



EDGEWOOD CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1161

METABOLOMIC ANALYSIS OF THE SECRETOME OF HUMAN EMBRYONIC STEM CELLS FOLLOWING METHYL PARATHION AND METHYL PARAOXON EXPOSURE, PHASE III: LC-MS-MS STRUCTURAL CONFIRMATION

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January 2014

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 h per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) XX-01-2014		2. REPORT TYPE Final		3. DATES COVERED (From - To) Jan 2012 - Mar 2012	
4. TITLE AND SUBTITLE Metabolomic Analysis of the Secretome of Human Embryonic Stem Cells Following Methyl Parathion and Methyl Paraoxon Exposure, Phase III: LC-MS-MS Structural Confirmation				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Madren-Whalley, Janna S.; Sekowski, Jennifer W. (ECBC); Palmer, Jessica A.; Donley, Elizabeth L. R.; West, Paul; Conard, Kevin (Stemina)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Director, ECBC, ATTN: RDCB-DRB, APG, MD 21010-5424 Stemina Biomarker Discovery, Inc., 504 South Rosa Road, Suite 150, Madison, WI 53719-1256				8. PERFORMING ORGANIZATION REPORT NUMBER ECBC-TR-1161	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Edgewood Biological Chemical Center, In-House Laboratory Independent Research Program, Aberdeen Proving Ground, MD 21010-5424				10. SPONSOR/MONITOR'S ACRONYM(S) ECBC ILIR	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: This technical report is the third of three reports from the U.S. Army Edgewood Chemical Biological Center (ECBC) In-House Laboratory Independent Research (ILIR) funded project "Molecular Toxicology of TICs in Human Embryonic Stem Cells". One of the goals of the study was to identify metabolites from the secretome of human embryonic stem cells (hESC) exposed to the toxic industrial chemicals (TICs) methyl parathion (MP) and methyl paraoxon (MPO). The non-targeted liquid chromatography followed by mass spectrometry (LC-MS) approach used in the Phase I work (Sekowski et al., ECBC-TR-1177) revealed hundreds of human metabolites and multiple metabolic pathways altered as a result of exposure to both chemicals. This was followed by Phase II work (Madren-Whalley et al., ECBC-TR-1178) in which twenty metabolites were down-selected for further structural confirmation. The purpose of this Phase III study was to confirm the identity of the down-selected subset of putative metabolites by comparing them with commercially available standards using liquid chromatography followed by tandem mass spectrometry (LC-MS-MS). Commercially available standards were available for nine metabolites. Of these nine, the identity of five metabolites was confirmed in this work.					
15. SUBJECT TERMS Human embryonic stem cells (hESC) Methyl parathion Methyl paraoxon Human metabolites Electrospray ionization (ESI) LC-MS-MS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Renu B. Rastogi
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code) (410) 436-7545

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PREFACE

The work described in this report was authorized under a U.S. Army Edgewood Chemical Biological Center (ECBC) In-House Laboratory Independent Research (ILIR) program during the 2012 fiscal year. The work was started in January 2012 and completed in March 2012.

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This report has been approved for public release.

Acknowledgments

The authors acknowledge the following individuals for their hard work and assistance with the execution of this technical program:

- Way Fountain (ECBC) and Laura Borland (Booz Allen Hamilton, Belcamp, MD) for their support of this project under the ILIR program.
- Kelley Betts (Science Applications International Corporation, McLean, VA) for her technical assistance in the preparation of this manuscript.

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METABOLOMIC ANALYSIS OF THE SECRETOME OF HUMAN EMBRYONIC STEM CELLS FOLLOWING METHYL PARATHION AND METHYL PARAOXON EXPOSURE, PHASE III: LC-MS-MS STRUCTURAL CONFIRMATION

1. INTRODUCTION

This technical report is the third of three reports from the U.S. Army Edgewood Chemical Biological Center In-House Laboratory Independent Research (ECBC ILIR) “Molecular Toxicology of TICs in Human Embryonic Stem Cells”. The overarching goal of the study was to provide a better understanding of the basic biological and toxicological mechanisms in human embryonic human cells (hESC) exposed to an organophosphate toxic industrial chemical (TIC), methyl parathion (MP). This work has broad implications on many Army goals from developing better toxicant screening tools, including organ-on-a-chip type applications, to medical applications including regenerative medicine applications and stem cell based therapies. It also supports U.S. Army Training and Doctrine Command (TRADOC) Pam 525-66 (March 08) *Force Operating Capabilities*, FOC-09-07: Army Health System Casualty Prevention, which states the importance of protecting the warfighter from TICs and toxic industrial materials (TIMs) in general.

Chemicals that have certain known effects in adults can have dramatically different toxic effects during embryonic and prenatal development. For example, the successful adult anti-epileptic drug, valproate, has a dramatic toxic effect on embryonic development; leading to neural tube deficits, autism, and cognitive dysfunction. In their 2007 study (Cezar et al., 2007) used non-targeted liquid chromatography followed by mass spectrometry (LC-MS) to examine the metabolites in spent medium from pluripotent hESC exposed to valproate and found a blockage of the serotonin production pathway. Therefore, an important part of any complete toxicological evaluation must include examination of the compound’s effect on human embryonic development. The use of hESC to explore human embryonic molecular toxicological endpoints is a promising development in the field of toxicology. Since pluripotent hESC contain the ability to differentiate into any somatic cell in the body, they provide a unique window into the influence of toxicants on the entire early human development process.

Early in the course of the study, we confirmed that hESC are not able to convert parathion to paraoxon. Thus, in order to understand the effect of both the parent compound and its metabolite, the testing of both MP and methyl paraoxon (MPO) was included in the study design.

The initial, Phase I, work (Sekowski et al., ECBC-TR-1177) employed LC-MS of the spent media from MP and MPO exposed hESC to identify the human metabolites altered by exposure to MP and MPO. The Phase I work revealed hundreds of putatively identified metabolites significantly altered in abundance by exposure to MP or MPO. These were linked to multiple metabolic pathways. Specific putative metabolite identifications were assigned based on comparison with the Stemina Biomarker Discovery, Inc. (Madison, WI) Metabolite Data Base (MetDB).

The follow-on, Phase II, work (Madren-Whalley et al., ECBC-TR-1178) down-selected the putatively identified metabolites for follow-on definitive structural confirmation using several criteria. That subset of down-selected mass features was determined by the following criteria: (1) impact on the arginine-proline metabolism pathway, (2) involvement with reactive oxygen species (ROS), and (3) good quality mass features suitable for structure confirmation analysis by liquid chromatography followed by tandem mass spectrometry (LC-MS-MS). In that work, twenty metabolites in fifteen metabolic pathways were down-selected for further structural confirmation.

The purpose of this Phase III study was to confirm the identity of the down-selected subset of putative metabolites by comparing them with commercially available standards using LC-MS-MS. Within our budgetary constraints and given availability of commercially available standards, a subset of nine of the twenty metabolites was chosen for confirmation. Of these nine, the identity of five metabolites was confirmed in this work. The investigators regrettably report that this Phase III work is incomplete, cut short due to budgetary constraints. Thus, more work is required to completely illustrate the exact metabolic changes that occurred in this hESC model system as a result of exposure to MP and MPO.

Exposure to MP has been suspected to be toxic in human development based on previous epidemiological and animal model based literature. However, the results reported here demonstrate for the first time that MP and its active metabolite, MPO, at all physiologically relevant concentrations tested, may disrupt at least six specific metabolic pathways in human embryonic development.

