Determination of Dicamba and 3,6-Dichlorosalicylic Acid (DCSA) in Liver, Kidney, Skeletal Muscle, Adipose Tissue and Milk.

**SUMMARY:** The residues of dicamba and 3,6-dichlorosalicylic acid (DCSA) are extracted from liver, kidney, skeletal muscle and adipose tissue by blending with ethyl ether and aqueous 10% sulfuric acid at high speeds. An aliquot of the extract is concentrated to approximately 1 ml on a 65°C water bath. The extract is further reduced to near dryness with a nitrogen stream at 50°C. The residues are derivatized using diazomethane, thereby converting the DCSA to the methyl ester of dicamba. The extracts are cleaned up by silica gel column chromatography utilizing a 5% ether in pentane eluent. The residues in milk are extracted in a separatory funnel using ether and 0.1 N HCl. The aqueous phase is separated and further extracted with 50 and 25 ml portions of ether. The three ether layers are combined, concentrated, and cleaned up as described above. Both residues (as the methyl esters of dicamba) were detected by EC-GC using a 10% OV-101 2 mm packed column. Results may be confirmed by EC-GC using an SE-30 wall coated open tubular capillary column (WCOT). Quantitation is based on a standard curve prepared by plotting peak height versus ng of parent compound injected. Recoveries are generally greater than 90% for dicamba and 80% for DCSA at 0.02 to 0.05 ppm.

**APPARATUS:**

- Bath, Hot water, 65°C.
- Beaker, 250 ml.
- Bottles, glass, with polyseal screwcap, 8 and 32 oz.
- Column, Chromatographic, 450 mm x 15 mm (i.d.), Teflon stopcock, fitted with water jacket, Scientific Glass Apparatus Co., Bloomfield, New Jersey.
- Condenser, Vigreaux.
- Cylinders, Graduated, 50 and 250 ml.
- Esterification Apparatus, See diagram p.4.
- Filter paper, glass fibre, GF/A, Whatman.
- Flask, Erlenmeyer with sidearm, 250 ml.
- Flask, Kuderna-Danish, 125 ml with 12 ml graduated distillation receiver tube.

- Funnel, Buchner.
- Funnel, Filter, 75 mm.
- Funnel, Separatory with Teflon stopcock, 125 ml.
- Gas Chromatograph, Hewlett-Packard, Model 5710A or 5700A equipped with 45Ni electron capture detector.
- Glass Wool.
APPARATUS (cont'd):

Homogenizer, Polytron, Brinkmann Instruments, Inc., Cantiague Road, Westbury, New York, 11590.
Oven, 250°C.

REAGENTS:
Carbitol, 2-(2-Ethoxethoxy) ethanol, Aldrich Chemical Co., Milwaukee, Wisconsin.
Diazald, Aldrich Chemical Co., Milwaukee, Wisconsin.
Ethyl Ether, with 2% ethanol preservative, "Distilled in Glass", Burdick and Jackson, Muskegon, Michigan.
Hexane, "Distilled in Glass", Burdick and Jackson, Muskegon, Michigan.
Hydrochloric Acid, reagent.
Methanol, Nanograde, Mallinckrodt.
Pentane, "Distilled in Glass", Burdick and Jackson.
Potassium Hydroxide, reagent.
Sodium Sulfate, anhydrous, granular, reagent.
Sulfuric Acid, reagent.

STANDARDS:
Velsicol Analytical Reference Standards: Dicamba, (2-Methoxy-3,6-dichlorobenzoic acid); DCSA, (3,6-Dichlorosalicylic acid).

PROCEDURE: A. Extraction of Tissues

1. Subdivide the tissue sample into approximately 1 g pieces (this facilitates blending).

2. Weigh 20 g of chopped tissue into a 250 ml beaker.

3. Add 150 ml* of ethyl ether and 5 ml of 10% H₂SO₄.

4. Blend at high speed with a Brinkmann "polytron" for 5 min.

5. Examine tissue for completeness of disintegration. This is conveniently done by compressing the tissue between the beaker wall and the polytron stem. The blending step is complete when the sample possesses a spongy consistency and is totally free of lumps (Note: If further blending is required, add additional ether).

*If a smaller sample is to be analyzed, reduce volume of solvents proportionately.
PROCEDURE (cont'd):

6. Rinse the "polytron" stem with ether and collect in the sample beaker.

7. Filter the solution using a Buchner funnel through filter paper covered by a 1 cm layer of Na₂SO₄.**

8. Rinse the beaker and pulp with approximately 50 ml of ether and filter.

9. Transfer the filtrate to a 250 ml graduated cylinder, rinse flask and adjust the volume of the organic layer to 200 ml with ether.

