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BENAICHE Ghania

TOPIC

Extraction and GC/MS Analysis of major Alkaloids found in the Family of Fabaceae

Publicly submitted the 04/07/2007 on the following committee:

Mr. Houcine SAADI	Pr. (M.B.U.M'sila)	President
Mr. Dehimi OUALI	Pr. (M.B.U.M'sila)	Supervisor
Mr. Mahmoud BOUNEKHEL	M.C. (F.A.U.Sétif)	Examiner
Mr. Abdelouahab BOUZIDI	M.C. (F.A.U.Sétif)	Examiner

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Dedicated to my parents

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Abbreviation

TLC: Thin layer chromatography
MeOH: Methanol
ATCC: American Type Culture Collection
UV: Ultra Violet
IR: Infrared
GC/MS: Gas chromatography Mass spectroscopie
HPLC: High Performance Liquid Chromatography
CC: Column Chromatography
Rf: Retarding Factor
RI: Retarding Index
Rel. Int: Relative intensity
EI-MS: Electronic Impact-Mass Spectroscopy
QA: Quinolizidine Alkaloids

Introduction

The use of plants by man is an ancient practice . Plants are particulary useful as medicines , flavours , foods , insect deterrents , ornamentals , fumigants , spices , and cosmetics [1] .

In fact , secondary metabolite , which are the products of plants , have always played a central role in plant identification by animals (including Man) , in the form of perceived tastes , smells , toxic effect , etc. , and by insects , who use chemical cues to perceive host and food plants .

Few , if any classes of secondary metabolite have played a great role in the evolution of organic chemistry than the alkaloids . The biological activity exhibited by many alkaloids ensured that plants containing them were highlighted in folklore and herbal medicine and consequently many of importance to man before any thought was given as to what it was that caused their bioactivity [2] .

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The alkaloids are structurallay the most diverse class of secondary metabolites , and over 5000 compounds are known , ranging from relatively simple structures and other complex . They are most commonly encountered in the plant kingdom , but representatives have been isolated from most other orders of organisms ranging from fungi to mammals [3] .

There is no absolute definition that can be applied to an alkaloid . All compounds that have any pretence to being an alkaloid must , obviously , contain nitrogen , but beyond this there are no absolutes . Most but not all , alkaloids are basic and are derived in part from an amino acid . In rationalising alkaloids one is left with a sort of collective definition in which characters such as basicity , source of nitrogen , physical form , propensity for biological activity can all be cited .

The most strict definition of an alkaloid would demand that the nitrogen originated from an amino acid and that the compound was basic .

One commonly used subterfuge designed to make the term alkaloid more definitive is to employ the term *pseudoalkaloid* and protoalkaloid to distinguish compounds that clearly fit some , but not all , of the traits normally associated with alkaloids [2] .

Of the 5000 or so known alkaloids , the majority occur in flowering plants , though 40 percent of all plant families have at least one species containing alkaloids , when the 10 000 plant genera are considered , only about 9 per cent of these have been shown to produce alkaloids .

There are two worthwile methods of classifying alkaloids ; one that links them to some parts of their chemical structures , and one based on their route of biosynthesis , emphasising the amino acid from which are derived . The alkaloids which are derived from ornithine and lysine are named " Quinolizidine alkaloids " [3]. They are characteristic secondary metabolites of the Family of Fabaceae (Leguminosae) and are especially abundant in the tribes Genisteae , Sophoreae and Thermopsideae [4] . The genus ***Cytisus*** is known to produce this type of alkaloids [5] .

As part of investigations on quinolizidine alkaloids in leguminous plants native to ALGERIA , we have studied the pattern of alkaloids of plant ***Cytisus purgans*** .

Our study is divided into three chapters : Firstly , there is data , much of it tabulated , concerning the studied plant (botanical Family , genus , systematic , …etc) , including also structures , plant sources , methods of extraction and separation as well as the biological activity and biosynthesis . The second chapter was concerned with the results which were obtained with their discussions , and , finally , we have described the experimental procedures which were applied .

Chapter One
Theoretical Study

I-1-Vegetal study:

I-1-1-The Family of Fabaceae:

Named also Leguminosae, this is a cosmopolitan family with about 700 genera and 18000 species, and is one of the most important families, is characterized by a large number of derived traits. Most taxa of this Family are herbaceous, sometimes shrubby and only very rarely trees. Typically, the leaves are pinnate and sometimes the terminal one is modified to form a tendril, used for climbing. Bipinnate leaves are not found in this family. The five sepals are at least basally united. The corolla is formed of five petals and has a very characteristic butterfly-like shape (papilionaceous), with the two lower petals fused and forming a keel-shaped structure, the two lateral ones protruding on both sides of the flower and the largest petal protruding above the flower, being particularly showy. The androecium of ten stamens generally forms a characteristic tubular structure with at least nine out of ten of the stamens forming a sheath. Normally, the fruit are pods, containing beans (technically called legumes) with two sutures, which open during the drying of the fruit [6].

This large Family is characterized by an impressive phytochemical diversity. Polyphenols (especially flavonoids and tannins) are common, but from a pharmaceutical perspective various types of alkaloids are prabably the most interesting and pharmaceutically relevant groups of compounds [7]. In the genera ***Genista*** and ***Cytisus*** (both commonly called broom) as well as ***Laburnum***, quinolizidine alkaloids, including cytisine and sparteine, are common [5]. The hepatotoxic pyrrolizidine alkaloids are found in this Family (e.g. in membres of the genus ***Crotalaria***) [8].

Other important groups of natural products are the isoflavonoids, known for their oestrogenic activity, and the coumarins used as anticoagulants. ***Glycyrrhiza glabra*** **L.** (licorice) is used because of its high content of the triterpenoid glycyrrhic acid, which, if joined to a sugar, is called glycyrrhizin (a saponin) and is used in confectionery as well as in the treatement of gastric ulcers (controversial). Last but not least, the lectins must be mentioned. These large (MW 40,000 – 150,000), sugar-binding proteins agglutinate red blood cells and are a common element

of the seeds of many species. Some are toxic to mammals, for example phasing from the common bean (***Phaseolus* spp.**), which is the cause of the toxicity of uncooked beans [5].

In ALGERIA it is presented by 53 genera and 339 species, which are mainly distributed in ***Genista*** and ***Cassia*** [9].

I-1-2-The Genus of *Cytisus*:

Cytisus is an ornamental genus , commonly called broom , it is presented by about 50 species mainly mediterranean , is native to central and south Europe , Canaries Isles and north Africa . The common name ' broom ' may have been given to the plant because of its growth habit.

The shrubs are 1-2 meters high and deciduous. The green branches are strongly angled and appear naked or almost so. The leaves are trifoliate, the yellow flowers are usually borne solitary in axils, blooming between April and June [10].

Six species represented in ALGERIA, the more abundant is ***Cytisus trifolus*** **L'her.** Other rare species which are ***Cytisus balansae*** **Boiss.** Et **Reut.** (***Cytisus purgans***), ***Cytisus fontanesi*** **Spach** (Ouarsenis, Bibans, …), ***Cytisus sessilifolius*** **L.** (Babor), ***Cytisus boeticus*** **Webb.** (north of ORAN) [11].

I-1-3-Definition and Systematic of Studied Plant:

Cytisus purgans is a shrub always more or less ramify, a trifoliolate and persistent leaves and a yellow flowers, native to central France, north central Spain and Portugal, Algeria and southern Morocco [12].

Cytisus purgans (***L.***) ***Benth***. (= ***Genista linifolia L.***) is distributed in clear forest of coast of Algiers (Algeria) which is known to name of ' **Chahmet el Atrous** ' and ' **Hisbane** '
[13].

Cytisus purgans (***L.***) ***Boiss***. (***Genista purgans L.***) , is distributed in seaside cliff of France [14].

Cytisus balansae Boiss. (***Cytisus purgans***) is a rare species of Algeria (Aures, Mahdids, and Lella Khadidja) [11].

I-1-4-Bibliographic Information concerning ' *Cytisus purgans* ':

A few studies concerning ' ***Cytisus purgans*** ':

- A study [15] of a condensed tannin content of several shrub species from a mountain area in northern Spain , and its relationship to various indicators of nutritive value , in this study , ***Cytisus purgans*** and seven other species were studied .

- Balandrin and coll. were studied a distribution of alkaloids in some species of the papilonaceous tribes Thermopsideae and Genisteae (includes ***Cytisus purgans***). Quinolizidine alkaloids were a main constituents [16].

- Pascol , J. and coll in Alkaloids of Orbanche rapum-genistea [17] were studied the variation of the amount of sparteine and lupanine in the host-parasite relation , in this study , the lupanine content of broomrape parasitizing providence broom (***Cytisus purgans***) was higher than the sparteine content , but the contents of both alkaloids changed similarly during growth .

- The same researchers in ' Two new ecotypes of Orbanche rapum-genistae thuill ' [18] were studied the Morphological and Biochemical characteristics of O. rapum-cytisi purgantis parasitizing ***Cytisus purgans***.

-Balandrin, R. and coll. were studied the application to the measurement of in situ symbiotic fixation, ***Cytisus purgans*** includes [19].

- Aurnhammer and coll. were studied the L-rhamnosyl-D-glucosides of pincombrin (I) , sarotanoside , and isosarotanoside , isolated by different workers from from ***Cytisus commutatus*** and ***Cytisus purgans*** [20].

- Adzet, T. and coll. were studied the alkaloids of some Genisteae species of Catalonia (Spain), includes ***Cytisus puragns*** [21].

- Plouvier, Victor. Was studied the structure of three flavonosides, isosarotanosides from ***Cytisus purgans*** [22].

- Finally, Paris, R. A. and Faugeras, G. were studied the use of electrophoresis in the separation of alkaloids and flavonic compounds from some legumes, includes ***Cytisus purgans*** [23].

To our knowledge, this the first report on ***Cytisus purgans*** of Algerian flora.

I-2-Chemical Study:

I-2-1-Introduction:

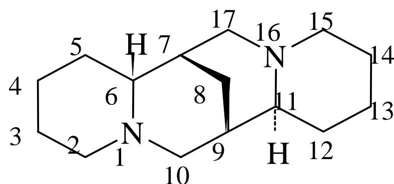
A. Structures:

Quinolizidine alkaloids (QA), of which more than 170 structures have been reported (Table I-1), are distinct from other alkaloids in that they contain at least one quinolizidine ring system [24]. According to the degree of substitution , at least eight groups can be distinguished (Figure I-1) : (**1**) the lupinine type ; (**2**) the leontidine type ; (**3**) the sparteine /lupanine /multiflorine type ; (**4**) the α-pyridone type ; (**5**) the matrine type ; the ***Ormosia*** type (**6**), (**7**) piperidine and dipiperidines , and (**8**) miscellaneous structures , which often occur together with QA [25] .

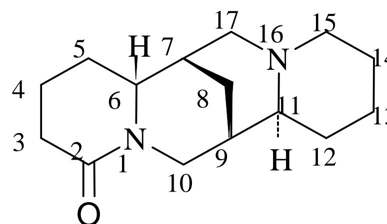
B. Nomenclature:

For practical purposes, QA are generally known by their trivial names, usually derived from the names of their source plants. Systematic chemical names are unwieldy and are infrequently used. A useful note on the systematic naming of the QA is given.

The sparteine is named as derived of diazocine (Dodecahydro-7, 14-methano-2H, 6H-dipyrido [1, 2-a: 1', 2'-e] diazocine, 9CI.) . The lupanine is named as derived of sparteine (2-Oxo-11α-sparteine). Systematics names can accordingly be derived for esters and hydroxylated compounds [26].



Dodecahydro-7, 14-methano-2H, 6H-dipyrido [1,2-a : 1',2'-e] diazocine, 9CI.



2-Oxo-11α-sparteine

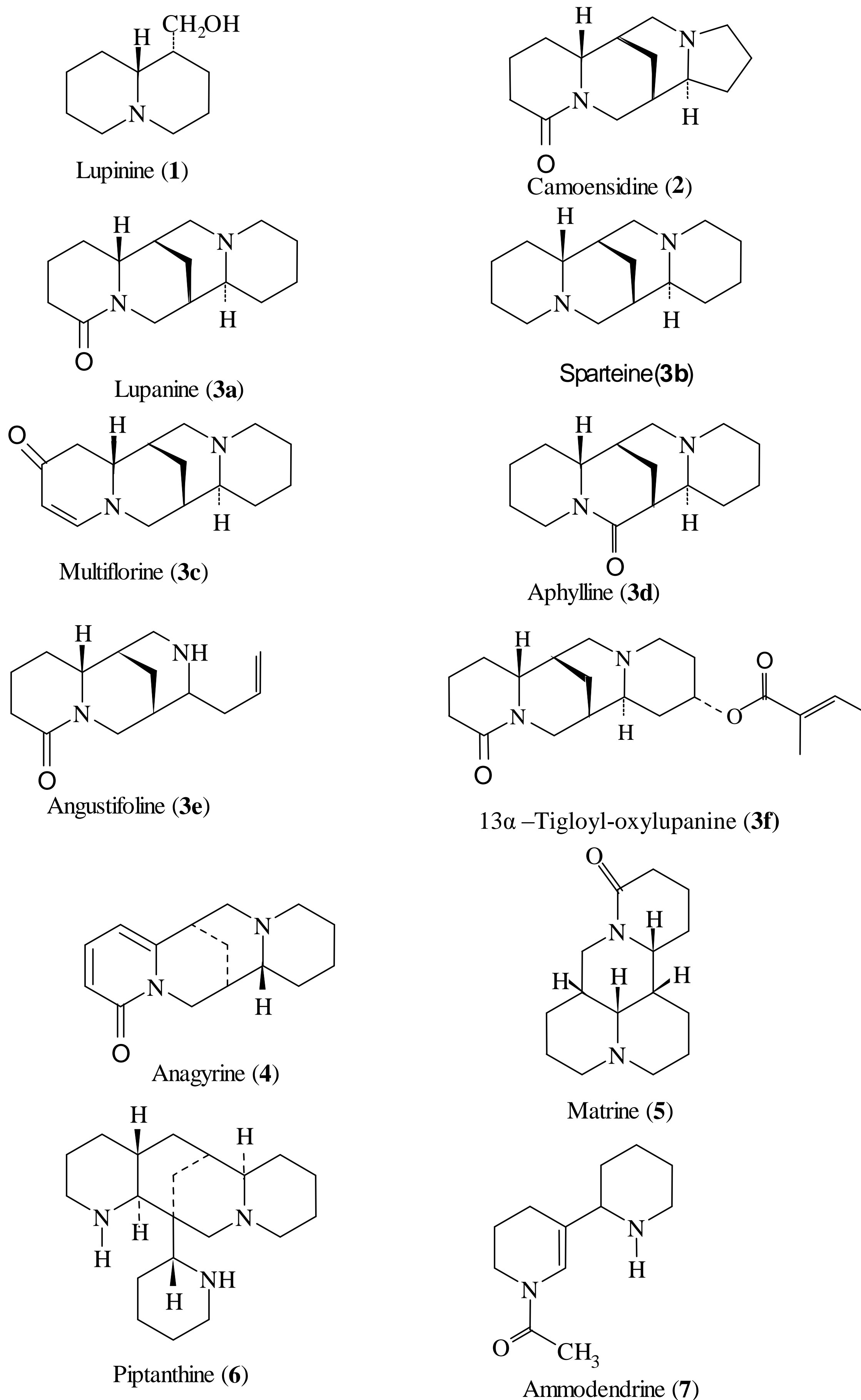


Figure I-1: Structural types of quinolizidine alkaloids [27].

C. Natural Distribution:

Quinolizidine alkaloids are mainly distributed within the Leguminosae. This is the third largest family of flowering plants after the Compositae and Orchidaceae, consisting of about 650 genera and 18,000 species. Quinolizidine-accumulating tribes are considered ' primitive ' within the Leguminosae and are the Sophoreae , Dalbergieae , Euchresteae , Thermopsidae , Genisteae , Bossiaeae , Brongniartieae , Podalyrieae , Liparieae and Crotalarieae [25] . However, restricted occurrences have been reported from a number of other families which are unrelated to the Leguminosae such as Berberidaceae (***Leontice***, ***Caulophyllum***), Solanaceae, Ranunculaceae (***Cimicifuga***), Chenopodiacae (***Anabasis***) and Rubiaceae (***Readea***). Parasitic or hemiparasitic plants can tap the phloem and xylem sap of their host plants. If the host plant contains QA an alkaloid transfer is plausible, as has been observed for a number of species of the Scrophulariaceae: ***Orobanche*** [28], Castilleja [29] and ***Pedicularis*** [30], but also in other families such in ***Viscum*** (Loranthaceae) [31], Cuscuta (Cuscutaceae) [32] and ***Osyris*** (Santalaceae) [33]. Table I-1 presents an overview of the distribution of some QA on the generic level.

Plant cell suspension cultures of ***Daucus carota*** (Umbelliferae) , ***Conium maculatum*** (Umbelliferae) , ***Atropa belladonna*** (Solanaceae) , ***Chenopodium rubrum*** (Chenopodiaceae) , ***Spinacia oleracea*** (Chenopodiaceae) and ***Symphytum officinale*** (Boraginaceae) have been observed to produce minute amounts of lupanine , especially when challenged with elicitors [34] . Using a very sensitive radioimmunoassay (RIA) (see section I-2-4) for lupanine we found traces of lupanine in c. 50 out of 100 plants studied [35]. The next step will be to determine the nucleotide sequences of the corresponding genes, to see whether and how the genes and plants are related. These surprising findings suggest that the genes of QA biosynthesis are widely distributed within plant kingdom, but are generally ' silent '. Only in the Fabaceae (Leguminosae) are the genes for biosynthesis, transport and storage turnred on, since here the alkaloids serve for chemical defence (see section I-2-D).

The different structural types (Fig. I-1) of QA are not evenly distributed within the Leguminosae; common to all tribes are QA of the lupanine (**3a**, **3e**, **3f**) and sparteine (**3b**) type. Multiflorine (**3c**), aphylline (**3d**) and derivatives are only present in a few genera.
α-Pyridone (**4**) are also common but more restricted than type **3a** and **3b** . Lupinine and derivatives (**1**) have been detected in a comparably small number of species. Leonitidane-type

Table I-1: Structures and natural distribution of quinolizidine alkaloids [27].

Alkaloid	Genus	Type
Lupinine (-)	*Lupinus , Thermopsis , Calpurnia , Virgilia , Genista , Anabasis[a] , Goebelia*	1
Camoensidine	*Maackia, Camoensia*	2
Lupanine	*Cytisus (+ , -) , Genista , Lupinus (+ , -) Diplotropis , Ormosia , Maackia (-) , Dalbergia , Templetonia (+) , Thermopsis , Baptisia (+) Laburnum , Melolobium , polhillia , Agyrolobium , Lebeckia , Dichilus , Wborgia , Rothia , Aspalathus, Rafnia, Pearsonia, Lotononis, Pedicularis[a], Pericopsis, Haplormosia. Bolusanthus, Virgilia, Cadia, Chamaecytisus, Echinospartum Ammodendron, Hovea (-), Liparia (-), Loutus , Petteria , Piptanthus (+) , Podalyria (+) Echinosophora (-) , Sophora (-) , Orobanche[a] , Spartium, Ulex , Anagyris , Retama (-) , Leontice[a] (-), Viscum[a], Thermopsis (+)*	3 a
Sparteine	*Leontice[a] (+) , Cytisus (- , +) , Genista (+ , -) , Thermopsis (+) Melolobium , Polhillia , Laburnum , Argyrolobium , Lebeckia , Spartidium , Wiborgia , Aspalathus , Rafnia , Pearsonia , Lotononis , Ormosia (-) , Pericopsis , Haplormosia , Bolusanthus , Sophora (+) , Cadia Chamaecytisus , Hovea (+) , Liparia , Piptanthus (+ , -) , Retama (+) , Adenocarpus (-) , Ammodendron (+) , Ammothamnus (+) , Anagyris (+) , Baptisia (+) , Keyserlingia (+) , Lupinus (- , +) , Thempletonia , Spartium , Chamaecytisus , Orobanche[a] .*	3b
Multiflorine	*Lupinus (-)*	3c
Aphylline	*Lupinus, Teline, Anabasis[a], Podalyria*	3d
Angustifoline	*Lupinus, Diplotropis, Ormosia, Cytisus*	3e
13 α-Tigloyloxylupanine	*Rothia , Ormosia , Calpurnia , Cadia , Lupinus, Cytisus , Orobanche*	3f
Anagyrine (-)	*Genista , Lupinus , Maackia , Sophora , Templetonia , Thermopsis , Baptisia , Teline , Ulex , Laburnum , Dichilus , Melolobium, Polhillia, Argyrolobium, Pearsonia, Castilleja[a] , Pedicularis[a] , Retama , Ormosia , Percopsis , Haplormosia , Bolusanthus , Clathrotropis , Osyris[a] , Chamaecytisus , Echinospartum , Hovea , Piptanthus , Anagyris , Cytisus , Petteria , Spartium , Ulex , Euchresta , Echinosophora , Viscum[a]*	4
Matrine	*Sophora (+) , Goebelia , Keyserlingia , Euchresta (+)*	5
Piptanthine	*Hovea (+) , Ormosia , Templetonia (+ , -) , Piptanthus*	6
Ammodendrine[b] (+)	*Lupinus , Ormosia , Maackia , Sophora , Templetonia , Cytisus , Retama , Thermopsis , Laburnum , Dichilus , Spartidium , Lebeckia , Rothia , Pearsonia , Lotononis , Pericopsis , Haplormosia , Castelleja , Genista , Echinospartum , Ammodendron , Liparia , Orobanche , Spartium , Baptisia*	7

The sign after plant refers to the optical rotation of the alkaloid. [a] not a member of the Fabaceae; [b] not a quinolizidine alkaloid , but co-occuring with QA .

alkaloids (**2**) seem to be very specialied and have as yet been found only in ***Camoensia***, ***Melolobium*** and ***Maackia*** (Table I-1). Matrines (**5**) are common in the genus ***Sophora*** and ***Ormosia*** type alkaloids are especially common in the genus ***Ormosia***. Although the alkaloids from Lycopodiales (***Lycopodium***), Lythraceae (***Heimia***, ***Decodon***), Nymphaeaceae (***Nuphar***) and from ladybird beetles (Coccinellidae) contain a quinolizidine ring system they are not reviewed here, due to lack of space [36].

