Detection of O-(3-Chloro-4-nitrophenyl) O,O-Dimethyl Phosphorothioate and Analysis Of Residues in Milk

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With minor modifications, the analytical method of Averell and Norris for parathion is applicable to the determination of O-(3-chloro-4-nitrophenyl) O,O-dimethyl phosphorothioate (chlorthion). The concentration-absorbance plot for pure chlorthion conforms to Beer's law in the region 20 to 300 γ of chlorthion in 100 ml.; the absorption maximum is at 545 mμ. The possibility of existing residues in milk when chlorthion is used as residual fly spray in dairy barns was investigated. Details of the analysis of milk fortified with chlorthion are given. Concentrations greater than 0.1 p.p.m. in 500 ml. of milk can be determined spectrophotometrically. Concentrations from 0.02 to 0.1 p.p.m. can be estimated by visual comparison using Nessler tubes. No residual chlorthion was found in milk from cows occupying treated barns when the visual comparative method sensitive to 0.02 p.p.m. was used.

THE ESTABLISHMENT of *O*-(3-chloro-4-nitrophenyl) *O,O*-dimethyl phosphorothioate (chlorthion, the common name adopted by Farbenfabrik Bayer) as a promising insecticide and its development as a substitute for some of the more toxic phosphates indicated the need for an analytical method for determining this material. The basic method of Averell and Norris (*1*) for the determination of parathion has been modified in some minor points for the preparation of the standard curve with pure chlorthion. Other modifications were necessary to apply the basic method to the analysis of milk.

Modifications of Method

Preparation of Standard Curve Aliquots of an ethanolic solution containing 30, 60, 120, 180, 240, and 300 γ of chlorthion (purified sample furnished by Farbenfabrik Bayer) are pipetted into 100-ml. round-bottomed standard-taper flasks. Ten milliliters of 99% isopropyl alcohol (*6*), 10 ml. of water, 1 ml. of 85% phosphoric acid, and 0.5 gram of powdered zinc are added; the chlorthion is reduced to the amine by gentle boiling on a steam bath for 30 minutes. The solutions are filtered through coarse, fritted funnels into 100-ml. volumetric flasks, and the reaction flask is rinsed with several portions of 0.1*N* hydrochloric acid, which are also passed through the filter until the volume of filtrate is about 60 ml. Color is produced by the method of Averell and Norris (*1*) for parathion—1 ml. of 0.25% sodium nitrite solution is added (10-minute wait), followed by 1 ml. of 2.5% ammonium sulfamate solution (10-minute wait), and 2 ml. of 1% *N*-1-naphthylethylenediamine dihydrochloride. After 10 minutes, 6 ml. of concentrated hydrochloric acid are added, and the colored solution is diluted to 100 ml. with water. The transmittance can be determined immediately; the color is stable for at least 4 hours.

Discussion of Modifications The modifications for chlorthion consist of a longer reduction period of the nitro moiety to the amine and of the presence of somewhat greater acidity in the final colored solution. A lesser acidity than that recommended here results in lower absorptivity and a shift of the absorption maximum toward shorter wave lengths. The absorption maximum does not deviate from 545 mμ when still greater amounts of acid are used. Absorption is also decreased if higher proportions of ethyl alcohol are used in diluting to volume after color formation; this is probably due to decreased acid strength in the organic solvent and the resultant effect on the acid-base tautomerism of the color body. Consequently, water is recommended as diluent if this does not result in turbidity due to organic matter from the substrate under investigation. If final ethyl alcohol concentrations greater than 50% by volume are necessary to obtain clarity, more concentrated hydrochloric acid than the 6 ml. recommended here may be necessary to obtain maximum absorption for a given chlorthion content. This emphasizes the necessity for an evaluation of the basic method when applied to a particular substrate.

The acid must be added after the formation of the color body, since coupling is extremely slow in highly acid solutions. Furthermore, the alcohol content of the solution during diazotization and coupling must not exceed 30% by volume, as auxiliary reactions may produce interfering colors even in reagent blank solutions.

The spectral characteristics of the color formed from chlorthion as obtained with a Beckman DU spectrophotometer are shown in Figure 1. The absorption maximum is at 545 mμ and the absorptivity index, A_s, at 545 mμ is 33,000. In Figure 2 is shown the standard curve obtained by developing color with pure chlorthion. The absorbance-concentration plot conforms to Beer's law in the region 20 to 300 γ of chlorthion per 100 ml. The reproducibility of the standard curve is shown by the conformance to the line of points obtained when a standard curve is produced at two different times.

