PHYTOCHEMICAL INVESTIGATION OF TRITICALE ERGOT

by

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A C K N O W L E D G E M E N T S

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A B S T R A C T

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Ergot grown on Triticale grain (Rosner cultivar) was investigated with regard to morphological characteristics, fat content and alkaloid content (quantitative and qualitative).

Soxhlet extraction of the powdered ergot sample using petroleum ether yielded 16.74% w/w of ergot oil. The total alkaloid content of the sample was determined by a colorimetric assay procedure to be 0.181%. The water-soluble and water-insoluble alkaloids constituted 20% and 80% of total alkaloids respectively.

The ergot alkaloids extracted from the sample were separated on a tartaric acid impregnated cellulose column into the peptide alkaloid fraction, the clavine alkaloid fraction and ergometrine. Subsequent thin-layer chromatography and paper chromatography of these fractions identified ergotamine, the ergotoxine group, ergometrine, a trace of ergometrinine, penniclavine, setoclavine, elymoclavine, agroclavine and possibly isopenniclavine.

The alkaloids from two of the cellulose column fractions were purified and characterized. The melting points, infrared, nuclear magnetic resonance, and mass spectra of the isolated alkaloids were recorded. The alkaloids were identified as ergotamine and ergometrine.

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I N T R O D U C T I O N

Claviceps purpurea

Claviceps purpurea (Fries) Tulasne (Family Hypocreaceae, Class Ascomycetes) is a parasitic fungus commonly occurring on grasses and cultivated cereals. Ergot of commerce is defined as the dried sclerotium, or resting stage of Claviceps purpurea - a hard, purplish, somewhat curved body, the size of which is usually determined by the size of the grain it parasitizes (1). The generic name Claviceps refers to the club-like character of the sclerotium, purpurea to its purple color (2).

Originally, the main sources of supply for ergot were Spain, Russia and the Balkan countries; however, Russia and the Balkans export very little ergot today. Currently, considerable ergot is being cultivated in Czechoslovakia, Germany, Hungary and Switzerland (2).

Other species of Claviceps are capable of producing ergots in the ovaries of many other members of the Gramineae family such as Triticum, Avena, Festuca, Lolium, Molinia and Nardus (3).

Alkaloids of Claviceps purpurea

Since galenical preparations of the crude drug are seldom employed in pharmacy in this country, ergot has been omitted from the official compendia. Nevertheless, the ergot alkaloids continue to enjoy widespread use as extremely important medicinal agents (2). More than a score of alkaloids have been isolated from the fungus,

Claviceps purpurea, growing as a parasite on various members of the Gramineae (grasses) family, the most important of which are ergometrine, ergotamine, and a mixture of ergocornine, ergocristine, and ergocryptine (6).

The average content of total alkaloids in the sclerotia varies between 0.025 and 0.4% but the alkaloid content of individual sclerotia is also variable; some sclerotia containing very little alkaloid, while others may contain as much as 1% (3). The qualitative and quantitative composition of the alkaloids obtained from different host plants or grown in saprophytic culture is influenced by a number of factors but especially by the identity of the strain of the organism involved (2). Both peptide alkaloids and nonpeptide (water-soluble) alkaloids were obtained from parasitically developed ergot sclerotia, while only the water-soluble type was produced commercially in saprophytic culture. In 1969, however, Amici et al. (4) succeeded in isolating three strains of Claviceps purpurea from sclerotia grown on rye which produced, under submerged saprophytic conditions, ergocryptine and ergotamine, ergocornine and ergosine, and ergocristine, respectively. Large scale production of lysergic acid derivatives in submerged culture was achieved in 1960 by Tonolo, Chain and co-workers in Italy (2). These investigators utilized a strain of Claviceps paspali which produced several simple lysergic acid derivatives (2).

The ergot alkaloids belong to the large, significant class of the indole alkaloids and possess a tetracyclic ring system named ergoline (Figure 1). All of the ergot alkaloids possess this characteristic base structure (5).

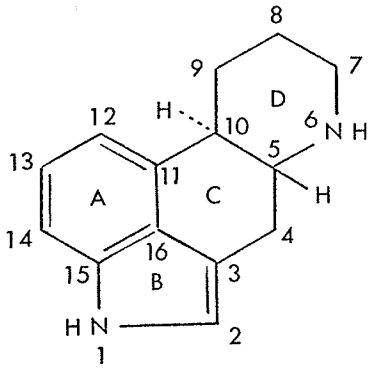


Figure 1. Ergoline

The natural ergot alkaloids can be classified into two main groups according to their basic chemical structure; the peptide alkaloids, and the clavine alkaloids (5).

The peptide or water-insoluble alkaloids, are derivatives of d-lysergic acid (Figure 2). Since this compound is readily converted to its isomer, d-isolysergic acid (Figure 3), corresponding isolysergic

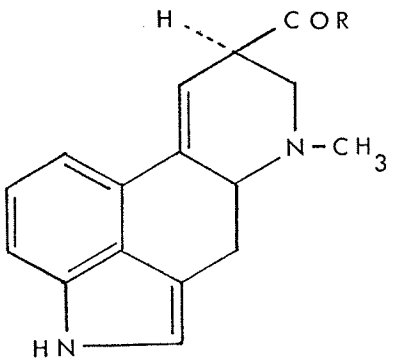


Figure 2. d-Lysergic Acid

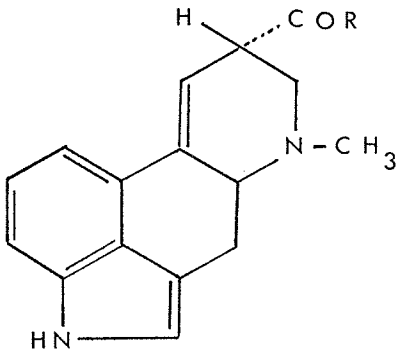


Figure 3. d-Isolysergic Acid

acid derivatives often accompany the d-lysergic acid alkaloids in the natural material or are produced during the course of extraction. These peptide alkaloids are acid amide derivatives of the basic d-lysergic or d-isolysergic structure and consequently yield, on alkaline hydrolysis, d-lysergic or d-isolysergic acid. The individual alkaloids in this group differ only in the type of side chain attached in the R position of the basic d-lysergic acid or d-isolysergic acid structure as shown in Table 1 (5).

Table 1. Types of Peptide Alkaloids (5)

Type of Alkaloid	R Substituent	Alkaloids in Group
1. Peptide Type	Cyclic Peptide Side Chain	Ergotamine, Ergosine, Ergocryptine, Ergocornine, Ergostine and their Isolysergic Acid Isomers
2. Alkalolamine Type	L-2-Aminopropanol	Ergometrine and its Isolysergic Acid Isomer
3. Amide Type	$-NH_2$	Ergine and its Isolysergic Acid Isomer
4. Carbinolamide Type	$-NHCHOHCH_3$	Lysergic Acid Methyl Carbinolamide

From the ergot sclerotium there have been isolated six isomeric pairs of the "peptide-type" ergot alkaloids, each pair comprising a laevorotatory, pharmacologically active alkaloid and its dextrorotatory

isomer which is practically inactive pharmacologically. On alkaline hydrolysis these alkaloids give rise to either d-lysergic acid or d-isolysergic acid as previously mentioned. Each alkaloid exhibits fluorescence under ultraviolet light and produces a characteristic deep blue color when reacted with p-dimethylaminobenzaldehyde (p-DMAB) (6). Tables 2 and 3 list the alkaloids of this group and some of their physical and chemical characteristics.

Figure 4 illustrates the basic chemical structure of the "peptide-type" ergot alkaloids, the isolysergic acid isomers differing only in the configuration at carbon C-8. Each alkaloid possesses the same basic structure, the individual alkaloids differing in structure only in the substituents at the R_1, R_2 and R_3 positions as indicated in Table 2 (5).

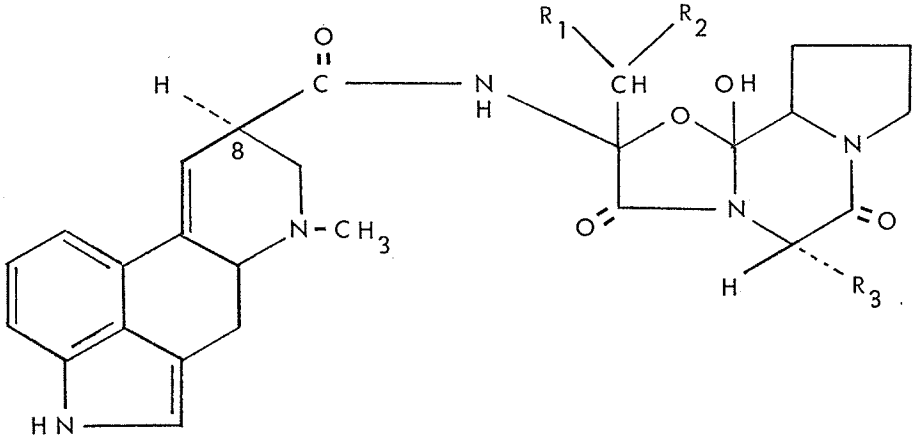


Figure 4. General Structure of Peptide Alkaloids

Ergometrine (ergobasine or ergonovine) and its isomer, ergometrinine can be classified as alkalolamine-type peptide alkaloids and possess an L-2-aminopropanol side chain (Figure 5). Some physical characteristics of these alkaloids are listed in Table 3 (5).

Table 2. Chemical and Physical Characteristics of Peptide Alkaloids (5)

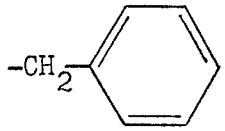
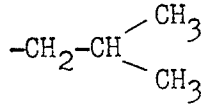
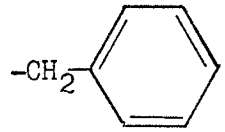
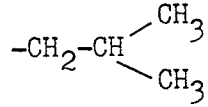
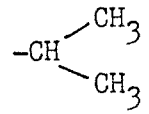
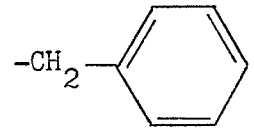
Alkaloid	R_1	R_2	R_3	Molecular Formula	Molecular Weight	α_D^{20}
Ergotamine	H	H		$C_{33}H_{35}O_5N_5$	581.7	-12.7
Ergotaminine	"	"	"	"	"	+397
Ergosine	H	H		$C_{30}H_{37}O_5N_5$	547.6	-8
Ergosinine	"	"	"	"	"	+420
Ergocristine	CH_3	CH_3		$C_{35}H_{39}O_5N_5$	609.7	-108
Ergocristinine	"	"	"	"	"	+462
Ergocryptine	CH_3	CH_3		$C_{32}H_{41}O_5N_5$	575.7	-117
Ergocryptinine	"	"	"	"	"	+479
Ergocornine	CH_3	CH_3		$C_{31}H_{39}O_5N_5$	561.7	-111
Ergocorinine	"	"	"	"	"	+488
Ergostine	H	CH_3		$C_{34}H_{37}O_5N_5$	595.7	-38
Ergostinine	"	"	"	"	"	+429

Table 3. Solubilities and Melting Points of Peptide Alkaloids (5)

Alkaloid	Crystallized from	Solubilities	M.P.[1]
Ergotamine	90% aqueous acetone	70 parts methanol, 150 parts acetone	213
Ergotaminine	hot methanol	pyridine, glacial acetic acid	242
Ergosine	ethyl acetate	slightly in methanol or acetone	225
Ergosinine	90% aqueous acetone	-----	228
Ergocristine	acetone or boiling benzene	40 parts boiling benzene	160-175
Ergocristinine	ethanol	slightly in chloroform	228
Ergocryptine	methanol or boiling benzene	slightly in ethanol or acetone	211
Ergocryptinine	hot ethanol	-----	241
Ergocornine	methanol or boiling benzene	ethanol, acetone	183
Ergocorninine	ethanol	slightly in methanol or ethanol	221
Ergostine	ethyl acetate or acetone	benzene	211
Ergostinine	boiling methanol or ethanol	benzene	215
Ergometrine	boiling ethyl acetate	water	162
Ergometrinine	acetone	-----	196

1. melting point in degrees centigrade

----- not reported

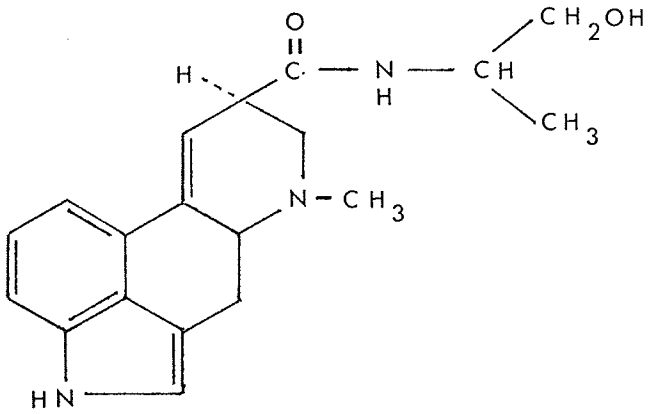


Figure 5. Structure of Ergometrine

The second main group of ergot alkaloids, the clavine alkaloids, are water-soluble ergoline derivatives which do not yield lysergic or isolysergic acid on alkaline hydrolysis and which have the general structure as illustrated in Figure 6 (5). The clavine alkaloids also

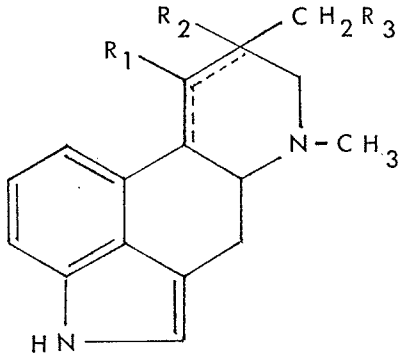


Figure 6. General Structure of Clavine Alkaloids

produce color with the van Urk reagent (p-dimethylaminobenzaldehyde), but in many cases the color so produced is not entirely the same as the deep blue produced by the peptide-type ergot alkaloids, but rather as violet-blue, purplish blue, or greenish or brownish in some

cases. Setoclavine and penniclavine, containing the C_9-C_{10} double bond conjugated with the indole ring system, show fluorescence under ultraviolet light as the peptide-type ergot alkaloids do, while the other clavine alkaloids do not fluoresce. Other physical characteristics and the substituents occurring at the R_1, R_2 and R_3 positions of the basic clavine structure are listed in Tables 4 and 5 (2, 5, 6).

The ergot alkaloids which are derived from lysergic acid and those clavine alkaloids possessing a double bond in the C_9-C_{10} position have the same characteristic ultraviolet absorption spectrum, distinguished by possessing an absorption maximum at 316-318 nanometers (nm) and a minimum at 268 nm (Figure 7). The alkaloids having a double bond at the C_8-C_9 position exhibit an absorption spectrum having an absorption maximum at 282-284 nm, another at 292 nm, and a minimum at 245 nm (Figure 7).

Table 4. Chemical and Physical Characteristics of Clavine Alkaloids (5)

Alkaloid	Constitutional Characteristics of Ring D	R_1	R_2	R_3	Molecular Formula	Molecular Weight	α_D^{20} 1
Agroclavine	C_8-C_9 Double Bond	H	H	H	$C_{16}H_{18}N_2$	283.3	-182
Elymoclavine	" "	H	H	OH	$C_{16}H_{18}ON_2$	254.3	-152
Molliclavine	" "	OH	H	OH	$C_{16}H_{18}O_2N_2$	270.3	+30
Setoclavine	C_9-C_{10} Double Bond	H	OH	H	$C_{16}H_{18}ON_2$	254.3	+174
Isosetoclavine	" "	H	OH	H	"	"	+107
Penniclavine	" "	H	OH	OH	$C_{16}H_{18}O_2N_2$	270.3	+151
Isopenniclavine	" "	H	OH	OH	"	"	+146
Festuclavine	No Double Bond in Ring D	H	H	H	$C_{16}H_{20}N_2$	240.3	-110
Pyroclavine	" "	H	H	H	"	"	-90
Costaclavine	" "	H	H	H	"	"	+44
Chanoclavine	Tricyclic - No Ring D	H	H	OH	$C_{16}H_{29}ON_2$	256.3	-240

1. in pyridine

Table 5. Solubilities and Melting Points of Clavine Alkaloids (5)

Alkaloid	Crystallized from	Solubilities	M.P.[1]
Agroclavine	acetone	water, benzene	205
Elymoclavine	boiling methanol	pyridine	245-290
Molliclavine	methanol or acetone	water	253
Setoclavine	methanol or acetone	water, ethyl acetate	229-234
Isosetoclavine	methanol	acetone	235
Penniclavine	methanol or acetone	acetone	223
Isopenniclavine	hot water	moderately in chloroform	164
Festuclavine	methanol	moderately in benzene, ether	243
Pyroclavine	benzene or methanol	moderately in chloroform, ethyl acetate	204
Costaclavine	acetone, methanol, or ethanol	moderately in chloroform, ethyl acetate	182
Chanoclavine	methanol or acetone	acetone, methanol	221

