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TITLE

PAY-OFF* Insecticide, flucythrinate (CL 222,705): Validation of GC Method M-1259 for the Determination of CL 222,705 Residues in Cattle Muscle, Liver, Kidney, and Fat

PURPOSE

To validate Method M-1259 for the determination of CL 222,705 residues in cattle muscle, liver, kidney, and fat.

SUMMARY

Method M-1259 was found to be satisfactory for the gas chromatographic determination of CL 222,705 residues in cattle muscle, liver, kidney, and fat. Recovery data to validate the procedure are presented in Tables I to IV. The validated sensitivity of the method is 0.05 ppm in all tissues.

Recoveries ranged from 79.1 to 94.8% for CL 222,705 in muscle with an average of 89.0%; from 71.0 to 102.6% in liver with an average of 82.7%; from 72.4 to 92.0% in kidney an average of 85.1%; and from 84.8 to 101.6% in fat with an average of 92.5% at fortification levels of 0.05 ppm to 0.5 ppm. Apparent residues in the controls were less than 0.001 ppm in muscle, liver and kidney, and 0.002 ppm in fat. Typical gas chromatograms are shown in Figure M-1259.A for muscle, liver, kidney, and Figure M-1259.B for fat.

D. Kim

DK/pms

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TABLE I

TYPE OF EXPERIMENT: RECOVERY STUDY
SAMPLE TYPE: BOVINE MUSCLE
ANALYZED FOR: CL 222,705
METHOD USED: M-1259
VALIDATED SENSITIVITY: 0.0500 PPM
RESPONSE MEASURED AS: PEAK AREA, INTEG. READX.001

$$\text{APP. PPM FOUND} = \frac{R(SAMP) * V1 * V3 * V5 * C(STD) * D.F.}{R(STD) * W * V2 * V4}$$

$$\%\ \text{RCVRY} = \frac{\text{PPM FOUND} * 100}{\text{PPM ADDED}} = \frac{\text{PPM FOUND} * 100}{FV * FC / W}$$

	FORTIFICATION			SAMPLE PREPARATION						STANDARD SOL'N		RESPONSES			
SAMP. NO.	FV (mls)	FC mcg/ml	PPM added	W (gms)	V1 (mls)	V2 (mls)	V3 (mls)	DIL. fac.	V4 (mcl)	V5 (mcl)	C(STD) (mcg/ml)	R(SAMP) (UNITS)	R(STD) (UNITS)	APPARENT PPM FOUND	PERCENT RECOVERY
54.10	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	379	< 0.001	
54.20	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	345	< 0.001	
54.30	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	311	< 0.002	
54.40	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	378	< 0.001	
54.50	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	370	< 0.001	
												AVERAGES		< 0.001	
55.10	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	327	379	0.043	86.3
55.20	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	318	345	0.046	92.2
55.30	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	311	341	0.046	91.2
55.40	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	357	399	0.045	89.5
55.50	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	327	401	0.041	81.5
												AVERAGES		0.044	88.1
56.10	1.00	1.00	0.100	10	150	150	8	1	5	5	0.125	377	410	0.092	92.0
56.20	1.00	1.00	0.100	10	150	150	8	1	5	5	0.125	367	387	0.095	94.8
												AVERAGES		0.093	93.4
57.10	1.00	2.00	0.200	10	150	150	16	1	5	5	0.125	327	377	0.173	86.7
57.20	1.00	2.00	0.200	10	150	150	16	1	5	5	0.125	355	397	0.179	89.4
												AVERAGES		0.176	88.1
58.10	1.00	5.00	0.500	10	150	150	40	1	5	5	0.125	398	425	0.468	93.6
58.20	1.00	5.00	0.500	10	150	150	40	1	5	5	0.125	325	411	0.395	79.1
												AVERAGES		0.432	86.4

N.M. = not measurable (less than 10 UNITS), corresponding for control samples, to the limits given above in the APPARENT-RESIDUE COLUMN. These limits are calculated by insertion of the number 10 into the calculation as the value for R(SAMP) wherever N.M. is indicated. For treated samples, N.M. is expressed as less than the validated sensitivity of the method.

CONTROL samples are indicated by a minus sign before the R(SAMP) value.

