CHARACTERIZATION OF CARBOHYDRATES USING GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS)

By

DANIEL DERBIE ASRES

A Thesis

Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science

Department of Chemistry

University of Manitoba

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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To my wonderful wife and

loving parents.

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ABSTRACT

Methylation analysis has been widely used for determination of carbohydrate structures by mass spectrometry. Permethylation of monosaccharides yields mixtures of anomeric pyranosides and furanosides. One part of this thesis discusses the influence of some of the permethylation reaction parameters on the proportions of isomeric products obtained. The ratios of three, five- and six- membered ring products obtained from two permethylated monosaccharides, D-galactose and L-fucose, have been determined as a function of reaction parameters. The method of Ciucanu and Kerek[80] (methyl iodide in dimethyl sulfoxide (DMSO) in the presence of sodium hydroxide (NaOH)) was used as a starting point. We have investigated the effect of mixing DMSO, substrate, and NaOH for various times prior the addition of methyl iodide on the proportion of products obtained. In summary, higher temperatures and longer reaction times favored the main pyranoside product. Gentler conditions (i.e., shorter reaction times and lower temperatures) significantly favored the formation of the main furanoside product.

Another part of this thesis describes methods of preparing, isolating characterizing permethylated pyranoside and furanoside species of D-(+)-galactose and L-(−)-fucose. Permethylated standards for analyses of this

type are not readily available from chemical companies, more particularly the α and β furanoside forms.

Preparation of the derivatives is accomplished using the Ciucanu and Kerek[80] permethylation procedure. This method, when applied to native monosaccharides, produces four isomers. Separation of these isomers was carefully optimized and successful using a combination of silica column chromatography (flash) and continuous-elution thin layer chromatography (TLC). Separations were monitored using conventional TLC techniques and GC/MS.

In the third portion of this thesis, two different sets of methods that involve methanolysis, permethylation, and acetylation, were developed to identify the components of GM_1, a glycosphingolipid. Both methods are similar except for the order of the steps in the procedures, and identical products were obtained.

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INTRODUCTION

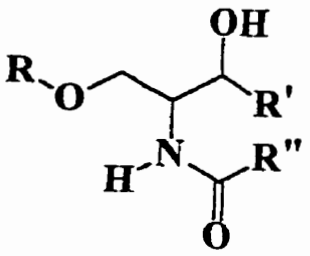
1.0 Classification and Structure of Sphingolipids

Sphingolipids are thermally labile, nonvolatile, complex molecules that are present in cell membranes of animals, plants, and in some lower forms of life. Sphingolipids contain three characteristic building block components; one molecule of sphingoid base, one molecule of fatty acid, and a polar head group. Each component has a different biological function.

Sphingolipids are classified according to their polar head groups as ceramides, glycosphingolipids, sphingomyelins, and phosphosphingolipids (Figure 1.1). The hydrophobic portion of sphingolipids contains a fatty acid (many possible types) that is amide-linked to a sphingosine chain or any other long chain aliphatic amine (sphingoid base). When this hydrophobic chain is attached to hydrogen (R = H in Figure 1.1) the resulting compound is a ceramide. Glycosphingolipids contain carbohydrates as their hydrophilic portion (polar head group). Sphingomyelins contain phosphorylethanolamine (phosphoryl choline) esterified at the ceramide 1-hydroxy group. Phosphosphingolipids have either aminoethyl phosphoric acid or N-methyl aminoethyl phosphoric acid as their water soluble polar heads.

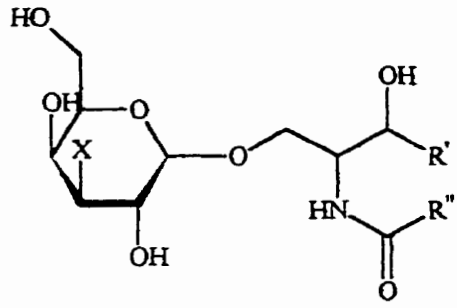
Glycosphingolipids themselves are classified as neutral glycosphingolipids, sulfatides, gangliosides, phosphoinositol-sphingolipids, depending on the substituent group of the carbohydrate (see Figure 1.2). Neutral glycosphingolipids are sphingolipids that contain hexoses, N-acetyl hexosamines and/or methyl pentoses as their carbohydrate chain. If the carbohydrate chain is a simple sugar, the glycosphingolipid is described as a cerebroside. When there is N-acetylneuraminic acid in the carbohydrate chain the sphingolipid is a ganglioside. Glycosphingolipids that contain sulfate esters are sulfatides, and those, which contain inositol monophosphate, are phosphoinositol sphingolipids.

The biological activities of sphingolipids vary substantially with their structures, so an understanding of these biological processes is intimately linked to the knowledge of structures involved. Determining the exact structures of complex sphingolipids requires elucidating the composition of the polar head group and determining the structure of both the sphingoid base and fatty acid chain in the ceramide unit. If the polar head group is a carbohydrate chain, its sequence and linkage positions also need to be determined.

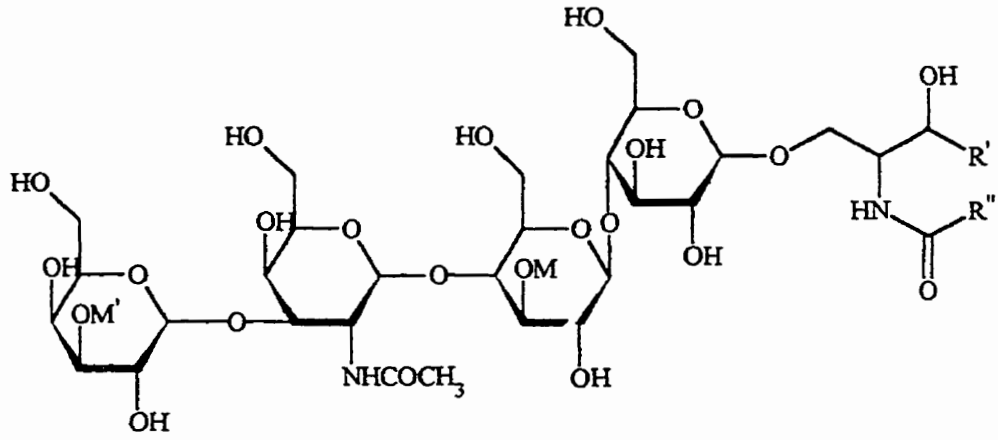


R group	Name
R = H	Ceramide
R = Carbohydrate	Glycosphingolipid
R = –P(=O)(O⁻)–O–CH₂–CH₂–N⁺(CH₃)₂–CH₃	Sphingomyelin
R = –P(=O)(OH)–O–CH₂–CH₂–N(H)–M (M = H or CH₃)	Phosphosphingolipid

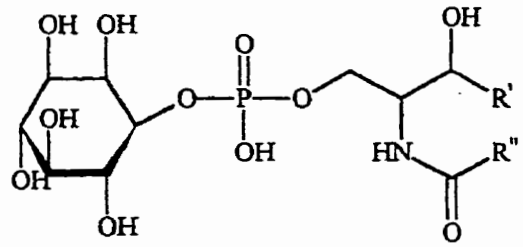
Figure 1.1: Basic structure of sphingolipids. R' can be equal to $CH{=}CH(CH_2)_{12}CH_3$ [for (4E)-sphingenine], $(CH_2)_{14}CH_3$ [for sphinganine], or other analogs. R" is an alkyl chain, and R is a polar head group.



X = OH Cerebroside
X = OSO_3H Sulfatide



M = H M' = NeuAc Monosialosyl ganglioside
M = M' = NeuAc Disialosyl ganglioside



Phosphoinositol-sphingolipid

Figure 1.2: Structures of cerebrosides, sulfatides, gangliosides, and phosphoinositol-glycosphingolipids.

1.1 Function and Occurrence of Gangliosides

The identification of gangliosides as sphingolipids and the determination of their constituents date back to the fundamental work of Klenk et al[1]. Gangliosides are found in human and animal brains. They are composed of sphingosine [(2S 3R)-2-amino trans-4-octadecene-1,3 diol], fatty acids, hexoses, and sialic acid i.e. N-acetylated or O-acetylated neuraminic acid. Gangliosides were first isolated and obtained in the pure form by the use of chromatographic methods[2]. The differences between gangliosides lie mainly in their carbohydrate moiety, but the nature of the sphingosine bases and fatty acids may also vary. Gangliosides are colorless crystalline substances which melt with decomposition. They are insoluble in non-polar solvents and their solubility in polar solvents (water, alcohols, etc) increases with the size of their sugar residue and their sialic acid content. In aqueous solutions, gangliosides form micelles having a molecular weights of about 200000-250000[3,4]. In dimethyl formamide or tetrahydrofuran, on the other hand, they form molecular solutions characterized by molecular weights of 1000-3000[5,6]. Gangliosides occur in high concentration in the brain and spleen of an organism. Brain gangliosides are qualitatively the same in most of the species[7], but spleen gangliosides show strong inter-specific variations, and so do the gangliosides of the visceral systems in general. Gangliosides are

also found in the erythrocytes, leukocytes, serum, kidneys, adrenal glands, placenta, milk, blood vessel wall, intestines, lungs, and in the lenses of the human eye.

The functional role of gangliosides is still speculative but they have been implicated in several neurological mechanisms[8-11]. They act as toxin binding receptors[12] and intermediates in cell-cell interaction[13]. Accumulation of gangliosides in the brain, due to genetic deficiency of enzymatic metabolism leads to severe neurological dysfunction[14].

1.2 Review of analytical methods for ganglioside analysis

1.2.1 Gas chromatography

Gas chromatography (GC) has been extensively used for the separation of carbohydrates and related polyhydroxyl compounds. Since these compounds are not sufficiently volatile for gas chromatography, many studies have been directed to preparing suitably volatile derivatives.

A number of reports have been made regarding determination of various sugars in the form of polymethyl ethers, polyacetyl esters, and polytrimethylsilyl ethers. Extensive reviews by Bishop[15], Kircher[16], and Wells et al.[17] compare separations achieved with various derivatives, and describe useful conditions for the determination of these compounds. An

important area of application of these techniques, in the biomedical field, is useful for the determination of various carbohydrates that occur in complex glycolipids. The first step consists of cleaving the glycosphingolipid into its sub-components. Wiegandt et al.[18, 19] pioneered the release of oligosaccharides from glycosphingolipids and gangliosides. According to their method, oligosaccharides are released by ozonolysis of the double bond of sphingosine followed by hydrolysis in aqueous sodium carbonate. Ohasi and Yamakawa[20] have been reported the GC analysis of the oligosaccharide portion of the glycosphingolipids, as trimethylsilyl derivatives of glycitols. Another method for releasing oligosaccharides from glycosphingolipids is based on the selective oxidation of the double bond of the sphingosine using periodate and osmium tetraoxide as a catalyst followed by a sequence of reactions catalyzed by sodium methoxide[21, 22], which results in cleavage of the glycoside-lipid bond. Both methods produce an intact oligosaccharide, which might be difficult to analyze by gas chromatography. However, the oligosaccharide portion of these glycosphingolipids can then be degraded to monomeric carbohydrates by hydrolysis or methanolysis and further derivatized to volatile forms of monosaccharides[23, 24]. The above degradation procedures break the ceramide portion of the ganglioside into different pieces, hence it is difficult to characterize the ceramide portion at

the same time. Enzymatic cleavage is also another possible method for degrading the carbohydrate portion of glycosphingolipids[25].

It has been suggested that methanolysis can be used for complete cleavage of glycosphingolipids into its sub-components[26, 27]. The products of methanolysis of glycosphingolipids are sphingoid bases and their methyl derivatives, fatty acid methyl esters, and methyl glycosides. These components can be separated by solvent extraction and analyzed by gas chromatography.

Methyl glycosides can be analyzed directly by GC[23, 28], or they can be converted into more volatile forms, which are even more suitable for GC analysis. Because of solvent equilibrium mixtures of pyranosidic, furanosidic, and anomeric forms of the glycosides, upon methanolysis, each derivatized saccharide produces more than one peak on the chromatogram[23, 24, 29]. Only a single peak is observed if the saccharides are converted to alditol acetates[30]. Fatty acid methyl esters can easily be analyzed without further treatment. Sphingosines are determined once they are converted to their trimethylsilyl forms[31, 32].

1.2.2 Mass spectrometry

Mass spectrometry (MS) has made a significant contribution to solving

structural problems in glycosphingolipid chemistry. Various researchers have been involved in developing derivatization procedures for MS and in employing soft ionization techniques[33] and collision induced dissociation (CID)[34-37].

Sweeley and Dawson[38] pioneered the determination of neutral glycosphingolipids and gangliosides as intact molecules by derivatization to their trimethylsilyl (TMS) ether forms followed by GC-electron ionization (EI) MS. Using their method, information about the masses of the monosaccharide units, the sphingoid base, and the N-acyl chain can be obtained. However, no intact molecular ions can be observed. Keranen[39] used methylation and acetolysis, followed by aqueous hydrolysis, to degrade the carbohydrate moiety into partially methylated alditols and hexosaminitol acetates, and to cleave the glycosidic bonds. A successful determination of the composition and linkage of carbohydrates can then performed by GC-EIMS of the acetates.

Freedman et al.[40] were able to determine gangliosides as intact molecules by converting them to their permethylated derivatives. Their spectra provided fragment ions, which arose from cleavages of glycosidic bonds and yielded information about the composition and the sequence of the carbohydrate chain. Urdal and Hakomori[41] used direct probe EIMS instead of GC-EIMS

of permethylated derivatives to characterize the tumor-associated gangliotriaosyl ceramide. The mass spectra of the permethylated gangliosides shows that the differences in the tumor associated gangliotriaosyl ceramide result from the variation in N-acyl composition of the gangliosides.

Wood et al.[42] demonstrated the use of field desorption ionization mass spectrometry (FDMS) to study a mixture of cerebrosides. Later Kushi et al.[43] and Handa[44] studied gangliosides using FDMS. The FD spectra showed weakly abundant $(M+Na)^+$, $(M+Na-H_2O)^+$ ions and several abundant fragment ions that arose from sequential cleavages of glycosidic linkages. The fragment ions provided information about the carbohydrate sequence. The $(M+H)^+$ ions, however, were not detected. After permethylation, the $(M+Na)^+$ ions became the base peaks but fragment ions were not detected, except for those derived from the loss of the N-acetylneuraminic acid (NeuAc) group.

Fast atom bombardment mass spectrometry (FABMS) has been extensively used for structural elucidation of glycosphingolipids[45, 38]. Egge and co-workers[46-48] have successfully studied the sequence and linkage positions of carbohydrate groups in $(M-H)^-$ and $(M+H)^+$ ions of underivatized and derivatized glycosphingolipids. They investigated the composition of the

$(M-H)^-$ ions of native monosialosyl lactosyl ceramide (GM_3) and also measured the *m/z* values of molecular ions of gangliosides from human Gaucher spleen. FABMS produced abundant $(M+H)^+$ and $(M-H)^-$ ions, but no fragment ions arising from cleavage of ceramide were observed. Thus, direct characterization of the ceramide portions of the individual gangliosides by FABMS was not possible. In addition, the contents of FABMS spectra depend on the type of the matrix used in the analysis[49, 50]. Several groups have investigated the characterization of underivatized gangliosides by FABMS in conjunction with CID[51]. Costello and co-workers[37] developed a systematic nomenclature for fragments observed in the CID mass spectra of $(M+H)^+$ and $(M-H)^-$ ions of glycosphingolipids. They also reported that $(M-H)^-$ ions of gangliosides decomposed by high energy CID predominantly yield sugar fragments that contain sialic acid units and other fragments that contain the ceramide moiety (C, B, and Y ion series)[51]. The lower energy CID spectra studied by Kasma and Handa[52] show mainly fragments of the Y ion series. Consequently, high energy CID of gangliosides provides more structural information than low energy CID. It should be noted, however, that the fewer but more characteristic peaks that appear in the low energy CID spectra make interpretation of the spectral data easier.

Derivatization of gangliosides prior to FAB and or CID-MS has been shown to be advantageous. Pahlsson and Nilsson[53] and Levery et al.[54] found that after permethylation, the abundance of $(M+H)^+$ ions significantly increased, and high mass gangliosides ($m/z \leq 5700$) could be desorbed.

FABMS analyses of both derivatized[55] and underivatized gangliosides generally require microgram quantities of sample, and the mass range is limited to several thousands of units depending on the instrument used and on the information needed. Another disadvantage of FABMS is that fragment ions which are diagnostic of the structure in the low mass region are obscured by matrix ions, and the CID technique can not be applied to low abundance ions in the molecular region. Electrospray ionization mass spectrometry (ESIMS) overcomes these problems and has been shown to be applicable to biological samples with high molecular weight[56], including intact gangliosides[57-60]. Ii et al.[61] reported on the characterization of gangliosides based on negative and positive ion ESI and CID-MS. They were able to determine the positional isomers of glycoconjugates and distinguish the two isomeric disialogangliosides, GD_{1a} and GD_{1b} by CID-MS/MS. In general, ESI requires lower amounts of materials than FAB for mass spectral analysis. So far, ESI and matrix assisted laser desorption/ionization coupled with time of flight analyzers (MALDI-

TOFMS) have achieved the highest sensitivity for characterization of derivatized glycosphingolipids[62-64]. Egge et al.[65] demonstrated that MALDI is useful for the molecular weight determination of native and permethylated neutral glycosphingolipids. Later, Juhasz and Costello[62] found that the application of MALDI to ganglioside analysis depends on the ionization mode (positive or negative) and on the choice of matrix and derivatization.

1.3 Derivatization methods

Gas chromatography and conventional ionization methods in mass spectrometry [EI and chemical ionization (CI)] are not adequate for the analysis of carbohydrates and polyhydroxy compounds because of the low volatilities and thermal labilities of these compounds. These techniques therefore require derivatization of these compounds or degradation to their sub-components for analysis to be possible.

Underivatized glycosphingolipids can be analyzed by soft ionization methods, such as, FAB, ESI, and MALDI. Intact derivatized glycosphingolipids are also more suitable for soft ionization methods and yield better signals.

So far, a number of different derivatization methods such as trimethyl silylation[66], permethylation[67], peracetylation[68], benzylation[69], and formation

of 2-aminopyridine derivatives[70] have been described. In this thesis, permethylation has been used for derivatizing monosaccharides and a ganglioside, GM_1. Hence, this method will be discussed in more detail.

1.3.1 Permethylation

Permethylation is a common procedure to derivatize carbohydrates and carbohydrate-containing compounds for analysis by gas chromatography and mass spectrometry. The aim of permethylation is to achieve etherification of all free hydroxyl groups.

An earlier permethylation procedure suggested by Denham and Woodhouse[71] and Haworth[72] used dimethyl sulfoxide (DMSO) and sodium hydroxide. Purdie and Irvine[73] reported the use of silver oxide and methyl iodide. Both the Haworth and Purdie techniques require repetitive methylations to obtain complete etherification (permethylation).

Kuhn and coworkers[74] improved Purdie's technique, by using silver oxide and methyl iodide as methylating agents, and dimethyl formamide (DMF) as a solvent. This procedure promoted complete methylation in a single step. Some modifications using barium hydroxide[75, 76] or sodium hydroxide[77] were reported to be better than using silver oxide. With these bases, the reaction

could be performed in DMSO, which is a better solvent than DMF for polysaccharides.

A simple and convenient method for permethylation of carbohydrates and carbohydrate-containing compounds is probably the Hakomori method[78]. In this method, carbohydrates are treated with the strong base methyl-sulfinylmethyl sodium in DMSO. The hydroxyl functions are thus ionized to give the corresponding polyalkoxide ions, which then react readily with methyl iodide. These two step reactions are shown below:

$$R\text{-}OH + CH_3SO\text{-}CH_2^- Na^+ \rightarrow R\text{-}O^- Na^+ + CH_3SOCH_3$$

$$R\text{-}O^- Na^+ + CH_3I \rightarrow R\text{-}O\text{-}CH_3 + NaI$$

Complete methylation may be accomplished in one step. Isolation of the methylated carbohydrate is simple and the procedure is suitable for both macro and micro-scale preparations. In spite of low yields (0.3 mol of permethylated derivative per mol of sugar), the Hakomori method has been used extensively in structural investigation of carbohydrates. The use of potassium tert-butoxide instead of sodium hydride improved stability of the reagent but did not substantially increase the yield of the reaction[79].

A procedure introduced by Ciucanu and Kerek[80] has a better permethylation yield (98% ± 2) upon a very short reaction time (6-7 min). This method is experimentally easier and yields cleaner products, and is therefore preferred

to other methods for many applications. The method is based on the use of solid bases such as powdered sodium hydroxide (NaOH) in DMSO and methyl iodide. In this procedure, methylation of carbohydrates occurs through the successive, base-catalyzed ionization of hydroxyl groups followed by reaction with the methylating agent. The methylating technique described by Ciucanu and Kerek[80] for carbohydrates is well adapted to the analysis of glycosphingolipids[67] and for other carbohydrate-containing biological compounds. Because it is simple, fast, and reliable producing good yields. It completely methylates all hydroxyl, carboxyl groups and amido nitrogens and has few by-products.

1.4 Methanolysis

The first step in the analysis of carbohydrate-containing biological compounds by GC/MS is cleavage into monosaccharide units. This is usually performed by hydrolysis with aqueous acid or by methanolysis. Formolysis[81] (with 90% formic acid at 100°C) is another possible method. Carbohydrate chains can also be cleaved selectively, for example 1→6 linkages of carbohydrates can be cleaved using acetolysis[82].

Methanolysis converts all carbohydrates to methyl glycosides or methyl ketals (neuraminic acid) and converts all free carboxyl groups such as those

in neuraminic acid and hexuronic acid to methyl esters. Methanolysis is performed with 0.5-1N methanolic HCl at 80-100°C[24, 27]. This reagent can be prepared by bubbling gaseous HCl into methanol. Methanolysis is a satisfactory procedure for the determination of all monosaccharides, and it enables all components to be analyzed together in a single procedure which is advantageous over other methods. Methanolic HCl can be removed by rotary evaporation. However, during this procedure, extensive loss of monosaccharides can take place, so it is preferable to neutralize the acid by silver carbonate before rotary evaporation. It has been observed that methanolysis can cause de-N-acetylation of aceto amido sugars. This can be overcome by re-N-acetylation using acetic anhydride.

In general all monosaccharides are reasonably stable in 2 M methanolic HCl at 85°C and 1M methanolic HCl at 100°C. However 3% of hexuronic acids can be lost in 2M acid at 85°C, and 9% in 1M acid at 100°C[27]. At higher concentrations of methanolic acid (2-4 M), all monosaccharides undergo destruction to various extents[27].

1.5 Goals of the present research

Most methylation analysis procedures for oligosaccharides yield mixtures of monosaccharides mainly composed of permethylated pyranoside and

furanoside species. Each of these species has a characteristic retention behavior in chromatography and a characteristic mass spectrum. Therefore, it is necessary to have permethylated monosaccharide standards to characterize those chromatographic and spectral behaviors. Hence, the objective of the first part of this research was to investigate the influence of some of the permethylation reaction parameters on the proportion of isomeric products obtained for monosaccharides. The ratios of three five-membered and six-membered ring products obtained for two permethylated monosaccharides, D-galactose and L-fucose, have been determined as a function of different reaction parameters such as temperature and time of reaction. In addition, column chromatographic and thin layer chromatographic methods were developed to isolate individual isomers of permethylated D-galactose and L-fucose with separation monitored using gas chromatography/mass spectrometry. This technique produced permethylated monosaccharide standards for further identification of unknowns.

The second part of this thesis focuses on developing a method for qualitative determination of gangliosides. In this case, two methods have been used to characterize a monosialoganglioside, GM_1. The first method involves the analysis of native and derivatized GM_1 by ESI-MS. In this method the

molecular weight of the ganglioside is determined. Due to fragmentation of GM_1 some peaks that indicate the nature of the carbohydrate and of the ceramide chain also appear in the spectrum.

The other method used gas chromatography/mass spectrometry to determine the individual components which make up the ganglioside. Thus the goal of this research was to investigate the performance of the GC/MS method for the analysis of ganglioside species and to develop GC/MS conditions for analysis of ganglioside sub-components. A combination of both methods can give detailed information about the type of ganglioside, hence this research emphasizes a structural investigation of gangliosides by combining information obtained from these methods.

1.6 Mass Spectrometric Techniques Related to This Research

1.6.1 Instrumentation

All mass spectrometers consist of three basic components: the ion source, the mass analyzer, and the detector. Ions can be produced from the sample in the ion source by many ionization methods. The mass analyzer separates ions according to their mass to charge ratio (*m/z*). The ions strike the detector and produce signals proportional to their relative abundances. A plot of the relative abundance of ions versus *m/z* ratios is recorded ; this

graph is known as a mass spectrum. A schematic representation of the components of a mass spectrometer is shown below.

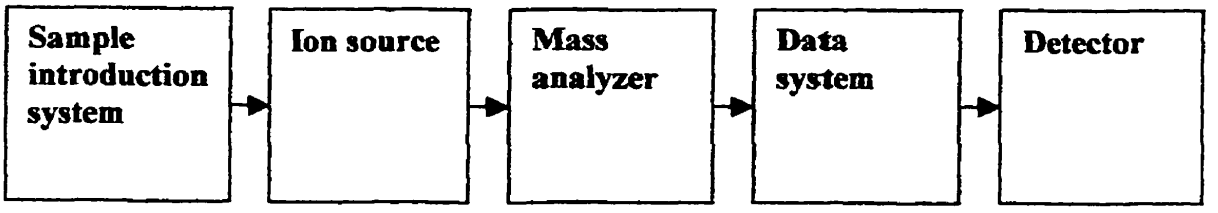


Figure 1.3: Schematic representation of a mass spectrometer.

1.6.2 Methods of ionization

Since J. J. Thomson developed the first mass spectrometer in 1912[83], the field of mass spectrometry has shown tremendous advances in instrumentation and applications. The formation of ions is an essential part of mass spectrometry. Typically, mass spectrometers have used electric and magnetic fields to separate ions according to their *m/z* ratios. Until recently, mass spectrometry was limited to relatively low molecular weight and volatile species that could be analyzed as gases, by EI [84] or CI [85]. More recently, newer ionization techniques, such as FAB [86], secondary ionization mass spectrometry (SIMS) [87], and plasma desorption ionization [88], have been developed for the characterization of various involatile compounds in the low kilo Dalton range (mass < 5 kDa). Currently, MALDI [89] and ESI [90] have emerged as effective bioanalytical tools for ionizing large biological

molecules. This chapter reviews only the ionization methods used in this research.

Table 1.1: Methods for ionization of bioorganic compounds

Ionization technique	**Acronym**	**Means of ionization**
Electron ionization	EI	Electron beam/electron transfer
Fast atom/ion bombardment	FAB, SIMS	Ion desorption/proton transfer
Matrix assisted laser desorption/ionization	MALDI	Photon adsorption/proton transfer
Electrospray ionization	ESI	Evaporation of charged droplets

1.6.2.1 Electron Impact Ionization

The electron impact ionization source was first used by Dempster in 1918[91] and subsequently developed by Nier[92] in 1947. A schematic diagram of the electron ionization source is shown in Figure 1.4.

A stream of electrons, emitted from a heated tungsten or rhenium filament are accelerated (usually 70 keV) towards the ionization region, where a small fraction of them interact with the gaseous sample molecules, and the remainder impinge on the trap electrode. Usually, a constant ionizing

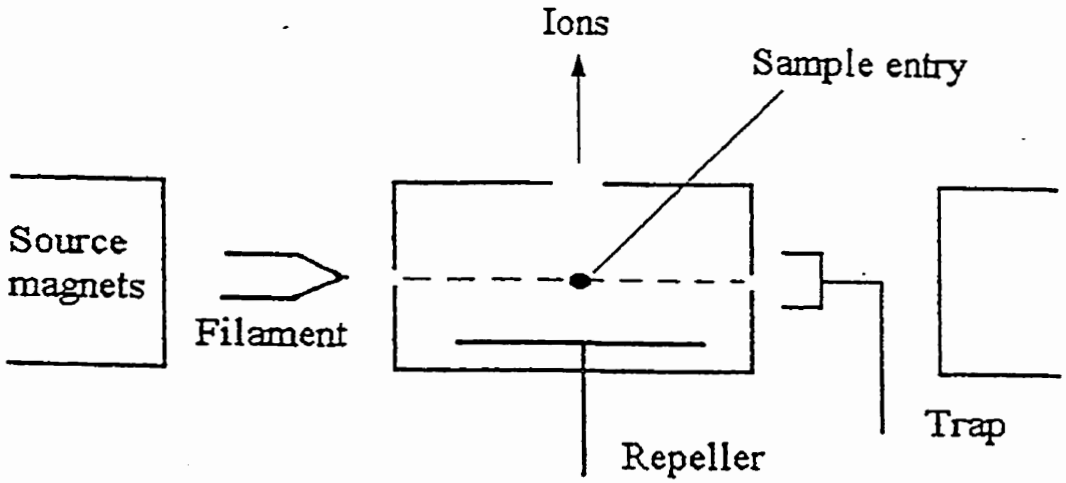


Figure 1.4: Schematic diagram of an electron impact source.

electric current is maintained by a feedback circuit to the filament power supply to maintain a constant trap current. The samples to be analyzed must be in the gas phase at a pressure of less than 10^{-4} torr in the ion source. Higher pressures will lead to ion/molecule interactions between the primary ions and the neutral molecules. Such reactions can distort the mass spectrum by producing new species such as the protonated molecule $(M+H)^+$. Gaseous sample molecules entering the ionization region interact with the beam of electrons to form positive and negative ions. It is estimated that only 1 in 100 molecules in the ion source is ionized[93, 94]. Usually more positive ions, which are in the form of radical cations, are formed rather than negative ions.

