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RECOMMENDED METHODS FOR THE DETERMINATION OF RESIDUES OF PYRETHRINS AND PIPERONYL BUTOXIDE

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RECOMMENDED METHODS FOR THE DETERMINATION OF RESIDUES OF PYRETHRINS AND PIPERONYL BUTOXIDE

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Abstract - Methods are recommended and described for determining Pyrethrin I, Cinerin I, Pyrethrin II, Cinerin II and Jasmolin II with or without combination with synergists such as piperonyl butoxide, in milk, fish and meat, fat and meat by-products of cattle, goats, hogs, horses, sheep, poultry tissues and eggs using g.l.c. and t.l.c. It is probably adaptable to cereals, fruit and oilseeds.

INTRODUCTION

The recommendations are directed mainly to the Codex Committee on Pesticide Residues and the Joint Meeting of the Food and Agriculture Organization Working Party of Experts on Pesticide Residues and the World Health Organization Expert Committee on Pesticide Residues, but they are also intended for any other interested body.

The recommendations are intended as a guide to the analyst who should make such modifications to the procedure as are required by his own laboratory circumstances, the equipment and materials available and the samples being analysed. It is strongly emphasized that the experience of the analyst in residue work is crucial to obtaining acceptable results.

The recommendations cover the pyrethrin insecticides composed of a mixture of Pyrethrin I, Cinerin I, Pyrethrin II, Cinerin II, and Jasmolin II; with or without combination with synergists such as Piperonyl Butoxide.

The recommended method for pyrethrins applies only to milk, fish and meat, and meat by-products of cattle, goats, hogs, horses, sheep, poultry, tissues and eggs. It is probable that, with suitable modifications in the extraction and/or clean-up procedure, the method would be adaptable to cereals, cereal products, fresh and dried fruit, and oilseeds. However, such modifications to the method have not yet been validated or collaboratively studied and cannot be recommended at this time.

Since pyrethrins applied to cereals and fruit are always used in conjunction with a synergist, usually piperonyl butoxide at a ratio of 1:10 w/w, and since piperonyl butoxide residues are more stable than those of pyrethrins, an index of pyrethrin residues can be obtained indirectly by the determination of residual piperonyl butoxide.

RECOMMENDATIONS

A. Pyrethrins

The recommended method is that described in the United States Food and Drug Administration (FDA), Pesticide Analytical Manual, Volume II, (Ref 1). Other residue methods under development such as the gas chromatographic method of Bruce (Ref 2), which requires a specially constructed detector cell, or the liquid chromatographic approach of Schmit *et al* (Ref 3), using an ultraviolet detector, show promise but cannot be recommended due to lack of sufficient study and evaluation. The need for a special or modified electron capture detector is questionable in view of the successful use of a conventional tritium foil detector in the recommended method.

Outline of the method

The insecticide is extracted from the samples with a mixture of ethyl alcohol, ether, and hexane and partitioned between acetonitrile and petroleum ether. Clean-up of the extracts is effected by Florisil column and thin-layer chromatography. The determination is accomplished by gas chromatography using an electron capture detector. Because of the limited availability of the Pesticide Analytical Manual in some locations the method is reproduced in its entirety as the Appendix.

Development of the method

The method was developed by the Kenya Pyrethrum Company, Minneapolis, USA, and validated, with further refinements and modifications, by the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA).

Interference with the method

If the thin-layer chromatographic clean-up is omitted, the gas chromatograms show elevated baselines, but recoveries were higher at the sensitivity limit. Chlorinated pesticides are eluted from the Florisil column in the 10% ether in hexane fraction wheras the pyrethrins were recovered in the subsequent 50% ether in hexane eluate. Recoveries in milk by FDA laboratories ranged from 50% at the 0·01 mg kg^{-1} level to 88% at the 0·1 mg kg^{-1} level. Sensitivity was 0·05 mg kg^{-1} on a whole milk basis.