A number of insects are spcialised on host plants which contain QA. Whereas some Coleoptera and Pentatomidae have been shown to excrete the dietary alkaloids [37], a number of oligophagous aphids (e.g. ***Macrosiphum albifrons***, ***Aphis genistae*** and ***A***. ***cytisorum***) actively store QA in their bodies and seem to use them for their own defence against predators [38]. Larvae of the moth ***Uresiphita reversalis*** store cystisine and N-methylcytisine, which is present in their host plant ***Teline monspessulana*** [39]. The uptake of QA seems to be specific and carrier mediated [40].

D. Biological Studies:

1- Biosynthesis and metabolism in plants and cell cultures:

Although most studies on quinolizidine alkaloids have been concerned with their phytochemical characterisation, the knowledge of their biological background is already substantial. The biosynthesis from lysine via cadaverine to lupanine, sparteine, matrine, lupinine and cytisine has been studied by using radioactive isotopes, or more recently by using ^{13}C- and ^{2}H-labelled precursors [41]. In addition, a number of the enzymic steps involved have been characterised [42]. But many steps are still unclear and hardly anything is konwn about the pathways leading to the many substitutions and variations of the quinolizidine ring skeletons.

Quinolizidine biosynthesis takes place in the aerial green parts of legumes. Its intracellular site is the chloroplast [43]. Biosynthesis is regulated by light; thus QA concentrations display a diurnal rhythm, with a stimulated production period during the day [44].These alkaloids are translocated by the phloem to other parts of the plant [45], and they accumulate predominantly in epidermal and subepidetrmal cell layers [46]. The subcellular site of QA storage is the vacuole, into which the alkaloids are pumped by a selective carrier system [47]. Especially rich are the seeds, which can store up to 8% of their dry weight as alkaloids. During germination the alkaloids are translocated from the cotyledons to the newly formed tissues, where they are partly degraded, obviously serving as nitrogen storage compounds [48].

They also disappear from senescing leaves during the vegetation period, indicating that they are not end products but metabolically mobile compounds [49].

Quinolizidine alkaloids metabolism has been studied in cell and organ cultures of more then 14 legume species [50]. Quinolizidine alkaloid formation is reduced in undifferentiated cells cultured *in vitro*, this seems to be caused by a reduced expression of the biosynthetic enzymes, by limited storing and transport capacity, and by an increased degradation activity of the cells [28]. Whereas the differentiated plants produce a variety of QA, the alkaloid patterns of the cell cultures are very simple since lupanine is always the main alkaloid [51]. Callus of ***Sophora flavescens*** accmulate matrine as the main product, and those of ***thermopsis lupinoides*** lupanine. Alkaloid formation is correlated with light and greening of the cell culture. In organised tissues QA production can be observed in shoots and leaves but not in roots [52], which supports the veiw that the genes of QA metabolism are regulated in a tissue-specific way, as is the case with many other cellular phenomena [52].

2- Chemical ecology and biological activity:

It can be shown experimentally that pure QA or QA mixtures are biologically active [25], in that they inhibit the multiplication of potato-X virus [53], the growth of Gram-negative and Gram-positive bacteria [54] and of certain fungi [55]. Quinolizidines deter or repel feeding of a number of herbivores (nematodes , bees , caterpillars , beetles , aphids , locusts , snails , rabbits , cows) or are directly toxic or mutagenic to them [56] . The toxic effects may be due to inhibition of K^+ ion channels, to interactions with the acetylcholine receptor, to inhibition of protein syntheis and to other mechanisms which have not been elucidated [57]. Thus QA are broad range bioregulators. Their concentration in the plant is sufficiently high to perform their inhibitory effects observed *in vitro.* In adition QA contents can be increased by wounding: this effect was highest under greenhouse conditions, but also measurable in the field [58]. In this context, QA localisation in epidermal tissues can be interpreted as a strategically important adaptation, since this tissue has to ward off small herbivores and pathogens in the first instance. Plant breeders have selected lupin mutants which produce only minute amounts of alkaloids. These so-called ' sweet ' lupins have a dramatically reduced fitness under natural conditions as compared to their ' bitter ' wild forms. They are preferentially eaten by rabbits, leaf miners, aphids or beetles and are vulnerable to other pathogens [59]. Thus it seems well established that the ecological function of QA is that of defence against herbivores, but also against microorganisms. Some evidence also suggest activity against competing plants [60]. It is known that QA have toxicological and

pharmacological activities. They interact with Ach receptors as agonists and some inhibit Na + and K+ channels; which might lead to respiratory paralysis and ventricular arrest at high doses.

Investigations of any possible antidiabetic effect of Lupins , in general , has until now mainly focused on the QA and a series of alkaloids have been reported as characteristic constituents and are generally thought to be responsible for the hypoglycaemic activity of the seed powder . Some reports show that Lupin alkaloids such as Lupanine, Sparteine, Multiflorine and N-methylcytisine, in fact, do posses hypoglycaemic activity [61].

I-2-2- Extraction and Separation:

A. Extraction:

In the following a laboratory routine for QA extraction which has been successfully used several thousand times in the author's laboratory is described.

1-Stability of alkaloids:

It is important to ensure that, as far as possible, alkaloids isolated from plants or other sources are qualitatively and quantitatively the same as those which were present in the original material. There are several ways in which alkaloids might be altered during isolation procedures.

Alkaloids may be changed by heat or by enzymeic action when harvested plants are stored and dried. Bull and call. Reported the loss of 50-80% of alkaloids during drying of some, but not all, ***Crotalaria*** and ***Heliotropium*** species [62]. On the other hand, Pderson [63] compared the alkaloid content of fresh leaves with that of dried leaves from several species of the Boraginaceae; allowing for the loss in weight on drying there were no changes in total alkaloid content, suggesting that enzymeic oxidation or reduction of alkaloids could take place during the early stages of drying.

If fresh green plant material cannot be extracted straight away, it should be derived as quickly as possible to minimize enzyme action and fungal decomposition. A warm environment, with plenty of circulating air, is best, direct suglight should be avoided because of possible overheating, the alkaloids are not especially light-sensitive. After drying, the plant should be stored in a cool place [8].

2-Fresh plant material:

It is common practice to dry plant material prior to extraction. Since QA may be modified during the drying process (e.g. hydrolysis of ester alkaloids), it is recommended that material is freeze-dried (which avoids decompartmentalisation and thus enzymic breakdown processes) or that fresh material or material stored frozen at -20°C is used.

The extraction of QA is based on the fact that the free alkaloidal base does not dissolve in water but in an apolar organic solvent, such as ether or methylene chloride. In contast, the charged alkaloids (most are charged molecules under acidic conditions) are soluble in water but insoluble in apolar organic solvents. Both the free base and the salts dissolve in MeOH.

For most analytical purposes it is convenient to take 5 or 10g of the fresh or frozen material and to homogenise it in about 15 ml 0.5_M HCI. Homogenisation can be performed by pestle and mortar (use quartz sand for grinding), with a Waring blender or an Ultraturrax. Great care should be taken that the mortar, blender or Ultraturrax is cleaned rigorously after use in order to avoid contamination (QA stick to surfaces!). The homogenate is transferred to a beaker and is left standing for at least 30 min (overnight incubations are also appropriate). In the next step the homogenate is centrifuged for 10 min at 5000 to 10 000 x g and the clear supernatant is collected. For quantitative work the pellet is resuspended in 0.5_M HCI and centrifuged again. Both supernatants are then pooled, made alkaline by 4_M NH_4OH (pH 12–14), and left standing for 15 min. It is recommended that solid phase extraction be used as the next step, since this procedure can easily be standardised and applied to the extraction of many samples concomitantly. Chemelut (Analytichem) or Extrelut (Merck) are modified kieselgur powders which have a high adsorption capacity for water (c. 14g bind 20ml water). Commercial extraction columns (Merck or Analytichem) (which can be reused after thorough cleaning) are filled with c. 14g matrix material. Up to 20 ml of the homogenate (determine the exact volume!) can be applied on the column. It will take c. 10 min for the homogenate to be adsorbed the collumn matrix. The column is then eluted 2 to 3 times with 20ml methylene chloride. The eluate is collected in a flask and evaporated in a rotavapor under reduced pressure at 40°C. When the solvent has been evaporated the crude alkaloid extract is dissolved in a small volume of methylene chloride and transferred quantitavely to a sample vial. The solvent can be evaporated in these vials either by delivering a steady stream of nitrogen gas or just by leaving the samples overnight in a fume cupboard. Then the vials are closed and stored if possible at 4°C or even better at – 20°C until analysis.

Alternatively, extraction can be performed by liquid-liquid extraction: methylene chloride is added to the alkaline homogenate and mixed in a shaking funnel. The lower organic phase is collected. The process is repeated at least twice. The organic phases are pooled and processed as described above. Depending on the material, emulsion will be encountered using the liquid-liquid precedure but not with the solid- phase extraction [27].

3-Seeds and dried material:

Dry material, which needs to be milled into a fine powder prior to extraction, can be extracted by two procedures.

a) About 500mg of plant material is suspended in 15 ml 0.5 M HCl and left standing under continuous stirring (magnetic stirrer) for at least 3h. The following steps are identical to those outlined above.

b) About 500mg or more of plant powder are extracted with MeOH for several hours, which is conveniently performed with a Soxhlet apparatus. The methanolic extract is then evaporated to dryness under reduced pressure in a rotavapor. For some applications (HPLC, TLC) this extract can be used directly. Normally, these extracts need to be further purified: the dried residue is dissolved in 15ml 0.5 M HCl and extracted at least three times with methylene chloride (until a clear organic phase is obtained) in a shaking funnel to remove lipid material. Then the aqueous phase is made alkaline with 4N NH_4OH and extracted with methylene chloride using either solid-phase extraction or liquid-liquid extraction as described above [27].

B. Detection of Alkaloids:

Numerous reagents are recommended for simple ' spot test ' identification of alkaloids, most working best in a dilute aqueous acid solution of the alkaloid. Pride of place among these must go to Dragendorff's Reagent but there are many others, such as iodoplatinic acid, Mayer's Reagent, Hagar's Reagent and Wagner's Reagent. Mixing a drop of extract with a drop of the Reagent will lead to the development of a precipitate if alkaloids are present.

Great care must be taken with these spot tests because, while alkaloids will generally be characterised by strong reactions, other types of natural products are able to react to some degree (flavonoids, coumarines, tannins). So-called ' false positives ' can be eliminated by taking the alkaloid-containing acid extract, making it basic and extracting the alkaloid into an organic

solvent. This is followed by separation of the organic phase and its back extraction into acid again. If the new acid extract still gives a positive reaction then the presence of alkaloid base is confirmed; although if the alkaloid had been non-basic or quaternary and hydrophilic it would be lost. In addition to these general colour reagents there are many others that are said to be specific to certain alkaloid types. Today, their use is very limited [2].

Table I-2: Some common spray reagents for detecting alkaloids [2].

Dragendorff Reagent	**(a)** 0.85g bismuth (III) nitrate in 10ml glacial acetic acid and 40ml water. **(b)** 8g potassium iodide in 20ml water. Mix (a) and (b) in equal portions to use as spray or spot test reagent.
Cerium sulphate	0.1g cerium (IV) sulphate in 4ml water. Add 1g trichloroacetic acid, boil and add dropwise 97% sulphuric acid until solution clarifies. Heat to produce colours.
Cobalt (II) thiocyanate	Ammonium thiocyanate (3g) and cobalt (II) chloride (1g) in 20ml water.
Iodoplatinic acid	**(a)** 5% hexachloroplatinic (IV) acid solution **(b)** 10% potassium iodide solution. Mix 5ml (a) with 45ml (b).
Sodium tetraphenylboron	1% sodium tetraphenyloboron solution in ethyl methyl ketone saturated with water.

C. Chromatography:

1-Thin Layer Chromatography:

Since most plants contain complex mixtures of up to 30 or more individual alkaloids, Thin Layer Chromatography (TLC), with its limited resolution power, is helpful primarily for initial analysis of the major alkaloids and for preparative work. A quantitative TLC system has been described by Karlsson and Peter [64].

The following solvent systems are adequate for silica gel G plates:

a) MeOH –28% NH_4OH (65:1).

b) $CHCL_3$–MeOH–28% NH_4OH (85:15:1).

c) Cyclohexane –diethylamine (70:30).

In Table I-2 R_f Values of some representative QA are tabulated. They can be visualised with ' Dragendorff's ' reagent as orange red spots; KI/I_2 produces dark brown stains. Plates need to be dried abd heated (20min at 110°C) prior to visualisation in order to remove the ammonia or diethylamine which interfere with the colour reaction [27].

Table I-3: Selection of TLC R_f values [27].

Alkaloid	R_f value		
	System 1	**System 2**	**System 3**
Lupinine	0.25	0.36	0.52
Camoensidine	0.16	0.31	0.39
Lupanine	0.30	0.69	0.51
Sparteine		0.07	0.93
Multifloine	0.27	0.35	0.26
Aphylline	0.60	0.80	0.52
Angustifoline	0.41	0.63	
$13\alpha-$ Isolupanine	0.51	0.69	0.50
Anagyrine	0.49	0.66	0.30
Matrine	0.26	0.52	0.35
Ammodendrine	0.17	0.23	0.37
Retamine	0.25	0.18	0.65

System 1 : MeOH –28% NH_4OH (65:1) .

System 2: $CHCL_3$–MeOH–28% NH_4OH (85:15:1).

System 3 : Cyclohexane –diethylamine (70:30).

2-Liquid Chromatography:

(a) Low-pressure Chromatography. For preparative work QA can be separated by liquid chromatography on silica (70-230 or 230-400 mesh, Merck) with the following solvent systems: CH_2CL_2–MeOH –25% NH_4OH (70:30:1) [65] or CH_2CL_2–MeOH –25% NH_4OH (90:9:1) [66] or diethyl ether–MeOH–25% NH_4OH (100:30:1) [66]. Arslanian and coll. [67] used a basic AL_2O_3 column (Merck, Type T) with solvents of increasing polarity, starting with ether, then 10% MeOH in ether, 50% MeOH in ether, and finally MeOH.

(b) High Performance Liquid Chromatography. High performance liquid chromatography (HPLC) has been employed to the analysis of QA mixtures. Saito and *coll.* [68] Used an Inertsil ODS (5µm(column (150 x 4.6mm(with the solvent system acetonitrile–mM potassium phosphate buffer (pH 5.5) (1:9). Murakoshi and coll. [66] used LiChrosorb Si 100 (Merck; 10µm, 0.3 x 50cm) with the solvent system 15% MeOH in Et_2O–H_2O–28% NH_4OH (500:10:3). Saito and coll. [68] separated 22 of these alkaloids on LiChrosorb Si 60 (5µm) (250 x 4.6mm) with the solvent systems 15% or 25 % MeOH in diethyl ether–5% NH_4OH (25:1), or 50% MeOH in diethyl ether–5% NH_4OH (25:2). R_t values are presented in [68] and [69].

Detection can be performed using refractive index (RI) or UV detectors. Since most QA have no sensitive chromopore, UV detection is performed between 205 and 220nm. α-Pyridone alkaloids (Fig. I-1, type **4**), such as cytisine or anagyrine, have an aromatic ring and can be monitored at 310nm [68].

Using preparative columns, HPLC is a convenient technique for isolating sufficient amounts of individual alkaloids (only spateine, lupinine, α-isolupanine and cytisine are commercially available) for spectroscopic or biochemical purposes [69]. Wink have successfully employed a semi-preparative column (300 x 7.8mm; 10µm material, µ-Bondapak $C_{18;}$ waters), using an isocratic system with the solvent system 0.01M phosphate buffer–20% MeOH–20% isopropanol [65]. Murakoshi and coll. [66] and Ohmiya and coll. [69] used Lichrosorb Si 100 (Merck ; 10µm , 0.3 x 50 cm) with the solvent system 15% MeOH in Et_2O–H_2O –28% NH_4OH (500:10:3) .

3-Gas –Liquid Chromatography.

Until 1980 most gas–liquid chromatography (GC) applications to separate QA mixtures involved packed columns. However , best results can be obtained using the new generation of fused silica capillary columns with bonded phases : The capillary columns (dimensions 15m x 0.23mm to 30m x 0.32mm) have a higher number of theoretical plates (> 70 000) , which allow the separation of complex mixtures (Fig. I-2) and even of enantiomers , epimeric at C-11 or C-6 , such as sparteine and α-isospartiene , lupanine and α-isolupanine ,13-hydroxylypanine and 13-epihydroxy-lupanine , anagyrine and thermopsine ,13α-tigloyloxylupanine and 13β-tigloyloxylupanine , and of *cis* and *trans* isomers , such as 13α-Angeloyloxylupanine and 13α-Tigloyloxylupanine , as well as the *trans*- and *cis*-cinnamic acid esters [70] . Furthermore, even nanogram amounts of QA can be detected by FID (flame ionisation detector) or more sensitively and specifically by a nitrogen specific detector (abbreviated PND or NPD). Therefore, for most biochemical and phytochemical applications, capillary GLC seems to be the method of choice for QA analysis. A further convenience is the possibility of performing GC-MS analyse [27] (section I-2-3-B).

As a liquid phase several silicone derivatives (0.1µm or 1µm films) are employed , good resolutions have been obtained using DB-1 or DB-5 columns (J & W Scientific) , but equivalent products of other manufacturers also work . Split injection techniques are usually appropriate. On-column injection does not provide significant advantages for most applications. Helium is routinely used as carrier gas, but hydrogen or nitrogen will also work. The injector temperature is usually set to 250°C, that of the detectors to 300°C [71].

Hydroxylated QA, sush as 13-hydroxylupanine or 4-hydroxylypanine, may be derivatised by TMS prior to injection to avoid tailing and to achieve better quantification. Care should be taken not to use the PND for these derivatives, since the PND would soon be destroyed [27].

Some authors give relative retention indices for QA (e.g. [72]). However, Kovats retention indices (RI) [73] give better comparative information and are helpful in identifying individual alkaliods in a GC profile. Some representative RI indices are given in Table I-4.

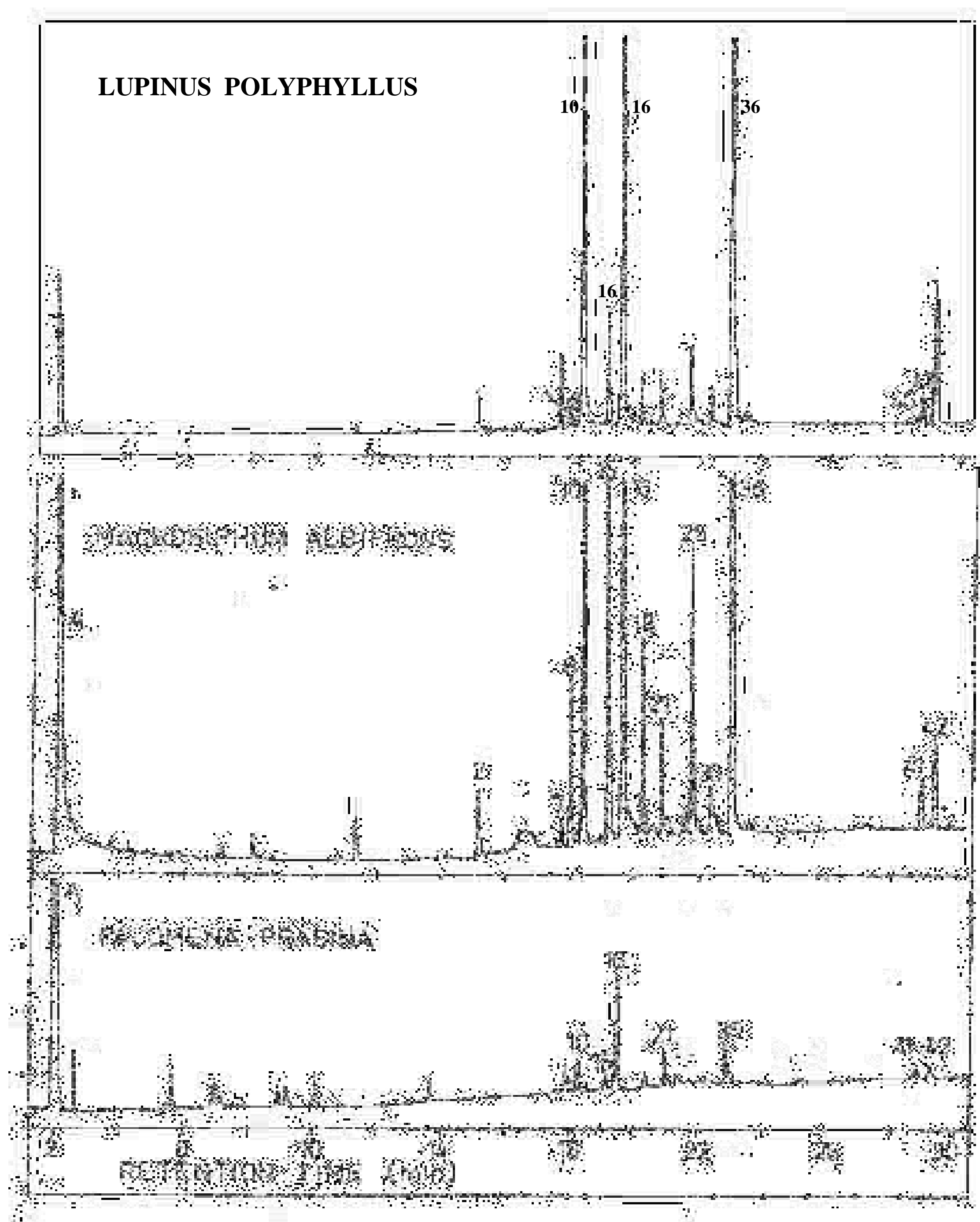


Figure I-2-Separation of QA of *Lupinus polyphyllus* and two infesting insects by capillary GLC [27].
Key : 2, ammodendrine; 7, 'isoangustifoline'; 8, tetrahydrohombifoline; 9, 'dehydroangustifoline'; 10, angustifoline ; 15, dehydrolupanine; 16, lupanine; 18, 11,12-12,13-didehydromultiflorine (*N*-methylalbine); 21, nuttaline; 25, multiflorine; 36, 13α-hydroxlupanine; 40, 13α (2-methylbutyryl)oxylupanine; 41, 13α-angeloyloxylupanine; 42, 13α-tigloyloxylupanine .

