

US EPA ARCHIVE DOCUMENT

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AMERICAN CYANAMID COMPANY
AGRICULTURAL RESEARCH DIVISION
PRODUCT DEVELOPMENT
P. O. Box 400
Princeton, New Jersey 08540

Recommended Method of Analysis

PAY-OFF* flucythrinate (CL 222,705): The GLC Determination of
CL 222,705 Residues in Cattle Milk

A. Principle

The bulk of the milk solids are precipitated by the addition of acetone to the sample. After filtration and evaporation of the acetone, CL 222,705 is extracted from the aqueous phase with hexane. Many coextractives are removed by liquid partitioning between hexane and acetonitrile and final clean up is achieved with Florisil column chromatography. Quantitation of CL 222,705 is effected by gas chromatography using an electron capture detector and the external standardization technique.

B. Apparatus

1. Gas Chromatograph: An instrument suitable for use with glass columns and equipped with an on-column injection system should be used. The Tracor Model 222 or equivalent is suitable when equipped with an appropriate electron capture detector and electronic integrator.
2. Recorder: Hewlett Packard Model 3380A recording integrator.
3. Detector: Tracor Nickel-63 high temperature linear pulsed, electron capture detector.
4. Gas Chromatographic Column: 120-cm borosilicate glass tube (2-mm ID, 6-mm OD) bent to fit the chromatograph.
5. Glass Wool: Silane treated (Applied Science Laboratories, No. 14502).
6. Microsyringes: (Hamilton Company, Series No. 700), 10-mcl.
7. Funnels, Filtering: Buchner-type Porcelain Coors 490. Inside diameter 70 mm.

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8. Funnels, Plastic: Disposable, polypropylene (Ace Scientific Supply Company, Incorporated, Linden, New Jersey).
9. Funnels, Separatory: Squibb-type with stopcock of Teflon (Kontes Glass Company, No. K-636030), 500-ml capacity.
10. Flasks, Filtering: With side Pyrex-tube (Corning 5340), 500-ml capacity.
11. Filter Paper, Glass Fiber: (H. Reeve Angel Company, Grade 934AH), 70-mm.
12. Flasks, Round-Bottom: # 24/40 (Kontes Glass Company, No. K-601000), 300-ml, 500-ml capacity.
13. Flasks, Volumetric: (Kontes Glass Company, No. K-621500), 10-, 50-, 100-, and 1,000-ml capacity.
14. Beakers: Pyrex, 500-ml capacity.
15. Graduated Cylinders: (Corning Glass Work, No. 3022), 5-, 10-, 100-, and 1,000-ml capacity.
16. Pipettes, Volumetric: (Corning Glass Work, No. 7100), 1-, 2-, 5-, 10-, 20-, 50-, and 100-ml capacity.
17. Chromatographic Tubes: With reservoir and stopcock of Teflon (Kontes Glass Company, No. K-420380), Size 213, 10 mm X 250 mm.
18. Flash Evaporator: Buchler Instrument, Model PF-10DN or equivalent with a heated water bath in which evaporation flasks can be partially submerged.
19. Analytical Balance: Capable of weighing to the nearest 0.1 milligram.
20. Sartorius Balance or equivalent.
21. Waring Blendor: Or other suitable laboratory blendor with one-quart jar.

C. Reagents

1. Analytical Standard: CL 222,705 analytical grade, known purity, obtainable from American Cyanamid Company, Agricultural Research Division, P. O. Box 400, Princeton, New Jersey 08540.
2. GLC Packing: 3% SP 2401 on 100/120 mesh Supelcoport, Supelco, Incorporated, Cat. No. 1-1978.

3. Solvents, Specially Purified: "Distilled in Glass", Burdick and Jackson Laboratory, Incorporated, Muskegon, Michigan).
 - a. Acetone
 - b. Hexane
 - c. Toluene
 - d. Acetonitrile
4. Florisil: 60/100 mesh (Fisher Scientific Company, Cat. No. F-100). This material in brown bottles as supplied by the manufacturer has been found to be satisfactory. Keep the containers tightly closed except when removing the adsorbent.
5. 10% Sodium Chloride Solution: Dissolve 100 grams of sodium chloride in 1,000 ml distilled water.

D. Preparation of Standard Solutions

1. CL 222,705 Standard

Accurately weigh by difference using an analytical balance 10 mg (+ 1 mg) of CL 222,705 standard of known purity into a 100-ml volumetric flask. Dissolve the material in 100 ml hexane and mix. Designate this solution which contains approximately 100 mcg of CL 222,705/ml as Standard Solution A.

Transfer by pipet a 10-ml aliquot of Standard Solution A to a 100-ml volumetric flask. Dilute to the mark with hexane and mix. Designate this solution which contains approximately 10 mcg CL 222,705/ml as Standard Solution B.

Transfer by pipet a 2.5-ml aliquot of Standard Solution A to a 50-ml volumetric flask. Dilute to the mark with hexane and mix. Designate this solution which contains approximately 5 mcg CL 222,705/ml as Standard Solution C.

