Determination of Heptachlor Epoxide in Fat and Milk

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Earlier work by Davidow and others has shown that the insecticide heptachlor is altered, when ingested by dairy animals, to its epoxide (1,4,5,6,7,8,8-heptachloro-2,3-epoxy-2,3,-3a,4,7,7a-hexahydro-4,7-methanoindene). As heptachlor epoxide is found primarily in the fat, methods were developed for the determination of microgram quantities in butterfat and beef fat. The Polen-Silverman reagent which was originally developed for the determination of heptachlor is also a sensitive color-forming reagent for heptachlor epoxide. Methods are presented for the separation of heptachlor epoxide from butterfat and beef fat by chemical and chromatographic techniques and for the subsequent spectrophotometric analysis of the epoxide.

THE INSECTICIDE, heptachlor, which is used extensively for the control of certain forage insects, is metabolized by animals to an expoxide (*1, 7*). This metabolite has been identified as 1,4,5,6,7,8,8 - heptachloro - 2,3, - epoxy-2,3,3*a*,4,7,7*a* - hexahydro - 4,7 - methanoindene, more commonly known as heptachlor epoxide (Figure 1). Dairy animals fed heptachlor metabolize it to the epoxide which is stored in the fatty tissues of these animals and is excreted in their milk (*2, 3*). Therefore, to evaluate properly the safe level at which heptachlor can be applied to forage crops, sensitive and accurate methods were developed for the determination of microgram quantities of heptachlor and heptachlor epoxide in animal fat and milk. The basis for these methods is the procedure described for the analysis of heptachlor epoxide on alfalfa (*9*).

Reagents

PENTANE, colorimetric grade (Phillips Petroleum Co.). Check the residue from 2 liters of pentane evaporated from a 50° C. bath not to develop more absorbance at 410 mμ, when treated with Polen-Silverman reagent, than 2.5 γ of heptachlor epoxide.

DIETHYL ETHER, colorimetric grade (Eastman Kodak Co.). Same specification as for pentane.

FLOREX XXS, 60/90-mesh (Floridin Co.) activated and standardized for chromatography. Determine optimum activation experimentally for each commercial batch. Agitate the Florex for 15 minutes with excess pentane; allow it to settle. Decant the excess pentane and remove the residual solvent by application of suction from a water aspirator. Perform a trial activation on pre-treated Florex by heating it in an oven for 24 hours at 180° C. Check the activation by performing a complete analysis for heptachlor epoxide, measuring the recovery and interference upon fat fortified with known amounts of reference heptachlor epoxide. High interference means that the temperature of activation is too low. Low recovery means that the activation temperature is too high. Repeat the pretreatment and activation of portions of a particular batch of Florex until maximum recovery and minimum interference occur upon analysis of a synthetic sample of heptachlor epoxide in fat.

HEPTACHLOR EPOXIDE reference standard, melting point 160–161.5° C., minimum assay 99% (Velsicol Chemical Corp.)

POLEN-SILVERMAN REAGENT. Dissolve 33 grams of 85% potassium hydroxide (reagent grade) in 28 grams of water. Add an equal volume of butyl Cellulose (distilled from potassium hydroxide pellets) and 30.5 grams of ethanolamine (freshly distilled). Dilute to 1 liter with butyl Cellosolve. The reagent is stable if stored in tightly stoppered bottles.

MODIFIED POLEN-SILVERMAN REAGENT. Dilution (1 to 1) of Polen-Silverman reagent with benzene (reagent grade) renders it stable for about 1 month. Fresh reagent should be prepared frequently and calibration curves should be adjusted if required.

BENZENE-ISOPROPYL ALCOHOL solution, 4 to 1 by volume.

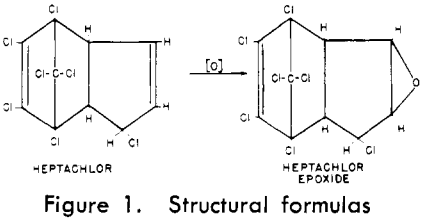

Figure 1. Structural formulas

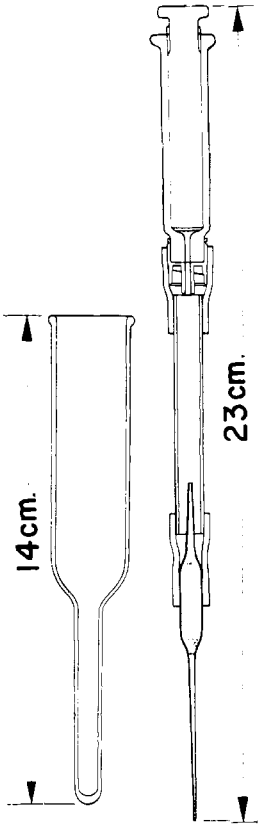

Figure 2. Special micro-equipment

Left. Reaction tube
Right. Filter-pipet assembly

Special Apparatus

A more complete description of the apparatus listed is given by Ordas, Smith, and Meyer (*5*) in their method for the determination of heptachlor and chlordan residues.

