GC/MS EXAMINATION OF LYCOPODIUM EXTRACTS FOR ALKALOID CONTENT

BY

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Abstract

Plants of the Lycopodium species have long been known to elaborate alkaloids. Over one hundred alkaloids have been isolated from these plants, but no efficient method exists to quickly screen plants to determine their alkaloid content. In this research it has been found that gas chromatography-mass spectrometry (GC/MS) is a suitable method to achieve this purpose. Seven species of Lycopodium were examined; L. australianum, L. scariosum, L. fastigiatum, L. deuterodensum, L. clavatum var.borbonicum, L. flabelliforme and L. lucidulum, of these L. australianum, L. scariosum and L. fastigiatum have been examined for the first time in this research. New alkaloids were found in some of the species examined. Two new alkaloids, des-N-methylfastigiatine and fastigiatine, were isolated from L. fastigiatium and their structures determined by X-ray analysis. Since these alkaloids represent a new ring system extensive mass spectrometric and nuclear magnetic resonance experiments were undertaken.

A data base of the mass spectra of known Lycopodium alkaloids was compiled and found to be useful in computer searches of GC/MS data for the identification of known alkaloids. Chemical ionization mass spectrometry with methane as the reagent gas was found to provide information on some of the functional groups present in the Lycopodium alkaloids.

Acknowledgements

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Introduction

1.1 General introduction

A recent and satisfactory definition of an alkaloid is the one given by S. W. Pelletier in his book, "Alkaloids Chemical and Biological Perspectives", John Wiley and Sons, Inc, 1983; 'An alkaloid is a cyclic organic compound containing nitrogen in a negative oxidation state which is of limited distribution among living organisms". One objective of this research was to develop an analytical method for rapid qualitative and quantitative screening of Lycopodium plants for the presence of alkaloids. The information gained would be of importance in the chemotaxonomy of the order Lycopodiales. A second objective was to examine the structures of any new alkaloids discovered providing that enough compound could be isolated. New alkaloids are of general interest since they may help to illuminate the pathways of biosynthesis and often stimulate others to undertake research into their synthesis and pharmacology.

Gas chromatography/mass spectrometry (GC/MS) provides a very powerful tool for the analysis of mixtures and the identification of components. GC/MS was used in pursuing the first objective of this research. Mass spectra of pure substances are reproducible and are useful for the identification on less than a microgram of compound; thus large quantities of plant material do not have to be extracted to

undertake an analysis of alkaloid content. Gas chromatography (GC) using fused silica columns (FSC) with a flame ionization detector (FID) and a nitrogen phosphorus detector (NPD), provides a sensitive and selective method of separation and analysis of mixtures. From a GC examination, retention indices can be determined and used to identify components of a mixture. If the eluate of a GC column is introduced into a mass spectrometer a mass spectrum can be obtained for each component of a mixture. Thus two methods of identification and a separation procedure are combined in a single experiment.

From data obtained in this research and from the literature, a library or data base of the mass spectra of the known Lycopodium alkaloids was compiled. The library was then used in the identification of the components of each extract using a computer program.

When a new alkaloid was detected, liquid chromatography (LC) was employed in an attempt to isolate sufficient amounts of the compound for structural analysis. Fractions that were collected from LC experiments were examined by GC-MS. High resolution mass spectrometry and nuclear magnetic resonance techniques were used to gain structural information on the new alkaloids.

Figure 1 shows a flow chart that outlines the scheme used to identify new or previously known alkaloids.

The thesis has been organized along the following lines. This chapter deals with the structures and occurrence of the Lycopodium alkaloids; taxonomy and chemotaxonomy of the Lycopodium species are also discussed. A brief account of the type of information that can be

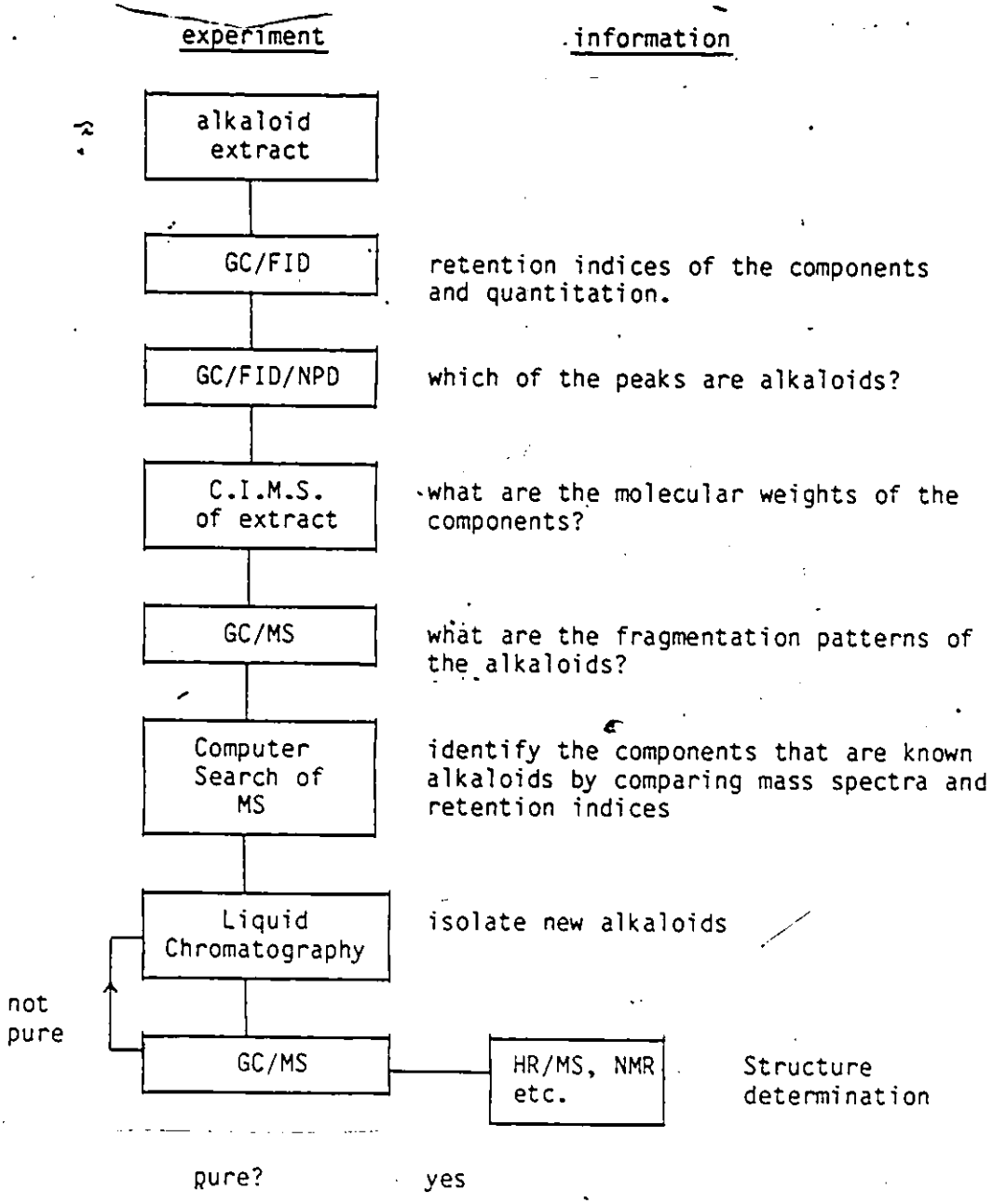


Figure 1 Flowchart of methods used.

obtained from mass spectrometry is also given using, examples drawn from the Lycopodium alkaloids. In Chapter 2, the experimental section, the source of materials, and the methods and techniques used in this investigation are described. Chapter 3 deals with results obtained from the examination of various Lycopodium species. Finally in Chapter 4 the results are summarized. Mass spectra of the lycopodium alkaloids are presented in the Appendix.

1.2 Botanical description

Species of the order Lycopodiales grow in the arctic, temperate and tropical zones.[1] Some of the tropical species are epiphytes, that is they gain physical support from other plants but are not parasitic upon them. Plants of the order are usually of small size, seldom growing larger than 30 centimeters in height, but some species with climbing stems such as L. volubile may reach a length of 2 meters. Plants consist of creeping rhizomes (a horizontal stem) which may be on the surface or below ground[1]; forked roots arise from the rhizomes as well as upright dichotomous branches which are usually covered in many, small, spirally arranged leaves.[1] Leaves which bear structures in which spores are produced (sporangia) are termed sporaphylls. They can be located at any point on the stem or only at the tips of the branches, to form cones or strobili. The strobili resemble clubs and hence the name club moss is used to describe the order. Some species resemble wolf feet. The word Lycopodium is derived from the Greek for wolf (lykos) and foot (pod). A typical plant is shown in Figure 2.

1.3 Historical introduction

In 1881 Karl Böedeker isolated an alkaloid from Lycopodium complanatum which he named lycopodine and assigned to it the incorrect formula $C_{32}H_{52}N_2O_3$.[2] Eleven years later Arata and Canzoneri isolated an alkaloid from L. saururus which they named pillijanine $C_{15}H_{20}N_2O$.[3] Orechoff reported the presence of alkaloids in L. annotinum in 1934[4] and in the following year Muszynski confirmed Orechoff's work and found alkaloids in three other European species.[5] The correct formula for lycopodine, $C_{16}H_{25}NO$, was determined in 1938 by Achmatowicz and Uzieblo;[6] they also isolated two new alkaloids from L. clavatum which they named clavatine and clavotoxine. In 1942 Deulofeu and DeLanghe isolated saururine, $C_{10}H_{19}N$, and sauroxine, $C_{17}H_{26}N_2O$, from L. saururus but did not find any alkaloid with the properties of pillijanine.[7]

Modern research began in the 1940's when Manske and Marion examined ten species of Lycopodium plants and were able to isolate thirty-five new alkaloids.[8-17] Since then many new alkaloids have been isolated from other species and by the application of new techniques to the species previously examined. At present there are about one hundred Lycopodium alkaloids of known structure.

Fractional crystalization and fractional distillation were the first methods employed to investigate alkaloid content. Liquid chromatography, including column chromatography, thin layer chromatography and counter current distribution have also been used to separate alkaloids found in plants of the order Lycopodiales. Recently gas chromatography using packed columns and gas chromatography/mass spectrometry were examined for their suitability to determine alkaloid content but an exhaustive study was not undertaken.[18]

1.4 Structure of the Lycopodium alkaloids

In order to have a reference list of alkaloids of established structure, the structure and physical properties of the Lycopodium alkaloids were compiled in Table 1. The alkaloids are classified according to their structural type and arranged within each structural group by molecular weight along the lines used by Nyembo.[18] Alkaloids which have been reported but for which the structures are not known are listed in Table 2. Alkaloids which have proven to be molecular complexes are listed in Table 3.

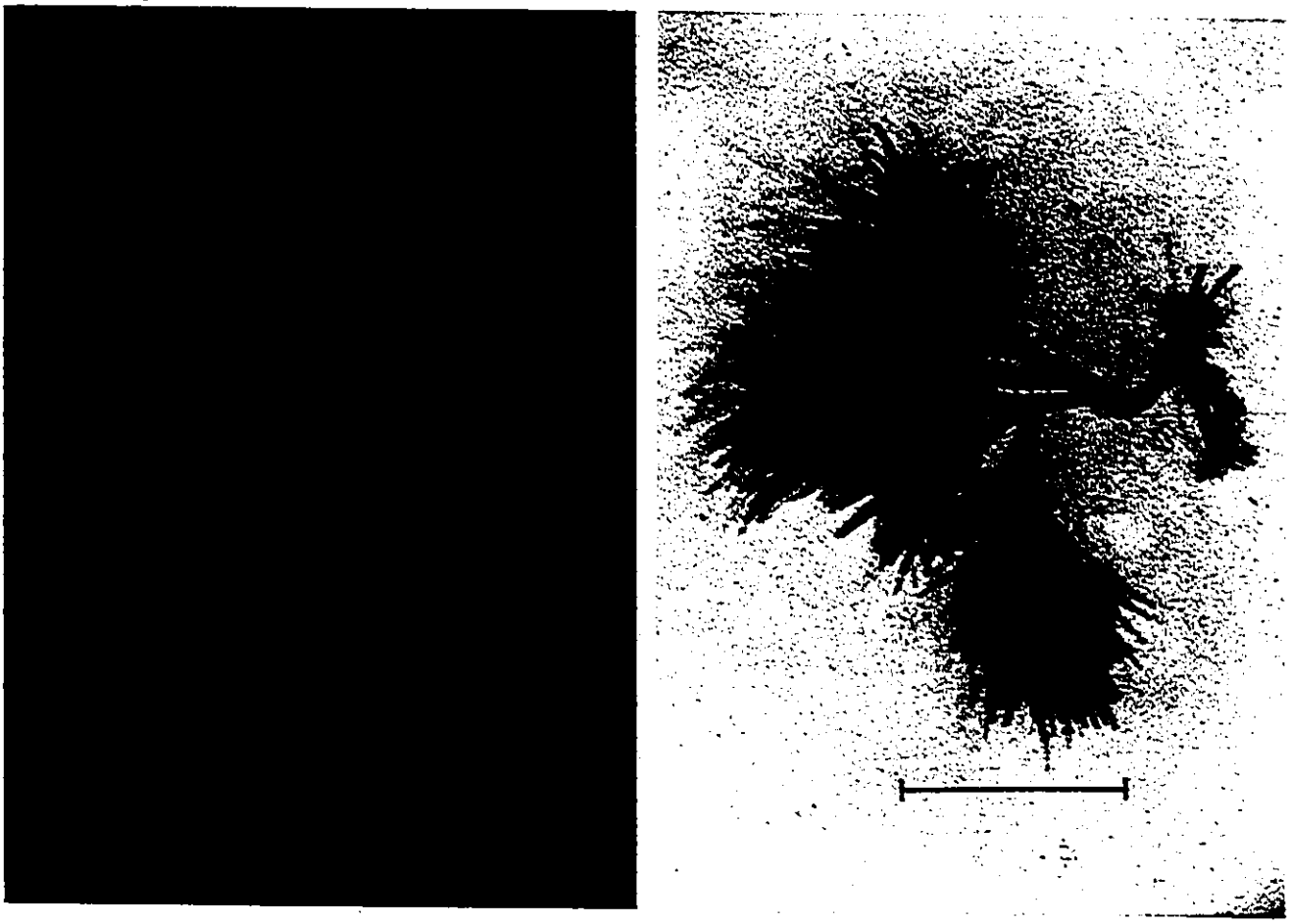
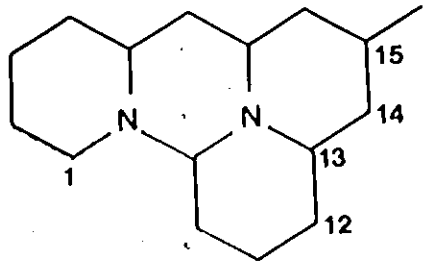


Figure 2. Plants of the genus lycopodium: L. clavatum and L. obscurum

TABLE 1

Structure of the Lycopodium alkaloids

Cernuane skeleton



Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
dihydrodeoxycernuine		$C_{16}H_{28}N_2$	248	✓			18	19	64-5
anhydrolycocernuine	Δ12,13; 1, = O	$C_{16}H_{24}N_2O$	260	✓			18	19	137-8
cernuine (L32)	1, = O	$C_{16}H_{26}N_2O$	262	✓	✓	1	19	19,20, 21,22	106
dihydroxydeoxylycocernuine	12, OH (R)	$C_{16}H_{28}N_2O$	264	✓			18	19	193-4
carolinianine	Δ14,15; 1, = O; 12, OH (R)	$C_{16}H_{24}N_2O_2$	276	✓	✓	2	23	23	198.5-201.4
lycocernuine (L33)	1, = O; 12, OH (R)	$C_{16}H_{26}N_2O_2$	278	✓	✓	1	23	19,20 21,22	218

MW = molecular weight
MS = mass spectrum (✓ indicates the mass spectrum is in the appendix)
RI = retention index (✓ indicates the retention index has been determined)
S = sample source
MSL = mass spectrum literature reference
STR = structure reference
MP = melting point in °C
The absolute configurations of the alkaloids are given in the diagram

Table 1 continued.

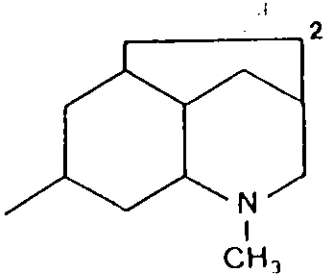
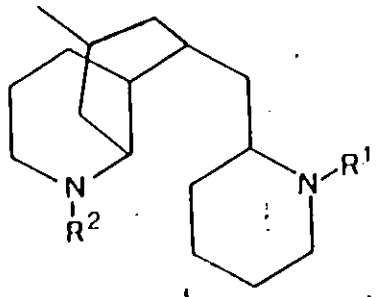
Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
Lucidulane skeleton									
luciduline (L21)	2, = O	$C_{13}H_{21}NO$	207	✓			24	24	Oil
dihydroluciduline	2, OH (R)	$C_{13}H_{23}NO$	209	✓			24	24	65-70
Phlegmarane skeleton									
phlegmarine	$R^1 = R^2 = H$	$C_{16}H_{30}N_2$	250					25	
N_β-methylphlegmarine	$R^1 = H$; $R^2 = CH_3$	$C_{17}H_{32}N_2$	264					25	
N_α-methylphlegmarine	$R^1 = CH_3$; $R^2 = H$	$C_{17}C_{32}N_2$	264					25	
N,N-dimethylphlegmarine	$R^1 = R^2 = CH_3$	$C_{18}H_{34}N_2$	278	✓			25	27	
N_α-acetyl-N_β-methylphlegmarine	R^1 = Ac; $R^2 = CH_3$	$C_{19}H_{34}N_2O$	306	✓	✓	1	18	25	

Table 1 continued.

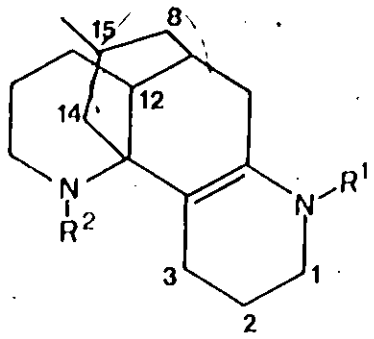
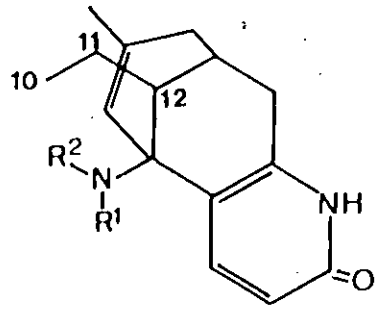
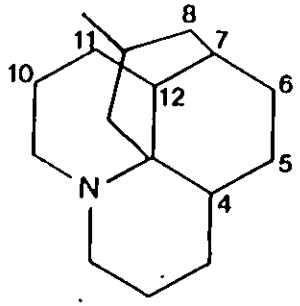
Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
Lycodane skeleton									
lycodine	ΔN,1; Δ2,3; R^2 = H	$C_{16}H_{22}N_2$	242	✓	✓	1	28	29,30 31	118
N-methylycodine	ΔN,1; Δ2,3; R^2 = CH_3	$C_{17}H_{24}N_2$	256	✓	✓	3		30,32	92-2
de-N-methyl-α-obscurine	R^1 = R^2 = H; 1, = O	$C_{16}H_{24}N_2O$	260	✓	✓	1	33	32	270-2
erythreine	Δ14,15; R^1 = H; R^2 = CH_3; 1, = O	$C_{17}H_{22}N_2O$	270					34	
β-obscurine (L6)	Δ2,3; R^1 = H; R^2 = CH_3; 1, = O	$C_{17}H_{24}N_2O$	272	✓	✓	1		35,36 32,37	322-3
α-obscurine (L6)	R^1 = H; R^2 = CH_3; 1, = O	$C_{17}H_{26}N_2O$	274	✓	✓	1	28	35,36 32,37	282-3
sauroxine	R^1 = H; R^2 = CH_3; 1, = O; 12, H(S)	$C_{17}H_{26}N_2O$	274	✓			38	38	200-1
hydroxy-des-N-methyl-α-obscurine	R^1 = R^2 = H; 1, = O; 12, OH (S)	$C_{16}H_{24}N_2O_2$	276	✓			33	39	300-5
flabellidine (L5)	R^1 = Ac; R^2 = H	$C_{18}H_{28}N_2O$	288	✓	✓	1	33	39	

Table 1 continued.

Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
									
selagine	Δ11,12; $R^1 = R^2 = H$	$C_{15}H_{18}N_2O$	242	✓			28	40,41	224-6
erythreidine	Δ10,11; $R^1 = R^2 = CH_3$	$C_{17}H_{22}N_2O$	270					34	

Lycopodane skeleton



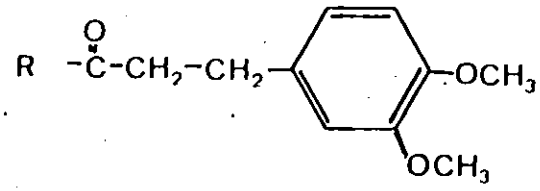


Table 1 continued.

Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
anhydrodihydrolycopodine (L14) (L4)	Δ4,5	$C_{16}H_{25}N$	231	✓			28	42	238
anhydrolycodoline	Δ11,12; 5 = O	$C_{16}H_{23}NO$	245	✓	✓	1	18	43,44	
anhydrodeacetylpaniculine (P5)	Δ4,5; 10, OH(R)	$C_{16}H_{25}NO$	247	✓			26	26,45	175-8
lycopodine (L13)	5, = O	$C_{16}H_{25}NO$	247	✓	✓	1	28	*	116
dihydrolycopodine (complanatine) (L1)	5, OH (R)	$C_{16}H_{27}NO$	249	✓	✓	1	28	46,43	168
acrifoline (L27)	Δ11,12; 5, OH(R); 8, = O	$C_{16}H_{23}NO_2$	261	✓	✓	1	28	57,58,59	97-104
gnidioidine	Δ11,12; 5, = O; 8, OH(R)	$C_{16}H_{23}NO_2$	261	✓			18	60	
lycophlegmine	Δ11,12; 5, = O; 10, OH(S)	$C_{16}H_{23}NO_2$	261	✓			27	27	121-3
serratidine	Δ11,12; 5, = O; 7, OH(R)	$C_{16}H_{23}NO_2$	261	✓	✓	4		61	143-4
annofoline	5, OH(R), 8, = O	$C_{15}H_{25}NO_2$	263	✓			28	62,63,44,64,54	156-7
clavolonine (L34)	5, = O; 8, OH(R)	$C_{16}H_{25}NO_2$	263	✓	✓	1	28	65,54	238
flabelliformine (clavatine)	4, OH(R); 5, = O	$C_{16}H_{25}NO_2$	263	✓	✓	1	28	66	210-1
L20	5, = O; 6, OH(S)	$C_{16}H_{25}NO_2$	263	✓	✓	1		67	258-9
L23 (pseudoselagine) (isolycodoline)	5, = O; 12, OH(R)	$C_{16}H_{25}NO_2$	263	✓			68	68	161-2
lucidioline	Δ11,12; 5, OH(S); 6, OH(S)	$C_{16}H_{25}NO_2$	263	✓	✓	3	69	69	230-3
lycodoline (L8)(L30)	5, = O; 12, OH(S)	$C_{16}H_{25}NO_2$	263	✓	✓	1	28	70,71	180-1
lycofoline (Base H)	Δ11,12; 5, OH(R); 8, OH(R)	$C_{16}H_{25}NO_2$	263					59,72	144-5
deacetylfawcettiine	5, OH(R); 8, OH(R)	$C_{16}H_{27}NO_2$	265	✓	✓	1		65,54	203-4
deacetyllycoclavine (P4)	5, OH(S); 6, OH(S)	$C_{16}H_{27}NO_2$	265	✓			26	26	
deacetylpaniculine	5, OH(R); 10, OH(R)	$C_{16}H_{27}NO_2$	265	✓			26	26	172-4
flabelline	Δ4,5; 5, NHAc	$C_{18}H_{28}N_2O$	288	✓	✓	1	33	73	185-7.5
acetyldihydrolycopodine (L2) (L3)	5, OAc(R)	$C_{18}H_{29}NO_2$	291	✓	✓	1	28	43,54	95-6

* 9, 42, 43, 44, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56

Table 1 continued.

Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
acetylacrifoline (L12)	Δ11,12; 5, OAc; 8, = O	$C_{18}H_{25}NO_3$	303					57,58, 59	119-20
lycoverticine	Δ4,5; 5, NHAc; 12, OH(S) OH(R)	$C_{18}H_{28}N_2O_2$	304	✓			60	60	222-5
α-lofoline	5, OAc(R); 8, OH(S)	$C_{18}H_{29}NO_3$	307	✓	✓	1	28	54,74	211-2
fawcettine (β-lofoline) (Base C) (Base J)	5, OAc(R); 8, OH(R)	$C_{18}H_{29}NO_3$	307	✓	✓	1		65	166-7
lycoclavine	5, OAc(S); 6, OH(S)	$C_{18}H_{29}NO_3$	307	✓	✓	1	28	75	212-3
paniculine (P2)	5, OAc(R); 10, OH(R)	$C_{18}H_{29}NO_3$	307	✓			26	26,45	
lycofawcine (Base L)	5, OAc(R); 8, OH(R); 12, OH(S)	$C_{18}H_{29}NO_4$	323	✓	✓	5	76	77,78	
diacetyllycofoline (Base N)	Δ11,12; 5, OAc(R); 8, OAc(R)	$C_{20}H_{29}NO_4$	347					76 59	140
acetylfawcettine (Base K)	5, OAc(R); 8, OAc(R)	$C_{20}H_{31}NO_4$	349					43,54	117
acetyllofoline	5, OAc(R); 8, OAc(S)	$C_{20}H_{31}NO_4$	349	✓			79	79	
acetyllycoclavine	5, OAc(S); 6, OAc(S)	$C_{20}H_{31}NO_4$	349					75	144-5
acetyllycofawcine (Base O)	5, OAC(R); 8, OAc(R), 12, OH(S)	$C_{20}H_{31}NO_5$	365					76,77	181-2
lycognidine	5, OR* (S); 6 OH(S)	$C_{27}H_{38}NO_5$	457	✓	✓	2	60	78 60	

Table 1 continued.

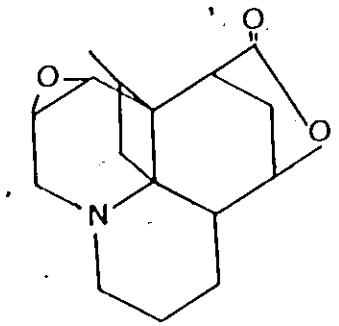
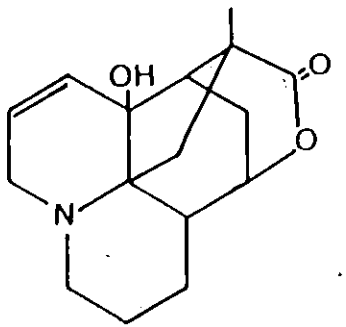
Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
annotinine (L7)		$C_{16}H_{21}NO_3$	275	✓	✓	1	28	80 to 103	232
annotine (L11)		$C_{16}H_{21}NO_3$	275	✓	✓	1	104	105,106 107,108	174

Table 1 continued.

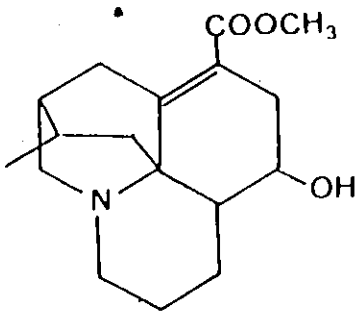
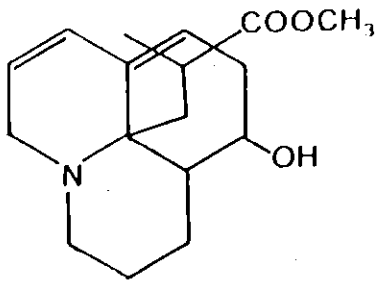
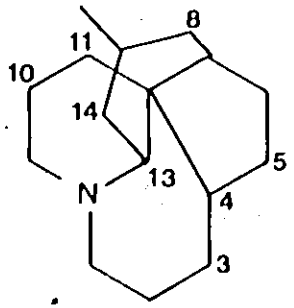
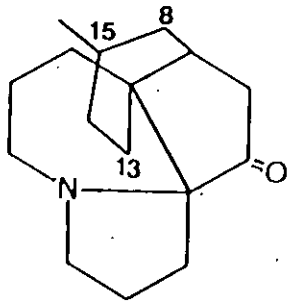
Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
annopodine		$C_{17}H_{25}NO_3$	291	✓	✓	3	109		212
lyconnotine		$C_{17}H_{25}NO_3$	291	✓			110	110,111	123

Table 1 continued.

Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
Fawcettidane skeleton									
fawcettidine (Base F)	Δ13,14; 5, = O	$C_{16}H_{23}NO$	245					112,113	
epidihydrofawcettidine	Δ13,14; 5, OH	$C_{16}H_{25}NO$	247	✓			27	27	156-7
alolycopine	Δ3,4; Δ13,14; 5, = O; 8, OH(S)	$C_{16}H_{21}NO_2$	259	✓			114	114	53-6
anhydroaposerratinine	Δ13,14; 5, = O; 8, OH(S)	$C_{16}H_{23}NO_2$	261	✓			60	60	151
lycothunine	Δ10,11; 5, = O; 13, OH(S)	$C_{16}H_{23}NO_2$	261					115	
8-deoxyserratinidine	Δ13,14; 5, NHAc	$C_{18}H_{28}N_2O$	288					27	198-9
serratinidine	Δ13,14; 5, NHAc; 8, OH(S)	$C_{18}H_{28}N_2O_2$	304	✓	✓	4		113	232-4
Serratinane skeleton									
8-deoxy-13-dehydro serratinine	13, = O	$C_{16}H_{23}NO_2$	261	✓			27	27	110-2
8-deoxyserratinine	13, OH(S)	$C_{16}H_{25}NO_2$	263	✓	✓	4	116	117;113	
serratine	13, OH(S); 15, OH(S)	$C_{16}H_{25}NO_3$	279					118,119	
serratinine	8, OH(S); 13, OH(S)	$C_{16}H_{25}NO_3$	279	✓	✓	4	116	*	244-5
serratanidine	8, OH(R); 13, OH(S); 15, OH(R)	$C_{16}H_{25}NO_4$	295	✓	✓	4	116	127,119	

* 116, 120 to 126

Table 1 continued.

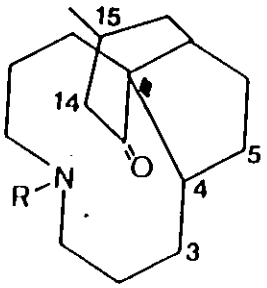
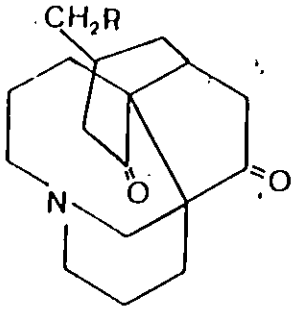
Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
fawcettimane skeleton									
fawcettimine (Base A)	R = H; 5, = O	$C_{16}H_{25}NO_2$	263					128	
alopecuridine	R = H; 4, OH(R); 5, = O	$C_{16}H_{25}NO_3$	279	✓	✓	3	129	129	171-2
lycophlegmarine	R = CH_3; Δ3,4; Δ14,15; 5, OH(S); 14, OCH_3	$C_{18}H_{27}NO_3$	305					115	011
									
lycoflexine (lycobergine)	R = H	$C_{17}H_{25}NO_2$	275	✓	✓	1	34	130	130-1
saurudine	R = OH	$C_{17}H_{25}NO_3$	291	✓			34	34	

Table 1 continued.

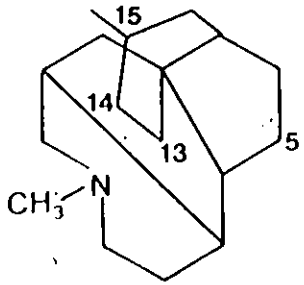
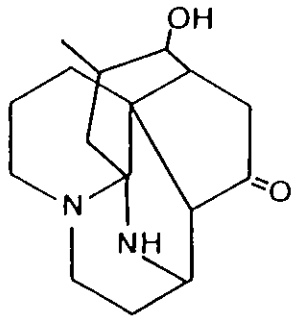
Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
Magellanane skeleton									
5-dehydromagellanine	Δ14,15; 5 = O; 13, = O	$C_{17}H_{23}NO_2$	273	✓			131	131	125-6
magellanine	Δ14,15; 5, OH(S); 13, = O	$C_{17}H_{25}NO_2$	275	✓	✓	1	132	132	165-6
paniculatine	5, = O; 13, OH(S)	$C_{17}H_{27}NO_2$	277	✓	✓	1	133	133	181
									
base R		$C_{16}H_{24}N_2O_2$	276					134	129-30

Table 1 continued.

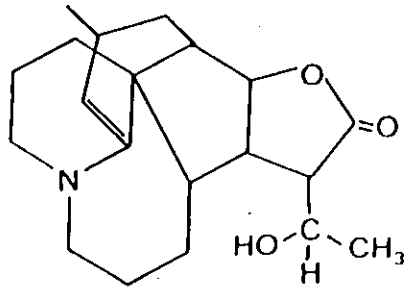
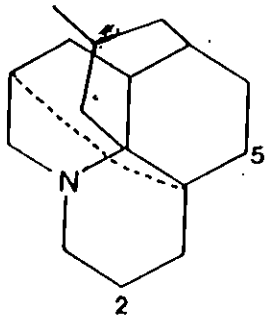
Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
megastachine		$C_{20}H_{29}NO_3$	331	✓	✓	2	135	135	167-9
Inundatane skeleton									
dehydrolycopecurine	5, = O	$C_{16}H_{23}NO$	245	✓	✓	2	136	136	57-9
lycopecurine	5, OH(R)	$C_{16}H_{25}NO$	247	✓	✓	3	137	137	239-41
inundatine	2, OH(R); 5, = O	$C_{16}H_{23}NO_2$	261	✓			136	136	174-7
isoinundatine	2, = O; 5, OH	$C_{16}H_{23}NO_2$	261	✓			136	136	255
debenzoylalopecurine	2, OH(S); 5, OH(R)	$C_{16}H_{25}NO_2$	263					138	230-2
acetyldebenzoylalopecurine	2, OAc(S); 5, OH(R)	$C_{18}H_{27}NO_3$	305	✓			138	138	238-40
alopecurine	2, $OCOC_6H_5$(S); 5, OH(R)	$C_{23}H_{29}NO_3$	367	✓	✓	3	138	138	244-5

Table 1 continued.

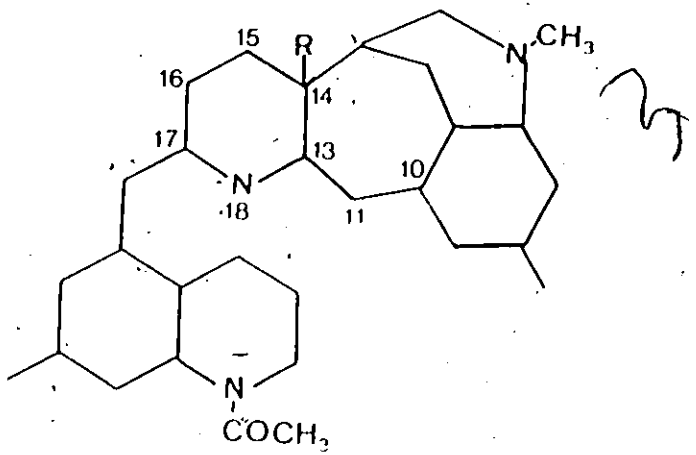
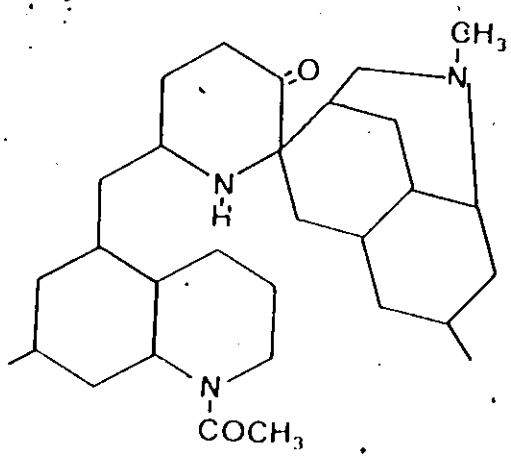
Name	Structure	Formula	MW	MS	RI	S	MSL	STR	MP
Lucidane skeleton									
lycolucine	Δ10,11; Δ17,18; Δ15,16; Δ13,14	$C_{30}H_{43}N_3O$	461					139	198-200
dihydrolycolucine	Δ17,18; Δ15,16; Δ13,14	$C_{30}H_{45}N_3O$	463					139	
lucidine B (serratanine A)	Δ13,18	$C_{30}H_{49}N_3O$	467	✓			139	139	
serratanine B (oxolucidine B)	Δ13,18; R = OH(S)	$C_{30}H_{49}N_3O_2$	483					140	
spirolucidine		$C_{30}H_{49}N_3O_2$	483	✓			141	141	

Table 2

Alkaloids of undetermined structure

Name	Molecular Formula	Molecular Weight	Source	Reference
Base 168	$C_{16}H_{23}NO_3$	277	L. clavatum	142
Base 258	---	263	L. clavatum	142
Base E	$C_{17}H_{25}NO_2$	275	L. fawcettii	143
Base G	$C_{18}H_{27}NO_3$	305	L. fawcettii	143
---	$C_{10}H_{19(21)}NO$	169 (171)	L. annotinum	105
---	$C_{16}H_{23(25)}NO$	245 (247)	L. annotinum	105
Base IV	$C_{16}H_{23}NO$	245	L. annotinum	144
Base V	$C_{17}H_{25}NO_2$	275	L. annotinum	144
Base VI	$C_{16}H_{23}NO_2$	261	L. annotinum	144
Base VII	$C_{20}H_{29}NO_4$	347	L. annotinum	144
Base VIII	$C_{16}H_{21}NO_3$	275	L. annotinum	144
Base IX	$C_{17}H_{25}NO_2$	275	L. annotinum	144
Base X	$C_{17}H_{25}NO_3$	291	L. annotinum	144
Base XI	$C_{18}H_{25}NO_3$	303	L. annotinum	144
Base XII	$C_{18}H_{25}NO_4$	319	L. annotinum	144
-	$C_{16}H_{25(27)}NO_2$	263 (265)	L. clavatum	75
-	$C_{18}H_{27}NO_3$	305	L. contigum	145
			L. thyoides	145
Borbonicine	$C_{17}H_{24}N_2O$	272	L. clavatum var. borbonicum	18

Table 2 continued.

Name	Molecular Formula	Molecular Weight	Source	Reference
Dehydrolycocaroline	$C_{32}H_{51}N_3O$	493	L. carolinianum	18
Gnidine	$C_{29}H_{51}N_3$	441	L. gnidioides	18
Gnidinine	$C_{29}H_{51}N_3O$	457	L. gnidioides	18
LE1	$C_{28}H_{47}N_3O$	441	L. erythraeum	34
LO1	$C_{31}H_{49}N_3O_2$	495	L. obtusifolium	146
LO2	$C_{30}H_{49}N_3O$	467	L. obtusifolium	146
LO3	$C_{29}H_{49}N_3$	439	L. obtusifolium	146
LS14	$C_{30}H_{49}N_3O$	467	L. saururus	34
Lucidine A	$C_{30}H_{49}N_3O$	467	L. lucidulum	139
LV1	$C_{29}H_{45}N_3$	435	L. verticillatum	60
Lycocaroline	$C_{32}H_{53}N_3O$	495	L. carolinianum	18
Lycodiflexine	$C_{35}H_{50}N_2O_4$	562	L. clavatum	18
Lycoserrine	$C_{16}H_{26}N_2$	246	L. serratum	117
Lycoserramine	$C_{16}H_{25}NO_2$	263	L. serratum	17
L10	$C_{16}H_{27}NO$	249	L. annotinum	10
L15	$C_{20}H_{31}NO_4$	349	L. tristachyum	11
L16	$C_{16}H_{25}NO$	247	L. obscurum	12
L17	$C_{18}H_{27}NO_3$	305	L. obscurum	12
L18	$C_{11}H_{19}NO$	181	L. clavatum	13
L19	-	-	L. clavatum	13
L22	$C_{16}H_{27}NO$	249	L. lucidulum	14

Table 2 continued.

Name	Molecular Formula	Molecular Weight	Source	Reference
L24	$C_{16}H_{25}NO$	247	L. lucidulum	14
L 25	$C_{16}H_{25}NO_2$	263	L. lucidulum	14, 67
L26	$C_{15}H_{25}NO$	235	L. sabinaefolium	15
L28	$C_{17}H_{27}NO_2$	277	L. annotinum	16, 144
L29	$C_{16}H_{23}NO_2$	261	L. annotinum	16, 144
L31	$C_{20}H_{29}NO_4$	347	L. annotinum	16, 144
L35	$C_{14}H_{21}NO_2$	235	L. densum	147
Pillijanine	$C_{15}H_{24}N_2O$	248	L. saururus	3
Saururine	$C_{10}H_{19}N$	153	L. saururus	7

Table 3

Molecular Complexes

Name	Molecular Formula	Components	Source	References
Annotoxine	$C_{32}H_{44}N_2O_5$	Acrifoline + Annotine	L. annotinum	74, 105, 106, 148
Clavatoxine	$C_{32}H_{50}N_2O_4$	Flabelliformine + Lycodoline	L. clavatum	6
Isoerythreine	$C_{34}H_{44}N_2O_2$	Erythreidine + Erythreine	L. erythraeum	34
Isolycopodine	$C_{32}H_{48}N_2O_3$	Acrifoline + Lycopodine	L. annotinum L. selago	149 150
L9	$C_{36}H_{56}N_2O_5$	Acetyllofoline + Lycopodine	L. annotinum	10
--	$C_{32}H_{52}N_2O_3$	Dihydrolycopodine + Flabelliformine	L. clavatum	75

Table 4

Occurrence of the Lycopodium alkaloids

Subgenus Urostachys

Selago section
1. L. erythraeum Lawalree
2. L. gnidioides L.f.
3. L. lucidulum michx.
4. L. obtusifolium
5. L. saururus Lam.
6. L. selago L.
7. L. serratum var. thunbergii
8. L. serratum var. serratum
9. L. verticillatum L.

Phlegmaria section
10. L. megastachyum
11. L. ophioglossoides
12. L. phlegmaria L.
13. L. phlegmaria L. (Sri Lanka)

	1	2	3	4	5	6	7	8	9	10	11	12	13
lucidiuline			14,24										
dihydroluciduline			24										
lycodine			67				117,153						
N-methyllycodine	34												
erythreine	34												
ß-obscurine.						150							
α-obscurine						150							
sauroxine					6,34								
selagine	34	18,60			34	40,150							
erythreidine	34												
phlegmarine												25	
N_{β}-methyl-phlegmarine												25	
N,N-dimethyl-phlegmarine													27
anhydrolycodoline		18,60			34								
lycopodine	34		14		34	149,151 152		117				25;154 155	
dihydrolycodine					34	149,151 152							
acrifoline												25	
gnidioidine		18,60											

Table 4 continued.

Subgenus Urostachys	Selago section							Phlegmaria section					
	1	2	3	4	5	6	7	8	9	10	11	12	13
lycophlegmine													
serratidine													27
clavolonine							117						
flabelliformine					34		117						
L20			67										
L23			14,67			150							
			14,67			149 to 152							
lucidioline		18,60	67										
lycodoline			67		34	149,151	117,153	117			18		
						152						25	
lycoverticine													
lycoclavine		18,60							60				
lycognidine		18,60											
fawcettidine													
epidihydro-fawcettidine													27
anhydroapo-serratinine													27
lycothunine									60				
8-deoxyserratinidine								117					
serratinidine													27
8-deoxy-13-dehydro-serratinine							117	117					
8-deoxyserratinine													27
serratine							117						
serratinine							117,153	117					
serratanidine								117					
lycophlegmarine								117					
lycoflexine													27
saurudine					34			117				25	27
megastachine					34								
										135			

Table 4 continued.

Subgenus Urostachys

	Selago section						Phlegmaria section					
	1	2	3	4	5	6	7	8	9	10	11	12	13
lycolucine			139										
dihydrolycolucine			139										
lucidine B			139				117,153						
oxolucidine							117,153						
spiroluciduline			141										
gnidine		18											
gnidinine		18											
LE1	34												
LO1				146									
LO2				146									
LO3				146									
LS14					34								
lucidine A			139										
LV1									60				
LV2									60				
lycoserrine							117						
lycoserramine			14					117					
L22			14										
L24			14										
L25			14,67										
pillijanine					3								
saururine					7								
nicotine			14										

Table 4 continued.

Subgenus Lepidotis

1) Cernua section
1. L. cernuum L.

2) Inundata section
2. L. alopecuroides L.
3. L. carolinianum L. var. affine
4. L. inundatum L. (Asia)
5. L. inundatum L. (Europe)

3) Lateralia section
6. L. laterale R. Br.

	1	2	3	4	5	6
dihydrodeoxycernuine	19		157			
anhydrolycocernuine			23	158	18	
cernuine	19,17		157			159
dihydrodeoxylycocernuine			18			
carolinianine			23			
lycocernuine	19,17		23	158	18	159
N_o-methylphlegmarine	25					
anhydrolycodoline		156			18	
lycopodine		156		158	136,18	
clavolonine		156			18	
lycodoline		156			136,18	
fawcettidine		138				
alolycopine		138				
lycoflexine					18	
alopecuridine		156				
dehydrolycopecurine					136,18	
lycopecurine		138				
inundatine					136,18	
isoinundatine					136,18	
debenzoylalopecurine		156				
acetyldebenzoylalopecurine		138				
alopecurine		156				
borbonicine					18	
dehydrolycocaroline			18			
lycocaroline			18			
nicotine	17					

Table 4 continued.

Subgenus Lycopodium

Lycopodium section

1. L. annotinum var. acrifolium, Fern.
2. L. annotinum L. (Canadian)
3. L. annotinum L. (European)
4. L. clavatum var. borbonicum
5. L. clavatum L. (Canadian)
6. L. clavatum var. inflexum
7. L. clavatum (Jamaican)
8. L. clavatum var. megastachyon
9. L. clavatum L. (Polish)
10. L. contiguum Klotzsch
11. L. obscurum L. var. dendroideum

	1	2	3	4	5	6	7	8	9	10	11
N_α-acetyl-N_β-methyl-phlegmarine				25							
lycodine		29,74									30, 31
de-N-methyl-α-obscruine					32				142		
α-obscurine		10,74, 106	144,148 149		32				142		12
β-obscurine		10,74 106	144								12
anhydrolycodoline				25							
lycopodine	16	10,74, 106	105,148 149	25	13	161,130	77,162	75	6,142	145,157	12, 31
dihydrolycopodine				25		130	162	75	142	157,18	
acrifoline	16	74,105, 106	105,148 149								
annofoline		160					162	75	142	145,18	
clavlonine								75	6,142		
flabelliformine									6,142		
lycodoline	16	10,74, 160	148,149	25		161,130					
lycofoline											

Table 4 continued.

Subgenus Lycopodium

Lycopodium section

	1	2	3	4	5	6	7	8	9	10	11
deacetylfawcettine							162			18	
acetyldihydrolycopodine				25			162			157,18	
acetylacrifoline		10,74, 106									
acetyllycofoline		10									
α-Lofoline		160,74									
fawcettiine		160,74					162			145,157, 18	
lycoclavine								75			
acetylfawcettiine										145,157, 18	
acetyllofoline		10									
acetyllycoclavine								75			
annotinine	16	10,74, 106	105,148, 149								
annotine		74,105, 106	105,148, 149								
annopodine		74,109									
fawcettimine						130	162				
lycoflexine				25		130					
lyconnotine		74									
base 168									142		
base 258									142		
borbonicine				18							

Table 4 continued.

Subgenus Lycopodium

Lycopodium section

	1	2	3	4	5	6	7	8	9	10	11
$C_{16}H_{23}NO_4$			105								
$C_{31}H_{42}N_2O_5$			105								
$C_{10}H_{19(21)}NO$			105								
$C_{16}H_{23(25)}NO$			105								
IV $C_{16}H_{23}NO$			144								
V $C_{17}H_{25}NO_3$			144								
VI $C_{16}H_{23}NO_2$			144								
VII $C_{20}H_{29}NO_4$			144								
VIII $C_{16}H_{21}NO_3$			144								
IX $C_{17}H_{25}NO_2$			144								
X $C_{17}H_{25}NO_3$			144								
XI $C_{18}H_{25}NO_3$			144								
XII $C_{18}H_{25}NO_4$			144								
$C_{16}H_{25(27)}NO_2$										145	
$C_{18}H_{27}NO_3$								75			
L10		10									
L16											12
L17											12
L18					13						
L19					13						
L28	16		144								
L29	16		144								
L31	16		144								
Nicotine			144		13						

Table 4 continued.

Subgenus Lycopodium

2) Complanata section

1. L. alpinum L.
2. L. carolinum Lawalree
3. L. complanatum L.
4. L. fawcettii Lloyd et Underwood
5. L. flabelliforme
6. L. isleri (Rouy) Lawalree
7. L. sabinaefolium Willd.
8. L. sitchense Rupr.
9. L. thyoides Humb. F. Bonpl. et Willd
10. L. tristachyum Pursh

	1	2	3	4	5	6	7	8	9	10
lycodine			164	78	33					164
N-methyl-α-obscurine			32							
de-N-methyl-α-obscurine	163			78	33					
β-obscurine					8					
α-obscurine					8			18	18	
hydroxy-de-N-methyl-obscurine					33					
flabellidine					8				157,18	
anhydrodihydrolycopodine					8					11
anhydrolycodoline		34								
lycopodine	163	157,34	2		8	18,157	11,15	18	18,145, 157	11,164
dihydrolycopodine		157			8				18	
clavolonine	163				33			18	18	
flabellifomrine					66					
lycodoline		34		143						
lycofoline				77						
deacetylfawcettine				143					18	
flabelline					33					

Table 4 continued.

Subgenus Lycopodium

2) Complanata section

	1	2	3	4	5	6	7	8	9	10
acetyldihydrolycopodine					8			145,157 18		
acetyllycofoline				77						
fawcettiine				77,143				145,157 18		
lycoclavine	163									
lycofawcine				78						
diacetyllycofoline				77						
acetylfawcettine				77				145,157 18		
acetyllycofawcine				77						
annotinine					33					
fawcettidine				143						
fawcettimine				77,143						
lycoflexine		34								
base R				78						
base E				143						
base G				143						
base I				77						
$C_{18}H_{27}NO_3$								145		
L15									11	
L26						15				
nicotine						15			11	

Table 4 continued.

Subgenus Lycopodium
not further classified.[197]

1. L. magellanicum Palisot de Beauvois
2. L. paniculatum Desvaux
3. L. volubile Forst.
4. L. densum Labill.
5. L. confertum var. confertum
6. L. prostatum

	1	2	3	4	5	6
lycodine	131					
N-methyllycodine	131					
α-obscurine	132					
flabellidine		26				
lycopodine	132,131	133	165	147		
anhydrodeacetylpaniculine		26				
dihydrolycopodine		133	165			
clavolonine	132			147		
lycodoline						167
deacetylfawcettiine	132					
deacetyllycoclavine		26				
deacetylpaniculine		26			45,166	
acetyldihydrolycopodine	131	133				
lycoclavine	132	26				
paniculine		26			45	
acetylfawcettiine	131					
5-dehydromagellanine	131					
magellanine	132					
paniculatine		133				
L35				147		

1.5 Occurrence of the Lycopodium alkaloids

The sources of the alkaloids are tabulated in Table 4, where the species are grouped together according to subgenus and section. Some of the material in this section and the previous section has appeared in earlier reviews,[168-180] but is included here for completeness.

1.6 Taxonomy

Several classification systems of the order Lycopodiales have been proposed.[181-202] All of the systems recognize the genus Phyloglossum which contains only one species, P. drummondii, found only in Australia, New Zealand and Tasmania. However there is a divergence of opinion on how other members of the order should be classified. Wilce recognizes only one genus, Lycopodium, which is further split into three subgenera on the basis of spore shape.[197] Wilce's classification system was used to organize Table 4 and is described below.

Wilce recognized three subgenera, Urostachys, Lepidotis and Lycopodium. The subgenus Urostachys contains more than four hundred species which have spores of the foveolate-fossulate type. The spores are characterized by having a minutely pitted surface (foveolate) that progresses through a series of intermediate stages to a grooved surface (fossulate). The sub-genus, Urostachys, can be further divided into two sections acccording to spore shape, the Selago section and the Phlegmaria section.

The subgenus, Lepidotis (Lycopodiella), contains forty species which have spores of the rugulate type (wrinkled or corrugated surface).

The subgenus is divided into three sections featuring two spore types; the Cernua section, with spores of the cernua type, and the Inundata and Lateralia sections, with spores of the carolinianum type.

The subgenus, Lycopodium, contains sixty species, which is divided into seven sections, comprising three spore groups. The reticulate group of spores have a network-like surface and contains four types of spores; the clavatum type, the scariosum type, the fastigiatum type and the volubile type. The Lycopodium and Complanata sections have clavatum type spores. The baculate (with rod shape projections) and scabrate (rough surfaces) group of spores contain only one species each. Some of the plants which comprise each of the subgenera are listed in Table 5.

1.7 Chemotaxonomy

Braekman et al.[157,203] have observed that the distribution of alkaloids in the genus Lycopodium is in good agreement with the classification proposed by Wilce.[197] The subgenus Urostachys elaborates alkaloids of the lucidulane, phlegmarane, fawcettidane, lycodane, lycopodane and lucidane type. It is the only subgenus from which lucidane alkaloids have been isolated. The subgenus Lepidotis elaborates alkaloids of the cernuane, phlegmarane, lycodane, lycopodane and inundatane type; the subgenus is distinct in the formation of cernuane and inundatane alkaloids. The subgenus Lycopodium forms alkaloids of the phlegmarane, fawcettidane, lycodane and lycopodane type. The major alkaloids are of the lycopodane type with minor amounts of alkaloids of

Table 5

Plants of the genus *Lycopodium*

Plants of the subgenus *Urostachys*

Selago section	*Phlegmaria* section
L. australianum≠	*L. megastachyum**
L. celanicum	*L. ophioglossoides**
*L. erythraeum**	*L. phlegmaria**
L. fontinaloides	
*L. gnidioides**	
*L. lucidulum**≠	
L. miniatum	
*L. obtusifolium**	
*L. saururus**	
*L. selago**	
L. squarrosum	
L. verticillatum	

Plants of the subgenus *Lepidotis*

Cernua section	*Inundata* section*	*Lateralia* section*
*L. cernuum**	*L. alopecuroides**	*L. laterale**
L. convolutum	*L. carolinianum**	
L. eichleri	*L. inundatum**	
L. pendulinum		

Plants of the subgenus *Lycopodium*

Lycopodium section	*Complanata* section	*Scariosum* group	*Fastigiatum* group	*Volubile* group
*L. annotinum**	*L. alpinum**	*L. caneris*	*L. fastigiatum*≠	*L. volubile**
*L. clavatum**≠	*L. carolinum**	*L. gayanum*	*L. magellanicum**	
*L. contiguum**	*L. complanatum**	*L. holtonii*	*L. paniculatum**	
*L. obscurum**	*L. fawcettii**	*L. jussiaei*	*L. spurium*	
	*L. flabelliforme**≠	*L. scariosum*≠		
	*L. issleri**			
	*L. sabinifolium**			
	*L. sitchense**			
	*L. thyoides**			
	*L. tristachyum**			

L. deuterodensum≠ (baculate) *L. casuarinoides* (scabrate)

* examined for alkaloid content
≠ examined for alkaloid content in this thesis.

the three other ring systems. The Complanata section, elaborates mainly a single alkaloid, lycopodine. Braekman et al. have noted that only about ten percent of the known species of the genus Lycopodium have been examined, and that generalizations based on alkaloid content are tenuous at best.

The chemotaxonomy of the genus Lycopodium has also been investigated by examining phenolic acids[204] and dihydric phenols.[205] Flavanoid distribution has also been examined.[206-209] Some of the classifications based on alkaloid content are supported by the pattern of distribution of other types of natural products.

1.8 Mass spectrometry

Mass spectrometry (MS) is a very useful technique in structural studies of organic compounds and has been used in the examination of the structure of many Lycopodium alkaloids. The primary process in mass spectrometry involves ionizing a sample to produce ions. These ions are then accelerated by a potential field into a mass analyzer where the path of the ion is deflected in a magnetic field (see section 3.4.6.2 for further discussion). The mass analyzer separates the ion beam, acccording to the mass-to-charge ratio (m/z). The detection of the ions depends on the radius (r) of the ion path in the magnetic field (of strength B) and the accelerating potential (V_{acc}).

$$m/z = \frac{B^2 r^2}{2V_{acc}}$$

In this research both electron ionization and chemical ionization were used to generate ions.

1.8.1 Electron ionization mass spectrometry

Electron ionization (EI) is the most common form of mass spectrometry. Electrons emitted from an incandescent filament interact with vaporized sample molecules to form ions. These ions are then accelerated into the mass analyzer. A mass spectrum is a plot of the relative abundance of an ion versus its mass to charge ratio, m/z; the most abundant ion is referred to as the base peak. The molecular ion ($M^{+\cdot}$) is defined as that ion, the mass of which is the sum of the masses of all elements in the molecule, using the most abundant isotope in each case.[210] Thus the m/z value of the molecular ion is the molecular weight of the compound. Since organic compounds are seldom isotopically pure the mass spectra obtained are representative of the natural abundance of the elements contained in the compound. It is thus possible to deduce the presence of some elements (particularly chlorine and bromine) in a compound. If the m/z ratio is measured with sufficient accuracy (high resolution mass spectrometry) it is possible to calculate the elemental composition of an ion and thus the molecular formula of the compound. Mass spectrometry can also be used to detect functional groups and substituents. For example the loss of 17 and 18 mass units from the molecular ion usually indicates the presence of a hydroxyl group. The loss of a substituent can often be verified if a metastable peak is observed. Metastable peaks (m^{*}) arise from ion decompositions in a field

free drift region of a mass spectrometer. For the decomposition $M_1^+ \rightarrow M_2^+ + M_3$ where M_3 is a neutral fragment a metastable peak may be found at $M^* = M_2^2/M_1$. The carbon skeleton of an organic compound can in some instances be deduced from a mass spectrum.

Examples of how mass spectra can be interpreted in terms of structure are presented below using some of the Lycopodium alkaloids as examples.

1.8.1.1. Electron ionization mass spectrometry of alkaloids with a cernuane skeleton

Alkaloids of the cernuane type have two nitrogens and hence an even molecular weight. Losses from the D-ring characterize the mass spectra of these alkaloids (Figure 3). Initial fragmentation occurs between C-9 and C-10 which is followed by the loss of CH_3CH_2 (M-29), C_3H_6 (M-42), and C_3H_7 (M-43) via pathways 1, 2 and 3, respectively, when R = H. If R = OH as in lycocernuine, the corresponding fragmentations via pathways 2 and 3 yield ions at M-58 and M-59.

In the case of carolinianine which has a double bond between C-14 and C-15 and R = OH, the fragment, C_3H_6O (M-58), is lost from the D-ring with no loss of a proton from the C-ring. The presence of a $\Delta^{12,13}$ double bond makes the loss of the D-ring atoms more difficult, resulting in an intense molecular ion as in the case of anhydrolycocernuine. The loss of a methyl group is seen in all cases, as is the loss of the hydroxyl group at C-12 if one is present. The mass spectra of the cernuane alkaloids were reported in the following references.[18,19,23]

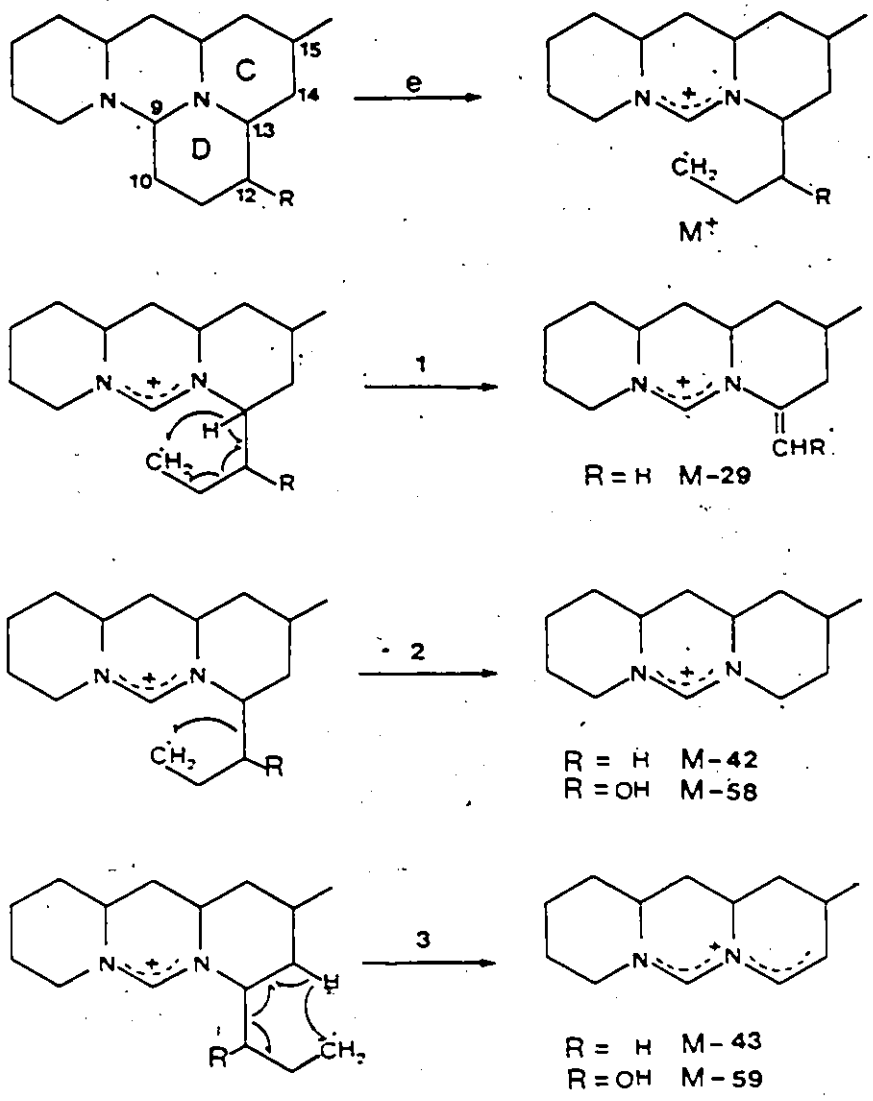


Figure 3 Fragmentation of cernuane type alkaloids.

It is important to remember that structures drawn in proposed fragmentation schemes such as Figure 3 may not correspond to the actual structure of the ion. They are however useful constructs in interpreting spectra and deducing structures.

1.8.1.2 Electron ionization mass spectrometry of alkaloids with a lycopodane skeleton

The mass spectra of lycopodane alkaloids have been extensively examined.[28] Alkaloids of the lycopodane type can be classified into three categories: (1) those bearing a hydrogen at C-12, (2) those bearing a hydroxyl group at C-12, and (3) those bearing a double bond between C-11 and C-12. These classifications are useful in the interpretation of their mass spectra.

1.8.1.2.1 Lycopodanes with a hydrogen at C-12

A general fragmentation scheme of lycopodane type alkaloids with a hydrogen at C-12 is shown in Figure 4. Generally the molecular ion is weak and the base peak corresponds to the loss of the bridge atoms and a proton, that is C_4H_9 (M-57) (pathway 2). The stereochemistry at C-12 has little effect on the fragmentation pattern, but the intensity of the ion corresponding to the loss of the bridge atoms can be altered depending on the stereochemistry.[38] If the bridge is substituted (for example a hydroxyl group at C-8) the loss of the bridge and the substituent will be observed (M-73). The loss of C_3H_7 (M-43) from the bridge atoms is observed in all cases as a low intensity ion (pathway 1). The loss of ethylene after the loss of the bridge atoms is observed and may follow or

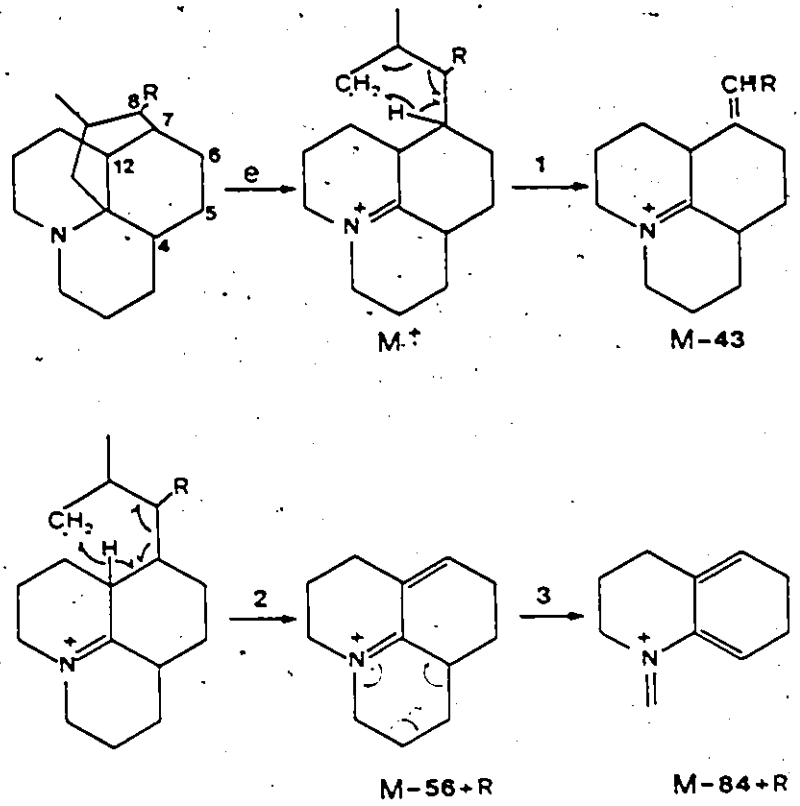


Figure 4 Fragmentation of lycopodane type alkaloids with a hydrogen at C-12.

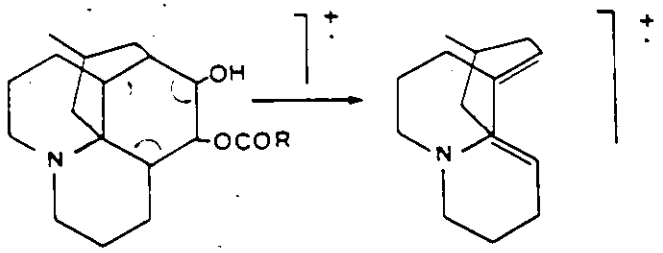


Figure 5 Fragmentation of lycopodane type alkaloids with a hydroxyl at C-6 and an ester at C-5.

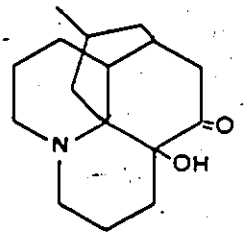


Figure 6 Structure of flabelliformine.

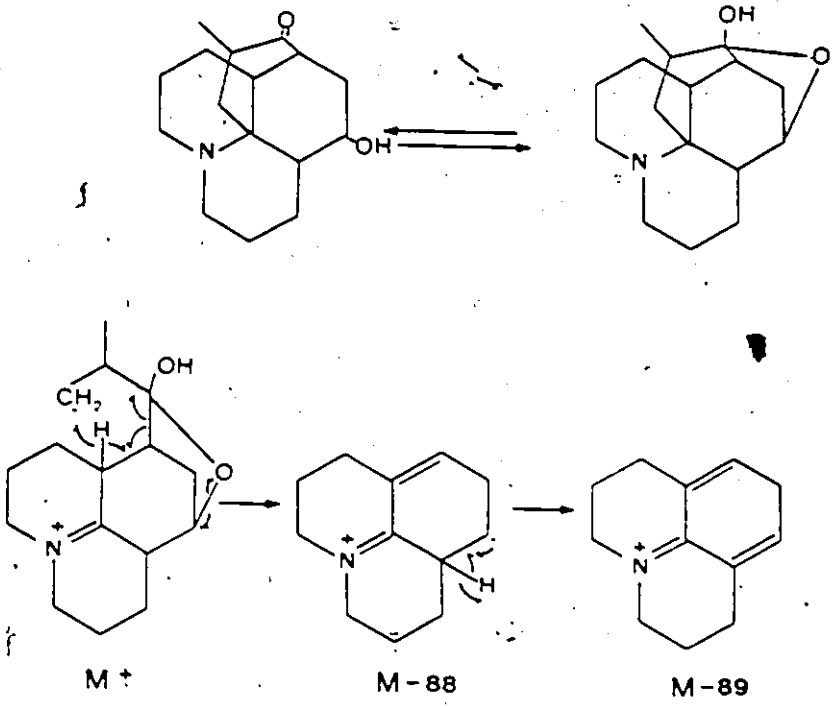


Figure 7 Fragmentation of annofoline.

precede the loss of a functional group at C-5 or C-6 (pathway 3). Functional groups on the lycopodane skeleton can give characteristic fragmentation patterns; some examples are given below. Alkaloids with a hydroxyl group at C-6 and an ester at C-5 have been reported to lose (HOCH = CHOCOR).[28] A possible route for the fragmentation is shown in Figure 5. These alkaloids also undergo the expected fragmentations of the lycopodane-type alkaloids with a hydrogen at C-12.

If a hydroxyl group is present at C-4 as in flabelliformine (Figure 6) a loss of 42 (possibly C_3H_6 or C_2H_2O) from the ion that has already lost the bridge atoms is observed.[28]

Annofoline exists as an equilibrium mixture of the hydroxyketone and the hemiketal. The intense ion at (M-88) can be explained as a concerted loss of $(CH_3)_2CHCO_2H$ from the molecular ion as shown in Figure 7.[28] Annofoline also fragments as the hydroxyketone as expected.

Flabelline (Figure 8) is interesting since it is only one of the two alkaloids of the lycopodane type bearing a nitrogen containing substituent. Flabelline can be distinguished from its isomer flabellidine (Figure 8) (lycodane skeleton) by the presence of an ion at m/z = 172 (M-116). This ion arises from the loss of acetamide after the loss of the bridge (M-57). Metastable peaks indicate that acetamide can be lost by two pathways,[33] in the first case the initial loss of ketene is followed by the elimination of ammonia and in the second case the loss of CH_3CONH is followed by the loss of a hydrogen atom. Acetamide may also be lost in a concerted elimination, however this fragmentation was not supported by a metastable peak.

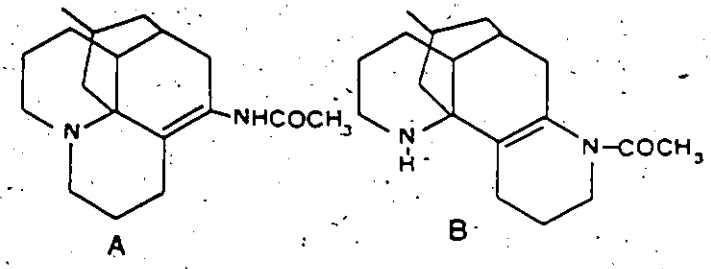


Figure 8 Structure of flabelline (A) and flabellidine (B).

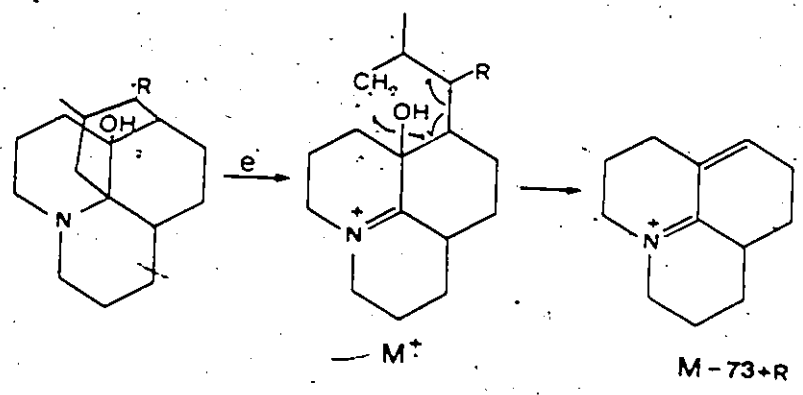


Figure 9 Fragmentation of lycopodane alkaloids with a hydroxyl group at C-12.

1.8.1.2.2 Lycopodanes with hydroxyl at C-12

If a hydroxyl group replaces an H at C-12 a more intense molecular ion is observed and it is usually the base peak. The ion (M-73), corresponding to the loss of the bridge and the hydroxyl group at C-12 is always present. A general fragmentation scheme of this type of alkaloid is shown in Figure 9. If a hydroxyl group is present at C-8 as well as at C-12 as in lycofawcine (Figure 10), the loss of the bridge and the hydroxyl group at C-12 is observed as an intense peak, while the molecular ion is very weak.[76]

1.8.1.2.3 Lycopodanes with a double bond between C-11 and C-12

If a double bond is present between C-11 and C-12 and the bridge is not substituted the molecular ion is the base peak. The ion corresponding to the loss of C_3H_7 (M-43) is also intense. Anhydrolycodoline (Figure 11) is an example of an alkaloid that undergoes this type of fragmentation.[18] If a hydroxyl group is present at C-8 along with a double bond between C-11 and C-12 ions at M-17, M-59, M-71, M-72 and M-73 are observed as in the case of gnidioidine (Figure 11).[18] A fragmentation scheme accounting for this pattern is shown in Figure 12. If a ketone is present at C-8 and a double bond exists between C-11 and C-12 the loss of CO (M-28) and the loss of the bridge is observed. An example of this fragmentation is observed in the case of acrifoline (Figure 13).[28] Lucidioline (Figure 13) has hydroxyl groups at C-6 and C-7 and can undergo a loss of CHOH = CHOH (M-60) to form the base peak at m/z 203.[69] This is analogous to the loss of CHOH = CHOCOR in lycoclavine discussed previously.

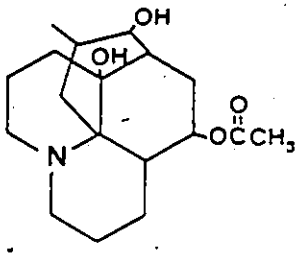


Figure 10 Structure of lycofawcine.

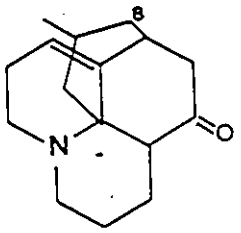


Figure 11 Structure of anhydrolycodoline (8 = H) and gnidioidine (8 = OH).

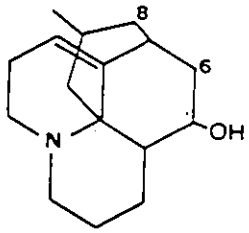


Figure 13 Structure of acrifoline (8 = 0) and lucidioline (6= OH).

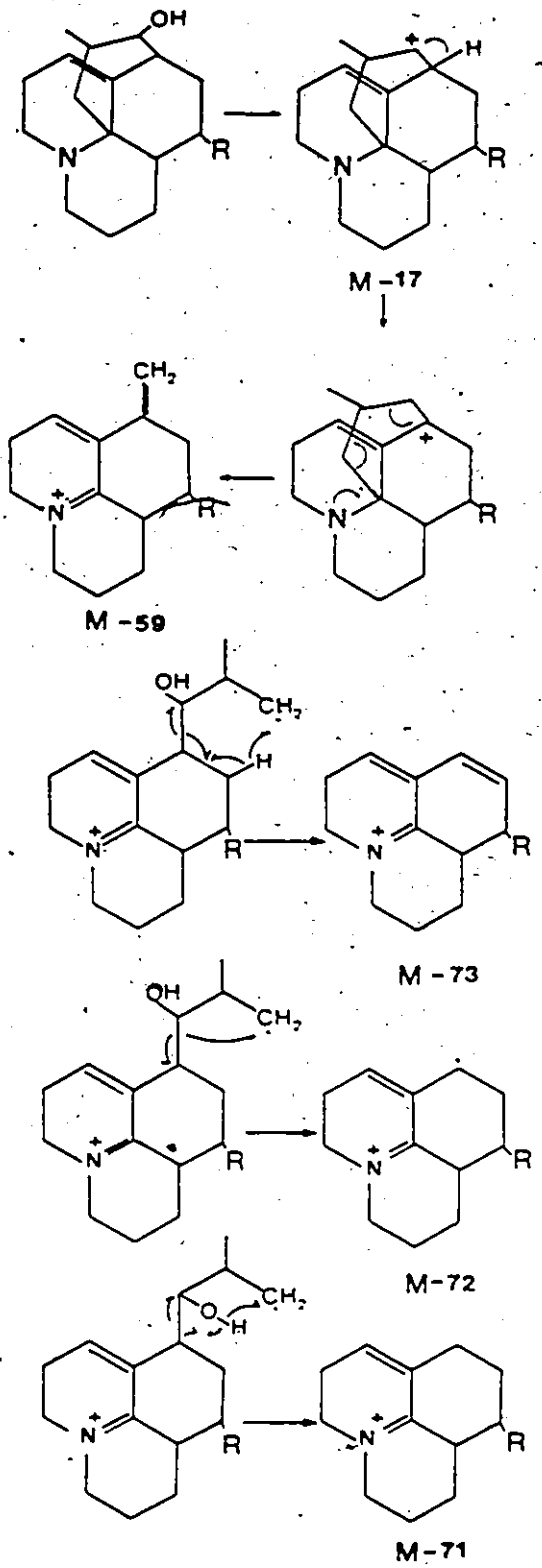


Figure 12 Fragmentation of lycopodane type alkaloids with a hydroxyl group at C-8 and a double bond between C-11 and C-12.

1.8.2 Chemical ionization mass spectrometry

Chemical ionization (CI) is a milder form of ionization than electron ionization; not as much energy is imparted to the molecule and less fragmentation results. A reagent gas such as methane or ammonia at about 1 torr is bombarded with electrons to form reagent ions. The reagent ions can transfer charge in the form of a proton to vaporized sample molecules to form $(M+H)^+$ ions. Reagent ions can also add onto the sample molecule to form other adduct ions. Some of the reactions which can take place in chemical ionization mass spectrometry are listed below,[212] where R represents the reagent gas and M represents the sample.

Reagent ion formation

$$R + e^- \rightarrow R^{+\cdot} + 2e^-$$
$$R^{+\cdot} + R \rightarrow RH^+ + (R-H)^{\cdot}$$
$$R^{+\cdot} + R \rightarrow (R-H)^+ + RH^{\cdot}$$

Protonation

$$RH^+ + M \rightarrow R + (M+H)^+$$
$$(R-H)^+ + M \rightarrow (R-2H) + (M+H)^+$$

Hydride abstraction

$$(R-H)^+ + M \rightarrow R + (M-H)^+$$

Charge exchange

$$R^{+}\cdot + M \rightarrow R + M^{+}\cdot$$

Adduct formation

$$R^{+}\cdot + M \rightarrow RM^{+}\cdot$$

The $(M+H)^{+}$ ion is usually very intense; thus it can be used to determine the molecular weight of a compound. Functional groups such as OH are easily lost and the residual fragment is often observed as an intense ion if methane is used as the reagent gas.

1.8.2.1 Chemical ionization mass spectrometry of alkaloids

At present very little information is available on chemical ionization mass spectrometry of alkaloids. Fales et al.[211] have examined the chemical ionization mass spectra of a group of alkaloids and in all cases an $(M+H)^{+}$ ion was observed. In those cases where a hydroxyl group was present the loss of water from the $(M+H)^{+}$ ion was observed. When more than one hydroxyl group was present only the loss of one group was observed. The same investigators examined colchicine, an alkaloid with an acetamide group. They reported that the amide linkage was stable to chemical ionization with methane. They also observed that alkaloids which contained an $OCOCH_3$ group lost acetic acid under chemical ionization with methane. The chemical ionization mass spectra of some Lycopodium alkaloids has been examined in this research and will be discussed in a later chapter.

CHAPTER 2

Experimental

2.1 Materials

Samples of alkaloids examined in this thesis were provided by 1. Dr. D. B. MacLean, McMaster University, Hamilton, Ontario, Canada. 2. Dr. J. C. Braekman, Université Libre de Bruxelles. 3. Dr. W. A Ayer, University of Alberta, Edmonton, Alberta, Canada. 4. Dr. Y. Inubushi, Kyoto University, Sakyo-Ku, Kyoto, Japan. 5. Dr. R. H. Burnell, Université Laval, Québec, Quebec. The source of each sample is designated in Chapter I, Table 1.

All *Lycopodium* species collected in New Zealand were obtained through the Department of Scientific and Industrial Research, Botany Division, Christchurch, New Zealand. *L. australianum* (Herter) Allan was collected at Travers Range, Nelson and Malte Brun Range and Mt. Cook, South Island, New Zealand. *L. deuterodensum* Herter was collected at Huia, Auckland, North Island, New Zealand. *L. fastigiatum* R. Br. was collected at Mt. Robert, Nelson Lakes National Park, South Island, New Zealand. *L. scariosum* Forst. f. was collected at Black Hill, Lake Rotoiti, South Island, New Zealand. *L. lucidulum* and *L. flabelliforme* were collected near Algonquin Park in the Haliburton region of Ontario, Canada. *L. clavatum* var. *borbonicum* was obtained through Dr. J. C. Braekman; the plant material was collected in Zaire, Africa by Dr. L. Nyembo.

2.2 Extraction of Plant material

Dried plant material was extracted with methanol in a Soxhlet apparatus for 48 hours. The methanolic extract was then filtered and taken to dryness by a stream of nitrogen. The residue was heated on a steam bath with 5% HCl and left to stand for up to 24 hours, then filtered over Celite. The Celite was washed with 5% HCl until the filtrate was negative to Dragendorff's reagent. The filtrate was then basified with concentrated NH_4OH and extracted with $CHCl_3$. Removal of the $CHCl_3$ gave a crude alkaloid extract which was filtered through a pad of alumina to remove strongly polar material. The mobile phase used was EtOAc:MeOH = 9:1. The amount of each plant extracted was as follows: L. australianum 75 g, L. clavatum var. borbonicum 5100 g, L. deuterodensum 2259 g, L. fastigiatum 2077 g, L. flabelliforme ~ 200 g, L. lucidulum 377 g and L. scariosum 2468 g.

2.3 Gas chromatography

2.3.1 Packed column

Both a Varian 3700 and a Pye 104 gas chromatograph were used. Glass columns 6 ft long with an outside diameter of 0.25 in and an inner diameter of 2.0 mm (Chromatographic Specialties) were used. The columns were silylated by the following method:

1) wash column with water
2) wash column with acetone

3) dry column
4) wash column with 1% aqueous KOH
5) wash column with methanol
6) wash column with toluene
7) wash column with 5% dimethyldichlorosilane in toluene and let stand for 15 minutes
8) drain column and rinse with toluene
9) wash immediately with methanol
10) dry column in an oven

Columns were packed with 1% SP-2100 on 100/120 Supelcoport (Supelco) or 3% OV-1 on 100/120 gas chrom Q (Chromatographic Specialties) by connecting the injector end of the column to a pressurized resevoir (40 psi, nitrogen) of packing material while the detector end was connected to a vacuum line. When the column was uniformly packed both ends were plugged by a small amount of silanized glass wool. Columns were conditioned by heating overnight at the maximum temperature recommended for the packing. During conditioning the column was not connected to the detector to prevent contamination.

Nitrogen (prepurified, Canadian Liquid Air) was used as the carrier gas with a flow rate of 30 mL/min. Hydrogen (prepurified, Canadian Liquid Air) and air (dry, Canadian Liquid Air) for the flame ionization detector had flow rates of 30 and 300 mL/min, respectively. The injection port was heated to 250°C. The Varian flame ionization detector (FID) was heated to 300°C; the Pye FID does not have a heater. The temperature program used was 100°C to 280°C at 10°C per minute.

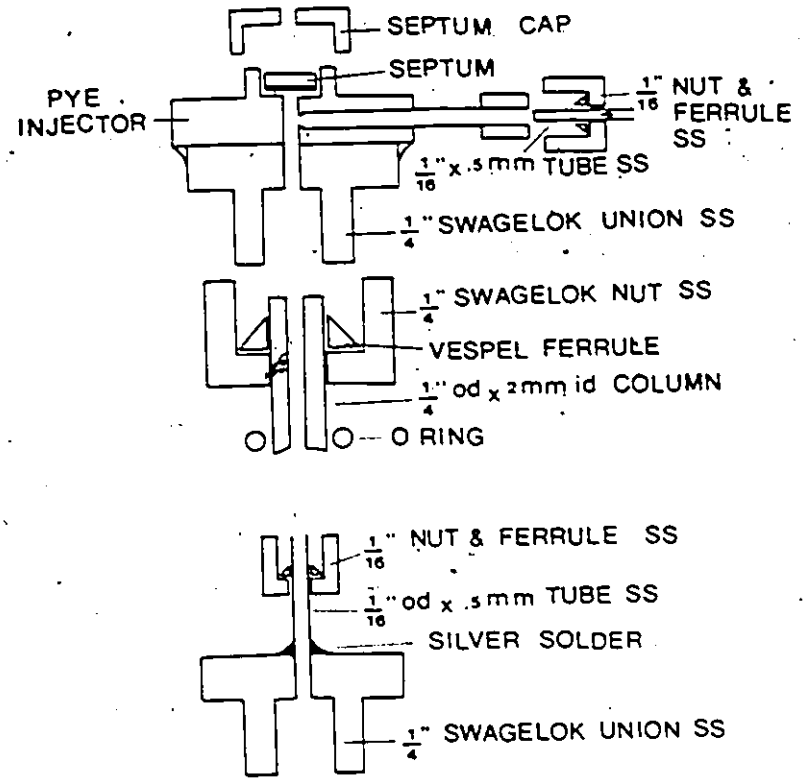


Figure 14 Packed column injector and detector modifications.

Sample concentrations were typically 0.001 g/mL with 1 to 5 microliters of sample injected.

The Pye column connections to the injector and detector were modified so that columns could be connected with 0.25 in stainless steel Swagelok nuts and 0.25 inch Vespel ferrules (Mandel). The thread on the injector port was cut off and replaced with one-half of a 0.25 in stainless steel Swagelok union. Teflon lined septa were used in the injector port (Microsep F-532, Mandel). A short length of 1/16 in stainless steel tubing was silver soldered to one-half of a 0.25 in stainless steel Swagelok union. A 1/16 in nut and ferrule were used to connect the union to the detector inlet port, see Figure 14.

2.3.2 Capillary Column

The Pye 104 gas chromatograph was modified for use with fused silica capillary columns (FSC). An on-column injector (P. D'Agostino personal communication) was built and used for all injections, see Figure 15. A nitrogen makeup gas "T" (SG-2517 Mandel) was connected to the Pye detector port. All columns used were thirty meters long and of the wide bore type. The stationary phase thickness that was selected was the thinnest available in order to reduce column bleed. J&W columns with the following stationary phases; DB-1, DB-5 and DB-17 were tested (Chromatographic Specialties). Injections were made using a J&W 10 uL syringe with a fused silica needle (Chromatographic Specialties). Typical sample concentrations were 0.001 g/mL with one microliter being injected on column. Helium (high purity, Canadian Liquid Air) at 15 psi (Matheson regulator) was used as the carrier gas. The helium was passed

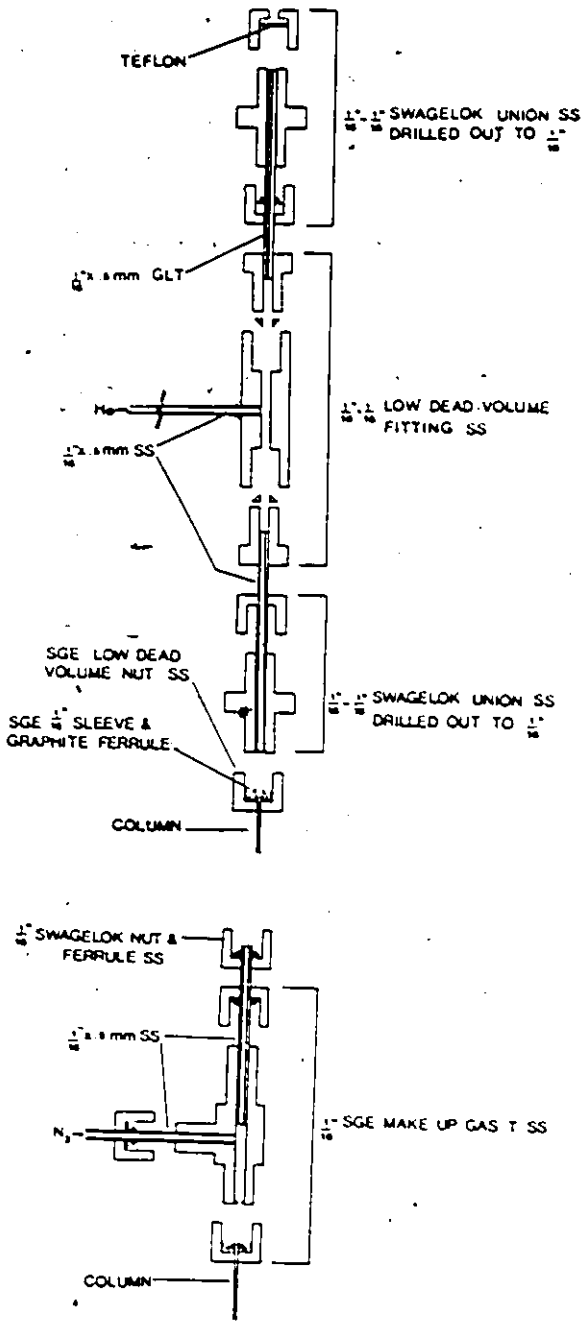


Figure 15 On-column injector.

through a gas purifier containing self-indicating drying agent and molecular sieves (Alltech Associates) and an oxy-trap (Alltech Associates) prior to entering the column. Hydrogen (prepurified) and air (dry) for the flame ionization detector had flow rates of 30 and 300 mL/min, respectively. The nitrogen (prepurified) makeup gas had a flow rate of 30 mL/min. The temperature program used was 50°C to 300°C at 10°C per minute. Chromatograms were recorded on a Kipp and Zonen chart recorder (Johns Scientific).

2.3.3 Gas chromatography with a fused silica column and a nitrogen phosphorus detector

A Varian 3700 gas chromatograph with a nitrogen phosphorus detector (NPD) and flame ionization detector (FID) was used. Helium was used as the carrier gas. The lead current was 5.60 amps with a bias voltage of -4.0 volts. The column eluent was split into two streams. Output from each detector was recorded on the same chart recorder.

2.3.4 Retention indices of Lycopodium alkaloids

The retention indices of authentic alkaloid samples were calculated relative to hydrocarbon standards. Hydrocarbons C-16, C-18, C-20, C-22, C-26, C-28, C-30, C-32, C-34 and C-36 (Alltech) were dissolved in hexane (0.001 g/mL) and co-injected with the alkaloids. The distance between the alkaloid peak and adjacent hydrocarbon peaks was used to calculate the retention index of the alkaloid. For example, a compound eluting half-way between C-18 and C-20 would have a retention index of

1900. When possible, the retention index was measured three times so that a standard deviation could be calculated.

2.4 Mass spectrometry

2.4.1 Probe samples

2.4.1.1 Electron ionization

A Vacuum Generators (VG) Micromass 7070F mass spectrometer was used throughout. For electron ionization an electron energy of 70 ev was used for all samples. The ion source was heated to 200°C. An accelerating voltage of 4 Kv was used. The mass range 40 to 500 was scanned at two seconds per decade. Samples were introduced via a sample probe.

2.4.1.2 Chemical ionization

Chemical ionization mass spectrometry was carried out with methane or ammonia as reagent gases. The source pressure was maintained at 1 torr in the chemical ionization mode. An electron energy of 70 ev and an accelerating voltage of 4 Kv was used.

2.4.2 Gas chromatography-mass spectrometry (GC/MS)

2.4.2.1 Packed column

A Varian 3700 gas chromatograph interfaced to a VG micromass 7070F mass spectrometer was used. Modified Varian 3700 glass columns were employed. About five centimeters had to be cut off the detector end of the column. The column was connected to the injector and detector with 0.25 inch Vespel or graphite ferrules and 0.25 inch stainless steel

Swagelok nuts. The detector end of the column was connected to a 1/16 inch glass lined stainless steel tubing which led to the jet separator interface. The jet separator interface (VG) was heated to 250°C, as was the injector port. Helium was used as the carrier gas at 10 psi. The temperature program was 150°C to 300°C at 10°C per minute. Typical sample concentrations were 0.001 g/mL with 1 to 5 microliters being injected. The jet separator divert valve was left shut until the solvent had eluted. Mass spectrometer operating conditions were the same as with probe samples.

2.4.2.2 Gas chromatography-mass spectrometry with fused silica columns (FSC/MS)

A Varian 3700 gas chromatograph interfaced to a VG 7070F mass spectrometer was used. An on-column injector previously described as well as a J&W on-column injector were used. A J&W 10 µL syringe with a fused silica needle was used for all injections. Sample concentrations were 0.001 g/mL with 1 microliter being injected on column. Helium was used as the carrier gas. The helium was passed through a gas purifier and oxy-trap (Alltech Associates) before entering the column. The detector end of the column was inserted through a heated (250°C) glass lined stainless steel tube to within a few millimeters of the ion beam. The temperature program used was 50°C to 300°C at 10°C per minute. The ion source was kept in the tripped position until the solvent eluted. The mass range was scanned at one second per decade.

2.4.2.3 Chemical ionization GC/MS with fused silica columns

Chemical ionization GC/MS with fused silica columns was done by operating the mass spectrometer in the chemical ionization mode. All other conditions were as described for GC/MS with FSC.

2.4.3 Data acquisition

Data from the mass spectrometer were collected and manipulated by a Digital PDP 8 computer. Data were stored on Digital RL02 disk packs. Vacuum Generators (VG) data system 2000 software was used throughout.

2.5 Blanks

Blanks were prepared for each liquid chromatography and extraction technique used. All blanks were examined by FSC/FID and then by FSC/MS if components were to be identified.

2.5.1 Extraction blank

Methanol (500 mL) was refluxed in a Soxhlet extractor for 48 hours. The methanol was then evaporated and the flask extracted with 500 mL of 10% HCl (aq). The acid was neutralized with concentrated ammonia (aq) and extracted with chloroform. The chloroform was reduced in volume to 1 mL. The extract was examined by FSC/FID but no peaks were observed in the chromatogram.

2.5.2 Preparative layer chromatography blanks

2.5.2.1 Silica

A Silica 60 (without fluorescence indicator) preparative layer plate 2 mm thick and 20 cm square (Merck) was developed with chloroform:methanol = 9:1. A band 3 cm wide was cut from the plate and extracted with methanol (200 mL). The methanol was filtered to remove particles of silica and reduced in volume to 1 mL. The extract was examined by FSC/FID but no peaks were observed in the chromatogram.

2.5.2.2 Alumina

An Alumina F254 (type T) preparative layer plate 1.5 mm thick and 20 cm square (Merck) was developed half-way with ethyl acetate:methanol = 9:1, allowed to dry and then developed with ethyl acetate. A band 3 cm wide was cut from the plate and extracted with methanol (200 mL). The methanol was filtered to remove particles of alumina and reduced in volume to 1 mL. The extract was examined by FSC/FID but no peaks were observed in the chromatogram.

2.5.3 Column chromatography blanks

2.5.3.1 Silica flash chromatography

A glass column 0.5 m × 2 cm was packed with silica [Kieselgel 60 (230-400 mesh) (Merck)] and eluted with 500 ml of chloroform:methanol = 9:1. The solvent was reduced in volume to 1 mL and the extract examined by FSC/FID but no peaks were observed in the chromatogram.