The production of ions can be classified under the following schemes;

Ionization

$$ABC + e \rightarrow ABC^+ + 2e \qquad (1)$$

Dissociation ionization

$$ABC + e \rightarrow AB^+ + C + 2e \qquad (2a)$$

$$ABC + e \rightarrow AC^+ + B + 2e \qquad (2b)$$

Ion pair formation

$$ABC + e \rightarrow AB^+ + C^- + e \qquad (3)$$

Electron capture

$$ABC + e \rightarrow ABC^{-} \quad (4)$$

Dissociative electron capture

$$ABC + e \rightarrow AB^{-} + C \quad (5)$$

Reaction (1) occurs when the energy of bombarding electrons exceeds the ionization energy of the molecule ABC. Greater electron energy is required for Reactions 2a and 2b because such reactions involve a chemical bond rupture. Under normal operating conditions (20-70 eV) Reactions 1, 2a, and 2b are dominant processes with a minor contribution from Reaction 3. The electron capture reactions (Reactions 4 and 5) are usually resonance processes, which have significant cross sections only over a very narrow range of energies usually, in the range 0-10 eV.

1.6.2.2 Electrospray Ionization

Dole[95] proposed the use of electrospray as a source of gas phase ions and for their analysis by mass spectrometry in 1968. Despite the promising early evidence of Dole, application of ESI to mass spectrometry waited until Yamashita and Fenn[96] introduced modern ESI-MS in 1984. Aleksandrov[97] and co-workers independently reported the coupling of an ESI source to magnetic sector mass spectrometers at approximately the same time. The

characteristic feature of ESI which distinguishes it from other ionization methods is that it is performed under atmospheric pressure and it generally imparts multiple charges to larger molecules. The formation of multiply charged ions leads to substantially lower *m/z* ratios. This allows the analysis of larger molecules by conventional mass spectrometers with a limited mass range.

ESI is an excellent way of ionizing samples in solution and has been used to study a wide range of compounds including proteins[98], glycoconjugates[99], nucleotides[100] (including DNA, RNA and oligonucleotides), fullerenes[101], synthetic polymers[102], and inorganic transition metal complexes[103].

The process of ESI involves three major steps: (1) formation of charged droplets at the electrospray capillary tip; (2) shrinkage of charged droplets by solvent evaporation and (3) production of gas phase ions from the very small and highly charged droplets.

In this process, a fine aerosol of highly charged droplets are created by applying a voltage of +2 to +4 kV to the metal capillary tip (Figure 1.5). The charged droplets produced shrink by solvent evaporation until they reach the Rayleigh stability limit[90] while the charge remains constant. At the Rayleigh limit, the electrostatic repulsion becomes just sufficient to overcome the surface tension that holds the droplets together, and fission of

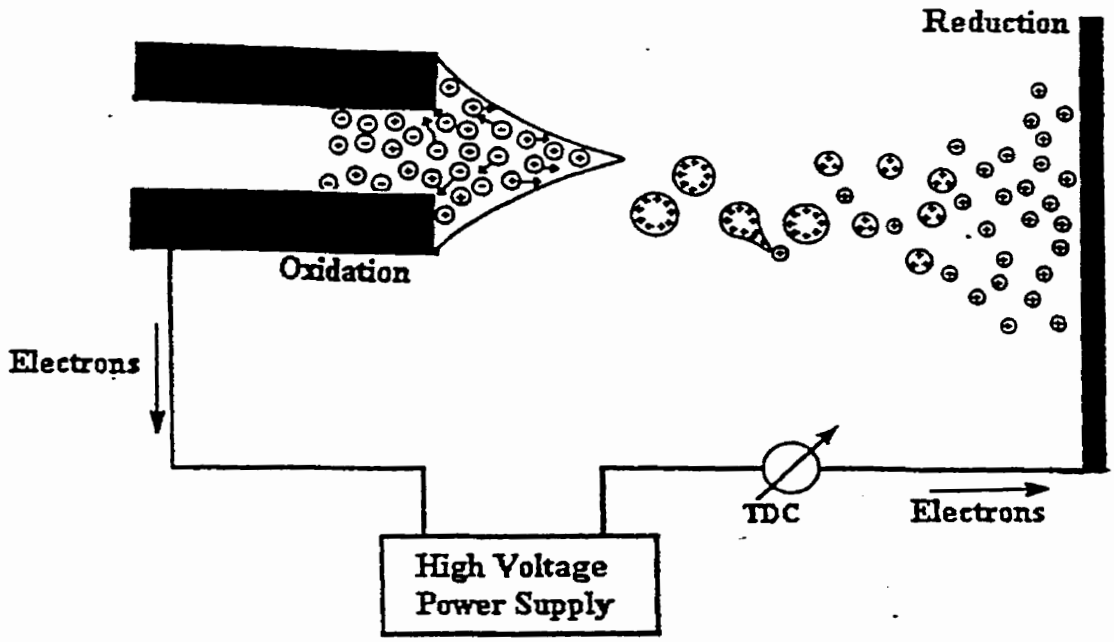


Figure 1.5: Schematic representation of charged droplet formation.

the droplets occurs. Such fragmentation is generally referred to as *coulombic fission*. It is unclear whether ions escape from droplets (i.e. field ionization) or solvent evaporates to leave ions (i.e., droplet evaporation), and the two mechanisms are likely be experimentally undistinguishable[104].

1.6.3 Mass Analyzers

Mass analyzers distinguish ions over a particular mass range by making use of appropriate electric fields, sometimes in combination with magnetic fields or field-free regions. The first mass analyzers, made in the early 1900s, used magnetic fields to separate ions according to their *m/z* ratios. Modern analyzers whose designs include variations on the early magnetic methods now offer high accuracy, high sensitivity, high mass range and an ability to give structural information.

The choice of mass analyzers depends on a number of interrelated factors such as mass range, resolving power, accuracy of mass measurements, ion transmission, sensitivity, scanning speed, and ease of use with ancillary equipment (such as chromatographic equipment). No one mass analyzer is suitable for all applications and the choice of instrument is determined by the type of problem under investigation.

Several types of mass analyzers exist. These include magnetic sectors, quadrupole mass filters, ion traps, ion cyclotron resonance devices, and time of flight analyzers. This chapter reviews double focusing, quadrupole mass filter, and ion trap mass analyzers, which were used in this research.

1.6.3.1 Double Focusing Mass Analyzer

The separation of ions according to their mass to charge ratios can be achieved with electric and magnetic fields alone or combined[105]. A single magnet can be used to deflect a beam of ions based on their *m/z* ratios. This type of instrument is known as single focusing mass analyzer. The resolution of this instrument is low, because it focuses ions of the same mass with different kinetic energies at different mass values. The resolution of the instrument can be improved by placing an electric sector before the magnetic analyzer. This combination is known as double focusing mass analyzer, as represented schematically in Figure 1.6. The combined use of electric and magnetic sectors gives a double focusing property. The ion beam is focused first for translational energy using the electric sector and then the mass-to-charge ratio is analyzed by the magnetic sector.

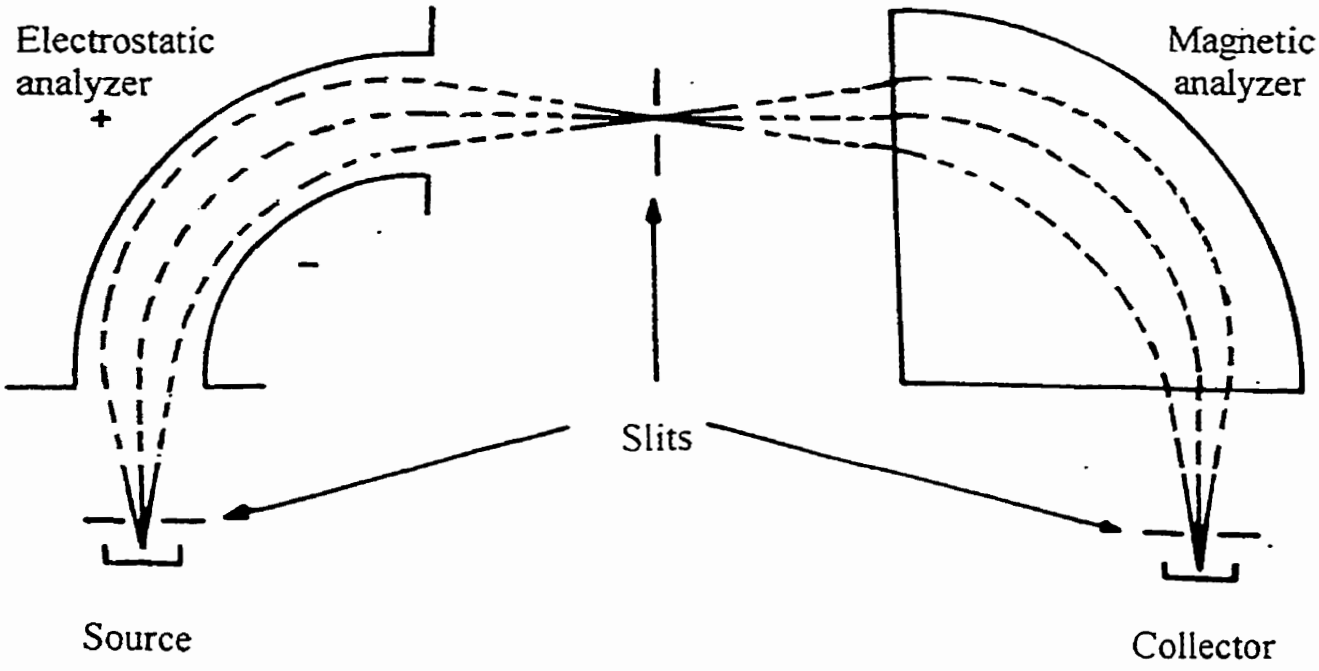


Figure 1.6: Schematic representation of a double focusing mass analyzer.

Velocity selection is achieved by passing ions through a radial electric field of radius R and strength E. The force on the ion is zE and to follow a path of radius R the necessary condition is:

$$zE = mv^2/R \quad (1)$$

Where m is the mass, v is the velocity and z is the charge of the ion.

Ions produced in the ion source are accelerated by a potential V, then the kinetic energy of the ions is given by

$$KE = 1/2\ mv^2 = zV \quad (2)$$

A combination of these two equations give

$$R = 2V/E \quad (3)$$

Equation (3) shows that all ions of the same energy, regardless of mass will be focused for a given value of E. Therefore, if the field E is kept constant, the electric sector focuses the ions according to translational energies. Accelerated ions which possess a translational energy, zV pass through a magnetic field of strength, B, experience a force given by

$$F = Bzv \quad (4)$$

This force causes the ions to follow an arc of a circle of radius r, thus the ions possess a centrifugal force that can be expressed as

$$F = mv^2/r \quad (5)$$

Combination of equations (2), (4), and (5) give

$$m/z = B^2r^2/2V \quad (6)$$

Since, for a given instrument, r is constant then

$$m/z = k\,(B^2/V) \quad (7)$$

Thus as shown in in equation 7 it is possible to focus ions of different *m/z* by varying either B or V. There are certain advantages and disadvantages to both methods[106], but usually V is kept constant and magnetic scanning is employed.

1.6.3.2 Quadrupole Mass Analyzers

A quadrupole mass analyzer contains four equidistant rod electrodes arranged symmetrically relative to the z-axis, as shown in Figure 1.7. The rods are electrically connected in opposite pairs to radio frequency RF and DC potentials, which form a hyperbolic electrostatic field[107-109]. Opposite electrodes have identical charges. DC voltages which are opposite in sign and AC voltages which are 180° out of phase of each other are applied to each electrode pair. For example in Figure 1.7 the potential applied to the horizontal pair of electrodes is:

$$V_+ = U + A\,\mathrm{Cos}\,\omega t \quad (1)$$

Where U is the applied DC voltage and A and ωt are the amplitude and frequency ($\omega = 2\pi f$) of the AC voltage, respectively. Similarly the

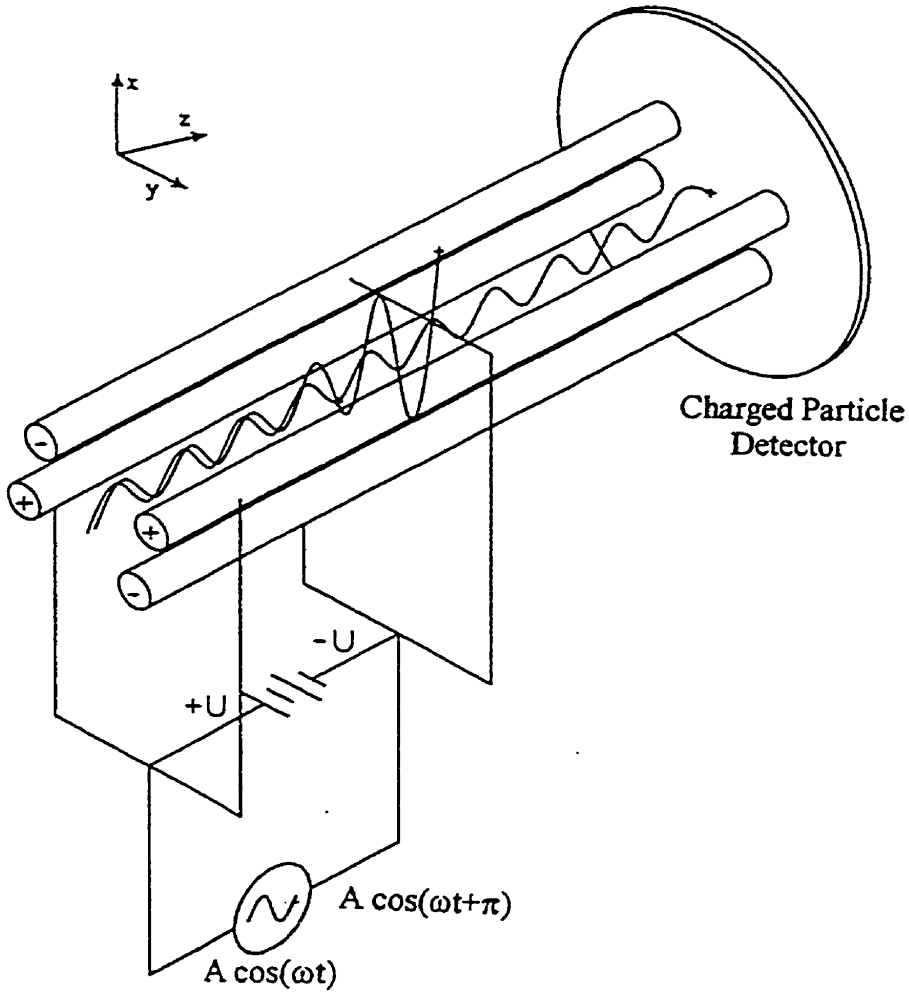


Figure 1.7: Schematic representation of a typical quadrupole mass analyzer. a time varying electric potential consisting of a DC component (U) and an AC component (A cosωt) is applied to oppositely paired elements.

potential applied to the vertical pair of electrodes is:

$$V_{-} = -U + A \cos(\omega t + \pi) \quad (2)$$

The superpositions of these potentials in space results in a quadrupolar electric field which can be expressed as:

$$V_{tot} = V (x^2 + y^2)/ r_o^2 \quad (3)$$

Where x, y, and r_o are the x-coordinate, the y-coordinate, and the radius of curvature of the quadrupole elements, respectively. Differentiating Equation 3 with respect to time and making a number of substitutions give a series of equations known as Mathieu equations[110, 111] that describe the conditions of stable trajectory. An accelerated particle that enters the mass analyzer oscillates in a complex manner, according to its *m/z* and the applied RF/DC ratios. For every value of these ratios, ions of only one *m/z* are able to pass completely through the filter and reach the detector. The others, having trajectories of greater amplitude, collide on one of the charged rods and discharged themselves. A fixed RF/DC ratio will allow ions of only one *m/z* value to pass through the filter. If the RF/DC ratio is gradually varied, (scanned) it is possible to obtain a full mass spectrum.

1.6.3.3 Quadrupole ion trap mass analyzers

In the early 1950s, Wolfgang Paul and his co-workers developed the quadrupole mass filter and the quadrupole ion trap, which both could be used to determine the *m/z* ratios of ions[112, 113]. A quadrupole ion trap is a three dimensional analogue of the quadrupole mass filter. This device is roughly the size of a tennis ball and consists of three hyperbolic shaped electrodes (Figure 1.8): two end-cap electrodes that are normally at ground potential and a ring electrode between them to which a radio frequency (RF) voltage, often in the MHz range, is applied to generate a quadrupolar electric field[114, 115].

Ions are trapped due to a RF field created by applying a RF voltage to the ring electrode. Trapping of ions is assisted by the presence of helium gas at a pressure of approximately 0.001 torr. This gas collisionally cools ions and forces them into the center of the ion trap[116]. Ions above a certain minimum *m/z* ratio are trapped and cycle in a predominantly sinusoidal motion. The magnitude of the RF voltage determines the amplitude and the frequency of the motion and the minimum *m/z* ratios of ions. This is described by the Mathieu parameter[116], q, which is expressed by $q=4V/(m/z)\omega^2 r_o^2$, where, V is the RF amplitude, *m/z* is the mass to charge ratio, ω is the RF angular frequency and r_o is the radius of the ring electrode.

Ion trajectories become unstable when q = 0.908. This creates a well-defined low mass cut-off value for a given value the amplitude of the RF voltage, V. Thus ions above the cut-off value will be trapped and ions below the cut off *m/z* value will be ejected.

Mass analysis of ions with quadrupole ion traps were originally accomplished by using either one of two techniques " mass selective stability detection" or "mass selective storage"[117]. Due to limited mass range and resolution, these methods of mass measurement were not practical for many analytical purposes. George Stafford and co-workers[118] developed a better technique known as "mass selective instability mode of operation". In this procedure, a change in operating voltages (additional RF voltage) is used to cause trapped ions of a particular *m/z* to adopt unstable trajectories. To record the mass spectrum, this RF voltage is increased with time so that ions of successively greater *m/z* ratios develop unstable trajectories and exit through perforations in an end cup, and become detected with an electron multiplier.

A variant of the mass selective instability mode is resonant ejection[114, 116]. In this mode, ions are brought into resonance by applying a fixed frequency auxiliary field across the two end-cap electrodes. The ions may then absorb sufficient power to exit the ion trap and be detected at the external detector.

This mode of mass instability enhances the resolution and sensitivity of the ion trap mass spectrometer. The other significant advantage of this method is that it increases the mass range of the instrument.

1.6.3 **Collision Induced Dissociation Mass Spectrometry (CID-MS)**

Collision induced dissociation (CID) is a widely employed mass spectrometric technique for probing analyte structures[119]. CID involves inelastic collisions of translationally excited ions with neutral inert gaseous atoms or molecules to generate fragment ions. The following generalized equation describes the fragmentation process by CID.

$$M^+ + N \rightarrow (M^+)^* \rightarrow X^+ + Y^+ + Z^+.$$

Where M^+ represents the "parent ion", N is the inert gaseous atom or molecule, $(M^+)^*$ is the activated ion with excess internal energy and X^+, Y^+, Z^+ are "product ions" or "fragment ions" or "daughter ions". ESI typically uses a triple quadrupole mass spectrometer with CID to perform structural analysis of sample molecules. The triple quadrupole mass spectrometer (QQQ) is composed of a series of three quadrupoles, each with different functions. A schematic representation of a triple quadrupole mass spectrometer is shown in Figure 1.9.

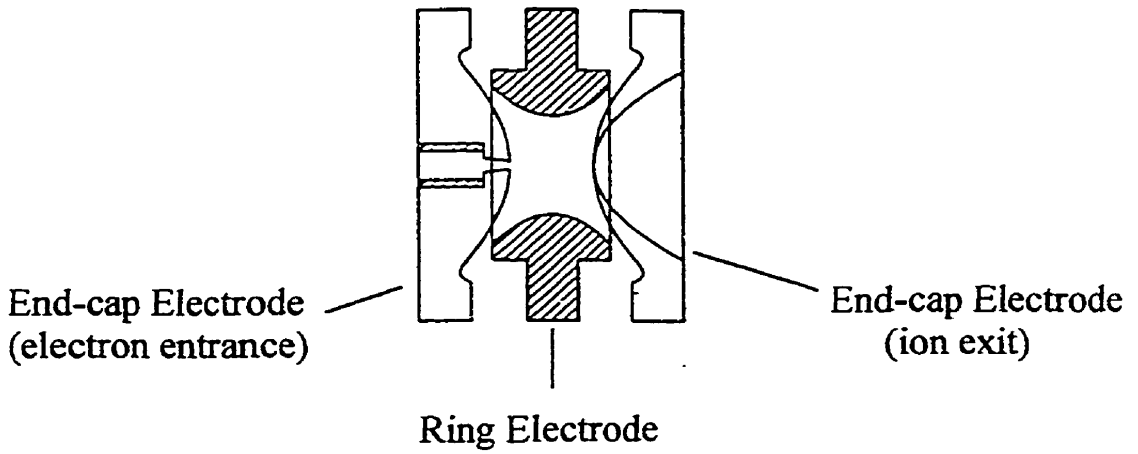


Figure 1.8: Schematic diagram of ion trap electrode.

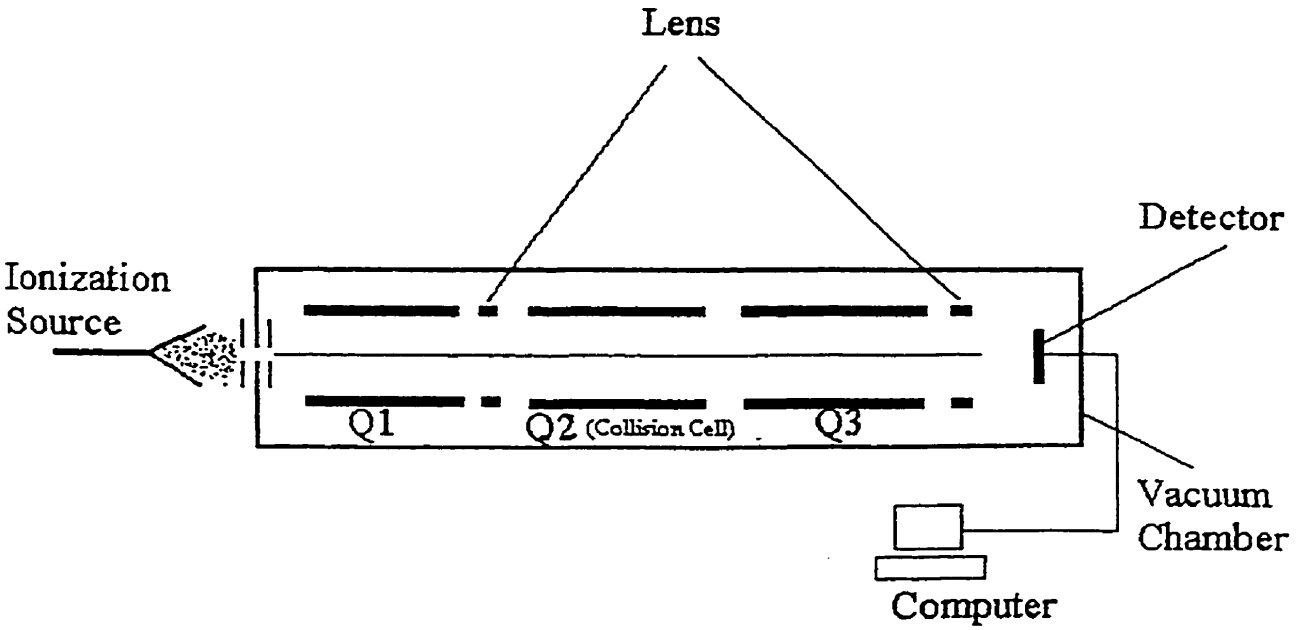


Figure 1.9 : Simplified schematic diagram of a triple quadrupole mass spectrometer.

The first quadrupole, Q1, is used to select ions with one *m/z* value of interest and introduce those ions into Q2. Q1 is operated by application of the appropriate RF and DC voltages. The second quadrupole, Q2, does not function as a mass analyzer. Instead it serves as a collision cell and is operated with a RF field only. Daughter ions, or fragment ions, are generated in this cell by colliding the parent ions with molecules of an inert gas (e.g. argon, helium). The emerging daughter ions are then accelerated to the third quadrupole, Q3, that serves to analyze them according to their *m/z* values. Ions exiting Q3 are then detected.

EXPERIMENTAL

2.0 Introduction

The experimental section of this thesis is divided into three parts. The first part describes permethylation of monosaccharides to produce fully methylated sugars. These methylated sugars are then analyzed by GC/MS, to determine the proportions of different glycoside isomers produced by permethylation.

In the second part, chromatographic methods were developed and adapted for isolating isomers of methylated monosaccharides (β and α pyranosides and furanosides). The isomers are then characterized by GC/MS.

The third part of this thesis describes the characterization of glycosphingolipids. Here, glycosphingolipid samples are permethylated, and their molecular weights are determined by ESI/MS to ensure complete methylation. The permethylated samples are then subjected to methanolysis and analyzed by GC/MS and ESI/MS.

2.1 Reagents

Monosaccharide samples D-galactose (99.9%) and L-fucose (99%) were obtained from Sigma Chemicals (St. Louis, MO) and used without further purification. Dimethylsulfoxide (DMSO), methyl iodide and solvents such as

heptane, cyclohexane, methanol, & dichloromethane were purchased from Fisher Scientific (Anachemia Fairlawn, NJ). Isopropanol, sodium hydroxide and chloroform were obtained from Mallinckrodt (Paris, KY). Deuterated chloroform was purchased from Cambridge Isotope Laboratories (Andover, MA).

Methyl-D-galactopyranoside (α and β) and methyl-L-fucopyranoside (α and β) were obtained from Sigma Chemicals. Methyl-D-galactofuranoside (α and β) and β-methyl-L-fucofuranoside were purchased from Color Your Enzyme (Kingston, ON, Canada).

Aluminum sheet TLC plates coated with 0.25 mm of silica gel 60 F_{254} were purchased from E. Merck (Darmstadt, Germany). Silica gel 60, 230-400 mesh, 40-63 microns, used for column chromatography was obtained from Mallinckrodt.

Monosialoganglioside (GM_1) was obtained from Sigma and, 3N methanolic HCl was purchased from Supelco (Bellefonte, PA).

2.2 Permethylation

Permethylation is a common procedure to derivatize carbohydrates for analysis by gas chromatography and mass spectrometry. Two procedures are considered to be most suitable for permethylating carbohydrates and

carbohydrate containing compounds. The first, which was originally described by Hakomori[78], uses the anion of DMSO (dimsyl anion $DMSO^-$) to remove hydroxyl protons from the sample prior to their replacement with methyl groups. The second procedure, which was introduced by Ciucanu & Kerek[80], uses solid sodium hydroxide base in DMSO and methyl iodide. The latter procedure is experimentally easier and yields a cleaner product and has therefore been used in this research.

2.2.1 Permethylation of Hexoses

Permethylation of D-galactose and L-fucose was performed according to the method used by Larson et al.[67], which is adapted from Ciucanu and Kerek's[80] method. Briefly, 4-5 mg samples of sugars were placed in screw-cap culture tubes and 450-1000 μL of DMSO and 32-82 mg of powdered sodium hydroxide were added to each sample. The mixtures were sonicated at room temperature for 0-60 min. Methyl iodide (100-200 μL) was then added and the mixtures were sonicated for 15-180 min at 15-50 °C.

The permethylated products were then recovered with chloroform (2 mL) extraction. The organic layers were washed four times with 2 mL of distilled deionized water. Chloroform was evaporated *in vacuo* and the permethylated products were dissolved in dichloromethane for further analysis by GC/MS.

2.2.2 Permethylation of reference standards

Permethylation of methyl-galactopyranosides and furanosides (α and β), and of β-methyl-L-fucofuranoside also followed the same procedure, however the mixtures were sonicated for a fixed time of 30 minutes after the addition of DMSO, powdered sodium hydroxide and methyl iodide.