1. melting point in degrees centigrade

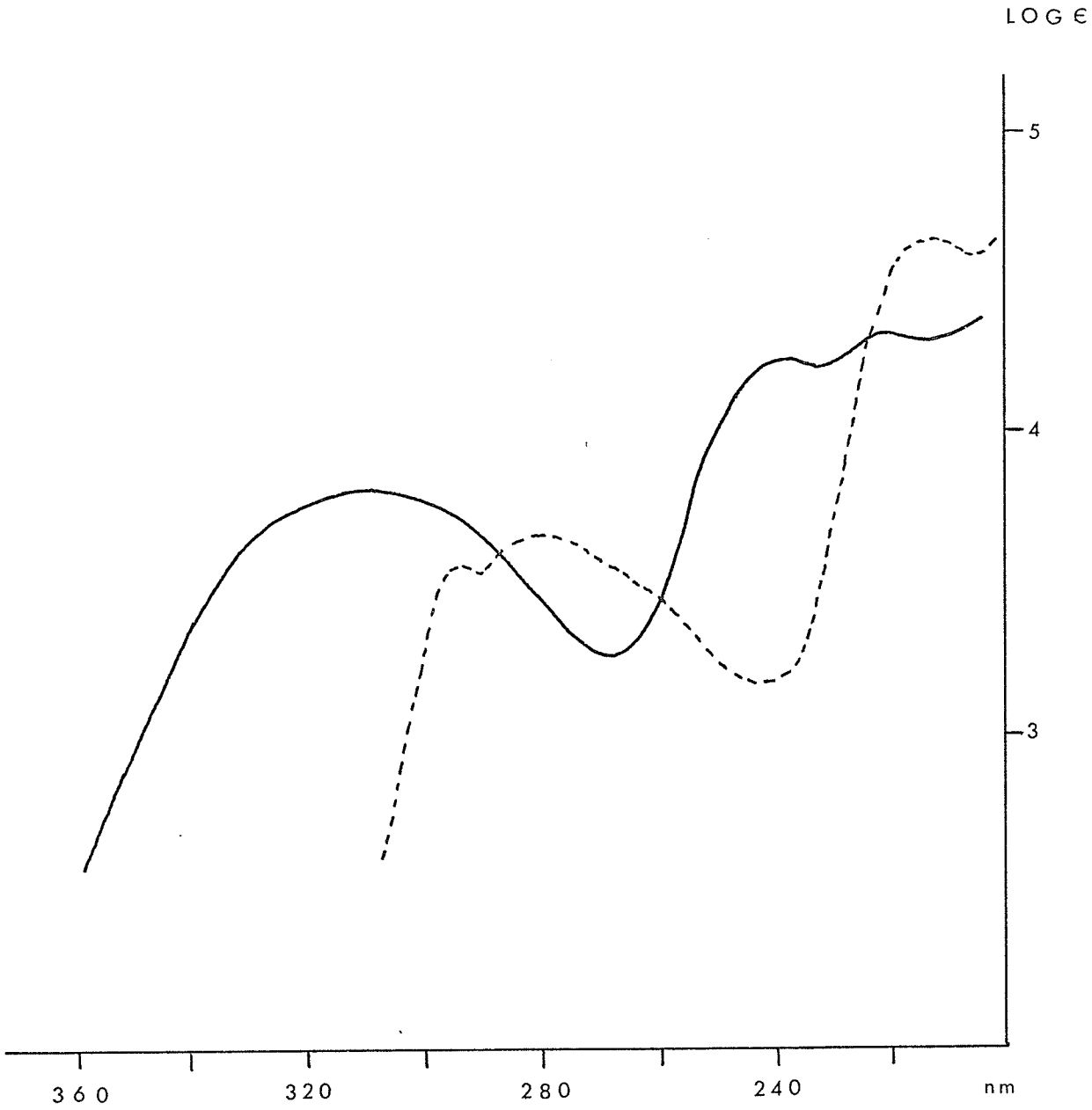


Figure 7. Ultra-violet Absorption Spectrum of Ergot Alkaloids

- - - - - - - - Alkaloids with C_8-C_9 double bond

———————— Lysergic acid derivatives and clavine alkaloids with C_9-C_{10} double bond

Triticale

Rye, wheat, barley and many other members of the Gramineae family have proven to be excellent hosts for the ergot fungus. Recently, Triticale has been tested for its susceptibility to ergot. Triticale is a man-made species derived from a cross between durum wheat (Triticum) and its distant cousin, rye (Secale) (Plate 1). It is similar to bread wheat in many respects, but has heads up to twice as large and a higher protein content. It is a plant that when compared with wheat grown under comparable conditions is similar in tillering capacity, has a considerably larger spike (thus more florets) and produces a kernel about the size of durum wheat. Theoretically, therefore, it has a potential for producing higher ergot yields than wheat (7).

The synthesis of wheat-rye hybrids is not a new development. The first report of a wheat-rye cross came from England in 1875 and although this hybrid was sterile, it was only a few years later, in 1890, that a fertile hybrid was reported from Germany (7).

Despite these early reports of success in producing the hybrid combination between these two species, it was not until the 1930's that an active interest, from an agricultural standpoint, was taken in this hybrid. Dr. Arne Muntzing, a Swedish plant breeder, foresaw the value of combining into one hybrid species the high quality characteristics of wheat with the hardy, competitive traits of rye. A number of other workers in various parts of the world have experimented with Triticale, but all have been on a rather limited scale. Today, apparently, only two large scale programs involving this species are underway - one in Hungary under the direction of Dr. Kiss and the other in Canada at the University of Manitoba. At the University of Manitoba a research team of professional

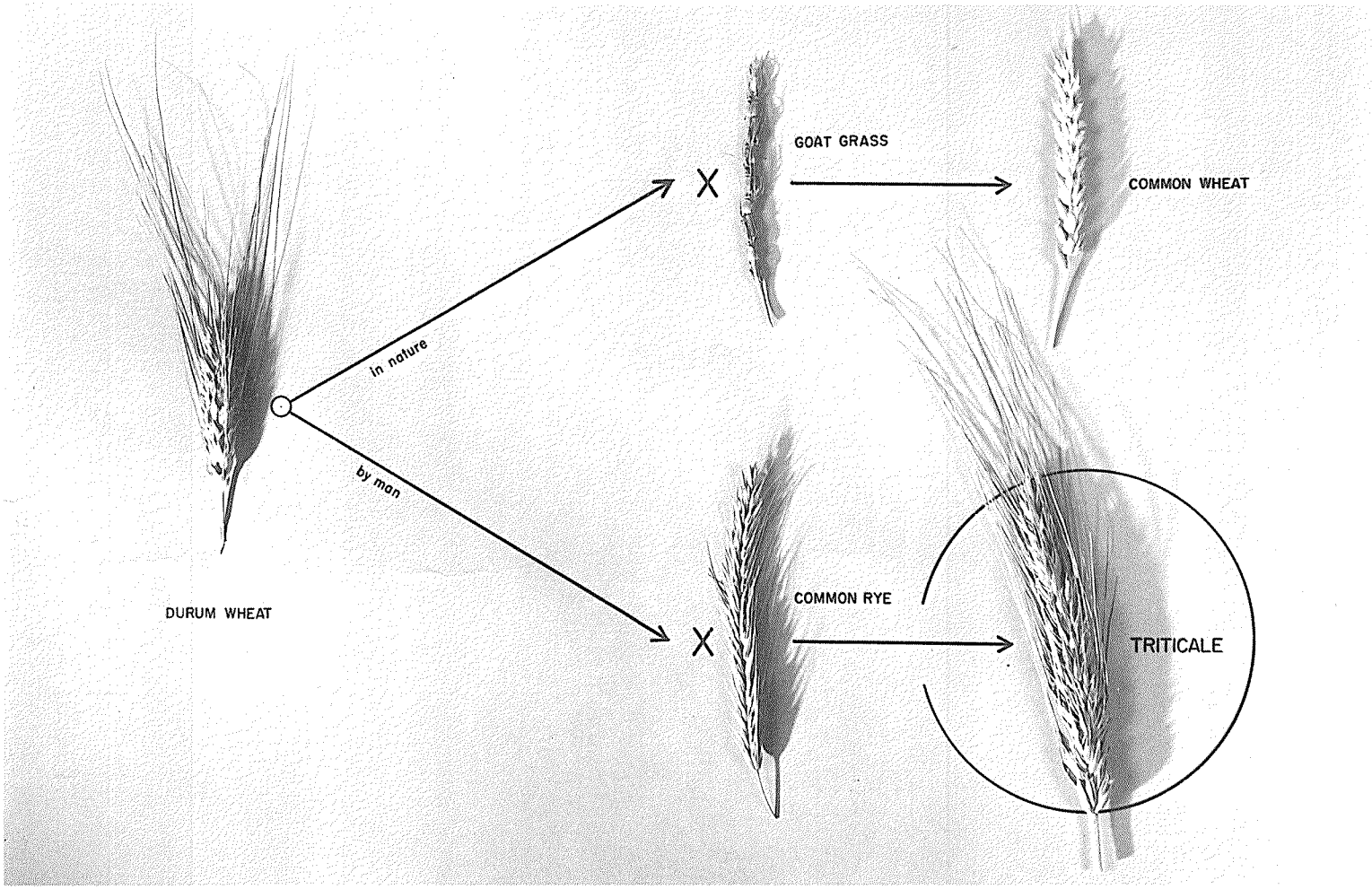


Plate 1. Wheat-rye hybridization in formation of Triticale

and technical personnel are exploiting the potential agronomic value of new interspecific and intergeneric hybrids synthesized from both wild and domesticated forms of wheat and its grass-like distant relatives (7).

Since its initiation in 1954, research with Triticale at the University of Manitoba has proceeded through three more or less distinct stages:

(1) An introduction and appraisal period from 1954 to 1959 during which an extensive number of "raw" Triticale hybrids were collected from the world over, accessioned, and evaluated under field conditions at Winnipeg.

(2) An initial hybridization phase from 1959 to 1962 during which the first intercrosses between promising amphiloids were made.

(3) A testing and improvement program which began in 1962 and continues to the present.

The initial production of new wheat-rye hybrids is beset by a number of difficulties. First, because of the distant relationship between these two species, crossability between them is low - less than 15 percent under field conditions depending upon the genotype of the parent. For example, the tetraploid wheat species (e.g. durum) are generally much more difficult to cross with rye than are the hexaploid (common) wheat species. Moreover, because the hybrid embryos that are successfully initiated are the results of wide crosses, incompatability systems inhibit their full development in vivo. In order to sustain their growth, it is necessary to excise them from their maternal tissue 10 to 15 days after fertilization and to culture them on artificial nutrient media (7).

Having successfully overcome the barrier to its early development, the young hybrid seedling possesses only a haploid set of chromosomes; a single set from each parent which because of their distant relationship do not associate (pair) during the process of gamete production. Without chromosome pairing, the hybrid is sterile (7).

The property of the chemical colchicine to double the chromosome number and thereby restore fertility is vital to the success of this program. Although no method of application devised to date approaches even a moderately high level of efficiency, at least some of the treatments are effective - each chromosome in the original hybrid cell is made to duplicate in the absence of a subsequent cycle of cell division. The result is that each new cell henceforth contains a double chromosome complement. More important, however, is that each chromosome is now in a doubled state and has a partner with which to pair - a requisite for normal sperm and egg production and fertility (7).

When Triticale becomes a crop of commerce, initially its main use will no doubt be as a feed grain. In addition, however, this new crop species will find its way into the manufacture of products for human consumption. The use of Triticale in the distilling industry has already received extensive testing on a commercial scale and results appear promising. In addition, commercial brewing trials rated Triticale high as a potential malting grain. Breakfast cereals including flakes, puffs, shredded cereals, as well as pancake flour, have all been produced by industry on an experimental basis and have been rated as very acceptable (7).

One of the problems encountered with the Manitoba Triticale however, has been its susceptibility to attack by ergot (Plate 2). A study was undertaken, therefore, at the Faculty of Pharmacy in conjunction with the Department of Plant Science to determine how this Triticale ergot compared with ergot obtained from other hosts. The ergot was examined with regard to morphological features, fat content, and alkaloidal content (quantitative and qualitative).

Plate 2. Triticale head

Ergot infected Triticale head

E X P E R I M E N T A L

I. MATERIALS

Solvents

Acetone, A.S.C. (Fischer Scientific Co.)

Ammonium Hydroxide, C.P. Reagent (C.I.L.)

Benzene, A.S.C. (Fischer Scientific Co.)

Carbon Tetrachloride, A.S.C. (Fischer Scientific Co.)

Chloroform, N.F. (Fischer Scientific Co.)

Diethylamine, Reagent (Fischer Scientific Co.)

N,N-Dimethylformamide, Reagent (Baker and Adamson)

Ethanol, 95% (Commercial Alcohols, Ltd.)

Ether, U.S.P. (Fischer Scientific Co.)

Ethyl Acetate, A.S.C. (Fischer Scientific Co.)

Hydrochloric Acid, C.P. Reagent (C.I.L.)

Methanol, A.S.C. (Fischer Scientific Co.)

Petroleum Ether, A.S.C. (b.p. 30-60 and 60-80°C) (Fischer Scientific Co.)

Sulfuric Acid, C.P. Reagent (C.I.L.)

Chemicals

Agroclavine (Aldrich Chem. Co. Inc.)

Calcium Chloride, Anhydrous (McArthur Chem. Co. Ltd.)

p-Dimethylaminobenzaldehyde (Calbiochem.)

Elymoclavine (Aldrich Chem. Co. Inc.)

Ergocristine (Pierce Chemical)

Ergometrine maleate, B.P. (British Drug Houses)

Ergotamine Tartrate (Sigma Chem. Co.)

Ergotaminine (Sigma Chem. Co.)

Ergotoxine Ethanesulfonate (Pierce Chemical)

Potassium Hydroxide, pellets, A.S.C. (Fischer Scientific Co.)

Sodium Carbonate, granular (McArthur Chem. Co. Ltd.)

Sodium Chloride, Lab. reagent (British Drug Houses)

Sodium Nitrite, U.S.P. (McArthur Chem. Co. Ltd.)

Tartaric Acid, granular, Reagent (McArthur Chem. Co. Ltd.)

Adsorbents

Aluminum Oxide G, TLC (Pleuger Chromatographic Products, Belgium)

Corn Starch (Canada Starch Co. Ltd., Montreal)

Schleicher and Schuell No. 123 Cellulose Powder, Column Chromatography (Schleicher and Schuell Co.)

Silica Gel GF, TLC (E. Merck, AG, Darmstadt)

Silica Gel, blue indicator, (analytical reagent) (Mallinckrodt Chem. Co., St. Louis)

Silicic Acid, analytical reagent (Mallinckrodt Chem. Co., St. Louis)

Whatman No. 1 Chromatography Paper (Whatman Co.)

Whatman No. 3 MM Chromatography Paper (Whatman Co.)

Instruments

Beckman IR-8 Infrared Spectrophotometer (Beckman Instruments Inc., U.S.A.)

Unicam SP600 Spectrophotometer (Unicam Instruments Ltd., Cambridge)

Varian A-56/60A NMR spectrophotometer (Varian Associates, California)

MS-9 Mass Spectrophotometer (Associated Electrical Industries)

Equipment

Chromatographic Pyrex Glass Jars, 30 x 60 cm., fitted with stainless steel rack, glass rods and trough (Canadian Laboratory Supplies)

Thomas Comminutor, Wiley Mill, Intermediate Lab. Model (A.H. Thomas Co., U.S.A.

TLC Developing Tank (Desaga, Germany)

Freeze Dryer (Lyophilizer) Model No. 10-100 (Virtis Research Equipment, U.S.A.

Fractomat Automatic Fraction Collector (Buchler Instruments, U.S.A.)

Long Wave UVL-22 (Black light lamp) (Ultraviolet Products)

Rinco Rotary Evaporator (Rinco Instrument Co., Inc. U.S.A.)

Short Wave SL-2537 (Ultraviolet lamp) (Ultraviolet Products)

TLC Spreader (Quickfit Instruments, England)

Thomas Hoover Capillary Melting Point Apparatus (A.H. Thomas Co., U.S.A.)

II. MORPHOLOGICAL EXAMINATION

The morphological features of the Triticale ergot (Rosner cultivar) are quite similar to those of rye ergot (8), except the sclerotia are somewhat shorter in length (Plate 3). The Triticale ergot measured in length from 10 mm. to more than 19 mm. (average length 13.6 mm.). The thickness ranged from 3 mm. to 6 mm. with an average thickness 3.9 mm. The average weight of a sclerotium was 128 mg.

In outline, individual sclerotia appeared lanceolate, cylindrical, angular and tapered to blunt or pointed ends. Several grains occurred twisted, but most were straight or curved. The surface consisted of deep furrows running longitudinally, some extending the entire length of the sclerotium. Transverse fissures were numerous and deep in some sclerotia, giving each a rough and scaly appearance.

The color varied from purple to purple-brown with lighter brown areas caused by deep furrows sometimes showing conspicuously. The inner areas upon fracturing showed grayish white to pink coloration.

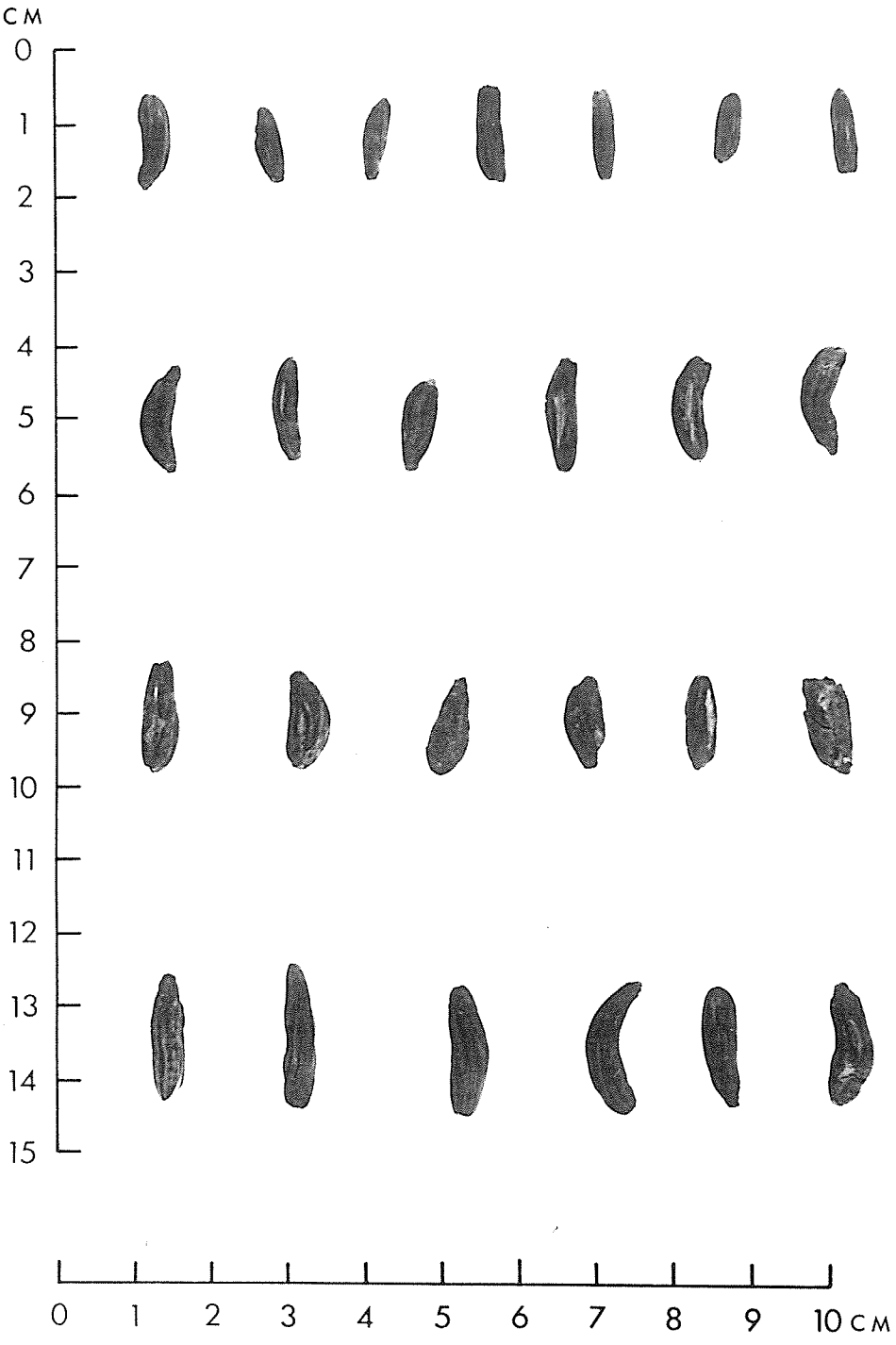


Plate 3. Typical sclerotia of Triticale ergot

III. FAT ASSAY

The ergot alkaloids are more or less soluble in organic solvents with the exception of petroleum ether in which they are practically insoluble (3). This solvent was therefore used for the removal of fats and oils from the powdered sclerotia prior to extraction of the alkaloids with ether.