I-2-3-Characterisation:

A. Ultraviolet and Infrared Spectroscopy:

Only the α-pyridone alkaloids with an aromatic ring A and the cinnamoyl derivatives have a reasonable chromophore; thus UV spectroscopy does not normally provide much information for the identification of QA [74]. Until *c*. 1970 infrared spectroscopy was an important method for the identification of QA; such data are found in many papers [75]. Since MS and NMR are more powerful methods, infrared spectroscopy has been replaced by these to a large degree. Optical rotation measurements and circular dichroism have been employed to determine the absolute configuration of QA by, among others, [76] and [77].

B. Mass Spectroscopy:

Following the pioneering work of Schumann and coll. [78] and Neuner-Jehle and coll. [79 , 80] who worked out the principal fragmentation patterns of the most important QA , mass spectrometry (MS) developed into a convenient method of QA characterisation [81] . Mass spectrometry is widely used today, since QA usually provide distinctive fragmentation patterns in the electron impact mode (EI-MS). Chemical ionisation (CI-MS) , field desorption (FD-MS) and fast atom bombardment (FAB-MS) are suitable for identifying molecular ions of QA esters and tricyclic alkaloids , whose molecular ions are usually obscure or absent in EI-MS spectra . A major advantage of MS is the possibility it gives of combining the high resolution power of capillary GC with sensivity and informativity of EI- or CI-MS. Work using GC-MS was very much facilitated after 1980 by the development of new GC capillary columns, the development of new methods to position the GC column outflow near the MS ion source and, most importantly, by the improved possibilities of data processing. During a GC-MS run, MS spectra are recorded continuously, thus a typical GC-MS run consists of up to 1000 spectra. These data can easily be processed by computer, with background subtraction or the profiling of individual and charateristic fragment ions (‘ion chromatography ’), which are helpful to select the relevant MS spectra. A normal plant extract contains a number of hydrocarbons and contaminants, such as phthalic acid derivatives, which can be identified because of their distinctive m/z 149 fragment [82].

In Table I-4 the molecular ion and the four most informative fragments and their abundances are tabulated. For CI-MS data the molecular ion ($M + H^+$) is given. The data for

FAB-MS were reported, among others, [41]. Field desorption and chemical ionisation techniques are also helpful in the direct analysis of alkaloid mixtures [83].

Table I-4 :Identification of representative quinolizidine alkaloids using capillary GLC and mass spectrometry. For each compound the following information is provided (is available) : name ; Kovats retention index (RI) ; molecular ion M^+ ; EI-MS , five significant fragments and their relative abundane (%) ; CI-MS [27] .

Compound	RI		EI – MS					CI-MS
Lupinine	1422	169	152 (100)	169 (90)	148 (82)	97 (85)	136 (70)	170 (100)
Camoensidine	2080	234	122 (100)	135 (56)	234 (52)	233 (44)	84 (52)	
Lupanine	2165	248	136 (100)	149 (60)	248 (40)	150 (34)	219 (8)	249 (100)
Sparteine	1785	234	137 (100)	98 (90)	234 (44)	193 (25)	84 (10)	235 (100)
Multiflorine	2310	246	134 (100)	246 (65)	148 (20)	110 (15)	217 (5)	247 (100)
Aphylline	2180	248	136 (100)	220 (45)	124 (40)	248 (35)	191 (20)	249 (100)
Angustifoline	2083	234	193 (100)	112 (50)	150 (15)	55 (20)	134 (5)	235 (100)
13α-Acetyl-oxylupanine	2450	306	246 (100)	134 (70)	148 (30)	112 (39)	55 (30)	307 (100)
13α- Angeloyloxylupanine	2733	346	246 (100)	134 (30)	148 (15)	112 (12)	55 (10)	347 (100)
Anagyrine	2390	244	98 (100)	244 (40)	146 (20)	160 (15)	136 (15)	245 (100)
Matrine	2265	248	248 (100)	205 (80)	96 (70)	150 (60)	247 (75)	249 (100)
Ammodendrine[a]	1865	208	165 (100)	136 (60)	123 (60)	208 (55)	191 (50)	209 (100)

Laser desorption mass spectrometry (LAMMA 1000) is a soft ionisation method which produces significant $(M – H)^+$ ions and informative fragment ions. This method has been used to localise the site of QA storage in plant tissue; sparteine could be located in the epidermal and subepidermal cell layer of ***Cytisus scoparius*** stems. Lupanine is accumulated in the epidermis of petioles of ***Lupinus polyphyllus*** [84].

Alkaloid extracts of many legumes contain piperidine alkaloids such as ammodendrine, *N*–methylammodendrine, hystrine or smipine. These alkaloids also derive biogenetically from lysine via cadaverine [85]. Mass spectral information is provided in Table I-4.

C. Nuclear Magnetic Resonance Spectroscopy:

Proton and carbon-13 nuclear magnetic resonance (NMR) techniques are essential and extremely powerful methods for the structure elucidation of new QA and are often used in combination with MS techniques. The only disadvantage is that some NMR techniques still require relatively large amounts of pure compounds (>10mg), which need to be first isolated from complex mixtures. In consequence , NMR techniques are usually employed for the analysis of the major alkaloids , which can be purified more easily , whereas most minor alkaloids , which are often very important for chemosystcmatics or other biological questions , have only been caracterised by MS , since in GC-MS studies only µg amounts are required . A representative cross-section of ^{13}C NMR data for QA is illustrated in Fig.I-3 [27].

Data for ^{2}H and ^{1}H NMR have been reported [86].

D. X-Ray Crystallography:

In a number of recent investigations QA have been studied crystallographically by X-ray diffraction techniques [67].

I-2-4-Qantification:

Many biochemical and agricultural problems (e.g. determination of QA contents in Lupin seeds, which are used for animal nutrition), require either the quantification of total alkaloids or of every single QA. For total alkaloid analysis a number of different methods are available. Prior to analysis the alkaloids need to be extracted, preferably by solid phase extraction as described above. The methods that can be used for analysis are: (1) gravimetry; (2) titrimetry with *p*-toluenesulphonic acid, and (3) photometry with bromocresol purple or I_2/KI [87].

Immunological methods such as radioimmunossay (RIA) and enzyme-linked immunossay (ELISA) have been established only recently. These allow the quantification of nanogram amounts of lupanine-type alkaloids. In principle, antibodies are raised in rabbits against bovine serum albumin to which 13-hydroxylupanine is coupled over a succinic acid

bridge. These antibodies are selective for lupanine-type alkaloids, but discriminate sparteine, lupinine, cytisine and tricyclic alkaloids (Fig.I-4). A respective RIA, SPA (scintillation proximity assay) and ELISA has been worked out [88]. A comparable ELISA has also been developed in Australia [27].

Methods (1) – (3) listed above work if the alkaloid contents in the extracts are in excess 100μg g^{-1}. Radioimmunossay and ELISA techniques are especially suitable for large screenings, when only minute amounts of material are available, and if the alkaloid sample consists mainly of lupanine-type alkaloids (as is the case in many lupins) [27].

If individual alkaloids in the extracts are to be analysed, then HPLC, or preferably GC, is the method of choice. If possible, reference alkaloids should be used to work out calibration procedures [71]. For practical purposes the commercially available alkaloids (sparteine, lupinine, cytisine, and α-lupanine) are usually adequate. Quantification can either be performed by the internal or external standard procedure [27].

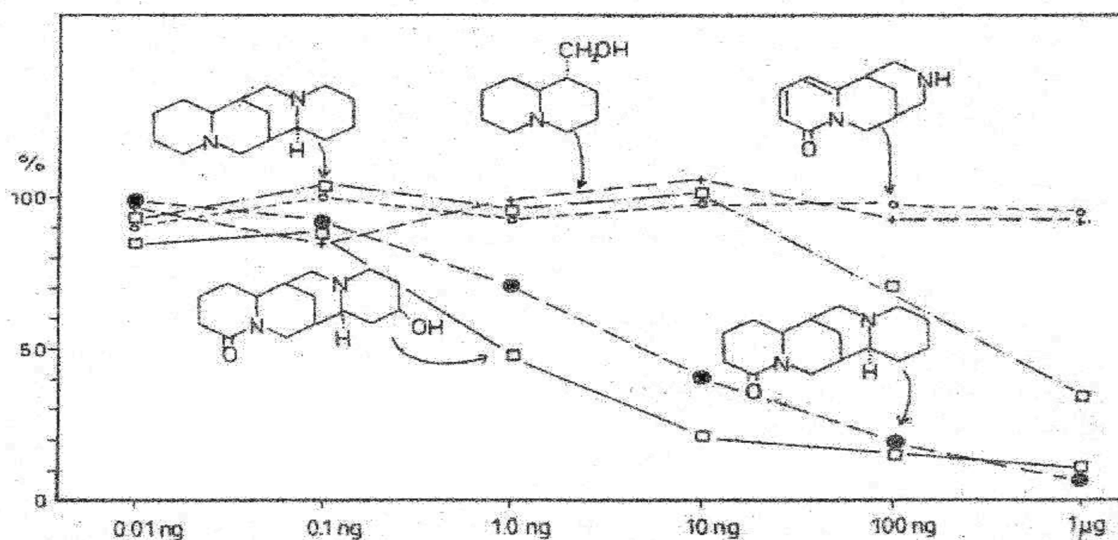


Figure I-3-Selectivity and sensitivity of a lupanine RIA. Scintillation proximity assay (SPA) was performed with fluomicrospheres coupled to anti-rabbit antibodies according to the procedure of Amersham. Specific antibodies were raised in rabbits which detect lupanine and 13α-hydroxylupanine, whereas lupinine and cytisine are completely discriminated. Sparteine is recognised at higher alkaloid concentrations [27].

Chapter two

Results and Discussion

Our research on quinolizidine alkaloids from the plant ***Cytisus purgans*** revealed the presence of twelve (12) known compounds which were confirmed with the use of GC/MS analysis, as well as previous QA spectral data. The identification of components was accomplished using computer search in commercial libraries.

13α-Hydroxylupanine was isolated from the MeOH extract of aerial parts of ***Cytisus purgans*** by preparative TLC and was identified using UV and IR data.

Finally, we are tested the antimicrobial activity of MeOH extract of this plant against three species of bacteria and two species of fungi.

II-1-GC/MS Analysis:

The crude MeOH extract of aerial parts of ***Cytisus purgans*** was subjected to GC/MS analysis, and it gave peaks as shown in Figure II-1.

Capillary GC in combination with mass spectrometry was found to be the method of choice for the analysis of complex mixtures of QA [89]. This technique allows much more precise alkaloidal identifications to be made than by comparative TLC and GLC methods, and enables even trace constituents to be analysed [90]. In this context, many studies have been published. Wink and coll. [89] have studied the pattern of QA in 56 species of the genus ***Lupinus*** using this method, 102 compounds were identified. K. B. Torres and coll. [91] have studied the alkaloid profile of leaves and seeds of ***Lupinus hintonii*** **C. P. Smith** using GC/MS, 19 compounds were detected.

We have studied the QA patterns of ***Cytisus purgans*** by this method and could determine the structures of 12 alkaloids according to their mass fragmentations patterns.

Twelve (12) known quinolizidine alkaloids were identified which eleven (11) were were of sparteine-type which are:

Compound **1** : Sparteine .

Compound **2** : $\Delta^{11,12}$ Sparteine .

Compound **4** : Retamine .

Compound **5** : 17-Oxosparteine .

Compound **6** : α-Isolupanine .

Compound **7** : Lupanine .

Compound **8** : 13α-Hydroxylupanine .

Compound **9** : 13α-Acetyloxylupanine .

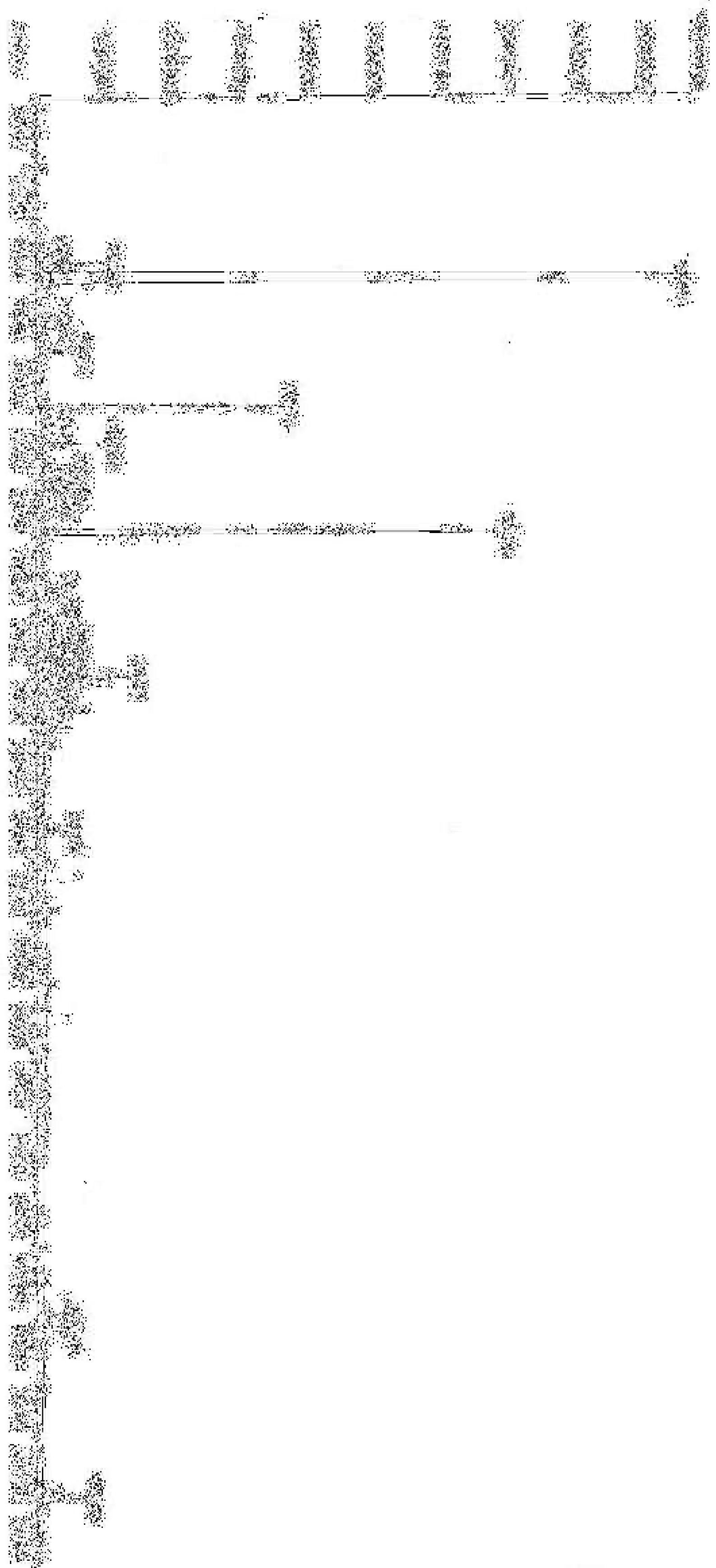
Compound **10**: 13α-Propyloxylupanine.

Compound **11**: 13α-Angeloyloxylupanine.

Compound **12**: 13α-Tigloyloxylupanine.

And one (1) was a piperidine alkaloid which is:

Compound **3**: Ammondendrine.



II-1-1-The Sparteine:

The mass spectrum of compound **1** (**R_t = 16.83min, M_w = 234g/mol**) (Fig.II-2) showed a molecular ion $[M]^+$ at m/z 234 (23.77 % rel. int.). A base peak at m/z 193 (22.13 %) corresponding to $[M - 41]^+$ indicating the loss of $C_3H_5^{\bullet}$ which also showed at m/z 41 (10.65 %) (allylic cation $CH_2 \cdots CH \cdots CH_2^{+}$). An other base peak at m/z 137 (100 %) indicating the cleavage of the compound. The peaks at m/z 110 (18.85 %), m/z 98 (64.75 %) and m/z 55 (9.01 %) corresponding to fragmentation of lupinine ion respectively [92].

It is known also that the fragment at m/z 98 (64.75 %) arises from ring A following the fragmentation pattern of the sparteine-type alkaloids [93].

These results indicate that compound **1** might be the sparteine ($C_{15}H_{26}N_2$).
The scheme II-1 showed the possible pathway of fragmentation of sparteine.

II-1-2-The $\Delta^{11, 12}$ Sparteine:

The mass spectrum of compound **2** (**R_t = 17.96min, M_w = 232g/mol**) (Figure II-3) showed a molecular ion $[M]^+$ at m/z 232 (61.47 %). The pattern of fragmentation of this compound is very similar to those of compound **1** except for the $[M]^+$ which is low of two (2) units of mass.

These results indicate that compound **2** might be $\Delta^{11, 12}$ Sparteine ($C_{15}H_{24}N_2$).

The scheme II-2 showed the possible pathway of fragmentation of $\Delta^{11, 12}$ Sparteine.

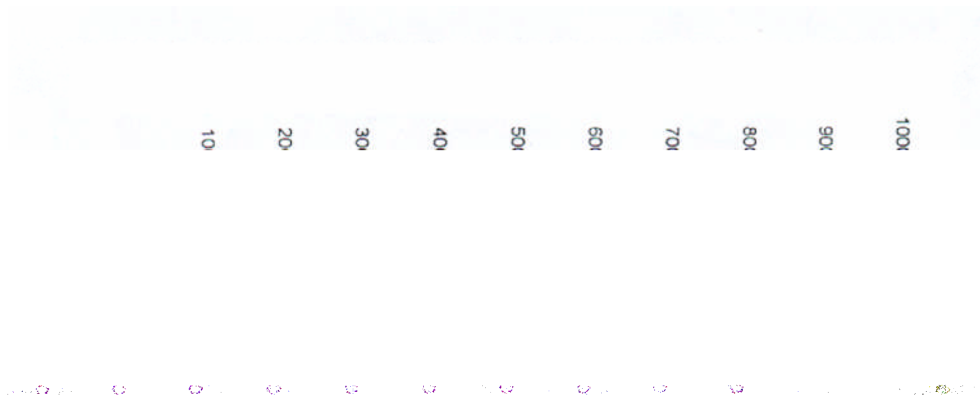
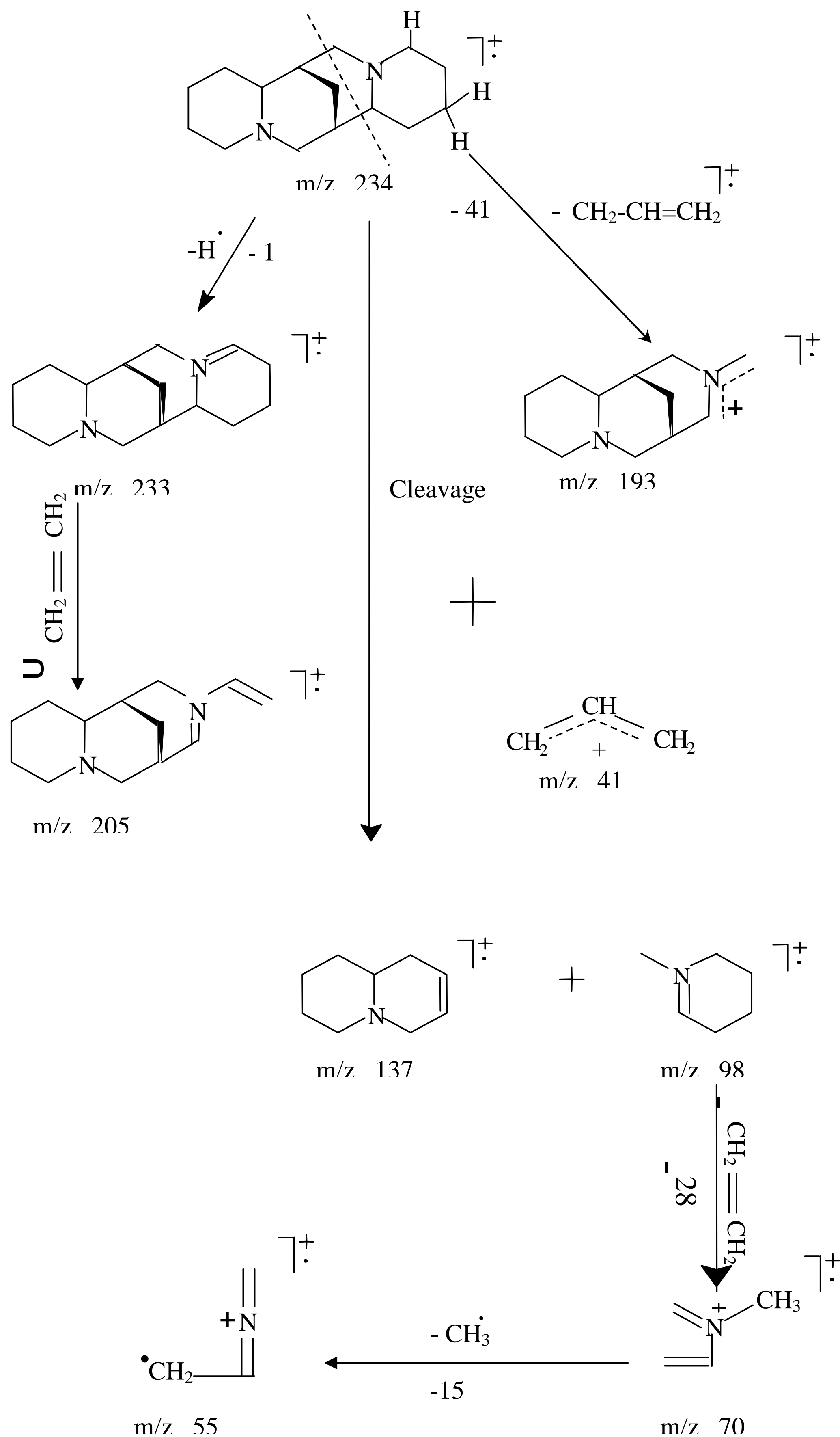


Figure II-2-Mass spectrum of Sparteine.



