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The free amino acid content of the intracellular pools in higher marine fungi was determined by thin-layer and gas-liquid chromatography. The organisms investigated include:

Ascomycetes; Nais inornata, Leptosphaeria oraemaris, Corollospora maritima, Haligena elaterophora, Halosphaeria mediosetigera, Halosphaeria appendiculata, Halosphaeria quadriremis, Lignincola Laervis and Remispora hamata, and Fungi Imperfecti; Zalerion maritimum and Culcitalna archraspora.

All organisms investigated were found to contain: alanine, glycine, valine, proline, leucine, isoleucine, serine, threonine, hydroxyproline, aspartic acid, glutamic acid, methionine, phenylalanine, ornithine, lysine, tyrosin, tryptophan, cystine, cysteine and histidine.

The widespread occurrence of hydroxyproline is of particular interest. This compound was identified in all species examined and since it has been reported to occur only rarely in terrestrial fungi, it may have chemotaxonomical value in delineating obligate marine fungi from terrestrial forms.

The detection of hydroxyproline was considered in light of the experimental procedures employed. The use of water as the extracting solvent, a long extraction period (48 hours) and two different analytical procedures facilitated the identification of the compound.

The role of fungi in the chemical ecology of the marine environment was considered.

FREE AMINO ACIDS IN CULTURED HIGHER MARINE FUNGI

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FREE AMINO ACIDS IN CULTURED HIGHER MARINE FUNGI

INTRODUCTION

Fungi occur indigenously in oceans and estuaries and are known to play an integral role in the marine ecosystem (Johnson and Sparrow, 1961). However, little is known of the biosynthetic potential of these organisms, or of their exact chemical roles in the marine economy.

As saprophytes, marine fungi are believed to perform the same functions as their terrestrial counterparts, that is, to reduce and transform organic wastes (Johnson and Sparrow, 1961). These fungi have been shown to contribute to the decay of algal remains and are a major cause of the deterioration of wood and cordage submerged in sea water (Barghoorn, 1942). Since this early study, the ability of marine fungi to produce degradative enzymes has been demonstrated repeatedly (Meyers, 1968; Kohlmeyer, 1972). The observation that marine fungi can degrade organic material in the sea has prompted speculation into their role as detritus decomposers. Conceivably fungi may be directly involved with bacteria in transforming marine hemicellulosic and cellulosic materials into nutritive products utilizable by marine animals.

The nutritional relationships of various marine animals to decay fungi and other heterotrophic microorganisms has been shown by studies of the red mangrove (Rhizophora mangle) ecosystem (Heald, 1971; Odum, 1971). These investigators have illustrated that arthropods, molluscs

and fishes ingest decaying mangrove leaves, but digest only the microflora absorbed to the ligno-cellulosic leaf particles rather than the leaf matrix itself. Investigations into the food webs based on eel-grass (Zoster spp.), turtle grass (Thalassia testudinum) and cord-grass (Spartina alterniflora) indicates a process similar to that of the mangrove ecosystem (Wood et al., 1969; Fenchell, 1970; Meyers, 1971).

The contribution of marine fungi to detritus production seems to be of major significance in marine ecosystems since the most biologically productive marine environments are based on detritus formed from the decay of sea grasses, salt marsh vegetation and mangroves. Various researchers have shown that a diverse mycoflora is associated with each of these higher plants (Meyers et al., 1965; Kohlmeyer, 1969; Kohlmeyer and Kohlmeyer, 1964; Lee and Baker, 1972).

As parasites, marine fungi have also had great impact on the marine ecosystem. In 1935, eel-grass along the Atlantic coast was parasitized by a Labyranthula species which resulted in its near disappearance (Renn, 1935). This event was illustrated graphically by the death of many species of scallops, mussels and game birds which feed on this species of grass. Direct parasitism by fungi on human food sources in the sea has been demonstrated also. Couch (1942) reported that Lagenidium callinectes was found parasitizing the blue crab. Clams and oyster larvae have also been found to be parasitized by a "sirolpidium-like" fungus (H. C. Davis et al., 1954). Since these reports, marine fungi have been shown to be parasites of numerous marine animals. More specifically, various fungi have been

shown to elaborate enzymes which degrade chitin and keratin which are major structural components of many marine animal exoskeletons (Kohlmeyer, 1972).

To date, approximately 200 species of higher marine fungi, mostly Ascomycetes and Fungi Imperfecti, have been shown to occur saprophytically or parasitically on higher plants and sea weeds in littoral and netritic environments (Kohlmeyer and Kohlmeyer, 1971). Of this group, approximately one-half are lignicolous or wood rotting fungi (Jones, 1968; Hughes and Chamut, 1971).

As indicated previously, the biological role of marine fungi, although incompletely understood, has been somewhat delineated in an ecological point of view. However, substantial chemical or biochemical information concerning these fungi is lacking. The metabolic contributions of fungi to the chemical and nutritive aspects of the sea is an almost totally neglected field of investigation. Before the role of fungi in the marine ecosystem can be established, full knowledge of the chemical activities, biosynthetic potential and primary and secondary metabolites of these organisms should be at hand. This type of data would not only contribute to a better understanding of the biology of the marine environment but also should yield other dividends of basic and applied scientific interest.

The first published accounts concerning marine fungal metabolities occurred relatively recently. In 1957, Schafer and Lane identified twelve amino acids in Lulworthia floridana. These investigators also implicated the nutritional use of these amino acids by marine wood-boring animals. Since that time a small body of information

on marine fungal metabolites has accumulated. Many of these metabolites could serve as sources of energy and precursors for vitamins and hormones in marine vertebrates and invertebrates (Sebrell and Harris, 1971).

At the present time, knowledge of the metabolic products of higher marine fungi include, as previously mentioned, the demonstration of cellulolytic enzymes and the production of various amino acids. Other investigations have illustrated the occurence of keratinolytic and chitinolytic enzumes (Kohlmeyer, 1972).

Recently, investigators have reported the presence of numerous triglyceride fatty acids in several marine fungi. Species of Corollospora and Zalerion were shown to produce oleic, palmitic and linoleic acids; with lesser amounts of stearic,palmitoleic, caproic, myristic, behenic, and linolenic acids (Block et al., 1973).

The presence of several sterols have also been reported to occur in marine fungi. Ergosterol has been identified in a variety of marine Ascomycetes and Fungi Imperfecti (Kirk, 1966; Kirk and Catalfomo, 1970). Also cholesterol has been demonstrated in the marine fungus, Labyrinthula (Vishniac, 1957).

Reports on the occurence of organic sulfur and nitrogen containing compounds in marine fungi are very few. Recently, however, numerous lignicolous marine fungi have been shown to contain choline and choline sulfate ester (Kirk and Catalfomo, 1970; Catalfomo et al., 1973). Other than these studies, little is known of nitrogen containing metabolites elaborated by these fungi.

It is well known that ocean waters contain variable concentrations of amino acids (Degens et al., 1964; Pockington, 1972). The source of these amino acids is incompletely determined. Therefore, it is the objective of this study to identify the free amino acids present in various marine Ascomycetes and Fungi Imperfecti in an attempt to better understand the contribution of these organisms to the marine ecosystem. A secondary objective of this investigation is the possible application of such information to the taxonomy of marine fungi which has been mainly floristic in nature. The application of chemical knowledge to taxonomic problems has proved of value in delineating species of terrestrial fungi (Maag, 1959, Catalfomo and Tyler, 1961).

The studies of one investigator concerning the classification of marine Ascomycetes from cytochemical and histochemical data seems to indicate that specific chemical characteristics of ascospores may be used as critera for identification (Kirk, 1966). In the taxonomic classification of marine ascomycetes much emphasis is placed on ascospore characteristics. The most important aspect being the presence or absence of appendages, and if present, their type and insertion. Due to the great variability of these appendages, the taxonomy of the marine ascomycetes is a most perplexing problem in systematic marine mycology (Kirk, 1966). Thus it seems most logical to attempt to apply chemical data as a taxonomic aid.

TAXONOMIC CONSIDERATIONS OF INVESTIGATED MARINE FUNGI

Opinions in the literature differ greatly as to what is meant by "Marine Fungi". A strict definition is met with two mutually contributory difficulties. Firstly, the mechanisms and physiological adaptations which allow marine fungi to exist in saline environments are only dimly understood. Secondly, there are numerous aquatic fungi that do exist equally well in saline and fresh water environs (Kohlmeyer and Kohlmeyer, 1964).

Probably the most accepted definition presented describing "Marine Fungi" is that of Johnson and Sparrow (1961) who write

> ...Mere chance occurrence of a fungus in saline waters does not make it a marine of brackish water one. Neither does its morphology, for not a single species of which we are aware is structually modified for existence in salt water. The requirement, by a fungus, for a specific salt or group of salts in the ocean waters would probably constitute a valid criterion for using the label "marine", but as far as is known, no such demonstration has been convincingly made. Conceivably, there are physiological reasons why a fungus is marine, but these have not yet come to light. Of course, there are species which occur exclusively in a salt water environment. or so we judge from the literature and from our own observations. These could be considered true marine fungi and defined somewhat as follows: capable of developing to reproductive maturity even though exposed at some point in their growth to salinities of $30^{o}/_{oo}$ (parts per thousand) or more, either while continually submerged or intermittently inundated by tidal waters...

The fungi examined in this investigation do meet the preceding definition and have been placed taxonomically in one of two classes. The majority of organisms studied belong to the class Ascomycetes. This class is distinguishable by the following as described by

Johnson and Sparrow (1961).

> ...The chief feature is the production of an ascus, a saccate cell typically bearing internally a definite number of spores arising by free cell formation following meiotic division of a diploid nucleus. Further, the majority of Ascomycetes produce a complex, generally prosenchymatous fructification, the ascocarp, within which the asci are borne. In the salt-water inhabiting Ascomycetes, the usual ascocarp is a perithecium. ...Perithecial shape, number and length of necks, coloration, and positions (that is, superficial or innate), are variable characteristics.

Two organisms examined in this investigation have been placed in the class, Fungi Imperfecti (form-class Deuteromycetes). According to Johnson and Sparrow (1961), this class is characterized by

> ...Septate mycelium and the production of asexual reproductive units--conidia--abstricted endo- or exogenously from a specialized hypha, the condidiophore, are the principal characteristics of the Fungi Imperfecti. Numerous variations occur in conidial insertion, septation and color. Many form species, although in the same form genus, are totally unrelated, as evidenced by their differing perfect states. The great majority of Fungi Imperfecti are very likely the conidial stages of Ascomycetes, whose alliance with the ascogenous stage has not as yet been established...

Further and more specific taxonomic treatment is beyond the scope of this dissertation, however; all organisms were authoratively classified and identified by Dr. P. W. Kirk, Departments of Biology and Oceanography, Old Dominion University, Norfolk, Virginia. The species of Marine Fungi examined in this investigation include: Ascomycetes; Leptosphaeria oraemaris linder, Corollospora maritima Werdemann, Haligena elaterophora Kohlm., Lignincola laevis Hohnk, Nais inornata Kohlm., Remispora hamata (Hohnk) Kohlm, Halosphaeria

appendiculata (Linder) H. quadriemis (Hohnk) Kohlm., H. mediostetigera Cribb et Cribb, and Fungi Imperfecti; Zalerion maritimum (Linder) Anastasiou., Culcitalna archraspora Meyer et Moore.

EXPERIMENTAL

All organisms analyzed for free amino acids in this investigation were supplied from the culture collection of Dr. Philip Catalfomo, School of Pharmacy, Oregon State University. Pure culture isolation and authorative identification of the organisms was provided by Dr. P. W. Kirk, Departments of Oceanography and Biology, Old Dominion University, Norfolk, Virginia. The cultures prior to this study were maintained on solid media slants designated 5M and described in Table I (Kirk and Catalfomo, 1970).

Inoculum for liquid culture was produced by inoculating each organism from stock cultures with a sterile platinum spade into two 250 ml. Erlenmeyer flasks containing 100 ml. of sterile medium 6M (Table I). All media was autoclaved for twenty minutes at 127°C. before use.

TABLE I

DESCRIPTION OF MAINTENANCE AND LIQUID CULTURE MEDIA

Medium 5M-Maintenance Medium (Kirk and Catalfomo, 1970)

Glucose (Difco, Detroit, Michigan)	10 Gm.
Yeast Extract (Difco)	1 Gm.
Rila Marine Mix*	40 Gm.
Agar (Difco)	20 Gm.
Distilled water	To volume-1L.

Medium 6M-Liquid Culture Media

Glucose (Difco)	10.0 Gm.
Yeast Extract (Difco)	1.0 Gm.
Rila Marine Mix	40.0 Gm.
Ammonium Succinate (Reagent Grade)	1.0 Gm.
Distilled water	To volume-1L.

*Synthetic Sea Water Compound, Rila Products, Teaneck, N. J.

The inoculum flasks were incubated on a rotary shaker (240 r.p.m.) for five days at 25°C. The inoculum culture was then aseptically transferred to a sterile Waring blender and homogenized for fifteen seconds. The homogenate was aseptically pipetted, in 2.0 ml. aliquots, into eight 500 ml. Erlenmeyer flasks containing 200 ml. of sterile 6M media. These cultures were then incubated on a large rotary shaker (160 r.p.m.) at 25°C. for seven days. A duplicate culture set Zalerion maritimum was incubated for fourteen days.

After incubation, the cultures were harvested by separating the mycelium from growth media using a Buchner suction filtration apparatus fitted with Whatman No. 3 filter paper. The mycelium was washed thoroughly with cold distilled water. Particular care was taken to move all traces of culture medium. The washed mycelium was placed in 150 mm. evaporating dishes and dried in a forced air dryer at 40°C for 48 hours.

The dried mycelia was then ground to a 40 mesh powder in a Thomas mill. The powder was weighed (Table II) and placed in ointment jars. The jars were sealed and stored in a refrigerator at 5° centigrade until extraction.

TABLE II

DRY WEIGHTS OF POWDERED MYCELIA EXTRACTED

Isolate	Organism	Dry Weight (Gm.)
R-2	*Lignicola laevis* Hohnk	15.2
R-4	*Nais inornata* Kohlm.	13.4
R-6	*Zalerion maritimum* (Linder) Anastasiou	19.1
R-13	*Leptosphaeria oraemaris* Linder	10.6
R-19	*Corollospora maritima* Werdermann	12.3
R-524	*Halosphaeria mediosetigera* Cribb et Cribb	11.9
R-572	*Halosphaeria quadriremis* (Hohnk) Kohlm	11.4
R-577	*Remispora hamata* (Hohnk) Kohlm.	9.9
R-601	*Haligena elaterphora* Kohlm.	15.5
R-605	*Halosphaeria appendiculata* Linder	13.8
F-1	*Culcitalna archraspora* Meyers et Moore	8.4
R-6-(14 days)	*Zalerion maritimum* (Linder) Anastasiou	12.7

I. Extraction Procedures

The method used for extraction of the free amino acids from powdered mycelia was essentially the combined methods of Heathcote *et al.*, (1972) and Catalfomo *et al.* (1973). The ground mycelia was placed in cellulose extraction thimbles (33 x 94 mm. Whatman, Balston Ltd., England), covered with glass wool, and inserted into Soxhlet extractors. The Soxhlet apparatuses used were heated on a six hot plate bank (Lab-Line Inc. Melrose Park, Ill.).

Each sample of powdered mycelia was first extracted with 200 ml. of low boiling petroleum ether (30-60°C.) for 24 hours. After this defatting procedure, the thimbles containing the defatted mycelia were placed in a hood and allowed to dry. The petroleum ether extracts

were stored in a refrigerator at $5^{o}C.$ awaiting further study.

After the defatted mycelium had dried for 12 hours in a laboratory hood, it was placed back in the Soxhlet extractors and extracted with 200 ml. of double distilled water for 48 hours. This fraction contained the free amino-acids and was the subject of the amino-acid analysis. The extracted mycelial marcs were dried and stored in a refrigerator at $5^{o}C.$

When the aqueous extracts had cooled sufficiently, they were immediately evaporated to dryness on a Rinco^{R} rotary flash evaporator at $40^{o}C.$ with vacuum supplied by a water aspirator. When the extracts had reached dryness, they were taken up with three successive 25 ml. washes of methanol. The 75 ml. methanolic solution of free amino acids was then stored in a refrigerator at $5^{o}C.$ until later treatment. This procedure precluded bacterial growth in the extracts.

II. Desalting Procedures

Before chromatographic analysis, the amino acid extracts were desalted on a cation exchange resin column. This procedure removed inorganic salts, proteins, and peptides which may interfere with the amino acid analysis (Heathcote _et al_., 1972; Pockington, 1972).

The resin chosen for this procedure was a polyvinyl sulfonate resin, Dowex^{R} 50W x 12(H^{+}form), 100-200 mesh; available through Dow Chemical Corporation, Midland, Michigan. The resin was preconditioned before use by swirling in double distilled water for several hours

before being placed in glass columns.* This allowed the resin to attain its maximum swollen state.

The columns used for the desalting were standard 50 ml. burettes (Pyrex). These columns were not only convenient but precluded column volume calculations as the volume was marked on them. The burettes were washed and rinsed three times with double distilled water. A resin bed support was placed in the delivery end of the burette by inserting one-half inch of glass wool above which was placed a layer of three millimeter diameter glass beads. The support was washed with double distilled water before insertion. The glass bead layer was adjusted in thickness so the top edge was even with the 50 ml. mark on the burette.

The column was filled by slurring the resin beads from a beaker with the use of a stream of distilled water from a wash bottle. Excess water was decanted from the resin before placement in the column to produce a thick slurry. This was done to avoid stratification of resin particles by particle size due to settling. Double distilled water was added to the column to a depth of several inches before the resin was added to minimize the formation of air pockets.

The burettes were filled to approximately one-half inch above the zero milliliter mark. The filled burettes were than allowed to settle for 24 hours. The resin was backwashed with distilled water. This was done by attaching surgical tubing to the delivery end of

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*Dow Chemical Corporation Tech. Bull. A Laboratory Manual for Ion Exchange. Products and Systems Department Pub. (1972)

the burette and forcing distilled water through the tubing and resin column with a wash bottle. Care was taken to avoid injecting air into the resin column. The backwash procedure was done several times until a reproducible resin bed depth occurred after settling. The resin bed was finally adjusted to the zero milliliter mark on the burette giving an exact 50 ml. volume of resin or approximately 40 gms. of resin.

Before desalting, the methanolic solution containing the extracted amino acids was taken to dryness in a rotary flash evaporator as described previously. The dry residue was taken up in 50 ml. of distilled water and placed in a beaker. This solution was adjusted to pH 7.0, adding 0.1N sodium hydroxide dropwise and monitored by a pH meter.

