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Bacterial Cell Wall Constituents in the Body Fluids of Patients in Disease and in Health

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A thesis submitted in partial fulfilment of the requirements of the Council for National Academic Awards for the degree of Master of Philosophy

May 1990

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Bacterial Cell Wall Constituents in the Body Fluids of Patients in Disease and in Health. By P. Cheetham, May 1990.

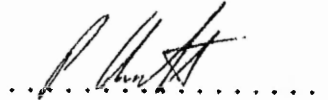
ABSTRACT

The development of a method for the detection and measurement of the amino sugar subunits of peptidoglycan in the body fluids of patients involved a number of stages. A number of derivatisation procedures were tested to find the most suitable. The gas chromatography conditions were investigated to obtain the optimum results. A combination of the most effective derivatisation reagent and chromatography conditions were used to examine pure chemicals, bacterial extracts and clinical specimens. The feasibility of using gas chromatography for detecting the amino sugars in body fluids was thus studied.

Results obtained with two of the derivatisation reagents were promising when used with the pure chemicals. The examination of bacterial extracts demonstrated a lack of sensitivity of the methods used. The examination of clinical samples also illustrated this lack of sensitivity and the interference from other compounds. The examination of amino sugars in body fluids is not a feasible alternative to conventional culture and microscopy techniques.

DECLARATION

This work has not been accepted for any other degree and is not currently being submitted in candidature for any other degree. The work submitted here was carried out by the candidate himself and due acknowledgement has been made of any assistance received.

Signed 

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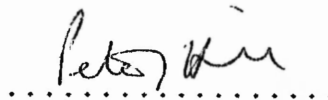
Signed 

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INTRODUCTION

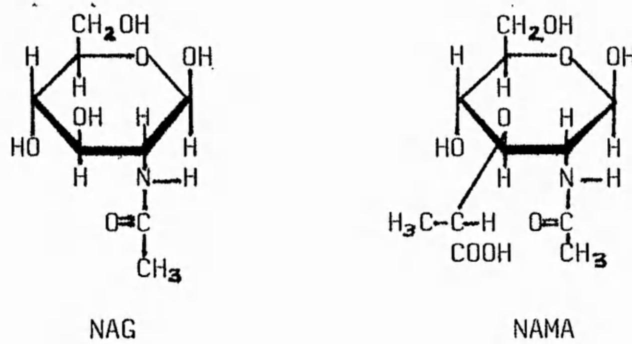
A major difference between bacterial and mammalian cells is the presence of a rigid cell wall in bacteria . The structure and functions of the bacterial cell wall are responsible in many ways for the unique character of bacteria. The mammalian cell has a cell membrane and is maintained in an osmotically stable environment and thus has no need for a rigid cell wall. This cell, functioning as part of a greater being, may or may not be specialised in nature, however it will be provided with its requirements by the body as a whole. Conversely, bacteria are free living single celled organisms which may need to survive in a variety of environments either favourable or unfavourable. Consequently the presence of a cell wall is essential in protecting the cell from forces it may be subjected to when living in an osmotically unfavourable environment. In this introduction, a brief description of the structure of the bacterial cell wall will be given, indicating how it may vary according to species and how this affects the nature of the organism. An account of the ways in which gas chromatography has been used in elucidating the structure of bacterial cell walls and a review of bacterial cell wall breakdown is also included.

i Bacterial Cell Wall Structure

During the late nineteenth century the presence of a relatively stiff outer coating surrounding bacteria was recognised (1). It had been noticed that motile rod shaped organisms did not bend when they bumped into objects and also that when placed in concentrated solutions of neutral substances the cytoplasm shrank away from the outer membrane which maintained the bacterium's shape. The Gram stain has been used for many years in the classification of bacteria into Gram positive and Gram negative, however there was no indication of its mechanism for at least fifty years after the discovery of this differential staining technique. Only after the isolation of bacterial cell walls and studies of their structure and chemical composition have the fundamental differences between Gram positive and Gram negative cell walls become clear. Electron microscopy has revealed that Gram positive cell walls are about five times thicker than Gram negative walls and show little or no fine structure. However the Gram negative walls show an outer membrane very similar to the inner cytoplasmic membrane common to both types (2).

The rigidity of bacterial cell walls is due to a

peptidoglycan polymer. This comprises of chains of two β 1-4 linked amino sugars arranged alternately (Figure 1). Firstly 2- acetamido-2-deoxy-D-glucose, commonly known as N-acetyl glucosamine (N.A.G.) and secondly 2-acetamido-2-deoxy-3-O-[D-1'-carboxyethyl]-D-glucopyranose commonly known as N-acetyl muramic acid (N.A.M.A.). The amino sugar N-acetyl muramic acid is a compound found only in the bacterial cell wall in nature. The polymer chains of the amino sugars are cross-linked by short peptide chains. The carboxyl group of muramic acid is substituted by these peptide chains which may themselves be cross-linked to form a rigid three dimensional structure.



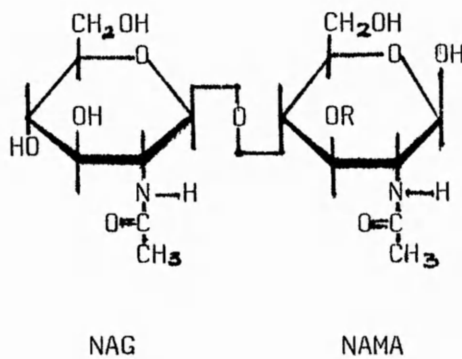


Figure 1. The structures of N-acetyl glucosamine and N-acetyl muramic acid. N.A.M.A. is linked to N.A.G. by a β 1-4 glycosidic bond.

The following is a peptide chain, found in *Staphylococcus aureus*, linking two amino sugar polymers: L-alanine-D-glutamic acid-L-lysine-D-alanine. The tetrapeptides of peptidoglycan are composed of alternate L and D isomers of the amino acids. This is in itself unusual as proteins are composed of only the L series of amino acids. In addition a number of unusual amino acids may be incorporated into the chain, including diaminopimelic acid, ornithine and homoserine, none of which occur in proteins. It is becoming clear that peptidoglycan is a unique polymer which accounts to a great extent for the nature of bacteria. A schematic illustration of the peptidoglycan of *Staph. aureus* is shown in Figure 2.

The importance of peptidoglycan to the viability of bacteria is illustrated by the number of potent antimicrobials that interfere with its synthesis. For example cycloserine is a structural analogue of D-alanine preventing its incorporation into the polymer; the penicillins and cephalosporins inhibit the final stage of the cross-linking reaction. Similarly bacitracin prevents the assembly of the completed peptidoglycan polymer in one of the later stages. These antibiotics are relatively non-toxic because similar reactions do not occur in man.

The peptidoglycan is the backbone of the cell wall and thus if it is malformed or not formed at all then the bacterial wall will weaken and finally rupture. This will lead rapidly to cell lysis and death unless it is maintained in an osmotically favourable environment. The final structure of peptidoglycan resembles a three dimensional net of amino sugar chains crosslinked in all directions by peptide chains. This produces a very strong but flexible basis for the bacterial cell wall to be built around.

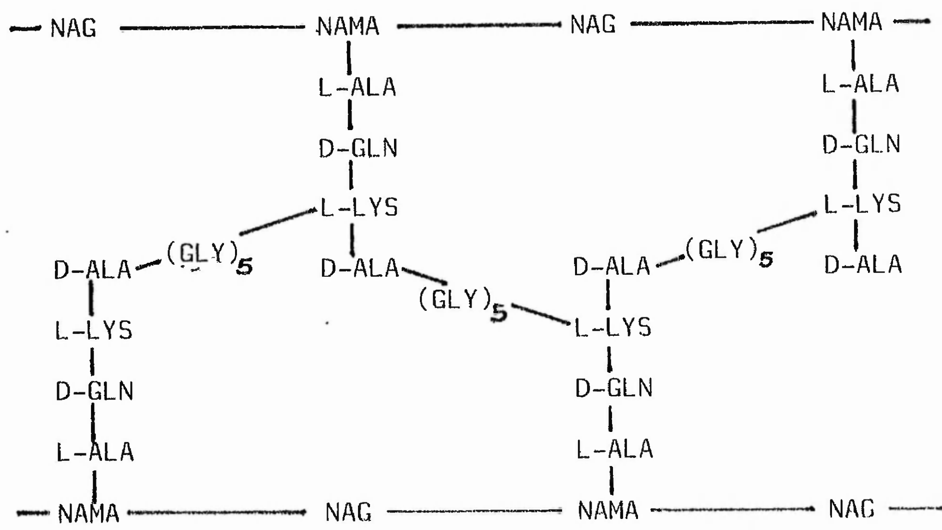


Figure 2 The peptidoglycan structure of <u>Staphylococcus aureus</u> .

Key: NAG=N-acetyl glucosamine; NAMA=N-acetyl muramic acid; L-ALA=L-alanine; D-ALA=D-alanine; D-GLN=D-glutamine; L-LYS=L-lysine; GLY=glycine:

Peptidoglycan is the only structure common to both Gram positive and Gram negative cell walls. In Gram positive walls the other major components are the teichoic and teichuronic acids (Greek: teichos = wall). Between 10 and 50 per cent of the dry weight of cell wall may be teichoic acids possibly of different structural types. In their simplest form teichoic acids are long chains of either glycerol or ribitol units joined by phosphodiester bonds. These may be substituted by sugars, amino sugars, choline or D-alanine, all of which may be antigenic determinants. The varied patterns of substitution can produce a range of antigenic types even within the same species. Figure 3 shows the formation of ribitol teichoic acid by a membrane bound enzyme in Staph aureus .

A lack of phosphate in the environment prevents synthesis of teichoic acid. Under these conditions an alternative acidic polymer can be formed by glycosidically linking uronic acid residues. In this case the resultant polymer is a teichuronic acid (Figure 4).

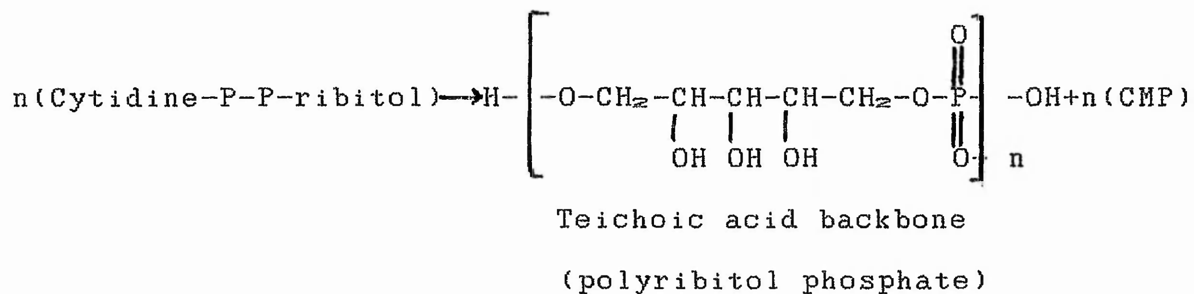


Figure 3. The formation of ribitol teichoic acid in *Staph aureus*.

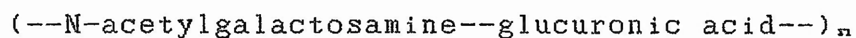


Figure 4. The repeating subunit of teichuronic acid from *Bacillus licheniformis*.

The exact function of the teichoic acids is not fully understood although they may be involved in the transport of metal ions, e.g. calcium and magnesium, through the cytoplasmic membrane. They are covalently attached to the muramic acid of the peptidoglycan by a short glycerol linkage unit. The synthesis of these compounds is linked with the synthesis of the peptidoglycan and their composition is dependent to some extent on the conditions in which the organism grows (as described previously).

The cell envelope of Gram negative bacteria does not contain teichoic acids however it is more complex than that of the Gram positive bacteria. The peptidoglycan layer of the Gram negative wall is surrounded by an outer membrane, which is composed of phospholipids, proteins and lipopolysaccharides. The phospholipids occur on the inside of the membrane, the lipopolysaccharides on the outside of the membrane while the proteins pass all the way through (Figure 5). Bacteria have a cytoplasmic membrane which resembles a bilayer on electron microscopy. The outer membrane of Gram negative cells is also a bilayer membrane thus giving them two membranes as opposed to the one

present in Gram positive cells. The lipid section of the molecule is toxic to the host, contributes to the barrier role of the outer membrane and may also confer some rigidity. The aqueous layer between the outer membrane and the peptidoglycan, the periplasmic space, contains many proteins that bind and transport sugars and other nutrients. This periplasmic space is distinctive of Gram negative cell walls (3).

The lipopolysaccharides present in the outer membrane are very unusual. They consist of lipid A and core oligosaccharide (Figure 6).

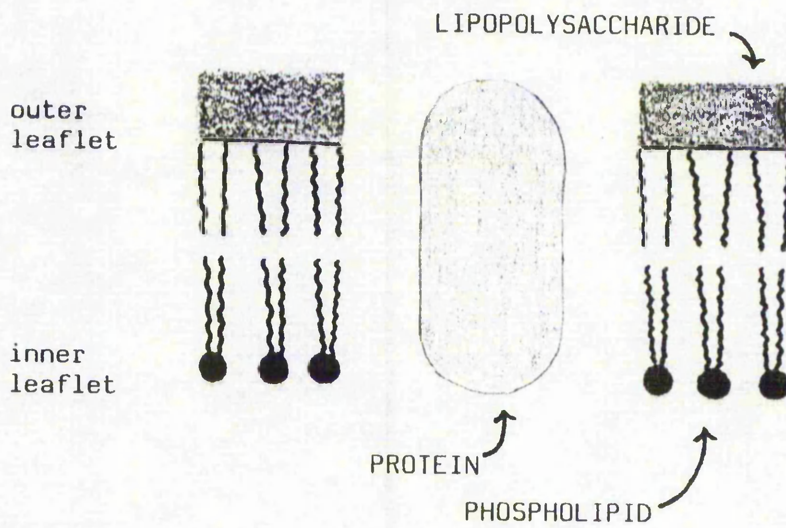


Figure 5. An example of a typical Gram negative cell wall outer membrane.

Figure 6. An example of a typical lipopolysaccharide molecule from the outer cell membrane of a Gram negative cell wall.

KEY. ABE: abequose, EtN: ethanolamine, GAL: galactose, GLU: glucose, GLN: glucosamine, HEP: heptulose, KDO: 2-keto -3-deoxyoctonate, MAN: mannose, NAG: N-acetyl-glucosamine, RHA: L-rhamnose.

The lipid A part of the molecule is hydrophobic while the core oligosaccharide and the O side chain are highly hydrophilic. The latter two regions contain a number of sugars that are rarely seen elsewhere in nature: an eight carbon sugar, 2-keto-3-deoxyoctonate, a seven carbon sugar, heptulose, and two six carbon sugars with $-CH_3$ instead of $-CH_2OH$ at C-6, L-rhamnose and abequose.

The proteins present in the outer membrane are of two kinds. By far the most abundant is a small lipoprotein which contains only fifty eight amino acids. It is an α helical protein linked to the peptidoglycan of the cell wall and with three fatty acids covalently attached to it. The tying of the outer membrane to the peptidoglycan by the lipoprotein confers rigidity and stability on the structure. The second type of protein is porin, a transmembrane protein. Channels in the membrane are formed by trimers of porin through which small polar molecules can diffuse rapidly, for example the monosaccharide metabolites.

The outer layer of a bacterium, the cell wall, is responsible for its antigenicity. In Gram positive cells

the teichoic and teichuronic acids are the antigenic determinants. Substitutions of the sugars, choline, amino sugars and D-alanine can produce a variety of these acids which will react with different antibodies. This allows the recognition of different types of organisms often within the same species which can be very useful particularly to the epidemiologist. Gram negative cells do not contain teichoic acids; in such organisms it is the lipopolysaccharides found in the outer part of the membrane which are responsible for their antigenic nature. The antigenicity is due to the polysaccharide portion of the molecule, which is negatively charged because of the phosphorylation of some of the sugars. The O side chains of the molecules are variable (the one illustrated in Figure 6 is just an example), leading to many antigenic variants. The hydrophilic nature of the cell surface, because of the polysaccharide part of the molecule, renders it less susceptible to phagocytosis. In addition Gram negative bacteria can rapidly mutate to alter the structure of their O side chains thus rendering ineffective the antibodies the host may have raised against it.

The structure of bacterial cell walls, particularly the peptidoglycan of Gram positive cells, was studied by early workers using a variety of methods including

chromatographic techniques. (4, 5). In 1964 Salton wrote a comprehensive account of the methods, both physical and chemical, for isolating bacterial cell walls (6).

A number of workers have used gas chromatography to study the structure of bacterial cell walls. Vacheron and others studied the cell walls of Nocardia species (7) while others examined the polysaccharide from the cell wall of the related Micromonospora species, (8). The glycans from the cell wall of Streptococcus faecalis have been studied by Pazur (9,10,11). Similarly the peptidoglycan of Streptococcus pneumoniae has also been examined (12). The linkage between the polysaccharide and peptidoglycan in the cell wall of Lactobacillus casei was analysed by Knox (13). The K 15 antigen of Vibrio parahaemolyticus, 2-amino-2-deoxyguluronic acid, was demonstrated by Reistad to be present in the cell walls of Halococcus species, another halophilic organism using this technique (14). The use of pyrolysis gas chromatography mass spectrometry has been used to study cell walls (15). Both the polysaccharide from the cell wall (16) and a fragment of the cell wall (17) of Micrococcus lysodeikticus have been examined. It has been shown that the cell walls of Leptospira species contain the sugar 4-O-Methylmannose in their cell walls (18). The 2-keto-3-deoxyoctanate from the

lipopolysaccharide of _Neisseria oblongata_ (19) and the polysaccharide from the lipopolysaccharide of _Pseudomonas alcaligenes_ (20) have been studied using gas chromatography. Similarly the fatty acid content of cell wall lipid from _Salmonella enteritidis_ has recently been studied (21). The polysaccharide content of the cell walls of _Planococcus_ species and _Staphylococcus_ species have been shown to be very similar (22). Finally the effect of the temperature of incubation on cell chemistry has been studied for _Bacillus coagulans_ (23) a thermophilic species.

The bacterial cell wall is a very strong, yet flexible structure which can protect the cell from quite extreme conditions. If the cell needs to be disrupted to investigate its internal structure or that of its wall then vigorous measures need to be taken. There have been a number of comprehensive studies of the available techniques (6, 24, 25), thus only a brief description of the methods used will be included here. The methods employed and their complexity will depend on the investigations to be undertaken. If for example it is desired to study the different fractions of the cell wall then a number of procedures will be involved (5, 24, 26) or a specific procedure may be required if only one component is to be

examined (27). For convenience, the methods may be grouped into those using physical techniques, those using enzymes or antibiotics and thirdly, those using chemicals.

A number of workers have used fairly rigorous physical techniques to disrupt bacterial cells, e.g. the use of crude physical stress typified by grinding the bacteria in a paste. However more effective techniques have been developed. The ability of sonic and ultrasonic waves to disrupt bacterial structures, including cell walls, has been utilised (28). Pressure cell disintegration and decompression disintegration techniques use a rise or fall in pressure to disrupt the organism. The combination of low temperature and raised pressure in cold shear methods have been used widely in the past (22, 29). Often the method developed for cell wall disruption is a combination of physical and chemical methods and may include drying the organism bringing about changes in osmotic pressures.

Araki used the proteolytic enzyme trypsin in combination with lysozyme to break down the peptidoglycan of Bacillus cereus (28). It is also possible to use antibiotics to disrupt cells. The most effective of these are the penicillins. These kill bacteria by interfering with cell wall synthesis. Penicillin prevents the formation of

peptidoglycan by inhibition of the transpeptidase that links the peptidoglycan strands via the D-alanine chains.

A widely used technique involves the heating of bacteria for a period of time in the presence of a chemical, usually an acid. The exact details of the chemical extraction of bacteria vary from worker to worker. Knox used relatively mild acid hydrolysis when studying the polysaccharide and mucopeptide of Lactobacillus casei , 0.05mol/L H_2SO_4 at 60°C for 3 hours (13). Alternatively Johnsen (27) used a variety of conditions including 6mol/L HCl at 105°C for 18 hours. The choice of conditions used appears to depend to some extent on personal preference and on the nature of the cell under investigation. Acid hydrolysis of bacteria has been widely used by many workers some of which are referred to here (8, 9, 10, 12, 13, 14, 17, 18, 23, 27, 28, 30, 31, 32, 33). Hydrochloric acid at concentrations between 0.1 and 6mol/L has been used while some workers have used sulphuric acid, trichloroacetic acid and unusually hydrogen fluoride. The temperatures used vary between 4°C to 105°C while times of extraction range from 2 hours to 48 hours. The diversity of conditions used makes the evaluation of the most effective technique very difficult (see Table 1). In addition, both Johnsen (27) and Ohno (12) used sodium hydroxide when studying peptidoglycan.

ACID CONCENTRATION	TEMPERATURE	DURATION (hours)	REFERENCE
0.2mol/L H_2SO_4	60°C	3	13
2mol/L H_2SO_4	100°C	5	18
0.1mol/L HCl	60°C	24	28
0.1mol/L HCl	100°C	2	27
0.1mol/L HCl	100°C	6	9
0.1mol/L HCl	100°C	48	32
0.5mol/L HCl	100°C	8-12	17
2mol/L HCl	85°C	18	33
2mol/L HCl	100°C	2	9
3mol/L HCl	100°C	3	27
4mol/L HCl	100°C	16	17
6mol/L HCl	100°C	4-24	23
6mol/L HCl	100°C	15	31
6mol/L HCl	105°C	2	14
6mol/L HCl	105°C	18	27

Table 1. Conditions of acid hydrolysis used by a number of workers.

In trying to develop a method for the breakdown of bacterial cells for investigation there is certainly no shortage of previous work. However the technique finally chosen may well be arrived at by trial and error rather than by development of scientific theory.

ii The Theory of Gas Liquid Chromatography

James and Martin in 1952 (64) described a technique called gas liquid partition chromatography which has over the years been developed and is now termed gas liquid chromatography. Initially they used the method to separate out a mixture of fatty acids. In its simplest form the major interest in gas liquid chromatography is its use as a separation technique. The basis of all chromatographic methods is the distribution of the sample between two phases. The movement of one of these phases, the mobile phase, in relation to the other, the stationary phase, will also result in the movement of the sample. This movement will be less than that of the mobile phase and it is this difference which leads to the retention of the sample relative to the solvent it is dissolved in. The sample also spreads out as a consequence of this movement to give a gaussian distribution of concentration. The ability to

differentiate or resolve two or more compounds depends on the separation of the peaks produced and the width of each peak, this is described as the efficiency of the column.

In gas liquid chromatography the mobile phase is a gas whilst the stationary phase is a liquid. The retention times of a sample can be altered by changing a number of factors. The liquid phase is coated onto a support material which is packed into a column. The alteration of either of these may give rise to changes in retention times. Increasing the temperature of the column will result in a faster elution of the sample. Alteration of the gas phase and the gas velocity will also result in changes in retention times. The distribution coefficients of two compounds must be different under the analysis conditions for separation to occur.

$$\text{Distribution coefficient} = \frac{\text{Conc sample in liquid phase}}{\text{Conc sample in gas phase}}$$

The rate of progress of a sample through a column is dependent on the type of liquid phase used. The retention time of a compound in a column will depend on its

solubility in the liquid phase which will directly affect the distribution coefficient. Optimum separation is obtained with good solubility, this usually requires the use of a stationary phase of a similar nature to the compounds being separated. In general this means using polar liquid phases, e.g. substituted silicones, to separate polar compounds while using non-polar liquid phases, silicone ethers, for the separation of non polar compounds. If the temperature of the column is raised the equilibrium between liquid and gas phases will move towards the mobile phase. The distribution coefficient will decrease and samples will be eluted more rapidly.

The elution of a sample as a narrow peak allows the separation of a number of samples in a short time. The narrower the peaks, the larger the number of samples that can be separated in a given time and hence the greater the efficiency of the column. The efficiency of a column can be calculated and expressed by the Van Deemter equation.

$$H = A + \frac{B}{u} + Cu$$

H = Efficiency : A = Eddy diffusion : u = Gas velocity :

$\frac{B}{u}$ = Longitudinal diffusion : Cu= Resistance to mass transfer

A uniform gas flow through a column is dependent on the particle size of support material that the liquid phase is coated onto and how tightly this is packed into the column.

$$A = 2 R d_p$$

d_p = average particle size : R = packing factor

The sharpness of a peak will depend on the longitudinal diffusion of the sample within the gas phase during analysis. This is affected by the choice of the carrier gas.

$$\frac{B}{u} = \frac{2 L D_{gas}}{u}$$

D_{gas} = solute diffusivity in gas

L = tortuosity correction

Resistance to mass transfer will affect the efficiency of the column and is affected by a variety of factors.

$$Cu = \frac{8}{\pi^2} \times \frac{k'}{(1+k')^2} \times \frac{df^2}{D_{liq}} \times u + \frac{vdp^2u}{D_{gas}}$$

$$k' = \frac{\text{proportion of solute in liquid phase}}{\text{proportion of solute in gas phase}}$$

df = thickness of liquid film

D_{liq} = diffusivity of solute in liquid phase

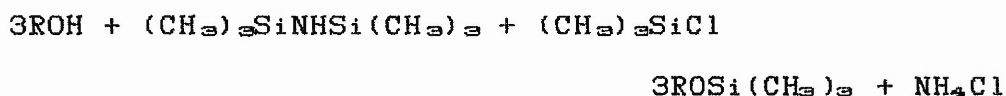
v = constant

A variety of factors can greatly affect the efficiency and resolution of an analysis by gas liquid chromatography. The selection of these can greatly affect the quality and speed of the results obtained.

Gas liquid chromatography can only be used for the separation of volatile compounds. There are many compounds unsuitable for analysis by gas liquid chromatography

because of their lack of volatility. These include carbohydrates, carboxylic acids, amines and amimo acids all of which it is often useful to assay. It is possible to derivatise these compounds to form volatile products. These can then be analysed by gas liquid chromatography satisfactorily.

A very popular way of increasing a compound's volatility is by silylation. This is the tranfer of a methylsilyl group to the compound to be examined to increase its volatility and stability. The simplest reagent of this type to use is a mixture called TRI-SIL (Pierce Chemicals) which is trimethylchlorosilane and hexamethyldisilazane in dry pyridine. This can be used to derivitise sugars, alcohols, phenols and some amines e.g.



$$3ROH + (CH_3)_3SiNHSi(CH_3)_3 + (CH_3)_3SiCl \qquad 3ROSi(CH_3)_3 + NH_4Cl$$

<u>Key</u>

ROH = carbohydrate (nonvolatile)

$(CH_3)_3SiNHSi(CH_3)_3$ = hexamethyldisilazane

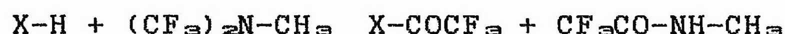
$(CH_3)_3SiCl$ = trimethylchlorosilane

$ROSi(CH_3)_3$ = trimethylsilyl carbohydrate (volatile)

NH_4Cl = ammonium chloride

There are variety of silylation reagents all of which have a methylsilyl group in one form or another. One of the newest is N-Methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide, M.T.B.S.T.F.A., which donates the tert - butyldimethylsilyl moiety to confer volatility.

Acylation agents are an alternative group of derivatisation reagents. One of these is N-Methyl-bis(trifluoroacetamide), M.B.T.F.A., which trifluoroacetylates a variety of compounds including sugars and amines. The products of reaction are very volatile.



$$X\text{-}H + (CF_3)_2N\text{-}CH_3 \quad X\text{-}COCF_3 + CF_3CO\text{-}NH\text{-}CH_3$$

Key

$X\text{-}H$ = amine or sugar

$(CF_3)_2N\text{-}CH_3$ = M.B.T.F.A.

$X\text{-}COCF_3$ = trifluoroacetylated compound

$CF_3CO\text{-}NH\text{-}CH_3$ =

N - methyltrifluoroacetamide (volatile byproduct)

Fluorinated anhydrides e.g. heptafluorbutyric acid anhydride, and fluorinated acylimidazoles e.g. heptafluoro -butyrylimidazole, derivitise in a similar way. The

advantage of all the acylating agents is that they fluorinate the derivatised compound enabling the use of both Flame Ionisation and Electron Capture Detectors. However the production of volatile by-products can cause problems on analysis.

iii Use of Gas Liquid Chromatography in Microbiology

The use of gas chromatography in microbiology has been widespread for a number of years. An attempt will be made here to discuss the areas in which it has been most widely used. As described gas liquid chromatography is a separation technique, as are all chromatographic methods, hence its use in microbiology largely involves the detection of a compound or group of compounds from the microbe.

