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INSECTICIDE RESIDUES

Colorimetric Estimation of Malathion Residues in Animal Products

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A colorimetric method for the estimation of spray residues of malathion on plant material has been adapted to the analysis of animal products. This has led to a more detailed study of the basic procedure, and a number of improvements and modifications have been made. Methods for the determination of residues in meat, fat, liver, milk, and eggs are presented.

In connection with a program to extend the commercial application of the organic phosphorus insecticide, malathion [*S*-(1,2-dicarbethoxyethyl)-*O,O*-dimethyldithiophosphate], to insect control on livestock and poultry, suitable methods were needed for determining residues in edible products obtained from the treated animals. The colorimetric method reported for the determination of malathion residues on plant material (*4*) has been adapted successfully, with a number of improvements and modifications, for this purpose. This method is based upon the alkaline decomposition of malathion in ethyl alcohol–carbon tetrachloride solution to sodium dimethyl dithiophosphate, sodium fumarate, and ethyl alcohol. The sodium dimethyl dithiophosphate is extracted into an aqueous solution, converted to a copper complex, which is extracted into carbon tetrachloride with the formation of a yellow color, the intensity of which is a measure of the malathion present. Amounts as low as 20 γ of malathion may be determined by the revised procedure. These methods will be used to obtain the residue data now required for federal and state insecticide licensing and registration purposes.

The methods described were developed for the determination of malathion residues in meat, fat, liver, milk, and eggs. The applicability of the methods was tested by analyzing prepared samples containing added amounts of the insecticide. Results of these analyses are shown in Tables I, II, and III. The method for analysis of pork meat was used as described; for beef and chicken meat, the sample size and volumes of all reagents were doubled, except that 30 ml. of carbon tetrachloride was used to extract the final colored copper-dithio complex. These modifications were made in order that a Klett-Summerson photoelectric colorimeter with 4-cm. cells might be used in place of a spectrophotometer without loss of sensi-

Table I. Recovery of Malathion from Meat

Type	Sample Represented by Extract Analyzed, G.	Malathion, P.P.M. Added	Malathion, P.P.M. Found	Recovery, %
Beef	200	0.24	0.16	67
	200	0.24	0.16	67
	200	0.47	0.37	79
	100	0.47	0.39	83
	200	0.47	0.35	74
	200	0.52	0.39	75
	200	0.94	0.77	82
	100	0.94	0.77	82
	200	0.94	0.67	71
	200	1.03	0.80	78
	200	1.18	0.93	79
	100	1.88	1.63	87
	100	2.06	1.75	85
	160[a]	0.47	0.33	70
	163[a]	0.94	0.72	77
	100[a]	1.88	1.41	75
Chicken (fowl)	200	0.24	0.17	71
	200	0.24	0.17	71
	200	0.47	0.40	85
	200	0.47	0.33	70
	180	0.47	0.32	68
	200	0.94	0.72	77
	200	0.94	0.64	68
	100	1.88	1.50	80
Chicken (fryer)	200	0.24	0.16	67
	200	0.47	0.37	79
	200	0.94	0.78	83
Pork	98	0.50	0.41	82
	98	0.50	0.41	82
	120	1.00	0.95	95
	120	1.00	0.87	87
	31	5.00	5.00	100

[a] Extracts stored for 2 weeks in dark, under refrigeration, before analysis.

tivity. The method was used as described for pork and chicken fat, but for beef fat, Versene was used to eliminate interference of copper in the reagents, and 10% sodium chloride solution was used to prevent emulsion formation.

Procedure

Reagents Malathion, purified sample. Obtainable from American Cyanamid Co., Stamford, Conn.

Carbon tetrachloride. ACS reagent grade preferred; technical grade distilled from glass may be used if appropriate tests are run to prove its suitability.

Ethyl alcohol, absolute or formula 2B (anhydrous).

Carbon disulfide solution, 1 ml. of C.P. carbon disulfide in 200 ml. of ACS reagent grade carbon tetrachloride.