TABLE II

TYPE OF EXPERIMENT: RECOVERY STUDY
SAMPLE TYPE: BOVINE LIVER
ANALYZED FOR: CL 222,705
METHOD USED: M-1259
VALIDATED SENSITIVITY: 0.0500 PPM
RESPONSE MEASURED AS: PEAK AREA, INTEG. READX.001

$$\text{APP. PPM FOUND} = \frac{R(SAMP) * V1 * V3 * V5 * C(STD) * D.F.}{R(STD) * W * V2 * V4}$$

$$\%\ \text{RCVRY} = \frac{\text{PPM FOUND} * 100}{\text{PPM ADDED}} = \frac{\text{PPM FOUND} * 100}{FV * FC / W}$$

	FORTIFICATION			SAMPLE PREPARATION						STANDARD SOL'N		RESPONSES		APPARENT	PERCENT
SAMP. NO.	FV (mls)	FC mcg/ml	PPM added	W (gms)	V1 (mls)	V2 (mls)	V3 (mls)	DIL. fac.	V4 (mcl)	V5 (mcl)	C(STD) (mcg/ml)	R(SAMP) (UNITS)	R(STD) (UNITS)	PPM FOUND	RECOVERY
45.10	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	357	< 0.001	
45.20	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	475	< 0.001	
45.30	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	448	< 0.001	
45.40	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	520	< 0.001	
45.50	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	520	< 0.001	
												AVERAGES		< 0.001	
46.10	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	372	475	0.039	78.3
46.20	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	559	545	0.051	102.6
46.30	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	527	530	0.050	99.4
46.40	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	328	448	0.037	73.2
46.50	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	384	448	0.043	85.7
												AVERAGES		0.044	87.8
47.10	1.00	1.00	0.100	10	150	150	8	1	5	5	0.125	325	458	0.071	71.0
47.20	1.00	1.00	0.100	10	150	150	8	1	5	5	0.125	355	458	0.078	77.5
												AVERAGES		0.074	74.2
48.10	1.00	2.00	0.200	10	150	150	16	1	5	5	0.125	349	438	0.159	79.7
48.20	1.00	2.00	0.200	10	150	150	16	1	5	5	0.125	362	438	0.165	82.6
												AVERAGES		0.162	81.2
49.10	1.00	5.00	0.500	10	150	150	40	1	5	5	0.125	355	396	0.448	89.6
49.20	1.00	5.00	0.500	10	150	150	40	1	5	5	0.125	349	408	0.428	85.5
												AVERAGES		0.438	87.6

N.M. = not measurable (less than 10 UNITS), corresponding for control samples, to the limits given above in the APPARENT-RESIDUE COLUMN. These limits are calculated by insertion of the number 10 into the calculation as the value for R(SAMP) wherever N.M. is indicated. For treated samples, N.M. is expressed as less than the validated sensitivity of the method.

CONTROL samples are indicated by a minus sign before the R(SAMP) value.

B.K.

TABLE III

TYPE OF EXPERIMENT: RECOVERY STUDY
SAMPLE TYPE: BOVINE KIDNEY
ANALYZED FOR: CL 222,705
METHOD USED: M-1259
VALIDATED SENSITIVITY: 0.0500 PPM
RESPONSE MEASURED AS: PEAK AREA, INTEG. READX.001

$$\text{APP. PPM FOUND} = \frac{R(SAMP) * V1 * V3 * V5 * C(STD) * D.F.}{R(STD) * W * V2 * V4}$$

$$\%\ \text{RCVRY} = \frac{\text{PPM FOUND} * 100}{\text{PPM ADDED}} = \frac{\text{PPM FOUND} * 100}{FV * FC / W}$$

	FORTIFICATION			SAMPLE PREPARATION						STANDARD SOL'N		RESPONSES		APPARENT	PERCENT
SAMP. NO.	FV (mls)	FC mcg/ml	PPM added	W (gms)	V1 (mls)	V2 (mls)	V3 (mls)	DIL. fac.	I4 (ml)	V5 (mcl)	C(STD) (mcg/ml)	R(SAMP) (UNITS)	R(STD) (UNITS)	PPM FOUND	RECOVERY
61.10	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	397	< 0.001	
61.20	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	397	< 0.001	
61.30	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	409	< 0.001	
61.40	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	409	< 0.001	
61.50	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	435	< 0.001	
												AVERAGES		< 0.001	
62.10	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	345	409	0.042	84.4
62.30	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	354	424	0.042	83.5
62.20	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	390	424	0.046	92.0
62.40	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	353	422	0.042	83.6
62.50	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	314	434	0.036	72.4
												AVERAGES		0.042	83.2
63.10	1.00	1.00	0.100	10	150	150	8	1	5	5	0.125	213	278	0.077	76.6
63.20	1.00	1.00	0.100	10	150	150	8	1	5	5	0.125	252	278	0.091	90.6
												AVERAGES		0.084	83.6
64.10	1.00	2.00	0.200	10	150	150	16	1	5	5	0.125	236	278	0.170	84.9
64.20	1.00	2.00	0.200	10	150	150	16	1	5	5	0.125	253	278	0.182	91.0
												AVERAGES		0.176	87.9
65.10	1.00	5.00	0.500	10	150	150	40	1	5	5	0.125	288	351	0.410	82.1
65.20	1.00	5.00	0.500	10	150	150	40	1	5	5	0.125	298	331	0.450	90.0
												AVERAGES		0.430	86.0

