

**AN SOP DEVELOPED
FOR THE TESTING
INTEGRITY PROGRAM,
INC.**

**Detection of Citalopram (Celexa)
And
Metabolites in Horse Urine After Oral
Administration**

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INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) have become the standard of antidepressant therapy over tricyclic antidepressants due to safety considerations. Although not approved for these purposes, the SSRIs have been used in racing animals to combat obsessive-compulsive behavior and to promote mild sedation. Citalopram (Celexa, Forest Pharmaceuticals), a racemic bicyclic phthalane derivative and a newly approved SSRI, enhances serotonergic neurotransmission through selective and potent inhibition of neuronal serotonin reuptake.¹ In humans, approximately 12% of an oral dose is excreted unchanged in the urine and 12% as the active metabolite desmethylcitalopram. Minor inactive metabolites include didesmethylcitalopram, citalopram-N-oxide, and a deaminated propionic acid derivative.^{2,3} Approximately 65% of the drug is unaccounted for, suggesting fecal elimination or other metabolic pathways.⁴ Interestingly, escitalopram, (Lexapro, Forest Pharmaceuticals) approved in August, 2002 as an SSRI, is the pure S-enantiomer of racemic citalopram. Applicability of this SOP to escitalopram has not been established.

This SOP details extraction and thin layer chromatography protocols for detecting citalopram in horse urine and GC/MS and LC/MS/MS techniques for confirmation of parent drug. Only traces of the metabolites reported in humans are detected in horse urine, but a major unidentified metabolite is present.

SCOPE

This SOP describes screening and confirmation of parent citalopram and an unidentified metabolite in horse urine after an oral dose. Screening is accomplished by thin layer chromatography (TLC) after either solid phase extraction (SPE) or liquid/liquid (L/L) extraction. Confirmation is by gas chromatography/mass spectrometry (GC/MS) and/or liquid chromatography/mass spectrometry (LC/MS).

LIMITATIONS

The primary metabolite, which is present in greater abundance than parent, remains as yet unidentified. While parent is relatively stable, this metabolite undergoes decomposition during frozen storage.

Since no immunoassay is currently available to detect citalopram, screening is limited to instrumental techniques or TLC.

ADMINISTRATION

140 mg (seven 20 mg tablets) orally as CELEXA (Forest Pharmaceuticals) at TVMDL. 240 mg PO at University of Pennsylvania New Bolton Center.

ANALYTICAL STANDARD

Sigma Chemical Company (citalopram hydrobromide), #C7861.

IMMUNOASSAY

Immunoassay tests specific for citalopram and/or its metabolites are not available. The battery of ELISA kits available from Neogen Corporation were tested on administration urine. All produced negative results.

SOLID PHASE EXTRACTION

The columns used in this protocol are from United Chemical Technologies, Inc. (XTRACT, 500 mg, # XRDAF515).

Sample Preparation

1. To 5 ml of sample in a numbered 20 x 125 mm glass tube, add 2 ml of 1M acetate buffer (pH 5).
2. Add 1 ml beta-glucuronidase to each tube. Mix well.
3. Place tubes in test tube rack and cover loosely with foil. Set into 65°C (\pm 5°C) water bath. Set timer for 2-4 hours.
4. Remove tubes from bath and add 5 ml 0.2 M phosphate buffer (pH 6.5) into the sample tube and mix. (Note: pH of sample should be between 5.5 and 6.5)

Column Preparation

All solid phase extraction manifolds are used according to manufacturer's instructions. Traps should be installed between manifold and vacuum source.

1. Prepare numbered 16 x 125 disposable screw-top glass tubes labeled **B** (Basic drugs) for sample collection.
2. Place the appropriate number of stopcocks on the manifold.
3. Insert the appropriate number of SPE columns (numbered accordingly) on stockcocks.
4. Add 5 ml methanol to each SPE syringe bed and aspirate. Do not allow the column to go dry during steps 4-6. Use minimum vacuum.
5. Immediately add 5 ml deionized H₂O to each column and aspirate.
6. Add 3 ml 1.0 N acetic acid (HOAc) to each column and aspirate. At this time the column is activated.

SPE Sample Washes and Extraction

1. Apply the sample to the activated column at a rate of approximately 1-3 ml/min.
2. Add 5 ml 0.2 M phosphate buffer (pH 6.5) to each column and aspirate (Wash)
3. Add 2 ml 1.0 N HOAc to each column and aspirate. (Wash) Dispose of waste.
4. Dry the column under strong vacuum (e.g., 12-15 in) for ~10-40 minutes.
5. Release vacuum.
6. Add 5 ml hexanes to each column and aspirate. (Wash) Dispose of waste.
7. Add 5 ml DCM containing 1% MEOH to each column and aspirate (this fraction contains most acid/neutral drugs and is discarded).

8. Add 7 ml methanol to each SPE column and aspirate. (Wash)
9. Insert numbered 16 x 125 mm screw-top glass tubes labeled with **B** (Basic drugs) into the manifold.
10. Mix thoroughly 80:20:2 DCM:ISO:NH₄OH (ISO is isopropanol) prior to use. **NOTE:** (Mixture should not be allowed to sit in an open container and is to be prepared fresh daily.) Elute the Basic/Enzyme hydrolyzed drugs with 5 ml 80:20:2 DCM:ISO:NH₄OH. Flow rate should not exceed 1 to 3 ml/minute.

Apply vacuum as necessary to recover residual solvent from the column.

11. Remove tubes and evaporate solvent at 45°C ± 5°C with nitrogen.

LIQUID/LIQUID EXTRACTION

Enzyme Hydrolysis Procedure

1. To 5 ml of urine in a 16 x 125 mm screw-top glass tube, add 2 ml of 1M acetate buffer (pH 5).
2. Add 1 ml beta-glucuronidase to each tube. Vortex each tube.
3. Cover tubes with foil (or loosely cap), place in a small test tube rack and set in the water bath or incubating oven (approximately 60°C ± 5°).
4. Incubate tubes for 2-4 hours.
5. At end of heating period, place tubes in cool water for minimum of 5 minutes.
6. Add 0.5 ml ascorbic acid (10%) solution to each tube.
7. Add 0.3 ml of ammonium hydroxide (NH₄OH):dH₂O (50:50) to each tube. Vortex 3 seconds.
8. Adjust each tube to pH 8.5-9.2 using 6N HCl (1 drop at a time) or 0.1-0.2 ml NH₄OH:dH₂O (50:50).
9. Add 5 ml dichloromethane (DCM):isopropanol (ISO) (10:1) to each tube.
10. Cap tightly and rotorack slowly for 5 minutes.
11. Centrifuge at approximately 2000-2500 rpm for 5 minutes.
12. Aspirate aqueous layer and carefully transfer solvent layer to clean screw-top tube.
13. Add 3.0 ml 0.5 M sulfuric acid (H₂SO₄).
14. Cap tightly and rotorack slowly for 5 minutes.
15. Aspirate and discard bottom layer.
16. Add 0.2 ml ascorbic acid (10%) solution to each tube.
17. Add 0.6 ml NH₄OH:dH₂O (50:50) to each tube.
18. Vortex each tube for three seconds.
19. Adjust each tube to pH 8.5-9.2 using 6N HCl (1 drop at a time) or 0.1-0.2 ml NH₄OH:dH₂O (50:50).
20. Add 5 ml DCM:ISO (10:1) to each tube.
21. Cap tightly and rotorack slowly for 5 minutes.
22. Aspirate aqueous layer and carefully transfer solvent layer to clean 13 x 100 mm disposable glass test tube.
23. Evaporate at 45°C ± 5°C using N₂.

