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Linkage Analysis and Compositional Studies of β-Glucan from *Saccharomyces Cerevisiae* and Compositional Studies of Mannan from *Candida Albicans*

A thesis

presented to

the faculty of the Department of Chemistry

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Chemistry

---

by

Clara Arthur

August 2015

Dr. Cassandra T. Eagle, Chair

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Keywords: Monosaccharide compositional analysis, Linkage analysis, Partially methylated alditol acetate, Alditol acetate, *Saccharomyces cerevisiae*, *Candida albicans*

ABSTRACT

Linkage Analysis and Compositional Studies of β-Glucan from *Saccharomyces Cerevisiae* and Compositional Studies of Mannan from *Candida Albicans*

by

Clara Arthur

The efficacy of a novel carbohydrate extraction procedure was investigated with methylation analysis and alditol acetate method by Gas Chromatography-Mass Spectrometry. A published extraction procedure for β-glucans was compared to one developed in house. Both procedures gave a dominant glucose peak in the Gas chromatogram indicative of successful β-glucan isolation. Further linkage studies showed four linkage positions for β-glucans isolated with the published method; terminal, 1,3-linkage, 1,6-linkage and 1,3,6-linkage, while β-glucans isolated using the new method showed six linkage positions; terminal, 1,3-linkage, 1,6-linkage, 1,4-linkage, 1,2,3-linkage and 1,3,6-linkage. Diminishing β-glucan linkage peaks in the chromatogram for the published method indicated structure degradation. The results for mannan isolated with 50 mM base gave mannose as a dominant component compared to mannan isolated with 50 mM acid. Base extracted mannan also indicated a good yield of mannan in hyphal form of Candida albicans. This has not been reported with other published isolation methods.

DEDICATION

I dedicate this work to the Almighty God Jehovah, my parents, Mr. and Mrs. Arthur, my siblings, and Mr. Patrick Agbanyo.

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LIST OF ABBREVIATIONS

GPI	Glycosylphosphophatidylinositol
TFA	Trifluoroacetic acid
FTIR	Fourier-Transform Infrared Spectrometer
GC-MS	Gas Chromatography-Mass Spectrometer
$NaBD_4$	Sodium Borodeuteride
NMR	Nuclear Magnetic Resonance

CHAPTER 1

INTRODUCTION

The Yeast Cell Wall

Yeasts are eukaryotic microorganisms (cells contain a nucleus and other organelles) that belong to a group of organisms called fungi. Yeasts are mostly unicellular which typically measure 3-40 µm in diameter. Some yeasts cause diseases whiles others exist in mutual or commensal relationship with their host. There are a wide variety of applications for yeast including wine, bread, beer, cheese and whiskey. Yeast is also employed to produce large quantities of certain hormones and enzymes to heal wounds and reduce inflammation.[1]

The fungal cell wall plays an important role in the physiological adaptation of the fungus to its environment. The yeast's cell wall serves several essential functions for the organism; such as the ability to hold its shape, the regulation of water flow into and out of the cell, and the prevention of the entry of foreign bodies.[2] It constantly changes during cell division, growth and morphogenesis. The dynamic cell wall dictates the organism's protection or susceptibility to the environment.[3,4,5,6]

A true fungal cell wall consists of three main components; lipids, proteins and carbohydrates (glucan, mannan and chitin). The carbohydrate component of the cell wall has been demonstrated to have extensive applications in medicine.[4,7,8] The carbohydrate content of the cell wall varies depending upon the type of fungal species. *Schizosaccharomyces pombe* is one fungal species which lacks chitin in its cell wall. The pathogenic (disease causing) fungus *Candida albicans* and the saprophytic (feeds on dead plant and animal remains) fungus *Saccharomyces cerevisiae* each contain glucan, mannan and a very small amount of chitin.[9]

Theories exist as to how the individual components of the cell wall are cross-linked together to provide the valuable functions of the cell wall, however the exact nature of this cross-linking has yet to be established.[10,2,11] A pictorial representation of a proposed structure of the yeast cell wall containing mannoproteins (outermost layer), β-glucans, β-glucans+chitin, and a plasma membrane that contains lipids is depicted in Figure 1. The figure demonstrates one theory of how the yeast cell wall components are believed to be connected. Insights on how the nature of the individual components of the cell wall are connected may lead to new therapeutics.[9,12]

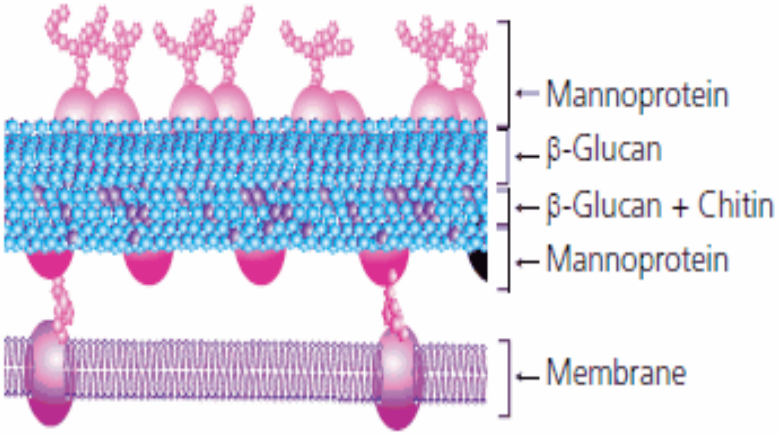

Figure 1: Typical structure of a yeast cell wall[12]

The Greene research group is primarily concerned with developing effective and milder procedures for isolating β-glucan and mannan from the cell wall of fungal species for structural and physicochemical characterization. This research could have future application in the commercial scale production of fungal cell wall carbohydrates. In view of this, the fungal species used in this research, together with a description of the cell wall carbohydrates and their applications are briefly discussed. The various failed and/or successful isolation procedures available for extracting these carbohydrates from the fungal cell walls are also introduced briefly.

The research described in this thesis focuses primarily on determining the efficiency of developed/optimized carbohydrate isolation procedures. Monosaccharide compositional analysis was conducted to determine the sugar components of the isolated β-glucan and mannan while linkage analysis was conducted to determine if the native structure of β-glucans were retained using a milder optimized approach.

Glucan

Glucan is a polysaccharide made up of repeating units of glucose monomers that are linked by glycosidic bonds. They are classified into two categories, based on the type of intrachain linkage that exists in the polysaccharide.[13] Alpha (α) linked glucans such as starch have their glucose units linked together by alpha glycosidic bonds. Beta (β) linked glucans, on the other hand, are linked by beta glycosidic bonds. Alpha glycosidic bonds, depicted as a red colored bond in Figure 2, points in the opposite direction (trans) to the hydroxyl group on carbon-6, while β-glycosidic bonds (blue) point in the same direction (cis) as the hydroxyl group on carbon-6.

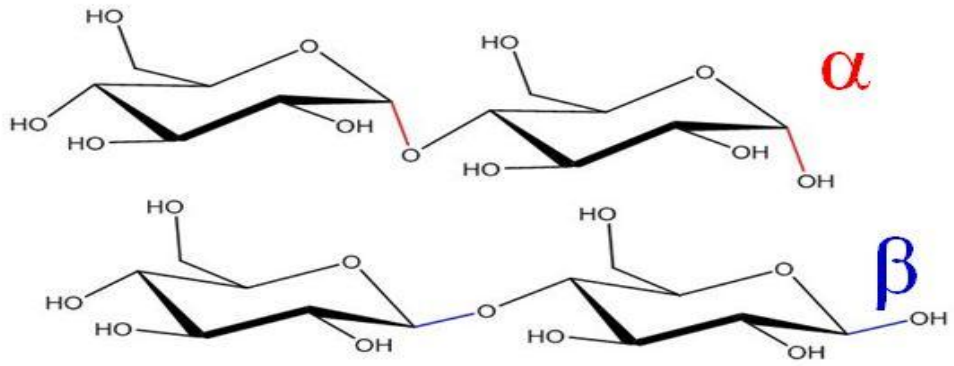

Figure 2: A simplified structure of alpha and beta glucan

The prevalent type of glucan found in the yeast cell wall is the β-linked glucans. The presence of a mixed (1,4)-α-(1,3)-β-glucan in yeast cell wall has also been reported.[11] Beta (β)-glucans typically comprise 50-55% of the cell wall polysaccharide content.[14] They are known to play an important role in several health related applications. Therapeutic applications of β-glucans include the treatment of acute renal failure, post-surgical infections, burns caused by heat, X-ray or UV radiations, pressure ulcers and hospital-acquired pneumonia.[11] Beta (β)–glucans have been shown to stimulate the innate immune system and reduce infectious complications in humans.[13] β-glucans are also believed to play an important part in the recognition and response of the innate immune system to fungal pathogens.[15] They also have potential medicinal uses in diseases such as cancer, infection, sepsis shock and arthritis.[16,17,18]

Not all β-glucans have the ability to induce immune activity. This characteristic is dependent on the chemical structure of the β-glucan.[19] Beta (β)-glucans derived from fungi and yeast consist of a (1, 3)-β-linked backbone with (1, 6)-β-linked side chains. The primary (1, 3)-linked backbone and (1,6)-side chain structure appears to be essential for the fungus to be recognized by the innate immune system. Yeast β-glucans are known also to be highly branched polysaccharides with large molecular weights.[20] Figure 3 shows an example of the primary structure of biologically important β-glucans which consist of a linear backbone of glucose monomers connected to each other via a 1,3-linkage and glucan branches on the backbone with a 1,6-linkage.

