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TOTAL RESIDUE METHOD FOR U-36,059 [1,5-DI-(2,4-DIMETHYLPHENYL)-3-METHYL-1,3,5-TRIAZAPENTA-1,4-DIENE] IN SWINE TISSUE

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SYNOPSIS

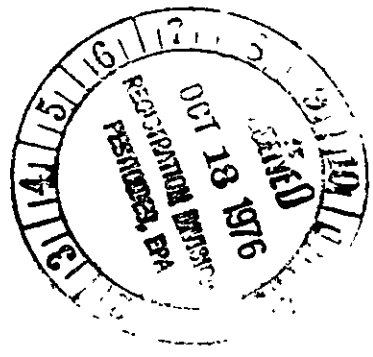
A total residue procedure has been developed for the determination of U-36,059 and its metabolites in swine tissue. The procedure includes the base hydrolysis of U-36,059 and its major metabolites to 2,4-dimethylaniline, steam distillation and continuous extraction of the aniline into an organic solvent, aqueous partitioning for clean-up, and derivitization with heptafluorobutyric anhydride. The heptafluorobutyrylamide derivative of 2,4-dimethylaniline is then analyzed by gas-liquid chromatography.

The method was evaluated by linear regression analysis and by analysis of variance techniques. Recoveries for muscle, liver and kidney tissues were found to be 75-85% with recovery in fat around 65%. Analysis of variance showed the regression factor to be highly significant for all four tissues. Percent recoveries and peak height responses at the low level of fortification (0.05 ppm) relative to the respective blank tissue sample indicated the procedure to be sensitive down to 0.05 ppm.

INTRODUCTION

The miticide U-36,059 has shown efficacy for the control of mites on swine. As a result, residue methods were developed for analysis of fat, muscle, liver and kidney swine tissue. Two methods for the total residue analysis of U-36,059 in soil and fruit crops have been developed and are presently in use[1,2]. Direct application of these methods to swine tissue gave poor recoveries.

The method described in this report is a modification of the total residue method for U-36,059 in oranges. The addition of acid hydrolysis improved recoveries in swine tissue with only a slight increase in chromatogram complexity.



EQUIPMENT

Gas Chromatograph.

A Tracor Model MT-220 Gas Chromatograph equipped with ^{63}Ni electron capture detector.

Gas Chromatographic Column.

A glass U-tube, 6mm o.d., 3 mm i.d., 6 ft. in length, packed with 3% OV-17 on 100-120 mesh Gas Chrom Q.

Syringe.

Precision Scientific 10 µl capacity.

Distillation/Extraction Head.

Constructed according to the design of W. Heizler of CIBA, Ltd. (2) Illustrated in Figure 1.

Special Glassware.

1000 ml one neck, round bottom flask

250 ml one neck, round bottom flask

350 mm Friedrich Condenser

Heating Block.

Reacti Therm - Pierce Chemical Company.

Meat Grinder.

Household hand-operated or power operated grinder.

REAGENTS

2,2,4-Trimethylpentane.

Distilled in glass - Burdick & Jackson.

Heptafluorobutyric Anhydride.

Pierce Chemical Company.

Sodium Bicarbonate.

Analytical Reagent Grade - The Mallinckrodt Chemical Company.

Sodium Hydroxide.

50% aqueous solution - J. T. Baker Chemical Company

1.0N aqueous solution - The Mallinckrodt Chemical Company.

Hydrochloric Acid.

0.1N aqueous solution - The Mallinckrodt Chemical Company.

Sulfuric Acid.

Concentrated - The Mallinckrodt Chemical Company.

Antifoam.

Antifoam A Silicone Defoamer - Dow Corning

Analytical Standard U-36,059.

Reference 11905-VLR-85 The Upjohn Company

Analytical Standard - Heptafluorobutyrylamide Derivative of 2,4-Dimethylaniline.

Reference JLN-XI-41 The Upjohn Company

PROCEDURE

Sample Preparation.

Grind tissue in a meat grinder and package in 50g quantities for analysis. Store the packaged sample at -20°C.

Acid Hydrolysis.

Place 50g of tissue in a one liter, one neck, r.b. (round bottom) flask. Add 200 ml of deionized water and 4 ml of concentrated sulfuric acid. Add approximately 0.2 ml of antifoam and swirl to mix. To prevent bumping add 2 or 3 boileezers and 4 or 5 glass boiling beads to the flask. Attach a reflux condenser and heat to reflux for 2 hours. Remove from heat and cool in an ice bath until flask is below room temperature. Add 40 ml of 50% NaOH solution and continue to cool in ice bath. Add 1 ml of antifoam and 2 or 3 more boileezers and swirl to mix.

