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TITLE: Application of Atmospheric Pressure Chemical Ionization Gas Chromatography in Urine Organic Acid Analysis

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LAY ABSTRACT

Inborn errors of metabolism (IEM) are a class of genetic diseases that when left untreated, cause reduced quality of life and sometimes death in newborns. Urine organic acid (UOA) analysis is used for detection using an instrument called GC-EI-MS (Gas Chromatography Electron Impact Mass Spectroscopy). This project explores how a new instrument, the Agilent 7890 GC and the Waters' Xevo TQ-S MS, can detect these genetic diseases using a technique called APCI (Atmospheric Pressure Chemical Ionization) while still being accurate and sensitive.

UOAs are isolated from urine and run through the new machine. When compared to the currently used technique, results were promising but further optimization is needed. Using the new machine, various UOA compounds that were elevated and/or decreased in newborns with genetics diseases were identified and quantified. With clear avenues for future work, the APCI technique can greatly improve newborn diagnosis of IEMs.

ABSTRACT

Inborn errors of metabolism (IEM) cause significant morbidity and mortality when left untreated. Urine organic acid (UOA) analysis is often a first-line investigation when an IEM is suspected. UOAs are usually qualitatively analyzed via the current gold standard, GC-EI-MS (Gas Chromatography-Electron Impact-Mass Spectroscopy). The Agilent 7890 GC in tandem with the Waters' Xevo TQ-S MS contains an easily interchangeable LC-ESI (liquid chromatography-electrospray Ionization) and GC-APCI (Atmospheric Pressure Chemical Ionization) instrument set-up, while maintaining accuracy and sensitivity in both LC and GC applications. Utilizing this novel GC-APCI instrument, this project aims to develop and validate a new UOA method for clinical use. Furthermore, utilizing the machine's MRM mode would increase sensitivities thus allowing for hopefully quantitative analysis.

Chemical standards and patient urine samples were extracted via a liquid-liquid ether extraction and derivatized with BSTFA for proper GC elution. Results were compared on the current gold standard GC-EI-MS instrument and the new GC-APCI-MS instrument. Initial instrument suitability and method setup was then optimized. Source moisture levels were modified to explore the wet proton transfer and the dry charge transfer mechanism using $[M+H]^+$ and $[M+*]^+$ ion peak ratios, respectively. Elution times and APCI ion mass spectra profiles of UOA metabolites of interest were identified from full scan mode in preparation for MRM mode analysis. Exploration into the wet and dry mode settings of the APCI source determined that the former induced via methanol had greater peak areas and signal-to-noise ratios. Suitable MRMs were determined for

clinically relevant organic acids from which a quantitative assay was developed for methyl malonic acid and several other compounds.

The Waters' Xevo TQ-S micro with Agilent 7890 GC demonstrated promising GC-APCI-MS detection of urine organic acids. With clear avenues for future work, the APCI technique hints at great benefits for biochemical genetic laboratories.

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My supervisory committee consisted of Dr. Philip Britz-McKibbin from the Department of Chemistry and Chemical Biology at McMaster and Dr. Joseph Macri from the Hamilton General Hospital. Both of them receive my immense gratitude for providing their input and advice on the analytical and clinical portions of research. My committee meetings were extremely helpful thanks to their critical yet constructive questions and feedback, pushing my research to a high standard. I highly recommend both of them to any future graduate students pursing degrees in similar branches of science.

Lastly, I send my deepest thanks to my family and friends for all their love and support throughout the process. In between all the work and sometimes stress, they always gave me comfort and made it fun. Special thanks to my parents, Shanthi and Rohitha, and my little brother, Sanjula, for always pushing me to do my best while making sure I took time to rest. Once again, thank you to everyone who had a positive impact on me during this time and my life leading me to this moment. I would like to dedicate the merit of this work to the patients and families afflicted by these diseases studied. I truly hope that the findings contribute to having better outcomes and lives for all. Thank you kindly.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABR – Auditory Brain Stem Response

ACHDNC – Advisory Committee on Heritable Disorders in Newborns and Children

APCI – Atmospheric Pressure Chemical Ionization

BPPE – 4-Bromo Diphenyl Ether

BSTFA – N, O - Bis (Trimethyl Silyl) Trifluoroacetamide

CLSI – Clinical and Laboratory Standards Institute

DoE – Design of Experiments

EI – Electron Impact

ESI – Electrospray Ionization

IEM – Inborn Errors in Metabolism

GC – Gas Chromatography

LC – Liquid Chromatography

LOB – Limit of Blank

LOD – Limit of Detection

LOL – Limited of Linearity

LOQ – Limit of Quantification

MCM – Methyl Malonyl-CoA Mutase

MMA – Methyl Malonic Acid

MRM – Multiple Reaction Monitoring

MS – Mass Spectroscopy

MTBSTFA - N-Methyl-N-Tert-Butyl-Dimethyl Silyl Trifluoroacetamide

OEA – Otoacoustic Emissions

PKU – Phenylketonuria

QC – Quality Control

RSD – Relative Standard Deviation

S/N – Signal to Noise Ratio

SOP – Standard Operating Procedure

TBDMS – N-Tert-Butyl-Dimethyl Silyl

TMCS – Trimethyl Chlorosilane

TMS – Trimethyl Silyl

UOA – Urine Organic Acid

DECLARATION OF ACADEMIC ACHIEVEMENT

The work done for this thesis was completed solely by Devanjith Ganepola with contributions and assistance from the following individuals. Initial hypothesis of utilizing APCI for clinical applications such as UOA analysis was presented in a poster authored by Lori Beach, a Clinical Biochemist at the IWK Health Centre in Halifax, Nova Scotia. While no research or data was used from the IWK Health Centre in this thesis, permission was granted to continue with the proposal with the goal to improve patient diagnosis and care. Laboratory training and safety was taught by Lyse Wortel, who was also the main contact for the purchase of materials and repairs for the instrument used. Maintenance of the Agilent 7890 GC and the Waters' Xevo TQ-S MS was primarily handled by service engineers at Waters Corporation. Data obtained and analyzed on the GC-EI-MS by members of the Biochemical and Genetics Lab in the Department of Pathology and Molecular Medicine of McMaster Children's Hospital. All experimentation, data analysis, and extraneous data was done by the author.

1 - INTRODUCTION

1.1 - Inborn Errors in Metabolism

Multiple studies observing the major causes of infant deaths estimate that genetic errors are one of the main underlying complications in 20-50% of cases.[1,2,3] These statistics also underestimate the issue as certain populations around the world do not have access to comprehensive genetic testing facilities. Early detection of these genetic errors greatly decreases infant mortality rates while increasing chances of longer life expectancy and better quality of life.[2] One class of genetic conditions are inborn errors in metabolism (IEM) where problems in certain metabolic pathways prevent the breakdown, biosynthesis, storage, and conversion of metabolites. Though cases of IEMs individually are rare, their combined incidence ranges from 1/2500 to 1/1500 births.[3] For most IEMs, multiple organ systems are often affected adding to the difficulty of clinical diagnosis due to the non-specificity of signs and symptoms.[4] These symptoms range from feelings of "unwell", lethargy, feeding problems, vomiting, abnormal breathing, hypotonia and seizures. Even more of a problem would be metabolic disorders involving glucose, protein, and fat breakdown which have clinical manifestations after an initial asymptomatic stage as it may take time for toxic metabolites to build up. However, once symptoms show and progress, it may be too late as permanent damage might have already occurred. Fortunately, with early detection, many IEMs can be treated using modified diet, medication, or surgery allowing for a relatively normal lifestyle.[3,4]

1.2 - Newborn Screening

Newborn screening was introduced for the diagnosis of phenylketonuria (PKU) in the 1960s and was later extended to a few other disorders.[20,21,23,24] In current times, the Advisory Committee on Heritable Disorders in Newborns and Children (ACHDNC) issues regular advisements known as the Recommended Universal Screening Panel (RUSP) for which lists the 35 core conditions and 24 secondary conditions that screening programs should be testing.[23,25] Many A large number of these conditions are IEMs. Screening usually begins shortly after birth with a non-invasive hearing test. The two standard methods used to detect hearing loss are otoacoustic emissions (OEA) test and auditory brain stem response (ABR) test.[26,27] The former confirms whether sounds are reflected back from the ear canal and the latter detects the presence or absence of brain's response to sounds using electrodes placed on the baby's head. In addition to hearing tests, the majority of newborn screening disease targets are tested using a blood spot collected via the heel prick method.[27,28] Should any of these tests come back positive or if the baby presents clinically with symptoms suggestive of a metabolic disorder, the newborn will likely undergo a number of investigations, one of which being urine organic acid (UOA) analysis. This is due to these diseases causing the accumulation or decline of certain metabolites in the blood and this is often reflected in the urine as well.[4,5] Urine samples are usually collected in the morning at volumes of 10-20 mL. While only as little as 2 mL is needed for a UOA analysis, since creatinine is often used to normalize many compound peaks, creatinine concentrations need to be adequate in the same. Thus, more urine is needed when creatinine levels are low.[34,35,36]

1.3 - Key Clinical Metabolites and Diseases

While UOA analysis looks for numerous compounds important in diagnosing a multitude of disorders, one of the key metabolites of interest for this project is methyl malonic acid's (MMA) indicative of methyl malonic aciduria.[29] Most cases involve mutations causing a reduced or lack of function in the enzyme, methyl malonyl-CoA mutase (MCM), or the biosynthesis of its cofactor, adenosyl cobalamin, which in is responsible for the breakdown of amino acids, certain lipids, and cholesterol.[29] This disorder results in increased MMA, 3-OH propionic and methyl citric levels; decreased free carnitine and increased propionyl and methyl malonyl carnitine levels; and increased glycine and alanine levels, which is reflective in tests looking for UOA, acyl carnitines and amino acids, respectively.[29,30] Once diagnosed, treatment usually involves dietary adjustments that reduce intake of isoleucine, threonine, methionine, and valine. In addition, Vitamin B12/cobalamin and carnitine supplements are taken as well.[30] High levels of MMA can also be due to low Vitamin B12/cobalamin intake, difficulty in its absorption from food in the ileum, or reduced transport to the tissues.[29,30]

1.4 - Urine Organic Acid Properties and Extraction/Derivatization Mechanism

There are few key characteristics of UOA metabolites that must be understood for their analysis. Their molecular weight range is on the smaller side with most in the range of 50-300 Daltons. Among these clinically relevant UOAs, there are many isomers which introduces the challenge of coelution as GC separation is primarily boiling point and therefore molecular weight dependent.[7] Lastly, most UOAs contain alcohol or carboxylic acid functional groups adding another layer of difficulty of making direct GC

volatilization problematic due to the high boiling point of such polar compounds.[7,8] These latter two traits are the reason for the extraction and derivatization protocols used to prepare patient samples for analysis. Detailed steps can be found in the methodology but the important points to note are the addition of NaOH and hydroxylamine-HCl for the deprotonation and oxidation of the organic acids, respectively, as well as for keeping them in the aqueous layer to ensure non-polar compounds can be removed in the first ether wash. Afterwards, the addition of HCl reprotonates the acids while the NaCl salt reduces polar interactions allowing the UOAs to enter the ether phase to be extracted. Finally, the alcohol and carboxylic acid groups are derivatized with BSTFA (N, O-Bis(trimethylsilyl)trifluoroacetamide) with 1% TMCS (trimethylchlorosilane) in an SN2 reaction, thus greatly reducing the polarity of the compounds due to the TMS (trimethylsilyl) group making them optimal for GC separation.[31,32] Due to TMS derivatization, the resulting molecular ion peak are displaced depending on the number of TMS groups on the molecule. For example, methylmalonic with a molecular weight of 118.09 g/mol can be observed to have 191.29 m/z or 263.49 m/z given its two potential carboxylic acid groups as points of TMS reaction. Going forward, it is important to keep this in mind when trying to identify ion peaks of unknown UOAs.

1.5 - Gas Chromatography Separation Science

The current gold standard detection of UOAs is GC-EI-MS (Gas Chromatography-Electron Impact-Mass Spectroscopy) after which compounds in chromatograms are identified through cross reference to a mass library of clinical UOAs of interest.[5,6] GC operates on the principle of maximum separation relative to a dispersive

transport. Herein, the sample of interest is volatilized and transported through the mobile phase by the carrier gas, usually an inert gas such as helium or hydrogen. This mixture passes through, and separates based on the interactions with the often silica column with a non-polar inner coating, the stationary phase. While the analyte-column interactions play an important role, the initial separation is caused by the changing oven temperature.[9] As compound concentrations in the gas phase are a function of the vapor pressure, compounds with greater boiling points elute later.[10] As increased molecular weight and polarity both play a role in increasing boiling point, generic GC separation science can be simplified to being dependent on those analyte qualities.[9] As an analytical tool, GC follows the concepts demonstrated in the Van Deemter Plot, $H\ (plate\ height) = A + \frac{B}{u} + (Cs + Cm)u$, showcased by the A term (Eddy Diffusion), B term (Longitudinal Diffusion), and the C term (Mass Transfer). This relationship is summarized in Figure 1 with the dashed line indicating the culmination of the Van Deemter equation. The important point to take away is the lack of the Eddy Diffusion term as GC columns are hollow resulting in the dashed line to be shifted downwards by a fixed amount. This allows for smaller theoretical plate height and greater immediate efficiencies in separation for GC at all points in the curve when compared to other methods such as LC.[9]

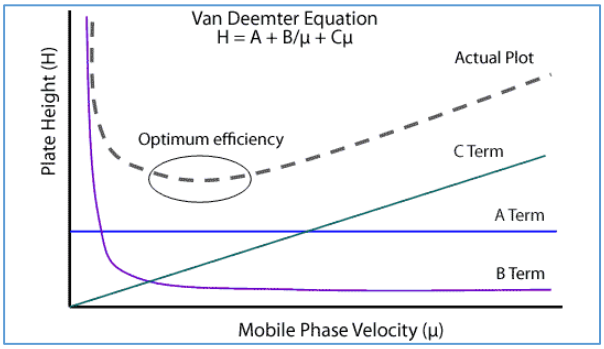

Figure 1 - A plot demonstrating the relationship of flow rate (u) and plate height (H) based on each of the terms in the Van Deemter Equation. The terms are as follows: A term (Eddy Diffusion), B term (Longitudinal Diffusion), and the C term (Mass Transfer). The dashed line indicates the resulting relationship when all the terms are combined.

While LC may be more flexible of a technique, there are numerous factors to consider such as solvents, analyte-column interactions, and more. In the regards to IEM detection via UOAs, GC has historically been the chosen separation system allowing for a greater resource for understanding the separation science specific to this group of analytes.[7] Given the volatile nature of UOAs, the complexity of urine as a sample matrix, and the numerous runs required in clinical settings with newborn patients, this further supports the idea of using GC separation.[6,8]

1.6 - Atmospheric Pressure Chemical Ionization Mechanism

While the use of GC in the field of biochemical genetics has been prominent through the decades, the use of APCI as the mass spec ionization method as opposed to EI or ESI is quite novel in clinical settings.[5,6,12] APCI is similar to Electrospray Ionization (ESI) in that it is a softer ionization compared to EI, giving less in-source fragmentation. This leads to greater retention of molecular ions, thus potentially increasing sensitivity, and the identification ability of UOA compounds.[13,14] However, the innovative nature of the APCI technique is in its mechanism of ionization. Once volatilized eluted compounds

exit the GC capillary, it is mixed with nitrogen gas in the atmospheric pressured ionization chamber. Here, the corona pin discharge causes the ionization and excitation of first the nitrogen which then interacts with the compound directly to produce a $[M+^{\circ}]^{+}$ peak.[14] However, in the presence of water or methanol in the source through modifier vials, the ionized nitrogen reacts with either of those two compounds first. These series of reactions then continue producing a $[M+H]^{+}$ peak instead.[14] These pathways of interaction are called dry and wet mode which result in either the charge exchange or proton transfer mechanism, respectively, as summarized in Figure 2 and 3.

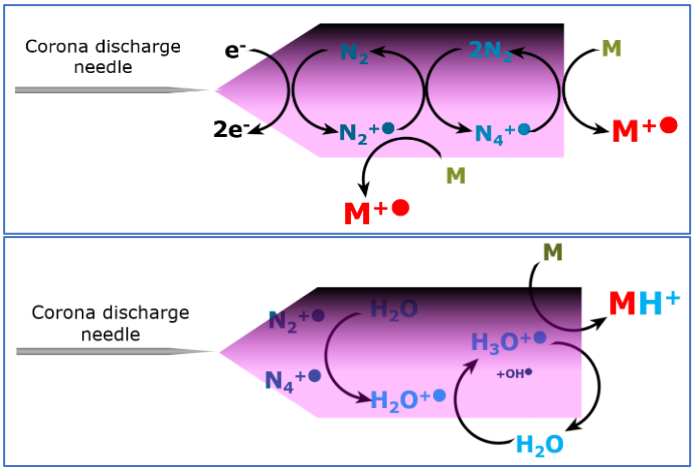

Figure 2 - Brief diagram of the charge exchange (on the left) and proton transfer (on the right) mechanism caused by the dry and wet modes of the source enclosure, respectively.

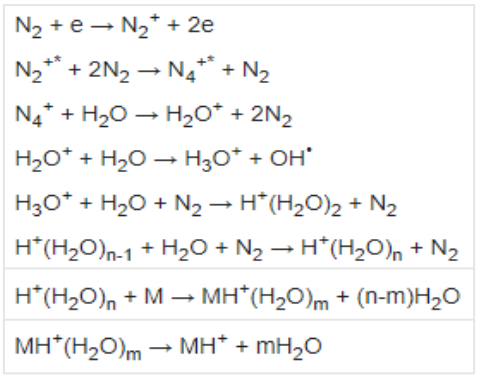

Figure 3 - Proton transfer mechanism breakdown occurring during the APCI's wet mode. The charge exchange mechanism found in the dry mode would instead continue from the second step of the top box where N4+ would react with and ionize the product producing a [M+°]+ ion.

According to the literature, the wet (proton transfer) mode seems to be more suited for relatively polar compounds like UOAs due to their affinity for accepting the proton from the excited methanol or water intermediate.[34,35] As such, this mode will likely be used in the project going forward but differences in sensitivity and specificity need to be observed.

2 - SPECIFIC AIMS AND HYPOTHESIS

Given the prevalence of genetic errors in populations around the world, the need for an efficient method of detection is paramount. Along side UOA analysis by GC-MS, biochemical genetic testing laboratories often run LC-MS methods for other patient tests such as for acyl-carnitines, peroxisomal metabolites, and more.[2] With the most expensive component of this setup being the mass spectrometer, the idea of a shared mass spec for both LC and GC applications is highly favourable.[9,10] The Waters' Xevo TQ-S mass spec in combination with the Agilent's APGC 7890 achieves this by utilizing the principles behind the APCI technique allowing for a shared mass spec environment with an interchangeable LC-ESI and APCI-GC source. In this way, UOA and other biochemical genetic testing methods are made possible on a single instrument, without losing sensitivity and specificity.[11] The introduction of these types of dual instruments into the genetic testing lab environment can greatly impact the field as this would save labs large amounts of funding which could be appropriated to other projects and machines.

Herein, the following Masters thesis project will explore the fundamental properties of APCI-GC-MS using the above mentioned instrument, adapt existing GC-EI-MS methods for UOA analysis to APCI-GC-MS, design appropriate quality control measures, and begin to validate such methods according to the Clinical & Laboratory Standards Institute (CLSI) guidelines.[12] A significant area of preliminary research would be instrument optimization for UOA samples to achieve the adequate separation, sensitivity, and resolution needed before validation. One benefit of APCI is the method's softer ionization compared to EI, allowing for greater retention of molecular ions, thus

potentially increasing sensitivity, and identification ability of UOA compounds.[13,14] In addition, ion adducts that could cause interference both in noise and confusion in identifying from the mass spectrum can be reduced as well. However, these differing fragmentation spectra require the reconstruction of a mass reference library specific to APCI. Despite the tedious nature of this task given the hundreds of compounds observed in a single UOA run, the workload can be greatly reduced using reference points of known high concentration metabolites. By retaining similar GC separation parameters and using the same column as the current assay, the elution order remains largely unchanged other than a few shifts. Using this already well understood elution, other UOA compound peaks in the library can thus be predicted and confirmed efficiently. Going forwards, the APCI mechanism should be further explored while observing its relationship to source enclosure moisture levels giving [M+H] or [M+°] peaks in wet or dry conditions, respectively.[15,16] With the proper optimization and successful method transfer, it is hypothesized that future UOA analysis can be instead be conducted on this GC-APCI-MS machine as the new gold standard due to its convenience for running LC and GC applications on the same machine while maintaining adequate precision and accuracy.

In addition, the current UOA analysis is a qualitative method with the final interpretation being done by a trained biochemical geneticist.[20,21] By elevating the method to a more quantitative one with this new APCI technique, UOA metabolites could automatically be quantified allowing for diagnosis based on healthy and diseased compound reference intervals. This would greatly reduce possible errors in diagnosis and

the workload. The following project will achieve this by introducing MRM mode in the runs to increase sensitivity which matches perfectly with the softer ionization technique of APCI as it allows for ions with large peak areas to be chosen. The final goal is to also validate the quantitative aspects of this novel instrument based on CLSI guidelines.

3 - METHODOLOGY

2.1 - Patient Sample Extraction and Derivatization

The rationale behind the following extraction procedure via a liquid-liquid ether extraction is to isolate the UOAs from the urine sample as this matrix contains numerous other bodily metabolites. Afterwards, the derivatization protocol is to label the compounds with TMS to reduce their polarity and lower their boiling point. This ensures that the compound elutes at a suitable time and temperature so that the GC run is not unnecessarily long, and the compound does not degrade. The following procedure was taken from the currently validated protocol for preparing patient samples for GC-EI-MS. This was done to ensure large variance in elution times and elution order do not occur when comparing chromatograms retrieved from the gold standard GC-EI-MS approach and the new GC-APCI-MS technique.

Urine sample extraction is a simple non-invasive procedure conducted on patients with approximately 2 mL of urine being needed for a successful extraction. Samples can be stored at room temperature or in the refrigerator for 1 or 7 days, respectively. A stock concentration of the internal standard of tropic acid is prepared to 2 mM by mixing 33.7 mg of tropic acid in 100 mL of distilled water. In a siliconized screw-top tube, 2 mL of urine is spiked with 50 uL of 2 mM tropic acid stock. 3 M sodium hydroxide (NaOH) is added to each tube and tested with pH paper until an optimal pH of 11-12 is reached (~3 drops of 3 M NaOH but can vary based on the patient sample). 2 mL of ether is added after which the solution is vortexed and centrifuged at 2500 rpm for 5 minutes. With a glass pipette, the upper ether layer is discarded. 100 uL of 1.4 M hydroxylamine-

hydrochloride is added in as a reducing agent and vortexed. The solution is then heated on the 60°C heating block for 30 minutes and allowed to cool for 5 minutes. A few grains of sodium chloride (NaCl) salt are added followed by 5-6 drops of 5M HCl to acidify the sample to a pH less than 1. 4 mL of ether is added after which the solution is again vortexed and then centrifuged at 2500 rpm for 5 minutes. The upper ether layer is now extracted using a Pasteur pipette into prepared reaction vials containing Na_2SO_4 salt. Another round of ether addition, centrifugation, and extraction into reaction vials is conducted. The reaction vials are placed on the 60°C heating block for 15 minutes until all the ether has evaporated off. BSTFA with 1% TMCS is removed from the refrigerator to warm to room temperature while the reaction vials cool. 87 uL of the BSTFA with 1% TMCS is added to the reaction vials and heated once again on the 60°C heating block for 15 minutes. The reaction vials are occasionally rotated to ensure that the BSTFA reagent coats the inside vial walls. The now derivatized samples are stable at room temperature for two weeks and even longer when refrigerated. In a clear glass auto-sampler vial each with a spring insert, pipette and mix 100 uL of hexane with 50 uL of the derivatized sample. Once the auto-sampler vials are sealed with a cap liner, the samples are now ready for analysis.

3.2 - Individual Metabolite and Quality Control Sample Extraction/Derivatization

In addition to analyzing patient samples, individual UOA metabolites were extracted and derivatized to determine retention times of key compounds. Appropriate amounts of each compound were diluted in first water to achieve 5 mM of stock solution. 40 uL of this was then diluted in 2 mL of a urine-like matrix to reach a final concentration

of 0.1 mM. A full list of extracted compounds and their dilution calculations can be seen in Table 1. External quality assurance samples were needed to assess the analytical separation method and ensure proper operation of the instrument. The supplier of these samples for the Biochemical and Genetics Lab at McMaster Children's Hospital is ERNDIM, an independent non-for-profit foundation based in Europe. Its goal is to reach a consensus between Biochemical Genetics Centres in Europe and around the world on reliable and standardised procedures for diagnosis, treatment, and monitoring of inherited metabolic diseases.[39] The major quality assurance sample obtained from ERNDIM are MCA samples which are an array of UOA metabolites made to specific concentrations. There are two levels these concentrations are made, designated as L1 and L2 for a lower and higher concentrated pooled sample respectively.[39] Table 2 outlines the metabolites and their concentrations found in MCA standards. These individual UOAs and MCA samples were then extracted and derivatized as normal similar to a patient sample. It is important to note that both types of samples are still spiked with 50 uL of the 2 mM tropic acid stock solution prior to extraction and derivatization.

Table 2 - Metabolite concentrations in MCA L1 and L2 standards.[39]

Metabolite	**Molecular Weight (g/mol)**	**Level 1** (umol/L)	**Level 2** (umol/L)
2-Methyl Citric acid	206.15	2	47
2-OH Glutaric acid	148.114	38	394
3-Methyl Glutaconic acid	144.125	16	93
3-Methyl Glutaric acid	146.141	7	49
3-OH 3-Methyl Glutaric acid	162.141	41	359
3-OH Glutaric acid	148.114	N/A	N/A
3-OH Isovaleric acid	118.131	9	46
4-OH Butyric acid	104.105	12	129
Adipic acid	146.14	18	265
Creatinine	113.12	3100	6100
Ethyl Malonic acid	132.115	13	208
Fumaric acid	116.07	7	217
Glutaric acid	132.12	15	225
Hexanoylglycine	173.21	5	36
Keto Glutaric acid	146.11	31	554
Methyl Malonic acid	118.091	11	237
Mevalonic acid	148.16	7	134
N-Acetyl Aspartic acid	175.139	24	413
Pyroglutamic acid	129.04	112	549
Sebacic acid	202.25	10	84
Tiglylglycine	157.167	14	57
Vanillactic acid	212.199	11	86
Isovalerylglycine	159.183	6	39
Suberic acid	174.2	15	173

3.3 - Extraction/ Derivatization Optimization

As mentioned earlier, the extraction and derivatization steps above where from the current validated protocol for preparing patient samples for GC-EI-MS. To avoid impacting the elution order when undergoing GC separation, substantial changes to the procedure were avoided. However, consideration was given into adjusting the times of

both the temperature and duration of the reducing agent step involving hydroxylamine-hydrochloride and the derivatization step using BSTFA. The procedure for both parts required the solution to be heated for 30 minutes at 60°C on the heating block. As such, the experimental procedure was adjusted to explore how UOA yield would be impacted by using either 60°C or 80°C on the heating block in combination for a duration of 15 or 30 minutes. A longer time duration was not explored because the sample preparation time was already quite long and so steps to either reduce time spent or improve yield were done instead.

As mentioned previously, the goal of derivatization is to make the extracted UOA metabolites more volatile, less reactive, and therefore, have better chromatographic resolution. With silylation being a very common example of derivatization used in this lab and other similar research, one major problem is its sensitivity to moisture. In the presence of even small amounts of water, the derivative is rendered unstable as the SN2 reaction greatly favours water instead returning the functional group back to an active carbon rather than TMCS. Other biochemical genetics labs used N-tert-butyl-dimethyl-silyl-N-methyl-trifluoroacetamide (MTBSTFA) which forms tert-butyl dimethyl silyl (TBDMS) derivatives, known for being more stable and less sensitive to moisture compared to BSTFA.[31,32,33] As such, an alternate derivatization procedure was followed where MTBSTFA was used instead and impact on peak area in chromatogram was analyzed.

3.4 - Method Transfer and APGC-MS Parameter Optimization

To maintain consistency between the gold standard to this projects GC-APCI-MS approach in terms of elution order and time, similar machine parameters and oven method ramping schemes were used. The software used to control the instrument and view the data was MassLynx 4.1 with TargetLynx being used to analyze the chromatograms. The column used was an Agilent J&W 30 m x 0.25 mm, 0.25 um film fused silica capillary GC column. As outlined in detail in Table 3, the oven method began at 75°C and ramped up in steps to a burn out temperature of 285°C, for a total run time of 45 minutes.

Table 3 - Oven method ramping protocol for patient samples.

Rate (°C/min)	Final Temp. (°C)	Hold Time (min)
0.01	75	0.50
5.00	80	0.00
2.00	84	0.00
12.00	96	0.50
3.50	110	0.00
3.50	141	1.00
3.50	225	0.50
95.00	285	1.00

The carrier gas was helium and the machine operated in split mode with a ratio of 1:10 to prevent the overloading of the column, particularly when analyzing individually extracted compounds. Most patient tests were initially run in the dry mode unless indicated, therefore giving $[M+^{\circ}]^{+}$ ions in the mass spectrum chromatogram. Going forward, this may change to running in wet mode using either methanol or water modifiers in the source enclosure. Other major mass spectrometer settings, some of which were optimized from experiments described later in this paper, include: 3 uA corona

current, 20 V cone voltage, 500 L/hr auxiliary gas flow, 60 L/hr cone gas flow, 150°C source enclosure temperature, and 50-650 m/z MS scanning range. Three settings of importance that could be further optimized were cone voltage, auxiliary gas flow and cone gas flow. Experimental APGC-MS conditions were adjusted to explore each of these three at the following ranges: 20 to 60 V at 5 V intervals for cone voltage, 300 to 600 L/hr at 100 L/hr intervals for auxiliary gas flow, and 60 to 150 L/hr at 30 L/hr intervals for cone gas flow. The above settings correspond to the runs conducted on most patient runs, isolated compound extractions, and ERNDIM/MCA samples.

3.5 - Sample Stability Tests

As mentioned previously, the extraction and derivatization procedure was taken from the current protocol for preparing patient samples for GC-EI-MS. The protocol indicates that the finished samples can be stored for two weeks at room temperature or in the fridge at 5°C before sample degradation may begin to occur and impact UOA analysis results. A simple experiment was designed to observe the impact of refrigeration for two days as well as for one week on the extracted samples. Results were compared to ones directly analyzed after extraction and derivatization.

3.6 - Wet and Dry Source Enclosure Optimization

Wet/dry tests were conducted on solutions of 4-bromo diphenyl ether (BPPE) and phenanthrene. 100 mg of each compound was diluted in a solution of 100 mL of hexane and serial diluted down to the Waters recommended concentration of 1000 pg/mL. In a clear glass auto-sampler vial with a spring insert, a 1:1 ratio of BPPE and phenanthrene

was made by pipetting 100 uL of each stock 1000 pg/mL solution. This sample was then run on the wet/dry method outlined in the next section.

For the analysis of the wet/dry conditions of the machine on the solutions of BPPE and phenanthrene, the APGC machine was run on splitless mode. The oven method began with being held at 55°C for 1 minute followed by a ramping temperature of 33°C/minute until a burnout of 280°C, for a total run time of 7.8 minutes. In addition, the cone voltage for these tests were set to 30 V. The $[M+^{\circ}]^{+}$ vs $[M+H]^{+}$ peaks were observed for BPPE (248 vs 249, and 250 vs 251 m/z; the shift due to bromine isotopes) and phenanthrene (178 vs 179 m/z).[15,16] One can refer to Figure 4 to observe the resulting peak distributions in dry and wet conditions, respectively. The peak ratios of phenanthrene were compared to Table 4 (reference data from Waters) to qualitatively determine source enclosure moisture levels. Moisture levels were analyzed after swapping from LC to the GC and from opening the source enclosure door to determine the length of time source moisture levels take to settle. This is to ensure future runs are not done too early as ion peaks would vary depending on the mode the instrument is in. It is hypothesized that machine sensitivity will differ in wet and dry mode as the former prefers polar analytes in the mechanism.[31,32] As such, patient and MCA sample results will be compared between runs done on both wet and dry mode to determine which mode is more optimal for clinical use.

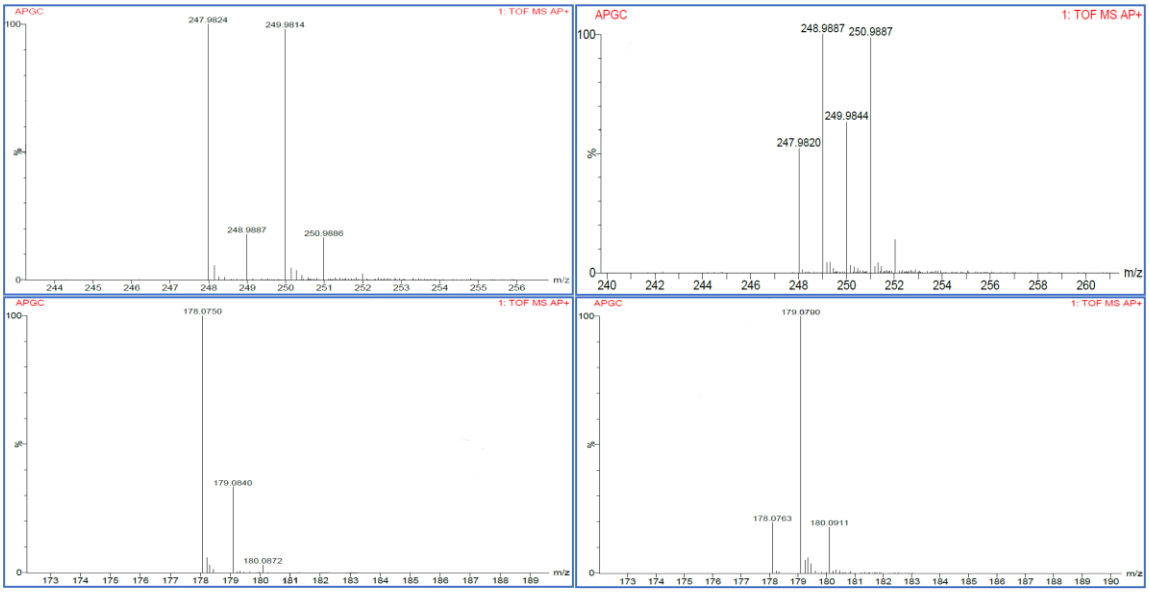

Figure 4 - Mass spectrum of the standards used to the test the wet/dry mode of the source enclosure. Molecular ion ratios of 4-bromo diphenyl ether (BPPE) in dry (top left) and wet (top right) mode. Corresponding molecular ion ratios of phenanthrene in dry (bottom left) and wet (bottom right) mode.

Table 4 - Percentage of charge transfer mechanism of phenanthrene based on $[M+\cdot]^+$ and $[M+H]^+$ relative mass ratio intensities.

Relative Intensity (%)		Charge Transfer (%)
178.078 m/z	**179.086 m/z**	
100	16	100
100	20	96
100	30	87
100	40	80
100	50	74
100	60	69
100	10	65
100	80	61
100	90	57
100	100	54
90	100	51
80	100	48
70	100	44
60	100	40
50	100	35
40	100	30
30	100	24
20	100	17
10	100	9
0	100	0

3.7 - UOA Library Building Process

A crucial step in the transition of this analytical method from exploratory research to clinical use is the building of mass reference and ion profile library specific to APCI. The gold standard method of GC-EI-MS has been used in the biochemical genetics field for diagnosis for decades.[21,22] As such, the GC separation science with that technique is well understood and the reference library is detailed and extensive to reflect that. Even

with a similar GC column and a successful transfer of the previous method on the new APGC machine, there will slightly differences in elution time but likely not in elution order of the metabolites. More importantly, the fragmentation patterns will be vastly different as APCI is a softer ionization compared to EI. This means that in addition to different retention times, the new library must also have the newly updated ion profiles for each of the metabolites. The currently used gold standard method analyzes over 300 compounds. Given the timeline of two years, this project will focus on building a library around the 24 compounds found in the MCA matrix and an additional 20 more that were readily available and were determined important to the preliminary version of this assay.

To begin making this database of compounds, rather than working with patient samples or even MCA samples, isolated extractions of the metabolites of interested must be analyzed first. This was to prevent matrix interferences and avoid the confusing nature of unlabelled patient sample chromatograms at the early stage as those can be revisited later in the research. More importantly, it was crucial that these steps were done properly because we had to know which peak belonged to which metabolite with high confidence to prevent returning to back to this stage in the future. Isolated extractions of the compounds were first analyzed in full scan mode looking for ions in the range 50-650 m/z. Due to APCI being a soft ionization technique, many of the metabolites will likely resolve in an unfragmented form. It is also important to keep in mind that protonation and derivatization of hydroxyls with the TMS group adds 73.2 m/z to the compound's molecular weight for singly derivatized compounds and 72.2 m/z for every addition TMS group added. With this knowledge, the molecular ion can be predicted, and mass searched

using the MassLynx software to the determine and confirm which peaks are the compound of interest.

After building an array of retention times and ion profiles of the metabolites, daughter/product scans of the two or three most prominent ions must be done at varying collision energies (10, 20, or 30 V) to determine the transition ions for MRM analysis. From this set of product ion scans, the one transition is chosen for quantitation and another one or two are chosen as confirmation transitions to ensure the correct metabolite is being analyzed. Once these transitions are finalized, samples can now be analyzed in MRM mode for increased sensitivity.

3.8 - UOA Quantification

With the goal of quantitation in mind, samples are run now solely run in MRM mode with the transitions determined from the section above. As a proof of concept of the potential quantitative abilities of the APCI instrument, MMA will be analyzed on a calibration curve scale from 10 uM to 10000 uM. This is because the clinical range of MMA presents itself in patient samples in healthy and diseased individuals varies greatly. In contrast, the other analytes that were quantitatively analyzed were only observed from 10 uM to 200 uM as their clinical range was smaller. With some analysis of urine matrix and water blanks, which are samples where extraction and derivatization was done on a 2 mL urine matrix and a 2 mL water solution in duplicate, LOD and LOQ can be calculated as well.

4 - RESULTS AND DISCUSSION

4.1 - Preliminary Experiments and Method Transfer

The goals of the initial experiments were to successfully transfer the GC and MS method of the old assay onto this new GC-APCI machine. Figure 5 and 6 showcases one of the first patient and MCA samples performed on the newly transferred method. It was expected that the patient sample would have similar eluting orders as the original given that the method was largely unchanged, and the machine conditions were very similar. Focusing in on Figure 6 of the MCA-L2, majority of the 24 compounds in the sample were adequately resolved and distinct as well. However, more work needed to be done in matching each peak to their corresponding metabolite. Though not shown here, it was important to note that MCA-L1, which contains the same batch of compounds but at greatly reduced concentrations, was not able to be properly analyzed as many peaks were not detectable. While sensitivities and noise do seem to be a problem, further work can be done to improve them but initial impressions on the technique were quite positive.

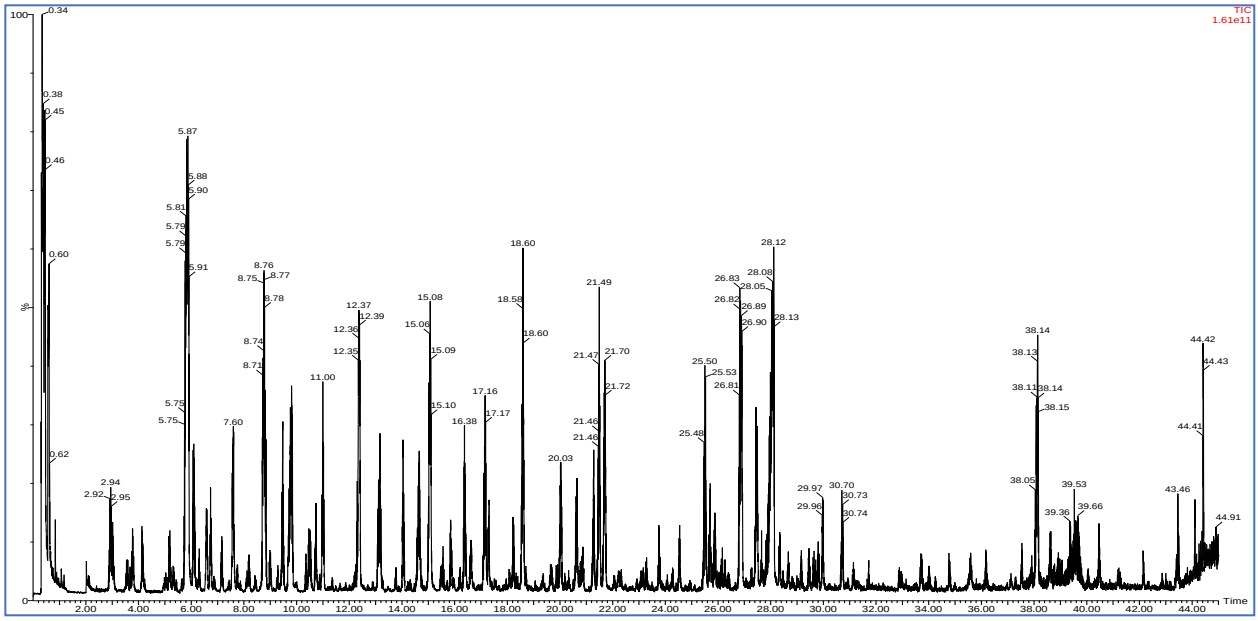

Figure 5 - Initial chromatogram of patient run using newly transferred oven method protocol.

