Physicochemical Properties, Chemical Constituents, Antioxidant and Antibacterial Activity of Honey from *Plebenia hylderbrandii* and *Meliponula bocandei* species of stingless bees against *Escherichia coli* and *Staphylococcus aureus*.

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A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science in Chemistry of Masinde Muliro University of Science and Technology.

July, 2021

DECLARATION

This research thesis is my original work prepared with no other than the indicated sources and support and has not been presented elsewhere for a degree or any other award.

Signature……... Date……..

Felistus Wavinya Kalungu

SCH/G/01-55509/2016

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance of Masinde Muliro University of Science and Technology a thesis entitled: '**Physicochemical Properties, Chemical Constituents, Antioxidant and Antibacterial Activity of Honey from *Plebenia hylderbrandii* and *Meliponula bocandei* species of stingless bees against *Escherichia coli* and *Staphylococcus aureus*.**'

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DEDICATION

This work is dedicated to my parents (Veronicah Kalunda & Josphat Kalungu), husband (Stephen Mwoni), siblings, classmates and friends for their immense love, encouragement and support. May the Almighty God bless them.

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ABSTRACT

Free radicals are natural byproducts of chemical processes, such as metabolism. They move through cells, disrupting the structure of other molecules and resulting in cellular damage. The damage coupled with pathogenic bacterial activity in the body is believed to contribute significantly to various health problems; macular degeneration, aging, bacterial diseases, cardiovascular problems, certain cancers, emphysema, Alzheimer's and Parkinson's diseases, ulcers and all inflammatory diseases, such as arthritis and lupus. Antioxidants protect key cell components from damage by neutralizing the free radicals. They occur naturally in the body or are consumed through the diet and hence help block cell damage. Honey particularly from the stingless bees is believed to be a source of antioxidants due its high pollen load and also possess antimicrobial compounds. It is good for scavenging free radicals, limiting microbes' proliferation and hence has potential for management of health problems that result from the effect of free radicals generated by the body cell. So far, there is no reported study on stingless bee honey constituent(s), potential as an antioxidant; neither is there any report on its antibacterial activity in Kenya. The aim of this research was to determine the chemical constituents, the antioxidant and antibacterial activity of honey from *Plebenia hylderbrandii* and *Meliponula bocandei* species of stingless bees against selected bacterial species viz; *Escherichia coli* and *Staphylococcus aureus*. The organic compounds of the honey were extracted using column extraction method and elution of the organic compounds done using acetone. Analysis of the organic compounds was done by use of liquid chromatography Electrospray ionization Quadrupole Time of flight Mass spectrometry (LC-ESI-QTOF-MS). The antioxidant activity of the honey was determined using Ferric Reducing Antioxidant Power (FRAP) assay and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) methods while the antibacterial activity was done by agar dilution method. Data obtained was analyzed statistically using Predictive Analytics SoftWare (PASW), Version 20. Thirty five (35) organic compounds including, O-glycosyl compounds, tricarboxylic acids and derivatives, isoquinoline derivatives, tannins, peptides and derivatives, flavonoids, terpene glycosides, coumarin derivatives, quinic acid derivatives, phenolic glycosides, anthracycline derivatives, porphyrins, pyridyl piperazine derivatives and indole derivatives were identified from the 2 honey samples. The honeys' physicochemical properties (pH, electrical conductivity, color intensity, moisture content) obtained were compared with East African Standards (EAS), European Union (EU) and Codex Alimentarius honey standards (2001). The Inhibition Concentration, IC_{50} values for the DPPH % scavenging activity ranged between 10.61-14.31 mg/ml. The FRAP results ranged from 585.82 µM for *Meliponula bocandei* to 911.36 µM for *Plebenia hylderbrandii* honeys Fe (II) /100 ml of honey. In addition, both honey types exhibited antibacterial activity against both *S. aureus* and *E. coli* but *Plebenia hyderbrandii* honey had a higher antibacterial effect than *Meliponula bocandei* honey against the two bacteria at the tested concentrations. This study reports for the first time the antioxidant activity and chemical composition of *Meliponula bocandei* and *Plebenia hylderbrandii* stingless bee honey samples from Western Kenya. Thus, it is evident that the potential of honey as an antioxidant results from an extensive range of compounds such as glycosides, quinolines, amino acids, flavonoids, among others. These natural antioxidants could be better alternatives to most synthetic antioxidants most of which have a lot of negative health repercussions. In addition, the two stingless bee honey possess bacteriostatic and bactericidal activity *in vitro*. Therefore, the various medicinal usages may be related to the honeys' chemical constituents, antioxidant and antibacterial activity. It is thus recommended that the honey harvested from the two stingless bees can be used as nutritional supplements in management of cancer, healing of wounds and inflammatory infections.

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LIST OF ABBREVIATIONS AND ACRONYMS

AEAC: Ascorbic acid equivalent antioxidant capacity

CAMNFF: Centre for African Medicinal and Nutritional Flora and Fauna

CAPE: Caffeic acid phenyl ester

CDC: Center for Disease Control and Prevention

CHD: Coronary heart disease

CRD: Completely Randomized Design

CRP: C - reactive protein

CVD: Cardiovascular disease

DNA: Deoxyribonucleic acid

DPPH: 2, 2-diphenyl-1-picrylhydrazyl

EAS: East Affrican Standards

EC: Electrical Conductivity

EU: European Union

FBG: Fasting Blood Glucose

FE: Ferrous Equivalent

FRAP: Ferric Reducing Antioxidant Power Assay

HDL-C: High-density Lipoprotein cholesterol

HPLC: High Performance Liquid Chromatography

HUS: Hemolytic Uremic syndrome

LC-ESI-QTOF-MS: Liquid Chromatography Electrospray Ionization Quadrupole Time of Flight Mass Spectrometry

LDL-C: Low-density Lipoprotein cholesterol

MGO: Methylglyoxal

MMUST: Masinde Muliro University of Science and Technology

MRSA: Methicillin Resistant *Staphylococcus aureus*

NFKB: Nuclear Factor –kappa beta

PASW: Predictive Analytics Software

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

TEAC: Trolox equivalent antioxidant capacity

TNF: Tumor necrosis factor

TLR-4: Toll-like receptor 4

UTI: Urinary tract infections

UV-VIS: UltraViolet-visible spectroscopy

VOCs: Volatile Organic Compounds

UV/VIS: Ultraviolet- visible Spectroscopy

WHO: World Health Organization

LIST OF SCHEMES

CHAPTER ONE

INTRODUCTION

1.1 Background information

Honey is a natural substance produced by both stingless and honey bees. It is a functional food with various biological properties, including; antioxidant, antibacterial, wound and sunburn healing potential (Alvarez-Suarez *et al*., 2013; Cooper, 2016). Further, properties like radical scavenging, anti-inflammatory, antidiabetic and antimicrobial activities have been reported (Gomes *et al*., 2010). It comprises of concentrated solution of complex sugar mixture whose main ingredients are glucose (31%), fructose (38%) and water (17-20%) (Aljadi & Kamaruddin, 2004; Alvarez-Suarez *et al*., 2016). It also has polyphenolic compounds such as flavonoids, phenolic acids, flavonols, cinnamic acid derivatives and catechins (Ferreira *et al*., 2009). These constituents have been reported to be responsible for honey's health benefits and its ability to reduce or inhibit formation of free radicals in humans (Ferreira *et al*., 2009). According to Alvarez-Suarez *et al*. (2012), free radicals can lead to oxidative damage induction to molecules essentially carbohydrates, proteins, DNA and lipids that can result to disease development. In addition, honey also possesses other minor constituents such as proteins, organic acids, minerals, vitamins and colloids. This composition is believed to influence its biological properties, which are correlated with its total flavonoid and phenolic content (Souza *et al*., 2009, Saxena *et al*., 2010; Khalil *et al*., 2011). A research done by Al-Mamary *et al*. (2002) indicated that there is a variation in the composition of honey from different sources. This variation mainly depends on varying entomological and floral origin, bee species among other external

factors including climate, type of soil and period of maturation (Alvarez-Suarez *et al*., 2018).

Apis mellifera honeys are commonly consumed worldwide because they are produced by bees, which are highly adaptable in various ecosystems (Meixner, 2010). However, stingless bee honeys are utilized in South America, Australia and Africa. Nevertheless, they have limited distribution worldwide compared to *Apis mellifera* honeys. This is due to their low industrial production, short shelf-life, and lack of official quality parameter standards, due to limited studies which involve these products (Souza *et al*., 2006; Guerrini *et al*., 2009). There is extremely limited documented information on stingless bee honey in Africa unlike *apis mellifera* honey and this prompted this study.

According to Bertoncelj *et al*. (2007), a rising concern in the determination of antioxidant potential of honey has been realized. These properties of honey are associated with antioxidant compounds like the polyphenols (Alvarez-Suarez *et al*., 2013; Santos-Buelga & González-Paramás, 2017). Reports show that the antioxidant activity is highly influenced by the botanical origin of the honey. Nevertheless, handling, processing and storage can also have effects on the antioxidant activity but to a lesser extent (Beretta *et al*., 2005). In addition, honey's antioxidant potential is strongly correlated with the existing total phenolic concentration and color of the honey (Bertoncelj *et al*., 2007; Beretta *et al*., 2005). It has been revealed that dark colored honey has higher total phenolic content and therefore higher antioxidant activities than the light-colored honey (Beretta *et al*., 2005; Bertoncelj *et al*., 2007). Antioxidant components of honey have been identified and quantified all over the world from the previous research (Ferreira *et al*., 2009; Gheldof *et al*., 2002).

Honey has been reported to possess antimicrobial activity which is attributed to its physicochemical parameters such as pH, Moisture content, electrical conductivity, color intensity, among others. Its low pH level results from organic acids concentration such as gluconic acid; which in combination with its high osmolarity prevents growth of microbes (Szweda, 2017). Its moisture content determines its ability to persist stability and to resist decay by yeast fermentation. Too much moisture content often causes fermentation of honey resulting to low shelf life and unpleasant flavor (Bogdanov *et al*., 2002). Besides, its electrical conductivity is related to organic acids, mineral content, some complex sugars, etc. (Warui *et al*., 2019). High ash and acid contents signify higher conductivity (Adenekan *et al*., 2010) hence can lead to high antibacterial activity. Honey's color intensity shows presence of pigments such as flavonoids and carotenoids; which are well-known to contribute to its antioxidant activity (Moniruzzaman *et al*., 2013). This in return contributes to its antibacterial activity.

The antimicrobial activity is also attributed to the presence of hydrogen peroxide and antimicrobial peptide; defensin-1; (Alvarez-Suarez *et al*., 2013; Santos-Buelga & González-Paramás, 2017). The hydrogen peroxide presence results into antimicrobial activity variation (Lusby *et al*., 2005; French *et al*., 2005). Moreover, it may also be associated with the hygroscopic nature of honey since it can dehydrate bacteria. The global burden of infectious diseases can be reduced by antimicrobial agents. Despite the development and spread of resistant pathogens, efficacy of antibiotics is still diminishing. This may pose a very severe threat to the public health, including all types of antibiotics, especially the main last-resort drugs, for which their frequency in resistance is rising all over the world (Levy & Marshall, 2004; Mandal *et al*., 2009). Thus, there is urgent need

for alternative antimicrobial strategies, which will lead to re-evaluation of the therapeutic use of ancient remedies, especially the use of honey (Mandal *et al*., 2010a; 2010b; Basualdo *et al*., 2007).

Nowadays, synthetic phenolic compounds including butylated hydroxytoulene and butylated hydroxyanisole have been used as antioxidants in therapeutic industries, food industries and cosmetics. However, these synthetic antioxidants are highly volatile and unstable at high temperatures (Papas, 1999). In addition, some of the synthetic antioxidants have been proven to be carcinogenic in nature and thus use of synthetic food additives has been strictly regulated. This has led to manufacturers shifting from using synthetic antioxidants to natural ones (Papas, 1999). These problems have necessitated the research for alternative effective, non-toxic natural compounds with antioxidative activity, which could be used as natural antioxidants for free radical scavenging in human body.

In Kenya, bioactive constituents, antioxidant and antibacterial activity has been documented for *Apis mellifera* honey (Mokaya *et al.,* 2020) but only antimicrobial activity on stingless bee honey has been reported (Muli *et al*., 2008). In Western part of Kenya, no study has been carried out on stingless bee honey; therefore, the study was set to scientifically evaluate physicochemical properties, chemical constituents, *in vitro* antioxidant and antibacterial activity (bacteriostatic & bactericidal effect) of *Plebenia hylderbrandii* and *Meliponula bocandei* honeys produced by stingless bees against *E. coli* and *S. aureus*. These two honey samples are new and have never been tested before for these activities. The bacteria (*E. coli* and *S. aureus*) used are commonly involved in causing urinary tract infections, diarrhea, septicemia, wound infections and community

acquired and nosocomial infections in humans (Makvana & Krilov, 2015; Monecke *et al*., 2011). *E. coli*, a gram-negative bacterium represents the other gram-negative bacteria while *S. aureus*, a gram-positive bacterium represents the other gram-positive bacteria since they are accepted test organisms. In addition, they are the most sensitive and prevalent organisms and have fast growth rates thus, allow organic compounds to penetrate fast within short period of time hence the reason for their study.

1.2 Statement of the problem

The damage of body cells and tissues due to free radicals contributes to the etiology of many chronic health problems such as carcinogenesis, atherosclerosis, mutagenesis, Parkinson's disease, Alzheimer's disease, cataracts, asthma, dementia, cardiovascular and inflammatory diseases. All these health problems are directly or indirectly linked to the highly reactive and potentially destructive free radicals generated in human body cells (Sastre *et al*., 1996). In addition, the damage to cells due to free radicals may also lead to pathological changes that are associated with aging (Ashok & Ali, 1999). Consequently, there is need to search for natural antioxidants compounds, which can be used to reduce the risks of these diseases by mopping off the free radicals generated in human body cells. Antimicrobial agents (such as antibiotics) have been reported as essentials in reduction of worldwide burden of infectious infections. Nevertheless, the effectiveness of the antibiotics has diminished due to development and spread of resistant pathogens. This bacterial resistance to the antimicrobial agents poses severe threat to public wellbeing. The increase in resistance frequencies is well documented globally in all categories of antibiotics, including the major last-resort drugs (Mandal *et al*., 2009). This led to urgent need for alternative antimicrobial control strategies to re-evaluate the therapeutic use of

ancient remedies, such as honey a decade ago (Mandal *et al*., 2010a; 2010b). Most research work have focused on honey from honey bee and little has been done on stingless bee honey especially from the stingless bees inhabiting Africa. The research in this thesis focused on honey from two species of stingless bees (*Plebenia hylderbrandii* and *Meliponula bocandei*) in Western Kenya.

1.3 Objectives

1.3.1 General objectives

To determine physicochemical properties, chemical constituents, antioxidant and antibacterial activity of *Plebenia hylderbrandii* and *Meliponula bocandei* honey samples against *Escherichia coli* and *Staphylococcus aureus*.

1.3.2 Specific objectives

The research was guided by the following specific objectives:

i. To determine the physicochemical properties and chemical constituents of honey from *Plebenia hylderbrandii* and *Meliponula bocandei* species of stingless bees.
ii. To determine the antioxidant activity of honey from *Plebenia hylderbrandii* and *Meliponula bocandei* species of stingless bees.
iii. To determine the antibacterial effect of different concentrations of *Plebenia hylderbrandii* and *Meliponula bocandei* honey samples on the growth of *E. coli* and *S. aureus*.

1.4 Hypothesis

Honey from *Plebenia hylderbrandii* and *Meliponula bocandei* species of stingless bees contain phenolic and flavonoid chemical constituents.

Honey from *Plebenia hylderbrandii* and *Meliponula bocandei* species of stingless bees possess antioxidant and antibacterial activity.

1.5 Justification

Health disorders such as inflammatory diseases have been reported as a result of oxidative stress caused by free radicals. Despite the fact that there are several synthetic antioxidants for free radical scavenging, their use has led to many problems such as toxicity to the users due to their carcinogenic nature (Papas, 1999). They have also been noted to be highly volatile and unstable especially at elevated temperatures. These problems have made researchers to rethink their use as they shift their attention to natural antioxidants. This is because these natural antioxidants are cheap, have high compatibility with their intake of diet and are safe in the human body with no harmful effects. Among the natural antioxidants, honey is acceptable. This acceptability is due to its cheapness, non-toxicity and compatibility with dietary intake to human beings.

Besides the problems associated with synthetic antioxidants, antimicrobial agents, including antibiotics, which have been used in reducing infectious diseases' worldwide burden have proven resistant to these infections hence diminishing their effectiveness. This calls for urgent need for alternative antimicrobial control strategies, which will consequently re-evaluate the therapeutic use of ancient medicine such as honey (Mandal *et al*., 2010a; 2010b; Basualdo *et al*., 2007). Honey is reported to possess healing property which is attributed to its antibacterial activity. In addition, it has a high viscosity which provides protective barrier thus preventing infection and also maintains a moist wound condition (Mandal & Mandal, 2011).

CHAPTER TWO

LITERATURE REVIEW

2.1 Stingless bees

Bees are flying insects closely related to wasps and ants, known for their role in pollination and, in the case of the best-known bee species, the western honey bee, for producing honey. They are classified into stingless bees and honeybees or stinging bees.

Stingless bees are the largest group of eusocial insects with no functional sting. They belong to *Apidae* family and *Meliponinae* subfamily. They comprise of over 600 species in the tribe meliponin (or sub-tribe meliponina) within 56 named genera and reside in all the tropics and subtropic regions of the world where they are abundant in species (Kwapong *et al*., 2010). They are active throughout the year though less active in cool weather with some species presenting diapauses (Ribeiro, 2002). Unlike the honey bees, stingless bees lack effective vestigial stings since they are greatly reduced hence are essentially harmless to human beings. Most of their species are shy and sensitive but when disturbed or attacked, they defend themselves by mass swarming, chasing and attacking, though they rarely attack individuals (Gloag *et al*., 2008). Although they do not sting, they are able to regurgitate a noxious, sticky chemical that they rub on trespassers and defend themselves by biting when disturbed. For instance, stingless bees of genus *oxytrigona* possess mandibular secretions that trigger painful blisters in humans. Due to their eusocial nature, they may have formidable large colonies made by the number of defenses despite their lack of sting.

Stingless bees exploit plant-based resources (Heard, 1999) including pollen, nectar, resin, leaves, latex, oils, scents and seed as they forage (Nunes-Silva *et al*., 2010). They produce honey which is less viscous and darker than *Apis* honey and has a stronger acid flavor. In addition, the honey possesses a stronger bacteriostatic effect (Krell, 1996). In Kenya, stingless bees are found in Mwingi, Kakamega forest, some parts of the Rift Valley, Nyanza and Arabuko Sokoke along the coast.

2.2 Honey

Honey is a natural health product which is produced by both stingless and *Apis mellifera* bees from secretions or plants' nectar and normally lacks additives or preservatives. Generally, it is composed of intricate mixture of carbohydrates and other infrequent substances like amino acids, flavonoids, proteins, vitamins , lipids, minerals, organic acids, aroma compounds, pigments, enzymes, waxes and other phytochemical substances (Gomes *et al*., 2010; Lazarevic *et al*., 2010). This composition depends highly on nectar floral origin foraged by the bees. Nevertheless, its specific composition is dependent on other factors like bees' species, agricultural practices, nectar composition of the plant source, seasonal and environmental conditions, climate, honey treatment during extraction and its storage (Iglesias *et al*., 2012).

Honey is a preferable functional food utilized since ancient times in ethnomedicine as a remedy for microbial diseases. According to WHO (2000), 75-80% of the world's population utilizes traditional folk medicine (Alves & Rosa, 2005). Many people depend on natural products from nature for medicinal purposes. This indicates that medicinal plants are fundamental elements of traditional medical practices (Assessment, 2005). Honey has been proved to treat burns, asthma, infected and chronic wounds, eye ailments

such as cataracts, skin ulcers and gastrointestinal disorders (Molan, 2001; Molan, 2002; Orhan *et al*., 2003). Its effectiveness partially results from its antibacterial activity, which is mainly contributed by hydrogen peroxide together with other non-peroxide factors including pH, flavonoids, phenolic acids and lysozyme (Yamani *et al*., 2016). The therapeutic property of honey is also partly dependent on its antioxidant capacity since some of these diseases result from oxidative damage. The presence of hydrogen peroxide and minerals in honey may result to very reactive hydroxyl radicals (McCarthy, 1995), which is evidence that honey has mechanisms to control the formation and removal of reactive oxygen species.

According to Küçük *et al*. (2007), honey is believed to contain natural antioxidants that help in food preservation and play a key role in human health by curbing damage, which result from oxidized agents such as oxygen thus reducing risks of immune-system decline, heart disease, cancer, cataracts and other various inflammatory diseases (National Honey Board, 2003). These antioxidants include both enzymatic substances such as catalase (Schepartz, 1966) and non-enzymatic substances such as α-tocopherol, ascorbic acid, amino acids, carotenoids, organic acids, proteins and Maillard reaction products, which result from condensation of amino acids or proteins and reducing sugras and responsible for darkening of honey (Bertoncelj *et al*., 2007; Baltrušaityte *et al*., 2007). Others include polyphenolic compounds such as flavonols, flavonoids, phenolic acids, cinnamic acid derivatives and catechins. In humans, honey consumption raises plasma antioxidants, which protect cells in the bloodstream, an indication that bioactivity and bioavailability of honey is efficient in antioxidant transfer from the honey to the plasma (Gheldof *et al*., 2003).

2.3 Stingless bee honey

Stingless bee honey is a valuable product produced by the stingless bees. It varies from the one produced by honey bees with regard to viscosity, taste, total acidity, water content, color and diastase activity (Chuttong *et al*., 2016; De Almeida-Muradian *et al*., 2014; Guerrini *et al*., 2009). It distinctively has a more fluid texture, higher protein content, higher acidity, higher electrical conductivity, higher moisture content, distinct sweetness with an acidic taste, lower diastase activity and slow crystallization (Chuttong *et al*., 2016; Kek *et al*., 2017; Abd Jalil *et al*., 2017). Moreover, it doesn't ferment in the nest due to the numerous resin chemicals that impart hydrogen peroxide and dark color to it despite its high water content (Greenwood, 1995). Its higher water content enables microorganisms to survive and be active. This makes it to naturally ferment inside the pots. Its tangled biological properties comprise of sugars; glucose and fructose, phytochemical compounds such as flavonoid and phenolic acid, and other bioactive compounds such as trace elements, organic acids, amino acids including phenylalanine, tyrosine, alanine and valine. In addition, it is also composed of proteins, vitamins and Maillard reaction products (Mustafa *et al*., 2018; Abu Bakar *et al*., 2017). All these constituents particularly the proteins may play a notable role in disease development signaling pathway.

It has been reported to boost immune system and improve cell functioning in erythrocytes involved with anticancer, antiseptic, antimicrobial, anti-inflammatory and wound healing (Abu Bakar *et al*., 2017; Zainol *et al*., 2013). Moreover, it is regarded in folk medicine as being powerful than honey bee honey for utilization as natural cure in handling common diseases (Garedew, 2003). Stingless bee honey has been used locally for medicinal

purposes for many ailments, particularly to the poor people with less access to modern medicine. For instance, it has been utilized to treat cough, sore throat, stomach disturbance, tonsillitis, intestinal and stomach ulcers, mouth disease, disease of mucus membrane, cold and as wound dressing (Garedew, 2003). However, stingless bee honey is more expensive than honey bee honey. This may be attributed by its medicinal value and its rearing labor intensiveness and harvesting process (Garedew, 2003).

Stingless bee honey has higher potential of antioxidant and flavonoid content than honey bee honey. According to Alvarez-Suarez *et al*. (2018), stingless bee honey contains larger content of flavonoids, ascorbic acid, phenolic compounds, carotenoids, protein and free amino acids as well as antioxidant capacity compared to *Apis mellifera* honey. In addition, a study carried out by Avila *et al*. (2018) confirmed that stingless bee honey has potential antimicrobial activity hence can be used as an alternative in the treatment of microbial infections and inflammation. This type of honey can act as a significant potential in food innovation, cosmetic and pharmaceutical industries and should be utilized in stimulation of new lines of research in consolidating its application and consumption. Despite its application, there is limited data on stingless bee honey. This is attributed to its low output/production. In western Kenya, for instance, no study has been carried out on stingless bee hney. This prompted the need for this study to determine the physicochemical properties, chemical constituents, antioxidant and antibacterial activity of honey from *Plebenia hylderbrandii* and *Meliponula bocandei* species of stinglees bees against *E. coli* and *S. aureus*. Moreover, these two honey samples are new and have never been tested before for these activities.

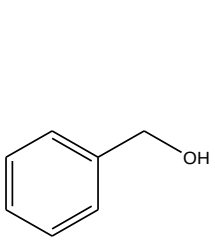
2.4 Chemical composition of honey

Honey composition depends mainly on entomological and floral origin, bee species among other external factors including climate, type of soil and period of maturation (Alvarez-Suarez *et al*., 2018).

Honey comprises of roughly 200 compounds, including phytochemicals, enzymes, proteins, pollen, pigments, vitamins, organic acids and amino acids, with sugar and water being the major content whereby sugars (glucose & fructose) consists of approximately 95% of honey's dry weight (Fallico *et al*., 2004; Escuredo *et al*., 2014; Da Silva *et al*., 2016). Moreover, other oligosaccharides and disaccharides, such as pentose, sucrose, maltose and maltotriose can also be present. Other components of honey include trace elements and minerals such as potassium, calcium, sodium, phosphorus, magnesium, sulphur, zinc, iron, manganese and copper. Various vitamins are found in honey and include riboflavin, thiamine, pantothenic acid, nicotinic acid, pyridoxine, biotin, ascorbic acid, folic acid and cyanocobalamin (Ciulu *et al*., 2011). Proteins and enzymes are the minor components, with enzymes playing essential roles in activities including antimicrobial activity and facilitation of calcium absorption (Ariefdjohan *et al*., 2008).

Honey also comprises of flavonoids and total phenolic compounds, which have been reported to ameliorate oxidative stress enabling it to possess antioxidant activity (Can *et al*., 2015; Flores *et al*., 2015). These flavonoids and phenolic acids highly determine the unique appearance, flavor and bioactivity of honey (Da Silva *et al*., 2016). The antioxidant activity is also contributed by phytochemicals present in honey such as polyphenols. Some of these phytochemicals include benzyl alcohol (**1**), methyl 3, 5-dimethoxy-4-hydroxybenzoate (**2**), 3, 5-dimethoxy-4-hydroxybenzoic acid (**3**), 3, 4, 5-

trimethoxybenzoic acid (**4**), 2-hydroxybenzoic acid (**5**), 2- hydroxy-3- phenylpropionic acid (**6**) and 1, 4-dihydroxybenzene (**7**) which have been reported to possess antimicrobial activity (Molan, 2001). The composition of honey varies in different unifloral honeys (Amiot *et al*., 1989).



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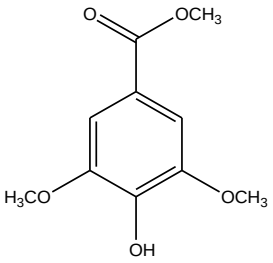

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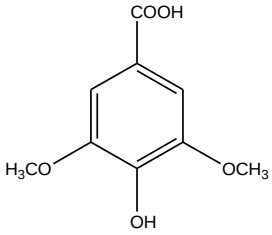

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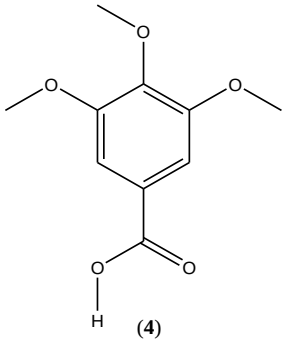

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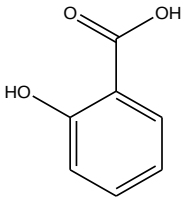

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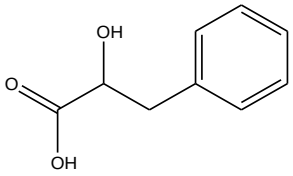

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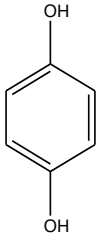


(**7**)

2.5 Volatile organic compounds (VOCs) in honey

VOCs are organic compounds with high vapour pressure at normal room temperature. Honey may consist of aromatic compounds at minimal concentrations in form of volatile mixtures. The volatile compounds in the mixture may have varying taste, aroma and

function, which results to the honey uniqueness. In monofloral honey, a lot of the volatiles come from the nectar of flowers. The volatile organic compounds which contribute to the aroma of honey differ in quantity and quality (Jerković & Marijanović, 2009). This is due to varying sources of nectar, honey microbes, processing of honey, storage condition and modification of plant compounds by bees (Jerković & Marijanović, 2009; Alissandrakis *et al*., 2005). The volatiles detected in honey include benzene derivatives, norisoprenoids, aldehyde, hydrocarbons, cyclic compounds, furan and pyran derivatives, terpenes and their derivatives and Sulphur organic compounds (Castro-Vázquez *et al*., 2006; Barra *et al*., 2010). VOCs composition in honey is enticed by floral origin and nectar composition. This could also be affiliated with the geographical origin of honey (Kucuk *et al*., 2007; Cuevas-Glory *et al*., 2008).

2.6 Physico-chemical properties of honey

The quality of honey can be determined through physico-chemical analysis (Cantarelli *et al*., 2008; Stolzenbach *et al*., 2011). This analysis provides useful information which can help verify honeys' authenticity along with its geographical and botanical origin (Stolzenbach *et al*., 2011). Among the physico-chemical parameters include; moisture content, pH, electrical conductivity and color intensity.

Moisture content is a significant parameter in determining the quality of honey and is the most crucial physicochemical parameter due to its effect on storage life of honey. It determines honey capability to remain stable and to resist spoilage by yeast fermentation. According to Bauer (1960), the moisture content of honey can vary after being removed from a bee hive due to storage conditions after extraction. Too much moisture content regularly causes fermentation of honey resulting to low shelf life and unpleasant flavor

(Bogdanov *et al*., 2002). Climatic conditions, degree of honey maturity reached in the hive and handling in the course of harvesting, processing and storage determine the water content level in honey (Silva *et al*., 2009; Yücel & Sultanog, 2013). Moisture content in honey varies depending on either botanical origin of the honey (Yücel & Sultanog, 2013) or conditions of weather during honey production (Yousif, 2007). Variations may also result from temperature, floral source, honey extraction method, storage conditions and relative humidity. Various researchers studied moisture content in honey and found that it ranges between the following; 13.4 % to 22.9% (White *et al*., 1962); 13.1% to 26.8% (Ibrahim, 1985); 14.2 % to 19% (Terrab *et al*., 2004) and13.0 %to 18.9% (Lazaridou *et al*., 2004).

All acids dissociate in aqueous solution releasing hydrogen ions or protons. This gives acids their sour taste and other characteristics. The strength of acidity is measured by the total concentration of hydrogen ions. However, the pH of honey is indirectly related to free acidity as a result of buffering action of minerals and several acids present. pH of honey range between 3.42 to 6.10 (White *et al*., 1962); 3.56 to 4.79 (Terrab *et al*., 2004); 3.72 to 4.64 (Serrano *et al*., 2004); 3.75 to 4.61 (Downey *et al*., 2005) and between 3.49 to 4.43 (Ouchemoukh *et al*., 2007). The low pH level of honey results from organic acids concentration such as gluconic acid which in combination with its high osmotic pressure prevents growth of microbes contributing to its antibacterial activity (Szweda, 2017).

Electrical conductivity (EC) of honey is interrelated to concentration of ash, organic acids, mineral salts, proteins and complex sugars (Chefrour *et al*., 2009). It may also be based on inorganic salts and polyols (Singh & Bath, 1997). The EC variation in honey is

dependent on floral origin and can be used in classification of honey as from either honeydew or nectar.

The color of honey reflects relatively the content of pigments with antioxidant activities (flavonoids, carotenoids etc.). High color intensity indicates high content of flavonoids and phenolic compounds (Moniruzzaman *et al*., 2013). It is also an indicator of the possibility of presence of condensation products of reactions between the amino acids or proteins and reducing sugars in honey.

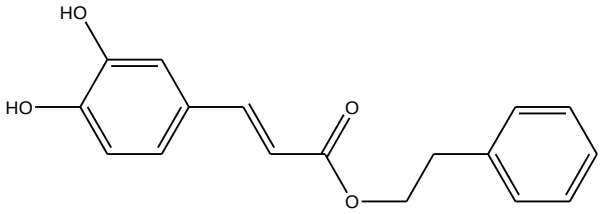
2.7 Antioxidants

Antioxidants are natural chemical substances in food that can avert or reduce oxidative stress of physiological processes. Human body constantly produces free radicals as a result of systematic utilization of oxygen. These free radicals produced lead to cell damage in the body. This causes different kinds of health complications including macular degeneration, diabetes, heart disease and cancer. Antioxidants being marvelous free radical scavengers assist in repairing and preventing cell damage triggered by these free radicals. Regular consumption of anti-oxidative foods have been reported to reduce the risk of chronic diseases such as cancer, coronary heart disease, and altitude sickness (Dembinska-Kiec *et al*., 2008; Yadav *et al*., 2016). The main sources of antioxidants traditionally are dietary foods and traditional herbs. Some of the reported antioxidants in food include β-carotene, vitamin C, polyphenols, flavonoids, vitamin E, vitamin A, peroxidase and glutathione in addition to enzymes such as superoxide dismutase, catalase and various peroxides (Yadav *et al*., 2016).

2.7.1 Antioxidant properties of honey

Honey exhibits strong antioxidant activity. The antioxidant capacity of honey highly depends on the floral source used in the collection of nectar, environmental and seasonal factors, in addition to the processing of the honey (Kıvrak & Kıvrak, 2017).

The components responsible for the antioxidant activity include proteins, vitamin E, vitamin C, amino acids, carotenoids, organic acids, trace elements and enzymes such as oxidase, catalase and peroxidase. In addition, it also contains polyphenols such as caffeic acid phenethyl ester (CAPE) (**8**), caffeic acid (**9**), chrysin (**10**), quercetin (**11**), galangin (**12**), kaempferol (**13**), acacetin (**14**), pinobanksin (**15**), pinocembrin (**16**) and apigenin (**17**). These have evolved as favourable pharmacological agents in cancer treatment (Jaganathan & Mandal, 2009).



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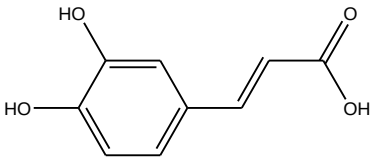

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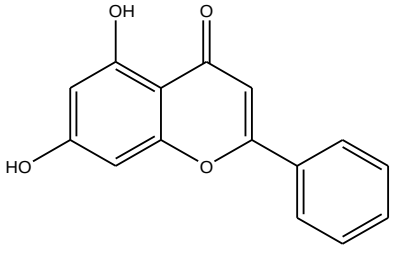

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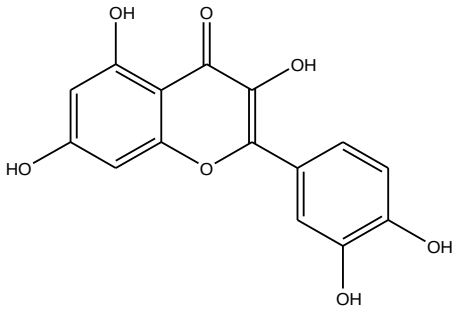

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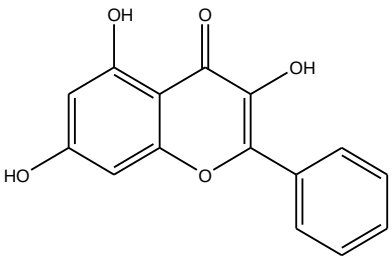

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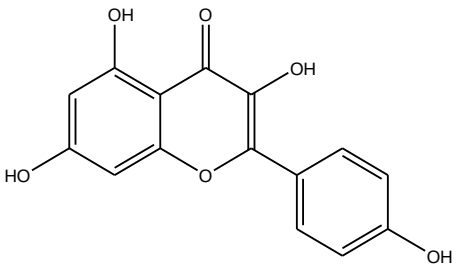

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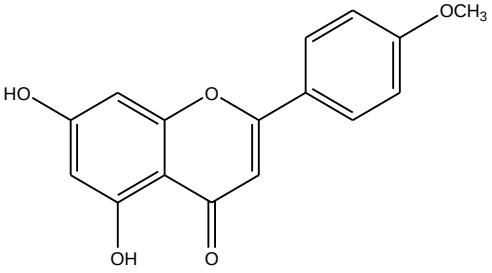

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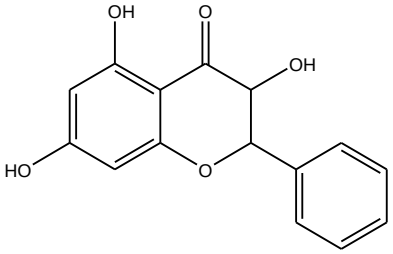

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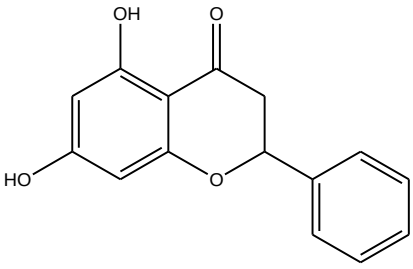

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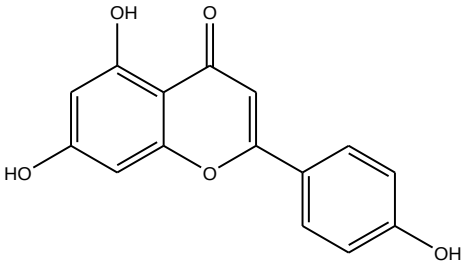


(**17**)

Honey also acts as an antidepressant during high intellectual, physical and emotional stress as a result of the antioxidant properties (Jaganathan & Mandal, 2009). A research done by Tsiapara *et al*. (2009) and Ahmed & Othman (2013) indicates that phenolic acid and flavonoid constituents are the main contributors for the antioxidant activity and medicinal effects in honey. Thus, the content of phenolic compounds can notably reflect the total honey antioxidant activity although it is a combination of comprehensive active

substances such as flavonoids, phenolic acids, proteins, enzymes, amino acids, carotenoids, among others (Beretta *et al*., 2005). However, according to Küçük *et al*. (2007), the level of phenolic components in honey is always not positively proportional to its antioxidant activity. This is due to the presence of fluctuating polyphenols' types. This leads to inconsistent scavenging activity of honey (Küçük *et al*., 2007). Research shows that polyphenols content is notably correlated with color of honey stipulating that darker honey has higher content of phenolic compounds than lighter colored honey. This in turn results to enhanced antioxidant activity (Estevinho *et al*., 2008; Wesołowska & Dżugan, 2017). Nevertheless, honey with higher water content has a better antioxidant potential (Frankel *et al*., 1998).

Honey provides antioxidants in a higly palatable form although it may not be the main source of dietary antioxidants. It may be consumed by people hesitant to take plant-derived antioxidants. Thus, it can be a flavorful, supplementary antioxidants source (Jaganathan & Mandal, 2009).

Erratic analytical methods have been used to determine antioxidant activity in honey. These include; FRAP (ferric reducing antioxidant power), DPPH (2, 2-diphenyl-1-picrylhydrazyl), AEAC (ascorbic acid Equivalent Antioxidant Capacity), TEAC (Trolox equivalent antioxidant capacity) (Chua *et al*., 2013). In this study, DPPH and FRAP methods were used in the determination of antioxidant potential of honey. According to Amarowicz *et al*. (2004), DPPH free radicals are believed not to be affected by explicit side reactions including enzyme inhibition and metal ion chelation. Moreover, honey contains bountiful free radical scavengers which can lower the disparity between antioxidant level and free radical production (Kishore *et al*., 2011)

2.7.2 Botanical origin and antioxidant activity of honey

According to Perez *et al*. (2007) and Nagai *et al*. (2006), honey contains notable antioxidant compounds such as organic acids, carotenoid derivatives, proteins, amino acids, Maillard reaction products, catalase, glucose oxidase, ascorbic acid, phenolic acids and flavonoids. The antioxidant property of honey depends on the botanical origin of the honey and varies from distinctive botanical sources (Kücük *et al*., 2007; Baltrusaityte *et al*., 2007). This is displayed in its biological and quality properties, thus it is important to study the characteristics of honey from varying regions of its origin.

2.7.3 Antioxidant activity in decreasing inflammation

Inflammation refers to a concise array of physiological responses to foreign organisms such as dust particles, viruses and human pathogens. It occurs in response to injuries in human body. There are two types of inflammations namely; acute inflammation and chronic inflammation. The classification depends on the diverse cellular mechanisms and inflammatory processes. According to Arulselvan *et al*. (2016), inflammation is a contributing factor in the progression of varying chronic disorders such as cancer, diabetes, eye disorders, cardiovascular diseases, obesity, arthritis, inflammatory bowel disease and autoimmune diseases. Production of free radicals from various environmental and biological sources is as a result of natural antioxidants' imbalance, which stimulates differing inflammatory associated disorders.

A research done by Van den besrg (2008) demonstrates that honey can neutralize free radicals. The antioxidant activity of honey helps to control the free radicals thus initiating healing in burns. Hence, honey dressing can be applied on burns in clinical tests. Honey's antioxidant activity may thus be responsible for its anti-inflammatory action since oxygen

free radicals participate in various inflammation aspects. The antioxidants in honey do not suppress the inflammation directly but it can reduce damage from the reactive oxygen species by scavenging the free radicals (Molan, 2001).

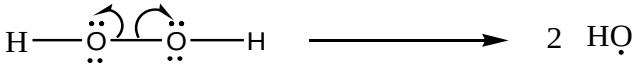
2.7.4 Free radicals, free radical scavengers and antioxidants

Free radicals are byproducts formed by cells during the oxygen metabolism. They are very unstable since they consist of unpaired electrons. They take electrons from other cells or give away unpaired electrons to cells by moving through the bloodstream. They may include reactive oxygen species (ROS) such as alkoxyl radical (RO·), hydroxyl radicals (·OH), hypochlorous acid (HOCl·), peroxyl radical (ROO·) and superoxide radicals ($O_2^{\cdot-}$) as well as reactive nitrogen species (RNS) such as peroxynitrite and nitric oxide (NO·) (Valko *et al*., 2007; Jackson & Loeb, 2001). Both the ROS and RNS result from both endogenous sources (peroxisomes, mitochondria, phagocytic cells, endoplasmic reticulum etc.) and exogenous sources (alcohol, pollution, heavy metals, tobacco smoke, transition metals, pesticides, industrial solvents, certain drugs such as paracetamol, radiation and halothane) (Phaniendra *et al*., 2015).

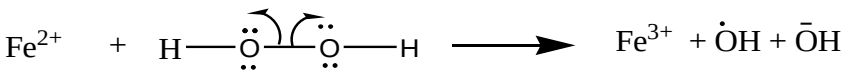
Free radicals can have adverse effects on various essential classes of biological molecules for instance; lipids, proteins and nucleic acids, thus modifying the normal redox status, resulting to amplified oxidative stress. The induced oxidative stress may be involved in numerous disease conditions like neurodegenerative disorders (Alzheimer's disease, Multiple sclerosis and Parkinson's disease), diabetes mellitus, several cancers (prostate, colorectal, lung, bladder, breast cancers), cardiovascular diseases (hypertension and atherosclerosis), cataract development, respiratory diseases (asthma) and rheumatoid arthritis (Phaniendra *et al*., 2015). For instance, although some of the ROS and RNS play

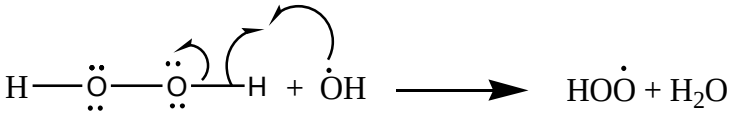
significant roles in cell physiology, they may travel through the cell disrupting other molecules' structures resulting to cellular damage, which is believed to cause aging and other health problems. ROS may also cause oxidation of DNA if present at inappropriate level leading to serious damages including double-stranded DNA breaks and impairments, which are commonly found in human tumors (De Bont & Van Larebeke, 2004). They may also result to great damage to DNA and cell membranes such as oxidation, which leads to decreased membrane fluidity, membrane lipid peroxidation and DNA mutations resulting to neurodegenerative disorders, heart disease, cancer and other diseases (Finkel & Holbrook, 2000).

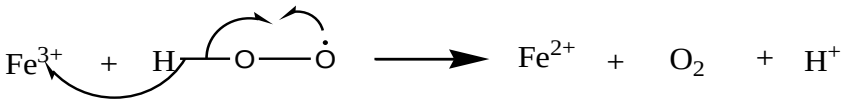
There are other compounds that are non-radicals; they lack unpaired electrons, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) yet can cause extensive oxidative cellular damage. Superoxides spontaneously, rapidly and enzymatically generate H_2O_2 and other free radicals. H_2O_2 is converted to reactive hydroxyl radicals (. OH) by homolytic cleavage of the peroxide bond as in the following illustration:

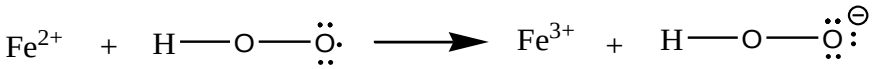


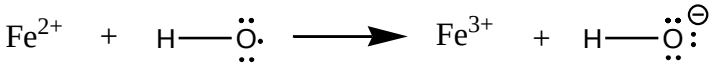
This is done either by interaction with some transition metal ions such as Fe^{2+}, Cu^{2+}, Co^{2+} and Ti^{3+}, the most important iron being Fe^{2+} or by exposure to UV light (Halliwell & Gutteridge, 1990). Fe^{2+} undergoes Fenton reaction as in the following illustrations:











This reaction recycles the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) form that creates more hydroxyl radicals from H_2O_2. Hydroxyl radicals have been reported to oxidize proteins and lipids and DNA scission.

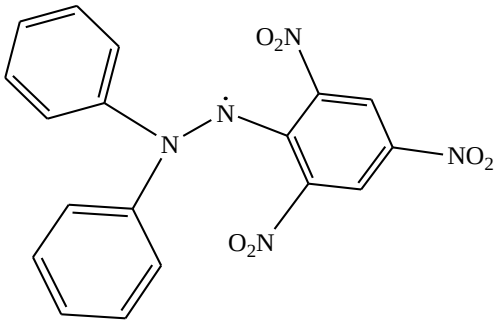
Nevertheless, free radicals are produced in human body and destroyed by immune system through the white blood cells. However, an average immune system is unable to destroy all free radicals hence requires assistance. Thus, foods with free radical scavengers are of great importance in enhancing immune system and this keeps the unstable cell level at desired limits. Free radical scavengers are organic compounds such as flavonoids, vitamins, enzymes or minerals that can damage free radicals by neutralizing them, thus reducing their destruction ability. They inhibit or delay cellular impairment primarily through their free radical scavenging activity (Halliwell, 1995). They hunt down these unstable molecules and damage them before they destroy cells within the body. They also inhibit the oxidation process required for the electrons to be transferred from one cell to another. Free radical scavengers, often called antioxidants, are normally found in dark

colored fruits and vegetables such as blueberries. Mammalian cells have defense mechanisms for detoxification of radicals. Various metabolic steps have been used in the destruction of toxic peroxides such as superoxide dismutase, catalase and glutathione peroxidase. Furthermore, non-enzymatic molecules such as thiols, disulfide-bonding and thiore-doxin have also antioxidant defense systems. Other antioxidant compounds are also found in food such as ascorbic acid, α-tocopherol and β-carotene as well as micronutrient elements such as Selenium and Zinc (Halliwell & Gutteridge, 1998). Lack of enough free radical scavengers within the body results to higher risk of housing free radicals leading to serious diseases in life later. Honey can take up these free radicals leading to better health.

2.7.4.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl)

2, 2-diphenyl-1-picrylhydrazyl (DPPH) (**18**) is a common free radical scavenger for other radicals. DPPH free radical scavenging is the basis of DPPH antioxidant assay; a decolorization assay that measures the capacity of antioxidants to directly scavenge DPPH radicals. It has been used in the determination of free radical scavenging activity in beekeeping products including honey (Meda *et al*., 2005), pollen (Leja *et al*., 2007) and propolis (Kumazawa *et al*., 2004). DPPH has also been used to determine the ability of compounds to act as free radicals' scavengers despite evaluation of antioxidant activity (Kirtikar & Basu, 2006). DPPH also monitors chemical reactions which involve radicals, particularly antioxidant assay. Antioxidant activity involving the use of this stable molecule is based on electron-transfer from antioxidant compounds to free radicals. The DPPH assay method is based on the reduction of DPPH from its purple coloring to yellowish color. Therefore, assessment of the antioxidant reducing power is assessed,

which is afterwards oxidized after electron donation and thus pro-oxidant substances are not detected (Brand-Williams *et al*., 1995). This method allows testing of both hydrophilic and lipophilic compounds unlike other methods which are restricted in the nature of antioxidants being quantified (Kulisic *et al*., 2004; Koleva *et al*., 2002). The DPPH radical produces dark purple color solution in solvents such as ethanol and methanol.



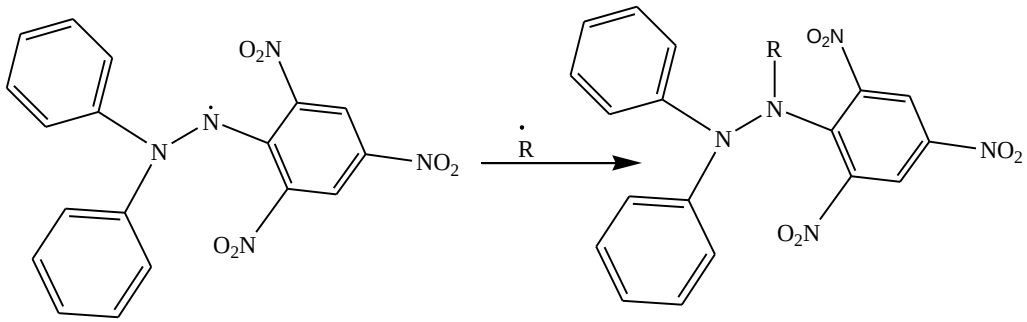
(**18**)

2.7.4.2 Mechanism of action of DPPH

DPPH is a stable free radical at room temperature which can accept hydrogen radical or an electron to become a stable diamagnetic molecule. Addition of DPPH in a chemical reaction which contains antioxidants results to DPPH reduction, an indication of radical nature of the reaction, which in turn reduces the absorbance from that of DPPH (Harbone, 1998). The DPPH methanolic solution shows a strong absorption band at 517nm as a result of its odd electron. DPPH radical reactions have deep violet color in solution. When neutralized, the color becomes pale yellow or colorless depending on the number of electrons captured (Ghosh, 1984).

The more the decolourization the more the reducing power, that is, the color of the solution gets lost with an increase in antioxidant concentration since the electrons are taken up

from the antioxidant by the DPPH radical (Calliste *et al*., 2001 & Calliste *et al*., 2010). This property gives room for visual monitoring of the reaction and thus the initial number of radicals can be noted from variation in the optical absorption at 517nm. This reactivity has been widely used to determine the ability of plant extracts/compounds to act as free radical scavengers (Chang *et al*., 2007). DPPH is a strong inhibitor of radical mediated polymerization as indicated in the scheme (**1**) below. This is because it is an effective radical trap.



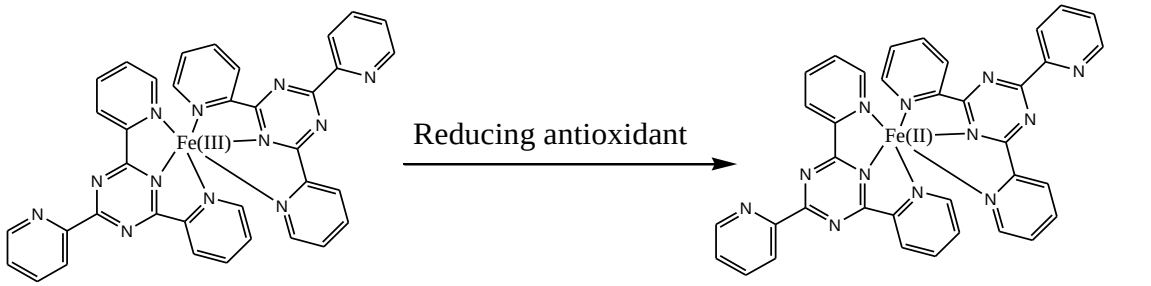
Scheme 1: Reaction of DPPH radical with other radicals (˙R = ˙H, alkyl radical etc.)

According to Sharma & Bhat (2009), methanol is suitable for DPPH assay because it possesses better sensitivity towards DPPH absorbance value. Furthermore, it can also be used in DPPH assay with antioxidant activity of polar and non-polar/less polar compounds in the crude extracts. Sharma & Bhat (2009) reported that methanol solvent also has a better IC_{50} value which may be as a result of the polarity of the reaction medium, pH of the reaction medium and chemical structure of the radical scavenger. All operation of the assay was done in the dim light (Ozcelik *et al*., 2003) to prevent degradation which results from photo-oxidation. This method has been providing rapid and easy way in the evaluation of antioxidants by spectrophotometric method (Huang *et al*., 2005), thus it can be used in the assessment of varying products at a time.

2.7.4.3 Ferric reducing antioxidant power (FRAP)

Ferric Reducing Antioxidant Power (FRAP) assay is a method used to measure antioxidant activity (Benzie & Strain, 1996). It uses antioxidants as reductants in a redox linked colorimetric method in which Fe^{3+} is reduced to Fe^{2+} (Alvarez-Suarez *et al*., 2010). The reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion at low pH causes formation of a colored ferrous-probe complex from a colorless ferric-probe complex. For instance, it is mostly based on reduction of Fe^{3+} -TPTZ (2, 4, 6-Tripyridyl-S-triazine) complex, a colorless complex, to Fe^{2+} -TPTZ, a blue colored complex, which is formed by action of electron donating antioxidants at low pH as in scheme 2 below.

Fe^{3+}-TPTZ + reducing antioxidant ⟶ Fe^{2+}-TPTZ (Intensive blue)



Fe^{3+}-TPTZ (colorless complex) Fe^{2+}-TPTZ (Intensive blue)

Scheme 2: Conversion of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ in FRAP assay

This is normally monitored by measurement of absorption change at a wavelength of 700nm. The measured reducing ability does not essentially reflect antioxidant activity; rather it provides a valuable total antioxidant concentration, without measuring and summing the concentration of all the antioxidants involved.

FRAP is a sensible screen for the capability to retain redox status in tissues or cells. The reducing power seems to have some relations to hydroxylation degree and conjugation

extent in polyphenols (Pulido *et al*., 2000). This type of assay is advantageous in that it is robust, simple, inexpensive, speedy, and does not require utilization of specialized equipment (Pulido *et al*., 2000). However, it has various weaknesses, one of them being its inability to measure thiols since their reduction potentials are normally below that of the Fe (III)/Fe (II) half reaction. Thus, their contribution to the total antioxidant ability can be considered negligible since only a few of these compounds are expected in honey. FRAP method also cannot detect compounds that have radical quenching /hydrogen atom transfer (HAT) action, especially, proteins and thiols and slow reacting polyphenolic compounds (Ou *et al*., 2002).

2.7.5 Health benefits of antioxidants in honey

Free radicals and reactive oxygen species (ROS) have been reported to contribute to aging processes and diseases (Schramm *et al*., 2003). It has been reported that foods containing antioxidants like honey can offer protection against the damaging effects of the free radicals and ROS. Thus, honey possess valuable effects on human health; including prevention of death of cells in various cancer cell lines (Jaganathan *et al*., 2015), enhancement of antioxidant defense system in humans (Schramm *et al*., 2003), protection of cardiovascular by prevention of Reactive Oxygen Species-induced low density lipoprotein oxidation (Schramm *et al*., 2003), among others. Honey antioxidant capacity is normally attributed to its phenolic and flavonoid compounds which possess various preventive effects against varying diseases such as cancer, cardiovascular diseases, neurological degeneration, inflammatory disorders, infectious diseases, aging and wound healing (Khalil *et al*., 2010).

2.7.6 Role of antioxidants in preventing cancer

Cancer is among the most important and dreadful disease in Kenya. Studies on the honey efficacy on different types of cancers shows that honey stimulates angiogenic action and has a debriding potential (Hawley *et al*., 2014; Kustiawan *et al*., 2014). Various studies from Malaysia on honeys reported good activities against different cancers, such as cervical (Fauzi *et al*., 2011), oral, bladder (Swellam *et al*., 2003), bone and breast cancers (Fauzi *et al*., 2011) and liver cancer (Baig & Attique, 2014). An investigation on cancer and honey by Othman, (2012) illustrates natural honey as a cancer "vaccine" because of the reported decrease in prolonged inflammation, a risk factor for cancer pathogenesis. Natural honey has been reported to posess anti-tumor activity through inhibition of proliferation and enhancement of early death of varying cancer cells as a result of its high antioxidant content (Othman, 2012). This is also attributed by the fact that natural honey is very cytotoxic against cancer or tumour cells whereas it is non-cytotoxic to normal cells. These cancer cells include sarcomas, carcinomas, cancer cell lines, bone and breast cancer, and experimental cancers (Kadir *et al*., 2013). The control and treatment of these varying cancer cells with the utilization of honey entails cancer pathways inhibitions including Mtor, $AKTPI_3$ and capsases by the bioactive honey constituents.

2.7.7 Antioxidants and cardiovascular diseases

Excess free radicals' level can cause damage to nucleic acids, membrane lipids, cellular proteins and finally cell death. Evidence shows that highly RNS and ROS of environmental or endogenous origin can have a notable role in the origin and progression of the varying cardiovascular disease (CVD) forms (Cai & Harrison, 2000). The formation of these ROS has been contributed by various mechanisms. Oxidative stress emanates

from imbalance between ROS/RNS generation and the antioxidant defenses activity (Madamanchi & Runge, 2007). Inflammation and oxidative stress have been the significant pathways entangled with endothelial dysfunction resulting to atherosclerosis. The inflammatory processes also play a major role in the CVD development although the inflammation cause is unknown (Huang & Glass, 2010). Generation of these ROS/RNS in vivo triggers actions which are opposed by coordinated antioxidant defense system lines. These include non-enzymatic and enzymatic antioxidants which repair oxidative cellular damage. Most endogenous antioxidants such as uric acid, glutathione, bilirubin, albumin, caeruloplasmin and N-acetylcysteine also exhibit antioxidant functions and often act synergistically with dietary antioxidants and against various types of free radicals.

The etiology and prevention of CVD has been associated with lifestyle factors such as nutrition. Optimal nutrition has been examined as non-pharmacological prevention and therapy of cardiovascular diseases (Ferrari *et al*., 2004). Honey has been used experimentally in the therapy of CVD. It has been applied in lowering levels of cholesterol in hyperlipidemic patients. For instance, persistent administration of honey in those patients showed significant reduction in their levels of lipids (Al-Waili, 2004). Yaghoobi *et al*. (2008) conducted research on honey effects on body weights, fasting blood glucose (FBG), total cholesterol, low-density lipoprotein cholesterol (LDL-C), triacylglycerol, high-density lipoprotein cholesterol (HDL-C) and C-reactive protein (CRP) among various patients. The outcome indicated that oral administration of honey for a month led to a decrease in LDL, cholesterol and triacylglycerols in obese patients. Moreover, 10% consumption of honey for a long period of time elevated HDL-C levels, an indication that consumption of honey has glycemic control health benefits and can also improve lipid

profile that can directly or indirectly result to a decrease in the cardiovascular disease occurrence (Chepulis & Starkey, 2008). Nevertheless, honey prevents low density lipoprotein oxidation *in vitro* and thus can prevent coronary heart disease. Some of the polyphenols in honey have been reported as favorable pharmaceutical drugs in treating cardiovascular diseases and include Caffeic acid phenyl ester (CAPE) (**8**), Quercetin (**11**), Galangin (**12**), Kaempferol (**13**), Acacetin (**14**) and Pinocembrin (**16**).

2.8 Anti-inflammatory activity of honey

According to Molan (2001), honey contains anti-inflammation properties. This is due to its scavenging ability of ROS responsible for inflammation induction. The honey's anti-inflammatory effects can be attributed to its antibacterial activity since bacterial cell wall components are strong inflammatory responses stimulators. Application of honey to wounds also removes slough from wounds thus decreasing inflammation.

Due to the rise in antibiotic resistance, honey has since been used in wound repair as a natural treatment (Lee *et al*., 2011). This may be attributed to its effectiveness in healing. Honey can reduce healing time through a binary effect on inflammatory response by suppressing proliferation and production on inflammatory cells at the site of the wound. This inhibits prolonged inflammatory response and triggers production of proinflammatory cytokine enabling the occurrence of normal healing (Tonks *et al*., 2007).

Wound healing constitutes an orderly progression of events, which require several interactions controlled by biologically active proteases, cytokines and growth factors, thus a tissue remodeling process (Majtan *et al*., 2010). Nuclear factor-kappa beta (NF-KB), a transcription factor, is an essential inflammation marker which boosts proinflammatory

activity. This results in an amplified inflammatory response and activation of gene codes for proinflammatory cytokines interleukin (IL)-6, IL-8 and tumor necrosis factor-α (TNF-α) through a toll-like receptor 4 (TLR4)-dependent pathway (Chung *et al*., 2004). This proinflammatory cytokine trigger production of nitric oxide, an inflammation mediator. The NF-KB activation and nitric oxide production are inhibited by the honey flavonoids (Molan, 2002). Impairment of healing leads to development of chronic wounds, which are characterized by reactive oxygen species and proinflammatory cytokines (Van den Berg *et al*., 2008).

2.9 Antimicrobial activity of honey

Honey is believed to possess antibacterial activity, hence can be used as medicine due to its effectiveness as a remedy (Molan, 2001). Honey's antimicrobial activity is attributed to its physicochemical parameters including high acidity resulting from low pH value, high osmotic pressure as a result of high sugar concentration, phytochemical factors such as flavonoids, low protein content, low water activity and hydrogen peroxide produced in honey resulting from glucose oxidase conversion (Molan & Cooper, 2000; Kwakman *et al*., 2010). High sugar concentration eradicates microorganisms, particularly bacteria, which are sensitive to high osmotic pressure and prevents more osmotolerant microorganisms from developing. Low pH, which results from high organic acid concentration along with high osmotic pressure eliminates most microorganisms' development. Hydrogen peroxide on the other hand protects ripening honey from the development of pathogenic microorganisms.

Other compounds responsible for the antimicrobial activity include bee defensin-1, complex carbohydrates and several phenolic and flavonoid compounds (Alvarez-Suarez

et al., 2013; Zainol *et al*., 2013; Bogdanov *et al*., 2008). Bee defensin-1 may play a significant role in bee larvae health and exhibits activity against gram-positive bacteria. However, hydrogen peroxide is the main contributing factor towards the antibacterial activity in honey (Alnaimat *et al*., 2012). It is produced by glucose oxidase action. The antimicrobial properties of honey were examined in a study *in vitro* and it was realized that methylglyoxal, bee defensin-1 and hydrogen peroxide had roles in definite mechanisms associated with honey bactericidal activity (Khan *et al*., 2007; Kwakman *et al*., 2010). These varied factors may account for failure of bacteria to be resistant to honey unlike the rapid induction of resistance perceived with conventional antibiotics (Blair *et al*., 2009).

A research done on Manuka honey reported that honey has antibacterial activity against pathogenic bacteria such as *Staphylococcus aureus* and *Helicobacter pylori*. This makes honey a functional food for wound and stomach ulcers treatment (French *et al*., 2005). Another investigation was carried out on honey from different sources for their inhibitory activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus cereus* and *Shigella sonnei* (Taormina *et al*., 2001). Disc assay method indicated that zones of inhibition of growth development is dependent on the concentration and type of honey alongside the pathogen tested. The growth inhibition of *L. monocytogenes*, *S. aureus* and *S. sonnei* in 25 % solutions of honey decreased by treatment of the solutions with catalase an indication that hydrogen peroxide is a contributor to antimicrobial activity. *Bacillus cereus* growth was affected to a minor extent in the study. However, darker colored honey had a better inhibitory effect than the light-colored honeys.

The antimicrobial potency among varying honeys is dependent on seasonal, botanical and geographical source besides processing, storage and harvesting conditions (Molan & Cooper, 2000). Honeys' antibacterial nature is dependent on differing factors such as high acidity, which inhibits pathogenic organisms (Pieper, 2009), low water content, phenolic compounds, high osmolarity, which inhibits bacterial growth, hydrogen peroxide and other non-peroxide constituents such as phytochemical components. Hydrogen peroxide is reported to be the main contributing factor towards honey's antimicrobial activity and its varying concentrations in distinctive honeys may result to diverse antimicrobial effects (Molan, 1992).

According to Taormina *et al*. (2001), antimicrobial activity of honey can also vary depending on its physical property alongside varying floral source. In addition, the varying plant sources can also contribute to the differing antibacterial activity (Lusby *et al*., 2005). Honey's antibacterial can range between concentrations of less than 3%-50% (v/v) and higher (French *et al*., 2005; Lusby *et al*., 2005). Furthermore, honey's bactericidal effect also depends on honey's concentration and bacteria nature (Basualdo *et al*., 2007; Adeleke *et al*., 2006). High concentration of honey compounds implies that it's a good antibacterial agent (Badawy *et al*., 2004). Research done by Taormina *et al*. (2001) reports that honey concentration of less than 25% is required for absolute inhibition of *Salmonella typhimurium* growth.

2.10 Bacteria used in the study

Two bacteria were used in this study. These include *S. aureus*, a gram-positive bacterium and *E. coli*, a gram-negative bacterium. These bacteria are a severe cause of a range of hospital- and community-acquired infections. *Staphylococcus aureus* is an etiological

infection agent liable for substantial mortality and morbidity levels. *Escherichia coli* is a common nosocomial pathogen that is belived to cause enterocolotis and urinary tract infections (UTI) (Raho & Abouni, 2015). According to Olivares Pacheco *et al*. (2013), *S. aureus* accounts for 18.8% of clinical infections necessitating hospitalizing and is the first common cause of infections followed by *E. coli* which accounts for 17.3%. In addition, *E. coli* is the most common bacterium and accounts for 38.6% among outpatient patients (Olivares Pacheco *et al*., 2013). In the current years, resistant *S. aureus* and resiatant *E. coli* strains to antibiotics have risen leading to a major concern to global public health. This thesis determined these two bacteria bracing the need for new antibacterial compounds.

2.10.1 *Staphylococcus aureus*

This is a gram-positive bacterium spherical in shape and approximately 1 µm in diameter. It is a versatile, ubiquitous and common deadly bacterial pathogen and grows in both anaerobic and aerobic conditions. Its cell division takes place in more than one plane and thus its cells form grape-like clusters. *Staphylococcus aureus* is found in the environment. Its habitats in humans include skin of warm-blooded animals and nasal membranes (Kuroda *et al*., 2001). It also colonizes gastrointestinal tracts, genitourinary tracts, perineum and pharynx (den Heijer *et al*., 2013). It's the causative agent for comprehensive infections in both humans and animals with a notable impact on public health (Luzzago *et al.*, 2014). In humans, it causes nosocomial infections, which lead to fatal diseases such as necrotizing fasciitis, pulmonary infections such as empyema and pneumonia, blood stream infections, food poisoning, meningitis, gastroenteritis, osteomyelitis, bone and joint infections, prosthetic device infections, bacteremia, infective endocarditis, toxinoses

including toxic shock syndrome, urinary infections and severe sepsis (Monecke *et al*., 2011; Shaw *et al*., 2004; Centers for Disease Control and Prevention (CDC), 2003). In addition, it is also the leading causative agent for skin and soft tissue infections including furuncles, impetigo, cellulitis, folliculitis, scalded skin syndrome, carbuncles and abscesses. Furthermore, it also causes diseases in animals such as mastitis in cow, dermatitis in dogs, botryomycosis in horses and septicemia and arthritis in poultry (Luzzago *et al*., 2014; Zunita *et al*., 2008). These infections are frequent in community-acquired and hospital acquired settings. However, their treatment is a challenge as a result of the emerging multi-drug resistant strains like the MRSA (Methicilin-Resistant *Staphylococcus aureus*) (Chamber, 2005; Boucher & Corey, 2008).

Staphylococcus aureus infections are mostly transmitted to other people through direct contact. Mucous and skin membranes are efficient barriers against these infections. Nevertheless, if breaching of these barriers takes place, for instance skin damage as a result of mucosal damage or trauma due to viral infections, *S. aureus* can obtain access to the bloodstream or the underlying tissues leading to infections.

It is believed that people with invasive medical devices or the immunocompromised are mostly vulnerable to *S. aureus* infections. This may also include hospitalized patients, health care workers, people who utilize needles regularly especially the diabetics and intravenous (IV) drug users (Tong *et al*., 2015).

2.10.2 *Escherichia coli*

This is a ubiquitous gram-negative bacterium particularly found in humans' and animals' intestines. It is a facultative anaerobe, which can ferment simple sugars like glucose to

form acetic, formic and lactic acids. This bacterial species has four strains some of which are believed to cause both intestinal and extra intestinal illnesses (Nataro *et al*., 2011; Quinn *et al*., 2011). These strains include enteropathogenic *E. coli*, enteroinvasive *E. coli*, enterohemorrhagic *E. coli* and enterotoxigenic *E. coli*. Enteropathogenic *E. coli* can cause nausea, severe diarrhoea, abdominal cramps, fever, headache, chills and vomiting. It has high mortality rates in most developing countries. Enteroinvasive *E. coli*, on the other hand, involves bacterial penetration and intestinal mucosa destruction. It causes fever, muscle pain, headache, chills, profuse diarrhoea and abdominal cramps. Enterotoxigenic *E. coli* comprises of enterotoxin producing strains once the organisms proliferate in the intestine. It is particularly responsible for traveller's diarrhoea. It also causes diarrhoea which can lead to dehydration. Enterohemorrhagic *E. coli* can cause bloody diarrhoea, acute abdominal cramps, vomiting and watery diarrhoea. It also causes hemolytic uremic syndrome (HUS) in children, which can lead to kidney failure and later result to death (Makvana & Krilov, 2015)

Escherichia coli can also lead to bacterial infections such as bacteremia, cholecystitis, urinary tract infection and cholangitis and clinical infections like pneumonia and neonatal meningitis. The transmission of most of the enteric infections caused by *E. coli* is through inter-human contacts especially those caused by Enteropathogenic *E. coli*, Enteroagrregative *E. coli*, and Enteroinvasive *E. coli* (Van den Beld & Reubsaet, 2012; Kabiru *et al*., 2015). This can be through fecal-oral route or hand to mouth contact. Improper food handling and poor hand washing may also spread these infections. The ones ascribed to Shiga toxin-producing *E. coli* or Enterotoxigenic *E. coli* are passed from one person to another through consumption of contaminated food or water (Qadri *et al*.,

2005; Caprioli *et al*., 2005). This may include sources of food including undercooked ground beef, ham, unpasteurized apple milk, turkey, sandwich meats, roast beef, cheese and raw vegetables.

