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Volume 1

Study Title

Captan: Determination of Residues of Tetrahydrophthalimide, and
cis-3-, trans-3-, cis-5-, and trans-5-Hydroxytetrahydrophthalimide
in Bovine Tissues and Milk Using Gas Chromatography with
Mass-Selective Detection

Captan Task Force Submitter's No.

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Data Requirement

Guideline §171-4(d)

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Study Completion Date

March 13, 1992

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CAPTAN 91-01

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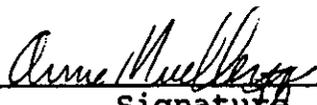
RR 92-018B

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Company: Captan Task Force

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Study Number: CAPT-90-AT-01
CAPTAN 91-01

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Tissues and Milk Using Gas Chromatography with Mass-
Selective Detection

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study meets the requirements for 40 CFR Part 160 with the
following exceptions:

1. The data given in section 11.3, Ruggedness Testing, was
not produced under GLP guidelines.
2. The ion ratios given in the table in section 4.1.5
Analytes, Ions Monitored, Analyte Retention time, and Ion
Ratio, were not produced under GLP guidelines.
3. The spectra given in Appendix C were not produced under
GLP guidelines.

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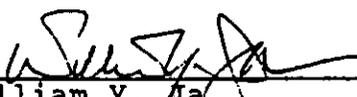
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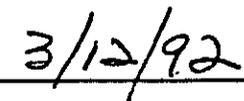
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Selective Detection

QUALITY ASSURANCE STATEMENT

In accordance with ICI policy and procedures for Good Laboratory Practice, the recovery data in this method has been audited by either the Quality Assurance Unit at the Western Research Center, Richmond, CA, or by the Quality Assurance Unit at Morse Laboratories, Inc., Sacramento, CA, U.S.A. The recovery data incorporated in the report accurately reflect the raw data produced to generate the recovery data. The data shown in this report were generated by the same method as described here. The recovery data was obtained during the conduct of study CAPT-90-AT-01 and study CAPTAN 91-01. Captan 91-01 was conducted by Technical Assessment Systems, Inc., Washington D. C.



William Y. Pa
Quality Assurance Officer



Date

CERTIFICATION OF AUTHENTICITY

Study Number: CAPT-90-AT-01
CAPTAN 91-01

I, the undersigned, hereby declare that the analytical portion of these studies were performed under my direction (as Study Director for the study CAPT-90-AT-01 and as Analytical Coordinator for the study CAPTAN 91-01) and that this report represents a true and accurate record of the procedures and results obtained.

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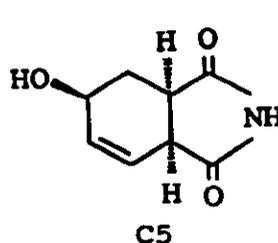
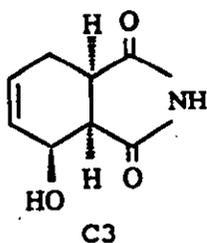
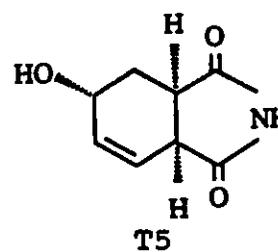
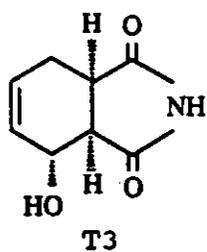
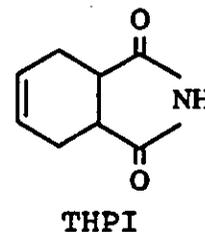
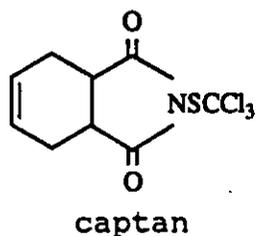
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1 INTRODUCTION AND SUMMARY

This report, hereafter referred to as a method, is intended for the determination of five possible metabolites of captan in bovine tissues and milk. The five metabolites are tetrahydrophthalimide (THPI), *trans*-3-hydroxytetrahydrophthalimide (T3), *trans*-5-hydroxytetrahydrophthalimide (T5), *cis*-3-hydroxytetrahydrophthalimide (C3), and *cis*-5-hydroxytetrahydrophthalimide (C5). THPI is the principal degradate and/or metabolite of captan. The hydroxy-THPI metabolites are believed to be formed from THPI (ref. 14.1). The chemical structures of captan and the five analytes are shown below.



1.1 Scope

This method is used to analyze for five possible metabolites of captan in adipose tissue (fat), kidney, liver, muscle, and milk from cows.

The method described here is based upon Method No. 166 (ref 14.2) and is identical in principle to Meth-18 (ref 14.3) and Meth-26 (ref 14.4). All of the recovery data shown in this method was generated by following either Meth-18 or Meth-26.

It should be noted that neither of the *cis* THPI isomers was found in the feeding study (CAPT-90-AT-01) where cows were dosed with up to 100 mg of captan/kg of feed for a 28-day period. It is therefore unlikely that either of the *cis* isomers will be found in tissues or milk.

1.2 Nomenclature

- 1.2.1 THPI: The IUPAC name is 1,2,3,6-tetrahydrophthalimide. The CAS name is 3a,4,7,7a-tetrahydro-1*H*-isoindole-1,3(2*H*)-dione. The CAS Registry number is 85-40-5.
- 1.2.2 T3 (trans-3-OH-THPI): The IUPAC name is *trans*-3-hydroxy-1,2,3,6-tetrahydrophthalimide. The CAS name is 3a,4,7,7a-tetrahydro-4-hydroxy-1*H*-isoindole-1,3(2*H*)-dione (3a.alpha.,4.alpha.,7a.alpha.)
- 1.2.3 T5 (trans-5-OH-THPI): The IUPAC name is *trans*-5-hydroxy-1,2,3,6-tetrahydrophthalimide. The CAS name is 3a,4,5,7a-tetrahydro-5-hydroxy-1*H*-isoindole-1,3(2*H*)-dione (3a.alpha.,5.alpha.,7a.alpha.)

- 1.2.4 C3 (cis-3-OH-THPI): The IUPAC name is *cis*-3-hydroxy-1,2,3,6-tetrahydrophthalimide. The CAS name is 3a,4,7,7a-tetrahydro-4-hydroxy-1*H*-isoindole-1,3(2*H*)-dione (3a.alpha.,4.beta.,7a.alpha.)
- 1.2.5 C5 (cis-5-OH-THPI): The IUPAC name is *cis*-5-hydroxy-1,2,3,6-tetrahydrophthalimide. The CAS name is 3a,4,5,7a-tetrahydro-5-hydroxy-1*H*-isoindole-1,3(2*H*)-dione (3a.alpha.,5.beta.,7a.alpha.)
- 1.2.6 The five analytes will hereafter be referred to as THPI, T3, T5, C3, and C5.

1.3 Principles

A known weight of sample is macerated with acetone to extract the analytes from the matrix. An aliquot of the acetone extract is diluted with ethyl acetate to force the water out of the acetone solution. The solvent mixture is passed through a bed of anhydrous sodium sulfate to remove particulates and water. The solvents are removed under a stream of dry nitrogen. The residuum is dissolved in hexane. The analytes are partitioned from the hexane into acetonitrile to separate them from the hexane-soluble lipids. The acetonitrile is removed under a stream of nitrogen. The residuum is dissolved in a mixed-solvent solution consisting of toluene and ethyl acetate. The mixed solvent solution is passed through a silica column to cleanup the extract. The solvents are removed from the eluate under a stream of dry nitrogen. The residuum is dissolved in acetonitrile for derivatization. The acetonitrile solution is treated with *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 10% trimethylchlorosilane (TMCS) to form the volatile trimethylsilyl derivatives of the captan metabolites for

gas chromatographic analysis. The acetonitrile solution is analyzed by using capillary gas chromatography with mass-selective detection for good selectivity and detectability.

2 MATERIALS

The equipment and reagents described below were used to generate the data and chromatograms presented in this method. Equipment with equivalent performance specifications, reagents or solvents of comparable purity, and other equipment or techniques may be substituted, except where noted.

2.1 Equipment

- 2.1.1 Usual laboratory glassware and support equipment.
- 2.1.2 High speed laboratory blender, e.g. PowrPulse™ Homogenizer (Craven Labs., #414). Blade for the PowrPulse™ Homogenizer (Craven Labs., #415)
- 2.1.3 Glass centrifuge bottles - 200-mL capacity (Baxter Scientific Products, #C4138-250)
- 2.1.4 Glass centrifuge screw-top tubes - 50-mL capacity (VWR, #21055-022). Screw caps, Teflon-lined, for the 50-mL centrifuge tubes (VWR, #16198-915)
- 2.1.5 Glass centrifuge screw-top tubes - 15-mL capacity (VWR, #21055-000). Screw caps, Teflon-lined, for the 15-mL centrifuge tubes (VWR, #16198-911)
- 2.1.6 Glass culture tubes - 13 x 100 mm with screw cap (VWR, #60826-188). Screw caps, Teflon-lined, for the 13 x 100 mm culture tubes (VWR, #60826-304)

- 2.1.7 Glass graduated cylinder - 100-mL capacity - glass stoppered (VWR, #24760-100)
- 2.1.8 Glass mixing cylinder - 100-mL capacity - glass stoppered (VWR, #24762-117)
- 2.1.9 Glass funnel - 5-cm diameter for steps 3.2.4 and 3.4.7 (VWR, #30232-027)
- 2.1.10 Glass funnel - 7-cm diameter for step 3.5.2 (VWR, #30232-060)
- 2.1.11 Glass 100-mL volumetric flask - glass stoppered
- 2.1.12 N-EVAP™ solvent reduction system (Organomation)
- 2.1.13 Maxi-Mix II vortex mixer (VWR, #58810-185)
- 2.1.14 Heating block with test tube holder (VWR, #13259-005 and #13259-162, respectively)
- 2.1.15 VAC ELUT™ SPS 24 vacuum manifold manufactured by Analytichem International, Inc. (Varian Associates, #1223-4004). Stopcocks for the vacuum manifold (Baxter Scientific Products, #9433)
- 2.1.16 Hewlett-Packard gas chromatograph Model 5890, equipped with capillary split/splitless inlet; Hewlett-Packard Model 7673A high-speed automatic sampler/injector; Hewlett-Packard Model 5970A Mass Selective Detector and data processing system.
- 2.1.17 Bonded, fused silica capillary chromatographic column, 10 m x 0.18 mm i.d., 0.3- μ m film thickness, Durabond (DB) 17; J & W Scientific, Inc., #121-1713

2.1.18 Gas chromatograph inlet liner - 78 mm x 4 mm i.d. silanized borosilicate straight glass insert (HP #19251-60540) containing a 5-6 mm silanized glass wool plug (lightly packed) in the middle. The insert must be silanized by treating with 5% DMCS (dimethylchlorosilane)(v/v) in hexane prior to use. An insert is silanized by immersing the clean dry liner completely in the DMCS/hexane solution for 10 min. Remove and allow to air dry in a fume hood before installing in the instrument.

2.1.19 Silanized glass wool for the gas chromatograph inlet liner (see 2.1.18) (Supelco, #2-0411)

2.1.20 Glass wool for filtration (VWR, #32848-003)

2.1.21 Syringe - 1000- μ L - Teflon-tipped plunger, one each (Supelco, #2-0740M). Syringe - 500- μ L - Teflon-tipped plunger, one each (Supelco, #2-0690M). Syringe - 250- μ L - Teflon-tipped plunger, one each (Supelco, #2-0689M). Syringe - 100- μ L - Teflon-tipped plunger, five each (Supelco, #2-0688M). Syringe - 50- μ L - Teflon-tipped plunger, one each (Supelco, #2-0687M). In order to protect against cross contamination, the five 100- μ L syringes will each be reserved for a specific task (1) for transfer of derivatization agent to reagent blanks and control samples, (2) for transfer of derivatization agent to samples, (3) for transfer of derivatization agent to standards, (4) for transfer of derivatization agent to fortified controls, and (5) for dilution of standards.

2.1.22 Hobart chopper

2.1.23 Autosampler vials (Hewlett-Packard, #5180-4198). Crimp caps for the autosampler vials (Hewlett-Packard, #5061-3370)

- 2.1.24 Gas chromatograph lower inlet seal for the HP5890 (Hewlett-Packard, #18740-20880)
- 2.1.25 Glass 125-mL separatory funnel with Teflon stopcock and glass stopper
- 2.1.26 Centrifuge equipped to accept the 200-mL centrifuge bottle (2.1.3) and capable of operating at 2000 rpm.

2.2 Reagents

- 2.2.1 Acetone: "OmniSolve" (EM Science) (VWR, #EMAX00116-1)
- 2.2.2 Ethyl Acetate: "OmniSolve" (EM Science) (VWR, #EMEX0241-1)
- 2.2.3 Acetonitrile: "High Purity" (Burdick & Jackson) (Baxter Scientific Products, #015-4)
- 2.2.4 Hexane: "OmniSolve" (EM Science) (VWR, #EMHX0296-1)
- 2.2.5 Toluene: "OmniSolve" (EM Science) (VWR, #EMTX0737-1)
- 2.2.6 Regisil RC-3; N,O-bis(trimethylsilyl) trifluoroacetamide containing 10% trimethylchlorosilane. Also known as BSTFA + 10% TMCS (Regis Chemical Company, #270131)
- 2.2.7 Sodium Sulfate: anhydrous, reagent grade, (Mallinckrodt) (Alameda Chemical, #8024-260)
- 2.2.8 BOND ELUT LRC™ disposable solid phase extraction columns (10.0-mL reservoir) containing 500 mg of unbonded silica (Varian, #1211-3036)
- 2.2.9 Dimethylchlorosilane (DMCS) (Pierce, #83400)

2.2.10 Reagent solutions

2.2.10.1 Toluene:ethyl acetate (90:10) v/v.

2.2.10.2 Toluene:ethyl acetate (95:5) v/v.

2.2.10.3 DMCS:hexane (5:95) v/v

2.2.10.4 Note on preparation of v/v solutions: measure each volume separately.

2.1.11 A cylinder of gas chromatograph quality dry nitrogen equipped with a regulator and an outlet suitable for manually directing a stream of nitrogen into a sample.