2. EXPERIMENTAL OVERVIEW

2.1 LC-MS-MS Data Acquisition

Samples were analyzed using electrospray ionization (ESI) in either positive or negative polarity (depending on the feature of interest), on an Agilent (Santa Clara, CA) 6520 or 6540 Quantitative-Time of Flight (Q-TOF) instrument, operated in high resolution, extended dynamic range mode. The same mass spectrometry (MS) source conditions and liquid chromatography (LC) method and gradient were used as was employed in the Phase I work. LC-MS-MS data were acquired using Agilent MassHunter Time of Flight (TOF) and Q-TOF Acquisition software B.04.0. Tandem mass spectrometry (MS-MS) spectra were acquired in a targeted fashion by inputting the desired precursor ion for the feature of interest into the software. A narrow precursor quadrupole isolation window [1.3 Da (Dalton)] setting was used and MS-MS was performed across the entire chromatographic run, acquiring MS spectra every 5 s to regain mass axis calibration using the internal reference ions. Data was acquired for three types of samples as shown in Table 1.

Table 1. MS-MS Sample Types and Solvents

Sample Type		Sample Solvent/Matrix
1	Spent media from Phase I MPO or MP dosed cells containing feature of interest	mTeSR 1 cell media dosed with MPO or MP
2	Reference standard: compound of interest	Dissolved in mTeSR 1 cell media at 3 different concentrations
3	Reference standard: compound of interest	Dissolved in 50:50 acetonitrile:water with 0.1% formic acid

2.1.1 Sample Type 1: Stored Samples from Phase I

LC-MS-MS acquisition was performed on stored samples from the Phase I work that originally showed the greatest MS signal strength for the features of interest. Prior to this analysis, samples from the Phase I study had been stored at 4 °C sealed in the original injection vials (containing the solvent originally used for dissolution (1:1 0.1% formic acid in water: 0.1% formic acid in acetonitrile). None of the features appeared to show significant signal loss as after storage. Retention time (RT) shifts from the original study from a minimum of 0% to a maximum of 10% were observed.

2.1.2. Sample Type 2: Commercially Purchased Reference Standards

Commercially available reference standards (Table 2) were used to verify the identity of the mass features of interest. The LC-MS-MS data was acquired for the reference standards at 3 different concentrations (0.001 mM, 0.01 mM and 0.1 mM) dissolved in mTeSR1 (Stemcell Technologies, Vancouver, BC, Canada) cell media under the same LC gradient conditions as those originally used in Phase I. Samples were dissolved in this media because the mTeSR1 can affect the chromatography and the RT of the features of interest. Three concentrations were used to help distinguish which extracted ion chromatographic peaks correspond to the reference standard in cases where multiple peaks are present, for example those caused by isobaric compounds.

Table 2. Commercially Available Reference Standards for Metabolites Chosen for MS-MS Structural Confirmation and Their Pathways

Metabolite Reference Standard	Pathway
Asymmetric dimethylarginine (ADMA). (NOS inhibitor).	Arginine and Proline metabolism
Ornithine	
L-proline	
<i>Trans</i> -4-hydroxy-L-proline	
4-Acetamidobutanoic acid	
<i>cis</i> -4-hydroxy-D-proline	
Ornithine	D-Arginine and D-Ornithine metabolism
L-Cystathionine	Gly, Ser and Thr metabolism
Choline	
5-Aminolevulinic acid	
Choline	Glycerophospholipid metabolism
Ornithine	Glutathione metabolism
L-cystathionine	Cys and Met metabolism

Cys, cysteine
Gly, glycine
Met, methionine

NOS, nitric oxide synthase
Ser, serine
Thr, threonine

2.1.3 Sample Type 3: Commercially Purchased Reference Standards in Acetonitrile

LC-MS-MS data was acquired for the same reference standards, in this case at a concentration of 0.1 mM dissolved in 50:50 acetonitrile: water with 0.1% formic acid. Data acquired under these conditions may show a different RT, but a cleaner reference MS-MS spectrum can sometimes be obtained. For the reference standard samples dissolved in 50:50 acetonitrile: water with 0.1% formic acid (sample type 3), data was acquired at three different collision energies (industry standard 10, 20 and 40 V), then an optimal collision energy was determined from that data by examining the quality of the spectra, including matching the spectra to public databases when available. This optimal energy was then employed in the MS-MS of the reference standards dissolved in mTeSR1 (sample type 2), and the original samples (sample type 1).

2.2. Data Analysis and Confirmation Criteria

Data analysis was performed using Agilent MassHunter Qualitative Analysis v B.04 software. The chemical structure of a mass feature of interest in a Phase I sample was considered a match with a reference standard if three criteria were satisfied:

1. The relative mass difference between the observed reference standard compound molecular ion and the molecular ion for the mass feature of interest are less than 20 ppm.
2. The MS-MS spectral peaks resulting from the collision induced dissociation of the same precursor ion for both the reference standard compound and molecular ion for the mass feature of interest are similar in mass (within 40 ppm) and abundance.
3. The RT for the peak corresponding to the mass feature of interest and reference standard as shown in the extracted ion chromatogram (EIC) are within 30 seconds and the elution profile is similar for both the reference compound and mass feature of interest.

3. RESULTS

Overall, the MS-MS spectra for the confirmed metabolites were high quality matches with the spectra for the reference standards. Some features showed different RTs from when first run in Phase I; however those that were confirmed showed consistent RTs for the reference standards. Five out of nine metabolites tested were confirmed; L-cystathionine, ADMA, choline, ornithine, and L-proline. *Cis*-4-hydroxy-D-proline, *trans*-4-hydroxy-L-proline and 5-aminolevulinic acid were not confirmed. 4-Acetamidobutanoic acid was not confirmed, but was determined to be plausible. The RT and elution profile for 4-acetamidobutanoic acid was similar to the reference standard, but the MS-MS spectra were only a partial match to the reference standard. A summary of the results is shown in Table 3. All the EIC plots, MS-MS spectra and data interpretation are shown in Figures 1 through 9.

Table 3. Summary of Metabolites and Their Structural Confirmation Results

Targeted Compound	Polarity	Precursor Ion Exact Mass	Phase I Feature RT (s)	Reference Standard RT in mTeSR1 (s)	Collision Energy	Confirmed by MS-MS
ADMA	Positive	203.1503	425	400	20	Yes
Ornithine	Positive	133.0972	514	465	10	Yes
L-proline	Positive	116.0706	511	465	20	Yes
* <i>Trans</i> -4-hydroxy-L-proline	Negative	130.0505	83	405	20	No
* <i>Cis</i> -4-hydroxy-D-proline				405	20	No
*5-Aminolevulinic acid				60-270	10	No
4-Acetamidobutanoic acid	Positive	146.081	101	100	20	No (Plausible)
L-cystathionine	Negative	221.0591	607	607	16	Yes
Choline	Positive	104.1075	50	55	20	Yes