Ferric chloride solution, 5 grams of C.P. $FeCl_3.6H_2O$ in 100 ml. of 1N hydrochloric acid.

Copper sulfate solution, 3.5 grams of C.P. $CuSO_4.5H_2O$ in 100 ml. of water.

Apparatus Beckman Model DU spectrophotometer (or equivalent).

Wide-mouthed glass-extraction jars, 1-gallon capacity or larger, with plastic screw caps lined with tin or aluminum foil.

Freeze-drying apparatus, capacity for condensing 2 liters of water from vapor phase without interruption.

Centrifuge bottles, borosilicate glass, 1000-ml. heavy-walled (Corning No. 1280).

Glass extraction tube. Glass tube, 15 inches by $1\frac{1}{2}$ inches with a glass stopcock sealed to one end.

Calibration Curves **Meat, Fat, Eggs, and Liver.** Dissolve approximately 40 mg. of purified malathion in 250 ml. of ethyl alcohol in a volumetric flask. Transfer a 25-ml. aliquot to a 250-ml. volumetric flask and dilute to volume with ethyl alcohol (1 ml. is equivalent to about 16 γ of malathion). Using this standard solution, take aliquots of 0, 0.5, 1, 2, 3, 6, and 9 ml. through the following procedure:

Transfer the aliquot to a 250-ml. separatory funnel containing 100 ml. of carbon tetrachloride and 1 ml. of the carbon disulfide solution, add ethyl alcohol until the total volume of alcohol present is 25 ml., then mix by gentle swirling. Add 75 ml. of aqueous 9% sodium sulfate solution, which has been acidified with 2.5 ml. of 12N hydrochloric acid, and shake vigorously for 1 minute. Filter the carbon tetrachloride layer through a fluted filter paper into a dry 250-ml. separatory funnel, but do not allow any of the aqueous layer to run onto the filter paper and do not wash the paper. Add 25 ml. of ethyl alcohol to the filtered carbon tetrachloride solution of malathion, then add 1 ml. of 6N sodium hydroxide and shake for exactly 1 minute. The procedure from this point on should be carried out without interruption, as aqueous alkaline and acid solutions of dimethyldithiophosphoric acid are stable for short periods only. Reaction periods of less than 30 seconds and more than 2 minutes produce low results.

Immediately, add 75 ml. of aqueous 9% sodium sulfate solution and shake vigorously for 1 minute. Discard the carbon tetrachloride layer. Add 25 ml. of carbon tetrachloride, shake vigorously for 30 seconds, and discard the carbon tetrachloride layer. Add to the separatory funnel 25 ml. of carbon tetrachloride, 2 drops of phenolphthalein solution, and 6N hydrochloric acid dropwise with swirling until the pink color disappears; then add 1 ml. of the 5% ferric chloride solution. Shake vigorously for 30 seconds and discard the carbon tetrachloride layer. Repeat the extraction twice more with 25-ml. portions of carbon tetrachloride and discard each portion. Add exactly 20 ml. of carbon tetrachloride and then 1 ml. of copper sulfate solution; shake vigorously for 1 minute and allow the phases to separate. Immediately, filter the carbon tetrachloride layer through cotton placed loosely in a funnel into a 5-cm. cell and measure the absorbance of the yellow solution (the color is usually not stable and should be measured within 2 or 3 minutes after extraction) at 418 mμ, using ACS reagent grade carbon tetrachloride as the reference. Prepare a calibration curve by plotting the absorbance of each of the aliquots *vs.* micrograms of malathion present. Fifty micrograms of malathion produces a color having an absorbance of approximately 0.124. The calibration curve between 25 and 150 γ is essentially a straight line with a slope of 3.2 (absorbance per milligram of malathion).