2.2.12 List of Suppliers

Alameda Chemical	West Sacramento, CA
Baxter Scientific Products	Deerfield, IL
Craven Labs	Austin, TX
Hewlett-Packard	Santa Clara, CA
J & W Scientific, Inc.	Folsom, CA
Organomation	Berlin, MA
Pierce	Rockford, IL
Regis Chemical Company	Morton Grove, IL
Supelco	Bellefonte, PA
Varian Associates	Harbor City, CA
VWR Scientific	Philadelphia, PA

2.3 Reference Materials

The analytical reference standards are available from ICI Americas Inc., 1200 South 47th Street, Box 4023, Richmond CA 94804-0023; Attention: Environmental Science Department Manager.

2.3.1 Reference Standards:

	<u>Standard</u>	<u>Purity</u>	<u>ICI Code</u>
2.2.3.1	THPI: tetrahydrophthalimide.	99.7%	ASW-1369-C
2.2.3.2	T3: trans-3-OH-THPI.	99.0%	MSW-1198-C
2.2.3.3	T5: trans-5-OH-THPI.	97.0%	MSW-1200-C
2.2.3.4	C3: cis-3-OH-THPI.	98.0%	MSW-1197-C
2.2.3.5	C5: cis-5-OH-THPI.	99.5%	MSW-1199-C

2.3.2 Reference standard solution preparation.

2.3.2.1 Prepare separate stock solutions of THPI, C3, T3, C5, and T5 at concentrations of 1000 μg of analyte/mL in acetonitrile. Take the purity of the reference standard material into account when making these stock solutions. Next, combine aliquots of all five analyte stock solutions together to prepare intermediate calibration solutions of 1.0 and 0.1 $\mu\text{g}/\text{mL}$, and dilute with acetonitrile.

For example, for a 1.0 $\mu\text{g}/\text{mL}$ intermediate calibration solution, transfer 100- μL from each stock solution into a 100-mL volumetric and dilute to the mark. For a 0.1 $\mu\text{g}/\text{mL}$ intermediate calibration solution, transfer 10-mL from the 1.0 $\mu\text{g}/\text{mL}$ intermediate calibration solution into a 100-mL volumetric and dilute to the mark. These two intermediate calibration solutions are used to prepare the four concentration levels of working calibration standards. The working calibration standards will be analyzed with the samples and be used to quantitate the samples.

However, before these four working calibration standards can be used, they must first be derivatized. These

working calibration standards are prepared (diluted down from the intermediate calibration solutions) and derivatized at the same time that a set of samples are derivatized (see 3.7.2).

- 2.3.2.2 When a set of samples is ready for derivatization, prepare the working calibration standards in autosampler vials. For the analysis of tissue samples, the four concentration levels are 0.1, 0.05, 0.025, and 0.005 $\mu\text{g/mL}$. For the analysis of milk samples, the four concentration levels are 0.2, 0.1, 0.05, and 0.01 $\mu\text{g/mL}$. The lowest concentration working calibration standard is set at a level that is half of the limit of quantitation (LOQ) (see section 11.2). (Note that the standards used to produce the milk data in Table 5 were 0.1, 0.05, 0.02, and 0.005 $\mu\text{g/mL}$, where the LOQ for this study was validated at 0.005 ppm).

Working calibration standards may be prepared at the time that samples are to be derivatized by suitable dilution of the intermediate calibration standards by weight or by using volumetric flasks. Alternatively, they may be prepared by the dilution sequence described below using the syringes specified in section 2.1.21 for all transfers. (1) For the 0.2 $\mu\text{g/mL}$ working calibration standard, transfer 1000 μL of acetonitrile to an autosampler vial. Remove 200 μL of acetonitrile from the vial. Then transfer 200 μL of the 1.0 $\mu\text{g/mL}$ intermediate calibration solution to the vial. Immediately cap the vial and invert to mix. (2) For the 0.1 $\mu\text{g/mL}$ working calibration standard, transfer 1000 μL of the existing 0.1 $\mu\text{g/mL}$ intermediate calibration solution to an autosampler vial. Immediately cap the vial. (3) For the 0.05 $\mu\text{g/mL}$ working calibration standard, transfer 1000 μL of acetonitrile to an autosampler vial. Remove 50 μL of

acetonitrile from the vial. Then transfer 50 μ l of the 1.0 μ g/mL intermediate calibration solution to the vial. Immediately cap the vial and invert to mix. (4) For the 0.01 μ g/mL working calibration standard, transfer 1000 μ L of acetonitrile to an autosampler vial. Remove 100 μ L of acetonitrile from the vial. Then transfer 100 μ l of the 0.1 μ g/mL intermediate calibration solution to the vial. Immediately cap the vial and invert to mix. Proceed with the derivatization step as discussed in 3.7.2.

In a similar manner, prepare proper levels listed above of the working calibration standards required for the analysis of tissue samples.

- 2.3.2.3 As a note concerning the transfer of solutions containing the analytes, use a glass barreled syringe equipped with a plunger that has a Teflon tip. Prior to the actual transfer, rinse the syringe several times with the solution to insure that the demand capacity of the glass wall active sites have been satisfied.[?] To prevent analyte carry-over when making more than one transfer, transfer the more dilute solution before rinsing and making a subsequent transfer of a more concentrated solution.

3 ANALYTICAL PROCEDURE

The preparation and extraction steps are different for the tissues and the milk prior to step 3.5 Solvent Partition/Cleanup, and will be discussed separately prior to step 3.5.

Store samples in either the original containers as purchased, or in glass containers equipped with Teflon-lined caps.

3.1 Preparation of the Milk Samples

- 3.1.1 Allow frozen samples to completely thaw. Samples, that have been stored in paper cartons or plastic jugs, may be thawed by placing them in lukewarm water (30-35°C) or leaving them overnight at room temperature. However, do not allow the temperature of the samples to exceed 8°C.
- 3.1.2 Mix the thawed samples well by shaking vigorously to generate a homogenous suspension. If necessary, the sample may be transferred to a larger vessel for mixing purposes.
- 3.1.3 Keep the samples in a refrigerated state (1-8°C) during the weighing process. Samples may be weighed 1-2 days prior to analysis. However, they must be stored frozen until extraction and must be allowed to thaw to a temperature of 1-8°C prior to extraction.

3.2 Extraction of Milk Samples

- 3.2.1 Weigh a 25.0 g sample into a 200-mL centrifuge bottle. If the sample had been previously frozen, allow it to thaw before adding acetone.
- 3.2.2 Add 75-mL of acetone to the bottle. A precipitate or curdle will form when acetone is added.

During this step of the analysis, initiate preparation of a "reagent blank" sample, starting with the 75-mL of acetone. The reagent blank sample is put through the entire process in exactly the same way as a milk sample. The reagent blank is designed to demonstrate that the solvents and reagents used throughout the procedure do

not significantly contribute to the chromatographic background response.

3.2.3 Blend for 5 min using a PowrPulse™ homogenizer

3.2.4 Centrifuge at approximately 1000 rpm for 6 min.

3.2.5 Decant the supernatant through a funnel containing glass wool into a 100-mL mixing cylinder to remove floating solids.

3.2.6 Proceed with step 3.5 Solvent Partition/Cleanup.

3.3 Preparation of the Tissue Samples

3.3.1 Grind the frozen tissue samples in a Hobart chopper while maintaining the sample in a frozen state by adding dry ice.

3.3.2 Allow all of the dry ice to sublime in a well ventilated area.

3.3.3 After the dry ice has dissipated, weigh the samples to be extracted (see 3.4.1). Samples may be weighed 1-2 days prior to extraction. However, they must be stored frozen until extraction and must be allowed to thaw for a minimum period prior to extraction.

3.4 Extraction of Tissue Samples

3.4.1 Weigh a 10.0 g sample into a 200-mL centrifuge bottle. If the sample has been preweighed, allow it to thaw to a temperature of 1 to 8° C for a minimum period.

3.4.2 Add 50-mL of acetone to kidney, liver, and muscle samples. Add 60-mL of acetone to the fat. Note that the fat procedure described here is different from the other three tissues. The need to alter the procedure was necessitated by the formation of a 'whipped cream' emulsion during the analysis of omental fat. Although other types of fat that do not form the emulsion have been successfully analyzed by following the procedure described for the other three tissues, the fat procedure described here assumes that all fat samples will form an emulsion.

Blend for 5 min using a PowrPulse™ homogenizer. The tissue will usually plug-up the blender blade fairly quickly. Stop the homogenizer after 15-30 seconds to remove plugged tissue using a clean instrument and then continue blending for 4.45 to 5.0 min. Repeat removal of tissue from the homogenizer blades as necessary. Blending of the fat may form an emulsion with a 'whipped cream' consistency.

During this step of the analysis, initiate preparation of a "reagent blank" sample, starting with the 50- or 60-mL of acetone. The reagent blank sample is put through the entire process in exactly the same way as a tissue sample. The reagent blank is designed to demonstrate that the solvents and reagents used throughout the procedure do not significantly contribute to the chromatographic background response.

3.4.3 Centrifuge the kidney, liver, and muscle at approximately 1000 rpm for 6 min to clearly separate the layers. Centrifuge the fat at approximately 2000 rpm for 15 min. For the muscle, kidney, and liver, continue on with the next step. For the fat, skip to step 3.4.9.

- 3.4.4 Decant the supernatant into a 100-mL graduated cylinder.
- 3.4.5 Add 10-mL of acetone to the centrifuge bottle. Use a stirring rod to thoroughly resuspend the sedimented material at the bottom of the bottle.
- 3.4.6 Centrifuge again at approximately 1000 rpm for 6 min.
- 3.4.7 Decant the supernatant through a funnel containing glass wool into the graduated cylinder discussed in 3.4.4 above to combine the acetone extracts.
- 3.4.8 Adjust the volume in the graduated cylinder to 60-mL using acetone. Stopper the cylinder and invert it to mix. Skip to step 3.5, Solvent Partition/Cleanup.
- 3.4.9 (This is continued from 3.4.3 for the fat only.) Decant the supernatant from the fat into a 100-mL mixing cylinder. (Note that no adjustment of the volume is necessary.)
- 3.4.10 Stopper the cylinder and invert it to mix.
- 3.4.11 If the solubility of the fat in acetone has not been previously determined, transfer 10-mL of the acetone extract to the 15-mL graduated centrifuge tube. If the factor has already been determined for a similar sample of fat, skip to step 3.5, Solvent Partition/Cleanup.
- 3.4.12 Evaporate the acetone from the 15-mL centrifuge tube until a constant volume is obtained using the N-Evap solvent reduction apparatus with the water bath maintained at 30 to 35°C. The time for this step should be 30 min or less.

- 3.4.13 Determine the volume of residuum in the 15-mL centrifuge tube. This volume represents the amount of fat that was soluble in the acetone. The final result reported for the fat sample will be adjusted to reflect this soluble fat.

For example, if 1.0-mL of fat residue remains in the 15-mL centrifuge tube, 10 % of the extract volume is not acetone $[(1.0 \text{ mL}/10 \text{ mL}) \times 100\%]$. The total liquid volume resulting from the extraction of the fat is therefore 10%, or 6 mL greater than the 60-mL of acetone that was added to the sample. In step 3.5, Solvent Partition/Cleanup, 6-mL of acetone extract is transferred for further workup for each tissue sample. Since 10 g of sample is extracted using 60-mL of solvent, this 6-mL represents 1 g of sample for the kidney, liver, and muscle. But since the final solvent volume resulting from the extraction of 10 g of fat was not 60 mL, but 66 mL, a 6-mL aliquot of the acetone fat extract contains about 10% less sample, or 0.91 g of fat $[(10 \text{ g}) \times (6 \text{ mL}/66 \text{ mL})]$. Therefore, for the fat samples, the calculated results expressed in units of μg , will be divided by 0.91 g to convert to the reported result of $\mu\text{g/g}$.

Proceed with step 3.5 Solvent Partition/Cleanup.

3.5 Solvent Partition/Cleanup

- 3.5.1 For the tissues, transfer 6.0-mL (= 1.0 g of sample, except for the fat as noted in step 3.4.13) of the acetone supernatant to a 125-mL separatory funnel. For the milk, transfer 8.0 mL (= 2.0 g of sample) of the acetone supernatant to a 125-mL separatory funnel.

3.5.2 Add 25-mL of ethyl acetate and shake for 1 minute to produce a homogenous solution. Filter the solution through a bed of approximately 25 g of anhydrous sodium sulfate held in place in a funnel by a plug of glass wool and into a 50-mL screw-top glass centrifuge tube. Note, the sodium sulfate will harden after contact with the solution. Next, wash (an easy single inversion is sufficient for these wash steps) the separatory funnel twice with 5-mL of ethyl acetate each time. Globules of sample may adhere to the walls of the separatory funnel. These globules will remain on the walls of the funnel after the ethyl acetate washes have been completed. Pass each wash separately through the sodium sulfate into the centrifuge tube. **THE ANALYSIS CAN BE DELAYED AND CAPPED SAMPLES STORED AT $4^{\circ} \pm 3^{\circ}\text{C}$ FOR UP TO TWO DAYS IF NECESSARY AFTER COMPLETION OF THIS STEP.**

3.5.3 Place the 50-mL screw-top centrifuge tube into the N-EVAP solvent reduction system filled with water that is maintained at 30° to 35°C . With the nitrogen flow rate set so as to cause a 2 mm dent on the surface of the solvent, reduce the solvent volume to 0.1 to 0.2 mL (see note below concerning an optional temporary discontinuation of the process during this step before the volume has dropped below 1 mL). Next, manually evaporate the remaining solvent with high purity, dry nitrogen. This is done by directing a stream of nitrogen from the nitrogen cylinder into the tube held at about a 30° angle towards the semi-liquid residue via a disposable Pasteur pipet attached with flexible tubing. Position the pipet tip about 1 inch above the surface of the residue. Use a flow rate of nitrogen that is about 2 L/min. The sample is sufficiently dry when no further reduction in the sample volume is observed.