B. Piperonyl butoxide (in grain and vegetable beans or peas)

The recommended method is that given in Official Methods of Analysis of the Association of Official Analytical Chemists (Ref 4). This colorimetric procedure applies to Alaska peas, barley, hulled rice, oats, pinto beans, and wheat. The colorimetric method developed by Secreast and Cail (Ref 5) for the low residues of piperonyl butoxide in flour is also recommended. The gas chromatographic method of Moore (Ref 6), although very sensitive, requires a special design of electron capture detector which is not commercially available and therefore cannot be recommended at this time.

Outline of the method

Grain or bean samples are extracted with chloroform and the residue hydrolysed with methanolic potassium hydroxide. Subsequent treatment with strong sulfuric acid liberates formaldehyde which is determined colorimetrically with chromotropic acid.

Flour samples are extracted with pentane and chromatographed on a Florisil column. Determination is colorimetric following treatment with a reagent prepared from tannic acid, acetic acid, and orthophosphoric acid (85%) (Jones *et al* (Ref 7); Williams and Sweeney (Ref 8)).

Development of the method

The methods are described in detail in references 4 and 5. The method in reference 4 has been collaboratively studied extensively and is Official Final Action (AOAC). The method in reference 5 has not been collaboratively studied but has found acceptance for routine use by the Stored-Product Insects Research and Development Laboratory, Agricultural Research Service, United States Department of Agriculture.

Interferences with the method

No clean-up is required for residues in flour greater than 6 mg kg^{-1} when using the colorimetric method of reference 5; however, smaller residues require a clean-up on Florisil. The sensitivity of the procedure is 0·2 mg kg^{-1} after clean-up and recoveries of 95% or better are obtained.

The AOAC method for grains (Ref 4), has a sensitivity of 0·5 mg kg^{-1} or 20 µg of piperonyl butoxide.

APPENDIX

Resumé of Methods - A clean-up procedure was developed to remove interfering materials from samples of milk and tissue extracts without causing deterioration of pyrethrins. Samples are extracted with a mixture of ethyl alcohol, ether, and hexane. Acetonitrile partition removes much of the extracted fat. The acetonitrile extract in hexane is chromatographed through Florisil to separate the pyrethrins from the chlorinated hydrocarbon pesticides. The final clean-up is accomplished using acetonitrile with Gelman I.T.L.C. Type A microfiber. When analyzed by g.l.c. with electron capture detection, the amount of pyrethrins is determined. The analysis of pyrethrins residues should be based upon the pyrethrins I because it is the most easily detected ester of pyrethrins.

PROCEDURE

Apparatus

7 in x 9 in x 12 in chromatographic tank containing a rack for suspending the I.T.L.C. Type A microfiber strips, five 150 ml beakers for acetonitrile, and one 150 ml beaker for water and 2 in x 8 in microfiber wick.

Macro. Virtis No. 23 Homogenizing Mill
Vacuum Rotary Evaporator
Kuderna - Danish apparatus
Conical centrifuge tubes, 15 ml graduated
Chromatographic columns. Kontes (K-420600) Chromaflex 22 mm x 330 mm
Moist chamber - Consists of a glass covered battery jar with a 150 ml beaker of water and a 2 x 8 I.T.L.C. Type A microfiber strip to serve as a wick.

500 µl Hamilton Syringe for transferring residues to microfiber (Gelman 5 cm x 20 cm) I.T.L.C. microfiber Type A)
Whatman No 12 folded filter paper
Gas Chromatograph with electron capture detector

Reagents

Petroleum ether - nanograde
Acetonitrile - nanograde
Acetone - nanograde
Hexane - nanograde
Ethyl ether - nanograde
Ethyl alcohol - 99·8% redistilled in glass
Reagent grade Sodium chloride - heated 450°C overnight to eliminate interference
Reagent grade Sodium sulphate, anhydrous - heated 450°C overnight to eliminate interference
Florisil - heated 450°C overnight, cooled and partially deactivated by adding 5% water by wt. Mixed and stored in a glass stoppered flask.
Sodium oxalate

Extraction:

(A) Animal Tissues

10 g of chopped tissue or 2 g of fat, 50 ml of ethyl alcohol, and 50 ml of ether are homogenized 5 minutes in a 500 ml Virtis flask with the Virtis Mill. Add 100 ml of hexane and 50 g of anhydrous sodium sulphate and blend 1 - 2 minutes in the Virtis Mill. Decant the solvent through a Whatman No 12 folder filter paper into a 1000 ml flask. Wash the residue in the Virtis flask twice with 100 ml of hexane and filter into the 1000 ml flask. The flask is attached to the rotary evaporator and the solvent evaporated. A water bath at 50°C will accelerate removal of the solvents. Quantitatively transfer the extract with 5 portions of 5 ml of petroleum ether to a 125 ml separatory funnel. Proceed with Acetonitrile Partitioning described below.