Scheme II-1-Possible pathway of fragmentation of Sparteine [94].

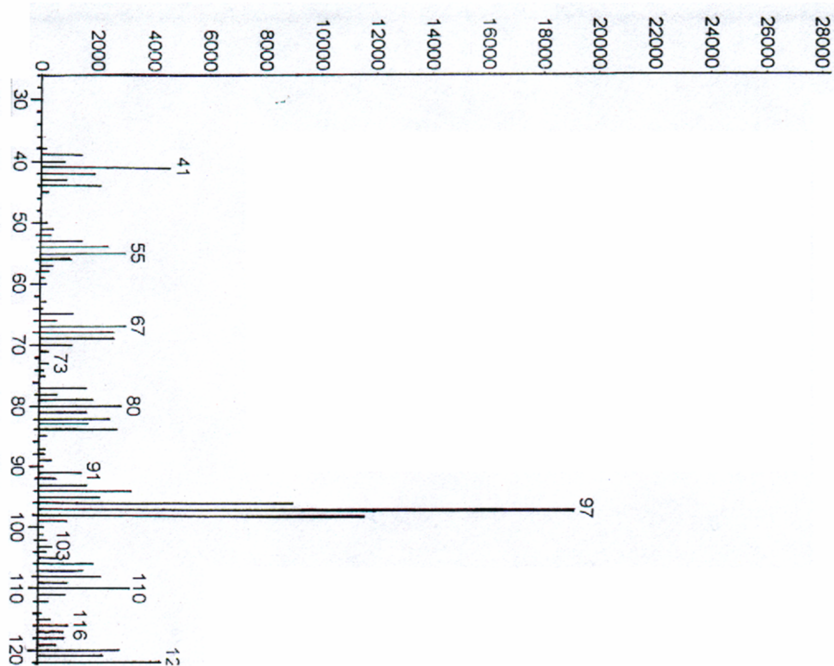
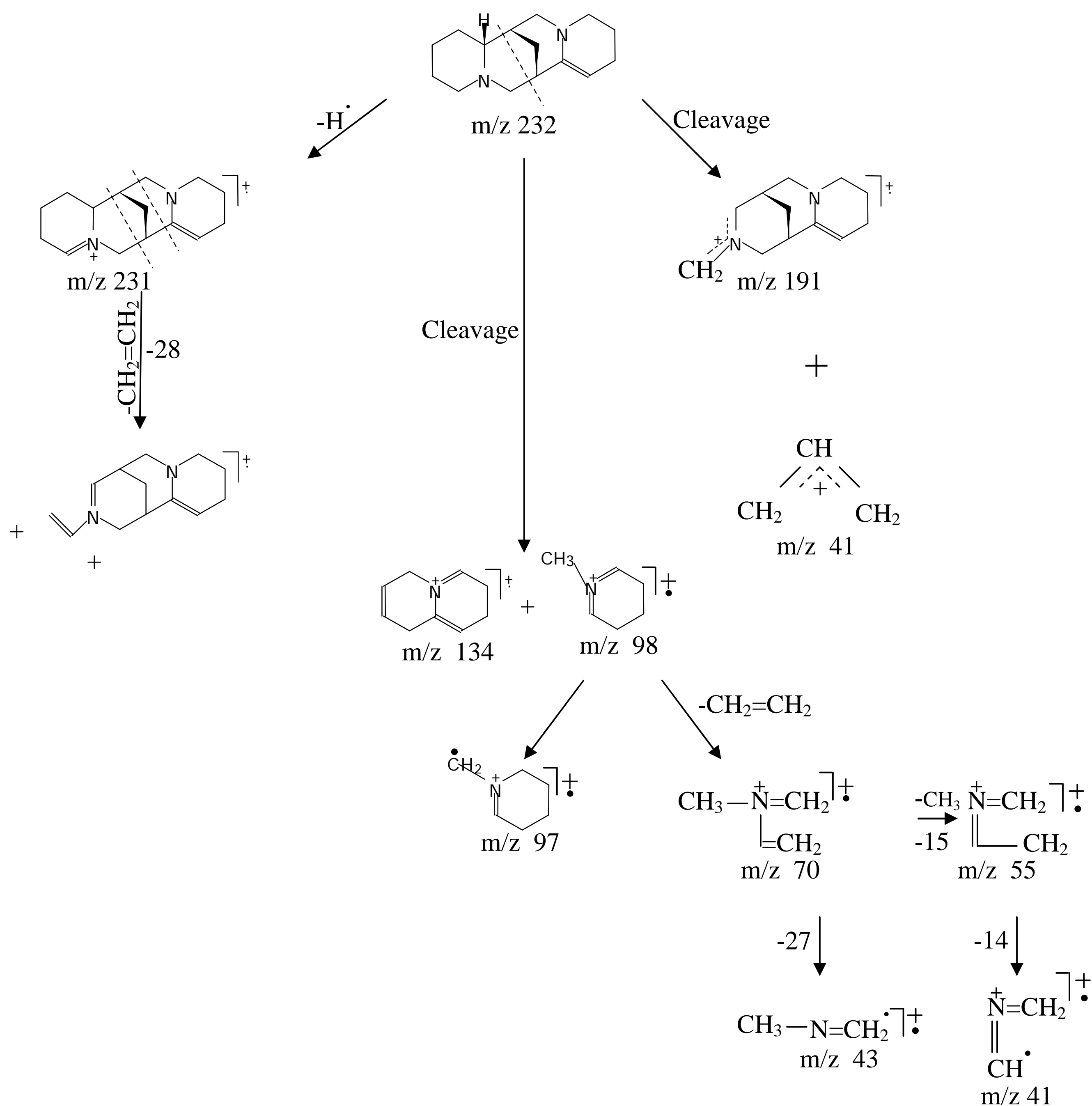


Figure II-3-Mass spectrum of $\Delta^{11,12}$ Sparteine.



Scheme II-2-Possible pathway of fragmentation of $\Delta^{11,12}$ Sparteine [94].

II-1-3-The Ammodendrine:

The mass spectrum of compound **3** (**R_t = 18.97min, M_w = 208g/mol**) (Figure II-4) exhibited a molecular ion $[M]^+$ at m/z 208 (51.63 %). A characteristic fragments ions at 165 $[M - MeCO]^+$ (100 %) and m/z 191 $[M - OH]^+$ (61.47 %) indicated the presence of N-acetyl group. The peaks at m/z 180 $[M - CH_2{=}CH_{2}]^+$ (8.2 %) and m/z 179 $[M - CH_2\text{-}CH_3]^+$ (50.81 %) also suggested the presence of N-acetyltetrahydropyridine [95].

The peaks at m/z 123 (57.37 %) and m/z 85 (19.67 %) resulting from the cleavage of compound at position C_5-$C_{2'}$. The fragment at m/z 56 (9.83 %) resulting from the opening of pyridine ring.

Consequently, the compound **3** might be the ammodendrine. The scheme II-3 elucidated the possible pathway of fragmentation of this compound.

II-1-4-The Retamine:

Concerning compound **4** (**R_t = 20.74min, M_w = 250g/mol**). A molecular ion at m/z 250 (4.1 %), the presence of a hydroxyl group was indicated by the fragments at m/z **233** (27.86 %) and m/z 232 (43.44 %) in the EI-mass spectrum (Figure II-5), corresponding to $[M - OH]^+$ and $[M - H_2O]^{+\cdot}$ respectively [96].

A base peaks at m/z 134 (44.26 %) and m/z 98 (100 %) indicating the cleavage of compound. The other peaks are very similar at those of sparteine.

By combination of this results and mass spectral data to those of reference data stored in our data library [27, 89], compound **4** could be the retamine (12α –hydroxy-β-isosparteine) .

The scheme II-4 elucidated the possible pathway of fragmentation of this compound.

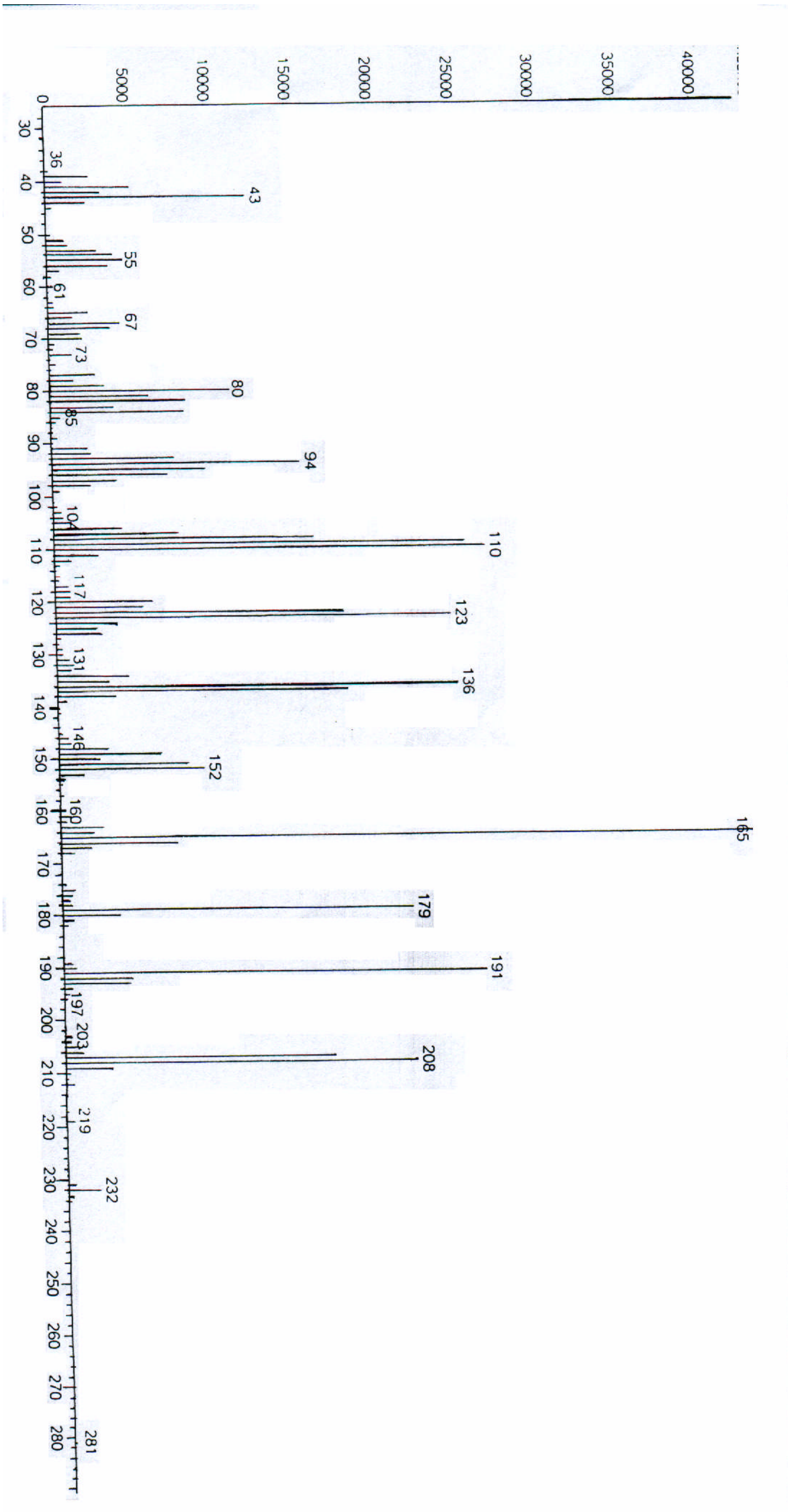
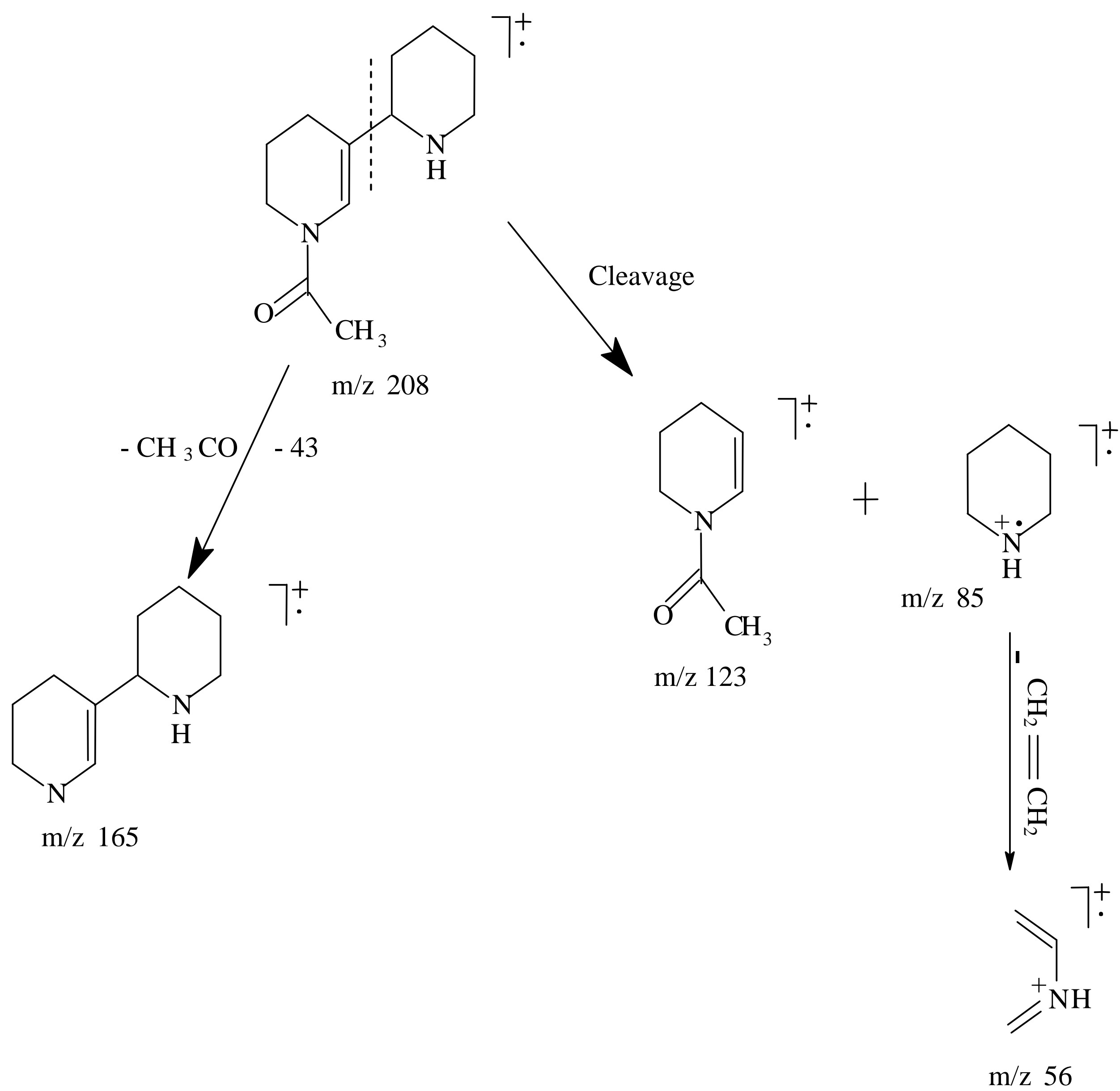


Figure II-4-Mass spectrum of Ammodendrine.



Scheme II-3-Possible pathway of fragmentation of Ammodendrine [94].

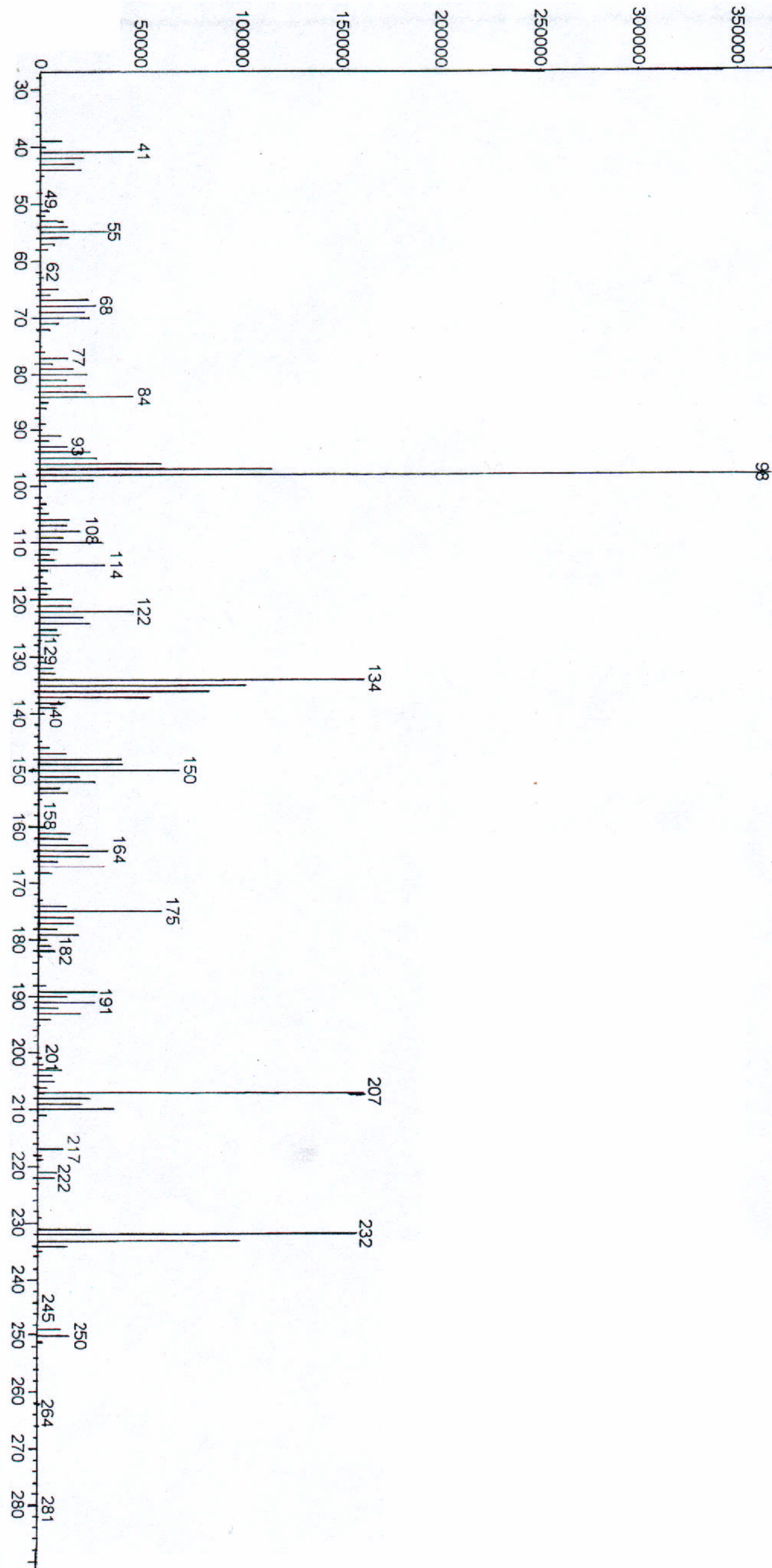
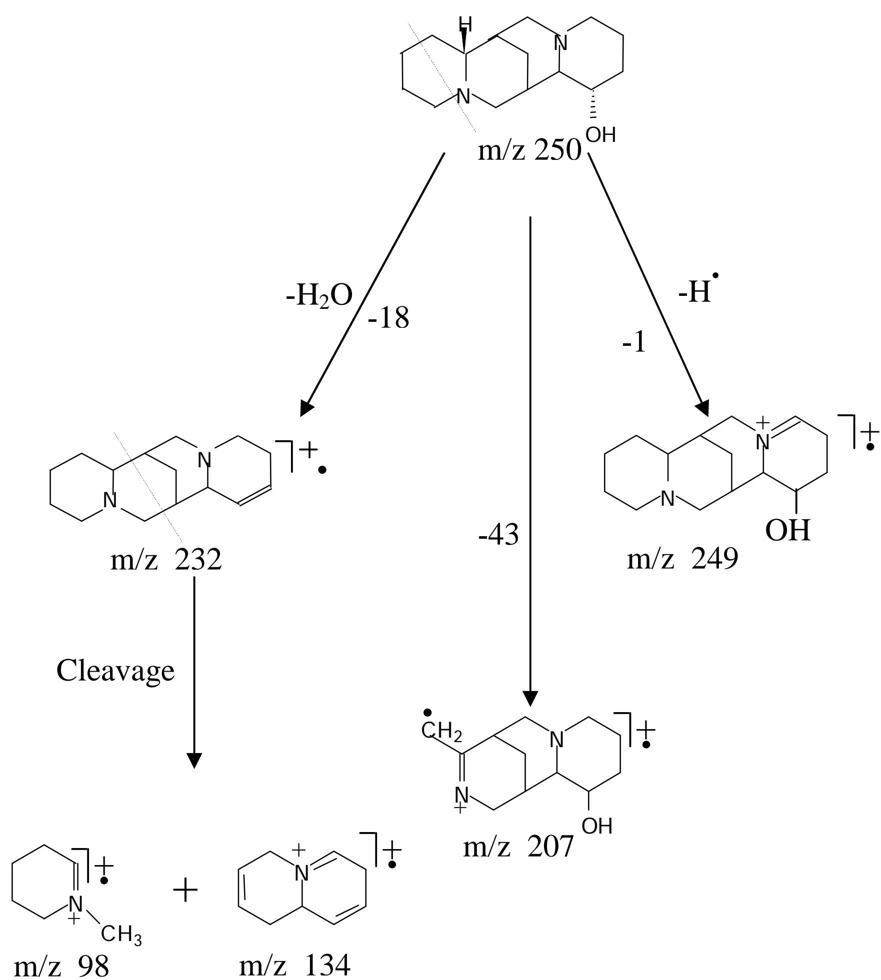


Figure II-5-Mass spectrum of Retamine.