2.5.3.2 Dry column chromatography blank

A glass column (0.5 m × 2 cm) packed with alumina (Woelm dry column grade neutral alumina, activity III) was eluted with 500 mL of ethyl acetate. The ethyl acetate was reduced in volume to 1 mL and examined by FSC/FID. Many small peaks were observed but the extract was not examined by GC/MS.

2.5.3.3 Alumina column blank

A glass column (0.5 m × 2 cm) was packed with neutral alumina (activity I) and eluted with 100 mL each of benzene, benzene:ether = 1:1, ether, ethyl acetate and methanol. Examination of the extract by FSC/FID revealed several peaks. The total ion chromatogram (TIC) obtained by FSC/MS is shown in Figure 16. Compound A was identified as 2,6-di-tert-butyl-4-methylphenol from its mass spectrum shown in Figure 17. This compound is added to solvents such as diethyl ether to act as a stabilizer.[213] Antioxidants of this type react with radicals formed in a solvent to prevent the production of peroxides. The ion at m/z 205 is due to the loss of a methyl group from the additive while the peak at 207 is due to column bleed. The ion at m/z 177 will be explained below.

Compound B was identified as 2,5-di-tert-pentylhydroquinone (DAH) from its mass spectrum shown in Figure 18. The ion at m/z 221 is due to the loss of an ethyl group from the molecular ion; the oxidation product of (DAH) is the quinone (DAQ). The base peak of DAQ is m/z 177; thus DAQ may coelute with compound A. The structures of A and B are shown in Figure 19. The mass spectra of stabilizers has been reviewed.[213]

2.6 NMR Spectroscopy

Nuclear magnetic resonance was done using a Bruker WM-250 250 MHz spectrometer (this Department) and a 400 MHz (South-Western Ontario Regional NMR Facility, University of Guelph) instrument. Carbon and proton spectra were obtained on the 250 MHz instrument. Carbon/proton and proton/proton COSY spectra were obtained on the 400 MHz instrument. All samples were dissolved in $CDCl_3$ and run in 5 or 10 millimeter tubes.

2.7 Isolation of unknown C from L. australianum

Polar residues were removed from the crude extract using dry column chromatography. A column 50 cm × 2 cm was packed with alumina for dry column chromatography and developed with ethyl acetate. The column was then sectioned into four sections and the alumina extracted with methanol. Only the fourth fraction (bottom of the column) contained alkaloids.

This purified extract was then chromatographed on an alumina column 20 cm × 1 cm. The column was eluted with 100 mL each of benzene, benzene:ether = 1:1, ether, ethyl acetate and finally methanol. Five fractions were collected and examined by FSC/MS. Fractions 1 and 2 contained only dioctyl phthalate. Fraction 3 contained unknown C, while fraction 4 contained only cernuine. Fraction 5 contained many unidentified polar residues. Fraction 3 was taken to dryness yielding 0.8 mg of unknown C.

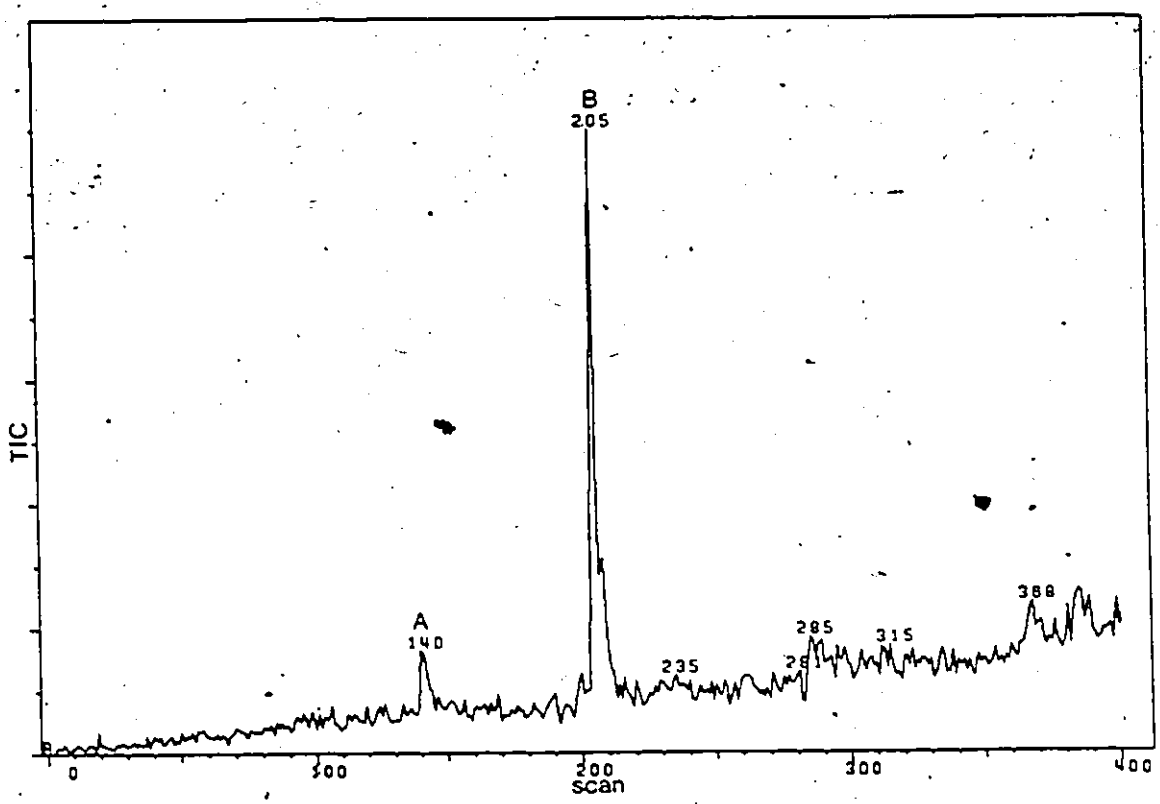
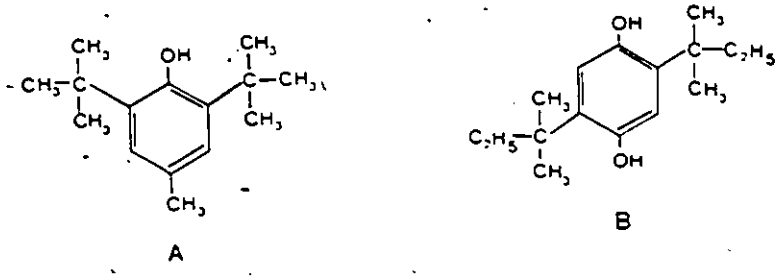


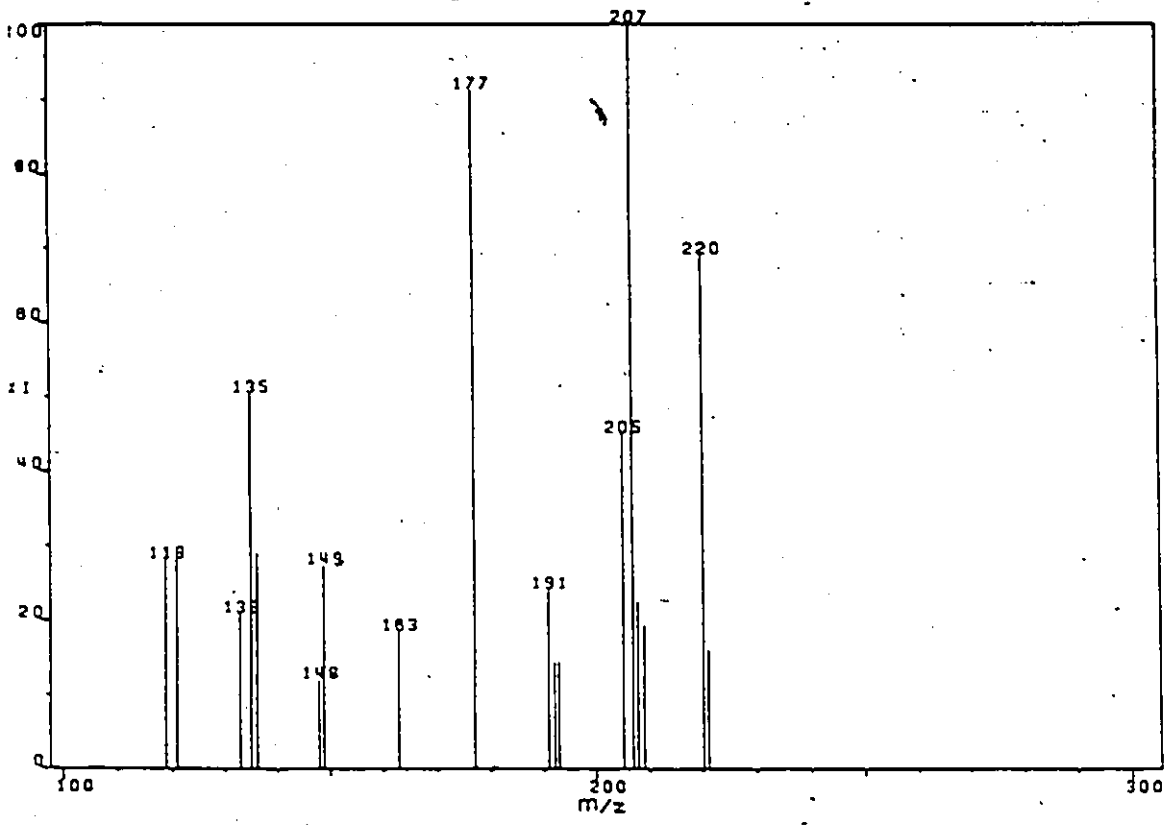
Figure 16 FSC/MS TIC for alumina column blank.



A B

Figure 19 Structure of components A and B.

Figure 17 Mass spectrum of component A.



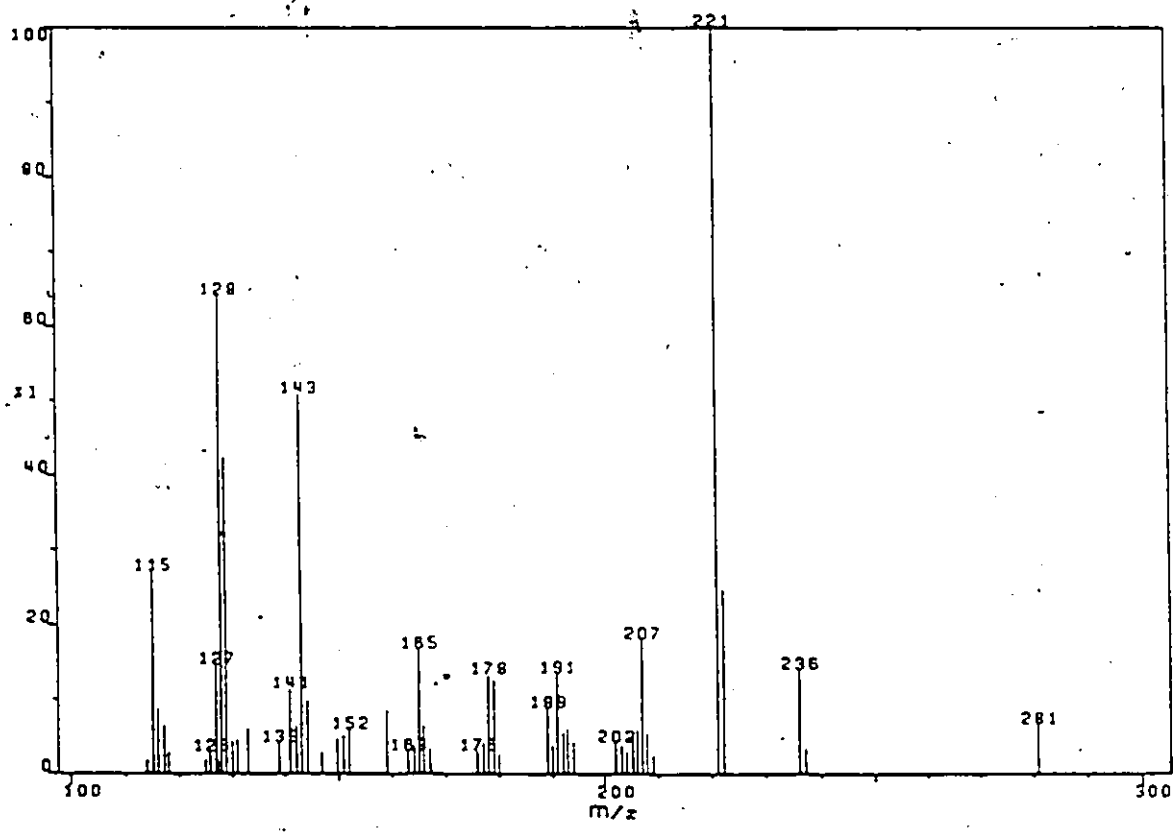


Figure 18 Mass spectrum of component B.

2.8 Isolation of the alkaloids of L. clavatum var. borbonicum

A portion of the crude extract (2.2 g) was loaded onto a column (2 m × 4 cm) filled with dry basic alumina (activity III). The column was developed with ethyl acetate and then sliced into 10 sections each 20 centimeters long. Each section was then extracted with methanol. Removal of the methanol provided a total of 1.47 g of material.

The fractions were then examined by GC/MS using a packed column (3% OV-1). The results are listed in Table 6.

2.9 Isolation of alkaloids from L. deuterodensum

The crude extract was chromatographed on an alumina column (50 cm × 2 cm). The column was developed using 250 mL each of benzene, benzene:ether = 1:1, ether, ethyl acetate and methanol. Twenty-five fractions (50 mL each) were collected and examined by FSC/MS. Lycopodine, clavolonine, lycodine, lycodoline, flabelline and lycoflexine were identified, however none of the unknowns detected by GC/MS were obtained. The contents of each fraction are tabulated in Table 7.

2.10 Isolation of the alkaloids of L. fastigiatum

The crude extract was chromatographed on an alumina column (50 cm × 2 cm). The column was developed with 250 mL each of benzene, benzene:ether = 1:1, ether, ethyl acetate and methanol. Fractions (125 mL) were collected and examined by FSC/MS. The results are tabulated in Table 8. Fraction 10 contained unknowns L and M along with

Table 6

Alkaloids from L. clavatum var. borbonicum

Alkaloid	Fraction										
	1	2	3	4	5	6	7	8	9	10	
Compound I	✓										
N_α-acetyl-N_β-methylphlegmarine	✓										
lycodine		✓	✓								
dihydrolycopodine	✓	✓	✓	✓							
lycodoline	✓	✓	✓	✓							
lycoflexine	✓	✓	✓	✓	✓	✓	✓	✓			
lycopodine				✓	✓	✓	✓	✓	✓	✓	
anhydrolycodoline							✓	✓	✓	✓	
acetyldihydrolycopodine	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
weight/grams	.08	.08	.15	.17	.13	.05	.06	.04	.09	.62	1.47

Table 7

Alkaloids from L. deuterodensum

compound	fraction																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
dioctyl phthalate				✓	✓	✓	✓																		
lycopodine				✓	✓	✓	✓	✓	✓	✓	✓														
flabelliformine						✓	✓	✓	✓	✓	✓														
lycodoline				✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓										
clavolonine									✓	✓	✓	✓	✓	✓	✓	✓									
methylester of ferulic acid											✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
flabelline																						✓			
lycoflexine																								✓	

Table 8

Alkaloids from L. fastigiatum

Alkaloids	Fraction										
	1	2	3	4	5	6	7	8	9	10	+
anhydrolycodoline	✓	✓									
lycopodine		✓	✓	✓	✓	✓	✓				
acetyldihydro-lycopodine						✓	✓	✓	✓		
lycoflexine								✓	✓		
unknown M									✓	✓	
unknown L									✓	✓	
lycodoline										✓	
dihydrolycopodine										✓	

lycoflexine and dihydrolycopodine. Fraction 10 was rechromatographed on a silica column (50 cm × 2 cm) which was developed with chloroform:-diethyl amine (98:2) and the fractions collected were examined by FSC/MS. Fractions 1 and 2 (first eluted) contained unknowns L and M while fractions 3 and 4 contained dihydrolycopodine and small amounts of the unknowns. All fractions after and including fraction 5 contained only dihydrolycopodine.

In order to separate unknowns L and M fraction 1 was applied to a preparative (1.5 mm) alumina plate (20 cm × 20 cm). The plate was developed half-way with ethyl acetate:methanol = 9:1, allowed to dry and then developed with ethyl acetate: Two bands were observed under ultraviolet light. The bands were cut off and extracted with methanol and filtered to remove alumina particles. The band with R_f 0.67 was unknown M while the band with R_f 0.30 was unknown L.

2.10.1 Conversion of unknown L into unknown M

Formaldehyde 37% (1 mL) was added to a small amount of unknown L. (10 mg) dissolved in methanol. Sodium borohydride was added to the solution until the reaction was complete. Water was added to the reaction mixture and then the mixture was extracted with chloroform. Mass spectrometry showed that unknown M is the N-methyl derivative of unknown L. Compound L could not be obtained in a crystaline form. Compound M recrystallized from ether gave a melting point of 143-6°C, other physical properties that were determined are: $[\alpha]_D^{23}$ + 289.9 (c 1.362, $CHCl_3$), λ_{max}^{MeOH} 224 (nm), log ε = 3.78, $\nu_{max}^{CHCl_3}$ 1620 cm^{-1}.

2.11 Quantitation

The percentage of each alkaloid in the extract was determined by internal normalization. Peaks generated by chromatography using capillary columns are generally very narrow. As such, peak heights are a good measure of peak area, and can be used to determine the relative ratio of components. The percentage of each alkaloid was calculated by using peak heights from FSC/FID experiments. However some plants of the Lycopodium species elaborate eighty to ninety percent of one alkaloid, typically lycopodine. Using peak heights to calculate relative ratios of components might in such cases underestimate the percentage of the main components, as their peaks would be considerably wider. Peak areas were also used to calculate relative ratios of components. Peak areas were determined by using a Spectra Physics Autolab Minigrator, the operating parameters of the integrator are listed in Table 9.

The internal standard method was also used for quantitation. Annotinine was chosen as an internal standard. Annotinine has a structure similar to other Lycopodium alkaloids, but its retention index is distinctly different from most alkaloids of interest in this study. Stock solutions of 1.0 mg/mL of a lycodane alkaloid (lycodine), two lycopodane alkaloids (lycopodine, dihydrolycopodine), a cernuane alkaloid (lycocernuine) and annotine were prepared. The stock solutions were diluted by the factors of 0.5, 0.25, 0.1 and 0.01. One mL of each dilution was mixed with 1 mL of internal standard solution. All concentrations are tabulated in Table 10. Each dilution was examined three times by FSC/FID. The ratio of alkaloid to internal standard was determined by an integrator. Response curves were calculated by fitting

Table 9

Spectra-physics Autolab minigrator operating parameters.

value	parameter
1 PW	peak width in seconds
50 SS	peak detection threshold
250 T1	time after injection in seconds to start integration (eliminates solvent peak)
600 T2	time after which PW and SS parameters are doubled to account for wider peaks
5 BL	baseline test parameter
20 TP	tailing peak parameter
1 SP	spike rejection if equals 1
1 PL	plateau rejection if equals 1

NB: The numbers represent settings of the various functions (denoted by the two letter code) that were used to operate the integrator.

the data to a straight line using linear regression analysis. Analysis of variance showed that the equations calculated had no significant lack of fit. Raw data are listed in Table 11. The response curve equations are listed in Table 12. Analysis of variance (ANOVA) tables are shown in Table 13.

2.12 Library of EI mass spectra of the Lycopodium alkaloids

A library of mass spectra of the alkaloids was prepared by running authentic samples when available and storing the spectra directly in a data base. The library was supplemented by entering literature spectra *via* a keyboard into the data base. The actual spectra are listed in the Appendix, where they are arranged firstly according to structural type and secondly, by molecular weight. The references for literature spectra used are listed in Table 1 of the Introduction. The sources of authentic samples are also listed in Table 1. In all cases when an authentic sample existed as well as a literature spectrum they were compared to verify that the sample was of good quality.

A library search program (VG datasystem 2000) was used to search the data base to find the best match to an unknown spectrum. The software uses three different equations to determine a measure of "fit". The closer the value is to 1000 the better the unknown matches the library spectrum.

The purity fit equation uses the intensity of the masses in both the unknown and the library.

Table 10

Stock solutions used for internal standard quantitation

Alkaloid	Concentration in miligrams/milliliter					
	Stock	1.0	0.5	0.25	0.1	.01
lycodine	0.9050	0.4525	0.2262	0.1131	0.0452	0.0045
lycopodine	0.9100	0.4550	0.2275	0.1138	0.0455	0.0046
dihydrolycopodine	1.7200	0.8600	0.4300	0.2150	0.0860	0.0086
annotine	1.4500	0.7250	0.3625	0.1812	0.1450	0.0145
lycocernuine	2.6900	1.3450	0.6725	0.3362	0.1345	0.0134
lucidulum 1	1.7100	0.8550	-	-	-	-
lucidulum 2	12.1800	6.0900	-	-	-	-
annotinine	1.4160	0.7080				

Table 11

Raw data for calibration curves.

lycodine		dihydrolycopodine		lycopodine		lycocernuine		annotine	
x	y	x	y	x	y	x	y	x	y
1.565	1.565	0.8232	0.8164	1.556	1.591	0.5264	0.6341	0.9766	1.191
1.565	1.552	0.8232	0.8221	1.556	1.533	0.5264	0.5264	0.9766	0.9950
1.565	1.564	0.8232	0.7161	1.556	1.608	0.5264	0.5766	0.9766	0.9461
3.129	3.096	1.646	1.642	3.112	2.970	1.053	1.123	1.953	1.822
3.129	3.052	1.646	1.459	3.112	3.170	1.053	1.124	1.953	2.261
3.129	2.809	1.646	1.442	3.112	3.270	1.053	1.174	1.953	1.765
6.260	6.292	3.293	3.273	6.224	6.203	2.106	2.434		
6.260	6.316	3.293	2.818	6.224	5.896	2.106	2.316		
6.260	5.960	3.293	2.708	6.224	6.224	2.106	2.410		
15.65	15.19	8.233	7.863	15.56	15.12	5.264	6.848		
15.65	16.05	8.233	7.481	15.56	15.17	5.264	5.334		
15.65	15.82	8.233	7.795	15.56	16.53	5.264	5.526		
156.5	146.3	82.33	88.37	155.6	152.5	52.64	59.42		
156.5	152.9	82.33	90.91	155.6	151.0	52.64	60.20		
156.5	149.0	82.33	91.78	155.6	159.3	52.64	54.58		

Table 12

Calibration curves for internal standard quantitation

lycodine	$y = 0.9533 \times + 0.2544$	$R^2 = 0.9995$
dihydrolycopodine	$y = 1.1040 \times - 0.6090$	$R^2 = 0.9994$
lycocernuine	$y = 1.1025 \times + 0.0374$	$R^2 = 0.9973$
lycopodine	$y = 0.9912 \times + 0.0503$	$R^2 = 0.9852$
annotine	$y = 0.9272 \times + 0.1385$	$R^2 = 0.8717$

Table 13

Analysis of variance

Anova lycodine

Source	df	SS	MS	f ratio
Source				
Regression	1	61844.4	61844.4	
Residual	14	23.63	1.688	
Lack of fit	4	1.0864	0.2716	.1205 (not significant)
Pure error	10	22.547	2.2544	
Total	15	61868.1		

Anova lycopodine

Source	df	SS	MS	f ratio
regression	1	72143.7	72143.7	
Residual	14	40.619	2.9014	
Lack of fit	4	0.072	0.018	0.004 (not significant)
Pure error	10	40.547	4.055	
Total	15	72184.3		

Anova dihydrolycopodine

Source	df	SS	MS	f ratio
Regression	1	24701.1	24701.1	
Residual	14	9.367	0.6691	
Lack of fit	4	2.794	0.6985	1.063 (not significant)
Pure error	10	6.573	0.6573	
Total	15			

Anova lycocernuine

Source	df	SS	MS	f ratio
Regression	1	10241.9	10241.9	
Residual	14	19.936	1.4240	
Lack of fit	4	0.0255	0.0064	0.003 (not significant)
Pure error	10	19.910	1.9910	
Total	15			

Anova annotine

Source	df	SS	MS	f ratio
Regression	1	14.67	14.67	
Residual	5	0.1809	0.0362	
Lack of fit	1	1.97×10^{-5}	1.97×10^{-5}	0.0004 (not significant)
Pure error	4	0.1809	0.0452	
Total	6			

$$\text{purity fit} = \frac{1000\ (\Sigma Ium \times ILm)^2}{\Sigma\ Iu^2 \times \Sigma\ IL^2}$$

The mixture fit uses only the intensity of the masses in the unknown which also occur in the library.

$$\text{mixture fit} = \frac{1000\ (\Sigma Ium \times ILm)^2}{\Sigma\ Ium^2 \times \Sigma\ IL^2}$$

The reverse fit uses only the intensity of the masses in the library which occur in the unknown.

$$\text{Reverse fit} = \frac{1000\ (\Sigma Ium \times ILm)^2}{\Sigma Iu^2 \times \Sigma ILm^2}$$

Iu = Intensity of a peak in the unknown

IL = Intensity of a peak in the library

Ium = Intensity of a peak in the unknown which matches a peak in the library

ILm = Intensity of a peak in the library which matches a peak in the unknown

In order to speed up the search process the library mass spectra or master files are encoded into search files. Search files can be created such that the 6, 8, 10, 12, 15, 16 or 20 largest peaks are stored. The intensity of the stored peak is normalized to 1000. Search files can also be created such that the largest peak every 7 a.m.u. in

the range 20 to 509, the largest peak every 14 a.m.u in the range 20 to 999 or the two largest peaks every 14 a.m.u. in the range 20 to 509 is stored. Since this would result in a large number of stored peaks the intensities are stored as logs to reduce storage space.

At run time it is possible to omit masses from the search. It is useful to ignore peaks that arise from column bleed or background, such as 207 and 281. It is also useful to ignore the ions arising from the major components since they may mask the presence of compounds which elute close to the major components. For example the ions from lycopodine may mask the presence of dihydrolycopodine.

Search files in this study were created using the six largest peaks.

CHAPTER 3

Results and Discussion

3.1 Introduction

The main objective of this research was to develop a fast method of screening extracts for alkaloid content. GC/MS was selected as the method to be investigated. Both retention indices and mass spectra can be used to identify an alkaloid if its retention index or mass spectrum has been previously recorded. How closely two mass spectra match each other can be determined by a computer program. Thus with a list of retention indices and a data base of mass spectra, GC/MS data can be used to identify previously known alkaloids and recognize new compounds. GC/MS analysis can be carried out on sub milligram quantities; therefore only a few grams of plant material need be extracted. Thus many different plants could be quickly extracted and analysed for alkaloid content. This would be useful in examining the chemotaxonomy of the genus Lycopodium. Quantitation of any new alkaloids found would indicate how much plant material (Kg's) should be extracted to isolate sufficient alkaloid for structural analysis. Any new alkaloids found may help to illuminate the pathways of biosynthesis of the Lycopodium alkaloids.

3.2 Mass spectrometry

3.2.1 Electron ionization mass spectrometry of the alkaloids

The electron ionization mass spectra of 43 Lycopodium alkaloids have been measured. These mass spectra along with 32 from the literature are presented in the Appendix in order of structure and molecular weight. The data presented in the Appendix were entered into a data base for computer searching of GC/MS data.

3.2.2 Chemical ionization mass spectrometry of the alkaloids

The chemical ionization mass spectra of 34 alkaloids have been measured using methane as the reagent gas. Authentic alkaloid samples were introduced into the mass spectrometer via a probe. All of the alkaloids examined gave adduct ions at $(M + 1H)^+$ and in most cases at $(M + 29)^+$. The spectra are recorded in Table 14 according to structural type and increasing molecular weight. In all cases where the alkaloid contained a hydroxyl group the loss of water from the $(M + H)^+$ ion was observed. The loss of water from the $(M + H)^+$ ion can be explained by protonation of the hydroxyl oxygen, followed by the elimination of H_2O. Similar behavior is observed when $OCOCH_3$ groups are present, acetic acid being lost from the $(M + H)^+$ ion. In the case of flabelline (Figure 8), which contains a secondary acetamide group, the ion at $(M + H)^+ - 59$ can be attributed to the loss of NH_2COCH_3. Serratinidine (Figure 20) also contains an acetamide group, and behaves similarly. The results obtained in this study with respect to the loss of protonated functional groups parallel the observations of Fales et al. Lycopodium alkaloids lacking the groups discussed above gave strong ions at $(M + H)^+$ and occasionally

less intense ions corresponding to the loss of the bridge atoms. It was noted that alkaloids with a lycodane skeleton undergo a fragmentation under chemical ionization that is not observed under electron ionization conditions as shown in Figure 21. The ion "a" of m/z 150 may arise from the cleavage of the lycodane skeleton in the manner illustrated. If R_1 is a methyl group the ion a shifts to m/z 164.

The α-amino ketones serratinine and 8-deoxyserratinine (Figure 22) exhibit the loss of 29 mass units from the $(M + H)^+$ ion, this may be due to the loss of HCO. The loss of CO is observed for these alkaloids in the electron ionization mode. The chemical ionization fragmentation may be diagnostic for the identification of a serratinane skeleton.

Chemical ionization has proven useful in confirming the molecular weight of the alkaloids investigated and in determining the molecular weight of compounds such as unknown C from L. australianum section 3.4.4, which did not exhibit a molecular ion in the electron impact ionization mode.

3.3 Retention indices of the alkaloids

Retention indices are commonly used in the identification of compounds. In this study the retention indices of the alkaloids were determined relative to hydrocarbon standards on three different fused silica columns. The results obtained using a new and old DB-1 30 m column and a DB-5 30 m column are recorded in Table 15. A DB-17 30 m

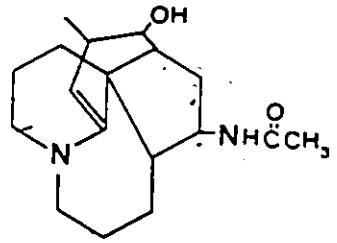


Figure 20 Structure of serratinidine.

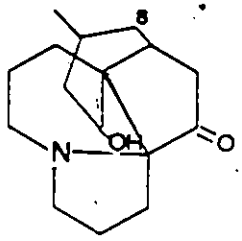


Figure 22 Structure of 8-deoxyserratinine (8 = H), serratinine (8 = OH).

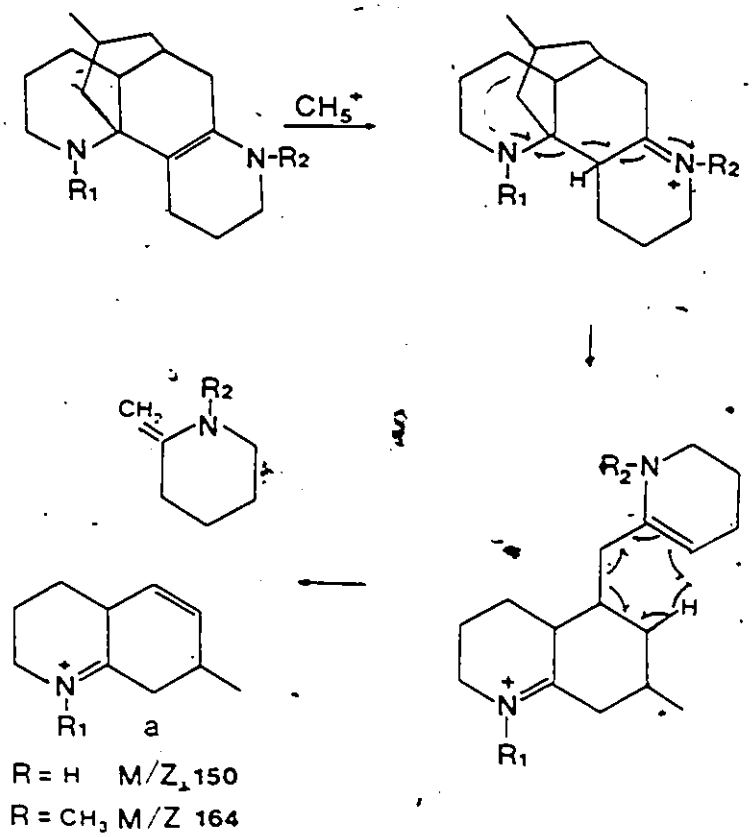


Figure 21 . Fragmentation of a lycodane skeleton under chemical ionization with methane.

Table 14

Chemical Ionization Mass Spectra of the Lycopodium alkaloids

	Name	(M+29)/ int	(M+2)/ int	(M+1)/ int	(M+)/ int	(M-1)/ int	(M-15)/ int	(M-17)/ int	(M-Bridge) int	additional ions
1	lycocernuine	307/15	-	279/100	278/40	277/40	263/25	265/25	-	
2	lycodine	271/15	244/20	243/100	242/25	241/25	227/6	-	185/15	150/25
3	N-methyl-Lycodine	286/10	258/20	257/100	256/35	255/25	-	-	199/30	164/10
4	des-N-methyl-α-obscurine	289/15	262/20	261/100	260/15	259/10	-	-	203/30	150/5
5	β-obscurine	301/15	274/20	273/100	272/10	271/5	-	-	215/10	
6	α-obscurine	303/15	276/20	275/100	274/30	273/10	-	-	217/25	164/5
7	flabellidine	317/20	290/20	289/100	288/30	287/26	273/15	-	231/40	150/15
8	lycopodine	276/15	249/20	248/100	247/30	246/25	232/5	-	190/20	
9	dihydro-lycopodine	278/5	-	250/75	249/75	248/80	234/20	232/80	192/100	
10	serratidine	290/10	263/15	262/100	261/25	260/20	-	244/60	-	
11	clavolonine	292/20	265/15	264/80	263/15	262/30	-	246/100	190/70	
12	flabelli-formine	292/10	265/20	264/100	263/40	262/30	248/15	246/25	206/20	
13	L20	292/15	265/20	264/100	263/30	262/25	248/25	246/60	206/20	
14	lucidioline	292/10	265/20	264/100	263/70	262/60	248/15	246/95	203/40	
15	lycodoline	292/10	265/20	264/100	263/50	262/25	248/5	246/25	206/15	
16	deacetyl-fawcettine	-	-	266/20	265/15	264/30	-	248/100	192/80	
17	flabelline	317/10	290/20	289/100	288/25	-	-	-	231/45	(M-$NHCOCH_3$) 230/15
18	acetyldihydro lycopodine	-	-	292/30	291/20	290/15	-	-	234/25	(M-$OCOCH_3$) 232/100
19	α-Lofoline	336/3	-	308/20	307/10	306/25	-	290/40	234/55	(M-$OCOCH_3$) 248/100, (M-64) 238/35, (M-133) 174/35
20	β-Lofoline	-	-	308/25	307/5	306/10	-	290/20	234/100	(M-$OCOCH_3$) 248/80, (M-133) 174/35
21	lycodonine	336/2	-	308/30	307/35	306/30	292/10	-	250/35	(M-$OCOCH_3$) 248/100
22	lycognidine	-	-	458/20	457/25	-	-	440/10	400/10	(M-209) 248/100

Table 14 continued.

	Name	(M+29)/ int	(M+2)/ int	(M+1)/ int	(M+)/ int	(M-1)/ int	(M-15)/ int	(M-17)/ int	(M-Bridge) int	additional losses
23	annotine	304/10	277/15	276/100	275/10	-	-	258/50	-	
24	annotinine	304/10	277/15	276/100	-	-	-	-	-	(M-42) 233/20
25	annopodine	320/25	293/25	292/100	291/50	290/60	-	274/70	-	(M-43) 248/25
26	serratinidine	333/20	306/20	305/100	304/40	303/20	-	287/60	-	(M-$NHCOCH_3$) 246/20
27	8-deoxyserratinine	292/20	265/20	264/100	263/5	262/25	-	246/15	-	(M-28) 235/35, (M-111) 152/30
28	serratinine	308/10	281/15	280/100	279/5	278/25	-	262/35	-	(M-28) 251/30, (M-45) 234/15, (M-127) 152/30
29	seratanidine	-	-	296/100	-	-	-	278/70	-	
30	alopecuridine	308/5	281/15	280/80	279/20	278/15	-	262/100	-	
31	magellanine	305/15	277/15	276/100	275/50	274/45	-	258/20	-	
32	paniculatine	306/20	279/25	278/100	277/40	276/50	-	260/80	-	(M-44) 288/40, (M-61) 270/60
33	megastachine	-	333/40	332/100	331/30	-	-	-		
34	lycopecurine	276/10	249/15	248/100	247/90	246/60	232/40	230/95	-	

Table 15

Retention indices of the alkaloids

	alkaloid	DB-1 new	DB-1 old	DB-5
1	lycodine	1920-2	1930-1	1968-2
2	N-methyllycodine	1975-5	1934-4	2031-5
3	dehydrolycopecurine	1985-2	2000-	2052-2
4	lycopodine	2015-7	2030-	2065-5
5	flabelliformine	2070-3	2034-1	2130-4
6	dihydrolycopodine	2000-	2035-2	2070-2
7	lycopecurine	2000-	2035-2	2070-5
8	acrifoline	2044-1	2082-	2104-2
9	acetyldihydrolycopodine	2085-2	2098-2	2130-3
10	serratidine	2085-4	2130-20	2146-4
11	lucidioline	2130-5	2157-15	2188-5
12	lycodoline	2133-2	2159-	2200
13	8-deoxyserratinine	2145-8	2165-3	2221-7
14	L20	2154-2	2174-1	2224-1
15	lycoclavine	2236-2	2242-	2305-4
16	magellanine	2283-5	2268-3	2316-4
17	paniculatine	2249-5	2272-	2325-2
18	clavolonine	2237-5	2299-	2330-3
19	flabellidine	2278-2	2304-	2349-1
20	annotinine	2299-7	2328-	2411-4
21	α-lofoline	2307-2	2329-2	2360-1
22	des-acetylfawcettiine	2250-10	2351-4	2305-4
23	annopodine	2338-8	2357-2	2413-2
24	des-N-methyl-α-obscurine	2273-5	2367-16	2360-2
25	serratinine	2348-15	2369-12	2431-7
26	annotine	2349-4	2382-	2452-4
27	megastachine	-	2384-	-
28	flabelline	2314-5	2422-13	2398-2
29	α-obscurine	2343-2	2422-6	2420-2
30	lycocernuine	2456-5	2473-	2547-6
31	carolinianine	2472-9	2500-2	2572-7
32	serratinidine	2561-1	2639-11	2652-3
33	lycognidine	3467-9	-	3552-7

NB - retention index is followed by the standard deviation.

column was also investigated but proved to be too polar for separation of the alkaloids. Authentic samples of 32 alkaloids were run in triplicate when sufficient sample was available and the standard deviation calculated. As the DB-1 column aged (2 years) retention indices typically increased by 1%; perhaps this was due to more active sites being exposed. Since the DB-5 column is more polar than the DB-1 column the alkaloids were retained longer on the former. These results established that the DB-1 column was preferred for GC/MS analysis since the alkaloids elute at a lower temperature resulting in a quicker analysis and less column bleed.

3.4 Examination of plant extracts

Seven species of Lycopodium were examined in this thesis. L. flabelliforme and L. lucidulum have been studied extensively and have been found to be rich in alkaloids. L. flabelliforme elaborates mainly alkaloids of the lycopodane type with minor amounts of the lycodane type.[8,33,66] L. lucidulum is the only known species which elaborates alkaloids of the lucidulane type[14,24] and is rich in alkaloids of the lucidane type.[139,141] These species were examined to test experimental procedures. L. clavatum var. borboncium has been previously examined and has been found to elaborate N_{α}-acetyl N_{β}-methyl phlegmarine.[18] An unsuccessful attempt was made to isolate this alkaloid in order to investigate its stereochemistry. L. deuterodensum has also been previously examined;[34,147] however reexamination using modern techniques was felt to be worthwhile. The other three species, L. australianum,

L. fastigiatum and L. scariosum have not been previously studied. L. australianum has been placed in the selago section of the subgenus Urostachys.[197] Since only six members of this section have been investigated it was chemotaxonomically interesting to determine its alkaloid content. L. fastigiatum belongs to the fastigiatum group of the subgenus Lycododium.[197] The production of magellanane type alkaloids is unique to this group.[26,131,132,133] The presence of magellanane alkaloids in L. fastigiatum would lend credence to its taxonomic assignment. L. scariosum is a member of the scariosum group of the subgenus Lycopodium.[197] Prior to this report none of the members of this group had been examined for alkaloid content.

3.4.1 Examination of L. flabelliforme

An extract of L. flabelliforme was prepared to test experimental procedures. L. flabelliforme has been examined in various laboratories, and the results of previous work are presented in Table 16. The extract was examined by GC and GC/MS as shown in Figures 23 to 26.

Figure 23 shows the chromatogram obtained from GC analysis of the extract and co-injected hydrocarbon standards using FSC/FID. Several of the trace components detected by GC/MS were not observed in Figure 23 and as such no retention indices could be calculated. The extract was also examined by FSC/FID/NPD, as shown in Figure 24 to determine which components were nitrogen containing.

The total ion current chromatograms (TIC) from GC/MS experiments using packed and fused silica columns (FSC), shown in Figures 25 and 26 respectively, are plotted versus scan number. The retention indices and

computer library search fit values are tabulated in Table 17. The percent of each component in the extract has been calculated from peak heights and areas from FSC/FID; the results are also tabulated in Table 17.

Peak A was identified as anhydrodihydrolycopodine from its mass spectrum shown in Figure 27 and peak B as lycodine by its retention index and mass spectrum shown in Figure 28. The major alkaloid of L. flabelliforme was identified as lycopodine whose mass spectrum is shown in Figure 29. Eluting very close to lycopodine is dihydrolycopodine. Its fragmentation pattern is analogous to that of lycopodine with major ions being shifted two atomic mass units. Figure 30 shows the mass spectrum of a mixture of lycopodine and dihydrolycopodine taken from the trailing edge of the peak. When the mass spectrum of lycopodine is subtracted from that of the mixture, the mass spectrum of dihydrolycopodine can be clearly seen, as shown in Figure 31.

Component E has a molecular weight of 263. Care must be taken to identify this alkaloid since eleven of the known Lycopodium alkaloids have a molecular weight of 263. The mass spectrum of component E is shown in Figure 32. Since the base peak is at m/z 190 (M-73) this implies that a hydroxyl group is present on the (C_4H_9) bridge as in the case of clavolonine and lycofoline. The double bond at C-12 of lycofoline would prevent the easy loss of the bridge (see section 1.8.1.2.3) and a much more intense molecular ion would be expected than that found in the mass spectrum of clavolonine. On the basis of its mass spectrum component E was identified as clavolonine. Authentic lycofoline was not available so that a comparison of retention indices to clavolonine could not be made.

Component F has a molecular weight of 288 and an intense ion at m/z 231, one of the components of peak G has the same ions. These ions are characteristic of the isomeric alkaloids flabellidine and flabelline.

The mass chromatogram of the GC/MS scans which contain both ions 288 and 231 is shown in Figure 33. The two peaks obtained correspond to peaks F and G in the TIC shown in Figure 25. Thus component F whose mass spectrum is shown in Figure 34 was identified as flabellidine on the basis of its retention index. Peak G can be assigned to a mixture of flabelline and a compound H with a molecular weight of 260. The mass spectrum of the mixture of components G and H is shown in Figure 35. The mass spectrum of flabelline was subtracted from Figure 35 to yield Figure 36 from which it was possible to identify component H as des-N-methyl-α-obscurine from the significant peaks at m/z 260, 217, 203 and 175. When the mass spectrum of des-N-methyl-α-obscurine is subtracted from the mass spectrum shown in Figure 35 the mass spectrum of flabelline becomes more apparent as shown in Figure 37 with significant ions at m/z 288 and 231.

Component I was identified as α-obscurine from its mass spectrum shown in Figure 38 and component J was identified as dioctyl phthalate from its mass spectrum.

All the alkaloids discussed above gave $(M + H)^+$ ions when the extract was examined by chemical ionization mass spectroscopy. The results obtained with ammonia as a reagent gas are shown in Figure 39. The ion at m/z 232 is identified as the $(M + H)^+$ ion of anhydrodihydroly-

copodine, however it may also arise from the loss of water from the (M+H) ion of dihydrolycopodine. The ion at m/z 190 comes from the loss of the bridge carbons from lycopodine.

The alkaloids that were previously reported as being found in L. flabelliforme that were not detected in this study may be present in quantities below the detection limit of the detectors used, or may not be present in the particular sample that was extracted. The season in which a sample is obtained along with the site of collection has been found to influence alkaloid content.[18]

3.4.2 Examination of L. lucidulum

The alkaloids listed in Table 16 have been reported to be present in L. lucidulum which is the only source of lucidulane alkaloids. It was of interest to determine if the high molecular weight lucidane alkaloids present in this species could be detected and identified by GC/MS.

The analysis of the extract by FSC/FID and FSC/FID/NPD gave the chromatograms shown in Figures 40 and 41. Figures 42 and 43 show the TIC of a FSC/MS experiment with the capillary interface at 250°C and 350°C, respectively. Lucidane alkaloids are only observed when the interface is at the higher temperature. The retention indices and the computer search fit values for the components of L. lucidulum are tabulated in Table 19. The percentage of each component in the total alkaloid was calculated from peak heights and areas from FSC/FID and is also tabulated in Table 19.

Component A was identified as dihydroluciduline from its mass spectrum shown in Figure 44; dihydroluciduline was first characterized as

Table 16

Alkaloids that have been reported in L. flabelliforme

Alkaloid	Reference
dihydrolycopodine	8
acetyldihydrolycopodine	8
anhydrodihydrolycopodine	8
flabellidine	8
α-obscurine	8
β-obscurine	8
lycopodine	8
nicotine	8
flabelliformine	66
flabelline	73
clavolonine	33
lycodine	33
annotinine	33
des-N-methyl-α-obscurine	33
hydroxy-des-N-methyl-α-obscurine	33

Table 17

Retention indices, computer search fit values and percent total alkaloid for the components of L. flabelliforme

	Compound	R.I.	A.R.I.	Pure	Mix	Reverse	# of scans	Pk.h.	Int.
A	anhydrodihydro-lycopodine	1764	-	747	778	926	6	0.6	-
B	lycodine	1920	1930	780	848	910	12	2.9	1.0
C	lycopodine	2000	2030	854	819	942	9		
D	dihydrolycopo-dine	2000	2000	824	840	954	16	90.8	94.8
E	clavolonine	-	2300	613	676	710	23	1.1	-
F	flabellidine	2311	2304	584	668	837	17	2.9	
G	flabelline	-	2422	580	762	730	6		4.2
H	de-N-methyl-α-obscurine	-	2367	615	794	772	1	1.7	
I	α-obscurine	-	2422	511	756	648	1		
								100.0	100.0

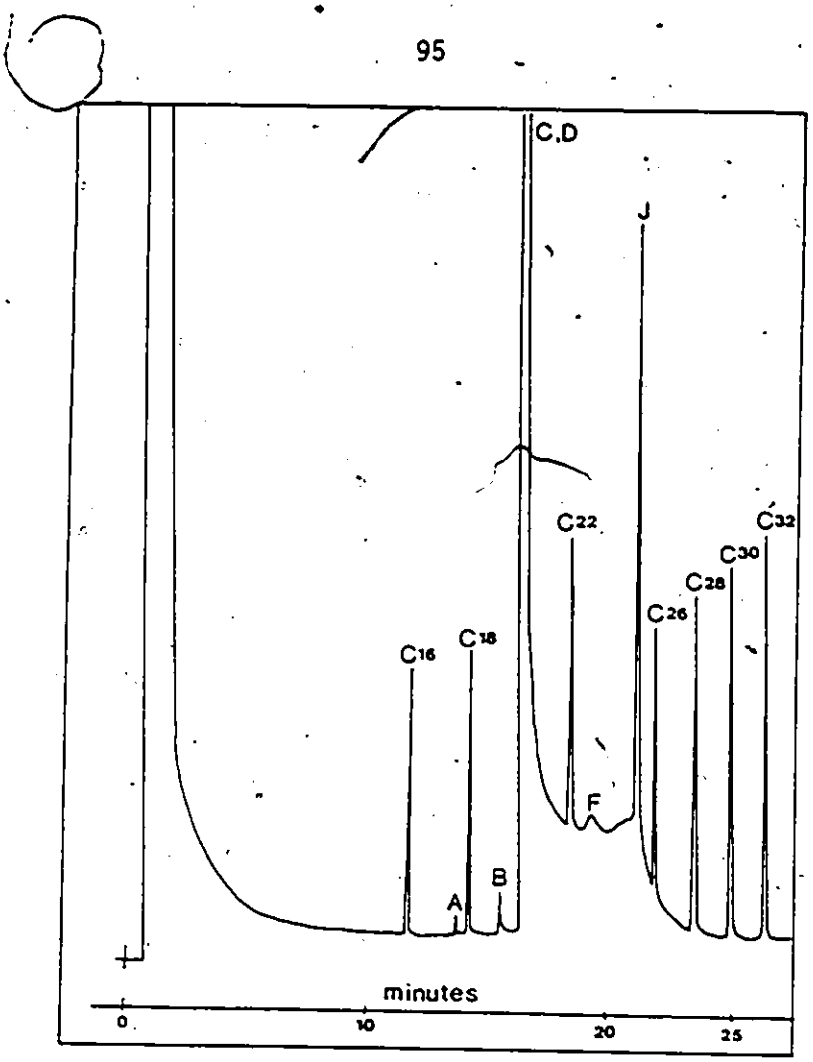


Figure 23 FSC/FID chromatogram of L. flabelliforme extract with hydrocarbon standards.

A anhydrodihydrolycopodine
B lycodine
C lycopodine
D dihydrolycopodine
E clavolonine
F flabellidine
G flabelline
H des N-methyl-α-obscurine
I α-obscurine
dioctyl phthalate

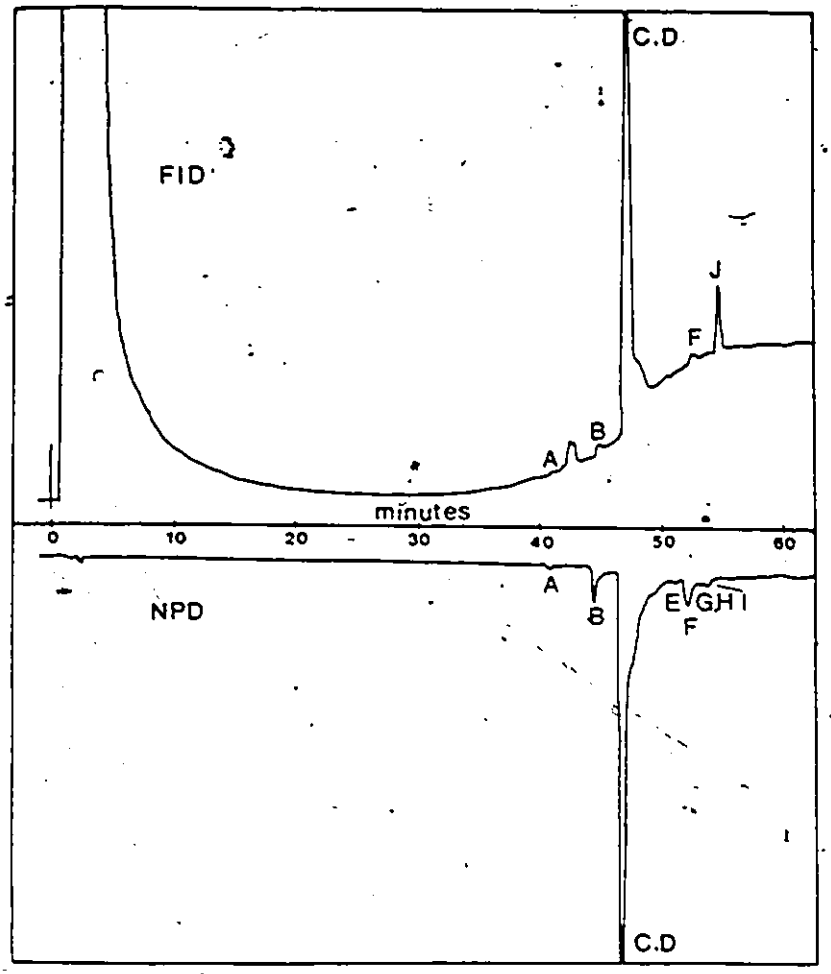


Figure 24 FSC/FID/NPD chromatogram of L. flabelliforme extract.

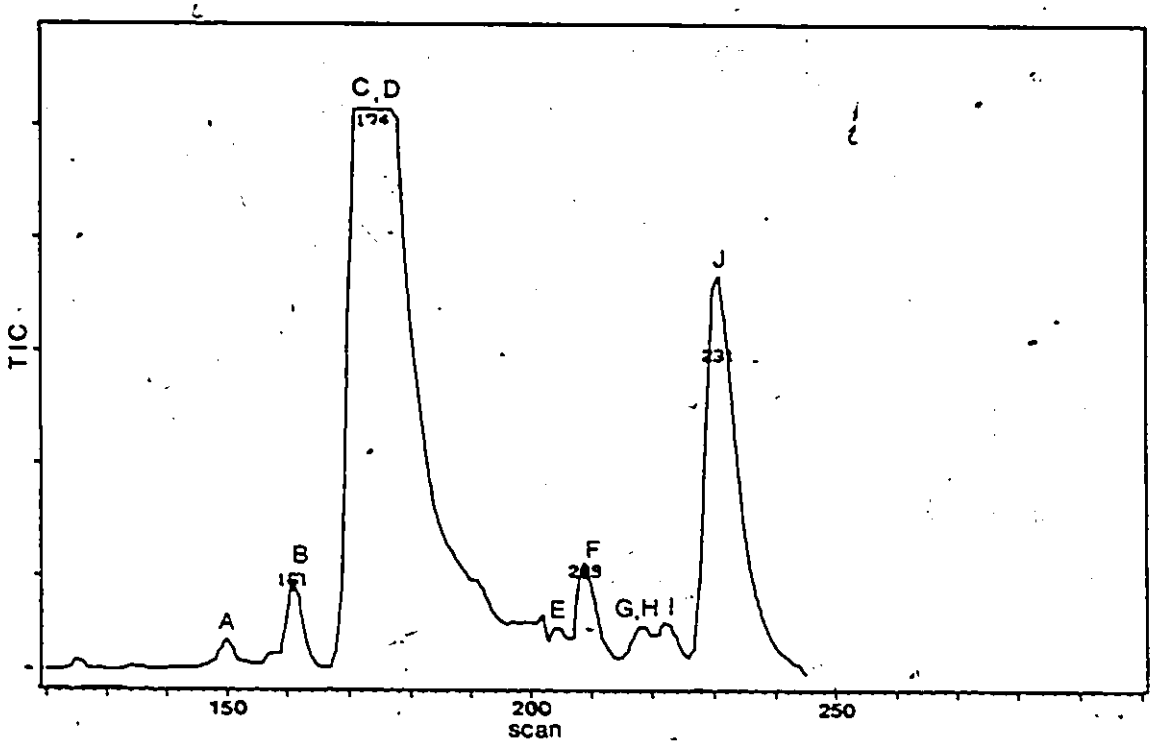


Figure 25 . GC/MS TIC of L. flabelliforme extract.

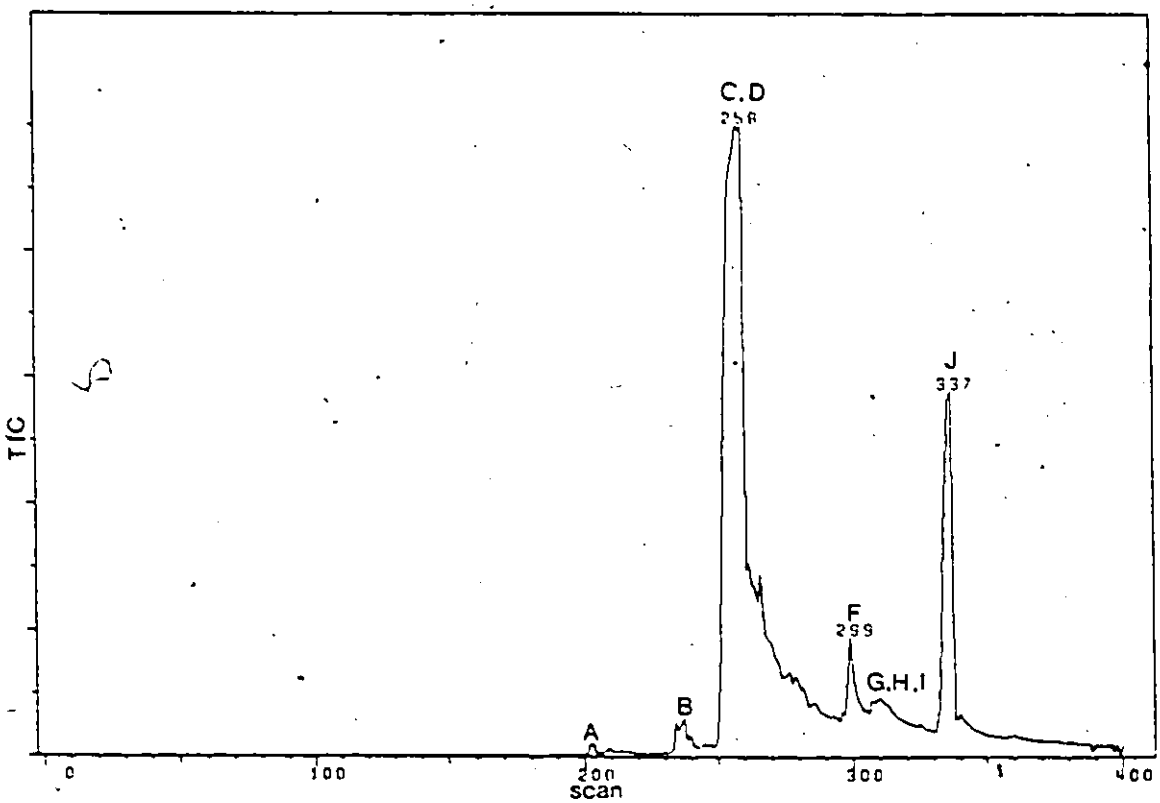
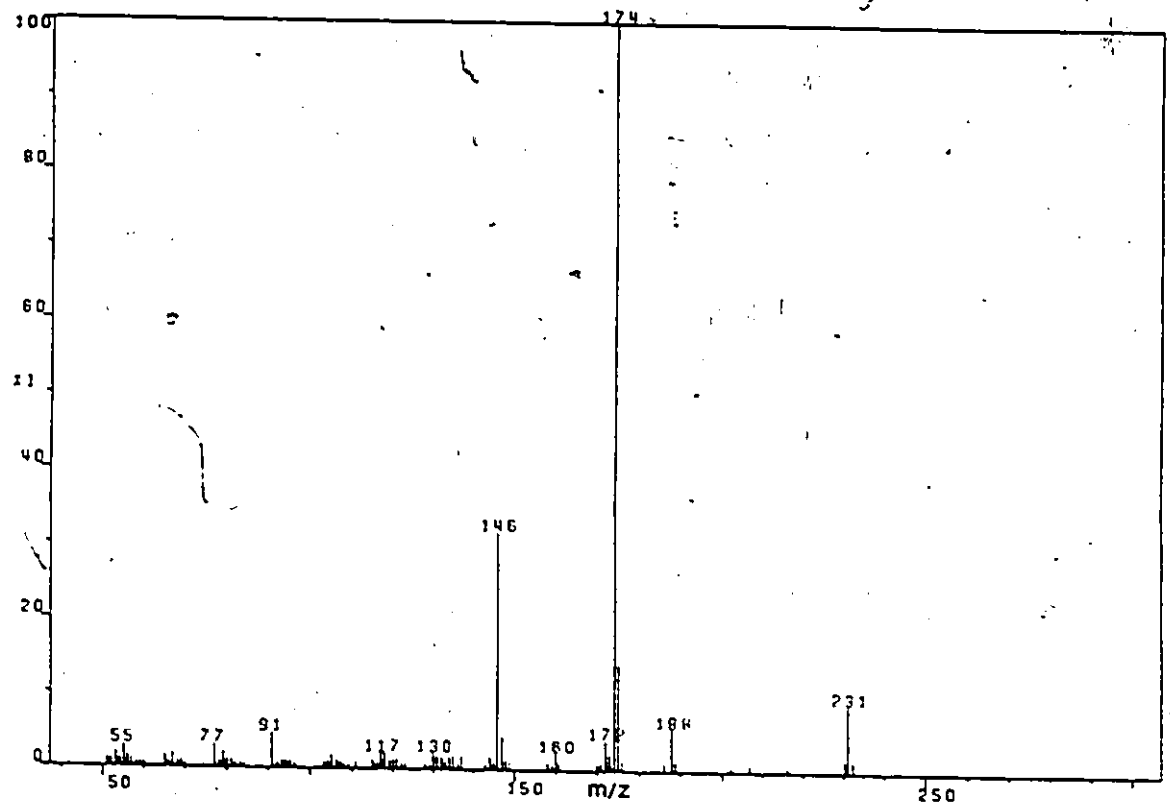


Figure 26 FSC/MS TIC of L. flabelliforme extract.

Figure 27 Mass spectrum of component A (anhydrodihyrolycopodine).



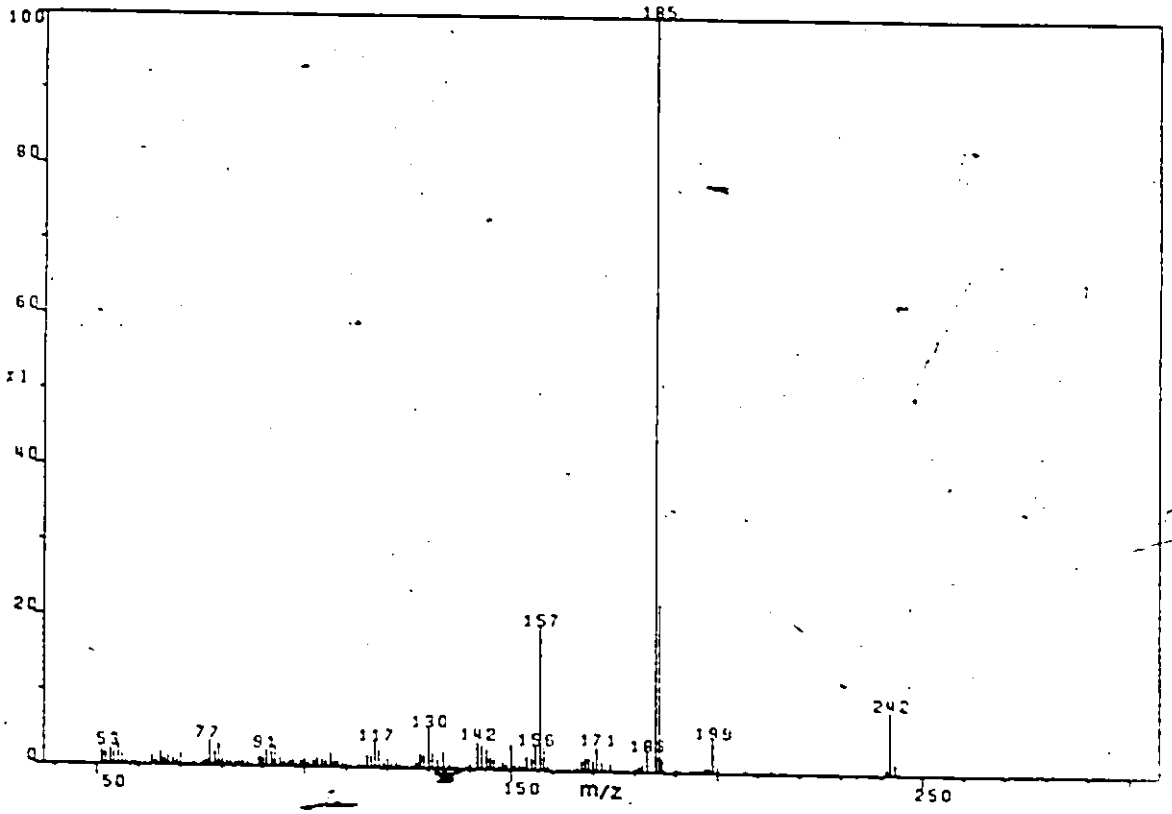
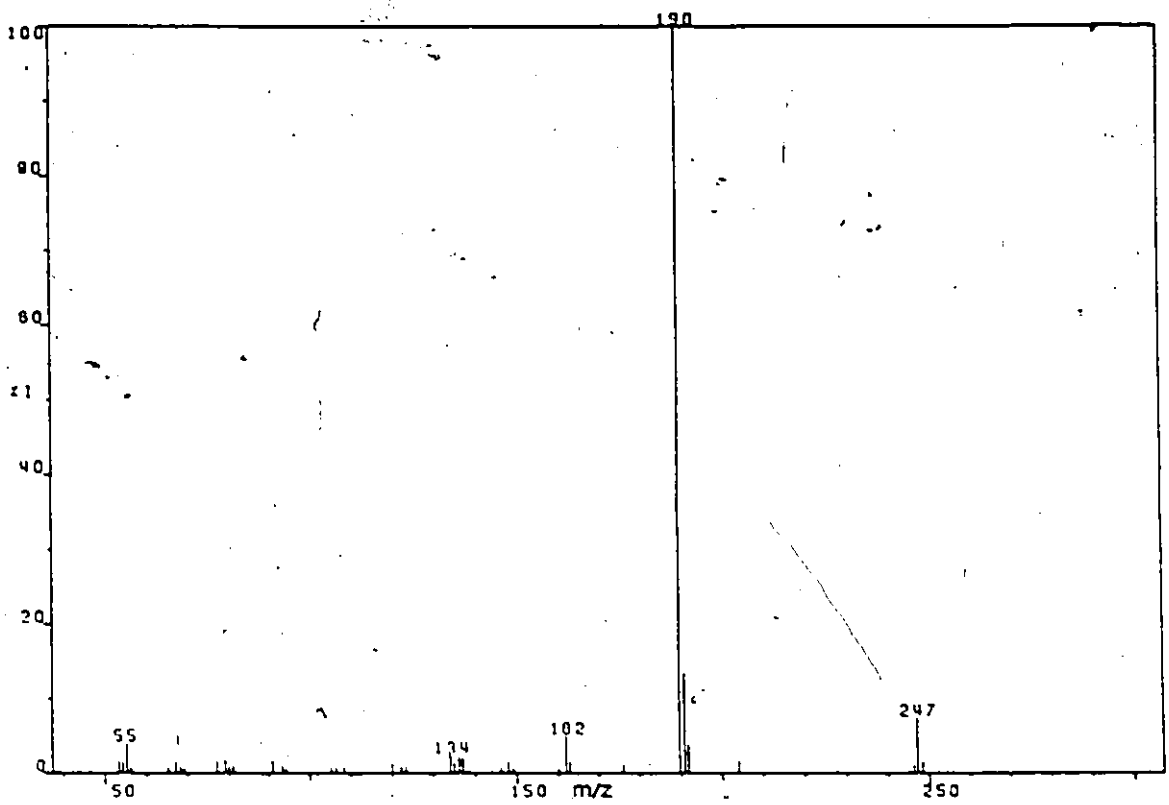


Figure 28 Mass spectrum of component B (lycodine).

Figure 29 Mass spectrum of component C (lycopodine).



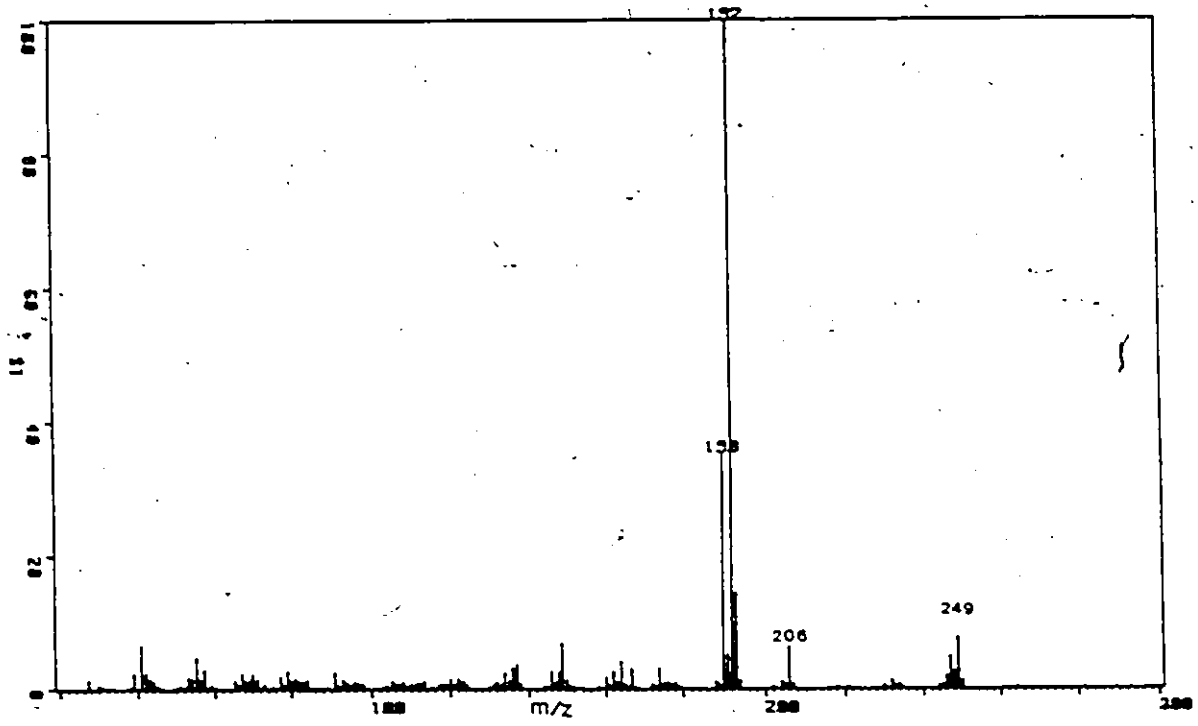
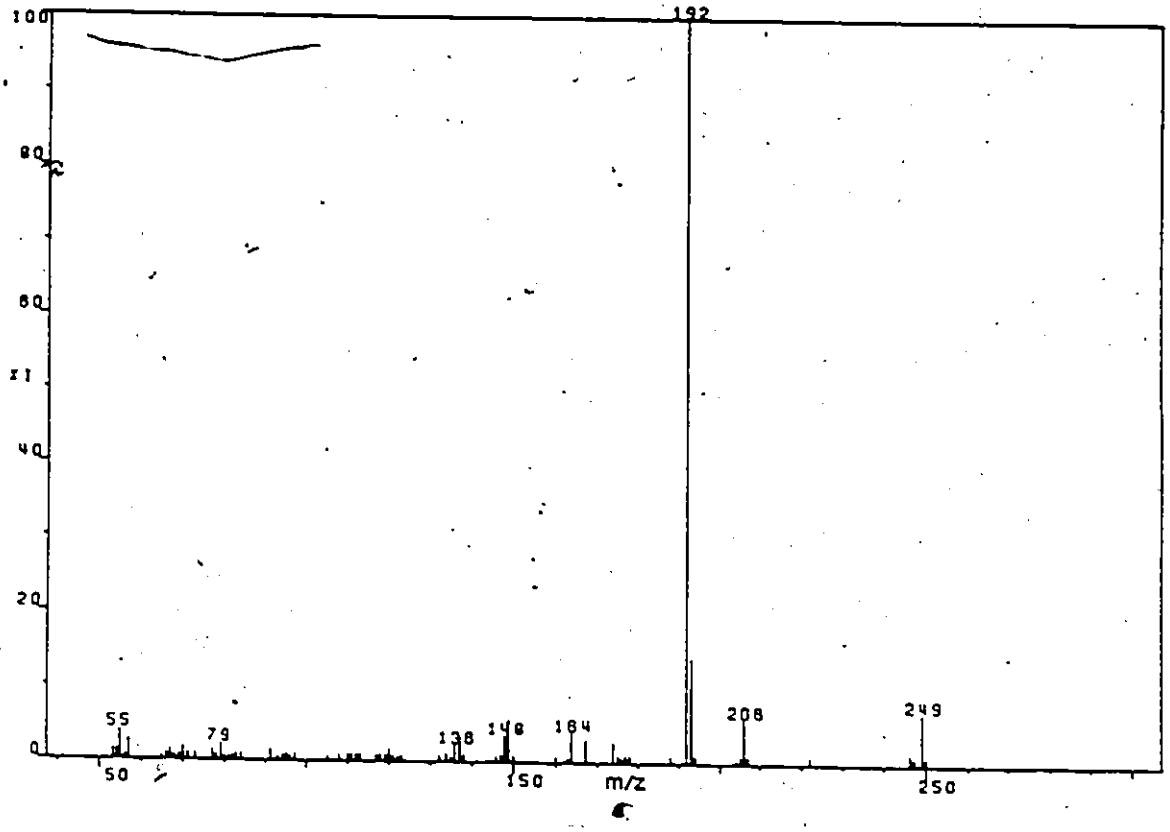


Figure 30 Mass spectrum of components C and D (lycopodine and dihydrolycopodine).

Figure 31 Mass spectrum of dihydrolycopodine (derived from Figure 30).



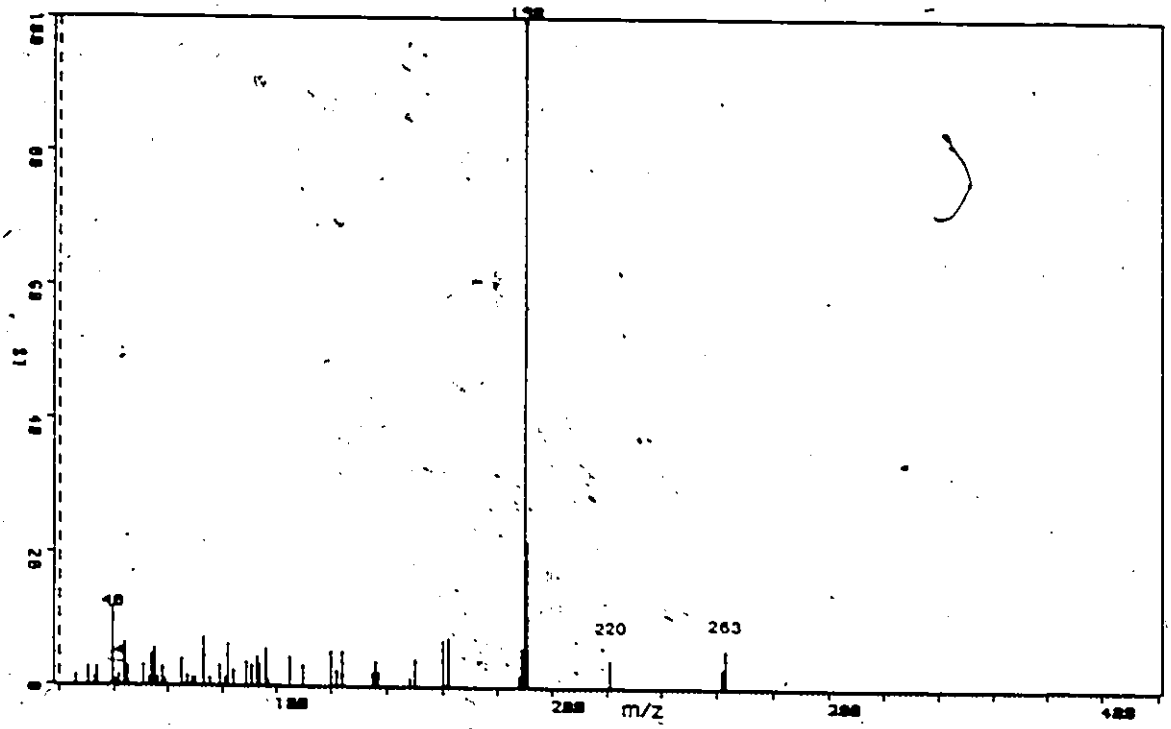
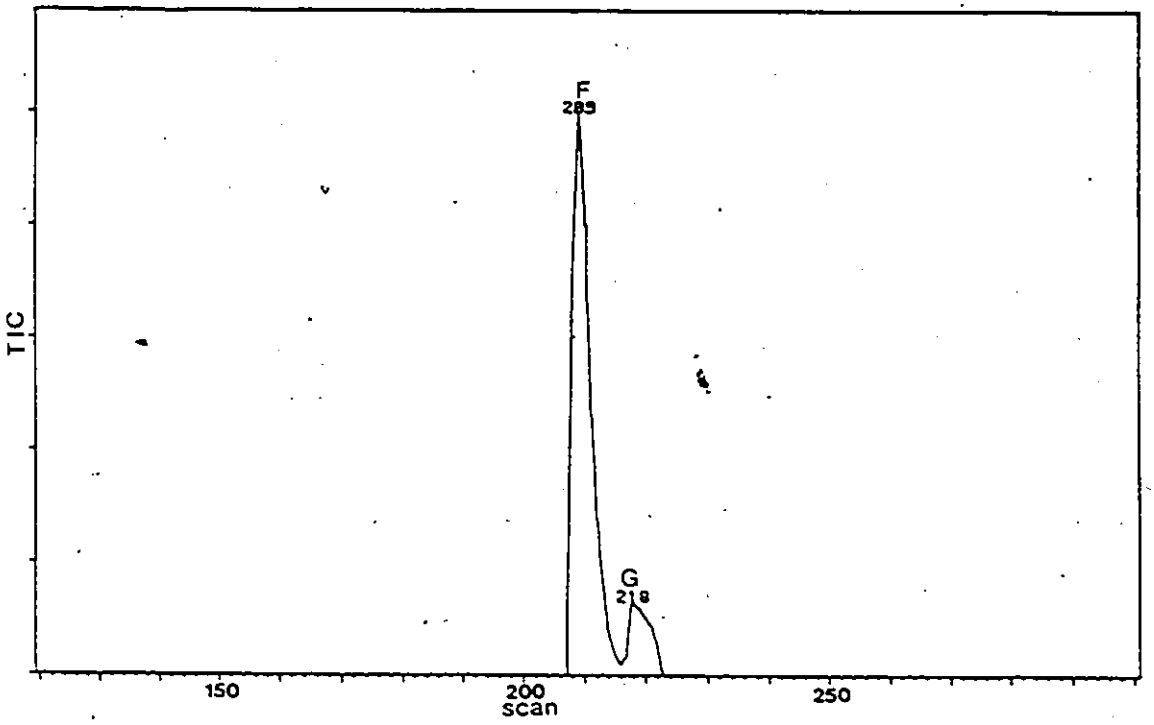


Figure 32 Mass spectrum of component E (clavolonine).

Figure 33 Mass chromatogram of the GC/MS scans which contain both ions m/z 288 and m/z 231.



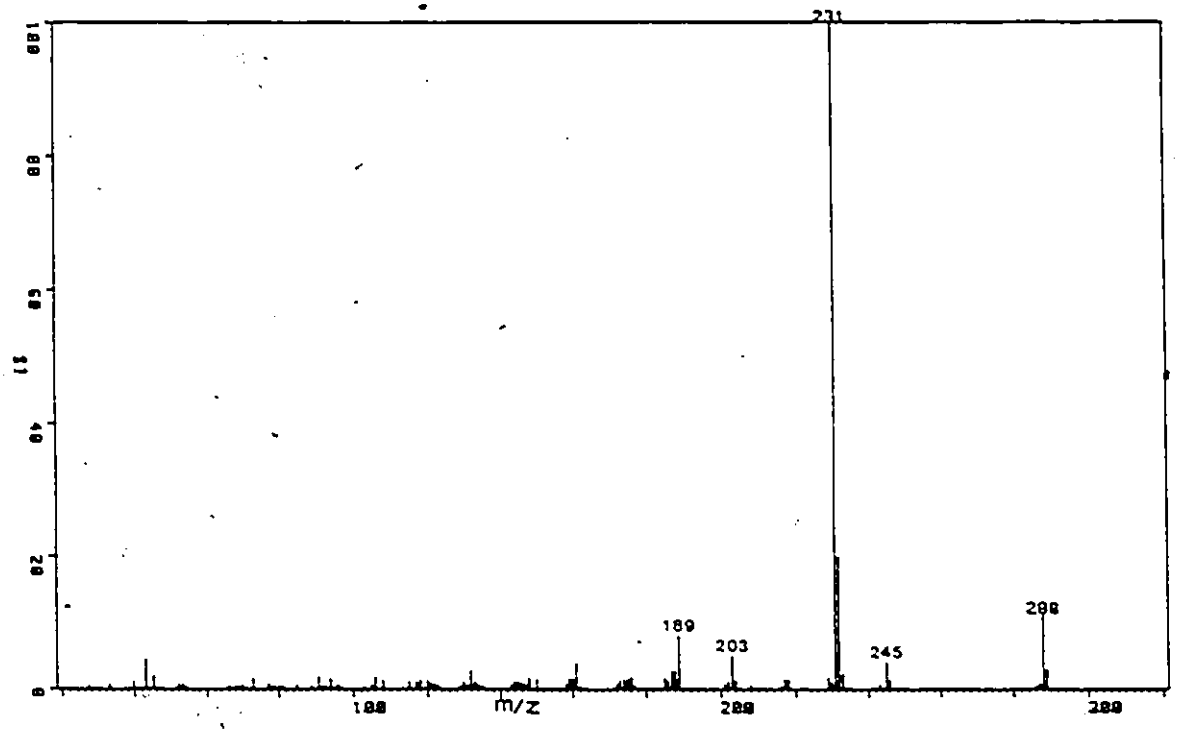
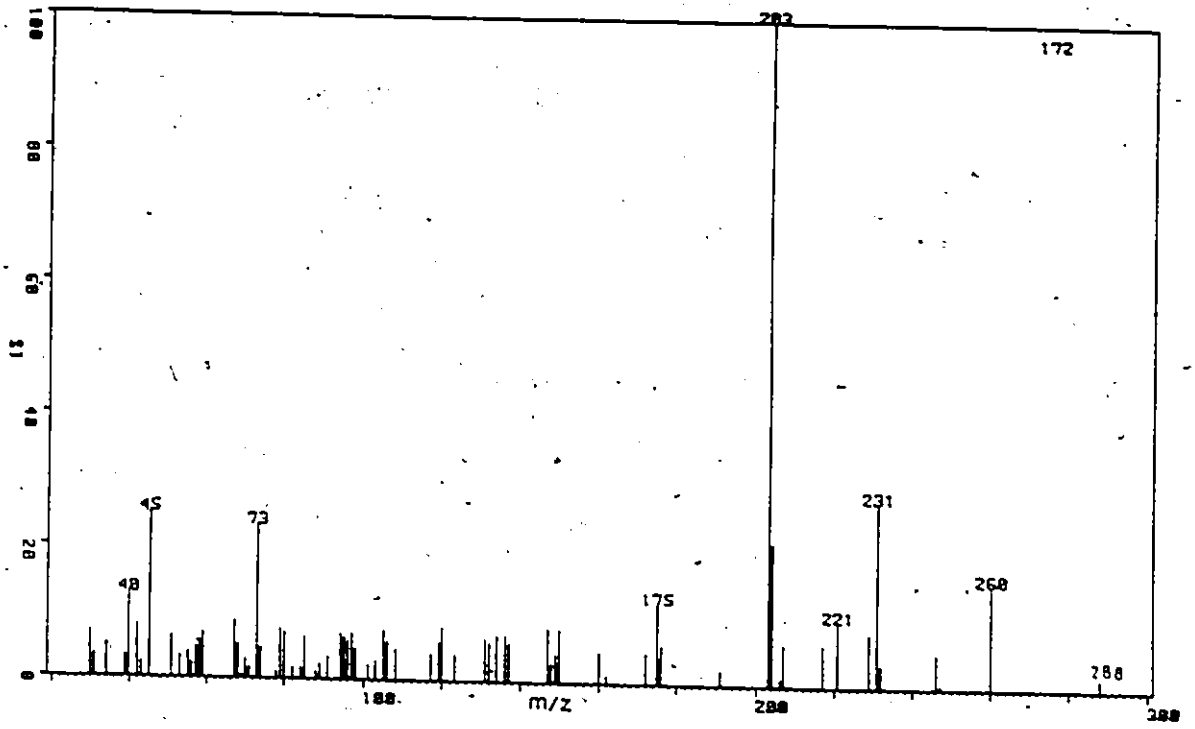


Figure 34 Mass spectrum of component F (flabellidine).

Figure 35 Mass spectrum of component G and H (flabelline and des-N-methyl-α-obscurine).



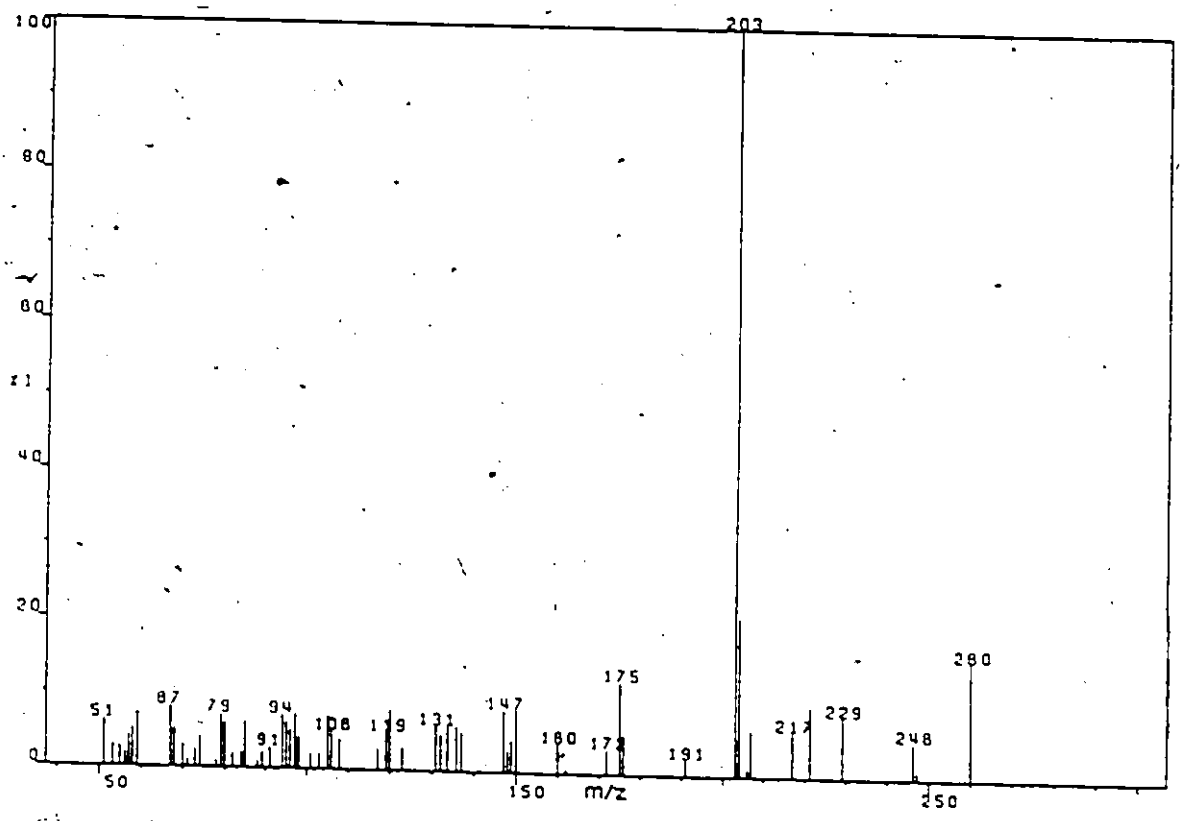
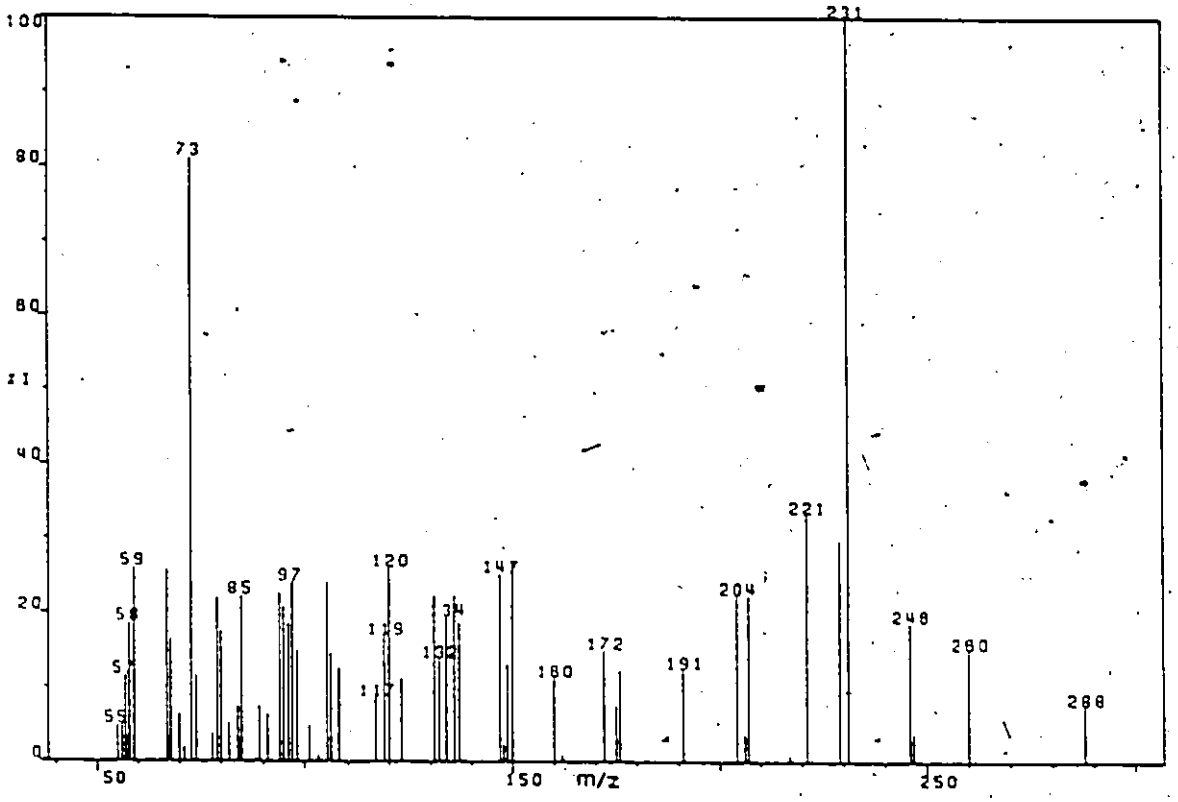


Figure 36 Mass spectrum of des-N-methyl-α-obscurine (derived from Figure 35).

Figure 37 Mass spectrum of flabelline (derived from Figure 35).



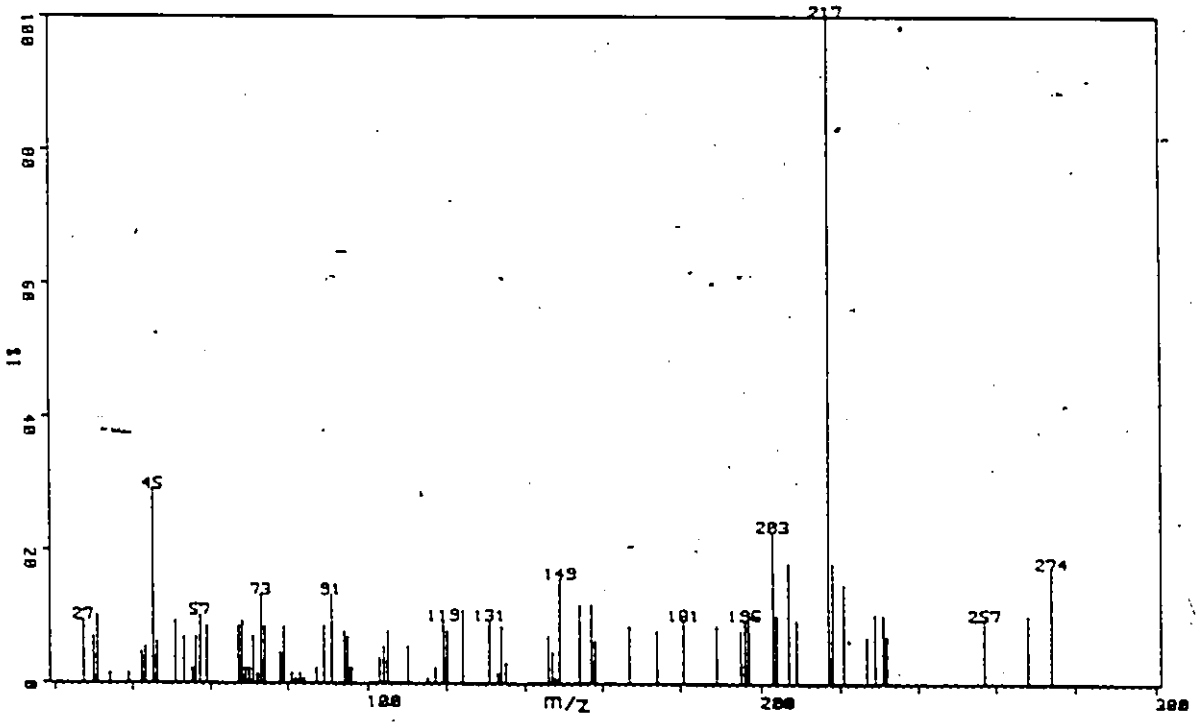


Figure 38 Mass spectrum of component I (α-obscurine).

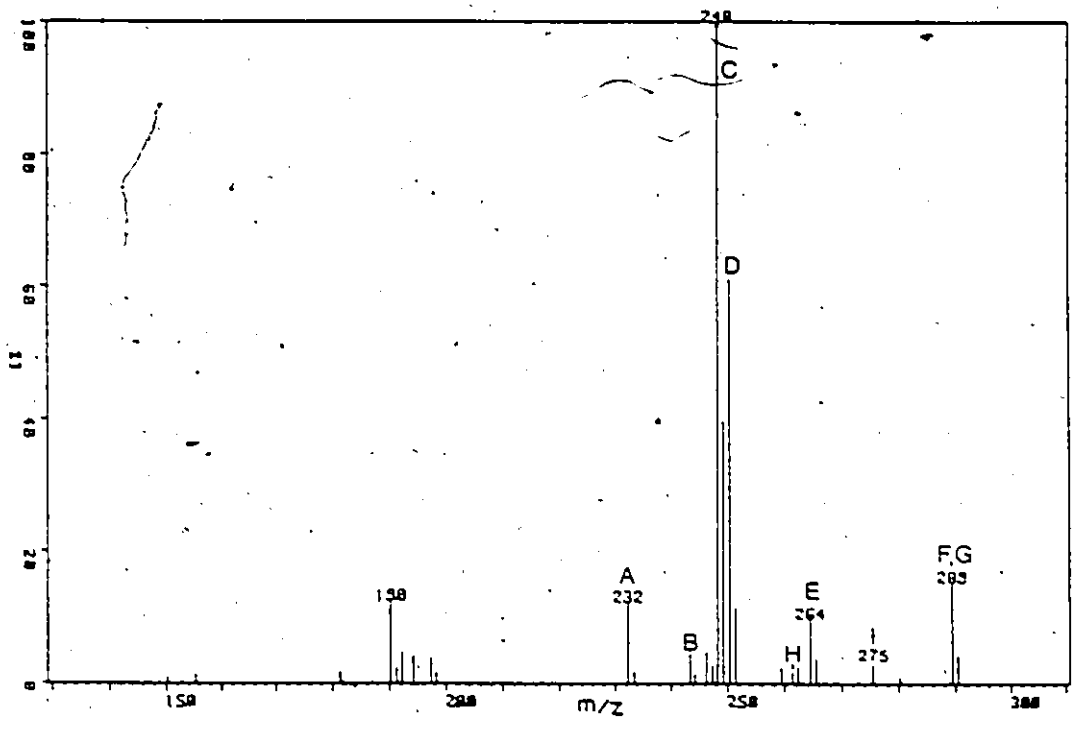


Figure 39 Chemical ionization of L. flabelliforme extract using ammonia as the reagent gas.

a derivative of luciduline.[24] Luciduline (component B), identified by its mass spectrum shown in Figure 45 elutes after dihydroluciduline. Also detected in the extract was methyl ferulate as component C; it was identified from its mass spectrum shown in Figure 46. Methyl ferulate has a molecular weight of 208 and the major fragments correspond to the loss of a methoxy group and methanol. The literature mass spectrum (EPA/NIH Mass Spectral Data Base, Volume 2, Heller and Milne) is shown in Figure 47. Ferulic acid has been found previously in L. clavatum, L. annotinum and L. selago.[214] Component D was identified as lycodine and peak E as N-methyllycodine, from their retention indices and mass spectra shown in Figures 48 and 49, respectively.