2.2.3 Permethylation of glycosphingolipids[67]

A solution of glycosphingolipid, monosialoganglioside (GM_1) (1 mg/mL) was evaporated to dryness in a screw-cap culture tube. DMSO (1.5 mL), powdered sodium hydroxide (30-80 mg), and methyl iodide (1 mL) were added and the mixture was sonicated for about 2-3 hours at room temperature. The product was then extracted using two 2 mL portions of chloroform and washed 4-5 times with distilled deionized water. The solvent was evaporated *in vacuo*. The dried sample was dissolved in 1 mL of acetonitrile and analyzed by ESI/MS.

2.3 Methanolysis of glycosphingolipids

A complete release of sugars from many biological materials can be effected by methanolysis with 1-2 N anhydrous methanolic HCl at 85-100 $^{\circ}$C for 3 hours or more[24, 27]. Methanolysis appears to be as efficient as hydrolysis at

cleaving glycosidic bonds and also causes less destruction of carbohydrate than does aqueous acid hydrolysis.

To a methylated glycosphingolipid (0.5-1 mg) dried *in vacuo*, methanolic HCl (1.5 mL) was added, and the sample was heated at 90 °C for 6-14 hours. Powdered silver carbonate (ca. 100 mg) was added to neutralize the sample. The sample was centrifuged, and the supernatant was transferred to another tube and dried *in vacuo*. Chloroform (ca. 2 mL) was added and the sample was washed four times with deionized water. The solvent was evaporated *in vacuo* and the final product was analyzed by GC/MS, using dichloromethane as the solvent.

2.4 Instrumentation

2.4.1 Gas chromatography/mass spectrometry (GC/MS)

Analyses were performed on a Varian 3400 gas chromatograph (Varian Chromatographic Systems, Walnut creek, CA) coupled to a Finnigan Mat 800 ion trap detector (ITD) (Finnigan Corp., San Jose, CA) equipped with a ITD 4.10 data system. The second system used was a HP 5890 gas chromatograph (Hewlett Packard Canada, Calgary, Alberta) coupled to a HP5988 single quadrupole mass analyzer. The third system used was a HP 5890 gas chromatogram (Hewlett Packard) coupled to a VG 7070E-HF

double focusing mass spectrometer (VG Analytical Ltd., now Micromass, Manchester, England). For both GC/MS systems, scans were effected on a range of 50-650 Da. Each gas chromatograph was equipped with a 30 m × 0.25 mm i.d. DB-5 column (J&W Scientific Inc., Folsom, CA) with a 0.25 mm film thickness.

The transfer lines to the three mass spectrometers were maintained at 275 °C. All samples were injected as solutions in dichloromethane. In each case, the injection port temperature was 260 °C and the helium carrier gas flow rate was 20 mL/min.

The temperature program used for permethylated sugars was as follows: the initial temperature of 80 °C was held for 10 min, then the temperature was increased to 260 °C at the rate of 20 °C/min, held at 260 °C for 10 min, increased to 265 °C at the rate of 2 °C/min and finally held for 2 min.

The monosaccharide standard mixture and methanolysis products obtained from glycosphingolipids were analyzed using the third GC/MS system described above. The column temperature program was, as follows: the initial temperature of 100 °C was held for 2 min, then the temperature was increased to 280 °C at the rate of 10 °C/min, and finally held at 280 °C for 2 min.

2.4.2 Thin layer chromatography

Experiments to characterize permethylated galactose and fucose were carried out with 0.25 mm. silica-coated aluminium plates with 8-12 successive elutions. Each elution took 25-30 minutes and the plates were dried at room temperature for 10-15 minutes. The plates were developed in isopropanol-ethyl acetate-hexanes (1:9:40). Visualization of plates was performed by spraying 5% H_2SO_4 in ethanol and heating the plates for 10-20 min. at 80-110 °C[120, 121].

Preparative separations of permethylated galactose and fucose isomers were performed using continuous TLC elution and a mixture of isopropanol-ethyl acetate-heptane (1:9:40). Separation of the permethylated galactose isomers required 14-18 hours of continuous development, whereas in the case of fucose, only 7-9 hours. Samples of permethylated sugars (5-10 mg) were loaded onto 1mm thick 20 × 10 cm silica coated glass plates. A type of continuous elution chromatography, as described by Truter[122] and Eijnden[123] was used. In this method, a few centimeters of the top of the plate are exposed to the outside of the TLC chamber, thus causing the solvent to evaporate. The chamber is sealed with a cover, which has a narrow space at the center to let the plate out at the top. Development was carried out in a circular glass jar (16 × 11.5 cm i.d.).

Bands were scraped off the plates and dichlormethane was added to the powder to dissolve the product. The resulting solution was then filtered and processed for GC/MS. A small part of the plate on one side was sprayed by 5% H_2SO_4 solution and heated for 20-30 min at 80-100 °C, to locate the positions of the products.

2.4.3 Column chromatography

A 0.7 x 20 cm glass column, packed with 100 g of silica which resulted 13 cm length of packing, was used for column chromatographic separation. The best solvent found after optimization was a mixture of 3:1 cyclohexane ethyl acetate. The flow rate was 0.3-0.5 mL/min. The amount of sample (permethylated sugar) loaded onto the column for one separation was on the order of 5-7 mg.

2.4.4 Electrospray ionization mass spectrometry

Solutions of native GM_1 and permethylated GM_1 (20 μL) were injected into the mass spectrometer. The instrument used for these analyses was a Quattro-LC from Micromass (Manchester, UK) equipped with a Z-Spray™ electrospray source. Solutions were injected using a 20 μL loop, and the carrying solvent was at a flow rate of 10-12 μL/min (600-800 μL/h). A

syringe pump (KD scientific Inc, Boston, MA) was used to provide the carrying solvent (acetonitrile-water 50:50). The samples were sprayed with 3.50 kV electrospray needle voltage, and the cone-voltage (declustering voltage) was set at 60 V. The source block temperature was set at 110 °C and the desolvation temperature, at 130 °C.

Mass spectrometric measurements were taken in the positive ion mode for methylated samples and the negative ion mode was used for native GM_1. The scan rate was set at 300 u/s.

DISCUSSION

3.0 Discussion of the influence of reaction conditions on monosaccharide permethylation products

3.0.1 Permethylation products of D-(+)-galactose

Reducing sugars are usually stable in their crystalline state, but in aqueous solution, the hemiacetal linkage opens and reforms to give products with different ring sizes and anomeric configurations[124]. As a result, sugar solutions may be very complex and their composition can vary with the type of solvent, temperature, pH, and concentration[125].

Perlin[126] and MacKie[127] published two articles that describe the influence of solvents on solution equilibra of sugars. These articles showed that in solvents other than water, the α:β pyranose ratio is higher (if the α-anomeric hydroxyl group is axial) and that there is a greater proportion of furanose forms than in water. Maple and Allerhand[128] and Frank and coworkers[129] determined the composition of glucose solutions over a wide range of temperatures. Angyal[130] reported that an equilibrated D_2O solution of glucose contains 64% β-D-glucopyranose and 36% α-D-glucopyranose, at 20°C. He also reported the proportions of α and β glucopyranosides observed when D-glucose is dissolved in DMSO, without a base. The relative amounts reported were 45% α-glucopyranose and 55%

β-glucopyranose, with no mention of other two possible isomers (α- and β-furanoses). Similar proportions were found when D-glucose was dissolved in pyridine. In this solvent, the amount of β-glucopyranose decreased by 2% and both α- and β-glucofuranosides make up for that 2%. These proportions, although measured in a non-basic environment, reflect the numbers reported by Ciucanu and Kerek[80] and our laboratory for permethylated derivatives[131]. In the same publication, Angyal[130] reported the following proportions for the forms of D-galactose in pyridine: 31% α-galactopyranose, 46% β-galactopyranose, 5% α-galactofuranose, and 18% β-galactofuranose in comparison to 30%, 64%, 2.5%, and 3.5% respectively in water[124]. Unfortunately, no such data were given for galactose in DMSO. According to Ferrier and Collins[132], aprotic solvents [e.g. N,N-dimethyl formamide (DMF) and DMSO] do not solvate sugars as well as water does, and as a consequence they enhance the anomeric effect and yield a higher proportion of five-membered ring compounds. The figures given above for galactose in pyridine *vs.* water agree with this statement.

It has been suggested that a basic environment will influence the rate of mutarotation in aqueous solutions. Hence, it will affect the proportions of sugar forms in solution. It is difficult to evaluate the pH values of a basic solution in DMSO but typically, DMSO solutions of NaOH are more basic

than the corresponding aqueous solutions. In assuming that the permethylation method of Ciucanu and Kerek[80] was performed in a more basic environment, substrates such as D-glucose (pKa=12.28) and D-galactose (pKa=12.30) should be in their monoanionic forms[75], if not dianionic[133]. The proportions of anomeric species as opposed to electrically neutral species have not been discussed in detail in the literature. The ability of hydroxide ions *vs.* silver, barium and strontium oxides to act as acid receptors has been discussed as a possible factor influencing the proportions of ionic/nonionic substrate molecules in methylation reactions[134].

Walker et al.[75] reported complete methylation of reducing sugars using silver oxide and methyl iodide in DMF solvent. In their procedure, a mixture of at least two and sometimes four completely methylated glycosides (pyranose and furanose forms) were obtained. According to their report, galactose yielded 80% methyl-2,3,5,6-tetra-O-methyl-α-D-galactofuranoside (αgf), 10% methyl-2,3,4,6-tetra-O-methyl-β-D-galactofuranoside (βgf), and 10% of (α- and β-) methyl-2,3,4,6-tetra-O-methyl-D-galactopyranoside (αgp and βgp). Gee[135] reported the separation of fully methylated galactosides by thin layer chromatography. She observed three spots in order of decreasing R_f values, corresponding to βgf, αgf overlapping with βgp, and αgp.

According to our permethylation procedure D(+)-galactose yielded two major products and two minor components. A composition of (7±2)% βgf (Product 1), (61±2)% of βgp (Product 2), and (32±2)% of αgf (Product 4) was found when using Method 2 in Table 3.1. A very small proportion of αgp (Product 3) was obtained, and this compound co-eluted with 4. Figure 3.1a shows the GC/MS total ion current (TIC) obtained from permethylation using Method 2 as a bench mark for starting the present investigation. A similar trace was obtained from Method 1, which involves a longer sonication time than Method 2, both before and after addition of methyl iodide. The sonication of DMSO, galactose, and NaOH mixture was aimed at making the solution as homogeneous and base saturated possible. Interestingly, a shorter sonication time (Method 3, Table 3.1) was found to have an important impact on the proportions of Products 2 and 4. Previous reports demonstrated the importance of temperature[125, 136] and pH[80] on mutarotation and anomerization of sugars. Smirnyagin and Bishop[137] studied the kinetics of glycosylation of D-galactose, and their results confirmed an earlier observation for pentoses[138]. According to their results when D-galactose is subjected to methanolysis in methanolic HCl, galactofuranosides are preferentially formed first and their relative concentrations decay as they isomerize into galactopyranoses or anomerize.

These authors also showed the effect of temperature on the relative concentrations of galactosides (furanose and pyranose forms) for periods of time of more than 200 h.

Collins and Ferrier[132] reported that when galactose is heated under reflux in methanol containing 2% HCl, 12 h or more are necessary to reach equilibrium between the different glycoside forms. It seems reasonable to assume that a similar situation exists for anionic galactose in basic solution, in which case the first sonication time involved in Methods 1 to 3 would be too short for an equilibrium to be reached. If the addition of CH_3I "interrupts" the progression towards equilibrium, a systematic variation in the proportions of permethylated galactosides should be observed, which appears to be the case from Method 3 to Method 1. The proportions of Product 1 and 2 increases, while Product 4 becomes less abundant.

In methanolic HCl solution, glycosides would be far from equilibrium after 45 min or 1 h of reflux, which corresponds more with the times used by us for the first sonication. Even if our experimental conditions are far different from the methanolysis experiment used by the above researchers[137, 138], this rationalization helps in understanding that our starting material (galactose) in DMSO is changing with time and temperature. Thus Method 3 yields different proportions of products relative to Method 2, and 1, which involve

Meth. No.	Quant. Gal. (mg)	Vol. DMSO (μL)	Quant. NaOH (mg)	1st Sonic. (min)	Vol. CH_3I (μL)	2nd Sonic. (Reac'n time, min)	Temp. (°C)	Prod. 1 (Rel. Conc., %)	Prod. 2 (Rel. Conc., %)	Prod. 4 (Rel. Conc., %)
1	4.7	450	35.7	60	100	180	20-50	12	67	22
2	4.9	450	35.6	45	100	85	20-50	7	61	32
3	4.7	450	35.5	20	100	85	20-50	7	45	48
4	5	500	31.8	0	100	85	20-50	9	20	71
5	5	500	31.8	0	100	15	20-50	10	23	68
6	5.3	500	82.4	0	100	15	20-25	5	32	63
7	8.2	1000	81.9	0	100	10	10-14	5	14	81

Table 3.1: Experimental conditions of permethylation of D-(+)galactose, based on the method of Ciucanu and Kerek[80]; proportions of products relative to the most abundant one (± 2%)

Product 1: Methyl-2,3,5,6-tetra-O-methyl-β-D-galactofuranoside (βgf)
Product 2: Methyl-2,3,4,6-tetra-O-methyl-β-D-galactopyranoside (βgp)
Product 4: Methyl-2,3,5,6-tetra-O-methyl-α-D-galactofuranoside (αgf)

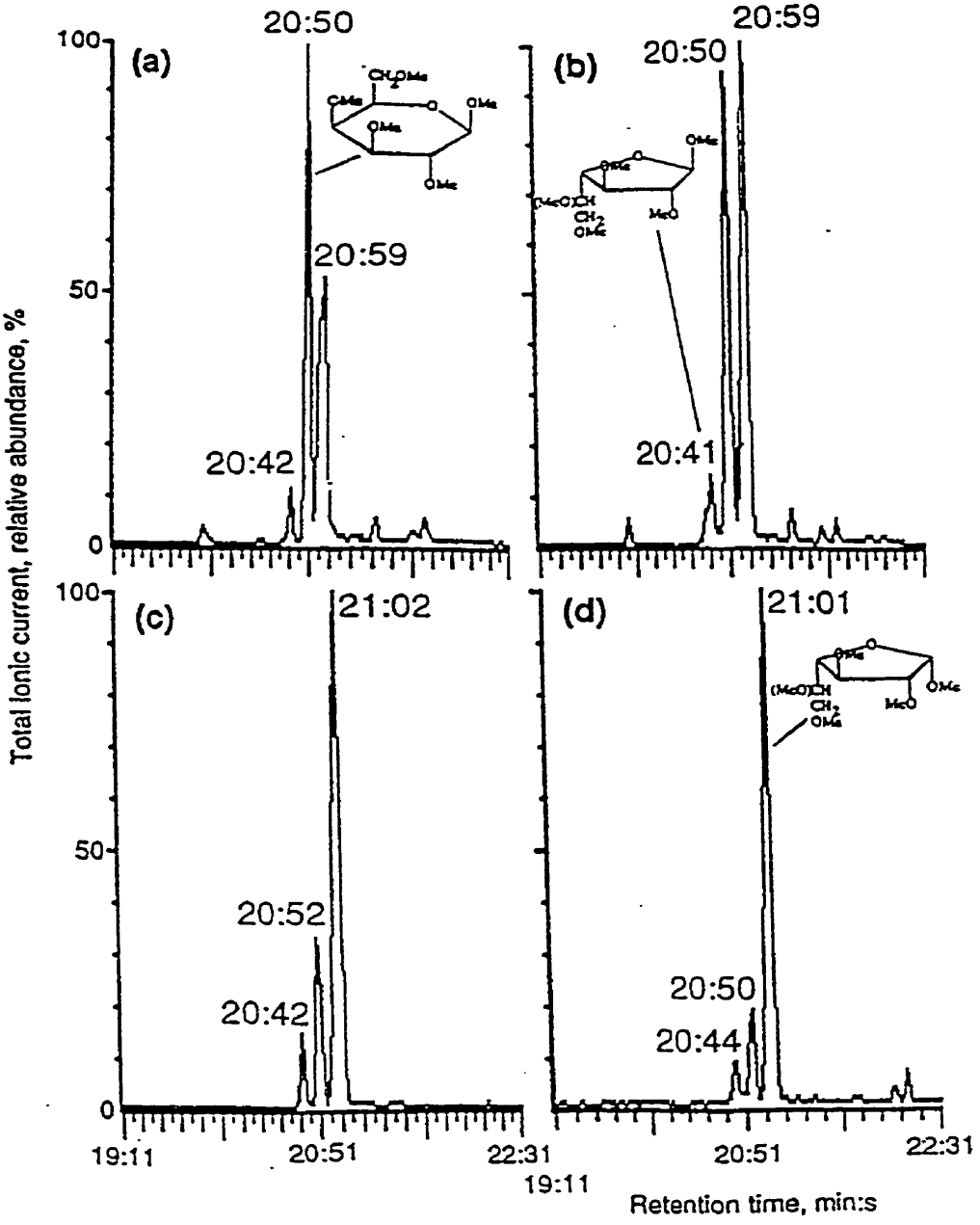


Figure 3.1: GC-MS total ion chromatograms obtained for the permethylation products of D-galactose. Referring to Table 3.1, (a) Method 2, (b) Method 3, (c) Method 5, and (d) Method 7. The three time-labelled peaks on each chromatogram correspond to Products 1, 2, and 4, in retention time order.

longer pre-reaction sonication times. The sonication time was further reduced to zero with yet another increase in the proportion of αgf (Product 4) to 71% of the total (see Method 4, Table 3.1).

In the fifth experiment (Method 5, Table 3.1) the reaction time with CH_3I was reduced to 15 minutes. The results were very similar to those of method 4, indicating that the reaction was complete after 15 minutes. Figure 3.1c illustrate these results. Collins and Ferrier[132] indicated the possibility that permethylated hexosides may mutarotate in pyridine, but no mention was made of DMSO. The similarity in proportion of products obtained with Method 4 (85 min reaction time) and Method 5 (15 min) suggests that no mutarotation takes place once permethylation is complete.

As pointed out by Ciucanu and Kerek[80], there is a strong relationship between pH of the starting solution (mixture of monosaccharide, DMSO, and NaOH) and equilibrium between anomeric species in solutions. The effects of bases on hexoses in aqueous solutions are well documented[139]. In our experiment when the amount of NaOH was increased (see Method 6, Table 3.1), there was a small increase in the amount of βgp (r.t. 20:50 min), at the expense of Products 1 and 4. Our results seem to indicate that high pH values (longer pre reaction sonication, NaOH saturation) favors formation of permethylated β-galactopyranoside. In the opposite situation, a lower NaOH

concentration (no pre-reaction sonication) seems to favor production of permethylated galactofuranoside (α or β) and yield anomer proportions that are in a better agreement with those reported by Walker et al.[75].

Epimerization, which is one of the common effects of basic environments, did not seem to occur in our experiments. The permethylated products obtained with Methods 1-7 had GC retention times and mass spectra that characterized fully methylated galactosides.

In aqueous solutions where the concentration of the base exceeds 1%, sugars may be converted into isomeric deoxyaldonic acids (saccharinic acid, isosaccharinic acids) [139]. Also, prolonged treatment of hexoses with bases may yield 3-carbon fragments such as 2-hydroxypropionaldehyde, pyruvic acid, methylglyoxal, and lactic acid[139]. No such information could be found for solutions of sugars in DMSO. However, these facts may explain the presence of side products after long first sonication and reaction times. We made no further attempts to identify the side products, present at low level relative to the fully methylated galactosides (Figures 3.1a and 3.1b).

In previous experiments (Methods 1-6, Table 3.1), the reactions were started at room temperature (ca. 20-25°C); however the temperature of sonication bath gradually increased, reaching 50°C after 1 h of reaction. Holding the temperature of this reaction constant at 50, 30, 20, 15, and 10°C showed that

low temperature (10°C and 15°C) favored formation of αgf (Product 4) and almost eliminated the six membered ring species, βgp (Product 2) (Figure 3.1d). Temperatures above 20°C (Methods 4 and 5) all yielded approximately the same proportion of products. Although the reaction appeared to be complete after 15 minutes at the lower temperatures, we felt that it was safer to allow 1 h for permethylation. We believe that a lower starting temperature will modify the initial proportions of galactosides, as observed by Smirnyagin and Bishop[137] for methyl glycosides at 25°C and 44°C. Conducting permethylation at low temperature such as 10-15°C is also likely to slow down by-product formation.

Method 7 (Table 3.1) provided a 81% relative yield of αgf (Product 4) (Fig 3.1d), indicating that this method might be preparatively useful. Vain attempts were made to prepare βgp (Product 2) exclusively. Large concentrations of NaOH and long sonication times before addition of methyl iodide were investigated, however very similar results to those obtained with Method 1 were obtained. The only effect associated with excess NaOH and long sonication permethylation-times was the appearance of small amounts of unidentified side products, possibly saccharic-type acids and smaller fragments as discussed above.

The effect of altering the amounts of DMSO and methyl iodide relative to other reagents was also investigated. No significant changes in the proportions of the products were observed.

Standard permethylated α and β galactopyranosides and galactofuranosides were prepared as described above (see experimental) and characterized by GC/MS. Permethylated α-galactopyranosiae (αgp) had a retention time of 21 min, the same as permethylated α-galactofuranoside, (αgf) thus it was difficult to identify each of them using GC. However, the mass spectra of these compounds were very different in the *m/z* 80-120 region (see Figures 3.2a-3.2d). Permethylated α-galactofuranoside produces an abundant ion at *m/z* 101 and permethylated α-galactopyranoside, at *m/z* 88. Based on these differences and by comparing their spectra with NIST library spectra[140], Product 4 was identified as αgf. A mixture of the four permethylated standards was run, and three peaks appeared on the GC-chromatogram, with αgf and αgp appearing at the same retention time (Figure 3.3). The mass spectrum of this peak showed almost equally intense peaks at *m/z* 88 and *m/z* 101. This can be an evidence for the presence of the two compounds, and it can be used as an indicator to detect the presence of αgp in our sample. The standard permethylated β-galactopyranoside came out at 20:50 min. and therefore it was possible to positively identify Product 2 as permethylated

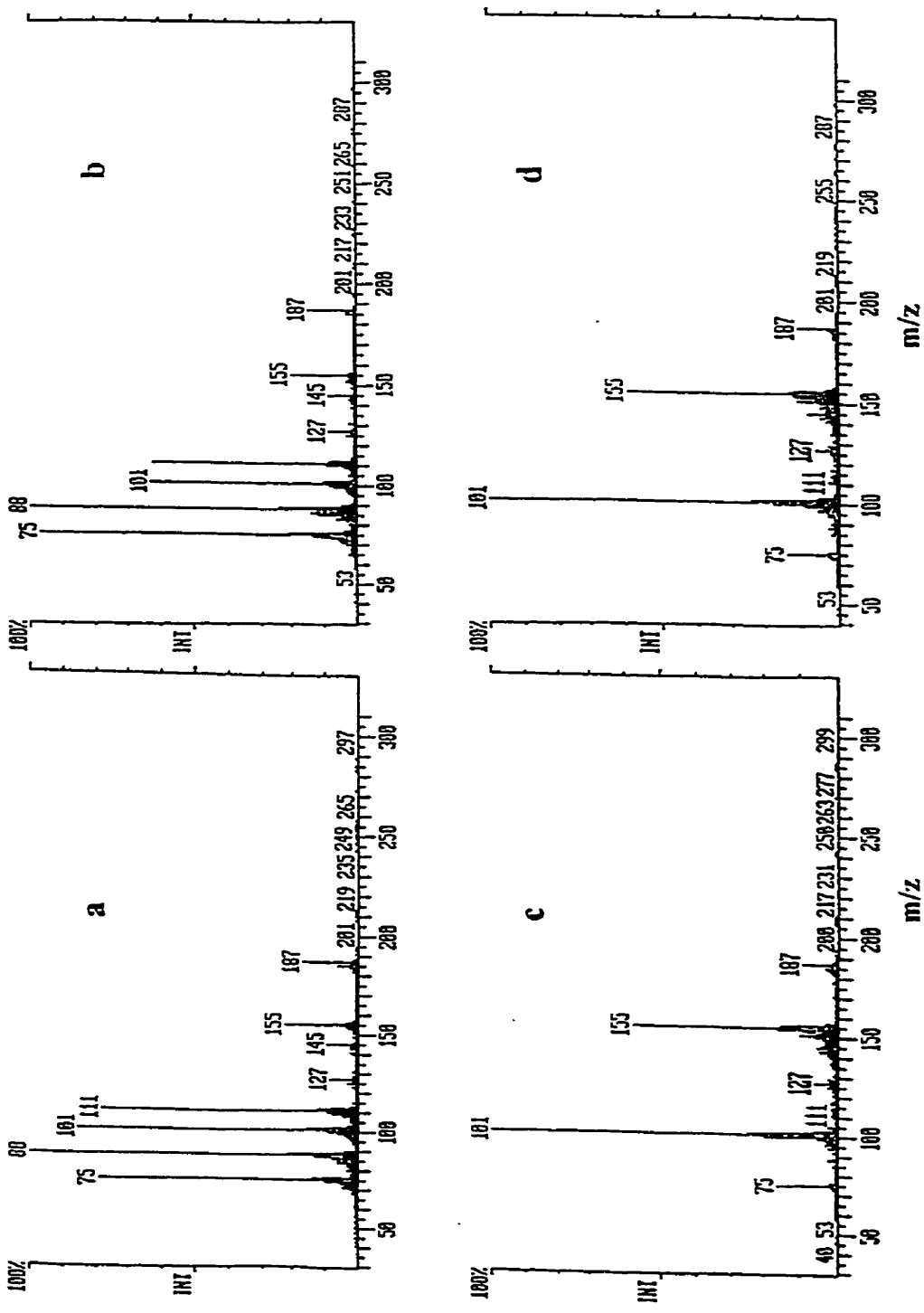


Figure 3.2: Mass spectra of permethylated galactose standards obtained using the Finnigan MAT ion trap system. a) Permethylated β-galactopyranoside, b) Permethylated α-galactopyranoside, c) Permethylated β-galactofuranoside, d) Permethylated α-galactofuranoside.

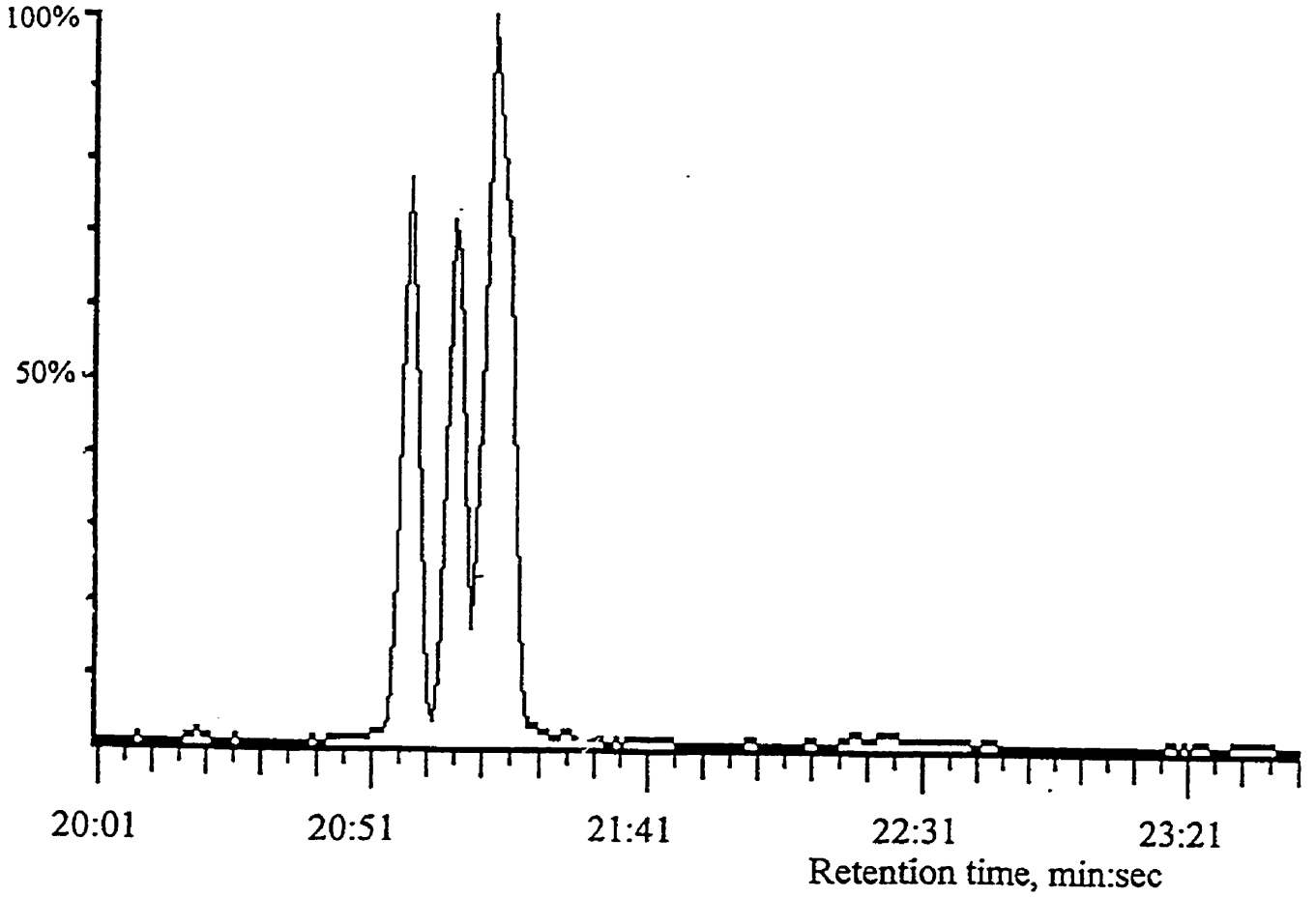


Figure 3.3: GC-MS total ion chromatogram obtained for the permethylated galactose standards mixture (α and β permethylated galactopyranosides and furanosides).