Twenty-five milliliters of the neutralized extract was placed on the resin column and allowed to percolate into the resin bed. The water column above the resin was adjusted to one-half inch above the resin by draining off some of the water (resin void volume). The resin was then washed with double distilled water until the effluent was free of anions as shown by a negative test for chloride with silver nitrate test solution (Pockington, 1972). Periodic checks of the eluate for amino acids during the wash was done by spotting filter paper with eluate and spraying with ninhydrin spray reagent (cf. TLC section). The spotted filter paper was developed in an oven at 105°C. for 30 minutes. This test was to insure no amino acids were washed from the column during the wash

procedure. The eluate was then stored in a refrigerator for further study.

Amino acids were eluated from the washed column with 200 ml. of 2M ammonium hydroxide. The volume ratio of ammonia to resin was 4:1 (Pockington, 1972). The column was finally washed with double distilled water until the ninhydrin test described above showed no amino acids in the eluate. The flow rate for both of these procedures was adjusted to 3 ml./min.

The collected amino acid fraction and final water wash was then combined and taken to dryness with a rotary flash evaporator. The dried residue was taken up in 50 ml. of methanol and stored at $5^{o}C$ until chromatographic analysis.

The resin was then regenerated for further use. This was done by emptying the resin from the columns and washing it repeatedly with double distilled water until the wash water was clear of any coloration. The resin was then swirled in 2% hydrochloric acid solution for 1 hour and finally rewashed with double distilled water. The resin was placed back in the burette columns as described previously. After backwashing and settling, 100 ml. of 10% HCL solution was passed through the column at a rate of 3 ml. per minute. This procedure regenerated the resin columns for further use. The regenerated column was finally washed with 200 ml. of double distilled water at a flow rate of 3 ml. per minute. The regenerated columns were then used in the same manner as described previously for further desalting operations.

III. Chromatographic Procedures

A. Thin-layer Chromatographic analysis

The thin-layer chromatographic analysis method used in this study was a combination of that described by Haworth and Heathcote (1969), Heathcote *et al*. (1972) and E. Stalh (1970). The method utilized two-dimensional development of cellulose thin-layer plates in two different solvent systems. Amino acids were detected with ninhydrin spray reagent (cf. TLC section).

Cellulose plates were prepared by mixing 8.0 gm. of cellulose powder, MN 300 (Macherey Nagel and Company Ltd., Cambridge, England), with 45 ml. of ethanol (95% USP). This suspension was shaken for two minutes and applied to five clean, 20 cm. by 20 cm., chromatography plates with a plate spreader (slit width 4 mm.). The plates were then allowed to dry for 15 minutes at room temperature and finally placed in an oven at 105°C. for ten minutes.

The dry plates were then allowed to cool to room temperature and amino acid samples were applied to them immediately. Spots of amino acid standards and samples were placed at a position 2.0 cm. up from the lower left edge of the cellulose layer and 2.0 cm. inside from the left edge of the layer. The amino acid solutions were applied to the plates with capillary tubes (0.5 mm. ID by 75 mm., Scientific Products, Inc.) and the spots dried in a stream of warm air. The spots during the application procedure were not allowed to expand over 0.5 cm. in diameter.

For comparison, individual amino acid standard solutions were prepared by placing 1.0 mg. of each appropriate amino acid in 5 ml. of double distilled water. These solutions were then warmed on a hot plate to insure all of the amino acids were completely solubilized. The standard amino acid solutions prepared were of: alanine, glycine, valine, ornithine, proline, leucine, isoleucine, serine, threonine, hydroxyproline, aspartic acid, glutamic acid, methionine, phenylalanine, tyrosine, tryptophan, cysteine, cystine, and histidine. Standard amino acid mixtures were prepared by placing 1.0 mg. of each of the above amino acids in 10 ml. of double distilled water. This solution was also warmed on a hot plate to insure complete solubilization. These solutions were then used for spotting on the cellulose plates as described above. All amino acid standards were obtained from Nutritional Biochemical Corporation, Chicago, Illinois.

The mycelial extracts were prepared for thin layer chromatographic analysis by evaporating the methanolic amino acid solutions to dryness in a rotorary flash evaporator and taking up the dry residue with 50 ml. of double distilled water. This solution was warmed on a hot plate to insure the residue was completely solubilized. The amino acid solution produced above was then applied to the cellulose plates as described previously.

The solvent systems chosen for two dimensional development of the plates was a combination of those described by Heathcote _et al_. (1972) and E. Stahl (1970) with slight modification. For development in the first dimension (direction A); Isoprophyl alcohol,

butanone, and 1N hydrochloric acid in water (60:15:25 v/v) was used. The second dimension (direction B) was developed using butyl alcohol, acetic acid, and distilled water (40:20:20 v/v) as a solvent system. All solvents were of reagent grade purchased from Mallinckrodt Chemical Corporation and the acids were obtained from J. T. Baker Chemical Corporation.

Before development, chromatography tank saturation was achieved employing a modified method of Sankoff and Sourkes (1963). In this procedure, solvent from the previous day's development was allowed to remain in the tank. Prior to use, the old solvent was removed and replaced with 100 ml. of fresh solvent. Also, 20 cm. by 20 cm. pieces of Whatman No. 3 filter paper were saturated with appropriate solvent and applied to the chromatography tank walls to aid in producing a solvent saturated atmosphere within the tank.

Two spotted chromatographic plates were placed in each tank with their lower edges dipped into the solvent. The plates were first developed in direction A for 4.0 hours with the solvent from ascending 19 cm. from the lower edge of the plate. The plates were then taken out of the tanks and allowed to dry for 15 minutes at room temperature and then placed in an oven at 105°C. for 10 minutes. The plates were allowed to partially dry before placement in the oven to avoid possible charring of the cellulose in the presence of both acid from the solvents and heat. After drying, the plates were cooled to room temperature and turned 90° counter-clockwise. The plates were then placed in another chromatography tank as above and developed in direction B with the solvent system described. The solvent front was

allowed to ascend 19 cm. in direction B (3.0 hours) and the plate removed from the tank. The plate was dired in the same manner as indicated above.

The amino acid and other reacting substances were located on the chromatographic plates by spraying with ninhydrin reagent. This reagent was prepared according to the method of E. Stahl (1970) using 300 mg. ninhydrin reagent (Olin-Matheson Chemical Co.), 100 ml. butanol, and 3 ml. acetic acid.

The plates were sprayed with ninhydrin solution so as to saturate the entire plate. The plates were then dried for 15 minutes at room temperature and placed in an oven at 105°C. for 30 minutes. After the 30-minute period in the oven, the plates were inspected for coloration and all spots scored with a needle probe. The plates were finally placed on a laboratory bench at room temperature for 24 hours and reinspected to insure slow developing spots would be determined. All amino acid chromatographic patterns of samples and standards were reported by tracing the patterns on onion skin typing paper. These tracings were used later in pattern comparisons. Also, relative spot intensity and color was noted on the tracings along with the amino acid application point.

B. Gas-Liquid Chromatographic Analysis

The development of gas-liquid chromatography (GLC) methods for qualitative and quantitative analysis of amino acids in complex physiological fluids and biological substances has recently been perfected to relatively great sophistication (Zumalt *et al.*, 1970).

Due to the great sensitivity, speed and relative simplicity of GLC analysis, this method was chosen as the major analytical procedure in this study of marine fungi amino acid pools.

The instrument used in this investigation was a Hewlett-Packard, Model 402, dual column gas-liquid chromatograph. The GLC was equipped with dual flame ionization detectors and a Hewlett-Packard strip-chart recorder. The column oven of this instrument had a programable temperature control which was used in this study. All gasses used by the instrument were supplied in compressed gas bottles by Liquid Air, San Francisco, California.

1. Column Preparation. The column utilized in the analysis was a 1/8 inch ID by 6 ft., borosilicate glass column. Before packing was added, the column was silanized with Sylon^R (10% dimethyldichlorosilane in toluene; available from Supelco, Inc., Bellefonte, Penn.). This was accomplished by filling the column with Sylon^R and allowing it to stand for 15 minutes. After the standing period, the Sylon^R was removed and the column was washed with toluene, then methanol, and finally with three washes of acetone. A stream of nitrogen was used to dry the empty column after the wash procedure. A small amount of glass wool was also treated by soaking it in 25 ml. of Sylon^R for 15 minutes. It was rinsed in a similar manner as described above. This was later used for plugging of the ends of the packed column.

The column was packed with 3% OV-1 (dimethylsiloxane) on Gaschrom-Q^R support (100-120 mesh). The packing was purchased premixed from Applied Science Laboratories, State College, Pa. The packing was poured into the column (1.8 gm. per foot of column) and packed

into place with the aid of a vibrator (Gehrke and Leimer, 1971). The column was packed in such a manner as to avoid having packing material in the area of the column that was in contact with the flash heater manifold. This was to avoid interference during the analysis from degrading column material because of the high temperature of that area of the column. The ends of the column were plugged with the silanized glass wool to hold the packing in place. The column was placed in the chromatograph oven and flushed with carrier gas (nitrogen, 50 ml. per min. flow rate). The oven was then heated to 300°C and the column allowed to condition at this temperature for 24 hours with a nitrogen flow of 10 ml. per minute traveling through it. After this initial conditioning period nitrogen was allowed to flow through the column at the same rate between periods of actual instrument use (Gehrke and Leimer, 1971).

2. Amino Acid Derivatization Procedures. The low volatility of amino acids has prevented their direct analysis by gas-liquid chromatography, however; the preparation of volatile derivatives has now circumvented any problems concerning amino acid volatization. The methods used in producing amino acid derivatives in this study are a combination of those of Gehrke *et al*. (1970) and Bergstrom *et al*. (1970).

The amino acid derivatives produced for this study were N-trimethylsilyl trimethylsilyl esters (TMS) using bis (trimethylsilyl) trifluroacetamide (BSTFA) as a silylation reagent. FSTFA was acquired from Supelco Inc., Bellefonte, Penn. The TMS derivatives of amino adics produced with this reagent are very volatile and the reaction

products formed in the derivatization reaction with BSTFA do not interfere with GLC analysis of these compounds.

Preparation of the TMS derivatives was carried out in the following manner. One milligram of pure standard amino acid was placed in a ¼ inch by 2 inch glass screw-cap vial. One milliliter of anhydrous methylene chloride was added to the vial which was then placed in a sand bath and heated to 70°C. with the aid of a hot plate. The methylene chloride was allowed to evaporate off and the procedure was repeated two more times. The purpose of this step was to azeotropically remove any moisture which might be present in the sample (Gehrke et al., 1970). The sample was then placed under a stream of dry nitrogen for 30 minutes while still in the sand bath at a temperature of 70°C. This procedure was carried out for all amino acid samples used in this investigation.

Mycelial extracts were prepared for derivatization by placing 1 ml. of the methanolic amino acid solution in vials as described above. This solution was allowed to evaporate to dryness on the sand bath at 70°C. Once dry, the samples were treated in the same manner as the standard amino acid samples.

A second series of standard amino acids was also made. This set contained alanine as an internal standard. This series was prepared in exactly the same manner as the other standard series except 1 mg. of alanine was added to each vial prior to dehydration and derivatization procedures.

The standard amino acid series for GLC analysis was composed of the following amino acids: alanine, glycine, valine, proline, leucine, isoluecine, serine, threonine, hydroxyproline, aspartic acid, glutamic acid, methionine, phenylalanine, tyrosine, ornithine, tryptophan, cystine. Histidine and cysteine were omitted for reasons discussed subsequently. Standard amino acid mixtures of all the above amino acids were prepared by dissolving 1 mg. of each amino acid in 50 ml. of methanol. A chromatographic standard mixture was prepared by placing 1 ml. of the stock solution above in a glass vial as described. This mixture was then dehydrated by the above indicated procedure prior to derivatization.

All samples and standard amino acid mixtures were derivatized by adding 0.25 ml. of BSTFA to each vial. The vials were then sealed and placed on sand bath at 135°C. for 1 hour. Only the bottoms of the vials were buried in the sand to allow plenty of space for atmospheric cooling, and thus, refluxing to occur.

After refluxing, the derivatized solutions were allowed to cool to room temperature and then subjected to the GLC analysis. When the analysis lasted more than one day, the derivatives were stored in a refrigerator at 5°C. overnight. The next day they were again refluxed at 135°C. for 1 hour before analysis. If a standard or sample was needed for more than three days, a freshly derivatized one was prepared.

3. GLC Operating Conditions and Procedures. Prior to actual sample analysis, a preliminary investigation was done to ascertain the optimum operating conditions for amino acid analysis with GLC.

The object of this preliminary study was to determine conditions and procedures to resolve amino acid mixtures and insure reproducibility, and overall efficiency.

The operating conditions finally decided upon are as follows: For the analysis, the column oven was programmed for a 7.5^{o}C. increase per minute. There was no initial isothermal period or no final isothermal period as have been used by many investigators using this type of analysis (Gehrke et al., 1970, 1971; Zumalt et al., 1970; Bergstrom, 1970; Pockington, 1972). It was found in the preliminary study that the oven could not be held reliably at high isothermal temperature after a programmed run with this instrument. Also, it was found that derivatization reaction products came off the column prior to any of the amino acid derivatives if an initial isothermal period was omitted. Thus, initial and final isothermal periods were not included in the regime. The initial column temperature decided upon was 75°C. At the point of sample injection into the column, the 7.5^{o}C/min, increase program was started. This increase in temperature occurred until a final column oven temperature of 300°C was reached. The analysis at that point was complete and the oven automatically shut off.

For the analysis, gas flow rates were adjusted to the following. The carrier gas (nitrogen) was set at a flow rate of 45 ml. per minute Hydrogen was adjusted to 25 ml. per minute and air (breathing air quality) was set at 35 ml. per minute. All gas input pressures were set at 20 psi.

The injector port flash heater was adjusted to 200°C. and the detector temperature was set at 250°C. as indicated by Pockington (1972). Detector range was set at 10^2 and attenuation was adjusted to 16 for standard mixture and individual standard analytical runs, while extract samples were run at an attenuation of 4.

For all analytical runs, exactly 5 microliters of sample were injected directly into the column with a Hamilton syringe. After each analytical run, the column oven was allowed to cool to 75°C. A subsequent analytical run was not made until the oven stablized isothermally at 75°C. for 10 minutes.

RESULTS

Extracts containing the free amino acids of all organisms previously described were subjected to thin-layer and gas-liquid chromatographic analysis. The results of both methods produced strong collaborative data.

Identification of the amino acids on TLC plates was accomplished by chromatographing solutions of standard amino acids singly, in combination with extracts and comparing these with chromatograms of extracts without additions. These were also compared with plates spotted with mixtures of standard amino acids.

Thin-layer chromatograms of all species of marine fungi studied showed similar amino acid patterns with exception of 3 amino acids, hydroxyproline, phenylalanine, and methionine. Hydroxyproline was only detected in three organisms; *Lignincola laevis*, *Leptosphaeria eraemaris*, and *Remispora hamata* by this analytical method. Also phenylalanine was not shown to be present in *Lignincola laevis* or *Halosphaeria quadriremis*, and methionine was not detected by this method in extracts of *Zalerion maritimum* (incubated for 7 days) or *Halosphaeria* quadriremis. All other amino acids included in the standard mixture were shown to be present in all the organisms with this method. The results are summarized in Tables III-XVI and Figures 1-26.

Gas-liquid chromatography results indicated all of the amino acids included in the standard mixtures were present in the investigated organisms.

The results of the gas-liquid chromatography analyses was interpreted by the method of Gehrke et al. (1970). Relative retention times were determined for the individual amino acids in standard mixtures and in extract samples by dividing the retention time of the internal standard (alanine) in each sample into the retention time of major peaks for each sample as indicated on the GLC stripchart. This was also done with the individual standard amino acid samples which contained alanine as an internal standard. Relative retention times for standard amino acid mixtures were then tabulated and compared to the relative retention times of the major detector responses or peaks from the mycelial extract samples. When the relative retention time of a standard amino acid peak matched the relative retention times of unknown peaks, the appropriate amino acid was considered to be present.

GLC detector response was considered major if the marker pen deflection was greater than 0.1 millivolt or greater than 1/10 of full scale deflection. If an emerging peak from a mycelial extract had a relative retention time that matched a standard amino acid and had a detector response greater than 0.1 mv., the peak was considered to be the particular amino acid. If the detector response was less than 0.1 mv., the presence of the amino acid was considered equivocal, and if detector response was not indicated at an appropriate retention time the amino acid was considered absent.

The above criteria were used to determine all amino acids except phenylalanine, ornithine, lysine, tryptophan, tyrosine and cystine.

These amino acids when derivatized seemed to have relatively low detector responses when compared to the other amino acids at equal milligram concentration. Therefore, a detector response of 0.025 mv. was considered indicative of the presence of these amino acids. All relative retention time calculations were based on an average of three analytical runs. The results of GLC analysis are summarized in Tables III-XVI and Figures 1-26.

TABLE III

GLC RELATIVE RETENTION TIMES OF FREE AMINO ACIDS FOUND IN INVESTIGATED MARINE FUNGI*

Amino acid Standards	Organism (Isolate Code**)												
	STD.	R-2	R-4	R-6	R-13	R-19	R-524	R-572	R-577	R-601	R-605	R-6 14/d	F-1
Ala	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Gly	1.06	1.06	1.05	1.06	1.06	1.05	1.06	1.05	1.07	1.08	1.06	1.07	1.08
Val	1.46	1.44	1.45	1.47	1.45	1.46	1.45	1.46	1.48	1.44	1.47	1.47	1.49
Pro	1.66	1.68	1.68	1.67	1.68	1.67	1.68	1.69	1.68	1.68	1.69	1.65	1.67
Leu	1.75	1.74	1.75	1.75	1.74	1.74	1.73	1.74	1.75	1.76	1.78	1.74	1.73
Ile	1.84	1.86	1.85	1.83	1.83	1.86	1.86	1.86	1.86	1.83	1.84	1.86	1.87
Ser	2.09	2.10	2.09	2.07	2.08	2.10	2.10	2.10	2.08	2.10	2.07	2.07	2.08
Thr	2.24	2.23	2.26	2.21	2.23	2.21	2.23	2.25	2.23	2.21	2.26	2.26	2.25
Hpr	2.49	2.52	2.51	2.51	2.50	2.51	2.48	2.51	2.52	2.48	2.49	2.52	2.47
Asp	2.57	2.60	2.56	2.58	2.57	2.59	2.58	2.59	2.60	2.56	2.57	2.59	2.58
Met	2.68	2.70	2.67	2.69	2.69	2.69	2.68	2.69	2.67	2.70	2.71	2.67	2.69
Glu	2.93	2.95	2.94	2.96	2.94	2.94	2.94	2.90	2.94	2.93	2.94	2.95	2.92
Phe	3.12	3.13	3.10	3.11	3.10	3.13	3.11	3.11	3.10	3.14	3.12	3.10	3.11
Orn	3.51	3.48	3.49	3.50	3.50	3.51	3.48	3.53	3.50	3.52	3.51	3.52	3.54
Lys	3.86	3.89	3.84	3.85	3.86	3.88	3.89	3.88	3.88	3.84	3.85	3.88	3.86
Tyr	4.08	4.10	4.09	4.08	4.07	4.09	4.06	4.09	4.08	4.10	4.08	4.07	4.09
Try	4.16	4.14	4.17	4.18	4.16	4.18	4.15	4.16	4.14	4.16	4.19	4.18	4.19
Cys	4.65	4.68	4.68	4.67	4.64	4.66	±4.69	4.63	4.66	4.68	4.66	±4.61	4.63

*Relative retention time calculations based on three analytical runs. Note Results Section.
**Note Table II for organism isolate codes.