Milk. Dissolve approximately 62.5 mg. of purified malathion in 250 ml. of ethyl alcohol in a volumetric flask. Transfer a 10-ml. aliquot to a 250-ml. volumetric flask and dilute to volume with ethyl alcohol (1 ml. contains 10 γ of malathion). Using this standard solution, take aliquots of 0, 1, 2, 3, 4, 7, and 10 ml. through the procedure for the analysis of carbon tetrachloride extracts from milk, but use 6N sodium hydroxide instead of 9N. Prepare a calibration curve by plotting the absorbance of each of the aliquots *vs.* micrograms of malathion. Forty micrograms of malathion produces a color having an absorbance of approximately 0.084.

Table II. Recovery of Malathion from Animal Fat and Milk[a]

Type	Malathion, P.P.M. Added	Found	Recovery, %
Beef fat	0.8	0.6	75
	0.8	0.6	75
	1.2	0.9	75
	1.9	1.4	74
	1.9	1.4	74
	1.9	1.3	68
	1.9	1.6	84
	3.1	2.4	77
	3.8	3.2	84
	7.7	7.7	100
Pork fat	1.1	0.9	82
	1.2	1.1	92
	1.2	1.0	83
	1.2	1.1	92
	3.0	2.9	97
	3.7	3.8	103
	4.0	3.5	88
	4.5	4.4	98
	5.5	4.8	87
Chicken fat	2.3	2.1	91
Milk, pasteurized	0.016	0.013	81
	0.020	0.018	90
	0.031	0.024	77
	0.054	0.040	74
	0.063	0.042	67
	0.064	0.045	70
	0.075	0.054	72
	0.108	0.073	68
	0.215	0.156	73
Milk, raw whole	0.019	0.025	132
	0.029	0.027	93
	0.029	0.022	76
	0.047	0.043	92
	0.066	0.052	79
	0.079	0.053	67
	0.079	0.066	84

[a] Based on 20-gram samples of animal fat and 1000-ml. samples of milk.

Table III. Recovery of Malathion from Eggs and Liver[a]

Type	Malathion, P.P.M. Added	Found	Recovery, %
Yolks	0.22	0.15	68
	0.22	0.20	91
	0.22	0.17	77
	0.22[b]	0.15	68
	0.24	0.30	125
	0.49	0.64	131
	0.49	0.50	102
	0.49	0.47	96
	0.49	0.36	73
	0.49[b]	0.35	71
	0.66	0.53	80
	0.99	0.82	83
	0.99[b]	0.84	85
	0.99	0.87	88
	0.99	0.84	85
	0.99	0.96	97
	1.01[c]	0.75	74
	1.48	1.22	82
	1.98	1.87	94
	1.98	1.65	83
Whites	0.24	0.18	75
	0.24	0.20	83
	0.24	0.16	67
	0.51	0.37	73
	0.51	0.47	92
	0.51	0.41	80
	0.51	0.55	108
	0.99	0.91	92
	1.92	1.67	87
	1.98	1.85	93
Liver, beef			
Raw	1.5	1.36	91
Cooked	1.5	1.32	88
	0.8	0.88	110
	1.60	1.90	119
Liver, chicken	1.50	1.30	87
	1.50	1.22	81

[a] Based on 50-gram samples.
[b] Carbon tetrachloride extract stood overnight in refrigerator before analysis.
[c] 100-gram sample.

The calibration curve between 20 and 100 γ is essentially a straight line with a slope of 3.14 (absorbance per milligram of malathion).