2.11 Medicinal property of honey

Honey has been known since ancient times due to its therapeutic and nutritive values and hence it's highly valued as an alternative medicine. The healing properties is owing to the fact that it offers antioxidant and antibacterial activity, sustains moist wound environment which can promote healing and it possesses high viscosity that can assist in providing protective barrier to intercept the microbial infection (Lusby *et al*., 2002). It has been reported to accelerate wound healing (Van der Berg *et al*., 2008). Topical application of honey swiftly clears wound infection and therefore accelerates healing of intense infected surgical wounds. Furthermore, healing of infected wounds which, don't respond to conventional therapy such as antiseptics and antibiotics is also promoted by application of honey (Ahmed *et al*., 2003). This includes wounds infected with Methicillin-resistant *Staphylococcus aureus* (MRSA) (Natarajan *et al*., 2001). Honey can act as a debriding agent when application is done directly onto the wound. According to Efem (1988), honey boosts granulation, tissue thickness, oedema and epithelialization around wounds. In addition, it has gained acceptance as an agent in treating inflammations, cough, ulcers, bed sores, and other skin infections, which result from wounds and burns (Cooper *et al*., 2002a; 2002b; Lusby *et al*., 2005). Honey's antibacterial activity accelerate the new tissue growth to enhance wound healing (Lusby *et al*., 2002). It possesses well known antibacterial effects against various microorganisms such as *Shigella spp*., *Escherichia coli*, *Salmonella spp.* and *Helicobacter pylori* (McGovern *et al*., 1999).

Honey can also be used successfully on infected skin graft donor sites and skin grafts (Misirlioglu & Eroglu, 2003). It has immunological property that is also relevant for wound repair and thus exerts pro-inflammatory and anti-inflammatory effects (Mandal & Mandal, 2011). Honey can modulate production and quenching of free radicals. This may help resolve the inflammation state epitomizing chronic wounds (Henriques *et al*., 2006). For the past years, it has been proved to be a hepatoprotective, cardioprotective and antioxidant agent (El Denshary *et al*., 2012). It has also been confirmed to have protective effects against gastrointestinal diseases (El-Arab *et al*., 2006). It has also anti-cancer activities against cervical, breast (Fauzi *et al*., 2011), osteosarcoma (Ghashm *et al*., 2010) and prostate cancers (Samarghandian *et al*., 2011). Traditionally, honey has been used in ameliorating thyroid disturbances (Adewoye & Omolekulo, 2014) and as a hypolipidemic (Adnan *et al*., 2011) and anti-diabetic agent (Erejuwa *et al*., 2012b).

2.12 Mechanism of antibacterial activity of honey

Honey's beneficial role is ascribed to its antibacterial activity, which results from its acidity (low pH), high viscosity, low water content, high osmolarity and presence of H_2O_2 and non-peroxide constituents such as phytochemical constituents including methylglyoxal (Mavric *et al*., 2008).

The low pH of honey results from conversion of glucose by glucose oxidase into gluconic acid and hydrogen peroxide. Several honey types generate H_2O_2 when diluted as a result of glucose oxidase activation which oxidizes glucose to gluconic acid and H_2O_2. This low pH (between 3.2 and 4.5) may contribute to the honey's bactericidal activity. This is because most bacteria thrive in a pH that ranges between 6.5 and 7.5. The acidity is as a result of organic acids especially gluconic acid (Küçük *et al*., 2007; Cavia *et al*., 2007).

The low pH can also be inhibitory to various bacterial microorganisms (Koochak *et al*., 2010). The minimal pH for pathogenic bacteria growth includes *Salmonella spp.* (4.0), *Escherichia coli* (4.3), *Streptococcus pyogenes* (4.5) and *Pseudomonas aeruginosa* (4.4) and hence in concentrated honey acidity can be an efficient antibacterial factor. The pH can be an efficient antibacterial factor in concentrated honey. However, the pH alone cannot hamper growth of most bacterial species when diluted by body fluids or in food (Molan, 1992).

The high sugar concentration of honey triggers hypertonic conditions which may result to microbial cell walls' lysis and thus eradicates microorganisms such as bacteria which are sensitive to high osmotic pressure and hinder development of more osmotolerant microorganisms. The antibacterial activity can be decreased on dilution of honey by human body fluids at the infection site (Albaridi, 2019).

According to Wahdan (1998), non-peroxide components such as phenolic acids and flavonoids also contribute to the antimicrobial activity in honey. The antimicrobial agents' concentration in honey is dependent on the relative catalase levels originating from flower pollen and glucose oxidase synthesized by the bee (Weston, 2000).

CHAPTER THREE

MATERIALS AND METHODS

This chapter outlines the methods that were employed in the study to collect the relevant information and data.

3.1 Collection of samples

Samples of *Plebenia hylderbrandii* and *Meliponula bocandei* stingless bee honey were supplied by the Centre for African Medicinal & Nutritional Flora and Fauna (CAMNFF) at the Directorate of Science Park Innovation & Incubation Center (SPIIC) of Masinde Muliro University of Science and Technology (MMUST). Random sampling was used to obtain the honey samples.

3.2 Preparation and packing of filters

A glass column of length 14.0 cm and internal diameter 1.0 cm was cut and one end sealed with glass wool. Porapak Q porous polymer beads (750mg) were packed into the column and the other end sealed with glass wool to keep the porapak Q in place inside the glass column and also to prevent it from spilling.

3.3 Cleaning of the filters

Round bottomed flask, soxhlet and fractionating column were thoroughly cleaned and baked in an oven at 130-150 ℃ for 24 hours to avoid contamination of the filter samples to be cleaned. Dichloromethane was put in the quick fit flask, and then anti-bumping stones were added into it to prevent the dichloromethane from spilling over to the filtrate once the boiling point (B.P) was attained. The filters were placed in the Soxhlet and round

bottomed flask was placed on a heating mantle at 42^0C. The porapak Q adsorbent filetrs were then cleaned for 24 hours after which the water bath was turned off and left to cool.

3.4 Activation of the filters

The filters were removed from the soxhlet using forceps to avoid contamination and wrapped in aluminium foil. Handling of the filters was done by use of forceps and cotton gloves to prevent contamination with sweat/body volatiles. The oven was set at 130-150ºC and left to bake for 30 minutes. The temperature was then adjusted to 95 ºC and allowed to cool down from 150 ºC to 95 ºC for 1 hour. The filters were placed in the oven for 24 hours for activation at 95 ºC after which they were removed and kept in an empty, clean glass jar and sealed with Teflon tape for safe storage at room temperature until when they were needed for use.

3.5 Extraction of honey organic compounds

Honey sample (100ml) was passed through the previously cleaned and activated porapak Q column. The column was then inverted and washed with 20 ml of distilled water, followed by 10ml dichloromethane and finally 10ml methanol in order to remove sugars which may prevent elution of the organic compounds. Adsorbed organic compounds were then eluted with 100 ml of acetone. The eluate was Rota-evaporated (20 degrees Celsius) until dryness and then taken up in 200 µl of acetone. Two different replicates were prepared from each sample for the purposes of ensuring consistency in collection of organic compounds.

3.6 Physico chemical parameters

3.6.1 Color intensity

The honey samples' mean absorbance was determined using the method of Beretta *et al*. (2005). Concisely, dilution of the honey samples was done using warm deionized water (45–50 °C) to 50% (w/v). Filtration of the resulting solution was done using a 0.45 μm filter in order to remove large particles. Absorbance was measured at 450 and 720 nm by use of a spectrophotometer and the difference of the absorbance expressed as mAU.

3.6.2 Moisture content

The moisture content of the honey samples was determined by use of Karl Fischer method. About 50 ml of methanol in a beaker was used in Karl Fischer titrations. Sensors were fitted till deeper in the methanol inside the beaker then closed tightly. The start button was pressed after which the Karl Fischer reagent got pumped from the reserve bottle to the methanol till all the water molecules in the Karl Fischer reagent and methanol mixture got exhausted. Using the syringe, 10 microliters of purified water in the already dehydrated methanol was added then the calibration button pressed in the Karl Fischer machine. The Karl Fischer reagent was pumped in the mixture of methanol and the 10 microliters of water in the beaker. When the 10 microliters of water got exhausted, the machine stopped then gave the Karl Fischer factor (x3). After determining the Karl Fischer factor the water content in the beaker was removed by pressing the start button in the Karl Fischer machine. The samples already weighed in the 10ml volumetric flask were added to the methanol Karl Fischer solution in the beaker then the tare weight taken from the balance. The volume exerted in the Karl Fischer-methanol solution was determined by subtracting the tare weight from the previous weight of 10ml volumetric flask and the honey sample.

The sample already in the machine was dehydrated by the Karl Fischer reagent and when the water content got exhausted the sensors stopped the machine from pumping more of the Karl Fischer reagent into the sample solution. Then the moisture content was read from the output screen in milliliters (titre). The % water content was determined by the following formular: Titre value * Kf factor *100/ (Weight sample in mg). The readings were obtained in triplicate.

3.6.3 pH

A 10% (w/v) solution of honey was prepared in deionized water and the pH measured by use of a pH meter (Bogdanov, 2009).

3.6.4 Electrical conductivity (EC)

A 20% (w/v) solution of honey was suspended in deionized water and the EC determined using a conductivity meter (Bogdanov *et al*., 1999).

3.7 Analysis of chemical constituents

3.7.1 Analysis/identification of organic compounds

The extracted organic compounds were analysed using liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS). The samples were analyzed by Agilent1260 Infinity HPLC chromatographic system (Agilent, Germany) hyphenated to Agilent 6530 Quadrupole Time of Flight (Agilent, Singapore)). 10µl of the sample was injected into Agilent Zorbax SB-C18 column (4.6 mm × 150 mm, 1.8 µm) and the temperature was kept at 40ºC. Separation was done using 1% formic acid in water and 1% formic acid in acetonitrile and the flow rate was kept at 0.400ml/min. Full scan MS (MS1) was used as the acquisition mode and the mass ranged

between 100-1000 m/z with scan rate =1 (spectra/min). The set mass spectrometer parameters were as follows; Gas temperature = 300ºC; Gas flow = 10 l/min; Nebulizer = 35 psig; Sheath Gas temperature = 350 ºC and Sheath Gas flow = 11 l/min. The MS spectra were recorded in positive ion mode. Metlin database was then used in the structure elucidation by use of simple search at a tolerance of 10 ppm, collision energy of 25-45 eV and Tandem MS (MS/MS) tolerance of 5-1200 Da.

3.7.2 Structure elucidation techniques

LC-ESI-QTOF-MS was used for identification, quantification and mass analysis of the non-volatile organic compounds in the samples. Metlin database was used to elucidate the structures by use of simple search at a tolerance of 10 ppm coupled with rationalization of the various mass fragment ions through fragmentation patterns.

3.8 Antioxidant activity experimental design

Factorial (5x2) experimental approach in a Completely Randomized Design (CRD) was used to test for the the antioxidant activity of the two stingless bee honey samples. The factors included; 5 concentrations (20mg/ml, 25mg/ml, 30 mg/ml, 35mg/ml and 40mg/ml) and 2 honey species (*Plebenia hylderbrandii* & *Meliponula bocandei*). A total of 3 treatment calibrations were replicated three times to have a total of 9 treatments. Two methods were used to determine the antioxidant activity, namely; DPPH method and FRAP method.

3.8.1 Determination of free radical scavenging activity with DPPH (2, 2-diphenyl-1-picrylhydrazyl)

It involves investigative electro donating capability of antioxidant health-promoting dietary supplements. 2, 2-Diphenyl-1-picrylhydrazyl radical is composed of stable free radical molecules and have been used to monitor chemical reactions involving radicals.

3.8.1.1 Ascorbic acid standard curve preparation

Ascorbic acid was prepared in six different concentrations (0, 2, 4, 6, 8 and 10 mg/l) using methanol as the solvent. Pure methanol was termed as 0 mg/l concentration of the Ascorbic acid. In this experiment, free radical scavenging activity of Ascorbic acid was tested. An equal volume of DPPH solution in methanol (concentration 20mg/l) was added into each of the prepared Ascorbic acid concentrations (2ml of the Ascorbic acid solution was added to 2ml of DPPH solution). The absorbance of the mixture was measured at 517 nm after 15 min of incubation at 25 ºC using UV-VIS spectrophotometer. This was done in triplicates for each of the test samples. Methanol (2 ml) was added to DPPH (20 mg/l; 2ml) and used as negative control, while methanol was used as blank.

3.8.1.2 Preparation of samples for the DPPH free radical scavenging activity

Methanolic honey solution (2 ml) at five different concentrations, ranging from 20 to 40 mg/ml (20, 25, 30, 35, and 40) was added to 2ml of the DPPH solution for each of the five concentrations.

The absorbance of each of the prepared methanolic honey and DPPH solutions was measured at 517 nm after 15 min of incubation at 25 ºC. Methanol (2ml) added to DPPH solution (2ml) was used as negative control while pure methanol acted as blank.

A graph of % standard scavenging activity against honey concentration in ppm was plotted and drawn for each of the test honey samples. The effective concentration providing 50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against honey concentration.

3.8.2 Ferric reducing antioxidant power (FRAP) assay

This assay involves use of antioxidants as reductants in the redox linked colorimetric method. Normally, Potassium ferric chloride complex is reduced into ferrous chloride complex. This is monitored by change in absorption and thus give the total antioxidant concentration.

Ferric reducing antioxidant power assay was done according to the method as described by Oyaizu (1986) with some modifications. Varying concentrations of water honey solutions (2.5ml) were mixed with 2.5 ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide (2.5 ml). Incubation of the reaction mixture was done for 20 minutes at 50^0C after which 10% trichloroacetic acid (w/v) (2.5ml) was added. Centrifugation of the mixture was done for 8 minutes at 1000rpm. Top layer (5ml) was mixed with deionized water (5ml) and 0.1% of ferric chloride (1ml). Hydrated ferrous sulphate ($FeSO_4.7H_2O$); concentrations 200, 400, 800, 1200 and1600 µM; was prepared and used as the standard. The absorbance of the obtained solution was measured by UV-VIS spectrophotometer at 700 nm. The absorbance of the samples were compared to the $FeSO_4$ standard curve and the FRAP values were expressed as Ferrous Equivalent (FE), the concentration of chemical which gave the same absorbance as 1 µmolar ferrous ion. Higher absorbance indicates higher reducing power. The concentration of the extract

providing 50% of absorbance (EC_{50}) was calculated by interpolation from the graph of absorbance against extract concentration.

3.9 Bioassay

3.9.1 Test microorganisms

The antibacterial activity of each honey sample was determined by use of two bacterial strains, namely; one Gram-positive bacteria (*Staphylococcus aureus*; ATCC 25923) and one Gram-negative bacteria (*Escherichia coli*; ATCC 25922). The bacterial strains were supplied by University of Nairobi, Medical School, Microbiology Department.

3.9.2 Media Preparation

The Nutrient agar media was prepared to the manufacturer's preparation instructions followed by autoclaving at 121 ºC for 15 minutes at 15 psi.

3.9.3 Inoculum preparation

Each bacterial strain was sub-cultured overnight at 37 ºC in Soya bean casein Digest medium (Tryptone soya Broth; HIMEDIA). The bacterial growth was harvested using saline sterile water, its absorbance adjusted at 580 nm and diluted to attain viable cell count of 10^4 CFU/spot by use of spectrophotometer.

3.9.4 Agar dilution bioassay and its experimental design

A (12x2x2) factorial experiment was arranged in Completely Randomized Design (CRD). The factors included; 12 concentrations; 1.18%, 1.76%, 2.35%, 2.94%, 3.53%, 4.71%, 5.88%, 7.06%, 8.24%, 9.41%, 10.59% and 11.76% (v/v) for *Plebenia hylderbrandii* honey and 4.71%, 5.88%, 7.06%, 8.24%, 9.41%, 10.59%, 11.76%, 12.94%, 14.12%, 15.29%, 16.47% and 17.65% (v/v) for *Meliponula bocandei* honey; 2 bacteria (*E. coli* & *S. aureus*)

and 2 honey samples (*Plebenia hylderbrandii* & *Meliponula bocandei*). Agar dilution bioassay was performed to determine the antibacterial activity of the two honey species.

3.9.5 Antibacterial activity of honey samples

The antibacterial activity was done in steps according to the method described by NCCLS, (1997). Preparation for honey samples for assay was carried out according to the method described by Mandal *et al*. (2010) with some modifications. Honey was stored in the dark at room temperature until used for antimicrobial assays. Under aseptic conditions, different honey concentrations in dimethyl sulfoxide (DMSO) 1.18%, 1.76%, 2.35%, 2.94%, 3.53%, 4.71%, 5.88%, 7.06%, 8.24%, 9.41%, 10.59% and 11.76% (v/v) were prepared for *Plebenia hylderbrandii* honey while 4.71%, 5.88%, 7.06%, 8.24%, 9.41%, 10.59%, 11.76%, 12.94%, 14.12%, 15.29%, 16.47% and 17.65% (v/v) were prepared for *Meliponula bocandei* honey. Molten nutrient agar was distributed (15ml each) in sterile culture tubes and autoclaved at 121ºC for 15 minutes. Honey at different concentrations were put in sterile culture plates and the sterile molten agar (55ºC) was dispensed into the respective culture plates and swirled thoroughly for uniform mixing. The mixture was left for 5 minutes to set, followed by inoculation of the microorganisms on the culture plates by use of a sterile transfer loop as shown in appendix A.II.1. After inoculation with 10^4 CFU/spot, the culture plates were incubated for 24 hours at 37ºC. A nutrient agar plate with DMSO (without honey) was similarly inoculated as negative control to control the appropriate growth of the organisms. Nevertheless, a nutrient agar plate with ampicillin was used as positive control. The experiments were done in triplicate. Results have been reported in terms of bacterial growth on the agar plates.

3.9.6 Interpretation of results

The partial inhibitory concentration (PIC) was reported as the lowest concentration of honey that retarded growth as compared to the negative control plate while the minimum inhibitory concentration (MIC) was described as the lowest concentration of honey required for inhibiting the visible growth of the isolates. The minimum bactericidal concentration (MBC) was determined by further sub-culturing the last plate, that exhibited visible growth, and all the plates in which there was no growth on agar medium. The MBC was thus the lowest concentration of honey required to produce sterile culture.

3.10 Data analysis

Statistical analyses were conducted using Predictive Analytics SoftWare (PASW), Version 20. All tests were carried out in quadruplicate and results obtained expressed as mean values ± standard deviation (SD). The confidence limits used in this study were based on 95% (i.e $p < 0.05$)

CHAPTER FOUR

RESULTS

4.1 Physico-chemical parameters

In this study, color intensity, moisture content, pH and electrical conductivity of *Plebenia hylderbrandii* honey and *Meliponula bocandei* honey were examined. The mean results are presented in table 4.1.

Table 4.1: Selective Physico-chemical parameters values for the investigated honey samples

Type of Honey	**Colour Intensity (mAU)**	**Moisture content (MC) (%)**	**pH**	**Electrical conductivity (EC)** (mS/cm)
P. hylderbrandii	1.11 ± 0.002^a	27.79 ± 0.023^a	3.54 ± 0.012^a	1.00 ± 0.001^a
M. bocandei	1.20 ± 0.004^a	24.54 ± 0.042^a	3.99 ± 0.006^a	0.42 ± 0.002^a

The number of replicates (N) for all the physicochemical properties in each honey type was 3.

From the results in this table, similar letter 'a' accompanying each mean in each column indicate that there is no significant difference between the physicochemical properties in the two samples under study.

4.1.1 Color intensity

The color intensity of the honey samples ranged between 1.11 ± 0.002^a to 1.2 ± 0.004^a mAU. *Meliponula bocandei* honey had a higher color intensity of 1.2 ± 0.004^a mAU while *Plebenia hylderbrandii* honey had 1.11 ± 0.002^a mAU. There was no significant difference between the two samples.

4.1.2 Moisture content

The Moisture content of the honey samples ranged from 24.54 to 27.79%. *Plebenia hylderbrandii* honey had higher moisture content (27.79± 0.023[a] %) than *Meliponula bocandei* honey (24.54 ± 0.042[a] %). The means were not significantly different as indicated by similar letters.

4.1.3 pH

All the analysed honey samples were acidic in character. The pH results of the honey have been presented in the table 4.1 on the previous page. *Meliponula bocandei* honey had higher pH (3.99 ± 0.006[a]) than *Plebenia hylderbrandii* honey (3.54 ± 0.012[a]). There was no significant difference in the pH between the two honey samples.

4.1.4 Electrical conductivity

The honey samples' electrical conductivity (EC) ranged between 0.42 to 1.00 mS/cm. *Meliponula bocandei* honey had EC of 0.42 ± 0.002[a] mS/cm while *Plebenia hylderbrandii* honey had EC of 1.00 ± 0.001[a] mS/cm. There was no significant difference in the EC between the two honey samples.

4.2 Chemical composition

In this study, LC-MS combined with ESI-QTOF experiments were performed to assign the chemical constituents of honey samples. The LC chromatograms for the *Plebenia hylderbrandii* and *Meliponula bocandei* stingless bee honey samples are as presented in figures 4.1 and 4.2, respectively. Molecular ion signals in positive mode, retention time (tR), fragments ion masses of the identified compounds are as shown in tables 4.2 to 4.5. The molecular masses of the identified compounds comprised of H^+ ($M + H^+$) and ACN

(M+ACN+H$^+$) adducts for the two honey samples. The H$^+$ and ACN$^+$ adducts were as a result of solvents (formic acid and acetonitrile) respectively, used in the analysis.

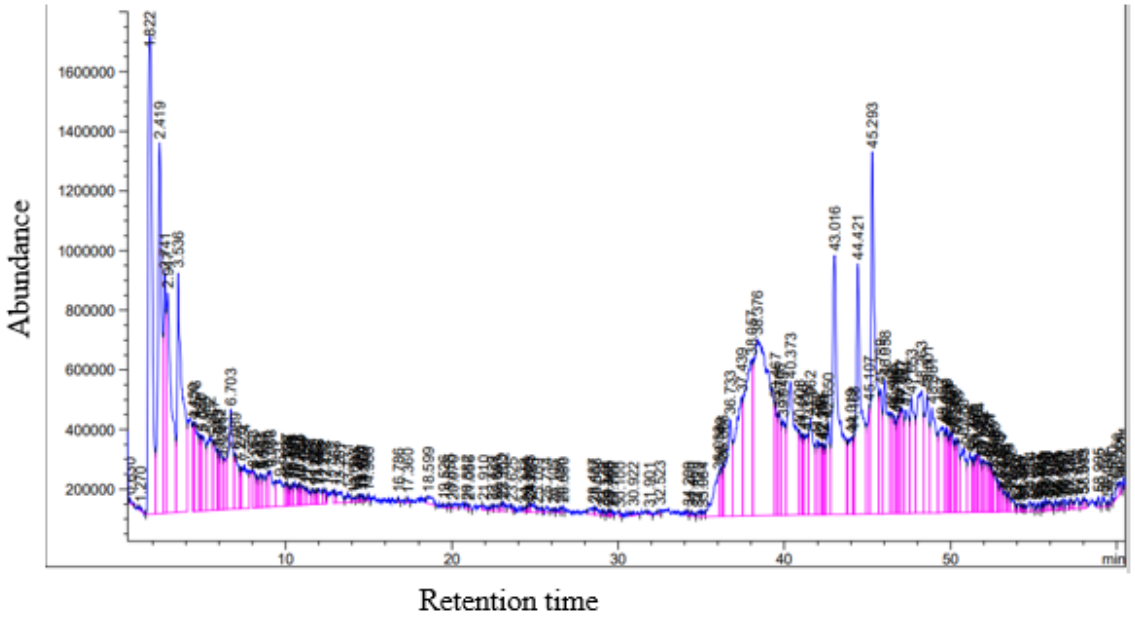


Fig. 4.1: LC chromatogram for *Plebenia hylderbrandii* honey

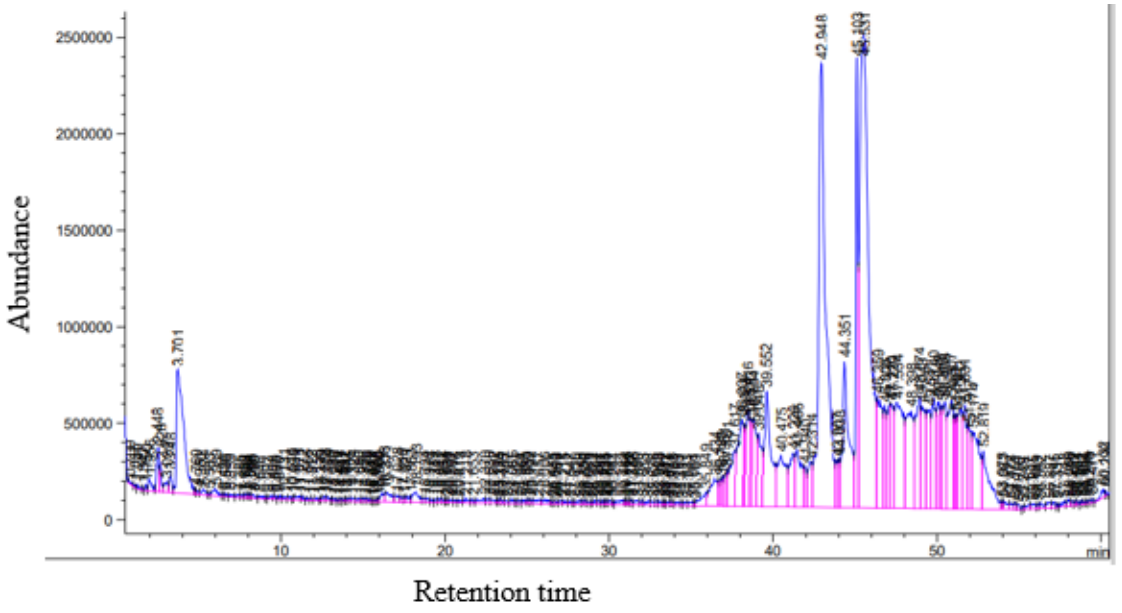


Fig. 4.2: LC chromatogram for *Meliponula bocandei* honey

Table 4.2: $[M+H]^+$ Ions and fragment ions of identified compounds for *Plebenia hylderbrandii* honey by LC-ESI-QTOF-MS

#	tR (min)	+ve molecular ion $[M+H]^+$	Fragment ions (m/z)	Name of identified compound
1	25.39	283.1	85, 71, 60, 56	Arabinofuranobiose
2	32.23	307.1	83, 71, 60, 56	Starch acetate
3	13.70	336.2	72, 71, 60	6-O-(N-Heptylcarbamoyl)methylglucoside
4	7.51	339.1	197, 131, 85, 71, 59	N-(4-Hydroxy-8-phenoxyisoquinoline-3-carbonyl)glycine
5	24.63	347.1	307, 279, 71, 60, 59	Methyl 6-O-galloyl-beta-D-glucopyranoside
6	4.87	394.2	328, 302, 262, 228, 202,131, 85, 71, 59	N-(3-carboxypropanoyl)-L-phenylalanyl-L-lysine
7	8.45	431.1	262, 113, 97, 85, 71, 59	4-(7-Hydroxy-4-oxo-4H-1-benzopyran-3-yl)phenylbeta-D-glucopyranosiduronic acid
8	4.25	443.3	366, 330, 298, 247, 195, 144, 83, 72, 56	Ala-Lys-Lys-Pro
9	43.97	549.2	517, 401, 341, 74, 71, 60	Met-His-Met-Met
10	46.83	551.2	532, 511, 397, 357	Genipin 1-beta-gentibioside
11	36.01	565.3	520, 476, 364,251, 85, 60, 56	Lys-Met-Met-Arg
12	37.20	613.3	608, 565, 564, 521, 503, 477, 432, 319, 251, 72, 56	Asp-His-Leu-Thr-Gln
13	50.47	557.3	71, 60	Tyr-Tyr-Val-Leu

Table 4.3: $[M+H]^+$ Ions and fragment ions of identified compounds for *Meliponula bocandei* honey by LC-ESI-QTOF-MS

#	tR (min)	+ve molecular ion $[M+H]^+$	Fragment ions (m/z)	Name of identified compound
14	52.43	295.1	60	Tuliposide B
15	52.93	323.1	297, 60	Gly-Gly-Ser-Cys
16	7.60	353.1	85, 71	Psoralidin oxide
17	48.32	354.1	261	(S)- (-)-Acenocoumarol
18	1.55	355.1	89, 71	Cryptochlorogenic acid
19	39.57	423.2	229, 207, 71, 57	4-Heptylumbelliferyl-beta-glucoside
20	18.17	520.3	165, 85, 71, 59	His-Lys-Val-His
21	41.60	609.2	306, 207, 163, 71, 59	4-(4-Hydroxyphenyl)-2-butanone O-[2-galloyl-6-cinnamoyl glucoside
22	39.15	785.3	741, 696, 423, 395, 393, 377, 362, 225, 163, 71, 59	antibiotic MA 144U5
23	36.30	608.3	565, 520, 476, 242, 170, 85, 72, 59	Tryptophylalanyltryptophylphenylalaninamide

Table 4.4: $[M+ACN+H]^+$ Ions and fragment ions of identified compounds for *Plebenia hylderbrandii* honey by LC-ESI-QTOF-MS

#	tR (min)	+ve molecular ion $[M+ACN+H]^+$	Fragment ions (m/z)	Name of identified compound
24	4.87	394.2	394, 302, 262	pongagallone A
25	50.47	557.3	71, 60	Leu-Gln-Gln-Gln
26	39.72	564.2	520, 448, 445, 414, 269, 180, 71, 61	Glu-Asp-Met-Glu
27	43.03	638.2	341, 319, 60	2-Butenedioic acid(2Z)-bis [2-(4-benzyol-3-hydroxyphenoxy)ethyl ester
28	5.09	688.3	345, 288, 247, 157, 113, 85, 71, 60, 56	tetra-tert-butyl 4,4'-carbonyldi(benzene-1,2-dicarboperoxoate
29	38.20	740.3	652, 635, 608, 592, 564, 547, 503, 476, 447, 360, 316, 251, 163, 71, 59	pentacarboxyporphyrin

Table 4.5: $[M+ACN+H]^+$ Ions and fragment ions of identified compounds for *Meliponula bocandei honey* by LC-ESI-QTOF-MS

#	tR (min)	+ve molecular ion $[M+ACN+H]^+$	Fragment ions (m/z)	Name of identified compound
30	25.05	189.1	72, 60	N,N-Di(methylcarbamoyl)hydroxylamine
31	42.23	349.2	261, 205, 187, 71, 60	ethyl 4-(6-acetamido-3-aminopyridin-2-yl)piperazine-1-carboxylate
32	50.04	397.1	353, 71, 60	Cys-Met-Cys
33	18.17	520.3	177, 165, 149, 131, 85, 71, 60, 59	D-Alanyl-O-{10-[4-ethylbenzoyl)oxy]decanoyl}-L-serine
34	37.45	653.3	610, 608, 566, 564, 520, 503, 477, 432, 326, 291, 263, 153, 85, 71, 60	4-[5-Benzyloxy-2-(4-benzyloxy-phenyl)-3-methyl-indol-1-ylmethyl]-phenoxy}-acetic acid ethyl ester
35	37.91	696.3	653, 613, 608, 566, 564, 525, 476, 337, 307, 223, 171, 71, 59	Tyr-Phe-Tyr-Tyr

4.3 Antioxidant activity by DPPH method

4.3.1 Absorbance and % DPPH scavenging activity of Standard

The absorbances of the DPPH Standard obtained from antioxidant assay were as presented in table 4.6.

Table 4.6: Absorbance values of the DPPH standard

Conc. of ascorbic acid (ppm)	Mean Abs. ± S.D
0	0.266 ± 0.02572^{a}
2	0.22671 ± 0.06087^{a}
4	0.13914 ± 0.04846^{b}
6	0.05186 ± 0.04445^{c}
8	0.00886 ± 0.00273^{c}
10	0.00586 ± 0.00488^{c}

S.D stands for standard deviation
Means with same letters are insignificantly different

Ascorbic acid is an antioxidant hence a hydrogen donor and a radical scavenger. The DPPH free radical was reduced to DPPHH in the presence of an antioxidant molecule (Ascorbic acid). This led to change of color from purple to yellow from the lowest concentration to the highest concentration. The resulting discoloration is stoichiometric with respect to the number of electrons captured. The more the discoloration, the more the reducing ability. The antioxidant effect is proportional to the disappearance of DPPH radicals in the test samples.

A graph of absorbance against concentration of the DPPH standard was as illustrated in fig. 4.3 below.

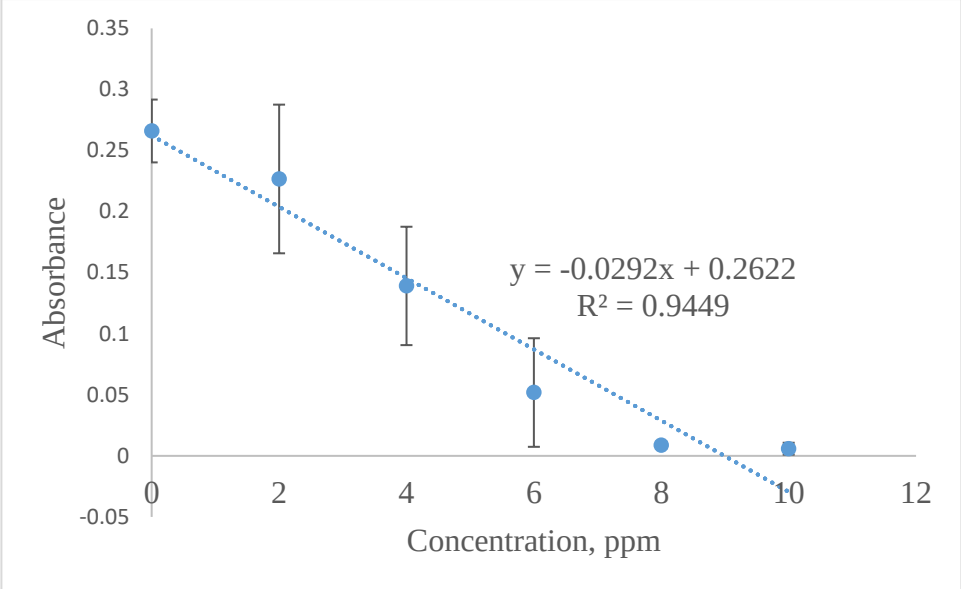


Fig. 4.3: A graph of absorbance against concentration of DPPH standard

The absorbance decreased with increase in the concentration of the standard sample. Lower absorbance of the highest concentration of the Ascorbic acid indicated higher free radical activity.

DPPH Standard % scavenging activity was calculated using the following formula:

(Ac-As/Ac)* 100.

Whereby;

Ac- Absorbance of control

As- Absorbance of standard.

Absorbance (Abs) of the negative control was found to be 0.266 ± 0.025684.

The results for the scavenging activity of DPPH standard are summarized in table 4.7.

Table 4.7: Percent (%) scavenging activity of DPPH standard

Concentration of Ascorbic acid (ppm)	**% Scavenging activity**
0	0
2	14.77
4	47.69
6	80.50
8	96.67
10	97.80

The % Scavenging activity increased with increase in concentration of Ascorbic acid. The standard curve was plotted using the DPPH standard % scavenging activity versus concentration of the Ascorbic acid. The standard curve is shown on fig. 4.4 below.

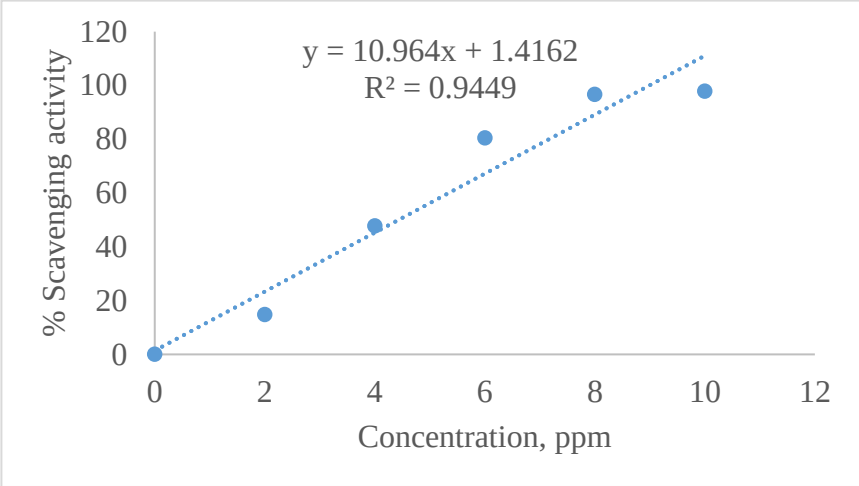


Fig. 4.4: A graph of % scavenging activity of DPPH standard against concentration of Ascorbic acid

IC_{50}, which is the concentration of the standard required to inhibit 50% of the DPPH free radical was calculated from the ascorbic acid calibration curve using the generalized linear

equation y=mx+ c; whereby y is the % activity, 50; m is the slope; c is the y-intercept and x is the concentration required.

From the graph;

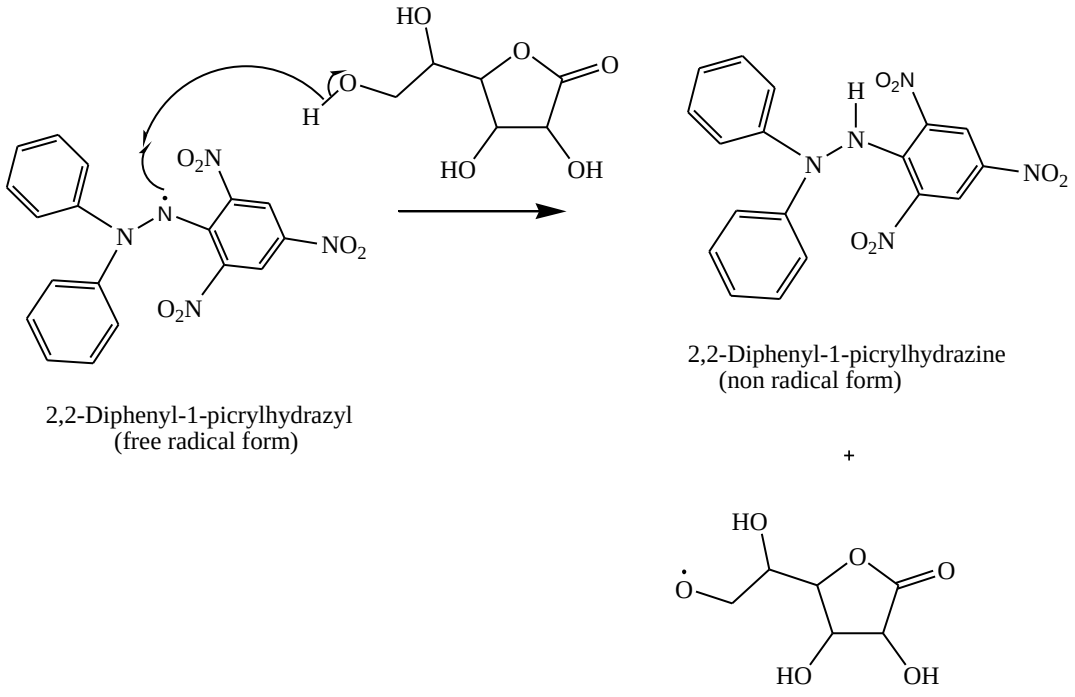
50=11.419x-8.1767

X = (50+8.1767)/11.419

= 5.09 ppm

Thus; IC_{50} of the Ascorbic acid was 5.09 ppm.

The reaction mechanism for Ascorbic acid with DPPH is illustrated in scheme 3.



Scheme 3: Reaction mechanism of ascorbic acid with DPPH

4.3.2 Absorbance and DPPH % Scavenging activity of honey samples

The samples' absorbance were obtained and the corresponding graphs of absorbance against concentration drawn accordingly as presented in tables 4.8, 4.9 and 4.10 as well as figures 4.5, 4.6 and 4.7 in the subsequent pages.

4.3.2.1 Absorbance of *Plebenia hylderbrandii* honey

This honey had high pollen load and so formed two layers. The two layers got separated by decantation and their absorbances studied separately.

4.3.2.1.1 Absorbance of *Plebenia hylderbrandii* honey (Bottom layer)

Table 4.8 presents the mean results of the absorbance of the bottom layer of *P. hylderbrandii* honey diluted in five different concentrations. From the absorbance results presented on table 4.8, the absorbance curve is as shown on fig. 4.5. From the graph, the values of % scavenging activity for the various dilutions of the sample were calculated as tabulated on table 4.11.

Table 4.8: Absorbance of *Plebenia hylderbrandii* honey (bottom layer)

Conc. of sample (mg/ml)	Mean Abs. ± S.D
20	0.08014 ± 0.00227^{a}
25	0.06057 ± 0.01713^{ab}
30	0.05086 ± 0.02393^{ab}
35	0.04671 ± 0.02472^{b}
40	0.04257 ± 0.02114^{b}

N = 7; Means with same letters are insignificantly different

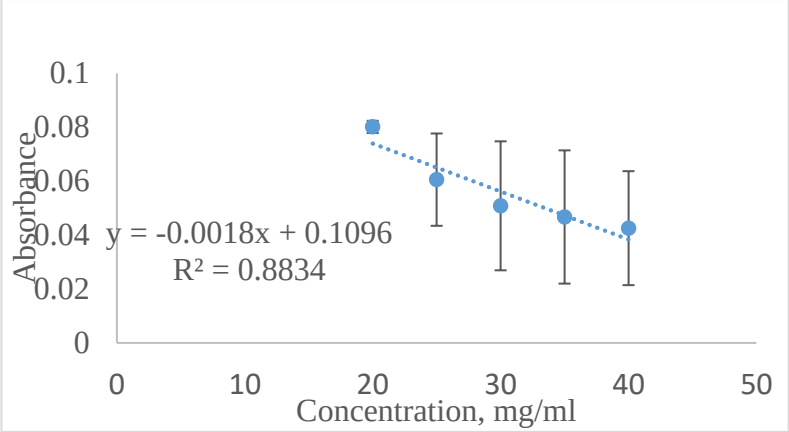


Fig. 4.5: A graph of absorbance against concentration of *Plebenia hylderbrandii* honey (bottom layer)

4.3.2.1.2 Absorbance of *Plebenia hylderbrandii* honey (Upper layer)

The absorbance of five dilutions of the bottom layer of *P. hylderbrandii* was determined and the results obtained (table 4.9) used to plot the absorbance curve for the honey sample (fig. 4.6). From the graph, the values of % scavenging activity for the various dilutions of the sample were calculated as tabulated on table 4.11

Table 4.9: Absorbance of *Plebenia hylderbrandii* (upper layer)

Conc. of sample (mg/ml)	Mean Abs. ± S.D
20	0.01529 ± 0.00138^{a}
25	0.01371 ± 0.00138^{ab}
30	0.01243 ± 0.00127^{b}
35	0.00943 ± 0.00162^{c}
40	0.00733 ± 0.001862^{c}

N = 7; Means with same letters are insignificantly different

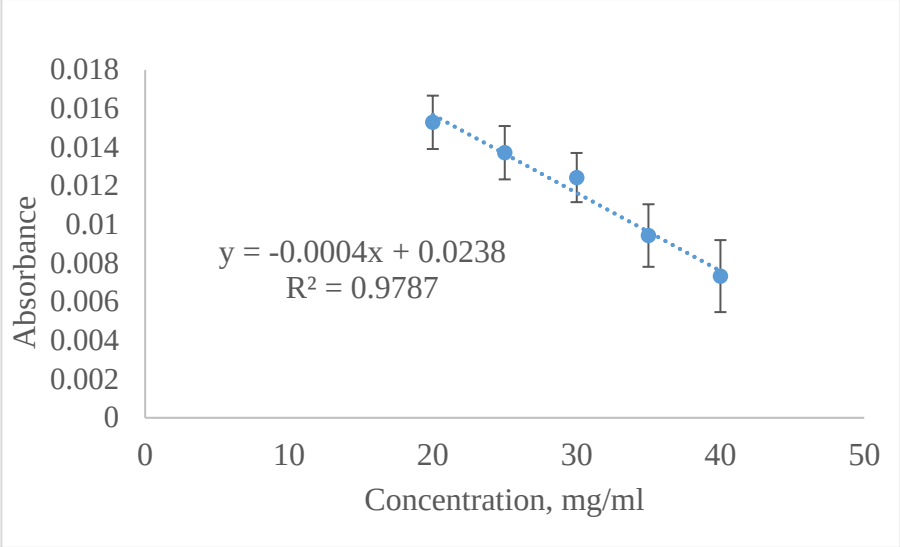


Fig. 4.6: A graph of absorbance against concentration of *Plebenia hylderbrandii* honey (top layer)

4.3.2.2 Absorbance of *Meliponula bocandei* honey

The absorbance of five dilutions of *M. bocandei* was determined and the results obtained (table 4.10) used to plot the absorbance curve for the honey sample (fig. 4.7). From the graph, the values of % scavenging activity for the various dilutions of the sample were calculated as tabulated on table 4.11

Table 4.10: Absorbance of *Meliponula bocandei* honey

Conc. (mg/ml)	Mean Abs. ± S.D
20	0.03929 ± 0.00867[a]
25	0.03629 ± 0.00634[a]
30	0.03014 ± 0.00647[ab]
35	0.02486 ± 0.00438[bc]
40	0.019 ± 0.00346[c]

N=7; Means with same letters are insignificantly different

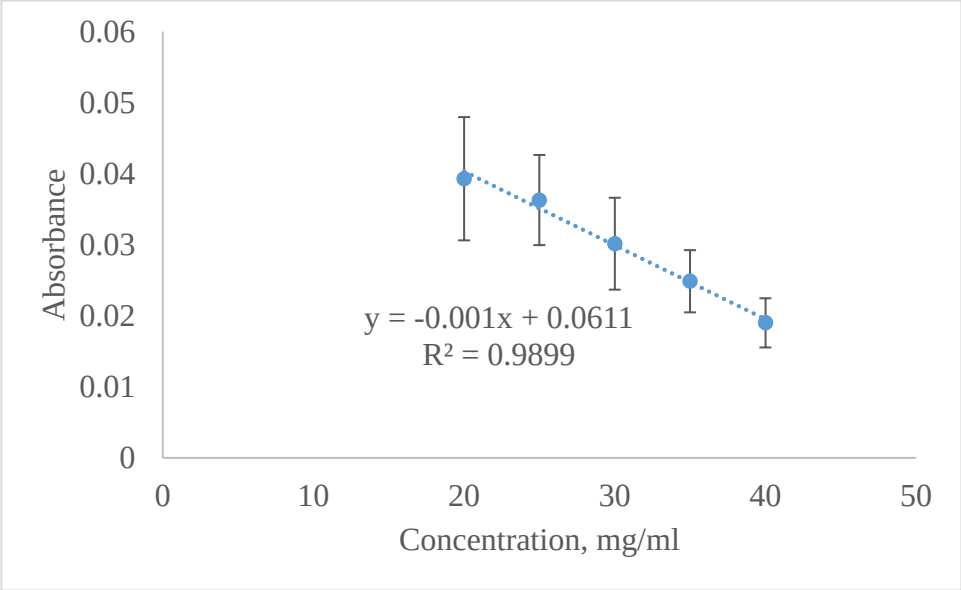


Fig. 4.7: A graph of absorbance against concentration of *Meliponula bocandei* honey

From the results on tables 4.8, 4.9 and 4.10, the DPPH scavenging activities (%) of the samples were calculated using the following formula;

DPPH Scavenging activity (%) = {(A control-Asample)/A control} *100

Whereby; A control- Absorbance of control

A sample- Absorbance of sample

The samples' % Scavenging activities were obtained and tabulated as shown on table 4.11.

Table 4.11: % scavenging activity of the investigated honey samples

% Scavenging activity			
Honey type / Concentration of honey samples (mg/ml)	*P. hylderbrandii* honey (Lower layer) (%)	*P. hylderbrandii* honey (Upper layer) (%)	*Meliponula bocandei* (%)
20	69.87	94.25	85.23
25	77.23	94.85	86.36
30	80.88	95.33	88.67
35	82.44	96.45	90.65
40	84.00	97.24	92.86

The % Scavenging activity increased with increase in concentration of the honey samples. The graphs for % Scavenging activity of the honey samples were plotted using the samples' % scavenging activity versus their concentration as presented on figures 4.8, 4.9 and 4.10 for *P. hylderbrandii* honey (lower layer), *P. hylderbrandii* honey (upper layer) and *M. bocandei* honey, respectively.

Plebenia hylderbrandii honey (Lower layer)

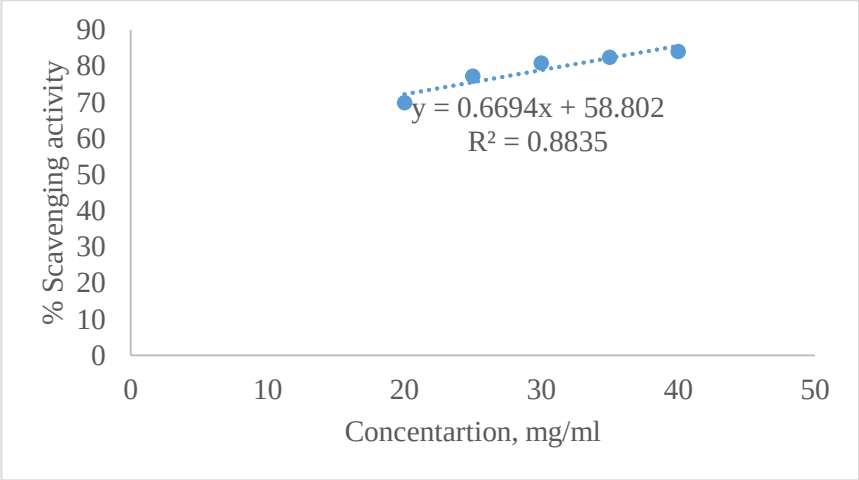


Fig. 4.8: A graph of % scavenging activity against concentration of *Plebenia hylderbrandii* honey (bottom layer).

Plebenia hylderbrandii honey (Upper layer)

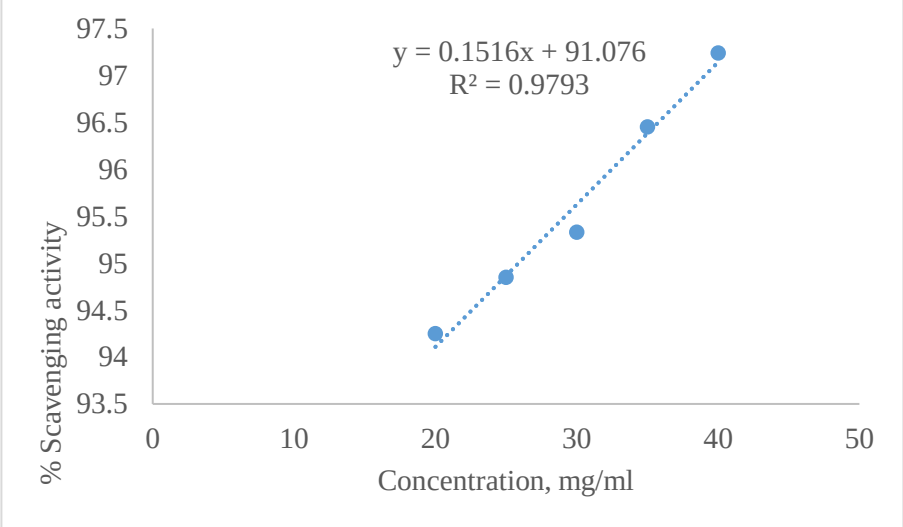


Fig. 4.9: A graph of % scavenging activity against concentration of *Plebenia hylderbrandii* honey (top layer)

Meliponula bocandei

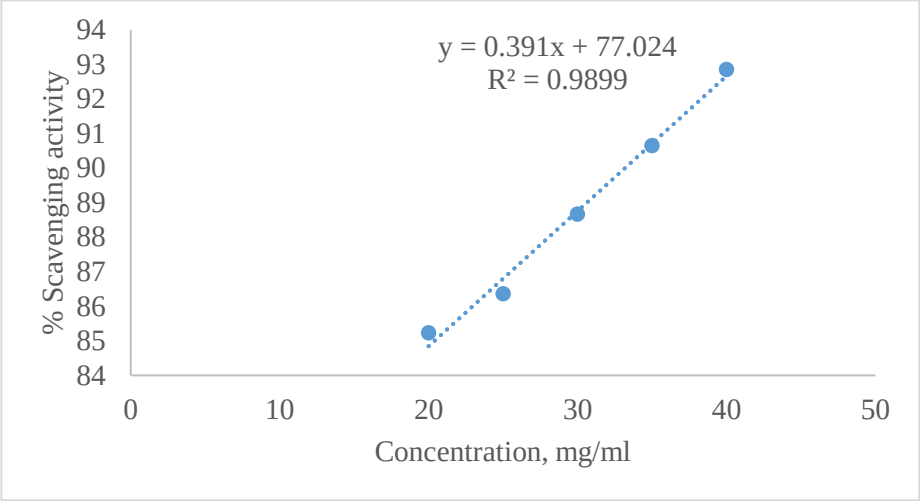


Fig. 4.10: A graph of % scavenging activity against concentration of *Meliponula bocandei* honey

The IC_{50}, which is the concentration of the sample required to inhibit 50% of the DPPH free radical was calculated from the samples' calibration curves (fig. 4.8, 4.9, 4.10) and the values obtained are presented on table 4.12 and fig. 4.11 below.

Table 4.12: IC_{50} values of the honey samples

Honey sample	IC50 value
Plebenia hylderbrandii honey (Lower layer)	14.31 mg/ml
Plebenia hylderbrandii honey (Upper layer)	10.61 mg/ml
Meliboca bocandei honey	11.73 mg/ml

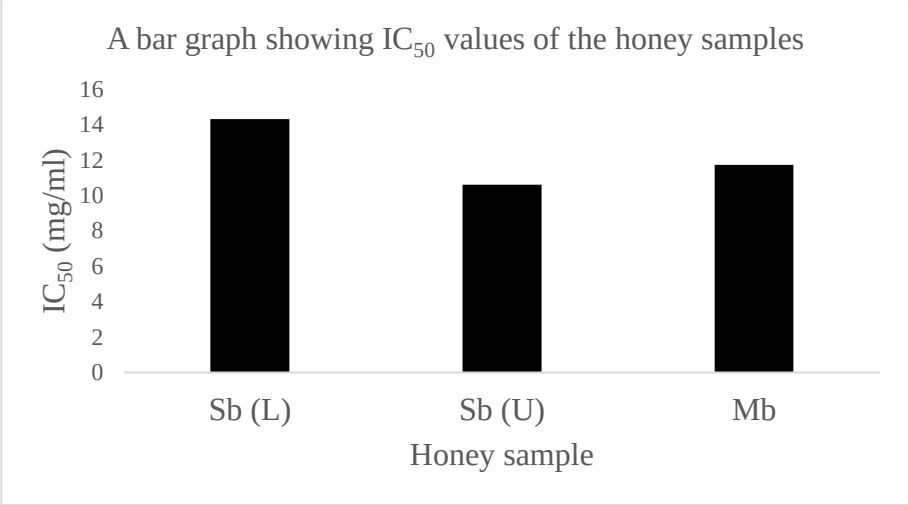


Fig. 4.11: A comparison graph of IC_{50} values among the honey samples

4.4 Antioxidant activity by Ferric Reducing Antioxidant Power Assay

4.4.1 Absorbance and concentration of $FeSO_4.7H_2O$ standard

The absorbances of the $FeSO_4.7H_2O$ standard obtained were as presented in table 4.13. A graph of absorbance against concentration of the $FeSO_4.7H_2O$ standard was as illustrated in fig. 4.12.

Table 4.13: Absorbance values of FRAP standard

Concentration (µmol)	Absorbance
200	2.5675
400	3.1115
800	4.324
1200	5.172
1600	6.6385

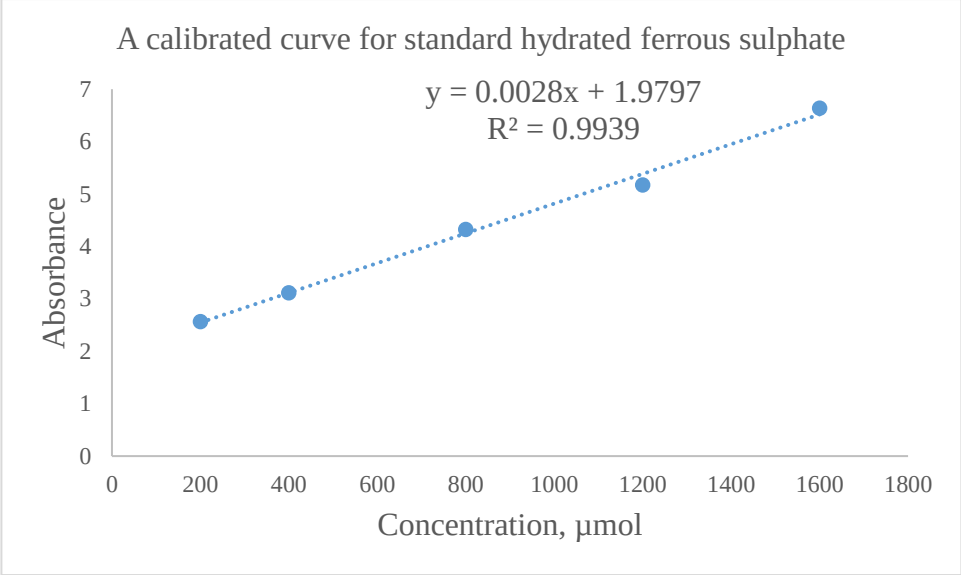


Fig. 4.12: Calibration graph for $FeSO_4.7H_2O$

4.4.2: Absorbance and concentration of *Plebenia hylderbrandii* and *Meliponula bocandei* honeys

Table 4.14 presents the mean absorbances and concentrations of the honey samples. The concentrations were calculated from the equation from the graph (fig. 4.12); y = 0.0028x + 1.9797 where the values of the mean absorbance (y) were replaced and hence the values of the concentration (x) obtained.

Table 4.14: Absorbance values of the investigated samples by FRAP method

Honey sample	Mean Absorbance	Mean Concentration (µmol)
P. hylderbrandii	4.5325 ± 0.004723[a]	911.7143 ± 2.385495
M. bocandei	3.621 ± 0.03924[a]	586.1786 ± 19.81921

N=4; Means with same letters are insignificantly different

4.5 Antimicrobial activity

The results of the *in vitro* susceptibility of the test microorganisms to the honey samples were varying as shown in tables 4.15-4.20. *Plebenia hylderbrandii* honey had a bactericidal activity at a concentration of 4.71 % (v/v) and 2.35 % (v/v) for *E. coli* and *S. aureus* respectively (appendices A.I.9 & A.I.10). *Meliponula bocandei* honey, on the other hand, had a bactericidal activity at a concentration of 16.47 % (v/v) for *S. aureus* (appendix A.I.15) but had no bactericidal activity for *E. coli* since none of the concentrations in this study showed any inhibition. From the results, it is evident that both honeys had a higher bactericidal activity for *S. aureus* than for *E. coli* (Table 4.17-4.20; appendix A.I.17).

Partial inhibition for *Plebenia hylderbrandii* honey was observed from 1.18 % - 2.94% (v/v) for *E. coli* (appendix A.I.5) and 1.18% (v/v) for *S. aureus* (appendix A.I.6) indicating their Partial Inhibitory Concentration (PIC) (table 4.15); while complete inhibition was stronger for *S. aureus* than *E. coli* at Minimum Inhibitory Concentrations (MIC) of 1.76% (v/v) and 3.53% (v/v), respectively (table 4.15; appendices A.I.8 & A.I.7). On the other hand, for *Meliponula bocandei* honey, partial inhibition was observed at a concentration of 10.59- 17.65 and 9.41 - 15.29 % (v/v) for *E. coli* and *S. aureus* respectively (appendices A.I.12 & A.I.14) while complete inhibition was observed at a Minimum Inhibitory Concentration (MIC) of 16.47 % (v/v) for *S. aureus* (table 4.16; appendix A.I.15). As for *E. coli*, no complete inhibition was detected since none of the concentrations from the study showed inhibition (table 4.16). The Minimum Bactericidal Concentration (MBC) value for *Plebenia hylderbrandii* honey was 4.71% (v/v) and 2.35% (v/v) for *E. coli* and *S. aureus* respectively (table 4.17) while for *Meliponula bocandei* honey the MBC value was 16.47% (v/v) for *S. aureus* but it wasn't detected for *E. coli* since none of the tested concentrations showed inhibition (table 4.18). Table 4.19 illustrates the comparison of PIC, MIC and MBC between the two bacteria and honey samples. Appendices A.I.11, A.I.13, A.I.16 and A.I.18 also compares the growth of the *E. coli* and *S. aureus* on the two honey samples at concentration 4.71% (v/v).

The positive control had a wide PIC range between 0.118 - 0.5882% (w/v) against *E. coli* while that for *S. aureus* was only at 0.05882% (w/v) (table 4.20). Its positive control had

a higher MIC value of 1.176% (w/v) against *E. coli* than for *S. aureus*, which had a lower value of 0.088% (w/v). Similarly, its MBC value was 1.176% for *E. coli* compared to 0.118% (w/v) for *S. aureus*. This indicates that the positive control had a higher bactericidal activity for *S. aureus* than for *E. coli*. This is evident from the antibacterial plates as shown in appendices (fig. A.I.1 & A.I.2). On the other hand, the negative control had no antibacterial effect on the growth of *E. coli* and *S. aureus* since heavy growth was observed in all the culture plates (table 4.21; appendices A.I.3 & A.I.4)

Table 4.15: PIC & MIC (% v/v) of *Plebenia hylderbrandii* honey against bacterial pathogens

Bacterial strain	Honey concentrations (% v/v) of *Plebenia hylderbrandii* honey against bacterial pathogens												MIC (%v/v)	PIC %(v/v)
	11.76	10.59	9.41	8.24	7.06	5.88	4.71	3.53	2.94	2.35	1.76	1.18		
E. coli (25922)	_	_	_	_	_	_	_	_	+	+	+	+	3.53	1.18-2.94
S. aureus (25923)	_	_	_	_	_	_	_	_	_	_	_	+	1.76	1.76

\+ means minimum growth; _ means no growth; _ indicates MIC values

Table 4.16: PIC & MIC (% v/v) of *Meliponula bocandei* honey against bacterial pathogens

Bacterial strain	Honey concentrations (% v/v) of *Meliponula bocandei* honey against bacterial pathogens												MIC (%v/v)	PIC %(v/v)
	17.65	16.47	15.29	14.12	12.94	11.76	10.59	9.41	8.24	7.06	5.88	4.71		
E. coli (25922)	+	+	+	+	+	+	+	++	++	++	++	++	N/D	10.59-17.65
S. aureus (25923)	_	_	+	+	+	+	+	+	++	++	++	++	16.47	9.41-15.29

N/D means not detected; + means minimum growth; ++ means moderate growth; _ means no growth; _ indicates MIC value

Table 4.17: MBC (% v/v) of *Plebenia hylderbrandii* honey against bacterial pathogens

Bacterial strain	Honey concentrations (% v/v) of *Plebenia hylderbrandii* honey against bacterial pathogens							MBC (%v/v)
	5.88	4.71	3.53	2.94	2.35	1.76	1.18	
E. coli (25922)	_	_	+	+	N/D	N/D	N/D	4.71
S. aureus (25923)	_	_	_	_	_	+	+	2.35

N/D means not detected; + means minimum growth; _ means no growth; _ indicates MBC values

Table 4.18: MBC (% v/v) of *Meliponula bocandei* honey against bacterial pathogens

Bacterial strain	Honey concentrations (% v/v) of *Meliponula bocandei* honey against bacterial pathogens			MBC (% v/v)
	17.65	16.47	15.29	
E. coli (25922)	N/D	N/D	N/D	N/D
S. aureus (25923)	_	_	+	16.47

N/D means not detected; + means minimum growth; _ means no growth; _ indicates MBC values

Table 4.19: The in vitro antibacterial activity: PIC, MIC and MBC % (v/v) of honey produced by stingless bees in nutrient agar by agar dilution method against *E. coli* and *S. aureus*

Honey type	Bacterial strain	Antibacterial activity of honey % (v/v)		
		PIC	MIC	MBC
P. hylderbrandii	*E. coli* (25922)	1.18 – 2.94	3.53	4.71
	S. aureus (25923)	1.18	1.76	2.35
M. bocandei	*E. coli* (25922)	10.59 – 17.65	N/D	N/D
	S. aureus (25923)	9.41 – 15.29	16.47	16.47

N/D means not detected

Table 4.20: The in vitro antibacterial activity (PIC, MIC & MBC) of positive control

Positive Control	Bacterial strain	Antibacterial activity of honey % (w/v)		
		PIC	MIC	MBC
Ampicillin	*E. coli* (25922)	0.118-1.176	1.176	1.176
	S. aureus (25923)	0.05882	0.088	0.118

Table 4.21: The in vitro antibacterial activity of negative control

Negative control	Bacterial Strain	Growth
	E. coli (25922)	+++
	S. aureus (25923)	+++

+++ means heavy growth

CHAPTER FIVE

DISCUSSION

5.1 Physico-chemical Parameters

Honey's color intensity is represented by the ABS_{450}. It shows pigments' presence such as flavonoids and carotenoids, which are well-known to contribute to antioxidant activity (Moniruzzaman *et al*., 2013) for *Plebenia hylderbrandii* and *Meliponula bocandei* honey varieties. In this study, the honeys' ABS_{450} values ranged between 1.11 ± 0.002^{a} to 1.2 ± 0.004^{a} mAU. In comparison, the ABS_{450} values were conveyed to be between 724 and 1188 mAU in Algerian honey samples (Khalil *et al*., 2012); 25 and 3413 mAU in Italian honey samples (Beretta *et al*., 2005); 70 and 495 mAU in Slovenian honey samples (Bertoncelj *et al*., 2007); 524 and 1678 mAU in Indian honey samples (Saxena *et al*., 2010) and between 254 and 2034 mAU in Bangladesh honey samples (Islam *et al*., 2012). The high color intensity in the investigated honey samples indicates their purity and antioxidant properties (Moniruzzaman *et al*., 2014). The variation of the color intensity for the samples may have resulted from the difference in concentration of the pigments as well as flavonoids, which have absorption maxima at 450 nm (Mendiola *et al*., 2008). The honey color may also have been influenced by biochemical properties during ripening (Beretta *et al*., 2005). The colorimetric characteristics may also vary depending on the area of origin of the honeys. Color intensity correlates with antioxidant activity in that high color intensity implies high antioxidant activity (Saxena *et al*., 2010).

Moisture content is a valuable parameter significant for honey shelf life and is critical in prevention of microbial spoilage. It also helps in determining quality of honey and its

ability to resist fermentation and crystallization during storage (Saxena *et al*., 2010). It is dependent on season of the year, climatic conditions and maturity degree of the honey sample (White, 1975). In addition, moisture affects some physical properties like glucose crystallization and viscosity (Bogdanov *et al*., 2004). Karl Fischer (KF) titration method, a commonly used method, was used to determine the moisture content. Although it is time consuming and expensive, it is the most accurate method for moisture content determination (Bogdanov *et al*., 2004). However, the values obtained may have been dependent on the experimental conditions of titration such as temperature and solvent utilized. The moisture content of the honey samples were 24.54 and 27.79%. *Meliponula bocandei* honey had moisture content (24.54 ± 0.042^{a} %) within the acceptable range of moisture content of 15.7-26.7% (White, 1975) while *Plebenia hylderbrandii* honey had slightly higher Moisture content (27.79 ± 0.023^{a} %) than the standard value. These values were similar to those of research conducted by Neupane *et al*. (2015) whose moisture content values ranged between 20.12 to 29.1% for stingless bee honey. The results also corresponded to the ones from a study done by Jimenez *et al*. (2016), whose MC content values ranged between 20.61 to 28.04% for stingless bee honey. The higher moisture content observed in *Plebenia hylderbrandii* honey might be due to the decrease in sugar content of the sample as the harvest season was wet with high humidity. Nevertheless, open storage and processing may lead to absorption of moisture resulting to an increase in moisture content. Increased moisture content is also an indicator of adulteration and can cause honey fermentation resulting to low shelf life and nasty flavor (Bogdanov *et al*., 2002). This fermentation results from the osmotolerant yeasts' activity and the resultant carbon dioxide and ethyl alcohol formation (Saxena *et al*., 2010). On the other hand, low moisture content in honey confers a protective influence against microbial attack,

particularly at times of long-term storage (Saxena *et al*., 2010). In addition, it also guarantees better quality allowing them for longer duration storage. However, results of this study were lower compared to the values 28.46%, 30.28%, 31.35% and 31.54% obtained from a research carried out from Oyo, Osun, Ekiti, Lagos and Ogun (Adebiyi *et al*., 2004). Nevertheless, they were more than those obtained in Azerbaijan (16.0 to 18.6%), India (14.6 to 17.2%) (Asadi-Dizaji, 2014) and 12.1 to 13.2% in Benin (Azonwade, 2018). Moisture content in honey varies depending on varying environmental conditions such as honey sample's floral origin, climate, nectar's water content, storage conditions and processing conditions (Rossant, 2011). Subsequently, moisture content is an intricate function of a huge number of variables for instance; handling practices and extraction and hygroscopic nature, which is dependent on the time of the year, climatic conditions, degree of maturation, geographical origin, and its primary moisture of nectar (Rossant, 2011).

pH is a measure of acidity or basicity of an aqueous solution. The pH values in honey are very significant during storage and extraction since their acidity can have effect on its stability and texture. Generally, honey is acidic in nature regardless of its variable geographical origin (Saxena *et al*., 2010). This is due to the presence of organic acids that contribute to the honey flavor and stability against microbial spoilage. The main acid found in honey is gluconic acid which is believed to be in equilibrium with its esters or its lactones and inorganic ions like chlorides and phosphates. Other acids present in honey include tartaric acid, citric acid, butyric acid, oxalic acid, formic acid, maleic acid, succinic acid, lactic acid and aromatic acids (Přidal & Vorlova, 2002). *Plebenia hylderbrandii* honey had a pH of 3.54 ± 0.011^{a} while *Meliponula bocandei* honey had a

pH of 3.99 ± 0.006[a]. The pH values of the two types of honeys investigated were within the acidic range of pH 3.4 to 6.1 for pure honey (Codex Alimentations, 2001), thus the results from the honey investigated were consistent with the international standards. These results were more or less similar to the Algerian honey (3.58-4.72) (Zerrouk *et al*., 2011). Honey samples from Spanish, Turkish and Brazilian had pH values ranging between 3.63 to 5.01, 3.67 to 4.57 and 3.10 to 4.05 respectively (Kayacier & Karaman, 2008). The variation in acidic values may be due to disparity of various minerals and acids in honey. It may also result from the bee flavor, bee salivary secretion, and the fermentative and enzymatic processes during the raw materials' processing bearing in mind that acids in honey originate from flowers and digestive bees' secretions. The significance of acidic range in foods can't be overemphasized. It inhibits the honey samples from persistent contamination by several micro-organism species and hence helps in ensuring a longer shelf life. Low pH in the acidic range might be an indication of good shelf life (Lawal *et al*., 2009).

