UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Químicas e Ingeniería

TÍTULO: Determination of the biological activities of the components present in the venom from the endemic Ecuadorian scorpion *Teuthraustes aff. atramentarius*

Trabajo de integración curricular presentado como requisito para la obtención del título de Químico

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Dedicatoria

Todos mis logros los dedico en primer lugar al Divino Niño Jesús por las bendiciones de sabiduría y paciencia que han sabido guiar mis pasos.

La presente tesis, asimismo como todos mis logros, se los debo a mi familia. Infinitas gracias a mis padres y hermana por el constante apoyo en todos los proyectos emprendidos. Hoy soy la persona y el profesional que soy, gracias al ejemplo constante de mi familia.

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Abstract

Ecuador, in spite of its small land area, harbors a great diversity of scorpion species. Most of the endemic Ecuadorian scorpions have an abundant presence within the big cities. The endemic scorpion species *Teuthraustes aff. atramentarius* is largely distributed in the Andean region and has a great presence in the metropolitan area of Quito. The significant interaction of these scorpions with humans and their activities may lead to accidental stings. There is no information on the toxicity and biological activities of the venom of any species of scorpions in the country.

This study describes the biological activity of the components present in the venom of the Ecuadorian endemic scorpion *Teuthraustes aff. atramentarius*. The venom was fractionated by Reversed-Phase High-Performance Liquid Chromatography, and the molecular weights of the separated components were determined by mass spectrometry. Separated components were tested to determine their enzymatic, antibacterial, and toxic activities. The venom of *Teuthraustes aff. atramentarius* presented hyaluronidase activity, and several fractions showed toxicity against insects. The venom did not present any antibacterial properties against *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Escherichia coli* strains.

One of the toxic components was sequenced, and its amino acid sequence has similarities with an insecticidal component isolated from the African scorpion *Hadogenes troglodytes*.

Key-words: scorpion, venom, enzymatic activity, toxicity.

Resumen

Ecuador, a pesar de su pequeña superficie territorial, alberga una gran diversidad de especies de escorpiones. La mayoría de los escorpiones endémicos ecuatorianos tienen una presencia abundante dentro de las grandes ciudades. La especie de escorpión endémica *Teuthraustes aff. atramentarius* está ampliamente distribuida en la región andina y en el área metropolitana de Quito. La interacción significativa de estos escorpiones con los humanos y sus actividades puede provocar picaduras accidentales. A pesar de esto, no hay información sobre la toxicidad y las actividades biológicas del veneno de ninguna especie de escorpión en el país.

Este estudio describe la actividad biológica de los componentes presentes en el veneno del escorpión endémico ecuatoriano *Teuthraustes aff. atramentarius.* El veneno fue fraccionado por cromatografía líquida de alta eficacia en fase reversa y las masas moleculares de los componentes fueron determinadas por espectrometría de masas. Los componentes separados se probaron para determinar sus actividades enzimáticas, antimicrobianas y tóxicas. El veneno de *Teuthraustes aff. atramentarius* presentó actividad de hialuronidasa, así como varias fracciones presentaron toxicidad contra insectos. El veneno no presentó ninguna propiedad antimicrobiana contra cepas de *Staphylococcus aureus*, *Pseudomonas aeruginosa* y *Escherichia coli*.

Uno de los componentes tóxicos fue secuenciado y tiene similitud de secuencia con un componente insecticida aislado del veneno del escorpión africano *Hadogenes troglodytes*.

Palabras clave: escorpión, veneno, actividad enzimática, toxicidad

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List of Abbreviatures

aff.: affinis

$CaCL_2$: calcium chloride

cDNA: complementary deoxyribonucleic acid

Da: Daltons

DNA: deoxyribonucleic acid

ESI-MS: Electrospray Ionization Mass Spectrometry

H. troglodytes: *Hadogenes troglodytes*

HA: hyaluronic acid

KDa: kilo Daltons

Kv: voltage-gated potassium channel modulators

LD_{50}: median lethal dose

MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry

mAU: milli-absorbance units

MHA: Mueller Hinton agar

MHB: Mueller Hinton broth

mRNA: messenger ribonucleic acid

MS: mass spectrometry

Nav: voltage-gated sodium channel modulators

P. transvaalicus: Parabuthus transvaalicus

PAGE: Polyacrylamide Gel Electrophoresis

PLA_2: phospholipase A_2

RP-HPLC: Reversed-Phase High-Performance Liquid Chromatography

SDS: sodium dodecyl sulfate

T. asthenes: Tityus asthenes

T. obscurus: Tityus obscurus

T. aff. atramentarius: Teuthraustes affinis atramentarius

TEMED: tetramethyl ethylenediamine

TFA: trifluoroacetic acid

UK: United Kingdom

UNAM: Universidad Nacional Autónoma de México

USA: United States of America

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1. Introduction and Justification

1.1. Scorpions: morphology, diversity, and scorpionism in Ecuador

Scorpions are venomous predatory arthropods belonging to the class Arachnida and the order Scorpiones. It harbors about 2,100 extant species fitting in 19 families, of which three include species hazardous for humans [1]. Scorpions are known as the most antique arachnids that first appeared in the late Silurian period 435 million years ago and have not changed on its main characteristics throughout time [2].

The morphology of scorpions fits the common characteristics of the class Arachnida. They have a segmented body, chelicerae (appendages near the mouth) and eight eyes, and eight walking legs (Figure 1ab) [3]. In general, the scorpion is segmented in two major sections: the prosoma or cephalothorax and the opisthosoma, which is subdivided in mesosoma and tail-like metasoma [2].

The prosoma houses eight eyes that are distributed in groups of four on each side and the eight walking legs in the same fashion. From the prosoma are also originated the chelicerae and a pair of pedipalps (pincers).

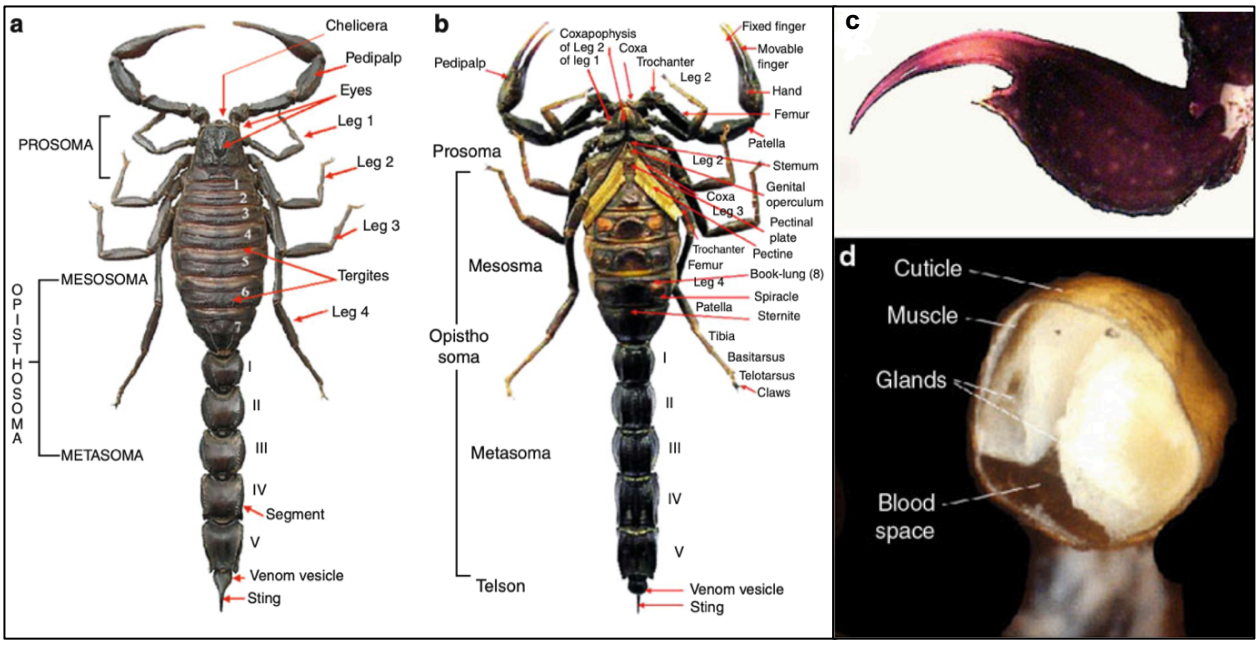

Figure 1. **Morphology of scorpions.**
a) *Androctonus crassicauda* dorsal view. b) *Androctonus crassicauda* ventral view. c) *Tityus obscurus* telson with subaculear protuberance close-up. d) Section of the venom vesicle of *Euscorpius tergestinus*. Reprinted from "Introduction to Scorpion Biology and Ecology", by Stockmann, 2015, Toxinology. Copyright 2015 by Springer Science+Business Media Dordrecht.

The mesosoma is the upper section of the opisthosoma. On its ventral side contains pectines, which are two comb-like appendages that protect the genital operculum and 3 to 4 pairs of spiracles connected to book-lungs for breathing.

The metasoma is divided into subsections called sternites that are covered by tergites (scale-like exoskeleton) on its dorsal side. Finally, the metasoma or tail is divided into five well-articulated segments. It ends in the telson, which contains a pair of venom glands that are connected to a sharp stinger at the end of section [4,5].

Scorpions have adapted to a wide range of environmental conditions and are widely distributed across the world, being present in all continents except Antarctica [6]. The most widespread scorpion family is Buthidae, with currently over 1127 species dispersed throughout the world. The most important genera within this family are *Androctonus*, *Buthus* in northern Africa and Asia, *Parabuthus* in southern Africa, *Centruroides* in North America, and *Tityus* in South America [2]. The individuals from these genera are known for harboring the most dangerous scorpions for humans and are responsible for several envenomation cases reported worldwide [7]. Mexico is considered the most diverse country in terms of scorpions, housing over 281 described species that represent 12% of the whole scorpion diversity of the world [8].

Ecuador, regardless of its small size, it is also a very diverse country ranking as the 10th most diverse country in the world [9]. Such biodiversity is echoed to scorpions variety, being the tropical rainforest and Andean moors acknowledged centers of diversity of unique species of scorpions [10].

Indeed, Ecuador harbors over 47 species of scorpions, of which 33 are endemic, and are comprehended on four families and seven genera. The Andean region accommodates 22 species in spite of the high altitudes of the cities. The coastal and Amazonic regions house 14 and 11 scorpion species. The Amazonic region has a higher content of Buthidae family members, which encompass species of clinical interest [11].

The most representative genera of endemic Ecuadorian scorpions are *Tityus* (Buthidae) and *Teuthraustes* (Chactidae) with 8 and 13 endemic species respectively [11]. For the genus *Teuthraustes*, Ecuador has been described as a center of endemism, since the country houses 13 species over the 24 described for the genus. These scorpions are mainly found in the Andean region of Ecuador as well as in Colombia, Venezuela, Brazil, and Peru [12]. The most abundant and widespread scorpion in the Andean region of Ecuador is *Teuthraustes atramentarius*, being

present in the provinces of Imbabura, Pichincha, Cotopaxi, Tungurahua, Chimborazo and certainly the most abundant scorpion in the metropolitan area of Quito [11,13].

Despite the great scorpion diversity that Ecuador displays, there is no information on the toxicological aspects of any Ecuadorian endemic scorpions, even though there are over 16 species that are considered of clinical importance [11]. Scorpionism, which is the phenomenon of grouped cases of envenomation by scorpion stings, has become a public health issue in many countries. The incidence of scorpion stings envenomation has been majorly reported in northern Sahara, Middle East, Mexico, Brazil, and Venezuela [14].

However, Ecuador has had isolated cases of scorpionism. The most known cases occurred in 2014 in Manabí, where over 30 cases of stings occurred, and two infants of less than two years old died by cardio-respiratory failure caused by *T. asthenes* stings [15]. Other known cases occurred in 2015-2016 in Morona Santiago, where over 20 cases of scorpion stings happen, and three infants (3-4 years old) died by pulmonary edema, and cardio-respiratory failure produced by *T. asthenes* and *T. obscurus* stings [16].

Yet, popular knowledge reports a greater incidence of scorpion stings but without any signs or symptoms of envenomation. Among the most reported cases, there are stings of *Teuthraustes atramentarius* in the city of Ambato and Quito as well as various reports of stings by *Centruroides margaritatus* in Guayas province [11,17].

1.2. Composition of scorpion venoms.

Venomous animals are widely spread throughout the animal kingdom. Various animals produce venoms through specialized glands and injection apparatus; many others have toxic substances on tissues or specialized cells. Individuals of different species have evolved and developed these substances that are secreted with many different biological functions that go from defense to digestion aid [18].

Animal venoms are exceptionally rich and complex mixtures of molecules that interact to perform various biological actions. Venoms are commonly aqueous solutions of a vast number of components, mainly of peptide-protein nature[18]. The toxicity of the venoms directly depends on the composition, which in turn is particular for the needs of each individual or species [19].

In the case of scorpions, all of them are known to be venomous [2]. These arachnids have a pair of specialized glands that are connected to a wounding apparatus to inject the venom (Figure 1cd). These glands have evolved to produce enough venom and with different compositions to be able to sting several times, depending on the situations [5,20]. Therefore, a vast amount of components and different venom compositions are secreted by these arachnids, depending on whether it is hunting or needs to defend itself from threats [19].

Studies on the variation of the composition of the venom from individuals of the same species have proven the fact that an individual can actually vary the composition of its venom depending on the conditions of the sting. Nisani (2011) reported the use of MALDI-TOF MS to determine the molecular weight of the main components of the venom from *Parabuthus transvaalicus* stings conducted under different threat conditions and found that some components successively appeared over the different threat levels. Still, others were characteristic of each condition (Table 1) [20].

Table 1. **Metering by mass spectrometry of the venom components from *Parabuthus transvaalicus* stings at different levels of threat.**

Comparison of molecular weight values of venom composition among different *P. transvaalicus* stings under two levels of threat (high and low) analyzed by MALDI-TOF MS [20].

Stinging sequence									
1st sting		2nd sting		3rd sting		4th sting		5th sting	
High	Low	High	Low	High	Low	High	Low	High	Low
	4084.11[a]					**4081.41[a]**			**4083.16[a]**
									4291.41
			4748.21		4748.59			4748.40	
5048.92	5050.58	5049.13	5048.53	5048.92	5049.45	5048.72	5049.27	5048.17	
5256.17		5257.38	5256.92	5257.33	5257.22	5256.71	5257.46	5257.11	
						6543.09[b]	**6544.30[b]**	**6543.33[b]**	**6545.21[b]**
		6604.21[c]	**6604.06[c]**	**6604.18[c]**	**6604.12[c]**	**6603.88[c]**	**6604.53[c]**	**6603.80[c]**	**6604.82[c]**
		6645.26[d]	**6644.97[d]**	**6645.11[d]**	**6645.33[d]**	**6644.99[d]**	**6645.57[d]**	**6644.97[d]**	**6646.02[d]**
						6811.13			
7219.37[e]	**7221.38[e]**	**7220.14[e]**	**7219.42[e]**	**7220.14[e]**	**7220.05[e]**	**7220.27[e]**	**7220.40[e]**	**7219.72[e]**	**7221.25[e]**
			7222.08		7222.15				
7277.73	7279.55				7278.07		7278.99		7279.38
7298.65	7300.67		7298.91				7299.99		7300.23
		7335.10	7334.38	7335.04	7334.83	7335.28	7335.77	7334.69	
7390.39	7391.93	7391.27	7390.55	7391.14	7390.93	7391.01	7391.72	7391. 05	7392.07
									7428.05
	7445.92						7445.88		
									7505.55
7514.76	7516.75	7515.60	7514.98	7515.63	7515.29	7515.44	7516.20	7515.50	

Majorly, scorpion venoms presented over 70% of peptide-nature components, of which the majority are low molecular weight peptides. These complex peptide mixtures have been reported from 72 up to more 600 different components. It is known that those venoms containing large variety peptides have components of low molecular weight (<1500 Da), and virtually nothing is

known about their functions [21]. The other 30% of the venom are water, inorganic salts, and some traces of sugars and free ions [22].

Among the protein components of the venoms, voltage-gated ion channel modulators are strongly present in scorpion venoms [23]. These toxins are specialized in regulating the flow of ions across voltage-gated ion channels within the cell membrane. These channels are responsible for the communication of the neural systems. Hence, the toxins are dedicated to interrupt inter-neuron communication to induce systemic neural system arrest upon their targets [24,25].

The most common voltage-gated ion channel modulators present in scorpion venom are sodium and potassium channel modulators [26,27]. These neurotoxins are responsible for an increasing number of human pathologies, including autoimmune disorders and inflammatory neuropathies and neural system arrest [28,29].

Other known components found in scorpion venoms are enzymes. The most common enzymes found in scorpion venoms are PLA_2, hyaluronidase, serine proteases, and metalloproteases. These components are believed to have a direct influence on the envenomation syndrome acting as mediators for the inflammatory response or spreading factors for the venom distribution within the organism after the sting [28].

To determine whether the venom of a specific scorpion species is toxic enough to be considered harmful to humans is necessary to study its composition. The utilization of various experimental methods is essential in the acquisition of knowledge on the composition of venom. These methods allow the testing of the biological activities of the different components present in the venom.

1.3. Study of the biological activities of the components present in scorpion venoms

Initially, to be able to study scorpion venoms is important to have methods for extracting venom from the individuals. The "milking" process for scorpion requires to take into account various factors, such as the size of the specimen, venom dose per sting as well as the wellness of the individuals in captivity [30].

For this, various mechanisms and devices have been reported in the literature over the years. The first method used is the sting through parafilm [31]. The most common method for extraction of the venom is the electrical stimulation of the telson. The secretion of the venom is

controlled by muscles-like fibers surrounding the venom glands; electrical stimulation can cause a constant and strong contraction of these muscles, and thus, the venom glands are depleted from its venom content at the time [32].