The ergot sclerotia were ground in a Thomas comminutor to pass through a 0.037 inch mesh. Quadruplicate eighty gram samples of the powdered ergot were extracted by a continuous extraction in a Soxhlet apparatus with petroleum ether (b.p. 30°-60°) for 48 hours. The hot petroleum ether extract was filtered into four round-bottom flasks, each extraction apparatus rinsed with three portions of petroleum ether, added to the filtered solvent, and the solvent removed completely in vacuo. The fat samples so obtained were stored in a desiccator over anhydrous calcium chloride for 72 hours, the amount of fat extracted was determined, and the percentage total fat in the sample calculated.

These same four samples of ergot were then extracted in the same manner for an additional 48 hour period, this time using diethyl ether and the percentage of fat extracted was calculated.

Results and Discussion

The fat extractions using petroleum ether removed an average of 16.74% of the dry weight of the powdered ergot (Table 6). An

Table 6. Results of Fat Assays

Sample No.	Weight of sample (gm.)	Petroleum ether extracted fat %	Diethyl ether extracted fat %	Total fat extracted %
1.	78.31	16.21	4.37	20.58
2.	79.48	16.54	4.07	20.61
3.	75.53	16.20	4.35	20.55
4.	75.43	18.02	4.07	22.09
		average=16.74		average=20.95

additional 4.21% of fat was extracted using diethyl ether to yield a total of 20.95% of extracted fat. Fat extractions utilizing diethyl ether were found to extract some of the ergot alkaloids as well, thus all subsequent fat extractions were carried out using only petroleum ether for a period of 48 hours.

The fat content of ergot appears to depend upon a number of factors, perhaps the most important one being the nature of the host infected. The fat content as shown in Table 7 may range from as low as 9% for Couch grass ergot to 26% for rye and wheat ergots. The geographical source of a sample of ergot also seems to be an important factor in fat content. Table 7 illustrates that two samples of ergot, both grown on the same host (rye) may vary in fat content from 15 to 25% depending on the location in which it was grown (Minnesota and Nebraska, respectively). These variations in fat content may be due to many variables such as soil conditions, length of growing season, seasonal temperatures, and moisture availability (8,9).

Table 7. Comparison of Fat Content[1] of Ergot Samples from Various Hosts and Locations

Host	Geographical Origin	Fat % w/w	Reference
Secale cereale (rye)	Minnesota	25.7	8
Secale cereale	Nebraska	15.6	9
Tetraploid rye	Germany	23.6	10
Triticum durum (wheat)	Minnesota	25.8	8
Domestic hybrid[2]	Minnesota	24.8	8
Hordeum vulgare (barley)	Nebraska	15.6	9
Agropyron repens (couch grass)	Ohio	9.2-15.9	11
Bromus inermis (smooth brome)	Nebraska	13.5	12
Triticale	Manitoba	16.74	

1. All experimental results obtained by Soxhlet extraction using petroleum ether for 15-30 hours.
2. Cross between Durum wheat and giant wild rye.

Variations in the fat content of ergot may also be caused by mechanical differences such as storage conditions, the method of fat extraction, and the age of the ergot at the time of extraction (8,9). All these factors must be considered if comparisons between the fat content of different samples are to be meaningful.

According to Markwell (13), rye ergot contains 30-40% of fixed oil or fat while ergot of the wheat-rye hybrid investigated by Youngken, et al. (8) contains 25% fat. The ergot grown on Triticale ergot averaged 16% fat. This low fat content may be attributed to

environmental conditions, strain variability of the organism, or a combination of both factors. It is not necessarily a disadvantage since the fat content of ergot is closely associated with the instability of the drug (i.e. the instability of the drug is proportional to the fat content) (12).

Triticale ergot from the same source was extracted by Artes (14) in a Soxhlet apparatus for 44 hours using petroleum ether (b.p. 38-45°C). The fat extracted comprised 12.3% by weight of the dry sclerotia and a number of physical and chemical tests were carried out to better characterize the fat. The results of these tests are found in Table 8, with the characteristics of two samples from different ergot. The oil was separated into saponifiable and unsaponifiable fractions by Artes and the sample yielded 2.4% unsaponifiable matter. Thin-layer chromatography of the unsaponifiable fraction by Artes indicated the presence of ergosterol.

Table 8. Characteristics of Ergot Oil

	Triticale Ergot (14)	Ergot A[1]	Ergot B[2]
Acid Value (mgm. KOH/gm. oil)	11.8	30.6	18.5
Saponification number	193.2	220.5	184
Unsaponifiable matter	2.4%	2.6%	5.7%
Saponification equivalent	290.4	280.0	304.6
Refractive index (n_D^{25})	1.4692	---	---
Specific gravity	0.911	---	---

1. Rye host (15)

2. Rye host (16)

--- not recorded

Gas-liquid chromatography of the saponifiable fraction (as the methyl esters) revealed the presence of nine fatty acids (Table 9). The investigation revealed a somewhat larger amount of myristic and palmitic acids in the Triticale ergot than that reported in the oil of other sources. This was consistent with the fact that the oil sample was liquid at room temperature.

Table 9. Comparison of Ergot Oil Fatty Acid Composition

Fatty Acids	Triticale ergot[1]	Ergot C[2]	Ergot D[3]
	(weight % methyl esters)		
Myristic	5.29	0.1	0.85
Palmitic	29.57	19.9	23.71
Hexadecenoic	4.91	6.5	3.74
Stearic	8.41	4.3	3.15
Oleic	23.08	22.5	20.71
Linoleic	15.87	14.3	12.18
Arachidic	1.58	trace	0.91
Linolenic	0.67	trace	---
Ricinoleic	8.85	32.3	33.92

1. Triticale ergot (14) - analysed by GLC
2. Claviceps paspali ergot (17) - analysed by TLC
3. Rye ergot (18) - analysed by GLC

--- not recorded

IV. ALKALOIDAL ASSAY

Alkaloid determinations were carried out according to the method described by Sim (6). Four 200 gm. samples of powdered ergot were first defatted as previously described and then made basic to a pH 10-11 by formation of a paste using sodium carbonate and distilled water. The paste was then spread over a uniform thickness and allowed to air dry. The dry powder was reground to pass through a 0.037 inch sieve and then extracted by means of a continuous extraction in a Soxhlet apparatus using diethyl ether. The extraction was continued until exhaustion of the alkaloids from the sample (approximately 36 hours).

The ether extract was removed after extraction was complete, the apparatus washed with three successive portions of ether and concentrated to approximately 200 ml. *in vacuo*. The concentrated ether extract was then transferred to a 500 ml. separatory funnel and shaken with six successive 75 ml. portions of a 2% tartaric acid solution. The aqueous solution containing the alkaloid tartrates was then diluted to 500 ml. in a volumetric flask. This solution was then assayed to obtain the total alkaloid content of the ergot sample (Figure 8).

The commonly used colorimetric assay procedure involves reaction of the ergot alkaloids with *p*-dimethylaminobenzaldehyde in the presence of sulfuric acid and a mild oxidizing agent (sodium nitrite). This reaction was first described by van Urk (19) as a means of detecting indole compounds, and later modified and improved for use as a quantitative reagent (20,21,22). The reagent used was prepared

according to Stahl (23), by dissolving 100 mg. of p-dimethylaminobenzaldehyde in a mixture of 50 ml. of sulphuric acid and 50 ml. of distilled water.

Suitable aliquots (0.1 ml.) of the 2% tartaric acid solution were diluted to 2 ml. with distilled water, and each sample mixed with 2 ml. of the freshly prepared van Urk reagent. After 15 minutes, 0.1 ml. of a freshly prepared 0.1% solution of sodium nitrite was added to each sample and the blue color developed after 5 minutes was measured for optical density in a Unicam SP600 spectrophotometer at a wavelength of 590 nm.

A reference graph was prepared using United States Pharmacopoeia (USP) reference standard ergometrine maleate in concentrations ranging from 2-100 µg/ml. in distilled water. The same procedure used in the assay was followed in determining the values for the reference graph. The optical density readings of the unknown solution were then compared to those given by the standard graph, and the quantity of total alkaloids in the ergot sample was calculated from the data obtained.

For the estimation of the water soluble alkaloids, the water insoluble alkaloids were first removed from the tartaric acid solution containing the total alkaloids by making the solution alkaline with ammonium hydroxide and extracting the water insoluble alkaloids using six successive 100 ml. portions of carbon tetrachloride (6). The remaining aqueous solution (containing the water soluble alkaloids) was then saturated with sodium chloride and extracted with eight successive 75 ml. portions of diethyl ether. The ether extract was then re-extracted using eight successive 50 ml. portions of a 2% tartaric acid solution

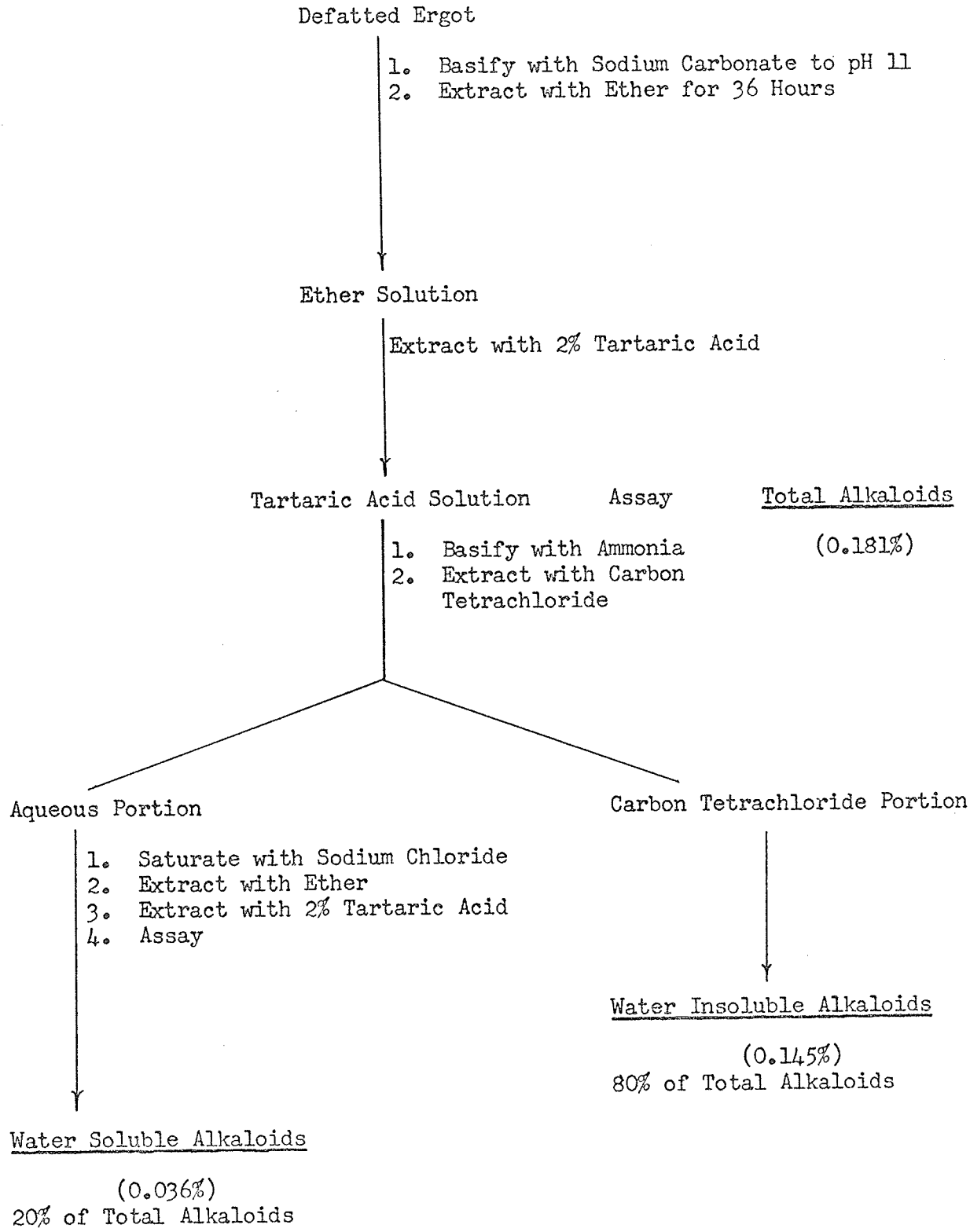


Figure 8. Procedure for Alkaloid Assay

and this solution made up to 500 ml. in a volumetric flask. Suitable aliquots (one ml.) of this tartaric acid solution were then treated as previously described for total alkaloids and the quantity of water soluble alkaloids determined (Figure 8).

The quantity of water insoluble alkaloids in the sample was then determined by subtracting the quantity of water soluble alkaloids present from the total alkaloids in the sample.

Results and Discussion

The quantitative alkaloid content of a particular sample of ergot will be dependent upon variable conditions such as climate, length of growing season, storage conditions employed, age of the sample at time of assay, moisture content and fat content (both increase alkaloid deterioration), and the methods employed in the actual assay (8,12).

Meinicke (24) reported on the alkaloid content of ergot produced by inoculation of various wild grasses with a specific strain of Claviceps purpurea. The results of his experiments indicate that the host may influence the alkaloid content of the parasite in a quantitative manner only. It is apparent therefore, that quantitative reports of the alkaloid content of ergot sclerotia formed on different grasses are not comparable, one with another, unless it is known that both hosts were infected with the identical strain of the fungus.

As shown in Table 10, of ergot grown in the United States, ergot of wheat may contain only 0.06% total alkaloids while ergot of rye may have 0.35% and barley 0.54%. Even with ergot grown on the

Table 10. Comparison of Alkaloid Content of Ergot on Different Hosts and from Different Locations

Host	Geographical Source	1	2	3	4	Reference
Rye	U.S.A.	0.686	0.63	0.045	6.8	9
"	U.S.S.R.	0.47	0.37	0.10	21.0	25
"	Poland	0.019	0.015	0.004	5.3	26
"	Germany	0.003	0.002	0.001	33.0	27
"	France	0.045	0.04	0.005		28
" (wild)	U.S.S.R.	0.05			4-14	29
" (cultivated)	U.S.S.R.	0.40			4-14	29
"	India	0.12	0.117	0.024	20	30
"	Sweden	0.015				31
" (normal diploid)	Germany	0.028				10
" (tetraploid)	Germany	0.035				10
Wheat	U.S.A.	0.058				8
"	U.S.S.R.	0.15				8
Barley	U.S.A.	0.542	0.515	0.027	4.9	9
Couch Grass	U.S.A.	0.202	0.198	0.004	4.9	11
Smooth Brome	U.S.A.	0.297	0.111	0.026	8.7	12
Rye-Wheat hybrid	U.S.A.	0.109				8
Triticale	Manitoba	0.181	0.145	0.036	20	

1. % w/w total alkaloids

2. % w/w water insoluble alkaloids

3. % w/w water soluble alkaloids

4. water soluble alkaloids as % of total alkaloids

same host differences in total alkaloid content may occur depending on the geographical source of the sample. For example, in Table 10, Polish ergot of rye yielded only 0.02% total alkaloids while Russian ergot yielded 0.47% and American 0.68%.

The ergot grown on Triticale in Manitoba was found experimentally to contain an average of 0.181% total alkaloids. This figure was below the minimum requirement for ergot set by the British Pharmacopoeia, 1948, which states that ergot must contain no less than 0.2% total alkaloids. The alkaloid yield was, however, above the requirements of the National Formulary XI and the International Pharmacopoeia. Both sources state that ergot must contain not less than 0.15% total alkaloids. The alkaloid yield from Triticale compared favourably with results obtained by Youngken et al. (8). They obtained a 0.109% yield from a rye-wheat hybrid in the United States.

The variation in the percentage of water soluble alkaloids in samples from different sources is demonstrated in Table 10. The water soluble alkaloid fraction may constitute from 5-33% of the total ergot alkaloids in a sample of ergot. The Triticale ergot was assayed and found to contain approximately 20% of water soluble alkaloids. This figure is above the minimum requirements set by the British Pharmacopoeia 1948, the National Formulary XI, and the International Pharmacopoeia which are 15%, 6.6%, and 15.3%, respectively.

V. COLUMN CHROMATOGRAPHY

A column for the separation of the crude alkaloid mixture extracted from the ergot sample was prepared and developed according to the method used by Voigt and Kaehler (32).