One of the major alkaloids of L. lucidulum is lycopodine, component F, the mass spectrum of which is shown in Figure 50. Component G which lies on the trailing edge of the lycopodine peak was identified as flabelliformine from its retention index and mass spectrum shown in Figure 51. Figure 52 shows the result of subtracting the mass spectrum of lycopodine from the mass spectrum of the mixture shown in Figure 51 providing a cleaner mass spectrum of flabelliformine. The mass spectrum of component H, obtained by subtracting the mass spectrum of lycopodine and flabelliformine from the mass spectrum in Figure 51 is shown in Figure 52. It has a molecular weight of 261 with ions at m/z 244 (M - 17), 218 (M - 43), 204 (M - 57) and 176 (M - 85). Such a pattern is diagnostic of a lycopodane skeleton. The loss of 17 and 57 mass units indicates that a hydroxyl group is present, but not on the lycopodane bridge. The only known alkaloid that meets these conditions is lycophlegmine which has a double bond between C-12 and C-11, a

hydroxyl group at C-10 and a ketone at C-5. The literature mass spectrum of lycophlegmine has the same ions as Component H, but the intensities are different suggesting that H may have a different substitution pattern.

Peaks I, J and K have been identified as L23, lycodoline and L20, respectively. The alkaloids L23 and lycodoline are stereoisomers and their mass spectra differ only in the intensity of the ion with m/z 190. The mass spectra of L23, lycodoline and L20 are shown in Figures 54, 55 and 56.

The alkaloids lucidine B and spirolucidine, components L and M, were identified from their mass spectra shown in Figure 57 and 58. However lucidine A (of unknown structure), lycolucine and dihydrolycolucine were not definitively identified. Ions that would arise from their fragmentation were detected in the region between the hydrocarbon standards C-36 and C-40, but they were of low intensity so that a positive identification could not be made. The alkaloid lucidioline was not detected although it has been reported in this species.

Component H, dihydroluciduline and N-methyllycodine have not previously been reported in L. lucidulum.

3.4.3 Examination of L. clavatum var. borbonicum

Nyembo[18] has reported the following alkaloids in L. clavatum var. borbonicum: anhydrolycodoline, lycopodine, dihydrolycopodine, acetyldihydrolycopodine, lycodoline, lycoflexine, borbonicine, lycodiflexine and N_{α}-acetyl-N_{β}-methylphlegmarine.[18]

Table 18

Alkaloids of L. lucidulum

alkaloid	reference
luciduline	14, 24
lycodine	67
lycopodine	14
L20	14, 67
L23	14, 67
lucidioline	67
lycodoline	67
flabelliformine	67
lucidine A	139
lucidine B	139
lycolucine	139
dihydrolycolucine	139
spirolucidine	141

Table 19

Retention indices, computer search fit values and percent total alkaloid for each component of L. lucidulum

	Compound	R.I.	A.R.I.	Pure	Mix	Reverse	# of scans	Pk.h.	Int.
A	dihydroluci-duline	1600	-	792	839	933	5	1.4	0.5
B	luciduline	1600	-	769	802	944	8		
D	lycodine	1917	1930	772	897	847	8	4.1	1.1
E	N-methyllyco-dine	1969	1975	588	836	698	5	-	-
F	lycopodine	2000	2030	791	913	859	15	39.5	36.3
G	flabelliform-ine	2065	2070	538	586	795	3		
H	unknown 261	2065	-	-	-	-	-	0.5	
I	L23	2103	-	701	702	930	4	2.9	1.5
J	lycodoline	2137	2133	682	682	938	4		
								2.4	
K	L20	2148	2154	842	890	930	10		
L	lucidine B	3763	-	536	766	648	2	19.3	25.7
M	spirolucidine	3814	-	-	-	-	-	29.9	34.9
								100.0	100.0

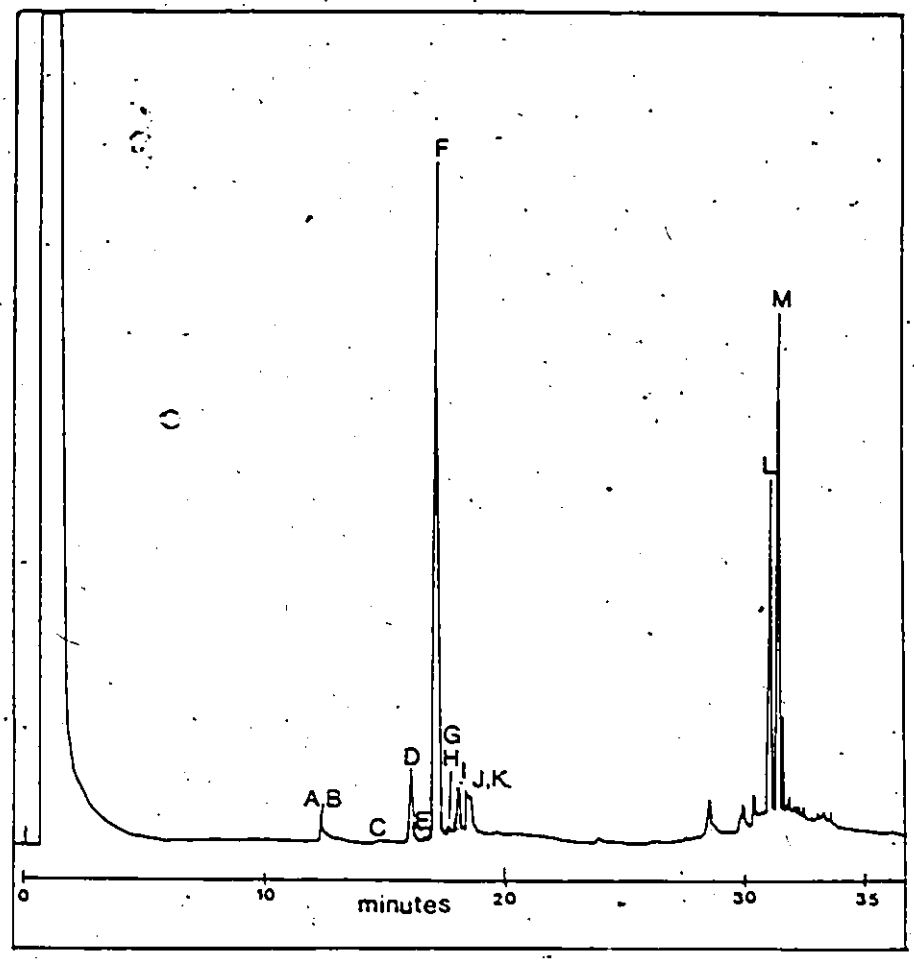


Figure 40. FSC/FID chromatogram of L. lucidulum extract.

- dihydrolucidine
- lucidine
- methyl ester of ferulic acid
- lycodine
- N-methyllycodine
- lycopodine
- clavolinine
- unknown (m)
- L23
- lycodoline
- L20
- Lucidine P
- spirolucidine

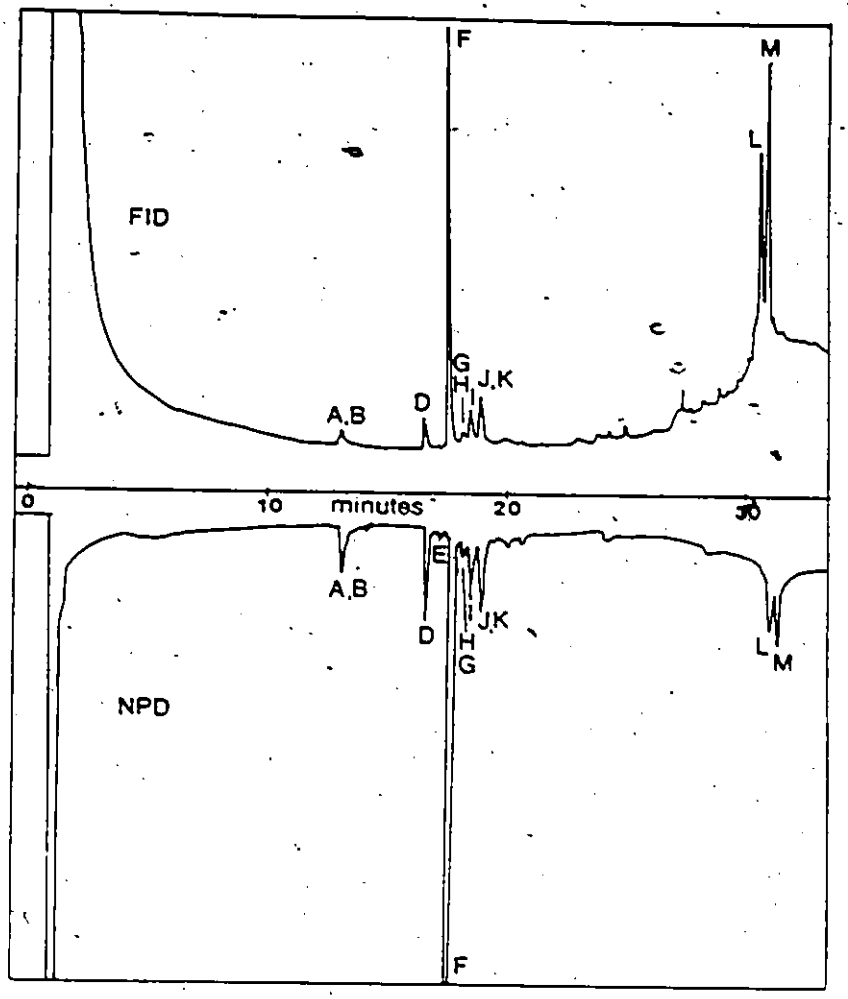
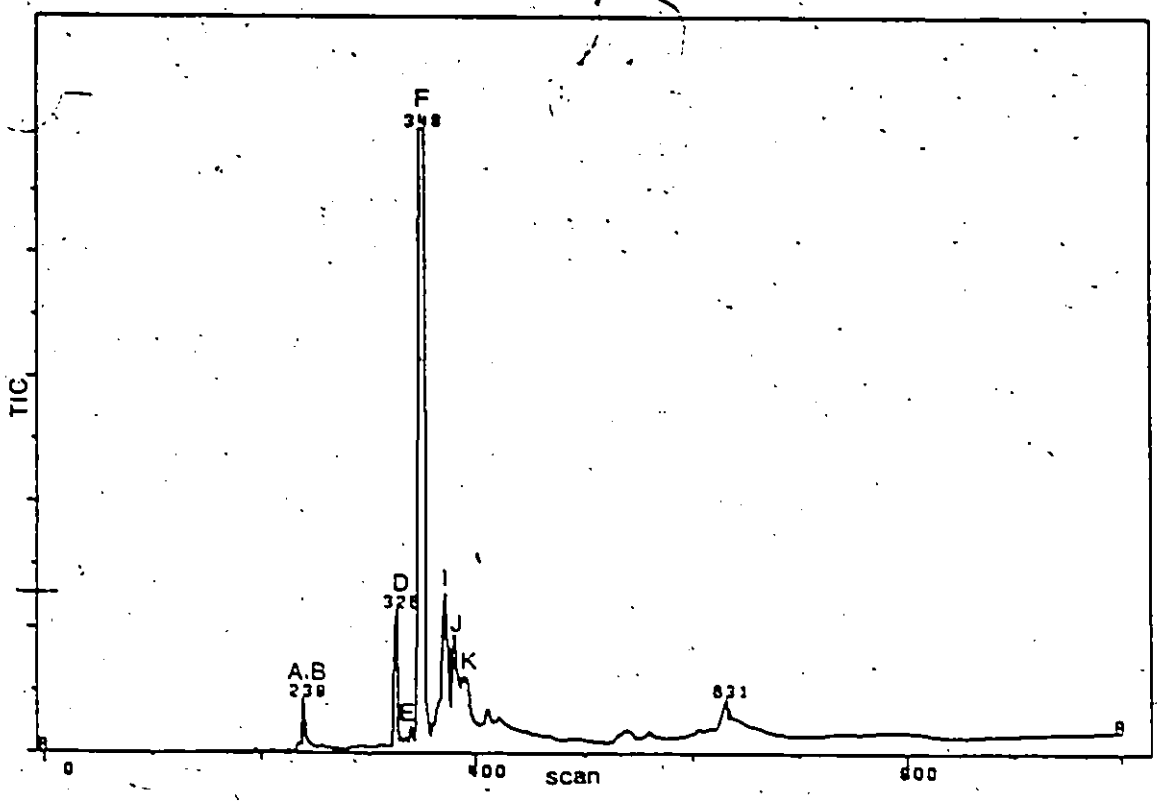


Figure 41 FSC/FID/NPD chromatogram of L. lucidulum extract.

Figure 42 FSC/MS TIC of L. luciculum extract.



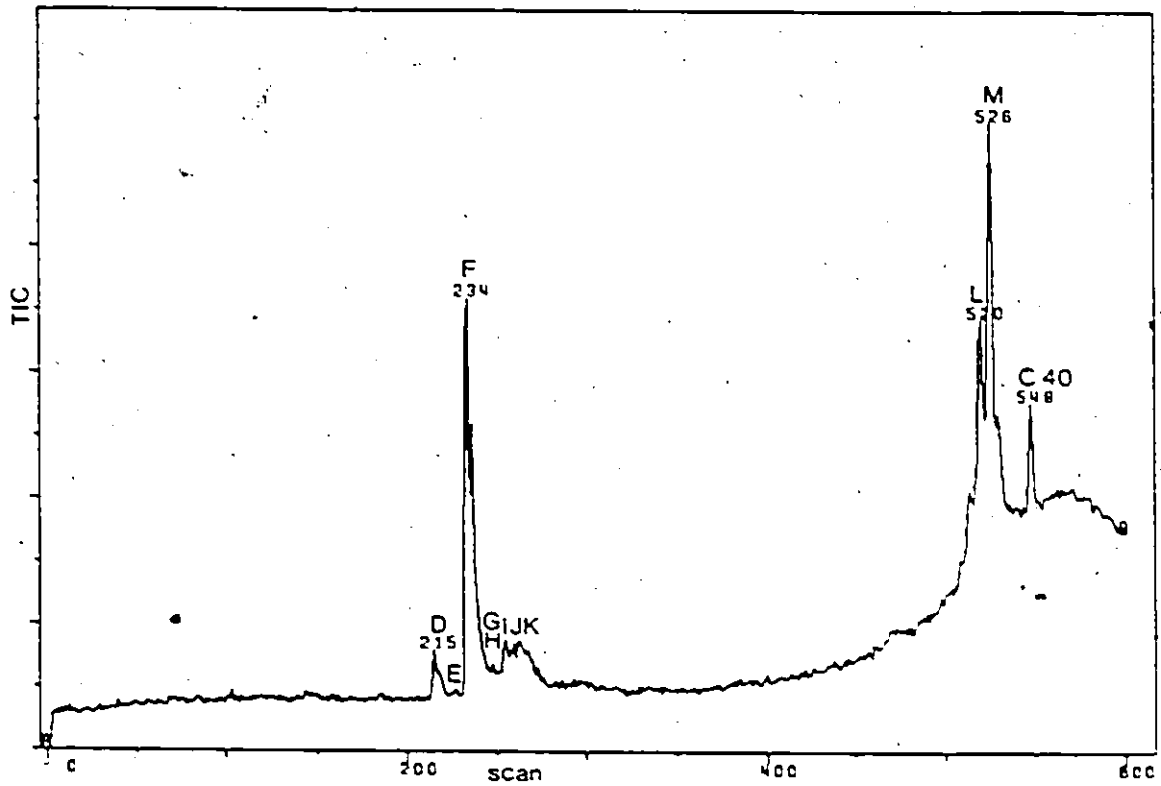
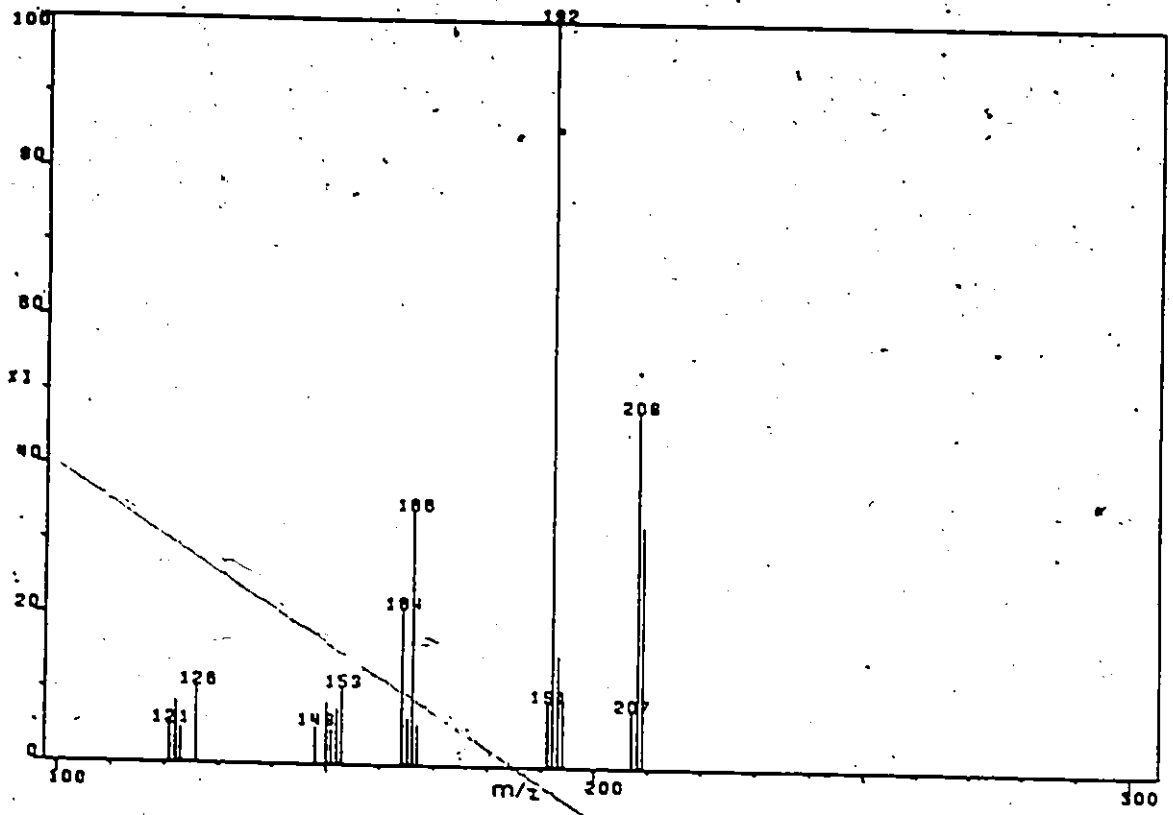


Figure 43 FSC/MS TIC of L. luciculum extract with high temperature interface.

Figure 44 Mass spectrum of component A (dihydroluciduline).



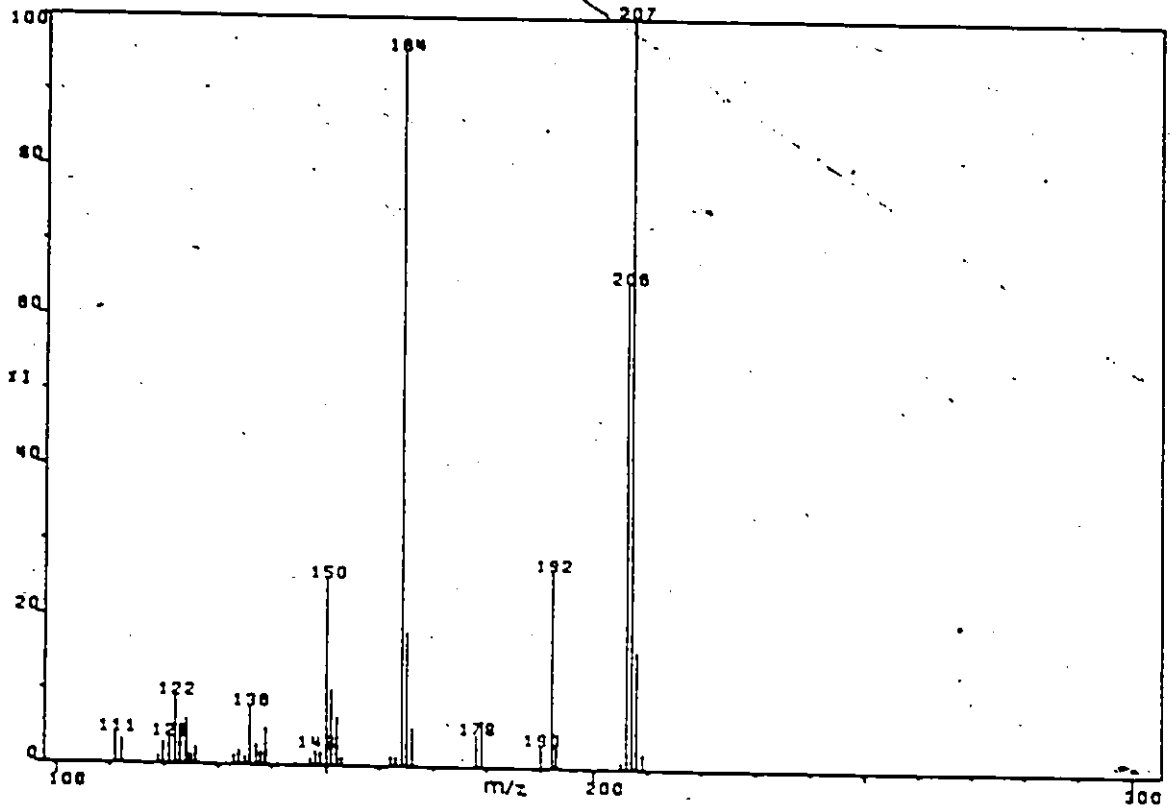


Figure 45 Mass spectrum of component B (luciduline).

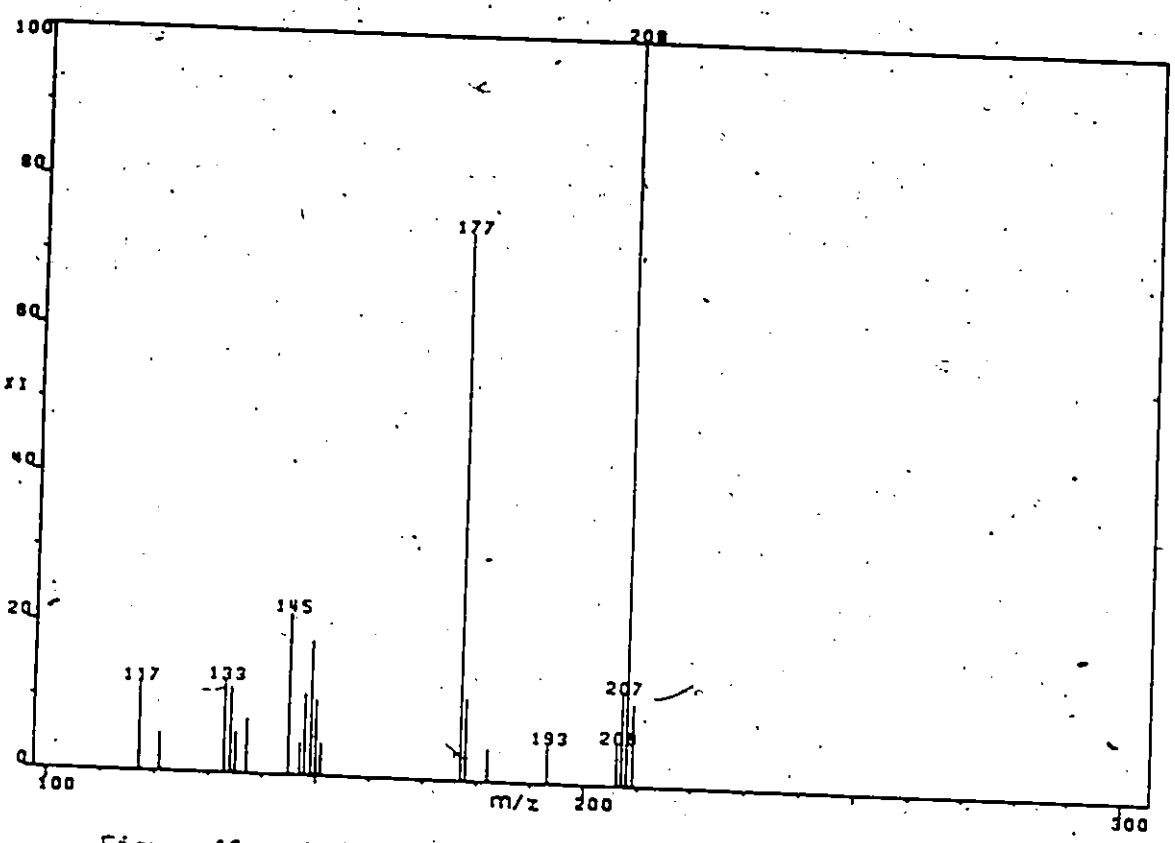


Figure 46 Mass spectrum of component C (the methyl ester of ferulic acid).

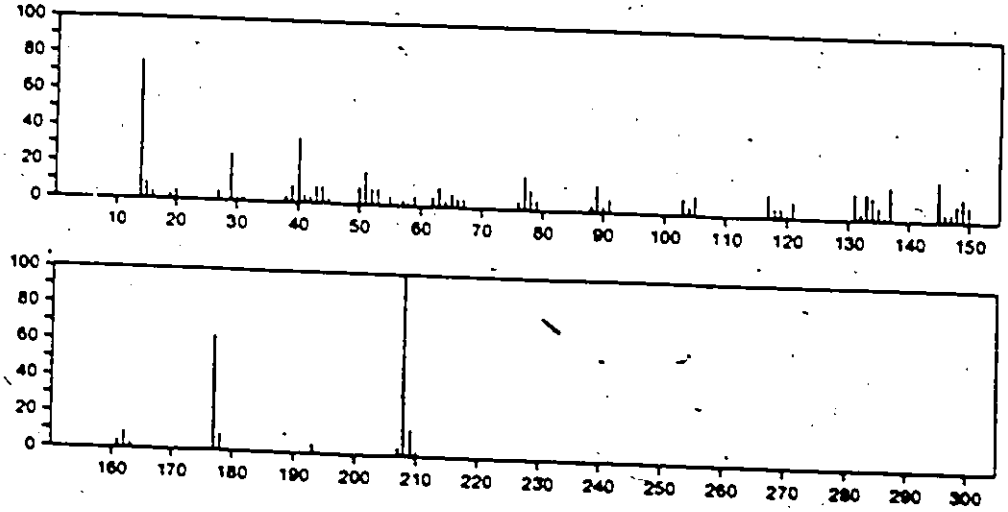
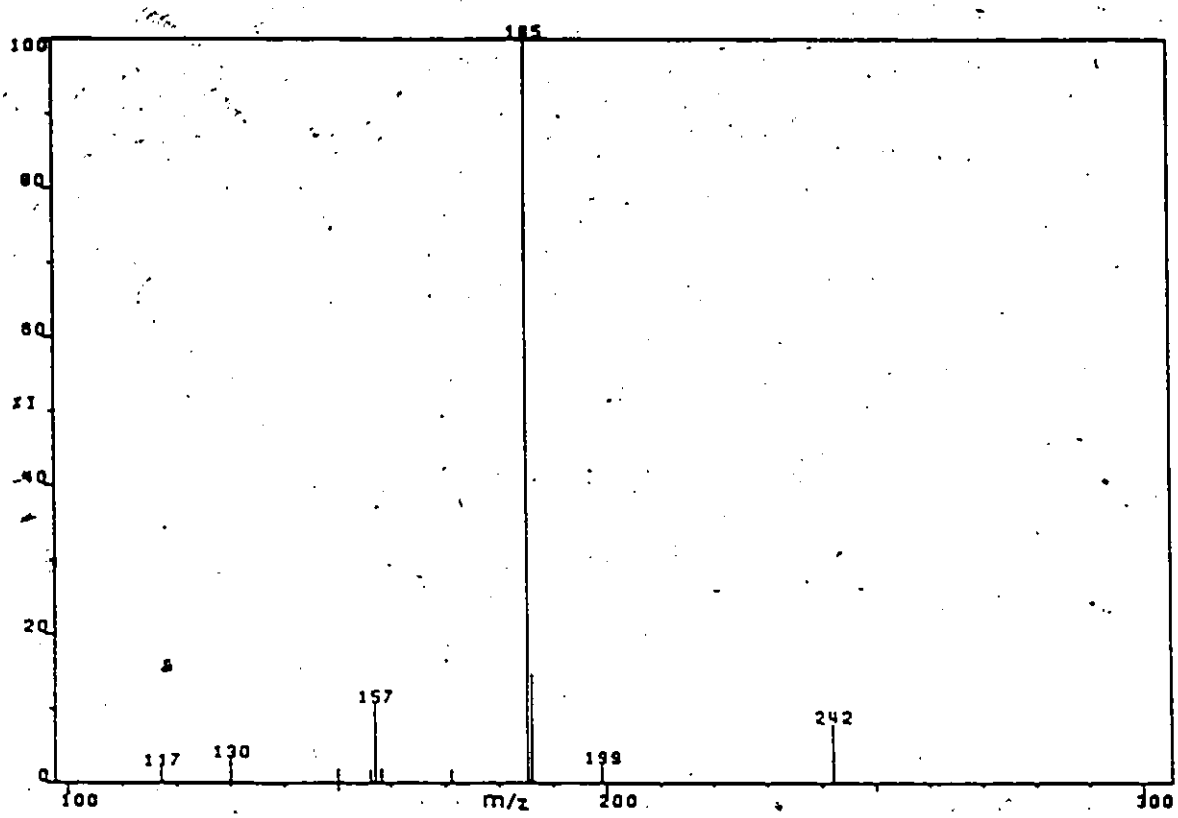


Figure 47 The literature mass spectrum of the methyl ester of ferulic acid.

Figure 48 Mass spectrum of component D (lycodine).



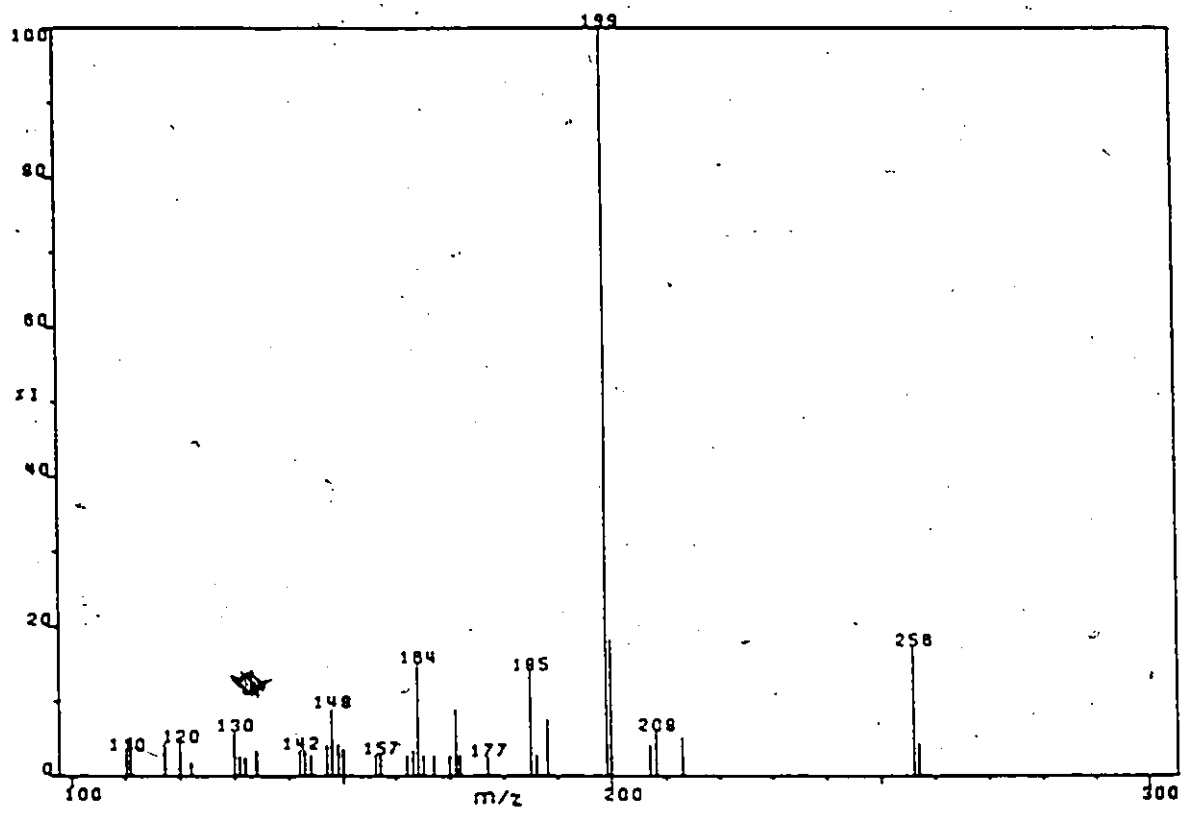
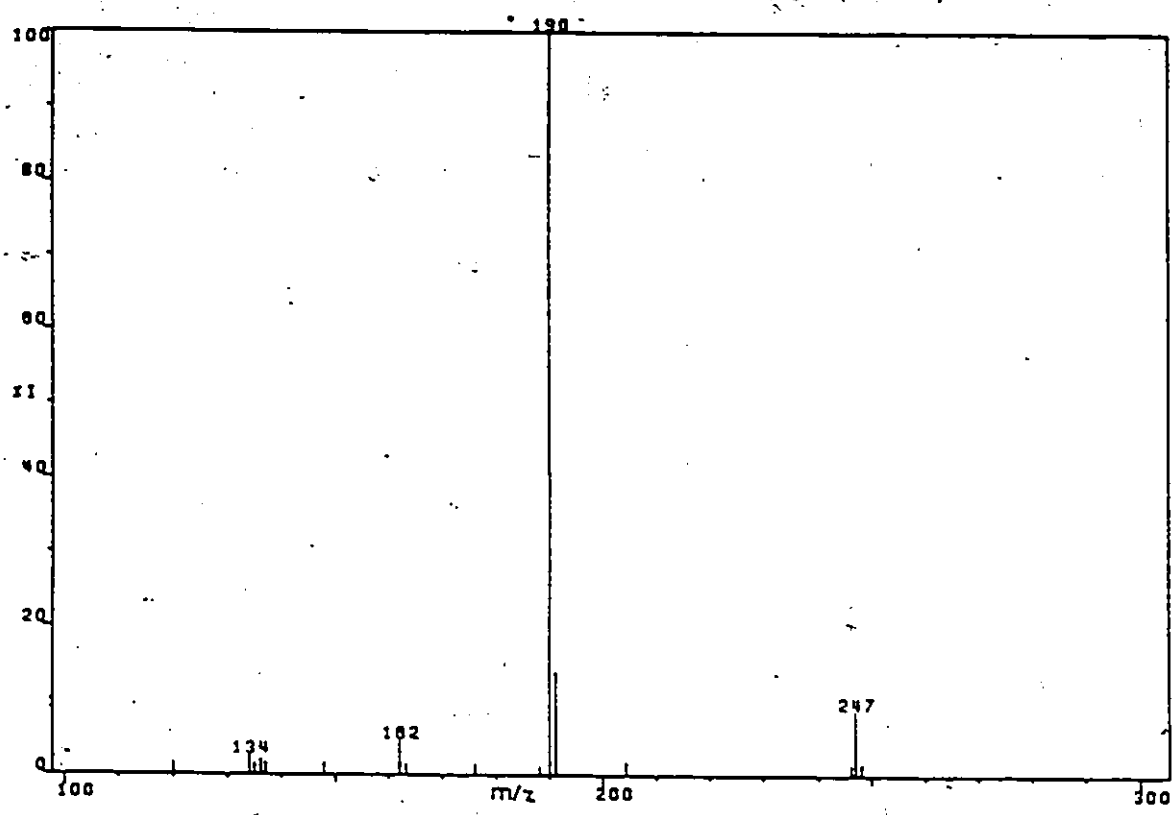


Figure 49 Mass spectrum of component E (N-methyllycodine).

Figure 50 Mass spectrum of component F (lycopodine).



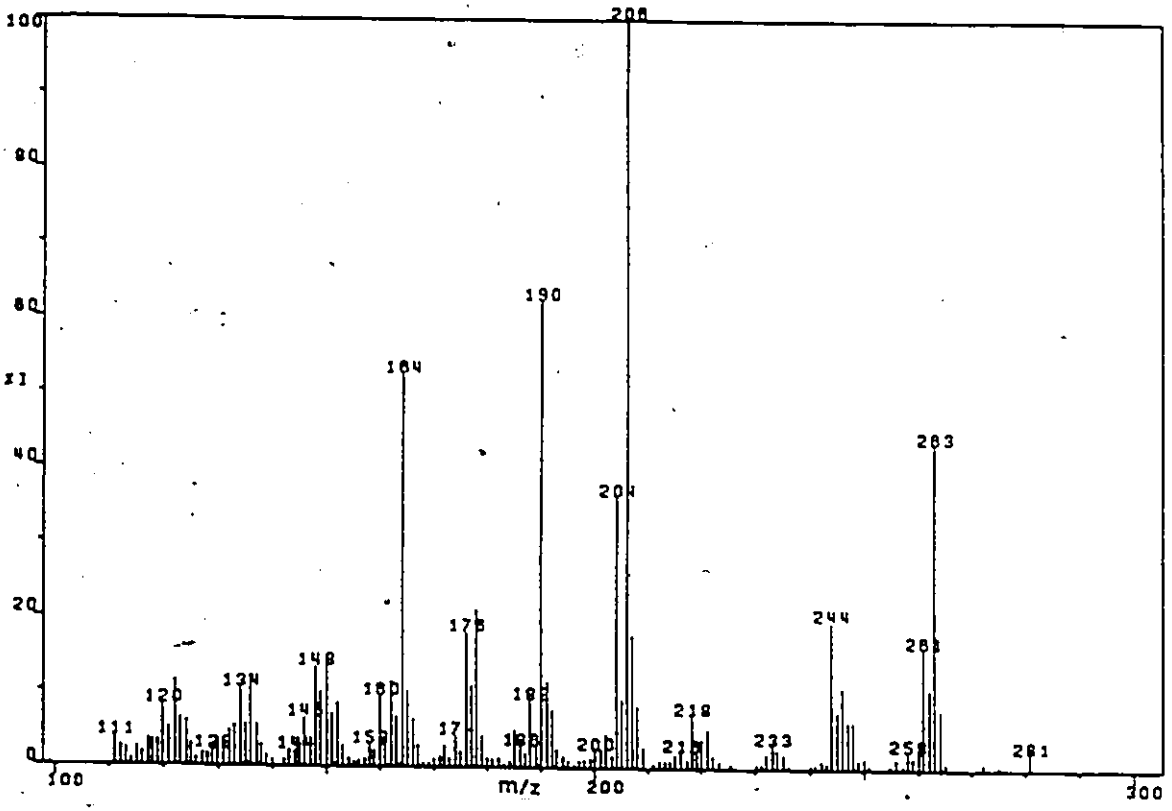
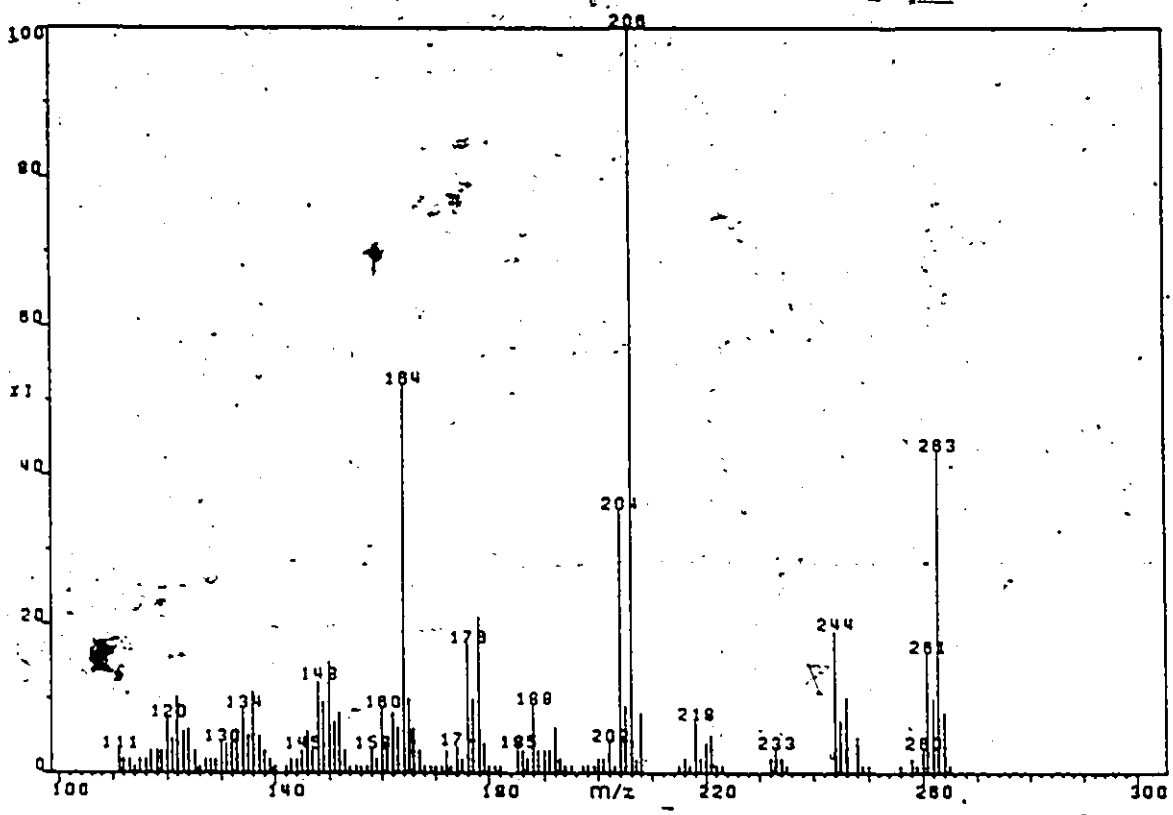


Figure 51 Mass spectrum of component G (flabelliformine).

Figure 52 Mass spectrum of flabelliformine (derived from Figure 51).



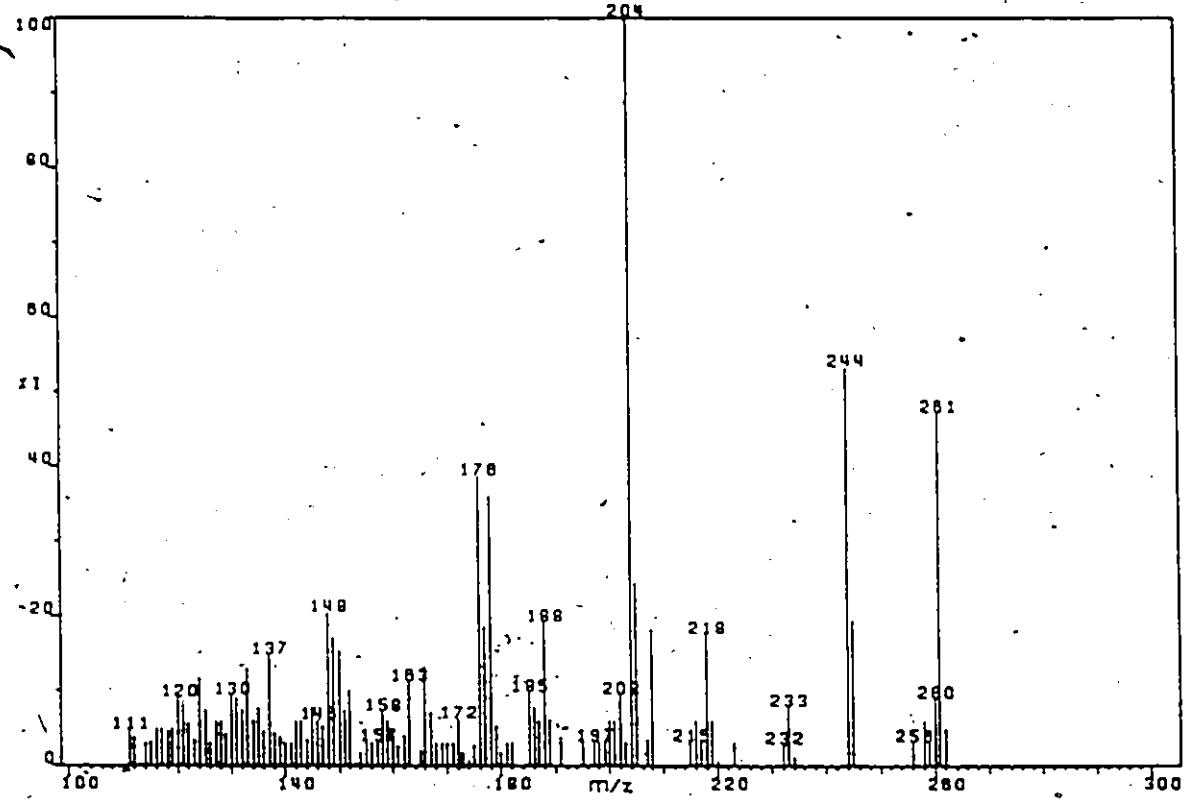
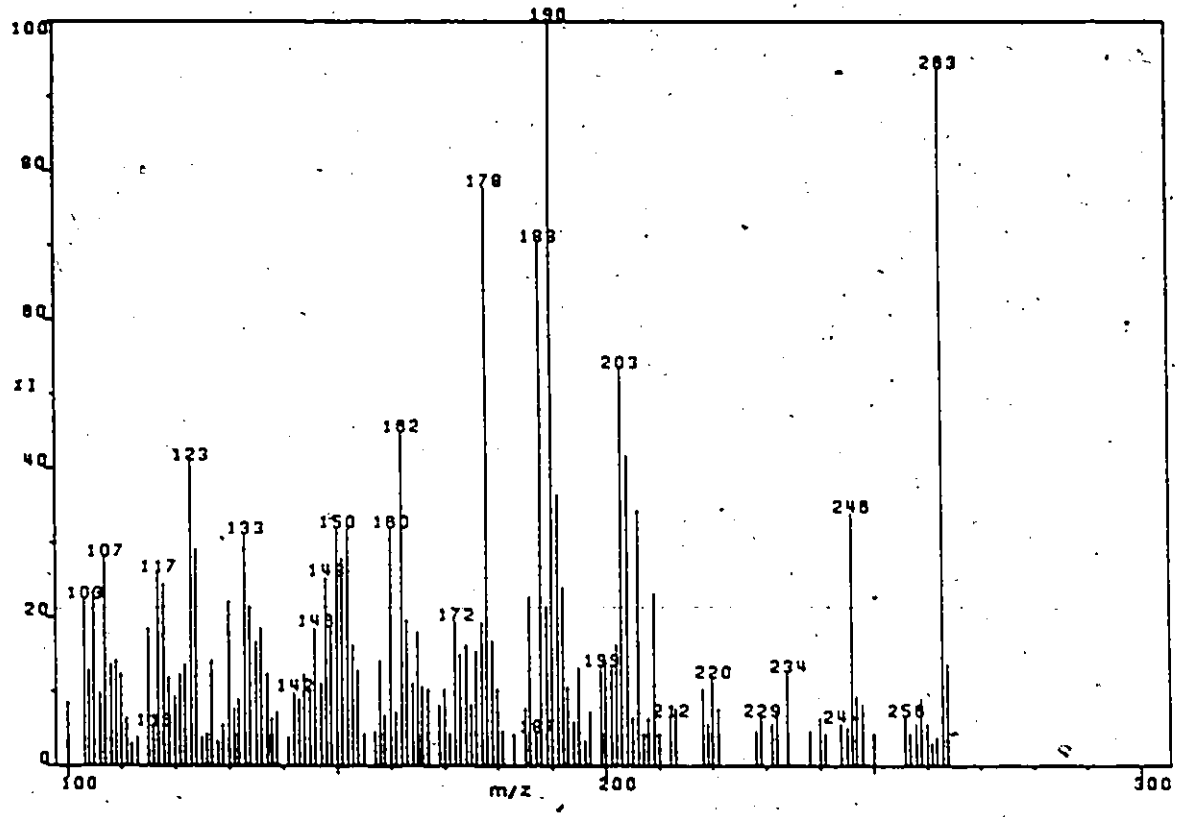


Figure 53 Mass spectrum of component H (derived from Figure 51).

Figure 54 Mass spectrum of component I (L23).



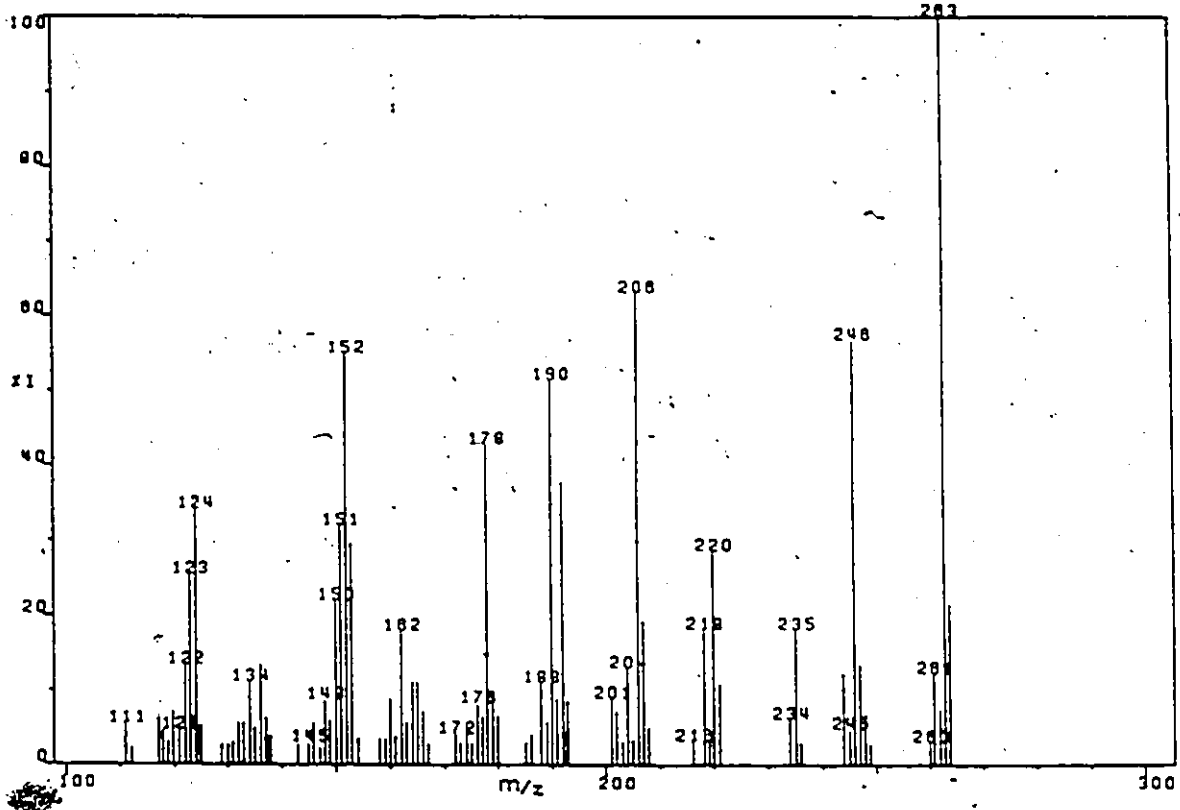
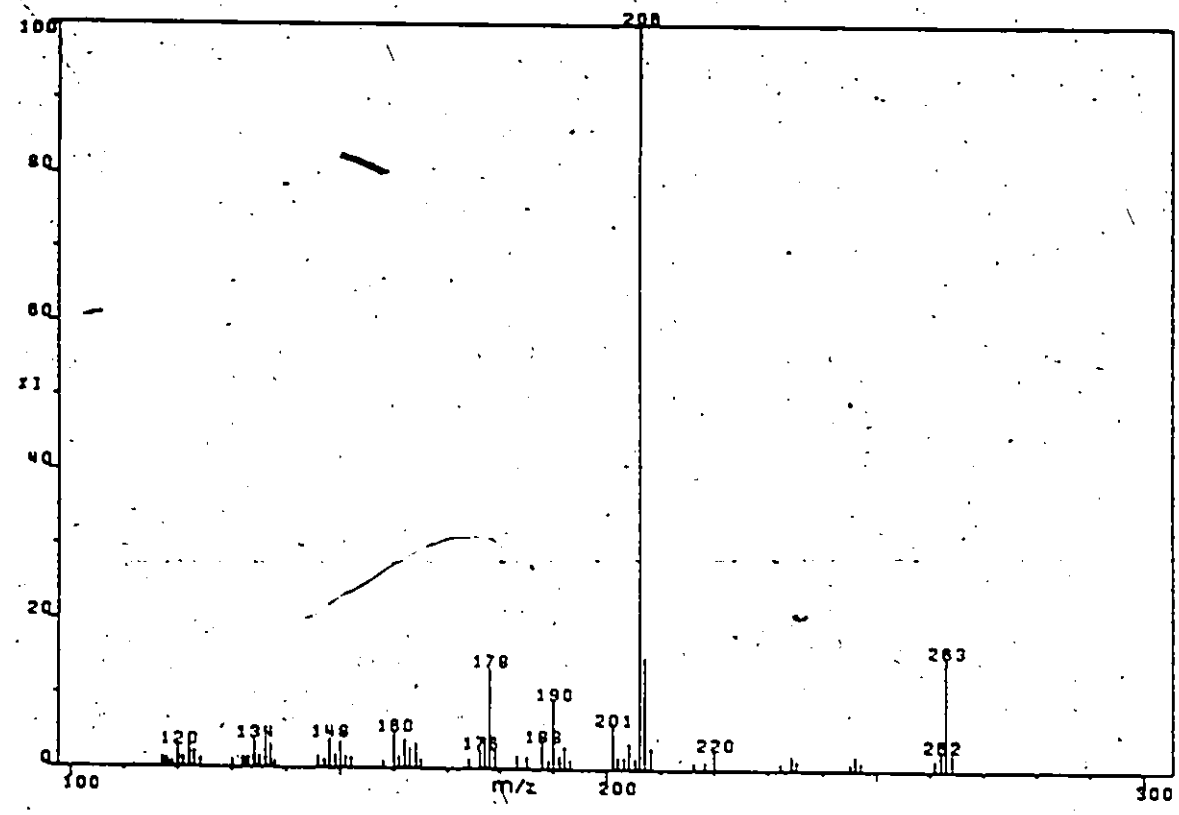


Figure 55 Mass spectrum of component J (lycodoline).

Figure 56 Mass spectrum of component K (L20).



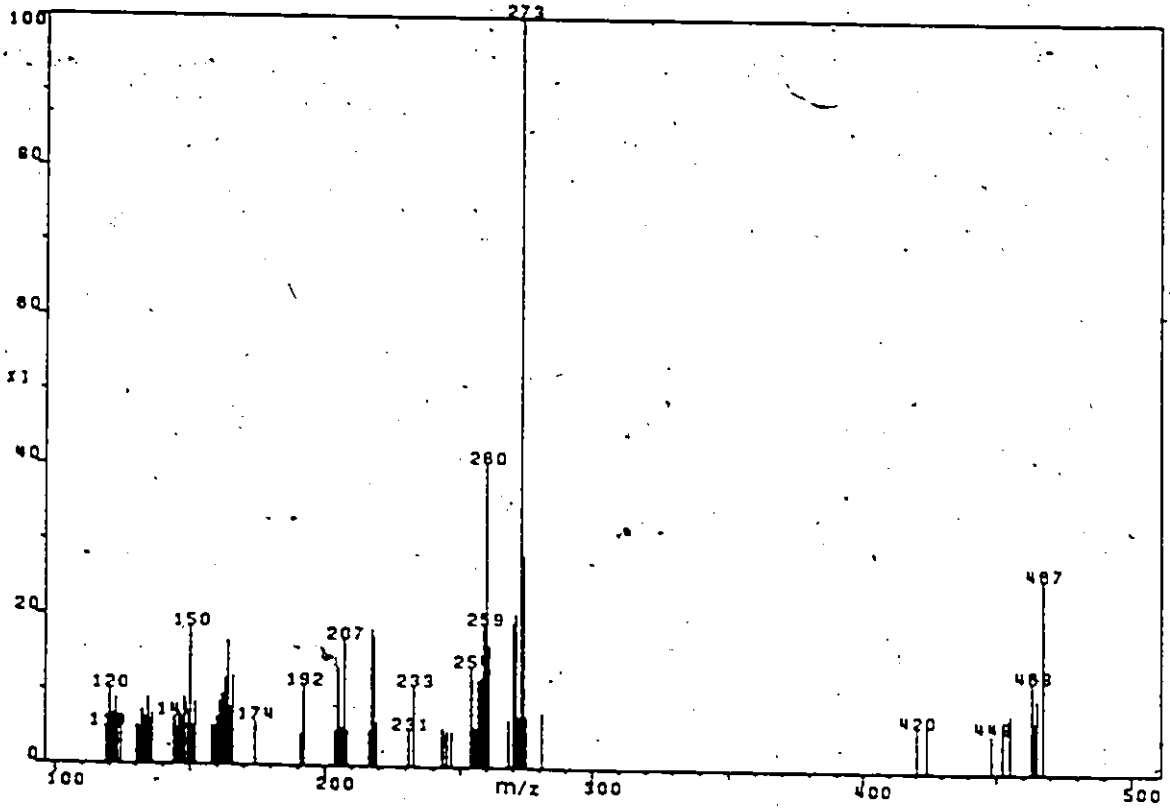


Figure 57 Mass spectrum of component L (lucidine B).

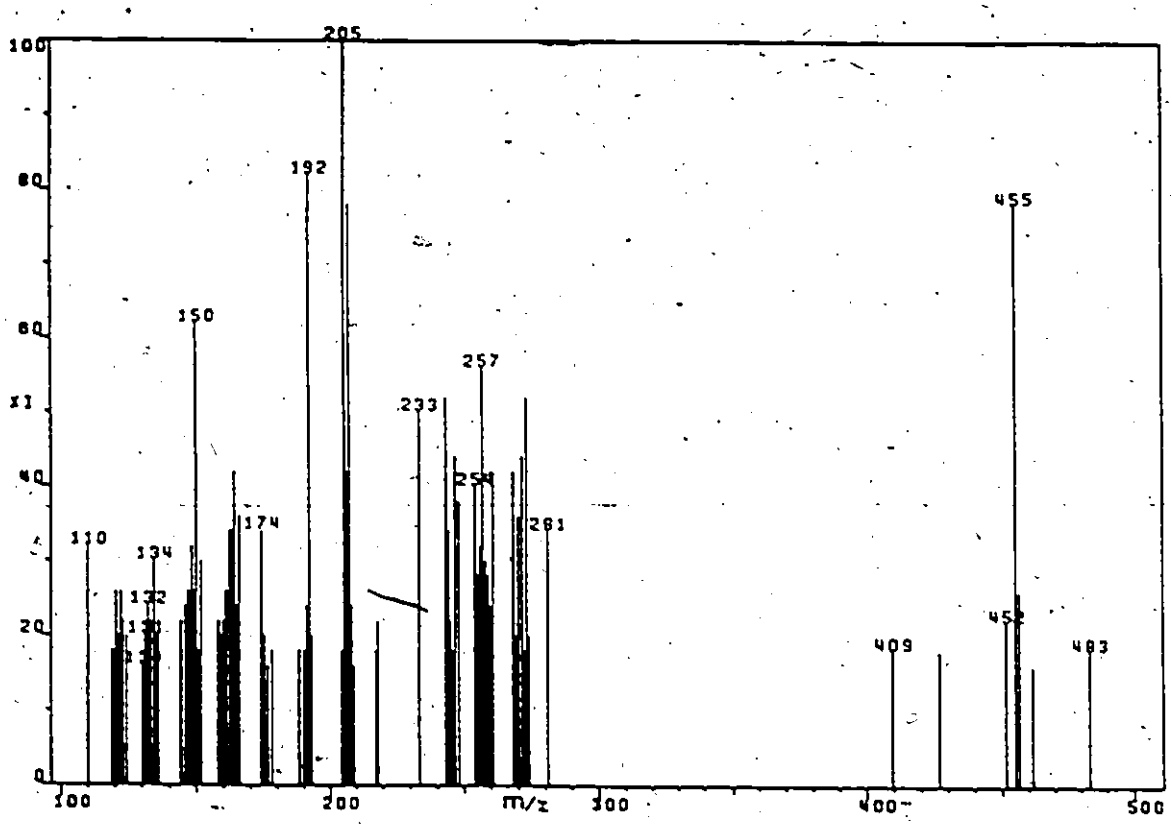


Figure 58 Mass spectrum of component M (spiroluciduline).

The extract obtained in this study was examined by GC using both a FID and NPD detector as shown in Figures 59 and 60. The extract was also examined by GC/MS and FSC/MS and the TIC which resulted are shown in Figures 61 and 62. The retention indices and computer library search fit values are tabulated in Table 20. The percent of each component in the extract has been calculated from peak heights and areas from FSC/FID; the results are also reported in Table 20.

The first alkaloid to elute, component A, was identified by its retention index and mass spectrum shown in Figure 63 as lycodine. Component B which elutes just after lycodine is anhydrolycodoline, the mass spectrum of which is shown in Figure 64. The major alkaloid of L. clavatum was identified from its retention index and mass spectrum shown in Figure 65 as lycopodine. Component D which coelutes with lycopodine as shown in Figure 66, was identified as dihydrolycopodine from its retention index and mass spectrum. Figure 67 shown the result of subtracting the mass spectrum of lycopodine from the mass spectrum in Figure 66. Component F was identified from its retention index and mass spectrum (Figure 68) as acetyldihydrolycopodine. The small peak, E, that elutes before F represents a mixture of flabelliformine and acetyldihydrolycopodine as shown in the mass spectrum in Figure 69. Figure 70 shows the result of subtracting the mass spectrum of acetyldihydrolycopodine from the mass spectrum in Figure 69 and shows the characteristic ions of flabelliformine at m/z 263, 205 and 164.

Component G, which has a molecular weight of 263 from its mass spectrum shown in Figure 71, was identified as lycodoline. Component H, also has a molecular weight of 263 as seen in its mass spectrum shown in

Figure 72. A base peak at m/z 206 indicates that the bridge is unsubstituted. Since the molecular ion is of low intensity this indicates that no functional groups are present at C-12. Computer searching of the library of Lycopodium alkaloid mass spectra enabled component H to be identified as L20. Component I has a molecular weight of 279 and a base peak at m/z 262 indicating the loss of a hydroxyl group. The mass spectrum of Component I is shown in Figure 73. The identity of component I has not been established but it may prove to be alopecuridine. Component J, lycoflexine, can be identified by its mass spectrum shown in Figure 74. Eluting after lycoflexine is an alkaloid which Nyembo named borbonicine. The mass spectrum of borbonicine shown in Figure 75 is not intense and is contaminated by lycoflexine. The structure of borbonicine has not been fully established. The last alkaloid to elute, L, was identified as N_α-acetyl-N_β-methylphlegmarine by its mass spectrum (Figure 76).

The extract was also examined by chemical ionization mass spectrometry. The results obtained from using methane and ammonia as reagent gases are shown in Figure 77 and 78. All the alkaloids that were identified by GC-MS gave $(M + H)^+$ ions.

The dimeric alkaloid, lycodiflexine reported by Nyembo was not found in any of the GC/MS analyses, possibly because of its high molecular weight. The alkaloids lycodine, flabeliformine and L20 were not reported by Nyembo nor was the compound with the molecular weight of 279.

L. clavatum var. borbonicum was initially extracted to isolate N_α-acetyl-N_β-methyl phlegmarine to verify its stereochemistry which had

Table 20

Retention indices, computer search fit values and percent total alkaloid for each component of L. clavatum var. borbonicum

	Compound	R.I.	A.R.I.	Pure	Mix	Reverse	# of scans	Pk.h.	Int.
A	lycodine	1935	1930	913	913	993	1	4.9	3.5
B	anhydrolycodoline	1935	-	536	536	905	1		
C	lycopodine	2000	2030	846	866	923	4	84.4	80.8
D	dihydrolycopodine	2000	2000	810	927	851	1		
E	flabelliformine	2073	2070	688	737	843	1	0.5	0.4
F	acetyldihydrolycopodine	2086	2085	885	885	995	1	4.9	10.0
G	lycodoline	2120	2133	568	568	937	2	0.8	1.6
H	L20	2149	2154	766	857	880	1	1.1	0.7
I	unknown mol wt 279	2182	-	-	-	-	-	0.4	0.9
J	lycoflexine	2263	-	622	849	725	1	0.8	0.2
K	borbonicine	2278	-	409	409	971	-	0.8	0.5
L	N_α-acetyl-N_β-methylphlegmarine	2380	-	603	628	951	2	1.4	1.4
								100.0	100.0

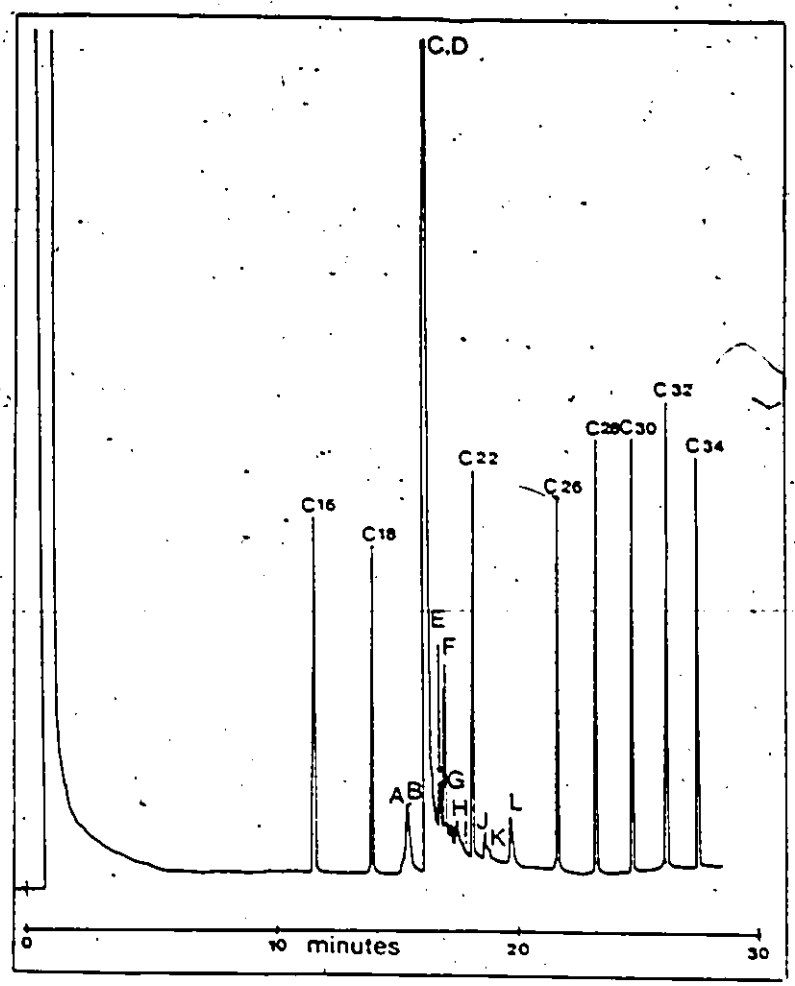


Figure 59 FSC/FID chromatogram of L. clavatum var. borbonicum extract with hydrocarbon standards.

A lycodine

B anhydrolycodine

C lycopodine

D dihydrolycopodine

E flabelliformine

F acetyldihydrolycopodine

G lycodoline

H L26

I unknown 276

J lycoflexine

K borbonicine

L N-n-acetyl-N-B-methylphlegmarine

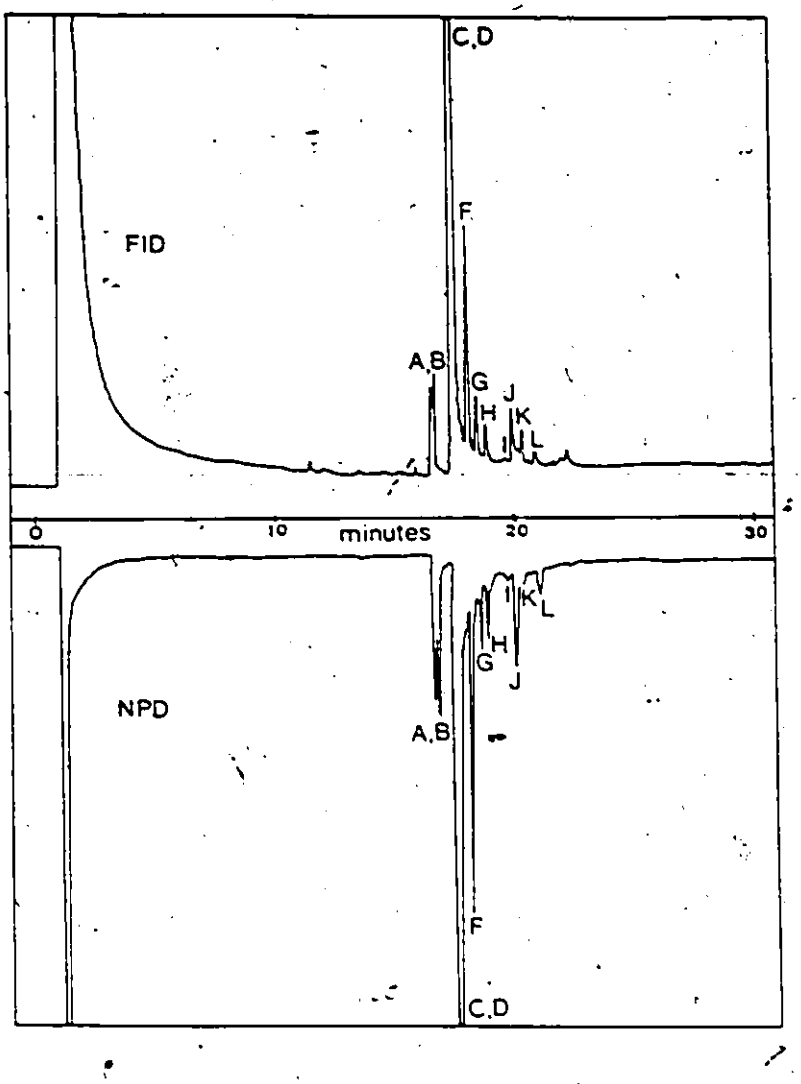
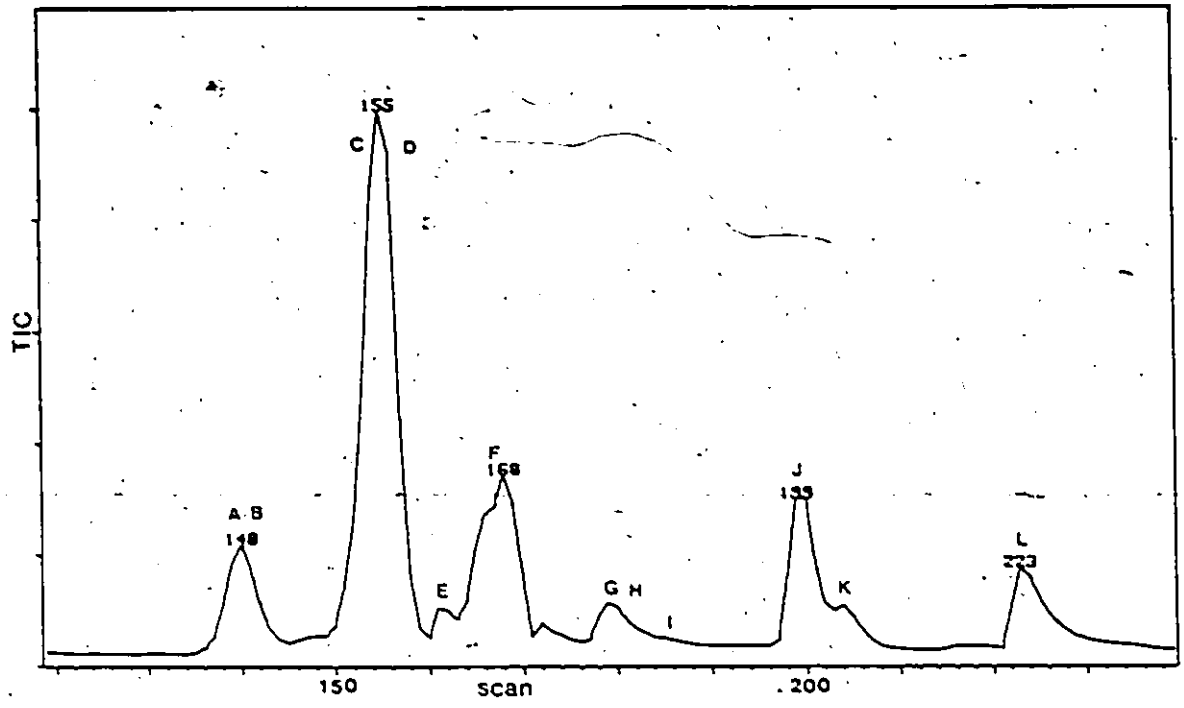


Figure 60 FSC/FID/NPD chromatogram of L. clavatum var. borbonicum extract.

Figure 61 GC/MS TIC of L. clavatum var. borbonicum extract.



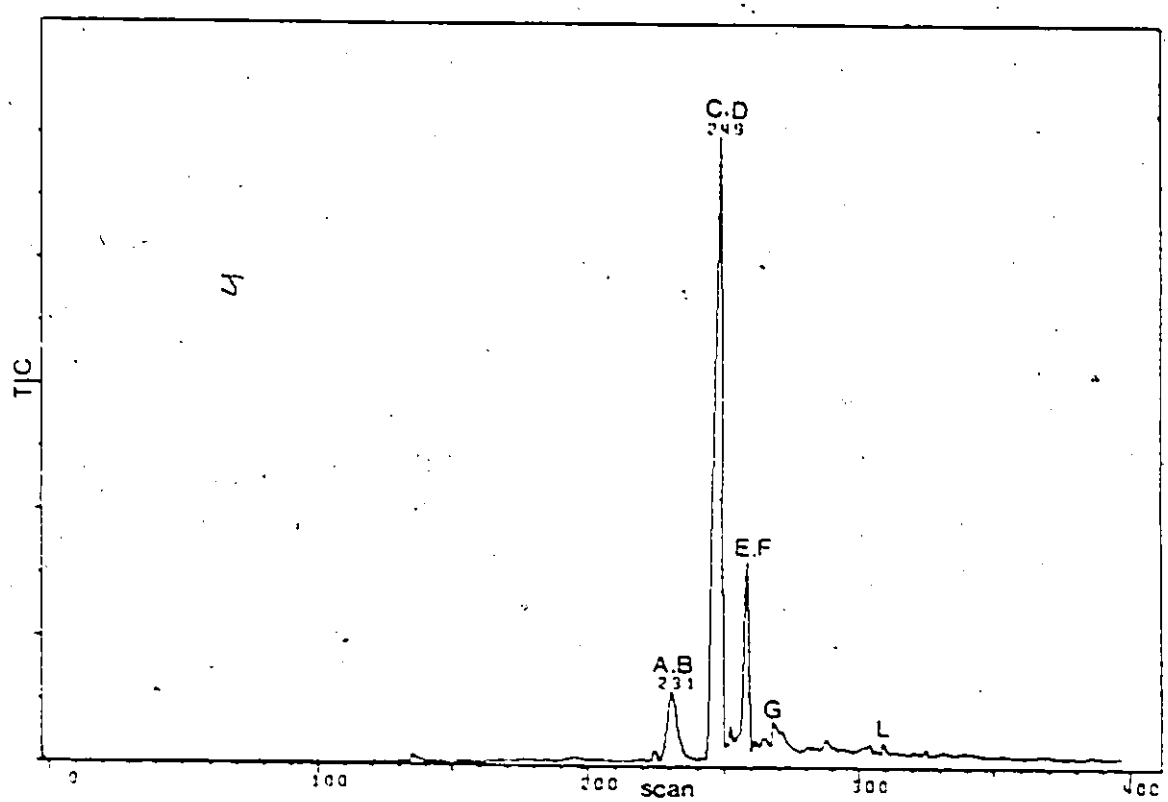
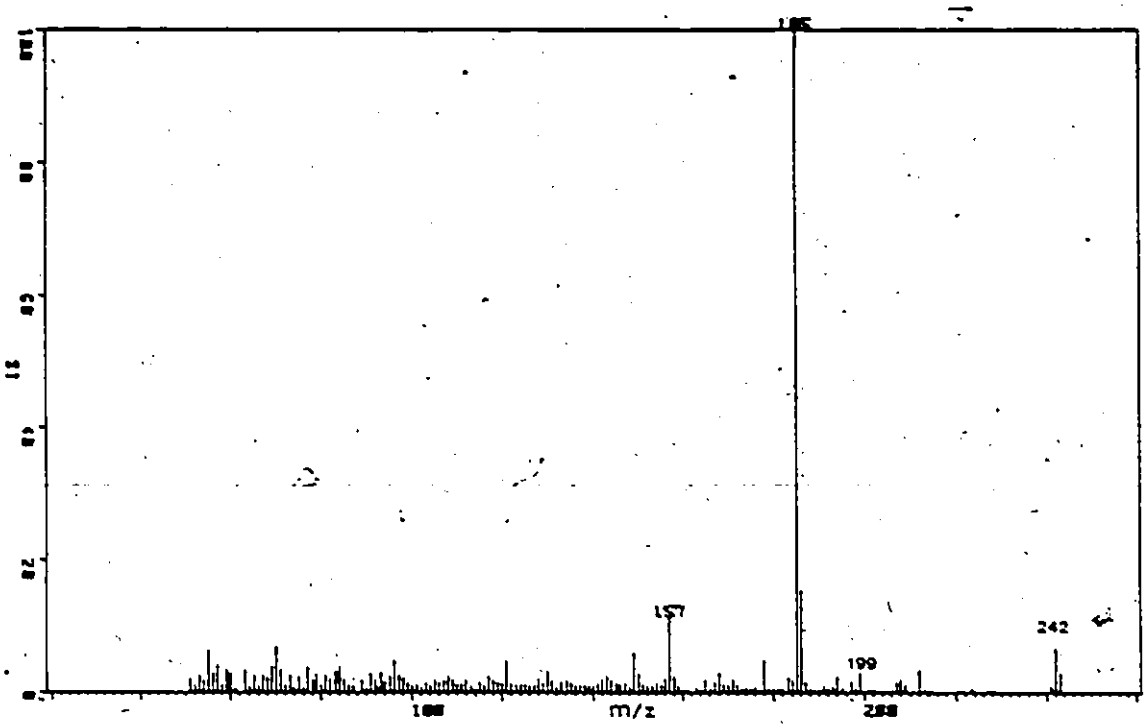


Figure 62 FSC/MS TIC of L. clavatum var. borbonicum extract.

Figure 63 Mass spectrum of component A (lycodine).



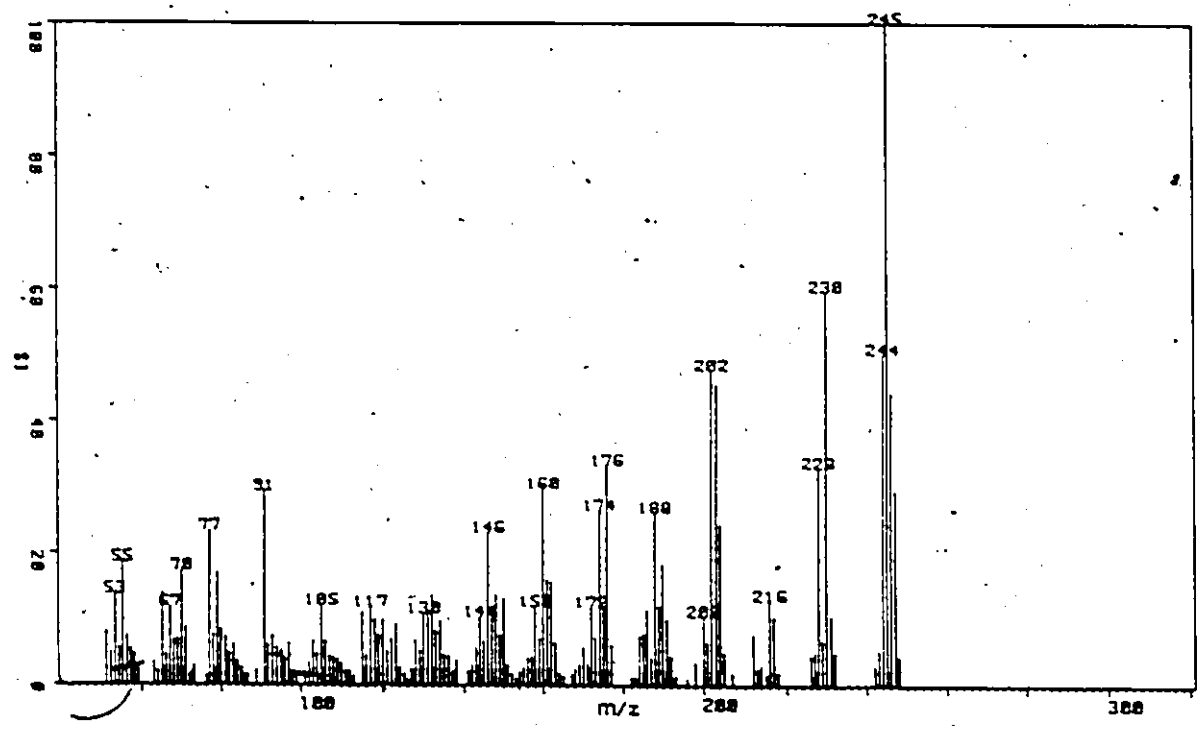
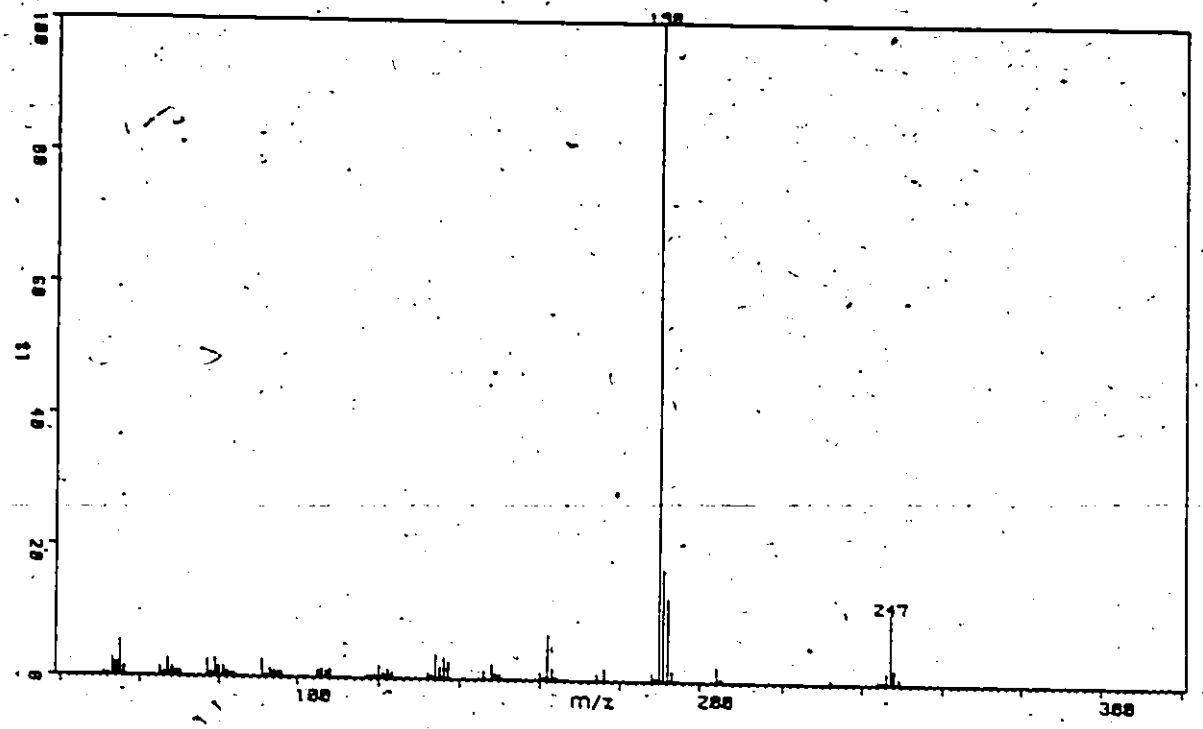


Figure 64 Mass spectrum of component B (anhydrolycodoline).

Figure 65 Mass spectrum of component C (lycopodine).



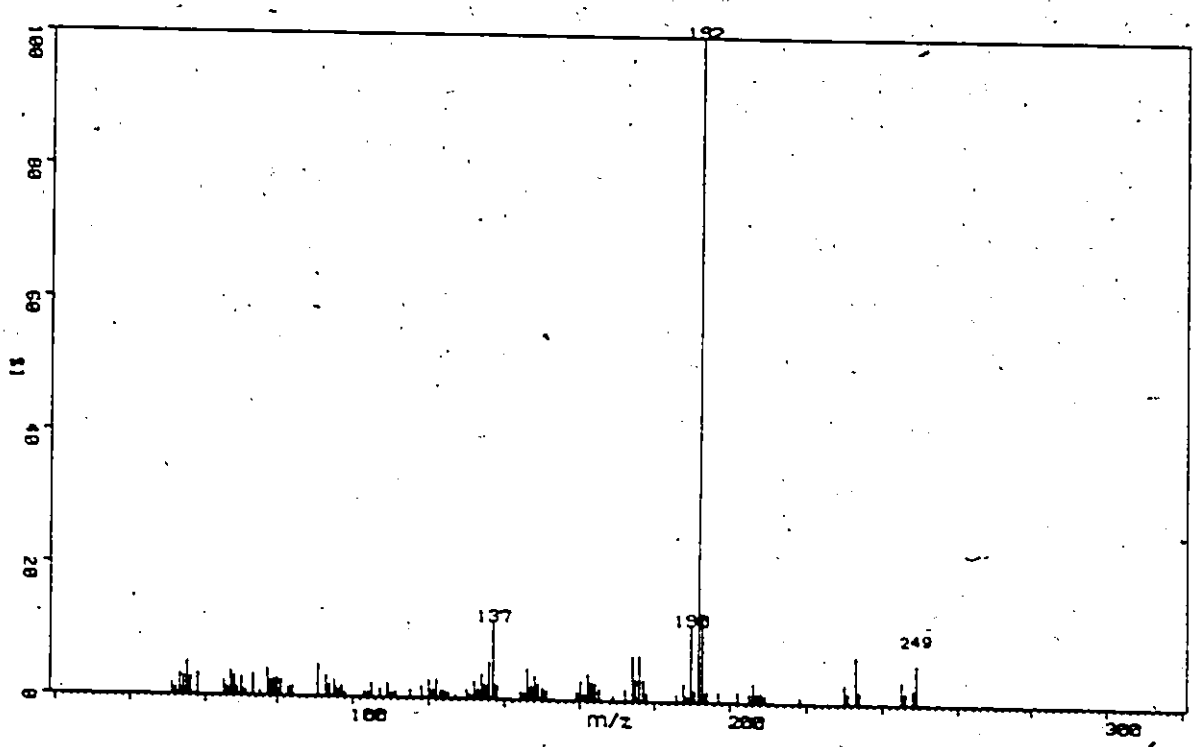
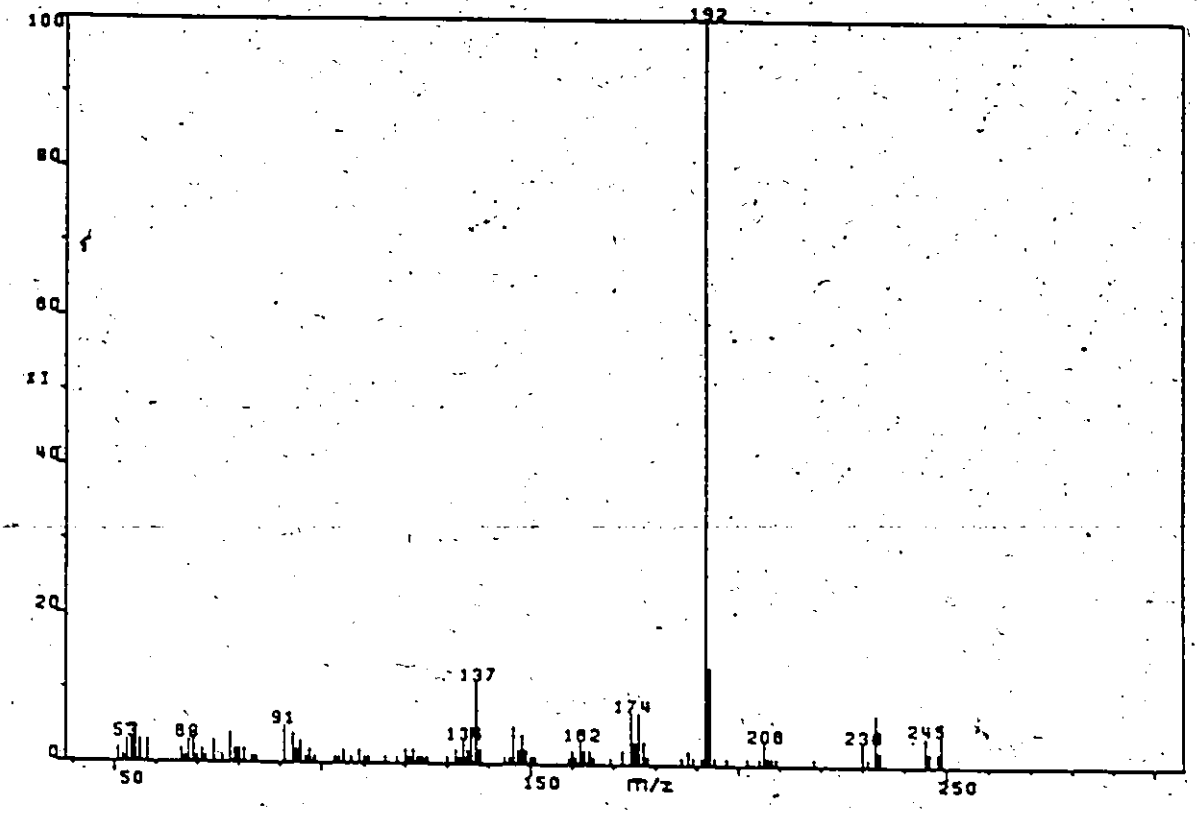


Figure 66 Mass spectrum of component D (dihydrolycopodine).

Figure 67 Mass spectrum of dihydrolycopodine (derived from Figure 66).



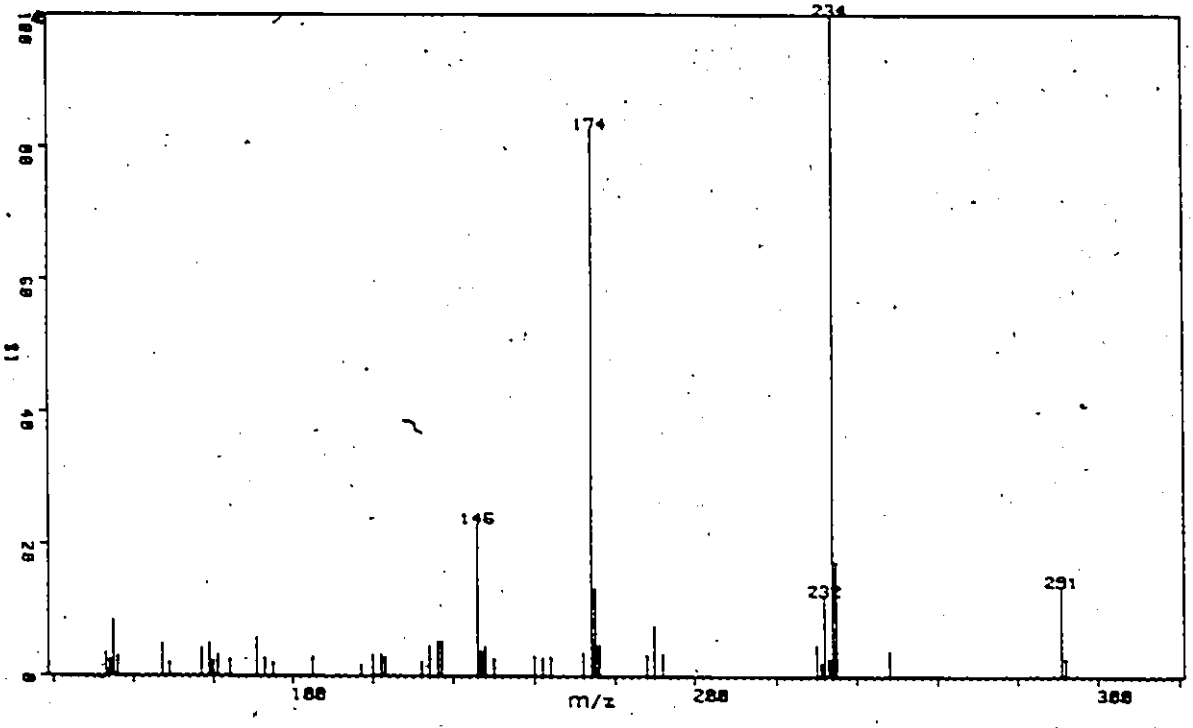
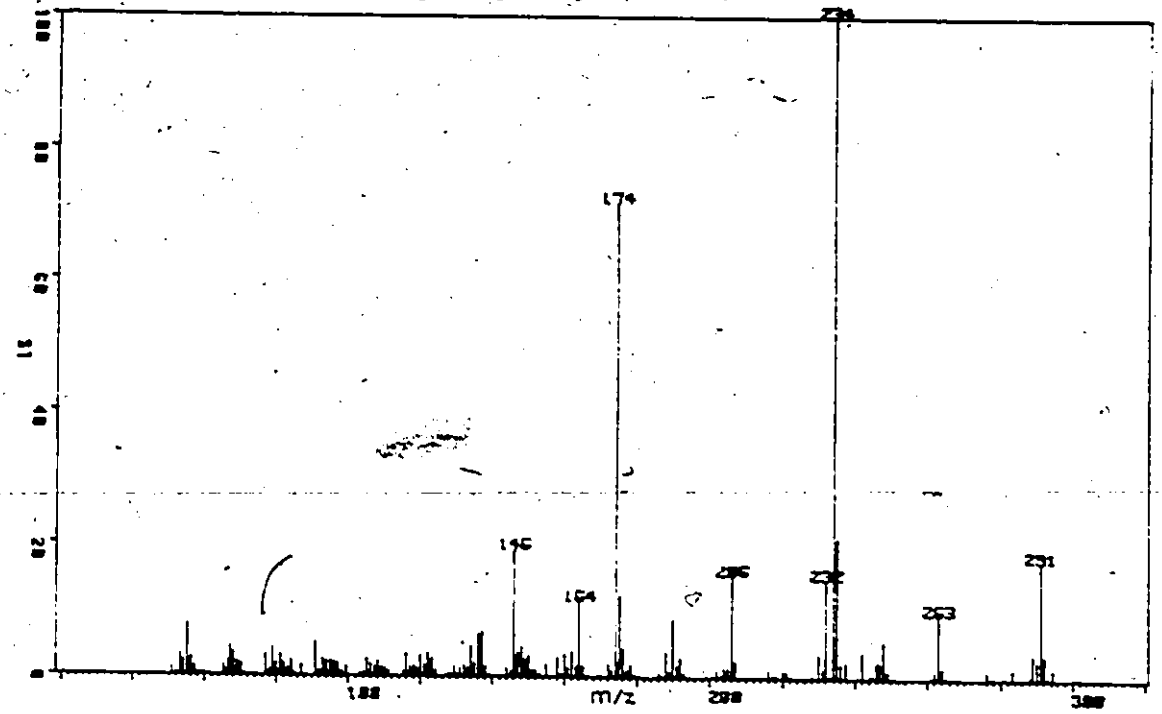


Figure 68 Mass spectrum of component F (acetyldihydrolycopodine).

