%, w/w on Gas-Chrom Q, 100–120 mesh, 1 m × 2 mm I.D.). The temperatures of the injector, column and detector were 200, 180 and 250°C, respectively. Alternative GC conditions have been described by Gee *et al.*[3].

Determination of low levels

To determine exceptionally low levels of chlorophenols, successive 20-μl aliquots of cleaned-up extracts were injected and the eluate fractions which spanned the PCP and 2,3,4,6-TCP peaks were combined. A methanolic solution of 2,3,5-trichlorophenol (0.025 mg/l, 50 μl) was added as internal standard, the mixture was taken just to dryness and the residue dissolved in 25 μl of methanol. A 20-μl portion was chromatographed on the analytical column.

Confirmation of identity

Eluate fractions from the porous silica column which apparently contained chlorophenols were collected (corresponding fractions from successive injections being combined if necessary) and concentrated to a volume of 1 ml. An aliquot was removed for additional confirmatory tests if required, and the remainder taken to dryness in a stream of nitrogen at 35°C. The residue was dissolved in 0.1 ml of the reversed-phase eluant: methanol–water (75:25, v/v) containing tetrabutylammonium phosphate (0.005 *M*, 1.69 g/l; this ion-pairing reagent was used because TBAH attacked the column packing). Aliquots (20 μl) of this solution were chromatographed on the reversed-phase column at an eluant flow-rate of 1 ml/min.

The identities of chloroanisoles could be confirmed by injecting 20-μl aliquots of the methanolic solutions prepared for GC onto the reversed-phase column and eluting with methanol–water (90:10, v/v, 1 ml/min).

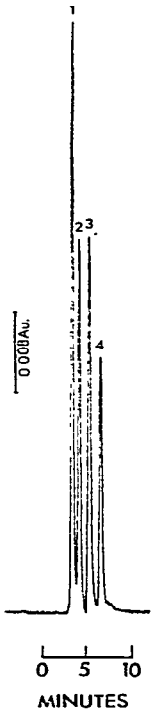
RESULTS AND DISCUSSION

Separation and calibration

Preliminary experiments showed that PCP and the three TCPs could be separated on a 250 mm column of porous silica with methanol as eluant. Trichlorophenols (and pentachloro- and tetrachloroanisoles) were eluted later as a series of incompletely resolved peaks. A typical chromatogram of PCP and the TCPs on a freshly prepared column is shown in Fig. 1. Resolution decreased slowly with use, PCP and 2,3,5,6-TCP being eluted together but resolved from the 2,3,4,6-isomer. Such resolution is still adequate for routine analyses, since in practice 2,3,5,6-TCP normally contributes negligibly to the chlorophenol residue.

Since the chlorophenols were eluted before many larger but less polar molecules, separation was evidently neither by size-exclusion nor by adsorption. Its mechanism was not investigated. Ion-pairs formed by reaction of the sodium chlorophenates with tetrabutylammonium hydroxide or phosphate were dissociated when chromatographed in the same way and gave peaks at retention times identical to those of the free phenols.

When extracts of tissues, fluids and eggs obtained as described above and spiked with the chlorophenols were chromatographed, PCP, 2,3,5,6-TCP and 2,3,4,6-TCP were sufficiently resolved from extracted materials for quantitative measurement. 2,3,4,5-TCP usually appeared as a leading shoulder on the large peak produced by substrate extractives. Fig. 2 shows chromatograms of extracts from chicken liver, with and without added PCP and 2,3,4,6-TCP, obtained from a column which had been in use for some months. Ion-pair chromatography on a C_{22} column also effectively resolved PCP from 2,3,4,6-TCP (but not 2,3,4,6- from 2,3,5,6-TCP). This sepa-