Powdered cellulose No. 123 (Schleicher and Schuell) was impregnated with a 0.2% tartaric acid solution, pressed down between filter paper and spread out to dry at a temperature of 60°C. The readily crumbled, agglomerated mass was then powdered by grinding in a porcelain mortar and pestle and sifted through a screen (mesh 120, aperture 0.0049 inch) prior to use (32).

A cylindrical glass column 60 cm. long and 4.5 cm. in diameter, the upper end of which contained a bulbous one litre solvent reservoir, was used. The lower end possessed a teflon stopcock and was fitted with a plug of cotton wool. A wet slurry was made with the impregnated cellulose using the solvent system diethyl ether/acetone/water (2:4:2) (E / A / W), and the column was packed by successive additions of the slurry until a column free from air bubbles was obtained. The first litre of eluted brownish colored liquid was rejected and the column allowed to settle overnight with a head of solvent in the reservoir (32).

The basic free alkaloid residue (1.5 gm.) obtained from previous extractions was dissolved in a minimum volume of the solvent E / A / W (2:4:2) and sufficient tartaric acid impregnated cellulose added to make a stiff paste. The solvent was removed in vacuo and the dry powder was added to the top of the column. This layer was compressed and a round piece of filter paper cut to fit the internal diameter of the column

was placed on top, followed by a layer of small glass beads 1/4 inch thick (32).

The development of the column was completed in about 10-12 hours using the solvent system previously described (E / A / W 2:4:2). With the aid of ultraviolet lamps the fluorescent bands of alkaloids could be followed as they moved down the column. When the column was fully developed it appeared to have several ultraviolet fluorescing and one absorbing band (Figure 9) (32).

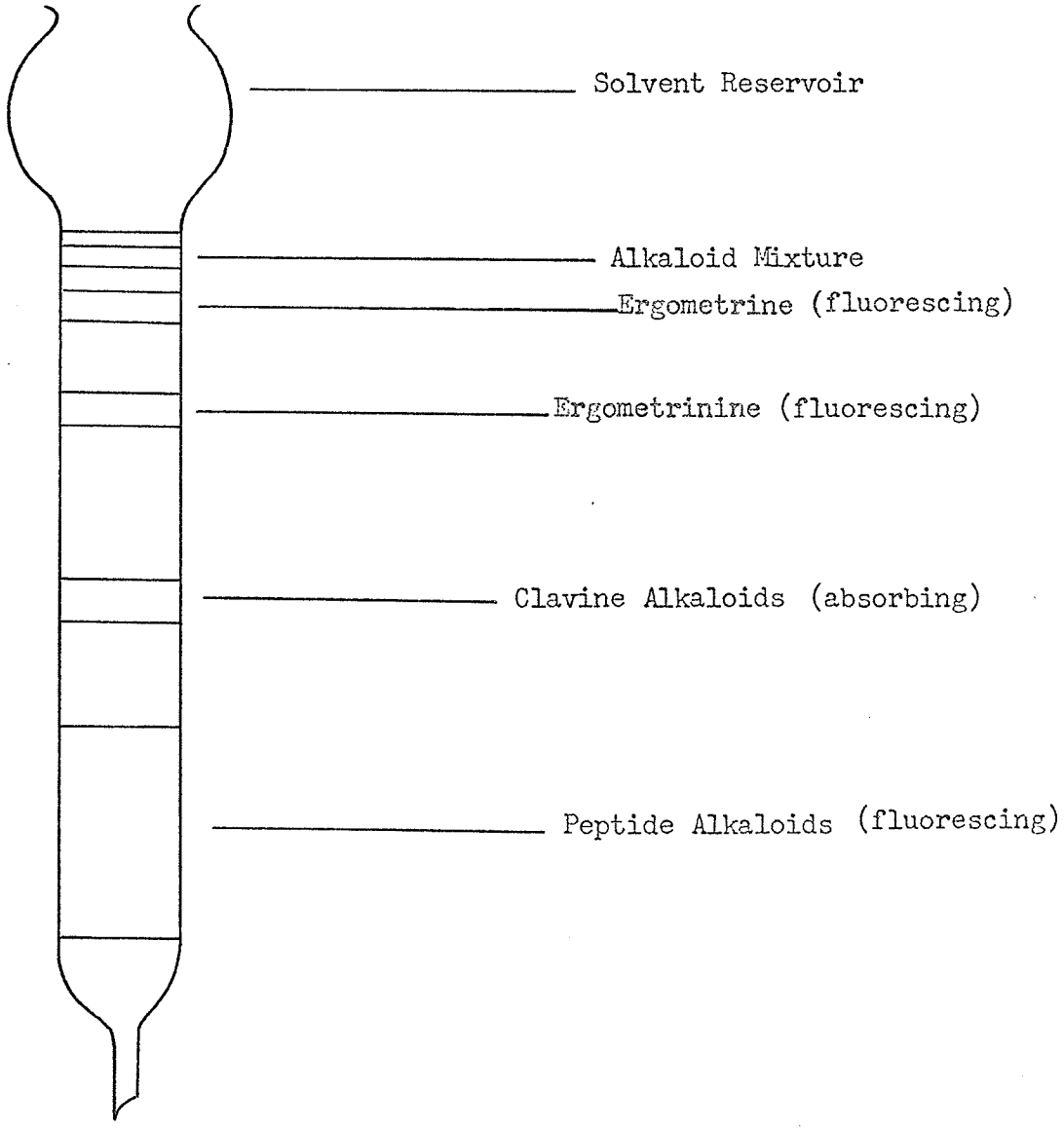


Figure 9. Development of Cellulose Column

After development of the column was completed, fractions of 10 ml. each with an elution time of 6-7 minutes were collected using an automatic fraction collector. Using the ether/acetone/water (2:4:2) system as elution solvent the bands were eluted in the following order:

1. peptide alkaloids
2. clavine alkaloids
3. ergometrinine
4. ergometrine

After separation of the peptide and clavine fractions rapid elimination of ergometrine from the column was accomplished by the addition of 10% aqueous ammonia (one drop in each 100 ml.) to the elution solvent (32).

Every second fraction removed from the column was assayed for alkaloid content using the van Urk's reagent previously described and the optical density readings obtained using the Unicam SP 600 spectrophotometer. The tube number versus alkaloid content per tube (in mg.) was plotted and the results interpreted (Figure 10).

Results and Discussion

The column procedure proved to be very successful in the separation of the groups of ergot alkaloids. According to the results illustrated on the graph (Figure 10) the tubes were divided into suitable fractions as shown in Table 11.

The alkaloids were eluted from the column beginning with tube 66. The first fluorescent band was eluted from tubes 67 through to 100 having a heavy concentration of alkaloids. This fraction accounted for

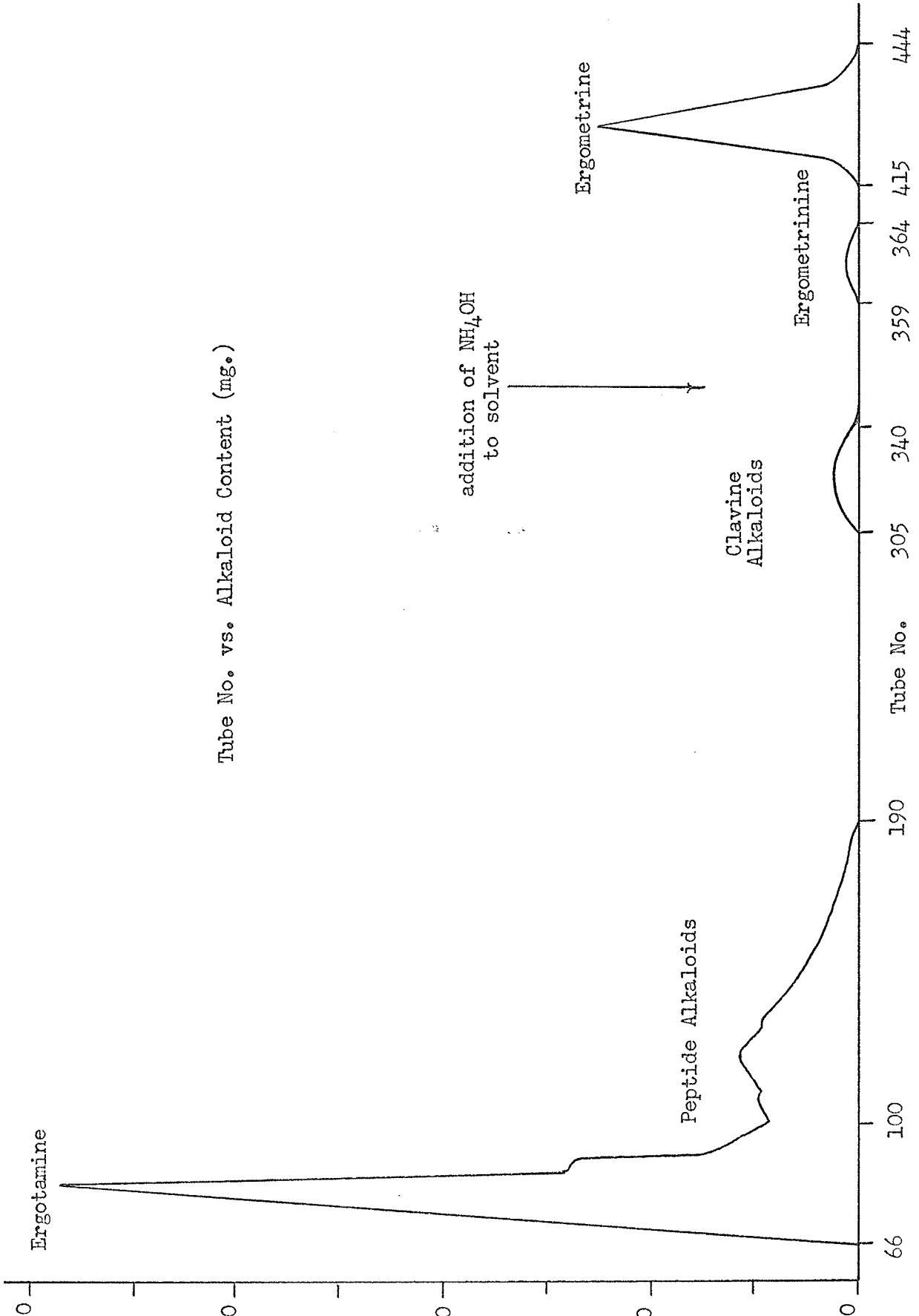


Figure 10. Elution of Alkaloids from Cellulose Column

Table 11. Fractions Collected from Cellulose Column

Tube No.	Fraction
1 - 66	Blank
67 - 100	Ergotamine
101 - 190	Peptide Alkaloids
191 - 304	Blank
305 - 340	Clavine Alkaloids
341 - 358	Blank
359 - 364	Ergometrinine
365 - 414	Blank
415 - 444	Ergometrine
445 - 460	Blank

approximately 760 mg. of alkaloids, (68% of total alkaloids). A second band was eluted in tubes 101 - 190 and accounted for a further 320 mg. of alkaloids (13% of total alkaloids). These first two bands were later shown to contain the peptide alkaloids.

Tubes 191 - 304 were devoid of alkaloids. Tubes 305 - 340 produced positive results with van Urk reagent but they were so slight that these tubes were combined into a single fraction, concentrated under reduced pressure, and assayed again. The fraction accounted for about 25-30 mg. of alkaloids (3% of total alkaloids) and was later shown to contain the clavine alkaloids.

Tubes 341 - 358 were devoid of alkaloids, but tubes 359 - 364 exhibited a slight positive reaction with van Urk reagent. According

to Voigt and Kaehler (32) ergometrinine should be eluted from the column at this point, but the quantity present was not sufficient to allow further identification by thin-layer chromatography.

Tubes 365 - 414 were devoid of alkaloids. The band which fluoresced with the highest intensity under ultraviolet light (short wave) was eluted in tubes 415 - 444 after an elution time of 8-10 hours. This fraction accounted for some 200 mg. of alkaloids (16% of total alkaloids) and was later shown to contain ergometrine. The column development was continued for another 2 hours, but tubes 445 - 460 were devoid of alkaloids. At this point the development of the column was stopped.

The assay results accounted for about 1.13 gm. of the 1.5 gm. of crude alkaloid mixture applied to the column. Of this quantity, 1.08 gm., or 81% appeared to be peptide alkaloids, while the clavine alkaloids (0.025-0.030 gm.) and ergometrine (0.20 gm.) made up the remaining 19%. The results obtained from the column separation of the alkaloids compared favourably with the results obtained in the alkaloid assay of the ergot sample, i.e. colorimetric assay of the sample determined the water soluble alkaloids to constitute 20% of total alkaloids; the peptide alkaloids constituting the remaining 80%.

VI. THIN-LAYER CHROMATOGRAPHY

Preparation of Adsorbents

1. Silica Gel GF plates

Glass plates (20 cm. x 20 cm.) were covered with a 0.25 mm. thick layer of silica gel GF for TLC (E. Merck, AG, Darmstadt) by spreading a well stirred mixture of 30 gm. of the adsorbent and 60 ml. of distilled water with a TLC spreader. After drying in air for one hour the plates were activated at 110°C for 30 minutes, then stored in a desiccator over silica gel for not more than two days prior to use.

2. Aluminum Oxide G plates

Plates made using this adsorbent were prepared in the same manner previously described for the silica gel GF plates.

3. Silicic acid plates

Silicic acid was used to make "alkaline" TLC plates which gave excellent separation of the clavine alkaloids. Silicic acid (25 gm.) which had been passed through a No. 100 sieve was stirred with 80 ml. of a 1% solution of potassium hydroxide (KOH). To this suspension was added 1.3 gm. of corn starch in 10 ml. of a 1% KOH solution. This mixture was heated at 70°C for 15 minutes with vigorous stirring. During the heating an additional 10-20 ml. of the 1% KOH solution was added to produce a mixture which could be readily poured.

The suspension was then allowed to cool slightly and spread over the surface of six 10 x 20 cm. glass plates to make a 0.25 mm. thin-layer. The plates were dried in an oven for two hours at a temperature not exceeding 75°C and stored in a desiccator until used (33).

Preparation of van Urk Spray Reagent

The spray reagent used was a modified van Urk's reagent. It was freshly prepared by dissolving 100 mg. of p-dimethylaminobenzaldehyde in a mixture of 50 ml. of hydrochloric acid and 50 ml. of ethanol (23).

All solvents were mixed immediately before use and 100 ml. of the solvent mixture to be used was poured into the bottom of the glass TLC tanks which were previously lined with Whatman No. 1 filter paper. The tanks were shaken, and in this way allowed to equilibrate for one hour at room temperature before the plates were quickly inserted.

Reference alkaloids in their base form were dissolved in suitable organic solvents (methanol or chloroform/isopropanol (3:1)) to contain approximately 0.5-0.7 µg. of alkaloid in the applied 2-3 µl. of solvent and were applied 1.5 cm. from the lower edge of the plate.

Basic TLC Separation of Column Fractions

An aliquot of the solution in every second tube collected from the column was run in a TLC system used in the general separation of the ergot alkaloids. This system consisted of methanol/chloroform (2:8) and had previously been used for the separation of ergot alkaloids

(34, 35, 36). As a rule this system was used as the basic system since all the alkaloids were uniformaly distributed in it. The contents of the tubes proven by TLC in this system to possess the same alkaloids were combined into fractions A to J as shown in Table 12.

Table 12. Fractions Combined from Tartaric Acid Impregnated Cellulose Column

Tube No.	Fraction	Alkaloids[1]
1-66	A	-
67-100	B	+
101-190	C	+
191-304	D	-
305-340	E	+
341-358	F	-
359-364	G	+
365-414	H	-
415-444	I	+
445-460	J	-

1. Alkaloids = + Positive with van Urk's reagent
- Negative with van Urk's reagent

The TLC results obtained with each fraction run in the methanol/chloroform (2:8) system are illustrated in Figure 11. Using this TLC system fractions A, D, F, H and J were found to be devoid of alkaloids and were therefore discarded. Fraction B chromatographed as a single spot with an R_f value of 0.66 and ran uniformly with the reference ergotamine. In fraction C a spot

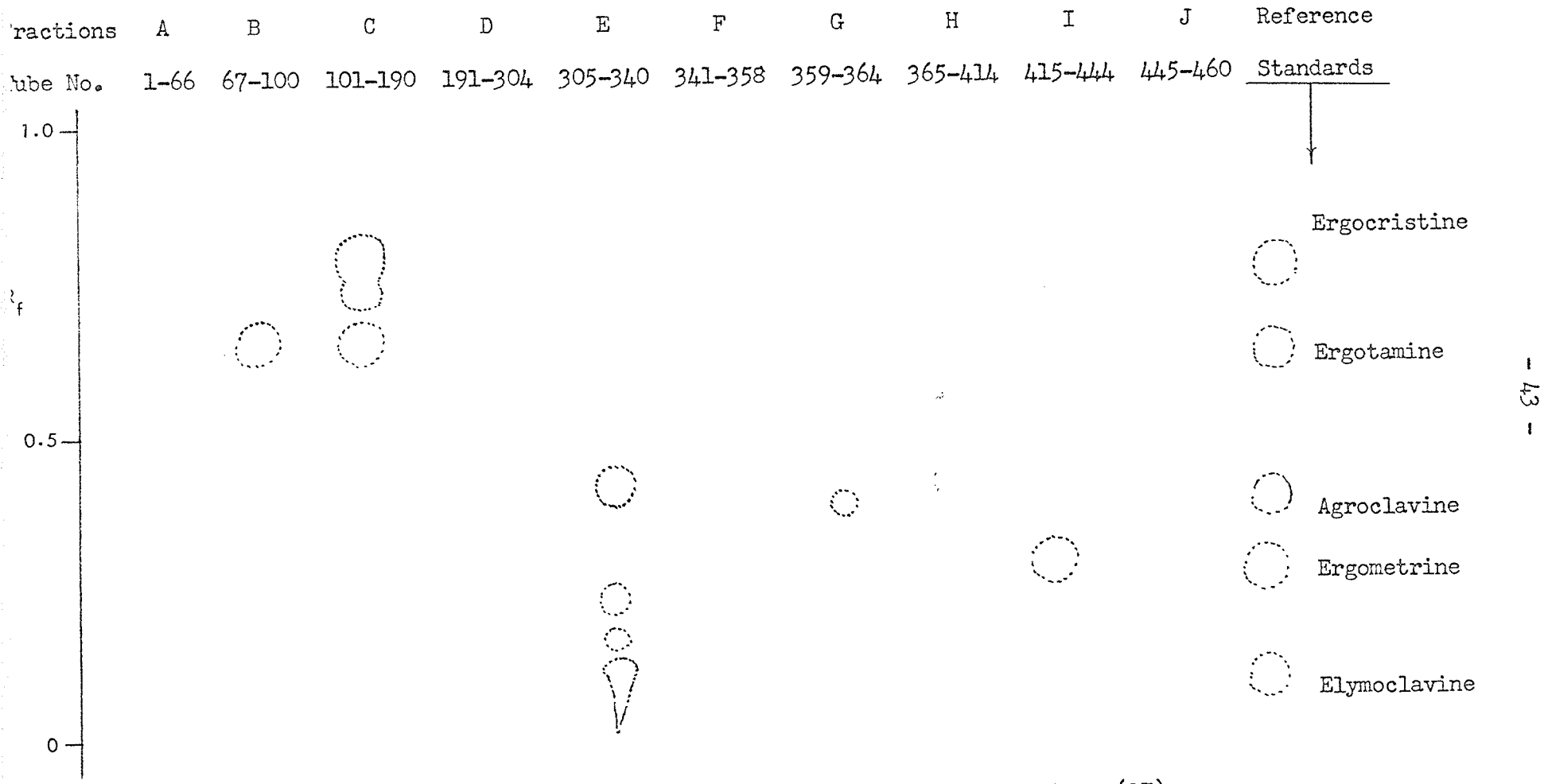


Figure 11. Basic TLC of Cellulose Column Fractions (37)

Solvent System : methanol/chloroform (2:8)

Adsorbent : silica gel GF

appeared with an R_f value of 0.76 which corresponded to the R_f of the reference ergocristine. Fraction E, subsequently proven to contain the clavine alkaloids gave evidence of three or four spots but no definite conclusions could be drawn since this system did not give a good separation of the clavine alkaloids. Although fraction G produced a slightly positive reaction with the van Urk's reagent, subsequent TLC of the fraction did not produce any definite identification. Fraction I showed one spot on TLC in this system, having an R_f value of 0.30 and running uniformly with the reference ergometrine.