*Isomers

4. MS-MS SPECTRA AND DATA INTERPRETATION

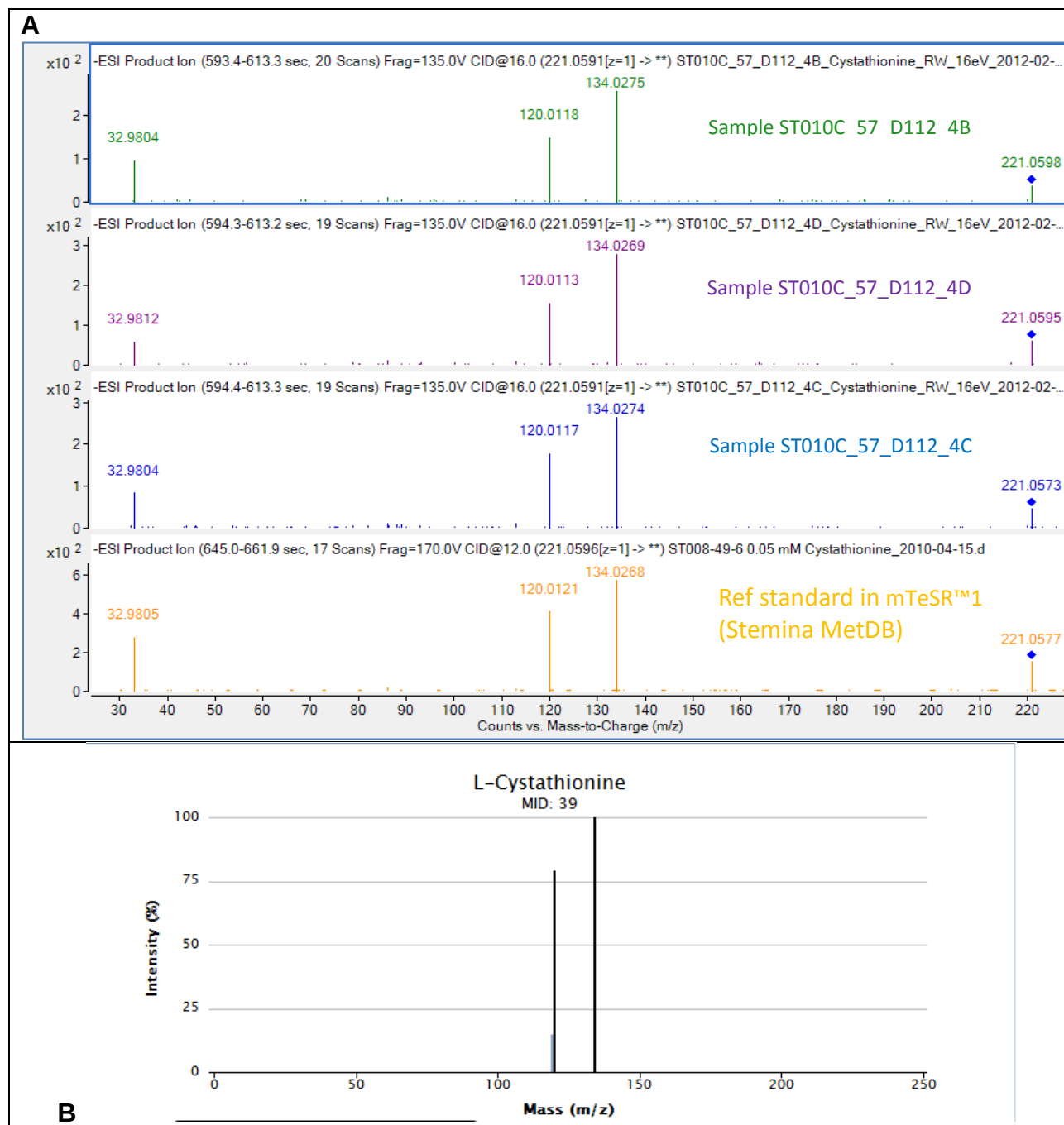


Figure 1. ESI-MS/MS spectra for L-cystathionine.

Compound is confirmed. RT (600 s) and MS-MS spectra for the species analyzed in negative ion mode which generated a precursor at mass over charge ratio (m/z) 221.0596 in original samples match Stemina MetDB reference standard. Representative samples shown in (A), ST010C_57_D112_4B, 4C, and 4D are replicates from 50 μ M MP exposures. (B) Metlin public MS-MS spectrum for L-cystathionine (M-H)⁻ precursor.

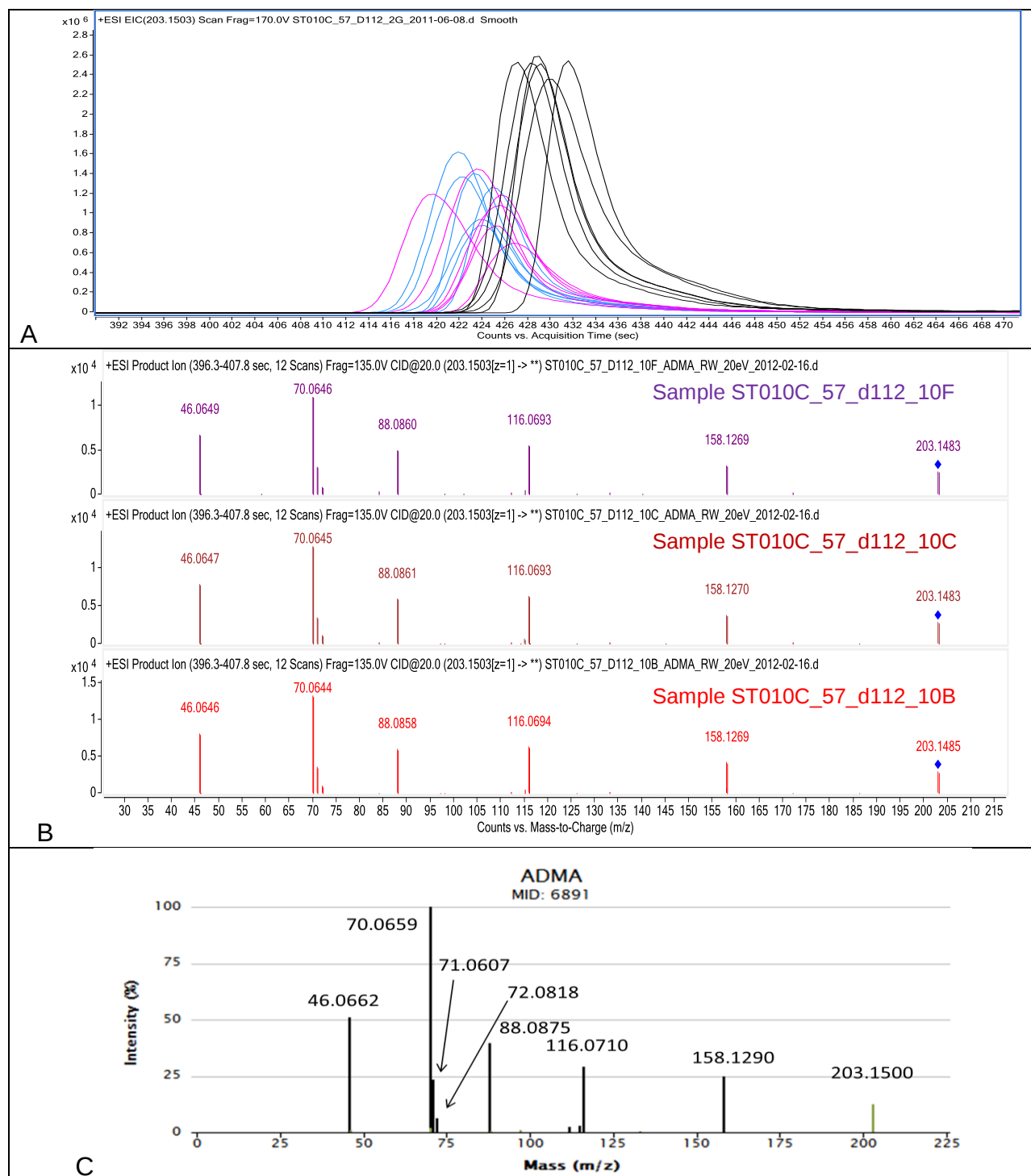


Figure 2. ESIpos EICs and MS-MS spectra for ADMA.