Cream Separator. DeLaval No. 9.

Microreaction Tubes, microtransfer pipet, and syringe (Figure 2). Draw out a piece of 6-mm. glass tubing to a capillary with a 1-mm. bore. The pipet has a length of 10 cm. with a volume of approximately 1 ml. Attach the pipet to a 1-ml. hypodermic syringe with a piece of Tygon tubing.

Microcuvettes, quartz microcells, 1.5 × 10 × 25 mm., 0.375 ml. (Pyrocell Manufacturing Co., New York).

Treatment of Samples

Milk (Separation of Butterfat). Remove most of the aqueous phase from the milk sample with the cream separator. Mix the cream with anhydrous sodium sulfate (reagent grade), and grind thoroughly in the food grinder. Enough anhydrous sodium sulfate should be used so that the mixture will be friable. Store the cream-sulfate mixture in polyethylene bags for at least 24 hours at 18° F. or lower.

Extract the mass with pentane (colorimetric grade) for 4 hours in a Soxhlet extractor fitted with a glass wool plug. Strip off the pentane from the extract on a steam bath. Avoid excessive heating to prevent loss of heptachlor epoxide. Use a stream of dry air or nitrogen to remove the last traces of solvent.

Animal Fat. Slice samples of animal fat into small pieces and mix with anhydrous sodium sulfate. Grind the mixture with a food grinder. Transfer the mass to a polyethylene bag, and store at 18° F. or lower for at least 24 hours. Use the same procedure for the extraction of animal fat samples as described for butterfat.

Removal of Interfering Substances

Chemical Treatment. If samples are to be analyzed for heptachlor epoxide only, an alkaline saponification removes a major portion of the fat. When analyses for both heptachlor and heptachlor epoxide are required, the alkaline treatment will destroy much of the heptachlor, if present. An acid treatment which gives high recovery of heptachlor is used in this case.

Saponification (Heptachlor Epoxide Only). Melt the fat sample on a steam bath and weigh up to 20 grams (10 grams are preferred) of the melted fat into a 250-ml. Erlenmeyer flask. Add to the flask 15 ml. of 95% ethyl alcohol and 10 ml. of 50% aqueous potassium hydroxide, and heat at reflux for 5 minutes. Wash down the condenser with 15 ml. of distilled water. Cool the solution to room temperature and transfer to a 500-ml. separatory funnel. Wash the flask with three 5-ml. portions of pentane, and transfer the washings to the separatory funnel.

Add 200 ml. of pentane to the funnel. Shake the funnel thoroughly for 1 minute. Allow the layers to separate, and remove the alcohol (lower) layer. Save the alcohol layer, as it will be re-extracted. Wash the pentane extract with 100 ml. of distilled water acidified with 1 to 2 ml. of 1*N* hydrochloric acid. The acid is used to prevent the formation of emulsions. Discard the water layer and rewash the pentane with another 100 ml. of water. Discard the second water wash and transfer the pentane extract to a 500-ml. Erlenmeyer flask.

Return the alcohol layer to the separatory funnel and extract with 100 ml. of pentane. Discard the alcohol and wash the pentane layer with water as previously described.

Combine the pentane extracts in a 500-ml. Erlenmeyer flask and dry with anhydrous sodium sulfate. Filter the dried pentane extract through coarse filter paper or a glass wool plug into a 500-ml. Erlenmeyer flask fitted with a ground-glass joint. Attach a distillation column to the flask, and concentrate the extract on a 50° C. water bath to about 10 ml.

Acid Treatment (Both Heptachlor and Heptachlor Epoxide). If fat samples are to be analyzed for both heptachlor and heptachlor epoxide, the alkaline treatment described above may not be used as it will result in a substantial loss of heptachlor. Substitute the following treatment with strong sulfuric acid:

Melt the fat on a steam bath and weigh up to 20 grams (10 grams are preferred) into a 500-ml. separatory funnel containing 350 ml. of pentane. Add to the solution 35 ml. of a 1 to 1 mixture of 15% fuming sulfuric acid and concentrated sulfuric acid (reagent grade). Shake the funnel for about 1 minute, and allow 10 minutes for the layers to separate. Discard the acid (lower) layer.