N.M. = not measurable (less than 10 UNITS), corresponding for control samples, to the limits given above in the APPARENT-RESIDUE COLUMN. These limits are calculated by insertion of the number 10 into the calculation as the value for R(SAMP) wherever N.M. is indicated. For treated samples, N.M. is expressed as less than the validated sensitivity of the method.

CONTROL samples are indicated by a minus sign before the R(SAMP) value.

D.F.

TABLE IV

TYPE OF EXPERIMENT: RECOVERY STUDY
SAMPLE TYPE: BOVINE FAT
ANALYZED FOR: CL 222,705
METHOD USED: M-1259
VALIDATED SENSITIVITY: 0.0500 PPM
RESPONSE MEASURED AS: PEAK AREA, IN TEG. READX.001

$$\text{APP. PPM FOUND} = \frac{R(SAMP) * V1 * V3 * V5 * C(STD) * D.F.}{R(STD) * W * V2 * V4}$$

$$\%\ \text{RCVRY} = \frac{\text{PPM FOUND} * 100}{\text{PPM ADDED}} = \frac{\text{PPM FOUND} * 100}{FV * FC / W}$$

	FORTIFICATION			SAMPLE PREPARATION						STANDARD SOL'N		RESPONSES		APPARENT	PERCENT
SAMP. NO.	FV (mls)	FC mcg/ml	PPM added	W (gms)	V1 (mls)	V2 (mls)	V3 (mls)	DIL. fac.	V4 (ml)	V5 (ml)	C(STD) (mcg/ml)	R(SAMP) (UNITS)	R(STD) (UNITS)	PPM FOUND	RECOVERY
71.10	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	244	< 0.002	
71.20	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	277	< 0.002	
71.30	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	275	< 0.002	
71.40	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	275	< 0.002	
71.50	0	0	0	10	150	150	4	1	5	5	0.125	-N.M.	233	< 0.002	
												AVERAGES		< 0.002	
72.10	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	237	267	0.044	88.8
72.20	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	208	262	0.040	79.4
72.30	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	211	233	0.045	90.6
72.40	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	216	233	0.046	92.7
72.50	1.00	0.50	0.050	10	150	150	4	1	5	5	0.125	206	243	0.042	84.8
												AVERAGES		0.044	87.2
73.10	1.00	1.00	0.100	10	150	150	8	1	5	5	0.125	278	303	0.092	91.7
73.20	1.00	1.00	0.100	10	150	150	8	1	5	5	0.125	288	301	0.096	95.7
												AVERAGES		0.094	93.7
74.10	1.00	2.00	0.200	10	150	150	16	1	5	5	0.125	231	259	0.178	89.2
74.10	1.00	2.00	0.200	10	150	150	16	1	5	5	0.125	310	305	0.203	101.6
												AVERAGES		0.191	95.4
75.10	1.00	5.00	0.500	10	150	150	40	1	5	5	0.125	213	223	0.478	95.5
75.20	1.00	5.00	0.500	10	150	150	40	1	5	5	0.125	181	197	0.459	91.9
												AVERAGES		0.468	93.7

N.M. = not measurable (less than 10 UNITS), corresponding for control samples, to the limits given above in the APPARENT-RESIDUE COLUMN. These limits are calculated by insertion of the number 10 into the calculation as the value for R(SAMP) wherever N.M. is indicated. For treated samples, N.M. is expressed as less than the validated sensitivity of the method.

CONTROL samples are indicated by a minus sign before the R(SAMP) value.