Compound is confirmed. MS-MS spectra are an excellent match to those of reference standard and public databases. (A) EIC of m/z 203.1503 ($M+H$)⁺ for original samples showing ADMA fold change. Pink = 180 μ M MPO, Blue = 130 μ M MPO, and Black = control cells. RT is consistent. (B) MS-MS spectra for precursor at m/z 203.1503 for samples ST01C_57_D112_B, C, and F (130 μ M MPO). (C) Metlin public MS-MS spectrum for ADMA ($M+H$)⁺ precursor.

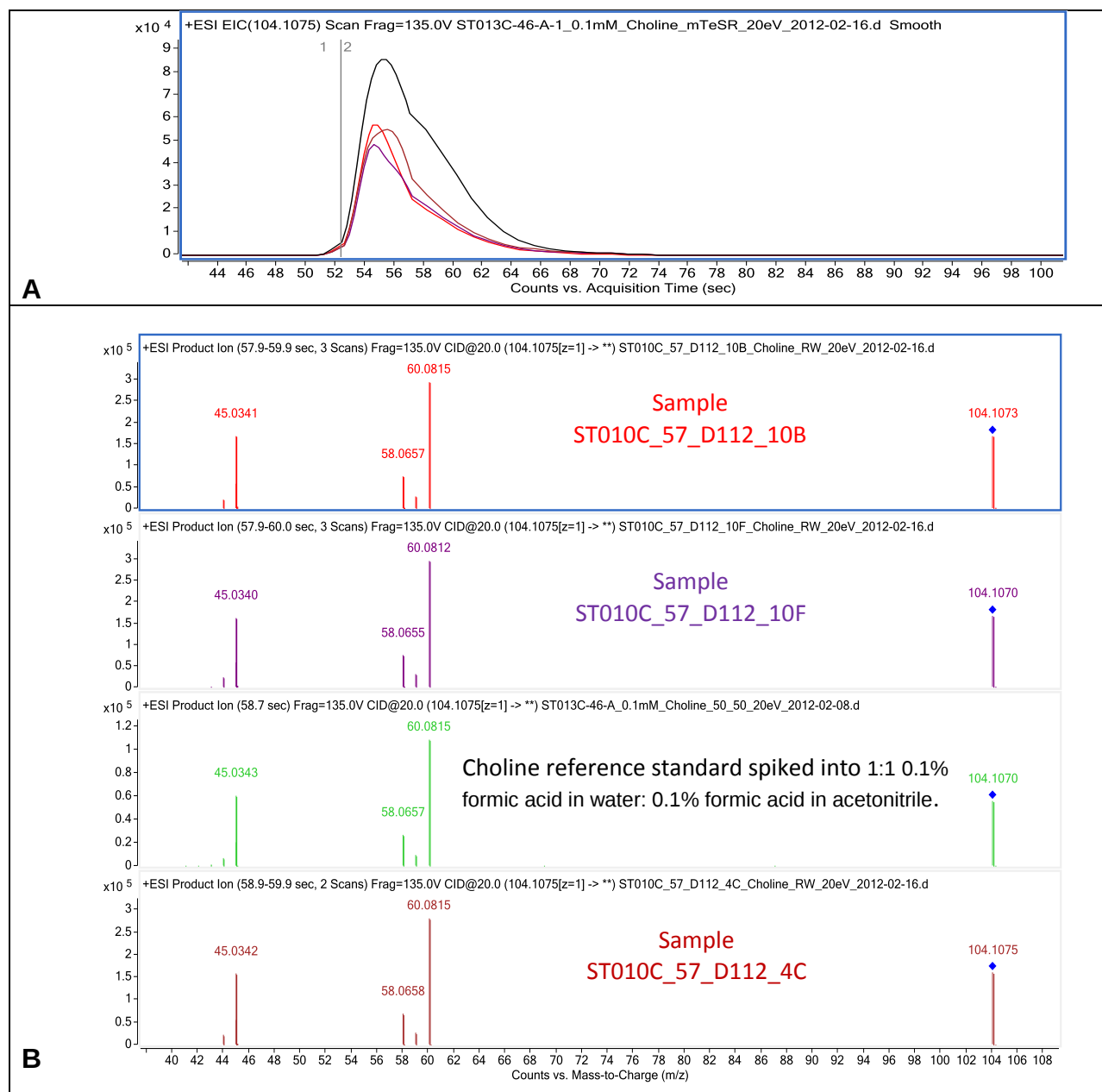


Figure 3. ESIpos MS-MS spectra for choline.

Compound is confirmed. RT and MS-MS spectra for precursor at m/z 104.1075 in original samples match those of reference standard as shown above. (A) EICs of m/z 104.1075 M^+ for: Black = 0.1 mM choline reference standard in mTeSR 1, Red = sample ST010C_57_D112_10B = 130 μ M MPO, Purple = sample ST010C_57_D112_10F = 130 μ M MPO, and Dark Red = sample ST010C_57_D112_4C = 50 μ M MP. (B) MS-MS spectra for precursor M^+ at m/z 104.1075. Spectra are labeled with sample information. MS-MS spectra are a good match with Metlin public spectrum (not shown).