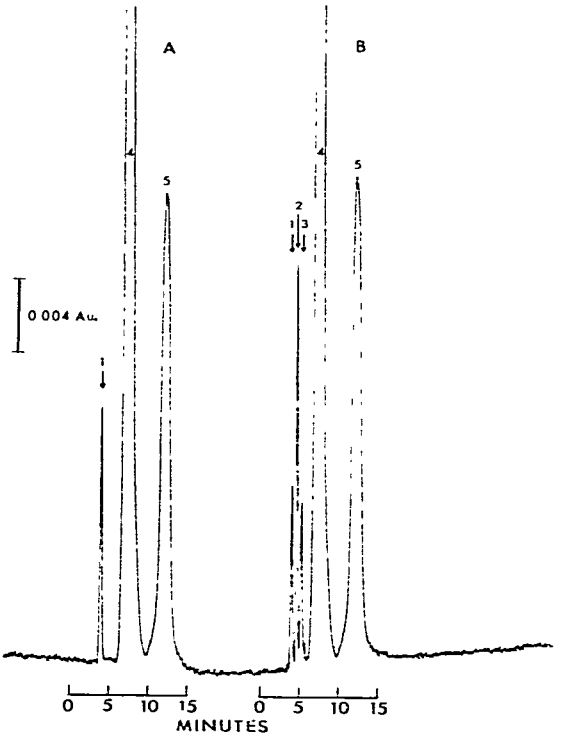


Fig. 1. Separation of chlorophenols by HPLC on porous silica. 1 = Pentachlorophenol; 2 = 2,3,5,6-tetrachlorophenol; 3 = 2,3,4,6-tetrachlorophenol; 4 = 2,3,4,5-tetrachlorophenol.

Fig. 2. HPLC on porous silica of extracts from (A) unfortified liver and (B) liver containing 0.5 mg/kg added pentachlorophenol and 2,3,4,6-tetrachlorophenol. 1, 4, 5 = Co-extractives; 2 = pentachlorophenol; 3 = 2,3,4,6-tetrachlorophenol.

ration was useful for confirmation of identity, but a lower limit of determination could be attained with the porous silica column.

The UV detector at 216 nm responded linearly to injections of 0.1–400 ng of PCP, 2,3,4,6-TCP and 2,3,5,6-TCP. Since the response decreased slowly with ageing of the deuterium lamp, direct comparison of sample and standard peaks was more reliable than reference to a calibration curve.

Extraction and clean-up

In some published methods for determining PCP in animal tissues[2,3,5–10] the compound is extracted or steam-distilled directly from a neutral or acid medium, in others[1,3,4] the tissue is first digested in alkali. Gee *et al.*[3] obtained higher recoveries of chlorophenols added to chicken liver and muscle when alkaline digestion preceded steam distillation. The advantages of dissolution in alkali should be most marked when analysing fibrous tissues.

In exploratory experiments PCP, 2,3,4,6-TCP and 2,3,5,6-TCP were adequately recovered from fortified non-fatty tissues and fluids after alkaline digestion followed by ion-pair extraction. The extracts were sufficiently free from interfering

co-extractives for determination by HPLC and were not significantly improved by pre-extracting the alkaline digest with organic solvents before adding the ion-pair reagent. Further clean-up on a "Sep-pak" silica cartridge before chromatography was advantageous however because it extended the effective life of the HPLC column and removed compound(s) which were eluted from it some 20 min after the main group of co-extractives. The time needed for clean-up was considerably less than that saved at the HPLC stage.

Ion-pair extraction failed with egg yolks and fat owing to interference from co-extractives which were not removed by the silica cartridge. Clean-up on Florisil and C_{18} cartridges was also inadequate. The chlorophenols could be determined in these substrates however after extraction of acidified homogenates with ethyl acetate–hexane mixtures (15% ethyl acetate was needed for efficient extraction) and clean-up on Florisil cartridges. (Extracts of egg yolk, but not fat, could also be effectively cleaned up on silica cartridges. Florisil could not be used for ion-pair extracts because the chlorophenols were retained on the cartridge.)

The acid extraction procedure was later shown to be applicable to liver, although ion-pair extraction was more convenient. Recoveries from serum and egg white were poor however, probably owing to protein binding; PCP is known to be firmly bound by some proteins, including albumins[22]. Bevenue *et al.*[23] could extract PCP from spiked plasma with benzene only by prolonged agitation of the mixture with warm acid (although Hoben *et al.*[4] successfully used milder conditions). Binding would be broken by acid hydrolysis before extraction, as in procedures designed to measure conjugated as well as free PCP[11].

Recoveries

In recovery experiments, solutions (0.1 ml) of the compounds to be analysed were well mixed with individual portions (5 g, 2 g or 1 ml) of the substrates and the solvent allowed to evaporate before digestion or extraction. Four separate samples of each substrate at each fortification level were analysed.

Recoveries of chlorophenols from spiked serum, liver, muscle, egg white and whole egg determined after alkaline digestion, and from spiked egg yolks and fat after solvent extraction of acidified homogenates, are shown in Table I. The results are presented as the mean percentage recovery at each level of each compound in each substrate, with the 95% confidence interval for each combination of compound and substrate. (Statistical analysis had justified the application of a single 95% confidence interval to all fortification levels of each compound–substrate combination.)

