### SYNTHETIC PYRETHRINS/C-ELISA

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DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structure

Rendered fat is extracted with a mixture of acetonitrile/water, centrifuged, and chilled. The acetonitrile phase is diluted with 2% NaCl and the synthetic pyrethrins are partitioned into hexane. A portion of the hexane extract is further purified using alumina column chromatography. The column eluate is concentrated, adjusted to 6% acetonitrile in phosphate-buffered saline, and analyzed with a competition enzyme-linked immunosorbent assay (c-ELISA).

2. Applicability

This method is applicable to bovine, porcine, and poultry fat. Synthetic pyrethrins are used for their insecticidal action. The monoclonal antibody used in this assay will detect the following pyrethroid insecticides: permethrin, cypermethrin, deltamethrin, phenothrin, and fenpropathrin. Flucythrinate and fenvalerate are only weakly detected, and tetramethrin is not recognized by the antibody. Refer to section J.7 for sensitivities to various pesticides. Identification and quantitation of the individual pyrethroids detected is not possible, because ELISA response is integrated over the spectrum of all detectable compounds.
DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Centrifuge (Sorval T6000B with adapter for 50 mL tubes).

b. Centrifuge tubes, 50 mL polypropylene disposable, with cap (Beckton-Dickinson Falcon 2098).

c. Water bath, heated circulating (Freas model 260).

d. 125 mL glass separatory funnel with stopper.

e. Centrifuge tube, glass, 15 mL graduated, with glass stopper.

f. Pasteur pipet, disposable, 5.25" (Scientific Products, Cat. P5202-1).

g. Pipetter, 50-200 µL adjustable, 8-channel, with tips (Titertek digital multichannel pipette, Flow Laboratories).

h. Pipetters, 2-20, 10-100, and 100-1000 µL adjustable single-channel, with tips (Gibson Pipetman P-20, P-100, P-1000).

i. Immulon II 96-well microtiter plates (Dynatech Laboratories).

j. Sealing tape, 8.26 x 13.34 cm, for microtiter plates (Dynatech Laboratories, Cat. 001-010-3501).

k. Skatron 96-well microtiter plate washer (Skatron, Inc.).

l. Incubator, mechanical convection (Precision Scientific Model 4EM).

m. Soxhlet extraction apparatus (Kontes 585000-023).

n. Extraction thimble, cellulose, 43 x 123 mm (Whatman 2800432).

o. Filter paper, Whatman #1, 24 cm.

NOTE: Equivalent apparatus may be substituted for those specified.

2. Instrumentation

a. Bio-Tek Model 310-96 well-plate reader with software (Bio-Tek Instruments, Inc.).

b. IBM PC/AT or compatible computer.
DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

1. Reagent List

a. Acetonitrile (ACN).
b. Hexane.
c. Benzene.
d. Dichloromethane (DCM).
e. Polyoxyethylene-sorbitan (Tween 20, Sigma Chemical Co.).
f. Sodium chloride, reagent grade.
g. Potassium phosphate, monobasic, reagent grade.
h. Potassium phosphate, dibasic, reagent grade.
i. Sodium azide (Sigma Chemical Co.).
j. Magnesium chloride hexahydrate.
k. Diethanolamine, 98% (Sigma Chemical Co.).
l. p-Nitrophenyl phosphate, 5 mg tablets (Sigma Chemical Co.). Store in a freezer at -10°C.
m. Alumina oxide (Bio-Rad acid alumina AG-4 100-200 mesh). Extract with dichloromethane for 24 hours in a soxhlet apparatus. Dry at 130°C for 24 hours; then store at 110°C.
n. Ovalbumin, crude powder, grade II (Sigma Chemical Co.).
o. Anti-mouse IgG (whole molecule) alkaline phosphatase conjugated, affinity isolated antigen, specific antibody developed in goat (Sigma Chemical Co.). Store at 4°C.
p. Py-1 antibody, 1 mg/mL. Monoclonal anti-pyrethroid antibody is referred to as Py-1 (Stanker, L. H., et al., J. Agr. Food Chem. 37:834-839, 1989). Py-1 is produced by the corresponding hybridoma cell line as an ascites fluid. The antibody is purified and stored at -20°C as a stock solution at a concentration of 1 mg/mL in small aliquots. Once an aliquot is thawed, it should be stored at 4°C. When used in the competition ELISA described in this method, using permethrin as a competitor, an I_50 of 1.55 ng ± 0.6 ng should be observed. (For source of supply, contact Chemistry Division, FSIS.)

