DERIVATIZATION AND CHROMATOGRAPHIC SEPARATION OF ACID INDOLE AUXINS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

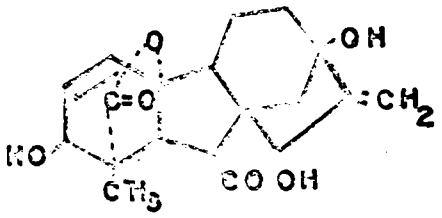
INTRODUCTION

Biological Use

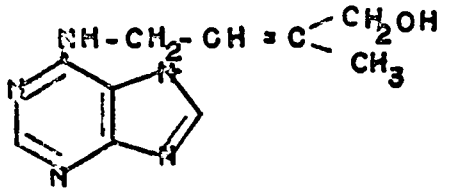
For many centuries, the regulation of plant growth remained a totally mysterious phenomenon. In the 1750's, however, Duhamel Du Monceau made the first step toward understanding this phenomenon by finding that root formation was related to descending plant sap.(1) Since then, great strides have been made toward uncovering the secrets of plant growth by many workers who have examined the complexities of substances which control this growth. Although exactly defining a plant growth substance is difficult, a suitable effort has been made by Hill who states:

> "A plant growth substance (or plant hormone) is an organic substance which is produced within a plant and which will at low concentrations promote, inhibit or qualitatively modify growth, usually at a site other than its place of origin. Its effect does not depend upon its calorific value or its content of essential elements."(2)

Five major endogenous plant hormone categories (gibberellins, cytokinins, abscisic acid, ethylene, and auxins) are being studied. Gibberellins are substances which have the gibbane structure and generally are chemically related to gibberellic acid (figure 1). Presently 42 gibberellins have been isolated from plants and chemically characterized.(3) They all share an ability to cause stem elongation when applied to intact plants. They show the greatest effect with certain genetic dwarfs such as dwarf strains of maize and peas.



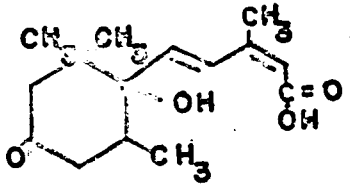
Gibberellic Acid



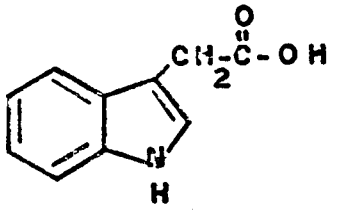
Zeatin-a naturally occurring cytokinin from Zea mays

$$CH_2 = CH_2$$

ethylene - a gaseous hormone



Abscisic Acid



3-Indole acetic acid

Figure 1. Plant Hormone Structures[4]

Cytokinins are substances which are derived from adenine; are involved in apical dominance and senescence; and are characterized by their ability to interact with auxins to promote cell division, particularly cell differentiation, in tissue cultures. Most substances known to inhibit plant growth are similar to abscisic acid (ABA) in their physiological properties. ABA acts as an inhibitor in many plant growth phenomena, particularly bud dormancy in woody plants and the control of abscission of leaves in the cotton plant. Ethylene has been implicated in many growth responses, typically leaf senescence, abscission, and ripening of fruits. Auxins are defined as those substances chemically related to 3-indole acetic acid (IAA), and are characterized by their ability to promote cell enlargement in excised plant parts such as Avena coleoptile. This definition over-simplifies the complexities of auxin activity and chemistry, and many workers over the past fifty years have directed their efforts toward discovering the structure and mode of action of auxins.(2,4)

Charles Darwin in the 1880 first intimated the existence of a substance which was transmitted from the apex of a monocot seedling to the basal regions and induced the plant to bend toward the light (the phototropic effect).(2) Boysen-Jensen in 1910 and Daal in 1919 verified this effect and noted that the effect must be caused by the downward movement of a growth-promoting substance on the shaded side of the seedling.(1) It was not until 1926 that F.W. Went clearly isolated this hormone from plant tissues. This was shown by allowing the chemical to diffuse from a seedling tip into an agar block. When

this block was placed on one side of a decapitated oat coleoptile, and the seedling left for a period in darkness, it gave a quantitative measurement of the hormone by the degree of curvature which occurred. The hormone which Went isolated was given the name "auxin" (from the Greek "auxein", to grow).[4] During the 1930's and 1940's, many studies were carried out using this agar block bioassay for the analysis of auxin where it was assumed, often tacitly, that this growth promotor was a single substance.[5] Upon the isolation of 3-indole acetic acid (IAA) from immature corn kernels[6,7] the endogenous auxin was considered to be IAA.

From this initial work and subsequent studies of a great number of plant species, auxins have come to mean substances related to IAA. Many indole compounds have IAA-like effects, possibly due to their enzymatic conversion to IAA. Further, many synthetic growth hormones which do not have an indole framework can also have IAA-like activity. An example of this phenomenon is 2,4-dichlorophenoxy-acetic acid, whose activity has been postulated to come from structural similarities to IAA.[8]

Most of the studies of the effects of auxins have resulted from the exogenous application of these compounds. These effects include apical dominance, cambial activity, fruit development, leaf abscission, peroxidase induction, rooting, senescence, phototropism, geotropism, and tendril coiling.[8] In the phenomena where auxin causes directional growth such as phototropism, geotropism and tendril coiling, the probable mechanism is that of cell extension rather than increased

cell division. Studies of cell enlargement and the mechanisms of auxin action have been linked since the discovery of auxin. This has been primarily because auxins are often used exogenously to cause cell enlargement in excised plant sections. In the intact plant the auxin is synthesized in the stem apex and moves downward to the site of cell enlargement. The mechanism of action of IAA in this case has been postulated to be the reduction of cross-linking between the structural biopolymers in the cell walls.(9)

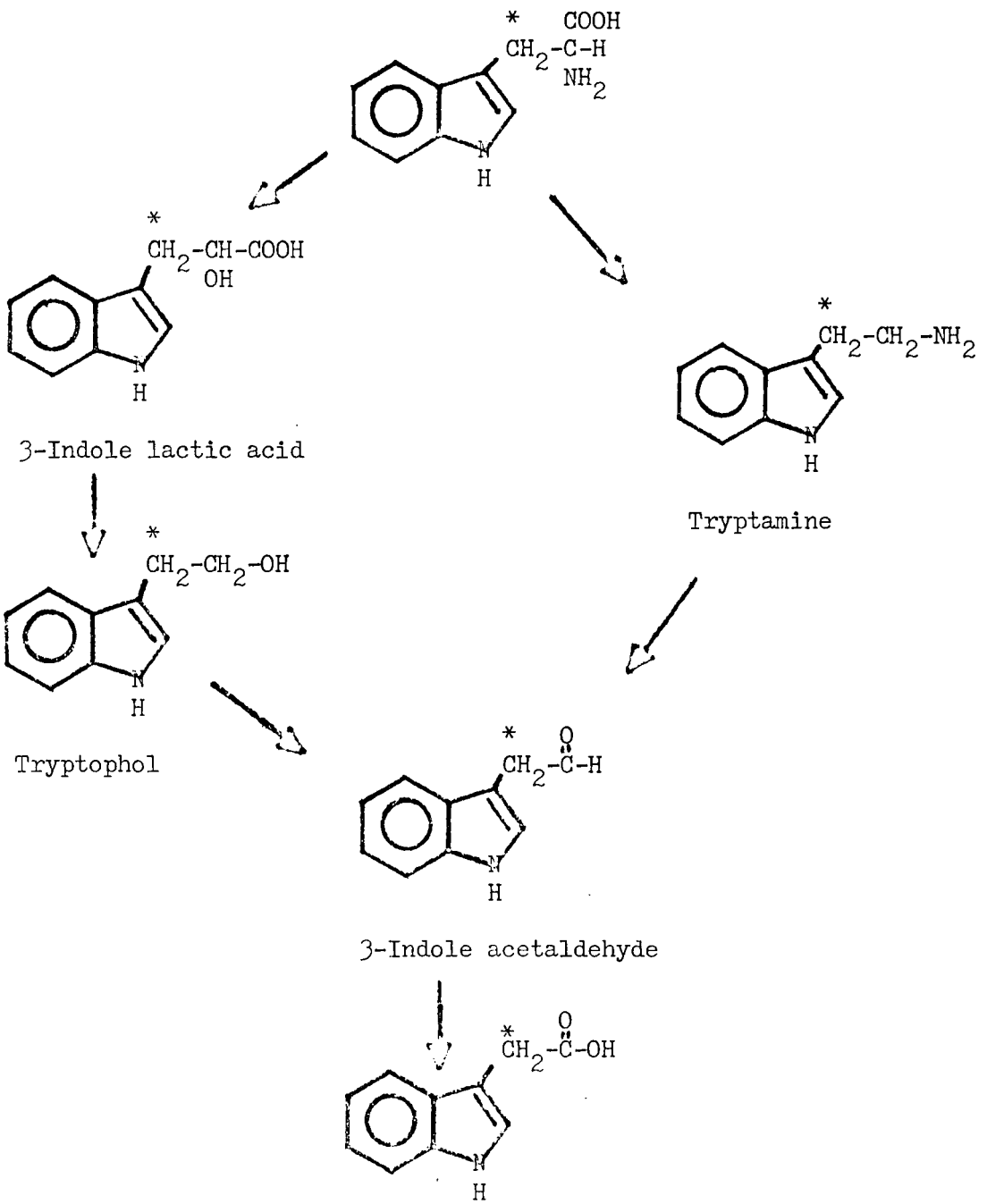
3-Indole butyric acid (IBA) has exhibited auxin-like activity in many of the above phenomena.(9) The most effective use of IBA is in the rooting of plant cuttings.(10) Cuttings of various plants that do not normally root spontaneously may be dipped into solutions or powders of IBA and when planted in a sand rooting bed will develop extensive roots.

3-Indole propionic acid (IPA) has also been shown to have auxin-like activity similar to IAA and IBA. However, it has been shown that this synthetic indole exhibits biological activity somewhat different from IAA.(4) IPA has been reported in Brassica and Nicotiana(11) and has been demonstrated to be highly active in root initiation of tomato plant stem cuttings.(12) The detection of IPA in the root promoting fraction of tomato cotyledon extracts has recently prompted interest in this compound as a natural auxin.(13)

Another area of interest in auxins has been the controversy of the biosynthesis of IAA from tryptophan (TRP). A large number of workers have determined that 3-indole acetic acid is produced in

higher plants, as well as micro-organisms, by the oxidative metabolism of TRP (see figure 2). A thorough study and review was made by Wightman who showed that when tomato, tobacco, pea, wheat and barley plants are supplied with TRP-3-C^{14}, the presence of radioactive IAA in experimental tissue can be readily detected.(14) However, Libbert et al.(15) have shown that in some plants this conversion of TRP to IAA appears to be carried out only by the bacteria present in the plant tissue. This was shown by comparing the IAA production from TRP in plants grown under non-sterile and sterile conditions with no IAA being produced from TRP in the sterile condition. These plants also had lower concentrations of IAA than the non-sterile control plants. Thus, at the present time uncertainty still remains as to the exact route of IAA biosynthesis.(2)

Although the developmental effects of IAA and other auxins are most dramatic in plants, these compounds also show significant effects in animal systems. The first chemically identified auxin, IAA, was isolated from human urine(16) before it was isolated from plants. Aside from the development of analytical techniques to determine low levels of auxin-like compounds in humans, relatively little work has been done on the functions of these compounds in animals. IAA has been shown to induce tryptophan pyrrolase and tyrosine transaminase in intact and adrenalectomized rats possibly due to its similarity to TRP.(17) Dye et al. have shown that tumors may be induced on plants with IAA,(18) and it is this work which has caused interest in IAA as a possible cancer promotor in animals.



*Carbon^{14}

Figure 2. Possible Biosynthetic Pathways[14] of 3-Indole Acetic Acid

Presently, research on the associations of IAA to cancer growth has been reported but sufficient data on this area has not yet been published.(3)

Much of the information gathered on auxins has been obtained not from chemical analysis but rather from bioassays. A bioassay is any system by which a minute amount of an applied substance can be measured by noting an organisms response, e.g. changes in size or root number, to that substance. A standard dosage response curve is obtained and an unknown quantity of that material can be determined from the amount of response it achieves.