2. Gas Chromatographic Working Standard

Transfer by pipet a 5-ml aliquot of Standard Solution B to a 100-ml volumetric flask. Dilute to the mark with hexane and mix. Designate this solution which contains approximately 0.5 mcg CL 222,705/ml as Standard Solution D.

E. Preparation and Conditioning of the Gas Chromatography Column

1. Pack the gas chromatographic column as follows: Insert a glass-wool pledget at the exit end of the tube and attach this end to a vacuum line. Attach a funnel to the entrance end. Apply a slight vacuum to the tube making sure that the glass-wool pledget remains in place. While vibrating the tube with an electric vibrator or by rapid hand tapping, add the packing in small quantities until the tube is filled to within 6 cm of the entrance end. Remove the vacuum line and funnel. Insert a glass-wool pledget at the entrance end compressing it only enough to hold the packing in place.

2. Condition the column overnight at a temperature approximately 25°C higher than the oven temperature specified below. This conditioning step should be conducted with the exit end of the column disconnected from the detector, but with the carrier gas flowing at the recommended rate.
3. Connect the exit end of the column to the detector and set the controls to provide the conditions listed below. Allow the instrument to come to equilibrium.
4. Repeatedly inject alternate 4-microliter portions of Standard Solution D and a sample extract, processed as described below, until the detector has been adjusted for optimum response and resolution of the two peaks has been obtained as illustrated in Figure M-1254.A. Continue with the standard injections until the response is reproducible.

F. Gas Chromatography Conditions*

1. Column Oven Temperature	190°C
2. Injection Port Temperature	245°C
3. Detector Temperature	300°C
4. Gas Flow Rate	30 ml/min
5. Retention Time	7 and 8 minutes (approximately)
6. Chart Speed	0.25 inch/min

G. Linearity Check

The gas chromatograph should be checked for linearity at least weekly and whenever the column new or used is newly installed in the instrument.

1. Transfer 0.5-, 1.0-, 1.5- and 2.5-ml of Standard Solution B to 10-ml volumetric flasks. Dilute to the mark with hexane. These solutions will have concentrations of CL 222,705 of 0.5, 1.0, 1.5 and 2.5 mcg/ml, respectively.
2. When employing peak height as a measure of chromatographic response, determine the appropriate attenuation setting and injection aliquot (between 3 and 6 mcl) of working standard to yield a peak height of approximately 2 inches (51 mm) on the recorder. The conditions so determined should be used for all standards in the study.
3. When employing digital integration for peak area measurements, determine the appropriate injection aliquot (between 3 and 6 mcl) of working standard to yield an area of at least 10,000 counts. The conditions so determined should be used for all standards in the the study.
4. Make at least two injections of this solution at each concentration.

*These conditions listed above are for the Tracor 222, on other instruments minor changes in operating parameters may be required to obtain equivalent performance and resolution of the two peaks as shown in Figure M-1254.A.

5. Plot average height (or area) obtained for a given solution against concentrations (in mcg of CL 222,705) to demonstrate a linear relationship between peak height (or area) and concentration of CL 222,705 the concentration range examined. Significant departure from linearity over this concentration range indicates instrumental or operational difficulties which must be corrected before proceeding.

H. Florisil Suitability Test

Prior to the recovery test, each lot of Florisil should be checked for suitability.

1. Prepare the chromatographic column as described in Section J.4.a.
2. Transfer by pipet a 2-ml aliquot of the Standard Solution D which contains 0.5 mcg CL 222,705/ml into a flask and add 2 ml of hexane and follow the procedure as described in Section J.4.b., and c.
3. Dissolve the residue in 2 ml of hexane and inject 5 μ l into the GLC versus an external CL 222,705 standard using the conditions described in Section F.
4. Using the appropriate calculation, a recovery of at least 85% can be considered satisfactory and the lot of Florisil that was tested can be used for recovery tests and residue analyses.

I. Recovery Test

The ability of the instrumentation and operator to perform the procedure satisfactorily should always be demonstrated by recovery tests before analysis of the unknown samples is attempted.

1. Weigh a 50-g sample of milk into a 16-ounce narrow-mouth bottle.
2. Add by pipet the volume of the fortification solution containing the number of micrograms of CL 222,705 appropriate to the sample size and fortification level to be tested.
3. Mix the sample well and allow to stand for 15 minutes.
4. Continue the extraction, partitioning and cleanup procedures as described in Section J.1., 2., and 3.

J. Analysis of Milk

1. Extraction

Weigh 50.0 g + 1 g of well-mixed milk into a 16-ounce narrow-mouth bottle. Add 150 ml of acetone to the bottle, stopper tightly, and shake the contents vigorously for 2 minutes. Filter the sample by suction through a glass-fiber filter using a porcelain filtering funnel fitted onto a 500-ml filtering flask.

Transfer the filtrate to a 500-ml evaporating flask and evaporate on a rotary-film evaporator at 35°C. Water droplets appearing on the evaporator trap are an indication of completion of acetone evaporation.