q. Coating antigen stock solution, 5 mg/mL; 3-phenoxyacetic acid conjugated to bovine serum albumin (3-pba-BSA) in PBS-7. The quality of each lot of conjugate must be evaluated in a c-ELISA using permethrin as a competitor and the monoclonal antibody Py-1. An I_50 of 1.55 ng ± 0.6 ng should be observed. Store at -20°C in small aliquots. (For source of supply, contact Chemistry Division, FSIS.)

NOTE: All solvents should be certified for use in pesticide analysis.
C. REAGENTS AND SOLUTIONS (Continued)

2. Solutions List

| a. | Tween 20, 1% solution. Dilute 1 mL Tween 20 to 100 mL with distilled water. |
| b. | Tween 20, 0.05% wash solution. Dilute 1 mL of Tween 20 to 2 L with distilled water. |
| c. | Phosphate buffered saline, pH 7.0 (PBS-7). Combine 9.65 g Na₂HPO₄, 1.9 g NaH₂PO₄, and 42.5 g NaCl. Add 9 L of distilled water and adjust the pH to 7.0 using 1 N HCl or NaOH. Dilute to 10 L with distilled water. |
| d. | Assay buffer. Dilute 1 mL of 1% Tween 20 solution to 1 L with PBS-7 and mix thoroughly. Store at 4°C. |
| e. | Phosphate buffered saline, pH 9.0 (PBS-9). Add 61.4 g NaCl and 18.76 g Na₂HPO₄ to 6 L of distilled water, and adjust the pH to 9.0 using 1N NaOH. Adjust volume to 7 L. Store at 4°C. |
| f. | Diethanolamine buffer, 10%. Add 97 mL of diethanolamine, 0.2 g NaN₃, and 0.1 g MgCl₂·6H₂O to 800 mL distilled water. Adjust pH to 9.8 with 6N HCl and adjust final volume to 1 L. The buffer is filtered (Whatman #1) and stored at 4°C. |
| g. | pba-BSA, 5 µg/mL solution. Dilute 100 µL of the 5 mg/mL pba-BSA stock solution to 100 mL with distilled water. This provides enough solution to coat ten 96-well plates. Prepare fresh prior to coating plates. |
| h. | Ovalbumin, 3% in PBS-9. Add 4.5 g of ovalbumin powder to a 200-250 mL flask or bottle containing 150 mL of PBS-9 buffer at 30-40°C, cap, and mix until dissolved. Filter through glass wool and collect in a stoppered bottle. Store at 4°C. |
| i. | Py-1 antibody, 1:5000 dilution. Dilute 3 µL Py-1 antibody (1 mg/mL) to 15 mL with assay buffer. Prepare immediately before use. |
| j. | Antimouse IgG, 1:500 dilution in assay buffer. Dilute 30 µL of antimouse IgG, 5 mg/mL, to 15 mL with assay buffer. Prepare immediately before use. |
| k. | p-Nitrophenyl phosphate solution, 1 mg/mL. Dissolve three 5 mg tablets of p-nitrophenyl phosphate in 15 mL 10% diethanolamine buffer. Prepare immediately before use. |
| l. | ELISA control solution. 6% ACN in assay buffer. Dilute 6 mL acetonitrile to 100 mL with assay buffer. |
| m. | Extraction solvent, 85% acetonitrile/water. Add 850 mL acetonitrile to a 1 L volumetric flask or mixing cylinder and dilute to volume with distilled water. |
| n. | 2% NaCl solution. Add 20 g NaCl to a 1 L volumetric flask or mixing cylinder and dilute to volume with distilled water. |
| o. | 6% acetonitrile/assay buffer. Add 6.0 mL of acetonitrile to a 100 mL volumetric flask and dilute to volume with assay buffer. |
**DETERMINATIVE METHOD**