Two major bioassays have been used to characterize auxin activity. In the oat or wheat coleoptile straight growth test, the seedlings are grown in the dark until the coleoptiles are 2-3 cm long. The apical 3-4 cm of the coleoptiles are removed, and a segment of a standard length is cut from the remaining part and floated in the solution to be tested. The increased length of this standard after a given period, usually 24 hours, in the dark is taken as the quantifiable response. A second test, usually used for rapid screening of auxin-like activity, is the Went pea curvature test. Sections of the stems of dark-grown pea seedlings are floated for 24 hours on solutions of the test substances. The sections are slit lengthways for about two-thirds of their length before use, and the degree of bends of these two arms gives a rapid visual estimate of the activity of the test substances relative to standard and control solutions.(2)

Previous Analytical Work

In addition to the use of auxin bioassays, a large number of analytical techniques have been applied to the analysis of the 3-indole acids (IAA, IPA, and IBA). Phosphorescence measurements have been carried out on 3-indole acid containing biological mixtures at 3-indole acid concentrations of down to 10^{-8} M.[19] Many investigators have found fluorescence to be a useful analytical technique for slightly purified biological extracts containing 3-indole acid[11,20,21] This technique yields good quantitative data by excitation of the mixture at 275 - 285 nm and monitoring the fluorescence at 365 - 375 nm.[11] Colorimetric techniques in which the 3-indole acids are derivatized to form chromophores have been used for quantitative analysis of these compounds in relatively pure plant extract.[22,23] A commonly used technique for the quantitation of isolated 3-indole acids utilizes their strong UV absorbance.[24] This characteristic of the 3-indole acids allows their detection at about 10^{-5} g levels once they are separated by liquid chromatographic techniques.

Separation techniques which utilize the above quantitation methods for detection have found great use in many laboratories. Thin layer chromatography is at present the most widely used technique for separation and quantitative work involving the common 3-indole acids.[25,26,27] Silica gel[25], cellulose[25], and polyamide[27] are the major stationary phases used. A wide variety of aqueous and non-aqueous mobile phases, the exact composition depending on other

substances in the mixture to be separated, have been utilized. Paper chromatographic separation of biological samples of 3-indole acids has also been widely used.[28] This technique yields poor sensitivity yet provides excellent preparative scale characteristics. Classical column chromatography has also proven useful for preparative scale separations of 3-indole acids. Sephadex[29], ion exchange resins[24], and silica gels[30] are the most widely stationary phases for liquid chromatographic separation. High pressure liquid chromatographic separations have been attempted[31], but these techniques are presently in the early stages of development and retention times on the order of hours are still common.

Since 1966, corresponding to the introduction of N,O bis(trimethysilyl)acetamide (BSA), gas chromatographic analysis of the acid indoles (IAA, IPA, IBA, and TRP) has proven to be the most accurate analytical method available. Many investigators have been using diazomethane to form methyl derivatives of these 3-indole acids.[11] However, most methylation reactions are tedious, and it is for this reason that the trimethylsilylation derivatization reaction has been receiving increasing attention. Initial chromatographic problems such as selecting an inert column material, glass[32], finding the most effective silylating agent, BSA[33,34], and selecting the most efficient liquid phase, OV-17[33], have already been overcome. Studies of the trimethylsilylation and gas chromatographic analysis of the amino acid analog of the 3-indole acids, tryptophan, are generally carried out as a part of amino acid derivative stud-

ies.[35,36] However, complete work on the structures of the TRP as well as IAA, IPA, and IBA - BSA reaction products, as well as optimum silylation conditions for the IAA, IPA, IBA and TRP mixture, has not yet been done.

Methods for Analysis of Derivatives

Although a thorough structural study of this system has not been carried out, many studies of the structures of other trimethylsilyl (TMS) derivatives have been made. The techniques used for these studies range from indirect, e.g. parallel reactions, to comprehensive, e.g. gas chromatography-mass spectrometer coupling. In the initial work on BSA, Klebe et al.[37] reacted several compounds with BSA and isolated the primary reaction product by the removal of the solvent, BSA, and N-trimethylsilyl acetamide through vacuum distillation. The postulated structures for the products were then verified by elemental analysis (C,H, and N). Most subsequent studies on the reaction of BSA with these and similar compounds have not determined the structures of the reaction products but rather have relied on this earlier publication. Aside from the obvious possibility of the decomposition of the trimethylsilyl derivatives through this procedure, the experimental methods do not take into account the identification of intermediates and decomposition products which might be present in these reaction mixtures.

Studies of the derivatives of indole acids in biological samples has led to indirect approaches to the determination of the structures of the intermediate derivatization products. In one case,[38] the

intermediate trimethylsilylation product of the reaction of IAA with BSA was compared with the one product of the reaction of BSA with pure indole. Due to the differences in reaction times between these two samples it was assumed that the intermediate silylation product for the IAA has a structure where the acid group proton was replaced by a trimethylsilyl group. The authors then assumed that the second and final reaction product of the IAA - BSA reaction resulted from the slow replacement of the indolic nitrogen proton of the mono trimethylsilyl IAA by a trimethylsilyl group.

More direct structure determination methods can be applied to the study of trimethylsilylation reactions. Perhaps the most direct method is that of direct coupled gas chromatography - mass spectrometry (GC/MS) using an interface which does not cause sample decomposition. Alternatively, the chromatographic effluent containing the sample may be collected in a trap. This trapped derivative, after testing for decomposition, can be introduced into a mass spectrometer for structural analysis.

Although not universally useful, Nuclear Magnetic Resonance (NMR) spectroscopy may also be used to study the structures of trimethylsilyl derivatives. Earlier workers[37] had problems with the NMR technique due to the lack of solubility of the starting materials in a solvent suitable for silylation. Acetonitrile has been found to be a useful silylating solvent, and this solvent proves to be a good solvent for the 3-indole acids. Although acetonitrile has a strong NMR response at a chemical shift of 1.94 ppm, if no interfence with

the peaks of interest is found, one can use this peak as the frequency reference. One major problem is encountered in determining 0 ppm from the usual standard, tetramethylsilane. Since trimethylsilyl groups tend to be in a variety of environments in most trimethylsilylation reactions, these distort the tetramethylsilane peak sufficiently to preclude its effective use as a frequency standard.

Statement of Our Problem

Considering the importance of gas chromatographic analysis of trimethylsilyl 3-indole acids by researchers in the field of plant growth substances, an in-depth study of the derivatization method was chosen as the subject of this thesis work. The structures of the silylation reaction intermediates, the major product and the decomposition products for the trimethylsilylation of IAA, IPA, IBA, and TRP by BSA were to be throughly investigated. This would be accomplished through the correlation of NMR data of the individual reaction mixtures together with their gas chromatographic behavior. With the objective of identifying the major products obtained, on all glass effluent splitting and trapping device would be designed, installed and evaluated.

In conjunction with the structural study, optimum conditions for the trimethylsilylation of the four indole acids would be examined. This would include reaction temperature, reaction time and reagent concentrations. Included in this study would be an examination of the effects of the storage of the derivative for the four indole acids.

Finally, the feasibility of using high speed liquid chromatography for the separation and analysis of IAA, IPA, IBA, and TRP was to be examined.

EXPERIMENTAL

Instrumentation

Three gas chromatographs were utilized for the work in this study, and these will be listed in order of decreasing use.

1. A Bendix model 2600 equipped with dual flame ionization detectors, linear temperature programmer, and separate heater controls for detector, injection port, and column oven was used for the derivative study (see table I for complete conditions). The forced air oven contained two 3 foot "U" shaped glass columns of $\frac{1}{4}$ inch outside diameter and generally 4 mm inner diameter. In all cases, the optimum hydrogen flow rate, 35 ml/min and the optimum flow rate for air, 1.6 standard cubic feet per hour, were used. The carrier gas, pure helium, was maintained at a flow rate of about 35 ml per minute. All three gases were passed through 3 feet of $\frac{1}{4}$ inch tubing containing 5Å molecular sieve prior to entry into the gas chromatograph. This was to remove any traces of hydrocarbons or water present in the gas cylinder.

2. A Bendix model 2500 gas chromatograph with the same characteristics as listed for the Bendix model 2600 with the exception of a flow rate of 45 ml per minute for hydrogen and the use of 6 foot "U" shaped glass columns was also used. Work on this instrument included installation and use of the sample splitting device and examination of biological extracts.

3. A Hewlett-Packard model 700 gas chromatograph with a model 240 temperature programmer, thermal conductivity detector, and dual

TABLE I - Standard Gas Chromatographic Conditions

Column:	3% OV-17 on Supelcoport 80/100 mesh
Column length:	3' x 1/4" o.d. glass
Oven temperature	
initial:	150°C
program rate:	20°C/min
final:	210°C
Detector temperature:	250°C
Injection port temperature:	155°C
Carrier gas:	Helium at 32 ml/min
Hydrogen flow rate:	35 ml/min
Air flow rate:	1.6 S.C.F.H.
Recorder range:	1.0 mv full scale

column capability was used in preparative scale separations. Samples of both BSA and N-trimethylsilyl acetamide were trapped using this chromatograph. Attempts were made to use this instrument in trapping the 3-indole acid derivatives.

The liquid chromatographic separation of the 3-indole acids was developed on a homemade liquid chromatograph. This chromatograph consisted of an external gradient device, a 3000 psi Milton Roy reciprocating pump (model #HDBI-3012), a Dupont low dead volume injection port, an off-line reservoir pulse dampener, and a Pharmacia UV photometer (254 manometers).

All nuclear magnetic resonance spectra were run on a JEOL JNM-PS-100 MHz high resolution spectrometer using the proton mode. The instrument tuning was accomplished using ethyl benzene and the sweep offset was adjusted using the tetramethylsilane in the ethyl benzene sample. Tetramethylsilane could not be used for the spectra relative frequency reference due to the abundance of trimethylsilyl groups in BSA, its decomposition products. Thus the ubiquitous solvent, acetonitrile, with a relative frequency of 1.94 ppm served as the relative frequency reference.

Reagents

BSA was obtained from Regis Chemical Co., Morton Grove, Illinois in 1 ml ampules; the ampules were kept at 4°C until use. Excess BSA was kept at 4°C under an inert atmosphere in an air tight container and used within one day. As an assurance of purity, all BSA reagents were chromatographed prior to use to check for decomposition products.

Acetonitrile (Regis Chemical Company, Morton Grove, Illinois, batch #270010) was used both as the silylating solvent and solvent for the starting materials in every case. As complete dryness of the silylating solvent is critical, many precautions were taken to assure that the solvent was water-free. First, the bottle was opened as infrequently as possible and all acetonitrile transfer vessels and containers were kept dry. Second, the acetonitrile was stored over 5Å molecular sieve which had been dried for 36 hours at 170°C in a forced air oven. Although more complex drying methods may be used[39], given less than 2 per cent by weight of water in acetonitrile and a water to molecular sieve adsorption ratio of 30 per cent by weight, the water content in the acetonitrile was kept at less than one part in one thousand by the molecular sieve method.

The four starting materials were obtained primarily from two sources. D-tryptophan (lot 7125) and 3-indole propionic acid (lot 083C-2760) were ordered through Sigma Chemical Company, St. Louis. 3-Indole acetic acid (lot 7125), 3-indole butyric acid (lot 8256), and 3-indole propionic acid (lot 2008) were obtained from the Nutritional Biochemicals Corp., Cleveland. The compounds (except IPA) were of sufficient purity to be used without recrystallization. A second lot of IPA proved to be of adequate purity. Although several sources suggest drying the samples by azeotropic distillation of the water with methylene chloride under a stream of dry N_2[35], in this work the samples were dried at 105°C for 2 hours prior to trimethylsilylation. Some thermal decomposition problems were encountered

with the Nutritional Biochemical Corporation IPA, but these were solved by obtaining a purer batch of this compound from Sigma Chemical Company.

Columns

Although QF-1 was tried as a liquid phase, OV-17 showed the best peak shape and resolution for the trimethylsilyl acid indoles in concurrence with Horning et. al.[33] The OV-17 was obtained from Applied Science Laboratories, Inc., State College, Pa. while the solid support used (Supelcoport 80/100 mesh) was obtained from Supelco, Inc. Silanized spun glass wool, obtained from Analabs, Inc., North Haven, Conn., was used to retain the packing with the gas chromatographic column.

Excellent reviews of column preparation may be found in Research and Development[40] and Basic Gas Chromatography.[41] Borosilicate "U"-shaped glass tubing, $\frac{1}{4}$ inch o.d., 4 mm i.d. and 3 feet in length were given washings and silane treatment as described in (40). For the study comparing silanized versus non-silanized columns, one prepared column did not receive silane treatment. A three per cent by weight liquid phase loading of OV-17 on Supelcoport was obtained by the method of vacuum coating given in Basic Gas Chromatography. Columns were packed by the introduction via funnel of small quantities of stationary phase into the column followed by gently tapping the column on a padded wooden table top and gently vibration with an electric vibrator. To assure uniform packing, the column was then

gently tapped for an additional 5 minutes at the end. The columns were then conditioned overnight at 275°C at low carrier gas flow.