THIS SOLVENT REDUCTION STEP CAN BE DELAYED AND CAPPED SAMPLES STORED AT $4^{\circ} \pm 3^{\circ}\text{C}$ FOR UP TO TWO DAYS IF NECESSARY ONLY IF THE SOLVENT VOLUME HAS NOT BEEN REDUCED TO LESS THAN 1 mL. If the solvent volume has been reduced to less than 1 mL, the procedure should be continued through step 3.5.5. This 3.5.3 solvent reduction step may take from 3 to 4 hr.

- 3.5.4 Add 5-mL of hexane to the centrifuge tube and vortex for 1 min to dissolve all of the residuum. Add 5-mL of acetonitrile, cap the tube, and shake mixture for 1 min.
- 3.5.5 Allow the phases to separate. Draw off and discard the upper, hexane layer using a disposable Pasteur pipet. Add another 5-mL of hexane to the remaining acetonitrile layer in the tube. Cap the tube, and shake for 1 min. Remove and discard the upper, hexane layer as before. **THE ANALYSIS CAN BE DELAYED AND CAPPED SAMPLES STORED AT $4^{\circ} \pm 3^{\circ}\text{C}$ FOR UP TO TWO DAYS IF NECESSARY AFTER COMPLETION OF THIS STEP.**
- 3.5.6 Reduce the volume of the remaining acetonitrile by use of the N-EVAP maintained at 30 to 35°C. Reduce the solvent volume to 0.1 to 0.2 mL, and complete the evaporation using nitrogen following the procedure described in 3.5.3.
- 3.5.7 Add 2-mL of toluene:ethyl acetate (95:5) to the centrifuge tube. Vortex to dissolve residuum.
- 3.6 Adsorption Column Cleanup
- 3.6.1 Place the required number of silica BOND ELUT™ LRC solid-phase extraction columns into the VAC ELUT SPS 24 Vacuum Manifold. With the manifold set to the "waste"

position, pre-wet the BOND ELUT LRC extraction columns with approximately 10-mL of toluene. For this step, and for the subsequent steps, adjust the flow rate of each column to be no more than 2 to 3 drops/sec. Allow the vacuum to cause the solution to enter the column packing; do not allow the top of the solvent to drop below the top of the frit that holds the silica gel in place. Use the manifold stopcocks to control the flow and to stop the flow when the solvent has reached the top of the frit.

3.6.2 Use a disposable Pasteur pipet to transfer the toluene:ethyl acetate (95:5) solution containing the analytes from step 3.5.7 (Solvent Partition/Cleanup) onto the BOND ELUT LRC extraction column. The vacuum manifold should be in the "waste" position. Control the flow rate and level of solvent as described in 3.6.1.

3.6.3 Add 5-mL of toluene:ethyl acetate (90:10) to the 50-mL centrifuge tube, swirl the tube and then transfer the solvent to the BOND ELUT LRC extraction column. The vacuum manifold should be in the "waste" position. Control the flow rate and level of solvent as described in 3.6.1.

3.6.4 With the vacuum manifold in the "collect" position, add 5-mL of ethyl acetate to the column to elute the analytes and collect the eluate in a 13 x 100 mm screw-cap culture tube equipped with a Teflon-lined cap. **THE ANALYSIS CAN BE DELAYED AND CAPPED SAMPLES STORED AT 4° ± 3°C FOR UP TO TWO DAYS IF NECESSARY AFTER COMPLETION OF THIS STEP.**

3.6.5 Using the N-EVAP maintained at 30 to 35°C, reduce the ethyl acetate volume to 0.1 to 0.2-mL. Complete the evaporation of the samples one at a time with nitrogen following the manual procedure described in 3.5.3. Note

that it is important to complete the evaporation, add the acetonitrile as described in 3.6.6, and then immediately cap the samples one at a time in order to minimize contact of the sample with trace water in the room air. Trace amounts of water present in humid air could enter the culture tube and subsequently interfere with quantitative derivatization.

- 3.6.6 Immediately add 1-mL of dry acetonitrile, cap the tube, and vortex for 30 sec to dissolve the residuum. Note that it is important to insure that the contents of the acetonitrile storage bottle is handled in a manner that minimizes contact of the solvent with room air, e.g., leave the container uncapped only for the minimum period of time necessary.

3.7 Derivatization

- 3.7.1 Use a disposable Pasteur pipet to transfer the acetonitrile sample extract to a labeled autosampler vial, one at a time. Cap and crimp seal each vial immediately after the transfer. **THE ANALYSIS CAN BE DELAYED AND CAPPED SAMPLES STORED AT $4^{\circ} \pm 3^{\circ}\text{C}$ FOR UP TO TWO DAYS IF NECESSARY AFTER COMPLETION OF THIS STEP.**
- 3.7.2 Prepare the working calibration standards as discussed in 2.3.2.2. Also, prepare several vials of derivatized acetonitrile to be available for diluting samples that have a response above the calibration range (see 10).
- 3.7.3 Remove the cap from the sample vial and add 70 μL of BSTFA + 10% TMCS to the sample or working calibration standard. Cap with a new cap and crimp seal the vial immediately. Invert the vial to mix. Complete one vial at a time. Do not add the derivatization agent to a

number of vials greater than the capacity of the heating block. There are 12 wells in the heating block described in the Apparatus Section.

It is recommended that for each set of samples, a new 1-mL ampule of derivatization agent be opened. After opening the ampule, transfer the contents to an autosampler vial and immediately cap it. The 70- μ L aliquots are removed from the vial when needed by piercing the vial cap with the syringe needle. An opened ampule of the derivatization agent has successfully been used throughout a 4-wk period when kept stored at $20^{\circ} \pm 5^{\circ}\text{C}$ in autosampler vials where the punctured cap was replaced before storage. The autosampler vials are of limited capacity and consequently limits the amount of humid air that can enter the vial and degrade the derivatization agent. When additional aliquots are needed from a vial of derivatization agent, remove the required amount of derivatization agent by syringe, then immediately replace the pierced cap, and return the vial to storage. Do not use the derivatization agent if it is colored (brown or yellow) or if it contains particulate. Unused portions of the derivatization agent must be discarded in a safe manner.

- 3.7.4 Derivatize all of the samples in the set together, including the working calibration standards, by heating for 30 min at $115^{\circ} \pm 2^{\circ}\text{C}$ in a heating block.
- 3.7.5 Submit derivatized samples and standards for GC-MSD analysis within 24 hr.
- 3.7.6 Because there has been no sample division after step 3.5.1, the extractives in the final 1-mL of acetonitrile plus derivatization agent submitted for analysis is

equivalent to 2 g for the milk and is equivalent to 1 g for the tissues (see 3.4.13 for exceptions).

4 INSTRUMENTATION

Follow the manufacturer's instructions for operation of the gas chromatograph and mass-selective detector. The conditions below were found to allow generation of acceptable data. In general, these conditions should not be significantly altered.

4.1 Description of the instrumentation.

4.1.1 Gas chromatograph.

Model:	See section 2.1.16
Column:	See section 2.1.17
Inlet liner:	See section 4.1.6
Carrier:	Helium, 6 lb/sq. inch at column head
Inlet Temperature:	230°C
MSD Interface Temperature:	238°C

4.1.2 Oven temperature profile.

Initial Oven Temperature:	60°C
Initial Time:	1.0 min
Program Rate:	20°C/min
Final Oven Temperature:	265°C
Final Time:	5.5 min

4.1.3 Other conditions.

Volume Injected:	1 µL
Split Valve Off:	0.78 min
Total Run Time:	18.5 min between injections

4.1.4 Mass-selective detector.

Model:	See section 2.1.8
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Mode: Low resolution, selective ion monitoring (SIM)

Dwell Per Ion: 100 msec

Tune Program: Hewlett-Packard standard autotune program using perfluorotributylamine

EM Voltage Offset: + 400 relative to Autotune, depending on the sensitivity of the instrument

EM Voltage Absolute: The value as obtained from the Autotune program plus the offset value

Ion Source Temperature: Not selectable on the HP 5970A. The Hewlett-Packard factory preset source temperature is about 170°C

EM Temperature: Not selectable on the HP 5970A. The Hewlett-Packard estimated EM temperature is from 70° to 90°C

4.1.5

Analytes, ions monitored, analyte retention time, and ion ratio.

Analytes (as their trimethylsilyl derivatives)	m/z	Retention Time (min)	Ion Ratio (smaller ion/larger ion)*
THPI	208 & 223	8.11	0.81 (223/208)
T3	296 & 311	8.77	0.36 (311/296)
T5	296 & 311	9.02	0.43 (296/311)
C3	296 & 311	9.20	0.048 (311/296)
C5	296 & 311	9.38	0.31 (311/296)

* Ion ratios were determined from analysis of a 0.1 µg/mL reference standard. Representative full scan spectra of the derivatives are shown in Appendix C.

Group 1 of ions 208 and 223 should start about 6 min and end at about 8.4 min. Group 2 of ions 296 and 311 should start at about 8.4 min and end about 11 min. Once the exact retention times of the analytes have been established, the start time for the Group 1 ions can be moved closer to the retention time of THPI and the end

time for the Group 2 ions can be moved closer to the retention time of C5.

4.1.6 Inlet specifications. The borosilicate glass inlet (specified in 2.1.18) is to be silanized and packed with silanized glass wool (specified in 2.1.19) prior to its use. The insert should be new and must be silanized by treatment with a solution of 5% DMCS in hexane. The treatment conditions are given in section 2.1.18.

4.1.7 Note on the Hewlett-Packard Model 7673A Autosampler. This high-speed injector is capable of making injections taking about 0.1 sec. It was found that quantitative analysis was unsuccessful when the injections were made either manually or made by a slower auto-injector. It is therefore important that no substitution be made for this equipment. ✓

The syringe is adversely affected by the excess derivatization agent in samples and standards. A metal plunger that is corroded by hydrochloric acid generated from the derivatization agent, will tend to "freeze" in the glass barrel. This is not normally a problem within a set of samples if the following syringe wash cycle is included in the program: (1) wash bottle #1: 2 washes with acetonitrile followed by (2) wash bottle #2: 2 washes with acetonitrile, and where injections are made on a regular basis as would be done during an auto-sequence. However, after running a set of samples, if the syringe is allowed to remain motionless for several hours, the metal to glass surface interface of the syringe may be damaged beyond recovery. It is recommended that a new syringe be installed before running a set of samples if efforts to manually wash the used syringe are not successful. (One laboratory

reported success with a methanol post-auto-sequence wash to give the syringe longer life.)

- 4.1.7 Note on the capillary column. A new column should be used for this procedure. It is acceptable for a used column to be used only if a standard chromatogram similar in appearance and response to the standards in section 16, Appendix A, can be generated using the used column.
- 4.1.8 Note on the initial oven temperature. The 60°C initial oven temperature should not be significantly altered. The technique employed for the sample introduction is commonly identified as the Grob Splitless Solvent Effect, where the initial oven temperature is 10° to 15°C below the boiling point of the sample solvent. This technique is fundamental to the success of this method. It is therefore important to continue to employ this technique for this method.

5 CALIBRATION AND ANALYSIS

Calibrate the gas chromatograph by using the working calibration standards discussed in section 2.3.2.2 and derivatized in section 3.7.1. Use the 0.1, 0.05, 0.025, and 0.005 µg/mL standards for the tissues, and use the 0.2, 0.1, 0.05, and 0.01 µg/mL standards for the milk.

It is recommended that a high level working calibration standard (0.2 µg/mL) be prepared several days prior to the day of the analysis. Use this standard to determine that the instrument is operating correctly. Compare the chromatograms obtained to the chromatograms shown in Appendix A. If the standard chromatograms obtained are not similar in appearance to those shown in Appendix A,

there is a problem with the procedure or the instrument which should be corrected prior to the analysis of samples.

Prior to the start of the analysis, make several injections of a medium level working standard (0.05 $\mu\text{g}/\text{mL}$) to determine the actual retention times of the analytes and ensure that the system is operating correctly. The magnitude and reproducibility of the response of the analytes may change or improve after samples with matrix have been injected.

Unless making injections to equilibrate the column or to determine reproducibility, make single injections from each standard or sample vial. A typical analytical scheme for the tissues should include injections in the following order. The first four injections are intended for column equilibration only:

- 5.1 The 0.05 $\mu\text{g}/\text{mL}$ standard (a medium level standard).
- 5.2 Any tissue sample.
- 5.3 Any tissue sample.
- 5.4 Any tissue sample

The following injections are intended for analyte linearity determinations.

- 5.5 The 0.1 $\mu\text{g}/\text{mL}$ standard.
- 5.6 The 0.05 $\mu\text{g}/\text{mL}$ standard.
- 5.7 The 0.025 $\mu\text{g}/\text{mL}$ standard.
- 5.8 The 0.005 $\mu\text{g}/\text{mL}$ standard.

The following injections are intended for data collection.