Over the years gas chromatography has been widely used for the identification of bacteria and also in the classification of bacteria. Almost certainly its most widespread use has been in the identification of anaerobic bacteria. This relies on the ability of these organisms to produce fatty acids and alcohols when grown in culture media. The most important of these are the volatile fatty

acids: acetic through to caproic. These are produced to a greater or lesser extent by all the anaerobes and are very easily detected by gas chromatography following a simple ether extraction. The patterns of volatile fatty acid production can be very helpful in identifying individual or groups of anaerobic bacteria. In addition the detection of the non volatile fatty acids, lactic through to succinic, following methylation, is of great value in the identification of the non-sporing anaerobic gram positive bacilli. The detection of the alcohols is of less value. This subject has been comprehensively covered in many publications. One of the most useful is the laboratory manual of The Virginia Polytechnic by Holdeman and Moore (34).

As long ago as 1964 Yamakawa and Ueta (30) used gas chromatography to try and characterise the Neisseriaceae. Using methanolysis they examined various Neisseria species for fatty acid and neutral monosaccharides. They hoped that these would prove characteristic for various Neisseria species. Their conclusions were that this could be used to characterise not only the genus but also the species within the genus. They did not however examine any other group of organisms. Later work in the 1970s by Jantzen et al (33) seems to confirm their findings. They

claimed that the grouping of the Neisseraceae strains, by gas chromatography of the cell walls, precisely reflected the classification established by genetic associations. They claim a correlation between lipid composition and genetic compatibility and also a group specific heptose. Their conclusions are that the gas chromatographic study of Neisseriaceae provides information of considerable value in classification and identification of bacteria.

A study of the polysaccharide from the cell wall of an actinomycete (Micromonospora species) was carried out by Tabaud-Therisod et al (8). They used gas chromatography in conjunction with mass spectrometry to identify the carbohydrates in the cell wall. On acid hydrolysis the cell walls yielded mannose, xylose, glucose, galactose, arabinose, glucosamine, phosphoric acid and glycerol. In addition for the first time they demonstrated galactitol although previous work had shown the presence of other polyols such as ribitol and glycerol.

When Legionella pneumophila was first isolated in 1976 by McDade and others (35) the nature of the organism was uncertain. A number of workers used gas chromatography in an attempt to elucidate the nature of the cell wall of the bacterium. This work has been instrumental in recognising

the true bacterial nature of the organism and the classification of this group of previously unrecognised bacteria. Many workers have been involved in this important investigation, far too great a number to be included here. However early work carried out in Atlanta using gas chromatography deserves a mention. In 1977 Moss and others used gas chromatography to investigate the fatty acid composition of the Legionnaires disease bacterium (36), very similar to the work Jantzen was doing with the Neisseriaceae as previously described. Two years later the demonstration of diaminopimelic acid in the cell wall of _Legionella pneumophila_ (37) provided additional evidence that the organism was a bacterium.

The use of gas chromatography to investigate a new group of organisms such as the Legionellae may be expected, however it has been widely used on much better known groups of organisms. Typical of this is the work performed by Brooks and others to differentiate between _Clostridium sordellii_ and _Clostridium bifermentans_ (38). Previously these organisms had always been very difficult to separate by conventional identification techniques. These workers demonstrated that the two organisms produced different profiles of amine production by using gas chromatography. Work by Fugate et al (39) investigated the rapid

recognition of *Clostridium botulinum* by gas chromatography of methyl esters of extractable fatty acids. Farshtchi and Moss examined trimethylsilyl derivatives of whole cell hydrolysates (40). They claimed to be able to differentiate between *Clostridium sporogenes*, *Clostridium perfringens*, *Listeria monocytogenes* and *Neisseria meningitidis* by comparing profiles.

This is not a complete account of the use of gas chromatography in the identification and classification of bacteria but gives a brief idea of its use. An equally important area of use for gas chromatography in microbiology is its use for the rapid detection of bacterial disease.

One of the first and most widely accepted uses of gas chromatography was in the rapid detection of anaerobic infections. This procedure relies on the detection of volatile acids in specimens, particularly pus samples, and depends on the ability of anaerobic organisms to produce volatile fatty acids as by-products of metabolism. The use of these in identification of anaerobes has previously been discussed. A number of workers have used this technique (41, 42) for a prompt diagnosis of anaerobic infections allowing the instigation of appropriate chemotherapy

without delay.

Tuberculous meningitis is a serious life threatening condition. Although it may demonstrate typical clinical signs it is often difficult to confirm the diagnosis in the laboratory. If the sample of cerebrospinal fluid (C.S.F.) collected from a patient with suspected tuberculous meningitis is negative on direct microscopy, a not uncommon occurence, then it may be a number of weeks before a culture result is obtained, if at all. Consequently there has been some interest over the last ten years in using gas chromatography to rapidly diagnose tuberculous meningitis. In 1977 Brooks and others (43) tentatively described a compound called 3-(2'-ketohexyl)indoline in the C.S.F. of patients suffering from tuberculous meningitis using gas chromatography and mass spectrometry. In the same year Craven et al (44) suggested that this could be used in the rapid diagnosis of lymphocytic meningitis. Reports by two separate groups of workers in July 1987 in separate journals (45, 46) both suggest that the detection of tuberculostearic acid, 10-methyloctadecanoate, in the C.S.F. of patients with tuberculous meningitis could be very useful in early diagnosis. In addition Brooks and others (47) used gas chromatography in conjunction with culture for rapid differentiation of the major causative

agents of bacterial meningitis. This relied on the derivatisation of spent culture media and comparison of the gas chromatographic profiles obtained. Brooks et al (48) have also used gas chromatography to differentiate Rocky Mountain Spotted Fever from meningococcal meningitis and some viral illnesses by looking at profiles of serum samples. Other workers in the United States (49) examined C.S.F. for fatty acids and for sugars. They found that the pattern of fatty acids and sugars differed from normal according to the cause of the meningitis. They examined experimental meningitis in dogs caused by _Streptococcus pneumoniae_, _Haemophilus influenzae_, _Staphylococcus aureus_ and _Neisseria meningitidis_, and human cases of _H. influenzae_ and _Strep. pneumoniae_ meningitis. They claimed to be able to differentiate between the different causes of meningitis. In this country an attempt at rapid diagnosis of meningitis (50) by looking for a bacterial by-product in the C.S.F., lactic acid, at first appeared promising. Further studies revealed many false positive results due to lactic acid production by red and white blood cells in the C.S.F.

The concept of gas chromatographic profiles used by Brooks and others to investigate meningitis has also been used on other samples. The same group of workers have looked at

profiles of infective and noninfective arthritis (51) and also at pleural effusions (52). With synovial fluids they demonstrated different profiles from patients with streptococcal, staphylococcal and gonococcal arthritis. In addition these were different from those obtained from post trauma and normal fluids. Using similar techniques they were able to differentiate between pleural effusions from patients with congestive heart failure, tuberculosis and some other types of bacterial empyemas and pleural effusions.

Early work by Mitruka and others (53) attempted to obtain "microbiological fingerprints" of metabolic products of organisms. They did not attempt to identify these compounds detected by chromatography. A few years later these workers used the same methods to detect organisms in mixed culture. (54)

A number of workers have used gas chromatography as a scientific tool for studying the actual structure of the bacterium. Previously discussed is the examination of bacterial cell walls in the Neisseriaceae (30, 33), actinomycetes (8) and legionellae (36, 37). In the early 1970s gas chromatography and mass spectrometry were used in conjunction to characterise the capsular polysaccharide of

klebsiellae (55). From this work it was shown that the type 9 capsular polysaccharide was constructed of pentasaccharide repeating units. Later workers examined the surface polysaccharide antigen of Eubacterium saburreum (32). Polysaccharide C of Staphylococcus epidermidis, a wall associated teichoic acid, has been studied by gas chromatography (27). Again gas chromatography was used with mass spectrometry or alone to study the structure of bacterial endotoxins. Davis et al (56) studied the polysaccharide portion of the lipopolysaccharide of Salmonella species, Escherichia coli and Proteus mirabilis. Later workers also utilised mass spectrometry to examine the lipopolysaccharide of Pseudomonas species (20).

Gas chromatography has been used in other areas of microbiology apart from the investigation of bacterial disease. In recent years many workers have tried to use gas chromatography to diagnose systemic fungal infections. These can be very difficult to diagnose reliably using culture techniques hence the search for a rapid and reliable method. Early work relied on comparing gas chromatography profiles of serum (57). Subsequently, more specific attempts have been made at finding a compound in the serum indicative of fungal infection, for example a

number of workers have measured the level of mannose or arabinitol in serum as an indicator of systemic infection (58, 59, 60, 61, 62, 63).

The work described here is an attempt to apply gas liquid chromatography to the detection of the amino sugars from bacterial cell walls in the body fluids of patients. If this proved to be possible the resultant rapid diagnosis of serious bacterial infections would be of great clinical value. In addition if these levels could be used to monitor the success of treatment it may be possible to shorten the course of antimicrobial treatment thus reducing costs and the possibility of side effects.

The research was carried out in three areas: (1) investigation of the amino sugars and their extraction from bacteria, (2) an assessment of the derivatisation reagents most suitable and (3) the development of the optimum gas chromatography conditions. Initial work involved the development of the optimum gas chromatography together with the most suitable derivatisation procedure using N acetyl glucosamine , this being considerably cheaper than N acetyl muramic acid. This method was then used to examine negative clinical samples, these same samples with amino sugar added and bacterial extracts.

MATERIALS AND METHODS

1 SAMPLES

i Cultures and Extracts

The bacteria used were fresh clinical isolates of *Staphylococcus aureus* , *Streptococcus pyogenes* and *Streptococcus pneumoniae* . These were grown overnight at 37°C in 15mL of Brain Heart Infusion broth (Oxoid Ltd.) and then centrifuged at 2000 r.p.m. for 30 minutes. The deposits of these cultures were collected and treated by one of the following methods. 5 mL of 6mol/L hydrochloric acid (BDH Ltd.) was added to a deposit and the mixture heated at 105°C for 2 hours. Alternatively 2mol/L hydrochloric acid was used and heated for 2 hours at 105°C. Alkali hydrolysis was performed on the extracts using 1mol/L sodium hydroxide (BDH Ltd.) and heated for 3 hours at 100°C. *Staphylococcus aureus* was treated with the enzyme lysostaphin (Sigma Chemicals). Lysostaphin solution, 0.5mL, (1 mg/mL or 110 units/mL) was added to 0.5 mL of *Staphylococcus aureus* deposit. This mixture was then incubated at 37°C for 1 hour, and then either boiled

for 3 hours with 1mol/L sodium hydroxide or 6mol/L hydrochloric acid. After hydrolysis all extracts were freeze dried in 0.2 mL amounts, to both concentrate and dry them. They were then in a suitable state for derivatisation.

ii Clinical Samples

The cerebrospinal fluids used were collected when the patients had a myelogram. Culture performed on the samples onto, horse blood agar (Oxoid Ltd.) and chocolate agar (Oxoid Ltd.) incubated at 37°C for 48 hours in 5% carbon dioxide and trypticase soya broth (Oxoid Ltd.) incubated at 37°C for 48 hours failed to produce any bacterial growth. Aliquots, 0.2 mL, of these specimens were freeze dried. The serum sample used was sent to the laboratory for routine antenatal syphilis testing and again freeze dried in 0.2 mL amounts.

iii Reference Sugars

The sugars mannitol, sorbitol, dulcitol, glucose, arabinose, cellobiose, maltose, N-acetyl glucosamine (BDH Ltd.) and N-acetyl muramic acid (Sigma Chemicals) were reacted in their dry state in measured amounts with TRISIL.

2 DERIVATISATION TECHNIQUES

i Silylation

To a known quantity of dry chemical , freeze dried extract of bacteria or freeze dried clinical sample, in crimp cap vials (Phase Sep Ltd.), 0.5 mL of the reagent TRISIL (Pierce Chemical Company), was added. The mixture was shaken vigorously for 30 seconds then left to stand for 5 minutes at room temperature prior to injecting 1µL into the gas chromatograph using a 5µL S.G.E. syringe (65).

To 0.01 g of N-acetyl glucosamine was added 0.1 mL of acetonitrile (BDH Ltd.) and 0.1ml of N-Methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (Regis Chemical Company). The mixture was incubated at room temperature for 20 minutes before injecting 1µL into the gas chromatograph using a 5µL S.G.E. syringe (65).

ii Acylation

A known amount of N-acetyl glucosamine (BDH Ltd.), in a crimp cap vial (Phase Sep Ltd.), was dissolved in 0.1 mL of benzene. To this 0.1 mL of 0.05mol/L trimethylamine (BDH

Ltd.) in benzene (BDH Ltd.) and 0.01 mL of heptafluorobutyric acid anhydride (Pierce Chemical Company) was added. The vial was capped and heated at 50°C for 15 minutes. After cooling 1 mL of water was added and shaken for 1 minute. 1 mL of 5% aqueous ammonia (BDH Ltd.) was added and shaken for a further 5 minutes. After centrifuging 1µL of the benzene layer was injected into the gas chromatograph using a S.G.E. syringe (65).

N-Methyl-bis(trifluoroacetamide), MBTFA (Pierce Chemical Company), 0.5 mL and pyridine (BDH Ltd.), 0.5 mL, were added to dry amino sugar or freeze dried extract and incubated at 65°C for 1 hour. The reaction mixture, 1 µL, was injected onto the gas chromatograph using a S.G.E. syringe (65).

3 CHROMATOGRAPHY CONDITIONS

i Equipment

The gas chromatograph used throughout was a Packard 437A dual channel (Chrompack Ltd.) with flame ionisation detector on one channel and electron capture detector on

the other. Chromatographs were recorded using either a Packard single channel chart recorder or a Shimadzu computing integrator. For all analyses the detector temperature was 225°C and the injector temperature was 250°C. In Figure 7 a 1 metre glass column packed with 10% SE30 on Chromosorb W 80 - 100 mesh (Phasesep Ltd.) was used. All other chromatograms were performed on a 2 metre glass column packed with 10% OV11 on Chromosorb W 100 - 120 mesh (Phasesep Ltd.).

ii <u>Conditions</u>

The carrier gas used for all analyses was oxygen-free nitrogen (British Oxygen Company). The carrier gas flow was set using a bubble flow meter (Phasesep Ltd.) at three different rates: 20 mL/min, 30 mL/min and 40 mL/min. The oven was operated isothermally at both 200°C and 210°C. It was also operated using four different temperature programmed runs: (i) 75°C isothermally for 1 minute and then rising at 10°C/min to a final temperature of 250°C, (ii) the oven rising at 10°C/min from 125°C to 225°C or, (iii) from 125°C to 250°C, (iv) starting at 140°C rising at 4°C/min to a final temperature of 200°C.

RESULTS

The results are divided into two sections; method development and application. These are again further divided into subsections.

1 METHOD DEVELOPMENT

i Derivatisation Techniques

One of the simplest derivatisation techniques to use is silylation with TRISIL (Pierce Chemicals) or a similar mixture (Figure 7). The method has a number of advantages in use; the reagents are all present in the one mixture, the reaction time for most compounds is short (5 minutes), reaction conditions are easily obtained (incubation at room temperature), the product is available in small volumes which means that shelf life is not a problem once the vial is opened. The disadvantages are; it cannot be used with the electron capture detector, unwanted reaction byproducts

can be formed, it is rapidly inactivated by very small amounts of water, the smell of pyridine can be very unpleasant for fellow workers. However the simplicity of use allows the rapid production of results without a great deal of preparation.

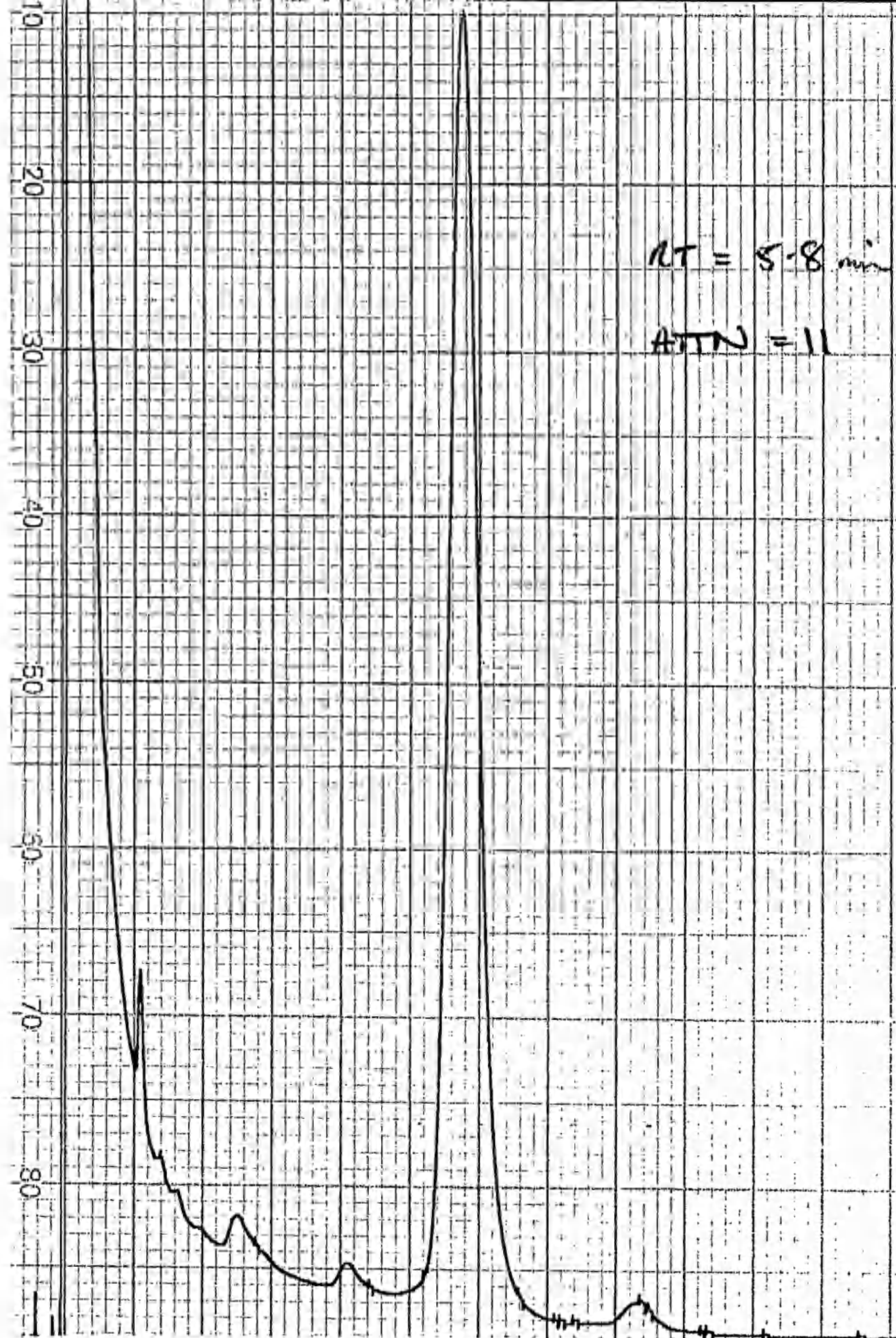


Figure 7. 0.01 g of N-acetyl glucosamine reacted with 0.5 mL of TRISIL for 5 minutes at room temperature and 1 μL injected. G.L.C. condtions: 1 metre column of SE30, oven 200°C, F.I.D. detector 225°C, injector 250oC, carrier gas nitrogen, gas flow 40 mL/min

The electron capture detector only detects halogenated compounds therefore it is necessary to use a halogenated derivatising agent when examining amino sugars by gas liquid chromatography. One such compound is heptafluorobutyric acid anhydride (Figure 8). Acylation of amines is quantitative within a few minutes and the trimethylamine used as a catalyst does not cause disturbances in the chromatogram at high sensitivity unlike pyridine. The procedure is more complex than for TRISIL involving the addition of four reagents at different stages plus an incubation and centrifugation step. The chemicals used in the reaction are particularly unpleasant to use especially trimethylamine. The analysis time is very rapid which can give rise to problems of separation. The method allowed the use of the electron capture detector however even at low sensitivity a high number of peaks additional to the amino sugar appeared.

N-methyl-N-(tert-butyldimethylsilyl)- trifluoroacetamide (M.T.B.S.T.F.A.) is an alternative reagent. This has a number of advantages similar to TRISIL because it is a silylation reagent. Addition of reagent in equal volume with acetonitrile 20 minutes incubation at room temperature prior to injection is a simple technical procedure. In

addition it is fluorinated allowing its use with an electron capture detector. However it failed to produce any peaks when reacted with an amino sugar (Figure 9).

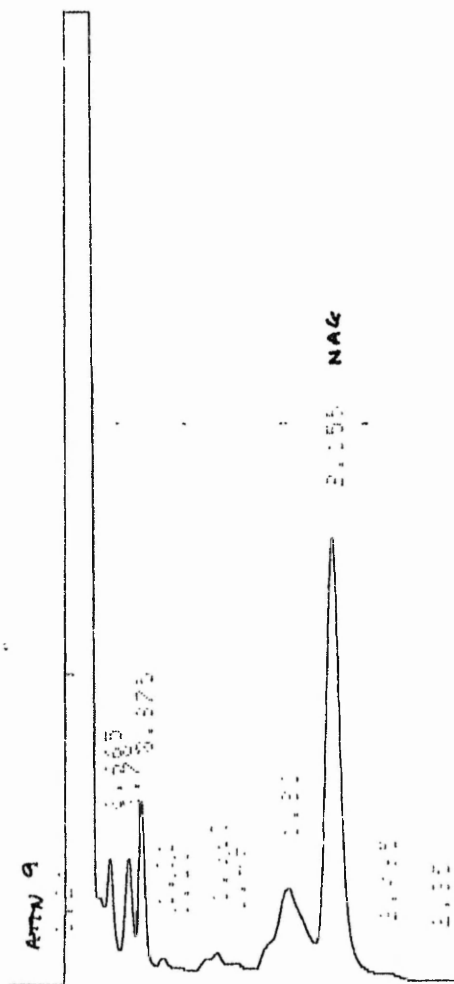


Figure 8. 0.001 g N-acetyl glucosamine dissolved in 0.5 mL benzene reacted with 0.1 mL 0.05mol/L trimethylamine in benzene and 10 uL of heptafluorobutyric acid anhydride for 15 minutes at 50°C. After the addition of water and 5% aqueous ammonia involving shaking and centrifugation 1 μL of the benzene layer was injected. G.L.C. conditions: 2 metre column of OV11, oven 210°C, E.C.D. detector 225°C, injector 250°C, carrier gas nitrogen, gas flow 20 mL/min.

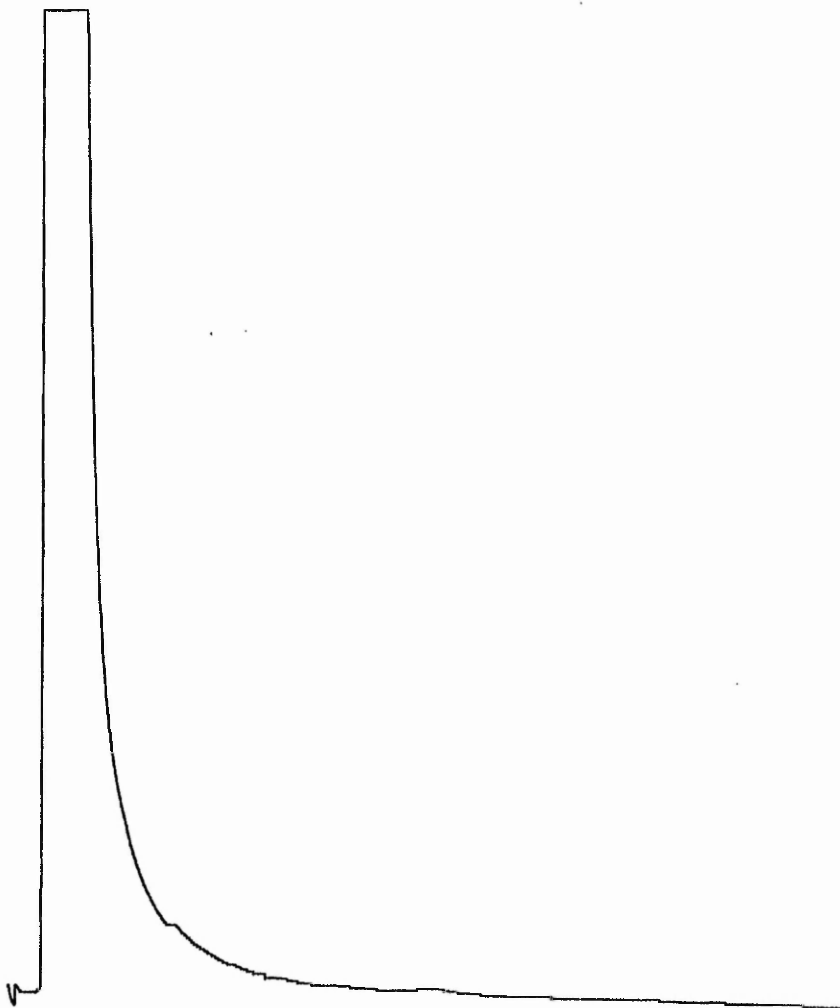


Figure 9 . 0.01 g of N-acetyl glucosamine reacted with M.T.B.S.T.F.A. for 20 minutes at room temperature and 1μL injected. G.L.C. conditions: 2 metre column of OV11, oven 200°C, E.C.D. detector 225°C, injector 250°C carrier gas nitrogen, gas flow 40 ml/min.

Trifluoroacetylation of sugars using N-methyl-bis (trifluoroacetamide) (M.B.T.F.A.) gives quantitative results with highly volatile derivatives. This is a much easier method of producing fluorinated derivatives for gas liquid chromatography with an electron capture detector than heptafluorobutyric acid anhydride (Figure 10). The preparation step of aliquoting reagent and pyridine to the sample is very simple. Following incubation for one hour at 65°C the reaction mixture can be injected directly onto the column. The technique is almost as simple to use as TRISIL although the reaction time is much longer and the smell of pyridine can still be a problem. Unlike TRISIL it can be used with flame ionisation and electron capture detectors. In addition it does not appear to give the multiple derivatives obtained with heptafluorobutyric acid anhydride.