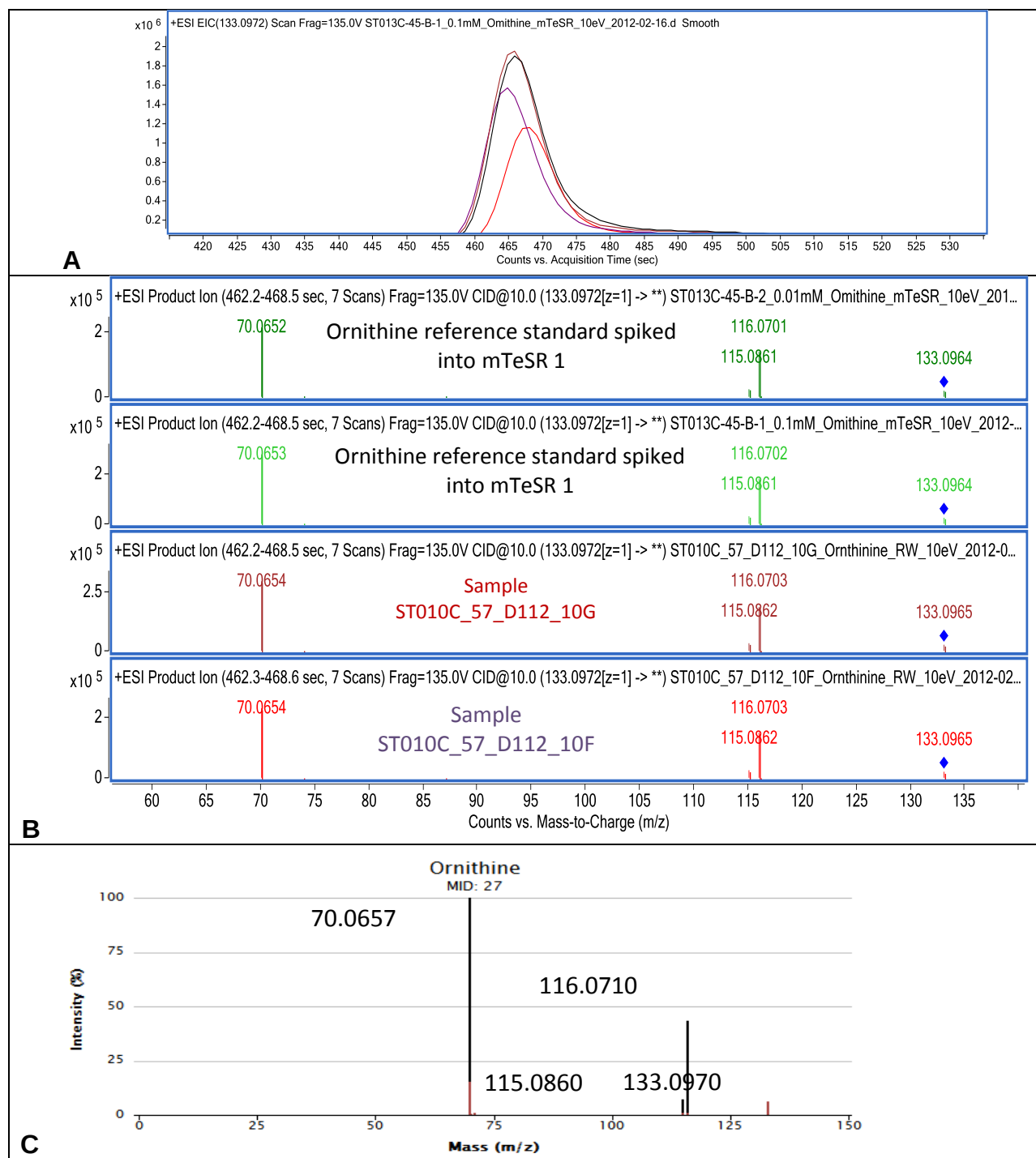


Figure 4. ESIpos MS-MS spectra for ornithine.

Compound is confirmed. RT and MS-MS spectra for $(M+H)^+$ precursor at m/z 133.0972 in original samples match those of reference standard as shown above. (A) EICs of m/z 133.0972 for: Black = 0.1 mM reference standard in mTeSR 1, Red = ST010C_57_D112_10E = 130 μ M MPO, Purple = ST010C_57_D112_10F = 130 μ M MPO, Dark Red = ST010C_57_D112_10F = 130 μ M MPO. (B) MS-MS spectra for precursor $(M+H)^+$ at m/z 133.0972. Spectra are labeled with sample information. (C) Metlin public ESIpos MS-MS spectrum for ornithine $(M+H)^+$ precursor.

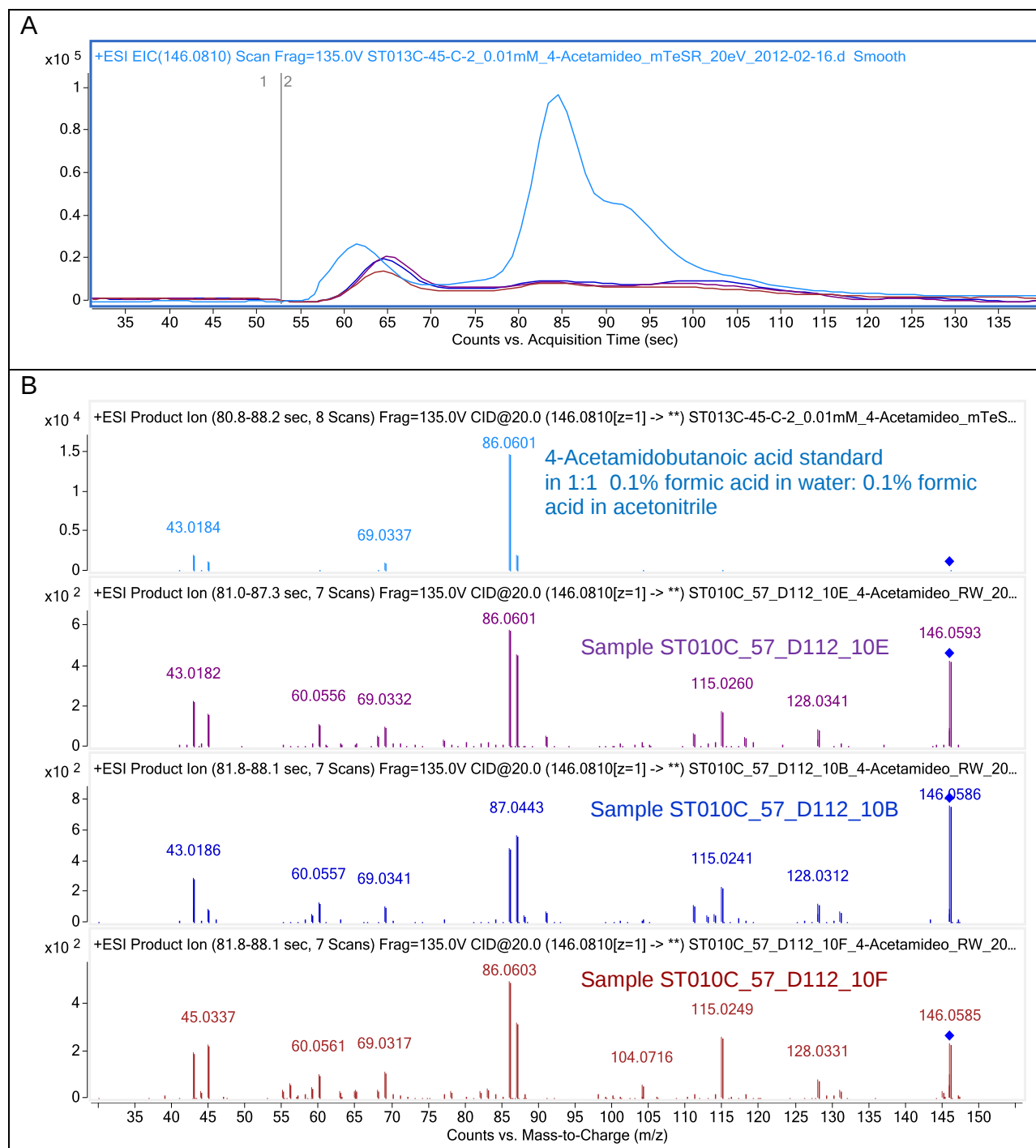


Figure 5. ESIpos EICs and MS-MS spectra for 4-Acetamidobutanoic acid.

Compound is not confirmed but is plausible. RT and elution profile are similar but MS-MS spectra are only a partial match to the reference standard. (A) EIC of m/z 146.0810 ($M+H$)⁺ for 4-Acetamidobutanoic acid reference standard. Light Blue = 4-Acetamidobutanoic acid reference standard in 1:1 0.1% formic acid in water: 0.1% formic acid in acetonitrile (control), Purple = ST010C_57_D112_10E = 130 μ M MPO, Dark Blue = ST010C_57_D112_10B = 130 μ M MPO, Dark Red = ST010C_57_D112_10F = 130 μ M MPO. (B) MS-MS spectra are from the same samples shown in A. Public MS-MS spectra are not available.

