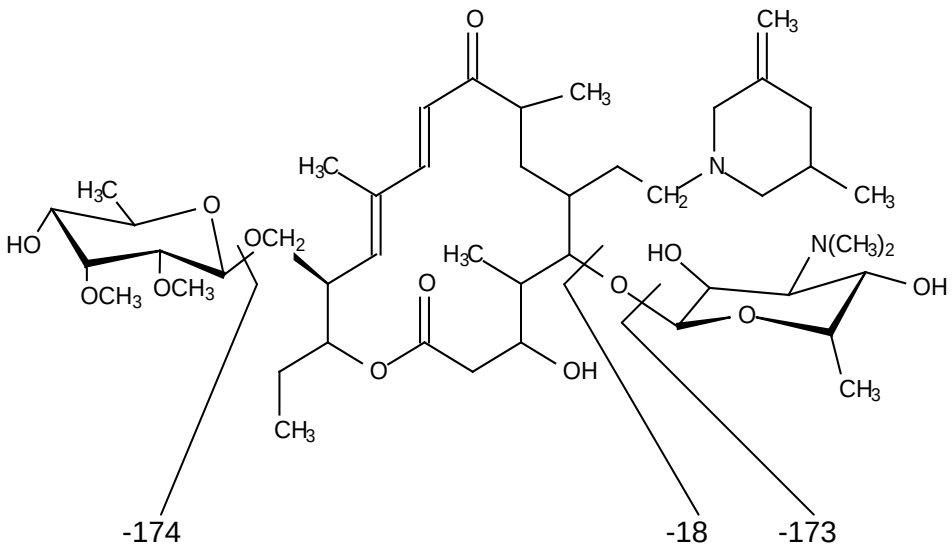
Confirmation of Tilmicosin by APCI-LC/MS^3

CONTENTS

A. INTRODUCTION

1. Theory and Structures

This procedure utilizes extracts from the determinative procedure (CLG-TIL1). The sample is diluted with 20/80 acetonitrile/water + 0.05% TFA solution and analyzed by APCI-LC/MS^3. The positive ion MS^1 spectrum produces a protonated ion for tilmicosin-869 Da. This ion becomes the precursor ion for the MS^2 scan which produces mainly an ion at 696 Da (869 minus the-amino sugar). This ion (696) becomes the second precursor for the MS^3 scan which produces a number of fragment ions including the aglycone at 522 Da. Not only is the MS^3 scan (190-875 Da.) used for confirmation but the ion ratio of 678/522 is utilized as well.



Tilmicosin 869.5717 (M+ H)

2. Applicability

This method is applicable to beef liver.

B. EQUIPMENT

1. Apparatus

a. H.P. model 1050 HPLC equipped with a quaternary pump and auto-injector.

b. Thermo-Finnigan LCQ — LC/MS equipped with an APCI inlet.

c. Analytical column — Zorbax-2.1x50 mm SB-Aq-5µ particle size.

d. Guard column is a 0.5µ frit.

NOTE: An equivalent may be substituted for any apparatus listed above.

C. REAGENTS AND SOLUTIONS

1. Reagents
   a. Water—HPLC Grade.
   b. Acetonitrile— Burdick & Jackson HPLC quality.
   c. Trifluroacetic acid (TFA)— Sigma-T-6508.

2. Mobile phase solutions
   a. Mobile phase A (20/80 acetonitrile/water + 0.05% TFA)— Mix 200 ml of acetonitrile with 800 ml deionized water and 0.5 ml TFA.
   b. Mobile Phase B (40/60 acetonitrile/water + 0.05% TFA)— Mix 400 ml of acetonitrile with 600 ml deionized water and 0.5 ml TFA.
   c. Mobile Phase C (95/5 acetonitrile/water + 0.05% TFA)— Mix 950 ml of acetonitrile with 50 ml of deionized water and 0.5 ml TFA.

   NOTE: An equivalent may be substituted for any reagent or solution listed above.