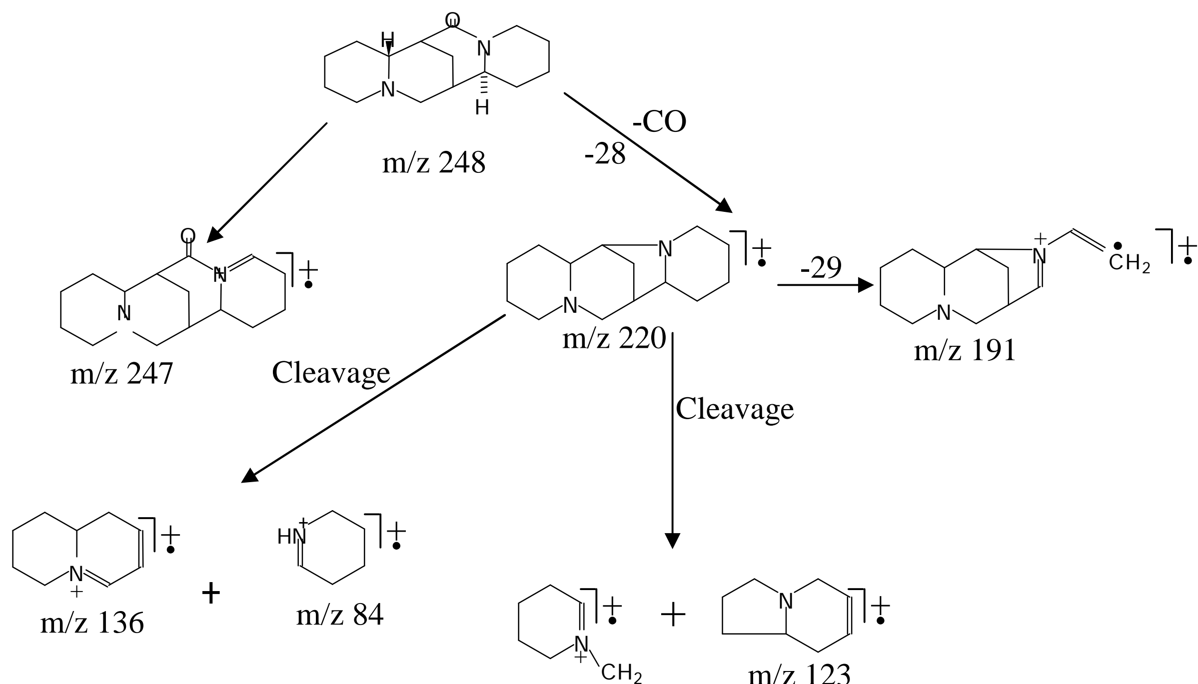
Scheme II-4-Possible pathway of fragmentation of Retamine [94].

II-1-5-The 17-Oxosparteine:

The compound **5** (**R_t = 22.96min, M_w = 248g/mol**) (the mass spectrum was shown in figure II-6) presents a molecular ion $[M]^+$ at m/z 248 (95.90 %), an other base peak at m/z 191 (25.40 %) corresponding to $[M - CH_3\text{-}CH_2]^+$. The other fragment ions were at m/z 136 (61.47 %), 110 (88.52 %), 98 (91 %) and m/z 97 (100 %).

Consulting our reference data [27, 89, and 91], compound **5** might be 17-Oxosparteine.

The scheme II-5 presented the possible pathway of fragmentation of this compound.



Scheme II-5-Possible pathway of fragmentation of 17-Oxosparteine [94].

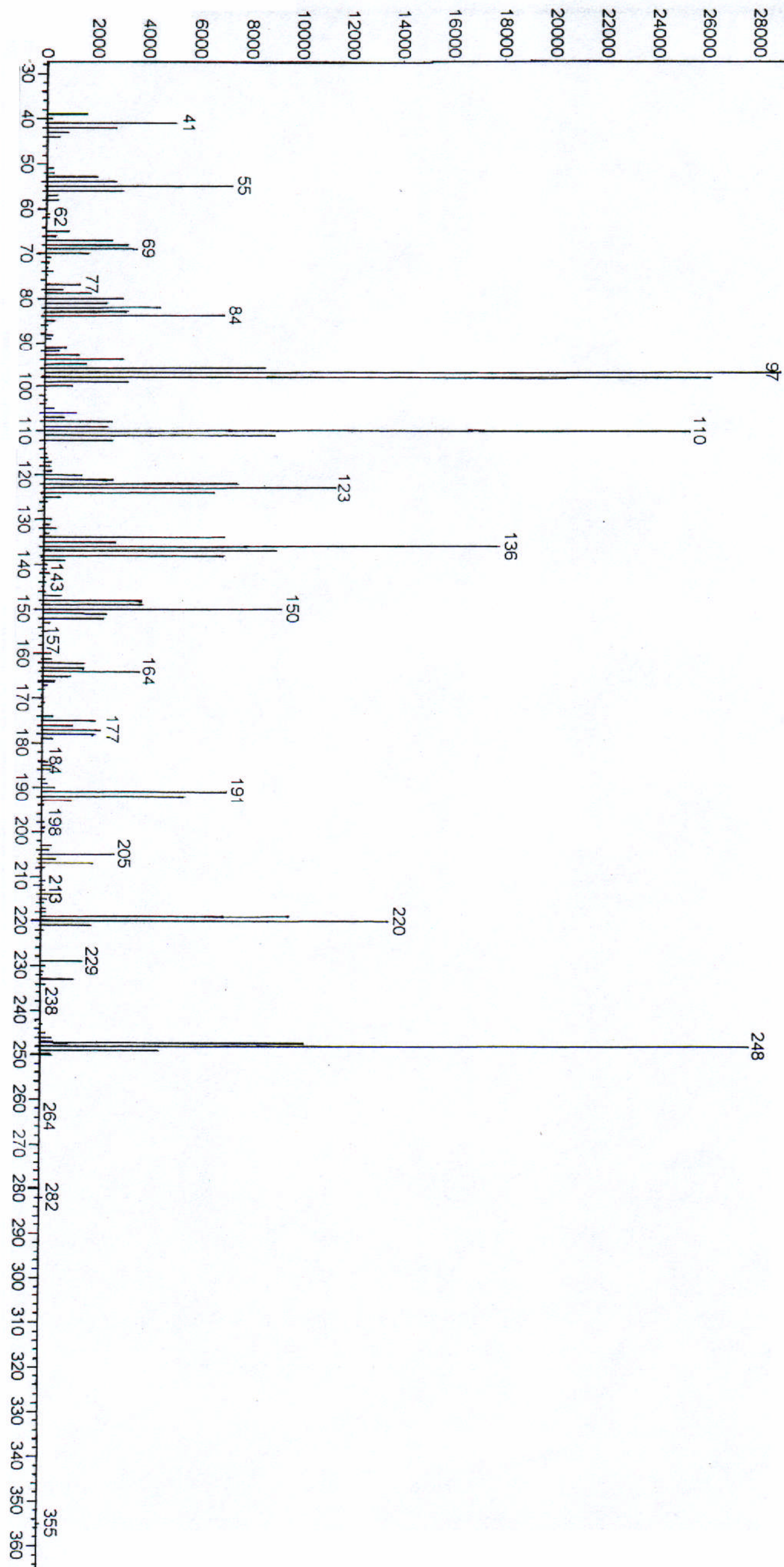


Figure II-6-Mass spectrum of 17-Oxosparteine.

II-1-6-The α-Isolupanine and Lupanine:

The mass spectrum of compound **7** (**R_t = 24.72min, M_w = 248g/mol**) (figure II-8) showed a molecular ion $[M]^+$ at m/z 248 (69.67 %). A base at m/z 247 (50.81 %) corresponding of $[M - H]^+$, an other peak at m/z 219 (11.47 %) indicating the loss of 28 units of mass $[M - CO]^+$. The predominant ions at m/z 149 (57.37 %), 136 (100 %), 110 (19.67 %), 98 (20.49 %), 84 (10.65 %), 55 (16.39 %) and m/z 41 (9.83 %) are characteristic of lupanine [27, 89, 91].

The fragment pattern of compound **6** (**R_t = 23.57min, M_w = 248g/mol**) (figure II-7) is characterised by a molecular ion $[M]^+$ at m/z 248 (70.49 %). The mass spectrum of this compound presents peaks at: m/z 219 (10.65 %), 149 (54.91 %) 136 (100 %), 110 (18.03 %), 98 (27.86 %), 84 (13.11 %), 55 (19.67 %) and m/z (13.11 %).

The pattern of fragmentation of compound **6** was very similar to that of compound **7**, but the two differed in their retention times (R_t) values, this indicate that compound **6** is an isomer of compound **7**. These were identified by means of their mass spectral fragmentation data which clearly distinguish different stereoisomers [91].

As results, compound **6** and compound **7** were the α-isolupanine and lupanine, respectively.

The scheme II-6 elucidated a possible pathway of fragmentation of lupanine.

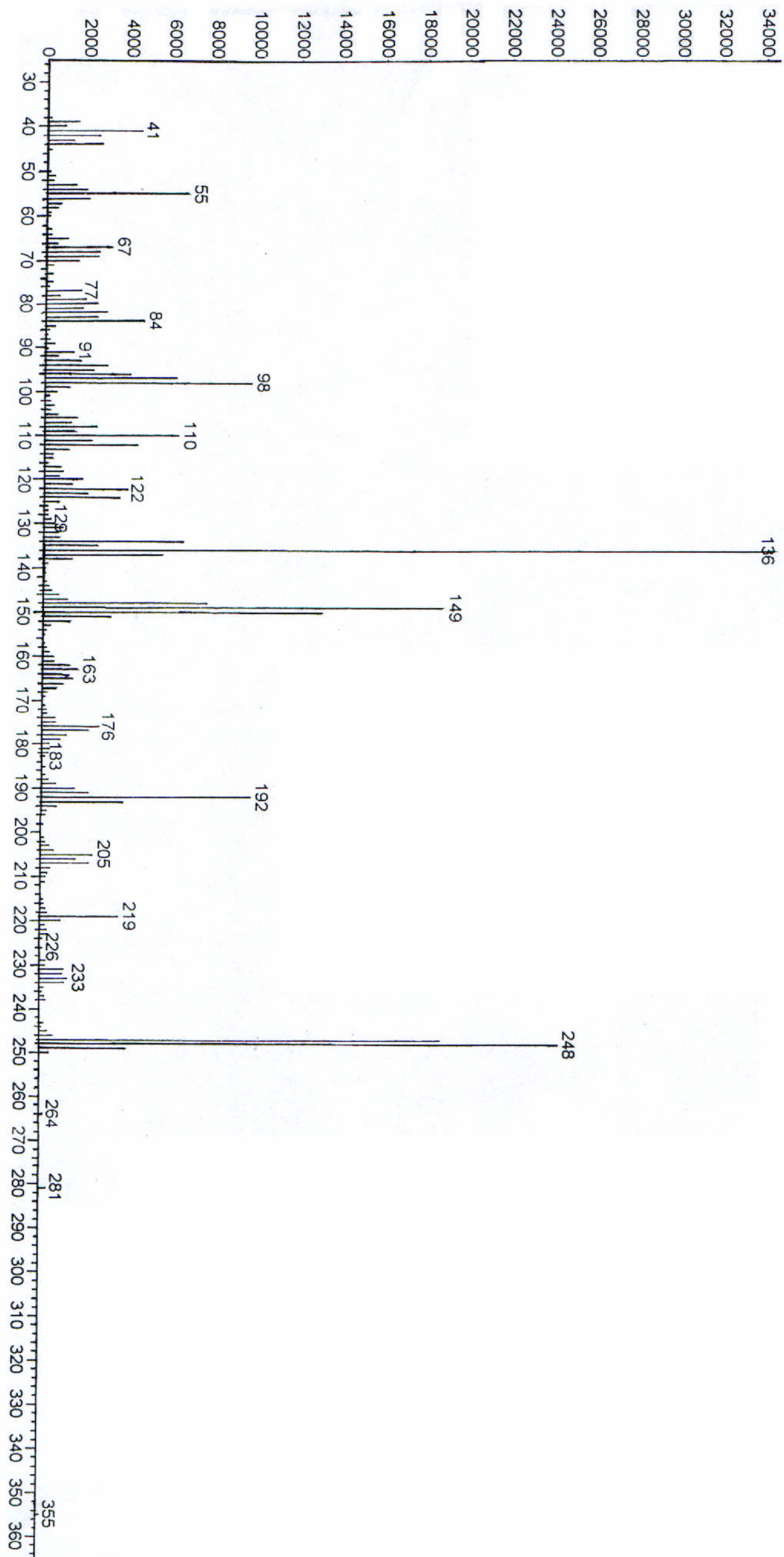


Figure II-7-Mass spectrum of α-Isolupanine.

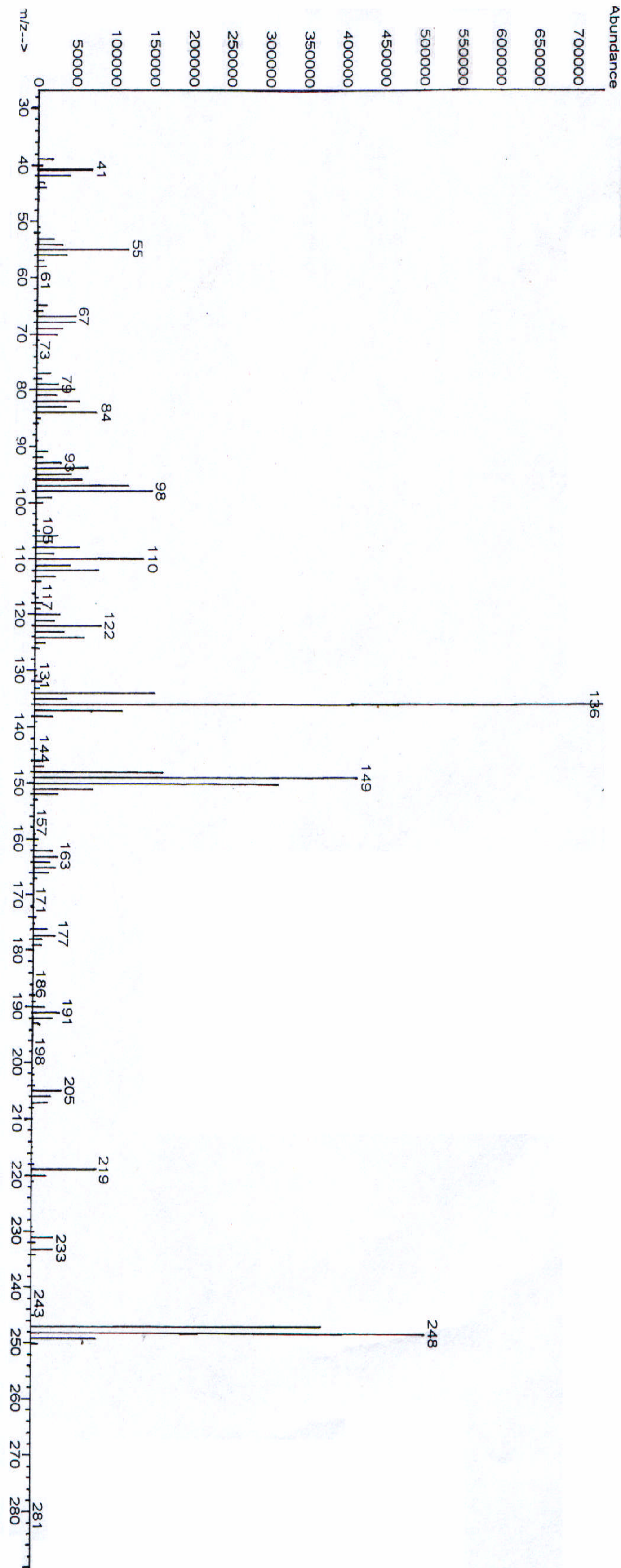
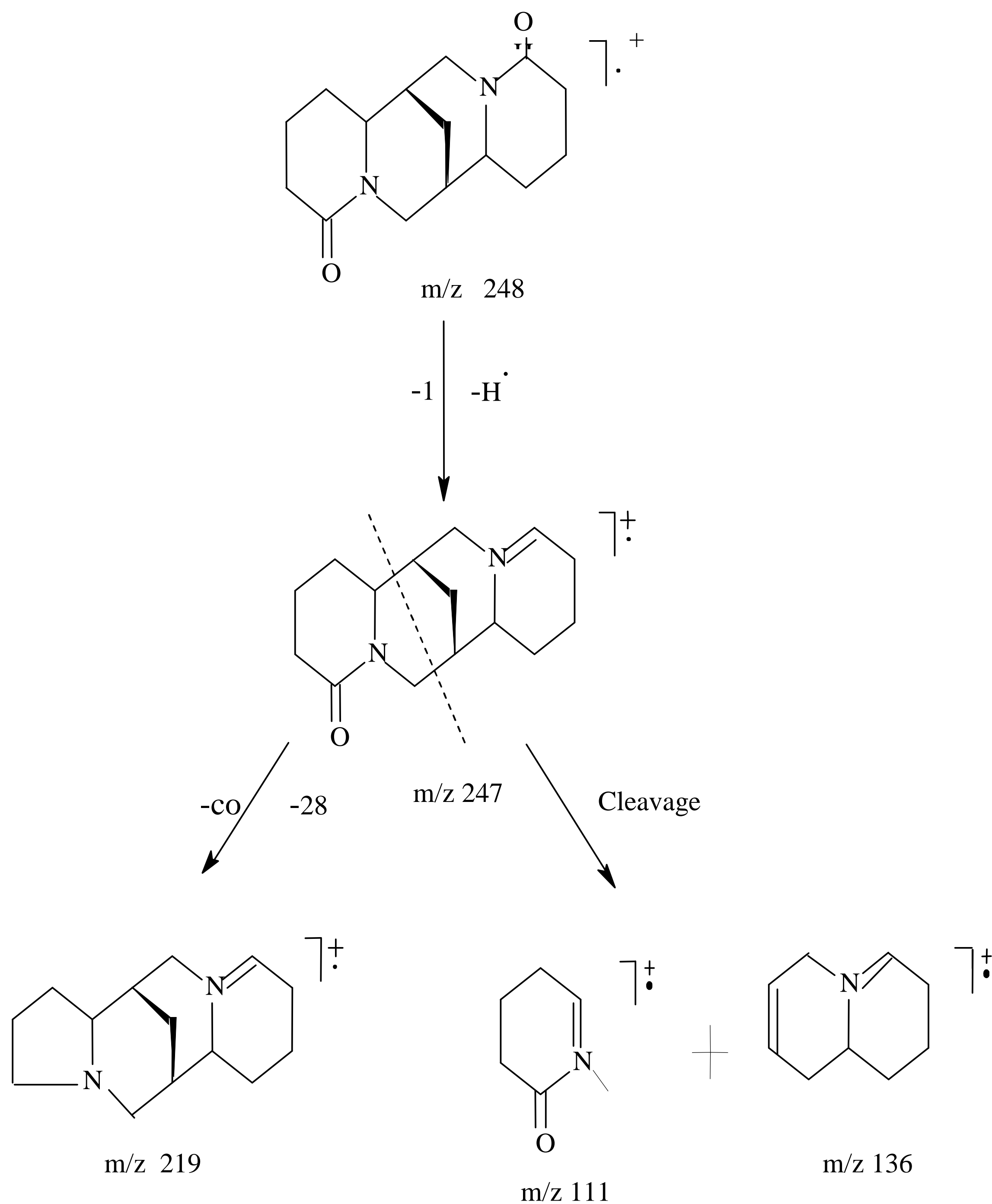
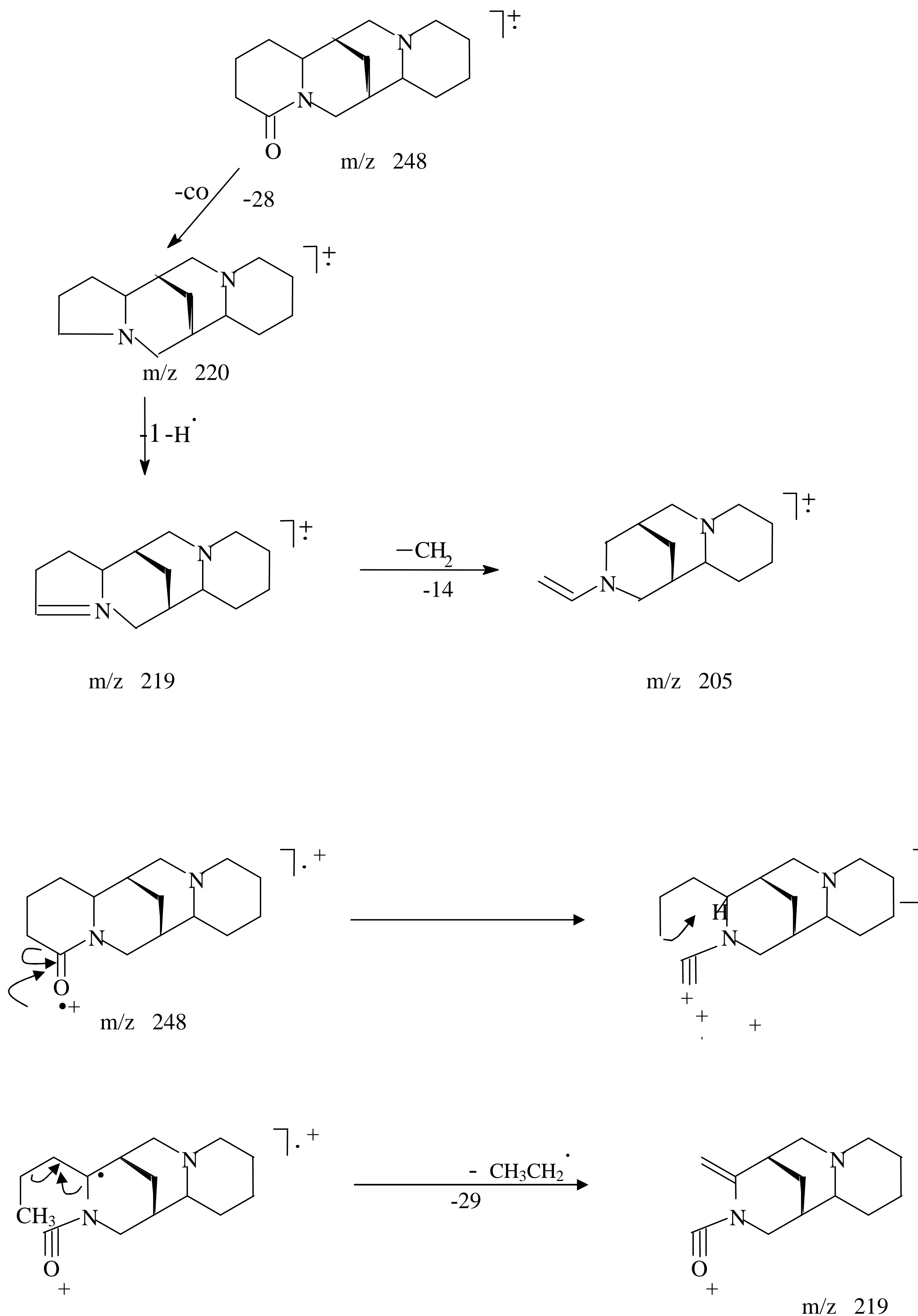