Table 13 compares the R_f x 100 values of the ergot alkaloids reported in literature with those found experimentally. Although the methanol/chloroform (2:8) system was very effective in separating groups of alkaloids from one another it was not as effective in separating individual alkaloids in each group.

Thus preliminary TLC of the fractions showed that the column system employed was effective in separating the peptide alkaloids, the clavine alkaloids, and ergometrine as described by Voigt and Kaehler (32). The crude alkaloid mixture before purification on the column showed several spots when chromatographed in this system. The individual fractions were separated using TLC systems which proved most effective. The solvent systems employed were chosen from those reported in the literature as being the most effective systems.

TLC of Fraction B

The solvent systems and adsorbents chosen for the TLC of fraction B are outlined in Table 14 with the results obtained. Fraction B chromatographed as a single spot in the five systems tested.

Table 13. R_f x 100 Values of Ergot Alkaloids in Methanol/Chloroform (2:8) on Silica Gel GF

Alkaloid	Reported R_f x 100 (37)	Experimental R_f x 100
Ergocristinine	83	
Ergocorninine	82	
Ergostinine	81	
Ergocryptinine	81	
Ergotaminine	78	73
Ergosinine	76	
Ergocornine	75	
Ergocristine	75	74
Ergocryptine	74	
Ergostine	72	
Ergotamine	65	64
Ergosine	63	
Setoclavine	48	
Isopenniclavine	41	
Agroclavine	39	41
Ergometrinine	39	
Ergometrine	27	30
Festuclavine	23	
Penniclavine	21	
Pyroclavine	20	
Elymoclavine	17	17
Costaclavine	05	
Chanoclavine	05	

Table 14. TLC Results of Fraction B Obtained from Cellulose Column

Solvent System ⟶	1			2			3			4			5		
R_f x 100 ⟶ Alkaloids ↓	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
Ergotaminine	78	73		90	89		60	61		72	70		71	70	
Ergocristine	75	74		87	88		47	45		75	64		72	70	
Ergotamine	65	64	63*	75	76	78	24	23	24*	54	54	59*	57	59	60
Ergometrine	27	30		40	40		08	05		30	31		21	20	

1. methanol/chloroform (2:8); silica gel GF (37)
2. methanol/chloroform/conc. ammonia (20:80:0.2); silica gel GF (37)
3. benzene/N,N-dimethylformamide (13:2); silica gel GF (38)
4. chloroform/ethanol/acetone (6:4:4); silica gel GF (39)
5. ethanol/chloroform (4:96); aluminum oxide G(37)

a. reported R_f x 100

b. R_f x 100 of reference alkaloids found experimentally

c. R_f x 100 of alkaloids in fraction B

* also co-chromatographed with reference ergotamine

There was no evidence of more than one component in fraction B. The fraction was co-chromatographed with reference ergotamine and ran as a single spot in systems 1, 3 and 4 (Table 14). Fraction B was stored at -20°C prior to subsequent analysis.

TLC of Fraction C

Preliminary TLC of fraction C after removal from the column, using the methanol/chloroform (2:8) system, showed two spots, both of approximately equal color intensities when sprayed with van Urk reagent. One spot had an Rf value corresponding to that of reference ergotamine (0.65) and the second spot had a higher R_f of 0.78, which could possibly have been the ergotoxine group of alkaloids (ergocornine, ergocristine, and ergocryptine). In some trials, the R_f 0.78 spot spread out into what appeared to be heavy tailing and the possibility of a third component was noted. Fraction C was therefore run in an

Table 15. TLC Results of Fraction C Obtained from Cellulose Column

Solvent System ⟶	1		2	
R_f x 100 Values ⟶	a	b	a	b
Alkaloids ↓				
Ergometrine	27	0*	0*	0*
Ergotamine	65	67	31	35
Ergocristine	75	74	56	60
Ergotaminine	78	74	64	66

1. methanol/chloroform (2:8); silica gel GF (37)

2. benzene/N,N-dimethylformamide (13:2); silica gel GF (38)

a. R_f x 100 of reference alkaloids

b. R_f x 100 of alkaloids in fraction C

* remains at origin

additional solvent system to see if any further separation of the spots could be obtained. The results obtained using both the systems are outlined in Table 15.

The benzene/dimethylformamide system proved very effective in the separation of fraction C. What appeared to be two compounds in the methanol/chloroform system separated into three individual spots using the benzene/dimethylformamide system. This system demonstrated the presence of three alkaloids or alkaloid groups, corresponding to ergotamine, ergotaminine, and ergocristine reference compounds.

TLC of Fraction E

The four solvent systems used in the TLC separation of fraction E are outlined in Table 16. The first system, employing ethyl acetate/ethanol/dimethylformamide (EED) was especially suited for the separation of the clavine alkaloids and gave the best results. The silicic acid plates however, were difficult and time consuming to prepare, did not always spread evenly, and uniform results were difficult to obtain. The long development time (3 hours) was yet another disadvantage to this system. Five individual spots were obtained when fraction E was chromatographed in this system. Four of the spots had R_f values which corresponded to penniclavine, elymoclavine, agroclavine and setoclavine reference compounds. The fifth spot was barely visible in most cases, but when present had an R_f value corresponding to that of isopenniclavine.

Table 16. TLC Results of Fraction E Obtained from Cellulose Column

Solvent System ⟶	1		2		3		4	
R_f x 100 ⟶	a	b	a	b	a	b	a	b
Alkaloids ↓								
Penniclavine	46	44*	05	02*	07	10	35	35*
Isopenniclavine	22	20	08		10		56	59
Ergometrine	37		21		02		40	
Elymoclavine	29	28*	29	29*	11	13	34	37*
Setoclavine	84	84*	56	54*	31	33	66	65*
Isosetoclavine			60		38		68	
Festuclavine			60		48		51	
Agroclavine	74	72*	63	62*	51	53	67	70*
Pyroclavine			68		59		63	

1. ethyl acetate/ethanol/N,N-dimethylformamide (13:1:1); silicic acid (33)
2. ethanol/chloroform (4:96); aluminum oxide G (37)
3. diethylamine/chloroform (1:9); silica gel GF (37)
4. methanol/chloroform/conc. ammonia (20:80:0.2); silica gel GF (37)

a. R_f x 100 of reference alkaloids

b. R_f x 100 of alkaloids in fraction E

* also co-chromatographed with reference alkaloids

The second system (ethanol/chloroform) separated fraction E into four definite spots, three having R_f values similar to agroclavine, setoclavine and elymoclavine reference compounds. The fourth spot had an R_f value similar to the penniclavine reference. The presence of a fifth alkaloid in this system could not be detected, probably because penniclavine and isopenniclavine ran as one spot.

The third system used (dimethylamine/chloroform) separated fraction E into three spots, two having R_f values corresponding to the setoclavine and agroclavine reference compounds. The third spot exhibited tailing and was thought to consist of more than one compound. The system was not very effective in the separation of penniclavine, isopenniclavine and elymoclavine.

The fourth system (methanol/chloroform/conc. ammonia) proved very effective in the separation of the clavine fraction. Using this system, four spots were observed in fraction E, and their R_f values corresponded to the penniclavine, isopenniclavine, setoclavine and agroclavine reference standards. The penniclavine and elymoclavine reference compounds ran as a single spot in this system.

According to the results obtained with these four TLC systems, the clavine fraction appeared to consist of four or possibly five alkaloids: agroclavine, elymoclavine, penniclavine, setoclavine, and isopenniclavine. Of these alkaloids, agroclavine appeared to be present in the largest concentration judging from the color intensity of the spots after spraying with van Urk's reagent. In two of the systems there appeared to be some material which remained at the origin and gave a faintly positive result when sprayed with van Urk's spray reagent.

The presence of these alkaloids in fraction E was confirmed by co-chromatography of fraction E in systems 1, 2 and 4 (Table 16) with reference penniclavine, setoclavine, agroclavine, and elymoclavine.

GLC of Fraction E

Analysis of fraction E by gas chromatography was attempted using a Beckman GC-5 Gas Chromatograph. A 0.5% Amine 220 Chromosorb G Hp AW - DMCS column was employed with a column temperature of 165°C. Results were unsuccessful and further identification of the alkaloids in fraction E was attempted using paper chromatography (page 54).

TLC of Fraction I

From the preliminary TLC of fraction I in the methanol/chloroform system, it was thought to contain ergometrine. Four systems previously used were chosen for their ability to separate ergometrine from the other alkaloids and fraction I was chromatographed in these systems to determine whether it contained a single alkaloid. The results are listed in Table 17.

Fraction I behaved as a single compound in the four systems in which it was chromatographed. Fraction I was co-chromatographed in systems, 1, 2 and 3 (Table 17), with reference ergometrine and ran as a single spot in these systems. Fraction I was stored at -20°C until required for further study.

Table 17. TLC Results of Fraction I Obtained from Cellulose Column

Solvent System ⟶	1		2		3		4	
R_f x 100 ⟶	a	b	a	b	a	b	a	b
Alkaloids ↓								
Elymoclavine	17		34		29		29	
Ergometrine	27	29*	40	45*	21	19*	37	41
Agroclavine	39		67		63		74	
Ergotamine	65		75		57		49	

1. methanol/chloroform (2:8); silica gel GF (37)
2. methanol/chloroform/conc. ammonia; silica gel GF (37)
3. ethanol/chloroform (4:96); aluminum oxide G (37)
4. ethyl acetate/ethanol/N,N-dimethylformamide (13:1:1); silicic acid (33)

a. R_f x 100 values of reference alkaloids

b. R_f x 100 values of fraction I

* also co-chromatographed with reference ergometrine

VII. PAPER CHROMATOGRAPHY

A number of solvent systems have been devised for the paper chromatography of the ergot alkaloids. As the constituents of fractions C and E were not separated or identified satisfactorily on TLC it was the thought that perhaps paper chromatography of these fractions would aid in their separation and identification. Two solvent systems were chosen, one for the separation of the water-insoluble alkaloids in fraction C, and the second for the separation of the water-soluble alkaloids in fraction E.

Formamide/Benzene/Petroleum Ether (FBPE) System

The FBPE system (40, 41) was reported to be useful for separating the water insoluble peptide-type ergot alkaloids. Whatman No. 1 filter paper was dipped in a formamide/absolute alcohol (1:1) solution and blotted before the alkaloids were applied. The alkaloids were applied in their base form dissolved in chloroform/isopropanol (3:1). The chromatograph was developed by the descending technique over a 10-12 hour period using benzene/petroleum ether (b.p. 60-80°C) 4:1 as the developing solvent.

The alkaloid mixture from fraction C either remained at the origin or was streaked along the entire length of the paper.

Formamide/Benzene/Pyridine (FBP) System

This system has been frequently reported for the separation of the water-soluble clavine-type ergot alkaloids (42,43). The alkaloids in their base form were dissolved in methanol or in chloroform/isopropanol (3:1) for spotting on Whatman No. 3MM filter paper which was previously dipped in a formamide/methanol (1:3) mixture containing 4% benzoic acid. A benzene/pyridine (6:1) mixture was used for equilibrating the chamber and as the developing solvent. The chromatograph was allowed to develop in a descending manner over a 3-4 hour period for the determination of R_f values, but for better separation the chromatograph was developed over an 8 hour period, in which case the solvent front was over-run.

The system proved effective in separating the clavine alkaloids in fraction E. The development of a chromatograph in this system is illustrated in Figure 12. Fraction E separated into 4 spots having R_f values corresponding to setoclavine, elymoclavine, agroclavine and penniclavine reference compounds.

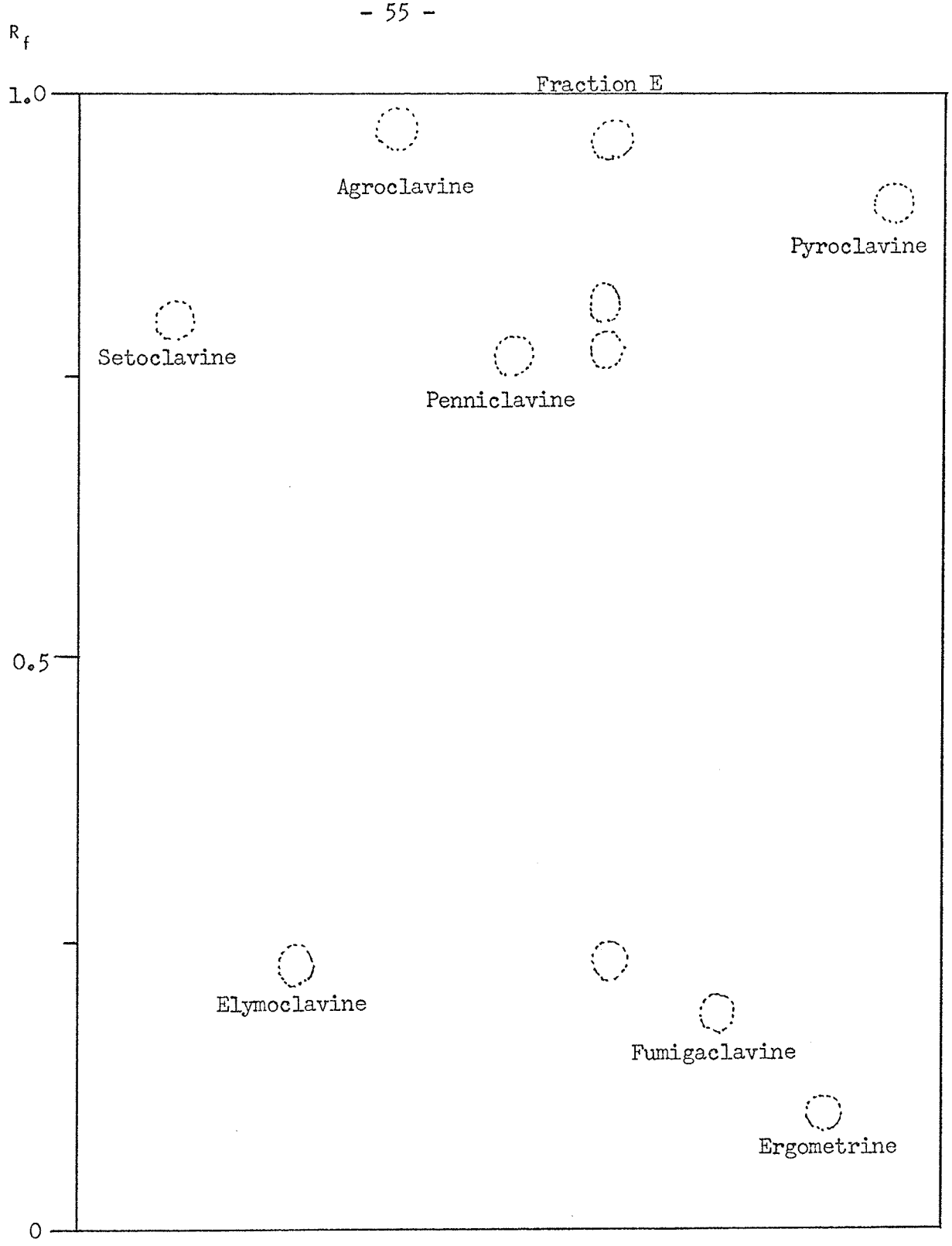


Figure 12. Paper Chromatography of Clavine Alkaloids in the Formamide/Benzene/Pyridine System

VIII. PURIFICATION OF ISOLATED ERGOT ALKALOIDS

Fraction B (Ergotamine)

To fraction B sufficient maleic acid was added to convert the free base to the maleate salt. This greatly enhanced the stability of the alkaloid during the subsequent steps. Fraction B was concentrated under reduced pressure to remove all organic solvents. The remaining aqueous solution was frozen and lyophilized (3).