Figure 69. Mass spectrum of component E and F (flabelliformine and acetyldihydrolycopodine).



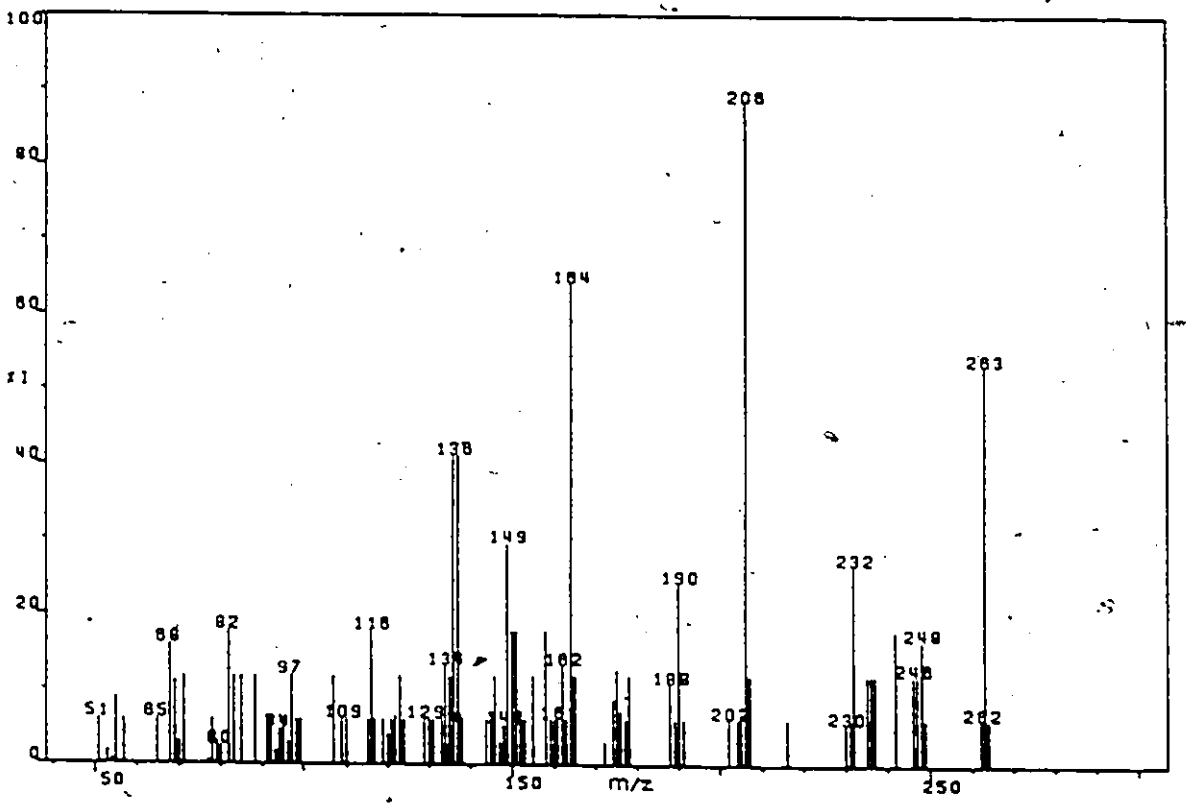
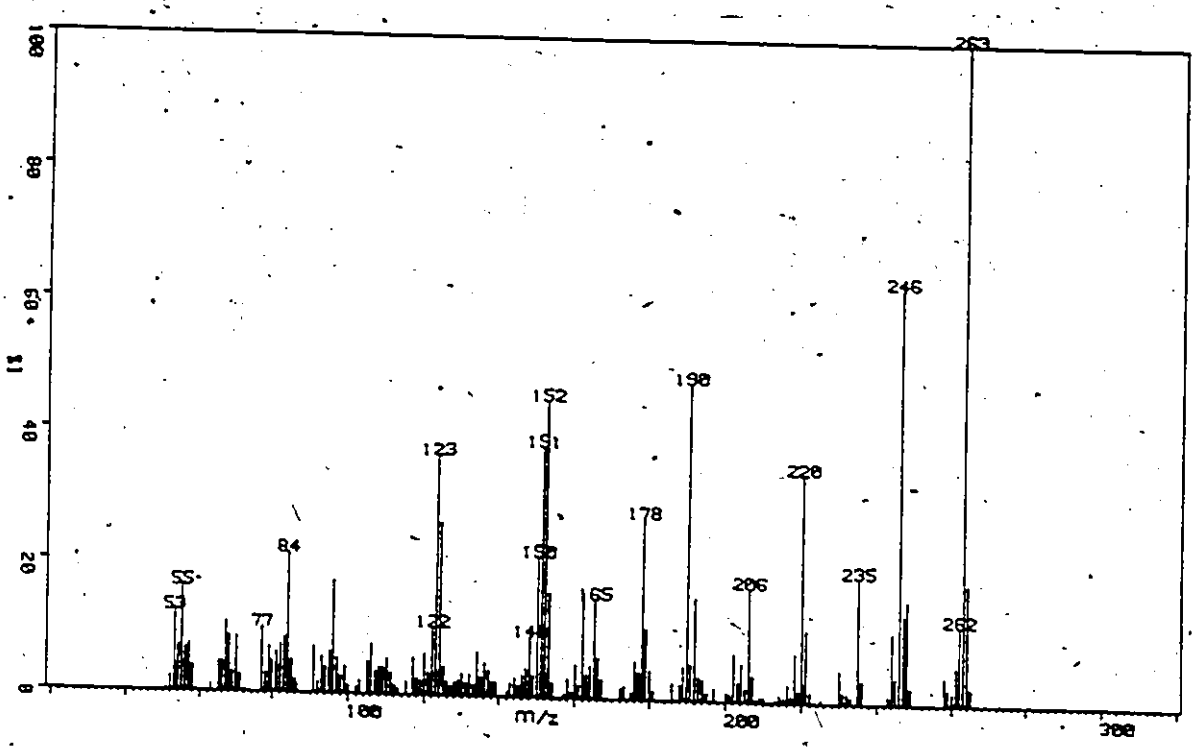


Figure 70 Mass spectrum of flabelliformine (derived from Figure 69).

Figure 71 Mass spectrum of component G (lycodoline).



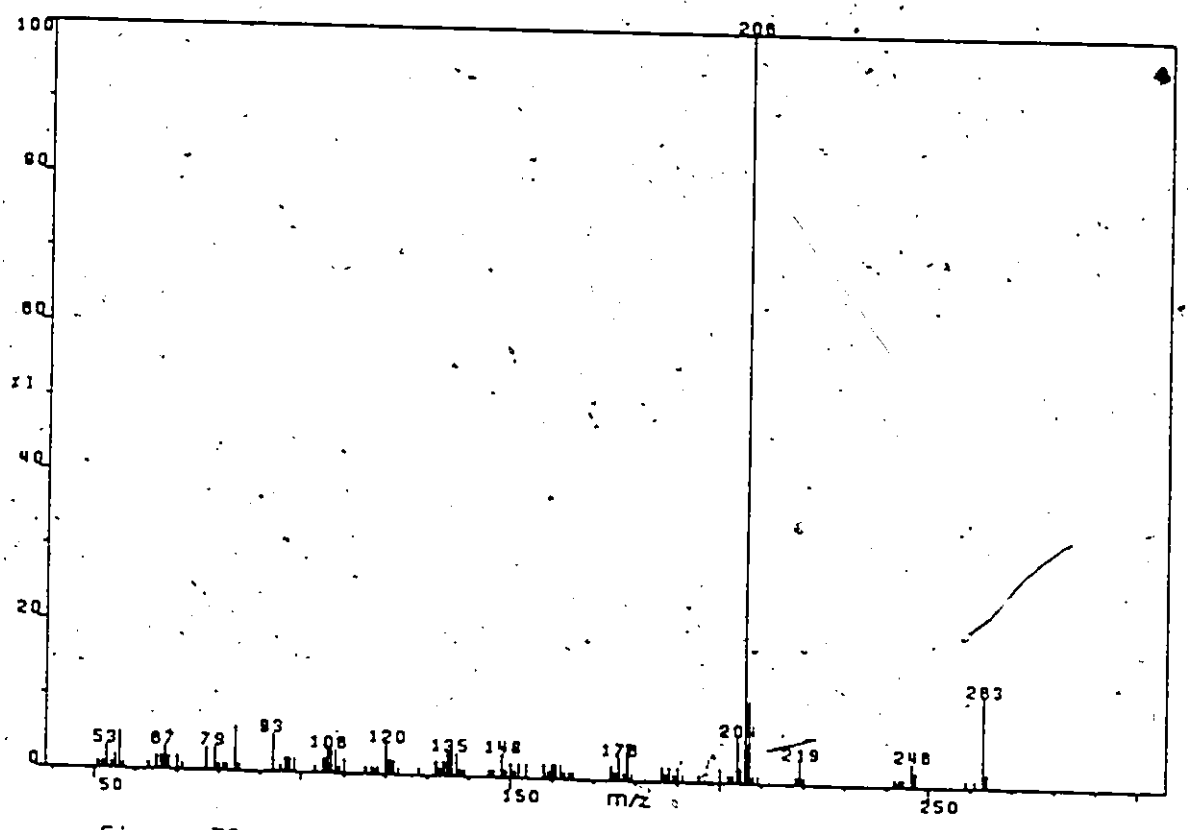
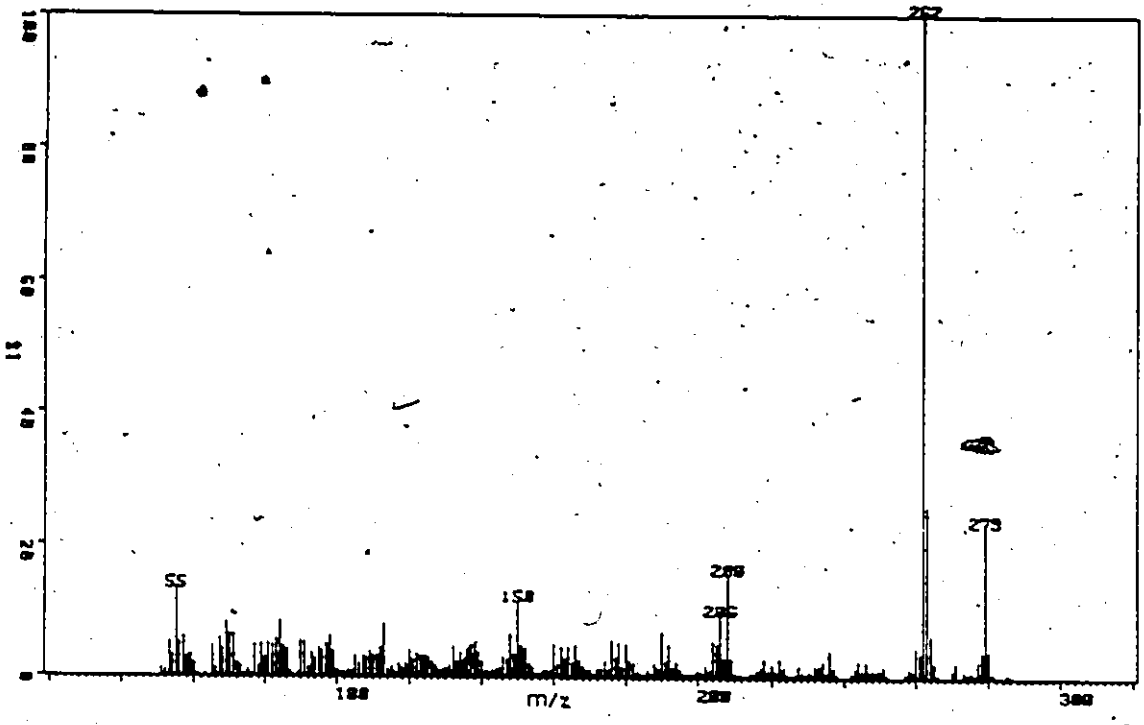


Figure 72 Mass spectrum of component H (L20).

Figure 73 Mass spectrum of component I.



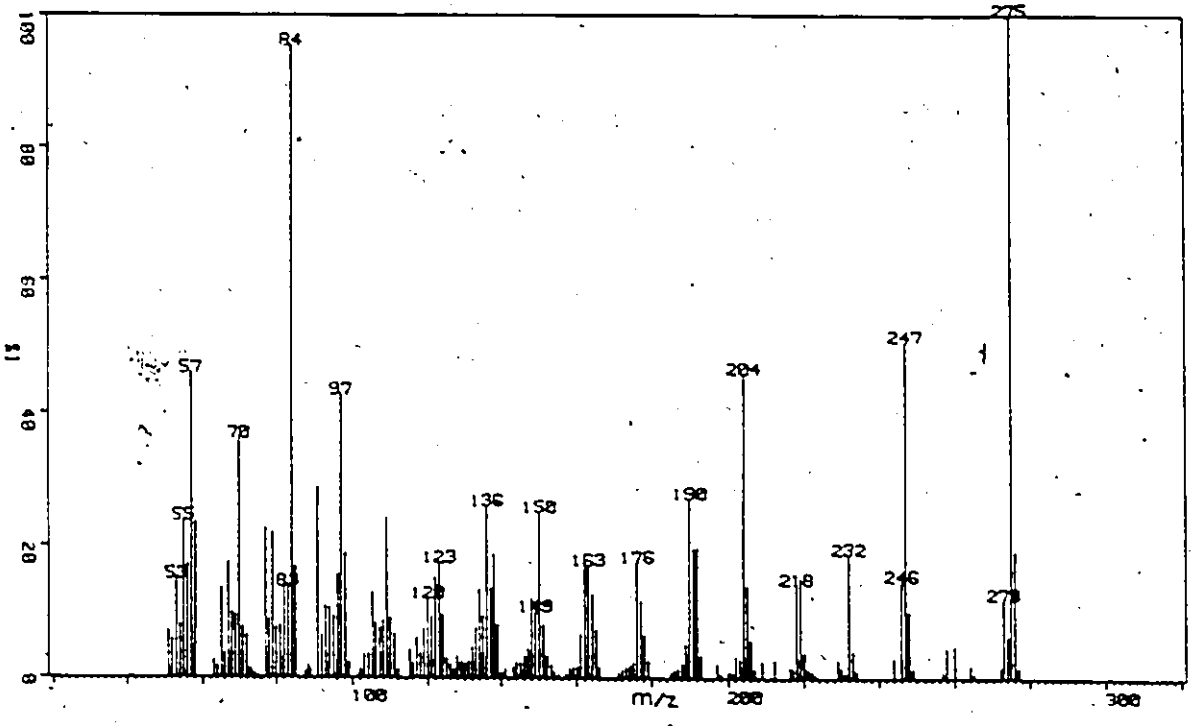
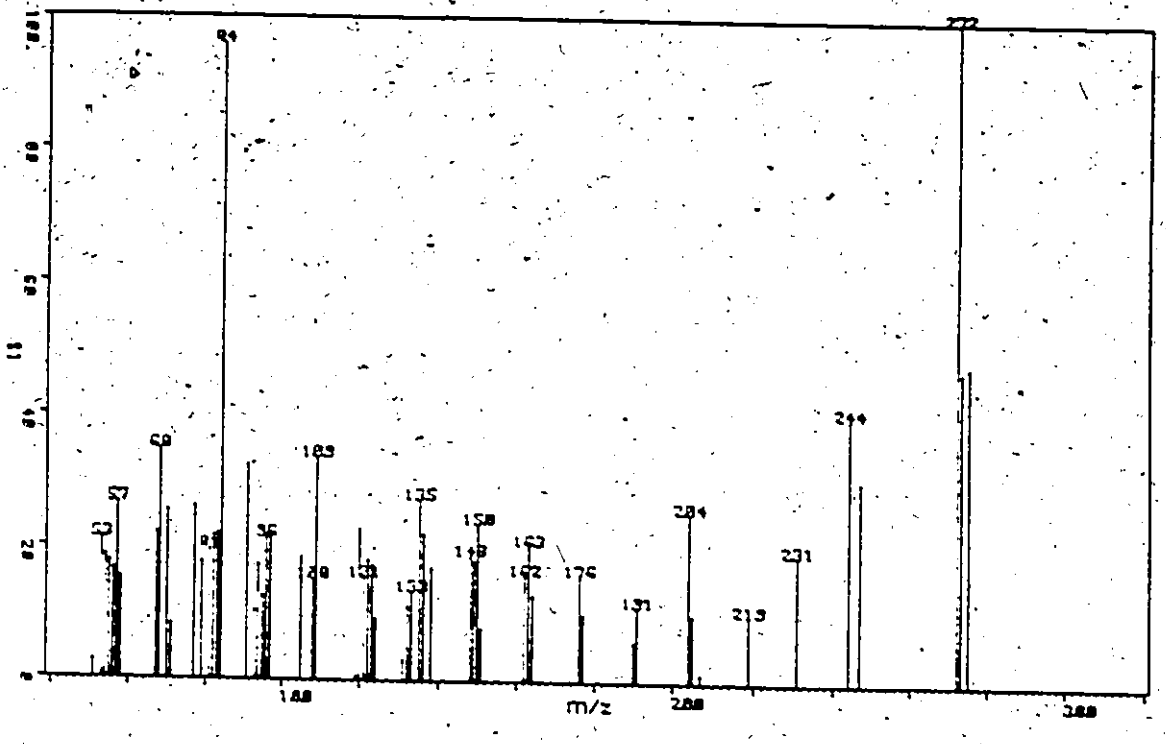


Figure 74 Mass spectrum of component J (lycoflexine).

Figure 75 Mass spectrum of component K (borbonicine).



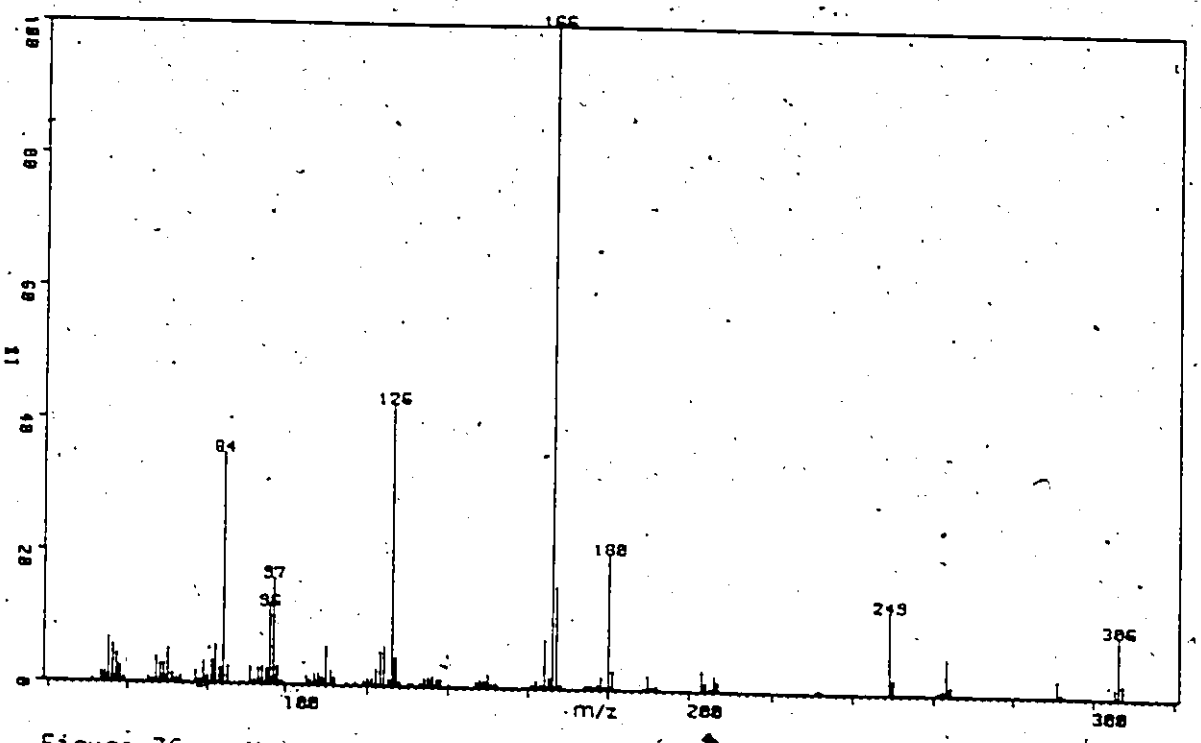
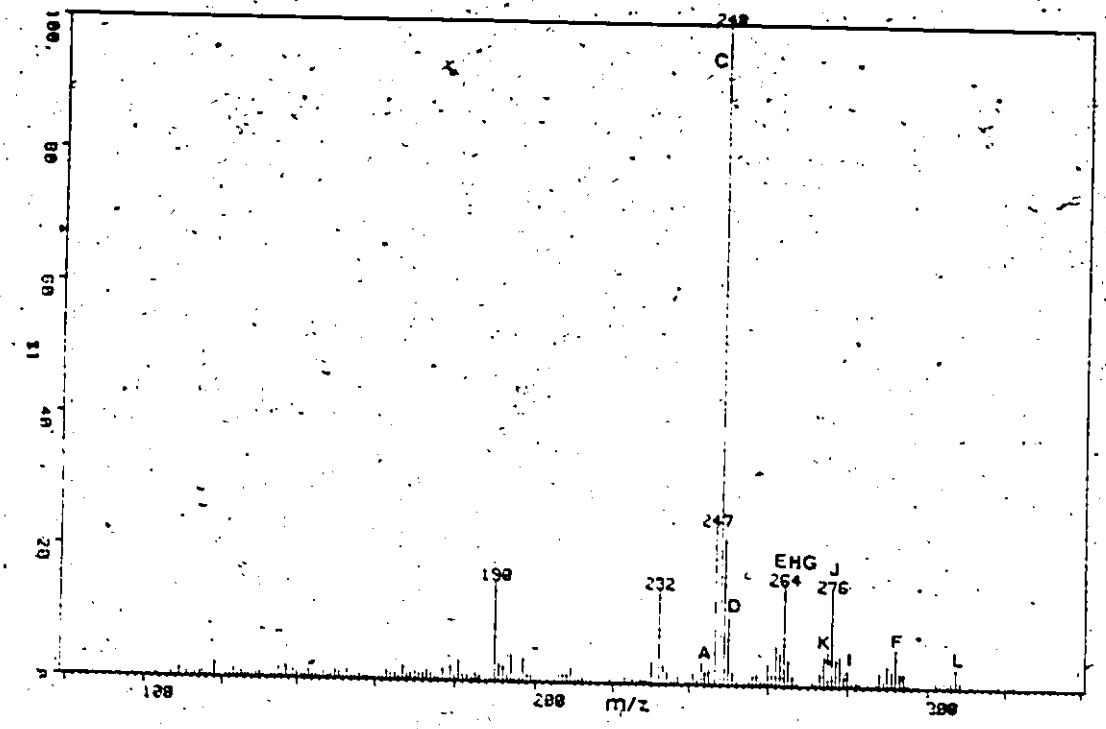


Figure 76 Mass spectrum of component L (N_{α}-acetyl-N_{ε}- methylphlegmarine).

Figure 77 Chemical ionization mass spectrometry of the L. clavatum var. borbonicum extract using methane as the reagent gas.



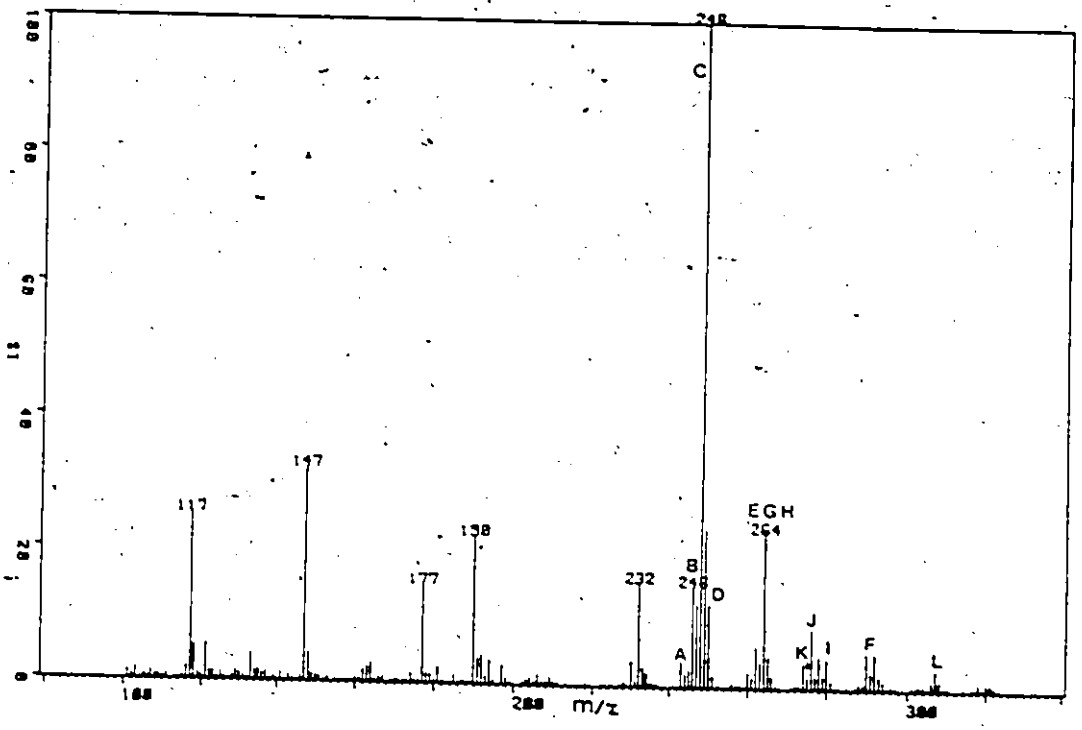


Figure 78 Chemical ionization mass spectrometry of the L. clavatum var. borbonicum extract using ammonia as the reagent gas.

been inferred on the basis of synthesis.[215,216] Based on the amount of plant extracted and a 1.4% abundance of this alkaloid in the total alkaloid content, about 100 milligrams could be expected. Using the liquid chromatography methods outlined in the experimental (section 2.8) it was not possible to isolate N_α-acetyl-N_β-methyl phlegmarine in pure form.

3.3.4 Examination of Lycopodium australianum

The results of the examination of the alkaloid extract by gas chromatography are illustrated in Figures 79 and 80. The retention indices of each component and the relative percentage of each component in the mixture are recorded in Table 21. It is apparent from Figure 80 that A, B and C are nitrogen containing but that D is not. The extract was also examined by GC/MS and FSC/MS; the TIC are shown in Figure 81 and 82. The retention index of A suggests that it is lycodine. The E.I. mass spectra of A, B and D shown in Figures 83, 84 and 85, respectively, suggest that they are lycodine, cernuine and dioctyl phthalate. The fit with spectra of authentic samples as measured by the library search software are recorded in Table 21. Since the fit values are close to the maximum of 1000 this indicates that the assignments are highly probable. The CI spectra of A and B (shown in Figures 86 and 87) obtained from FSC-MS in the CI mode show the pseudo molecular ions at (M + H) and (M + C_2H_5), in the case of A where ions appear at m/z 243 and 271, respectively, and in the case of B at m/z 263 and 291, respectively. These data conclusively demonstrate that peaks A and B correspond to lycodine and cernuine.

Compound C is apparently a new alkaloid. Its retention index and EI mass spectrum (shown in Figure 88) do not correspond to any of the known Lycopodium alkaloids. The EI mass spectrum has a single intense ion at m/z 166 but is otherwise uninformative. The composition of m/z 166 as measured by high resolution mass spectrometry was $C_{11}H_{20}N$ (found 166.164, calculated 166.160, deviation 4.6 mmu).

A probe spectrum run on a sample of C isolated by liquid chromatography showed an ion of low intensity (< 1% of m/z 166) at m/z 344. A molecular ion at m/z 344 is substantiated by the C.I. spectrum of C (Figure 89) which shows an $(M + H)^+$ ion at m/z 345. The fragment ion, m/z 166, is still the most intense peak in the spectrum and less intense ions are observed at m/z 344, 343 and 223, but an ion at $(M + C_2H_5)$ is not observed. Usually the ion at $(M + 29)^+$ is of low intensity (about 10% of the $(M + H)^+$ ion); thus it is not unusual that it is not observed.

An ion at m/z 166 is a feature of the mass spectra of phlegmarane alkaloids[172,177] where it has been attributed to the fragment shown in Figure 90. It is possible therefore that C contains the part structure shown in Figure 91. With the data available however it is not possible to draw any firm conclusions about the structure of X.

Wilce[202] has placed L. australianum in the subgenus Urostachys, section Selago. This is the first reported isolation of cernuane type alkaloids from the subgenus Urostachys. Cernuane alkaloids have previously been found to be elaborated only by plants of the subgenus Lepidotis.[16,18,19,23,158,159] However plants of the subgenus

Table 21

Retention indices, computer search fit values and percent total alkaloid for each component from L. australianum

	Compound	R.I.	A.R.I.	Pure	Mix	Reverse	# of scans	Pk.h.
A	lycodine	1921	1930	811	841	915	20	3.0
B	cernuine	2268	-	678	716	932	16	82.0
C	unknown	2422	-	-	-	-	-	15.0
								100.0

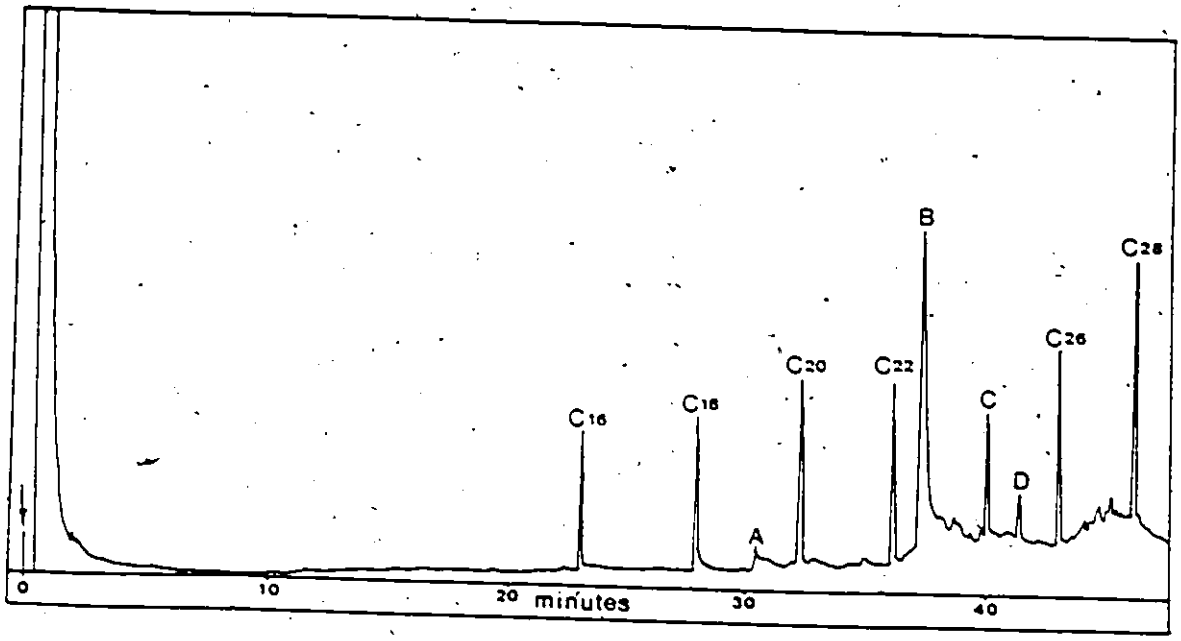


Figure 79 FSC/FID chromatogram of L. australianum extract with hydrocarbon standards.

A Lycodine

B cernuine

C unknown 344

D dioctyl phthalate

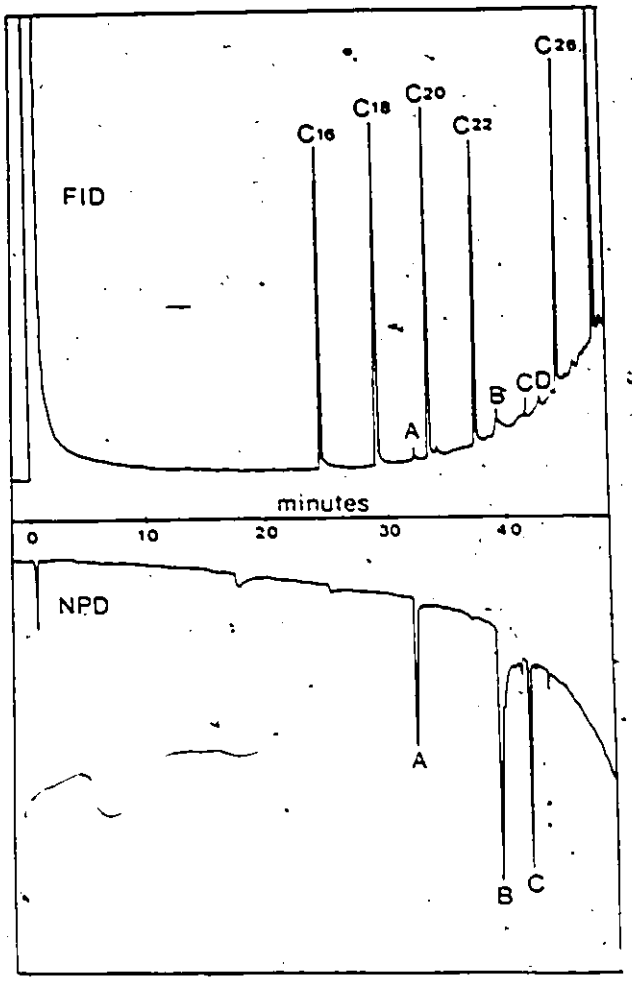
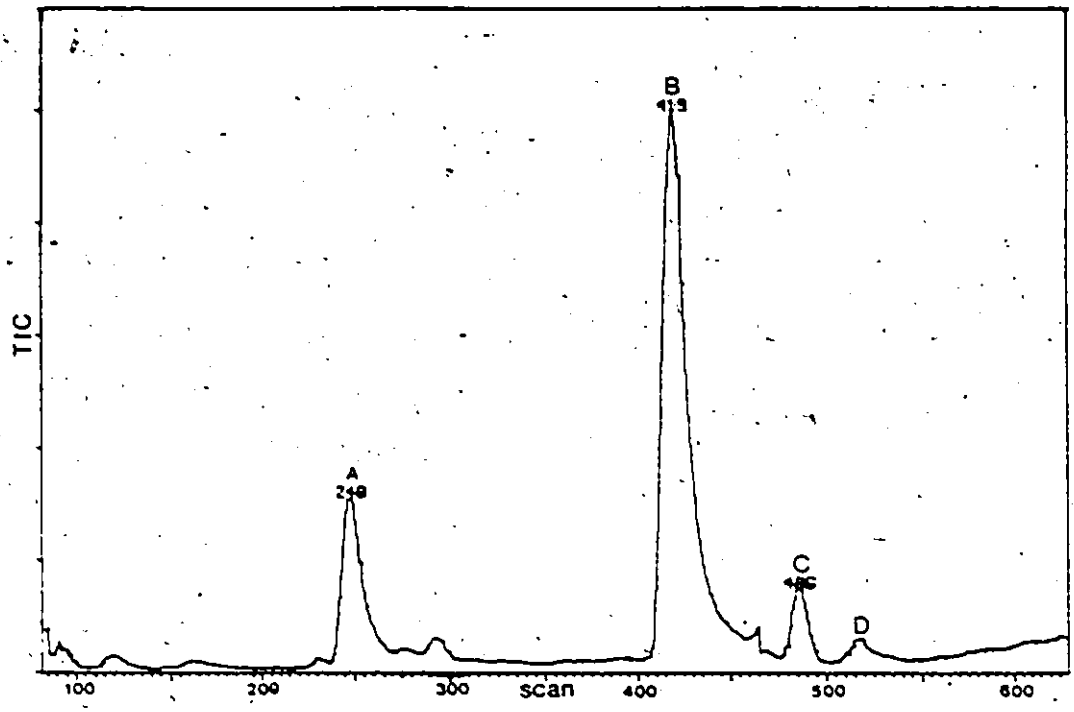


Figure 80 FSC/FID/NPD chromatogram of L. australianum extract with hydrocarbon standards.

Figure 81 GC/MS TIC of L. australianum extract.



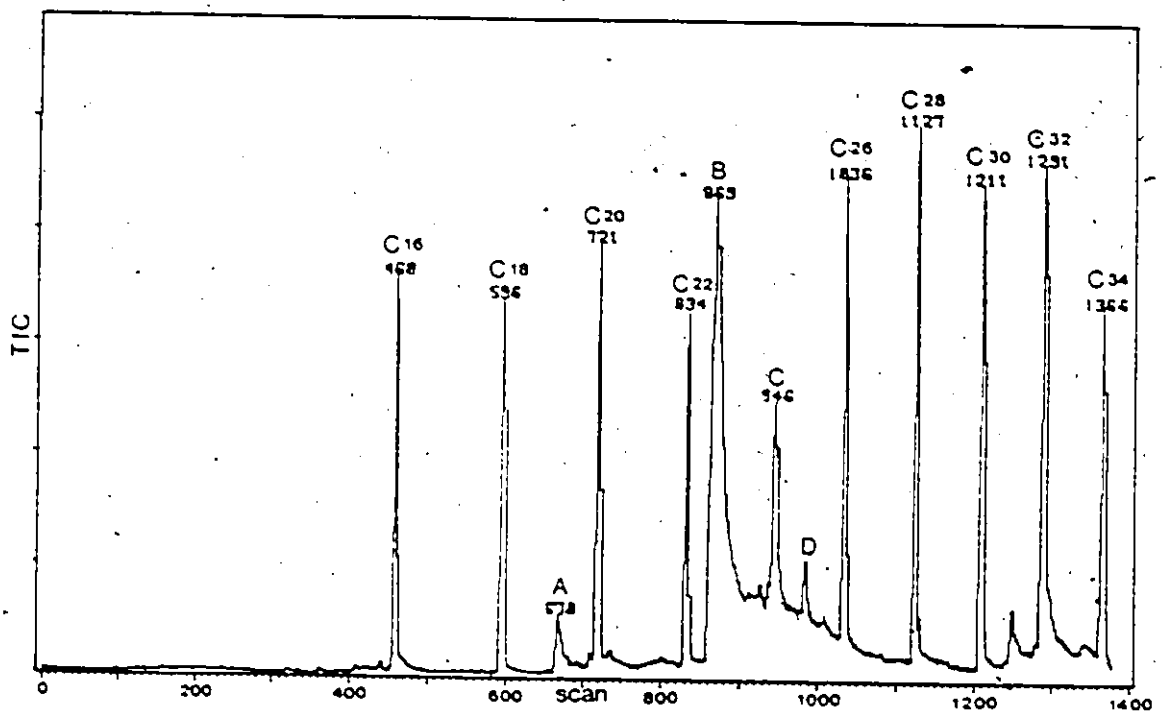
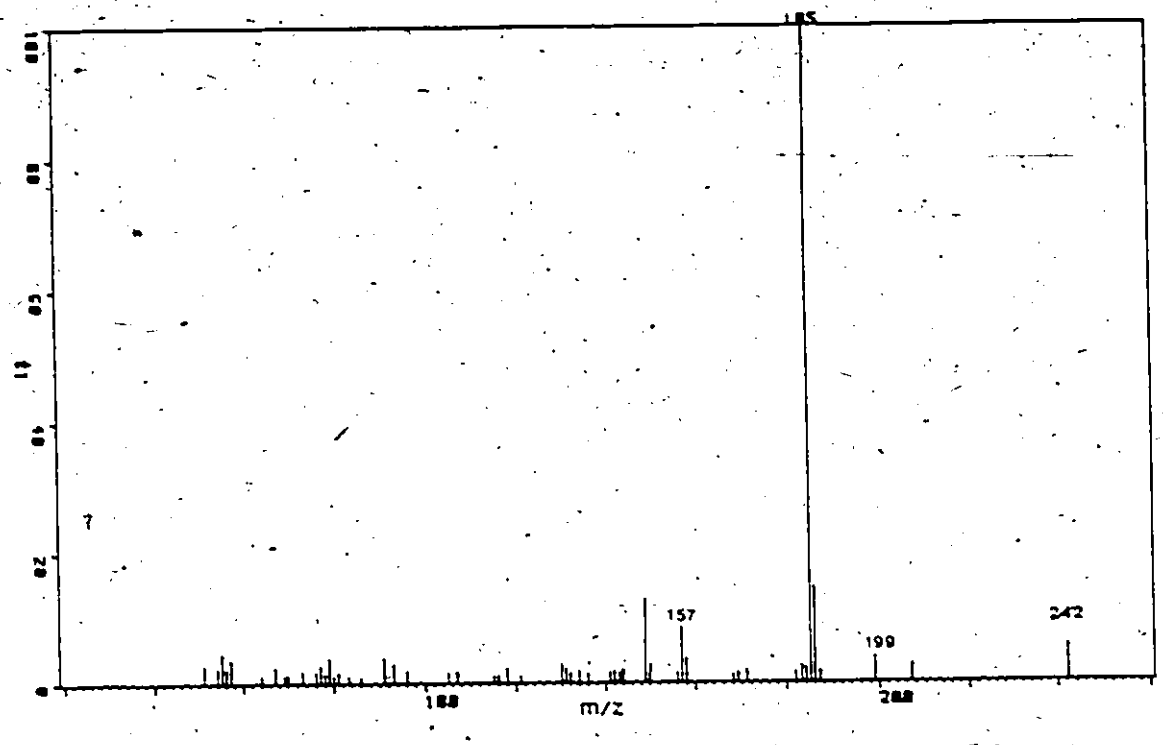


Figure 82 FSC/MS TIC of L. australianum extract with hydro carbon standard.

Figure 83 Mass spectrum of component A (lycodine).



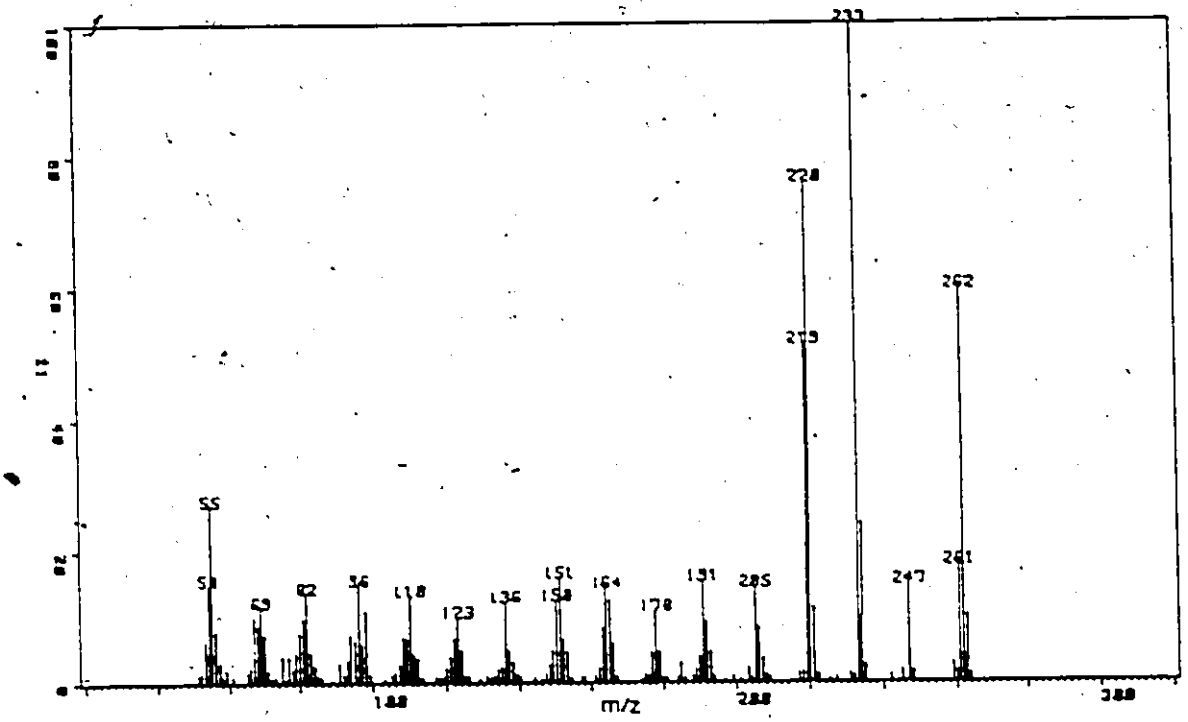
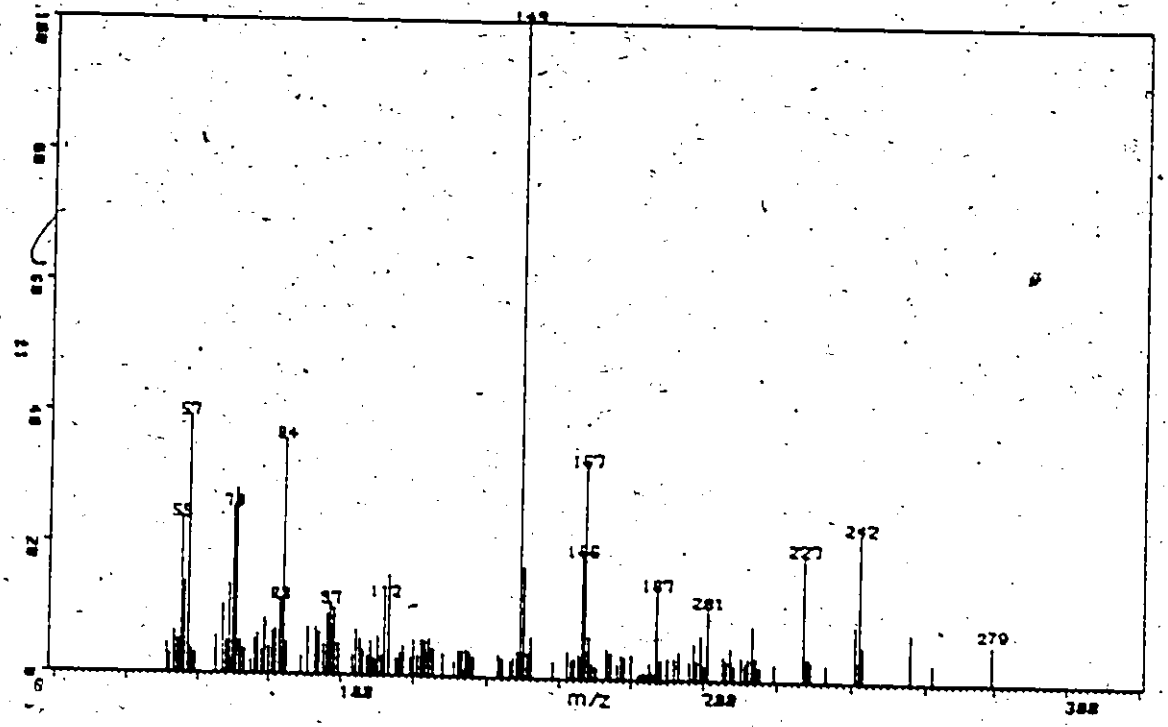


Figure 84 Mass spectrum of component B (cernuine).

Figure 85 Mass spectrum of component D (dioctyl phthalate).



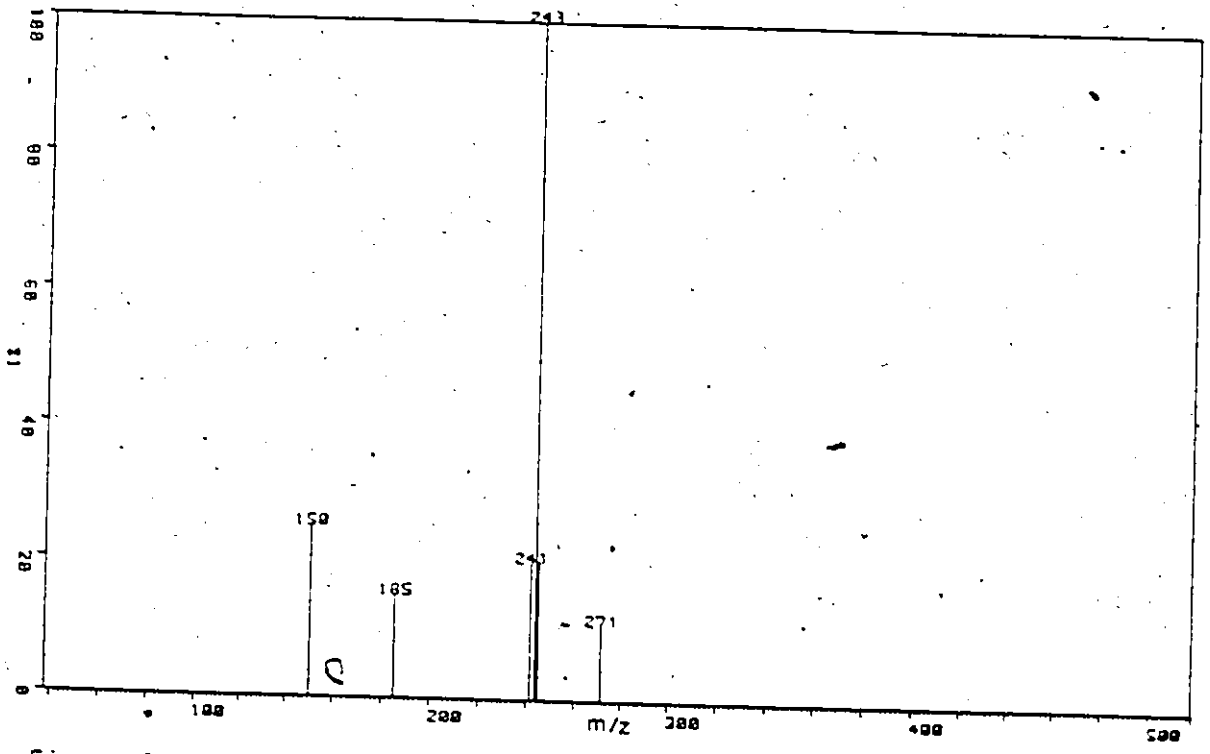
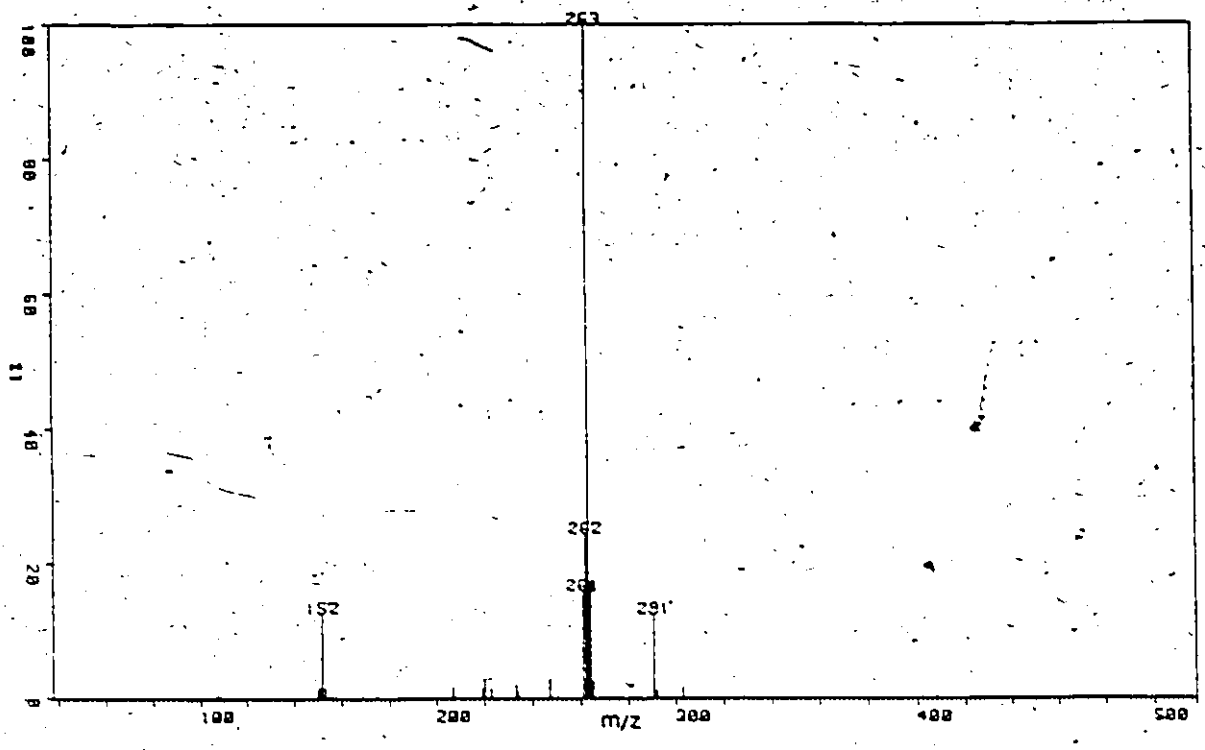


Figure 86 Chemical ionization mass spectrum of component A (lycodine).

Figure 87 Chemical ionization mass spectrum of component B (cernuine).



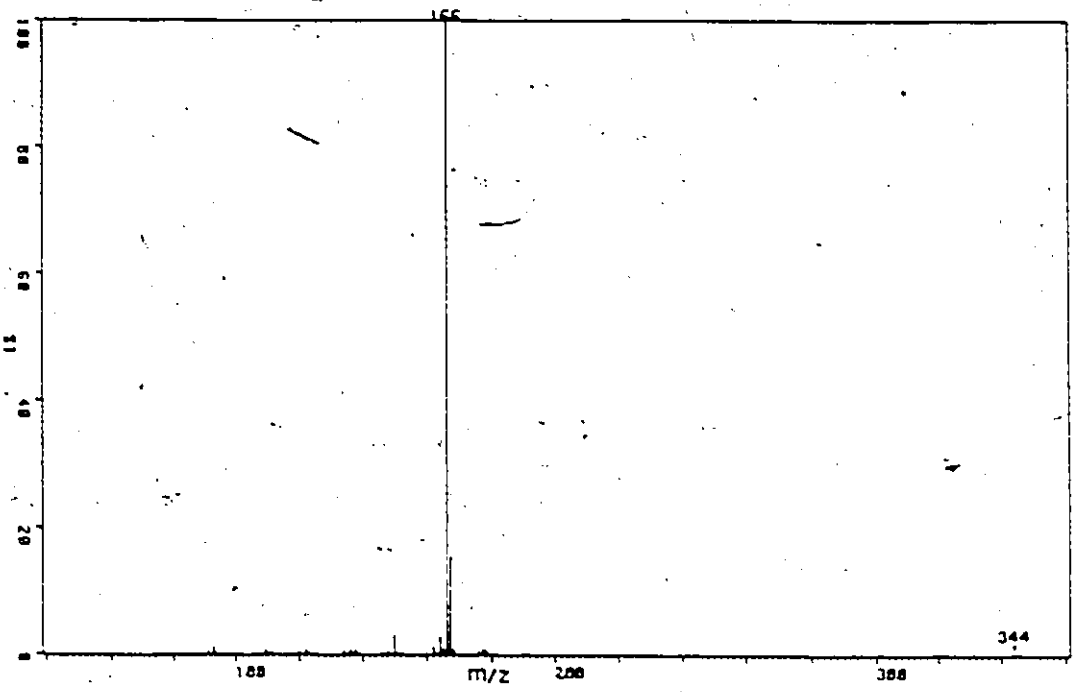


Figure 88 Mass spectrum of component C.

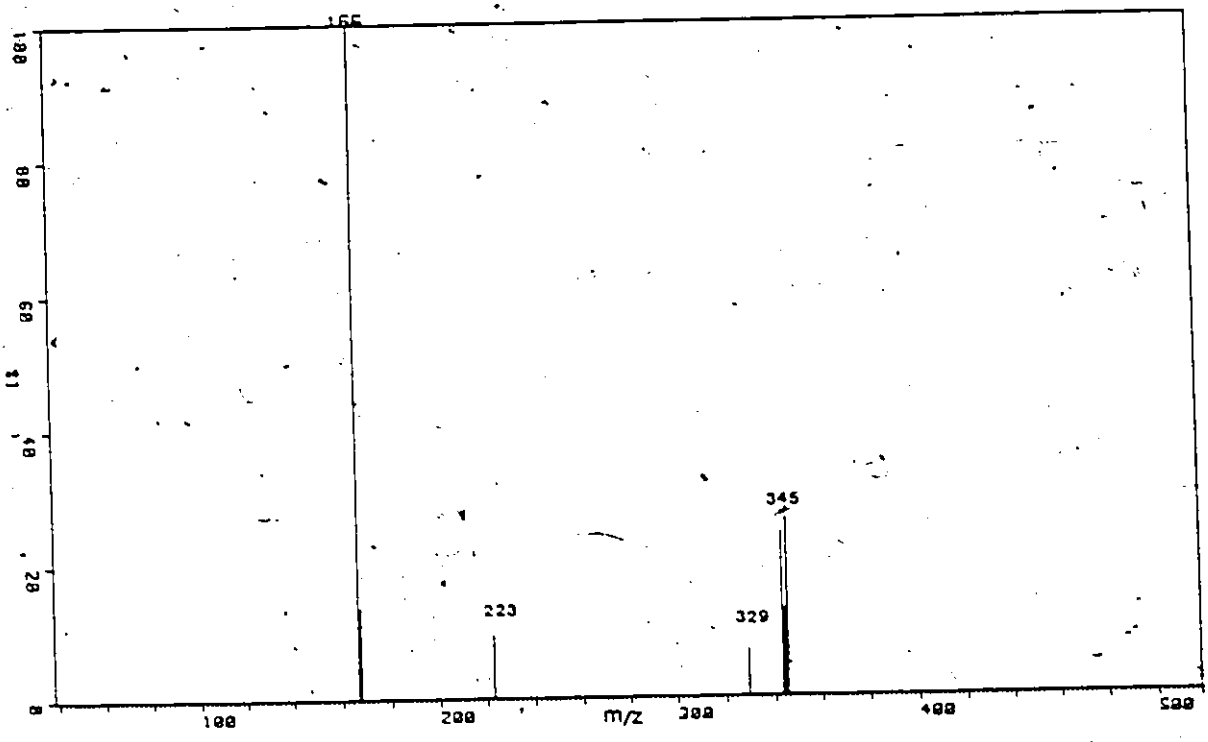


Figure 49 Chemical Ionization mass spectrum of compound C using methane as the reagent gas.

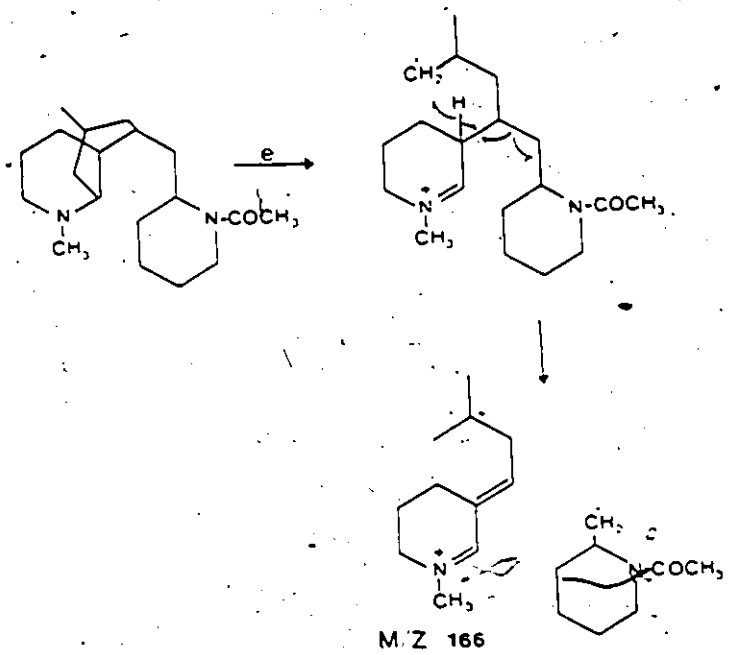


Figure 90 Fragmentation of phlegmarane type alkaloids.

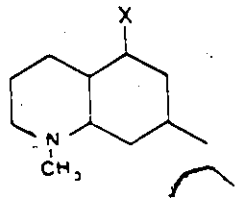


Figure 91 Partial structure of component C .

Urostachys have been found to be rich in phlegmarane type alkaloids.[25,27] Compound C may prove to be a new skeletal type.

3.4.5 Examination of L. deuterodensum

L. deuterodensum or L. densum has been previously examined and found to contain lycopodine, clavolonine (L34) and L35.[147] Alkaloid L35 melting at 235°C, was assigned the formula $C_{14}H_{21}NO_2$ on the basis of elemental analysis. This alkaloid does not correspond in composition or properties to ~~any~~ Lycopodium alkaloid of known structure.

The extract was examined by GC and GC/MS as shown in Figures 92 to 95. The results of GC with a FID and NPD detector are shown in Figures 92 and 93 while the TIC obtained from GC/MS and FSC/MS are shown in Figures 94 and 95. The retention indices and computer search fit values are tabulated in Table 22. The percent of each component in the extract has been calculated from peak heights and areas from FSC/FID; the results are also tabulated in Table 22.

The first alkaloid (component A), identified by its retention index and its mass spectrum, shown in Figure 95, is lycodine. Component B which elutes along with lycodine has been identified from its mass spectrum shown in Figure 97 as anhydrolycodoline. Figure 98 shows the result of subtracting the mass spectrum of lycodine from the mass spectrum in Figure 97. Component C, the major alkaloid in the extract, was identified by its retention index and mass spectrum shown in Figure 99 as lycopodine. Eluting after lycopodine is component D with a molecular weight of 263. From the retention index and mass spectrum,

shown in Figure 100, the compound was identified as flabelliformine. The ion at m/z 190 in this mass spectrum arises from traces of lycopodine. Figure 101 shows the result of subtracting the mass spectrum of lycopodine from the mass spectrum in Figure 100. The next peak, E, contains several components as evident from the mass spectrum shown in Figure 102. An alkaloid with molecular weight of 263 present in this mixture may be identified as lycodoline or L23. These two alkaloids differ in that the hydroxyl group at C-12 is in the alpha position (L23) or in the beta position (lycodoline). By comparing the literature mass spectra of lycodoline and L23 with the ions attributed to E in Figure 102, component C was tentatively identified as lycodoline. Figure 103 shows the result of subtracting the mass spectrum of lycodoline from Figure 102. The ions at m/z 271, 243 and 163 can then be attributed to unknown F. The intense loss of 28 may indicate the presence of an α-amino ketone. Another component elutes after component F with an apparent molecular weight of 261, however due to the very low intensity of the mass spectrum (which is not included) it could not be identified.

From its retention index and its mass spectrum it was possible to identify clavolonine as one of the coeluting components of the peak labelled H and I. The other component (I) was identified as lycoflexine from the mass spectrum shown in Figure 104. Figure 105 shows the result of subtracting the mass spectrum of clavolonine from the mass spectrum in Figure 104, the characteristic peaks at m/z 275 and 84 being indicative of lycoflexine. The characteristic ions of clavolonine at m/z 263, and 190 are observed in Figure 106 when the mass spectrum of lycoflexine was subtracted from the mass spectrum in Figure 104.

Peak J was identified as flabelline from its mass spectrum shown in Figure 107 and its retention index which more closely matches that of flabelline than its isomer, flabellidine. The peak labelled K,L contains two unknowns with molecular weights of 279 and 304, as shown in Figure 108 and 109, taken from the leading and trailing edge of the peak, respectively. Component K has a mass spectrum that is similar to that of component I found in L. clavatum. The mass spectrum of component K most closely fits that of alopecuridine. However sufficient amounts of pure alopecuridine were not available to test the indentity of Component K. Component L represents a mixture, however one of the major components can be assigned as having a molecular weight of 304. Peak M contains an unknown with a molecular weight of 272, whose mass spectrum shown in Figure 110 is quite unlike the mass spectra of most Lycopodium alkaloids.

Chemical ionization mass spectrometry of the extract with methane and ammonia gave the results shown in Figure 111 and 112. The alkaloids that were identified by their EI spectra yielded $(M + H)^+$ ions.

The unknowns represent only 4.3% of the total alkaloids (by integration) and attempts to isolate sufficient pure samples for structural elucidation were not successful.

3.4.6 Examination of L. fastigiatum

Examination of the extract by FSC/FID/NPD and FSC/FID gave the results shown in Figures 113 and 114. The TIC chromatograms of the GC/MS and FSC/MS experiments are shown in Figures 115 and 116. The retention indices and computer search fit values are listed in Table 23. The

Table 22

Retention indices, computer search fit values and percent total alkaloid for each component of L. deuterodensum

Compound	R.I.	A.R.I.	Pure	Mix	Reverse	# of scans	Pk.h.	Int.
A lycodine	1936	1930	754	808	933	1	2.9	1.1
B anhydroly-codoline	1954	-	324	443	724	1		
C lycopodine	2018	2030	711	829	927	18	76.6	93.6
D flabelliformine	2091	2070	615	615	994	1	1.2	0.1
E lycodoline	2145	2133	524	524	989	1		
F unknown mol wt 271	2145	-	-	-	-		5.3	3.4
G unknown mol wt 261	2145	-	-	-	-	-		
H lycoflexine	2286	-	459	509	857	1	5.3	0.7
I clavolonine	2308	2300	767	767	980	1		
J flabelline	2384	2422	591	865	665	11	2.0	0.2
K unknown mol wt 279	2416	-	-	-	-	-	4.7	0.4
L unknown mol wt 304	2416	-	-	-	-	-		
M unknown mol wt 272	2526	-	-	-	-	-	2.0	0.5
							100.0	100.0

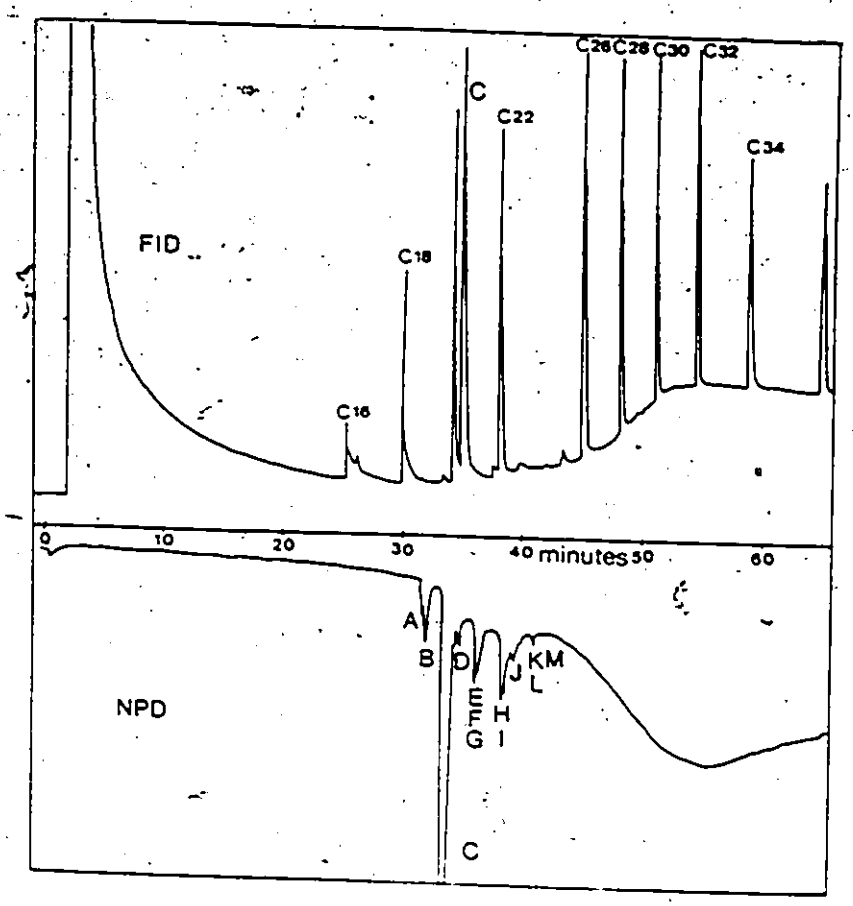


Figure 92 FSC/FID/NPD chromatogram of L. deuterodensum extract with hydrocarbon standards.

A lycodine

B anhydrolycodoline

C lycopodine

D flabelliformine

E lycodoline

F unknown 271

G unknown 261

H lycoflexine

I clavolonine

J flabelline

K unknown 229

L unknown 304

M unknown 272

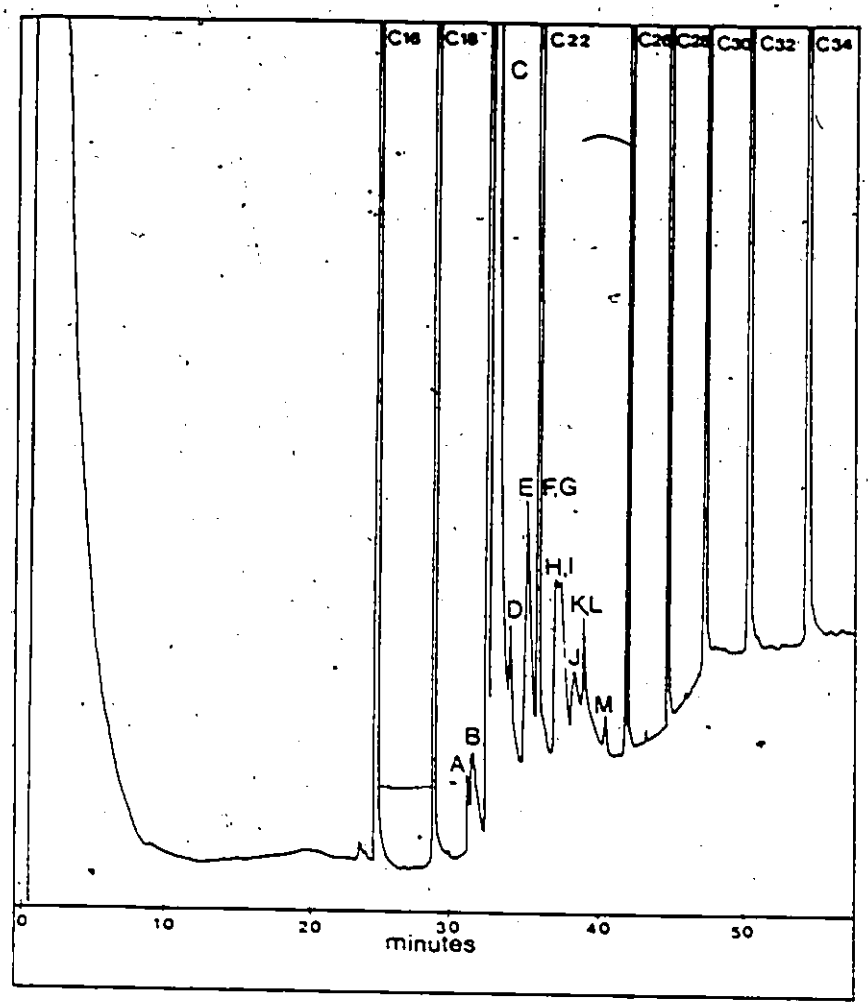
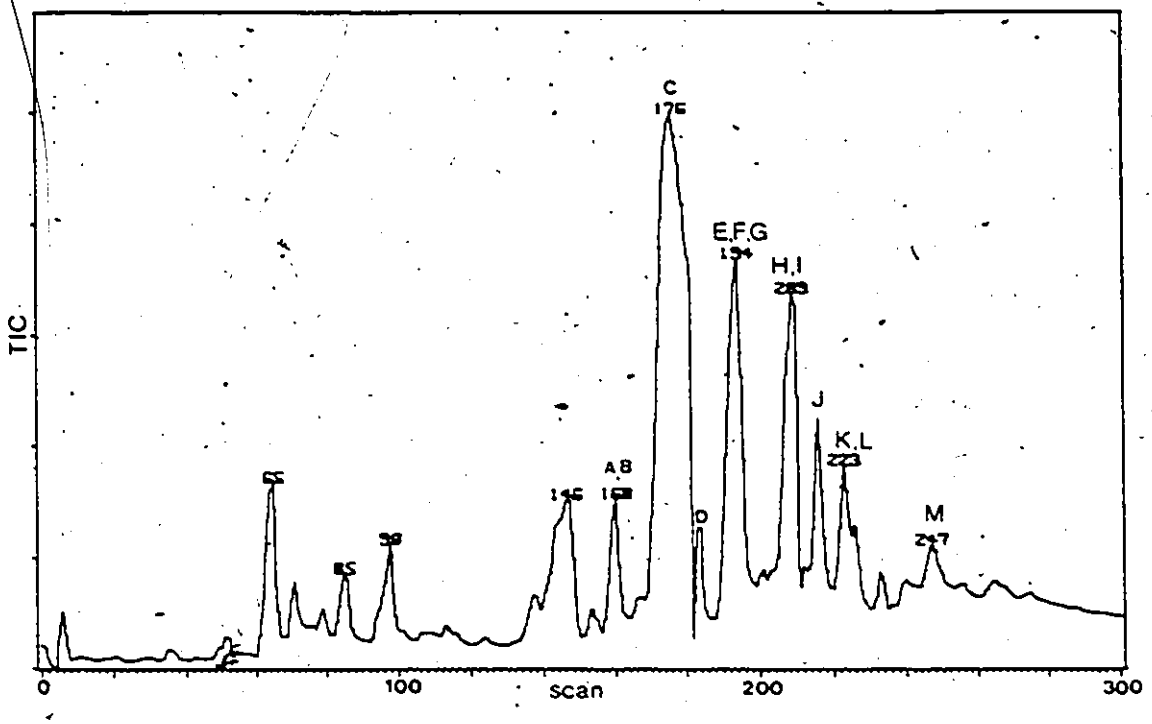


Figure 93 FSC/FID chromatogram of L. deuterodensum extract with hydrocarbon standards.

Figure 94 GC/MS TIC of L. deuterodensum extract.



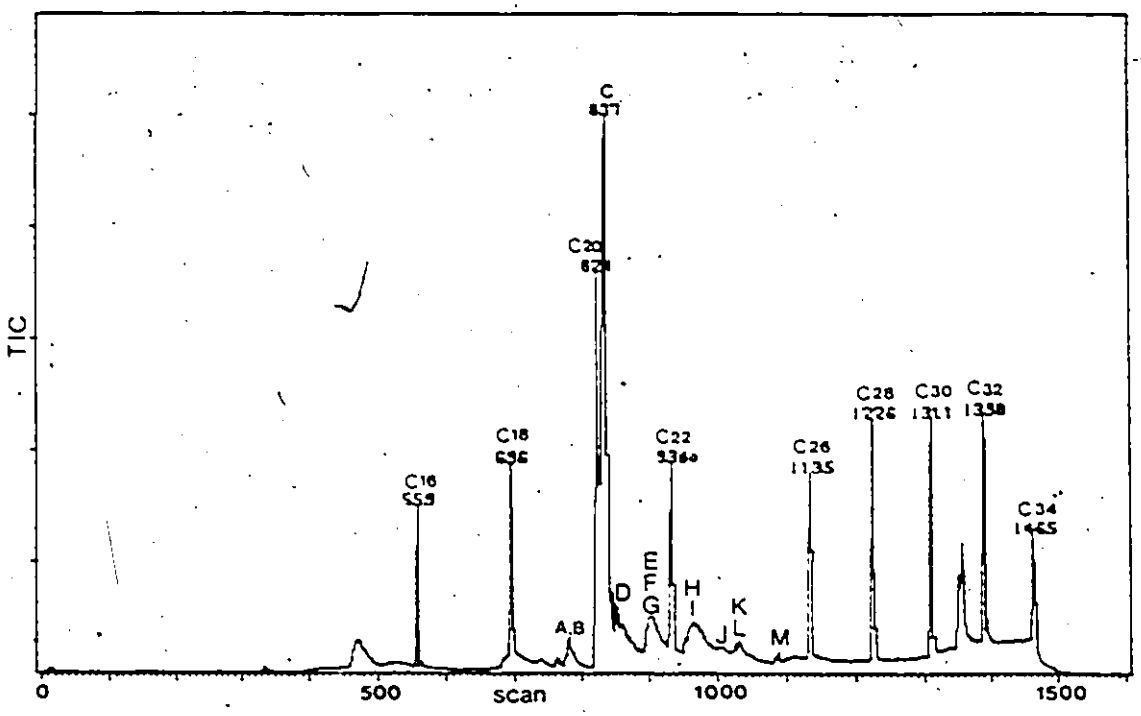
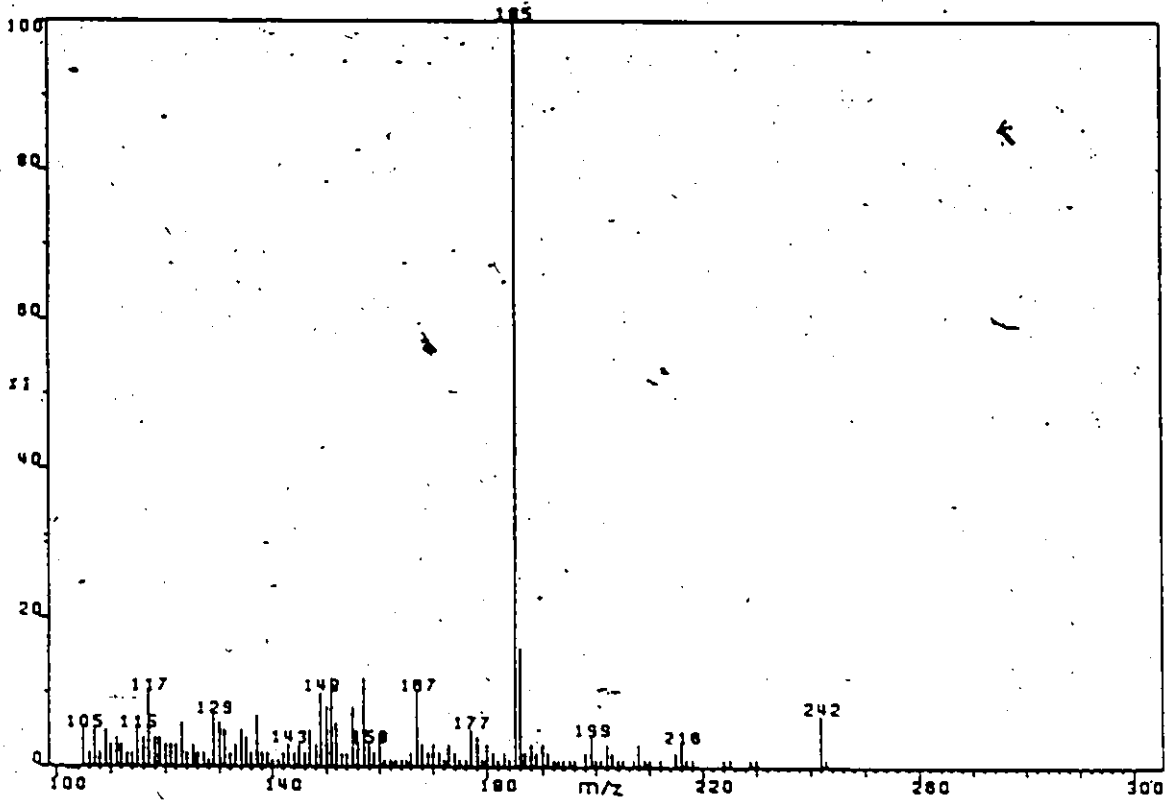


Figure 95 FSC/MS TIC of L. deuterodesnum extract.

Figure 96 Mass spectrum of component A (lycodine).



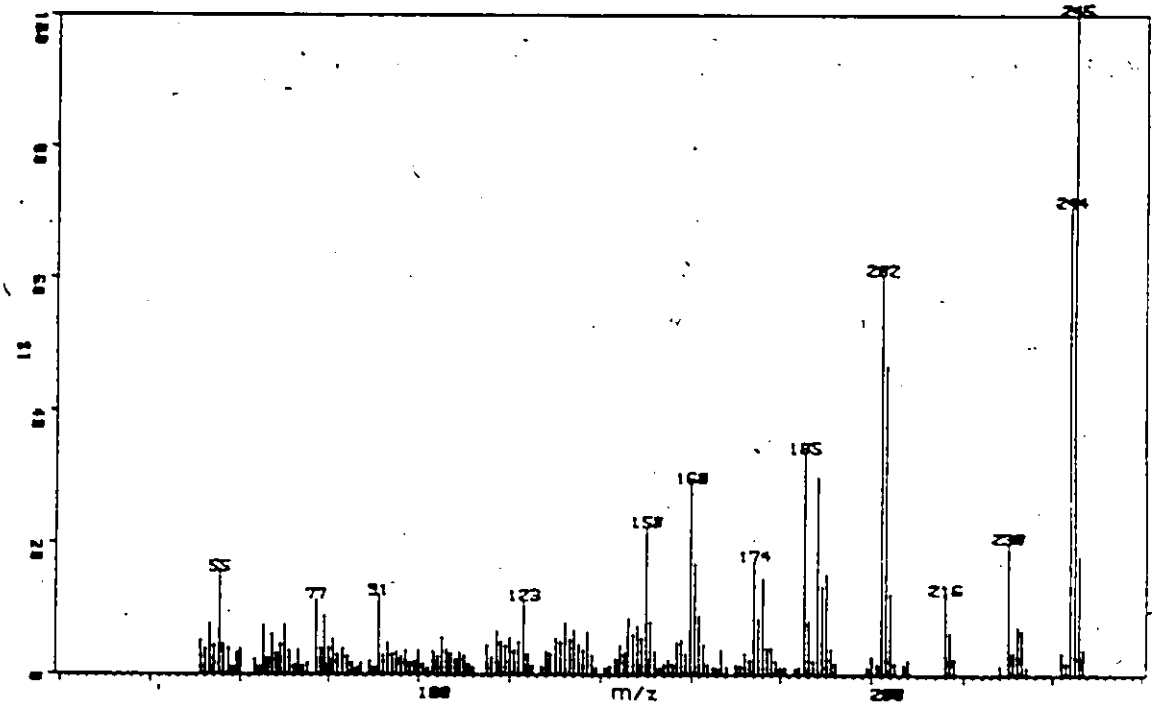
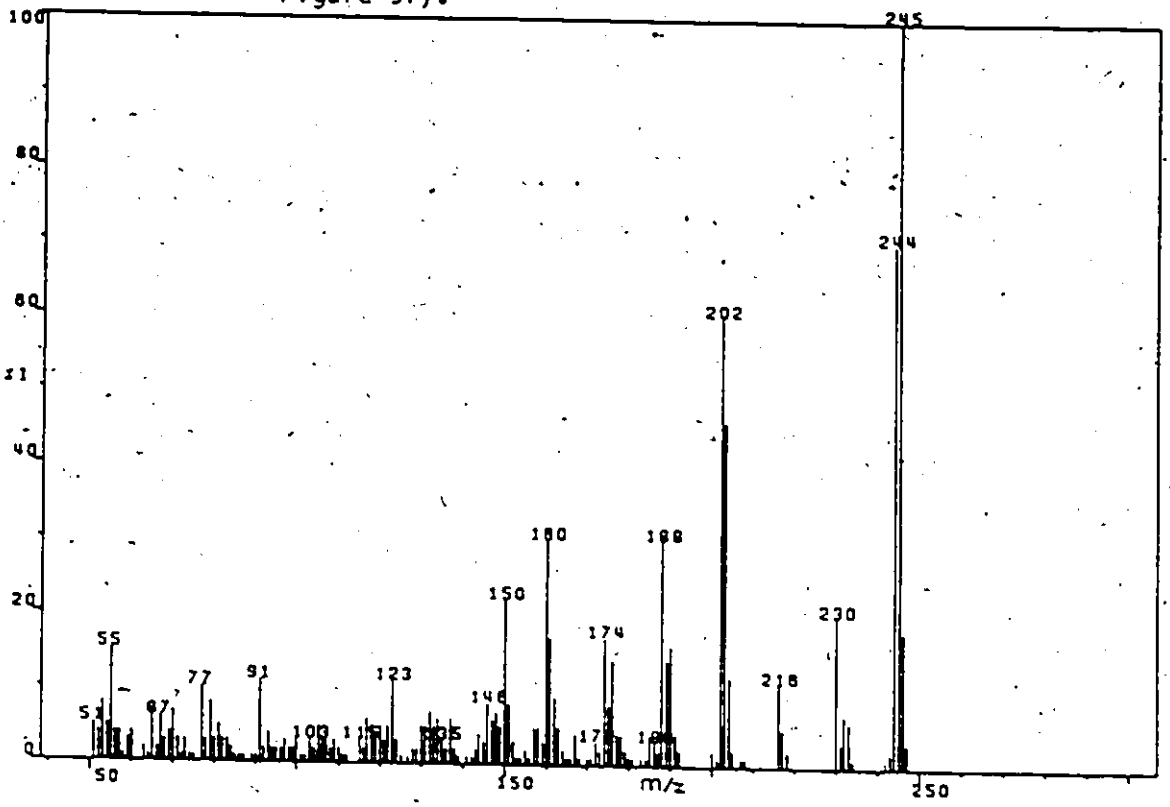


Figure 97 Mass spectrum of component B (anhydrolycodoline).

Figure 98 Mass spectrum of anhydrolycodoline (derived from Figure 97).



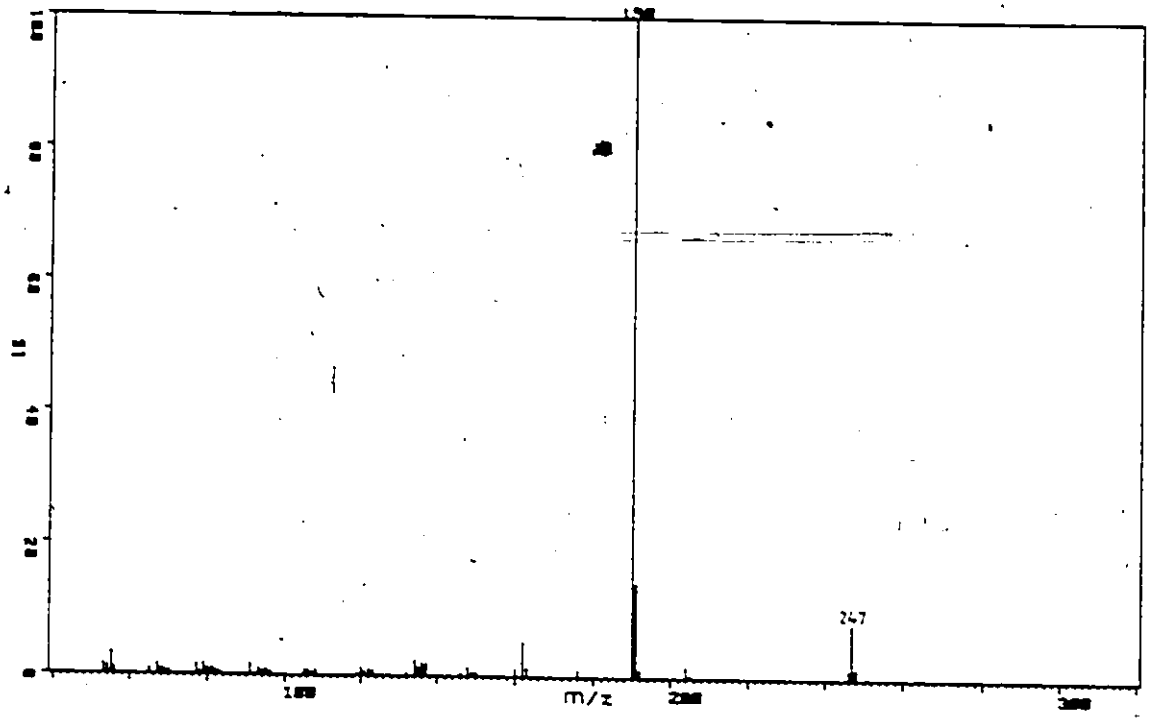
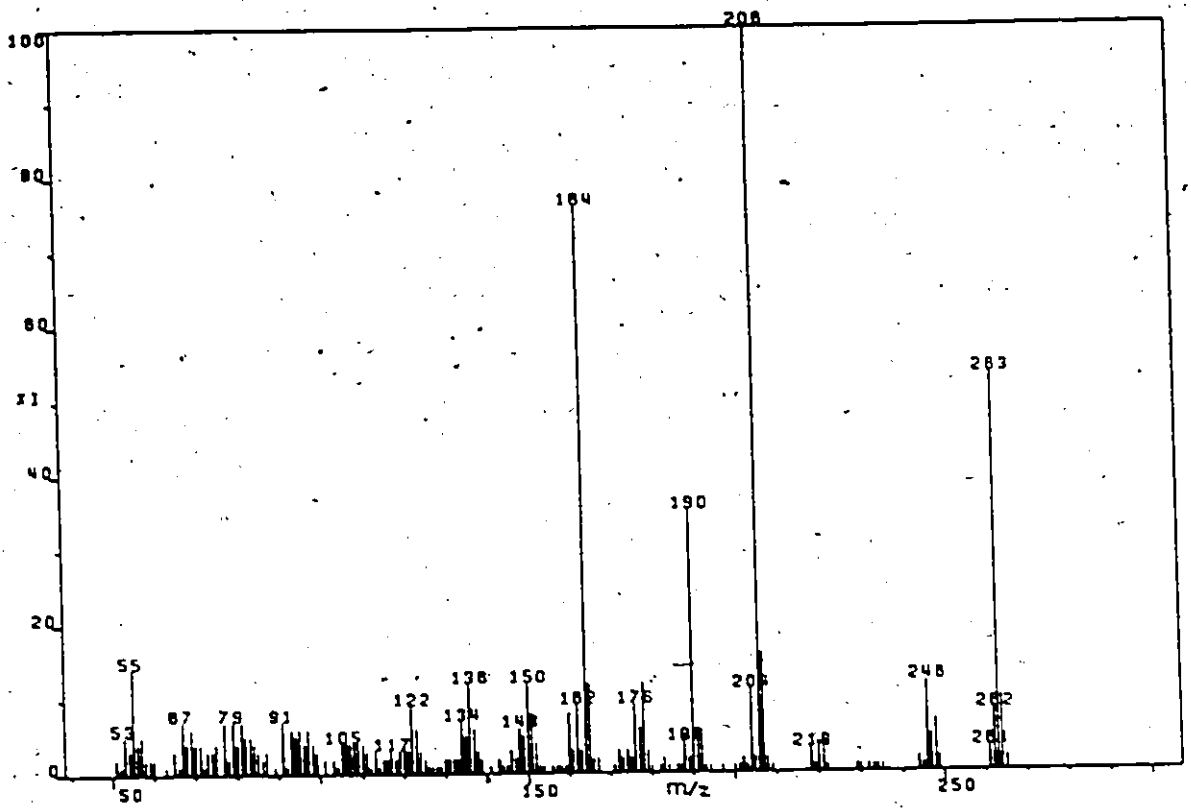


Figure 99 Mass spectrum of component C (lycopodine).

Figure 100 Mass spectrum of component D (flabelliformine).



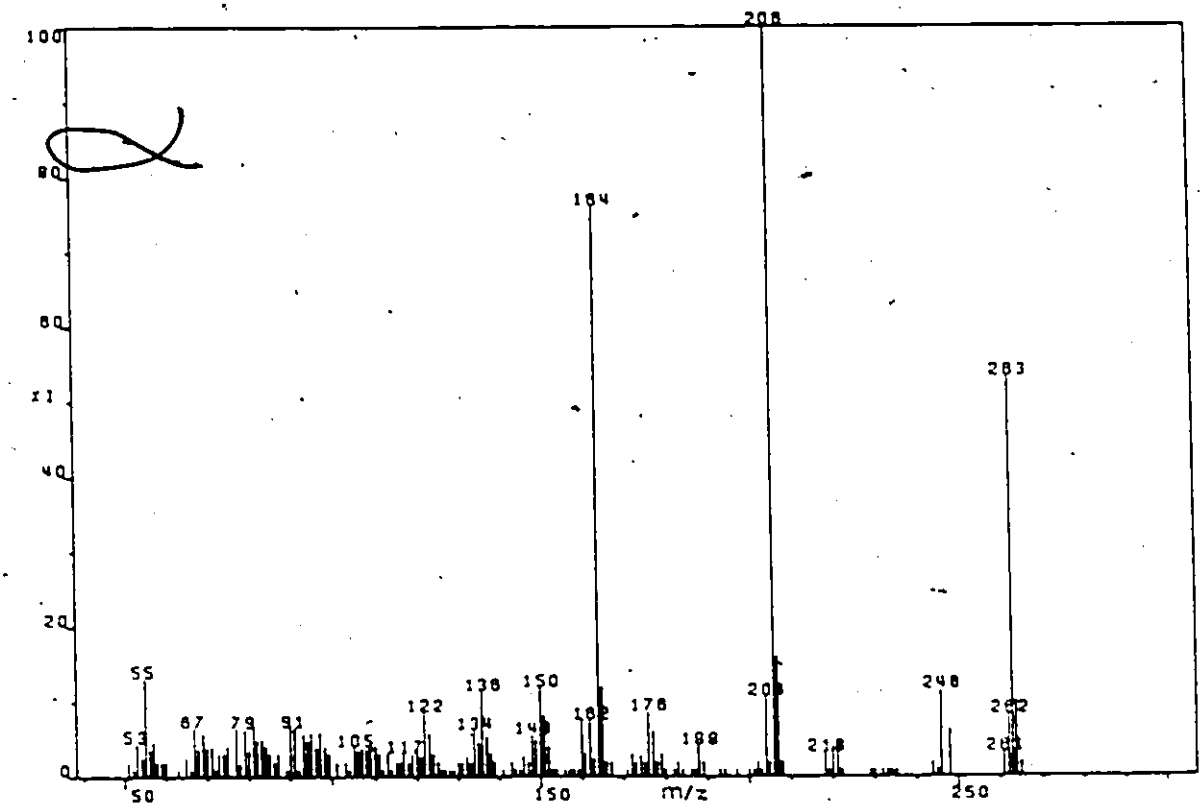
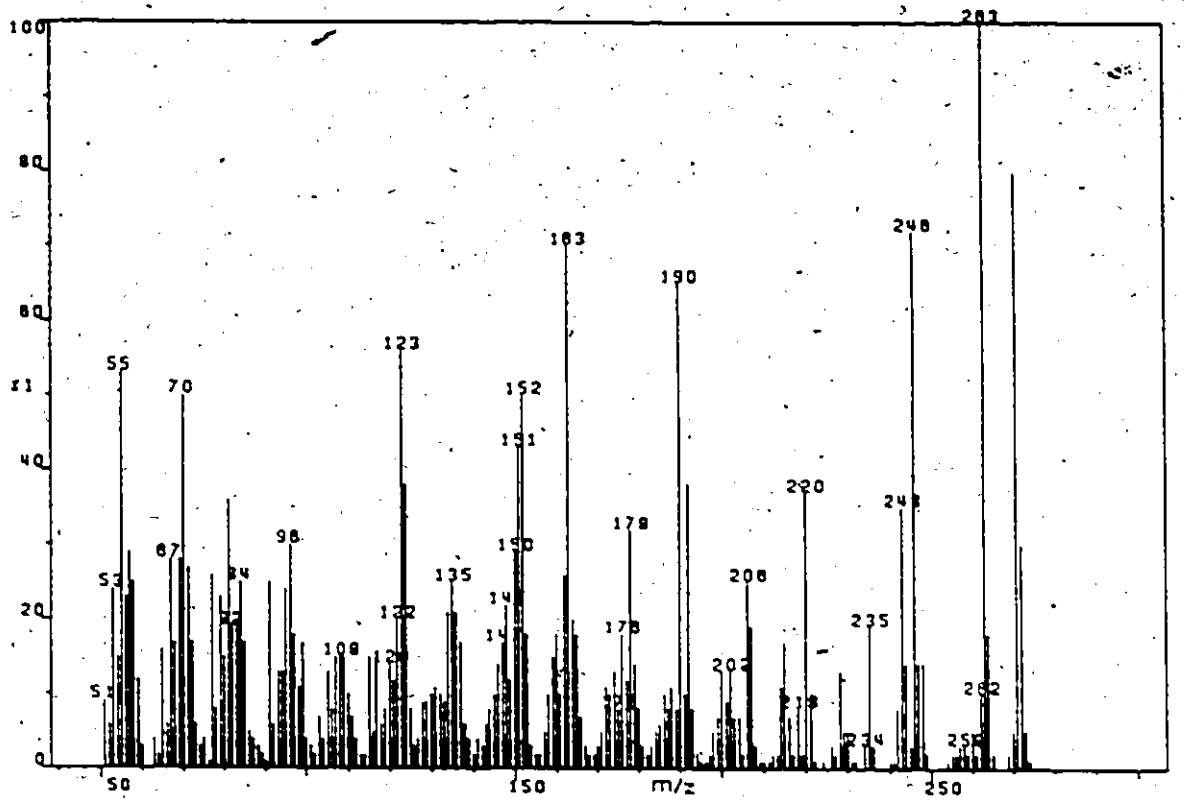


Figure 101 Mass spectrum of flabelliformine (derived from Figure 100).