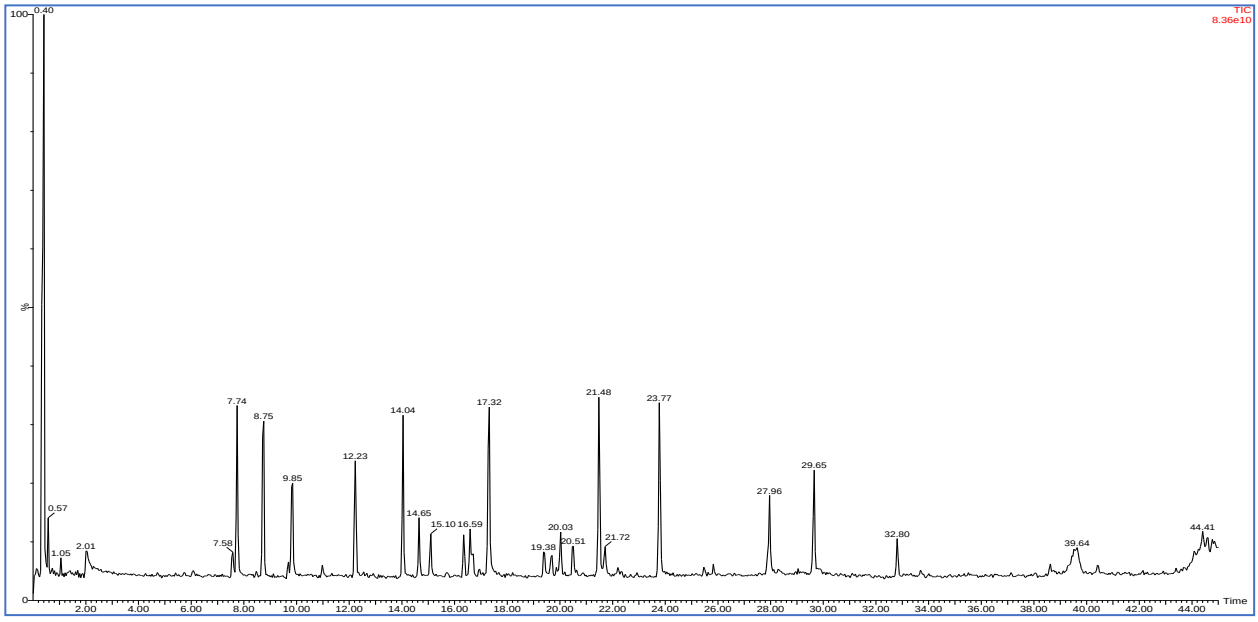

Figure 6 - Initial chromatogram of MCA-L2 run using newly transferred oven method protocol.

4.2 - Internal Standard Confirmation

The next major step was confirming the identity of the test's internal standard, tropic acid. The goal of an internal stand is to improve the precision and accuracy of results particularly when volume errors are difficult to predict and control. The internal standard should behave similarly to the analyte in its extraction, derivatization, and elution. However, it still needs to be distinguished apart from the analytes of interest in the method. With a proper internal standard, the ratio of internal standard signal to analyte signal can be plotted to correct for errors that may occur all the way upstream as far as sample preparation. As such, tropic acid was extracted and derivatized alone in a urine matrix solution of 2 mL, of which 50 uL was from the stock 5 mM of tropic acid. In addition, two other solutions containing 100 uL and 200 uL of tropic was prepped as well. This is to see if changes in concentration are reflective in the chromatogram. Figure 7 shows tropic acid being resolved at the time of 20.11 minutes.

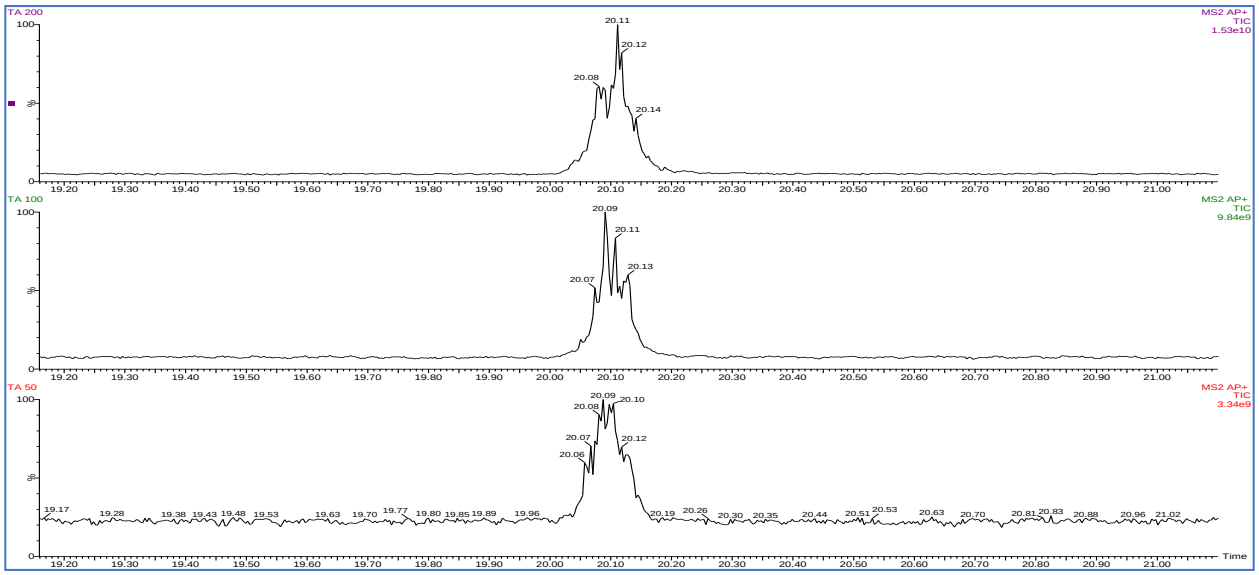

Figure 7 - Zoomed chromatogram of tropic acid at varying extraction volumes (200 uL - top, 100 uL - middle, 50 uL - bottom) with a retention time of 20.11 minutes.

This compound was confirmed by observing the predicted mass spectrum for the 311 m/z peak, indicative of tropic acid with two TMS groups (Figure 8). This retention time and m/z was relatively consistent across all patient samples spiked with tropic acid as well. The increasing concentration of tropic acid in the sample was reflected in both the chromatogram and mass spectrum as seen in the increasing peak height and peak area. This supports the possibility of transitioning this current qualitative method to a quantitative method in the future.

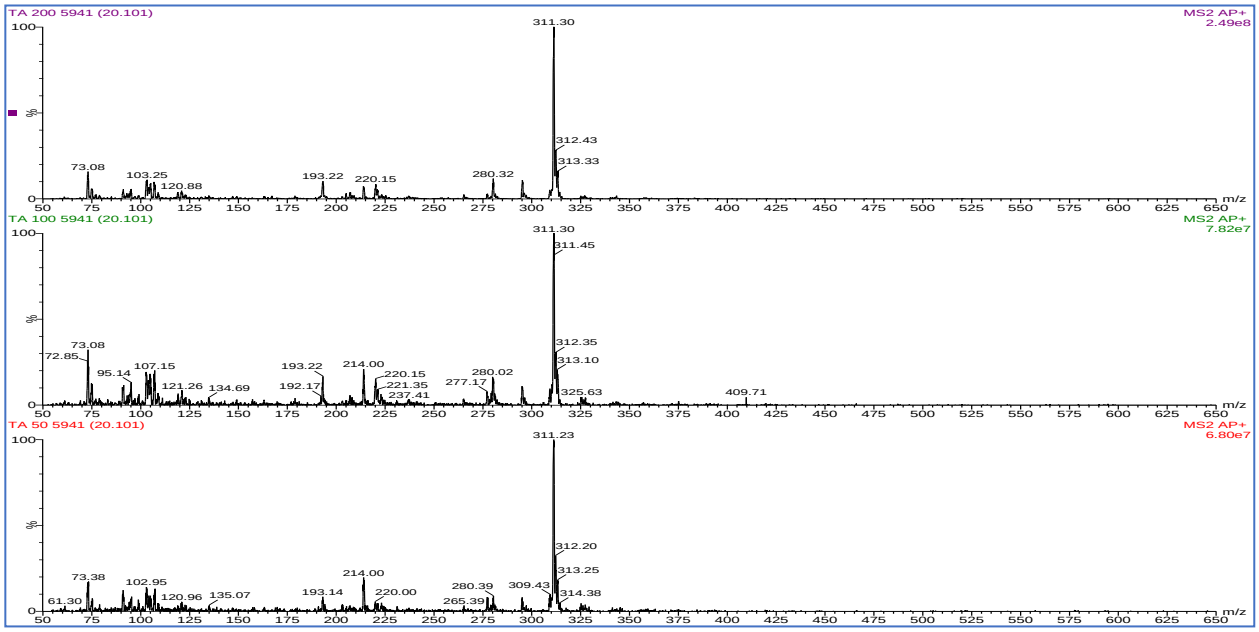

Figure 8 - Mass spectrum of tropic acid at varying extraction volumes (200 uL - top, 100 uL - middle, 50 uL - bottom).

4.3 - Mass Spectrometer Parameter Optimization

There were various parameters to be optimized before going forward in the research. Though many were explored, the ones mentioned in this paper include the cone voltage, auxiliary gas, and cone gas. Though not affecting the separation, the cone voltage is a key parameter affecting the degree of fragmentation observed in the mass spectrum.

The current goal was to reduce source fragmentation and obtain the largest molecular ion peak. As seen in Figure 10, increasing cone voltage introduced more fragmentation as expected and consistent with the current understanding of the parameter.[9] A cone voltage of 20 V was determined to be the most optimal going forward. A similar thought process was applied to the auxiliary gas and cone gas conditions. Figure 11 shows that decreasing auxiliary gas seemed to reduce sensitivity. As such, auxiliary gas of 600 L/hr was chosen. The same can not be applied to the cone gas as that is more tied to turbulence in the APCI source.[13] Increasing cone gas was seen to decrease peak height as seen in Figure 12 and so, the cone gas parameter of 60 L/hr was decided.

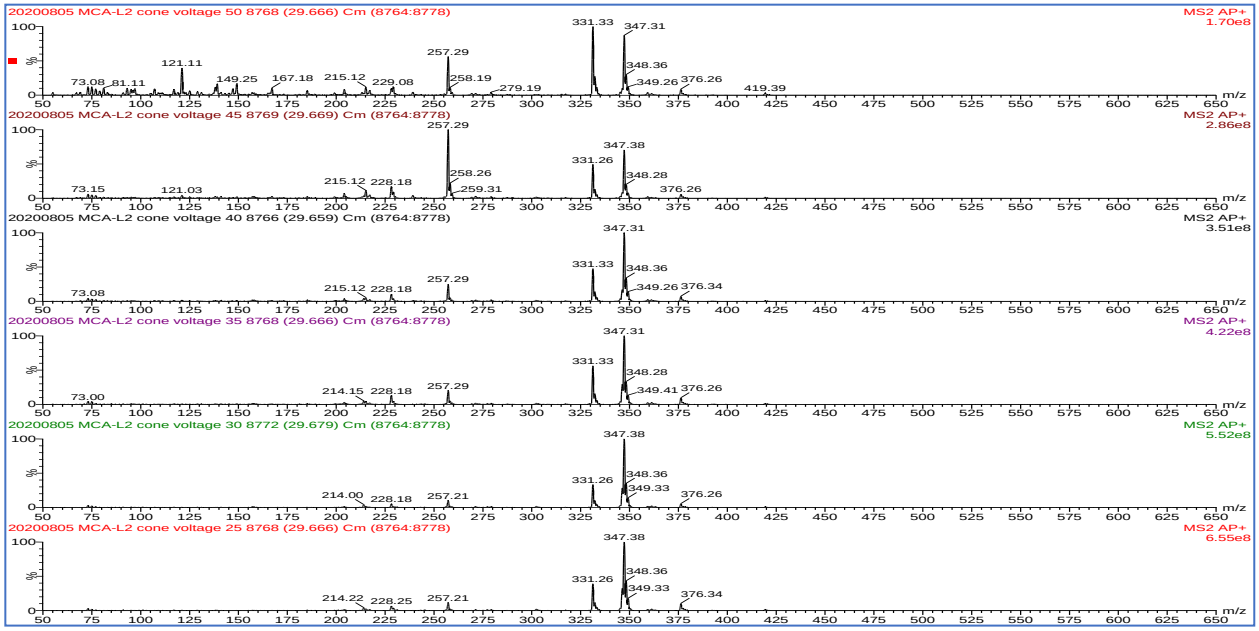

Figure 9 - Effects of increasing cone voltage from 25 V to 50 V on MCA-L2 samples.

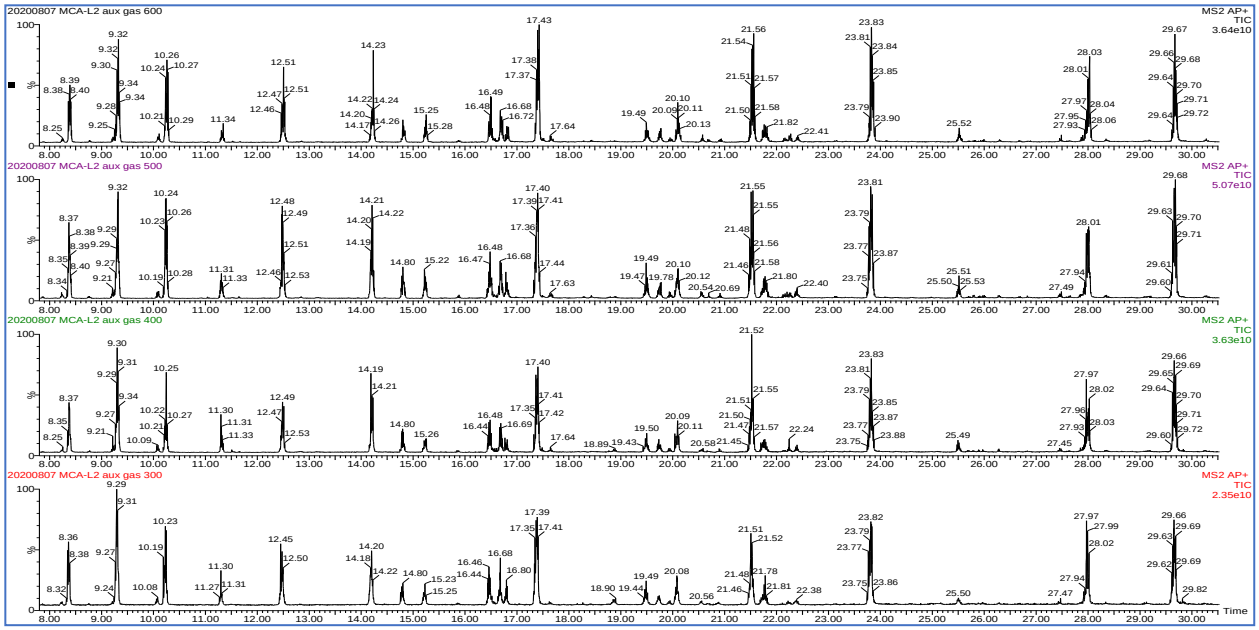

Figure 10 - Effects of increasing auxiliary gas from 300 L/hr to 600 L/hr on MCA-L2 samples.

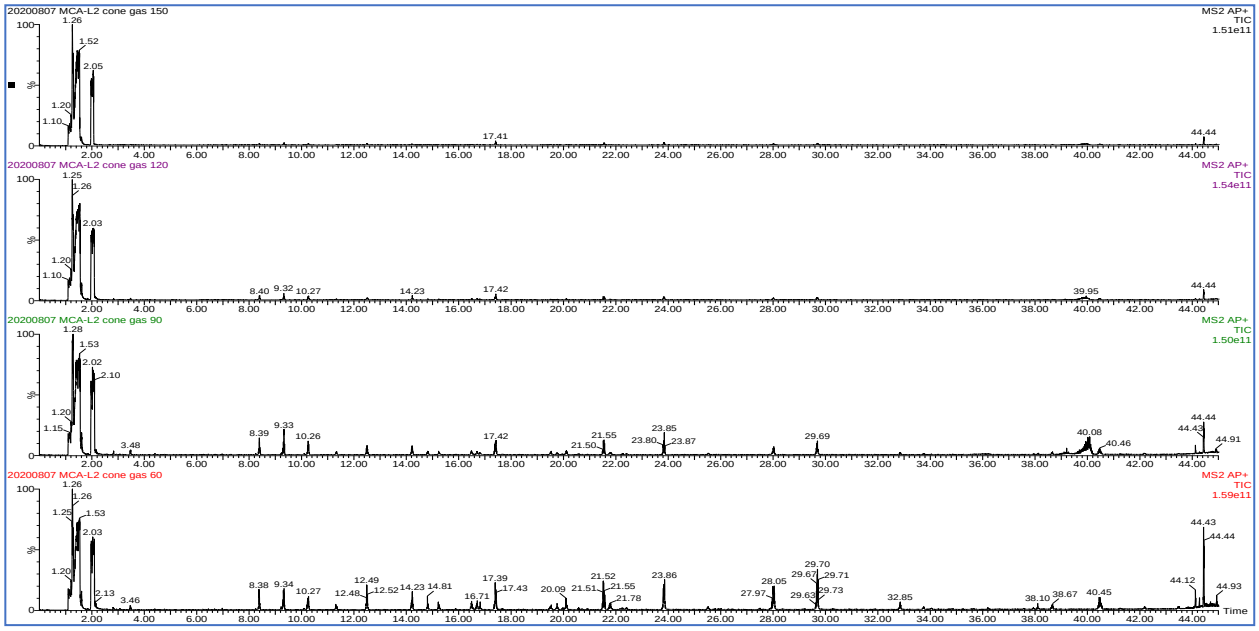

Figure 11- Effects of increasing cone gas 60 L/hr to 150 L/hr on MCA-L2 samples.

4.4 - Extraction/Derivatization Optimization

Given the complexity of changing and optimizing extraction and derivatization procedures, only limited exploration of this was undertaken. Increasing the reaction temperatures in the reducing agent step of hydroxylamine-HCl and the BSTFA derivatization to 80°C was observed and compared to the original 60°C. This is because increase heat can be a catalyst in many reactions. In addition, the reaction time was reduced to 15 minutes as a result hoping the 80°C had a positive effect on the reaction progression. This experimental procedure was repeated both for the original derivative, BSTFA, and the alternative derivative of interest, MTBSTFA, to determine if changing the derivative for future experimentation would be a good investment. Results are summarized in Table 5 and 6 below shown as a ratio of the topic acid peak area between the changed and original procedure conditions.

Table 5 – Ratio of topic acid peak area signal to standard reaction conditions of 60°C for 30 minutes. BSTFA was used as the derivative.

BSTFA	Reaction time: 15 min	Reaction time: 30 min
Temp: 60°C	91.14%	100.0%
Temp: 80°C	87.39%	NA

Table 6 - Ratio of topic acid peak area signal to standard reaction conditions of 60°C for 30 minutes. MTBSTFA was used as the derivative.

MTBSTFA	Reaction time: 15 min	Reaction time: 30 min
Temp: 60°C	72.93%	100.0%
Temp: 80°C	85.37%	N/A

As seen in the results above, reducing the reducing step and derivatization step reaction time to 15 minutes from 30 minutes causes a decrease of 8.86% of the signal. Similarly, increasing the reaction temperature to 80°C from 60°C causes a slightly greater decrease in signal of 12.61%. While the reaction time results were expected due to less time allowing for the reduction/derivatization to go towards completion, the increased temperature still causing even more of a decrease in signal was surprising. While the reasoning is not fully understood, this may be due to degradation of the sample due to heat. Now looking at the results for using the alternative derivative of MTBSTFA, it is important to note that the ratio was to its own original conditions at 60°C for 30 mins and not to the original conditions with BSTFA. If that was done instead, the signal ratio would be less than 0.1% indicating that MTBSTFA should not be used going forward, at least with these reaction conditions. Another major reason for not using MTBSTFA was the large shift in retention time downstream due the TBDMS group greatly increasing the molecular weight and thus boiling point. For example, the observed peak of tropic acid elutes at 20.11 minutes when derivatizing with BSTFA and at 27.8 minutes when with MTBSTFA. Since the internal standard, tropic acid, is usually considered a midpoint in the method, many latter metabolites likely do not elute in the 45 minute method and would require extensively modified GC methodology. Due to these reasons, the procedure of extraction and derivatization with BSTFA was unchanged going forward with future experiments.

4.5 - Sample Stability Tests

While it was understood that samples that were successfully extracted and derivatized were stable for one week at room temperature and even longer in the refrigerator, the stability was verified for this project. Figure 13 shows the results of storing a patient sample for 2 days in the fridge. The separation remained largely unchanged, but more noise seemed to occur in the post-refrigeration sample for reasons unsure. Similarly, Figures 14 and 15 demonstrate the same concept but using individually extracted compounds pre- and post-one week in the fridge, respectively. No significant changes were observed and so it was concluded that the stability of the samples seems to be the slightly worse than the old assay. As such, samples should not be stored for longer than one week.

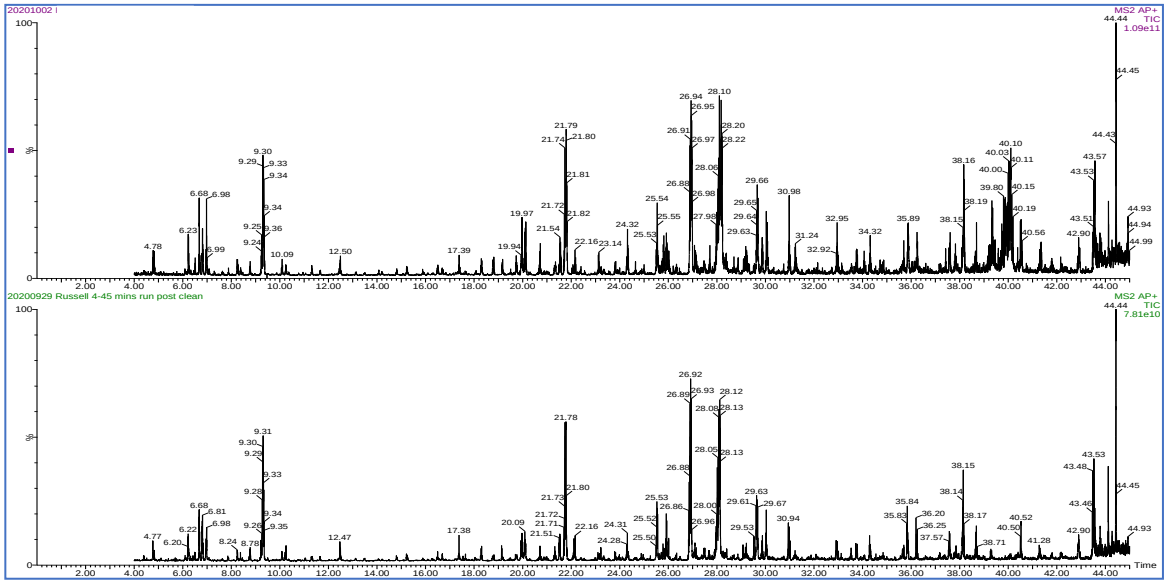

Figure 12 - Comparison of patient samples pre and post (bottom and top) two days of fridge storage.

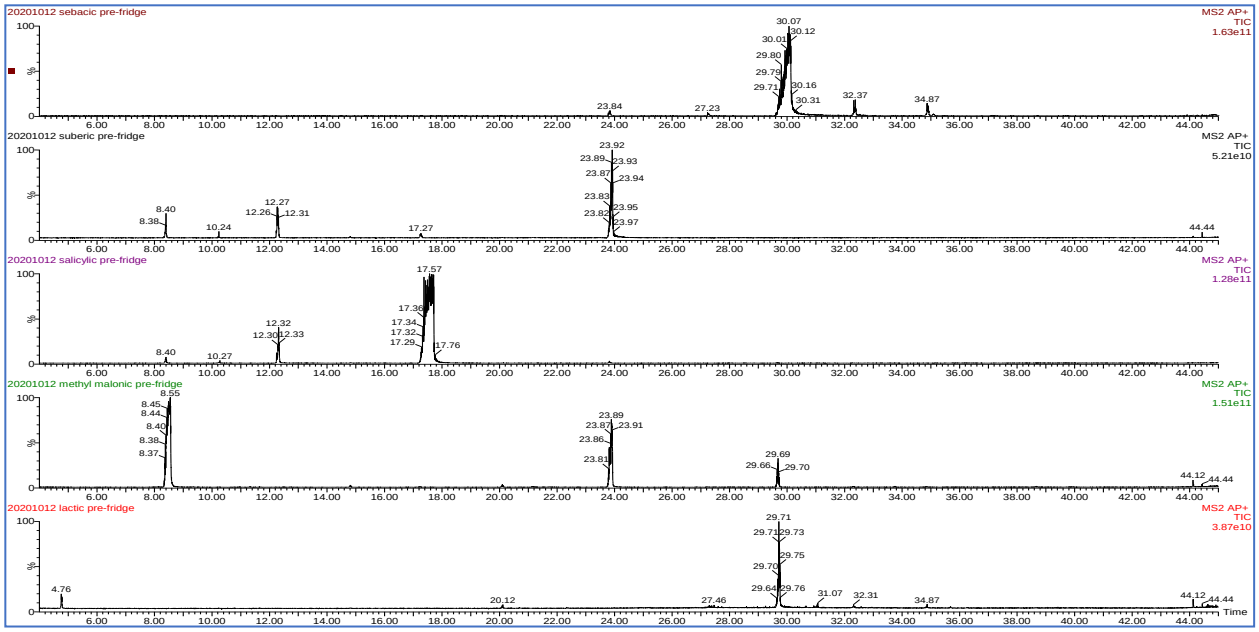

Figure 13 - Individually extracted compounds prior to one week of fridge storage.

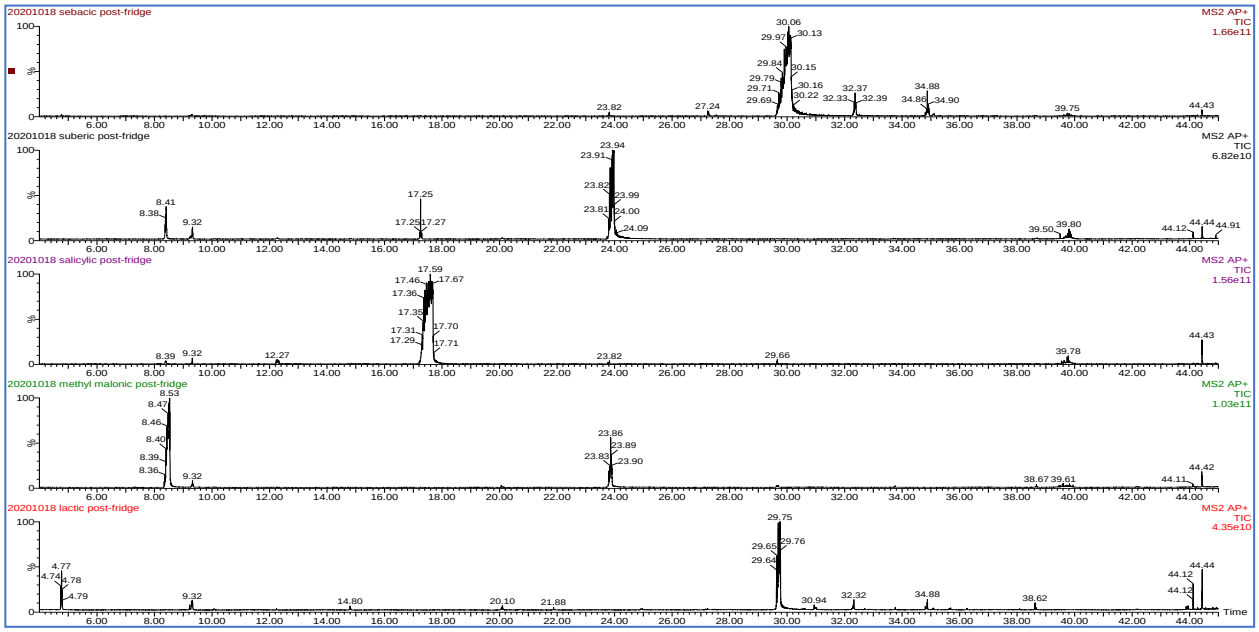

Figure 14 - Individually extracted compounds post one week of fridge storage.

4.6 - Wet and Dry Source Enclosure Optimization

The next set of experiments involved exploring the wet and dry conditions of the source using standards of BPPE and phenanthrene. With modifiers, the machine operates in dry mode by default, but it takes time for this mode to settle in, particularly if the source enclosure door has been opened or swapped from LC to APGC, as both introduce moisture into the source. These two compounds were used to determine these moisture levels to ensure the machine was operating in the desired mode before experimentation. Figure 16 shows the retention times of the BPPE and phenanthrene to be approximately 6.03 and 5.70, respectively. Their corresponding mass spectrums are also quite consistent with the proper mass ratios of a source without any modifiers that has been newly opened, which is considered as a wet environment (Figure 17 and 18). According to the ratios in Table 4, when the source door is swapped from LC to APGC or just opened, source moisture levels hover around the 30-40% charge transfer mechanism. It is important to note that below 50% is considered wet mode.

Similar observations of peak ratios were completed on an hourly basis, and it was determined the source enclosure achieves equilibrium after approximately 8 hours. At this settled equilibrium source moisture levels were calculated to 85-90% charge transfer mechanism with it being considered dry mode at levels greater 80%. Therefore, following a swap over from LC to GC mode of the machine, it is recommended to wait at least 8 hours before conducting any patient runs or other tests in dry mode.

Determining whether dry or wet mode was more suited for the samples was a key goal of these sets of experiments. In general, there seems to be greater sensitivity at the

cost of slightly more noise when in wet mode as seen in the patient samples in Figure 19 and 21. Additionally, after zooming into the chromatogram (Figure 20 and 21), one can see cleaner resolution across almost all the peaks further supporting the idea of continuing the project in wet mode. While the reasoning is currently uncertain as to why wet mode causes increased sensitivity, it is hypothesized that this is due to the proton transfer mechanism outlined in Figure 3. UOAs are compounds that are generally polar in nature. As such, they are good electron donors to the electron accepting proton. Further experimentation is warranted to explore why this mechanism is favoured for these analytes.

As the wet mode in the source can be induced by the addition of methanol or water modifiers, it would be important to differentiate the impact of each modifier on the chromatogram. More research needs to be done as there seems to be mixed results. Some patient samples show very little change between methanol and water modifiers (Figure 23 and 24) while others show a slight improvement in sensitivity when using water as opposed to methanol (Figure 25 and 26). This could likely be a case-by-case basis so it might be beneficial to observe the effects on methanol and water modifiers on individually extracted compounds first before moving on to observing patient data. Ultimately, methanol was chosen the modifier for inducing wet mode under the recommendation of service engineers at Waters.

4.7 - Pre and Post Septum and Liner Change

The next set of experiments was more of a maintenance of the machine by changing the septum and liner in the GC unit. As seen in the top chromatogram seen

below, there is a large improvement in the signal to noise ratio following the replacement of these parts. The old assay often had the septum and liner changed every one to two months as part of its maintenance. As such, a similar maintenance protocol should be adopted for this APCI technique. In general, more research should be into the maintenance related changes on this machine as using this for clinical samples is quite novel especially for complex and dirty matrix like urine.

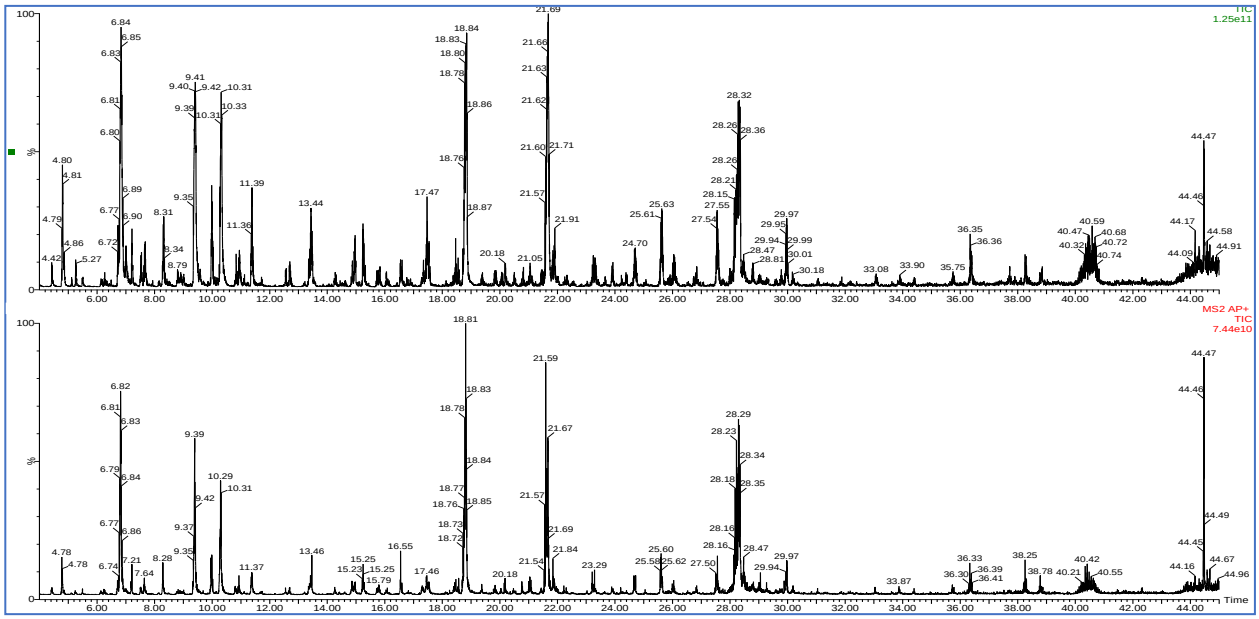

Figure 15 - Comparison of pre- (bottom) and post- (top) septum and liner change on patient samples.

4.8 - Isolated Compound Extractions

To work towards completing a mass spectrum library specific to APCI, certain UOA metabolites were chosen to be individually extracted, derivatized, and analyzed to serve as markers when predicting other UOA peaks in patient samples. One example as mentioned previously was tropic acid, the internal standard used in the protocol. Another compound of interest was lactic acid, the first UOA that elutes in a list of 300 compounds

that are analyzed. Having a retention time of 4.77 (Figure 12) and confirmed via its mass spectrum at 235 m/z, this was important in understanding when the first analytes would begin resolving on the chromatogram. Since the elution ordered remained unchanged, this led to concluding that the first 4 minutes of the run was deemed unnecessary as it did not have any compounds of interest. As such, the run was set to scan from 4 to 45 minutes to remove the large peaks initially seen at the beginning of the runs which often affected the relative peak heights across the whole chromatogram. It is important to note that the original retention times of tropic acid and lactic acid in the old assay was 2.77 minutes and 16.13 minutes, respectively. This indicates a shift of at least 2 minutes downstream for all metabolites allowing for the prediction of where certain compounds would be. As the run progresses, this shift margin seems to increase by 4 minutes at the 20.11 minute mark for tropic acid. Though it is unsure why this occurs, more research can be done on this topic to increase the predictive ability of UOA identification on the new assay, which will be explored later in the report.

A similar process of isolated extractions, derivatization, and analysis was conducted with each of the compounds found in MCA. Of the 24 compounds, 20 were fully resolved. The four compounds that were not fully resolved were mevalonic acid, vanillactic acid, isovalerylglycine, and creatinine. The first three had problems with increase baseline noise making it difficult to identify peaks. When conducting mass searches using the predicted m/z, no apparent peaks were shown indicating that these compounds may have different fragmentation patterns or there could have been problems with the original sample that caused interferences. As for creatinine, this did not seem to

chromatographically resolve on the GC-APCI-MS at all. This was not too much of a problem as this was expected since it occurred with the previous gold standard GC-EI-MS method as well. However, it would have been helpful as creatinine is often used to normalize the concentration of many metabolites in the human body.[34,25] Currently, creatinine is often quantified colorimetrically through the modified Jaffe method in blood and urine.[25] However, there are validated GC-MS methods that are able to quantify it but usually involve addition sample workup. In a report by Tsikas et al., they derivatized creatinine and an internal standard (methyl-trideuterated creatinine) with pentafluorobenzyl bromide, allowing to it resolve and be analyzed using Selected-Ion Monitoring (SIM) on a GC machine.[36.37] Further research into incorporating this analysis could be warranted.

An isolated extraction of particular importance is that of MMA. From early on, it was clear that MMA was a cleanly resolving compound. Given that it is in the array of compounds used in MCA samples and an important marker in some metabolic diseases, it was one of the first compounds following lactic acid that were successfully added to the library. Figure 16 shows the chromatogram of the isolated extraction of MMA eluting at 8.05 minutes. In addition, the ion profile confirms this given the 263, 247 and 218 m/z peaks as well. The 263 m/z will likely be the product ion used in further experiments for transitions in MRMs while 247 m/z and 218 m/z will act as confirmation peaks of the compound. Continuing with the other MCA compounds, Table 7 summarizes the retention times and ion profiles for the other 19 compounds found in the sample. It also

shows the previous retention times for those compounds on the old GC-EI-MS instrument as a comparison. This will be significant later on as this shift in retention time is analyzed.

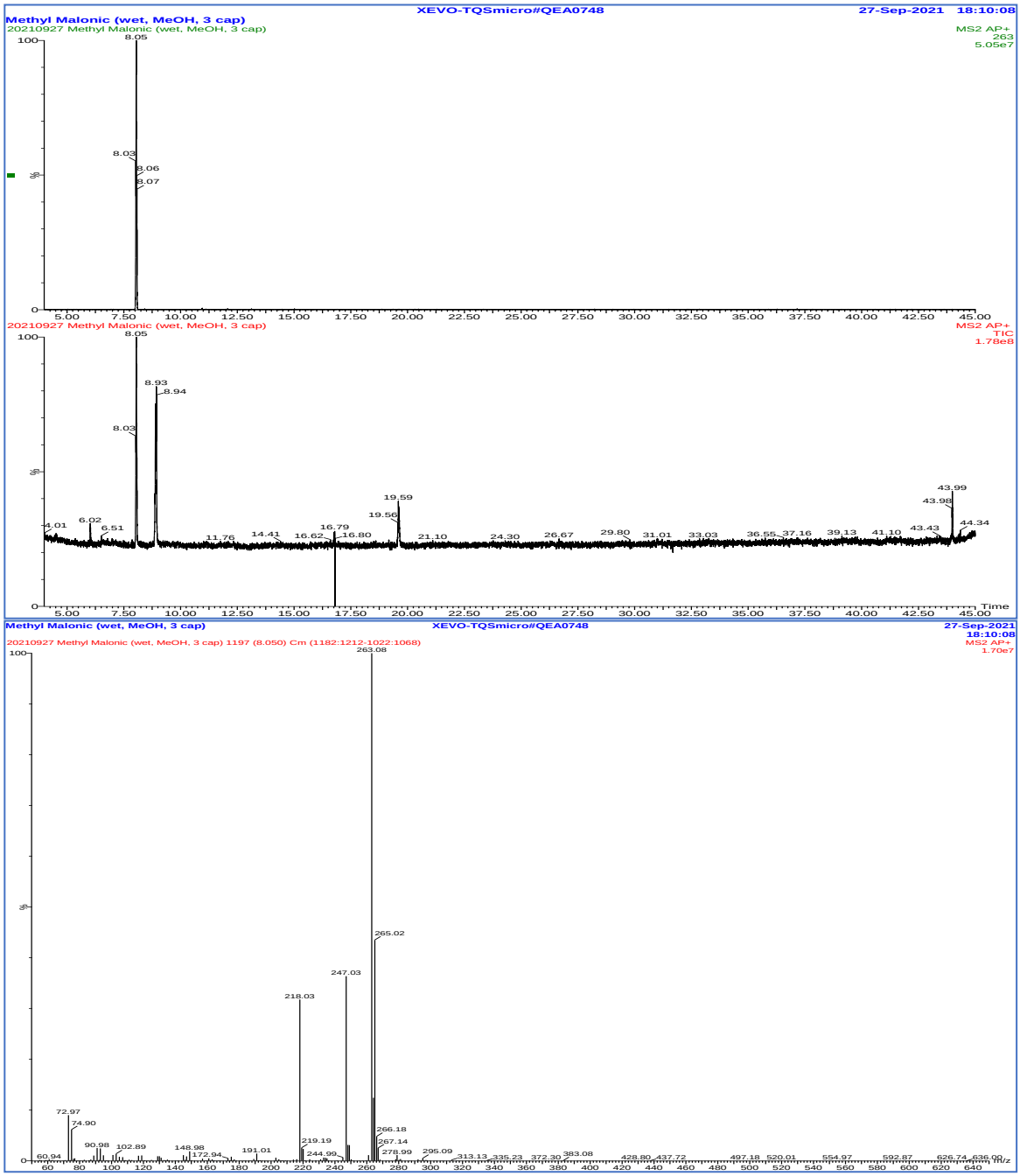

Figure 16 - Isolated extraction of MMA showing chromatogram (above) and ion profile (below)

Table 7 – Retention times and ion profile of UOA metabolites in MCA sample. Data was summarized from chromatograms and ion profiles in Appendix 8.2 - For Section 4.7 - Isolated Compound Extraction.