By the same procedure as for chlorthion, standard curves for its isomer, *O*-(2-chloro-4-nitrophenyl) *O,O*-dimethyl

phosphorothioate, and for *O,O*-dimethyl *O* - *p* - nitrophenyl phosphorothioate (methyl parathion) were prepared without difficulty. A distinct difference exists between the wave length of maximum absorption of the chlorthion color (545 mμ) and of the latter two (560 mμ) as obtained from a Beckman DU spectrophotometer. And because the parathion color has maximum absorption at 555 mμ (*1*), chlorthion should be qualitatively differentiable from most *p*-nitrophenyl phosphorothioates unsubstituted in the position adjacent to the nitro group.

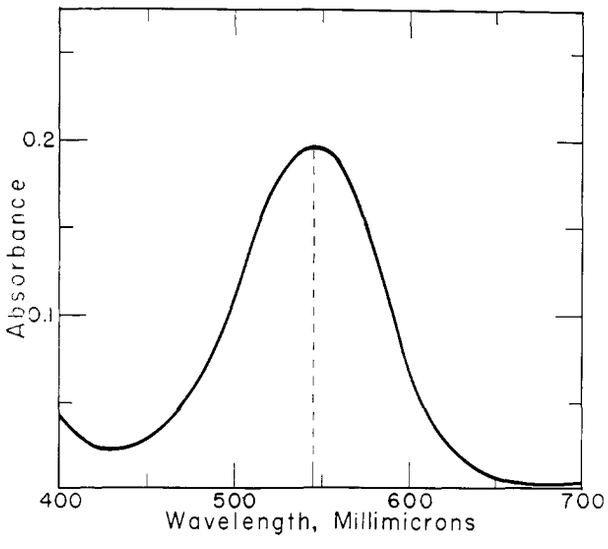
Analysis of Milk

As laboratory and field studies have shown chlorthion to be a promising fly-control insecticide (*3*), which will soon be commercially available for such purposes, it was necessary to investigate the possibility of existing residues in milk when this material was used for fly control in dairy barns. As a result, barns of three different dairies were treated with chlorthion, and milk samples from cows occupying these barns were collected and analyzed for residual chlorthion. Control samples were obtained before spraying, while samples for chlorthion analyses were collected at intervals of 24 and 72 hours after spraying. The milk was processed immediately, essentially by the method of Dahm *et al.* (*2*) (Method I), and the chlorthion determined in triplicate.

Subsequently, a method for extraction was developed that required blending in a Waring Blendor for only 1 minute (Method II) as contrasted with 6 hours' extraction in the case of Method I. Recoveries of chlorthion by both methods were the same.

Figure 1. Spectral characteristics of color produced from chlorthion

Beckman DR recording attachment for Beckman DU spectrophotometer, 1-cm. cells, 168 γ of chlorthion in 100 ml.



Treatment of Dairy Barns Treatment of the dairy barns was carried out in a standard commercial manner, using a high-pressure (600 pounds per square inch) spray rig and orchard-type guns with No. 5 spray disks. All accessible interior surfaces on which flies were likely to rest were sprayed, until the runoff point was reached, with a water spray containing 32 pounds of the 25% chlorthion wettable powder and 100 pounds of powdered sugar per 100 gallons. Approximately 25 gallons were used for each barn; a typical barn is 35 × 60 feet and has room for 28 cows. Milk rooms were not treated, and the animals were not in the barn at the time of treatment. The only precautions taken involved the removal or the covering of the feed cart and milk-handling equipment and a thorough water rinse of the mangers after spraying.

Extraction of Chlorthion from Milk **Method I** (*2*). One half of a 500-ml. milk sample is stirred in a Waring Blendor with 2.5 grams of reagent grade sodium chloride, then 250 ml. of 95% ethyl alcohol are added and stirring is continued for 1 minute. The mixture containing very finely divided milk solids is transferred to a specially designed liquid-liquid extractor (*3*) whose distributor tube, fitted with paddles, is revolved at 75 r.p.m. to prevent settling of the solids. The remaining half of the sample is treated similarly; the mixture is then extracted with 350 ml. of hexane for 6 hours at a reflux rate estimated at 1 liter per hour. If, after cooling, the extract solution contains some solids that had passed over during extraction, it is filtered through a short (1-inch) column of anhydrous sodium sulfate directly into a Kuderna-Danish evaporative concentrator (developed at the laboratories of Julius Hyman & Co., Denver, Colo.) (*4*), that is fitted with a 200-ml. round-bottomed standard-taper flask. After the volume is reduced to about 25 ml., 50 ml. of isopropyl alcohol are added, and the evaporation is continued until all the hexane is evaporated.