Base Extraction/Distillation.

Remove flask from ice bath and attach to arm B (Figure 1) of the distillation/ extraction head. Use teflon sleeves at all ground glass joints; wet each joint with deionized water before connecting. To arm C, attach a 250 ml r.b. flask containing ~~85 ml ± 10 ml~~ of iso-octane (2,2,4-trimethylpentane) and 4 or 5 glass boiling beads. Attach a water condenser to the top of the apparatus. Add

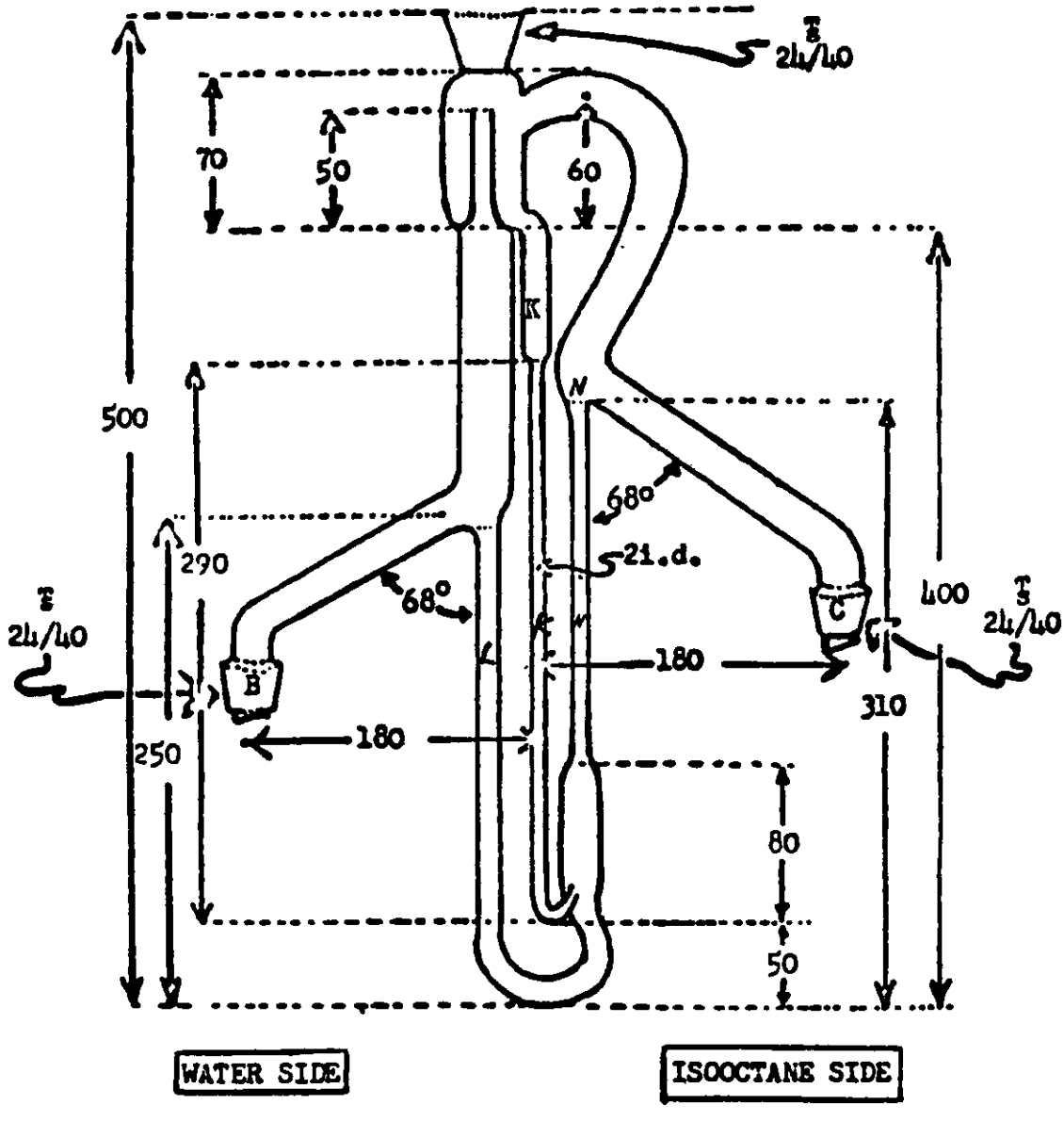


Figure 1, Heitzler Modification of the Bleidner Distillation-Extraction Apparatus.

enough deionized water to fill tubes K and L half full. Place heating mantles on both flasks and heat to reflux adjusting the rate until there is an equal flow of the two phases through tube K. Allow the extraction to continue for two hours: Be sure that the rates remain equal.

