QUANTITATIVE DETERMINATION OF CARBAZOLE ALKALOIDS FROM CRUDE PLANT EXTRACT (*Glycosmis pentaphylla* of family *Rutaceae*) USING GAS LIQUID CHROMATOGRAPHY

BY

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A THESIS SUBMITTED TO THE POST-GRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, IN PARTIAL FULFILMENT OF THE REQUIREMENT'S FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN (PHARMACEUTICAL CHEMISTRY

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D E C L A R A T I O N

This is to certify that the work reported in this Thesis was carried out by me under the supervision of Dr. A. MUSTAPHA and Dr. B.K. CHOWDHURY in the Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, A.B.U., Zaria.

The works of other investigators were acknowledged and referred to accordingly.

I solemnly declare that no part of this Thesis has been submitted elsewhere for a degree.

MGarba

(MAGAJI GARBA)

CERTIFICATION

This Thesis entitled "QUANTITATIVE DETERMINATION OF CARBAZOLE ALKALOIDS FROM CRUDE PLANT EXTRACT (*Glycosmis pentaphylla* of family *Rutaceae*) USING GAS LIQUID CHROMATOGRAPHY," meets the regulations governing the award of the degree of Master of Science (Pharmaceutical and Medicinal Chemistry) of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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D E D I C A T I O N

This work is dedicated to the following people;

1. Dr. Mohammed Bello Abdulkadir
2. Abdullahi Dahuwa Bello and
3. Sa'ida Mukhtar (My Wife)

for everything they did for me during the fight to accomplish this work.

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ABSTRACT

Quantitation of carbazole alkaloids (Carbazole, 3-Methyl Carbazole.Glycozoline and Glycozolidine) was carried out using Gas-Liquid Chromatography (GLC). This was -the first time a quantitative work was undertaken on carbazole alkaloids©

A stock solution of 1mg/ml in chloroform was prepared of each of the above mentioned carbazole alkaloids© Appropriate working concentration were prepared by suitable dilutions of various stock solutions .with chloroform. Heptazolidine , (lmg/ml) in chloroform, was used as the internal standard (i.s.) The plant extract (Glycosmis Pentaphylla of family Rutaceas) solution (unknown sample) was prepared by drawing a drop (weighed 23mg) from the light Petroleum extract by using a clean glass rod and diluting it to 5ml with chloroform. The I.S. (1ml) was added to all solutions prior to analysis© All samples (3ul) were chromatographed on a Bye Unicam series 204 chromatograph equipped with FID and Temperature programmer© A single point internal standard method was employed for the analysis of result. TLG was performed of carbazole, 3 - methyl carbazole and the Crude plant extract©

Single point method employed was faster and more convenient that the traditional individual calibration curve method. The concentrations of the alkaloids were found as:-

1. Carbazole - 17.35 ug/ml (0.4% w/w)
2. 3-Methyl Carbazole - 186.67 • (4.06% *)

4. Glycozolidine - 189.70 " (4.1 2% ")

TLC results added supportive evidence that the plant extract contains carbazole and 3 - methyl-carbazole (all identified for the first time from this plant species)$_c$ GLC was found to be a very convenient means of finding the levels of components in a plant extract.

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ABBREVIATIONS AND SYMBOLS (CONTINUED)

et.al = and others

µg. = Micro gram

µl. = Micro litre

pk = Peak

HT = Height

PC = Paper chromatography

ng = nanogram

A.B.U. = Ahmadu Bello University

LTD. = Limited

Fac. = Faculty

Dept. = Department

^{o}C = degrees celsius

MIN. = Minute

HR. = Hour

CONC. = Concentration

FID = Flame ionisation Detector

Psi = pound per square inch

U.V. = Ultra violet

S.D. = Standard deviation

$\bar{d}$ = Mean

Min. = Minute

hr. = hour

MS-GC-SIM = Mass Spectrometry-Gas Chromatography-Selected Ion Monitoring

ABBREVIATIONS AND SYMBOLS USED

GLC	=	Gas Liquid Chromatography
HPLC	=	High Performance Liquid Chromatography
TLC	=	Thin Layer Chromatography
GSC	=	Gas Solid Chromatography
OBS	=	OBCISSA
CAL.	=	CALCULATED
=	=	Equal
FIG	=	Figure
MG.	=	Milligram
CM^2	=	Square Centimeter
CM	=	Centimeter
$\propto$	=	Proportional
E.g.	=	Example
Σ	=	Sum total
Δ	=	Delta
I.S.	=	Internal Standard
$\pm$	=	plus or Minus
$W_{\frac{1}{2}}$	=	half width
h	=	height
%	=	Percentage
m	=	meter
mm	=	millimeter
Ml	=	millilitre
Vol.	=	Volume
$\simeq$	=	approximately
$>$	=	greater
$<$	=	less than
$\sqrt{}$	=	square root

in the carrier gas must be detected by suitable means, now generally by instrumental methods which are adapted to automatic recording devices. (D. AMBROSE, 1971.)

The following diagram is an assembly of GLC apparatus;

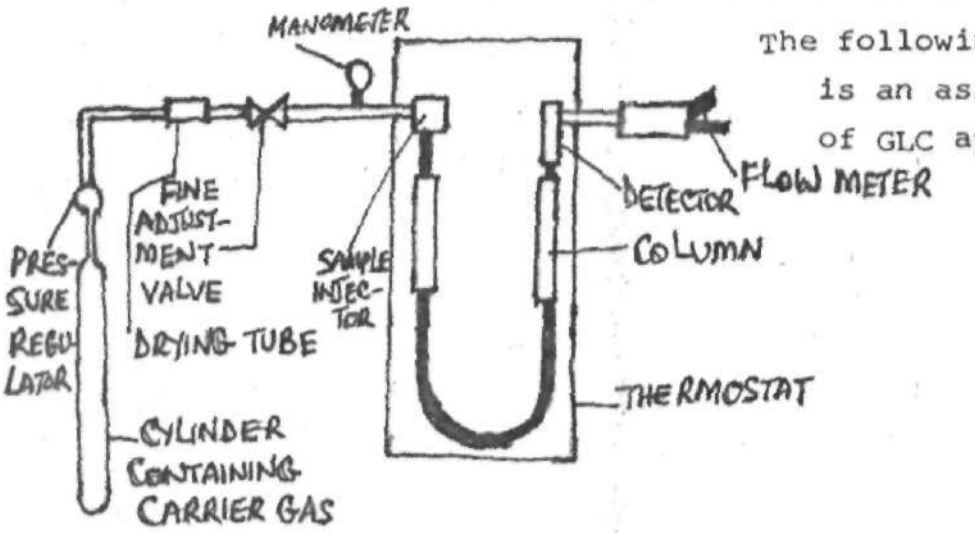


Fig. 1.1: APPARATUS FOR GAS CHROMATOGRAPHY
(AFTER D. AMBROSE, 1971, PAGE 5)

Qualitative and quantitative analyses can be carried out by GLC depending on the relative residence time of a given component in a column system which is an identification characterisitic or the magnitude (area) of the detected response for the compound as it emerges from the column which is proportional to its concentration. (T. KUWANA, 1978.)

1.3 QUANTITATIVE ANALYSIS:

Quantitative works on GLC are more and more increasing in number and are mainly on biological samples and extracts of plants or other samples or extracts. With integral detectors, quantitative analysis is simple; the detector response, is directly proportional to the mass of material, and the step heights permit analysis to be undertaken in accordance with the property being determined. However, only few types of integral detectors are in use and most literature report the less straightfoward quantitiative interpretation of chromatograms obtained by use of differential detectors. (D.AMBROSE,1971)

.../3.

CHAPTER 1: INTRODUCTION

1.1 CHROMATOGRAPHY:

"Chromatography is a process which allows the resolution of mixtures by effecting separation of some or all their components in concentrated zones or in phases different from those in which they are originally present, irrespective of the nature of the force or forces causing the substances to remove from one phase to another". (T. I. WILLIAMS, 1952.)

1.2 GAS CHROMATOGRAPHY:

Gas chromatography is a special form of the general chromatographic techniques, in which the moving phase is a gas and the stationary phase may be either a liquid or a solid. Hence, the system is divided into gas-liquid or gas-solid chromatography respectively. Gas-liquid chromatography is commonly used . The technique is suitable for separation of materials which are volatile without decomposition and it has been used at temperatures ranging from below or around room temperature, for the analyses of gases up to 300^{o}C or above, for example, for long-chain fatty acids. The sample to be analysed is introduced into the moving gas stream and is carried by it down the column. This column contains the active liquid (or solid, in case of gas solid chromatography) of low volatility held upon an inert support; the active solid or the involatile liquid forms the stationary phase. The constituents of the sample distribute themselves between the two phases. In general, their adsorptions or solubilities differ and they are therefore, carried along the column at different rates, emerging at the end in distinct zones (peaks) separated substantially by the pure carrier gas. On emerging, the vapour

.../2.

1.3.1 Principles of Quantitative Analysis:

When a chromatogram is obtained with differential detector which has a linear response, the displacement from the baseline at any instant is a measure of the concentration of a component in the carrier-gas stream at the outlet of the column, and the integral of the curve, that is, the area of each peak is proportional to the amount of that component. A quantitative analysis can then be obtained by measurement of the area of each peak (D. AMBROSE, 1971).

Standard conditions as required for qualitative analysis by retention measurements are also required for quantitative analysis since the peak dimensions are influenced by both the sample size and operating conditions. The accuracy of the analysis is directly dependent on the measurements of the resulting peaks and on the sample injected. The use of an internal standard eliminates the effect of many of minor variations, provided of course that these do not occur during determination. (J.K HAKEN, 1974.)

If the areas of the peaks are Ax A_1, A_2, A_3..., the concentration Cx of any component X is given by

$$Cx = \frac{ax\ Ax}{\Sigma a_1 A_1 + a_2 A_2 + a_3 A_3 + \cdots}$$ (D. AMBROSE, 1971.)

Where ax_1 a_2, a_3... are calibrated constants which relate each peak area to the amount of component. For an ideal detector, these constants would either all be the same or bear a simple relationship to the molecular structure of each component, and Cx would be obtained in terms of molar or weight concentration according to the characteristics of

..../4.

the detector. This method is known as internal normalisation.

Ideal chromatogram provides that all the peaks are symmetrical (Gaussian Curves), then the peak height of any one compound would be proportional to the peak area and the following equation;

$$Cx = \frac{ax\ Ax}{\Sigma a_1 A_1 + a_2 A_2 + a_3 A_3 + \cdots}$$

would equally be applicable to measurements of peak heights. Real chromatograms however, are not ideal and real detectors differ too, from ideal. Detectors are only linear over a limited range - their response is frequently not simply related to the weight or molecular weight of components and peaks are often unsymmetrical. Further complication resutls when peaks are not completely resolved. In such situation therefore, the analyst's task is to relate the behaviour he finds to the ideal. Detectors in use (which have been tried) have an approximately linear response at sufficiently low concentrations. Failure of linearity is not a difficulty in quantitative analysis since care is taken to ensure that the operations are carried out in the linear range (D. AMBROSE, 1971.)

1.3.2: Requirements For Accurate Analysis

Errors of most operation comprising a gas chromatographic analysis - sample injection, recording of the detector signal and measurements of peaks, arise independently of one another and the overall precision of the analysis is dependent upon the precision ofeach operation involved. Accuracy can only be finally checked after adequate precision has been attained, by the analysis of synthetic mixtures and comparison of the results with their known compositions (D. AMBROSE, 1971)

.../5.

represented by a peak is proportional to the peak area integrated against eluent gas volume. A 3% change in temperature of flow controller will lead to a 1% change in peak area sensitivity. Kathorometer response is therefore stronly affected by experimental conditions. With hydrogen and helium, the detector response is much less dependent upon the individual component than it is with nitrogen, and as in general, helium has replaced most other gases as carrier in analysis, the problem of calibration when Katharometers are used has been simplified.

The flame-ionization detector is typical of mass-flow-dependent detectors; it is sensitive to the amount of component passing through the detector and the response is proportional to the rate of flow of the sample. The amount of each component is proportional to the peak area integrated against time, and the peak height is proportional to gas flow-rate divided by the peak volume. Thus the flame-ionization detector also demands those controls of conditions for its best performance even if the demands are not quite so rigorous as they are for the katharometer.

1.3.2.3 Peak Measurements

Measurements of peaks are tedious and of considerable importance. A lot of errors can be made during measuring, plannimetering, weighing and cutting. Electronic integrators are some of the advances made to counteract this problem (D.AMBROSE, 1971.)

1.4 METHOD OF ANALYSIS

The technique of quantitative method of analysis is beset by a number of errors and these must be minimised

..../7.

carrier gas of high enough purity is supposed to be used (and can be obtained commercially.) Impurities are not supposed to be introduced into the gas by volatile materials present in connecting lines and through leaks in the apparatus. Thorough purging of the whole flow system, including the cylinder - reducing head, is necessary when apparatus is first set in operation. There is a desire to put a drying agent in the flowline. Small variations in the flow will have little effect on the separations but may affect detector response.

1.3.2.1: Sampling And Sample Injection:

A problem common to all methods of analysis is sampling, fractionation of volatile components can easily occur during each operation. Care must be taken so that volatile components are not lost in the process of injection into the column. Since most injections are made by means of a syringe, and while care and practice may reduce the variation in total quantity injected in replicate analysis, there is always the chance to get systematic error through loss of the most volatile component. Bad technique may lead to loss of the least volatile components if they donot immediately evaporate into the carrier stream. Leaks in the injector septum must be guarded against and sealed injection systems appear to be advantageous in avoiding flass losses.

1.3.2.2: Recording Of The Detector Signal:

Detectors such as katharometer is typical of concentration - dependent ones; the response to a given concentration is unaffected by slow change and the total amount of component

..../6.

(a_1 = M1/A1 where M is the amount of component and A the peak area; the units may be Mg/Cm^2) once these factors are known, the actual amount of component in an unknown sample may be calculated from the peak size, and its concentration from the sample size (V.G. BEREZKIN and V.S TATARINSKII, 1973.)