**C. REAGENTS AND SOLUTIONS (Continued)**

<table>
<thead>
<tr>
<th>3. Preparation of Microtiter Plates</th>
<th>a. Using a multichannel pipet, add 100 µL of a 5 µg/mL solution of pba-BSA in distilled water to each well of a 96-well microtiter plate.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>b. Place the plate, uncovered, in an incubator at 37°C, and allow the pba-BSA solution to evaporate overnight. Make sure that all traces of moisture are gone before removing plate.</td>
</tr>
<tr>
<td></td>
<td>c. Store &quot;coated&quot; plates in a sealed container at 4°C, and use within 4 weeks.</td>
</tr>
</tbody>
</table>
DETERMINATIVE METHOD

D. STANDARDS

1. Source
Permethrin (mixed isomers) and other synthetic pyrethroids may be obtained from:

Director
Environmental Monitoring Support Laboratory
Office of Research and Development
Cincinnati, OH 45268

2. Preparation of Standards
a. Permethrin stock standard, 0.5 mg/mL permethrin. Accurately weight out 25 mg of the permethrin standard (mixed isomers) into a 50 mL beaker and dissolve in ca. 30 mL acetonitrile. Transfer quantitatively to a 50 mL volumetric flask and dilute to volume with acetonitrile.

b. Permethrin intermediate standard, 10 µg/mL. Pipet 1.0 mL of permethrin stock standard into a 50 mL volumetric flask and dilute to volume with acetonitrile.

c. Permethrin fortification standard, 2.5 µg/mL. Pipet 5 mL of 10 µg/mL permethrin standard into a 25 mL volumetric flask. Pipet 15 mL acetonitrile into the flask (20 mL final volume) and mix.

d. Permethrin ELISA standard, 0.3 µg/mL. Prepare a 0.30 µg/mL standard to be used in constructing a standard curve by adding 60 µL of the 10 µg/mL permethrin standard into a 15 mL centrifuge tube and diluting to 2 mL with assay buffer.

3. Storage Conditions
Store all standards at 4°C.

4. Shelf Life Stability
a. Stock, intermediate, and fortification standards are stable for at least 6 months.

b. Permethrin ELISA standard must be prepared fresh daily.
DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation
   Pass chilled sample through a food chopper twice. Plug a powder funnel with glass wool. Put approximately 100 g of the comminuted fat into the funnel, place the funnel in a beaker, and render at 100-110°C overnight or until the fat ceases to drop.

2. Sample Extraction
   a. Add 2.5 g of rendered fat into a 50 mL centrifuge tube. Add 25 mL 85% acetonitrile/water extraction solvent, cap securely, and place tube into a 55°C water bath for 5 min. Shake sample vigorously for 3 min. A mechanical shaker may be used for this operation if it has a cycle speed greater than 200 strokes/min.
   b. Immediately centrifuge sample for 2 min at 3000 rpm. Chill sample until fat has solidified. (Do not chill to point of phase separation or freezing of water phase.)
   c. Remove 10 mL of the liquid phase and place in a 125 mL separatory funnel. No suspended particles of fat should be transferred.
   d. Add 50 mL 2% NaCl solution and swirl. Add 15.0 mL hexane, stopper, and shake for 2 min.
   e. Carefully drain and discard the aqueous fraction.
   f. Drain and discard the 10 mL wash. A small amount of hexane may be drained to displace residual water in the stopcock.
   g. Drain the hexane phase into a 15 mL graduated centrifuge tube. Adjust volume to 15 mL with hexane.
   h. Transfer the hexane eluate to a 1 cm x 0.5 cm id column of alumina topped with 2 cm sodium sulfate. Avoid transferring any residual water that may be in the tip of the centrifuge tube. If less sensitivity is required, a suitable aliquot of the eluate may be diluted to 15 mL and applied to the column.
   i. Wash the column with two 5 mL portions of hexane. Discard the washes.
   j. Elute permethrin from the column into a 15 mL centrifuge tube using 5 mL benzene.
   k. Evaporate the organic solvent to dryness under a stream of nitrogen in a 40°C water bath. Dissolve the residue in 60 μL of acetonitrile, then dilute to 1.0 mL with assay buffer.
   l. Analyze sample extracts for permethrin concentration using the competition ELISA described in section F.
DETERRMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