β-galactopyranosde (βgp). The βgf standard had a retention time of 20:43 min, which confirmed the identity of Product 1 as permethylated β-galactofuranoside (βgf). The mass spectra of the standard permethylated compounds are similar to those of our sample. A comparison of the mass spectra with NIST library data is also a supporting evidence for our characterization.

3.0.2 Permethylation products of L-fucose

It has been reported that partial methylation of L-fucose using Fischer's procedure[141] yields 6% of methyl-α-L-fucofuranoside, 13% of methyl-β-L-fucofuranoside, 54% of methyl-α-L-fucopyranoside and 27% of methyl-β-L-fucopyranoside. Similarly, using our permethylation procedure, a mixture of four isomers was obtained, two pyranosides and two furanosides. Figure 3.4 show the GC/MS TIC trace obtained for permethylated L-(–)fucose. As shown, a minor peak, which appears first in the chromatogram, corresponds to methyl-2,3,4-tri-O-methyl-β-L-6-deoxygalactofuranoside (βff, Product 5). This product has not been taken into account in the following discussion since it constitutes less than 3% of the total product mixture. The percentage figures presented in Table 3.2 do not include this minor product. As reported in Table 3.2, three major products with different proportions were

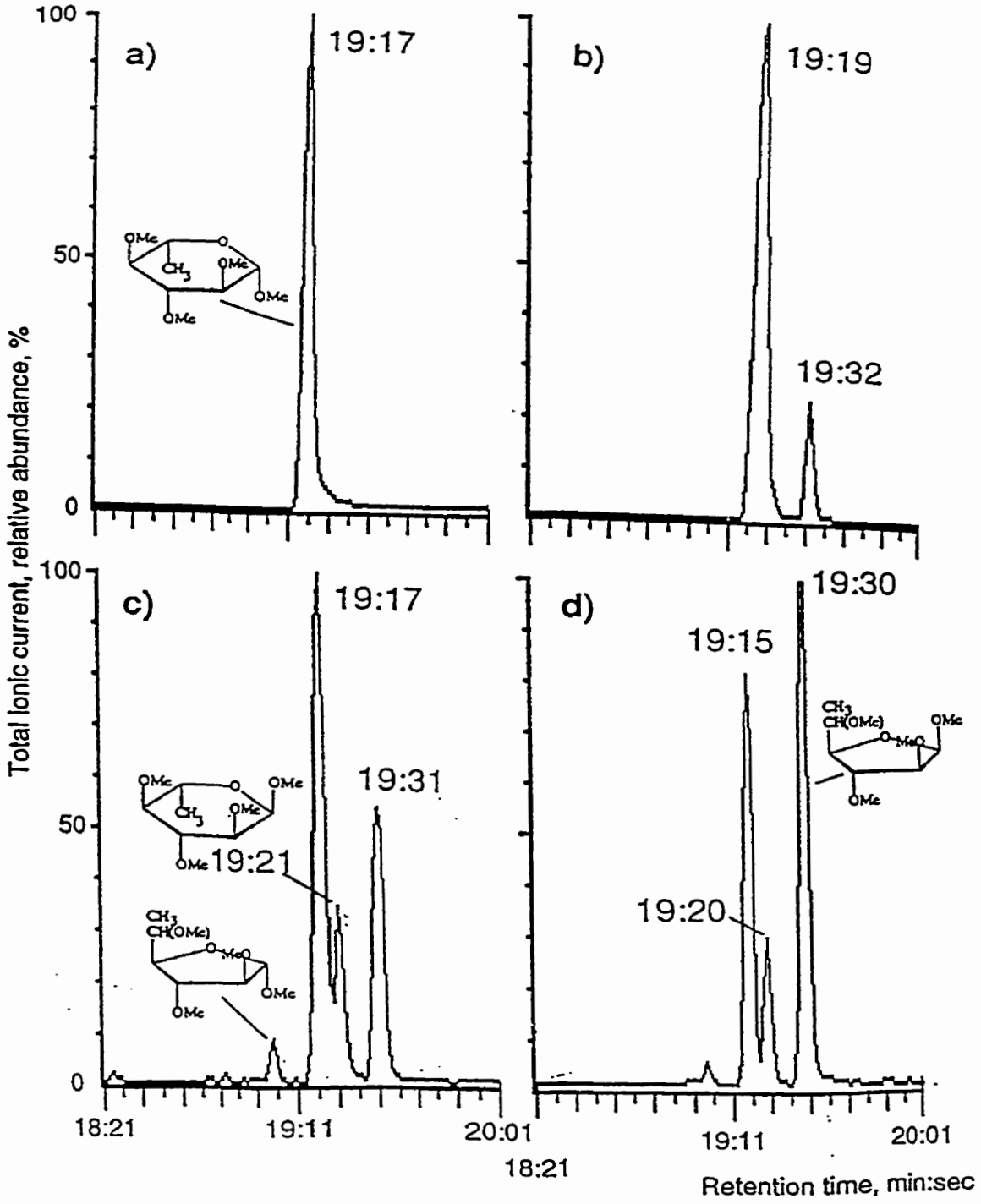


Figure 3.4: GC-MS total ion chromatograms obtained for permethylation products of L-fucose. Referring to Table 2, a) Method 9, b) Method 10, c) Method 15, and d) Method 16. The time-labeled peaks on the chromatogram correspond to Products 6, 7, 8.

Meth. No.	1st Sonic. (min)	2nd Sonic. (React'n time, min)	Temp. (°C)	Prod. 6 (Rel. Conc., %)	Prod. 7 (Rel. Conc., %)	Prod. 8 (Rel. Conc., %)
8	60	60	20-50	100	0	0
9	30	15	20-30	100	0	0
10	20	15	20-27	79	4	17
11	10	15	20-25	44	10	46
12	0	15	20-23	29	12	59
13	0	15	20-23	28	14	58
14	0	25	20-33	40	13	47
15	0	60	20-50	54	14	32
16	0	60	10-20	36	12	52

Table 3.2: Experimental conditions of permethylation of L-(–)fucose, based on the method of Ciucanu and Kerek[80]; proportions of products relative to the most abundant one (± 2%)

Fucose: 4.5-4.8 mg. DMSO: 450 μL. NaOH: 50 mg. CH_3I: 100 μL.

Product 6: Methyl-2,3,4-tri-O-methyl-β-L-6-deoxygalactopyranoside (βfp)
Product 7: Methyl-2,3,4-tri-O-methyl-α-L-6-deoxygalactopyranoside (αfp)
Product 8: Methyl-2,3,5-tri-O-methyl-α-L-6-deoxygalactofuranoside (αff)

obtained by permethylation of L-fucose. These are: methyl-2,3,4-tri-O-methyl-β-L-6-deoxy-β-galactopyranoside (βfp, Product 6), methyl-2,3,4-tri-O-methyl-α-L-6-deoxygalactopyranoside (αfp, Product 7), and methyl-2,3,4-tri-O-methyl-α-L-6-deoxygalactofuranoside (αff, Product 8).

The permethylated fucopyranosides, Products 6 and 7 were identified by comparing the spectra obtained on the HP system with those from the NIST library[140]. No library spectra were available for permethylated fucofuranosides, and these compounds were tentatively identified due to the similarity of features from galactose to fucose. Permethylated galacto or fucofuranosides each yield a main peak at *m/z* 101. The systematic identification of the fucosides by comparison with standard compounds will be discussed below.

Permethylation of our standards methyl-6-deoxy-β and α-L-galactopyranosides yielded products with retention times of 19:17 and 19:20 respectively. Product 6 was therefore identified as permethylated β-L-fucopyranoside (βfp) and Product 7 as permethylated α-L-fucopyranoside (αfp). The mass spectra obtained for these standards matched those of samples perfectly. Permethylation of an impure methyl-β-L-fucofuranoside standard produced a mixture of 80% βff, and 10% of each of Product 7 and 8. The permethylated β-L-fucofuranoside anomer had a retention time of

19:04 min and produced the minor peak appearing in the chromatograms (Figure 3.4, c and d) and will therefore not be discussed as part of the major products.

No standard was obtained for methyl-6-deoxy-α-galactofuranoside. However, Product 8 was identified as permethylated α-fucofuranoside (αff), by default and by comparison of the mass spectra of the furanose forms of permethylated galactose and fucose.

Varying the reaction parameters for the permethylation of L(–)-fucose was based on the results obtained for D(+)-galactose. Hence, the relative amounts of DMSO, CH_3I, NaOH and fucose used remained constant in all experiments. These quantities are indicated below Table 3.2. Parameters subjected to variations were: time of first sonication, reaction time, and temperature. Table 3.2 lists nine different methods, along with the corresponding proportions of products (Products 6, 7, and 8) obtained.

In Methods 8 to 11 fucose, NaOH, and DMSO were sonicated prior to the addition of CH_3I. The general trend shows increasing proportions of Products 7 and 8 relative to 6 with shorter pre-reaction sonication time. In Methods 13-15, the first sonication step was omitted and the reaction time was increased. Longer reaction times (25 min, Method 14 and 60 min, Method 15) had the effect of increasing the proportions of Product 6 *vs.* 7

and 8 i.e. the effect was similar to that of the longer first sonication time. This result suggests that longer sonication times produce strongly basic solutions, which favor the formation of Product 6.

Lowering the reaction temperature (Method 16) favored the formation of Product 8 and decreased the relative concentration of Product 6 from that observed using Method 15. The relative proportions of Product 7 were not significantly changed with Methods 11 to 16.

The results obtained from Methods 8 and 9 (e.g. Figure 3.4a) suggest that longer mixing times of the (fucose/NaOH/DMSO) solution yielded almost 100% of permethylated β-fucopyranoside. These methods are therefore advisable to use if preparation of βfp as a single isomer is desired. Shorter mixing times of the ternary solution (fucose/NaOH/DMSO), as in Methods 10-13 showed, a change in the proportion of fucosides, with a tendency towards more αfp (Product 7) and αff (Product 8) relative to the previously predominant βfp. Figure 3.4b illustrates this trend, here Product 7 is a minor constituent and has not be resolved from Product 6. The slight shoulder at the right of the peak assigned to Product 6 led us to assign a proportion of 4% to Product 7.

The results obtained for fucose are in agreement with the general trend observed for the galactose permethylation reactions. However, much less

information is available about the chemistry of fucose in solution than for galactose and interpretation of the results is more difficult.

In Methods 9 to 13, it is shown that a 15-min reaction time was used for complete methylation of the sugar. In such circumstances, we assumed that no further isomerization took place once the products were formed. In Methods 14 and 15, the reaction times were deliberately pushed above 15 minutes. In these cases isomerizaion seemed to occur, either during permethylation itself, or after the reaction was completed.

It could also be possible that one (or more) of the isomers was selectively being degraded. This would have changed the ratio of isomers and give the impression that equilibration between isomers was occurring. This process overall favored Product 6, to the detriment of 7 and 8 (Figure 3.4c).

Lowering the reaction temperature as in Method 16 seemed to slow down the process and the proportions were pushed in favor of Product 8. Figure 3.4d shows the corresponding chromatogram.

3.0.3 Comparison of mass spectra of galactose and fucose products

In our publication[131, 142], we discussed the GC/MS data for permethylated monosaccharides and observed the presence of two or more isomeric products. Similar results have been reported earlier[135, 143]. We observed that

D-galactose and L-fucose each yielded two major products and one minor product based on the set of reaction conditions. Pyranoside-type products yielded EI mass spectra that correspond quite well to the spectra found in the NIST library[140], while furanoside type products could not be readily identified from the same collection of reference spectra.

The EI spectra taken at the apex of pyranoside type product peaks were somewhat different from those obtained for furanoside-type products. The main difference is the occurrence of abundant ions at *m/z* 88 (Figure 3.6 and 3.9) in the case of pyranosides and the occurrence of *m/z* 101 in the case of furanosides (Figures 3.5, 3.7, 3.8, and 3.10). The possible structures of *m/z* 101 and *m/z* 88 ions are shown in Figure 3.11 and 3.12, respectively.

A comparison of mass spectra of permethylated monosaccharides obtained using the Finnigan Mat 800 ion trap GC/MS system with those obtained using the HP 5988A GC/MS instrument disclosed that the former in general produced less fragmentation than the HP instrument, even though similar ionization conditions (EI, 70 eV) were used. The nature of the fragment ions was different from one instrument to the other. These differences agree well with the fact that some EI-formed ions produced in the trap undergo CI or cooling while still in the trap, thus producing different extents and patterns of fragmentation relative to EI-formed ions travelling through a magnetic

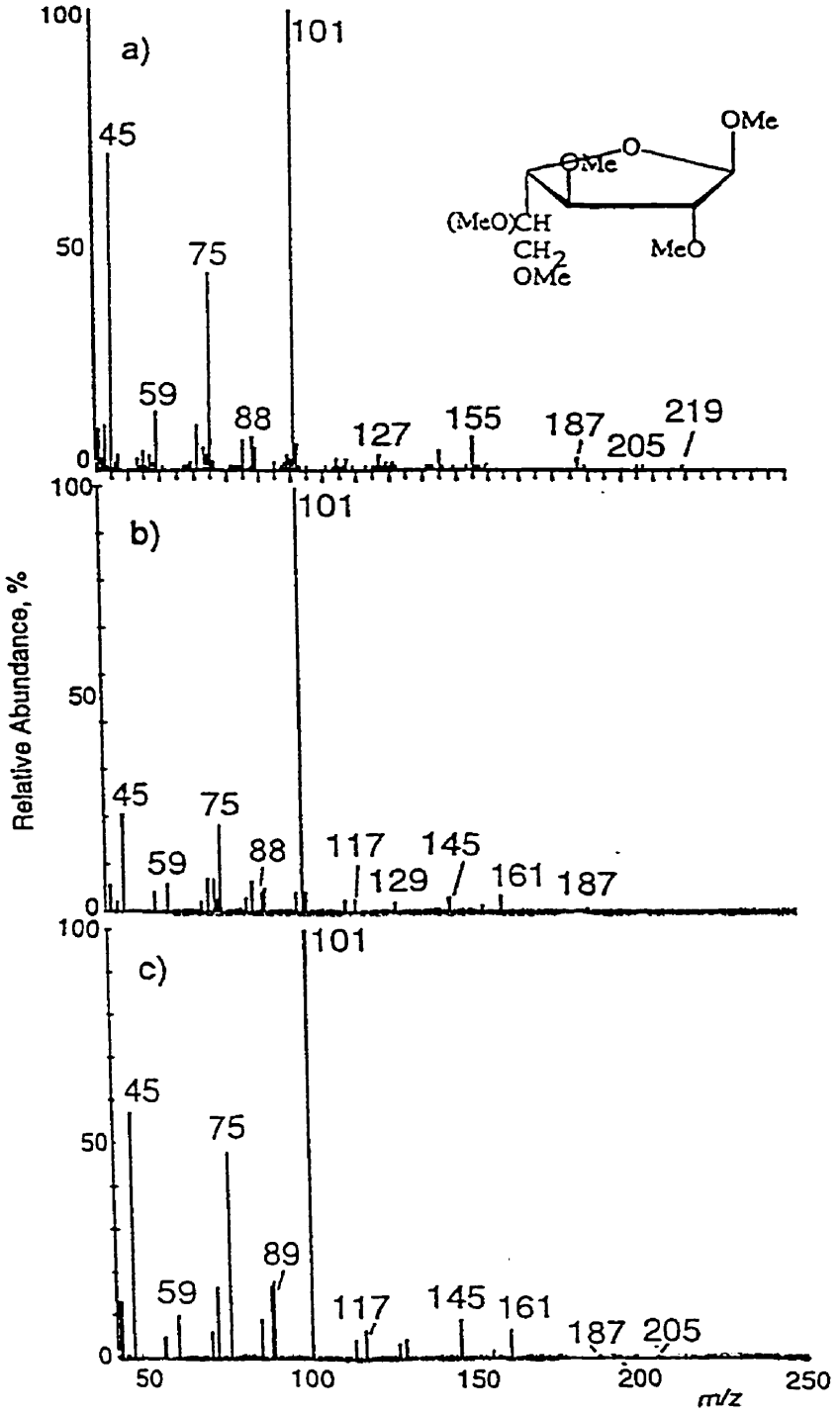


Figure 3.5: Mass spectra of Product 1, identified as permethylated β-D-galactofuranoside (βgf). a) Obtained using the Finnigan MAT ion trap system, b) obtained with the Hewlett Packard system, and c) from the spectral library[140].

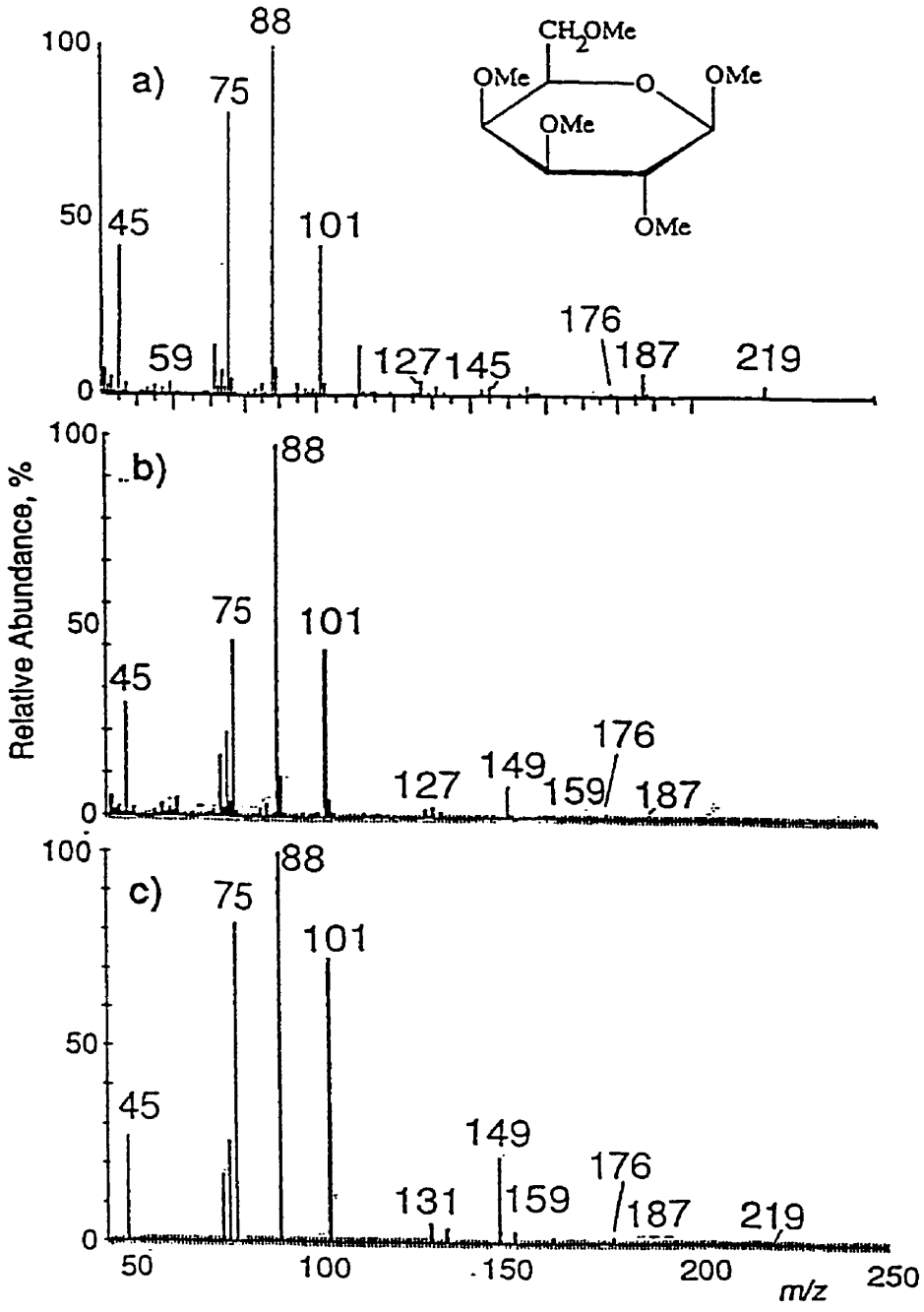


Figure 3.6: Mass spectra of Product 2, identified as permethylated β-D-galactopyranoside(βgp). a) Obtained using the Finnigan MAT ion trap system, b) obtained with the Hewlett Packard system, and c) from the spectral library[140].

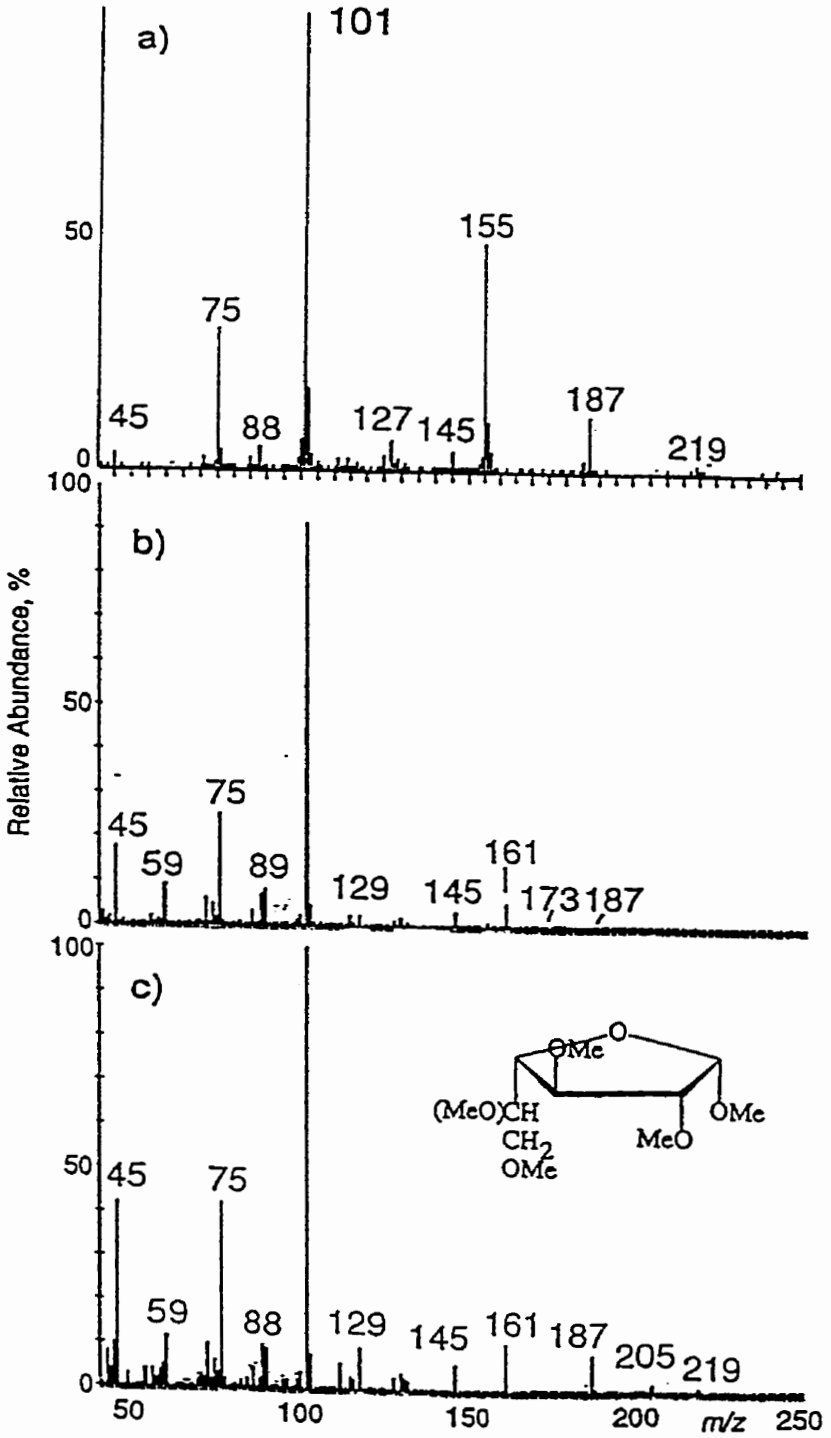


Figure 3.7: Mass spectra of Product 4, tentatively identified as permethylated α-D-galactofuranoside (αgf). a) Obtained using the Finnigan MAT ion trap system, b) obtained with the Hewlett Packard system, and c) from the spectral library[140].

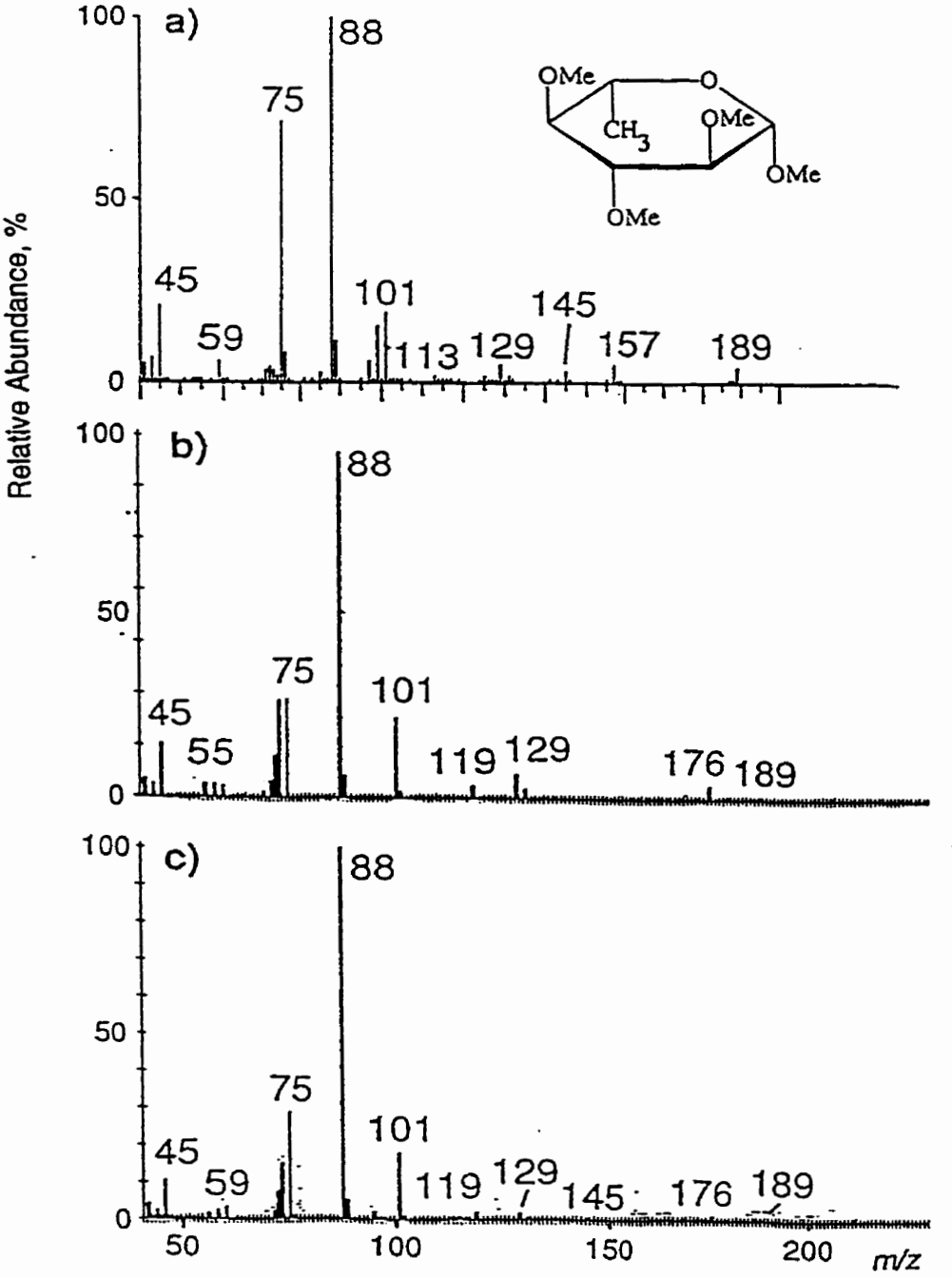


Figure 3.8: Mass spectra of Product 6, identified as permethylated β-L-fucopyranoside (βfp). a) Obtained using the Finnigan MAT ion trap system, b) obtained with the Hewlett Packard system, and c) from the spectral library[140].