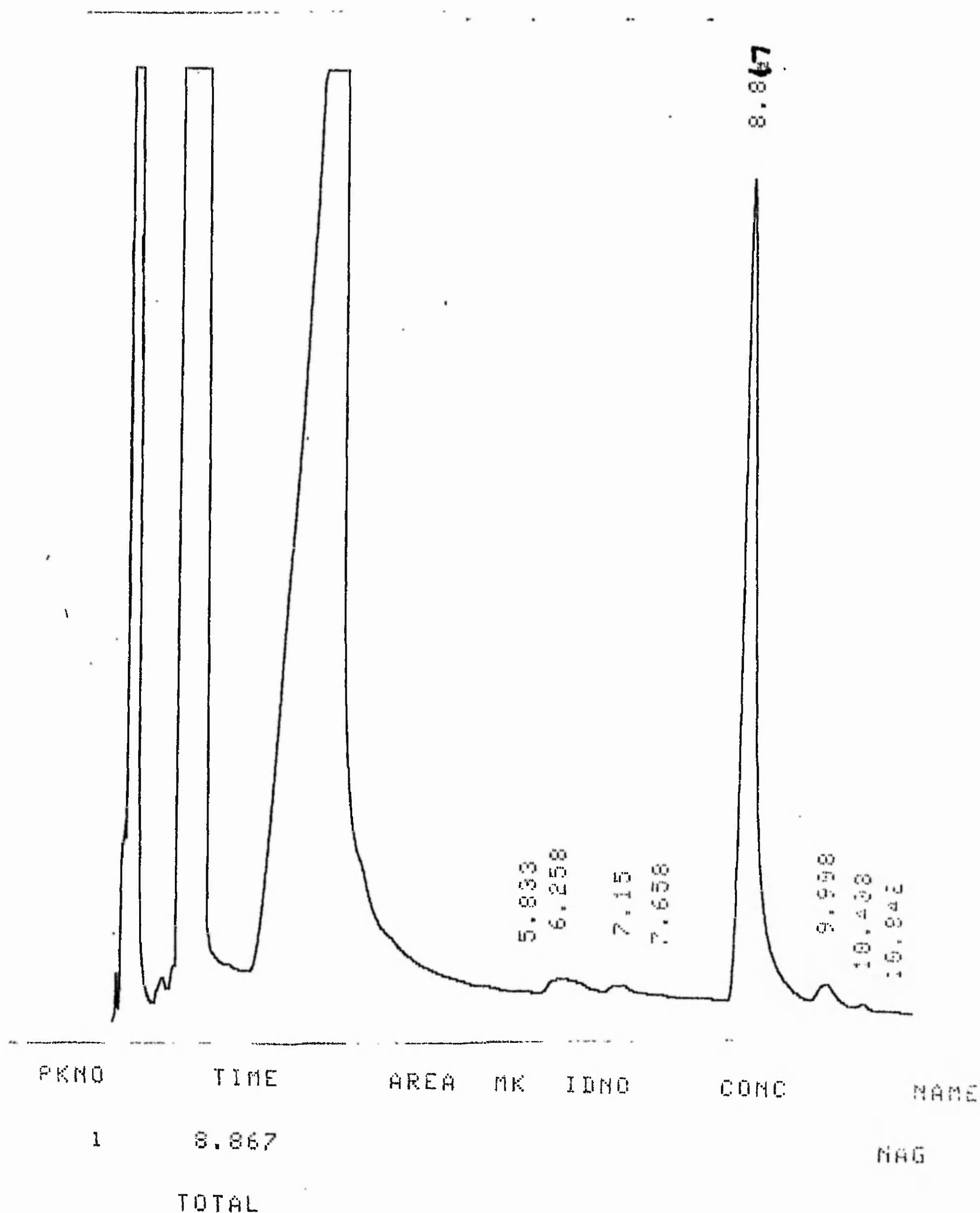


Figure 10. 0.01 g N-acetyl glucosamine reacted with 0.2 mL M.B.T.F.A. and 0.2 mL pyridine at 65° C for 1 hour and 1µL injected. G.L.C. conditions: 2 metre column of OV11, oven 75° C isothermal for 1 min and then 10° C/min rise to 250°C, E.C.D. 225°C, injector 250°C, carrier gas nitrogen, gas flow 40 ml/min.

ii Chromatography Conditions

Initially two different column lengths were compared for retention times. A one metre column filled with SE30, a non polar silicone ether, ran isothermally at 200°C with a gas flow of 40 mL/min of nitrogen gave a retention time of 5.8 minutes for N-acetyl glucosamine reacted with TRISIL (Figure 11). A two metre column of a similar stationary phase, OV11, under the same conditions gave a retention time of 9 minutes for N-acetyl glucosamine derivitised with TRISIL (Figure 12). Using the OV11 column the flow of carrier gas was halved to 20 mL/min this resulted in an increased retention time of 14.25 minutes (Figure 13). Increasing the gas flow by 20 mL/min to 60 mL/min gave a reduced retention time of 7.5 minute (Figure 14). Using the smallest gas flow at 20 mL/min of nitrogen under temperature programmed conditions starting at a lower initial temperature, 140°C rising to 200°C at 4°C/min, increased the retention time to 19.5 minutes (Figure 15). This longer retention time may be preferable when trying to separate a number of compounds. A similar retention time can be obtained on the same column with a slightly increased gas flow, 30 mL/min of nitrogen, but starting the analysis at a lower temperature. Starting isothermally at 75°C for one minute then rising at

10°C/min to a final temperature of 250° C giving a retention time of 18 minutes (Figure 16). The results above illustrate the difference in retention times possible using the same derivatisation reagent but varying the oven temperature and gas flow.

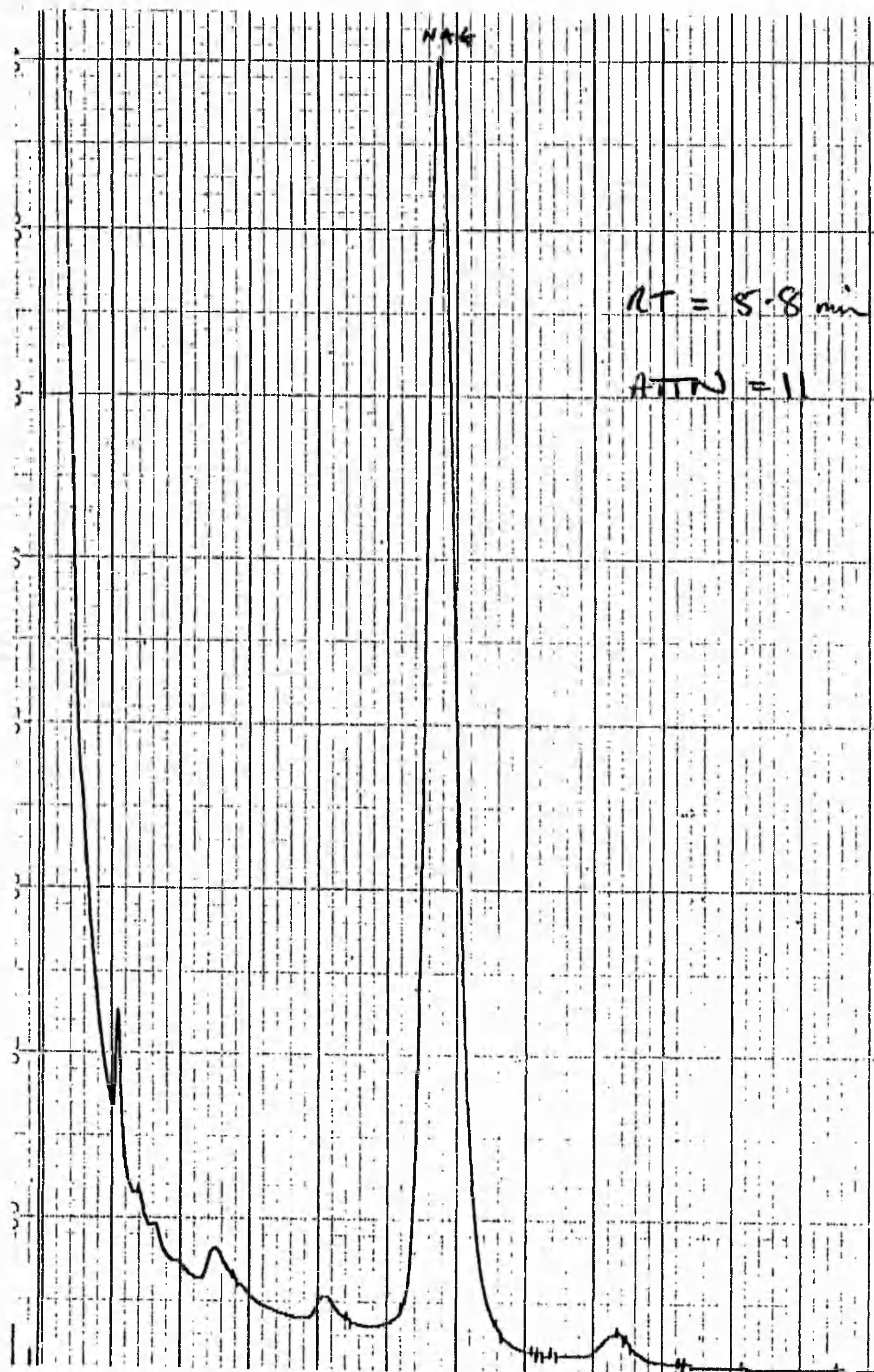


Figure 11. 0.01 g N-acetyl glucosamine reacted with TRISIL. Column: 1 metre packed with SE30. G.L.C. conditions: oven 200°C, carrier gas nitrogen 40 mL/min, detector 225°C, injector 250°C. Chart speed 60 cm/hr.

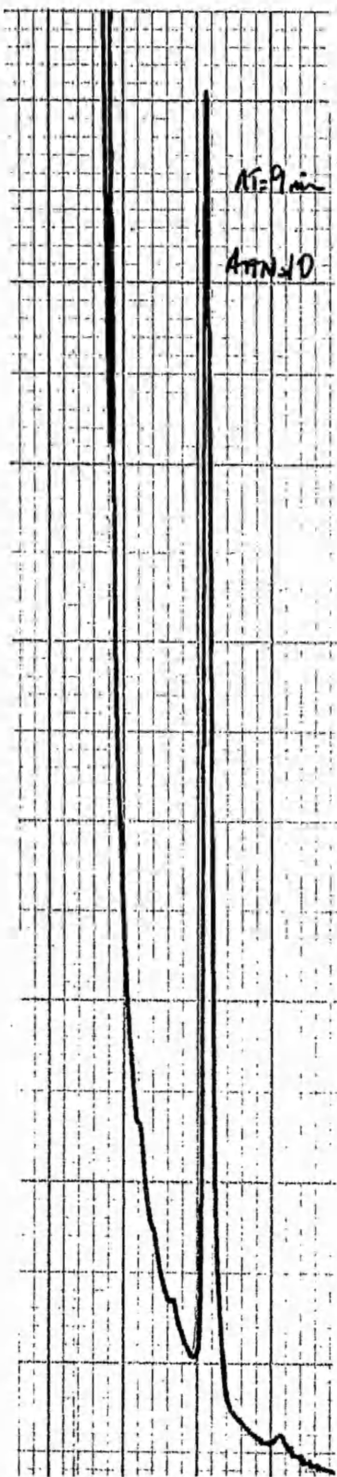


Figure 12. 0.001 g N-acetyl glucosamine reacted with TRISIL. Column 2 metres packed with OV11. G.L.C. conditions: oven 200°C, carrier gas nitrogen 40 mL/min, detector 225°C, injector 250°C. Chart speed 12 cm/hr.

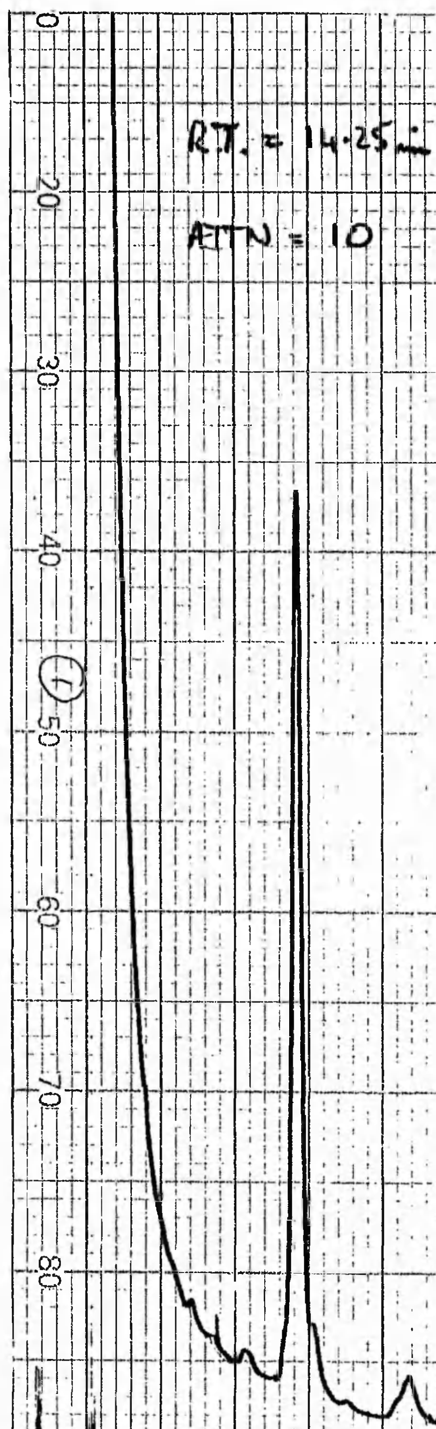


Figure 13. 0.001 g N-acetyl glucosamine reacted with TRISIL. Column 2 metres packed with OV11. G.L.C. conditions: oven 200°C, carrier gas nitrogen 20 mL/min, detector 225°C, injector 250°C. Chart speed 12 cm/hr.

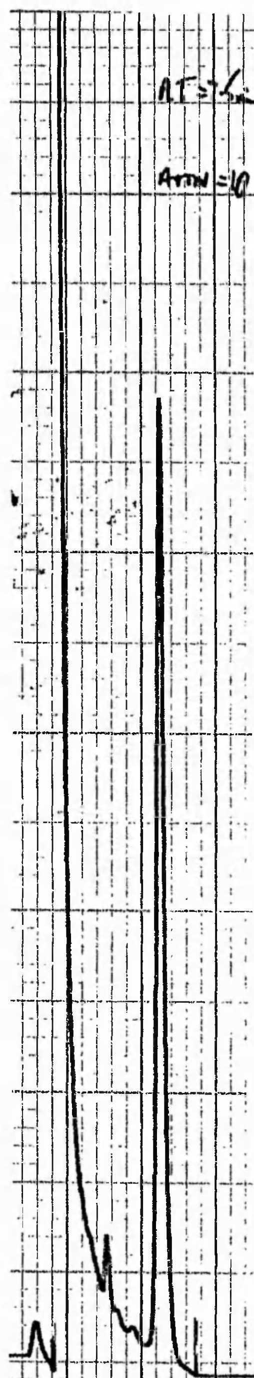


Figure 14. 0.001 g N—acetyl glucosamine reacted with TRISIL. Column 2 metres packed with OV11. G.L.C. conditions: oven 200°C, carrier gas nitrogen 60 mL/min, detector 225°C, injector 250°C. Chart speed 12 cm/hr.

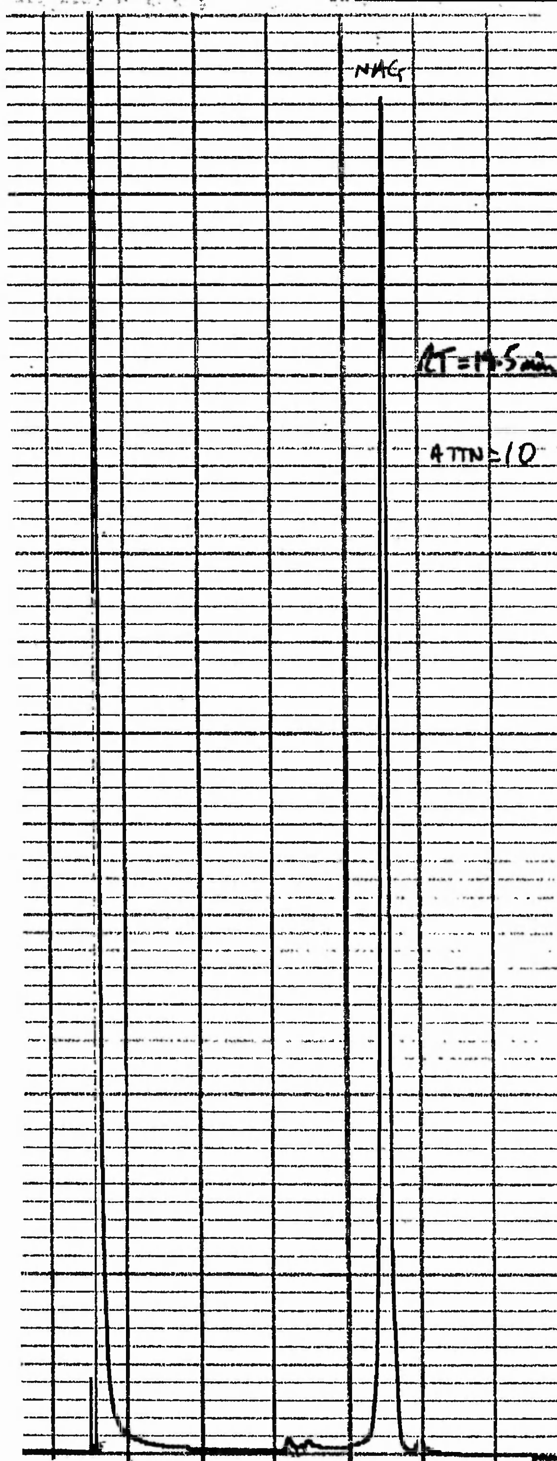
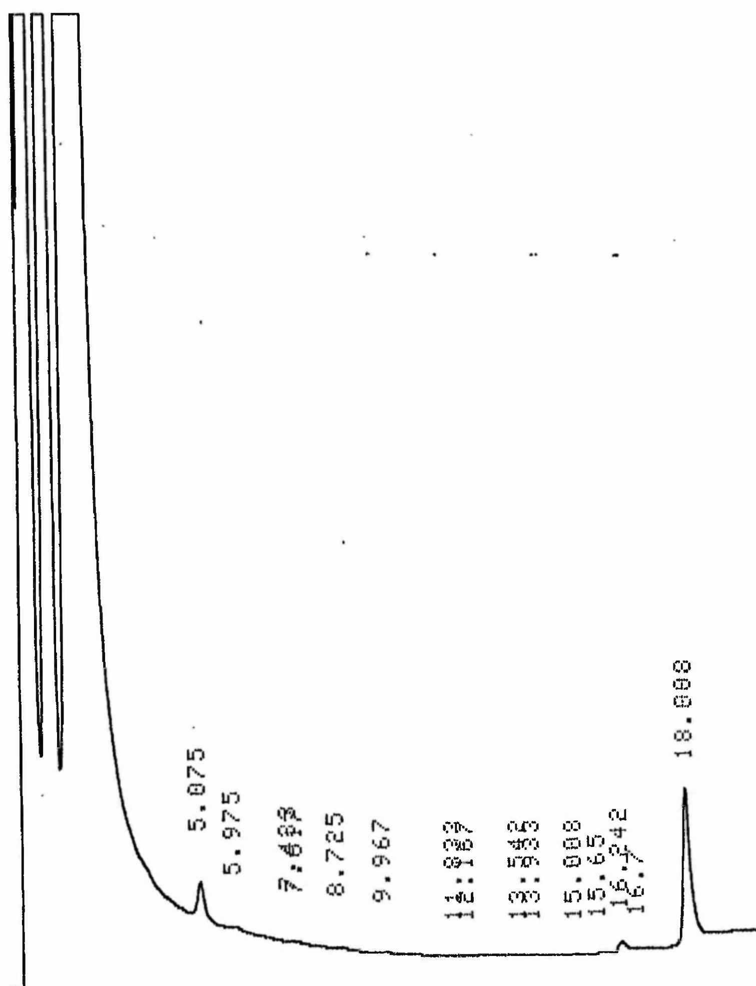


Figure 15. 0.001 g—N acetyl glucosamine reacted with TRISIL. Column 2 metres packed with OV11. G.L.C. conditions: oven 140°C to 200°C rising at 4°C/min, carrier gas nitrogen 20 mL/min, detector 225°C, injector 250°C. Chart speed 12 cm/hr.



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	18.008	508666		1	100	NAG
	TOTAL	508666			100	

Figure 16. 0.001 g N-acetyl glucosamine reacted with TRISIL. Column 2 metres packed with OV11. G.L.C. conditions: oven 75°C for 1 min then rising at 10°C/min to 250°C, carrier gas nitrogen 30 mL/min, detector 225°C, injector 250°C.

Acylation of N-acetyl glucosamine with heptafluorobutyric acid anhydride produces highly volatile derivatisation products. These compounds are much more volatile than those obtained by silylation and therefore have greatly reduced retention times. A gas flow of 20 mL/min of nitrogen and an oven temperature of 200°C on the OV11 column gives a retention time of 2.1 minutes compared with 14.5 minutes when TRISIL is used under the same conditions (Figure 17).

The trifluoroacetylation of primary and secondary amines and carbohydrates by N-methyl-bis(trifluoroacetamide) produces very volatile derivatives. A temperature programmed run from 125°C to 250°C rising at 10°C/min with a gas flow of 30 mL/min gives a retention time of 2.3 minutes for N-acetyl glucosamine (Figure 18). If the gas chromatograph conditions are altered to those in Figure 16 then a retention time of 9.1 minutes is obtained (Figure 19). This is about half of the retention time obtained with TRISIL under the same conditions.

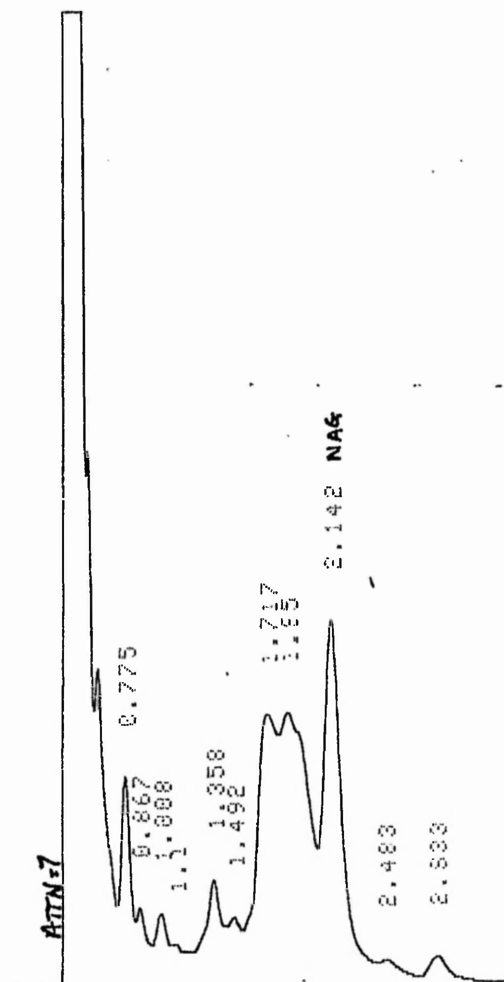
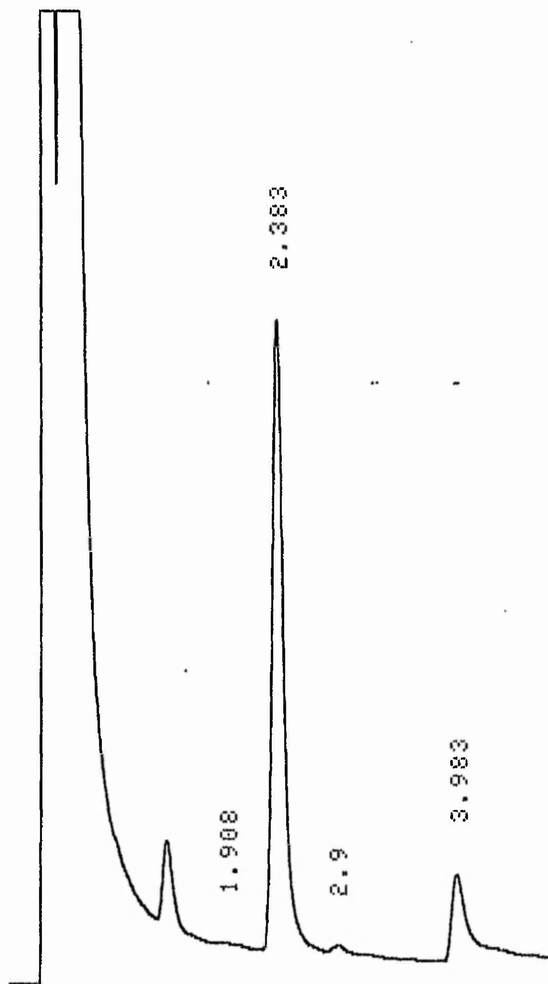
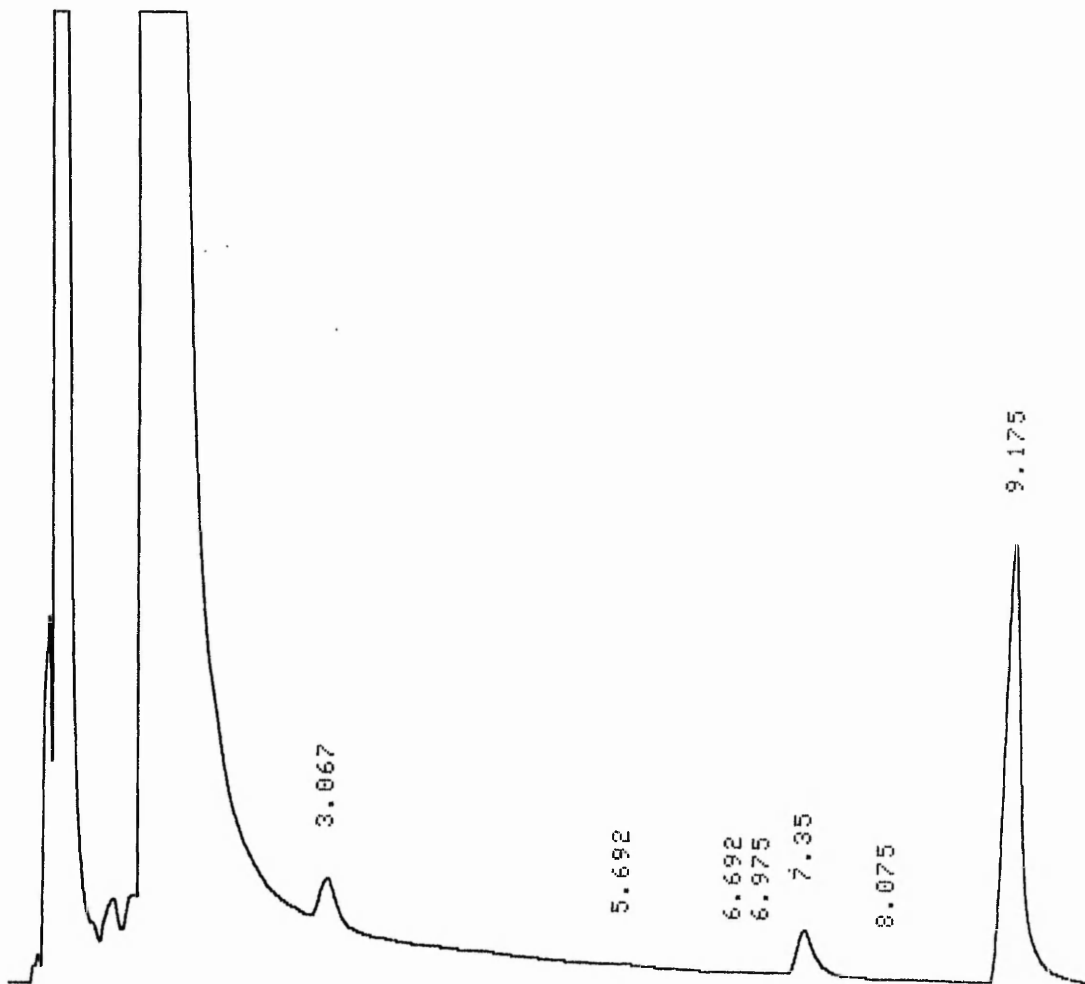


Figure 17. 0.001 g N—acetyl glucosamine reacted with heptafluorobutyric acid anhydride. Column 2 metres packed with OV11. G.L.C. conditions: oven 200°C, carrier gas nitrogen 20 mL/min, detector 225°C, injector 250°C.



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.383	2341235		1	100	NAG
	TOTAL	2341235			100	

Figure 18. 0.001 g N-acetyl glucosamine reacted with N - Methyl - bis - (trifluoroacetamide) . Column 2 metres packed with OV11. G.L.C. conditions: oven 125°C to 250°C rising at 10°C/min, carrier gas nitrogen 30 mL/min, detector 225°C, injector 250°C.



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	9.175	2421807		1	100	NAG
	TOTAL	2421807			100	

Figure 19. 0.001g N-acetyl glucosamine reacted with N - Methyl - bis - (trifluoroacetamide) . Column 2 metres packed with OV11. G.L.C. conditions: oven 75°C for 1 minute rising to 250°C at 10°C/min, carrier gas nitrogen 30 mL/min, detector 225°C, injector 250°C.

iii <u>Sensitivity</u>

The Flame Ionisation Detector is a stable easily used detector but it is not extremely sensitive. Levels of 1 g/L of N-acetyl glucosamine reacted with TRISIL are very easily detected as can be seen in Figure 20. The trace in Figure 21 shows the ability to detect a level of 0.1 g/L although problems are beginning to arise due to the peak eluting on the tail of the solvent peak. A longer retention time for N acetyl glucosamine is needed to facilitate the quantitation of the peak. At levels of 0.01g/L of N- acetyl glucosamine the lower range of sensitivity has been reached (Figure 22).

The Electron Capture Detector is more sensitive than the Flame Ionisation Detector but is more unstable and difficult to use. Acylating 1 g/L of N- acetyl glucosamine with heptafluorobutyric acid anhydride produces a reasonable peak although there is quite a lot of background noise (Figure 23). Increasing the sensitivity of the detector to examine a concentration of 0.001 g/L results in N-acetyl glucosamine peak virtually disappearing amongst the background (Figure 24).