CAUTION

If the flow of the organic phase becomes too rapid, the organic phase will begin flowing through tube L instead of tube N. This will cause the flask containing the iso-octane to boil dry resulting in possible loss of sample and damage to the flask.

At the end of two hours turn off the heating mantles and allow the flasks to cool to room temperature.

Aqueous Partition.

Pour the iso-octane from the 250 ml r.b. flask into a 100 ml mixing cylinder. Rinse the flask three times with fresh iso-octane into the mixing cylinder and adjust the total volume to 100 ml. Mix thoroughly by inverting the mixing cylinder repeatedly. Pipet 2 ml of the sample into a 15 ml screw cap centrifuge tube. Extract three times with 1 ml volumes of 0.1N HCl and combine aqueous extracts. Add 1.0 ml of 1.0N NaOH to the extracts and shake to mix. Extract resultant aqueous solution three times with 3 ml volumes of iso-octane and combine. Dilute combined organic extracts to 10 ml.

NOTE: The centrifuge tubes should be shaken for one full minute during each extraction.

Derivatization.

Pour 4 ml of the organic extract into a 15 ml screw cap centrifuge tube and add 10 µl of HFBA (heptafluorobutyric anhydride). Shake tube and heat to 50°C for one hour. Remove from heat and allow to cool. Add 4 ml saturated aqueous $NaHCO_3$ and shake for one full minute. Sample the organic layer and dilute with iso-octane to give a solution containing approximately 5 ng/ml based on 2,4-dimethylaniline.

Gas Chromatography.

GAS CHROMATOGRAPH - Tracor MT-220

COLUMN - 6 ft., 3% OV-17 on 100-120 mesh gas Chrom Q

DETECTOR - electron capture ^{63}Ni

DETECTOR MODE - Rf

DETECTOR VOLTAGE - 40V

PULSE RATE - 240 µsec

PULSE WIDTH - 6 µsec

DETECTOR TEMPERATURE - 265°C

INLET TEMPERATURE - 205°C

OUTLET TEMPERATURE - 250°C

OVEN TEMPERATURE - 114°C

CARRIER GAS - N_2

PURGE GAS - N_2

CARRIER GAS FLOW - 40 ml/min

PURGE GAS FLOW - 0.6 on rotometer

ELECTROMETER SENSITIVITY - 10x4

CHART SPEED - 0.25 in/min

Standard Preparation and Analysis:

Prepare in iso-octane a solution of 13.09 ng/ml of heptafluorobutyrylamide derivative of 2,4-dimethylaniline. This is equivalent to 5.0 ng/ml or 5.0 ppb of 2,4-dimethylaniline. Run a dosage response curve daily by injecting 1, 2, 3, 4, 5 and 6 µl of the standard into the chromatograph . Inject the standards by the "plug" method of injection as follows: The syringe is rinsed with iso-octane effectively filling the needle of the syringe; the appropriate amount of standard is drawn up into the barrel (It is often easier to draw in more of the standard than is required and adjusting the volume by pushing out the excess); and an additional amount of iso-octane is drawn into the barrel to give a total injection size of 7 µl.

Sample Analysis.

Inject 6 µl of the diluted sample by the "plug" method of injection described above. Smaller injection sizes may be used if the sample was not sufficiently diluted. Sample injections should be interspersed with standard injections.

Caluations.

Generate a standard curve by linear regression analysis of peak height vs. concentration of standards. Calculate the amount of 2,4-dimethylaniline on column from the regression line equation and determine the level of U-36,059 total residue in the tissue samples as follows:

$$PPM = \frac{(\text{picogram of 2,4-dimethylaniline on column})(\text{dilution})(100\text{ml})(1.21)^{*}}{(\mu\text{l injected})\ (\text{sample weight in grams})} \times \frac{10^{3}\mu\text{l}}{\text{ml}} \times \frac{1\mu\text{g}}{10^{6}\text{pg}}$$

*correction factor for molecular weight difference between U-36,059 and 2,4-dimethylaniline

Evaluation of Method.

Fat, muscle, liver, and kidney swine tissues were each fortified at 0, 0.05, 0.10, 0.50 and 1.00 ppm of U-36,059. The samples were analyzed according to the procedure outlined above. The results were evaluated by linear regression analysis of ppm added (x) vs. ppm found (y). To determine the significance of the regression factor the variation was partitioned into that due to error and that due to regression by analysis of variance. The results of the analysis of variance and linear regression analysis are given in the appendix and summarized in Table 1.