The best site for the application of the electricity is the base of telson, and with no more than 30 V for no longer than 3 to 5 seconds. Over 30 V applied, individuals present stiffness in the articulations, and further death was observed for various specimens from *Androctonus* and *Mesobuthus* species. The best results on venom collection and for maintenance of individuals was 25 V for 3-5 seconds [33].

Once the venom is collected, the study of it can be taken from two approaches: the molecular characterization of the components and the determination of the biological activities of them (Figure 2) [34].

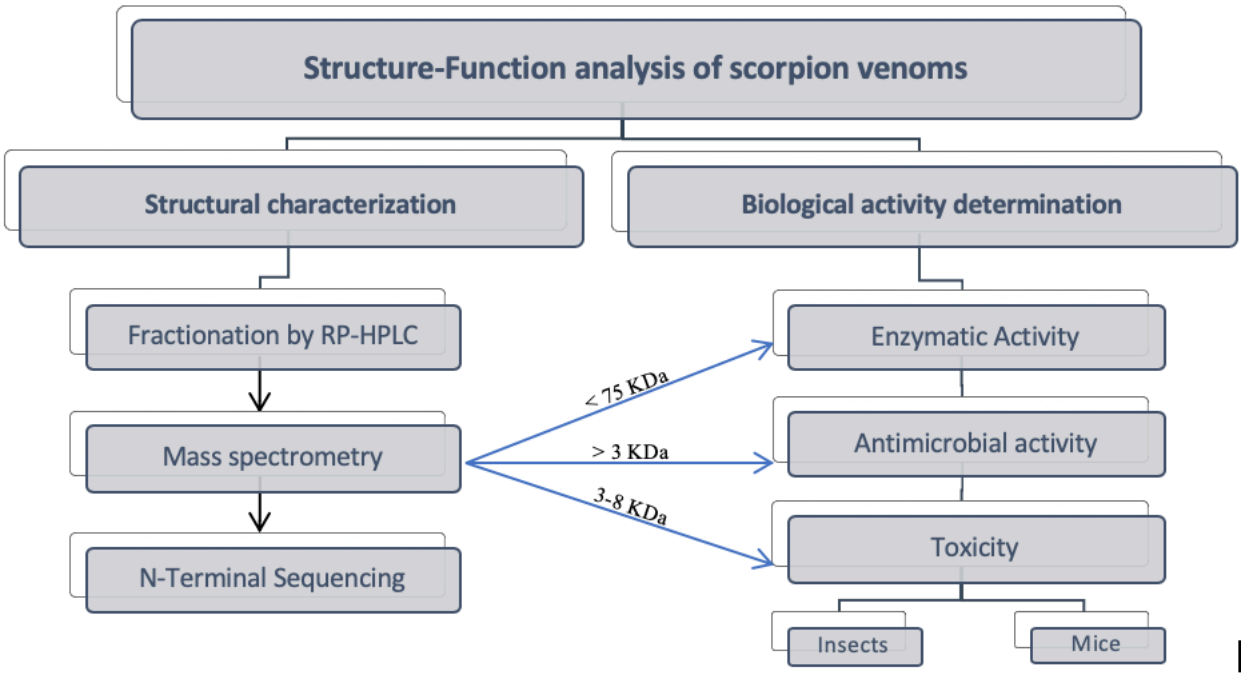

Figure 2. **Overview scheme of a structure-function analysis approach for the study of scorpion venoms**

The molecular characterization comprehends various analyses that focus on the determination of the identity of the components present in the venom. A common approach to gathering information on the number of components is chromatography separation. RP-HPLC is often selected for the separation of the components. The separation follows the hydrophobicity of the molecules present in the venom that gives an appreciation of the possible size or type of molecules that conforms to the venom suspension [35].

Commonly, molecules bigger in size tend to be more hydrophobic than those of lower molecular weights. Additionally, after several studies performed among scorpion venoms, it is known that the common toxins present in the venoms elute first than those highly hydrophobic components, which usually are of enzymatic nature [36].

Following the separation, the identification of the molecular weights of the components is commonly performed by mass spectrometry. The mass fingerprinting of the venoms gives an idea of the composition of the venom and provides suitable information to select fractions for further analysis, such as sequencing [37].

Three known methods can achieve the sequencing of the components: Edman degradation, mass spectrometry, and prediction from mRNA sequences [38]. Edman degradation follows up a reaction where the peptide attached to a solid matrix reacts with phenyl isothiocyanate that cleaves the N-terminal of a peptide giving amino acids as sub-products identified orderly by HPLC [39].

Tandem mass spectrometry or Ms/Ms is used for the sequencing of proteins. The method uses two or more mass spectrometers coupled together to introduce another fragmentation step and identify the amino acids that form a peptide by its molecular weights [40].

Prediction from the mRNA sequence follows the fact that the body synthesizes proteins following the transcription of genes into mRNA, and this is further traduced to proteins. From the mRNA collected from the venom glands, a cDNA library is constructed and further analyzed by bioinformatics to predict the protein sequences to be traduced by the collected RNA [41].

Starting from the molecular characterization, it is necessary to submit the components found in the venom to several tests to find out the biological function that each component has in the effects generated by the venom. Scorpion venoms possess many components with very different biological functions that participate in the envenomation mechanism or stabilize the venom suspension as well as others with a yet unknown function [28].

Gathering information from the molecular characterization and the common components known to be present in scorpion venoms, some targeted studies can be performed to determine its biological activity.

Understanding the enzyme composition of the venoms gives information on the probable envenomation symptoms [28]. A commonly used method to determine the enzymatic activity of the components is zymography. This electrophoretic technique uses SDS-PAGE conditions with

polyacrylamide gels copolymerized with specific substrates to test the specific enzymatic activity of the separated proteins [42]. This method is commonly used to test hyaluronidase and protease [43]. Furthermore, in the case of protease, some inhibitors may be added to test with precision an specific kind of protease presents such as serine or metalloproteases [44].

Other qualitative methods to determine PLA_2 activity have been described. A specific plate test uses and agarose gel mixed with egg yolk in Triton X-100 in a Petri dish and incubated with venom samples to determine the activity of all kinds of phospholipases [45]. Another technique consists of a colorimetric assay using a titration reaction to determine the digestion of phospholipids by PLA_2 [46].

The enzymatic activities can also be determined quantitatively. A method developed by Pukrittayakamee et al. (1988), test the hyaluronidase activity by measuring the optical density of a titration reaction and, by certain approximations, determine the amount of HA hydrolyzed per minute and milligram of protein [47].

Having an idea of the enzymatic activities that promote the envenomation syndrome of a scorpion sting, can be helpful with the development of antivenins and improve its efficacy through the inhibition of the enzymatic promotors of the envenomation [43,48]. The development of a venom specific antivenin serum for *Hemiscorpius lepturus* was enhanced by adding to the antibody serum, inhibitors for the gelatinolytic, caseinolytic, and hyaluronidase enzymes found in the venom of the scorpion [48 49].

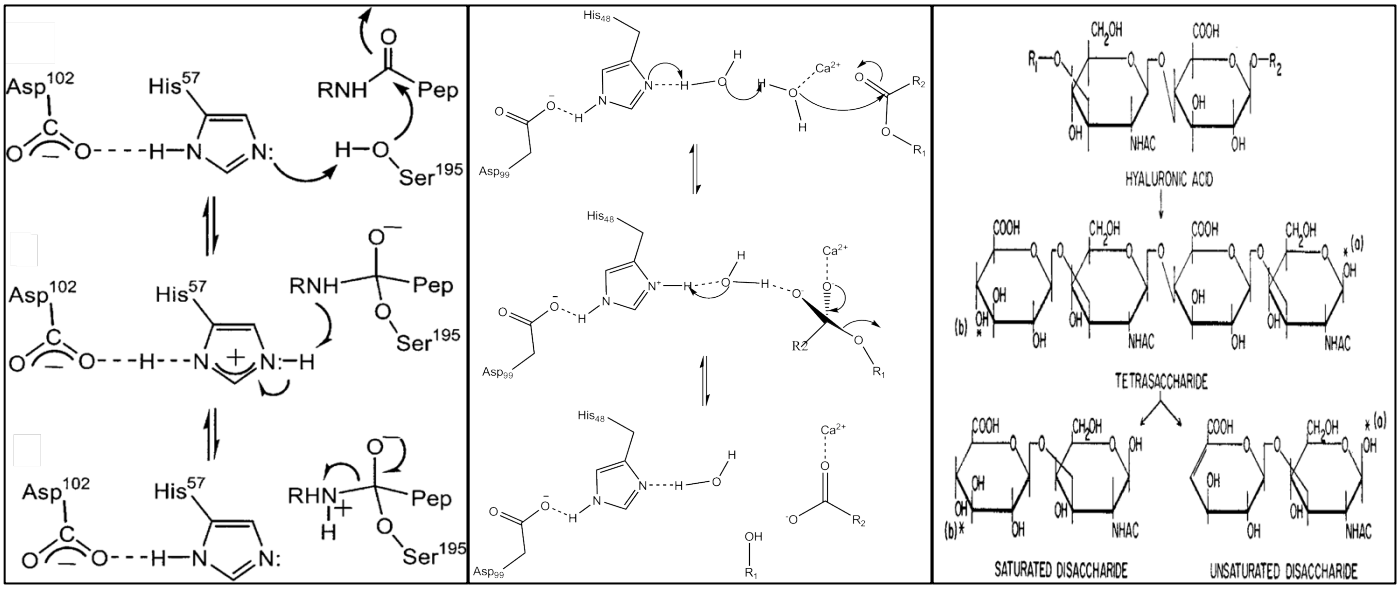

Figure 3. **Mechanisms of action of enzymes commonly found in scorpion venoms.**
a) Mechanism of hydrolysis of peptide bonds by action of a serine protease. b) Mechanism of hydrolysis of the second fatty acid in a phospholipid by action of a PLA_2. c) Mechanisms of degradation of HA by action of a hyaluronidase.
Reprinted from Lehninger, 2005, Freeman, " Principles of Biochemistry" .

Antibacterial properties have been found on some scorpion components. It is proposed that the presence of antibacterial peptides in the venom of some species is to constantly fight pathogenic infections that may reduce or constrain the production of venom [50].

The most common approach to screen the antibacterial properties of venom components is the agar method. This method consists of growing bacterial strains in Petri dishes filled with agar and incubated them with samples of target peptides, expecting a halo of growing inhibition. This method is commonly used along with *E. coli* ought to its higher susceptibility to dish assays [51].

Furthermore, to evaluate the strength of the antibacterial activity of certain components is necessary to find a minimal inhibitory concentration. For this, serial micro dilutions of the protein of interest in culture broth are tested against gram-positive and gram-negative bacterial strains as well as clinical isolates in a 96-well microplate. Then, the optical density of the solutions is measured to find the minimal concentration of the protein of interest that inhibits the growth of a certain bacteria strain [52].

The crescent problem of bacteria resistant to antibiotics has become a world public health crisis and reducing the clinical value of classical antibiotics [53]. Several antibacterial peptides have been isolated and presented important contributions to the fight against antibiotic-resistant bacteria and may suppose the future of the treatment of pathogenic infections [54].

For example, Vaejovine is an antibacterial peptide isolated from the scorpion *Vaejovis mexicanus*. After peptide purification, the isolated fraction containing the active compounds was cloned and sequenced to be further replicated by organic synthesis. Antibacterial assays have shown activity against *E. coli*, *S. aureus*, and other common nosocomial strains that cause clinical infections [55].

Finally, another important biological activity to test is the toxicity of the components found in scorpion venoms. Several approaches may be taken to determine whether a component is toxic or not depending on the target of the study.

The most common essay is to test the components of scorpion venoms against their natural prey like insects or other arachnids [56]. To achieve this, insects like crickets and arachnids like spiders are injected in the abdominal section with the fractions of interest, and the response of the organism is evaluated. Mainly, the parameters tested are paralysis considered the incapacity of standing up or death and the time of the effect after being injected [57].

However, to have a track of the toxicity of the components against mammals, the use of mice has been standardized [58]. To assess the direct toxicity of the venom, mice are injected intracranially, and the response expected is death. The time from injection to death is also evaluated. Additionally, intraperitoneal injections may be used to test lethality as a LD_{50} and intramuscular injections for other effects like local necrosis or muscular stiffness [59,60].

2. Problem Statement

The endemic Ecuadorian scorpion *Teuthraustes atramentarius* is the most abundant scorpion along the Andean region of Ecuador, especially over the metropolitan area of Quito. Their presence within big cities and their constant contact with humans and their activities lead to accidental stings all over the region. The lack of scientific knowledge on the toxicity and biological activity of the venom produced by these scorpions may cause trouble with the handle and clinical treatment of the stings. Having information on the biological activities that take part in the envenomation syndrome may aid in the treatment of these scorpion stings.

3. Objectives

3.1. General objective

- To determine the biological activities of the components, present in the venom of *T. aff. atramentarius.*

3.2. Specific objectives

- To find the components with enzymatic activity present in the venom of *T. aff. atramentarius.*
- To detect the components with antibacterial activity against gram-positive and gram-negative bacterial strains present in the venom from *T. aff. atramentarius*.
- To identify the toxic fractions of the venom from *T. aff. atramentarius.*

4. Methodology

- Reagents, Equipment, and Solutions

All chemicals used in this study were of analytical grade. All solvents used for chromatography were of HPLC grade.

- Chemicals

2-mercaptoethanol (Sigma-Aldrich, USA), acetonitrile (FisherScientific, Spain), acrylamide (Bio-Rad, USA), agarose (Bio-Rad, USA), ammonium persulfate (Sigma-Aldrich, USA), ampicillin (Sigma-Aldrich, USA), bromophenol blue (FisherScientific, Spain), $CaCl_2$ (Sigma-Aldrich, USA), Coomassie blue R250 (Bio-Rad, USA), ethanol and formamide (Fisher Bioreagents, USA), glacial acetic acid and hyaluronic acid (FisherScientific, Spain), isopropanol, methanol and MHB (ThermoScientific, USA), N,N'-methylenebisacrylamide (Bio-Rad, USA), NaCl (Sigma-Aldrich, USA), polybrene (Sigma-Aldrich, USA), Rhodamine 6G (ACROS Organics, UK), SDS (Bio-Rad, USA), sodium acetate (Fisher Chemicals, UK), Stains-All (Sigma-Aldrich, USA), TEMED (Bio-Rad, USA), TFA (Sigma-Aldrich, USA), Tris-HCl pH 10 (Sigma-Aldrich, USA), Triton X-100, (Bio-Rad, USA), Molecular weight standard for Tris-HCl SDS-PAGE (Bio-Rad Precision PlusProtein DualXtra standard).

- Other Materials

Egg yolk (Obtained from commercial eggs), gelatin (Bio-Rad, USA. 1706537), *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442, *E. coli* ATCC 25922 (Strains from Sigma-Aldrich, USA)

- Solutions and Buffers
    - HPLC Solution A: 0.1% aqueous TFA.
    - HPLC Solution B: 0.1% TFA in acetonitrile.
    - Reducing conditions sample buffer: 10% glycerol, 2.5% SDS, 50 mM Tris-HCl pH 6.8, 5% 2-mercaptoethanol and 0.002% bromophenol blue in water.
    - Tris-HCl pH 6.5 buffer: 5mL of 1 M Tris-HCl, pH 6.5, 12.5 mL of 20 mM $CaCl_2$ and 250 µL Triton X-100 in water.
    - Egg yolk reaction mixture: 0.2g agarose in 10 mL 0.2 M Tris-HCl, 1 mL of 20 mM $CaCl_2$, 0.5% aqueous Rhodamine 6G solution, 100 µl Triton X-100, 2mL of egg yolk solution in water.
    - Acetate buffer pH 3.6: 92.6% 0.2 M glacial acetic acid, 7.4% 0.2 M sodium acetate.
    - Formamide buffer: 5% formamide, 20% isopropanol and 15 mM Tris-HCl pH 7.95 buffer.

- Equipment
    - Nanodrop-1000 micro-spectrophotometer (Thermo Scientific, USA)
    - 1290 Infinity II HPLC system (Agilent Technologies, Mexico)
    - Reversed-phase C18 column (Sigma-Aldrich Supelco C-18 reverse phase column, 25cm, 4.6mm, 5µm)
    - Savant SpeedVac SPD2030 vacuum concentrator (ThermoFisher, Spain)
    - LCQ Fleet ion trap mass spectrometer (Thermo Scientific, USA)
    - Shimadzu PPSQ-31A automated gas-phase protein sequencer (Shimadzu, Japan)
    - Sunrise™ microplate reader (Tecan Ltd., USA).

- Venoms

Venom samples from species *Brachypelma vagans, Bothrops ammonytoides, Tityus pachyurus,* and *Centruroides granosus* were gently donated by Dr. Gerardo Corzo from the Institute of Biotechnology, UNAM, Cuernavaca, Mexico.

4.1. Scorpions

A pool of 20 scorpions was collected in Ambato, Tungurahua, Ecuador, within the locations comprehending the following coordinates: Ficoa [1°14'32.1"S 78°37'58.2"W] and Izamba [1°14'2"S 78°35'19.0"W].

Individuals were kept in captivity in individual recipients emulating their natural habitat and stored within the laboratory of Yachay Tech University at room temperature. Scorpions were fed with spiders once a week and hydrated twice a week with a humid sponge.

4.2. Venom extraction.

The venom of *T. aff. atramentarius* was obtained by electrical stimulation of the telson, applying 24V in intervals of 5 seconds by modified tweezers (Figure 2). Venom extraction was performed once every two weeks upon all individuals kept in captivity.

Venom extracted was dissolved in water and stored at -20 ℃. After several extractions, collected venom was dissolved in distilled water in proportion 1:5, centrifuged to 7000 g for 5 min, the supernatant was collected and lyophilized. The dry material was kept at -20 ℃.