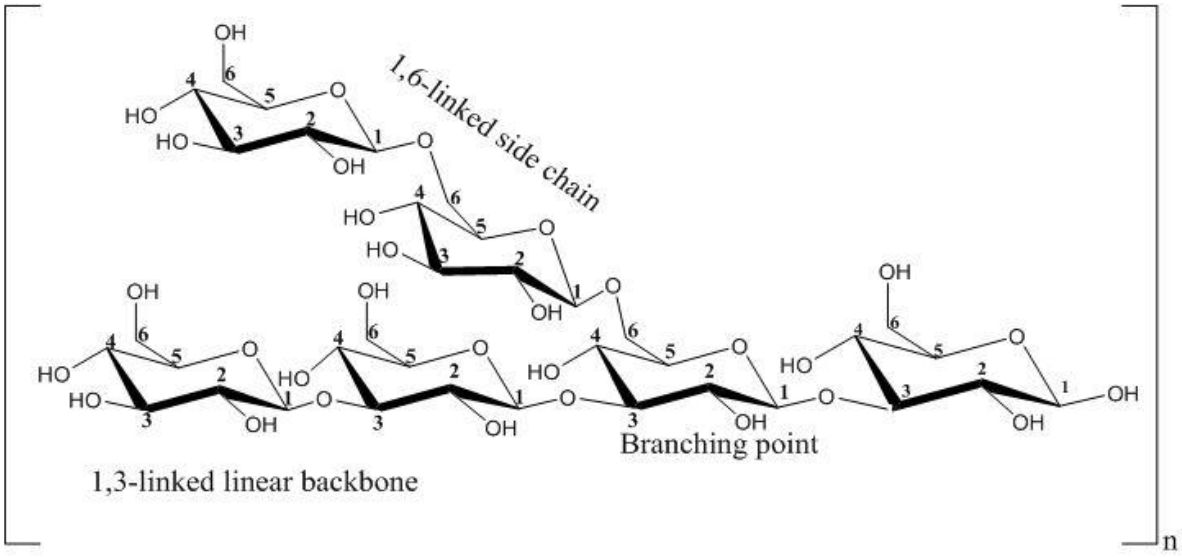

Figure 3: Chemical structure of medically important β-glucan

Several reports from researchers suggest that certain features such as primary structure, solution conformation, physicochemical parameters, and the molecular weight of the glucan dictate how glucans interact with the immune system.[17, 21,22] In depth structural analysis is essential to understand the biological pathway of the fungi in the body. The study of how individual glucose units are linked to each other may help to determine how the immune system is able to recognize β-glucans while in presence of many other cell wall carbohydrates, proteins, and lipids. There is also evidence of a heavy cross-linking between yeast glucan and other components of the cell wall; however, there is no known confirmation of how the other components are linked together in the cell wall.[8] Linkage information of β-glucans may help in understanding how the cell wall holds itself together through this cross-linking. Such information can help in understanding the problem of recurrent fungal infections and to pave way for new antifungal therapies.[12, 23]

Mannan

Mannan is a complex macromolecule consisting of repeating mannose units. Mannans can be obtained from a variety of sources such as ivory nut, algae, and fungal cell walls. In wine making, mannans obtained from yeast have been reported to play an important role in the quality of wine production.[24,25] This includes maximizing sweetness, improving foam quality of sparkling wine, reduce unpleasant feel that follows wine tasting (i.e. red wine) and protection against tartaric instability.

Mannan from fungal species such as *C. albicans* have been found to play a key role in the pathogenicity of this organism.[26] Mannans are believed to possibly mask β-glucans from recognition by the immune system and to exhibit antisuppressive properties.[27] Mannans obtained from *C. albicans* yeast consist of repeating mannose units linked in to fungal proteins through either N-acetyl-D-glucosamine dimer bridge (N-linked) or through a hydroxyl group of the fungal proteins (O-linked). The N-linked polymers are composed of a linear α-1,6 linked backbone of mannose units, with branches of α-1,2, α-1,3, mannans and a phosphodiester bond. The O-linked polymers are unbranched oligomers consisting of two to six mannose unit bonded together via a α-1, 2 and occasional terminal α-1, 3 linkages. Mannoproteins are believed to be attached to glucans or chitins in the cell wall covalently via their sugar residues or glycosylphosphophatidylinositol (GPI) links which may link the mannoproteins to the plasma membrane in the cell wall.[23,28,29]

Chitin

Chitin is a long chain polymer containing repeating unit of N-acetyl glucosamine, glucose with N-acetyl group attachment. It has some physicochemical similarities to cellulose. It is also found in the exoskeleton of insects and bound to proteins in crustaceans.[2] Chitin is present

in small or large amounts in some fungal species while absent in others. It is found in smaller quantities (0.1-2%) in the cell of *C. albicans* and *S. cerevisiae*. Chitin serves an important function of providing structural support to the cell wall, and possibly to coordinate electrochemical processes in fungal cells. It is also involved in immune recognition of pathogenic fungi by pattern recognition receptors.[30,31]

Cell wall Components of *Saccharomyces Cerevisiae*

Saccharomyces cerevisiae is one of the most studied model organisms in fungal species. It is used commercially in brewing, baking, and in aquaria to provide CO_2. The cell wall accounts for 15-30% of the cell's dry weight.[32] The cell wall is composed of an inner and an outer layer. The inner layer, composed of β-glucan and chitin, provides mechanical strength and gives the cell its shape. The outer layer consists of a matrix of mannans connected to proteins (mannoproteins) which are believed to be linked covalently to the glucan and chitin network via a glucan linkage. The outer membrane aids in cell-cell recognition and protects the inner layer and plasma membrane (containing lipids) from foreign bodies such as enzymes that degrade the cell wall.[9,33]

The cell wall contents are classified into five main components; glucan, mannans, chitin, lipids and proteins. Beta (β)-glucans and mannans are major components of the cell wall. Minor components include lipids and chitin-which contributes to insolubility of the fibers. The cell wall carbohydrate is composed of 50-55 % β-1,3 glucan, 5-10 % β-1,6-glucan, 35-40 % mannan/mannoprotein, and 1-2% chitin. Table 1 below, describes average dry weight portion of each of the different cell wall carbohydrate components observed in *S. cerevisiae*.[10]

Table1: Percent Dry Weight Composition of Carbohydrate Content Seen in *S. cerevisiae* Cell Wall

Macromolecule	**Cell wall % dry weight**
1,3-β-glucan	50-55
Mannoprotein	35-40[a]
1,6-β-glucan	5-10
Chitin	1-2[b]

a. Mannoproteins are heavily glycosylated, this value includes proteins
b. This value represents glucans including bud scars

Cell wall Components of *Candida albicans*

Candida albicans is a polymorphic (displaying multiple distinct morphologies) fungal species that can live in the human body without causing any harm. However, an overgrowth of *C. albicans* in the body can cause a variety of mental and physical health conditions including morbidity and death especially in immunocompromised patients. *C. albicans* has three distinct morphologies: yeast (unicellular), pseudohyphae, and hyphae (filamentous) forms. The yeast form is round or oval in shape while the hyphae and pseudohyphae form are threadlike or filamentous in shape as represented in Figure 4. The switching of *C. albicans* between single round cell forms (yeast) into a filamentous form plays an important role in the organism's ability to cause infection. Switching between morphologies can be stimulated by environmental factors such as decreased oxygen, limiting nutrients, neutral pH and high temperature. [34,35,35b]

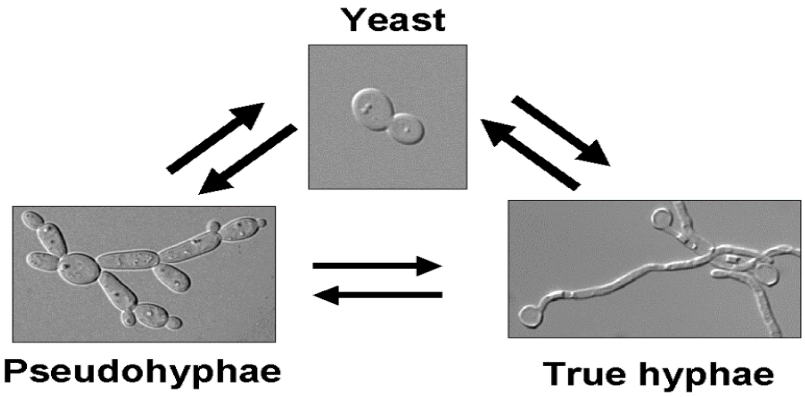

Figure 4: The three morphological stages observed in C. albicans. The organism's ability to switch between these morphologies is essential for virulence