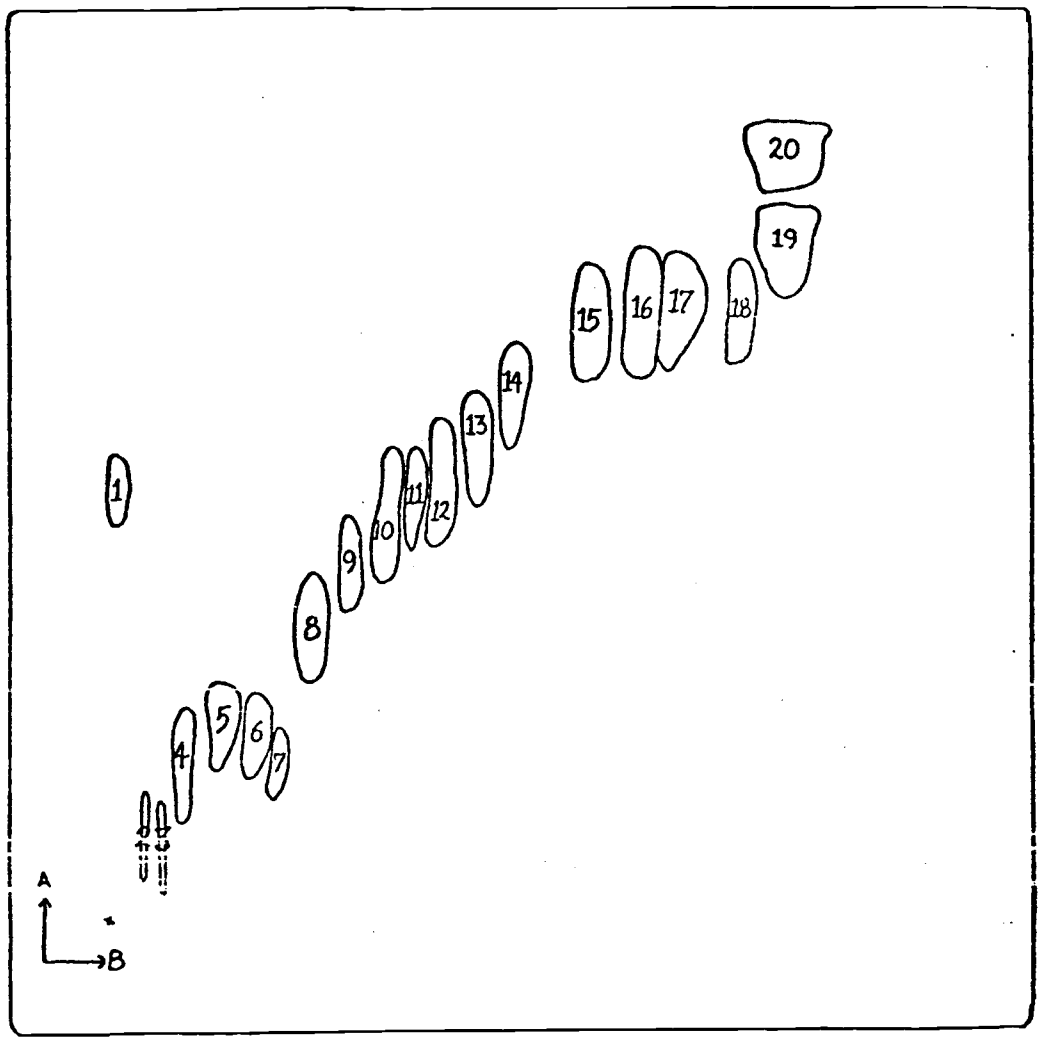

FIGURE 1. Two-dimensional chromatogram of a standard mixture of amino acids. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid water (40:20:20 v/v). 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine, 17. methionine, 18. phenylalanine, 19. leucine, 20. isoleucine.

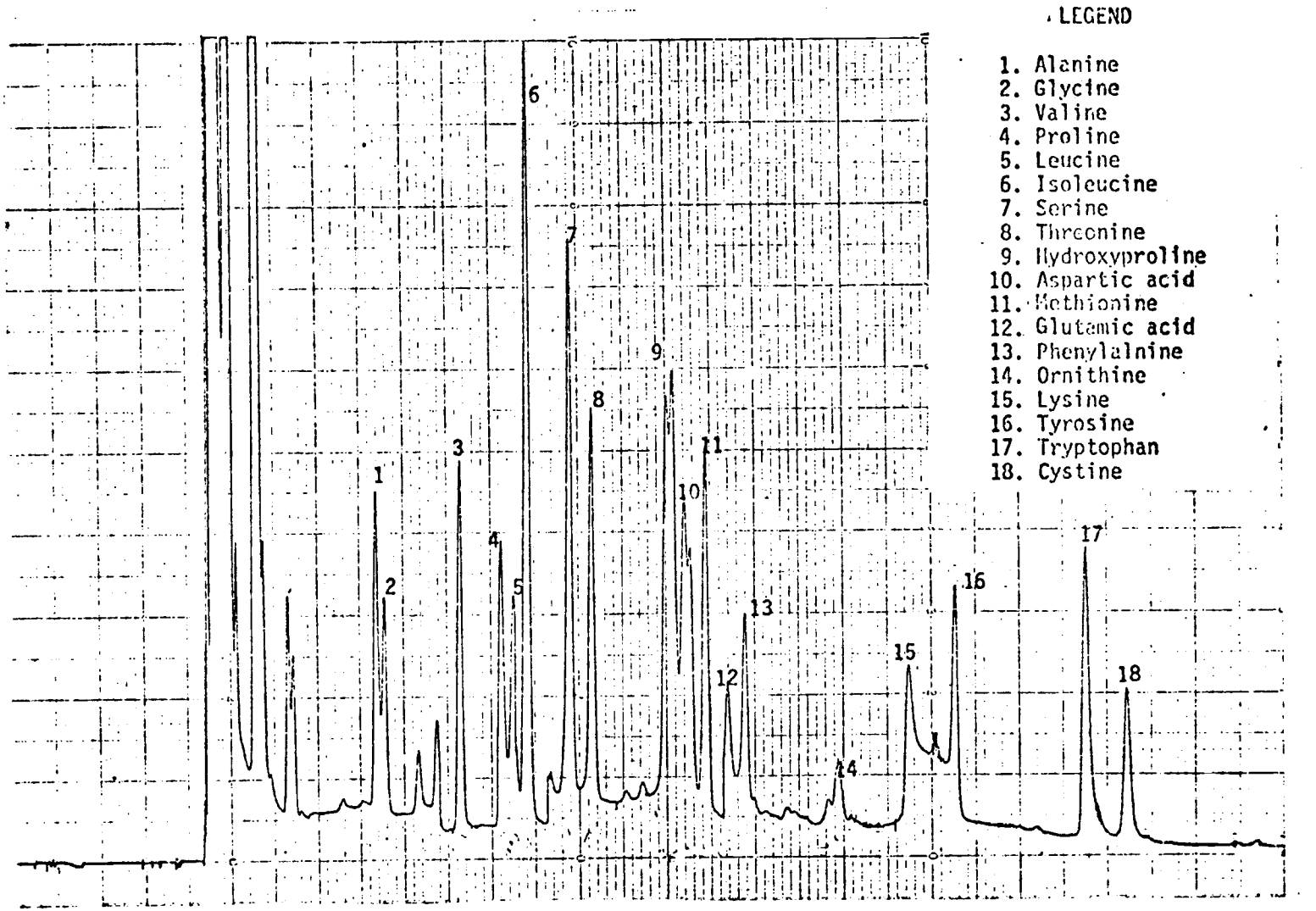

Figure 2. GLC spectrum of a standard amino acid mixture.

TABLE IV

THE FREE AMINO ACIDS FOUND IN *Lignicola laevis* Hohnk.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	±	+
Proline	+	+
Leucine	+	+
Isoleucine	+	+
Serine	+	+
Threonine	+	+
Hydroxyproline	+	+
Aspartic acid	+	+
Methionine	±	+
Glutamic acid	+	+
Phenylalanine	-	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	+	+
Cysteine	+	*
Histidine	+	**

Symbols: +, present; - absent, ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

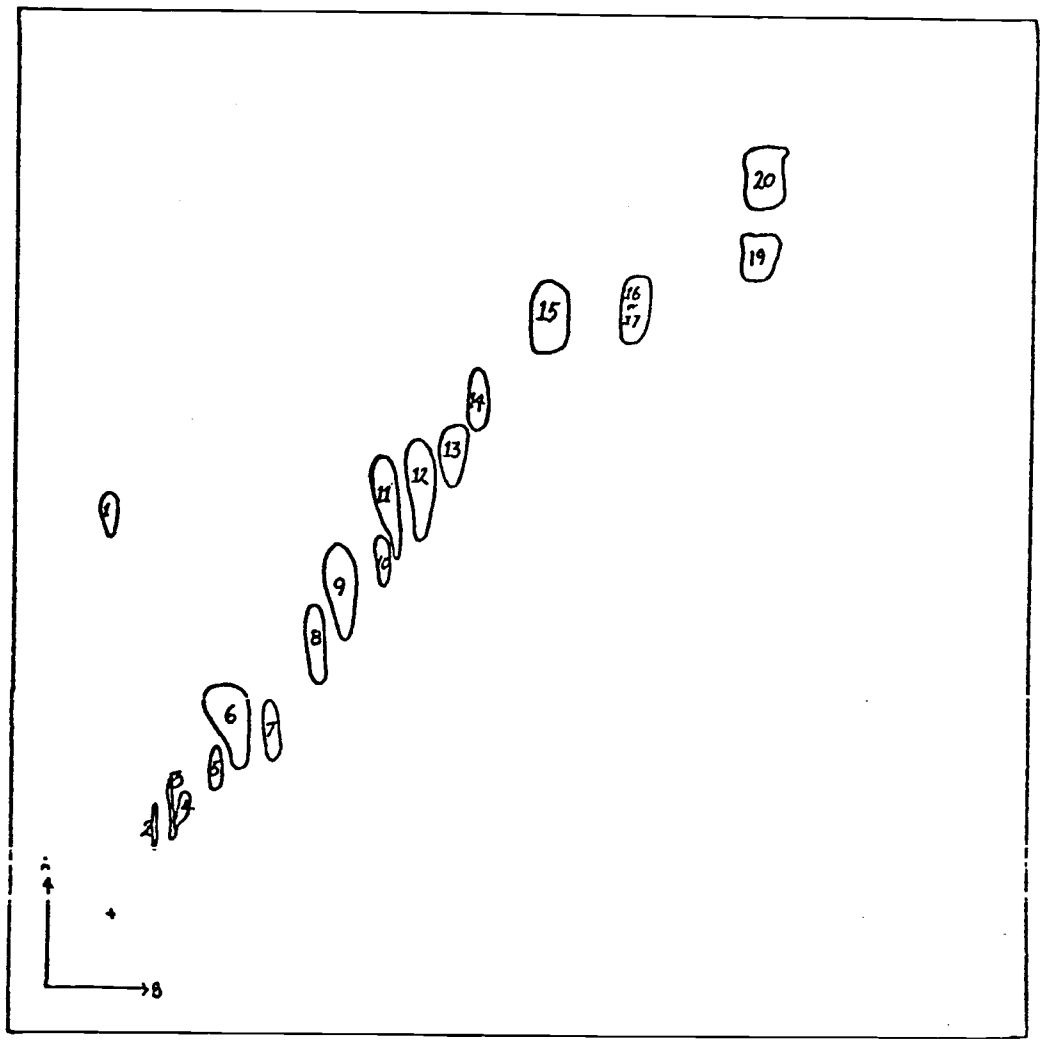

FIGURE 3. Two-dimensional chromatogram of the free amino acids of Lignicola laevis Hohnk. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid-water (40:20:20 v/v). 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine. 17. methionine, 18. phenylalanine, 19. leucine, 20. isoleucine.

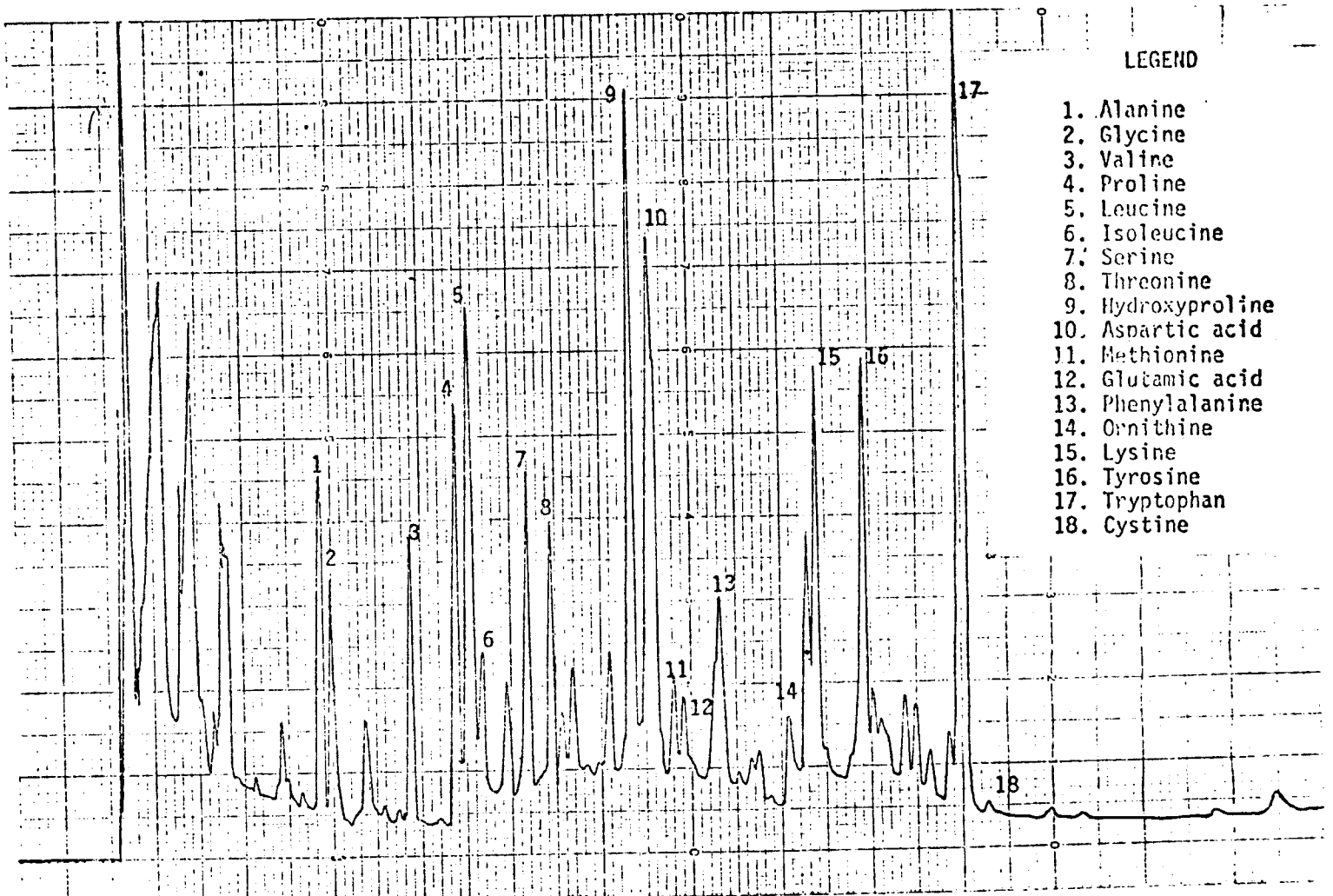

Figure 4. GLC spectrum of the free amino acids found in Lignicola laevis Hohnk.