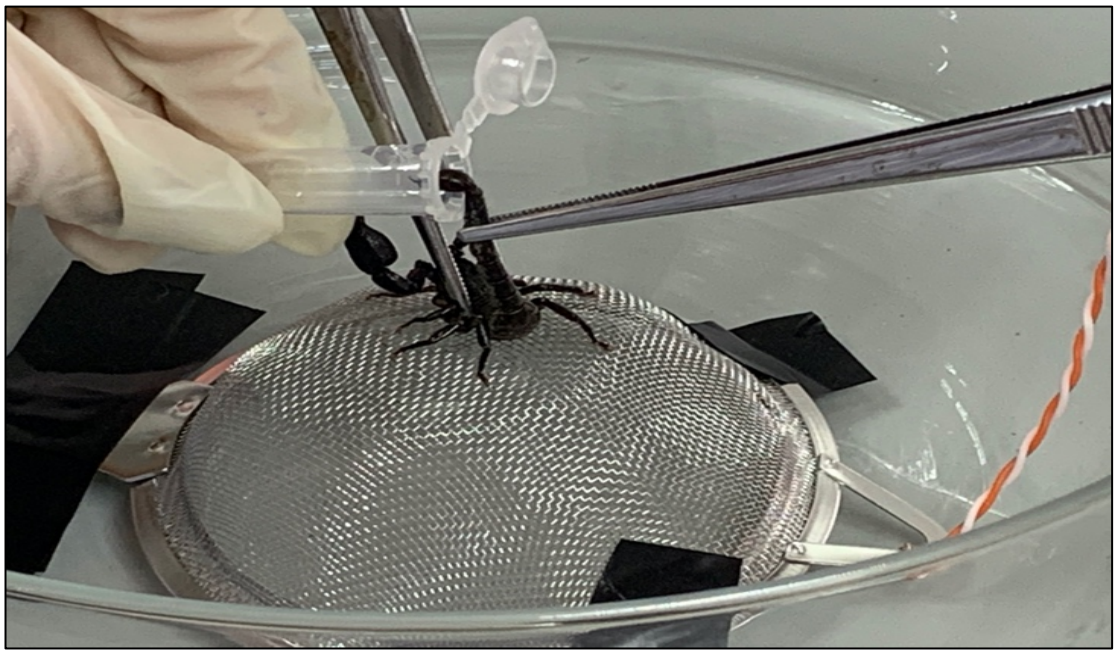

Figure 4. **Extraction of the venom by electrical stimulation of the telson from a *Teuthraustes aff. atramentarius* individual.**

4.3. Venom quantification

Lyophilized venom was weighted and resuspended with 700 µL of 0.1% aqueous TFA. The protein concentration was determined by measurement of the absorbance of the venom solution at λ = 280nm using a Nanodrop micro-spectrophotometer, assuming 1 mAU equals to 1 mg/mL.

4.4. Fractionation of the venom by Reversed-Phase High-Performance Liquid Chromatography

Sample of 2 mg was fractionated by HPLC system equipped with a reverse-phase C18 column and eluted with a linear gradient from 0% to 60 % HPLC Solution B for 75 minutes at 1mL/min flow rate [61]. The eluted fractions were scanned by absorbance measurement at λ = 230nm. Fractions were collected in 1.5 mL micro-tubes and dried under a high vacuum in a vacuum concentrator. Fractions were resuspended in 50µL of water, and their concentration was determined by absorbance measurement at λ = 280nm using a Nanodrop micro-spectrophotometer assuming 1 mAU equals to 1mg/mL and stored at -80 °C [61].

4.5. Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis

The crude venom from *T. aff. atramentarius* was analyzed by SDS PAGE following the method described by Laemmli (1970) [62], using 4% polyacrylamide stacking gel and 12.5% polyacrylamide separating gel. 10 µg of the molecular mass standard was run in parallel to estimate the molecular weights of proteins present in the venom. Alongside with *T. aff. atramentarius* venom sample was analyzed in the same gel samples of the venom from the Panamanian scorpion species *Tityus pachyurus* and *Centruroides granosus*. Electrophoresis was conducted for 15 minutes at 50 V in the stacking gel and 45 minutes at 120 V in the separating gel. The gel was stained with 1% Coomassie blue R250 in a 10% methanol solution and distained after 2 hours with an aqueous solution with 10% methanol and 10% glacial acetic acid and scanned with an Epson Artisan 830 flat scanner to a Macintosh computer for further analysis.

4.6. Mass Spectrometry for determination of the molecular weights of the fractions.

The venom fractions obtained by RP-HPLC were reconstituted to a final concentration of 500 pmol/ 5 µL of 50% acetonitrile with 1% acetic acid in water. Resuspended fractions were applied directly into the spectrometer using an MS syringe pump delivery system. Samples were eluted at 10 µL/min and split to introduce 5 % of the sample to the nano-spray source. The spray voltage was set to 1.5 kV and the capillary temperature to 150 °C [63]. All spectra were acquired by positive-ion mode and analyzed on an Xcalibur data system. Average molecular masses have an error of ±1 Da due to instrumental error window [61].

4.7. N-Terminal sequencing

Edman degradation was performed on an automated gas-phase sequencer. Samples of 15 µg of purified fractions obtained by RP-HPLC were dissolved in 10 µL of 37% (v/v) acetonitrile solution and applied to TFA-glass fiber membrane pre-cycled with polybrene [61].

4.8. Tests for enzymatic activities

4.8.1. Phospholipase A_2 activity test

Phospholipase A_2 activity of *T. aff. atramentarius* venom was measured by the method described by Habbermann and Hardt (1972) [45] with some modifications. Briefly, egg yolk solution was made by dissolving 2g of egg yolk in Tris-HCl buffer pH 6.5 at a concentration of 10% w/v. A Petri dish was filled with 20 mL of the reaction mixture agarose gel. Venom samples (5, 10 and 20 µg), positive control (15 µg *Bothrops ammonytoides* venom sample) and negative control (10 µL dH_2O) were loaded in agarose gel pots and incubated for 3h at 37 °C. Positive results are shown as decolorized halos around sample pots.

4.8.2. Protease activity test

Protease activity of *T. aff. atramentarius* venom was determined by zymography as described by Leber and Balkwill (1997) [42] using 12.5% polyacrylamide gel with 1.5 mg/mL gelatin concentration and 4% polyacrylamide stacking gel in non-reducing conditions (sample buffer does not contain 2-mercaptoethanol). Positive control (5µg of *Bothrops ammonytoides* venom), 5 µg of whole venom sample, and 5 µg of major fractions were loaded into gel pots, and

electrophoresis was conducted under the same conditions for SDS-PAGE. After electrophoresis, the zymogram was incubated for 1h in 5% Triton X-100 with 0.1M Tris HCl buffer pH 8, then 1h in 0.05% Triton X-100 with 0.1M Tris HCl pH 8 buffer and incubated overnight. Positive results are shown as decolorized bands on activity sites due to the degradation of gelatin.

4.8.3. Hyaluronidase activity test

Hyaluronidase activity of *T. aff. atramentarius* venom was evaluated by zymography with the method described by Steiner and Cruce (1992) [64] with some modifications. Electrophoresis gels were 12.5% polyacrylamide with 0.5 mg/mL HA concentration and 4% polyacrylamide stacking gel. A sample buffer for non-denaturing conditions was used. Positive control (10 µg of *Brachypelma vagans* venom*)* and 10 µg of whole venom samples were loaded, and electrophoresis was conducted in the same conditions for SDS-PAGE. After electrophoresis, the zymogram was incubated for 1h in acetate buffer pH 3.6 with 0.15M NaCl and 5% Triton X-100, then another 1h in the same buffer with 0.05% Triton X-100 and incubated overnight. Afterward, the zymogram was stained for 5h with 0.1% Stains-All solution in the formamide buffer. After that, the gel was distained with formamide buffer. Positive results are shown as decolorized bands on activity sites due to the degradation of HA.

4.9. Antibacterial activity assays

Two assays were performed to evaluate the antibacterial properties of the venom of *T. aff. atramentarius.* Both methods are described and approved by The Clinical and Laboratory Standards Institute (2010) [52]

4.9.1. Bacterial growth inhibition in serial micro dilutions

The first assay consists of the evaluation of different concentrations of venom in MHB against gram-positive *Staphylococcus aureus* ATCC 6538 and gram-negative *Pseudomonas aeruginosa* ATCC 15442 bacterial strains. First, in a 96-well microplate, rows of pots to be used were filled with 25 µL of MHB. The first pot of each row contained 50 µg of venom from *T. aff. atramentarius* diluted with an additional 25 µL of MHB. Serial dilutions were performed to a final volume of 25 µL in each pot. Both bacterial strains were cultured in MHB to an optical density range of 0.08-0.13 at 600 nm, followed by a 1:100 dilution in the culture broth. After

that, 50 µL of each bacterial sample was dispensed into the sample pots. Serial dilutions of 10 µg of Ampicillin were used as a positive control. Plates were incubated at 37 ℃ overnight. Antibacterial activity was determined by measuring absorbance at λ = 595 nm using a microplate reader.

4.9.2. Bacterial growth inhibition in agar

The second test consists of a tryout on the antibacterial activity of some fractions purified by RP-HPLC from the venom of *T. aff. atramentarius* against gram-negative *E. coli* ATCC 25922 strain. *E. coli* was cultured on MHB for 24 hours and inoculated on MHA on Petri dishes. After solidification of the agar, 5 µL of selected fractions are dispensed over the agar on marked sections and 1 µL of Ampicillin 10 mg/mL solution as a positive control. After fully dried at a vacuum concentrator, samples were incubated overnight at 37 ℃. Positive results appear as a non-growth section on the site of the application of the sample.

4.10. Toxicity assays

4.10.1. Insects

Crickets (*Acheta domesticus*) were injected intrathoracically, between the second and third pair of legs, with 2 µg of purified fractions from the venom of *T. aff. atramentarius* that falls in the range of molecular weights from 1 to 8 KDa. Crickets were evaluated up to 5h after injection, and toxicity assessment was appraised considering paralysis as the loss of the ability to standing up after placed upside down or death. It was considered mild toxicity if the crickets recovered from paralysis up to 30 min after injection. Moderate toxicity was considered if the crickets recovered from paralysis up to 2-3h after injection and severe toxicity if the cricket recovered after 5h from the injection. The lethal effect is pondered if the crickets died after injection. A volume of 5 µL of dH_2O was injected as the negative control.

5. Results, Interpretation, and Discussion

The individuals collected in the vicinities of the city of Ambato were identified as *T. aff. atramentarius* as an *affinis* species to *Teuthraustes atramentarius,* considering its coloration and general appearance [65]. Ought to the lack of a systematic revision on the genus, it is impossible to assign the individuals as a defined species due to little morphologic variations between closely related species found in similar locations [66].

Several extractions were performed upon all individuals, and the crude venom obtained was lyophilized, obtaining an approximate 6.4 mg of crude venom dry material. The crude venom sample protein concentration after being resuspended in water was determined and gave a result of 11.85 mg/mL of protein content in 700 µL of venom solution.

The resuspended venom was used for chromatography. From a 2 mg sample injected in the chromatograph, 115 fractions were collected manually from elution that gave a total of 1.7 mg of recovered material. The fractions that gave absorbance values over 400 mAU were considered major components (Figure 4).

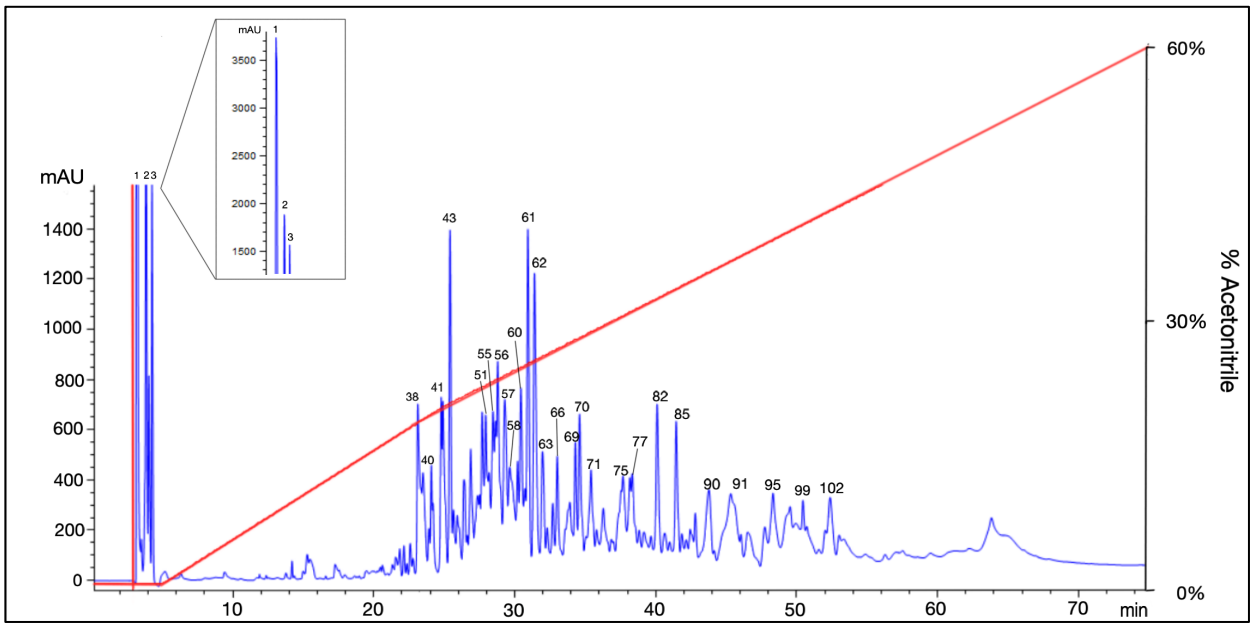

Figure 5. **Separation of crude venom from *Teuthraustes aff. atramentarius* by Reversed-Phase High-Performance Liquid Chromatography**
Sample 2 mg of crude venom of *T.aff. atramentarius* were injected for chromatography. Numbers over some fractions were placed to idetify major components for further analysis.

The most used technique for separating components of scorpion venoms is RP-HPLC. This method exploits the hydrophobicity of the molecules present in a mixture giving the chance of profiling the venom components by the interaction of the hydrophobic regions (amino acids) of each molecule with the stationary phase [67,68]. Due to the type of components that have been found in scorpion venoms, 0-60% acetonitrile in water gradient is commonly used, giving the chance to compare the profiles generated by venom from different species [69,70].

In general, from this gradient, the components that elute at first are mostly heterocyclic compounds that give high absorbance values and are associated with pain generation (fractions 1,2 and 3) [36]. Among those components that elute between 25-35 minutes, there are voltage-gated potassium channel blockers (*Kv*) with weights ranging between 3-9 Da. After them, the components eluting at 30-45 minutes may contain voltage-gated sodium channel blockers (*Nav*) with average molecular weights between 6-8 Da. Finally, the components that elute after 50 minutes are common fractions with enzymatic activities [61,70].

From the analysis of the chromatogram (Figure 4), the venom of *T. aff. atramentarius* appears to contain several fractions that may show toxic activity as well as some fractions of high hydrophobicity that generally have some enzymatic activity [67]. The venom from *T. aff. atramentarius* also stands out for its large number of components.

According to Rodriguez de la Vega (2013) [21], scorpion venoms with large amounts of components are known for being less toxic than those with less. This can be evaluated with scorpion species already studied and that have shown toxicity against humans. Very common scorpions in South America like *Tityus pachyurus* are also present in the Ecuadorian territory, which have caused important clinical cases throughout the country [16].

These species have also been studied by RP-HPLC and using the same gradient. Thus, their profiles can be compared. *Tityus pachyurus* and *Tityus stigmurus,* respectively, are those with a smaller number of components with the difference that *Tityus stigmurus* present larger absorbance values for each component. These two are the most toxic venoms against humans present in South America, with lethal doses LD_{50} of less than 1 mg/kg in mice (Figure 5ab).

The venom profile of *Centruroides granosus* presents a little increase in the number of components, and thus as expected, its toxicity is a little less than the species mentioned before, with a LD_{50} of 2.2 mg/kg (Figure 5c). Hence, due to the large number of components present in the venom of *T. aff. atramentarius*, it may suggest its toxicity might be low enough not to harm

humans. The number of components and the appearance of a strictly major component commonly shows the presence of a predominant toxic fraction. Hence, the toxicity and amount injected of that component with each sting are larger, and its effects significant increase [71].

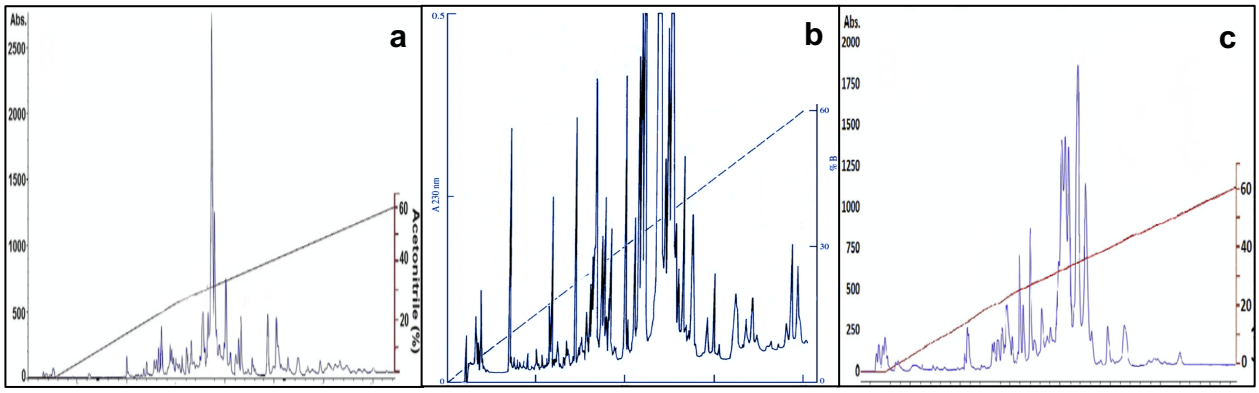

Figure 6. **Comparison of Reversed-Phase High-Performance Liquid Chromatography profiles of different scorpions species**
a) *Tityus pachyurus* venom profile. b) *Tityus stigmurus* venom profile. c) *Centruroides granosus* venom profile. All chromatograms were made in 0-60% acetonitrile in water gradient.
a, c) Reprinted from "Venoms of *Centruroides* and *Tityus* species from Panama and their main toxic fractions" by Salazar et al., 2018, Toxicon, 141, 79-87. b) Reprinted form "Proteomic analysis of the venom from the scorpion Tityus stigmurus: Biochemical and physiological comparison with other Tityus species." by Batista et al., 2007, Toxicology and Pharmacology,

The components of the venom were also separated by SDS-PAGE. Electrophoresis was performed with samples of venom from Ecuadorian scorpion *T aff. atramentarius* and Panamanian scorpions *Tityus pachyurus* and *Centruroides granosus* alongside with a molecular weight standard (Figure 6).