Analysis of the *C. albicans* cell wall has demonstrated that it is composed of 80- 90% carbohydrates such as mannan (linked covalently with proteins to form glycoproteins), β-glucan and chitin. It also consists of a plasma membrane containing lipids (1-7%). Beta (β)-glucans constitute about 47-60% of the cell wall dry weight, mannans/mannoproteins constitute 40% and chitin constitute for 0.9-6% of total carbohydrate in the cell wall. These values vary depending upon the morphological stage of the organism.[28]

Isolation of Beta (β)-Glucan and Mannan from Fungal Cell Wall

Determination of the molecular structure of the fungal cell wall components is essential in resolving the organism's ability to invade host tissues, and their potential use as therapeutics for immunocompromised patients. An efficient isolation procedure is imperative to be able to isolate mannan and glucan from the cell wall for structural analysis. Several extraction procedures have been developed over the years by researchers to isolate β-glucans and mannans from the cell wall of yeast. The classical extraction procedure for β-glucans historically results in the degradation of the structure of these important carbohydrates for structural studies. The

classical method for the isolation of mannans has thus far only been successful in the isolation of mannan in the yeast form and not the other morphogenic forms.[36,37,38]

The classical procedure for isolating glucans is successful in extracting β-glucan; however, it is time consuming and degrades the glucan to a large extent. The classical procedure is complex and employs high concentration and volume of reagents (i.e. strong acid such as hydrochloric acid, base, and ethanol) with multiple sequential extractions using these reagents. Starting from the early 1940s, scientists were primarily interested in the purity (high glucan content) of the glucans and not its native structure. This led to a widespread acceptance of the classical approach.[39] Several publications later reported the immune stimulating activity of β-glucans and its ability to be recognized by the innate immune system. This led to the need for isolating β-glucans which is a true reflection of the β-glucan found in the cell wall.[40,41]

Water insoluble glucans such as those obtained from *S. cerevisiae* exist as a stable triple helix and a highly branched polysaccharide in its native state.[20, 42] Structural analysis (^{1}H NMR and ^{13}C NMR) of water insoluble yeast glucans isolated with the classical method indicate a small degree of polymerization and branching frequency. This small degree of polymerization and branching using the classical method is not a true reflection of the reported triple helical and highly branched structure of the β-glucan in its native state. A milder extraction procedure that would maintain as much of the native structure as possible was necessary in order to better resolve the native structure of β-glucans and how it interacts with the immune system.[43] A milder isolation procedure was optimized in house by a member of the Greene research group (Shadrack Asare). This procedure involved single extraction of dried yeast in ethanol, 0.1 N phosphoric acid and 0.1N sodium hydroxide.

The existing isolation procedure for mannan has been successful in isolating mannan from yeast *C. albicans*; however, these procedures have been unsuccessful in the hyphal form of *C. albicans*. A common procedure which has proved successful in isolating yeast mannan involves precipitation of mannan with Fehlings copper solution. This procedure is not only unable to isolate hyphae mannan but also leaves traces of copper bonded to the mannan.[44] A novel method developed by another member of the Greene research group (Francis Kwofie) is successful in isolating both hyphal and yeast mannan from the cell wall of *C. albicans.* It is a simple and straightforward procedure that employs acetone, water and sodium hydroxide (50 mM) or phosphoric acid (50 mM).

Determining Efficiency of Developed Isolation Procedure by Nuclear Magnetic Resonance Spectroscopy

One method to determine the structure of glucans and mannans is nuclear magnetic resonance (NMR) spectroscopy. In determining the efficacy of the developed methods, the extracted sample is dissolved in a suitable solvent and analyzed using ^{1}H NMR or ^{13}C NMR. The spectra acquired are then compared against a spectral library using chemical shift values to determine the identity of the compound. The insolubility of β-glucans poses a challenge during NMR analysis due to the inability to detect undissolved substances. Also, the presence of a significant amount of other cell wall content can lead to unresolved spectra. This results in an incomplete structural analysis of the isolated cell wall carbohydrate.

Due to these limitations, a second method must be employed to gain more information on the structure of the carbohydrates. The alditol acetate and partially methylated alditol acetate method of carbohydrate analysis by Gas Chromatography Mass Spectrometry (GC-MS) is mostly employed. These methods convert the insoluble polysaccharides into a soluble form for

analysis.[45] The alditol acetate and partially methylated alditol acetate method is used in conjunction with NMR to provide a better understanding and confirmation of NMR results.

Monosaccharide Compositional Analysis (Alditol Acetate method) by Gas Chromatography-Mass spectroscopy

Monosaccharide compositional analysis by the alditol acetate method is a common method for the analysis of the monosaccharide composition of carbohydrates. This process breaks down the polysaccharide into small units (volatile alditol hexaacetate) through series of derivitisation steps for easy identification by GC-MS. It has an advantage over methods such as trimethysilyation or tifluoroacetylation because it produces only a single peak in the chromatogram for each sugar derivative. The alditol acetate method also gives extremely stable compounds once it has been derivatized, allowing for post derivatization clean up and long term storage of samples.[46]

Analysis of the monosaccharide composition can determine the exact carbohydrate components present in the extracts. A succesful isolation of β-glucans will exhibit a dominant glucose peak (glucitol hexaacetate after derivatization) in the chromatogram while that of mannan will exhibit a dominant mannose peak (mannitol hexaacetate after derivatizationderivatization). The monosaccharide compositional analysis gives information about other cell wall carbohydrates present in the extracts as well. Common contaminants that are observed are plasticizers, contaminating solvents used, pthalate esters from Eppendorf pipet tips,[46] and siloxanes from the GC injector septa or column. Pthalate esters are identified in the MS by a diagnostic m/z 149 and siloxanes by m/z of 207. The derivatization process primarily involves three steps:

- Acid Hydrolysis

- Reduction
- Acetylation

Acid Hydrolysis

Polysaccharides are susceptible to acid hydrolysis resulting in monomeric units such as glucose, mannose etc. Sulfuric acid (H_2SO_4), Hydrochloric acid (HCl) and Trifluoroacetic acid (TFA), are the most common acids used in hydrolysis of polysaccharides to monosaccharides.[46,47] Carbohydrates tend to be very unstable in strong acids at high temperatures; thus, high temperatures are avoided since this could lead to sample degradation and extraneous peaks in the gas chromatogram.[46] The most desirable acid of the three acids used is TFA as it is has lowest vapor pressure, and therefore is easiest to remove during post dervitization cleanup.

Reduction

Reduction is achieved with a reducing agent such as sodium borohydride ($NaBH_4$) or sodium borodeuteride ($NaBD_4$) in aqueous solution. This process can be completed in under two hours at room temperature at 37 ^{o}C, or overnight at 4 ^{o}C. Some researchers suggest the use of the reducing agent in dimethysulfoxide (DMSO) instead of an aqueous medium to achieve a more stable derivative; however, DMSO is difficult to remove and can result in extraneous peaks in the gas chromatogram. Reduction converts the monosaccharides formed from hydrolysis to acyclic alcohols. $NaBD_4$ is employed by most researchers as a reducing agent in order to mark the anomeric carbon with C-1 producing an asymmetric derivative. Reducing the aldehyde group to an alcoholic hydroxyl simplifies the gas chromatogram since the α- and β-configurations of monosaccharide no longer exist.[46]

Acetylation

The final step in the monosaccharide composition analysis is the acetylation of all the hydroxyl groups on the alcohol formed. This process is inhibited by the borate produced in the reduction step. The borate is hereby removed as volatile tetramethyl borate gas by multiple evaporations of the sample after reduction with methanol-acetic acid or methanol-hydrochloric acid. Addition of a catalyst such as pyridine or sodium acetate is employed to aid in acetylation. The samples are dried after acetylation and dissolved in chloroform or dichloromethane and extracted with water, or passed through an anhydrous sodium sulfate column. This reduces contaminants such as organic acids or bases and salts.[46] Figure 5 shows the reaction for each chemical derivatization step discussed aboved in monosaccharide compostional analysis of polysaccharides.