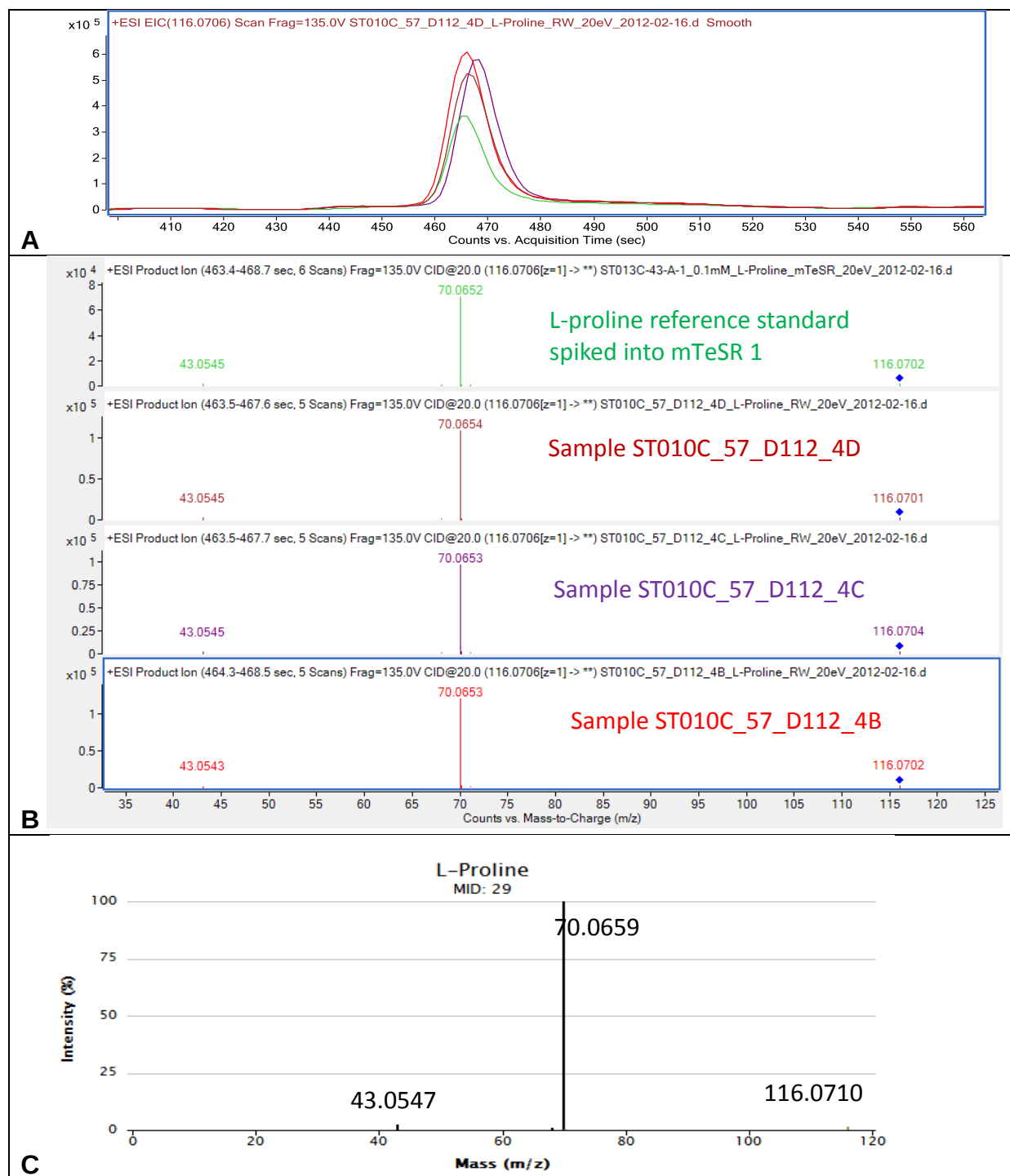


Figure 6. ESIpos MS-MS spectra for L-proline.

Compound is confirmed. RT and MS-MS spectra for $(M+H)^+$ precursor at m/z 116.0706 in original samples match reference standard as shown above. (A) EICs of m/z 116.0706 $(M+H)^+$ for L-proline. Green = 0.1 mM L-proline reference standard in mTeSR1 = control, Red = ST010C_57_D112_4B = 50 μ M MP, Purple = ST010C_57_D112_4C = 50 μ M MP, Dark Red = ST010C_57_D112_4D = 50 μ M MP. (B) MS-MS spectra for $(M+H)^+$ precursor at m/z 116.0706. Spectra are from the same samples as listed in A. (C) Metlin public ESIpos MS-MS spectrum for L-proline precursor at m/z 116.0706.

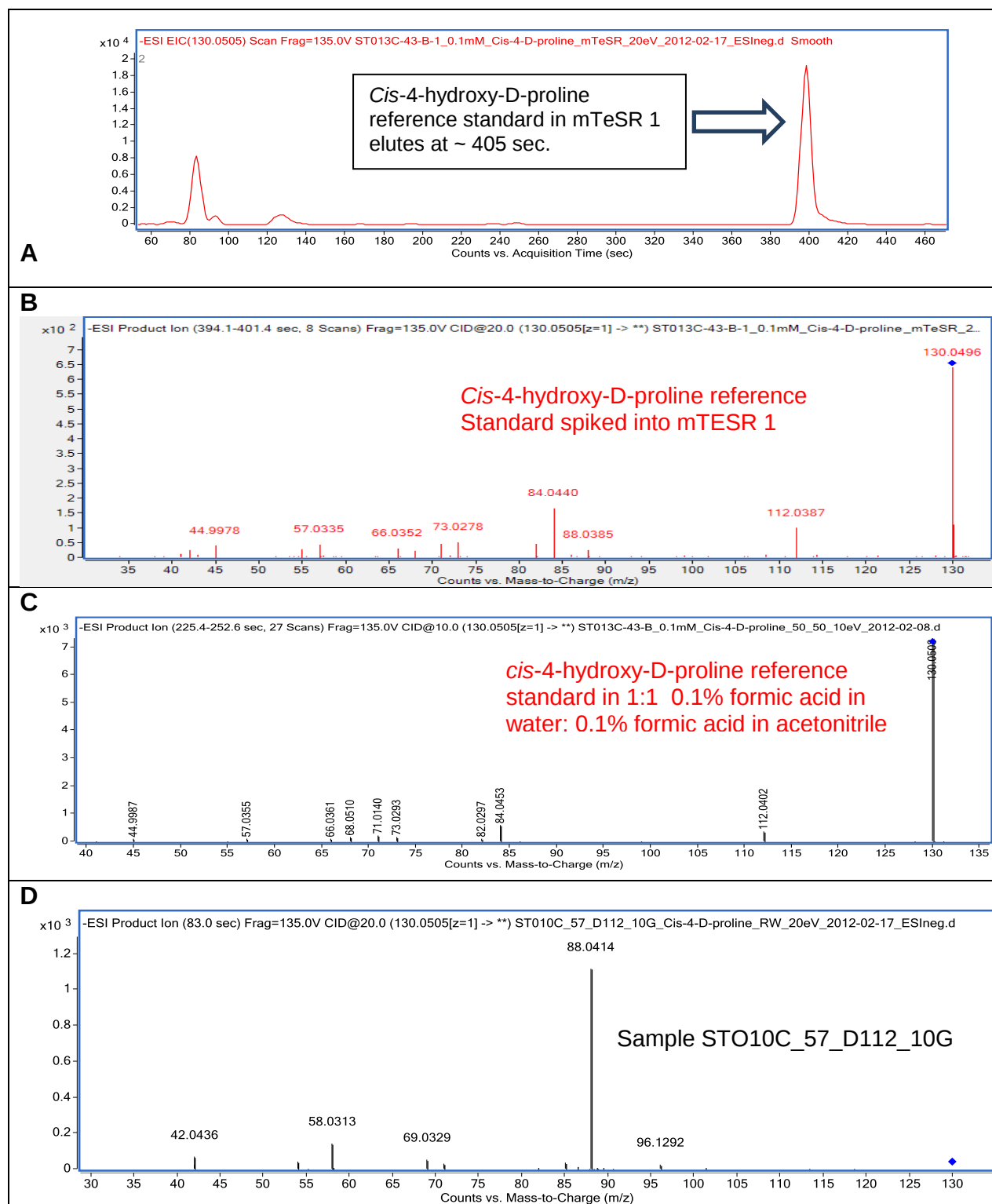


Figure 7. ESI-MS/MS spectra for *cis*-4-hydroxy-D-proline.