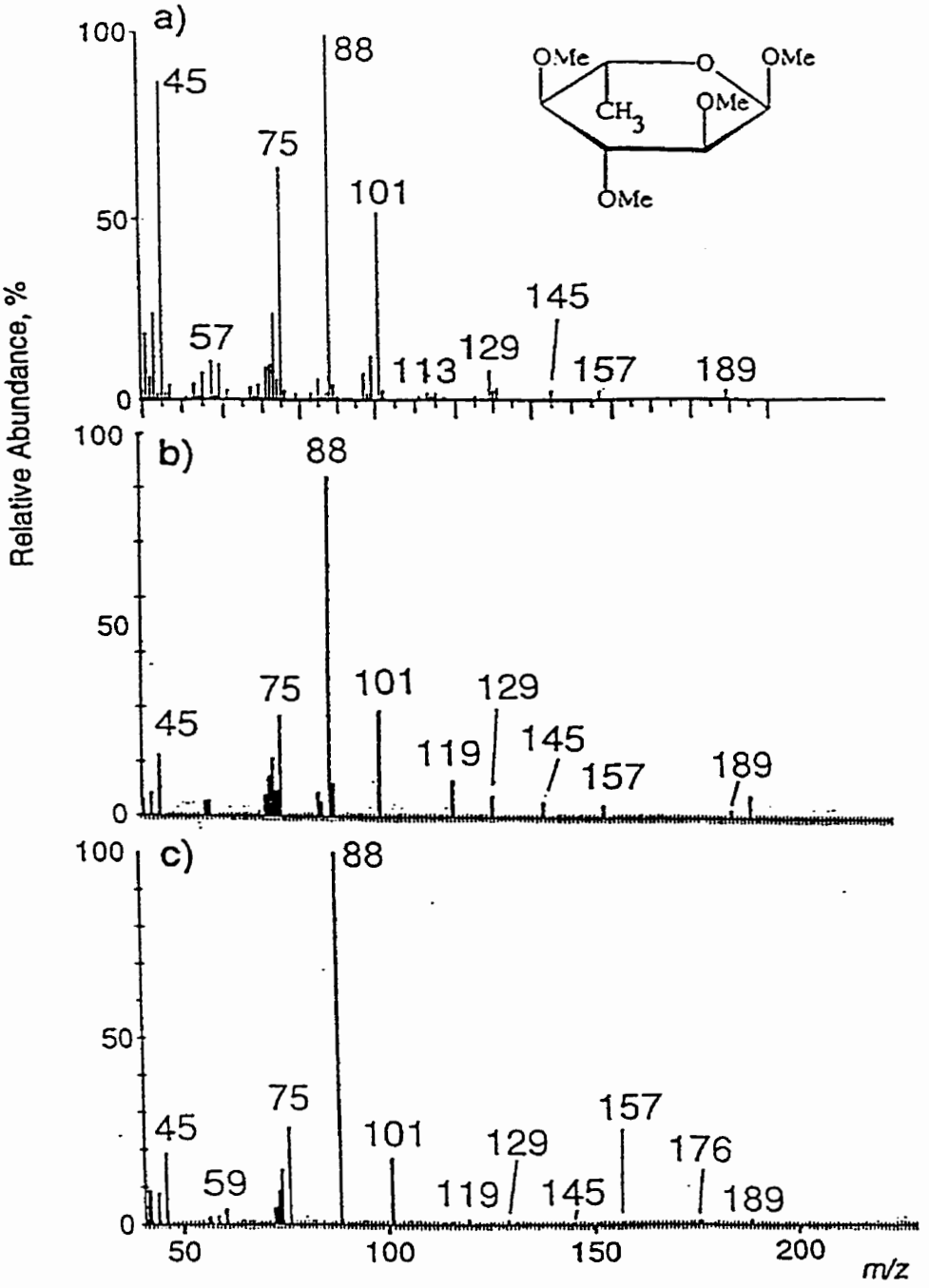


Figure 3.9: Mass spectra of Product 7, identified as permethylated α-L-fucopyranoside (αfp). a) Obtained using the Finnigan MAT ion trap system, b) obtained with the Hewlett Packard system, and c) from the spectral library[140].

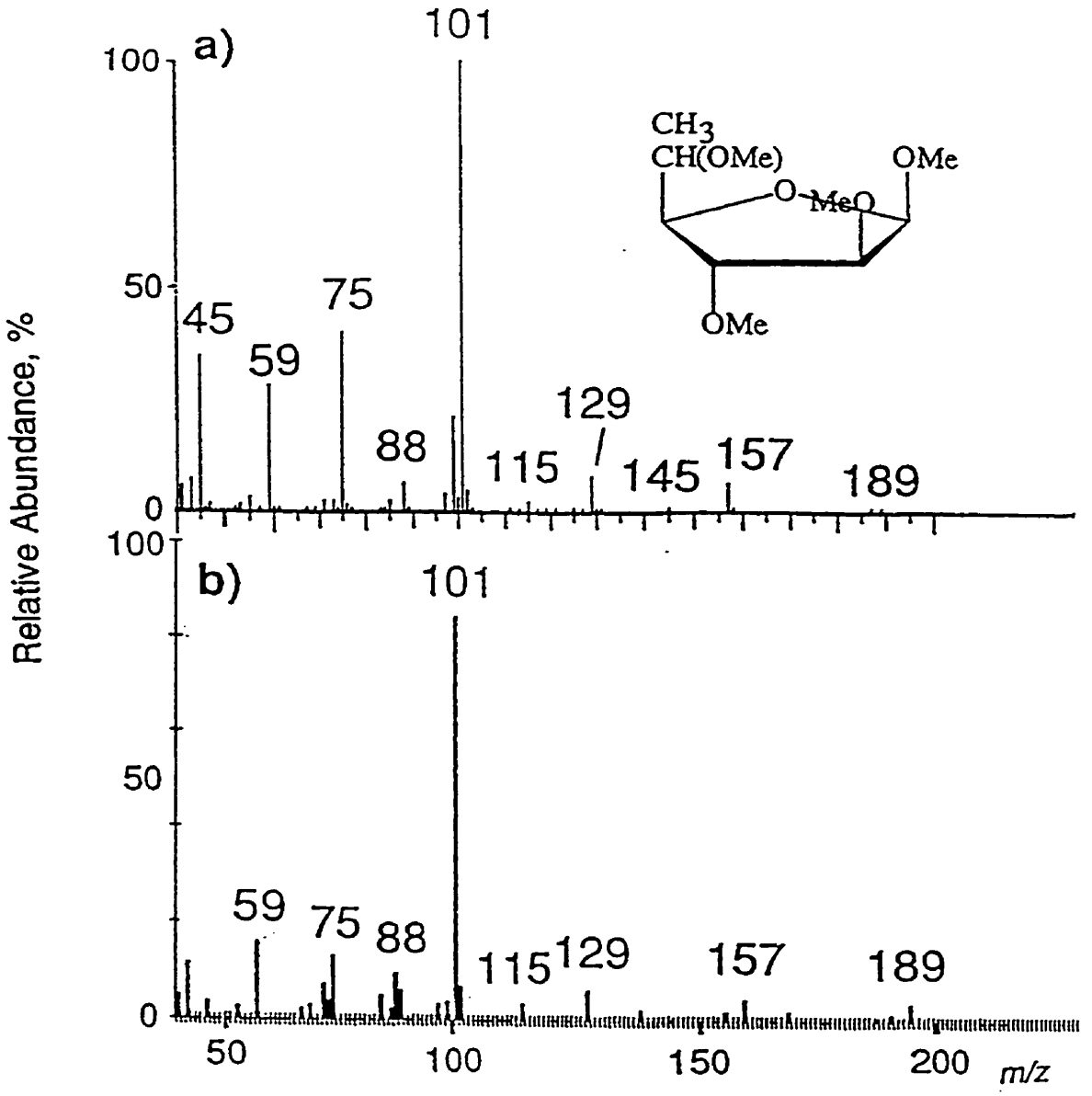


Figure 3.10: Mass spectra of Product 8, identified as permethylated α-L-fucopfuranoside (αff). a) Obtained using the Finnigan MAT ion trap system, b) obtained with the Hewlett Packard system.

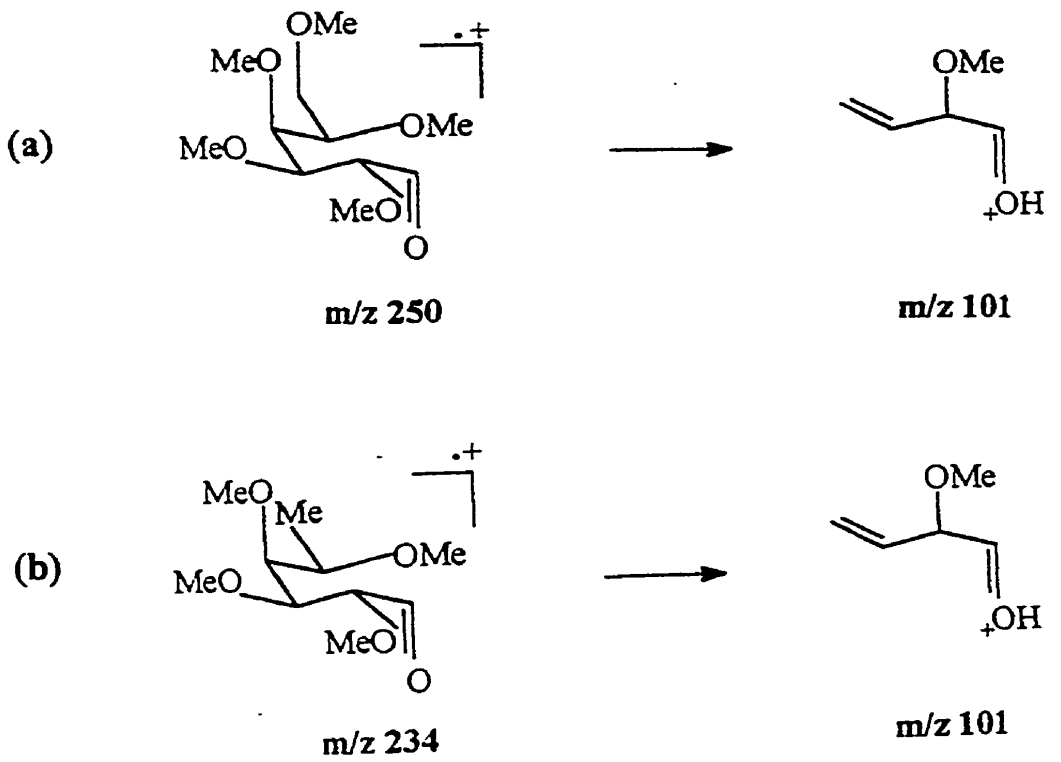


Figure 3.11: Suggested structures for the *m/z* 101 ions observed in the case of permethylated (a) D-(+)-galactose and (b) L-(–)-fucose.

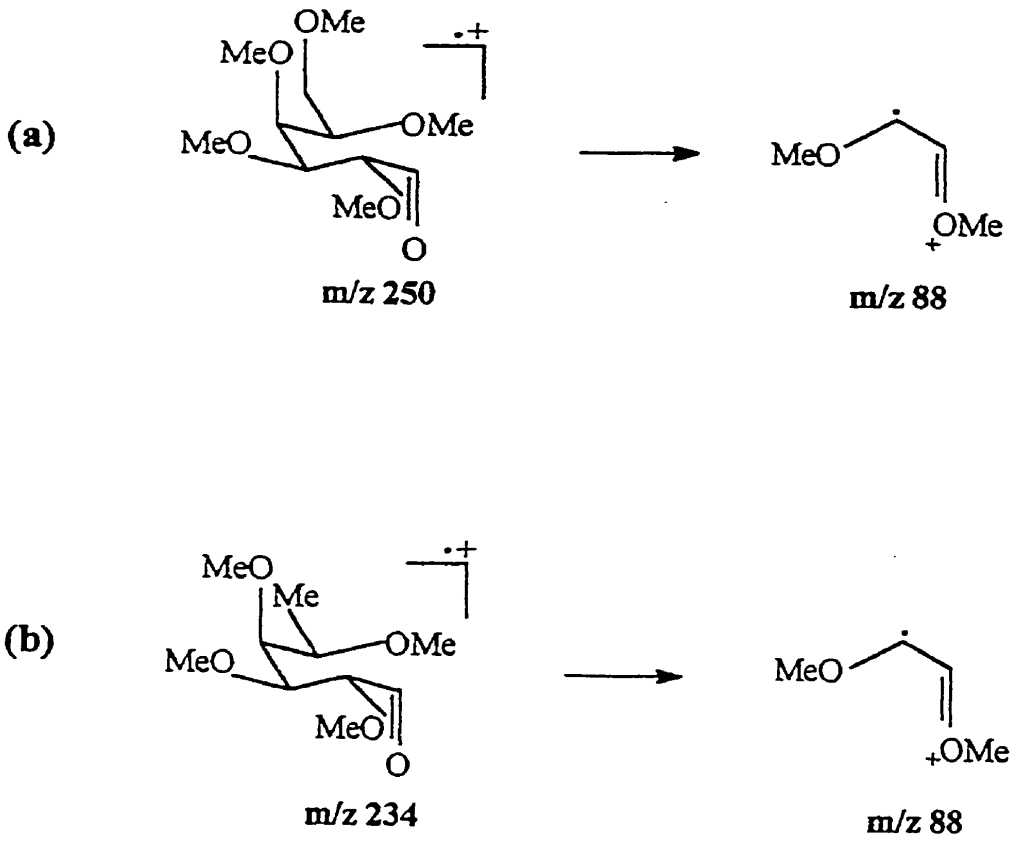


Figure 3.12: Suggested structures for the *m/z* 88 ions observed in the case of permethylated (a) D-(+)-galactose and (b) L-(–)-fucose.

sector or a quadrupole mass analyzer. The HP spectra matched the NIST library spectra[140] much better and more reliably than the Finnigan MAT spectra (see Figures 3.5 - 3.10). In general, we found that the EI spectra obtained with the ion trap system were not always reliable for identification of unknowns, using libraries and this for diverse classes of compounds. From the HP data obtained with a quadrupole analyzer, we assigned the permethylation products of D-galactose and L-fucose as pyranosides and furanosides. Figures 3.5 - 3.10 Shows comparative spectra for pyranoside and furanoside products from methylation of D-galactose and L-fucose.

3.1 Discussion on the isolation and characterization of permethylated galactosides and fucosides

In the previous part of this thesis, we discussed, based on GC/MS data, the relative abundance of permethylated galactoside and fucoside isomers formed with different set of reaction conditions. The results showed that for either galactosides or fucosides, four isomers were produced although only three peaks were resolved in the case of galactosides. The galactoside isomers observed by GC/MS were, by increasing retention time order: βgf (Product 1), βgp (Product 2), αgp (Product 3), and αgf (Product 4). Compounds 3 and 4 co-eluted. In the case of the fucosides, the GC retention

order was similar: βff (Product 5), βfp (Product 6), αfp (Product 7), and αff (Product 8). The four compounds were resolved.

For the purpose of the work discussed here, permethylation reaction conditions were chosen so as to produce selective isomers, as described previously in this chapter (Tables 3.1 and 3.2). However, a variation of the conditions did not have a significant influence on the amounts of Products 1, 3, and 5 obtained. These three compounds were produced in very low amounts and thus it was difficult to isolate as pure substances at the mg level.

The isolation of individual compounds was performed by continuous or repeated TLC with prior optimization of solvent conditions using small TLC plates. The type of separation sought was equivalent to or better than earlier published by Gee[135]. Her work showed that the separation of the four permethylated isomers of galactose was limited by co-elution of Products 2 and 4. Although a number of reports mention the use of TLC and column chromatography for the separation of monosaccharide permethylation mixtures[144-147], no details were given about elution order, chromatographic behavior, or resolution. The best solvents in our hands were a 1:9:40 isopropanol-ethylacetate-heptane mixture and a 1:9:40 isopropanol-ethylacetate-hexanes mixture. Hexanes is a distillation fraction like

petroleum ether and contains many hydrocarbons. A 3:1 cyclohexane-ethylacetate system nearly produced the same results. Using the isopropanol-ethylacetate-heptane solvent mixture the compounds were eluted as follows: βgf (Product 1, $R_f = 0.280$), αgf (Product 4, $R_f = 0.195$), βgp (Product 2, $R_f = 0.190$), and αgp (Product 3, R_f=0.145), for the galactosides, and βff (Product 5, $R_f = 0.565$), αff (Product 8, $R_f = 0.310$), βfp (Product 6, R_f= 0.245) and αfp (Product 7, $R_f = 0.150$), for the fucosides. The main challenge encountered during optimization, as reported previously[135], was the separation of Compounds 2 and 4. The conditions found to perform the best separation for permethylated galactoside isomers were applied even more successfully to separation of permethylated fucoside isomers, and no further optimization was required.

The 1:3 ethyl acetate-heptane solvent mixture yielded similar results with slight variation in the R_f values. Figure 3.13 shows the TLC elution patterns obtained for a) permethylated galactoside isomers and b) for permethylated fucoside isomers using the 1:9:40 isopropanol-ethyl acetate-hexanes solvent mixture. Both elution patterns were obtained by repeated development, and 12 successive developments were performed for permethylated galactoside isomers and 8 successive developments for permethylated fucoside isomers. Less than eight successive developments might have been enough in the

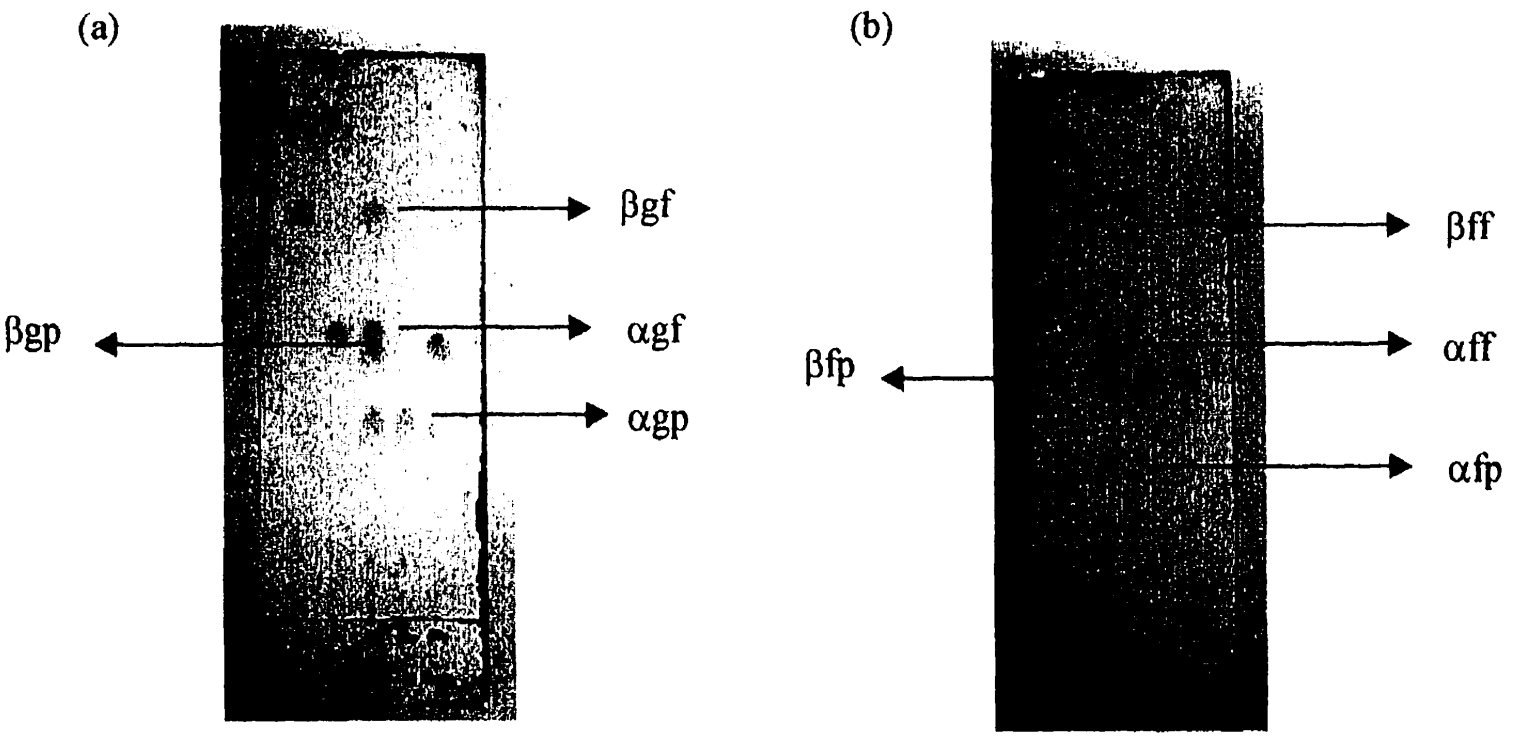


Figure 3.13: Thin-layer chromatographic patterns for the elution of a) a mixture of permethylated galactoside isomers (12 consecutive elutions) and b) a mixture of permethylated fucoside isomers (8 consecutive elution). Eluent : 1:9:40 isopropanol-ethyl acetate-hexanes

latter case, since the R_f values of permethylated fucosides are quite different from each other.

Column chromatographic separation was attempted using 1:3 ethyl acetate-heptane. On a 10 cm silica column, 0.7 cm i.d., the elution volumes for galactosides and fucosides were between 85 and 105 mL. The elution order was the same as observed by TLC. From the permethylated galactoside mixture, it was possible to isolate βgf (Product 1), and αgp (Product 3). The isolation of βgp (Product 2), and αgf (Product 4) was however not satisfactory when 5-7 mg of the total mixture were applied onto the column. GC/MS of the corresponding collected fractions showed a slight overlap between the peaks of the two compounds. The separation was complete when 1mg of total sample was applied onto the column, which however defeated the practical purpose of this application. On the other hand, fucoside isomers could be separated using the same conditions, whether 1 or 7 mg of the mixture were applied onto the column. Since column chromatography was not completely appropriate for the separation of glycoside isomers, the use of continuous elution TLC on glass-coated plates was investigated, and found to be the most efficient method for separating galactoside and fucoside isomers. Development for 14-18 h using 1:9:40 isopropanol-ethyl acetate-heptane as the eluent yielded a reasonably good

separation of 2 and 4, with quantities of material ranging from 5 to 30 mg. After 18 h, compound 1 had moved to very top of the plate, along with impurities, and was unrecoverable as a pure component. This loss, i.e. migration of 1 all the way to the top, was necessary in order to allow the separation of 2 and 4.

In the case of fucoside isomers, less time was required, i.e. only 7-9 hr. The time of development may vary depending on the dimension of the tank and the amount of solvent used. With a large tank, it was possible to effect the separation within a shorter time. Successive developments using 1:9:40 isopropanol-ethyl acetate-hexanes also allow the separation of 2 and 4 in a shorter period of time. This way, 8-12 h were required for separating permethylated galactosides and 4-6 h for permethylated fucosides. This solvent cannot be used for continuous development. Since hexanes are highly volatile the proportion of the solvent changed after a short period of time and the solvent only moved up to half of the glass plate. Table 3.3 lists each of the 8 compounds discussed in the study, along with the method, which is most efficient for their isolation.

Figure 3.14 shows GC/MS TIC traces obtained for each of the individual permethylated galactoside standards. Out of the four, only βgf (Product 1)

Table 3.3: Best isolation method found for individual permethylated galactosides and fucosides.

Compound No.	**Name of Compound (permethylated)**	**Compound better isolated by...**
1	β-galactofuranoside	Column chromatography
2	β-galactopyranoside	Continuous-elution TLC
3	α-galactopyranoside	Either of the above
4	α-galactofuranoside	Continuous-elution TLC
5	β-fucofuranoside	Column chromatography
6	β-fucopyranoside	Continuous-elution TLC
7	α-fucopyranoside	Either of the above
8	α-fucofuranoside	Continuous-elution TLC

contained a significant impurity of 4 (Figure 3.14a). These standards were mixed and an injection of the mixture produced the TIC trace shown in Figure 3.15a. This trace showed the co-elution of Products 3 and 4 . In the same figure, traces b) to e) show the elution each standard, once isolated using the continuous TLC method. Since these four compounds have similar chromatographic behaviors, it was not possible to perform isolation with 100% efficiency. Compound 1 was isolated with the highest purity level. Compounds 2, 3, and 4 contained very low levels of other components due to band broadening on the plate because of long hours of development. Successive developments using 1:9:40 isopropanol-ethyl acetate-hexanes showed a better separation efficiency.

The same experiment was repeated with a mixture of permethylated galactosides obtained from permethylation of D(+) galactose under conditions described previously in Method 3.1 . In this particular case permethylation favored formation of compounds 2 and 4 as major products, while 1 and 3 were produced in minimal quantities. Figure 3.16a shows the GC/MS TIC trace obtained for the permethylation products, and traces b) to e) correspond to the products isolated using the same methods as the standards. Compounds 1, 2, and 4 show reasonable levels of purity but Compound 3, the least abundant product in the mixture, contained

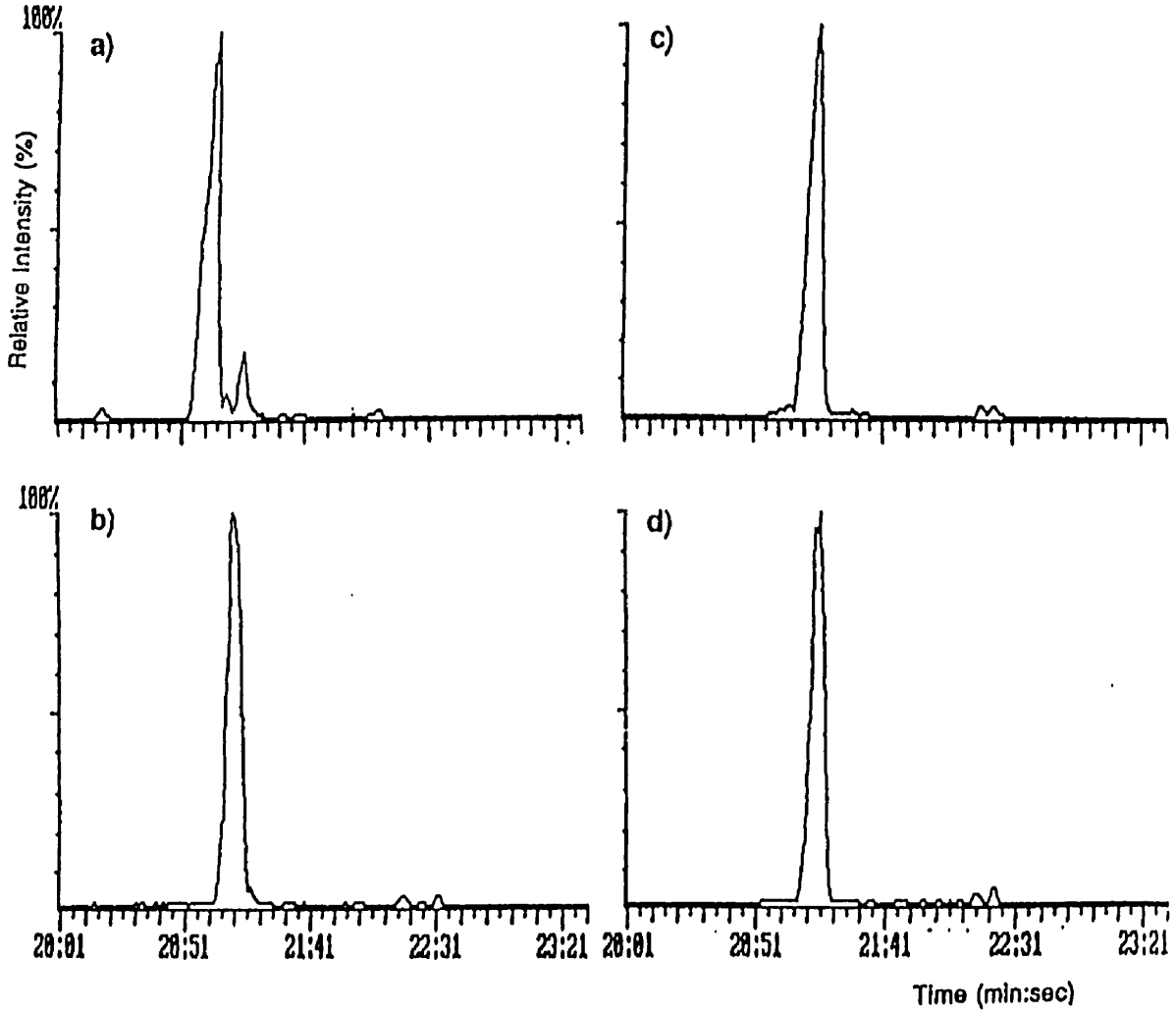


Figure 3.14: GC-MS total ion chromatograms of individual permethylated galactoside standards. a) β-galactofuranoside (βgf), b) β-galactopyranoside (βgp), c) α-galactopyranoside (αgp), and d) α-galactofuranoside (αgf).