For *T. aff. atramentarius* lane, there are a considerable number of bands from 25 to 150 KDa, as well as some components of greater molecular weights than 250 KDa were retained at the top of the gel (Fig 6 - lane 3). For *T. pachyurus* (Fig 6 - lane 1) and *C. granosus* (Fig 6 – lane 2), there are more bands in lower molecular weights less than 15 KDa than the Ecuadorian specimen and a slightly similar amount for greater molecular weights.

In the same fashion, most of the toxic components present in scorpion venoms are of low molecular weight. Ion channel modulators, especially sodium and potassium channel blockers with molecular weights less than 8 KDa, are extensively present in venoms from other species [72].

Comparing the electrophoretic profile of the venom from *T. aff. atramentarius* with the ones from *T. pachyurus* and *C. granosus* (Figure 6), the content on high molecular weight components from the Ecuadorian endemic scorpion is considerably higher than those other scorpions.

This may suggest that the venom from the Ecuadorian scorpion is not rich in those low molecular weight toxins that are highly present in the venom of the other species. This can be because of the venom of *T. aff. atramentarius* may not be focused on directly kill its prey by envenoming but to facilitate consumption or digestion of it [73].

On the other hand, the concentration of polyacrylamide used in the gel is designed to give a widespread overview of the components and their approximate molecular weights. To specifically separate low molecular weight peptides, gels with a higher concentration of polyacrylamide is required [74].

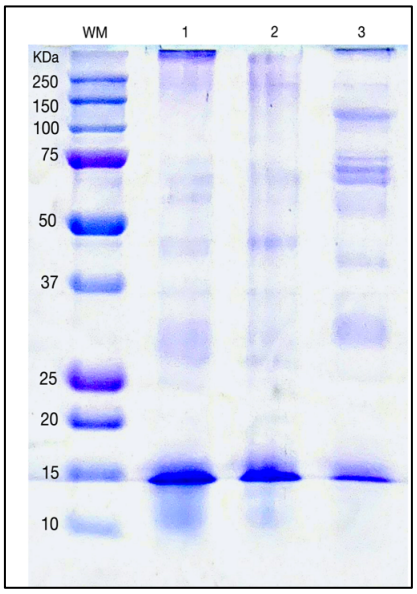

Figure 7. **Separation of crude venom from *Teuthraustes aff. atramentarius* by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.**
Lane WM) Molecular weight marker. Lane 1) Panamanian scorpion *Tityus pachyurus* venom. Lane 2) Panamanian scorpion *Centruroides granosus* venom. Lane 3) Ecuadorian scorpion *T. aff. atramentarius* venom.

To have accurate information about the molecular weights of the components present in the venom of *T. aff. atramentarius*, the major fractions were analyzed by mass spectrometry. Average molecular masses of major components in the venom determined by mass spectrometry shows that the main components of the venom are of low molecular weights with ranges between 1 to 10 KDa (Table 2). Some fractions also presented two values of average masses, which indicates that those fractions are mixtures of components and require further purification.

This suggests that the elevated content of high molecular weight components showed in the electrophoretic profile are of minor components with no particular incidence in the toxicity of the venom.

The major components of the venom of *T. aff. atramentarius* vary between the range of voltage-dependent ion-channel blockers with low molecular weights that commonly correspond to at least 20% of all components founds in the venom from different species. And some high molecular weight components are shown in the electrophoretic profile that may correspond to enzymes or lysozymes also identified in venoms from other species [22] .

Table 2. **Average molecular masses for components present in the venom of *Teuthraustes aff. atramentarius*.**
Average molecular masses determined by mass spectrometry of the major fractions from the venom of *T. aff. atramentarius* separated by RP-HPLC

FRACTION	RETENTION TIME (MIN)	MOL. WT. (DA)
38	23.2	4129.99 / 3630
40	23.9	4061.6 / 6979.3
41	24.2	2370.8 / 4060.75 / 771.3
42	24.7	4108.34
43	25.2	1194.66
51	27.6	3223.61
55	28.6	3781.58 / 3224.38
56	28.7	3795.10 / 3810.40
57	29.2	3810 / 3794.25
58	29.7	5901.00 / 8686.81
60	30.8	8684
61	31	2860.4
62	31.2	1430.3 / 4369
63	31.4	4369.66
66	32.7	1125.2
69	34.3	2199 / 1260.6
70	34.6	2710.51 / 2581.00
71	35.2	7094.53 / 5335.88
74	36.2	10448.13 / 9456.8
75	36.9	1238.5
77	38.1	1145.55
82	40.1	3473.77
85	41.4	3683.65 / 4096.60
90	43.3	8670.8
91	44.3	6744.3 / 8583
95	47.6	15332.83
99	50.4	2193.8
102	52.1	4215.24 / 3801.49

Combining the information obtained by SDS-PAGE and mass spectrometry, the venom of *T. aff. atramentarius* have components in a very wide range of molecular weights. The huge variety of components with very different molecular weights present in the venom of *T. aff. atramentarius* opens up great possibilities on the distinct biological functions of each of the components.

Scorpion venoms contain various enzymes that take part in the envenomation syndrome symptoms. *T. aff. atramentarius* venom was tested for phospholipase A_2, hyaluronidases, and protease (gelatinase), which are the most common activities reported for scorpion venoms [75].

For PLA_2 activity, venom from *T. aff. atramentarius* was tested in three concentrations, and all three samples tested negative for PLA_2 activity (Figure 7). Phospholipases A_2 are responsible for the cleavage of phospholipids by hydrolyzing the bond between the second fatty acid tail and the glycerol molecule [76].

These enzymes are present in venoms of many animals, including bees, wasps, and snakes. They also have been found in many scorpion species, mainly from the *Buthidae* family. Some products of the hydrolysis are involved in many physiological responses, including severe inflammation and pain after the sting [77].

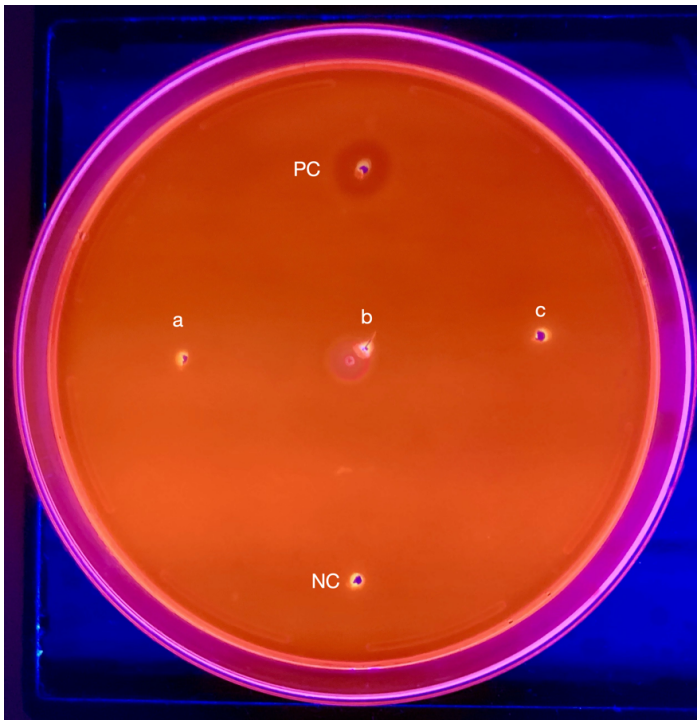

Figure 8. **Determination of phospholipase A_2 activity by egg yolk method.**
Petri dish with egg yolk agar to test phospholipase A_2 activity. PC) Positive control: *Bothrops ammonytoides* venom sample, a) 5 µg sample, b) 10 µg sample, c) 20 µg sample of venom from *T. aff. atramentarius*, NC) Negative control: water

In the case of protease, gelatinase activity was analyzed by zymography upon crude venom sample and fractions 60, 61, 62, 69, 70, 75, 82. All fractions and crude venom resulted in negative for gelatinase activity (Figure 8).

The most common proteases found in scorpion venoms are serine proteases and metalloproteases. These enzymes catalyze the proteolysis or breakdown of proteins into single peptides or amino acids [78]. These enzymes are commonly involved in swallowing and digestion processes. They have shown inflammation and coagulation responses in mammals after stings [79,80]. This suggests that the eating and digestion processes of the scorpion *T. aff. atramentarius* is adjuvated by other enzymes from their digestive juices instead of the venom [81].

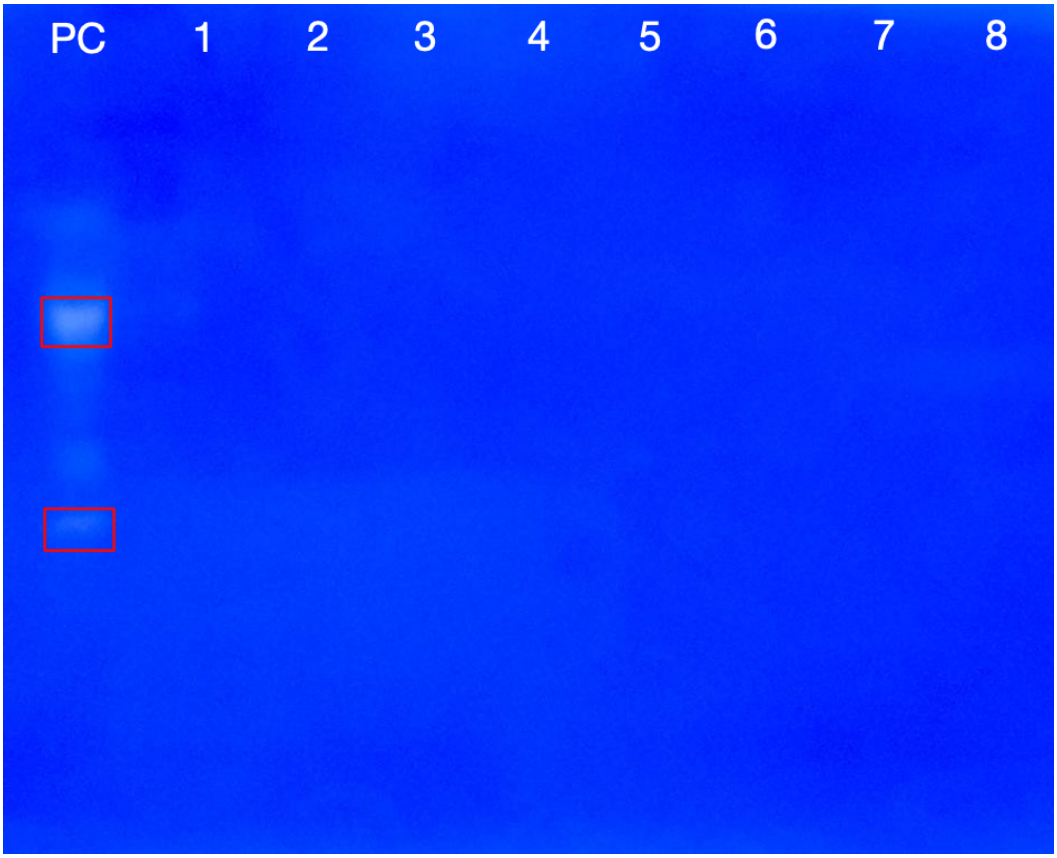

Figure 9. **Zymogram for detection of gelatinolytic (proteolytic) activity**
PC) Positive control: *Bothrops ammonytoides* venom sample, lane 1) crude venom sample of *T. aff. atramentarius* and fractions separated by RP-HPLC lane 2) 60, lane 3) 61, lane 4) 62, lane 5) 69, lane 6) 70, lane 7) 75, lane 8) 82

At last, hyaluronidase activity was evaluated by zymography upon crude venom and RP-HPLC fractions 95, 97, 99, 100, 102, 103, 113. Crude venom and fractions 102 and 113 resulted in positive for hyaluronidase activity (Figure 9).

Hyaluronidases are other important enzymes present in scorpion venoms. These enzymes are known for being spreading factors in many venoms of different species [82]. They act by hydrolyzing the glycosidic bonds in hyaluronic acid. These enzymes act as a spreading factor by degrading the hyaluronate present in the extracellular matrix and providing easy diffusion paths to bloodstream facilitating envenomation [83].

These enzymes are known to be ubiquitous for all venoms and the venom of *T. aff. atramentarius* it is no exception. The zymogram showed 2 fractions with molecular weights of approximately 35 KDa and 75 KDa, respectively, with hyaluronidase activity.

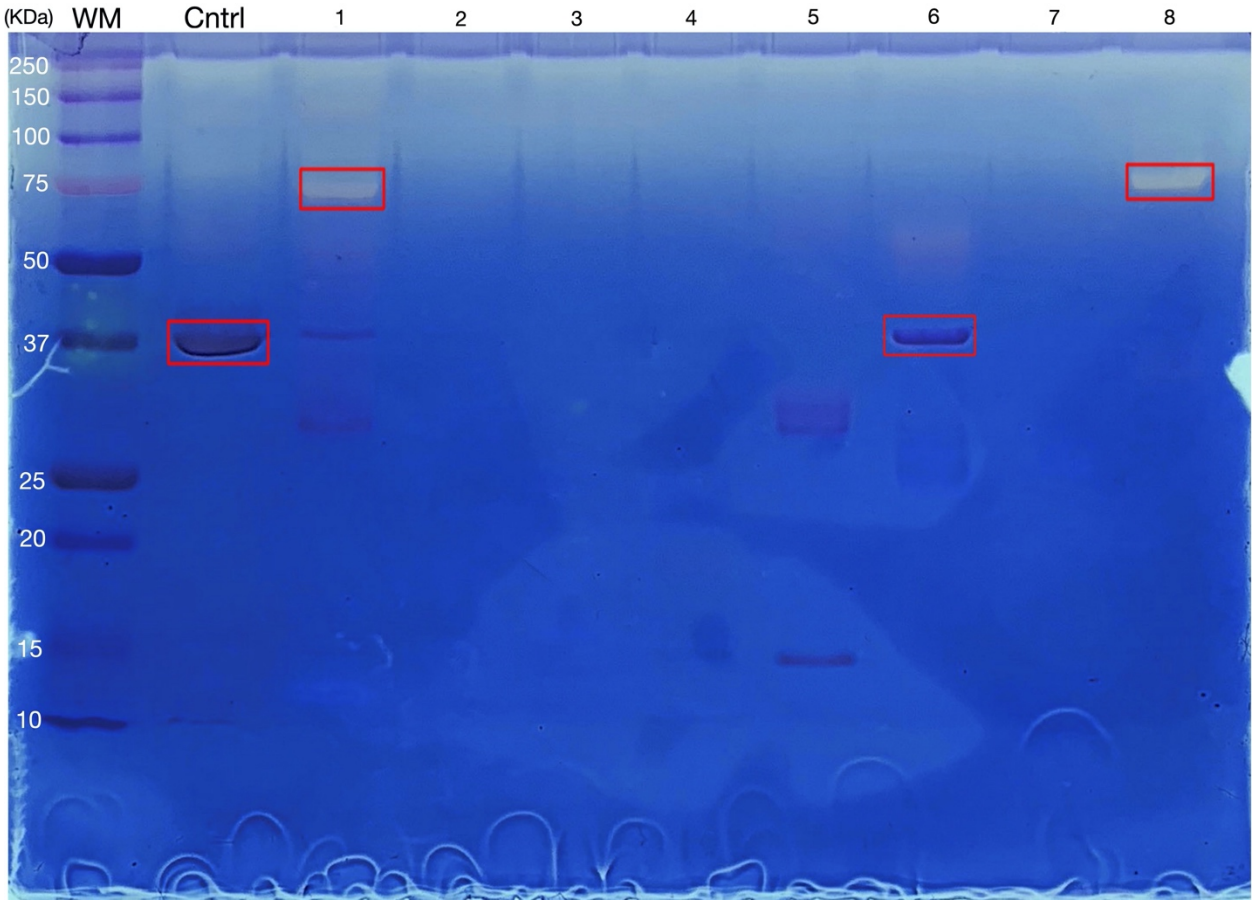

Figure 10. **Zymogram for detection of hyaluronidase activity**
WM) Molecular weight marker, Cntrl) Positive control*: Brachypelma vagans* venom sample, lane 1) crude venom sample of *T. aff. atramentarius* and fractions separated by RP-HPLC lane 2) 95, lane 3) 97, lane 4) 99, lane 5) 100, lane 6) 102, lane 7) 103, lane 8) 113.

Some venom components from other scorpion species studied before have shown antibacterial properties. It is proposed that the presence of antibacterial peptides could be to protect the venom glands from pathogenic infections and for the preservation of paralyzed wounded preys [50].

The venom of *T. aff. atramentarius* was tested first for a minimal inhibitory concentration against gram-positive *Staphylococcus aureus* ATCC 6538 and gram-negative *Pseudomonas aeruginosa* ATCC 15442 bacteria strains. The used methodology test various concentrations against the growing bacteria in a liquid medium so as to find a MIC for inhibiting the growth of a specific organism. The MIC is referred to as the minimal concentration of antibacterial agents by which the OD of bacteria solution is reduced to 0.05 mAU.