Compound is not confirmed. RT and MS-MS spectra do not match those of reference standards. A. EIC of m/z 130.0505 ($M-H$)⁻ for reference standard in mTeSR™1 shows compound eluting at 405 sec. MS-MS at this RT is an excellent match for Metlin MS-MS spectrum (data not shown). Original feature of interest eluted at 83 sec. where another isobaric peak can be seen above. B. MS-MS spectrum of reference standard in mTeSR 1. C. MS-MS

spectrum of (M-H)⁻ precursor at m/z 130.0505 for reference standard in 1:1 0.1% formic acid in water: 0.1% formic acid in acetonitrile. D. MS-MS spectrum for precursor at m/z 130.0505 at RT 83 sec in sample ST010C_57_D112_10G (130 μ M MPO).

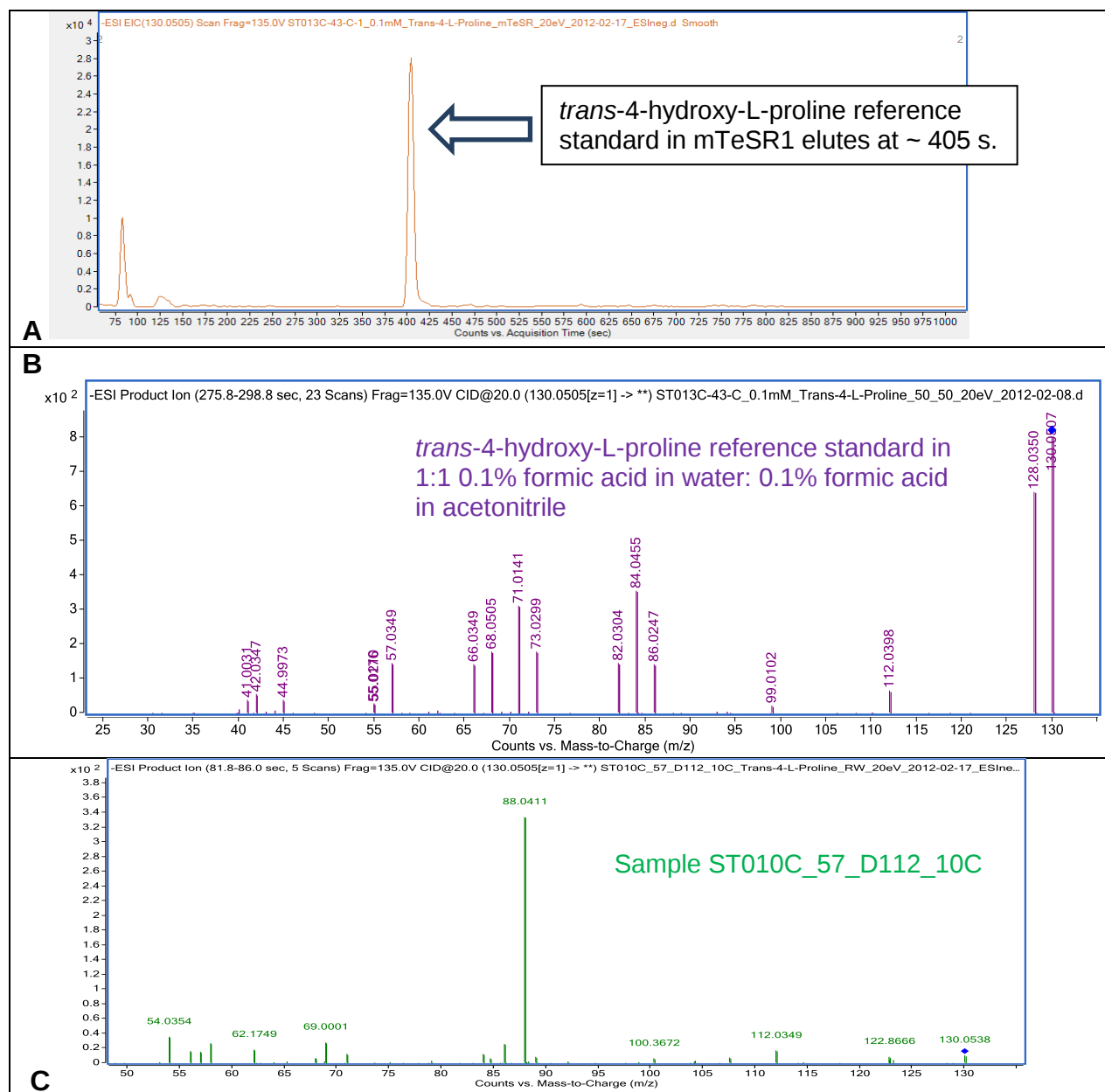


Figure 8. ESI neg EICs and MS-MS spectra for *trans*-4-hydroxy-L-proline.

Compound is not confirmed. RT and MS-MS spectra for original samples do not match those of reference standards. A. EIC of m/z 130.0505 (M-H)⁻ for reference standard in mTeSR 1 which elutes at 405 sec. Original feature of interest eluted at 83 sec, where another isobaric peak can be seen above. B. MS-MS spectrum for (M-H)⁻ precursor at m/z 130.0505 for reference standard in 1:1 0.1% formic acid in water:0.1% formic acid in acetonitrile. Reference standard spectrum is a good match for Metlin public database MS-MS spectrum, but is not a match for C. the MS-MS spectrum for precursor @ m/z 130.0505 at RT 83 sec in sample ST010C_57_D112_10C (130 μ M MPO).