Electrical conductivity is among the most important parameters for determining honeys' physical characteristics (Serrano *et al*., 2004). It is also a significant parameter for validation of unifloral honeys (Mateo & Bosch-Reig, 1998). This parameter can also be used to classify honey into honeydew or nectar (blossom) (Belay *et al*., 2013). In addition, it can be an indicator for routine determination of honey's botanical origin. The honey samples' electrical conductivity ranged between 0.42 to 1.00 mS/cm. *Meliponula bocandei* honey had EC of 0.42 ± 0.002[a] mS/cm, which was below the maximum value of 0.8 mS/cm as indicated in EU and Codex directive while *Plebenia hylderbrandii* honey had EC of 1.00 ± 0.001[a] mS/cm, which was above the maximum value of 0.8 mS/cm. EAS

has no specific required level for electrical conductivity of honeys. According to Pita-Calvo & Vázquez (2017), honeydew honey has an EC above 0.8mS/cm while blossom honey has EC less than 0.8mS/cm. Thus, *Plebenia hylderbrandii* honey can be classified as honeydew honey and *Meliponula bocandei* honey as blossom honey. Comparatively, the results from the investigated samples were in agreement with research carried out by Saxena *et al*. (2010) whose honey EC ranged between 0.33 to 0.94 mS/cm. A study of EC of some honey samples obtained from Apata, Ayeye, Iwo Road, Bere and Omi-Adio found out EC values were 0.27 mS/cm, 0.31 mS/m, 0.34 mS/m and 0.53 mS/m (Adenekan *et al*., 2010). Electrical conductivity of honey differs depending on its geographical and botanical origin. However, it is closely related to mineral content, proteins, organic acids, polyols and some complex sugars (Terrab *et al*., 2003; Warui *et al*., 2019; Saxena *et al*., 2010). Higher acid and ash contents indicate higher conductivity (Adenekan *et al*., 2010). Some plants have stronger nectars than others and even at low honey contamination, stronger nectar can alter its physicochemical and sensory properties (Persano *et al*., 1995). A range of between 0.21 to 1.61 mS/cm in a previous study was obtained in Algerian honeys (Ouchemoukh *et al*., 2007).

5.2 Chemical composition of the honey samples

Honey is believed to contain chemical compounds which play crucial roles in human body. For instance, it is believed to possess antioxidant, anti-inflammatory and antibacterial activity, among others. This study focused on non-volatile compounds which possess antioxidant activity. The antioxidant capacity of honey modulates production of free radicals, thus enabling protection of cell components from their detrimental action (Alzahrani *et al*., 2012).

Identification of compounds is a cumbersome process as a result of absence of web-based database. However, many published literature are used for guiding in the assigning and identifying the chemical compounds. In this study, liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry (LC-ESI-QTOF-MS) experiments were done to assign the chemical constituents in honey. Molecular ions signal in positive mode, retention time and fragment ion masses of the identified compounds were reported. The identified compounds were verified by Metlin database at a tolerance of 10ppm, collision energy of 25-45 eV and MS/MS tolerance of 5-1200Da.

Twenty-three (23) compounds were positively identified from *Plebenia hyderbrandii* (fig. 11-23) and *Meliponula bocandei* (fig. 24-33) honeys through ions generated from $[M+H]^+$ spectra with proton as an adduct.

Compound 1 had a molecular ion at m/z 283.1 and fragment ions at m/z 85, 71, 60 and 56 corresponding to arabinofuranobiose with retention time 25.39 min (fig. 5.1).

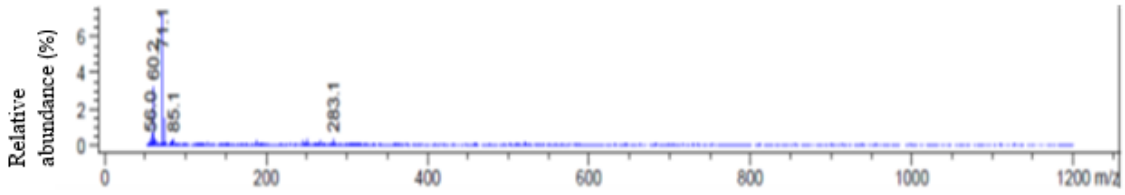
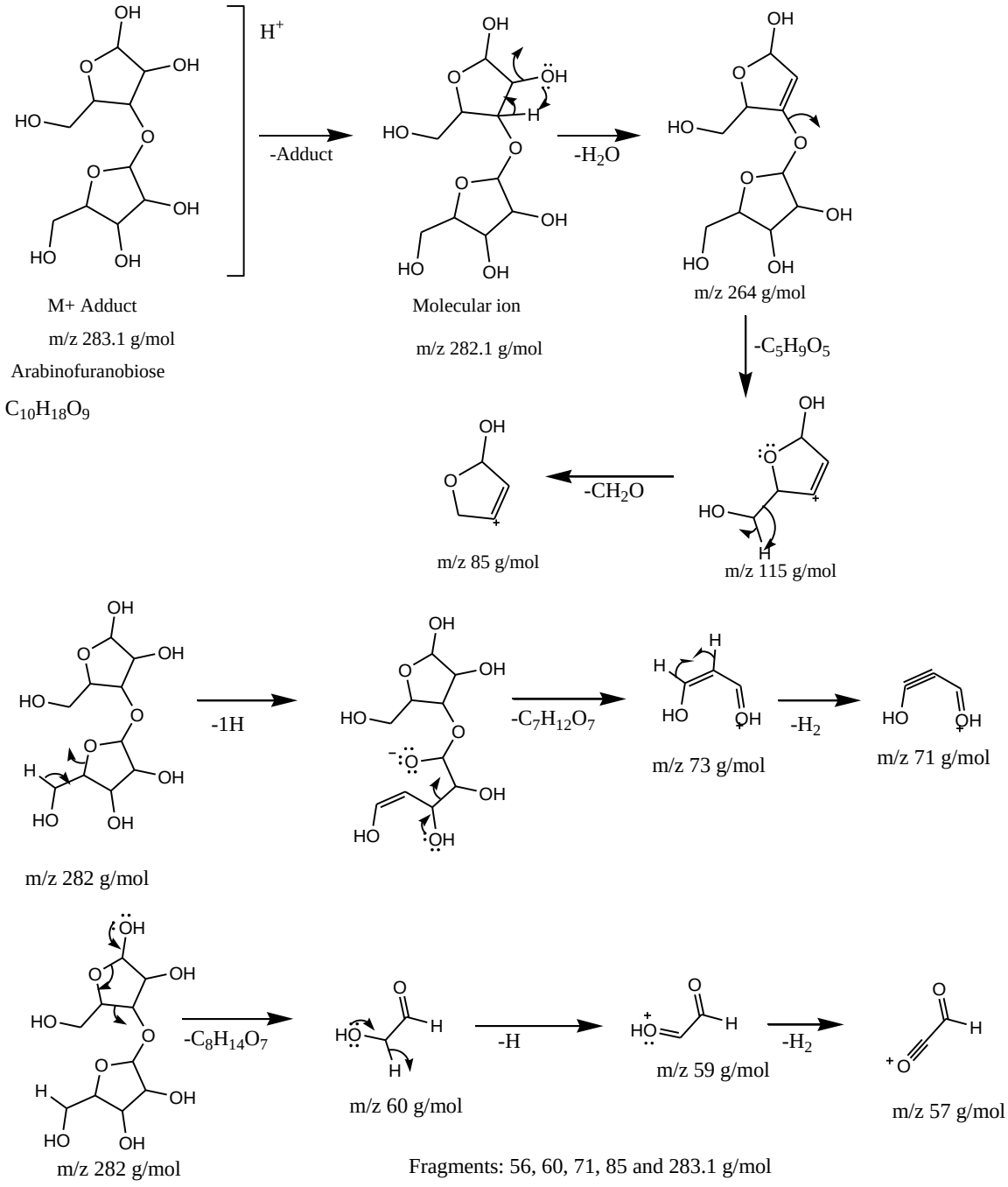


Fig. 5.1: The mass spectrum of compound 1

The fragment ion at m/z 85 ($C_4H_5O_2$) appeared due to the consecutive loss of H_2O, $C_5H_9O_5$ and CH_2O while the signal at m/z 71 resulted from that of H, $C_7H_{12}O_7$ and H_2. On the other hand, m/z 60 fragment was as a result of loss of $C_8H_{14}O_7$, which after further loss of

2H and H_2 resulted into m/z 56 fragment. The various fragment ions and fragmention pattern of compound 1, arabinofuranobiose, is illustrated in the scheme 4 below.



Scheme 4: Fragmentation pathway for arabinofuranobiose (compound 1).

Arabinofuranobiose, an o-glycosyl compound, has never been identified in previous research from honey. It was isolated from sugar beet arabinan by enzymatic degradation (Westphal *et al*., 2010). According to Minic *et al*. (2006), XYL3 enzyme released L-arabinose from arabinoxylan, (1 5)-α-L-arabinofuranobiose, debranched arabinan and sugar beet arabinan. Being a disaccharide consisting of fructose molecules, it has been rediscovered that identical fructose possess tolerance curves in normal and diabetic subjects (Renold & Thorn, 1955).

Compound 2 had a molecular ion of m/z 307.1 and fragment ions at m/z 83, 71, 60 and 56 with retention time of 32.23min and corresponded to starch acetate (fig. 5.2).

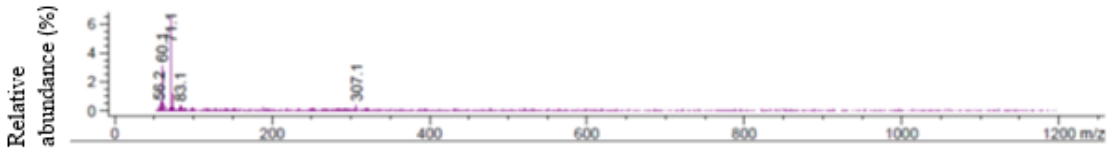
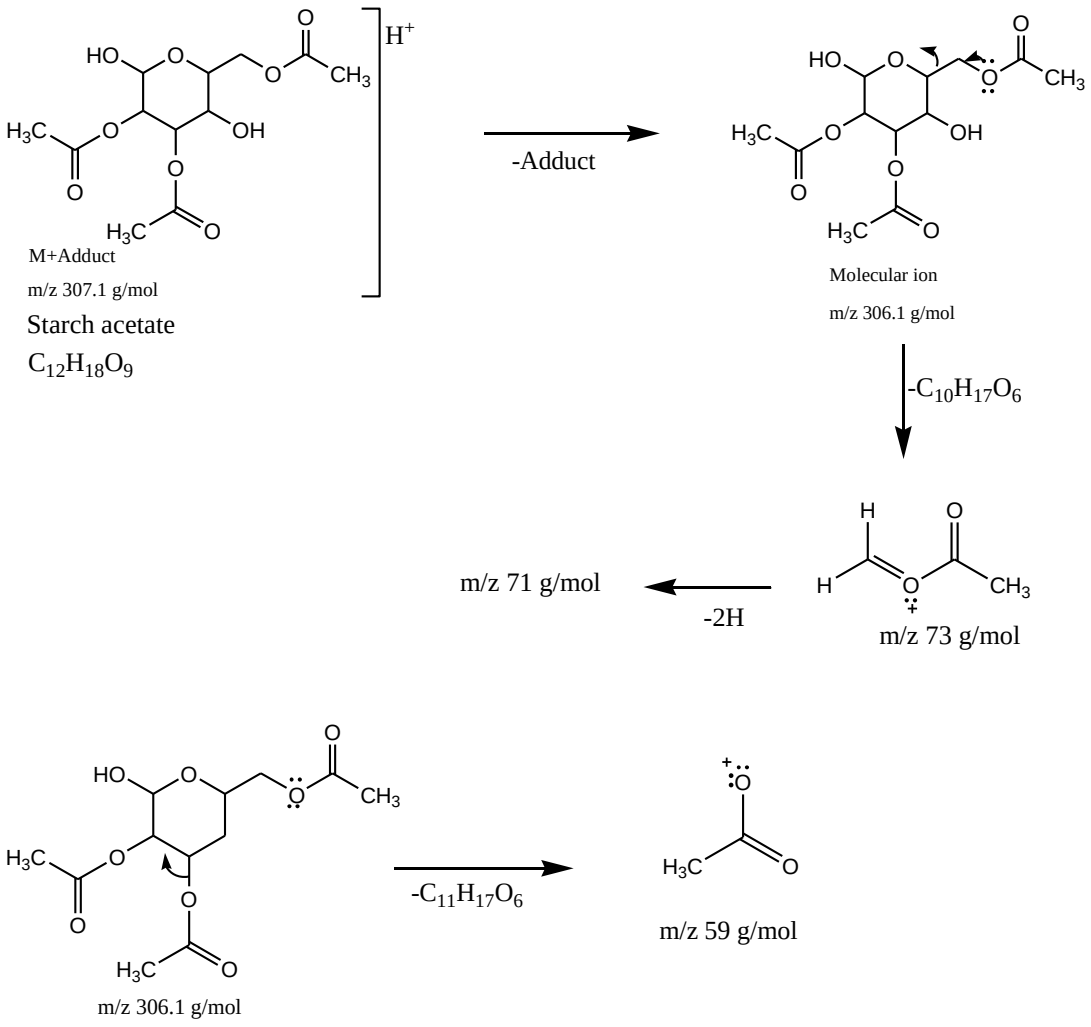
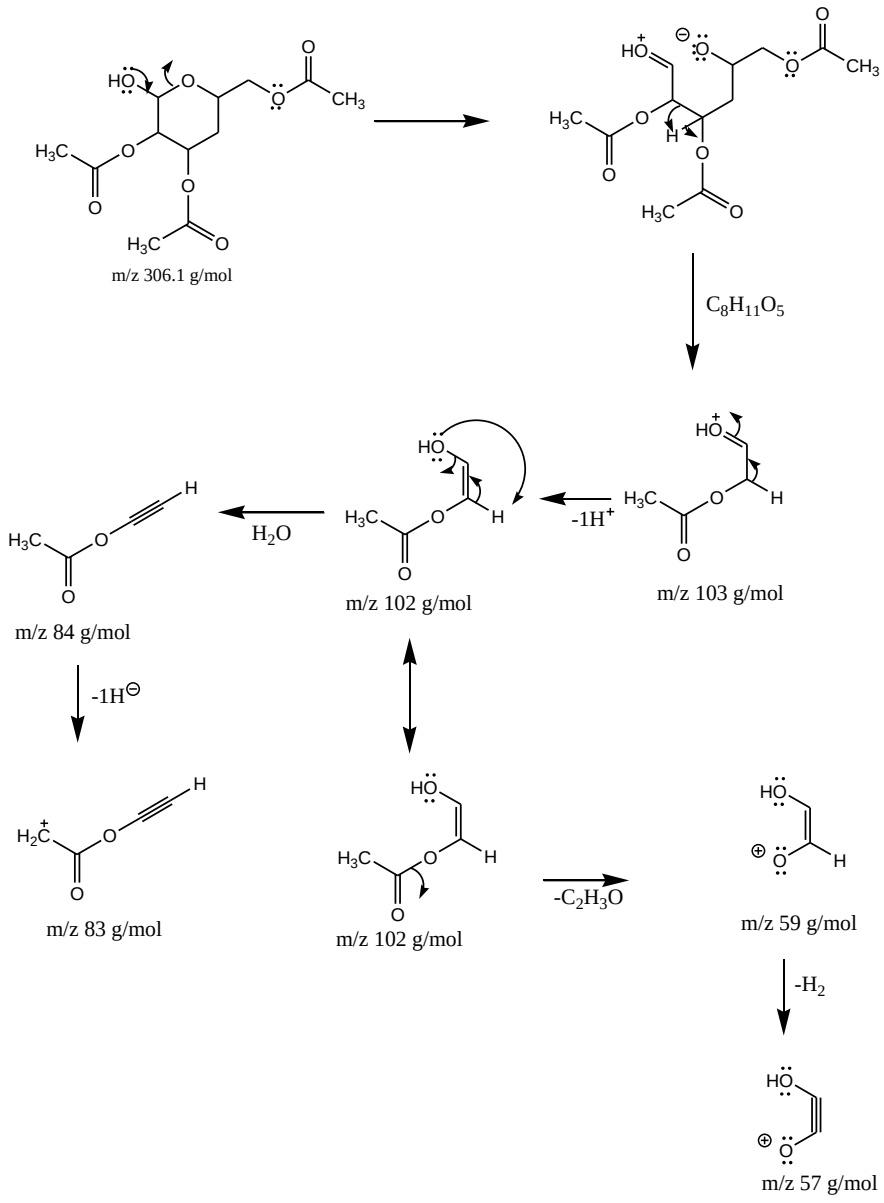


Fig. 5.2: The mass spectrum of compound 2

Fragment m/z 83 resulted from the loss of $C_8H_{11}O_5$, H^+, H_2O and H^- while m/z 71 fragment resulted from the loss of $C_{10}H_{17}O_6$ and H_2. Fragment m/z 60 was as a result of loss of $C_9H_{18}NO_2$ and C_4H_7O while m/z 56 resulted from the loss of $C_8H_{11}O_5$, $2H^+$, C_2H_3O and H_2. The various fragment ions and fragmention pattern of compound 2, starch acetate, is illustrated in the scheme 5 on pages 84 & 85.



Scheme 5: Fragmentation pathway for starch acetate (compound 2; continued)



Scheme 5: Fragmentation pathway for starch acetate (compound 2)

Starch acetate, a tricarboxylic acid derivative, was synthesized in cassava in preparation for biodegradable films (Larotonda *et al*., 2004). Starch esters such as starch acetate has been used as food additives in food industries (Tian *et al*., 2018).

Compound 3 displayed a molecular ion at m/z 336.2 with fragments 72, 71 and 60. It had a retention time of 13.70 min and corresponded to methyl glucoside bound to a heptylcarbamoyl (fig. 5.3).

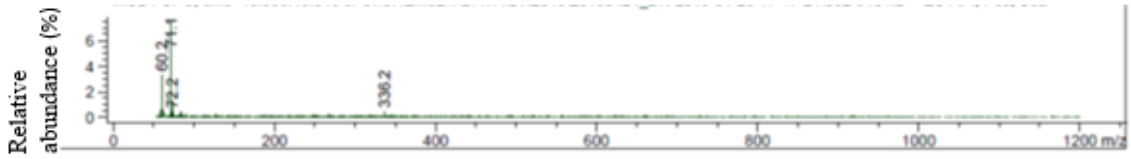
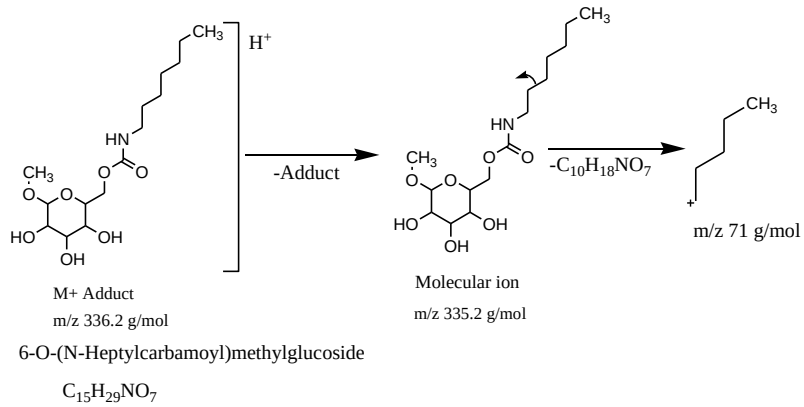
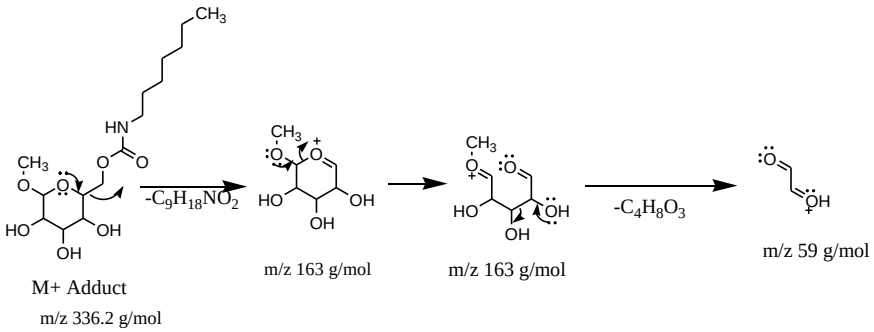


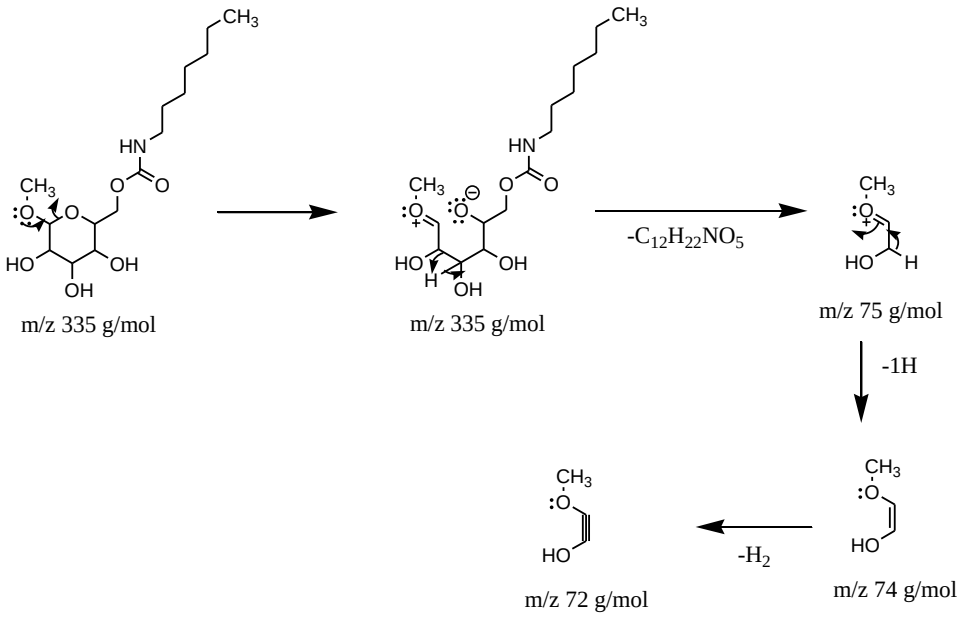
Fig. 5.3: The mass spectrum of compound 3

M/z 72 fragment appeared due to loss of $C_{12}H_{22}NO_5$, H and H_2. M/z 71 fragment was due to loss of $C_{10}H_{18}NO_7$ while m/z 60 resulted from loss of $C_9H_{18}NO_2$ and $C_4H_7O_3$ molecules. The various fragment ions and fragmention pattern of compound 3, 6-O-(N-Heptylcarbamoyl) methylglucoside, is illustrated in the scheme 6 on pages 86-87.





Continuing scheme



Fragments: 60, 71, 72 and 336.2 g/mol

Scheme 6: Fragmentation pathway for 6-O-(N-Heptylcarbamoyl) methylglucoside (compound 3).

α –methyl-D-glucoside, an o-glycosyl compound, has been used as a substrate for inducer and growth of production of amylase by *Aspergillus* sp., in both solid state and submerged fermentation (Moreira *et al*., 2001). 6-O-(N-Heptylcarbamoyl) methylglucoside has been synthesized from methyl-alpha-D-glucopyranoside by a low cost and simple procedure (Plusquellec *et al*., 1989). Truchado *et al*., 2015 characterized o-glycosyl compounds from *Tetragonul*a *carbonaria* pot-honeys from Australia. Polysaccharide extracts from mushroom showed antioxidant and antibacterial activities (Ren *et al*., 2014) proving that carborhydrate derivatives may have potent antibacterial and antioxidant potencies.

Compound 4 was characterized as N-(4-Hydroxy-8-phenoxyisoquinoline-3-carbonyl) glycine ($C_{18}H_{14}N_2O_5$ m/z 339.1) at a retention time of 7.51 with fragments 197, 131, 85, 71 and 59 (fig. 5.4).

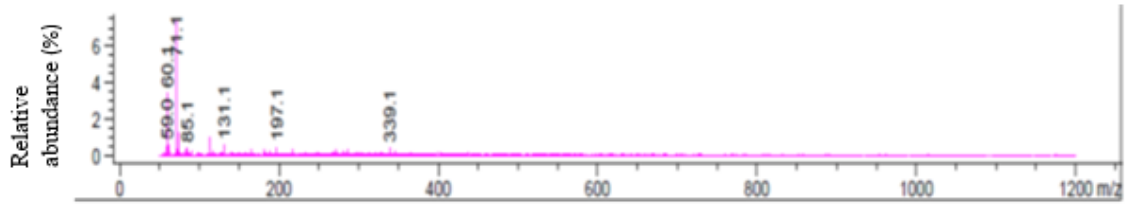
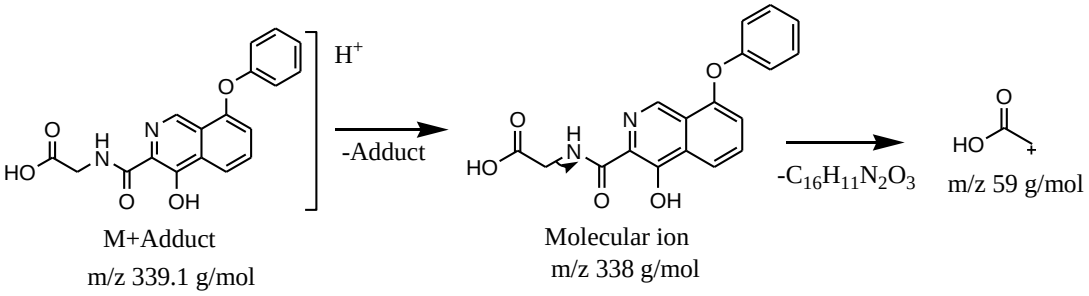


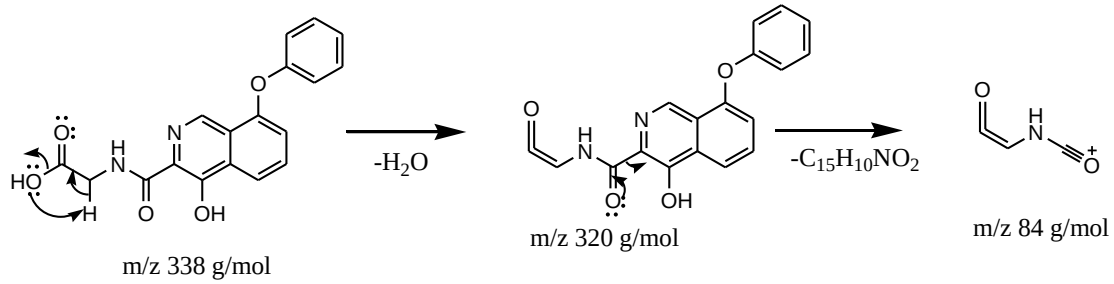
Fig. 5.4: The mass spectrum of compound 4

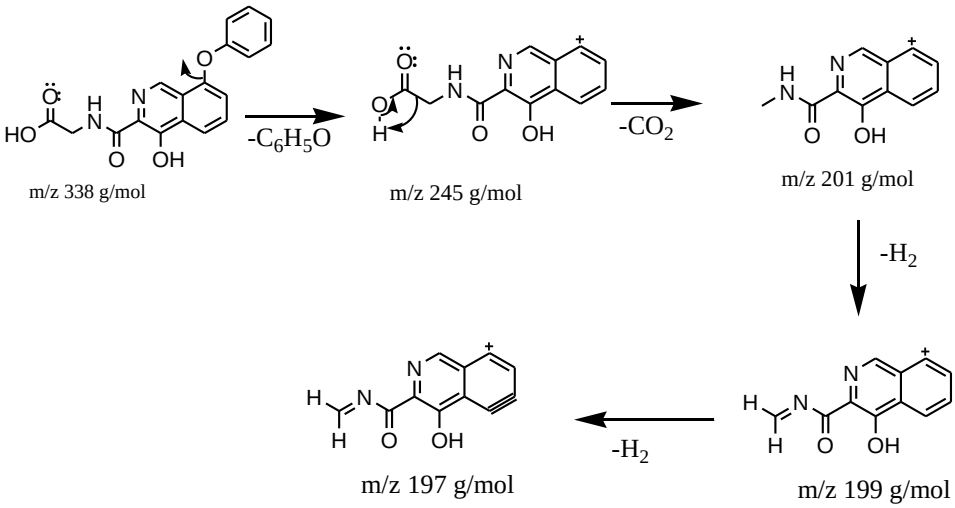
M/z 197 fragment resulted from the loss of phenoxide ion (C_6H_5O), decarboxylation (loss of CO_2) and dehydrogenation (loss of $2H_2$). M/z 131 was characterized by the loss of $C_4H_4N_2O_3$, C_6H_5, H_2 and H. M/z 85 fragment resulted from loss of $C_{15}H_9NO_2$ and dehydration (loss of H_2O). The fragment of m/z 71 was as a result of $C_{16}H_{10}NO_3$, H_2 and H. Lastly, m/z 59 fragment resulted from abstraction of $C_{16}H_{11}N_2O_3$. The various fragment ions and fragmention pattern of compound 4, N-(4-Hydroxy-8-phenoxyisoquinoline-3-carbonyl) glycine, is illustrated in the scheme 7 on pages 89-90.



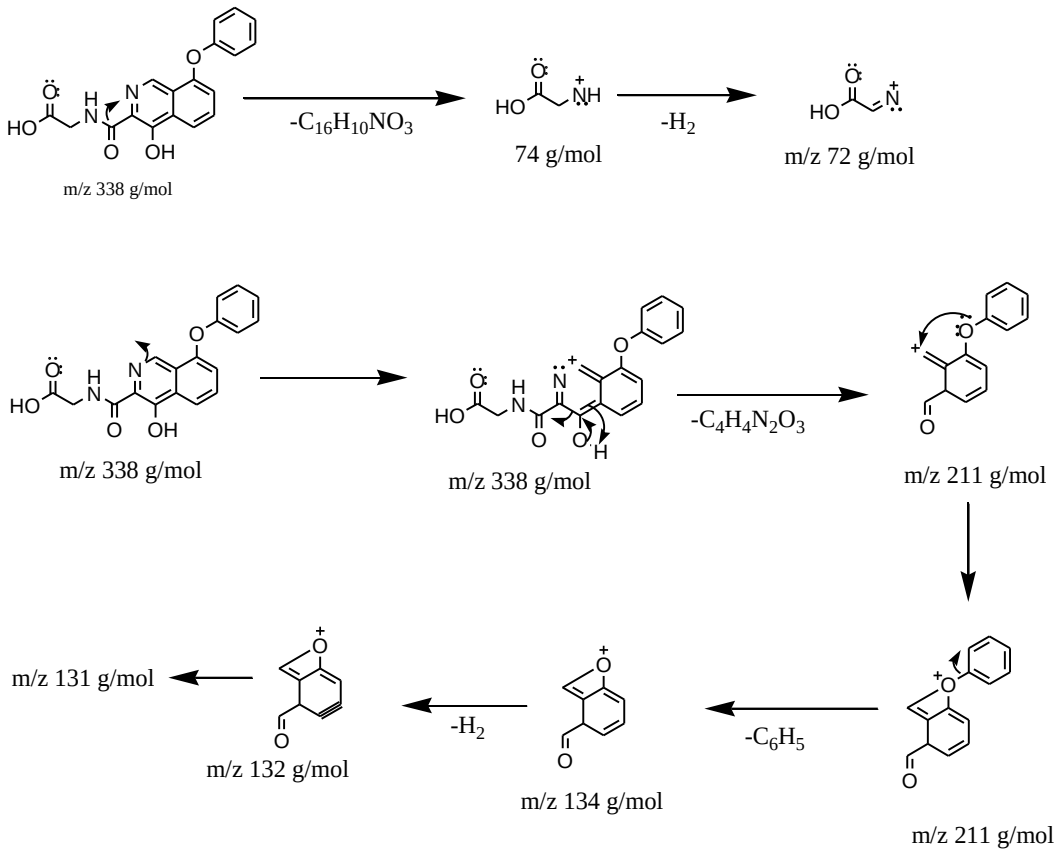
N-(4-Hydroxy-8-phenoxyisoquinoline-3-carbanoyl)glycine

$C_{18}H_{14}N_2O_5$





Continuing scheme



Fragments: 59, 71, 85, 131, 197 and 339 g/mol

Scheme 7: Fragmentation pathway for N-(4-Hydroxy-8-phenoxyisoquinoline-3-carbonyl) (compound 4).

N-(4-Hydroxy-8-phenoxyisoquinoline-3-carbonyl) is an isoquinoline alkaloid derivative bonded to glycine amino acid. Isoquinoline alkaloids were also isolated from McRB honey, refferred to as mad honey, produced by honey bees from *Macleaya cordata* (Willd.) R. Br (McRB) nectar (Zhao *et al*., 2018). A related compound, tertiary isoquinoline, has been isolated from roots of *Thalictrum flavum* (Ropivia *et al*., 2010). In addition, isoquinoline derivatives, asimilobine, nomuciferine and annonaine were also isolated from the fruit of *Annona muricata* (Hasrat *et al*., 1997). Isoquinoline alkaloids

possess enormous sorts of medicinal properties such as antifungal, antiviral, antioxidant, enzyme inhibition, anticancer and antispasmodic properties (Dey *et al*., 2020).

Compound 5 displayed the protonated molecular ion at m/z 347.1 at a retention time 24.63 min with fragments 307, 279, 71, 60 and 59. This compound corresponded to methyl 6-O-galloyl-beta-D-glucopyranoside (fig. 5.5).

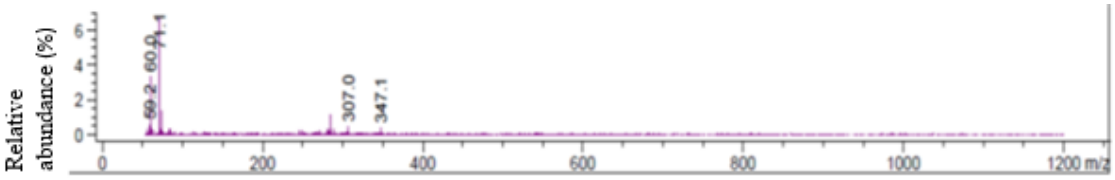
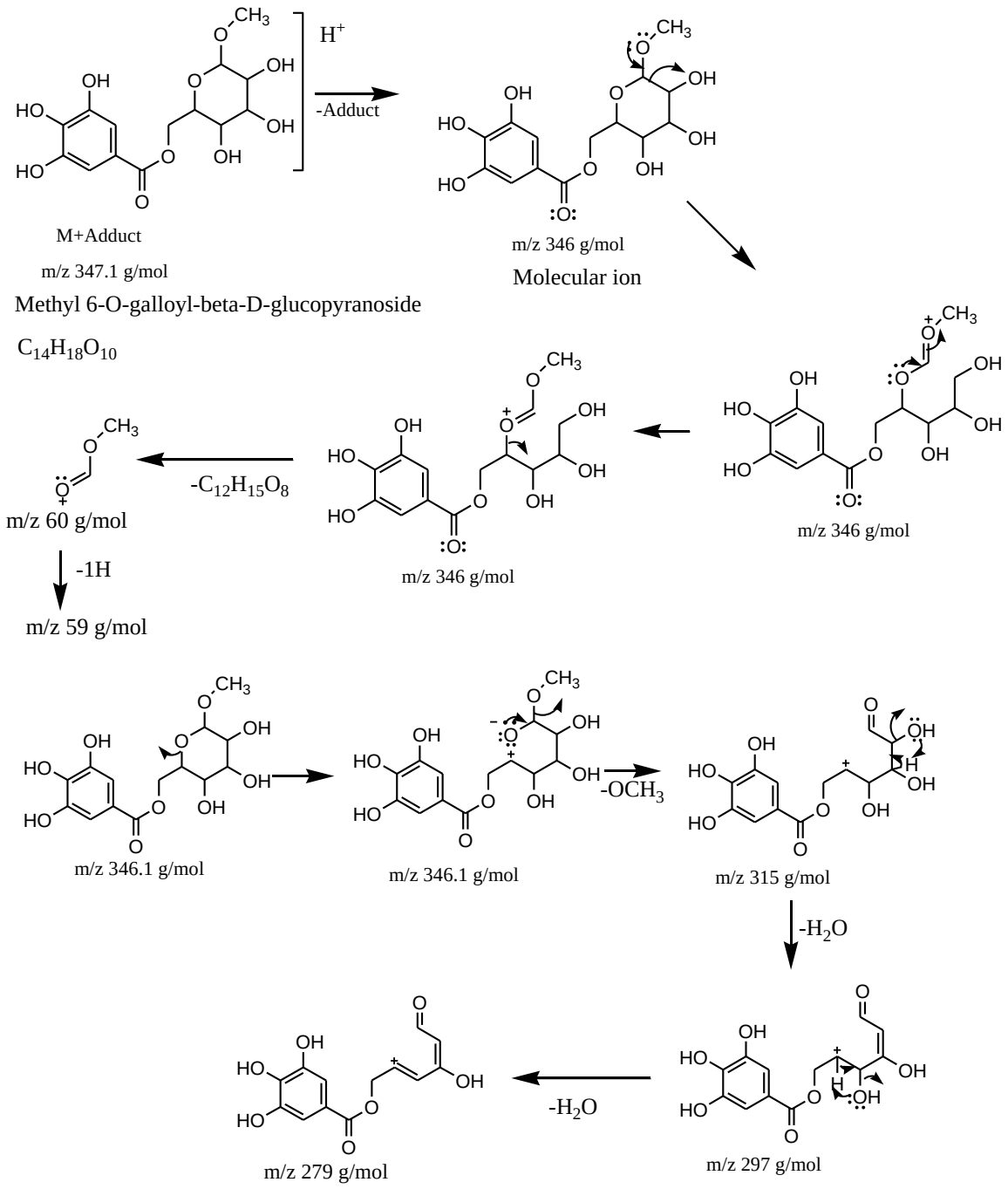
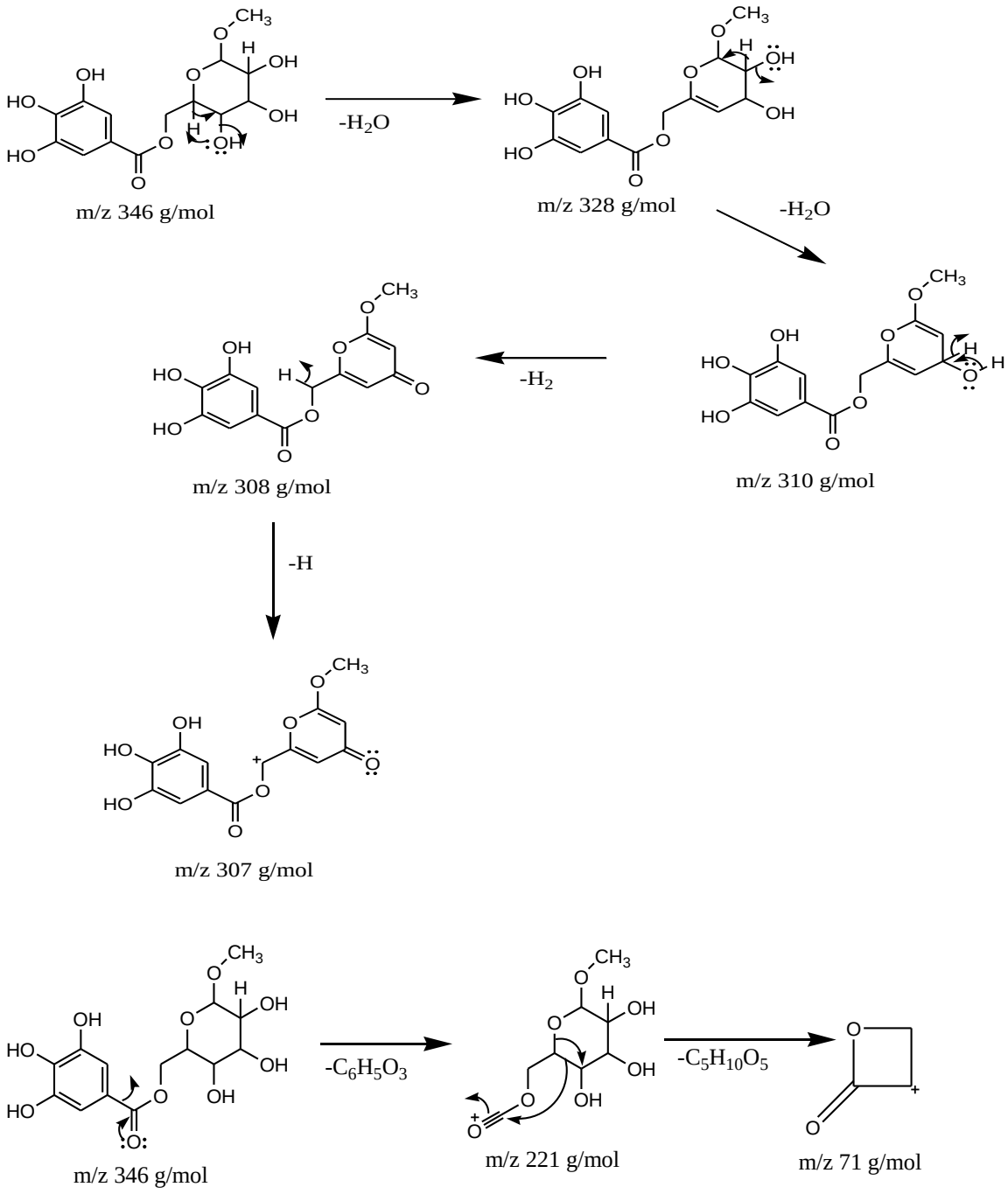


Fig. 5.5: The mass spectrum of compound 5

Fragment m/z 307 resulted from dehydration (loss of two water molecules), dehydrogenation (loss of H_2), and loss of H. M/z of 279 was due to abstraction of a methoxy group and two water molecules. M/z 71 was attributed to loss of $C_6H_5O_3$ and $C_5H_{10}O_5$ molecules. The fragment m/z 60 resulted from loss of $C_{12}H_{15}O_8$. Finally, fragment 59 was attributed to the loss of $C_{12}H_{15}O_8$ and H. The various fragment ions and fragmention pattern of compound 5, methyl 6-O-galloyl-beta-D-glucopyranoside, is illustrated in the scheme 8 on pages 92-93.



Continuing scheme



Fragments: 59, 60, 71, 279, 307 and 347.1 g/mol

Scheme 8: Fragmentation pathway for methyl 6-O-galloyl-beta-D-glucopyranoside (compound 5)

6-O-galloyl-beta-D-glucopyranoside belong to a class of organic compounds called tannins, naturally occurring polyphenol biomolecules. Related tannins, 1-*O*-Galloyl-*β*-d-glucose (β-Glucogallin) and 1, 2, 3, 6-Tetra-O-galloyl-β-d-glucose were isolated from fruit extract of *Phyllanthus emblica* Linn. or *Emblica officinalis Gaertn* (Variya *et al*., 2016) and from methanolic seeds extract of *Cornus officinalis* (Duan *et al*., 2004) respectively. Due to the phenolic moiety in 6-O-galloyl-beta-D-glucopyranoside, it possesses antioxidant, anti-inflammatory, anticancer and antimicrobial properties (Cianciosi *et al*., 2018).

Compound 6 displayed a protonated molecular ion at m/z 394.2 at a retention time of 4.87 min with fragment ions m/z 328, 302, 262, 228, 202, 131, 85, 71 and 59. This corresponded to N-(3-Carboxypropanoyl)-L-phenylalanyl-L-lysine (fig. 5.6)

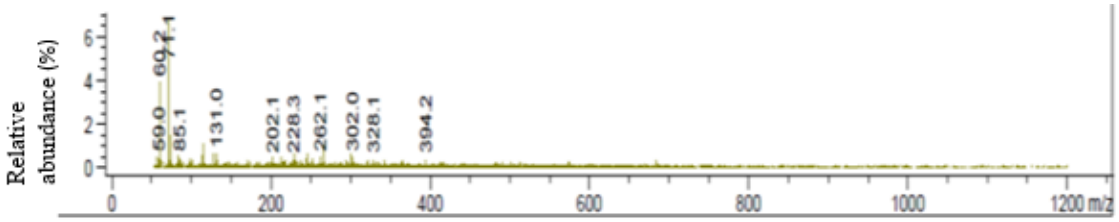
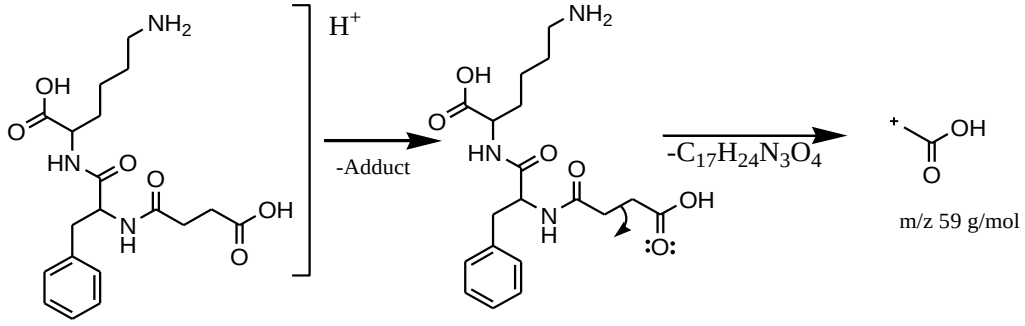


Fig. 5.6: The mass spectrum of compound 6

Dehydroxylation (loss of –OH), decarboxylation (loss of CO_2) and dehydrogenation (loss of H_2) yielded to fragment m/z 328. The m/z 302 fragment resulted from the loss of CO_2 (decarboxylation), C_2H_6N, H_2 and H. In addition, loss of $C_6H_{12}NO_2$ and H resulted to m/z 262 fragment. M/z 228 fragment was as a result of $C_3H_5O_2$ loss. Nevertheless, loss of $C_6H_{13}N_2O_2$, CO_2 (decarboxylation) and H_2 (dehydrogenation) led to the fragment m/z 202. Consequently, loss of $C_{13}H_{14}N_2O_4$ and $C_{17}H_{24}N_3O_4$ molecules resulted to m/z values 131 and 59 respectively. M/z 85 fragment resulted from loss of $C_{13}H_{14}N_2O_4$, CO_2 and H_2.

Lastly, m/z 71 fragment was attributed by loss of $C_{15}H_{17}N_2O_6$ and H. The various fragment ions and fragmention pattern is illustrated in the scheme 9 on pages 95-98.



M+Adduct

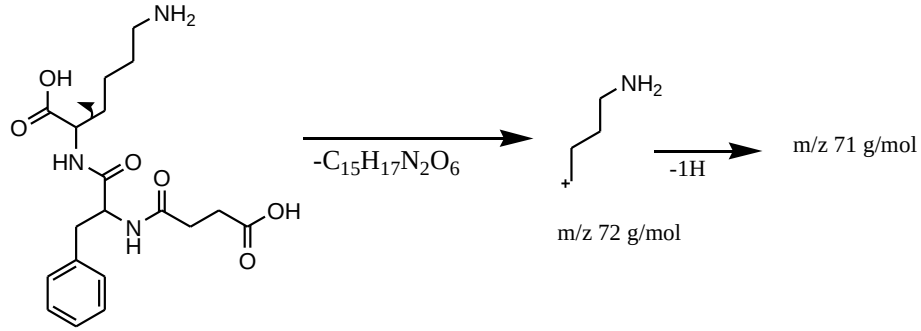
m/z 394.2 g/mol

Molecular ion

m/z 393.2 g/mol

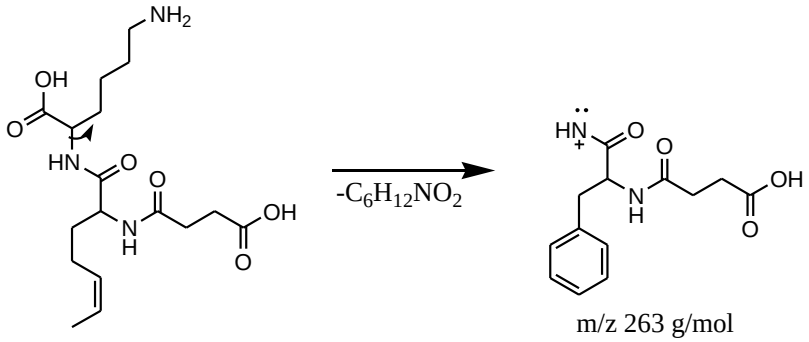
N-(3-Carboxypropanoyl)-L-phenylalanyl-L-lysine

$C_{19}H_{27}N_3O_6$



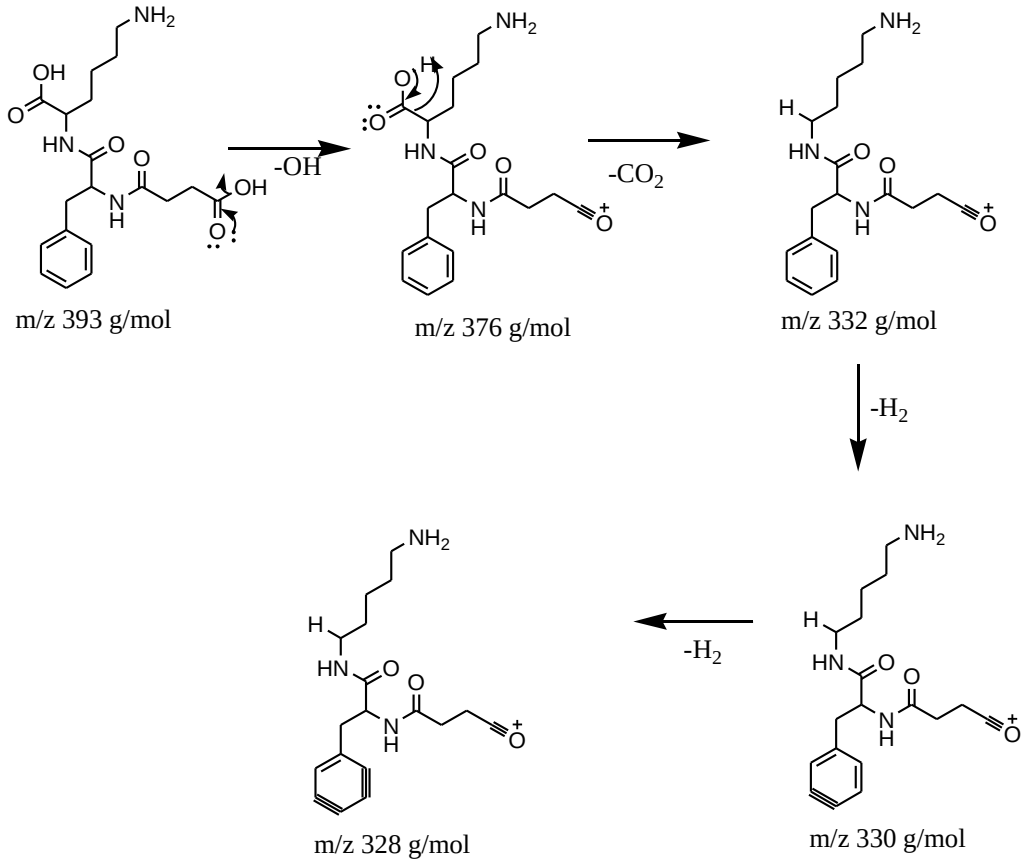
m/z 72 g/mol

m/z 393 g/mol

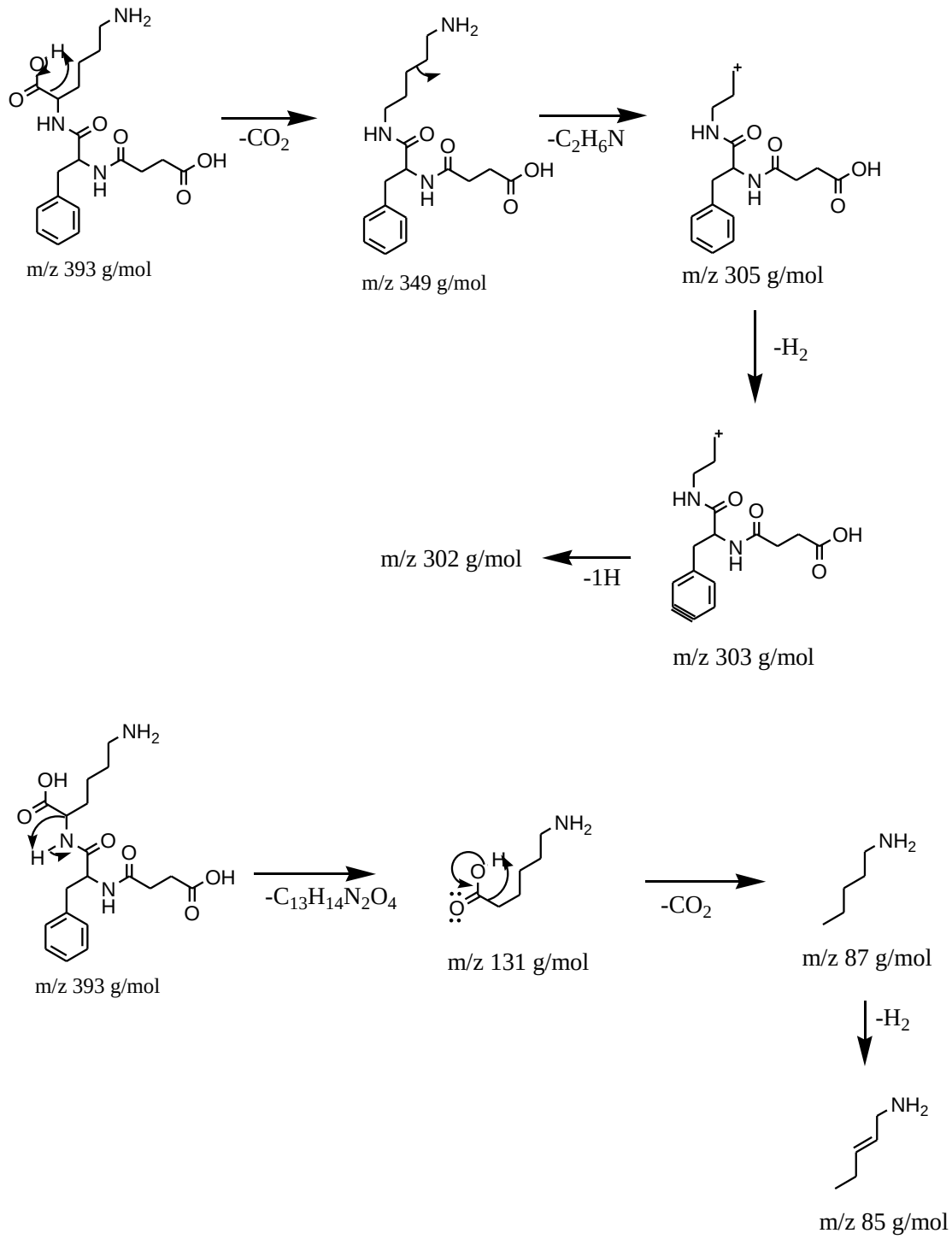


m/z 263 g/mol

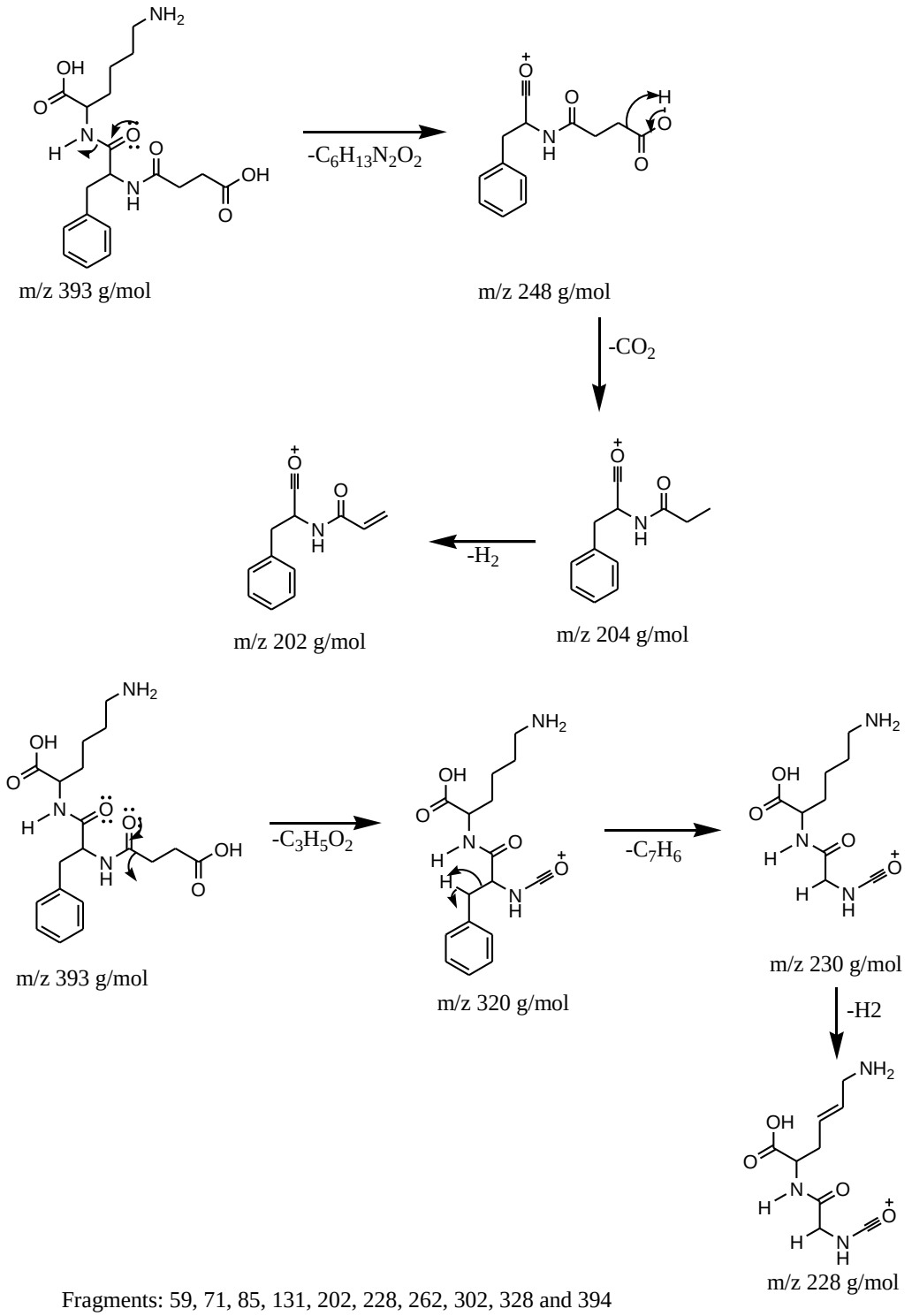
Continuing scheme



Continuing scheme



Continuing scheme



Fragments: 59, 71, 85, 131, 202, 228, 262, 302, 328 and 394

Scheme 9: Fragmentation pathway for N-(3-Carboxypropanoyl)-L-phenylalanyl-L-Lysine (compound 6)

N-(3-Carboxypropanoyl)-L-phenylalanyl-L-lysine is an amino acid derivative, with phenylalanine and lysine amino acids. Phenylalanine, proline, alanine, glutamic acid, leucine, tyrosine, histidine, threonine, glycine, arginine, aspartic acid, serine, lysine and valine were detected as the most common amino acid in forest honeys from Indonesia (Noor *et al*., 2019). Phenylalanine and lysine were also dominant amino acids in 31 Spanish honeys from 5 diverse botanical origins (Hermosín *et al*., 2003). Amino acids are believed to possess antioxidant activity (Hayes *et al*., 1977)

Compound 7 had a protonated molecular ion of m/z 431.1 at a retention time of 8.45 min with fragment ions at 262, 113, 97, 85, 71 and 59 corresponding to 4-(7-Hydroxy-4-oxo-4H-1-benzopyran-3-yl) phenylbeta-D-glucopyranosiduronic acid (fig. 5.7)

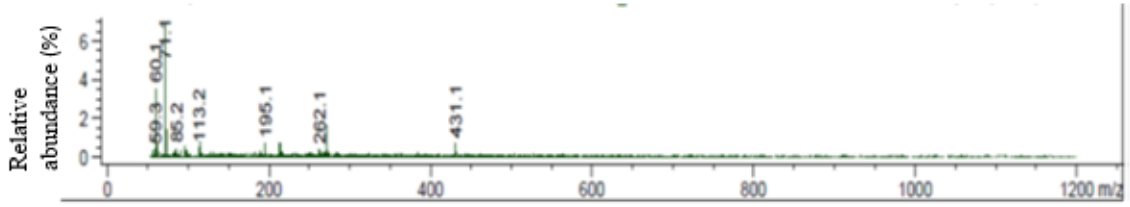
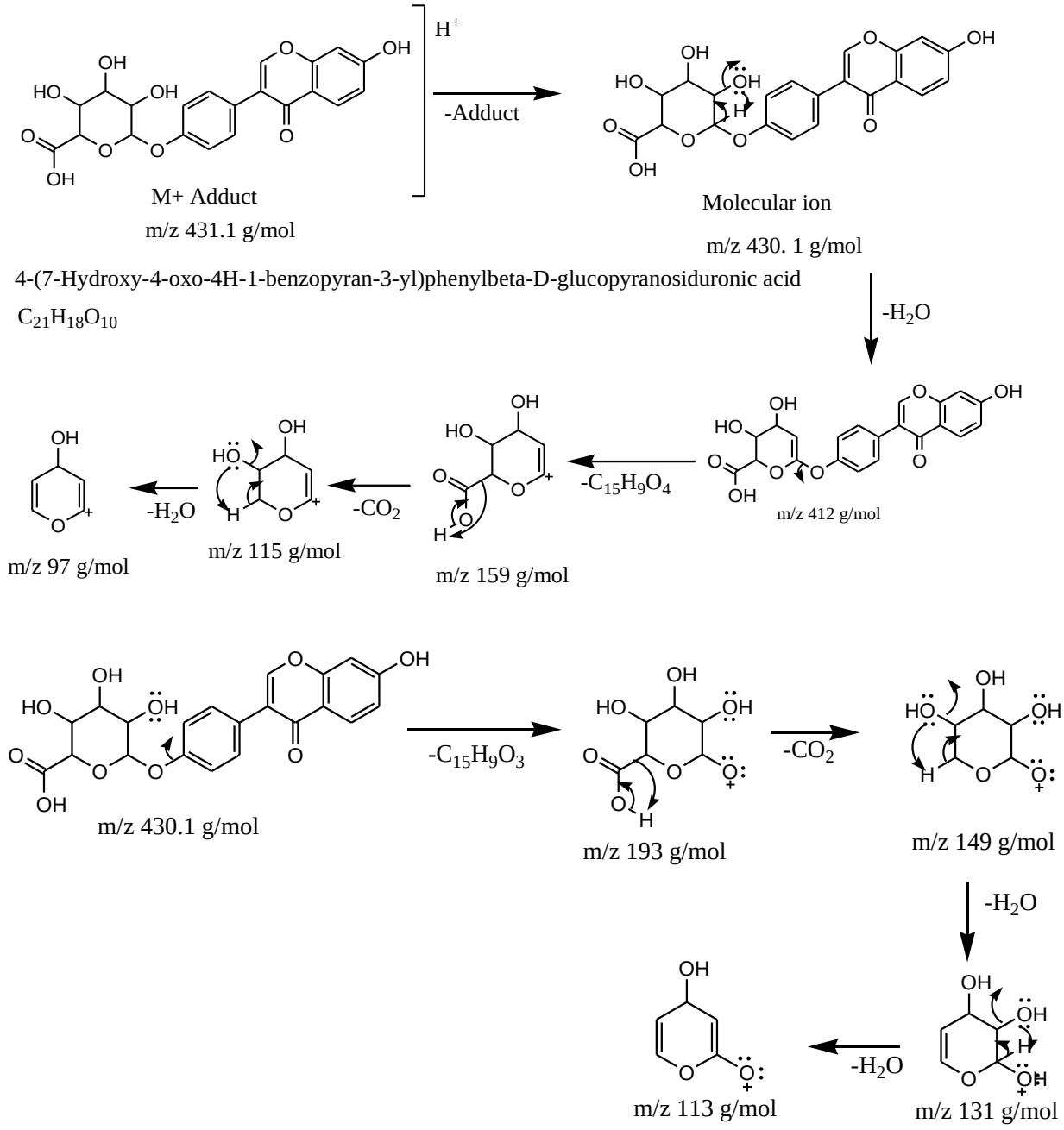
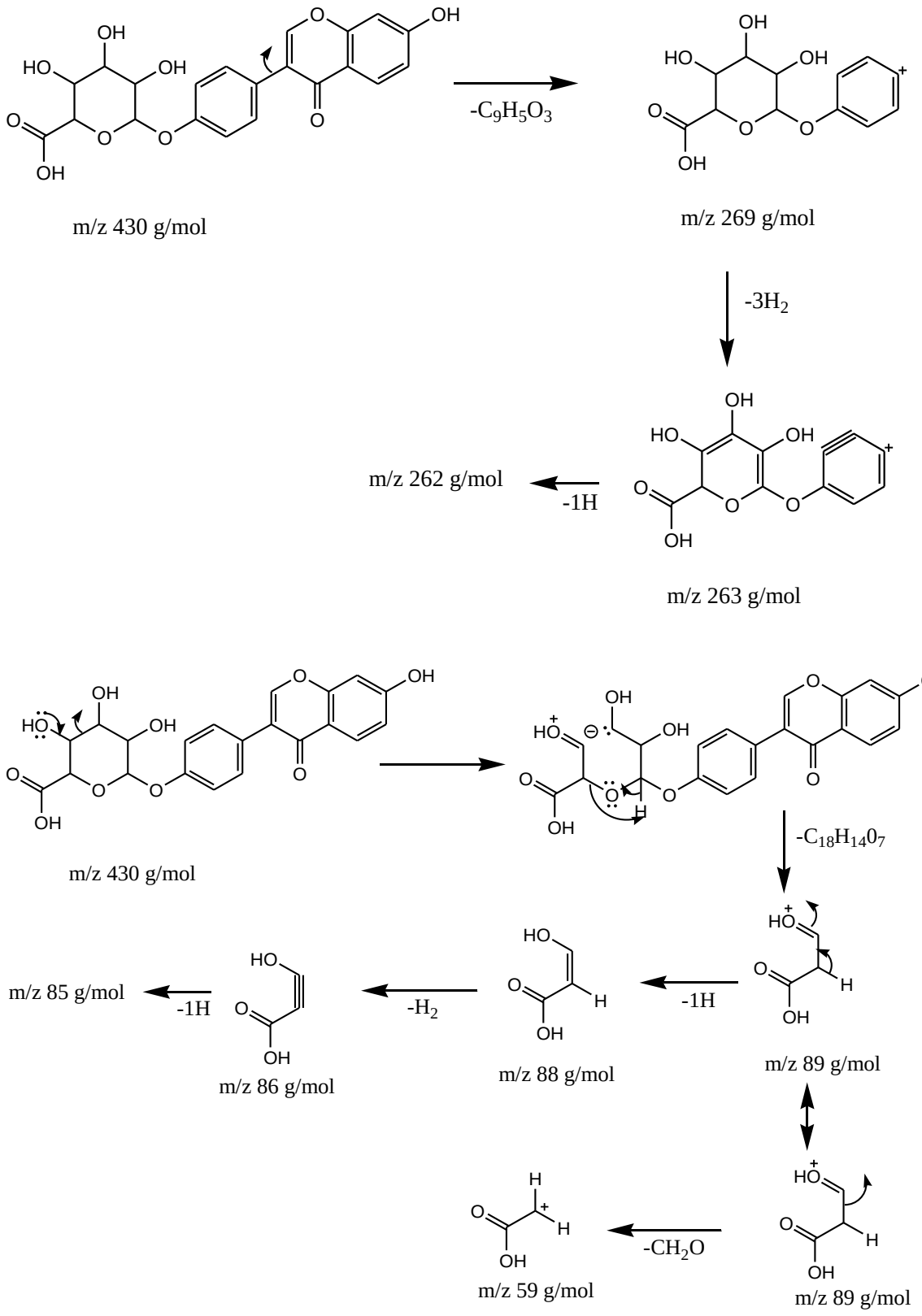


Fig. 5.7: The mass spectrum of compound 7

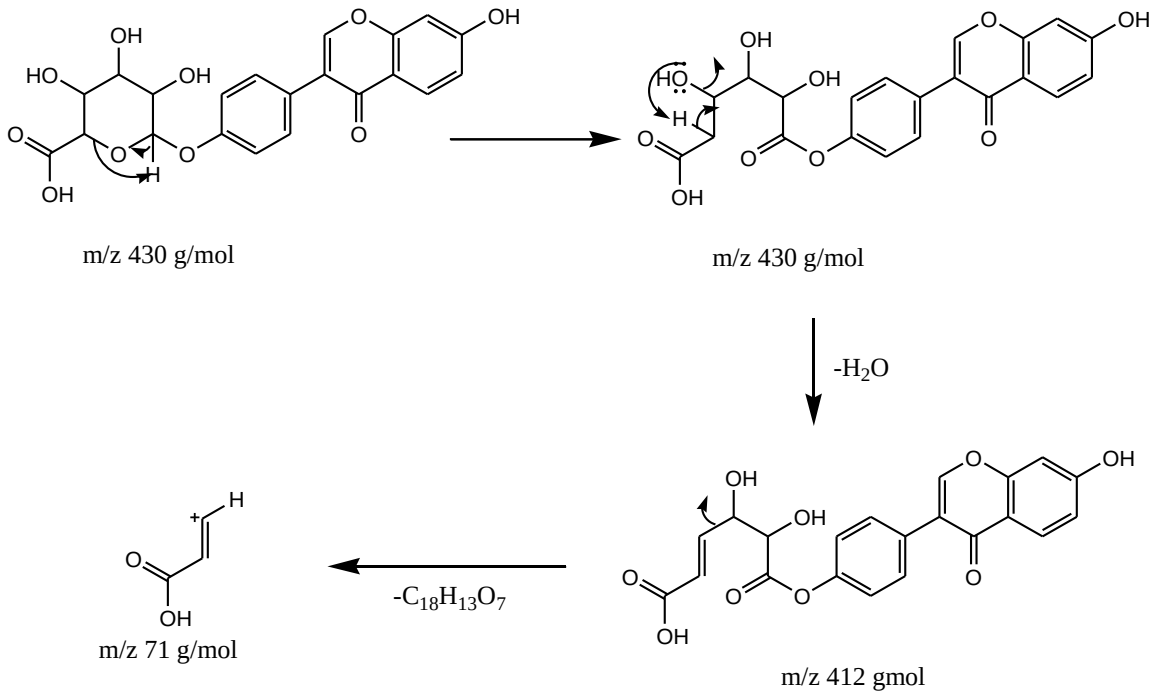
M/z 262 fragment was yielded from loss of $C_9H_5O_3$, $3H_2$ and H. M/z 113 fragment was as a result of $C_{15}H_9O_3$, CO_2, and two H_2O molecules abstraction. In the same way, m/z 97 fragment resulted from loss of two H_2O molecules, $C_{15}H_9O_4$ and CO_2. This compound also yielded a fragment at m/z 85 due to loss of $C_{18}H_{14}O_7$, H_2 and 2H. Similarly, the m/z 71 fragment resulted from the loss of $C_{18}H_{13}O_7$ and H_2O. The last fragment m/z 59 was attributed to the loss of $C_{18}H_{14}O_7$ and CH_2O as illustrated on scheme 10 on pages 99-101.



Continuing scheme



Continuing scheme



Fragments: 59, 71, 85, 97, 113, 262 and 431.1 g/mol

Scheme 10: Fragmentation pathway for 4-(7-Hydroxy-4-oxo-4H-1-benzopyran-3-yl) phenylbeta-D-glucopyranosiduronic acid (compound 7)

4-(7-Hydroxy-4-oxo-4H-1-benzopyran-3-yl) phenylbeta-D-glucopyranosiduronic acid also known as Daidzein 4'-β-D-Glucuronide is an isoflavone glucuronide and belongs to a class of organic compounds known as flavonoid derivatives. Isoflavones are believed to primarily occur in legumes especially soybean, alfafa, red clover and white clover (Křížová *et al*., 2019). Daidzein derivative phaseollin has been isolated from french bean (*Phaseolus vulgaris*) (Dakora *et al*., 1996). The identified compound has an isoflavone moiety which is responsible for its antioxidant, antidiabetic, anti-inflammatory, antimicrobial and anti- cancer activities (Dini, 2019).