In this case, several concentrations of crude venom were tested against the bacteria strains mentioned above (Figure 10a). For both organisms, all tested concentrations of crude venom from *T. aff. atramentarius* resulted in negative in the inhibition of the growth of both bacterial strains. Serial dilutions of ampicillin (Figure 10b) were used as positive control and showed different MIC values for each organism.

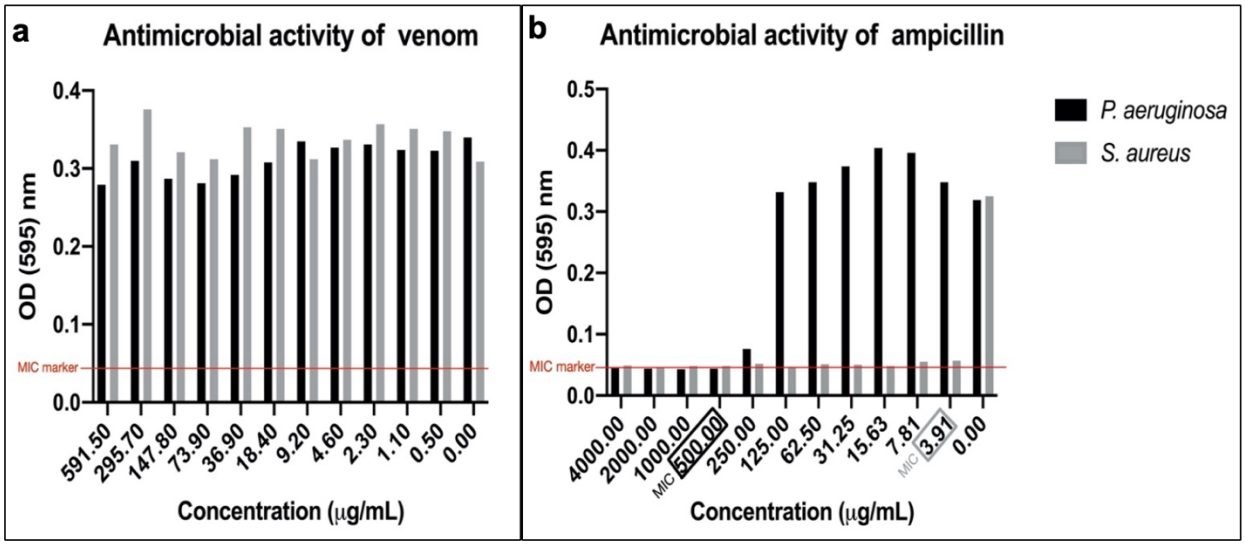

Figure 11. **Antibacterial activity of venom from *Teuthraustes aff. atramentarius* in serial micro dilutions against *Staphylococcus aureus* and *Pseudomona aeruginosa*.**

a) Optical density measurement of growing *Staphylococcus aureus* and *Pseudomona aeruginosa* in crude venom from *T. aff. atramentarius* solutions with different concentrations. B) Positive control: Optical density measurement of growing *Staphylococcus aureus* and *Pseudomona aeruginosa* in ampicillin solutions with different concentrations

The antibacterial activity of the purified fractions from the venom of *T. aff. atramentarius* were also tested. Qualitative analysis was performed on the major fractions from the venom purified by RP-HPLC against *E. coli* in solid medium. All fractions that assessed 300 mAU or more in the chromatogram were selected for this test. The 40 fractions selected resulted negative in the inhibition of *E. coli* growth (Figure 11).

The absence of antibacterial peptides in the venom may suggest that this species of scorpions fight pathogenic infection with its hemolymph circulation instead of directly protecting the venom gland as well as suggests that the consumption of their preys is immediately and there is no need of conservation of wounded preys [73].

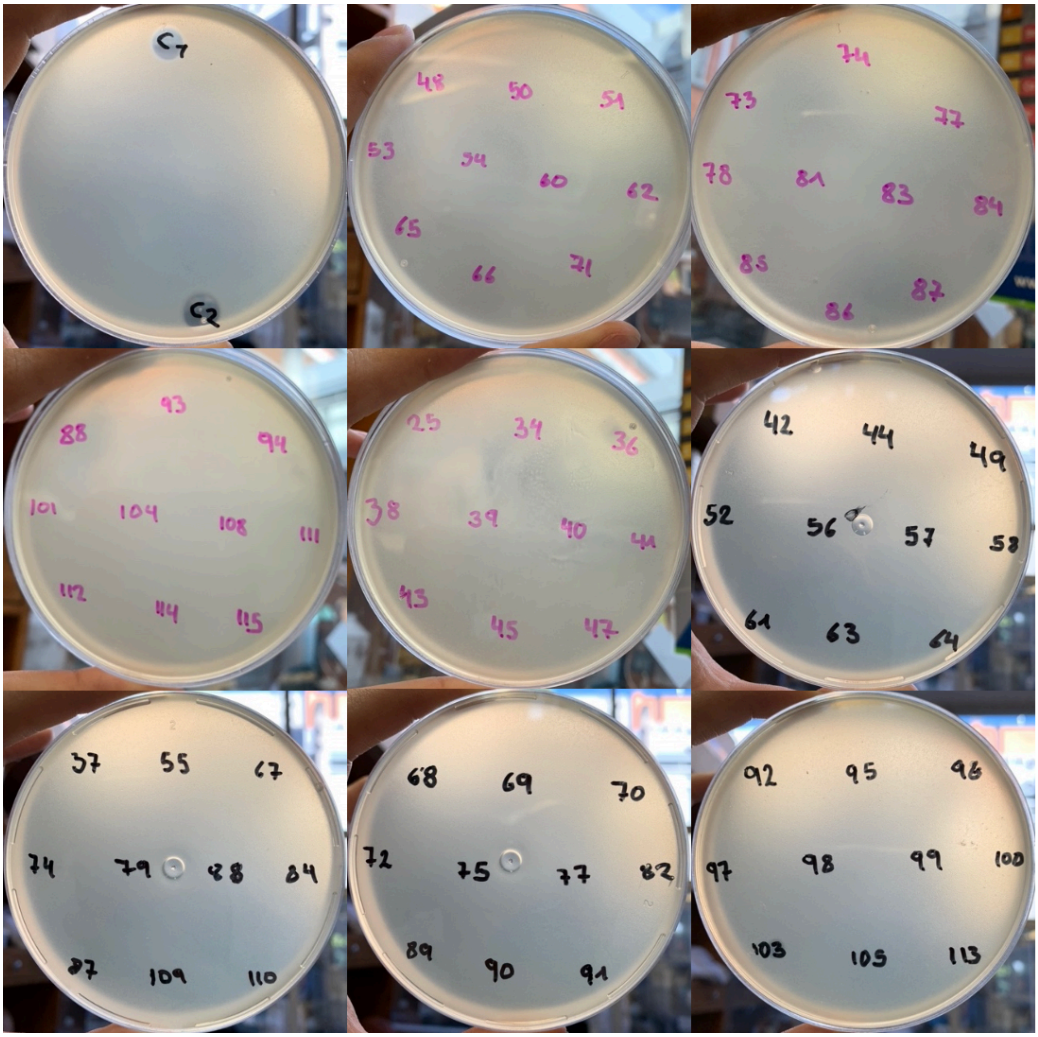

Figure 12. **Antibacterial activity of venom *from Teuthraustes aff. atramentarius* against *Escherichia coli* in solid medium.** Positive control c1) Ampicillin 2 µL, c2) Ampicillin 4 µL. 5 µg samples of numbered fractions were applied over the agar for diffusion.

Finally, the toxicity of the venom was evaluated in crickets. The selection of the targets for toxicity assessment was evaluated first on insects, as being them its natural prey. Batista (2007) describes voltage-gated sodium channel blockers with an average molecular weight of 6-8 KDa and voltage-gated potassium channel blockers with molecular weight range 2-9 KDa as the most common toxins present in scorpion venoms [70].

Based on this information and the average molecular weights of major components from the venom of *T. aff. atramentarius* shown in Table 2, some fractions were selected for the toxicity test on crickets, and some of the fractions generated numerous toxicity reactions upon the insects injected (Table 3). Fractions **43** with 1194.66 Da and **91** with 6744.3 / 8583 resulted lethal for crickets and may correspond to the main toxins present in the venom of the Ecuadorian endemic scorpion *T. aff. atramentarius.* Fractions **38** with 4129.99 Da and **61** with 2860.4 Da resulted in severe toxicity. Other toxic fractions generated moderate and mild toxicity upon crickets.

Martin-Eauclaire et al., 2019 describe voltage-gated sodium channel modulators as toxins with a low molecular weight ranging between 6 to 8 KDa and lethal for insects injected intrathoracically and for mammals injected intracranial [85]. The molecular weight of the fraction **91** and its biological activity falls in the description of this kind of toxins. The other fractions that caused lethal and severe toxicity fall in the description of voltage-gated potassium channel blockers with molecular weights range between 2-9 KDa and are the most common toxins in scorpion venoms [86].

Table 3. **Toxic fractions and level of toxicity of some fractions of the venom from *Teuthraustes aff. atramentarius*.**
Toxic fractions for crickets, its level of toxicity, and their corresponding average molecular masses determined by mass spectrometry. Lethal: cause death within 2 hours of injection. Severe: causes paralysis with recovery after 5 hours from injection time. Moderate: causes paralysis with recovery after 2-3 hours from injection time. Mild: cause paralysis with recovery after 30 minutes from injection time.

NUMBER OF FRACTION	ACTIVITY ON CRICKETS (4 µG)	AVERAGE MM (±1 DA)
38	Severe	4129.99 / 3630
43	Lethal	1194.66
51	Moderate	3223.61
61	Severe	2860.4
62	Mild	1430.3 / 4369
72	Moderate	7094.53
82	Mild	3473.77
85	Moderate	3683.65 / 4096.60
91	Lethal	6744.3 / 8583
95	Mild	15332.83
99	Mild	2193.8

For those fractions that generated moderate and mild conditions, their molecular weights oscillate around 4 KDa and 7-8 KDa and fall on the voltage-gated ion channel toxins described above. In general, most of the toxic fractions induced mild to moderate toxicity, which gives us an idea that the objective of the venom more than killing is to paralyze its prey long enough for the scorpion to be able to eat it. Furthermore, due to the average molecular masses of the active fractions, we can deduce that the venom of *T. aff. atramentarius* have more voltage-gated potassium channel blockers rather than sodium channel blockers. The literature explains that *Nav* blockers are responsible for the toxicity that may harm human beings by scorpion stings [87]. This is congruent with the local knowledge on the stings of T. *aff. atramentarius* on the site of a collection of the specimens, which is said that cause hard to severe local pain on site of sting but no additional more serious complications, which is known to be caused by *Nav* blockers.

Finally, the fraction **51** was selected for sequencing. The fraction showed great purity in the mass spectrum, as well as a good amount recovered from chromatography.

Edman degradation analysis performed on the selected fraction and resulting in a 27 amino acid peptide sequence with a theoretical molecular weight of 3225.7 Da that is in accord with the 3223.6 Da experimental molecular weight determined by mass spectrometry (Table 4).

The cysteines present in the structure may form two disulfide bonds. This type of peptides has been rarely observed among scorpion venom components [88]. Disulfide-bridged peptides have been found on snake venoms disintegrins. These molecules have a straight role in inhibiting cell adhesion and corroding the extracellular matrix as part of the envenomation syndrome [89].

This suggests that some toxins present in the venom of *T. aff. atramentarius* might have another way of action other than voltage-gated ion channel modulators. This also represents the uniqueness of the composition of the venom from this species inhabiting only the Andean region of Ecuador, since most scorpion venoms do not present any cysteine-patterned peptides in their composition [90].

Table 4. **Sequence of the peptide from the fraction 51 of the venom from *Teuthraustes aff. atramentarius***

SPNFCRNKCYKEYIPNNCVSYCEKVLN

This sequence has been compared to previously reported sequences finding that is congruent to a peptide section of HtC_4Tx1, a toxin isolated from the African endemic scorpion *Hadogenes troglodytes* [88]. This peptide has 78 amino acids and two disulfide bridges, and it is 79% consistent with the peptide found in the venom from *T. aff. atramentarius* (Table 5).

Table 5. **Comparison of the sequences isolated from *Teuthraustes aff. atramentarius* and *Hadogenes troglodytes***

T. aff. atramentarius	SPNFCRNKCYKEYIPNNCVSYCEKVLN	27 aa
	SP FCRNKC KEYIP NC+ YC K	
H. troglodytes	SPVFCRNKCLKEYIPTNCIEYCTKRGL	69 aa

This scorpion is the only species that have reported the presence of several components with disulfide bridges [88]. The consistency of this similar component may be because of a common ancestor between the species. *T. aff. atramentarius* belongs to the family Chactidae, and *H. troglodytes* belong to the family Hemiscorpiida (Figure 12). Both families belong to the suborder Iurida[1]. A common ancestor belonging to this order might have evolved separately, maintaining biological similarities after Pangea separation, where Africa and South America were together [91].

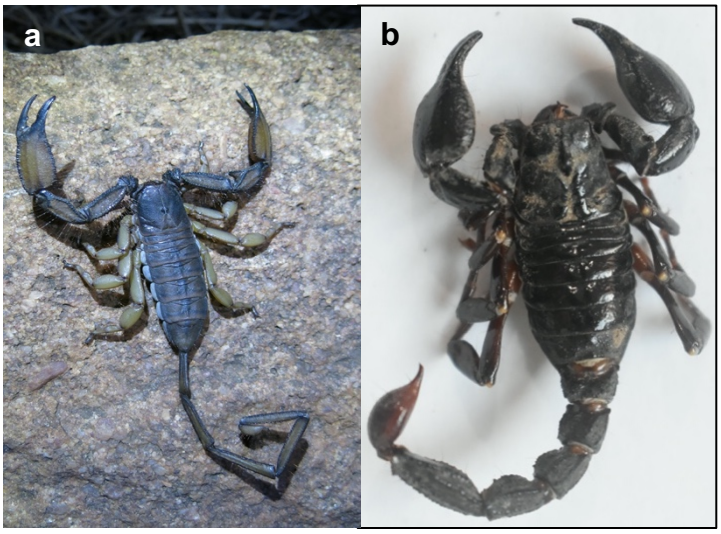

Figure 13. **Comparison of *Hadogenes troglodytes* and *Teuthraustes aff. atramentarius.***
a) Dorsal view of *H. troglodytes*. Reprinted from: Institute of Entomology of the Cape Town University b) Dorsal view of *T. aff. atramentarius*.

6. Conclusions and Recommendations

6.1. Conclusions

The venom from *T. aff. atramentarius* have a great variety of components, estimated to be over 120 with molecular weights varying between 1 KDa and 100 KDa.

The venom showed hyaluronidase activity at estimated molecular weights of 37 and 75 KDa and did not show any phospholipase A_2 and protease activity. The presence of hyaluronidase displays a good venom distribution mechanism.

The venom did not show any antibacterial activity, which suggests immune response against infections is delivered by other fluid-like hemolymph instead of venom.

The venom presented various components with toxic activity against insects. Most of the toxic components have molecular weights between 2 to 6 KDa.

One peptide that showed toxicity upon crickets was isolated and sequenced. The peptide showed a similar sequence to the toxin HtC_4Tx1 found in the African scorpion *H. troglodytes.*

6.2. Recommendations

- It is recommended to sequence all the peptides that showed toxicity upon insect and compare the sequences obtained to peptides already reported.
- It is necessary to test the fractions that showed toxicity upon insects, against mice to prove toxic activity upon mammals.
- It is proposed to test the antibacterial activity of the venom of *T. aff. atramentarius* against other bacterial strains.
- It is suggested to do the transcriptomic analysis of the venom glands to identify all the components present in the venom and as well as isolate all proteins of interest.