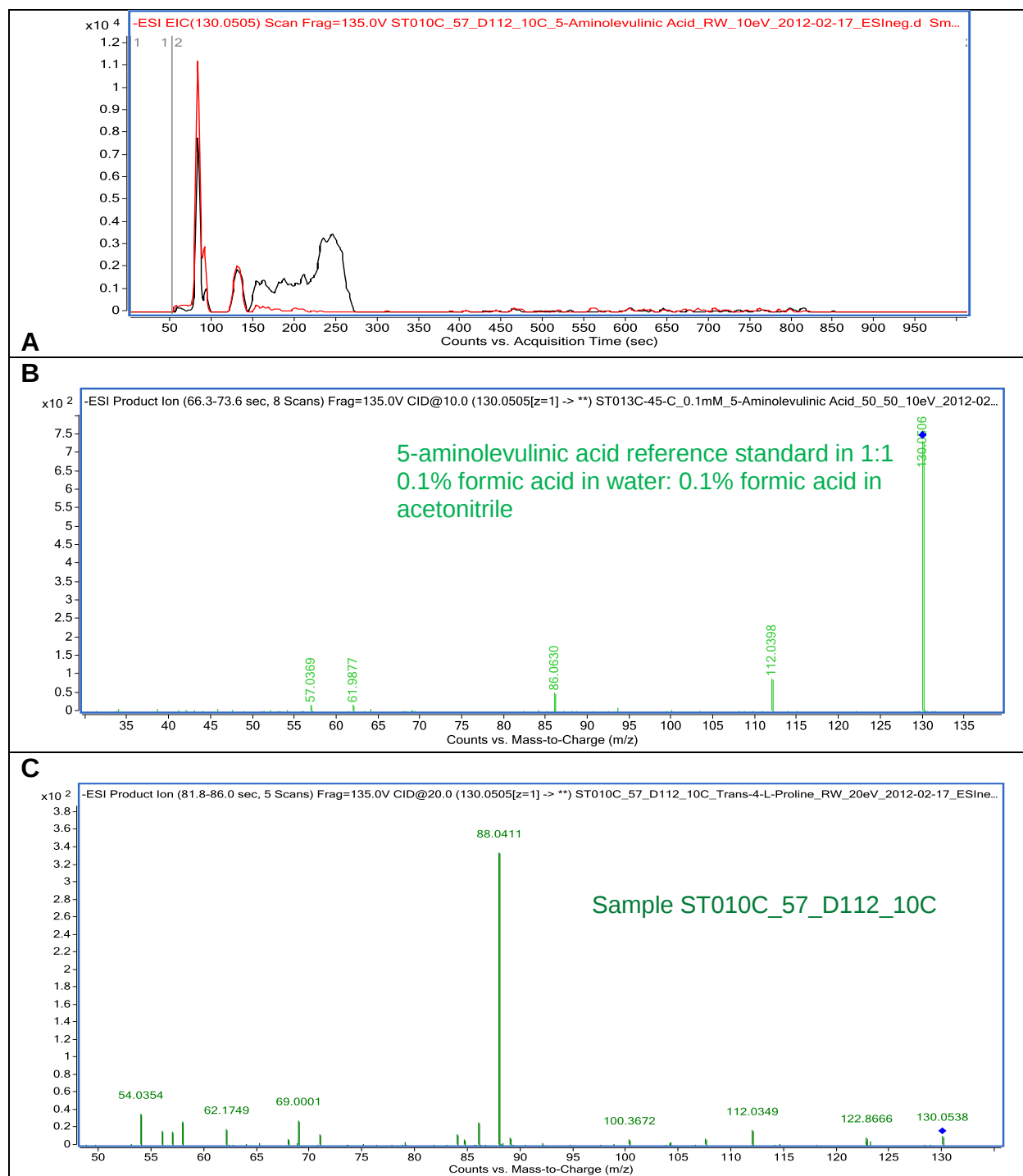


Figure 9. ESI neg EICs and MS-MS spectra for 5-aminolevulinic acid.

Compound is not confirmed. Elution profile and MS-MS spectra do not match those of reference standard. (A) EIC of m/z 130.0505 ($M-H$)⁻ for 5-aminolevulinic acid. Black = reference standard in mTeSR 1 (control), Red = ST010C_57_D112_10C (130 μ M MPO). (B) MS-MS spectrum of ($M-H$)⁻ precursor at m/z 130.0505 for reference standard in 1:1 0.1% formic acid in water: 0.1% formic acid in acetonitrile. (C) MS-MS spectrum for precursor at m/z 130.0505 at RT 83 s in sample ST010C_57_D112_10C (130 μ M MPO). Massbank Project (Yamagata, Japan) public MS-MS spectrum (not shown) is available but not diagnostic.

5. CONCLUSION

The purpose of this Phase III study was to confirm the identity of the down-selected subset of putative metabolites by comparing them with commercially available standards using LC-MS-MS. Within the constraints of the project budget, and the availability of commercially available standards, a subset of nine of the 20 metabolites was chosen for confirmation. Of these nine, the identity of five metabolites was confirmed in this work: L-cystathionine, ADMA, choline, ornithine, and L-proline. These metabolites participate in six metabolic pathways: (1) arginine and proline, (2) D-arginine and D-ornithine, (3) glycine, serine, and threonine, (4) glycerophospholipid, (5) glutathione, and (6) cysteine and methionine.

The significant fold changes in the abundance of ADMA could have multiple effects depending on the timing of the changes in the developing embryo. Maternal plasma ADMA levels are known to be decreased in normal pregnancy and to increase with gestational age. It is also increased in pregnancies developing preeclampsia (Huang et al., 2012).

Significant fold changes in ornithine would be expected to have several cellular consequences. Since ornithine is a critical component of the urea cycle, and the urea cycle has two important functions, (1) to produce urea and dispose of excess nitrogen, and (2) to participate in the production of arginine biosynthesis, a disturbance in the correct level of ornithine would have serious consequences in both of these processes critical for normal cellular metabolism and growth.

Choline was significantly decreased in abundance in the MPO exposed cells and increased in the MP exposed cells. Choline is known to play a role in the glycerophospholipid pathway, and is important for the normal functioning of all cells. In development, in particular, choline is critical for normal fetal brain development. (S.H. Zeisel, 2006).

Changes in the levels of L-proline in response to MP and MPO exposure could be linked to serious consequences related to the regulation of cell pluripotency. A seminal paper from Rathjen laboratory at the Australian National Laboratory in Acton, Australian Capital Territory (Washington et al., 2010) describes the first evidence that amino acids play a direct role in the regulation of pluripotency and cell differentiation in embryonic stem cells (murine). This work identified the key ingredient in the cell medium, MEDII, described in earlier work (Rathjen et al., 1999) as able to promote the formation of early primitive ectoderm like cells (EPL) from pluripotent embryonic stem cells.

L-cystathionine was found to be significantly and differentially changed by exposure to MP and MPO. L-cystathionine is a precursor of the amino acid cysteine. Thus, reduced abundance L-cystathionine would restrict the availability of the cysteine in the developing embryonic cells. Cysteine is a critical component of many proteins, forming the disulfide bonds within and between protein molecules. It is a significant determinant of the tertiary structure of most proteins (Murphy et al., 2006).

Exposure to MP, and other organophosphate (OP) pesticides, is associated with multiple problems related to human pregnancy and development based on many epidemiological

and animal model based studies. Rauch and colleagues (2012) report an inverse relationship of the level of maternal exposure to OP pesticides with gestational age and birth weight of the baby. Other work (Rauh et al., 2011; Bouchard et al., 2011) has documented the neurodevelopmental delays and reduced intelligence quotient (IQ) in children from mothers with documented exposure to OP pesticides during pregnancy. Earlier work by Bouchard and colleagues (Bouchard et al., 2010) details a significant positive association between the presence of OP metabolites in the urine of pregnant mothers and the occurrence of attention deficit-hyperactivity disorder (ADHD) in their offspring.

The investigators regrettably report that this Phase III work is incomplete, cut short due to budgetary constraints. Thus, more work is required to completely illustrate the exact metabolic changes that occurred in this hESC model system as a result of exposure to MP and MPO. However, the results reported here demonstrate for the first time that MP and its active metabolite, MPO, at all physiologically relevant concentrations tested, disrupt the normal abundance of at least five confirmed metabolites that play key roles in cellular growth, fetal development, and pregnancy and likely influence numerous other developmental endpoints through their modification of at least six specific metabolic pathways important to normal human embryonic development and pregnancy.

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ACRONYMS AND ABBREVIATIONS

ADHD	attention deficit-hyperactivity disorder
ADMA	asymmetric dimethylarginine
APG	Aberdeen Proving Ground
Cys	Cysteine
Da	Dalton
ECBC	U.S. Army Edgewood Chemical Biological Center
EIC	extracted ion chromatogram
EPL	early primitive ectoderm like cells
ESI	electrospray ionization
Gly	glycine
hESC	human embryonic stem cell
ILIR	in-house laboratory independent research
IQ	intelligence quotient
LC	liquid chromatography
LC-MS	liquid chromatography followed by mass spectrometry
LC-MS-MS	liquid chromatograph/mass spectrometer/mass spectrometer
Met	methionine
MetDB	Metabolite Data Base
MP	methyl parathion
MPO	methyl paraoxon
MS	mass spectrometry
MS-MS	tandem mass spectrometry
<i>m/z</i>	mass over charge ratio
NOS	nitric oxide synthase
OP	organophosphate
PPM	parts per million
Q-TOF	quantitative- time of flight
ROS	reactive oxygen species
RT	retention time
Ser	serine
Thr	threonine
TIC	toxic industrial chemical
TIM	toxic industrial material
TOF	time of flight
TRADOC	U.S. Army Training and Doctrine Command