A different acylating agent, N-methyl-bis (trifluoroacetamide), when used with the Electron Capture Detector is no better. At a concentration of 10 g/L of N-acetyl glucosamine the peak is well separated from the solvent peak (Figure 25). As the sensitivity is increased the background begins to interfere at a concentration of 1 g/L (Figure 26).

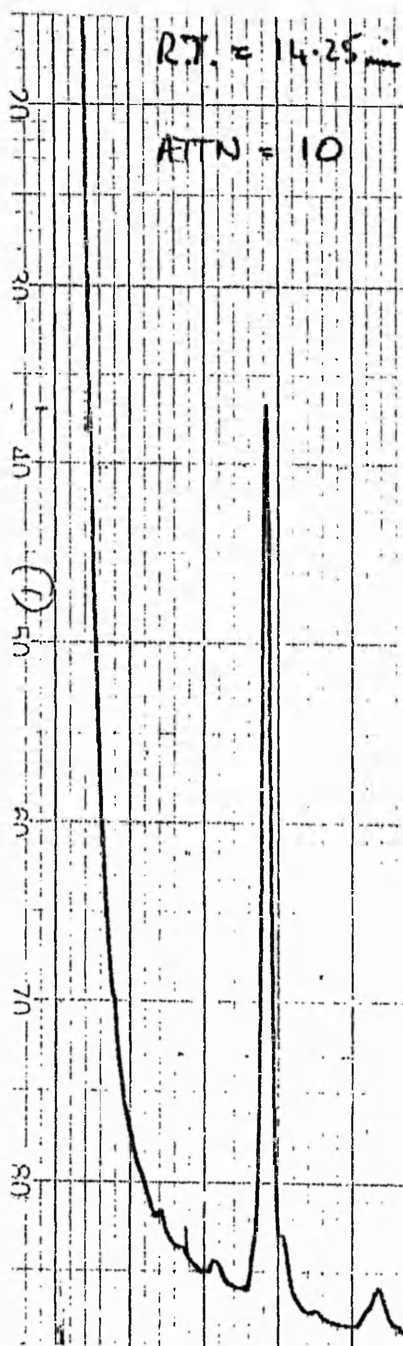


Figure 20. 1 g/L of N- acetyl glucosamine reacted with TRISIL. Column: 2 metre OV11. G.L.C. conditions: oven 200°C, carrier gas nitrogen 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C.

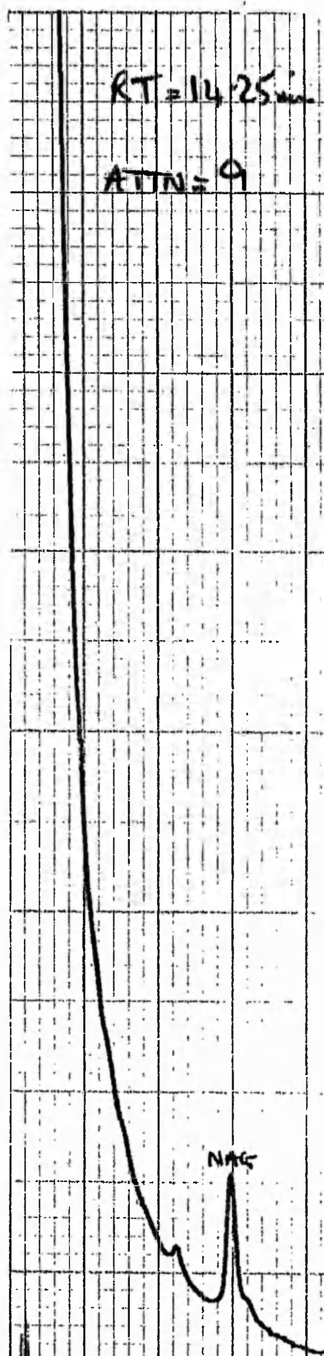


Figure 21. 0.1 g/L of N— acetyl glucosamine reacted with TRISIL. Column: 2 metre OV11. G.L.C. conditions: oven 200°C, carrier gas nitrogen 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C.

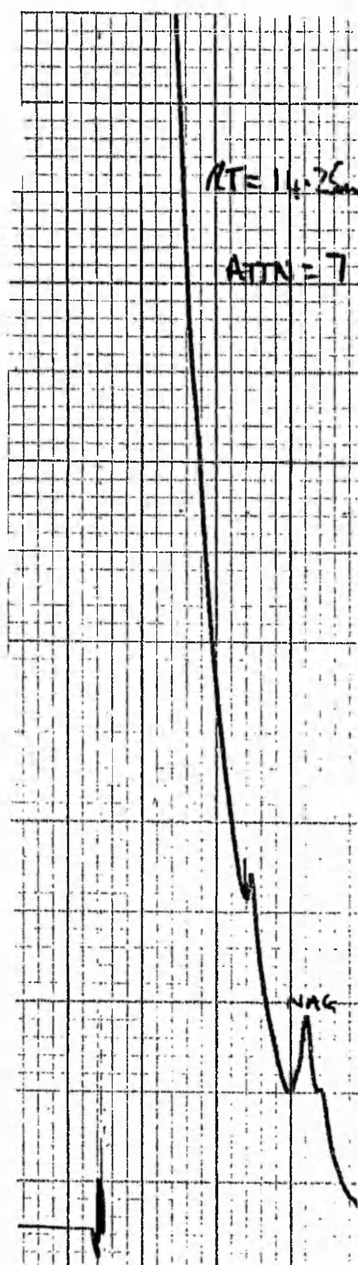


Figure 22. 0.01 g/L of N-acetyl glucosamine reacted with TRISIL. Column: 2 metre OV11. G.L.C. conditions: oven 200°C, carrier gas nitrogen 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C.

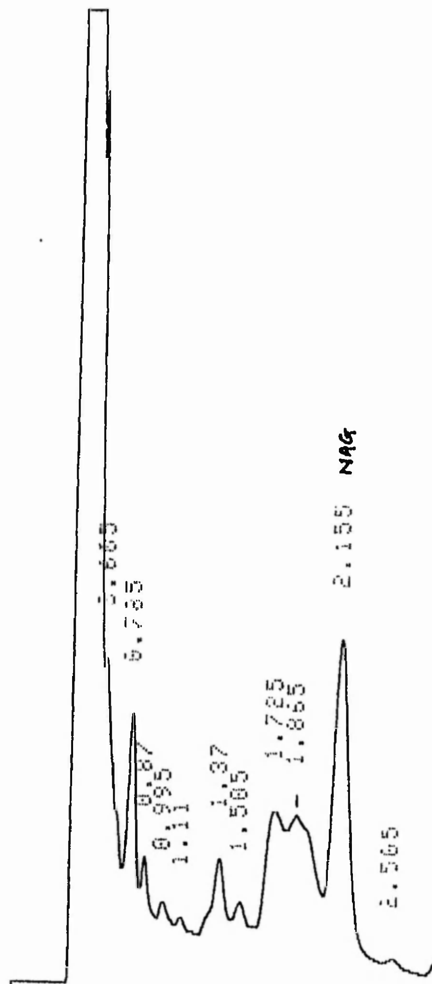


Figure 23. 1 g/L of N- acetyl glucosamine reacted with heptafluorobutyric acid anhydride. Column: 2 metre OV11. G.L.C. conditions: oven 200°C, carrier gas nitrogen 20 mL/min, Electron Capture Detector 225°C, injector 250°

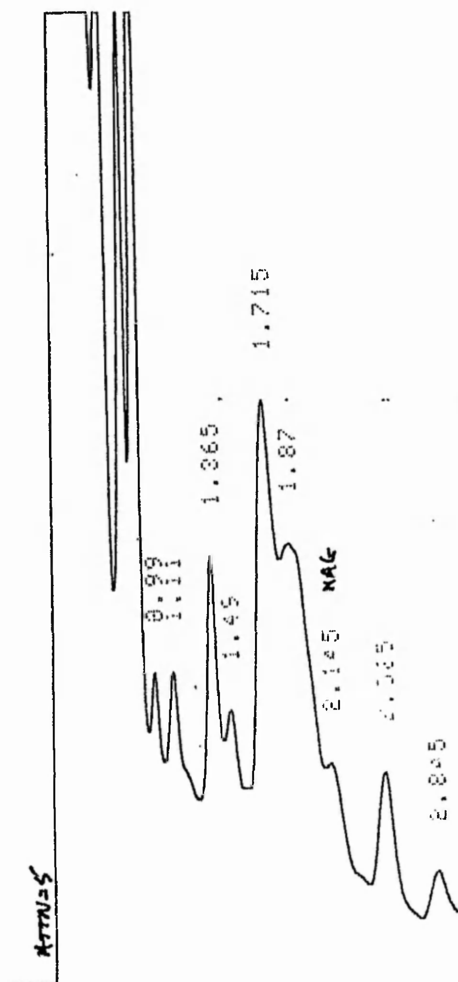
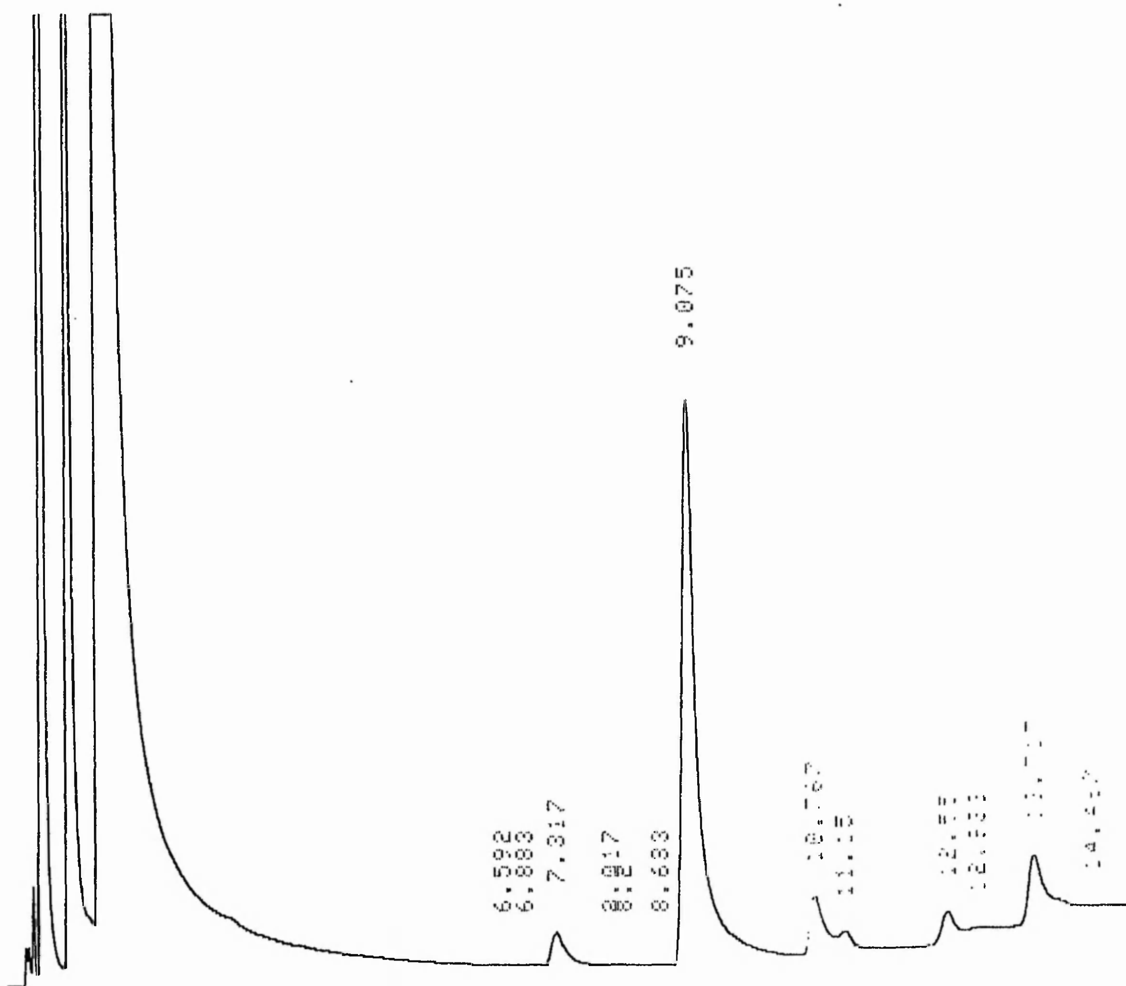
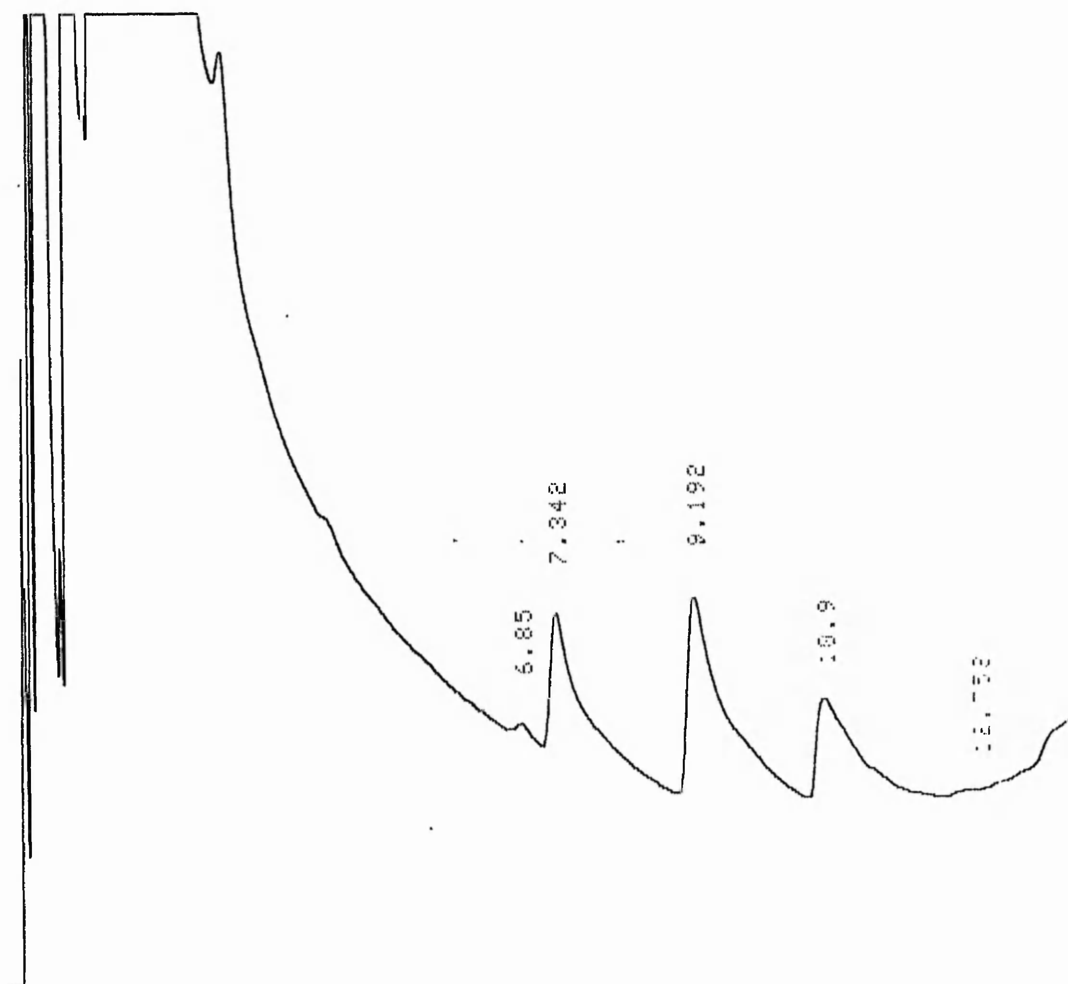


Figure 24. 0.001 g/L of N-acetyl glucosamine reacted with heptafluorobutyric acid anhydride. Column: 2 metre OV11. G.L.C. conditions: oven 200°C, carrier gas nitrogen 20 mL/min, Electron Capture Detector 225°C, injector 250°C.



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	9.075	1047822		[illegible]	100	[illegible]
	TOTAL	1047822			100	

Figure 25. 10 g/L of N-acetyl glucosamine reacted with N - methyl - bis (trifluoroacetamide). Column: 2 metre OV11. G.L.C. conditions: oven 75°C for 1 minute then rising at 10°C/min to 250°C, carrier gas nitrogen 20 mL/min, Electron Capture Detector 225°C, injector 250°C.



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	9.192	110215		1	100	NAG
	T [illegible]	110215			100	

Figure 26. 1 g/L of N-acetyl glucosamine reacted with N - methyl - bis (trifluoroacetamide). Column: 2 metre OV11. G.L.C. conditions: oven 75°C for 1 minute then rising at 10° C/min to 250°C, carrier gas nitrogen 20 mL/min, Electron Capture Detector 225°C, injector 250°C.

2 APPLICATIONS

i Bacterial Extracts

The deposit from a 15mL overnight broth culture of Staphylococcus aureus hydrolysed for two hours at 105°C with 6mol/L hydrochloric acid, freeze dried and then acylated with heptafluorobutyric acid anhydride. This was then injected onto the chromatograph, using the Electron Capture Detector, giving an extremely small peak at the retention time for N-acetyl glucosamine (Figure 27). When comparing this with the chromatograph of N-acetyl glucosamine performed subsequently it is possible that the peak in the bacterial extract is not due to N-acetyl glucosamine but could be a baseline fluctuation (Figure 28).

Acid hydrolysates of Staphylococcus aureus and Streptococcus pyogenes , 2mol/L hydrochloric acid for two hours at 105°C, were freeze dried and derivatised with TRISIL. When comparing the trace of the staphylococcal extract, Figure 29, with that for N-acetyl glucosamine, Figure 30, there is again a very small peak at the same

retention time. Similarly the streptococcal extract also demonstrates a small peak at the same point, Figure 31. The chromatographs of the organism extracts in addition contain a large number of larger unidentified peaks.

An alternative to acid hydrolysis is alkali hydrolysis with 1mol/L sodium hydroxide for three hours at 100°C. This was performed on the deposits of overnight broth cultures of *Streptococcus pyogenes* and *Streptococcus pneumoniae* . These extracts were freeze dried prior to derivatisation with N-methyl-bis(trifluoroacetamide) and examination using the Electron Capture Detector. Comparison of the *Strep. pyogenes* trace, Figure 32, and the *Strep. pneumoniae* trace, Figure 33, with the one of N-acetyl glucosamine, Figure 34, shows a double peak at 10.7 and 11. 1 minutes which is common to all three traces. Also the main N-acetyl glucosamine peak at 9 minutes occurs greatly reduced in the other two traces.

Enzymic degradation of *Staph. aureus* with lysostaphin was followed by either acid hydrolysis with 6mol/L hydrochloric acid for 3 hours at 100°C or alkali hydrolysis with 1mol/L sodium hydroxide for 3 hours at 100°C. These two extracts were freeze dried then derivitised with N-methyl

-bis(trifluoroacetamide). These were then injected into the chromatograph using the Electron Capture Detector. The N-metyhl-bis(trifluoroacetamide) derivatisation of both the acidic and alkali extract have peaks at 8.5 minutes, Figures 35 and 36, corresponding to N acetyl glucosamine Figure 37. The acidic extract was also derivitised with TRISIL for examination using the Flame Ionisation Detector Figure 38. The chromatogram of N-acetyl glucosamine, Figure 39, has major peaks at 12.1 and 15.6 minutes. One of these peaks, at 12.0, occured in Figure 38. Both peaks appear in Figure 40 where N-acetyl glucosamine is added to the acidic extract although the relative sizes appear to be altered.

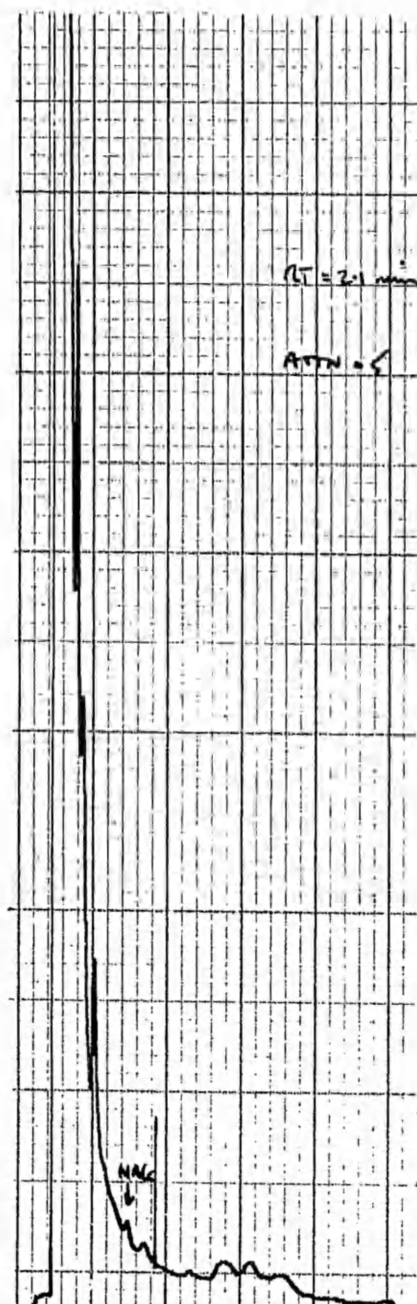


Figure 27. Acid hydrolysate of <u>Staphylococcus aureus</u> derivatised with heptafluorobutyric acid anhydride. Column: 2 metres OV11. G.L.C. conditions: oven 200°C, nitrogen carrier gas 20 mL/min, Electron Capture Detector 225°C, injector 250°C. Chart speed 30 cm/hr.

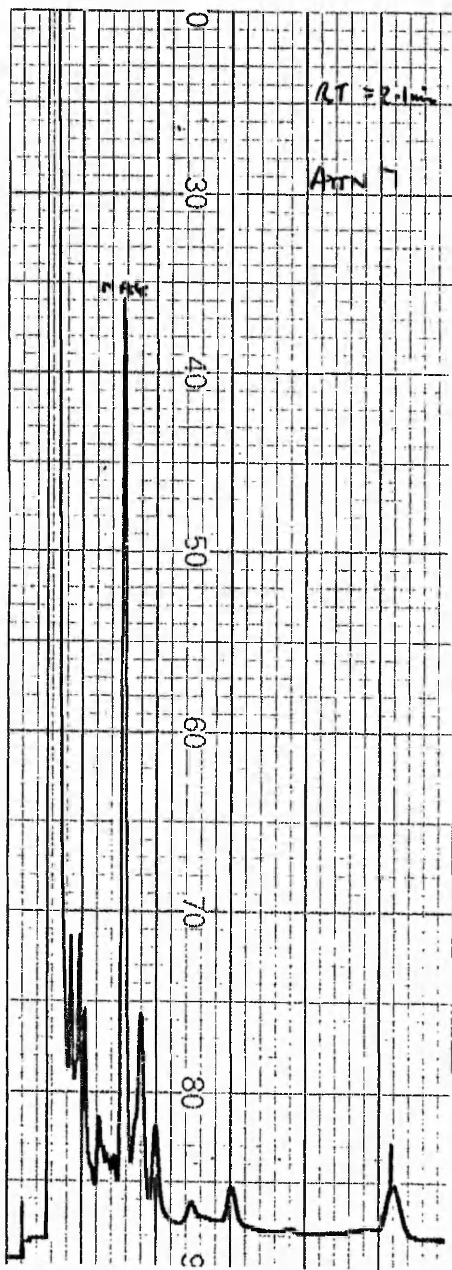


Figure 28. 1 g/L N-acetyl glucosamine derivatised with heptafluorobutyric acid anhydride. Column: 2 metres OV11. G.L.C. conditions: oven 200°C, nitrogen carrier gas 20 mL/min, Electron Capture Detector 225°C, injector 250°C. Chart speed 30 cm/hr.

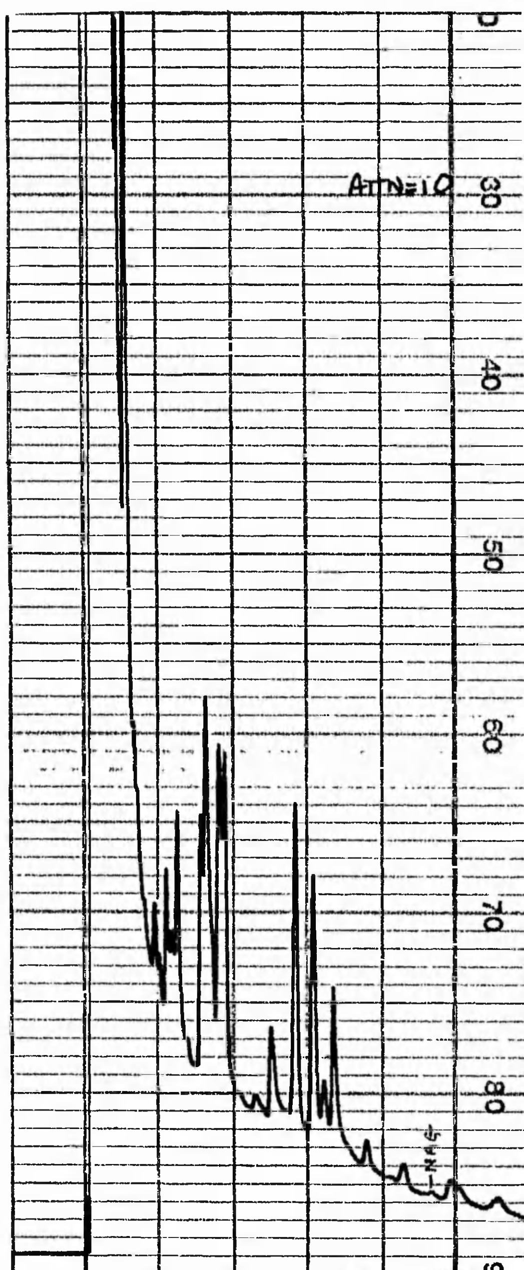


Figure 29. Acid hydrolysate of <u>Staphylococcus aureus</u> derivatised with TRISIL. Column : 2 metres OV11. G.L.C. conditions : oven rising from 140°C to 200°C at 4°C/min, nitrogen carrier gas 20 ml/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12 cm/hr.

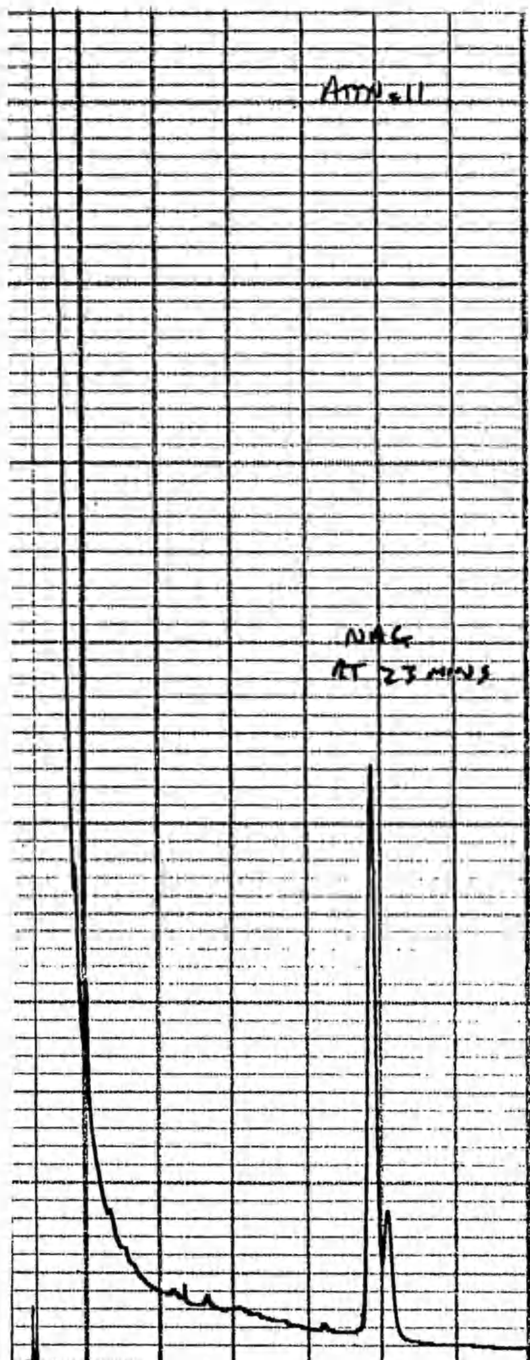


Figure 30. 1 g/L N-acetyl glucosamine derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven rising from 140°C to 200°C at 4°C/min, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12 cm/hr.