Figure 102 Mass spectrum of component E (lycodoline).



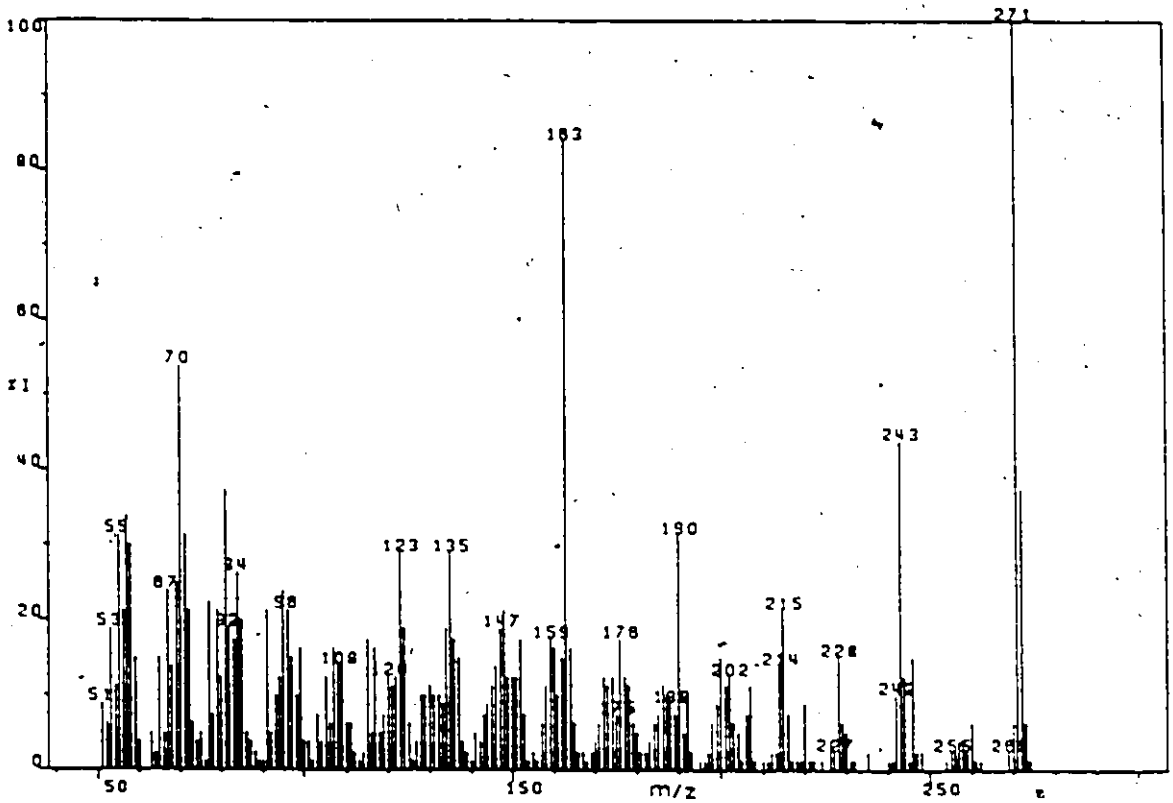
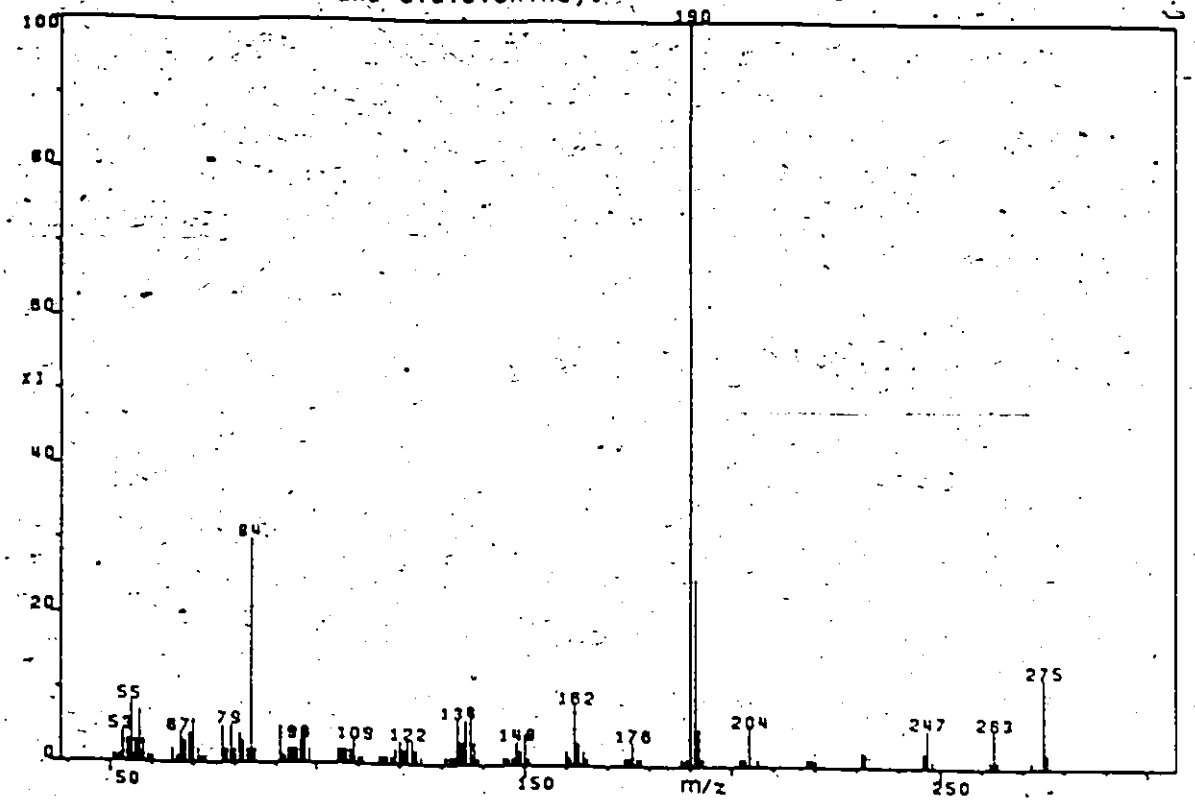


Figure 103 Mass spectrum of component F (derived from Figure 102).

Figure 104 - Mass spectrum of components H and I (lycoflexine and clavolonine).



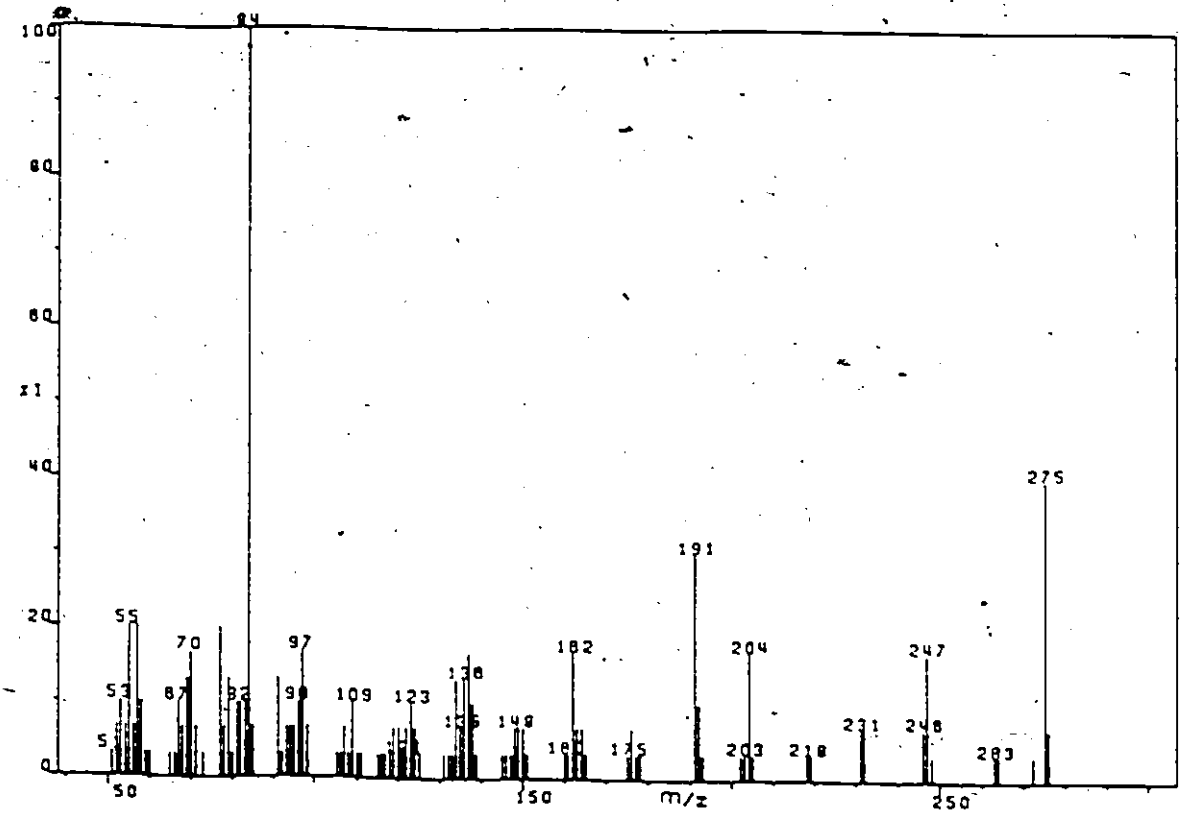
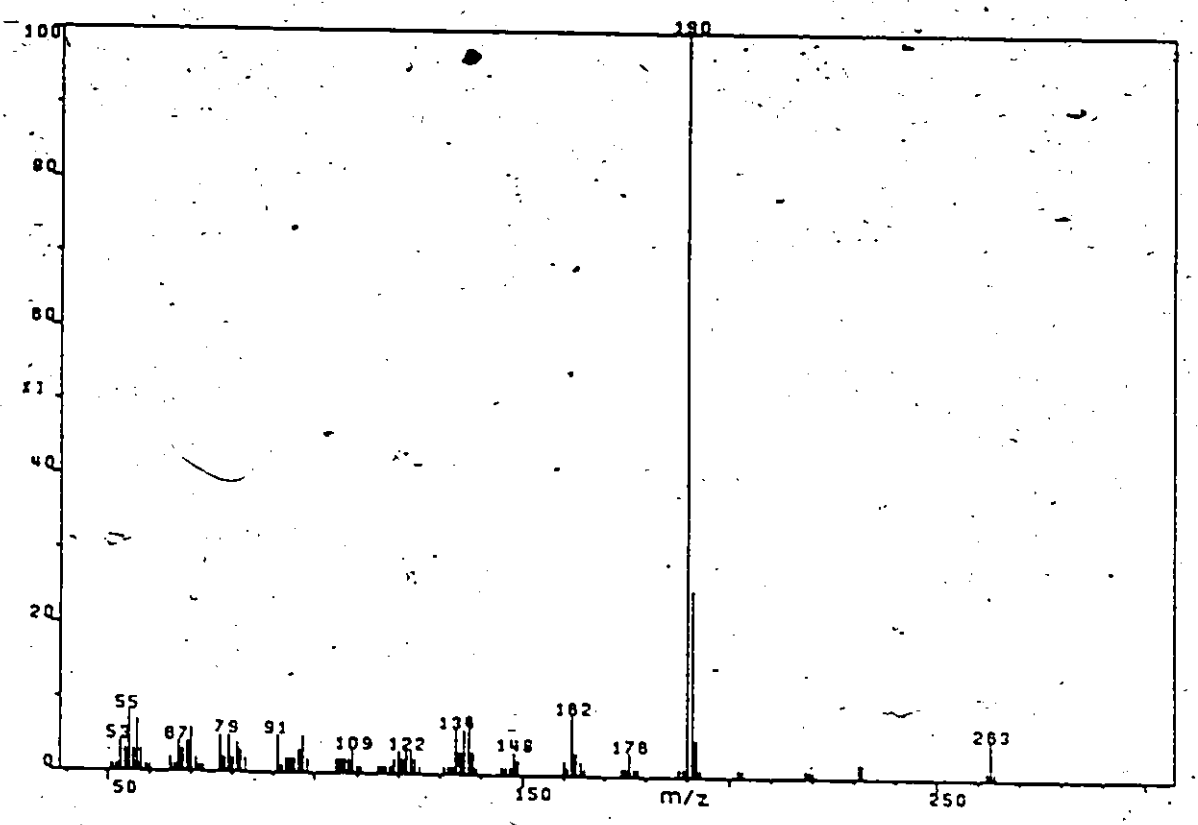


Figure 105 - Mass spectrum of lycoflexine (derived from Figure 104).

Figure 106 Mass spectrum of clavolonine (derived from Figure 104).



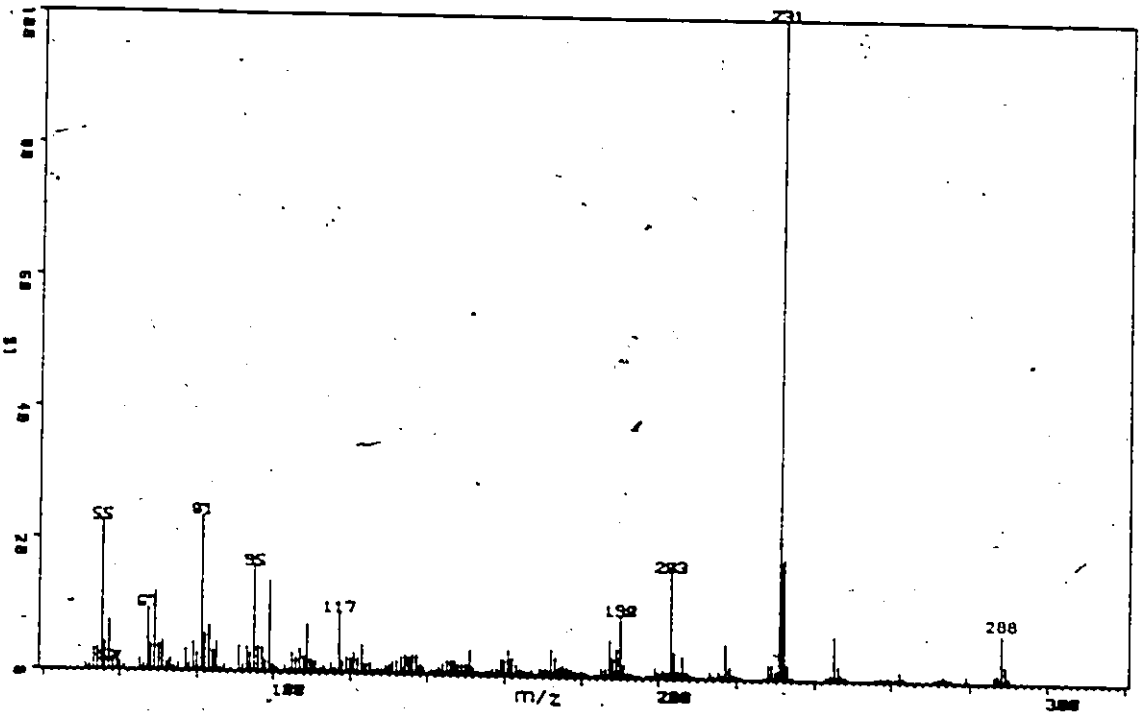
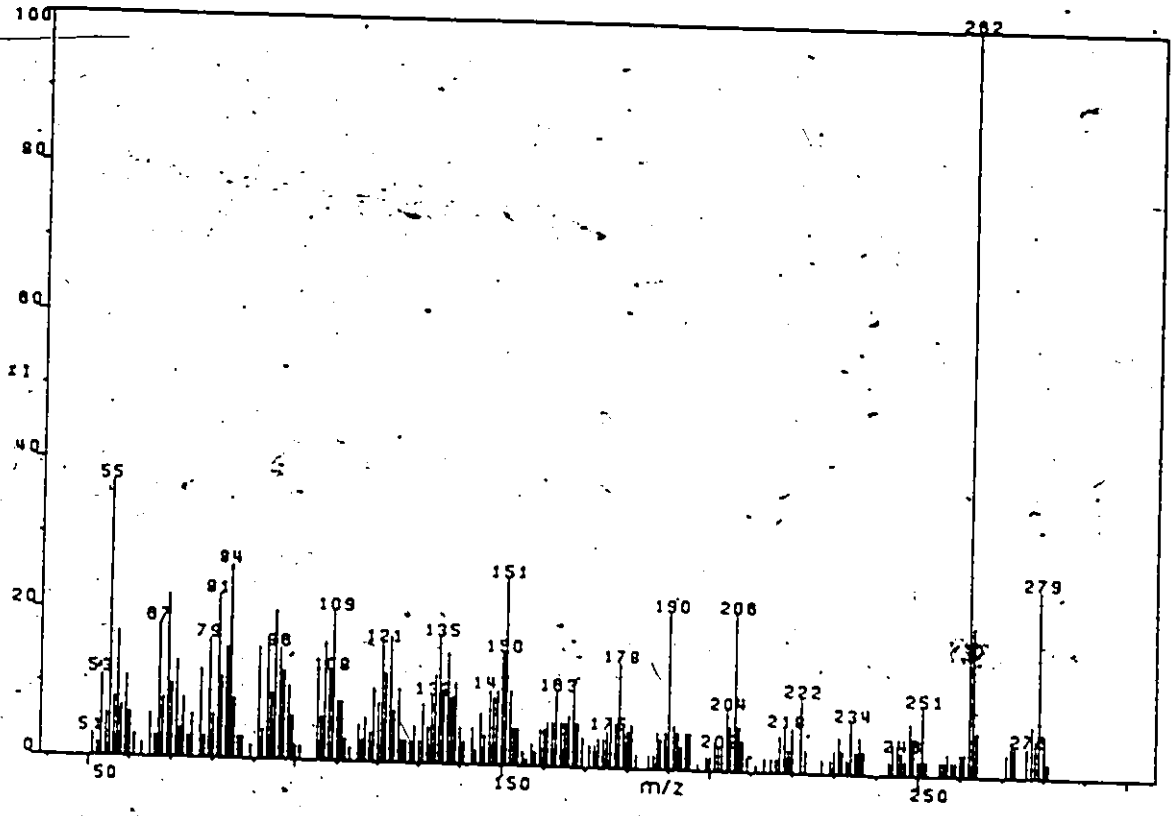


Figure 107 Mass spectrum of component J (flabelline).

Figure 108 Mass spectrum of component K.



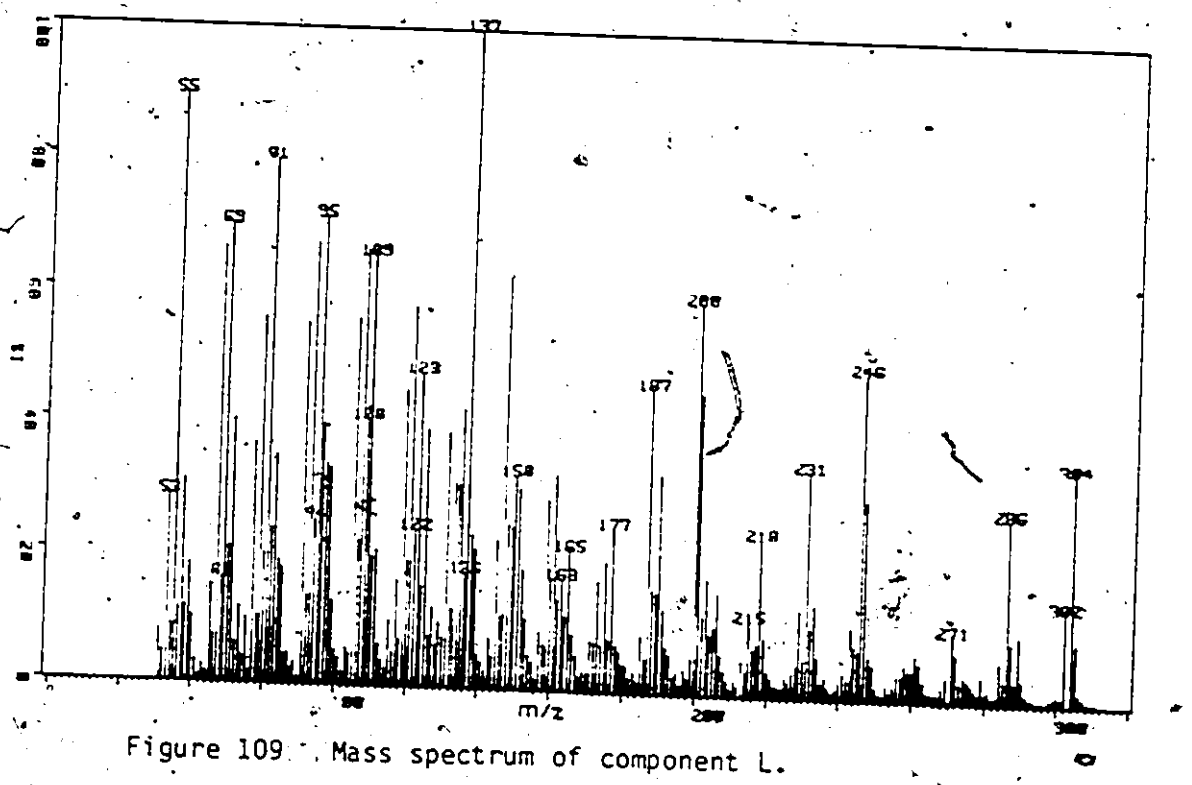
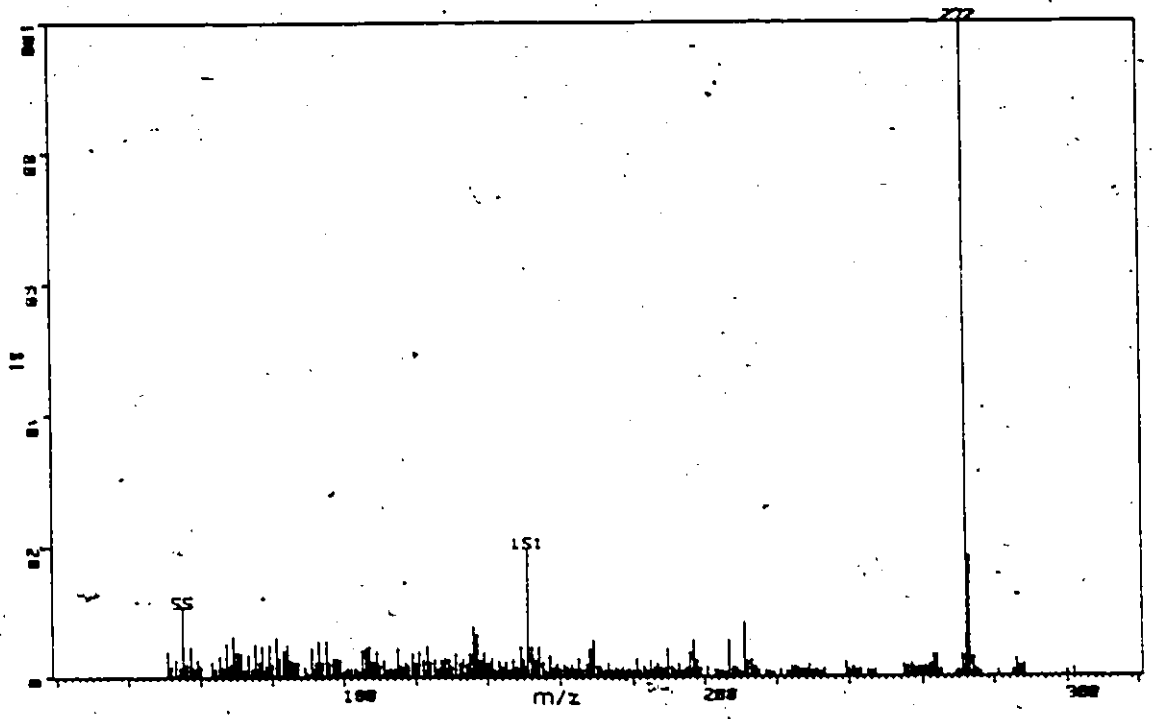


Figure 109 Mass spectrum of component L.

Figure 110 Mass spectrum of component M.



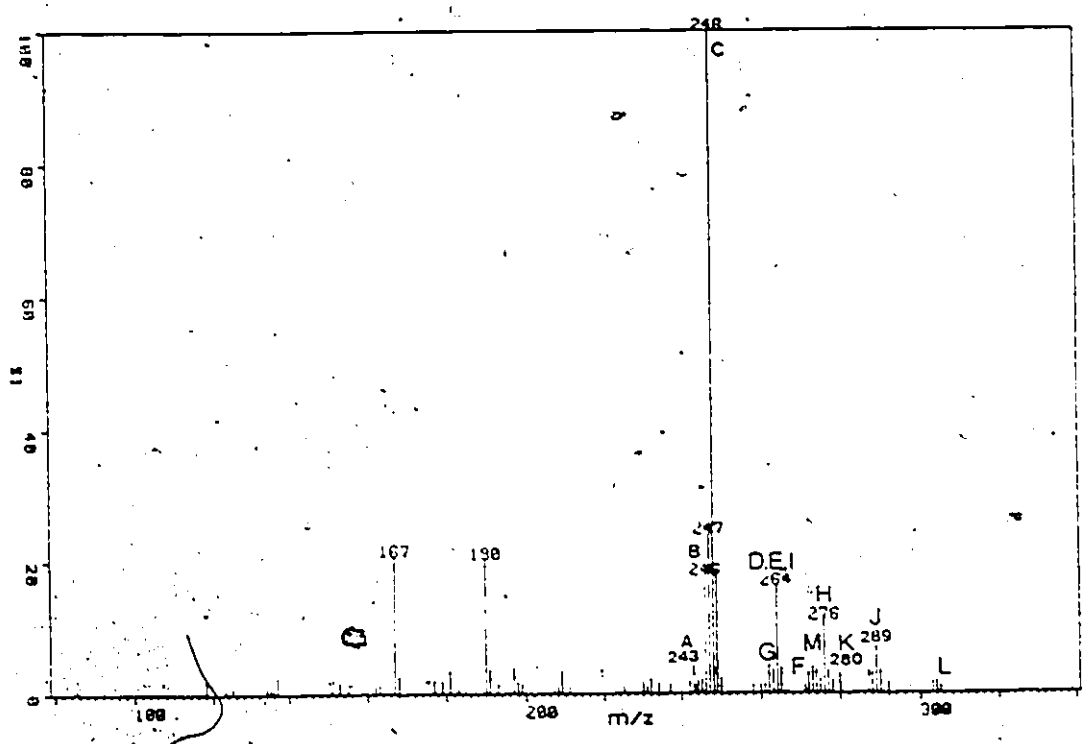


Figure 111 Chemical ionization mass spectrometry of L. deuterodensum extract with methane as the reagent gas.

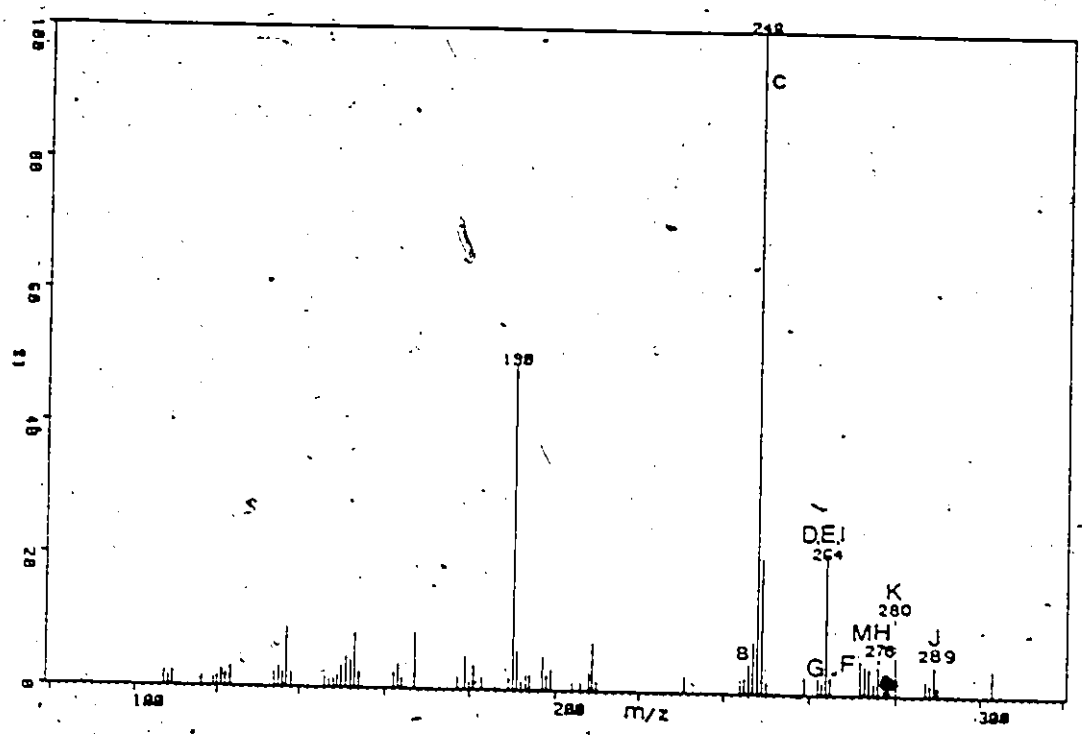


Figure 112 Chemical ionization of L. deuterodensum extract using ammonia as the reagent gas.

percent of each component in the extract was calculated from peak heights and areas from a FSC/FID experiment; the results are tabulated in Table 23.

Peak A in Figure 115 and the peak partially obscured by the C-18 hydrocarbon standard in Figure 116 were assigned to the methyl ester of ferulic acid by its mass spectrum shown in Figure 117. Peak B was identified as lycodine from its retention index and from its mass spectrum shown in Figure 118. Eluting after lycodine is the alkaloid anhydrolycodoline component C; its mass spectrum is shown in Figure 119. The major alkaloid D was identified as lycopodine from its retention index and mass spectrum shown in Figure 120. A small shoulder on the lycopodine peak was resolved in the FSC/MS analysis. From its retention index and mass spectrum the compound was identified as flabelliformine (component F) contaminated with lycopodine and dihydrolycopodine, (component E), as shown in Figure 121. Figure 122 shows the result of subtracting the mass spectrum of lycopodine and dihydrolycopodine from the mass spectrum in Figure 121. This now reveals more clearly the characteristic ions of the flabelliformine mass spectrum.

Component G was identified as acetyldihydrolycopodine from its retention index and mass spectrum shown in Figure 123. Figure 124 shows the result of subtracting the mass spectrum of lycopodine and dihydrolycopodine from the mass spectrum shown in Figure 123, the characteristic ions of acetyldihydrolycopodine at m/z 291, 234, 174, 146, are observed.

Peak H is comprised mainly of a compound with the molecular weight of 263. The mass spectrum shown in Figure 125 most closely matches the mass spectrum of lycodoline. In an attempt to identify other

components of peak H the mass spectrum of lycodoline was subtracted from the mass spectrum shown in Figure 125. The resulting spectrum was obviously a mixture and was uninterpretable.

Component I was identified as lycoflexine from its mass spectrum shown in Figure 126. Component J, which has a molecular weight of 273, determined from its mass spectrum shown in figure 127, has an unknown structure. High resolution mass spectrometry of the molecular ion at m/z 273 gave the composition $C_{17}H_{23}NO_2$ (found 273.172, calculated 273.173). This corresponds to magellaninone (5-dehydromagellanine); however since an authentic sample could not be obtained this assignment is tentative. The mass spectrum of component J is contaminated with clavolonine (component K) and flabelline or flabellidine. The ions at m/z 263, 190 and 162 indicate that clavolonine is present, and the ions at m/z 288 and 231 could arise either from flabellidine or flabelline. Figure 128 shows the result of subtracting the mass spectra of clavolonine and flabelline from the main spectrum in Figure 127. This may provide a more accurate representation of the spectrum of component J.

Components L and M elute closely together and have molecular weights of 286 and 300, respectively. Eluting with components L and M is component N with a molecular weight of 274. Ions at m/z 231 (M - 43), 217 (M - 57)and 189 (M - 85) indicate that compound N is α-obscurine. The mass spectrum of the mixture of components L, M and N is shown in Figure 129. Figure 130 shows the mass spectrum of α-obscurine after the mass spectra of components L and M have been subtracted from the mass spectrum shown in Figure 129.

Unknowns L and M were obtained in pure form by liquid chromatography and their mass spectra are shown in Figures 131 and 132; respectively. Their spectral properties indicate that they are new alkaloids. In the following section the structures of L and M are discussed.

FSC/MS done in the chemical ionization mode with methane as the reagent gas verifies the molecular weight of components J, L and M. Chemical ionization of the extract (probe sample) with methane and with ammonia as the reagent gas yields $(M + H)^+$ ions for the compounds which were identified, as shown in Figures 133 and 134, respectively.

3.4.6.1 The structure of components L and M

The relationship between L and M was established by forming the N-methyl derivative of component L. The spectral properties of the product were the same as those of component M. High resolution mass spectrometry on the molecular ions of L and M gave the compositions, $C_{18}H_{26}N_2O$ and $C_{19}H_{28}N_2O$, respectively, indicating that they differ by CH_2.

Preliminary NMR (250 MHz) examination of compound M indicated the presence of a $N\text{-}CH_3$ group (s, 3H, 2.19 δ), a $N\text{-}COCH_3$ group (s, 3H, 2.00 δ) and a $CH\text{-}CH_3$ group (d, 3H, 0.77 δ, J = 6.4 Hz). A doublet of doublets (1H, 5.2 δ, J = 1.1 and 5.5 Hz) indicated one vinylic proton and therefore a trisubstituted double bond (Figure 135). See section 3.4.6.3 for a detailed NMR examination.

The proton NMR (80 MHz) spectrum of compound L lacked a $N\text{-}CH_3$ signal but showed signals for a $N\text{-}COCH_3$ group (s, 3H, 2.04 δ), a $CH\text{-}CH_3$ group (d, 3H, 0.82 δ, J = 8 Hz) and a vinylic proton (d, 1H, 5.148,

J = 4.8 Hz). The vinylic proton was not resolved into a doublet of doublets probably because the spectrum was run at lower resolution.

These data indicate that M is pentacyclic and contains the following groups N-CH_3, N-$COCH_3$, CH-CH_3 and a double bond (C=C ^{H}). Thus compounds L and M represent a new skeletal type.

Since very little pure M could be isolated, X-ray crystylography was used to determine its structure. Component M was crystallized from ether and an X-ray crystal structure was carried out by C. J. L. Lock and R. Faggiani of this Department. The details of the X-ray investigation will be published separately. The structure determined by X-ray analysis of compound M, given the trivial name fastigiatine, is shown in Figure 136. Compound L will be referred to as des-N-methylfastigiatine.

3.4.6.2 Mass spectrometry of fastigiatine

Since fastigiatine has a skeleton which is new, it was of importance to investigate its mass spectrometric fragmentation pattern in order that new representatives be readily identified. The mass spectrum of fastigiatine shown in Figure 132 is characterized by ions at m/z 300 ($M^{+\cdot}$), 285 (M - 15), 257 (M - 43), 176 (M - 124) and 124 (M - 176). The composition of the ions in the mass spectrum of fastigiatine has been investigated by high resolution mass spectrometry with the results shown in Table 24. The loss of a methyl group gives the ion at m/z 285, while the loss of C_3H_7 and CH_3CO gives the doublet at m/z 257. The ions at m/z 176 and 124 have the compositions $C_{11}H_{14}NO$ and $C_8H_{14}N$, respectively. These ions together account for the composition of fastigiatine.

Table. 23

Retention indices, computer search fit values and percent total alkaloid for each component of L. fastigiatum

	Compound	R.I.	A.R.I.	Pure	Mix	Reverse	# of scans	Pk.h.	Int.
B	lycodine	1948	1930	525	819	575	1	2.8	0.9
C	anhydrolyco-doline	1956	-	388	530	726	1		
D	lycopodine	2010	2030	897	964	926	16	73.2	72.0
E	dihydrolyco-podine	2010	2000	440	702	605	1		
F	flabelliformine	2059	2070	504	750	618	1	0.9	
G	acetyldihydro-lycopodine	2095	2085	806	806	950	1	12.4	9.5
H	lycodoline	2171	2159	620	620	976	1	0.7	0.8
I	lycoflexine	2373	-	618	724	836	1	1.2	6.0
J	mol wt 273	2394	-	-	-	-	-		
K	clavolonine	2394	2300	524	648	581	6		
L	mol wt 286	2514	-	-	-	-	-		
M	mol wt 300	2514	-	-	-	-	-	8.8	10.8
N	α-obscurine	2514	2422	825	825	998	1		
								100.0	100.0

Table 24

Accurate masses of selected ions of fastigiatine (M) and des-N-methyl fastigiatine (L)

Compound	ion	composition	calculated	observed	deviation in mmu
L	$M^{+\cdot}$	$C_{18}H_{26}N_2O$	124.1126	286.203	1.6
			286.204		
	$M-CH_3$	$C_{17}H_{23}N_2O$	271.181	271.182	-0.6
M	$M^{+\cdot}$	$C_{19}H_{28}N_2O$	300.2202	300.2204[1]	0.3
	$M-CH_3$	$C_{17}H_{25}N_2O$	285.1967	285.1967[1]	0.2
	$M-C_3H_7$	$C_{16}H_{21}N_2O$	257.1654	257.1625[2]	2.9
	$M-CH_3CO$	$C_{17}H_{25}N_2$	257.2018	257.2018[1]	0.0
	$M-C_8H_{14}N$	$C_{11}H_{14}NO$	176.1075	176.1076[3]	-0.1
	$M-C_{11}H_{14}NO$	$C_8H_{14}N$	124.1126	124.1125[3]	-0.1

1 ZAB RP 20,000
2 MS-50
3 MS-50 RP 10,000
Other VG 7070F RP 3,000

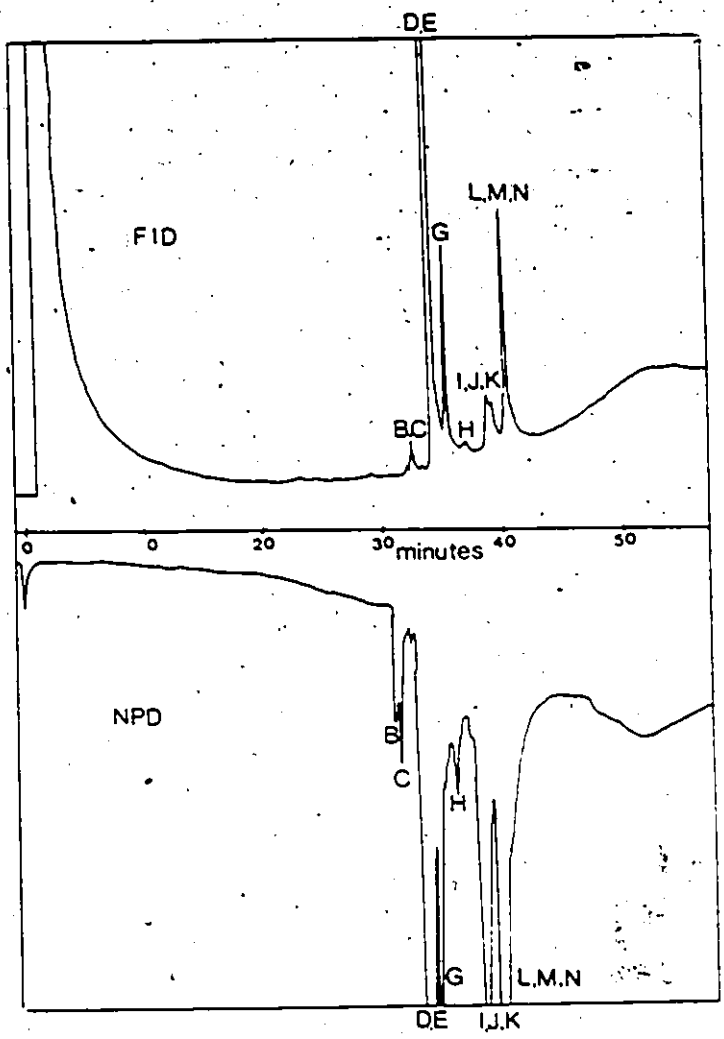


Figure 113 FSC/FID/NPD chromatogram of L. fastigiatum extract.

A methyl ester of ferulic acid
B lycocine
C anhydrolycodoline
D lycopodine
E dihydrolycopodine
F flabelliformine
G acetyldihydrolycopodine
H lycodoline
I lycoflexine
J unknown 273
K clavolonine
L unknown 286
M unknown 300
N α-obscurine

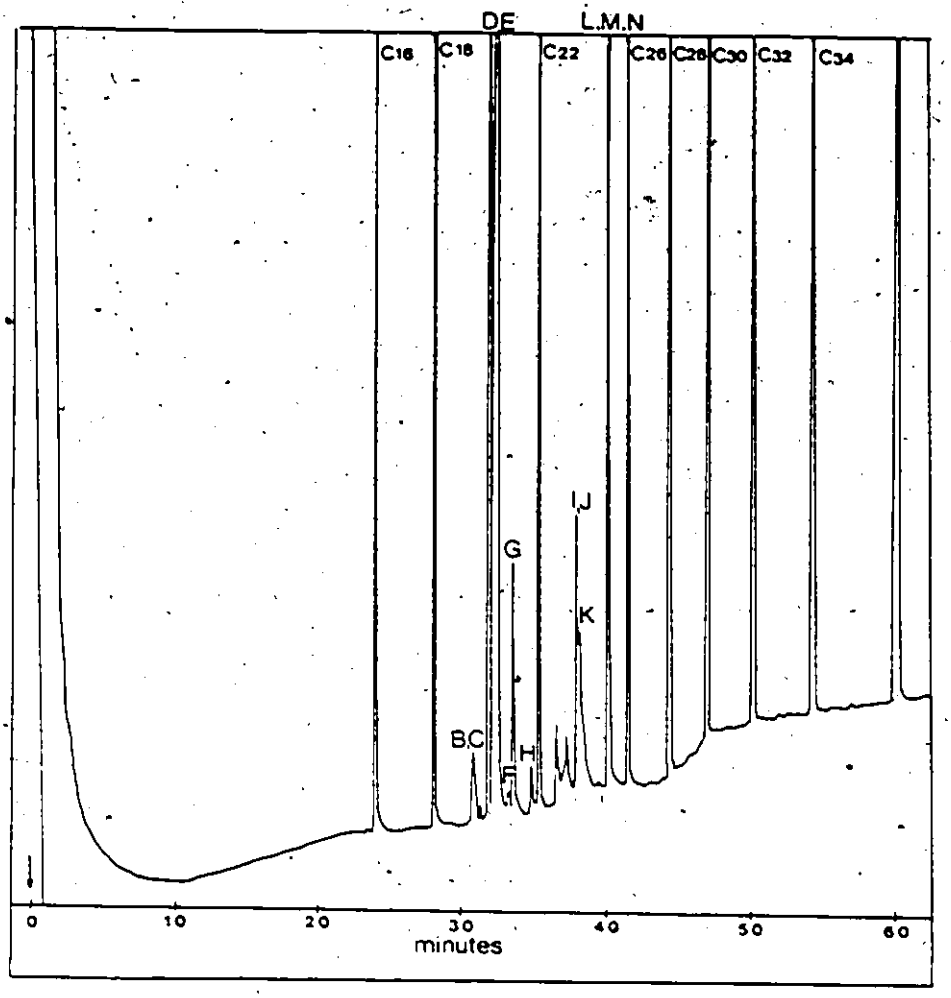


Figure 114 FSC/FID chromatogram of L. fastigiatum extract with hydrocarbon standards.

Figure 115 GC/MS TIC of L. fastigiatum extract

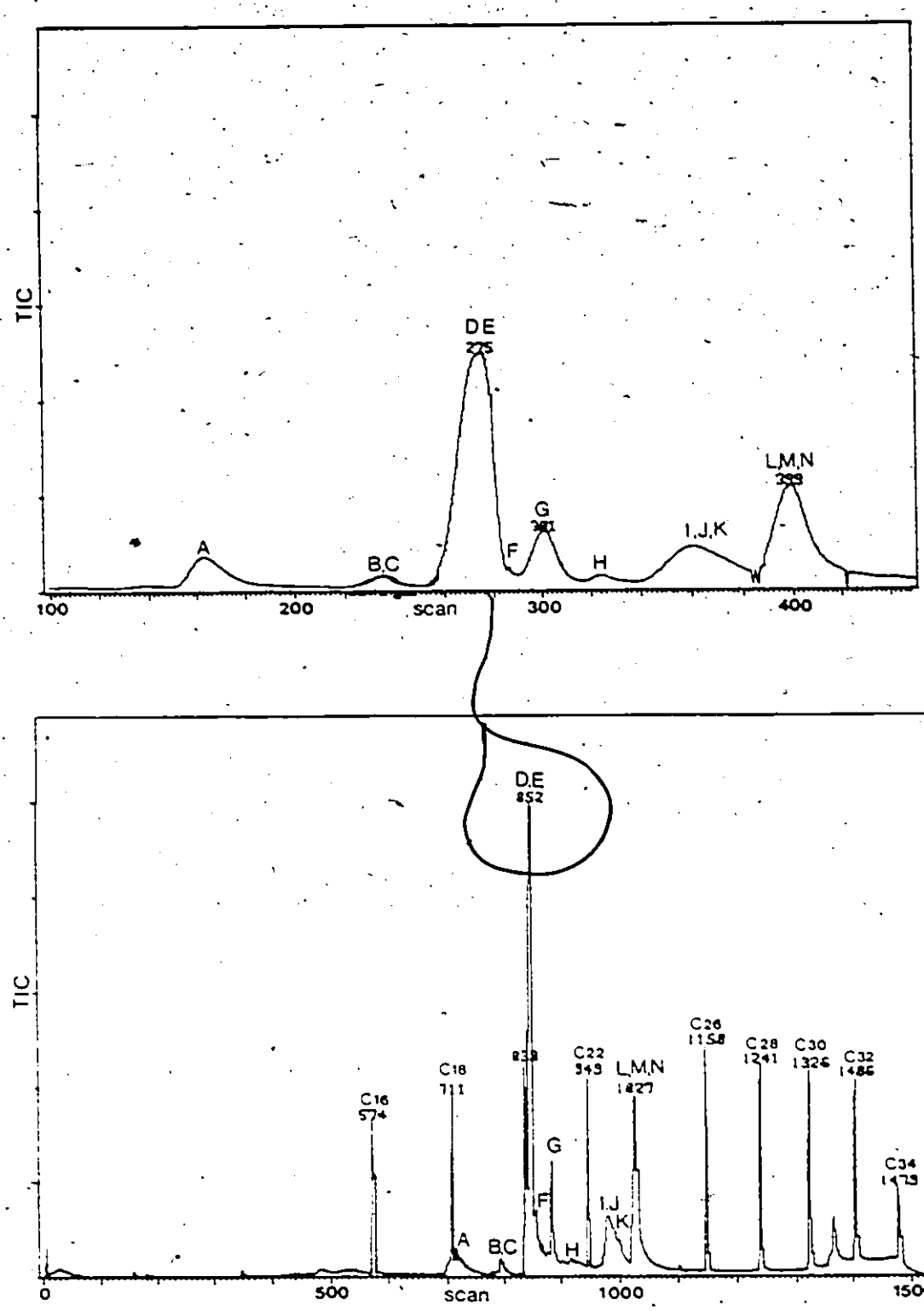
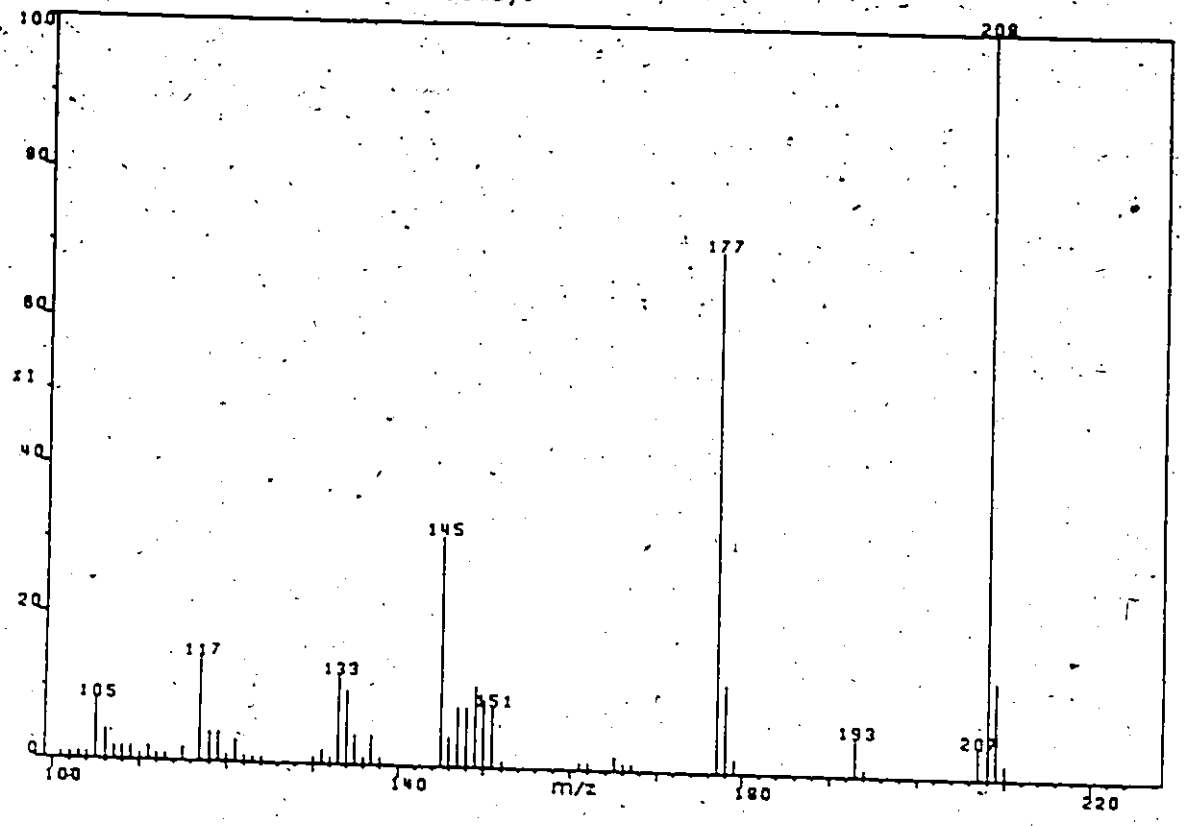


Figure 116 FSC/MS TIC of L. fastigiatum extract with hydrocarbon standards.

Figure 117 Mass spectrum of component A (the methyl ester of ferulic acid).



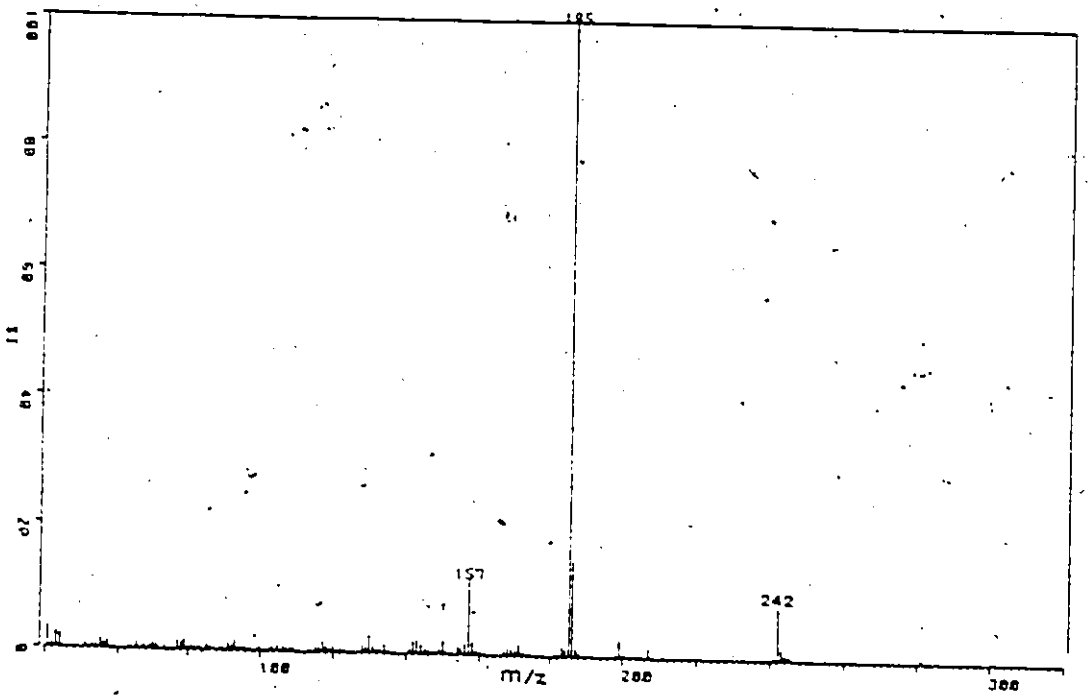
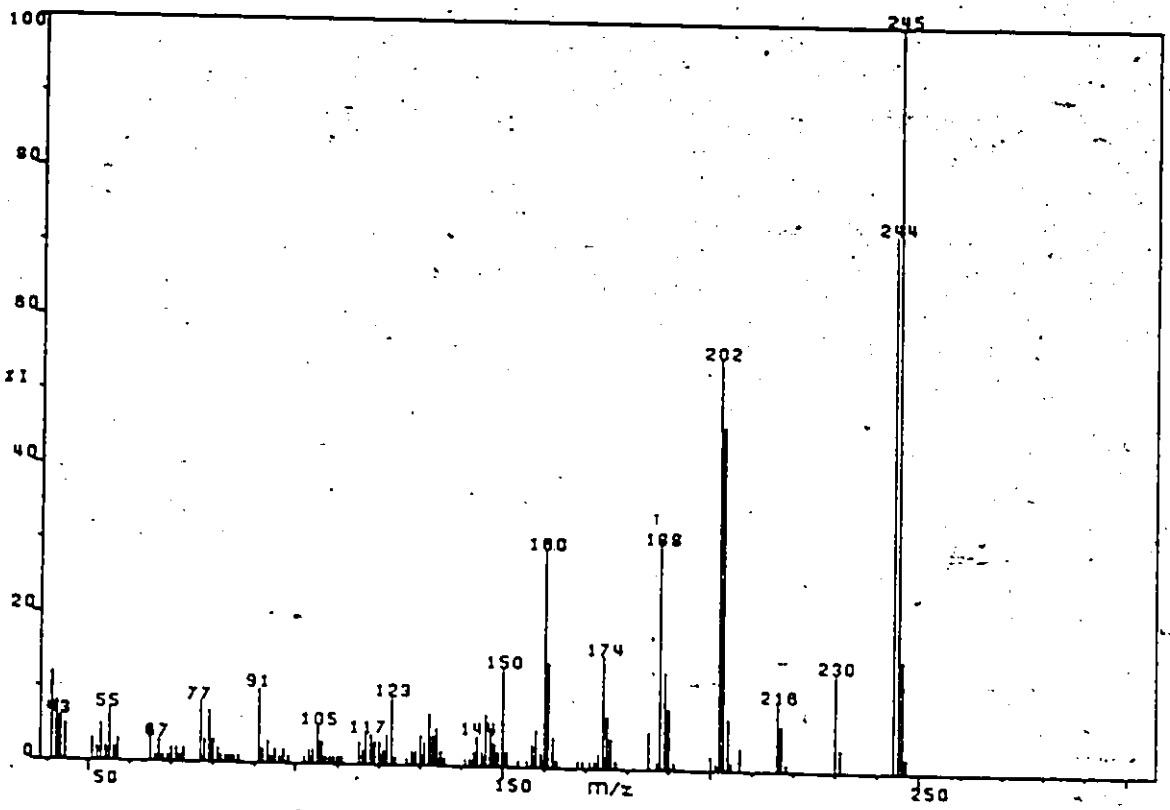


Figure 118 Mass spectrum of component B (lycodine).

Figure 119 Mass spectrum of component C (anhydrolycodoline).



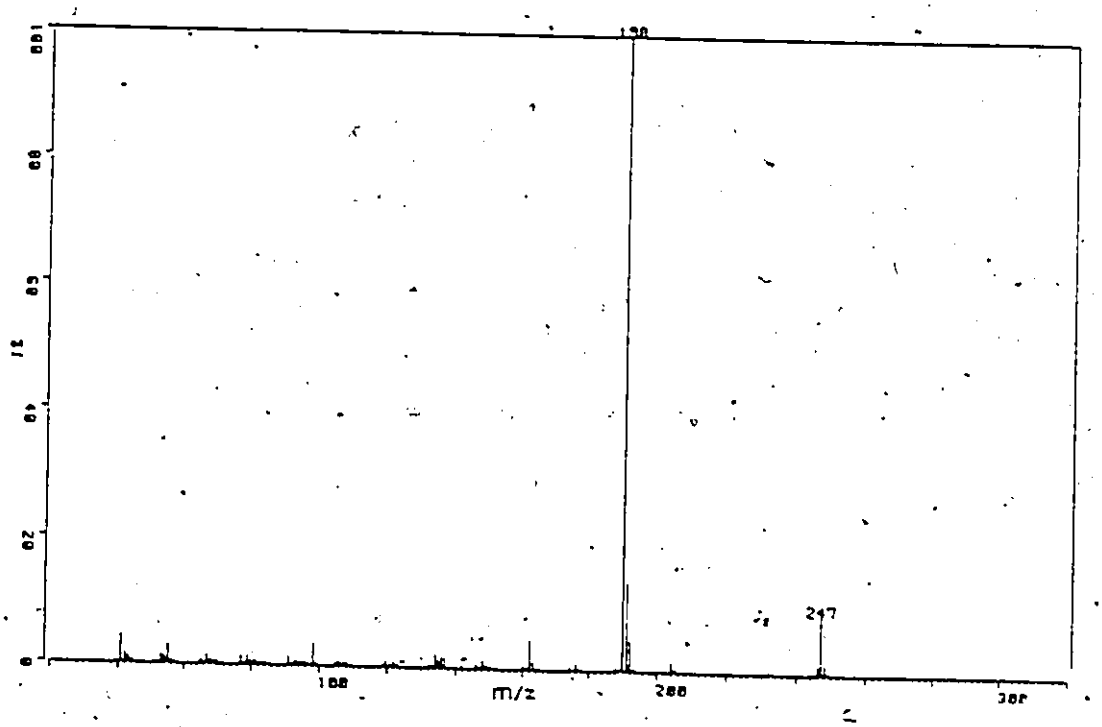
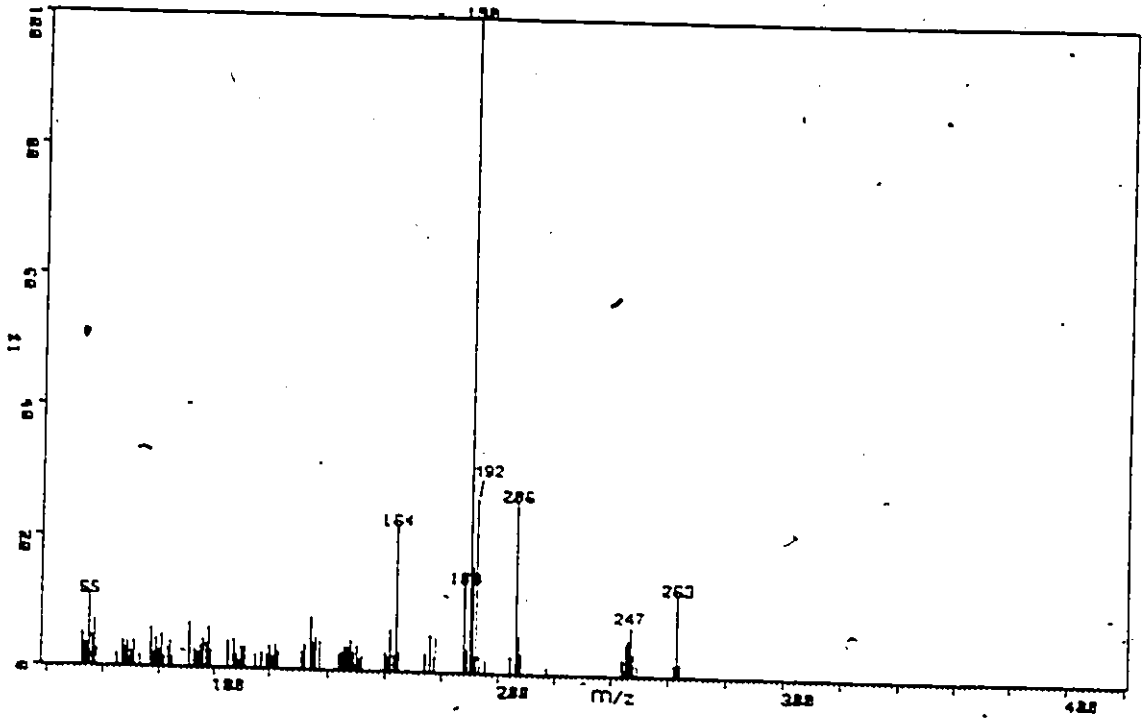


Figure 120 Mass spectrum of component D (lycopodine).

Figure 121 Mass spectrum of component D, E, F (lycopodine, dihydrolycopodine and flabelliformine).



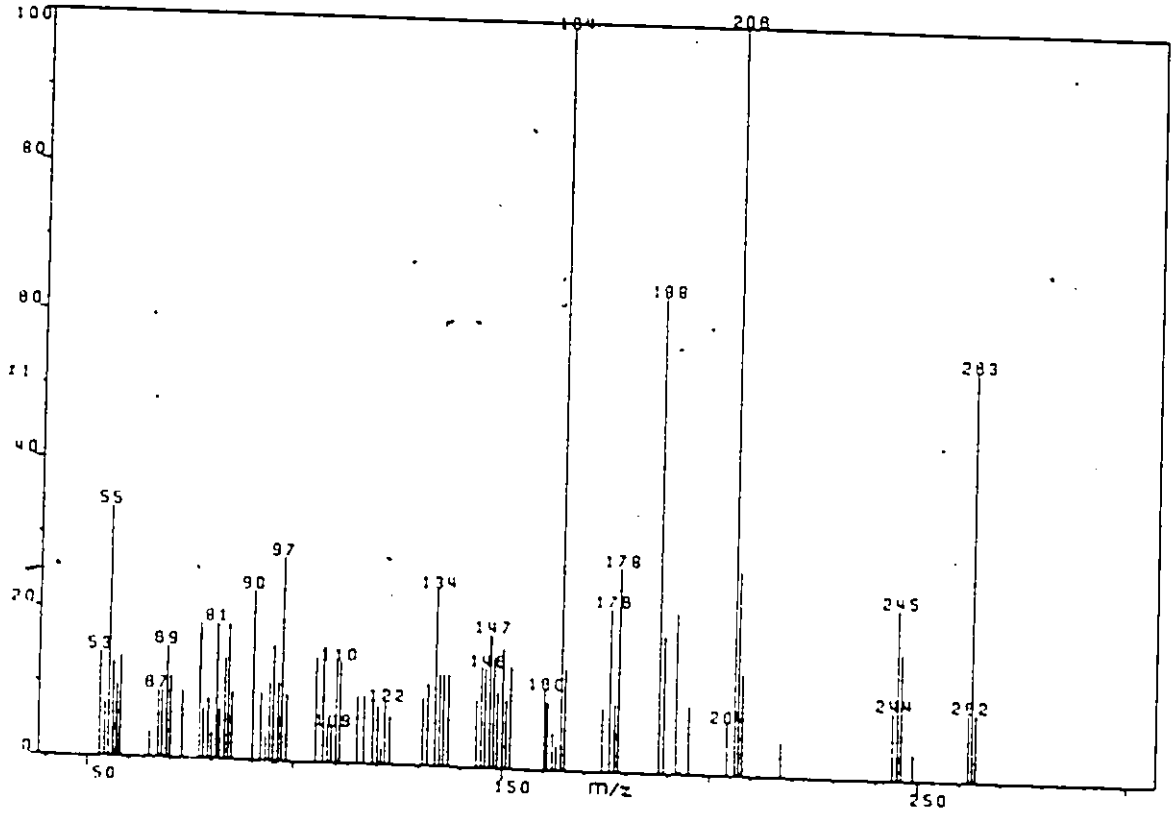
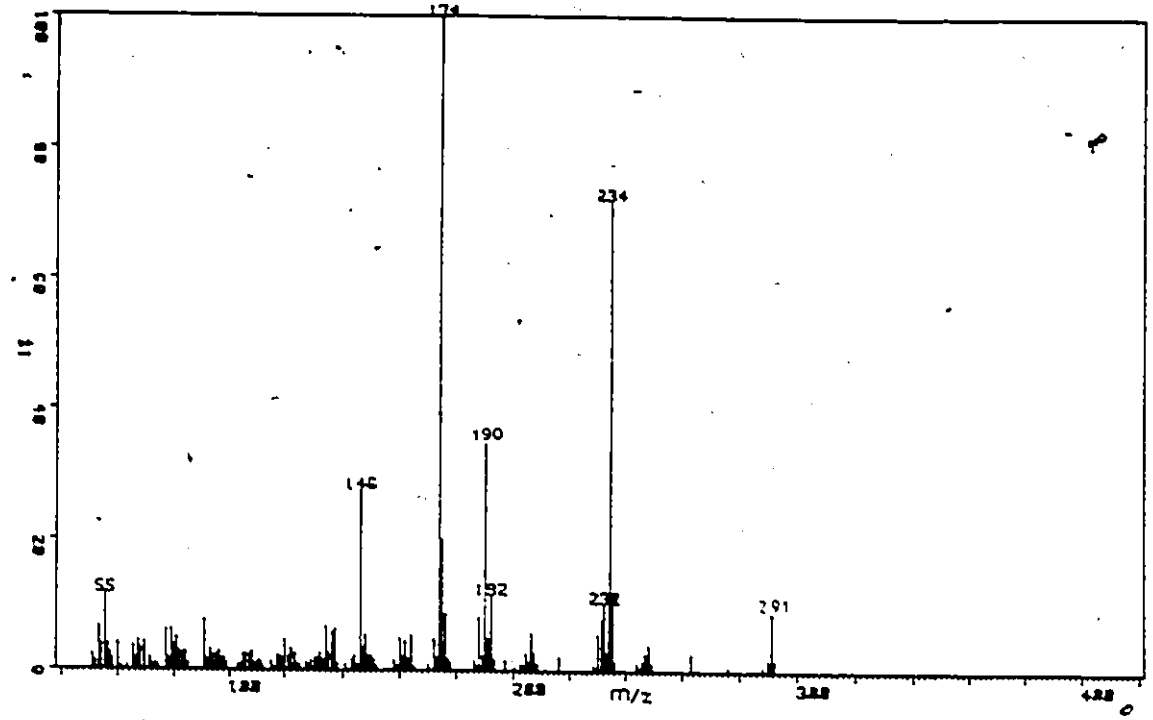


Figure 122 Mass spectrum of flabelliformine (derived from Figure 121).

Figure 123 Mass spectrum of component G (acetyldihydrolycopodine).



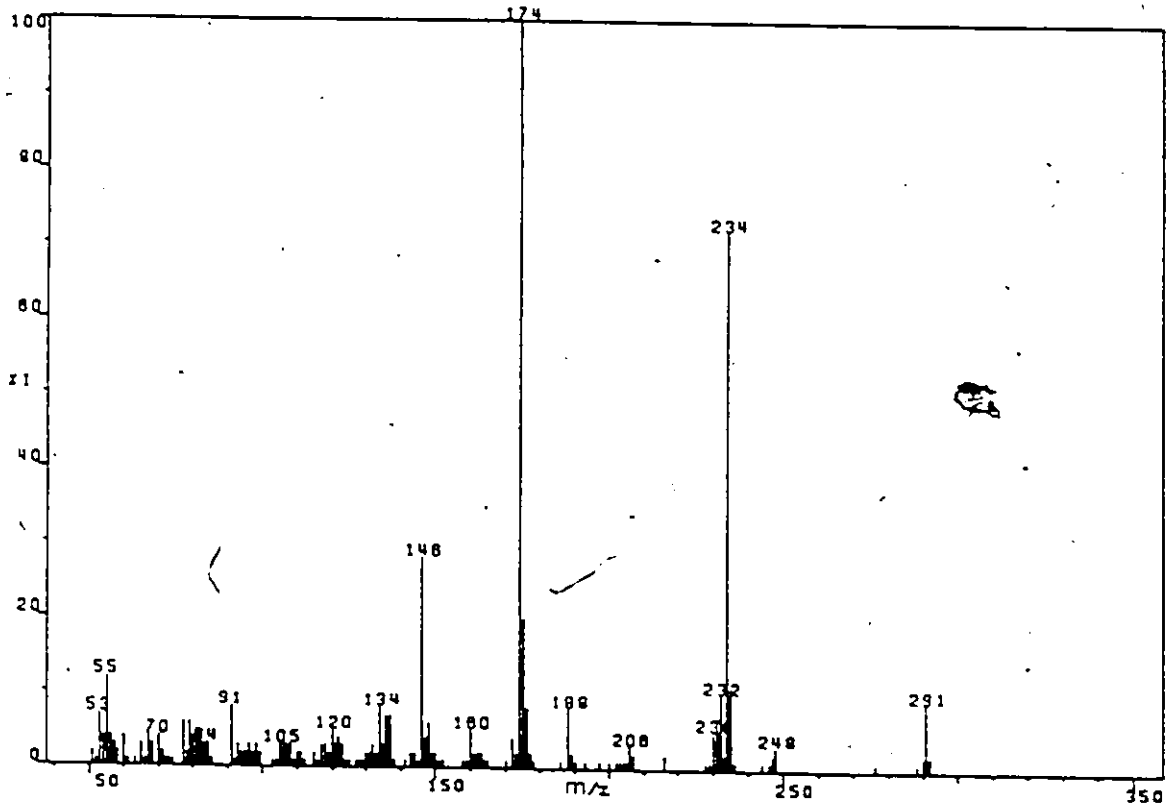
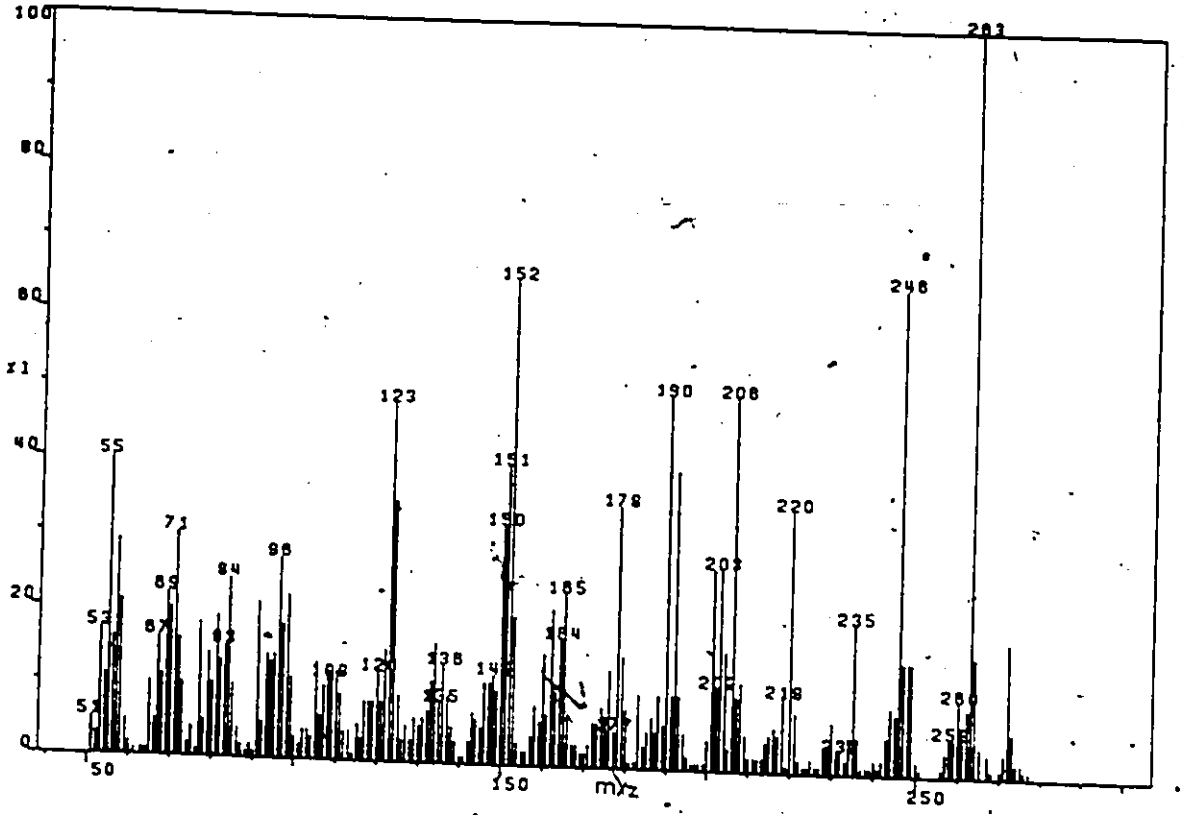


Figure 124 Mass spectrum of acetyldihydrolycopodine (derived from Figure 123).

Figure 125 Mass spectrum of component H (lycodoline).



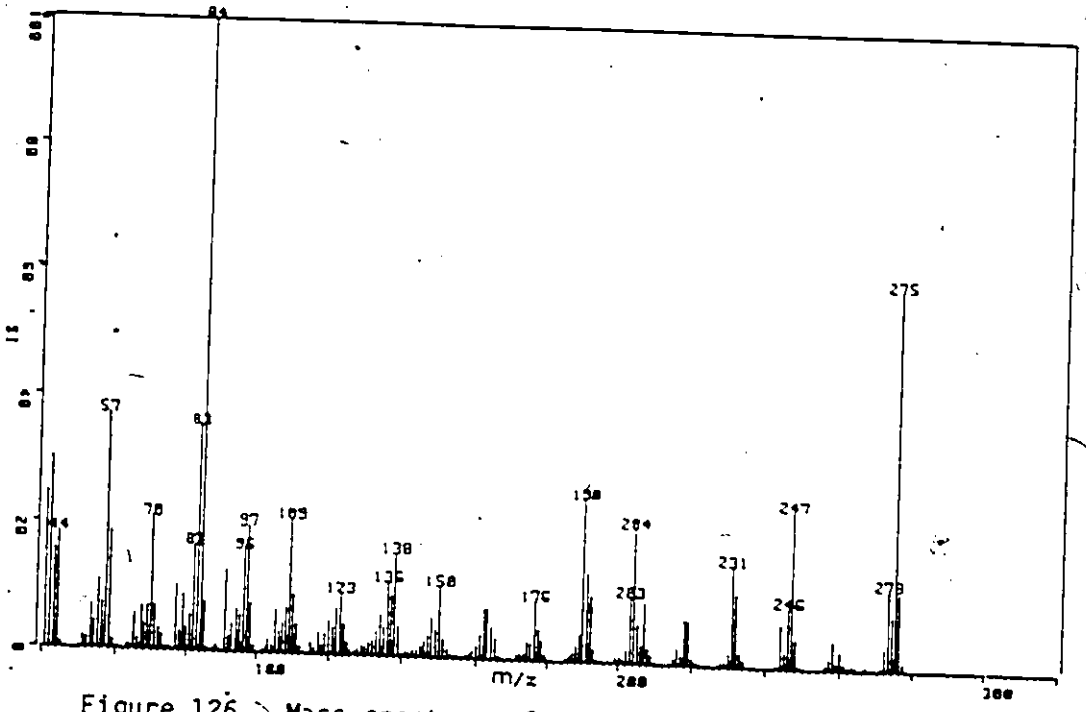
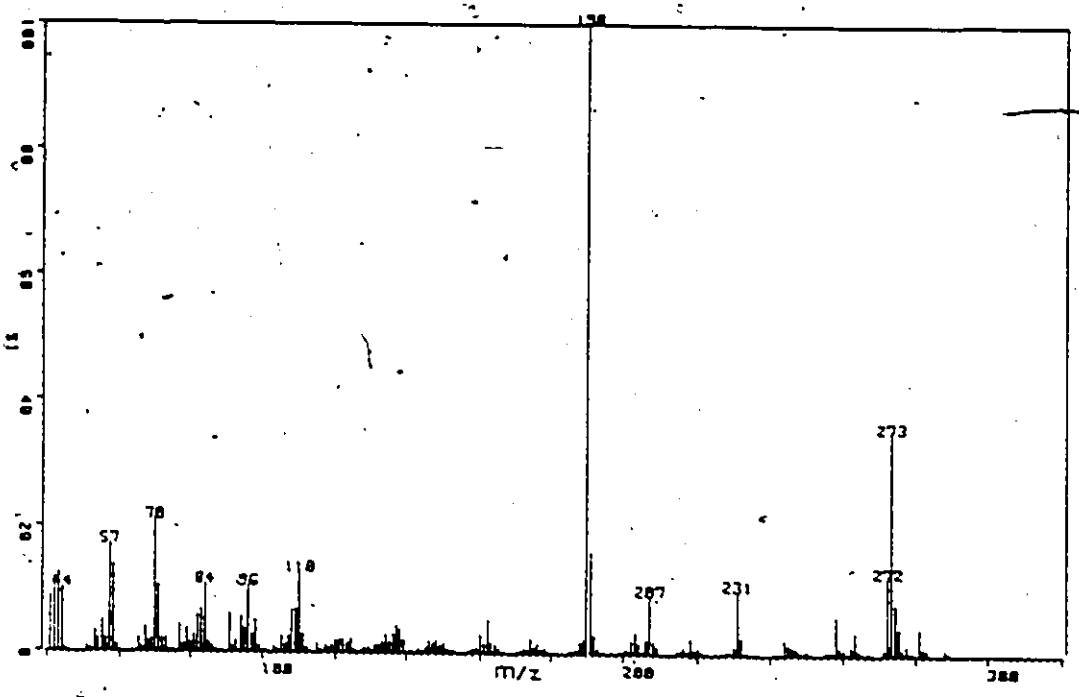


Figure 126 Mass spectrum of component I (lycoflexine).

Figure 127 Mass spectrum of component J and K (unknown J and clavolonine).



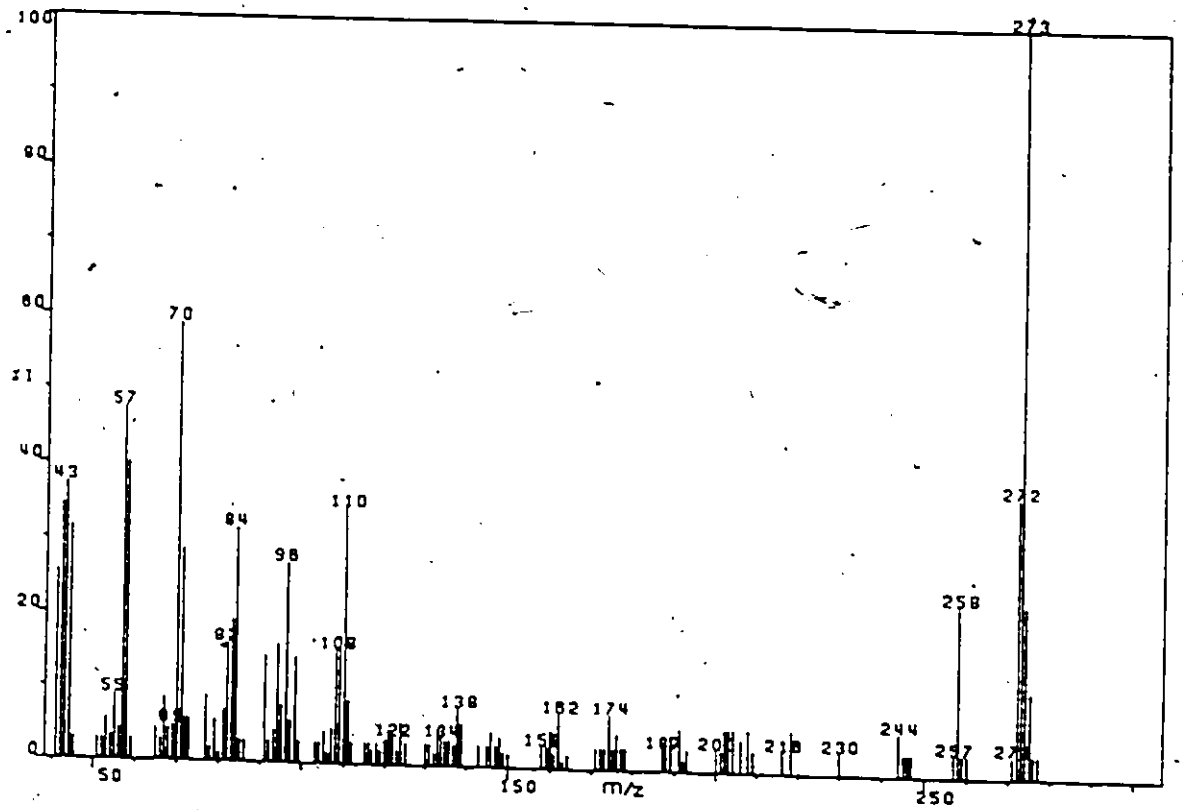
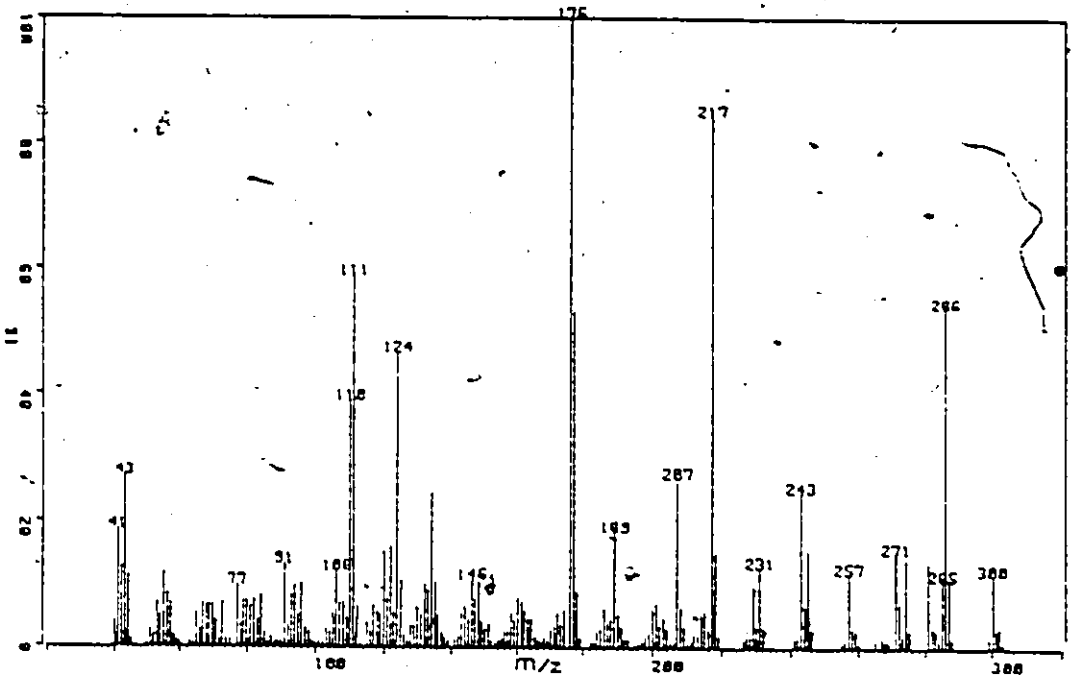


Figure 128 Mass spectrum of 5-dehydromagellanine? (derived from Figure 127.

Figure 129 Mass spectrum of components L, M and N (N = α-obscurine).



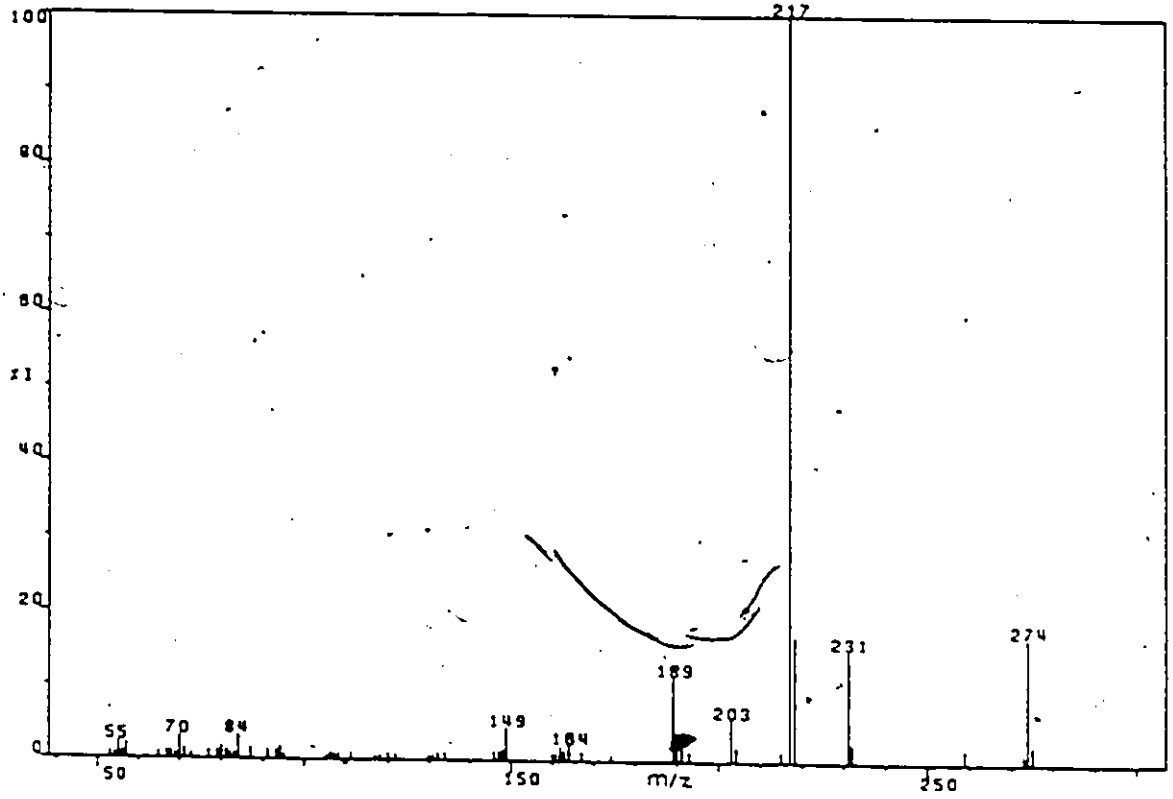
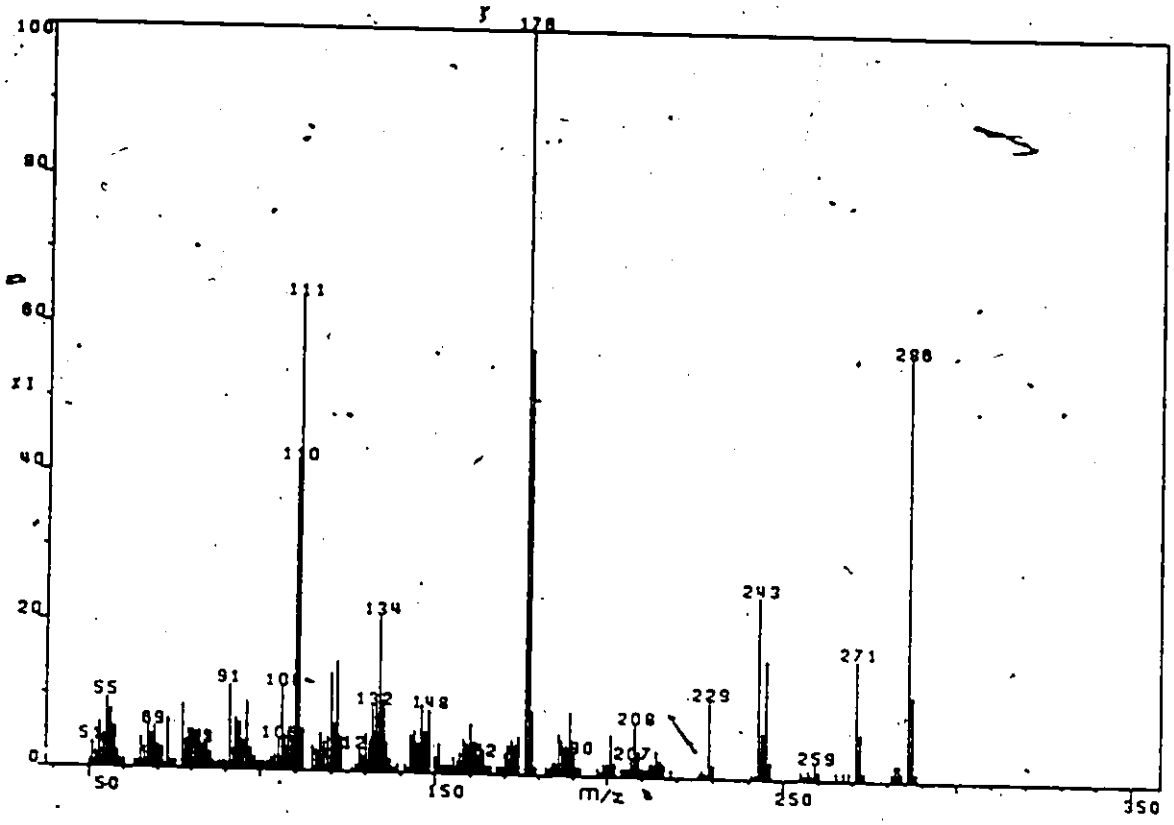


Figure 130 Mass spectrum of N α-obscurine (derived from Figure 129).

Figure 131 Mass spectrum of component L (des-N-methylfastigiatine).



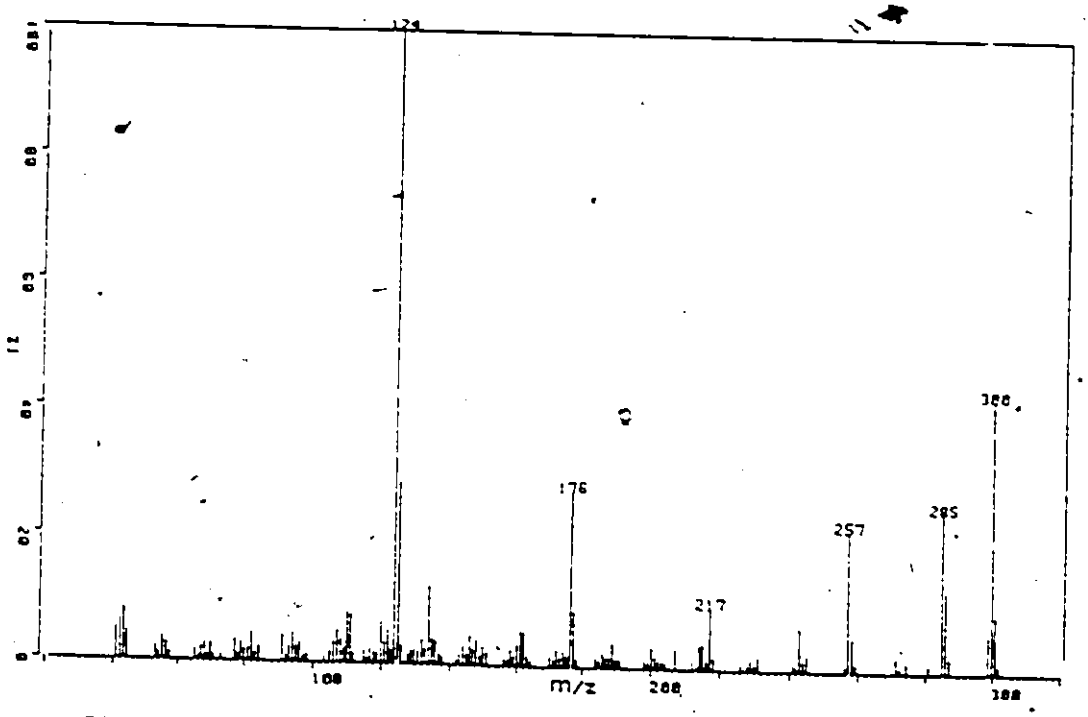
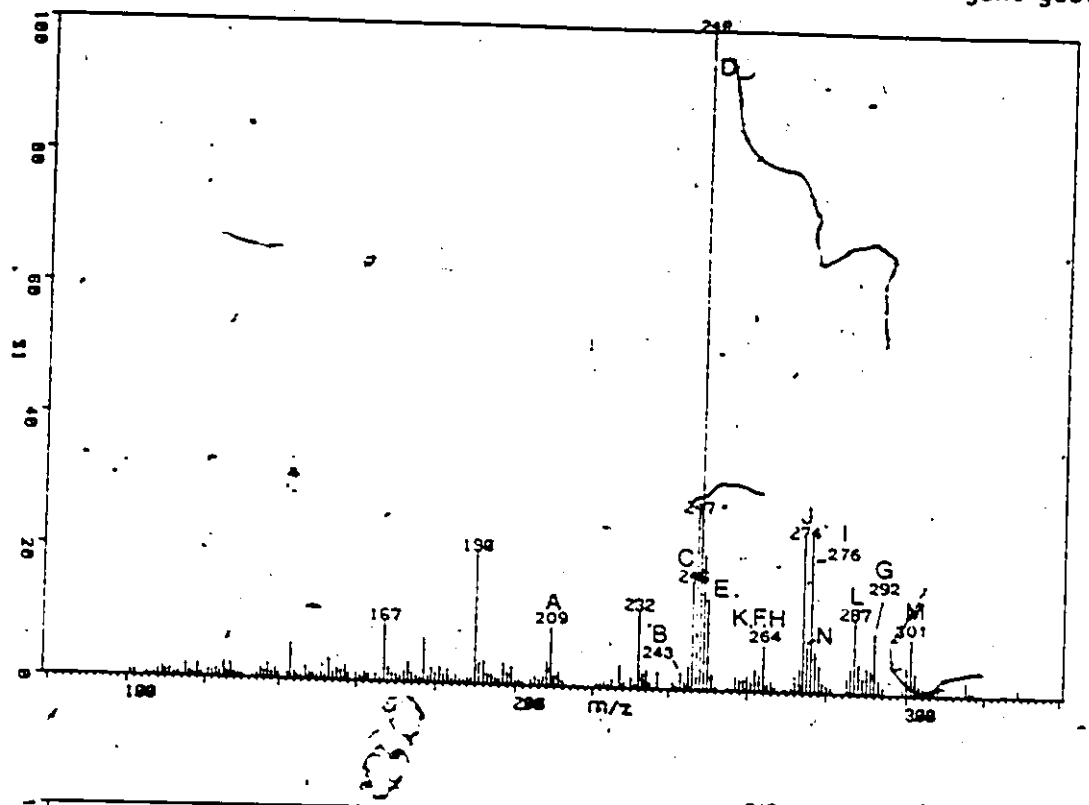


Figure 132 Mass spectrum of component M (fastigiatine).