M-1259
D. Kim/pms
09/27/82

AMERICAN CYANAMID COMPANY
AGRICULTURAL RESEARCH DIVISION
PRODUCT DEVELOPMENT
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Recommended Method of Analysis

PAY-OFF* Insecticide, flucythrinate (CL 222,705): GLC Method for the Determination of CL 222,705 Residues in Cattle Muscle, Liver, Kidney, and Fat

A. Principle

The CL 222,705 is extracted from muscle, liver, kidney and fat with methanol:methylene chloride. 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. 14501.

6. Microliter Syringes: (Hamilton Company, Series No. 700), 10 mcl.

7. Funnels, Filtering: Buchner-type, Porcelain Coors 490. Inside diameter 70 mm.

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R.K.

8. Funnels, Plastic: Disposable, polypropylene (Ace Scientific Supply Company, Incorporated, Linden, New Jersey).

9. Funnels, Separatory: Squibb-type with Teflon stopcock (Kontes Glass Company, No. K-636030), 500-ml capacity.

10. Flasks, Filtering: With side Pyrex-tube (Corning 5340), 250-ml capacity.

11. Filter Paper, Glass Fiber: (H. Reeve Angel Company, Grdae 934AH), 70 cm.

12. Flasks, Round Bottom: ⧫ 24/40 (Kontes Glass Company, No. K-601000), 300- and 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 equivalet 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.

P.K.

3. Solvents, Specially Purified: "Distilled in Glass", Burdick and Jackson Laboratory, Incorporated, Muskegon, Michigan.

   a. Methanol
   b. Hexane
   c. Acetonitrile
   d. Toluene
   e. Methylene Chloride

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. Methanol:Methylene Chloride (10:90): Add 100 ml methanol to a 1,000-ml volumetric flask and fill to the mark with methylene chloride. Stopper and mix well.

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 of 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 of CL 222,705/ml as Standard Solution C.

2. Gas Chromatographic Working Standards

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 of 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.

S.K.

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 equilibrium.

4. Repeatedly inject alternate 5-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-1259.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 volume 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 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-1259.A.

B.F.

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 over 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, 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 mcl 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 10-g sample of the ground control tissue and transfer to the blendor jar.

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 with extraction, partitioning, and clean-up procedures as described in Section J.2., 3., and 4.

J. Analysis of Animal Tissues

1. Sample Handling

Freeze the tissue sample at -20°F as quickly as possible after the animal has been sacrificed.

Pulverize sufficient dry ice in the bowl of the food chopper to chill the bowl thoroughly. Slowly add portions of the frozen animal tissue of approximately 50 g to the dry ice and continue chopping until the sample is reduced to a fine particle size. It may be necessary to add small pieces of dry ice along with the portions of animal tissue to keep the tissue frozen during this operation. Store the sample in a freezer until the excess carbon dioxide has completely dissipated.

2. Extraction

a. Weigh 10 g of the ground frozen tissue into a Waring blendor jar.

Add 150 ml of 10% methanol in methylene chloride and 20 g of anhydrous sodium sulfate (extract fat without sodium sulfate). Blend the sample for 3 minutes at moderate speed.

b. Filter the homogenate with gentle vacuum through glass fiber-filter paper. Quantitatively transfer to a 500-ml evaporating flask. Remove the solvent on a rotating evaporator with a water bath at 40°C.

3. Partitioning

a. Muscle, Liver, and Kidney

Dissolve the residue using two rinses with 50 ml each of hexane and transfer to a 250-ml separatory funnel. Rinse the flask with 100 ml of acetonitrile and transfer to the funnel. Stopper and shake for 1 minute. Allow the phases to separate and draw off the lower phase (acetonitrile) into a 250-ml separatory funnel. Add 100 ml of fresh hexane to the acetonitrile phase, stopper and shake for 30 seconds. Allow the phases to separate and draw off the acetonitrile phase into a 500-ml evaporating flask. Partition the original hexane phase with an additional 100 ml of acetonitrile. Stopper and shake for 30 seconds. Allow the phases to separate and draw off the lower phase (acetonitrile) into the 500-ml evaporating flask. Evaporate the combined acetonitrile extracts to dryness on a rotary evaporator at 40°C.