Figure II-8-Mass spectrum of Lupanine.



Scheme II-6-Possible pathway of fragmentation of Lupanine [94].

Scheme II-6-*Continued*.



II-1-7-The 13α-Hydroxylupanine:

The compound **8** (**R_t = 29.17min, M_w = 264g/mol**). The fragmentation pattern in the EI-mass spectrum (Figure II-9) indicated a possible lupanine skeleton.

The MS showed a molecular ion peak at m/z 264 (46.72 %) and fragment peaks at m/z 248 (5.73 %), 247 (37.70 %) and 246 (67.21 %) corresponding to $[M - O]^+$, $[M - OH]^+$ and $[M - H_2O]^+$, respectively , suggesting the presence of a hydroxyl group in the molecule .

The other main fragment ions at m/z 165 (43.44 %), m/z 152 (100 %) and m/z 134 (32.78 %) were characteristic of 13α-hydroxylupanine [66].

The scheme II-7 showed the possible pattern of fragmentation of this compound.

II-1-8-The 13α-Acetyloxylupanine:

The compound **9** (**R_t = 29.59min, M_w = 306g/mol**) presents a molecular ion peak at m/z 306 (6.55 %). The EI-MS (Figure II-10) showed the same fragment ion peaks with almost the same intensities as those of lupanine.

A base peak at m/z 246 (100 %) was indicative of the presence of an acetyl $[M - COOH]^+$ in the molecule.

The results suggesting that this compound is the 13α-Acetyloxylupanine.

The scheme II-8 showed the possible pathway of fragmentation of the compound.

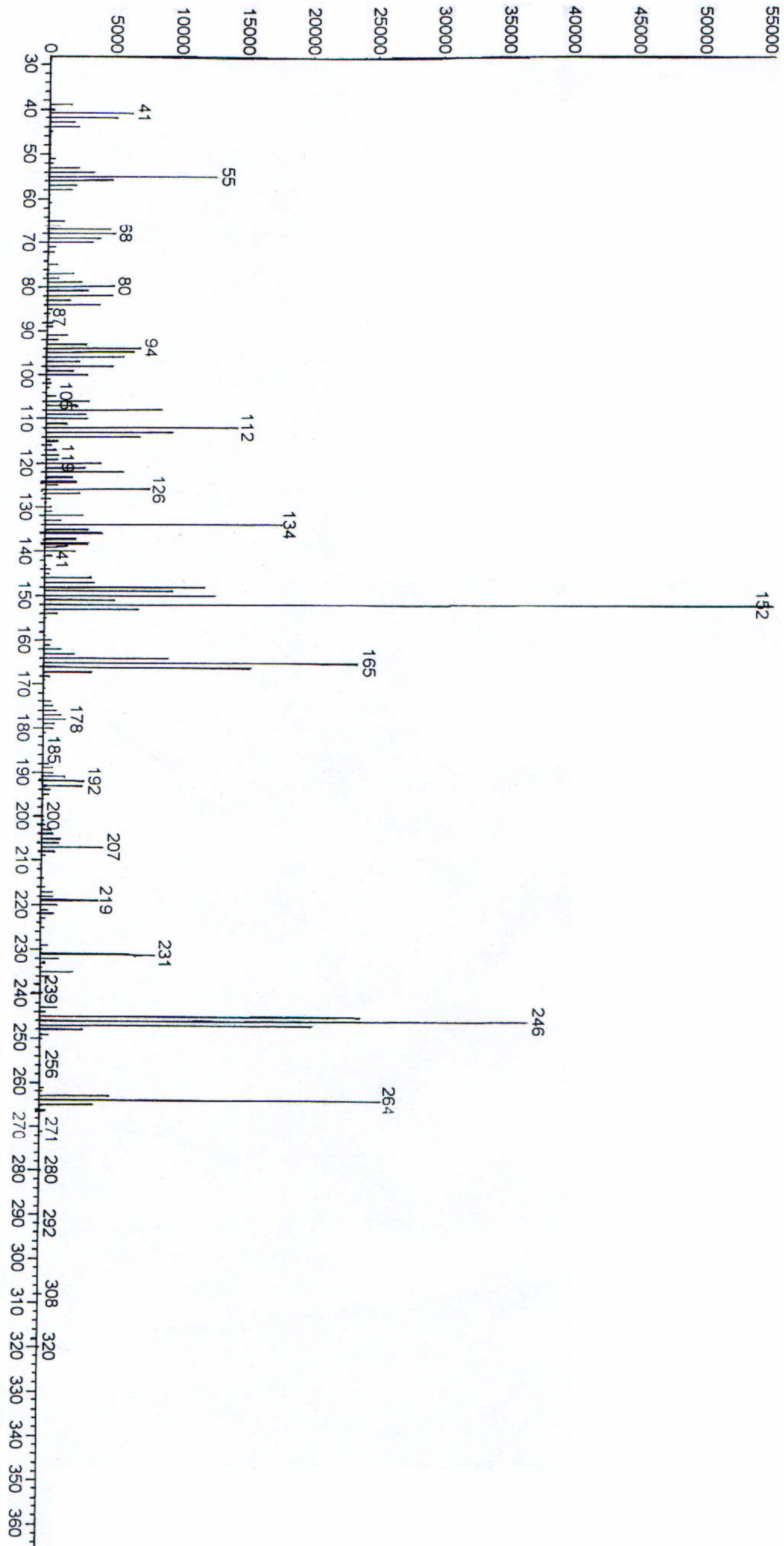
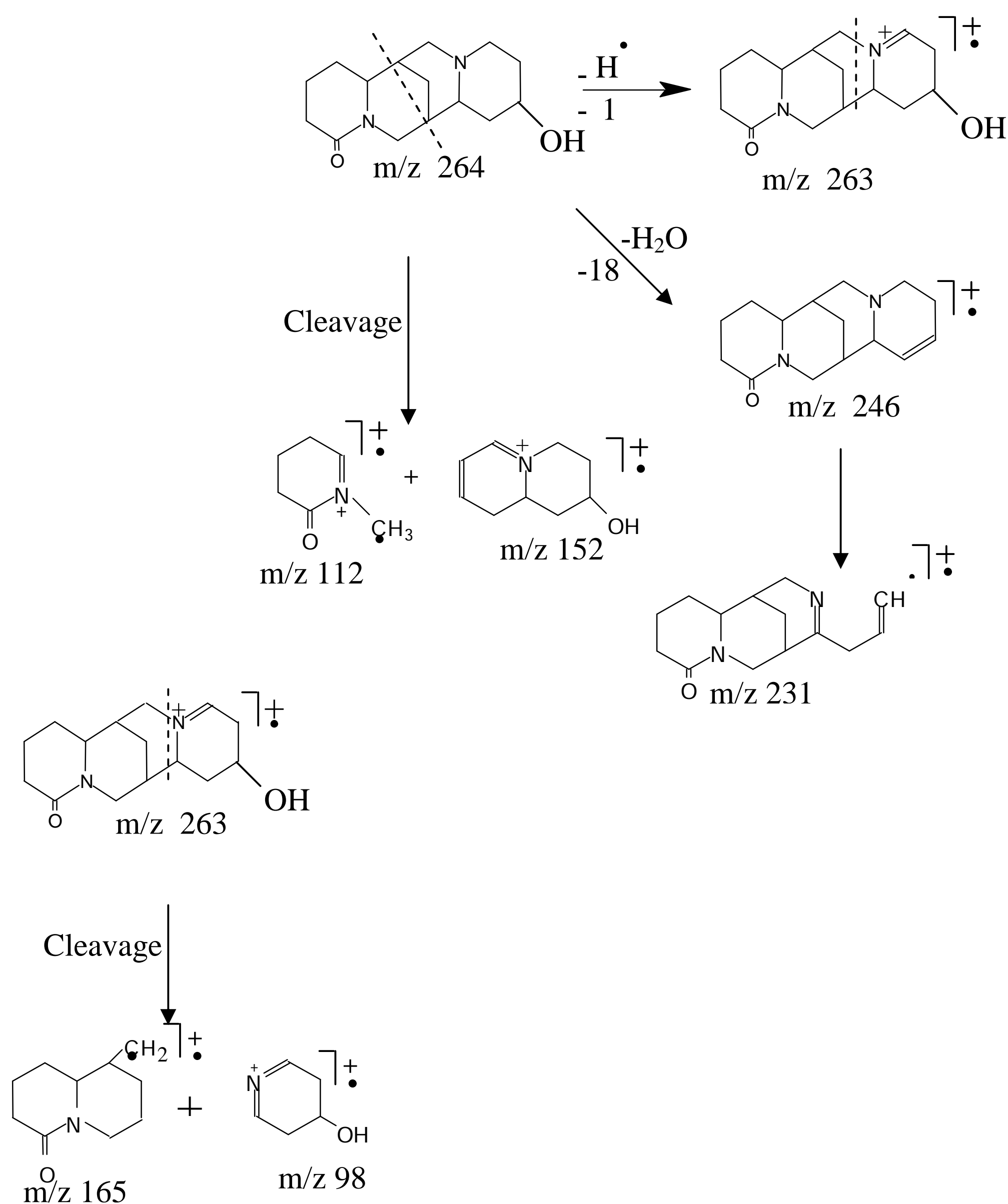


Figure II-9-Mass spectrum of 13α-Hydroxylupanine.



Scheme II-7-Possible pathway of fragmentation of 13α-Hydroxylupanine [94].

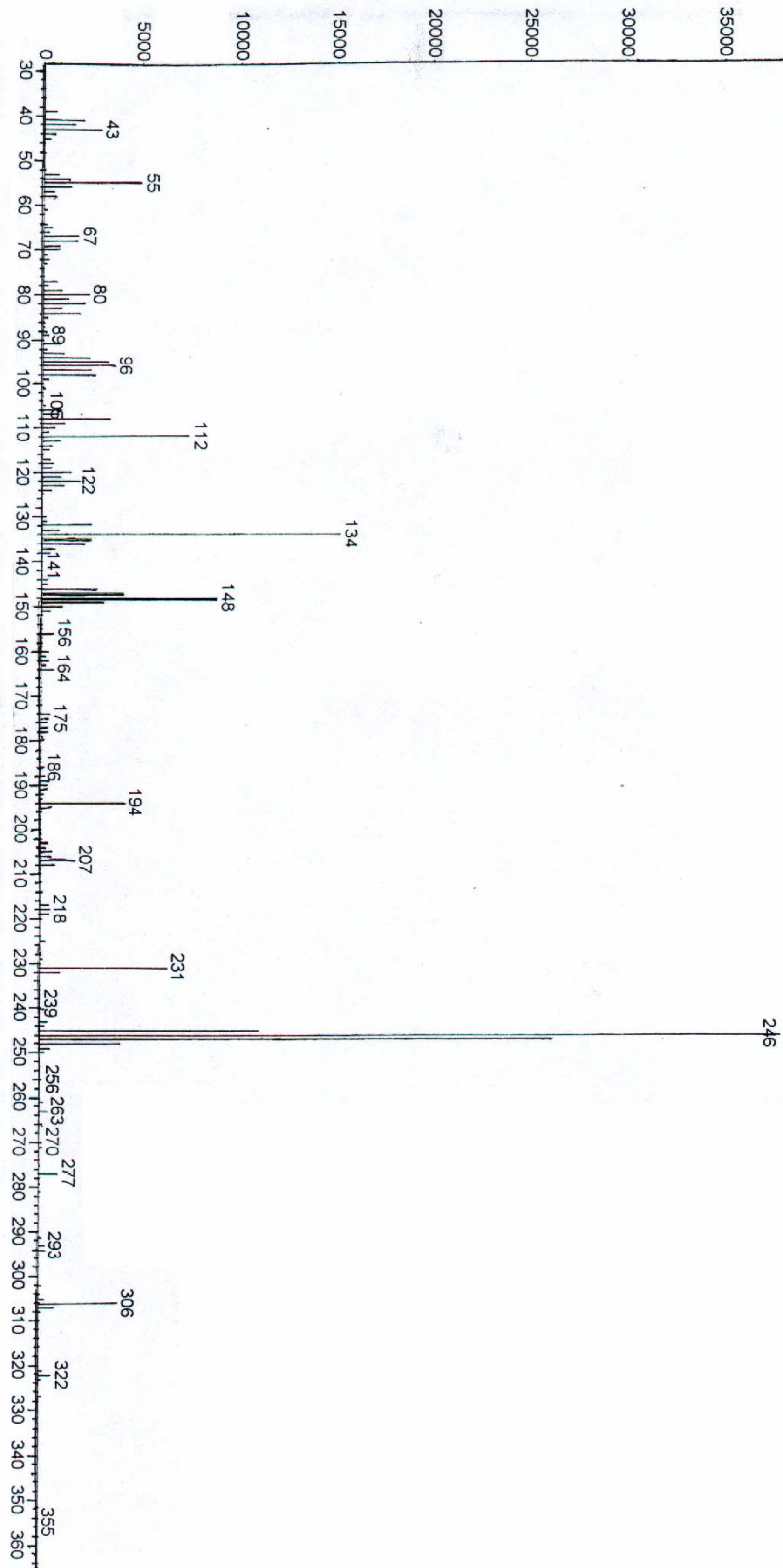
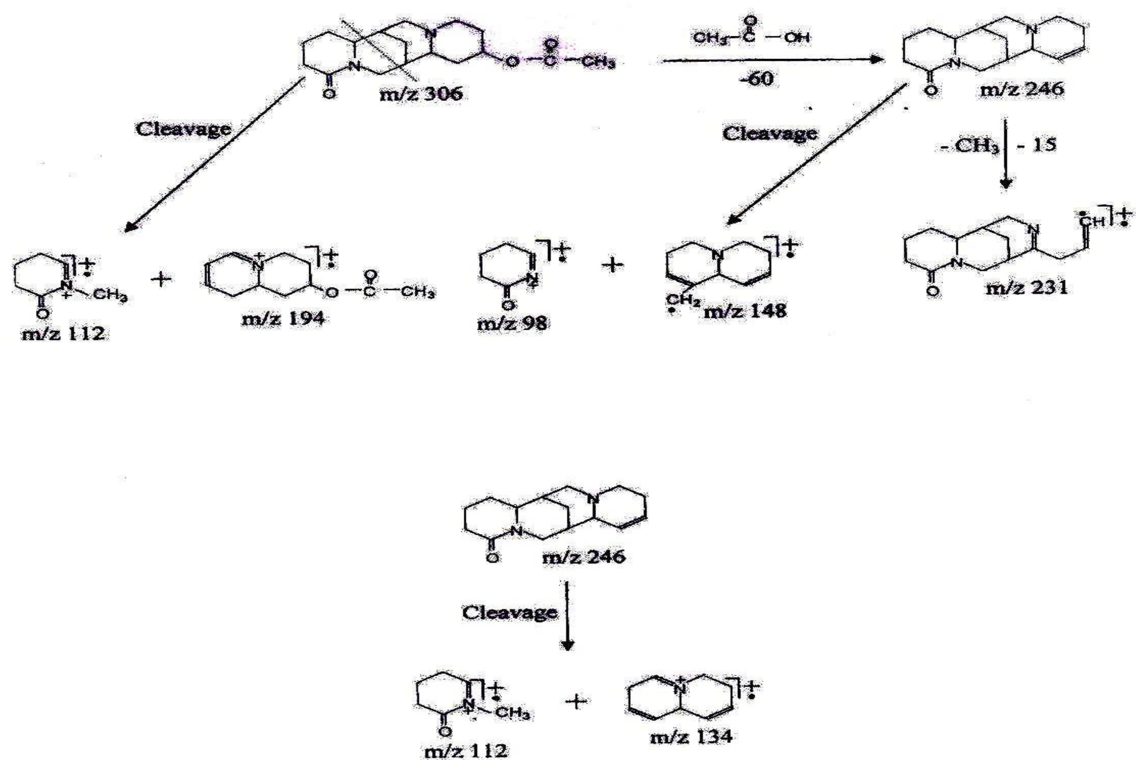


Figure II-10-Mass spectrum of 13α-Acetoxylupanine.



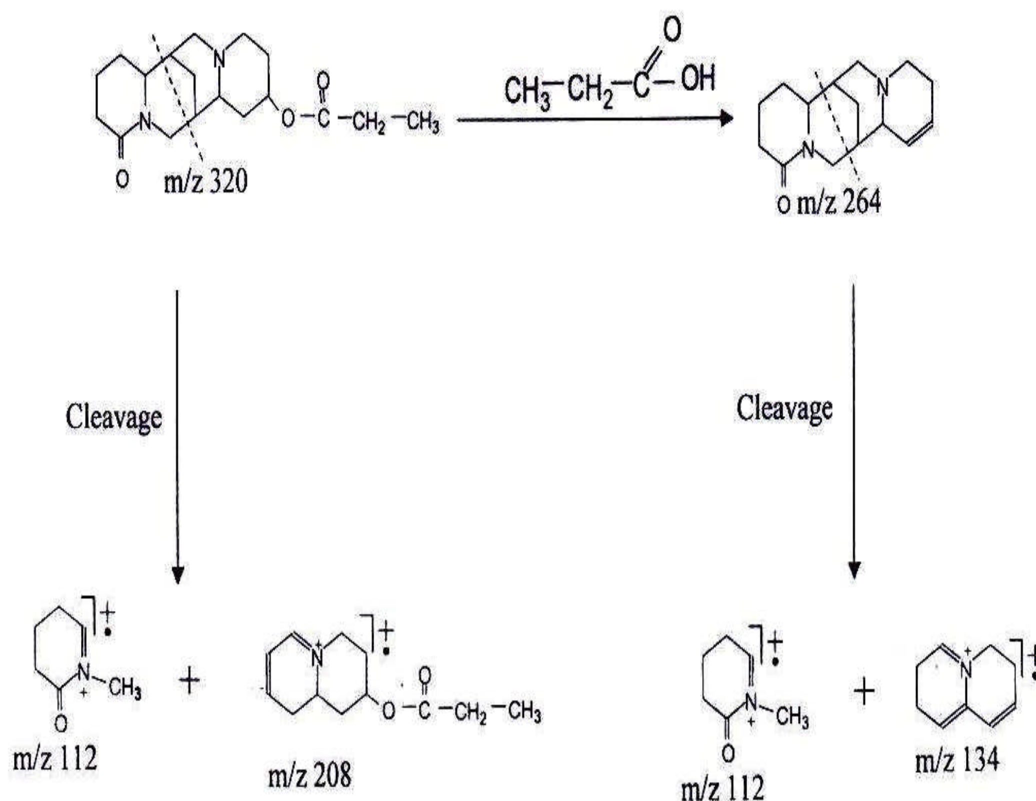
Scheme II-8-Possible pathway of fragmentation of 13α-Acetyloxylupanine [94].