Compound 8 displayed a protonated molecular ion of 443.3 at a retention time of 4.25 min with fragment ions of m/z 366, 330, 298, 247, 195, 144, 83, 72 and 56. This corresponded to Ala-Lys-Lys-Pro (fig. 5.8).

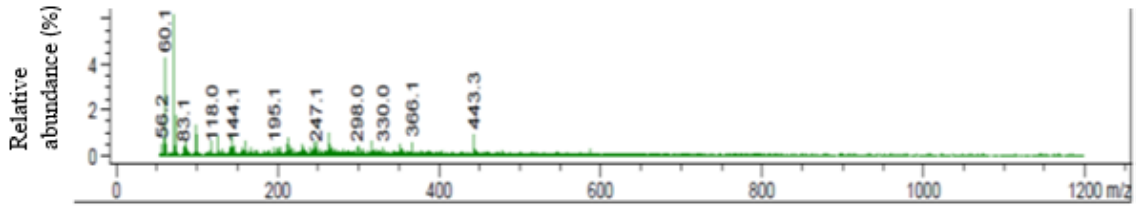
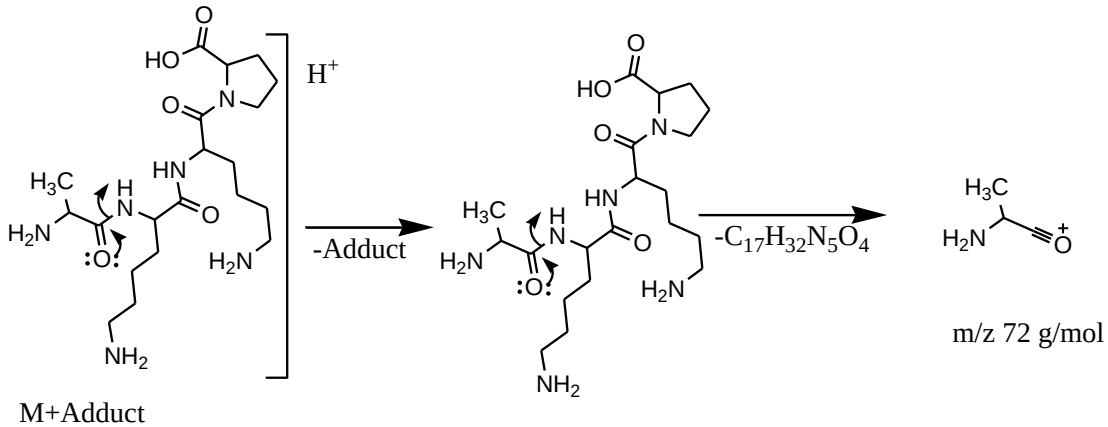


Fig. 5.8: The mass spectrum of compound 8

The fragment at m/z 366 resulted from loss of $C_4H_{10}N$ and two H_2 while for the one at m/z 330 was due to loss of C_2H_6N, C_3H_7N, five H_2 and H. The one at m/z 298 resulted from the loss of $C_6H_8NO_3$ and H_2. M/z 247 fragment was as a result of loss of $C_8H_{18}N_3O$, H_2O, $2H_2$ and H while m/z at 195 resulted from loss $C_{11}H_{20}N_3O_3$, $2H_2$ and H. In addition, m/z at 144 was as a result of $C_{11}H_{20}N_3O_3$ and C_3H_6N. Furthermore, m/z 83 fragment resulted from the abstraction of $C_{12}H_{19}N_3O_4$, $C_3H_7N_2O$, and H_2 while m/z 72 was due to abstraction of $C_{17}H_{32}N_5O_4$. The fragment m/z 56 was attributed to the loss of $C_{17}H_{30}N_5O_5$ and H_2 (scheme 11 on pages 104-107).



M+Adduct

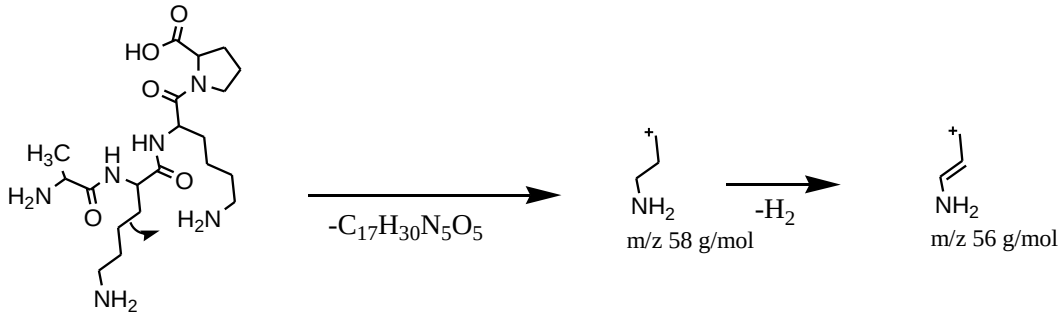
m/z 443.3 g/mol

Ala Lys Lys Pro

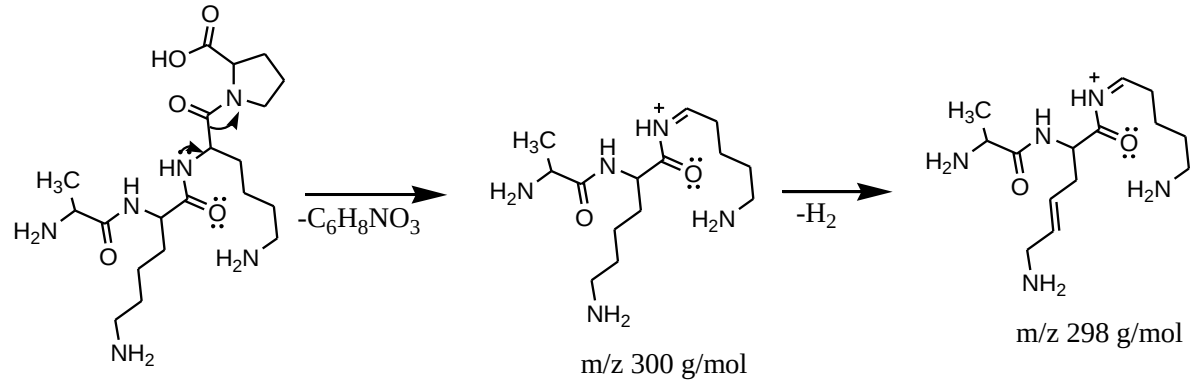
$C_{20}H_{38}N_6O_5$

Molecular ion

m/z 442.3 g/mol

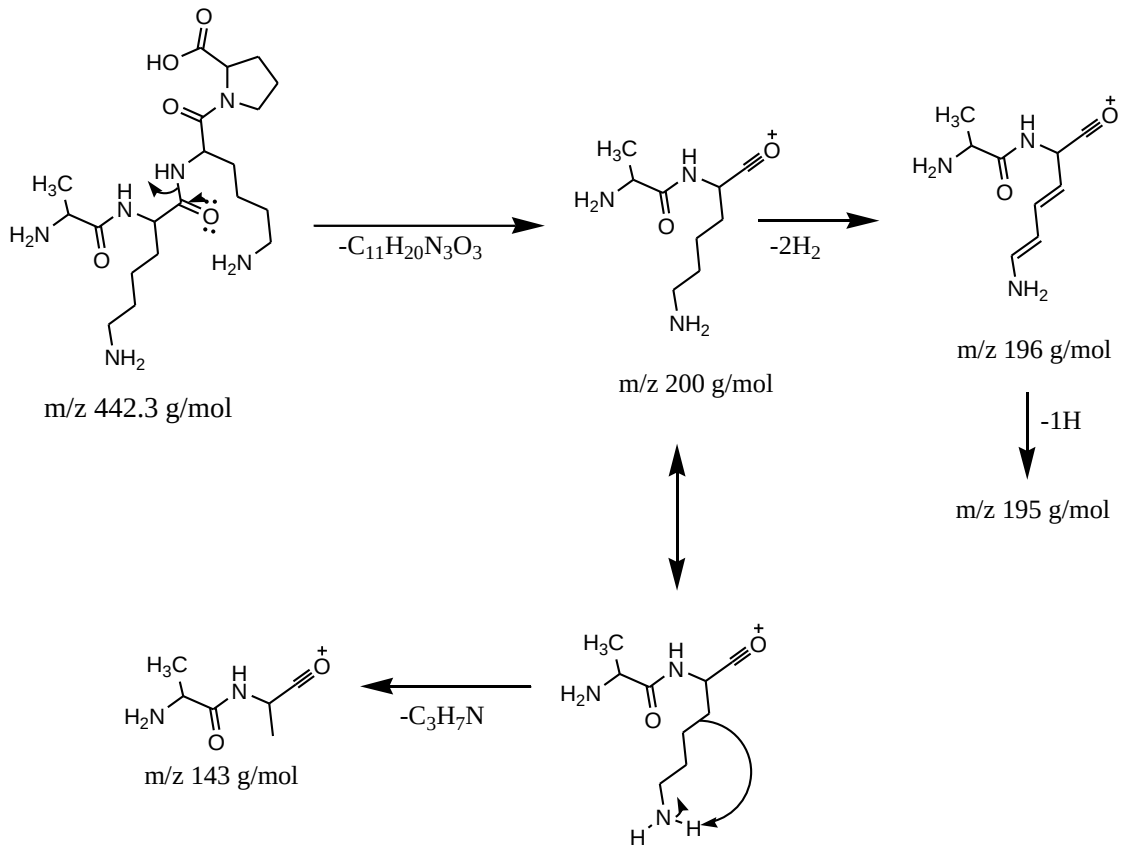


m/z 442.3 g/mol



m/z 442.3 g/mol

Continuing scheme



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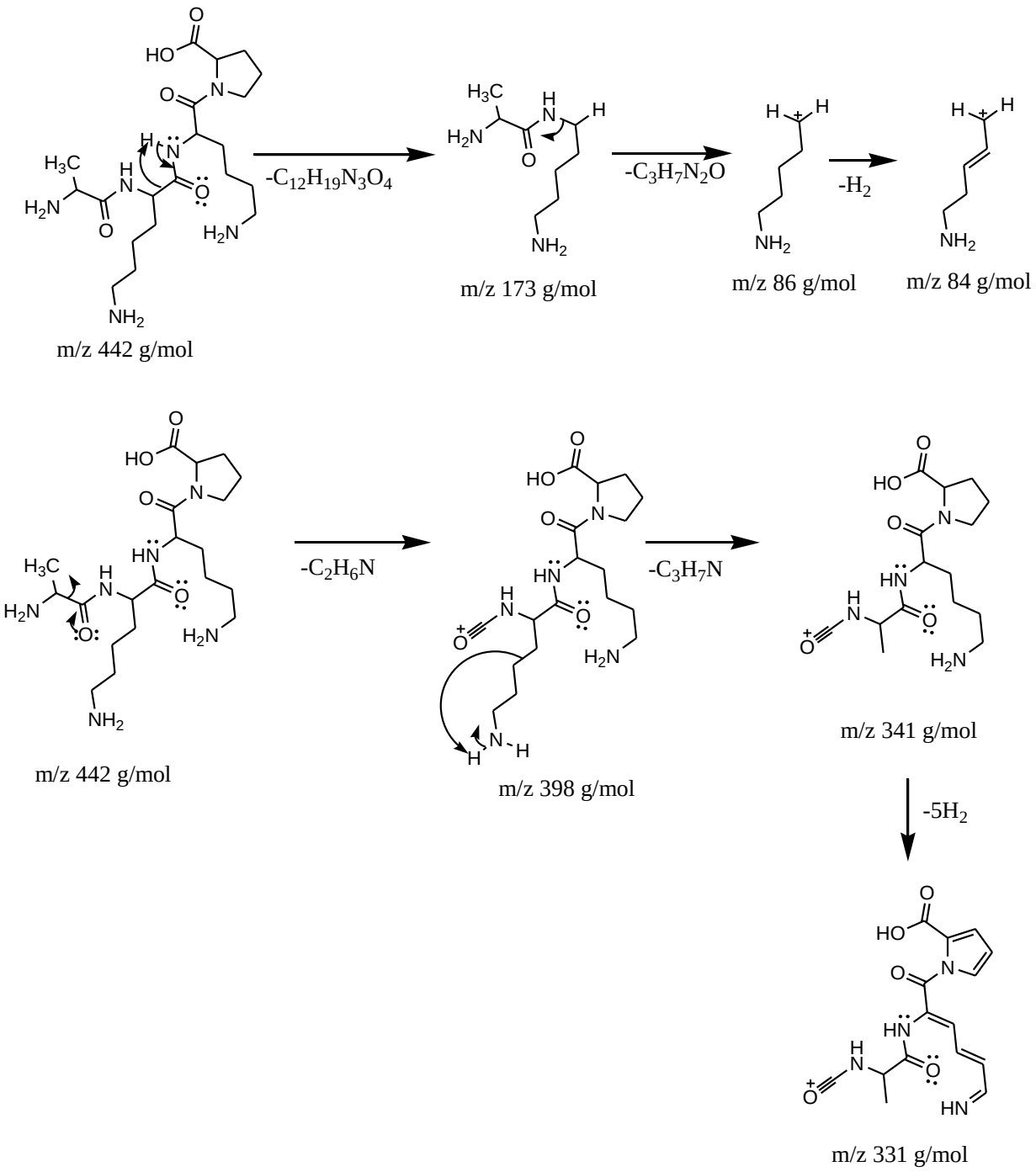
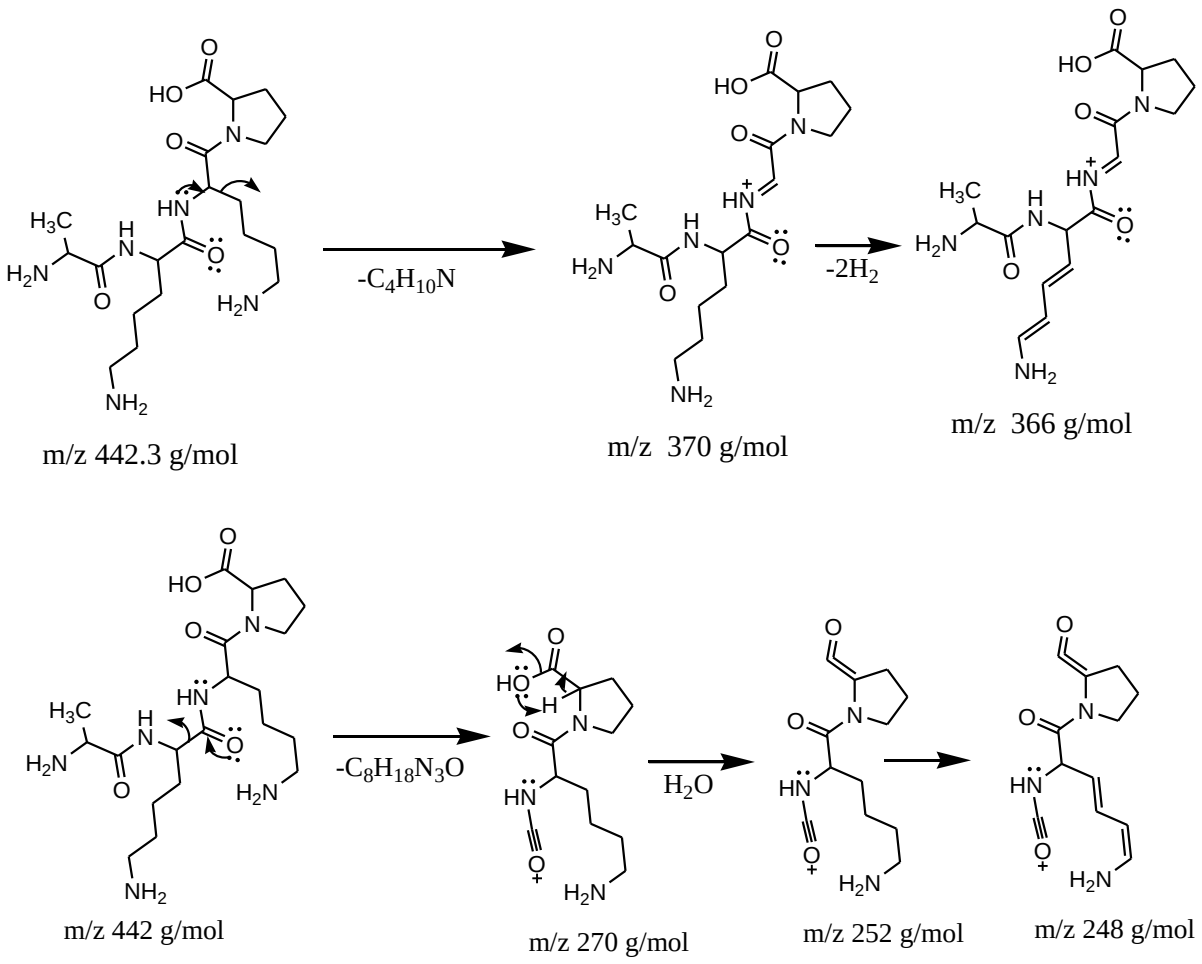


Continuing scheme



Fragments: 56, 72, 83, 144, 195, 247, 298, 330, 366 and 443.3 g/mol

Scheme 11: Fragmentation pathway for Ala-Lys-Lys-Pro (compound 8)

Ala-Lys-Lys-Pro is a tetrapeptide because it is a short chain of four amino acids. Alanine, lysine and proline amino acids present in this peptide were characterized in forest honeys from South Sulawesi in Indonesia (Noor *et al*., 2019). The same amino acids wer also obtained in Slovak and Polish honeys from Slovakia and Poland respectively (Kowalski *et al*., 2017). They were also detected from stingless bee honey (Shamsudin *et al*., 2019). Peptides and amino acids have antioxidant property (Hayes *et al*., 1977).

Compound 9 was detected at the retention time in 43.97min with m/z 549.2 and gave characteristic fragment ions at m/z 517, 401, 341, 74, 71 and 60. This corresponded to Met-His-Met-Met (fig. 5.9).

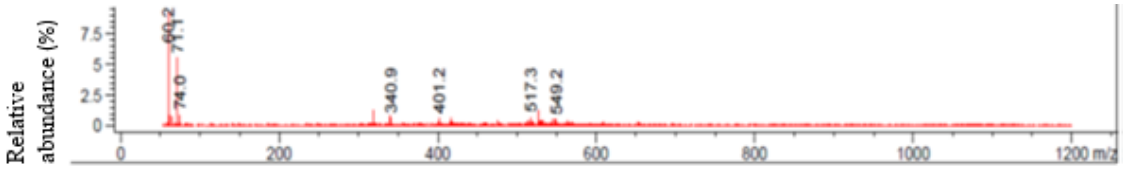
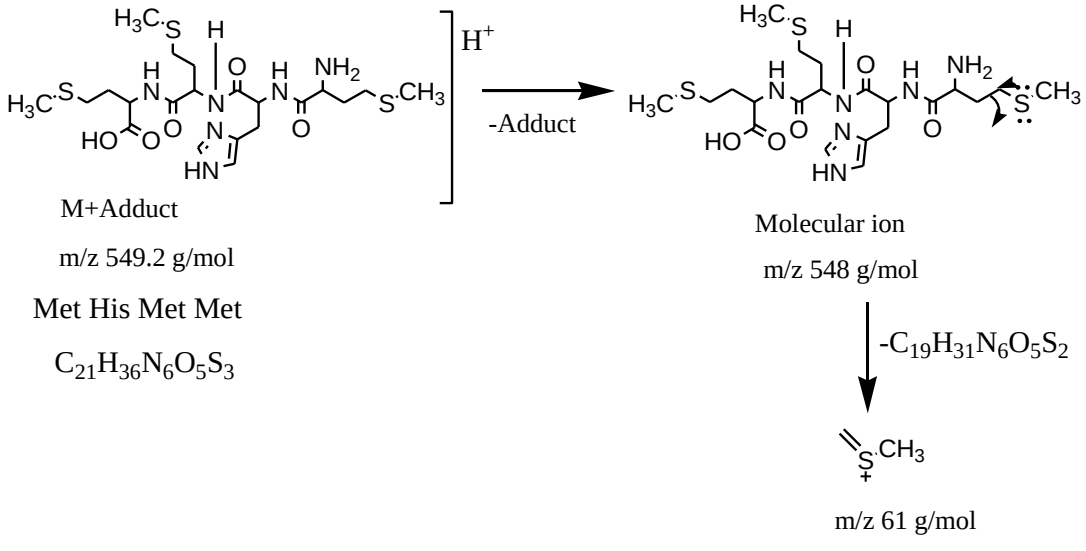
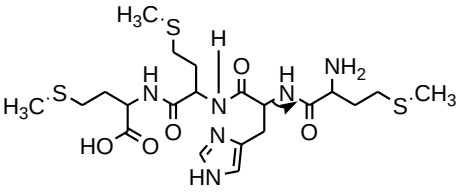


Fig. 5.9: The mass spectrum of compound 9

The Fragment at m/z 517 resulted from the loss of methyl (CH_3) and amine group (NH_2) while fragment m/z 401 was as a result of loss of $C_5H_{11}N_2O_5$. However, m/z 341 was related with loss of $C_6H_{10}NO_3S$, NH_3 and CH moeity while m/z 74 was related to $C_{18}H_{30}N_6O_5$ loss. Similarly, m/z 71 was produced due to loss of $C_{18}H_{29}N_6O_5S_2$ and two H_2 molecules while m/z 60 was produced due to loss of $C_{19}H_{31}N_6O_5S_2$ and H atom (scheme 12 on pages 108-110).

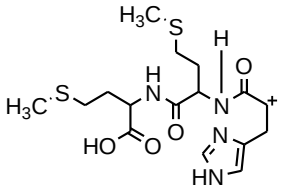


Continuing scheme

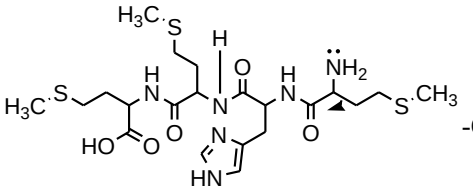


m/z 548.2 g/mol

$-C_5H_{11}N_2OS$

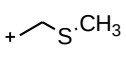


m/z 401 g/mol



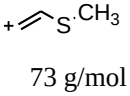
m/z 548.2 g/mol

$-C_{18}H_{29}N_6O_5S_2$



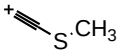
75 g/mol

$-H_2$

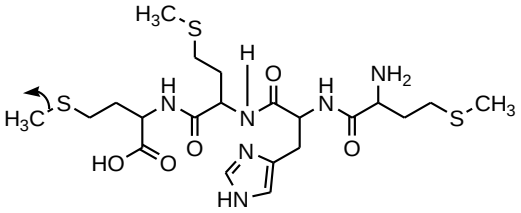


73 g/mol

$-H_2$

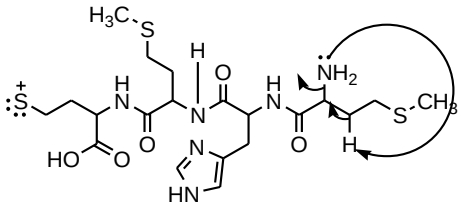


m/z 71 g/mol



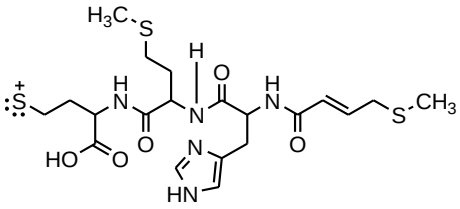
m/z 548 g/mol

$-CH_3$



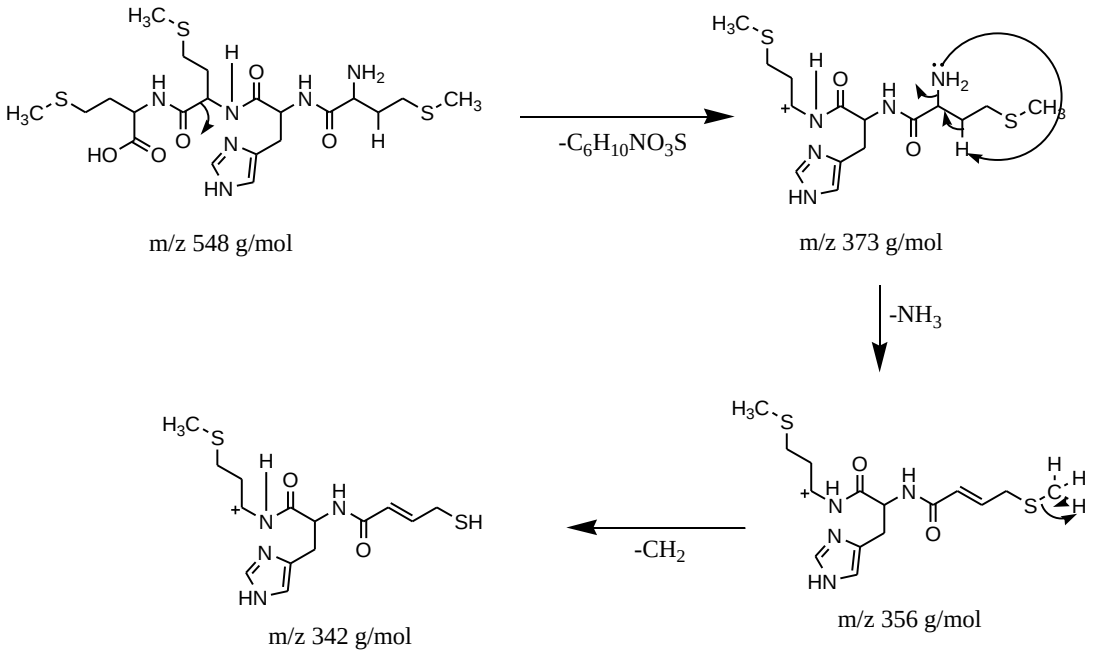
m/z 533 g/mol

$-NH_3$



m/z 516 g/mol

Continuing scheme



Fragments: 60, 71, 74, 341, 401, 517 and 548.2 g/mol

Scheme 12: Fragmentation pathway for Met-His-Met-Met (compound 9).

Met-His-Met-Met is a tetrapeptide comprising of methionine and histidine amino acids. These amino acids were determined in honey (Szeles *et al*., 2008; Biluca *et al*., 2019). Being a peptide, this possess antioxidant activity (Hayes *et al*., 1977).

Compound 10 displayed a molecular ion at m/z 551.2 at the retention time in 46.83min with fragments at m/z 532, 511, 397, 357 and 60. This corresponded to Genipin 1-beta-gentiobioside (fig. 5.10).

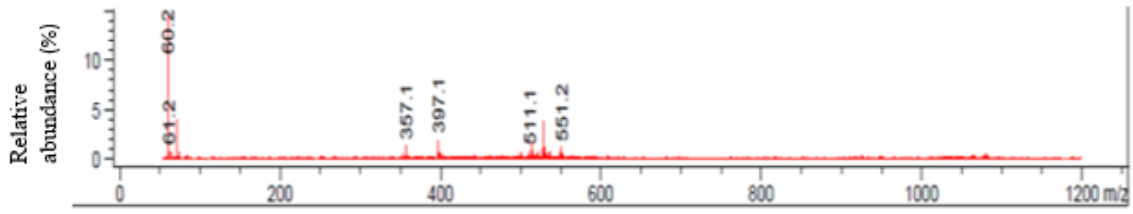
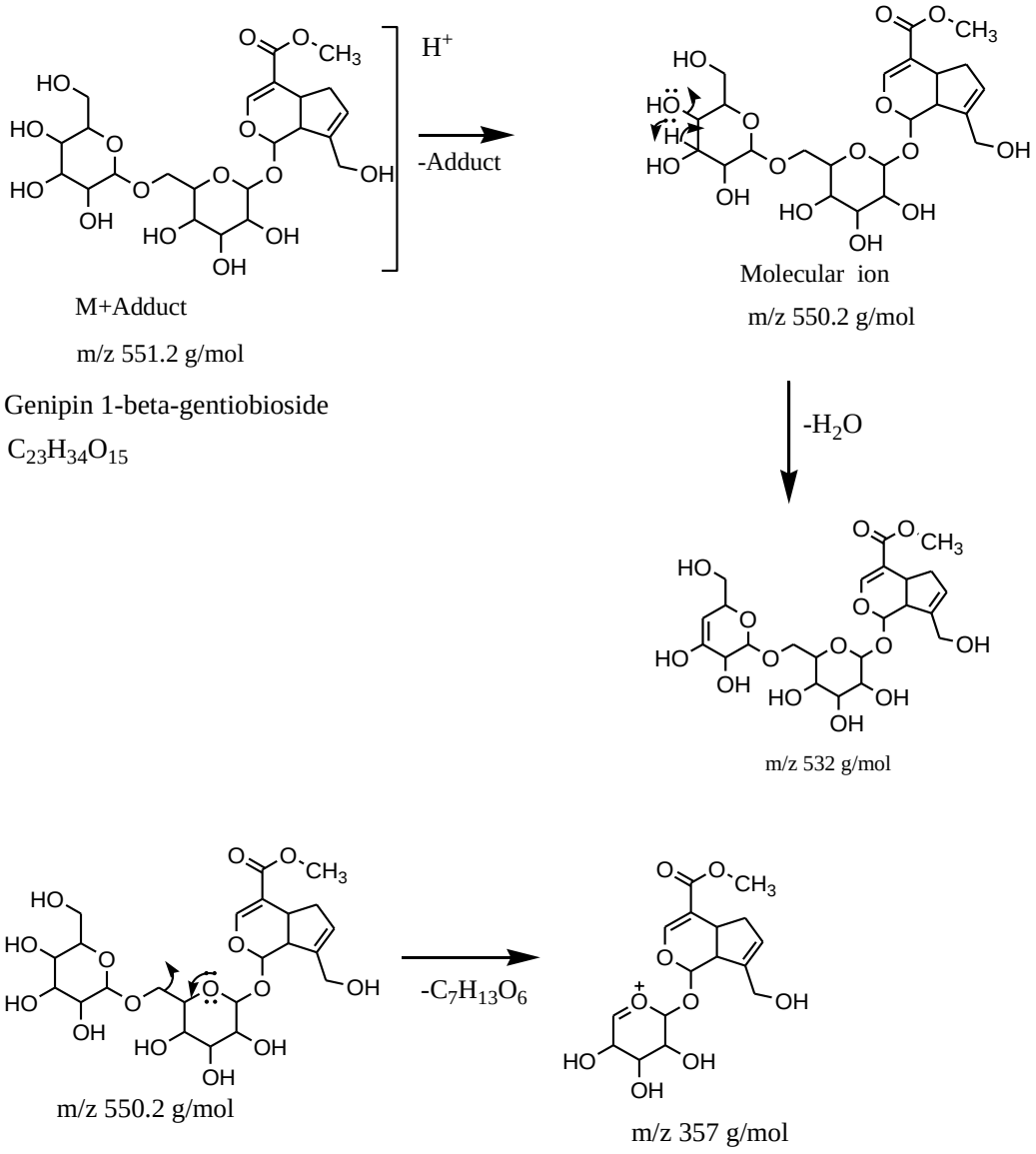
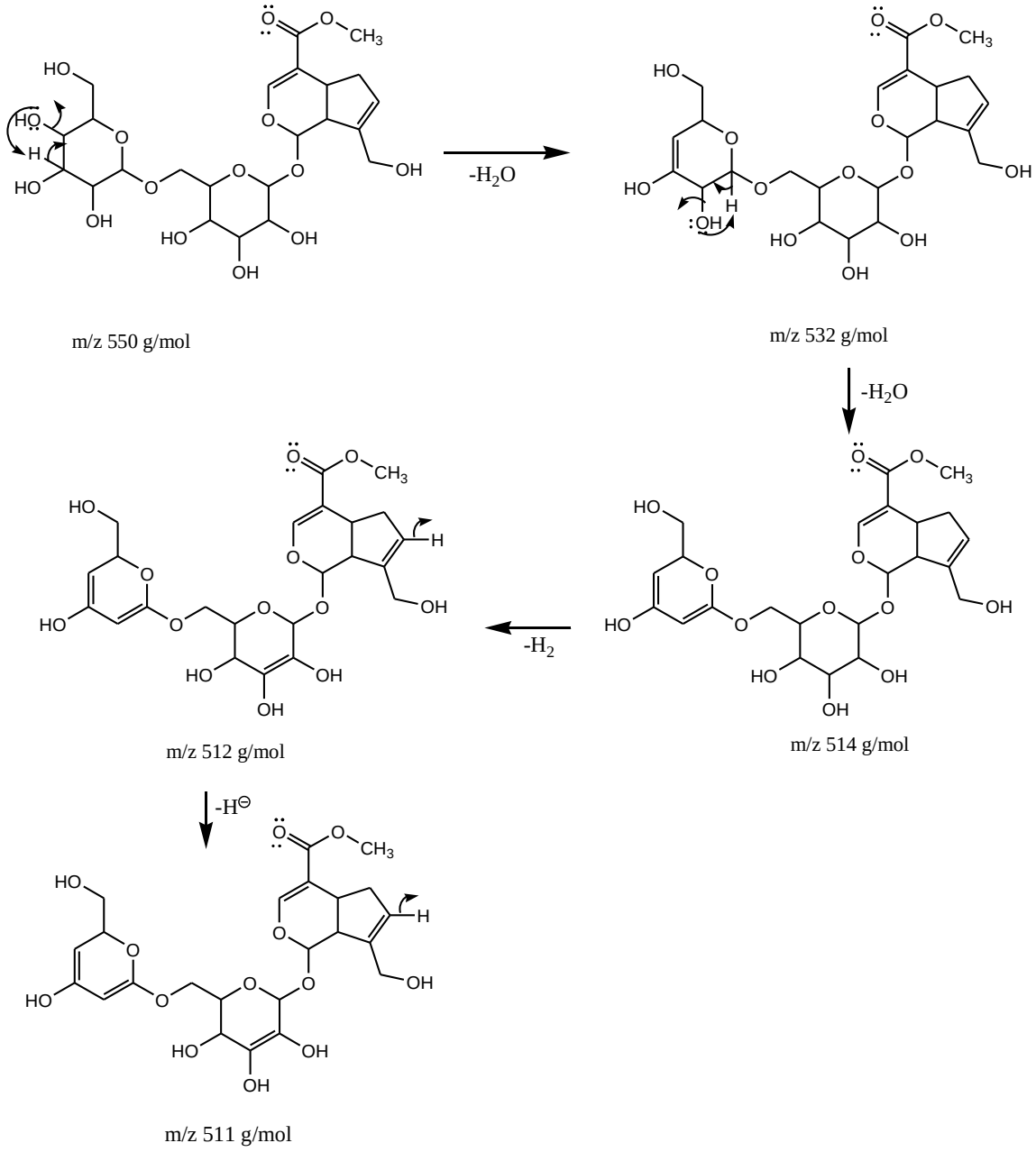


Fig. 5.10: The mass spectrum of compound 10

The fragment at m/z 532 was due to loss of water molecule while the one at m/z 511 resulted from loss of two H_2O molecules, H_2 and H^-. The fragment at m/z 397 was attributed by loss of CO_2, CH_2OH, four H_2O molecules and three H_2. Similarly, m/z 357 and m/z 60 were as a result of the losses of $C_7H_{13}O_6$ and $C_{21}H_{30}O_{13}$ respectively (scheme 13; pages 111-114).



Continuing scheme



m/z 550 g/mol

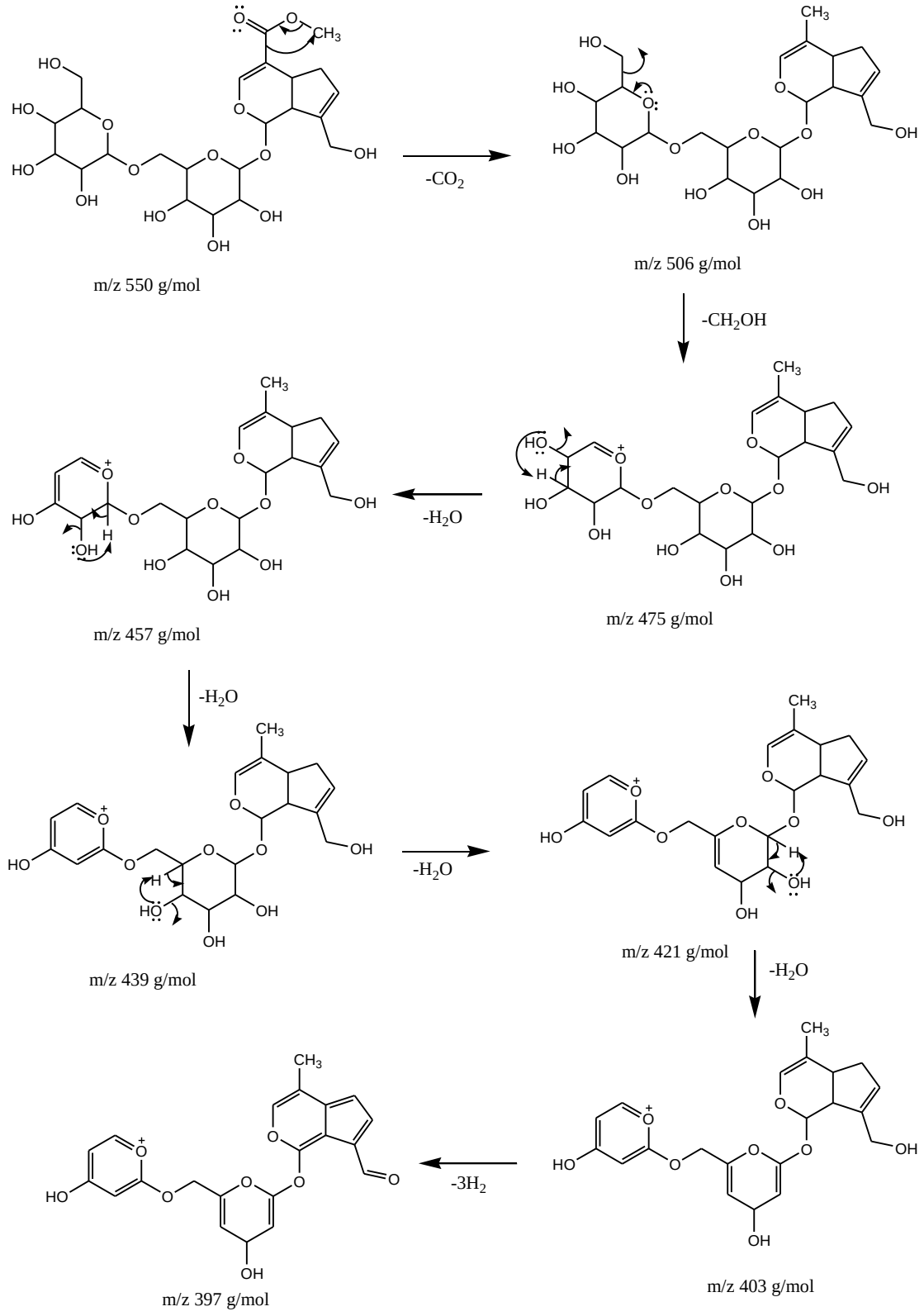
m/z 532 g/mol

m/z 514 g/mol

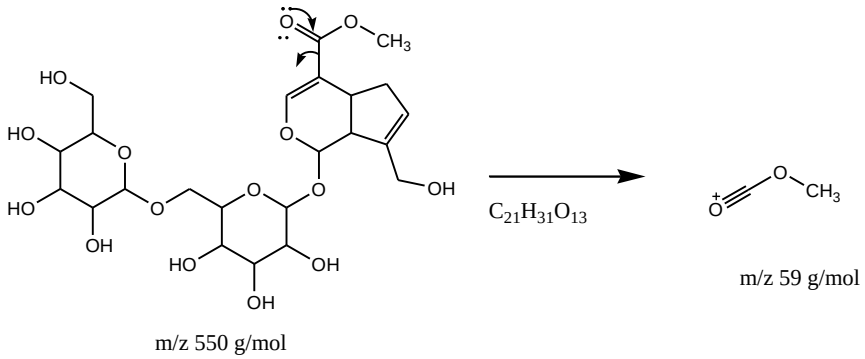
m/z 512 g/mol

m/z 511 g/mol

Continuing scheme



Continuing scheme



Fragments: 60, 357, 397, 511, 532 and 551.2 g/mol

Scheme 13: Fragmentation pathway for Genipin 1-beta-gentiobioside (compound 10)

Genipin 1-beta-gentiobioside is an iridoid glycoside. This compound was extracted from plasma samples by protein precipitation (Qu *et al*., 2013). Genipin-1-β-D-gentiobioside was also isolated from the fruits of *Gardenia jasminoides* ELLIS f. grandiflora (LOUR.) according to Endo & Taguchi (1973) and characterized according to Liu *et al.* (2006). Other iridoid glycosides were isolated from roots of *stachys geobombycis* (Zhou *et al*., 2019). Iridoids have been proven to have antitumor, hepatoprotective, anti-inflammatory, neuroprotective, hypolipidemic and hypoglycemic activities (Wang *et al*., 2020; Zhou *et al*., 2019).

Compound 11 displayed characteristic molecular ion at m/z 565.3 at the retention time in 36.01 min and gave characteristic fragment ions at m/z 520, 476, 364, 251, 85, 72, 60 and 56. This corresponded to Lys-Met-Met-Arg (fig. 5.11).

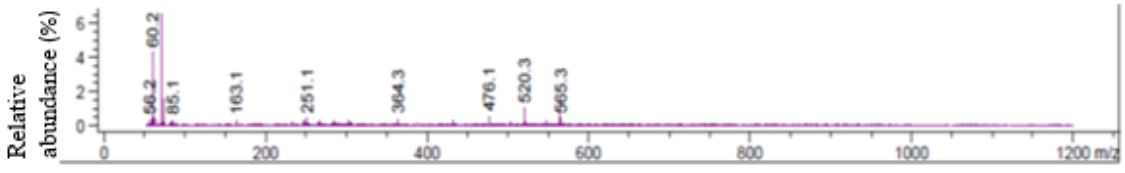
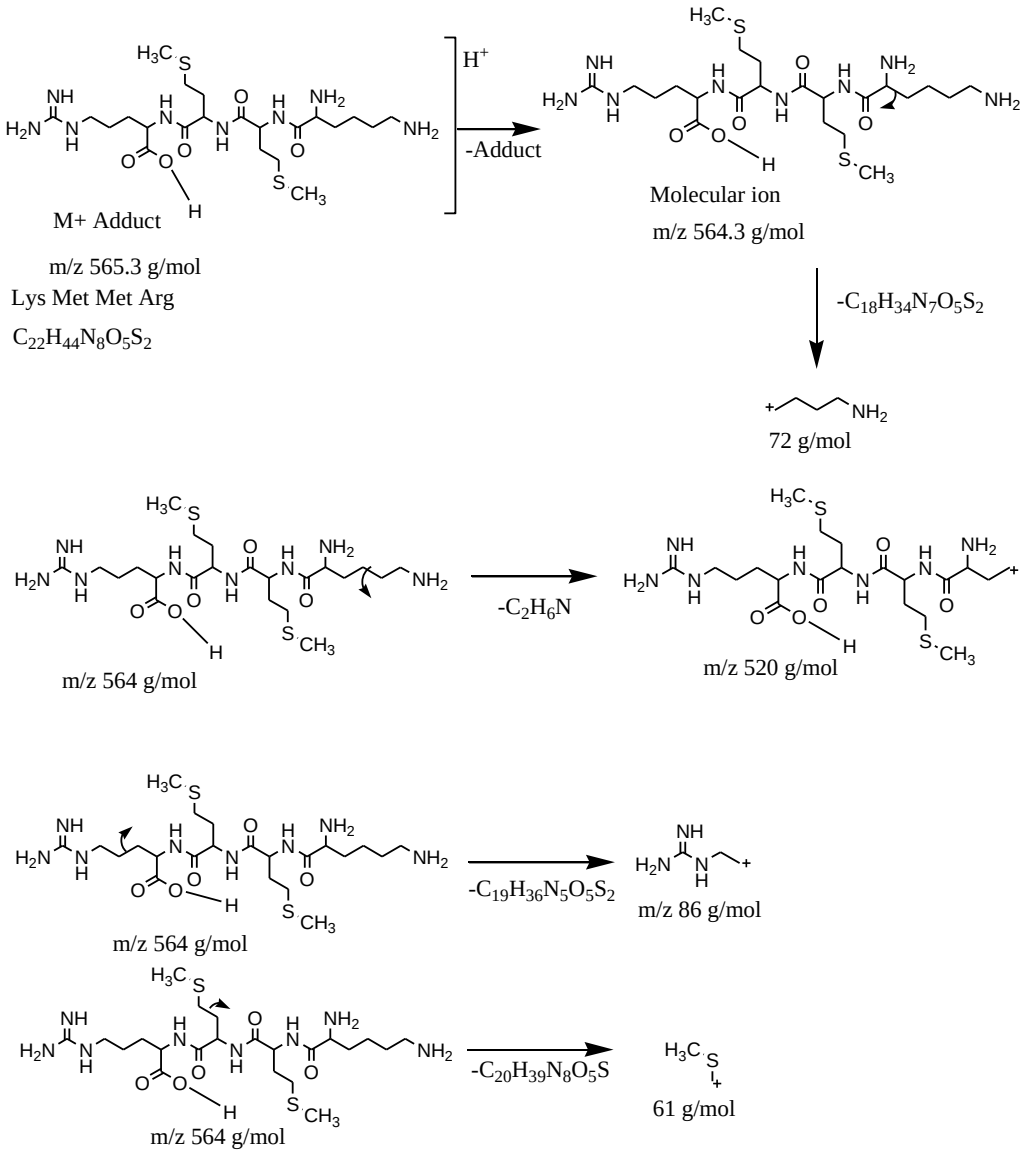
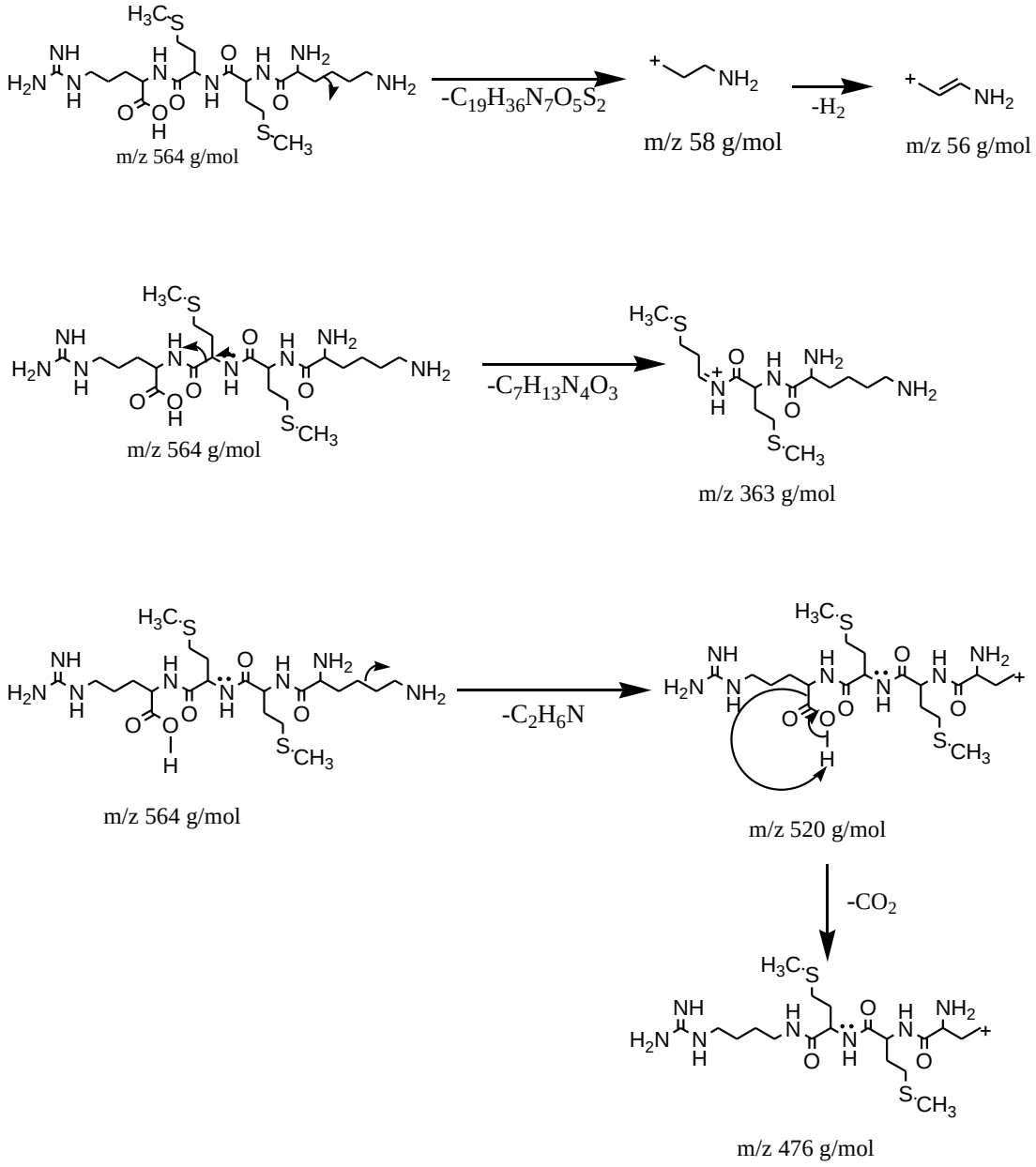


Fig. 5.11: The mass spectrum of compound 11

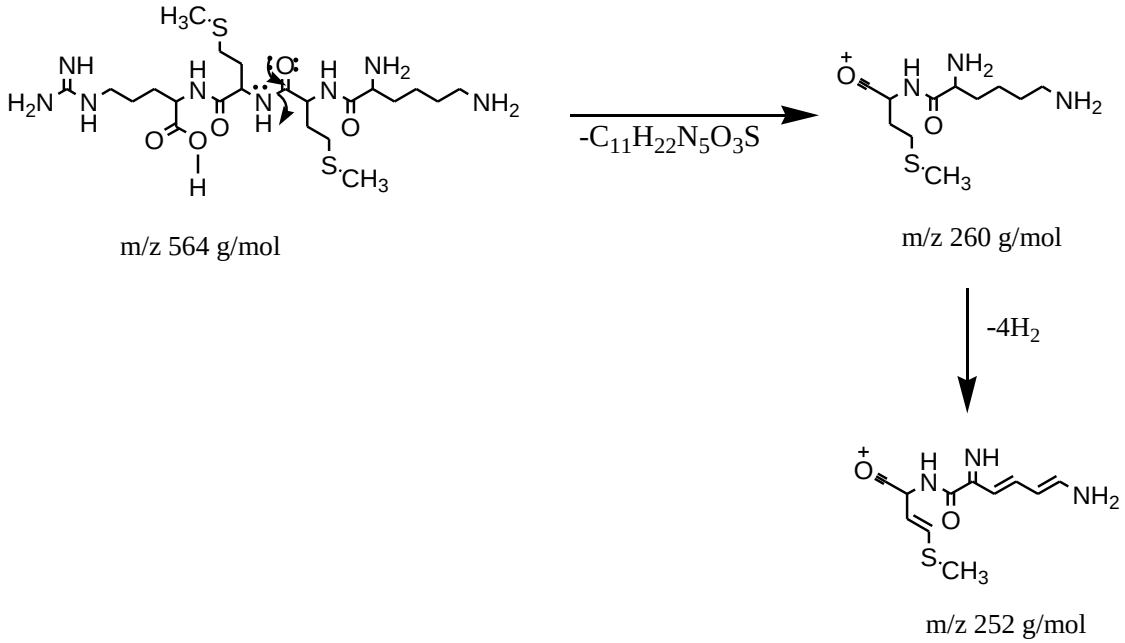
The fragment ion at m/z 520 was as a result of loss of C_2H_6N which on further loss of CO_2 led to the fragment m/z 476. M/z 364 fragment resulted from $C_7H_{12}N_4O_3$ loss while m/z 251 was due to loss of $C_{11}H_{22}N_5O_3S$, four H_2 and H. Similarly, loss of $C_{19}H_{37}N_5O_5S_2$ and H while m/z 72 resulted from loss of $C_{18}H_{34}N_7O_5S_2$. Finally, m/z 60 resulted from loss of $C_{20}H_{39}N_8O_5S$ and H while m/z 56 was due to loss of $C_{19}H_{36}N_7O_5S_2$ and H_2 (scheme 14; pages 115-117).



Continuing scheme



Continuing scheme



Fragments: 56, 60, 72, 85, 251, 364, 476, 520 and 565.3 g/mol

Scheme 14: Fragmentation pathway for Lys-Met-Met-Arg (compound 11).

Lys-Met-Met-Arg is a tetrapeptide comprising of lysine, methionine and arginine amino acids. These were characterized in forest honeys in South Sulawesi, Indonesia (Noor *et al*., 2019). They were also obtained in Slovak and Polish honeys from Slovakia and Poland respectively (Kowalski *et al*., 2017). They were also quantified from honey according to Kıvrak (2017). This compound possess antioxidant activity (Hayes *et al*., 1977)

Compound 12 was detected at the retention time in 37.20min with molecular ion at m/z 613.3 and gave characteristic fragment ions at 608, 565, 564, 521, 503, 477, 432, 319, 251, 72 and 56. This corresponded to Asp-His-Leu-Thr-Gln (fig. 5.12)

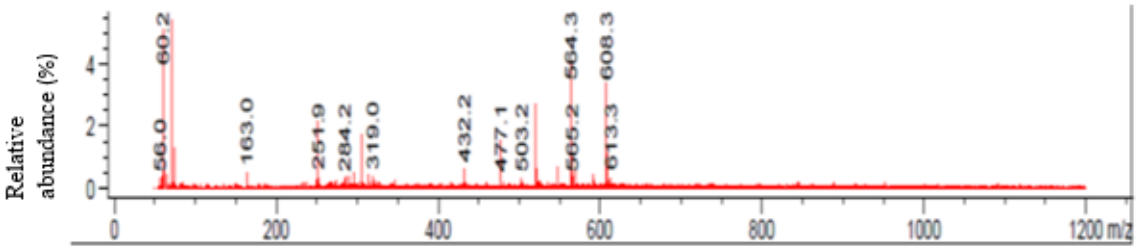
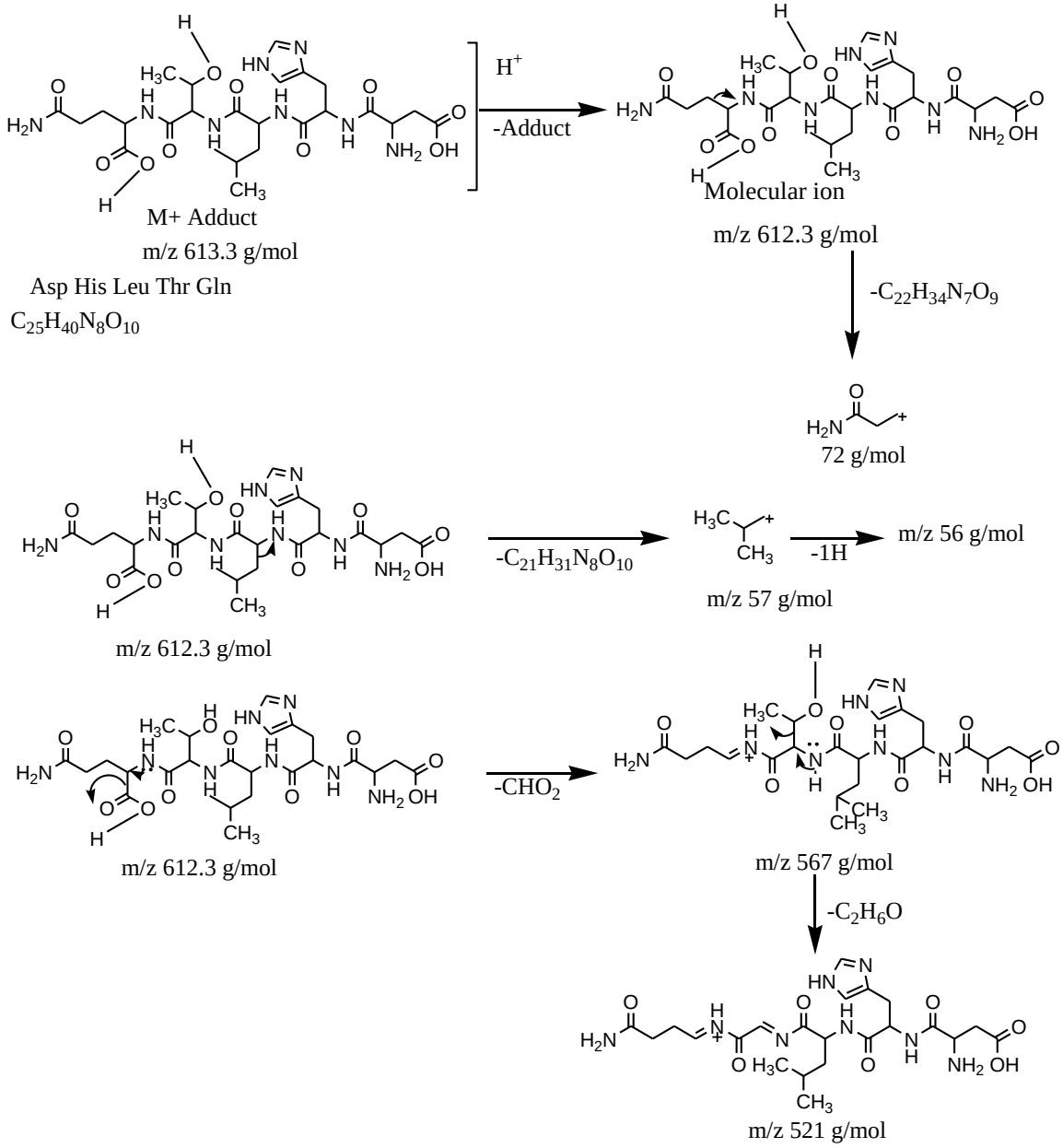
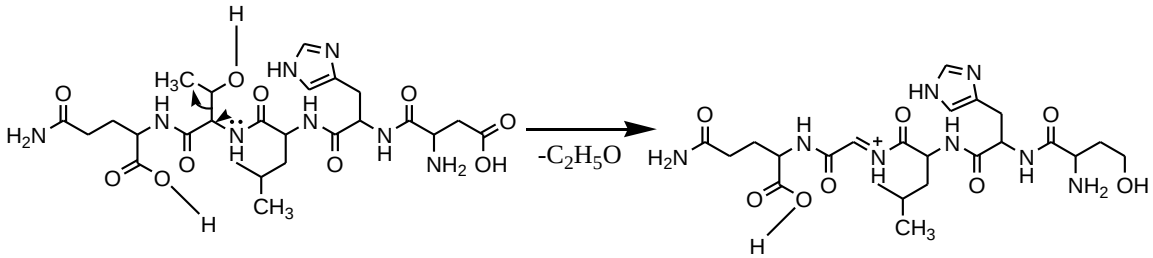


Fig. 5.12: The mass spectrum of compound 12

The fragment m/z 608 was as a result of two H_2 loss while that of m/z 565 resulted from loss of C_2H_5O, H_2 and H. Likewise, m/z 564 was attributed to loss of C_2H_5O, H_2 ad H while m/z 521 was as a result of loss of CHO_2 and C_2H_6O. The fragment 503 was due to $C_3H_6NO_2$, H_2O, H_2 and H loss while m/z 477 was as result of loss of $C_4H_7N_2O_3$ and two H_2 molecules. The fragment m/z 432 resulted from loss of $C_4H_7N_2O_3$, CO_2 and two H_2 molecules while m/z 319 resulted from $C_{10}H_{16}N_3O_6$, NH_3 and H_2. The fragment m/z 251 was generated due to loss of $C_{15}H_{27}N_4O_6$ and H_2 while m/z 72 was produced due to loss of $C_{22}H_{34}N_7O_9$. Finally, m/z 56 was generated due to loss of $C_{21}H_{31}N_8O_{10}$ and H (scheme 15 on pages 119-123).



Continuing scheme



m/z 612 g/mol

$-C_2H_5O$

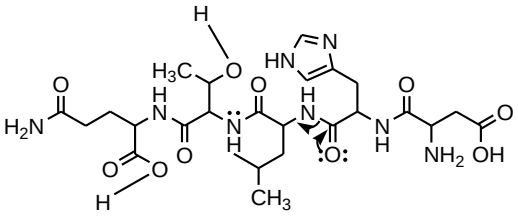
m/z 567 g/mol

$-H_2$

m/z 565 g/mol

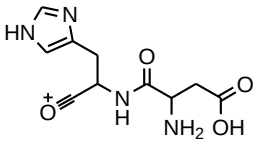
$-1H$

m/z 564 g/mol



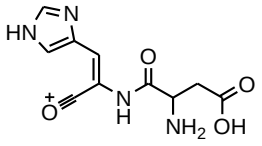
m/z 612 g/mol

$-C_{15}H_{27}N_4O_6$



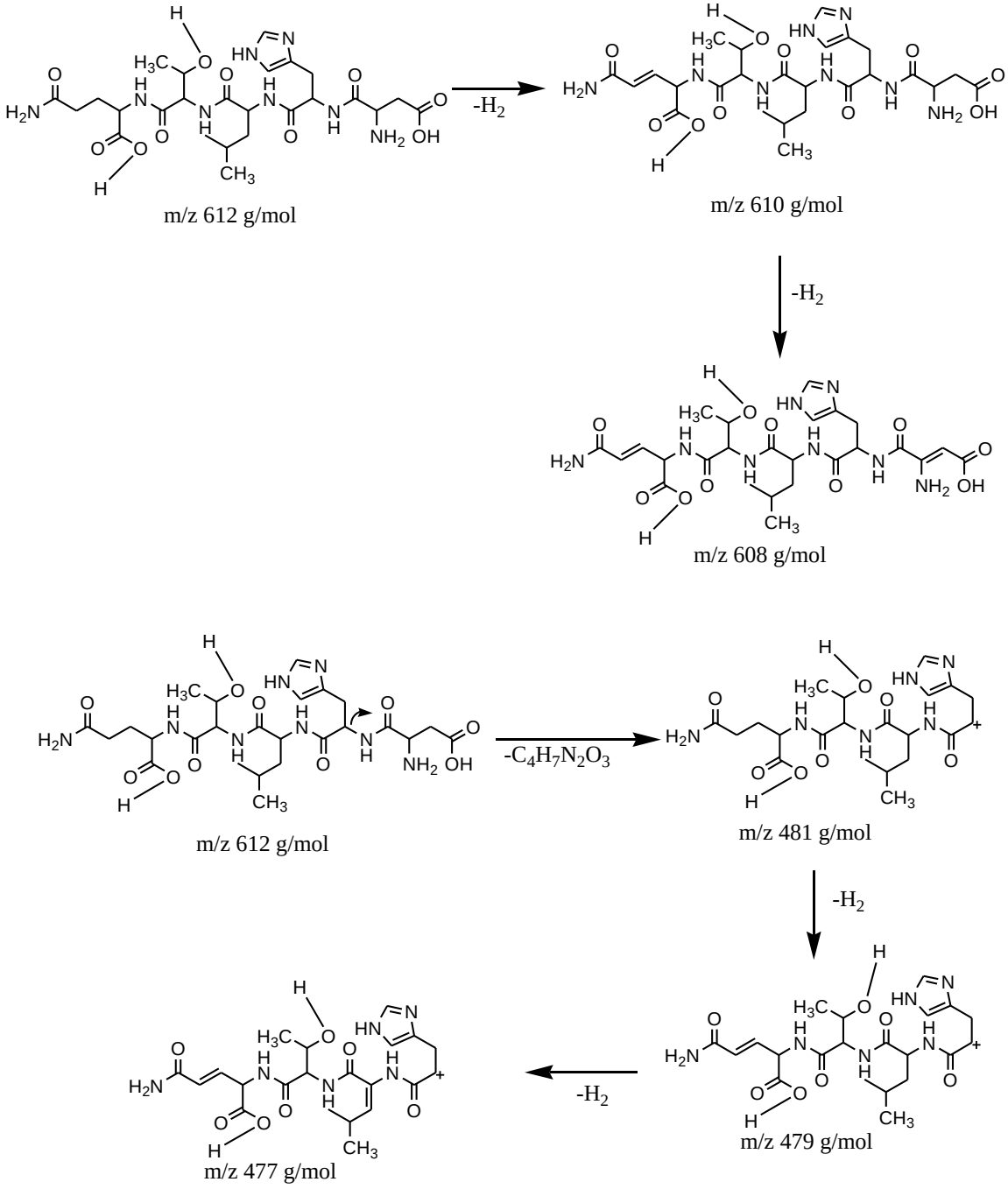
m/z 253 g/mol

$-H_2$

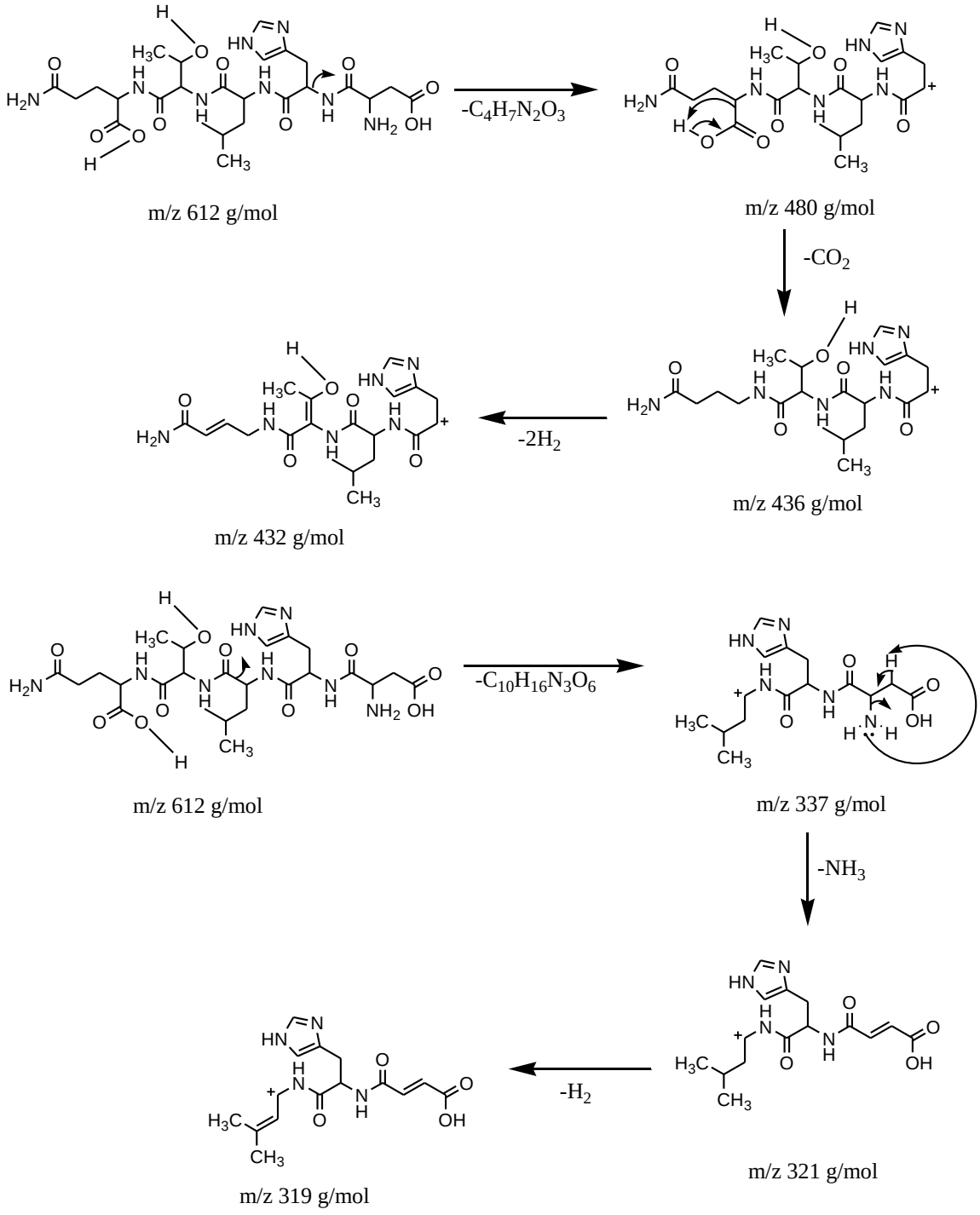


m/z 251 g/mol

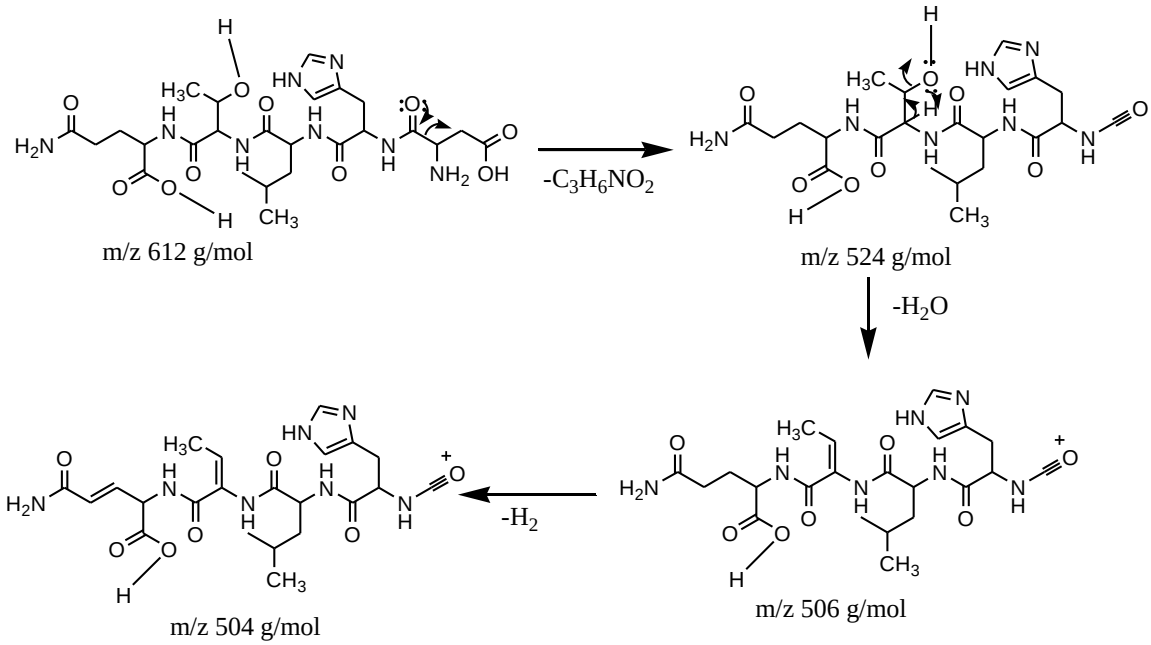
Continuing scheme



Continuing scheme



Continuing scheme



Fragments: 56, 72, 251, 319, 432, 477, 503, 521, 564, 565, 608 and 613 g/mol

Scheme 15: Fragmentation pathway for Asp-His-Leu-Thr-Gln (compound 12)

Asp-His-Leu-Thr-Gln is a pentapeptide consisting of aspartic acid, histidine, leucine, threonine and glutamine amino acids. All of them were identified from honey (Kıvrak, 2017). They were also characterised in Slovak and Polish honeys from Slovakia and Poland respectively (Kowalski *et al*., 2017). Aspartic acid, histidine, leucine and threonine were characterized in forest honeys in South Sulawesi, Indonesia (Noor *et al*., 2019). Being a peptide, it has antioxidant activity (Hayes *et al*., 1977).