Compound	Old EI-GC retention time (min)	New AP-GC retention time (min)	Most Abundant Ions	Parent Ion
3-OH Isovaleric acid TMS2	5.51	7.92	247, 263, 131, 173	263
Methyl Malonic acid TMS2	5.63	8.10	263, 265, 247, 218	263
4-OH Butyric acid TMS2	6.15	8.70	233, 249, 251	249
Ethyl Malonic acid TMS2	7.16	9.87	232, 277, 279, 261	277
Fumaric acid TMS2	9.13	12.09	245, 263, 261	261
Glutaric acid TMS2	10.72	13.80	277, 261, 187	277
Sebacic acid TMS1	25.80	14.59	275, 291	275
3-Methyl Glutaric acid TMS2	11.28	14.60	291, 275, 201, 293	291
3-Methyl Glutaconic acid TMS2	13.08	14.86	289, 199	289
3-OH 3-Methyl Glutaric acid TMS2	16.59	16.07	307, 175, 291	307
3-Methyl Glutaconic acid TMS2	13.08	16.30	199, 288, 244	289
Adipic acid TMS2	13.73	16.97	275, 291	291
Pyroglutamic acid TMS2	13.91	17.26	215, 274, 155, 279	274
Tiglylglycine TMS1	15.16	19.07	230, 185, 170	230
2-OH Glutaric acid TMS3	15.92	19.48	365, 279, 349, 247	365
3-OH Glutaric acid TMS3	15.96	19.52	365, 349, 367	365
3-OH 3-Methyl Glutaric acid TMS3	16.59	20.07	363, 379, 279, 277	379
Keto glutaric acid TMS3	17.51/18.18	21.10	377, 378, 379, 362	363
Hexanoylglycine TMS1	18.04	21.31	246, 201	246
Hexanoylglycine TMS2	18.16	21.68	318, 302, 200	318
N-Acetyl-Aspartic acid TMS2	18.34	22.10	320, 275, 304, 160	320
N-Acetyl-Aspartic acid	18.34	22.50	392, 391, 274,	392

TMS3			376	
Suberic acid TMS2	19.72	23.35	302, 319	319
2-Methyl Citric acid TMS3	23.59	26.24	423, 407, 305	423
2-Methyl Citric acid TMS4	23.93	27.70	495, 479, 377	495
Sebacic acid TMS2	25.80	29.39	347, 331, 215	347
Mevalonic acid TMS2	9.70	N/A	N/A	N/A
Vanillactic acid TMS2	29.03	N/A	N/A	N/A
Isovalerylglycine TMS1	13.14	N/A	N/A	N/A
Creatinine	N/A	N/A	N/A	N/A

In addition to the compounds in MCA, 19 more metabolites of interested were also successfully resolved with their retention times and ion profiles summarized below in Table 8. These compounds were chosen for their clinical relevance in certain genetic diseases but also for being readily available in the lab. It is important note that D3 MMA, MMA with three deuterated hydrogens was resolved at a slightly shifted retention time to MMA at 8.16 minutes with a 266 m/z parent ion. This will be discussed later in the paper as this was chosen as an alternative internal standard to tropic acid.

Table 8 – Retention times and ion profile of UOAs of interest. Data was summarized from chromatograms and ion profiles in Appendix 8.2 - For Section 4.7 - Isolated Compound Extraction.

Compound	**Old EI-GC retention time (min)**	**New AP-GC retention time (min)**	**Most Abundant Ions**	**Parent Ion**
N-Acetyl Leucine TMS2	11.15	15.6	246, 201, 158, 318	318
D3 Methyl Malonic acid TMS2	NA	8.16	266, 250, 221	266
Lactic acid TMS2	2.77	4.79	235. 219. 191	235
Homogentisic TMS3	23.7	28.01	385, 384, 295, 267	385
Salicylic acid TMS2	13.48	17.57	267, 195, 283	283

Caprylic/Octanoic acid TMS3	6.81	32.23	361, 345, 271	361
Azelaic acid TMS2	22.88	26.83	333, 317	333
Succinic acid TMS2	8.09	11.36	263, 173, 73, 247	263
Glycolic acid TMS3	3.05	5.12	205, 149, 147, 293	293
Citric Acid TMS3	NA	26.19	409	409
Citric Acid TMS4	23.59	27.5	481, 363, 465	481
Vanillic acid TMS2	21.61	25.82	313, 241, 297	313
Phenyl Acetic acid TMS2	7.5	11.01	91, 209, 193, 281	281
Methyl Succinic TMS2	8.38	11.85	187. 277. 261. 349	349
Phenyl Lactic TMS2	15.77/17.8	19.86	193, 295, 311, 383	311
2-OH Isobutyric acid TMS3	2.81	4.9	233. 205. 321. 147	321
Pimelic acid TMS2	16.72	21.1	289, 305, 215, 377	305
Maleic acid TMS2	7.75	11.06	261. 245. 263	261
2-OH Phenyl Acetic TMS2	15.27	21.85	296. 179. 369	296

4.9 - UOA Library Building - Full Scan Mode

With 40 compounds now properly resolved, a similar process could be continued for the other metabolites until a comprehensive APCI library can be made. While possible, this would require much more time than the allotted 2 years available for this thesis project as the original assay can analyze for over 300 compounds in a single patient compound. However, a greater obstacle than time was resources and funding. Some isolated metabolites are readily available for purchase at reasonable price ranges, but not all compounds were within the budget. Even more of problem was that there are some metabolites in urine that have not been isolated making it difficult to analyze just one compound at a time. To circumvent this issue, a predictive equation was made based on the consistent shift in retention time when comparing the old GC-EI-MS to the new GC-

APCI-MS. With a steady shift of 2 to 3 minutes downstream, plotting the old retention time against the new retention time resulted in Figure 17, which confirms a strong linear relationship between the two values as seen with the R-squared of 0.9802. As such, the time range a compound might elute in the new machine can be predicted based on the well understood previous library of the old machine. Now looking at full scans of patient samples instead of isolated extractions, one can predict where certain compounds might elute and confirm using the peaks ion profile. This would greatly decrease the time required for building the mass reference library specific to APCI as now at least all 300 compounds can now be narrowed down to where they might elute. It is important to note that two compounds were removed from this predictive equation for being outliers, Caprylic TMS3 (also known as octanoic acid) and Sebacic acid TMS1. However, these outliers can be explained as the previous retention times used as reference were for different TMS variants. This may be due to the machine having different ionization properties causing certain TMS compounds to be more or less detectable compared to EI ionization. As such, this does not warrant any concern to the validity of this model but could require some further research to investigate other TMS variants.

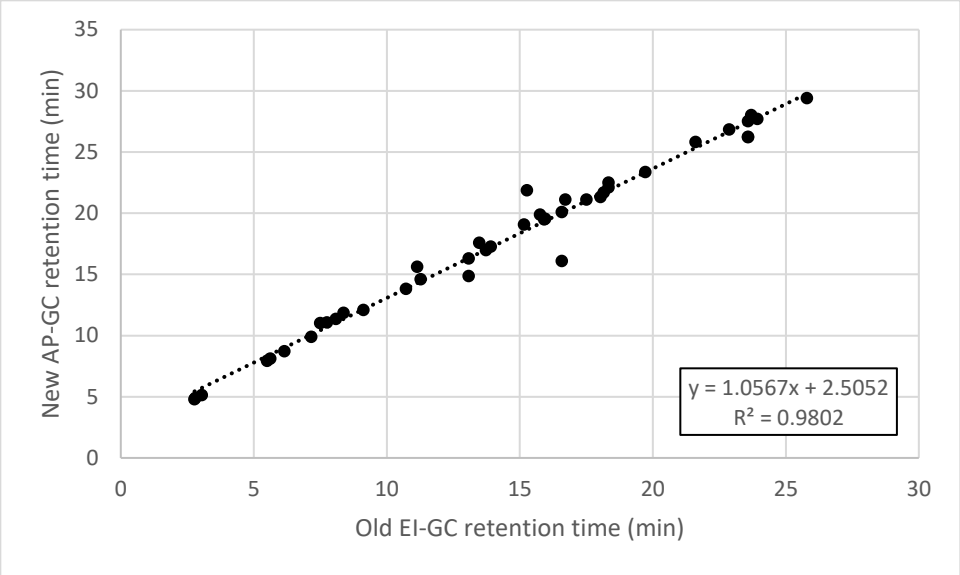

Figure 17 – Relationship between metabolite retention time on old EI-GC and new APCI-GC. Caprylic acid TMS3 (also known as octanoic acid) and Sebacic TMS1 were removed for being outliers.

4.10 - UOA Library Building - MRM Mode

One of the benefits of using the APCI technique is its softer ionization allowing for the retention of the parent molecular ion thus increasing sensitivity.[13] However, this same trait also makes it difficult to identify certain compounds. For example, though isobaric UOAs can be properly separated, they still need to be properly identified to complete the reference library of compounds. This can be solved through the introduction of using MRMs during the scan.[17] MRMs are a method used in tandem mass spectrometry where an ion from the first stage of mass spectrometry (MS1) is selected to be fragmented and detected in the second stage (MS2).[16,17] This is exceptionally powerful when used with APCI because the ion chosen from MS1 can be the molecular ion which is known to be present in high levels. This also removes interferents or any coeluting compounds as only the ion of interest goes forward through the mass spectrometer. In this

way, the method can achieve efficient separation using the GC, maintain great sensitive using the molecular ion from APCI, and accurately identify compounds.

With the goal of quantification in mind, the next stage of UOA library building specific to APCI was to run product ion scans on the molecular ion or two or three fragments of high abundance for each of the metabolite ion profiles determined previously. For each product ion explored, this was repeated at varying collision energies of 10V, 20V, and 30V. This was to make sure there was adequate fragmentation of the parent ion. For example, the transition of 263 to 263 m/z should be avoided for MMA because the goal of MRM transitions is to introduce specificity and sensitivity. The former is achieved because even if multiple analytes elute at a specific time, only the analyte of interest should continue and fragment in the MRM scan if the proper MRM transition is chosen. Using MMA as an example, this process for choosing the appropriate transition is seen in Figure 18. Similar to full scan mode, MMA will be analyzed for a quantifier daughter ion and a confirmation daughter ion. Below shows the product ion scans of MMA for 263 m/z and 247 m/z as the quantifier and confirmation ion, respectively. Usually decided by using the daughter ion of most abundance, the transitions 263 to 129 m/z and 247 to 147 m/z were selected for MMA.

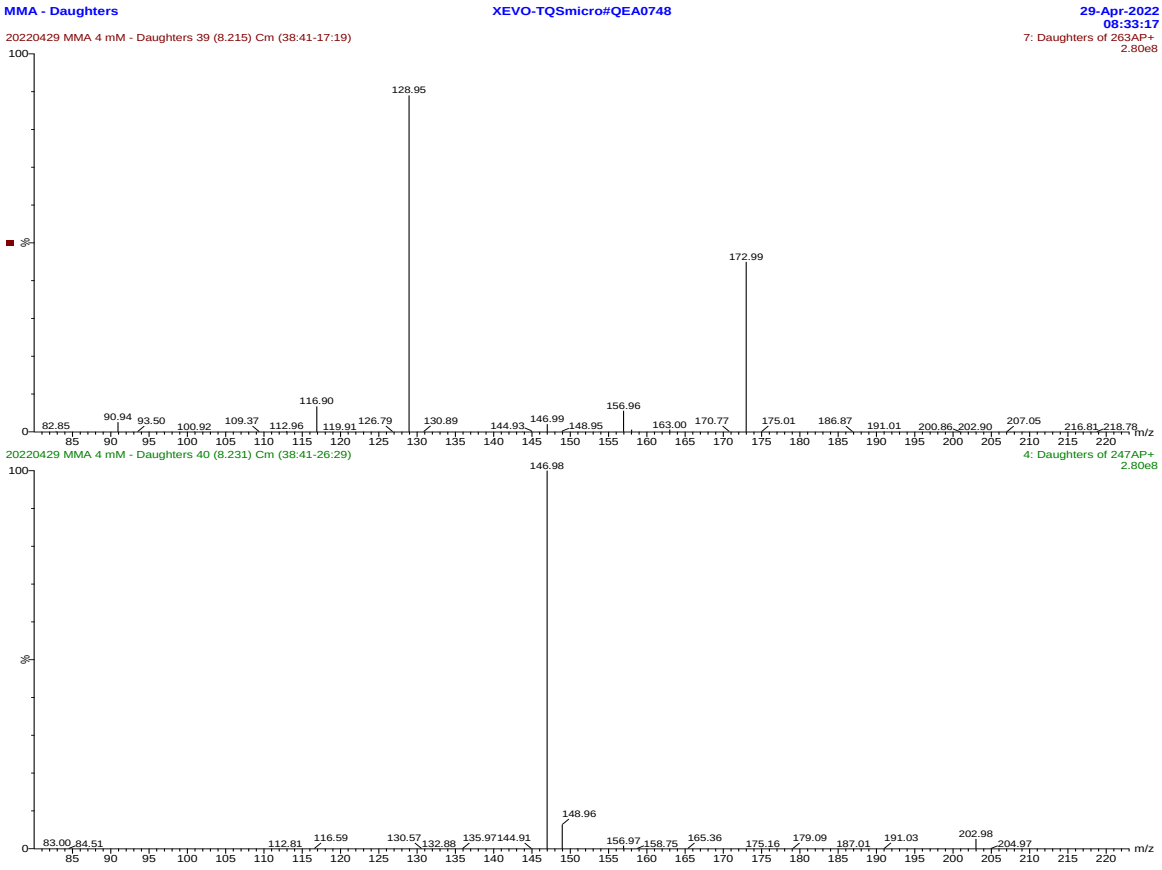

Figure 18 – Product ion scans of MMA at 263 m/z (above) and 247 m/z (below).

This process was repeated for all the MCA compounds that were resolved and a few additional metabolites of interested. The MRM transitions are summarized below in Table 9 with their specific collision energies as well. A prime example of the benefit of MRM scans as opposed to full scans is comparing 3-OH isovaleric TMS2 and MMA TMS2. Though both compounds are resolved separately by 0.2 minutes, MRM transitions can resolve problems of coelution even when they both have 263 m/z parent ions and similar fragmentation patterns. By using a 20V - 173 to 83 m/z for 3-OH isovaleric TMS2 and 10V – 263 to 129 m/z transition for MMA TMS2 for the quantifier transitions, you can remove interferences as the final ion analyzed in both are different. It is important to

note that there is not a problem if the confirmation transitions overlap as long as there are some unique ones.

Table 9 – MRM transitions for UOA metabolites of interest.

Compound	Collision Energy (V)	MRM Transition
3-OH Isovaleric acid TMS2	20	173 to 83
	30	247 to 147
	20	263 to 173
Methyl Malonic acid TMS2	10	263 to 129
	10	263 to 173
4-OH Butyric acid TMS2	10	249 to 159
Ethyl Malonic acid TMS2	10	277 to 143
Fumaric acid TMS2	10	261 to 245
Glutaric acid TMS2	10	277 to 143
Sebacic acid TMS1		275
3-Methyl Glutaric acid TMS2	10	291 to 201
	10	275 to 147
3-Methyl Glutaconic acid TMS2	10	289 to 199
3-OH 3-Methyl Glutaric acid TMS2	10	307 to 100
3-Methyl Glutaconic acid TMS2	10	289 to 199
Adipic acid TMS2	10	291 to 199
Pyroglutamic acid TMS2	10	274 to 230
Tiglylglycine TMS1	10	230 to 83
2-OH Glutaric acid TMS3	10	365 to 275
	20	365 to 129
	10	247 to 129
3-OH Glutaric acid TMS3	20	365 to 185
	10	349 to 185
3-OH 3-Methyl Glutaric acid TMS3	10	379 to 379
Keto glutaric acid TMS3	10	363 to 364
Hexanoylglycine TMS1	10	246 to 148
Hexanoylglycine TMS2	10	318 to 228
N-Acetyl-Aspartic acid	10	320 to 202

TMS2		
	10	320 to 230
	10	304 to 214
N-Acetyl-Aspartic acid TMS3	10	392 to 275
	10	376 to 147
Suberic acid TMS2	10	319 to 139
2-Methyl Citric acid TMS3	20	423 to 214
	10	305 to 214
2-Methyl Citric acid TMS4	20	495 to 287
	20	479 to 360
	10	377 to 287
Sebacic acid TMS2	10	347 to 121
D3 Methyl Malonic Acid TMS2	10	266 to 132
	10	250 to 147
Tropic acid	19	311 to 193

4.11 - Internal Standard Alternatives

Before transitioning to quantification, it is important to ensure that the current forms of internal validation are adequate. The one used in this project is tropic acid, a compound that is not found in patient samples but can be extracted, derivatized, and analyzed in manner similar to other UOAs. As mentioned previously, the goal of an internal stand is to improve the precision and accuracy of results particularly when volume errors are difficult to predict and control.[39] The internal standard should behave similarly to the analyte but should be easily distinguishable. Therefore, the most ideal internal standards would be isotopic variations of the current metabolites being analyzed.[40] Good examples of this would be using deuterated compounds or ones that use Carbon-13. The former is easier to make and thus, more readily available. As such, one deuterated compound explored in this project was D3 MMA. As seen below in Figure 19,

D3 MMA elutes at 8.16 minutes with an ion profile of 266, 250, and 221 m/z. This was as expected as MMA elutes at 8.12 minutes with an ion profile of 263, 247, and 218 m/z. Though coeluting, these two can be easily differentiated using MRM transitions of 266 to 132 m/z and 263 to 129 m/z, respectively. Given the success of using deuterated versions of UOA metabolites, it is recommended to further explore this avenue to introduce more internal standards through the chromatographic run. However, it is important to note that the deuterated compounds will have a slight loss in signal due the proton transfer mechanism that occurs when creating the $[M+H]^+$ fragment as seen in Figure 2 and 3.[31,32] In that mechanism, the deuterated hydrogen might be replaced with a regular hydrogen instead. This signal is likely further depreciated if the machine is operating in wet mode as this will occur even more. However, this signal decrease does not seem to be too substantial as seen in the full scan and MRM scan data of D3 MMA so far. This may change for other deuterated UOAs internal standards that might be introduced so it is important to keep this in mind.[31,32]

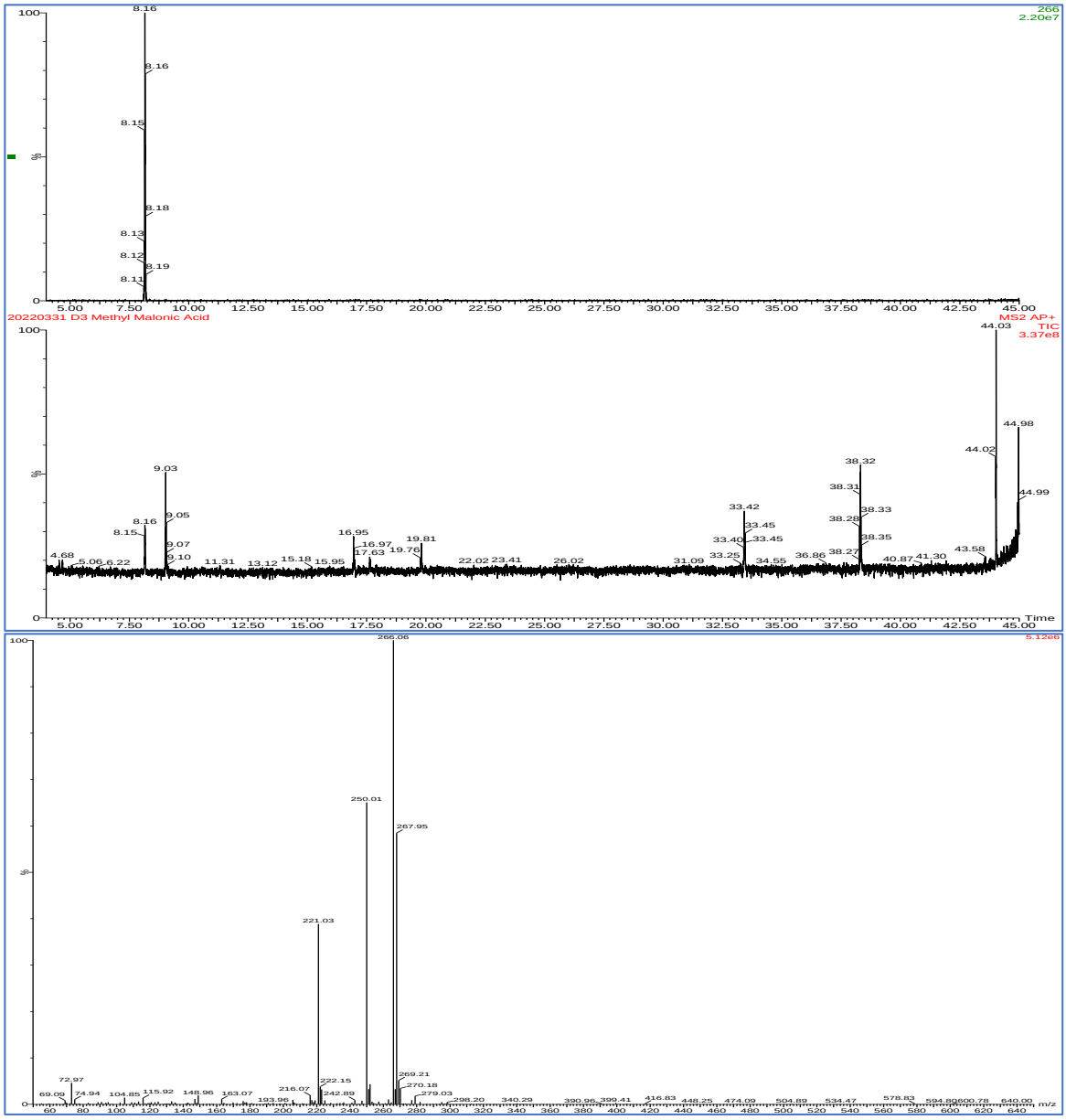

Figure 19 – Isolated extraction of D3 MMA showing chromatogram (above) and ion profile (below)

4.12 - UOA Quantification

Though the research thus far was quite substantial in establishing a qualitative assay for UOA analysis with this novel machine, there was the greater goal of pursuing quantification of these metabolites to have better understanding of the genetic diseases

and hopefully improve diagnosis. While quantification was possible using the full scan data of the UOAs, using the MRM scans would likely reduce interferences by having greater specificity and sensitivity. One of the first compounds analyzed quantitatively was MMA on a calibration curve from 10 uM to 10000 uM. This was because the clinical range of MMA when it presents itself in healthy and diseased patient samples varies greatly. MMA was analyzed at the concentrations: 10, 25, 50, 100, 250, 500, 1000, and every 1000 uM multiple up to 10000 uM. To reduce imprecision error, MMA peak areas were normalized against tropic acid peak areas as seen in Figure 20. The signal at 4000 uM was considered at outlier as the sample was more noticeably cloudy than other solutions, possibly due to error in the sample preparation. Removing that data point gives Figure 21 which has a strong correlation with a R-squared of 0.9984. Even among the lower concentrations as seen in Figure 22, the correlation is still quite strong with an R-squared of 0.9922. Though this needs to be confirmed via analysis of patient samples, this indicates that this instrument can be successfully used for quantification.

This MRM analysis of a calibration curve for each of the MCA compounds but only in the range of 10 uM to 200 uM as the clinical range of healthy and diseased patients is much smaller. The UOAs with notable quantitative data were tiglyglycine, hexanoylglycine, 4-OH butyric acid, glutaric acid, 3-methyl glutaconic acid, adipic acid, 3-methyl glutaric acid, suberic acid, keto-glutaric acid, 3-OH glutaric acid, 3-OH-3-methyl glutaric, and tropic acid. Quantitative data of calibration curves can be seen in Appendix 8.3 - For Section 4.11 - UOA Quantification.

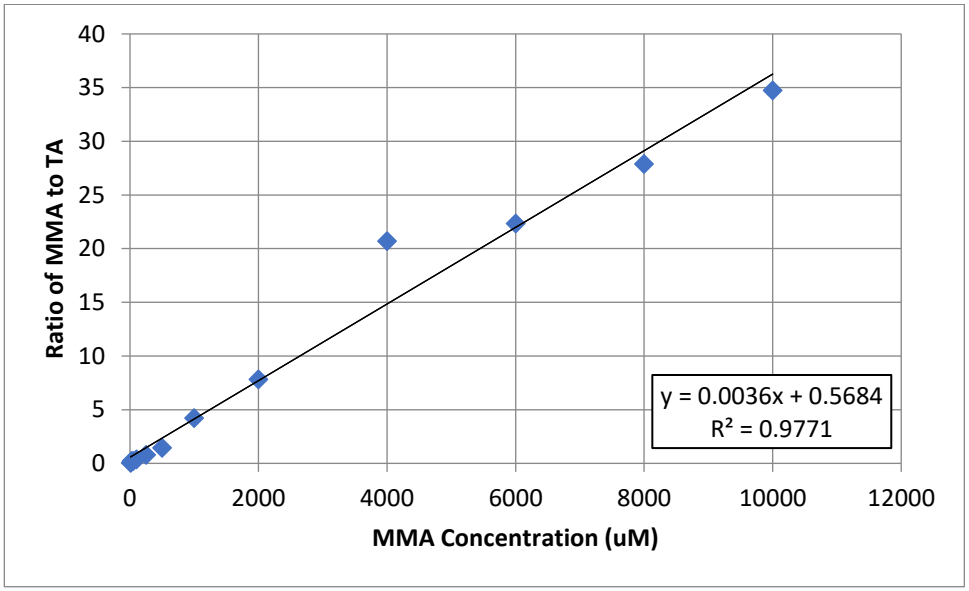

Figure 20 – Calibration curve of MMA from 10 to 10000 uM normalized to tropic acid.

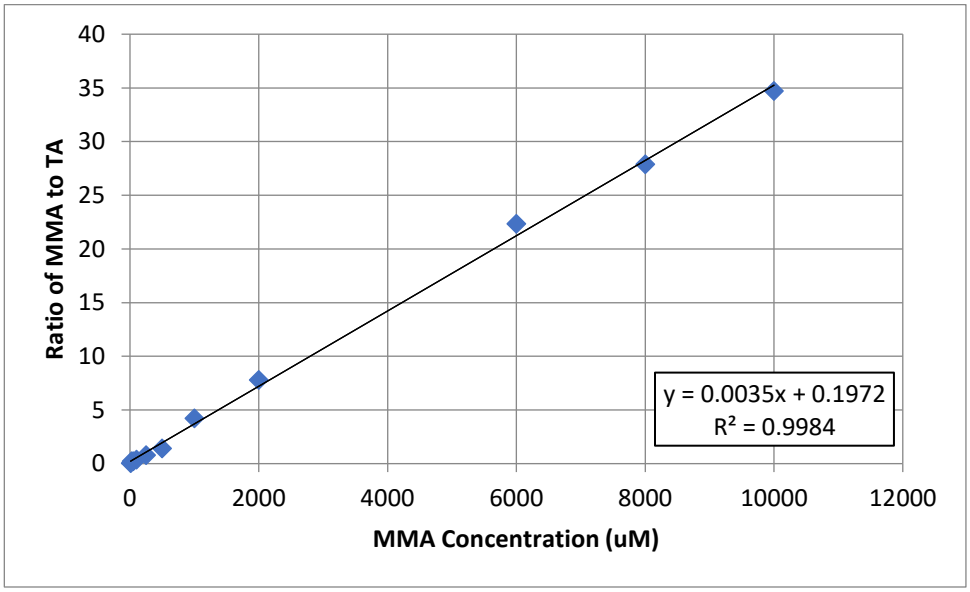

Figure 21 – Calibration curve of MMA from 10 to 10000 uM normalized to tropic acid. Data point at 4000 uM was removed as an outlier.

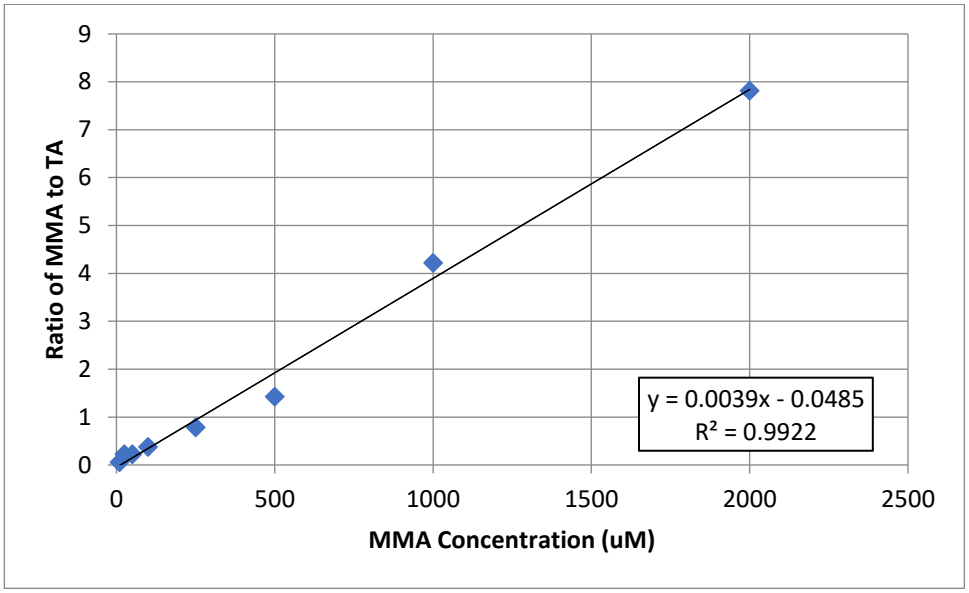

Figure 22 – Calibration curve of MMA from 10 to 2000 uM normalized to tropic acid.

5 - CHALLENGES AND FUTURE WORK

5.1 – Optimization of Extraction/Derivatization and Machine Maintenance

Despite the various optimization steps taken in the research above, more can be done to enhance peak resolution. Various other MS and GC parameters come into play in determining the chromatogram and corresponding mass spectrums. One example was the change seen above after changing the septum and liner, which greatly reduced trailing peaks on UOAs of high concentration. As per the recommendations of the Waters service technician for the machine, settings such as transfer line location, corona pin position, sample injector temperature and chromatographic column trimming are simple points of adjustment that can easily improve results. Similarly, settings that are considered standard can be changed as well such as solvents. One paper on pesticide residue analysis on an APGC system used a multi solvent system to reduce solvent effects as their extraction left them with a final extract in acetonitrile, which is considered as one of the less favourable GC solvents.[19] To solve this issue, they dissolved their samples in a combination of 50 uL of acetonitrile, 150 uL of acetone and 300 uL of hexane.[19] Similar concepts could be applied to this research as the current solvent being used is 100 uL hexane. It is important to note that for any new solvents being chosen, their boiling points should be below the analytes of interest to prevent any interference.

5.2 - Revisiting Method Optimization using Design of Experiments

The process used in this project to optimize the GC and MS method involved analysis of a single parameter or variable at a time. While the order the parameters were optimized (cone voltage, auxiliary gas, cone gas, etc.) were chosen to focus on the

settings with the largest impact first, this does not necessarily account for confounding variables. Despite this, this process was continued for two reasons. The first was because since this instrument was quite novel in clinical settings, deciding on the initial parameters to adjust were still unknown. The second and more important reason was that early on, the primary way of determining results was through observing tropic acid, MMA, and maybe a few other compounds at a qualitative level. This does not come near to level of analysis the final assay would be, which might look at over 300 compounds at a qualitative level and maybe 100 of which at quantitative level. As the research so far into this machine and for this project was primarily exploratory, this rudimentary way of optimization was deemed sufficient given the understanding, resources, and time at hand.

In these two years, much has changed in terms of understanding the instrument and building the method. As such, GC and MS optimization can be revisited with the newfound knowledge. A more optimal way of approaching this problem now would be to use Design of Experiments (DoE) which is a multivariant analysis technique that adjusts an array of multiple inputs at once while observing an array of multiple outputs.[47,48] This was not initially possible as mentioned earlier due to the lack of understanding of associated inputs and quantifiable outputs. However, that has now changed given the ability to now adjust many input parameters on the GC side (cone voltage, auxiliary gas, cone gas, etc.) and the MS side (corona current, parent ion analyzed, MRM transition, collision energy, etc.). More importantly, much of this data can be quantified as well due to the MRM analysis of UOAs. In this way, DoE can be used to create an array of inputs

that we explore all the minimums and maximums efficiently and tailor each to the various outputs of each of the different metabolites.[48]

5.3 – Further Exploration into Wet mode and Potential Source Modifiers

The research so far has indicated that the APCI in wet mode increases peak resolution and sensitivity at the cost of a minor increase in noise. This aligns with the literature on the topic and previous research done in the field as the wet mode is favourable for polar compounds with smaller molecular weights. This is due to the affinity UOAs have towards the final step of the proton transfer mechanism.[13] Wet mode can be induced using methanol or water and while tests under both conditions were completed and compared, it is still too early to determine which is more suitable for patient testing. As such, further research needs to be done by likely looking at key UOAs and the impact each condition has on its sensitivity. The source modifiers themselves can also be adjusted such as varying the degree of methanol or water in the vial, expanding the diameter of the capillary tube to increase the levels evaporation in the source, or adding more capillary tubes into the vials. The latter two are particularly promising because according to the ion ratios in the wet/dry tests of the source, using methanol or water at its current level seems to favour the proton transfer mechanism by only 85 to 90%. According to data collected by Waters, above 80% proton transfer is considered to operating in the wet mode regimen. There is more potential to increase this using the ideas mentioned previously. In doing so, machine sensitivity could be improved even further as a greater ratio of the analyzed $[M+H]^+$ would exist. Along the same lines, the current process in determining the moisture levels in the source involves a separate run

observing the ion ratios of BPPE and phenanthrene. It would be more efficient to find a UOA metabolite or incorporate a new compound into the patient sample that would demonstrate similar properties in acting as an internal test of source moisture levels all in the same run.

5.4 – Expanding the mass spectra reference library specific to APCI

With every novel analytical technique, certain challenges exist ranging from sensitivity issues to interferences and more. Fortunately, the research so far indicates that GC-APCI-MS has already solved one of the bigger problems facing analytical separation methods, adequate separation. After closer inspection of the chromatograms, it is apparent that its separation ability exceeds that of the old gold standard. Majority of the key UOA metabolites in the previous method often eluted in the first half of the 45 minute run. In this technique, one can see a relatively even distribution of peaks throughout the chromatogram with separation of compounds such as hippuric acid and azelaic acid who often coelute together in the old method. However, the major difficulty is identifying these peaks to their corresponding UOAs due to not having a mass spectra reference library specific to APCI. Completing such a library would be quite tedious given the over 300 analytes of interest in a single UOA run. However, the workload has been greatly reduced using reference points of known metabolites often present in high concentrations. With the elution order remaining largely unchanged, other UOA compounds peaks in the library can predicted as before or after these chromatogram markers and thus, this reference library could be completed in a smaller timeline. This can be further accelerated using the prediction equation mentioned previously to narrow the elution range one can

predict where the compound might be. Therefore, you can continue to use a set of patient samples rather than numerous isolated extractions to build the mass reference library. Unfortunately, this can not be used when building the MRM transition library as the transition will be unique for each parent ion, especially at different collision energies. Since the MRMs are mainly used for quantification, it is recommended that only UOA metabolites of interest that show benefit in quantification should reach this stage. The other compounds can be left at the full scan stage for identification.

5.5 – Analytical Method Validation

Though this project has progressed greatly since its first experiments, the goal was to create an analytical method that could be used in the clinical environment. As such, method validation via analytical and CLSI standards is one of the final steps needed. The two standards are slightly different in their calculation with the latter being stricter as the technique must meet criteria for patient diagnosis.[40,41,44] While some analytical measures of the APCI technique is given, it can not be currently fully validated given the lack of quantitative data. As such, this should be revisited in the future.

Each technique has measures with some level of uncertainty due to experiment error. This can be random (indeterminant) error or systematic (determinant) error. The former focus on the limitation of repeated measurements thus showing its precision and reproducibility and the latter involves flaws in the measurement caused by bias thus demonstrating the methods accuracy.[40,41]

Accuracy is the closeness of the measured result to the true value of the analyte in the matrix, usually calculated as percent accuracy. Ways to confirm this would be to

analyze standards of known concentration such as the compounds in MCA as well as repeated measures of blanks. The test results can be compared to another validated method for the same analyte, in this case GC-EI-MS.[4142] Lastly, round-robin experiments can be done using samples sent to multiple labs and compared to results on this instrument. For biochemical and genetics labs that conduct UOA analysis, these round robin experiments are organized by ERNDIM.[39]

In contrast, precision is the degree of variance when performing repeated measurements of an analyte in a sample. This is shown primarily using measures such as standard deviation and relative standard deviation (RSD) (coefficient of variance). In analytical methods, RSD should be ideally less that 10%.[42] Techniques with low precision result in lower confidence in differentiating between two signals. For example, the coeluting of compounds causes overlapping of peaks. Another measure of precision would be sample means and standard deviation. When used together, the confidence interval is known which is the interval where a true value falls into a range at a certain probability level.[42] In general, more replicates are beneficial but other factors such as time and resources need to be accounted for.

With the goal of quantification in mind, calibration solutions were run to create calibration curves. Fortunately, some metabolites showed a linear dependence between signal and analyte concentration allowing for a linear regression to give a line of best fit. Formatted as $y = mx + b$ with a R^2 (correlation of determination), this gives three important measures. The slope of m is a measure of sensitivity as it shows the change in signal to concentration. The y-intercept of b indicates the noise and detection limit of the

method. And lastly, R-squared shows the degree of linearity of the technique among a certain range of values.[43,44] Ideally, the technique should have been as sensitivity as possible but there are limits, particularly LOD (limit of detection), LOQ (limit of quantification), and LOL (limit of linearity). LOD and LOQ is the level the method can confidently state an analyte is present and quantified, respectively.[44] The two values can be calculated from the signal to noise ratio (S/N), usually approximated as three and ten times the standard deviation of the blank or approximately 3 or 10 times the S/N, respectively. While calibration curves can give acceptable lines of best fine, the linear range is from LOQ to LOL where reliable measures (RSD <10%) can be conducted.[44,45]

Another important part of method validation is quality assurance and quality control. The former is the use of processes, methodologies, and standards before an experiment to ensure it meets proper standards. The latter occurs during experimentation and analysis to confirm that standards are met. For example, actions such as using control charts, inter/intra-day variability, quality control (QC) samples, consistent standard operating procedures (SOPs).[40,41]

5.6 – CLSI Method Validation of Detection Capability

In comparison to general analytical method validation, CLSI guidelines for method validation is more rigorous as the technique will be used for patient samples. In this report, only EP17 – Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures will be shown as the each of the documents for the method validation sections above are quite extensive.[51,52]

According to CLSI guidelines for LOB and LOD, the minimal experimental design requires three days of testing, four blank samples, four low concentration positive samples, and two replicates per sample for each day. At this stage, only 3 days x 4 samples x 2 replicates = 24 replicates for each the blank and low-level sample are done. However, the sample design must be increased by a few factors to meet the minimum requirement of 60 total blank replicates and 60 total low level sample replicates. This is often achieved by increasing the number of samples, replicates, and/or days (i.e., 3 days x 5 samples x 4 replicates). It is not necessary to have the same number of blank and low-level samples, as long as the minimum number of requirements is met.[52]

In terms of the data analysis, α and β values for LOB and LOD must be decided, typically α = β = 0.05. Using the parametric approach, LOB is calculated using the equation below, where is M_B is the mean of the blank, SD_B is the SD of the blank, c_p is the multiplier given to the 95th percentile of a normal distribution, B is the total number of blank results, and K is the number of blank samples. It is important to note that 1.645 represent the 95th percentile of the normal distribution for α = 0.05, so if a different α is chosen, the multiplier needs to be changed as well.[52]

$$\text{LoB} = M_B + c_p SD_B$$

$$c_p = \frac{1.645}{1 - \left(\frac{1}{4(B-K)} \right)}$$

For LOD using the parametric approach, pooled SD for each of the low-level samples must be done first using the equation below, where SD_i is the SD of all the

results for the *i*th low-level sample, n_i is the number of results for the *i*th low-level sample, and J is the number of low-level samples.[51]

$$SD_L = \sqrt{\frac{\sum_{i=1}^{J}(n_i - 1)SD_i^2}{\sum_{i=1}^{J}(n_i - 1)}}$$

Afterwards, LOD can be calculated with the equation below. Similar to the equation LOB, the SD_L is the SD of the low-level sample, c_p is the multiplier given to the 95th percentile of a normal distribution, L is the total number of low-level results, and J is the number of low-level samples. Like before, the value of 1.645 must be adjusted if α value different that 0.05 is chosen.[52]

$$\mathrm{LoD} = \mathrm{LoB} + c_p \mathrm{SD_L}$$

$$c_p = \frac{1.645}{1 - \left(\frac{1}{4(L - J)}\right)}$$

In contrast to analytical validation as well as how LOB and LOD are calculated, LOQ depends on the specific acceptance goals used by the developer usually entailing requirements for bias, precision, total error goal, more. As such, LOQ can be essentially "chosen" by the developer if all these conditions are met but can only be equal to or greater than the LOD. Though there is no single definition for LOQ, the two more widely accepted definitions are from total error calculations from the Westgard model and Root Mean Square (RMS)/Variance model.[52]

The experimental design for LOQ involves testing over three days, four low level samples of known concentration (usually at varying levels near the LOQ goal), and three replicates per sample. Similar to before, experimental factors need to be increased to meet the minimum requirement of 36 total low level sample runs. Following the run of the above experiments, the average value and SD of each low-level sample across the replicates is done. Depending on which definition of LOQ is used, Westgard or RMS, the total error is calculated for each of the low-level samples. After removing the samples that do not meet the acceptable total error designated, the LOQ is the concentration of the lowest level sample of ones remaining.[52]

6 - CONCLUSION

Given the importance of detecting IEMs in infants to begin treatment as early as possible, an accurate detection is paramount. Currently, UOA analysis is one such diagnostic tool conducted on GC-EI-MS. With biochemical genetic labs often running tests on both LC and GC instruments, a shared machine would be highly beneficial to improve efficiency and save costs while maintaining accuracy and precision. The Waters' Xevo TQ-S mass spectrometer in tandem with the Agilent 7890 GC achieves this using the novel APCI technique allowing for a swappable LC-ESI and GC-APCI source. This thesis paper investigates this technique and its potential applications to the field of biochemical genetics. The oven method from the previous gold standard method was successfully transferred to the new machine along side many parameters being optimized as well such as corona current, cone voltage, and more. Initial patient samples tests have shown adequate separation with its added ability to isolate important isobaric metabolites. Extraction and derivatization optimization experiments determined that the current procedure did not need to be adjusted. In addition, research into alternative derivatives such as MTBSTFA showed the currently used BSTFA was more optimal. Sample stability tests showed that patient samples, QC solutions, and isolated extracted should only be stored in the fridge for one week before degradation begins. Wet and dry source conditions were used to explore the APCI's protonation ($[M+H]^+$) and charge transfer ($[M+^\circ]^+$) mechanism, respectively. Following the swapping from the LC to APGC mode on the instrument or opening of the source enclosure door, 8 hours was determined to be needed to allow source moisture levels to settle. The proton transfer mechanism in wet

mode induced by methanol or water modifiers was found to enhance peak height, signal-to-noise ratios, and therefore sensitivity. With this encouraging start, further research must be put into finalizing a UOA mass spectra reference library specific to APCI. Currently, 20 of 24 compounds of the MCA sample and 19 addition UOA are fully resolved with retention times and ion profiles. To further expand on this library in the future, a predictive model and equation was made to predict retention times on the new APCI machine from the previous gold standard. Though tropic acid as the current internal standard of chosen was suitable, research into deuterated forms of the metabolites such as D3 MMA show great merit in being introduced as addition internal standards as well. With the transition to MRMs, 28 metabolites were successfully advanced to this level. Calibration curves for the MCA compounds were made, 13 of which met adequate quantification levels. With clear avenues for future work, the project can easily be continued to develop a fully validated quantitative assay for UOA assay for IEM detection. Overall, the Waters' Xevo TQ-S micro with Agilent 7890 GC demonstrated promising GC-resolution separation of UOA metabolites using APCI-MS detection and is a great step towards expanding the field of biochemical genetic testing and improving newborn patient outcome.