Extraction and Determination of Malathion Residues

Meat. Macerate 300 grams of finely ground fat-free meat with 600 ml. of carbon tetrachloride in a Waring Blendor at high speed for 5 minutes. Transfer macerate to a 1-gallon, wide-mouthed jar, cap tightly, and agitate mechanically (preferably end over end) for 4 hours. Immediately, filter the slurry by vacuum and concentrate a 200-ml. aliquot to about 90 ml. over a steam bath with the aid of a jet of air. Transfer the concentrated extract to a 250-ml. separatory funnel, dilute to 100 ml. with carbon tetrachloride, add 1 ml. of the carbon disulfide solution, and 25 ml. of ethyl alcohol, and continue as for preparation of the calibration curve for meat beginning with the addition of the 9% sodium sulfate solution. For the alkali decomposition step use 9N sodium hydroxide instead of 6N used for the calibration curve. Extract the aqueous acidified layer containing the dimethyl dithiophosphoric acid four or more times with 25-ml. portions of carbon tetrachloride until the carbon tetrachloride layer is proved colorless by measuring absorbance at 418 mμ. Extractions at this point remove interfering colored substances originally present in the sample and also xanthates formed from carbon disulfide added previously. Obtain from the calibration curve the amount of malathion corresponding to the absorbance observed.

Milk. Measure a 1000-ml. sample of milk (milk samples containing malathion may be kept for several days under refrigeration without apparent decomposition of malathion) for analysis and transfer 500-ml. portions to two 1-liter heavy-walled centrifuge bottles. Rotate the stoppered bottles slowly in a dry ice–acetone slush in a nearly horizontal position until the liquid has frozen in a uniform shell on the inside walls, and the shell has "cracked" away from the glass. Freeze-dry the material until a dry solid is obtained. Dried milk solids may be kept satisfactorily in the dark at room temperature for several days before extraction.

Transfer the milk solids to a 1-liter beaker and add sufficient carbon tetrachloride to form a thin slurry. Mix thoroughly, break up all lumps, and allow to stand for about 15 minutes. Filter the slurry by vacuum, transfer the solid to the funnel, and continue to apply vacuum until carbon tetrachloride is removed. Repeat the extraction of the milk solids twice more in a similar manner. Concentrate the combined filtrates to about 175 ml. over a steam bath with the aid of a jet of air passing across the surface. Protect carbon tetrachloride extracts of dried milk solids, egg whites, or egg yolks as much as possible from exposure to light. Extracts may be stored overnight under refrigeration.

Transfer the concentrated carbon tetrachloride extract to a 500-ml. separatory funnel, and add 2 ml. of the carbon disulfide solution and then carbon tetrachloride until the total volume of solution in the funnel is 200 ml. Add 50 ml. of ethyl alcohol and 150 ml. of 2% aqueous sodium chloride solution (cooled to about 10° C.) and shake vigorously for 30 seconds. Filter the carbon tetrachloride layer through a fluted filter paper into a dry 500-ml. separatory funnel, but do not allow the aqueous layer to run into the filter paper and do not wash the paper. Add 50 ml. of ethyl alcohol to the separatory funnel and mix by swirling. Add 2 ml. of 9N sodium hydroxide and shake for exactly 1 minute. Immediately, add 150 ml. of 2% aqueous sodium chloride solution (cooled to about 10° C.) and shake vigorously for 1 minute.

Draw off the carbon tetrachloride layer, including the small amount of suspended solids which are usually found at the interface, and discard it. Add 50 ml. of carbon tetrachloride to the separatory funnel, shake vigorously for 30 seconds, and discard the carbon tetrachloride layer. Add 50 ml. of carbon tetrachloride, one drop of phenolphthalein solution, and 6N hydrochloric acid dropwise with swirling until the pink color disappears, then add 2 ml. of 1N hydrochloric acid. Shake vigorously for 30 seconds and discard the carbon tetrachloride layer. Repeat the extraction of the aqueous layer with 50-ml. portions of carbon tetrachloride until carbon tetrachloride layer is colorless; draw off carbon tetrachloride as completely as possible and discard it. Extractions at this point remove interfering colored substances originally present in the sample and also xanthates formed from carbon disulfide added previously.

Add exactly 20 ml. of carbon tetrachloride and 2 ml. of the copper sulfate solution and shake vigorously for 1 minute. Immediately, filter the carbon tetrachloride layer through a cotton plug, placed loosely in a funnel, into a 5-cm. cell. Immediately measure the absorbance of the yellow-colored solution at 418 mμ using ACS reagent grade carbon tetrachloride as the reference solution. From the similarly prepared calibration curve, read the amount of malathion corresponding to the absorbance observed and calculate the parts per million of malathion in the sample.