Compound 13 was detected at the retention time in 50.47 min with m/z of 557.3 and gave characteristic fragment ions at 71 and 60. This corresponded to Tyr-Tyr-Val-Leu (fig. 5.13) on the next page; page 124)

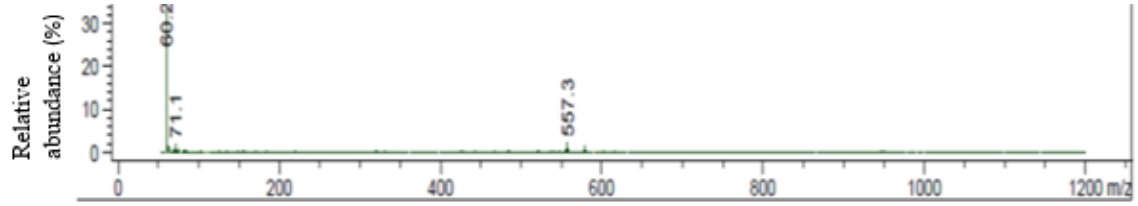
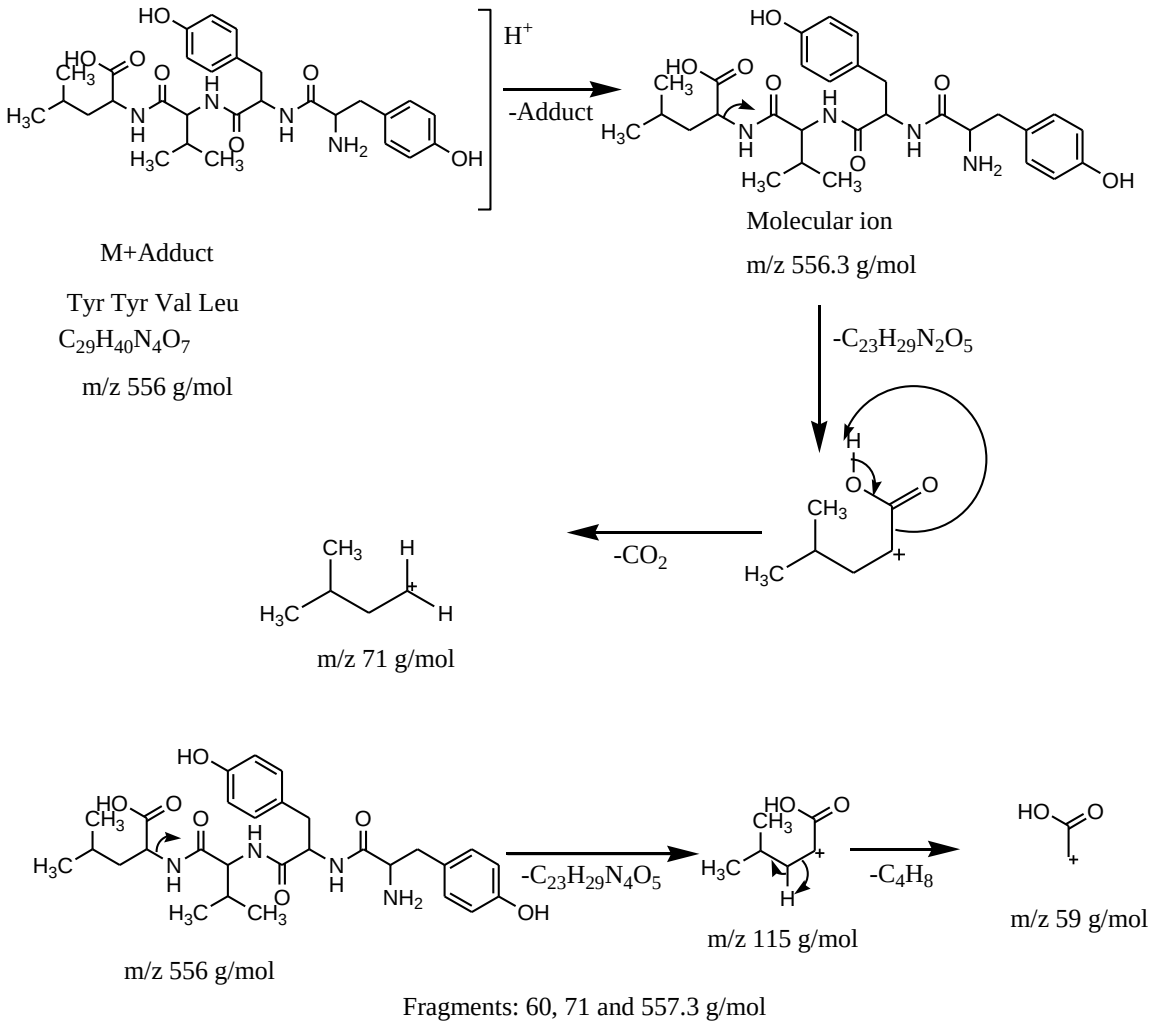


Fig. 5.13: The mass spectrum for compound 13

The m/z 71 fragment was due the loss of $C_{23}H_{29}N_2O_5$ and CO_2. On the other hand, m/z 60 resulted from loss of $C_{23}H_{29}N_4O_5$ and C_4H_7 (scheme 16).



Scheme 16: Fragmentation pathway for Tyr-Tyr-Val-Leu (compound 13)

Tyr-Tyr-Val-Leu is a tetrapeptide. It consists of tyrosine, valine and leucine amino acids. They were characterized in forest honeys in South Sulawesi, Indonesia (Noor *et al*., 2019). In addition, they were also identified from honey (Kıvrak, 2017). Valine and leucine were characterised in Slovak and Polish honeys from Slovakia and Poland respectively (Kowalski *et al*., 2017). Both peptides and amino acids possess antioxidant activity hence this compound has antioxidative properties (Hayes *et al*., 1977).

Compound 14 was displayed at a retention time of 52.43 min with a molecular ion of 294.1 and produced fragment at m/z 60. This corresponded to Tuliposide B (fig. 5.14).

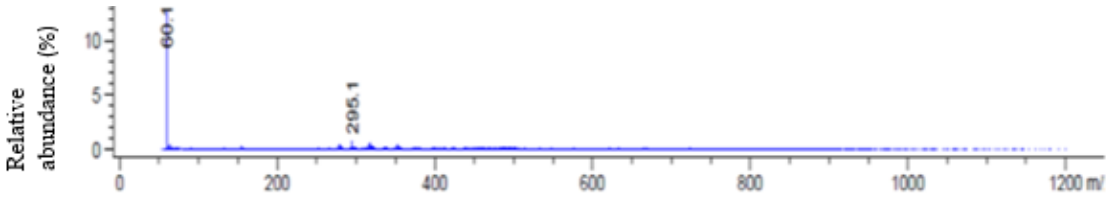
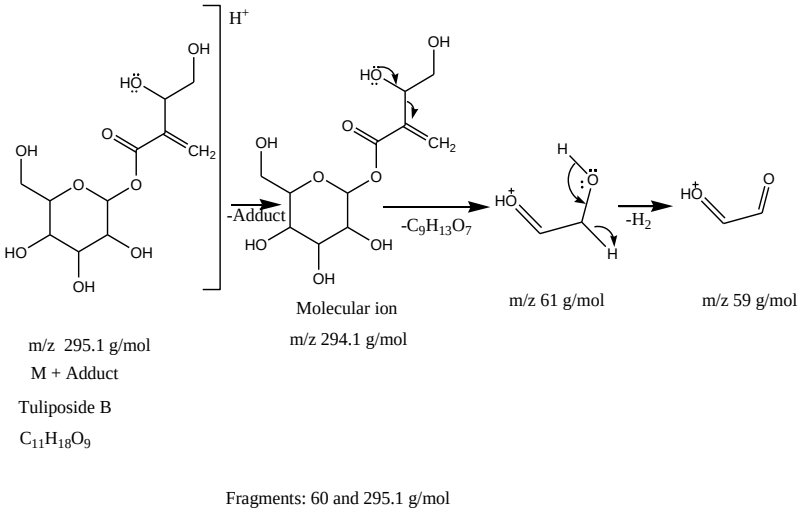


Fig. 5.14: The mass spectrum of compound 14

This fragment was produced due to loss of $C_9H_{14}O_7$ (scheme 17).



Scheme 17: Fragmentation pathway for Tuliposide B (compound 14)

Tuliposide B is a fatty acid derivative and an O-acyl-carbohydrate. This compound was isolated and quantified in *tulips* species (*tulipa*) (Christensen & Kristiansen, 1999). 6-Tuliposide B was isolated from tulip gums (Lubbe *et al*., 2012) and from leaves/stems and flowers of *Tulipa turkestanica* (Christensen, 1999). Apart from being an antioxidant, tuliposide B possess antibacterial activity (Shigetomi *et al*., 2010) and antifungal activity (Shigetomi *et al*., 2011).

Compound 15 displayed a molecular ion at m/z 322.1 with a retention time of 52.93min with fragment ions at m/z 297 and 60. This corresponded to Gly-Gly-Ser-Cys (fig. 5.15).

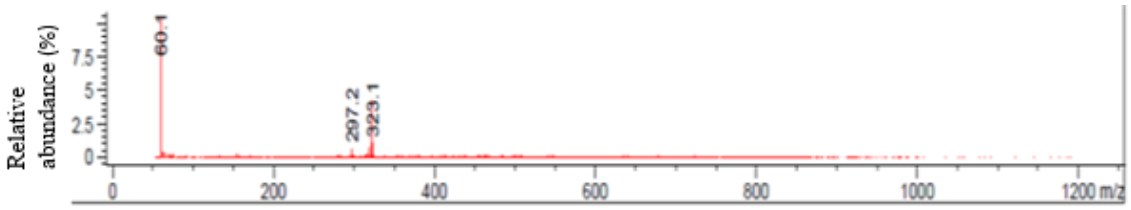
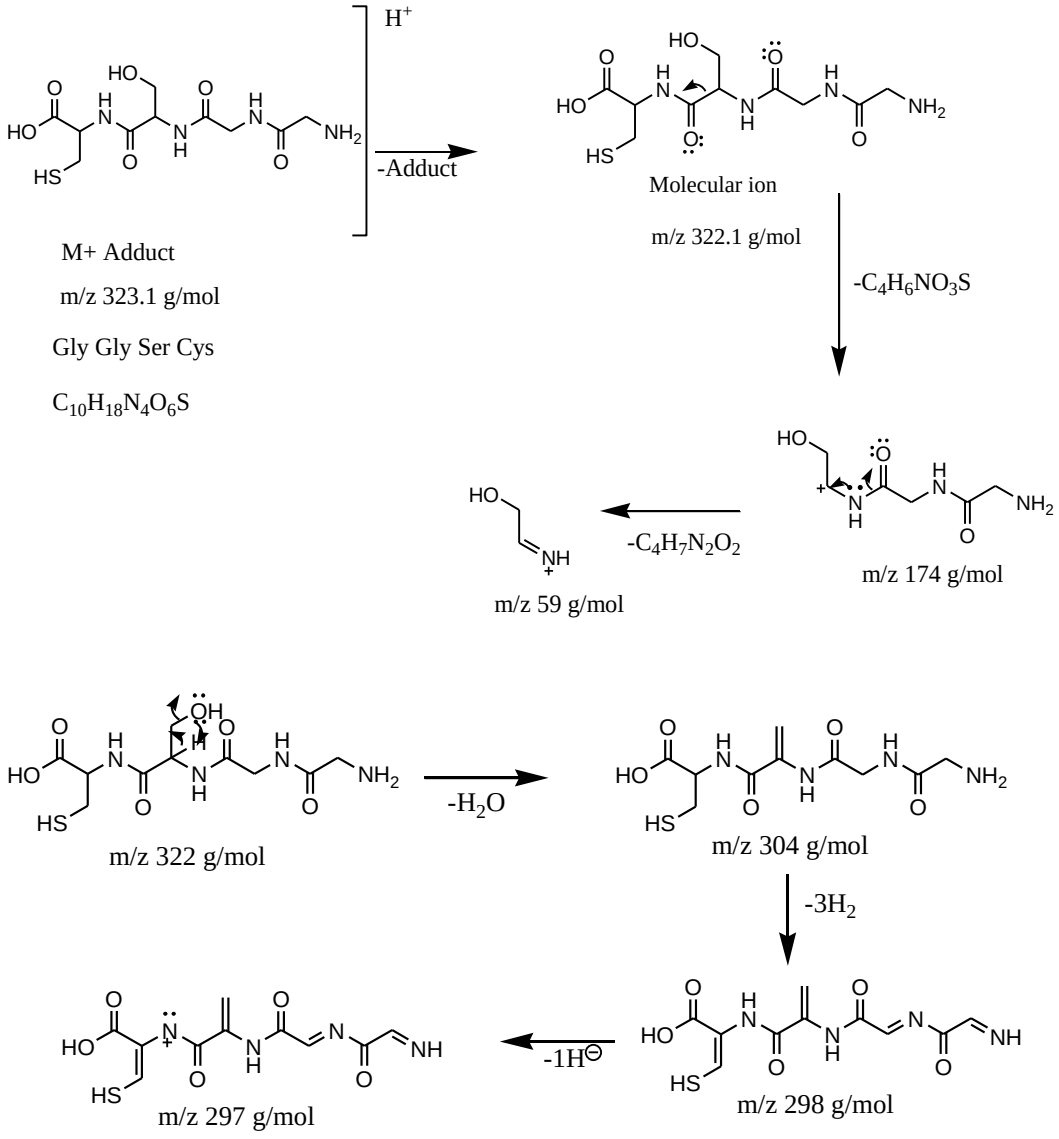


Fig. 5.15: The mass spectrum of compound 15

The fragment ion at m/z 297 was generated as a result of loss of H_2O, three $3H_2$ and H^- while fragment ion at m/z 60 was produced due to loss of $C_4H_6NO_3S$ and $C_4H_6N_2O_2$ (scheme 18; page 127).



Scheme 18: Fragmentation pathway for Gly-Gly-Ser-Cys (compound 15).

Gly-Gly-Ser-Cys is a tetrapeptide with glycine, serine and cysteine amino acids. Glycine and serine were identified in Slovak and Polish honeys (Kowalski *et al*., 2017; Noor *et al*., 2019). All of them were characterized in honey as described by (Kıvrak, 2017; Hermosín *et al*., 2003). Being a peptide, this compound has antioxidant potential (Hayes *et al*., 1977).

Compound 16 was detected at the retention time 7.60 min with a molecular ion at m/z 352.1 and fragment ions at m/z 85 and m/z 71. This corresponded to Psoralidin oxide and its profile is shown below (fig. 5.16).

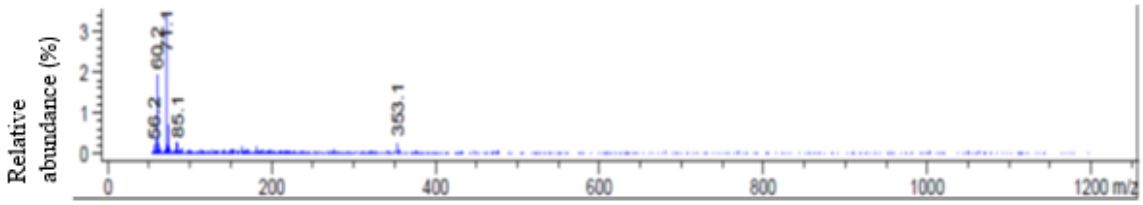
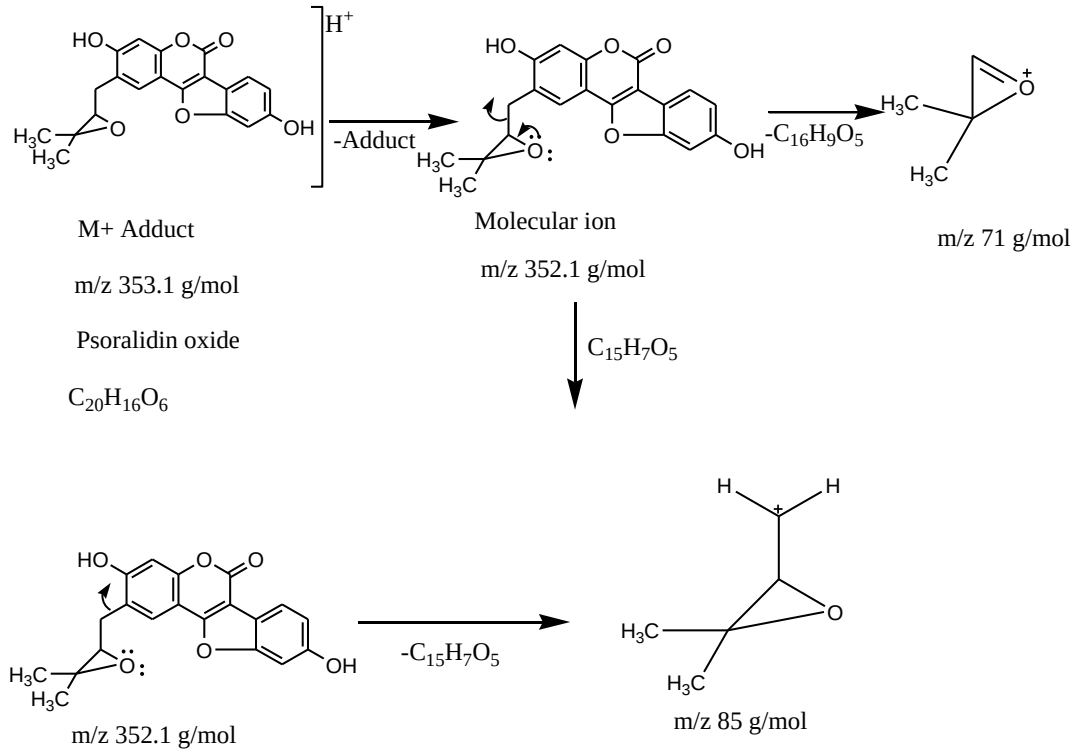


Fig. 5.16: The mass spectrum of compound 16

These fragments were generated as a result of loss of $C_{15}H_7O_5$ and $C_{16}H_9O_5$ respectively (scheme 19 below).



Scheme 19: Fragmentation pathway for Psoralidin oxide (compound 16)

Psoralidin oxide is a coumestan flavonoid. It has been isolated from *Psoralea corylifolia* (Gupta *et al*., 1980). Apart from antioxidant activity, Psoralidin oxide possess anticancer activity (Pahari & Rohr, 2009).

Compound 17 displayed a characteristic molecular ion of m/z 353.1 at a retention time of 48.32min with fragment m/z 261 (fig. 5.17).

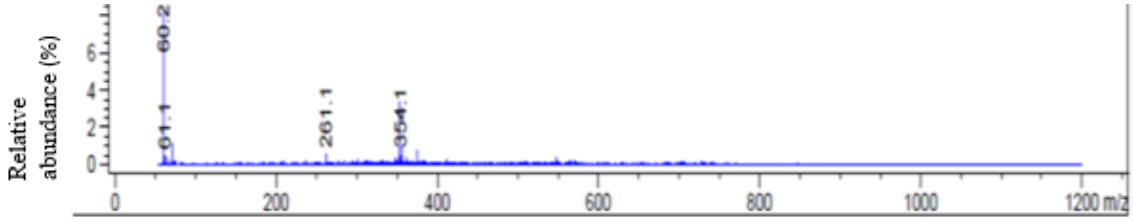
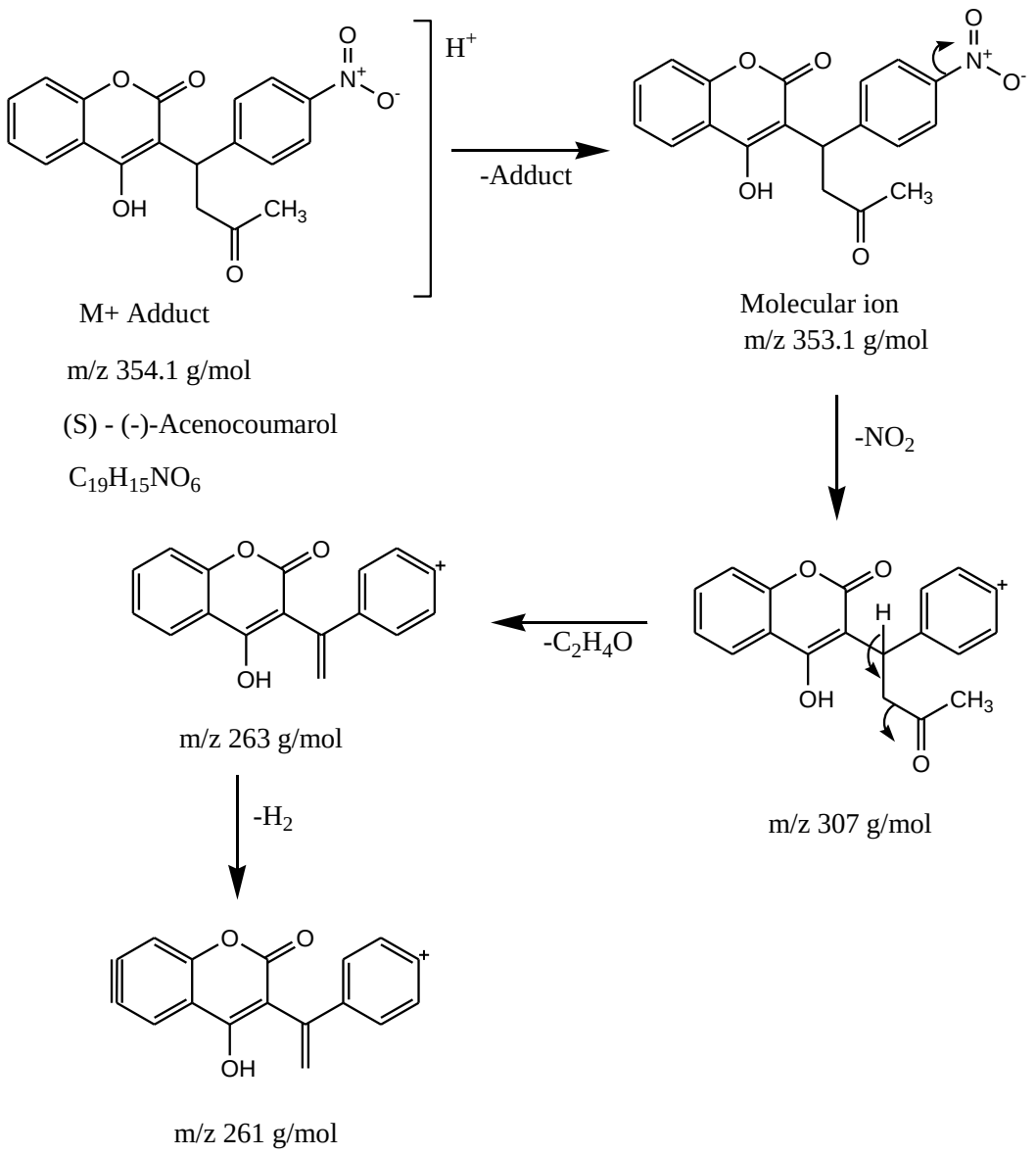


Fig. 5.17: The mass spectrum of compound 17.

The fragment resulted from the loss of NO_2, C_2H_4O and H_2 (scheme 20; page 130).



Scheme 20: Fragmentation pathway for (S) - (-)-Acenocoumarol (compound 17)

(S) - (-)-Acenocoumarol is a coumarin derivative, class of C6-C3 plant metabolites. 4-hydroxycoumarin enantiomers and 7-hydroxycoumarin derivative were isolated from the entire plant of *Ainsliaea fragrans* (Lei *et al*., 2015). Apart from antioxidant activity, (S) - (-)-Acenocoumarol possess anticoagulant properties (Trailokya *et al*., 2016).

Compound 18 was detected at a retention time of 1.55min with a characteristic molecular ion of m/z 354.1 and gave fragment ions of m/z 89 and 71. This corresponded to cryptochlorogenic acid. (fig. 5.18).

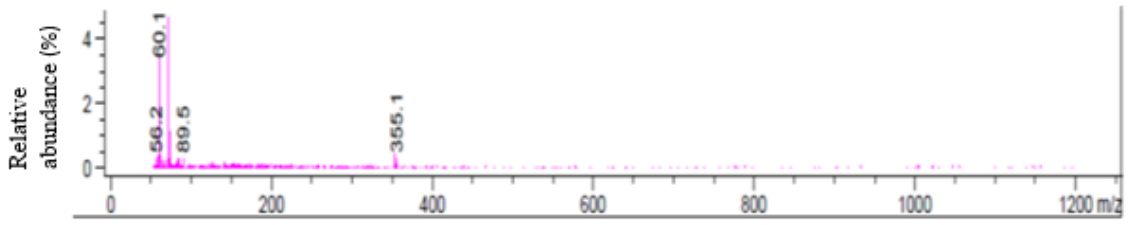
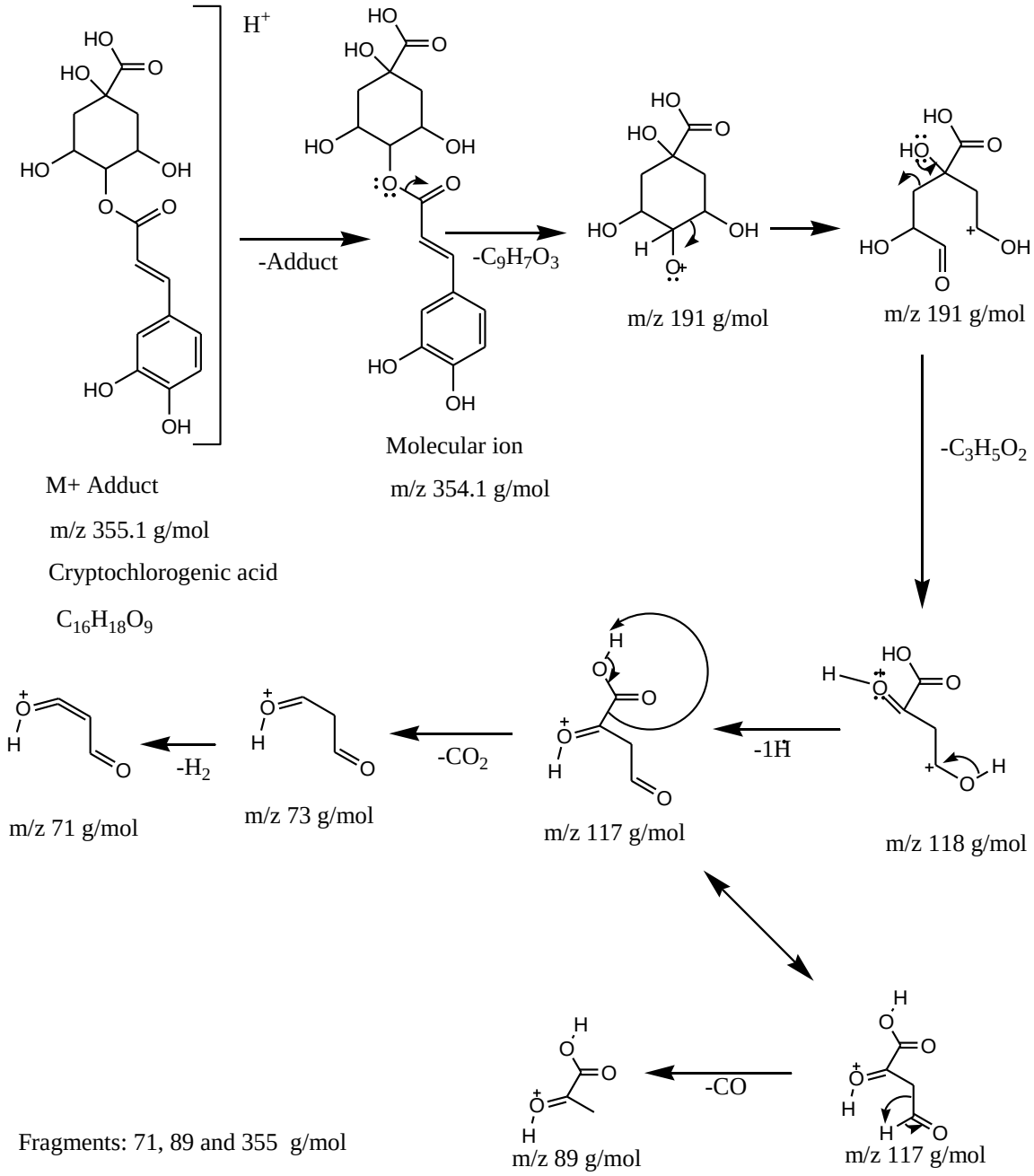


Fig. 5.18: The mass spectrum of compound 18

These fragments were due to loss of $C_9H_7O_3$, H, CO and $C_9H_7O_3$, $C_3H_5O_2$, H, CO_2, H_2 respectively (scheme 21 on the next page; page 132).



Scheme 21: Fragmentation pathway for cryptochlorogenic acid (compound 18)

Cryptochlorogenic acid, also known as 4-caffeoylquinic acid (4-CQA) or 4-O-caffeoylquinic acid, belongs to quinic acids and derivatives. It belongs to phenolic acids compounds, broadly classified as a polyphenol. It was first characterized in honey according to Shen *et al*. (2019). It was also isolated for the first time from the leaves of

Prunus domestica (Nakatani *et al*., 2000). In addition, it was also identified in the leaves of *Hibiscus sabdariffa* (Zhen *et al*., 2016). Caffeic acid derivatives including caffeoylquinic acids such as 4-caffeoylquinic acid (4-CQA), 5-caffeoylquinic acid (5-CQA) and 3-caffeoylquinic acid (3-CQA) have been isolated from leaves of *Morus alba* (medicinal plant) (Wang *et al*., 2019). Similar derivatives above together with 1,3-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid were isolated fom *Artemisia capillaris* (Tian *et al*., 2020). 4-O-caffeoylquinic acid was also quantified from leaves of sweet potato cultivars (Luo *et al*., 2013). Apart from antioxidant properties, cryptochlorogenic acid also possess antimicrobial and anticancer activities (Elansary *et al*., 2020; Naveed *et al*., 2018).

Compound 19 displayed a characteristic molecular ion at m/z 423.2 with a retention time of 39.57 min and produced fragment ions of m/z 229, 207, 71 and 57. This corresponded to 4-Heptylumbelliferyl-beta- glucoside (fig. 5.19).

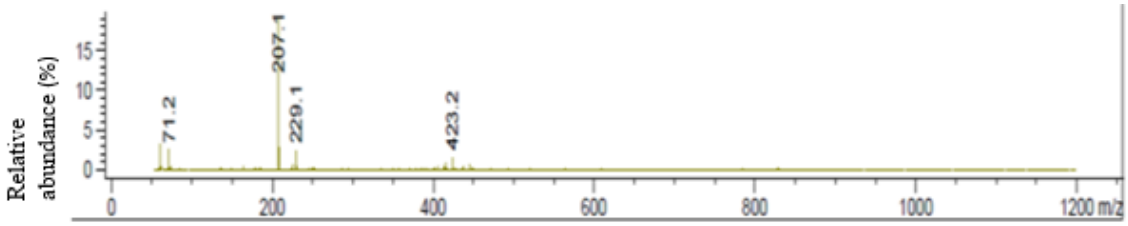
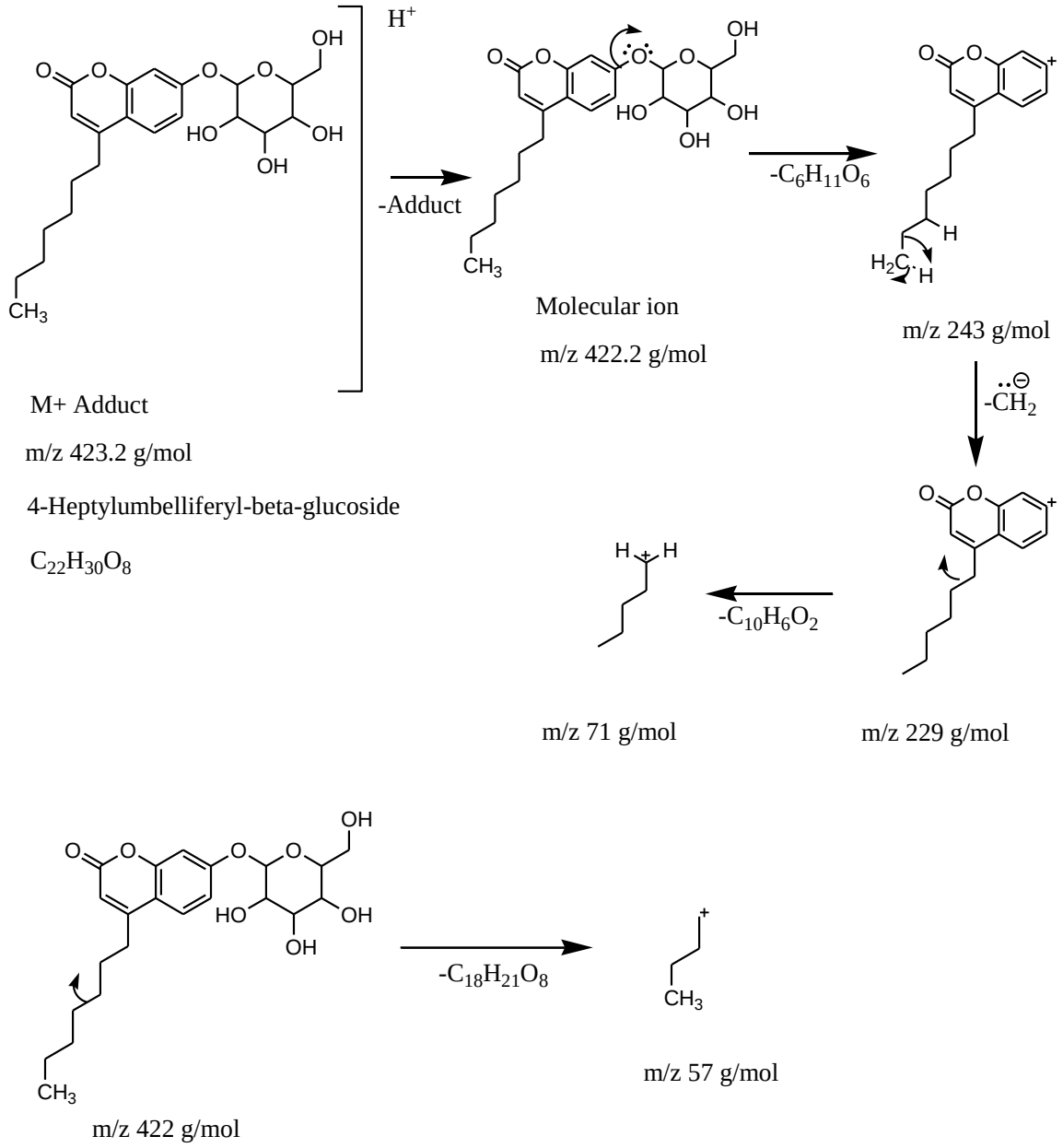
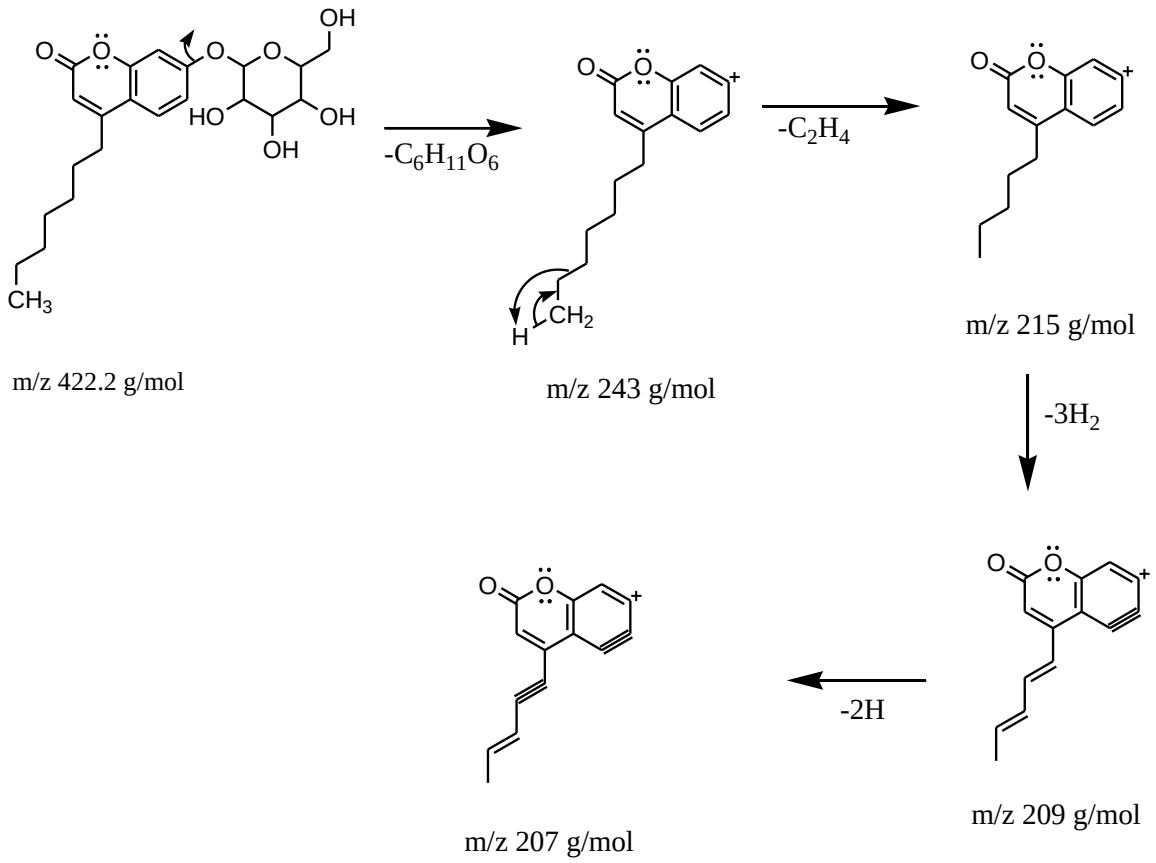


Fig. 5.19: The mass spectrum of compound 19

The fragment ion m/z 229 resulted from loss of $C_6H_{11}O_6$ and CH_2 while m/z 207 was due to loss of $C_6H_{11}O_6$, C_2H_4, $3H_2$ and 2H. Similarly, m/z 71 was attributed to loss of $C_6H_{11}O_6$, CH_2 and $C_{10}H_6O_2$ while m/z 57 was attributed to loss of $C_{18}H_{21}O_8$ (scheme 22 on pages 134-135 below).



Continuing scheme



Fragments: 57, 71, 207, 229 and 423.2 g/mol

Scheme 22: Fragmentation pathway for 4-Heptylumbelliferyl-beta- glucoside (compound 19).

4-Heptylumbelliferyl-beta- glucoside, a coumarin glucoside, is a beta-D-glucoside with a glycosidic bond linkage. Besides being a member of coumarins, it is also a monosaccharide derivative. This compound has been detected for the first time in honey. A related compound, Umbelliferone (7-hydroxyl coumarin) was isolated from *Diplostephium foliosissimum* medicinal plant (Morikawa *et al*., 2011). An extracellular β-glucosidase was isolated from *Issatchenkia terricola* (González-Pombo *et al*., 2011). 4-Heptylumbelliferyl-beta- glucoside has a wide range of pharmacological properties

properties such as antioxidant, anticancer, antitumor, anticoagulant, antibacterial, anti-HIV and anticoagulant due to its coumarin moiety (Xu *et al*., 2015).

Compound 20 was detected at a retention time of 18.17min with a molecular ion of m/z 519.3 and generated fragment ions of m/z 165, 85, 71 and 59. This corresponded to His Lys-Val-His (fig. 5.20 below).

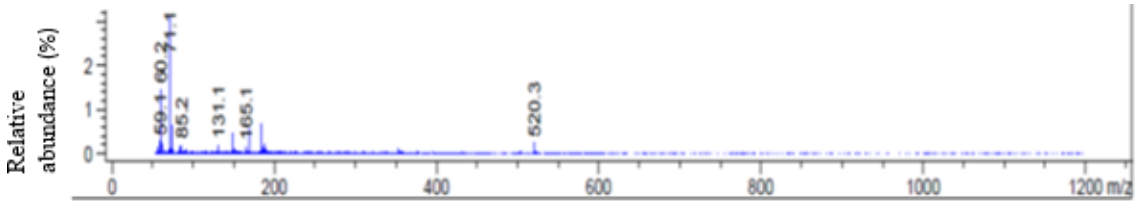
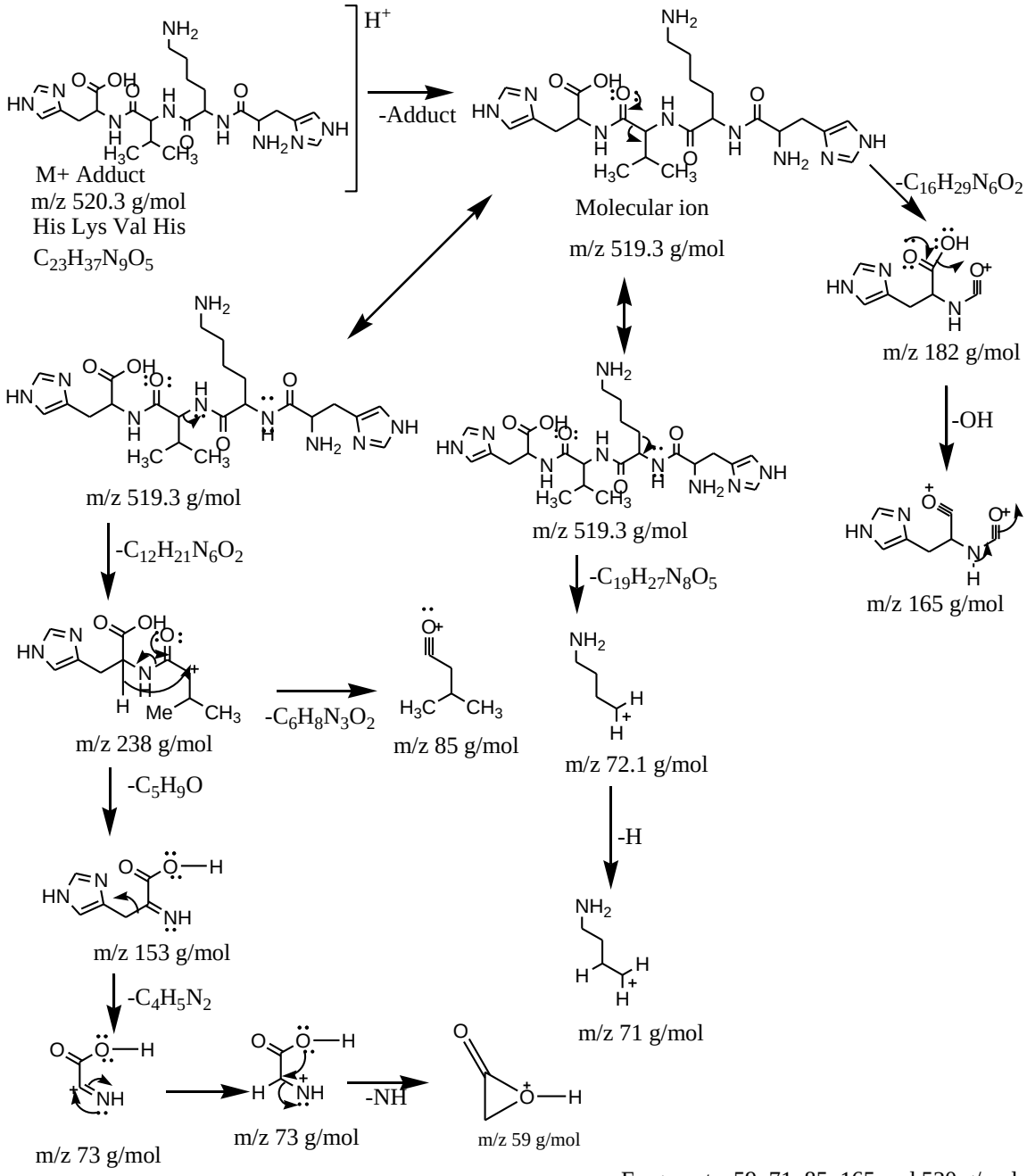


Fig. 5.20: The mass spectrum of compound 20

The fragment ion m/z 165 was related to the loss of $C_{16}H_{29}N_6O_2$ and OH while fragment ion 85 was related to the loss of $C_{12}H_{21}N_6O_2$ and $C_6H_8N_3O_2$. Likewise, fragment m/z 71 was due to loss of $C_{19}H_{27}N_8O_5$ and H. Finally, fragment m/z 59 resulted from the loss of $C_{12}H_{21}N_6O_2$, C_5H_9O, $C_4H_5N_2$ and NH (scheme 23 on the next page; page 137).



Scheme 23: Fragmentation pathway for His-Lys-Val-His (compound 20)

His-Lys-Val-His is a peptide consisting of histidine, lysine and valine amino acids. All these amino acids were identified in forest honeys from Soth Sulawesi in Indonesia (Noor *et al*., 2019). They were also characterized in honeys from Poland and Slovak (Kowalski

et al., 2017). Moreover, they were quantified in honeys as decribed by Kıvrak (2017). Peptides have antioxidative properties (Hayes *et al*., 1977).

Compound 21 displayed a characteristic molecular ion of 608.2 with a retention time of 41.60 min and generated fragment ions at m/z 306, 207, 163, 71 and 59. This corresponded to 4-(4-Hydroxyphenyl)-2-butanone O-[2-galloyl-6-cinnamoylglucoside] (fig. 5.21).

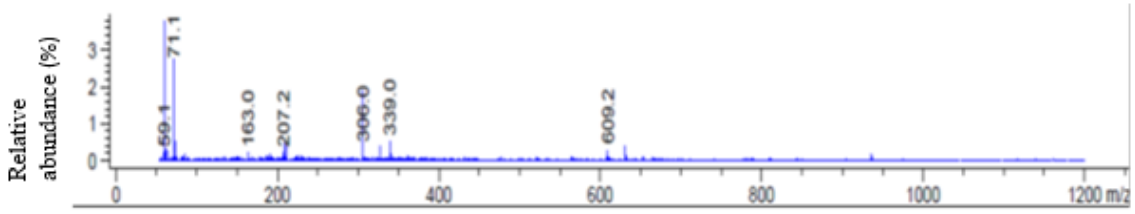
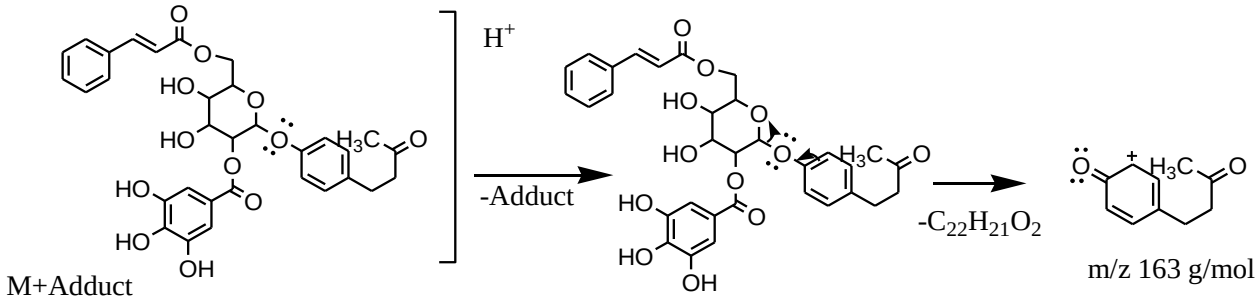


Fig. 5.21: The mass psectrum of compound 21

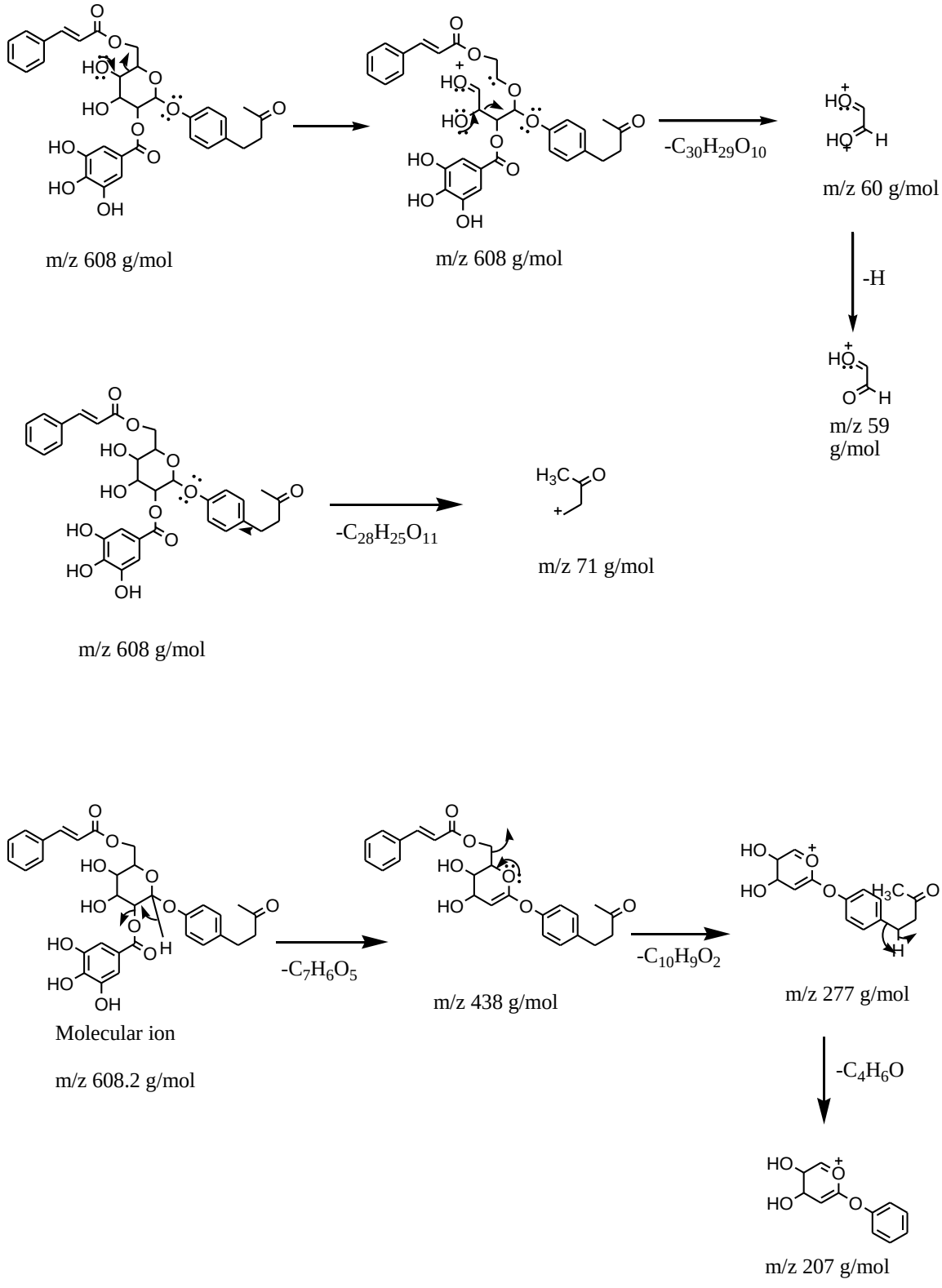
The fragment ion m/z 306 was due to loss of $C_7H_6O_5$ and C_9H_7O while fragment ion m/z 207 resulted from the loss of $C_7H_6O_5$, $C_{10}H_9O_2$, and C_4H_6O. Similarly, fragment ions m/z 163 and 71 were as a result of loss of $C_{22}H_{21}O_2$ and $C_{28}H_{25}O_{11}$ respectively. Finally, fragment 59 resulted from abstraction of $C_{30}H_{29}O_{10}$ an H (scheme 24 on pages 138-139).



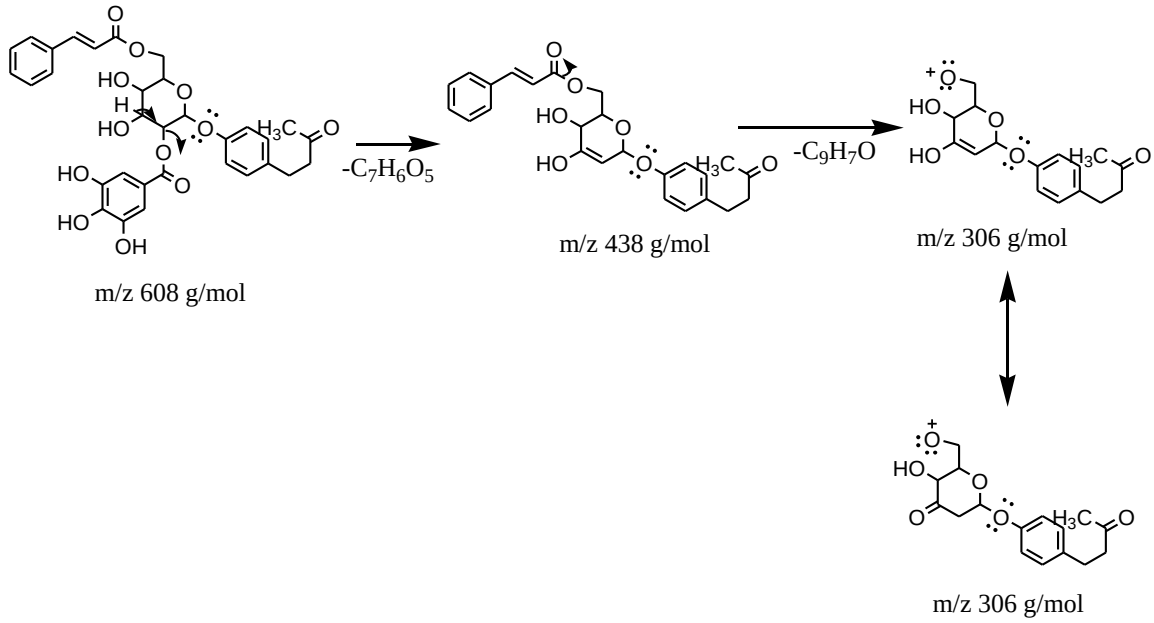
4-(4-Hydroxyphenyl)-2-butanone O-[2-galloyl-6-cinnamoylglucoside]

$C_{32}H_{32}O_{12}$

Continuing scheme



Continuing scheme



Fragments: 59, 71, 163, 207, 306 and 608 g/mol

Scheme 24: Fragmentation pathway for 4-(4-Hydroxyphenyl)-2-butanone O-[2-galloyl-6-cinnamoylglucoside] (compound 21)

4-(4-Hydroxyphenyl)-2-butanone O-[2-galloyl-6-cinnamoylglucoside] belongs to a class of phenolic glycosides. According to PubChem database, it has been isolated from commercial rhubarb, has been detected but not quantified in green vegetables. Phenolic glycosides have been isolated from *Clematis tashiroi* and were reported to possess antiiflammatory and antioxidant properties due to their phenolic moieties (Zhang *et al*., 2015).

Compound 22 was displayed at a characteristic molecular ion of m/z 784.3 with a retention time of 39.15 min and produced fragment ions at m/z 696, 423, 395, 393, 377, 362, 225, 163, 71 and 59. This corresponded to Antibiotic MA 144 U5 (fig. 5.22).

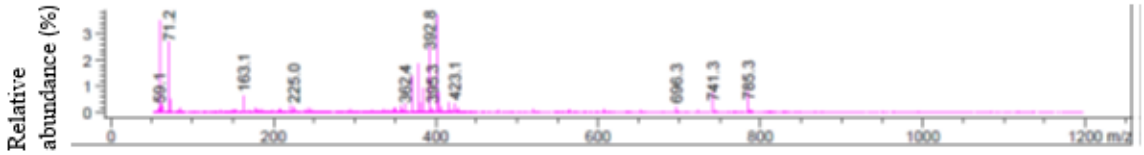
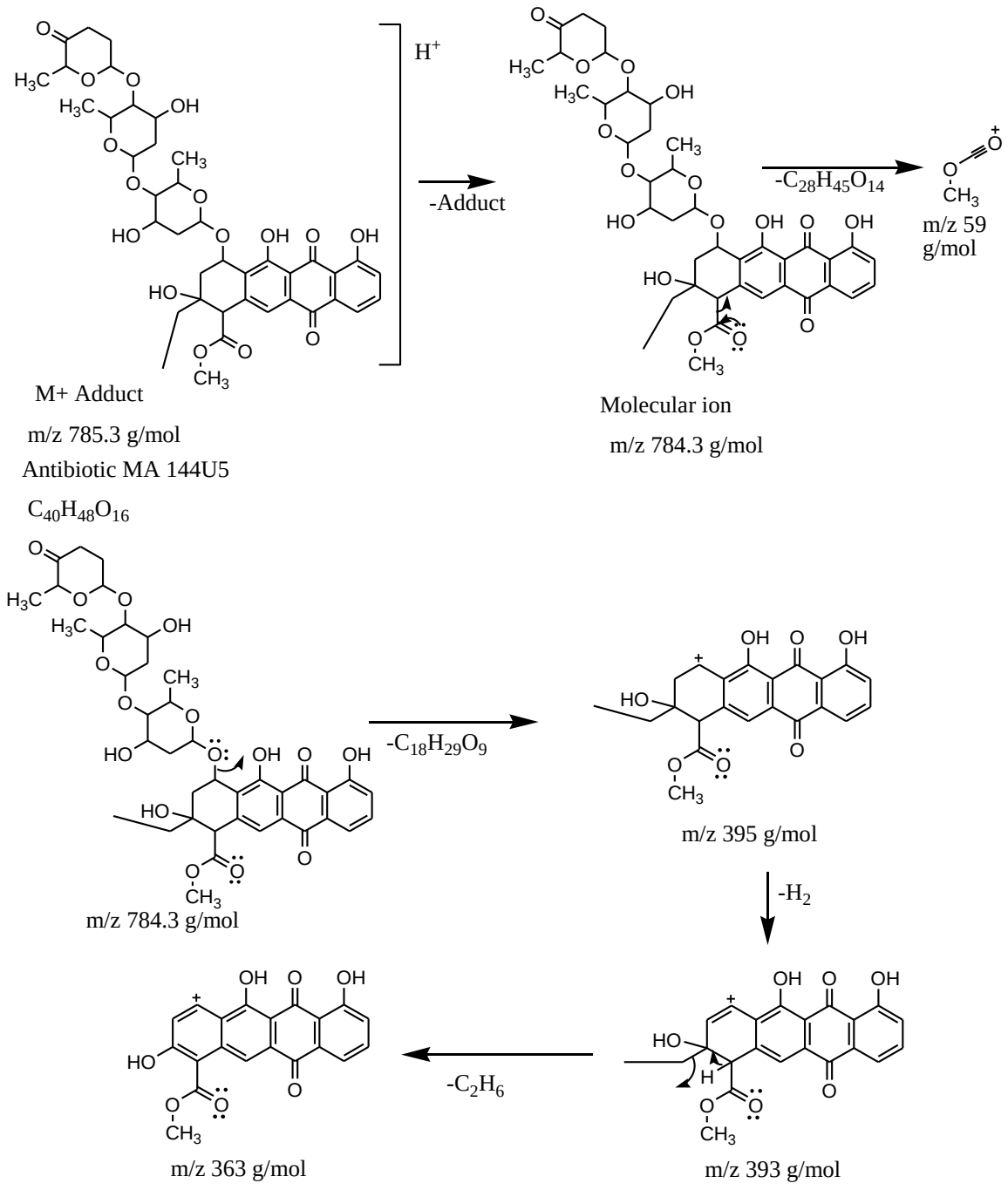
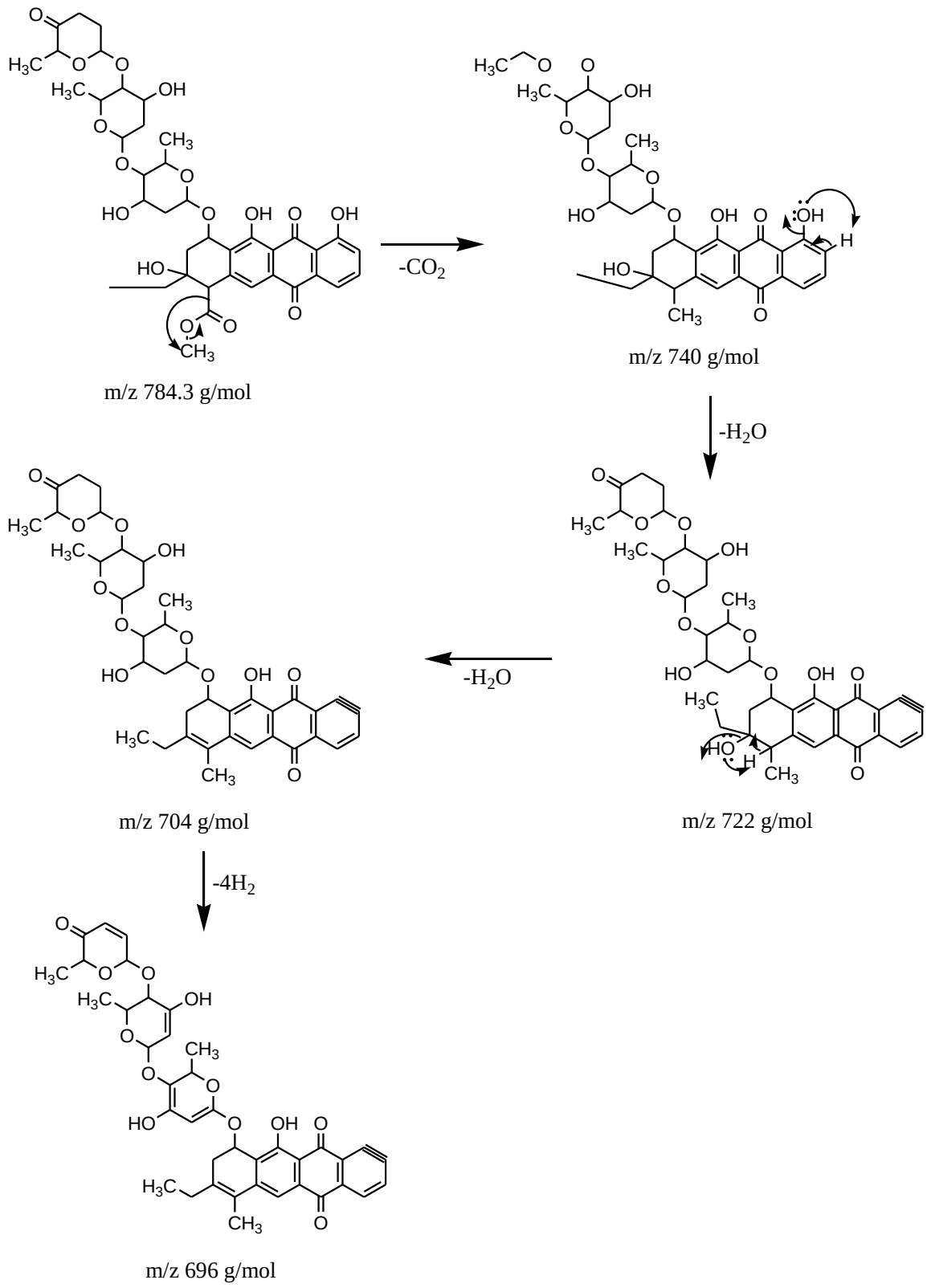


Fig. 5.22: The mass spectrum of compound 22

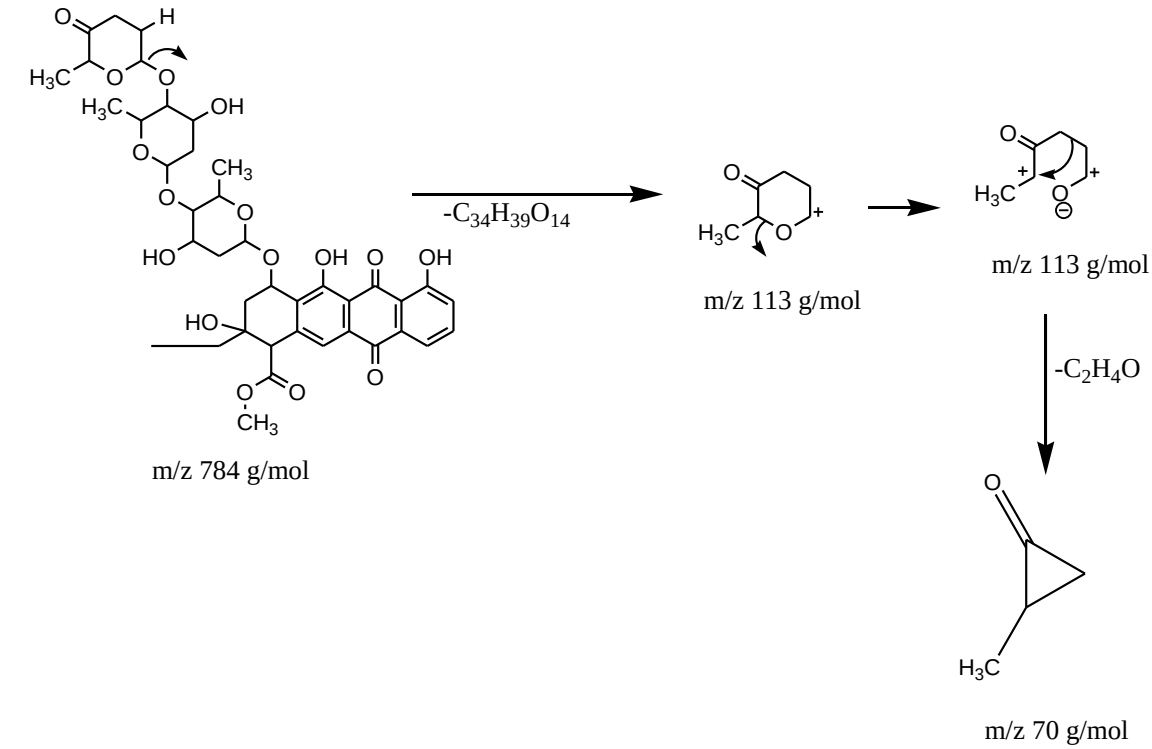
The fragment ion m/z 696 was due to decarboxylation (loss of CO_2), dehydration (loss of two H_2O molecules) and dehydrogenation (loss of H_2) while the m/z 423 was due to loss of $C_{12}H_{19}O_6$, $3H_2O$, CO_2 and $2H_2$. M/z 195 resulted from loss of $C_{18}H_{29}O_9$ whereby further loss of H_2 from that fragment leads to fragment m/z 393. Likewise, fragment m/z 377 was attributed to the abstraction of $C_{18}H_{29}O_9$ and H_2O molecule while m/z 362 due to abstraction of $C_{18}H_{29}O_9$, H_2, C_2H_6 and H. Similarly, the fragment m/z 225 was related to the loss of $C_{28}H_{29}O_{11}$ and H_2O while fragment m/z 163 was related to the loss of $C_{28}H_{29}O_{11}$, $C_3H_5O_2$, $2H_2$ and H^-. Lastly, the fragment ion at m/z 71 was attributed to loss of $C_{34}H_{39}O_{14}$ and C_2H_3O and while m/z 59 was due to $C_{28}H_{45}O_{14}$ (scheme 25 on pages 142-146).