Calibration curve for determining tetrafluoro-ethylene is shown below;

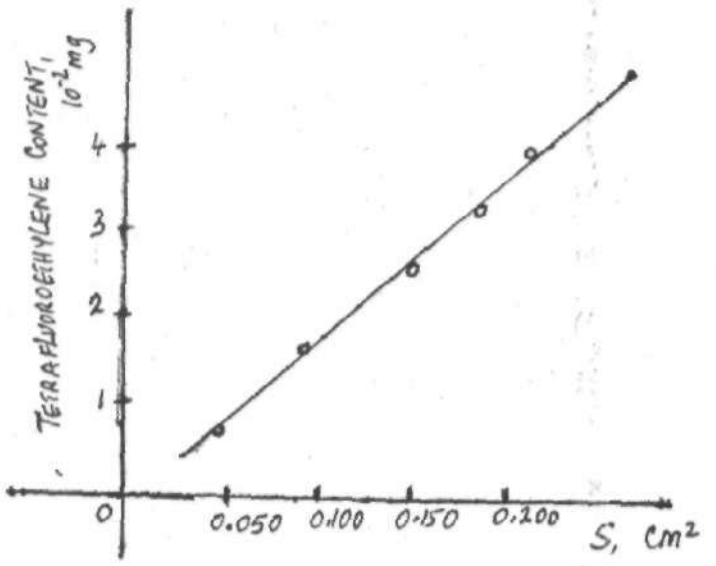


Fig. 1.2. Calibration Curve for determining Tetrafluoroethylene
(AFTER V.G. BEREZKIN and V.S. TATARINSKII, 1973, page 161)

This method is widely used and has the following advantages;

a) it is possible to measure only the relevant components,

b) it is possible to use mixtures containing components that are not recorded,

c) detector non-linearity is balanced out,

d) and the error of measurements is related only to the component being measured.

On the other hand, the method is very time-consuming and it is extremely difficult to know exactly the weight of solute injected.

1.4.2 Internal Standard technique:

This is often most generally used technique in quantitative analysis principally because the exact volume injected is not

...../9.

before a reliable estimate can be given. Factors which affect quantitation in GLC are; sampling and sample storage, adsorption and/or decomposition of the sample components, detector performance, associated performance of the amplifier and recorder, peak area measurements (which can be solved by integration) and calculation of results.

Several methods of measuremtns find application, and for each, good resolution of the peaks and a stable baseline is necessary. Early chromatographs often exhibited considerable drift of the baseline due to instability of the electronics or temperature control. The modern chromatographs show remarkably stable baselines when operated at high sensitivity, and drift is often due to improper conditioning of the column (J.K. HAKEN, 1974.)

Quantitative results can be obtained in several ways. The simplest may appear to be by absolute calibration by injection of the sample and of known amounts of the pure materials with the use of a calibration line or curve. This procedure requires precise sample injection and with a series of compounds, a series of calibrations is necessary. The alternative procedure employs an internal standard where chromatograms with a multiplicity of peaks can be determined without precise measurement of the sample. Derivatization method has more recently been described (J.K. HAKEN, 1974.)

1.4.1:Absolute Calibration Method

This is carried out by measuring the peak area obtained from known amounts of solute. The resulting data are plotted and calibration factor obtained from the slope of the resultant line. The calibration factors a_1, a_2,..., are found by determining peak areas or heights for known amounts of components

...../8.

Similarly for flame thermocouple detector;

a, $\alpha \frac{1}{\Delta HC_i}$ where ΔHC_i is the heat of combustion of component 1,

Then,

$$Cx = \frac{Ax/\Delta HC_x}{A_1/\Delta HC_1 + A_2/\Delta HC_2 + \cdots}$$

These two equations permit internal normalisation to be carried out without calibration (J.K. HAKEN, 1974)

When detectors which require calibration are used with liquid samples for which the making of exactly known blends is relatively easy, calibration is often carried out by obtaining the relative response for the components in synthetic mixture. This allows internal normalisation to be used for calculation of results according to the equation,

$$Cx = \frac{Ax\ ax/ay}{\Sigma A_1\ a_1/ay + A_2\ a_2/y_2 + \cdots + A_y + \cdots}$$

Component y is the one against which the relative response is measured and ax/ay = (mx/my) (Ay/Ax) where Mx/My is the ratio of the amounts of two components in a synthetic mixture. Several calibration runs should be made with mixture of differing composition (if possible through that of the unknown), and the calibration factors should be cross-checked and adjusted (V.G. BEREZKIN and V.S. TATARINSKII, 1973.)

The requirements of a substance as an internal standard are:

a) it must yield a completely resolved peak,

b) it should elute close to the components being measured to minimize any change in detector response due to variations in the operating conditions,

..../11.

required. Similarly, repeatability of injection is not important. Basically, the technique involves comparing the areas of the unknown peaks to the areas of known amount of added standard. A series of standard solutions are prepared containing the relevant components and the added standard, and these are chromatographed. The ratio of peak areas of components to standard are plotted against the known concentrations of the components, for each component of interest. All various calibrations can be carried out using the same solutions. When it is desired to analyse a sample containing these components, all that is necessary is to add a small fixed quantity of the internal standard and reading off the component's concentration from the calibration graphs.

In internal normalisation, for some detectors, the variation in the calibration factor is predictable, for eample, the density meter;

$$a, \propto \frac{1}{(M1 - MC)} \quad \text{(J.K HAKEN, 1974)}$$

M_1 and M_c = Molar weights of Component and carrier gas respectively.

The following equation

$$Cx = \frac{ax\ Ax}{\sum a_1 A_1 + a_2 A_2 + a_3 A_3 + \ldots}$$ can be rewritten thus,

$$Cx = \frac{\frac{Ax}{(M_x - M_c)}}{\sum \frac{A1}{(M_1 - Mc)} + \frac{A2}{(m_2 - m_c)} + \ldots}$$

The above equation also applies for the ionization cross-section detector if for Mc, Mx, M_1 M_2 ... are substituted the ionization cross-sections of the molecules Qc, Qx, Q1, Q_2....

..../10

c) it should not be present in the original sample,

d) it must not react with the sample,

e) the standard (I.S.) should be present in about the same concentration as the components measured to minimize the effect of non-linear response of the detector to varying amounts of material (J.K. HAKEN, 1974.)

1.4.3. Relative Detector Response Method:

Response factors of the unknown solutes are measured with respect to a given standard which may be added to the sample prior to analysis. The area (Ai) of a given peak is proportional to the concentration (Ci) of the respective solute, that is,

$$Ai = Fi\ Ci$$

fi = proportionality constant (depends upon chemical characterisitics of component i.)

Expressions. for areas of 2 peaks, S and X where S is standard and X is to be determined,

$$As = f_s\ c_s$$

$$Ax = fx\ cx$$

or

$$Cs = As/fs$$

$$Cx = Ax/fx$$

If Cs = Cx, then,

$$As/fs = Ax/fx \text{ whence}$$

$$fx = \frac{Ax\ fs}{As}$$

S being standard can be arbitrarily assigned figure say 1, hence,

$$fx = ax/As$$

..../12.

sample size with symmetrical peaks. The method is unsuitable with assymmetrical peaks or very narrow peaks; in the lattercases errors of up to ± 30% may be experienced! This method is based on the relation $A = hw_{\frac{1}{2}}$, where $w_{\frac{1}{2}}$ is the width at half-height of the peak and h is the height of the peak. A diagram of points of measurements in a peak is shown below;

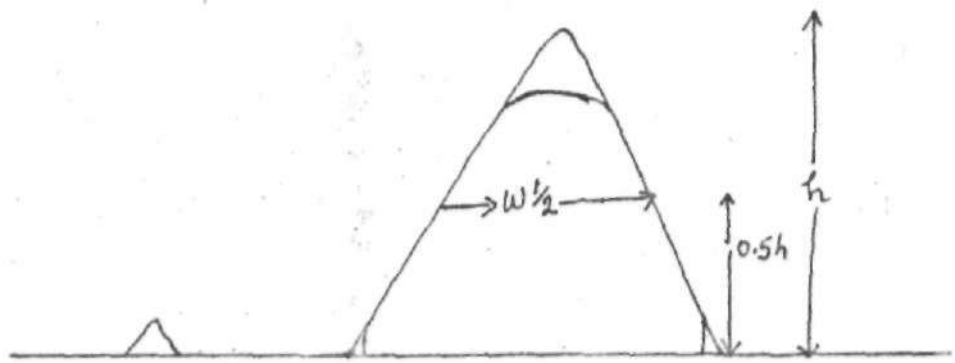


Fig. 1.3: Points of measurements on a chromatogram
(AFTER J.K. HAKEN, 1974. PAGE 74.)

1.4.4.3 Weighing:

The accuracy of peak area measurements determined by weighing the cut out peaks is dependent on the care exercised in cutting and on the constancy of the paper. The in homogenity of 1.25% reported by Janak (J.K. HAKEN, 1974), is considered high by many workers. The difficulty with the procedure is in uniformly cutting out the peaks. It is next most accurate method and may be the one of necessity for an assymmetrical peaks when other methods are not available.

1.4.4.4 Electromechamical or eletronic Integrators:

There are various types of electromechanical and electronic integrators. The ball-and-disc type which is conveniently incorporated in the recorder casing, has found widespread application and has been discussed by Perrine (J.K. HAKEN, 1974.), who indicated that it is capable of

..../14.

1.4.4. Measurements Of Peak Area :

For routine measurements where considerable accuracy is not required, simple measurement of the peak height may be made and where this can be tolerated a marked reduction in the time of analysis is achieved. The measurement of peak is usually conducted by a) the use of plannimeter b) the product of the peak height and the peak width at half height c) Weighing d) electromechanical or eletronic integrators (J.K. HAKEN, 1974.)

1.4.4.1 plannimeter - is a mechanical device that is designed to measure the areas of irregular peaks. It is not accurate for small peaks and is not any more precise than geometrical approximations for symmetrical peaks. It's effective use requires practice and considerable patience (T. KUWANA, 1978.)

Although slow, plannimetering is of considerable accuracy especially with large peaks and the use of fast recorder speeds has been recommended to increase the area of small peaks and decrease probable error. Dal Nogare and Juvet (J.K. HAKEN, 1974.), have reported that peak areas are reproducible to $\pm$ 0.1% cm^2. Therefore, for large, medium and small peaks of area 50, 20 and 2 cm^2 reproducibility of $\pm$ 0.2%, $\pm$ 0.5% and $\pm$ 1.0% are obtained respectively. The results of Janak studies (J.K. HAKEN, 1974.) of peak areas (0.25 to 6 cm^2) were in agreement with these findings.

1.4.4.2 product . of the peak Height and peak width at half - height:

The peak area determined as the product of peak height and half-peak width as described by Creme and Muller (J.K. HAKEN, 1974.), produces an area several percent less than the actual area, but the calculated area is proportional to the

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0.1 - 0.2% accuracy. The accuracy of any integrator is dependent on the accuracy of the recorder used and inaccuracies of up to 4% have been reported on certain parts of some recorder ranges. Others include low inertia dc motor as an integrator, voltage-to-frequency integrators using a digital counter. The use of integrators and computer-coupled gas chromatography has been the subject of several American Chemical Society Symposia during 1967, 1969 and 1971 (J.K. HAKEN, 1974.)

1.4.5 Dérivative Formation In GLC.

This is a recent useful technique to give additional evidence of identity or to improve gas chromatography characteristics. For identification purposes it is the indication that specific functional groups are present and that derivative formation is possible that provides the most useful information. Although retention characteristics are changed by such formation, the order of elution of a series of derivatives will be same as for the parent compounds. Silylation, formation of imines (Schiff base), acylation and methylation are the processes through which derivative reagents are formed (E.G.C. CLARKE, 1981.)

The qualitative and quantitative analysis of certain mixtures can be carried out advantageously by combining gas chromatography with chemical treatment. This is called chromatography or derivative formation in GLC. One of the variants of this mode of pperation is substraction chromatography, in which one or more components are eliminated selectively, resulting in the disappearance of the corresponding peaks. By comparison with conventional chromatogram, identification and quantitative analysis of the reacting compounds can be carried out readily.

The reagents employed act rapidly; they are located on the solid support and filled into a short piece of tubing attached to the main partition column. Examples of suitable reagents, are concentrated sulphuric acid, which is useful for the substraction of olefins in mixtures with other hydrocarbons, or silver nitrate which permit analysis, of secondary and tertiary alkylbromides in the presence of primary isomers.

The following reagents have been used successfully in reactive gas chromatography to quantify the level of impurities in some compounds; 40% Bromine, conc. Sulphuric acid, Toluene, Hydrogen, 20% diglycerol, 10% polyethylene glycol 400, carbon-dioxide, hydrogen sulphide and hydrogen cyanide (V.G. BEREZKIN and V.S. TATARINSKII, 1973.)

1.5 Columns

Column must be in a state and used at a temperature where column bleed causes a minimum of baseline movement. Temperature conditions must be steady because variation will cause change in gas flow and may cause change in the column bleed.

1.5.1 Column Technology:

Once a proper instrumentation has been selected to attempt to solve a problem, the choice of column which will perform the desired separation becomes the important consdieration. Factors that affect the separation (peak maxima) and the resolution (resolution of area of one peak from another) of a column include the liquid phase, the support, the mesh size of the support, the column length and diameter, the carier gas flow rate and the column .

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temperature (T. KUWANA, 1978.)

Any suitable tubing can be used to make the column. The materials most frequently used are glass, copper, stainless steel and (for moderate temperature) nylon. Internal diameters range from 3-6mm and for general analysis, a length of 1-2m is adequate. For convenience, the column is bent, usually into the form of Au, a U or a helix (or in some commercial instruments, a spiral.) In order to overcome difficulty in packing for helical or spiral column, the tube is packed when straight and then bent to suit convenience (copper is probably the easiest material to use.) Helical shape offers the advantage of being relatively compact for a given length of column and is easiest to heat. If copper tubing is difficult to bend without kinking, then it should be annealed by heating to redness in a flame (and may be quenched in water.) Copper tubing may react with the substances being analysed and for this reason a stainless steel tubing may be preferred (most commonly used in commercial instruments.)

Glass as column tubing has the advantages of being chemically inert and allowing inspection of the state of the packing both during the preparation of the column and subsequently after use. It is most used in laboratory-built equipment. The simple configuration is a simple U or in a series of Us connected after packing. Capillary connectors are used so that empty space in the column is kept to a minimum otherwise the efficiency is decreased. Helical columns have the outside path longer than the inside. If this difference were excessive some loss of efficiency might be expected (D. AMBROSE, 1971.)

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The superior performance characteristics of glass as column material have been recognised for a long time; the surface is more inert and defects in the columns are more apparent and can be diagnosed (W. JENNINGS, 1978.)

Much of the previous discussion has been directed to the use of packed columns. However, capillary columns are used for an increasing number of analyses, especially for mixtures with 20 or more components. Such columns are also called open tubular columns. They are prepared by use of 5-50-m length of tubing with an internal diameter of 0.2 - 0.5mm and can be divided into 3 types:

1.5.1.1 Classical Open-tubular Columns

Here the coating of the liquid phase is applied to the internal wall of the capillary tubing (WCOT - Well coated open tubular.)