Mean recoveries of all the chlorophenols from all substrates except whole eggs were within the range 73–108% and most were above 90%. Mean recoveries from whole eggs lay between 50 and 84%, but only that of PCP at 10 mg/kg exceeded 75%. Poor recoveries were to be expected, since egg whites and yolks require different extraction procedures for their analysis. It appears that yolks and whites should generally be analysed separately, although analyses of whole eggs might be sufficient for some screening purposes.

Recoveries of pentachloroanisole and 2,3,4,6-tetrachloroanisole by GC from serum, liver, fat and whole eggs are shown in Table II, expressed in the same way as those in Table I. Recoveries, except from whole eggs, were generally lower than those of the phenols, with most mean values lying between 70 and 80%. Since in practice

TABLE I

DETERMINATION OF CHLOROPHENOLS BY HPLC: RECOVERIES FROM SPIKED SUBSTRATES

Column, 250 × 5 mm I.D., "Hypersil" porous silica (5 μm); mobile phase, methanol; flow-rate, 1 ml/min. Extraction and clean-up as described in text. Four separate samples at each level analysed. Spiked before digestion or homogenization.

Substrate and fortification level (mg/kg)	*Percentage recovery, mean ± 95% confidence interval, of*		
	Pentachlorophenol	*2,3,4,6-Tetrachlorophenol*	*2,3,5,6-Tetrachlorophenol*
Serum*			
10	97 ± 5.5	82 ± 6.2	
5	102 ± 5.5	79 ± 6.2	
1	104 ± 5.5	91 ± 6.2	90 ± 9.5
0.5	108 ± 5.5	97 ± 6.2	
0.1	83 ± 5.5	94 ± 6.2	93 ± 9.5
Liver*			
10	90 ± 6.3	94 ± 9.9	
5	88 ± 6.3	84 ± 9.9	
1	81 ± 6.3	73 ± 9.9	93 ± 7.7
0.5	86 ± 6.3	73 ± 9.9	
0.1	93 ± 6.3	92 ± 9.9	89 ± 7.7
Muscle*			
10	97 ± 3.0	95 ± 3.8	
1	85 ± 3.0	82 ± 3.8	
0.1	93 ± 3.0	86 ± 3.8	
Egg white*			
10	97 ± 6.8	97 ± 5.6	
1	94 ± 6.8	96 ± 5.6	
0.1	87 ± 6.8	86 ± 5.6	
Whole egg*			
10	84 ± 10.5	70 ± 5.1	
1	75 ± 10.5	56 ± 5.1	
0.1	62 ± 10.5	50 ± 5.1	
Fat**			
10	97 ± 4.3	96 ± 2.2	
5	105 ± 4.3	90 ± 2.2	
1	101 ± 4.3	86 ± 2.2	102 ± 4.6
0.5	102 ± 4.3	92 ± 2.2	
0.1	88 ± 4.3	92 ± 2.2	92 ± 4.6
Egg yolk**			
10	100 ± 3.7	102 ± 5.7	
1	94 ± 3.7	86 ± 5.7	98 ± 6.9
0.1	78 ± 3.7	96 ± 5.7	94 ± 6.9

* Samples digested in alkali. Chlorophenols extracted as ion pairs with tetrabutylammonium hydroxide.

** Samples homogenized with acid. Chlorophenols extracted with ethyl acetate–hexane (15:85, v/v).

TABLE II

DETERMINATION OF CHLOROANISOLES BY GC: RECOVERIES FROM SPIKED SUBSTRATES

Column, 1 m × 2 mm I.D., 3%, w/w XE-60 on Gas-Chrom Q, 100–120 mesh; column temperature 180°C. Extraction and clean-up as described in text. Four separate samples at each level analysed. Spiked before digestion or homogenization.

Substrate and fortification level (mg/kg)	*Percentage recovery, mean ± 95% confidence interval, of*	
	Pentachloroanisole	*2,3,4,6-Tetrachloroanisole*
Serum*		
10	78 ± 2.3	77 ± 8.9
1	73 ± 2.3	73 ± 8.9
0.1	79 ± 2.3	80 ± 8.9
Liver*		
10	75 ± 3.8	72 ± 5.0
1	77 ± 3.8	78 ± 5.0
0.1	70 ± 3.8	70 ± 5.0
Whole egg*		
10	81 ± 2.6	71 ± 10.8
1	78 ± 2.6	73 ± 10.8
0.1	58 ± 2.6	76 ± 10.8
Fat**		
10	119 ± 2.3	91 ± 10.1
1	82 ± 2.3	84 ± 10.1
0.1	60 ± 2.3	69 ± 10.1

* Chloroanisoles extracted from alkaline digest

** Chloroanisoles extracted from acid homogenate.

TABLE III

RECOVERIES OF LOW LEVELS OF CHLOROPHENOLS AFTER CONCENTRATION AND RE-INJECTION OF COLLECTED ELUATES

Chromatographic conditions as in Table I. Substrates spiked with 5 μg/kg of chlorophenols before extraction. Chlorophenol-containing eluate fractions from 10 injections combined, concentrated and re-injected under same conditions.