Wash the pentane layer twice with 100-ml. portions of distilled water and discard the water layers. Dry the pentane solution with anhydrous sodium sulfate. Filter the pentane extract into an Erlenmeyer flask, wash the sulfate, and filter with additional small portions of pentane to complete the transfer. Concentrate the combined extracts to 10 ml. by heating on a 50° C. water bath, and distilling the excess pentane through a column.

Chromatography. Prepare a chromatographic column by inserting a small plug of glass wool into a 50-ml. Mohr buret. Pour 10 grams of Florex (activated and standardized) into the column with gentle tamping to pack the adsorbent properly. Wet the column with pentane. Carefully wash the concentrated extract from the alkaline treatment or acid treatment into the chromatographic column with a few milliliters of pentane. Elute the column with 200 ml. of pentane. Discard the first 50 ml. of elutriate. Collect the next 150 ml. which contain heptachlor, if any. Heptachlor may be determined in this fraction by the method of Ordas, Smith, and Meyer (*5*).

Now elute the column with 200 ml. of 2% ethyl ether (colorimetric specifications) in pentane. Be careful not to allow the column to dry between additions of eluent. Collect the ether-pentane elutriate in a 500-ml. Erlenmeyer flask fitted with a ground-glass joint. Attach a distilling column to the flask and concentrate the contents to about 15 ml. on a 50° C. water bath.

Transfer the concentrate to a microreaction tube (Figure 2) and evaporate to dryness on a 40° C. water bath.

Spectrophotometric Determination of Heptachlor Epoxide

Add 0.5 ml. of the modified Polen-Silverman reagent to the contents of the microreaction tube and heat in a 100° C. bath for exactly 15 minutes. Remove the reaction tube from the bath, and cool immediately to room temperature by immersing the tube in a beaker of cold water. Dilute the colored reaction product formed to 0.5 ml. with the benzene–isopropyl alcohol solution, and transfer the solution to a microcuvette by use of the special microtransfer pipet (Figure 2). Determine the absorbance of the solution at 410 mμ (Beckman Model DK-1 spectrophotometer or equivalent), using the benzene–isopropyl alcohol solution as reference. The heptachlor epoxide concentration is determined from a calibration curve derived from measurements made upon fat containing known amounts of heptachlor epoxide.

Detection of Interference

The shape of the heptachlor epoxide absorption curves is used to establish the presence of interference. The following criterion has been established to determine if a valid measurement for heptachlor epoxide has been obtained.

For absorbance readings greater than those obtained for uncontaminated fat (about 0.3 absorbance), the absorption curve must have a peak at 410 mμ to be a true measure of heptachlor epoxide. If a recording spectrophotometer is not available, the presence of a peak is determined by taking absorbance readings at 390, 410, and 430 mμ. The absorbance reading at 410 mμ must be greater than the average of the readings at 390 and 430 mμ. If no peak is found,

either no heptachlor epoxide is present or else interference is predominant and no valid quantitative estimation of heptachlor epoxide can be made on the solution; the sample must be reanalyzed.

Calibration

Fat with Zero Heptachlor Epoxide (Blanks). Analyze by the complete procedure (treatment of samples, removal of interfering substances, and spectrophotometric determination) at least five samples of uncontaminated fat from different sources. Estimate the mean absorbance (410 mμ) and the 95% confidence absorbance range for the blank samples (zero heptachlor epoxide) using standard statistical methods for analysis of variance (*8*, *10*).

Fat Containing Heptachlor Epoxide. Accurately prepare a standard solution of approximately 5 γ of heptachlor epoxide per milliliter of colorimetric grade pentane. Pipet aliquots of from 1 to 6 ml. of the standard solution into separate Erlenmeyer flasks. Add to each a constant weight (10 grams are suggested) of control sample fat and analyze by the complete procedure from removal of interfering substances up to and including spectrophotometric determination.

Working Curve. Construct a smooth curve through plotted points of micrograms of heptachlor epoxide added *vs.* absorbance of the reaction product at 410 mμ. The curve is not necessarily a straight line over its entire length nor does it pass through the origin. It may be used to determine heptachlor epoxide at absorbance values higher than the upper 95% confidence limit determined for the blanks.

Extended Range. For determination of heptachlor epoxide in higher concentrations, the range may be extended. When absorbance of the color complex exceeds about 1.0, one may use smaller samples or greater dilution in the final solution upon which absorbance is read. Whichever course is chosen, the technique used for calibration and for measurement of unknowns must be the same. The conditions suggested give an absorbance value of roughly 1.0 at a heptachlor epoxide concentration of 3 to 3.5 p.p.m. in fat.