1.5.1.2 PLOT (Porous-layer open tubular) Columns

Here the internal wall of the tubing is treated to make it more porous, a larger amount of liquid phase can be applied.

1.5.1.3 SCOT (Support-Coated Open Tubular) Columns:

These have support materials coated on the internal wall to increase the capacity for liquid phase.

The great advantage of open-tubular columns is their high resolving power; Separation efficiencies that are 5-30 times better than for packed columns are common. There are two reasons which account for the limited use of capillary columns inspite of their superior ability to separate complex mixtures. One is that, most commercial gas chromotographs must be modified because of the slow gas flow rates needed for capillary columns. Low dead volume injectors and detectors are also required. Another is the low capacity of capillary

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columns, which requires that only a small amount of sample be used (T. KUWANA, 1978.)

1.5.2 Column Support Materials:

Of different supports which have been tried, those prepared from Kieselguhr (diatomaceous earth) have been most widely used. This was first available as a filter-aid (Celite 545), or as thermal insulating bricks (sterchamol, celite 22) in which clay is added as a binding agent. The manufacturers of celite now supply products, chromosorb, specifically intended for chromatography.

The aim of a support in the column is to provide a thin liquid film with as large an interface as possible between the gas and liquid phase so as to facilitate partition between them. The support for the liquid should have a high specific surface if a good performance, that is, sharp separation, is to be obtained from the column but many solids of high specific surface exhibit adsorptive effects which would interfere with the partition and lead to peaks of unsatisfactory shape. The support should therefore, have a low adsorptive activity in addition to a large surface, and it [illegible] also has been found desirable for the individual particles to be as nearly as possible of the same size.

The simplest way of grading is by sedimentation, and this is probably the best first step if support is prepared from the raw filter-aid or crushed bricks; the granules are added to a beaker containing about 20cm depth of water, and that part is rejected which has not settled in a given time, say 2-3 min; the process is then repeated until a clear supernatant layer is obtained. For columns of high efficiency, a closer grading is required. Wet or dry sieving is the

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process generally used. The mildest, although slowest, method of grading is probably wet sieving; the material is placed in the top one of a stock of successively fined sieves which is gently rocked from side to side as a stream of water is allowed to percolate down wards. Suitable fractions are 40/60, 60/80, 80/100, 100/120 and 120/150 BS mesh. The two types of support, from the filter-aid and from brick, are respectively white and pink. The bulk density of the white is 0.15-0.18g/ml, and of the pink about 0.38g/ml, so that columns prepared from the two different supports with the same proportions by weight of stationary phase will contain widely differing amounts of liquid. Adsorption poses great problem and manifested itself in failing of the peaks. Reduction of adsorption have been attempted in the following ways;

1.5.2.1 By Choice of a stationary phase which itself contains suitable functional groups for the formation of hydrogen-bonds with the support. This is achieved by the incorporation in the stationary phase of a tailing reducer, e.g., presence of 0.1% of a polar material such as Alka Terge T in squalane eliminated tailing of alcohols and ketones.

1.5.2.2 By removal of the adsorption sites by chemical reaction with the silanol group. Supports have been treated with trimethylchlorsilane and with dimethyl dichlor silane, but an easier and equally effective reagent to use is hexamethyldisilazane. The treatment does bot leave the surface completely inactive and the performacne of column treated in this way may be improved further by the addition of 0.1% polyethyleneglycol (PEG 400) to the

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stationary phase.

1.5.2.3 By removal of the adsorptive sites with acids or alkali (KOH 2%.)

1.5.2.4 By Coating the support with a solid which will cover the adsorptive sites.

1.5.2.5 Other Supports:

Of other supports materials, perhaps polytetrafluoroethylene (PTFE) is the most important becuase although so far the columns packings made from it are not as efficient as those made from Kieselguhr, its adsorptive capacity is lower than that of any other suitable material. Glass beads, common salt, sand and unglazed tile have all been used as support materials, but in general they give columns of low efficiency. Any way of improving the efficiency of glass beads columns will offer obvious advantages over Kieselguhr because of their mechanical strength (D.AMBROSE, 1971.) Graphatized thermal carbon black is an excellent non-specific adsorbent which retains compounds in relation to their polarizability and geometric configuration. They are effective for separation of isomers and stereoisomers. Alumina and silica gel, unlike above ones, are polar adsorbents. The general order of retention is similar for both adsorbents. Porous polymer beads are made from polymeric materials. First used for gas chromatography by Hollis (1966) (D.AMBROSE, 1971), and are commercially available as porapaks and chromosorbs. They have good mechanical strength and are resistant to breakage, give highly efficient separations and do not bleed

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or change retention characteristics with time when used below their decomposition temperature (T.KUWANA, 1978.)

1.5.3 Stationary Phases:

Over the years a large number of substances have been used as stationary phases and now on a single substance, the number of stationary phases is now several hundred. Often choiceof stationary phase is arbitrary and has been arrived at by a process of trial and error. Many workers have tried mixed phases to obtain variation in the polarity of columns. In 1966, Rohrrchneider described a method for classifying liquid phase by the determination of the retention indices of a number of organic solvents. Substances used were benzene, ethanol, methyl ethyl Ketone, pyridine and nitromethane. The sum of the retention indices for these compounds was used as a constant to compare the ability of stationary phases to separates different classes of compounds and to show which phases were similar in behaviour.

In 1970, (E.C.G. CLARKE, 1981), this method was refined and practical problem resolved by Mc Reynolds, who studied the retention indices of 68 compounds and by statistical analysis showed that the higher homologous, butanol, methylethyl-ketone and nitropropane could be used to replace ethanol, methyl, methylethyl ketone and nitromethane with no loss in accuracy.

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TABLE 1.1: Common Liquid Phases Used As Stationary Phases:

Name	MC Reynold's Constant
OV-1	222
OV-101	229
OV-17	886
OF-1	1497
OV-225	1813
SE-30	217

Sequalane = reference phase

Absolute = 3221

(AFTER E.C.G. CLARKE, 1981.)

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There are now new liquids phases in use:

These are as follows;

TABLE 1.2:

Phase	Temperature limit (°C)
SF 96	350
Apiezon L and M	250
Dexsil 300	350
20% phenylsiloxane	350
20% Phyenylsiloxane Dinonylphthalate	150
SP 2250	300
SP 2401	250
Carbo Wax 20m	250
DEGS	225
SP 2340	250
Silar 10 CP,	250
TCEP	175
FFAP	-•
EGSS-X	-
Porapak Q	-
FS 1265	-
UCON Polar 2000	-
Carbowax 400	-
Dowfax 9N9	-
Versamid 900	-
Ethofat	-
XF 1150	-
DC 11	-
STAP	-
XE 60	-

(AFTER T. KUMANA, 1978, pages 23 - 28)

Also from supelco (1977) special stationary phase series was used. These were the SP series.

These are:

SP-2510, SP-2510 DA, SP-2401 DB, SP-2100 DB.

•Temperature limit not given

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SP - 2250 DB, SP-2401 were all used to achieve separation of many classes of compounds. Very recently (the Supelco Reporter, International, Vol. V, No.2), reported a new single 0.53mm I.D. capillary column, SPB-5 was introduced which can replace all of the packed columns used in methods 604, 607, 609, 611 and 612.

1.5.4 Column packing:

As the final step before the stationary phase is added the support should be dried at 100-150°C. If stationary phase is a liquid at room temperature, the required proportions of the liquid and support may be stirred together; the liquid is adsorbed by the Kieselguhr to give a fairly free-flowing powder. This method may not give the most even distribution and a higher efficiency will be obtained if the liquid is dissolved in sufficient volatile solvent to form a slurry with the support; the slurry is then poured into a flat dish as a thin layer and the solvent is gently evaporated. Alternatively, the evaporation may be carried out in a larger filter flask which is warmed while a current of dry air is passed through it. A specialised apparatus is used. (D. AMBROSE, 1971.)

The stationary phase must be completely dissolved before it is blended with the support. After the solvent has been evaporated, the packing may, with advantage, be heated under vacuum for some hours at a temperature slightly higher than the intended maximum operating temperature as this assists the conditioning process

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described below. Before packing, the empty tubing must be cleaned to remove any residual contaminants from the manufacturing process or the previous packing. Even packing is desirable so that the gas flow does not vary either across the column or irregularly along its length. A funnel is attached to one end of the column by rubber tubing, the other end is plugged with a load of glass or silica wool and sufficient packing to fill 5-10cm of the column is placed in the funnel after sieving (final) and is agitated so that it moves down into the column. Impregnation with the stationary phase may be deferred until after the column has been packed with dry support. Description of column packing is no substitute for experience. The aim is a high efficiency accompanied by a low pressure drop and the highest eifiiencies are to be obtained with the finest particles. The necessary skill in packing columns may be acquired by practice. Various methods should be used and the efficiencies of the columns obtained compared in order to determine the best method. A newly packed column should be conditioned before being put into use by passing carrier gas through it at the highest intended operating temperature; this will remove volatile contaminants which otherwise would affect the detector and cause movement of the baseline. Conditioning may also lead to chemical reaction between components of the stationary phase which will help to stabilize its performacne. If this operation is done in the chromatography, a steady baseline will show when conditioning is complete. The duration of column conditioning ranges from 12-24 hours (D. EMBROSE, 1971.)

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1.5.5 Column Performance and Efficiency:

Column performance is measured by the number of theoretical plates, which may be determined from the dimensions of peaks. Although most workers recognise the imperfection of the theoretical plate concept, it is still widely used in reporting column effectiveness and measuring column efficiencies.

The fundamental assumptions of this theory are that the partition coefficient is independent of concentration and that the rate of diffusion within a single phase along the length of the column is negligible compared with the speed of equilibrium between phases. A theoretical plate may be defined as a layer, at right angles to the column, of such a thickness that the solute at its mean concentration in the stationary phase in this layer is in equilibrium with its vapour in the mobile phase leaving the layer. Column is imagined as divided into n of these elementary plates, each of height h. If unit weight of a volatile solute is placed on the first plate and distributed between the phase, the concentrations XL_1, XL_G are related by the partition coefficient K as follows;

$$XL_1 = KX_{G1}$$

If a and b are cross-sectional areas of the mobile phase and stationary phase respectively,

then;

$$h\,(a\,X_{G1} + bXL_1) = 1 \qquad V_h = \text{effective volume of the plate.}$$

$$\text{or } XG_1\,(a + Kb) = 1$$

$$\text{or } XG_1\,Vh = 1 \qquad Vh = h(a + Kb)$$

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Consider dv of the mobile phase passing along the column to plate 2. If takes with it

$dv \times G_1 = dv/h$ and leave

$1 - dv/h$.

Expressions in the subsequent development are simplified if we put $dv/vh = Z$ and $1 - dv/vh = y$. Quantity y redistributes between the phases, so that the concentration in the mobile phase XG_1 is given by

$X'G_1\ Vh = y$ and when the next increment dv passes to plate 2 it carries $dvx'G_1 = y^{dv/vh} = yz$ and leaves $y - yz = y^2$ (since $Z = 1-y$.) Simultaneously the first infinitesimal amount passes from plate 2 to plate 3. Distribution on plate 2 gave $XG_2\ V_h = Z$. The amount removed tc plate 3 is therefore $dv \times G_2 = {}^{dv}/_{vh} = Z^2$ and the amount left on plate 2 is there-fore $Z-Z^2 = yz$. The total amount on plate 2 is therefore 2yz. Continuation of this argument leads to the following table (which shows the amount of solute on each plate after successive volumes dv of mobile phase have passed.);

TABLE 1.3:

	Number of Plate				
dv	1	2	3	4	5
0	1	0	0	0	0
1	y	z	0	0	0
2	y^2	2yz	z^2	0	0
3	y^3	$3y^2z$	$3yz^2$	z^3	0
4	y^4	$4y^3z$	$6y^2z^2$	4y z3	z^4 and so on.

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(AFTER D. AMBROSE, 1971, Pages 140 - 163)

Thus it is seen that the contents of each plate may be expressed as a term in the expression $(y + z)^m$ so that, when a volume $V = mdv$ has passed, the amount on each plate $Qn+1$ is given by

$$Qn+1 = \frac{m!\ y^{m-n} z^n}{n!\ (m-n)!}$$

When m is large $\frac{m!}{(m-n)!} = m^n$

and $y^{m-n} = (1 - dv/vh)^{m-n} \approx 1 - \frac{mdv}{Vh}$

$$= e^{-mdv/vh}$$

$$= e^{-mx}$$

$$\therefore\ Qn+1 = \frac{(mz)^n e^{-mz}}{n!}$$

In GLC, we are interested in only volumes of $n > 100$ (D. AMBROSE, 1971.)

1.5.5.1 Effect of Changes In Column perfromance:

1. Increase in column length, that is, an increase in the number of plates of constant height, leads to a proportional increase in retention volume, whereas the peak width increases and the peak maximum is reduced as the square root of the number of plates. It is therefore advantageous to increase the length of the column for a difficult separation.

2. For a given number of plates, the rentention volume and the peak widths are proportional to the effective volume of each plate; that is for 2 columns of identical plate number, the column with smaller HETP (Height Equivalent to a Theoretical Plate), that is, the shorter column, will have the smaller peak width. In assessing the

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efficiency of a column, the HETP is important in addition to the plate number.

3. On a given column, the peak width is proportional to the retention volume, that is, peaks gradually broaden from beginning to end of the chromatogram.

4. For a given retention volume, the peak width is proportional to $1/\sqrt{n}$, that is, of 2 columns, the one with the higher plate number gives the sharper peak and a better separation between substances with retention volumes close together.

5. The peak width is independent of the size of the sample provided the column is not over loaded.

The continued use of the plate model for the development of chromatographic theory has been adversely criticized on the grounds that deductions of equations is no more valid, on account of the simplifying assumptions made, than an intuitive assumption of a Gaussian form for the peak. The application of the random theory was put forward (D. AMBROSE, 1971.)

1.5.5.2. The Rate Theory

Plate theory ignored the mechanism of the column process and therefore provides no information about the value of the HETP, in terms of the column variables. The rate theory of which the random walk model is a simplified representation, is based on the continuous nature of the chromatographic process which essentially is one of diffusion. Account is taken of diffusion phenomena in the following ways;

1. The column packing, if viewed on a sufficiently small scale, is irregular and the paths available to the gas are not therefore uniform.

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2. The solute diffuses longitudinally in the gas phase.
3. There is resistance to mass transfer between the phases.

Development of these ideas allows a quantitative expression to be given to the assumption that they are the causes of band spreading in the following equation for HETP (D. AMBROSE, 1971.)