The residue obtained was mixed continuously with two litres of warm benzene and made slightly alkaline with ammonia gas. It must be mildly basic as the alkaloids tend to oxidize readily. After one hour, the solution was filtered warm and the residue washed with benzene until free of alkaloids. The filtrate and washings were concentrated under reduced pressure to 25 or 30 ml. when ergotamine crystallized out. The base was purified by recrystallizing three times from aqueous acetone, dried, and stored at $-20^{\circ}C$, free from moisture and light (3).

Fraction I (Ergometrine)

Enough maleic acid was added to fraction I to stabilize the alkaloid bases as salts. The fraction was then concentrated under reduced pressure to remove all organic solvents and lyophilized. The residue obtained was dissolved in 10-15 ml. of distilled water, made

alkaline with ammonia, saturated with sodium chloride and extracted with diethylether. Theoretically, with the aqueous portion saturated with sodium chloride, the alkaloid base would be expected to dissolve in the diethylether portion. Therefore the diethylether portion was removed and concentrated under reduced pressure to obtain the basic alkaloid residue. When the diethylether portion was reduced to a small volume, however, it was evident that a small amount of water was present. The alkaloid residue therefore was not obtained, but was dissolved in the water which was present in the diethylether. The procedure was repeated unsuccessfully. Finally, the residue remaining after lyophilization, i.e. ergometrine maleate, was recrystallized three times from methanol. The sample was stored at -20°C, free from light and moisture (3).

IX. CHARACTERIZATION OF ERGOT ALKALOIDS

The isolated alkaloids from fractions B and I were recrystallized until the melting points were constant. The determined melting points and reported melting points are shown in Table 18. The purified alkaloids were submitted to mixed melting point determinations. The mixed melting point of reference ergotamine with the alkaloid from fraction B, and of reference ergometrine maleate with the alkaloid from fraction I (as the maleate salt) were not depressed.

Table 18. Melting Points of Isolated Alkaloids

	Determined M.P. °C	Reported M.P. °C (5)
Fraction B (Ergotamine)	211 - 212.0	212 - 214.0
Fraction I (Ergometrine Maleate)	188 - 189.0	188 - 190.0

The specific rotation of reference ergometrine maleate and of fraction I were measured in a 1% w/v solution of 95% ethanol. They were calculated to be $+65.0^{\circ}$ and $+64.1^{\circ}$, respectively.

The infrared absorption spectra of the isolated alkaloids were recorded and compared with the infrared spectra of reference alkaloids. The infrared spectra were obtained on a Beckman Model 8 spectrophotometer using samples prepared in Nujol as a thin film on a sodium chloride block.

The nuclear magnetic resonance spectra of the isolated alkaloids were recorded and compared with the spectra of reference alkaloids. The nuclear magnetic resonance spectra were measured on a Varian A-56/60A instrument, using a 10% solution of tetramethylsilane in carbon tetrachloride as an external reference in the case of ergotamine and the alkaloid obtained from fraction B. Deuterium oxide was employed as the solvent in the case of reference ergometrine maleate and fraction I (as the maleate salt).

The mass spectra of the isolated alkaloids and the reference alkaloids were recorded and compared. The mass spectra were taken on an Associated Electrical Industries MS-9 mass spectrophotometer using direct insertion of the sample.

Results and Discussion

The infrared absorption spectra of the isolated alkaloids and the reference alkaloids are shown in Figures 13-16. In all spectra, the peaks at 2900, 1450, and 1375 $cm.^{-1}$ were due to Nujol.

Ergotamine (Figure 13) and the alkaloid obtained from fraction B (Figure 14) showed similar absorptional behaviour (5). Both spectra had absorption bands at V_{max} 3500, 3250, 1700, 1575, 1050, 850, 750 and 655 $cm.^{-1}$ Both spectra showed 3 peaks with strong absorption at 655, 750, and 870 $cm.^{-1}$ due to the polynuclear structure of ergotamine. Polynuclear aromatic compounds like the mononuclear aromatics, show characteristic absorption in 3 regions of the spectrum. The most characteristic absorption of polynuclear aromatics is in

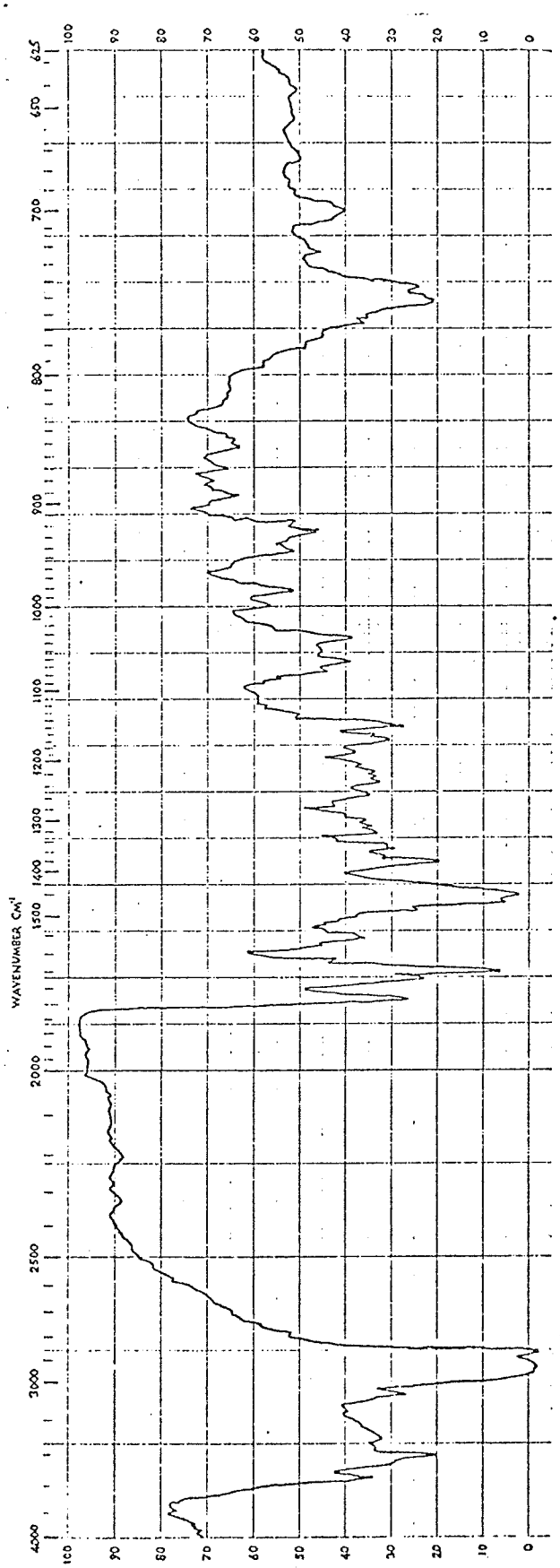


Figure 13. IR Spectrum of Ergotamine

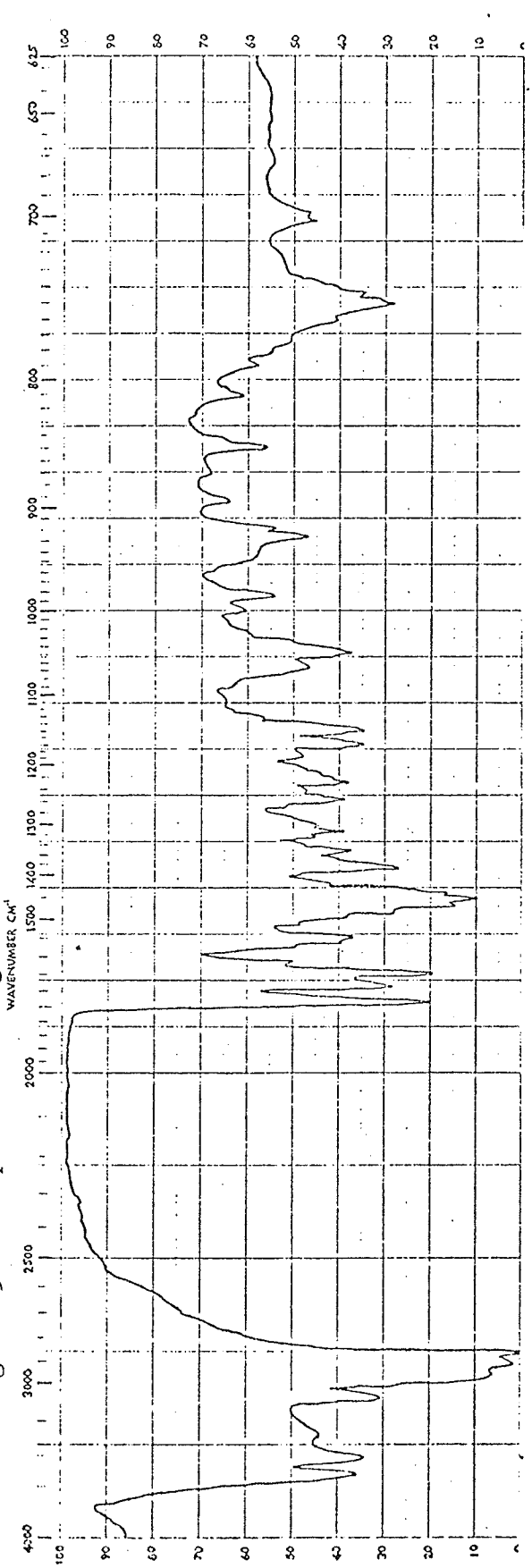


Figure 14. IR Spectrum of Fraction B

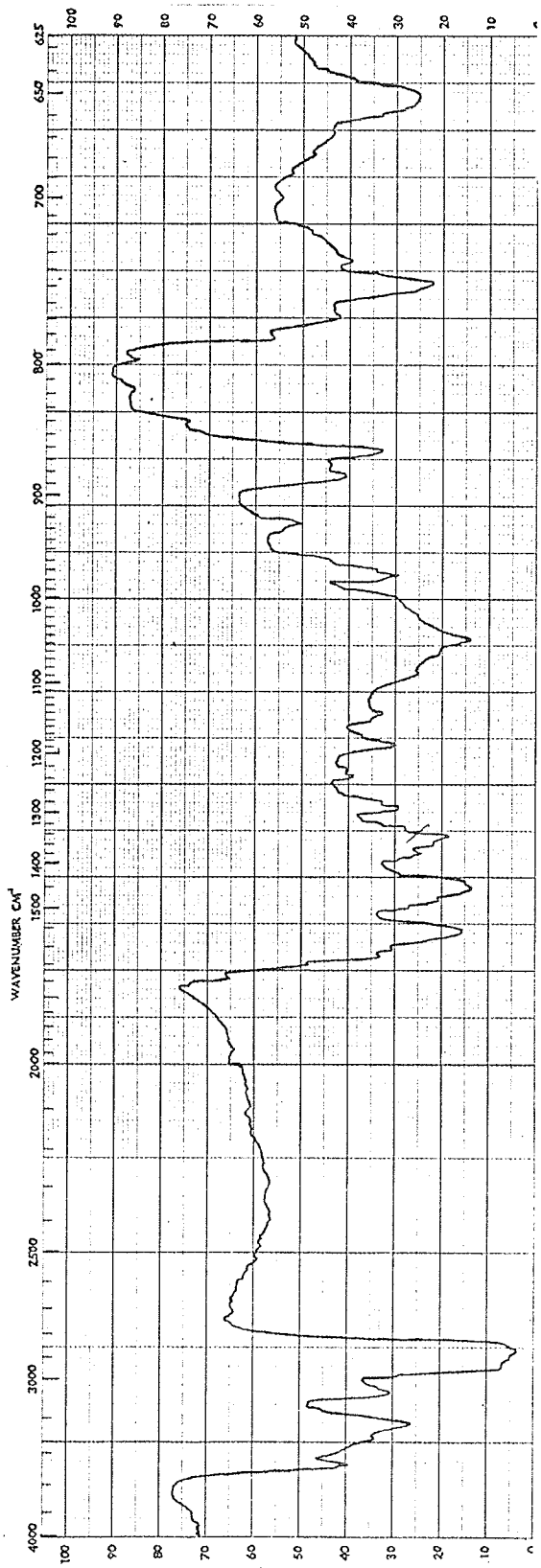


Figure 15. IR Spectrum of Ergometrine Maleate

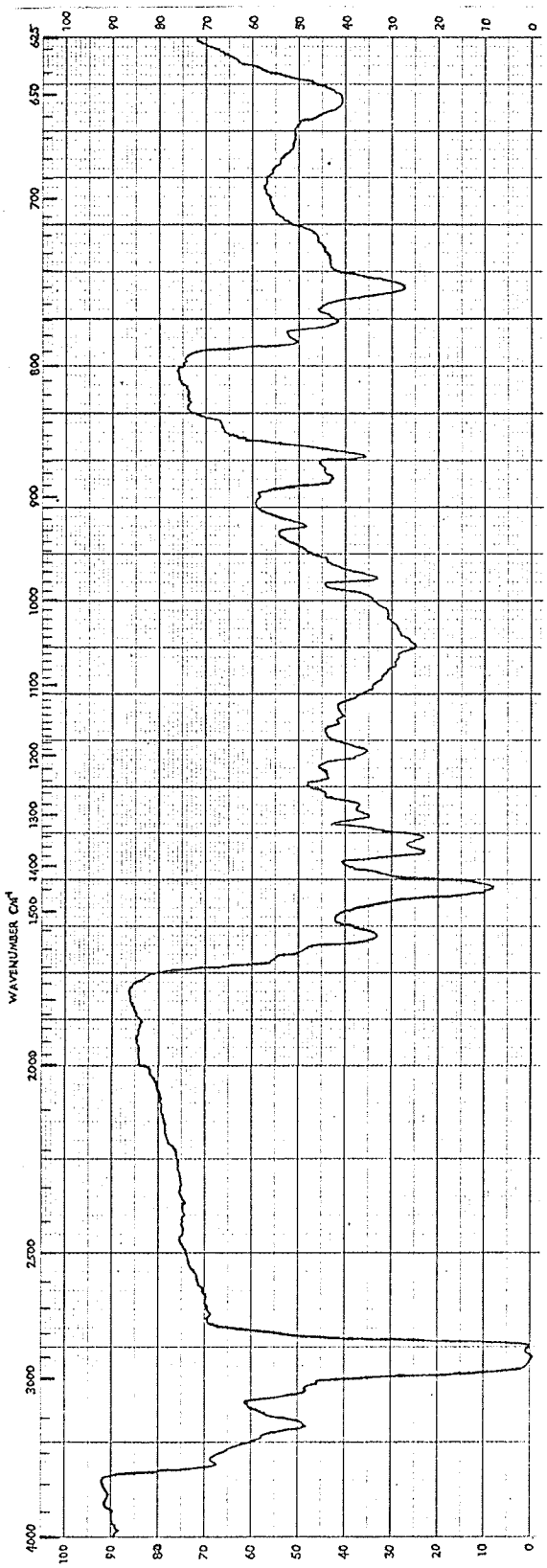


Figure 16. IR Spectrum of Fraction I

the 900-650 cm.$^{-1}$ region. The numerous intense bands in this region are characteristic of polynuclear structures (44).

The peak at 700 cm.$^{-1}$ in both spectra is due to the mono-substituted benzene in the structure of ergotamine, while the peaks between 1225 and 1100 cm.$^{-1}$ can be attributed to the tertiary alcohol group of ergotamine. All amides show a carbonyl absorption in the range 3500-3400 cm.$^{-1}$ Quite a pronounced absorption was observed in both spectra in the region 3600-3200 cm.$^{-1}$ possibly due to the amide groups present. The disubstituted amides in the ergotamine structure show absorption at 1725 and 1625 cm.$^{-1}$

Ergometrine maleate (Figure 15) and the alkaloid obtained from fraction I (as the maleate salt) (Figure 16) exhibited comparable absorptional behaviour (5). Both spectra showed 3 peaks in the region 900-650 cm.$^{-1}$ characteristic of polynuclear aromatic compounds. The strong absorption in both spectra in the range 1075-1050 cm.$^{-1}$ is due to the unbonded or free hydroxyl group of ergometrine.

Secondary and tertiary amines in ergometrine absorb strongly in the region 1650-1550 cm.$^{-1}$ as evidenced by two peaks at 1700 and 1575 cm.$^{-1}$ Both spectra exhibited the characteristic absorption in the region 3600-3200 due to the amide group in ergometrine.

The nuclear magnetic resonance spectra of the isolated alkaloids and the reference alkaloids are shown in Figures 17-20.