- 5.9 The reagent blank (see 3.2.2)
- 5.10 Tissue sample #1
- 5.11 Tissue sample #2

- 5.12 The 0.05 $\mu\text{g}/\text{mL}$ standard (a medium level standard)
- 5.13 Tissue sample #3
- 5.14 Tissue sample #4
- 5.15 Tissue sample #5
- 5.16 The 0.05 $\mu\text{g}/\text{mL}$ standard (a medium level standard)
- 5.17 Continue the pattern established from injection 5.13 through injection 5.16 until all of the samples have been analyzed.

The suggested injection sequence for the milk is the same as shown above for the tissues. Include the appropriate milk working standards as discussed above in this section.

The concentration level of the standard used throughout the run (5.12, and 5.16) should be selected with due regard to the expected residue levels in the samples. If the residue level in the sample is known, this working standard concentration should be similar to the concentration of the analyte in the sample. If no residues are expected in the samples, an appropriate standard concentration level would be the next to the lowest level prepared, i.e., 0.025 $\mu\text{g}/\text{mL}$ for the tissues and 0.05 $\mu\text{g}/\text{mL}$ for the milk. If a sample contains a residue higher than the highest standard, dilute the sample and reanalyze as discussed in section 10.

6 INTERFERENCES

Analysis of samples in two laboratories have shown that no significant impurities interfered with quantitation of the analyte peaks. However, on occasion low levels of impurities would interfere with THPI and C5. The levels observed were generally below the limit of quantitation.

The C5 interference was attributed to coextractives from the sample matrix. The THPI interference was attributed partially to coextractives from the sample matrix and partially to THPI present in the sample from laboratory contamination of samples with captan. Technical captan had been handled in the same laboratory that was used to prepare control samples containing the THPI interference. Captan readily breaks down to THPI in some matrices. Captan, formulated captan, captan treated samples or concentrated solutions of captan should not be present in the same laboratory used to prepare or analyze samples described here. Captafol should also not be present in the laboratory.

In a third laboratory which has run this method, all samples or standards analyzed that contained the derivatization agent gave chromatograms that had large background peaks; one peak significantly interfered with the C5 analyte. This interference can arise from either (1) off-specification derivatization agent or (2) cold-trapped contaminants present in the GC inlet system that react in-situ with the derivatization agent and subsequently volatilize.

As a preventative measure, (1) ensure that the derivatization agent is purchased directly from the manufacturer. The manufacturer has established a 2-year expiration date for unopened material and will not ship out-of-date material. (2) Prior to starting the method, add 70- μ L of the derivatization agent from a newly opened vial to 1-mL acetonitrile and analyze this sample. If large background peaks are observed, the GC inlet system may be contaminated and should be cleaned. Also, as discussed in section 5, before analyzing samples using this method, a standard chromatogram similar in

appearance to the ones shown in Appendix B should be generated. If there are significant differences between the generated chromatogram and the chromatograms found in Appendix B with respect to background, the problem with either the derivatization agent, the inlet system, or other factors should be determined and corrected prior to analyzing the samples.

One commonly overlooked source of contamination which can come from the HP5890 itself, is due to a dirty lower inlet seal (see 2.1.24). This seal is a metal disk with a small hole in the center located in the bottom of the injection port.² It is accessed by removing the retaining nut inside the oven. Replacement with a new seal, or cleaning of the used seal may eliminate much of the background.

7

CONFIRMATORY TECHNIQUES

Verification of a suspected residue can be confirmed (1) by an ion ratio technique, (2) by an alternative column technique (3) by re-extraction and re-analysis of the sample, or by a combination of the three options.

7.1

Ion Ratio Confirmation

Two ions are monitored for each analyte. The relative abundance of each ion is automatically stored by the Hewlett-Packard workstation. By following the workstation operating instructions, obtain the relative abundance for the suspected residue. Then, obtain the relative abundance for the same analyte in the working calibration solution that is the closest in concentration to the extract containing the suspected sample residue. Compare the ratio of the two ions monitored for both the

sample extract and the standard. If the ratios are nearly the same (within $\pm 10\%$ of each other), the analyte identity is confirmed. If the ratios are significantly different (greater than $\pm 40\%$ of each other), the analyte is not confirmed. If the ratio differences are intermediate (between $\pm 10\%$ and $\pm 40\%$), another confirmation technique should be conducted.

For example, let the ion ratio of the two ions monitored for the determination of T3 (296 amu and 311 amu) be 0.36 (311 abundance/296 abundance). This ion ratio value was obtained from the spectrum produced by a $0.1 \mu\text{g/mL}$ standard on the author's GC/MSD. Another instrument with its own autotune parameters and unique characteristics might give a different ratio. Therefore, the expected ion ratio for the analytes must be determined on the same instrument used for the sample analysis.

If the ion ratio value for the sample significantly varies from the ion ratio for the standard, the analyte identity is not confirmed. The boundary for a significant difference in the two values in the author's laboratory has been defined as $\pm 40\%$. Using the value of 0.36 for the expected ion ratio, a sample ion ratio that falls outside the range of 0.22 to 0.50 (0.36 ± 0.14) ($40\% \times 0.36 = 0.14$) is considered a negative confirmation result. To be considered confirmed, the difference in the two ratios should be within $\pm 10\%$; the acceptance range for the ion ratio in the sample is therefore between 0.32 and 0.40. For samples that do not fit within the two categories listed above, i.e., where the sample ratio is between $\pm 10\%$ and $\pm 40\%$, another confirmation technique should be used.

The table in section 4.1.5 lists the ratios obtained in the author's laboratory for the other analytes. Appendix C shows full scan spectra of the five trimethylsilyl derivatized analytes.

7.2 Alternative Column Technique

The column listed below is installed in the gas chromatograph. The samples are analyzed in the same manner as suggested in section 5. A residue is considered confirmed if the results from using two different columns agree.

7.2.1 Column: The alternative column is a 12 m x 0.2 mm i.d., 0.33- μ m film thickness Ultra 1 from Hewlett-Packard Company, Santa Clara, CA.

7.2.2 Oven temperature profile.

Initial Oven Temperature:	60°C
Initial Time:	1.0 min
Program Rate 1:	20°C/min
Final Oven Temperature 1:	190°C
Final Time 1:	5.5 min
Program Rate 2:	15°C/min
Final Oven Temperature 2:	270°C
Final Time 2:	1 min

7.2.3 Other conditions. The other conditions are the same as listed in section 4.

7.2.4 Retention times. Using the Ultra 1 column and the above temperature program, the analytes elute at the following retention times:

THPI	6.82 min
T3	8.31 min
T5	8.52 min
C3	8.57 min
C5	8.93 min

It should be noted that the separation between T5 and C3 is minimal. However, peak heights can still be measured to give adequate quantitation. In addition, as discussed in section 1.1, C3 and C5 may not be actual animal metabolites and a difficulty with the quantitation of T5 caused by C3 would therefore not occur.

7.3 Re-extraction and Re-analysis

The sample to be confirmed can be re-extracted and re-analyzed by either the intended method discussed in sections 4 and 5, or by the alternative column technique discussed in 7.2.

8 TIME REQUIRED FOR ANALYSIS

The time required for the analysis of five samples is approximately three 8 hr days.

- 8.1 Day 1: The five samples should be at or beyond step 3.5.3 after one day.
- 8.2 Day 2: The five samples should be at or beyond step 3.6.4 after the second day.
- 8.3 Day 3: The five samples should be completed after day 3.

9 MODIFICATIONS OR POTENTIAL PROBLEMS

9.1 The derivatization step is the key for the success of this method. The method is designed to minimize the levels of water or co-extractives in the sample that could interfere with the derivatization. It is important that the solvent removal steps prior to derivatization be conducted strictly according the instructions.

9.2 The N-Evap solvent reduction or drying procedure consumes a significant portion of the method time. It is included in the method because it has been demonstrated to be a proven reliable component during the analysis of a large number of samples.

The authors feel that the common rotary evaporator should not be used for solvent reduction. Supposition for possible failure of the rotary evaporator include:

- (a) The large surface area of the glass walls may be detrimental to subsequent removal of the analytes.
- (b) Venting of the system may reintroduce sufficient water vapor from the ambient air to interfere with the derivatization.
- (c) The analytes may be lost by volatilization.

Another alternative solvent evaporation procedure that has been proven to be successful for the analysis of milk samples used the vortex evaporator (HaakeBuchler Rotary Evapo-mix vortex evaporator; HBI Inc., Saddle Brook NJ; The Evapo-mix is no longer commercially available.). This device is equipped to accept the 50-mL centrifuge tubes.

This device removes solvent under an adjustable level of vacuum (the system is brought to a maximum vacuum of 27"

Hg within 2 minutes of the start of the evaporation) while spinning the solvent up on the walls of the 50-mL centrifuge tube maintained at 30° to 35° C. Several key features of this device which differentiate it from the standard rotary evaporator are:

- (a) The glass wall surface that each sample contacts is minimized.
- (b) When the procedure is finished, venting of the system allows ambient air to enter the system through a convoluted pathway. The introduction of water vapor from air may be reduced to a very low level.

10

CALCULATIONS

The concentration of the analytes in the original sample is calculated by using the external standard method, i.e., the response obtained for the analytes in the sample is compared to the response obtained for separate injections of a known amount of analyte (the working calibration standards). To use the calculations shown below, the injection volume for all calibration solutions and sample solutions must be fixed at the same volume. The peak height is the response that is usually measured.

A prerequisite for the calculation of residue levels in sample extracts is that the sample response must fall within the response range of the standards. If a sample has a response that is higher than the highest standard, dilute the sample to the acceptable range, and re-assay. The acetonitrile solvent used to dilute the sample must be as similar to the sample solvent as is possible. For this reason, at step 3.7.1, prepare several vials containing 1-mL of 'derivatized' acetonitrile. Add the 70 μ L of the derivatization agent to each vial containing

1-mL of acetonitrile, cap, and heat for 30 min at $115^{\circ} \pm 2^{\circ}\text{C}$ in a heating block. The acetonitrile is now ready for use for dilution of a sample.

As an example for dilution of a sample, let a tissue sample response for an analyte be equivalent to 0.4 ppm (which for a tissue is the same as $0.4 \mu\text{g/mL}$ in the solution analyzed). The highest level standard for the tissues is $0.1 \mu\text{g/mL}$. Therefore, the response of the sample is 4 times greater than the response of the highest standard. To have the response fall within the calibration curve, dilute the sample by at least a factor of 5. To make a 1 to 5 dilution, remove $200 \mu\text{L}$ from the vial containing $1000 \mu\text{L}$ of 'derivatized' acetonitrile (see 3.7.2). Transfer $200 \mu\text{L}$ of the sample to the 'derivatized' acetonitrile. Analyze this diluted sample.

There are several acceptable procedures for calculating the results.

- 10.1 Manually construct a calibration curve for each analyte (response versus concentration) on linear graph paper using standards 5.5, 5.6, 5.7, and 5.8. Connect the individual data points including zero as a data point using either a ruler since the response should be linear, or using a "boatman's" curve if the response is nonlinear. Use the graph to determine the μg of analyte in each sample. Since the final concentration of sample in the solvent is 1 g/mL for the tissues (the fat will be slightly different, see section 10.2.3), and 2 g/mL for the milk (see 3.7.6), divide the tissue μg by 1 g , and the milk μg by 2 g to report the results in terms of $\mu\text{g/g}$ or ppm.

The standards interspersed with the samples are intended to show that no drift in the system occurs after the calibrating standards have been injected.

10.2 Any equivalent calculation procedure to the calculation procedure in 10.1 is acceptable including using all of the standards to determine a single calibration factor, or to determine a calibration factor from the standards which bracket a group of samples.

10.2.1 Calibration factor determination. A single average calibration factor using all of the standards is calculated as follows. Calculate the response factor, F, for each of the standards:

$$F = \frac{C}{R}$$

where

C = concentration of calibration solution, $\mu\text{g/mL}$ (note, the purity need not be included in the calculation since it has been factored into the concentration of the stock calibration solution (see 2.3.2.1))

R = average response units (e.g., peak height) from the detector for the calibration solution

Determine the average (mean) value F(avg) for the standards.

10.2.2 Analyte in sample. Next, calculate the concentration of the analyte in the original sample (for the fat, see 10.2.2):

$$A = \frac{F(\text{avg}) \times R}{C_s}$$

where

- A = concentration of analyte in original sample ($\mu\text{g/g}$ or ppm)
- F(avg) = average response, ($\mu\text{g/mL}$)/response unit
- R = average sample response units from detector for sample
- Cs = concentration of milk or tissue extract in final derivatized sample. Use 1 g/mL for the tissues (except for fat) and 2 g/mL for the milk

10.2.3 Analyte in fat sample. The concentration (Cs) of fat in the final extract is calculated from the volume of fat residue that remains in the 15-mL centrifuge tube discussed in section 3.4.13. This volume represents the amount of fat that was soluble in the 10 mL of the acetone extract of the fat.

For example, if 1.0-mL of fat residue remains in the 15-mL centrifuge tube, 10 % of the extract volume is not acetone [(1.0 mL/10 mL)x100%]. The total liquid volume resulting from the extraction of the fat is therefore 10% greater, or 6 mL greater than the 60-mL of acetone that was added to the sample. In step 3.5, Solvent Partition/Cleanup, 6-mL of acetone extract is transferred for further workup for each tissue sample. Since 10 g of sample is extracted using 60 mL of solvent, this 6-mL represents 1 g of sample for the kidney, liver, and muscle. But since the final solvent volume resulting from the extraction of 10 g of fat was not 60 mL, but 66 mL, a 6-mL aliquot of the acetone fat extract contains about 10% less sample, or 0.91 g of fat [(10 g)x(6mL/66mL)]. This 0.91 g of fat extract is the final concentration of extract in the 1 mL of solvent that is

to be analyzed. Therefore, in 10.2.2, Cs for the fat is equal to 0.91 g/mL.