$h = A + B/u + C_L U + C_G U$, where A, B and C are constants (C_L relating to the liquid and C_G to the gas phases.), and u is the linear gas velocity in the column.

Practical investigation on factors affecting column performance;

a) Particle size - reduction in size of the particles led to a reduction in HETP, that is, reduction in the peak width. The performance of the finest particle was less affected by increase of the velocity above the optimum than was that of the coaser particles. It was necessary however, to keep the particle size as uniform as possible, since performance fell off when a wide range was used.

b) Carrier gas - the effect of changes in the carrier gas is shown as the value of the HETP was greater for hydrogen than for nitrogen or argon.

c) Sample size - reduction in the size of the sample led to reduction in the HETP. This finding leads to the conclusion that the smallest possible sample should be used if the highest efficiency is required.

Today packed columns of high performance can be used to separate a mixture of various components.

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1.5.5.3 Optimum conditions:

To summarize, the following factors contribute to the high efficiency of columns;

1. Low ratio of in let pressure to out pressure,
2. Operation at the optimum practical gas velocity - found by determination of column performance as a function of gas flow-rate, either in terms of HETP or in terms of the resolution of a pair of components for which α is small,
3. the use of dense carrier gas,
4. small column diameters,
5. A small proportion of stationary phase.
6. a small sample size. It is stated that if 5% of stationary phase is used, the sample should not contain more than 5ug of any one component,
7. a small range of particle size of the support,
8. study of the appratus and technique to ensure that their contributions to zone spreading are minimal - correct injection technique is most important (D. AMBROSE, 1971.)

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CHAPTER 2: LITERATURE REVIEW

A lot of work has been done in the area of quantitative Gas liquid chromatography over the years. However, the area which concerns the monitoring of drug metabolites in biological fluids has received considerable attention. Before the advent of GLC it was extremely difficult to obtain data on metabolic products, of considerable importance, in a quantitative way as was the case of an anti-tumor agent, 5-fluorouracil. With GLC, its most important metabolite, 5,6-dihydrofluorouracil , was identified and quantified (BRUIJN et al., 1983.)

KRYLOV and KHLEBINKOVA (1982) reported a quantitative gas liquid chromatographic determination of pemoline using trimethylanilinium hydroxide as internal standard. The study was carried out because pemoline is a major constituent of many drugs used as anti-depressants and also to ascertain the reason for acute toxicity often encountered in the use of such drugs. Quantitation of pemoline was done by the individual calibration curve method.

An estimation of methone, menthofuran, menthyl acetate and menthol in peppermint oil by capillary gas chromatography was reported by Sang (1982.) Commercial quantities of peppermint oil are produced in Tasmania and feasibility studies for peppermint and spearmint

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oil production are currently being made in Victoria,(Sang 1982.) Quantitative data are therefore required to evaluate the effect of factors such as varying photoperiod, temperature, plant density and time of harvest on the levels of menthone, menthofuran,methylacetate, and menthol in the growing plant. A spectro-physics (system 1) computing integrator with post-run calculation accessory was used to measure peak areas and compute the concentrations of the relevant compounds.

Bruijn et al.(1983) described a gas-chromatographic assay for the determination of 5-fluoro-uracil (5-FU) and 5,6- dihydrofluorouracil (FDHU), its major metabolic analog. The selectivity and sensitivity of the method allows the determination of both 5-FU and FDHU in 200ul of plasma. Temperature programmer was used to identify the metabolites of 5-FU and quantitation was carried out using calibration curve for each metabolite. The coefficient of variation (C.V.) for the assay ranges from 6.6% (n=3) at a level of 100 ng/ml to 1.6% at 5 ng/ml (n=3). The method was compared with a method described in the literature which used a packed column, and there appeared to be a good correlation coefficient.

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at levels higher than 2 ug/ml (correlation coefficient = 0.98, slope 0.927 and intecept - 0.550, n = 46.)

Improved gas-liquid chromatographic method for measuring fenfluramine and norfenflurmine in heparinised plasma was described in which the pharmacokinetic profiles of the two were produced (Morris et al.1983.) They also discovered that therapeutic monitoring of plasma levels is not only a check of patient compliance but may also avert reported dose-related side effects. This method is currently used in a long-term study involving measurements of steady-state plasma levels of the two drugs. Quantitation was carried out after obtaining the calibration curve for the standard. Ratios of peak heights of fenfluramine and its analog to the internal standard were linear over concentration range of 0.005 umol/l. Recovery of up to 82% was achieved.

A method for isolating and quantitating anti-convulsant drugs and certain metabolites of these drugs from biological fluids was used in South Bend Medical Foundation for the analysis of samples (Supelco, Inc., 1977.) Quantitation of this group of drugs was important because of their habit-forming tendencies. Calibration using a single point standard was used and was found to yield satisfactory results and eliminate the tedious preparation of a standard curve. Quantitative analysis was carried out using the following formula:

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$$Cu = \frac{Ru \times Cs}{Rs}$$

Where;

Cu = Concentration of unknown in ug/ml

Cs = Concentration of standard in ug/ml

Ru = Peak height ratio of drug in unknown

$$= \frac{\text{PK. HT. of unknown component}}{\text{PK. HT. of Internal Standard}}$$

Rs = Peak height ratio of drug in standard

$$= \frac{\text{PK.HT. of standard}}{\text{PK. Ht. of I.S.}}$$

This method is very good for drugs and plant extracts where there are many components and, most of the time, these components had to be analysed and quantitated. It would certainly be a very tedious, if not altogether too difficult exercise if an individual calibration curve method were to be followed. For example, this method was adapted in the present work and it was found to be highly convenient. More reasons will be given later why the choice of this method is justified.

A quantitative analysis of chloroquine and its major metabolites, desethylchloroquine was carried out using capillary gas-chromatographic method. Due to the present report which indicated that there are chloroquine-resistant strains of plasmodium, it is of immense importance to carry out a quantitative determination of chloroquine and its metabolites in plasma so as to determine correctly whether the resistance was due to

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underdosage or poor patient compliance (plasma steady state levels). quantitation was carried out by using calibration curve method (Churchill, 1983).

A quantitative analysis of melperone in human plasma using gas liquid chromatography was reported by Chen et al.(1983). Four healthy male volunteers were involved in the study. GC analysis was performed. Calibration and calculations were done on the basis of peak height ratios of melperone divided by the internal standard which were plotted against melperone concentrations used. The peak height ratio of each unknown was applied to this curve to determine the concentration. Alternatively, the equation of the line was found by linear regression and the concentration of the unknown calculated. Recovery % of malperone from plasma at 100 ng/ml was 78.5%

Erlane et al. (1983), carried out a GC assay of tocainide enantiomers using a chiral capillary column. Two healthy male volunteers were involved in the investigation. Blood samples were collected for analysis after 3 hr. Calibration curves were prepared for the various enantiomers and quantitation of enantiomers (synthesized) and tocainide was carried out with the help of those curves.

A gas-liquid chromatographic determination oftoloxatone in human plasma after oral administration was described.

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(Vajta et al.1983.) calibration curves were tested using a programme for regression analysis with atekronix 4052 desk computer. quantitative analysis was carried out using the area ratio method. Area ratios (y = Tx-TMS/I.S. - TMS) (Tx = toloxatone, I.S. = Internal standard, TMS = Trimethyl derivative) were plotted against concentrations of toloxatone.

Maksoud et al.(1983), reported an analysis of N-n- propylnorapomorphine in plasma and tissue by capillary gas chromatography - electron capture detection. Both apomorphine and its analog were synthesized with latter being used as internal standard. Morphine and analogs have a high incidence of drug dependence (addiction) and are of immense value as narcotic analgesics. Therefore, it is of interest to carry out a study which will make it possible to quantitatively determine the metabolites in plasma. quantitative analysis was carried out by comparing the retention time of the apomorphine in the plasma extract to that of the synthesized analog.

A simultaneous assay of triazolam and its main hydroxy metabolite in plasma and urine by capillary gas chromatography was described (Coassolo et al. 1983.) Triazolam and I-hydroxytriazolam were used as standards while estazolam was used as internal standard. Plasma was extracted with 50 ml of methanol containing the I.S. and quantitation of unknown samples was carried out by

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a least-squares linear regression curve from the peak area ratios versus the respective plasma concentrations. This standard curve was then used to interpolate biological samples. Same calculations could be performed using peak height ratios instead of peak area ratios.

A work on gas chromatographic determination of butafilolol in biological fluid has been reported (Jeanniot et at. 1983.) quantitation of butafilolol from plasma was achieved on the basis of a calibration. Standard curves were then run daily by spiking 1.0 ml of blank plasma with a known amount of the internal standard and increasing amounts of butafilolol. A least-squares regression between concentration and peak height ratio's of the drug to the 1.S. was calculated.

Measurement of a new monoamine oxidase inhibitor, meclobamide, by gas chromatography with nitrogen - selective detector was reported (Marguire et al 1983.) Quantitation was done by means of integration of the peak areas using a Hewlett-packard 3380 A integrator. The peak area ratio of meclobamide over the internal standard was calculated for each sample and standard curves were constructed using linear regression analysis.

Rosen et al. (1984), reported gas-chromatographic quantification and confirmation of four Fursarium mycotoxins in corn. Field corn was grounded with methanol and extracted with hexane. The final eluate was derivatized with ethyl-acetate. Quantitation of derivatized mycotoxins was

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carried out by comparing their computer-integrated peak areas to those of their correrponding deutro-derivatized internal standards. Excellent recoveries (91-103%) and precision (C.V., 1.6-8.7%) were obtained for all the mycotoxins except one (T-2 toxin), which gave an 80% recovery with 14.2% coefficient of variation.

Matisova et al.(1984), reported a quantitative analysis of hydrocarbons in gasoline by capillary gas-liquid chromatography. Quantitative analysis of 238 constituents of the gasoline fraction of crude oil has been performed. Quantitative analysis of hydrocarbons on squalane was done by choosing isothermal conditions in order to utilize published data on the characterization of the individual mixture constituents. The temperature of 58°C was selected by optimization to give the best compromise between analysis time and number of peaks resolved.

Quantitative analysis of aromatic amines in human urine by gas chromatography - Mass spectrometry-selected-ion monitoring (SIM) was reported (Carlucci et al. 1984.) N-phenyl-2- naphthylamine (PBNA), N-isopropyl-N-phenyl p-phenylenediamine (IPPD) and N- (1,3-dimethyl butyl) - N - phenyl-p-phenylenediamine (6PPD) are widely used anti-oxidants and antiozonants (generally at levels of 1-2%) in rubber industry, and workers may be exposed to them and as such develop various forms of cancer like bladder, lung, stomach etc. Hence, it is important to quantify the possible exposure to these chemicals in order to avoid or reduce

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contact with those chemicals for which the toxicological hazards are not yet clear. One of the simplest and most easily applicable systems is to measure urinary excretion of aromatic amines in exposed individuals by gas chromatography and HPLC. Calibration curves were constructed by derivatizing knonwn amounts (0,1, 0.2, 0.4, 1.0, 2.0 ng/ml) of aromatic amines. High sensitivity was achieved (MDC = 0.1 ng/ml) and there was no interferance.

Tong et al.(1984), reported a quantitative analysis of polycyclic aromatic compounds in diesel exhaust particulate extracts by gas-chromatographic analysis. Diesel cars emit more particulate matter than conventional automobiles and some compounds indentified in diesel exhaust particulate are mutogenic. Consquently there have been increasing investigations on chemical characterization and mutagenicity associated with diesel exhaust particulate (more so when a number of diesel engines are expected to be used up to the next 25 years.) A number of oxygenated polycyclic aromatic hydrocarbons (PAH), Oxy-PAH and some phthalate esters have been quantified in the extracts of three different diesel exhaust particulate samples. Quantitative analysis was carried out by the help of integrator. Integrated peak areas from gas chromatograms were used for component quantification.

Gamerith et al.(1985), reported a gas-chromatographic determination of free amino acids in fermentation .

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broth during growth of clostriduim oncolyticum M55. GLC is a conventional method for the analysis of amino acids especially when large number of samples are involved but derivatives have to be formed. Determination was carried out in the normal way using calibration curve method for individual sample.

The use of a new column in the quantitation and ana lyzing extractable priority pollutants by U.S. Environmental protection Agency(EPA) was reported recently (Supelco reporter, 1986) The method is capable of characterizing proteins without denaturation.. The column, 15m x 0.53mm I.D SPB-5 fused silica capillary column, provides a good resolution and shorter duration of analysis.

The present work, "quantitative determination of carbazole alkaloids from crude plant extract (Glycosmis pentaphylla) using gas-liquid chromatography," was undertaken in sequel to other works done with GLC in recent years (Chowdhury et al.1985, Roy et al. 1974, Dutta et al. 1969 and Chowdhury et al. 1985.) All the researchers have carried out qualitative works ranging from PC, TLC to GLC on carbazole alkaloids. However, none among them has undertaken any work on quantitative aspects, with GLC, on those alkaloids. It is of interest therefore, to develop a method for quantitative analysis for those alkaloids since they have been reported to be used for medicinal purposes which were said to be of repute (Chowdhury et al.(1986.)

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Throughout the literature, an individual calibration curve method was used in quantitating the various drugs' metabolites in plasma or urine. This is a very tedious exercise and would continue to take longer time of analysis if pursued, especially if an extract contains a lot of components of interest (which have to be determined simultaneously.) Further difficulties would arise if the components are present in very minute quantities (e.g., ng/ml as in all the cases) since it would be very difficult to isolate the individual components and then produce a calibration curve for each. This is beliguered by the fact that if concentrating a component is difficult to achieve. The lower the concentration of a component, the more effort has to be spent on work-up procedures prior to gas chromatography. Interference by compounds from the matrix can be considerable even if selective detectors are used. purification of the first extract is therefore essential. Furthermore, the volume of the extract is often rather large (2-10ml) and an increase in the concentration must be achieved before the measurement is made. Owing to the complexity of biological samples, the limited amount of sample available (especially from small animals) and the low concentration of the components, the gas-chromatographic procedure is usually preceded by extractive purification and derivatization (Schill et al . 1978.)

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Particular exampleswhere the adaption of individual calibration curve method would be highly inconvenient are the analysis of hydrocarbons (Tong et al. 1984). In gasoline, as much as 238 or more constituents were analysed quantitatively (Matisova et al. 1984)!! In such a situation a more convenient method which would provide faster means of analysis should have to be looked for, since very few, if any, will withstand the time and inconvenience it will take to analyse such a large number of constituents individually. Others include the quantification of amino acids in fermentation broth during growth of bacteria (Gamerith et al. 1985) and quantification of mycotoxins in corn (Rosen et al. 1984) where many components were determined individually. Furthermore, the choice of many internal standards for various components will affect the detector response since not all the internal standards have the same concentrations with the components being analysed. A single internal standard (provided it is chemically similar and has its concentration same as the components being analysed) will solve this problem. This will take care of a situation where two or more internal standards have to be used if the components being measured elute at widely different retention times and/or are present at greatly different concentrations (Haken, 1974.)