TABLE V

THE FREE AMINO ACIDS FOUND IN *Nais inornata* Kohlm.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	±	+
Proline	+	+
Leucine	+	+
Isoleucine	+	+
Serine	+	+
Threonine	+	+
Hydroxyproline	-	+
Aspartic acid	+	+
Methionine	±	±
Glutamic acid	+	+
Phenylalanine	+	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	+	+
Cysteine	+	*
Histidine	-	**

Symbols: +, present; - absent; ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

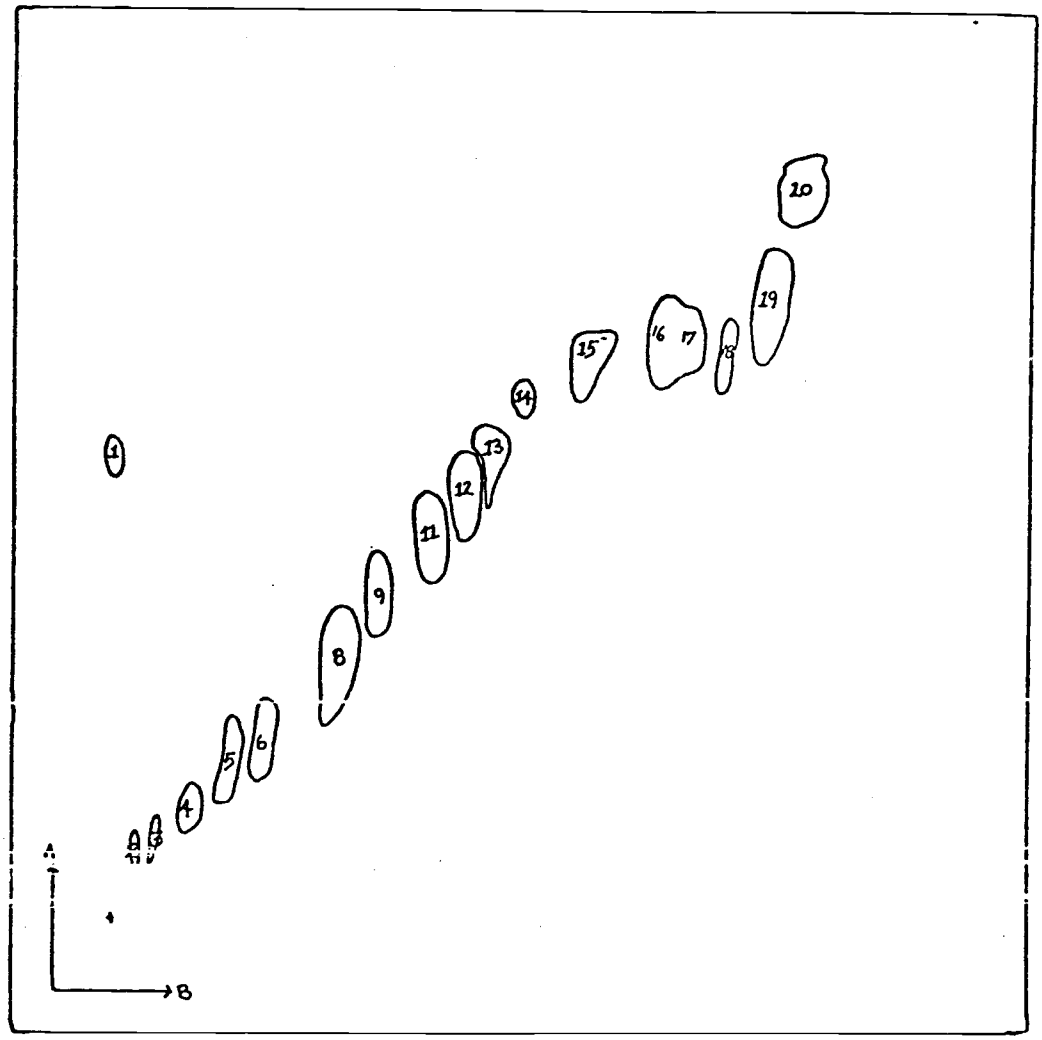

FIGURE 5. Two-dimensional chromatogram of the free amino acids of Nais inornata Kohlm. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid-water (40:20:20 v/v). 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine, 17. methionine, 18. phenylalanine, 19. leucine, 20. isoleucine.

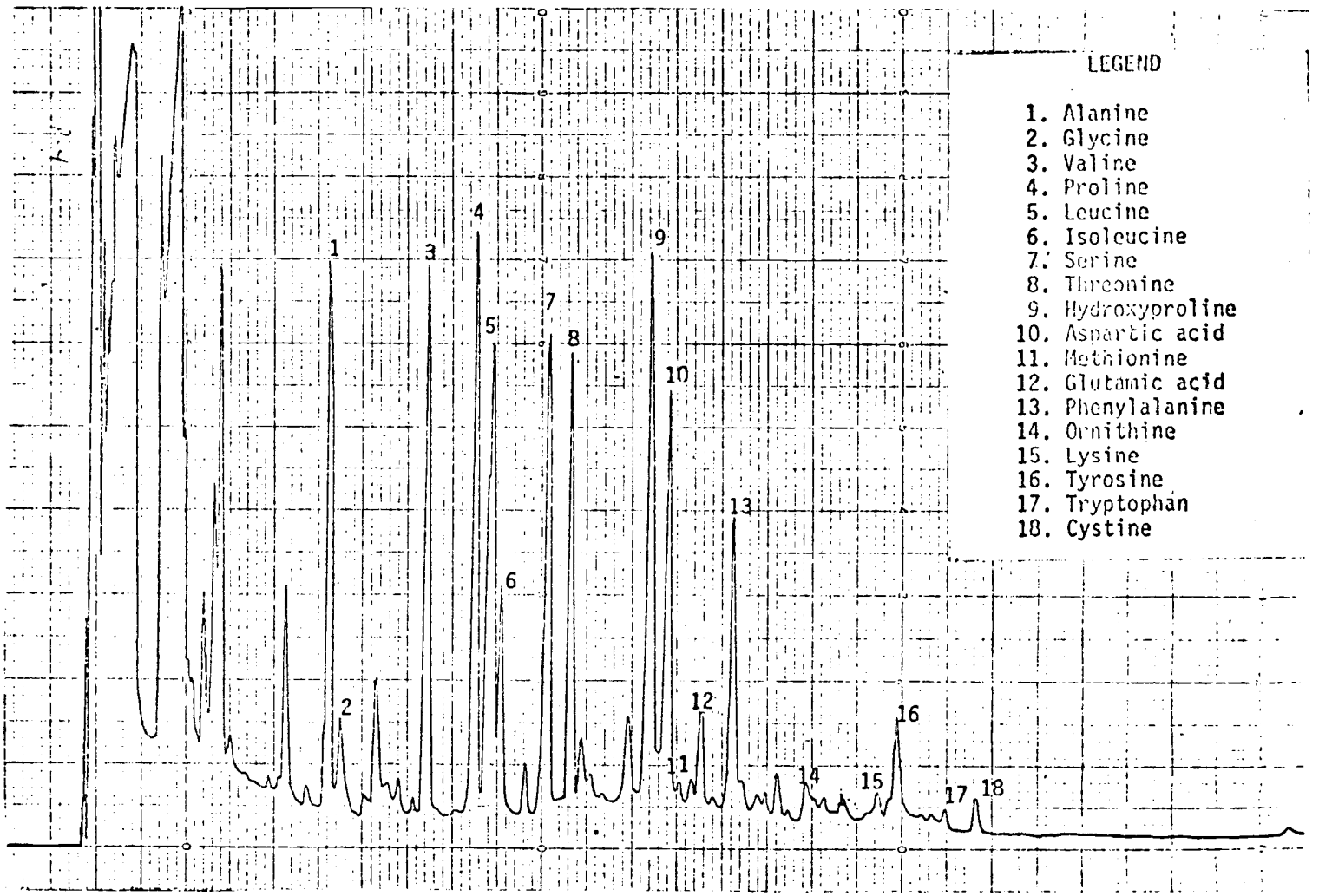

Figure 6. GLC spectrum of the free amino acids found in Nais inornata Kohlm.

TABLE VI

THE FREE AMINO ACIDS FOUND IN *Zalerion maritimum* (Linder) Anastasiou.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	+	+
Proline	+	+
Leucine	+	+
Isoleucine	+	+
Serine	+	+
Threonine	+	+
Hydroxyproline	-	+
Aspartic acid	+	+
Methionine	-	+
Glutamic acid	+	+
Phenylalanine	+	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	±	+
Cysteine	±	*
Histidine	+	**

Symbols: +, present; - absent; ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

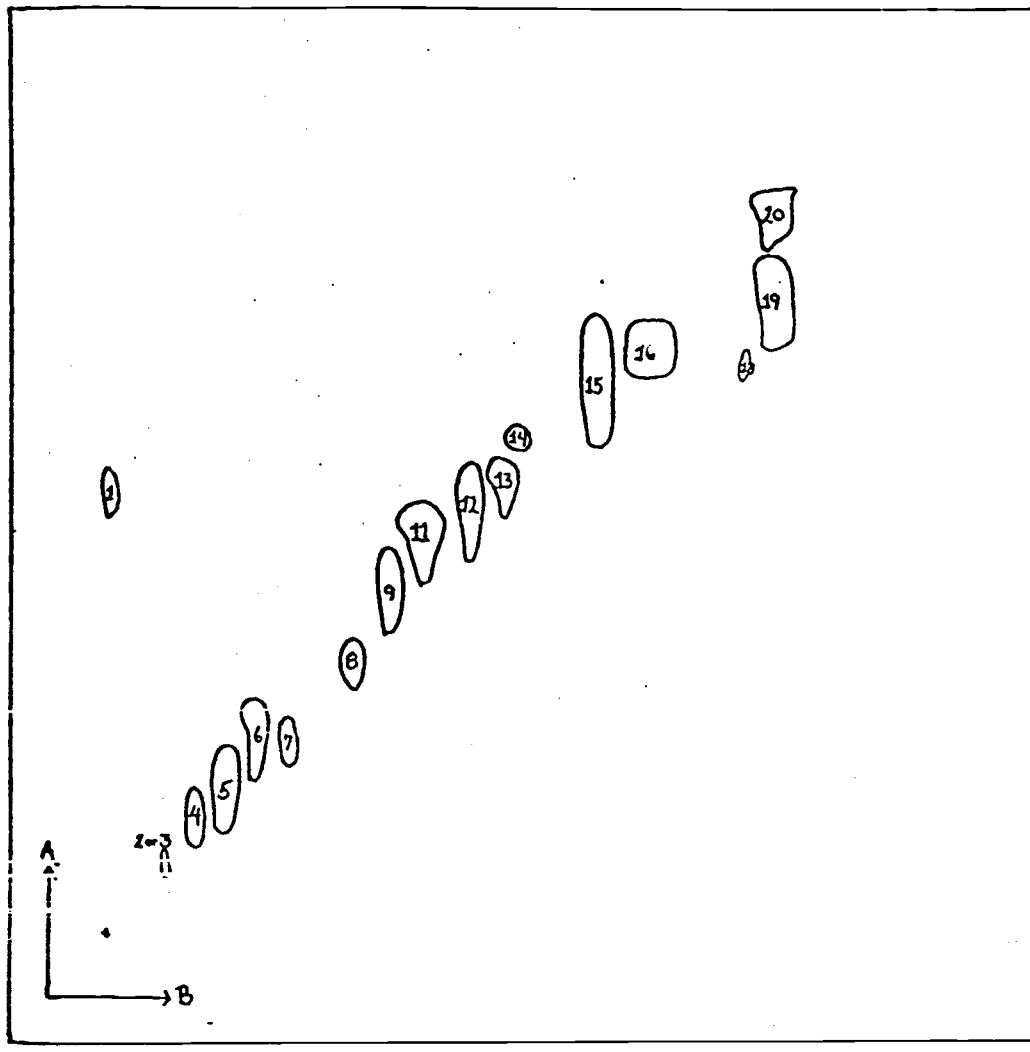

FIGURE 7. Two-dimensional chromatogram of the free amino acids of Zalerion maritimum (Linder) Anastasiou. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid-water (40:20:20 v/v). 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine, 17. methionine, 18. phenylalanine, 19. leucine, 20. isoleucine.

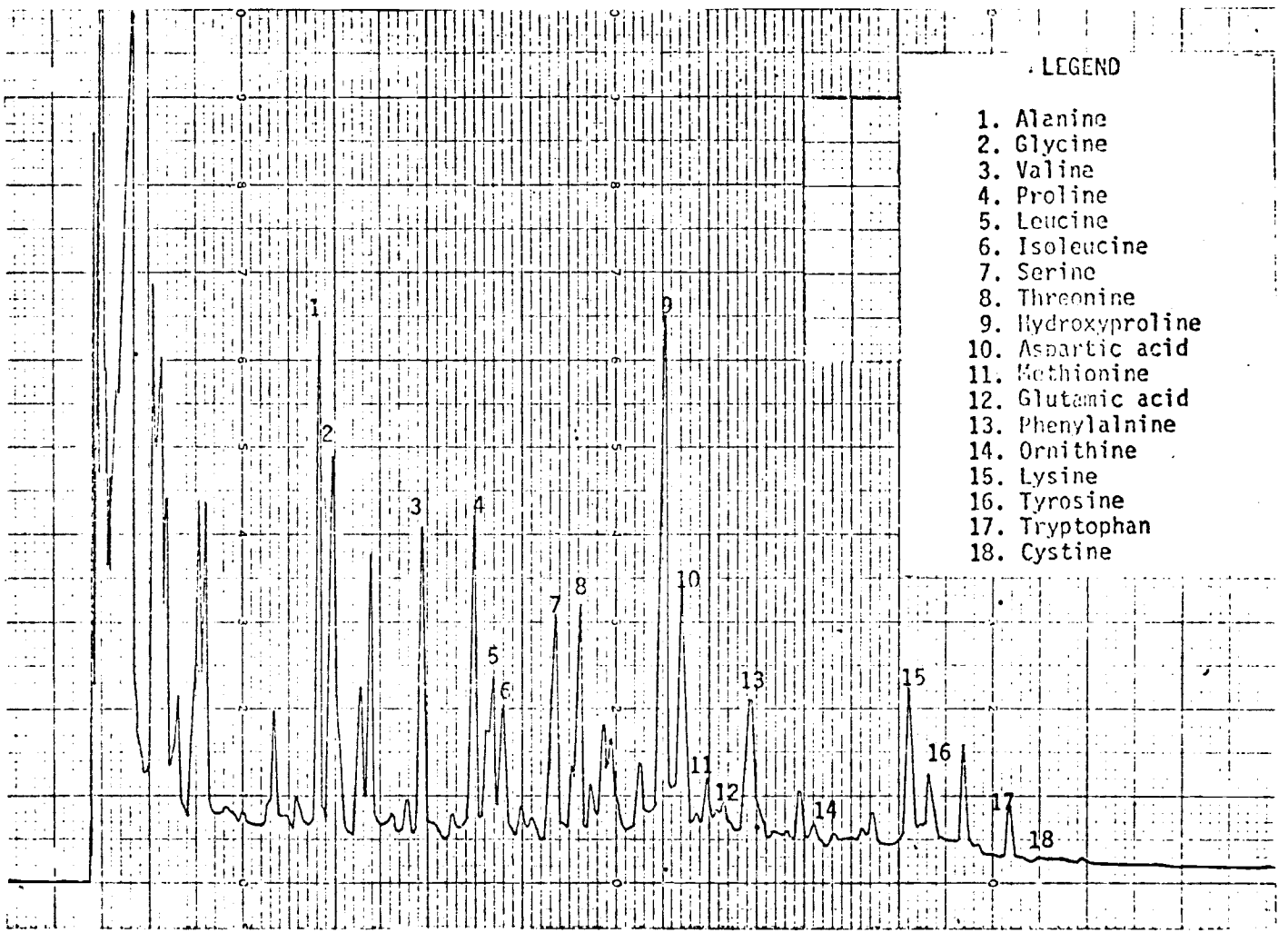

Figure 8. GLC spectrum of the free amino acids found in Zalerion maritimum (Linder) Anastasiou.

TABLE VII

THE FREE AMINO ACIDS FOUND IN *Leptosphaeria oraemaris* Linder.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	±	+
Proline	+	+
Leucine	+	+
Isoleucine	+	+
Serine	+	+
Threonine	+	+
Hydroxyproline	+	+
Aspartic acid	+	+
Methionine	±	+
Glutamic acid	+	+
Phenylalanine	+	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	+	+
Cysteine	+	*
Histidine	+	**

Symbols: +, present; -, absent; ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

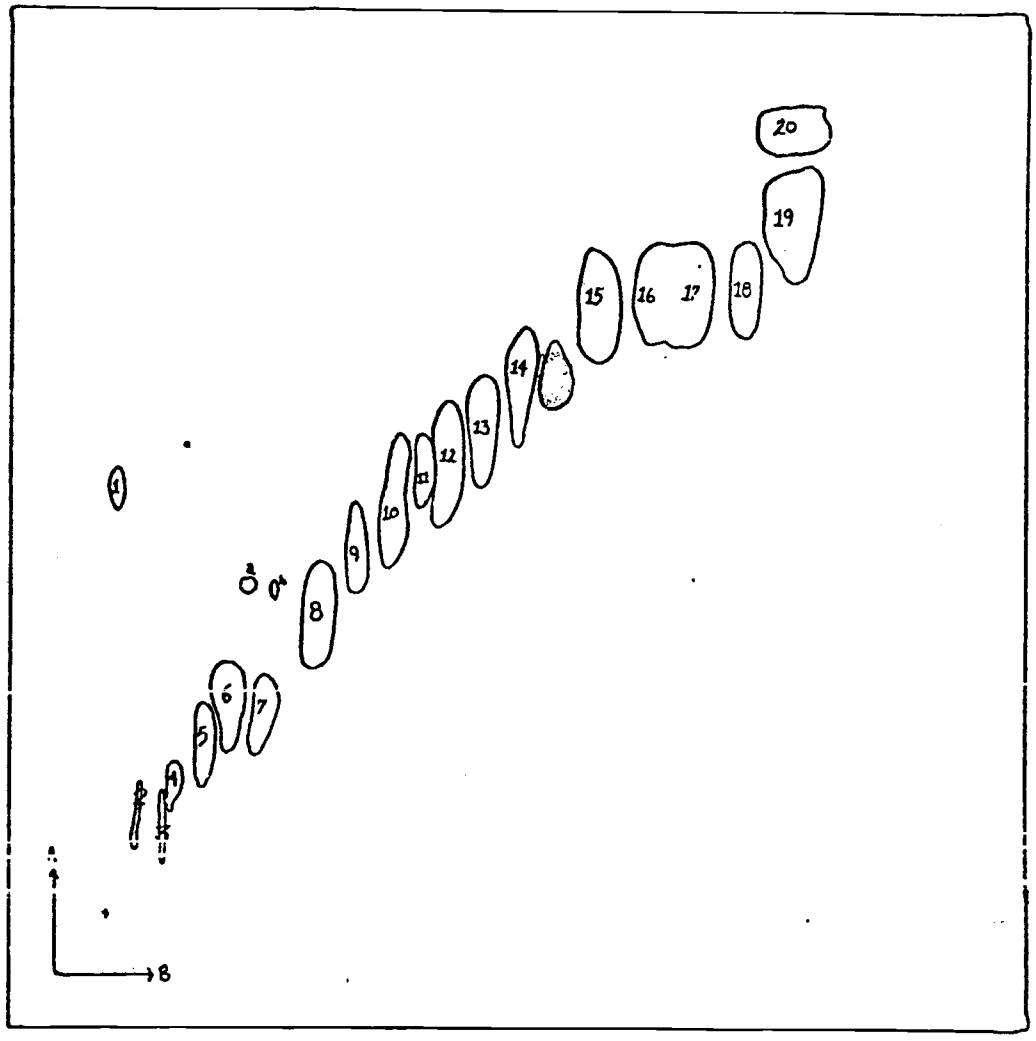

FIGURE 9. Two-dimensional chromatogram of the free amino acids of Leptosphaeria oraemaris Linder. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid water (40:20:20 v/v). 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine, 17. methionine, 18. phenylalanine, 19. leucine, 20. isoleucine, a and b unidentified ninhydrin-positive spots.