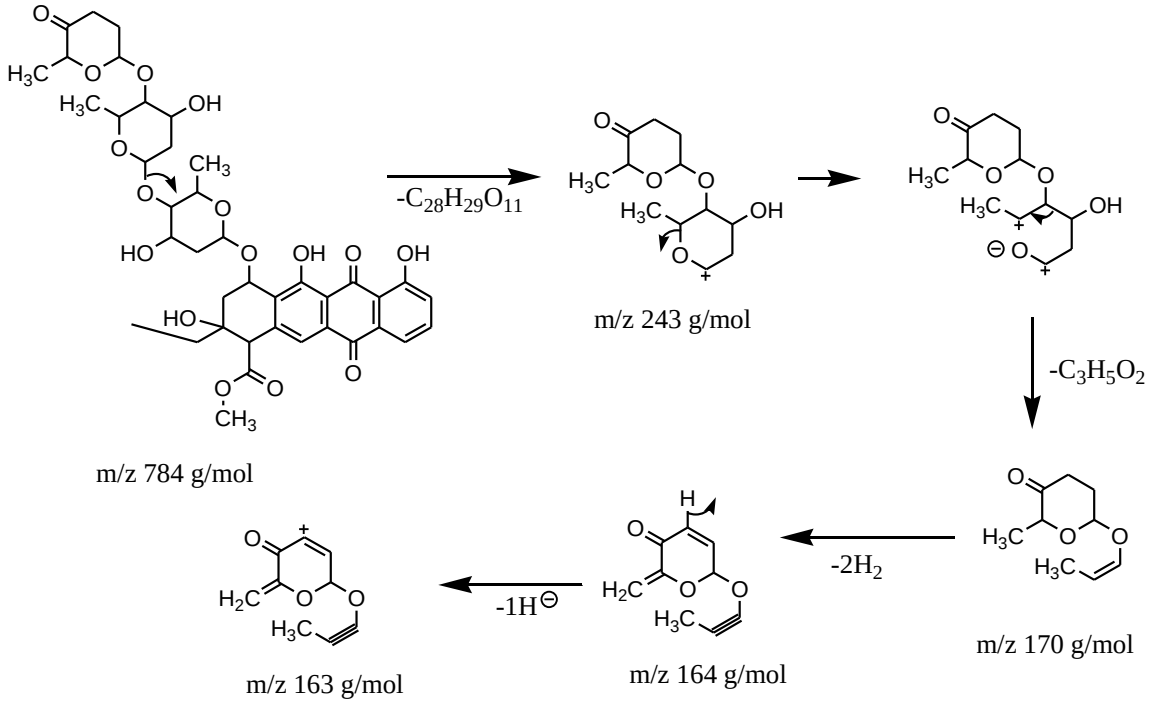


Continuing scheme

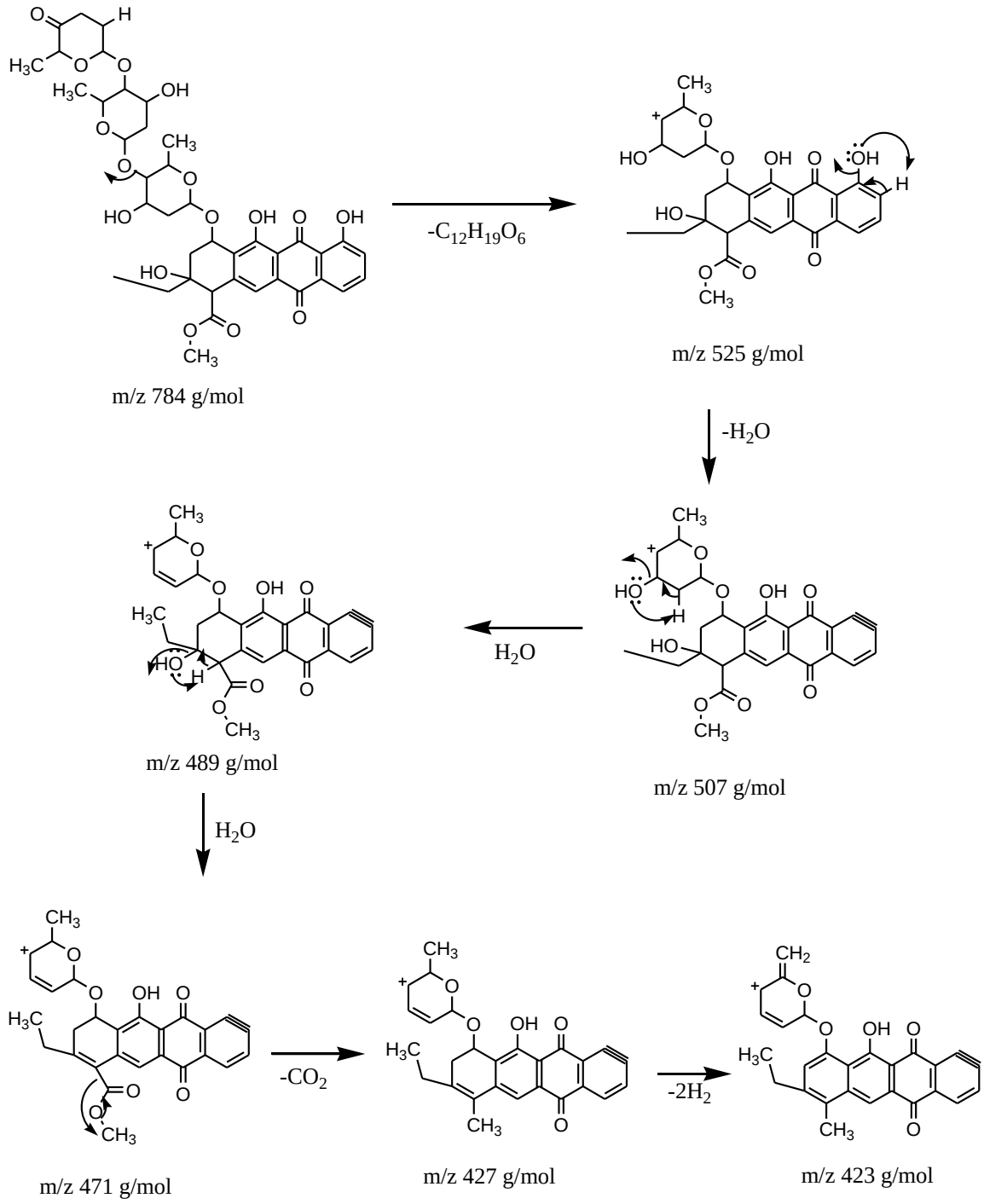


Continuing scheme

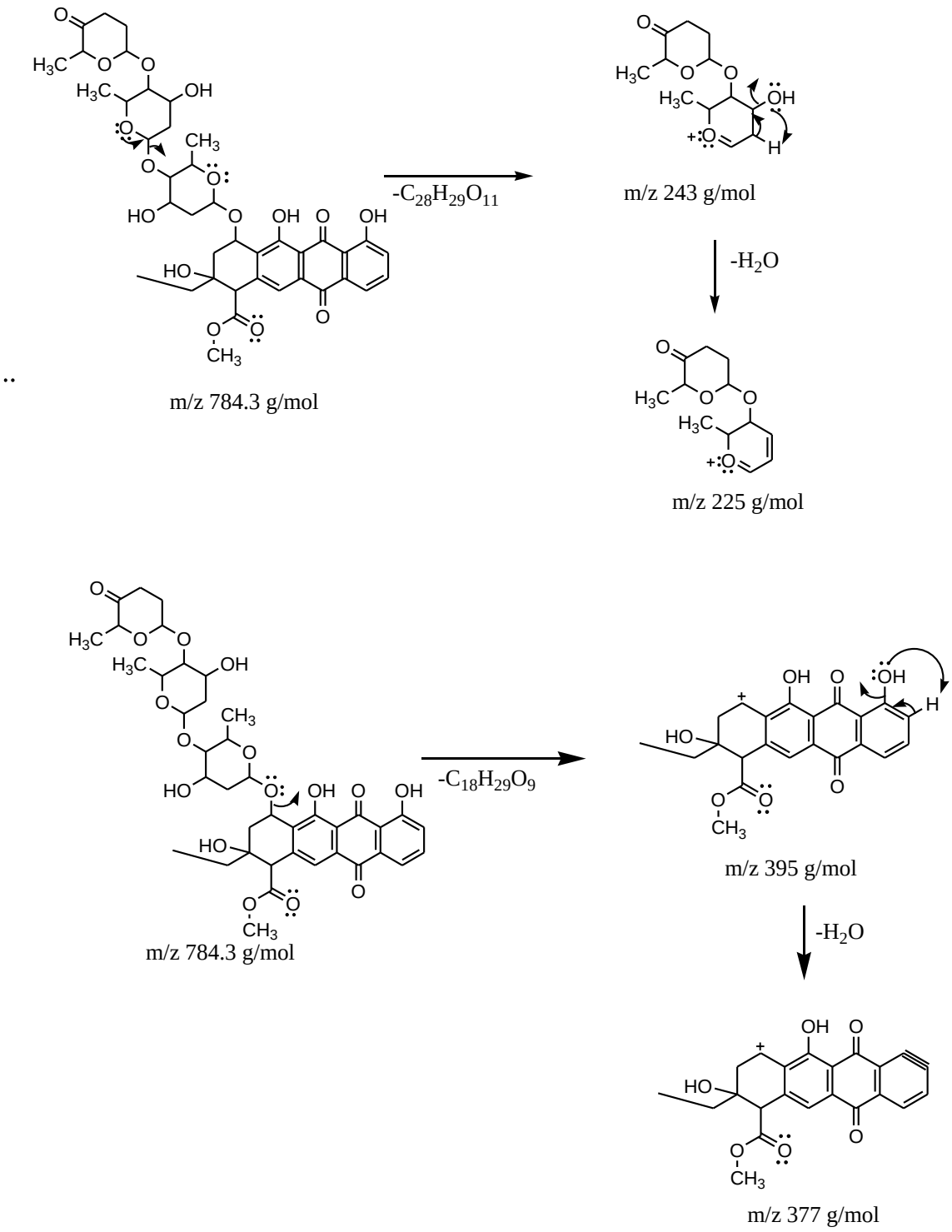




Continuing scheme



Continuing scheme



Fragments: 59, 71, 163, 225, 362, 377, 393, 395, 423, 696 and 785.3 g/mol

Scheme 25: Proposed fragmentation pathway for Antibiotic MA 144 U5 (compound 22).

Antibiotic MA 144 U5 belong to a class of anthracycline derivatives. Anthracyclines can be used to prepare anthracycline antibiotics, which are an essential group of antitumor drugs used in cancer therapy. This compound has not been previously isolated in honey. Two anthracycline compounds, Adriamycin (hydroxyl daunomycin or doxorubicin) and daunomycin (daunorubicin) have been isolated from *Streptomyces peucetius varcaesitue* (Běhal, 2000; Arcamone, 1981) and have been proved to exhibit wide spectrum of antitumor activity against human cancers (Myers, 1988; Gianni *et al*., 1983).

Compound 23 was detected at a retention time of 36.30 min with a characteristic molecular ion of 607.3 and generated fragment ions of m/z 565, 520, 476, 242, 170, 85, 72 and 59. This corresponded to Tryptophylalanyltryptophylphenylalaninamide (fig. 5.23).

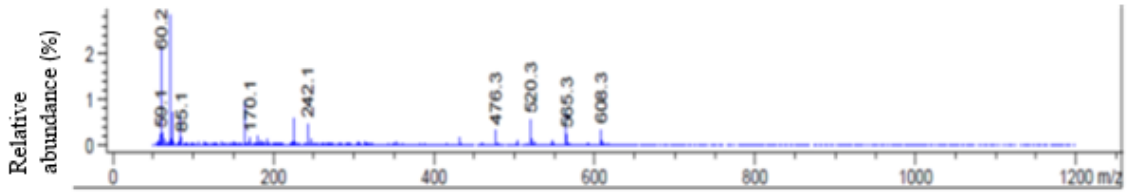
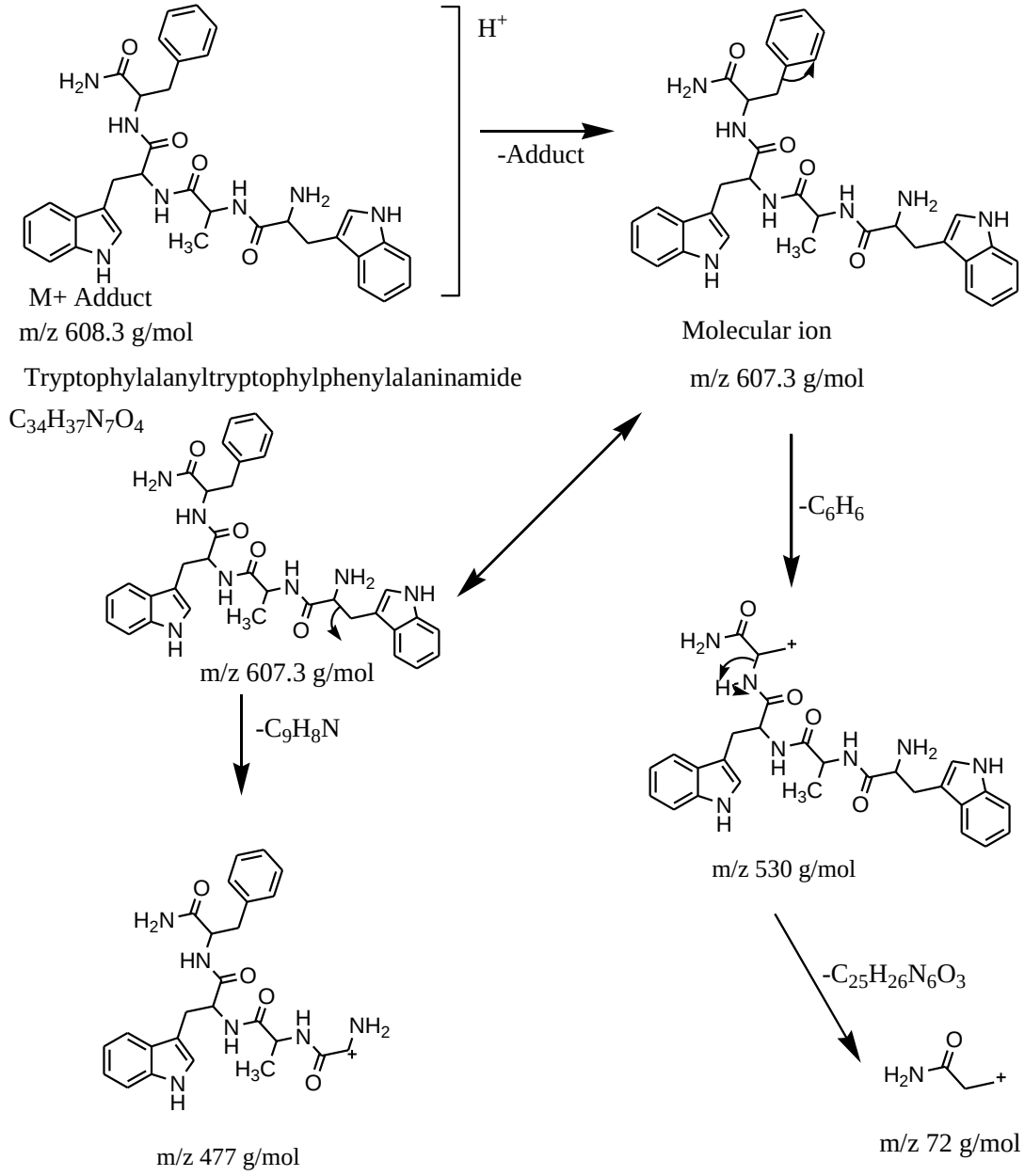
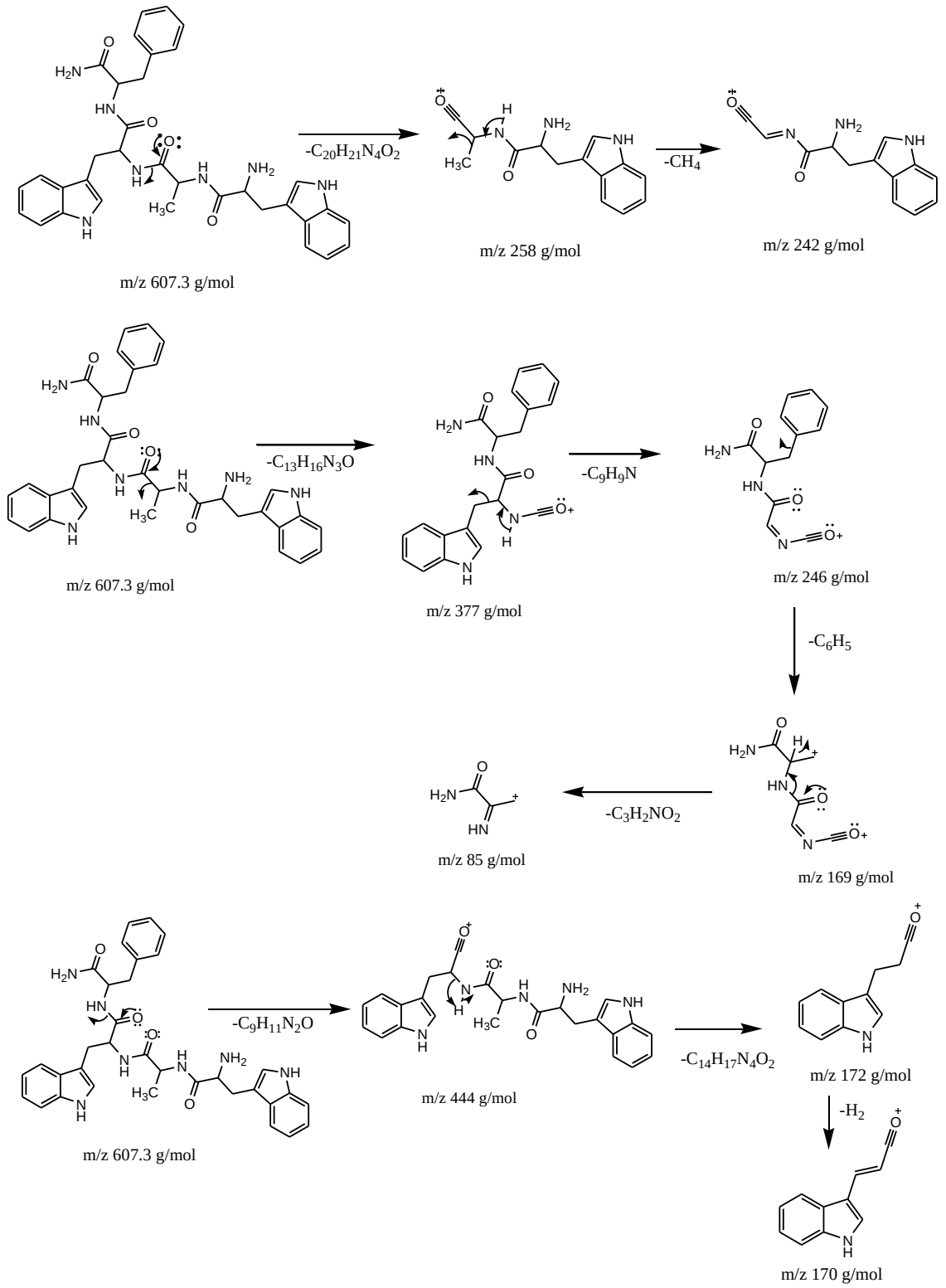


Fig. 5.23: The mass spectrum of compound 23

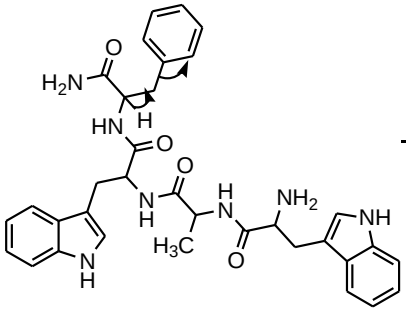
The fragment ion m/z 565 was as a result of loss of NH_3, CH_4, $4H_2$ and H^- while m/z 520 resulted from loss of C_6H_6, $4H_2$ and H^-. The fragment ion m/z 476 resulted from C_9H_8N and H while fragment ion m/z 242 was due to loss of $C_{20}H_{21}N_4O_2$ and CH_4. Likewise, m/z 170 was attributed to loss of $C_9H_{11}N_2O$, $C_{14}H_{17}N_4O_2$ and H_2 while m/z 85 was attributed $C_{13}H_{16}N_3O$, C_9H_9N, C_6H_5 and $C_3H_2NO_2$. Lastly, the fragment ion m/z 72 was due to loss of C_6H_6 and $C_{25}H_{26}N_6O_3$ while m/z 59 was due to loss of $C_{25}H_{28}N_6O_3$ and C_7H_5 (scheme 26 on pages 148-151).



Continuing scheme

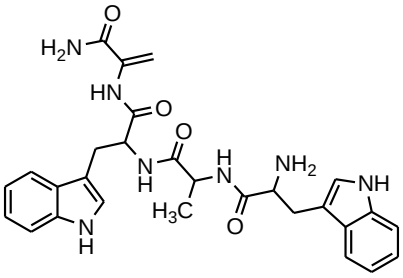

-C20H21N4O2
-CH4
m/z 607.3 g/mol
m/z 258 g/mol
m/z 242 g/mol
-C13H16N3O
-C9H9N
m/z 607.3 g/mol
m/z 377 g/mol
m/z 246 g/mol
-C6H5
-C3H2NO2
m/z 85 g/mol
m/z 169 g/mol
-C9H11N2O
-C14H17N4O2
-H2
m/z 607.3 g/mol
m/z 444 g/mol
m/z 172 g/mol
m/z 170 g/mol

Continuing



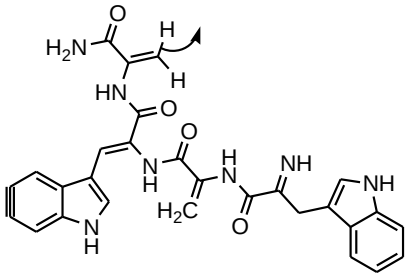
m/z 607 g/mol

$-C_6H_6$



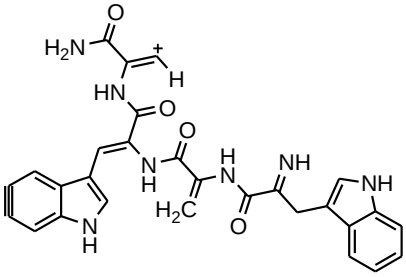
m/z 529 g/mol

$-4H_2$

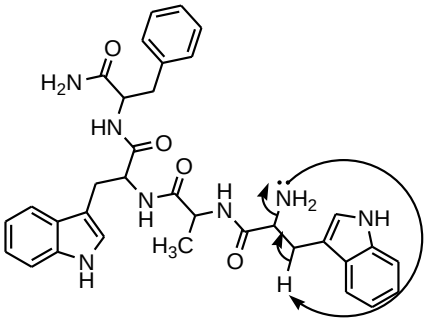


m/z 521 g/mol

$-1H^{\ominus}$

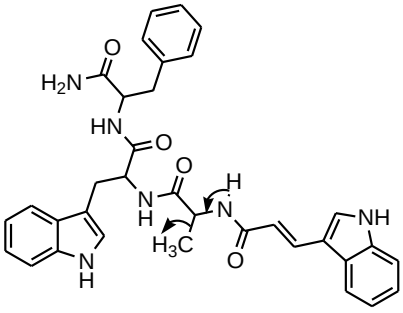


m/z 520 g/mol



m/z 607 g/mol

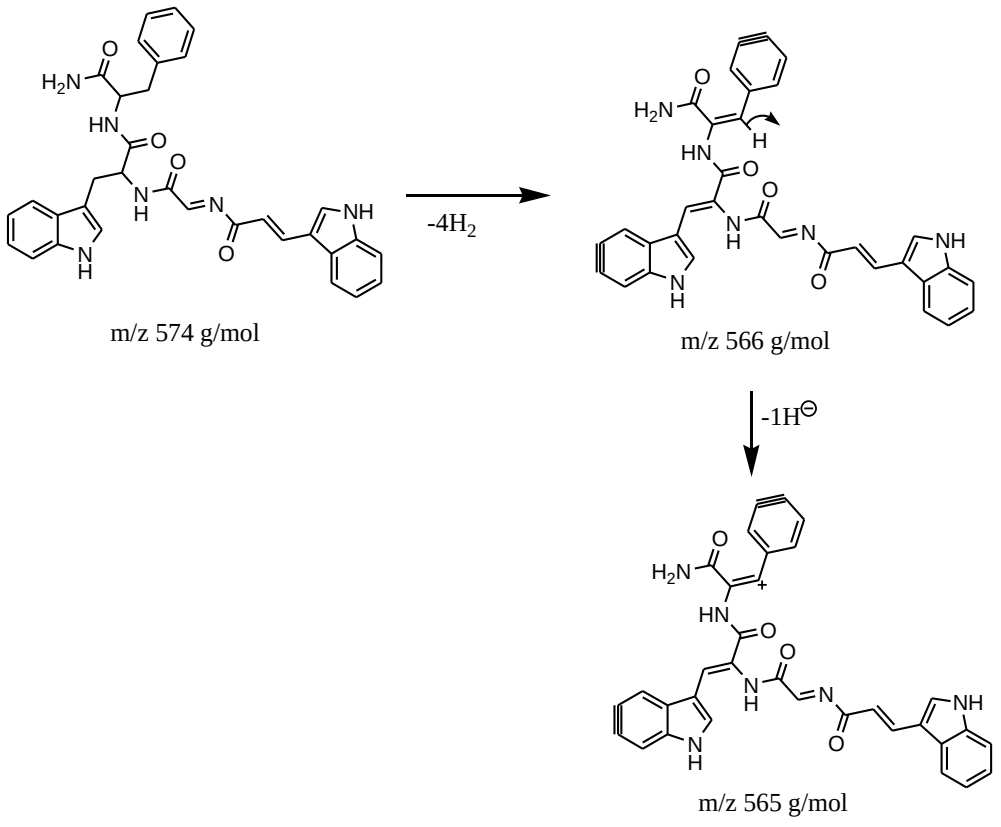
$-NH_3$

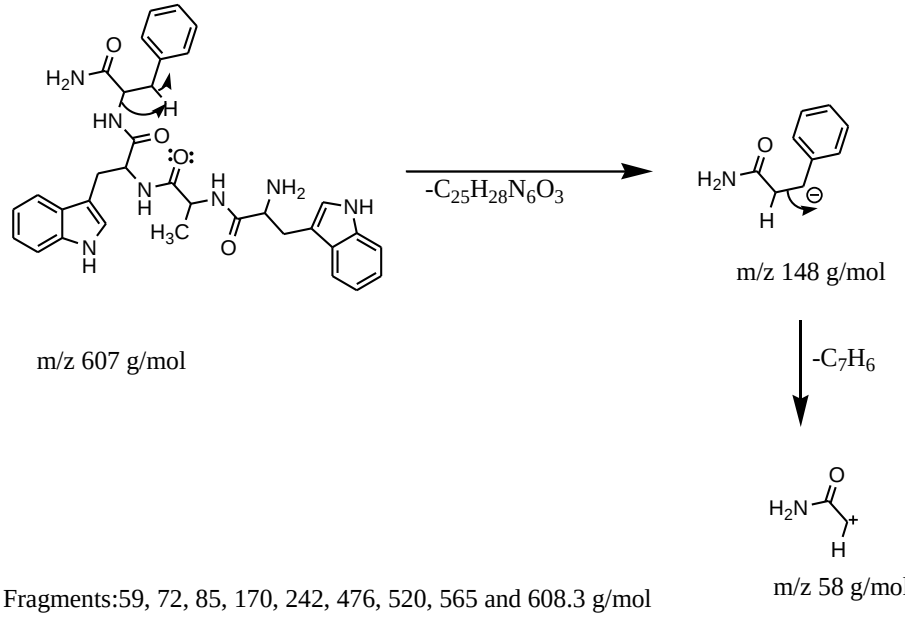


m/z 590 g/mol

$-CH_4$

Continuing scheme





Fragments:59, 72, 85, 170, 242, 476, 520, 565 and 608.3 g/mol

Scheme 26: Fragmentation for Tryptophylalanyltryptophylphenylalaninamide (compound 23).

Tryptophylalanyltryptophylphenylalaninamide is a peptide derivative with tryptophan and alanine amino acids. They were characterized in honey as decribed by Kıvrak (2017). These have also been identified in Polish and Slovak honeys (Kowalski *et al*., 2017). Amino acids such as tryptophan were reported to possess antioxidant activity (Meucci & Mele, 1997).

On the other hand, twelve (12) compounds were positively identified from *Plebenia hyderbrandii* (fig. 33-38) and *Meliponula bocandei* (fig. 39-44) honeys through ions generated from [M+ ACN + H] $^{+}$ spectra with proton and acetonitrile as the adducts.

Compound 24 was detected at a retention time of 4.87min with a characteristic molecular ion of m/z 352.2 and produced fragment ions of m/z 302 and m/z 262. This corresponded to Pongagallone A (fig. 5.24).

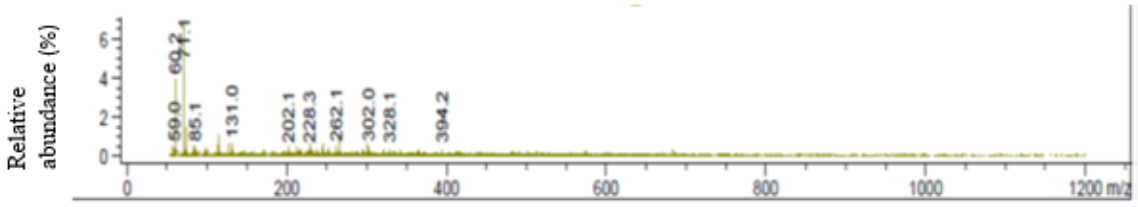
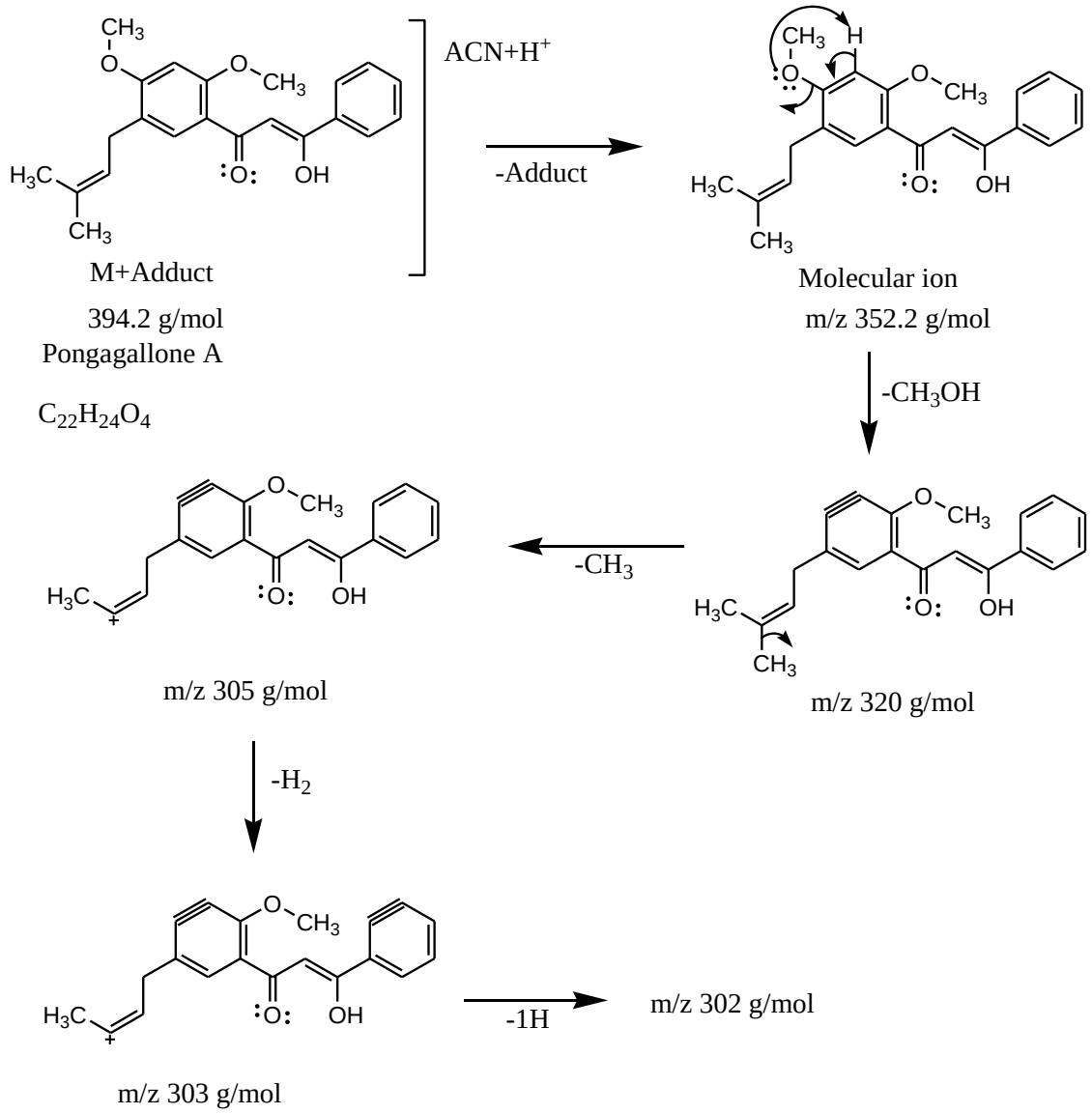
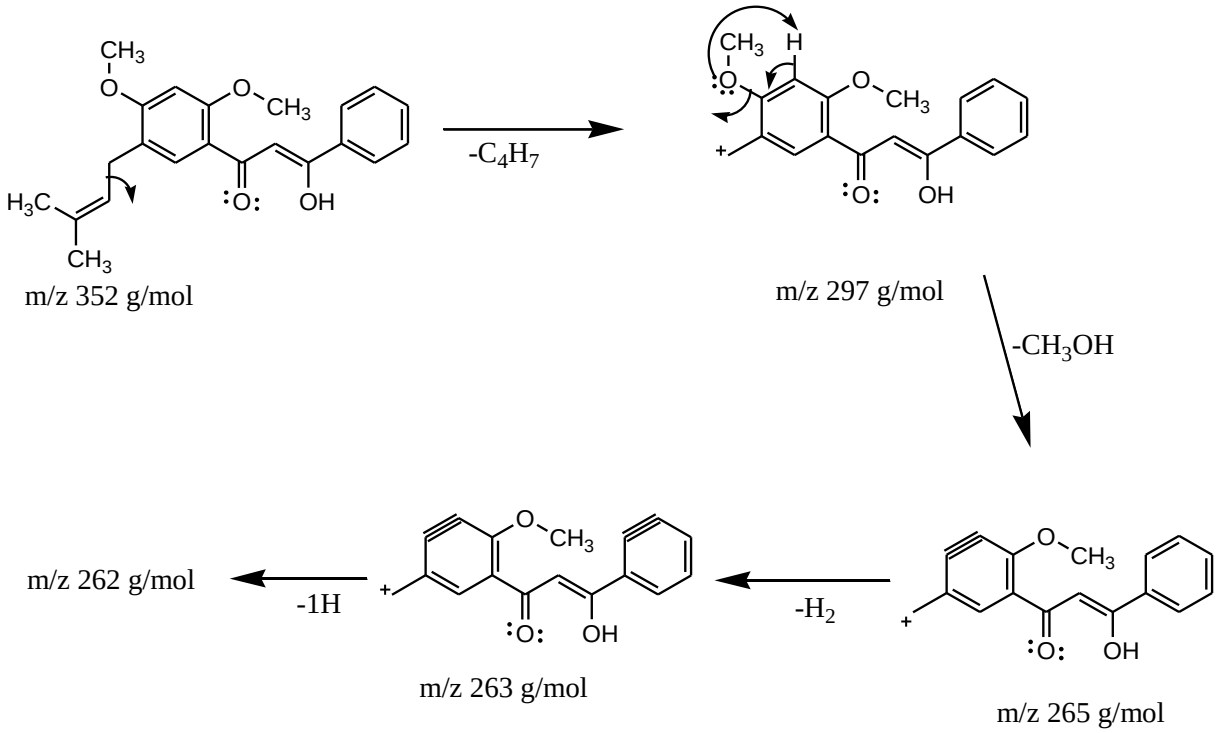


Fig. 5.24: The mass spectrum of compound 24

The m/z 302 fragment resulted from the loss of CH_3OH, CH_3, H_2 and H while m/z 262 fragment was attributed to the loss of C_4H_7, CH_3OH, H_2 and H (scheme 27 on pages 153 & 154).



Continuing scheme



Scheme 27: Fragmentation pathway for Pongagallone A (compound 24).

Pongagallone A, a chalcone, belong to the polyphenolic group of compounds, of the flavonoid family. It is believed to possess modulatory and cytoprotective functions, which may have therapeutic potential for several diseases. It has been identified on the infected leaves' galls of *Pongamia glabra* plant (Gandhidasan *et al*., 1986). It was also isolated from the seed kernel of *Pongamia pinnata* (Yadav *et al*., 2011). This is its first identification in honey. Apart from antioxidant activity, pongagallone A, being a chalcone also possess various biological effects such as antimicrobial, anti-inflammatory, antifungal, cytotoxic, chemopreventive and antitumor activities (Rozmer & Perjési, 2016).

Compound 25 displayed a molecular ion at m/z 515.3 at a retention time of 50.47 min and generated fragment ions of m/z 71 and 60. This corresponded to Leu-Gln-Gln-Gln (fig. 5.25).

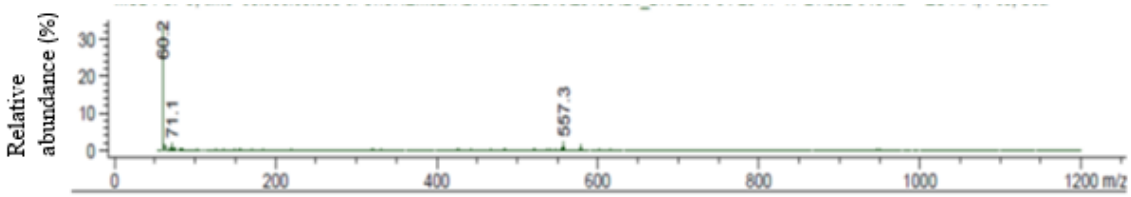
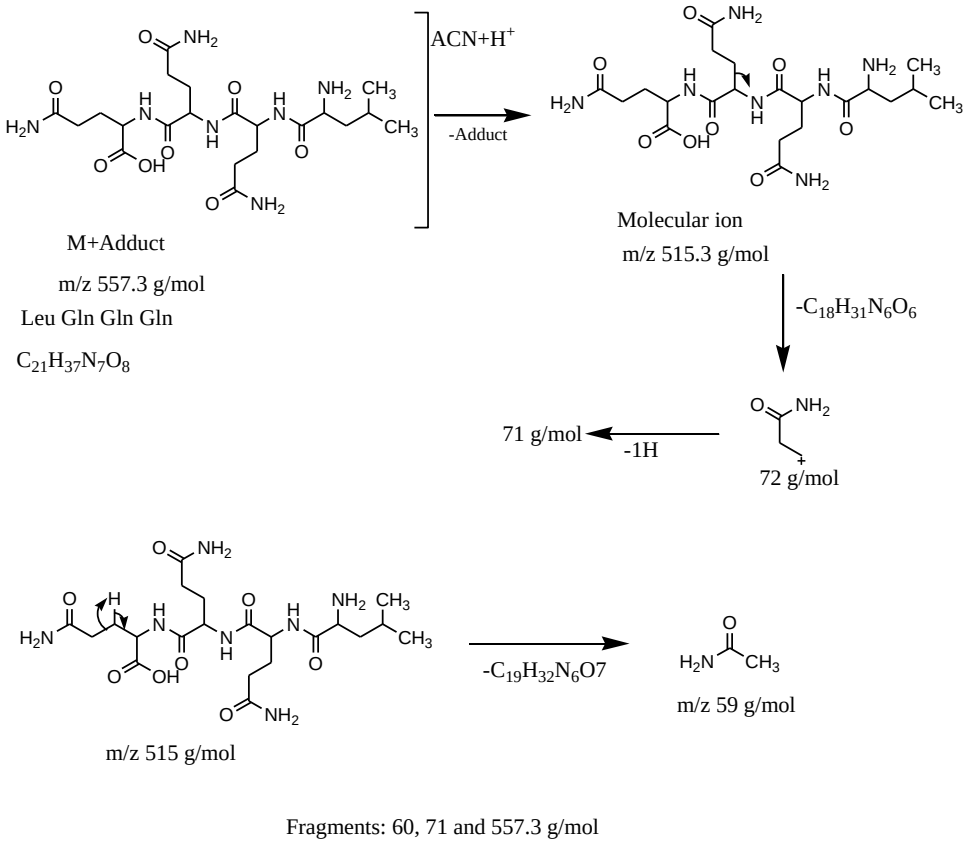


Fig. 5.25: The mass spectrum of compound 25

The m/z 71 fragment ion was as a result of loss of $C_{18}H_{31}N_6O_6$ and H while m/z 60 resulted from the loss of $C_{19}H_{31}N_6O_7$ (scheme 28 below).



Scheme 28: Fragmentation pathway for Leu-Gln-Gln-Gln (compound 25)

Leu-Gln-Gln-Gln is a tetrapeptide with leucine and glutamine amino acids. These were characterised in honey (Kıvrak, 2017). They were also isolated from Poland and Slovak honeys as described by Kowalski *et al*. (2017). Peptides and amino acids are reported to have antioxidant activities (Elias *et al*., 2008) and antimicrobial activity (Siebert *et al*., 2018).

Compound 26 had a characteristic molecular ion of 522.2 at a retention time of 39.72min and produced fragment ions of m/z 520, 448, 445, 414, 269, 180, 71 and 61. This corresponded to Glu-Asp-Met-Glu (fig. 5.26).

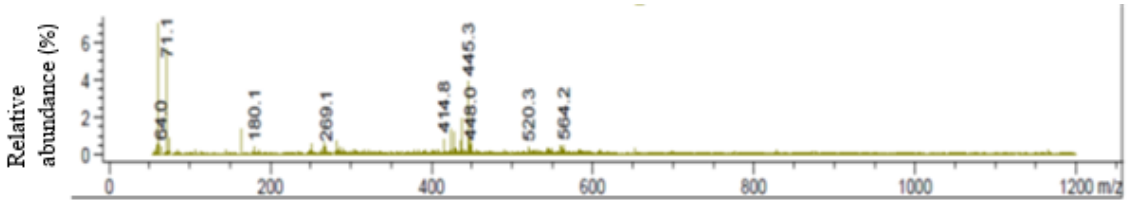
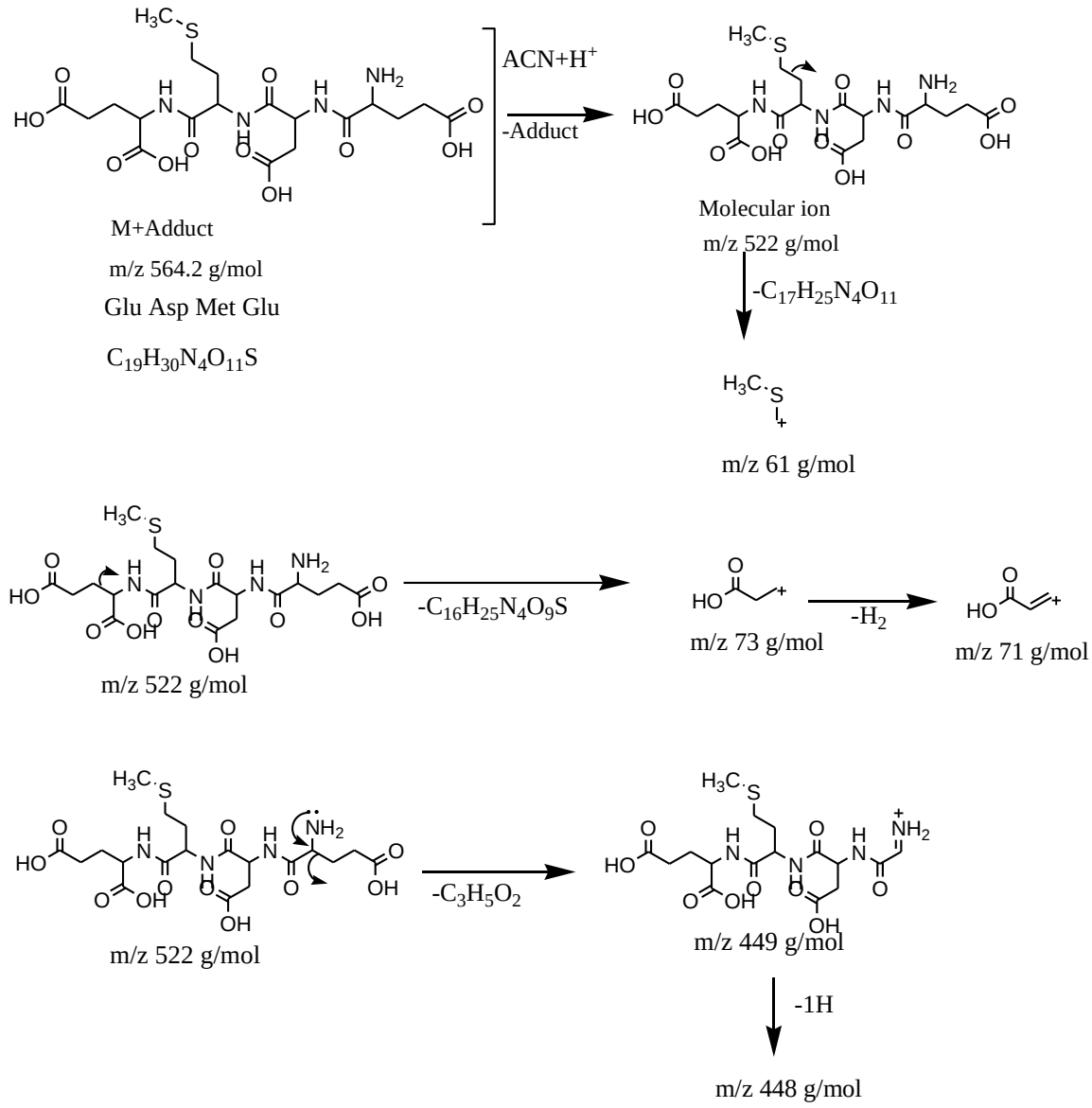
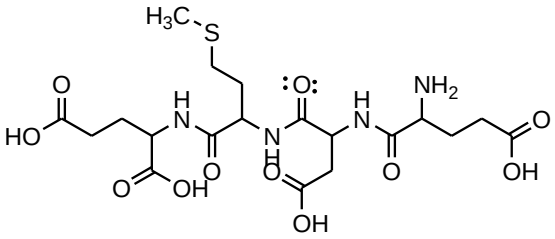


Fig. 5.26: The mass spectrum for compound 26

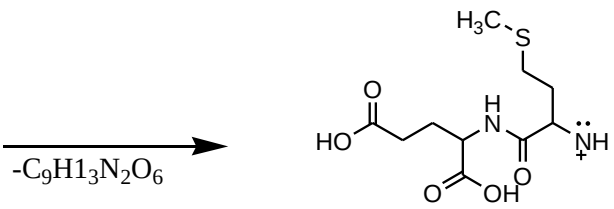
The fragment ion m/z 520 resulted from dehydrogenation (loss of H_2) while m/z 448 resulted from the loss of $C_3H_5O_2$ and H. The fragment ion m/z 445 was due to loss of $C_3H_5O_2$ and dehydrogenation (loss of two H_2) while m/z 414 resulted from loss of $C_4H_8NO_2$ and three H_2. M/z 269 was due to loss of $C_9H_{13}N_2O_6$ and $4H_2$ while m/z 180 was due to loss of $C_{11}H_{17}N_2O_6S$, dehydration (H_2O), ammonia (NH_3) and dehydrogenation (H_2). Similarly, m/z 71 resulted from the loss of $C_{16}H_{25}N_4O_9S$, and H_2 while the last fragment m/z 61 was attributed to loss of $C_{17}H_{25}N_4O_{11}$ (scheme 29 on pages 157-159).



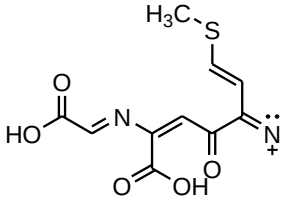
Continuing scheme



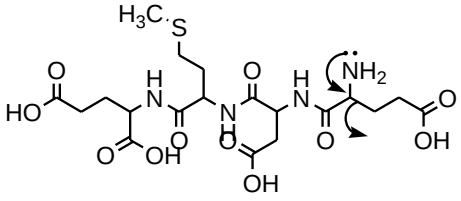
m/z 522 g/mol



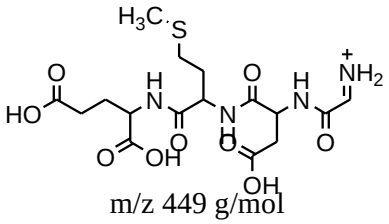
m/z 277 g/mol



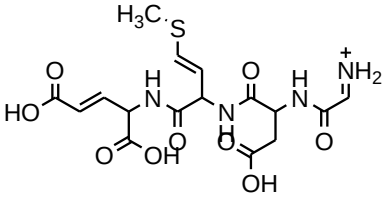
m/z 269 g/mol



m/z 522 g/mol

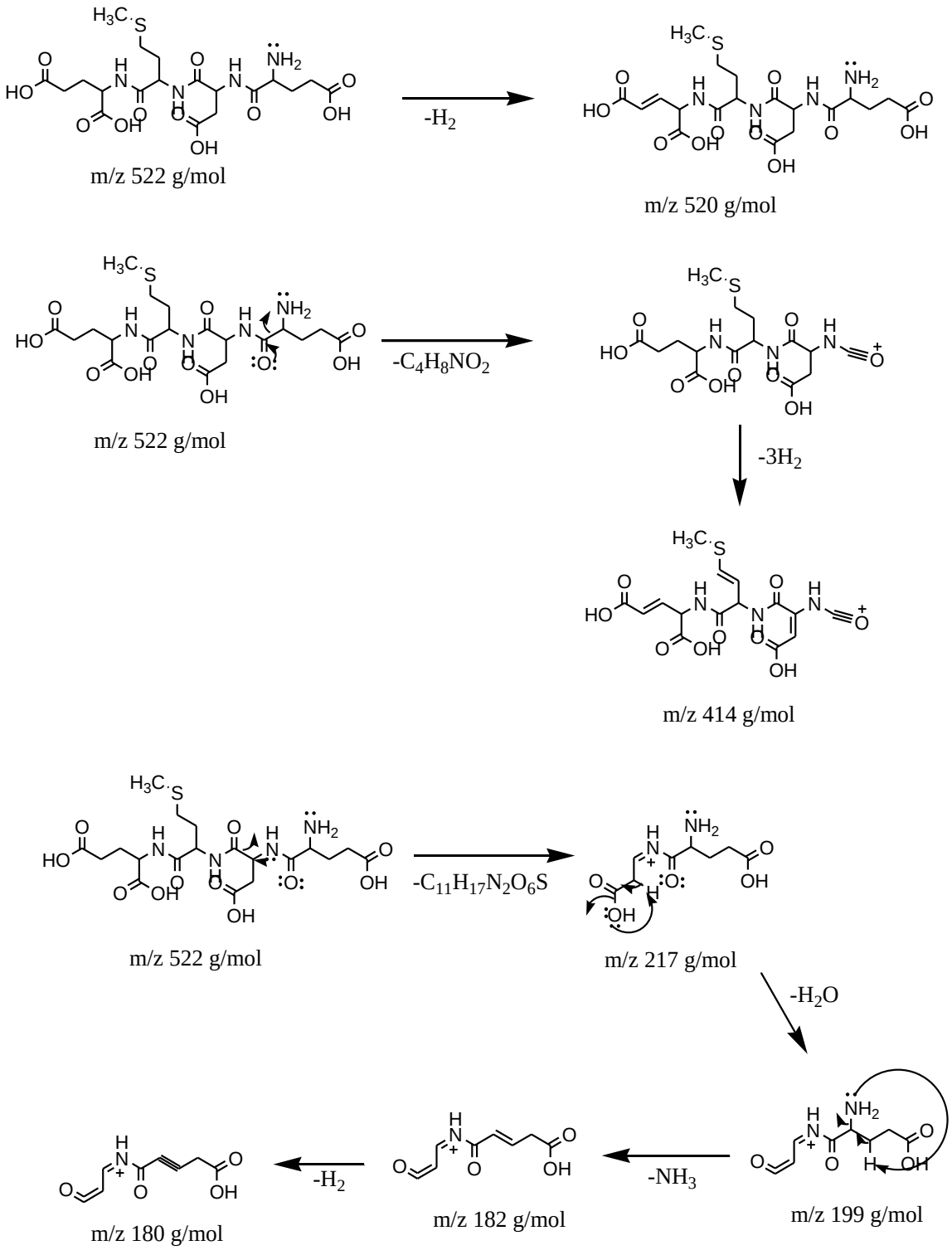


m/z 449 g/mol



m/z 445 g/mol

Continuing scheme



Fragments: 61, 71, 180, 269, 414, 445, 448, 520 and 564 g/mol

Scheme 29: Fragmentation pathway for Glu-Asp-Met-Glu (compound 26)

Glu-Asp-Met-Glu is a tetrapeptide consisting of glutamic acid, aspartic acid and methionine. All of them have been characterized in forest honeys from South Sulawesi, Indonesia (Noor *et al*., 2019). They have heen also isolated from Slovak and Poland honeys according to Kowalski *et al*. (2017). Peptides and amino acids possess both antioxidant activities (Elias *et al*., 2008) and antimicrobial activities (Coutinho *et al*., 2008).

Compound 27 had a retention time of 43.03 and a characteristic molecular ion of m/z 596.2 and generated fragment ions at m/z 341, 319 and 60. This corresponded to 1, 2, 3, 6-Tetra-O-benzoyl-alpha-D-threo-hexopyranose (fig. 5.27).

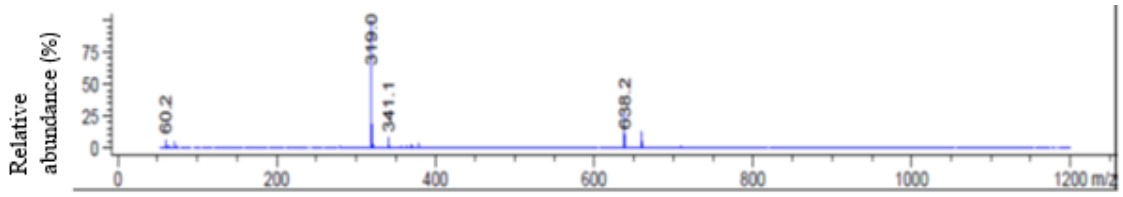
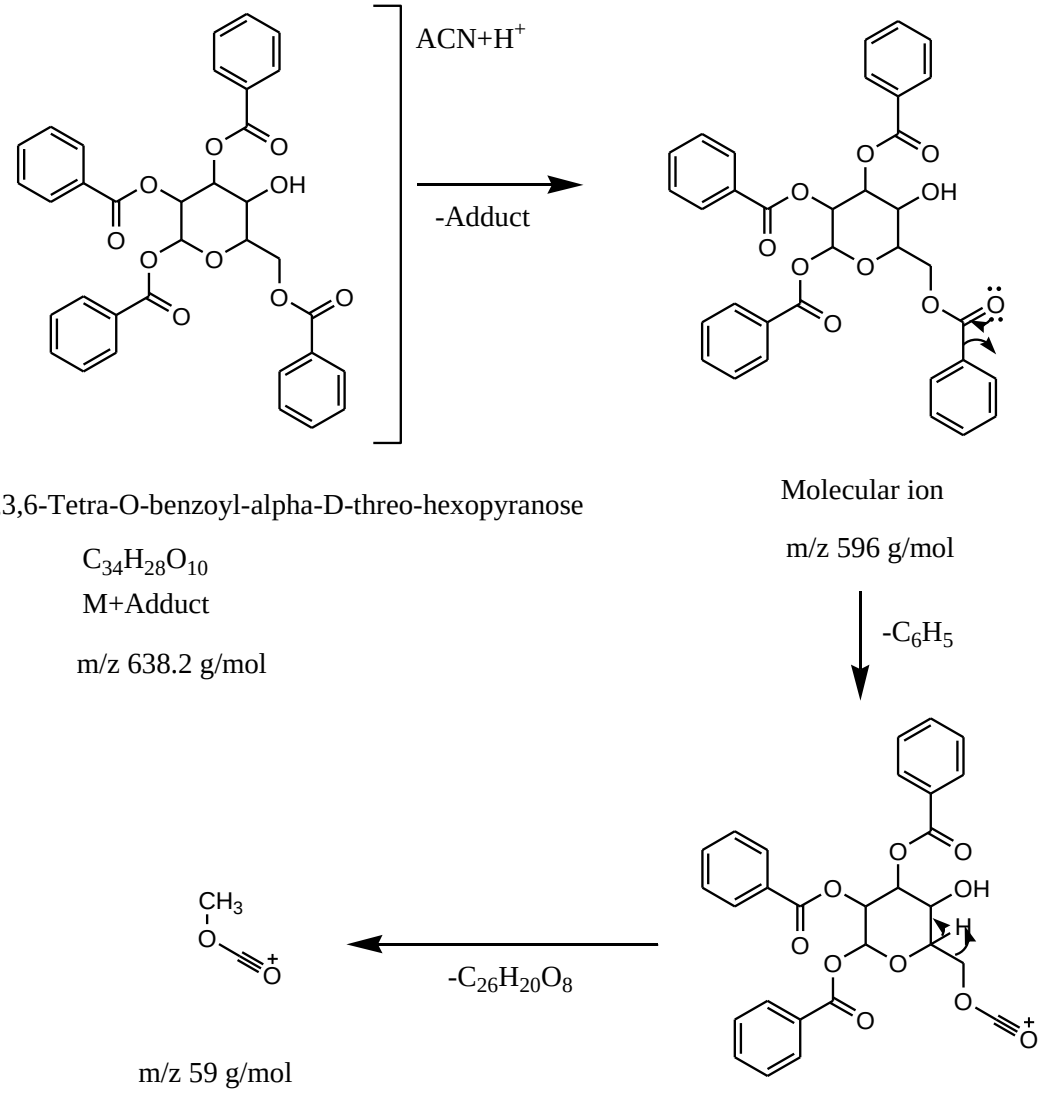


Fig. 5.27: The mass spectrum of compound 27

The fragment ion at m/z 341 was due to loss of $C_7H_5O_2$ and $C_8H_6O_2$, which on further loss of H_2O and two H_2 led to the fragment ion m/z 319. Similarly, m/z 60 resulted from the loss of C_6H_5 and $C_{26}H_{19}O_8$ (scheme 30 on pages 161 & 162).



1,2,3,6-Tetra-O-benzoyl-alpha-D-threo-hexopyranose

$C_{34}H_{28}O_{10}$

M+Adduct

m/z 638.2 g/mol

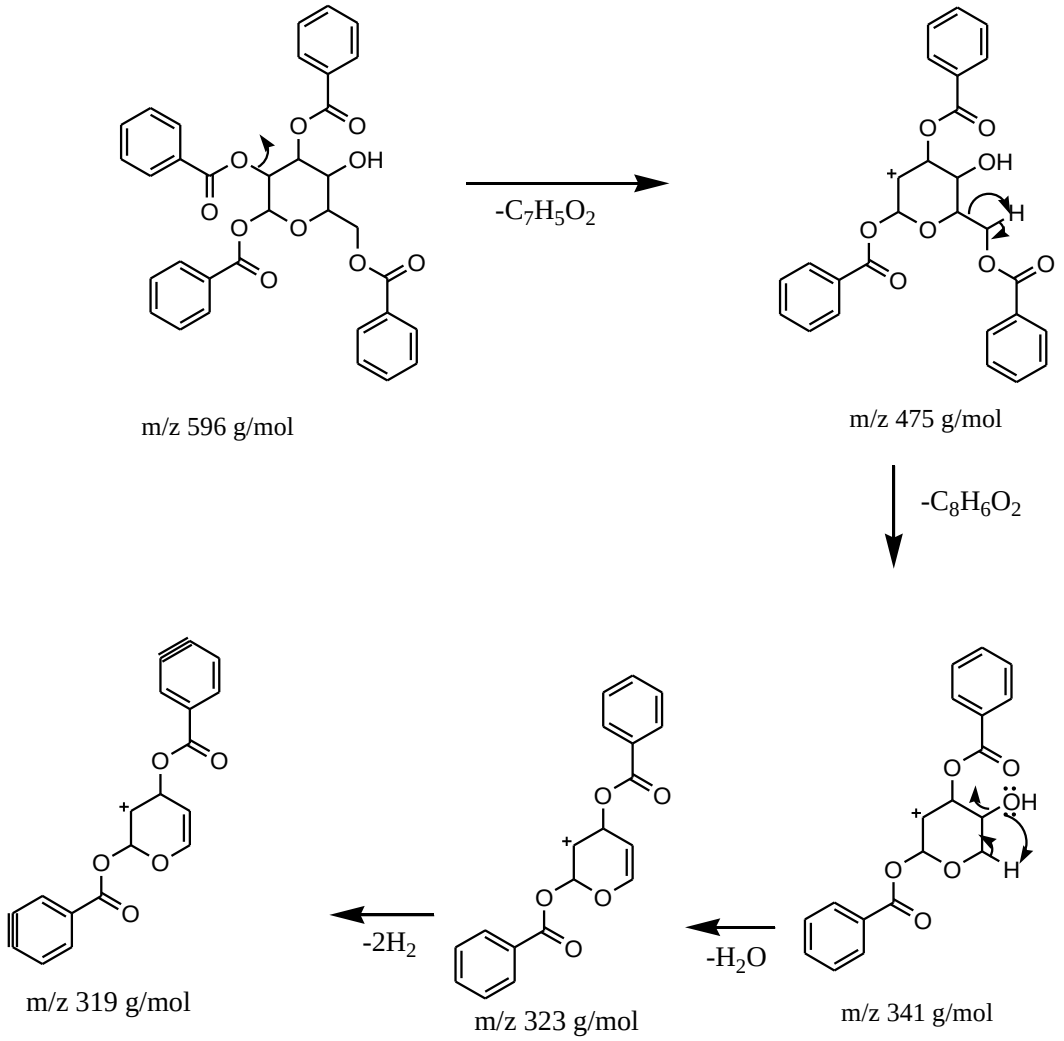
Molecular ion

m/z 596 g/mol

m/z 519 g/mol

m/z 59 g/mol

Continuing scheme



Fragments: 60, 319, 341 and 638 g/mol

Scheme 30: Fragmentation pathway for 1, 2, 3, 6-Tetra-O-benzoyl-alpha-D-threo-hexopyranose (compound 27)

1, 2, 3, 6-Tetra-O-benzoyl-alpha-D-threo-hexopyranose is an O-glycosyl compound. Its isolation is not documented anywhere. Being a carbohydrate derivative, it has diverse biological properties, which rather than antioxidant activity also include other properties such as anti-inflammatory, angiogenesis and anti-coagulant properties (Brooks *et al*., 2002).

Compound 28 was detected at a retention time of 5.09 min with a molecular ion of m/z 646.3 and produced fragments ions of m/z 345, 288, 247, 157, 113, 85, 71, 60 and 56. This corresponded to L-Seryl-L-tyrosyl-L-Prolyl-Serylglycyl-L-histidine (fig. 5.28).

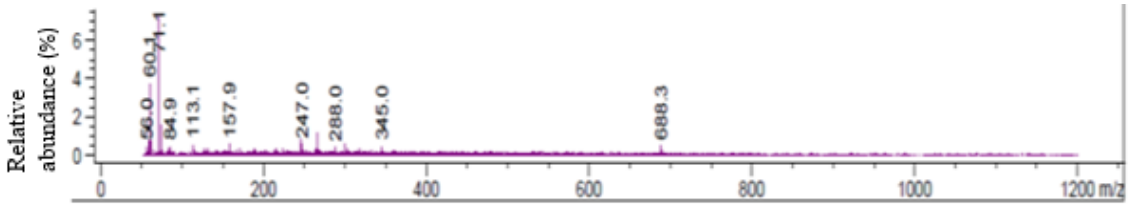
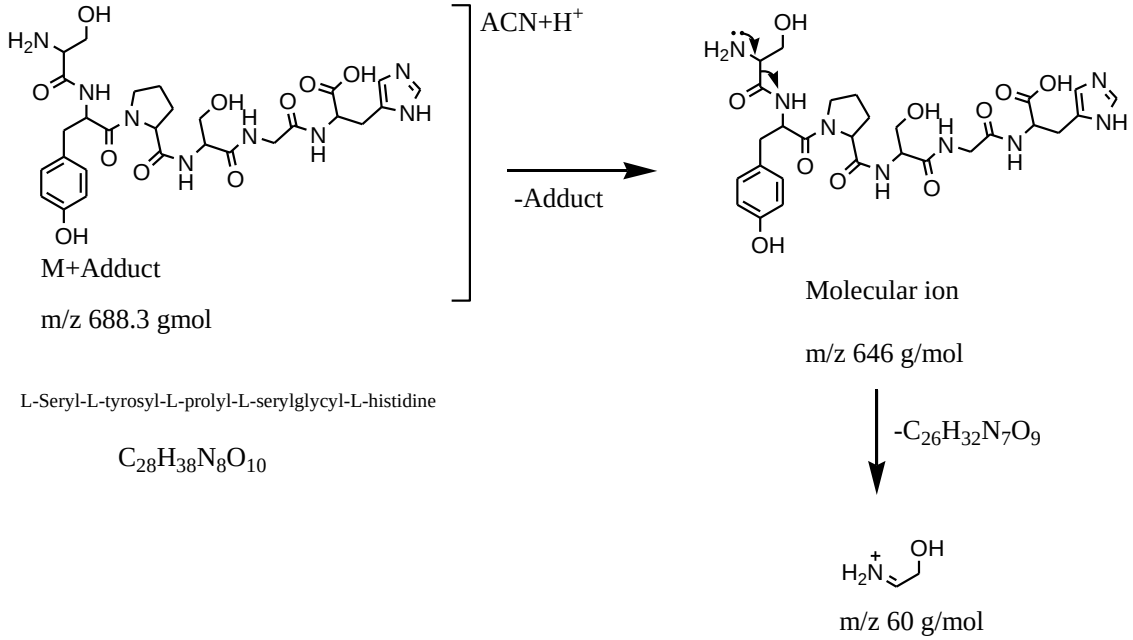
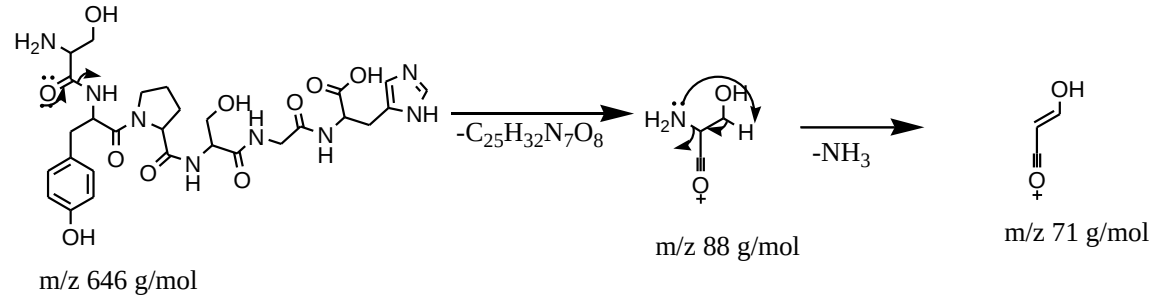


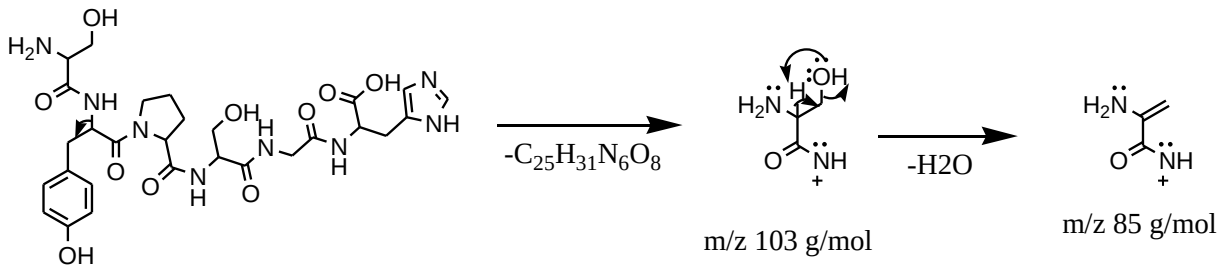
Fig. 5.28: The mass spectrum of compound 28

The fragment ion at m/z 345 was due to loss of $C_{11}H_{15}N_4O_5$ and H_2O while m/z 288 resulted from the loss of C_2H_6NO, $C_{11}H_{16}N_5O_5$, and H. m/z 247 was due to loss of $C_{16}H_{23}N_6O_6$, two H_2 while m/z 157 was due to loss of $C_{19}H_{27}N_4O_6$, $C_4H_4N_2$, H_2. Likewise, m/z 113 resulted from loss of $C_{16}H_{22}N_3O_4$, $C_8H_{11}N_4O_3$, H_2 and H while m/z 85 resulted from the loss of $C_{25}H_{31}N_6O_8$ and H_2O. Similarly, the fragment 71 resulted from abstraction of $C_{25}H_{32}N_7O_8$ and NH_3 while m/z 60 resulted from abstraction of $C_{26}H_{32}N_7O_9$. Finally, m/z 56 was due to loss of $C_{19}H_{27}N_4O_5$, $C_7H_7N_3O_3$ and H_2 (scheme 31 on pages 164-168).