In all cases, the extract - be it of plant or

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animal origin, may contain components of interest to be determined simultaneously. In polypharmacy for instance, drug,-drug interactions would continue to produce more complex metabolites which would add complexity to the isolation of individual component in appreciable amounts to be quantified using individual calibration curve method as would be the case by using antiepileptic and antitubaculosis drugs (which are used in combined dosages.) However, it would be very much easier to quantify the components simultaneously using a single point internal standard method.

2.1 Aims And objectives Of the Present Work:

As discussed in the literature, of all the workers who investigated carbazole alkaloids, none had undertaken a quantitative analysis of the alkaloids. This work would prove to be of importance since carbazole alkaloids are used as medicinal agents and as such their levels in plasma would help to design a suitable dosage range and also to study their toxicological properties.

Recent cases of quantitative analysis have tendenciously involved a mixture of many components to be analysed simultaneously. Due to the complexity of the components (isolation and concentration), individual calibration curve method would pose difficult processes and so the development of a method, which will make it possible to use a single

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point internal standard, would be of immense relief (taking into account the tedious nature and longer time of analysis posed by the individual calibration curve method).

In bioanalytical analysis, a sample from plasma or urine would be needed to be analysed very quicly and so the method to be used must be fast and convenient. Single point internal standard method would suit this type of situation or was the case in the Bend Medical Foundation in Indiana, U.S.A., where the method was used on many clinical samples for a long time (Supelco, Inc., 1977).

In the present work, a plant extract from Glycosmis Pentaphylla was encountered. The extract contained at least five components and all these were to be analysed simultaneously. Due to their concentrations (which were all in ug range), it would be difficult to analyse them individually by individual calibration curve method. It is therefore decided to use a single point internal standard method for quantitating the individual alkaloids present in the plant extract of Glycosmis Pentaphylla.

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CHAPTER 3: EXPERIMENTAL

3.1 Materials

The following materials were used in all the experiments of this work;

1.	Carbazole (a cream white amorphous substance.)	Supplied by Dr. B.K Chowdhury of the Dept. of Pharmaceutical and Medicinal Chemistry, Fac. of pharm. Sciences, A.B.U. Zaria.
2.	3-Methylcarbazole (white light substance in form of flakes.)	"
3.	Glycozoline (a white crystalline substance.)	"
4.	Glycozolidine (creamy crystalline substance.)	"
5.	Heptazolidine (cream larger crystalline substance which sticks together.)	"
6.	Light petroleum extract of Glycosmis Pentaphylla (dark oily green substance.)	"
7.	Chloroform (Analar, BDH chemicals LTd., Poole, England.)	
8.	Beakers, 250ml, 50ml capacity (Pyrex, England.)	

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9. Pipettes, 5ml, 2ml, 1ml capacity. All were calibrated. The 1ml pipette was graduated from 0.1ml-1ml. (Technico, B S700 B^{EX} 20^{O}C, Type 1, England.)

10. Silica gel plate (10 x 20cm, prepared in the lab.)

11. Solvent system: Petroleum ether (40-60^{O})-Acetic acid (10:1 ratio)

Petroleum ether (40-60^{O}c, Analar, BDH chemicals Ltd., Poole England.)

Acetic acid (Analar, BDH chemicals Ltd;, Poole, England.)

Carbazole, 3-methyl carbazole, Glycozoline, glycozolidine and Heptazolidine were all pure samples and were used as standards.

12. Screw-capped sample bottles.

They were washed (3 times) with soap solution and then rinsed with distilled water and finally dried in an oven at 150^{O}C.

13. Template and capillary tubes.

3.2. General Methods:

3.2.1 Solutions.

Preparation of solutions used in the experiments was carried out under stirctly clean conditons and with utmost care in measuring.

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3.2.1.1 Standard Solution

Standard solution was prepared of carbazole alkaloids investigated (carbazole, 3-methylcarbazole, glycozoline, glycozolidine), by dissolving 1mg of each in 1ml chloroform. From these solutions, working concentration standards were prepared by dilution with chloroform. In table 3.1 is shown the working concentration standards of the solutions prepared.

Table 3.1: Working Concentration Standards.

	Sample	Stock Solution	Amount taken from stock	Final Volume (chloroform)	Conc. of Working Std. µg/ml
1.	Carbazole	1mg/ml	0.3ml	1ml	400
2.	3-Methylcarbazole	"	0.3ml	"	400
3.	Glycozoline	"	0.5ml	"	500
4.	Glycozolidine	"	0.5ml	"	500

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3.2.1.2 Unknown Sample

The extract is from a plant genus Glycosmis Pentaphylla from Rutaceae family. It is also known as Glycosmis arboreae. From the light petroleum extract, one drop was withdrawn using a glass rod (weighed 23mg.) It was placedin a screw-capped sample bottle and 5ml of chloroform were added and then mixed thoroughly by shaking vigorously. This formed the unknown sample to be determined.

3.2.1.3 Internal Standard

Heptazolidine was used as an internal standard. 1mg of heptazolidine was dissolved in 1ml of chloroform. From this stock, 0.5ml were withdrawn by the help of a graduated pipette (0.1ml-1ml) and placed in a screw-capped sample bottle. This was made to 1ml volume by dilution with chloroform producing a working concentration of 500 µg/ml. 1ml of this solution was added to both the standard solution and unknown sample (prepared before) prior to analysis.

3.2.2. Analysis

The method of analysis adapted was similar to the one used by Supelco, Inc;, U.S.A., 1977. A microsyringe (10 µl) was used to inject 3 µl of each solution onto a gas chromatograph. Sample size in all cases were 3 µl. A temperature programming method was used and quantitation carried out by the following formula:

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$$Cu = \frac{RU}{RS} \times Cs$$

3.2.3 Gas Liquid Chromatography

Experiments on gas liquid chromatography were carried out with the following apparatus and conditions were as stated therein:

3.2.3.1 Apparatus and Conditions

A Pye Unicam Series 204 chromatograph equipped with flame-ionization detector (FID) and temperature programmer was used. A 3% OV-17 column (prepared, see appendix), 1.5mx 2mm I.D. glass packed with Gas chrom Q, 100/120 mesh size (part No. 0496-7649) was used. The column was conditioned for 24 hr. at 280°C and silanized with 3 volumes of, 10 µl each, hexamethyldisilazane. Preliminary works have stablished working conditions as; carrier gas (nitrogen) flow-rate was 28ml/min. at 19 psi; Air and Hydrogen inlet pressures were 8 and 20 psi respectively. Column ambient temperature was from 220°C, programmed to 292°C at 6°C per min. for 12 min. after 3min. initial delay. Injector and Detector temperatures were 300°C respectively. Perkin-Elmer recorder (56) was used

3.2.4 Thin Layer Chromatography

Thin layer chromatography (TLC) was carried out on silica get plate of the extract, mixture of carbazole and 3-methyl carbazole, carbazole (alone) and 3-methyl

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carbazole alone to ascertain the presence of carbazole and 3-methyl-carbazole (reported for the first time) to support the GLC findings of the two. Quite a lot of work has been done on TLC with carbozole alkaloids (Dutta *et. al.* 1969, Roy *et al.* 1974 and Chowdhury *et al.* 1986.)

The extract, carbazole and 3-methylcarbazole (mixture), carbazole (alone) and 3-methyl carbazole (alone) were spotted on the TLC plate (silica gel) using a capillary tube using a template to guide the applications. The spots were dried with a hot air blower and the plate was chromatographed in an ascending fashion to a level of 15 cm from the origin. It was dried and placed in an iodine chamber for 15 min. and finally viewed under U.V.

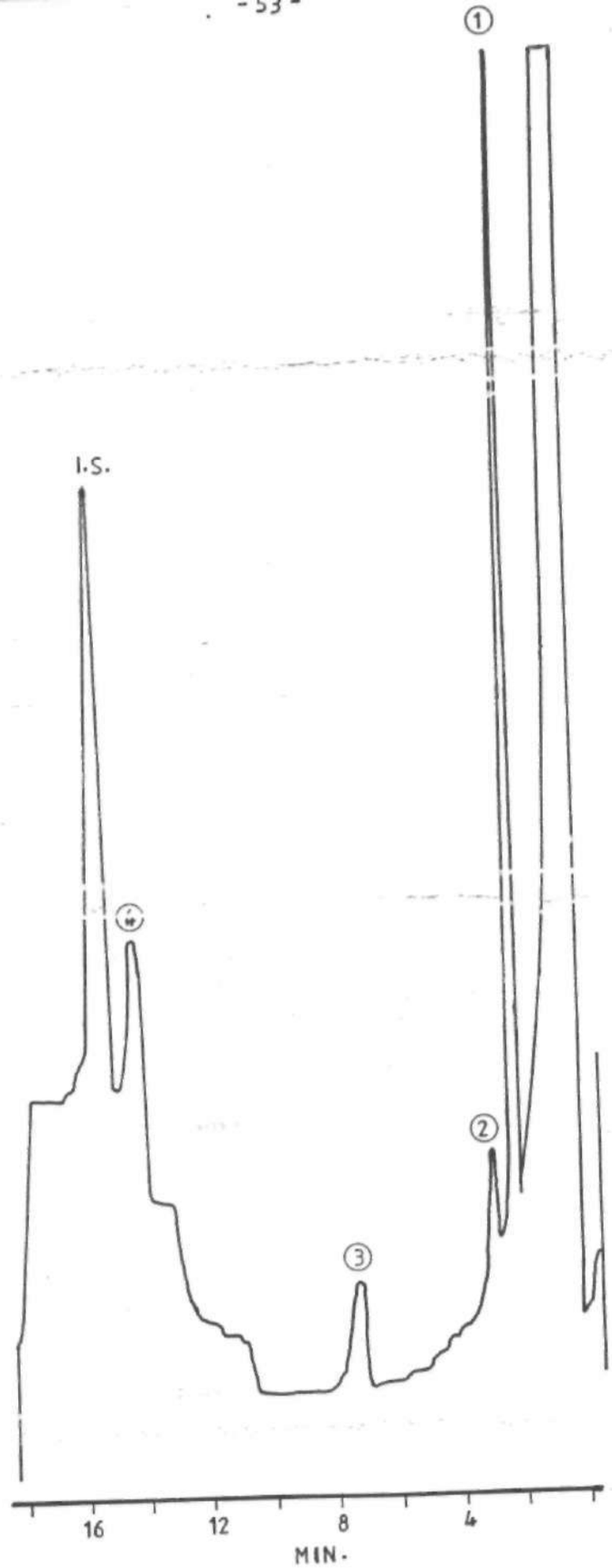


FIG. 4.1 GLC of standard solution injector and detector temperatures = 300°C, column temperature from 220° programmed to 292°C at 6°C/MIN. nitrogen flow-rate = 28 ml/MIN at 19 psi, H_2 and air inlet

CHAPTER 4: RESULTS AND DISCUSSION

4.1: RESULTS

Following are the results obtained from the experiments on GLC and TLC.

4.1.1: Gas Liquid Chromatography

In figs. 4.1, 4.2, 4.3, 4.4 and 4.5, and Tables 4.1, 4,2 and 4.3, are shown the Gas Chromatographic separations of carbazole alkaloids (Standard Solutions and Unknown Sample) and calibration curves for three carbazole alkaloids obtained by individual calibration curve run. The Tables (4.1, 4.2 and 4.3) show the various concentrations (in an increasing order) of the alkaloids and the corresponding peak height ratios of the resultant peaks.

4.12: Thin Layer Chromatography

In fig. 4.6 and Table 4.8 are shown the Thin Layer chromatogram of crude plant extract (E) carbazole and 3-methyl carbazole mixture (CMC) carbazole alone (C) and 3-methyl carbazole alone (MC), and the RF values of various spots obtained from the separation.