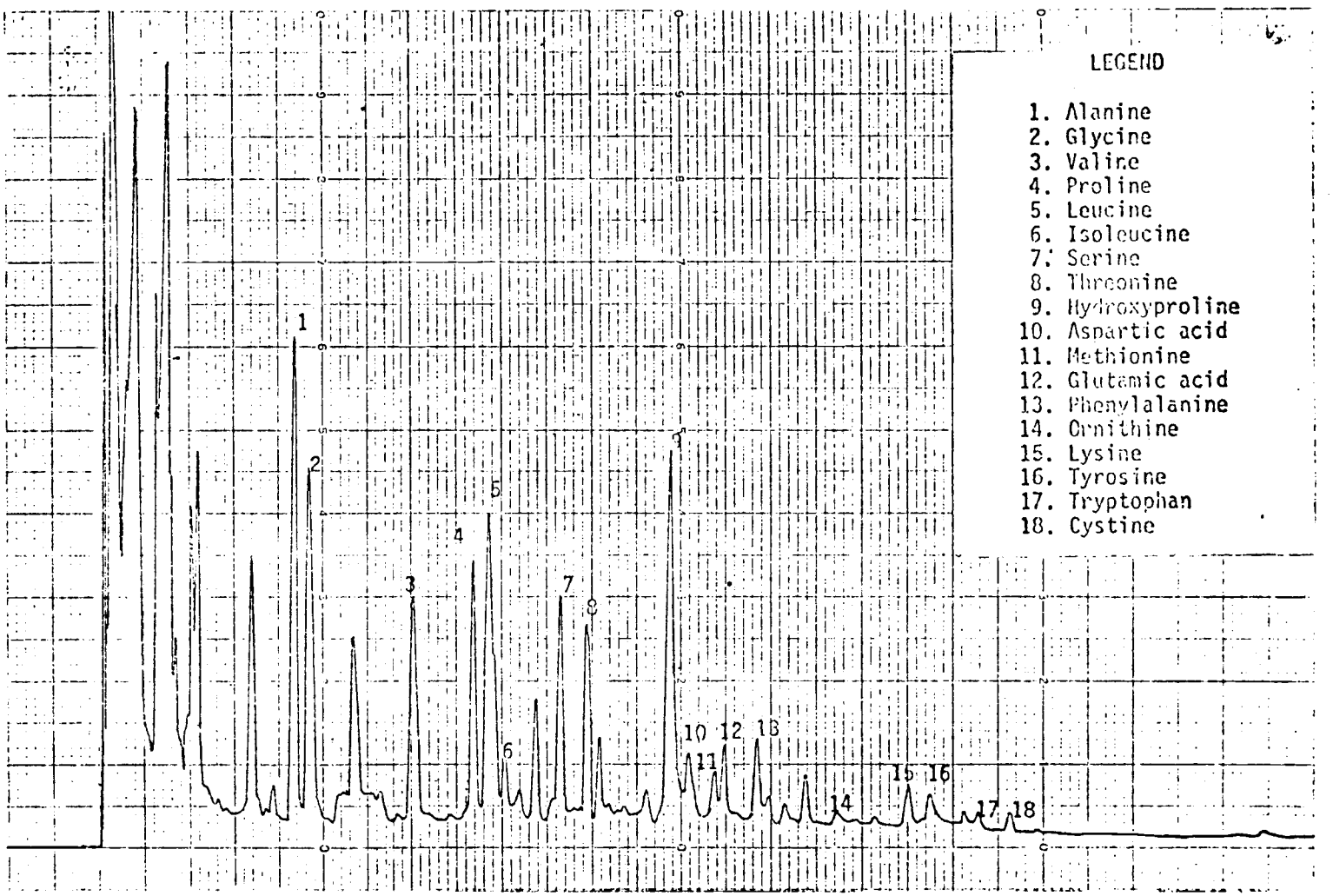

Figure 10. GLC spectrum of the free amino acids found in Leptosphaeria oraemaris Linder.

TABLE VIII

THE FREE AMINO ACIDS FOUND IN *Corollospora maritima* Werdermann.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	+	+
Proline	+	+
Leucine	+	+
Isoleucine	+	+
Serine	+	+
Threonine	+	+
Hydroxyproline	-	+
Aspartic acid	+	+
Methionine	+	+
Glutamic acid	+	+
Phenylalanine	+	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	+	±
Cysteine	+	*
Histidine	+	**

Symbols: +, present; - absent; ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

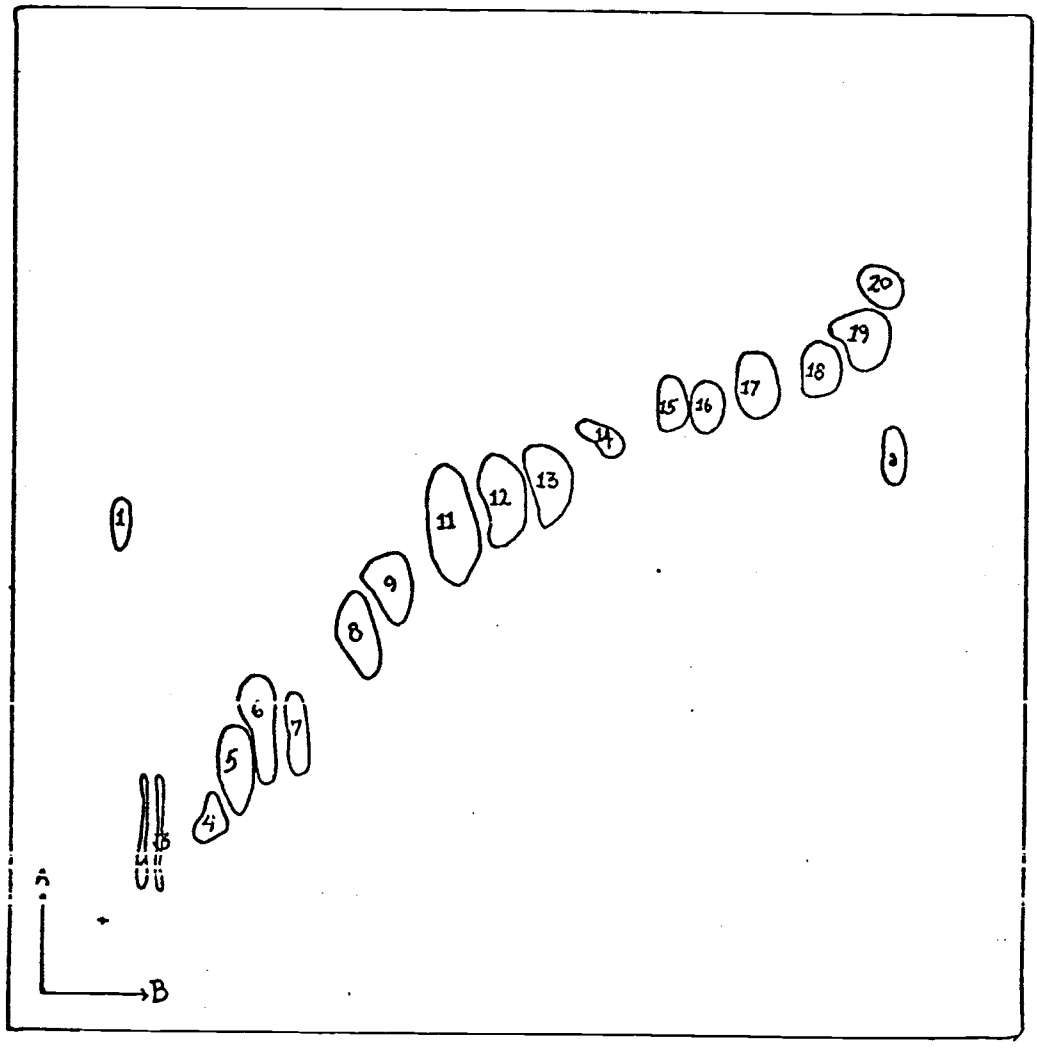

FIGURE 11. Two-dimensional chromatogram of the free amino acids of Corollospora maritima Werdermann. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid-water (40:20:20 v/v). 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16 valine, 17. methionine, 18. phenylalanine, 19. leucine, 20. isoleucine, a unidentified ninhydrin-positive spot.

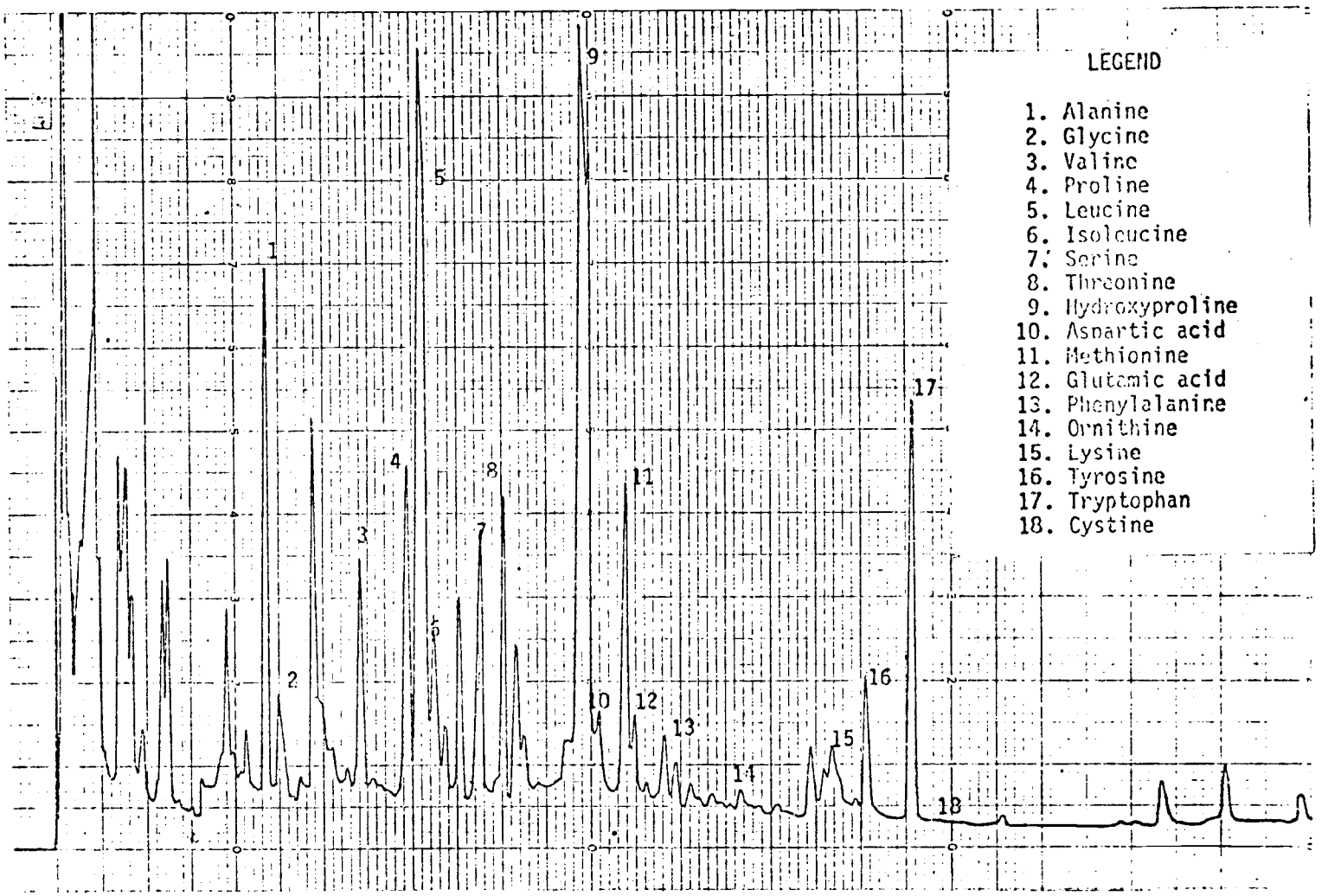

Figure 12. GLC spectrum of the free amino acids found in Corollospora maritima Werderman.

TABLE IX

THE FREE AMINO ACIDS FOUND IN *Halosphaeria mediosetigera* Cribb et Cribb.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	±	+
Proline	+	+
Leucine	±	+
Isoleucine	±	+
Serine	+	+
Threonine	+	+
Hydroxyproline	-	+
Aspartic acid	+	+
Methionine	±	+
Glutamic acid	+	+
Phenylalanine	+	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	±	*
Cysteine	±	*
Histidine	+	**

Symbols: +, present; - absent; ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

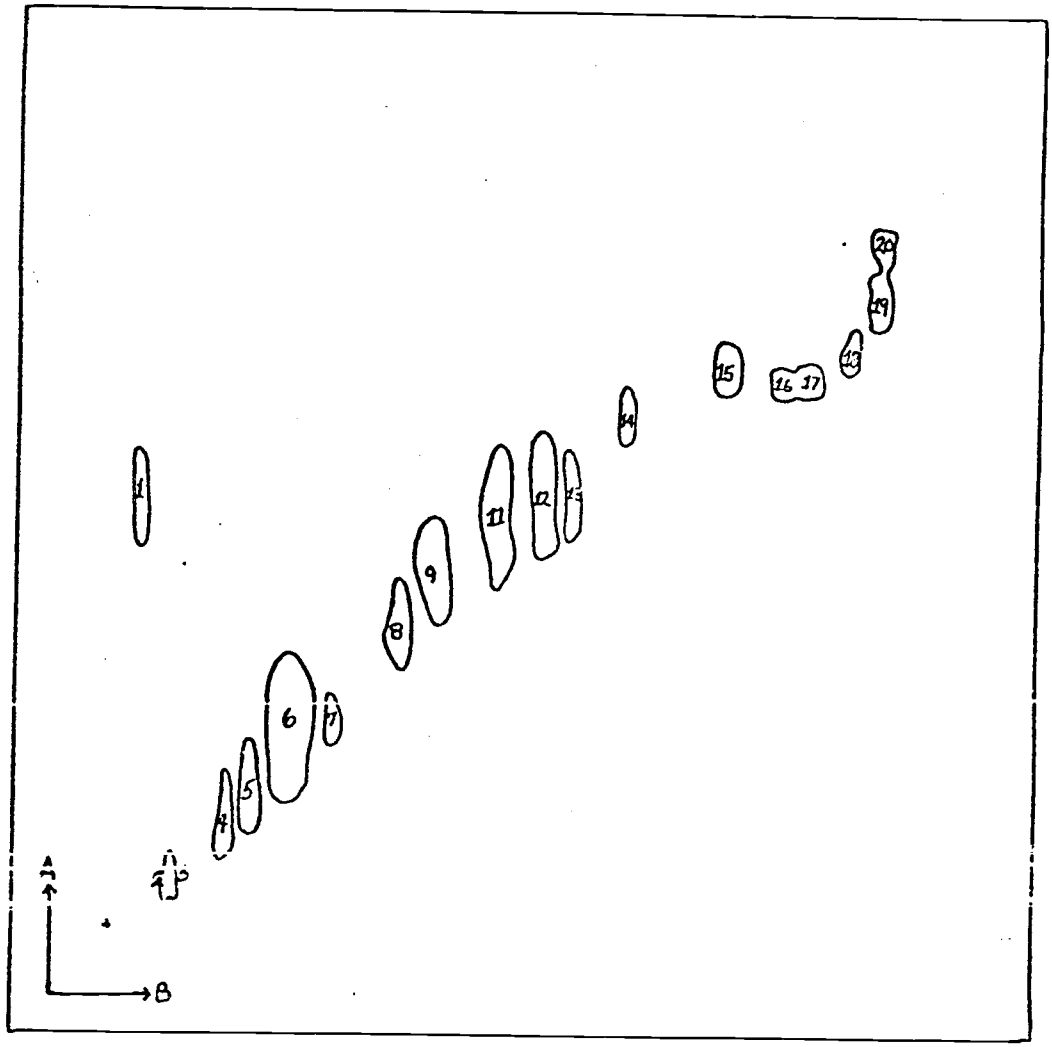

FIGURE 13. Two-dimensional chromatogram of the free amino acids of Halosphaeria mediosetigera Cribb et Cribb. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid-water (40:20:20 v/v). 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine, 17. methionine, 18 phenylalanine, 19. leucine, 20. isoleucine.

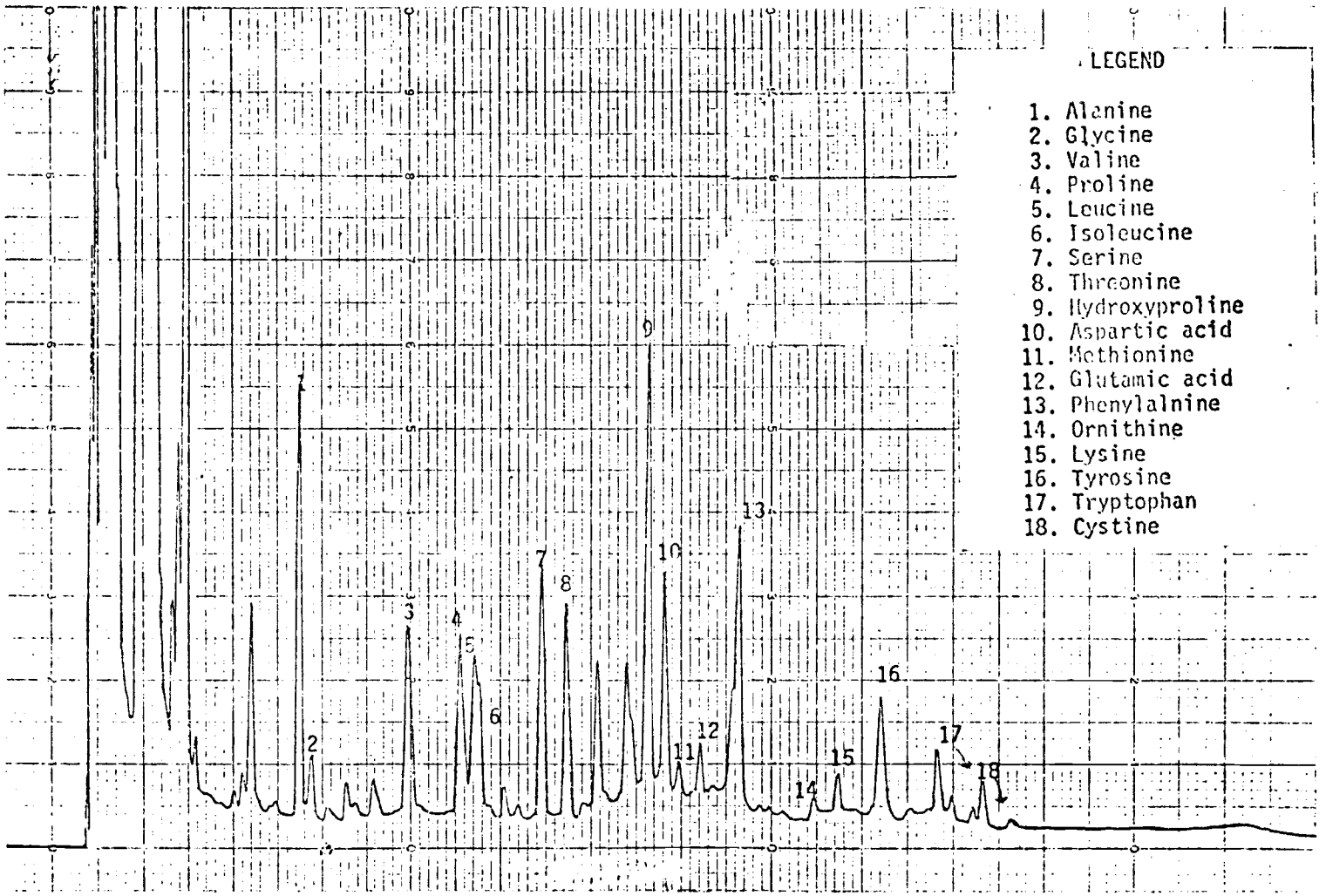

Figure 14. GLC spectrum of the free amino acids found in Halosphaeria mediosetigera Cribb et Cribb.