Silylation conditions

An excellent review on the general experimental considerations for silylation is available.[42] The guidelines proposed in this review were followed during this study with deviations being made as deemed necessary by convenience or situations peculiar to the acid indoles. Ideally elimination of atmospheric humidity would avoid trimethylsilyl derivative decomposition. This, however, was not possible and initial results were irreproducible since the derivative work was carried out during the humid summer months. Several precautions were found to be necessary to prevent high humidity from causing decomposition of the acid indole trimethylsilyl derivatives. First, the reaction vials were flushed with dry argon or nitrogen whenever the vials were opened. Further, since the use of NMR spectroscopy required transfering the mixture from the reaction vial to an NMR tube, all transfers were made by flushing both the NMR tube and the solution remaining in the reaction vial with dry gas. The most effective precaution was the use of minert™ teflon™ body valves, Regis Chemical Co. This was important when repeated injections of the reaction solution were made over a long period of time.

A variety of temperatures have been used to assure optimum reaction conditions for silylation reactions.[35,42] The major effect is the expected one of increased temperature increasing the rates of both the desired trimethylsilylation and the decomposition

of those derivatives. Until recently, no method was available for continuously and externally mixing the silylation reaction mixture in a reaction vial. However, Pierce Chemical Co., (Rockford, Illinois) has introduced a new 3 ml reaction vial with mini magnetic stirrer inside.[43] This development was too recent to be used in this study. Therefore, the temperature controls for optimum silylation required heating the bottom of the reaction vial while exposing the remainder of the vial to the atmosphere. In this fashion, refluxing and mixing of the reaction mixture takes place through convection currents. Satisfactory conditions for temperature in this case are achieved by placing the reaction vial on the injection port cover of the Bendix model 2600 as soon as the trimethylsilylation was initiated. These conditions resulted in an equilibrium temperature at the bottom of the solution of about 80°C and at the top about 60°C. Once the major product of silylation was achieved, the reaction vial was then kept in a container at 4°C and stored for longer periods in a cold room at the same temperature.

In two cases, following the slow silylation of IPA at 4°C and following the effect of room temperature on the silylation of IBA, non-refluxing silylation conditions were used.

Experimental Procedure

Although gas chromatographic ionization detectors provide the sensitivity to measure low concentrations of the acid indoles, NMR sample requirements limit this work to the use of relatively high sample concentrations. For practical spectra, at least 0.3 ml of

sample of 10 per cent by weight of acid indole are necessary. Therefore, for all structural determinations sample conditions of about 50 mg of acid indole in 0.7 ml of solution were used.

Since it was suspected that the 3-indole acids undergo trimethylsilyl substitution at two positions and that BSA optimally donates one trimethylsilyl group per molecule, a mole ratio of 2:1 (BSA: 3-indole acid) was used. However, since commercial BSA is only about 95% pure, approximately 30% excess BSA was used in the trimethylsilylation of the 3-indole acids. The optimum BSA:TRP ratio was expected from previous work[37] to be 3:1, but considering the possibility of replacing both α-amino protons a 4:1 ratio with 30% excess BSA was used.

Although the timing of individual steps varied for each acid indoles, the basic procedure remained the same. To an accurately weighed and carefully dried sample of the acid indole in a 1 ml tapered reaction vial (Regis Chemical Co., Morton Grove, Illinois), 0.5 ml of acetonitrile was added. The NMR spectrum was then obtained for the saturated solution of acid indole in acetonitrile. The solution was shaken vigorously; 0.3 ml of supernatent liquid was removed with a pasteur pipet and placed at the bottom of a 18 cm x 3 mm i.d. NMR tube. The tube was sealed under dry argon and the NMR spectrum recorded immediately. The solution was then returned to the reaction vial, the vial was flushed with dry argon and a minert valve was placed on it. In order to assure detection of artifacts resulting from any previous steps or reagents, a blank chromatogram of the dissolved starting material was then obtained.

BSA was injected into the vial, the reaction mixture was vigorously shaken and a chromatogram was obtained of this mixture after the reaction had proceeded for one minute. Thereafter, the mixture was sampled at 15 minute intervals.

For studies used to determine the optimum reaction time, the reaction vial was placed on the injection port cover, and the reaction was monitored chromatographically until the area of the single peak of interest did not increase with time. At that point, the reaction solution was cooled and placed in an NMR tube using the technique previously described. The NMR spectrum was then obtained immediately, followed by chromatography of the sample directly from the NMR tube to assure that no decomposition had occurred.

For the 25°C and 4°C studies of IBA and IPA, the procedure of transferring the sample solution from the reaction vial to the NMR tube and back was repeated several times during the reaction sequence. This transfer step was used on all samples after varying periods of storage and under various storage conditions to determine acceptable storage conditions and derivative longivity.

RESULTS AND DISCUSSION

BSA Results

Although studies on the structure of N,O bis(trimethylsilyl) acetamide and its products resulting from reaction with trimethylsilyl acceptors have previously been carried out[44], a verification of these structures in the reaction of BSA with the acid indoles was made. It was found that the reaction of BSA with the acid indoles gave a product with the same retention time as that obtained with the reaction of BSA with water. This reaction product and the starting material, BSA, were trapped and analyzed by infrared (IR). For BSA a strong O-Si band at 9.7 microns is observed, but in the reaction product this band disappears and amide carbonyl bands at 3.1 and 6.1 microns appear. This indicates that the reaction product is N-trimethylsilyl acetamide (MSA).

Since NMR spectroscopy was to be used as a method to determine the points of TMS attachment to the acid indoles, an NMR spectrum was obtained for commercial BSA which contained about 10 per cent MSA. Going downfield, a sharp nitrogen TMS peak appeared at δ =.2 ppm, a sharp oxygen TMS peak appeared at 1.8 ppm, a sharp terminal methyl proton peak appeared at 1.94 ppm, and sharp and broad peaks which were proportional to the amount of MSA present appeared at 2.02 and 7.2 ppm. This lower field peak corresponds to an amide proton (δ of 5-8 ppm) and a chemical shift of close to 5 ppm is expected in the less polar solvent, acetonitrile. Monitoring the relative areas of MSA to the compound being silylated can provide a check on the

number of TMS groups which have been transferred to the compound of interest. Of course, one must allow for a certain amount of BSA hydrolysis due to the residual moisture in the solvent. This will give a larger MSA peak than that due solely to the number of TMS groups being transferred to the acid indole.

Gas Chromatography of Indole Derivatives

As stated in the procedure section, evidence gathered on the derivatization of the acid indoles relies on experimentation using NMR and gas chromatography. However, for logical consistancy the gas chromatographic study of the derivatization reaction will be treated first, followed by the analysis of derivative structures by gas chromatographic and NMR spectral techniques.

Most trimethylsilylation reactions are sensitive to moisture, and precautions such as the removal of water from all solvents and apparatus are necessary. One variable which frequently cannot be controlled, however, is that of humidity. For this reason the results from the trimethylsilylation of the acid indoles are presented in two segments. The first segment will present the results of high humidity (about 70%) silylation while the second segment will present those obtained at low humidity (about 20%).

The 3-indole acids are members of a homologous series, but these compounds reacted with BSA differently when derivatized under high humidity conditions. Although derivatization under these conditions does yield irreproducible results, the data gathered from these reactions provides information on both the use of high

humidity silylations as a quantitative tool and on the nature of the reactions themselves. Thus the silylation at 70°C and standard reagent conditions (see procedure) of each of the indole acids, along with deviations in procedure used for that particular compound, will be reported on an individual basis in the following order: IAA, IPA, and IBA. The silylation of TRP, which is a more complex reaction, will be reported last.

The chromatogram for BSA and IAA obtained after reaction at 4°C and about 60% humidity for one minute showed three peaks with adjusted retention times of 3.6, 4.7, and 5.0 minutes and relative heights of 2, 47, and 51 per cent. These peaks were presumably O-TMS-IAA, N-TMS-IAA, and free IAA. The next two chromatograms were obtained after refluxing the mixture at about 70°C for 30 minutes. The first chromatogram after 12 minutes of reaction had two peaks of approximately equal height with $tr' = 3.5$ and 3.6 minutes while after 25 minutes a single peak at 3.5 minutes was present.

The reaction mixture was then cooled to 4°C in the screw capped reaction vial. Sampling after 3 and 12 days yielded chromatograms with 15 and 60 per cent decomposition of the primary reaction product to monosilyl IAA. Storage of the reaction mixture at room temperature in a screw top vial gave even more rapid decomposition. Subsequent work indicated that most of the decomposition resulted from the exposure of the derivatization solution to atmospheric moisture during sampling. When a minert^{R} sampling valve cap was used for the same reaction, the reaction solution could be stored for one week with less than five per cent decomposition of the primary reaction product.

The silylation of IAA by BSA has been previously carried out under similar reagent ratios (differing only by the use of a 10 fold excess of BSA) at room temperature under dry conditions.[38] The authors were not able to obtain a di-TMS derivative even after extended heating of the mixture. Although a 2-fold (and frequently a 10-fold) excess of BSA has been accepted as the optimum BSA:IAA ratio, a 30 per cent excess was sufficient for complete silylation of IAA within one half hour. Thus, any samples weighing less than 50 mg may be successfully monosilylated following the conditions given here.

Problems were initially encountered in the derivatization of IPA due to impurities in one batch of IPA tested. Since the only treatment prior to silylation was to be drying, a more pure sample was obtained. Chromatograms of IPA in acetonitrile on a well silanized[40] column showed a badly skewed peak at t_r' of 5.7 min. The tailing of this peak indicates adsorption of the highly polar IPA on the column surfaces during analysis. If a non-silylated glass column was used for the elution of solvated pure IPA no peak was observed. This is probably due to irreversible adsorption of IPA onto the column's glass surface and indicates the need for silanizing glass columns used with 3-indole acid derivatives.

IPA was derivatized under standard conditions (30 per cent excess BSA in acetonitrile at 70°C and high humidity). Two peaks at t_r' 4.3 (disilyl IPA) and 4.5 (monosilyl IPA) minutes with relative areas of 15 and 85 per cent appear after a reaction time of one

minute. Subsequent sampling of this mixture indicates that the mono-trimethylsilyl product is obtained at 95 per cent yield after 20 minutes. With the use of a minert™ valve this mono-TMS derivative was stored for one week at 4°C with less than 10 per cent decomposition.

Another study carried out using IPA was suggested by the work of Chambaz, et al.[45] They found that BSA can donate both silyl groups if the second silyl group donated replaces an acidic proton. To determine if it was possible to completely derivatize IPA using only one equivalent of BSA, a 1:1 molar IPA:BSA ratio with 30 per cent excess BSA was used. After one minute at room temperature and high humidity the mixture was sampled; the resulting chromatogram showed a peak of disilyl IPA and a peak of O-TMS IPA with an area ratio of about 1:10. This mixture was heated to about 70°C and sampled after 15 and 30 minutes. The resulting chromatograms had a slowly decreasing O-TMS IPA peak while the free IPA peak slowly increased from 7 per cent of the combined areas at 15 minutes to 18 per cent after 30 minutes. At this point the addition of an additional equivalent of BSA did not result in a single disilyl-IPA derivative but caused a repetition of the above results.

An unusual peak which appeared in the IPA in acetonitrile used as a blank offers possible alternative procedure to the normal derivatization methods. This peak had an adjusted retention time of 4.3 minutes and occurred after a syringe containing residual BSA was used to inject the IPA-acetonitrile mixture. When residual

BSA remained in the syringe during the sampling of separate solutions of IAA and IBA in acetonitrile, each sample resulted in a chromatogram with peaks corresponding to a trace quantity of the totally silylated 3-indole acid (t_r' 3.5 and 5.1) and the usual peak of the non-silylated 3-indole acid. Esquivel has noted the ease of "on-column" derivatization in his work with drugs of abuse.[46] It remains for another researcher to exploit this phenomenon for the acid indoles, but the ease of trimethylsilylation using this system shows promise.

Derivatization of IBA under the standard conditions of temperature and high humidity yielded chromatograms similar to those of high humidity derivatizations of IAA and IPA; however, the final mono-TMS product was obtained at 95 per cent yield after 25 minutes at refluxing temperature and decomposed at a faster rate than that for IPA or IAA.