(B) Milk (Results reported on milk basis)

To 100 g of fluid milk (4% butterfat) or 50 g of evaporated milk in a 1000 ml separatory funnel, add 100 ml of ethyl or methyl alcohol and 1 g of Na oxalate, and mix. Add 50 ml of ethyl ether and shake vigorously; then add 50 ml petroleum ether and shake vigorously. Allow to separate and drain off lower portion into another 1000 ml separatory funnel. Re-extract with another 100 ml portion of ether-petroleum ether (1:1). Combine with first extract and add 500 ml water. Swirl gently and drain off aqueous layer. Rewash ether-hexane layer twice with 100 ml water with a minimum amount of shaking and discard water. Pass ether through column of anhydrous sodium sulphate, 2 in deep, and collect eluate in a 500 ml flask. Wash column with small portions of petroleum ether and evaporate combined extracts in a vacuum rotary evaporator to obtain fat. Transfer fat into a small beaker with petroleum ether and remove the solvent with a stream of dry nitrogen. A water bath of 40°C will accelerate evaporation.

Weigh 2·0 g of extracted fat. Quantitatively transfer the butterfat to a 125 ml separator, using small portions of petroleum ether, so that the total volume of fat and petroleum ether in the separator is 25 ml. Proceed with Acetonitrile Partitioning described below.

Acetonitrile Partitioning

Add 30 ml of acetonitrile (saturated with petroleum ether), shake vigorously one minute, allow layers to separate and drain acetonitrile into a 1 l separator containing 700 ml of 2%

sodium chloride solution and 100 ml of petroleum ether. Extract petroleum ether solution in the 125 ml separator three more times in same manner using 30 ml portions of petroleum ether saturated acetonitrile each time. Combine all extracts in the 1 l separator. Stopper and invert 1 l separator, vent off pressure; while separator is inverted perform first extraction of pesticides by swirling inverted separator. (Swirl cautiously to minimize emulsions). Allow layers to separate and drain aqueous layer into a second 1 l separator. Add 100 ml of petroleum ether and shake vigorously 15 sec., let layers separate. Discard aqueous layer, combine petroleum ether in original separator and wash with two 100 ml portions of water. (If emulsions form, wash with 1 - 2% sodium chloride solution or add 5 ml of saturated sodium chloride solution to 1 l separatory). Discard washings and draw off petroleum ether through a 2 in column of anhydrous sodium sulphate into a Kuderna-Danish concentrator. Rinse separator and column three times with about 10 ml portions of petroleum ether. Evaporate to about 10 ml for transfer to Florisil column.

Florisil separation of pyrethrins from chlorinated hydrocarbons

A. Column Preparation

To the 22 mm x 320 mm chromatographic column add, in the following order:

1. 1/2 in of sodium sulphate.
2. 30 g Florisil containing 5% water.
3. Top with 1/2 in sodium sulphate.

B. Elution

Prewet column with 30 ml of hexane and place the receiving flask beneath column. Transfer the petroleum ether extract into the column and adjust elution rate to about 5 ml per minute. Rinse flask with two successive 5 ml portions of hexane to effect a quantitative transfer to the column. Allow the rinse to sink into the sodium sulphate layer, then rinse down the wall of the chromatographic column with 3 - 5 ml of hexane. It is usually necessary to prewet one or two such columns to establish the elution pattern of the Florisil column which effects the best separation of pyrethrins from the chlorinated hydrocarbons. (See FDA comments regarding this item).

Usually 125 to 150 ml of 10% ether in hexane will elute all of the interfering chlorinated hydrocarbons. This first fraction may be tested for chlorinated pesticides and discarded. The pyrethrins are eluted with 200 ml of ether-hexane (1:1). This second fraction will contain 50 to 100 mg of fat and must be removed to prevent contamination of the column and an unsatisfactory base-line on the chromatogram.