7 - REFERENCES

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8 - APPENDIX

8.1 – For Section 4.6 - Wet and Dry Source Enclosure Optimization

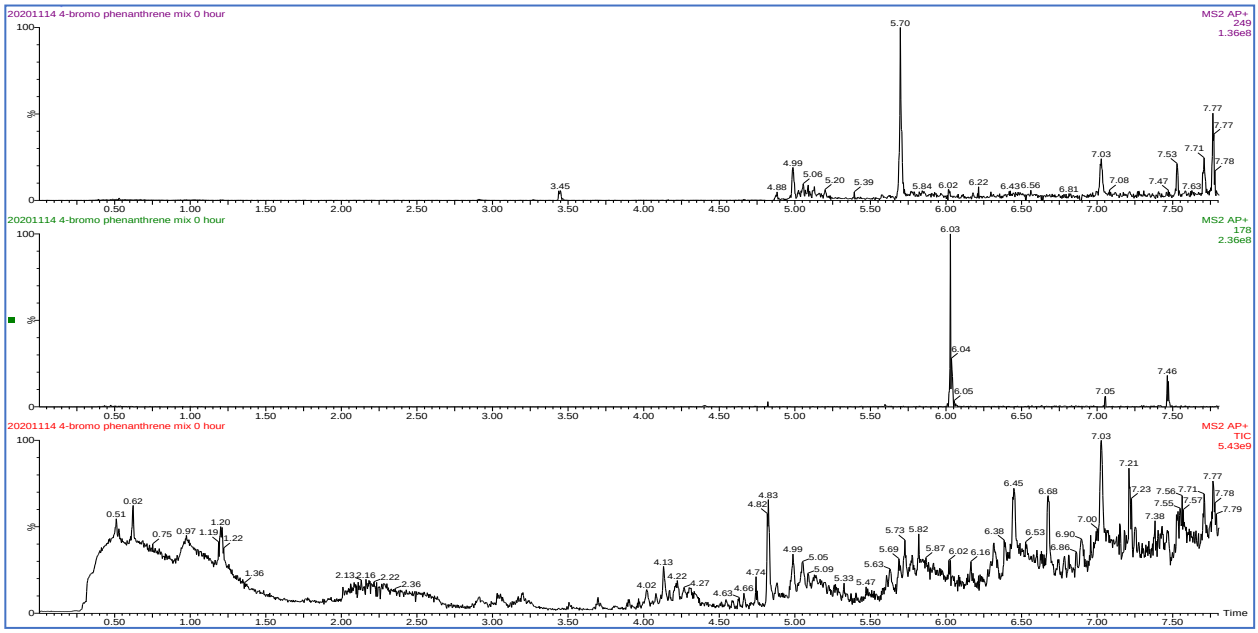

Figure 23 - Chromatogram of wet/dry tests determining the retention times of phenanthrene (top), BPPE (middle), and full scan (bottom).

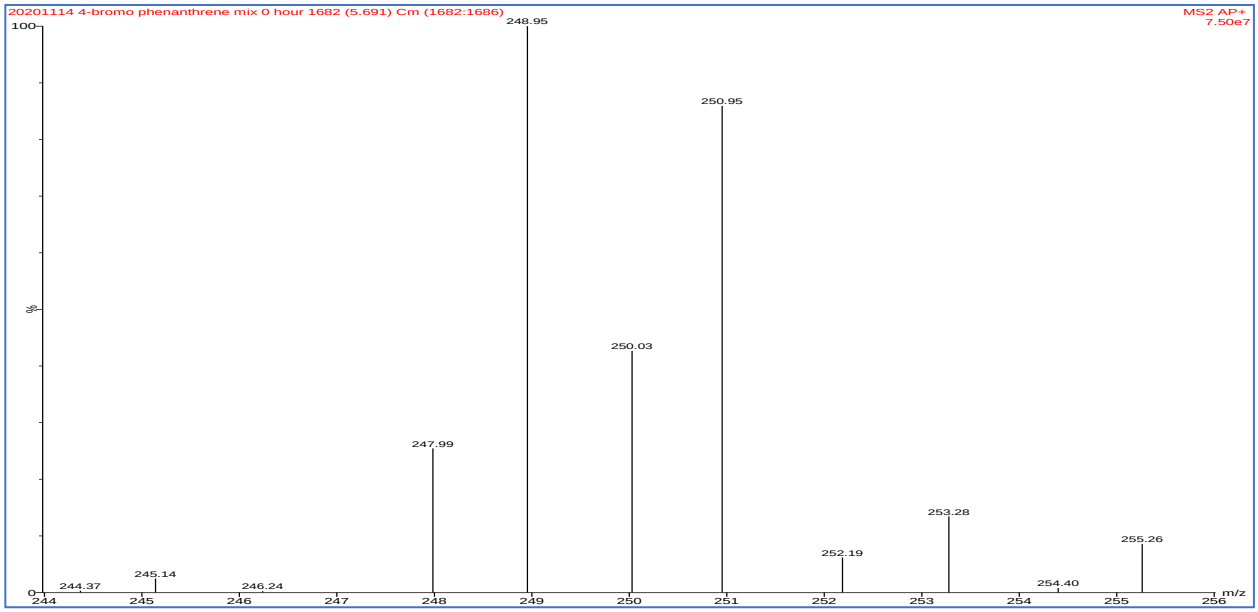

Figure 24 - Mass spectrum of BPPE ion ratios post zero hours of opening source enclosure.

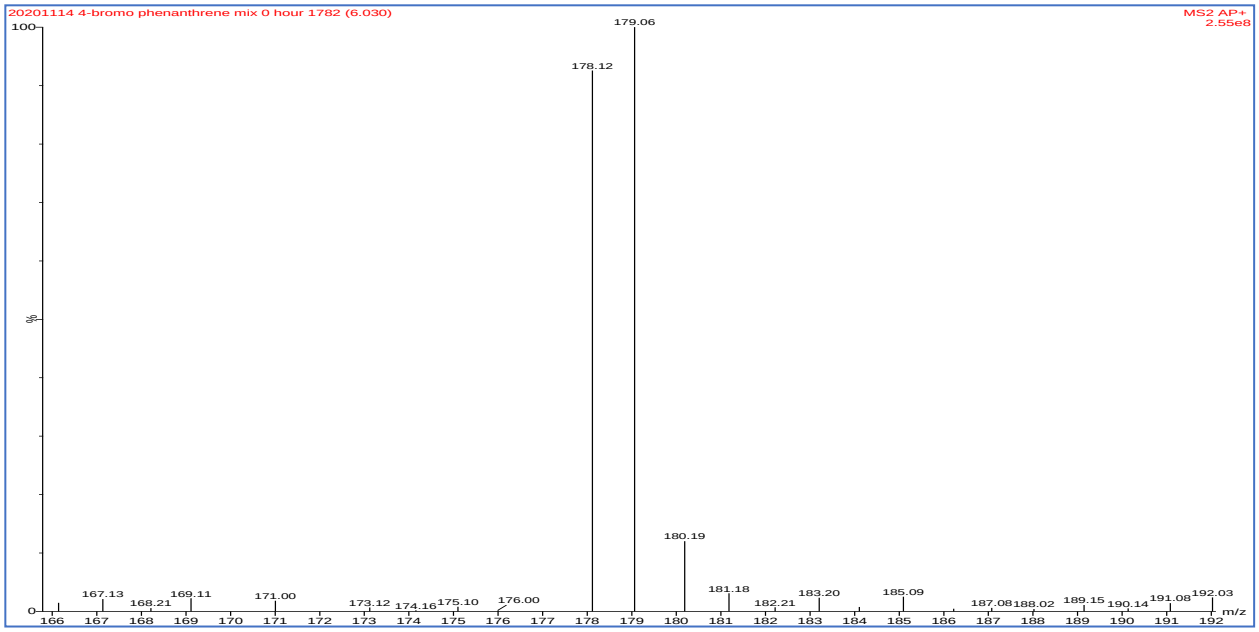

Figure 25 - Mass spectrum of phenanthrene ion ratios post zero hours of opening source enclosure.

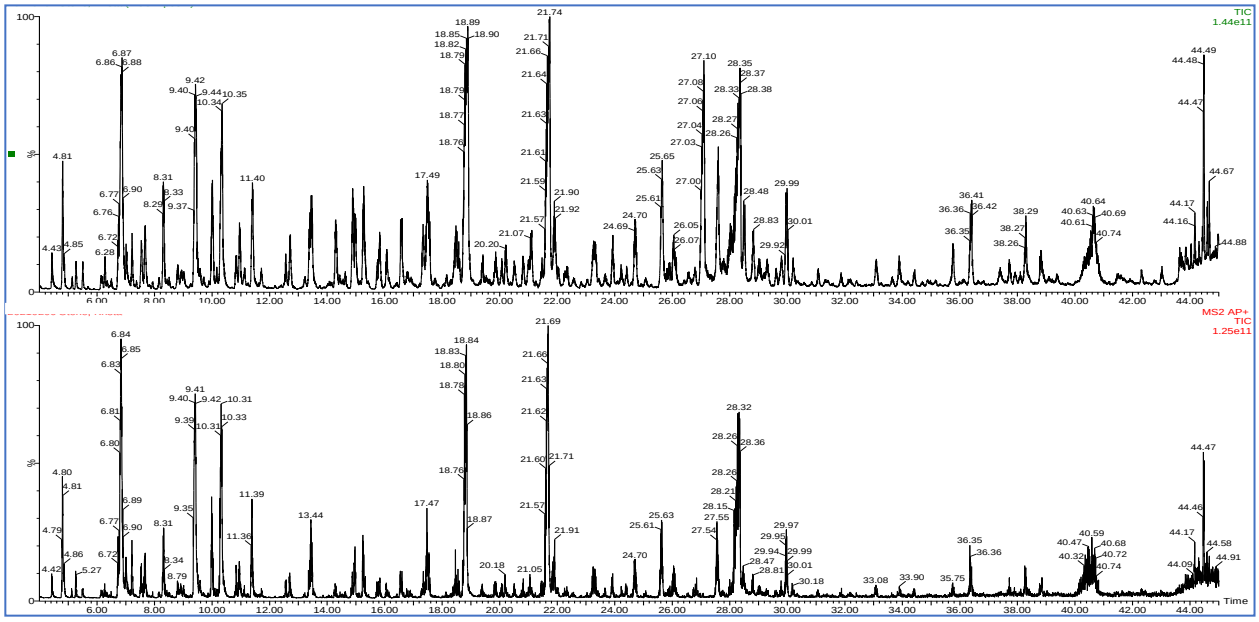

Figure 26 - Comparison of dry mode (bottom) and wet mode (top) on patient samples. Wet mode was induced using a methanol vial modifier.

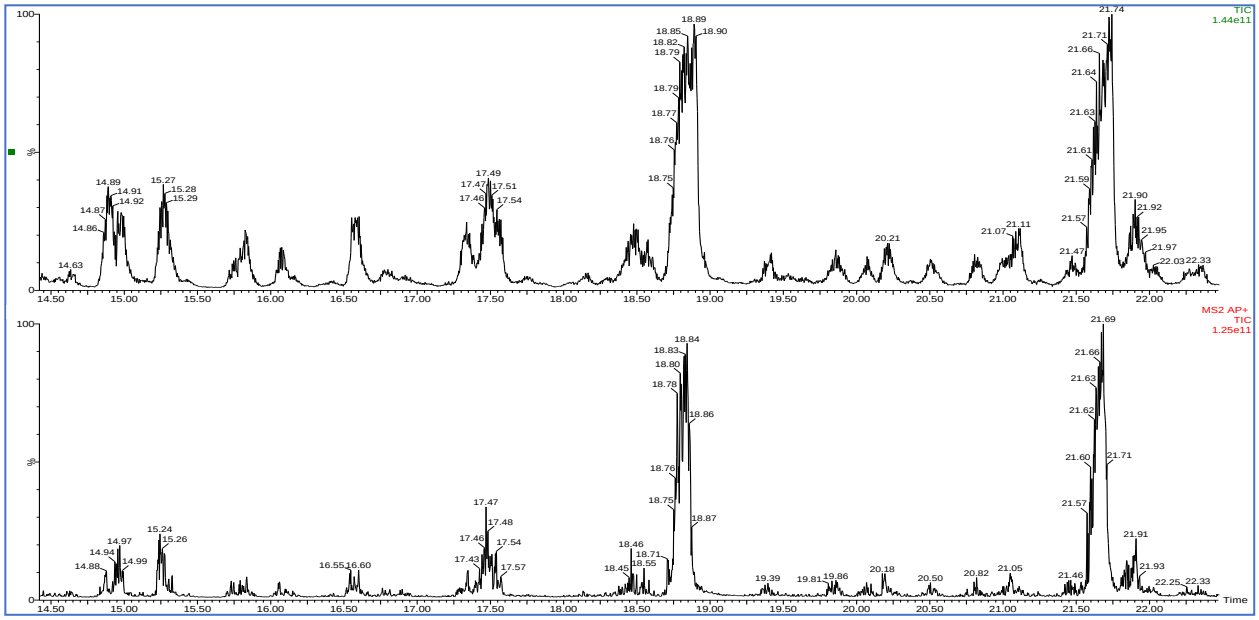

Figure 27 - Zoomed comparison of dry mode (bottom) and wet mode (top) on patient samples. Wet mode was induced using a methanol vial modifier.

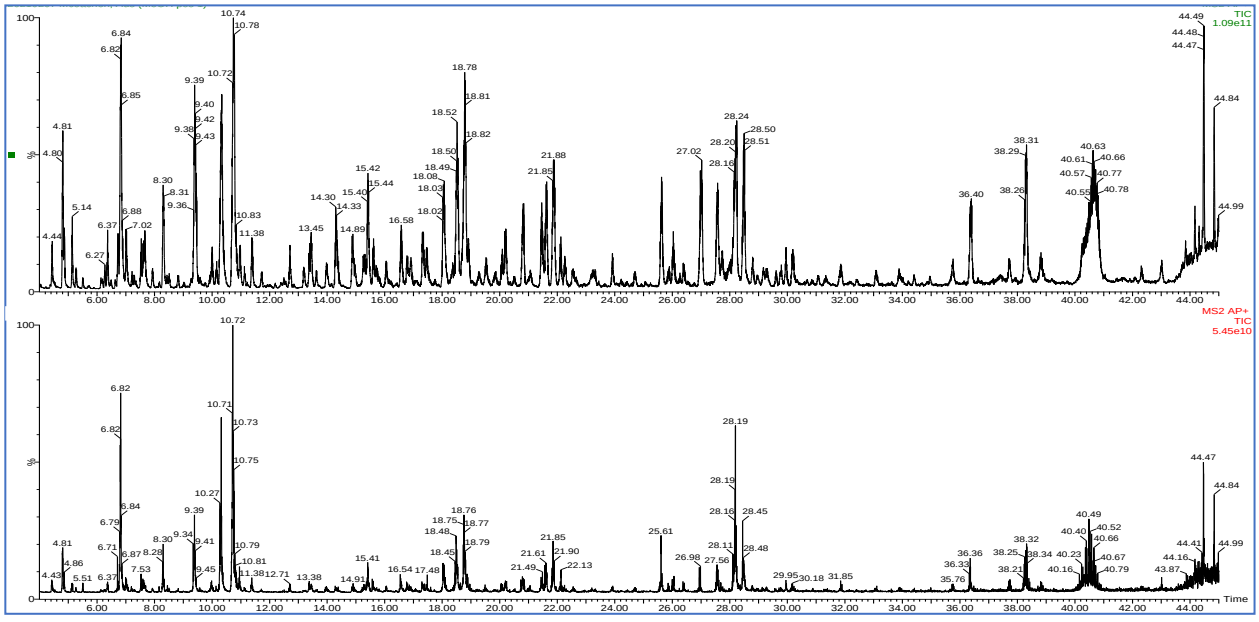

Figure 28 - Comparison of dry mode (bottom) and wet mode (top) on patient samples. Wet mode was induced using a methanol vial modifier.

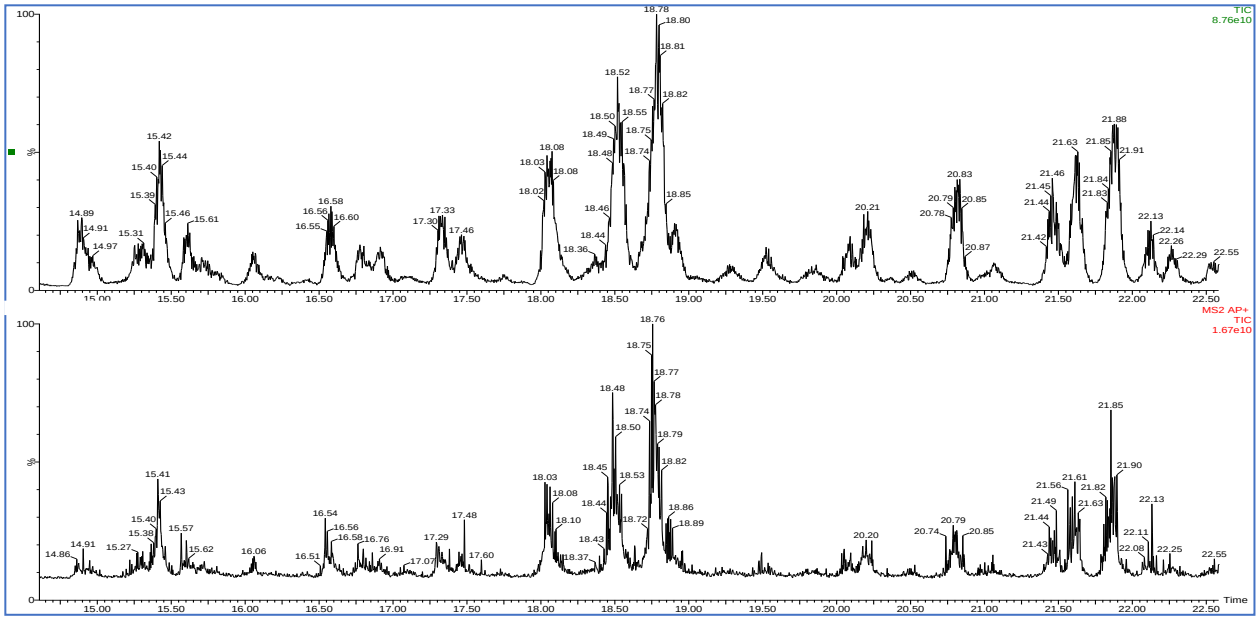

Figure 29 - Zoomed comparison of dry mode (bottom) and wet mode (top) on patient samples. Wet mode was induced using a methanol vial modifier.

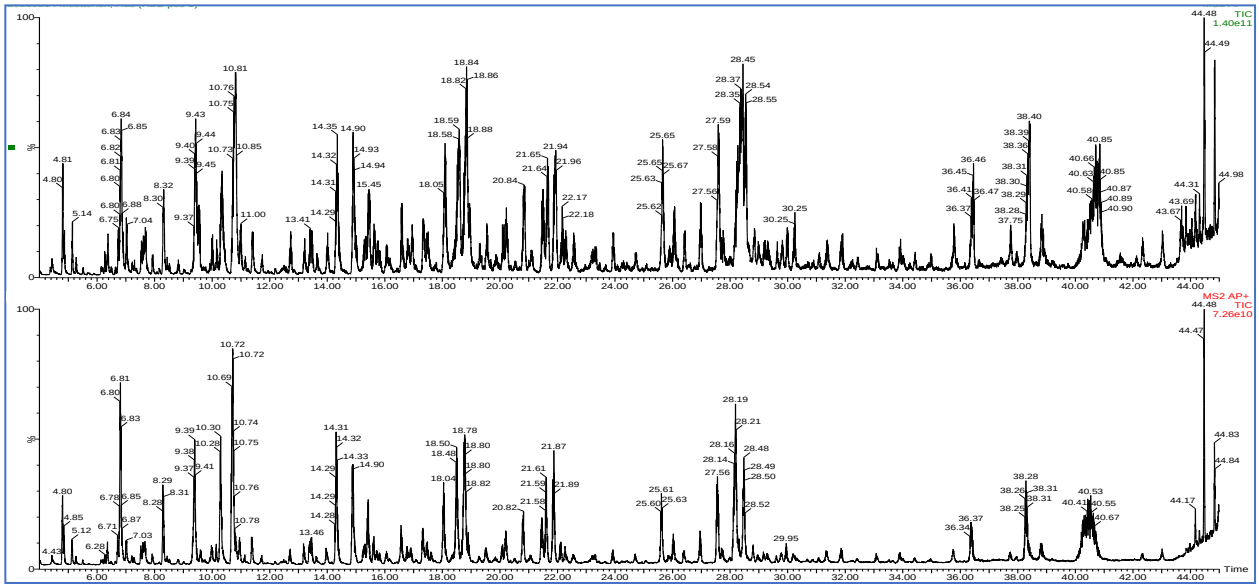

Figure 30 - Comparison of wet mode induced by methanol (bottom) and water (top) on patient samples.

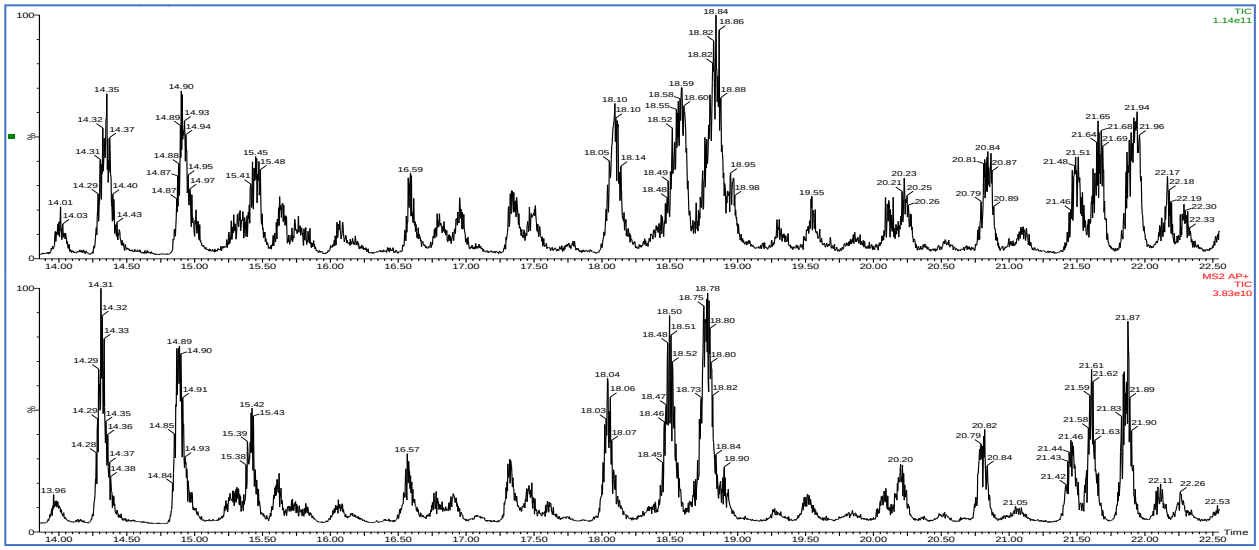

Figure 31 - Zoomed comparison of wet mode induced by methanol (bottom) and water (top) on patient samples.

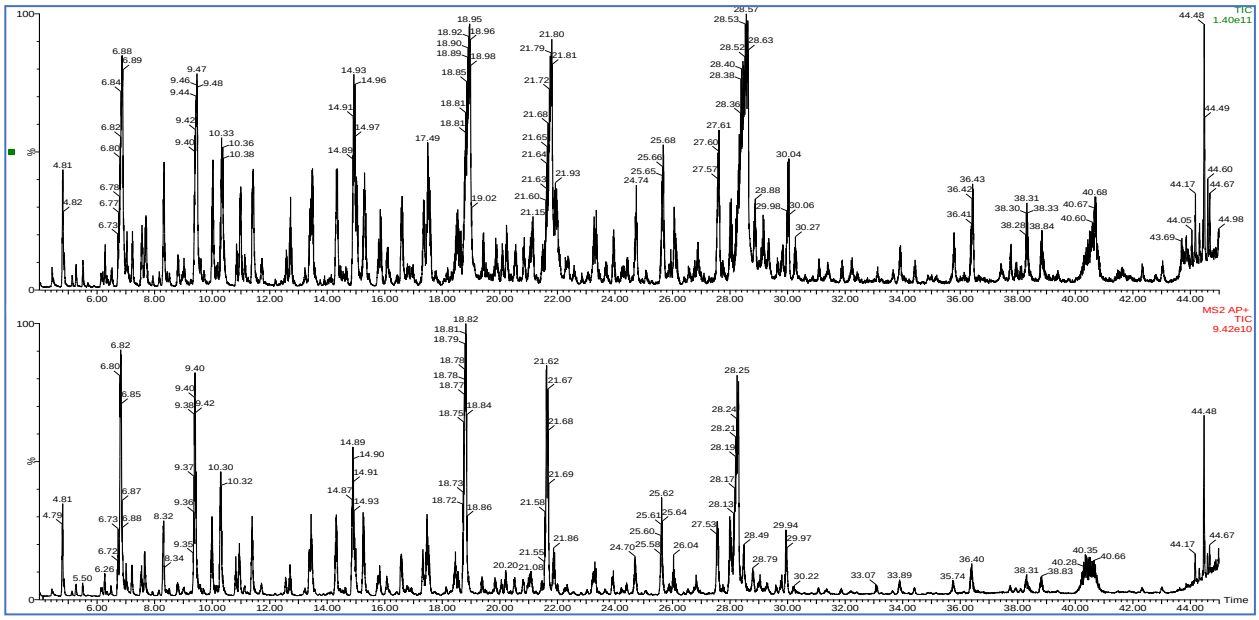

Figure 32 - Comparison of wet mode induced by methanol (bottom) and water (top) on patient samples.

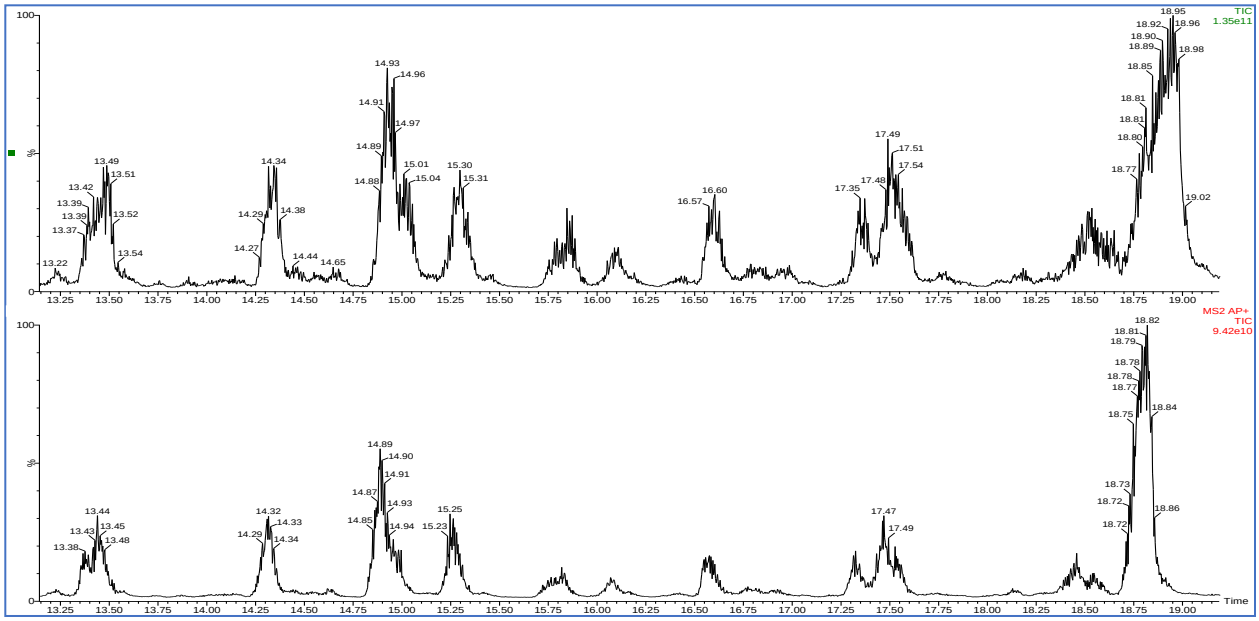

Figure 33 - Zoomed of wet mode induced by methanol (bottom) and water (top) on patient samples.

8.2 – For Section 4.7 - Isolated Compound Extractions

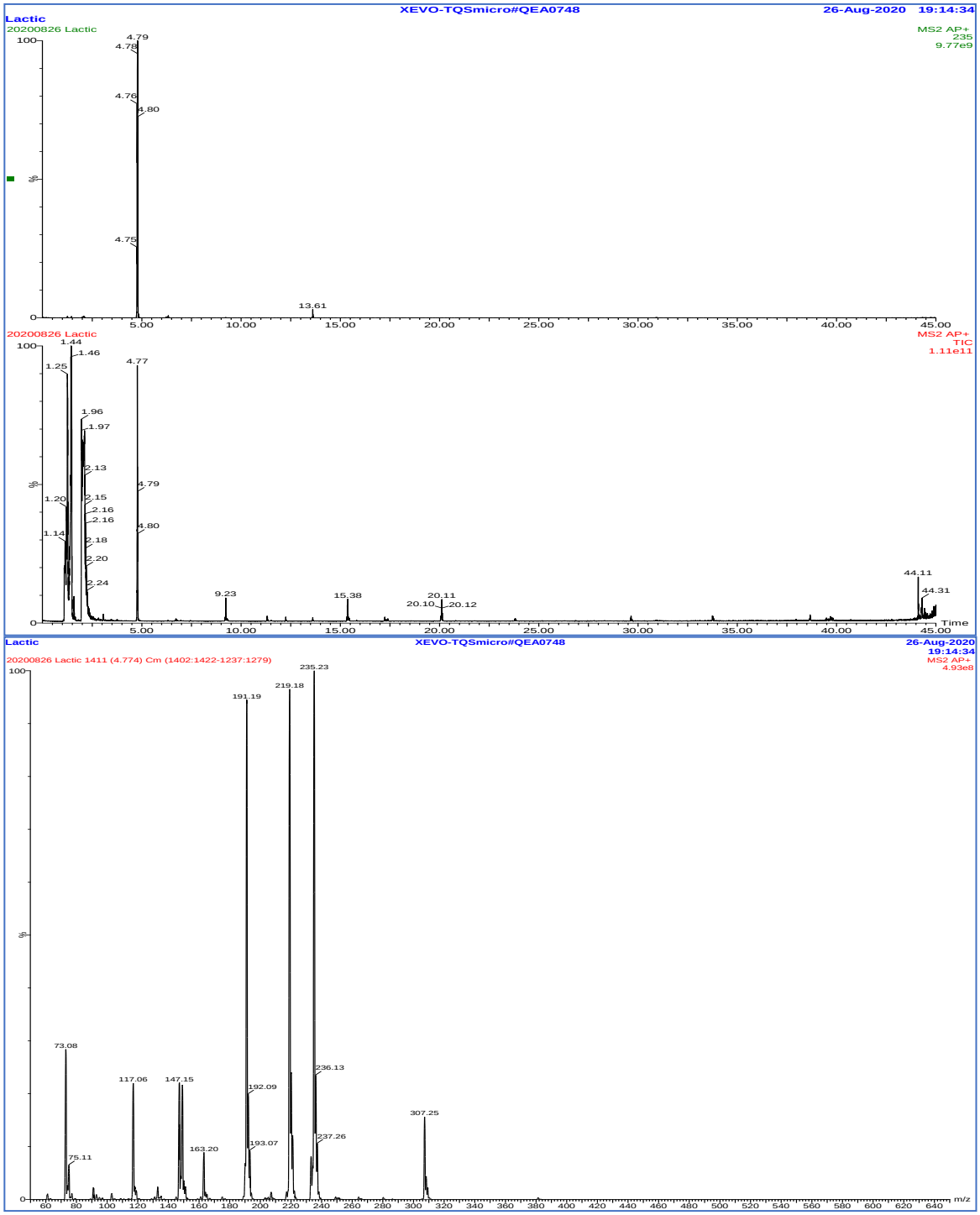

Figure 34 - Isolated extraction of lactic acid showing chromatogram (above) and ion profile (below)

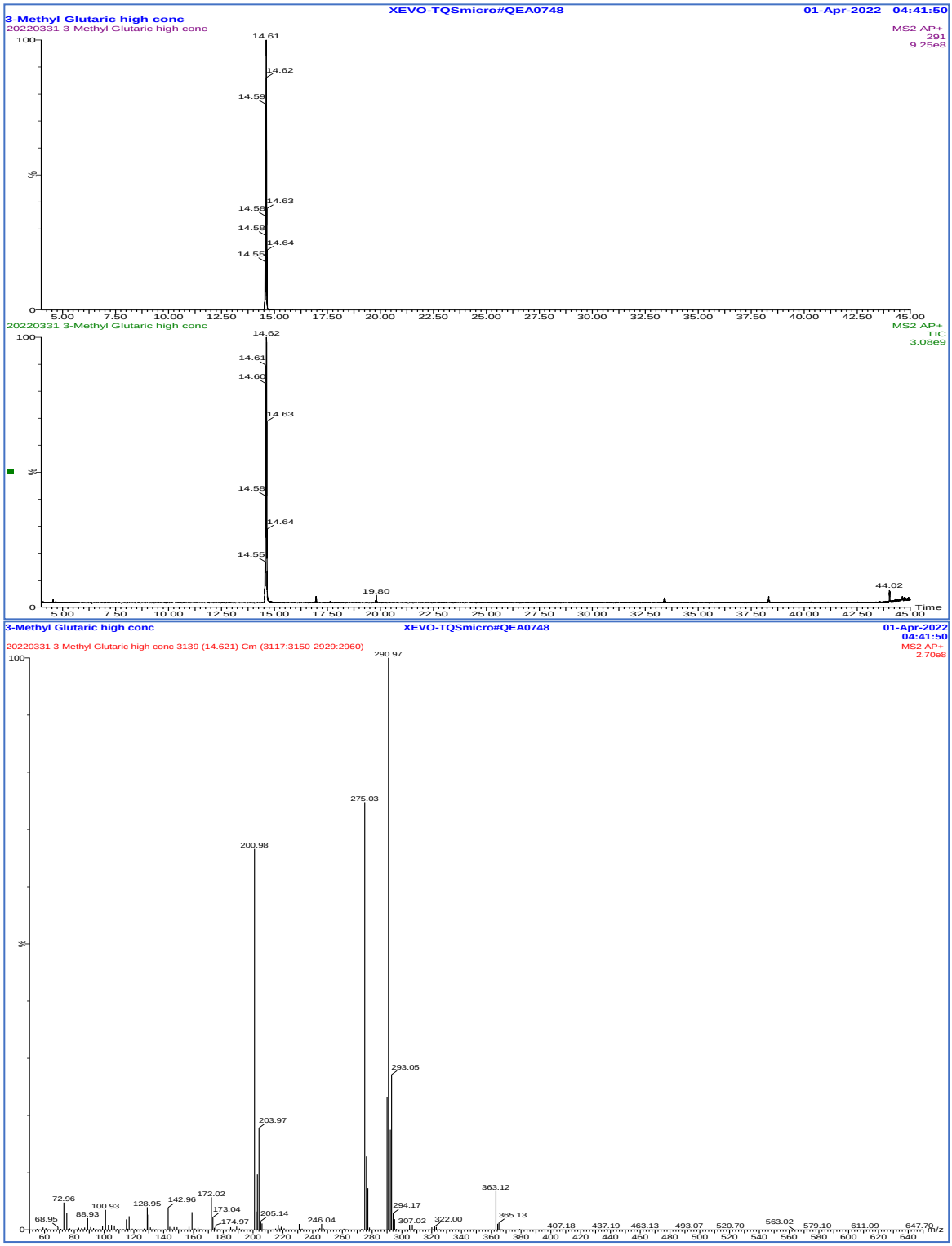

Figure 35 - Isolated extraction of 3-methyl glutaric acid showing chromatogram (above) and ion profile (below)

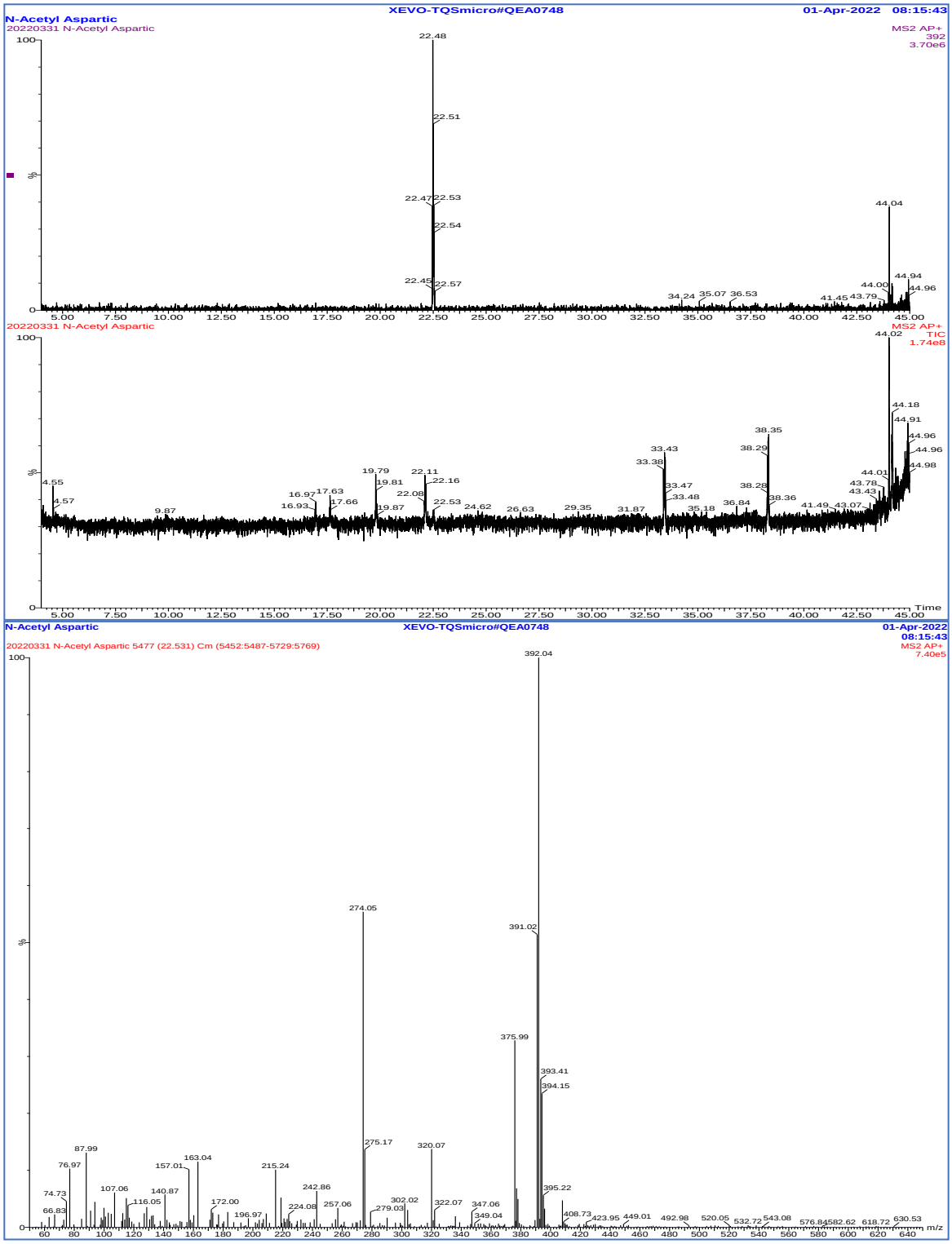

Figure 36 - Isolated extraction of N-acetyl aspartic acid TMS3 showing chromatogram (above) and ion profile (below)