TABLE X

THE FREE AMINO ACIDS FOUND IN *Halosphaeria quadriremis* (Hohnk) Kohlm.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	+	+
Proline	+	+
Leucine	+	+
Isoleucine	+	+
Serine	+	+
Threonine	+	+
Hydroxyproline	-	+
Aspartic acid	+	+
Methionine	-	+
Glutamic acid	+	+
Phenylalanine	-	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	+	+
Cysteine	+	*
Histidine	+	**

Symbols: +, present; - absent; ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

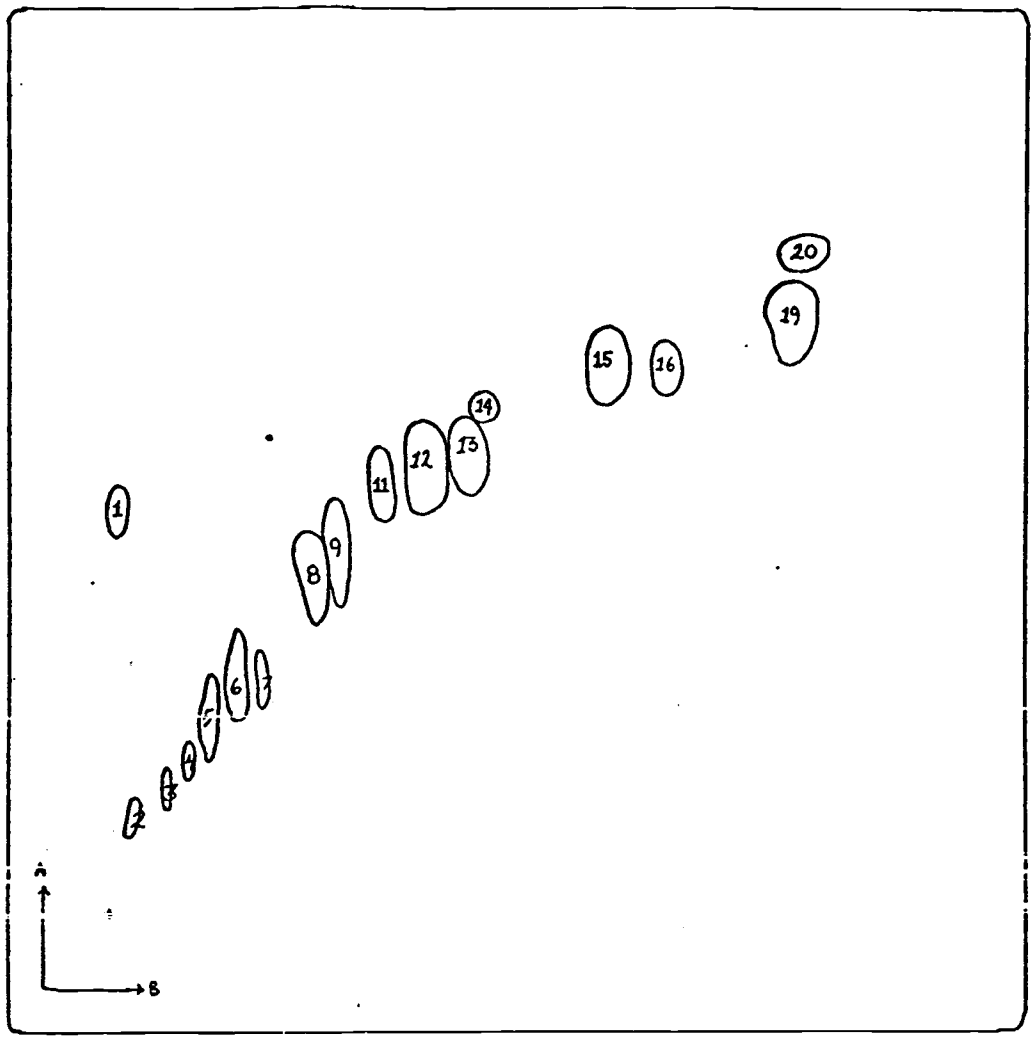

FIGURE 15. Two-dimensional chromatogram of the free amino acids of Halosphaeria quadriremis (Hohnk) Kohlm. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid-water (40:20:20 v/v). 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine, 17. methionine, 18. phenylalanine, 19. leucine, 20. isoleucine.

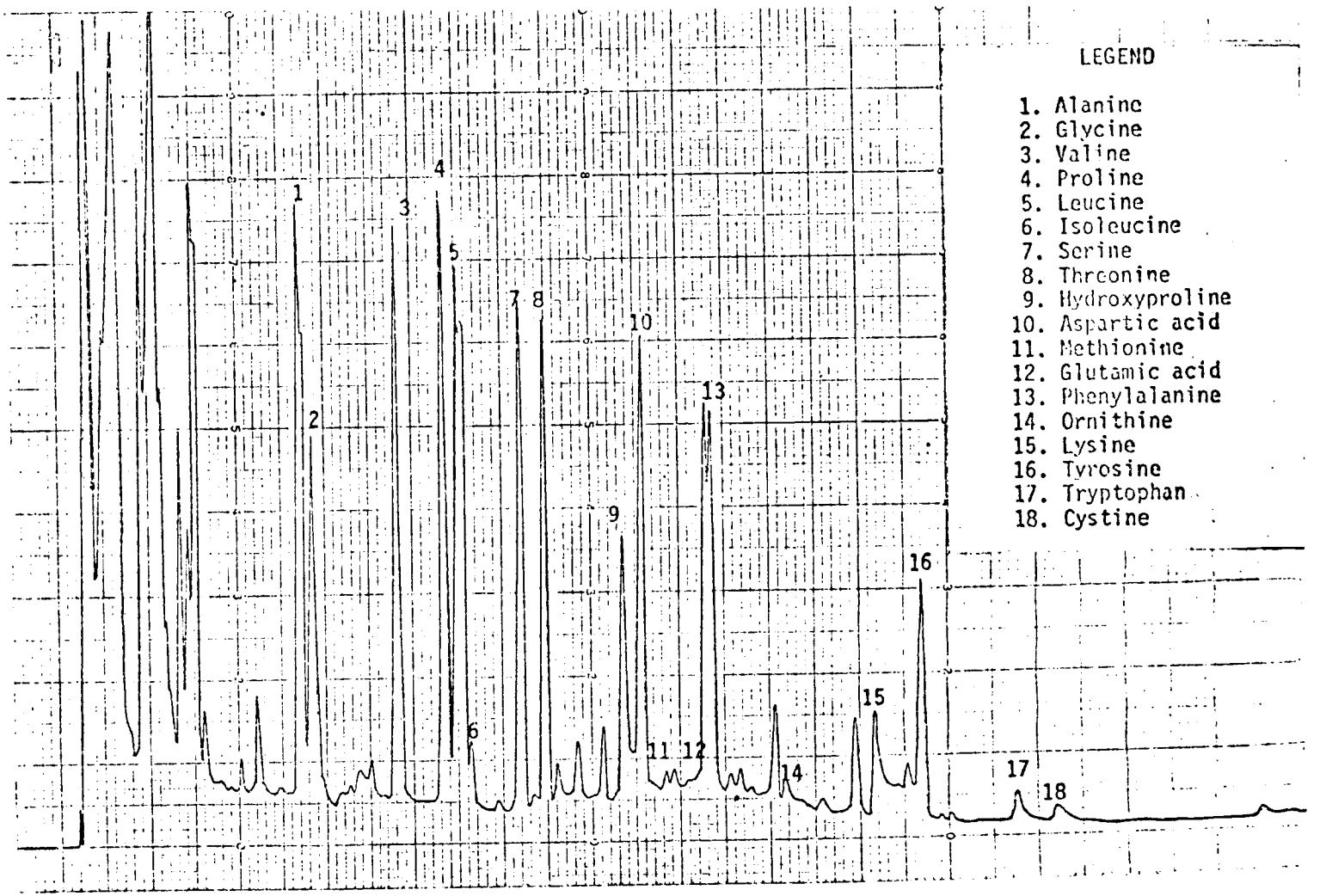

Figure 16. GLC spectrum of the free amino acids found in Halosphaeria quadriremis (Hohnk) Kohlm.

TABLE XI

THE FREE AMINO ACIDS FOUND IN *Remispora hamata* (Hohnk) Kohlm.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	±	+
Proline	+	+
Leucine	+	+
Isoleucine	+	+
Serine	+	+
Threonine	+	+
Hydroxyproline	+	+
Aspartic acid	+	+
Methionine	+	+
Glutamic acid	+	+
Phenylalanine	+	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	+	+
Cysteine	+	*
Histidine	+	**

Symbols: +, present; - absent; ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

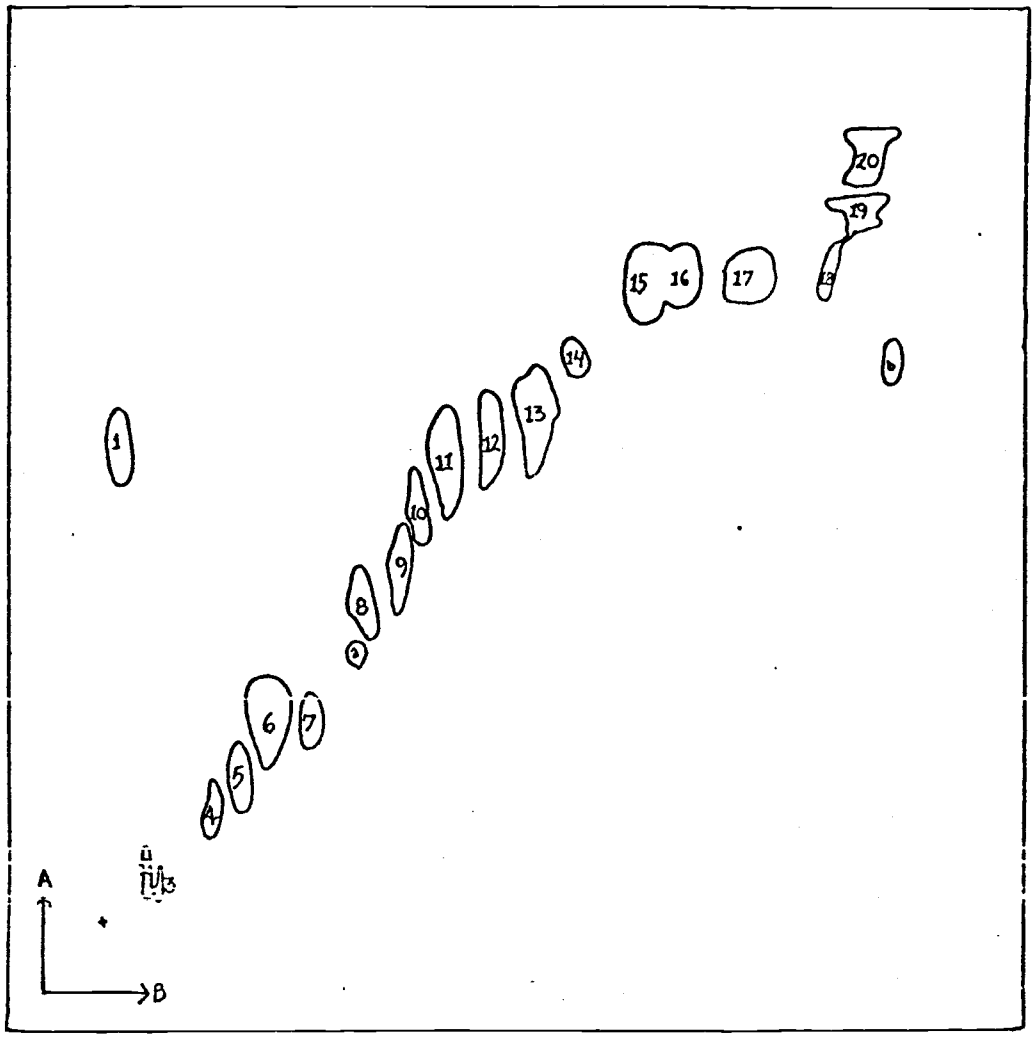

FIGURE 17. Two-dimensional chromatogram of the free amino acids of Remispora hamata (Hohnk) Kohlm. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid-water (40:20:20 v/v). 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine, 17. methionine, 18. phenylalanine, 19. leucine, 20. isoleucine, a and b, unidentified ninhydrin-positive spots.

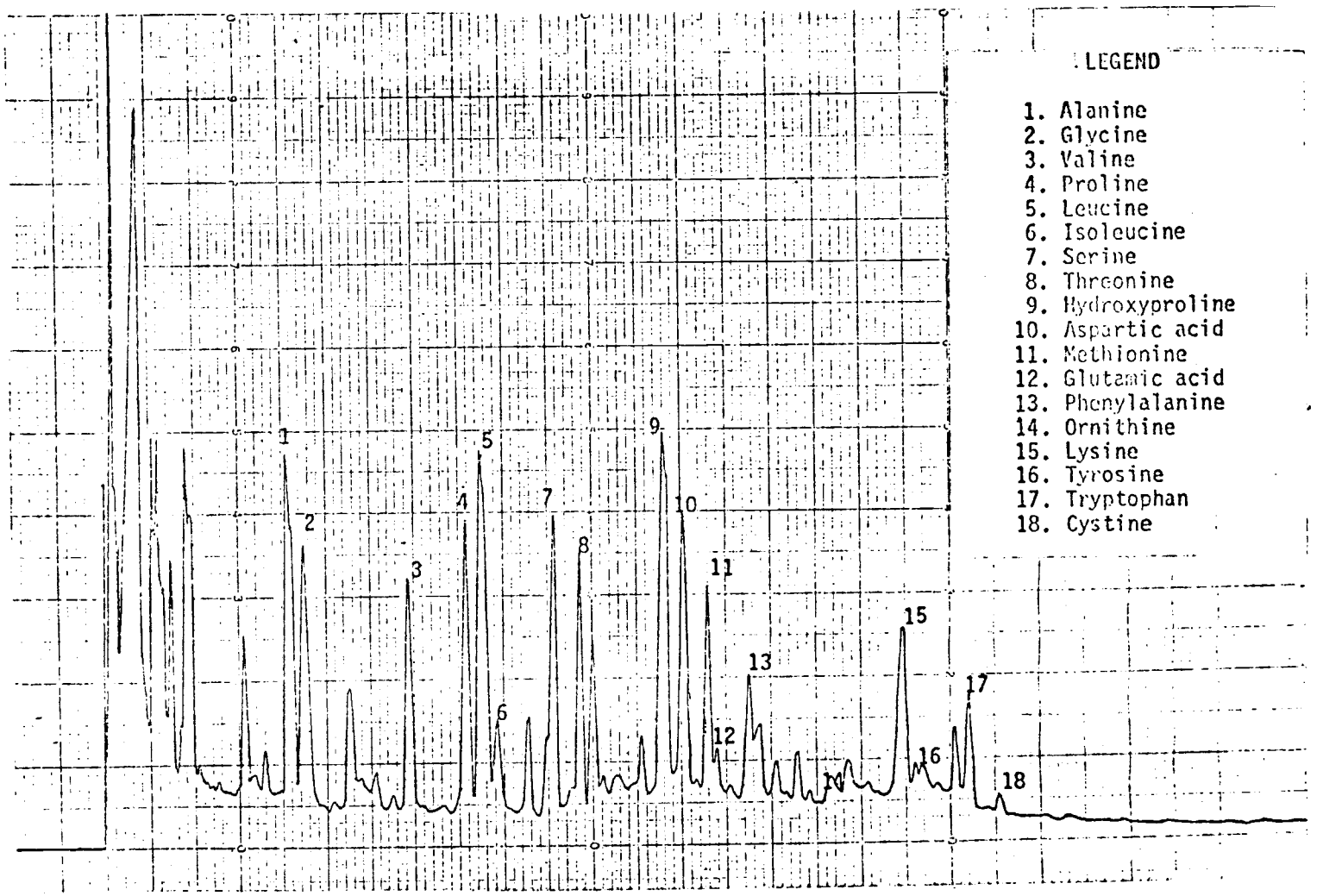

Figure 18. GLC spectrum of the free amino acids found in Remispora hamata (Hohnk) Kohlm.