THIN LAYER CHROMATOGRAPHY

The thin layer chromatography plates used in this protocol are silica gel 60 (10 cm x 20 cm) with a 254 nm fluorescent indicator (EM Science). Just prior to use, plates are activated by heating for 10 minutes at ~ 110°C.

1. Residues from either extraction protocol are dissolved in 20 ul ethyl acetate. Spot 2-5 ul on the TLC plate.
2. Develop the TLC plates in Prop-A solvent system to a height of at least 5 cm.
3. Remove plates from tank and allow to dry. Spray with Dragendorf's reagent, cover with glass plate to prevent spots from fading, and observe on light box.
4. Spray plates with NaNO₂ reagent, cover with glass plate to prevent spots from fading, and observe on light box.

After completion of spray sequence, parent citalopram appears as a brown spot at approximately Rf 0.35. An apparent metabolite appears at approximately Rf 0.25.

REAGENTS

TLC Solvent Systems

Prop-A

Solvent: chloroform 72 ml : methanol 18 ml : propionic acid 10 ml

Stock: chloroform : methanol (72:18)

Daily: Mix stock solution well. Dispense 90 ml stock into a 100 ml graduated cylinder. Add 10 ml propionic acid. Pour into tank, cover, mix thoroughly by tilting tank, and let equilibrate for 15 minutes.

Extraction Reagents

1.0M Acetate Buffer (pH 5.0)

Place 164.0 g **sodium acetate** or 272.0 g **sodium acetate trihydrate** in a 2 L flask. Add 66.0 ml **acetic acid**. Dilute to volume (2000 ml) with **deionized water**. Check pH (5.0 ± 0.1).

50% Ammonium Hydroxide NH₄OH:dH₂O (50:50)

Combine 250 ml **ammonium hydroxide** (NH₄OH) and 250 ml **deionized water**.

10% Ascorbic Acid

Dissolve 25 g of **ascorbic acid** in 250 ml **deionized water**. Store in refrigerator.

TIP Verified SOP 2003

Dichloromethane:Isopropanol DCM:ISO (10:1)

Combine 900 ml **DCM** and 90 ml **isopropanol**.

Beta-Glucuronidase “Patella vulgata” solution (5000 IU/ml)

Dissolve one bottle (1 million units) of beta-glucuronidase from Patella vulgata (e.g., Sigma) in 200 ml **deionized water**. Store in the refrigerator.

6 N Hydrochloric Acid (HCl)

Slowly add 258 ml concentrated **hydrochloric acid** (HCl) to 242 ml **deionized water** in a 1000 ml beaker. Slowly mix the solution, then allow to cool.

0.5 M (1.0 N) Sulfuric Acid (H₂SO₄)

Pour 500 ml **deionized water** into a 1000 ml vessel, slowly add 25 ml concentrated **sulfuric acid**. Dilute to 900 ml with **deionized water**.

Dichloromethane:Isopropanol:Ammonium Hydroxide DCM:ISO: NH₄OH (80:20:2)

Combine 2 ml **NH₄OH** to 20 ml **isopropanol** in a graduated cylinder (mix well). Add mixture to 80 ml **DCM**.

0.2 M Phosphate Buffer pH 6.5

Dissolve 87.04 g **potassium phosphate monobasic** (KH₂PO₄) in 3200 ml **deionized water**. Mix well. Adjust pH to 6.5 with **sodium hydroxide** pellets (approximately 11 g) or 5-10N Na or KOH.

TLC SPRAY REAGENTS

Dragendorf’s Spray

Reagent A: Add 2.0 g **bismuth subnitrate** to 25 ml **glacial acetic acid**. Dilute to 100 ml with **deionized water**.

Reagent B: Dissolve 40.0 g **potassium iodide** in **deionized water**; dilute to 100 ml with **deionized water**.

Spray: Mix 10 ml **Reagent A** and 10 ml **Reagent B**, add 20 ml **glacial acetate acid**; dilute to 100 ml with **deionized water**.

Unused A and B are stored at room temperature. Dragendorf’s is stable for two days.

Sodium Nitrite Spray (NaNO₂)

Dissolve 5.0 g **sodium nitrite** in deionized water. Dilute to 100 ml with **deionized water**.

LIMIT OF DETECTION BY TLC

Five ml aliquots of blank equine urine were spiked with varying amounts of citalopram, extracted by the liquid/liquid protocol (minus glucuronidase treatment), and analyzed by TLC as described in this SOP. The limit of detection for citalopram was 200 ng/ml urine.

STABILITY OF CITALOPRAM

Blank equine urine was spiked with citalopram at a concentration of 320 ug/ml. Citalopram was measured by HPLC (direct urine injection after diluting 1:10 with acetonitrile and centrifuging for 12 minutes at 12,000 g) using the following parameters: column, Econosphere 4.6 x 250 mm, 5 u C-18; solvent system, acetonitrile: 1% triethylamine and 1% tetrahydrofuran in H₂O, pH 4.2, 30:70; flow rate, 1 ml/minute; injection volume, 40 ul. Peak areas at 210 nm were measured at different times under various storage conditions and compared to determine the stability of citalopram.

Recovery of Citalopram After Storage at Various Temperatures

<u>Day</u>	<u>Frozen* (-20°C)</u>	<u>Room Temperature Plus Ambient Light (22°C)</u>	<u>Heated (65°)</u>
1	100.2%	101.6%	90.0%
2	99.3%	100.7%	76.6%
3	103.8%	102.6%	73.4%
5	101.1%	101.2%	57.0%
7	97.7%	99.1%	45.0%

* Each day represents a freeze/thaw cycle.

Hence, citalopram is stable for at least one week at room temperature and through five freeze/thaw cycles while frozen, but degrades rapidly when heated at 65°C.

CONFIRMATION

GC/MS

Gas chromatograph/mass spectrometer – All GC/MS work was done using a Hewlett Packard 5972 MS with a 5890 GC.

Column – An HP5 MS (30m/0.25 mm/0.25 um) capillary column was used.

Temperature program – Initial temperature 70°C (hold 1 minute), ramp rate 20°C/minute to 120°C, then 30°/minute to 300 °C.

Extraction – Five ml of urine were extracted by either SPE or L/L protocols as described in this SOP. After evaporation, residues were dissolved in 20 ul ethyl acetate and 1 ul was injected.

Derivatization – None required. Treatment with BSTFA does not improve chromatography or reveal additional metabolites.

Mass Spectra – Parent citalopram (Rt = 9.9. min) - 58 m/e (100), 238 m/e (14), 208 m/e (15), 324 m/e M+ (3). Metabolite – (Rt = 10.4 min) 58/m/e (100), 195 m/e (6), 252 m/e (12).