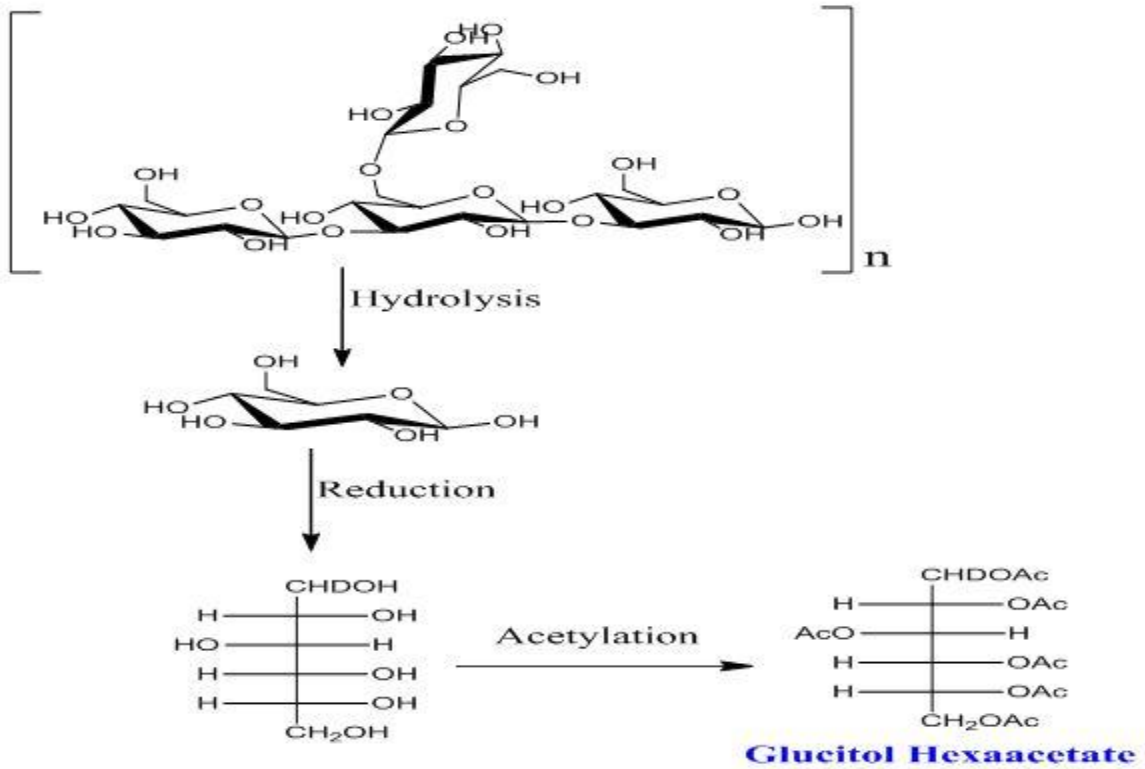

Figure 5: Reaction scheme for alditol acetate derivatization. Beta (β)-glucan, a complex polysaccharide found in fungal cell walls is converted to glucitol hexacetate

Methylation Analysis (Partially Methylated Alditol Acetate method) by Gas Chromatography-Mass Spectroscopy

Linkage analysis (methylation analysis) is employed to determine the various linkages that connect the monomers together to compose a polysaccharide. Linkage analysis conducted on isolated carbohydrates from the cell wall could reveal how the individual components of the cell wall are linked together. The procedure involves converting all the free hydroxy groups on the polysaccharide into methoxy groups followed by cleavage of the glycosidic linkages. The resultant products are then derivatized into partially methylated alditol acetates for analysis.[48] The methylation is completed with strong bases [sodium or potassium hydride (NaH/KH), potassium tert-butoxide [$(CH_3)_3COK$], or sodium or potassium hydroxide (NaOH/KOH)] in dipolar aprotic organic solvents.[49] The methylated polysaccharides are then derivatized chemically using the alditol acetate method as discussed above. The substitution pattern of O-methyl groups in the monomers reveal the carbon atoms in the polysaccharide that are not involved in the linkage. Figure 6 below demonstrates the chemical reaction involved in the derivatization of partially methylated alditol acetate.

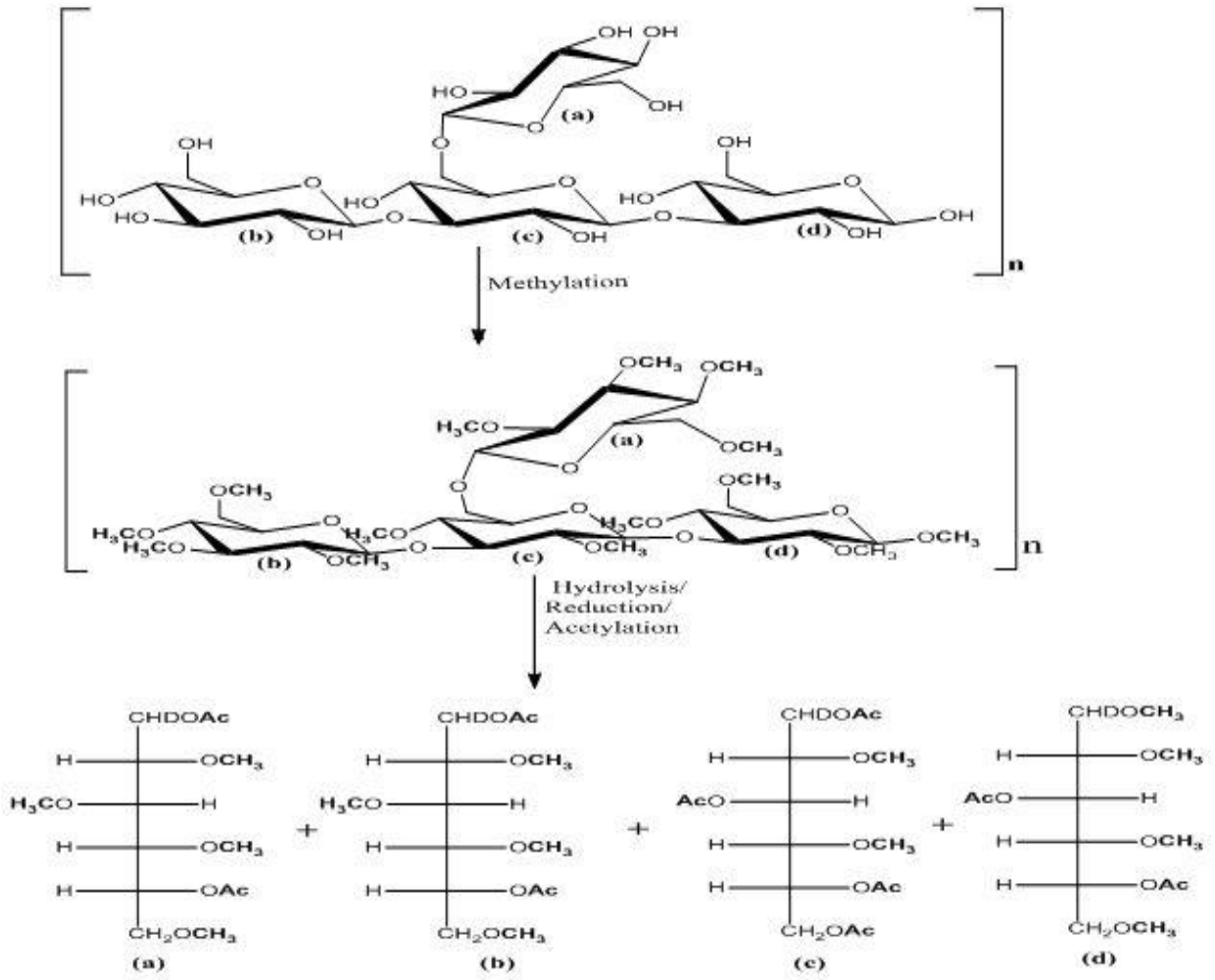

Figure 6: Reaction scheme for derivatization of partially methylated alditol acetates

Interpretation of Mass Spectrum Data for Linkage Analysis

The MS spectra obtained for the partially methylated derivatives are critically analysed using the following criteria: 1) Cleavage between two methoxylated carbons is favored over cleavage between a methoxylated carbon and an acetoxylated carbon and 2) Cleavage between a methoxylated carbon is favored over cleavage between contigious carbons resulting in an acetoxy group. These fragments are a result of the cleavage of the alditol chain. There are three main scenarios in the fragmentation of partially methylated alditol acetates in the electron impact

mode of the mass spectrometer as shown in Figure 7. In scenario 1, the methyl ether group exhibits a mild electron donating characteristic, thus resulting in positive ions for either fragment with equally strong frequency. In scenario 2, the acetyl group exhibits an intense electron withdrawing influence that results in a cation formed only by the methoxylated carbon. In scenario 3, the cleavage between contiguous acetylated carbons produces positive carbonium ions of extremely low frequency. Scenario 3 rarely happens except in fully acetylated derivatives. The positive charge always resides on the fragment with a methoxy bearing carbon atom adjacent to the cleavage point. Other fragments are possible but less common due to the loss of acetic acid (MW= 60 $gmol^{-1}$), methanol (MW=32 $gmol^{-1}$), ketene (MW =42 $gmol^{-1}$), and formaldehyde (MW= 30 $gmol^{-1}$).[46,50]