D. STANDARDS

1. Tilmicosin primary standard is available from Elanco Animal Health, a Division of Eli Lilly and Company, Greenfield, IN 46140-0708.

2. The Tilmicosin primary standard should be dried according to the following procedure immediately prior to use: Weigh approximately 40 mg of the standard into an actinic glass bottle; remove the lid and cover the mouth of the bottle with a piece of filter paper, secured with a rubber band; and place the bottle in a vacuum oven for three hours at 60 °C and a vacuum of 26 inches of mercury. After at least three hours, remove the primary standard from the oven and let cool to room temperature in a dessicator.

E. SAMPLE PREPARATION

1. The remaining extract from the determinative procedure (CLG-TIL1) is centrifuged @ 2500 rpm. for 5 min.

2. The extract is then diluted 1:1 with Mobile phase A and filtered through a 0.2µ nylon or ptfe Acrodisc into a 2 ml autosampler vial. Additional dilutions may be necessary.

F. ANALYTICAL PROCEDURE

1. Data Acquisition
   a. HPLC Conditions

      The following are examples of HPLC Conditions. The analyst should optimize these parameters for the instrument being used.

      Flow rate 0.6ml/min.

Mobile phase gradient profile

0.00 min.	100% Mobile phase A
8.00 min.	100% Mobile phase B
8.01 min.	100% Mobile phase C
12.00 min.	100% Mobile phase C
12.01 min.	100% Mobile phase A
17.00 min.	100% mobile phase A

b. MS Parameters

The following are examples of MS parameters. The analyst should optimize these parameters for the instrument being used.

APCI interface Parameters

Vap. Temp	470°C
Sheath flow	60 (unit of measure set by instrument)
Auxiliary flow	5 (unit of measure set by instrument)
Discharge current	5 µA
Capillary temp.	160°C
Capillary voltage	25 v
Tube lens offset	2.0 v

Acquisition parameters

Microscans	2
Injection time	200 ms

Precursor ions, isolation width, relative collision energy
869, 1.5, 33
696, 1.5, 35
Selected ions in MS^3 for ratioing---678,522.

c. MS Optimization

i. Averaged 'background spectrum' is taken and compared to previous run.

ii. Pure standard of tilmicosin is injected by flow injection to determine exact mass taken for precursor ion scans. 2-5µl of a 2.5µg/ml solution in methanol of tilmicosin is injected under MS^1 scanning conditions and the centroid of the 896 ion determined. Next, the same solution is injected under MS^2 scanning conditions using the 896 ion as precursor, and the centroid of the product ion at 696 determined.

iii A characteristic MS^3 full scan should be obtained using the 696 ion as precursor.

iv. The ion ratio 678/522 should be computed.

2. Confirmation Criteria

a. Retention time of unknown should be within ± 5% of the pure standard.

b. A reasonable match should exist between the MS^3 full scan of unknown and that of the pure standard. A reasonable match is defined as no large abundances of ions in the sample not found in the standard.

c. The ion ratio 678/522 of the unknown should match that of the pure standard. within ± 20% relative.

d. Tissue blank has no confirmable target compound.

3. Operational criteria for sample repeat injection

a. For unknown samples that will not confirm in the initial analyses and the system suitability has not been compromised, repeat the injection. Subsequently, an injection of the pure standard is made.

b. If upon re-injection the sample still fails to confirm, repeat the extraction using the determinative method.

c. If upon re-extraction the sample fails the confirmation, the sample should be reported out as non-detected for tilmicosin.