GC/MS chromatograms and complete spectra are included in the appendix.

Results – GC/MS analysis revealed parent citalopram and an unidentified metabolite. Additionally, two apparent metabolites (base MS peak of 58 m/e) were present in trace amounts at later hours (e.g. 12 hrs), but were unidentified due to the lack of reference standards and presence of contaminant MS peaks.

Both major compounds were detected from 2 to 8 hours, seen in trace amounts at 12 hours, and not detected at 24 hours. The predominant compound in all extracts was the unidentified metabolite. EH L/L extraction gave higher yield of both compounds than SPE. Basic extraction of urine without enzyme hydrolysis gave comparable results, indicating that enzyme treatment is unnecessary for detection of parent and the primary metabolite. However, cleaner extracts were obtained after enzyme treatment. Analysis of extracts from the TVMDL and Pennsylvania administrations yielded similar results. Attempts to purify the metabolite and obtain NMR spectra have as yet proved unsuccessful.

LC/MS

Liquid chromatograph/mass spectrometer – All LC/MS and MS/MS work was done using a Finnegan LCQ DUO.

Column – A Zorbax Eclipse XDB C-18 (4.6 x 150 mm) column was used.

Extraction – Fifteen ml of urine (enzyme hydrolyzed) were extracted by SPE as described. After evaporation, the residue was dissolved in 100 ul of CH₃CN:H₂O (1:1) and 5 ul were injected.

HPLC gradient program – Flow rate = 0.5 ml/min.

<u>Time (min)</u>	<u>(A) Methanol</u>	<u>(B) H₂O with 1% HAc</u>
0	20%	80%
10	50%	50%
15	95%	5%
17	95%	5%
18	20%	80%
20	20%	80%

MS conditions – Instrument mode, ESI MS; polarity, positive; capillary temperature, 270°; sheath gas flow, 80; auxiliary gas flow, 20; source voltage (kV), 4.5; capillary voltage, 10.

Results – LC/MS and MS/MS analysis confirmed the presence of parent citalopram (MS peak of 325 M+1; MS/MS product ions of mass 262 and 280) in administration urine. Other metabolites and possible structural assignments were as follows:

<u>Retention Time</u>	<u>MS Parent Ion (M+1)</u>	<u>MS/MS Product Ions</u>	<u>Assignment</u>
12.55 min	325	262,280,307	Parent (standard)
12.52 min	325	262,280,307	Parent (administration urine)
12.06 min	339	276,339,321,294	Major unidentified metabolite
12.22 min	311	262,280,293	Desmethyl
12.69 min	341	280,262	Hydroxy

In addition to the detection of parent citalopram, LC/MS studies confirmed the molecular weight (338) of the major unidentified metabolite as seen in GC/MS analysis. Reference standards for desmethyl and hydroxy metabolites are not available to confirm the presence of these compounds.

ESTIMATION OF CITALOPRAM IN HORSE URINE

The amount of citalopram in horse urine after the administration of Celexa (240 mg, PO, New Bolton) was estimated using GC/MS and the external standard method. Urine was extracted using the L/L protocol, and mass peak m/z 58 was measured for each collection time.

<u>Collection Time (Hr)</u>	<u>Approximate Concentration (ng/ml)</u>
2	1200
4	920
6	470
8	570
24	90

SUMMARY

Both L/L and SPE protocols are described for the detection of citalopram and metabolites in equine urine. The L/L enzyme hydrolysis procedure was superior to SPE for detection by both TLC and GC/MS. GC/MS and LC/MS studies demonstrated the presence of citalopram, a major unidentified metabolite, and possibly at least two minor metabolites (desmethyl, hydroxy).

REFERENCES

- 1) L. Pawloski, G. Nowak, Z. Gorka, H. Mazela: RO 11-2465 (cyan-imipramine), citalopram and their N-desmethyl metabolites: Effects on the uptake of 5-hydroxytryptamine and noradrenaline in vivo and related pharmacological activities. *Psychopharmacology* 1985, 86:156-163.
- 2) E. Oyeaug, E. T. Ostensen: High-performance liquid chromatographic determination of citalopram and four of its metabolites in plasma and urine samples from psychiatric patients. *Journal of Chromatography – Biomedical Applications* 1984, 308:199-208.
- 3) A. J. Jenkins, K. Gubanich: Disposition of citalopram in biological specimens from post mortem cases. *Journal of Forensic Sciences* 2002, 47(1):159-164.
- 4) R. J. Milne, K. L. Goa: Citalopram – A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in depressive illness. *Drugs* 1991, 41(3):450-477.

Figure 1: LCMS ESI MSMS Citalopram (standard)