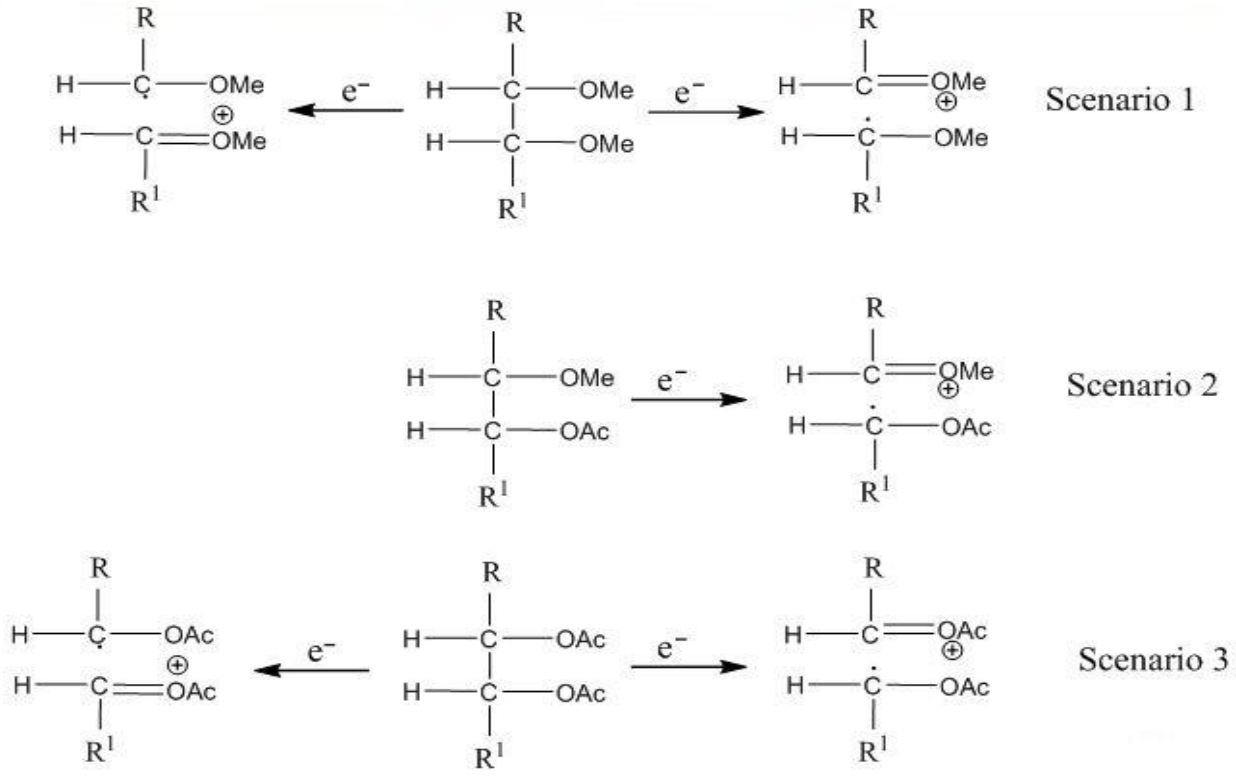

Figure 7: Formation of primary fragments by cleavage of the alditol chain in partially methylated alditol acetates

Research Aims

This research is twofold, the carbohydrate analysis using Gas chromatography-Mass spectrometry (GC-MS) of:

1. Beta (β)-glucans isolated from *Saccharomyces cerevisiae* and
2. Mannan isolated from *Candida albicans*

The aims of this research are listed below;

Aim 1

- Determine the sugar component of β-glucan isolated from *Saccharomyces cerevisiae* using an optimized method by a research group member
- Investigate the native structure retained in extracts isolated using this optimized method.
- Confirm absence or presence of 2,3 linkage in β-glucan isolated from *Saccharomyces cerevisiae*

Aim 2

- Investigate the sugar component of hyphal and yeast mannan isolated using acid and base extraction developed by another research group member
- Determine differences in sugar composition of hyphal and yeast mannan

CHAPTER 2

EXPERIMENTAL PROCEDURES

Materials

The yeast employed in the study of β-glucans was the commercially available *Saccharomyces cerevisiae* purchased from Red Star Yeast and Products Corporation in Wisconsin, USA. The laboratory grown yeast and hyphal form of *Candida albicans* cells were prepared by Kruppa and coworkers.[51] The monosaccharide standards were donated by V-Labs Inc. The standards are listed below:

- Glucitol Hexaacetate
- Mannitol Hexaacetate
- Galacitol Hexaacetate
- Xylitol Pentaacetate
- Arabinitol Pentaacetate

Standard N-acetyl-D-glucosamine, D-(+)-glucosamine hydrochloride, and unbleached chitin from MP Biomedicals were provided by Professors David L. Williams and Douglas Lowman from ETSU School of medicine, Department of Surgery. The extracted β-glucans from *S. cerevisiae* and mannan from *C. albicans* employed in this study were isolated by fellow graduate students in our research group (Shadrack Asare, Francis Kwofie, and James Elliot).

A Sorvall Legend RT+ general purpose centrifuge with a 230 V capacity, a four plate Super-Nuova multi-place stirrer and hot plate with integral controls and Fisher modular block dry-bath incubators were purchased from Fisher Scientific. Pierce Reacti-therm heating module with Reacti-Vap evaporating unit were used for stirring, heating and evaporation. Weighing of

sample and reagents were made with a METTLER TOLEDO's™ Basic Weighing balance donated by the ETSU chemistry department.

Reagents

Trifluoroacetic acid (TFA), Baker analyzed® reagent [F_3CCOOH, Specifications: Assay ≥99.0 %, Residue after evaporation ≤ 0.0100 %, Heavy metals (as Pb) ≤0.0010 %, Trace impurities (Fe)≤5 ppm], Dichloromethane [CH_2Cl_2, Specifications: Assay(exclusive of preservative) ≥99.8 %, Residue after evaporation ≤2.0 ppm], and Acetic anhydride, Baker analyzed® A.C.S. reagent[$(CH_3CO)_2O$, Specifications: Assay ≥98 %, Residue after evaporation ≤0.0020 %, Substances reducing permanganate (Cl, SO_4, PO_4) 3≥x≤5 ppm, Heavy metals(as Pb) ≤2 ppm, Trace impurities (Fe) ≤ 5 ppm] were purchased from Avantor. Glacial acetic acid, certified ACS [$C_2H_4O_2$, Assay: 99.9 %, Residue after evaporation <8 ppm, Heavy metals (as Pb) <0.5 ppm], Dimethyl sulfoxide, Certified ACS [C_2H_6OS, Specifications: Assay ≥99.0 %, Residue after evaporation <0.01 %],Methanol, Certified ACS [CH_3OH, Specifications: Assay ≥99.9 %, Residue after evaporation <1 ppm], Sodium sulfate [anhydrous, ACS grade] and Sodium acetate [anhydrous, Assay ≥99.4 %] were purchased from Fisher Scientific. Sodium borodeuteride [$NaBD_4$, 98 atom % D, Nitrogen flushed] and Methyl Iodide [99 %] were purchased from Acros. Anhydrous sodium hydroxide pellet was purchased from MP biochemicals.

Infrared Spectroscopy

A Fourier-transform infrared spectrometer (FTIR) was used to determine complete methylation of the polysaccharides. Spectra were recorded on the Schimadzu FTIR Prestige- 21

FTIR instrument. The solvents were evaporated from the samples and the resulting products were scanned with the FTIR from 4000 cm^{-1} to 750 cm^{-1}.

Gas Chromatography-Mass Spectroscopy

A Shimadzu GC-MS-QP2010 Plus GC system spectrometer was used to record GC-MS spectra of the derivatized sugars. The GC is equipped with a quadrupole MS detector. The retention times and fragmentation patterns were used to obtain compositional and linkage information. The final product obtained after derivatization was dissolved in 1 mL dichloromethane and analyzed with GC-MS. The GC-MS settings used for the monosaccharide composition and linkage analysis determination are given below.

GC-MS Settings for Compositional Analysis

Carrier Gas: Helium

Column: DB-5, 30 m x 0.25 mm x 0.25 μm

Injection volume: 1 μL

Injection temperature: 260^{o}C

Detector temperature: 270^{o}C

Run time: 85.05 min

Table 2: Temperature Programming Profile for Compositional Analysis

Oven Ramps	Rate (^{o}C/min)	Temperature (^{o}C)	Hold Time (min)
Initial	--	37	0.10
Ramp 1	20.0	160	20.00
Ramp 2	10.0	200	22.30
Ramp 3	20.0	250	30.00

GC-MS Settings for Linkage Analysis

Carrier Gas: Helium

Column: DB-5, 30 m x 0.25 mm x 0.25 μm

Injection volume: 1 μL

Injection temperature: 260 ^{o}C

Detector temperature: 270 ^{o}C

Run time: 105.05 min

Table 3: Temperature Programming Profile for Linkage Analysis

Oven Ramps	Rate (^{o}C/min)	Temperature (^{o}C)	Hold Time (min)
Initial	--	37	0.10
Ramp 1	20.0	140	30.00
Ramp 2	20.0	180	40.00
Ramp 3	30.0	230	30.00

Reagent Preparation

The reagents used in this research were prepared following standard laboratory procedures. All the reagents were prepared in the fume hood. The preparation of 4 M Trifluoroacetic acid and 5 % Acetic acid in methanol are described below;

Preparation of 4 M Trifluoroacetic Acid

The Trifluoroacetic acid (TFA) used is 99% reagent grade. The calculations below is used to prepare ~67 mL of 4 M TFA

Assay = 99 % Specific gravity = 1.53

Density = 1.53 g/ mL

Mass of TFA = 99 g in 100 g of solution

Volume = 100 g / 1.53 g/ mL = 65.63 mL

Number of moles = 99 g/ 114.03 gmol-1 =0.0868 mols

Molarity= (0.0868 mols/ 65.36 mL) X 1000 =13.3 M

Using dilution formula $M_1V_1=M_2V_2$

V_1= (4x 67)/13.3 =20 mL

Approximately 40 mL deionized water was measured using a 50 mL measuring cylinder and transferred into a 100 mL beaker. 20 mL of the 99 % (13.3 M) TFA were measured using a 10 mL graduated pipette and added into the water in the beaker and then 17 mL of deionized water added to make a total volume of 67 mL . The solution was placed on a stir plate and stirred for 3 min. The solution was then transferred from the beaker into 10 mL glass vials, labeled, and stored in the fume hood.