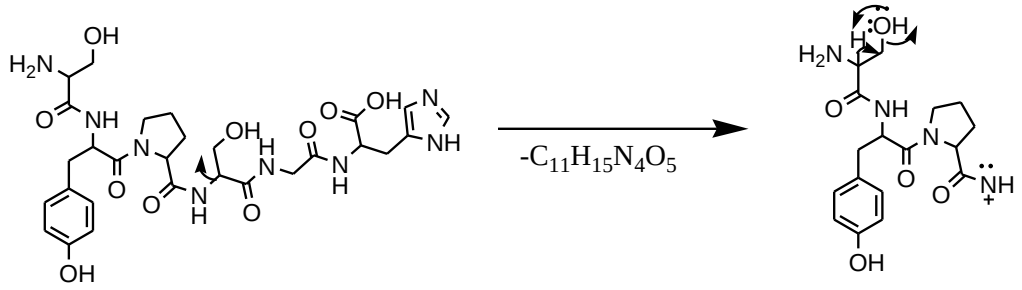
Continuing scheme



m/z 646 g/mol

m/z 103 g/mol

m/z 85 g/mol



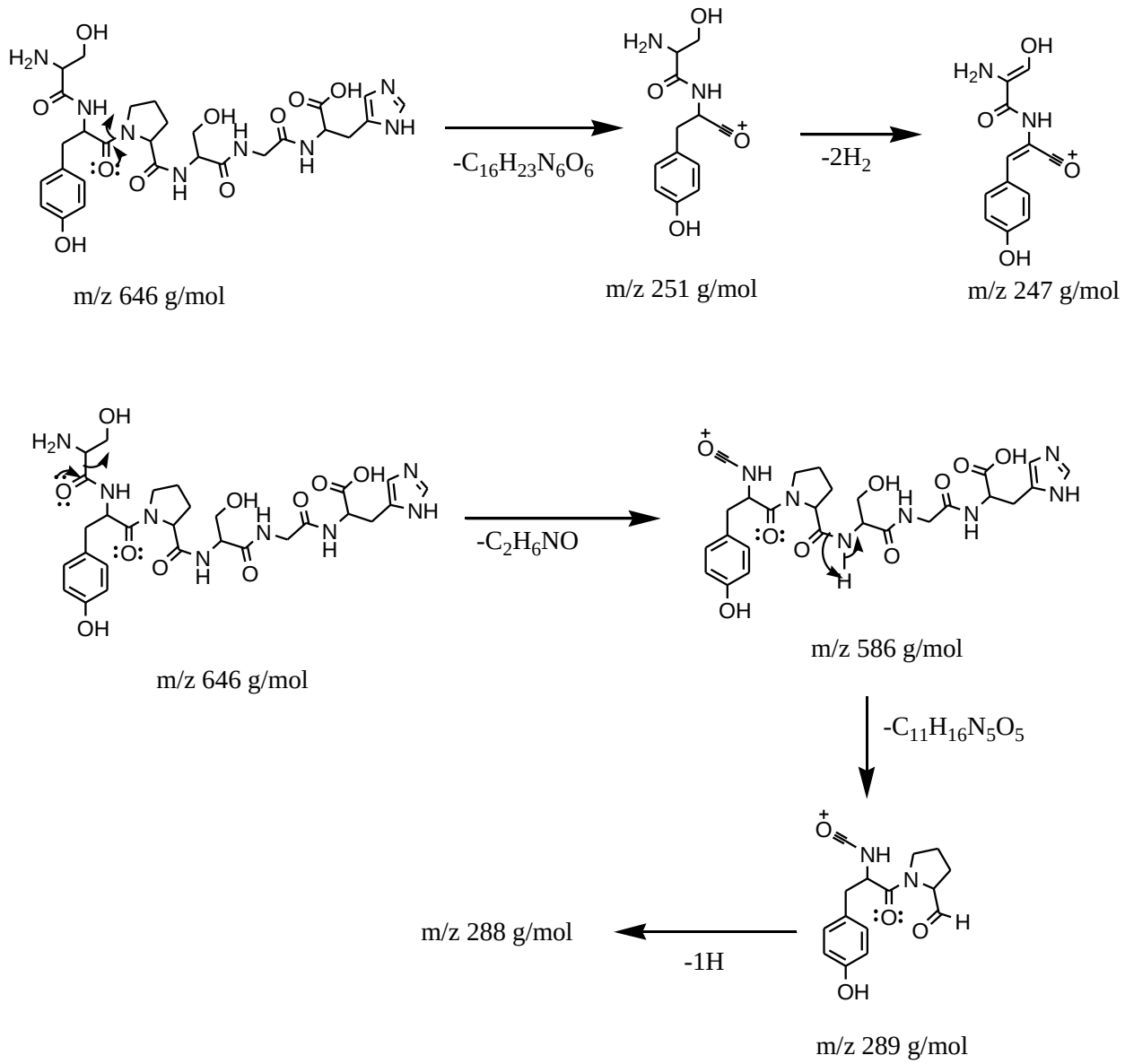
m/z 646 g/mol

m/z 363 g/mol

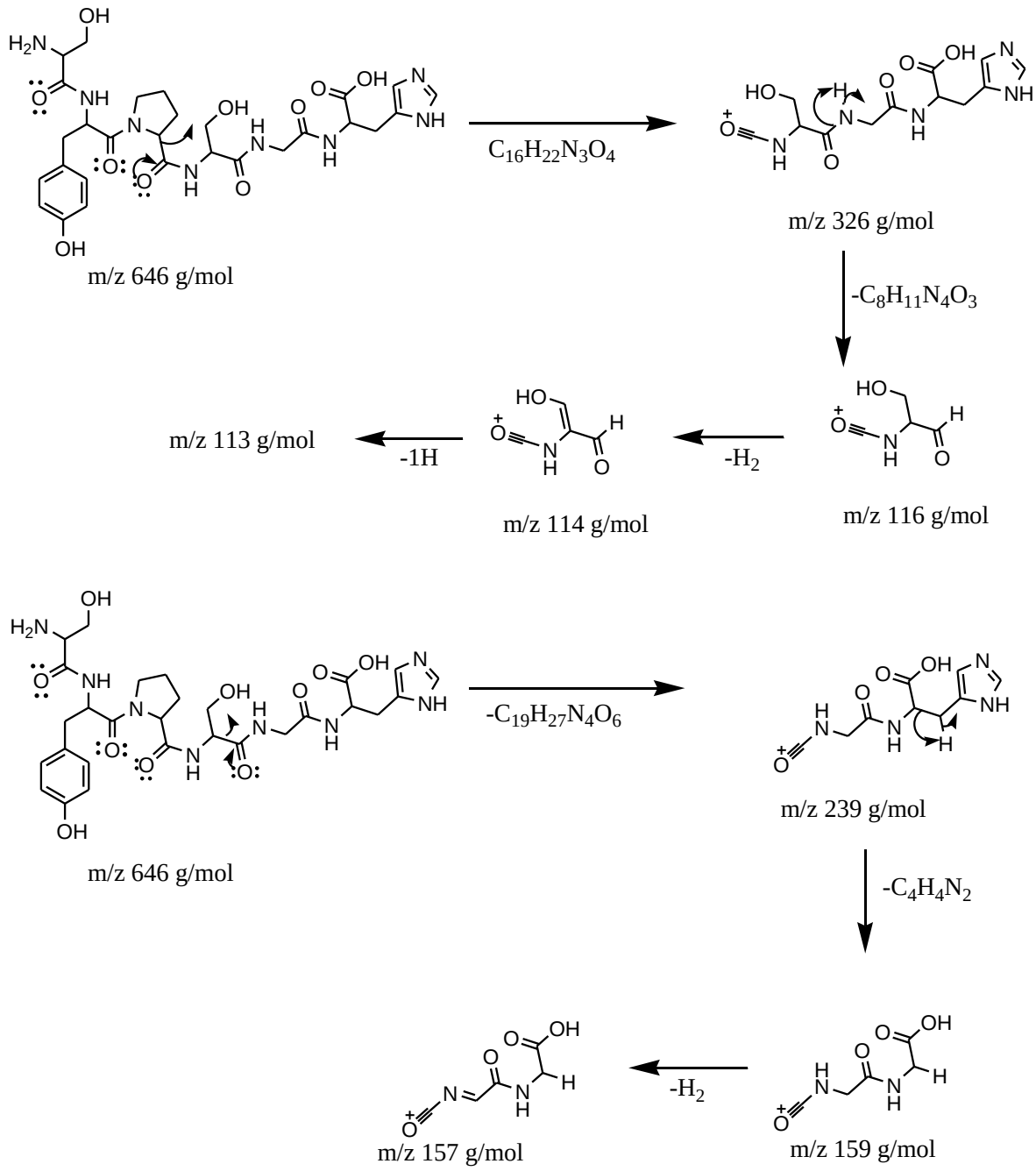
-H_2O

m/z 345 g/mol

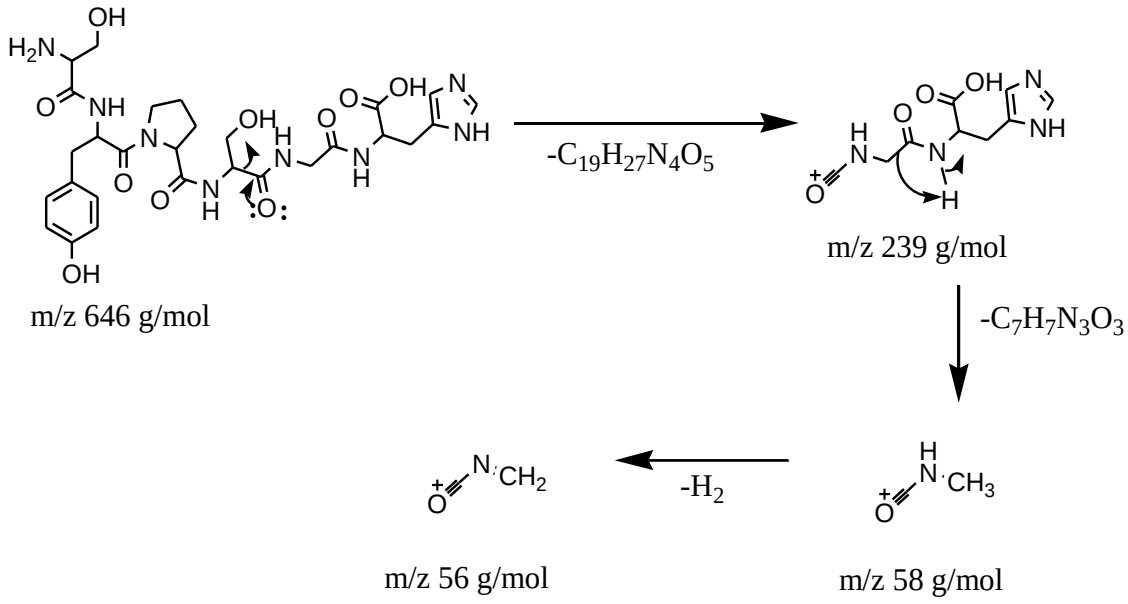
Continuing scheme



Continuing scheme



Continuing scheme



Fragments: 56, 60, 71, 85, 113, 157, 247, 288, 345 and 688 g/mol

Scheme 31: Fragmentation pathway for L-Seryl-L-tyrosyl-L-Prolyl-Serylglycyl-L-histidine (compound 28)

L-Seryl-L-tyrosyl-L-Prolyl-Serylglycyl-L-histidine is a peptide consisting of seryl, tyrosyl, prolyl, glycyl and histidine amino acids derivatives. Thus, it possess both antioxidant and antimicrobial activities (Coutinho *et al*., 2008).

Compound 29 was displayed at a retention time of 5.09 min with a characteristic molecular ion of m/z 698.3 and produced fragment ions at m/z 652, 635, 608, 592, 564, 547, 503, 476, 447, 360, 316, 251, 163, 71 and 59. This corresponded to Pentacarboxyporphyrin (fig. 5.29).

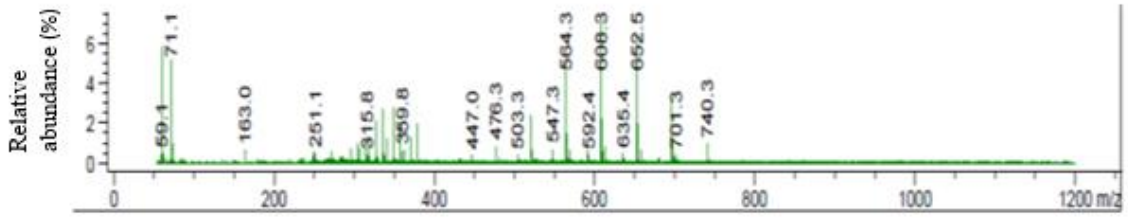
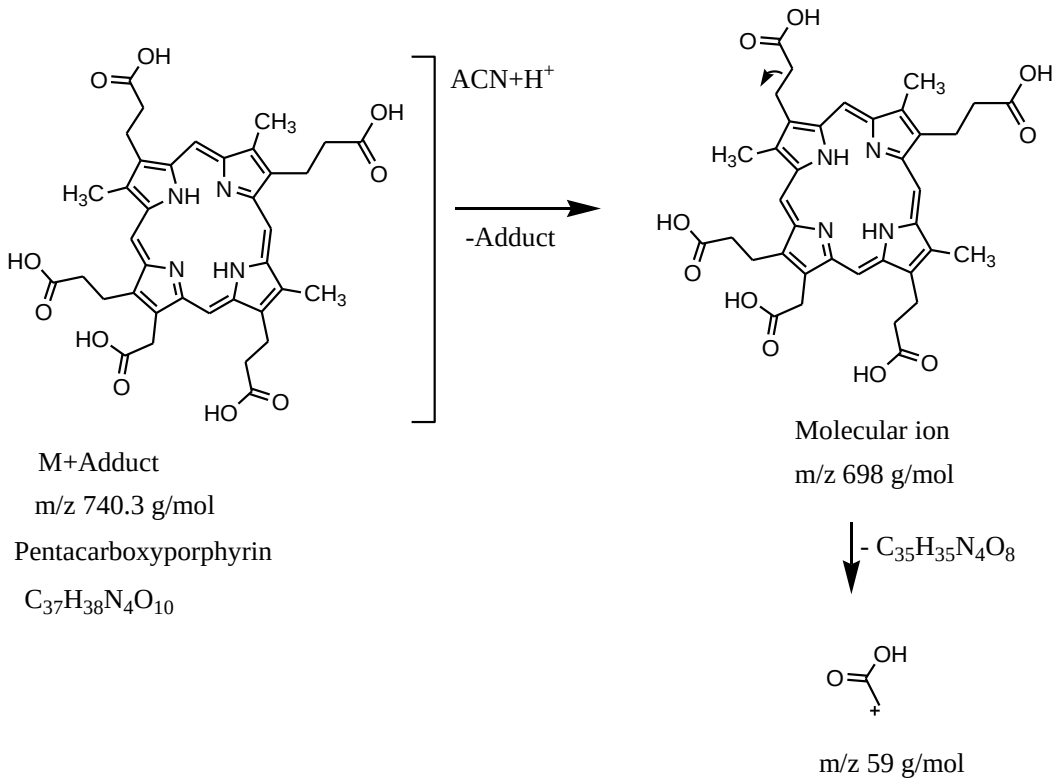


Fig. 5.29: The mass spectrum of compound 29

The fragment ion at m/z 652 resulted from loss of CH_2OH while m/z 635 fragment ion was due to loss of $C_2H_3O_2$ and $2H_2$. The fragment ion m/z 608 was due to abstraction of CO_2 and CH_2O_2; further loss of CO_2 from the m/z 608 led to the fragment m/z 564. Further loss of CH_3 and H_2 from the m/z 564 led to the m/z 547. Further decarboxylation (loss of CO_2) from the m/z 547 fragment resulted to the m/z 502 fragment. In addition, extra loss of C_2H_3 from the m/z 503 fragment led to the m/z 476 fragment. However, m/z 592 fragment resulted from abstraction of $C_2H_3O_2$, CO_2, H_2 and H. M/z 447 fragment resulted from the loss of CH_2OH, CH_3, H_2, two C_2H_4, decarboxylation (loss of three CO_2) while m/z 360 and 316 were as a result of $C_{19}H_{18}N_2O_4$ and $C_{20}H_{18}N_2O_6$ loss respectively. The fragment ion at m/z 251 resulted from the loss of $C_{20}H_{18}N_2O_6$, CO_2, CH_3, three H_2 while m/z 163 resulted from the loss of $C_{20}H_{18}N_2O_6$, C_8H_{10}, NO_2 and H. Finally, m/z 71 fragment ion resulted from loss of $C_{34}H_{33}N_4O_8$ and H_2 while m/z 59 resulted from loss of $C_{35}H_{35}N_4O_8$ (scheme 32 on pages 170-175).



M+Adduct

m/z 740.3 g/mol

Pentacarboxyporphyrin

$C_{37}H_{38}N_4O_{10}$

Molecular ion

m/z 698 g/mol

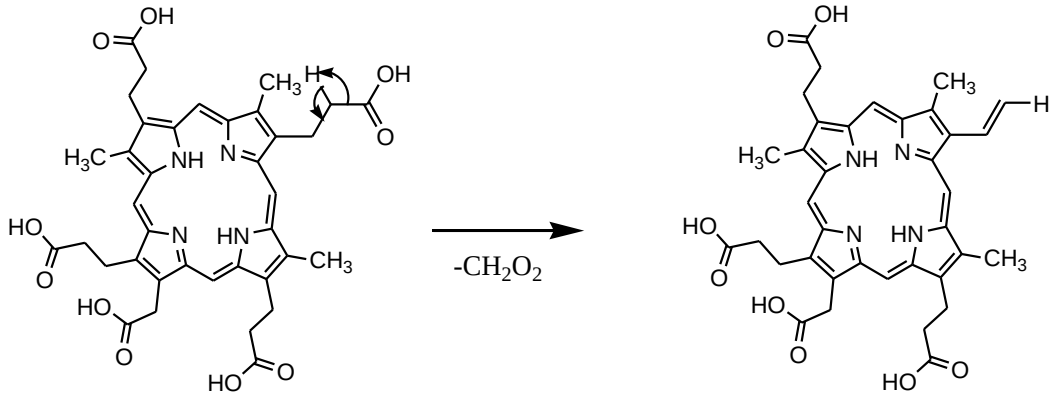
m/z 59 g/mol

m/z 73 g/mol

m/z 71 g/mol

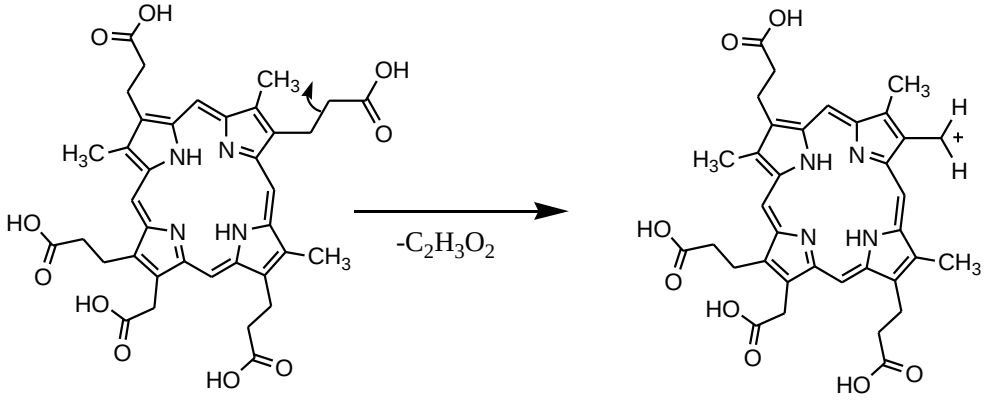
m/z 698 g/mol

Continuing scheme



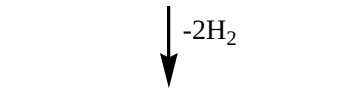
m/z 698 g/mol

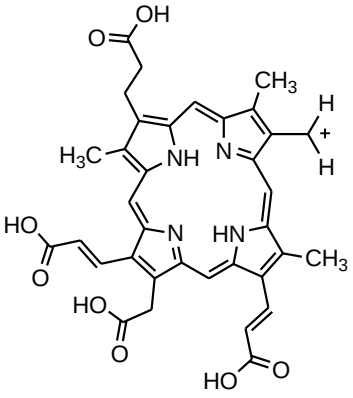
m/z 652 g/mol



m/z 698 g/mol

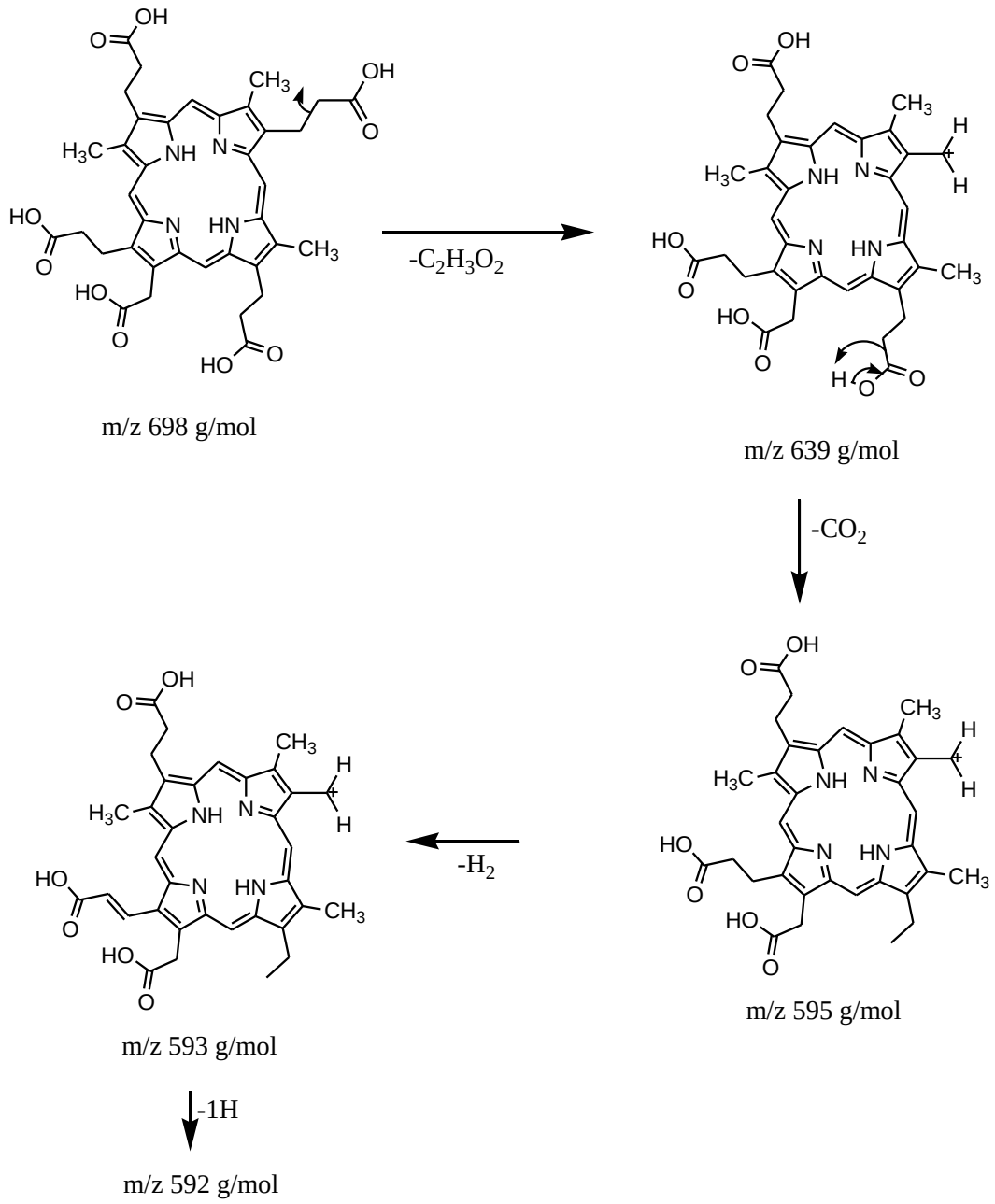
m/z 639 g/mol



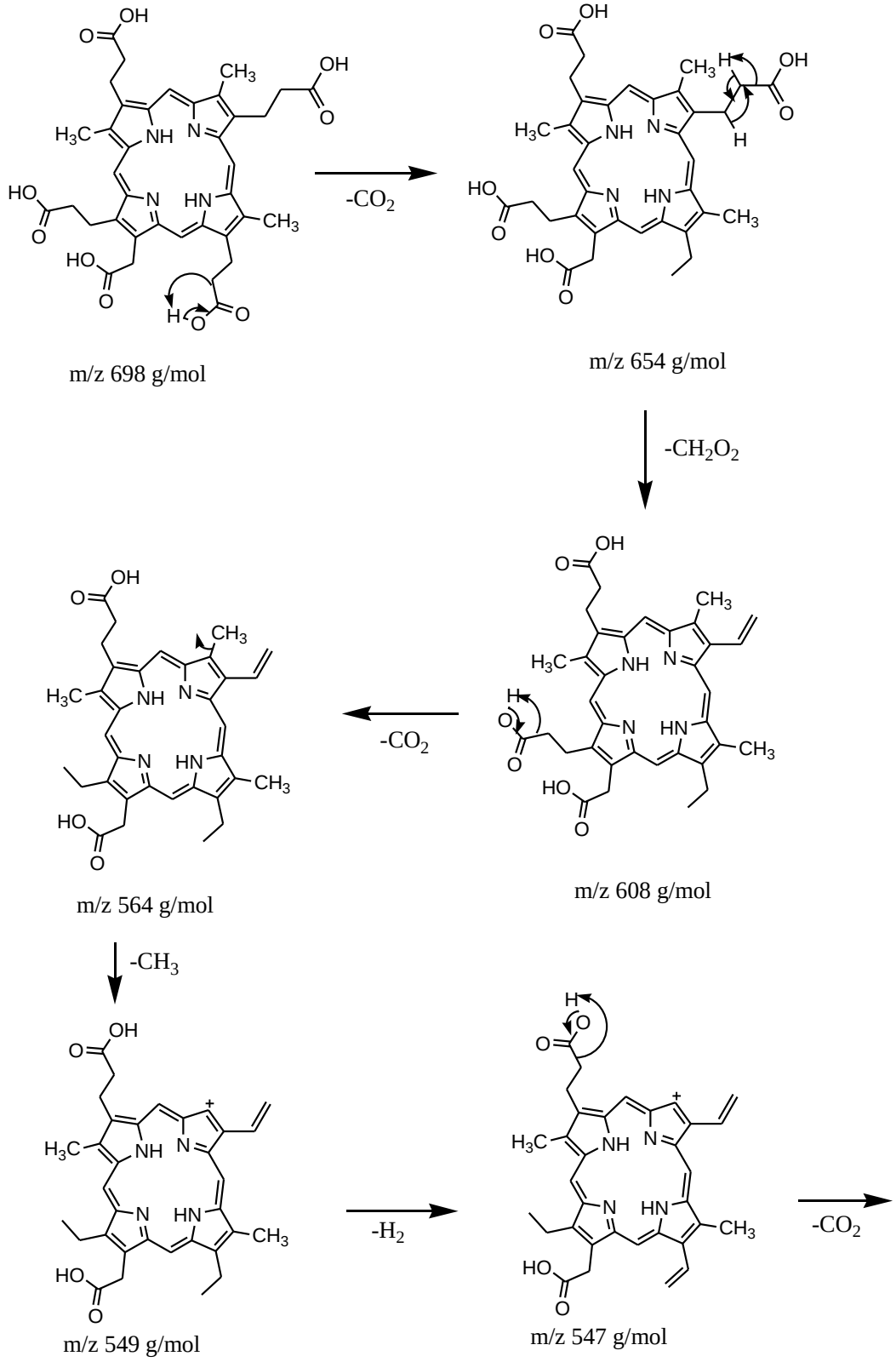


m/z 635 gmol

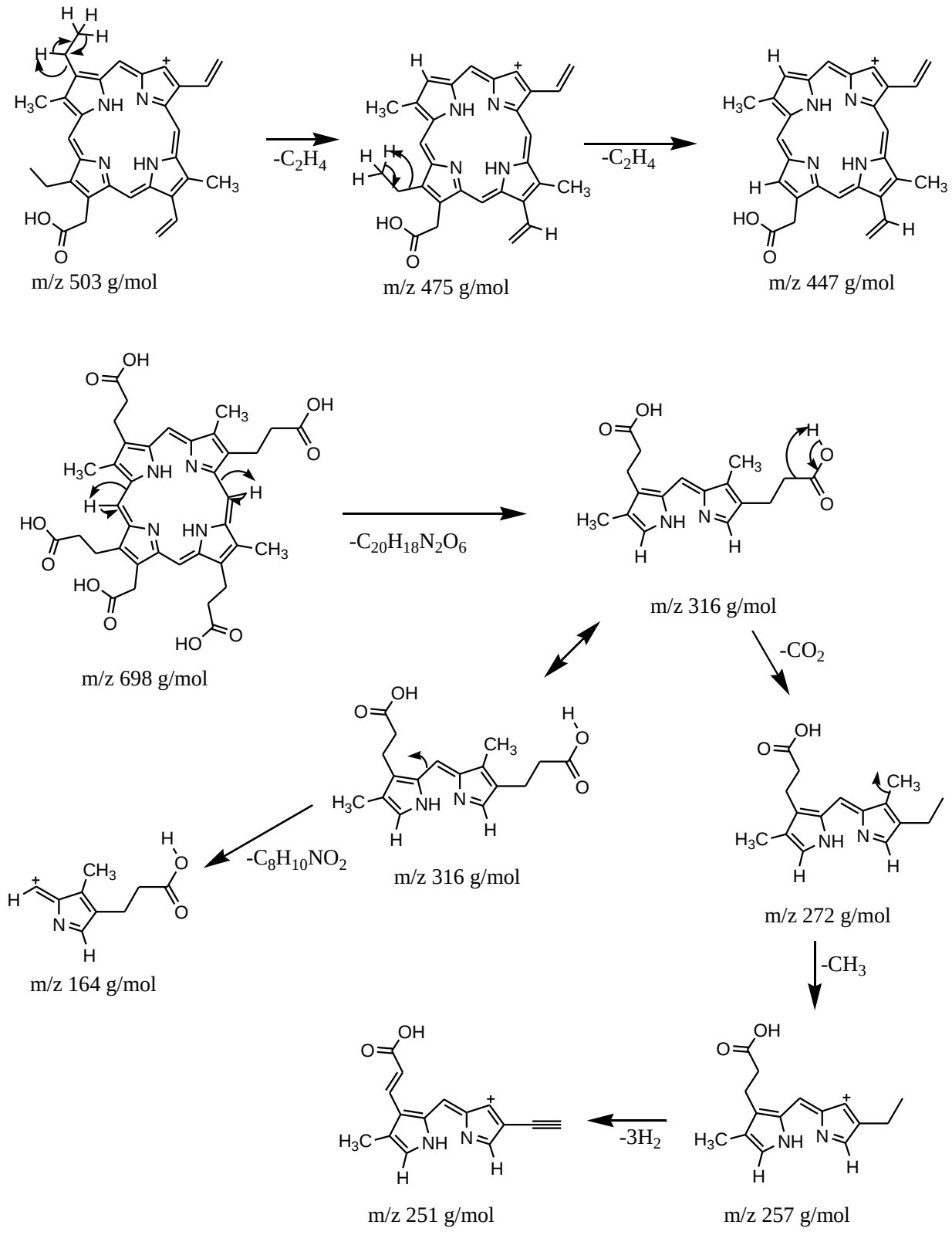
Continuing scheme



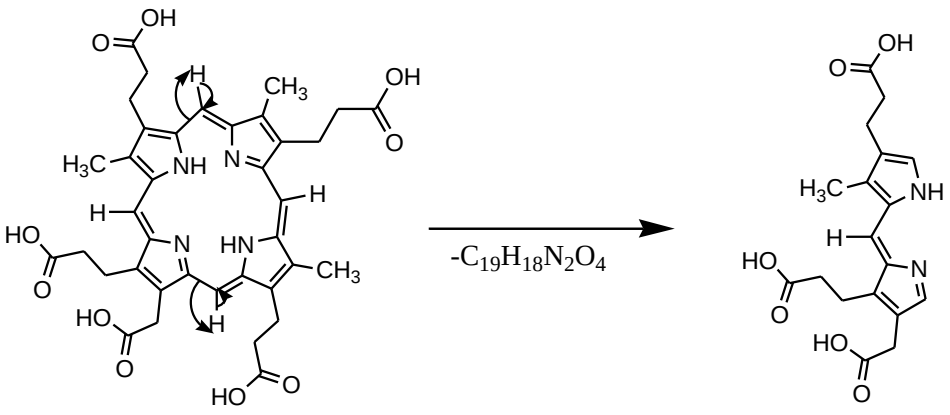
Continuing scheme



Continuing scheme



Continuing scheme



m/z 698 g/mol

mz 360 g/mol

Fragments: 59, 71, 163, 251, 316, 360, 447, 476, 503, 547, 564, 592, 608, 635, 652, 740.3 g/mol

Scheme 32: Fragmentation pathway for Pentacarboxyporphyrin (compound 29)

Pentacarboxyporphyrin belong to porphyrin class of organic compounds. This compound was extracted from red blood cells and plasma (Fyrestam, 2017). Related porphyrin derivatives including 5,10,15,20-tetrakis (4-aminophenyl) porphyrin and 5,10,15,20-tetrakis (4-hydroxyphenyl) porphyrin have been evaluated in their reaction with DPPH and were reported to possess antioxidant activity (Tesakova & Parfenyuk, 2017).

Compound 30 was characterized by a molecular ion of m/z 147.1 at a retention time at 25.05min and gave ions at m/z 72 and m/z 60. This corresponded to N, N-Di (methylcarbamoyl) hydroxylamine (fig. 5.30)

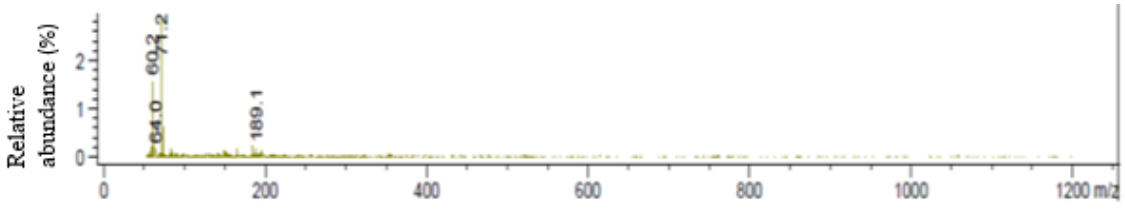
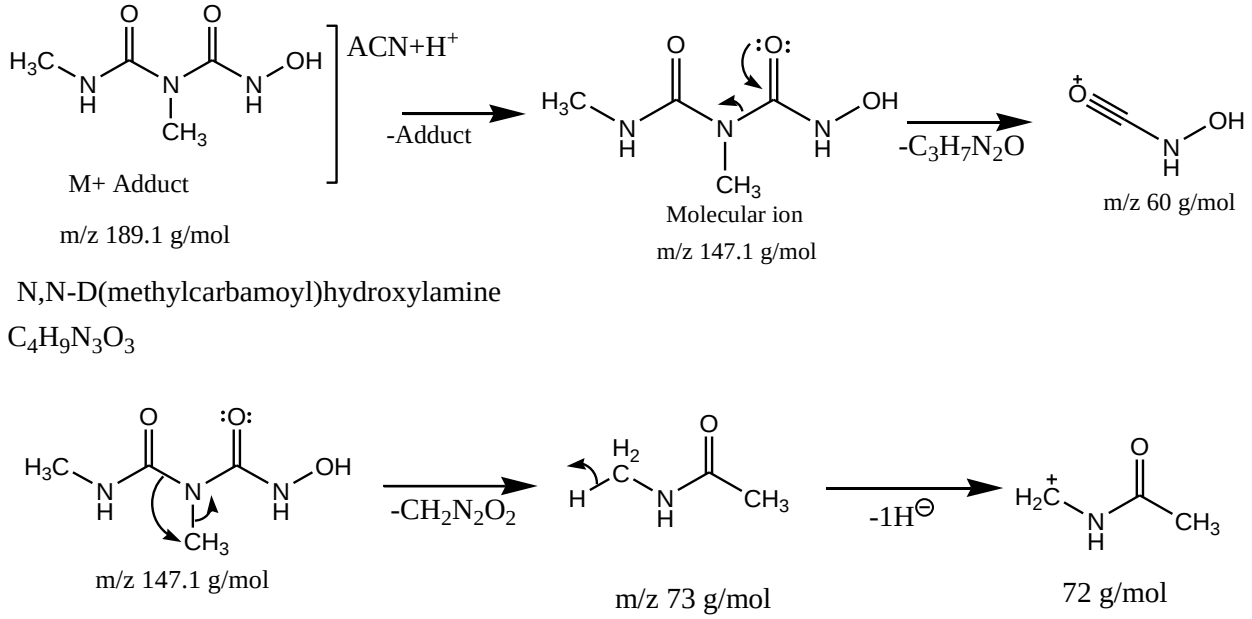


Fig. 5.30: The mass spectrum of compound 30

These fragments were related to the loss of $CH_2N_2O_2$, H and $C_3H_7N_2O$ respectively (scheme 33 below).



Fragments: 60, 72 and 189.1 g/mol

Scheme 33: Fragmentation pathway for N, N-Di (methylcarbamoyl) hydroxylamine (compound 30)

N, N-Di (methylcarbamoyl) hydroxylamine is not documented in literature. Its derivative, N-acetyl-O-methylcarbamoyl-hydroxylamine inhibits the enzyme ribonucleotide reductase of *E. coli* by interacting with its greater component protein R1 (Larsen *et al.*, 1992).

Compound 31 displayed a characteristic molecular ion of m/z 307.2 at a retention time of 42.23 min with fragment ions of m/z 261, 205, 187, 71 and 60. This corresponded to Ethyl 4-(6-acetamido-3-aminopyridin-2-yl) piperazine-1-carboxylate (fig. 5.31).

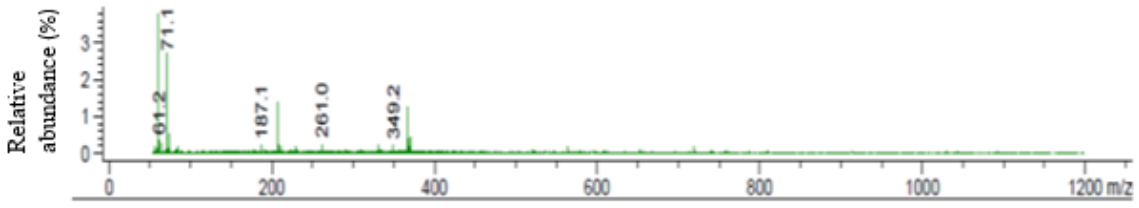
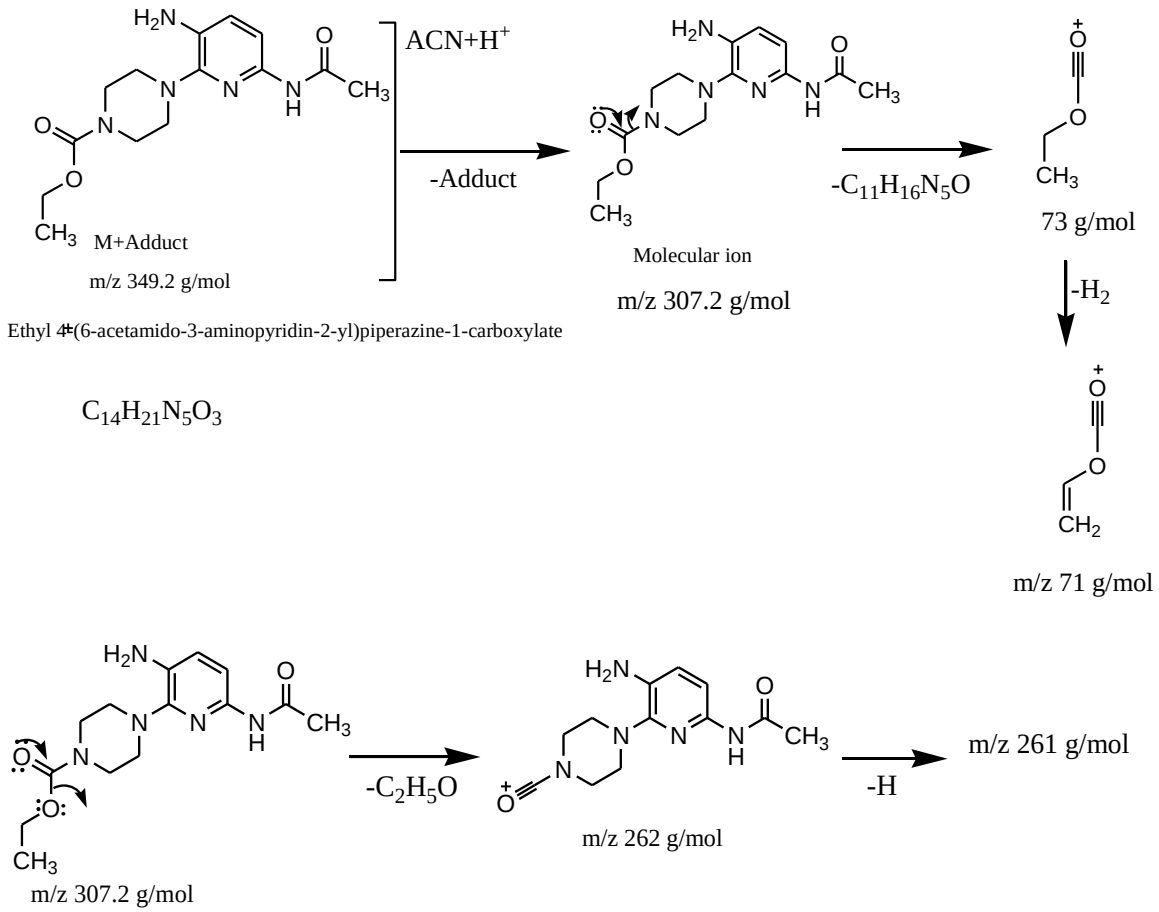
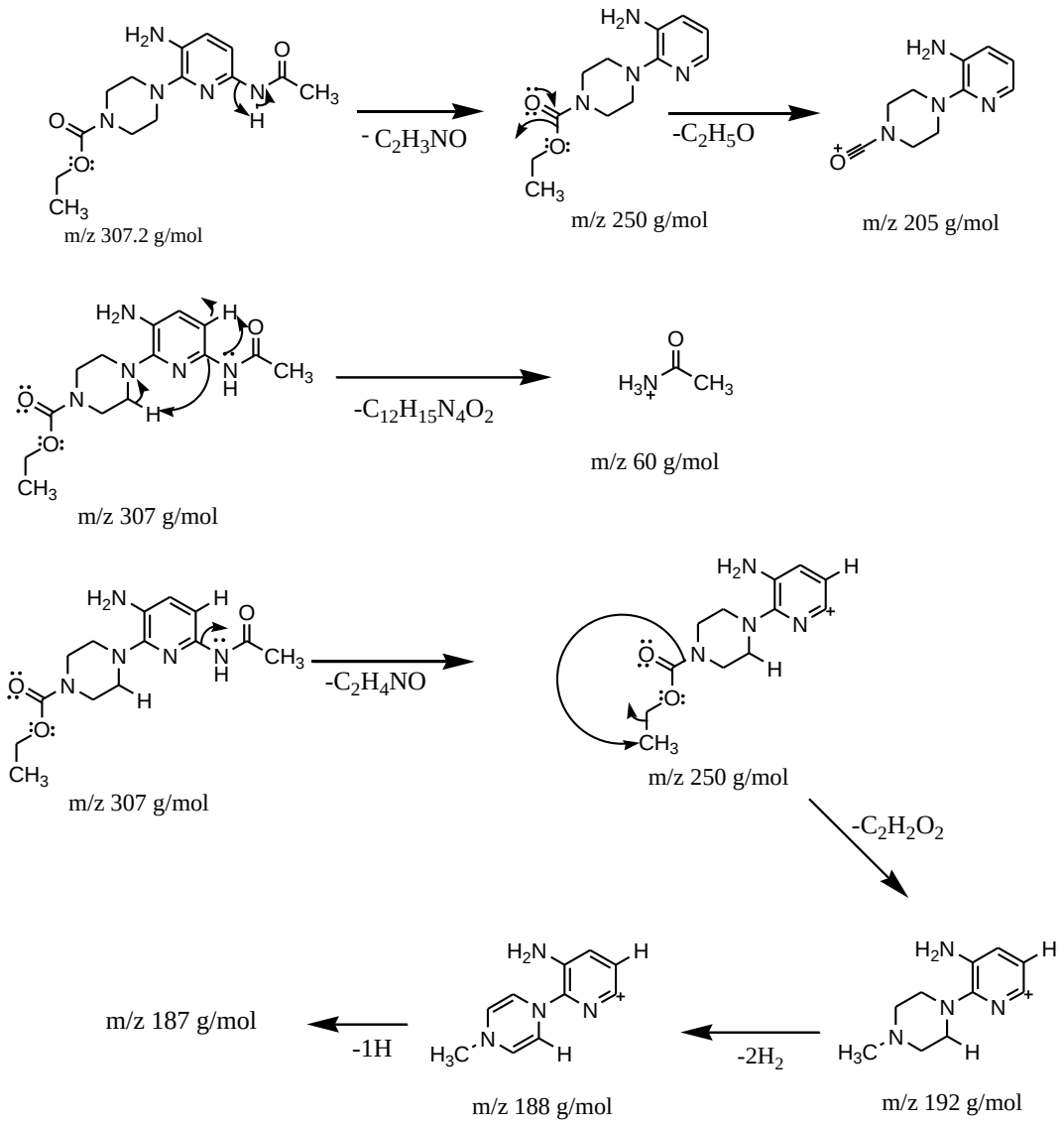


Fig. 5.31: The mass spectrum of compound 31

The fragment ion m/z 261 resulted from the loss of C_2H_5O and H while m/z 205 were due to loss of C_2H_3NO and C_2H_5O. Similarly, m/z 187 was attributed to loss of C_2H_4NO, $C_2H_2O_2$, $2H_2$ and H while m/z 71 was attributed to loss of $C_{11}H_{16}N_5O$ and H_2. Finally, m/z 60 was due to loss of $C_{12}H_{15}N_4O_2$ (scheme 34; pages 177 & 178).



Continuing scheme



Fragments: 60, 71, 187, 205, 261 and 349.2 g/mol

Scheme 34: Fragmentation pathway for Ethyl 4-(6-acetamido-3-aminopyridin-2-yl) piperazine-1-carboxylate (compound 31)

Ethyl 4-(6-acetamido-3-aminopyridin-2-yl) piperazine-1-carboxylate is a pyridyl piperazine derivative. No reported pyridyl piperazine derivatives have been reported in honey. A novel 1-(2-pyridyl) piperazine derivatives have been synthesized. They were tested as inhibitors of the histamine-induced bronchospasm in the guinea pig and of the

reaginic passive cutaneous anaphylaxis (PCA) in the rat (Catto *et al*., 1987). Some of the piperazine derivatives studied including phenyl piperazine, diphenylalkyl piperazine, benzylpiperazine (BZP), benzhydryl piperazine, trifluoromethylpiperazine (TFMPP) showed anti-depressant, anti-microbial, anti-parkinson, antipsychotic, antidiabetic, antioxidant, anticonvulsant, antiarrhythmic, anti-inflammatory, anxiolytic, antialzheimer, antiplatelet aggression, antihypertensive, antimalarial, and anti-histaminic antiproliferative activities (Singh *et al*., 2015).

Compound 32 had a molecular ion of m/z 355.1 with a retention time of 50.04 min and produced fragment ions of m/z 353, 71 and 60. This corresponded to Cys-Met-Cys (fig. 5.32).

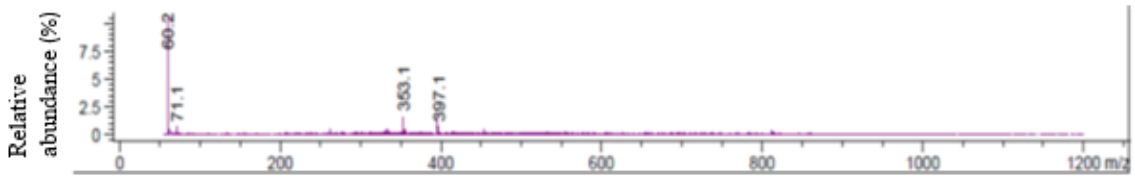
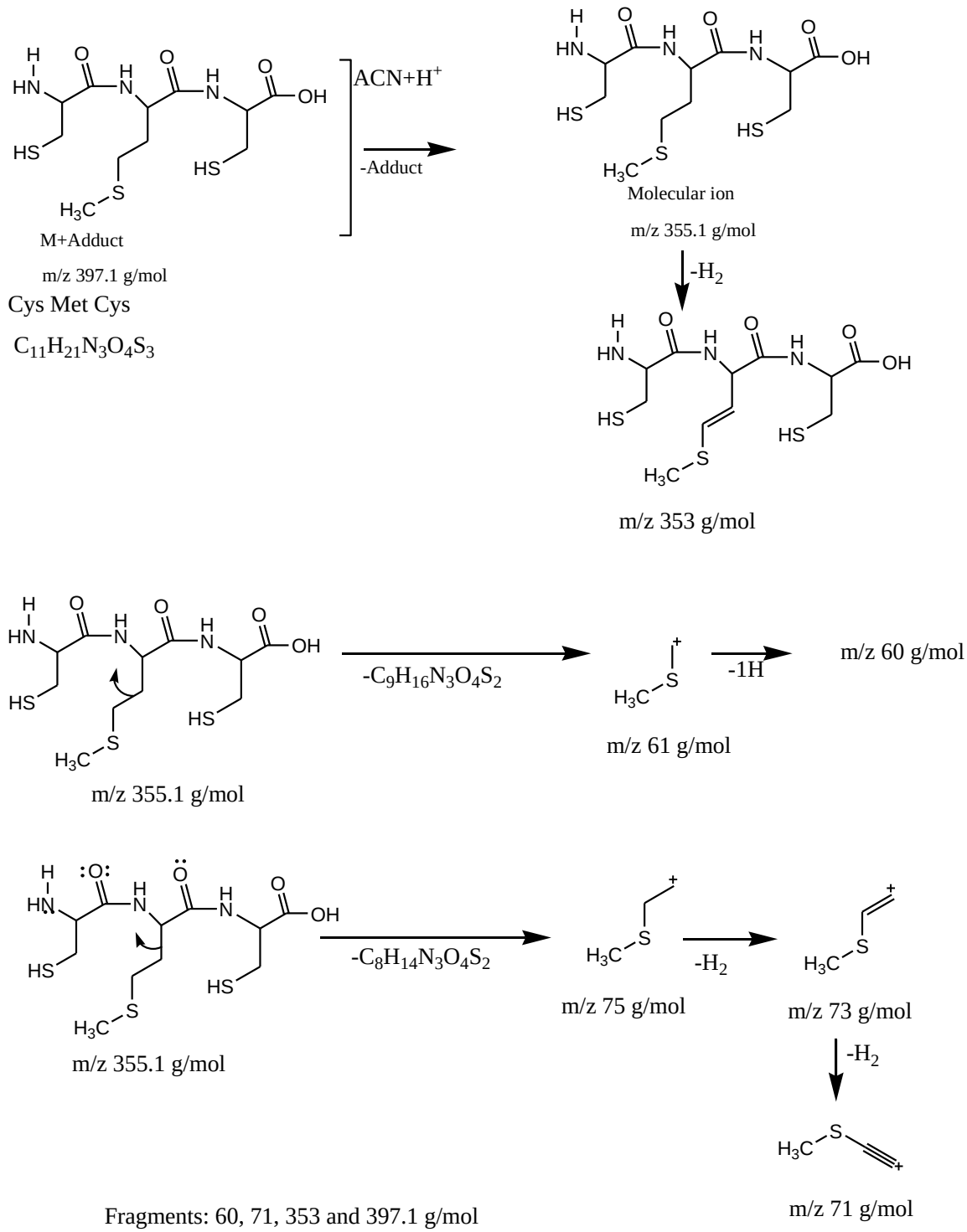


Fig. 5.32: The mass spectrum of compound 32

The fragment m/z 353 was due to dehydrogenation (loss of H_2). Likewise, m/z 71 was related to loss of $C_8H_{14}N_3O_4S_2$ and two H_2 while m/z 60 was related to loss of $C_9H_{16}N_3O_4S_2$ and H (scheme 35 on page 180).



Scheme 35: Fragmentation pathway for Cys-Met-Cys (compound 32)

Cys-Met-Cys is a tripeptide comprising of cysteine and methionine amino acids. Both amino acids have been characterized in honey (Kıvrak, 2017). They were also isolated from bee pollens from main floral sources in Al-Ahsa in eastern Saudi Arabia (Taha *et al*., 2019). Being a peptide, this compound possess antioxidant and antimicrobial activities (Coutinho *et al*., 2008; Elias *et al*., 2008).

Compound 33 displayed a characteristic molecular ion of 478.3 at a retention time 18.17min with fragment ions of m/z 177, 165, 149, 131, 85, 71, 60 and 59. This corresponded to D-Alanyl-O-{10-[(4-ethylbenzoyl) oxy] decanoyl}-L-serine (fig. 5.33).

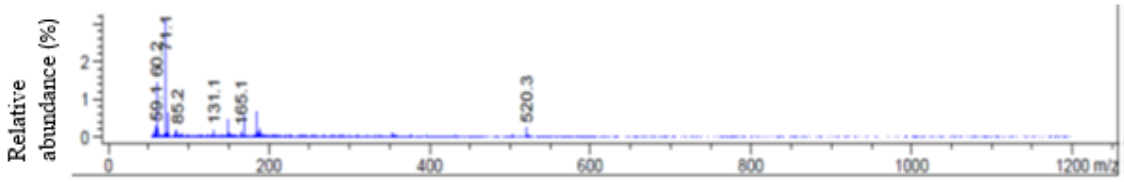
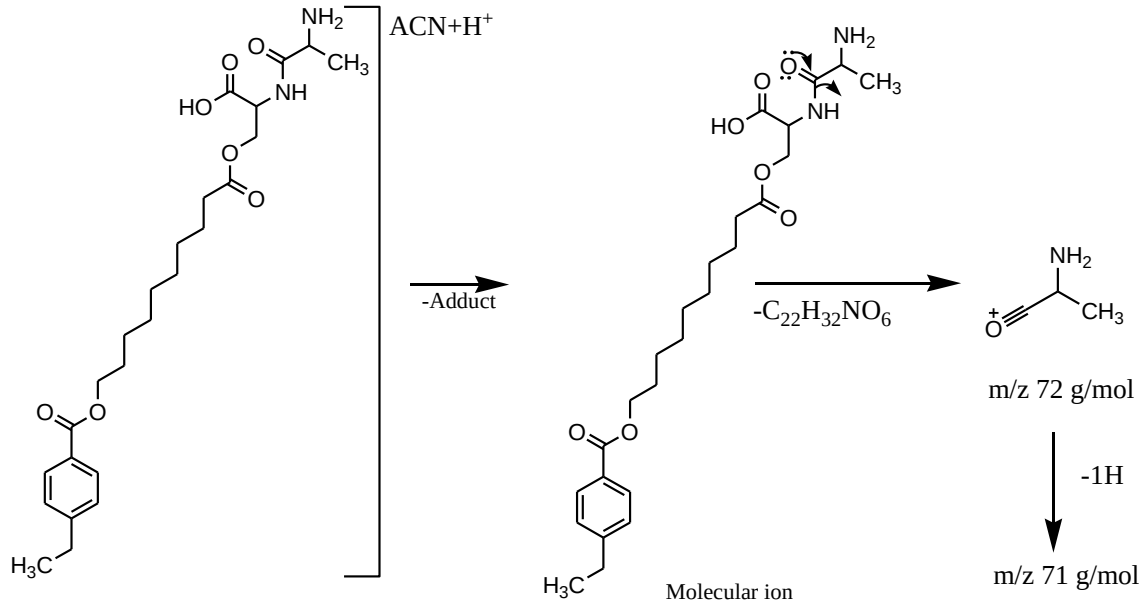


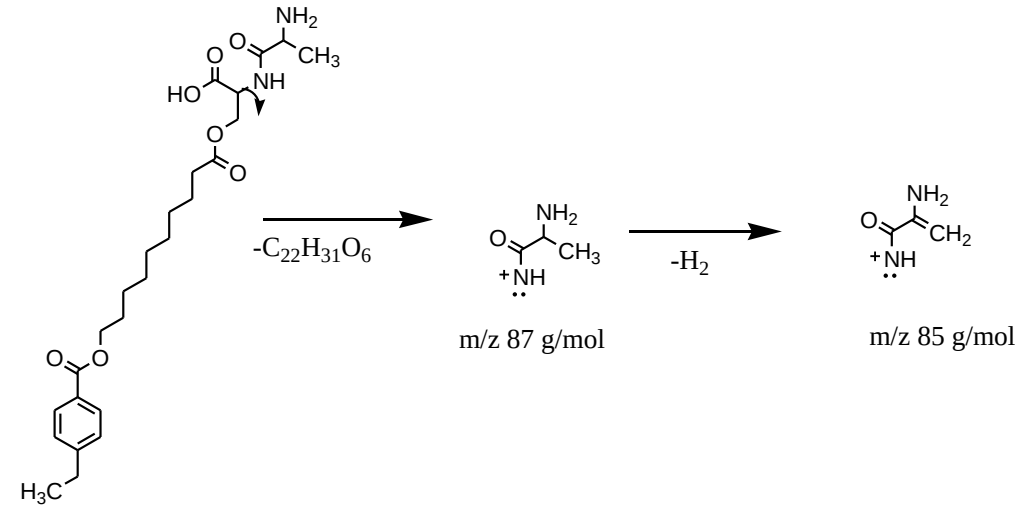
Fig. 5.33: The mass spectrum of compound 33

The fragment ion m/z 177 was due to loss of $C_{11}H_{13}O_2$ while m/z 165 was related to loss of $C_6H_{11}N_2O_4$, C_9H_8O and three H_2. M/z 149 was due to loss of $C_{16}H_{29}N_2O_5$ while m/z 131 resulted from loss of $C_{16}H_{29}N_2O_6$ and H_2. Similarly, m/z 85 was related to loss of $C_{22}H_{31}O_6$ and H_2 while m/z 71 was related to loss of $C_{22}H_{32}NO_6$ and H. The fragment 60 was due to loss of C_8H_9, $C_{15}H_{27}N_2O_5$ while m/z 59 was related to loss of C_8H_9 and $C_{15}H_{26}N_2O_5$ (scheme 36 on pages 182-184).

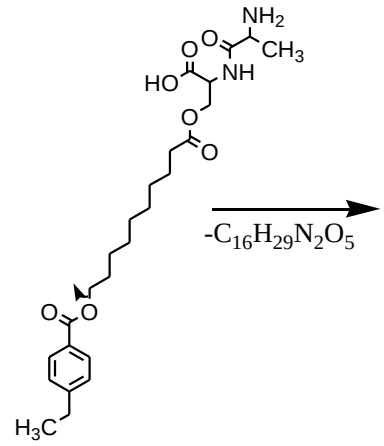


D-Alanyl-O-{10-[(4-ethylbenzoyl)decanoyl}-L-Serine

$C_{25}H_{38}N_2O_7$

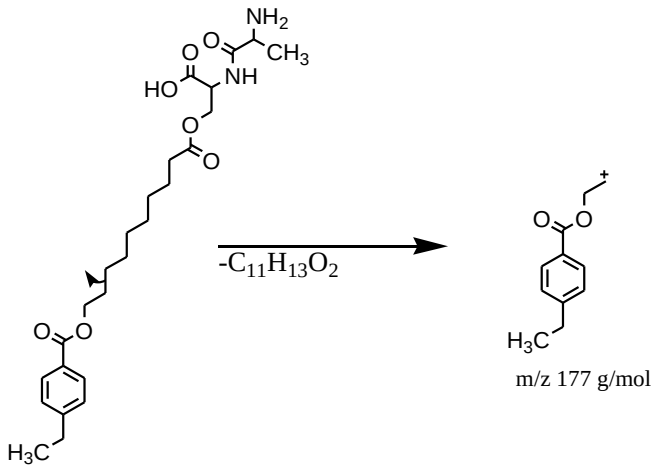


Continuing scheme



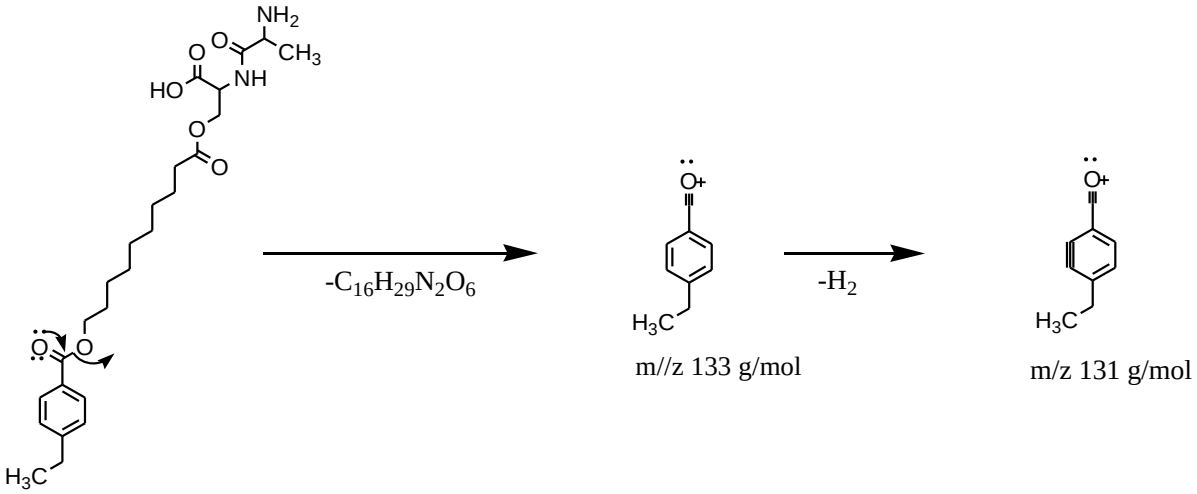
m/z 478 g/mol

m/z 149 g/mol



m/z 478 g/mol

m/z 177 g/mol

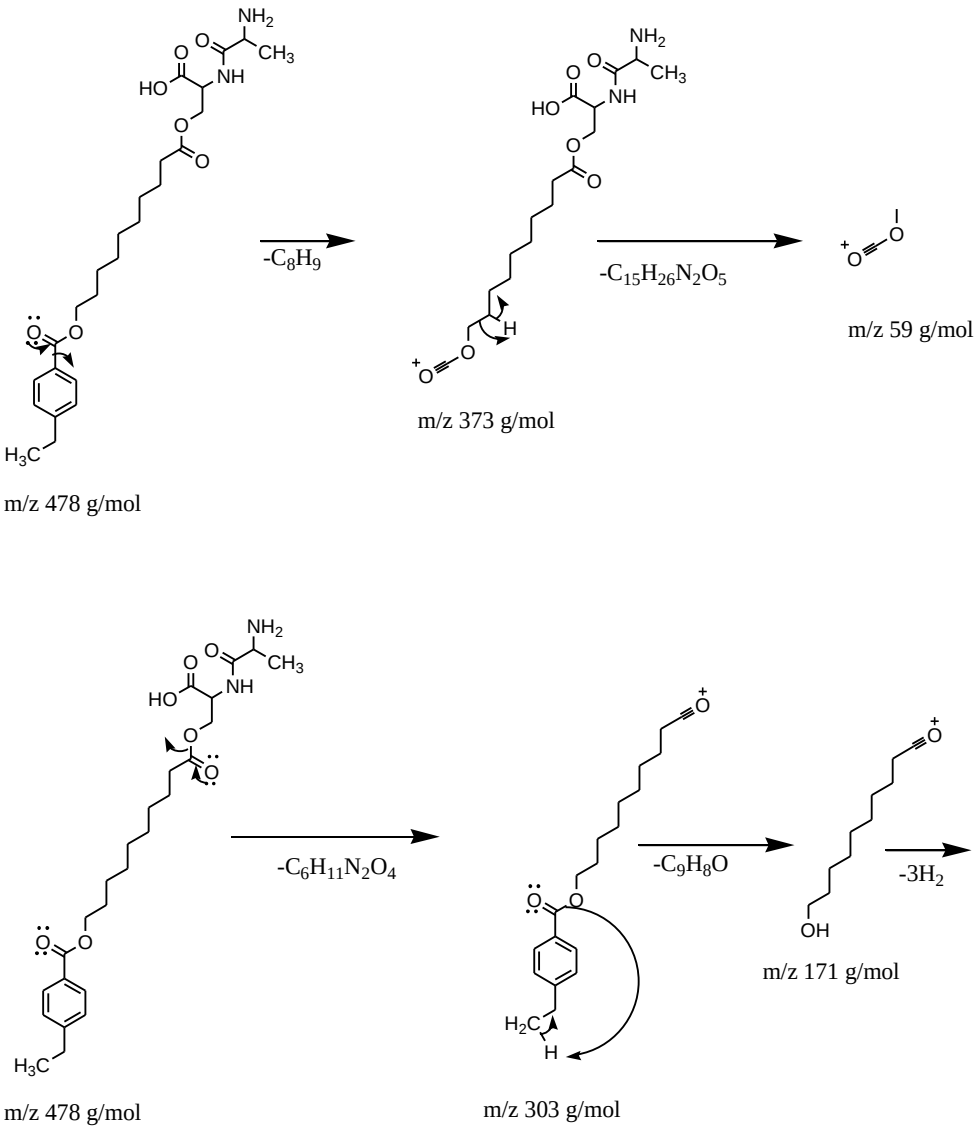


m/z 478 g/mol

m//z 133 g/mol

m/z 131 g/mol

Continuing scheme



Fragments: 59, 60, 71, 85, 131, 149, 165, 177 and 520.3 g/mol

Scheme 36: Fragmentation pathway for D-Alanyl-O-{10-[(4-ethylbenzoyl) oxy] decanoyl}-L-serine (compound 33)

D-Alanyl-O-{10-[(4-ethylbenzoyl) oxy] decanoyl}-L-serine is a peptide derivative with alanine and serine derivatives. These amino acids have been identified in forest honeys in South Sulawesi, Indonesia (Noor *et al*., 2019). They identification was also done in Polish and Slovak honeys (Kowalski *et al*., 2017). Kıvrak (2017) also confirmed their presence

in honey. Being a peptide it is believed to possess both antimicrobial and antioxidant activities (Elias *et al*., 2008; Coutinho *et al*., 2008).

Compound 34 was detected at a retention time of 37.45 min with a characteristic fragment ion of m/z 611.3 and produced fragment ions at m/z 610, 608, 566, 564, 520, 503, 477, 432, 326, 291, 263, 153, 85, 71, and 60. This corresponded to {4-[5-Benzyloxy-2-(4-benzyloxy-phenyl)-3-methyl-Indol-1-ylmethyl]-phenoxy}-Acetic Acid Ethyl ester (fig. 5.34).

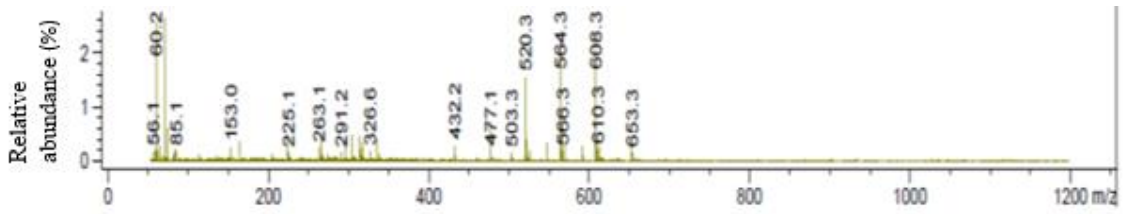
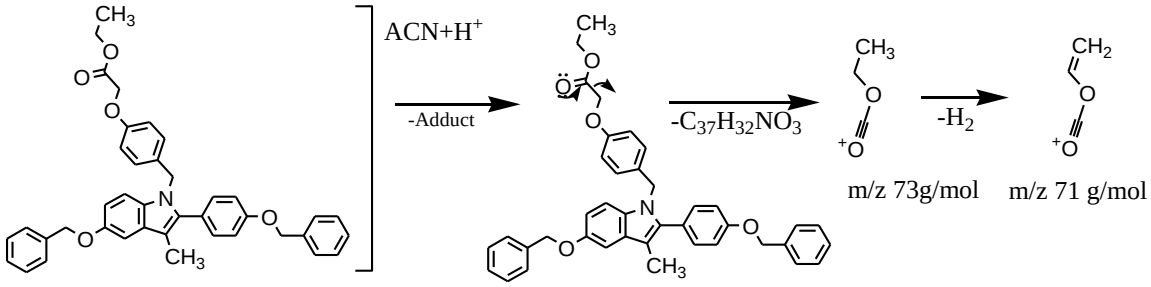


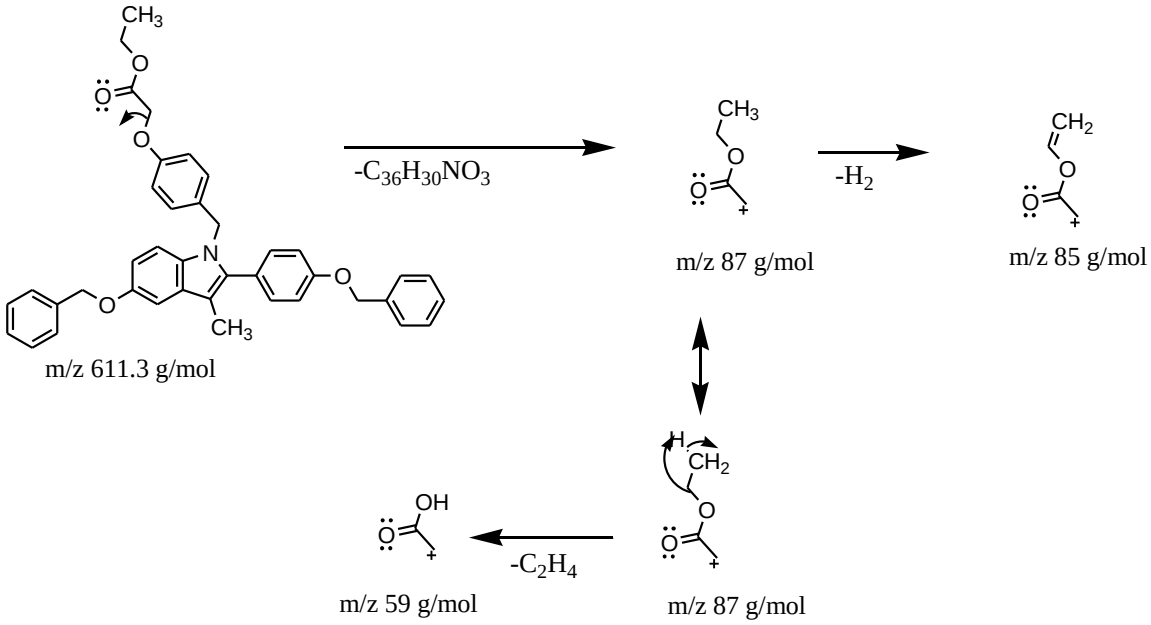
Fig. 5.34: The mass spectrum of compound 34

The fragment ion at m/z 610 resulted from the loss of H^- while m/z 608 resulted from the loss of H_2 and H^-. Likewise, m/z 566 was attributed to loss of C_2H_5O which further on further loss of H_2 resulted to m/z 564 while m/z 520 was attributed to loss C_7H_7 which on further loss of C_2H_3O led to the fragment ion m/z 477. Similarly, m/z 503 resulted from loss of C_7H_7O and H. In the same way, m/z 432 was due to loss of $C_{10}H_{11}O_3$ while m/z 326 was due to loss of $C_{11}H_{14}O_3$ and C_7H_7. Also, m/z 291 resulted from abstraction of $C_{13}H_{11}O$, C_7H_7, C_2H_5O and H_2 while m/z 263 was due to abstraction of $C_{13}H_{11}O$, C_7H_7, C_2H_5O and CH_2O. In addition, m/z 153 resulted from loss of $C_{27}H_{26}NO_4$ and CH_2O while m/z 85 resulted from loss of $C_{36}H_{30}NO_3$ and H_2. Also, m/z 71 was related to loss of $C_{37}H_{32}NO_3$ and H_2 while m/z 60 was related to loss of $C_{36}H_{30}NO_3$ and C_2H_4 (scheme 37 on pages 186-190).