4. Sample Chromatograms and Spectra

Refer to Section K, "Chromatograms and Spectra"

G. CALCULATIONS [Not Applicable]

H. HAZARD ANALYSIS

1. Method Title — Confirmation of Tilmicosin by APCI-LC/MS

2. Required Protective Equipment — Safety glasses, plastic gloves, and laboratory coat.

3. Hazards

Reagents	Hazard	Recommended Safety Procedures
Tilmicosin	Allergen. Eye irritant. May cause increased heart rate.	Wear plastic gloves, lab coat, & eye protection.
Acetonitrile	Highly flammable. Explosive hazard. Vapors mixed with air will explode if ignited. Irritating to skin and mucous membranes. Inhalation of high concentrations will cause narcosis and unconsciousness.	Keep tightly closed and away from fire. Use under a fume hood. Avoid breathing vapors.
Trifluoroacetic Anhydride (TFA)	Flammable and corrosive, may cause skin or respiratory irritation.	Avoid contact or prolonged exposure to vapors. Work in a fume hood. Keep away from flame or heat.

4. Disposal Procedures

Reagents	Hazard	Recommended Safety Procedures
Organic solvents	See above	Collect waste in tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. No false positives from blank tissues.
   b. Less than 10% false negatives at tolerance.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
None listed	

3. Readiness To Perform
   a. Phase I. Standards — On three separate days, inject standards at tolerance level and determine the ratios of the ions of interest
   b. Phase II. — On three different days, analyze a blank and samples spiked at tolerance and determine the ratios of the ions of interest.

      NOTE: Phases I and II may be performed concurrently.

   c. Phase III — Analyze 5 incurred or fortified tissues at levels $\geq$ 0.6 ppm.

4. Intralaboratory Check Samples
   a. Frequency- One weekly.
   b. Acceptability criteria

      If unacceptable values are obtained, then:

      i. Stop all official analyses for that analyst.
      ii. Take corrective action.

5. Sample Acceptability and stability

   Extracts must be clear and may be stored for up to two weeks if refrigerated.

6. Sample Set
   a. Standards.
   b. Tissue blank.
   c. Tissue fortified at level of interest with suspect drug.
   d. Samples

7. Sensitivity
   a. Lowest detectable level (LDL): Not Applicable
   b. Lowest reliable confirmation (LRC): 0.6 ppm.

J. WORKSHEET

The following is an example of a worksheet.

Analyst: _____
Date Started: _____
Date Completed: _____

Set Number : _____

Reviewed by: _____
(Initials and Date)

EQUIPMENT USED	ID NUMBER
Instrument Used (LMS #):	
Tube Lens Offset:	
Method File Name:	
Centrifuge (CNT#):	
External Standard:	
Injection Volume (uL):	
Mobile Phase "A":	
Mobile Phase "B":	
Mobile Phase "C":	

Confirmation Sample Analysis Data

Sample Number	RMS Lab. Number	R-222 Lab. Number	Tissue Code	Dilution Factor	TIL Ret. Vs. Standard Time (± 5%)	TIL Ion Ratio m/z (678/522) Ion Ratio ± 20% (vs. External std.)	Reasonable Mass Spectra match? Y or N?	Tilmicosin Result + or -

REMARKS:

K. CHROMATOGRAMS AND SPECTRA

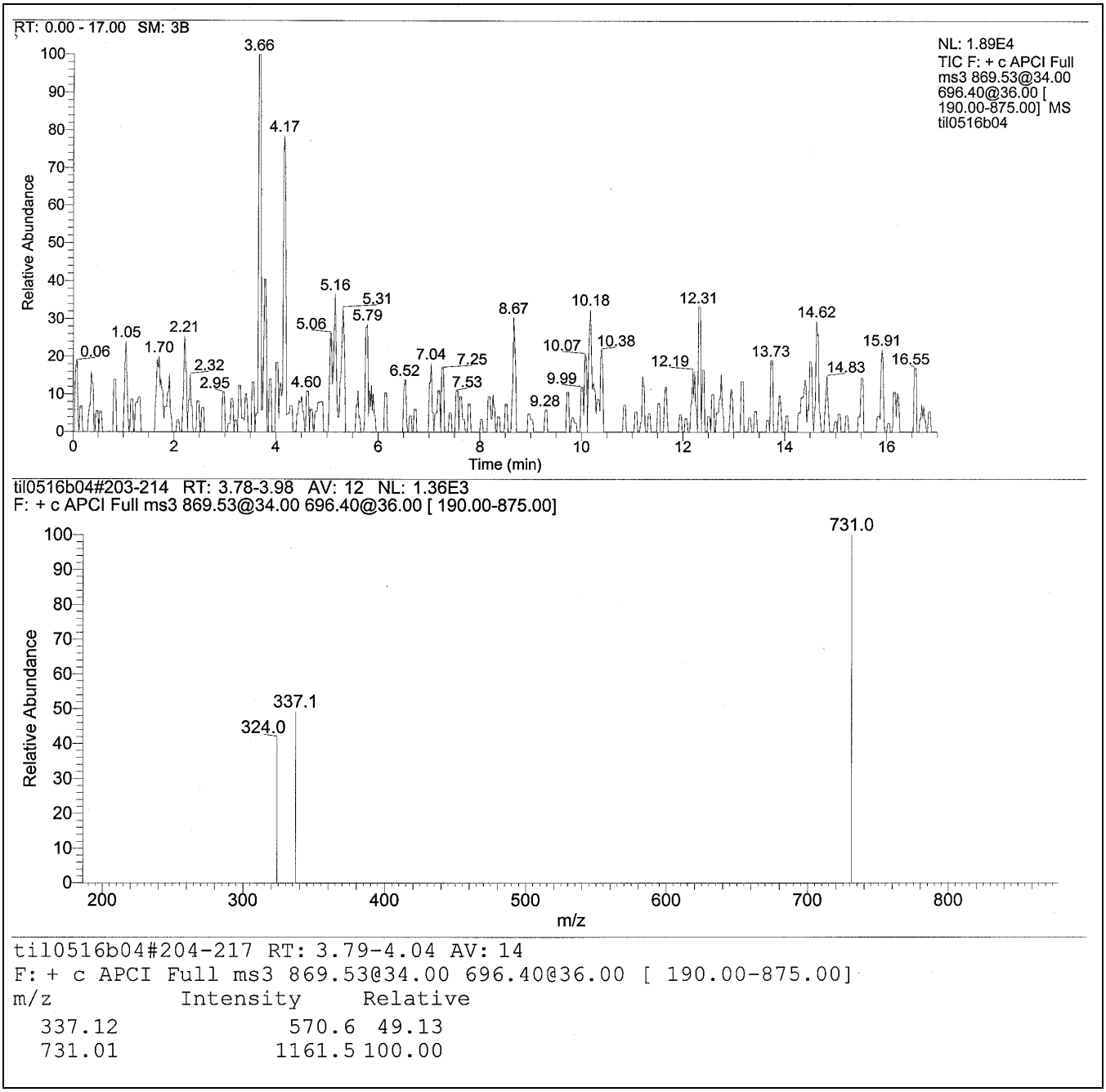