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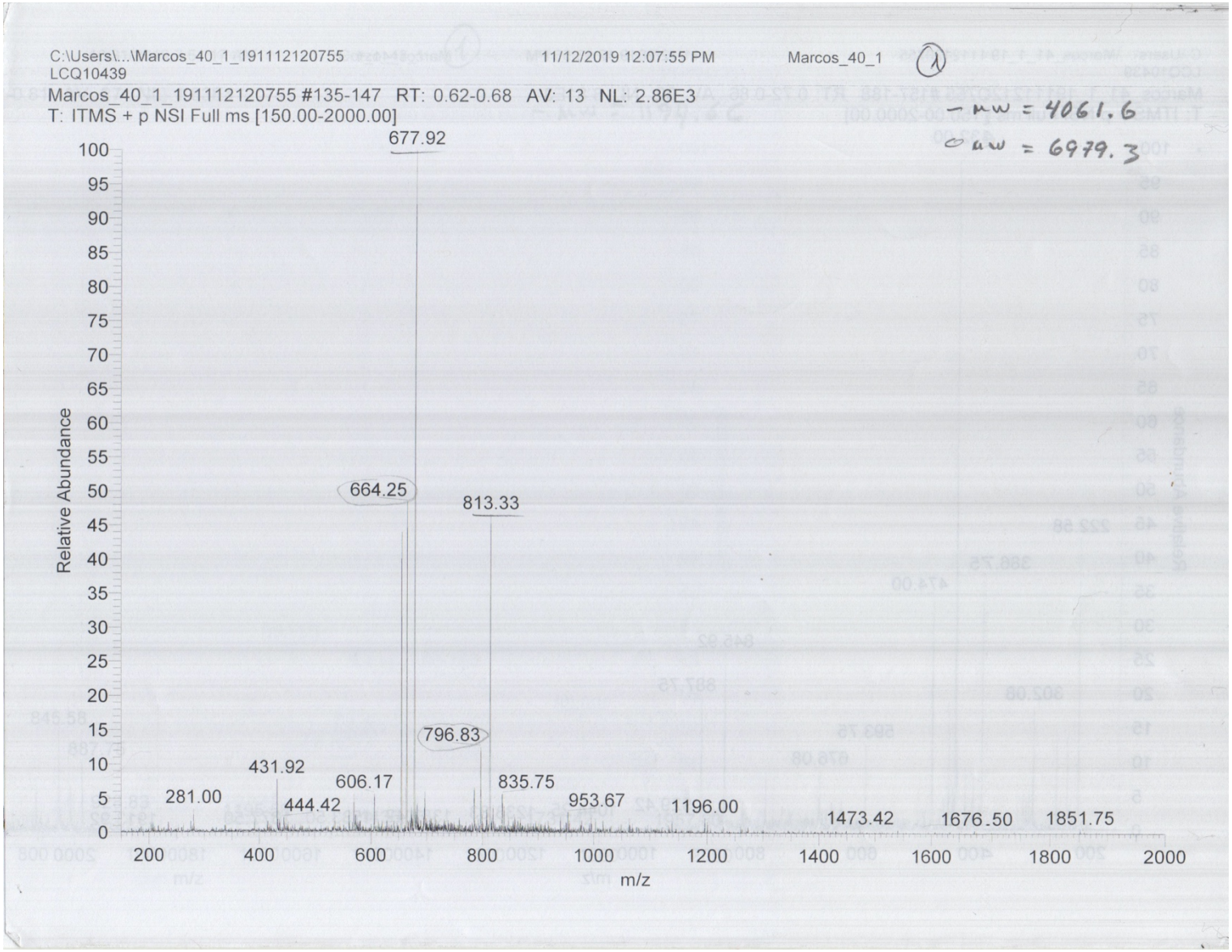
Annexes