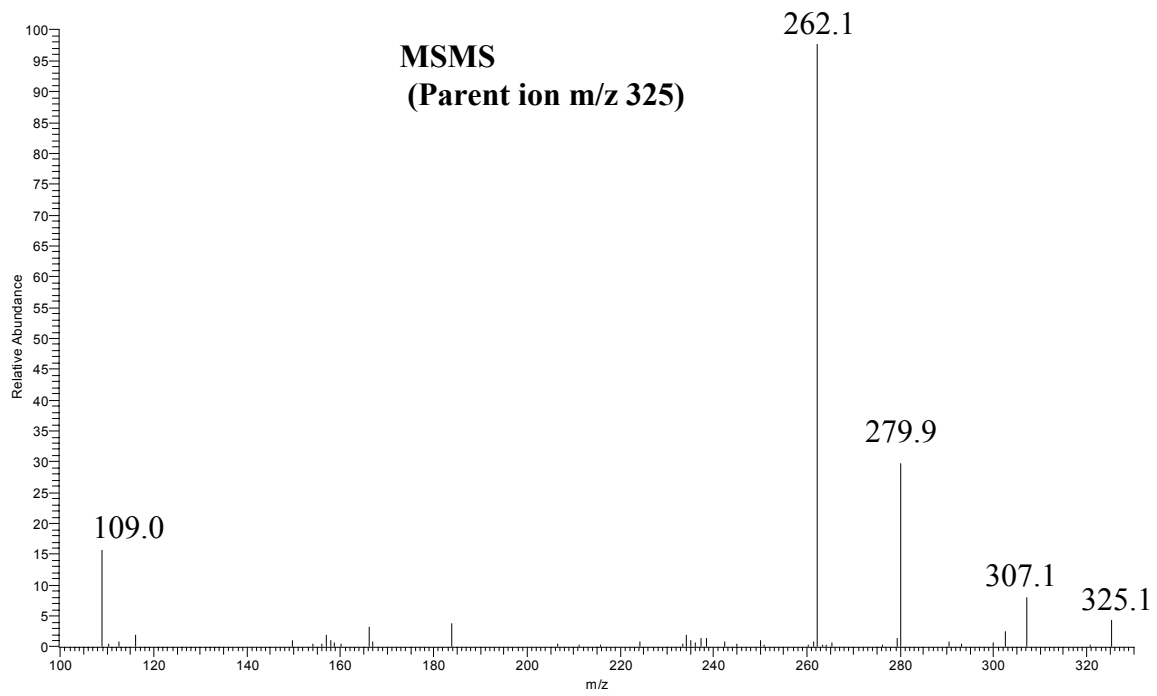
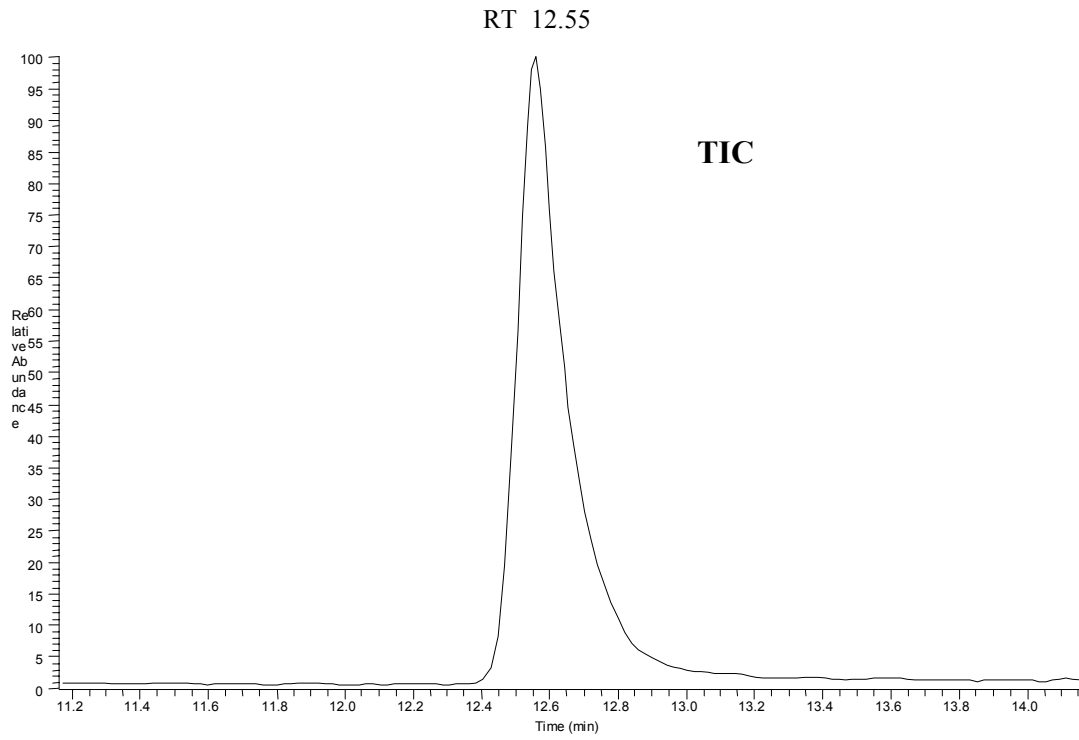


Figure 2: LCMS ESI MSMS Citalopram Admn. 4 Hr EH/SPE - Parent

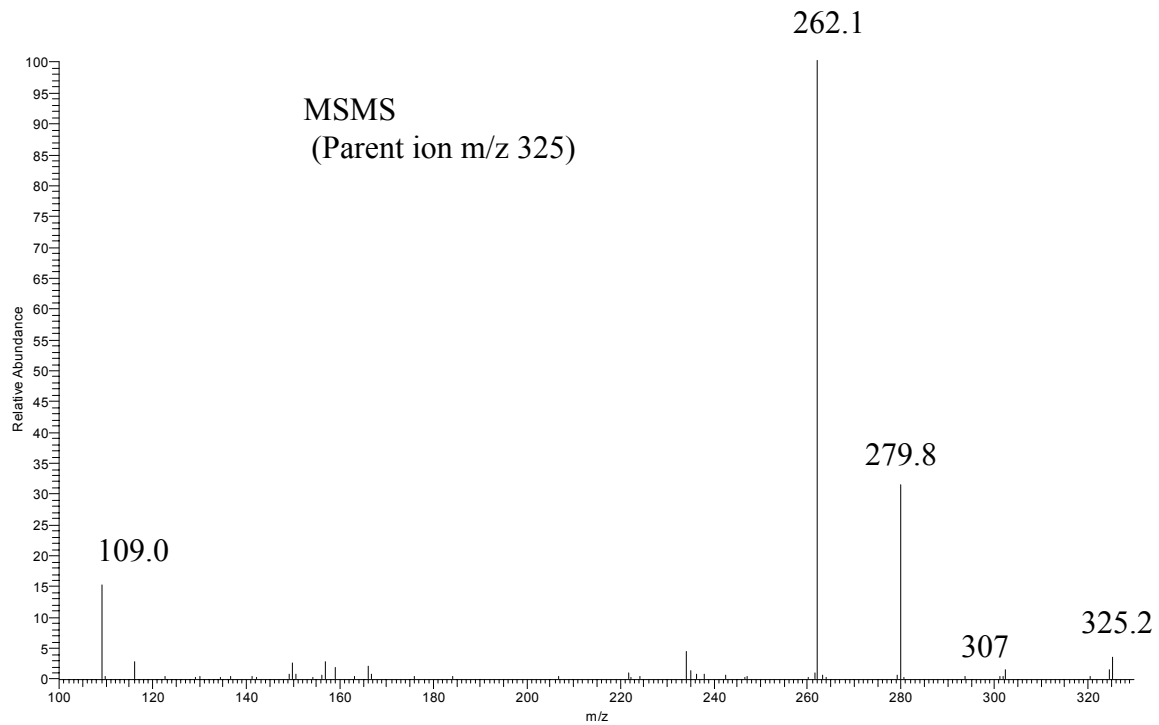
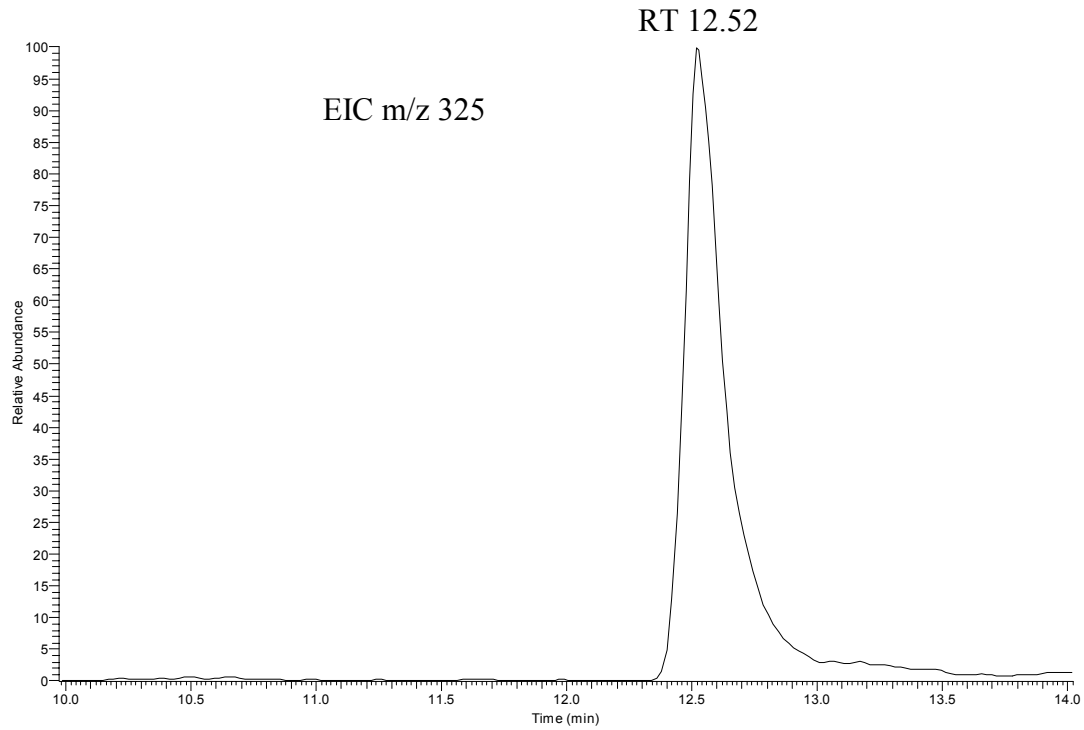