3. Flow Chart Summary

<table>
<thead>
<tr>
<th>Cleanup Flow Chart</th>
<th>ELISA Flow Chart</th>
</tr>
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<tr>
<td>2.5 g sample in 50 mL centrifuge tube</td>
<td>Microtiter plate</td>
</tr>
<tr>
<td>Add 25 mL ACN/water</td>
<td>Add 100 μL pba-BSA</td>
</tr>
<tr>
<td>Heat 5 min at 55° C</td>
<td>Dry overnight at 37° C</td>
</tr>
<tr>
<td>Extract 3 min</td>
<td>pba-BSA coated plate</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Add albumin solution</td>
</tr>
<tr>
<td>Chill to 4° C</td>
<td>Incubate 1 hr</td>
</tr>
<tr>
<td>Chilled sample</td>
<td>Wash 3X</td>
</tr>
<tr>
<td>Take 10 mL aliquot</td>
<td>Albumin-treated plate</td>
</tr>
<tr>
<td>Aliquot (10 mL)</td>
<td>Add standards, controls</td>
</tr>
<tr>
<td>50 mL 2% NaCl</td>
<td>Add 100 μL samples</td>
</tr>
<tr>
<td>15 mL hexane</td>
<td>Add 100 μL antibody</td>
</tr>
<tr>
<td>Shake 2 min</td>
<td>Seal plate</td>
</tr>
<tr>
<td>Discard aqueous phase</td>
<td>Incubate 1 hour (37° C)</td>
</tr>
<tr>
<td>Backwash 10 mL H₂O</td>
<td>Wash 7 x</td>
</tr>
<tr>
<td>Discard aqueous phase</td>
<td>Plate + residual antibody</td>
</tr>
<tr>
<td>Drain hexane on column</td>
<td>Add 50 μL IgG</td>
</tr>
<tr>
<td>Alumina column</td>
<td>Seal plate</td>
</tr>
<tr>
<td>Discard hexane eluate</td>
<td>Incubate 1 hr (37° C)</td>
</tr>
<tr>
<td>Wash 2 x 5 mL hexane</td>
<td>Wash 7x</td>
</tr>
<tr>
<td>(discard washes)</td>
<td>Plate reacted with IgG</td>
</tr>
</tbody>
</table>
| Elute 5 mL benzene | Add 100 μL p-nitroso-
| | phenyl phosphate |
| Benzene in 15 mL tube | Wait 30 min |
| Take to dryness | Read absorbances with |
| Dissolve in 60 μL ACN | plate reader |
| Add 940 μL buffer | Calculate results |
| Go to ELISA | |
DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Competition ELISA

NOTE: All solutions used should be brought to room temperature before being added to microtiter plates.

a. Add 400 µL of 3% ovalbumin solution to each well of an antigen-coated microtiter plate. Incubate the plate at 25-37°C for 1 hour. Wash the plate 3 times with 0.05% Tween 20/water solution. Remove residual wash solution by inverting the plate and rapping repeatedly onto an absorbent surface (e.g., paper towels).