II-1-10-The13α-Propyloxylupanine:

The mass spectrum of compound **10** (**R_t = 29.94min, M_w = 320g/mol**) (Figure II-11) showed a molecular ion peak $[M]^+$ at m/z 320 (5.73 %) and a base peak at m/z 246 (100 %) indicating the loss of 74 units of mass suggests the presence of a propyloxy group $[M - CH_3CH_2COOH]^+$ in the molecule. The other fragment peaks were similar to those of lupanine.

These results suggesting that compound **10** was 13α-Propyloxylupanine.

The scheme II-9 showed the possible pathway of fragmentation of 13α-Propyloxylupanine.



Scheme II-9-Possible pathway of fragmentation of 13α-Propyloxylupanine [94].

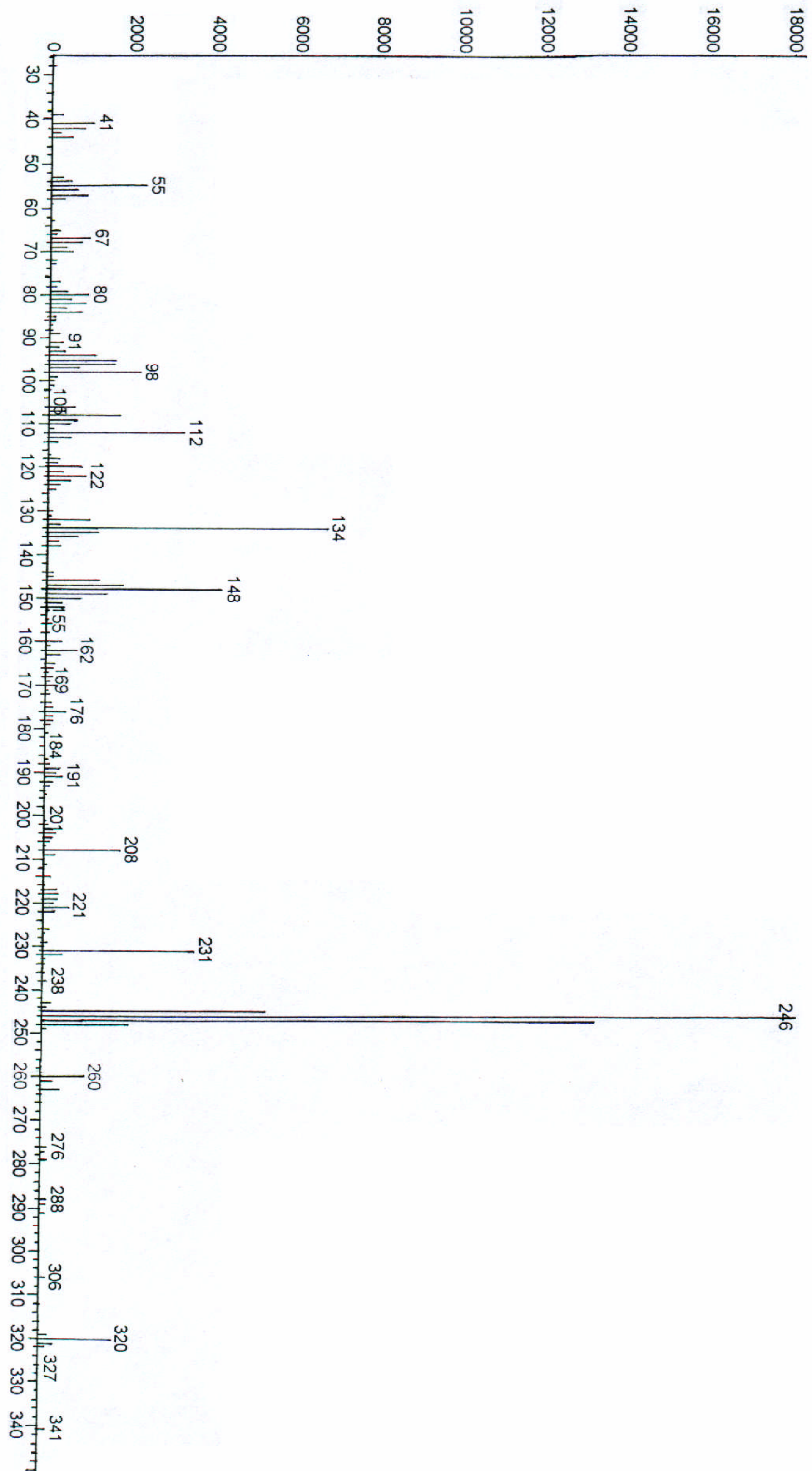


Figure II-11-Mass spectrum of 13α-Prpyloxylupanine.

II-1-11-The 13α-Angeloyloxylupanine and 13α-Tigloyloxylupanine:

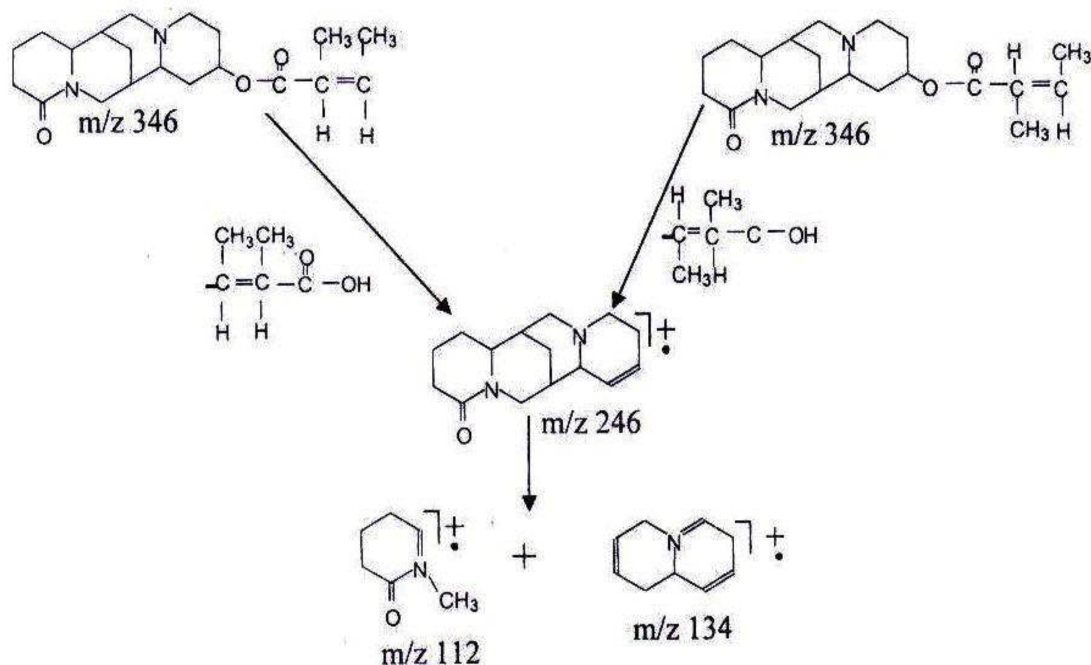
Concerning compounds **11** and **12**. The EI-mass spectrum of compound **11** (**R_t = 33.97min, M_w = 346g/mol**) (Figure II-12) presents a molecular ion $[M]^+$ at m/z 346 (0.9 %). The compound **12** (**R_t = 54.72min, M_w = 346g/mol**) (Figure II-13) showed a molecular $[M]^+$ at m/z 346 (0.05%).

Both compound **11** and **12** have the same pattern of fragmentation which was similar , also , of those of lupanine , but with a base peak at m/z 246 (100 %) indicating the loss of 100 units of mass $[M - C_5H_8O_2]^+$.

These results indicating that **11** and **12** are two compounds which have the same molecular weight but a different configuration which interpreted by the retention times different .

Consulting the literature data [27, 89, 91], **11** and **12** were 13α-Angeloyloxylupanine and 13α-Tigloyloxylupanine, respectively.

The scheme II-10 showed the possible pathway of fragmentation of these two compounds.



Scheme II-10-Possible pathway of fragmentation of 13α-Angeloyloxylupanine a 13α-Tigloyloxylupanine [94].

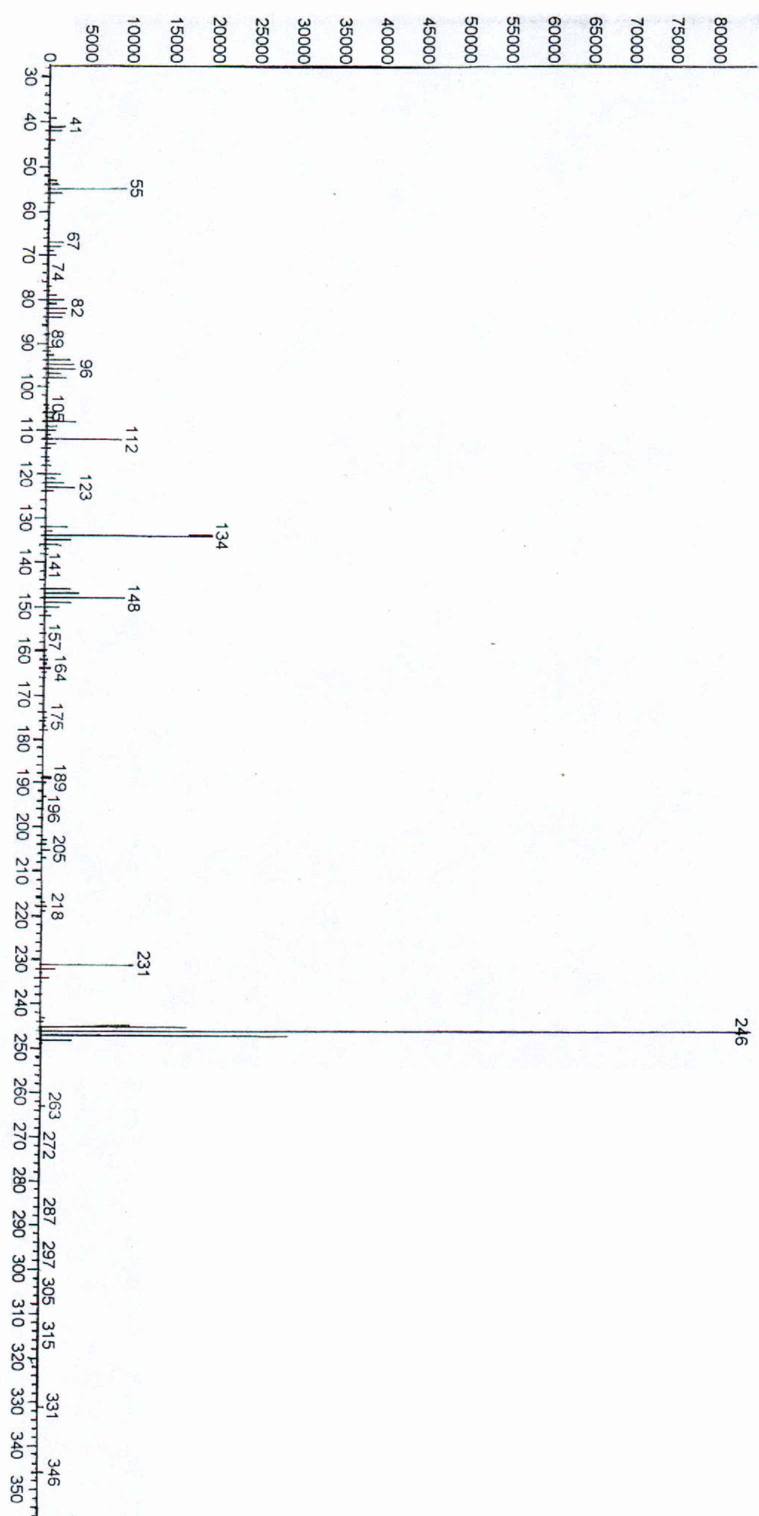


Figure II-12-Mass spectrum of 13α-Angeloyloxylupanine.

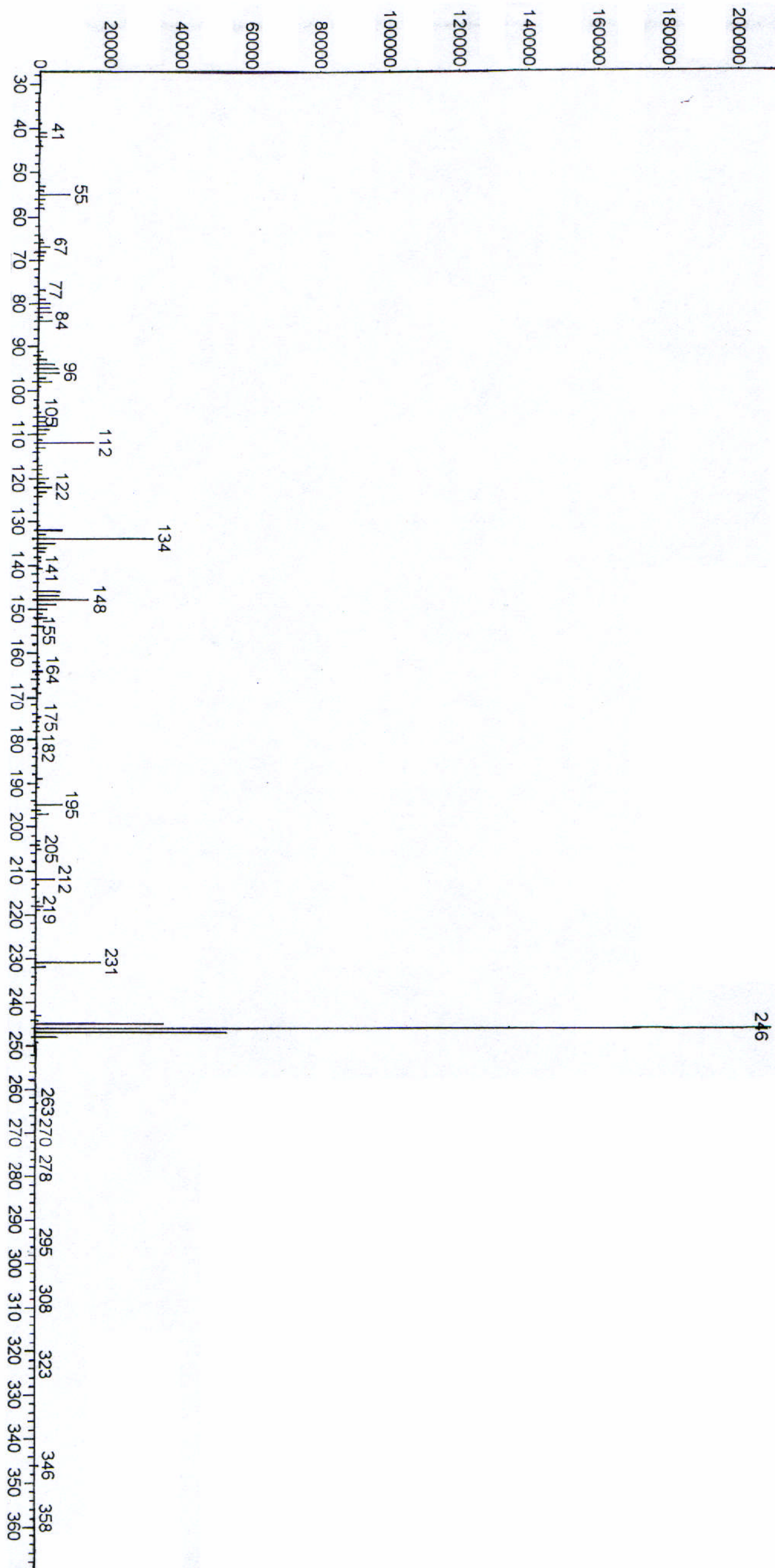


Figure II-13-Mass spectrum of 13α-Tigloyloxylupanine.

II-2-General Observations:

Cytisus purgans accumulates sparteine-type QA as the basic constituents together with a piperidine alkaloid the ammodendrine, this phenomenon is interesting from the viewpoints of chemotaxonomy of leguminous plants and biosynthesis of QA [89].

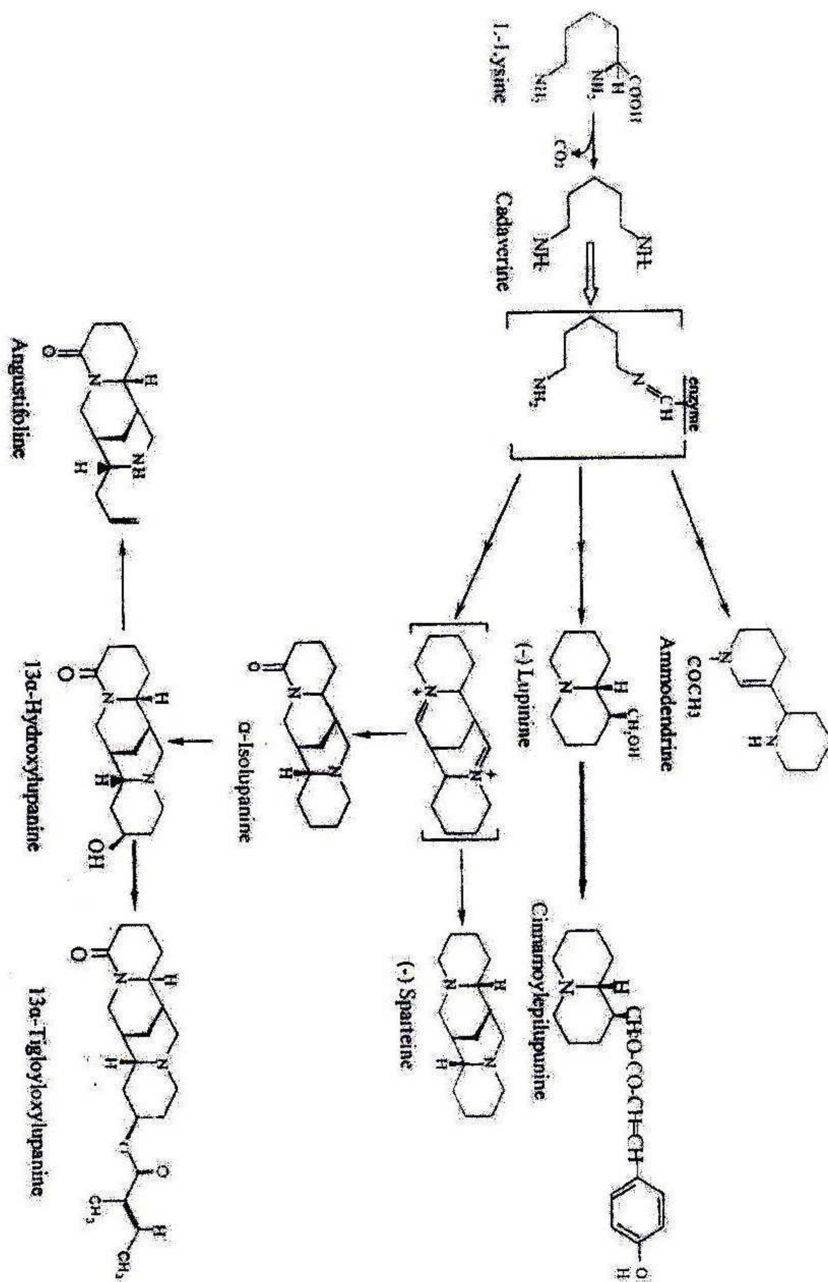
The concentrations of individual major alkaloids in the plant, which were shown in the chromatogram (Fig. II-1), indicate that the sparteine was the first major alkaloid (59.134 % of total alkaloids), the second major alkaloid was the lupanine (16.303 %).

The sparteine has been used as an oxytocic drug and is of interest because of its antiarrhythmic effect and inhibitory effect of natural killer cell growth [97]. Species which contain lupanine must considered potentially toxic, and unsuitable for forage [90].

Several QA in Fabaceae (Leguminosae) are present esterified with short chain carboxylic acids, e. g. as acetyl, tigloyl and angeloyl esters. Although the functions of these ester alkaloids in plant cells are still unclear, these ester alkaloids are assumed to be end products of alkaloid biosynthesis and storage forms of alkaloids. In particular, ***Lupinus*** species accumulate a variety of ester QA. Some of these ester alkaloids are specifically produced during seedling growth in some ***Lupinus*** plants. Subsequently the activities for 13α-Tigloyloxylupanine have been shown in cell-free extracts of ***Lupinus albus*** [98].

Cytisus scoparius accumulated only tigloyl esters; no angeloyl esters were detected in this plant [97]. On the other hand, ***Cytisus purgans*** contained both tigloyl and angeloyl esters.