Figure 3: LCMS ESI MSMS Citalopram Admn. 4 Hr EH/SPE - Metabolites

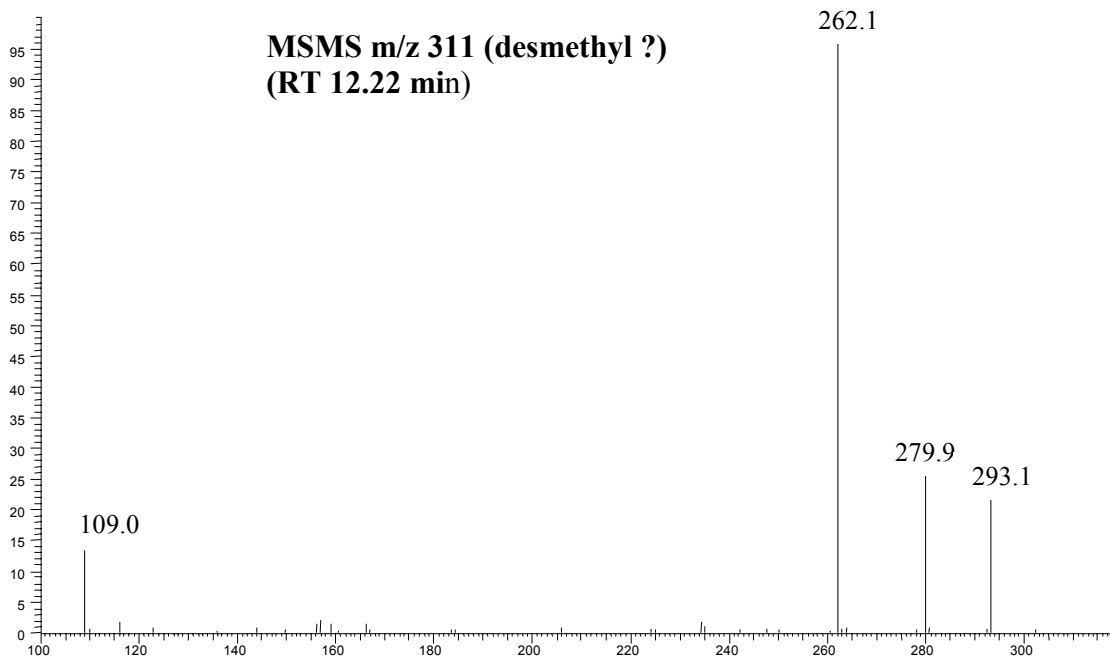
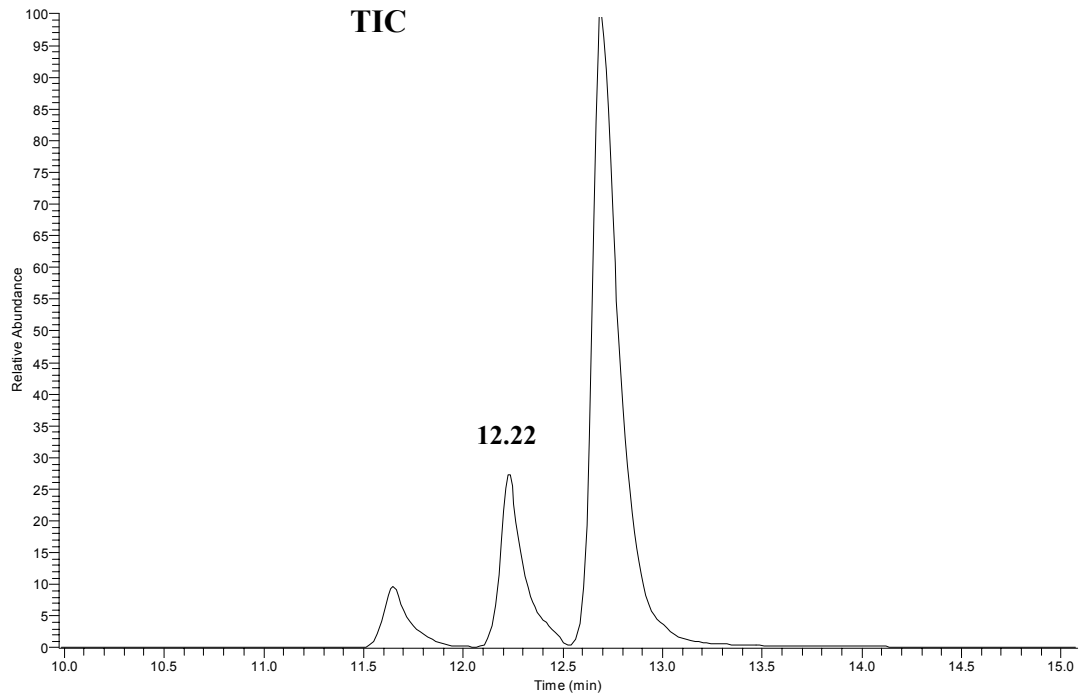