11 RESULTS/DISCUSSION

This method is suitable for the determination of five possible captan metabolites in milk and animal tissues. In order to validate the method during the analysis of samples generated from three residue studies conducted in 1989 through 1991, fortified control samples were analyzed concurrently with the samples from the three studies. Tables 1 through 5 show the matrices fortified, the levels of fortification, and the recoveries for these fortified control samples. Table 6 shows the results of the method validation conducted as a part of the study CAPT-90-AT-01.

11.1 Accuracy and Precision

The accuracy and precision of the method can be evaluated from the data shown in Tables 1 through 6. The tables show the mean recovery, the range of the recoveries, and the coefficient of variation for the recoveries.

11.2 Limits of Quantitation

The limit of quantitation (LOQ) for the tissues is 0.01 mg/kg. The LOQ for the milk is 0.01 mg/kg. The definition of LOQ is a "substance-specific level that a method must be able to routinely and reliably detect in specific sample matrices. It is not the lowest detectable level achievable, but rather the level that a method should reasonable quantify." This definition is

similar to the EPA's Contract-Required Quantitation Limit (CRQL) (ref. 14.5). ✓

The upper limit of quantitation is 0.1 mg/kg. Samples which have residues above 0.1 mg/kg are to be diluted and reanalyzed (see section 10). If a lower LOQ is required than the LOQ values listed above, the method must be validated at the lower level prior to the analysis of samples.

11.3 Ruggedness Testing

In a recent unprotocolled study conducted at WRC by the primary author, 1-^{mL} volumes of the 0.1 µg/mL working standards were fortified with 0.6-, 1.2-, 3-, and 6-µL of water just prior to the derivatization (see section 3.7.2). The response of the derivatized standards was found to decrease as the amount of added water increased (see also section 9). The following table shows the % of response as compared to a standard that had no added water.

<u>Description</u>	<u>% Recovery</u>
Standard + 0.6 µL water	100%
Standard + 1.2 µL water	30%
Standard + 3 µL water	0%
Standard + 6 µL water	0%

In an additional experiment, 10 µL of water was added to a 0.1 µg/mL derivatized standard that had previously produced a satisfactory chromatogram. Upon immediate reanalysis, there was no recovery of the analytes.

The presence of water in samples to be derivatized or analyzed must be minimized.

11.4 Limitations

The derivatized analytes are nominally stable for 24 hours (see section 3.7.5). Unforeseen delays, e.g., instrument malfunction, have led to instances where milk samples have been analyzed up to 48 hr and longer after derivatization. While the instrument was repaired, these samples were refrigerated in autosampler vials with unpunctured caps. Successful analysis of the samples followed.

On several occasions in one laboratory that was using the alternative solvent evaporation procedure where water contamination may have been a problem (see, 9.2 and 11.3) the response of the fortified controls gave recoveries that were lower than expected. The remedy, as judged by the success of the re-analysis of the fortified control samples, was to add 30- μ L of additional derivatization reagent, reheat the sample vials as described in section 3.7, and reanalyze the samples.

11.5 Safety Precautions

Personnel untrained in the routine safe-handling of chemicals and good laboratory practices must not attempt to use this procedure. Information on any specific chemical regarding physical properties, hazards, toxicity, and first-aid procedures can be found on the Material Safety Data Sheet (MSDS) accompanying the chemical, available from the supplier. In general, always wear safety glasses with side shields, work in a well ventilated area, avoid inhaling vapors, and avoid contact of the chemicals with skin and clothing. The flammable solvents used in this method, e.g., acetone,

acetonitrile, ethyl acetate, and toluene, should always be kept away from potential sources of ignition.

12

CONCLUSION

The method is specific for the analysis of five potential captan metabolites, namely tetrahydrophthalimide (THPI), trans-3-hydroxy-tetrahydrophthalimide (T3), trans-5-hydroxy-tetrahydrophthalimide (T5), cis-3-hydroxy-tetrahydrophthalimide (C3), and cis-5-hydroxy-tetrahydrophthalimide (C5). Only commercially available laboratory equipment and reagents are required. The analysis can be completed by one person in three 8-hr periods if an adequately ground sample is available and all glassware and equipment is set up and properly labeled.

13

RETENTION OF RECORDS

All of the raw data, the protocol, and final report for the study CAPT-90-AT-01 are located in the GLP Archive at the Western Research Center of ICI Americas, 1200 South 47th Street, Box 4023, Richmond, California 94804-0023.

All of the raw data and the protocol for the study CAPTAN 91-01 are currently located in the GLP Archive at Morse Laboratories, Inc. A draft report has been prepared by Technical Assessment Systems, Inc., Washington D.C. When the report is issued all of the raw data, the protocol, and the final report will be located in the GLP Archive at the Western Research Center of ICI Americas, 1200 South 47th Street, Box 4023, Richmond, California 94804-0023.

14 REFERENCES

- 14.1 Cheng, H. M. (1980) *Metabolism of [Carbonyl-14C]-Captan in a Lactating Goat*; Chevron Chemical Company, Agricultural Chemicals Division Research and Development Department, Richmond, California, File No: 721.14/Captan. MRID is 40658002.
- 14.2 Davy, G. S. (1989) *The Determination of Residues of Tetra-hydrophthalimide, 3-Hydroxy Tetrahydrophthalimide and 5-Hydroxy Tetrahydrophthalimide in Milk and Animal Tissues*; ICI Agrochemicals Residue Analytical Method No. 166. MRID is 41386501.
- 14.3 Westberg, G. L.; Clark, S. (1991) *Determination of Residues of Tetrahydrophthalimide, 3-Hydroxy Tetrahydrophthalimide and 5-Hydroxy Tetrahydrophthalimide in Milk*; Morse Laboratories, Inc., Sacramento, CA, SOP# Meth-18, revision 2.
- 14.4 Westberg, G. L.; Clark, S. (1991) *Determination of Residues of Tetrahydrophthalimide, 3-Hydroxy Tetrahydrophthalimide and 5-Hydroxy Tetrahydrophthalimide in Animal Tissues*; Morse Laboratories, Inc., Sacramento, CA, SOP# Meth-26.
- 14.5 Rules and Regulations, Federal Register 55(241): 51586, Friday, December 14, 1990)

djg/M92-108b.law/March 13, 1992

15 TABLES AND FIGURES

- Table 1 Recoveries From Fat (CAPT-90-AT-01)
- Table 2 Recoveries From Kidney (CAPT-90-AT-01)
- Table 3 Recoveries From Liver (CAPT-90-AT-01)
- Table 4 Recoveries From Muscle (CAPT-90-AT-01)
- Table 5 Recoveries From Milk (Captan 91-01)
- Table 6 Recoveries from Milk and Tissues for Method Validation (CAPT-90-AT-01)

Table 1. Recoveries from Fat (CAPT-90-AT-01)¹

Sample Number	Amount Added (ppm)	Amount Found (%)				
		THPI	T3	T5	C3	C5
F7001-189A2	0.05	95	114	110	115	108
F7001-189A2	0.01	109	121	109	121	138
56292 ²	0.20	94	101	103	94	90
56292	0.05	104	123	107	120	121
56292	0.01	106	117	112	117	128
56292	0.01	106	101	101	117	128
F7002-102	0.40	81	98	102	101	84
F7002-112	0.40	90	95	98	97	84
F7002-112	0.40	92	104	108	104	99
F7002-103	0.10	78	85	92	95	78
F7002-113	0.10	95	90	85	92	78
F7002-113	0.10	84	96	98	98	102
Number of Samples:		12	12	12	12	12
Range:		78-109	85-123	85-112	92-121	78-138
Mean:		94.5	103.8	102.1	105.9	103.2
Coefficient of Variation:		10.9	11.9	7.8	10.6	20.6

¹ The results are corrected for control sample background when necessary. The results for sample F7001-189A2 are adjusted upward by 13% to account for sample solubility in the extraction solvent; the results for sample 56292 are adjusted upward by 6% to account for sample solubility in the extraction solvent (see Section 3.4.11). No solubility correction was necessary for the samples with prefix F7002. These F7002 samples were extracted following the procedure for the kidney, liver, and muscle described in section 3.4.2.

² Sample number 56292 was purchased at a local market.

Samples numbers with a prefix F7001- are from the animal transfer study, CAPT-90-AT-01.

Sample numbers with a prefix F7002- are from the storage stability study, CAPT-90-SS-01, and are reported in study CAPT-90-AT-01.

Table 2. Recoveries from Kidney (CAPT-90-AT-01)¹

Sample Number	Amount Added (ppm)	Amount Found (%)				
		THPI	T3	T5	C3	C5
F7001-141A1	0.50	75	82	88	83	81
F7001-141A1	0.10	76	86	82	85	73
F7001-141A1	0.05	93	105	100	107	91
F7001-141A1	0.01	100	140	105	110	105
F7001-141A1	0.01	135	150	100	95	115
F7002-202	0.40	82	92	102	94	91
F7002-212	0.40	81	80	88	84	70
F7002-222	0.40	92	97	92	100	85
F7002-203	0.10	95	110	108	113	92
F7002-213	0.10	95	105	116	108	98
F7002-223	0.10	100	105	105	108	98
Number of Samples:		11	11	11	11	11
Range:		75-135	80-150	82-116	83-116	70-115
Mean:		93.1	104.7	98.7	98.8	90.8
Coefficient of Variation:		17.8	21.4	10.3	11.4	14.7

¹ The results are corrected for control sample background when necessary.

Samples numbers with a prefix F7001- are from the animal transfer study, CAPT-90-AT-01.

Sample numbers with a prefix F7002- are from the storage stability study, CAPT-90-SS-01, and are reported in study CAPT-90-AT-01.

Table 3. Recoveries from Liver (CAPT-90-AT-01)¹

Sample Number	Amount Added (ppm)	Amount Found (%)				
		THPI	T3	T5	C3	C5
F7001-157A1	0.05	77	67	59	67	NQ
F7001-157A1	0.01	70	50	50	60	NQ
57622 ²	0.50	85	80	75	82	69
57622	0.10	75	82	82	87	68
57622	0.05	NF	NF	NF	NF	77
57622	0.01	NF	NF	NF	NF	50
57622	0.01	95	95	95	115	115
F7002-302	0.40	68	67	61	76	54
F7002-312	0.40	77	69	61	82	56
F7002-322	0.40	73	62	54	66	51
F7002-303	0.10	72	83	75	90	72
F7002-313	0.10	95	88	80	105	70
F7002-323	0.10	88	70	68	82	60
Number of Samples:		11	11	11	11	11
Range:		68-95	50-95	50-95	60-115	51-115
Mean:		79.5	73.9	69.1	82.9	67.5
Coefficient of Variation:		12.2	17.5	19.7	19.8	27.0

¹ The results are corrected for control sample background when necessary.

² Sample number 57622 was purchased at a local market.

NF = Sample not fortified with the analyte.

NQ = Analyte not quantitated due to the presence of an interference.

Samples numbers with a prefix F7001- are from the animal transfer study, CAPT-90-AT-01.

Sample numbers with a prefix F7002- are from the storage stability study, CAPT-90-SS-01, and are reported in study CAPT-90-AT-01.

Table 4. Recoveries from Muscle (CAPT-90-AT-01)¹

Sample Number	Amount Added (ppm)	Amount Found (%)				
		THPI	T3	T5	C3	C5
F7001-174	0.50	107	98	92	97	98
F7001-174	0.10	112	102	100	105	105
F7001-174	0.01	75	95	90	95	80
58462 ²	0.05	96	116	112	105	104
58462	0.01	100	120	120	120	120
F7002-402	0.40	95	96	94	93	91
F7002-412	0.40	91	98	96	94	92
F7002-472	0.40	83	95	92	93	87
F7002-403	0.10	100	95	95	102	98
F7002-413	0.10	100	108	102	100	100
F7002-473	0.10	86	86	82	96	104
Number of Samples:		11	11	11	11	11
Range:		75-112	86-120	82-120	93-120	80-120
Mean:		95.0	100.8	97.7	100.0	98.1
Coefficient of Variation:		11.3	10.0	10.8	8.0	10.9

¹ The results are corrected for control sample background when necessary.

² Sample number 58462 was purchased at a local market.

Samples numbers with a prefix F7001- are from the animal transfer study, CAPT-90-AT-01.

Sample numbers with a prefix F7002- are from the storage stability study, CAPT-90-SS-01, and are reported in study CAPT-90-AT-01.

Table 5 Recoveries from Milk (CAPTAN 91-01)¹

Amount Added (ppm)	Amount Found (%)		
	THPI	T3	T5
0.1	105	108	110
0.1	90	100	96
0.01	88	90	88
0.01	110	115	108
0.01	88	90	90
0.01	96	88	88
0.01	93	175	75
0.01	97	112	108
0.01	100	102	107
0.01	102	120	120
0.01	102	105	107
0.01	103	115	110
0.01	92	100	90
0.01	88	105	105
0.01	100	115	120
0.01	100	115	128
0.01	90	95	97
0.01	93	82	82
0.01	90	92	92
0.01	110	103	115
0.01	88	103	115
0.01	113	126	122
0.01	107	105	115
0.01	103	108	118
0.01	105	108	105
0.01	105	103	98
0.01	100	105	105
0.005	80	90	80
0.005	84	96	84
0.005	96	110	100
0.005	106	126	124
0.005	100	100	116
0.005	100	110	96

Table 5 Recoveries from Milk (CAPTAN 91-01)¹

Amount Added (ppm)	Amount Found (%)		
	THPI	T3	T5
0.1	105	108	110
0.005	100	104	88
0.005	104	100	96
0.005	110	110	100
0.005	100	116	116
0.005	104	120	112
0.005	106	90	110
0.005	84	100	96
0.005	114	130	120
0.005	130	100	140
0.005	112	120	124
0.005	100	104	104
0.005	96	104	96
0.005	106	94	66
0.005	110	120	140
0.005	86	106	116
0.005	116	120	120
0.005	94	116	120
0.005	106	100	96
0.005	104	120	120
Number of Samples:	52	52	52
Range:	80-130	82-175	66-140
Mean:	100.1	107.5	105.7
Coefficient of Variation:	9.5	13.4	14.8

¹ The results are corrected for control sample background when necessary. The Morse Laboratories' sample identification number is 58520.