2. Partitioning

Transfer the remaining aqueous portion to a 250-ml separatory funnel. Add 50 ml of 10% sodium chloride solution to the separatory funnel. Add 50 ml of hexane, stopper, and shake for 1 minute. Allow the phases to separate and draw off the lower phase (aqueous portion) into a 250-ml separatory funnel. Add another 50 ml of hexane, stopper, and shake for 1 minute. Allow the phases to separate and draw off the lower phase (aqueous portion) into another 250-ml separatory funnel. Extract with another 50 ml of hexane. Two of 50 ml hexane extraction are combined with first hexane extraction into a 250-ml separatory funnel.

Partition the hexane with 80 ml of acetonitrile, stopper, and shake for 30 seconds. Allow the phases to separate, draw off the lower phase into a 500-ml round-bottom flask. Extract with another 80 ml of acetonitrile and combine into an evaporating flask. Evaporate the combined acetonitrile portions to near dryness on a rotary-film evaporator at 35°C.

3. Clean Up on Florisil

- a. Place a glass-wool pledget at the bottom of a 10-mm X 250-mm chromatographic tube. Measure 5 ml of Florisil using a 10-ml graduate cylinder and pour the material slowly into the column. Add approximately 30 ml of hexane to the column and drain the hexane to within 1 cm of the top of the Florisil.
- b. Dissolve the contents of the evaporating flask in 20 ml of hexane and transfer quantitatively to the Florisil column. Position a 250-ml beaker beneath the column and open the stopcock to provide a flow of 5 to 6 drops per second. When the liquid level drains to within 1 cm of the top of the packing, close the stopcock.
- c. Replace the beaker below the column with a 300-ml pear-shaped flask and elute the column with 100 ml of toluene using an effluent rate of 5 to 6 drops per second. When the flow ceases, transfer the flask to a rotary evaporator and evaporate at 35°C to dryness.
- d. Dissolve the residue in 4 ml of hexane (V3) for GLC analysis.

K. Gas Liquid Chromatographic Analysis

1. When employing peak height as a measure of chromatographic response, determine the appropriate attenuation setting and injection aliquot (between 3 and 6 µl) of working standard to yield a peak height for the CL 222,705 of approximately 2 inches (51 mm) on the recorder. The conditions so determined should be used for all samples and standards in the set.

2. When employing digital integration for peak area measurements, determine the appropriate injection aliquot (V5), (between 3 and 6 mcl) of working standard to yield an area of at least 10,000 counts for the CL 222,705. The conditions so determined should be used for all samples and standard in the set.
3. For each sample solution, make a trial injection (V4) under exactly the conditions found in Steps 1 and 2. If the CL 222,705 peak height (in mm) or area (in integrator units) exceeds twice that found for the standard in Steps 1 and 2, transfer by microliter syringe a 50-microliter portion of the sample solution to a graduated centrifuge tube. Keep the flask tightly stoppered throughout the rest of this step and the next. Dilute to the 1 ml mark with hexane and mix well. If the response still exceeds twice the standard response, dilute to the 10 ml mark and make a third trial injection. Continue to dilute by factors of 10 until the response is within twice the standard response.
4. From the dilutions made and the responses observed with the trial injections, estimate the total dilution necessary to match the response of the sample solution to that of working standard.
5. To the flask containing the undiluted sample solution, add the volume of hexane required to provide the dilution factor estimate in Step 3.
6. The following injection sequence should be used. Standard solution in duplicate, sample solution in duplicate, standard solution in duplicate, etc.

L. Calculations

1. When employing peak height as a measure of the chromatographic response, extend the baseline from the start of the first CL 222,705 peak to a point past the second CL 222,705 peak. Measure with a millimeter ruler the height of both peaks from the extended baseline to the apex of each peak and record the sum of the peak heights (the ratio between the peaks should be the same as in Figure M-1254.A). Perform this measurement for both the sample and standard solution.

For each sample average the total peak height for CL 222,705.

Average the total peak height of the standard solution before and following the two sample injections.

2. When employing digital integration for peak area as a measure of chromatographic response, add the area of both CL 222,705 peaks.

For each sample solution average the total area measurements of the CL 222,705.

Average the total peak area of the standard solution before and following the two sample injections.

3. Calculate the total contents of CL 222,705 in the sample by the following equation:

$$\text{CL 222,705 residue (ppm)} = \frac{R(\text{SAMP}) \times (V1) \times (V3) \times (V5) \times C(\text{STD}) \times \text{D.F.}}{R(\text{STD}) \times (W) \times (V2) \times (V4)}$$

Where:

R(SAMP) = Average peaks area or height for the sample solution in area units or millimeters.

R(STD) = Average peaks area or height for the standard solution in area units or millimeters.

V1 = Volume in ml of extraction solvent.

V2 = Volume in ml of (V1) taken for analysis.

V3 = Final volume in ml of sample solution used for GLC analysis (see Section J.3.d).

V4 = Volume of sample solution injected into GLC in ml.

V5 = Volume of standard solution injected into GLC in ml.

C(STD) = Concentration of standard solution used for GLC analysis in mcg/ml.

W = Weight of sample in grams.

D.F. = Dilution factor for sample solution obtained from the "trial dilution procedure" (see Section K., Step 3). Ignore if no dilution is required.

Figure M-1254.A: CL 222,705 Gas Chromatograms of Control and Fortified Milk