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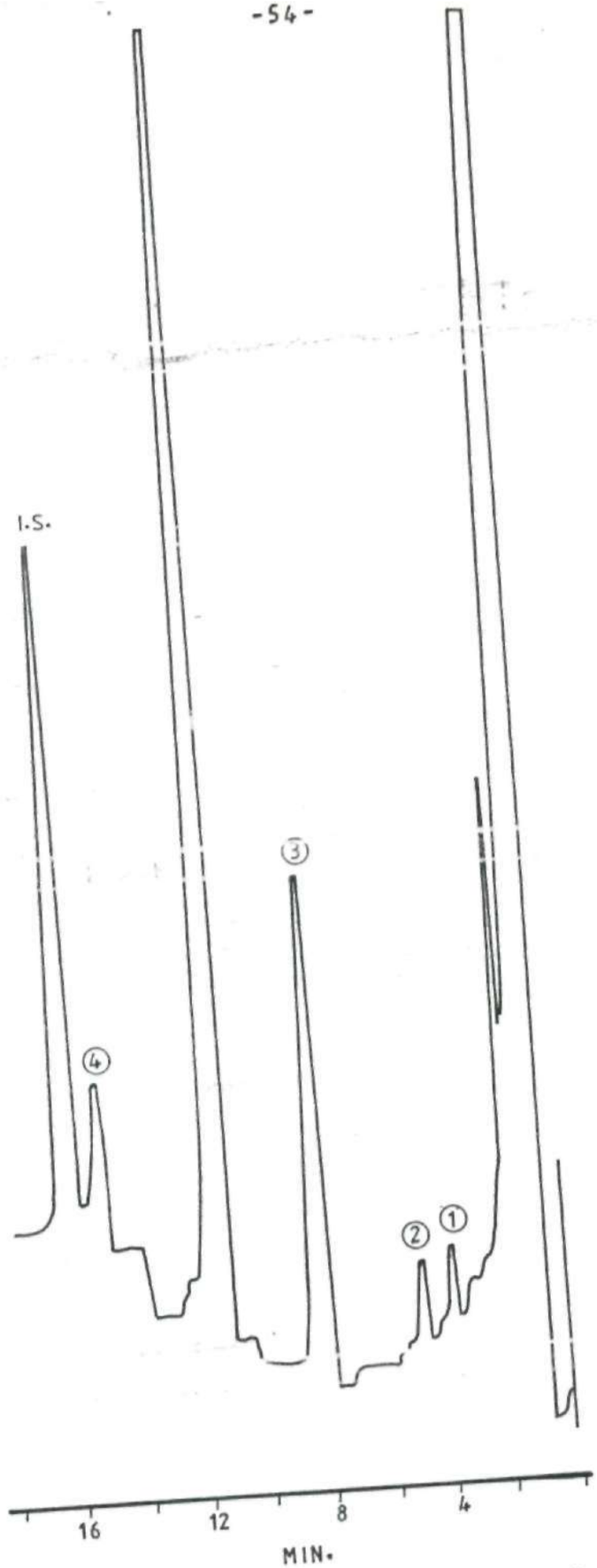
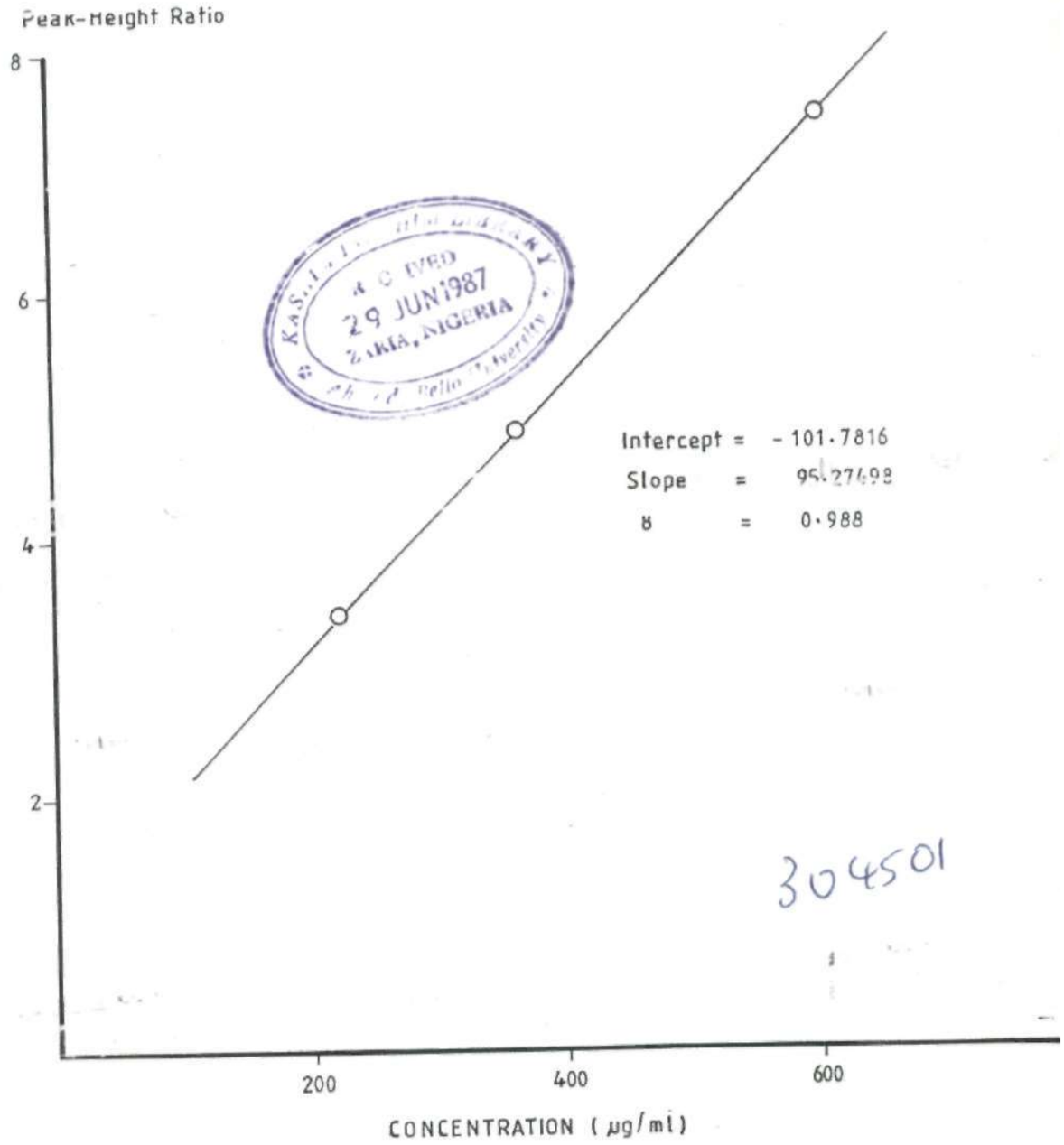


FIG. 4.2 GLC of unknown plant extract solution conditions same as in Fig. 4.1.



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FIG. 4.3 Calibration curve graph for Carbazole.

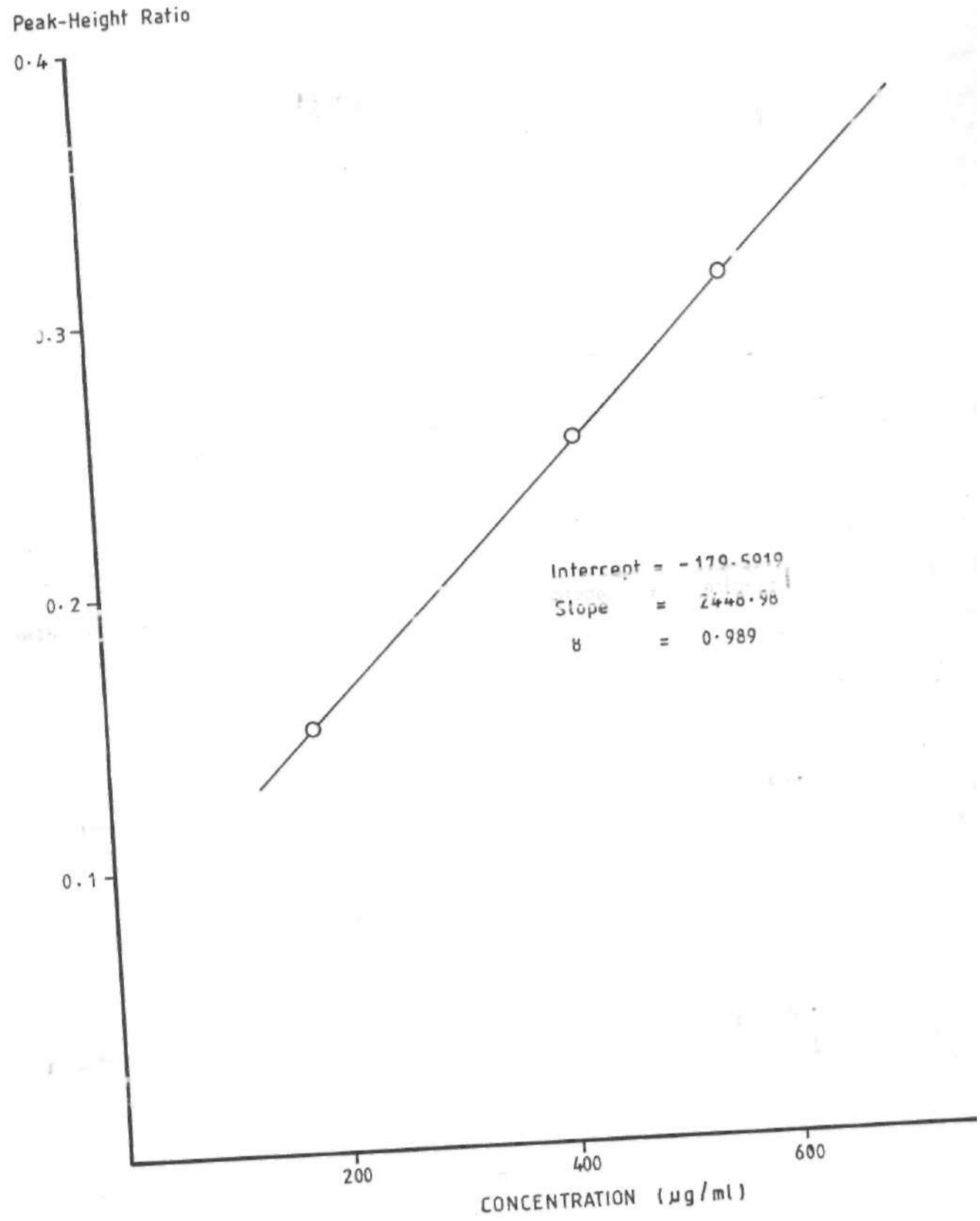
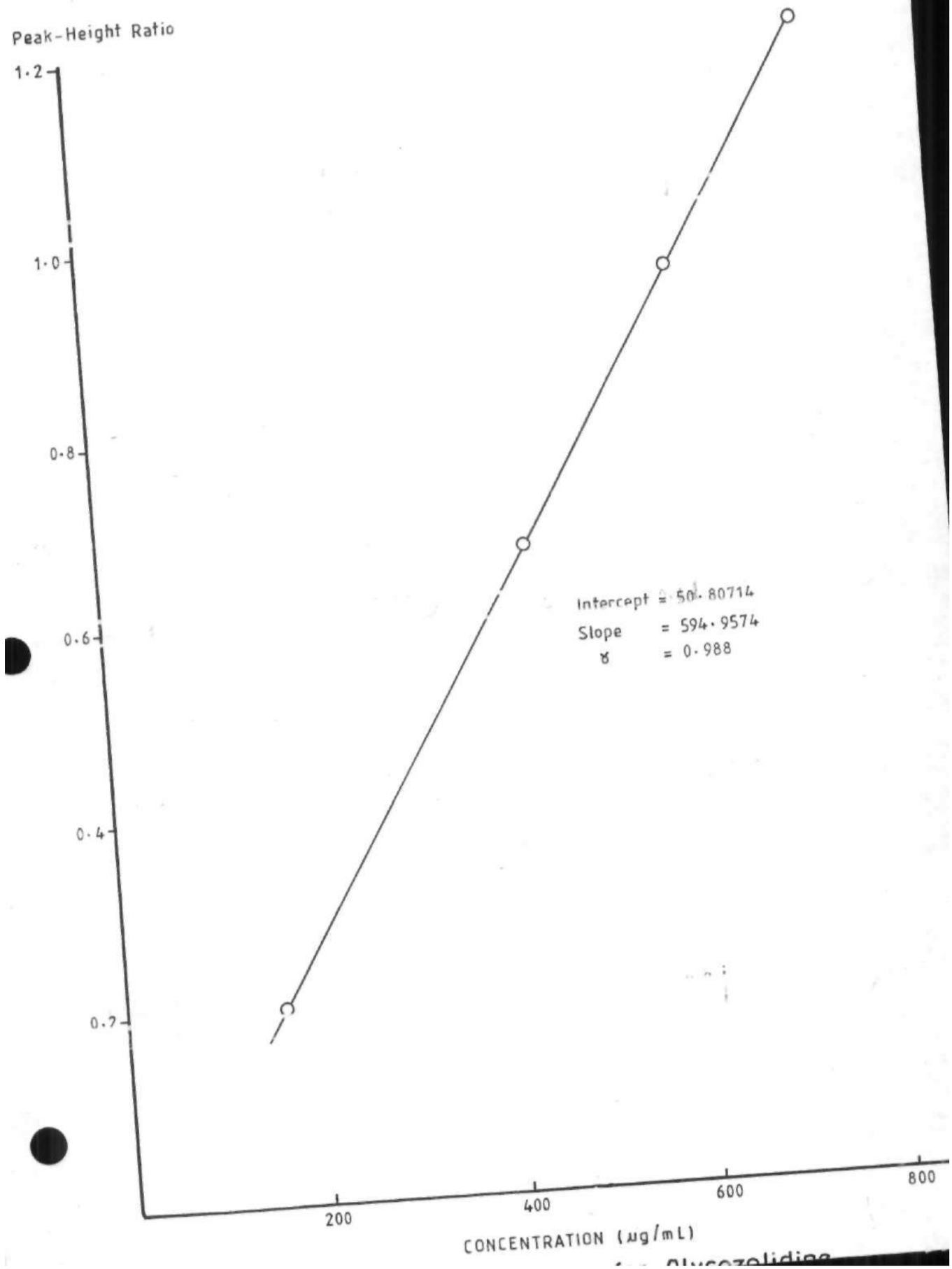


FIG. 4.4 Calibration curve graph for 3-Methyl Carbazole.


Peak-Height Ratio
1·2
1·0
0·8
0·6
0·4
0·2
Intercept = 50.80714
Slope = 594.9574
ϑ = 0.988
200
400
600
800
CONCENTRATION (µg/mL)