b. Refer to the plate map in section F.2 below. Add 100 µL of 6% acetonitrile in assay buffer to columns 1, 2, and 12, and to all wells in row A except A3. Add 200 µL of 0.3 µg/mL standard solution to well A3.

c. Prepare a standard curve by making seven serial 1:2 dilutions. Transfer 100 µL of standard from well A3 to well A4, and mix by repeated pipetting. Repeat the procedure for wells A4-A5, A5-A6, A6-A8 (A7 is a control), A8-A9, A9-A10, and A10-A11. Remove 100 µL from A11 and discard. (Standard in well A3 is equivalent to 300 ppb permethrin in the sample unless an aliquot is taken in section E.2, step j.)

d. All samples are analyzed in triplicate. Using the plate map in F.2 as a template, add 100 µL aliquots of each sample to each of the three specified wells. After all samples have been applied, any empty wells remaining can be filled with 100 µL of 6% acetonitrile in assay buffer. These will serve as additional controls.

e. Add 100 µL of a 1/5000 dilution of the 1 mg/mL stock solution of Py-1 in assay buffer to all wells except those in column 1. Column 1 is the no primary antibody blank.

f. Seal the plate using a plate sealer or other airtight wrap and incubate for 1 hour at 37°C.

g. Wash the plate 7 times with 0.05% Tween 20 solution. Remove residual wash solution as in step a above.

h. Pipet 50 µL of alkaline phosphatase conjugated goat-antimouse immunoglobulin diluted 1/500 in assay buffer into all wells of the plate.

i. Seal and incubate the plate at 37°C for 1 hour; then wash as in step g.

j. Add 100 µL of a 1 mg/mL solution of p-nitrophenyl phosphate (freshly prepared in 10% diethanolamine buffer) to all wells. Allow color to develop at room temperature for 30 minutes, or until the absorbance of the controls is in the range 1.0-2.0; then record the absorbances of all wells in dual wavelength mode at 405 nm (540 nm reference) using a plate reader.
### DETERMINATIVE METHOD

#### F. ANALYTICAL QUANTITATION (Continued)

2. **ELISA Plate Distribution Map**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>S</td>
<td>S/2</td>
<td>S/4</td>
<td>S/8</td>
<td>C</td>
<td>S/16</td>
<td>S/32</td>
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<td>19</td>
<td>20</td>
<td>21</td>
<td>C</td>
</tr>
</tbody>
</table>

- **B** = Blanks (no antibody; 6% acetonitrile/assay buffer)
- **C** = Controls (6% acetonitrile/assay buffer)
- **S** = Standard (30 ng/well)
- 1-21 = Samples applied to plate (each sample is applied three times)
DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

3. Competition ELISA
Flow Chart

COMPETITION ELISA

ADDITION OF MAB PY-1 AND UNKNOWN SAMPLE

STEP 1
ANTIGEN-COATED MICROTITER WELL

STEP 2

ADDITION OF MAB PY-1 AND UNKNOWN SAMPLE

KEY

\[
\begin{align*}
\circ &= \text{Immobilized 3-pba-BSA} \\
\gamma &= \text{Antibody} \\
\bullet &= \text{Permethrin in unknown sample} \\
\heartsuit &= \text{Reporter antibody} \\
S &= \text{Substrate} \\
P &= \text{Product}
\end{align*}
\]

STEP 3
ALLOW MAB TO PARTITION BETWEEN FREE & IMMOBILIZED ANTIGEN

STEP 4
WASH TO REMOVE NONBOUND PY-1

STEP 5
ADDITION OF SUBSTRATE

S \rightarrow P

WASH

SYNTHETIC PYRETHRINS/C-ELISA
Determination Method

G. Calculations

1. Procedure

NOTE: Since the ELISA antibody integrates its response over the entire spectrum of compounds that it recognizes, it is not possible to determine the identity or actual concentration of the pyrethroids present in a positive sample. All positives are quantitated as ppb permethrin.