TRP has the potential for 11 different silylation derivative combinations resulting from the possible replacement of four active protons by trimethylsilyl groups. Luckily, only three major derivatives were found. After reaction of TRP with BSA in acetonitrile for 30 minutes under the standard reflux conditions and high humidity, a predominant (95 per cent of the total peak area) peak at t_r' 6.7 min is obtained. If the mixture is heated in the same manner for another hour, the peak at t_r' 6.7 min is replaced by a peak at t_r' 5.9 min and then quantitatively replaced by a peak at t_r' 5.5 min. This derivative, when stored under previously determined acid indole storage conditions, is stable (with less than 10 per cent decomposition) for two weeks.

The silylation of the acid indoles appears to be more reproducible under low humidity conditions. In addition, the members of the 3-indole acid homologous series react with BSA in a similar manner under these conditions. Therefore, the results from these three silylations will be presented together.

One minute after the introduction of BSA into the solvated indole acid mixture, the chromatogram of the reaction mixture shows peaks at t_r' 3.6, 4.5, and 5.4 minutes (the monosilyl IAA, IPA, and IBA). After 15 minutes at about 85°C, the mixture gives a chromatogram which repeats the monosilyl peaks at slightly lower heights and now shows new peaks at t_r' 3.5, 4.3, and 5.1 minutes (the disilyl IAA, IPA, and IBA). These peaks have heights which are 3, 2, and 2 per cent, respectively, of the monosilyl peaks. After 2 hours at 85°C the series of disilyl peaks predominate and the heights of the monosilyl peaks are now 25, 60, and 95 per cent of the disilyl peak heights. After 4 hours this height ratio (monosilyl height/disilyl height) has changed to 5, 10, and 16 per cent. When the reaction has progressed for six hours the chromatogram produced is sufficient for quantitation as the heights of the monosilyl peaks are now less than 5% of those obtained for the disilyl peaks. Finally, after 7 hours less than one per cent of the monosilyl peaks remain (see figure 3).

A study similar to the one above except with a reflux temperature of 70°C yielded similar results. The reaction proceeded such that at 7.5 hours the three indole acids were 95 per cent in the disilyl form. After 10 hours the reaction had proceeded to 99 per cent completion.

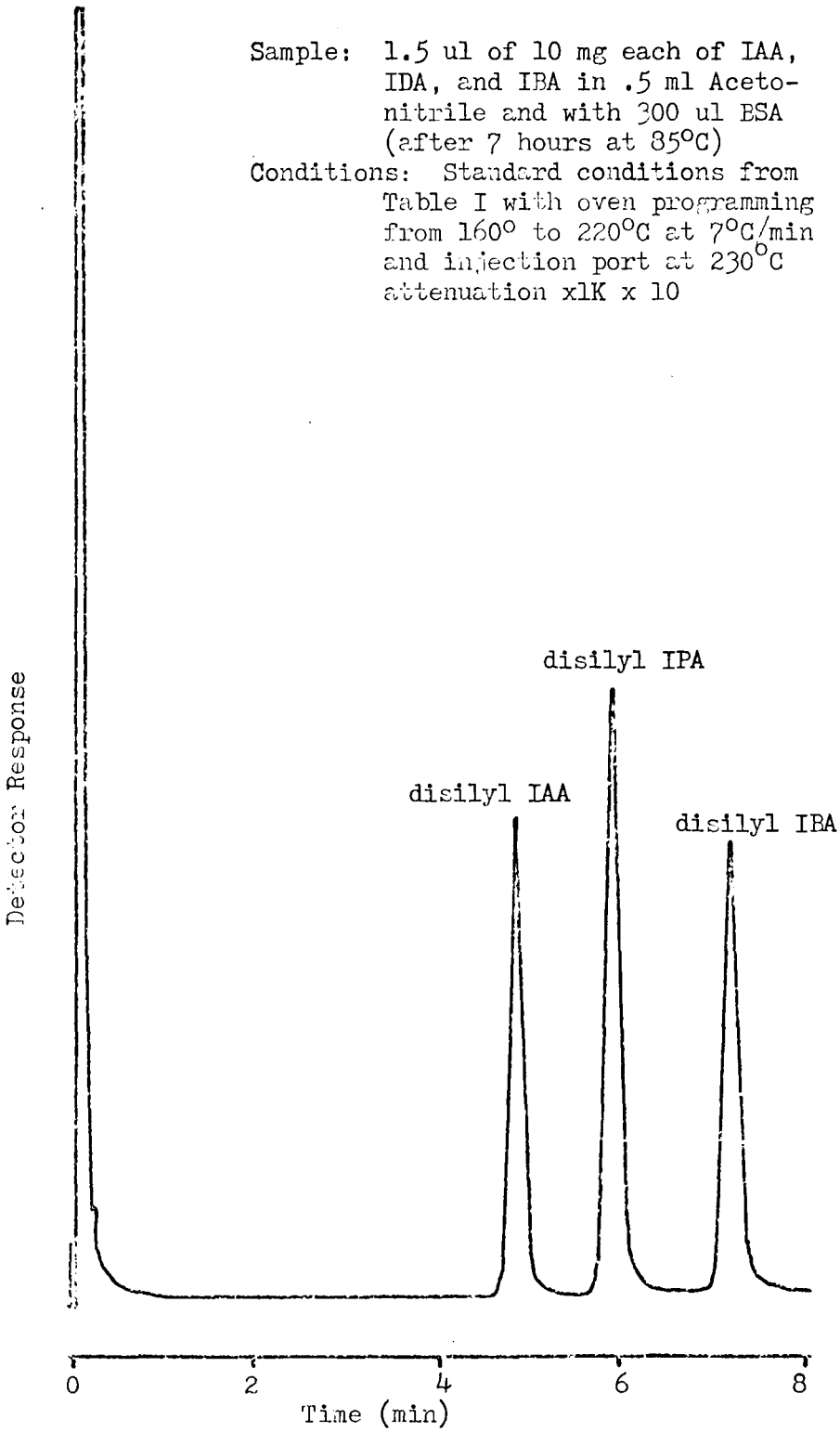


Figure 3. Gas Chromatogram of 3-Indole Acid Mixture

The silylation of TRP under low humidity occurs at a rate similar to that of the 3-indole acids under similar conditions. At 70°C and using standard TRP silylating procedure (see experimental section), the derivatization of TRP is 95 per cent complete with a single derivative peak (t_r' 5.5 min) in 9 hours. Reaction to 99 per cent occurs after 10 hours. The reaction intermediates observed are those observed for the high humidity silylation of TRP. A major difference is that the derivative at t_r' 6.8 minutes does not predominate at any time. Formation of the t_r' 5.9 minutes peak occurs immediately with some residual t_r' 6.8 min) and is slowly converted to the tr = 5.5 minute derivative.

Comparison of the derivatization reactions for acid indoles at high and low humidity leads to a major conclusion. The presence of water in the silylation solution results in an increased rate for both the N-trimethylsilylation of acid indoles and the N-protonation of the disilyl indoles. Previous researchers[34] came to the same conclusion and tried to increase the rate of the trimethylsilylation of these and similar compounds by breathing on the reaction mixture. It remains for other workers in this field to determine the amount of water which can be added to the reaction mixture to obtain a more reasonable time for the acid indole disilylation while assuring relatively long periods of stability of these disilyl derivatives.

Structural information about the trimethylsilylation of the acid indoles may be obtained from the interpretation of gas chromatograms of the acid indole derivatives. The three aspects of peak

symmetry, peak area, and peak position all contributed to identification of the peaks which were obtained during trimethylsilylation.

First, the peak shapes resulting from the separation of reaction mixtures on silanized and unsilanized glass columns gave an indication of the number of active protons present on the derivative. The shape of gas chromatographic peaks of indole acids with two active protons was badly skewed even on well silylated columns. The peaks resulting from monosilyl derivatives of these compounds showed only slight tailing. Finally, the di-TMS derivatives of these compounds had highly symmetrical peak shapes.

The use of peak areas was helpful, but not quantitative. Simply, if the same reaction mixture at two different times gave a single peak of different retention time, the peak with the larger area was the more completely trimethylsilylated derivative. This assumes the flame ionization detector is essentially a carbon counting detector.

The linear relation between t_r' and carbon number for temperature programming was also useful in identifying peaks. Thus, if the adjusted retention time any two 3-indole acid derivatives with the same number and points of attachment of the TMS groups were found, the adjusted retention time of third derivative could be obtained by linear extrapolation. It should be noted that for the free 3-indole acids, the ester-TMS 3-indole acids, and the N-TMS ester TMS 3-indole acids, plots of t_r' versus the side chain carbon number are linear (figure 4). In addition, the exact retention times for free, monosilyl, and

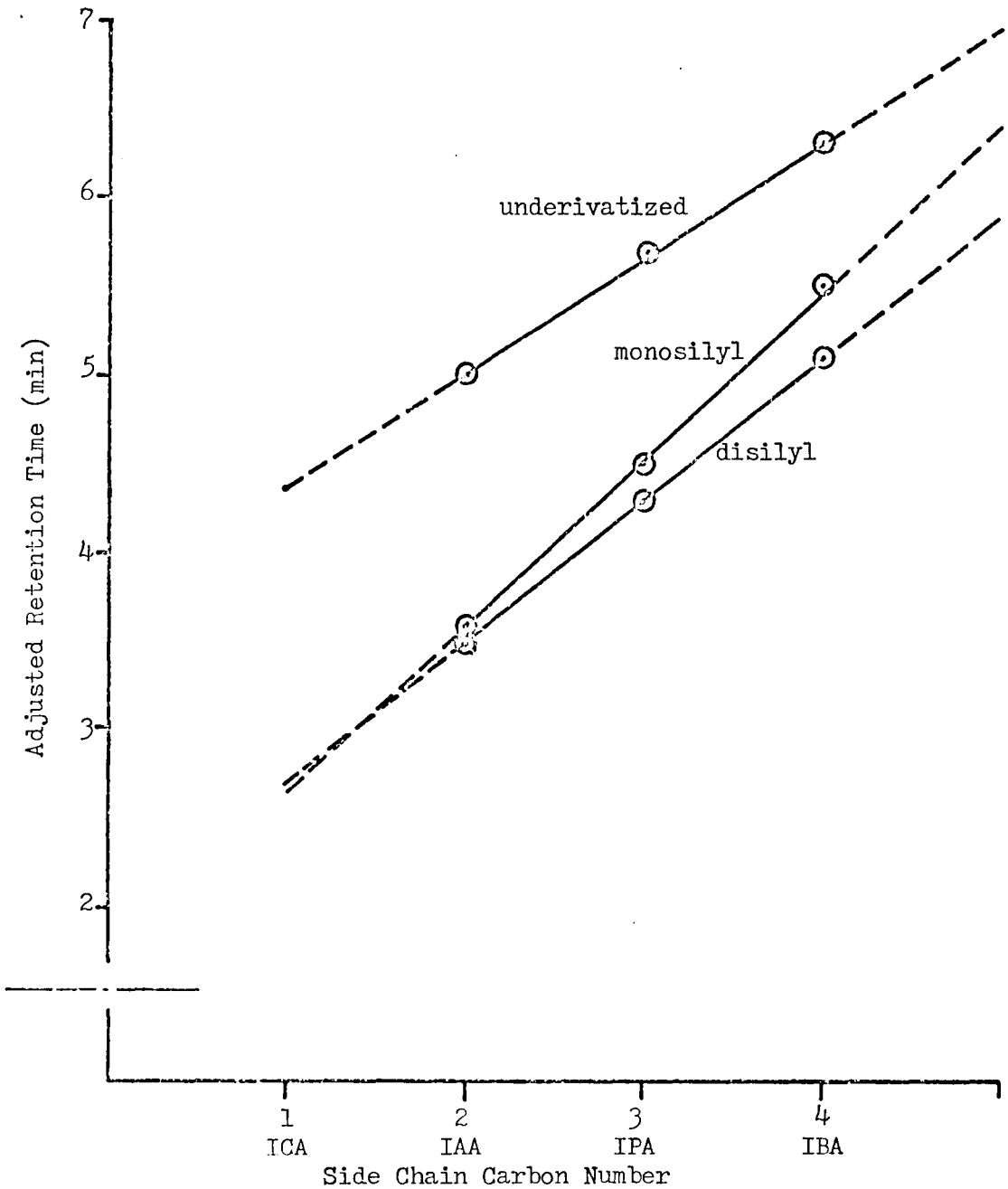


Figure 4. Plot of Carbon Number Versus Adjusted Retention Time for the 3-Indole Acids and Their TMS Derivatives (Using the Chromatographic Condition of Table I.)

disilyl 3-indole carboxylic acid (ICA) and 3-indole acids higher than 3-indole butyric acid may be found from these plots.