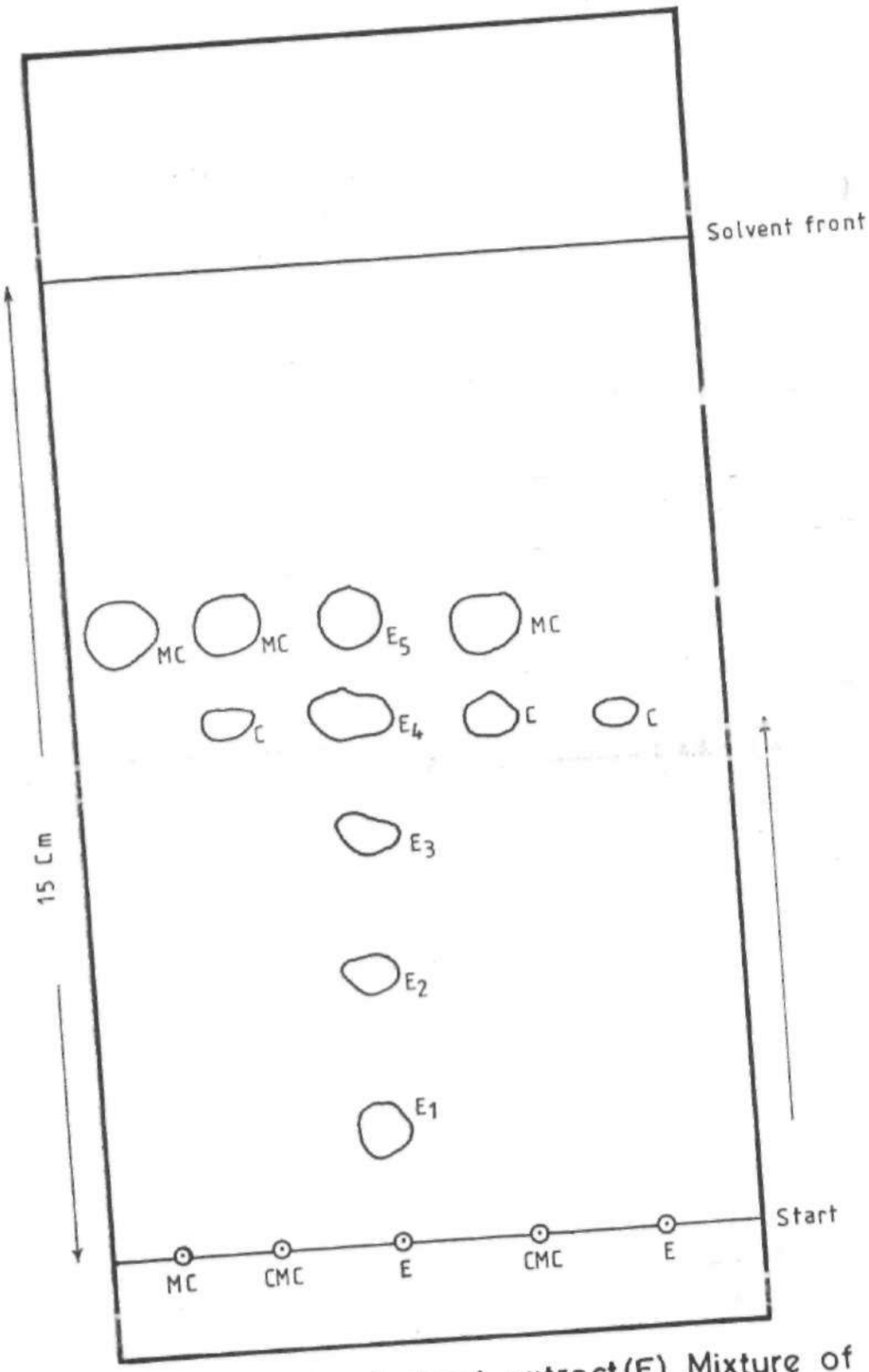
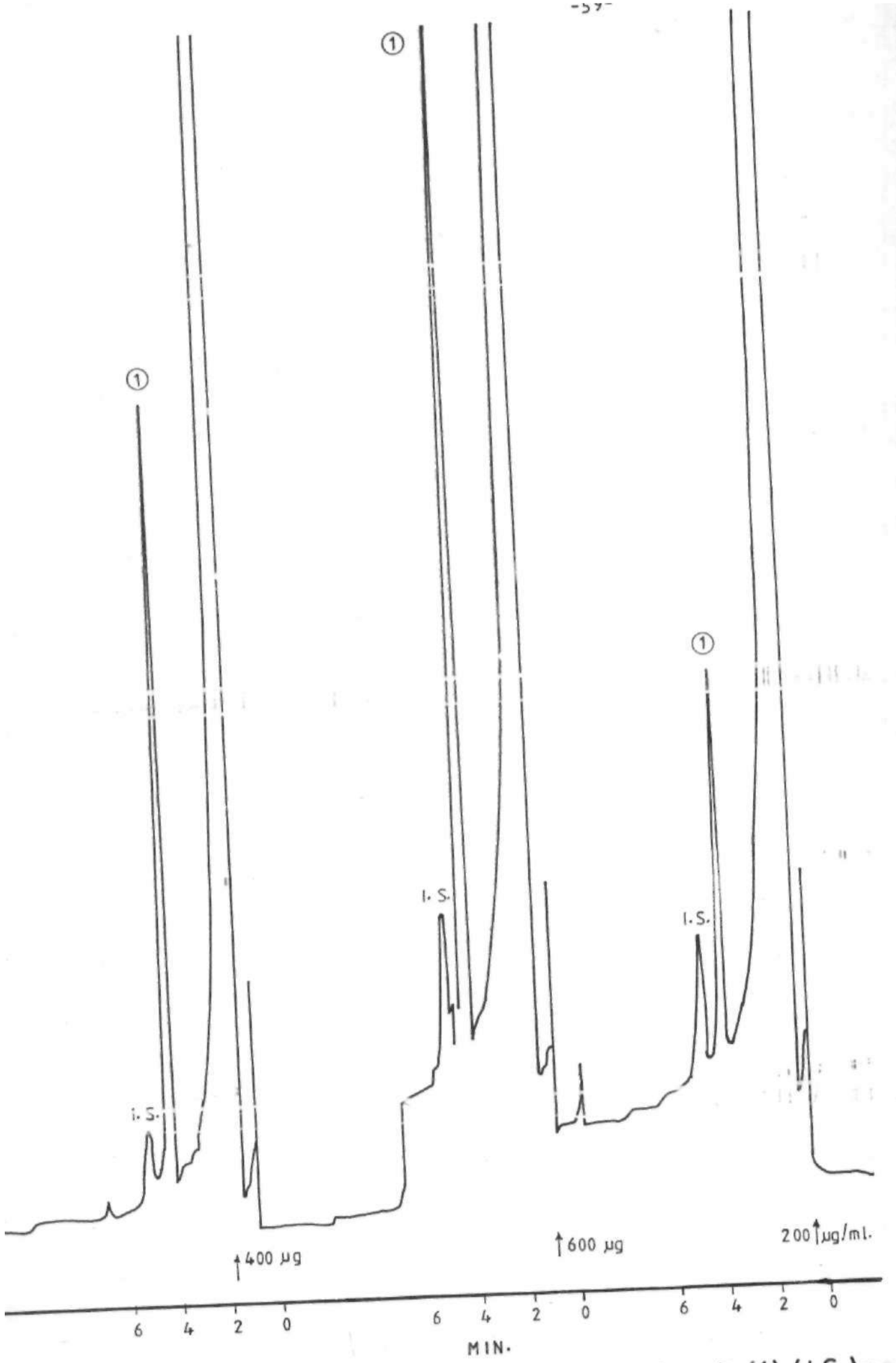


FIG. 4.6 TLC of plant extract(E) Mixture of Carbazole and 3-Methyl Carbazole (CMC) Carbazole alone (C) and 3-Methyl Carbazole alone (MC).


①
①
①
I.S.
I.S.
I.S.
400 µg
600 µg
200 µg/ml.
6
4
2
0
6
4
2
0
6
4
2
0
MIN.

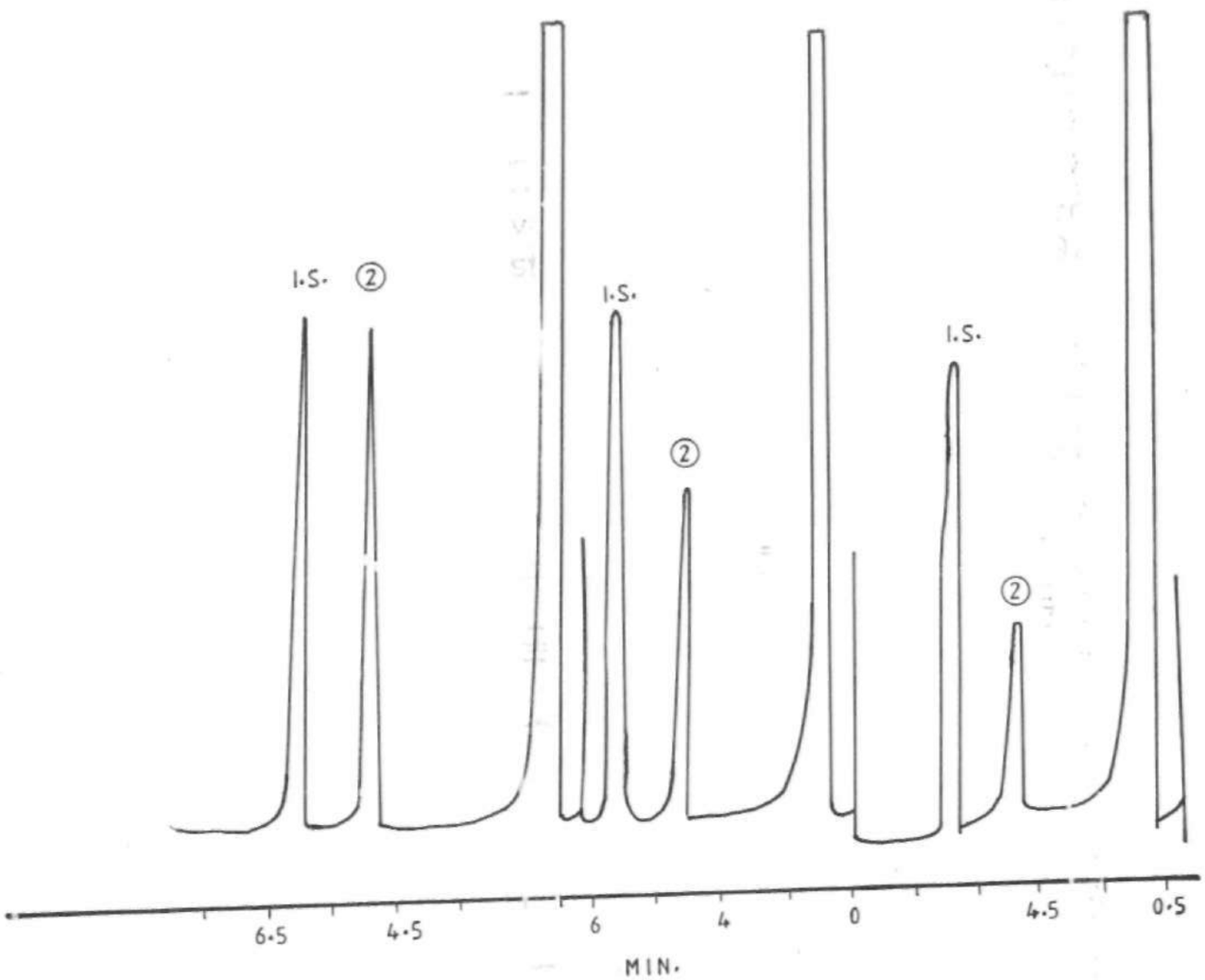


FIG. 4·8 Calibration curve (G L C) for 3-Methyl Carbazole (2) (I.S.) = Internal standard column temp. 220-292°C at 36°C / MIN.

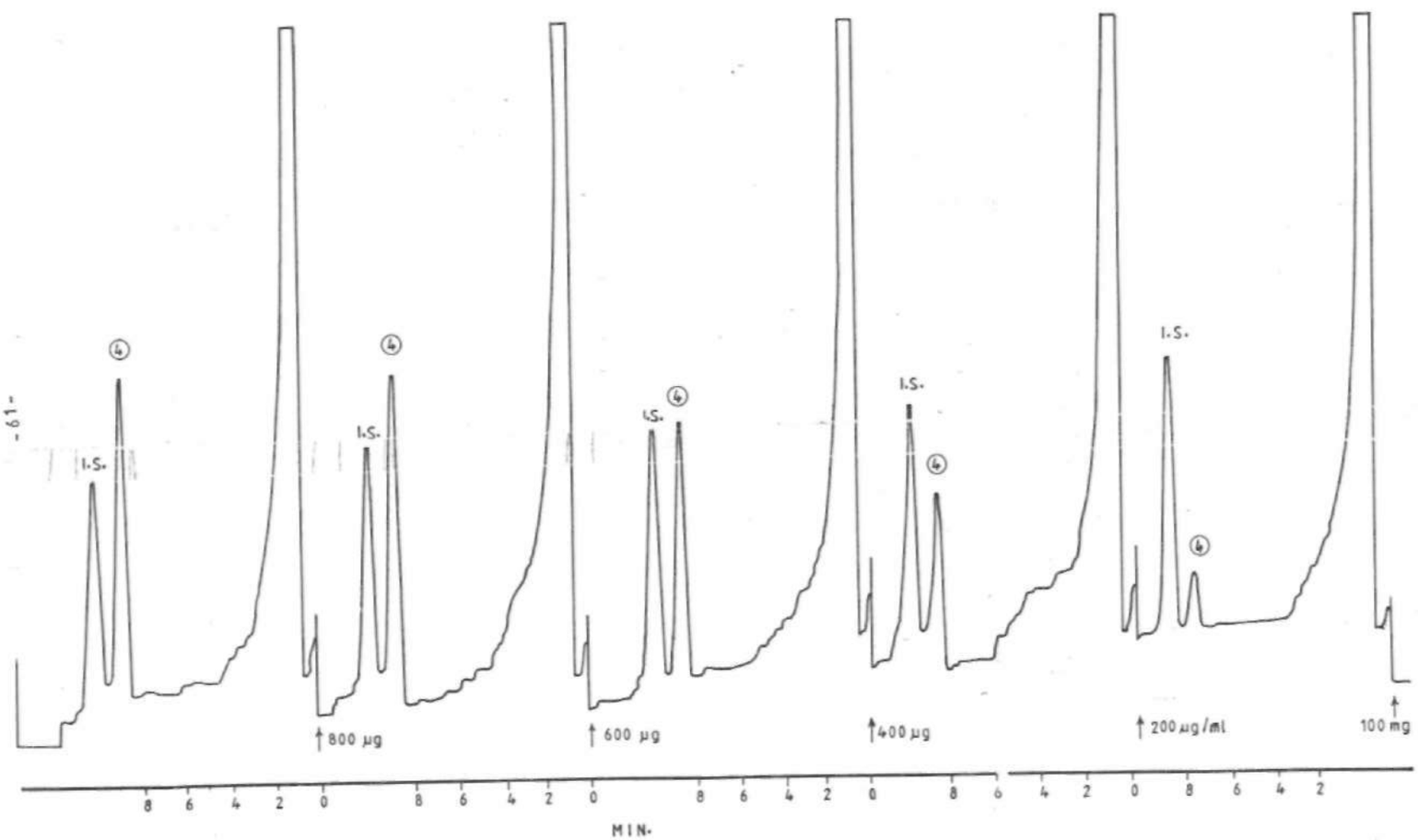


FIG. 4.9 GLC Calibration curve for Glycozolidine (4), (I.S.) = Internal standard isothermal run (158°C).

Table 4.1 Calibration curve table for carbazole

Conc. of carbazole (ug/ml)	peak-height Ratio
200	3.4
400	4.9
600	7.5

Table 4.2 Calibration curve table for 3-methyl carbazole

Conc. of 3-methyl carbazole	peak-height Ratio
200 ug/ml	0.15
400 "	0.25
600 "	0.31

Table 4.3: Calibration curve table for Glycozolidine

Conc. of Glycozoldine	Peak-height Ratio
200 ng/ml	0.20
400 "	0.67
600 "	0.95
800 "	1.20

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4.2 GENERAL DISCUSSION.

4.2.1 Gas Liquid Chromatography

From Figs. 4.1, and 4.2 which were the gas chromatographic Separations of Standard solution and unknown sample of carbazole alkaloids respectively, qualitative and quantitative analyses were carried out.

4.2.1.1 Qualitative Analysis

This was carried out from the results of retention times of carbazole alkaloids in the standard solution compared to same in the plant extract solution, (unknown.) It can be seen that carbazole, 3-methyl carbazole, Glycozoline and Glycozolidine bear same retention times in both Figs 4.1 and 4.2 respectively (Table 4.4.) Characterization of carbazole alkaloids was not difficult as such.(Also see Figs. 4.1 and 4.2.)