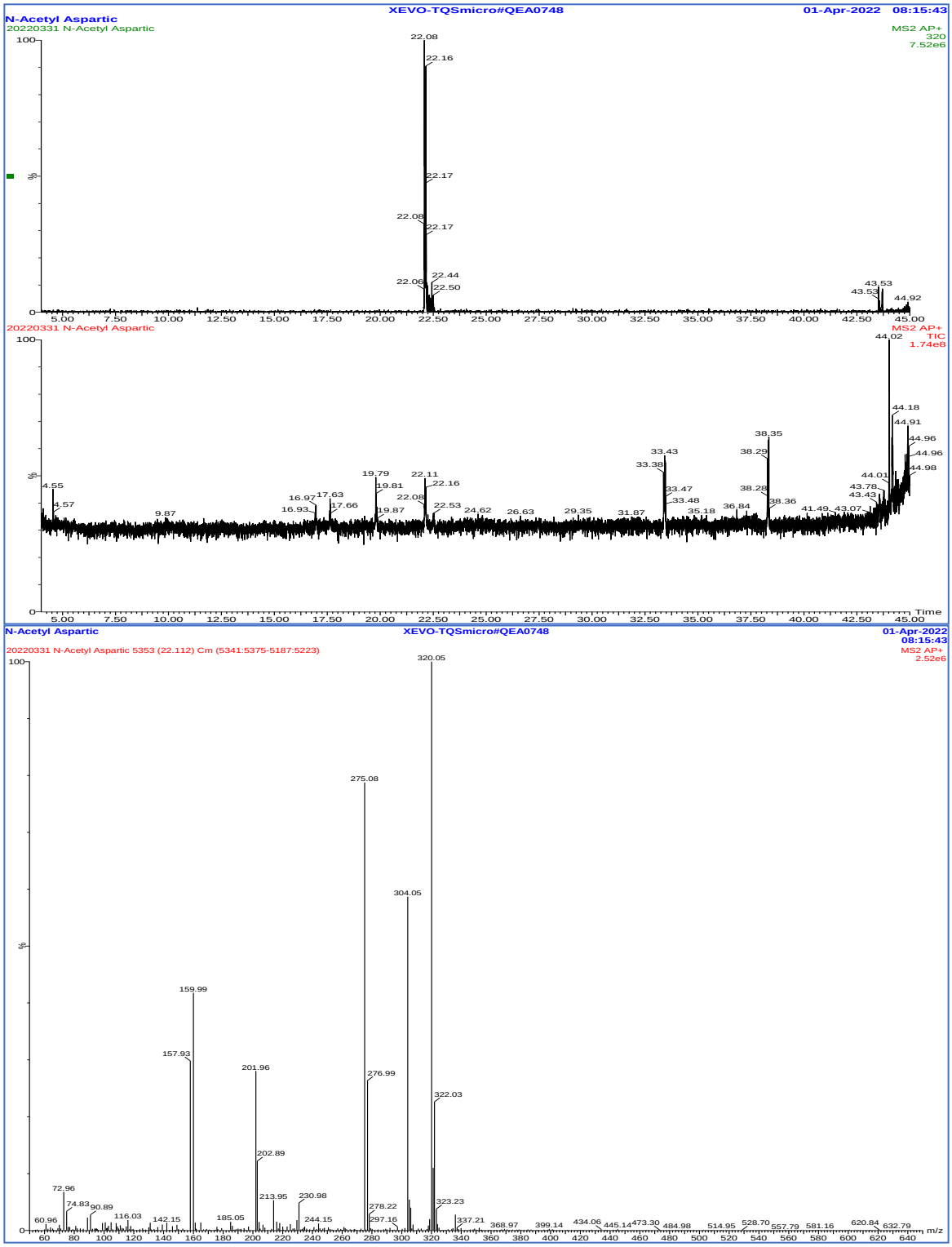

Figure 37 - Isolated extraction of N-acetyl aspartic acid TMS2 showing chromatogram (above) and ion profile (below)

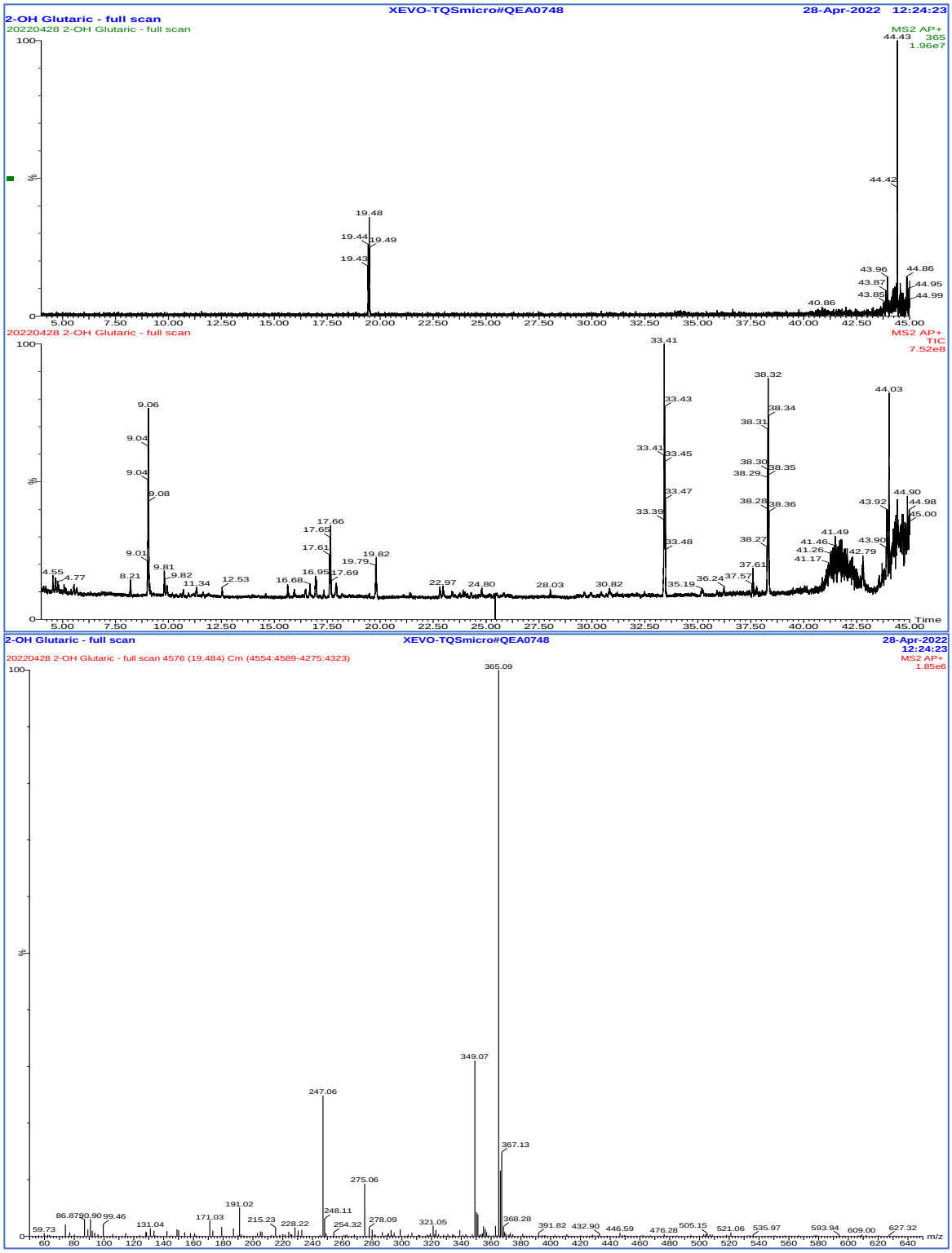

Figure 38 - Isolated extraction of 2-OH glutaric acid showing chromatogram (above) and ion profile (below)

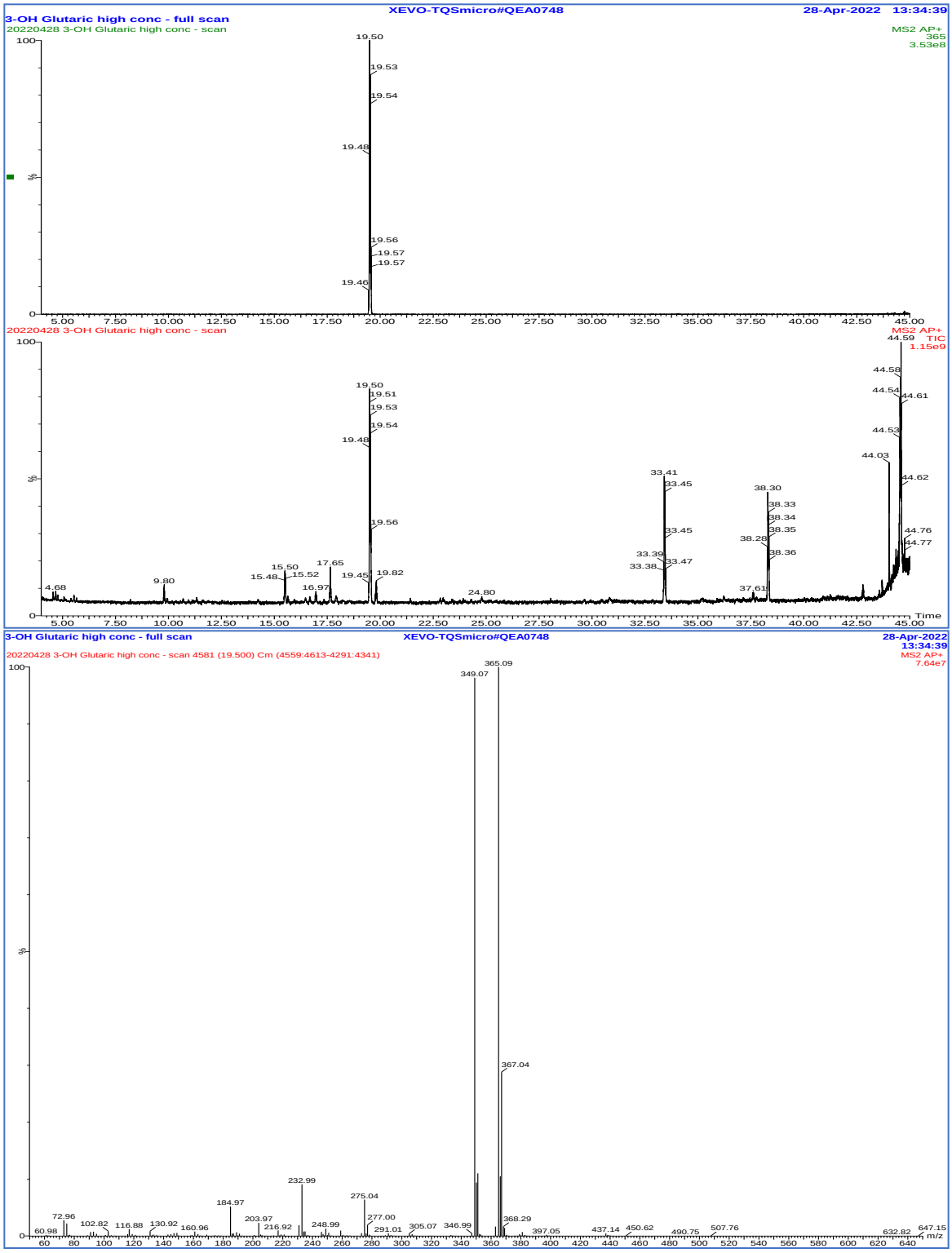

Figure 39 - Isolated extraction of 3-Oh glutaric acid showing chromatogram (above) and ion profile (below)

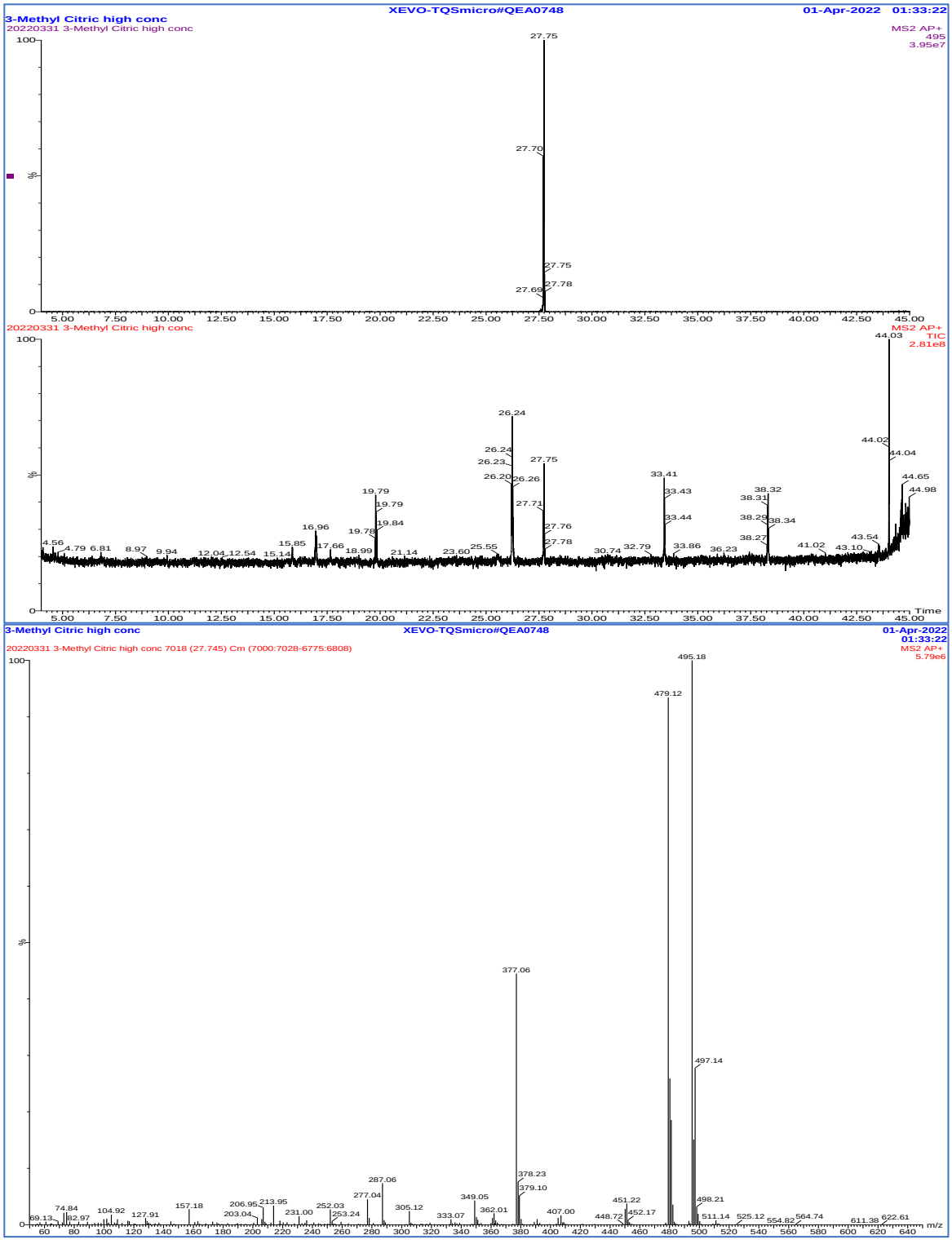

Figure 40 - Isolated extraction of 3-methyl citric acid TMS3 showing chromatogram (above) and ion profile (below)

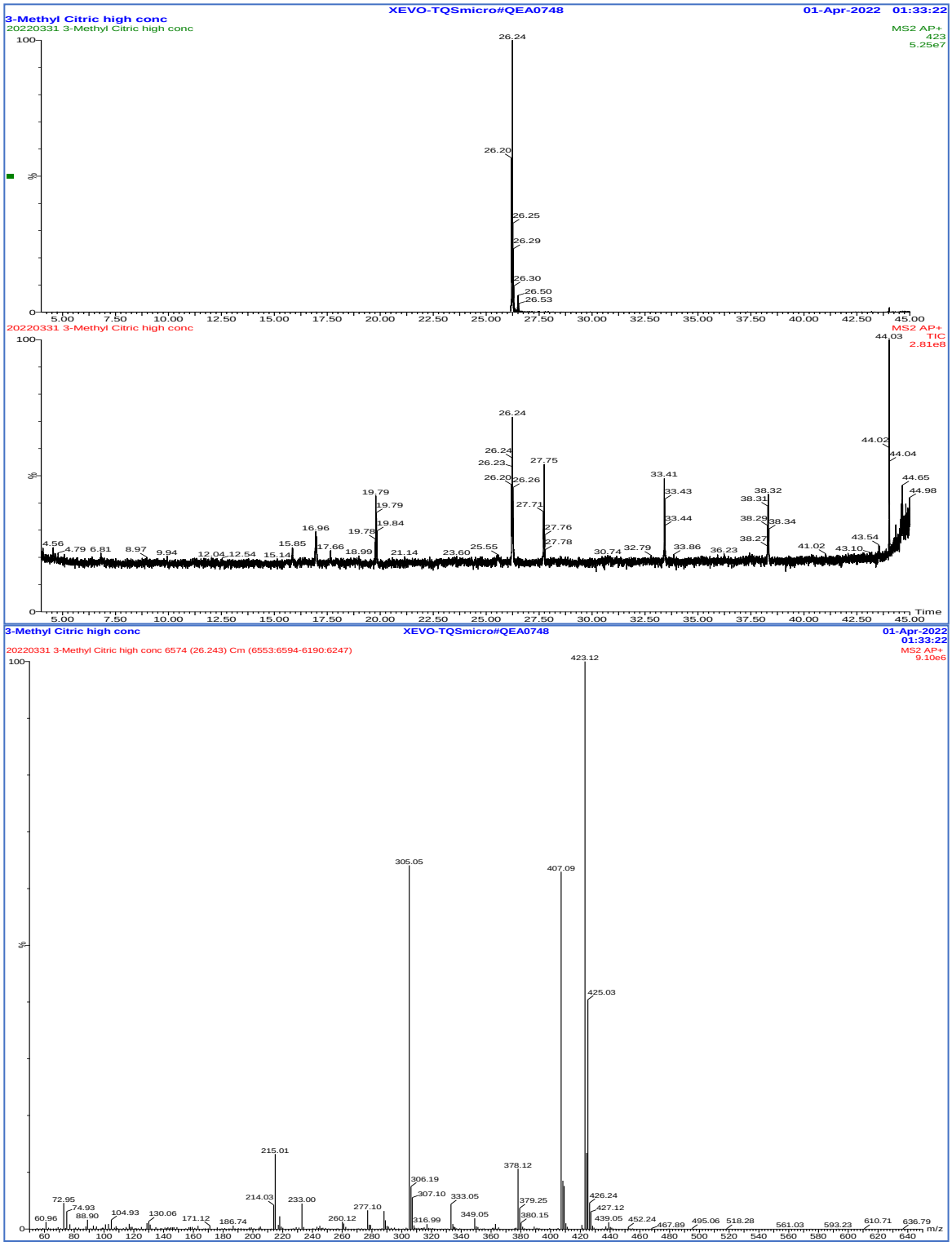

Figure 41 - Isolated extraction of citric acid TMS3 showing chromatogram (above) and ion profile (below)

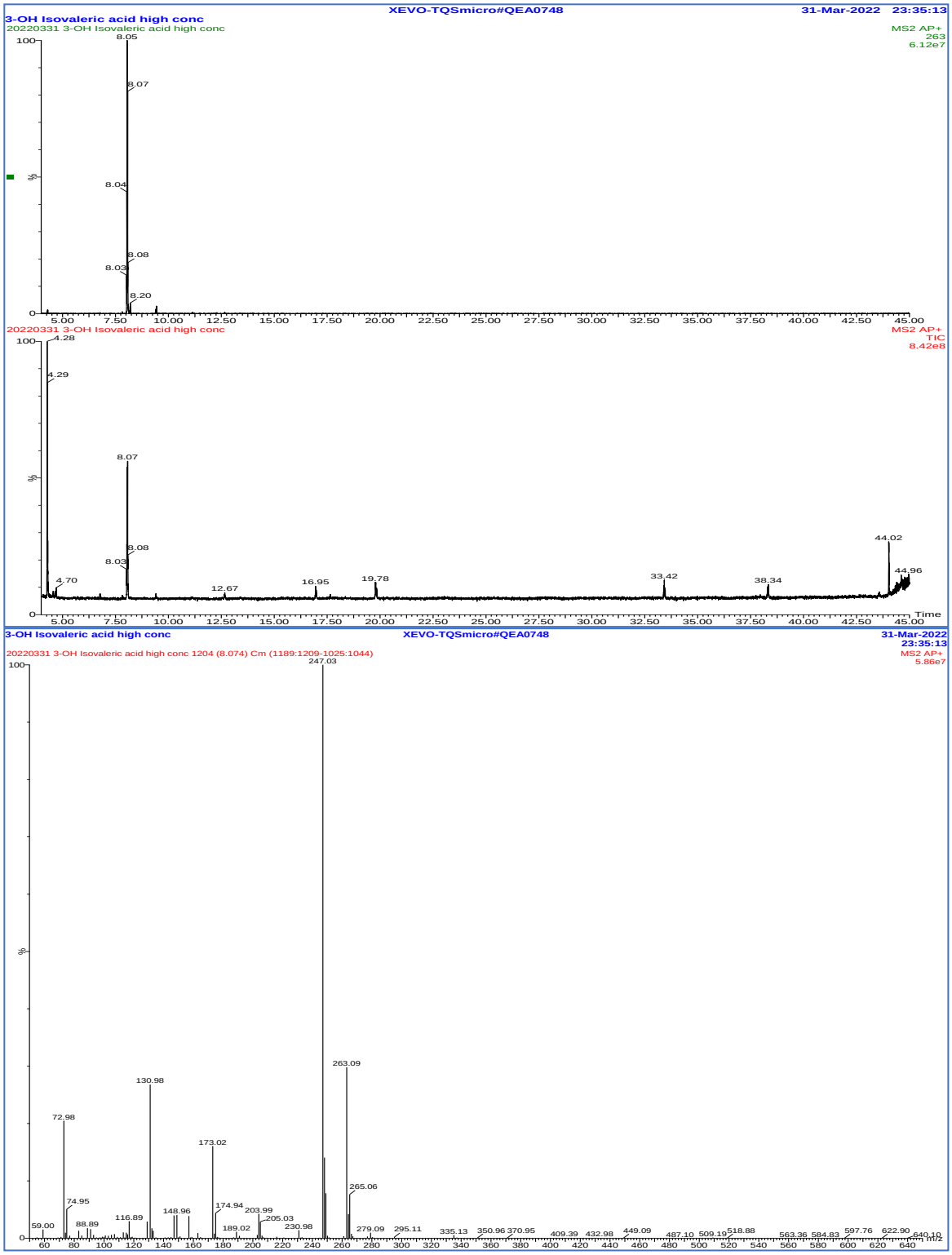

Figure 42 - Isolated extraction of 3-OH isovaleric acid showing chromatogram (above) and ion profile (below)

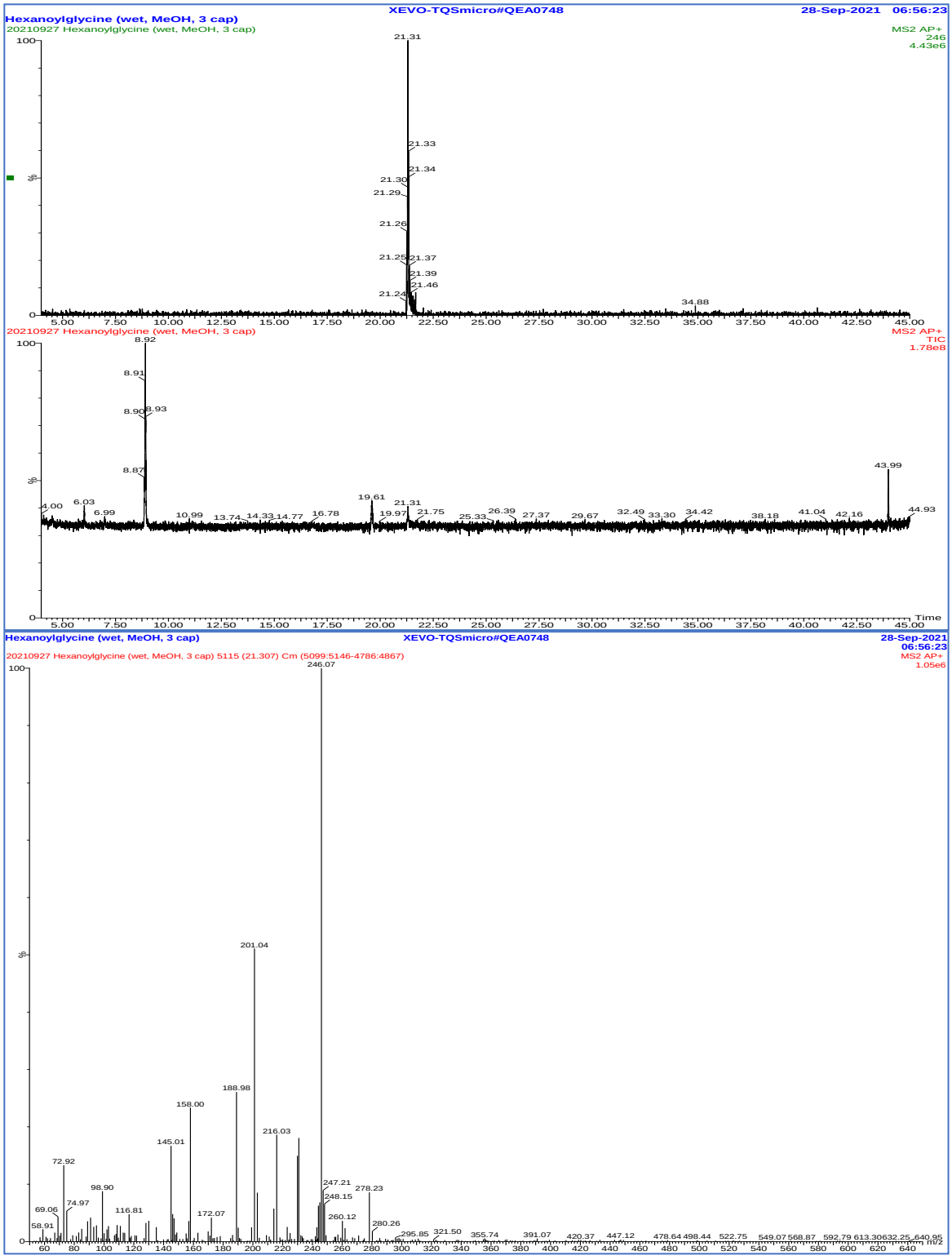

Figure 43 - Isolated extraction of hexanoylglycine TMS2 showing chromatogram (above) and ion profile (below)

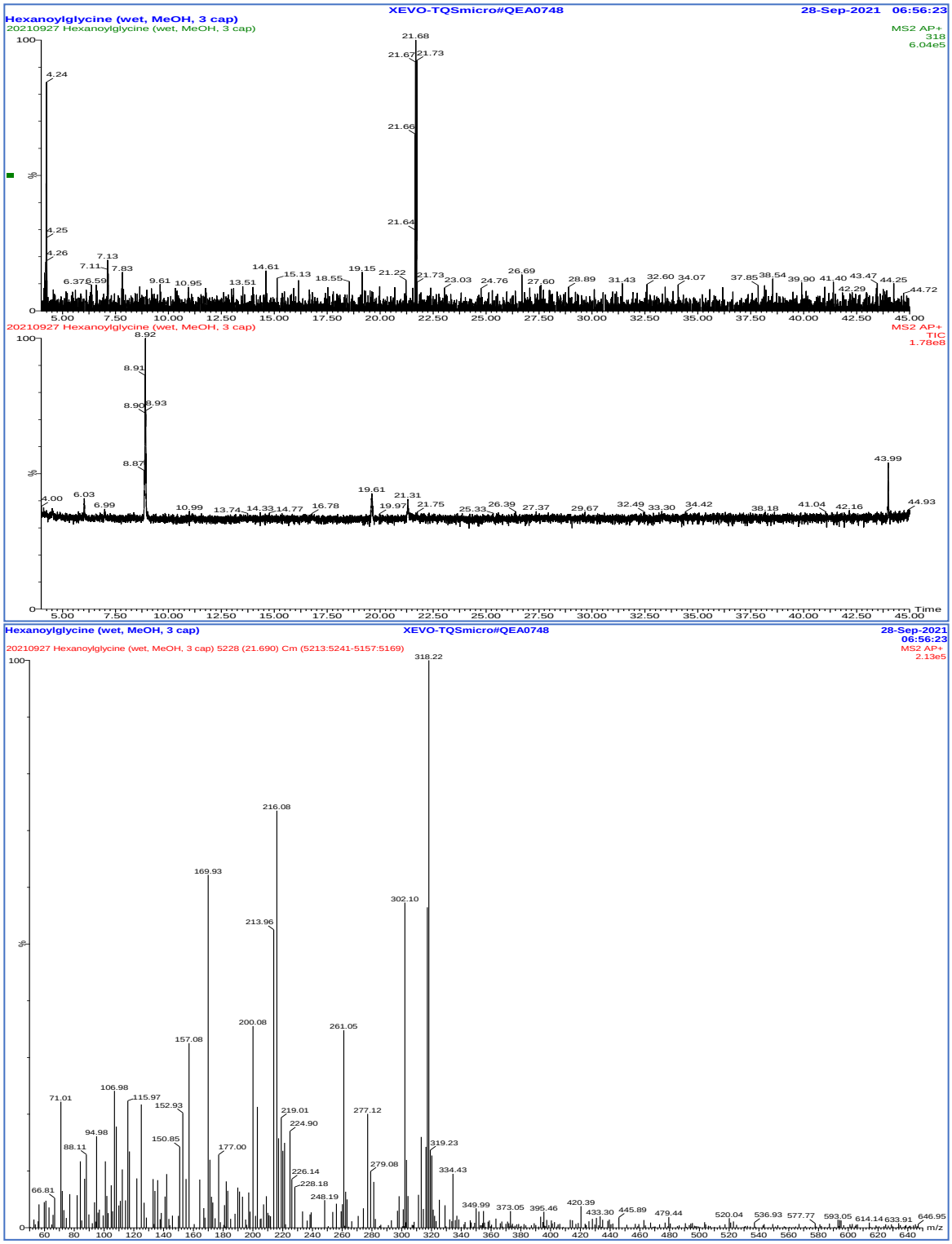

Figure 44 - Isolated extraction of hexanoylglycine TMS3 showing chromatogram (above) and ion profile (below)

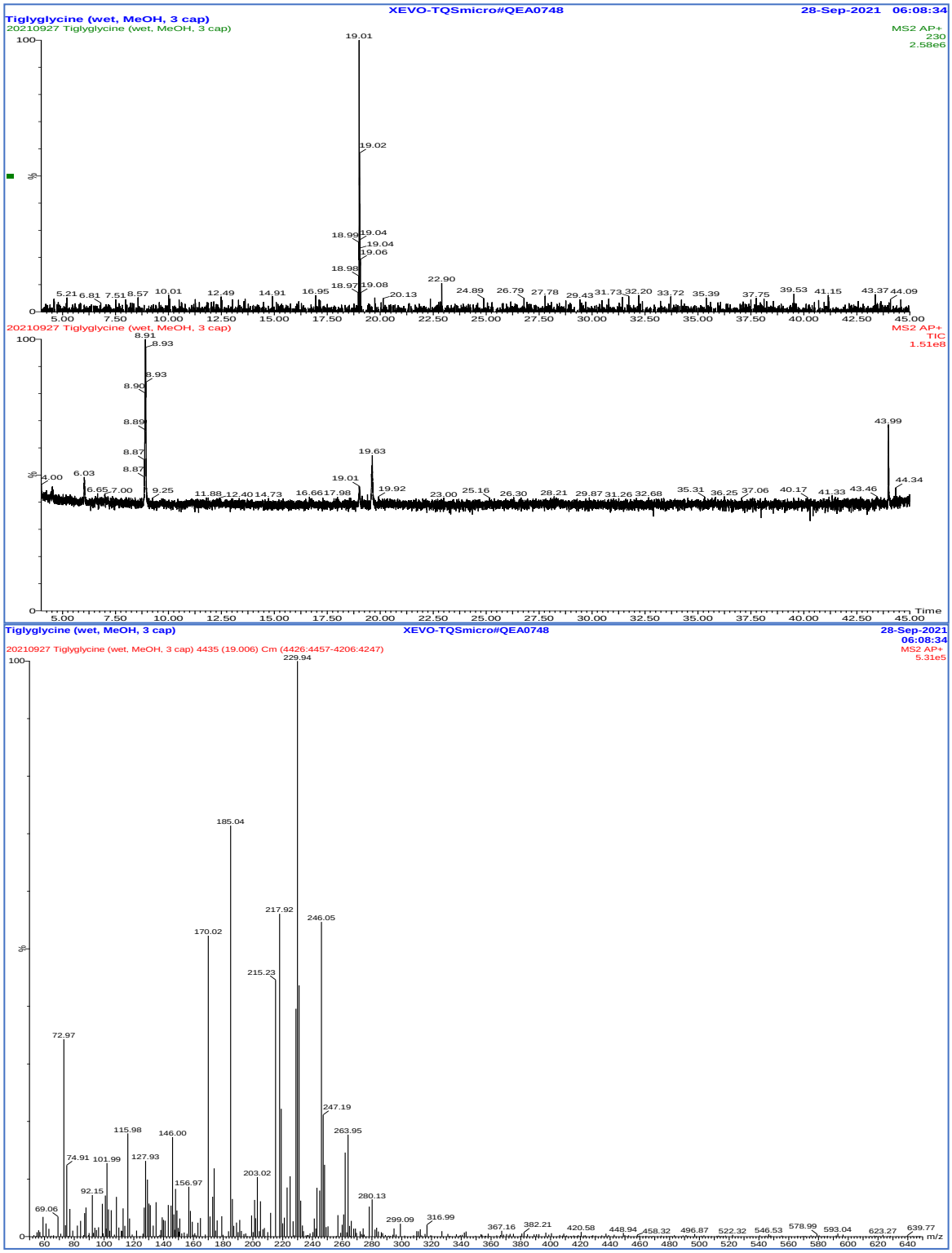

Figure 45 - Isolated extraction of tiglyglycine showing chromatogram (above) and ion profile (below)

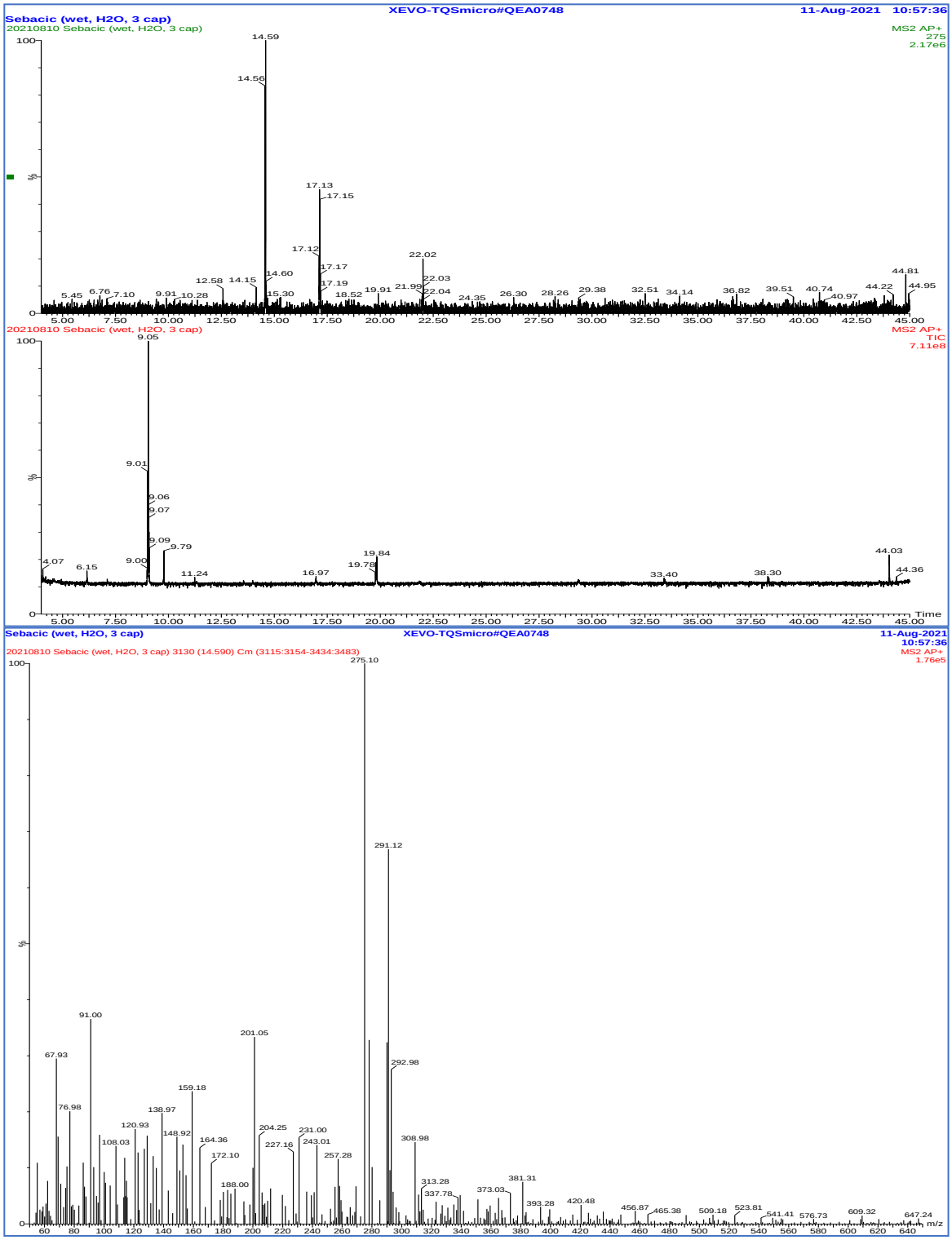

Figure 46 - Isolated extraction of sebacic acid TMS2 showing chromatogram (above) and ion profile (below)

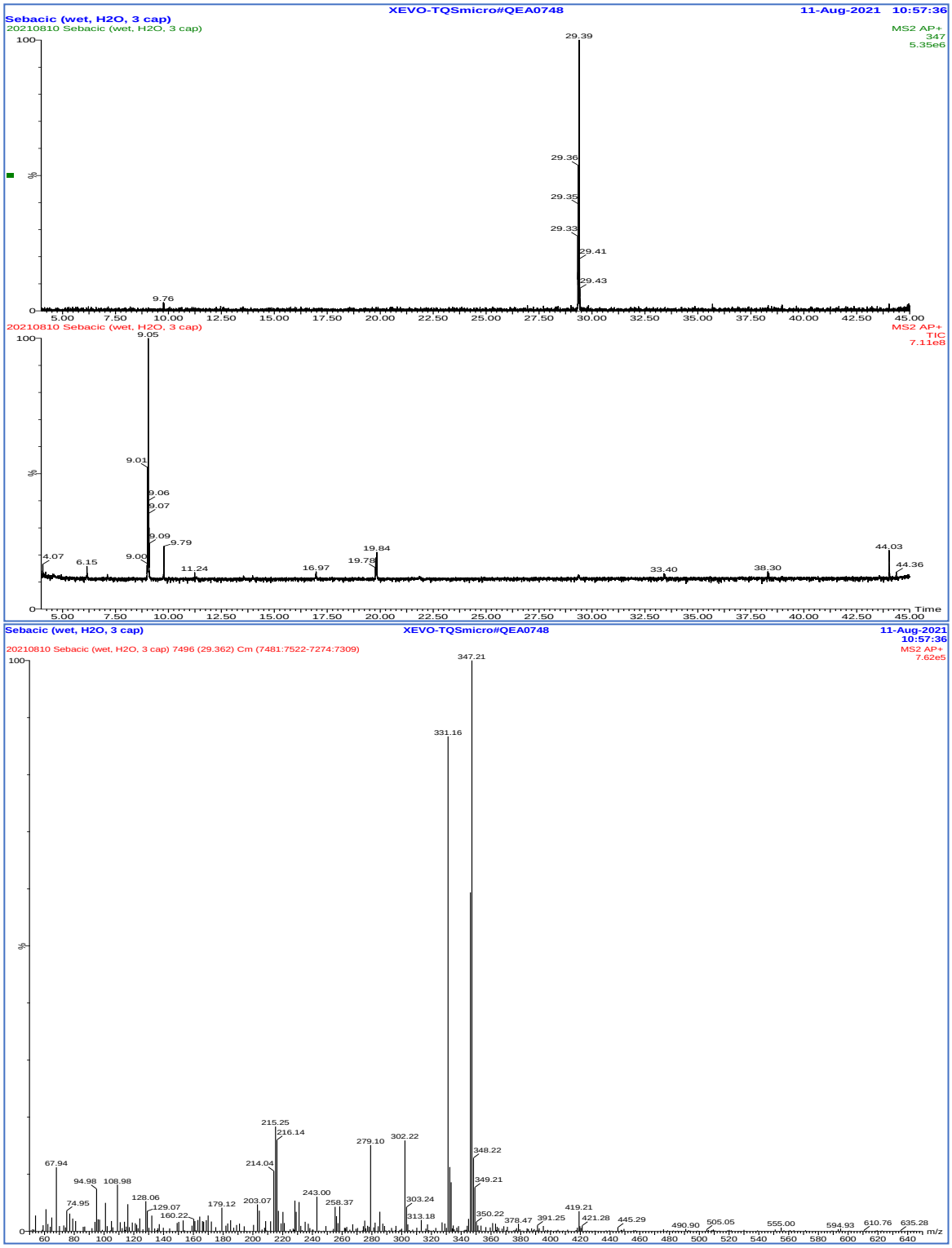

Figure 47 - Isolated extraction of sebacic acid TMS3 showing chromatogram (above) and ion profile (below)