10. Transfer 50 ml (5 g equivalent) of the non-adipose tissue or 10 ml (1 g equivalent) of the adipose tissue extract to a 125 ml KD concentrator through anhydrous sodium sulfate. Rinse the sodium sulfate with 15 ml ethyl ether.

11. Concentrate the extract to ca 0.5 ml in a 65°C water bath.

12. Evaporate the non-adipose tissue extract to near dryness in a 50°C water bath with a stream of nitrogen. The adipose tissue extract can only be concentrated to approximately 0.4 ml due to lipid coextractives.

13. Add 3 ml of ethyl ether, 0.1 ml methanol and proceed with derivatization.

B. Sample Partitioning Cleanup - Milk Samples Only

1. Transfer 5 g of the milk sample to a 125 ml separatory funnel and add 50 ml of ethyl ether.

2. Add 25 ml of 0.1 N HCl.

3. Separate and save both layers.

4. Extract the acidic aqueous phase with 50 and 25 ml portions of ethyl ether. Combine the 3 ether extracts and discard the aqueous phase.

5. Filter the ether extracts through anhydrous Na₂SO₄ into a 125 ml Kuderna-Danish set up.

**Occasionally the filter paper becomes obstructed during filtration of the adipose extracts. If this occurs transfer unfiltered extract to a freshly prepared funnel and continue filtration into same flask.
PROCEDURE (cont'd):

6. Rinse the Na$_2$SO$_4$ with 10 ml of ether.

7. Concentrate the extract to approximately 1 ml in a 65°C water bath.

8. Evaporate the solution to near dryness with a stream of nitrogen in a 50°C water bath.

9. Add 3 ml of ethyl ether, 0.1 ml of methanol and proceed with derivatization.

C. Sample Derivatization

Esterification: (Reference: Schlenk and Gellerman, Anal. Chem. 32, 1412, (1960)).

1. Set up tubes as shown in diagram below. Tube A serves to saturate the nitrogen flow with ether; the nitrogen flow transmits diazomethane. Add tube D when sample extract is colored.

2. To tube B (18 x 145 mm test tube) add 2 ml of 9 N KOH, 1.4 ml of carbitol, and 1.4 ml of ether.

ESTERIFICATION APPARATUS

trogen
ml/min.

---

Ether

2 ml 9N KOH
+ 1.4 ml Carbitol + 1.4 ml Ether + 0.1 ml MeOH

Sample in Ether

---

Ether

Indicator tube when sample
PROCEDURE (cont'd):

3. Adjust nitrogen flow rate to 30 ml/min.; add approximately 100 mg Diazald to tube B and seal stoppers in tubes A and B. Hold sample tube C so that the tubing is submerged in the solution until a yellow color is obtained (for colored extracts pass diazomethane for 1 minute after yellow appears in tube D).

4. Cap the KD tube for 15 minutes* and then reduce volume to 0.1 ml using a gently stream of nitrogen in a ventilated hood. Perform this last step at room temperature and do not permit the extract to go to dryness.

D. Derivatized Sample Cleanup

Column Preparation:

1. Set up the jacketed columns, fit each with a glass wool plug to contain the silica gel, and open the stopcocks.

2. To a 250 ml separatory funnel add the following:
   a) 70 ml of 5% ethyl ether in pentane.
   b) 20 g of 3% deactivated silica gel. (See Note)

3. Shake well and transfer slurry quickly to the column.

4. Rinse the separatory funnel with 10 ml of the 5% eluent and add to the column.

5. Cover the silica gel bed with 1 cm granular Na₂SO₄.

*If the yellow color completely fades during this interval, recharge the extract with diazomethane until the yellow color reappears, and proceed as in step 4 above. If incomplete methylation is suspected for colored extracts, recharge with diazomethane for 1 minute after yellow appears in tube D.

NOTE: Silica Gel Preparation.