a. Each row on the plate is treated as a separate experiment. Determine an average control value for each row by averaging all acceptable control values in the row. Any individual value that appears to be an outlier may be tested and discarded. Once average values have been determined, the percent inhibition for all wells in columns 3-6 and 8-11 can be calculated using the formula:

\[
\text{Percent inhibition} = \frac{\text{Abs. (control)} - \text{Abs. (sample)}}{\text{Abs. (control)} - \text{Abs. (blank)}} \times 100\%
\]

b. Average the three separate values of percent inhibition for each sample. Any individual value that appears to be an outlier may be tested and discarded.

c. Using semi-log paper, plot concentrations of the permethrin standards in ng/well (X axis) versus their percent inhibition values. Connect all points to form a standard curve. Concentrations (as permethrin) of all samples considered positive (see NOTE below) can be determined graphically by constructing a drop line from the sample’s percent inhibition value on the standard curve to the X axis.

Convert concentrations to ppb permethrin by dividing the permethrin concentrations in ng/well by the sample weight in grams/well. If no dilutions are made in section E.2, step j, this value is 0.1 g. If dilutions were made, this value may be calculated using the formula:

\[
g_{\text{sample/well}} = \frac{2.667(W)(A)}{1000V}, \text{ where}
\]

- \(W\) = sample weight in grams
- \(A\) = mL extract from E.2.i applied to alumina column
- \(V\) = final volume of extract in mL

NOTE: It is not uncommon for blank samples to exhibit high background readings. Percent inhibition readings as high as 15-20% have been observed with some blank fat samples. Samples giving inhibition values at or below this level range are likely to show a high incidence of false positives. Reliable detection of probable positive samples requires > 25% inhibition of response.
2. Standard Curves

Standard Curve for Permethrin

% Inhibition vs Permethrin (PPB)
Fortified Standard Curve in Poultry Fat

% Inhibition

bar = standard deviation
N = 15

Permethrin (PPB)
Fortified Standard Curve in Swine Fat

% Inhibition

Permethrin (PPM)

bar = standard deviation
N = 14
Standard Curves for Synthetic Pyrethroids

% Inhibition

Pyrethrins (PPB)

KEY
- phenothrin
- permethrin
- deltamethrin
- cypermethrin
- fenvalerate

1 10 100 1000 10000 100000

100

90

80

70

60

50

40

30

20

10

0

-10

-20
**DETERMINATIVE METHOD**

**H. HAZARD ANALYSIS**

<table>
<thead>
<tr>
<th>1. Method Title</th>
<th>Immunoassay Determination of Synthetic Pyrethrins by C-ELISA</th>
</tr>
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<tbody>
<tr>
<td>2. Required Protective Equipment</td>
<td>Safety glasses, plastic gloves, lab coat.</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>3. Procedure Steps</th>
<th>Hazards</th>
<th>Recommended Safe Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. Reagents</strong></td>
<td>Benzene, Acetonitrile, Hexane</td>
<td>These solvents are all flammable and can produce toxic effects through exposure to the skin, eyes, respiratory system, or ingestion. Benzene is a carcinogen.</td>
</tr>
<tr>
<td><strong>D. Standards</strong></td>
<td>Concentrated standards may be toxic. Exposure paths are the same as described above for reagents.</td>
<td>Prepare standards in a fume hood or glove box. Use protective gear.</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>4. Disposal Procedures</th>
<th>Reagent mixtures used in section E (see above)</th>
<th>Hold in designated cans awaiting disposal by the contractor or in-house specialist.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alumina columns (used)</td>
<td>(see above)</td>
<td>Allow columns to dry in a fume hood. Dispose of packing by pouring into a plastic bag and discarding.</td>
</tr>
</tbody>
</table>
DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

<table>
<thead>
<tr>
<th>Compound</th>
<th>Acceptable Recovery (%)</th>
<th>Analytical Range (ppb)</th>
<th>Repeatability %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permethrin</td>
<td>50-100</td>
<td>50-200</td>
<td>&lt; 30</td>
</tr>
</tbody>
</table>