A) Mass spectra of fractions of the venom from *T. aff. atramentarius* separated by RP-HPLC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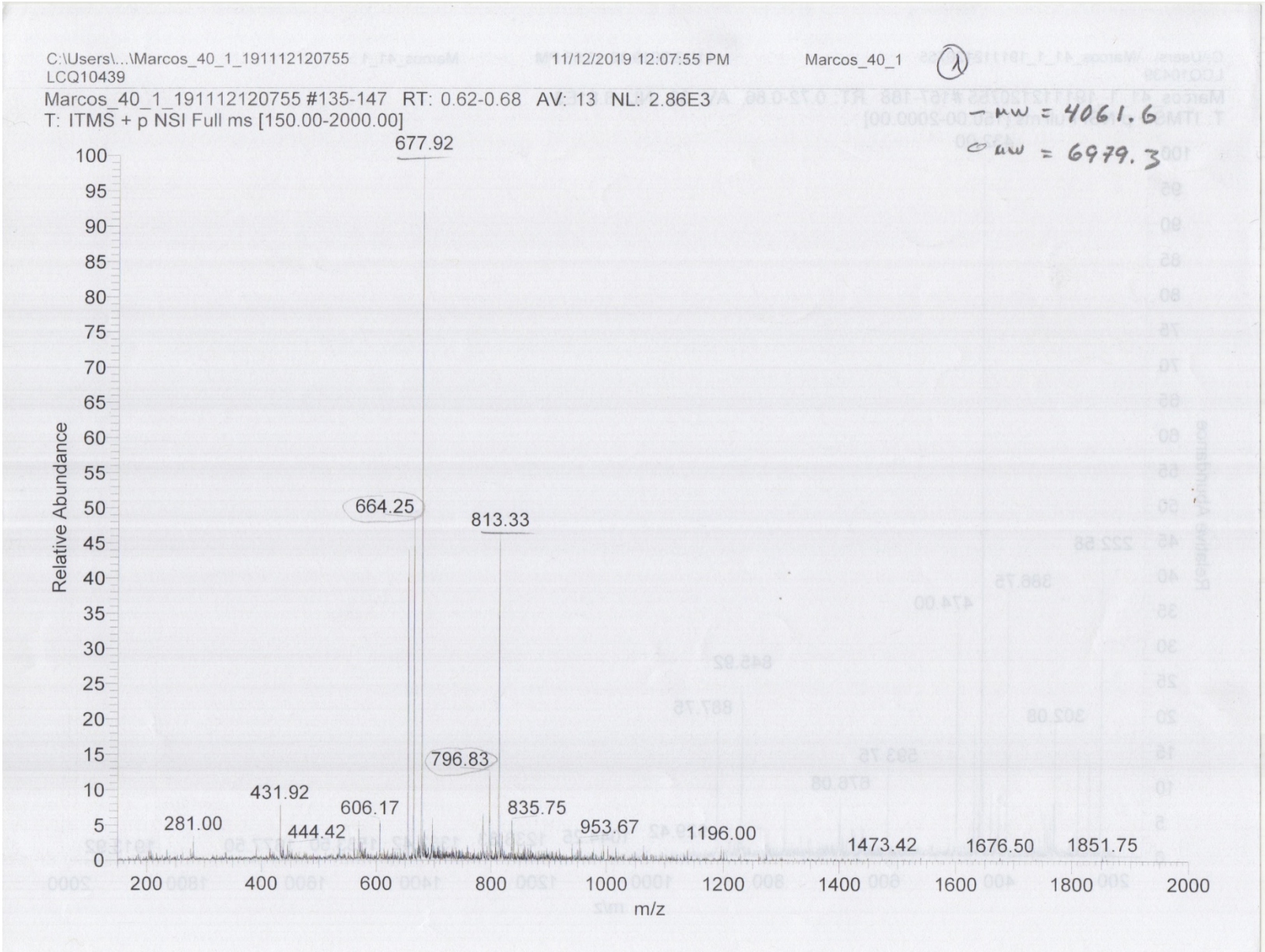
- MW = 4061.6
o MW = 6979.3
Relative Abundance
m/z
677.92
664.25
813.33
796.83
431.92
606.17
835.75
281.00
444.42
953.67
1196.00
1473.42
1676.50
1851.75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o MW = 6979.3
677.92
664.25
813.33
796.83
431.92
606.17
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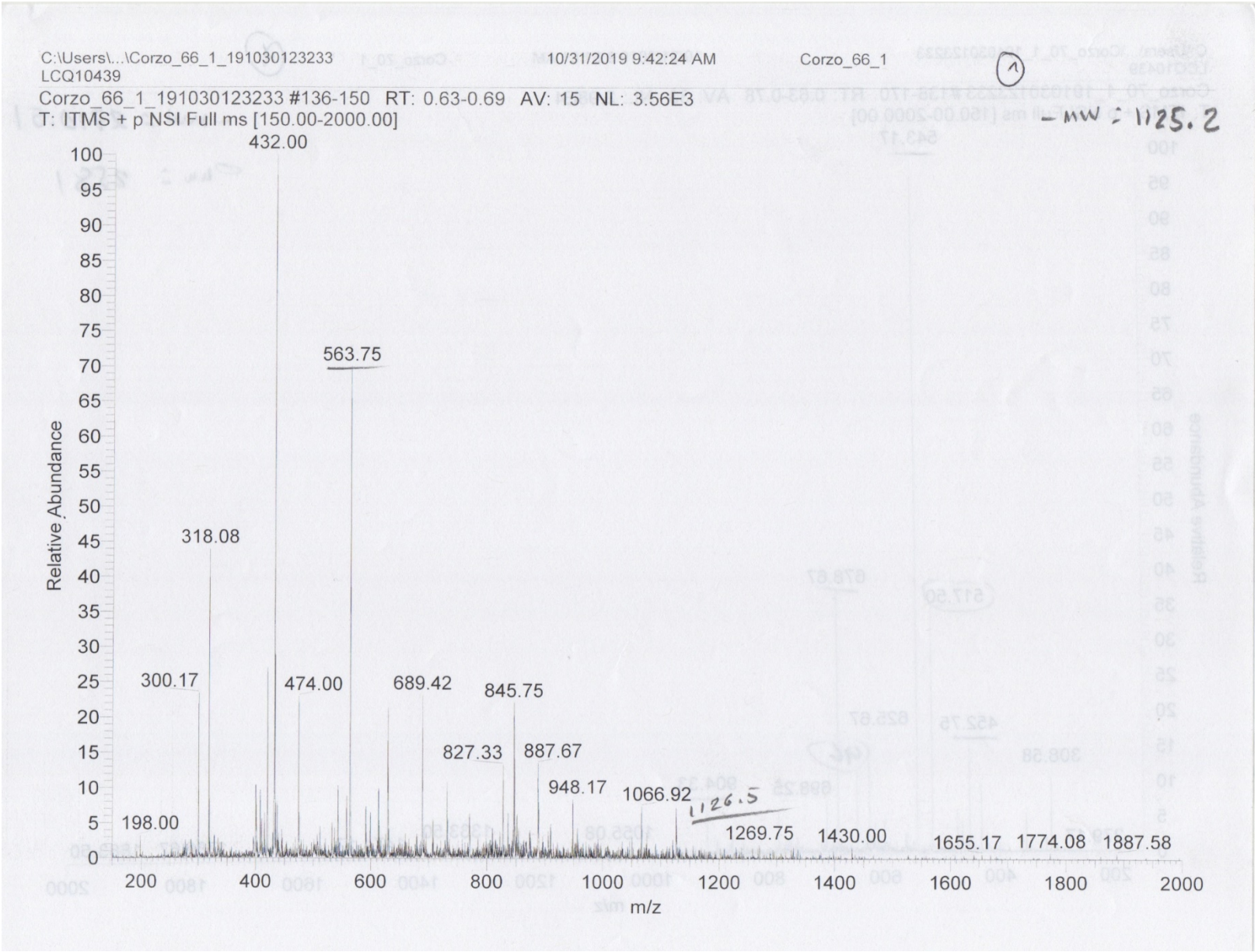
953.67
1196.00
1473.42
1676.50
1851.75
Relative Abundance
m/z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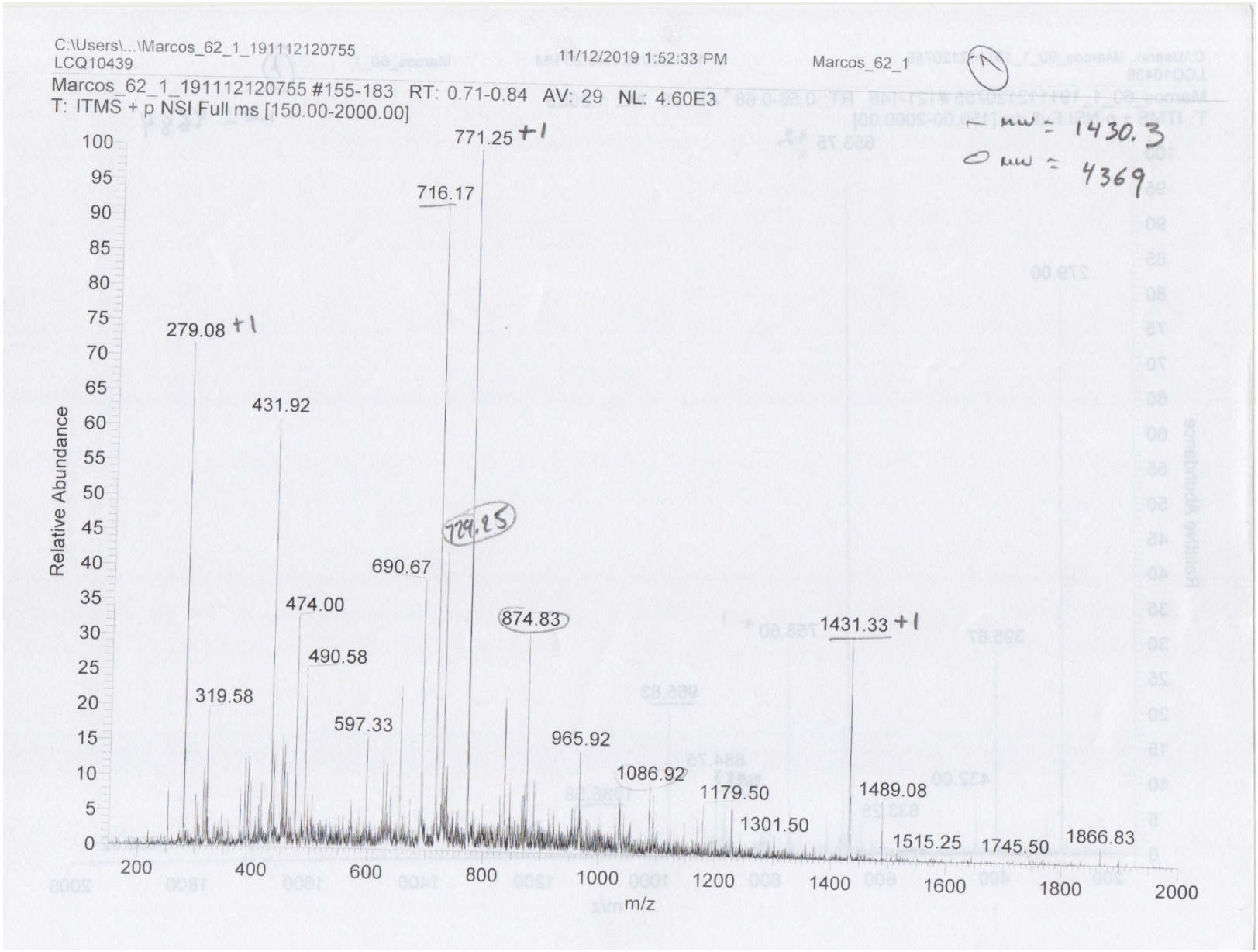
Relative Abundance
m/z
432.00
563.75
318.08
300.17
474.00
689.42
845.75
827.33
887.67
948.17
1066.92
1126.5
198.00
1269.75
1430.00
1655.17
1774.08
1887.58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o mw = 4369
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716.17
279.08 +1
431.92
729.25
690.67
474.00
874.83
1431.33 +1
490.58
319.58
597.33
965.92
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1745.50
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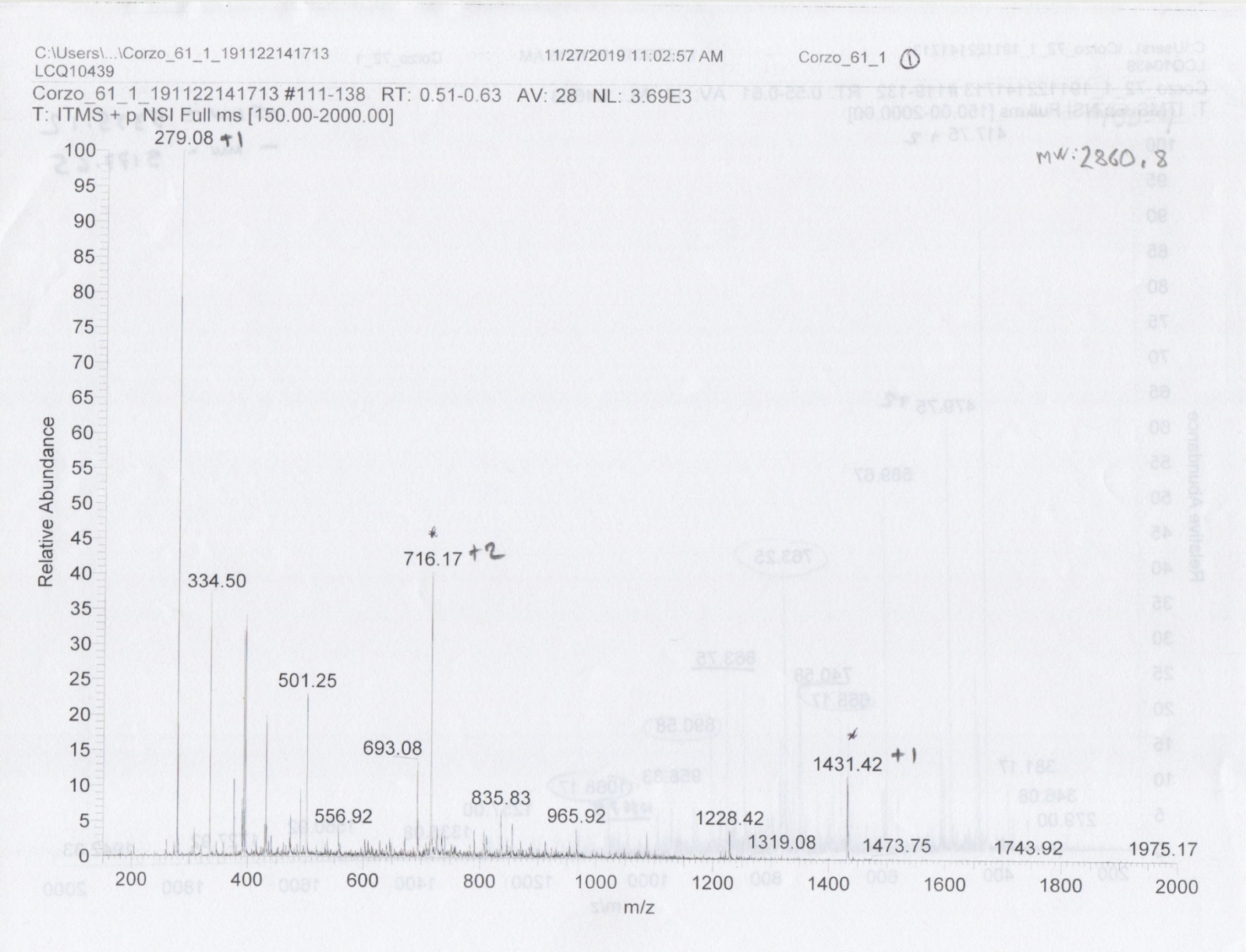
Relative Abundance
m/z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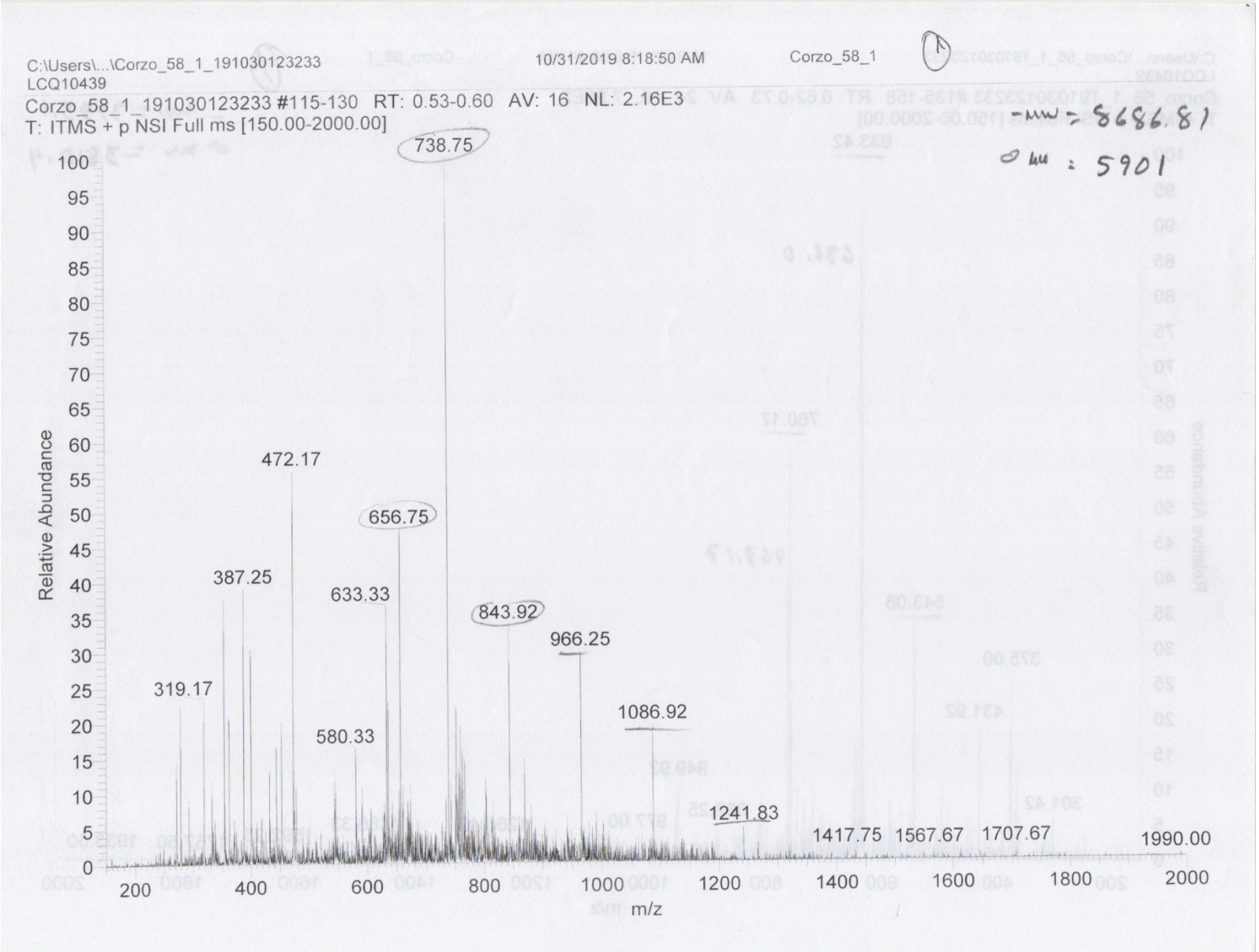
716.17 +2
1431.42 +1
334.50
501.25
693.08
556.92
835.83
965.92
1228.42
1319.08
1473.75
1743.92
1975.17
Relative Abundance
m/z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Σ MW = 5901
Relative Abundance
m/z
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472.17
656.75
387.25
633.33
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319.17
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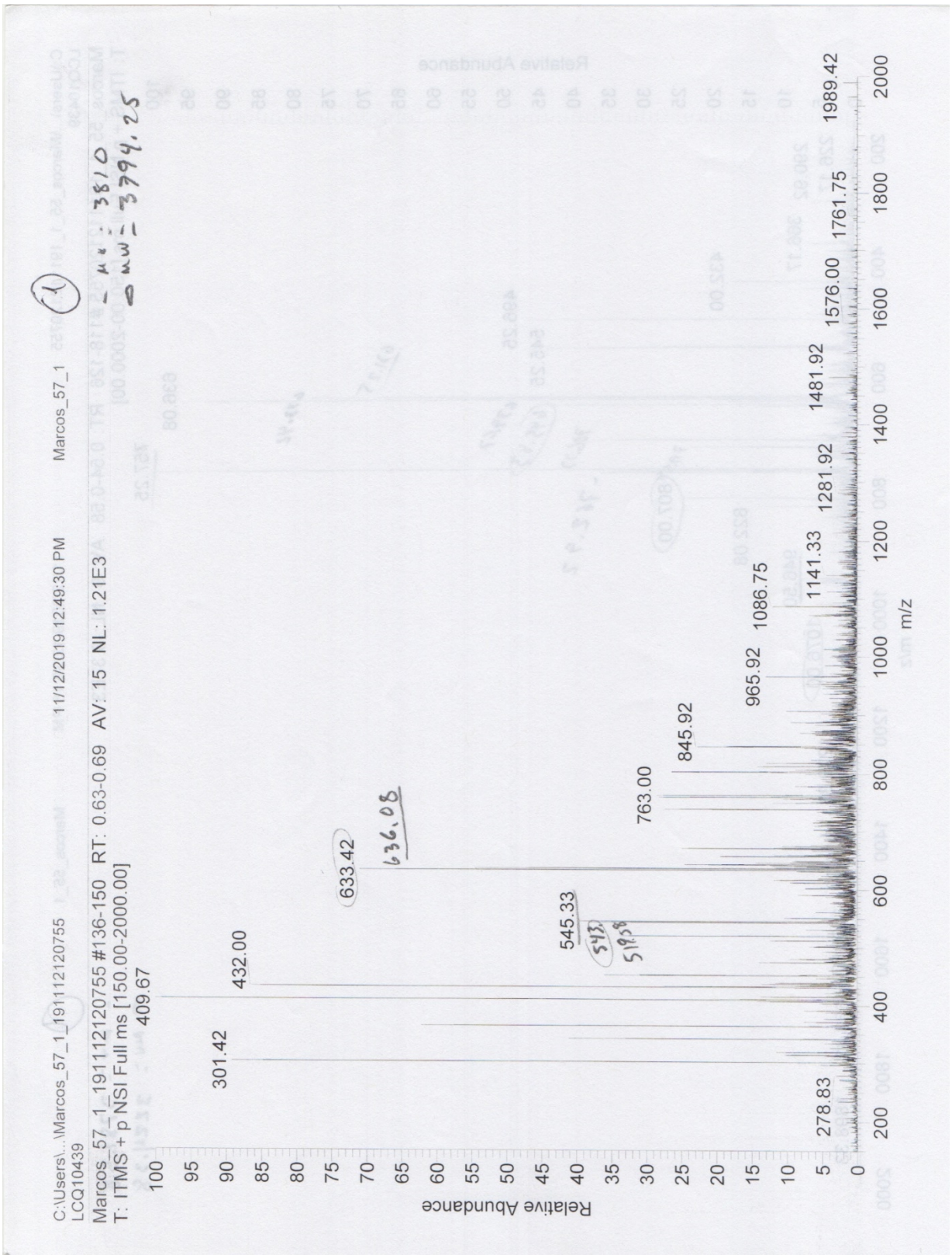
1417.75
1567.67
1707.67
1990.00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Relative Abundance
m/z
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301.42
432.00
633.42
545.33
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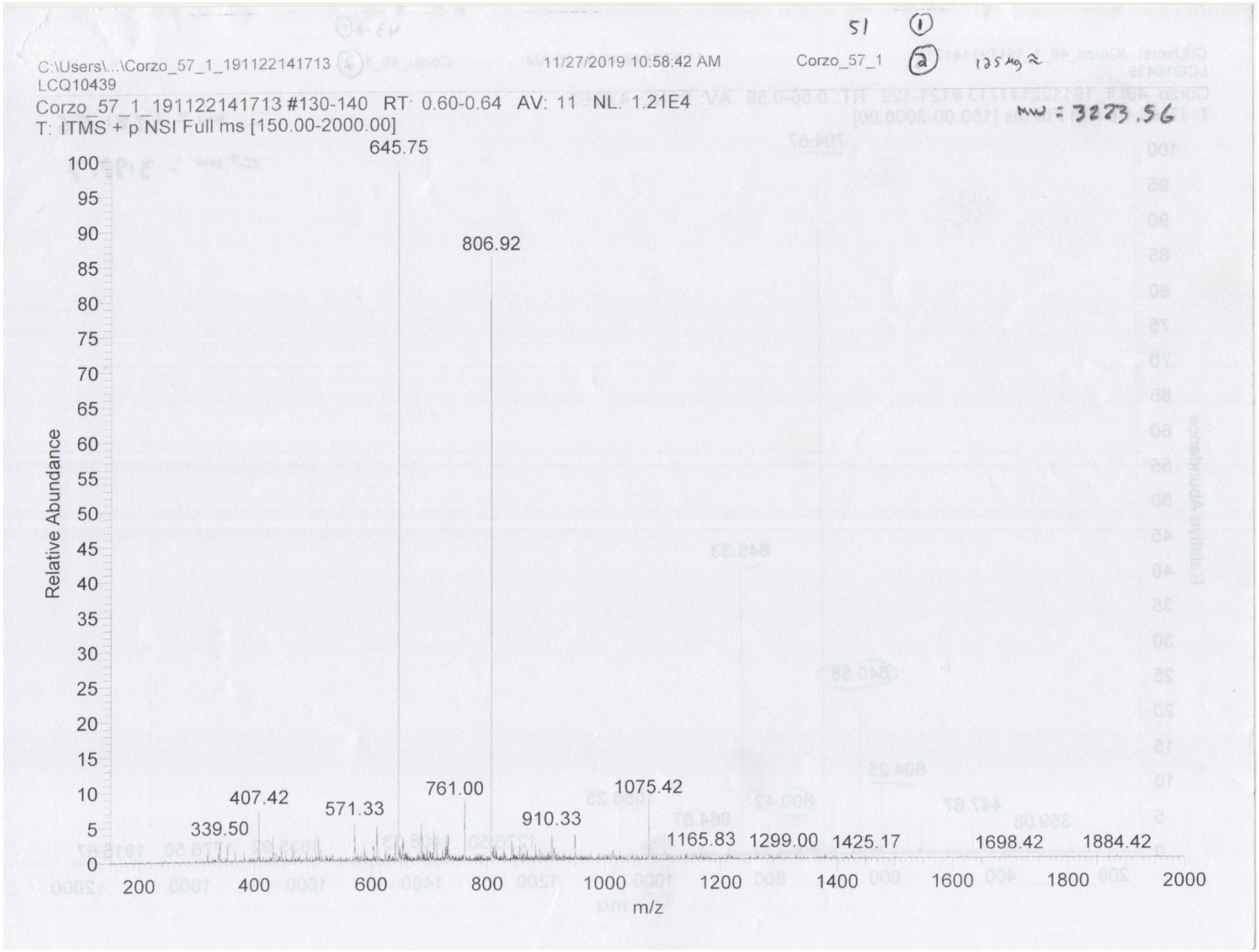
278.83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m/z
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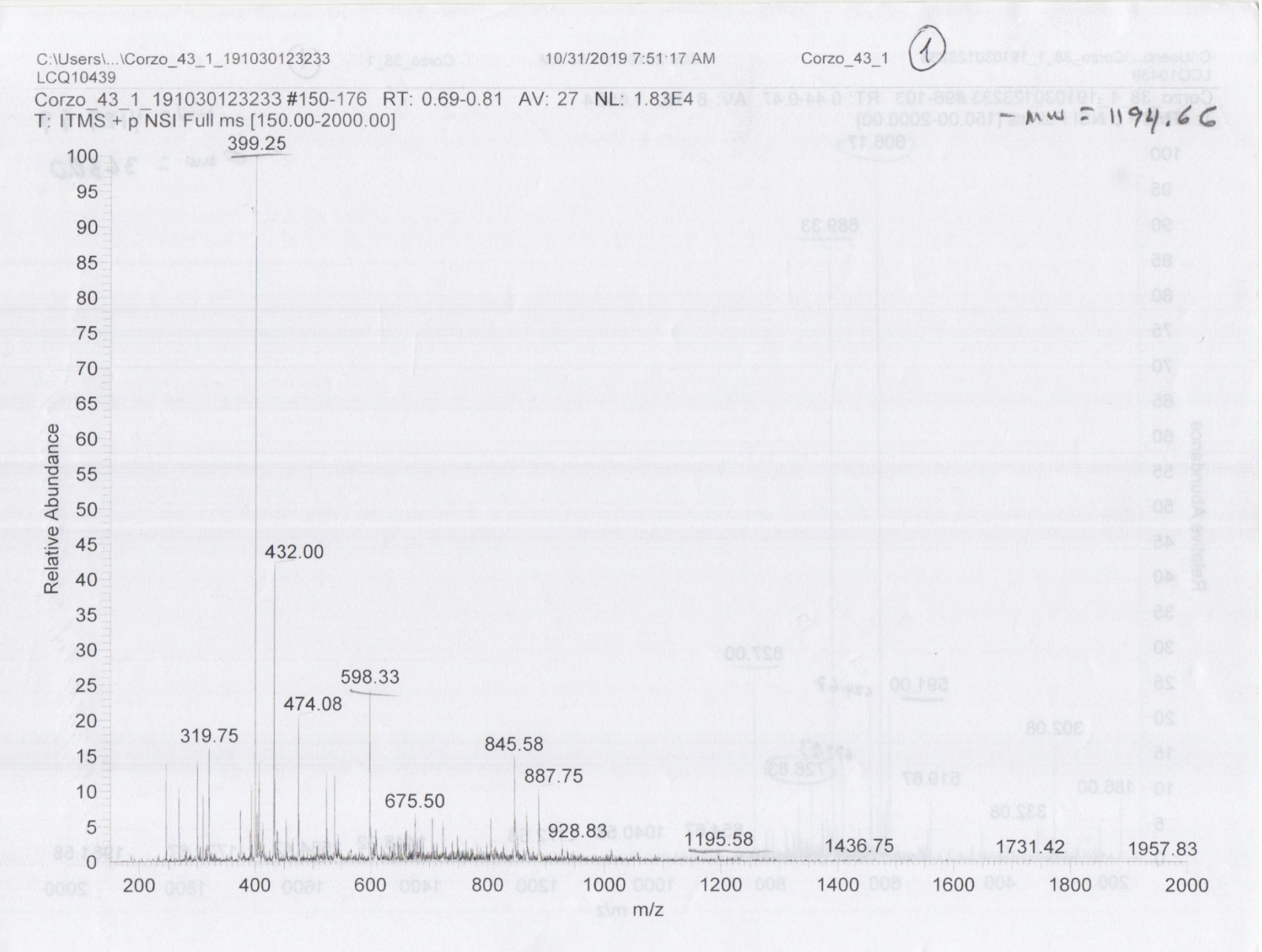
1884.42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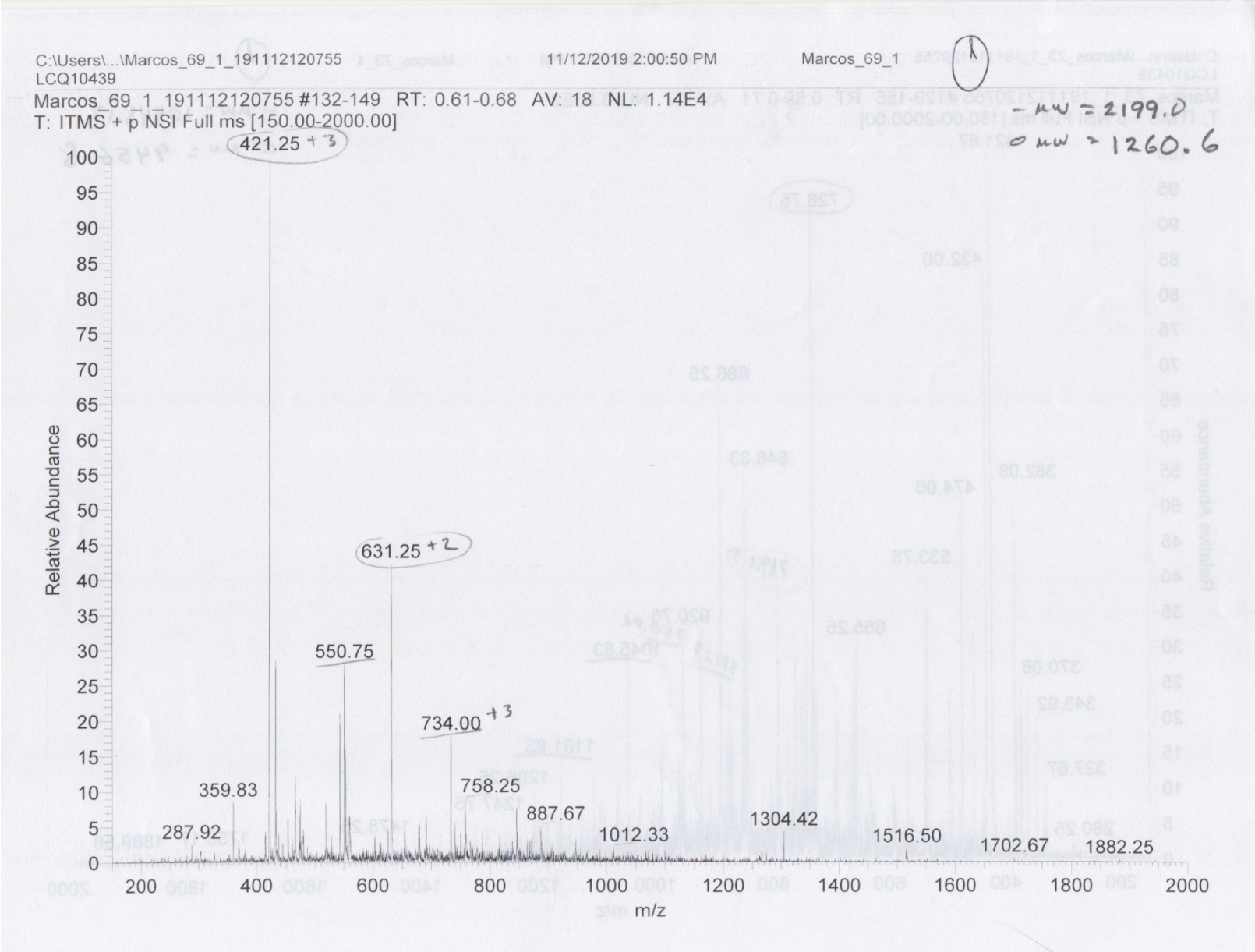
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o MW = 1260.6
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631.25 +2
550.75
734.00 +3
359.83
758.25
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287.92
1012.33
1304.42
1516.50
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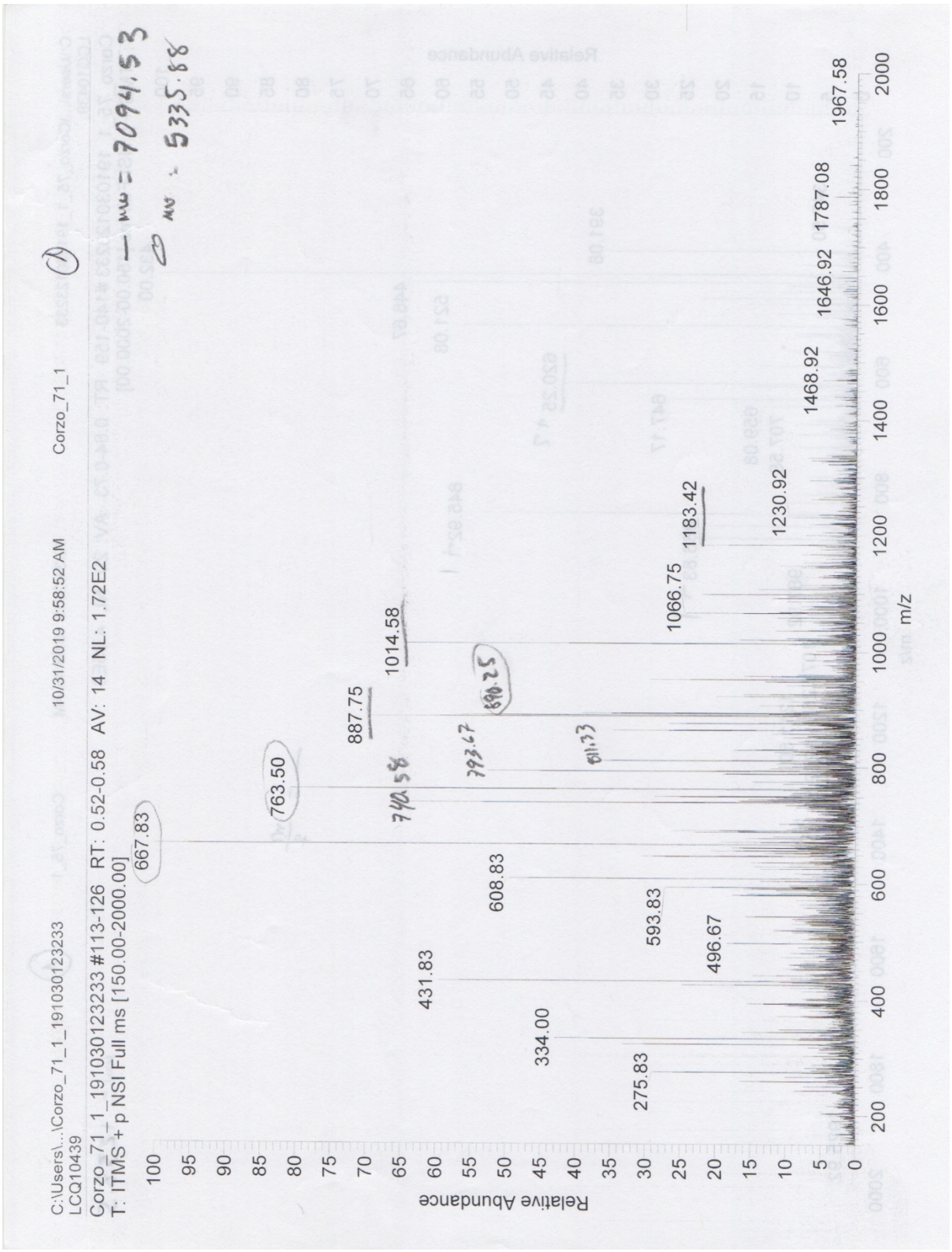
1882.25
Relative Abundance
m/z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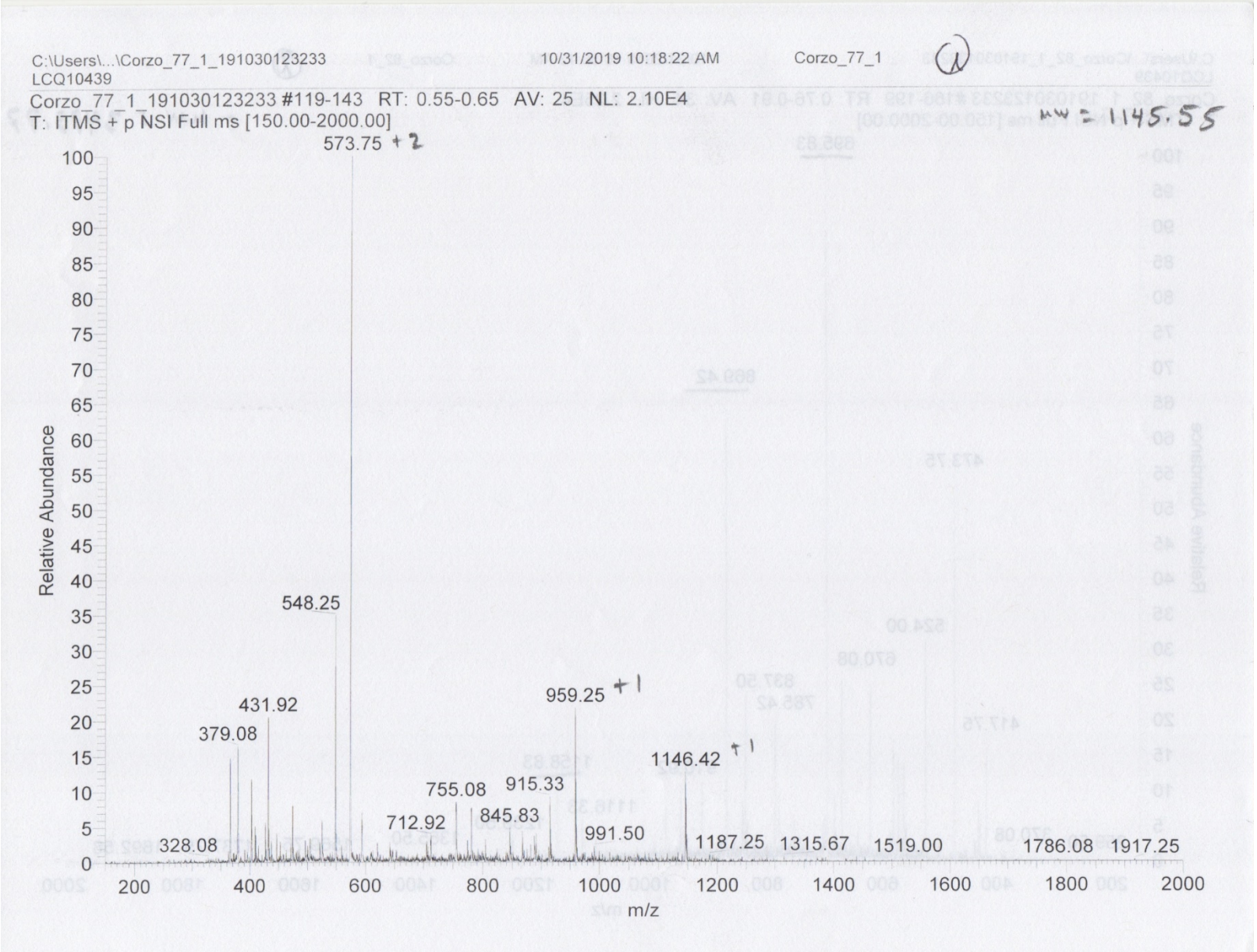
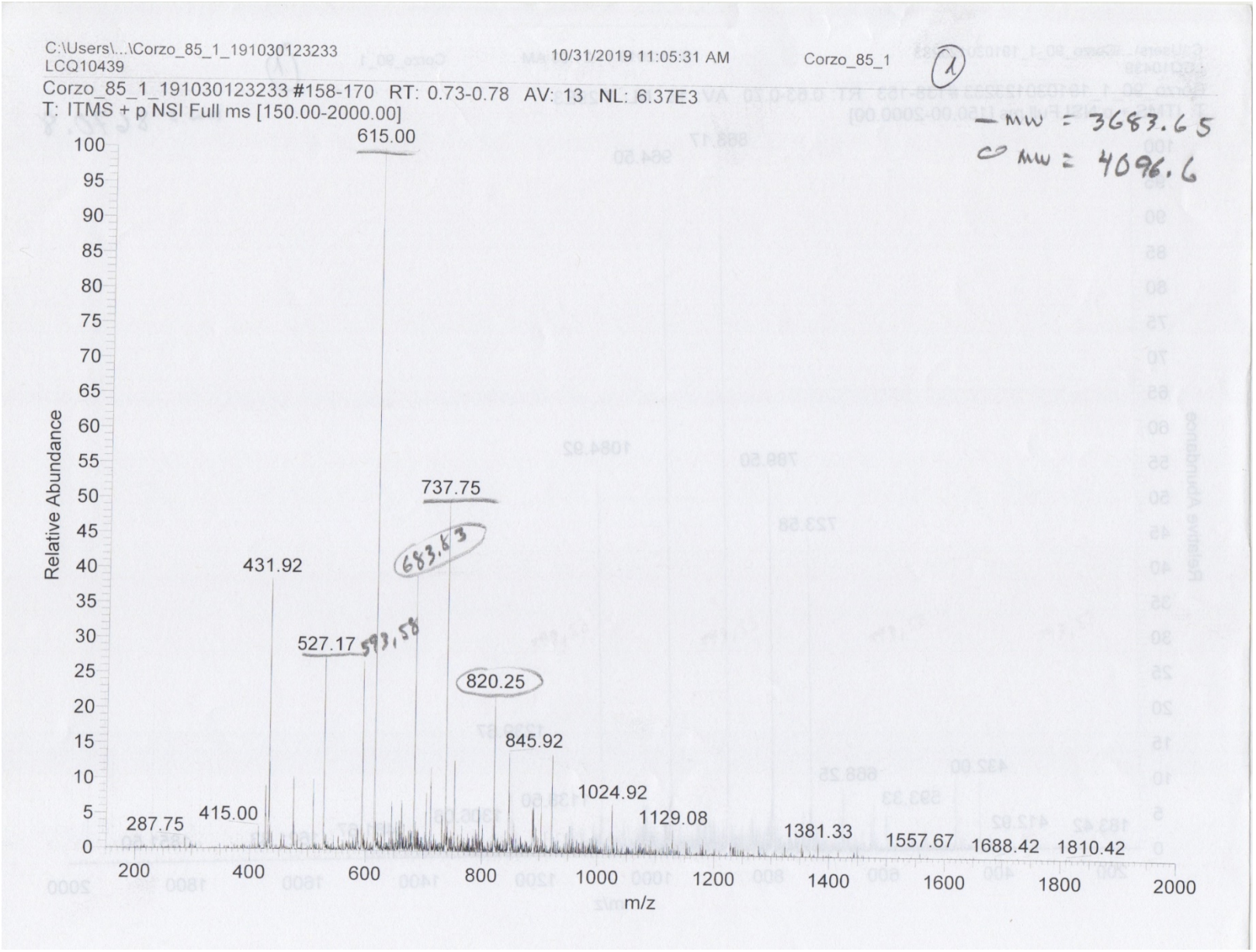
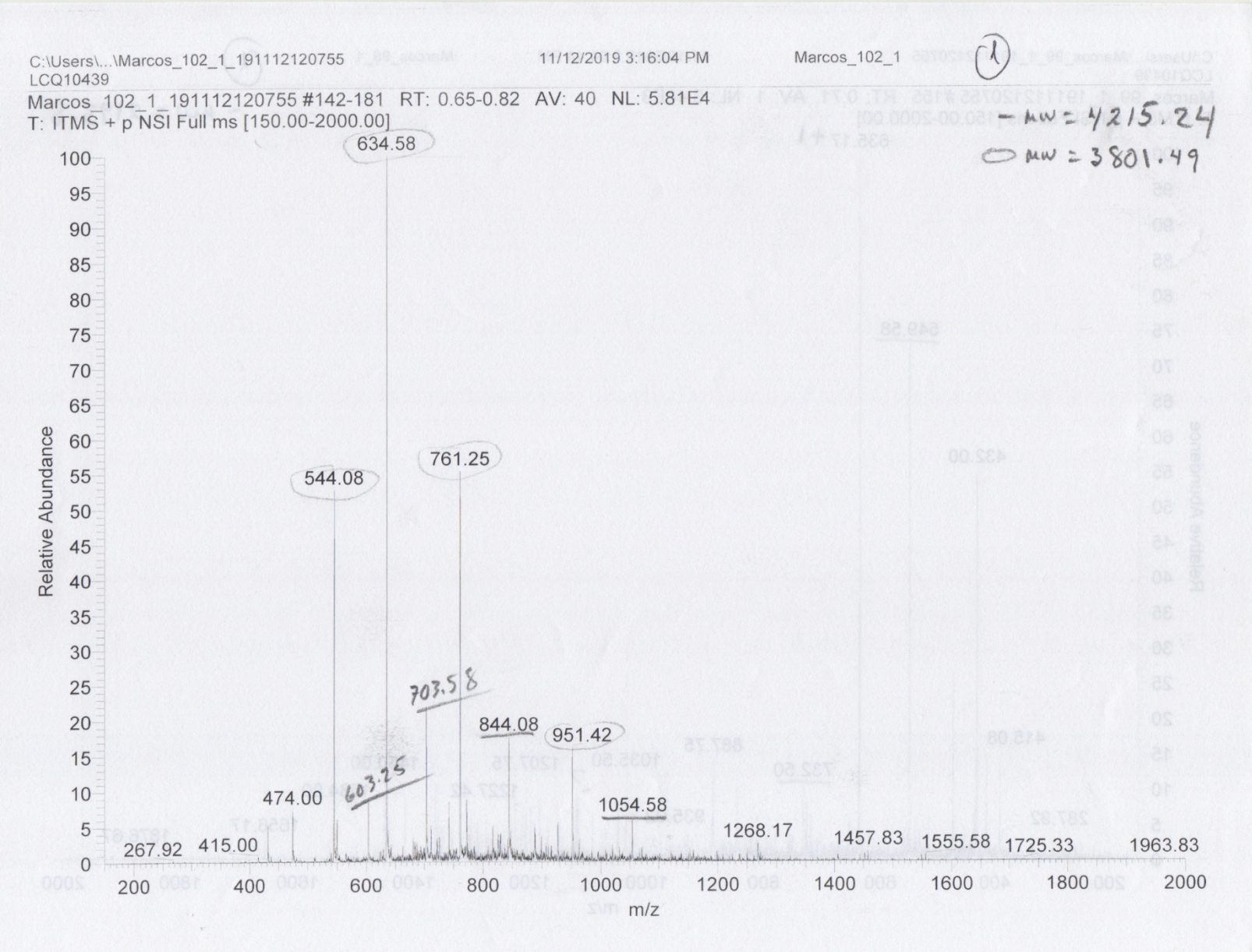
Corzo_71_1_191030123233 #113-126 RT: 0.52-0.58 AV: 14 NL: 1.72E2
T: ITMS + p NSI Full ms [150.00-2000.00]
Relative Abundance
m/z
667.83
763.50
887.75
1014.58
431.83
608.83
334.00
275.83
593.83
496.67
1066.75
1183.42
1230.92
1468.92
1646.92
1787.08
1967.58