Note that the standards used to produce the milk data in Table 5 were 0.1, 0.05, 0.02, and 0.005 µg/mL, where the LOQ for this study was established and validated to be 0.005 ppm.

Table 6. Recoveries from Milk and Tissues for Method Validation (CAPT-90-AT-01)¹

<u>Matrix</u>	<u>Amount Added (ppm)</u>	<u>Amount Found (%)</u>				
		<u>THPI</u>	<u>T3</u>	<u>T5</u>	<u>C3</u>	<u>C5</u>
Milk	0.01	95	110	105	110	105
Milk	0.01	80	100	85	95	80
Milk	0.01	85	105	105	100	95
Milk	0.5	78	90	86	91	87
Milk	0.5	83	95	91	98	91
Milk	0.5	87	100	96	102	91
Fat	0.01	85	92	95	100	90
Fat	0.01	100	102	95	110	105
Fat	0.01	85	72	75	80	65
Fat	0.5	72	89	86	91	71
Fat	0.5	82	100	98	98	82
Fat	0.5	74	80	79	85	69
Kidney	0.01	95	85	92	108	90
Kidney	0.01	95	95	82	108	100
Kidney	0.01	80	80	82	93	80
Kidney	0.5	84	81	80	89	68
Kidney	0.5	90	87	87	87	72
Kidney	0.5	90	88	90	86	73
Liver	0.01	90	85	70	95	80
Liver	0.01	90	90	80	100	100
Liver	0.01	80	90	80	95	90
Liver	0.5	64	58	53	65	48
Liver	0.5	79	77	69	81	64
Liver	0.5	67	69	61	74	58

Table 6. Recoveries from Milk and Tissues for Method Validation (CAPT-90-AT-01)¹

<u>Matrix</u>	<u>Amount Added (ppm)</u>	<u>Amount Found (%)</u>				
		<u>THPI</u>	<u>T3</u>	<u>T5</u>	<u>C3</u>	<u>C5</u>
Muscle	0.01	90	95	120	100	85
Muscle	0.01	115	105	120	115	115
Muscle	0.01	115	120	120	120	115
Muscle	0.5	80	90	89	85	81
Muscle	0.5	87	96	93	90	85
Muscle	0.5	79	98	95	90	89
Number of Samples:		30	30	30	30	30
Range:		64-115	58-120	53-120	65-120	48-115
Mean:		85.9	90.8	88.6	94.7	84.1
Coefficient of Variation:		13.2	14.0	17.7	12.7	19.0

¹ The results are corrected for control sample background when necessary.

The samples were purchased at a local market. The samples were fortified and analyzed at Morse Laboratories, Inc., under project # ML90-0173-CTF, in order to validate the method prior to the analysis of samples from the study CAPT-90-AT-01.

16 APPENDICES

A Representative Chromatograms

Figure 1 Sample Milk Chromatograms for the Analysis of THPI (8.10 min), T3 (8.76 min), T5 (8.01 min), C3 (9.19 min), and C5 (9.37 min), from the method validation data produced prior to starting the animal transfer study sample analysis (Table 6, CAPT-90-AT-01).

Figure 2 Sample Fat Chromatograms for the Analysis of THPI (7.63 min), T3 (8.30 min), T5 (8.55 min), C3 (8.71 min), and C5 (8.90 min) from the animal transfer study (Table 1, CAPT-90-AT-01).

Figure 3 Sample Kidney Chromatograms for the Analysis of THPI (7.63 min), T3 (8.31 min), T5 (8.55 min), C3 (8.72 min), and C5 (8.90 min) from the animal transfer study (Table 2, CAPT-90-AT-01).

Figure 4 Sample Liver Chromatograms for the Analysis of THPI (7.60 min), T3 (8.28 min), T5 (8.52 min), C3 (8.69 min), and C5 (8.87 min) from the animal transfer study (Table 3, CAPT-90-AT-01).

Figure 5 Sample Muscle Chromatograms for the Analysis of THPI (7.63 min), T3 (8.31 min), T5 (8.55 min), C3 (8.71 min), and C5 (8.90 min) from the animal transfer study (Table 4, CAPT-90-AT-01).

B Fortification

C Representative Spectra

Figure A THPI. Full scan spectra of 50 ng of the THPI trimethylsilyl derivative produced on the HP 5970A Mass Selective Detector.

Figure B T3. Full scan spectra of 50 ng of the T3 trimethylsilyl derivative produced on the HP 5970A Mass Selective Detector.

Figure C T5. Full scan spectra of 50 ng of the T5 trimethylsilyl derivative produced on the HP 5970A Mass Selective Detector.

Figure D C3. Full scan spectra of 50 ng of the C3 trimethylsilyl derivative produced on the HP 5970A Mass Selective Detector.

Figure E C5. Full scan spectra of 50 ng of the C5 trimethylsilyl derivative produced on the HP 5970A Mass Selective Detector.

Appendix A
Representative Chromatograms

Figure 1 Sample Milk Chromatograms for the Analysis of THPI (8.10 min), T3 (8.76 min), T5 (8.01 min), C3 (9.19 min), and C5 (9.37 min), from the method validation data produced prior to starting the animal transfer study sample analysis (Table 6, CAPT-90-AT-01).

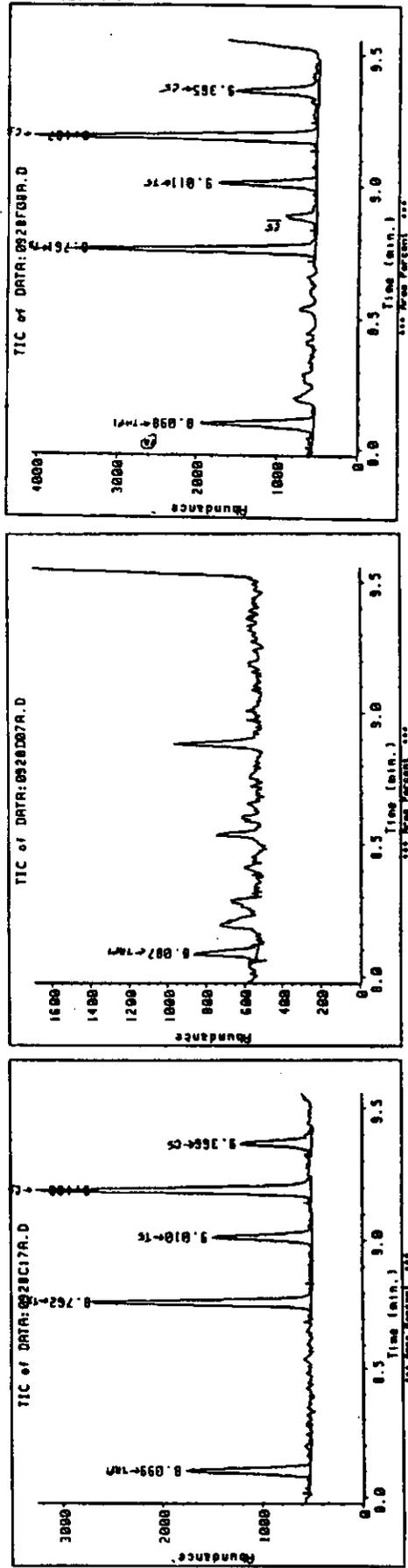
Figure 2 Sample Fat Chromatograms for the Analysis of THPI (7.63 min), T3 (8.30 min), T5 (8.55 min), C3 (8.71 min), and C5 (8.90 min) from the animal transfer study (Table 1, CAPT-90-AT-01).

Figure 3 Sample Kidney Chromatograms for the Analysis of THPI (7.63 min), T3 (8.31 min), T5 (8.55 min), C3 (8.72 min), and C5 (8.90 min) from the animal transfer study (Table 2, CAPT-90-AT-01).

Figure 4 Sample Liver Chromatograms for the Analysis of THPI (7.60 min), T3 (8.28 min), T5 (8.52 min), C3 (8.69 min), and C5 (8.87 min) from the animal transfer study (Table 3, CAPT-90-AT-01).

Figure 5 Sample Muscle Chromatograms for the Analysis of THPI (7.63 min), T3 (8.31 min), T5 (8.55 min), C3 (8.71 min), and C5 (8.90 min) from the animal transfer study (Table 4, CAPT-90-AT-01).

Figure 1. Sample Milk Chromatograms for the Analysis of THPI (8.10 min), T3 (8.76 min), T5 (8.01 min), C3 (9.19 min), and C5 (9.37 min)