The NMR spectra of fraction I (Figure 17) and ergometrine maleate (Figure 18) were not calibrated using an internal standard. For this reason the spectra obtained could not be compared except

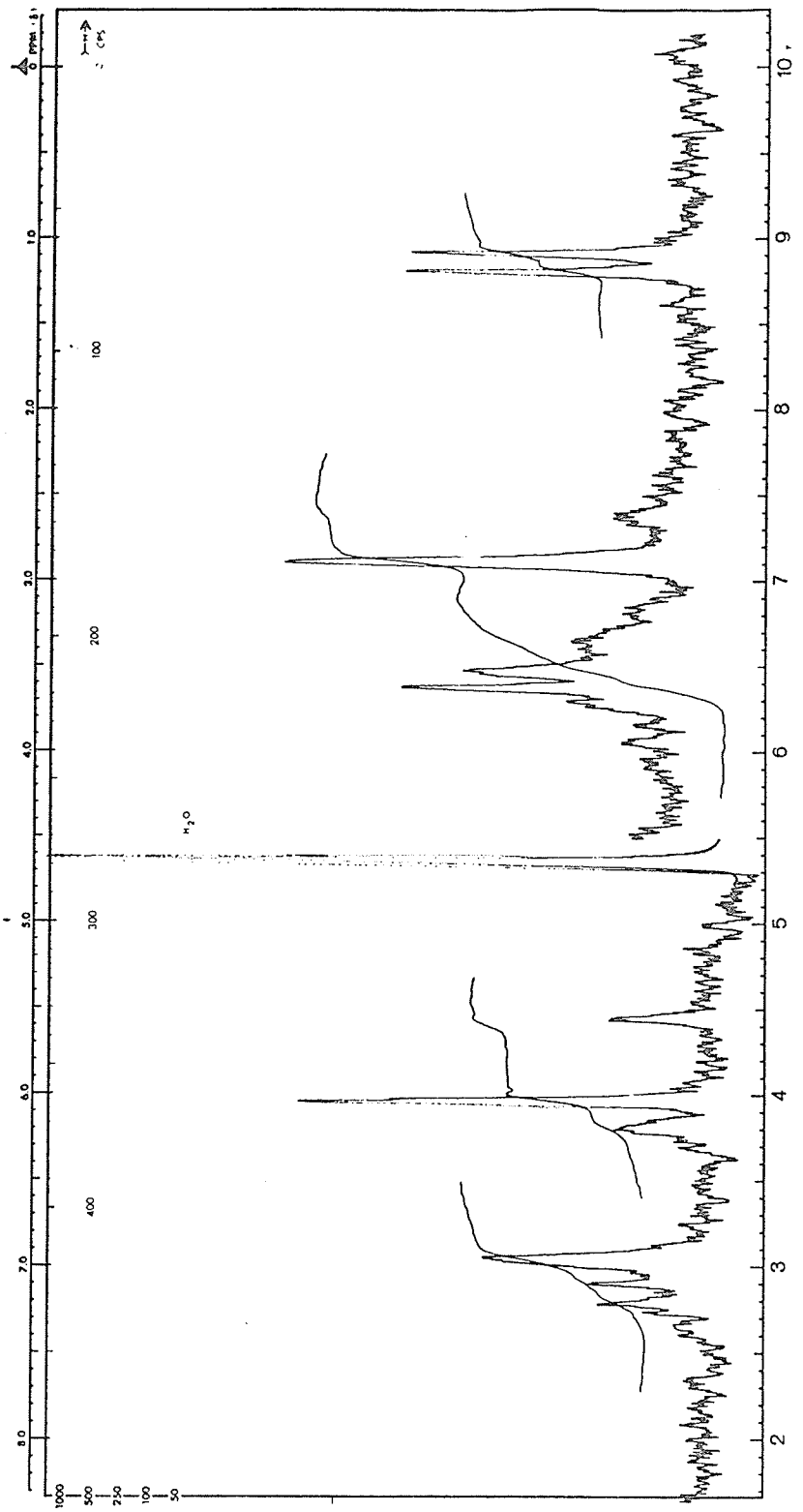


Figure 17. NMR Spectrum of Fraction I

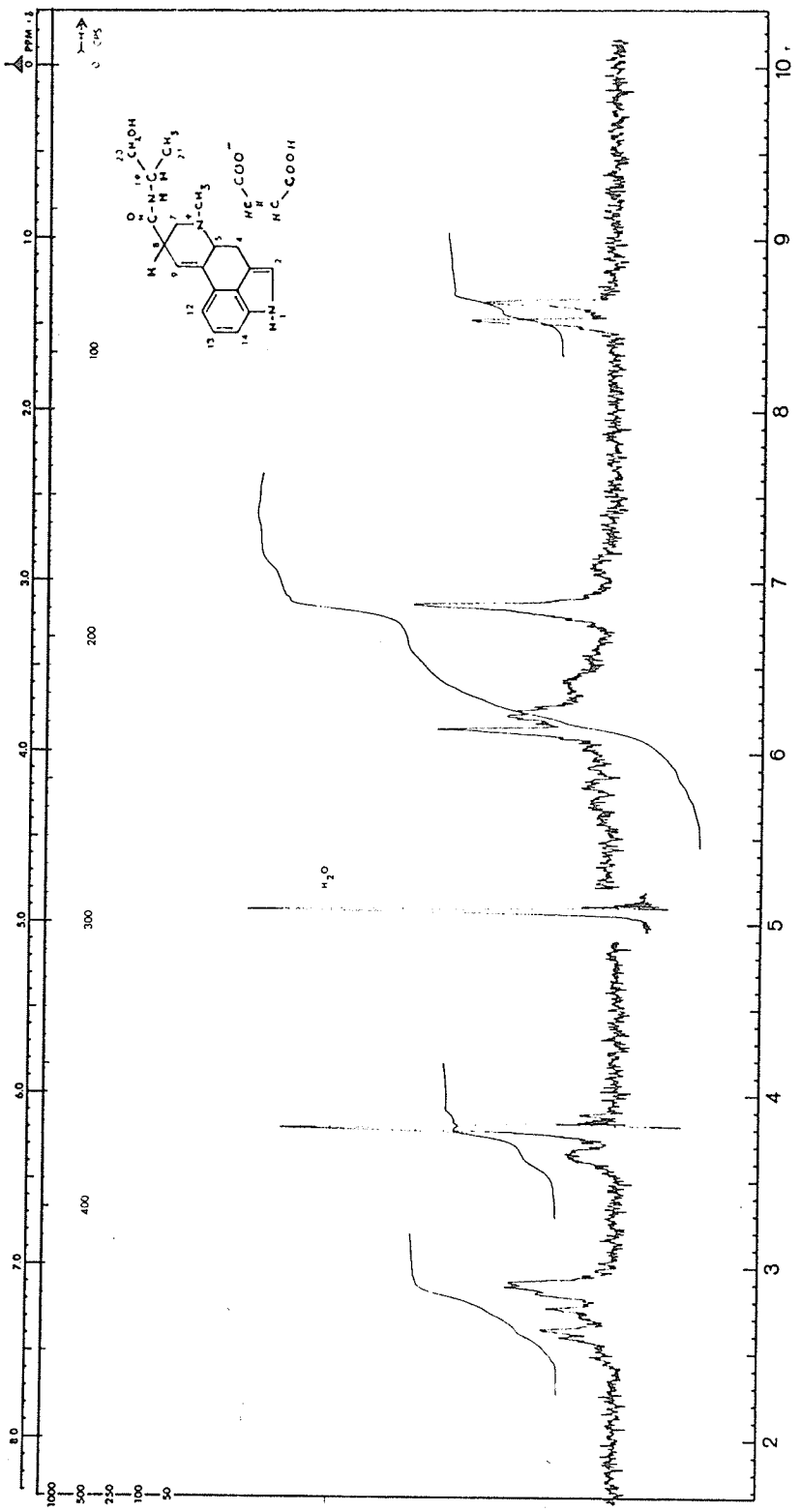


Figure 18. NMR Spectrum of Ergometrine Maleate

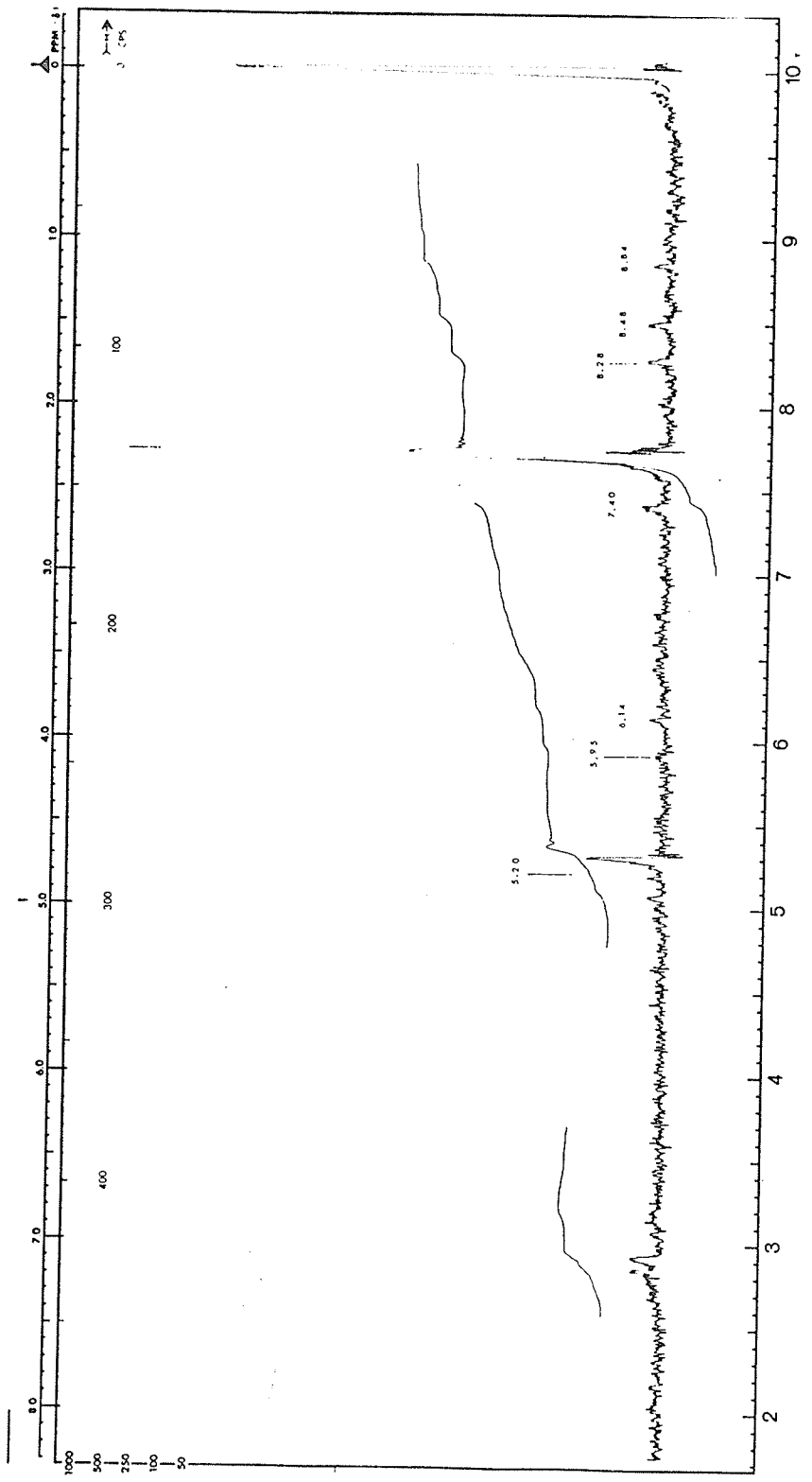


Figure 19. NMR Spectrum of Fraction B

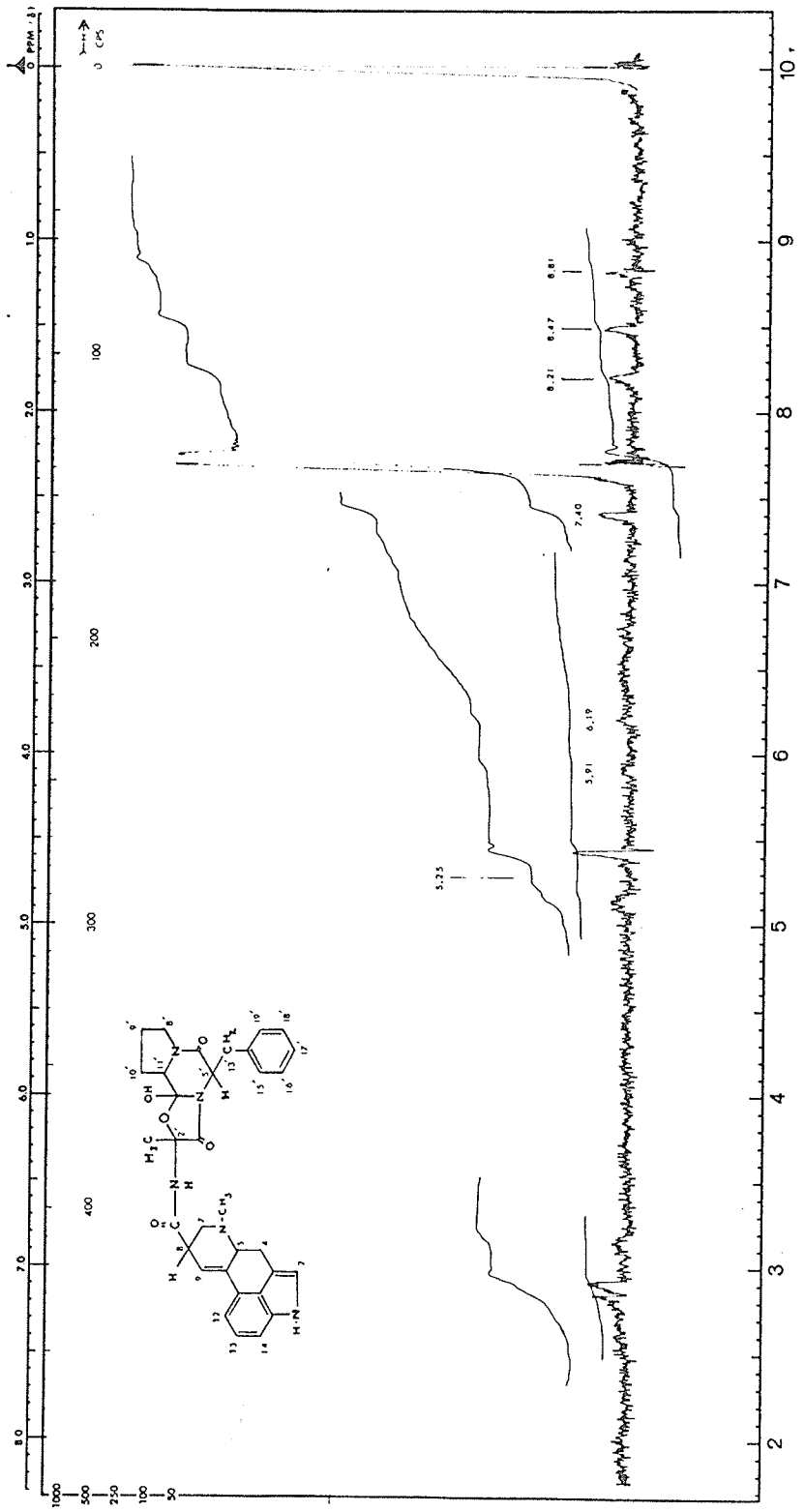


Figure 20. NMR Spectrum of Ergotamine

to note that the NMR spectra of both compounds exhibited a similar pattern.

The NMR spectra of fraction B (Figure 19) and of reference ergotamine (Figure 20) also exhibited a similar pattern.

The mass spectra of the isolated alkaloids and the reference alkaloids are shown in Figures 21-24.

Ergometrine maleate (Figure 21) and the alkaloid obtained from fraction I (as the maleate salt) (Figure 22) exhibited comparable spectra. Aside from the molecular peak of the ergometrine base (M=325) the spectra exhibited certain important peaks at m/e 307, 250, 235, 221, 194, 181, 167, 154, 113, 103, 56, 44, 28, and 18. The decomposition sequence of the compound appeared to occur as shown in Figure 25 using mainly retro-Diels Alder processes and ring expansions which lead to stable aromatic systems (45). The formation of these fully conjugated moieties seems to be the driving force for many of the observed ionic decompositions (45). Thus m/e 154 corresponding to (IX) is a prominent component of all ergot alkaloid spectra. These compounds also possess a m/e 167-168 pair which could be the 7, 6, 5-aromatic type system (X).

Similar mass spectra were exhibited by ergotamine (Figure 23) and the alkaloid isolated from fraction B (Figure 24). The spectra exhibited certain important peaks at m/e 337, 314, 267, 250, 244, 224, 195, 181, 167, 154, 125, 91, 70, 44, 28 and 18. The major peaks correspond to the alkaloid nucleus which then undergoes ionic decomposition previously outlined for ergometrine in Figure 25. The

two most important fragments occur at m/e 154 and 167. A peak corresponding to the complete peptide group (m/e 314) was observed which then farther fragments into the separate amino acids. A smaller peak, directly related to the alkaloid and a single attached amino acid (m/e 267) is likewise apparent.