Preparation of 95 % Acetic Acid in Methanol

In preparing 30 mL of 5 % acetic acid in methanol, 28.5 mL of 99.9 % methanol was measured using a 10 mL glass pipette into a 50 mL beaker, and 1.5 mL glacial acetic acid (99.9 %) added. The solution was set on a stir plate for 3 min. The solution was then transferred into 10 mL glass vials, labeled, and stored in a fume hood.

Evaporation of Solvent

The removal of solvents from the samples were initially done by leaving it in the aluminum block uncapped in the fume hood overnight, after approximately 8 hr, there was no visible residue of solvent observed. Later, the solvents were removed by blowing an inert gas such as nitrogen, over the sample vials in the aluminum block from 30 min to 2 hr depending upon the solvent employed. At the end of this period, there was no visible residue present.

Monosaccharide Compositional Analysis of Fungal Cell Wall Carbohydrate

The monosaccharide content of the cell wall carbohydrates was determined by converting the polysaccharide into volatile alditol acetates for analysis by GC-MS. The monosaccharide content of the following polysaccharides was determined in this study;

- β-glucans from *Saccharomyces cerevisiae*
- Mannan from *Saccharomyces cerevisiae*
- Yeast mannan from *Candida albicans*
- Hyphae mannan from *Candida albicans*
- Standard unbleached Chitin from MP biomedicals

Monosaccharide analysis of each of these samples was repeated at least twice to confirm the results. Table 4, below, details the number of analysis conducted on each sample type using this procedure. The monosaccharide analysis involves three main stages: hydrolysis, reduction and acetylation. Derivatization cleanup was required for a successful analysis. The various protocols involved in this procedure are described further in the subsequent subsections.

Table 4: Summary of Compositional Analysis Experiment Conducted on Each Sample for Confirmation of Results.

Sample	Extraction Condition	Repetition of Compositional Analysis
β-glucan	3X boiling in reagents	4
β-glucan	1X boiling in reagents	4
β-glucan	Using HCl	4
β-glucan	Using H_3PO_4	4
β-glucan	Using 1N reagent concentration	4
β-glucan	Using 0.1N reagent concentration	4
β-glucan	Using 0.01N reagent concentration	4
β-glucan	Using 0.0011N reagent concentration	4
β-glucan	Using less base	4
β-glucan	Using less acid	2
Yeast mannan	Base	4
Yeast mannan	Acid	4
Hyphal mannan	Base	6
Hyphal mannan	Acid	4
Chitin	Standard reference material	2

Trifluoroacetic Acid Hydrolysis

Acid hydrolysis was conducted on dry extracted samples to break the polysaccharide into its monomeric unit for ease of derivatization. Approximately 1 mg of the sample was weighed

into a clean 1 dram glass vial. Then 1 mL of 4 M Trifluoroacetic acid added. The glass vial was sealed with screw cap lined with Teflon. The sample was then placed in an aluminum heating block for four hours at 105 ^{o}C. The reaction was vortexed periodically to mix the vial's contents. After four hours, the sample was allowed to cool and the vial with the lid off was placed in the aluminum heating block (at 37 ^{o}C) and left in the fume hood to evaporate to dryness under air.

Sodium Borodeuteride Reduction

The reduction was carried out using sodium borodeuteride ($NaBD_4$) as a reducing agent. The sample after hydrolysis was then dissolved in 1 mL of deionized water and a few milligrams of anhydrous sodium borodeuteride were added. The vial was then sealed with the Teflon lined screw cap and left for 22 hours at room temperature with periodic vortexing. Glacial acetic acid was then added (3 drops, more was added if bubbling that ensued was vigorous) to the reaction vial the next day until no effervescence was observed. The glass vial with the lid off was placed in the aluminum heating block (at 37 ^{o}C) and left in the fume hood to evaporate to dryness under air.

Acetylation with Acetic Anhydride

After reduction, the dried sample was dissolved in acidic methanol (95 % methanol/5 % acetic acid) and evaporated to dryness to convert boric acid formed in the reduction stage to volatile methyl borate. This was repeated three times to completely remove boric acid which can interfere with the acetylation process. The samples were left to dry thoroughly after the last evaporation step to observe formation of a white powder in the vial. If, after the third evaporation, a white powder was not observed, a very small amount of anhydrous sodium acetate (NaOAc) was added followed 2 mL acetic anhydride. The vial was sealed and placed in an

aluminum heating block at 105 ^{o}C for 2 hours with periodic vortexing. After 2 hours the sample was allowed to cool. The vial (without the lid) was placed in an aluminum heating block (at 37 ^{o}C) and the solvent evaporated to dryness under air in the fume hood.

Column Construction

A sodium sulfate column was constructed by placing a small amount of glass wool at the bottom of a 5.75" borosilicate glass Pasteur pipette. The Pipette was filled with anhydrous sodium sulfate to about three inches high.

Post Derivatization

A clean 1 dram glass vial was placed underneath the column for collection and clamped in place. The partially methylated alditol acetate was then extracted with dichloromethane (1.5 mL) two times and applied to the sodium sulfate column. The extracted solution from the column was then evaporated to dryness in the heating block with trace heat under air and analyzed with GC-MS. Figure 8 below details the stages involved in the derivatization process for monosaccharide compositional analysis.

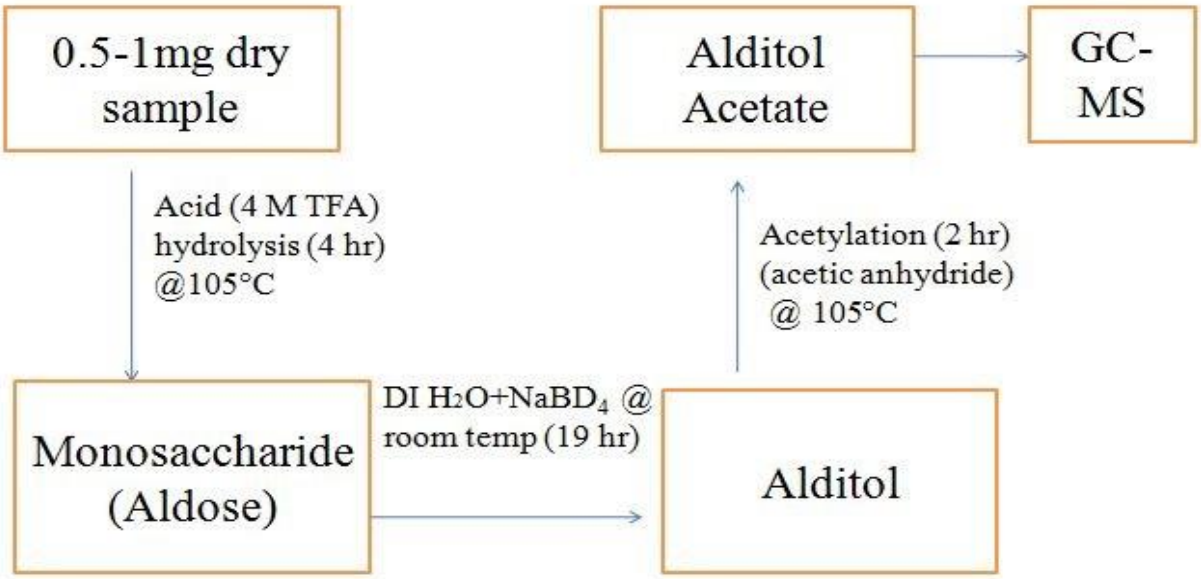

Figure 8: A Schematic diagram for monosaccharide compositional analysis of cell wall carbohydrate

Chemical Derivatization of N-acetyl Glucosamine and Glucosamine Hydrochloride

The derivatization of N-acetyl glucosamine and glucosamine hydrochloride into their respective alditol acetates was also conducted using the procedure described above. These standard samples were analyzed and used as control to determine the retention time and fragmentation of cell wall chitin (if present) in the extracts. No hydrolysis was required for theses samples as they were in the monosaccharide form initially. Each of these samples was duplicated to validate the compositional analysis method.