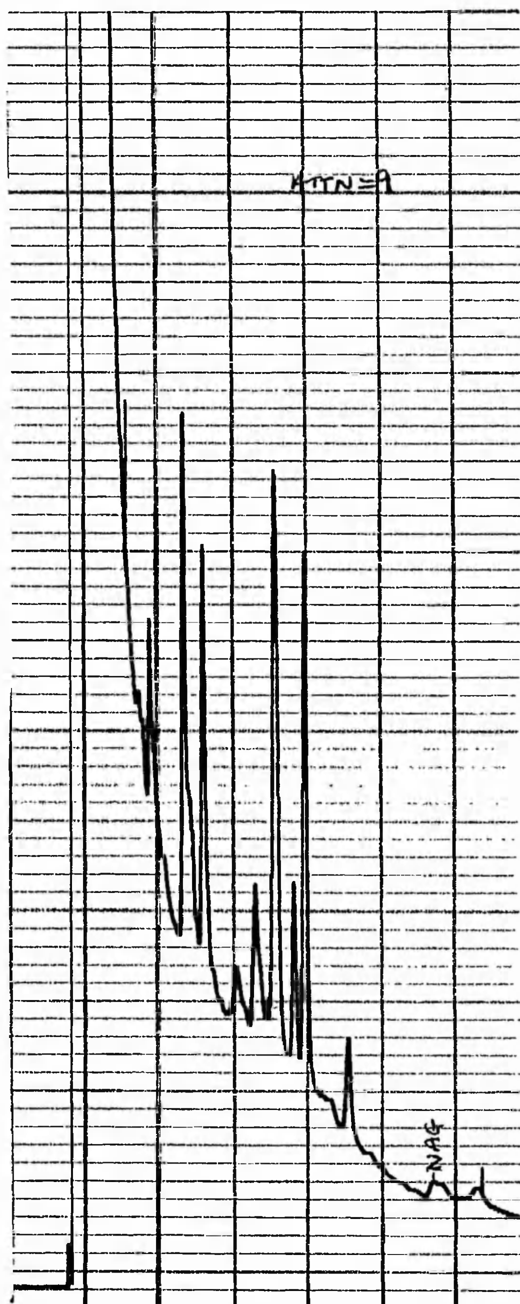


Figure 31. Acid hydrolysate of *Streptococcus pyogenes* derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven rising from 140°C to 200°C at 4°C/min, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12 cm/hr.

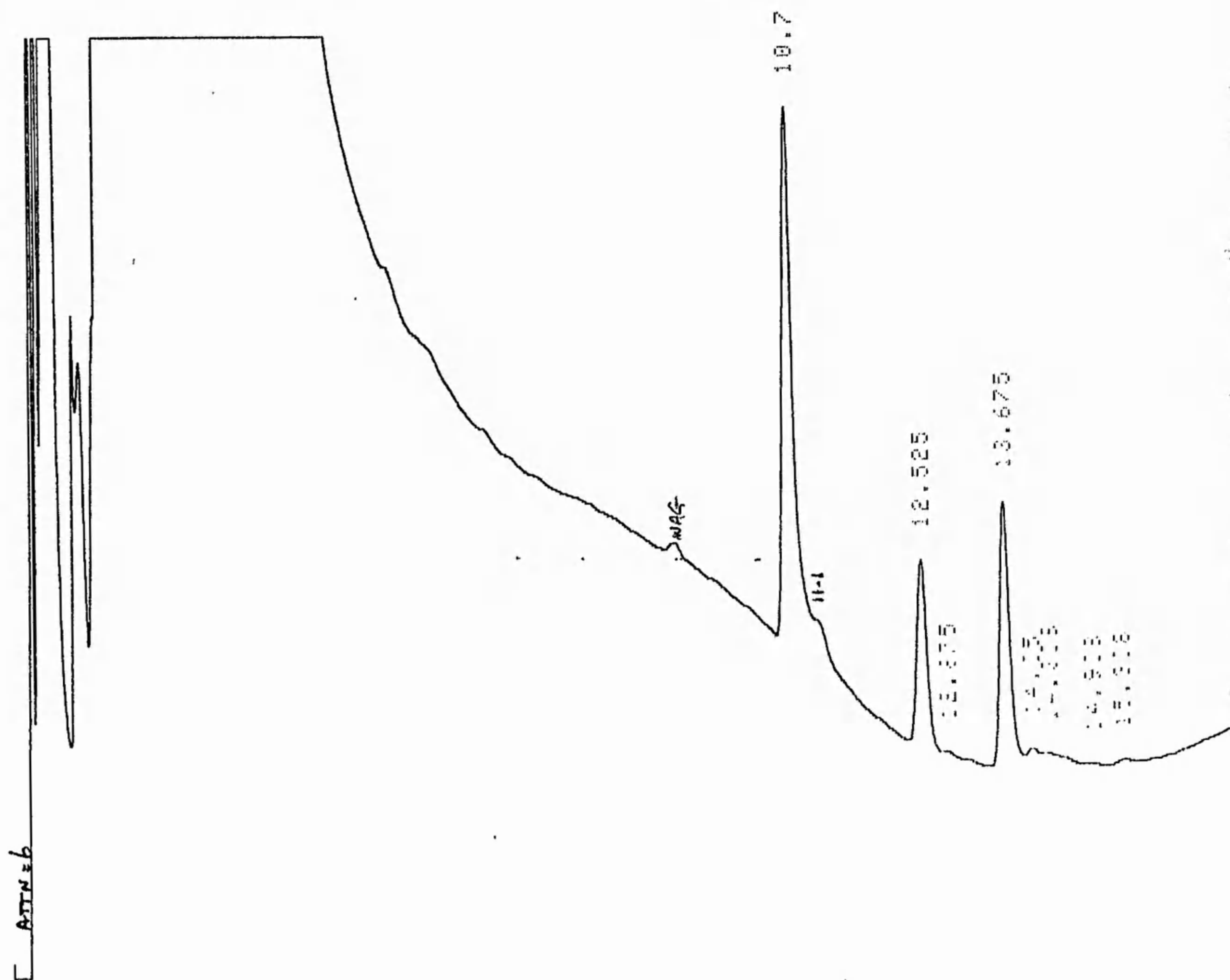


Figure 32. Alkali hydrolysate of <u>Streptococcus pyogenes</u> derivatised with N - methyl - bis (trifluoroacetamide). Column: 2 metres OV11. G.L.C. conditions: oven 75°C for 1 minute rising to 250°C at 10°C/min, nitrogen carrier gas 20 mL/min, Electron Capture Detector 225°C, injector 250°C.

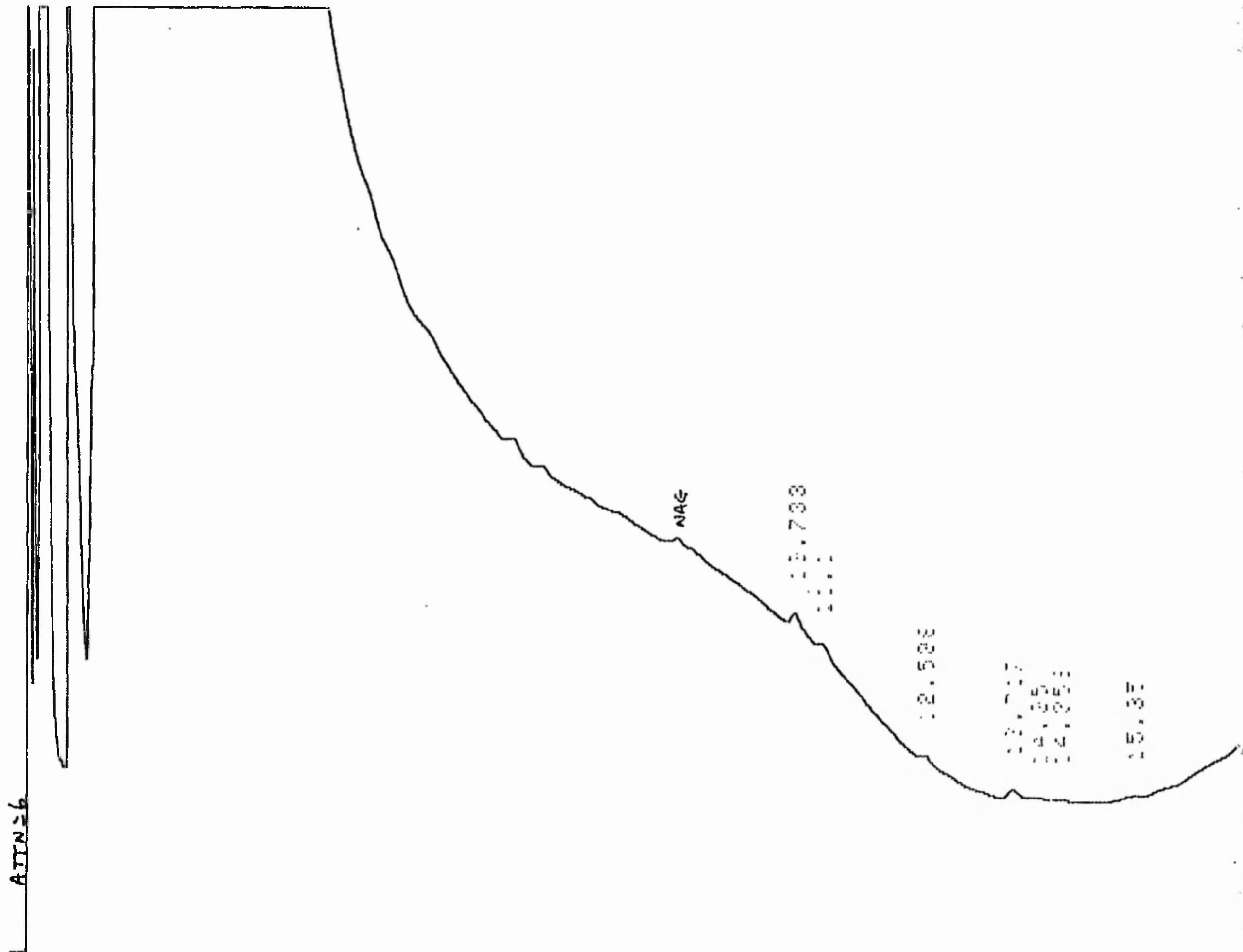


Figure 33. Alkali hydrolysate of Streptococcus pneumoniae derivatised with N - methyl - bis (trifluoroacetamide). Column: 2 metres OV11. G.L.C. conditions: oven 75°C for 1 minute rising to 250°C at 10°C/min, nitrogen carrier gas 20 mL/min, Electron Capture Detector 225°C, injector 250°C.

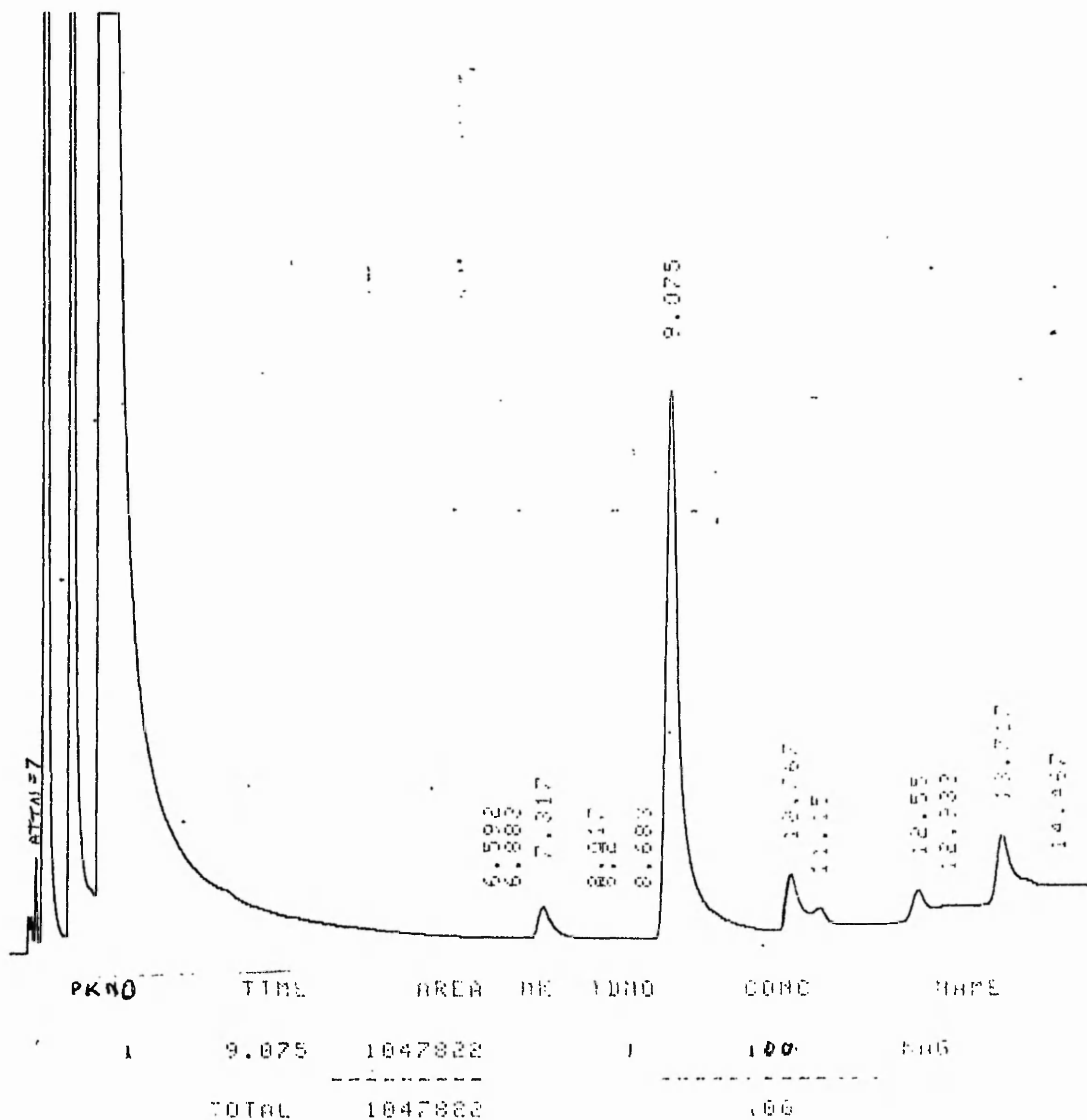
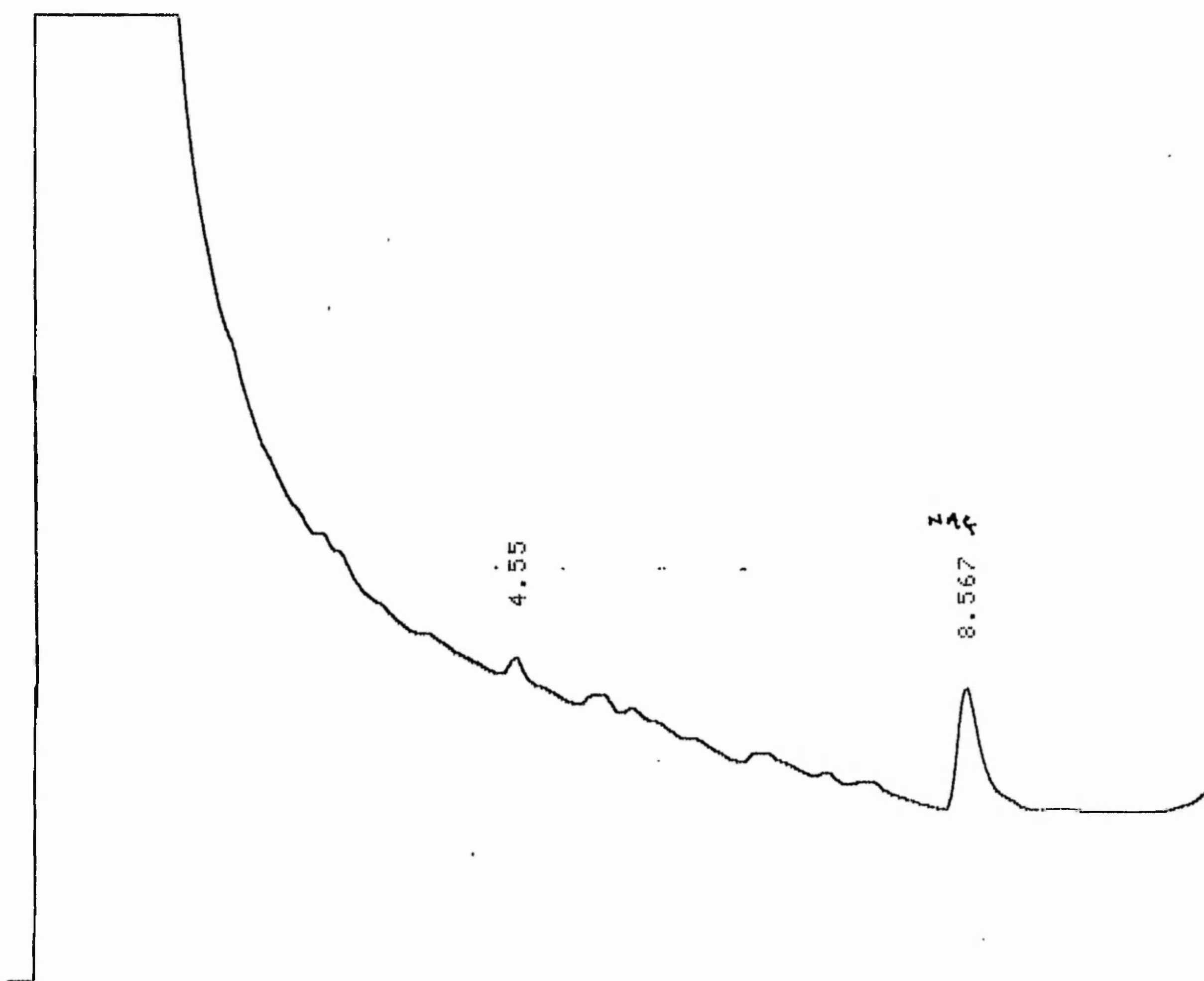


Figure 34. 10 g/L N-acetyl glucosamine derivatised with N - methyl - bis (trifluoroacetamide). Column: 2 metres OV11. G.L.C. conditions: oven 75°C for 1 minute rising to 250°C at 10°C/min, nitrogen carrier gas 20 mL/min, Electron Capture Detector 225°C, injector 250°C.

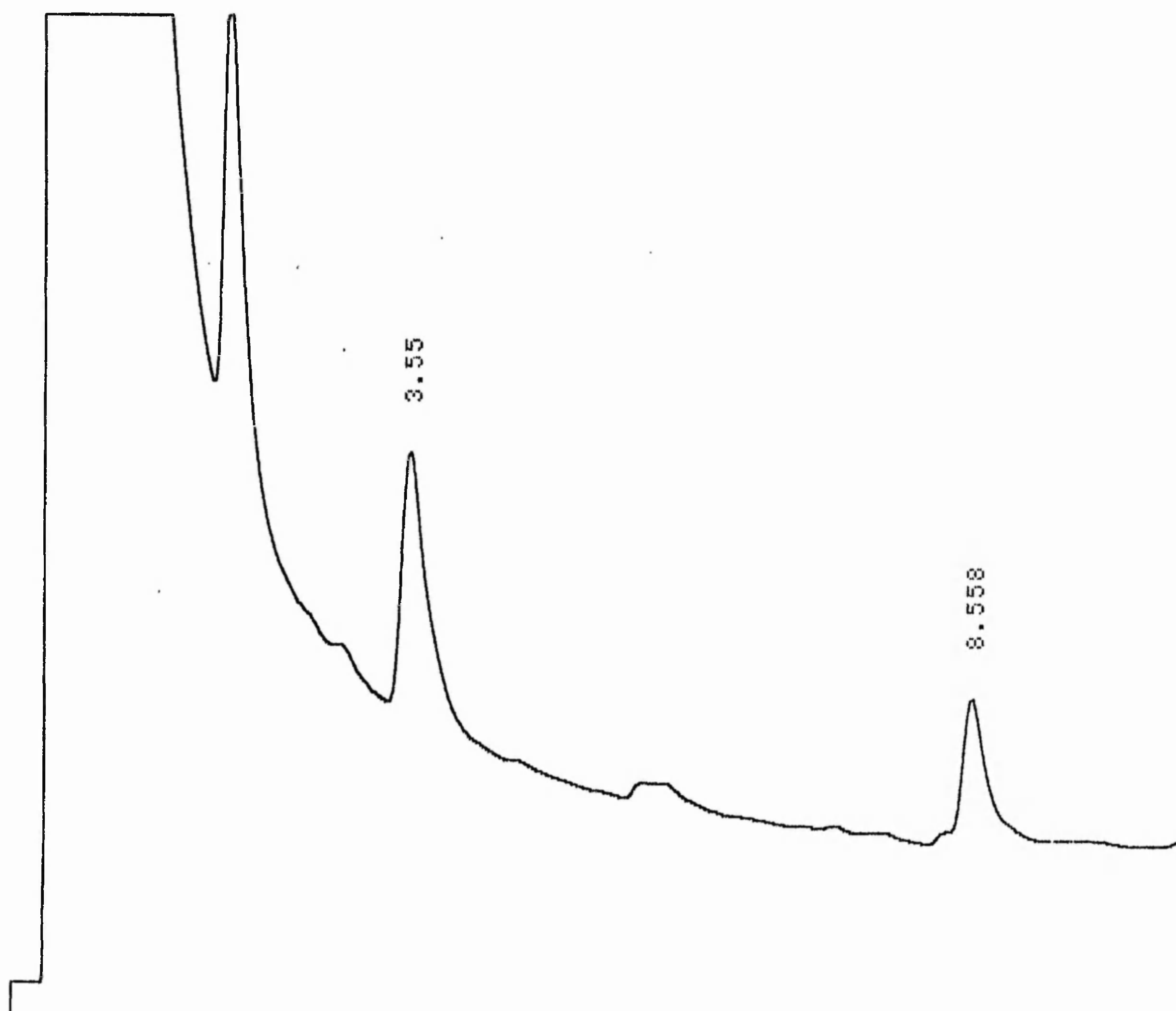
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.442	6066				
2	2.717	3156				
3	5.333	12678				
4	5.583	2662				
5	8.592	61023		1	100	NAG
	TOTAL	85586			100	

Figure 35. Enzymic acidic extract of <u>Staph. aureus</u> derivatised with N - methyl - bis (trifluoroacetamide). Column: 2 metres OV11. G.L.C. conditions: oven 125°C rising to 225°C at 10° C/min, nitrogen carrier gas 20 mL/min, Electron Capture Detector 225°C, injector 250°C.



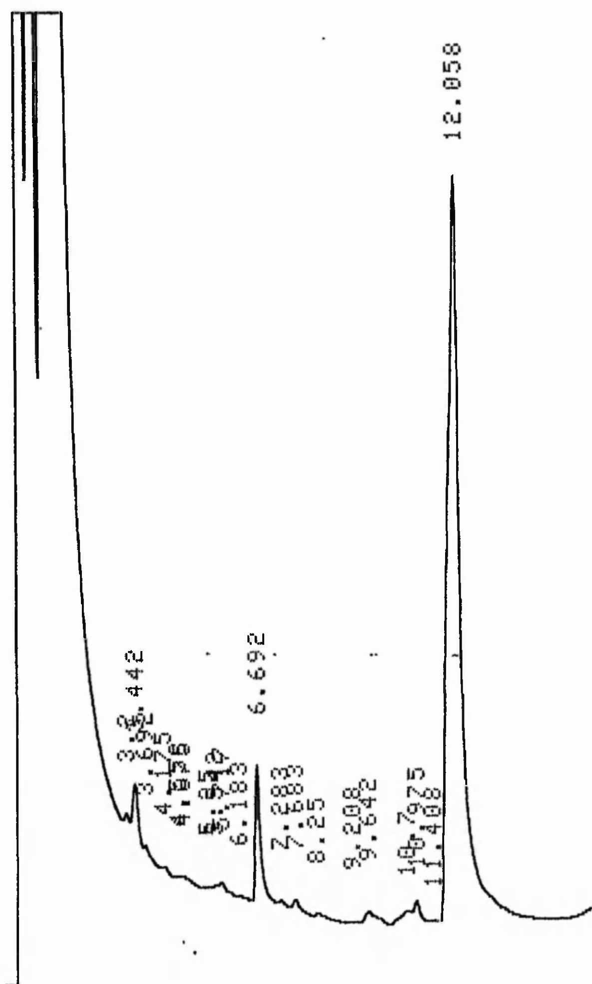
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	4.55	6011				
2	8.567	58635		1	100	NAG

Figure 36. Enzymic alkali extract of <u>Staph. aureus</u> derivatised with N - methyl - bis (trifluoroacetamide). Column: 2 metres OV11. G.L.C. conditions: oven 125°C rising to 225°C at 10°C/min, nitrogen carrier gas 20 mL/min, Electron Capture Detector 225°C, injector 250°C.



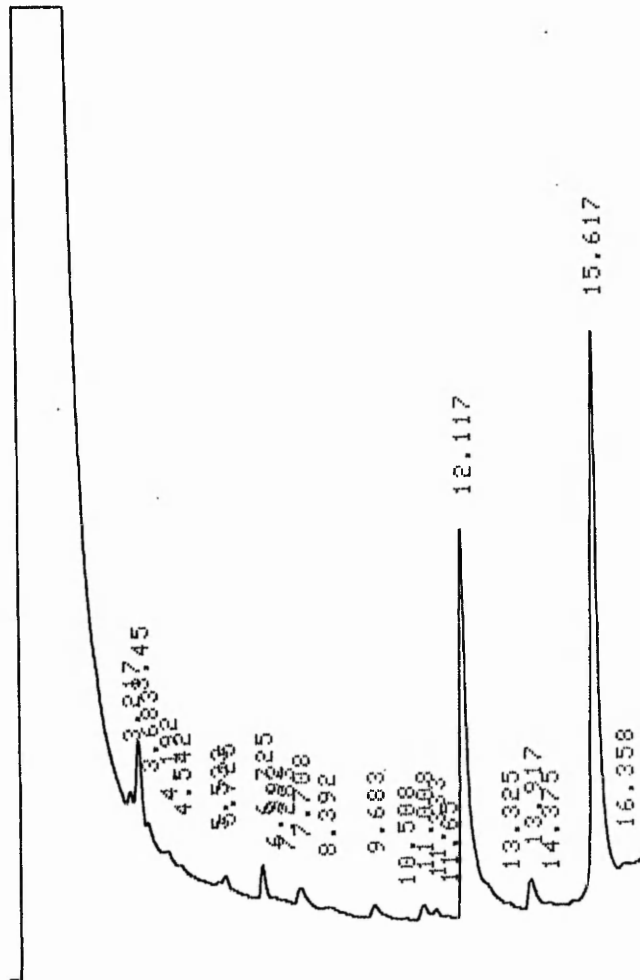
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.55	139468				
2	8.558	63001		1	100	NAG
	TOTAL	202469			100	

Figure 37. 1 g/L of N-acetyl glucosamine derivatised with N - methyl - bis (trifluoroacetamide). Column: 2 metres OV11. G.L.C. conditions: oven 125°C rising to 225°C at 10°C/min, nitrogen carrier gas 20 mL/min, Electron Capture Detector 225°C, injector 250°C.