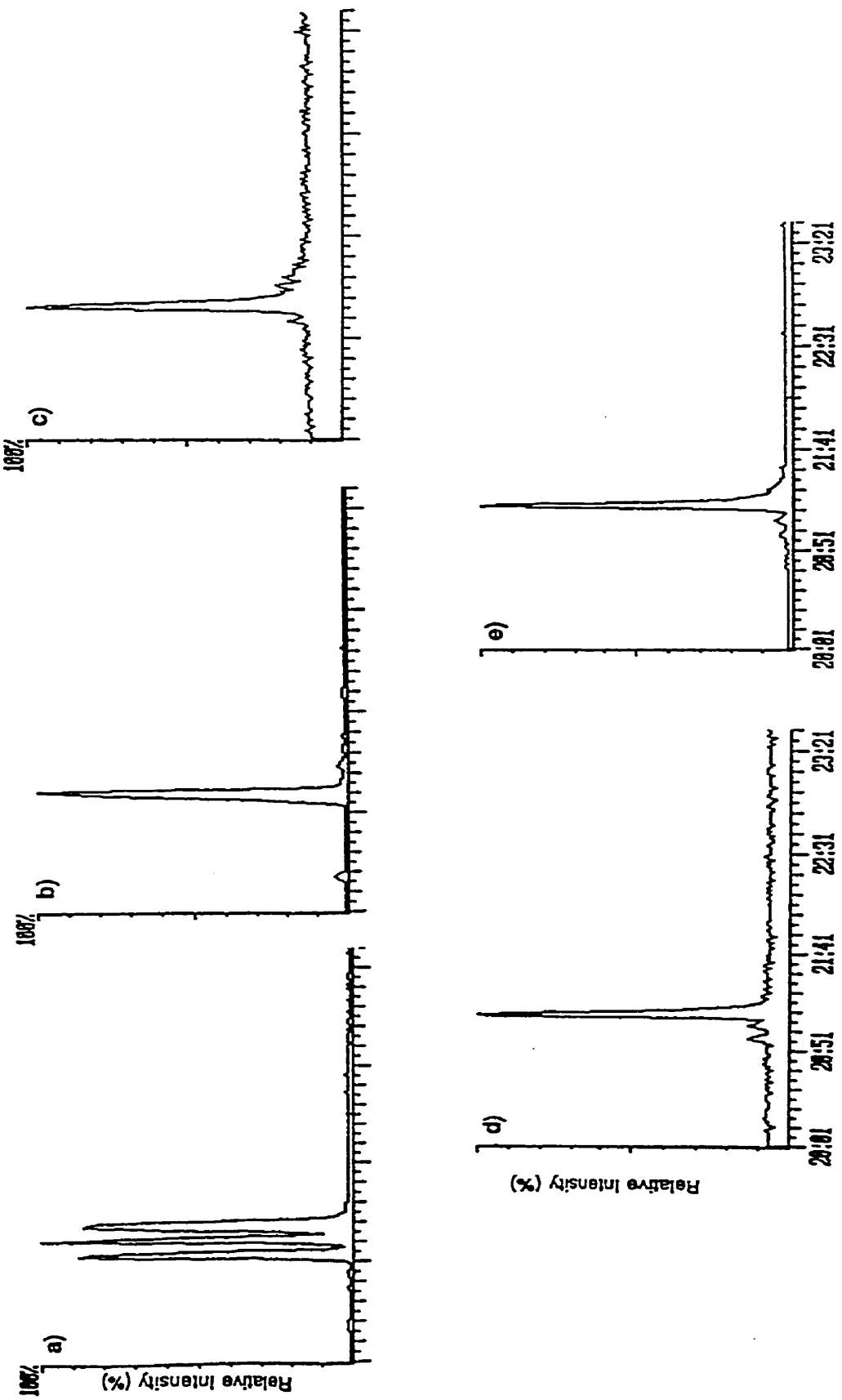


Figure 3.15: GC-MS total ion chromatograms of permethylated galactoside standards. a) Mixture of the 4 standards named thereafter, b) β-galactofuranoside (βgf) isolated from mixture, c) β-galactopyranoside (βgp) isolated from mixture, d) α-galactopyranoside (αgf) isolated from mixture, and e) α-galactofuranoside (αgp) isolated from mixture.

considerable amounts of 1 and 2 . Unfortunately, Compound 3 was the least abundant with each set of permethylation conditions used, and thus it was impossible to perfectly isolate this product from the rest of the permethylated mixture. Its preparation is however easily achieved by performing permethylation on commercially purchased 1-methyl-α-galctopyranoside. Both galactofuranosides were successfully isolated, which constituted an important step in our study since these compounds are not readily available commercially, or otherwise are very expensive.

The use of reversed phase high performance liquid chromatography (HPLC) as a preparative method for the separation of the isomers was also investigated. Detection based on ultraviolet absorption was made difficult by the absence of suitable chromophores on the glycosides, and refractive index detection became possible only when mg quantities of samples were loaded onto the column, at which point no separation was obtained. The only way to monitor the separation of the four isomers was by on line-HPLC-electrospray mass spectrometry, and injections of less than one picomole of each component were necessary to baseline resolve the isomers. The practicability of this approach was questioned, and finally the method was abandoned.

The conditions used for column chromatography and continuous elution TLC, as already optimized for the galactosides, were directly applied to the separation of furanosides. Figure 3.17a shows the elution (GC/MS) of the four permethylated fucoside standards. Figure 3.17d shows the GC/MS trace obtained for the permethylation products of fucose, in one specific set of permethylation conditions listed in Table 3.2. The isolation of the four main components from this product mixture resulted in the ion chromatograms presented in Figure 3.17,bcef. Similar chromatograms were obtained for the products isolated from the mixture of standards (not shown). Separation of permethylated fucosides by TLC or column chromatography was easier accomplished than in the case of galactosides, as shown by Figure 3.17. As shown in the chromatograms of 3.17bcef, Compounds 5 and 7 were well isolated (Traces b and e). However, Traces 3.17c and 3.17f, corresponding to Compounds 6 and 8, show the presence of a very low amount of the other isomer, due to a close elution and a slight overlap of these two components on TLC plates.

It was possible to prepare both permethylated fucopyranoside isomers from the 1-methyl fucopyranoside standards (α and β), but our method allows preparation of mg quantities of βfp from L-fucose, which is readily available and cheaper than 1-methyl-β-fucopyranoside. Preparation of permethylated

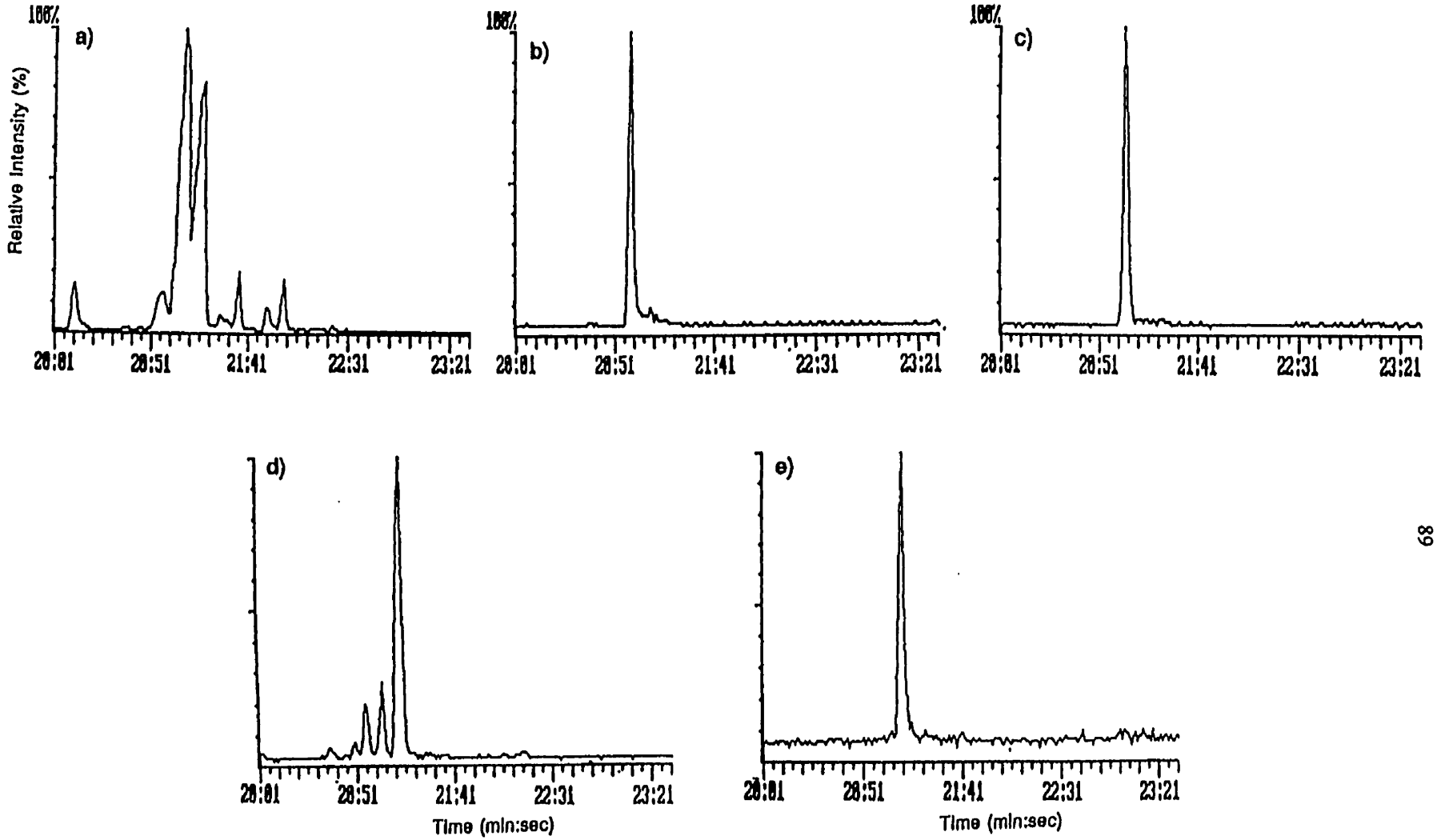


Figure 3.16: GC-MS total ion chromatograms of permethylation products of D-(+)-galactose. a)Product mixture, b) β-galactofuranoside (βgf) isolated from mixture, c)β-galactopyranoside (βgp) isolated from mixture, d) α-galactopyranoside (αgp) isolated from mixture, and e) α-galactofuranoside (αgf) isolated from mixture.

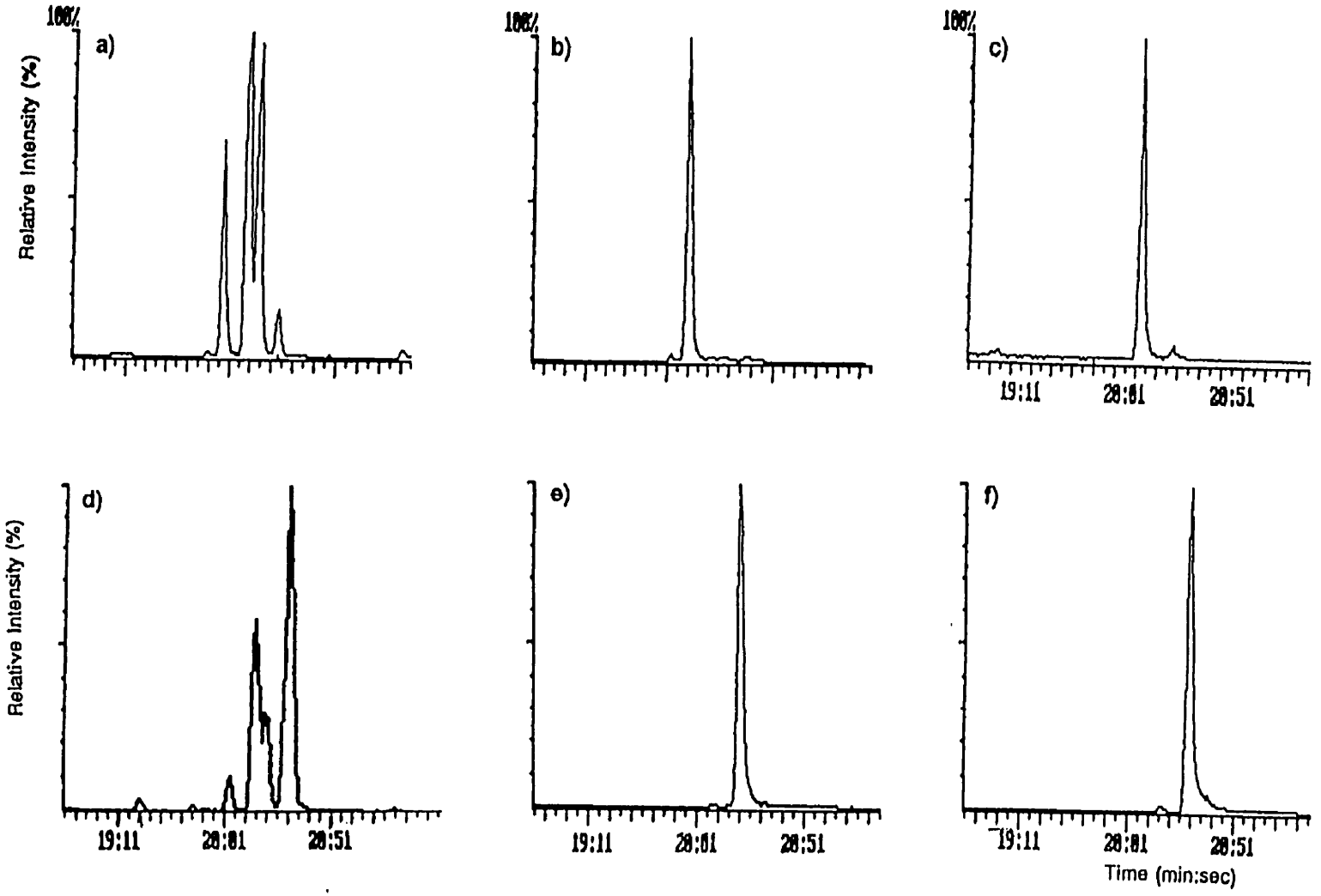


Figure 3.17: GC-MS total ion chromatograms of permethylation products of L-(–)-fucose and of the permethylated fucoside standards. a) Mixture of the 4 standards named as those isolated thereafter, b) β-fucofuranoside (βff) isolated from permethylation mixture, c) β-fucopyranoside (βfp) isolated from permethylation mixture, d) permethylation products of L-(–)-fucose, e) α-fucopyranoside (αfp) isolated from permethylation mixture, and e) α-fucofuranoside (αff) isolated from permethylation mixture.

fucofuranosides from 1-methyl fucofuranosides seems difficult or otherwise very expensive because these standards are not available from generic companies. However, we successfully isolated the two permethylated fucofuranoside isomers prepared by permethylating L-fucose. The permethylation procedure suggested in Table 3.2 and the separation method discussed is simple and inexpensive for preparing permethylated α-fucofuranose.

3.2 Results and discussion on the analysis of GM_1

The release of oligosaccharides for structural determination of the carbohydrate moieties of glycosphingolipids (GSL), was first reported by Wiegandt and Baschang[18, 19]. Their method involved ozonolysis of the double bond of the sphingolipid base followed by hydrolysis in aqueous sodium carbonate. The degradation was performed on amounts of GSL varying from twenty to several hundred milligrams. Later, Hakomori[21] introduced another method based on a selective oxidation of the double bond through osmium catalyzed periodate oxidation followed by sodium methoxide treatment. The oligosaccharides released using the above methods were analyzed by GC as the trimethylsilyl (TMS) derivatives of their monosaccharide hydrolysis products[23, 148].

The high sensitivity and resolving power of GC make it an ideal technique for the separation and analysis of GSL components. Two methods have been widely used to cleave GSL into their components. In one, the GSL are hydrolyzed with methanolic HCl to cleave glycosidic and amide bonds[26, 27]. This results in the production of sphingoid bases and their methyl derivatives, fatty acid methyl esters, and methyl glycosides. Fatty acid methyl esters are extracted by hexane and analyzed by GC. Sphingosine bases and their methyl derivatives, and methyl glycosides are treated with

TMS prior to GC analysis[31, 32, 148]. During methanolysis, methyl glycosides are produced from the various anomeric forms of hexoses, so several peaks are obtained in the GC trace of each component[23, 149]. However, the methanolysis-silylation treatment of N-acetylneuraminic acid produces only one peak on the GC recorder trace due to the formation of the 2-O-methyl ketal derivative of methyl neuraminate[23].

The second method involves the hydrolysis of GSL with aqueous acid. The hexoses produced are then reduced to their corresponding alditols and converted to acetate derivatives for GC analysis[150]. During the reduction step, the anomeric center is destroyed so that each component chromatographs as a single peak. Aqueous acid can bring about disruption of some hexoses so care is necessary during the hydrolysis step to keep such effects to a minimum.

In our attempt to analyze GM_1, we modified the first method and developed two different sets of methods. These involve methanolysis, permethylation and re-N-acetylation procedures.

In our first method, GM_1 was permethylated using the method developed by Ciucanu and Kerek[80] and later adapted for GSLs by Larson et al.[67]. The permethylated GM_1 was then methanolyzed by 0.75 N methanolic HCl.

Methanolysis products were re-N-acetylated using acetic anhydride and analyzed by GC/MS. Our second method involves methanolysis of GM_1 followed by re-N-acetylation and permethylation procedures. Both methods are similar except for the order of the steps in the procedures, and identical products were expected. A summary of the procedures used in both methods is given in Tables 3.4 and 3.5.

3.2.1 Discussion on permethylation of GM_1

The ESI mass spectrum of underivatized GM_1 can be obtained better in the negative ion mode than in the positive ion mode. The GM_1 ganglioside usually occurs in nature as a mixture of two species differing from each other in mass by 28 Da. This difference resides in the long chain base[64]. Thus, a pair of $[M+H]^+$ ion peaks, which contain the long chain base and separated by 28 u, was obtained in the ESI mass spectrum of native GM_1, shown in Figure 3.18.

Permethylated GM_1 also yielded a pair of $[M+H]^+$ ion peaks, with a difference of 28 u. The *m/z* difference between the permethylated GM_1 molecular ion peaks and the native GM_1 molecular ion peak clearly indicates complete methylation of the hydroxyl groups, amide nitrogens of amino sugars and long chain bases (i.e. 20 sites).

Table 3.4: Summary of permethylation and methanolysis procedures used for Method 1.

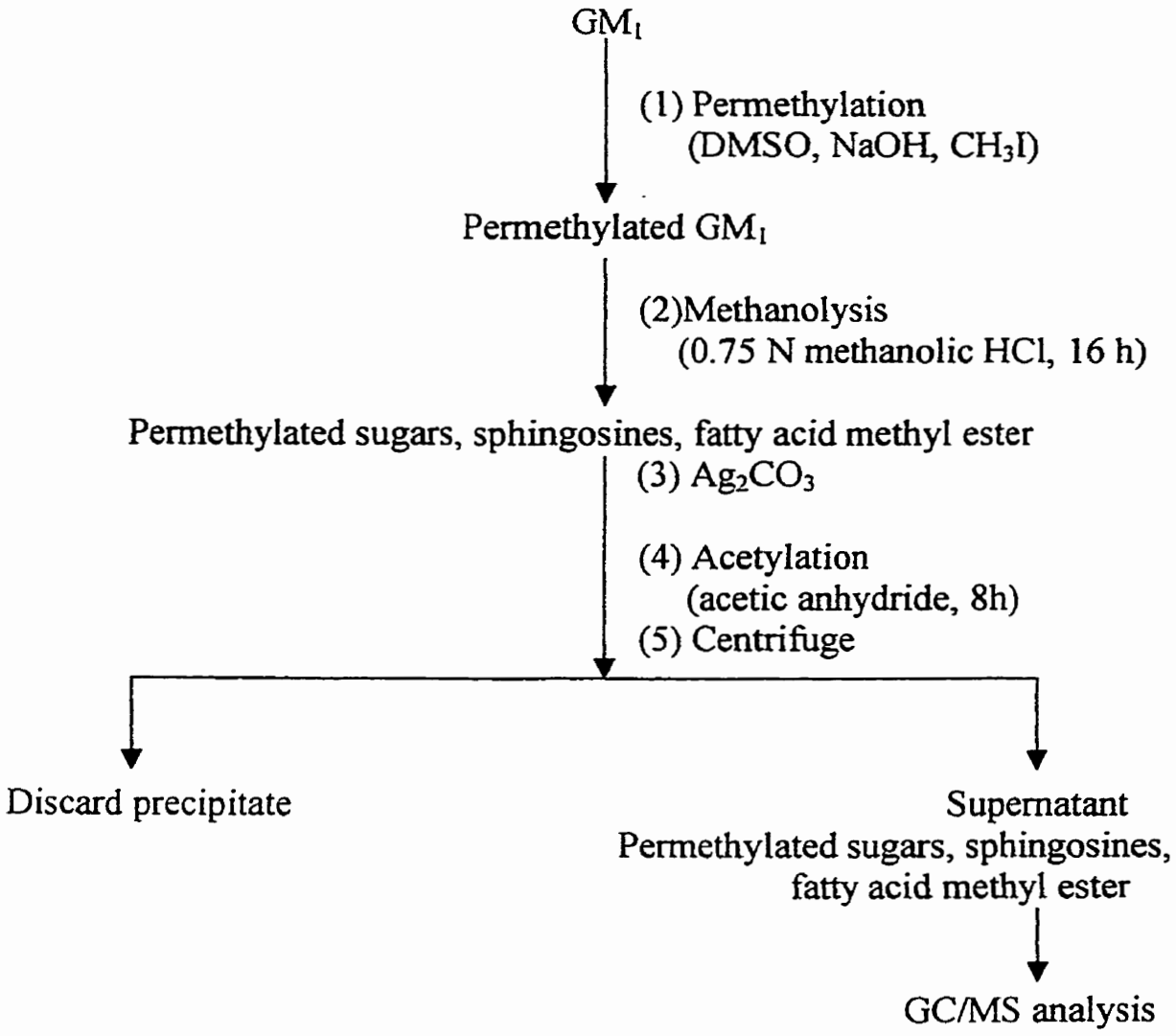
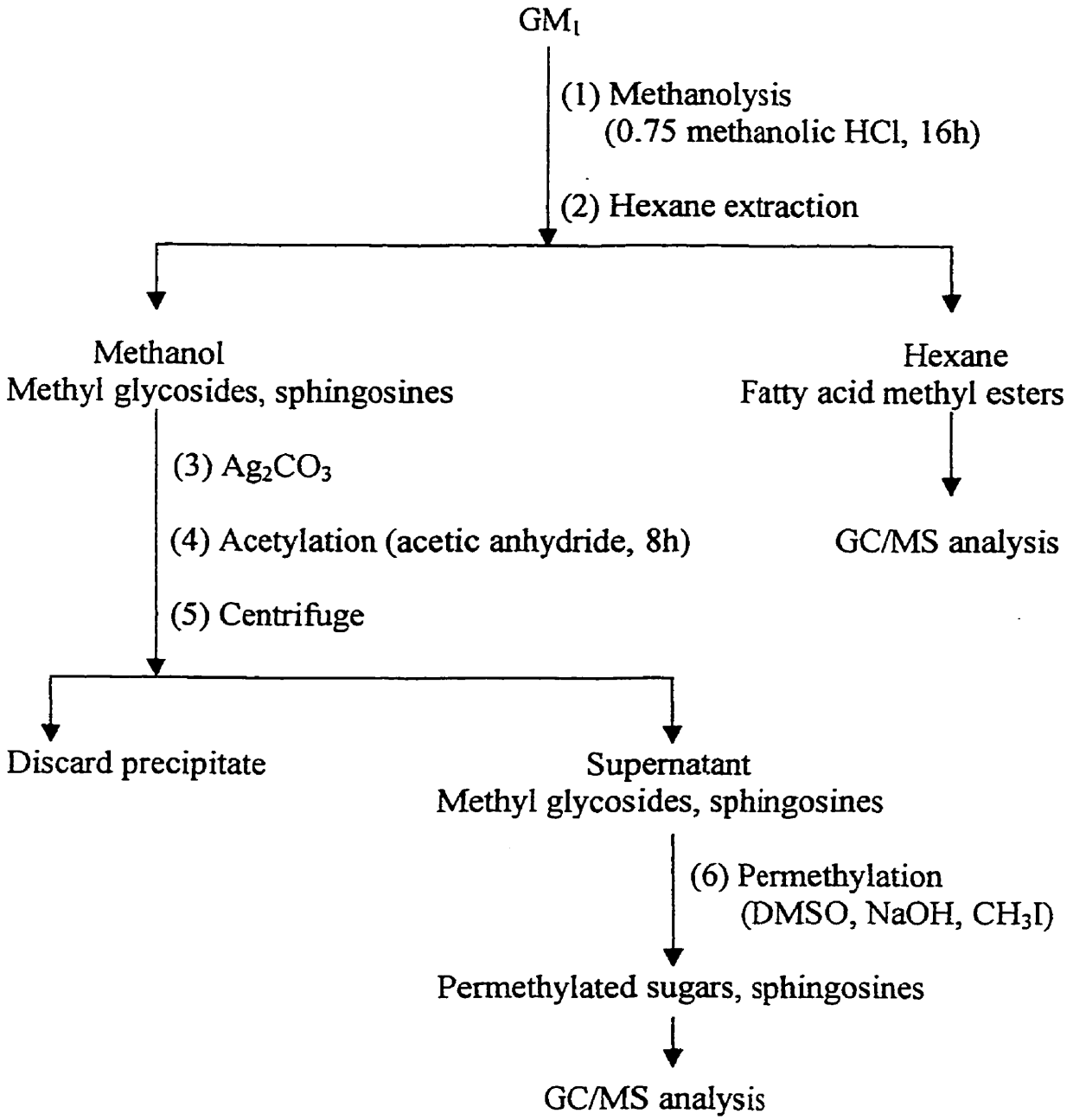


Table 3.5: Summary of methanolysis and permethylation procedures used for Method 2.



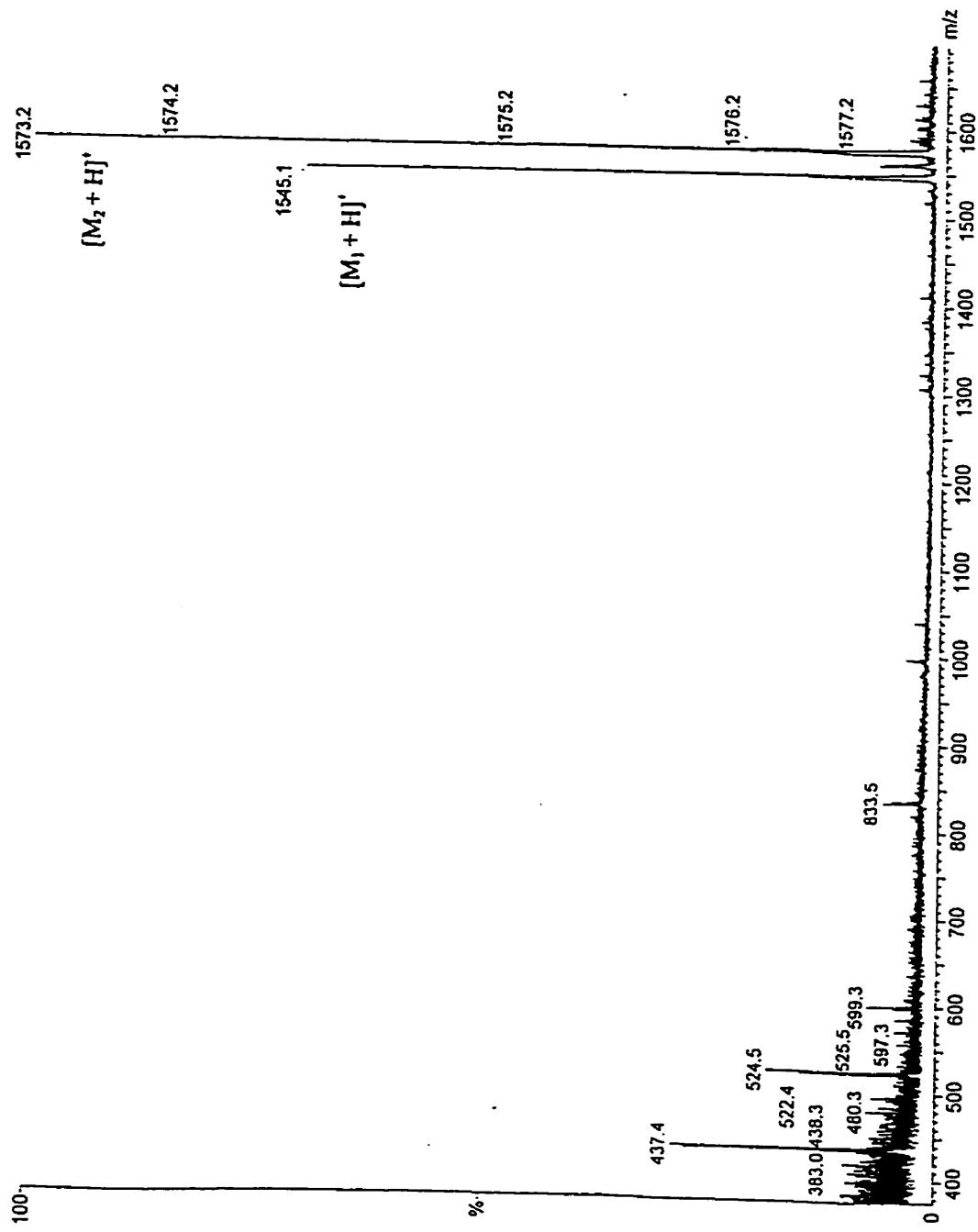


Figure 3.18: Negative ion ESI mass spectrum of native GM_1.