The alkaloid profile of ***Cytisus purgans*** supported the supposition of K. Saito and coll. which interested of this subject and posed a possible pathway of biosynthesis of these alkaloids. This supposition indicates that the quinolizidine alkaloids of lupin are formed from L-lysine (**1**) via cadaverine (**2**) as the first detectable intermediaire. The crucial steps of ring cyclization of cadaverine units are believed to occur as enzyme bound intermediates and subsequently to yield the initial bicyclic alkaloids e.g. (-) –lupinine (**4**) or the tetracyclic alkaloids such as (+) –lupanine (**7**, scheme II-1). These cyclic alkaloids are subsequently transformed by cellularenzymes through dehydrogenation, oxygenation or esterification. However, almost nothing is known on the regulatory mechanism biosynthesis. The scheme (II-10) elucidated the possible pathway of QA biosynthesis of lupin [98].



Scheme II-11-Possible biosynthetic pathway of quinolizidine alkaloids in bitter and sweet *Lupinus* plants [97].

II-3-Isolation of 13α-Hydroxylupanine:

13α-Hydroxylupanine (**M_w=264g/mol**) was isolated from the MeOH extract of aerial parts of ***Cytisus purgans*** as a colorless oil.

The compound give R_f values at 0.24 and 0.27 in the solvent systems: $CHCl_3$/MeOH/28% NH_4OH (85:15:1) and MeOH/NH_4OH (65:1), respectively.

The IR spectrum of compound **8** (Fig.II-14) showed a broad intense band at ~3320 cm^{-1} (O-H stretching, intramolecular, hydrogen bonding). Multiple bands at ~2960cm^{-1} and at ~ 2845cm^{-1} suggested quaternary nitrogen and combination overtones, embodying the C-H stretching the Bohlmann's bands characteristic for quinolizidine-type alkaloids [99].

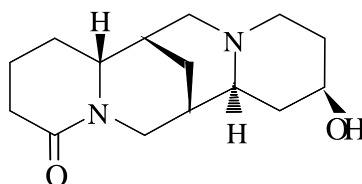
The peaks at ~1650 cm^{-1} and at ~1400 cm^{-1} indicating the presence of a conjugated carbonyl group and C-N stretching band, respectively [95].

The IR spectrum revealed, also, the presence of an intense peak at 1022 cm^{-1} due to the C-O stretching band.

The UV spectrum of compound **8** showed a pick at λ_{max}^{MeOH} (210 nm) concerning the transition of lone pairs of nitrogen atom and a transition at (323 nm) concerning the unsaturated system of the carbonyl group [99].

Thus **8** was presumed to be a sparteine –type quinolizidine alkaloids containing a hydroxyl and a carbonyl group in the molecule.

From the above data and consulting the reference [27], it was concluded that compound **8** may be the 13α-Hydroxylupanine.



13α-Hydroxylupanine

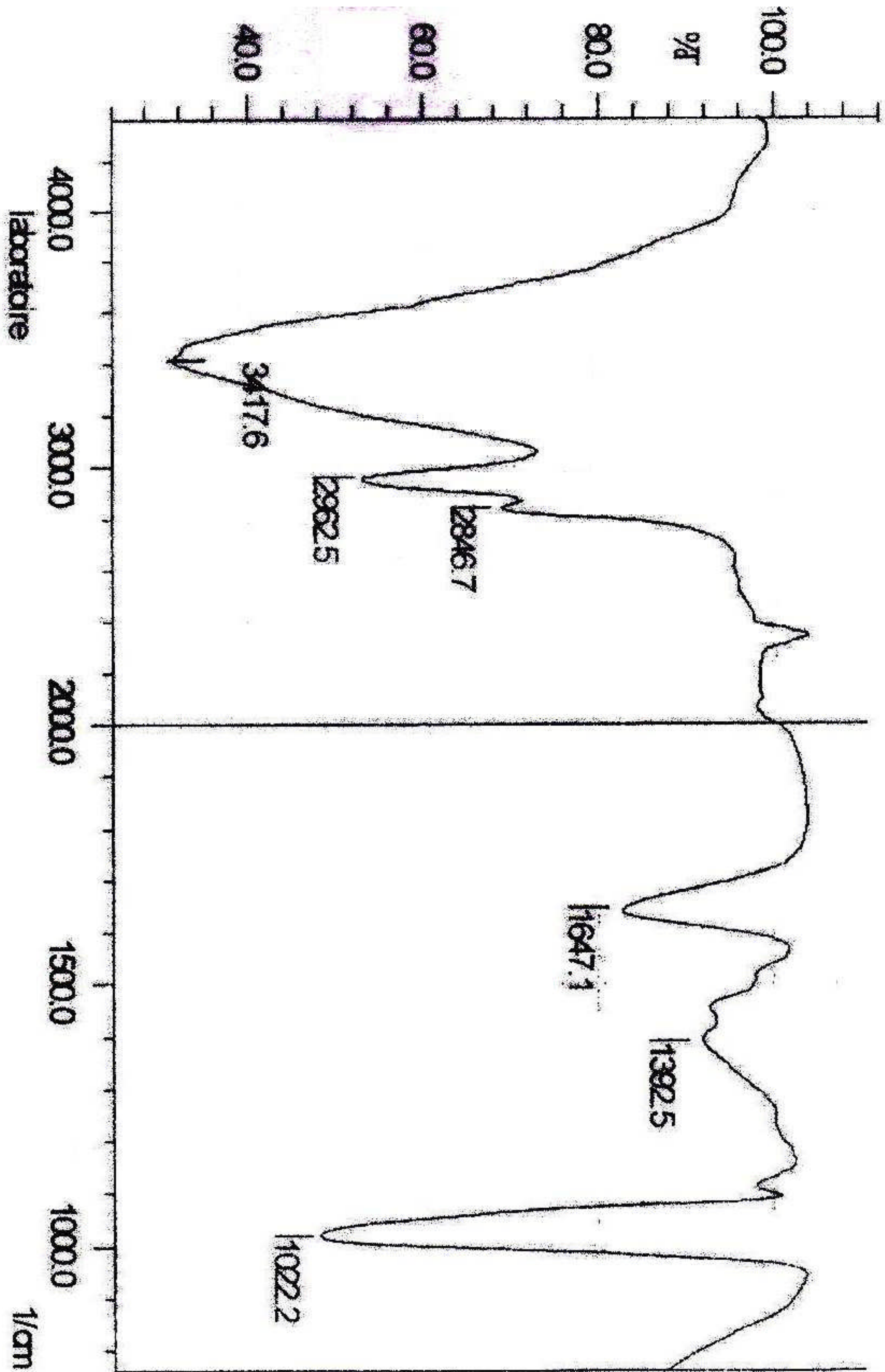


Figure II-14-The IR spectrum of 13α-Hydroxylupanine.

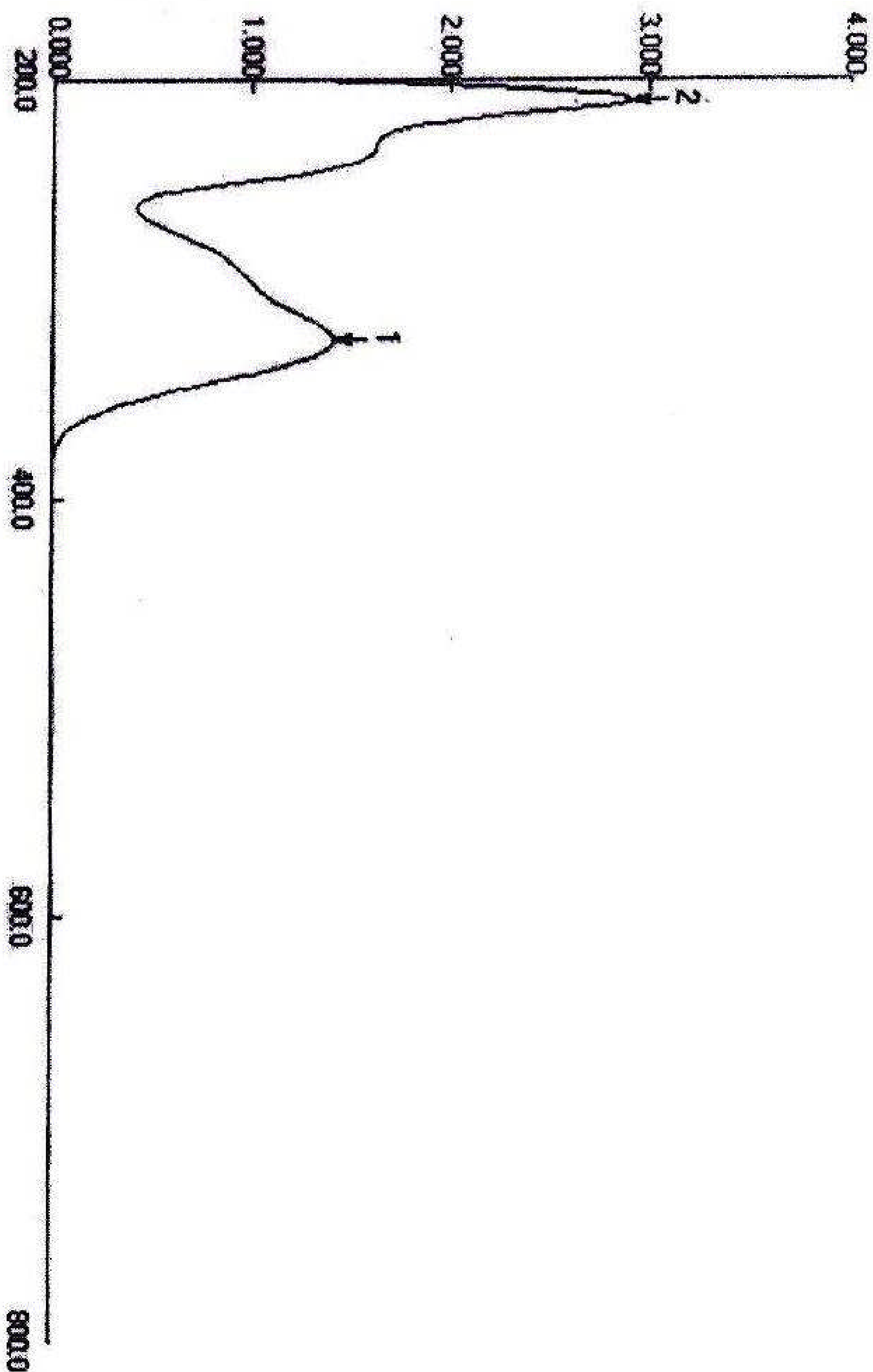


Figure II-15-The UV spectrum of 13α-Hydroxylupanine.

It is to be noted that this compound was also isolated from the aerial parts of ***Cytisus scoparious*** by Murakoshi and *coll*. [66], this is interesting for the relationship within ***Cytisus*** genus.

II-4-*In vitro* Antimicrobial Activity:

The antibacterial activity of the MeOH extract of ***Cytisus purgans*** was assayed in vitro by agar disc method against three bacterial species. The data in Table II-1 showed that ***Cytisus purgans*** exhibited antibacterial activity towards all bacteria strains. The maximum antibacterial activity was shown by ***Staphylococcus aureus*** (inhibition zone of 32 mm of diameter) followed by ***Pseudomonas aeruginosa*** and ***Escherichia coli*****,** respectively.
Similar results were also reported by Zellagui and *coll*. [100] which studied the antimicrobial properties of ***Genista microcephala***), according to the data, they obtained that this plant which contains also quinolizidine alkaloids showed a worth mentioning inhibition towards the same bacteria strains.

The antifungal activity of the MeOH extract of ***Cytisus purgans*** was also assayed in vitro by agar disc method against two fungal species: ***Aspergillus niger*** and ***Aspergillus flavus***. The results showed that the plant did not show any antifungal activity against these species, this is an indication that the organism has the potential of developing antibiotic resistance [101].

Table II-1- Inhibition zone of antibacterial activity of MeOH extract.

	Escherichia coli	**Pseudomonas aeruginosa**	**Staphylococcus aureus**
C_1	12	17	20
C_2	15	23	28
C_3	20	28	32
C	8	7	7.2

C_1: 40 mg/ml, **C_2**:60 mg/ml, **C_3**:80 mg/ml, **C**: Control.

However the MeOH extract showed negative results against fungi but showed high antibacterial activity, especially against ***Staphylococcus aureus*** which a pathogen is known to cause infectious disorders of the skin [101].

As a consequence of this study, we will try to isolate compounds responsible for the antimicrobial activity in the crude fraction.

The demonstration of antibacterial activity against both gram positive and gram negative bacteria may be indicative of the presence of broad spectrum antibiotic compounds. This will be of immense advantage in fighting the menace of antibiotic refractive pathogens that are so prevalent in recent times.

The high antibacterial activity exhibited by ***Cytisus purgans*** may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease chemotherapy and control. This investigation has opened up to drug development for human consumption possibly for the treatment of gastrointestinal, urinary tract and wound infections and typhoid fever. The effect of this plant on more pathogenic organisms and toxicological investigations and further purification however, needs to be carried out.

Our results could be evaluated as the first report on antibacterial features of ***Cytisus purgans*** growing in ALGERIA. Further research is necessary to determine the identity of the antibacterial compounds from within this plant and also to determine their full spectrum of efficacy. However, the present study of in vitro antimicrobial evaluation forms a platform for further phytochemical and pharmalogical studies.

Chapter Three

Experimental Procedures

III-1-Plant material:

Cytisus purgans was collected in June 2002 at AURES Mountains (2328m of altitude) at KHENCHELLA province in the north east of ALGERIA, and identified by Professor KAABECH Mohamed, Laboratory of biodiversity and phytogenetic resources, university of Ferhat ABBAS, Sétif, ALGERIA. A voucher specimen is deposited at the herbarium of laboratory of organic chemistry and phytochemistry of university Mohamed BOUDIAF of M'sila, ALGERIA.

III-2-Isolation of alkaloids:

The air-dried aerial parts of plant were cut into small pieces and were extracted with 98% MeOH three times at room temperature. The combined extracts were concentrated, acidified with 5% HCl and then, extracted with ET_2O three times. The aqueous layer was made alkaline with 25% NH_4OH to pH (9-10) and extracted with $CHCl_3$ six times. The $CHCl_3$ extracts were combined and dried over Na_2SO_4 and evaporated to dryness *in vacuo* [66].

III-3-GC/MS analysis:

The alkaloid extracts were separated and analysed by GC/MS with the equipment Hewlett-Packard 5890 gas chromatograph with a HP 5972 MSD detector. The column type used was DB1; 30m; 0.28mm i. d.; 0.25µm film thickness; split ratio 1:2; carrier gas: He; flow 1ml/min; injector temperature:250°c; over temperature program: 120°c; 3min isothermal ; auxiliary temperature was 280°c. The electron impact mass spectra were recorded at 70 eV ionisation energy, scan (50-550 VMA).

III-4-Isolation of compound 8:

Analytical TLC were done on silica gel 60 plates with fluorescent indicator UV_{254} (20x20,0.25mm, Merck) in following solvent systems: Toluene/Ethyl acetate/Triethylamine (70:20:10) and $CHCl_3$/MeOH/28%NH_4OH (85:15:1).

400 mg of total mixture alkaloids was subjected to preparative TLC in silica gel 60 plates (20x20,1mm, Merck) with the following solvent system: Toluene/Ethyl acetate/Triehtylamine (70:20:10) showed the presence of six alkaloidal constituents after monitoring under the UV (254 and 365nm). These constituents were further purified by preparative TLC on silica gel using the following solvent system: $CHCl_3$/MeOH/28%NH_4OH (85:15:1) to yield 23 mg of compound **8**.

III-5-Instrumentation:

The UV spectra were recorded in MeOH on a Shimadzu UV-240 spectrophotometer. The IR spectra were recorded in $CHCl_3$ on FT-IR 8300, Shimadzu Spectrophotometer.

III-6-Antimicrobial Assay:

III-6-1-Microorganisms:

The tested organisms used were bacteria and fungi. The bacteria used were two Gram-negative bacteria: ***Escherichia coli*** ATCC 25922 and **Pseudomonas aeruginosa** ATCC 27859 and one Gram-positive bacteria: ***Staphylococcus aureus*** ATCC 25923. These bacteria samples were provided by the Sétif’s hospital. Fungi included ***Aspergillus niger*** and **Aspergillus** ***flavus***. These were provided by the department of Microbiology, Faulty of Science, Sétif University. All the microorganisms were maintained at 4° c on nutrient agar slants [102].

III-6-2-Media:

The media were the Muller Hinton agar (MHA) (Aldrich) for bacteria, and Sabouraud Dextrose agar (SDA) (Aldrich) for fungi [102, 103 and 104].

III-6-3-Antimicrobial Activity:

The agar diffusion method was used for the determination of antimicrobial activities of the methanolic extract of ***Cytisus purgans***. , bacterial cultures were adjusted to 0.5 Mc Farland turbidity standards and inoculated onto Nutrient agar plates (diameter 9 cm). For the determination of antifungal activity, all the fungal isolates were adjusted to the concentration of 10^6 cfu/ml and were inoculated onto Sabauroud Dextrose Agar plates. Sterile discs (6 mm) were impregnated with 10µlof reconstituted crude extract and placed on the surface of Muller Hinton (MHA) for bacteria, concentrations used were: 40, 60 and 80mg/ml. For fungi, Sabouraud Dextrose agar (SDA) dispersed plates inoculated with the microbes, concentrations used for fungi were: 0.25, 0.5, 1, 20, 40, 60, 80, 1g/ml. Each extract was tested in triplicates. Control discs contained 10µl of methanol used as negative control. Agar plate containing bacteria was incubated at 37° C for 24 h. Fungi were incubated at 28° C for 48 h. Inhibition zones were recorded as the diameter of growth-free zones, including the diameter of the disc, in millimetres at the end of the incubation period[102, 103].

CONCLUSION

We have studied the alkaloid profile of the plant ***Cytisus purgans*** growing in Algeria. The crude MeOH extract of aerial parts of this plant was subjected to GC/MS analysis.

We have come to the conclusion that:

1)-The presence of tweleve (12) known QA , which eleven (11) were of sparteine-type and one (1) was a piperidine alkaloid (ammodendrine) . This phenomenon is interesting from the viewpoints of chemotaxonomy of leguminous plants and biosynthesis of QA.

2)-The sparteine was the first major alkaloid (59.134% of total alkaloids), the second major alkaloid was the lupanine (16.303%), consequently, ***Cytisus purgans*** must be considered potentially toxic and insuitable for forage.

3)-It is interesting that this plant accumulate both tigloyl and angeloyl esters of QA .

In the other hand, the isolation of 13α-Hydroxylupanine was carried out by the preparative TLC, and it was identified by their IR and UV spectra. Further research is necessary to determine the identity of the other constituents of the plant.

In this study, the antimicrobial influence of crude mixture alkaloids of MeOH extract of ***Cytisus purgans*** against bacteria and fungi was determined. The plant did not show any activity against any of the fungi at the tested concentration but showed high antibacterial activity.

As a consequence of this study, we will try to isolate compounds responsible for the antibacterial activity in the crude fraction.

Although much effort has been invested in the study of quinolizidine alkaloids world-wide, many questions are still open. The stereochemistry and absolute configuration of a number of QA need to be elucidated and the structures for some QA derivatives confirmed. A large number of QA-producing plants have not yet analysed with modern techniques, so that new QA can still be expected.

Most biochemical and molecular mechanisms underlying biosynthesis transport, storage and degradation need further studies, which will be facilitated by the modern phytochemical and molecular techniques.

It is to be noted that these results are preliminary. However, an expansion of this approach on more species will help establish if alkaloid distribution profiles will aid systematic studies at the specific level in ***Cytisus*** and other Fabaceae genera.

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الملخص

[illegible] GC/MS [illegible] *Cytisus purgans* [illegible] (**Fabaceae** [illegible] 12 [illegible] 11 [illegible] (**Lupin** .(**Ammodendrine**

.13α-Hydroxylupanine [illegible] *Cytisus purgans* [illegible]

Lupin alkaloids, Fabaceae, ***Cytisus purgans***, GC/MS , Antimicrobial activity.

Abstract

Through GC/MS study of methanolic extract of the aerial parts of ***Cytisus purgans*** (Family of **Fabaceae**) of Algerian Flora, twelve (12) known lupin alkaloids were identified which 11 (eleven) were a quinolizidine alkaloids of Sparteine-type and one (1) was a piperidine alkaloid (Ammodendrine).

In the other hand, the **13α-Hydroxylupanine** was isolated by preparative TLC.

The antimicrobial study showed that ***Cytisus purgans*** have a high antibacterial activity against all bacteria strains tested.

Key words: Lupin alkaloids, Fabaceae, ***Cytisus purgans***, GC/MS, Antimicrobial activity.

Résumé

Pendant l' étude par GC/MS de l'extrait méthanolique du parties aériennes du plante ***Cytisus purgans*** (Famille de **Fabaceae**) du Flore Algérienne , douze (12) alcaloïdes lupiniques (de type Lupin) ont été identifiées, dont onze (11) sont de type Sparteine (Sparteine-type) et un (1) est un alcaloïde piperidinique (Ammodendrine) .

Ainsi que, **13α-Hydroxylupanine** a été isolé en utilisant la TLC préparative.

L'étude de l'activité antimicrobienne a montré que ***Cytisus purgans*** a une activité antibactérienne contre toutes les souches bactériennes testées.

Mots clés : Lupin alkaloids, Fabaceae, ***Cytisus purgans***, GC/MS, Activité antimicrobienne.