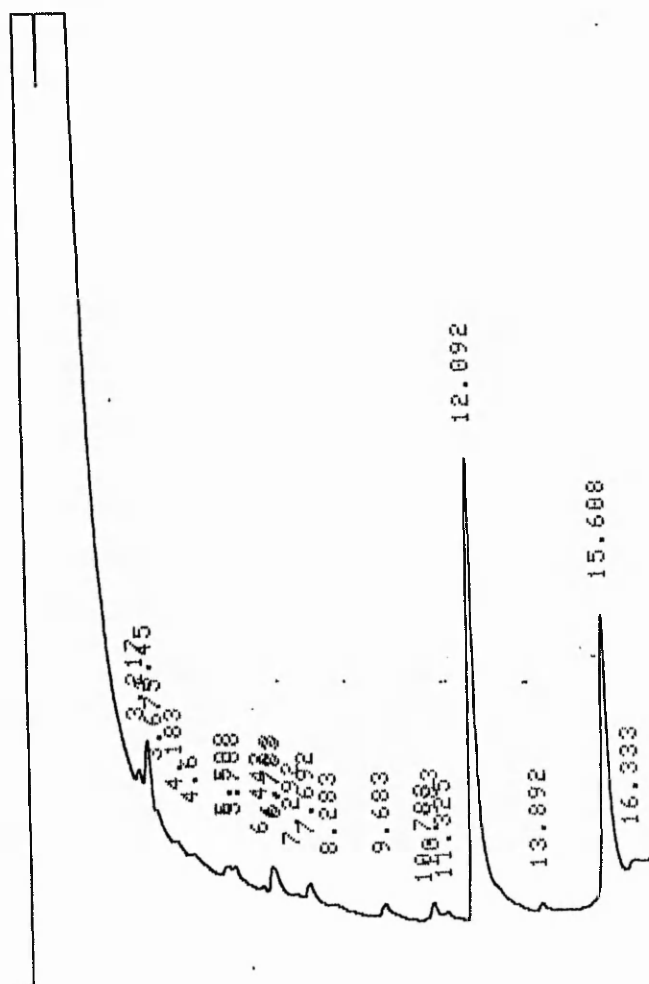
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	6.692	177113				
2	12.058	2361640	V			
	TOTAL	2538753			0	

Figure 38. Enzymic acidic extract of <u>Staph. aureus</u> derivatised with TRISIL Column: 2 metres OV11. G.L.C. conditions: oven 75°C for 1 minute rising to 250°C at 10°C/min, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, injector 250°C.



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.45	101169	V			
2	12.117	846148	S			
3	15.617	931047		1	100	NAG
	TOTAL	1878365			100	

Figure 39. 1 g/L of N-acetyl glucosamine derivatised with TRISIL Column: 2 metres OV11. G.L.C. conditions: oven 75°C 1 minute rising to 250°C at 10°C/min, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, injector 250°C.



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	12.092	959106	S			
2	15.608	402517		1	100	NAG
	TOTAL	1361623			100	

Figure 40. Enzymic acidic extract of <u>Staph. aureus</u> with 1 g/L of N acetyl glucosamine added prior to derivatisation with TRISIL Column: 2 metres OV11. G.L.C. conditions: oven 75°C for 1 minute rising to 250°C at 10°C/min, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, injector 250°C.

ii Chromatography of Sugars

A number of monosaccharides, disaccharides, sugar alcohols and amino sugars were examined using TRISIL derivatives by gas chromatography. The three sugar alcohols, mannitol, sorbitol and dulcitol, which are isomers of each other gave the same retention times of 13.5 minutes (Figures 41, 42, 43). The glucose gave three peaks at 11, 12.5 and 13.5 minutes (Figure 44). This was not of a high grade the peaks possibly due to contamination with the sugar alcohols. The five carbon sugar arabinose gave a shorter retention time of 7 minutes (Figure 45). Cellobiose, a disaccharide, had a long retention time of 23 minutes although sensitivity was greatly reduced due to solubility problems (Figure 46). The other disaccharide, maltose, gave three peaks in the same area as glucose, (Figure 47), perhaps due to instability. The two amino sugars N-acetyl glucosamine and N-acetyl muramic acid had markedly different retention times of 19.5 and 31 minutes respectively (Figures 48 and 49).

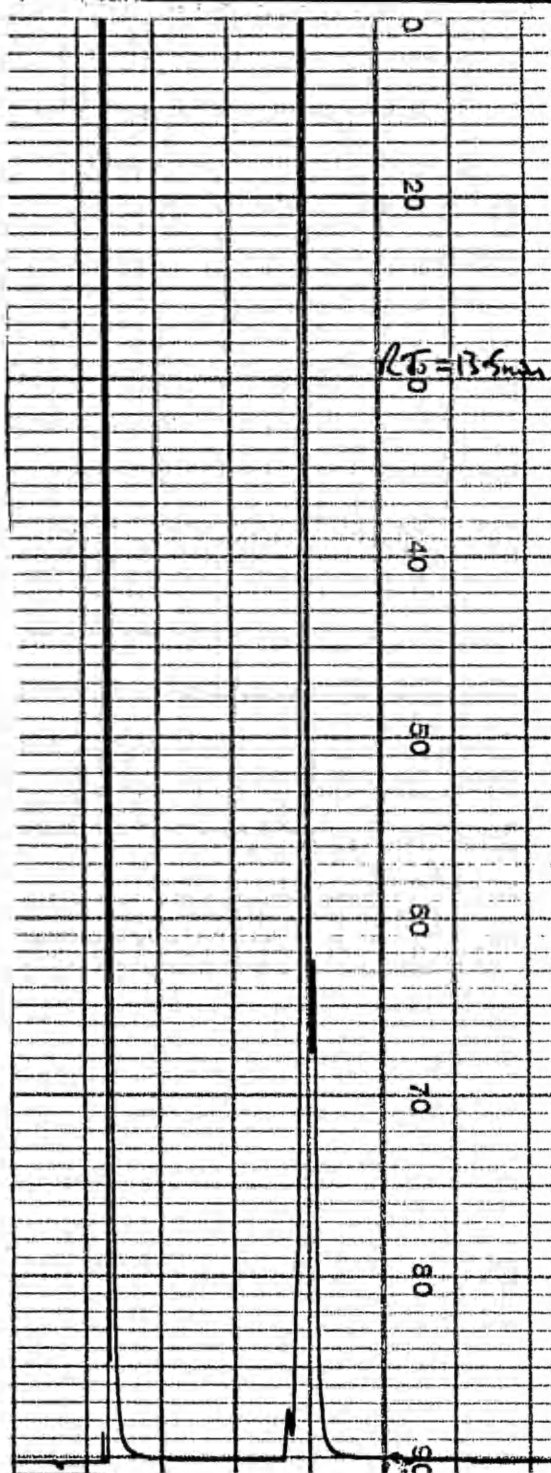


Figure 41. 1 g/L mannitol derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising to 200°C at 4°C/min, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12 cm/hr.

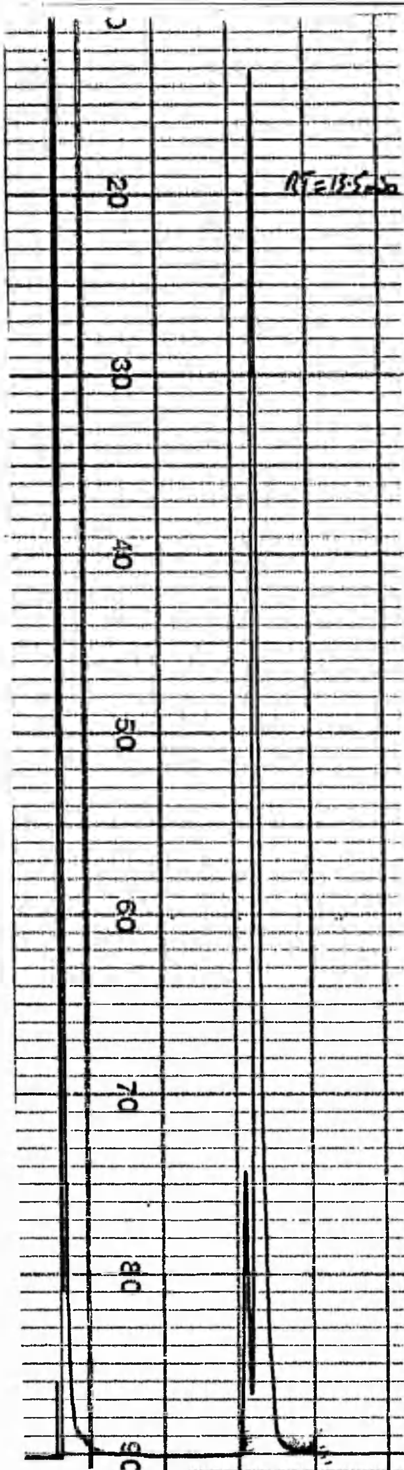


Figure 42. 1 g/L sorbitol derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising to 200°C at 4°C/min, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12 cm/hr.

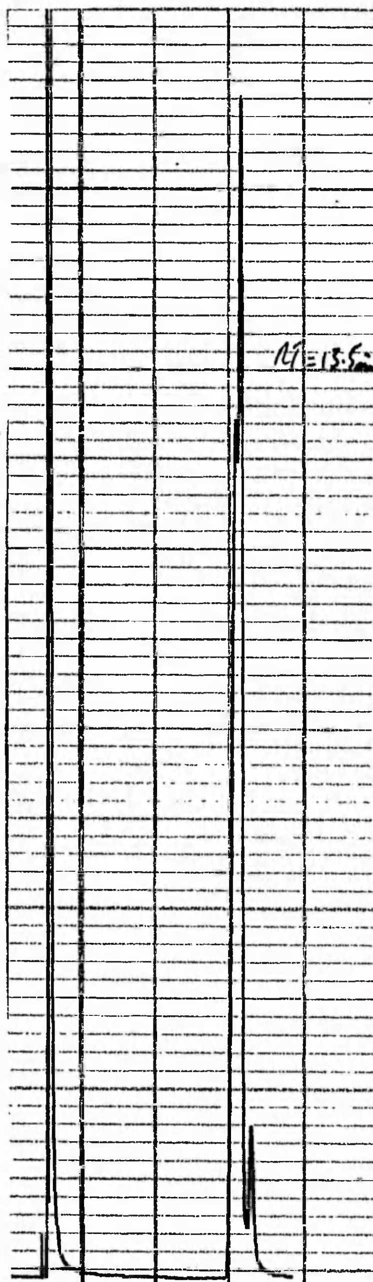


Figure 43. 1 g/L dulcitol derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising to 200°C at 4°C/min, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12 cm/hr.

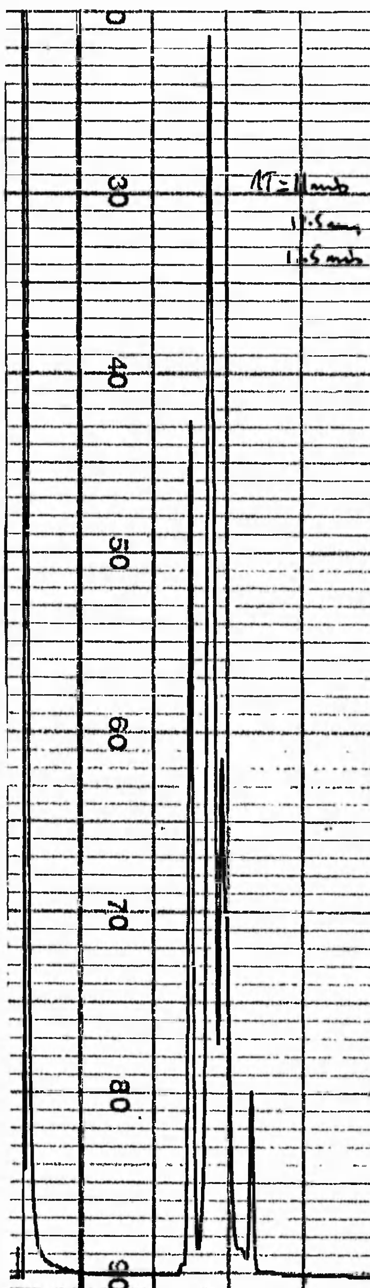


Figure 44. 1 g/L glucose derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising to 200°C at 4°C/min, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12 cm/hr.

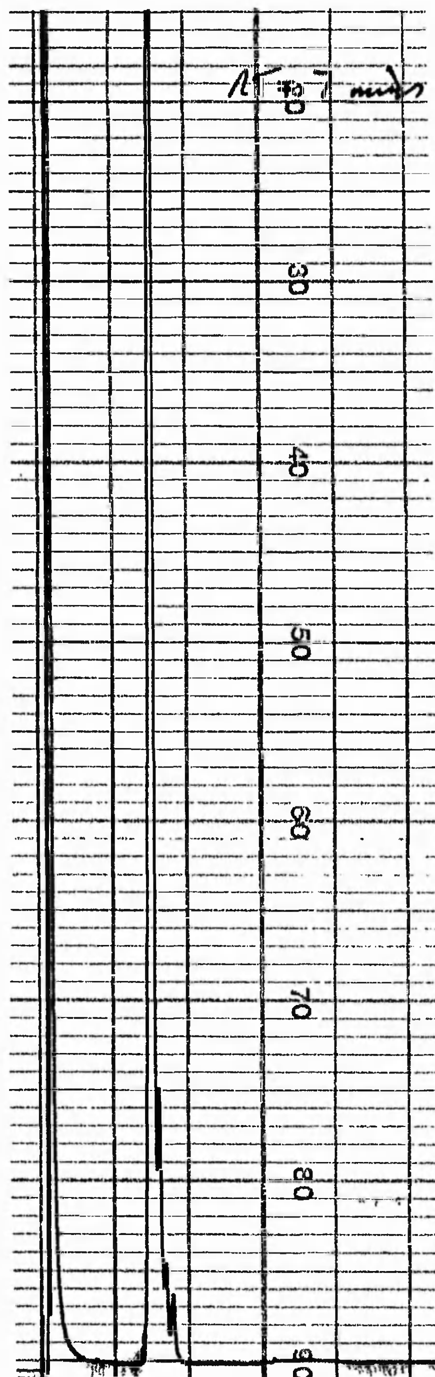


Figure 45. 1 g/L arabinose derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising to 200°C at 4°C/min, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12cm/hr.

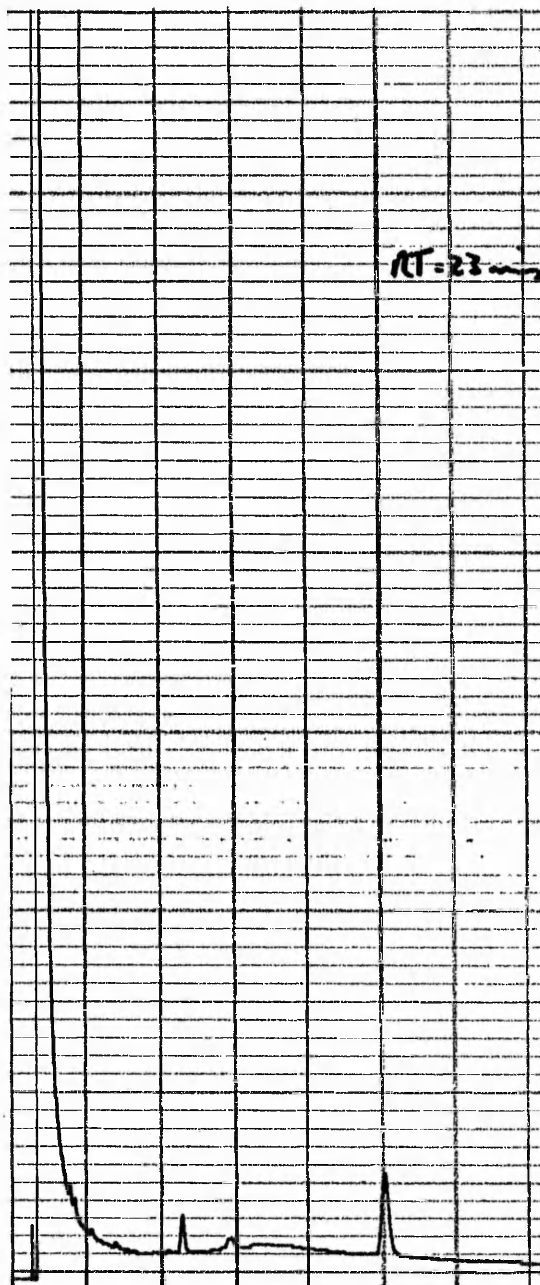


Figure 46. 1 g/L cellobiose derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising to 200°C at 4°C/min, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12 cm/hr.

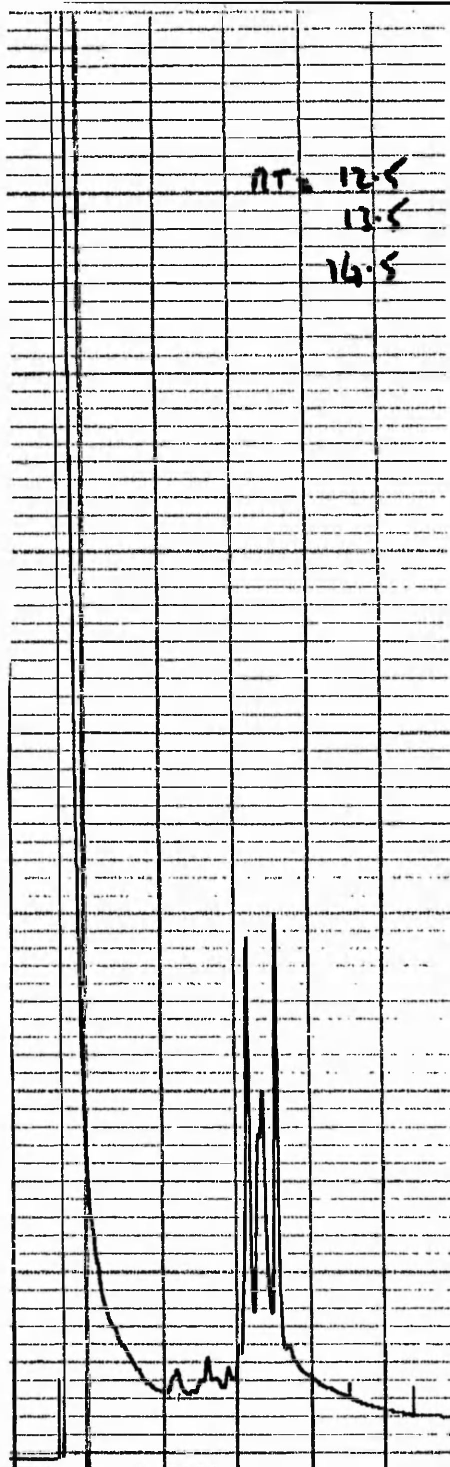


Figure 47. 1 g/L maltose derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising to 200°C at 4°C/min, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12 cm/hr.

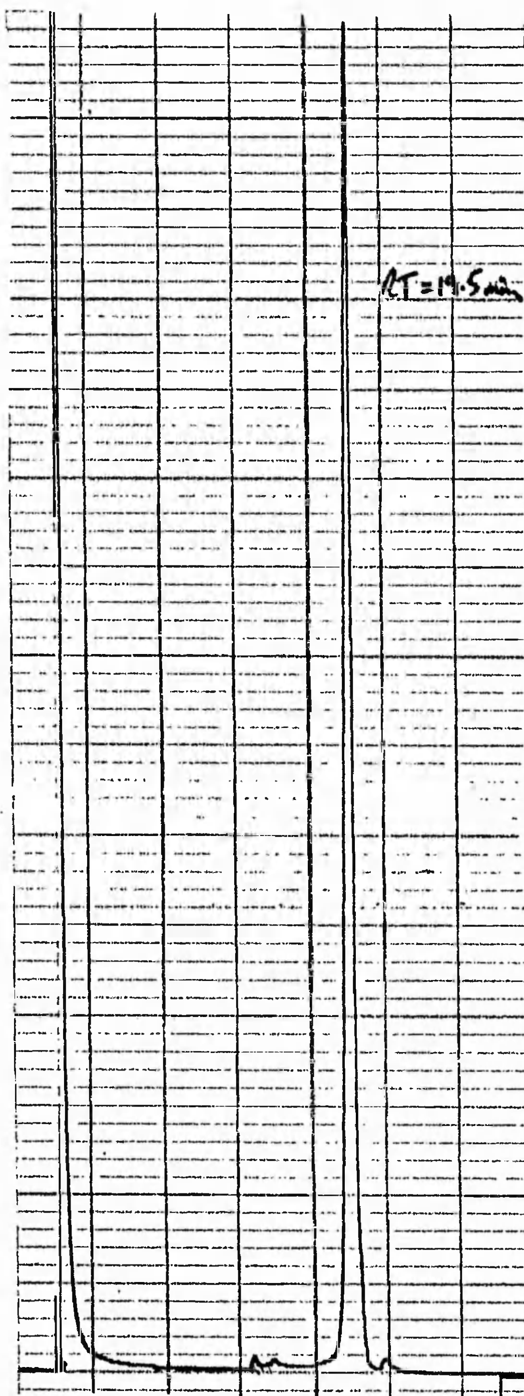


Figure 48. 1 g/L N-acetyl glucosamine derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising to 200°C at 4°C/min, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12 cm/hr.

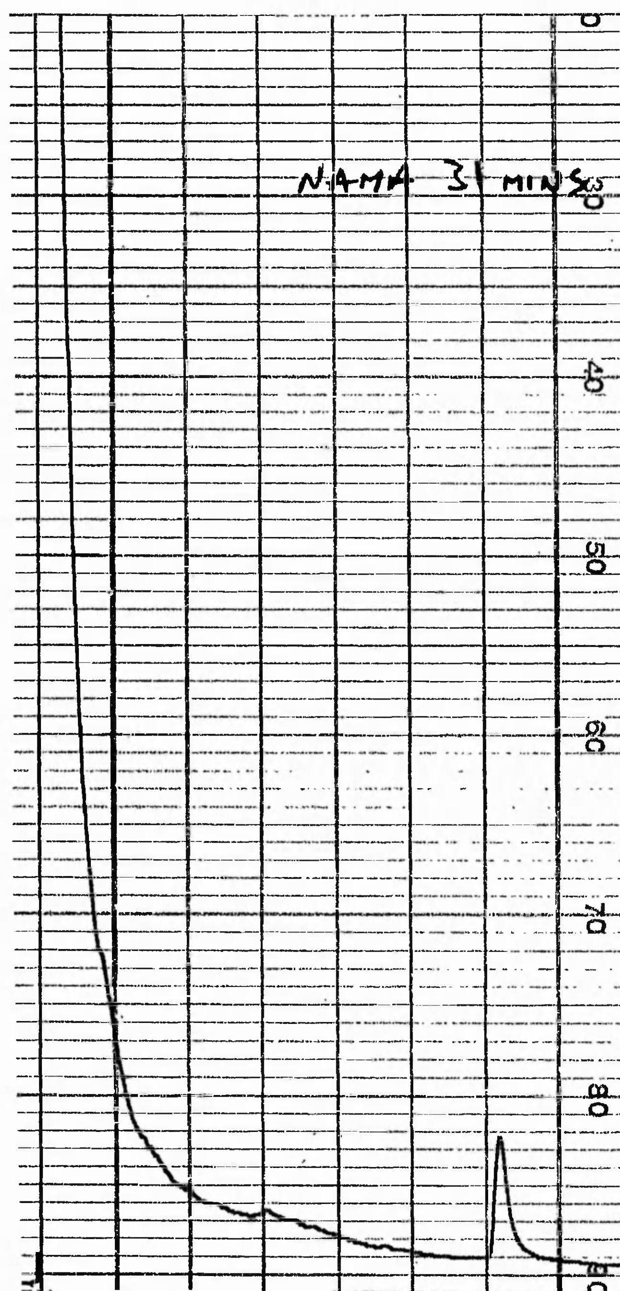


Figure 49. 1 g/L N-acetyl muramic acid derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising to 200°C at 4°C/min, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, injector 250°C. Chart speed 12 cm/hr.

iii Clinical Specimens

The chromatograph of TRISIL derivitised freeze dried serum containing 1 g/L of N-acetyl glucosamine (Figure 50) has a number of peaks including the N-acetyl glucosamine. However when compared with Figure 51 which is a chromatograph of N-acetyl glucosamine at the same concentration but in water there is a marked reduction in peak size. The process was repeated substituting another sugar, mannitol, for N-acetyl glucosamine without reduction in peak size (Figures 52 and 53).

Three specimens of cerebrospinal fluid (C.S.F.) were freeze dried. These were collected at myelogram and were negative by conventional bacterial culture techniques. These were derivitised with TRISIL and there traces compared with that of N-acetyl glucosamine (Figures 54, 55, 56, 57). All three C.S.F. traces failed to reveal a peak at the retention time for N-acetyl glucosamine. The samples with N-acetyl glucosamine added were treated in the same way. All three traces had the characteristic peak for N-acetyl glucosamine at 18.2 minutes (Figures 58, 59, 60). The peak at 16.4 occured on all three traces although the apparent increase in size is probably due to alteration of the attenuation. The enzymic extract of Staph. aureus previously used followed by acid hydrolysis was added to each sample. The resulting three chromatograms (Figures 61, 62, 63) do not have a peak at 18.2 minutes even when the attenuation was altered.

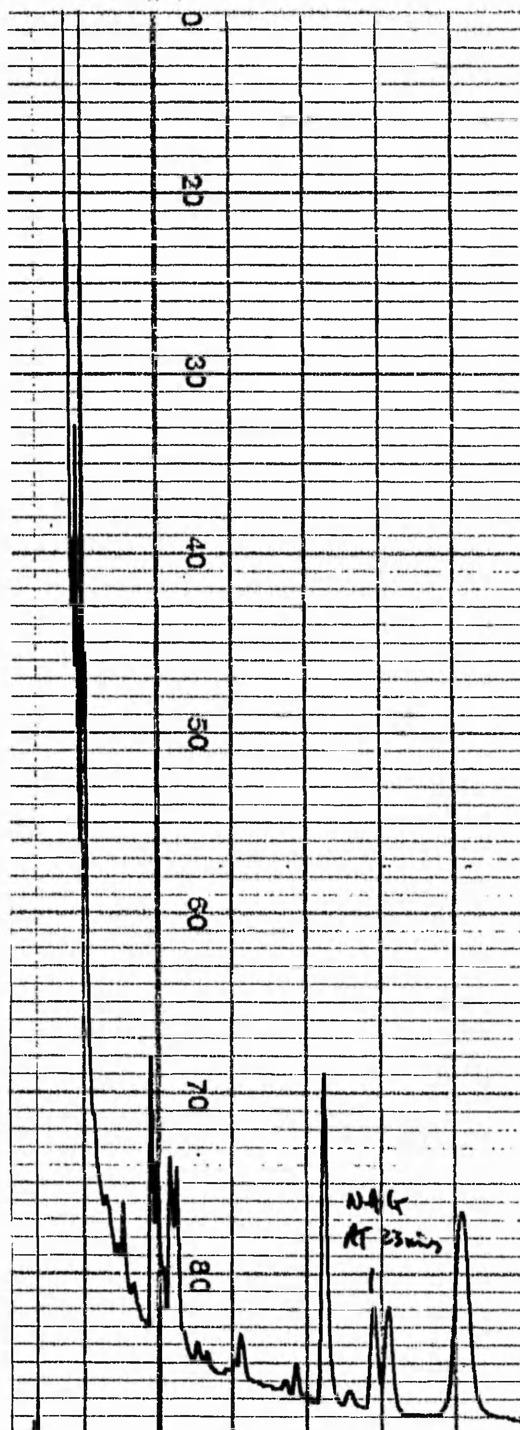


Figure 50. 1 g/L N-acetyl glucosamine in serum derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising at 4°C/min to 200°C, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, detector 250°C. Chart speed 12 cm/hr.

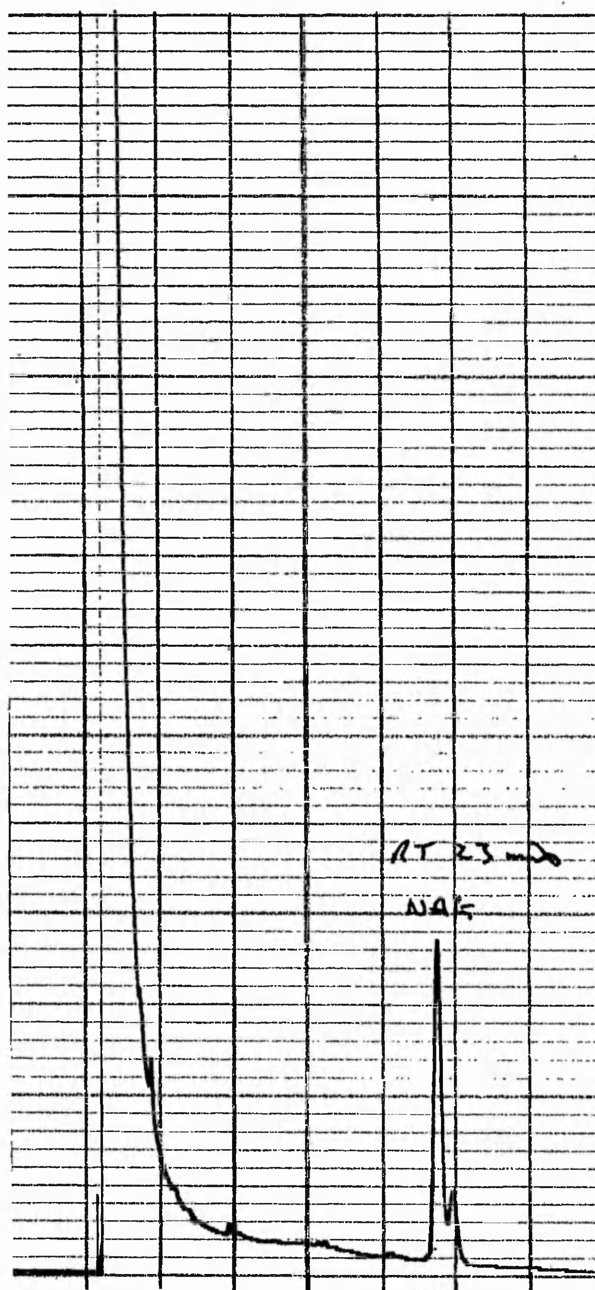


Figure 51. 1 g/L N-acetyl glucosamine in water derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising at 4°C/min to 200°C, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, detector 250°C. Chart speed 12 cm/hr.