Thin layer cleanup of second Florisil fraction containing pyrethrins

Evaporate the solvent from the second fraction with a vacuum rotary evaporator or a Kuderna-Danish apparatus. Transfer the fatty residue into a 15 ml conical centrifuge tube with 3 ml of petroleum ether. Carefully rinse the container with 3 more successive portions of petroleum ether to insure a complete transfer to the centrifuge tube. The petroleum ether in the centrifuge tube is evaporated with a slow stream of dry nitrogen. To assist the evaporation of petroluem ether the centrifuge tube is immersed in a water bath maintained at 40°C.

Rinse down the sides of the centrifuge tube with petroleum ether and adjust the volume to about 0•5 ml.

Although the type A microfibers are very clean it is advisable to further clean them by allowing 90% acetone to ascend to the top before storing in the moist chamber. After 12 h of equilibration in the moist chamber the microfiber will contain sufficient absorbed water for this cleanup procedure.

Transfer the 0•5 ml eluate concentrate by means of a 500 µl Hamilton syringe in a band measuring 1/2 in wide across the 5 cm width of the microfiber. This band should be about 1 1/2 in above the bottom edge of the strip. Rinse the centrifuge tube with 0•2 ml petroleum ether, and transfer this amount to the band. Repeat twice to complete transfer. Use a pencil and mark the strip 3/16 in above the fat band.

Rinse down the sides of the centrifuge tube with petroleum ether and adjust the volume to about 0•5 ml.

Although the type A microfibers are very clean it is advisable to further clean them by allowing 90% acetone to ascend to the top before storing in the moist chamber. After 12 h of equilibration in the moist chamber the microfiber will contain sufficient absorbed water for this cleanup procedure.

Transfer the 0•5 ml eluate concentrate by means of a 500 µl Hamilton syringe in a band measuring 1/2 in wide across the 5 cm width of the microfiber. This band should be about 1 1/2 in above the bottom edge of the strip. Rinse the centrifuge tube with 0•2 ml petroleum ether, and transfer this amount to the band. Repeat twice to complete transfer. Use a pencil and mark the strip 3/16 in above the fat band.

After drying 5 minutes the strip is hung in the 7 in x 9 in x 12 in chromatographic tank. The bottom edge is immersed 1/4 in into pure acetonitrile contained in the 150 ml beaker. The humidity is elevated in the chromatographic tank by means of a 150 ml beaker of water which has a 2 x 8 in microfiber wick. The pyrethrins develop with the acetonitrile and the fats remain at the application site. After the acetonitrile front has moved 4 in beyond the fat band the strip is removed from the tank, the solvent front marked with a pencil, and allowed to dry 10 minutes. Use a clean pair of scissors and cut on the line above the fat band. Discard this lower portion. The portion of the strip above and up to the solvent frons is cut across into 1/2 inch strips and dropped into a 125 ml Erlenmeyer flask. Pipette 5•0 ml of 80% hexane and 20% benzene mixture into the flask. Agitate in a swirling motion for 2 minutes. Add one ml of water to wet the microfiber which replaces hexane-benzene solvent absorbed in the fiber. Agitate briefly and transfer to a 10 ml volumetric flask. Rinse the flask 3 times with 1•5 ml of hexane and combine in the 10 ml volumetric flask. Make to 10•0 ml with hexane.

In the analysis of tissue fat samples (2 g) transfer the solvent to a graduated 15 ml centrifuge tube and reduce the volume of solvent to 1•0 ml with a stream of dry nitrogen. Adjust the volume to 2•0 ml with hexane.

G.L.C. Determination

For the determination of the residue, the gas chromatograph should be operated under the following parameters:

Column	:	1/8 in x 15 in packed with 1% QF-1 + 1% Epon 1001 on Aeropack-30 (70-80 mesh)*
Injection port temperature	:	210°C
Column temperature	:	180 - 190°F
Detector temperature	:	190°
Nitrogen gas flow	:	40 ml/min

Inject one or more microliters into the gas chromatograph depending upon its sensitivity.