Permethylation of Cell Wall β-Glucans

The first step in determining the linkage information of polysaccharides is methylation of all free hydroxyl groups on the polysaccharide. The extracted β-glucan (1 mg) was placed into a 5 mL conical bottom reaction glass vial equipped with a silicone adaptor lid and a triangular stir

bar. Dimethylsulfoxide (2 mL) was added and the reaction vial left on a stir plate overnight at room temperature. A few milligrams of powdered anhydrous NaOH (powdered NaOH was prepared using a clean, dry pestle and mortar) were added the next day and stirred for

10 minutes. Methyl Iodide (2.2 mL) was added to the reaction vial and a small amount of powdered anhydrous NaOH was added for the second time. The reaction vial was covered with the silicone adaptor lid and set on a stir plate for 4 hours at room temperature. The mixture was then allowed to settle for 5 min and the milky white liquid pulled off into a 15 mL centrifuge tube.

Dichloromethane (2 mL) was added to the vial to pull off any remaining sample left in the vial. Two mL of deionized water was then added to the sample in the centrifuge tube, centrifuged (6100 rpm) for 15 minutes and the deionized water layer removed after centrifugation. This was repeated three times to wash the samples of any impurities. After the last centrifugation, the dichloromethane layer was removed into a 5 mL reaction vial and left in the fume hood to evaporate to dryness under air.

Chemical Derivatization of Partially Methylated Polysaccharide

After methylation, the partially methylated polysaccharide was derivatized to partially methylated alditol acetate using the protocol for alditol acetate described above. Linkage analysis was conducted on β-glucan extracts that gave extremely dominant glucose content after analysis of the monosaccharide content. Figure 9 shows a schematic of the methylation and derivatization process involved in the protocol for linkage analysis described above.

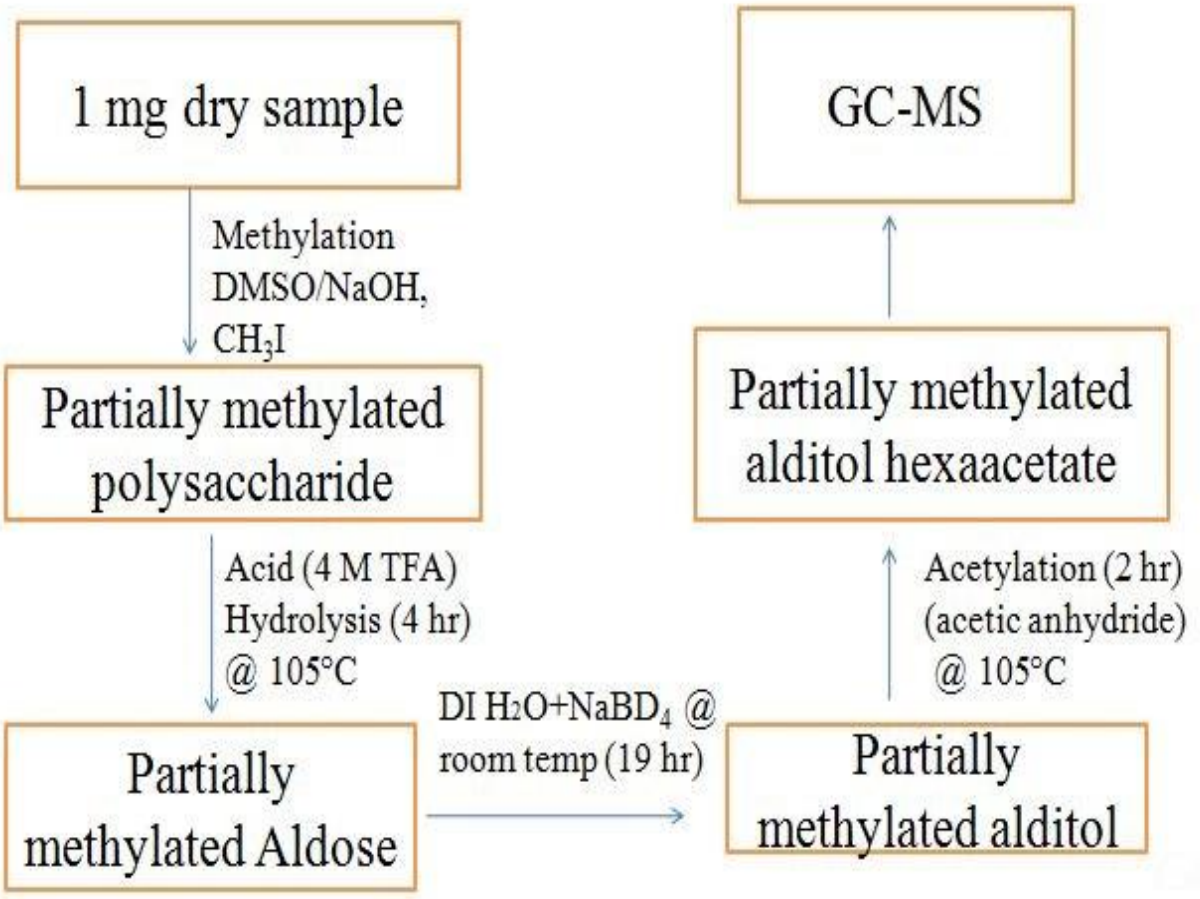

Figure 9: A Schematic diagram of linkage analysis for β-glucans from *S. cerevisiae*

CHAPTER 3

RESULTS AND DISCUSSION

There were several research aims presented in this thesis. The first aim was to determine the efficacy of a β-glucan isolation method optimized in house (completed by Shadrack Asare). The second aim was to determine the native structure retained in extracts isolated using this optimized method. The third aim was to determine if the unique 1, 2, 3-linkage in hyphal *Candida albicans*, could be found in the β-glucan from *Saccharomyces cerevisiae* using this novel method. The final aim was to investigate the efficacy of another method developed in house (by Francis Kwofie) for the isolation of hyphal and yeast mannan from *C. albicans* as well as to determine any differences in the sugar composition of hyphal and yeast mannan from *C. albicans*.

Determination of Monosaccharide Compositional Analysis Results

The compositions of β-glucan and mannan extracts were determined by chemical derivatization which converted the carbohydrates to volatile glucitol and mannitol hexaacetates respectively, which were then analyzed with GC-MS. Chitin, was converted to N-acetyl glucosaminitol hexaacetate (if present in the extract). Five standard alditol acetates were employed to ensure accurate identification of the monosaccharide's GC peaks observed during the analysis of β-glucan and mannan samples. Figure 10 demonstrates the GC separation of the alditol standards. Xylitol and arabinitol pentaacetate exhibited retention times of ~ 26.29 min and 27.68 min respectively. Mannitol, glucitol, and galacitol hexaacetate exhibited retention times of 34.96 min, 35.41 min, and 35.73 min respectively.

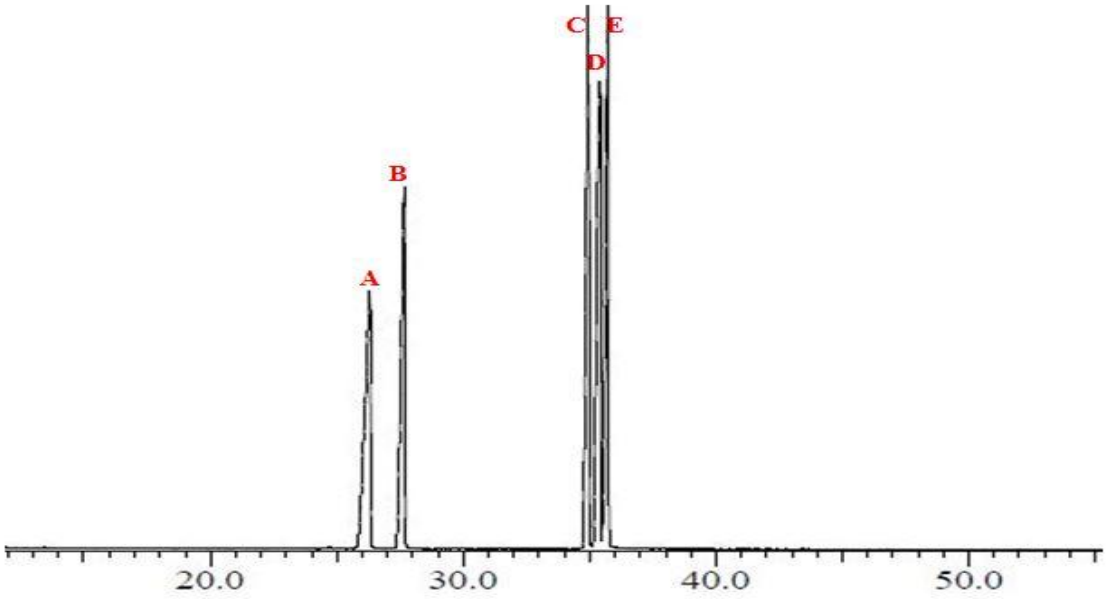

Figure 10: GC profile for a mixture of five different alditol acetates. A) Xylitol hexaacetate, B) Arabinitol hexaacetate, C) Mannitol hexaacetate, D) Glucitol hexaacetate, E) Galacitol hexaacetate

Each peak observed in the gas chromatogram for the standard alditol acetates exhibited a specific and representative fragmentation pattern in the MS spectrum which was used to identity each peak. Fragments are typically produced by cleavage of the alditol chain (primary fragments) or by elimination of ions (secondary fragments) such as acetoxy group (m/z 59), acetic acid (m/z 60), formaldehyde (m/z 30) and ketene (m/z 42).[46] Figure 11 details the interpretation of the mass fragmentation for glucitol hexaacetate (mannitol and galacitol hexaacetate have similar fragmentations). Significant ions such as m/z 145 (cleavage between C-2 and C-3), m/z 218 (cleavage between C-3 and C-4), m/z 289 (cleavage between C-4 and C-5) and m/z 361 (cleavage between C-5 and C-6) shows presence of glucitol hexaacetate. Secondary fragments such as m/z 375 (loss of acetoxy group from 434), and m/z 259 (loss of acetoxy and ketene from m/z 360) are also observed in the mass spectrum for glucitol hexaacetate.