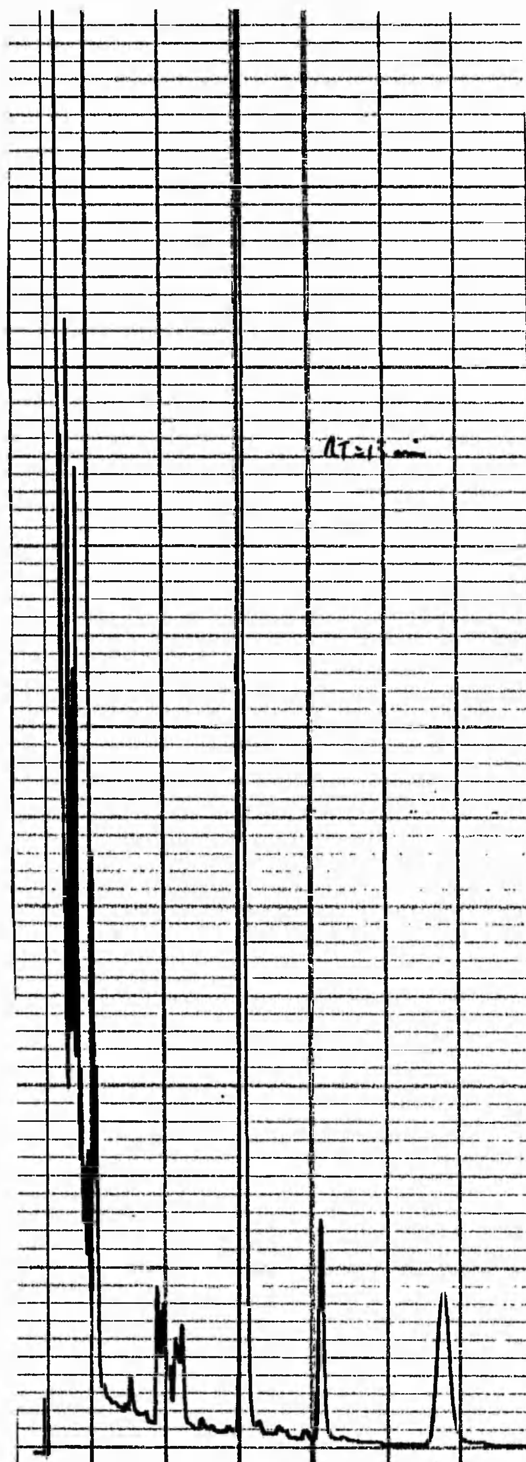


Figure 52. 1 g/L mannitol in serum derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising at 4°C/min to 200°C, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, detector 250°C. Chart speed 12 cm/hr.

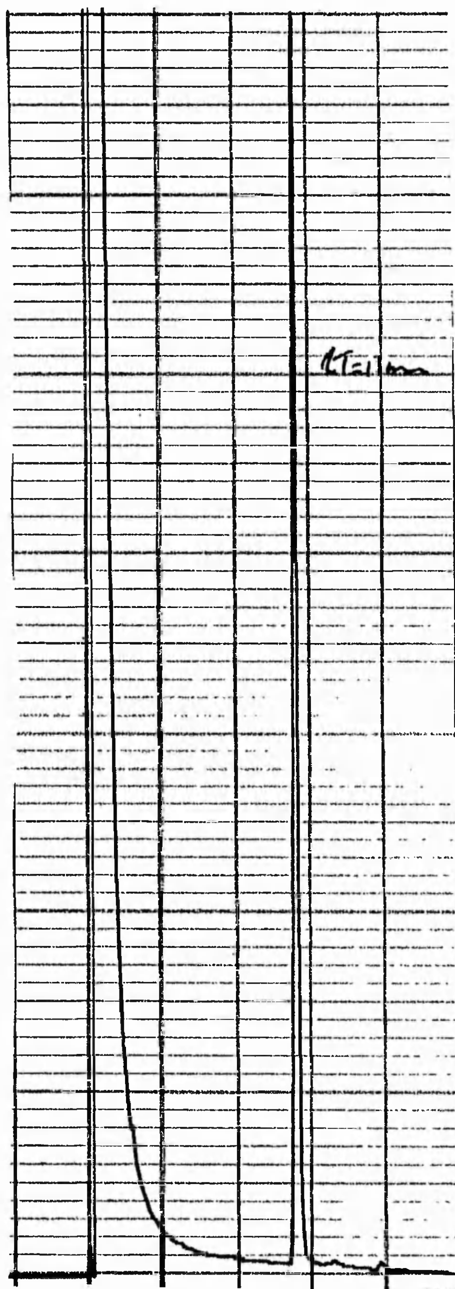
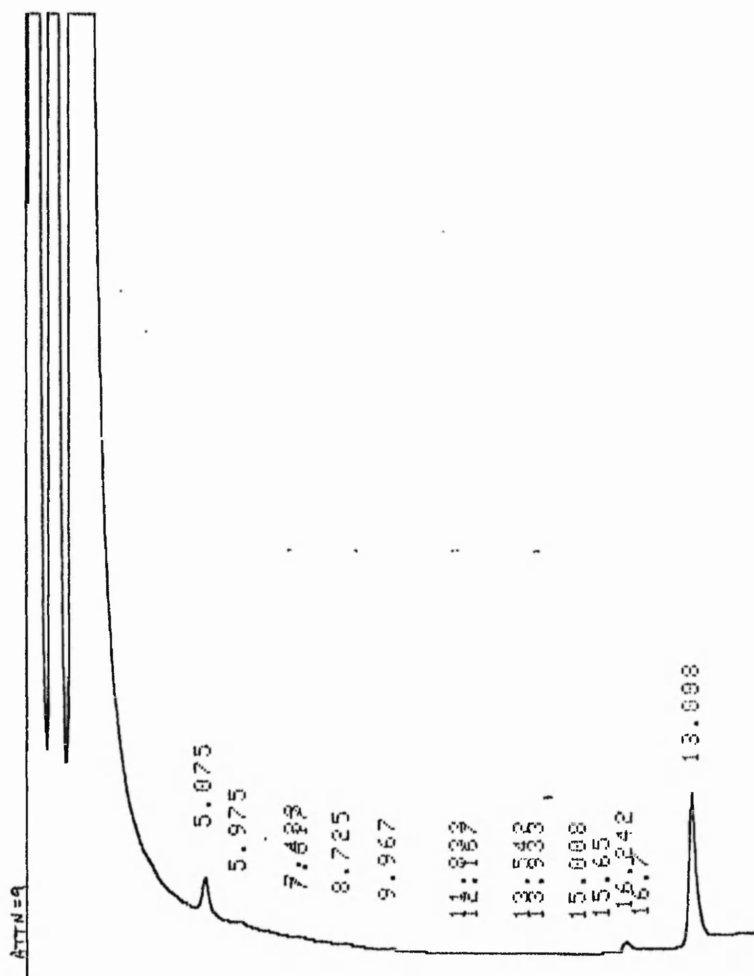


Figure 53. 1 g/L mannitol in water derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 140°C rising at 4°C/min to 200°C, nitrogen carrier gas 20 mL/min, Flame Ionisation Detector 225°C, detector 250°C. Chart speed 12 cm/hr.



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	18.008	508666		1	100	NAG
	TOTAL	508666			100	

Figure 54. 0.1 g/L N-acetyl glucosamine derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions: oven 75°C for 1 minute rising at 10°C/min to 250°C, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, detector 250°C.

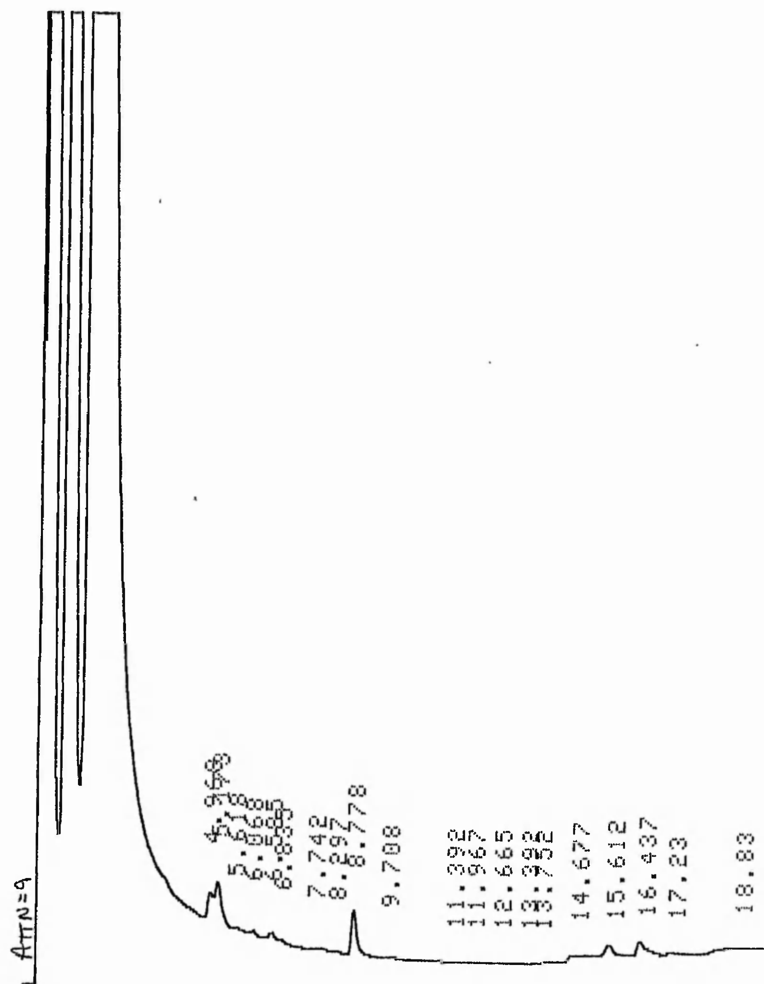


Figure 55. C.S.F. specimen (1) derivatised with TRISIL. Column: 2 metres OV11. G . L . C. conditions : oven 75°C for 1 minute rising at 10°C/min to 250°C, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, detector 250°C.

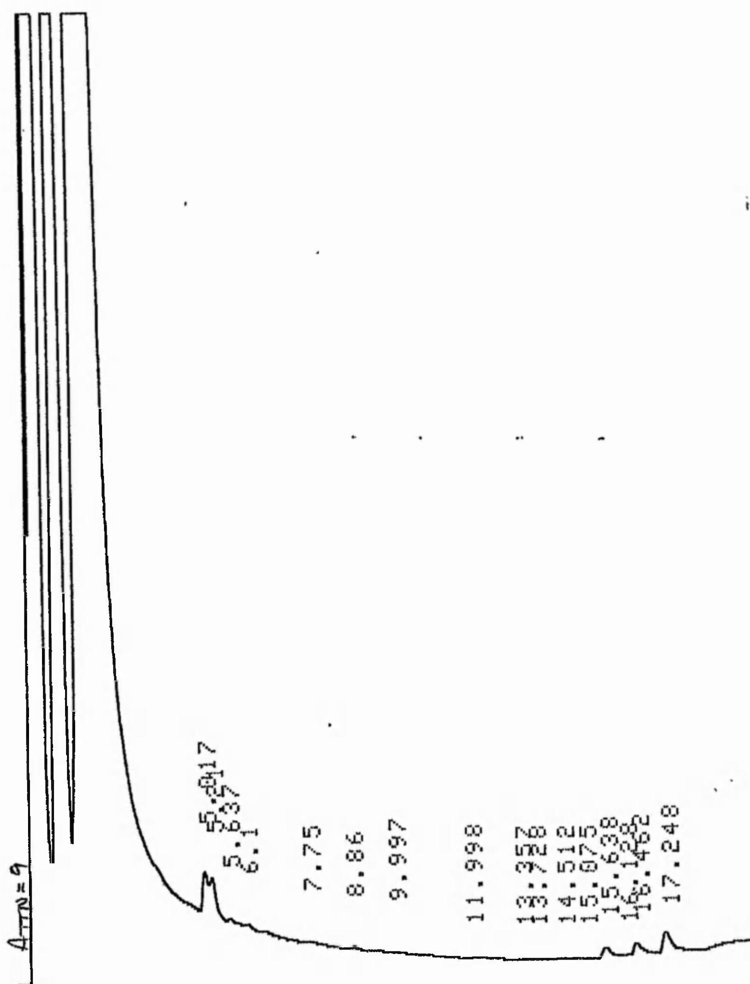


Figure 56. C.S.F. specimen (2) derivatised with TRISIL. Column: 2 metres OV11. G . L . C. conditions : oven 75°C for 1 minute rising at 10°C/min to 250°C, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, detector 250°C.

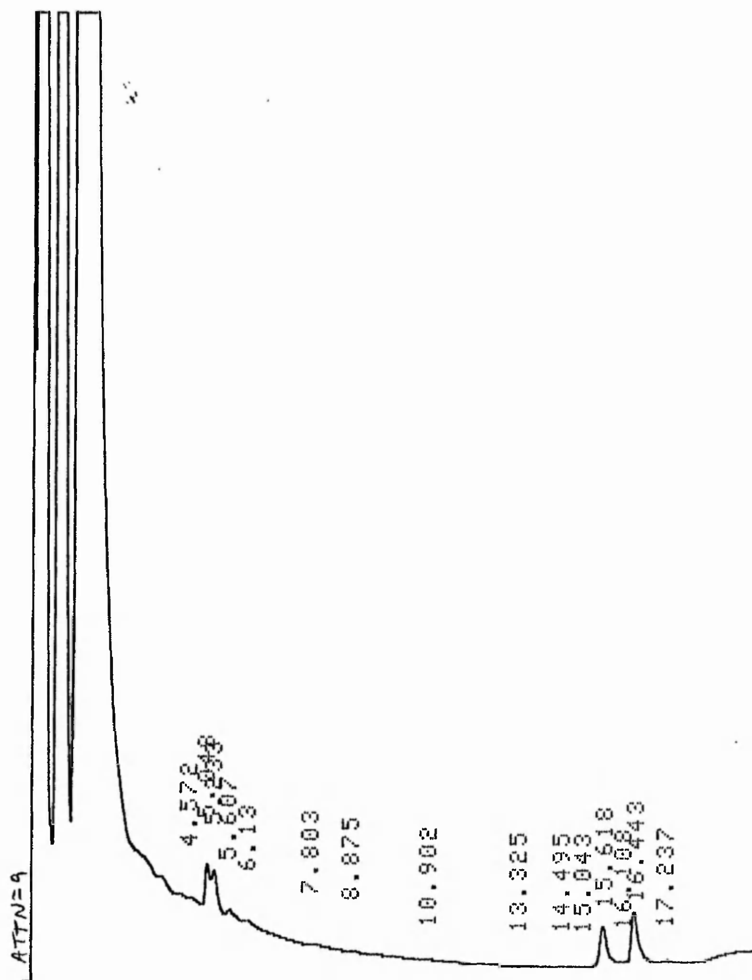
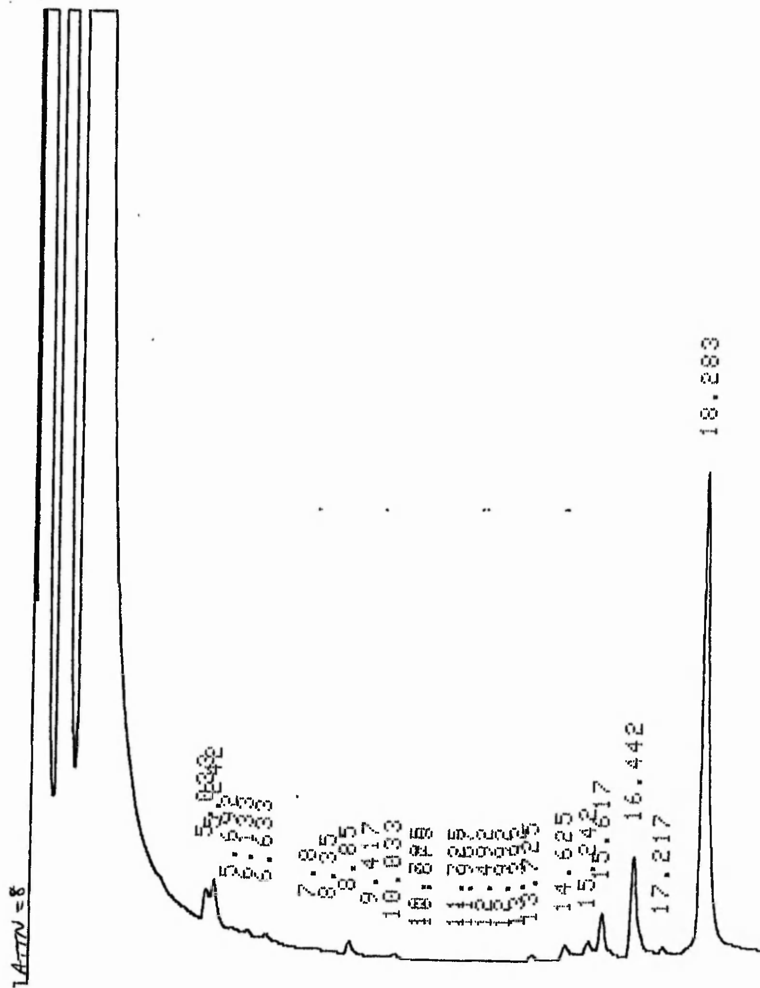
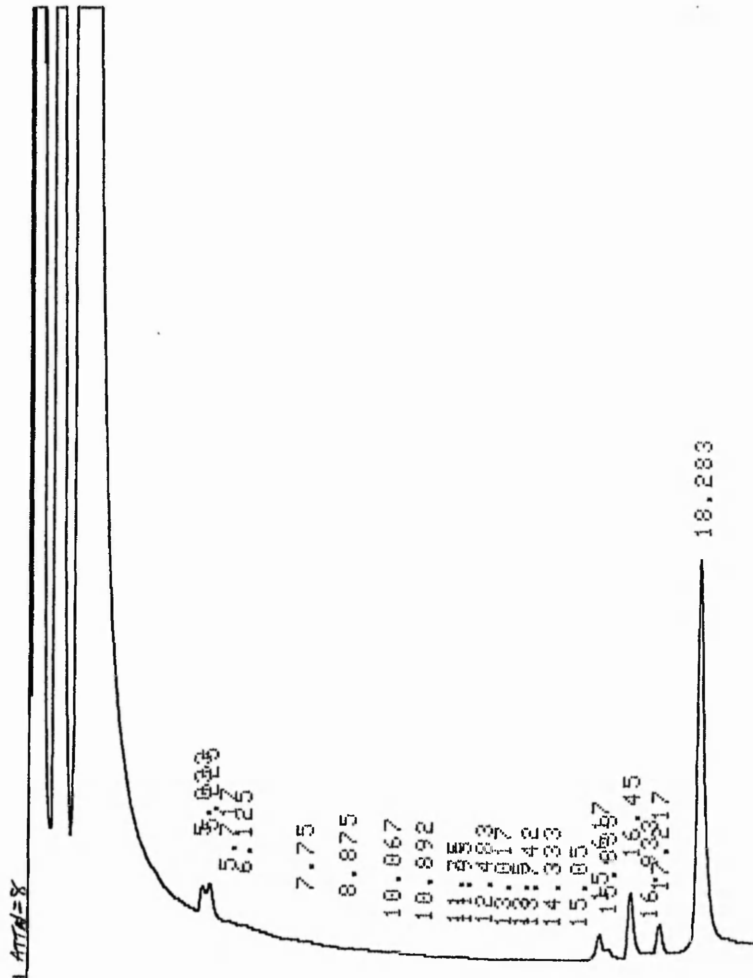


Figure 57. C.S.F. specimen (3) derivatised with TRISIL. Column: 2 metres OV11. G . L . C. conditions : oven 75°C for 1 minute rising at 10°C/min to 250°C, nitrogen carrier gas 30 ml/min, Flame Ionisation Detector 225°C, detector 250°C.



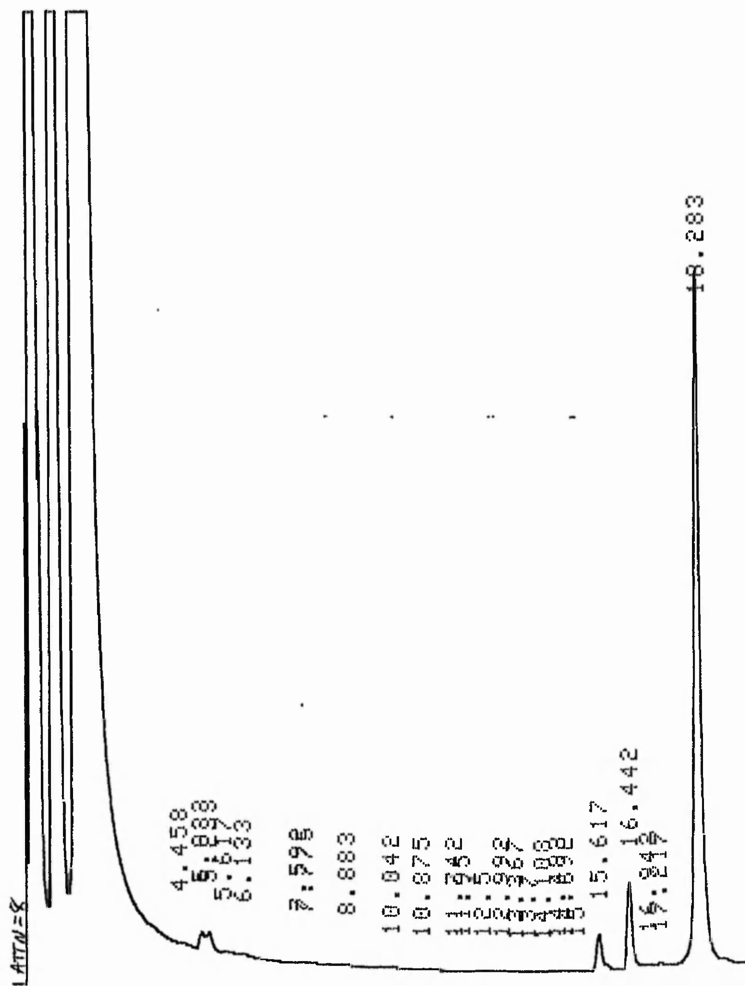
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	18.283	1837524	V	1	100	NAG
	TOTAL	1837524			100	

Figure 58. C.S.F. specimen (1) with 0.2 g/L of N-acetyl glucosamine added derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions : oven 75°C for 1 minute rising at 10°C/min to 250°C, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, detector 250°C.



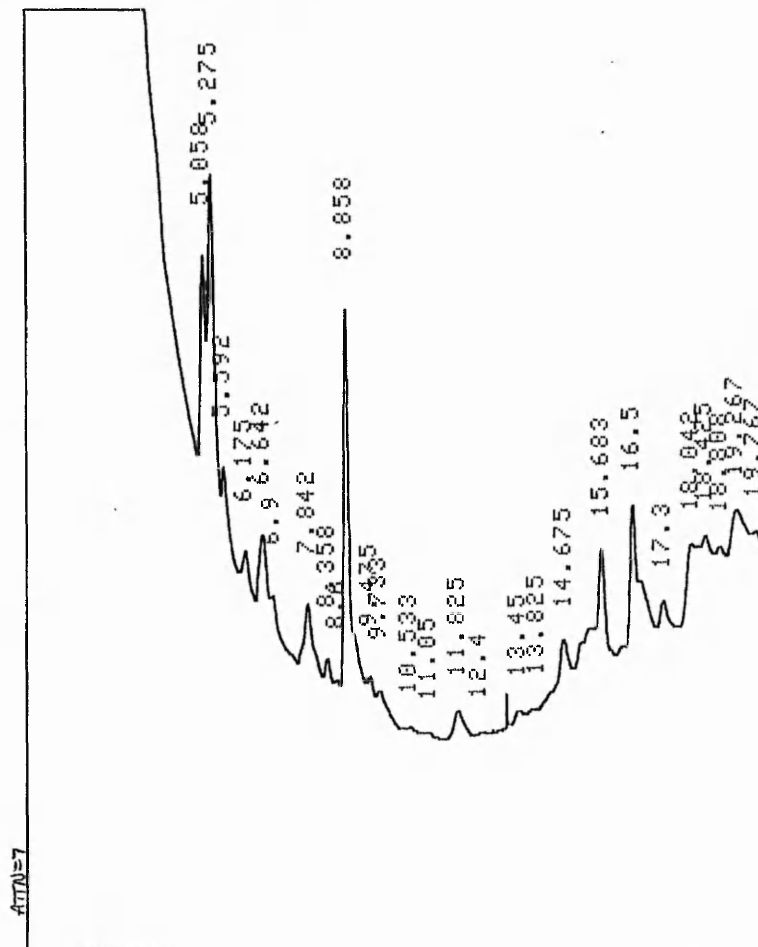
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	18.283	1554315	V	1	100	NAG
	TOTAL	1554315			100	

Figure 59. C.S.F. specimen (2) with 0.2 g/L of N–acetyl glucosamine added derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions : oven 75°C for 1 minute rising at 10°C/min to 250°C, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, detector 250°C.



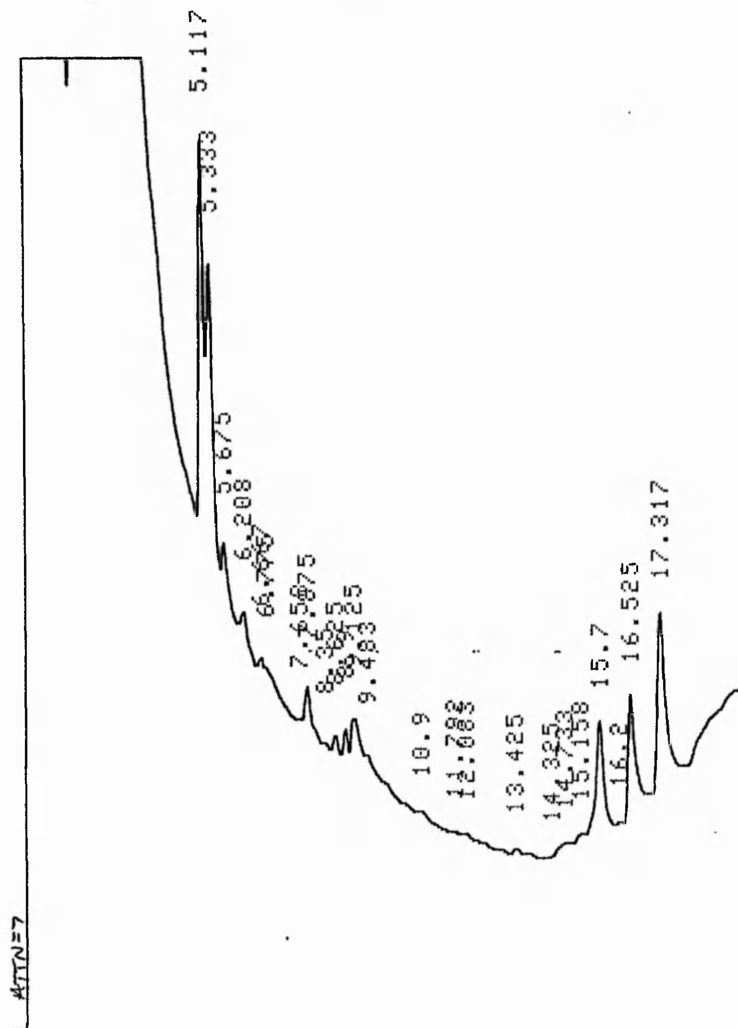
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	18.283	4600998	V	1	100	NAG
	TOTAL	4600998			100	

Figure 60. C.S.F. specimen (3) with 0.2 g/L of N-acetyl glucosamine added derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions : oven 75°C for 1 minute rising at 10°C/min to 250°C, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, detector 250°C.