b. Fat

Dissolve the residue in the 500-ml round-bottom flask using three rinses with 50 ml each of hexane and transfer to a 500-ml separatory funnel. Rinse the flask with 100 ml of acetonitrile and transfer to the funnel. Stopper and shake for 1 minute. Allow the phases to separate and draw off the lower phase (acetonitrile) into another 500-ml separatory funnel. Add 150 ml of fresh hexane, stopper and shake for 30 seconds. Allow the phases to separate and draw off the lower layer (acetonitrile) into a 500-ml round-bottom flask and discard the hexane layer. Partition the hexane layer (from the first separatory funnel) with a 100 ml of acetonitrile. Stopper and shake for 30 seconds. Allow the phases to separate, draw off the lower phase into another 500-ml separatory funnel and

add 150 ml of fresh hexane. Stopper and shake for 30 seconds. Allow the phases to separate and draw off the lower layer (acetonitrile) into a 500-ml round-bottom flask and discard the hexane layer. Evaporate the combined acetonitrile portions (200 ml) to near dryness on a rotary-film evaporator at 40°C.

4. Clean Up of Florisil

a. Place a glass-wool pledget at the bottom of a 10-mm X 250-mm chromatographic tube. Measure 8 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 eluate the column with 150 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 the eluate at 40°C to dryness. 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 mcl) 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 approximately 10,000 counts for the CL 222,705 peaks. The conditions so determined should be used for all samples and standards 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 the 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-1259.A). Perform this measurement for both the sample and standard solution.

   For each sample solution 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:

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

Where:

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

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

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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.4.c.).

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

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

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-1259.A: CL 222,705 Gas Chromatograms of Control and Fortified Tissues in Cattle