Table 1: SUMMARY OF EVALUATION

Tissue	Average % Recovery	Regression Equation	Standard Error (PPM)	F-Statistic	Significance of Regression Factor
Fat	64.7	y = 0.6940(x)-0.0063	±0.0146	1611.47	>99.99%
Muscle	76.3	y = 0.7304(x)+0.0064	±0.0140	1941.24	>99.99%
Liver	83.6	y = 0.6598(x)+0.0279	±0.0579	93.15	>99.90%
Kidney	76.4	y = 0.7464(x)+0.0049	±0.0172	1359.21	>99.99%

Discussion.

The gas chromatographic analysis produces chromatograms (see appendix) which, although are complicated with several peaks, are free of interference near the peak of interest. As may be expected the chromatograms from the fat analysis are the least complicated whereas those from the liver analysis are the most complicated of the tissues evaluated.

The evaluation of the method by linear regression analysis found the average recoveries for muscle liver and kidney to be between 75 and 85% with the recovery for fat found to be around 65%. The significance of the regression factor for all four tissues is better than 99.9% indicating a definite linear relationship between ppm added and ppm found.

REFERENCES

[1]Total Residue Method for U-36,059 [1,5-di-(2,4-dimethylphenyl)-3-methyl-1, 3,5-triazapenta-1,4-diene] in Apples, Pears and Soils. J. L. Nappier and R. E. Hornish, Report No. 315-9760-32, September 26, 1975.

[2]Total Residue Method for U-36,059[1,5-di-(2,4-dimethylphenyl)-3-methyl-1, 3,5-triazapenta-1,4-diene] in Oranges. J. L. Nappier, R. E. Hornish and R. E. Lane, Report No. 315-9760-70.

STATISTICAL EVALUATION OF FAT ANALYSIS

PPM ADDED	PPM FOUND	%RECOVERY
0	0	-
0.05	0.0283	56.7
0.10	0.0683	68.3
0.50	0.319	63.9
1.00	0.698	69.8

r = 0.999070

$\hat{C}1$ = -0.006284

$\hat{b}$ = 0.6939513

$(S.E.)_x$ = 0.423674

$(S.E.)_y$ = 0.294283

$\hat{S}_{y.x}$ = 0.014648

n = 5

SOURCE	DF	SS	MS	F	Sign. Level
regression	1	0.345766	0.345766	1611.42	$\alpha < 0.0001$
error	3	0.000644	0.000215		
Total	4	0.346410			

STATISTICAL EVALUATION OF MUSCLE ANALYSIS

PPM ADDED	PPM FOUND	% RECOVERY
0	0	-
0.05	0.035	70.4
0.10	0.084	83.8
0.50	0.391	78.2
1.0	0.727	72.7

r = 0.992288
$\hat{b}$ = 0.730404
$\hat{a}$ = 0 006367
$(S.E.)_x$ = 0.423674
$(S.E.)_y$ = 0.309692
$\hat{S}_{y.x}$ = 0.014047
n = 5

SOURCE	DF	SS	MS	F	Sign. Level
regression	1	0.383046	0.383046	1941.241	$\hat{\alpha} < 0.0001$
error	3	0.000592	0.000197		
total	4	0.383638			

STATISTICAL EVALUATION OF LIVER ANALYSIS

PPM ADDED	PPM FOUND	% RECOVERY
0	0	-
0.05	0.0403	81.6
0.10	0.0998	99.8
0.50	0.442	88.5
1.00	0.646	64.6

$r = 0.984276$

$\hat{b} = 0.659780$

$a = 0.027892$

$(S.E.)_x = 0.423674$

$(S.E.)_y = 0.283997$

$S_{y.x} = 0.057925$

$n = 5$

SOURCE	DF	SS	MS	F	Sign. Level
regression	1	0.312552	0.312552	93.152	$.0001 < \hat{\alpha} < .001$
error	3	0.010066	0.003355		
total	4	0.3226182			

STATISTICAL EVALUATION OF KIDNEY ANALYSIS

PPM ADDED	PPM FOUND	% RECOVERY
0	0	-
0.05	0.0378	75.7
0.10	0.0754	75.4
0.50	0.404	80.7
1.00	0.739	73.9

r = 0.988982
$\hat{b}$ = 0.746357
$\hat{a}$ = 0.004942
$(S.E.)_x$ = 0.423674
$(S.E.)_y$ = 0.316561
$\hat{S}_{y.x}$ = 0.017154
n = 5

SOURCE		SS	MS	F	Sign. Level
regression	1	0.399961	0.399961	1359.21	$\hat{\alpha} < 0.0001$
error	3	0.000883	0.000294		
total	4	0.400843			