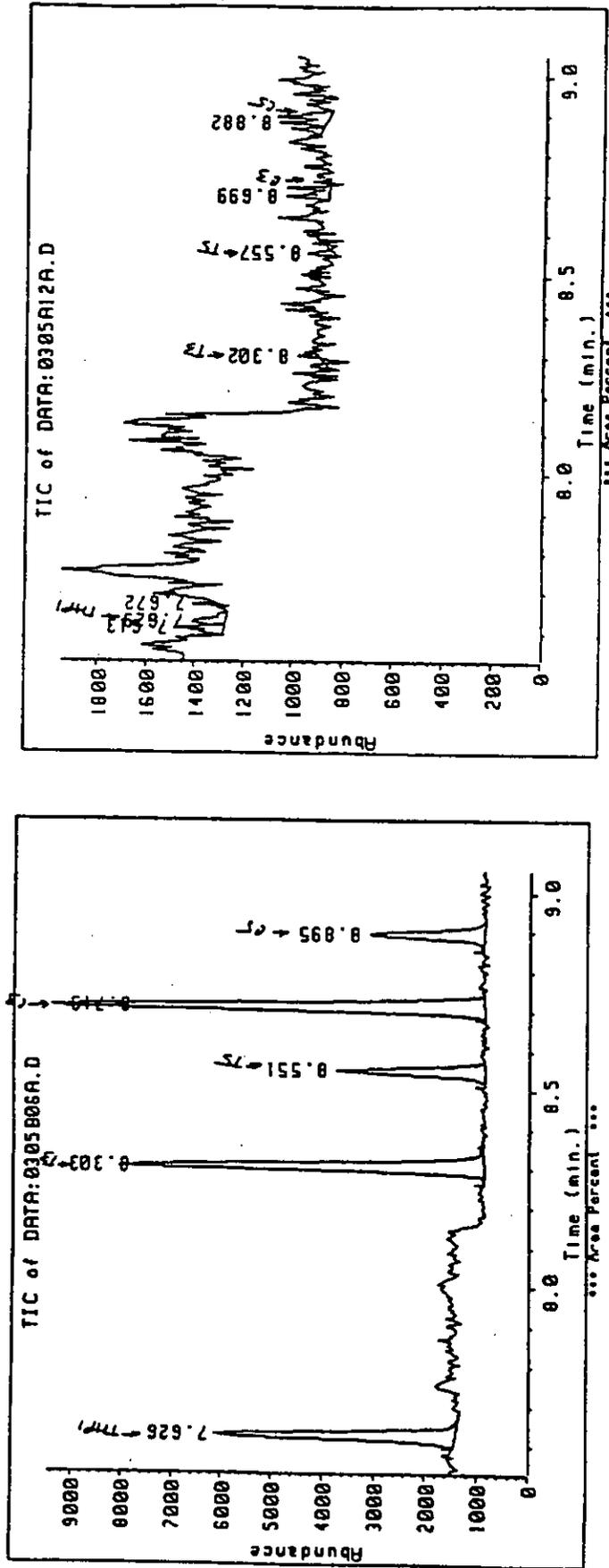


A. Calibration solution containing 0.02 µg/mL of THPI, T3, T5, C3, and C5

B. Untreated control milk (#57341)

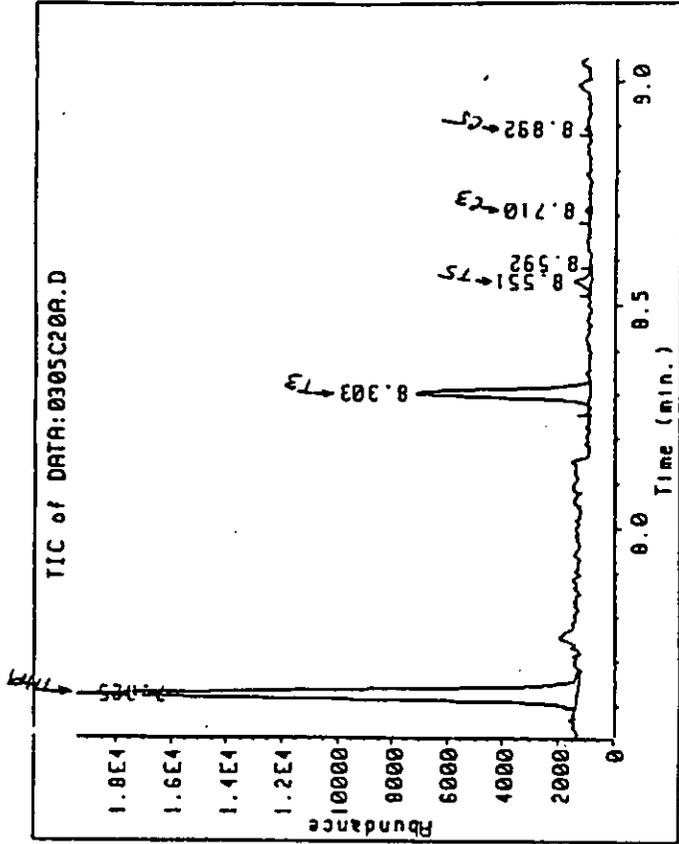
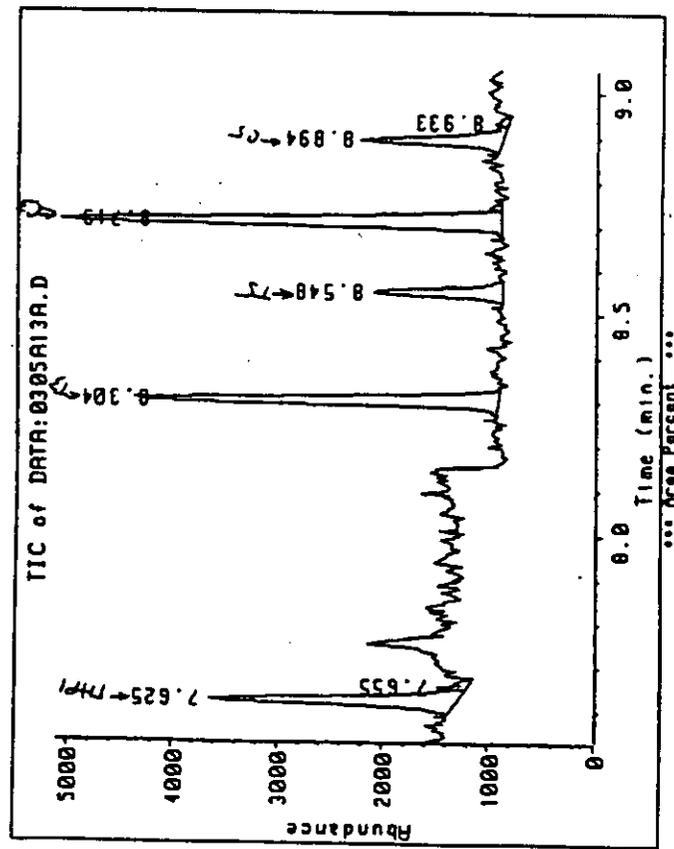
C. Untreated control milk (#57341) fortified at 0.01 ppm of THPI, T3, T5, C3, and C5

Figure 2. Sample Fat Chromatograms for the Analysis of THPI (7.63 min), T3 (8.30 min), T5 (8.55 min), T3 (8.30 min), T5 (8.55 min), C3 (8.71 min), and C5 (8.90 min)



- A. Calibration solution containing 0.02 µg/mL of THPI, T3, T5, C3, and C5.
- B. Untreated control fat (F7001-189A1 #1).

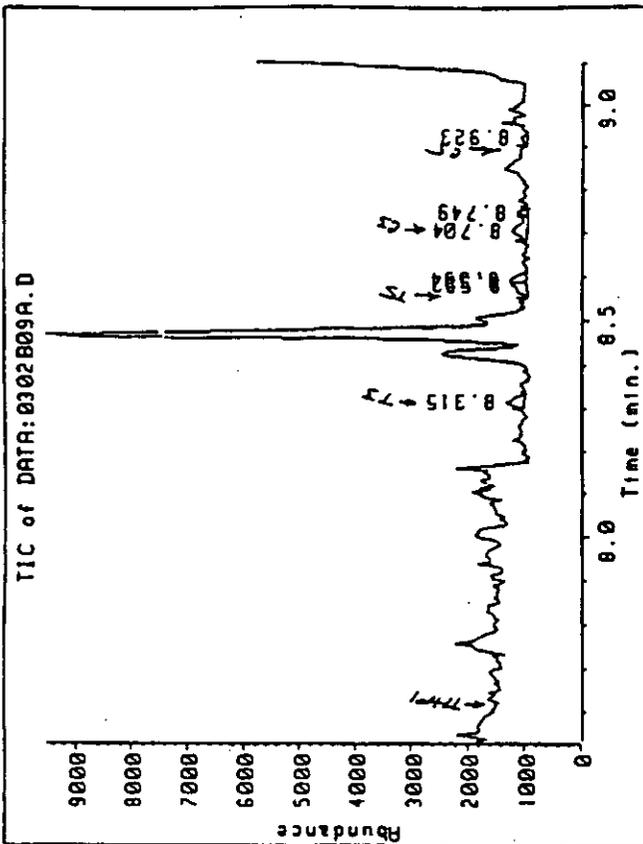
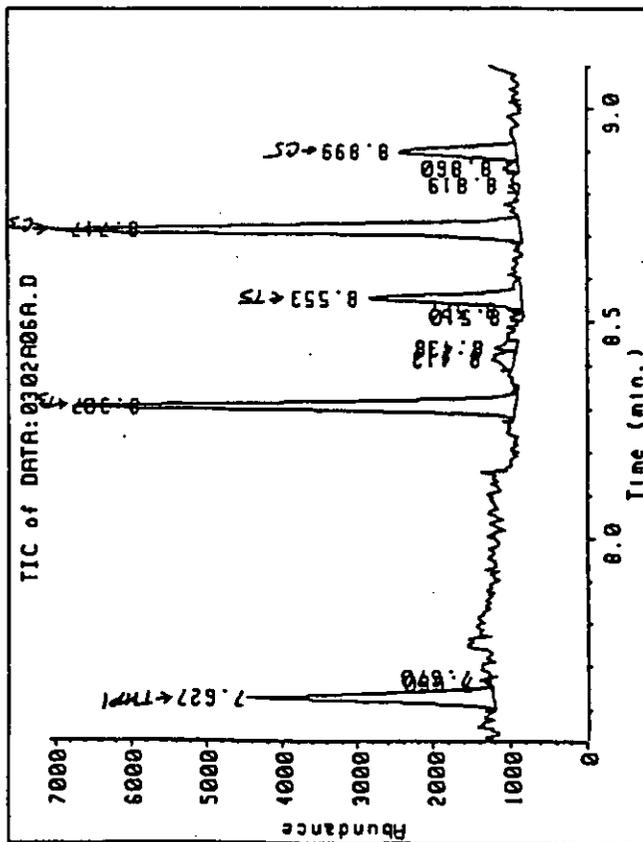
Figure 2. Sample Fat Chromatograms for the Analysis of THPI (7.63 min), T3 (8.30 min), T3 (8.30 min), T5 (8.55 min), C3 (8.71 min), and C5 (8.90 min) (Continued)



C. Untreated control fat (F7001-189A1 #1) fortified at 0.01 ppm of THPI, T3, T5, C3, and C5.

D. Treated test fat (F7001-203A1 #1) cow #1937, from the 10X treatment group. The fat contained 0.08 ppm of THPI, 0.02 ppm of T3, <0.01 ppm of T5, <0.01 ppm of C3, and <0.01 ppm of C5.

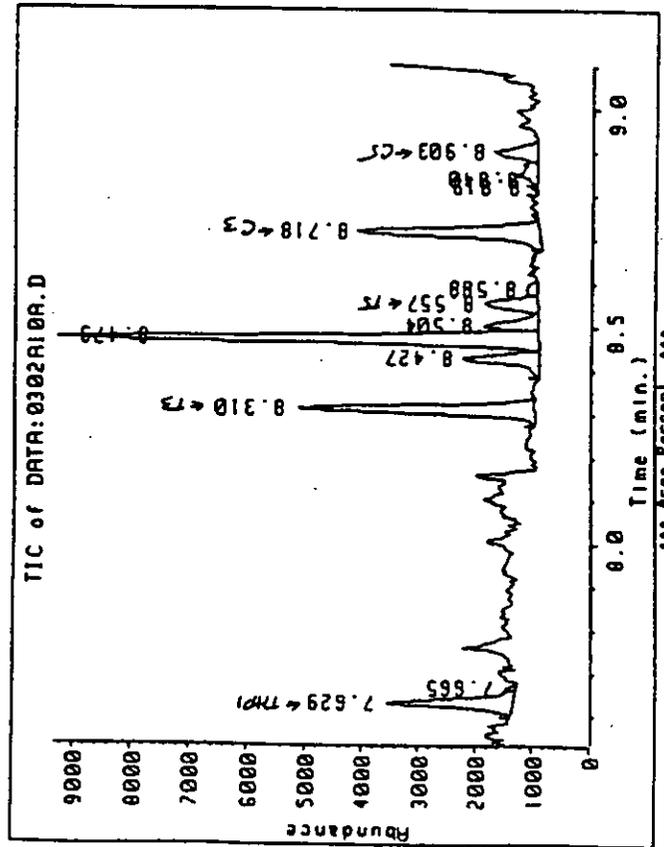
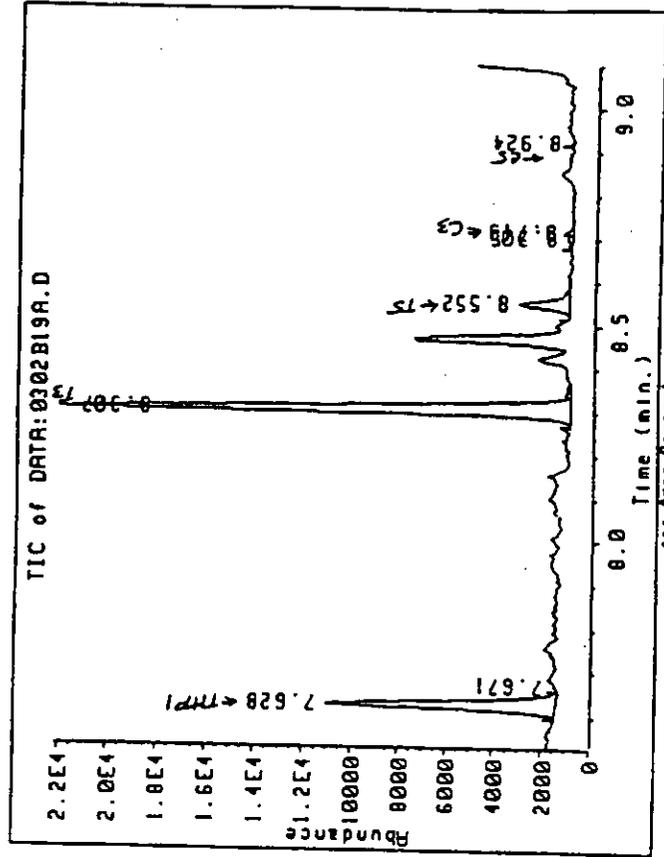
Figure 3. Sample Kidney Chromatograms for the Analysis of THPI (7.63 min), T3 (8.31 min), T5 (8.55 min), C3 (8.72 min), and C5 (8.90 min)



A. Calibration solution containing 0.02 µg/mL of THPI, T3, T5, C3, and C5.

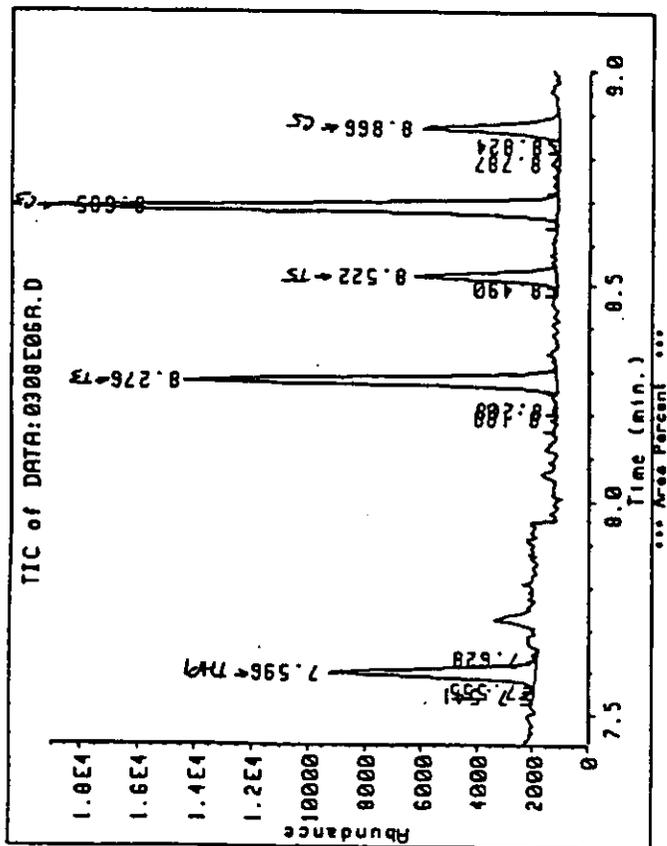
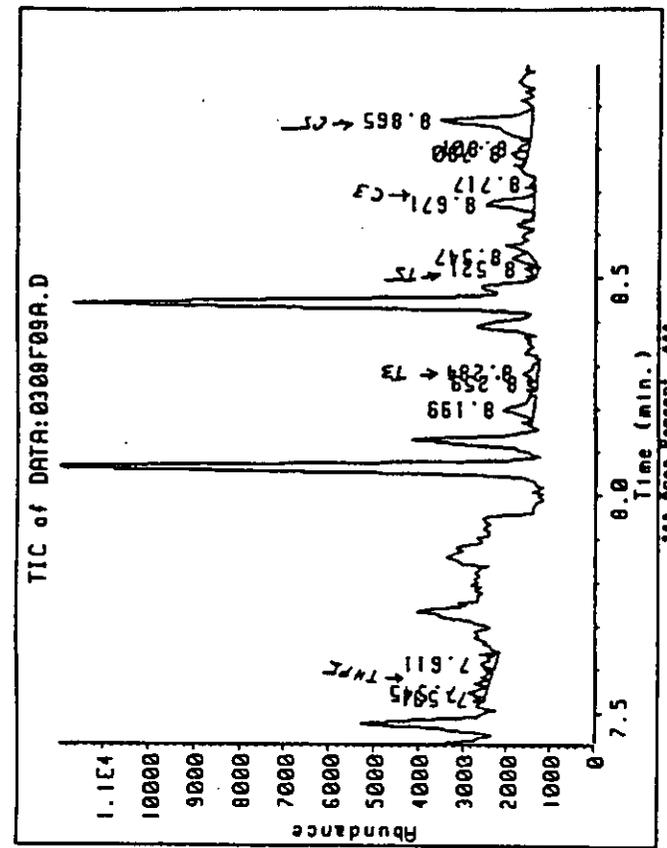
B. Untreated control kidney (F7001-141A1).