Table 4.4 Retention times of carbazole alkaloids

Sample	Retention Time (Min.)	
	Standard	Unknown
Carbazole (1)	3.0	3.0
3-Methyl Carbazole (2)	3.8	3.8
Glycozoline (3)	6.8	6.8
Glycozolidine (4)	14.2	14.2
Heptazolidine (5)	14.8	14.8

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Identification (which is a qualitative aspect of GLC) of a component depends on that component's retention time being similar to the retention time of an authentic sample which is regarded as the standard. In this way, all the unknown peaks were assigned (in Fig.4.2) and identified. However, in Fig.4.2, the presence of small peaks and a strong one in between peaks (3) and (4) might have been noticed. Since the unknown was a crude extract from a plant source, alot of such peaks are bound to appear. The small peaks near the solvent front cannot be assigned to be due to any of the carbazole alkaloids because carbazole (simplest member of the group which always elute first) eluted after 3min. In the chromatogram of the standard solution such peaks were not observed. This might be due to the impurity of the samples in the mixture. The strong peak between peaks (3) and (4) needs to be investigated further. In the normal authentic sample, Mukanol appeared between (3) and (4) but with a different retention time with this peak. Mukanol would have eluted at exactly 7 min. while this unidentified peak eluted at 12.00 min. It might be either an impurity in the extract (in high concentration) bearing similar structure with near peaks (4) and (3) or an ester of Mukanol or Glycozolidine since it is a common phenomenon that alkaloids occur in forms of their esters (e.g, Glycozoline and Glycozolidine.)

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In either case, further intensive work should be done which will involve synthesis and GC - mass spectrometry to fully identify and elucidate the structure.

4.2.1.2 Quantitative Analysis

Quantitation was carried out using the formula already discussed before (Supelco, Inc., 1977). The following table (Table, 4.5) shows the result obtained after applying the said formula to obtain the concentrations of unknown alkaloids in the plant extract. Percentage of each component in extract is also given in w/w.

Table 4.5: Table Analysis (Results).

Sample	Standard peake - height Ratio (Rs)	Extract Peake-height Ratio (Ru)	Conc. of working standard (in ug/ml) (Cs)	Unknow levels in Extract (in ug/ml) (Cu)	Percentage (w/w)
Carbazole (1)	1.50	0.065	400	17.33	0.4%
3-methyl carbazole (2)	0.12	0.056	400	186.67	4.06%
Glycozoline (3)	0.25	0.685	500	1370.00	29.30%
Glycozolidine (4)	0.34	0.129	500	189.70	4.12%
Heptazolidine (5)					
I. S.					

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This method (single point standard) was compared with the individual calibration cur ve method in order to see whether both would give similar result or not. A sample of three carbazole alkaloids (carbazole, 3-methyl carbazole and Glycozolidine) were quantitated and compared using both the single point and individual calibration methods. The results were subjected to paired t- test and table 4.6 shows the concentrations of the three carbazole alkaloids from the unknown extract solution obtained using the two methods:

Table 4.6: Concentrations of carbazole alkaloids used in paired t - test.

Alkaloid	Conc. in ug/ml	
	Single point	Individual
Carbazole (1)	17.33*	18.00*
3-methyl carbazole (2)	186.67*	190.00*
Glycozolidine (4)	189.70*	180.00*

* Represents P $\angle$ 0.05

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For calculations involved in the analysis of t-test, see (Appendix 2.)

This result therefore indicates that both methods are equally accurate for quantitating unknown component from an extract or mixture.

It must however be pointed out that the single point standard calibration method was found to be more convenient to use for reasons already given. It would have taken a researcher a much shorter time of analysis to obtain the concentrations of a many component-mixture with this method than if he were to employ an individual calibration curve method. It is therefore the view of this Author that the single point standard method is more preferred and recommended.

4.2.1.3 Linearity, Sensitivity and Reproducibility of the Method

The detector response over the concentrations used was linear (there was a linear response, as the conc. became higher so was the peak bigger.) Higher concentrations (above 500 µg) were not used in the experiments and therefore, linearity cannot be said to extend over those concentrations.

The sensitivity of the detector was equally high since as low a concentration as 0.01 µg/ml was detected!! The MDC (Minimum Detectable Concentration) at 256 x 1 and 128 x 1 were 0.05 µg/ml and 0.01 µg/ml respectively.

The reproducibility of the detector response was equally good too. Same retention times of various

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carbazole alkaloids were observed after many injections. In Table 4.7 is shown the retention times (3 runs) for each carbazole alkaloid, same retention times were recorded for each of the 3 runs.

Table 4.7 Retention times of carbazole Alkaloids (3 runs)

Sample			Retention Time (Min)					
			Standard		Unknown			
	1	2	3	Average	1	2	3	Average
Carbazole (1)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
3-Methyl Carbazole(2)	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
Glycozoline (3)	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8
Glycozolidine (4)	14.2	14.2	14.2	14.2	14.2	14.2	14.2	14.2
Heptazolidine (5)	14.8	14.8	14.8	14.8	14.8	14.8	14.8	14.8

The sensitivity and reproducibility recorded thus far would definitely allow the determination of carbazole alkaloids in plasma or any sample quite readily. Infact, concentrating their levels by extraction procedures to detectable limits was not found to be necessary.

Recalibration at the begining of each day was recommended (Supelco, Inc., 1977), but thiswas not found to be necessary because a single calibration at the beginning of the work was found to be sufficient.

Also the use of equeous calibration standards has been claimed unsuitable by several workers (Supelco, Inc., 1977.) However, in the analysis of samples and in-home quality control serum samples at the Medical Foundation, excellent

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results have been achieved by calibration with equeous standards (Supelco, Inc., 1977.) In the present work, this fact was further supported because equeous calibration standards were used sucessfully.

4.2.2 Thin Layer Chromatography

In Fig. 4.6, is shown the separations of plant extract (E) a mixture of carbazole and 3-methylecarbazole ((CMC) carbazole alone (C) and 3-methyl carbazole alone (MC). E4 and E5 spots with Rf values of 0.54 and 0.63 respectively correspond to carbazole and 3-methylcarbazole. E4 and E5 were therefore assigned as carbazole and 3-methyl carbazole respectively. E^1, E_2 and E_3 might have been the rest of the carbazole alkaloids as detected by the GLC (Glycozoline, unidentified peak and Glycozolidine as in Fig. 4.2.) In Table 4.8 is shown the Rf values obtained of various spots. This result gave a further evidence to support the detection of the two compounds by GLC.

Table 4.8: Rf Values of Carbazole Alkaloids

	Rf Values	
	Extract	Pure sample
Carbazole	0.54 (E_4)	0.54
3-methyl carbazole	0.63 (E_5)	0.63
	0.10 (E_1)	
	0.30 (E_2)	
	0.40 (E_3)	

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SUMMARY

Carbazole alkaloids were determined quantitatively using GLC from a crude plant extract. Single point standard calibration method was employed in the study and was found to be more advantageous than the commonly employed individual calibration curve method. Both methods gave similar results (concentrations of carbazole alkaloids quantified and (p<0.05.) The percentage composition (by weight) of carbazole, 3-methylcarbazole, Glycozoline and Glycozolindine found in the plant crude extract respectively were 0.4%, 4.06%, 29.30% and 4.12% (Table 4.5).

This was the first time a quantitative work was undertaken on carbazole alkaloids. Also it was the first time 3-Methyl Carbazole was detected from this plant species. Carbazole was first detected from plant source in this work and this will be of immense significance from the biogenesis point of view.

CONCLUSION

Quantitation of carbazole alkaloids in crude plant extract was successfully carried out and single point standard calibration method employed was found to be convenient and faster time of analysis was provided.

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APPENDIX1: PREPARATION OF 3% OV-17 GLC COLUMN

Materials:

1. Acetone (BDH Chemicals Ltd, Poole, England)
2. Ov-17 (part No. given in text from Pye Unicam, England) A highly viscous thick liquid
3. Gas Chrom Q ($^{100}/_{120}$ mesh size)-(Part No. given in text, from Pye Unicam Eng. land) which was the adsorptive support material.
4. Empty glass column 1.5m x 2mm I.D. glass.
5. Vacuum pump
6. Rubber band
7. Ultra sonic vibrator

Method:

0.15gm of Ov-17 was accurately weighed and placed in a round bottom-falsk. 40ml of acetone were measured and used to dissolve and dilute the Ov-17 in the flask by adding in aliquot portions while shaking the flask slowly. Then 15gm of Gas chrom Q ($^{100}/_{120}$) wered added in portions with constant shakings to ensure proper coating of Ov-17 (liquid phase) to the support. After adding all the support, the flask was allowed to stand for at least 30 min. after which theacetone was evaporated over steam. The wet coated support was then dried in an oven at 80-100°C, till a free-flowing powder was obtained. This was then filled into the column using the vacuum pump. The column was finally conditioned, after silazation, for 24 hr. at 280°C. by passing N_2 (6 psi) through it.

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APPENDIX 2: STATISTICAL ANALYSIS (PAIRED-t-TEST WITH CONCENTRATIONS OBTAINED FROM SINGLE POINT AND INDIVIDUAL METHODS.)

[redacted]loid	Concentration (ug/ml)			
	A (individual)	B (Single)	A-B(d)	d^2
Carbazole (1)	18.00	17.33	0.67	0.4489
3-Methylcarbazole (2)	190.00	186.67	3.33	11.0889
Glycozolidine (4)	180.00	189.70	9.70	94.0900
		Sum =	13.70	105.6278
		Mean ≙	5	

$n = 3$

$\bar{d} = 5$

$d = 13.7$

$(d)^2 = 187.69$

$d^2 = 105.6278$

$\frac{(d)^2}{n} = 62.56$

$\frac{SD^2}{n} = \frac{d^2 - \frac{(d)^2}{n}}{n-1} = 21.535$

$\frac{SD^2}{n} = \frac{21.535}{3} = 7$

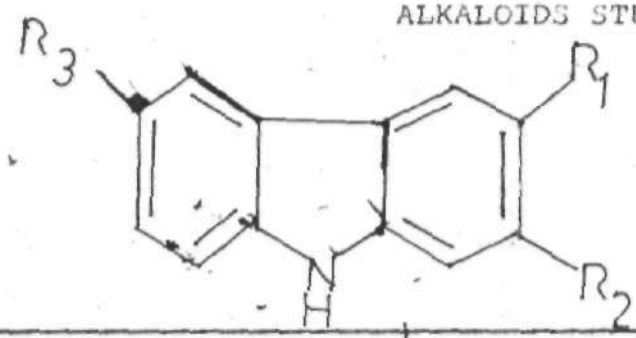
$\sqrt{\frac{SD^2}{n}} = 2.497$

$t = \frac{\bar{d}}{\sqrt{\frac{SD^2}{n}}} = \frac{5}{2} \simeq 3$

$p < 0.05$ which shows there was no significant difference between the results obtained by the two methods.

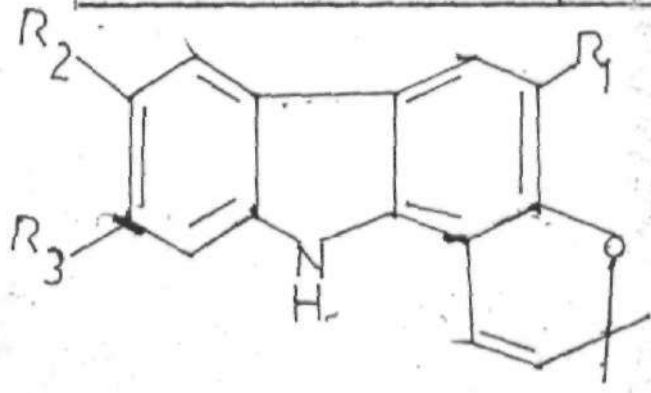
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APPENDIX 3: STRUCTURES OF CARBAZOLE ALKALOIDS STUDIED



Name	Substitution		
	R_1	R_2	R_3
Carbazole (1)	H	H	H
3-Methyl carbazole (2)	H	CH_3	H
Glycozoline (3)	H	CH_3	OCH_3
Glycozolidine (4)	OCH_3	CH_3	OCH_3

Name	Substitution		
	R_1	R_2	R_3
Heptazolidine (5)	OCH_3	CH_3	H



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APPENDIX 4: RESULTS OF CALCULATION

CARBAZOLE

Slope = 95.27498

Pearson's Correlation Coefficient = 0.9882144

Intercept on Y-Axis = -101.7816

Standard Deviation of points from the line = 43.29568

Error in Y value for each Data point:

X	Y (OBS)	Y (CAL)	ERROR
3.4	200	222.1534	-22.15337
4.9	400	365.0658	34.93417
7.5	600	612.7808	-12.78076

SUM OF ERRORS SQUARED = 1874.516

3-METHYL CARBAZOLE

Slope = 2448.98

Pearson's Correlation Coefficient = 0.9897435

Intercept on Y-Axis = -179.5919

Standard Deviation of points from the line = 40.4061

Error in Y value for each Data point:

x	Y (OBS)	Y (CAL.)	ERROR
0.15	200	187.7551	12.24493
0.25	400	432.653	-32.65303
0.31	600	579.5919	20.40917

SUM OF ERRORS SQUARED = 1632.652

GLYCOZOLIDINE

Slope = 594.9574

Pearson's Correlation Coefficient = 0.987791

Intercept on Y-Axis = 50, 80714

Standard Deviation of points from the line = 49.264448.

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Error in Y value for each Data point:

x	Y (OBS)	Y (CAL.)	ERROR
0.2	200	169.7986	30.20139
0.67	400	449.4286	-49.4286
0.95	600	616.0167	-16.01665
1.20	800	764.756	35.24396

SUM OF ERRORS SQUARED = 4853.98

APPENDIX 5: THIN LAYER CHROMATOGRAPHY

SOLVENT SYSTEM: PETROLEUM ETHER (40 - 60°) -
ACETIC ACID (10 : 1)

PLATE: SILICA GEL (10 x 20 CM)

(MC) = 3 - METHYL CARBAZOLE

(E) = PLANT EXTRACT

(CMC) = CARBAZOLE AND 3 - METHYL CARBAZOLE MIXTURE

(C) = CARBAZOLE

APPENDIX 6: PEAK-HEIGHT RATIOS EXTRAPOLATED
(OF UNKNOWN PEAKS)

ALKALOID	PEAK-HEIGHT RATIO
CARBAZOLE	0.115
3 - METHYL CARBAZOLE	0.165
GLYCOZOLIDINE	0.210

(FROM FIG. 4.2)