Substrate	*Percentage recoveries, individual values with means (M), of chlorophenols added to substrate at 5 μg/kg level*	
	Pentachlorophenol	*2,3,4,6-Tetrachlorophenol*
Serum	78, 84, 78, 81 (M = 80)	85, 85, 90, 90 (M = 87.5)
Liver	65, 69, 78, 67 (M = 70)	75, 80, 99, 80 (M = 83.5)
Egg yolk	72, 77, 71, 78 (M = 74.5)	76, 86, 77, 85 (M = 81)

the determination of chloroanisoles is likely to be ancillary to that of chlorophenols, such recoveries are probably adequate.

Chlorophenol contents of 0.1 mg/kg were readily determined in routine analyses with a detector in good condition, but determinations at much lower concentrations were possible if appropriate eluate fractions from several runs were combined, concentrated and re-injected. Table III shows recoveries from serum, liver and egg yolk fortified with PCP and 2,3,4,6-TCP at levels of 5 μg/kg: the chlorophenols eluted from ten 20-μl aliquots of each cleaned-up extract were re-chromatographed. Mean recoveries ranged from 70 to 88 % (the lowest individual recovery being 65 %) and can be regarded as satisfactory in view of the low levels of fortification. A chromatogram of the re-injected chlorophenol fraction from spiked liver is shown in Fig. 3: the chlorophenols are well separated from remaining co-extractives and it appears that levels below 1 μg/kg could be determined.

Confirmation of identity

Identities can conveniently be confirmed by reversed-phase HPLC on the C_{22} column. Solutions of both chlorophenols and chloroanisoles eluted from "Sep-Pak" cartridges were usually sufficiently clean for chromatography, but it was generally more convenient to chromatograph the chlorophenols after elution from the analytical column.

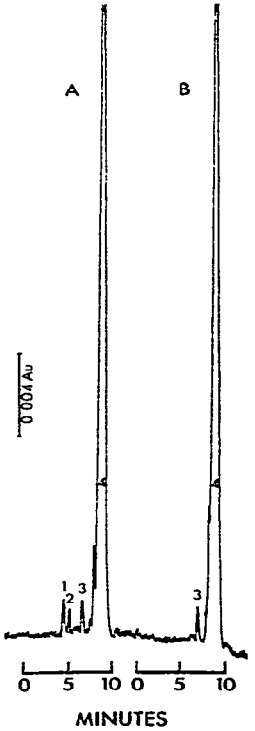


Fig. 3. Determination of chlorophenols in liver at 5 μg/kg level: combined eluate fractions from ten chromatographed aliquots of extract concentrated and re-injected. A, Liver spiked with 5 μg/kg of pentachlorophenol and 2,3,4,6-tetrachlorophenol. B, Unfortified liver. 1 = Pentachlorophenol; 2 = 2,3,4,6-tetrachlorophenol; 3 = 2,3,5-trichlorophenol (internal standard); 4 = co-extractives.

On the reversed-phase column, the tetrabutylammonium ion pair of PCP was well separated from that of 2,3,4,6-TCP but the latter was not resolved from 2,3,5,6-TCP. Since any coincidence on the porous silica column would be between 2,3,5,6-TCP and PCP, the reversed-phase separation would confirm identity. Pentachloroanisole, 2,3,4,5-tetrachloroanisole and 2,3,4,6-tetrachloroanisole were easily resolved, but the 2,3,4,6- and 2,3,5,6-isomers were eluted together.

CONCLUSIONS

It is concluded that PCP, 2,3,4,6-TCP and 2,3,5,6-TCP can be determined satisfactorily at residue levels in animal tissues and fluids, egg yolks and egg whites (but not whole eggs) by HPLC on porous silica with methanol as eluant. The corresponding chloroanisoles can be determined in the same sample by GC. Identities of both chlorophenols (as ion pairs) and chloroanisoles can be confirmed by reversed-phase HPLC. The limit of determination can be lowered some 100-fold at the cost of increased analysis time.

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