Figure 133 Chemical ionization mass spectrometry of L. fastigiatum extract with methane as the reagent gas.



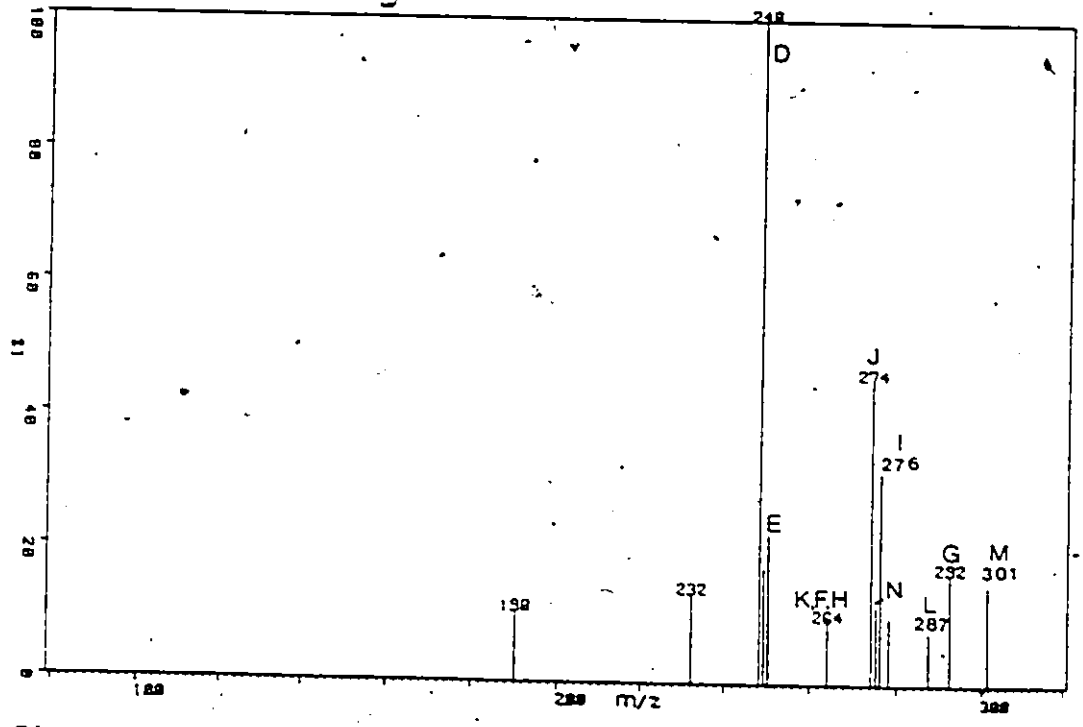


Figure 134 Chemical ionization mass spectrometry L. fastigiatum extract with ammonia as the reagent gas.

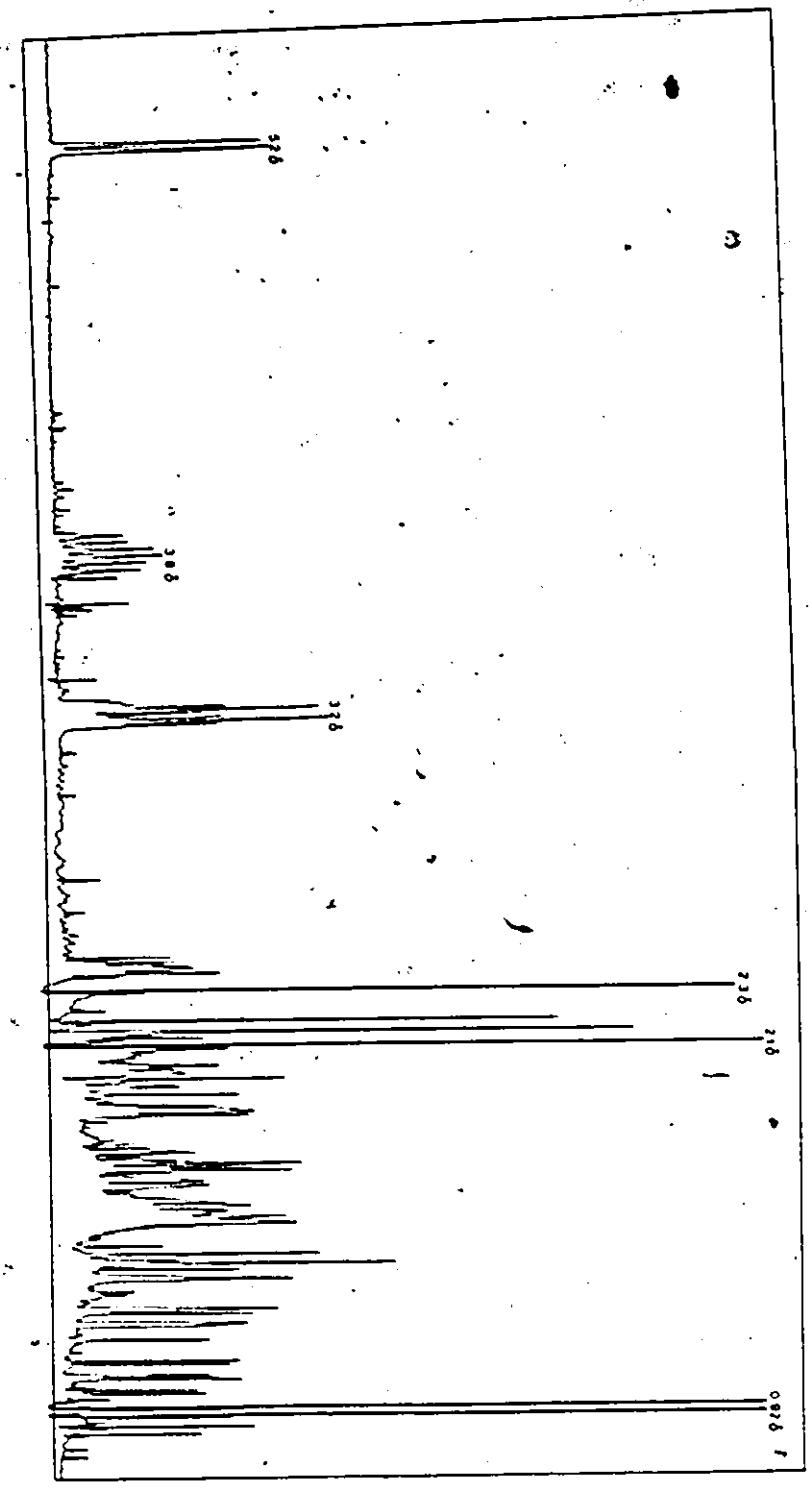
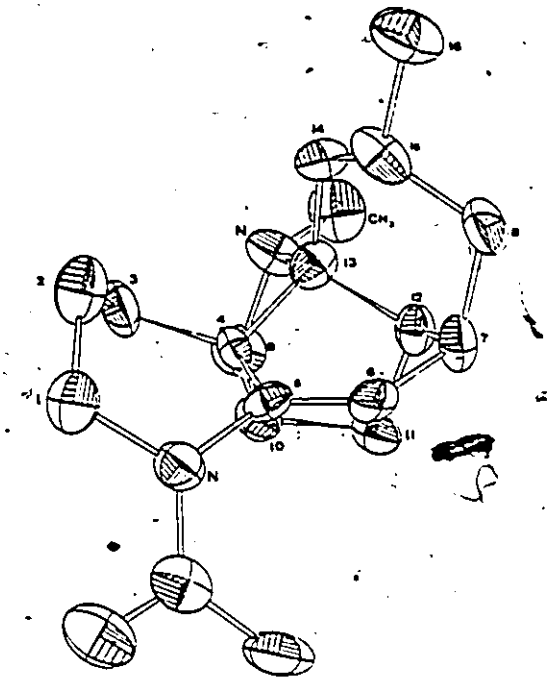


Figure 135 - Proton NMR of fastigiatine (250 mHz).



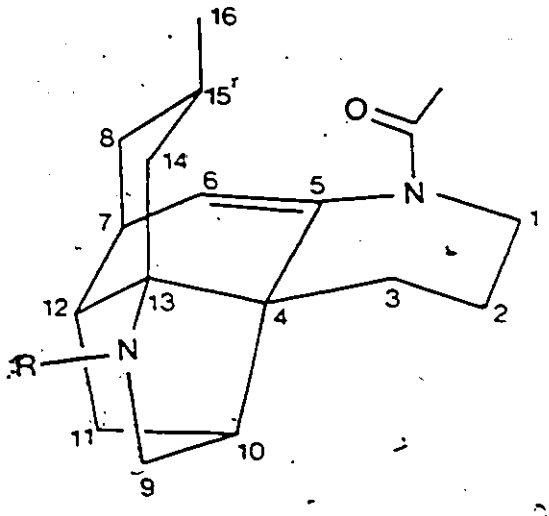


Figure 136 Structure of fastigiatine.

A B/E scan[217] of fastigiatine was carried out and is shown in Figure 137. A B/E scan detects all the fragment ions that are daughters of a selected parent ion. The only fragments that can be detected in a B/E spectrum are those which are formed in a field free region of the mass spectrometer. An ion of mass M and velocity v which is formed in the ion source will have a kinetic energy that is proportional to the accelerating voltage V_{acc}.

$$\frac{Mv^2}{2} = eV_{acc} \qquad (1)$$

In order for the ion to pass through an electric sector analyzer (ESA) with a potential of E and a radius of R_E, the deflection force (centripetal) K_E must balance the centrifugal force (K_C).

$$K_E = K_C \quad \text{or}$$

$$eE = \frac{Mv^2}{R_E} \qquad (2)$$

If the velocity of the ion, equation (3), derived from equation (1) is substituted into equation (2) one finds that the criterion for an ion to pass through the ESA is a function of its kinetic energy (equation 4).

$$v = \sqrt{\frac{2eV_{acc}}{M}} \qquad (3)$$

$$eE = \frac{M\left[\sqrt{\frac{2eV_{acc}}{M}}\right]^2}{R_E}$$

$$eE = \frac{2eV_{acc}}{R_E} \quad \text{or}$$

$$\frac{R_E E}{2} = V_{acc} \qquad (4)$$

For the ion to pass through a magnetic sector of strength B and radius R_M the magnetic force (centripetal) (K_B) must balance the centrifugal force.

$$K_B = K_C \quad \text{or}$$

$$evB = \frac{Mv^2}{R_M}$$

$$eB = \frac{Mv}{R_M}$$

$$eBR_M = J \qquad (6)$$

From equation (6) it is seen that the criterion for an ion to pass through the magnetic sector is a function of its momentum, J = Mv.

If a fragment ion is formed in the first field free region its velocity will be the same as its parent ion, however its kinetic energy would be reduced. For such an ion to pass through the ESA the potential, E must be reduced by a factor of M_1/M where M is the mass of the parent ion and M_1 is the mass of the fragment ion. Similarly the momentum of the ion would be reduced and for it to pass through the magnetic sector the field B must be reduced by a factor of M_1/M. If E and B are scanned downward from their initial values which permitted the detection of the parent ion, all of the daughter ions will be detected.

B/E scanning of fastigiatine shows that the ions at m/z 285 ($M-CH_3$), 257 ($M-C_3H_7$) or ($M-CH_3CO$) and 124 ($M-C_{11}H_{14}NO$) are formed directly from the molecular ion. Losses of 100 and 139 mass units from the molecular ion are observed; however these ions are not prominent in the normal EI spectrum. The ion with m/z 176 is not observed in the B/E spectrum, but this does not preclude its formation directly from the molecular ion if the fragmentation occurs in the ion source.

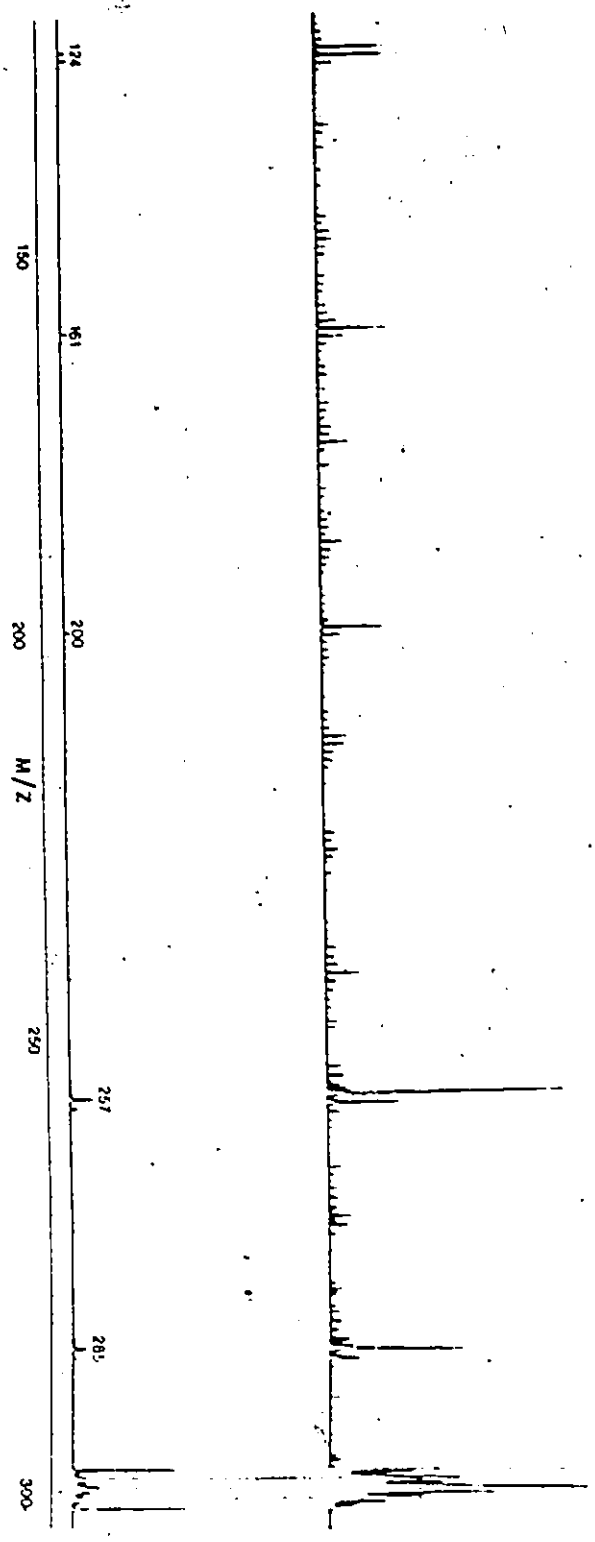


Figure 137. B/E mass spectrum of fastigiatine.

The ions at m/z 124 and 176 appear to arise from the fragmentation of fastigiatine into two parts, each of which can carry the charge. In the case of des-N-methylfastigiatine the ion corresponding to m/z 124 in fastigiatine is shifted by 14 mass units (CH_2) to m/z 110. Thus this ion arises from the portion of the molecule bearing the N-H group in the case of des-N-methylfastigiatine and bearing the N-CH_3 group in the case of fastigiatine. It is apparent that these ions cannot form in a simple manner. A fragmentation scheme for fastigiatine is proposed in Figure 138, in which the fragmentation is considered to be initiated by a retro Diels-Alder reaction resulting in the fragmentation of the bond between C-12 and C-7 and the bond between C-13 and C-4. Subsequent to the retro-Diels-Alder process the fission of the bonds between C-9, and C-10 and C-8, and C-15 is postulated and is predicted to be followed by the formation of bonds between C-9 and C-15 and between C-8 and C-10. The resulting ion can then fragment between C-11 and C-10 to form two species m/z 124 or m/z 176, depending on the fragment on which the charge becomes localized. The loss of a methyl group can arise from various locations on the molecular ion. The loss of CH_3CO is a well recognized loss from a N-$COCH_3$ group. The loss of C_3H_7 could arise in the same way as the similar loss of C_3H_7 from the bridge atoms in a lycopodane system.

3.4.6.3 NMR of fastigiatine

Since fastigiatine represents a new ring system a detailed study of its ^1H and ^{13}C NMR spectra was carried out. These studies should be

useful to other workers who might encounter this ring system in species yet to be examined.

From preliminary ^{1}H NMR examination fastigiatine was found to contain $N-CH_3$, $N-COCH_3$, and CH_3-CH groups and a trisubstituted double bond. From the ^{13}C NMR spectrum shown in Figure 139 and the spin sorted ^{13}C spectrum shown in Figure 140 and multiplicities obtained in off-resonance spectra, it was confirmed that four quaternary, three primary, seven secondary and five tertiary carbon atoms were present in fastigiatine. The assignment of signals to non-protonated carbon atoms was relatively simple. On the basis of their chemical shifts, peak 1 (170.0 δ) was assigned to the carbonyl carbon, (C-17) and peak 2 (139.2 δ) to the vinylic quaternary carbon C-5. The two tetrahedral quaternary signals, peaks 4 (65.4 δ) and 6 (55.0 δ) have been assigned to C-4 and C-13, respectively, on the basis of chemical shifts; however these assignments are not secure and may be reversed. Except in a few obvious cases the assignment of the ^{13}C signals of the protonated carbon atoms was not as straightforward and resort to more refined techniques had to be taken (see below).

The proton spectra of fastigiatine was also very complex as shown in Figures 135, 141 and 142. Minor differences in the chemical shifts among the spectra can be explained since the spectra were run by different operators on different instruments with different batches of $CDCl_3$. Again it was possible to assign definitively only a few of the resonances in the ^{1}H spectrum, for example, the vinylic proton and the three methyl groups. However by using two dimensional NMR methods assignments were made for the majority of the ^{1}H and ^{13}C signals in the respective spectra of the alkaloid.

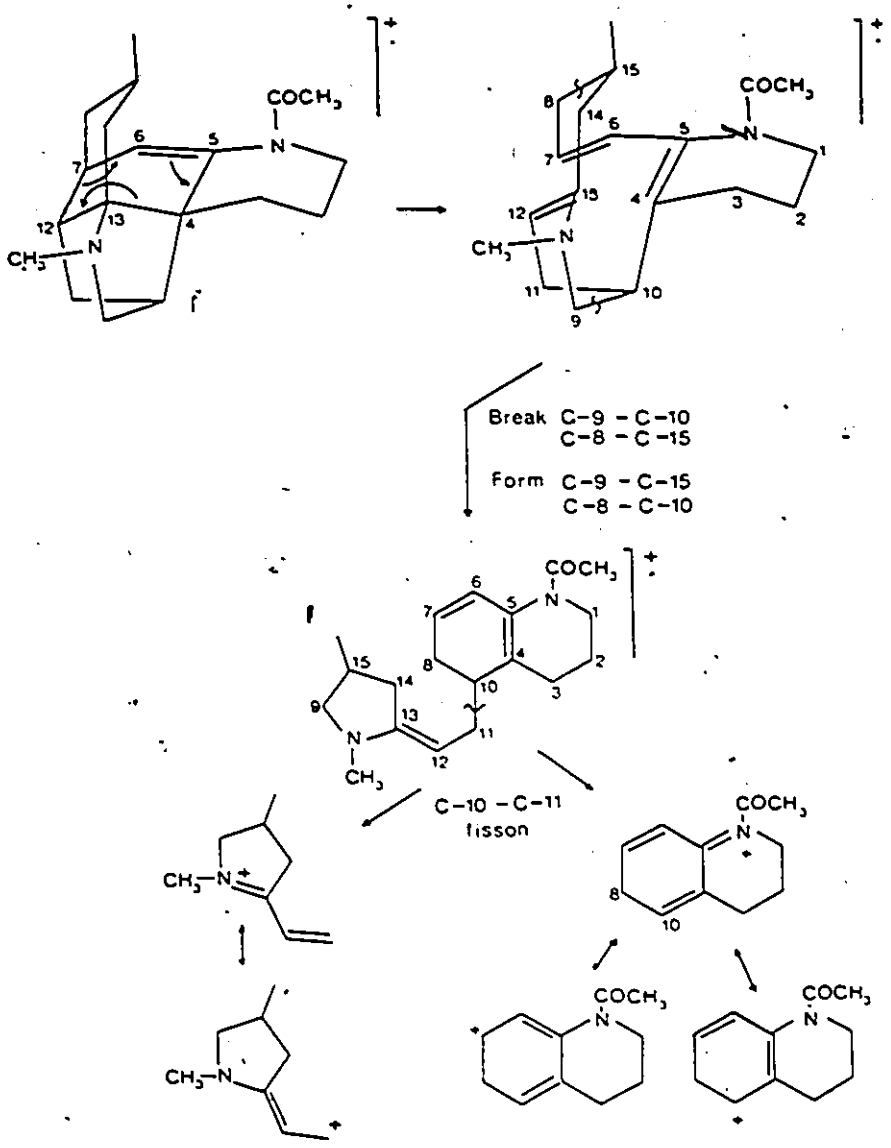


Figure 138 Fragmentation scheme of fastigiatine.

A $^{1}H/^{13}C$ heteronuclear correlated spectrum of fastigiatine is shown in Figure 141. If the $^{1}H/^{13}C$ spectrum is viewed along the horizontal axis a ^{13}C spectrum is observed, while a ^{1}H spectrum is observed along the vertical axis. With a spectrum of this type it is possible to interrelate the ^{13}C signal of a particular carbon atom with the signals arising from the proton or protons on the same carbon atom,[218] as demonstrated subsequently. A $^{1}H/^{1}H$ COSY spectrum of fastigiatine is shown in Figure 142. If the $^{1}H/^{1}H$ spectrum is viewed along the vertical, horizontal or diagonal axis a ^{1}H spectrum is observed. Those protons which couple exhibit signals symmetrically about the diagonal axis as illustrated in Figure 14.[218] The use of the 2D spectra in assigning the ^{13}C signals is discussed below.

The doublet at (0.92 δ, 3H, J = 6.5 Hz) in the proton spectrum of fastigiatine can be assigned to the protons on C-16 on the basis of its chemical shift, integrated area and multiplicity. Accordingly by making use of the $^{1}H/^{13}C$ spectrum (Figure 141) peak 17 (22.3 δ) can be assigned to C-16 of fastigiatine. The protons on C-16 are coupled to the proton on C-15 to form a multiplet at 1.9 δ as seen in the $^{1}H/^{1}H$ spectrum. With the chemical shift of the C-15 proton established, and referring now to the $^{1}H/^{13}C$ spectrum, peak 15 (25.5 δ) of the ^{13}C spectrum can be assigned to C-15 of fastigiatine.

From the proton spectrum (Figure 135) the singlet at 2.34 δ was assigned to the protons of the N-CH_3 group and the singlet at 2.15 δ to the methyl protons of the N-$COCH_3$ group. With this information the

$^{1}H/^{13}C$ spectrum can be used to assign the N-CH_3 group to peak 12 (35.0 δ) and the methyl of the N-$COCH_3$ group to peak 18 (21.5 δ).

Peak 3 (123.1 δ) in the ^{13}C spectrum can be assigned to the vinylic carbon, C-6, on the basis of its chemical shift and multiplicity. The proton on C-6 is coupled to the proton on C-7 and shows a long range coupling presumably to the proton on C-12 to form a doublet of doublets (dd, 5.2 δ, 1H, J = 1.1 and 5.5 Hz). Long range coupling, through more than three saturated bonds is often observed when the bonding system exists in the M or W conformation (H—C—C—C—H).[219] From the $^{1}H/^{1}H$ spectrum, couplings between the protons on C-6 and C-7 and between those on C-6 and C-12 are observed, which enables a chemical shift to be assigned to the protons on each of these carbon atoms. From the $^{1}H/^{13}C$ spectrum peak 9 in the ^{13}C spectrum can be assigned to C-7 and peak 10 (38.3) to C-12.

The protons on C-1 and C-9 should resonate downfield since they are adjacent to a nitrogen, and therefore the multiplets at 3.7, 3.3 and 2.3 δ in the ^{1}H spectrum can be attributed to the protons on C-1 and C-9. The protons on C-9 in fastigiatine should couple to each other and to the proton on C-10, a tertiary carbon. By examining the $^{1}H/^{1}H$ spectrum it may be observed that the multiplet at 3.3 δ couples to the multiplets at 2.3 δ and at 1.9 δ. With the determination of the chemical shifts of both C-9 protons and the proton at C-10, it was now possible by examining the $^{1}H/^{13}C$ spectrum to assign peak 5 (59.6 δ) to C-9 and peak 7 (45.5 δ) to C-10.

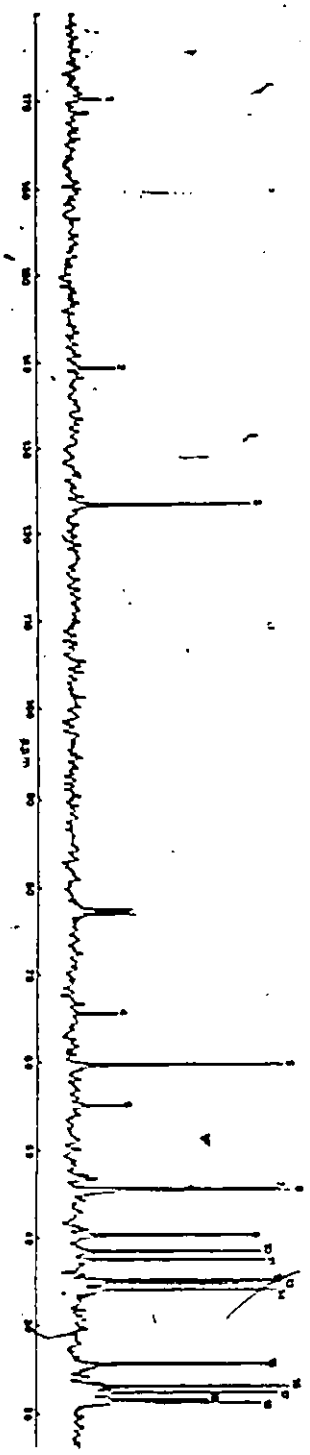


Figure 139 ^{13}C NMR spectrum of fastigiatine (250 mHz).

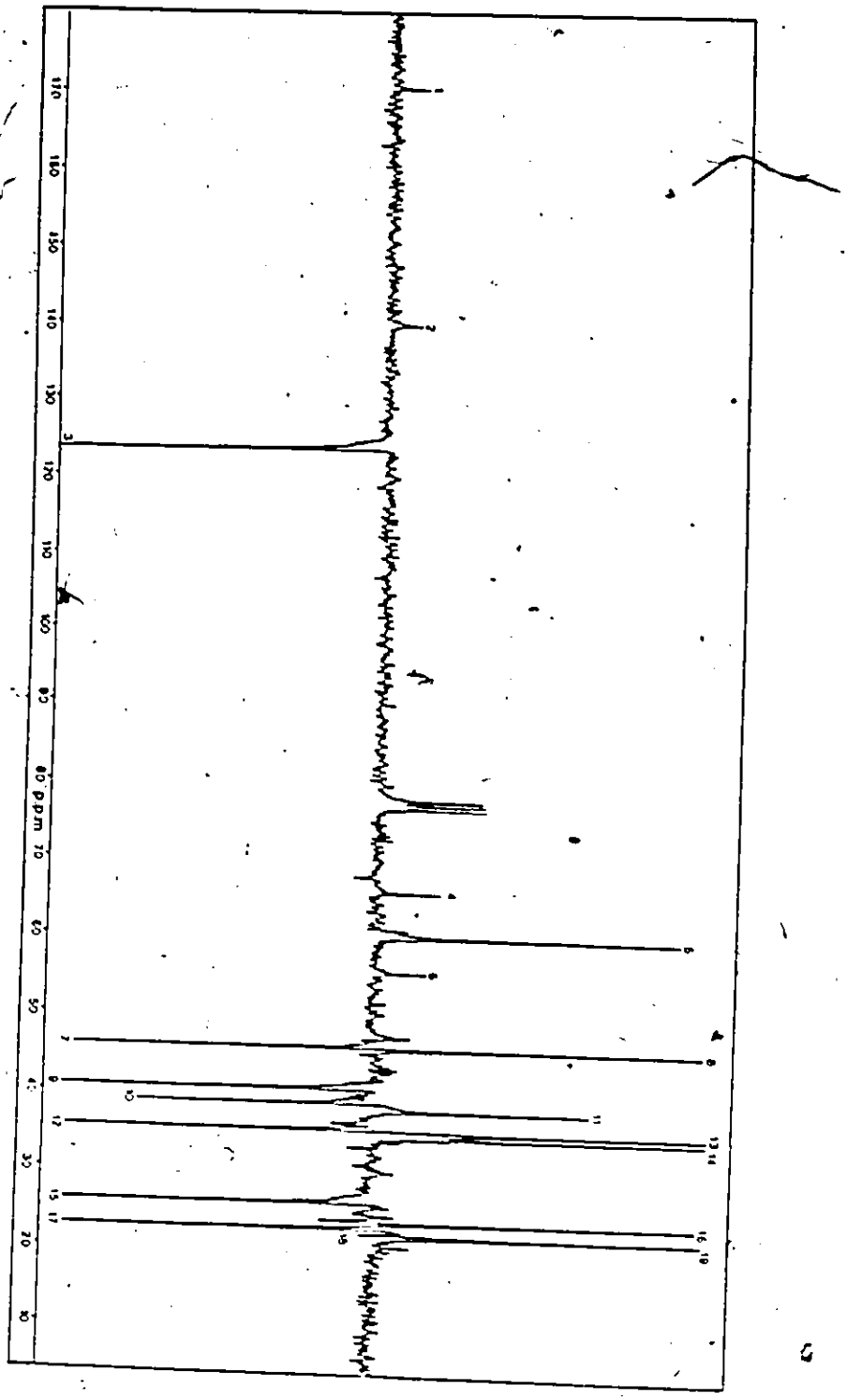
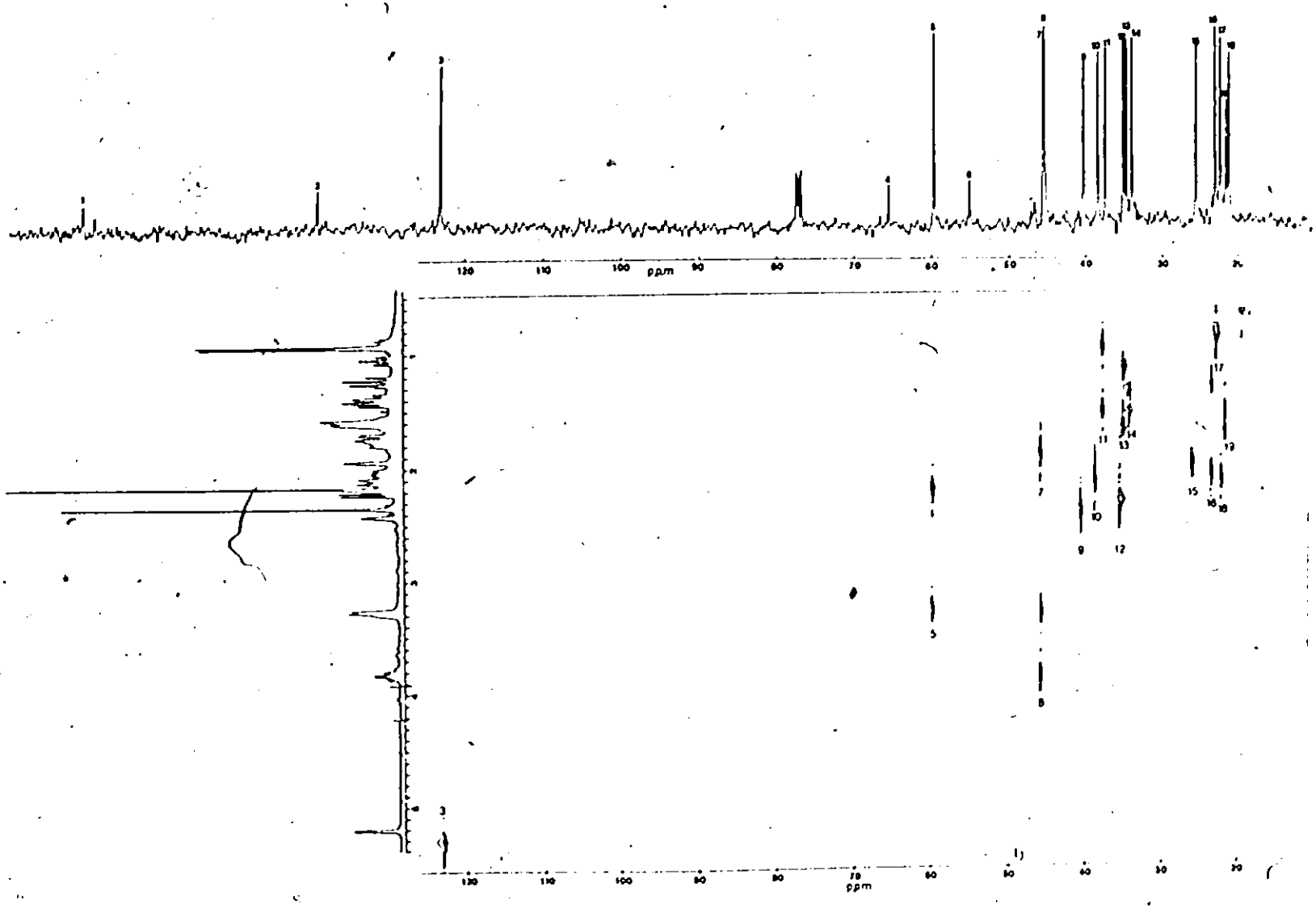


Figure 140 ^{13}C NMR spectrum of fastigiatine spin sorted C, CH_2 + ve and CH, CH_3 -ve (250 mHz).

Figure 141 Proton/^{13}C heteronuclear correlated spectrum of fastigiatine with the ^{13}C spectrum along the horizontal and the ^{1}H spectrum along the vertical axis (400 mHz).



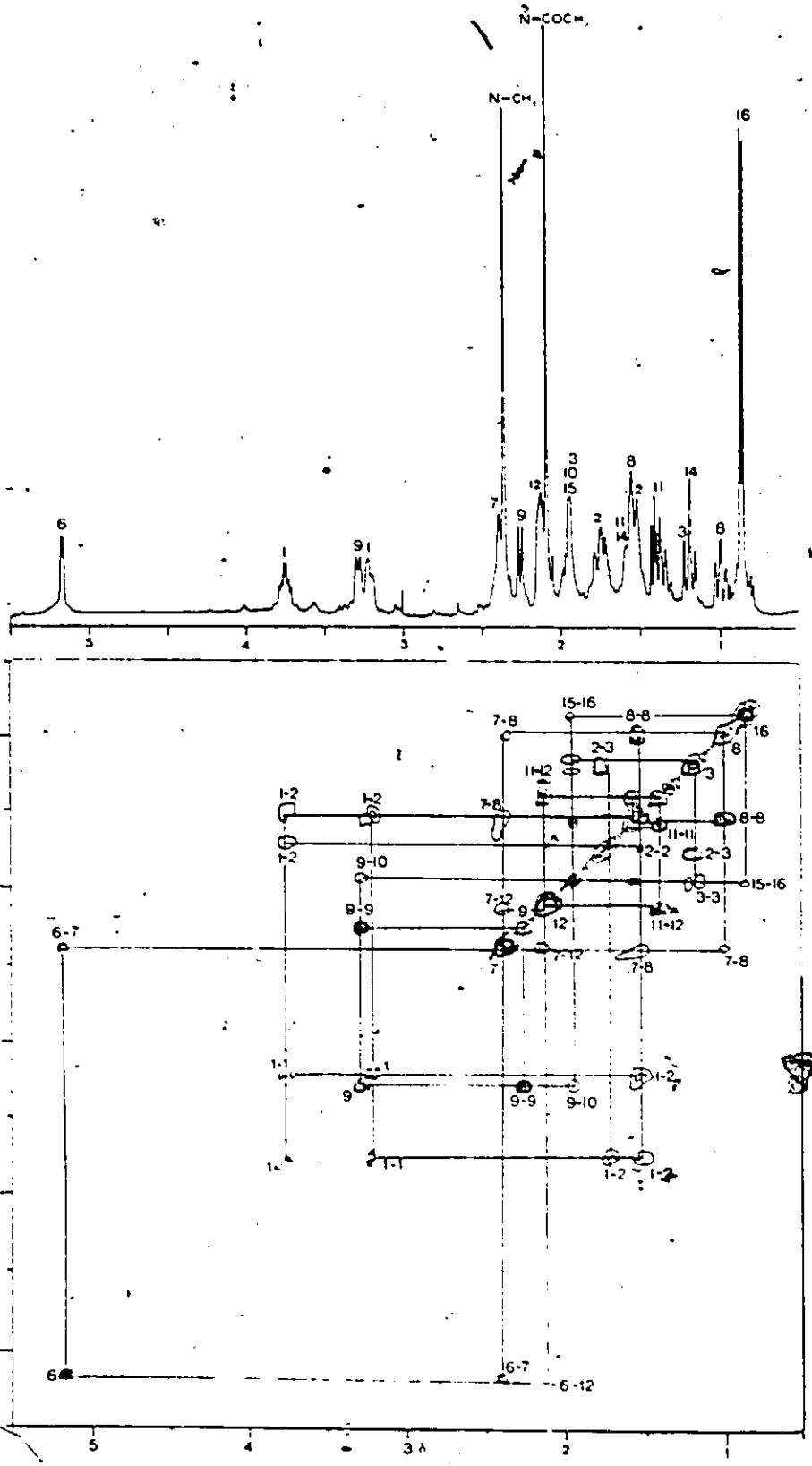


Figure 142 Proton/Proton COSY spectrum of fastigiatine (400 mHz).

The protons of C-1 in fastigiatine should couple to the protons of C-2. By examining the $^{1}H/^{1}H$ spectrum it can be observed that the multiplets at 3.7 and 3.3 δ couple to the multiplets at 1.5 and 1.7 δ. With the ^{1}H chemical shifts known it was possible to assign peak 8 (45.3 δ) to C-1 and peak 19 (21.1 δ) to C-2. Turning now to C-3 it is evident that the protons on C-2 will couple to the protons on C-3 and from an examination of the $^{1}H/^{1}H$ spectrum and the $^{1}H/^{13}C$ spectrum peak 16 (23.0 δ) was assigned to C-3.

The proton at C-7 couples to the protons at C-8 as seen from the $^{1}H/^{1}H$ spectrum and the chemical shift of each proton at C-8 could then be determined. Thus by examining the $^{1}H/^{13}C$ spectrum peak 11 (37.4 δ) can be assigned to C-8.

The assignment of the two remaining resonances was resolved in the following manner. The proton on C-10 was coupled to the two protons on C-11 and by analysing the $^{1}H/^{1}H$ spectrum and the $^{1}H/^{13}C$ spectrum C-11 can be assigned to peak 14 (33.9 δ). In like manner the protons on C-14 should couple to the proton on C-15, and by examining a slice of the $^{1}H/^{1}H$ spectrum along 1.9 δ the chemical shifts of the protons on C-14 were determined. In this way peak 13 (34.6 δ) in the ^{13}C spectrum was assigned to C-14. All assignments and ^{13}C chemical shifts are tabulated in Table 25.

3.4.7 Examination of L. scariosum

The chromatogram of the extract of L. scariosum by FSC/FID/NPD is shown in Figure 143. Peak B which gave a nitrogen response has a retention index of 2030 indicating that it may be lycopodine. The extract was also examined by GC/MS the TIC being shown in Figure 144. The major

Table 25

C^{13} assignments and chemical shifts of fastigiatine

peak #	δ	spin sort	carbon type	assignment
1	170.0	+	C	17
2	139.2	+	C	5
3	123.1	-	CH	6
4	65.4	+	C	4 or 13
5	59.6	+	CH_2	9
6	55.0	+	C	13 or 4
7	45.5	-	CH	10
8	45.3	+	CH_2	1
9	40.2	-	CH	7
10	38.3	-	CH	12
11	37.4	+	CH_2	8
12	35.0	-	CH_3	19 ($N-CH_3$)
13	34.6	+	CH_2	14
14	33.9	+	CH_2	11
15	25.5	-	CH	15
16	23.0	+	CH_2	3
17	22.3	-	CH_3	16 ($-CH_3$)
18	21.5	-	CH_3	18 ($NCOCH_3$)
19	21.1	+	CH_2	2

component of the mixture was found to be methyl ferulate from its mass spectrum shown in Figure 145. Compound B was identified by its mass spectrum shown in Figure 146 as lycopodine and compound C as dioctylphthalate. FSC/MS failed to disclose any other components.

L. scariosum is the first plant of the scariosum group in the subgenus Lycopodium to be examined. The low concentration of alkaloids may be a feature of that group. L. scariosum should be reexamined and other plants of the group examined to test these findings.

3.5 Internal standard quantitation

Lycodine and lycopodine were chosen to test internal standard quantitation since sufficient pure material was available for determination of response curves, and in L. lucidulum (the extract examined) both alkaloids eluted without contaminants. The percent of lycodine and lycopodine in the extract was determined through the use of annotinine as an internal standard. The raw data and calculations are tabulated in Table 26. Although lycopodine makes up 38% of the total alkaloid it only accounts for 13% of the extract. This implies that the extract contains components that do not pass through the gas chromatography column. These components could be inorganic salts or non volatile or highly polar organic compounds.

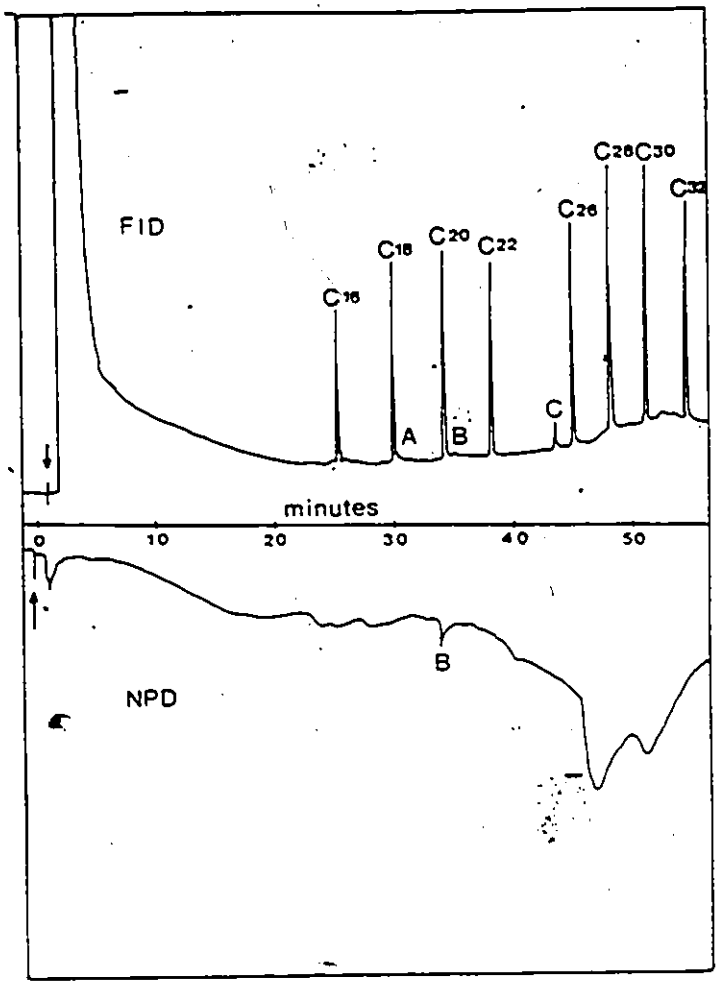


Figure 143 FSC/FID/NPD chromatogram of L. scariosum extract with hydrocarbon standard.

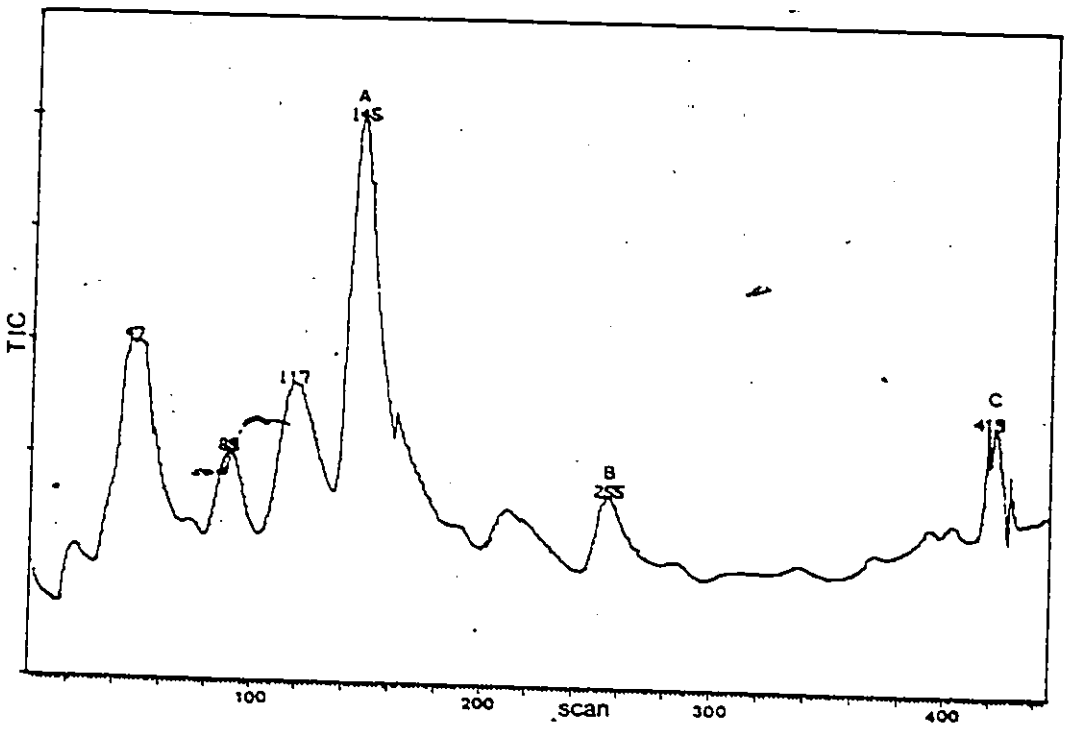
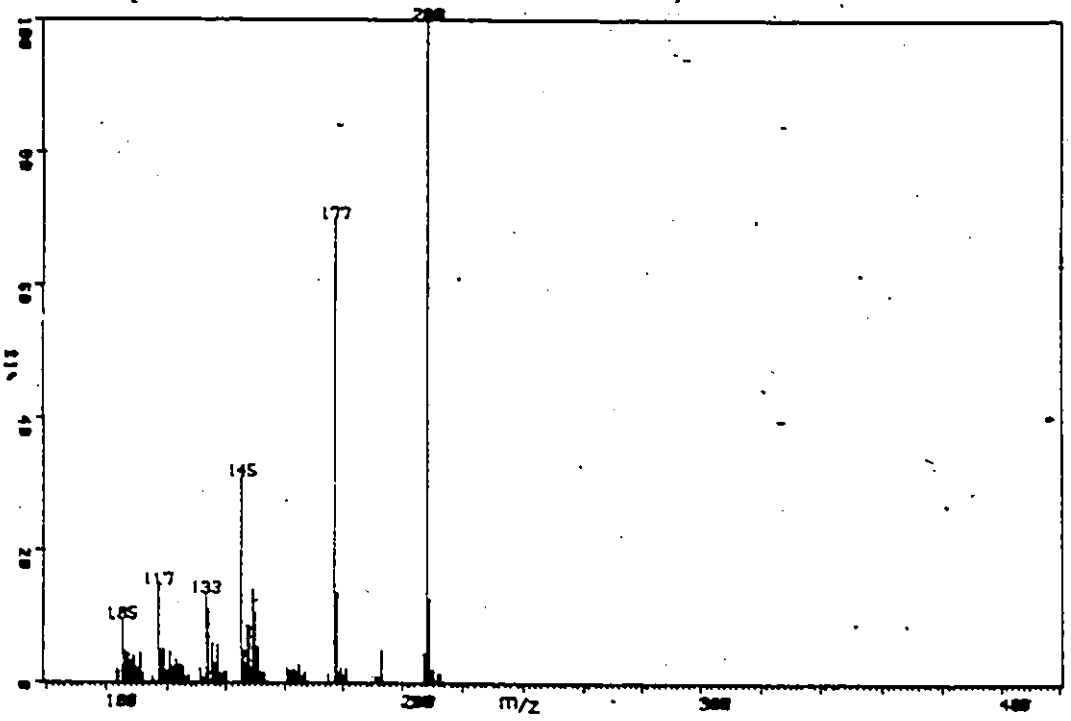


Figure 144 GC/MS TIC of L. scariosum extract.

Figure 145 Mass spectrum of component A (the methyl ester of ferulic acid).



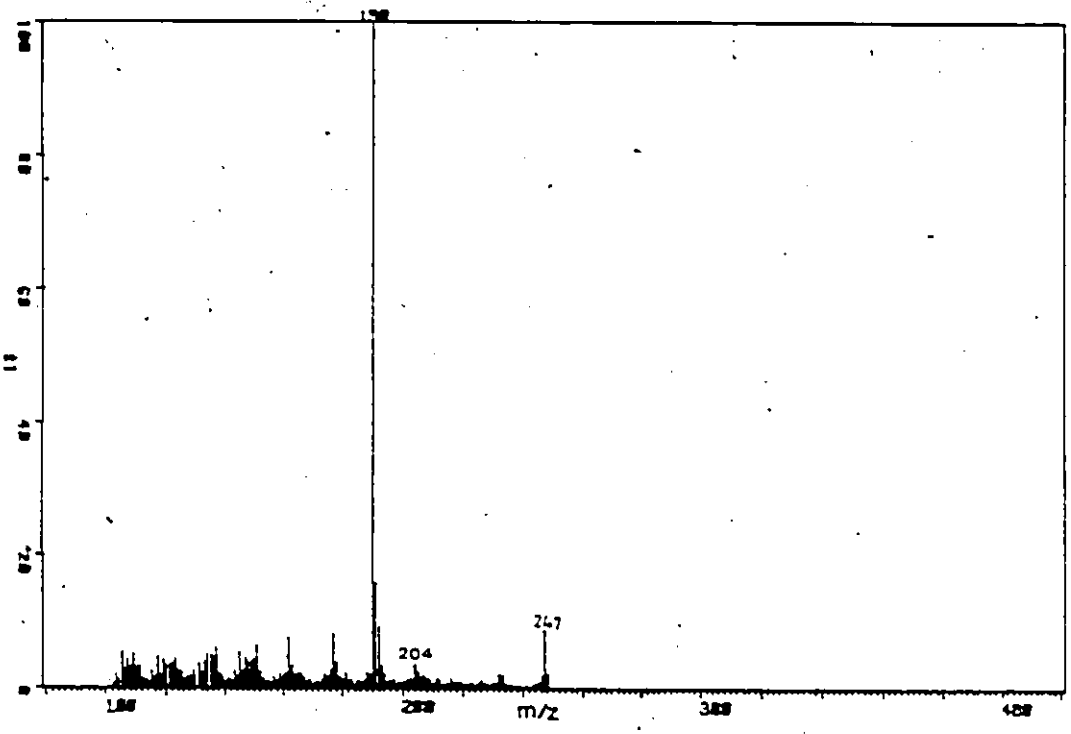


Figure 146 Mass spectrum of component B (lycopodine).

Table 26

Quantitation of lycodine and lycopodine in L. lucidulum

$$\frac{\text{alkaloid concentration of internal standard}}{\text{concentration of alkaloid}}$$

lycopodine	experimental	calibration	mg/ml of	%
	6.438	6.432	0.1101	12.9
	6.140	6.136	0.1154	13.5
	5.911	5.909	0.1198	14.0
		mean	0.1151	13.5
		standard deviation	0.0048	0.6
lycodine	30.83	29.64	0.0239	0.39
	25.10	24.18	0.0293	0.48
	25.67	24.72	0.0286	0.49
		mean	0.0273	0.45
		standard deviation	0.0029	0.06

CHAPTER 4

Conclusion

The retention indices of 33 authentic Lycopodium alkaloids were determined using DB-1 and DB-5 fused silica capillary columns. The electron ionization mass spectra of 44 authentic Lycopodium alkaloids were recorded. Thirty two mass spectra were obtained from the literature and entered into a computer data base along with the spectra obtained from authentic samples. The data base of 75 mass spectra was used as a library for a computer search program. After acquisition of data from gas chromatography-mass spectrometry the search program was used to identify components of the mixture which were known Lycopodium alkaloids. Retention indices were also compared to verify the identification of the alkaloids. The percent of each component in the alkaloid extract was calculated from peak heights and areas from gas chromatography. The internal standard method of quantitation was also investigated.

The chemical ionization mass spectra of 34 authentic Lycopodium alkaloids were recorded using methane as the reagent gas. It was found that the chemical ionization mass spectra could be used to establish the presence of OH, $OCOCH_3$ and $NHCOCH_3$ groups in a compound.

Seven Lycopodium species were examined for alkaloid content. L. australianum, which had not been previously examined, was found to elaborate lycodine, cernuine and an alkaloid of unknown structure. Wilce

placed L. australianum in the selago section of the subgenus Urostachys. This is the first time that a species of the subgenus Urostachys has been found to elaborate an alkaloid with a cernuane skeleton. Plants of the subgenus Urostachys are known to produce lucidane and phlegmarane type alkaloids. Unknown C from L. australianum might be related in structure to one of these two types of alkaloids.

L. clavatum var. borbonicum has been examined previously. The following alkaloids reported by others were detected; anhydrolycodoline, lycopodine, dihydrolycopodine, acetyldihydrolycopodine, lycodoline, lycoflexine, borbonicine and N_{α}-acetyl-N_{β}-methylphlegmarine. The dimeric alkaloid lycodiflexine was not observed. The alkaloids lycodine, flabelliformine, L20 and an unknown alkaloid with molecular weight 279 had not previously been reported to be elaborated by L. clavatum var. borbonicum.

L. deuterodensum has been previously examined and found to contain lycopodine, clavolonine and an alkaloid of undetermined structure, L35. Examination of L. deuterodensum disclosed the presence of the following alkaloids: lycodine, anhydrolycodoline, lycopodine, flabelliformine, lycodoline, lycoflexine, clavolonine, flabelline and five unknown compounds. The unknowns were present in low concentrations and could not be isolated for structural analysis. No alkaloid with the properties of L35 was detected. L. deuterodensum has been placed in a unique group of the subgenus Lycopodium. Plants of this subgenus elaborate alkaloids primarily of the lycopodane type and this was found to be the case for L. deuterodensum.

Examination of the alkaloids of L. fastigiatum revealed the presence of ten alkaloids of established structure, lycodine, anhydrolycodoline, lycopodine, dihydrolycopodine, flabelliformine, acetyldihydrolycopodine, lycodoline, lycoflexine, clavolonine and α-obscurine. Three new alkaloids were also detected, two of which have had their structure determined. The new alkaloids, named fastigiatine and des-N-methylfastigiatine, belong to a new skeletal type. The skeleton corresponds to a lycodane skeleton with an additional bond between C-10 and C-4. This is analogous to the inundatane skeleton which is a similarly modified lycopodane skeleton . L. fastigiatum has been placed in the fastigiatum group of the subgenus Lycopodium which also contains L. paniculatum and L. magellanicum. The latter species elaborate alkaloids with the magellanane skeleton, along with alkaloids of the lycopodane and lycodane type. There is some evidence for the presence of 5-dehydromagellanine in L. fastigiatum but its identity has not been firmly established.

L. flabelliforme has been extensively examined and the following alkaloids which have been reported in L. flabelliforme have been detected: dihydrolycopodine, anhydrodihydrolycopodine, flabellidine, α-obscurine, lycopodine, flabelline, clavolonine, lycodine and des-N-methyl-α-obscurine. Acetyldihydrolycopodine, β-obscurine, flabelliformine, annotinine, hydroxy des-N-methyl-α-obscurine and nicotine were not found. Perhaps these alkaloids were not detected because of seasonal variation in alkaloid production, or they may have been present at concentrations below the detection limits of gas chromatography- mass spectrometry. Nicotine, that was found in L. flabelliforme by earlier

investigators may have come from contamination by tobacco during the extraction.

L. lucidulum has been reported to contain luciduline, lycodine, lycopodine, lycodoline, L20, L23, lucidioline, flabelliformine, lucidine A, lucidine B, lycolucine, dihydrolycolucine and spirolucidine. All of these alkaloids were found with the exception of lucidioline, lucidine A, lycolucine and dihydrolycolucine. Dihydroluciduline and N-methyllycodine found in L. lucidulum had not previously been reported. It has been established that the high molecular weight alkaloids of L. lucidulum can be detected by GC/MS.

L. scariosum was found to elaborate only lycopodine. L. scariosum has been placed in the scariosum group of the subgenus Lycopodium. At present L. scariosum is the only member of the group which has been examined for alkaloid content. L. scariosum examined here had a very low alkaloid content, and should be reexamined along with other members of its group to test the results obtained.

The method of gas chromatography-mass spectrometry has proven very useful in detecting and identifying alkaloids in plants of the order Lycopodiales. The use of a data base search program has provided a quick method of screening plants for known or new alkaloids. Since only about ten percent of the known Lycopodium species have been examined the GC/MS method described in this thesis will enable future investigators to examine rapidly and quantitatively the remaining species of the genus using only small quantities of plant material. Such studies should provide useful information on the chemotaxonomy of the order and once a new alkaloid has been found, quantitation can be used to determine the

amount of plant material which must be extracted to provide sufficient alkaloid for a full structure determination.

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Appendix 1

Abbreviations

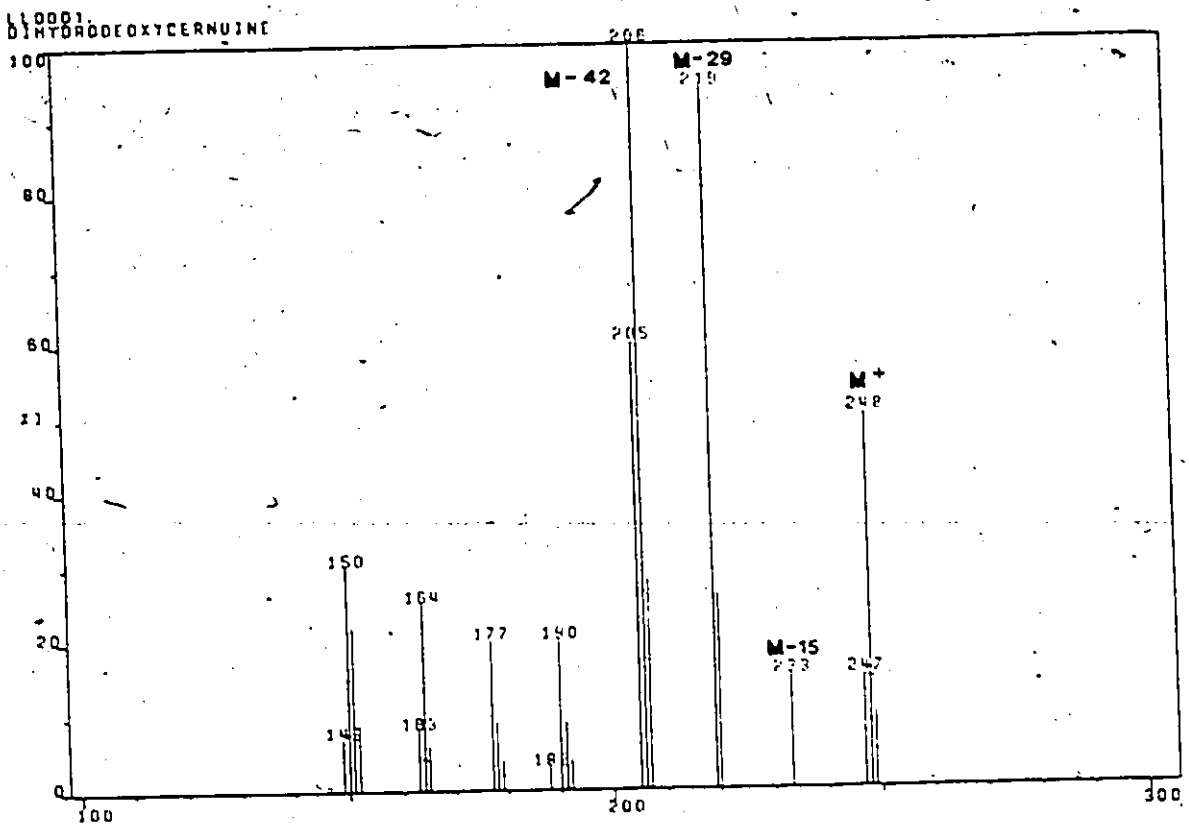
A.R.I.	authentic retention index
B	magnetic field strength
CI	chemical ionization
EI	electric ionization
E	electric field strength
FID	flame ionization detector
FSC	fused silica column
FSC/FID	gas chromatography using a fused silica column and a flame ionization detector
FSC/FID/NPD	gas chromatography using a fused silica column and a flame ionization detector and a nitrogen phosphorus detector
FSC/MS	gas chromatography-mass spectrometry using a fused silica column
GC	gas chromatography
GC/MS	gas chromatography-mass spectrometry
int	percent of total alkaloid fund by integrating peak areas from a gas chromatography experiment
LC	liquid chromatography
M	mass
(M*)	metastable ion
ms	mass spectrometry
m/z	mass to charge ratio
NPD	nitrogen phosphorus detector
PK_h.	percent of total alkaloid found from peak heights from a gas chromatography experiment

r	radius
R.I.	retention index
R_f	relativie front
TIC	total ion chromatogram
v	velocity
V_{acc}	accelerating voltage
VG	Vacuum Generators

Appendix 2

List of spectra

* spectrum 77a is the spectrum recorded in the literature.[129] Spectrum 77b is that obtained from a sample submitted by W. A. Ayer. Personal communication from W. A. Ayer to D. B. MacLean (June 15, 1983) indicates that the literature spectrum is incorrect.


LL0001
DIHYDRODEOXYCERNUINE
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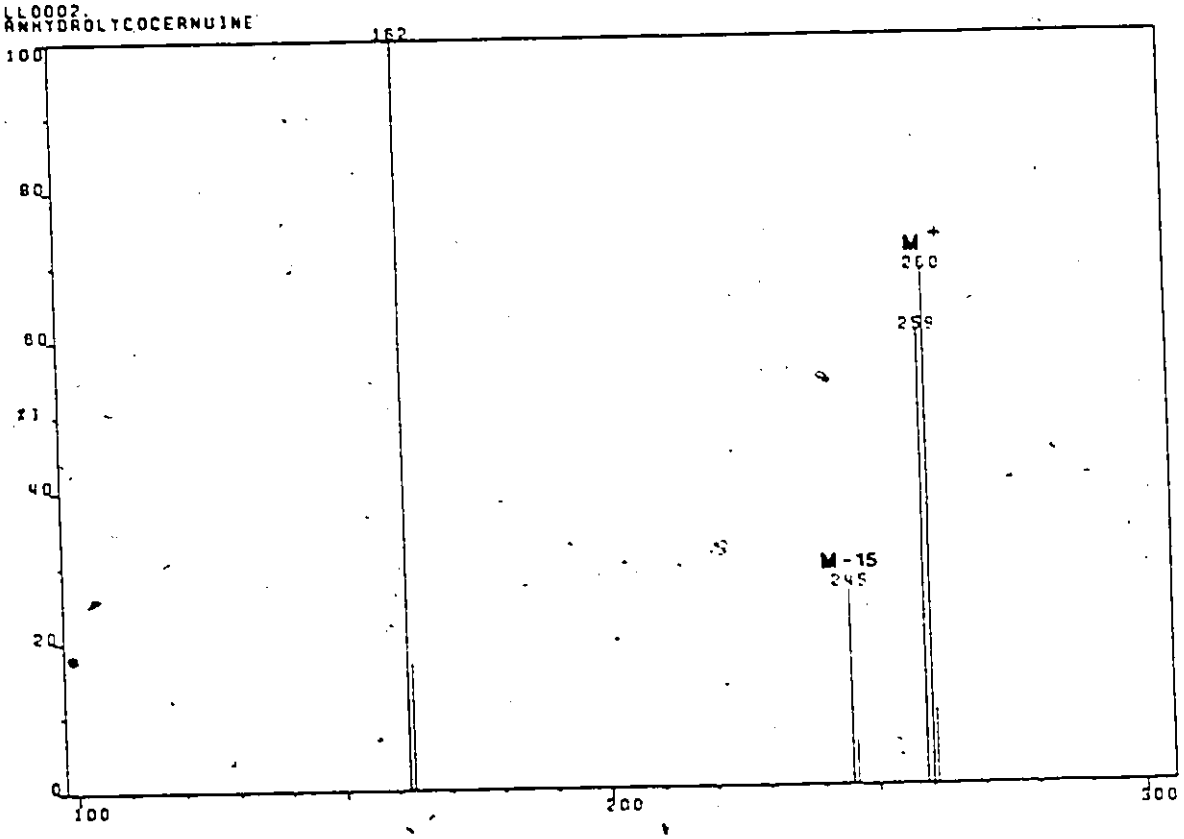
M-29
M+
248
M-15
223
247
205
150
164
177
140
100
200
300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ANHYDROLYCOCERNUINE
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M+
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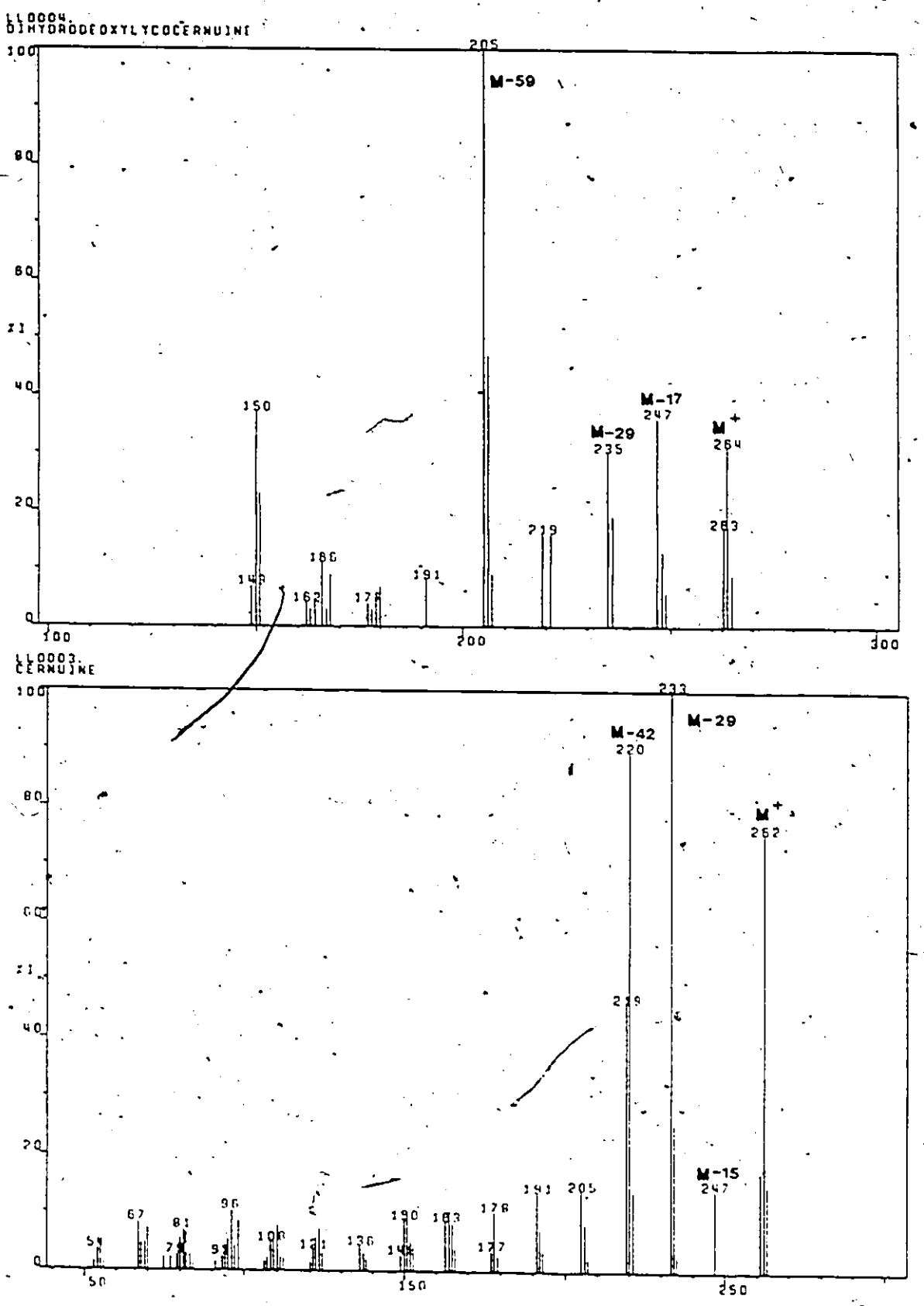
M-15
245
100
200
300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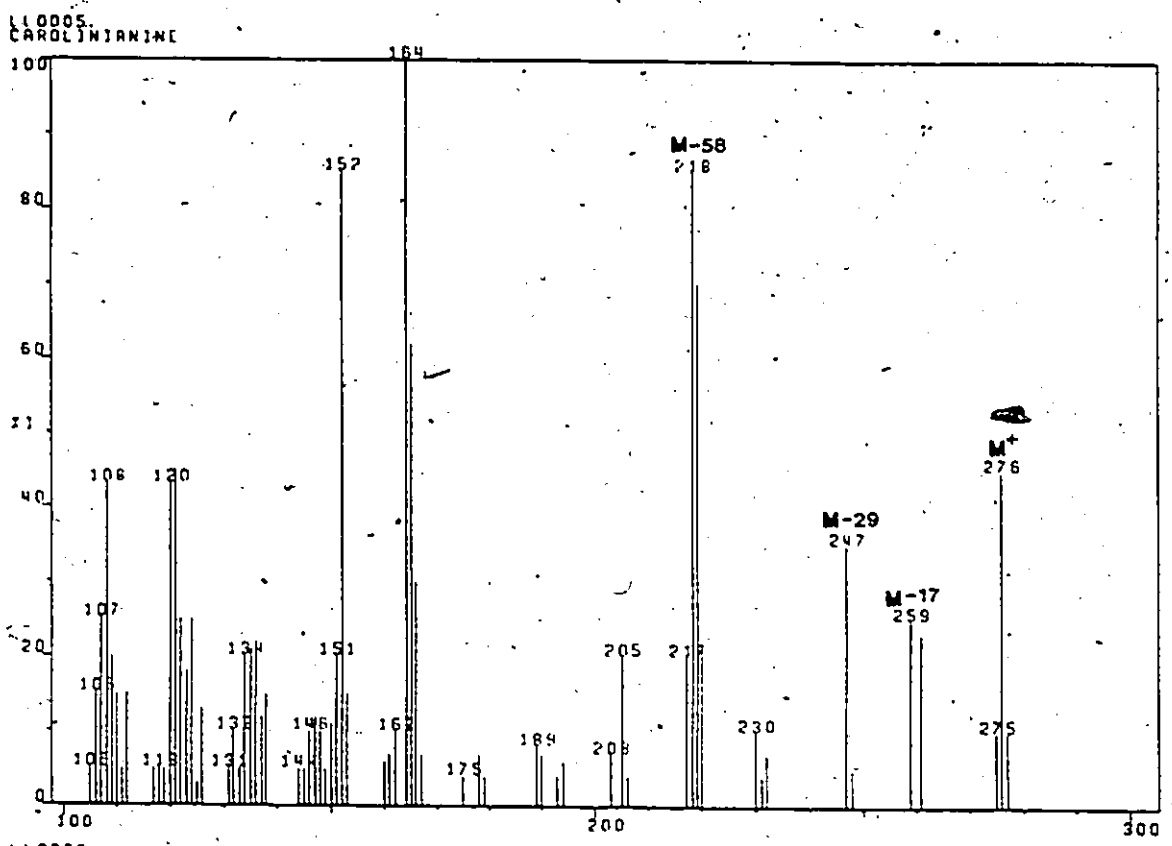
DIHYDRODEOXYLYCOCERNUINE
205
M-59
M-17
247
M-29
235
M+
264
150
219
263
180
191
148
162
178
100
200
300
LL0003.
CERNUINE
233
M-29
M-42
220
M+
262
219
M-15
247
205
191
190
183
178
177
67
81
96
54
50
150
250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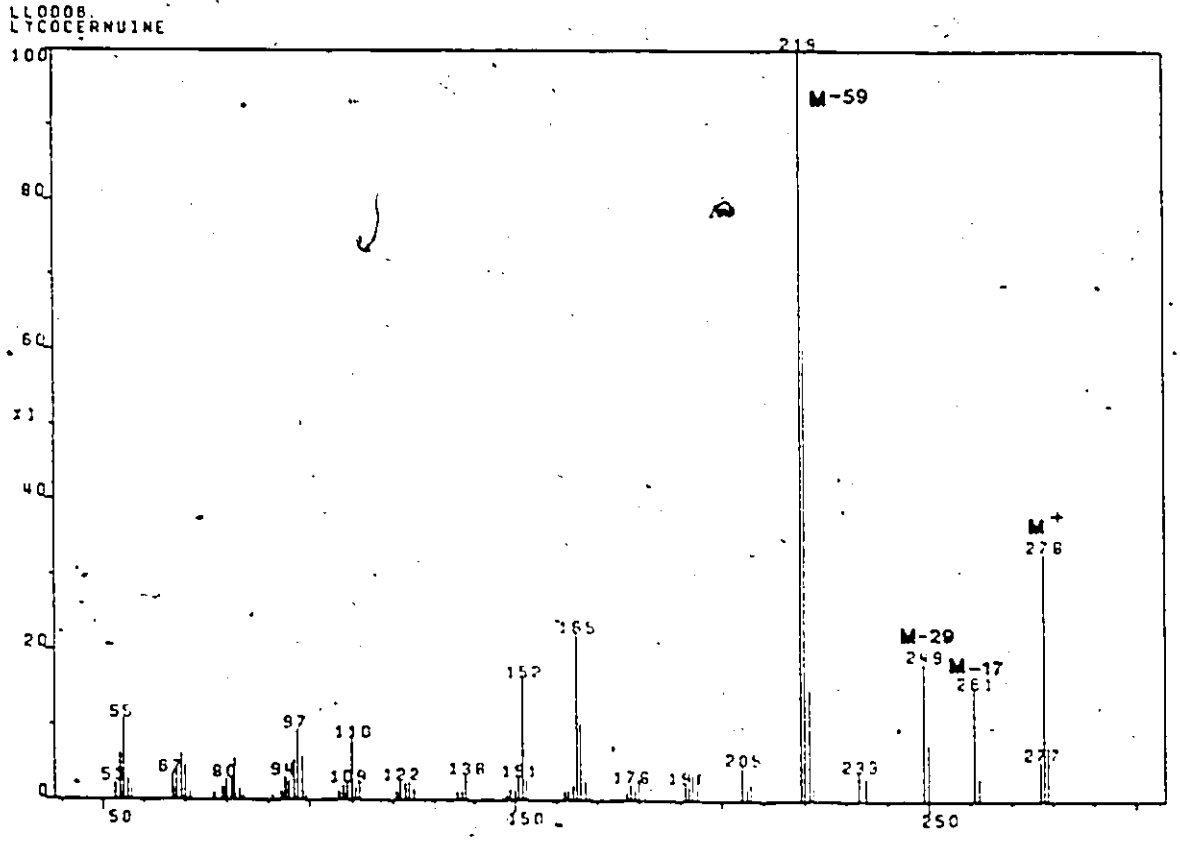
M-29
247
M-17
259
M+
276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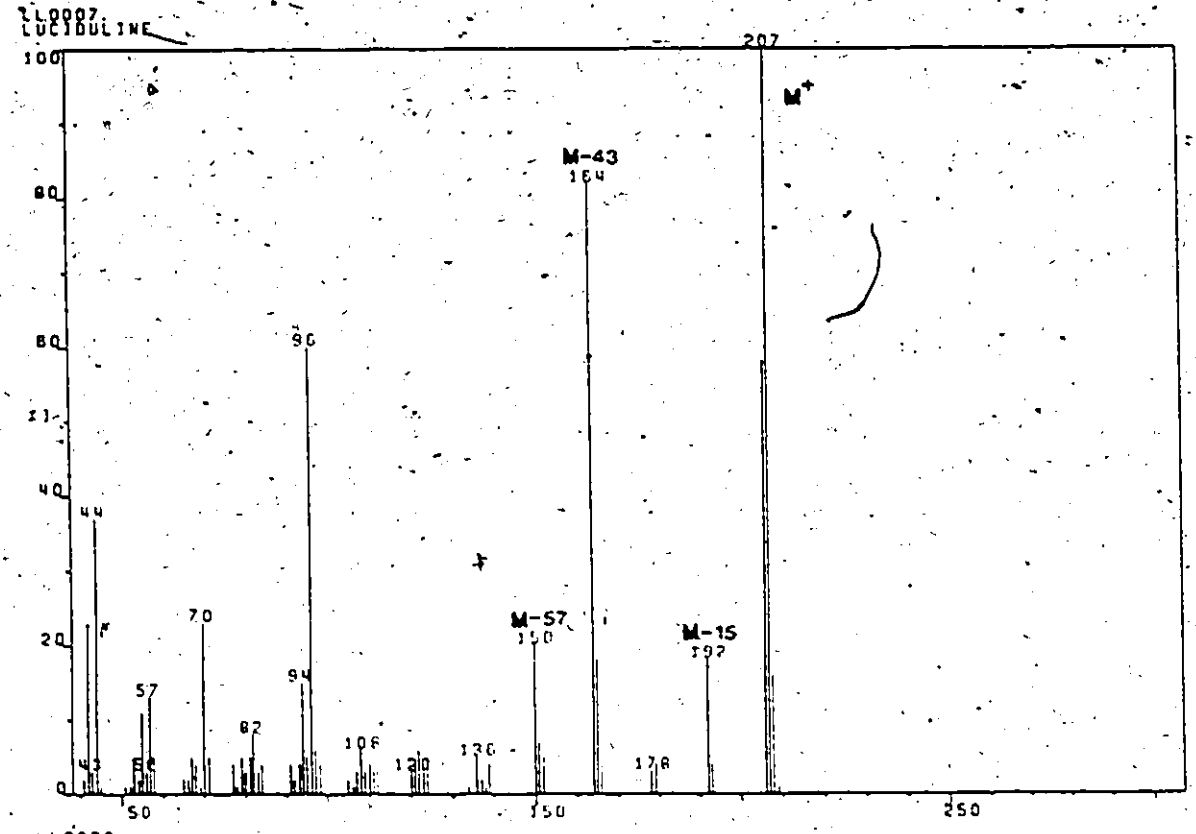
M-59
M-29
249
M-17
261
M+
276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80
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20
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207
M+
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150
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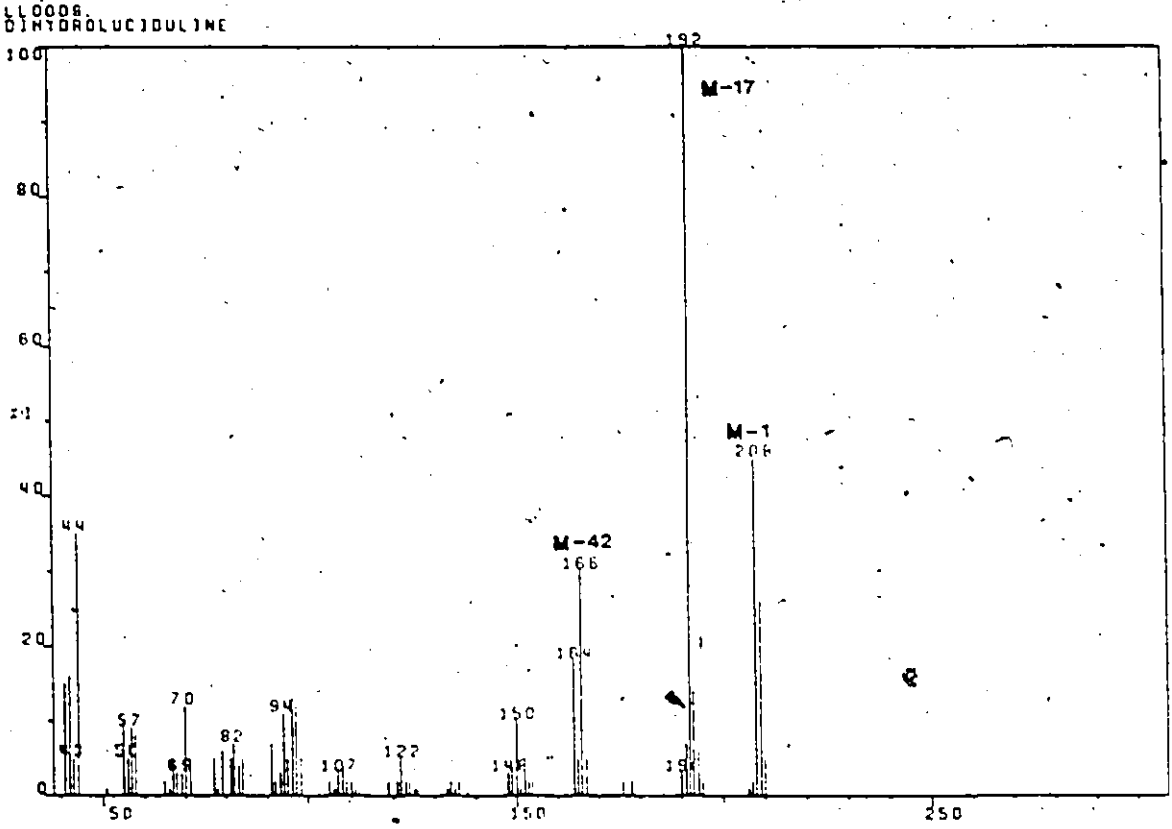
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70
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122
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150
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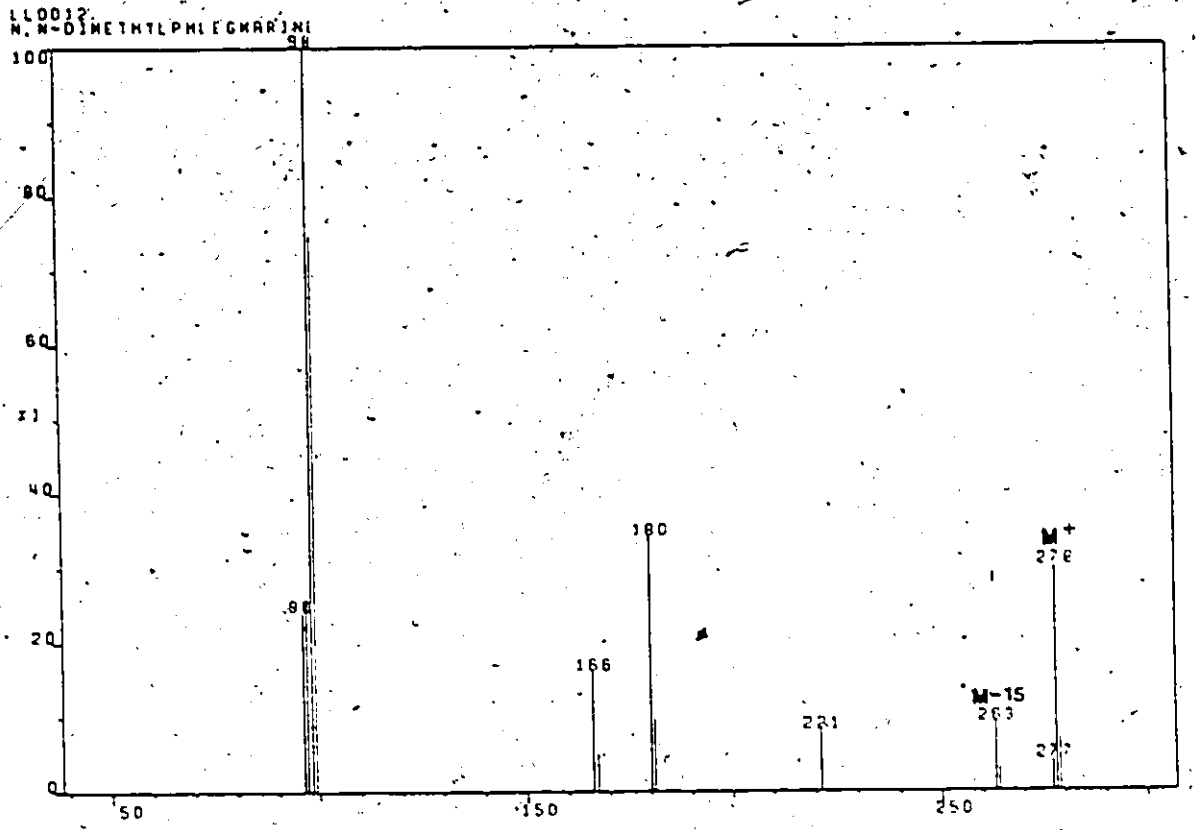
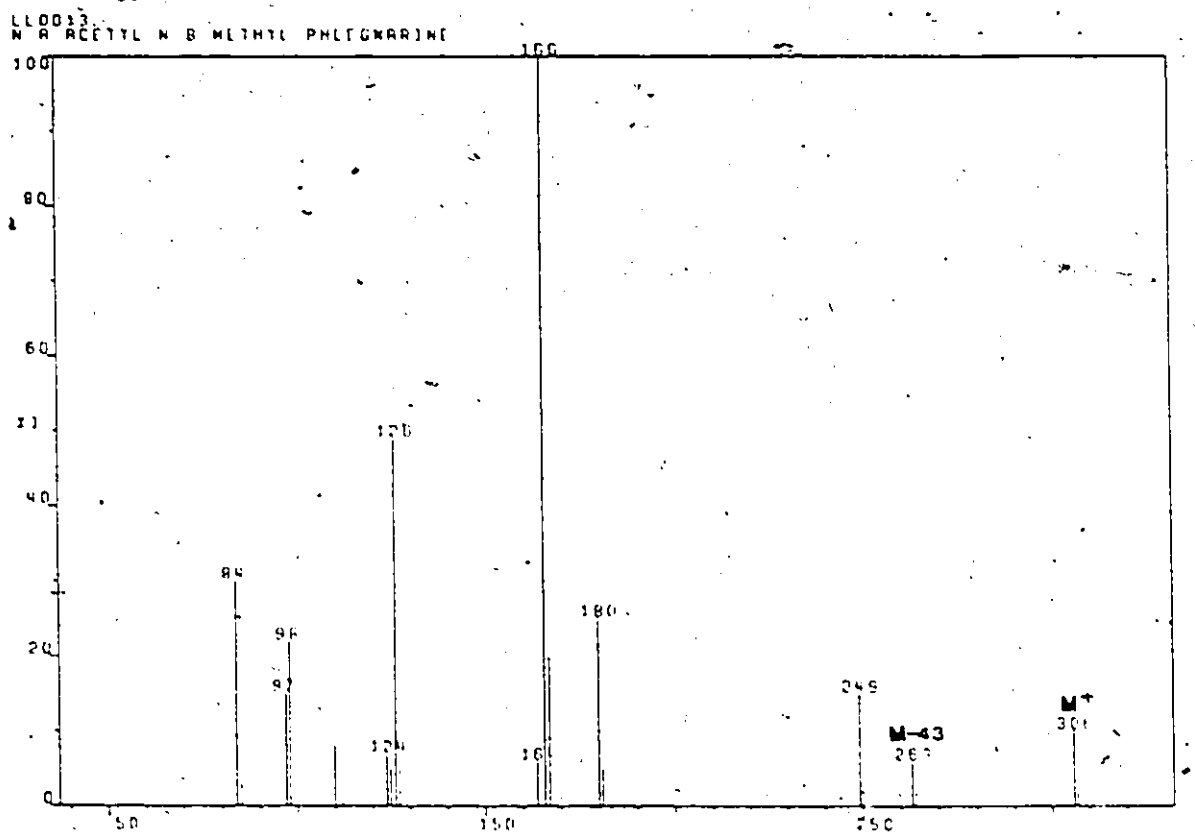
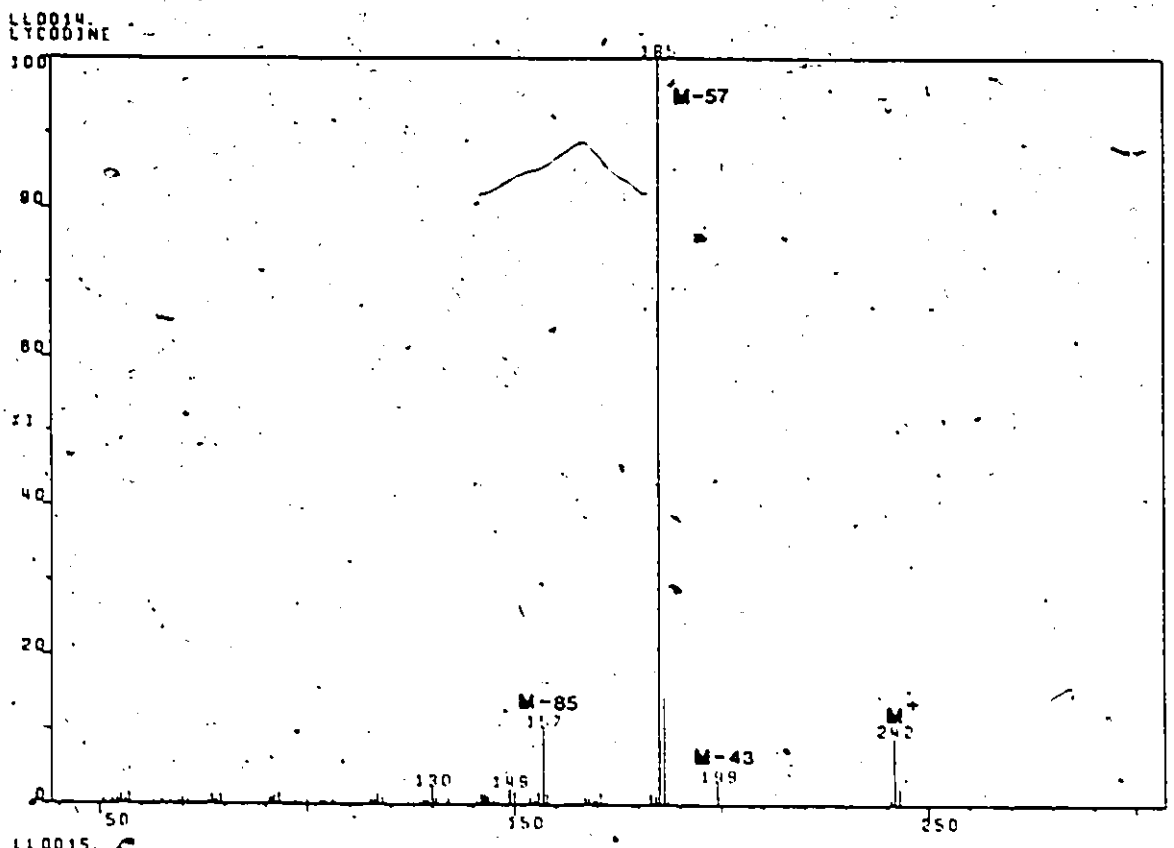
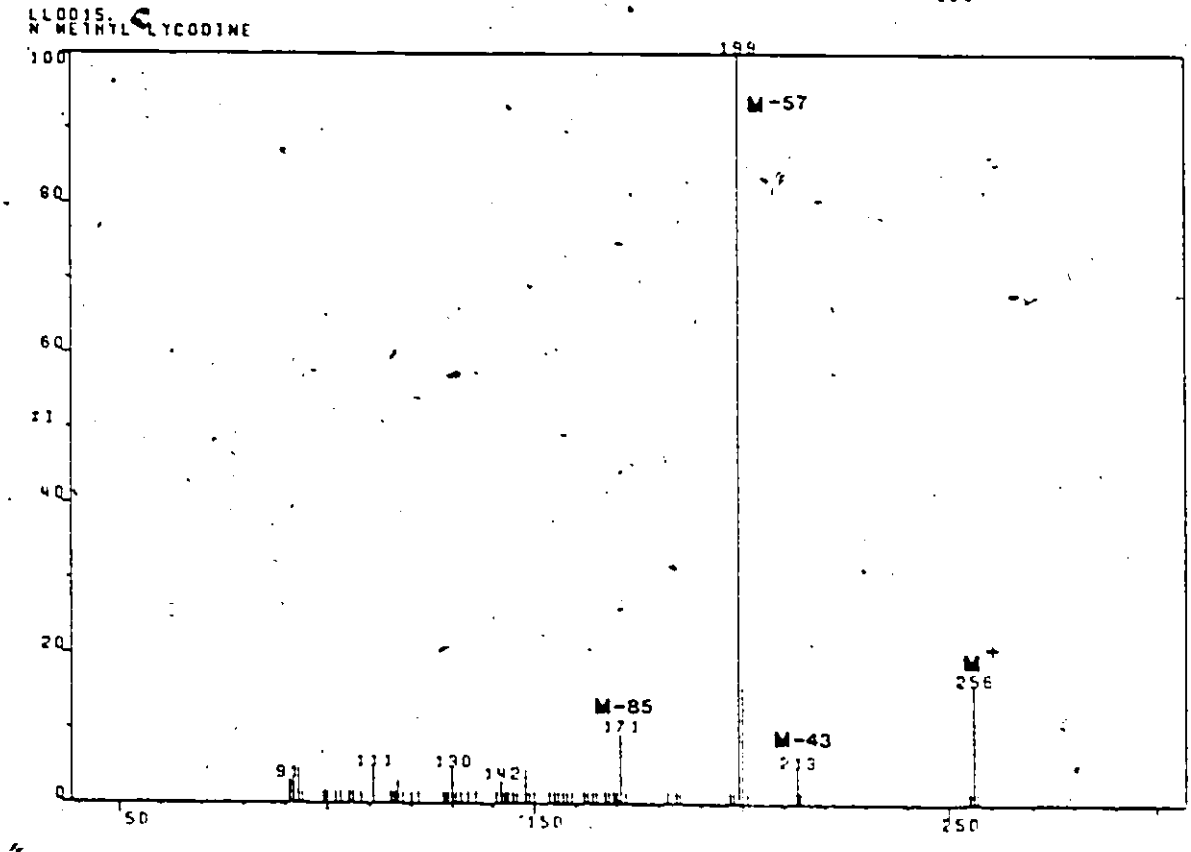
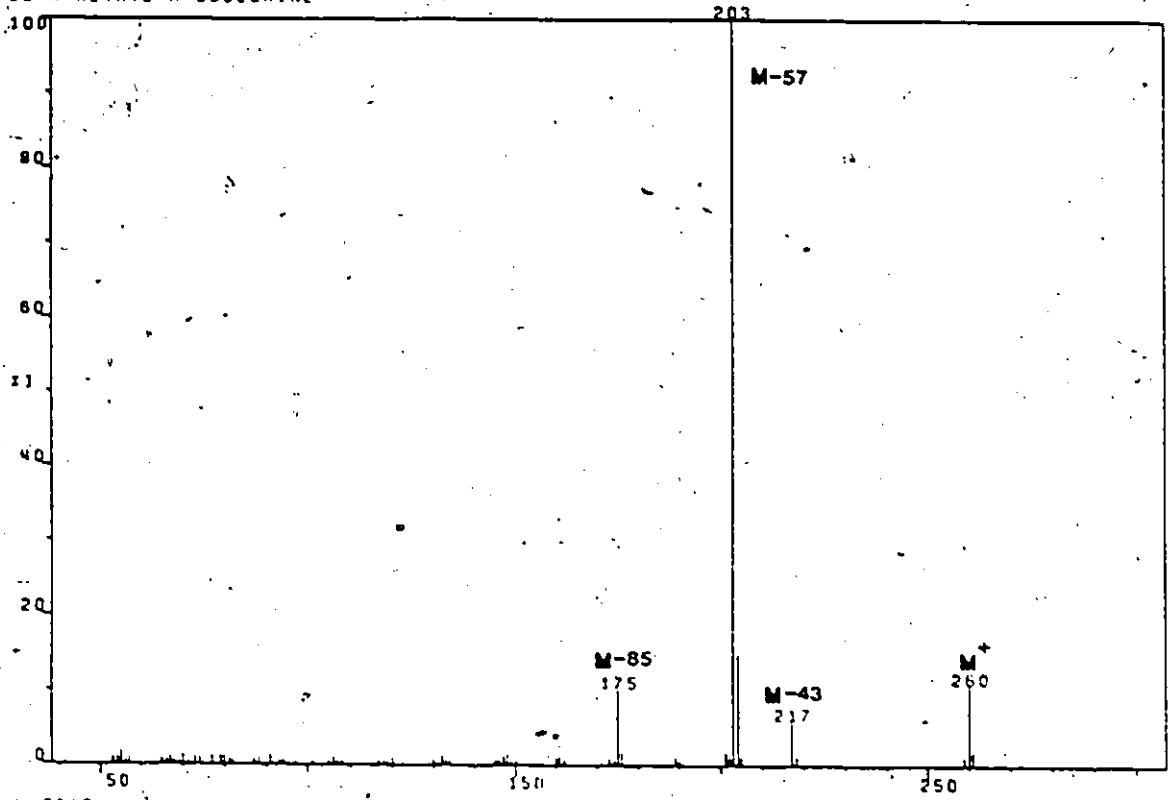