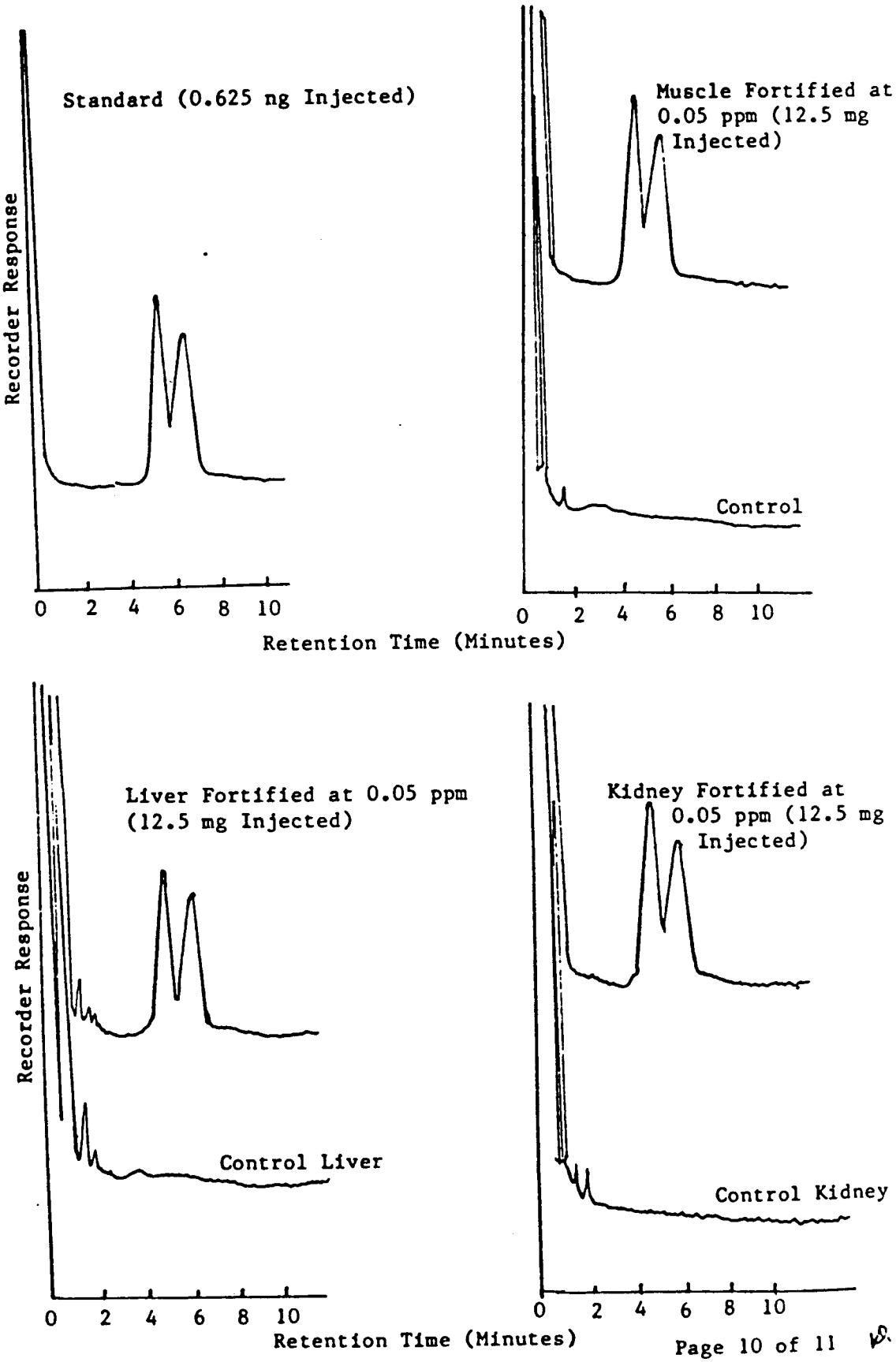
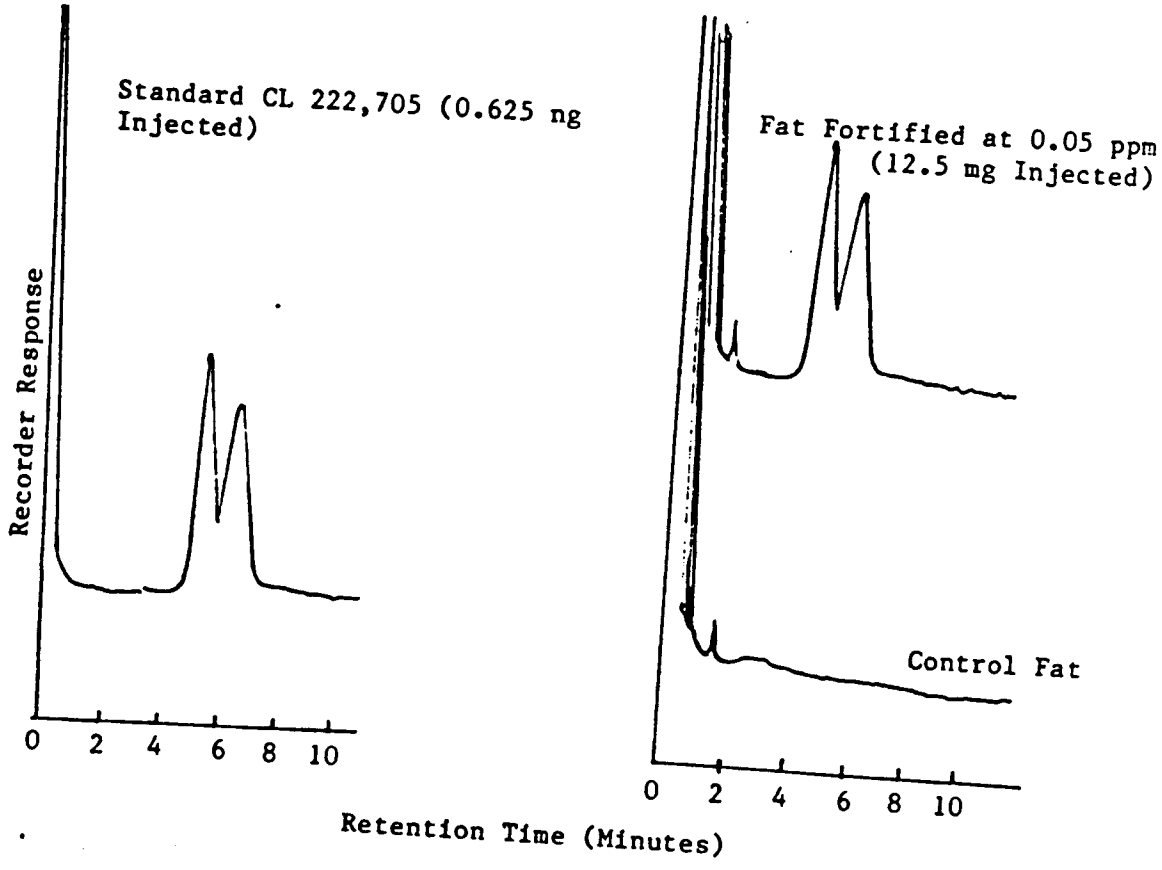


Figure M-1259.B: CL 222,705 Gas Chromatograph of Control and Fortified Cattle Fat



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