Minimum Range. Where an inadequate number of fat blanks are available to establish good statistical information on measurements at or near zero heptachlor epoxide content, a base-line technique and calibration is suggested. The principle described under detection of interference may be utilized.

Discussion

Reagent Sensitivity. The Polen-Silverman reagent, although developed for the colorimetric determination of heptachlor (*6*) (maximum absorption peak at 560 mμ), was also found to be a sensitive reagent for heptachlor epoxide. When reacted with Polen-Silverman reagent, heptachlor epoxide produces a bright yellow product with maximum absorption at 410 mμ. The reaction product of heptachlor epoxide and the Davidow reagent has the same qualitative absorption curve, but, as can be seen from Figure 3, the sensitivity of the Polen-Silverman reagent is about 2.5 times that of the Davidow reagent. Because of this greater sensitivity, and also because heptachlor residue procedures are standardized with Polen-Silverman reagent, this reagent was used for the determination of heptachlor epoxide.

Background Absorption. Interferences. The present method is complicated by rather high blanks—i.e., development of absorption at the wave length of the analysis even in the absence of heptachlor epoxide. The apparent measurement of heptachlor epoxide in blanks varies materially between the fat of different animals and also between specimens taken from the same animal at different times. This effect is too large to disregard or to treat by the conventional method of averaging the values determined on a small number of reference uncontaminated controls and then subtracting the mean value from all subsequent determinations. To illustrate the magnitude of variation in the blank absorption, statistical data from typical studies are cited in Table I. From these data, the least significant differences (*8*) at the 95% confidence level may be estimated. They are ca. 0.33 absorbance unit for butterfat and ca. 0.16 unit for beef fat. These statistical parameters must be applied to measurements intended to discriminate between the presence or absence of heptachlor epoxide in animal fat or in milk.

The response of the reagent to materials other than heptachlor epoxide may give rise to systematic errors for which statistical treatment of data will not correct. This situation may arise when changing batches of solvent or chromatographic sorbant, or as a result of changes in procedures. Two well-known brands of diethyl ether, each marked Reagent Grade, gave absorbance values (at 410 mμ) of 0.1 and 0.5 when treated directly with the reagent under identical conditions. Experimental verification of solvent and reagent conformance to specifications must be regarded as essential to the valid application of the present method.

Recovery. The interaction of a relatively large background absorption and varying recovery efficiencies through the range of measurements is illustrated in Figure 4. The comparison is made between absorbance values derived from reagent reacted directly with heptachlor epoxide (in pentane) and the values obtained upon complete analyses of fat samples to which known amounts of heptachlor epoxide have been added. The experimental curve for fats is neither linear over its range nor does it pass through zero absorbance for pure fat. The systematic discrepancy between the theoretical and experimental curves is eliminated by the calibration described in the present method. The data taken from this type of calibration may, therefore, be considered to be corrected to 100% recovery and corrected for background absorption (reagent blank).

Influence of Heptachlor. If hepta-

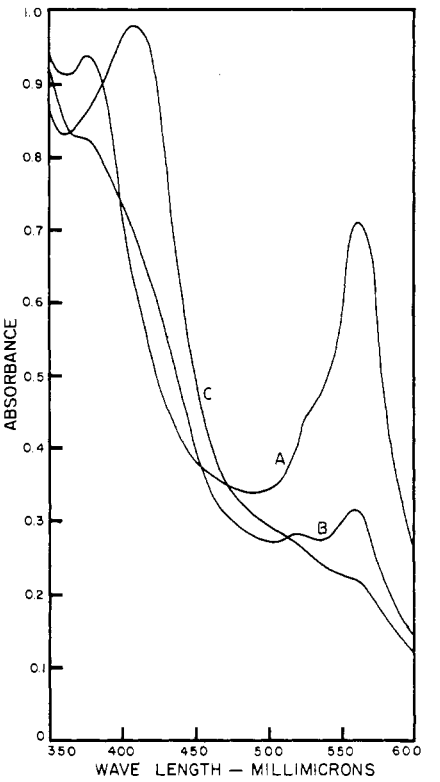

Figure 3. Absorption curves of color complexes (each with 20 γ of reactant)

A. 20 γ of heptachlor and 5 γ of heptachlor epoxide
B. 10 γ of heptachlor and 10 γ of heptachlor epoxide
C. 5 γ of heptachlor and 20 γ of heptachlor epoxide

Table I. Analysis of Variance

Background absorbance of fat plus reagent (zero heptachlor epoxide)