TABLE XII

THE FREE AMINO ACIDS FOUND IN *Haligena elaterophora* Kohlm.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	±	+
Proline	+	+
Leucine	±	+
Isoleucine	±	+
Serine	+	+
Threonine	+	+
Hydroxyproline	-	+
Aspartic acid	+	+
Methionine	±	±
Glutamic acid	+	+
Phenylalanine	+	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	+	+
Cysteine	+	*
Histidine	+	**

Symbols: +, present; - absent; ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

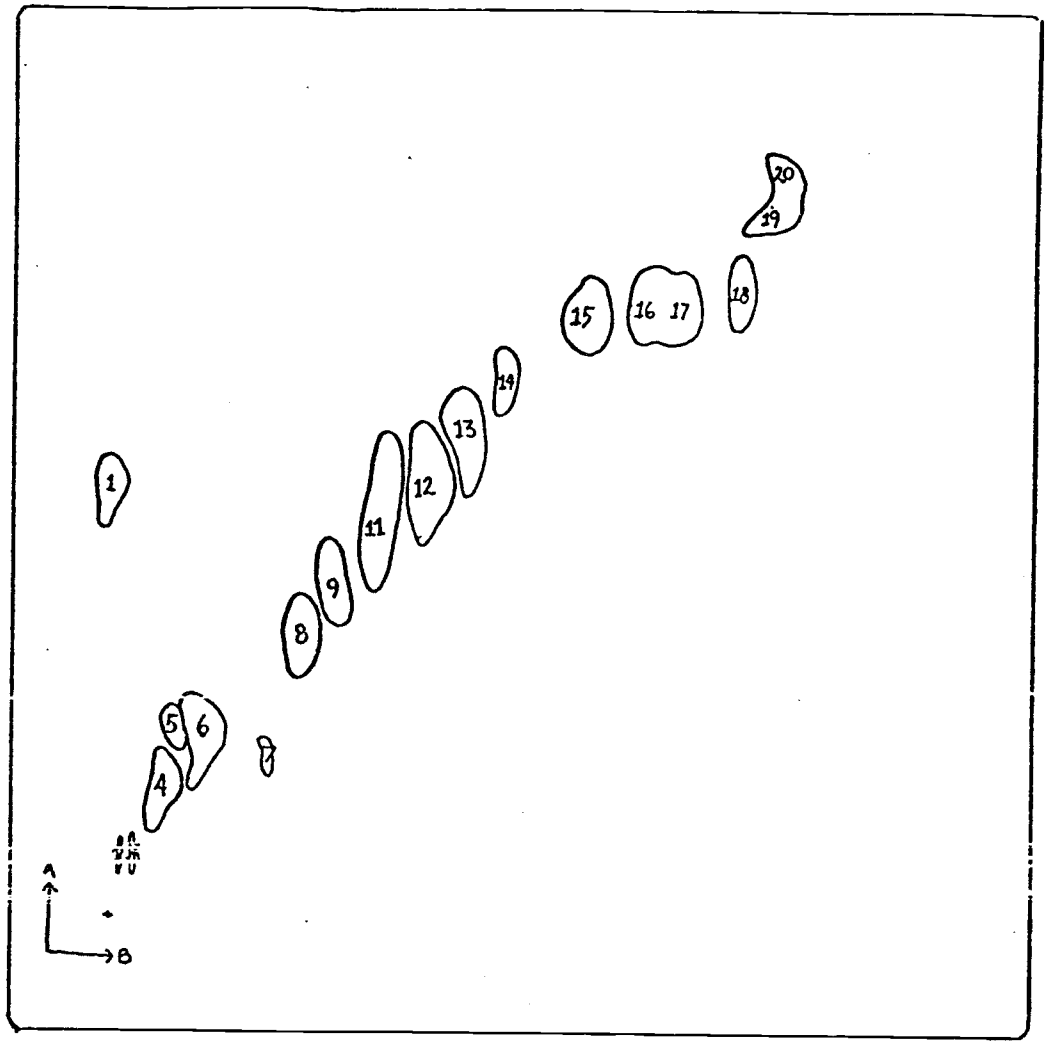

FIGURE 19. Two-dimensional chromatogram of the free amino acids of Haligena elaterphora Kohlm. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid-water (40:20:20 v/v). 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine, 17. methionine, 18. phenylalanine, 19. leucine, 20. isoleucine.

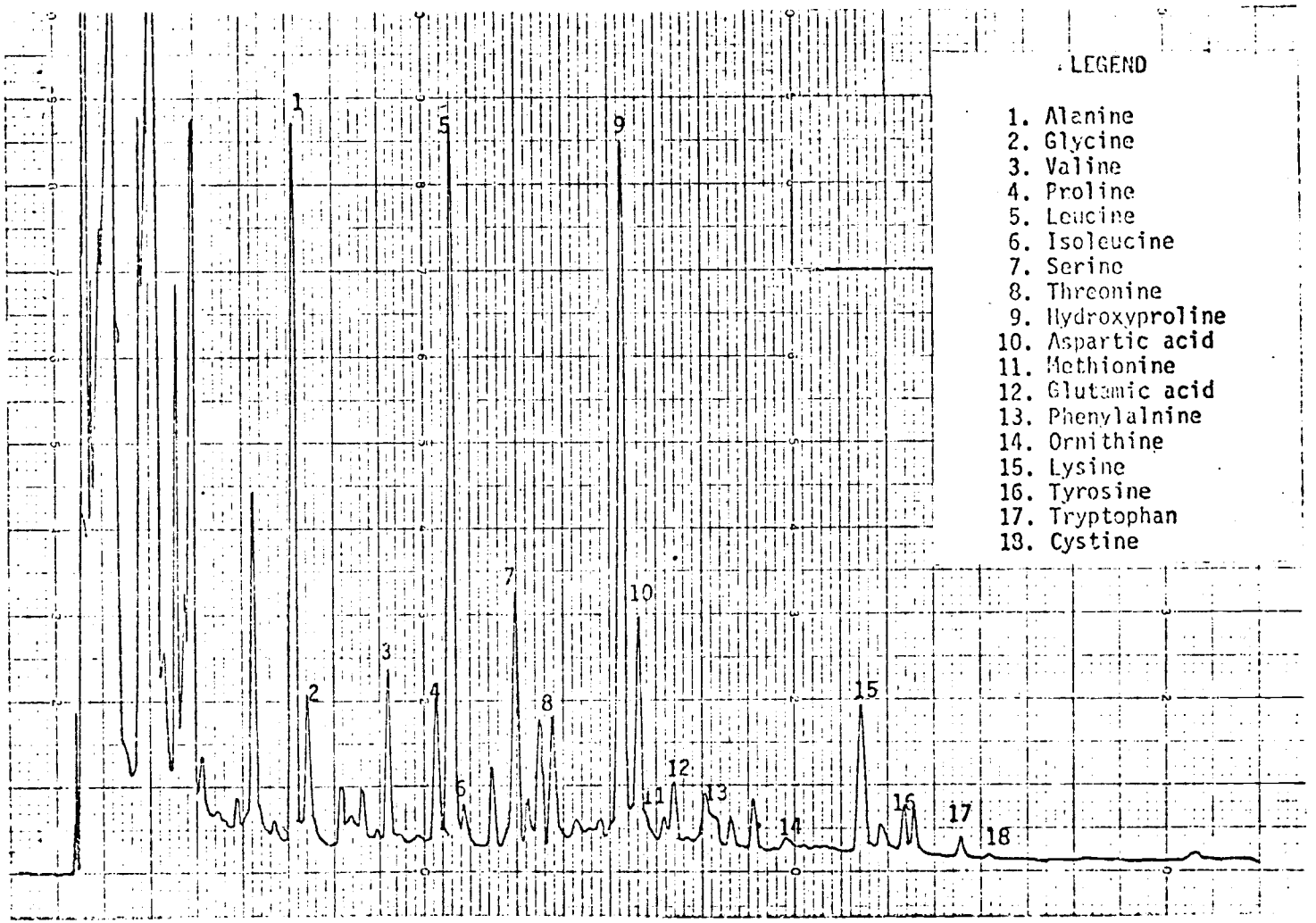

Figure 20. GLC spectrum of the free amino acids found in Haligena elaterphora Kohlm.

TABLE XIII

THE FREE AMINO ACIDS FOUND IN *Halosphaeria appendiculata* Linder.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	±	+
Proline	+	+
Leucine	+	+
Isoleucine	+	+
Serine	+	+
Threonine	+	+
Hydroxyproline	-	+
Aspartic acid	+	+
Methionine	±	+
Glutamic acid	+	+
Phenylalanine	+	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	+	±
Cysteine	+	*
Histidine	+	**

Symbols: +, present; - absent; ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

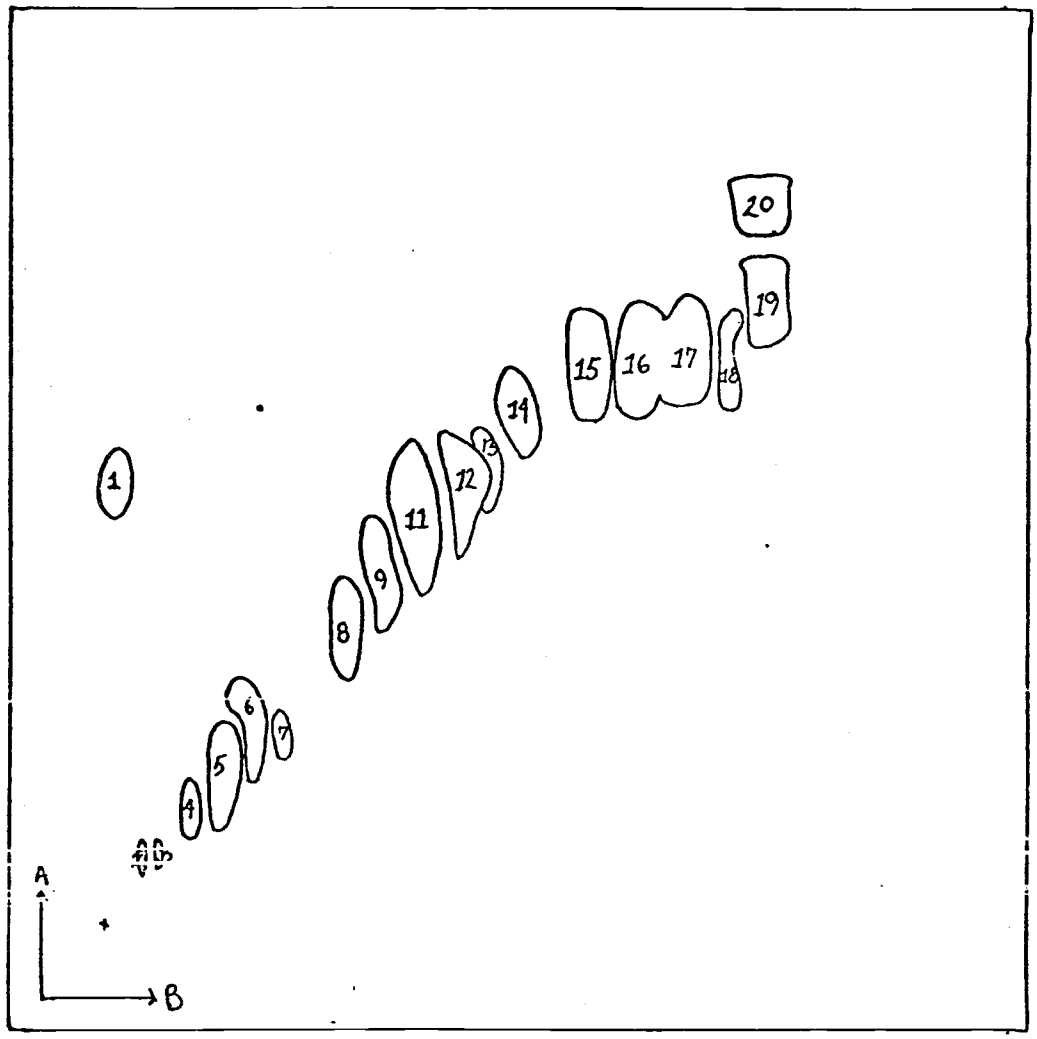

FIGURE 21. Two-dimensional chromatogram of the free amino acids of Halosphaeria appendiculata Linder. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid-water (40:20:20 v/v). 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine, 17. methionine, 18. phenylalanine, 19. leucine, 20. isoleucine.

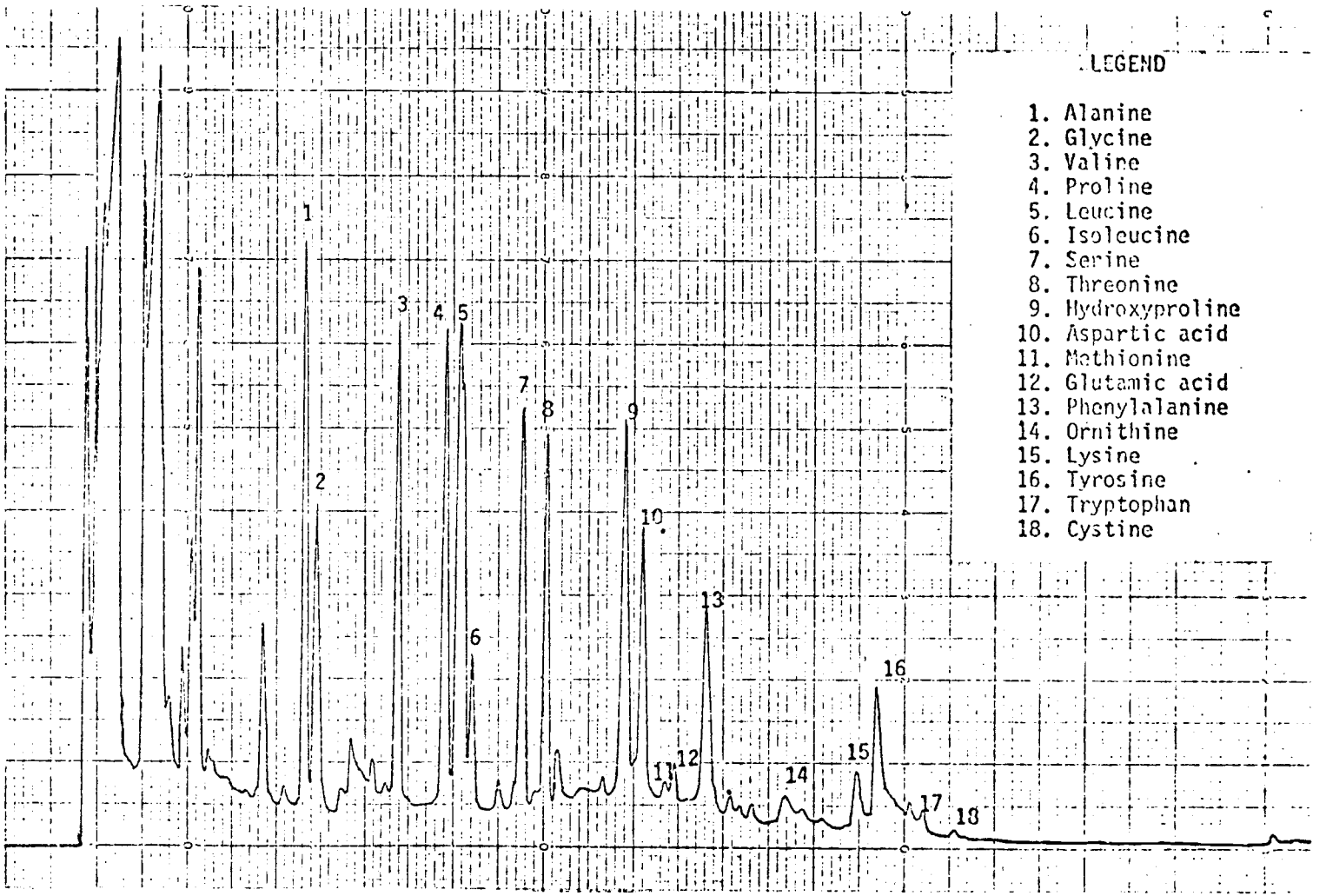

Figure 22. GLC spectrum of the free amino acids found in Halosphaeria appendiculata Linder

TABLE XIV

THE FREE AMINO ACIDS FOUND IN *Culcitalna archraspora* Meyers et Moore.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	+	+
Proline	+	+
Leucine	+	+
Isoleucine	+	+
Serine	+	+
Threonine	+	+
Hydroxyproline	-	+
Aspartic acid	+	+
Methionine	+	+
Glutamic acid	+	+
Phenylalanine	+	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	+	+
Cysteine	+	*
Histidine	+	**

Symbols: +, present; - absent; ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

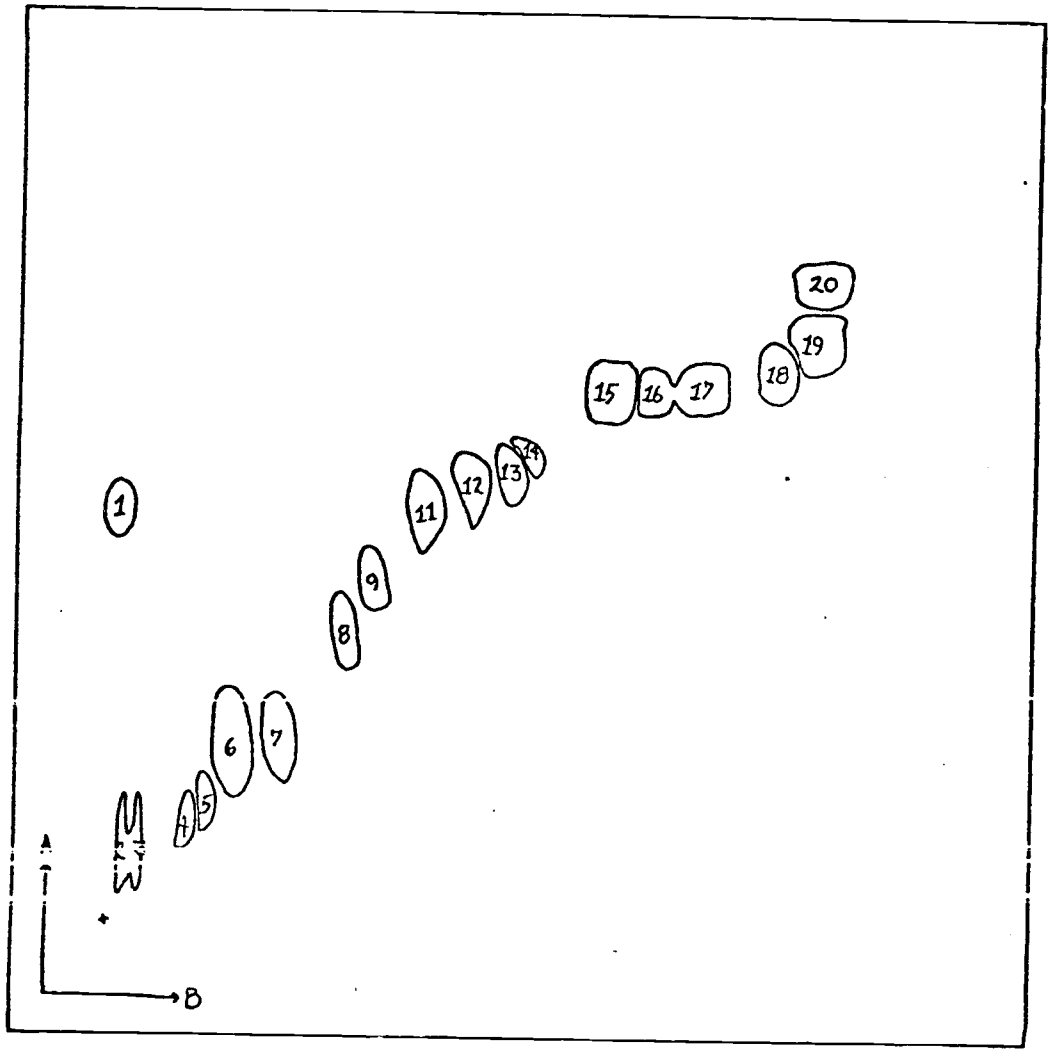

FIGURE 23. Two-dimensional chromatogram of the free amino acids of Culcitalna archrasnora Meyers et Moore. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid-water (40:20:20 v/v. 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine, 17. methionine, 18. phenylalanine, 19. leucine, 20 isoleucine.