1. Activate silica gel at 250°C for 2½ hours.

2. Transfer to 32 oz bottles and shake on platform shaker for ½ hour to cool.

3. Prepare the 3% deactivated silica gel by adding 6 g of distilled water to 194 g of silica gel. Shake for 15 hours before usage. The silica gel is good for 2 weeks.
PROCEDURE (cont'd):

Column Cleanup:

1. Add 5 ml of the 5% eluent to the methylated sample and transfer to the silica gel column.

2. Rinse the KD with 2 x 5 ml portions of the eluent and allow each rinse to penetrate to the top of the column.

3. Pass another 55 ml of the 5% eluent through the column and discard.

4. Elute the methylated dicamba and DCSA residues (both as the methyl ester of dicamba) with an additional 85 ml of the eluent and collect in a 125 ml KD set up.

5. Add approximately 2 ml hexane and concentrate the eluate on a 65°C water bath to about 2 ml. Dilute to a concentration of 0.5 g/ml for GC analysis.

E. Analysis

1. Gas Chromatographic Conditions:

   a. Packed Column

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Hewlett-Packard, Model 5710A with ¹³⁷\text{Ni} electron capture detector.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column*</td>
<td>1.8 m x 2 mm i.d. Pyrex with 10% OV-101 on Gas Chrom Q, 80-100 mesh.</td>
</tr>
<tr>
<td>Temperatures</td>
<td>oven, 160°C, injection port, 250°C, detector, 300°C.</td>
</tr>
<tr>
<td>Carrier</td>
<td>5% methane in argon, 30 ml/min.</td>
</tr>
<tr>
<td>Attenuation</td>
<td>x 128.</td>
</tr>
<tr>
<td>Chart Speed</td>
<td>0.25 in/min.</td>
</tr>
<tr>
<td>Retention Time</td>
<td>5.0 min.</td>
</tr>
</tbody>
</table>

*Note: Other liquid phases on Gas Chrom Q 80/100 or 100/120 mesh, have been shown to be suitable for the GC determination of dicamba. These are:

   a) 3\% SE-30 (1.2 m x 4 mm i.d.) at 135°C.
   b) 1.5\% OV-17 + 1.95\% QF-1 (1.8 m x 4 mm i.d.) at 155°C.
   c) 3\% OV-1 (1.8 m x 2 mm i.d.) at 130°C.
PROCEDURE (cont'd):

b. Capillary Column

Gas Chromatograph: Hewlett-Packard 5700A with *65*Ni electron capture.
Column: WCOT capillary, SE-30, 30 m x 0.25 mm i.d.
Temperatures:
oven, 165°C.
injector, 250°C.
detector, 300°C.
Carrier: helium at 0.71 ml/min.
Scavenger: 5% methane in argon at 30 ml/min.
Attenuation: x 16.
Retention Time: 5.6 min.

2. Preparation of Standard Solutions:

a. Weigh 10 mg of dicamba in a 100 ml volumetric flask. Dissolve and dilute to the mark with methanol. The concentration of the stock solutions is 1 x 10⁻⁷ g/µl.

b. Transfer 5 ml of the stock solution to a 50 ml volumetric flask and dilute to the mark with methanol (1 x 10⁻⁹ g/µl).

c. Methyleate 1 ml of the 1 x 10⁻⁹ g/µl solution as described on page 4. Concentrate to 1 ml under a stream of nitrogen in a well-ventilated hood.

d. Transfer to a 100 ml volumetric flask and adjust to the mark with hexane. The concentration of this GC standard is 1 x 10⁻¹⁰ g/µl.

e. Prepare a fortifying standard (1 x 10⁻⁹ g/µl) by making a 1:100 dilution of the 10⁻⁷ g/µl standard.

3. Quantitation

Prepare a standard curve by plotting peak height versus the quantity of parent compound injected (nanograms) on log log paper.
PROCEDURE (cont'd):

3. Quantitation (cont'd)

Read the quantity of compound in the aliquot of the injected sample extract from the standard curve. Calculate the concentration of the residue in the sample using the following expression:

$$\text{ppm} = \frac{\text{ng}}{\mu l} \frac{X}{W} \frac{V_s}{X}$$

where:

- ppm = concentration of the compound in the sample in parts per million.
- $\mu l =$ volume of sample extract injected into gas chromatograph in microliters.
- $V_s =$ volume of sample extract in milliliters from which GC aliquot was taken, allowing for all dilutions.
- ng = quantity of compound in injected aliquot as read from the standard curve in nanograms.
- W = weight of the sample taken for analysis in grams.

4. Recovery data

Values for recovery determinations of dicamba and DCSA from milk and tissue check samples fortified at 0.02 to 0.05 ppm are presented below. All milk and tissues were purchased at a store unless otherwise noted. Representative gas chromatograms are presented on pages 11-15.