Figure 4: LCMS ESI MSMS: Citalopram Admn. 4 Hr (EH/SPE) - Metabolites

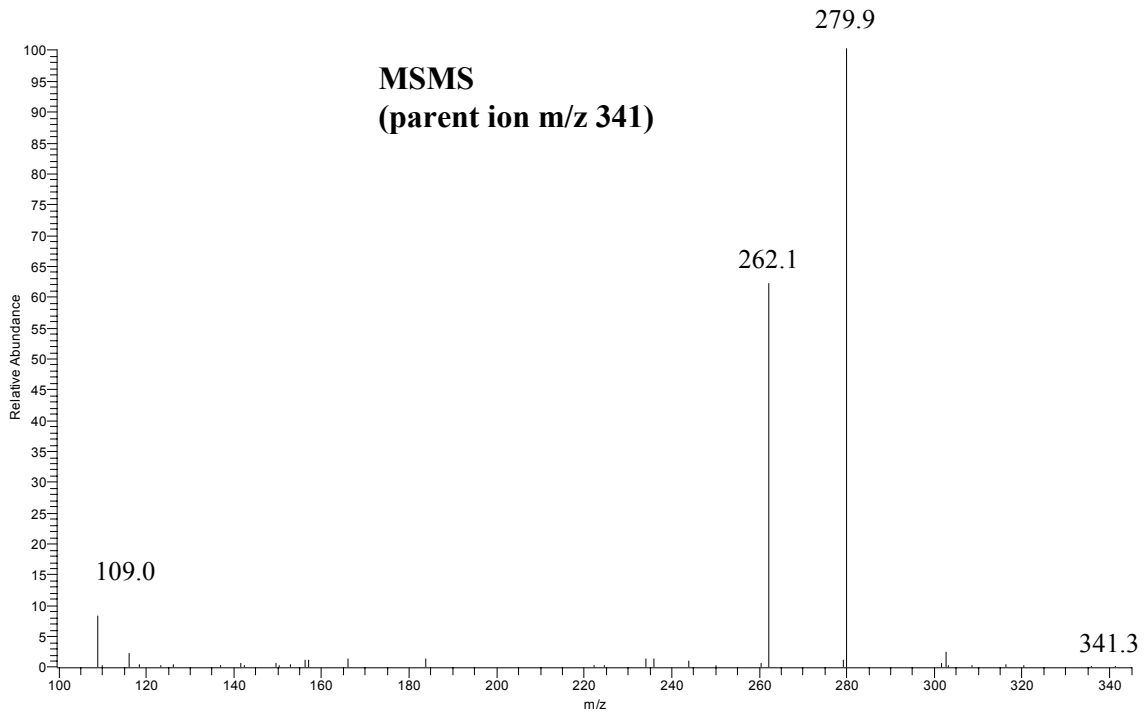
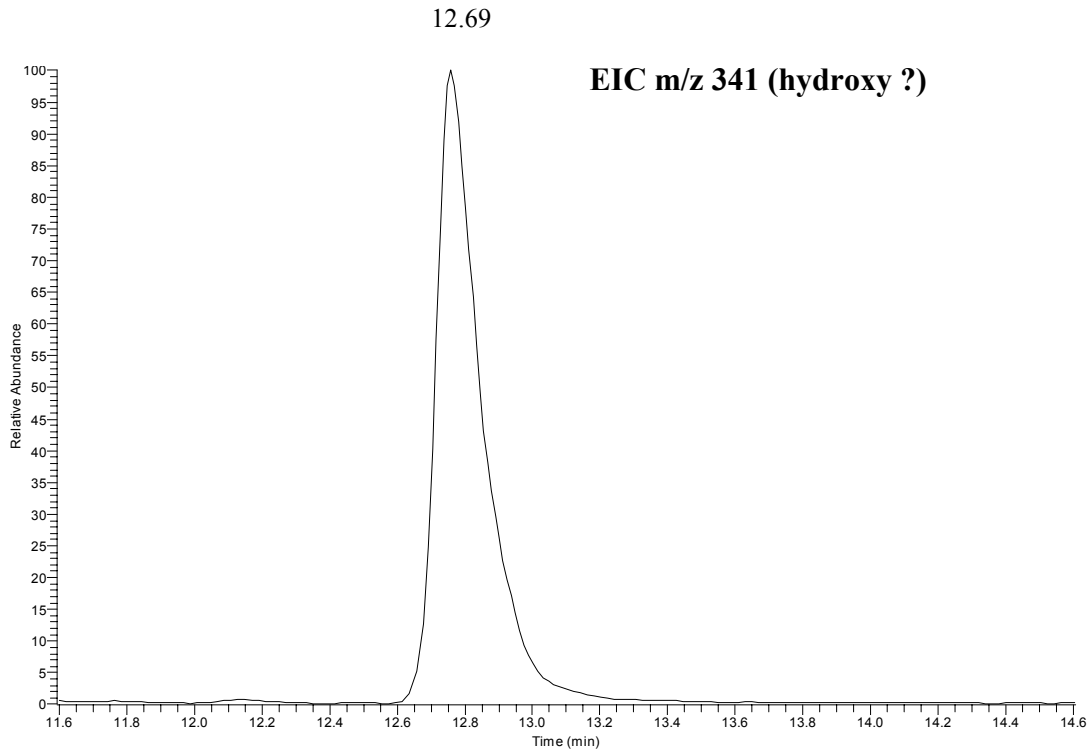