Basis: 10 grams fat—analysis by total method

	Butterfat	Beef Fat
	No. of Samples	
Absorbance	12	32
Mean value	0.239	0.171
Std. dev., single determination	0.107	0.074
Upper limit for zero heptachlor epoxide (95% confidence level)	0.475	0.321
Least significant difference (95% level)	0.333	0.155

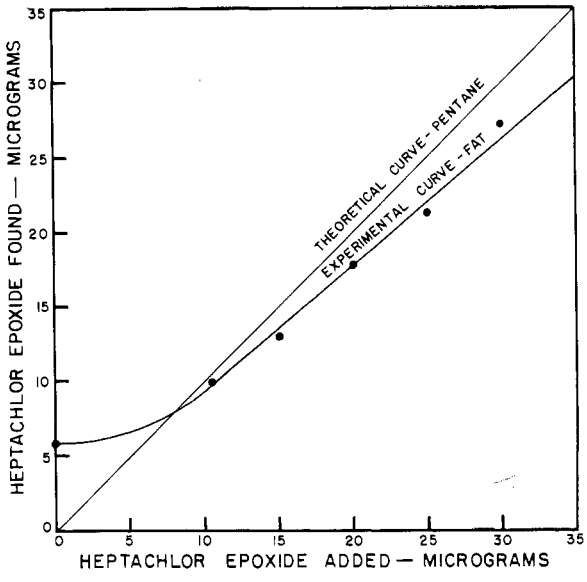

Figure 4. Recovery curve, uncorrected data

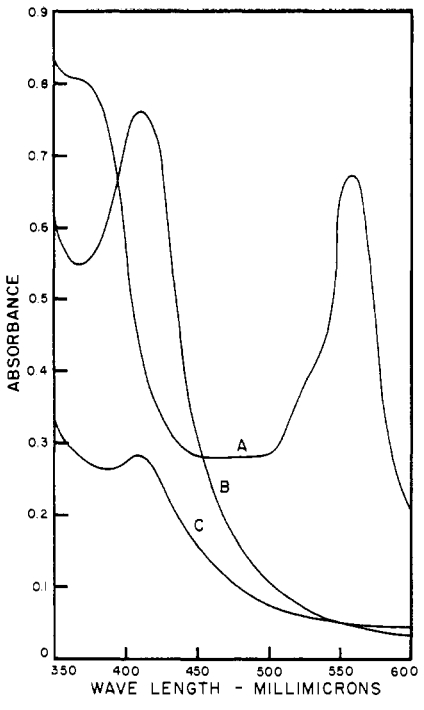

Figure 5. Absorption curves of heptachlor and heptachlor epoxide mixtures

A. Heptachlor (Polen-Silverman reagent)
B. Heptachlor epoxide (Polen-Silverman reagent)
C. Heptachlor epoxide (Davidow reagent)

chlor is present in samples to be analyzed for heptachlor epoxide, an enhancing effect will be exerted. This is illustrated in Figure 5. It is possible to analyze these mixtures in a single color development by applying calculations to measurements at the two characteristic peak wave lengths. A procedure involving chromatographic separation and separate color development is more reproducible and, hence, is preferred.

If a large amount of heptachlor is present in the sample, part of it may appear in the epoxide fraction. Figure 5 shows that the influence of heptachlor upon the heptachlor epoxide determination is significant, and erroneous results will be obtained if the proper corrections are not applied. A standard two-wave-length method for treating a two-component system must be used to make this correction (*4*).

As a general rule, heptachlor is absent under normal circumstances. When this fact has been indicated for a particular set of determinations, it will usually be more convenient to use saponification in preference to acid treatment in the cleanup. Slightly lower absorbance values result from acid treatment. By using acid treatment in the calibration, however, the appropriate correction to 100% recovery will yield the final analytical results. All things considered, the saponification step appears more convenient where a choice is available.

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INSECTICIDE RESIDUES

Procedure for Cleanup of Plant Extracts Prior to Analyses for DDT and Related Pesticides

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Pigments and waxes are extracted with pesticide residues from plant material, and interfere in quantitative and qualitative methods of analysis. Therefore, a cleanup procedure is needed, which will efficiently remove pigments and waxes from the organic solvent without removing or destroying the pesticides. Recently (*8*) stress has been placed on more efficient techniques for extracting pesticide residues from fruits and vegetables. These more vigorous extraction methods require more efficient cleanup procedures, because more pigments and waxes, as well as pesticides, are present in the extracts. Many cleanup procedures described in the literature are applicable in respect to one type of crop or one specific pesticide. A cleanup procedure was sought which would remove essentially all