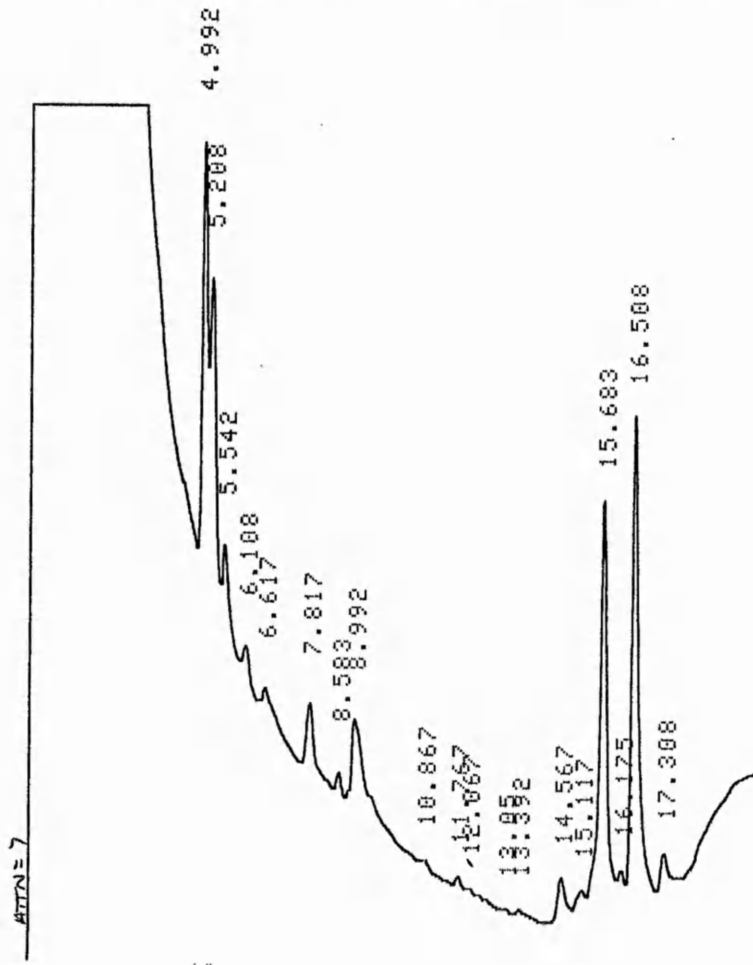
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.058	61529				
2	5.275	109603	V			
3	6.642	36152				
4	7.842	34000				
5	8.858	135412	S			
6	11.825	17669				
7	14.675	36338				
8	15.683	101221	V			
9	16.5	97764	V			
10	17.3	15627	V	1	100	NAG

Figure 61. C.S.F. specimen (1) with acidic extract of <u>Staph. aureus</u> added derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions : oven 75°C for 1 minute rising at 10°C/min to 250°C, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, detector 250°C.



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.117	117721				
2	5.333	95302	V			
3	17.317	87341	V	1	100	MAG
	TOTAL	300364			100	

Figure 62. C.S.F. specimen (2) with acidic extract of Staph. aureus added derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions : oven 75°C for 1 minute rising at 10°C/min to 250°C, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, detector 250°C.



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	4.992	130367				
2	5.208	98008	V			
3	5.542	15028				
4	6.617	14940				
5	7.817	26112				
6	8.992	72695				
7	14.567	19794				
8	15.683	192562	V			
9	16.508	206359	V			
10	17.308	13527		1	100	MAG

Figure 63. C.S.F. specimen (3) with acidic extract of Staph. aureus added derivatised with TRISIL. Column: 2 metres OV11. G.L.C. conditions : oven 75°C for 1 minute rising at 10°C/min to 250°C, nitrogen carrier gas 30 mL/min, Flame Ionisation Detector 225°C, detector 250°C.

DISCUSSION

The use of gas liquid chromatography to examine the subunits of a bacterial cell wall was attempted. This involved the investigation of derivatisation methods and gas chromatography conditions that can be used for the assay of the amino sugars, N-acetyl glucosamine and N acetyl muramic acid. The optimum technique could then be used for the examination of bacterial extracts and clinical specimens. Results obtained from the clinical samples could be used to determine if the patient has an infection, the effect of antimicrobial therapy, and to help assess the clinical status of the patient.

The first derivatisation method assessed was TRISIL (Pierce Chemicals) which is a mixture of trimethylchlorosilane and hexamethyldisilazane. This technique has a number of advantages. It is very simple to use with just one reagent addition followed by a short incubation step of 10 minutes at room temperature, requiring no special equipment or heater. An additional advantage is that the traces obtained with pure amino sugars give sharp peaks without interference from reaction byproducts (Figures 48 and 49). The disadvantage of the reagent is that it cannot be used

with an electron capture detector and therefore the sensitivity of the technique is limited to that of the flame ionisation detector. When used with TRISIL the flame ionisation detector is able to measure N-acetyl glucosamine at a sample concentration of 0.01 g/L (Figure 22).

In an attempt to increase the sensitivity of the assay, acylation of the amino sugar instead of silylation was investigated. Heptafluorobutyric acid anhydride is a very reactive reagent which fluorinates a compound rendering it volatile and suitable for use with electron capture detectors. There are a number of problems with this reagent. The method requires a number of additions including benzene and trimethylamine, both of which are unpleasant to use, and requires an incubation step at 50°C. At a N-acetyl glucosamine concentration of 1 g/L a number of reaction byproduct peaks appear (Figure 23) while at a concentration of 0.001 g/L the N acetyl glucosamine peak is all but lost amongst the other peaks (Figure 24). This technique fails to give a higher degree of sensitivity than TRISIL and involves the handling of unpleasant and toxic chemicals.

One of the latest silylation reagents, N-methyl-N-(tert-butyldimethylsilyl)-trifluoracetamide (MTBSTFA)

appeared to combine the advantages of the previous two reagents. It is easy to use with simple addition of reagent to solvent and incubation at room temperature. It is also a silylation reagent which can be used with electron capture detectors. Its use was investigated but reaction with N acetyl glucosamine failed to produce a peak on gas chromatography (Figure 9). This is because the reagent is unable to bind to the amino group due to stearic hindrance from the sugar molecule.

An alternative acylating reagent is N-methyl-bis(trifluoracetamide) (MBTFA). The trifluoroacetylation of the amino sugars with this reagent produced good results (Figure 10). The reaction procedure is quite simple although an incubation stage at 65° is required. The N-acetyl glucosamine peak is well separated from the solvent peaks and there is little interference from byproducts. The reagent is also suitable for both flame ionisation and electron capture detectors.

The conclusions drawn from the reagent assessment was that only two could be of use, i.e. TRISIL and MBTFA. The most promising MBTFA is more flexible because it can be used on both detectors. The optimum gas chromatography conditions were then investigated for both reagents. A 1 metre column

of SE30, a non polar silicone ether, and a 2 metre column of OV11, a similar stationary phase. The retention time with the 2 metre column when used with TRISIL was 50% longer than with the 1 metre column under the same conditions (Figures 11 and 12). The effect on retention time of changing carrier gas flow is clear from Figures 13 and 14, the better retention time being achieved with the lowest gas flow. The original retention time on the 2 metre column was more than doubled by using the low gas flow of 20 mL/min combined with a temperature programmed run (Figure 15). This longer retention time is preferred as it gives good separation from the solvent peak when using low concentrations of the amino sugars. The reaction product of N-acetyl glucosamine with MBTFA is much more volatile than that obtained with TRISIL. With the oven initially set at 125°C the retention time is only around 2 minutes (Figure 18). If the oven temperature is 75°C initially then a retention time of 9 minute is obtained (Figure 19). If TRISIL is used under the same conditions the retention time is doubled (Figure 16). These conditions are optimal for both MBTFA and TRISIL.

The most important factor in detecting bacteria in clinical samples is the sensitivity of the technique. The flame ionisation detector gives a good result at a concentration

of 1 g/L of N-acetyl glucosamine (Figure 20) however the lower limit of detection is 0.01 g/L (Figures 21 and 22). I think that if all the amino sugars from the bacterial cell wall were free in the clinical sample this level of detection would be insufficient. In the most overwhelming of infections the number of bacteria present in a sample of spinal fluid will be much less than the level detectable. The electron capture detector is more sensitive but from the results it is clearly more susceptible to interference from impurities in the reaction mixture. These can often be reaction byproducts or excess reagent. The effect is that as the sensitivity increases so does the interference from the baseline resulting in a reduction in the level of detection (Figures 23, 24, 25 and 26).

The deposit of an overnight broth culture of Staphylococcus aureus hydrolysed with hydrochloric acid failed to give a satisfactory peak for N-acetyl glucosamine when derivatised with heptafluorobutyric acid anhydride. A very small peak was obtained at the correct retention time but far too small to positively identify the compound (Figure 27 and 28). Acid hydrolysates of Staphylococcus aureus and Streptococcus pyogenes treated with TRISIL gave very similar results. Very small baseline peaks were obtained which cannot be definitely identified (Figures 29,

30 and 31). In addition the organism extracts contain a number of large unidentified peaks. The Streptococcus pyogenes and Streptococcus pneumoniae cultures treated with sodium hydroxide also failed to produce identifiable peaks for N-acetyl glucosamine when examined using electron capture detection (Figures 32, 33 and 34). The small double peak at 10.7 and 11.1 minutes and the peaks at 12.5 and 13.7 minutes in Figure 34 occur again in Figure 32. The alteration of the attenuation accounts for the increase in the peaks sizes and therefore they are probably reaction by-products. Contrary to this the peak sizes are decreased in Figure 33 rendering this argument invalid.

Due to the lack of success in detecting the amino sugar in both acid and alkali extracts of the organisms an alternative approach was adopted. A culture of Staphylococcus aureus was exposed to lysostaphin to enzymically degrade it prior to undergoing acid or alkali hydrolysis. These extracts were then derivatised using MBTFA before examination using an electron capture detector (Figures 35, 36 and 37). Both the acid and alkali extracts had peaks identifiable as N-acetyl glucosamine. When the acidic extract was repeated using TRISIL of the two peaks in Figure 39 only the peak at 12.1 can be seen in figure 38. Both peaks are again recognised in Figure 40 when

N-acetyl glucosamine is added. The two peaks demonstrated in Figurew 39 may be due to the two isomers of N-acetyl glucosamine which occur in aqueous solution. It is therefore possible that only one of the isomers is extracted in Figure 38. When equal volumes of the extraction mixture and N-acetyl glucosamine solution are reacted together peaks at 12.1 and 15.6 are demonstrable. The relative proportions are changed from Figure 39 probably due to the large amount of the substance in the extract eluted at 12.1 minutes.

It is important that the amino sugars can be easily differentiated from similar compounds by their retention times. A variety of sugars along with the amino sugars were derivatised with TRISIL, this was the only reagent available at the time, prior to examination under the same conditions. The three alcohols of glucose: mannitol, sorbitol and dulcitol, all gave the same retention time of 13.5 minutes (Figures 41, 42 and 43). General purpose reagent grade glucose gave three main peaks, one of those almost certainly due to the presence of one of its alcohols (Figure 44). The pentose arabinose gave a much shorter retention time of 7 minutes (Figure 45). The disaccharide cellobiose gave a long retention time although solubility lead to a small peak (Figure 46). The disaccharide of

glucose, maltose, gave a number of peaks similar to those of glucose (Figure 47). This is possibly because maltose is unstable when reacted with TRISIL or that the sugar had deteriorated on storage. The amino sugars found in bacterial cell walls, N-acetyl glucosamine and N-acetyl muramic acid, gave distinct retention times easily differentiating them from the other sugars examined (Figures 48 and 49).

A number of problems have been recognised so far, the most important being a lack of sensitivity and the interferance due to unknown compounds. These problems are likely to be worse when clinical samples are examined because these involve the introduction into the system of a wide variety of unknown compounds. The earlier experiments illustrated that there is little difference between the use of either detector for sensitivity although the electron capture detector is more susceptible to baseline interferance. It was therefore decided to use the flame ionisation detector in conjunction with TRISIL to examine clinical specimens. Comparison of Figures 50 and 51 demonstrate this interferance quite clearly. The chromatogram of N-acetyl glucosamine in serum has many more peaks than the pure compound which makes peak recognition more difficult. In addition the peak area is greatly reduced in serum possibly

due to interfering compounds or binding by serum proteins. This reduction of peak does not appear to happen with mannitol (Figures 52 and 53). The polar amino group of the amino sugar may result in greater binding to the protein which may be why N-acetyl glucosamine is serum protein bound while mannitol is not.

Three samples of cerebrospinal fluid that were negative by conventional techniques failed to demonstrate a peak for N-acetyl glucosamine when derivatised with TRISIL (Figures 54, 55, 56 and 57). These same samples examined using the E.C.D. gave a great deal of background interference with the baseline failing to settle within the analysis time. When N-acetyl glucosamine was added to these samples at a concentration of 0.2 g/L very good peaks were obtained for (Figures 58, 59 and 60). Finally the addition of the extract of Staphylococcus aureus , prepared by treatment with lysostaphin followed by acid hydrolysis, to each of the samples prior to derivatisation resulted in many unidentified peaks on all traces and the absence of an N-acetyl glucosamine peak, FIGURES 61, 62 and 63.

CONCLUSIONS

The derivatisation of the amino sugars to render them volatile proved to be possible with both TRISIL and MBTFA. They reacted readily with the compounds producing adequate resolution and retention times markedly different from those of other sugars. The separation of the compounds was helped by prolonging the retention times using temperature programmed oven control. The other two reagents tested, MTBSTFA and heptafluorobutyric acid anhydride, proved unsatisfactory for this project. The first failed to produce a peak with N-acetyl glucosamine while the second produced a number of background peaks. The flame ionisation detector used in conjunction with TRISIL was not sensitive enough to detect the amino sugars at the concentrations expected in clinical samples. Electron capture detector used with MBTFA also failed to detect low levels, which was partly due to baseline interference when the sensitivity of the detector was increased. When extracts of very large numbers of bacteria were prepared the levels of the amino sugars were on the limit of detection. The extracts also contained, in much larger amounts, a number of other compounds which may be polymers of the two sugars. When applied to clinical specimens the technique failed to

perform adequately because of the lack of sensitivity coupled with the interference from the sample. The best method is a great deal less sensitive than conventional microscopy and culture techniques. It is possible however to use either of the reagents or detectors to identify the amino sugars alone or when mixed with other sugars in high concentrations.

REFERENCES

1) Wilson, G., Miles, A. and Parker, M.T. (1984) Topley and Wilson's Principles of Bacteriology, Virology and Immunity. 7th Edition. Edward Arnold.

2) Meadow, P. (1978) The chemistry and composition of microorganisms. In: Essays in Microbiology, Chapter 6. Editors Norris and Richmond. John Wiley and sons.

3) Stryer, L. (1988) Biochemistry. 3rd Edition. W.H. Freeman and Co.

4) Heymann, H., Manniello, J.M. and Barkulis, S.S. (1964) Structure of streptococcal cell walls. J. Biol. Chem., 239,9.

5) Cummins, C.S. and Harris, H. (1956) The chemical composition of the cell wall in some Gram positive bacteria and its possible value as a taxonomic character. J. Gen. Microbiol. 14, 583-600.

6) Salton, M.R.J. (1964) The bacterial cell wall. Elsevier.

7) Vacheron, M.J., Guinand, M., Michel, G. and Ghuysen, J.M. (1972) Structural investigations on cell walls of Nocardia species. Eur. J. Biochem. 29, 156-166.

8) Tabaud-Therisod, H., Massot, J.C. and Vilkas, E. (1975) Cell walls of Micromonospora strain (Actinomycetales). Biochimie 57, 825-828.

9) Pazur, J.H., Anderson, J.S. and Karakawa, W.W. (1971) Glycans from Streptococcal cell walls. J. Biol. Chem. 246, 1793-1798.

10) Pazur, J.H., Cepure, A., Kane, J.A. and Hellerquist, C.G. (1973) Glycans from Streptococcal cell walls. J. Biol. Chem. 248, 279-284.

11) Pazur, J.H. (1982) B-D-Glucose 1-Phosphate. J. Biol. Chem. 257, 589-591.

12) Ohno, N., Yadomae, T. and Miyazaki, T. (1982) Identification of 2-amino-2-dextroglucose residues in the peptidoglucan of Streptococcus pneumoniae . Carbo. Res., 107, 152-155. Elsevier.

13) Knox, K.W. and Hall, E.A. (1965) The Linkage between Components of the cell wall of Lactobacillus casei. Biochem. J. 96, 302.

14) Reistad, R. (1974) 2-amino-2-deoxyguluronic acid: a constituent of the cell wall of Halococcus species, strain 24. Carbohydrate Research, 36, 420-423.

15) Hudson, J.R. Morgan, S.L. and Fox, A. (1982) Quantitative pyrolysis gas chromatography - mass spectrometry of bacterial cell walls. Analytical Biochemistry, 120, 59-65.

16) Hase, S., Tsuji, Y. and Matsushima, Y. (1972) Isolation of a disaccharide, 6-O-(Strontium 2-Acetamido-2-deoxy-B-D-monopyranosyluronate)-D-glucose. J. Biochem., 72, 1549-1555.

17) Nasir-Ud-Din and Jealoz, R.W. (1976) The chemical structure of a fragment of Micrococcus lysodeikticus cell wall. Carbohydrate Research, 47, 245-260. Elseier.

18) Yanagihara, Y., Kamisango, K., Takeda, K., Mifuchi, I. and Azuma, I. (1983) Identification of 4-O-methylmannose in cell wall polysaccharide of Leptospira. Microbiol.

Immunol. 27, 8,711-715.

19) Guerrant, G.O. and Moss, C.W. (1986) Detection by gas chromatography of 3-Deoxy-D-manno-2-octulosonic acid and L-Glycero-D-manno-heptose in whole cells of Neisseria elongata . J. Clin. Microbiol., 23, 425-430.

20) Lomax, J.A., Gray, G.W. and Wilkinson, S.G. (1974) Studies of the polysaccharide fraction from the lipopolysaccharide of Pseudomonas alcaligenes . Exchem. J. 139, 633-643.

21) Hansen, L.B., Kuhnley, L. and Kenner, C.T. (1977) Stable G.L.C. column for fatty acid analysis. Applied and Environmental Microbiol., 52, 480-481.

22) Endreson, C. and Oeding, P. (1973) Purification and characterisation of serology active cell wall substances from Planococcus strains. Acta. path. microbiol. scand., 81, 571-575.

23) Novitsky, T.J., Chan, M., Himes, R. and Akagi, J.M. (1974) Effect of temperature on the growth and cell wall chemistry of a facultative thermophilic bacillus. J. Bacteriol., 117, 858-865.

24) Park, J.T. and Hancock, R. (1960) A fractionation procedure for studies of the synthesis of cell wall mucopeptide and of the other polymers in cells of _Staphylococcus aureus_ . J. Gen. Microbiol., 22, 249-258.

25) Norris, J. Ribbons, A. (1971) Methods in Microbiology Volume 5B. Academic Press.

26) Schleifer, K.H. and Kandler, O. (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev., 36, 407-477.

27) Johnsen. G.S., Endresen, C., Grov, A. and Oeding, P. (1975) Studies on Polysaccharide C of _Staphylococcus epidermidis_ . Acta. path. microbiol. scand., 83, 226-234.

28) Araki, Y., Nakatani, T., Nakayama and Ito, E. (1972) Occurence of N - nonsubstituted glucosamine residues in peptidoglycan of lysozyme - resistant cell walls from _Bacillus cereus_ . J. Biol. Chem., 247, 6312-6322.

29) Yoshida, A., Heden, C.G., Cedergren, B. and Edebo, L. (1961) A method for the preparation of undigested bacterial cell walls. J. Biochemical and Microbiological Technology

and Engineering, 3, 151-159.

30) Yamakawa, T. and Ueta, N. (1964) Gas chromatographic studies of microbial components. Japan. J. Exp. Med., 34, 361-374.

31) Amano, K., Williams, J.C., McCaul, T.F. and Peacock, M.G. (1984) Biochemical and immunological properties of Coxiella burnetti cell wall and peptidoglycan - protein complex fractions. J. Bacteriol., 160, 982-988.

32) Hofstad, T. and Lygre, H. (1977) Composition and antigenic properties of a surface polysaccharide isolated from Eubacterium saburreum strain L452. Acta. path. microbiol. scand., 85, 14-17.

33) Jantzen, E., Bryn, K., Hagen, N., Bergen, T. and Bovre, K. (1978) Fatty acids and monosaccharides of Neisseriaceae in relation to established taxonomy. N.I.P.H. Annals. Oslo, 1, 59-71.

34) Holdeman, L.V., Cato, E.P. and Moore, W.E.C. (1977) Anaerobe Laboratory Manual. 4th Edition. Virginia Polytechnic Institute and State University.

35) McDade, J.E., Shepard, C.C., Fraser, D.W., Tsai, T.F., Redus, M.A., Dowdle, W.R. and the Laboratory Investigation Team. (1977) Legionnaires Disease: Isolation of bacteria and demonstration of its role in other respiratory diseases. N. ENGL. J. MED., 297, 1197-1203.

36) Moss, C.W., Weaver, R.E., Dees, S.B. and Cherry, W.B. (1977) Cellular fatty acid composition of isolates from Legionaires disease. J. Clin. Micobiol., 6, 140-143.

37) Guerrant, G.O., Lambert, M.S. and Moss, C.W. (1979) Identification of diaminopimelic Acid in the Legionnaires disease bacterium. J. Clin. Microbiol., 10, 815-818.

38) Brooks, J.B., Dowell, V.R., Farshy, D.C. and Armfield, A.Y. (1970) Further studies on the differentiation of Clostridium sordelii from Clostridium bifermentans gas chromatography. Can. J. Microbiol., 16, 1071-1078.

39) Fugate, K.J., Hansen, L.B. and White, O. (1971) Analysis of Clostridium botulinum Toxogenic types A, B and E for fatty and carbohydrate content. Applied. Microbiol. 21, 470-475.

40) Farshtchi, D. and Moss, C.W. (1969) Characterisation of

bacteria by gas chromatography: Comparison of trimethylsilyl derivatives of whole cell hydrolysates. Applied Microbiol., 17, 262-267.

41) Gorbach, E.L. (1974) Rapid diagnosis Bact. fragilis infections by gas liquid chromatography of clinical specimens. Clinical Research, 2, 442A.

42) Phillips, K.D., Tearle, P.V. and Willis, A.T. (1976) Rapid diagnosos of anaerobic infections by gas liquid chromatography of clinical material. J. Clin. Path., 29, 428-432.

43) Brooks, J.B., Choudhary, G.,Craven, R.B., Alley, C.C., Liddle, J.A., Edman, D.C. and Converse, J.D. (1977) Electron capture gas chromatography detection and mass spectrum identification of 3 - (2' - ketohexyl) indoline in spinal fluids of patients with tuberculous meningitis. J. Clin. Microbiol., 5, 625-628.

44) Craven, R.B., Brooks, J.B., Edman, D.C., Converse, J.D., Greenlee, J., Schlossberg, D., Furlow, T., Gwaltney, J.M. and Miner, W.F. (1977) Rapid diagnosis of lymphocytic meningitis by frequency-pulsed electron capture gas liquid chromatography: differentiation of tuberculous,

cryptococcal and viral meningitis. J. Clin. Microbiol., 6, 27-32.

45) French, G.L., Teoh, R., Chan, C.Y., Humphries, M.J., Cheung, S.W. and O'Mahony, G. (1987) Diagnosis of tuberculous meningitis by detection of tuberculostearic acid in cerebrospinal fluid. The Lancet, July 18, 117-119.

46) Brooks, J.B., Daneshvar, M.I., Fast, D.M. and Good, R.C. (1988) Selective procedures for detecting femtomole quantities of tuberculostearic acid in serum and C.S.F. by frequency-pulsed electron capture G.L.C. J. Clin. Microbiol., 27, 1201-1206.

47) Brooks, J.B., Kellog, D.S., Sheperd, M.E. and Alley, C.C. (1980) Rapid differentiation of the major causative agents of bacterial meningitis by use of frequency-pulsed electron capture G.L.C.: Analysis of acids. J. Clin. Microbiol. 11, 45-51.

48) Brooks, J.B., McDade, J.E. and Alley, C.C. (1981) Rapid differentiation of Rocky Mountain spotted fever from chickenpox, measles and enterovirus infections and bacterial meningitis by frequency-pulsed electron capture G.L.C.: Analysis of sera. J. Clin. Microbiol. 14, 165-172.

49) LaForce, F.M., Brice, J.L. and Tornabene, T.G. (1979) Diagnosis of bacterial meningitis by gas liquid chromatography. II Analysis of spinal fluid. J. Infect. Dis., 140, 453-461.

50) Ferguson and Tearle (1977) Gas liquid chromatography in the rapid diagnosis of meningitis. J. Clin. Pathol., 30, 1163-1167.

51) Brooks, J.B., Kellog, D.S., Alley, C.C., Short, H.B., Handsfield, H.H.and Huff, B. (1974) Gas chromatography as a potential means of diagnosing arthritis. I. Differentiation between staphylococcal, streptococcal, gonococcal and traumatic arthritis. J. Infect. Dis., 129, 660-668.

52) Brooks, J.B., Craven, R.B., Schlossberg, D., Alley, C.C. and Pitts, F.M. (1978) Possible use of frequency-pulse modulated electron capture G.L.C. to identify septic and aseptic causes of pleural effusions. J. Clin. Microbiol., 8, 203-208.

53) Mitruka, B.M., Jonas, A.M. and Alexander, M. (1970) Rapid detection of bacteremia in mice by gas chromatography. Infect. Immun., 2, 474-478.

54) Mitruka, B.M., Jonas, A.M., Alexander, M. and Kundargi, R.S. (1973) Rapid differentiation of certain bacteria in mixed populations by G.L.C. Yale J. Biol. Med., 46, 104-112.

55) Lindberg, B., Lonngren, J., Thompson, J.L. and Nimmich, W. (1972) Stuctural studies of the Klebsiella Type 9 capsular polysaccharide. Carbo. Res., 25, 49-57.

56) Davis, C.E., Freedman, S.D., Douglas, H. and Braude, A.I. (1969) Analysis of sugars in bacterial endotoxins by G.L.C. Anal. Biochem., 28, 243-256.

57) Miller, G.G., Witwer, M.W., Braude, A.I. and Davis, C.E. (1974) Rapid identification of Candida albicans septicaemia in man by G.L.C. J. Clin. Invest., 54, 1235-1240.

58) Kiehn, T.E., Bernard, E.M., Gold, J.W.M. and Armstrong, D. (1979) Candidiasis: Detection by G.L.C. of D arabinitol, a fungal metabolite, in human serum. Science, 206, 577-580.

59) Deacon, A.G. (1986) Estimations of serum arabinitol for diagnosing invasive candidosis. J. Clin. Pathol., 39,

842-850.

60) Roboz, J., Suzuki, R. and Holland, J.F. (1980) Quantification of arabinitol in serum by selected ion monitoring as a diagnostic technique in invasive candidiasis. J. Clin. Microbiol., 12, 594-602.

61) Monson, T.P. and Wilkinson, K.P. (1979) D-mannose in human serum, measured as its aldonitrile acetate derivative. Clin. Chem., 25, 1384-1387.

62) Marier, R.L., Milligan, E. and Yuan - Da Fan. (1982) Elevated mannose levels detected by G.L.C. in hydrolysates of serum from rats and humans with candidiasis. J. Clin. Microbiol., 14, 557-562.

63) Monson, T.P. and Wilkinson, K.P. (1981) Mannose in body fluids as an indicator of invasive candidiasis. J. Clin. Microbiol., 14, 557-562.

64) James, A.T. and Martin, A.J.P. (1952) Gas liquid partition chromatography: the separation and micro-estimation of volatile acids from formic acid to dodecanoic acid. Biochem. J., 50, 679-685.

65) Pierce, A.E. (1985) Derivatization methods. In: Handbook and General Catalogue, 97-124. Pierce Chemical Company.

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