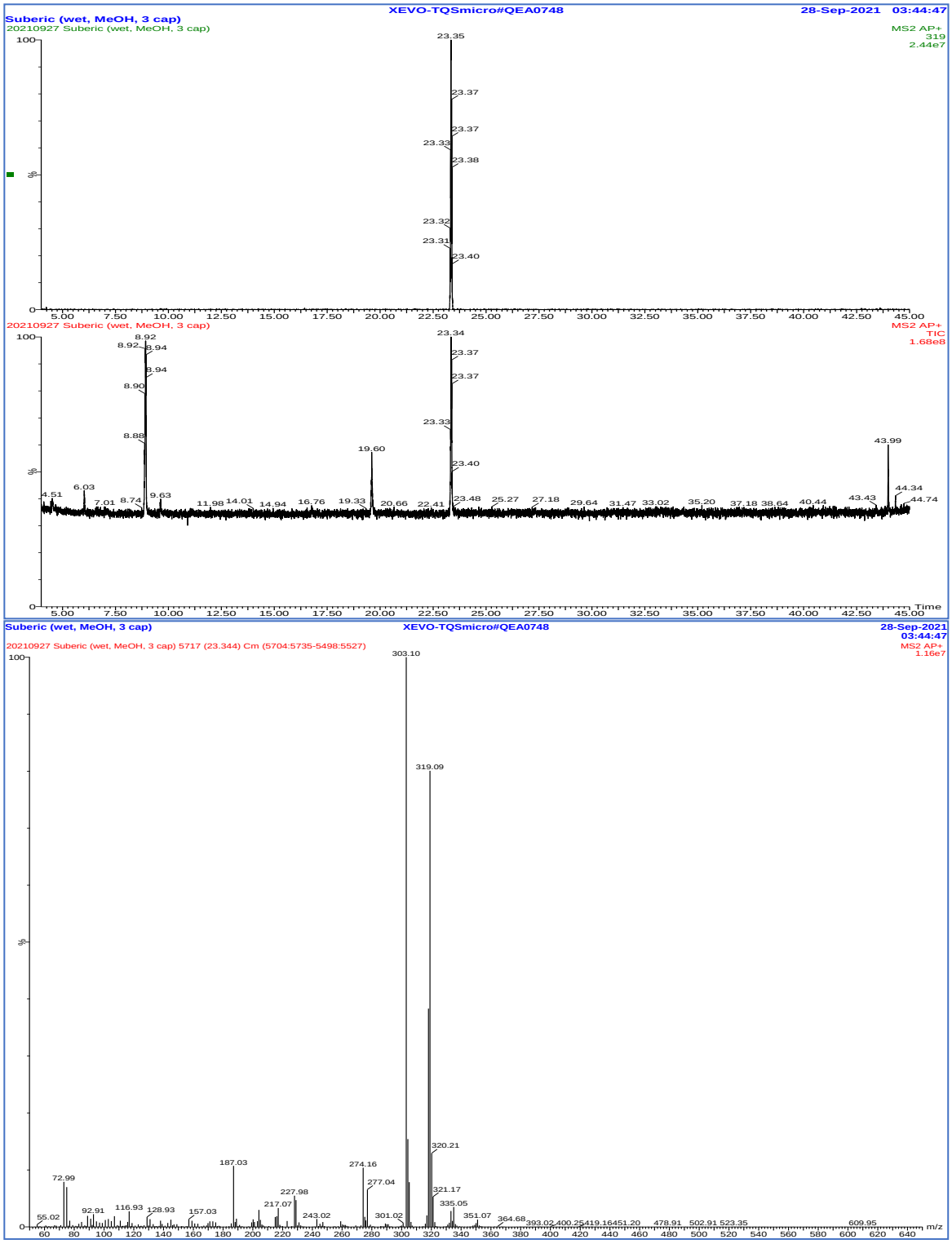

Figure 48 - Isolated extraction of suberic acid showing chromatogram (above) and ion profile (below)

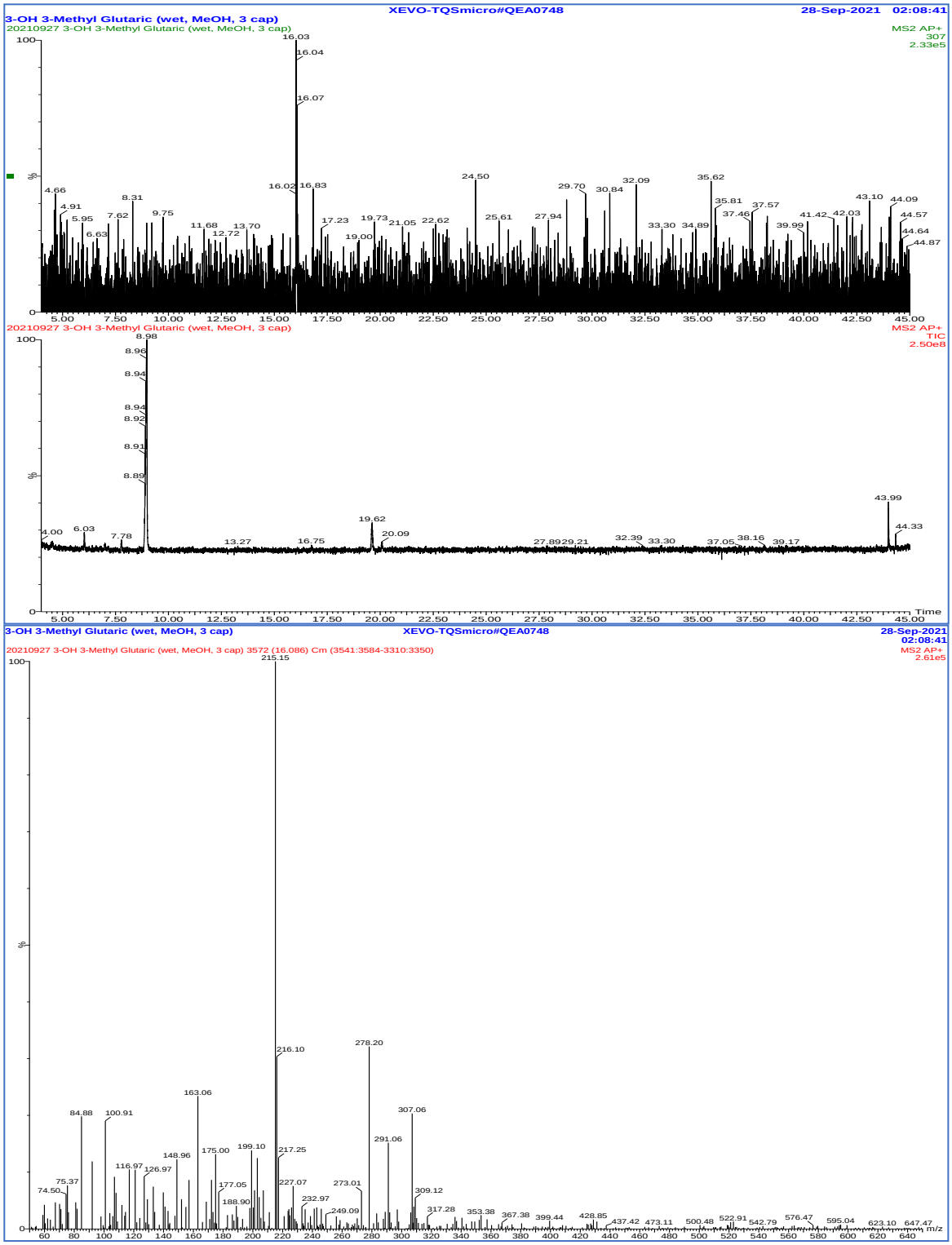

Figure 49 - Isolated extraction of 3-methyl glutaric acid showing chromatogram (above) and ion profile (below)

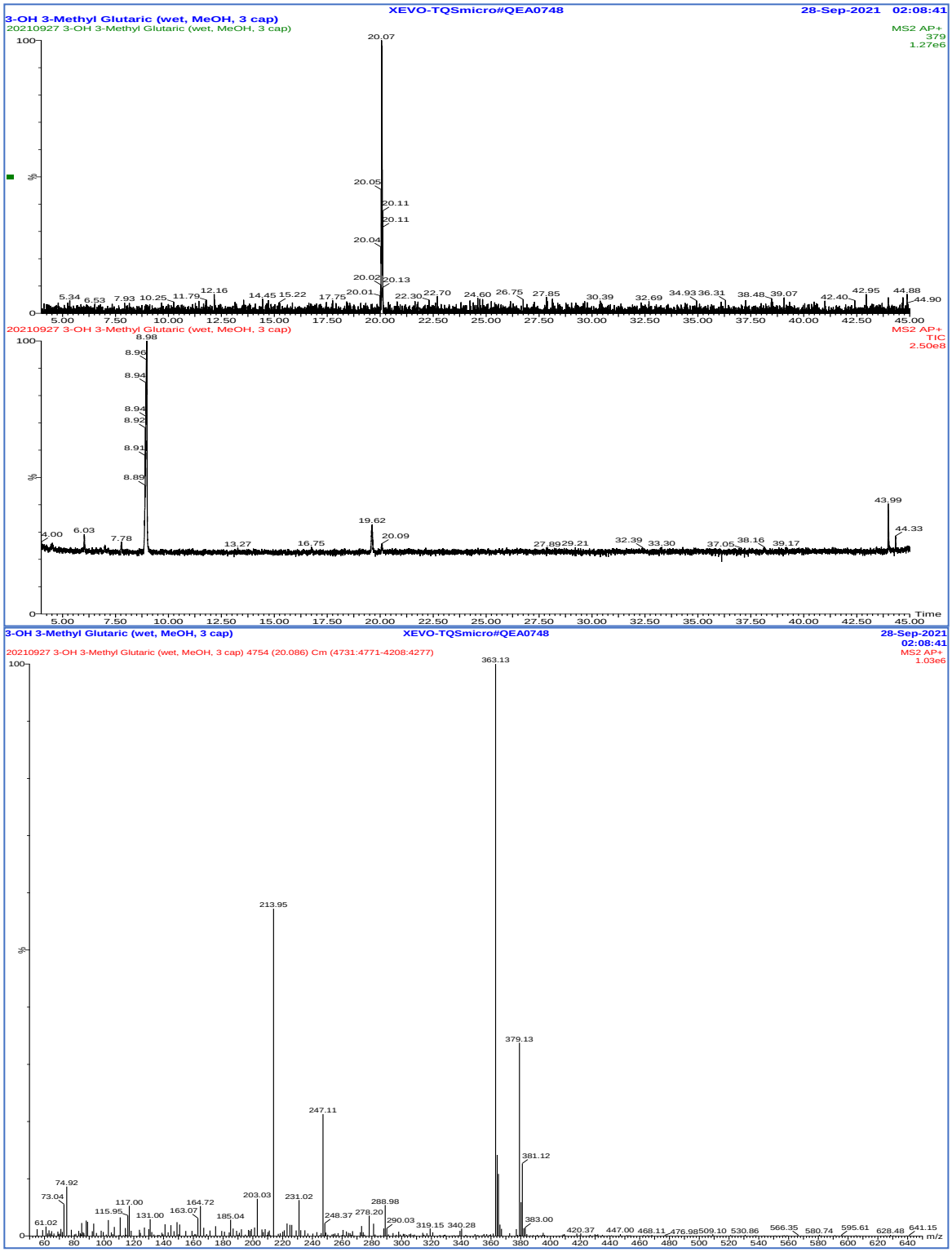

Figure 50 - Isolated extraction of 3-OH-3-methyl glutaric acid TMS3 showing chromatogram (above) and ion profile (below)

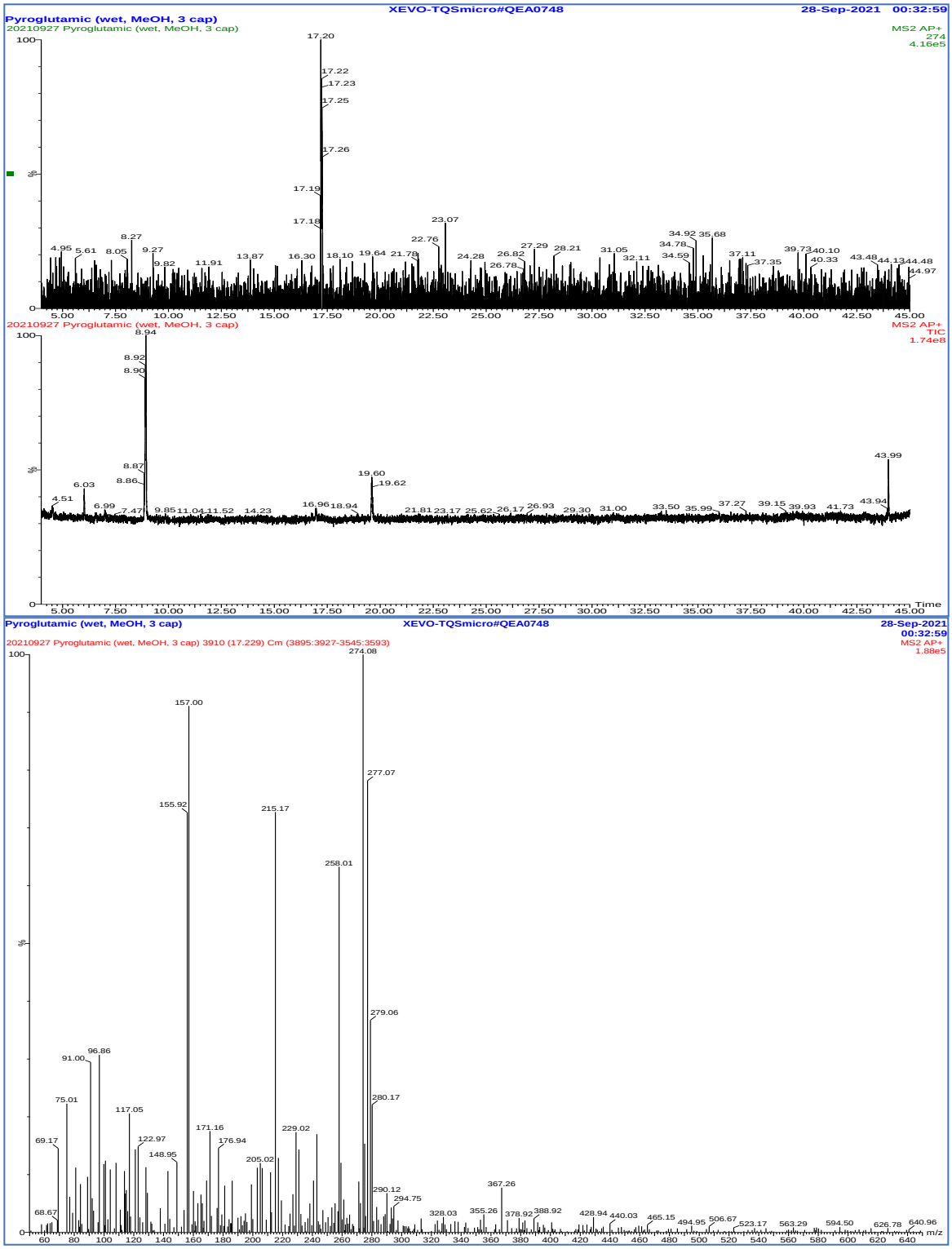

Figure 51 - Isolated extraction of pyroglutamic acid showing chromatogram (above) and ion profile (below)

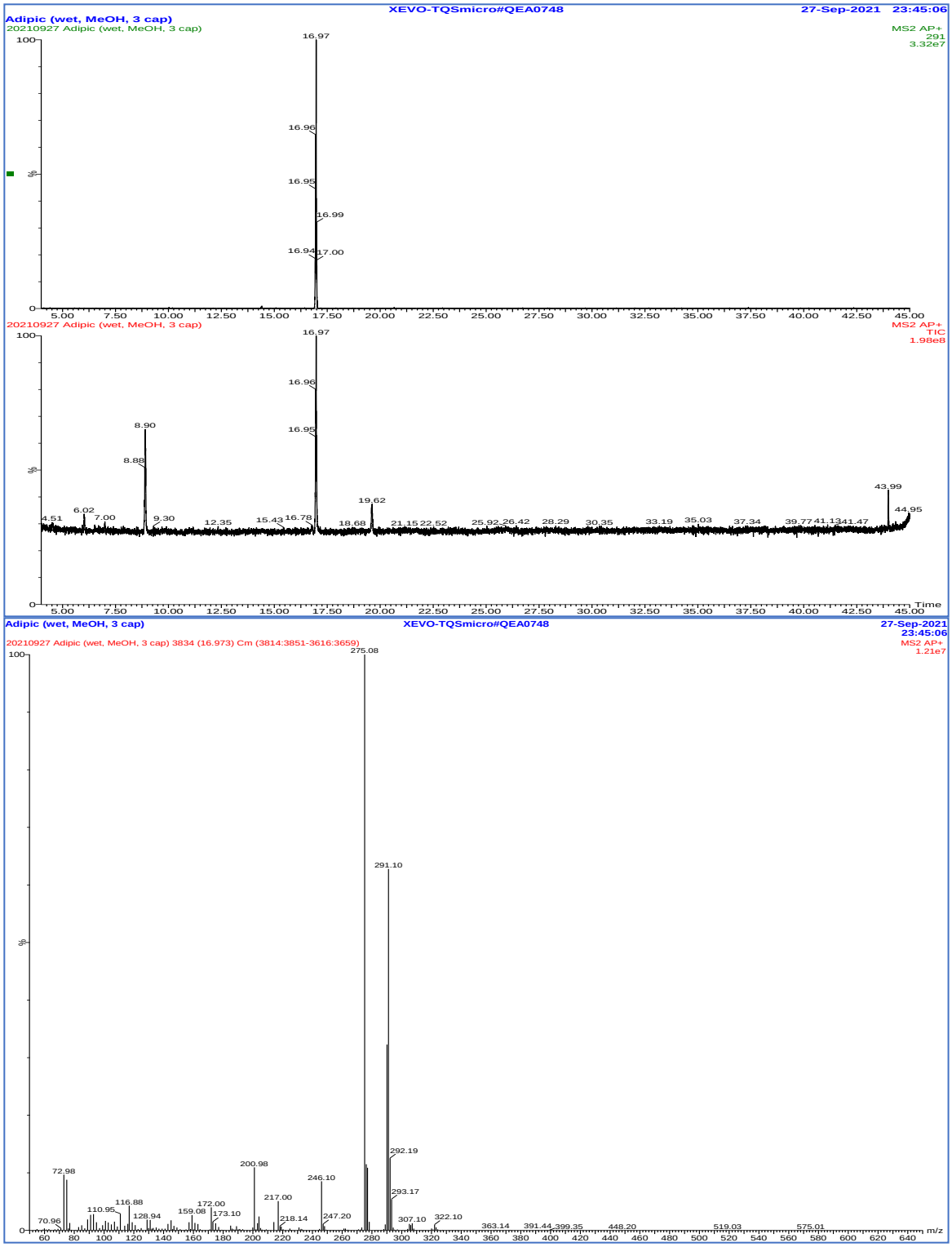

Figure 52 - Isolated extraction of adipic acid showing chromatogram (above) and ion profile (below)

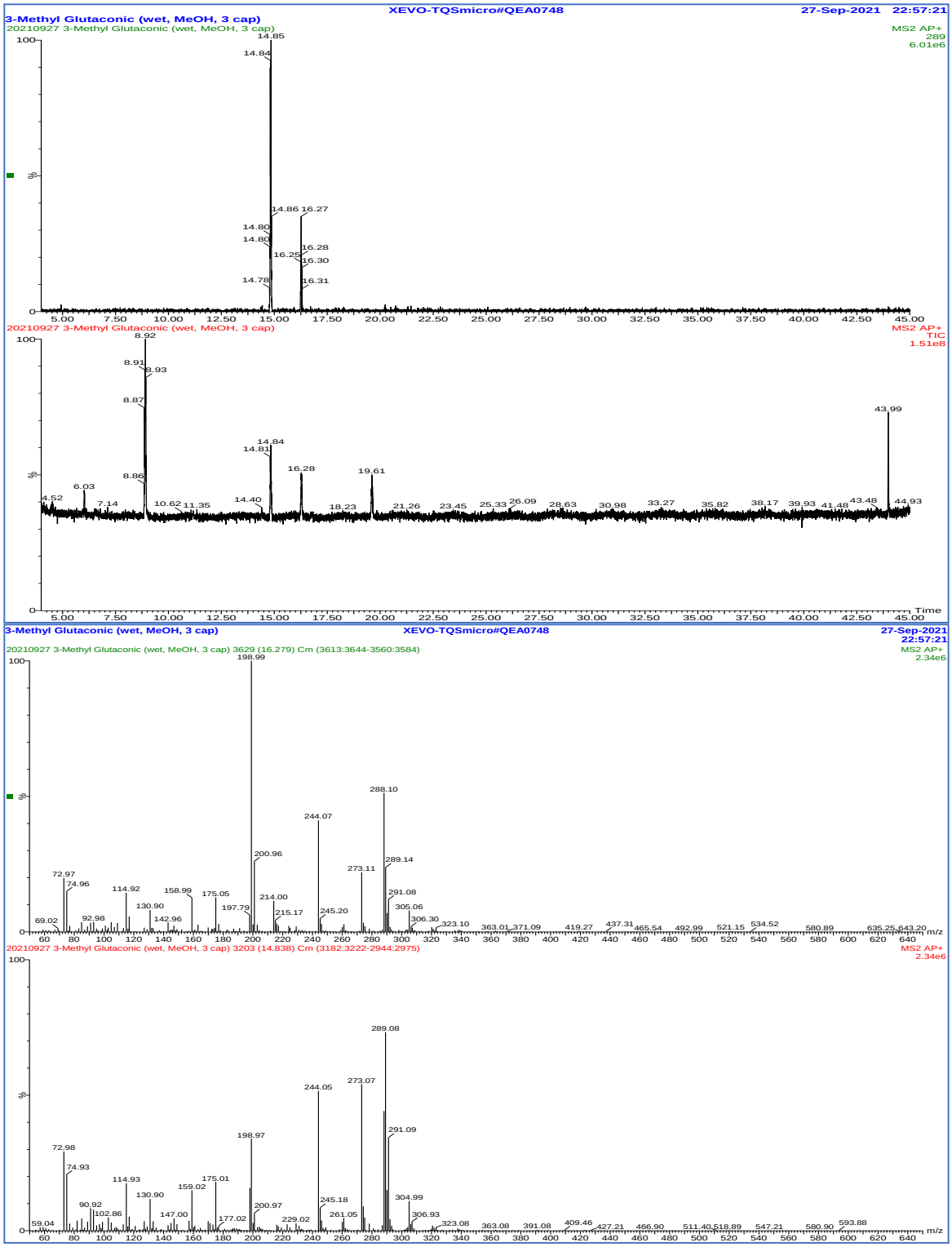

Figure 53 - Isolated extraction of 3-methyl glutaconic TMS2 acid showing chromatogram (above) and ion profile (below)

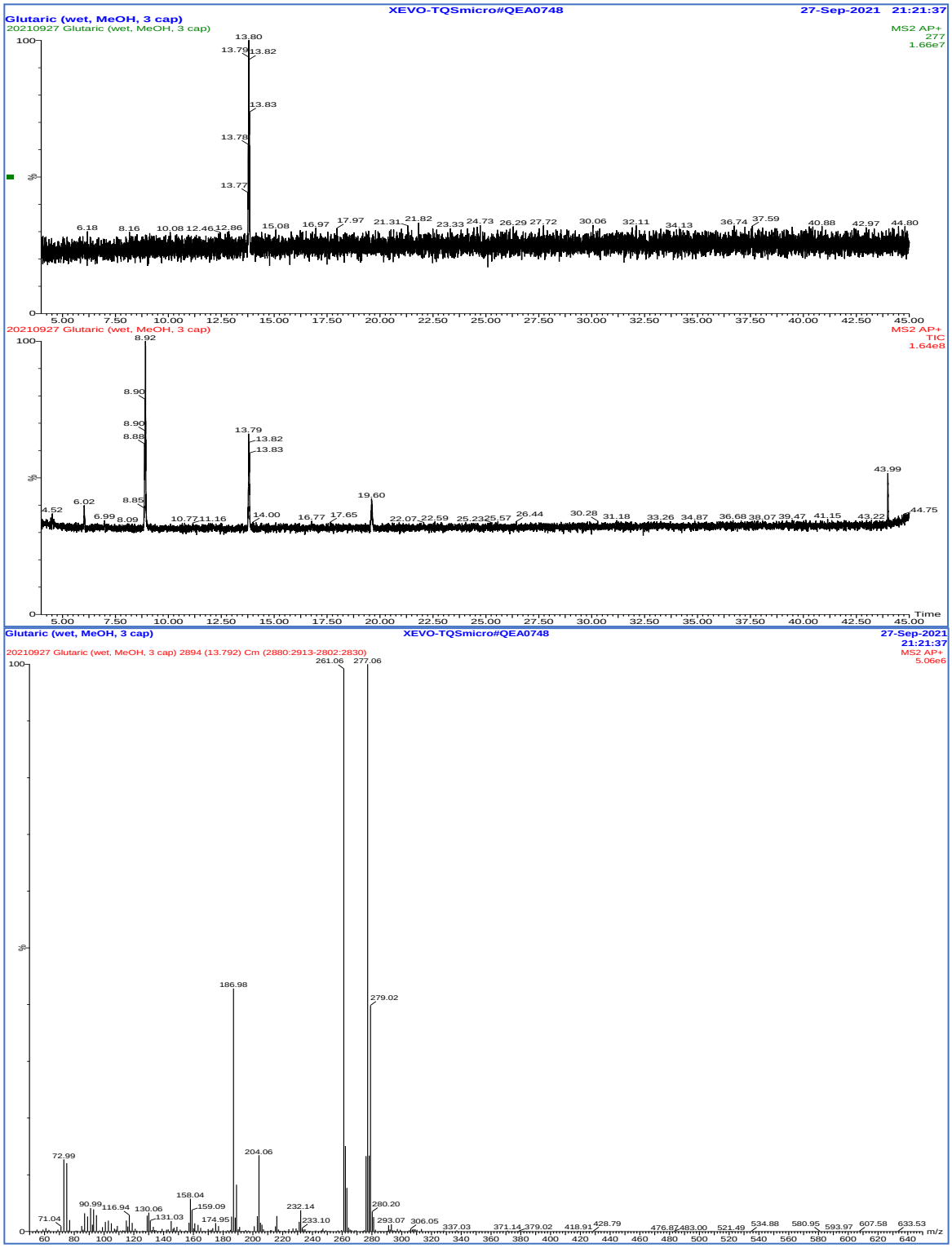

Figure 54 - Isolated extraction of glutaric acid showing chromatogram (above) and ion profile (below)

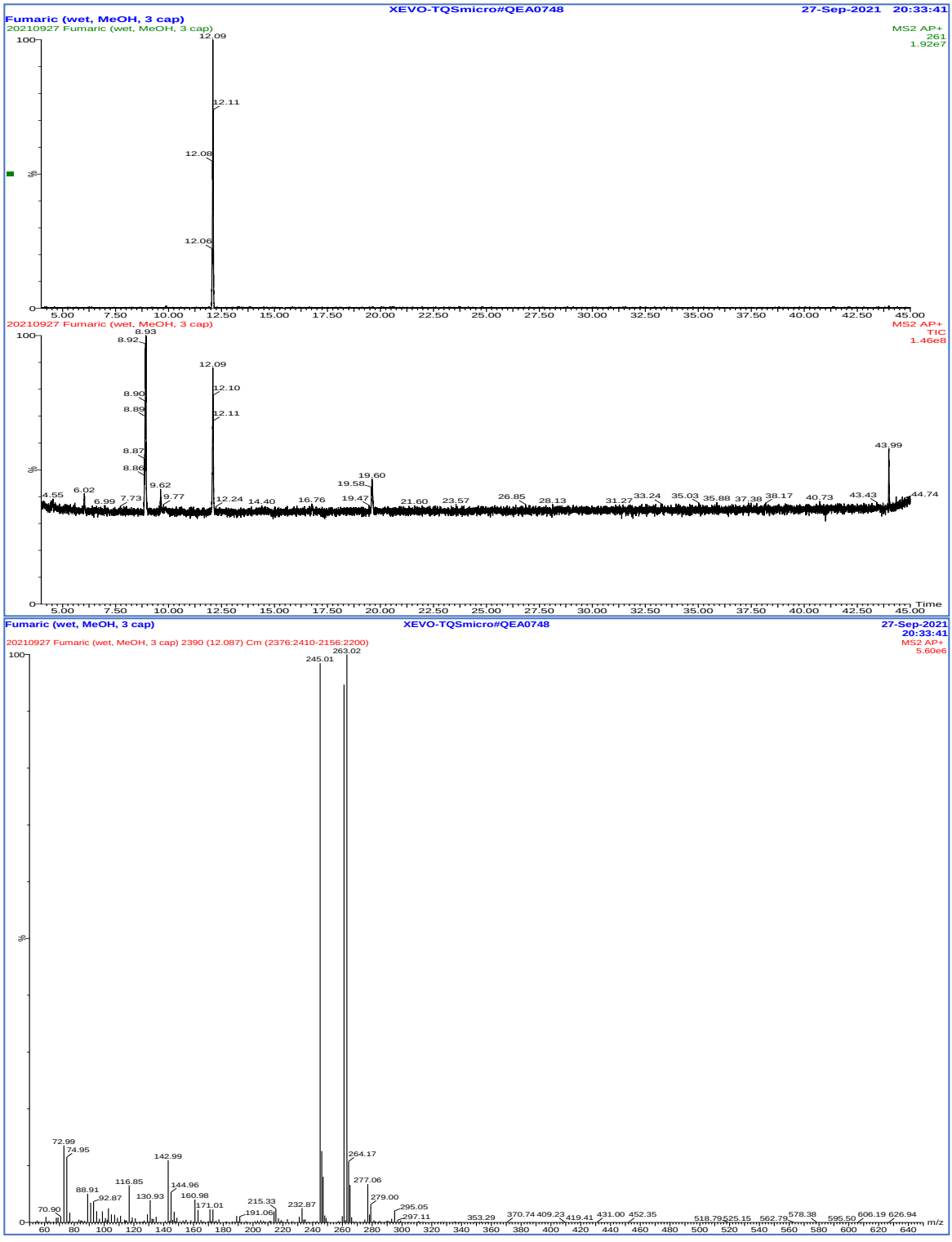

Figure 55 - Isolated extraction of fumaric acid showing chromatogram (above) and ion profile (below)

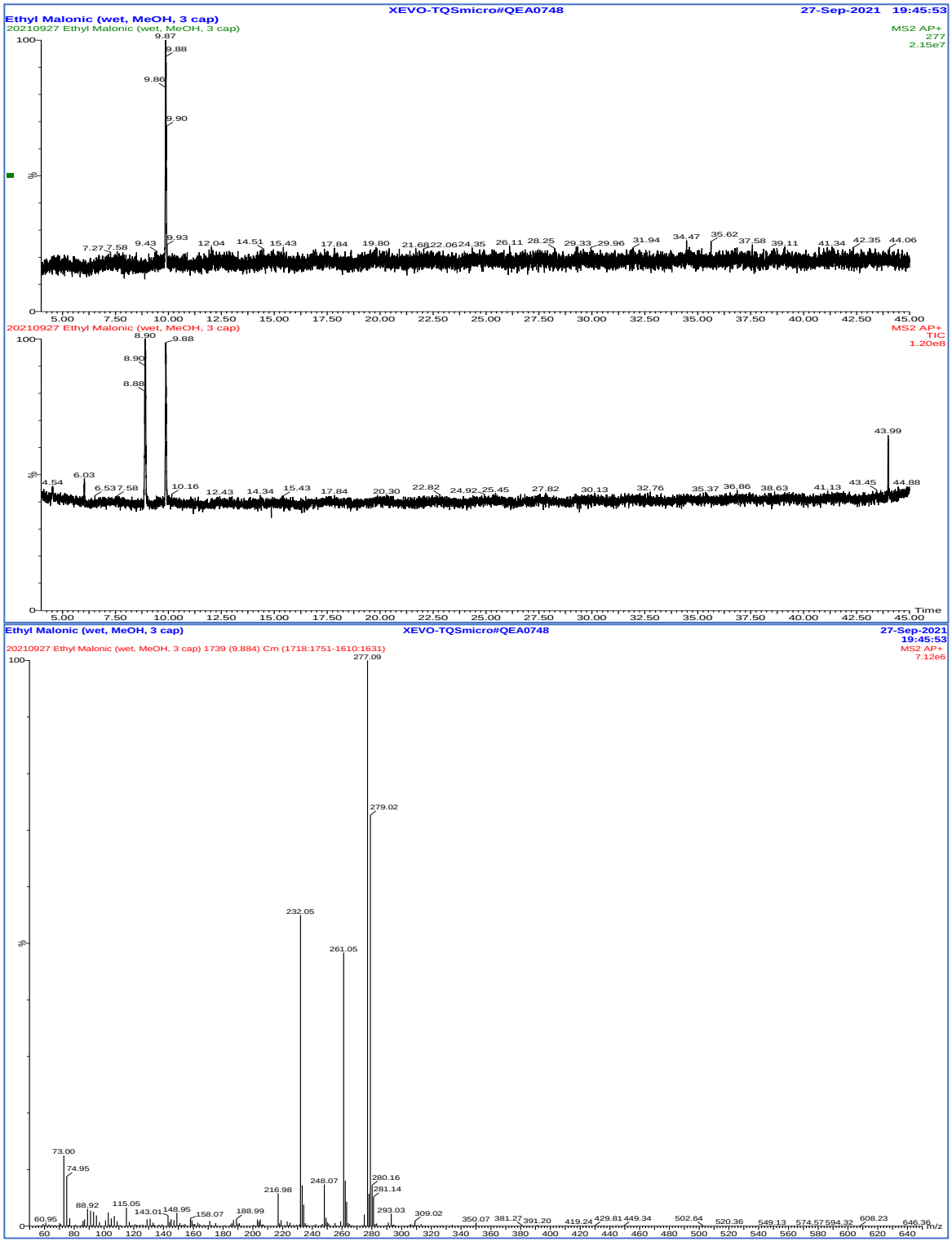

Figure 56 - Isolated extraction of ethyl malonic acid showing chromatogram (above) and ion profile (below)

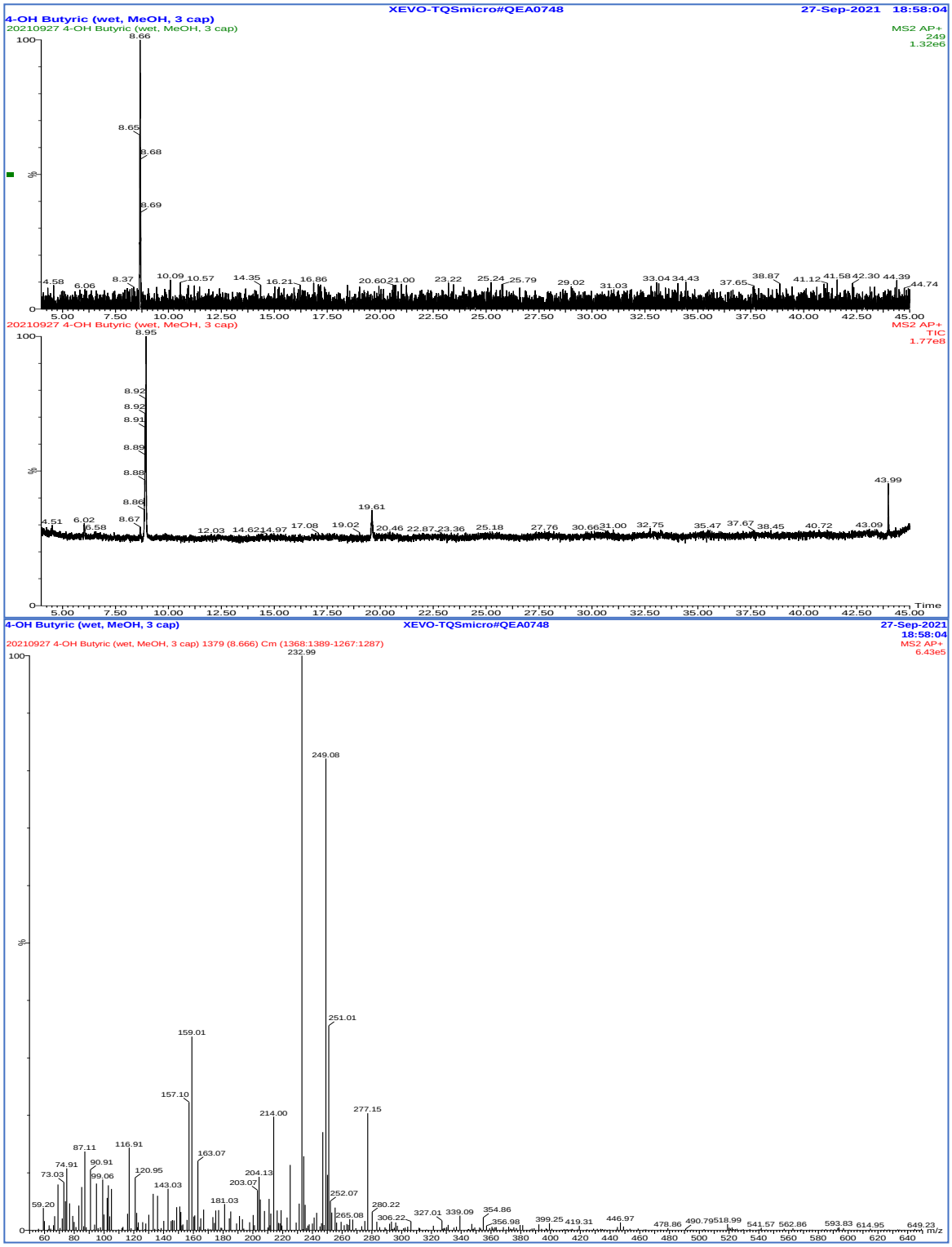

Figure 57 - Isolated extraction of 4-OH butyric acid showing chromatogram (above) and ion profile (below)

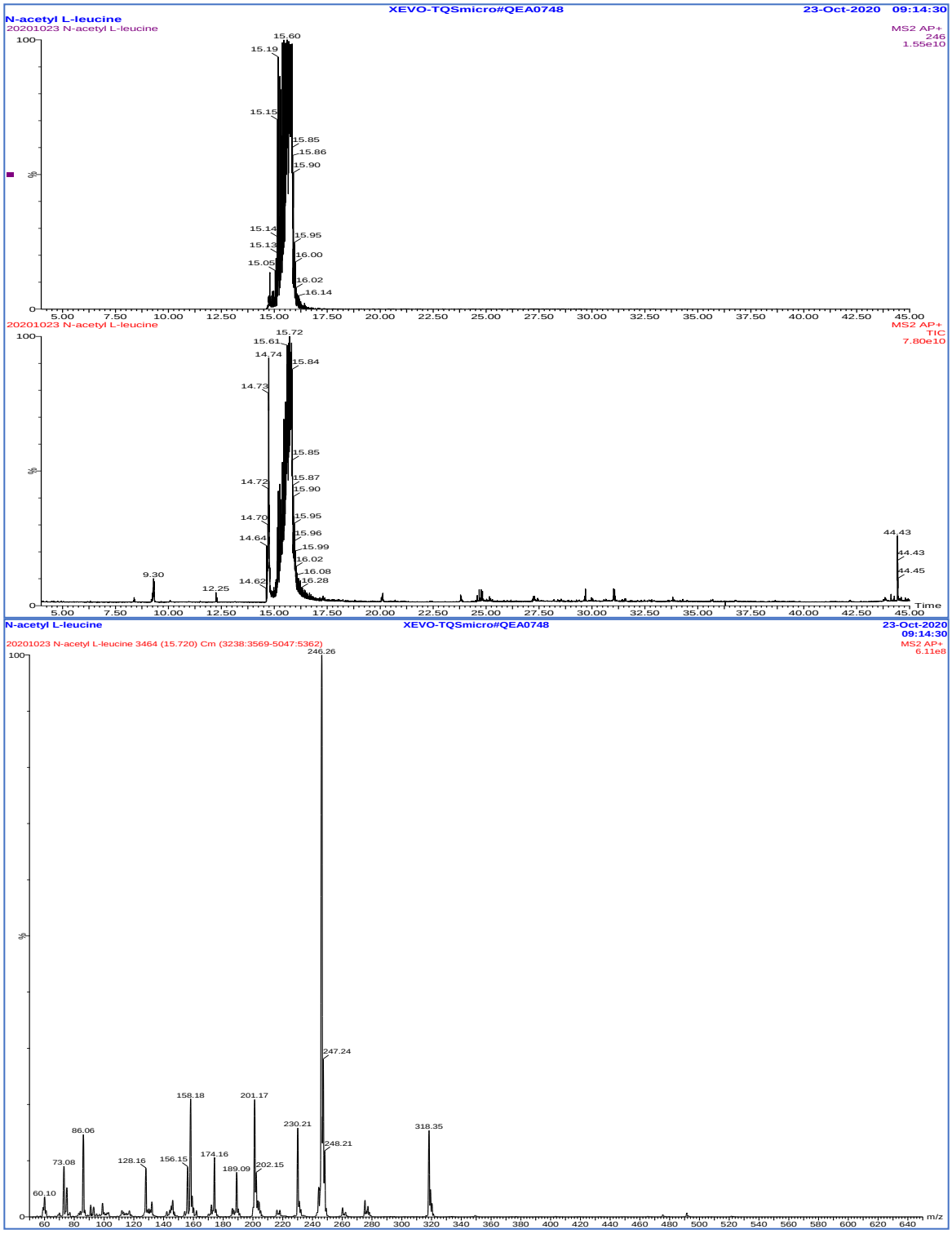

Figure 58 - Isolated extraction of N-acetyl Leucine showing chromatogram (above) and ion profile (below)

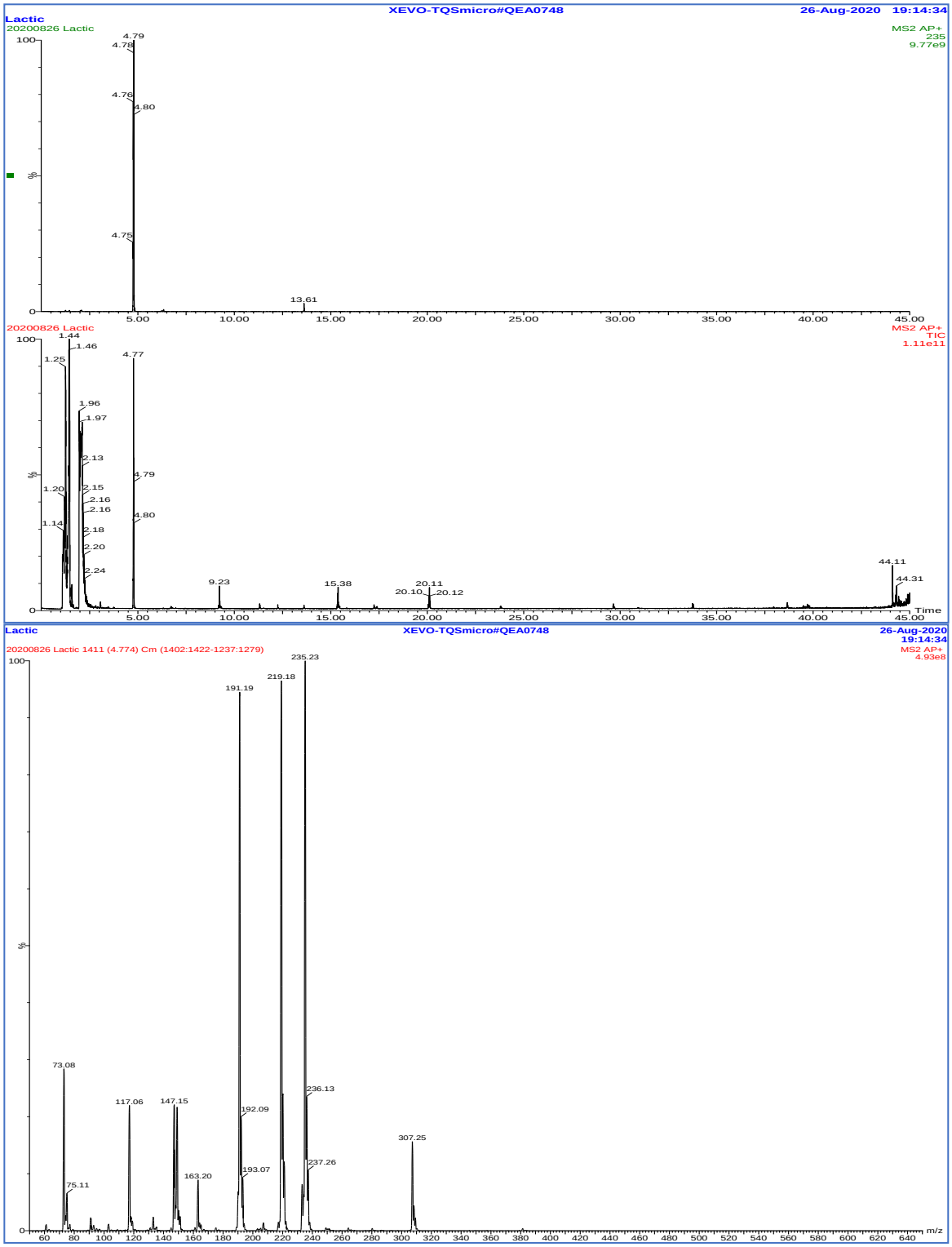

Figure 59 - Isolated extraction of lactic acid showing chromatogram (above) and ion profile (below)