Figure 3. Sample Kidney Chromatograms for the Analysis of THPI (7.63 min), T3 (8.31 min), T5 (8.55 min), C3 (8.72 min), and C5 (8.90 min) (Continued)



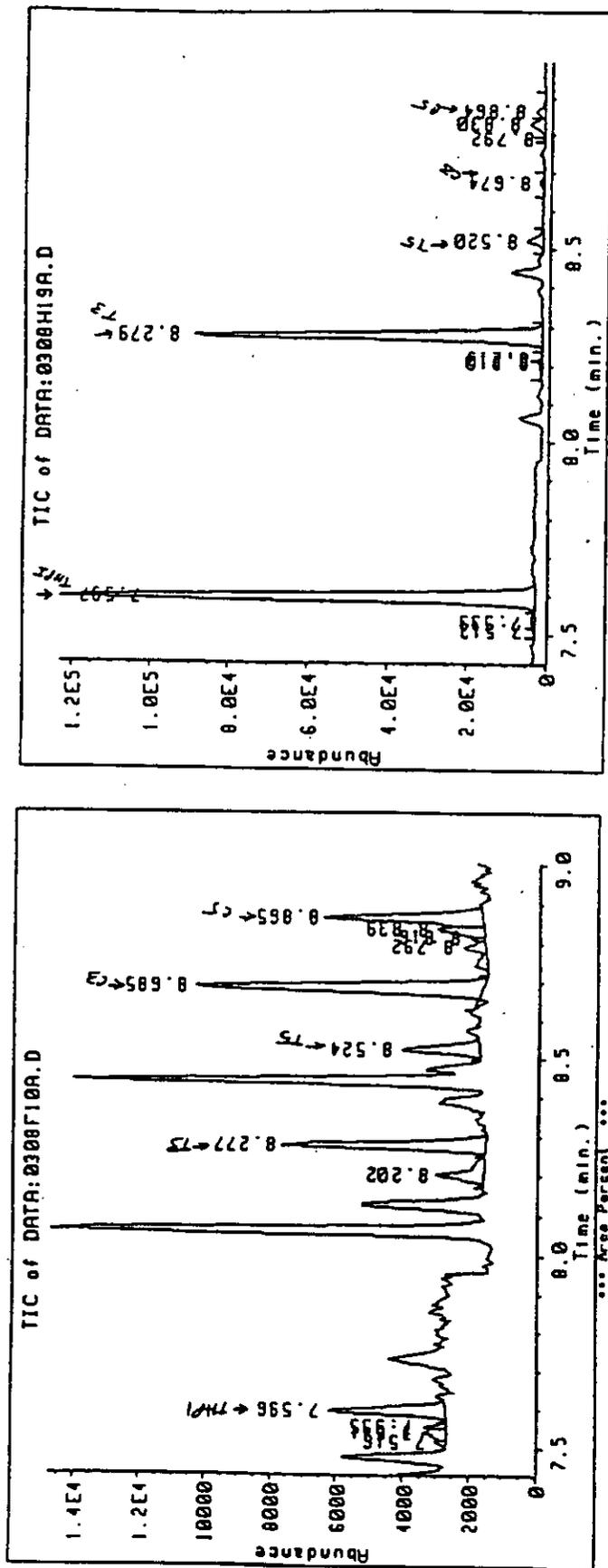
- C. Untreated control kidney (F7001-141A1) fortified at 0.01 ppm of THPI, T3, T5, C3, and C5.
- D. Treated test kidney (F7001-149A1) cow #583, from the 3X-treatment group. The kidney contained 0.06 ppm of THPI, 0.07 ppm of T3, 0.02 ppm of T5, <0.01 ppm of C3, and <0.01 ppm of C5.

Figure 4. Sample Liver Chromatograms for the Analysis of THPI (7.60 min), T3 (8.28 min), T5 (8.52 min), C3 (8.69 min), and C5 (8.87 min)



- A. Calibration solution containing 0.02 µg/mL of THPI, T3, T5, C3, and C5.
- B. Untreated control liver (#57622).

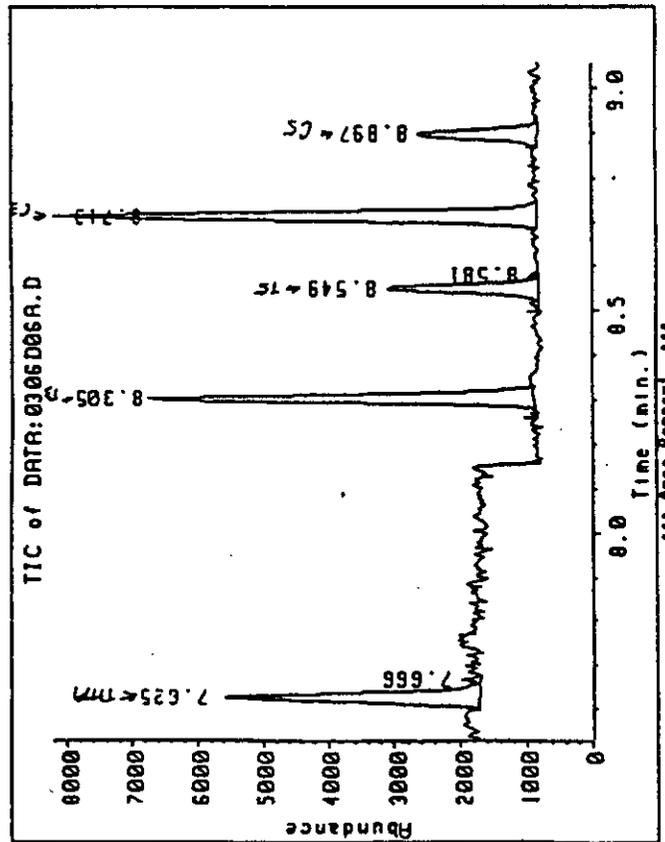
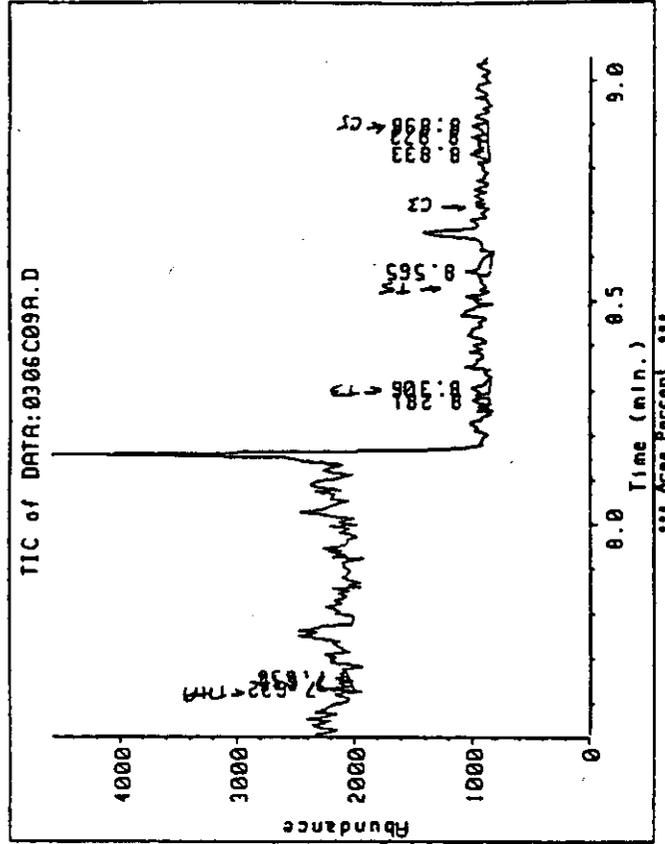
Figure 4. Sample Liver Chromatograms for the Analysis of THPI (7.60 min), T3 (8.28 min), T5 (8.52 min), C3 (8.69 min), and C5 (8.87 min) (Continued)



C. Untreated control liver (#57622) fortified at 0.01 ppm of THPI, T3, T5, C3, and C5.

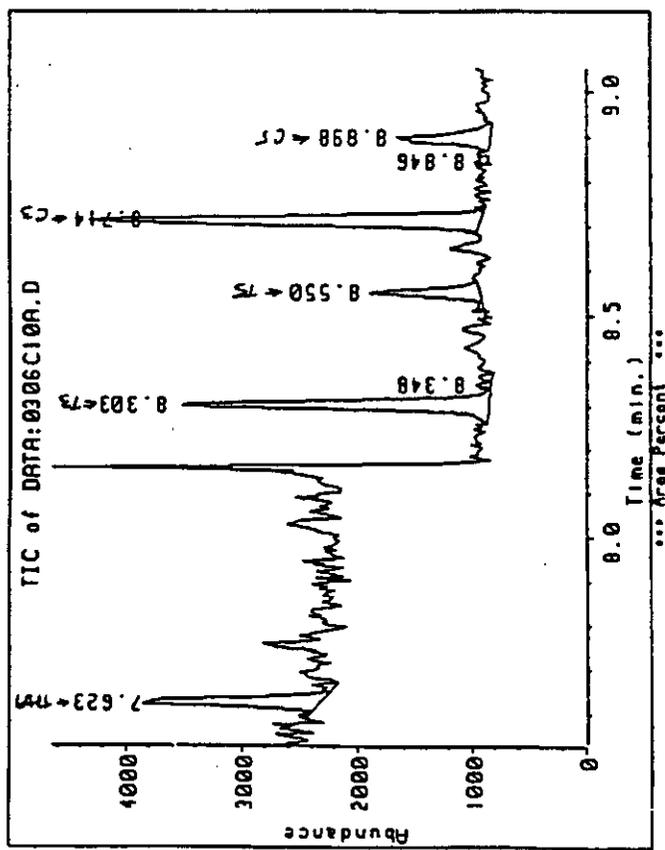
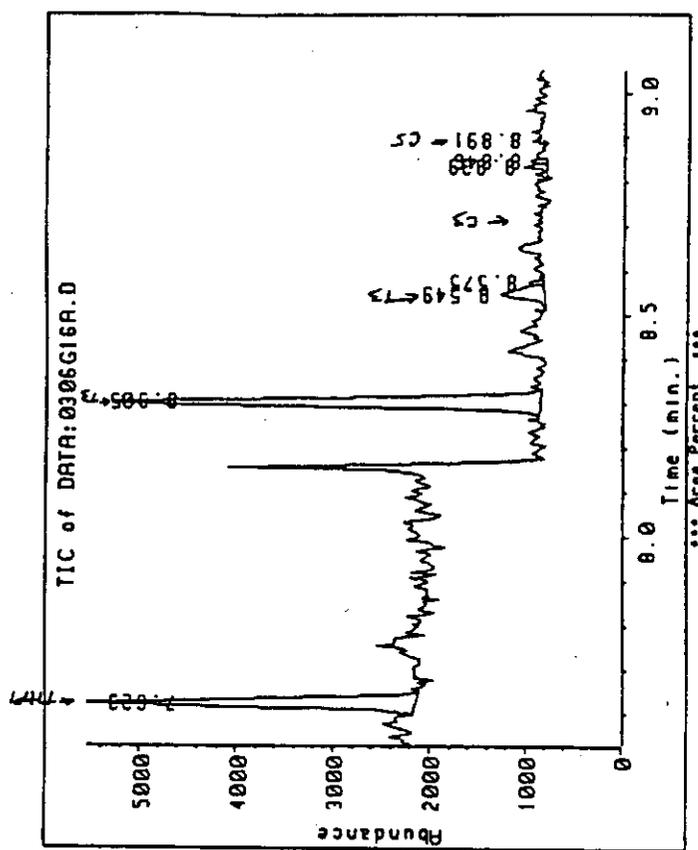
D. Treated test liver (F7001-171A2) cow #1937, from the 10X treatment group. The liver contained 0.28 ppm of THPI, 0.12 ppm of T3, 0.02 ppm of T5, <0.01 ppm of C3, and <0.01 ppm of C5.

Figure 5. Sample Muscle Chromatograms for the Analysis of THPI (7.63 min), T3 (8.31 min), T5 (8.55 min), C3 (8.71 min), and C5 (8.90 min)



- A. Calibration solution containing 0.02 $\mu\text{g/mL}$ of THPI, T3, T5, C3, and C5.
- B. Untreated control muscle (F7001-174A1).

Figure 5. Sample Muscle Chromatograms for the Analysis of THPI (7.63 min), T3 (8.31 min), T5 (8.55 min), C3 (8.71 min), and C5 (8.90 min) (Continued)



- C. Untreated control muscle (F7001-174A1) fortified at 0.01 ppm of THPI, T3, T5, C3, and C5.
- D. Treated test muscle (F7001-178A2) cow #625, from the 1X treatment group. The muscle contained 0.02 ppm of THPI, 0.02 ppm of T3, <0.01 ppm of T5, <0.01 ppm of C3, and <0.01 ppm of C5.