LL0012
N,N-DIMETHYLPHLEGMARINE
100
80
60
%
40
20
0
50
150
250
98
180
166
M+
276
M-15
261
221


LL0013
N-ACETYL N,O METHYL PHLEGMARINE
100
80
60
%
40
20
0
50
150
250
100
84
96
97
120
180
249
M-43
261
M+
303

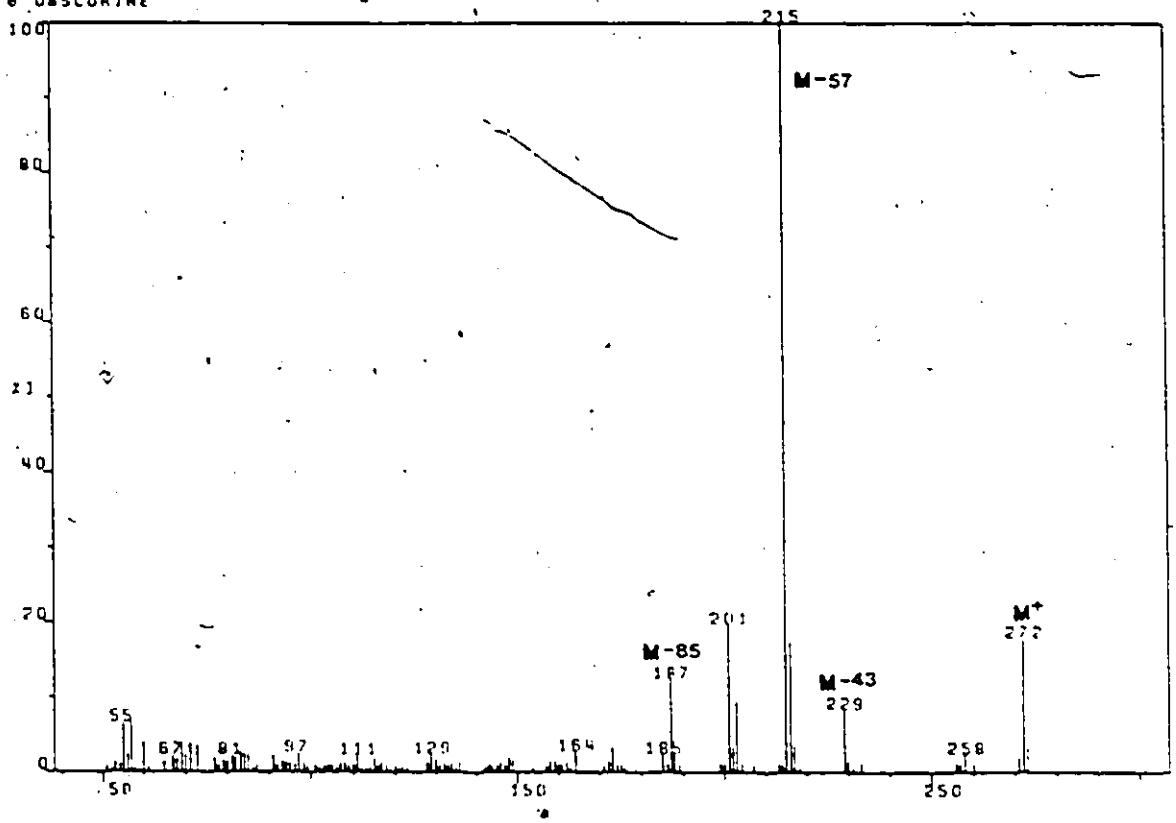

LL0014.
LYCODINE
100
80
60
40
20
0
M-57
M-85
M-43
M+
242
199
130
146
50
150
250


LL0015.
N METHYL LYCODINE
100
80
60
40
20
0
M-57
M-85
171
M-43
213
M+
256
199
93
111
130
142
50
150
250

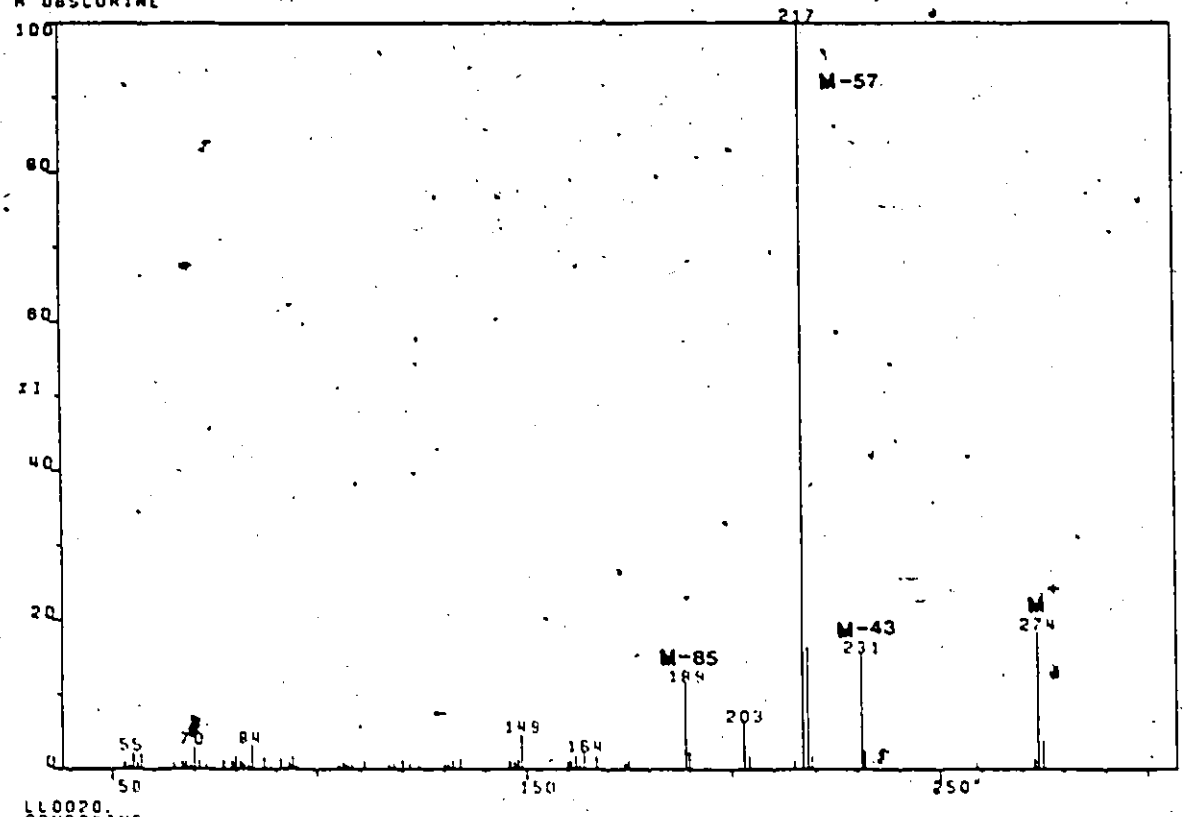
LL0018
DE N METHYL A OBSCURINE



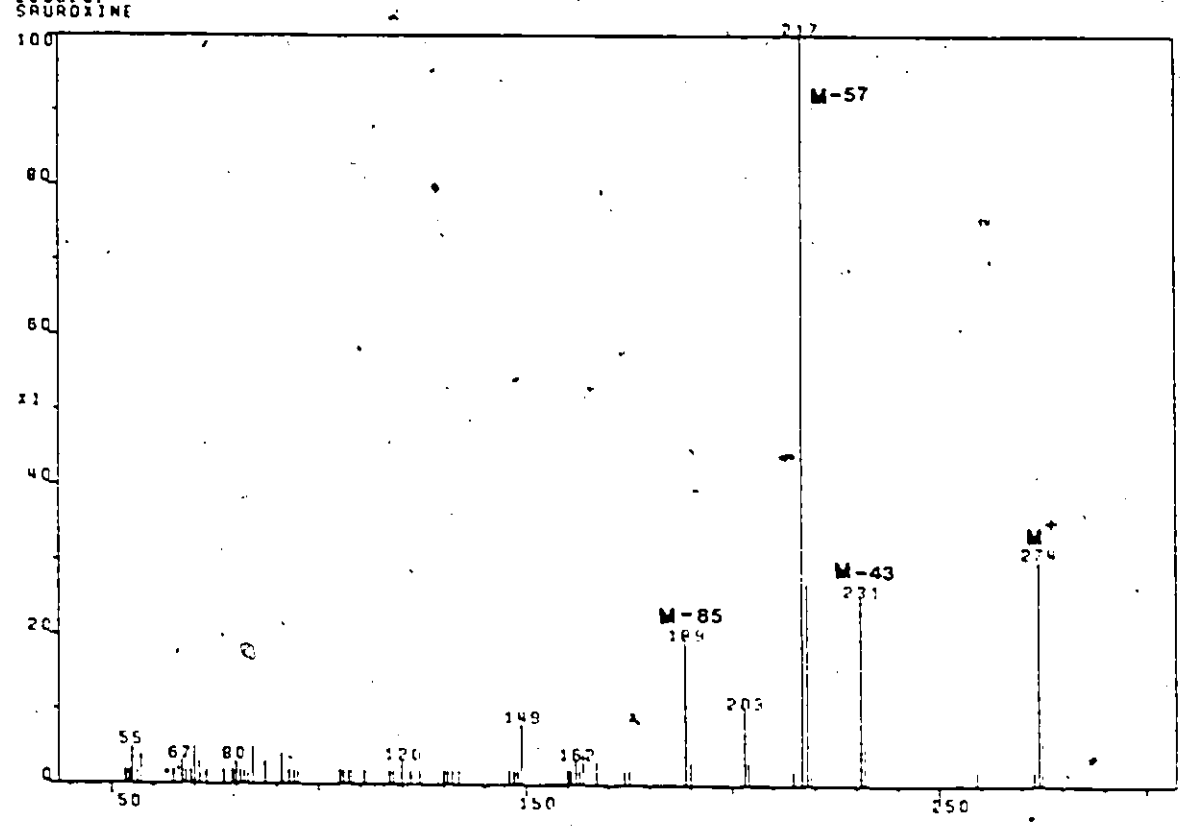
LL0016
8 OBSCURINE

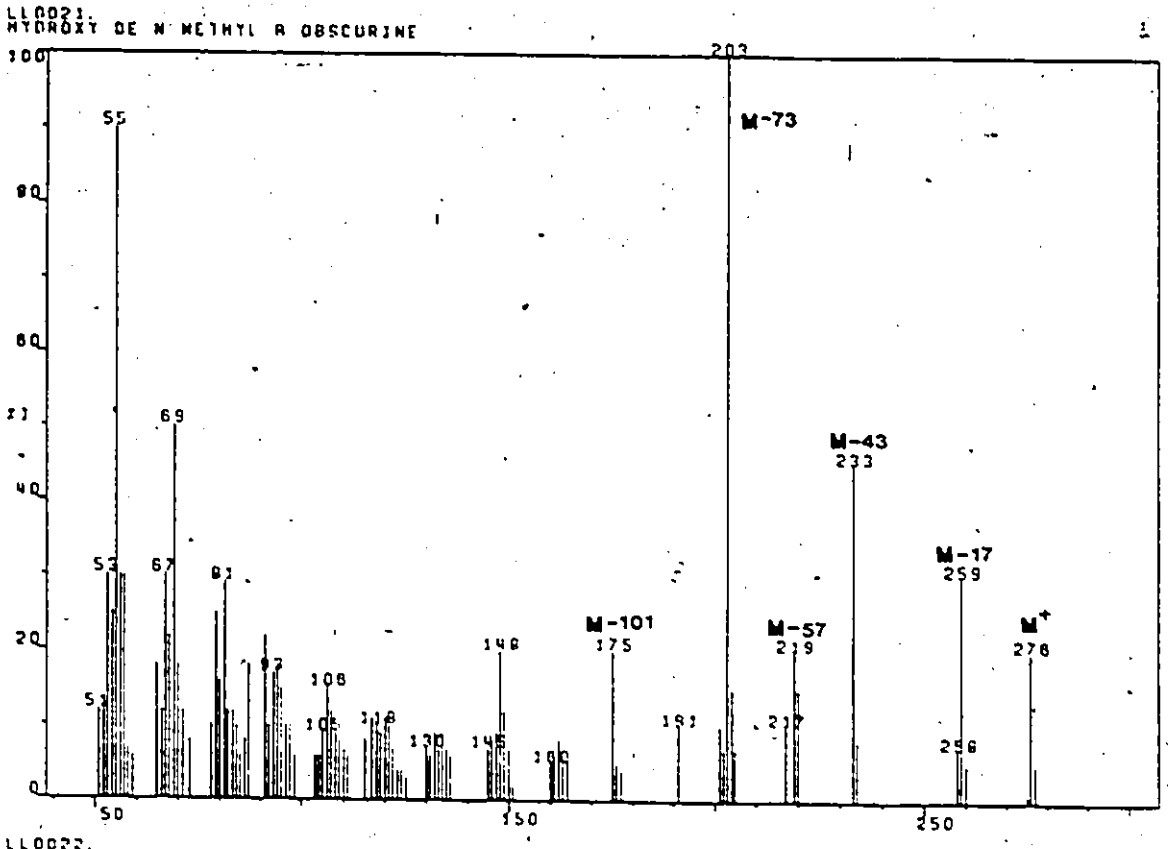


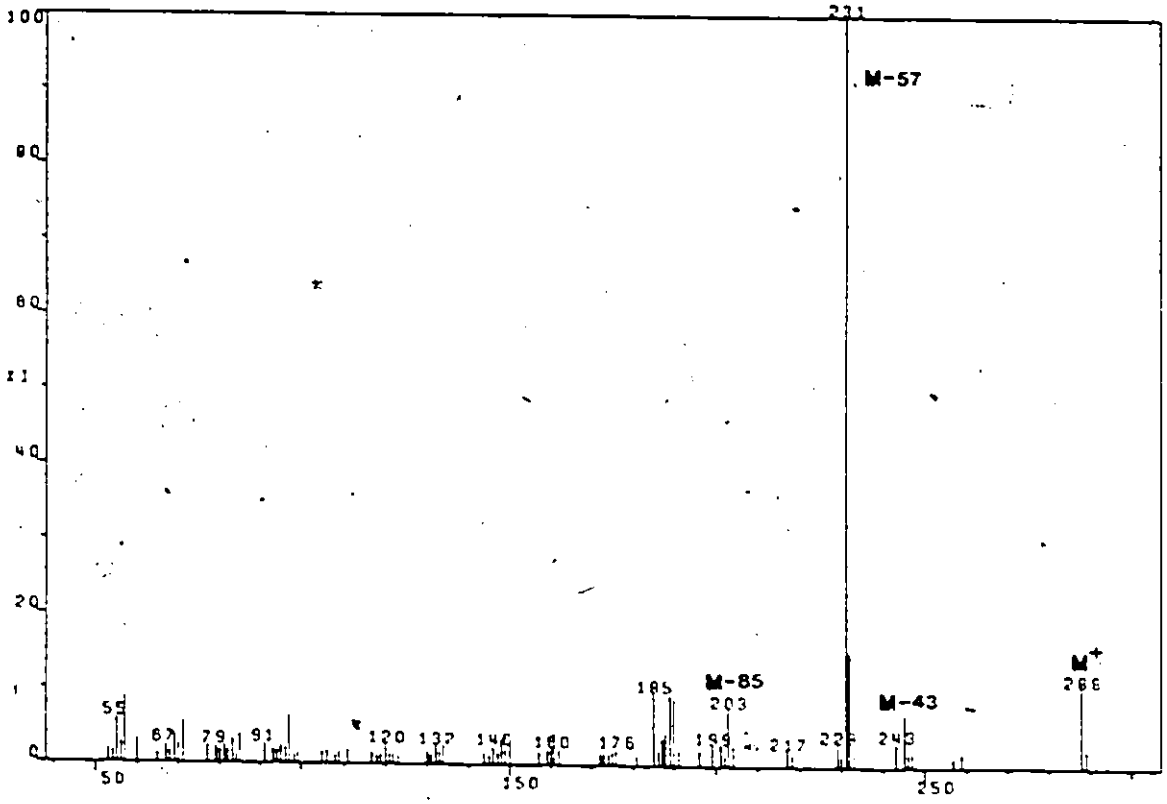
LL0019.
A OBSCURINE

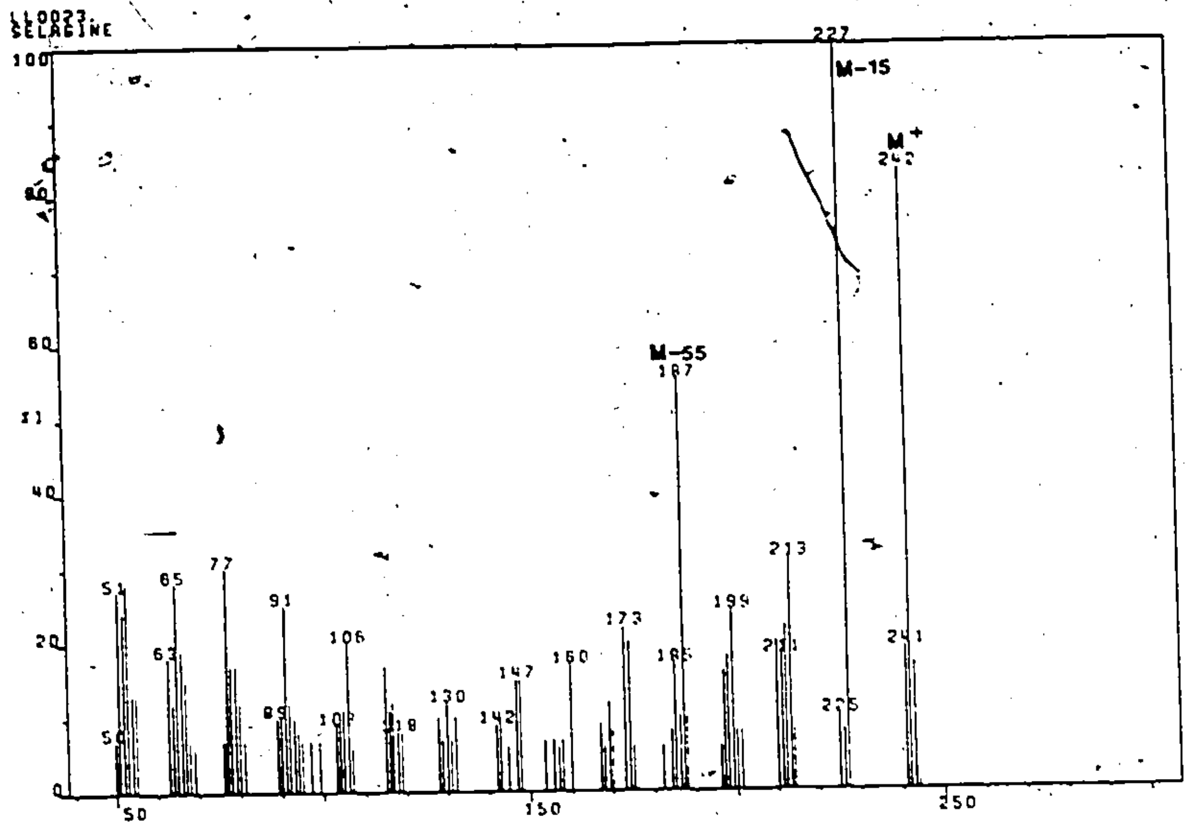


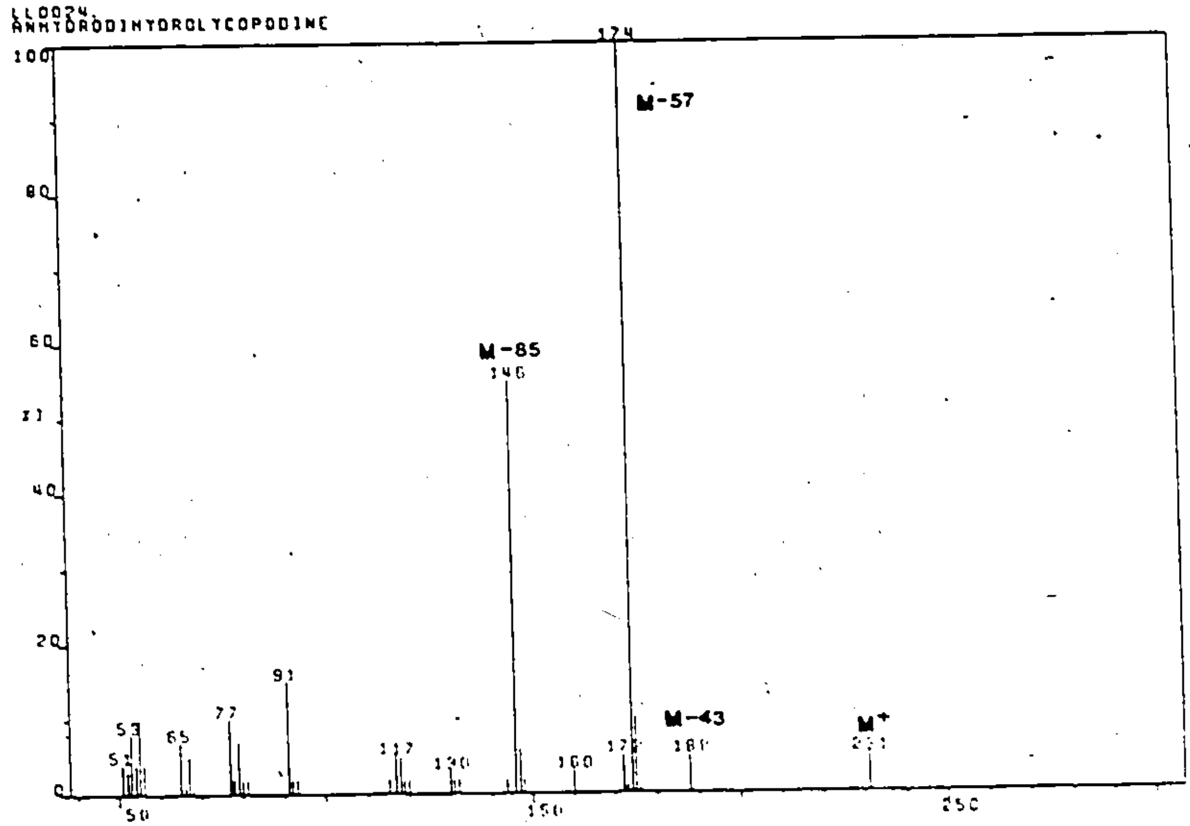
LL0020.
SAUROXINE

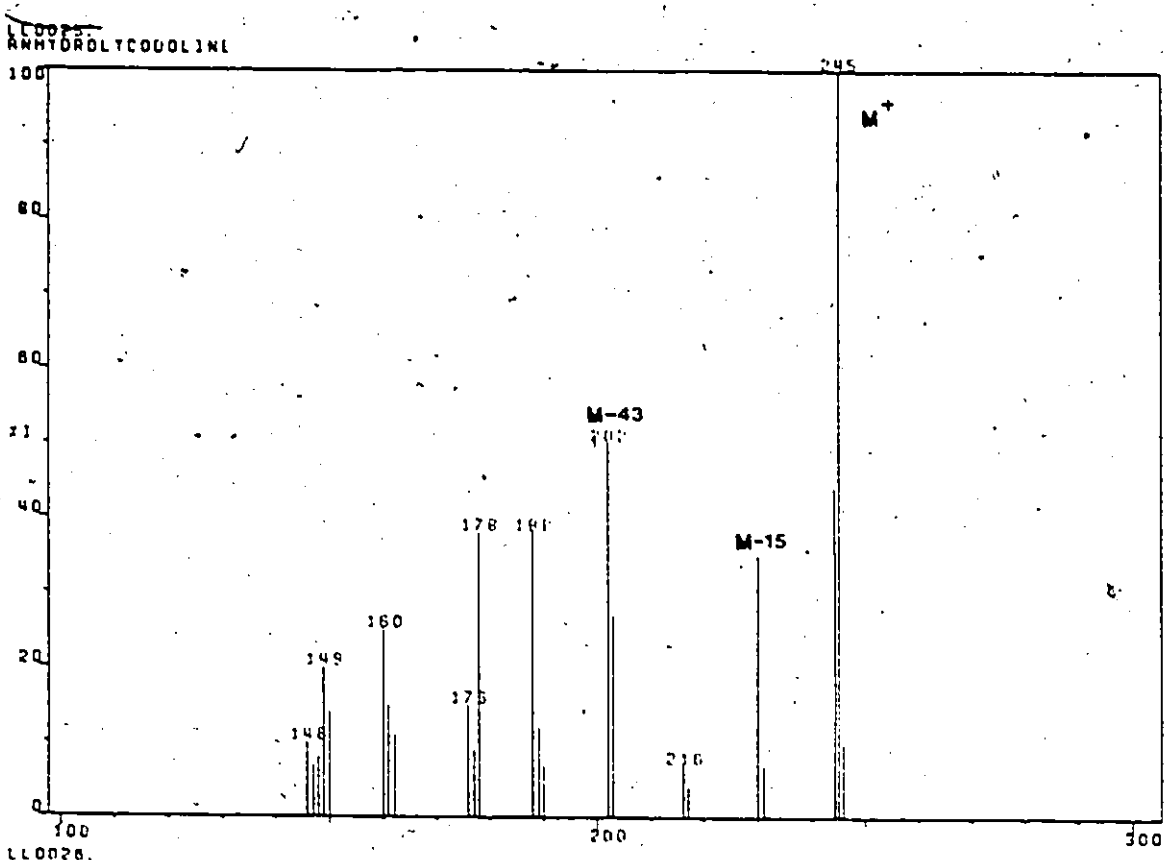


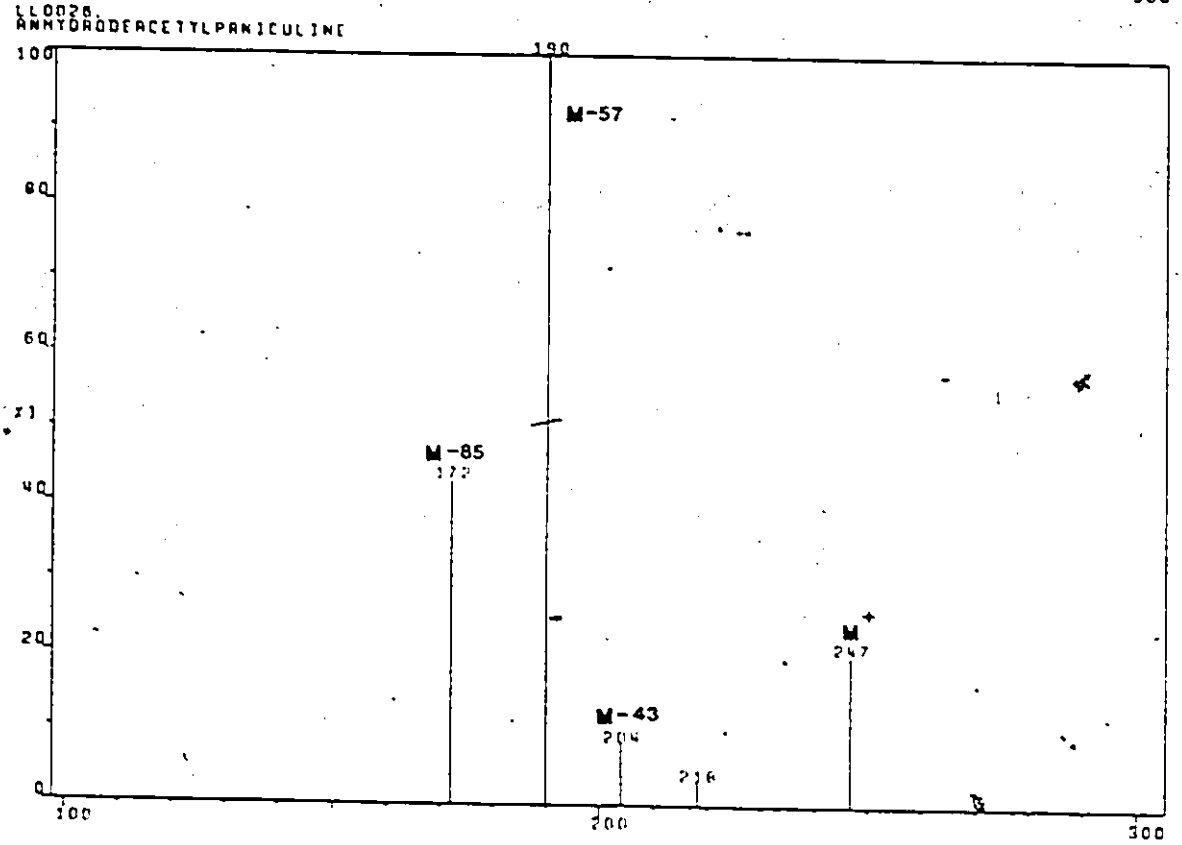

LL0021.
HYDROXY DE N METHYL A OBSCURINE
M-73
M-43
233
M-17
259
M-57
219
M-101
175
M+
276
55
69
53
67
81
92
108
148
130
145
191
217
256
50
150
250

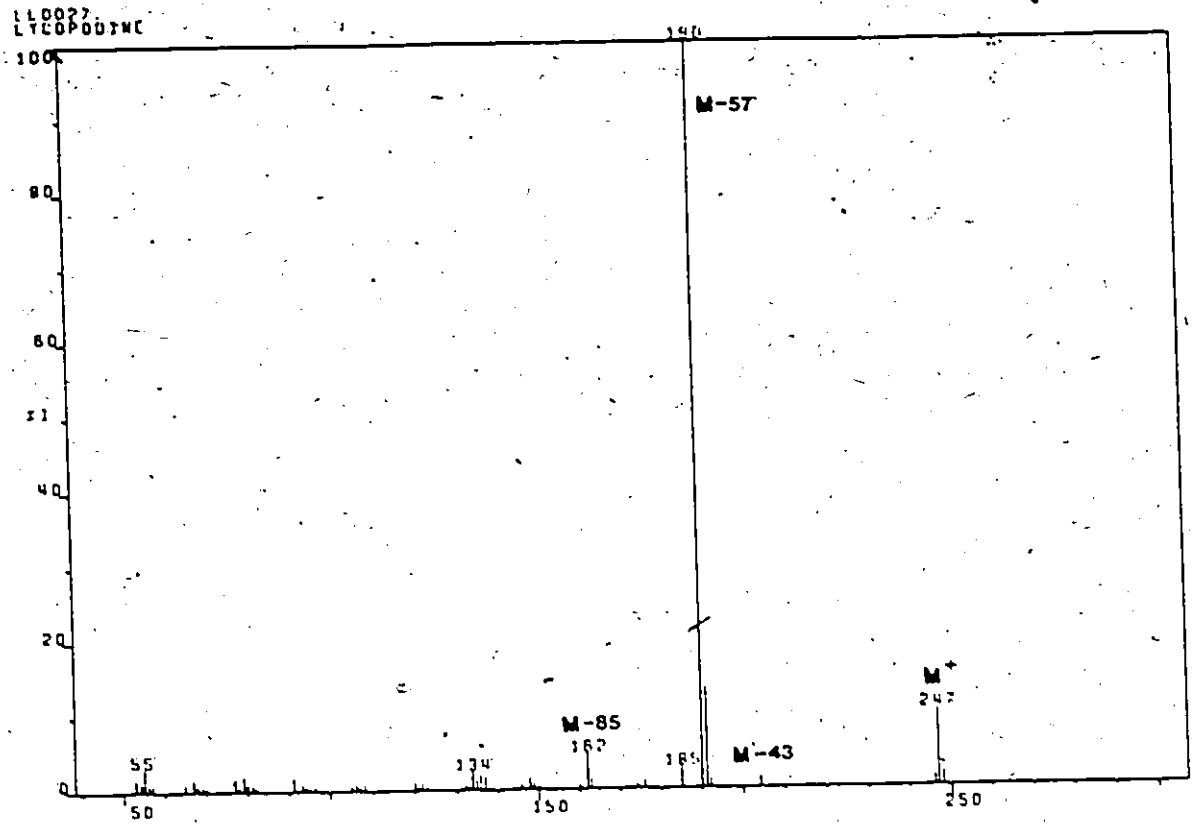

LL0022.
FLABELLIDINE
M-57
M-85
203
M-43
M+
286
55
67
79
91
120
132
146
160
176
185
199
217
228
243
50
150
250

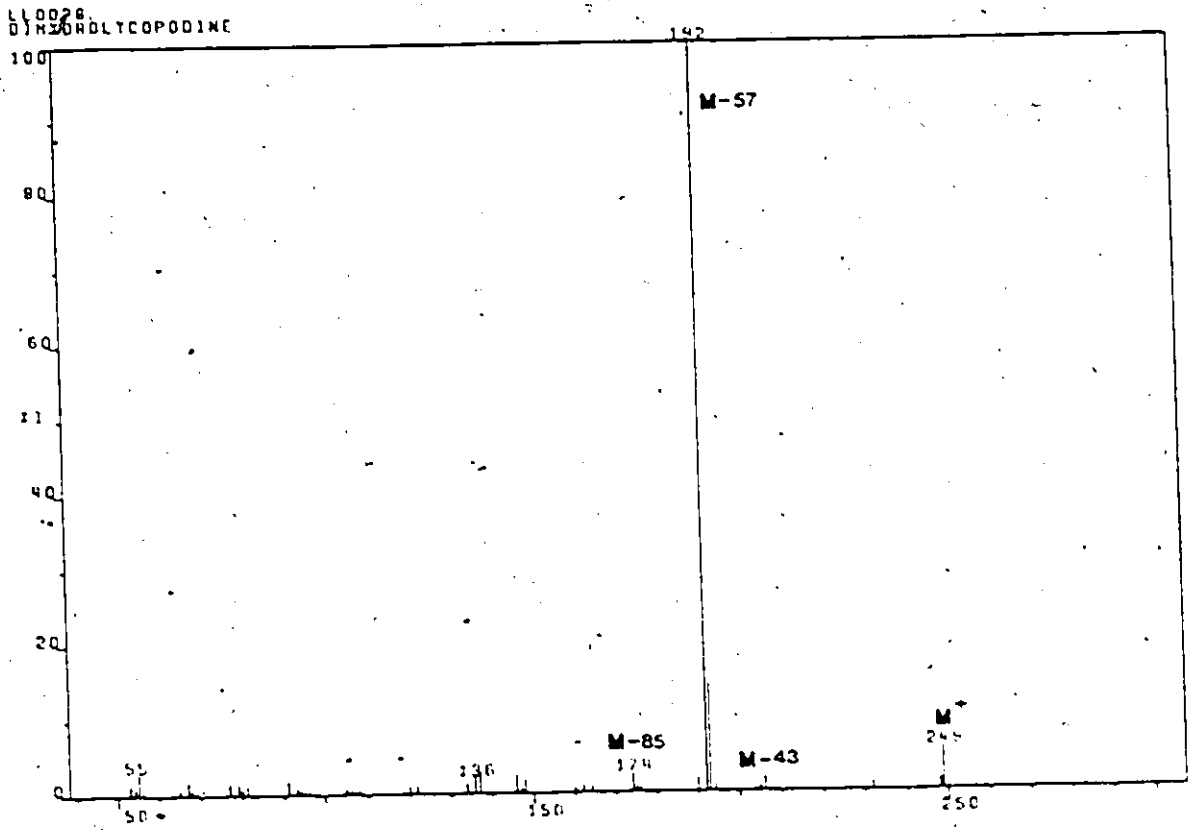

LL0023.
SELAGINE
227
M-15
M+
242
M-55
187
213
199
173
160
147
130
142
106
91
77
65
63
51
50
89
241
225
50
150
250

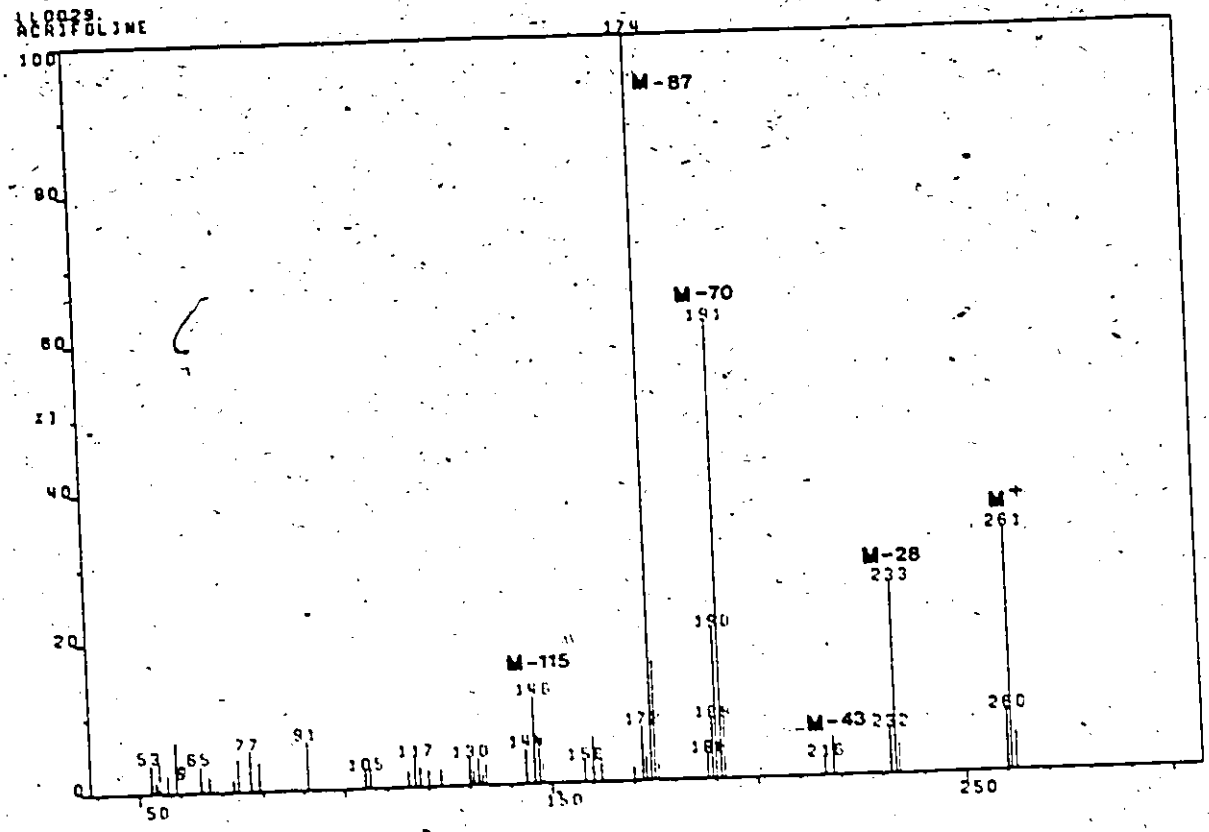

LL0024.
ANHYDRODIHYDROLYCOPODINE
174
M-57
M-85
146
M-43
M+
91
77
65
53
51
130
160
50
150
250

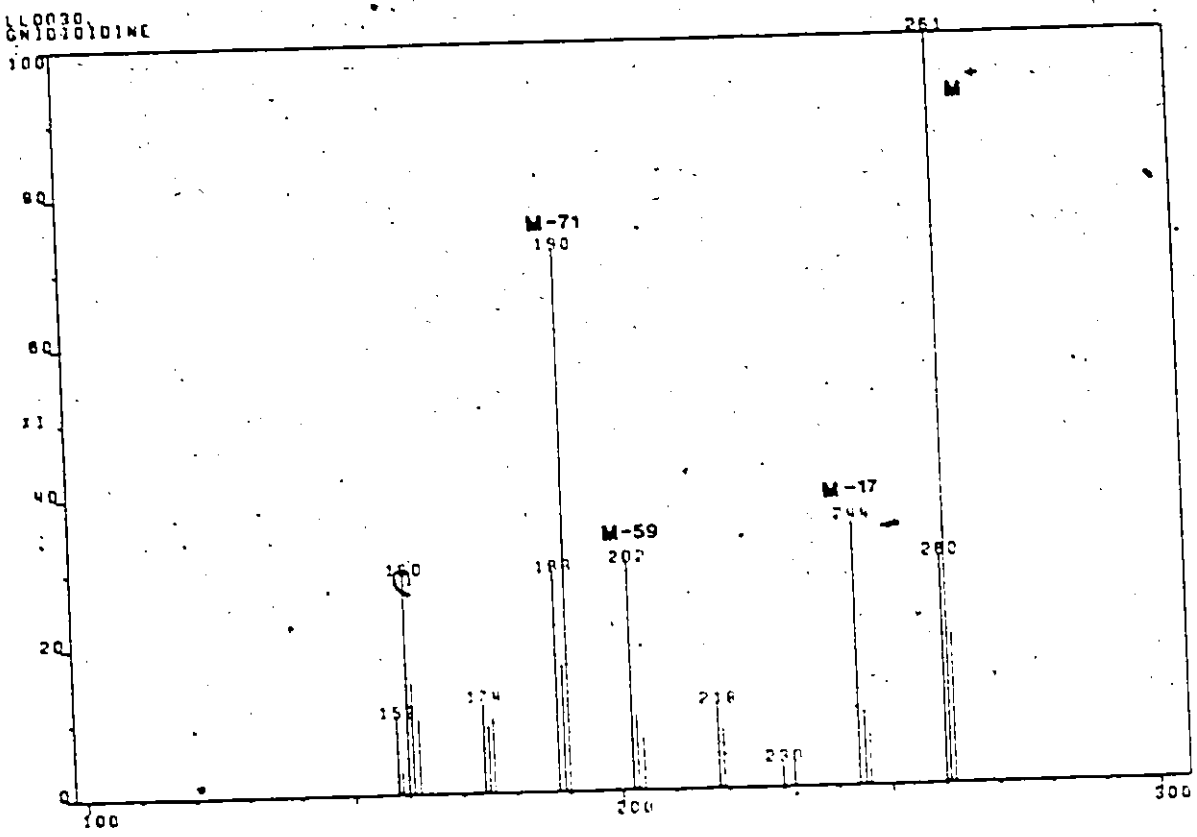

ANHYDROLYCODOLINE
100
80
60
40
20
0
100
200
300
M+
M-43
M-15
178
160
149
148
175
216

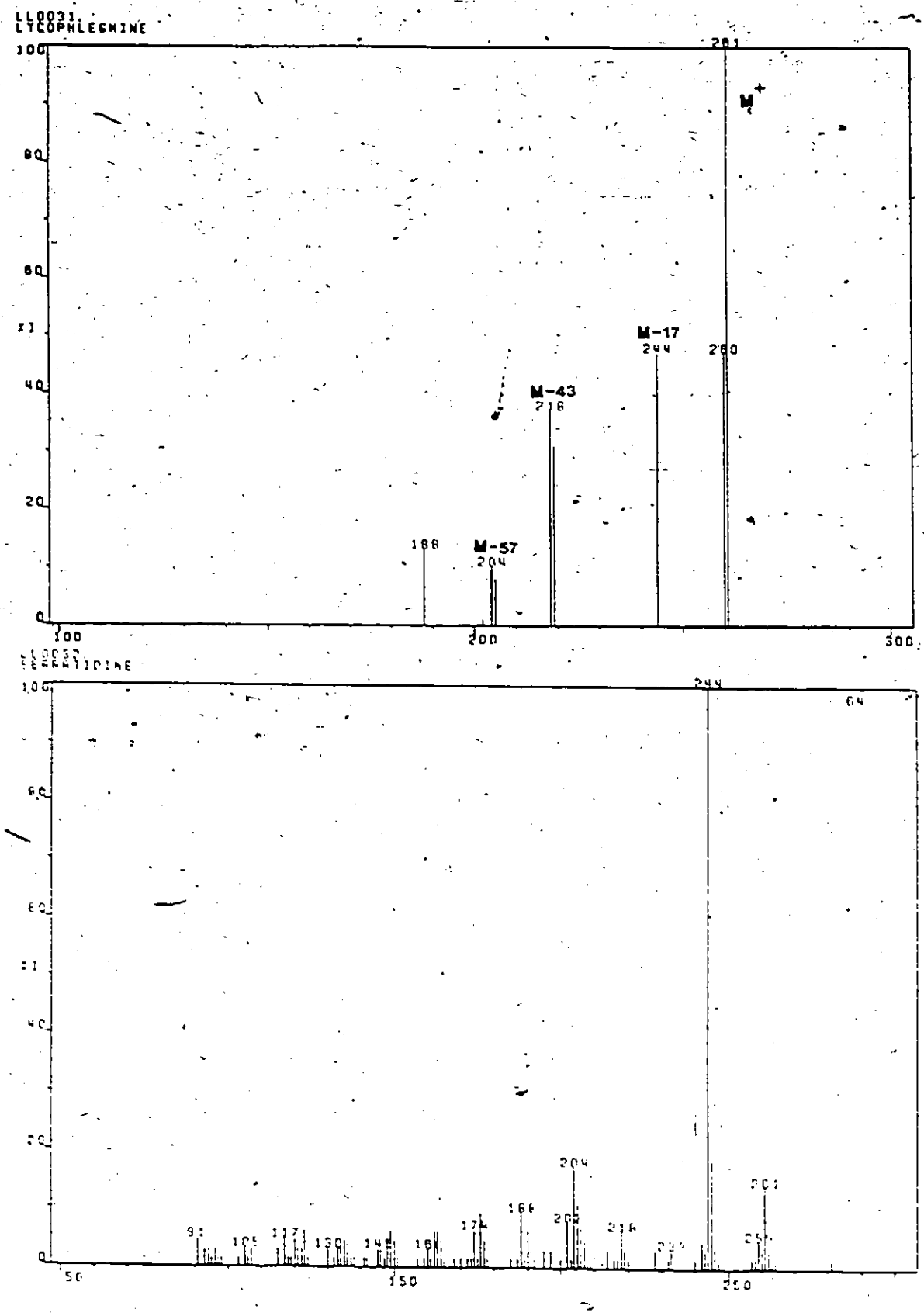

ANHYDRODEACETYLPANICULINE
100
80
60
40
20
0
100
200
300
M-57
M-85
M+
267
M-43
216


LL0027
LYCOPODINE
100
80
60
40
20
0
M-57
M-85
M-43
M+
247
55
134
162
185
50
150
250

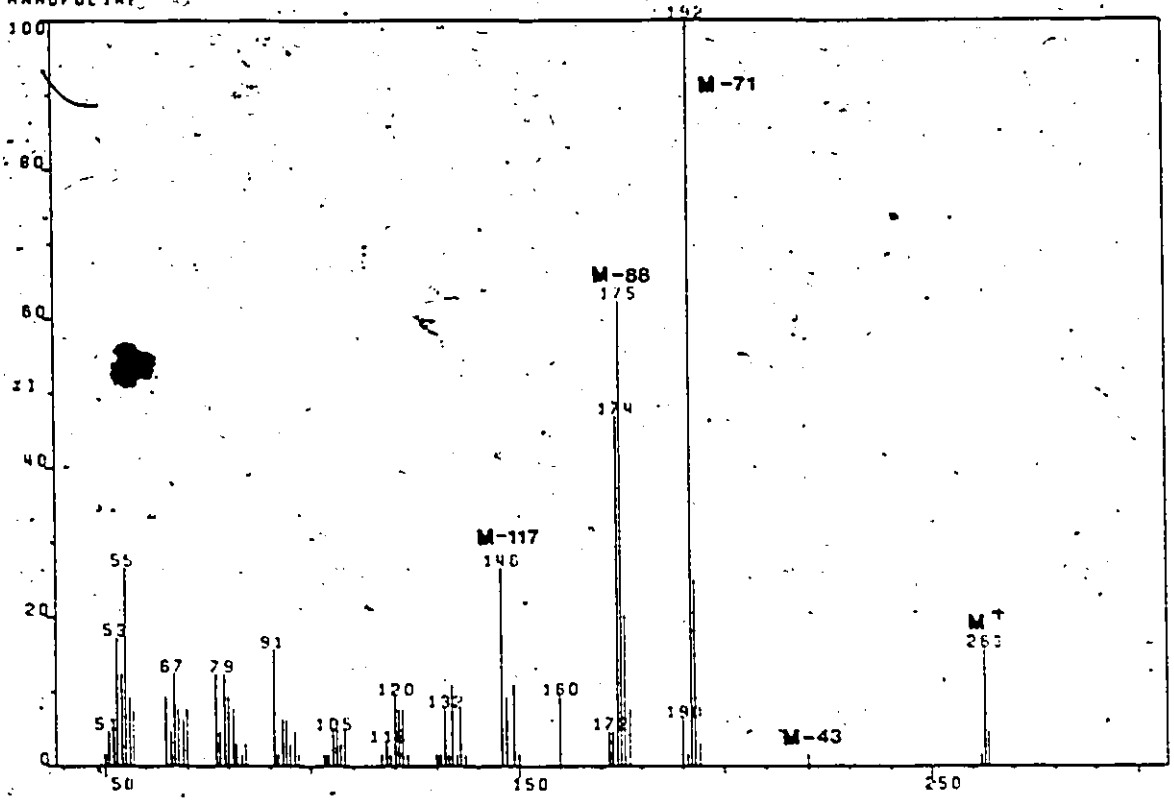

LL0028
DIHYDROLYCOPODINE
100
80
60
40
20
0
M-57
M-85
M-43
M+
245
55
136
174
50
150
250


LL0029
ACRIFOLINE
M-87
M-70
191
M+
261
M-28
233
M-115
146
M-43
218
260
250
150
50

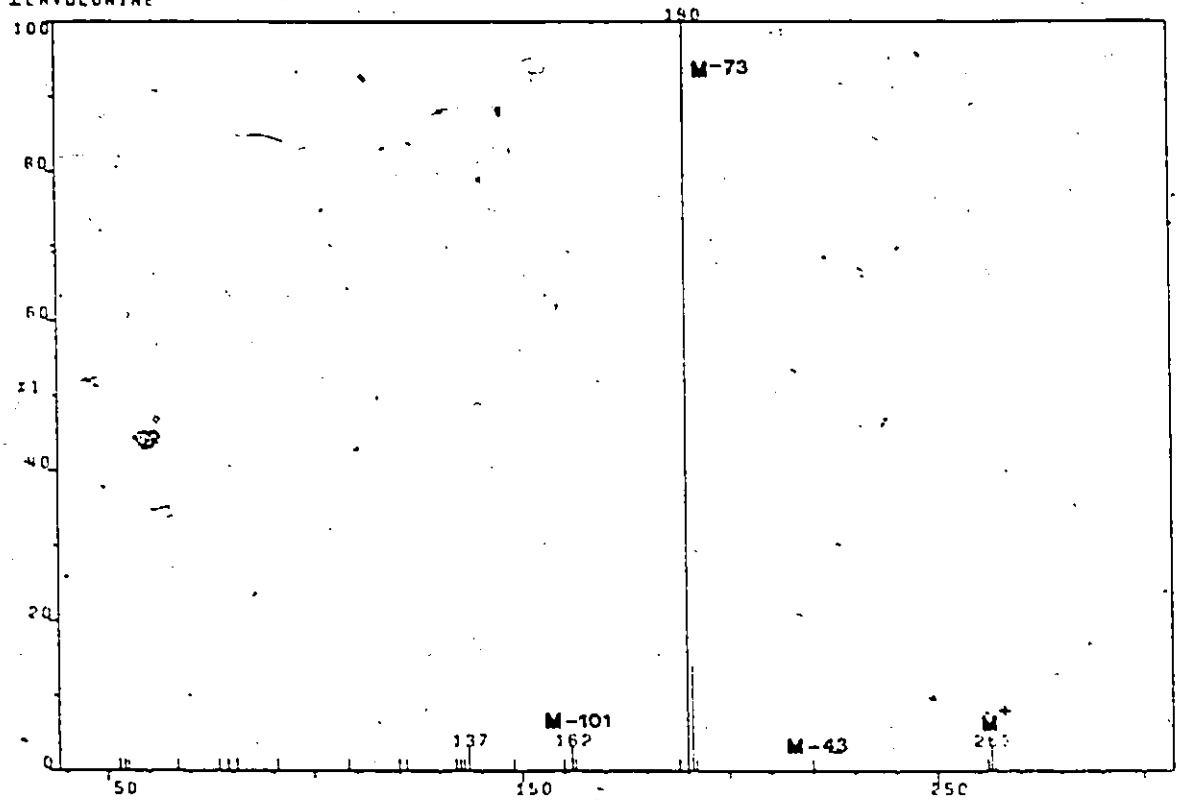

LL0030
CNIDICOLINE
261
M+
M-71
190
M-59
202
M-17
244
280
300
200
100

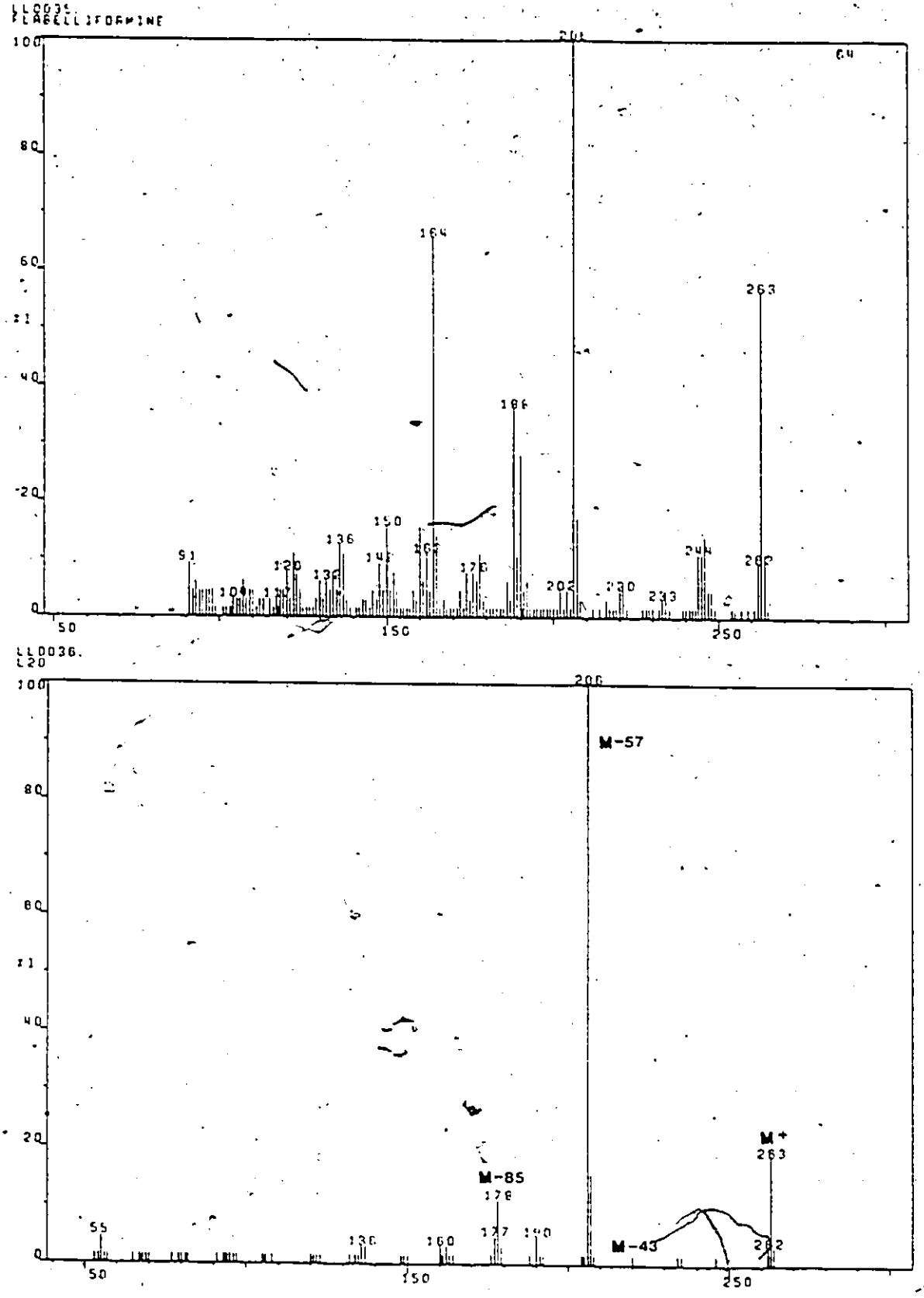

LL0031
LYCOPHLEGMINE
100
80
60
40
20
0
%I
281
M+
M-17
264
280
M-43
238
188
M-57
204
100
200
300
LL0032
SERRATIDINE
100
80
60
40
20
0
%I
244
64
204
201
166
91
105
117
130
145
161
174
204
216
50
150
250

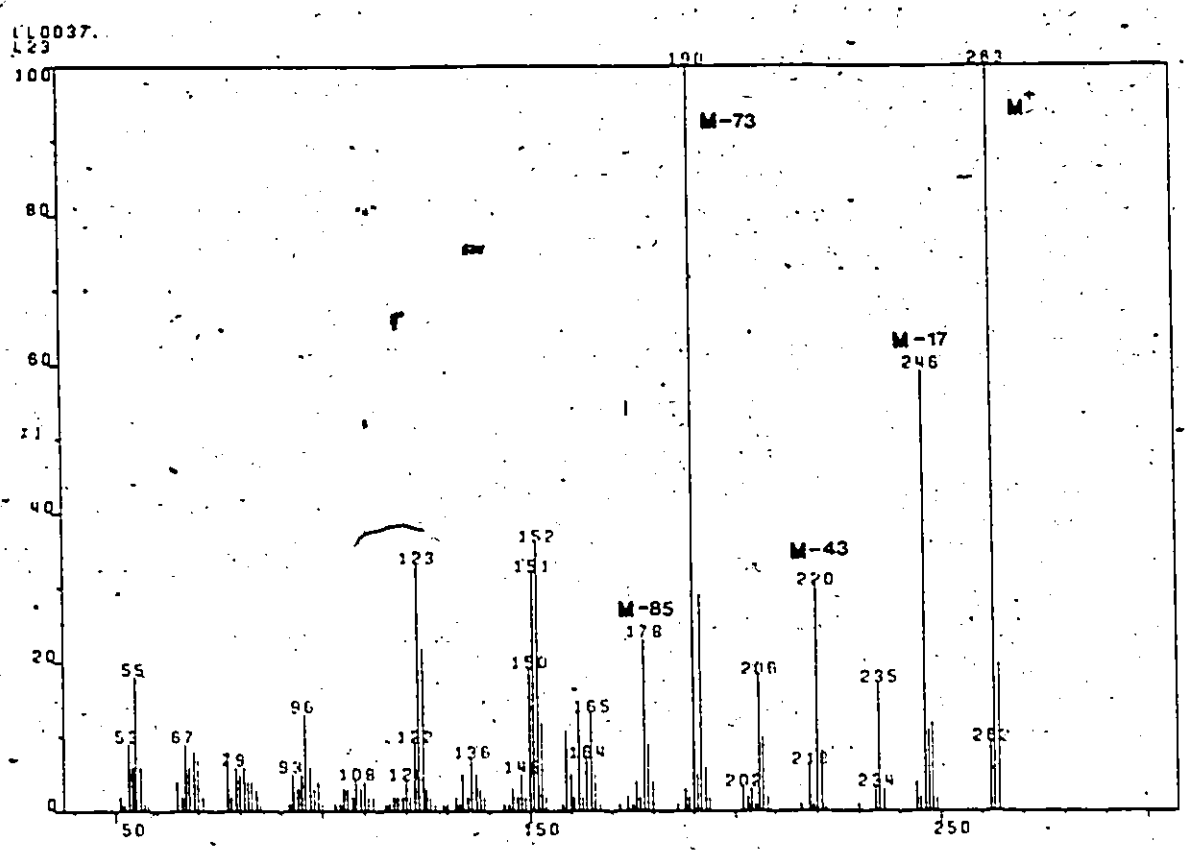
LL0033
ANNOFOLINE

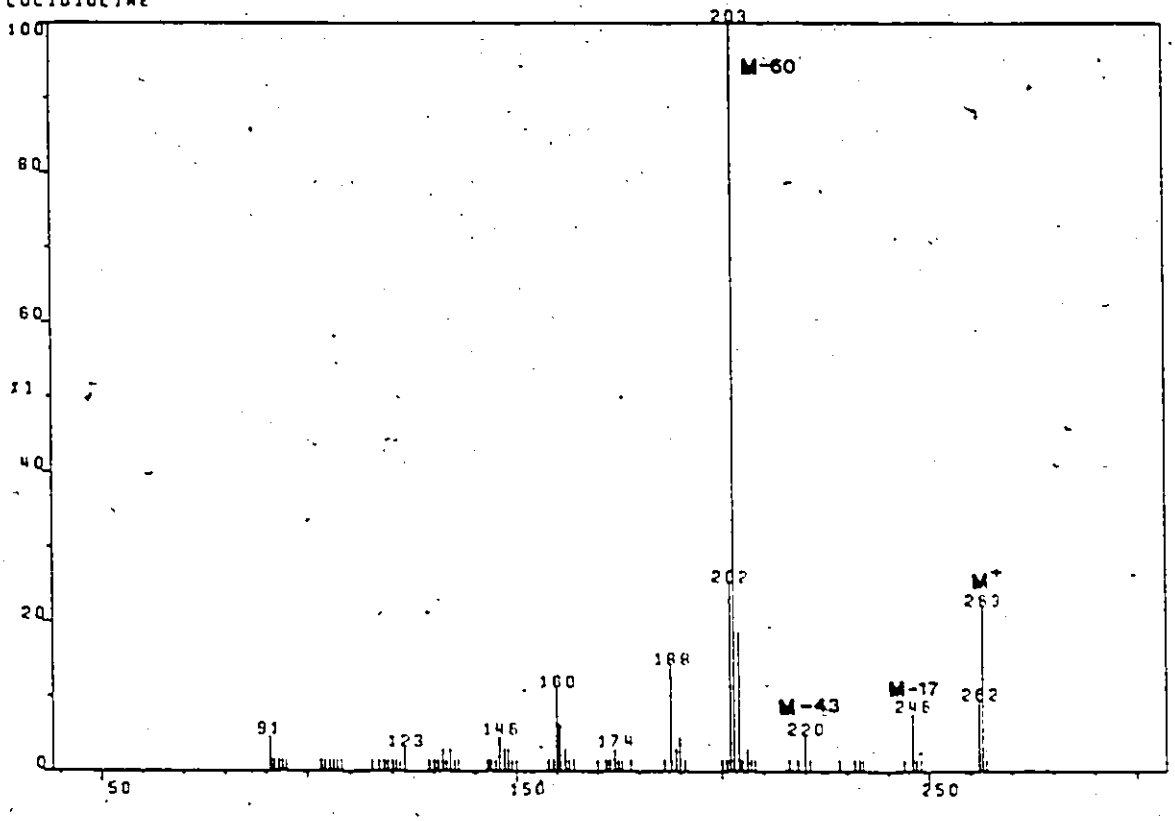


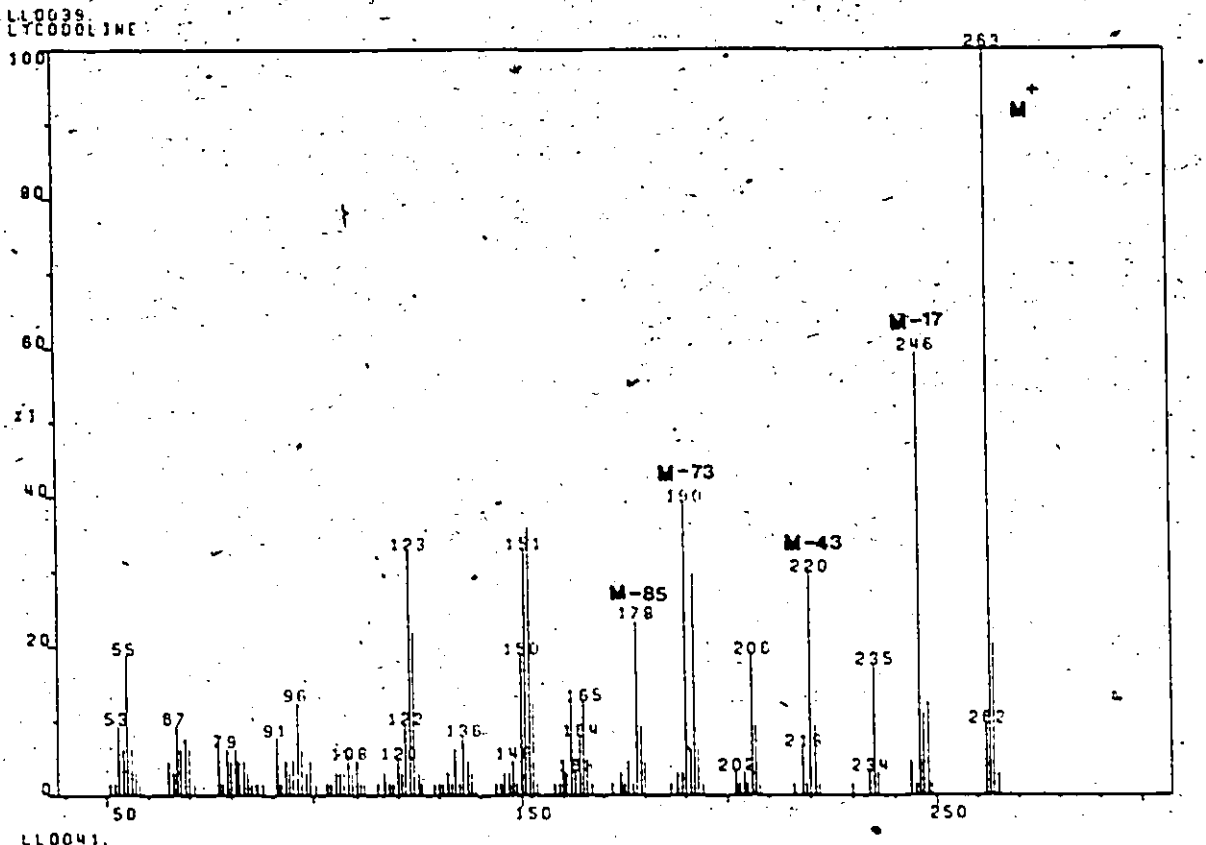
LL0034
ELAVOLONINE

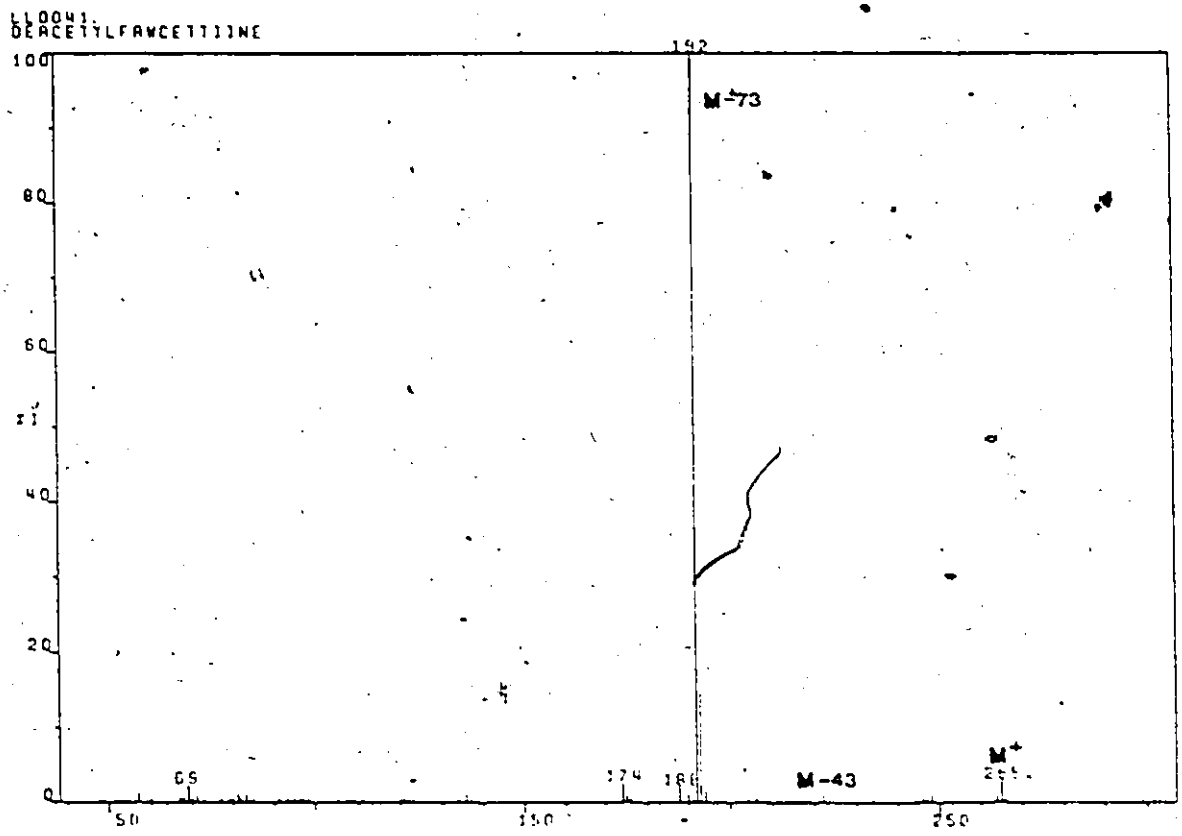


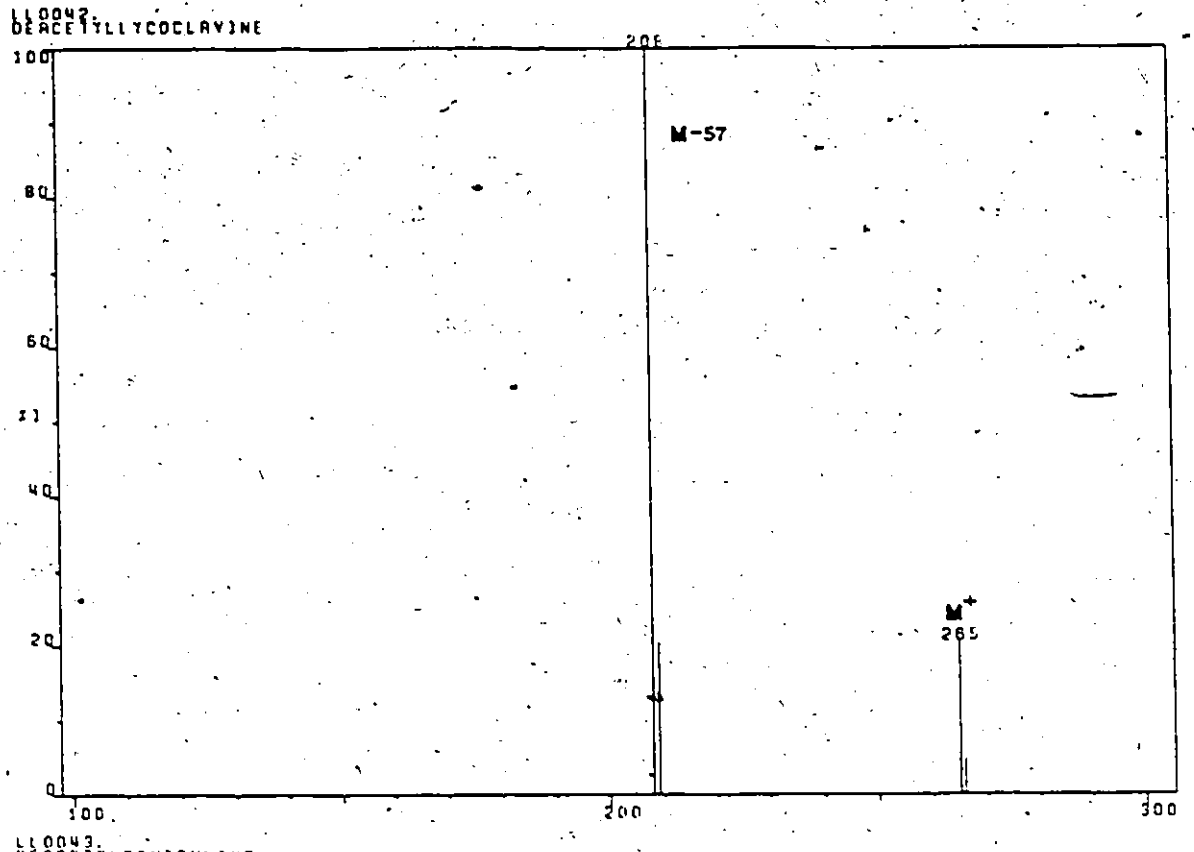

LL0035
PLABELLIFORMINE
100
80
60
40
20
0
50
150
250
263
164
106
150
136
91
120
244
202
230
233
LL0036.
L20
M-57
M-85
178
M-43
M+
263
55
136
160
177
190
282

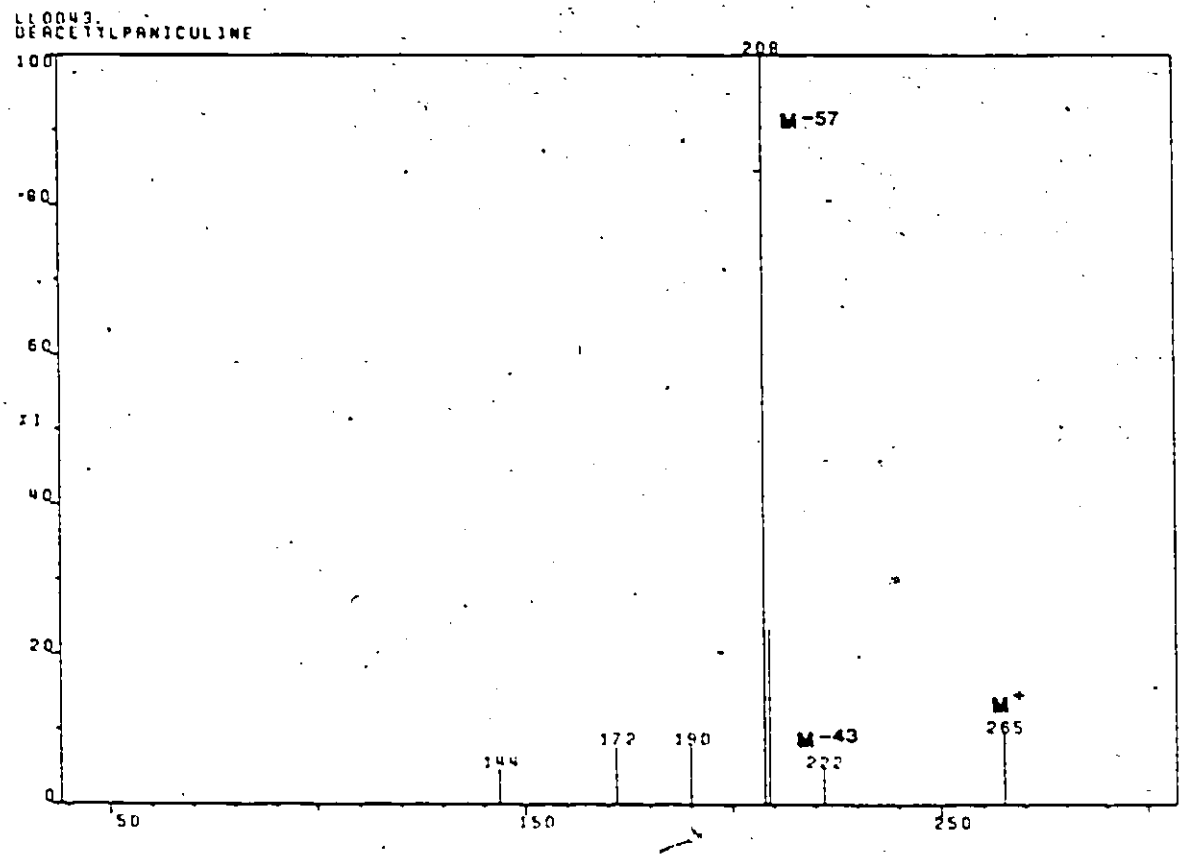

LL0037.
L23
M-73
M+
M-17
246
M-43
220
M-85
178
123
152
151
55
67
79
93
96
108
121
136
146
150
165
184
206
210
234
235
262
50
150
250

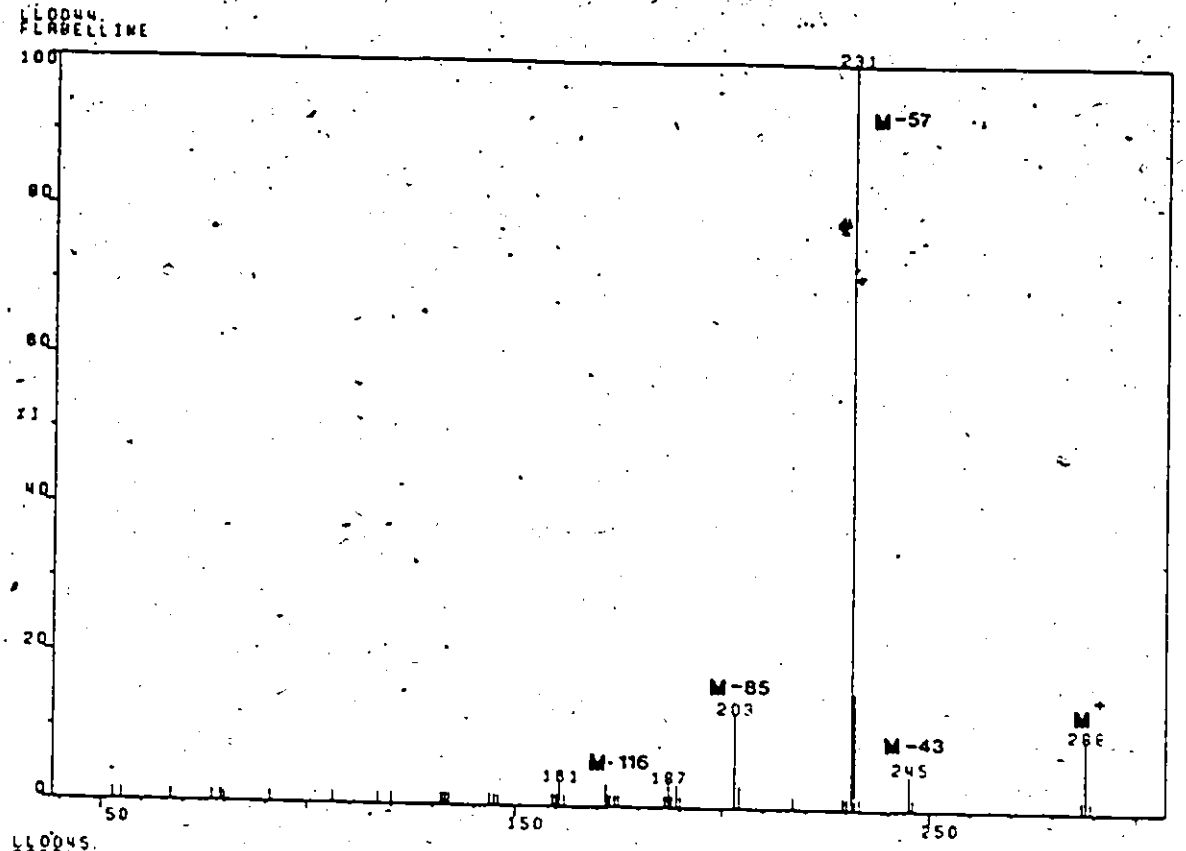

LL0038.
LUCIDIOLINE
M-60
203
202
M+
263
M-17
246
M-43
220
188
100
91
123
146
174
262
50
150
250

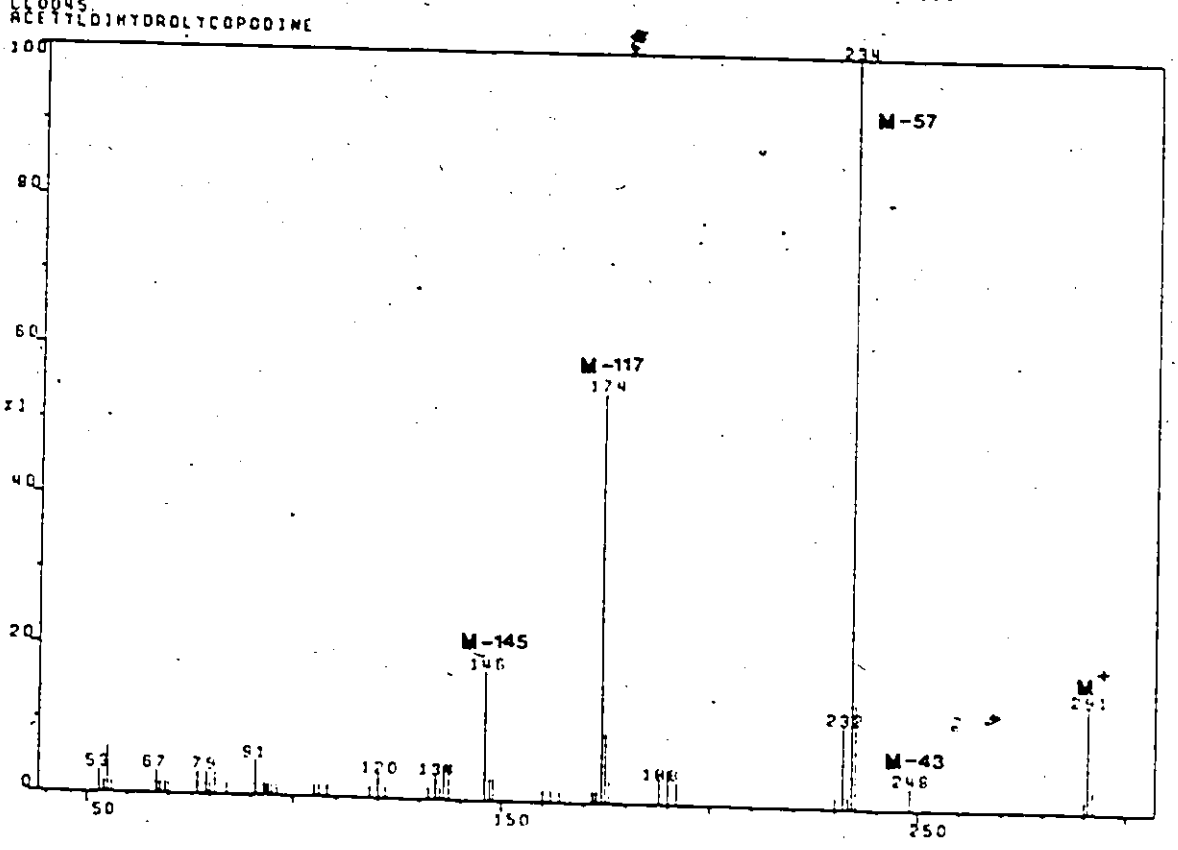

LL0039
LYCODOLINE
M+
M-17
246
M-73
190
M-43
220
M-85
178
263
55
53
67
79
91
96
123
136
150
151
165
178
200
220
235
262
100
80
60
40
20
0
50
150
250


LL0041
DEACETYLFAWCETTIINE
M-73
M-43
M+
65
174
188
192
100
80
60
40
20
0
50
150
250

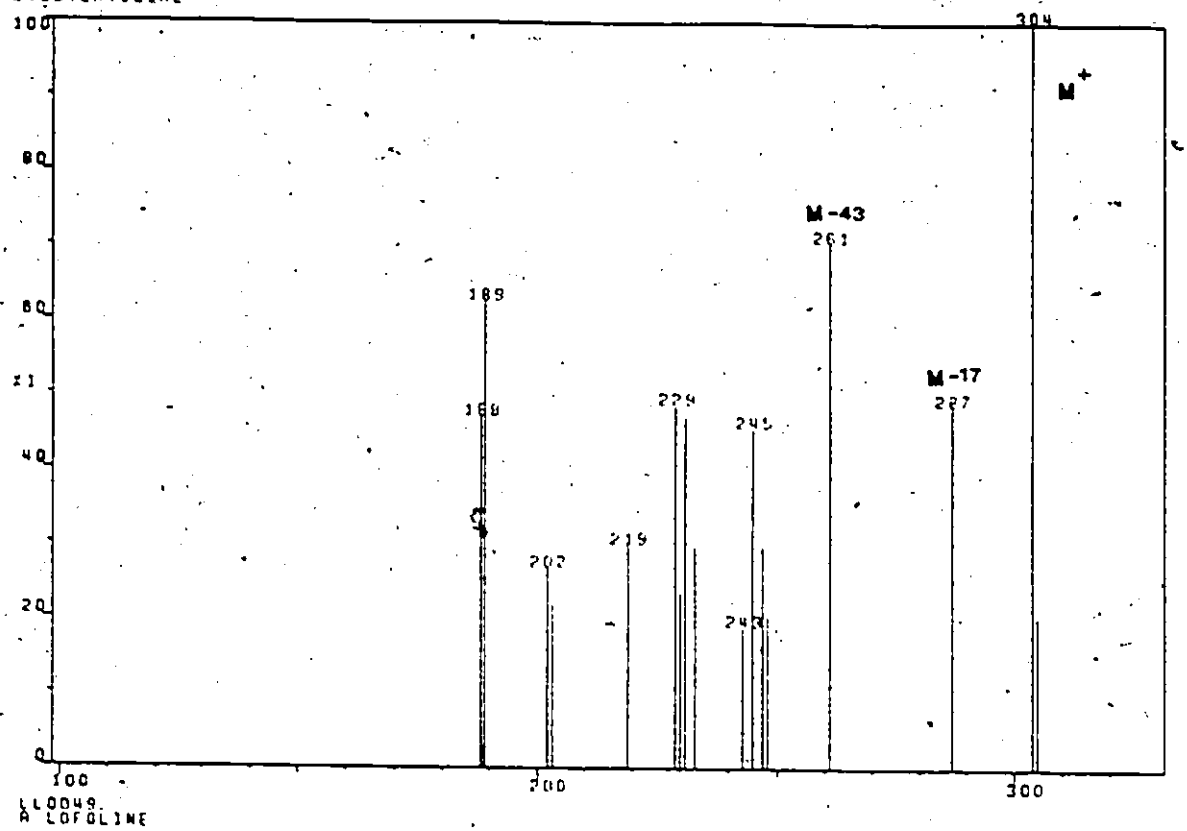

LL0042.
DEACETYLLYCOCLAVINE
100
80
60
40
20
0
208
M-57
M+
265
100
200
300


LL0043.
DEACETYLPANICULINE
100
80
60
40
20
0
208
M-57
M+
265
M-43
222
144
172
190
50
150
250

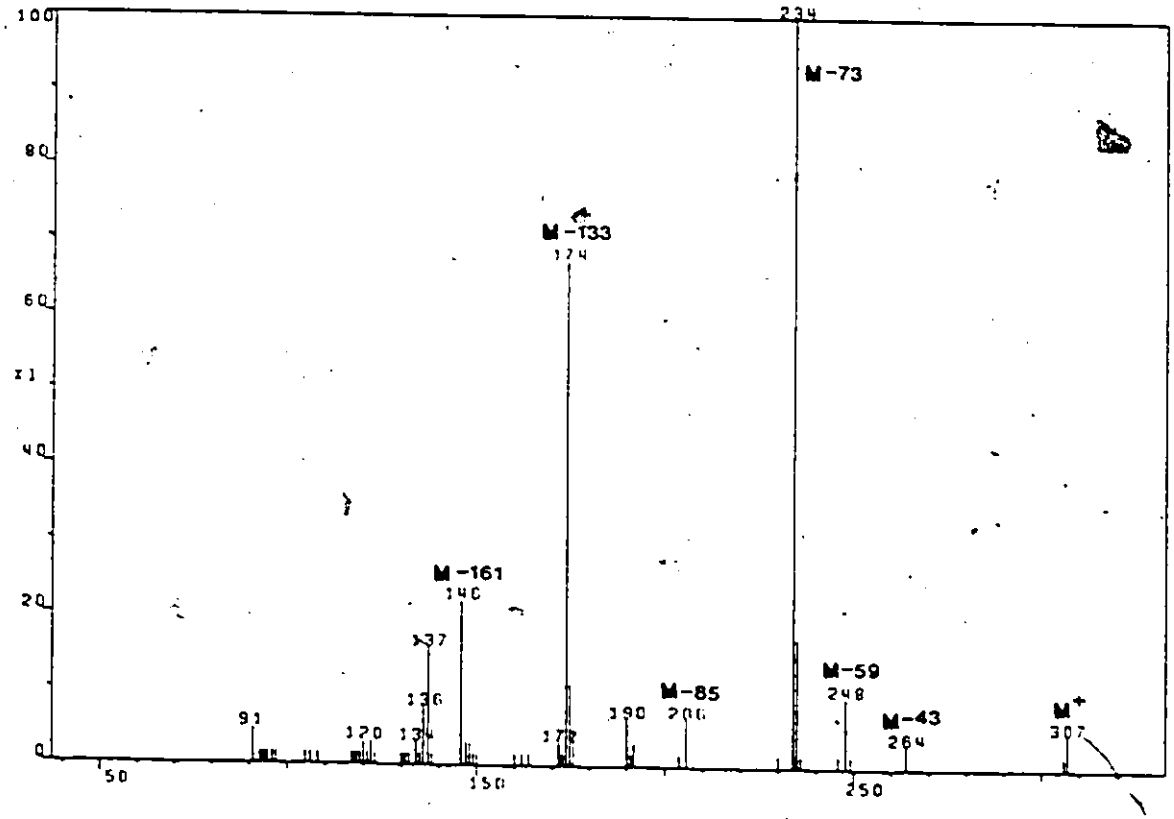

LLOOW4
FLABELLINE
100
80
60
40
20
0
231
M-57
M-85
203
M-116
181
187
M-43
245
M+
266
50
150
250


LLOOW5
ACETYLDIHYDROLYCOPODINE
100
80
60
40
20
0
234
M-57
M-117
174
M-145
146
53
67
79
91
120
138
166
232
M-43
248
M+
291
50
150
250