C:\Users\...\Corzo_77_1_191030123233
LCQ10439
10/31/2019 10:18:22 AM
Corzo_77_1
Corzo_77_1_191030123233 #119-143 RT: 0.55-0.65 AV: 25 NL: 2.10E4
T: ITMS + p NSI Full ms [150.00-2000.00]
MW = 1145.55
573.75 +2
548.25
431.92
379.08
328.08
712.92
755.08
915.33
845.83
959.25 +1
991.50
1146.42 +1
1187.25
1315.67
1519.00
1786.08
1917.25
Relative Abundance
m/z


C:\Users\...\Corzo_85_1_191030123233
LCQ10439
10/31/2019 11:05:31 AM
Corzo_85_1
1
Corzo_85_1_191030123233 #158-170 RT: 0.73-0.78 AV: 13 NL: 6.37E3
T: ITMS + p NSI Full ms [150.00-2000.00]
— MW = 3683.65
MW = 4096.6
615.00
737.75
683.63
431.92
527.17
593.58
820.25
845.92
1024.92
1129.08
1381.33
1557.67
1688.42
1810.42
287.75
415.00
Relative Abundance
m/z


C:\Users\...\Marcos_102_1_191112120755
LCQ10439
11/12/2019 3:16:04 PM
Marcos_102_1
Marcos_102_1_191112120755 #142-181 RT: 0.65-0.82 AV: 40 NL: 5.81E4
T: ITMS + p NSI Full ms [150.00-2000.00]
(1)
– MW = 4215.24
MW = 3801.49
Relative Abundance
m/z
634.58
544.08
761.25
703.58
844.08
951.42
603.25
474.00
1054.58
267.92
415.00
1268.17
1457.83
1555.58
1725.33
1963.83