The NMR data obtained on the acid indole derivative mixtures gave positive proof of derivative structures. A summary of this data is given on tables II-IV along with the structures of the derivatives formed and their retention times. A detailed explanation of both the chemical shifts and coupling constants may be in found in (47). One difficult assignment was the band found at 9 to 9.5 ppm in the spectra of IAA, IPA and IBA. Carboxylic acid protons generally absorb from 10.5 to 12 ppm[48] while indolic nitrogen protons generally absorb from 5 to 8.5 ppm. However, chemical shifts of up to δ=9.2 ppm (for 2 methyl ester indole in chloroform) have been obtained for the indolic nitrogen proton.[49] Since nitrogen protons tend to be affected more by solvents, and since the disappearance of this band occurred simultaneously with major splitting changes in the indole proton resonance pattern, the band at 9 to 9.5 ppm was assigned as the indolic nitrogen proton. The carboxylic acid proton exchanges so rapidly with the highly polar solvent that it is not observed (the spectra were scanned from δ=15 ppm to 0 ppm with no carboxylic proton being seen).

NMR data was used to provide structural information on the acid indole component of the derivatization mixture by two major methods. First and easiest, if the chromatogram of the mixture showed only one major peak, the NMR spectrum obtained at that time period (minus the acetonitrile, BSA, and MSA peaks) corresponded to the NMR spectrum of

TABLE II - Correlation of Structures with Retention Times and NMR Data for IAA

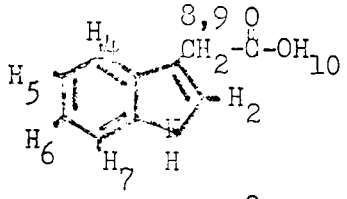
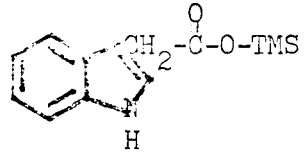
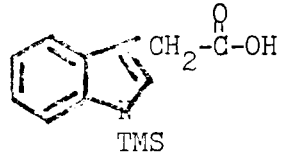
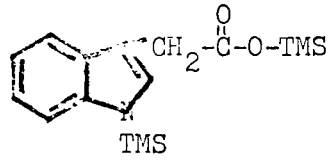
Structure	t'r	Chemical Shift (ppm)	Relative Integration
I. 3-Indole acetic acid			
I.a. 	5.0 min	δ_1 = 9.18 δ_{1}^{2-6} = 6.82-7.70 δ_7 = 7.29 $\delta_{8,9}$ = 3.64 δ_{10} = not observed	1 4 1 2 0
b. 	3.6 min	δ_1 = 9.22 δ_{1}^{2-6} = 6.82-7.70 δ_7 = 7.29 $\delta_{8,9}$ = 3.64 δ_{10} = not observed	1 4 1 2 0
c. 	4.7 min	NOT AVAILABLE	
d. 	3.5 min	δ_1 = not observed δ_{1}^{2-6} = 6.82-7.70 δ_7 = 7.40 $\delta_{8,9}$ = 3.64 δ_{10} = not observed	0 4 1 2 0

TABLE III - Correlation of Structures with Retention Times and NMR Data for IPA

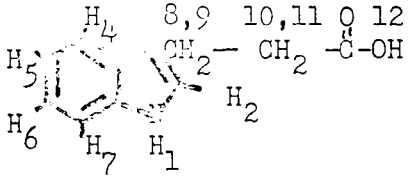
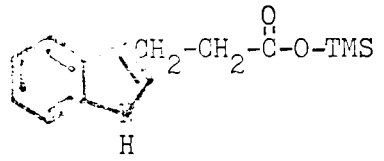
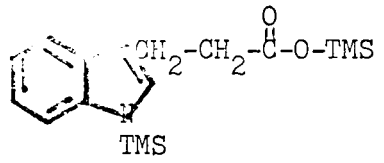
Structure	t'r	Chemical Shift (ppm)	Relative Integration
II. 3-Indole propionic acid			
a. 	5.7 min	δ_1 = 9.08 δ_{2-6} = 6.92-6.68 δ_7 = 7.36 $\delta_{8,9}$ = 2.66 $\delta_{10,11}$ = 2.66 δ_{12} not observed	1 4 1 2 2 0
b. 	4.5 min	δ_1 = 9.68 δ_{2-6} = 6.84-7.68 δ_7 = 7.36 $\delta_{8,9}$ = 3.04 $\delta_{10,11}$ = 2.66 δ_{12} not observed	1 4 1 2 2 0
c. 	4.3 min	δ_1 = not observed δ_{2-6} = 6.84-7.66 δ_7 = 7.54 $\delta_{8,9}$ = 3.04 $\delta_{10,11}$ = 2.66 δ_{12} not observed	0 4 1 2 2 0

TABLE IV - Correlation of Structures With Retention Times and NMR Data for IBA

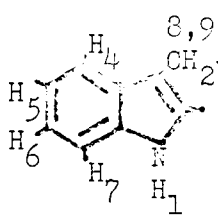
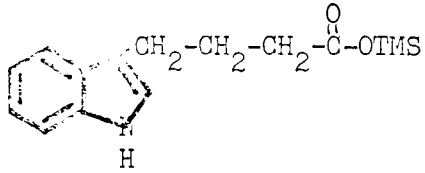
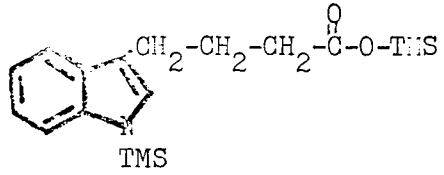
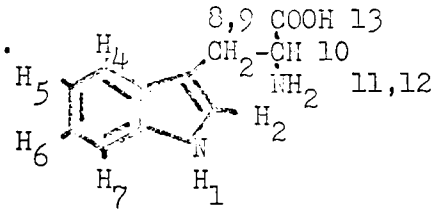
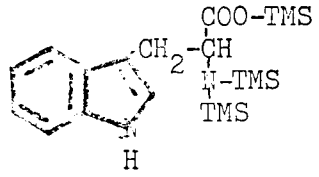
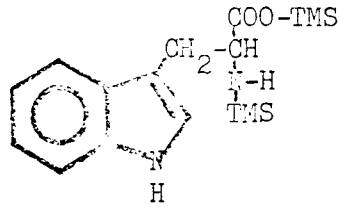
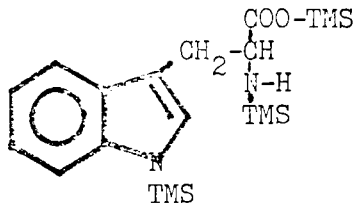
Structure	t'r	Chemical Shift (ppm)	Relative Integration
III. 3-Indole Butyric acid			
a.  8,9 10,11 12,13 O 14 CH_2-CH_2 -CH_2 -C-OH	6.3 min	δ_1 = 8.98 δ_{2-6} = 6.82-7.60 δ_7 = 7.28 $\delta_{8,9}$ = 2.68 $\delta_{10,11}$ = 2.24 $\delta_{12,13}$ = 2.52 δ_{14} = not observed	1 4 1 2 2 2 0
b.  CH_2-CH_2-CH_2-C-OTMS	5.5 min	δ_1 = 8.98 δ_{2-6} = 6.82-7.60 δ_7 = 7.28 $\delta_{8,9}$ = 2.68 $\delta_{10,11}$ = 2.24 $\delta_{12,13}$ = 2.52 δ_{14} = not observed	1 4 1 2 2 2 0
c.  CH_2-CH_2-CH_2-C-O-TMS	5.1 min	δ_1 = not observed δ_{2-6} = 6.82-7.58 δ_7 = 7.42 $\delta_{8,9}$ = 2.70 $\delta_{10,11}$ = 2.25 $\delta_{12,13}$ = 2.52 δ_{14} = not observed	0 4 1 2 2 2 0

TABLE V - Correlation of Structures with Retention Times and NMR Data for TRP

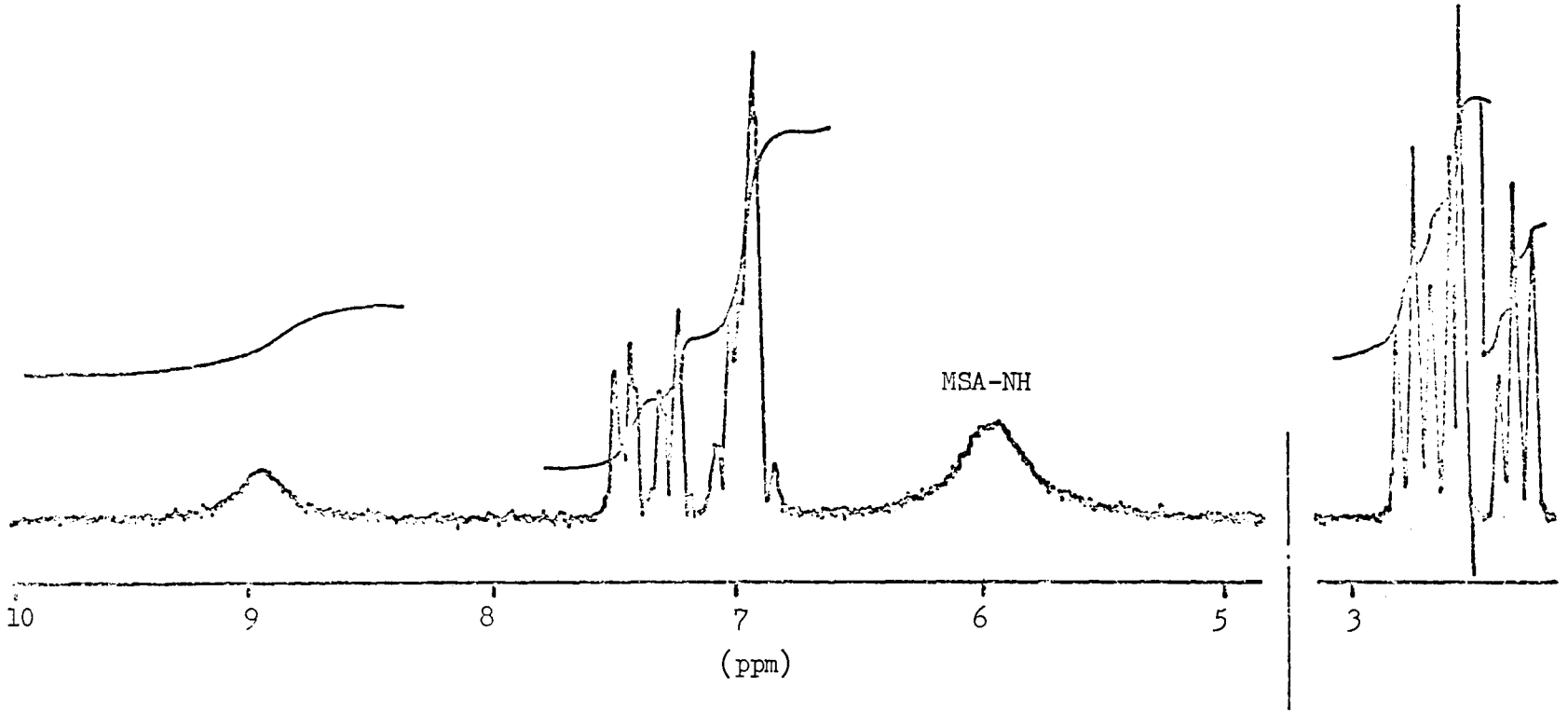
Structure	t'r	Chemical Shift (ppm)	Relative Integration
V. D-Tryptophan			
a. 	not eluted	*δ_1 = unobserved δ_{2-7} = 6.80-7.70 δ_8 = 3.03 δ_9 = 3.22 δ_{10} = not analogous $\delta_{11,12}$ = not analogous δ_{13} = 10.60	0 5 1 1 1 2 1
b. 	6.8 min	δ_1 = not observed $\delta_{4,7}$ = 6.90-7.20 $\delta_{2,5,6}$ = 7.32-7.70 δ_8 = 2.98 δ_9 = 3.04 δ_{10} = 3.66 $\delta_{11,12}$ = not observed δ_{13} = not observed	0 2 3 1 1 1 0 0

*D-Tryptophan data obtained from N-Acetyl DL Tryptophan, Sadtler Spectrum #NMR 13597M

TABLE V (con't)