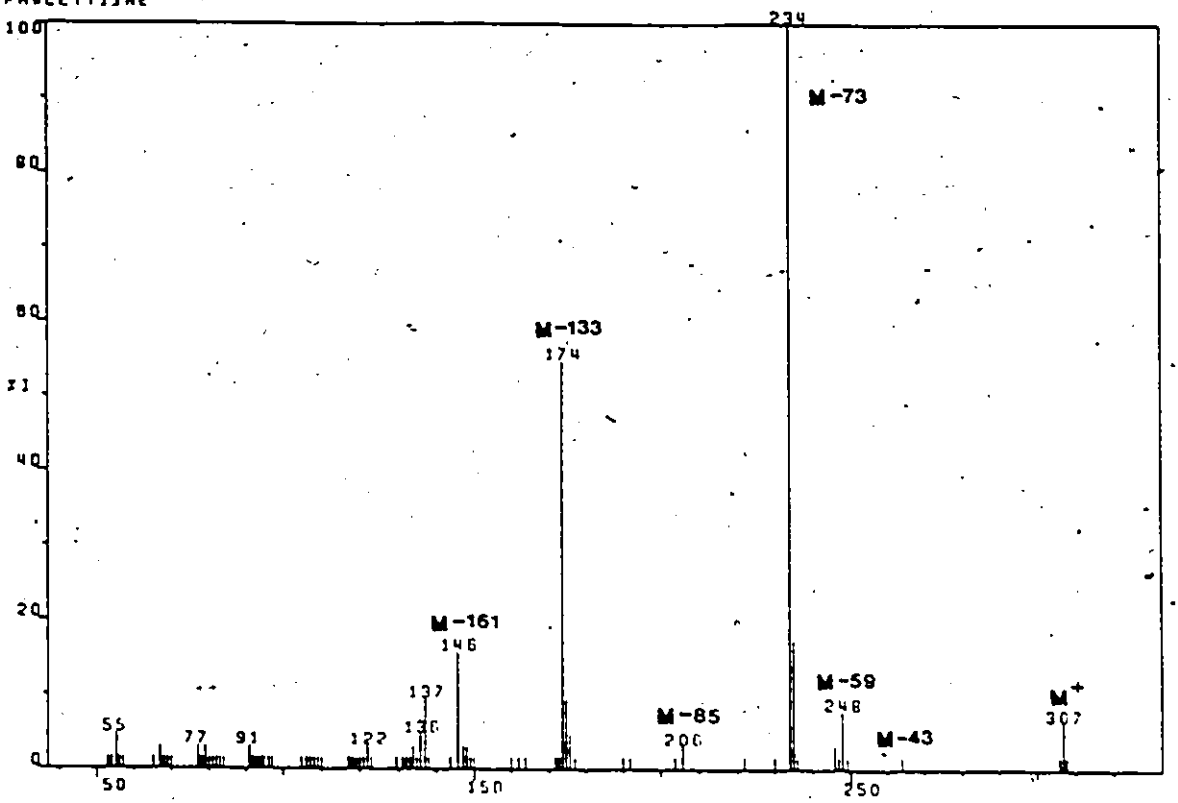
LL0047
LYCOVERTICINE



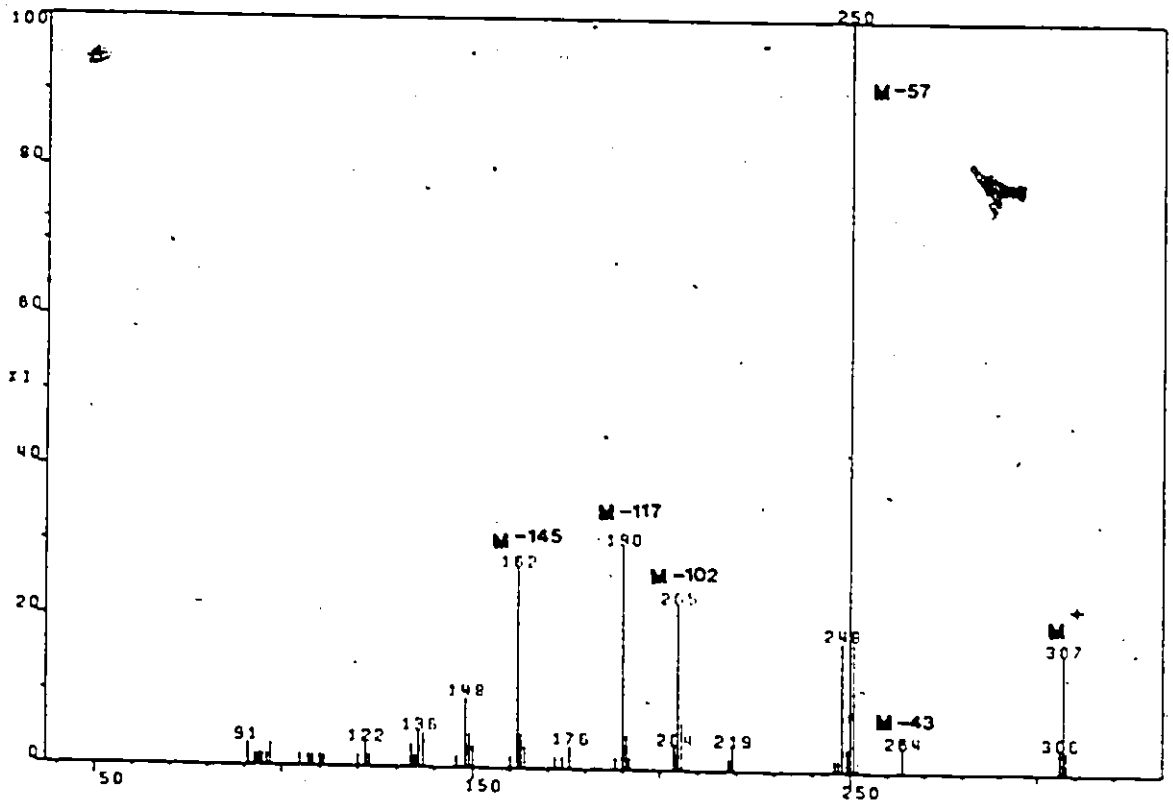
LL0049
A LOFOLINE

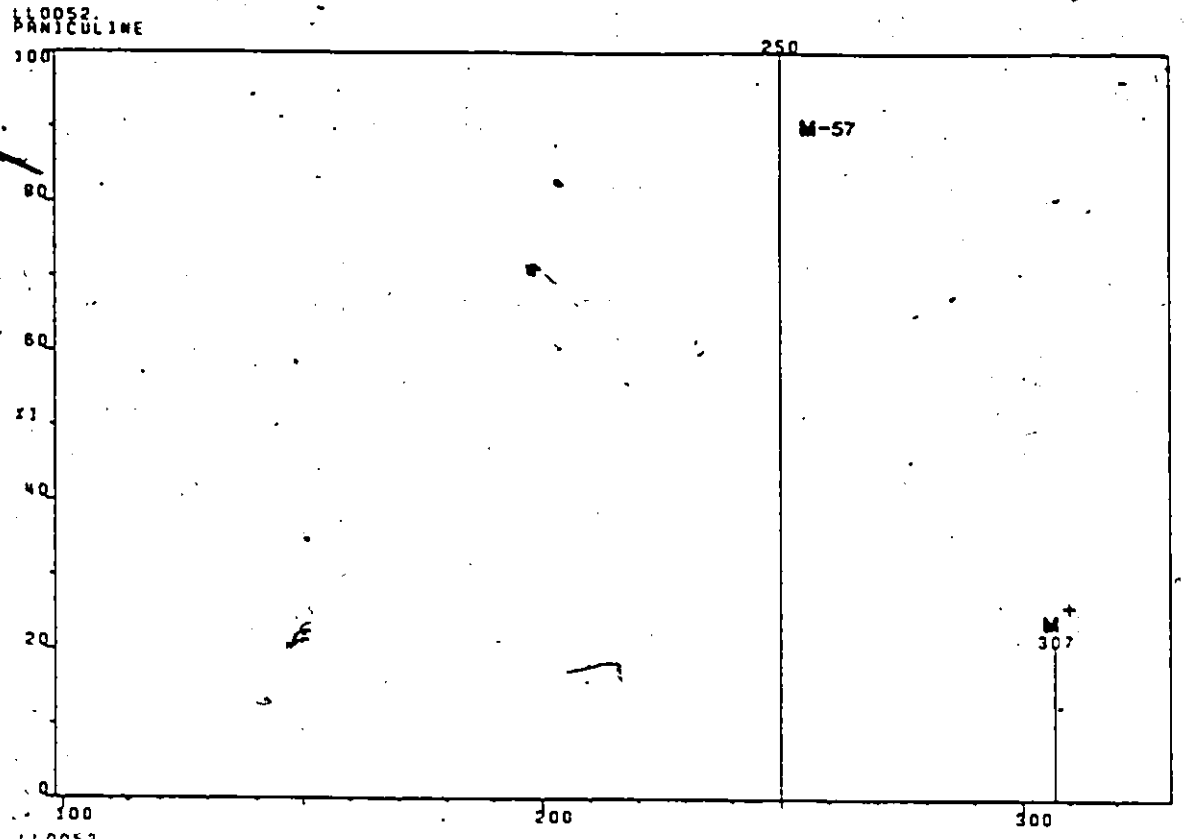


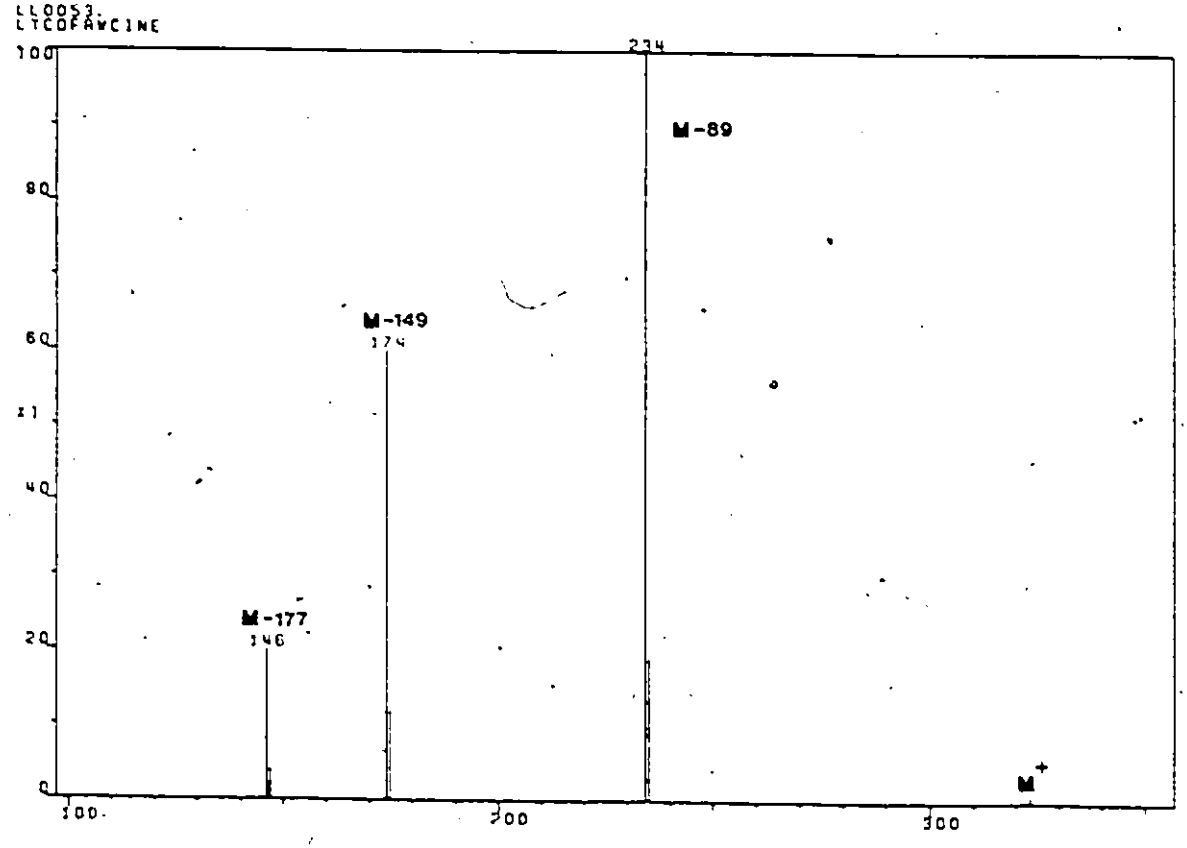
LL0050
FAWCETTIINE

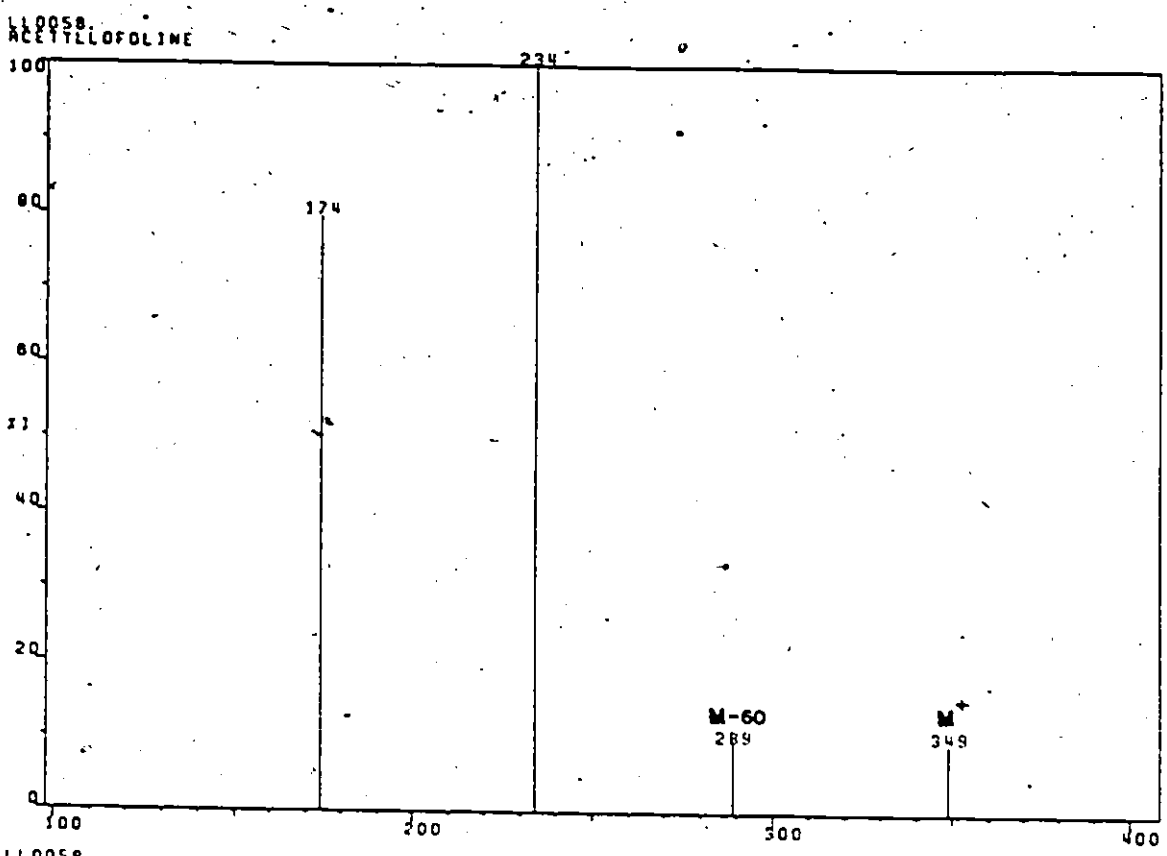


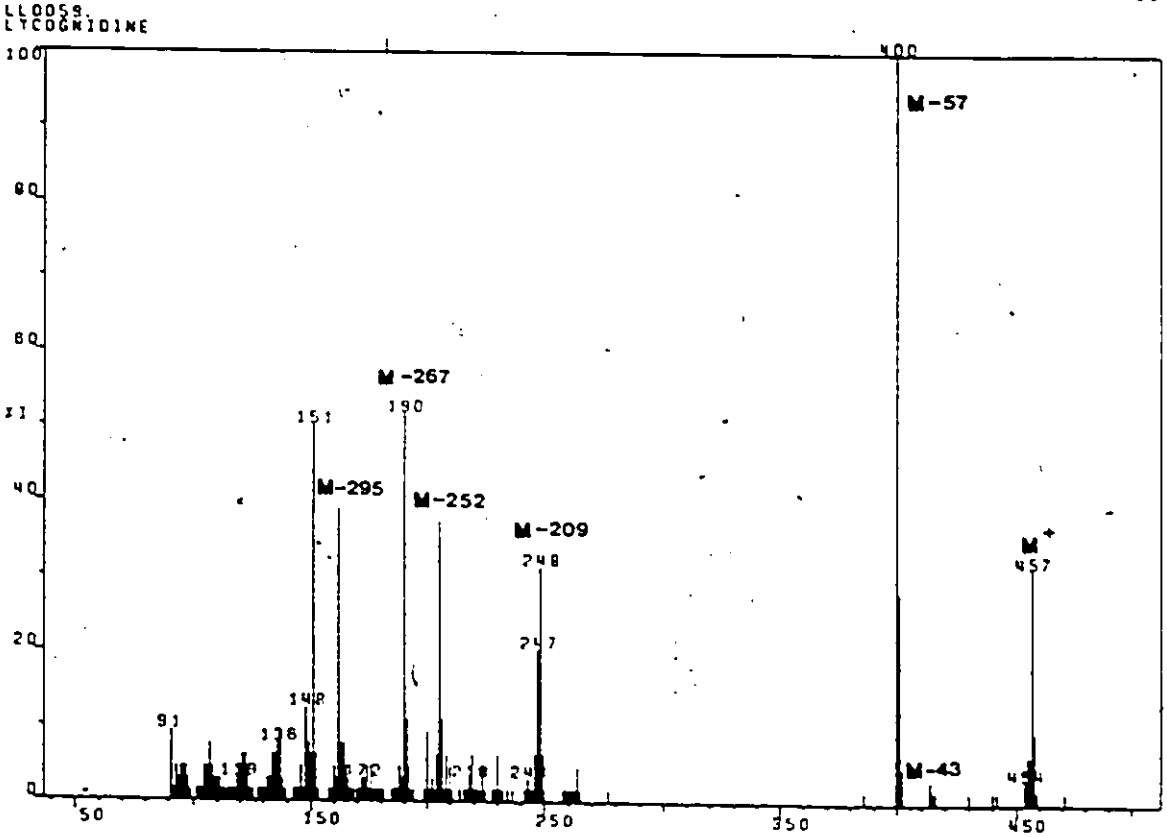
LL0051.
LYCOCLAVINE

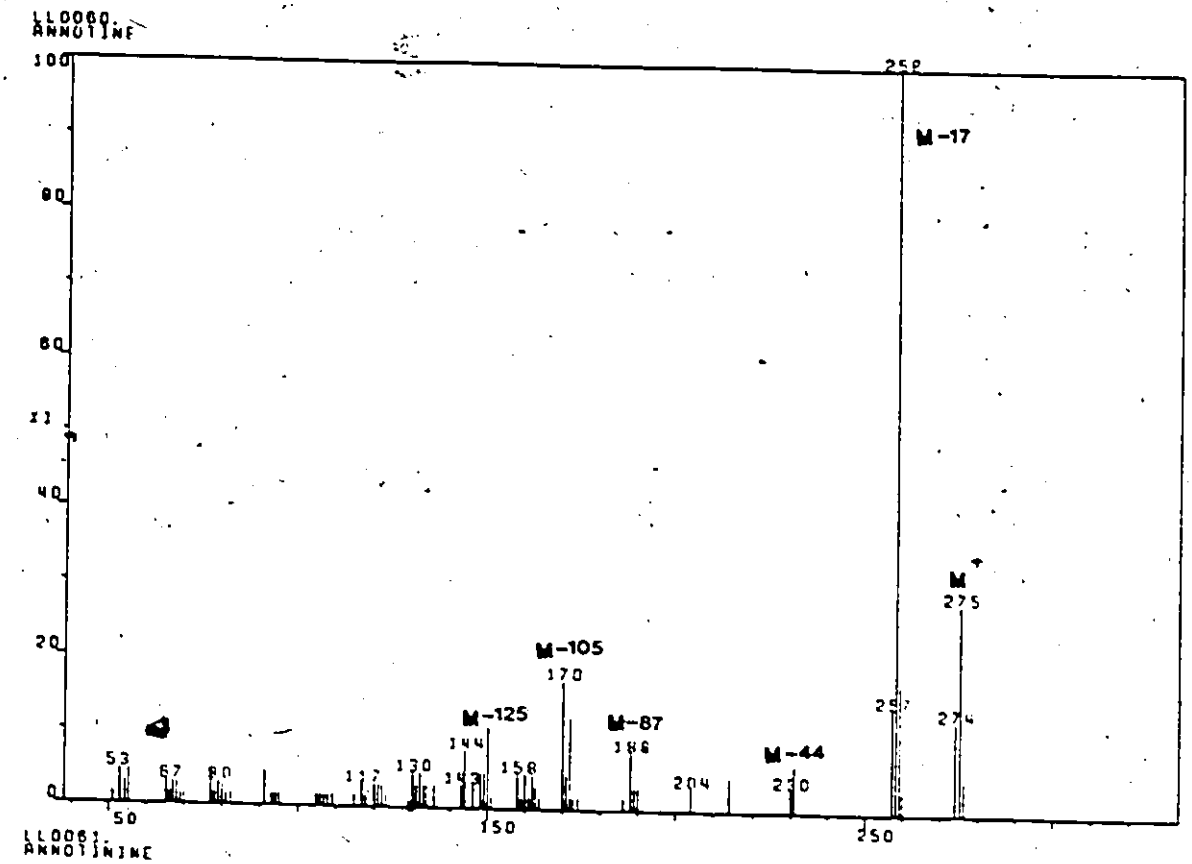


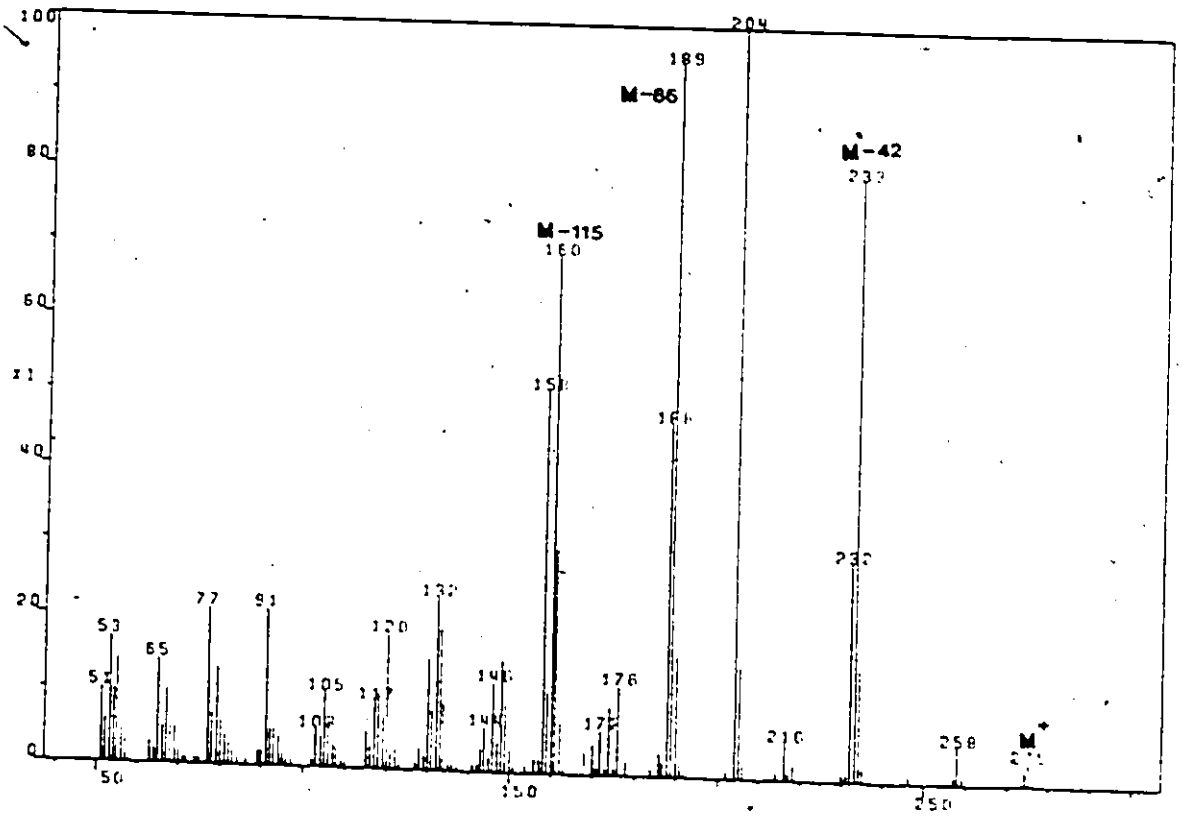

LL0052
PANICULINE
100
80
60
%I
40
20
0
250
M-57
M+
307
100
200
300


LL0053
LYCOPAVCINE
100
80
60
%I
40
20
0
234
M-89
M-149
174
M-177
146
M+
100
200
300

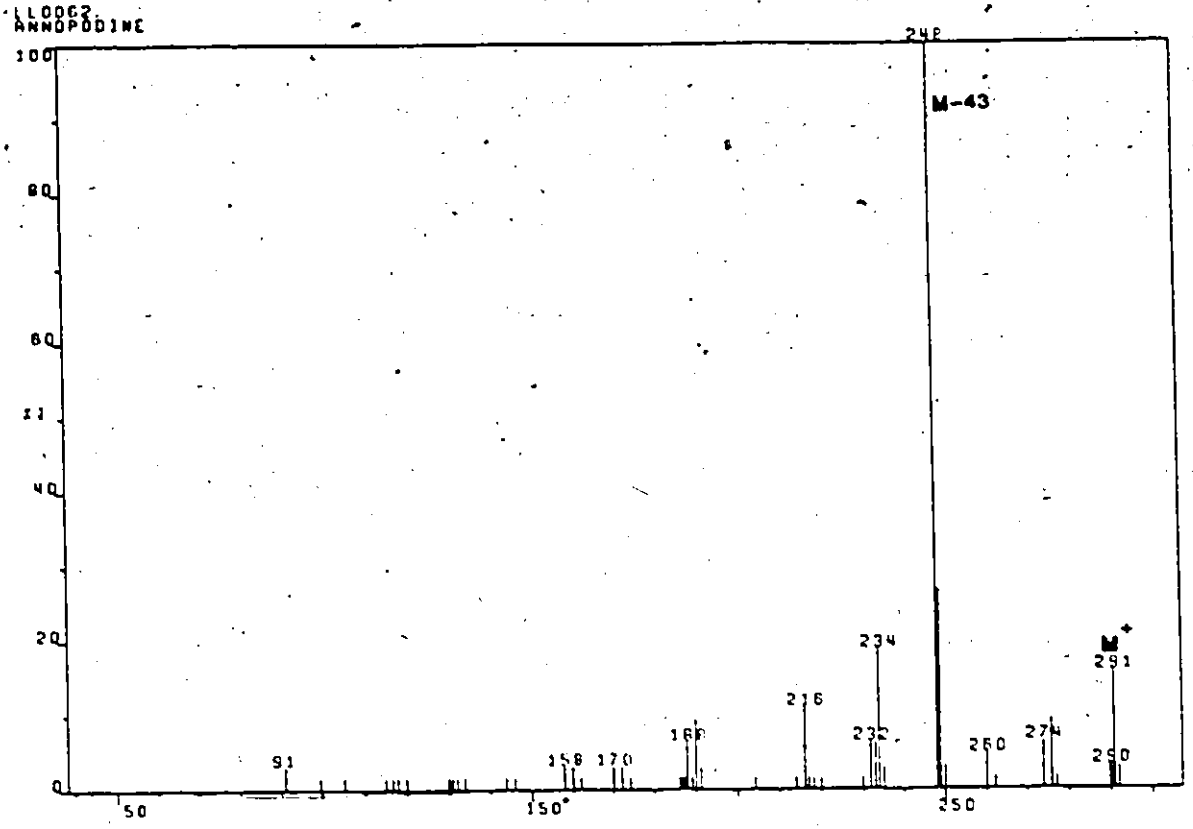

LL0058.
ACETYLLOFOLINE
100
80
60
%I
40
20
0
234
174
M-60
289
M+
349
100
200
300
400


LL0058.
LYCOGNIDINE
100
80
60
%I
40
20
0
400
M-57
M-267
190
151
M-295
M-252
M-209
248
247
M+
457
91
148
136
M-43
50
150
250
350
450

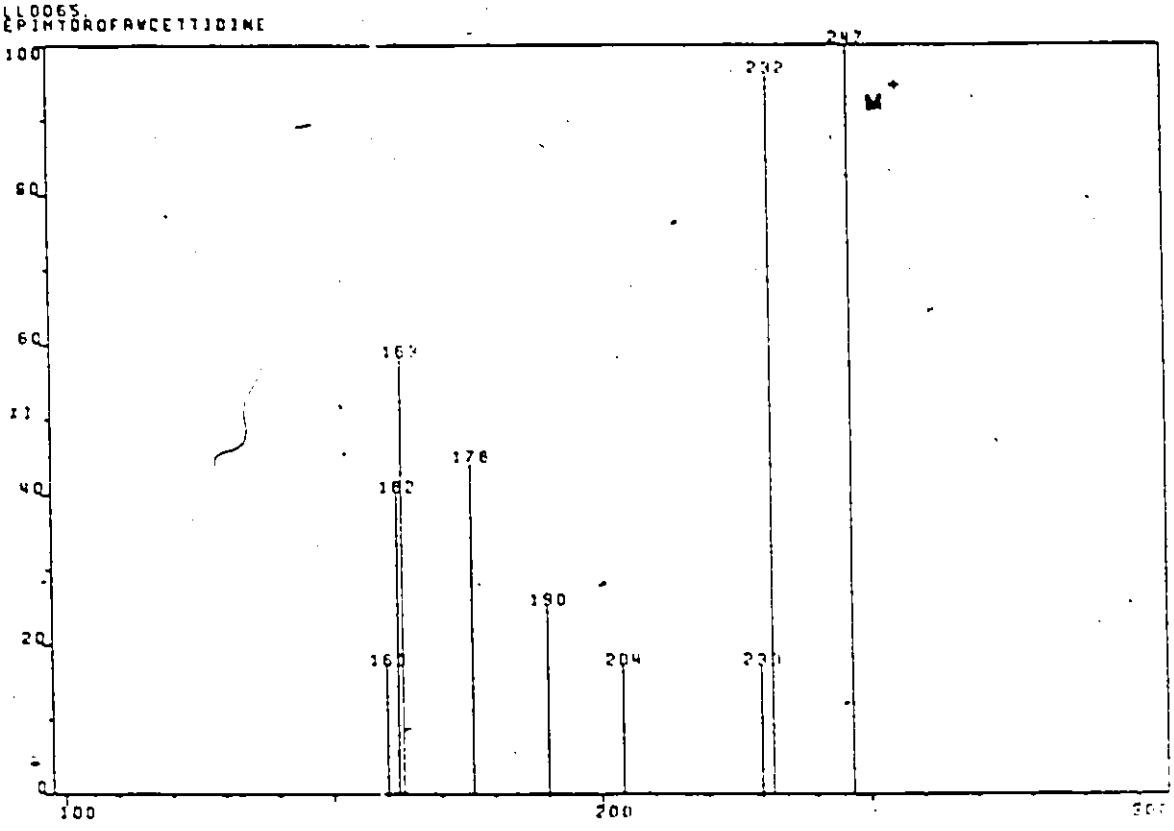

LL0060.
ANNOTININE
M-17
M+
275
M-105
170
M-125
144
M-87
186
M-44
230
53
67
80
117
130
158
204
50
150
250


LL0061.
ANNOTININE
204
189
M-86
M-42
232
M-115
160
53
65
77
91
120
105
176
216
258
M+
50
150
250

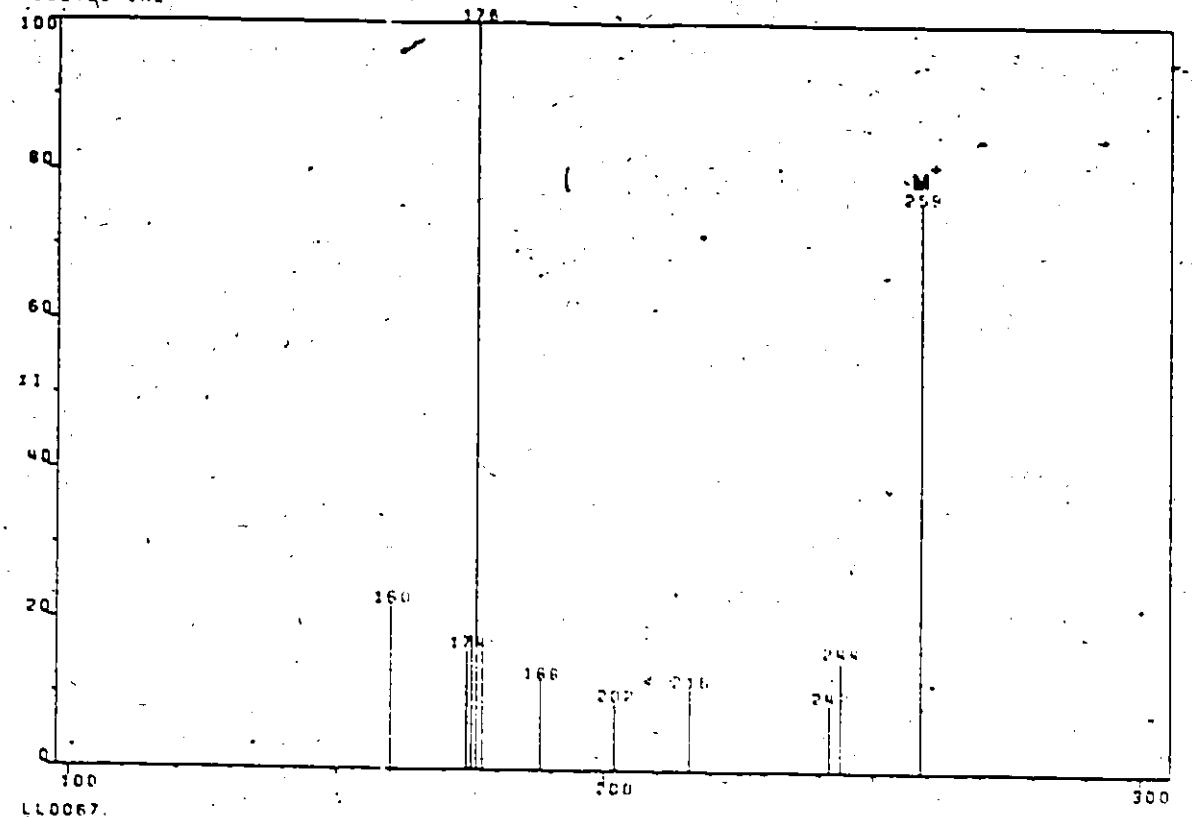
LL0062
ANNOPODINE



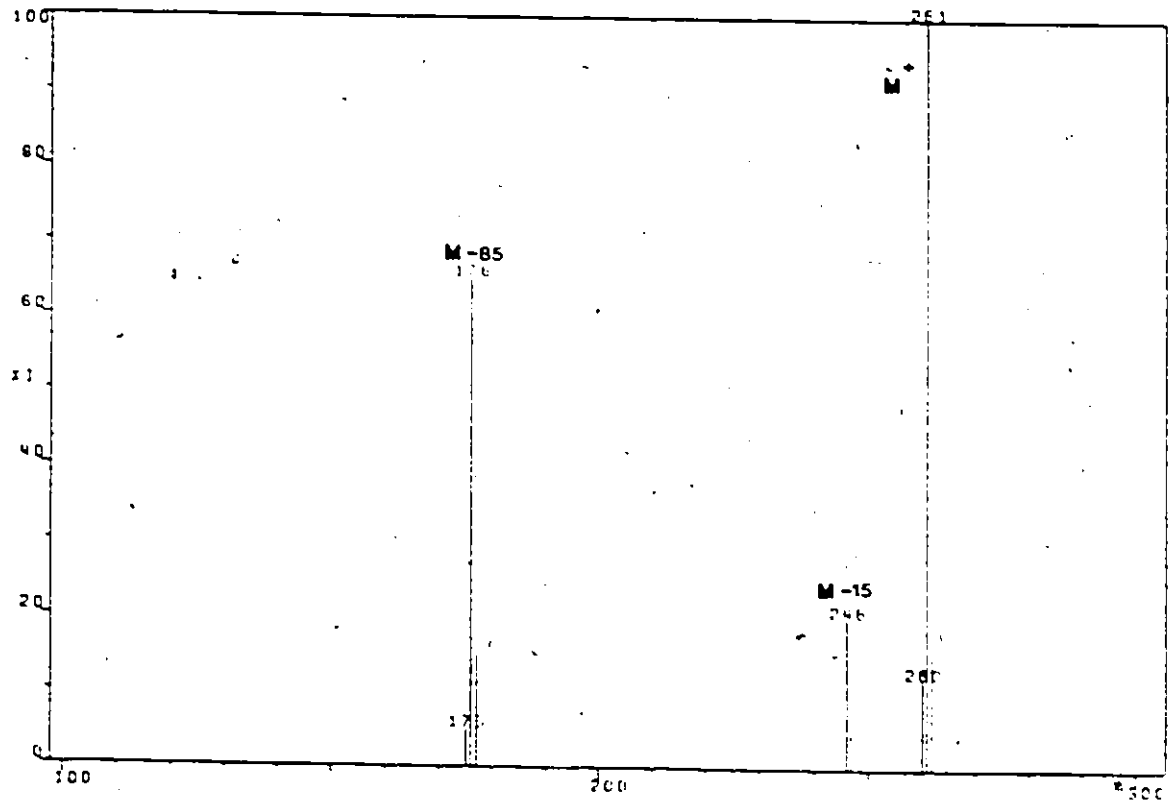
LL0065
EPIMTOROFAWCETTIDINE



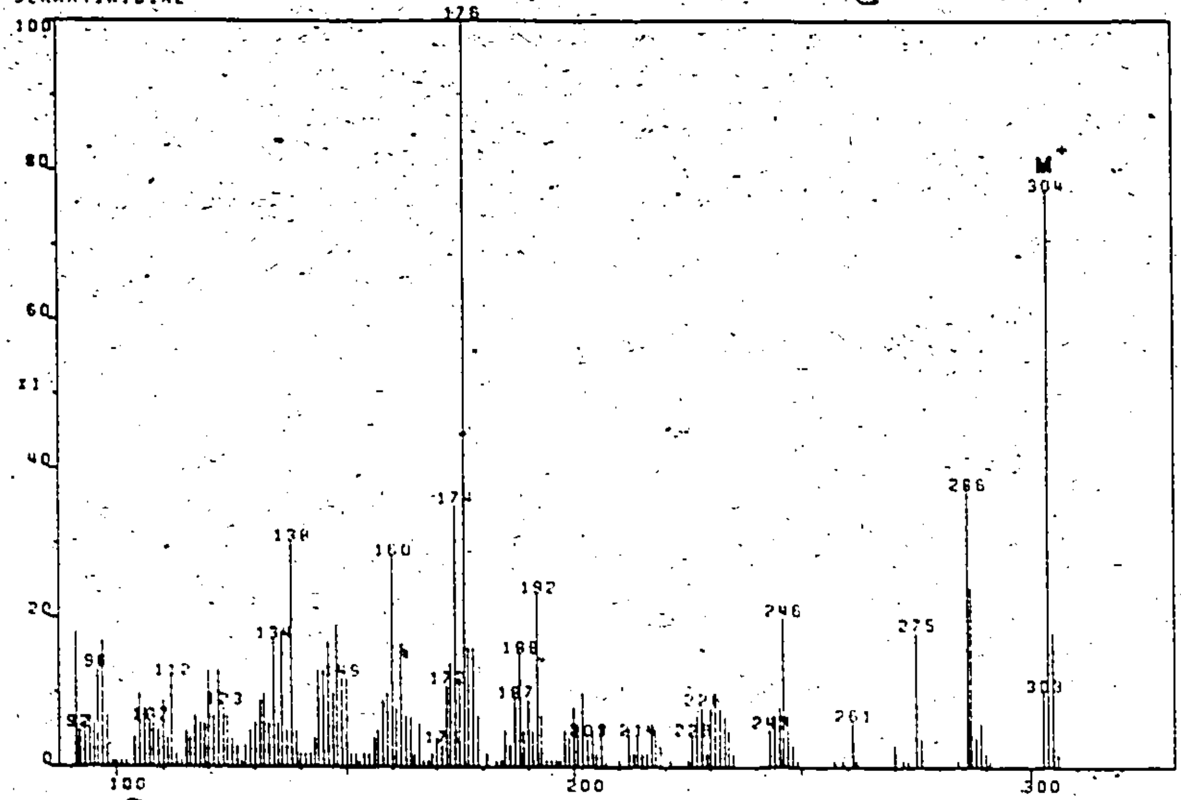
LL0068
ALOLTCOPINE



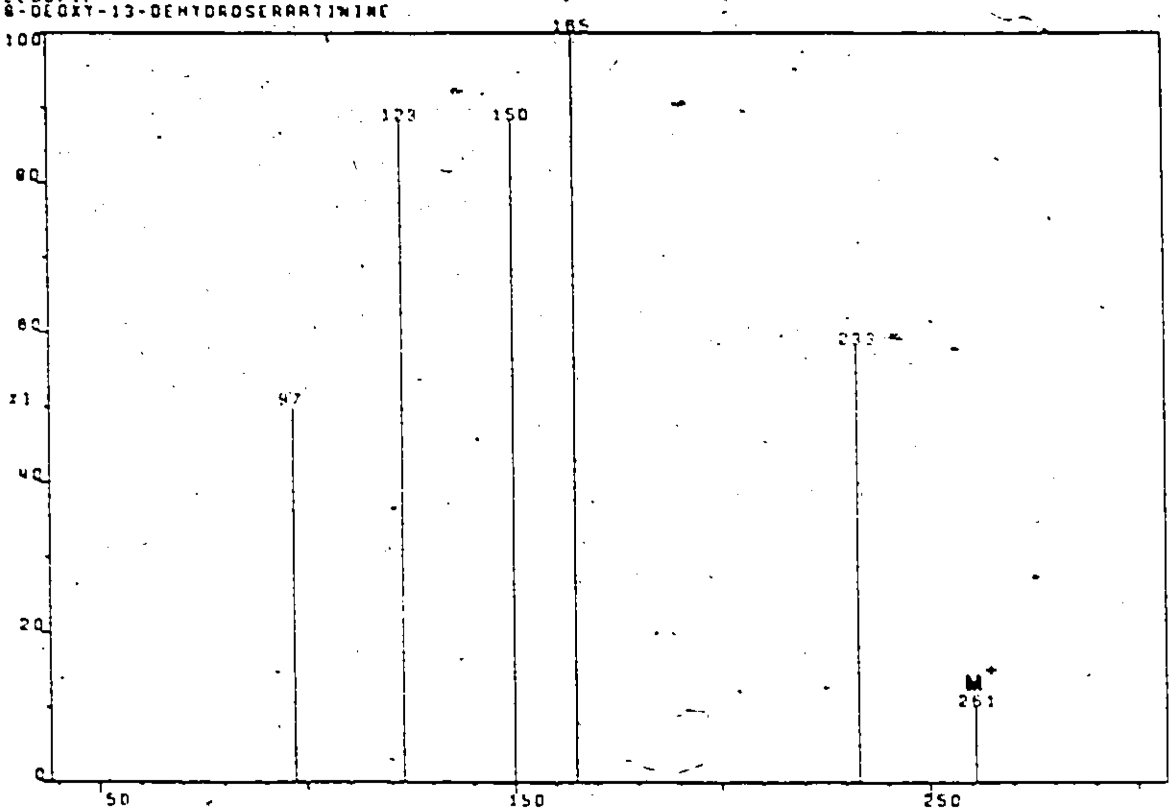
LL0067
ANHTDROAPOSERRATININE

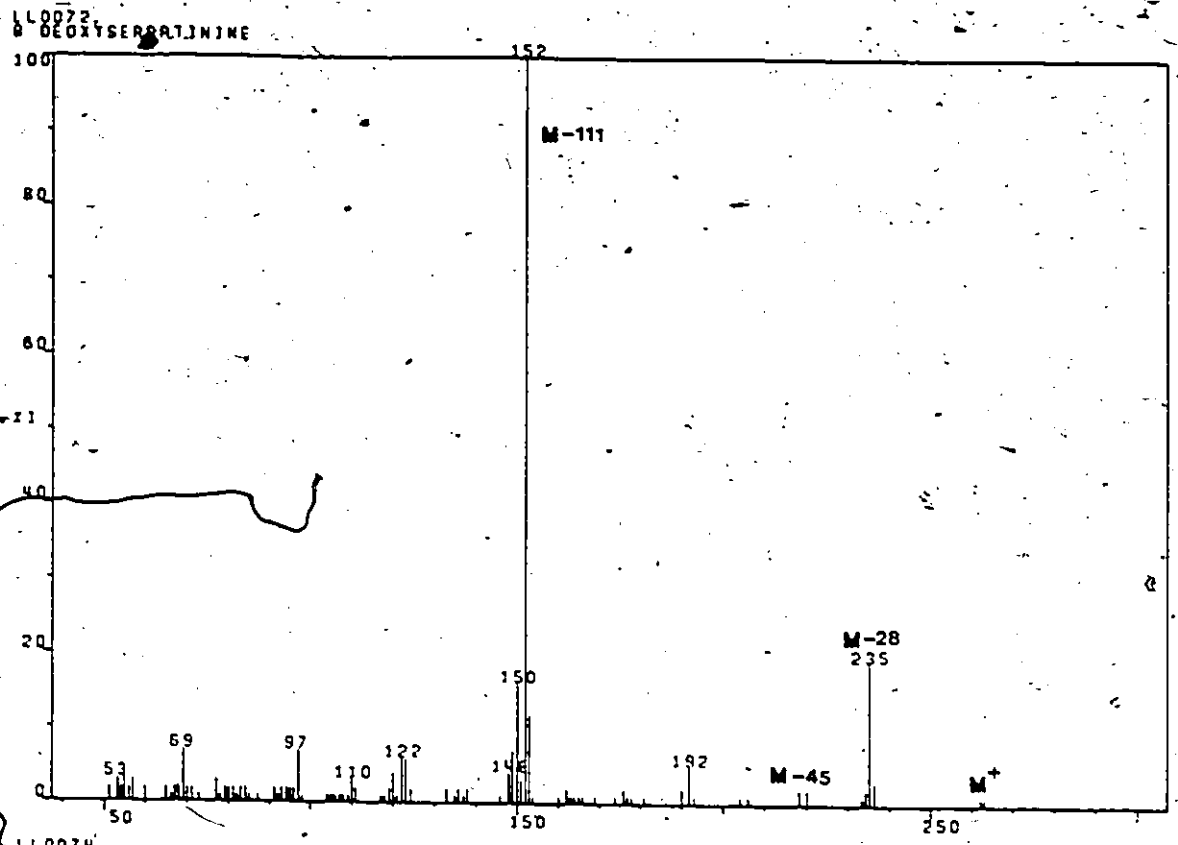


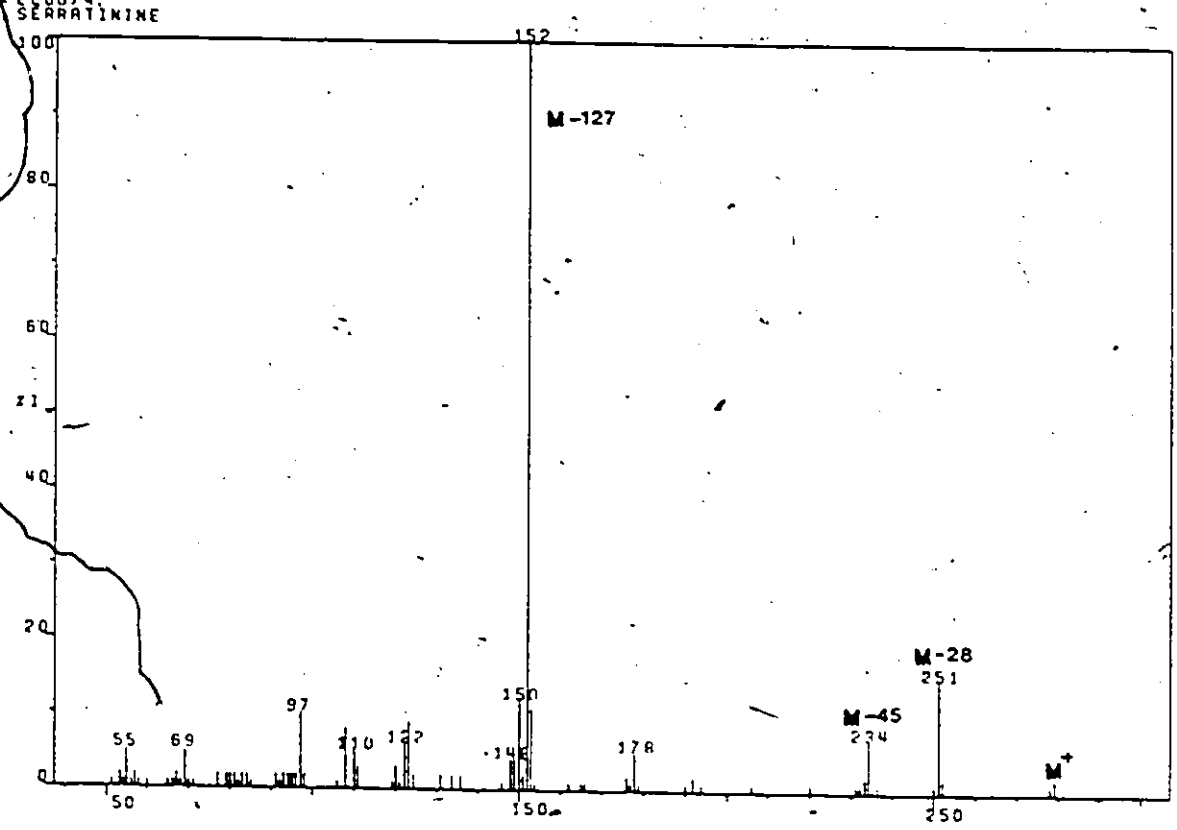
LL0070.
SERRATINIDINE



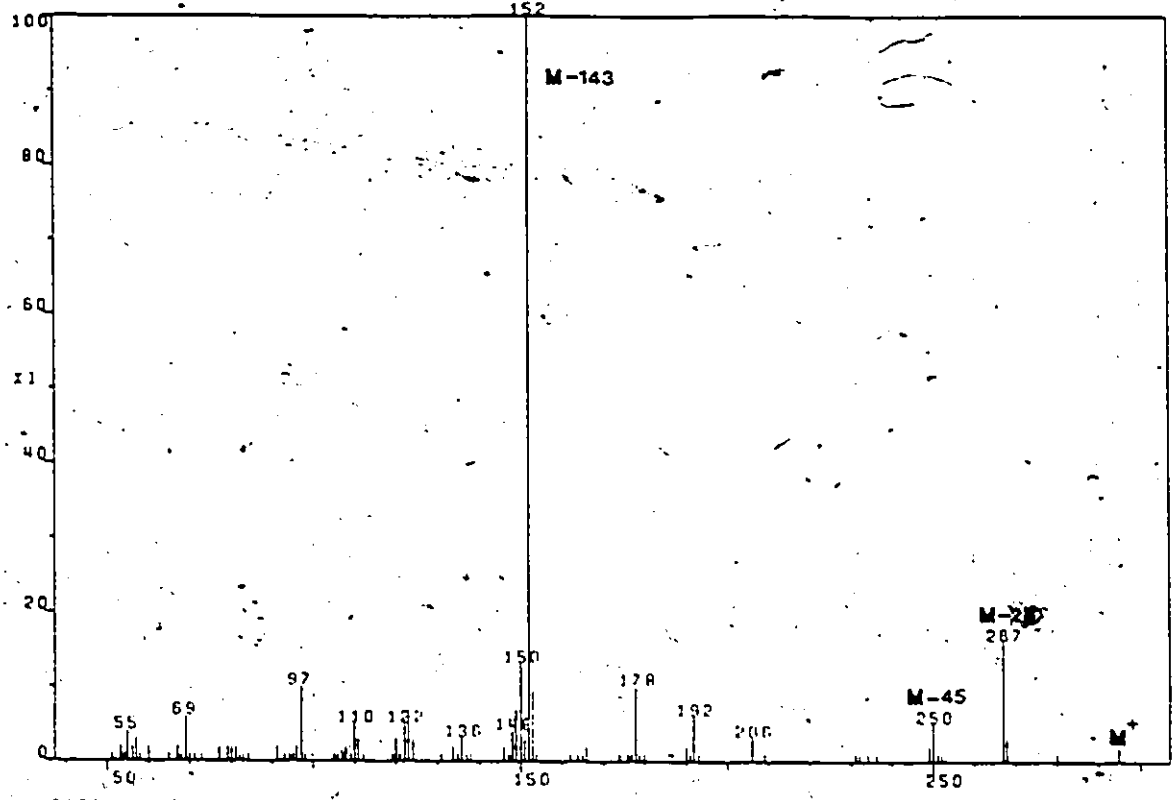
LL0071.
8-DEOXY-13-DEHYDROSERRATININE



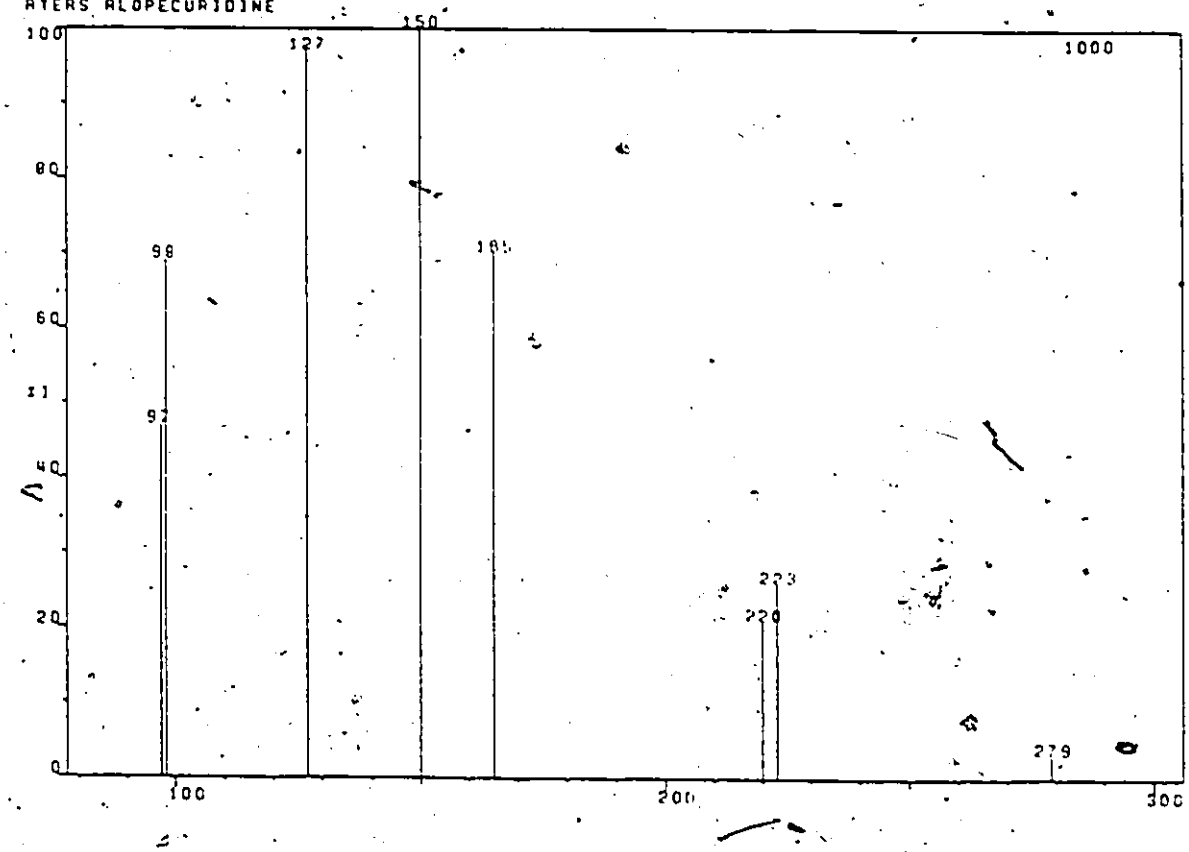

LL0072
DEOXYSERRATININE
100
80
60
40
20
0
RI
152
M-111
M-28
235
150
69
97
53
110
122
192
M-45
M+
50
150
250


LL0074
SERRATININE
100
80
60
40
20
0
RI
152
M-127
M-28
251
150
97
55
69
110
122
178
M-45
234
M+
50
150
250

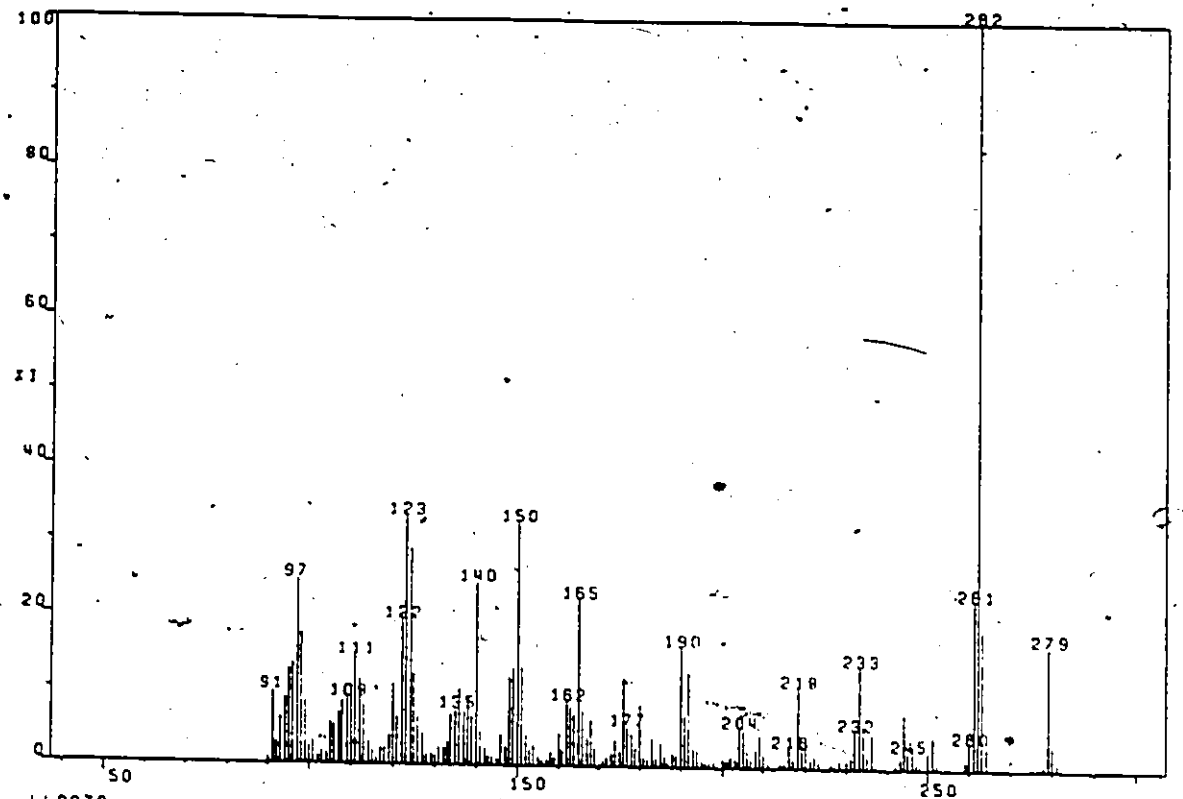
LL0075
SERRATANIDINE



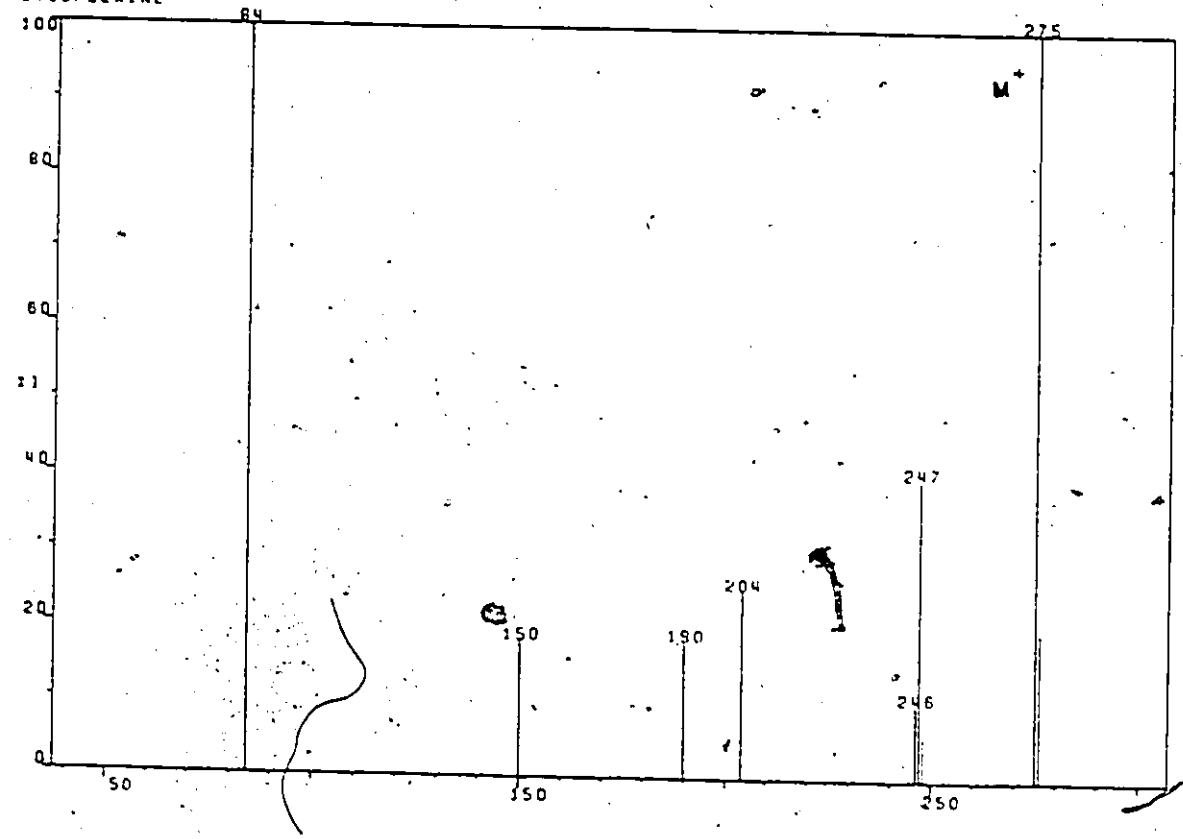
LL0100
ATERS ALOPECURIDINE

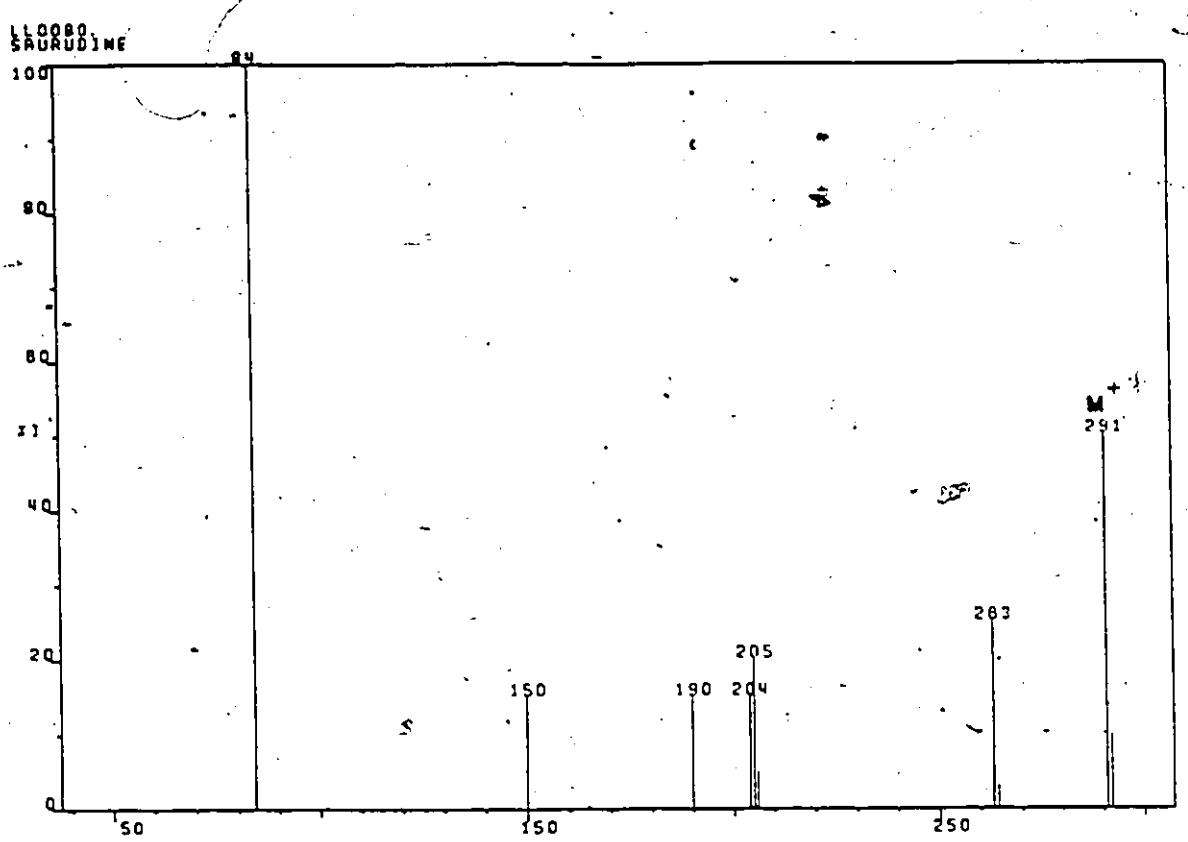


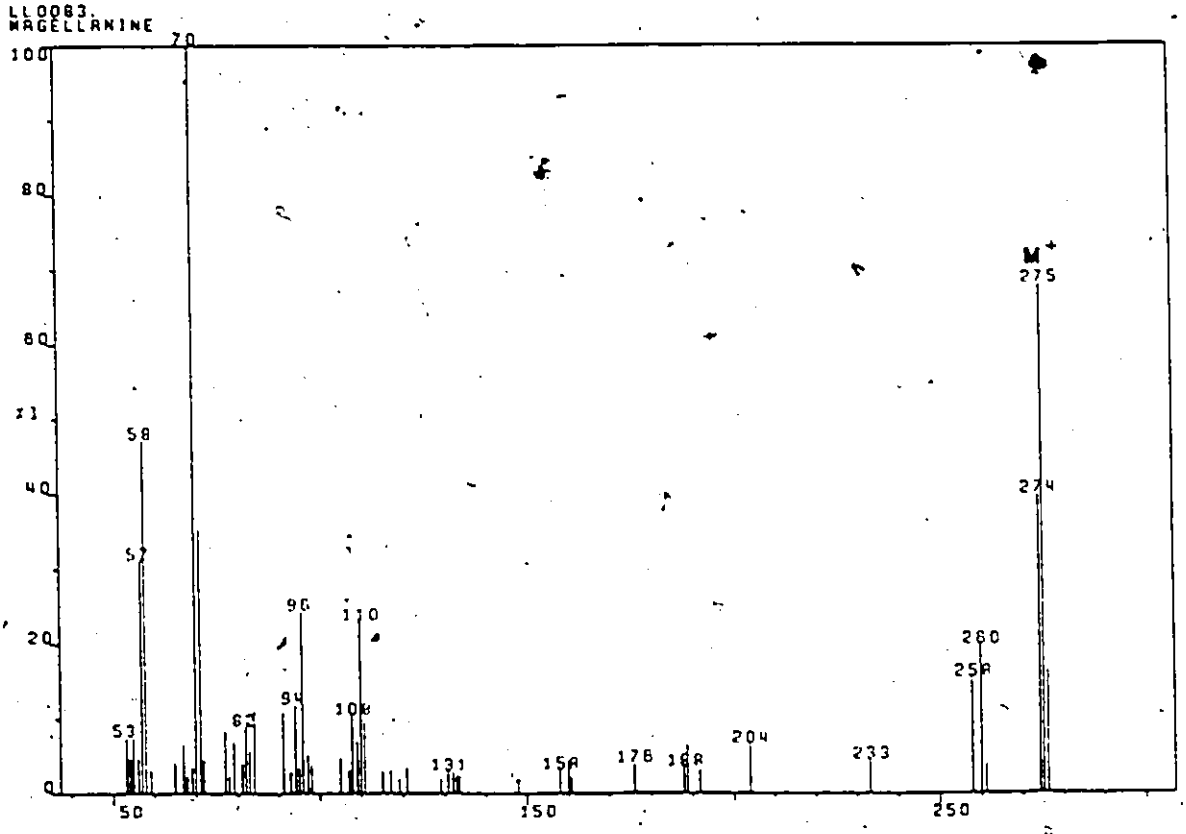
LL0077
ALOPECURIDINE

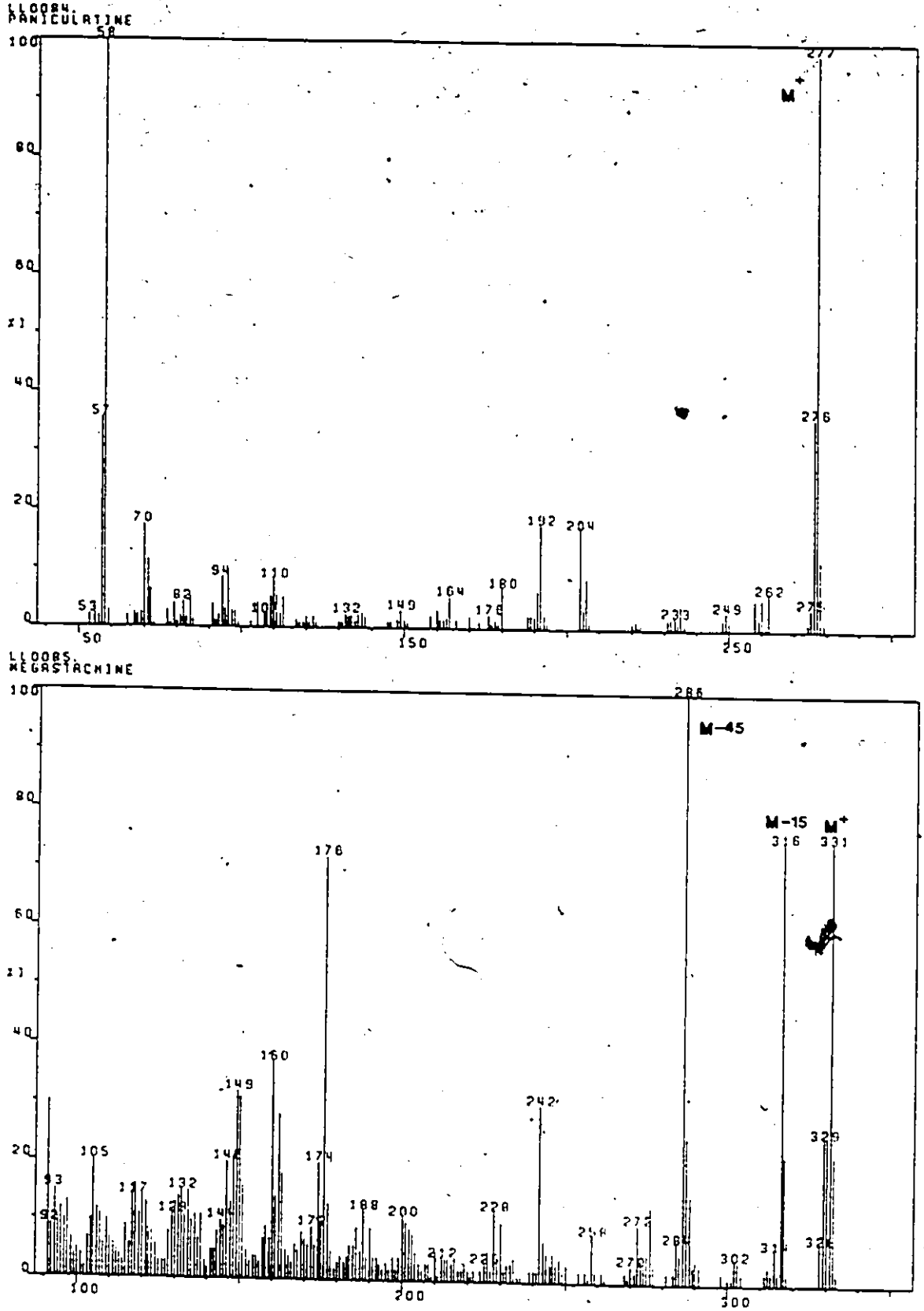


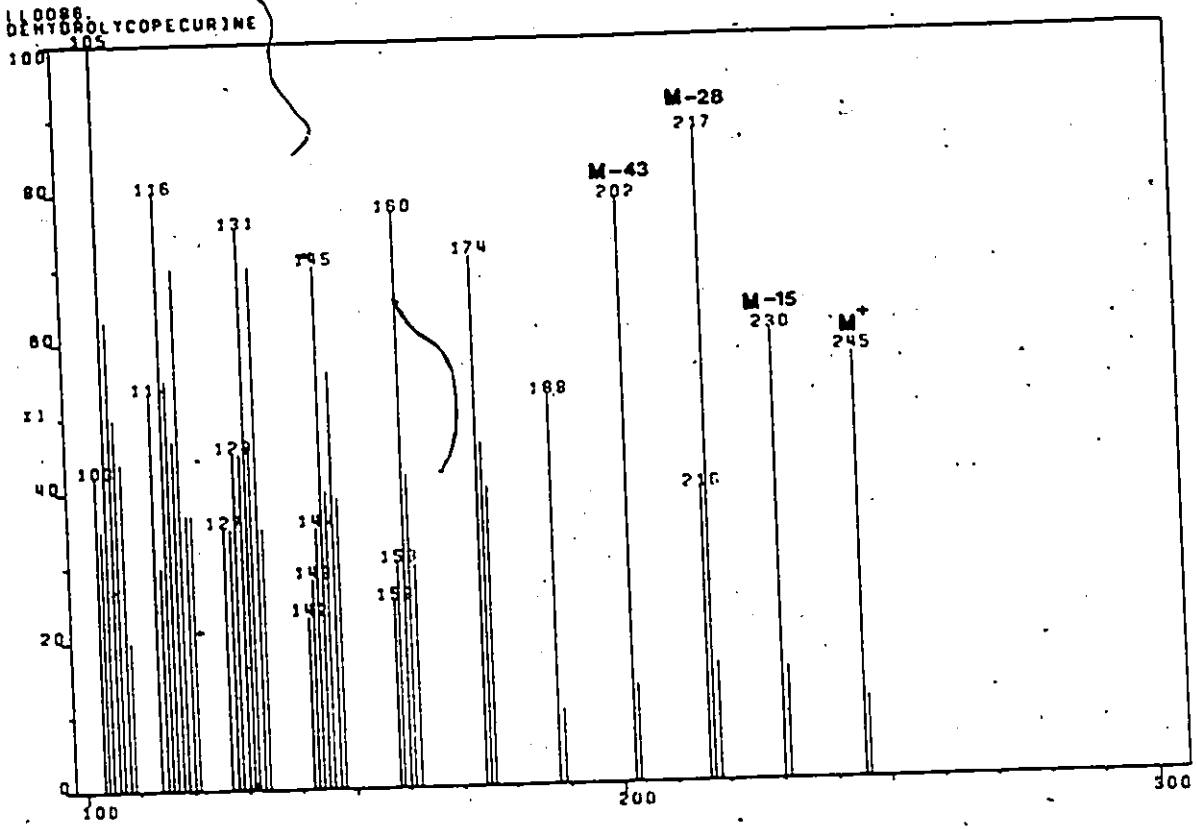
LL0079
LTCOFLEXINE

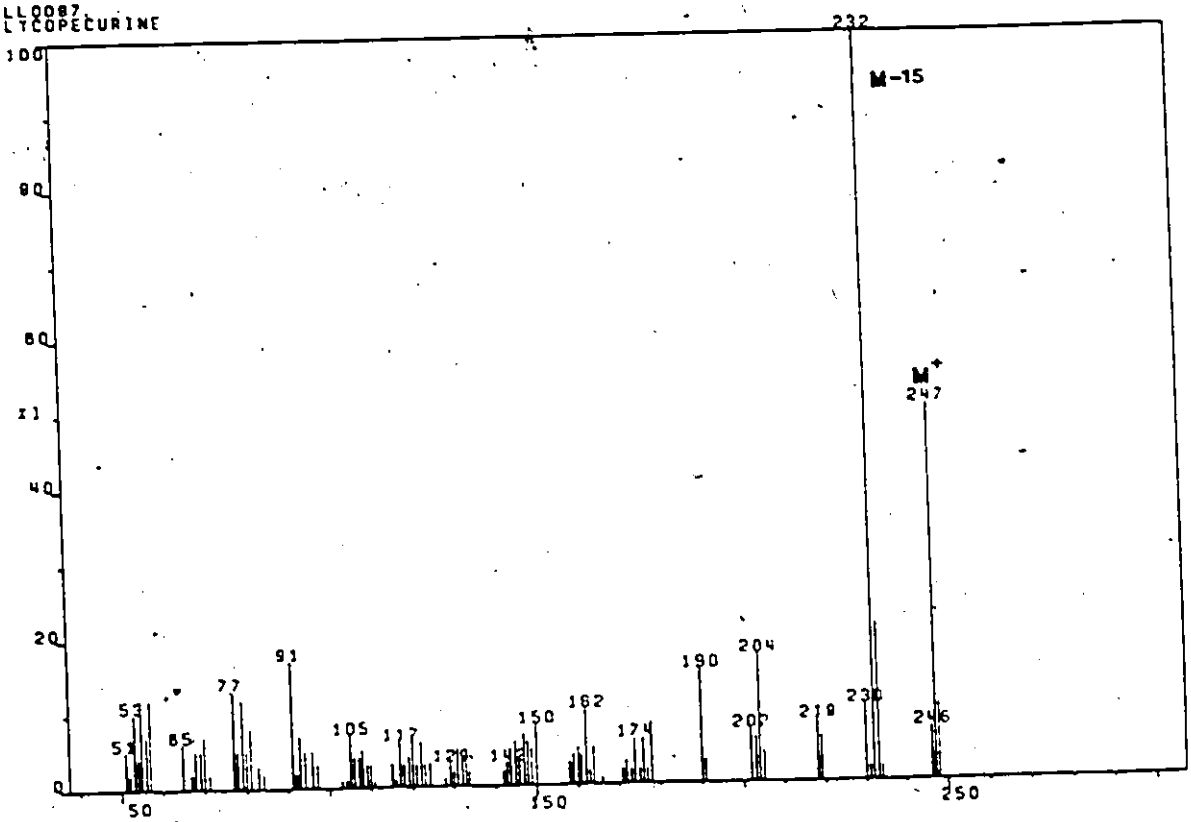


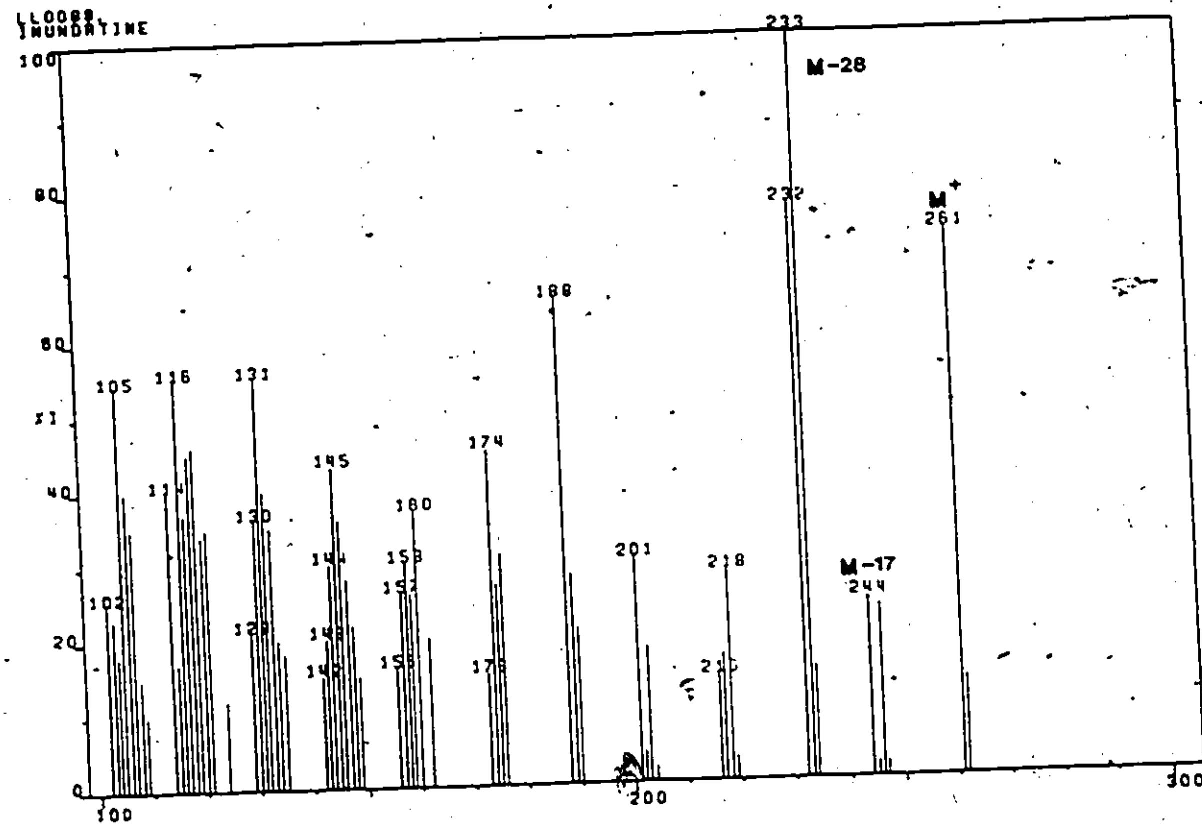

LL0080
SAURUDINE
84
M+
291
283
205
150
190
204
50
150
250

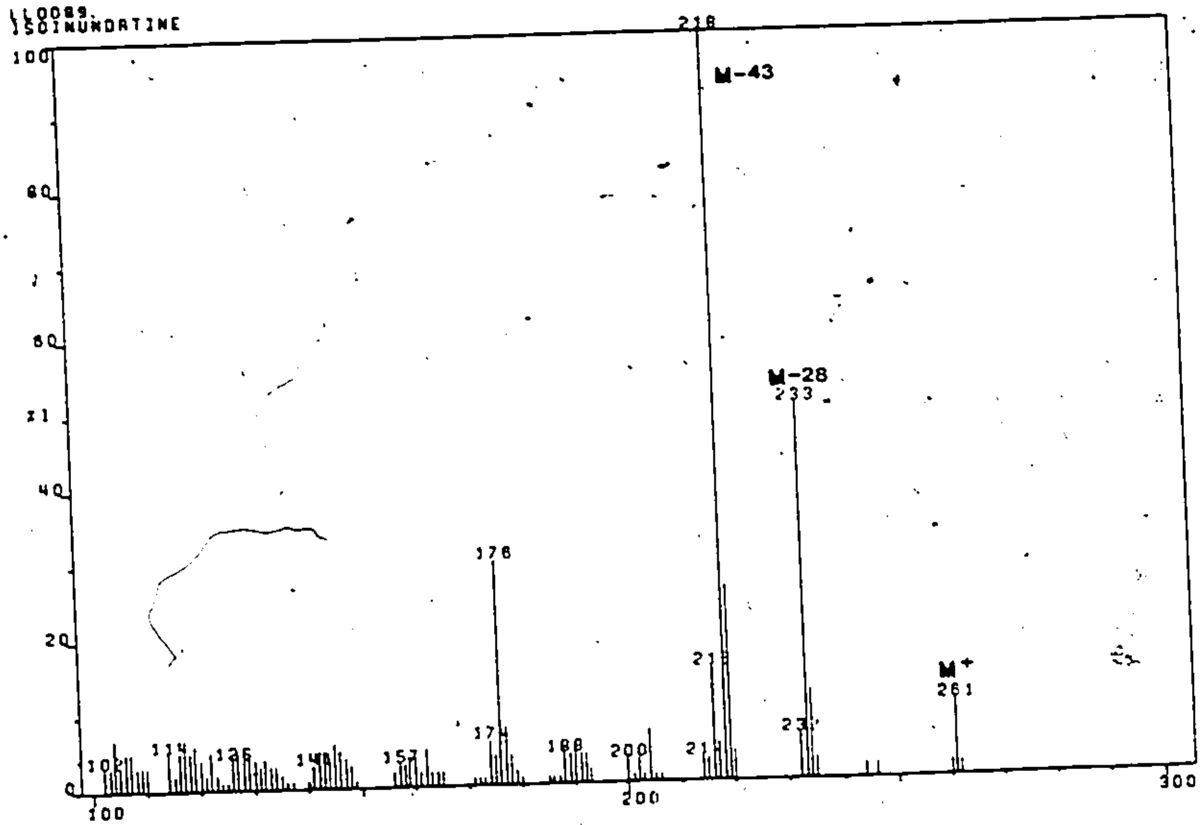

LL0083.
MAGELLANINE
70
M+
275
274
58
57
96
110
94
108
53
61
131
156
176
188
204
233
260
258
50
150
250


LLOOS4.
PANICULATINE
M+
277
276
58
57
70
94
110
192
204
LLOOBS.
MEGASTACHINE
M-45
M-15
M+
316
331
286
176
160
149
242
329
105
174
188
200
228
302

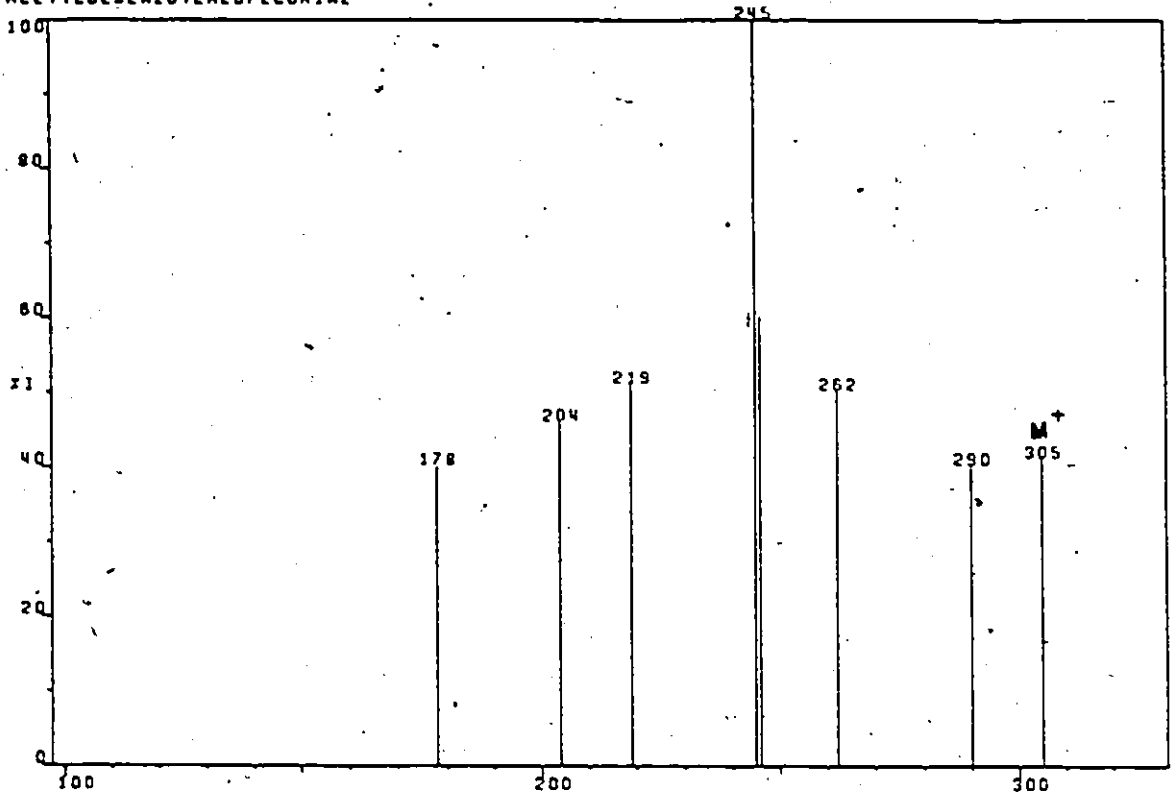

LL0086
DEHYDROLYCOPECURINE
M-28
217
M-43
202
M-15
230
M+
245
116
131
160
174
188
105
100
200
300


LL0087
LYCOPECURINE
232
M-15
M+
247
91
77
53
65
105
117
150
162
174
190
204
219
230
246
50
150
250

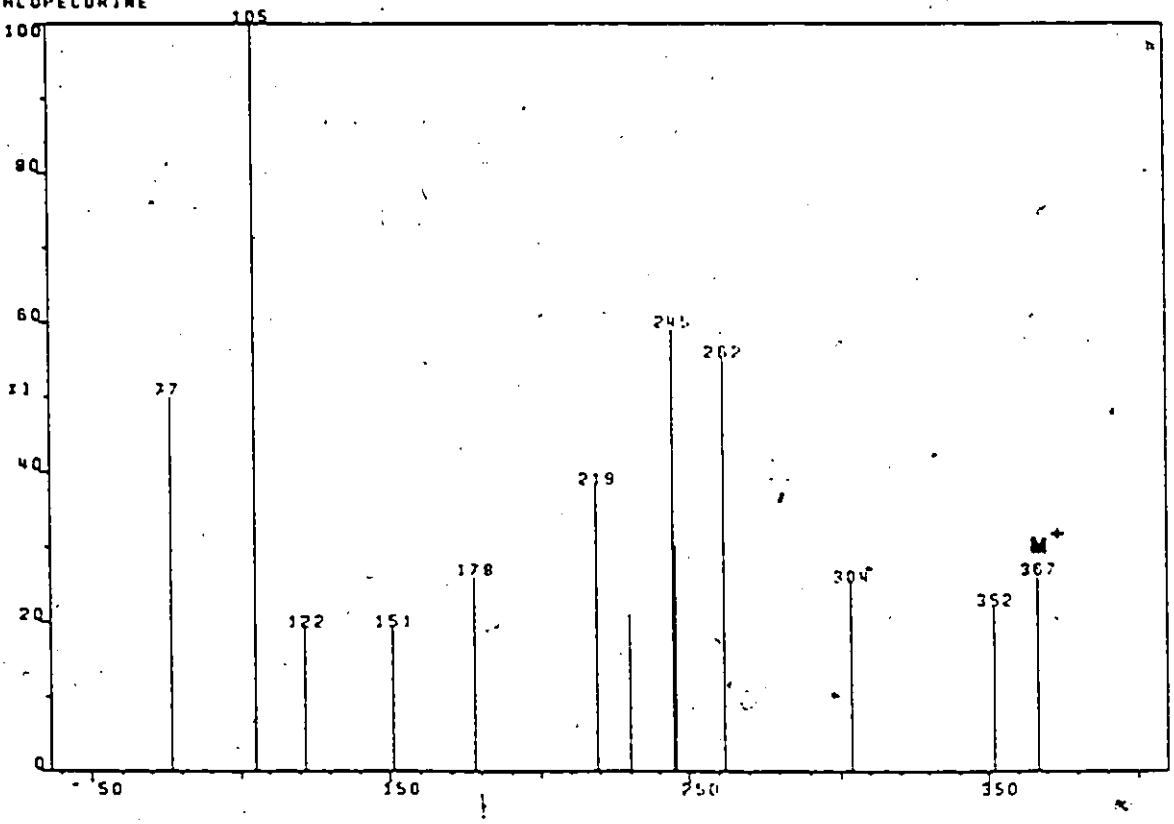

LL0089
INUNDATINE
M-28
M+
M-17
100
80
60
40
20
0
100
200
300
233
232
261
188
105
116
131
145
174
180
201
218
244

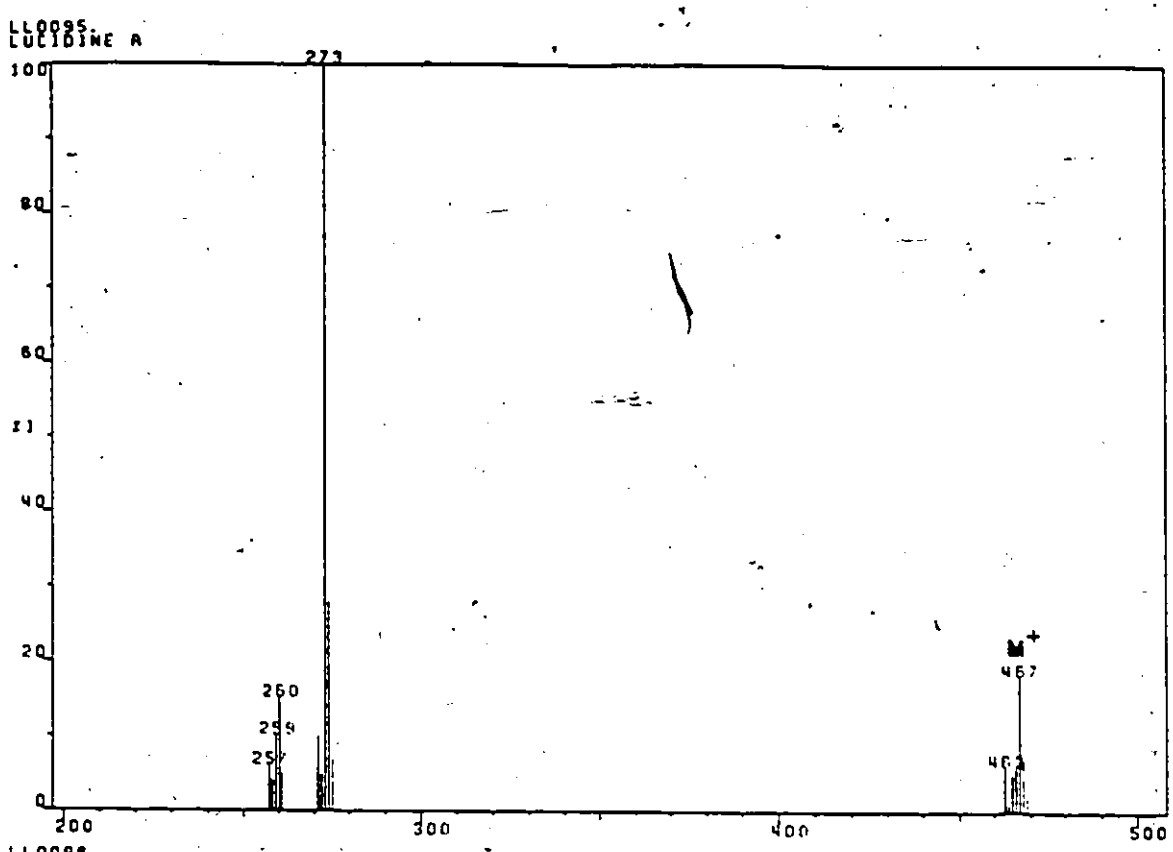

LL0089
ISOINUNDATINE
M-43
M-28
M+
218
233
261
176
100
200
300

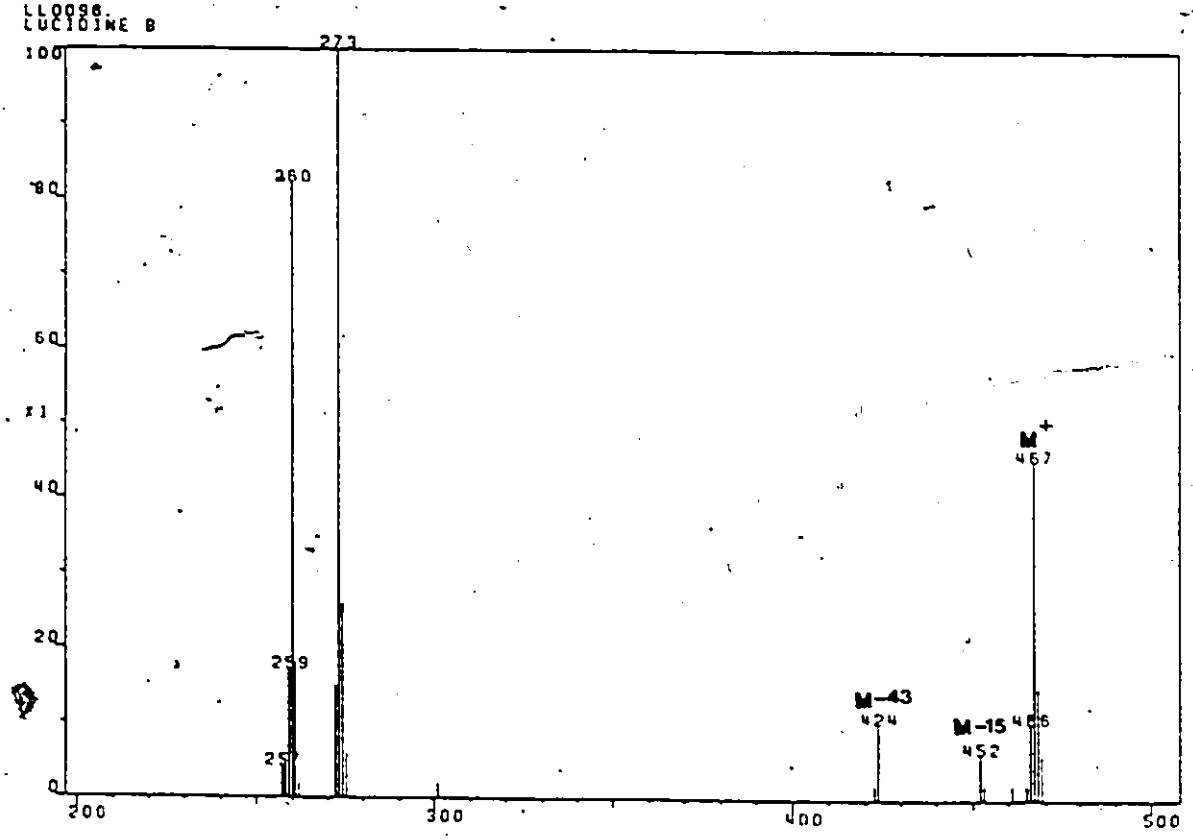
LL0091
ACETYLDEBENZOYLALOPECURINE



LL0092
ALOPECURINE




LL0095.
LUCIDINE A
100
80
60
%I
40
20
0
273
260
259
M+
467
200
300
400
500


LL0096.
LUCIDINE B
100
80
60
%I
40
20
0
273
260
259
M+
467
M-43
424
M-15
452
200
300
400
500