<table>
<thead>
<tr>
<th>Recovery</th>
<th>Dicamba</th>
<th>DCSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>$\leq 0.01$ ppm</td>
<td>--</td>
</tr>
<tr>
<td>Milk fortified 0.02 ppm dicamba</td>
<td>100% (97)*</td>
<td>--</td>
</tr>
<tr>
<td>Milk fortified 0.02 ppm DCSA</td>
<td>--</td>
<td>80% (71)</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>$\leq 0.01$</td>
<td>--</td>
</tr>
<tr>
<td>Skeletal Muscle fortified 0.02 ppm dicamba</td>
<td>95% (83)</td>
<td>--</td>
</tr>
<tr>
<td>Skeletal Muscle fortified 0.02 ppm DCSA</td>
<td>--</td>
<td>85% (78)</td>
</tr>
</tbody>
</table>

*Values in parenthesis represent recovery corrected for check background.
<table>
<thead>
<tr>
<th></th>
<th>Dicamba</th>
<th>DCSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>$&lt;0.01$</td>
<td>--</td>
</tr>
<tr>
<td>Liver fortified 0.025 ppm dicamba</td>
<td>105% (97)*</td>
<td>--</td>
</tr>
<tr>
<td>Liver fortified 0.02 ppm DCSA</td>
<td>--</td>
<td>96% (83)</td>
</tr>
<tr>
<td>Kidney</td>
<td>$&lt;0.01$</td>
<td>--</td>
</tr>
<tr>
<td>Kidney fortified 0.02 ppm dicamba</td>
<td>112% (112)</td>
<td>--</td>
</tr>
<tr>
<td>Kidney fortified 0.05 ppm dicamba</td>
<td>106% (106)</td>
<td>--</td>
</tr>
<tr>
<td>Kidney fortified 0.02 ppm DCSA</td>
<td>--</td>
<td>100% (100)</td>
</tr>
<tr>
<td>Kidney fortified 0.05 ppm DCSA</td>
<td>--</td>
<td>89% (89)</td>
</tr>
<tr>
<td>Adipose Tissue</td>
<td>$&lt;0.01$</td>
<td>--</td>
</tr>
<tr>
<td>Adipose Tissue check 787-09930 (Dicamba feeding study)</td>
<td>0.01</td>
<td>--</td>
</tr>
<tr>
<td>Adipose Tissue check 787-09930 fortified 0.05 ppm dicamba</td>
<td>115% (91)</td>
<td>--</td>
</tr>
<tr>
<td>Adipose Tissue check 787-09930 fortified 0.05 ppm DCSA</td>
<td>--</td>
<td>125% (103)</td>
</tr>
</tbody>
</table>

**DETECTION LIMIT:** The limit of detection for this method is 0.01 ppm.

*Values in parenthesis represent recovery corrected for check background.*
Figure 1. Representative standard curves for EC-GC quantitation of dicamba and DCSA. The curves show the relative EC/GC response of the two compounds as the methyl ester of dicamba. The individual residues can not be determined separately by this analytical method.
Chart Speed: 1 cm/min
Attenuation: x16
Carrier Flow: 20 mL/min
Carrier Gas: Nitrogen
Detector: Electron Capture
Temperature: Column 80-100 °C
Detector 0-30 °C
Purchased milk, and score purchased milk for protein and fat. Score purchased milk, and score purchased milk for protein and fat.
Figure 4. Representative chromatograms of DCSA standard, corrected.

4. 0.9 µg of DCSA

1.6 mg equivalent injected

0.05 ppm DCSA

Corrected 0.01 ppm DCSA

Ingestion of 1.0 mg equivalent

(Defluorine Feedings Study)

Ingestion of 2.4 mg equivalent

D. Store purchased muscle
C. Cow fat check 787-09990

B. Store purchased beef

A. 0.09 µg of DCSA

Detection Limit: 2.4 mg equivalent
Figure 5. Representative chromatograms of dexamethasone and DCSA standards.

A. 0.13 ng of dexamethasone. B. 0.13 ng of DCSA.

Standard.

D, Store purchased. D. Store purchased.

C, Store purchased. C. Store purchased.

B, Store purchased. B. Store purchased.

A, 0.13 ng of dexamethasone. A. 0.13 ng of DCSA.

Standard.

D, Store purchased. D. Store purchased.

C, Store purchased. C. Store purchased.

B, Store purchased. B. Store purchased.

A, 0.13 ng of dexamethasone. A. 0.13 ng of DCSA.

Standard.