Standard curves can be constructed for each pyrethrin ester if it is deemed necessary; however, greatest sensitivity is obtained for Pyrethrin I. It has been found in the analysis of minute residues that Pyrethrin I is the only ester that may be found. When residue analysis is based upon the detection of Pyrethrin I it is highly desirable to increase the length of the column to 30 to 40 inches so that the elution of Pyrethrin I is delayed enough to make peak height measurement more exacting. Resolution is increased and the Pyrethrin I peak is well removed from Cinerin I as well as peaks from interfering materials.

A 100 picogram injection of pyrethrins gave the following deflection as percent of full scale recorder response.

Cinerin I	9•0%		
Pyrethrin I	17•5%	=	37•8 picograms equivalent
Cinerin II	1•2%		
Pyrethrin II	1•6%		

Elution times relative to aldrin: (Submitted by Petitioner)

Aldrin	=	1•00
Cinerin I	=	2•955
Pyrethrin I	=	4•899
Cinerin II	=	21•12
Pyrethrin II	=	35•93
Lindane	=	1•10
Heptachlor epoxide	=	2•24
DDE	=	2•98
Dieldrin	=	3•92
Endrin	=	4•96
DDT	=	6•83

* This column is used for the analysis of Cinerin I, Pyrethrin I, Cinerin II and Pyrethrin II. A 1/8 in x 30 or 40 in column with the packing as specified above gives a better resolution for Pyrethrin I.

FOOD AND DRUG ADMINISTRATION COMMENTS:

Modifications to the Method

The procedure used during the method tryouts differed somewhat from the above method as written. The steps of the procedure as modified for the retrial are summarized below. The Burdick and Jackson "distilled - in glass" ethyl ether used throughout the second trial was stored under nitrogen (the bottle was purged with pure nitrogen after each use) and kept in a dark cabinet to inhibit peroxide formation.

1. Extraction - The procedure described in the FDA Pesticide Analytical Manual for the triple extraction of fat from milk (Ref 1, section 211•13i(1)), was used in place of the petitioner's extraction procedure because the latter led to difficulties with emulsions. A 2 g sample of the extract fat was carried through the subsequent steps of the procedure.

2. Acetonitrile Partitioning - As per petitioner's method without modification.

3. Florisil Column Chromatography - As per petitioner's method with special attention to the quality of the ethyl ether as noted above. The eluting mixtures of hexane/ether were prepared in small batches just prior to use. Pesticide Residue grade Florisil (60/100 mesh), heated overnight at 450°C and deactivated with 5% water before use, was used for the method tryout. The elution pattern for the batch of Florisil was determined using a mixture containing 100 µg each of pyrethrins, aldrin, heptachlor epoxide, dieldrin and endrin. The chlorinated pesticides were quantitatively eluted with 120 ml of 10% ether in hexane. No pyrethrins eluted with the 10% ether eluate and 95% of the added pyrethrins were recovered in the subsequent 50% ether in hexane eluate (200 ml). (Note: During the retrial, most samples were analyzed prior to t.l.c. cleanup. The 50% ether eluate of the Florisil Column was concentrated to 0•5 ml and diluted to 5•0 ml with petroleum ether before injecting 3 - 6 µl aliquots into the g.l.c. Samples were then re-concentrated to 0•5 ml for application to the t.l.c. micro-fiber sheets. Recovery of pyrethrins was higher, especially at the 0•02 ppm level, when the t.l.c. cleanup was omitted, but the gas chromatograms showed elevated baselines).

4. Thin Layer Chromatographic Cleanup - The procedure was followed as written to the point where the developed t.l.c. microfiber sheet is cut into 1/2 in wide strips. The strips were placed in a 125 ml Erlenmeyer flask and shaken for 2 min with 5•0 ml of 20% benzene in hexane. An amount of water sufficient to saturate the microfiber sheets (1•5 - 2 ml) was added to the flask and the flask was agitated for an additional 2 min. An aliquot of the mixed solvent (3 - 6 µl, representing 30 - 60 mg of milk) was analyzed by electron capture gas chromatography.