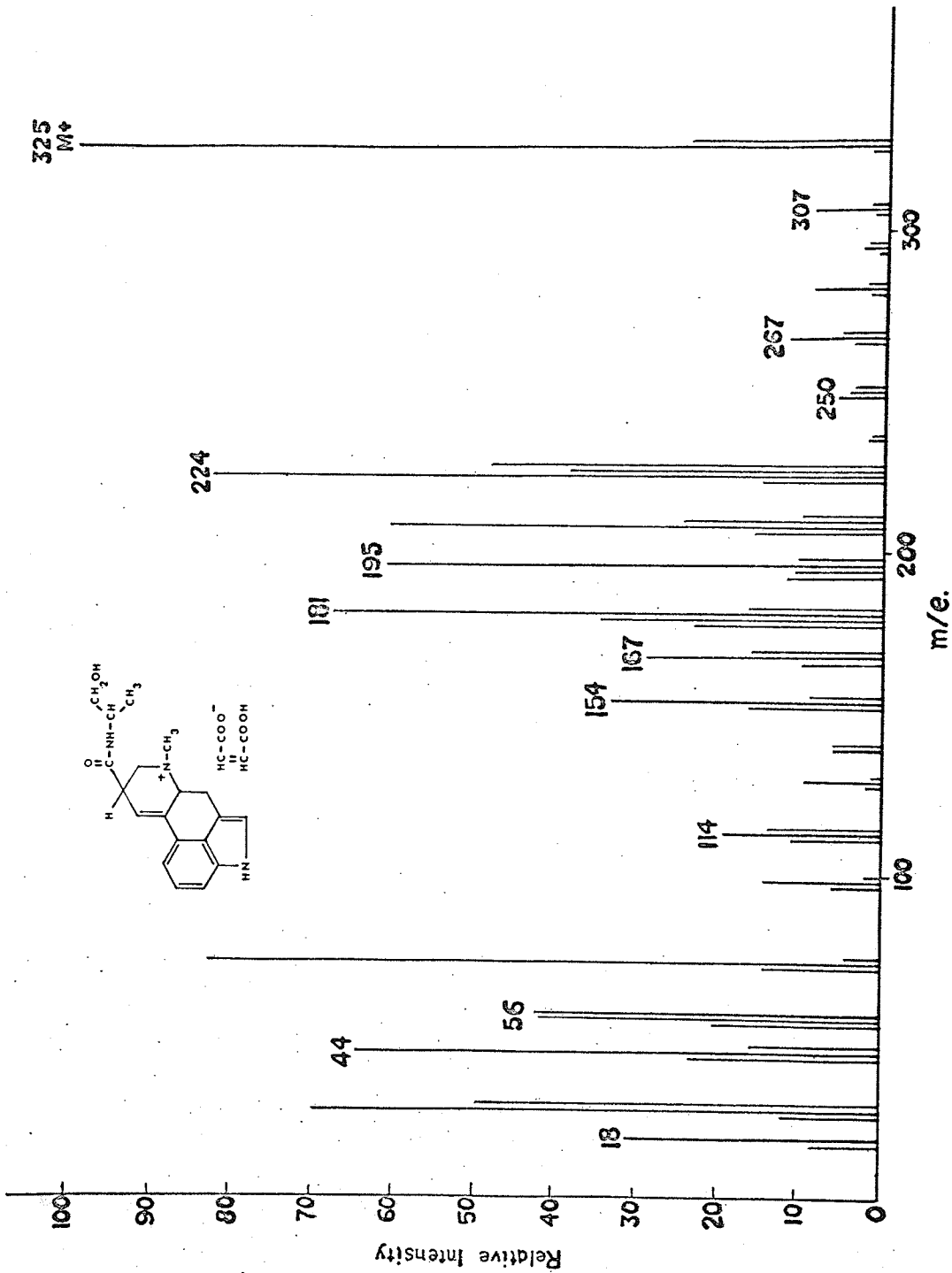


Figure 21. Mass Spectrum of Ergometrine Maleate

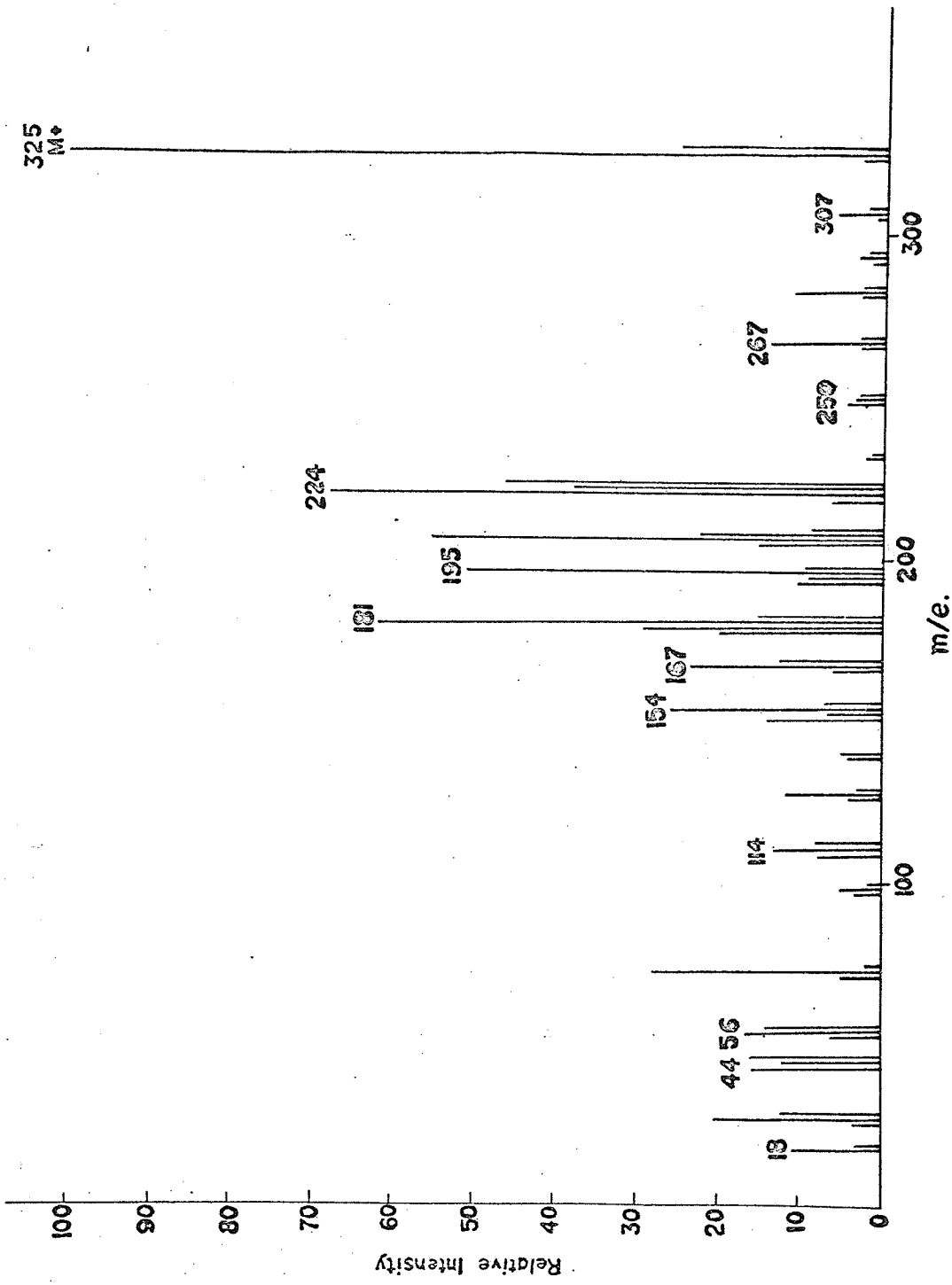


Figure 22. Mass Spectrum of Fraction I

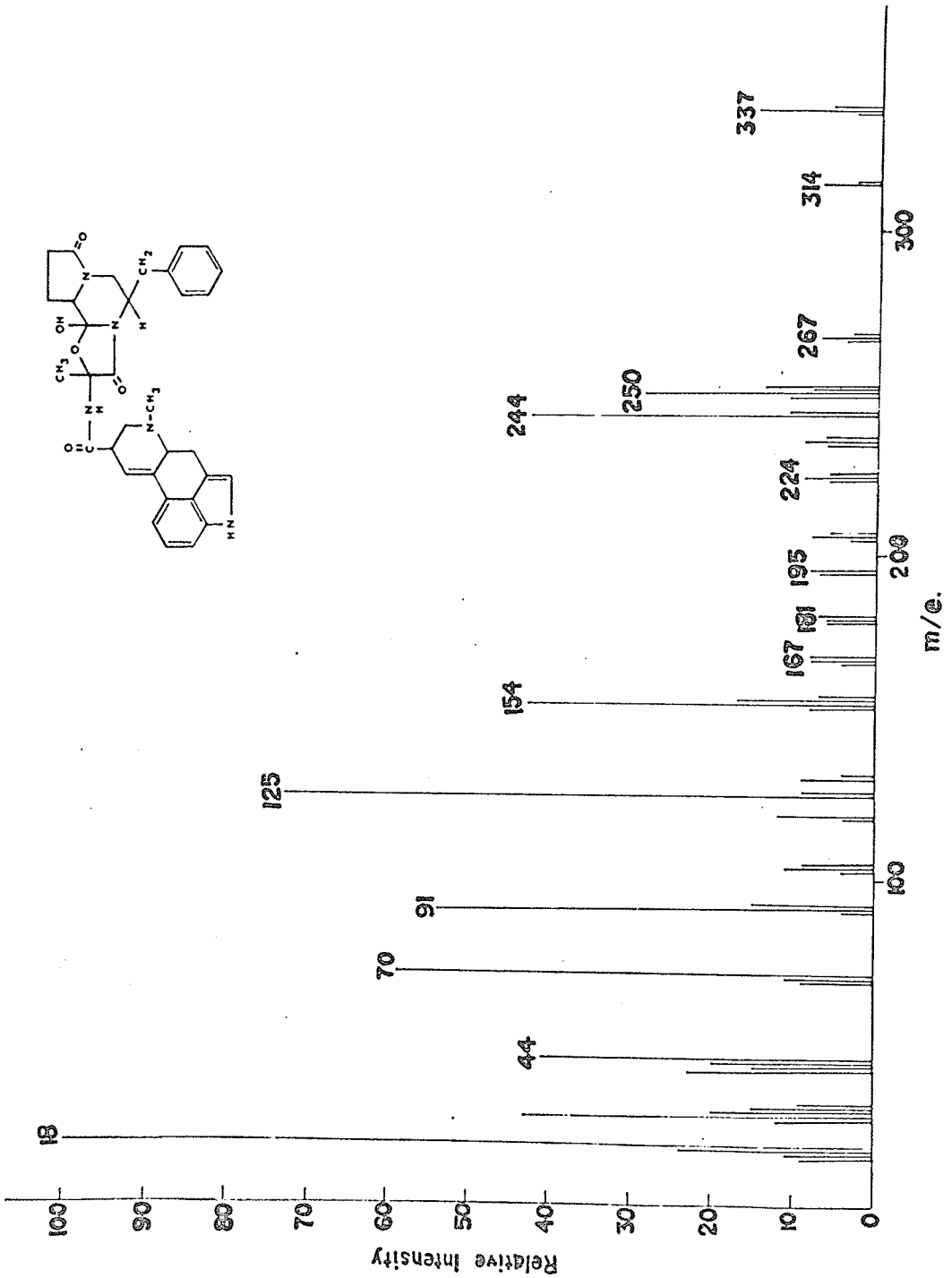


Figure 23. Mass Spectrum of Ergotamine

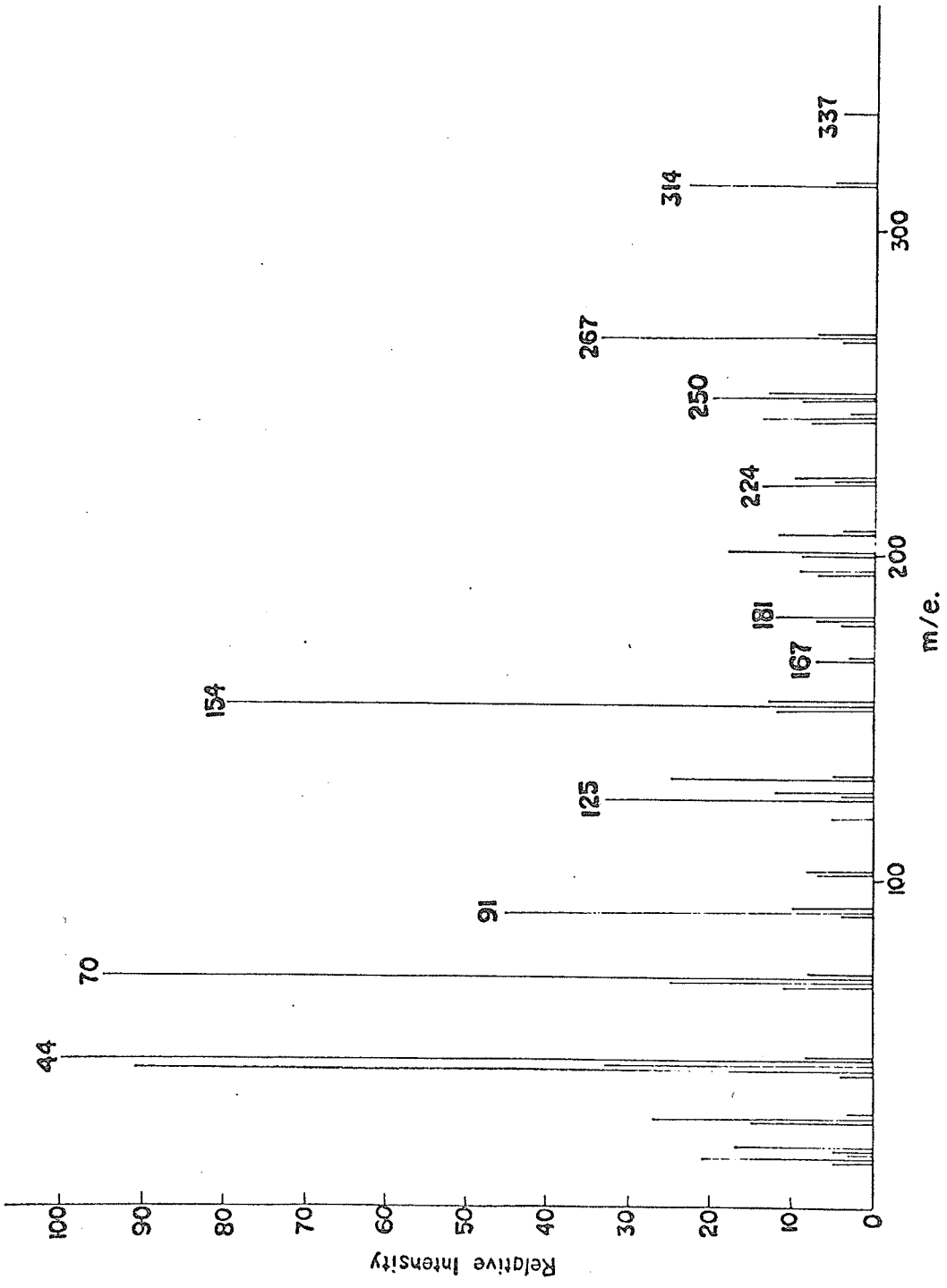


Figure 24. Mass Spectrum of Fraction B

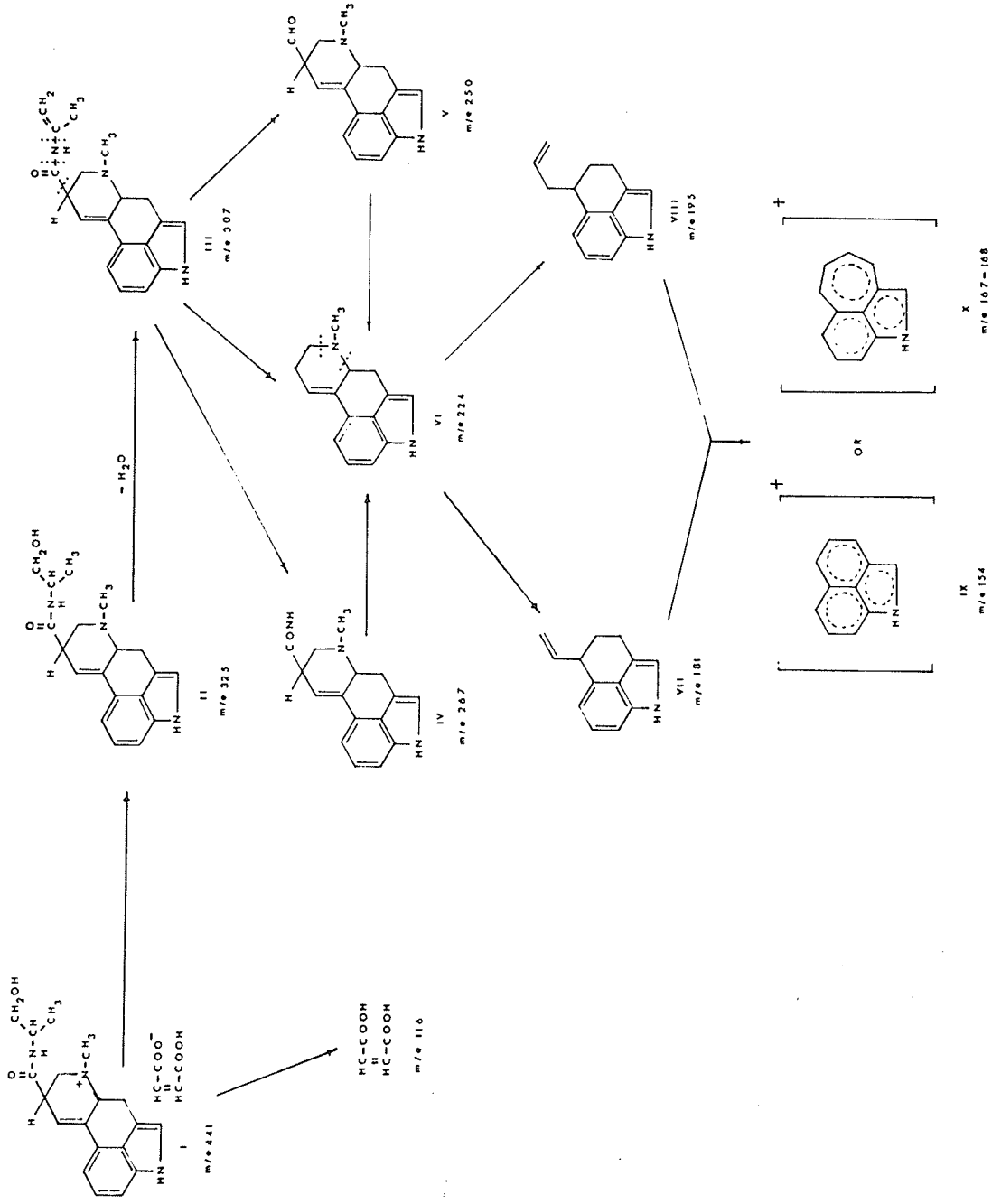


Figure 25. Decomposition Sequence of Ergometrine

S U M M A R Y

1. Ergot grown on Triticale grain had an average length of 13.6 mm. and an average thickness of 3.9 mm. The average weight of a sclerotia was 128 mg. Individual sclerotia appeared cylindrical, angular and tapered to blunt or pointed ends. Transverse fissures were numerous. The color varied from purple to purplish-brown.

2. Using a continuous Soxhlet extraction with petroleum ether, the fat content was found to be 16.74%.

3. The sample was shown by spectrophotometric assay to contain a total of 0.181% alkaloids. 20% of this total were assayed to be water soluble alkaloids. The water insoluble alkaloids were calculated by difference to constitute the remaining 80% of the total alkaloids.

4. A tartaric acid impregnated column was employed to separate the crude alkaloid mixture into ergotamine, the peptide alkaloids, the clavine alkaloids, and ergometrine. The column showed the clavine fraction to be about 3-4% of total alkaloids, while ergometrine was 16-17%.

5. Thin-layer chromatography of the fractions from the column identified ergotamine, the ergotoxine group, ergometrine, penniclavine, elymoclavine, setoclavine, agroclavine and possibly isopenniclavine.

6. Paper chromatography of the clavine fraction showed 4 spots which corresponded to penniclavine, setoclavine, elymoclavine, and agroclavine reference standards.

7. Two alkaloids were isolated and purified. Mixed melting point, infrared spectrophotometry, N.M.R., and mass spectra identified one as ergotamine and the other as ergometrine.

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