Egg Whites. Add 5 ml. of 6N hydrochloric acid to eight egg whites and stir until uniform. Grind a 50-gram portion with 200 grams of technical anhydrous sodium sulfate in a large mortar until a powder is obtained. A paste first forms but after it stands for about 20 minutes, a uniform powder can be obtained upon further grinding.

Transfer the powder to the glass extraction tube which contains a cotton plug in the bottom and pack the column firmly. Place a cotton plug on the top and attach a 500-ml. vacuum flask to the bottom and a 500-ml. separatory funnel to the top by means of rubber stoppers. Pass 600 ml. of carbon tetrachloride through the column at the rate of not more than 10 ml. per minute, using vacuum if necessary. Concentrate the extract over a steam bath to approximately 75 ml., with the aid of a jet of air, and determine the malathion by the procedure described for analysis of carbon tetrachloride extracts of meat. Protect carbon tetrachloride extracts of dried milk solids, egg whites, or egg yolks as much as possible from exposure to light. Extracts may be stored overnight under refrigeration.

Egg Yolks. Grind a 50-gram sample of well-mixed egg yolks with 200 grams of anhydrous technical sodium sulfate until a uniform powder is obtained and continue as described for egg whites beginning, "Transfer the powder to the glass extraction tube. . ."

Liver. Grind a 50-gram sample of finely divided liver with 200 grams of anhydrous technical sodium sulfate until a dry powder is obtained. Transfer the powder to the glass extraction tube and pass 600 ml. of a carbon tetrachloride solution containing 5% ethyl alcohol (by volume) through the column at the rate of not more than 10 ml. per minute—by using vacuum, if necessary. Measure the extract volume, A (for washing loss correction), and then shake for 1 minute with 300 ml. of 9% aqueous sodium sulfate containing 10 ml. of 12N hydrochloric acid. Centrifuge, if necessary, and transfer the carbon tetrachloride layer to a 1-liter separatory funnel and add 100 ml. of ethyl alcohol. Add 300 ml. of 9% aqueous sodium sulfate solution containing 2 drops of phenolphthalein indicator and 1-ml. increments of 0.5N sodium hydroxide, with swirling, until a pink color persists, even after shaking. Centrifuge, if necessary, and filter the carbon tetrachloride phase through paper. Measure volume, B [% washing loss = $100(1 - B/0.95A)$], and concentrate over a steam bath with the aid of a jet of air to about 80 ml. Transfer the concentrate to a 250-ml. separatory funnel, add 1 ml. of carbon disulfide solution (7 ml. of carbon disulfide per 100 ml. of carbon tetrachloride), and then add carbon tetrachloride until total volume of solution in the funnel is 100 ml. Add 25 ml. of ethyl alcohol, mix, then add 1 ml. of 6N sodium hydroxide, and shake for exactly 1 minute. Continue as described for preparation of the calibration curve for meat, fat, eggs, and liver,

beginning with the addition of 9% sodium sulfate solution.

Discussion

Carbon tetrachloride has been used in all cases to extract the malathion from samples; however, hexane may be used in the extraction step (*2*) and probably in the decomposition step. Limited data indicate that methanol may be substituted for ethyl alcohol; however, isopropyl alcohol was unsatisfactory. The amount of sodium hydroxide to be added for decomposition of malathion should equal 1 ml. of 6*N*, plus that consumed by any free acidity or ester hydrolysis of the sample and should be determined experimentally for each type of product. The concentration may vary, but the volume should be held constant at 1 ml. in order to keep the water content at a minimum.