Permethylation of GM_1 should be performed at room temperature (20 - 30 °C) because at higher temperature (> 30 °C), a pair of species (*m/z* 1954.1 and 1982.1) differing from the methylated GM_1 mixture by 126 u was obtained. These products seem to be a mixture of two permethylated GM_1 species, adducted with iodine. Figure 3.19 shows the result of permethylation of GM_1 at higher temperature. The two peaks at *m/z* 1828.3 and 1856.3 indicate the mass of pemethylated GM_1 obtained at lower temperature (20 - 30 °C). The spectrum of permethylated GM_1 shows clearer fragmentation patterns than that of native GM_1. It shows a pair of peaks separated by 28 mass units, corresponding to fragment ions, which contain the long chain bases (*m/z* 576 and 604). Other fragments such as *m/z* 464, which indicate hexoses connected to GalNAc, and *m/z* 376, that corresponds to permethylated sialic acid were also obtained.

3.2.2 Discussion on the standard mixture of permethylated Monosaccharides

Our preparation of a standard mixture by permethylation of galactose, glucose, N-actylgalactosamine, and N-acetylneuraminic acid produced a mixture of various anomeric forms of pyranosides and furanosides of

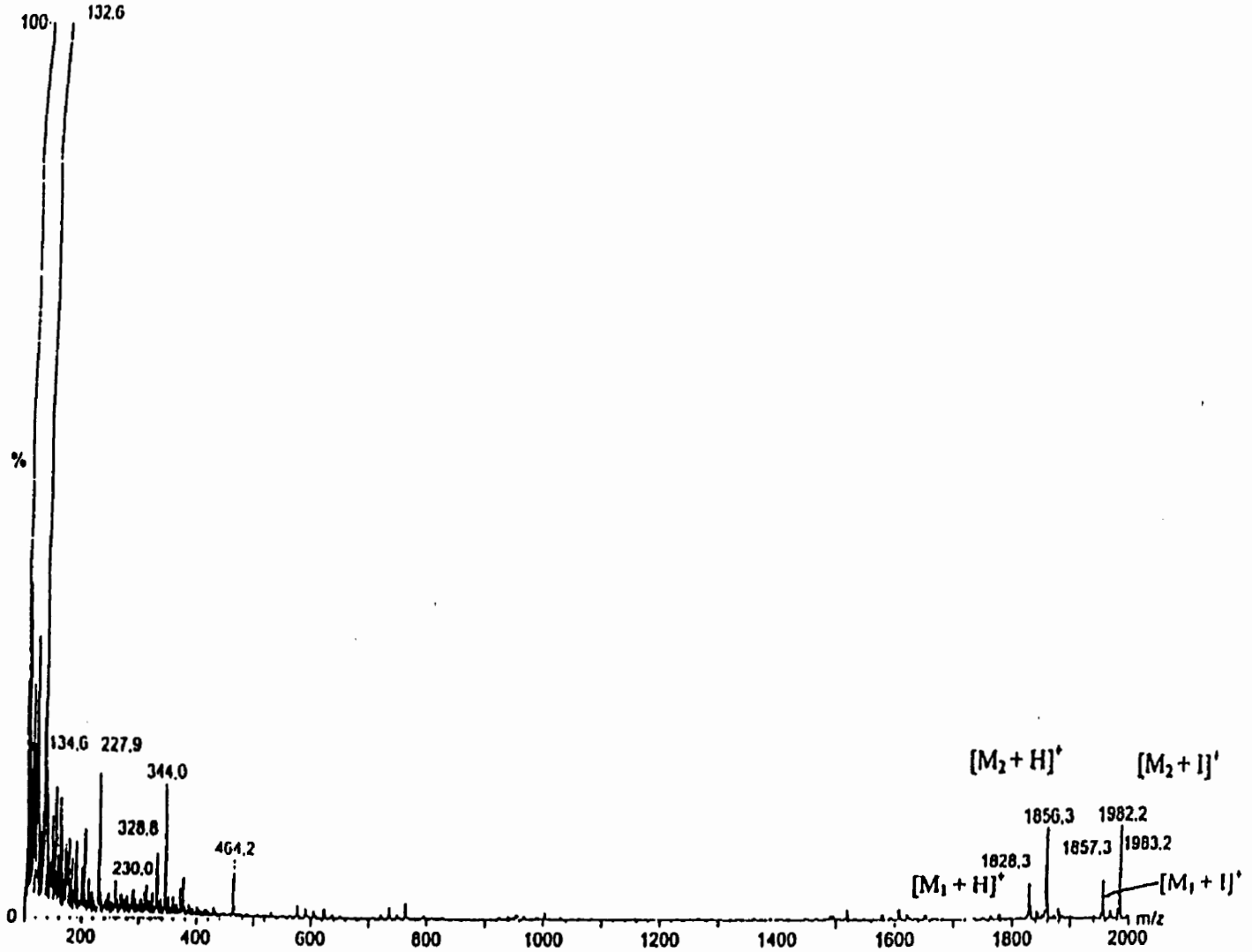


Figure 3.19: Positive ion ESI mass spectrum of permethylated GM_1. Permethylation is performed at higher temperature (30-40 °C). Peaks at *m/z* 1954.2, 1982.2, 1983.2 are absent from low temperature (25-30 °C) products.

permethylated sugars. The total GC/MS ion chromatogram of the standard mixture of permethylated sugars is shown in Figure 3.20.

Galactose produced three different permethylated isomers. These permethylated furanoside and pyranoside forms of galactose were identified based on our previous work[142] and from their mass spectral data. The permethylated galactoside isomers observed by GC/MS were, by increasing retention time order: β-galactofuranoside (βgf 17:13 min), β-galactopyranoside (βgp 17:27 min), and α-galactofuranoside (αgf 17:46 min).

Three well-separated peaks were found in the TIC trace of permethylated standard mixture and correspond to permethylated glucose isomers. The mass spectra at the maxima of the first (16:44 min) and the third peak (17:20) showed abundant *m/z* 88 ions. These spectra corresponded well with the NIST library spectra[140] of methyl-2,3,4,6-tetra-O-methyl (α and β)-D-glucopyranose. The spectrum corresponding to the second TIC peak at 17:05 min showed the predominant *m/z* 101 species and no *m/z* 88 ions. It is also very similar to the NIST library spectra[140] of α and β galactofuranose. This suggests the presence of a permethylated product that is different from the pyranoside anomers. This spectrum correspond to the library spectra[140] of methyl-2,3,5,6-tetra-O-methyl (either α- or β)-D-glucofuranose. Further

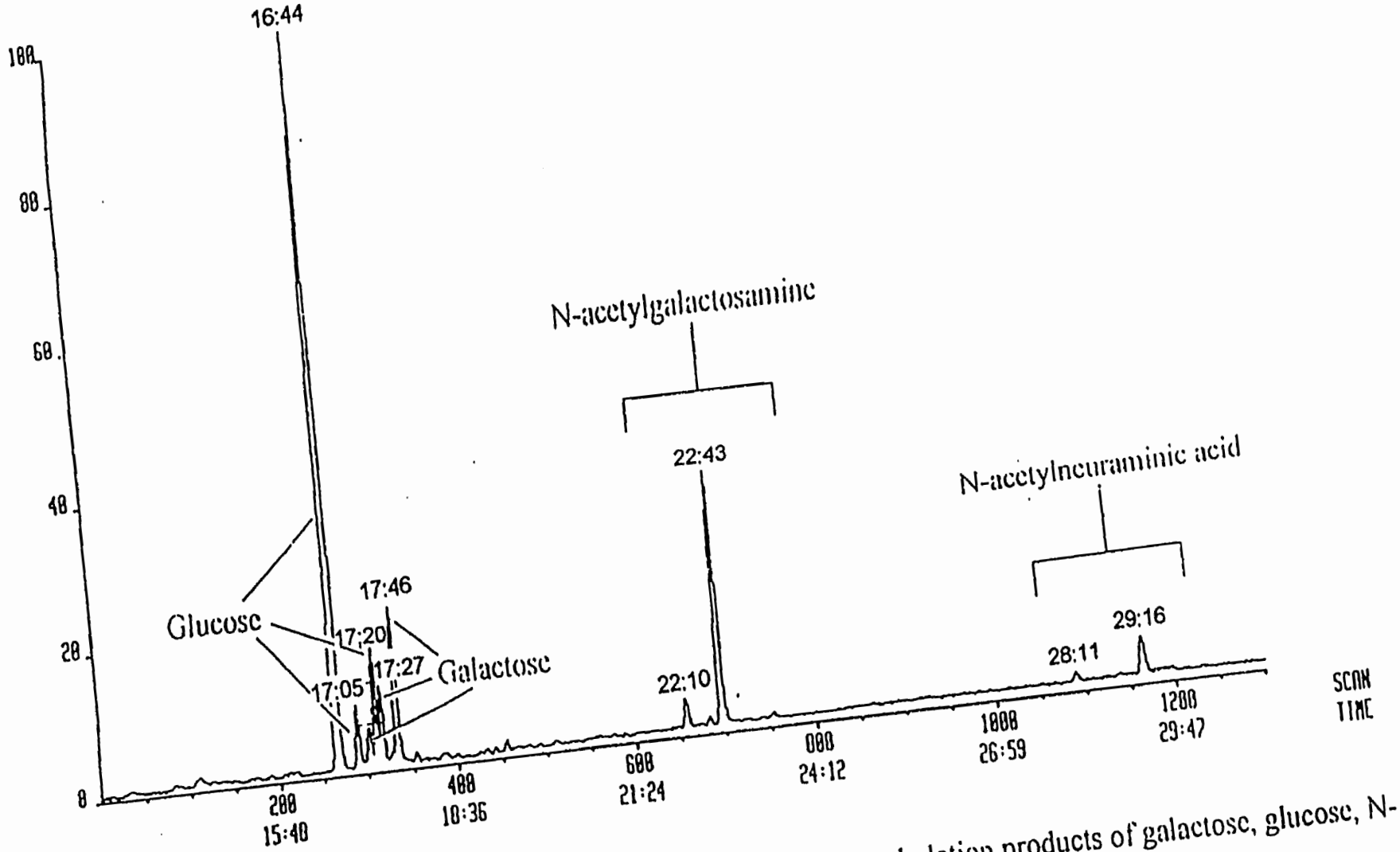
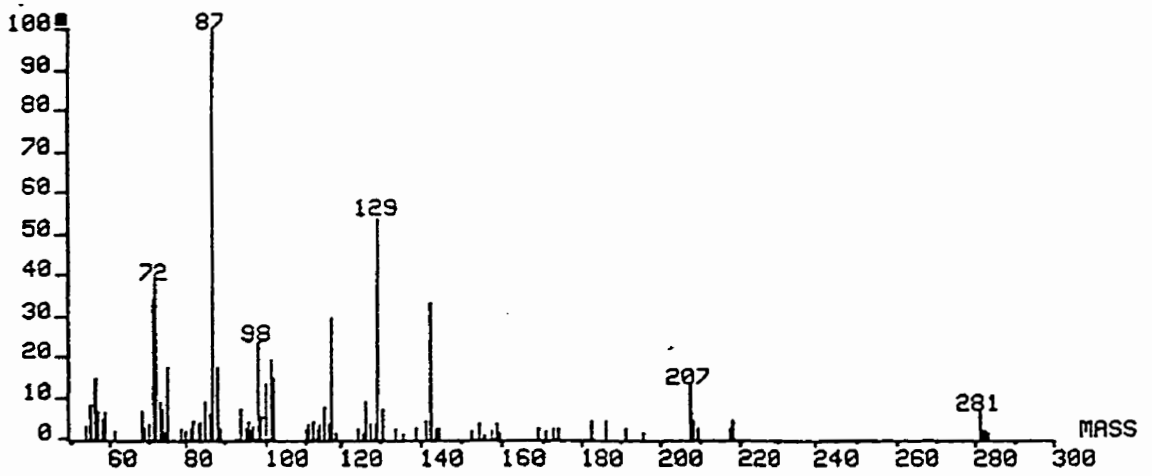


Figure 3.20: GC/MS total ion chromatogram obtained for the permethylation products of galactose, glucose, N-acetylgalactosamine, and N-acetylneuraminic acid.

characterization was not performed to identify the α and β pyranoside and furanoside forms of permethylated glucose.

Permethylation of N-acetylgalactosamine produced two isomers. The mass spectrum of the first peak (22:10 min) is quite different from that of the second peak (22:43 min). This suggests the presence of two tautomers of permethylated N-acetylgalactosamine that are different in structure. The spectrum of the first peak is very similar to the NIST library spectrum[151] of methyl 2-(acetylmethylamino)-3,4,6-tri-O-methyl-α-D-galactopyranoside. Both spectra showed abundant *m/z* 87 ions. The second spectrum showed a predominant peak at *m/z* 100. The spectra of these isomers show patterns similar to those of the pyranose and furanose forms of galactose, glucose and fucose. The most intense peak in the spectrum of the pyranosyl form of N-acetylgalactoseamine appears at one mass unit less than in the spectra of the corresponding forms of glucopyranose or galactopyranose. The second form of N-acetylgalactosamine also yielded an intense peak at one mass unit less than produced by glucofuranose or galactofuranose. This suggests that the second peak of permethylated N-acetylgalactosamine might correspond to one of its furanosyl products. The mass spectra of the standard permethylated N-acetylgalactosamine products and the library spectrum[151] are shown in Figures 3.21 (a), (b) and 3.22 respectively.

a)



b)

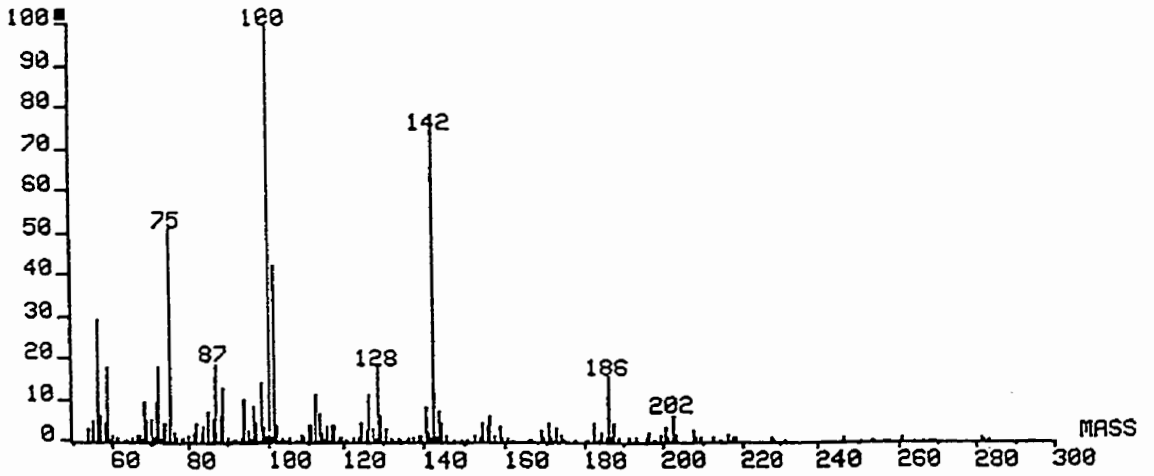


Figure 3.21: Electron impact mass spectra of permethylated N-acetylgalactosamine obtained from the GC/MS total ion chromatogram shown in Figure 3.20. (a) Peak at 22:10 min (b) peak at 22:43 min. Data obtained with the HP 5890 GC/VG 7070E-HF MS system.

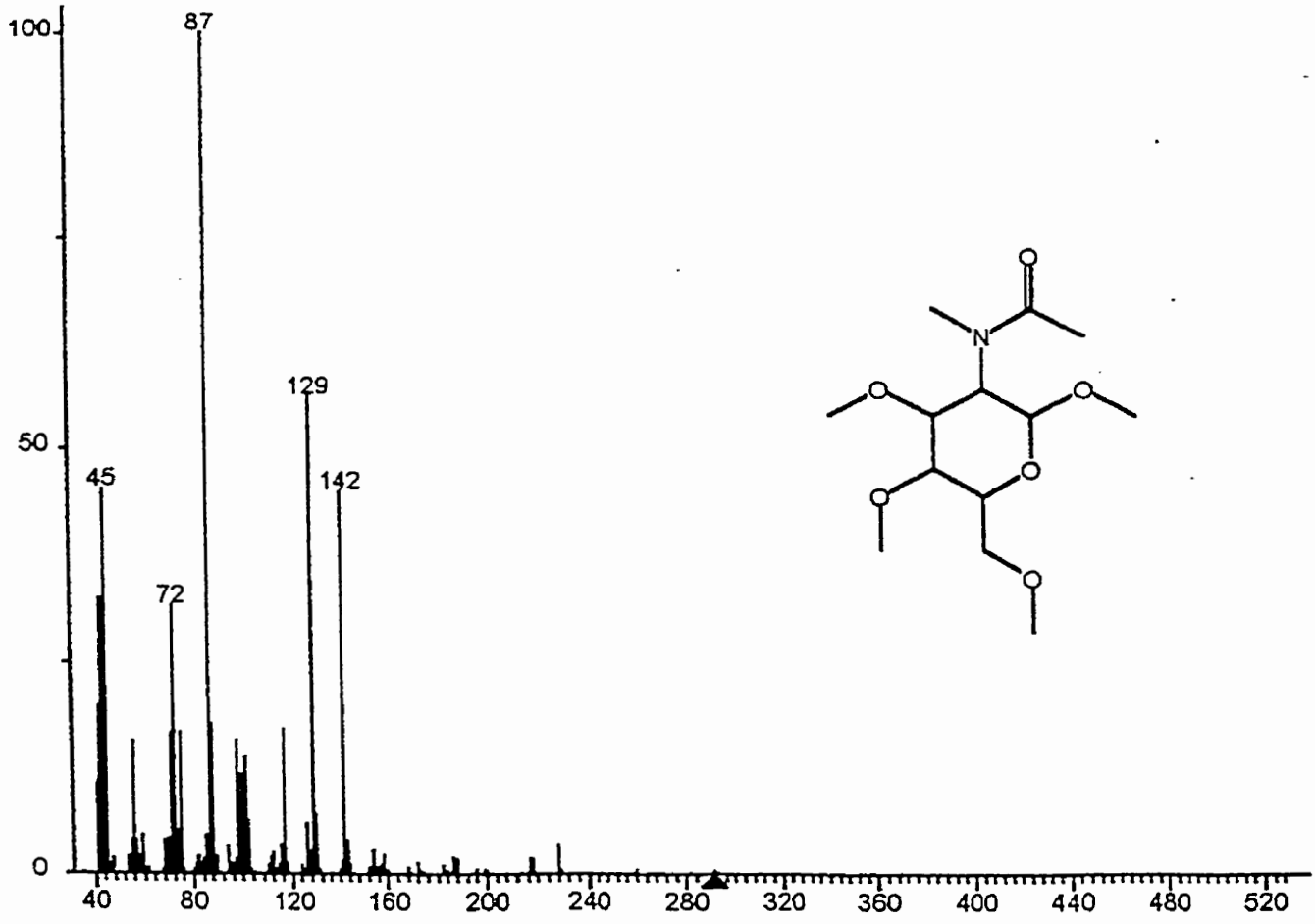


Figure 3.22: Electron impact mass spectrum of methyl 2-(acetylmethylamino)-2-deoxy-3,4,6,-tri-O-methyl-α-D-galactopyranoside obtained from the NIST spectral library[151].

Permethylation of N-acetylneuraminic acid yielded two isomeric forms. The mass spectrum taken at the apex of the most abundant peak (29:16 min) and the other spectrum (28:11 min) were quite similar to each other in spite of variations in the relative ionic abundance. No major structural differences can be deduced from comparison of these spectra; however, they can be used qualitatively to verify the presence of a sialic acid related residue upon methanolysis of an unknown glycosphingolipid.

3.2.3 Discussion on methanolysis and re-N-acetylation reactions of GM_1

3.2.3.1 Method 1

The ESI mass spectrum of methanolyzed GM_1 did not yield the $[M+H]^+$ ion peak for permethylated GM_1. However, pairs of peaks at *m/z* 766.6 and 794.7, 784.6 and 812.8, 818.7 and 842.8, 924.7 and 952.8 were observed. These peaks, all related to base containing fragments, are higher in mass than the predicted individual sub units of GM_1. Thus complete methanolysis of each of the glycosidic bonds did not occur. Based on the first method, we expected the following products: permethylated sugars (permethylated N-acetylgalactosamine, N-acetylneuraminic acid, galactose, and glucose), methyl derivatives of sphingosine bases, and fatty acid methyl ester. One important aspect of Method 1, which distinguishes it from Method 2, is its

potential to yield all the products in one single GC/MS chromatogram. We tried to accomplish this, however, our results show permethylated β-galactose (16:57 min), permethylated α-galactose, (17:06 min) fatty acid methyl ester (24:44 min) and very small amount of permethylated N-acetylgalactosamine (22:59 min). Thus permethylated glucose, N-acetylneuraminic acid and sphingosine base are missing. The total ion GC/MS chromatogram obtained for permethylated-methanolyzed GM_1 is shown in Figure 3.23. A better yield was obtained when a hexane extraction was performed after methanolysis, prior to evaporating the solvent mixture in *vacuo*. Permethylation-methanolysis treatment of GM_1 yielded two peaks that correspond to the α and β pyranoside forms of permethylated galactose. These products were identified by comparison of their retention times with those of the standard mixture and from their mass spectral data using NIST library spectra[140]. A very small peak was observed for the permethylated N-acetylgalactosamine product that was identified using the standard mixture and NIST library data[151]. The fatty acid methyl ester was identified as methyl ester octadecanoic acid by comparing its mass spectrum with NIST library data[151]. Since there are many isomers which have similar spectra, it would be difficult to identify the exact compound present in an unknown sample.

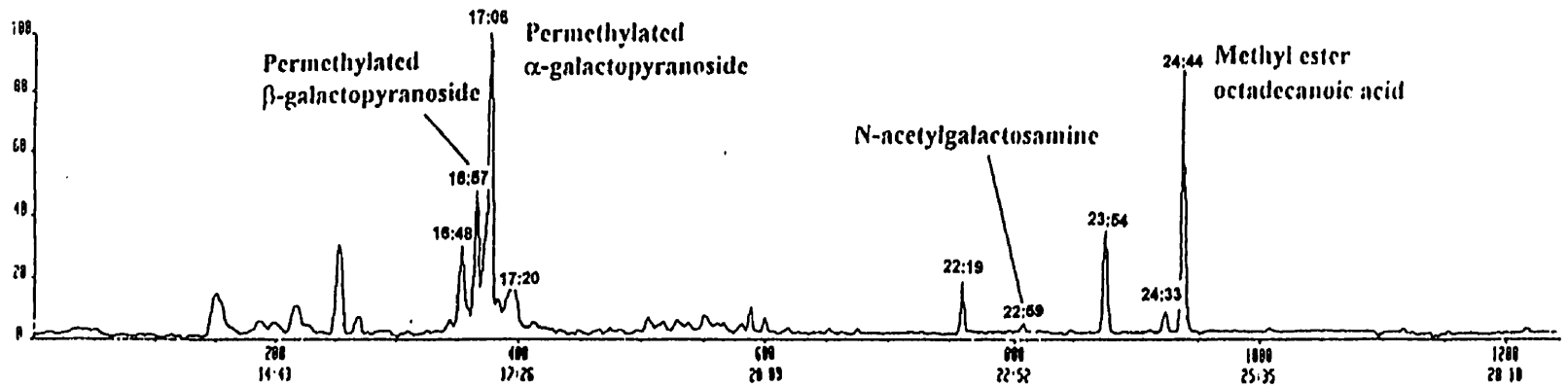
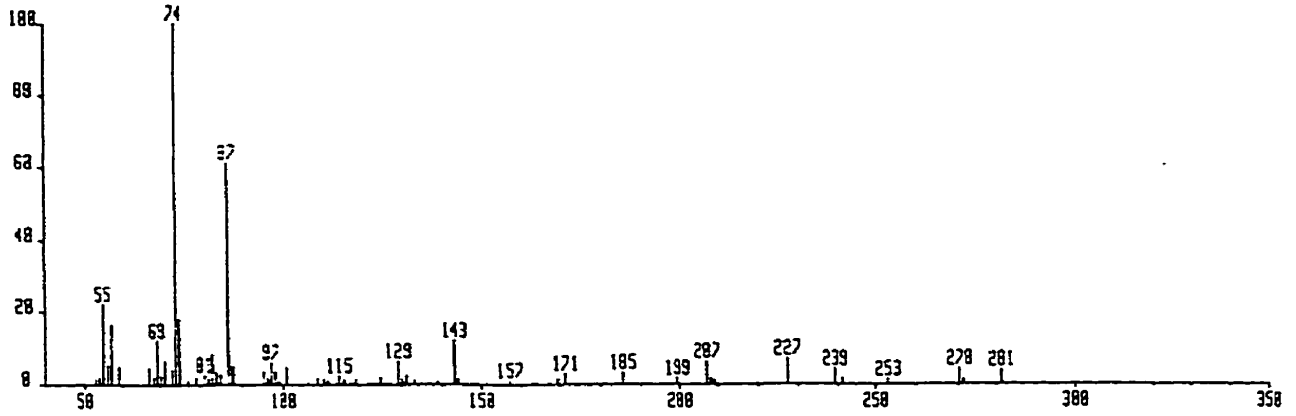


Figure 3.23: GC/MS total ion chromatogram obtained for permethylated- methanolyzed GM_1. Methanolysis was performed using 0.75 N methanolic HCl.

The mass spectrum of the fatty acid methyl ester peak (24:44 min) shown in Figure 3.24 (b) is quite similar to that of the peak observed at 22:19 min, shown in Figure 3.24 (a). This suggests the presence of another fatty acid methyl ester which is probably an isomer of methyl ester octadecanoic acid without the molecular ion peak. The NIST library spectrum[151] of methyl ester octadecanoic acid is shown in Figure 3.25. We were not able to identify peaks in the permethylated galactose region (16:48 min and 17:20 min) and those observed at 23:54 min and 24:33 min. We suspect that peaks at 23.54 min and 24.33 min are internal fragments of GM_1 containing two or more sub units, however, there are no library spectra to support our assumptions. The ESI mass spectrum of the methanolysis product mixture of GM_1 showed pairs of peaks of higher masses (*m/z* 766.6 and 794.7, 784.6 and 812.8, 818.7 and 842.8, 942.7 and 952.8) than those of the expected products. The mass difference within each pair indicated that the sphingosine base was contained in these ions. Peaks with *m/z* 924.7 and 952.8 describe the presence of non-volatile products which contain permethylated galactosamine, galactose, glucose and sphingosine bases [see Figure 3.27 (b)]. Since these products were not cleaved into their monosaccharide units, it was not possible to obtain peaks for permethylated glucose, permethylated galactosamine, and sphingosine bases on the GC chromatogram. In addition,

a)



b)

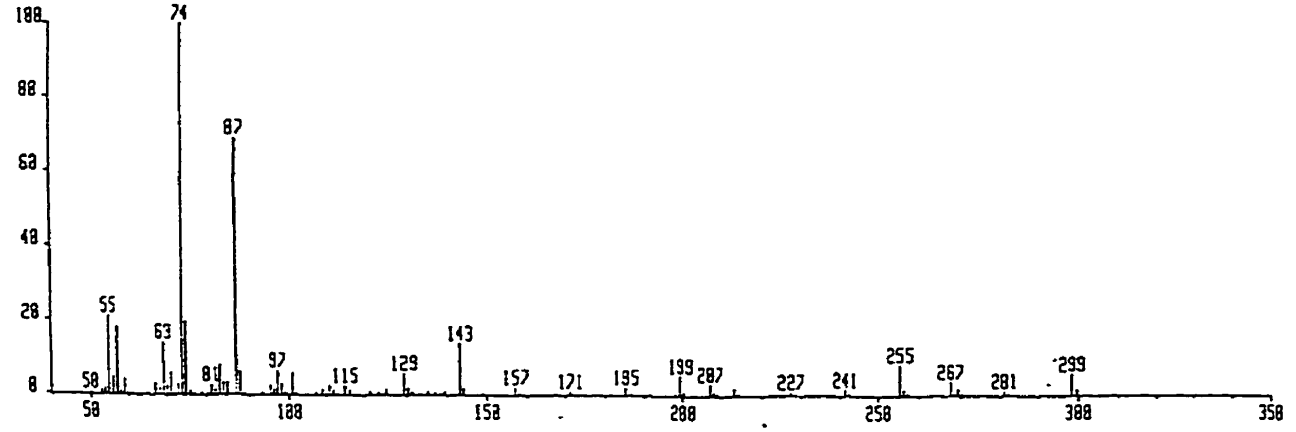


Figure 3.24: Electron impact mass spectra obtained from the GC/MS total ion chromatogram shown in Figure 3.23. (a) peak at 22.19 min; (b) peak at 24:44 min. Data obtained with the HP 5890 GC/VG 7070E-HF MS system.