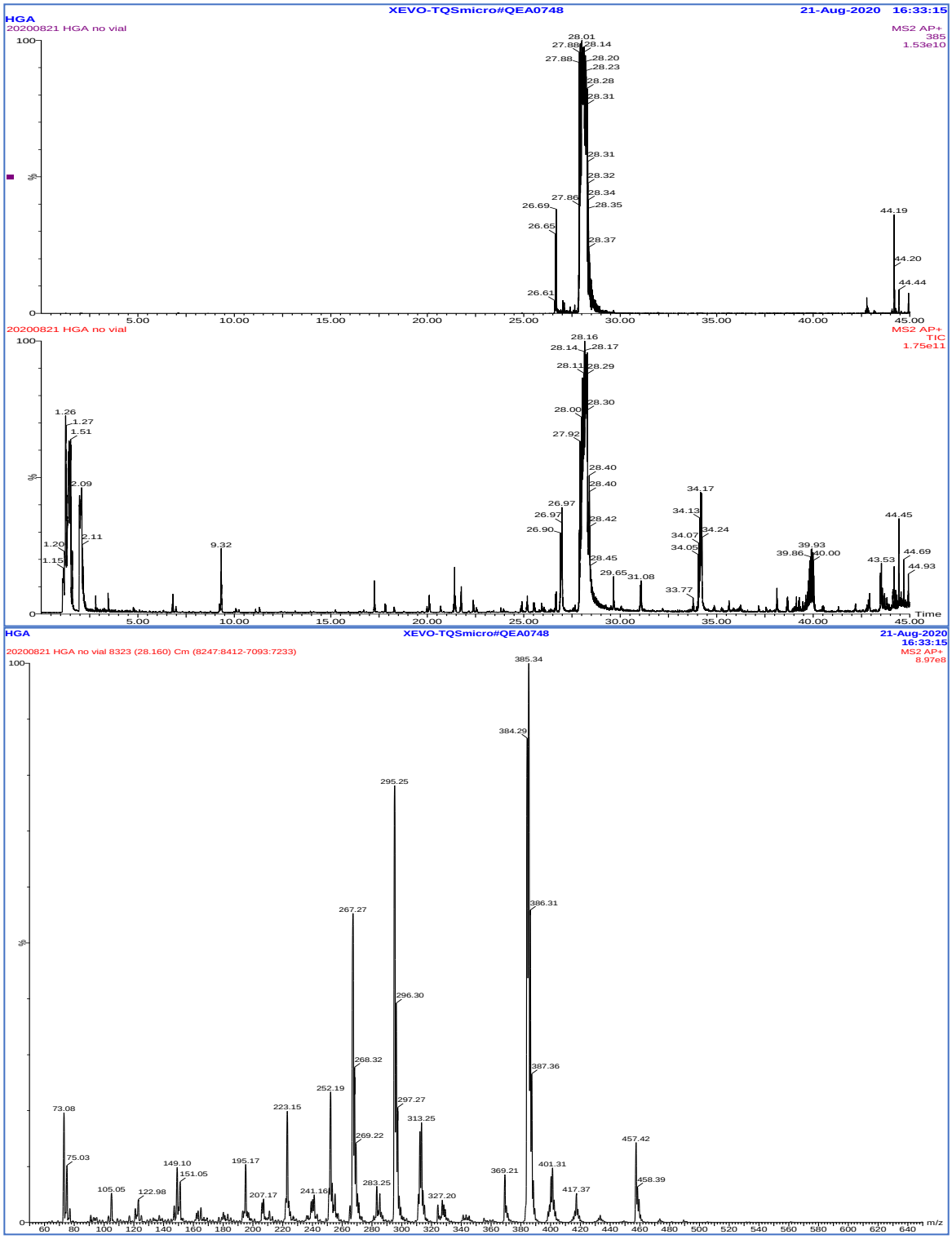

Figure 60 - Isolated extraction of homogentisic acid showing chromatogram (above) and ion profile (below)

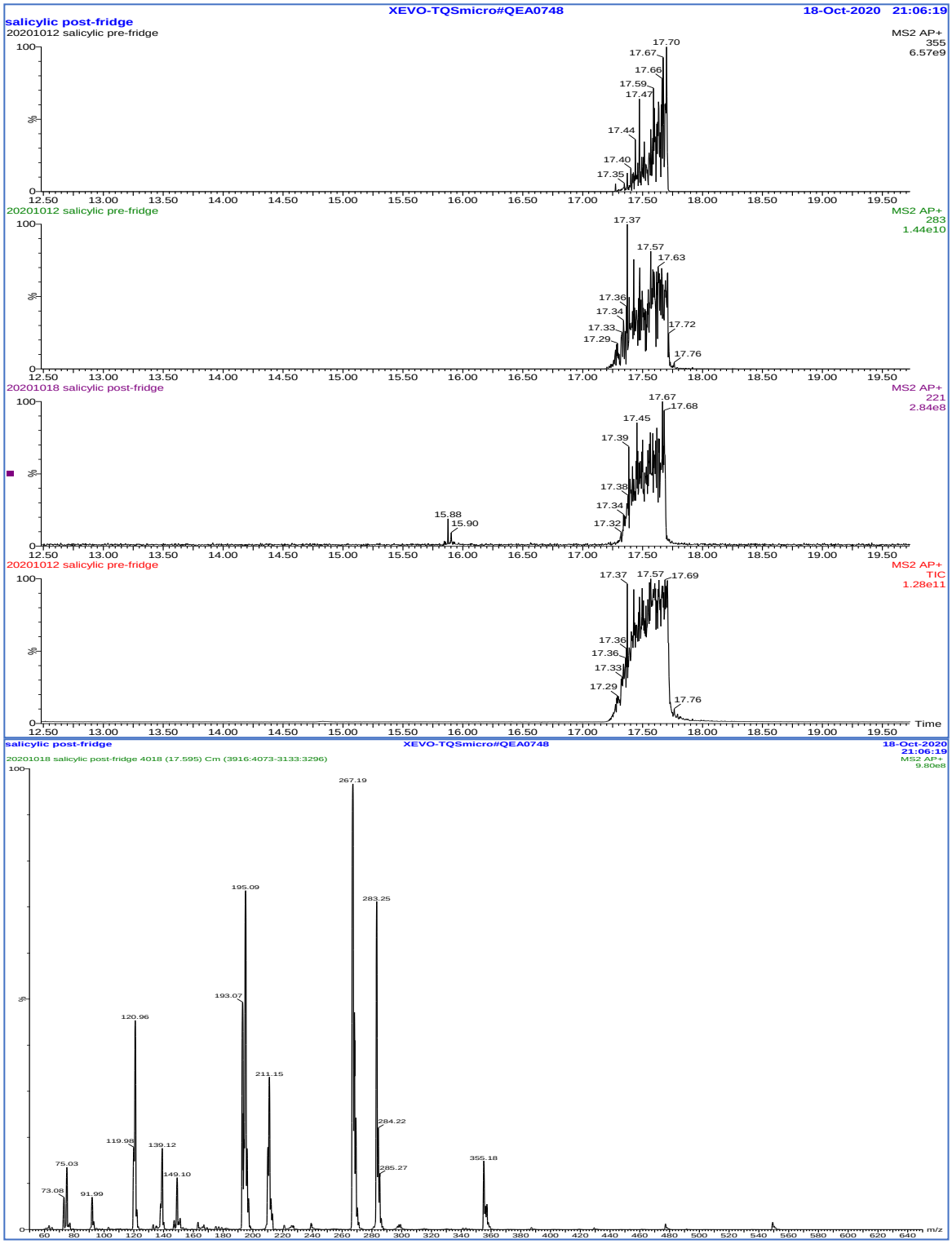

Figure 61 - Isolated extraction of salicylic acid showing chromatogram (above) and ion profile (below)

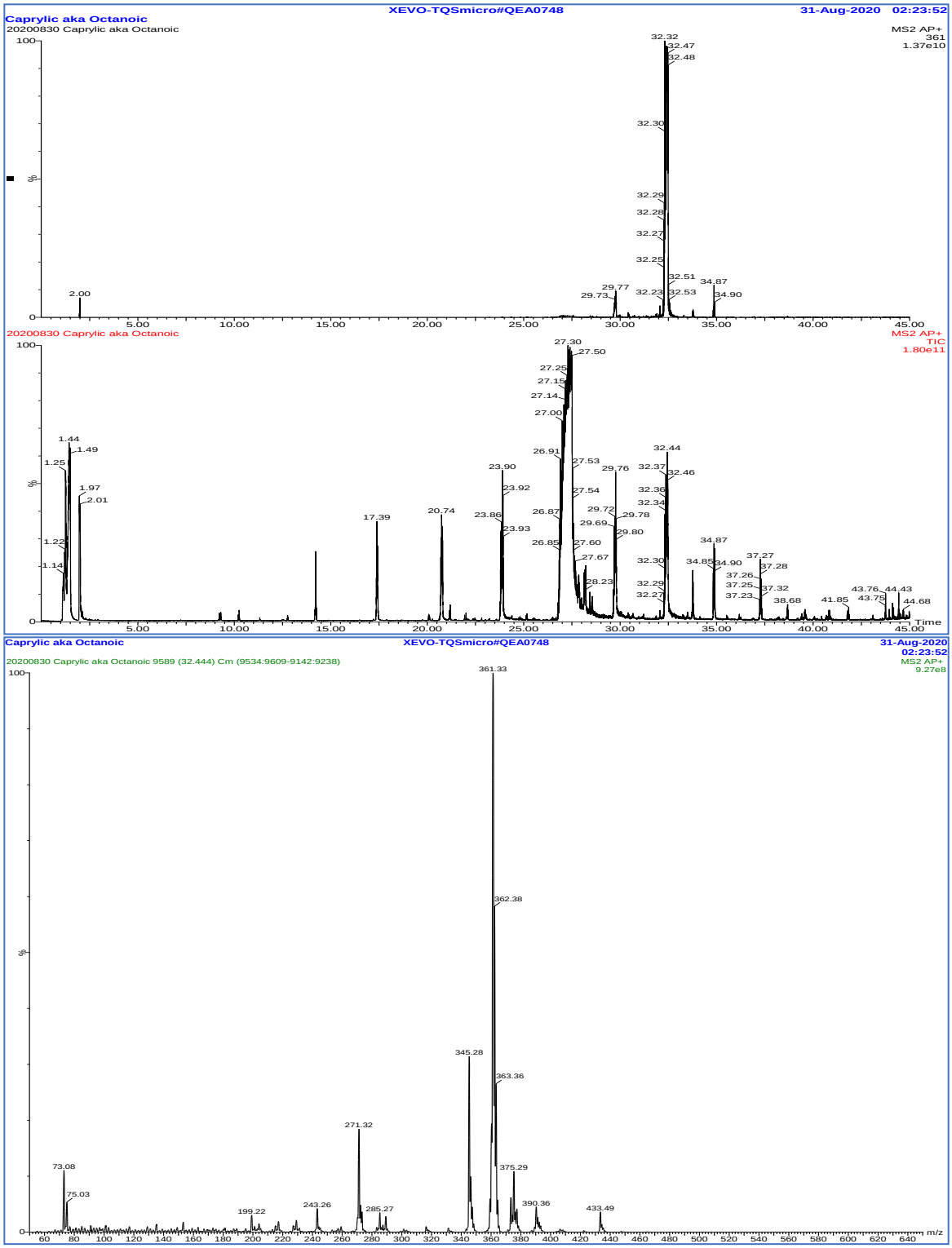

Figure 62 - Isolated extraction of caprylic acid (also known as octanoic acid) showing chromatogram (above) and ion profile (below)

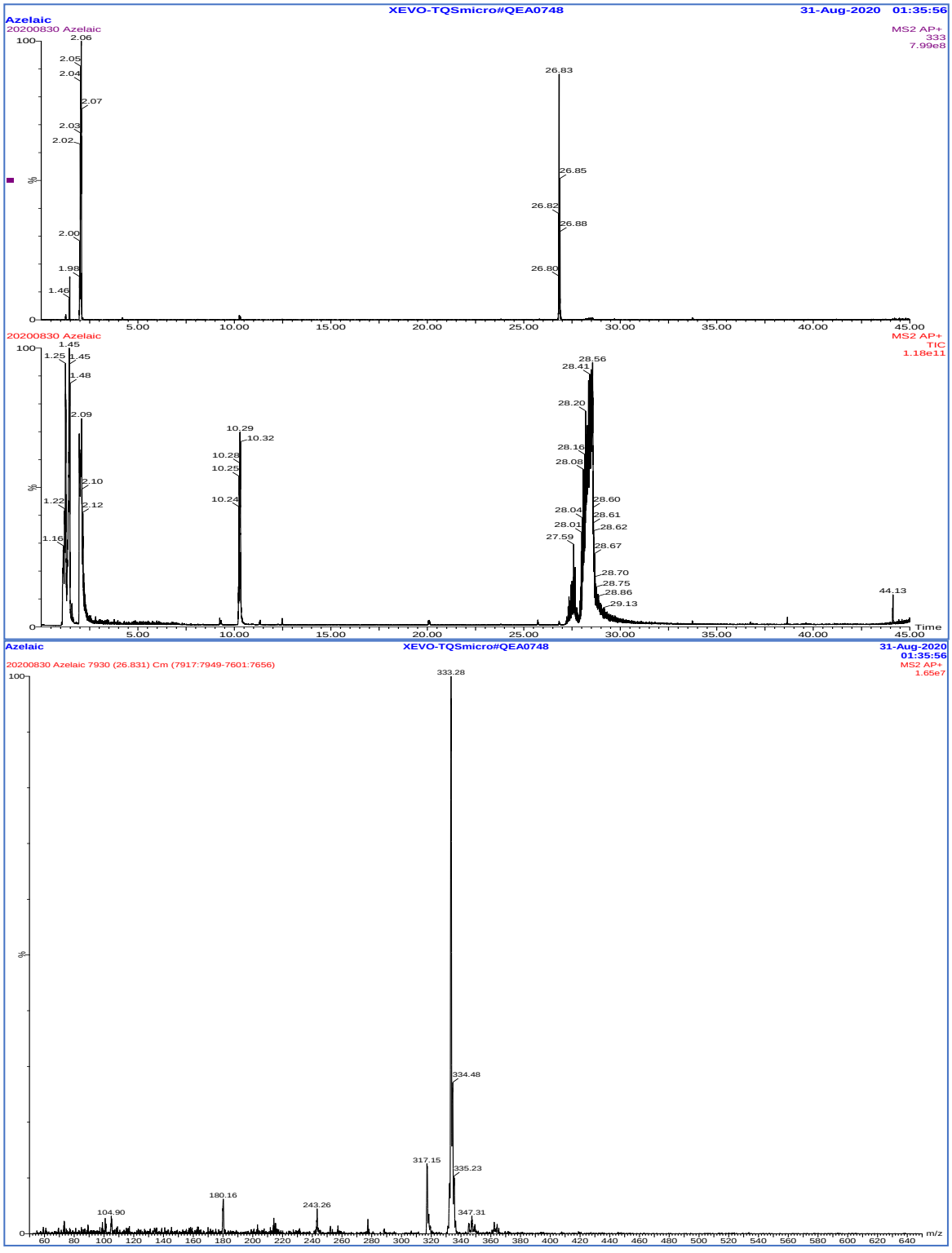

Figure 63 - Isolated extraction of azelaic acid showing chromatogram (above) and ion profile (below)

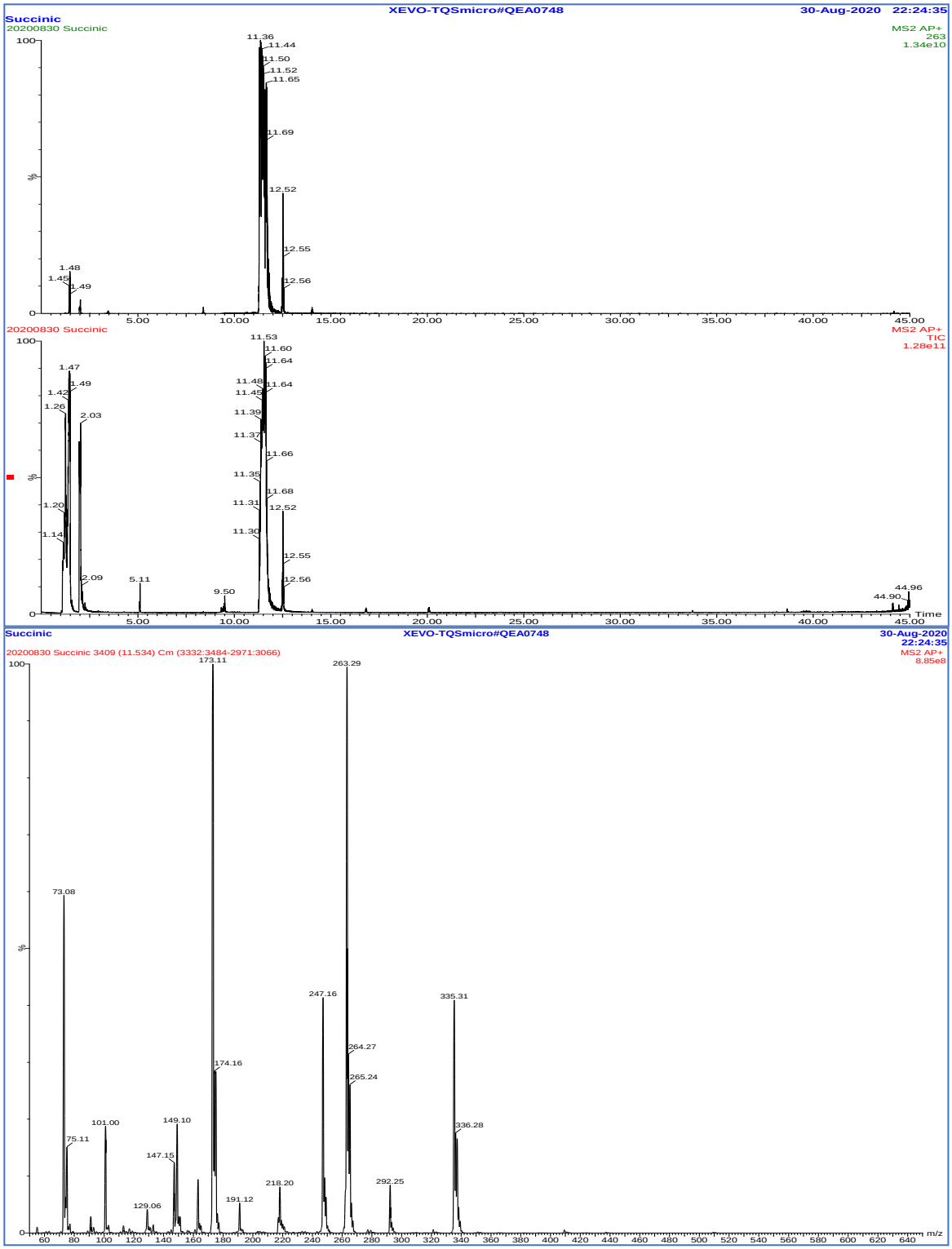

Figure 64 - Isolated extraction of succinic acid showing chromatogram (above) and ion profile (below)

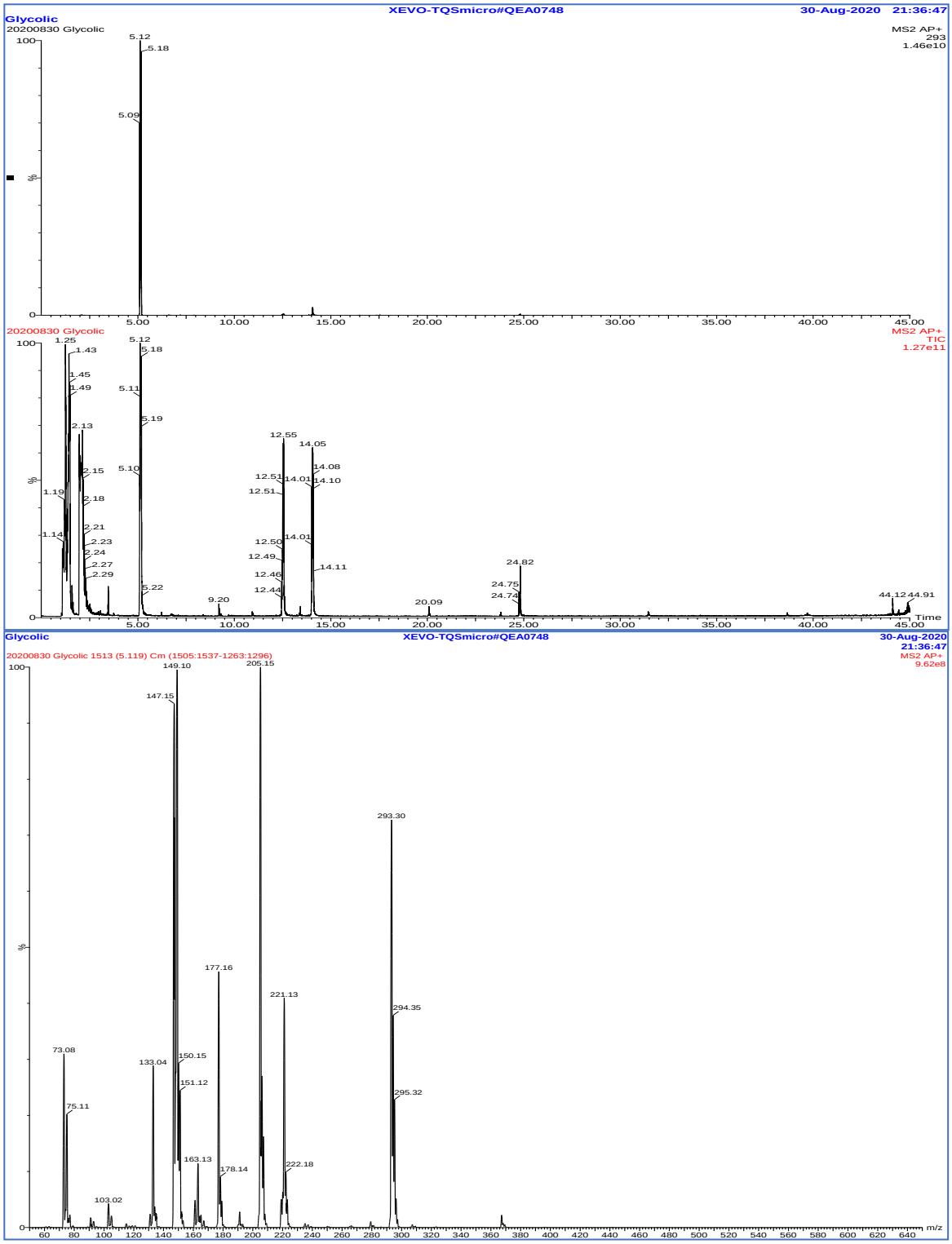

Figure 65 - Isolated extraction of glycolic acid showing chromatogram (above) and ion profile (below)

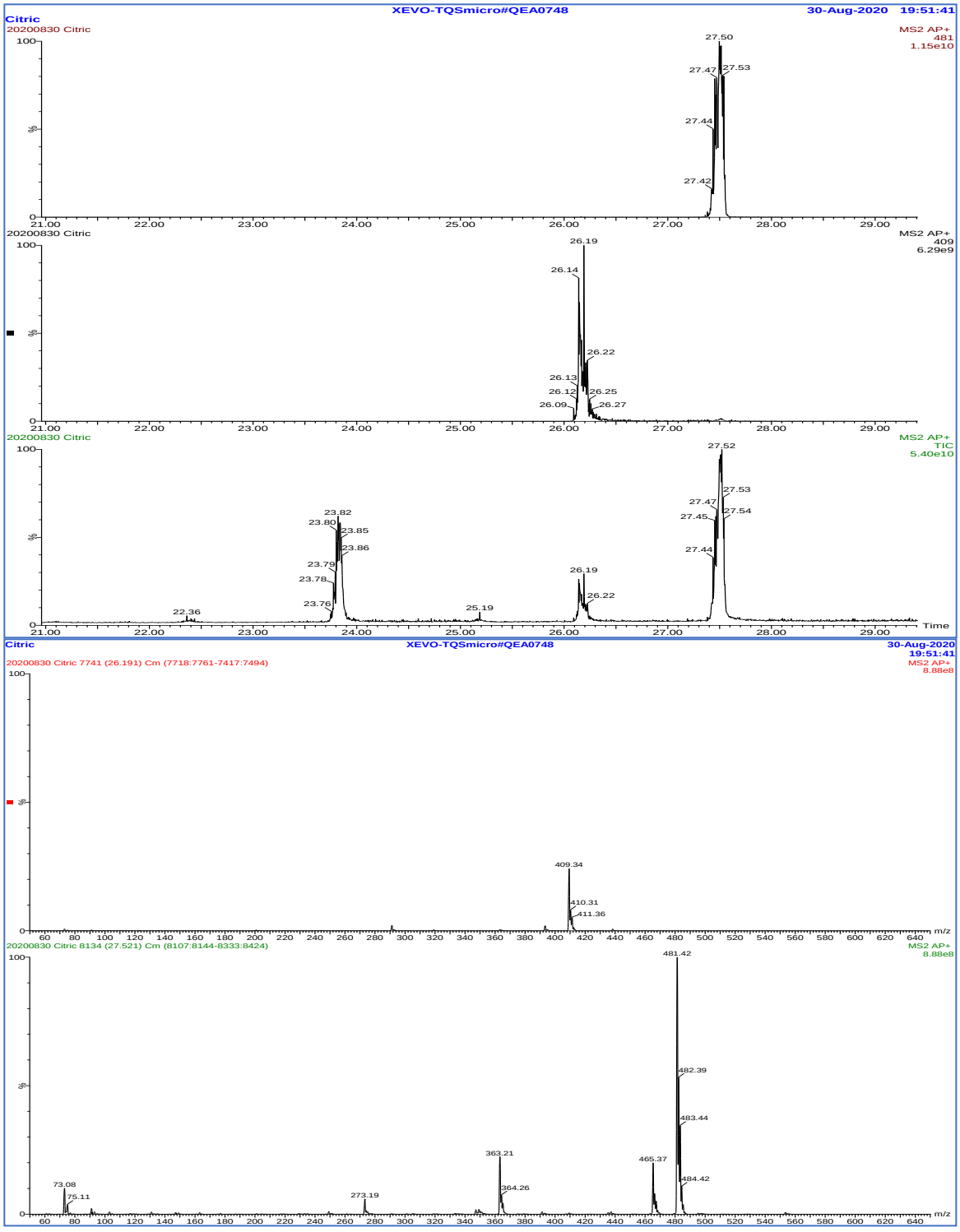

Figure 66 - Isolated extraction of citric acid showing chromatogram (above) and ion profile (below)

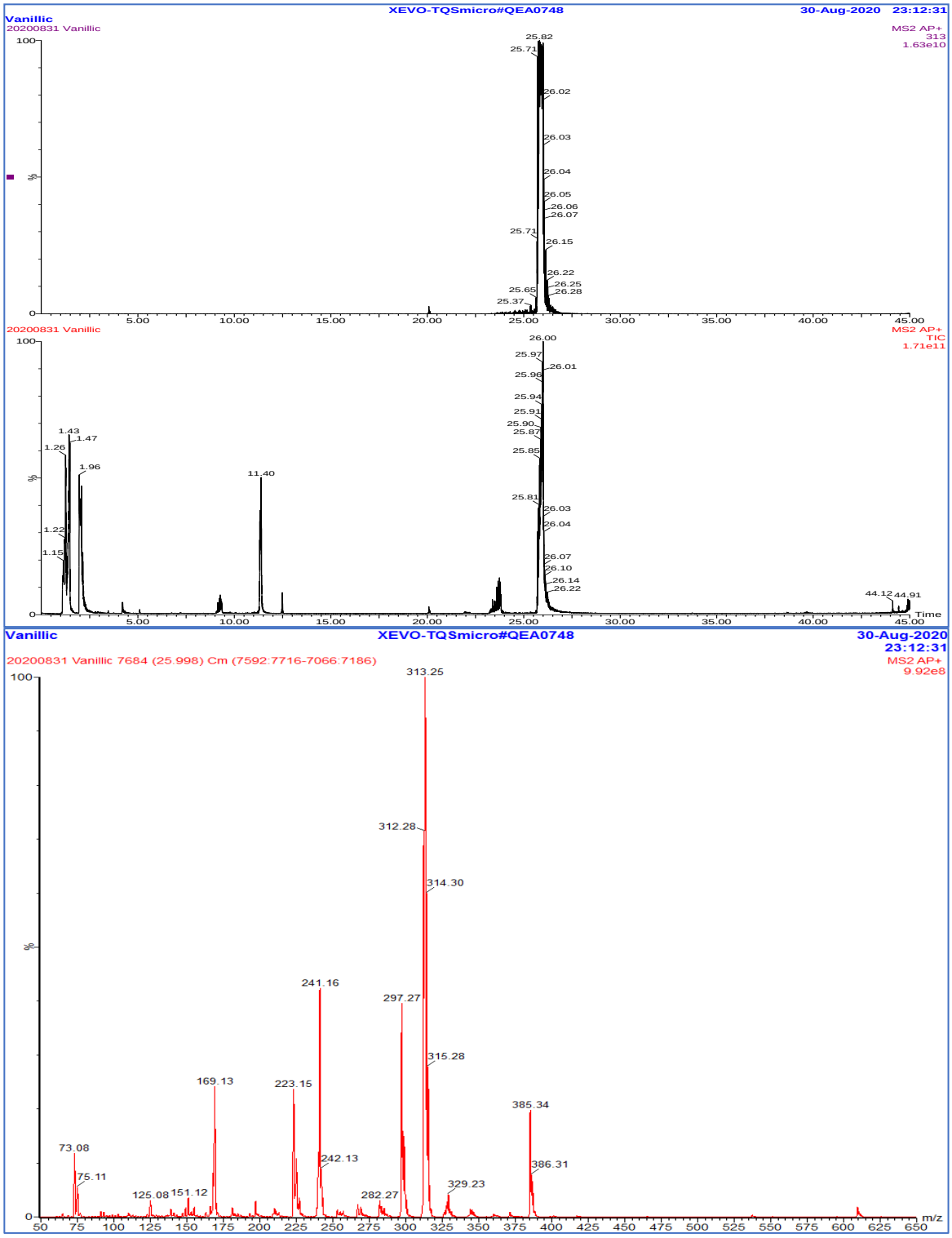

Figure 67 - Isolated extraction of vanillic showing chromatogram (above) and ion profile (below)

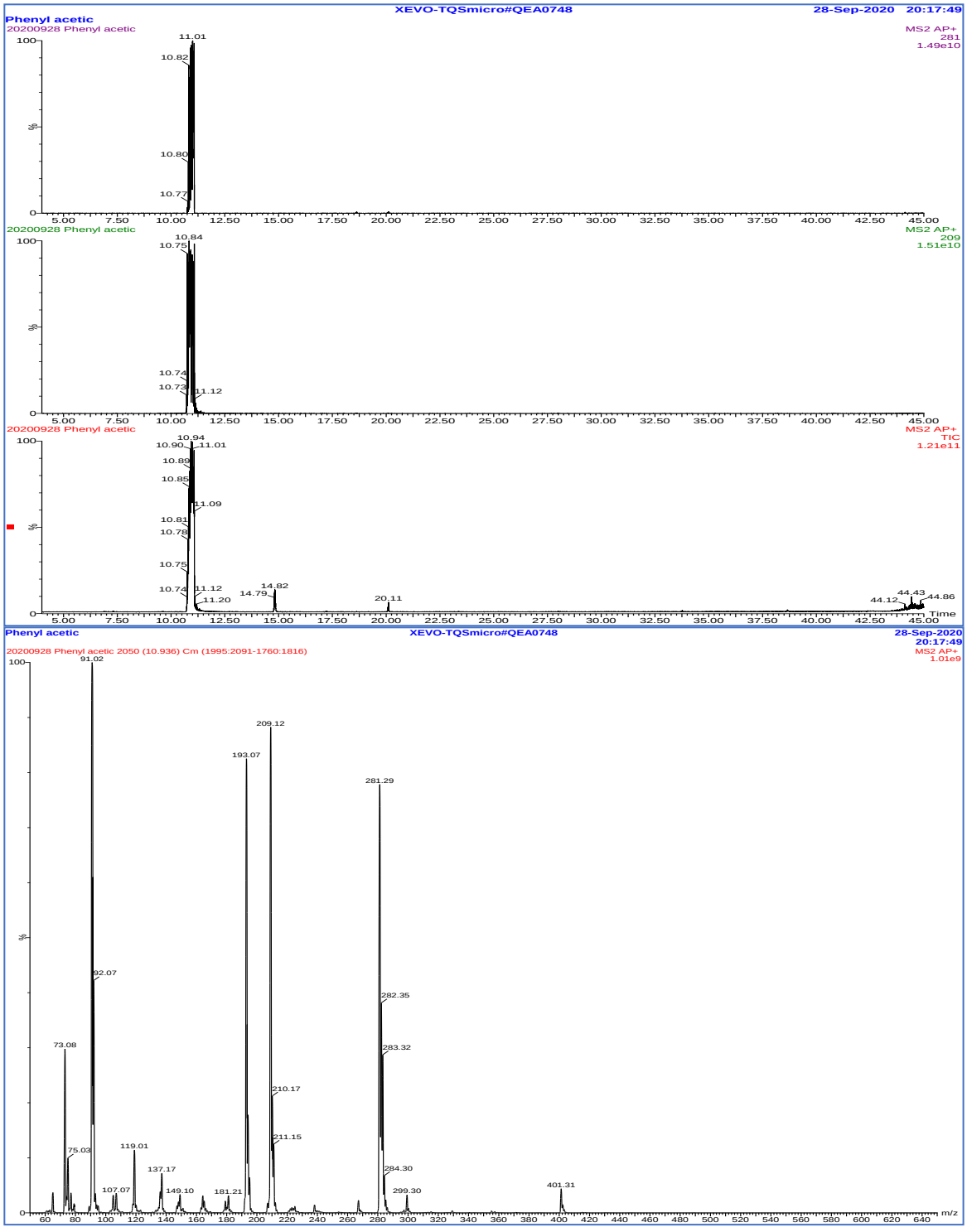

Figure 68 - Isolated extraction of phenyl acetic acid showing chromatogram (above) and ion profile (below)

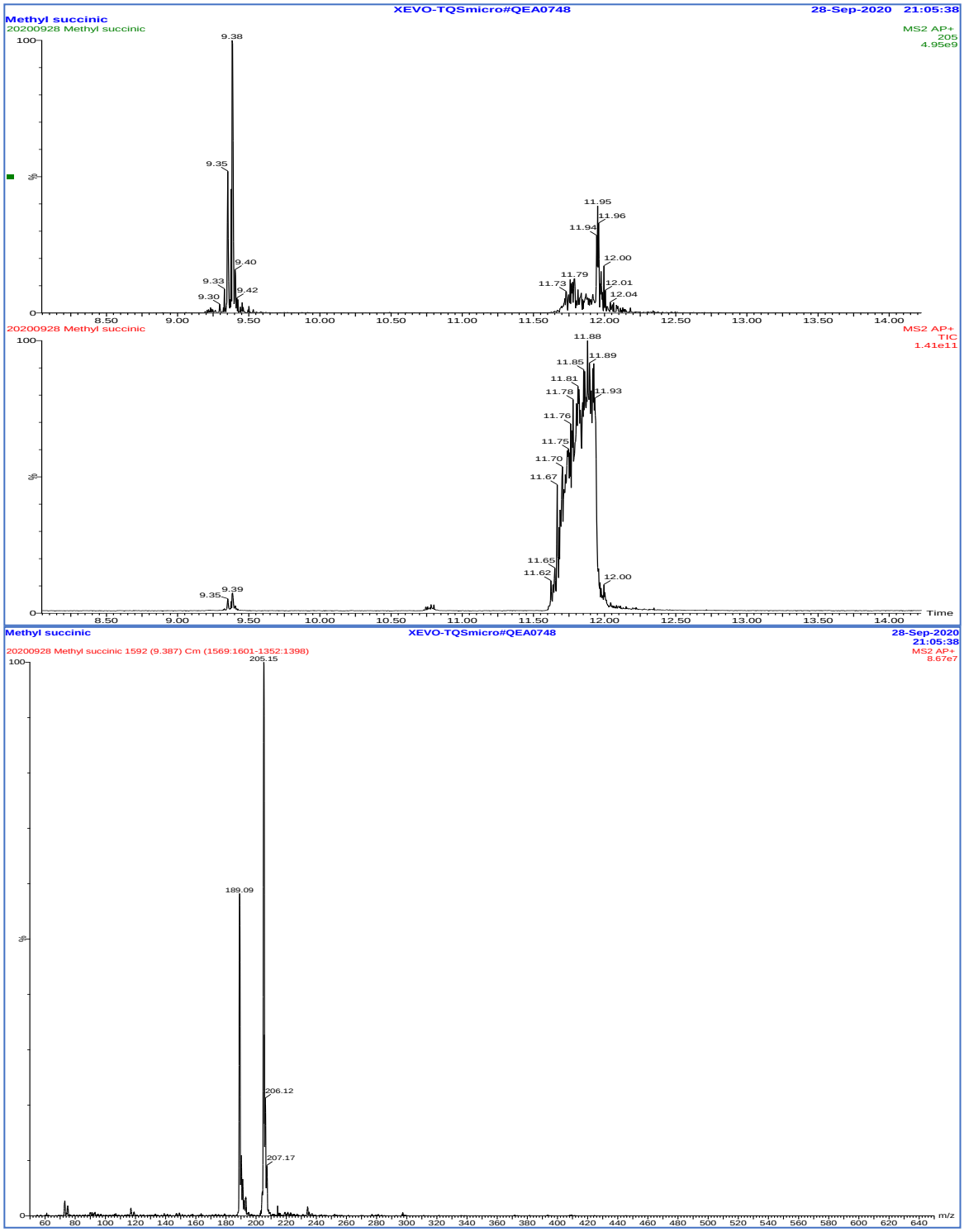

Figure 69 - Isolated extraction of methyl succinic acid TMS1 showing chromatogram (above) and ion profile (below)

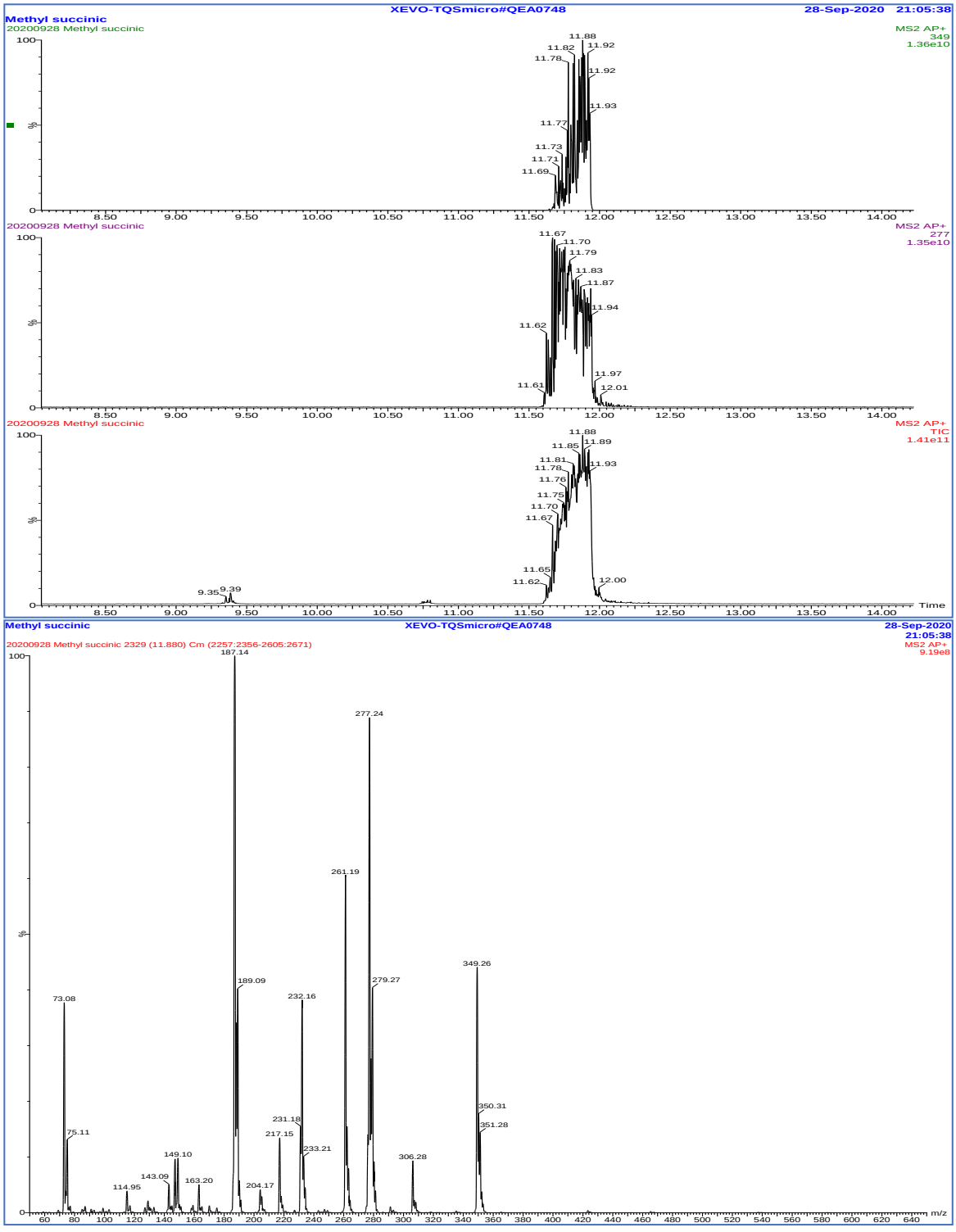

Figure 70 - Isolated extraction of methyl succinic acid TMS2 showing chromatogram (above) and ion profile (below)