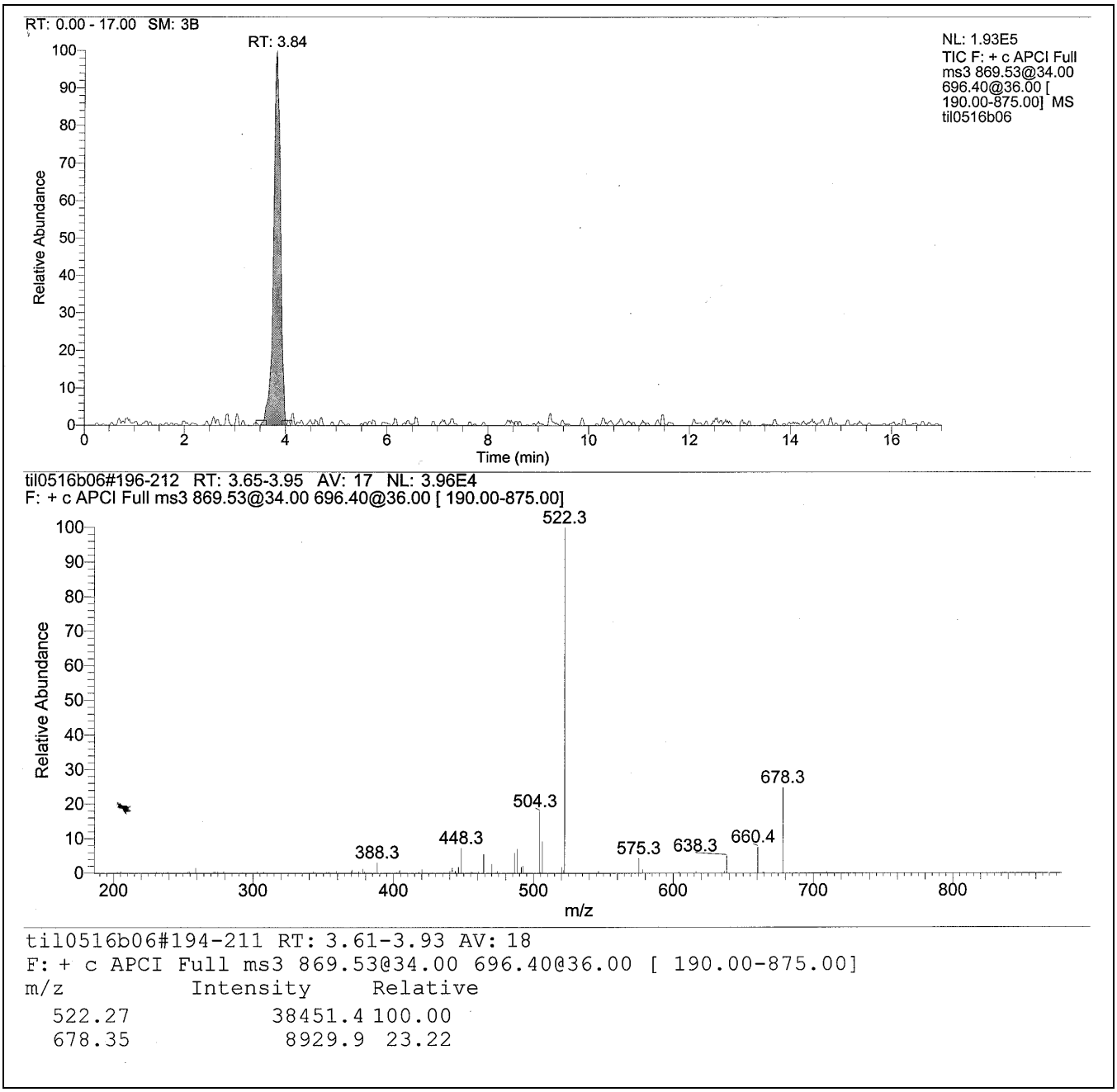