Structure	t'r	Chemical Shift (ppm)	Relative Integration
V. c. 	5.9 min	δ_1 = not observed	0
		$\delta_{4,7}$ = 6.90-7.20	2
		$\delta_{2,5,6}$ = 7.32-7.70	3
		δ_8 = 2.98	1
		δ_9 = 3.04	1
		δ_{10} = 3.66	1
		$\delta_{11,12}$ = 4.00	1
		δ_{13} = not observed	0
d. 	5.5 min	δ_1 = not observed	0
		$\delta_{4,7}$ = 6.90-7.12	1
		$\delta_{2,5,6}$ = 7.32-7.70	3
		δ_8 = 2.98	1
		δ_9 = 3.04	1
		δ_{10} = 3.66	1
		$\delta_{11,12}$ = 4.01	2
		δ_{13} = not observed	0

that compound. Figures 5 and 6 illustrate the use of this method for the determination of IBA derivative structures. Fortunately a majority of the derivatives could be identified in this manner. However, if two derivative peaks were observed in the chromatogram of a single acid indole, identification of the two peaks was more difficult. In all cases these two compounds differed only by the replacement of one proton by a trimethylsilyl group. Thus the NMR spectrum of the reaction mixture would show one particular peak which had an integration value of less than one proton. The chemical shift of this peak indicates the site of silylation which was present in one but not the other derivative. In addition, the actual proton value of that peak on the NMR spectrum will be same as the per cent of total derivative area for the gas chromatographic peak which corresponds to the less silylated derivative. An illustration of this method was the peak assignment for the low humidity IPA derivatives. After the IPA-BSA mixture had reacted at 85°C for 1.5 hours, the peak areas (for t_r' 4.3 and 4.5 minutes) from the chromatogram of the mixture were 33 and 66 per cent while the NMR spectrum showed that the indolic nitrogen proton was present at 65% of its expected height. This data indicates that the derivative at t_r' 4.3 minutes has a TMS group at the indolic nitrogen position while the derivative at t_r' 4.5 minutes has a proton in that position. When this method of peak identification was used, several comparisons of per cent derivative areas to per cent proton present were carried out for the same two peaks at different area ratios.

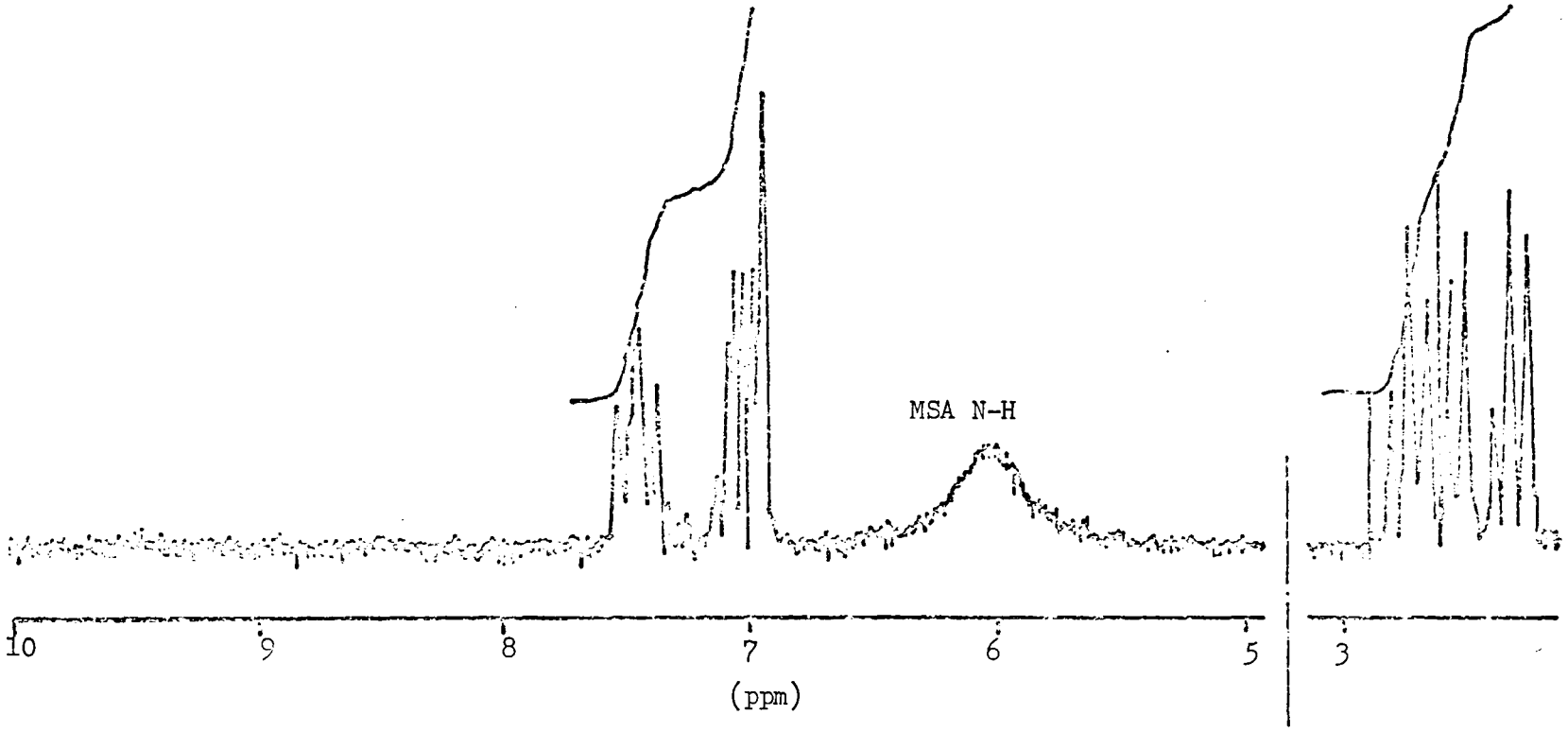


Conditions:

Solvent: acetonitrile
Concentration: 10 %
Temperature: ambient
RF level: 40

Spectrum amplitude: 10
Filter: 20 Hz
Sweep time: 250 seconds for 1080 Hz
Sweep offset: 220 Hz

Figure 5. NMR Spectrum of Monosilyl IBA



Conditions:
Solvent: acetonitrile
Concentration: about 10%
Temperature: ambient
RF level: 40

Spectrum amplitude: 10
Filter: 20 Hz
Sweeptime: 250 seconds for 1080 Hz
Sweep offset: 220 Hz

Figure 6. NMR Spectrum of Disilyl IBA

Trapping System

The trapping system shown in figure 7 resulted from many modifications of an initial commercial design. These modifications were made to provide a means by which the labile derivatives previously discussed could be isolated. All components of the system which contact the chromatographic effluent must be inert with respect to the compounds passing through the splitting system. For non-polar and thermally stable compounds a simple, heated tube of stainless steel coupled with a metallic splitting valve will suffice. The considerations for polar and thermal-labile compounds, especially trimethylsilylated, include careful thermostating and using component materials which are non-reactive.

The temperature should be uniform over the entire system; hot enough to prevent any condensation of sample; yet cool enough to avoid decomposition. This means that the entire splitter should be maintained at the maximum temperature at which the column will be operated. All materials contacting the chromatographic effluent should be teflon^{TM}, 316 stainless steel, or glass. Thorough cleaning procedures must be followed if stainless steel is used. Glass does not require extensive cleaning, but following the guidelines given in (40) peak shape will improve with simple cleaning procedures.

The second major consideration is choosing and maintaining an optimum split ratio for the system. For studies where large amounts of sample are available, the splitter should pass most of the chromatographic effluent (99%) to the trapping outlet. For studies where

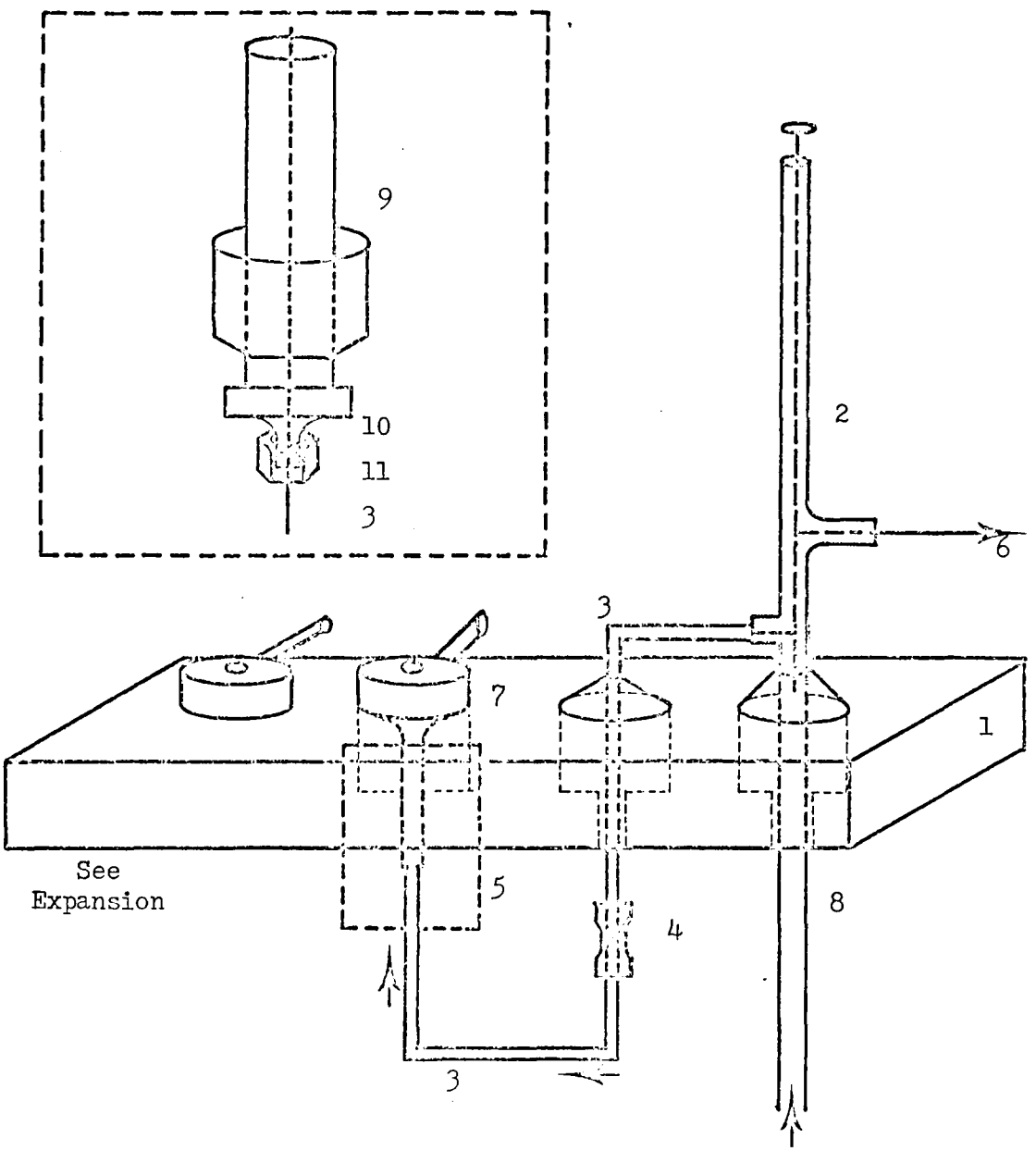


Figure 7. Diagram of on Column Flame Ionization Effluent Splitter

TABLE VI - Components of On Column Flame Ionization Effluent Splitter

1) Bendix 2500 detector block

2) Scientific Glass Engineering (SGE) all glass effluent splitter

3) 1/16" o.d. 0.5 mm i.d. SGE glass lined stainless steel tubing (GLT)

4) 316 stainless steel (SS) 1/16" to 1/16" union drilled out to GLT o.d.

5) System for going from 1/16" GLT into on column flame ionization detector (alternative is 9 mm of 1/4" glass tubing of SGE Glaslock G KLU/1/4"/1/16" 1/16" GLT to 1/4 glass connector).

6) Less than 1" of 1/16" GLT protruding for splitter

7) Flame ionization detector

8) 1/4" analytical glass "U" column

9) 1/4" o.d. 316 ss tube drilled out to fit o.d. of 1/16" GLT

10) 1/8" inlet

11) 1/8" to 1/16" teflon reducing ferrule

the components of interest are present in trace quantities and where a chromatogram of the separation is required, the effluent should be split about equally between the trapping outlet and the detector. Ideally, if quantitative work is to be done the split ratio should be such that all of the effluent reaches the detector.