Figure 5: LCMS ESI MSMS Citalopram Admn. 4 Hr EH/SPE

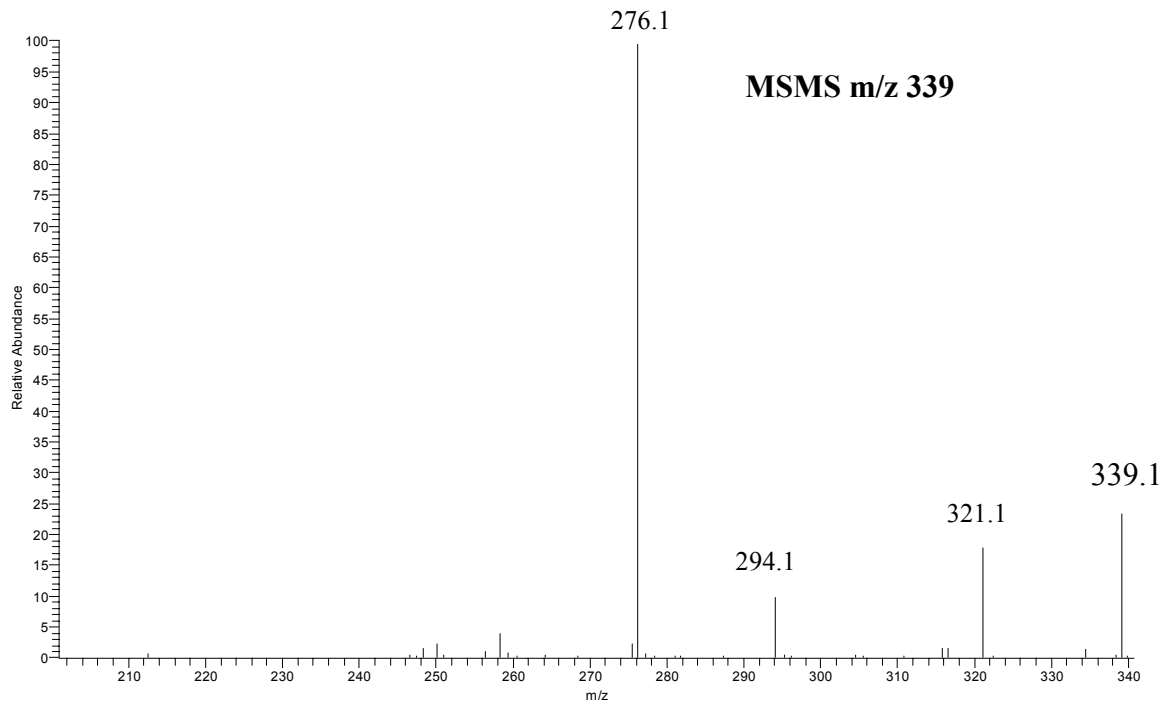
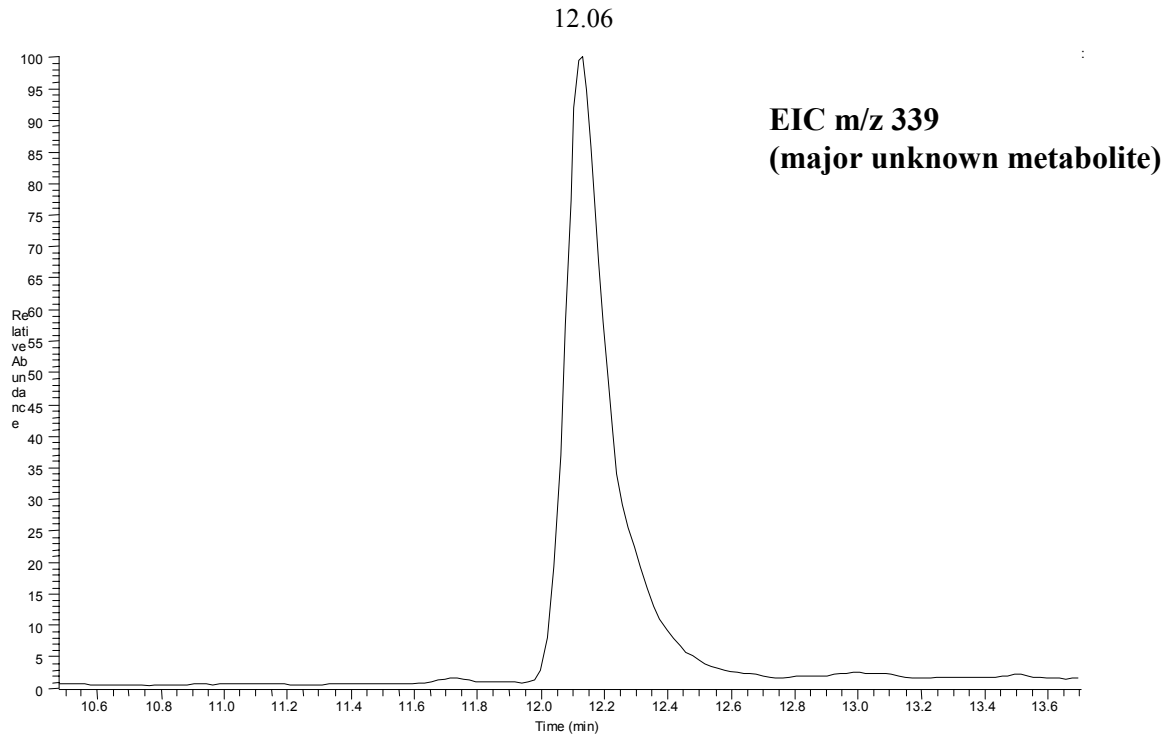