2. Critical Control Points and Acceptable Control Specifications

<table>
<thead>
<tr>
<th>Record</th>
<th>Acceptable Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagents, solvents (C.1)</td>
<td>Must be free of interfering substances</td>
</tr>
<tr>
<td>Sample weight (E.2.a)</td>
<td>2.50 ± 0.05 g</td>
</tr>
<tr>
<td>Column elution rate (E.2.j-l)</td>
<td>Less than 1.0 mL/min for all elution steps</td>
</tr>
<tr>
<td>Incubation temperature (F.1.g.j)</td>
<td>37° ± 3° C</td>
</tr>
<tr>
<td>Incubation conditions (F.1.g.j)</td>
<td>Plates must be tightly sealed prior to incubation steps</td>
</tr>
</tbody>
</table>

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Analyze permethrin standards at 50, 100, and 200 ppb with three replicates for each compound.

ii. Phase II: Recoveries—Fortified samples must include at least three levels with three replicates each at 50, 100, and 200 ppb using permethrin standards. Each set should include a reagent blank and tissue blank.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation—14 samples for each species monitored, including blanks and tissues fortified in the range 50-200 ppb.

   (a) Samples submitted by supervisor or LSO.

   (b) Report data to Chemistry Division, QSB.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

i. No false positives.

ii. No recovery missed if present at or above 50 ppb (fortified level).

iii. Refer to section J.1 above.
DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intra-laboratory Check Samples
   a. System, minimum contents.
      i. Frequency: At least one check sample biweekly per analyst.
      ii. Blind check samples or random duplicates chosen by the supervisor or LSO. Records to be maintained by the analyst and reviewed by the supervisor and LSO:
         (a) All duplicate findings.
         (b) All recovery values.
         (c) Running average, standard deviation, and CV for all recoveries.
         (d) CUSUM charts.
   b. Acceptability criteria.
      If unacceptable values are obtained, then:
      i. Stop all official analyses for that analyst.
      ii. Investigate and identify probable cause.
      iii. Take corrective action.
      iv. Repeat Phase II of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability
   b. Sample receipt size: Sufficient tissue for all quantitative and confirmational analyses and sample reserve portion (approximately 100 g).
   c. Sample receipt condition: Chilled to frozen.
   d. Sample storage:
      i. Time: 3 months.
      ii. Condition: Store at -4° C or below until analyzed.

6. Sample Set
   Each set must contain a tissue blank, a fortified blank at 50-200 ppb (100 ppb recommended), and the samples to be analyzed.

7. Sensitivity
   a. Lowest detectable limit (LDL), ppb: permethrin (mixed), 25; others, see 7.c.
   b. Lowest reliable quantitation (LRQ), ppb: permethrin (mixed), 50; others, see 7.c.
### J. QUALITY ASSURANCE PLAN (Continued)

c. Py-1 antibody sensitivity for selected pyrethroids.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$I_{50}$ Value (ng)</th>
<th>Method LDL (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permethrin (mixed)</td>
<td>1.5</td>
<td>0.025</td>
</tr>
<tr>
<td>Phenothrin</td>
<td>1.5</td>
<td>0.025*</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>15</td>
<td>0.25*</td>
</tr>
<tr>
<td>Fenpropathrin</td>
<td>17</td>
<td>0.28*</td>
</tr>
<tr>
<td>Cypermethrin</td>
<td>30</td>
<td>0.50*</td>
</tr>
<tr>
<td>Flucythrinate</td>
<td>120</td>
<td>2.0*</td>
</tr>
<tr>
<td>Fenvalerate</td>
<td>350</td>
<td>5.8*</td>
</tr>
<tr>
<td>Tetramethrin</td>
<td>&gt; 600</td>
<td>&gt; 10*</td>
</tr>
</tbody>
</table>

*Estimated limits calculated using $I_{50}$ values. Assumes similar recoveries for all compounds.