Method II. Five hundred milliliters of carbon tetrachloride and 250 ml. of milk are stirred in a Waring Blendor for 1 minute and transferred to a 2-liter separatory funnel. A second 250-ml. portion of milk is similarly treated and placed in the same funnel, and the mixture is allowed to separate for about 30 minutes. The carbon tetrachloride solution is then filtered by gravity through a 2-inch column of anhydrous sodium sulfate contained in a tall chromatographic column 1 inch in inside diameter and collected in a graduated cylinder; recoveries of solvent are always in the neighborhood of 85 to 90%. This volume is then taken as the aliquot of the milk sample being analyzed. The solvent is evaporated in a Kuderna-Danish evaporative concentrator to about 20 ml.; 50 ml. of methanol are added, and the solution is again evaporated to about 20 ml. to remove the last traces of carbon tetrachloride. Thirty milliliters of isopropyl alcohol are added to the flask residue.

Reduction To the extractives (obtained by either Method I or II) contained in isopropyl alcohol are added 1 ml. of 85% phosphoric acid and 0.5 gram of powdered metallic zinc; the mixture is then refluxed briskly for 30 minutes.

Separation of Milk Fats Excess zinc is filtered from the reducing solution by filtration with line vacuum through a medium, fritted funnel into a 1-liter separatory funnel. The reaction flask is rinsed alternately with several portions of 250 ml. of hexane and 150 ml. of a water-acetonitrile solution (3 to 1 by volume) containing 2 ml. of 5*N* hydrochloric acid; the washings are also passed through the filter. The remainder of the two solvents is then added to the separatory funnel, and the mixture is shaken briskly for about 30 seconds. The lower phase is withdrawn into a second 1-liter separatory funnel and equilibrated with 100 ml. of hexane; this procedure is repeated in a third separatory funnel and the lower phase is transferred to a fourth 1-liter separatory funnel. The hexane in each of the three separatory funnels is then extracted in turn with two 100-ml. portions of a solution of isopropyl alcohol–acetonitrile–water (1:1:2 by volume, 0.1*N* in hydrochloric acid); the lower phases from the third funnel are added to the fourth. Emulsions that form during extraction can always be broken by gentle swirling and standing. The hexane extracts are discarded.

The acid in the collected extracts is neutralized by the addition of 200 ml. of 4% sodium bicarbonate solution; the mixture is shaken, first cautiously, then more vigorously until the evolution of carbon dioxide ceases. The aqueous phase, containing precipitated zinc carbonate, is extracted twice with 150-ml. portions of hexane and discarded. The hexane extracts are washed in turn first with 100 ml. of 2% sodium bicarbonate and then with 75 ml. of saturated sodium

chloride solution. After the hexane is shaken with a small amount of anhydrous sodium sulfate (1 to 2 grams), it is transferred to a 500-ml. Erlenmeyer flask through a coarse, fritted funnel. Both funnels are washed with 50 ml. of fresh hexane. Fifty milliliters of 50% ethanolic 0.2*N* hydrochloric acid are added, and the hexane is evaporated completely on a water bath. A pinch of finely divided silicon carbide promotes smooth ebullition at the interface. In the final stages a gentle air stream is necessary to remove all traces of hexane. After it has cooled, the murky solution, now about 20 ml. in volume is filtered through a fine fritted-glass filter into a 100-ml. volumetric flask, and the Erlenmeyer flask rinsed with three 15-ml. portions of 0.1*N* aqueous hydrochloric acid solution that are also passed through the filter. The color development is then the same as for the preparation of the standard curve.