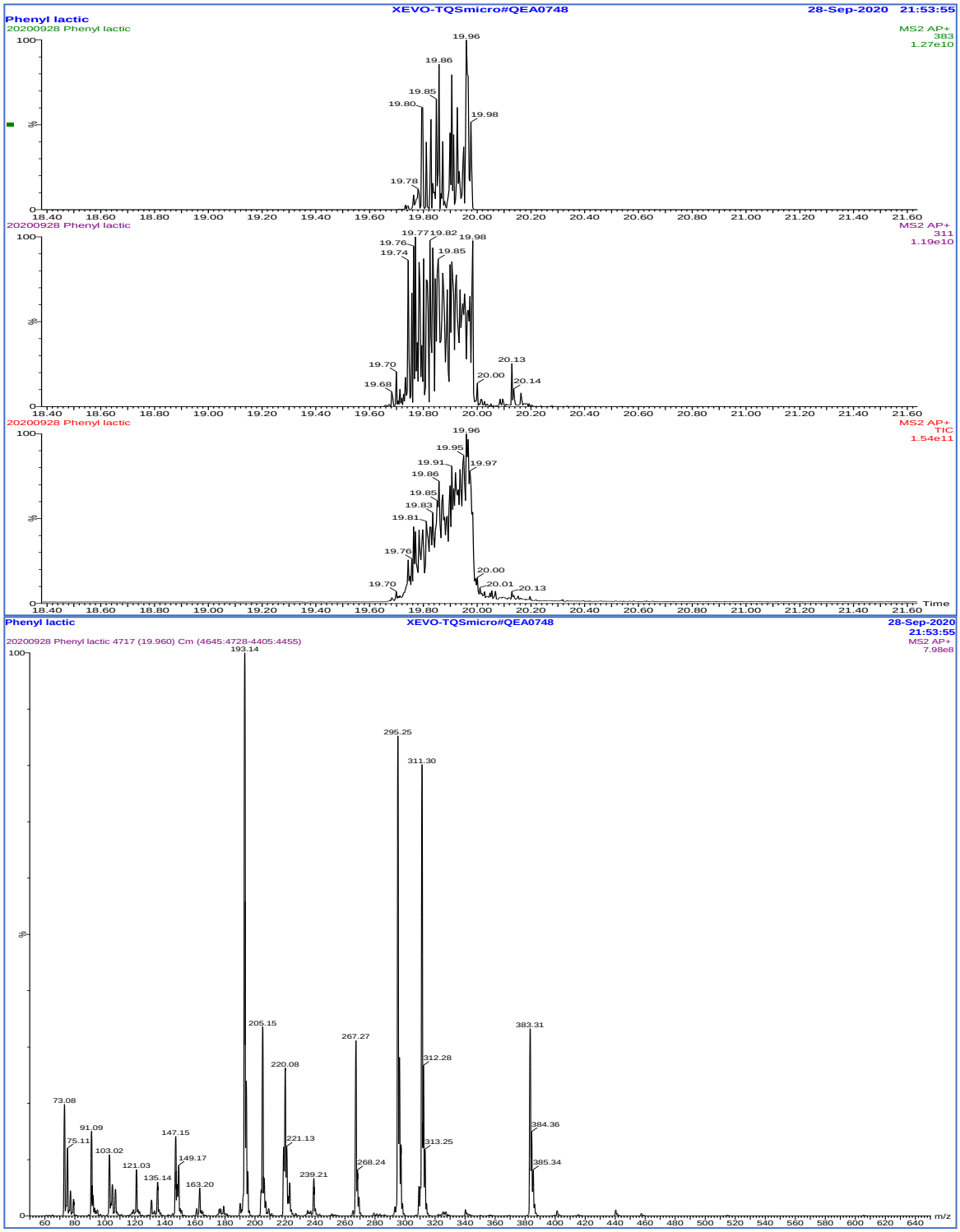

Figure 71 - Isolated extraction of phenyl lactic acid showing chromatogram (above) and ion profile (below)

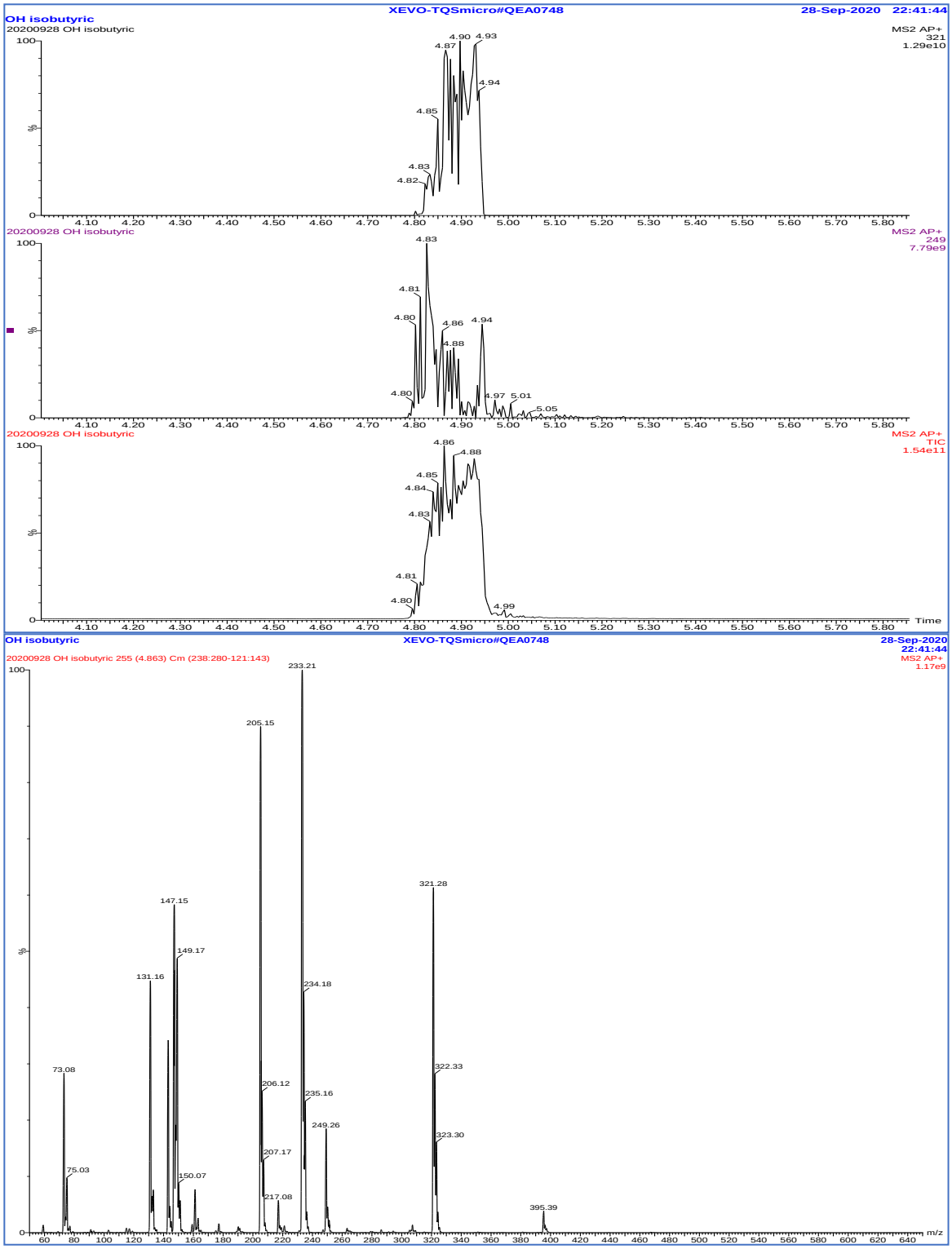

Figure 72 - Isolated extraction of 3-OH isobutyric acid showing chromatogram (above) and ion profile (below)

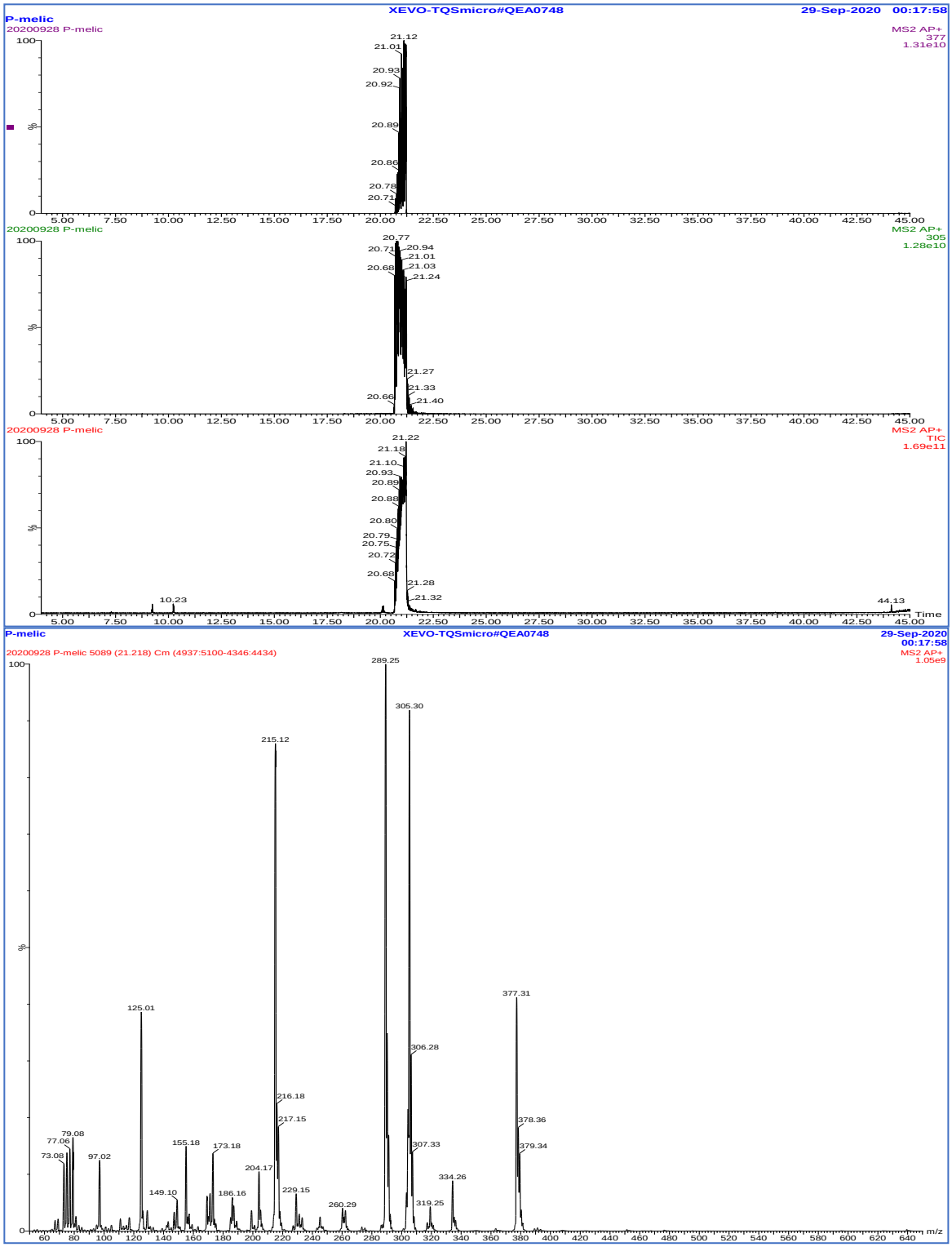

Figure 73 - Isolated extraction of p-melic acid showing chromatogram (above) and ion profile (below)

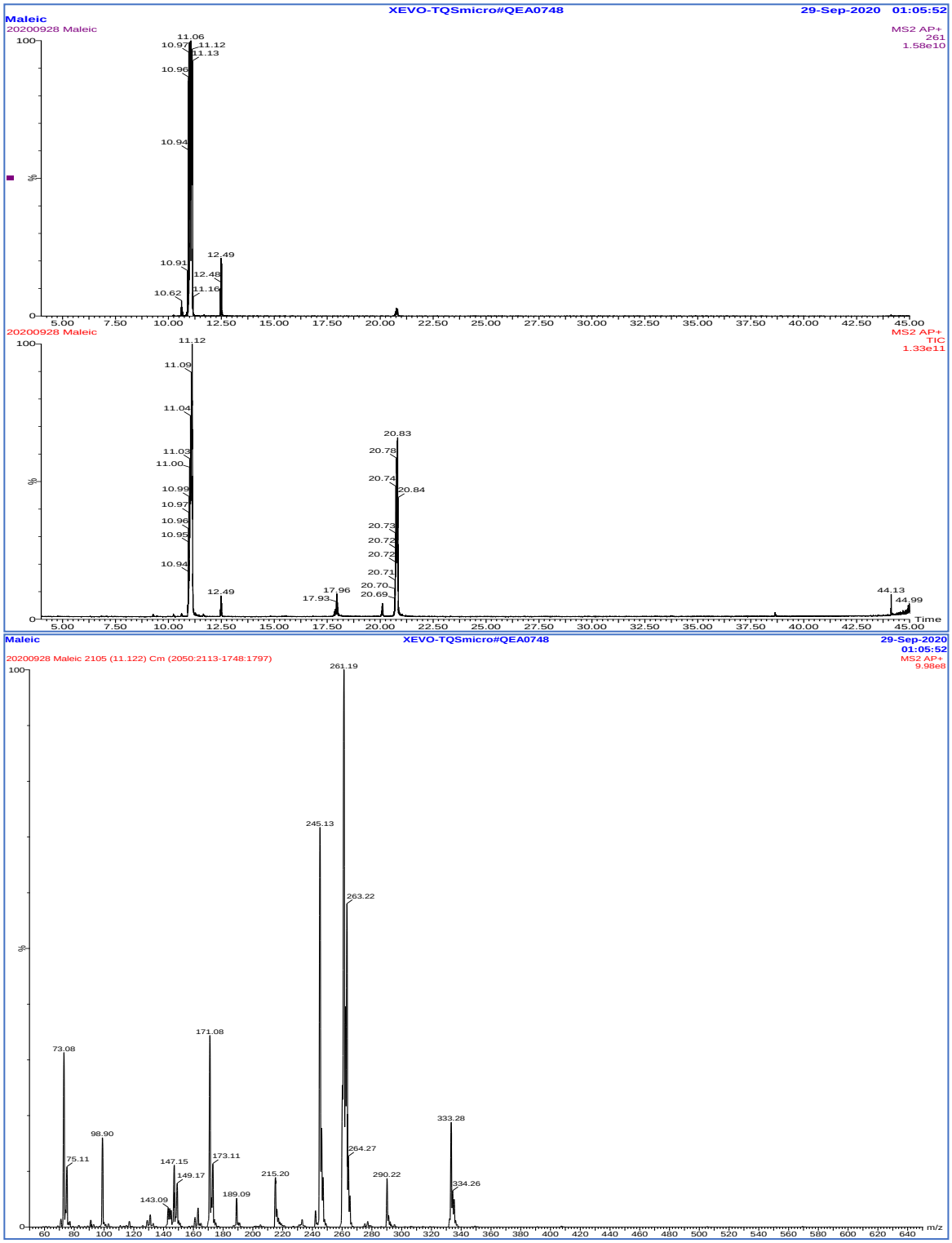

Figure 74 - Isolated extraction of maleic acid showing chromatogram (above) and ion profile (below)

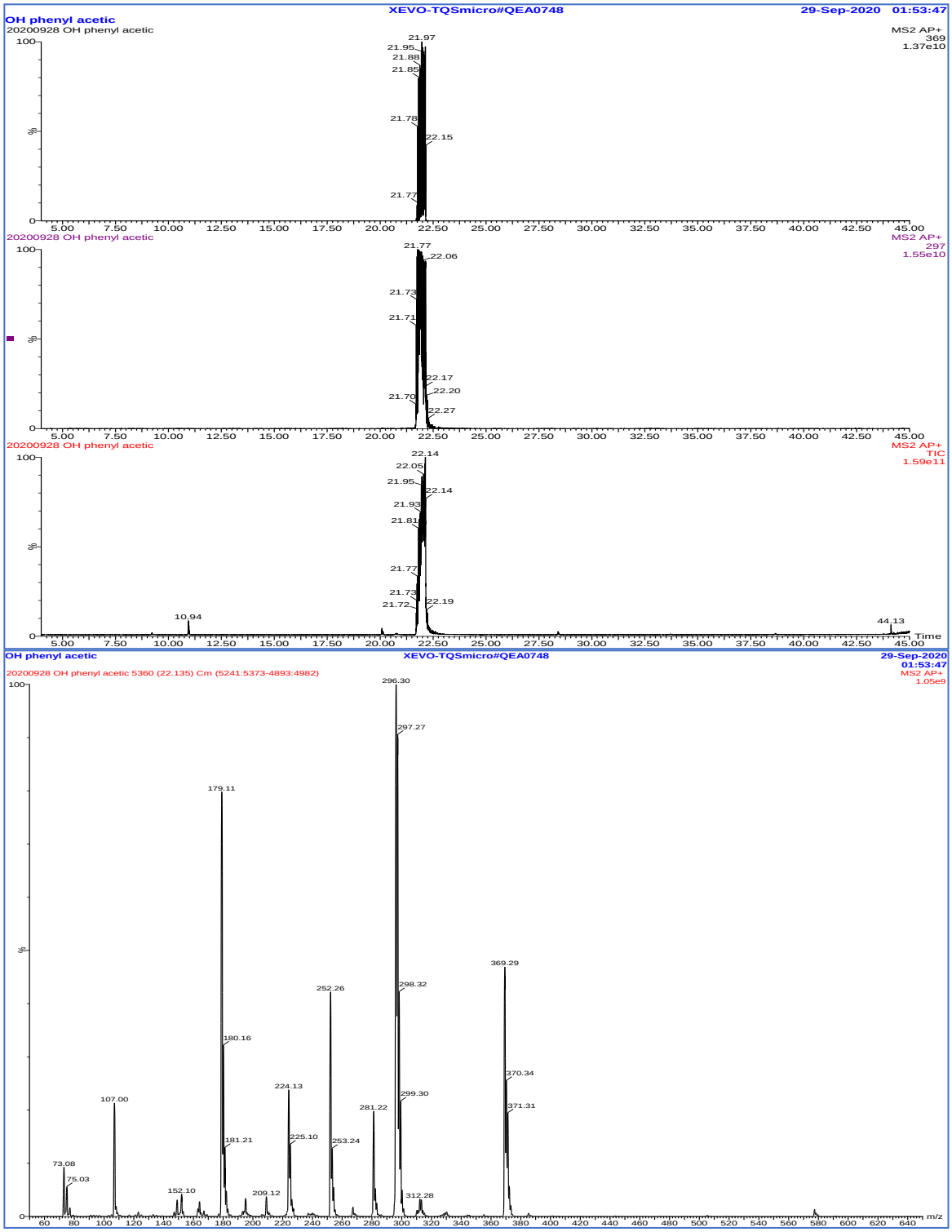

Figure 75 - Isolated extraction of 2-OH phenyl acetic acid showing chromatogram (above) and ion profile (below)

8.3 – For Section 4.11 - UOA Quantification

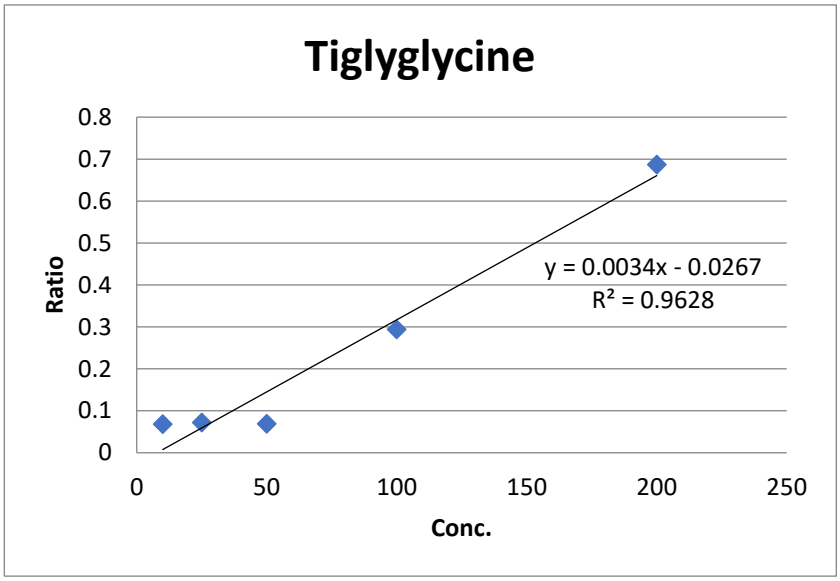

Figure 76 – Calibration curve of tiglyglycine from 10 to 2000 uM normalized to tropic acid.

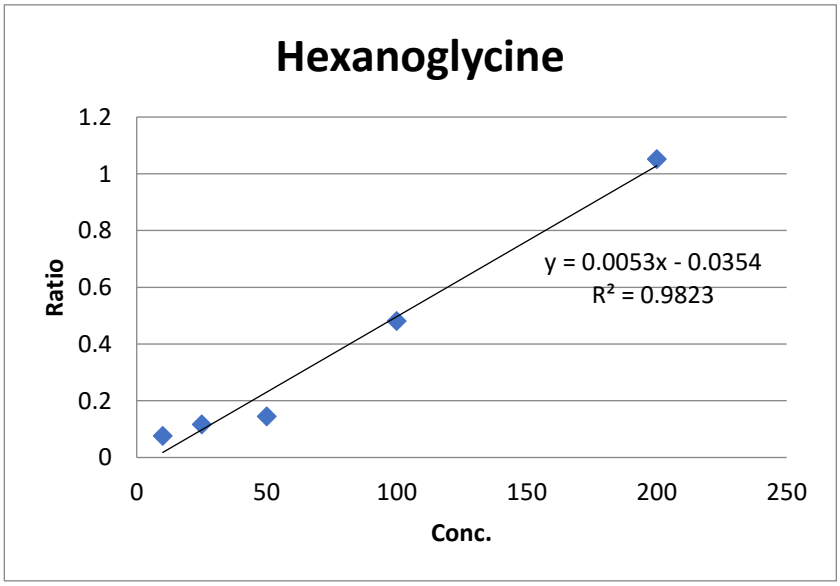

Figure 77 – Calibration curve of hexanoylglycine from 10 to 2000 uM normalized to tropic acid.

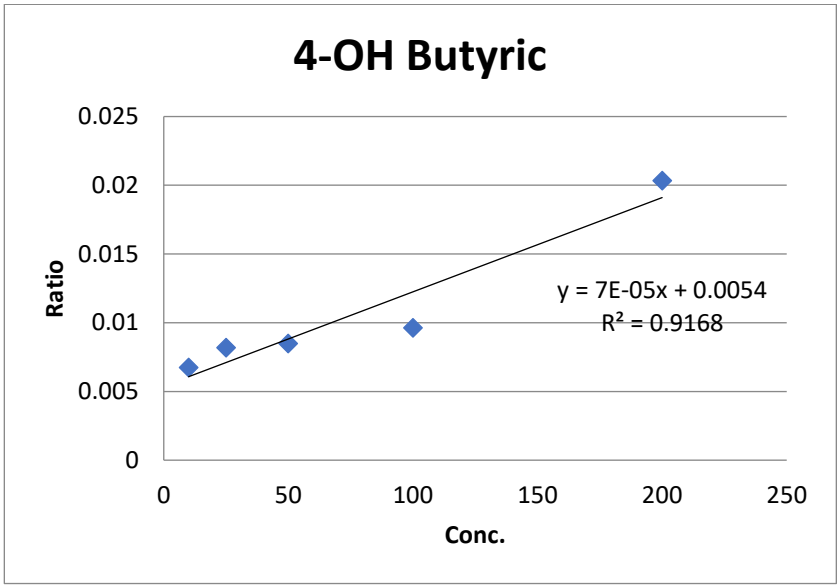

Figure 78 – Calibration curve of 4-OH butyric acid from 10 to 2000 uM normalized to tropic acid.

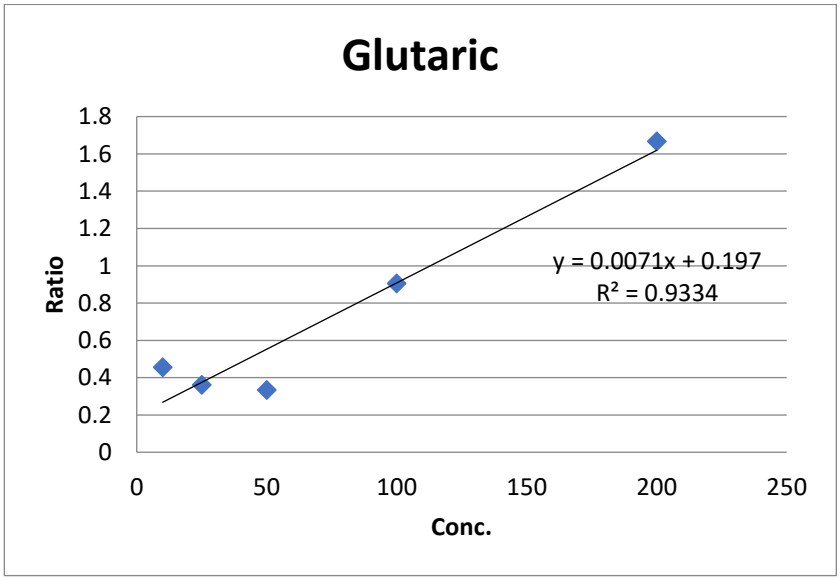

Figure 79 – Calibration curve of glutaric acid from 10 to 2000 uM normalized to tropic acid.

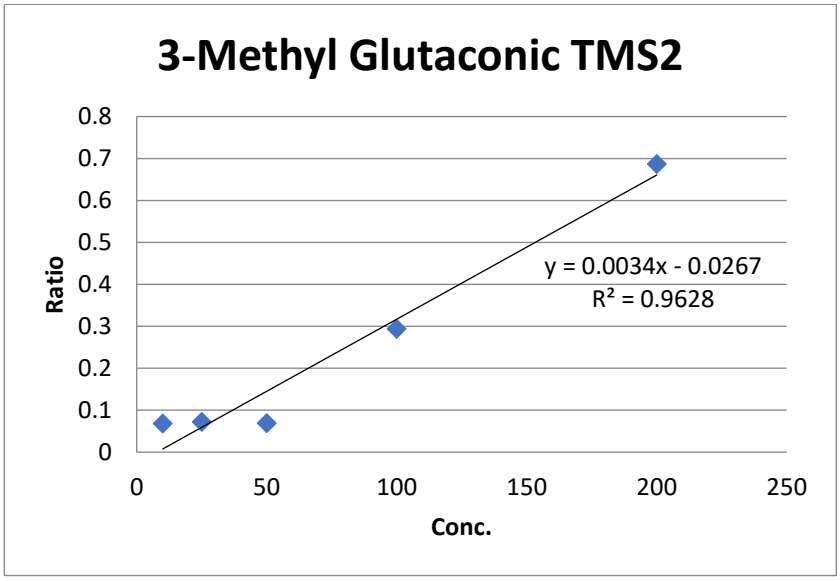

Figure 80 – Calibration curve of 3-methyl glutaconic acid TMS2 from 10 to 2000 uM normalized to tropic acid.

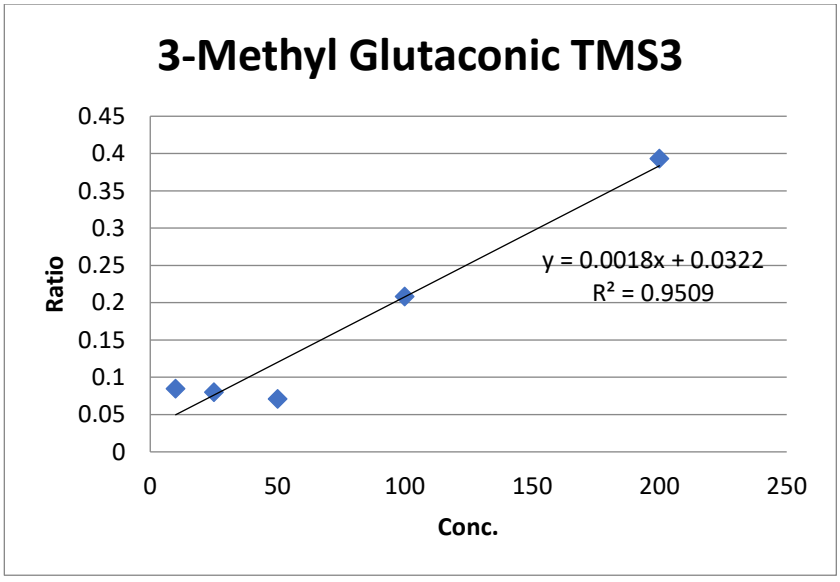

Figure 81 – Calibration curve of 3-methyl glutaconic acid from 10 to 2000 uM normalized to tropic acid.

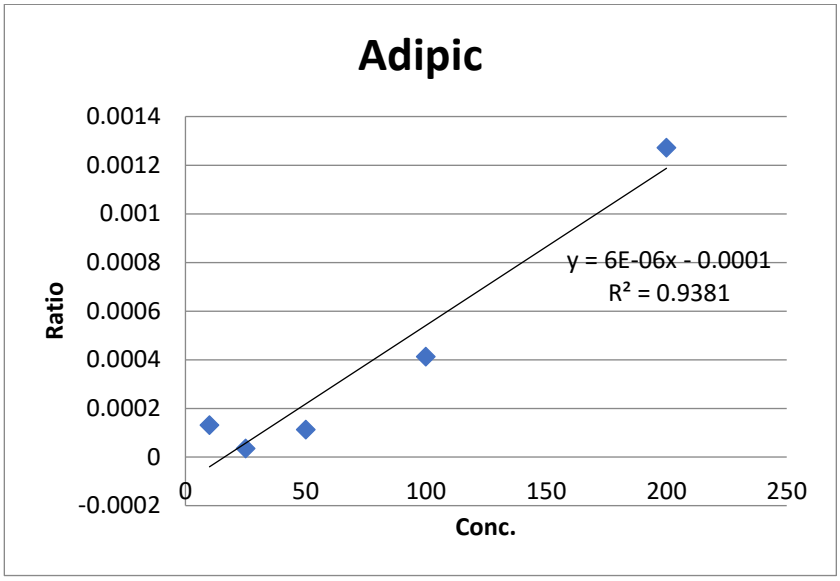

Figure 82 – Calibration curve of adipic acid from 10 to 2000 uM normalized to tropic acid.

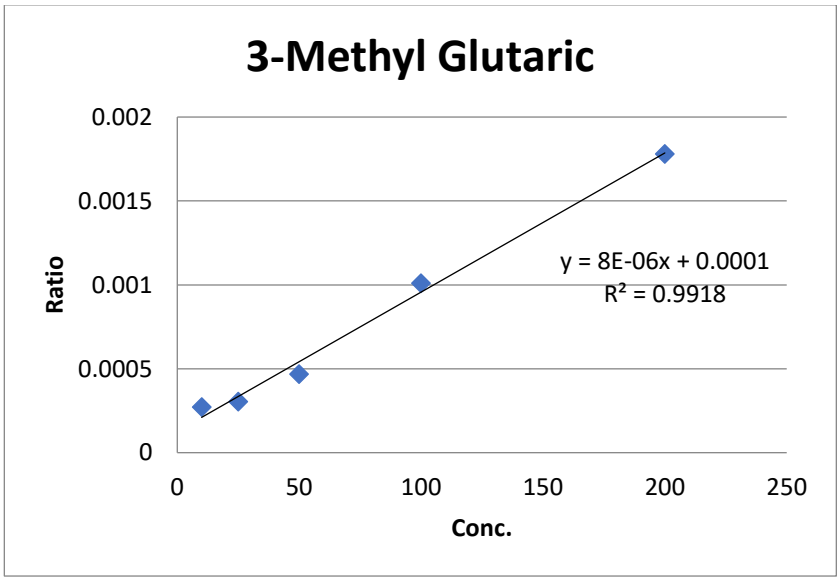

Figure 83 – Calibration curve of 3-mehtyl glutaric acid from 10 to 2000 uM normalized to tropic acid.

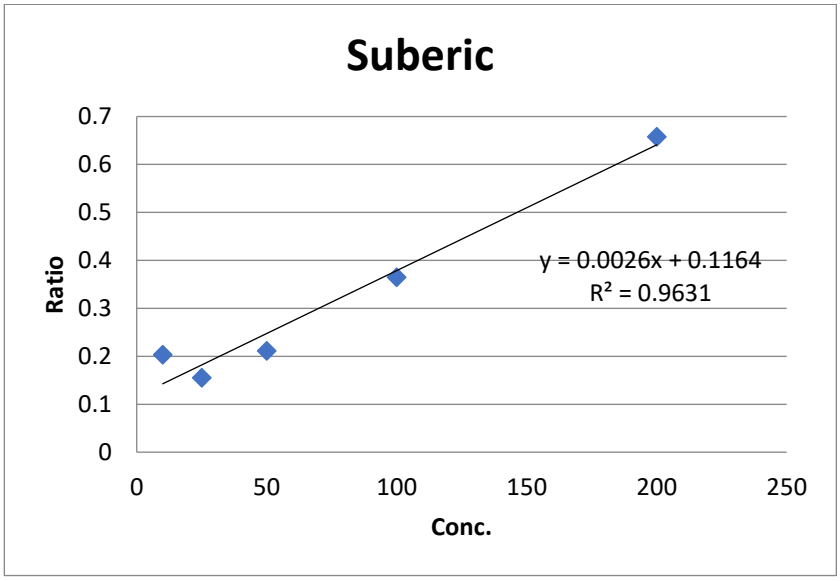

Figure 84 – Calibration curve of suberic acid from 10 to 2000 uM normalized to tropic acid.

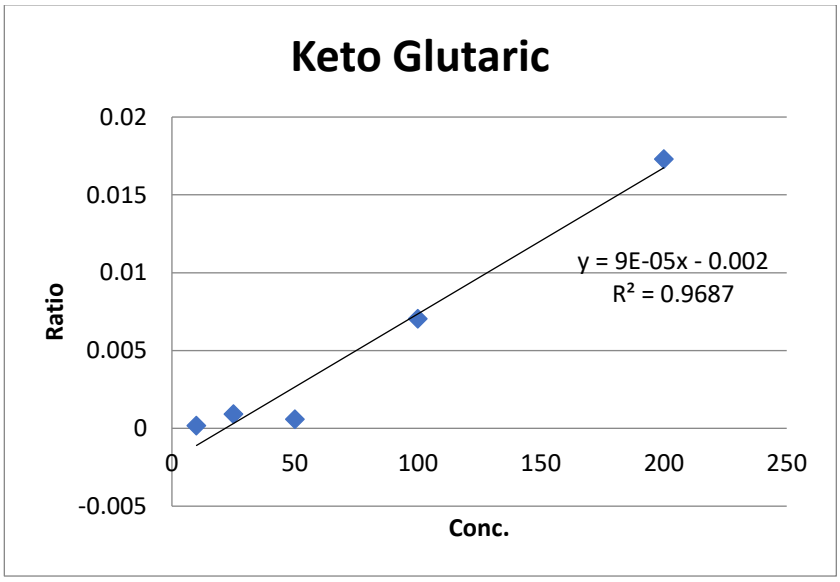

Figure 85 – Calibration curve of keto-glutaric acid from 10 to 2000 uM normalized to tropic acid.

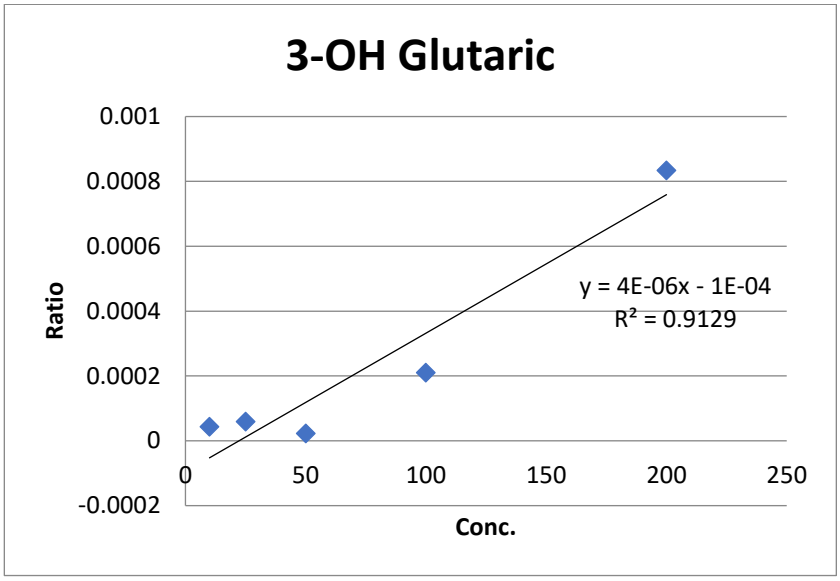

Figure 86 – Calibration curve of 3-OH glutaric acid from 10 to 2000 uM normalized to tropic acid.

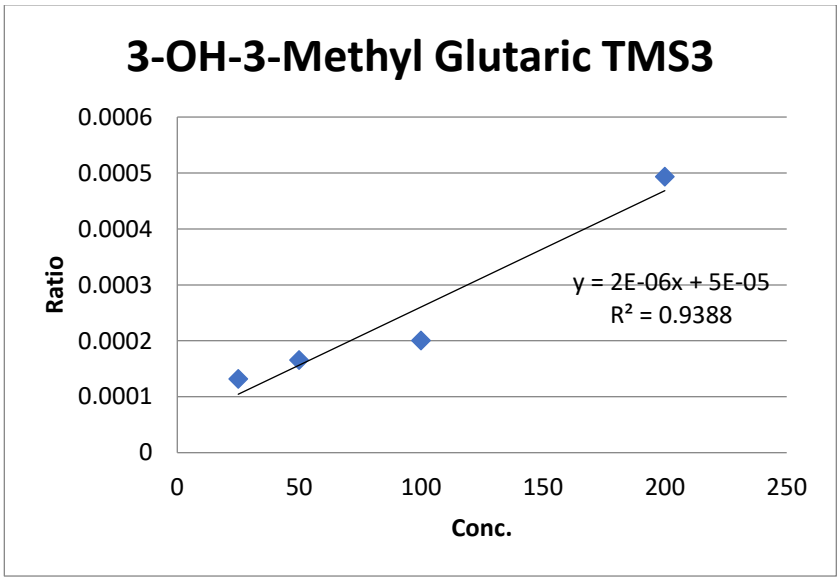

Figure 87 – Calibration curve of 3-OH-3-methyl glutaric acid TMS3 from 10 to 2000 uM normalized to tropic acid.

8.4 - Tables

Table 1 - Dilution calculations for individual extractions of UOAs of interest.

Compound	**MW (g/mol)**	**Mass of compound used in stock solution (mg)**	**Volume of stock solution (L)**	**Desired stock concentration (M)**
Tropic acid	166.17	41.5	0.050	0.005
2-Methyl Citric acid	206.15	51.5	0.050	0.005
2-OH Glutaric acid	148.114	37.0	0.050	0.005
3-Methyl Glutaconic acid	144.125	36.0	0.050	0.005
3-Methyl Glutaric acid	146.141	36.5	0.050	0.005
3-OH 3-Methyl Glutaric acid	162.141	40.5	0.050	0.005
3-OH Glutaric acid	148.114	37.0	0.050	0.005
3-OH Isovaleric acid	118.131	29.5	0.050	0.005
4-OH Butyric acid	104.105	26.0	0.050	0.005
Adipic acid	146.14	36.5	0.050	0.005
Creatinine	113.12	28.3	0.050	0.005
Ethyl Malonic acid	132.115	33.0	0.050	0.005
Fumaric acid	116.07	29.0	0.050	0.005
Glutaric acid	132.12	33.0	0.050	0.005
Hexanoylglycine	173.21	43.3	0.050	0.005
Isovalerylglycine	159.183	39.8	0.050	0.005
Keto glutaric acid	146.11	36.5	0.050	0.005
Methyl Malonic acid	118.091	29.5	0.050	0.005
Mevalonic acid	148.16	37.0	0.050	0.005
N-acetyl Aspartic acid	175.139	43.8	0.050	0.005
Pyroglutamic acid	129.04	32.3	0.050	0.005
Sebacic acid	202.25	50.6	0.050	0.005
Suberic acid	174.2	43.6	0.050	0.005
Tiglylglycine	157.167	39.3	0.050	0.005
Vanillactic acid	212.199	53.0	0.050	0.005