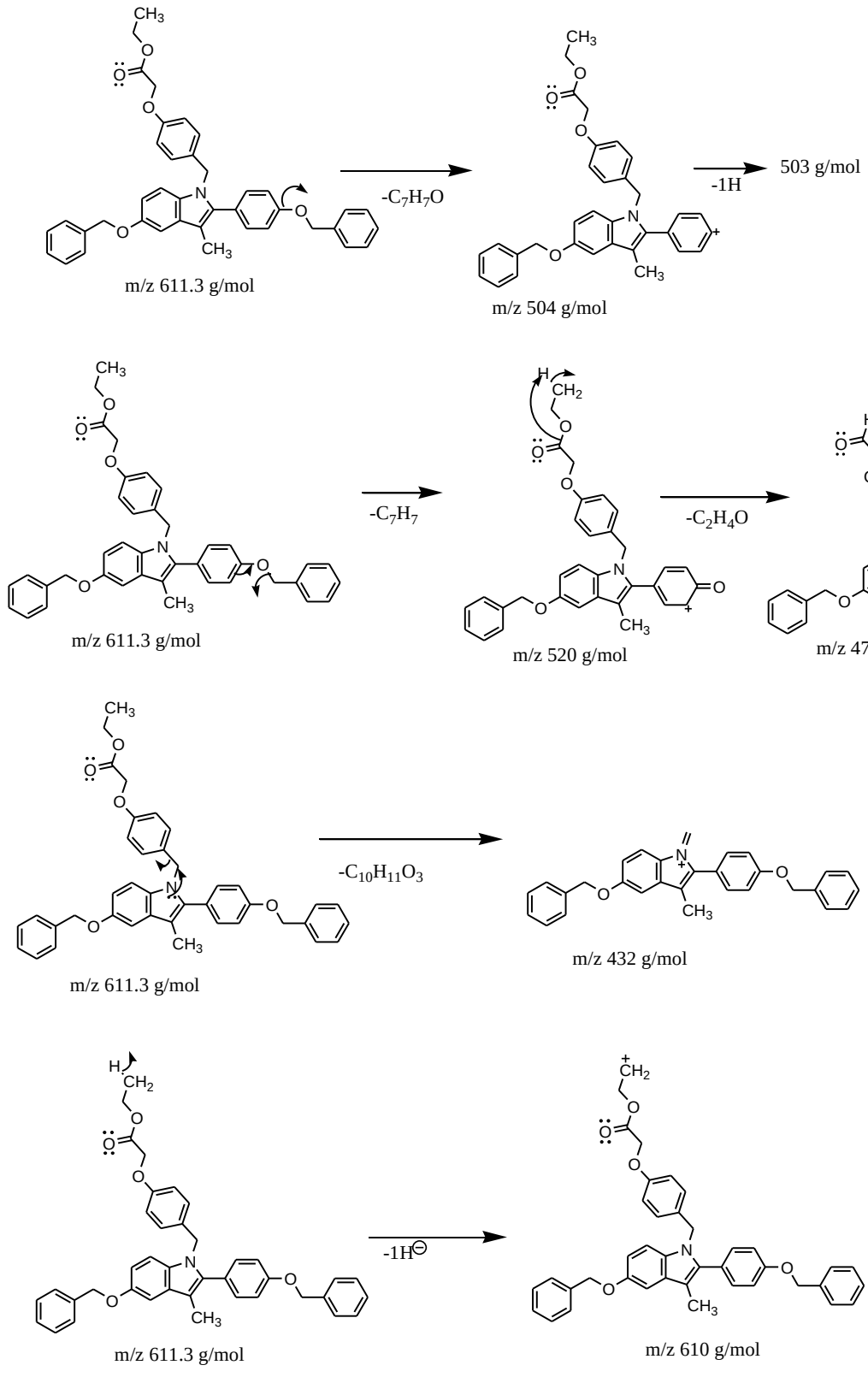


{4-[5-benzyloxy-2-(4-Benzyloxy-phenyl)-3-methyl-Indol-lylmethyl]-phenoxy}-acetic acid ethyl ester

$C_{36}H_{38}N_4O_8$



Continuing scheme



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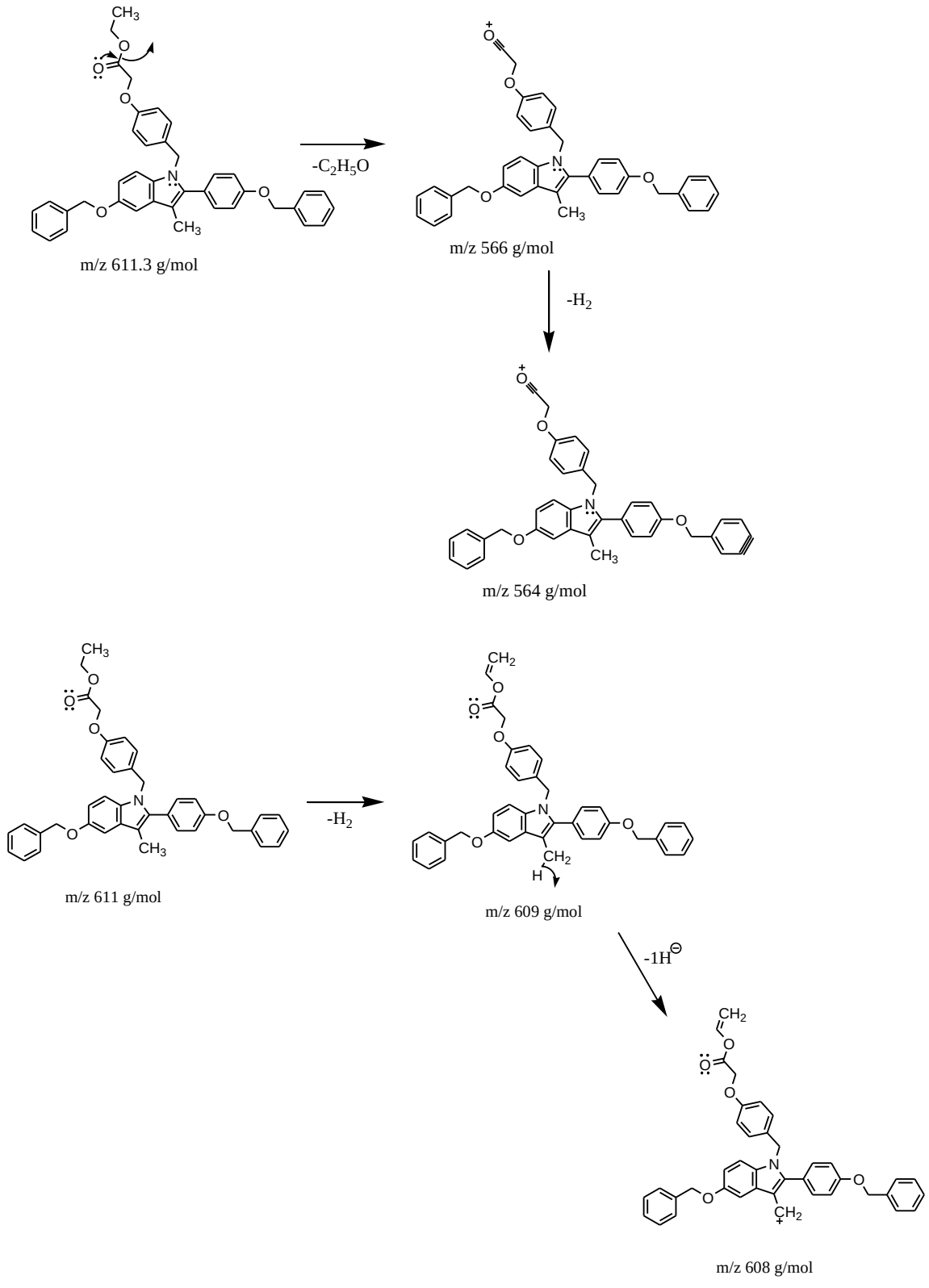
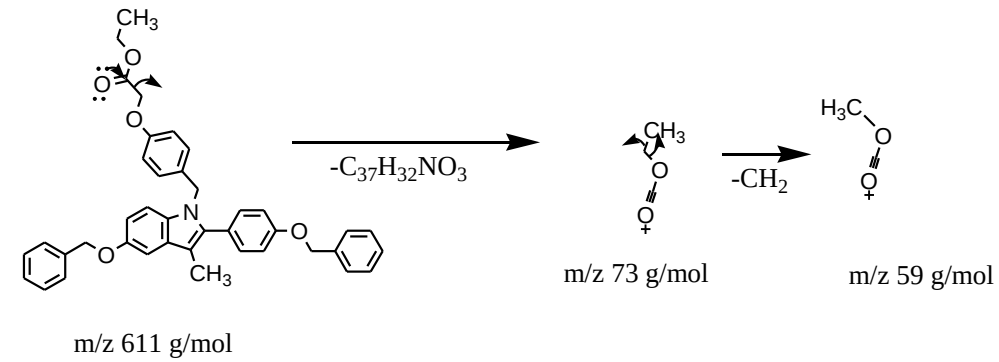
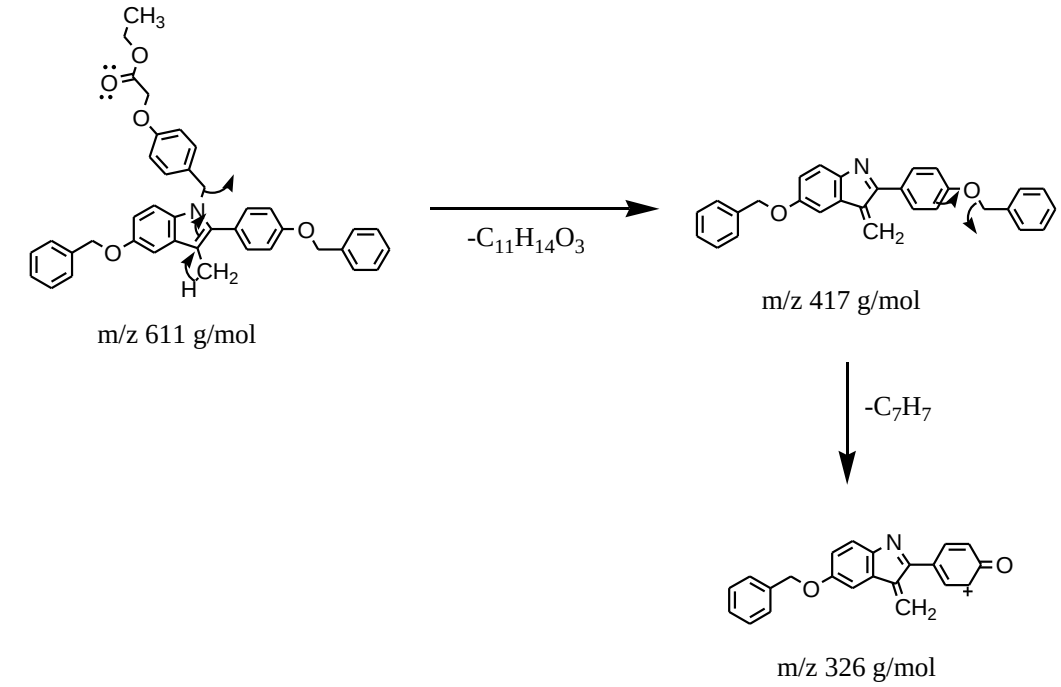
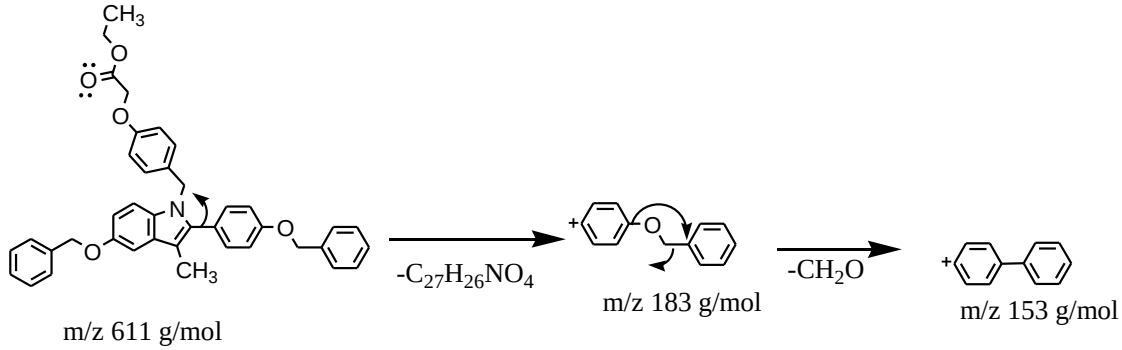


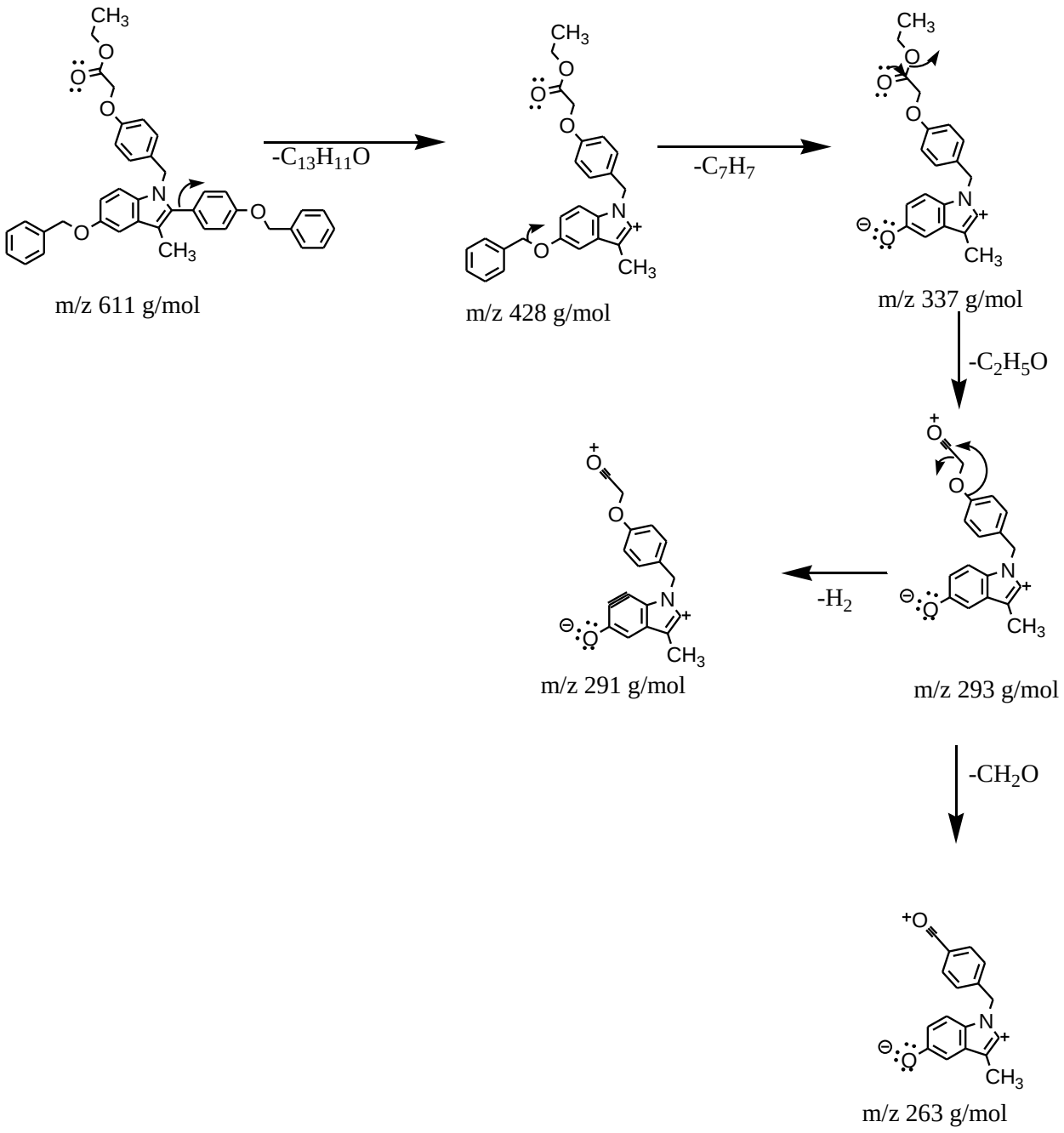
Continuing scheme







Continuing scheme



Fragments: 60, 71, 85, 153, 263, 291, 326, 432, 477, 503, 520, 564, 566, 608, 610 and 653 g/mol

Scheme 37: Fragmentation pathway for {4-[5-Benzyloxy-2-(4-benzyloxy-phenyl)-3-methyl-Indol-1-ylmethyl]-phenoxy}-Acetic Acid Ethyl ester (compound 34).

4-[5-Benzyloxy-2-(4-benzyloxy-phenyl)-3-methyl-Indol-1-ylmethyl]-phenoxy}-Acetic Acid Ethyl ester is an indole derivative compound. It was synthesized by Miller *et al*. (1999) and as estrogenic agent. The indole moiety makes it possess antimicrobial and antioxidant activities (Saundane *et al*., 2013).

Compound 35 had a characteristic molecular ion of m/z 654.3 with a retention time of 37.91min and produced fragment ions of m/z 653, 613, 608, 566, 564, 525,476, 337, 307, 223, 171, 71 and 59. This corresponded to Tyr-Phe-Tyr-Tyr (fig. 5.35).

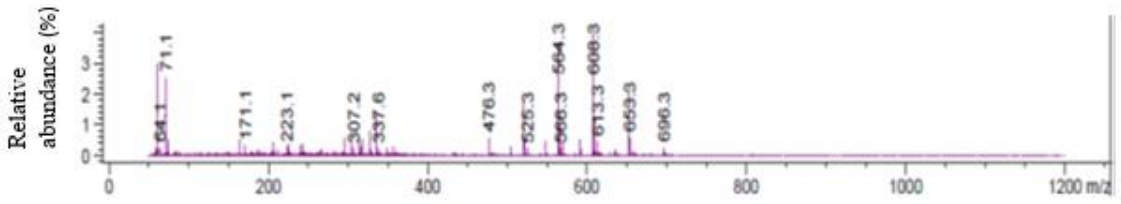
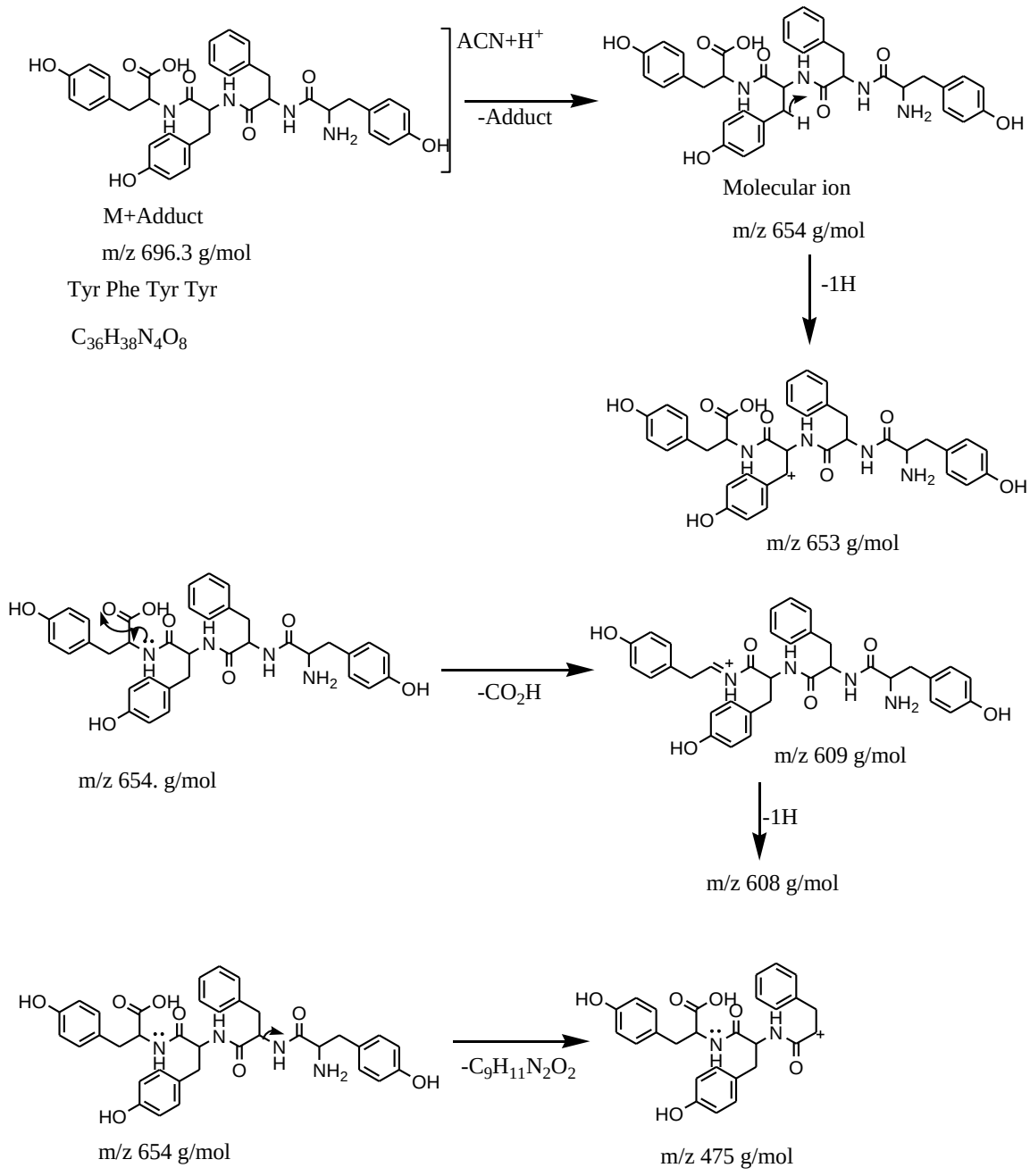
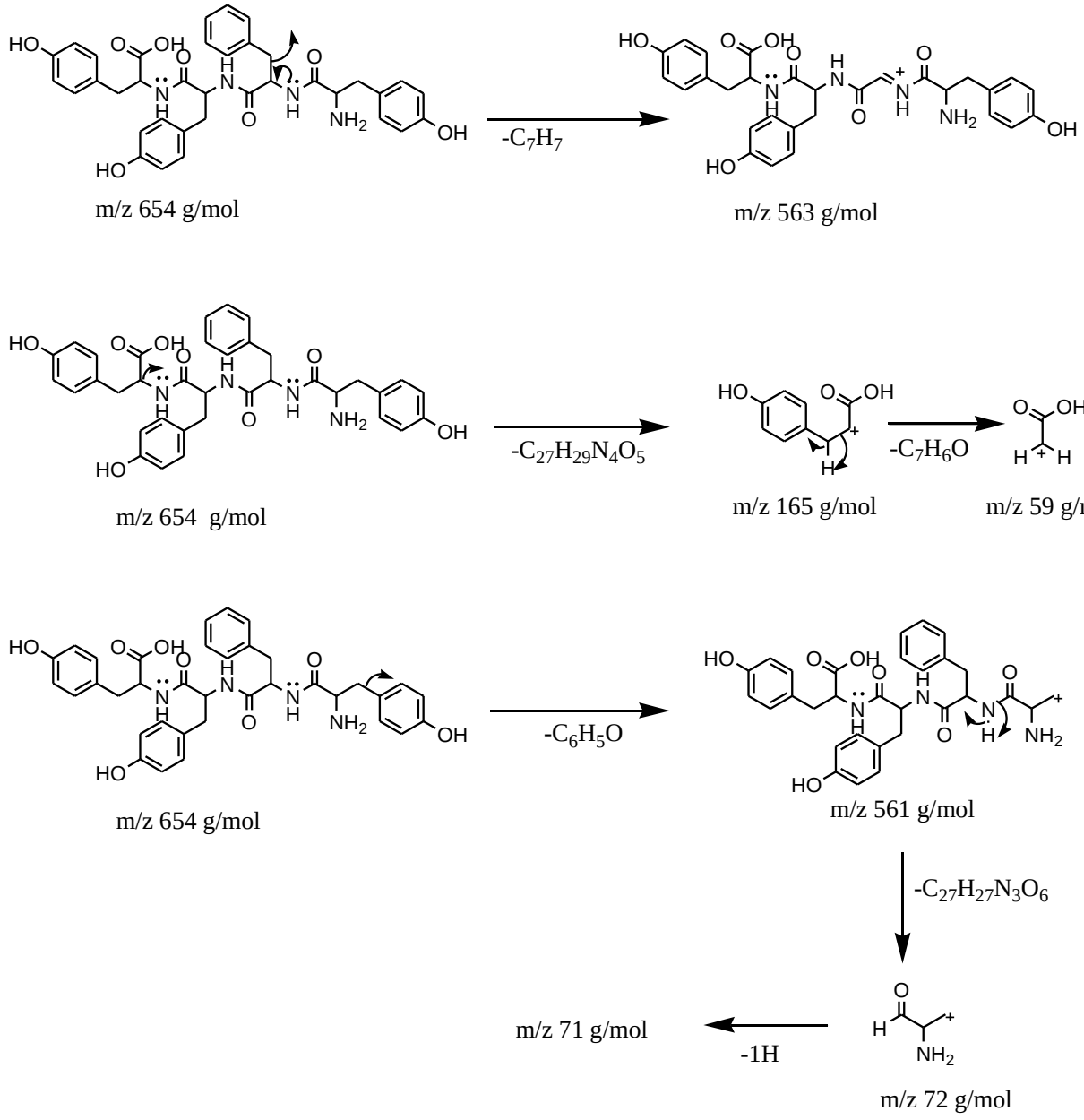


Fig. 5.35: The mass spectrum of compound 35

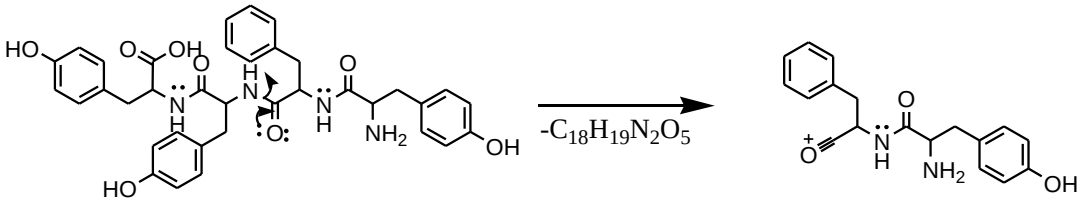
The fragment ion of m/z 653 resulted from loss of H while m/z 613 resulted from loss of NH_3 and dehydration (loss of $4H_2O$). M/z 608 due to loss of CO_2H and H while m/z 566 was due to loss of NH_3, H_2O, CO_2, $4H_2$ and H. Similarly, m/z 564 was due to loss of C_7H_6 while m/z 525 was due to loss of C_7H_7O, H_2O and two H_2. The fragment ion of m/z 476 was related to loss of $C_9H_{10}N_2O_2$ while m/z 337 was related to loss of $C_{18}H_{19}N_2O_3$ and three H_2. Also, m/z 307 was due to loss of $C_{18}H_{19}N_2O_5$ and two H_2 while m/z 223 was due to loss of C_7H_7O and $C_{18}H_{19}N_3O_3$. Likewise, m/z 171 resulted from the loss of $C_{26}H_{28}N_3O_4$ and dehydration (loss of H_2O) while m/z 71 was due to loss of $C_{27}H_{27}N_3O_6$, C_6H_5O and H. Finally, m/z 59 resulted from loss of $C_{27}H_{29}N_4O_5$ and C_7H_6O (scheme 38 on pages 192-197).



Continuing scheme



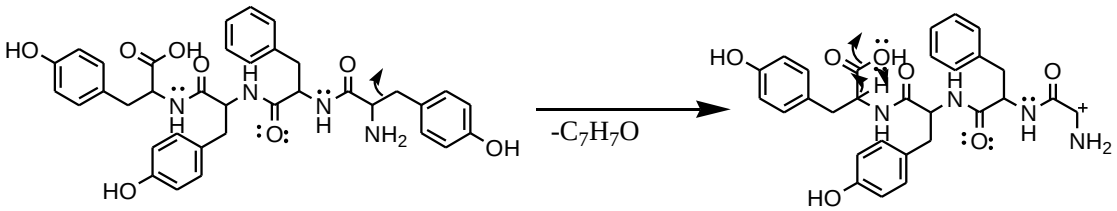
Continuing scheme



m/z 654 g/mol

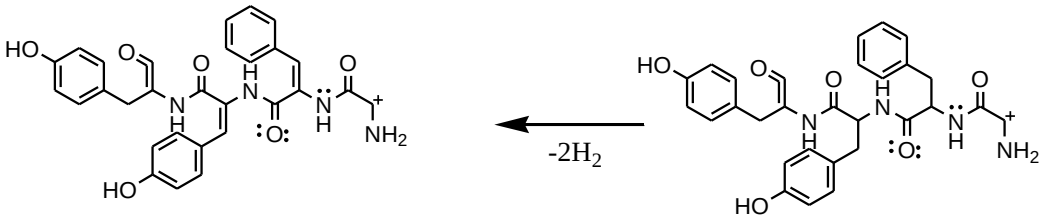
m/z 311 g/mol

m/z 307 g/mol



m/z 654 g/mol

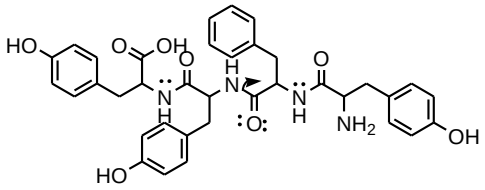
m/z 547 g/mol



m/z 525 g/mol

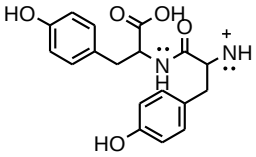
m/z 529 g/mol

Continuing scheme



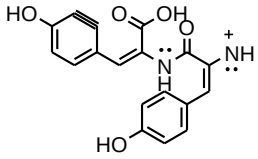
m/z 654 g/mol

$-C_{18}H_{19}N_2O_3$

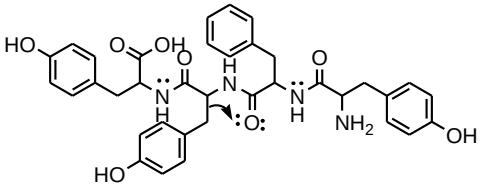


m/z 343 g/mol

$-3H_2$

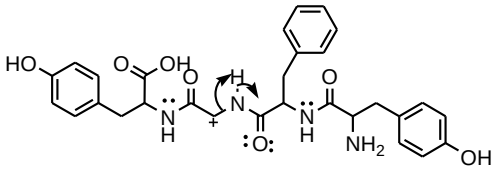


m/z 337 g/mol



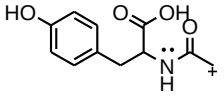
m/z 654 g/mol

$-C_7H_7O$



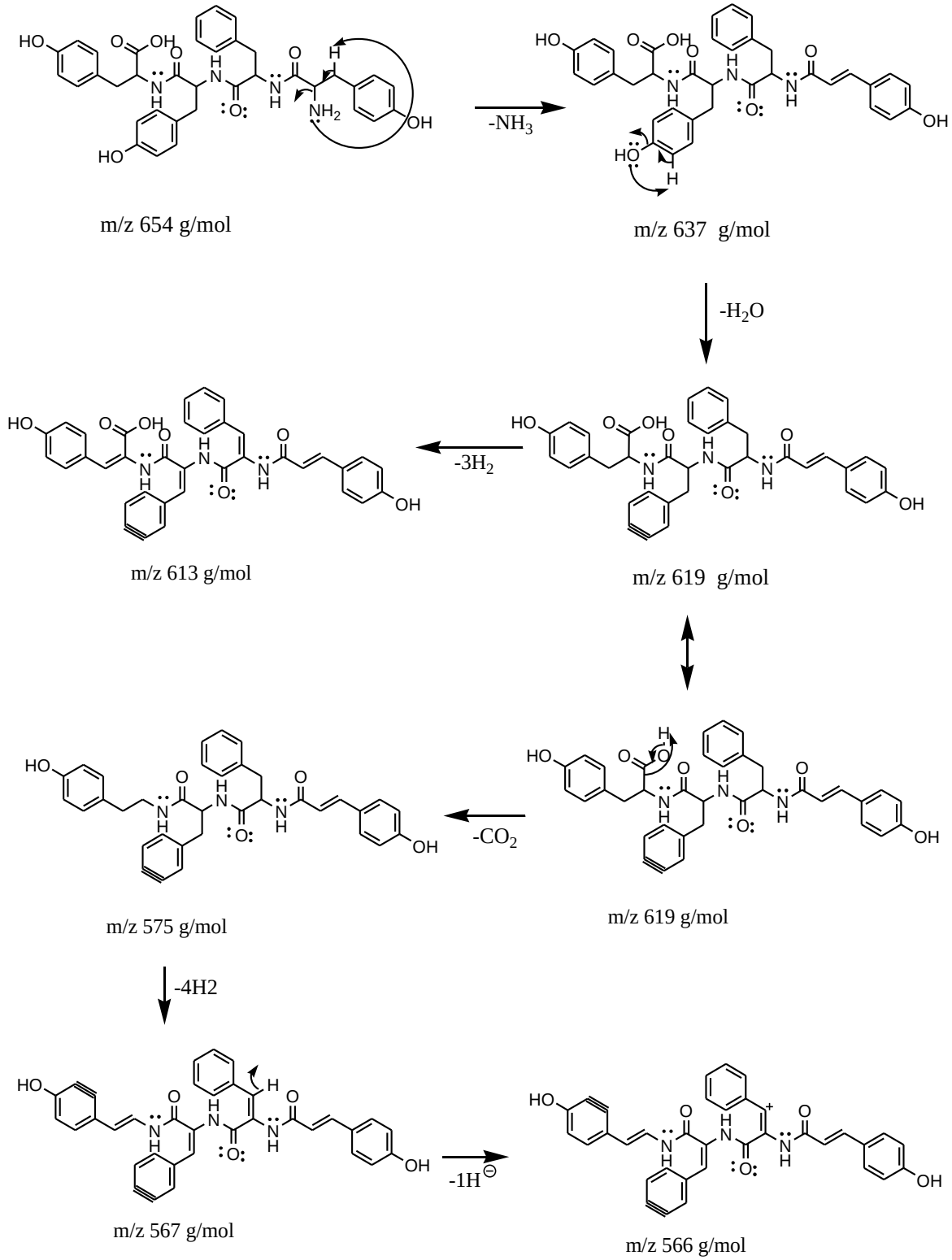
m/z 547 g/mol

$-C_{18}H_{20}N_3O_3$



m/z 222 g/mol

Continuing scheme



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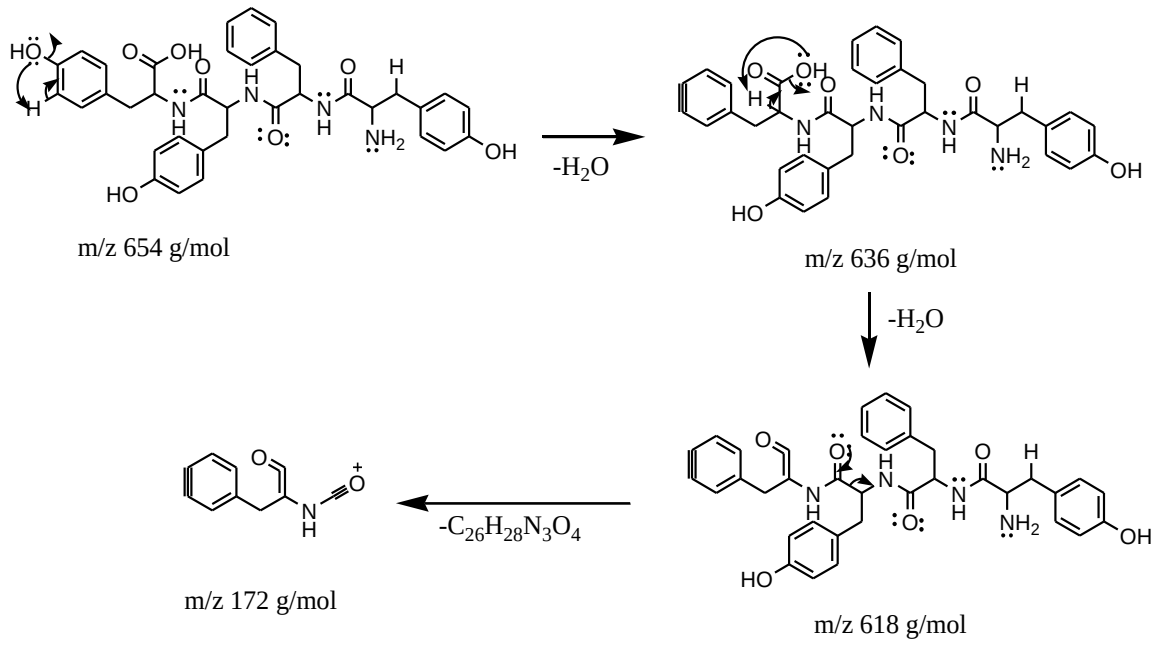


Fragments: 59, 71, 171, 223, 307, 337, 476, 525, 564, 566, 608, 613, 653, 696.3 g/mol

Scheme 38: Fragmentation pathway for Tyr Phe Tyr Tyr (compound 35)

Tyr-Phe-Tyr-Tyr is a tetrapeptide comprising of tyrosine and phenylalanine amino acids. Both were identified in honey as described by Kıvrak (2017). Their identification was also done on forest honeys from an area in South Sulawesi, Indonesia (Kowalski *et al*., 2017). Since it is a peptide it is believed to possess both antimicrobial and antioxidant activities (Elias *et al*., 2008; Coutinho *et al*., 2008).

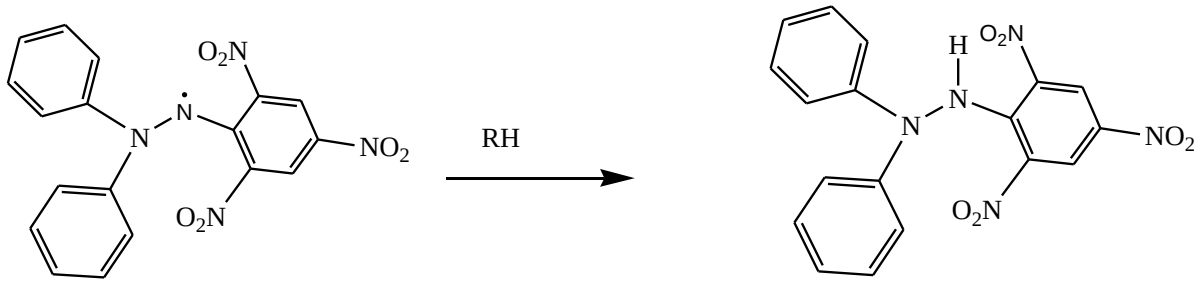
A research carried out by Mannina *et al*. (2015) reported that honey possess classes of compounds including; flavonoids such as quercetin rhamnosyl-hexosyl-rhamnoside, kaempferol rutinoside, quercetin hexosyl rutinoside, quercetin rutinoside (flavonols), pinobanksin acetate, pinobanksin methyl ether, pinobanksin butyrate, pinobaksin (flavanonols) and pinocembrin (flavanone); organic acids such as kynurenic acid, citric acid, 4-methoxyphenyllactic acid, phenyllactic acid; phenolic acids and their derivatives

such as caffeic acid isoprenyl ester, methyl syringate, p-hydroxybenzoic acid; Vitamins such as riboflavin, norisoprenoids such as abscisic acid; amino acids such as phenylalanine and other compounds such as 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione and dehydrovomifoliol ((±)-1'-hydroxy-4'-keto-α-ionone). These compounds possess the antioxidant properties hence contribute to the antioxidant activity. In addition, honey also contains other antioxidant compounds such as phenolic compounds, proteins, glucose oxidase, catalase, carotenoids and L-ascorbic acid (Beretta *et al*., 2005).

5.3 Radical scavenging activity

In this experiment, radical scavenging activity was measured by DPPH and FRAP methods. 2, 2-Diphenyl-1-picryl hydrazyl (DPPH) is a stable free radical with an odd electron at one atom of Nitrogen Bridge. In the presence of an antioxidant or hydrogen donating compound, the DPPH unpaired electron is paired off with a hydrogen donated by the free radical scavenging antioxidant and thus, reduced to its non-radical form (DPPHH) giving rise to a change of color which ranges from yellow to colorless. This decolourization is with respect to the number of electrons captured. The more the decolourization, the more the reducing ability. In this experiment, the DPPH unpaired electron was paired off with hydrogens donated by the free radical scavenging antioxidants from the honey. As a result, the purple colored odd electron DPPH was reduced to a yellow colored product, diphenyl hydrazine, in a concentration-dependent manner. The absorption decreased with increase in concentration of the test samples. Absorption decreases with respect to the number of electrons captured (Bastos *et al*., 2007). The absorbance decrease of the DPPH radical is caused by antioxidant molecules

and radical progresses which lead to the free radical scavenging by hydrogen donation (Sanja *et al*., 2009).



2,2-Diphenyl-1-picrylhydrazyl
(free radical form)

2,2-Diphenyl-1-picrylhydrazine
(non radical form)

Scheme 39: Reduction reaction of DPPH radical

In this spectroscopic method, "radical scavenging activity" or "percentage inhibition of free radical" term was employed. The absorbance of the reaction mixture without any antioxidant (negative control) was compared to the absorbance of the reaction mixture containing the DPPH radical and antioxidant sample. The results were also expressed using IC_{50} parameter for each honey sample. IC_{50} value refers to the concentration of the sample that is required to scavenge 50% of the free radicals present in the system. The IC_{50} is usually inversely proportional to free radical scavenging activity of samples. Thus, the lower the IC_{50}, the higher the free radical scavenging activity of honey under similar testing conditions since less amount of radical scavenger from honey is required to reduce DPPH (Chua *et al*., 2013).

According to Molyneux (2004), there has been doubts concerning DPPH direct determination obtained from calibration curve. This prompted use of ascorbic acid as the positive standard in this assay. Furthermore, ascorbic acid has proven as a radical scavenger and a good antioxidant. The IC_{50} for ascorbic acid in this assay was 5.09 ppm

with % inhibition 97.80% at 10ppm. The concentration of the standard used in this assay were 0, 2, 4, 6, 8 and 10ppm. It is evident that the IC_{50} value of the standard (ascorbic acid) was lower than all the honey samples. The lower the IC_{50} value, the higher the free scavenging activity of honey since lesser amount of radical scavenger from the honey is required to reduce the DPPH (Chua *et al*., 2013). In addition, it also implies a better ability of the sample to neutralize free radicals. From the results, *Plebenia hylderbrandii* honey (top layer) (IC_{50}=10.61 mg/ml) had the highest free scavenging activity, followed by *Meliponula bocandei* honey (IC_{50}=11.73 mg/ml) and lastly *Plebenia hylderbrandii* honey (bottom layer) (IC_{50}=14.31 mg/ml). The low IC_{50} value of the standard indicate that it possesses stronger free radical-scavenging compounds than the samples.

Previous study by Lu & Foo (2000) also used ascorbic acid as the positive control for DPPH free radical scavenging activity. Comparatively, these results were similar to a study carried out by Buratti *et al*. (2007) on multifloral honey whose IC_{50} values ranged between 8.0 to 12.0 mg/ml. Similar results were also reported from a research conducted by Stagos *et al*. (2018) who obtained values between 7.5 to 109.0 mg/ml. Another study carried out by Krpan *et al*. (2009) on acacia honey had higher IC_{50} values (111.05mg/ml) than those obtained in this study. This may have resulted from long storage which might have reduced the antioxidant activity of the honey samples. Moreover, Mokaya *et al*. (2020) did similar research on *Apis mellifera* honey and obtained IC_{50} values ranging between 8.20 to 186.85mg/ml. In other former studies, IC_{50} values were between 25.45 to 294.26 mg/mL (do Nascimento *et al*., 2018), 12.56 to 152.40 mg/mL (Can *et al*., 2015) and 4.2 to 106.72 mg/ml (Liberato *et al*., 2011). Thus, inference can be done that the tested Kenyan honey species had DPPH radical scavenging activity analogous to those reported

by other researchers. In the current study, the honey samples had greater DPPH radical scavenging activity, with IC_{50} values below 15mg/ml. Furthermore, the results concur with the research carried by Mokaya *et al*. (2020) that honey from Kakamega has high radical scavenging activity compared to other parts of the country such as Mt. Kenya. The studied honey samples possessed higher antioxidant activity compared to *Apis mellifera* honey, as described by Souza *et al*. (2006).

The same trend was also observed using % inhibition. The inhibition effect was enhanced with increasing concentration in the range of 20-40 mg/ml. *Plebenia hylderbrandii* honey (top layer) (97.24%) had the highest free radical scavenging activity, followed by *Meliponula bocandei* honey (92.86%) and lastly *Plebenia hylderbrandii* honey (bottom layer) (84%) at 40mg/ml. *Plebenia hylderbranddi* honey (top layer) had the highest antioxidant activity because it contained pollen which also possess antioxidant activity. This contributed to the higher scavenging activity values in the honey species. The free radical scavenging activity of the honey may have been due to presence of organic acids, enzymes, peptides and the products of Maillard reaction (Bertoncelj *et al*., 2007). In this study the free radical scavenging activity in *Plebenia hylderbrandii* honey may have been as a result of the presence of identified compounds such as pongagallone A (compound 24), peptides such as Ala-Lys-Lys-Pro (compound 8), complex sugars like genipin gentibioside (compound 10), arabinofuranobiose (compound 1), among others. In addition, in *Meliponula bocandei* honey, it may have been due to peptides such as His-Lys-Val-His (compound 20), 4-Heptylumbelliferyl-beta-glucoside (compound 19), (S)-(-)-Acenocoumarol (compound 17), among others. Comparatively, the antiradical activity of honey investigated by Neupane *et al*. (2015) was reported to range between 25.59% -

76.66%. These values were lower compared to the ones obtained in this study indicating higher free radical scavenging activity of the honey samples from this study. Another study carried out by Jimenez *et al*. (2016) on *Scaptotrigona Mexicana*, honeys obtained low antioxidant activity which ranged between 15.00 to 19.04%. From a previous study, the antioxidant activity of multifloral honey samples ranged between 64.2% - 80.9% (Bertonceji *et al*., 2007).

On the other hand, FRAP assay determines the total antioxidant activity of honey and employs an easily reduced oxidant in stoichiometric excess. It directly estimates the presence of reductones or antioxidants in a sample, depending on the capability of the analyte to reduce Fe^{3+} /Fe^{2+} couple (Lim & Tee, 2007; Beretta *et al*., 2005). The reduction of ferric ion results in the formation of a blue product. In this study, the two honey samples had high FRAP values of 911.36 and 585.82 µM for *Plebenia hylderbrandii* and *Meliponula bocandei* honeys respectively confirming their high antioxidant activities and high nutraceutical qualities.

Thus *Plebenia hylderbrandii* honey had more reducing power than *Meliponula bocandei* honey due to higher concentration of antioxidants. The reducing properties are commonly associated with presence of reductones (Duh, 1998). The difference in phenolic compounds, such as ferulic acid, quercetin, rutin, gallic acid and apigenin may also influence the honey's antioxidant activity hence the variation in the reducing potential (Can *et al*., 2015). It is reported that reductones' antioxidant activity is based on the breaking of free radical chain by donation of a hydrogen atom (Gordon, 1990). These reductones also react with certain peroxide precursors, thus inhibiting formation of peroxides. Comparatively, similar research has been done and the results obtained ranged

between 72.8 to 1501.4 µM Fe (II) (Beretta *et al.*, 2005). These results were similar to the ones in this study. However, a study carried out on Tualang honey by Mohamed *et al*. (2010) obtained a value of 322.1 ± 9.7 µM Fe (II), which was lower than the ones obtained in this study. Another study carried out by Bertoncelj *et al*. (2007) on Slovenian honey obtained values which ranged between 71.0 ± 10.2 to 426.4 ± 41.5. These were also lower compared to the honey samples in our study. Other previous studies obtained different FRAP values as follows: between 6.95 to 142.43 µM Fe (II) (Krpan *et al*., 2009), between 13.3 ± 0.1 to 19.4 ± 0.2 µM Fe (II) (Jerkovic *et al*., 2011), 40.22 ± 1.84 to 512.47.4 (Shamsudin *et al.*, 2019), 417.36 ± 0.52 to 668.53 ± 0.23 (Nweze *et al*., 2017), 209.28 to 653.76 µM Fe (II)/100g for Malaysian honeys (Moniruzzaman *et al*., 2013), 216.57 to 695.64 µM Fe (II)/100g for Italian honey (Perna *et al*., 2013), 287.45 to 403.54 µM Fe (II)/100g (Khalil *et al*., 2012) for Algerian honeys and between 267.19± 342.90 to 695.64 ± 428.31 for Southern Italian honeys (Perna *et al*., 2013); which were lower than the ones in this study.

Besides the nutritive value of honey, its antioxidant activity is also believed to be significant in human health (Badolato *et al*., 2017; Meo *et al*., 2017). For instance, the inflection of oxidative stress has been proposed as a mechanism through which honey employs chemopreventive effects against cancer (Badolato *et al*., 2017). However, previous reports indicate that botanical origin of honey affect their chemical constituents and antioxidant activities. Thus, honey from different botanical origins possess different antioxidant activity.

5.4 Antibacterial assay activity

This study demonstrates that honey from Kenyan stingless bee species *Plebenia hylderbrandii* and *Meliponula bocandei* have antibacterial activity. This activity varied between the two honeys and the individual test organisms were also found to differ in their susceptibilities. On the contrary, a research carried out on honey from Kenyan stingless bee species, *Dactylurina schimidti*, from Coast did not show any inhibitory effect against both *E. coli* and *S. aureus* in all the tested concentrations which ranged between 25% - 100 % (v/v) (Muli *et al*., 2008). A study done by Irish *et al*. (2008) reported that honey from Australian stingless bee species *Trigona carbonaria* possessed antibacterial activity. Another previous publication conveyed MICs of honey from various Guatemalan stingless bees including *Melipona beecheii*, *Geotrigona acapulconis*, *Tetragonisca angustula* and *Scaptotrigona spp.* ranging from 2.5- >10 % (v/v), with most of the MICs being 5 % (v/v) (Dardo´n & Enrı´quez, 2008). For *Apis mellifera* honeys, the MICs published for medical honey or Manuka honey ranged between 2-8 % (v/v) for Gram - positive cocci tested (Cooper *et al*., 2002b). In the current study, *Plebenia hylderbrandii* honey has a higher antibacterial effect than *Meliponula bocandei* honey. Moreover, substantial differences between the antibacterial activities displayed by the two honeys could be as a result of chemical constituent discrepancies in the stingless bee honeys tested. For instance, more of the identified compounds which contribute to antibacterial activity such as peptides, flavonoids, O-glycosyl compounds and phenolic acids (Wahdan, 1998) were from *Plebenia hylderbrandii* honey compared to *Meliponula bocandei* honey. These discrepancies may have also resulted from their feeding habits since they exploit plant-based resources (Heard, 1999) including pollen, nectar, latex, resin, scents, oil, seed and leaves during their foraging flight (Nunes-Silva *et al*., 2010), which are found in

diverse sites on broad diversity of crop plants. In addition, lower pH and higher electrical conductivity in *Plebenia hylderbrandii* honey may also have contributed to its higher antibacterial activity.

The study further shows that microorganisms may differ in their susceptibilities to honeys; with *S. aureus* being more susceptible when compared to *E. coli*. This finding is evidenced by the concentrations of the honeys that inhibited *S. aureus*, which were much lower than those that inhibited *E. coli*. It is also evident that the positive control was more active against *S. aureus* than *E. coli* as evidenced by its higher MIC and MBC values against *E. coli*. This is in accordance with Szweda (2017) who reported that Gram-negative bacteria such as *E. coli* are less sensitive to honey activity compared to Gram-positive bacteria, for instance, *S. aureus*. This is also in agreement with a study by Boorn *et al*. (2010) which also showed *S. aureus* as the most sensitive organism to honey having MIC ranges of 4% to >10% (w/v) for Gram-positive bacteria and 6% to >16% (w/v) for Gram-negative bacteria.

Honey have been proved to have antibacterial effect against *E. coli*. A study by Tan *et al*. (2009) reported that the MICs for tualang honey and Manuka honey for *E. coli* ranged from 17.5 % (v/v) to 22.5 % (v/v). On the same study, the MBC values ranged from 17.5% to 25% (v/v). Another study by Mulu *et al*. (2004) demonstrated that the concentration of honey which fully prevented *E. coli* growth was 6.5% (v/v). According to mandal *et al*. (2010), the bactericidal effect of the honey tested was at a concentration of 4 % (v/v). In this study, *Plebenia hylderbrandii* honey had a bactericidal effect at a concentration of 4.71 % (v/v) against *E. coli*. On the contrary, the bactericidal effect for *Meliponula*

bocandei honey against *E. coli* wasn't obtained since growth was observed in the concentration range tested. This calls for further research for its determination.

Generally, honey also demonstrates high *in vitro* anti-Staphylococcal activity as described by Szweda & Kot (2017) although other studies confirm that this activity vary based on different samples obtained from similar botanical sources, with MIC ranges of 3.12% and 6.25% (v/v) (Kuś *et al*., 2016). In this study, the percentage by volume of the two types of honey to completely inhibit *S. aureus* was 1.76 % (v/v) and 16.47 % (v/v) for *Plebenia hylderbrandii* and *Meliponula bocandei* honeys, respectively. A study by Mama *et al*. (2019) reported that the percentage by volume of honey to completely inhibit Methicillin-resistant *Staphylococcus aureus* (MRSA) growth was in the range of 18.7 to 37.5 % (v/v). The concentrations tested in the study ranged between 25 -100 % (v/v). On the contrary, a study carried out in Ethiopia, found out that the % v/v of honey that prevented growth of *S. aureus* was 6.5, which is higher than our result obtained from *plebenia hylderbrandii* honey whose inhibitory concentration was 1.76% (v/v) and lower than that of *Meliponula bocandei* whose inhibitory concentration was 16.47 % (v/v) for *S. aureus*. The concentrations from the study ranged between 10 -100 % (v/v) (Ahmed *et al*., 2014). Another study by Willix also reported that the percentage by volume of Manuka honey to completely inhibit *S. aureus* growth was 1.8 % (v/v) (Willix *et al*., 1992). Similarly, Sherlock *et al*. (2010) reported that inhibitory potential of Manuka honey from stinging bees on MRSA was only at concentrations above 12.5% (v/v) while Ulmo 90 honey was bactericidal on MRSA at concentrations of 3.1 % (v/v) and 6.3 % (v/v). These differences might be due to differences in the test methods used and test organisms, differences in the bee species or due to discrepancy in the antibacterial activities of honey in varying

geographical locations. This difference may also be attributed to their foraging behavior and feeding habits, which vary depending on the plant resources exploited by the bees.

Although the tested honey showed antibacterial effect, other studies have demonstrated that not all honeys have similar degree of antibacterial activity. Numerous previous reports of antibacterial activity of stingless bee honey are hard to compare with the current study due to methodological differences (Muli *et al*., 2008). In addition, most of these honeys were produced by bee species other than *Plebenia hylderbrandii* and *Meliponula bocandei* and from floral sources and regions discrete from those found in Kenya which has further limited the comparisons.

Moreover, a study carried out by Mohapatra *et al*. (2011) shows that honey was effective against both gram-negative (*P. aeruginosa*, *E. coli* and *S. typhi*) and Gram-positive bacteria (*Bacillus subtilis, S. aureus, Bacillus cereus, Micrococcus luteus* and *Enterococcus faecalis*); this effect was either bactericidal or bacteriostatic. In this study, *Plebenia hylderbrandii* honey had both bacteriostatic and bactericidal effect on *S. aureus* at concentrations above 1.76% (v/v) and 2.35% (v/v), respectively and on *E. coli* at concentrations above 3.53% (v/v) and 4.71% (v/v) respectively. However, *Meliponula bocandei* honey had both bacteriostatic effect at a concentration of 16.47 % (v/v) and bactericidal effect at a concentration of 16.47 % (v/v) on *S. aureus* but had neither bacteriostatic nor bactericidal effect on the growth of *E. coli* isolate because of the observed growth in all concentrations. In this study, the bactericidal effect was higher than that of Ulmo 90, which may have resulted from different geographical origins (Sherlock *et al*., 2010). However, in this study the honeys had a lower bactericidal activity on *E. coli* than on *S. aureus*. This could be attributed to the lower susceptibility of *E. coli*.

Antibacterial activity of honey varies depending on various factors such as honey bee, storage time, honey type and its concentration, type of microbe, honey components/characteristics and source of nectar on which the reared bees were fed (Irish *et al*., 2011). Tan *et al*. (2009) also indicated that honey has many sources of production and its antimicrobial activity may vary depending on the processing and origin. The antibacterial activity of stingless bee honey differs from that of *Apis* honey which is attributed to the storage of the specific bees. According to Ewnetu *et al*. (2013), stingless bee honey has higher antibacterial effects than *apis mellifera* honeys. In western Kenya, *apis mellifera* honey has been reported to possess antibacterial effects (Mokaya *et al*., 2020). However, no stingless bee honey has been explored for comparison purposes hence the purpose for this study.

In addition, the efficacy of honey against test microorganisms depends on the honey used, botanical origin variation, honey processing, geographical location and bee health (Sherlock *et al*., 2010). Honey also has various renowned characteristics that are believed to contribute to its total antimicrobial activity. These comprise of pH (being 3.2-4.5), H_2O_2 concentration, phytochemical factors and high osmotic effect (Cooper *et al*., 2002b). For instance, in this study, *Plebenia hylderbrandii* honey had a pH of 3.54 ± 0.012^{a} while *Meliponula bocandei* honey had a pH of 3.99 ± 0.006^{a}. These values are within the acceptable range hence their contribution to their antibacterial activity. However, *Plebenia hylderbrandii* honey had a lower pH hence its better antibacterial activity than *Meliponula bocandei* honey.

In comparison with the formerly published data, it is evident that *Plebenia hylderbrandii* and *Meliponula bocandei* honeys have similar activity to the other medicinal honeys and thus can be used as therapeutic agents.

CHAPTER SIX

CONCLUSION AND RECOMMEDATIONS

6.1 Conclusion

The physico-chemical parameters (pH, EC, MC, color intensity) for the honey samples were determined. They were within the recommended limits of honey except the electrical conductivity (EC) and moisture content (MC) for *Plebenia hylderbrandii* honey which were slightly above the standard values.

A total of thirty-five (35) organic compounds were identified from the two honey samples. The major compounds obtained were glycosides, quinolines, amino acids, phenolic compounds, flavonoids and peptides.

The honey samples exhibited high antioxidant activity by both DPPH and FRAP methods. The antioxidant activity obtained by the two methods may be attributed to the presence of peptides, isoquinoline derivatives, flavonoids, coumarin derivatives, phenolic glycosides, indole derivatives, etc. obtained in this study.

The honey samples had antibacterial activity against *E. coli* and *S. aureus*. The antibacterial activity shown by the bacteriostatic and bactericidal activities may be as a result of peptides, phenolic glycosides, flavonoids, etc. identified in this study.

6.2 Recommendations

More physicochemical properties such as hydroxymethylfurfural, redox potential, viscosity, total soluble substance (TSS) and specific rotation need to be done for the investigated honey samples.

Standards for the proposed organic compounds need to be sourced in order to compare with those of the identified samples. Volatile organic compounds in the investigated honey samples should be determined.

The identified compounds need to be isolated and tested for their individual antibacterial activities.

Other methods of determining antioxidant activities need to be performed for comparison.

Higher concentrations need to be done for *Meliponula bocandei* honey in order to determine its bacteriostatic and bactericidal activities against *E. coli*. In addition, there is need to test the activity of the honey samples with more Gram positive and Gram-negative microbes.

A similar study should be carried out on the bee bread of the two honey varieties.

Finally, the two honey varieties should be exploited as dietary supplement in the management of human disorders and diseases that arise from the generation of free radicals from the human body cells and tissues.

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LIST OF APPENDICES

Appendix I: Antimicrobial assay plates

Fig. A. I .1: Positive control (*E. coli*)

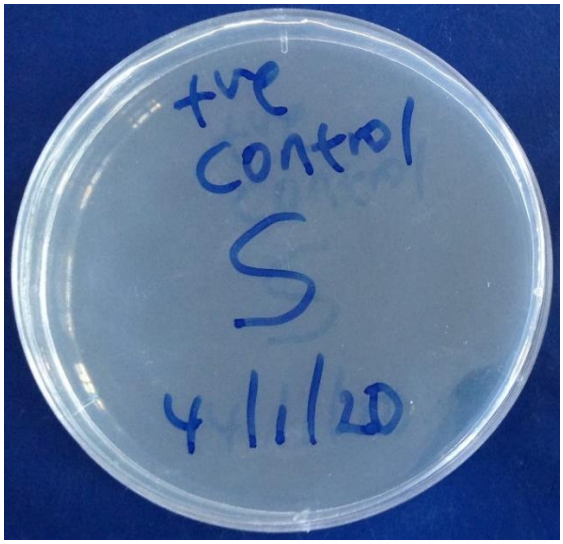


Fig. A. I. 2: Positive control (*S.aureus*)

Fig. A. I. 3: Negative control (*E. coli*)

Fig. A. I. 4: Negative control (*S.aureus*)

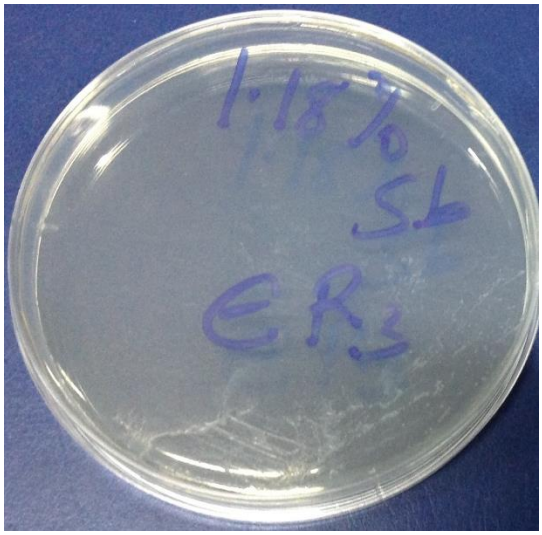


Fig.A.I.5: 1.18% (v/v) *P. hylderbrandii* honey honey against *E.coli* (PIC)

Fig. A. I. 6: 1.18% (v/v) *P. hylderbrandii* against *S.aureus* (PIC)

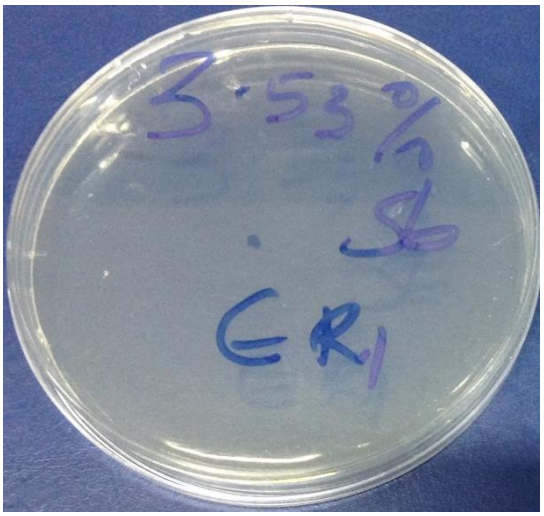


Fig. A. I. 7: 3.53% (v/v) *P. hylderbrandii* honey Against *E. coli* (MIC)

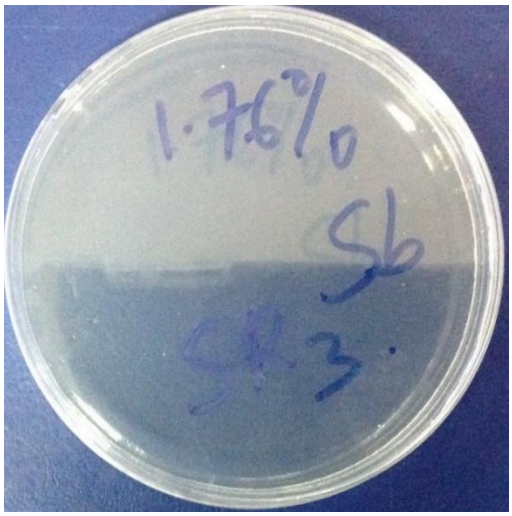
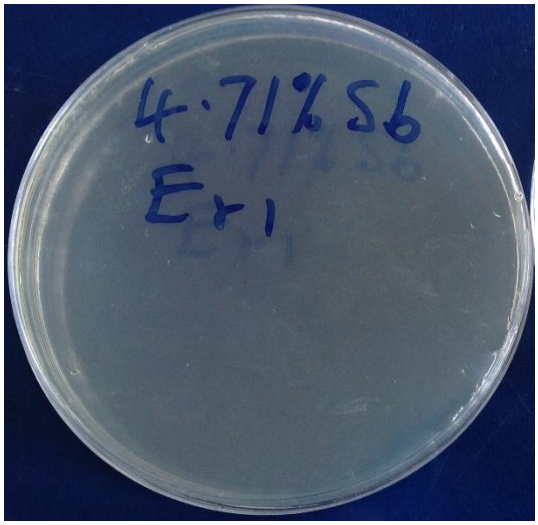


Fig. A. I. 8: 1.76% (v/v) *P. hylderbrandii* honey against *S.aureus* (MIC)



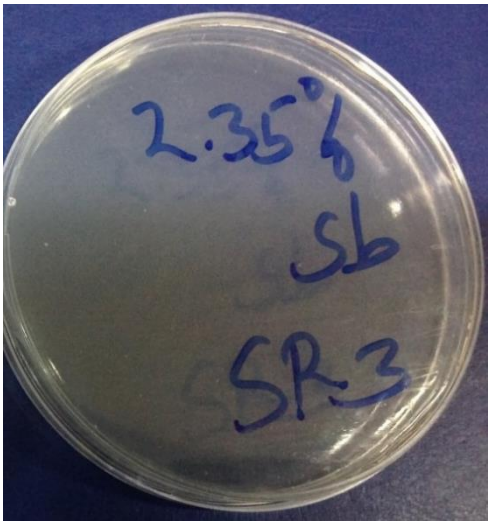


Fig. A. I. 9: 4.71% (v/v) *P. hylderbrandii* honey against *E. coli* (MBC)

Fig. A. I. 10: 2.35% (v/v) *P. hylderbrandii* honey against *S.aureus* (MBC)

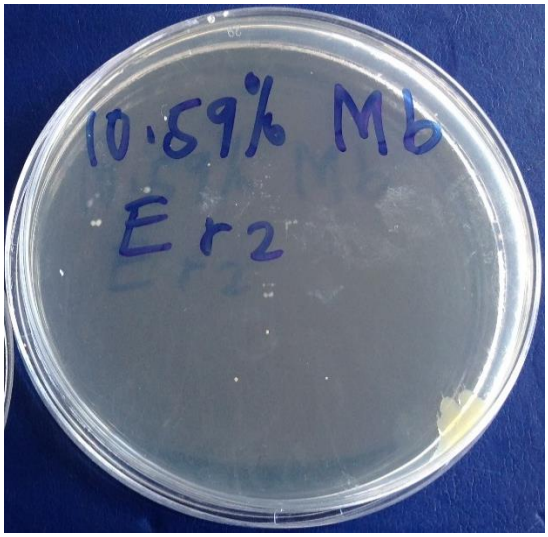


Fig. A. I. 11: 4.71% (v/v) *M. bocandei* Against *E. coli*

Fig. A. I. 12: 10.59% *M. bocandei* against *E. coli* (PIC)

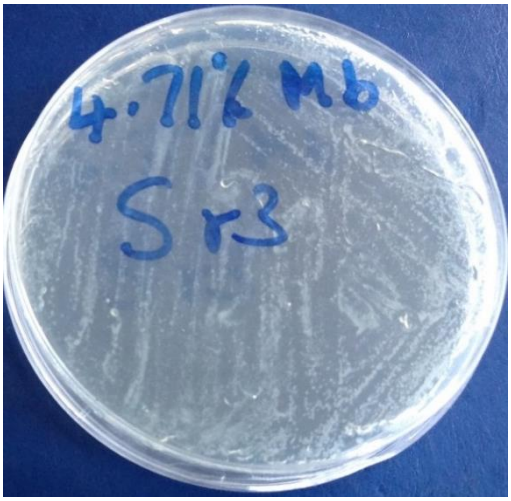


Fig. A. I. 13: 4.71% (v/v) *M. bocandei* against *S.aureus*

Fig. A. I. 14: 9.41% (v/v)*M.bocandei* honey against *S.aureus* (PIC)

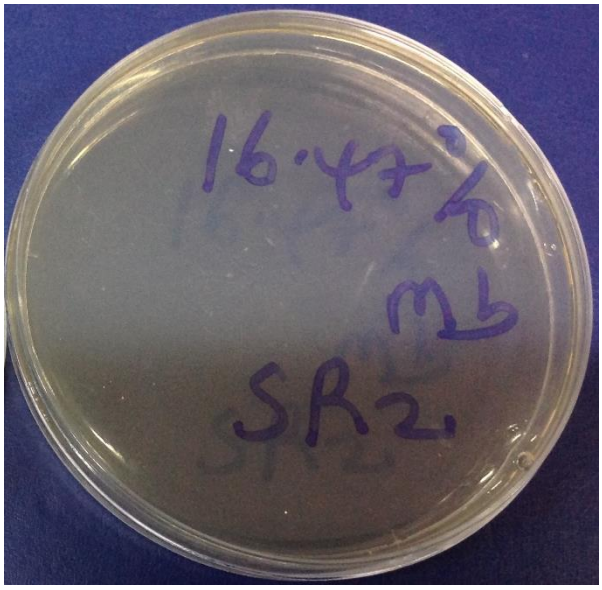


Fig. A. I. 15: 16.47% (v/v) *M. bocandei* against *S. aureus* (MIC, MBC)

Comparisons

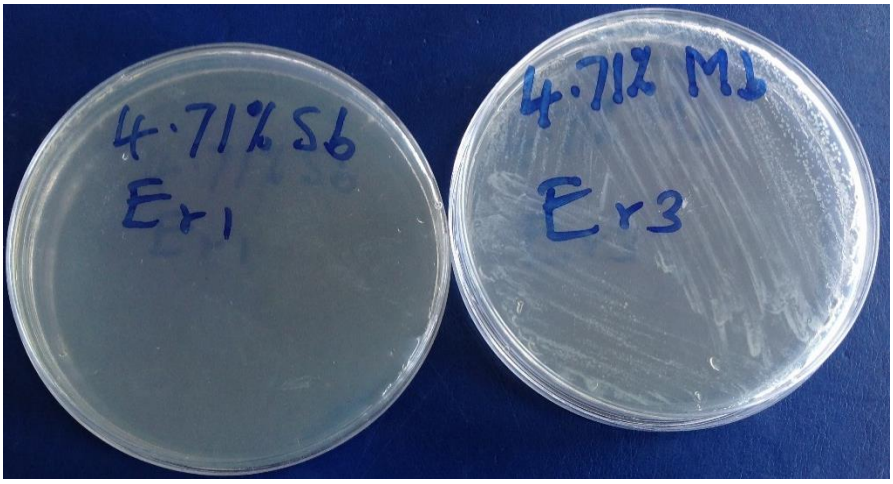


Fig. A. I. 16: 4.71% (v/v) *P. hylderbrandii* and *M. bocandei* honeys against *E. coli*.

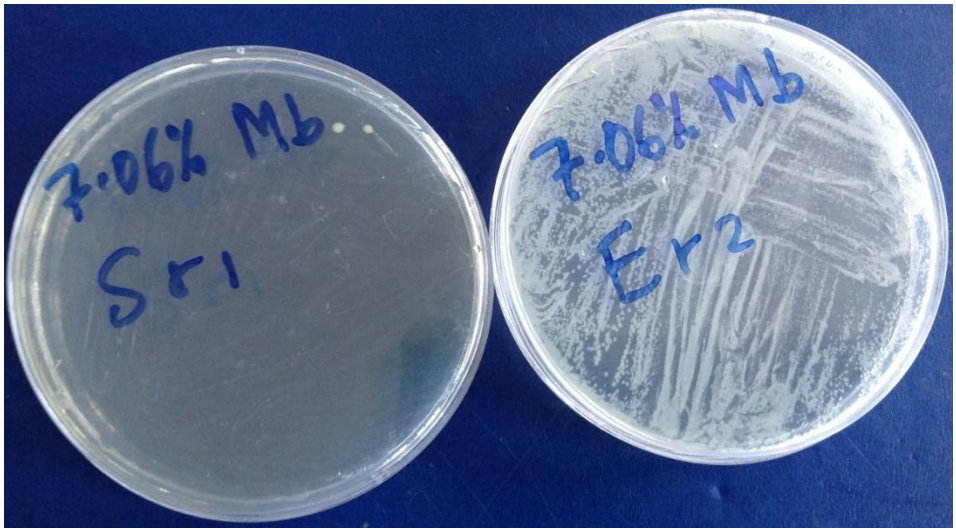


Fig. A. I. 17: 7.06% (v/v) *M. bocandei* honey against *E. coli* and *S. aureus*

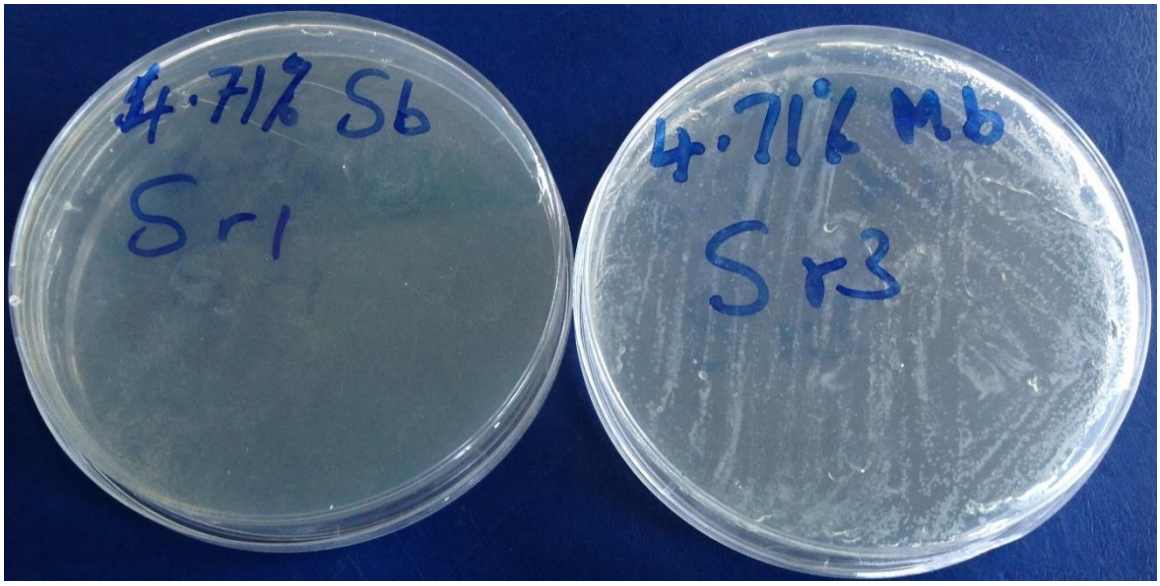


Fig. A. I. 18: 4.71%(v/v) *P. hylderbrandii* and *M. bocandei* honeys against *S. aureus*

Appendix II: Bioassay experiments

Fig. A. II. 1: Spreading organisms on culture plate