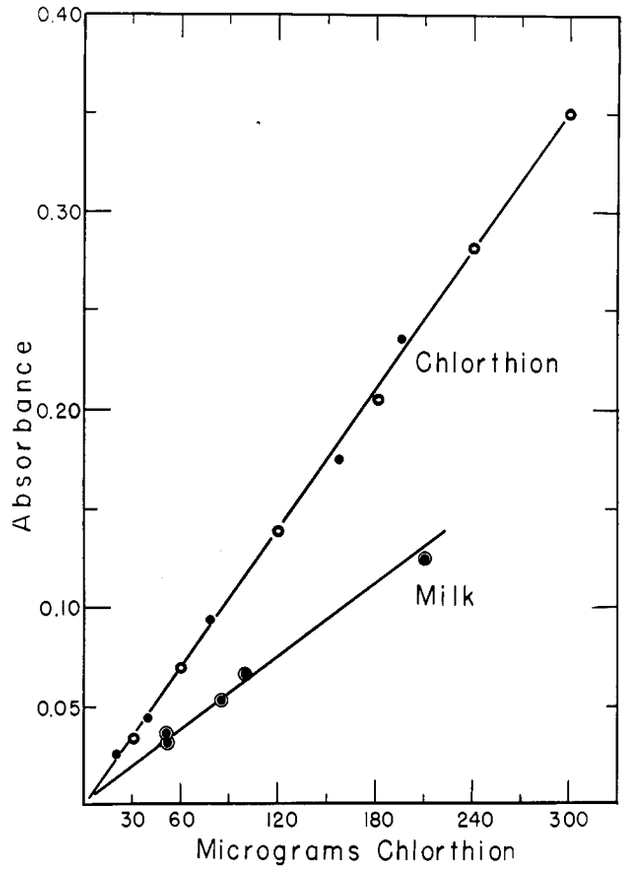


Figure 2. Standard curves for chlorthion (final volume 100 ml.)

○ Purified chlorthion, duplicate runs
⊙ Milk fortified with chlorthion
Beckman DU spectrophotometer, 1-cm. cells, λ = 545 mμ

Separation of Fats

A major difficulty in the analysis of milk is the separation of the various amounts of fats and other extractables that at times accompany chlorthion during its extraction with organic solvent. Often these may yield cloudy solutions in the final color development stage, which are not cleared by addition of alcohol. Serious losses occur when attempts are made to remove the fats with paraffin as suggested by Wilson *et al.* (*6*) and the semisolid nature of the paraffin-fat mixture makes adequate filtration impossible. Also, coextractable materials in the milk often yield high blank values and make the estimation of small quantities of chlorthion uncertain. Dahm *et al.* (*2*) in the investigation of parathion residues in milk considered that samples with transmittance values greater than 90% were not significantly different from the controls.

The solvent behavior of the chlorthion amine indicates that it is a very weak base and probably exists in equilibrium with the amine hydrochloride even in strongly acid solutions. Attempts to remove milk fats from the acid reduction mixture with carbon tetrachloride or chloroform resulted in complete loss of the amine from the aqueous phase. A single equilibration between 40 ml. of the solution obtained by filtration after the reduction of chlorthion (1 to 1 ethyl alcohol–water) and 100 ml. of hexane (petroleum ether, 60° to 70° C.) followed by two washings of the hexane phase with 15-ml. portions of 0.25*N* aqueous hydrochloric acid still resulted in only 70% recovery into the aqueous phase. Polar organic solvents such as acetonitrile and methanol proved, however, to be good solvents for the amine hydrochloride. Thus, three equilibrations of 100 ml. of hexane with 100-ml. portions of acetonitrile–isopropyl alcohol–water (1:1:2 by volume) (the water greatly reduces fat solubility), each portion acidified with 2 ml. of 5*N* hydrochloric acid, extracted 95% of the amine hydrochloride into the aqueous phase. The addition of the isopropyl alcohol is an aid in the prevention of intractable emulsions.

Consequently, a liquid-liquid extraction procedure for the separation of the amine hydrochloride from fats and other milk extractables has been developed. This resulted in solutions at the color-development stage that were always clear and the controls were consistently clear and water-white with transmittance values of 98 to 100% at 545 mμ when water was used as reference.

In the reduction of pure chlorthion to the amine, the use of ethyl alcohol and hydrochloric acid gave excellent analytical results. However, the use of 99% isopropyl alcohol as solvent affords a homogeneous liquid phase in the presence of relatively large quantities of milk fats. Phosphoric acid seems to give a more prolonged uniform reaction with zinc in isopropyl alcohol than does hydrochloric acid. This could be due to the relative solubilities of the two zinc salts in isopropyl alcohol or the porosity of the coating of salts on the metallic zinc.

When the extractive procedure described in Method II is used, it is absolutely essential that all carbon tetrachloride be removed before proceeding with the reduction step. If any is present, a vigorous initial reaction so modifies the chlorthion that no color is obtained in the presence of relatively large amounts. Final evaporation with methanol will remove last traces by azeotropic distillation.

Analytical results obtained from milk fortified with chlorthion are shown in Figure 2. Concentrations of chlorthion greater than 0.1 p.p.m. in 500 ml. of milk may be determined spectrophotometrically; over-all recoveries are all within the region of 50 to 60%. Concentrations from 0.02 to 0.1 p.p.m. could be approximated by visual comparison using Nessler tubes filled to 25-cm. depth.

No evidence of chlorthion was found in any of the milk samples from the treated dairies when the visual comparative method sensitive to 0.02 p.p.m. in 500 ml. of milk was used.

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