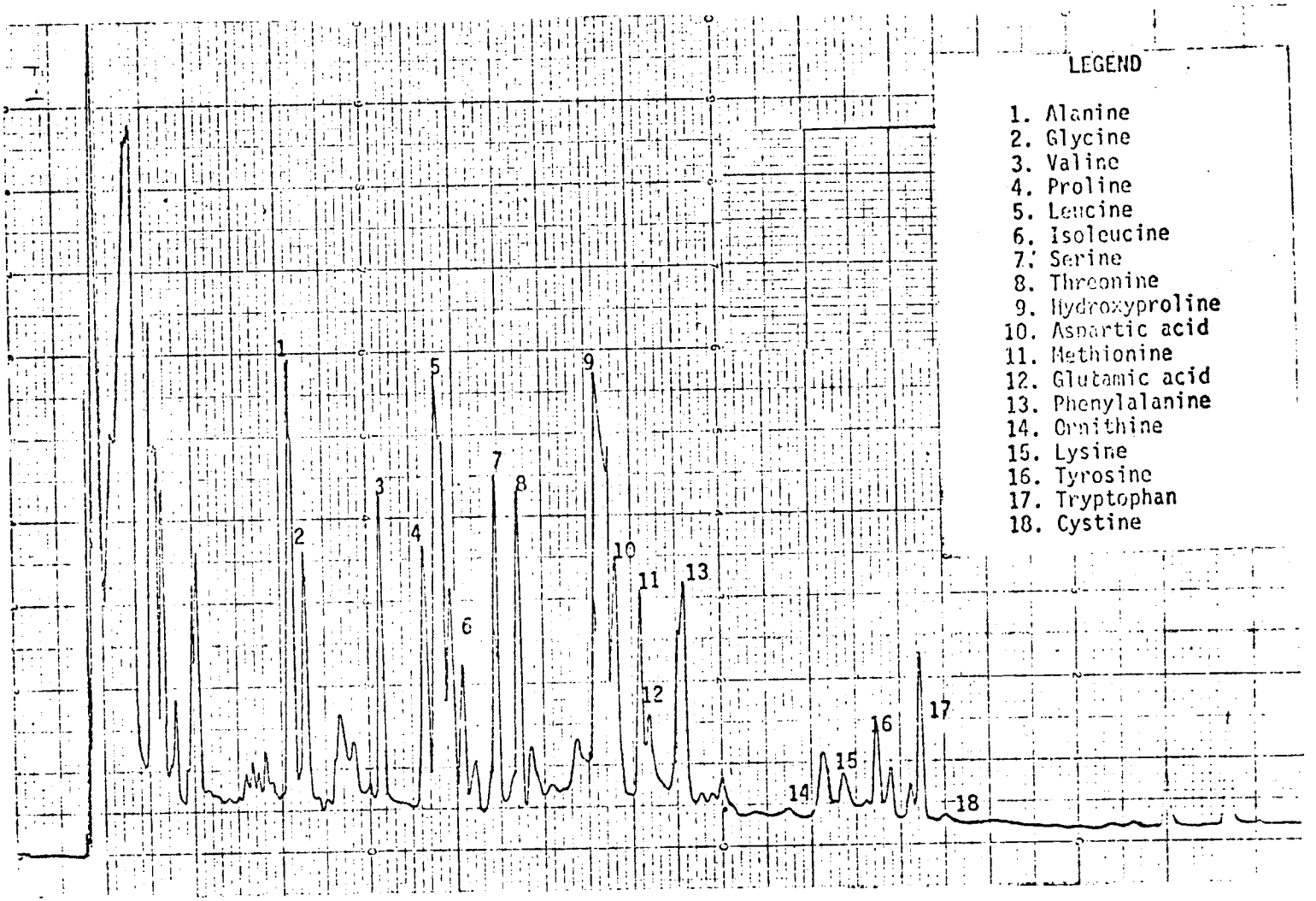

Figure 24. GLC spectrum of the free amino acids found in Culcitalna archraspora Meyers et Moore.

TABLE XV

THE FREE AMINO ACIDS FOUND IN *Zalerion maritimum* AFTER 14 DAY INCUBATION.

Amino Acid	Thin-layer Chromatography	Gas-liquid Chromatography
Alanine	+	+
Glycine	+	+
Valine	+	+
Proline	+	+
Leucine	+	+
Isoleucine	+	+
Serine	+	+
Threonine	+	+
Hydroxyproline	-	+
Aspartic acid	+	+
Methionine	+	+
Glutamic acid	+	+
Phenylalanine	+	+
Ornithine	+	+
Lysine	+	+
Tyrosine	+	+
Tryptophan	+	+
Cystine	+	+
Cysteine	+	*
Histidine	+	**

Symbols: +, present; - absent; ±, equivocal
*Not included in standard mixture for GLC analysis.
**Stable TMS derivatives could not be prepared for GLC analysis.

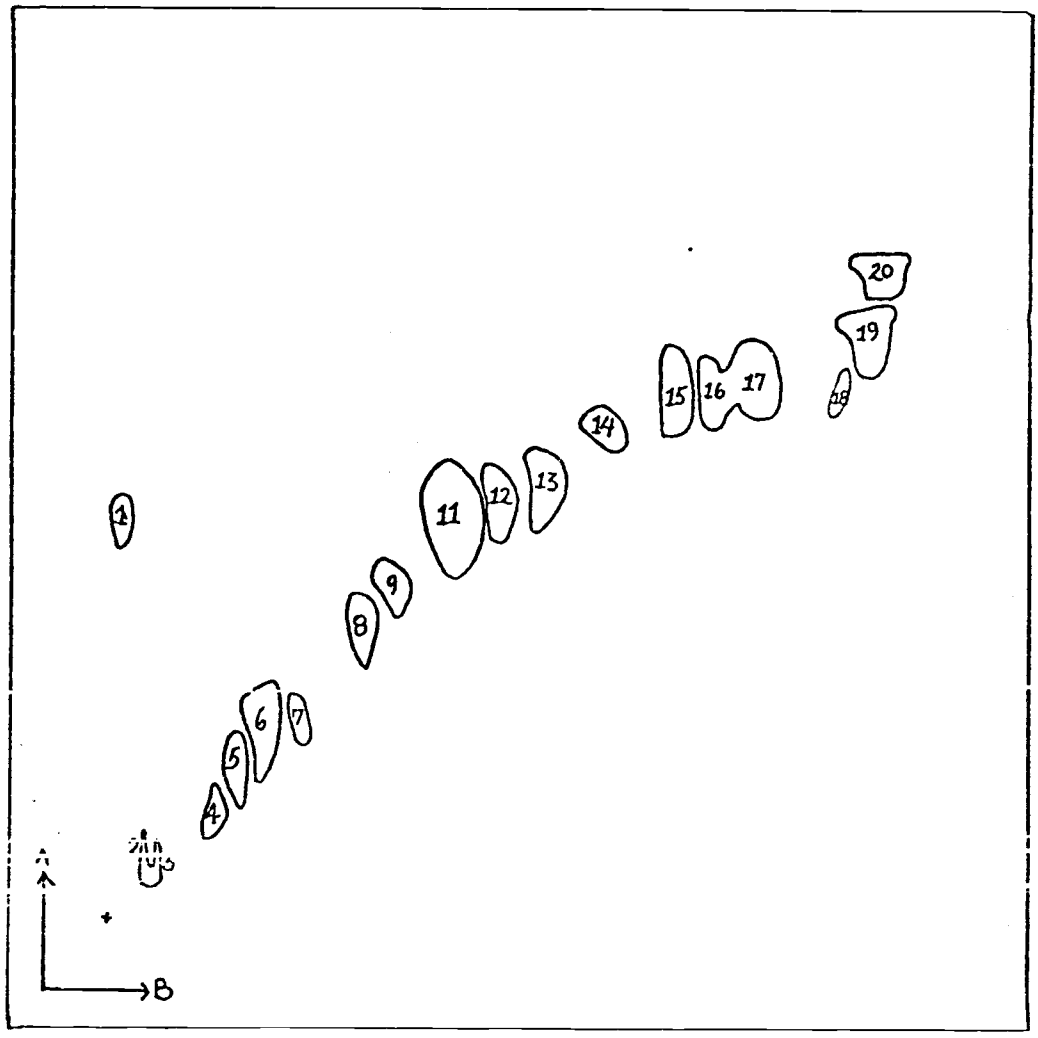

FIGURE 25. Two-dimensional chromatogram of the free amino acids of Zalerion maritimum (Linder) Anastasiou after 14 day incubation. The thin-layer cellulose plates were developed in Direction A with isopropyl alcohol-butanone-1N HCL (60:15:25 v/v) and developed in Direction B with butyl alcohol-acetic acid-water (40:20:20 v/v. 1. aspartic acid, 2. cystine, 3. cysteine, 4. ornithine, 5. lysine, 6. glycine, 7. histidine, 8. serine, 9. threonine, 10. hydroxyproline, 11. glutamic acid, 12. alanine, 13. proline, 14. tyrosine, 15. tryptophan, 16. valine, 17. methionine, 18. phenylalanine, 19. leucine, 20. isoleucine.

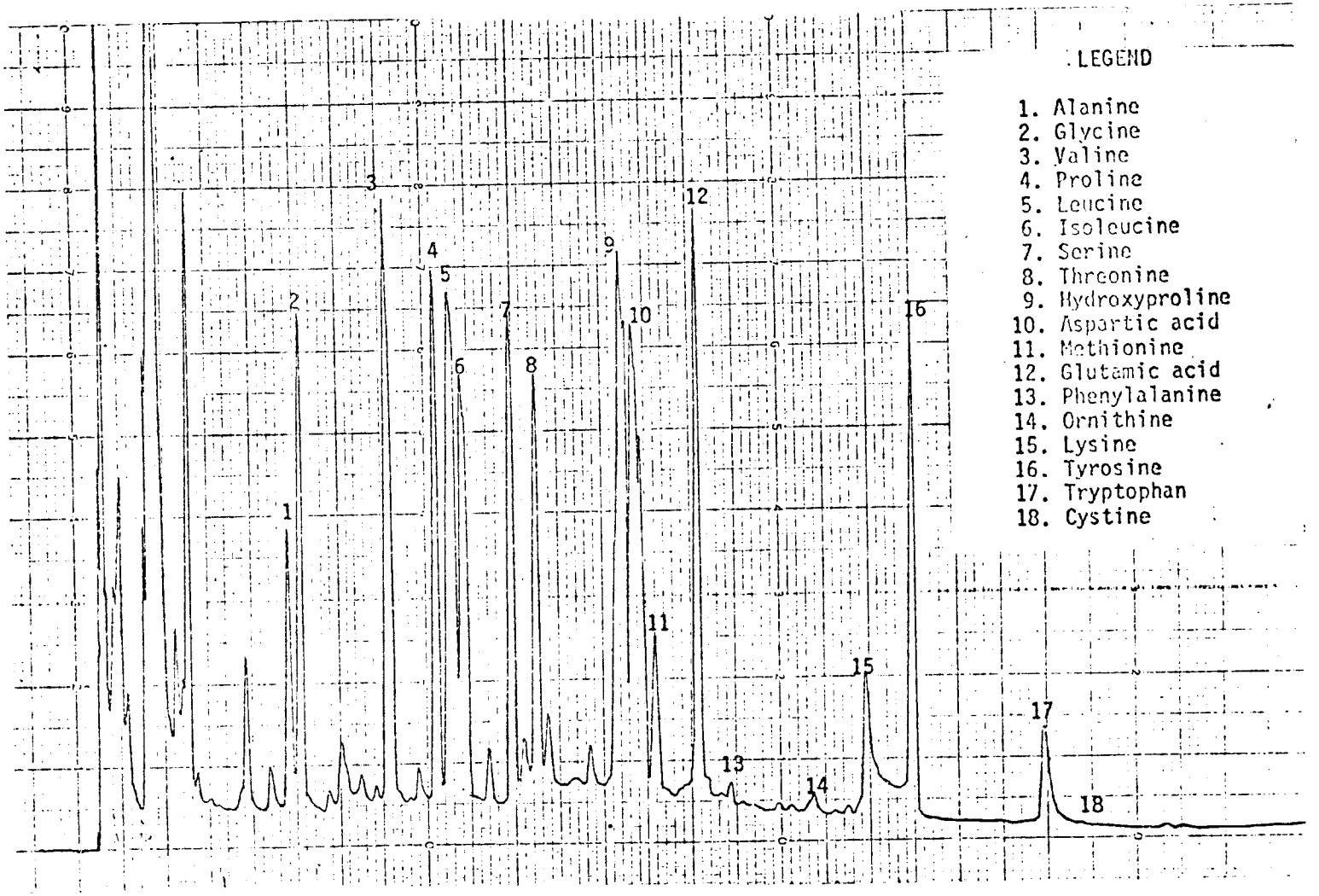

Figure 26. GLC spectrum of the free amino acids found in Zalerion maritimum (Linder) Anastasiou after 14 day incubation.

DISCUSSION

It is apparent from this investigation that the free amino acid content of higher marine fungi are qualitatively similar. The amino acids common to all species examined include: alanine, glycine, valine, proline, leucine, isoleucine, serine, threonine, hydroxyproline, aspartic acid, methionine, glutamic acid, phenylalanine, ornithine, lysine, tyrosine, tryptophan, cystine, cysteine and histidine.

This conclusion is based upon the collective data from TLC and GLC analyses which provided very similar but not identical results. The basic differences relate to the identification of phenylalanine, methionine and hydroxyproline via TLC analyses versus histidine and cysteine by GLC analyses. With regard to the former, these amino acids could not be identified with certainty in all species examined by TLC but were shown to be present when these same organisms were analyzed by GLC. Perhaps these results were due to concentrations not detectable by ninhydrin. On the other hand, TMS derivatives of histidine and cysteine are either too unstable or would otherwise obscure the GLC spectrum. Consequently, they were not included among the standards used in GLC analyses. However, both of these amino acids could be identified with certainty in all species examined by TLC.

Noteworthy is the occurrence of hydroxyproline in all species investigated. This finding may have implications relative to the role of fungi in the chemical ecology of the marine environment and may have taxonomic importance also.

It has been reported that fungi contribute to the pool of dissolved or suspended organic compounds in sea water (Kirk et al., 1972). These include such compounds as ergosterol, mannitol, triglyceride fatty acids and choline sulfate which occur in relatively large concentrations in marine fungi. However, it is generally believed that phytoplankton and algae are the prime sources of these materials (Hood, 1970).

Relative to amino acids, Pockington (1972) has reported the occurrence of these compounds in sea water and hydroxyproline was readily detected, whereas Degens (1968) noted that this amino acid is not ubiquitously present in seawater. Furthermore, Degen's investigations on amino acid content of phytoplankton and particulate matter in sea water indicated that hydroxyproline was not present as a free amino acid. Hydroxyproline is known to be a prime component of collagen-type proteins of animals including marine species (Degens, 1970). It is conceivable, considering the data that has been presented, that marine fungi represent an additional potential source of this amino acid in the marine environment.

Although it is felt that fungi contribute significantly to the wide variety of organics known to occur in sea water, including amino acids, the true significance of the contributions of these organisms cannot be assessed at this time. This is due to the lack of accurate biomass data and the lack of actual field evidence which is needed to consider to what extent, or under what conditions marine fungi add appreciably to the pool of organic matter in sea water.

As indicated previously, the apparent ubiquitous occurrence of hydroxyproline in higher marine fungi may have taxonomic significance. Numerous examples of the chemotaxonomic application of amino acid data have been noted in the literature. Maag (1959) was able to distinguish between two species of the fungus Emericellopsis by chromatographic comparison of their amino acid contents. Also several species of the genus Amanita, terrestrial fungi, have been described taxonomically using free amino acid and other chemical data (Catalfomo and Tyler, 1961).

Examples of species delimitation on the basis of chromatographically determined amino acid content are also found in the zoological literature. Buzzati-Traverso (1953) was able to show species differences from amino acid content of fish muscle. He also pointed out the possible application of this data to phylogenetic relationships. Also, Ball (1953) has shown that three species of Culex mosquitoes may be separated by comparing their amino acid contents.

In view of the above examples, it seems logical to attempt to utilize free amino acid data in marine fungal taxonomy. However, the amino acid content of the marine fungi examined in this study is strikingly similar. Therefore, it does not appear that this type of data will be useful in delineating marine fungal series *per se*. However, as stated earlier (Holden, 1961), hydroxyproline has been reported to occur only rarely in terrestrial fungi. Thus, it is possible that this data may be useful in designating marine fungi from true terrestrial forms.

It should be emphasized that the apparent absence of hydroxyproline from many terrestrial fungi may be due to experimental technique. Extraction procedures commonly cited in free amino acid determinations in terrestrial fungi have utilized common organic solvents. Recently, amino acids have been shown to be most efficiently extracted with hot water rather than common solvents such as methanol, ethanol and acidified butanone (Heathcote et al., 1972). Therefore, in previous studies hydroxyproline may have been extracted in undetectable concentrations.

The efficient extraction of hydroxyproline is very dependent on the extracting solvent as indicated by its solubility in common solvents. This amino acid is only slightly soluble in ethanol but very soluble in water (516.7 Gms./L. at 65°C). The method employed for amino acid extraction in this study was based on that of Heathcote et al. (1972) which utilized distilled water as the extracting solvent. This method was modified by extracting the fungal mycelia in Soxhlet extractors for 48 hours rather than the recommended 15 minute boiling period. The use of water as a solvent and the long extraction period may be responsible for determination of hydroxyproline in these organisms. Also the use of two separate analytical procedures, thin-layer and gas-liquid chromatography, may have contributed to this finding.

Finally, one must keep in mind that environmental factors may influence the free amino acid content in fungi. Several investigators have shown that changes in amino acid pool composition can occur if cultural conditions are varied. For example, Aspergillus oryzae has

been shown to produce ornithine only at lower culture pH, while at higher pH values most amino acids occured in greater quantity (Simonart and Chow, 1954). Also Sguros and Simms (1963) have shown culture media constituents can effect amino acid utilization in several marine fungi. However, there is some evidence in the present study to indicate that length of incubation does not effect the qualitative amino acid content of marine fungi since no differences were noted in 7 day and 14 day cultures of Zalerion maritimum. In view of these findings, further investigations concerning the effect of culture conditions on marine fungal growth and physiology must be carried out before accurate and comparable amino acid can be obtained.

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