Figure 1. Blank Beef Liver

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til0516b06#194-211 RT: 3.61-3.93 AV: 18
F: + c APCI Full ms3 869.53@34.00 696.40@36.00 [ 190.00-875.00]
m/z          Intensity     Relative
  522.27          38451.4 100.00
  678.35           8929.9  23.22
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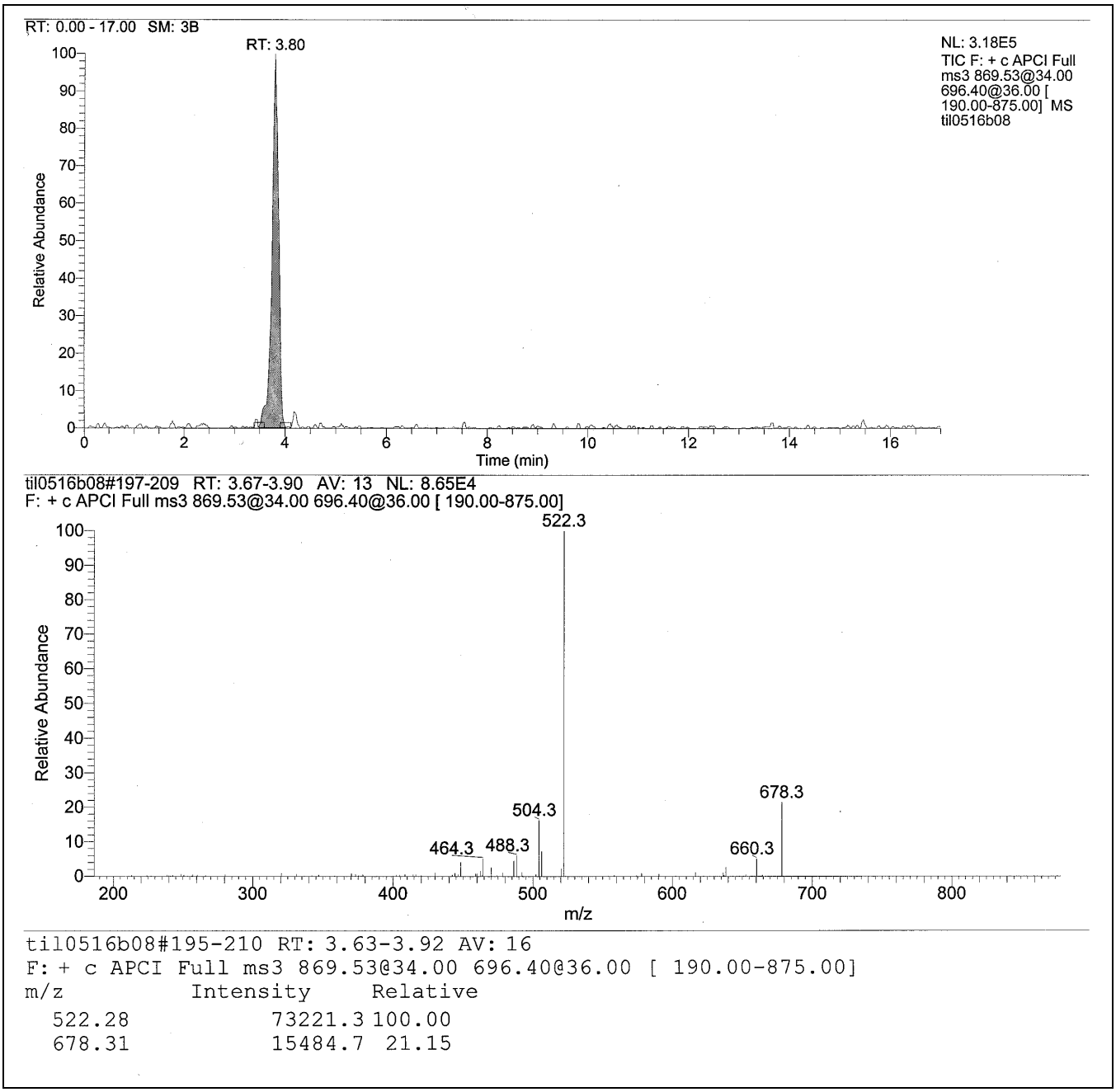
Figure 2. Beef Liver Recovery, 0.6 ppm



til0516b08#195-210 RT: 3.63-3.92 AV: 16
F: + c APCI Full ms3 869.53@34.00 696.40@36.00 [190.00-875.00]

m/z	Intensity	Relative
522.28	73221.3	100.00
678.31	15484.7	21.15

Figure 3. Beef Liver Recovery, 1.2 ppm

Approved by:	Date Approved:
Tom Mallinson	2/12/02
Terry Dutko	2/11/02
Jess Rajan	2/13/02
Bruce Cottingham	2/13/02