Usually, different techniques must be used to analyze each type of animal product—e.g., the whites and yolks of eggs were analyzed because the whites have a naturally occurring high alkalinity (pH 8.2 to 9.8 depending upon age) and must be acidified before extraction in order to prevent alkaline decomposition of the malathion. Because of this naturally occurring alkalinity, malathion would probably not be found in the whites of eggs from treated birds. In the development of a method for malathion in beef liver, data showed that malathion is probably converted by contact with raw beef liver macerate to a product which does not respond to the colorimetric procedure. This conversion does not take place if the liver is dried with anhydrous sodium sulfate, acidified to pH of about 3, or heated a few minutes at 80° C. before the malathion is added. Malathion will probably not persist without change for any length of time in livers of treated animals. The method reported, therefore, is satisfactory, provided the malathion is present at the time the sample is ground with anhydrous sodium sulfate. Use of sodium sulfate is a technique recommended by Jones and Riddick (*3*).

Emulsion difficulties with extracts of some animal products were minimized by a pre-extraction of concentrated carbon tetrachloride extracts with a strongly acidic sodium sulfate solution, and also after the alkali decomposition step by using an aqueous 9% sodium sulfate solution. Sodium sulfate was found preferable to sodium chloride because high chloride concentration caused decreased and somewhat erratic color development.

When determining malathion in the range of about 20 γ, interference due to trace amounts of metals, particularly copper, must be eliminated. Copper forms the carbon tetrachloride–soluble complex with the dimethyl dithiophosphoric acid which will be discarded before the color development step, thus producing low results. This interference may be eliminated by the addition of disodium ethylenediamine tetraacetate [(ethylenedinitrilo)tetraacetate, Versene, Sequestrene]. This technique was applicable to beef fat analysis but not for milk, so that a more reliable and generally applicable technique was developed. A small amount of carbon disulfide is added to the concentrated carbon tetrachloride extract taken for analysis. The carbon disulfide reacts with the ethyl alcohol and sodium hydroxide to produce sodium xanthate (CH_3CH_2—O—CSSNa) which forms a stable complex with copper. This complex is extracted and discarded, thus eliminating interference from copper. In addition, the xanthate probably serves to prevent oxidation of the dimethyldithiophosphoric acid.

Upon analysis of some products—eggs and liver, for example—a reducing material, probably a mercaptan, which interferes by reducing some of the copper to the cuprous ion, is extracted. Cuprous ion prevents formation of the colored cupric copper complex with the dithio acid. This interference may be eliminated by adding ferric chloride which prevents reduction of copper.

Control tests should be run whenever possible and suitable corrections made on samples known to contain no malathion. Controls on fat, meat, milk, eggs (whites or yolks), and liver have indicated approximate "apparent" malathion contents of 0.0, 0.0, 0.015, 0.05, and 0.2 p.p.m., respectively. Based upon results obtained by analysis of synthetic samples, the methods described appear satisfactory for determining malathion in fat, meat, milk, eggs (whites or yolks), and liver in concentrations down to 1.0, 0.5, 0.02, 0.5, and 1.0 p.p.m., respectively. The procedures described in detail are those which contain the latest improvements. Some of the results reported, however, were obtained before these modifications were made. Such cases have been indicated. The methods recommended for beef fat and milk have been applied by Claborn and associates (*1*) in connection with a series of experiments carried out by the U. S. Department of Agriculture on spraying cattle with malathion formulations.

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PLANT ANALYSES

Measurement of Microgram Amounts of Chlorine in Plant Materials

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RECENT DEMONSTRATIONS of chlorine as an essential plant nutrient (*2*) have required the development of methods for the measurement of amounts of chlorine in the microgram range. Severely chlorine-deficient plants may contain as little as 35 γ of chlorine per gram of dry weight. This low minimal value for chlorine concentration in plant tissue requires that chlorine be assigned the status of a plant micronutrient along with molybdenum, zinc, copper, boron, manganese, and iron, thus requiring methods of analysis of the order of sensitivity and accuracy similar to those available for these latter elements.

The most successful attempts to pro-

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