The splitting system devised and implemented in this work achieves uniform temperature, inert effluent surfaces, and reasonable split ratios. The entire splitting system which is not in the chromatographic oven was covered by 4 layers of 1 inch wide prefired asbestos tape (Bethlehem Apparatus Co., Hellerton, Pa.) saturated with plaster of paris. This surface was then wrappened evenly with 4 feet of 4 mm diameter heating tape, paying particular attention so that the splitter and all connecting tubing were well wrapped. The inlet thermocouple from the instrument was then buried under the asbestos and placed next to the glass splitter. Power for the heating tape was applied by means of a 0-125 V variac. Heat from the detector block (250°C) gave a reading of 70°C in the splitter with no variac current. The maximum suggested temperature for the splitting device was 300°C, and the heating system was easily able to achieve this temperature.

By using Scientific Glass Engineering (Victoria, Australia) glass lined stainless steel tubing, the entire effluent path was glass. To avoid adsorption from surface contaminants, the splitter system was cleaned using the procedures recommended in (40). Sample decomposition of trimethylsilylated cholestane, D-Tryptophan, and the three

3-indole acetic acids within the splitting system proved negligible. Each gave a single peak whose retention time and peak area was the same as the pure previous compounds chromatographed without a splitter. No decomposition peaks were noted. Peak shapes were generally good, however the IAA peak was slightly anti-langmuir in nature.

The split ratios for low and high boiling compounds were determined by first setting the split plunger (see figure 7) below the detector split (maximum detector split) and then above the trapping split (maximum trapping split). For methanol, the maximum effluent split to the detector was 100% and the maximum effluent split to the trapping outlet was 99.3%. For a high boiler disilyl IAA was used. The maximum split to the detector was again 100%, but the maximum split to the trapping outlet was 94.4%.

The split ratios in each case were determined by first obtaining a peak for the compound of interest with the trapping outlet sealed shut. With a simple glass capillary tube trapping device at the trap outlet and the splitting valve set for the maximum trapping flow, the same amount of that compound was again injected and the peak height determined. From the per cent of this sample effluent reaching the detector relative to that found when sealing the trap outlet the per cent of the sample effluent going through the trapping outlet was determined. This same technique was used to determine maximum per cent effluent flow to the detector by changing the split value to maximum detector flow.

Several precautions should be observed when using this splitting device for trapping highly unstable indole acid derivatives. The 1/16 inch glass lined tubing extending from the trapping effluent path (see figure 7) remains cooler than the rest of the splitter. Thus, if this tubing extends more than one inch from the heated and insulated splitter, high boiling compounds will condense in the glass lined tubing rather than in the trapping device. It is also essential that the device be continuously flushed with dry carrier gas and all atmospheric humidity excluded. If the atmospheric moisture is allowed to enter the trap, derivative decomposition takes place. Another possible source of decomposition is trace moisture in the solvent when the trapped condensate is being dissolved for further handling. The solvent should be dried by the addition of molecular sieve prior to use.

Liquid Chromatography of Acid Indoles

High speed liquid chromatographic separation of acids indoles allows for both easier sample collection and less care in sample preparation than the corresponding gas chromatographic separation. Complete resolution of all four free components (IAA, IPA, IBA, and TRP) was obtained on a Dupont Strong Anion Exchange (SAX) column with extremely strong solvent systems (0.01N HNO_3 in water). Elution using a 0.5 meter Pellionix strong anion exchange column and a .0028M to 1.00M KH_2PO_4 gradient produced baseline separation of a mixture of TRP, IAA, and IBA but the IPA peak was unresolved from IAA and IBA. Increasing the ionic strength and pH of the solvent and using the DuPont SAX column yielded a better IAA, IPA, IBA separation. It was

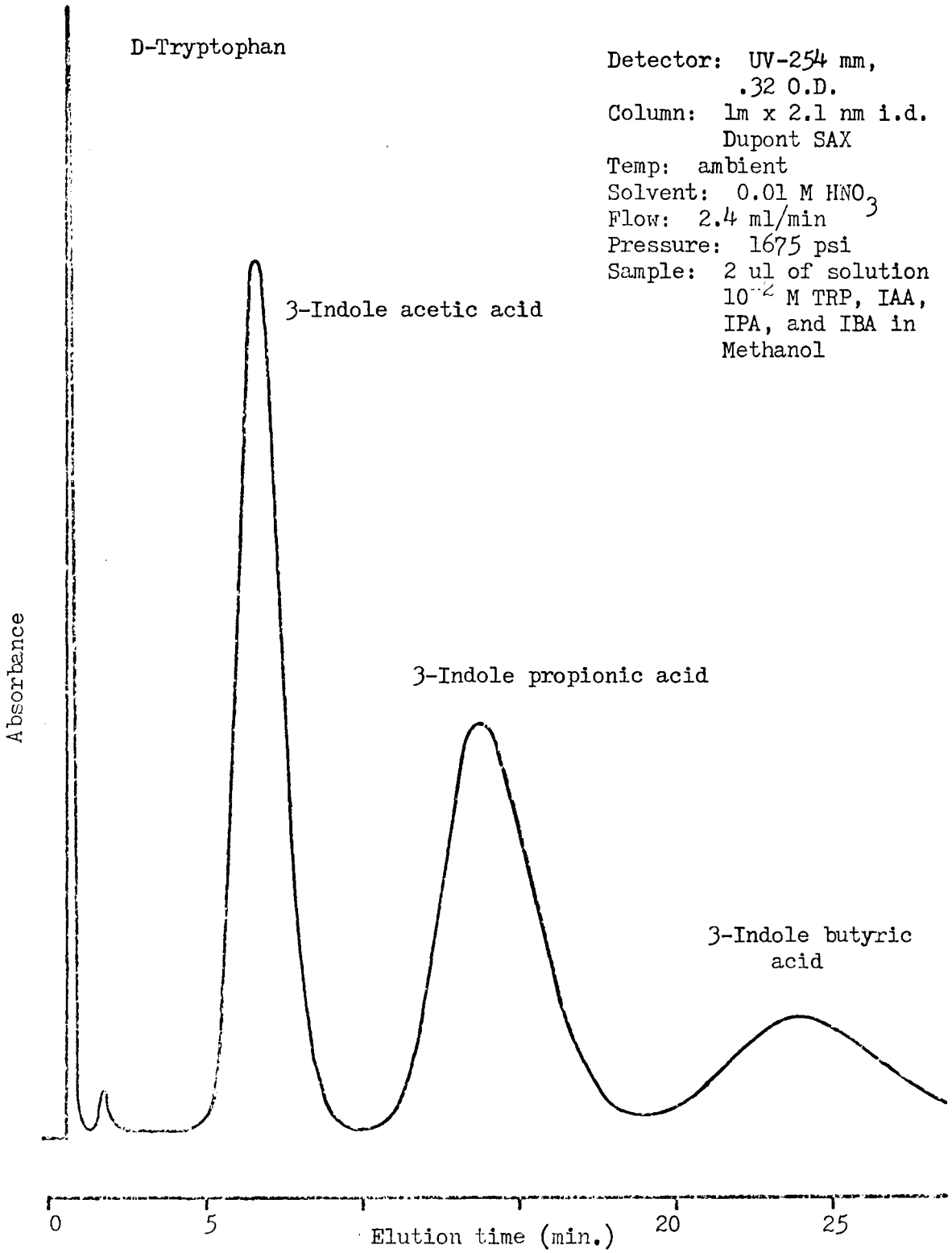


Figure 8 - Liquid Chromatogram of a Synthetic Acid Indole Mixture

not, however, until 0.01M HNO_3 (pH2) was used that the resolution became complete for all four components (figure 8). No degredation of the chromatographic system was observed during the short use of this solvent system. Although some decomposition of the indole acids during elution was possible with such a strong oxidizing solution, single peaks were obtained for each of TRP, IAA, IPA, and IBA and no decomposition products were observed. Unfortunately, the sensitivity of the UV photometer detection for the indole acids was very poor. At a pH of 2, the minimum detectable quantity is about 10^{-5} g (10^{-2} Molar) of the acid indoles.

CONCLUSIONS

Summary

1) When reacted with acid indoles, BSA is converted to N-trimethylsilyl acetamide.

2) The 3-indole acids form different stable TMS derivatives depending upon the room humidity. When derivatized at high humidity, the 3-indole acids are quantitively converted to mono-TMS derivatives appearing at t'r = 3.6, 4.5, and 5.4 minutes. The high moisture content of the silylation mixture causes the formation within one minute of the di-TMS derivatives of these compounds followed by their quantitative decomposition within 25 minutes to the mono-TMS derivative. Under conditions of low humidity, immediate formation of the mono-TMS derivatives of the 3-indole acids occurs. The total conversion of the mono-TMS derivatives to the di-TMS derivative occurs within 150 hours at 4°C, within 10 hours at 70°C, and within 7 hours at 85°C. Extrapolation of the time-reaction temperature curve implies that the reaction would be complete within 1 hour at 120°C.

3) Maximum longevity for the acid indole derivatives is obtained by the use of minert valve tops for the reaction vials, initially flushing the silylation vial with a dry gas, and storage of the derivatives at 4°C.

4) Optimum storage condition for the mono- or di-TMS 3-indole acid solutions allows the storage of these compounds for one week with the retention of 95 per cent of the major derivative peak in each case.

5) TRP reacts with BSA to form first a trisilyl (t'r = 6.8 min), then a disilyl (t'r = 5.9 min), and finally a trisilyl (t'r = 5.5 min) derivative. In all three derivatives the acid proton is replaced. In the first derivative both amine protons are also replaced, in the second derivative only one amine proton is replaced, and in the final, most stable derivative one amine proton and the indolic nitrogen proton are replaced.

6) The intermediate low humidity derivatization product, the high humidity major product, and the low humidity decomposition product in the cases of IAA, IPA, and IBA were shown to be the ester-TMS derivatives. The final high humidity derivative peak was proven to be the N-TMS, ester-TMS derivative of the 3-indole acids.

7) Although the standard gas chromatographic conditions given in table I were used for all derivative formation studies, these can be changed to optimize separation of the IAA, IPA, IBA and the IAA, IPA, IBA, TRP mixtures. The first mixture was optimally separated such that mono- and di-TMS derivatives are resolved in each case. This was done by following table I except that the column oven programming was from 160°C at 7°C/min. to 220°C. In the later mixture at an isothermal temperature of 160°C, the IBA derivatization intermediate and the final TRP derivative overlap but all other peaks are resolved. For high humidity work the injection port temperature must remain below 160°C, while under low humidity conditions injection port temperatures of up to 280°C may be used.

8) An original on-column effluent splitter was installed in a Bendix Model 2600 which gave a maximum detector split of 100% and a maximum trapping split for high boilers of 94%. The system has an all glass path and does not cause decomposition of TMS-derivatives.

9) TRP, IAA, IPA, IAA are completely resolved when separated in a liquid chromatograph using a Dupont SAX column with 0.01 M HNO_3 (pH2) as the mobile phase.

Biological Applications

The results presented in this study should help future investigators who wish to use gas chromatography for the analysis of acid indoles. Information on the conditions of time, temperature, and humidity necessary for a quantitative reaction should assist those workers who require rapid and complete conversions. Identification of all intermediate derivatization products and decomposition products found in the silylation of the acid indoles should assist those workers who wish to identify the peaks present in the complex chromatograms usually obtained from biological extracts (figure 8). Researchers interested in either or both quantitation and qualitation can benefit from understanding the optimum storage conditions of acid indole derivatives and length of time the derivatives may be stored under those conditions.