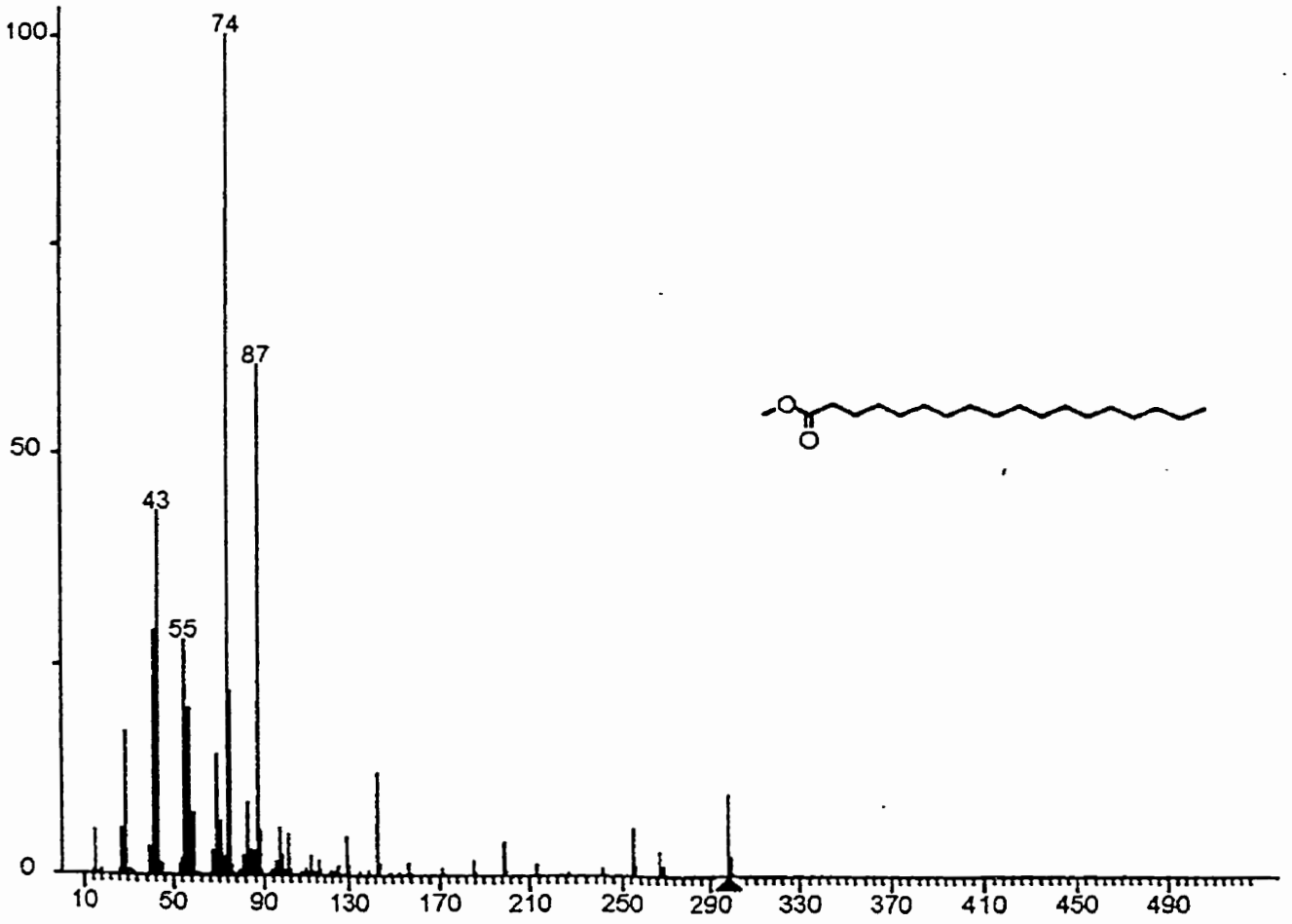


Figure 3.25: Electron impact mass spectrum of methyl ester octadecanoic acid obtained from NIST spectral library[151].

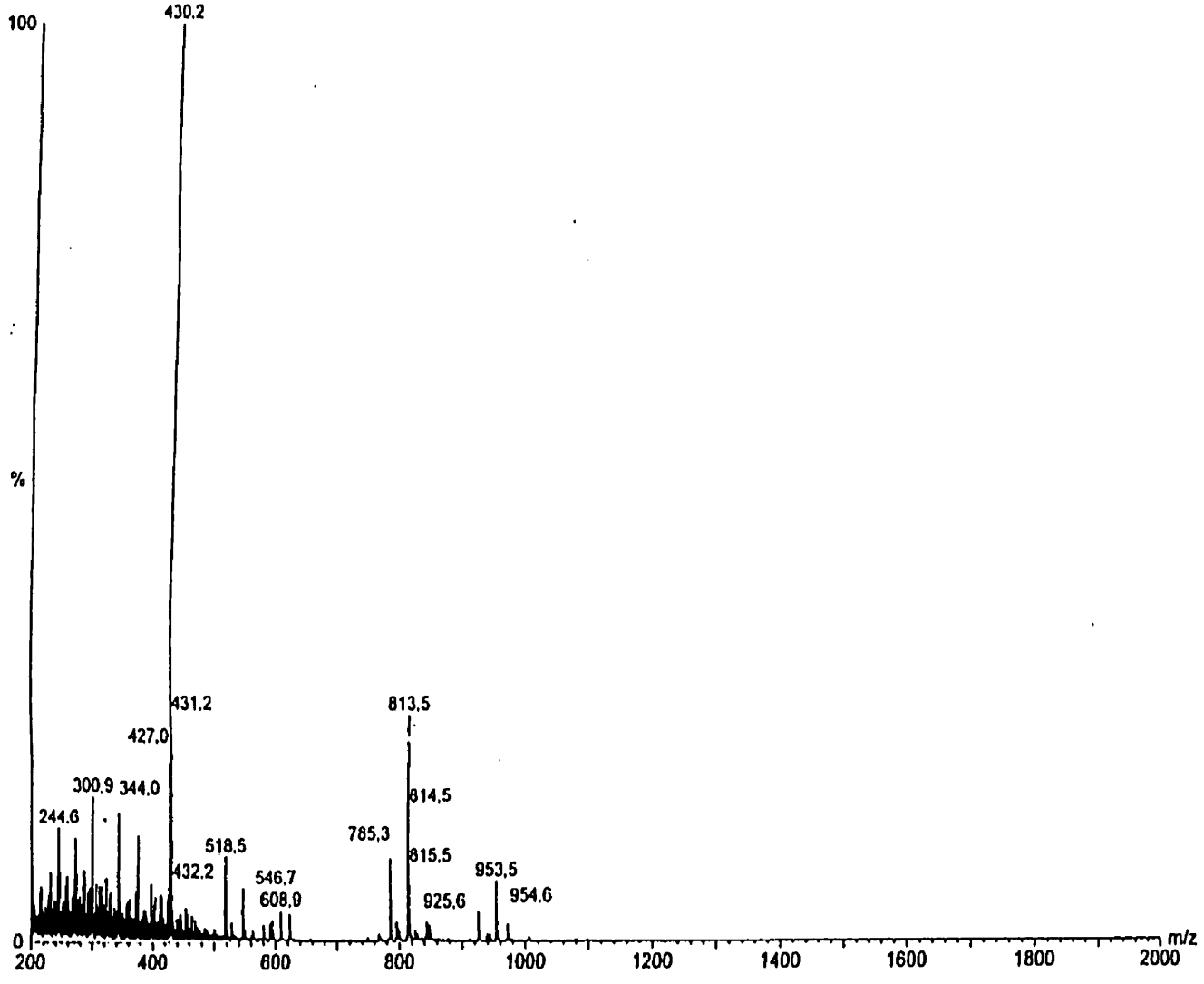
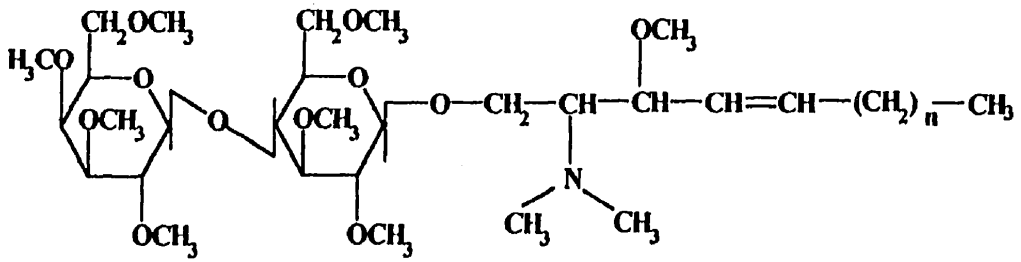


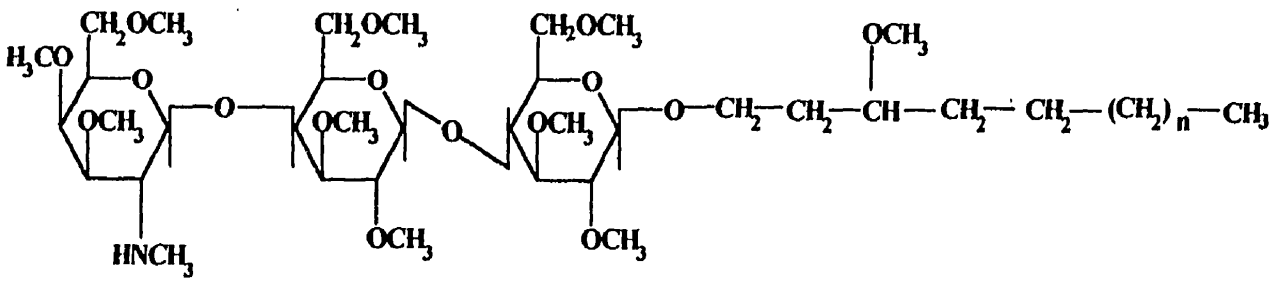
Figure 3.26: Positive ion ESI mass spectrum obtained for methanolyzed GM_1. Methanolysis was performed using 0.75 N methanolic HCl.

a)



*This fragment molecule produced a pair of sodium adduct ion peaks at m/z 786 ([763 + Na]$^+$) and 814 ([791 + Na]$^+$).

b)



*This fragment molecule yield a pair of peaks at m/z 926 ([925 + H]$^+$) and 954 ([953 + H]$^+$).

Figure 3.27: Possible structures of higher mass fragment molecules obtained after methanolysis of permethylated GM_1 using 0.75 methanolic HCl.

the nonvolatile products could not be detected by GC/MS. Peaks observed at *m/z* 784.6 and 812.3 in Figure 3.26 indicate the presence of a fragment molecule containing galactose , glucose and sphingosine bases (see Figure 3.27a). This is additional evidence for the incomplete methanolysis and for the small amount of permethylated N-acetylgalactosamine obtained. The ESI mass spectrum shown in Figure 3.26 indicates the presence of permethylated N-acetylneuraminic acid (fragments at *m/z* 430.2), however, we were not able to detect this in the GC/MS chromatogram. Like N-acetylgalactosamine, this molecule may also attached in some higher mass fragment molecules that could not be analyzed by GC/MS. A β(1→3) glycosidic cleavage and an amide cleavage are together responsible for the formation of permethylated galactose methyl ester octadecanoic acid. Thus the β(1→4) linkage that connects N-acetylgalactosamine to galactose and glucose, and the glucose-ceramide linkage seem to be more resistant to methanolysis than the other linkages. The ESI mass spectrum of the methanolysis product mixture and the possible structure of products obtained by incomplete methanolysis are shown in Figures 3.26, 3.27 (a), and (b), respectively.

Various researchers have suggested the importance of the re-N-acetylation procedure of amino sugars to recover the acetylated sugars. We thus tried re-

N-acetylating using dry acetic anhydride. However, no permethylated N-acetylneuraminic acid was obtained and there was no improvement in the recovery of N-acetylgalactosamine. As discussed above, galactosamine may have stayed linked β(1→4) to galactose. Thus re-N-acetylation could not possibly produce permethylated N-acetylgalactosamine, but rather the N-acetylated product from incomplete methanolysis. Our GC/MS chromatograms show the same peaks before and after re-N-acetylation and there is no significant change in the recovery of permethylated amino sugars. In this procedure, the mixture of permethylated and methanolyzed products was kept in acetic anhydride for 6-8 h at room temperature. This reaction time might not have been sufficient for re-N-acetylation of amino sugars. Thus it might be better to use a longer time (> 8 hours) for re-N-acetylation. Our results indicated that the treatment of GM_1 using 0.75 N methanolic HCl for 16-18 h at a temperature of 80-100°C did not yield a complete cleavage as desired. Thus modified conditions such as higher methanolic HCl concentration may be required for complete methanolysis. Our GC/MS results from the methanolysis products obtained using 1N methanolic HCl seem promising. All the permethylated sugar peaks are observed, along with some peaks which might correspond to the sphingosine bases. However, the intensity of the amino sugar peaks is very low and further work is required

to obtain a better recovery. In general , it seems better to use higher concentrations of methanolic HCl ($1 \leq 1.5$ N) for complete cleavage of GSLs into their components and more than 8 h of re-N-acetylation is required to increase the yield of recovery of amino sugars. Table 3.6 gives a comparison between expected and observed products from Method 1.

3.2.3.2 Method 2

According to Esselman et al.[26], methanolysis of native GM_1 yields methyl galactoside, methyl glucoside, methyl N-acetylaminogalactoside, methyl neuraminate, sphingosine bases and the fatty acid methyl ester. In Method 2, the first step is methanolysis of GM_1 using methanolic HCl. However, GC/MS analysis was not performed to monitor the formation of methanolysis products. Instead, we extracted the fatty acid methyl ester using hexane and analyzed it by GC/MS. The GC/MS total ion chromatogram of this analysis is shown in Figure 3.28. One very intense peak (24:44 min) that corresponds to fatty acid methyl ester and a very small peak at 22:19 min. are observed. Both these compounds are also obtained using Method 1 and show the same retention time with both methods. The mass spectrum of the most abundant peak is quite similar with the NIST

Table 3.6: Expected products and products obtained using Method 1.

(a) Procedures applied and expected products		(b) Procedures applied and products obtained
Intact GM_1		Intact GM_1
↓	Permethylation	↓
• Permethylated GM_1		• Permethylated GM_1
↓	Methanolysis	↓
• Permethylated galactose and glucose • Permethylated N-acetylgalactosamine and its de-acetylated analog • Permethylated N-acetylneuraminic acid and its de-acetylated analog • Permethylated Sphingosines • Methyl ester octadecanoic acid		• Permethylated galactose • Permethylated N-acetylgalactosamine • Methyl ester octadecanoic acid • Higher mass products
↓	N-acetylation	↓
• Permethylated galactose and glucose • Permethylated N-acetylgalactosamine • permethylated N-acetylneuraminic acid • Methyl ester octadecanoic acid • Permethylated sphingosines		• Permethylated galactose • Permethylated N- acetylgalactosamine • Methyl ester octadecanoic acid

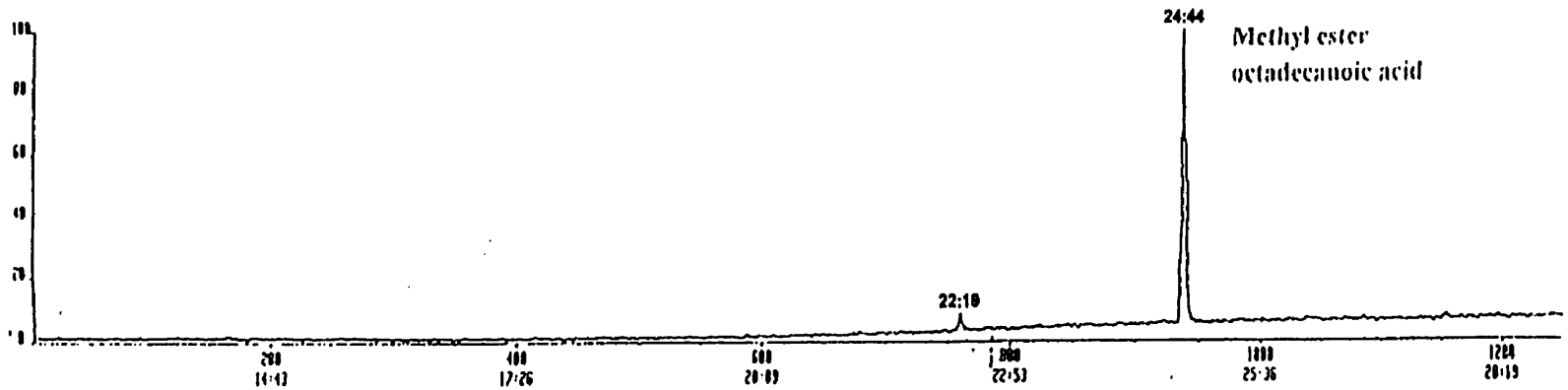


Figure 3.28: GC/MS total ion chromatogram obtained for native GM_1 after methanolysis treatment and hexane extraction.

library spectrum[151] of methyl ester octadecanoic acid. The other peak was identified as mentioned in the section on Method 1.

The methanolysis products of native GM_1 left after hexane extraction were then analyzed by GC/MS after acetylation and permethylation procedures. The GC/MS total ion chromatogram obtained after acetylation-permethylation treatment is shown in Figure 3.29. Method 2 yielded permethylated (β and α) glucopyranose (16:15 and 16.52 min), permethylated β-galactopyranoside (16:57 min), permethylated α-galactopyranoside (17:06 min), and a very small quantity of permethylated N-acetylgalactosamine (23:00 min). These products were identified as described in the section on Method 1. Permethylated N-acetylneuraminic acid and the sphingosine bases are missing. However, other peaks in the permethylated glucose and galactose region of the chromatogram (16:47 min, 17:12 min, and 17:19 min), and one at 23:53 min were obtained. All these peaks are also obtained using Method 1 and we were not able to characterize them.

A summary list of the products obtained using Method 2 in comparison with the expected products is given in Table 3.7.

A much better yield of permethylated N-galactosamine is obtained with Method 2 than Method 1. In addition, Method 2 yields permethylated

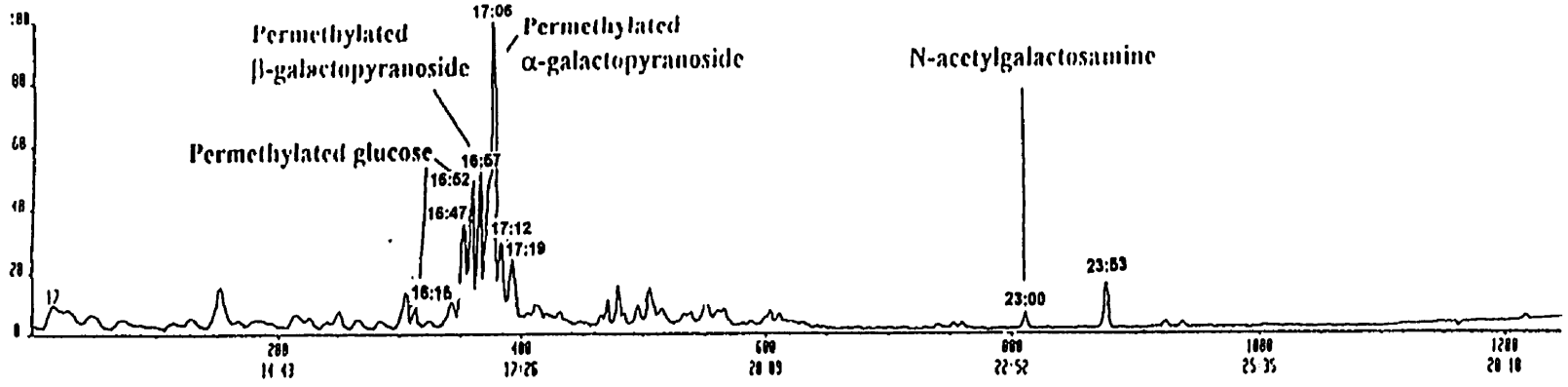
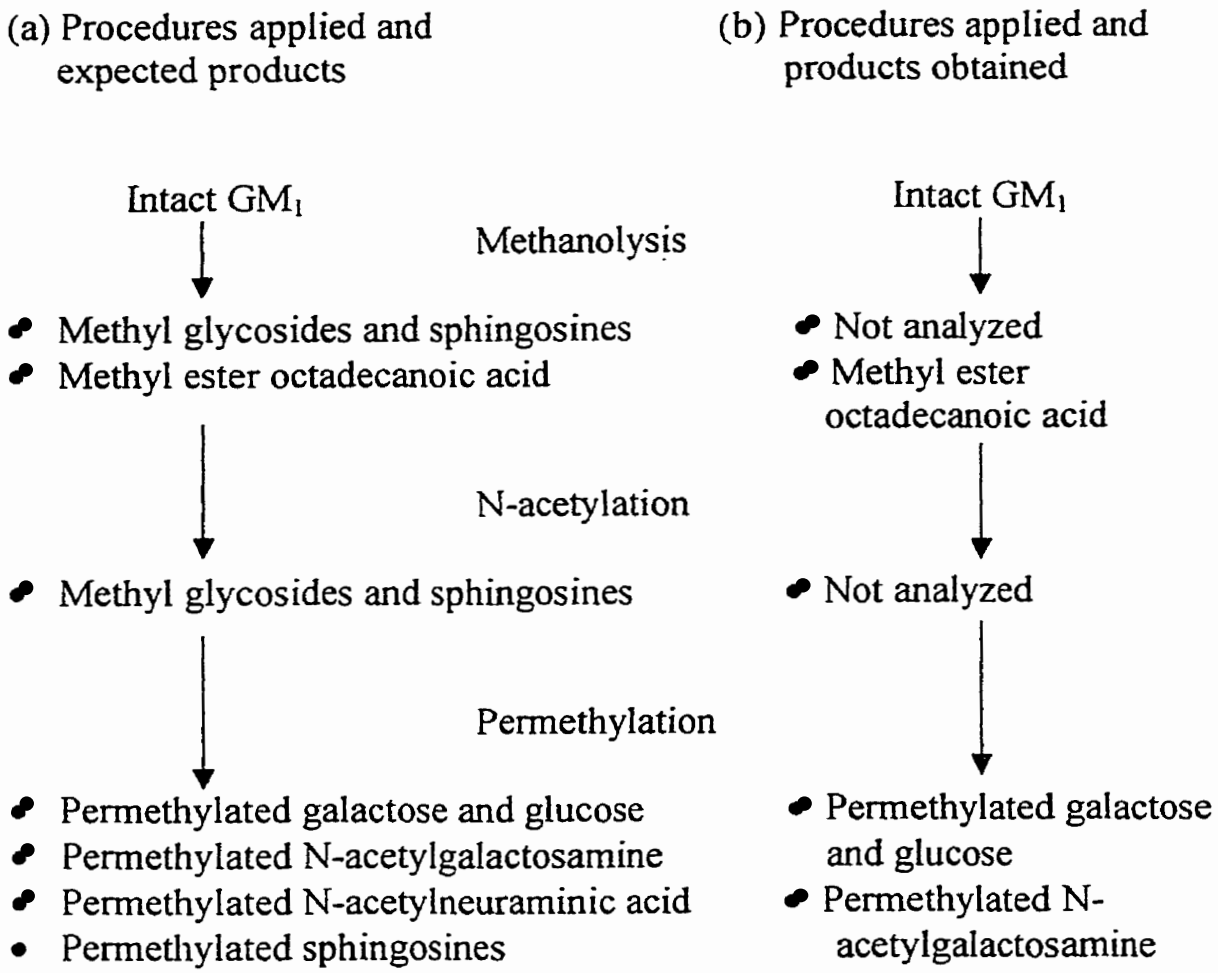


Figure 3.29: GC/MS total ion chromatogram obtained for native GM_1 after methanolysis, acetylation, and permethylation procedures.

Table 3.7: Expected products and products obtained using Method 2.



glucose, which is not obtained using Method 1. Thus it seems that more fragmentation occurred with Method 2 than Method 1. This suggests that methanolysis of permethylated GM_1 is more difficult than methanolysis of native GM_1. More concentrated methanolic HCl might be required for methanolysis of permethylated GM_1. Both methods failed to give the permethylated derivatives of sphingosine bases and N-acetylneuraminic acid. In fact in another trial with more concentrated methanolic HCl we were able to obtain permethylated N-acetylneuraminic acid. In any case the amount of permethylated N-acetylgalactosamine recovered is very low. Even though we performed re-N-acetylation to enhance the recovery of amino sugars, our results show no improvement in the yield of recovery of these sugars. A comparison of the products obtained using both methods are shown in Table 3.8.

Table 3.8: Products of GM_1 obtained after permethylation and methanolysis treatment.

Methanolysis products of GM_1	**Method 1†**	**Method 2‡**
Permethylated galactose	Yes	Yes
Permethylated glucose	No	yes
Permethylated N-acetylgalactosamine	No	yes
Permethylated N-acetylneuraminic acid	No	No
Fatty acid methyl ester	Yes	Yes
Sphingosine bases	No	No

† Method 1: A mixture of 0.5 mg of GM_1, 1 mL of DMSO, 40 mg of NaOH, and 0.5 mL of CH_3I was sonicated for 2 h at 20-30 °C. The permethylated GM_1 was then treated with 0.75 methanolic HCl for 16-18 h at 80-100 °C. Re-N-acetylation was performed with 1 mL acetic anhydride at room temperature for 6-8 h.

‡ The same amount of reagents and the same procedures were used for Method 2. However, methanolysis was performed prior to permethylation, and sonication of a mixture of methanolyzed sample, DMSO, NaOH and CH_3I was performed for 30 min.

CONCLUSION

It has been shown that permethylation conditions, based on the method of Ciucanu and Kerek[80], have a profound influence on the composition of the products obtained from permethylation of D-galactose and L-fucose. For both monosaccharides, permethylation produced a mixture of four isomers: two pyranoside and two furanoside α and β anomers. In each case, three compounds out of the four were predominant and well separated on the GC/MS chromatograms. The fourth component in each case is very minor and its proportions did not appear to vary from one set of conditions to another. Changes in the relative proportions of the three main compounds were reported as different reaction parameters were varied. The most influential parameter appeared to be the time the DMSO solution and NaOH were mixed by sonication before reaction with methyl iodide. The initial temperature of reaction also had an important effect. These two parameters influenced the initial proportion of the four possible isomers, or isomeric anions, of the monosaccharides in solution prior to permethylation.

It has been shown that care has to be taken when performing permethylation of sugars as a routine operation. The reaction conditions must be very carefully controlled in order to obtain reproducible meaningful results.

We have developed a practical and inexpensive method for the preparation

of a single isomers of permethylated D-(+)-galactosides and L-(–)-fucosides, to be used as standards in GC experiments as part of permethylation analysis. The products can be separated in mg quantities by silica flash column chromatography or continuous elution thin layer chromatography. Two of the galactosides, β-galactopyranoside and α-galactoforanoside, were difficult to separate and were not recoverable as pure species on a column. They were isolated almost as pure compounds, however, using continuous elution TLC. The fucoside isomers lent themselves better to separation, although there was still a slight overlap between β-fucopyranoside and α-fucoforanoside, although much less than the for the galactoside isomers. We prepared permethylated hexoside isomers of glucose , N-acetylgalactosamine, sialic acid using the Ciucanu and Kerek[80] method and we used these compounds as standards in the methanolysis-permethylation analysis of glycolipids.

The scope of this study was to develop a method to characterize the components of a glycosphingolipid. At this point in our study, GC/MS and ESI/MS have been used to characterize methanolysis products of native and permethylated glycosphingolipids. The comparison of retention times, peak patterns, and mass spectra provides sufficient information to identify the components of the glycosphingolipid. As shown, we have developed two

methods to identify the components of GM_1. These methods involve methanolysis, acetylation and permethylation procedures. Both methods yield almost identical products. Both methods failed to give amino sugars and sphingosine bases, so further studies are needed to improve the recovery of these compounds.

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