Figure 6: GCMS: Citalopram Standard

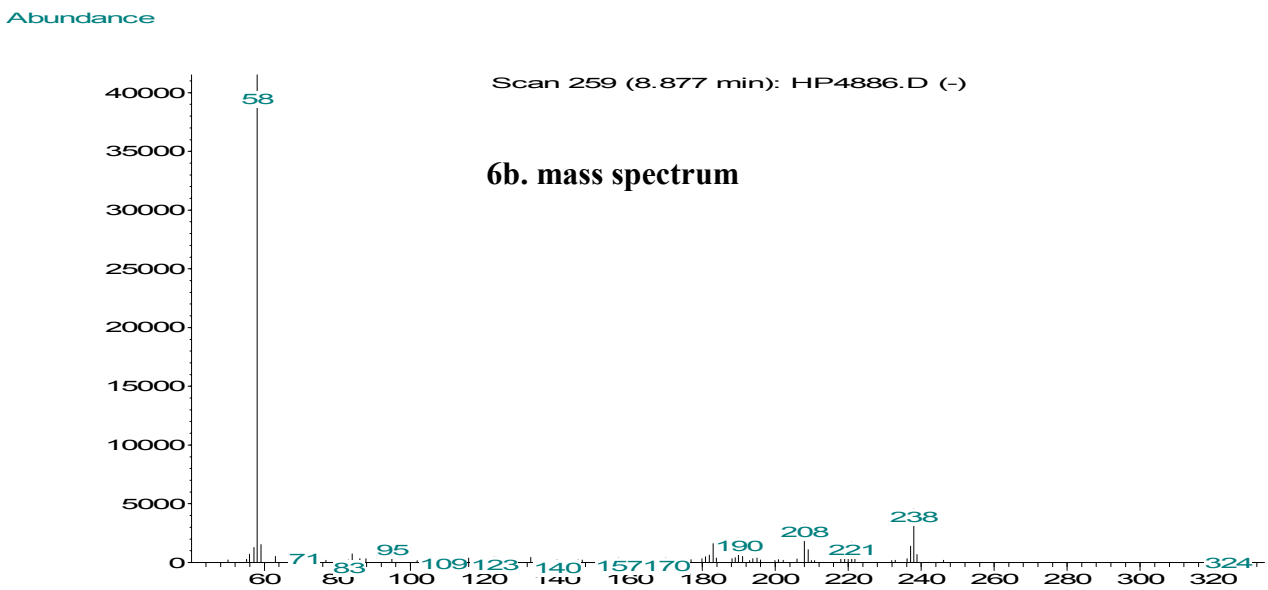
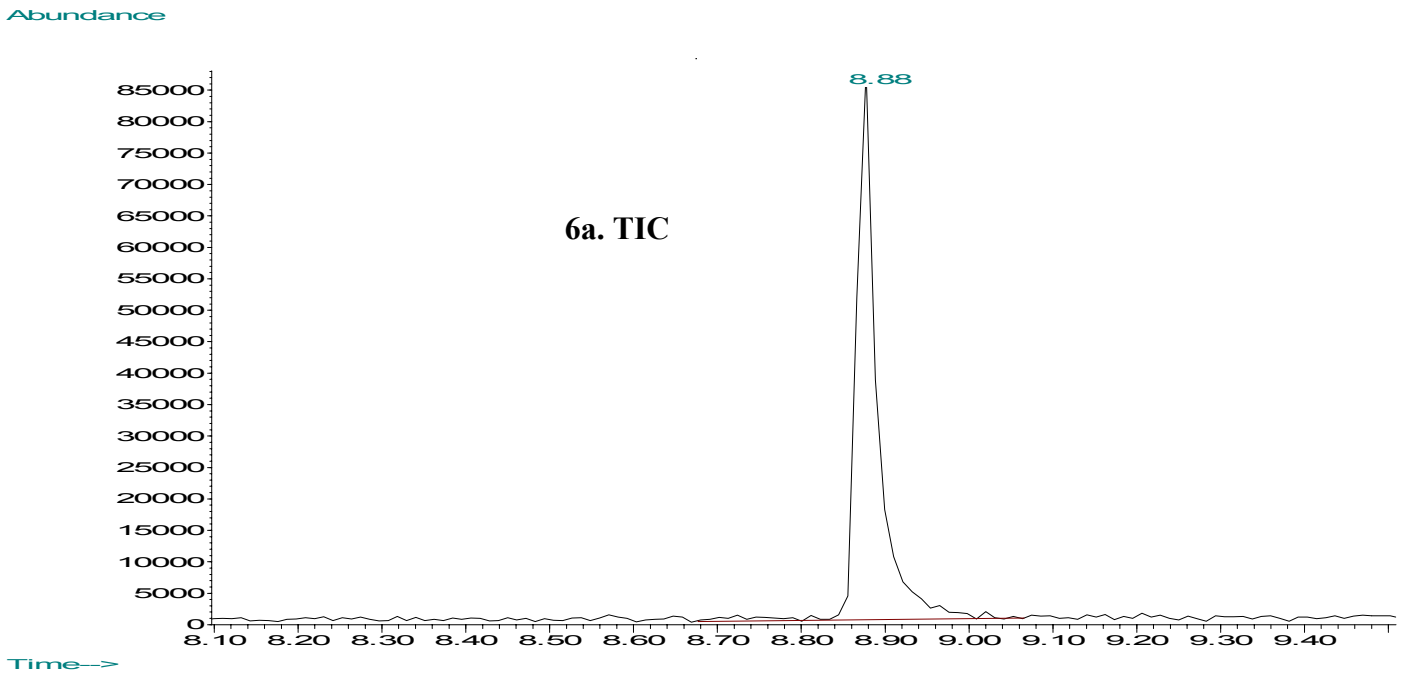
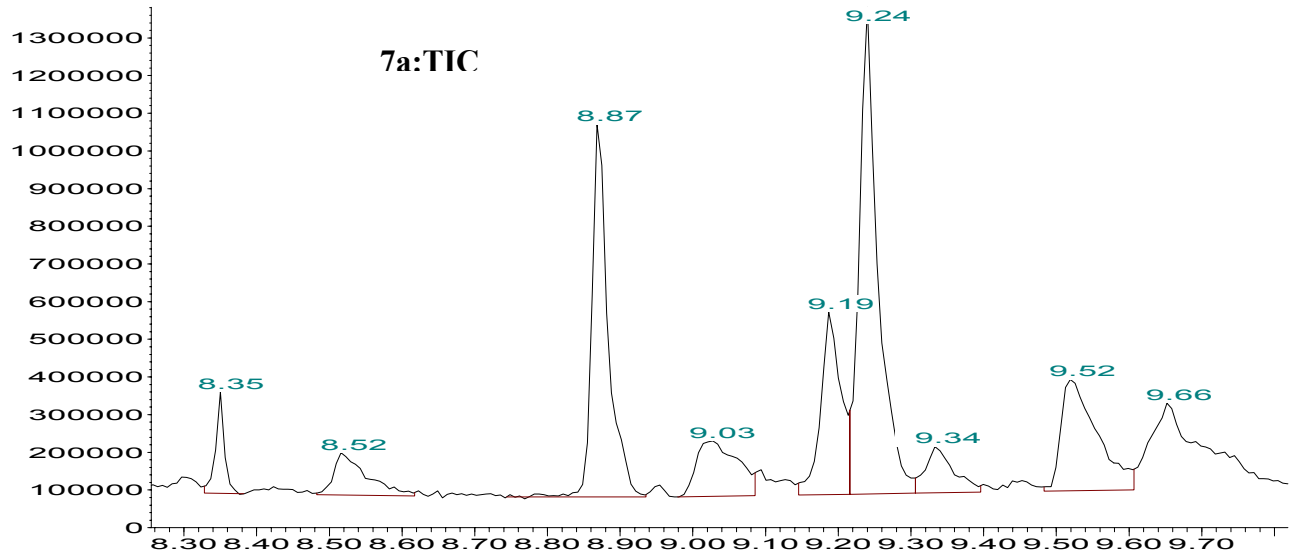


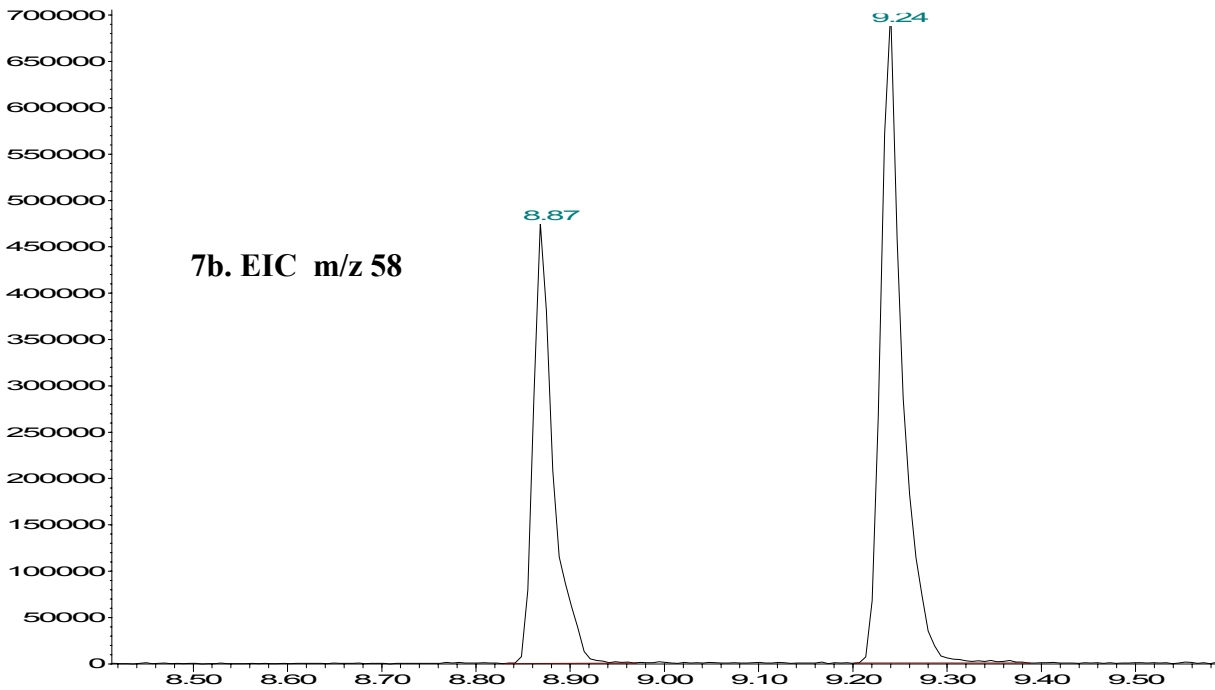
Figure 7: GCMS: Citalopram Admn. (6 Hr, EH/LL)

Abundance



Time-->

Abundance



Time-->

Figure 7: GCMS Citalopram Admn. (6 Hr, EH/LL) (contd):
Mass Spectra