Since the effluent splitting system was useful for the trapping of trimethylsilyl acid indole derivatives, it should be possible to use it to trap the major trimethylsilylation products from natural

Sample: 1/10 of R.F. 0.9 fraction from paper chromatogram of neutral indole fraction of tomato seedling extract silylated with 200 ml BSA

Conditions: 6 foot glass U column of 3% OV-17 on Chromosorb W 80/100 mesh. Oven 150°C to 220°C at 4°C/min; Detector 250°C; Injection port 240°C; 45 ml/min He

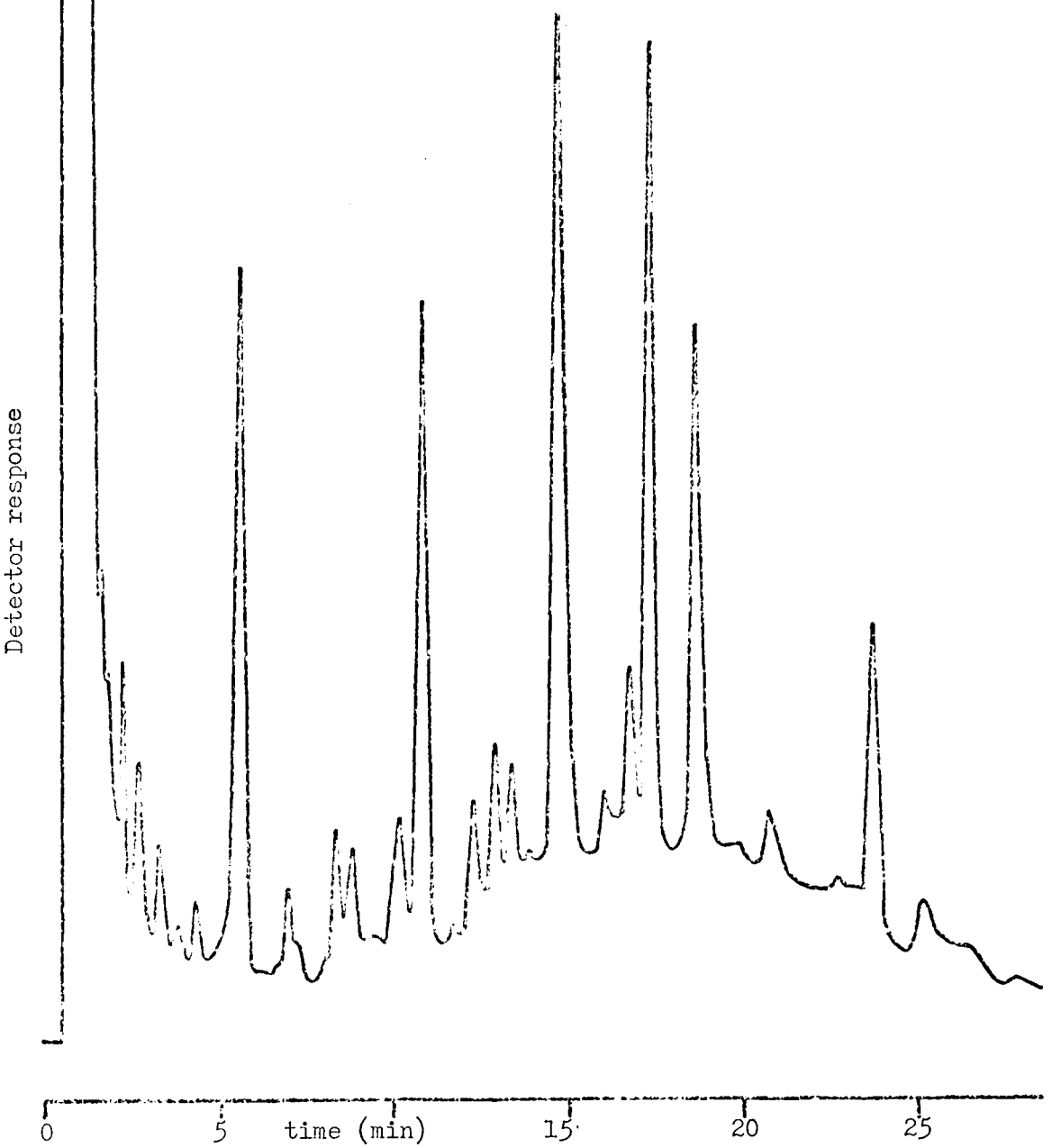


Figure 9 - Gas Chromatogram of a Biological Mixture Which Displays Auxin-Like Activity in the Rooting of Tomato Plants

product extracts. Pure samples not exceeding 100 ug may be obtained from a single run; this is sufficient to obtain a mass spectrum and thus positively identify the trapped compound.

Work Remaining

Although separation and quantitation of the acid indoles has been accomplished by liquid chromatography, works remains on extending the detection limit and optimizing the mobile and stationary phases. The first problem can be partially solved by the use of a fluorometer detector which previous studies[31] have indicated will detect down to 10^{-7} g of the acid indoles.

Two directions may be taken to alleviate the problem of high mobile phase strength. The first is the use of reverse phase (liquid-bonded liquid) chromatography, perhaps using Dupont ODS (octadecyl-silane) permaphase with methanol as the mobile phase. Alternatively, a weak anion exchange column or coupled weak anion and weak cation exchange columns would allow the use of a weaker solvent than 0.10 M HNO_3 for the mobile phase.

It should be noted that no study of the effect of BSA: aceto-nitrile acid indole ratios was made. Each worker will have different sample requirements in this area. Obviously the experimenter who has such a small sample that he must use BSA as both a solvent and silylating agent would have different problems from those workers who have sufficient sample to afford the use of a silylating solvent. Thus for the problem of mole ratio each researcher must tailor his ratio to fit the problems or sample at hand.

The phenomena of on-column derivatization was observed but not examined in this work. Since the reaction time (7 hours at 85°C) necessary to quantitatively obtain disilyl (trisilyl for TRP) derivatives of the acids indoles is a severely limiting experimental parameter, the use of on-column silylation would be appealing for the rapid analysis of these compounds.

Although the presence of moisture in silylation mixtures is to be avoided if possible, the addition of a small quantity of water to the acid indole silylation medium appears to catalyze both the disilylation and disilyl decomposition reactions. Since only one study was carried out under intermediate humidity, only the high and low humidity derivatizations of the acid indoles were examined. Thus it remains for another worker to examine the exact role of water in the kinetics of these reactions and determine that amount of water which will catalyze the disilylation reaction, however, not cause rapid decomposition of the disilyl derivative.

BIBLIOGRAPHY

1. Aung, L.H., Private Communication.

2. Hill, T.A., "Endogenous Plant Growth Substances," William Clowes and Sons, London (1973).

3. Yamaki, T.P., Shimojo, E., Yoshizawa, K., and Namekawa, K., "8th International Conference on Plant Growth Substances," S. Tamara, (ed.), U. of Tokyo Press, Tokyo (1973).

4. Phillips, I.D.J., "The Biochemistry and Physiology of Plant Growth Hormones," McGraw-Hill Book Co., New York (1971).

5. Scott, T.K. and Jacobs, W.P., "Regulateurs Naturels de la Croissance Vegetale," J.P. Nitsch (ed.), Edition du Centre National de la Recherche Scientifique, Paris (1963).

6. Berger, J., and Avery, G.S., Jr., Amer. Jour. Bot., 31, 199 (1944).

7. Haagen-Smit, A.J., Dandliker, W.B., Wittwer, S.H., and Murneek, A.E., Amer. Jour. Bot., 33, 118 (1946).

8. Galston, A.W., and Davies, P.J., "Control Mechanisms in Plant Development," Prentice-Hall, Inc., Englewood Cliffs, New Jersey (1970).

9. Bonner, J.F., and Varner, J.E. (ed.), "Plant Biochemistry," Academic Press, New York (1965).

10. Gentile, A.C., "Plant Growth," Natural History Press, Garden City, New York (1971).

11. Bayer, Margaret H., Plant Physiol., 44, 267 (1969).

12. Hitchcock, A.E., Contrib. Boyce Thompson Inst. 7, 87 (1935).

13. Aung, L.H., Physio. Plant., 26, 306 (1972).

14. Wightman, F., "Regulateurs Naturels de la Croissarce Vegetale," J.P. Nitsch (ed.), Edition du Centre National de la Recherche Scientifique, Paris (1963).

15. Libbert, E., Kaiser, W., and Kunert, R., Physiol. Plant. 22, 432 (1969).

16. Kogl, F., Haagen-Smit, A.J., and Erxleben, H., Z. Physiol. Chem., 228, 90 (1934).

17. Litwack, G., and Kritchersk, D., "Actions of Hormones on Molecular Processes," Wiley and Sons, Inc., New York (1964).

18. Dye, M.H., Clarke, G., and Wain, R.L., Proceedings of the Royal Society, 155, 478 (1962).

19. St. John, P.A., Brooks, J.L., and Biggs, R.H., Anal. Biochem., 18, 459 (1967).

20. Stowe, B.B., and Schilke, J.F., "Regulateurs Naturels de la Croissance Vegetale," J.P. Nitsch (ed.), Edition du Centre National de la Recherche Scientifique, Paris (1963).

21. MacNeil, J.D., Haeusler, M., Frei, R.W., and Hutzinger, O., Anal. Biochem., 45, 100 (1972).

22. Okamoto, T., Isogai, Y., and Koizumi, T., Chem. Pharm. Bull., 18, 1815 (1970).

23. Anthony, A., and Street, H.E., New Phytol., 69, 217 (1970).

24. Bak, M., and Carlson, J.R., Anal. Biochem., 34, 46 (1970).

25. Pillay, D.T.N., and Mekdi, Rafia, J. Chromatog., 32, 592 (1968).

26. Sagi, Ferenc, J. Chromatog., 39, 334 (1969).

27. Kung-Tsung Wang, Y., and Her-Hwey, L., Nature, 213, 213 (1967).

28. Stowe, B.B., and Thimann, K.V., Arch. Biochem. Biophys., 51, 499 (1954).

29. Raj, R.K., and Hutzinger, O., Anal. Biochem., 33, 471 (1970).

30. Powell, L.E., Plant Physiol., 35, 256 (1960).

31. Chilcote, D.D., and Mrochek, J.E., Clin. Chem., 18, 778 (1972).

32. Dedio, W., and Zalik, S., Anal. Biochem., 17, 244 (1966).

33. Horning, M.G., Boucher, E.A., and Moss, A.M., J. Gas Chromatog., 5, 297 (1967).

34. Coward, R.F., and Smith, P., J. Chromatog., 45, 230 (1969).

35. Gehrke, C.W., Nakamoto, H., and Zumwalt, R.W., J. Chromatog., 45, 24 (1969).

36. Gehrke, C.W., and Leimer, K., J. Chromatog., 57, 219 (1971).

37. Klebe, J.F., Finkbeiner, H., and White, D.M., J. Am. Chem. Soc., 88, 3390 (1966).

38. Grunwald, C., Mendez, J., and Stowe, B.B., "Biochemistry and Physiology of Plant Growth Substances," F. Wightman and G. Setterfield (ed.), Runge Press Ltd., Ottowa, Canada (1968).

39. Gordon, Arnold, J., and Ford, Richard A., "The Chemists' Companion - A Handbook of Practical Data, Techniques and References," John Wiley and Sons, New York (1972).

40. Leibrand, Roger J., and Dunham, Loren L., Research and Development, 24, 32 (1973).

41. McNair, H.M., and Bonelli, E.J., "Basic Gas Chromatography," Varian Aerograph, Walnut Creek, California (1968).

42. Handbook of Silylation, Pierce Chemical Company, Rockford, Ill. (1972).

43. Pierce Previews, Pierce Chemical Company, Rockford, Ill., February (1974).

44. Klebe, J.F., "Advances in Organic Chemistry - Methods and Results," Volume 8, E.C. Taylor, (ed.), Wiley Interscience, New York (1972).

45. Chambaz, E., Maume, G., and Horning, E.C., Anal. Letters, 7, 749 (1968).

46. Esquivel, B.E.H., Masters Thesis, Virginia Polytechnic Institute and State University (1973).

47. Remers, W.A., and Brown, R.K., "Heterocyclic Compounds - Indoles," part I, W.J. Houlihan (ed.) (1972).

48. Silverstein, R.M. and Bassler, G.C., "Spectrometric Identification of Organic Compounds," John Wiley and Sons, Inc., New York (1967).

49. Batterman, T.J., "NMR Spectra of Simple Heterocycles," Wiley-Interscience, New York (1973).

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DERIVATIZATION AND CHROMATOGRAPHIC SEPARATION OF ACID INDOLE AUXINS

by

Daniel G. Marsh

(ABSTRACT)

For many years silylation of polar and thermolabile compounds has been used to facilitate their gas chromatographic analysis. The three phytochemically significant indole acids; 3-indole acetic acid, 3-indole propionic acid, and 3-indole butyric acid along with an analog, D-tryptophan, are four compounds which have been analyzed by this method. A detailed study of the trimethylsilylation reaction of these compounds was the objective of this research.

Optimum conditions for the trimethylsilylation of the four acid indoles, including temperature, time of silylation, humidity and reagent excess were examined. Practical considerations for the gas chromatographic separation of these compounds, including column length, choice of liquid phase, and column temperature were studied and optimized. The effects of derivative storage were examined and optimum storage conditions, along with the stability of the primary reaction product, were determined. The structures of reaction side products and decomposition products were thoroughly investigated. To this end an all glass post column effluent splitter was designed and installed for the trapping of derivatives. Finally, the feasibility of using high speed liquid chromatography for the separation and analysis of these four compounds was also investigated.