5. Gas Chromatography - A Barber-Colman Model 5000 Gas Chromatograph equipped with a concentric type tritium source capture detector was usded for the method tryout. The instrument was operated under the following conditions:

Column	:	4•5 mm id x 4 ft, glass U-tube packed with 1% QF-1 + 1% Epon 1001 on 80/100 mesh Chromosorb W (H.P.)
Temperature	:	Column 190°C; Injector 210°C; Detector 200°C
Carrier Gas	:	Nitrogen at 80 ml - 1 min
Electrometer Sensitivity	:	1 x 10^{-9} amps full scale into a 5 MV recorder
Detector Voltage	:	Operated at an applied potential sufficient to give a peak 50% full scale on the recorder for 0•1 ng heptachlor epoxide. The detector used for the trial was operated at 30 V.

At these settings, the response for Pyrethrin I was 50% of full scale when 2•0 ng total pyrethrins was injected into the instrument. The response for Pyrethrin I was not linear for a series of standards, but the plot of peak height versus amount of pyrethrins was deemed useable in the range of interest. The following retention times were observed:

Cinerin I	3•2 min
Pyrethrin I	5•2 min
Cinerin II	12•5 min (broad)
Pyrethrin II	22•5 min (broad)

Recovery of Pyrethrins from Milk

Reagent blanks and control milk samples were free of interferences at the Pyrethrin I retention time both before and after t.l.c. cleanup. Elevated baselines were evident in the chromatograms of samples analyzed prior to t.l.c. cleanup, but the small amount of fat injected onto the glc column caused no apparent deterioration of the column during the tryout.

The recovery of pyrethrins from milk was calculated on the basis of the heights of the Pyrethrin I peaks in the sample chromatograms to the heights of the Pyrethrin I peaks in standards analyzed concurrently. The table below reports the results obtained by the entire procedure and by analysis prior to t.l.c. cleanup.

The method was tested in FDA Laboratories and the following recovery values were reported:

Sample	Pyrethrins Added ($mg\ kg^{-1}$)	Pyrethrins Found: Pre-t.l.c cleanup ($mg\ kg^{-1}$)	Recovery (%)	Pyrethrins Found: Complete Procedure ($mg\ kg^{-1}$)	Recovery (%)
1	none	nil	--	nil	--
2	none	nil	--	nil	--
3	none	nil	--	nil	--
4	0•10	0•081	81	0•080	80
5	0•10	0•090	90	0•088	88
6	0•10	*	*	0•087	87
7	0•05	0•036	72	0•030	60
8	0•05	0•036	72	0•033	66
9	0•05	0•035	70	0•032	64
10	0•02	0•012	60	0•010	50
11	0•02	0•011	55	0•009	45

* not analyzed prior to t.l.c. cleanup

ENVIRONMENTAL PROTECTION AGENCY COMMENTS:

This method is adequate for enforcement for residues in poultry and eggs. A sample of 20 g poultry tissue or eggs should be used for extraction.

REFERENCES

1. Pesticide Analytical Manual, Volume II, United States Department of Health, Education, and Welfare, Food and Drug Administration, Washington DC, Section 120•128. (revised 11/1/75).
2. W.N. Bruce, J. Agr. Food. Chem, 15 (1) 178-181 (1967).
3. J.A. Schmit, R.A. Henry, R.C. Williams, and J.F. Dieckman, J. Chromat. Sci, 9, 645-651 (1971).
4. Official Methods of Analysis of the Association of Official Analytical Chemists, 12th Edition (1975), Section 29•151 (page 551) and Section 29•093(c) (page 540).
5. M.F. Secreast and R.S. Cail, J. Agr. Food. Chem, 19 (1), 192-193 (1971).
6. J.B. Moore, Paper submitted to the International Symposium on recent advances in research with pyrethrum, the natural insecticide. Minneapolis, Minn., USA, August 30-31 (1971). (unpublished).
7. H.A. Jones, H.J. Ackerman, M.E. Webster, J. Assoc. Offic. Agr. Chem, 35, 771 (1952).
8. H.L. Williams, and J.P. Sweeney, J. Assoc. Offic. Agr. Chem, 39, 975 (1956).