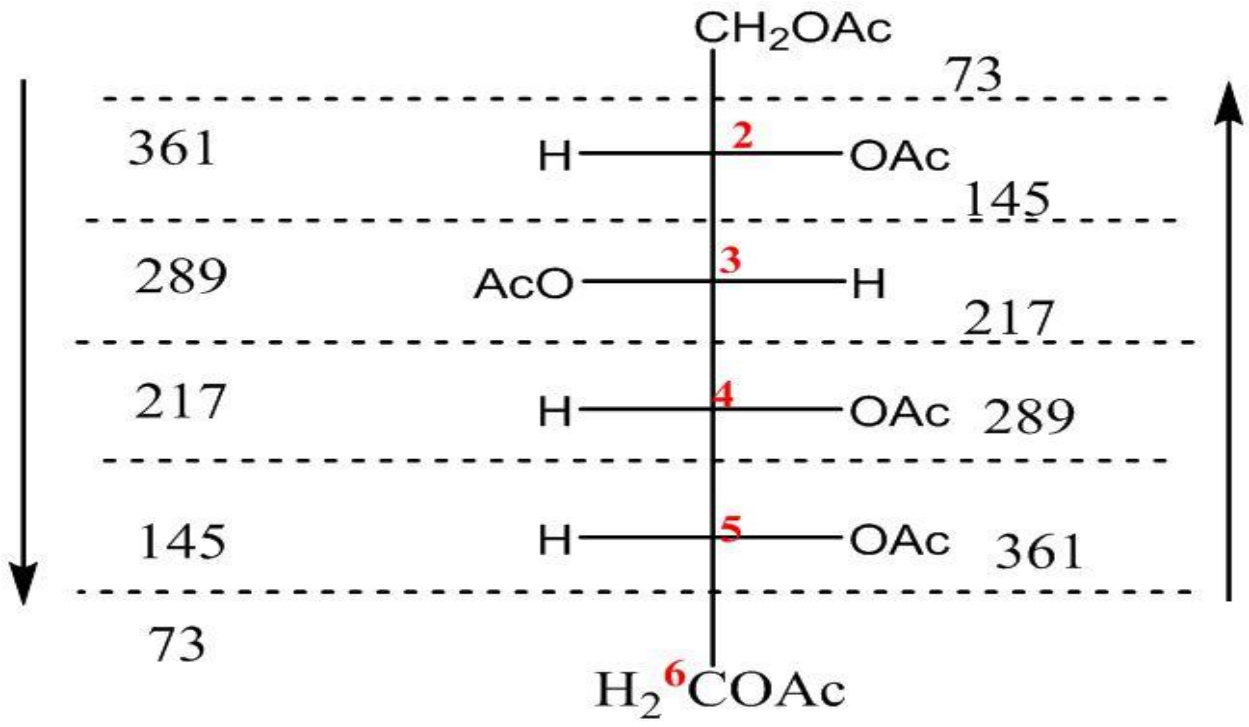

Figure 11: Interpretation of the peak fragmentation patterns found in the mass spectra for glucitol hexaacetate. The fragments are primarily as a result of cleavage of the alditol chain

Each alditol acetate produces significant m/z peaks with different intensities which help in confirmation of GC results. Table 5 details the retention times observed for the six alditol acetates and their significant ions in the MS spectrum.[46] Standard alditol acetates expected to be present (i.e. glucitol hexaacetate, mannitol hexaacetate and N-acetyl glucosamine) in the extracted glucan or mannan were run individually with the GC-MS. The peak retention times and fragmentation patterns of the standard alditol acetates were analyzed and used in identifying the sugar component of the derivatized β-glucan and mannan extracts.

Table 5: Retention Times and Significant Ions Produced by Six Standard Alditol Acetates

Alditol acetates	Retention time	Significant ions in mass spectrum(m/z)*
Xylitol hexaacetates	26.29	145/146,158, 217/218,289/290
Arabinitol hexaacetates	27.68	145/146,158, 217/218,289/290
Mannitol hexaacetates	34.96	139,145/146, 217/218,259/260,289,361,375
Glucitol hexaacetates	35.41	139,145/146, 217/218,259/260,289/290,360/361,375
Galacitol hexaacetates	35.73	139,145/146, 217/218,259/260,289/290,360/361,375
N-Acetylglucosaminitol hexaacetate	43.02	145/146,217/218,259/260,300,318,360,375

*All fragments reported are positive ions. The use of deuterium ion to mark C-1 during derivatization will result in doublets from symmetrical fragments.[46]

The mass fragmentation for glucitol hexaacetate and mannitol hexaacetate were similar due to them having the same chemical formula. They vary in the chirality of the carbons that form the molecule. The difference between the spectra for glucitol and mannitol was in the intensity of the m/z 139 and 259 m/z fragments (illustrated with blue arrows in Figures 12 and 13 below). These peaks had a lower intensity in glucitol hexaacetate than in mannitol hexaacetate. Figures 12-14 are a GC-MS of standard glucitol, mannitol and N-acetyl glucosaminitol hexaacetate, respectively. The significant ions observed are circled in red.

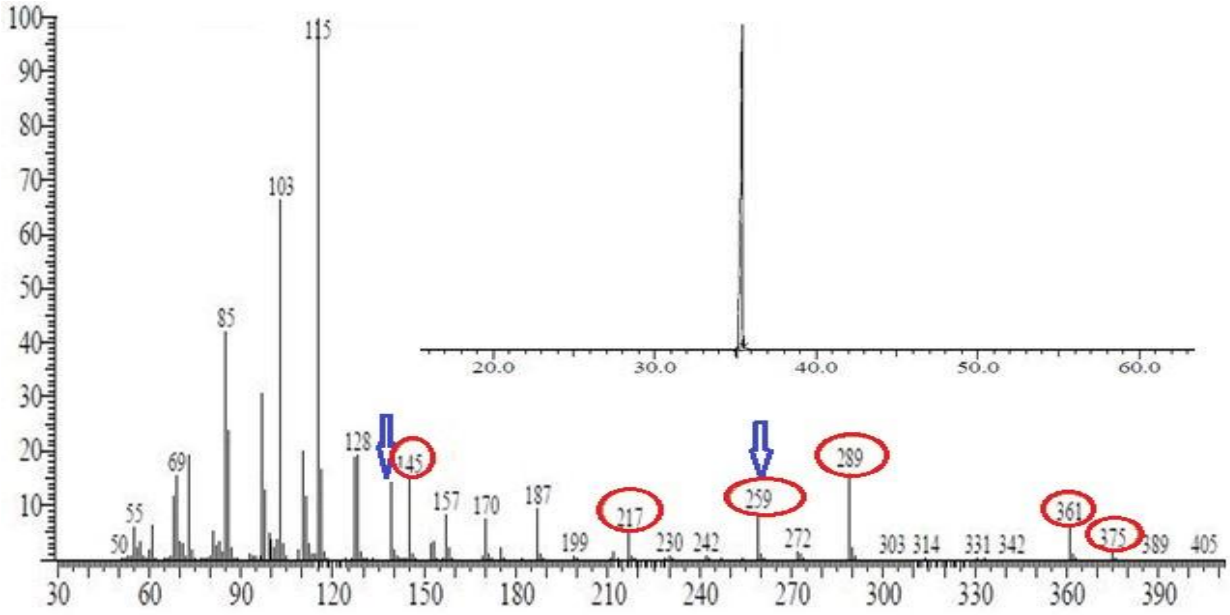

Figure 12: GC-MS of standard glucitol hexaacetate. GC profile shows a peak retention time of ~35.3 and MS spectrum showing m/z of ions produced after fragmentation, significant m/z highlighted in red. Blue arrows indicate m/z 139 and 259 which is lower in intensity for glucitol hexaacetate compared to mannitol hexaacetate

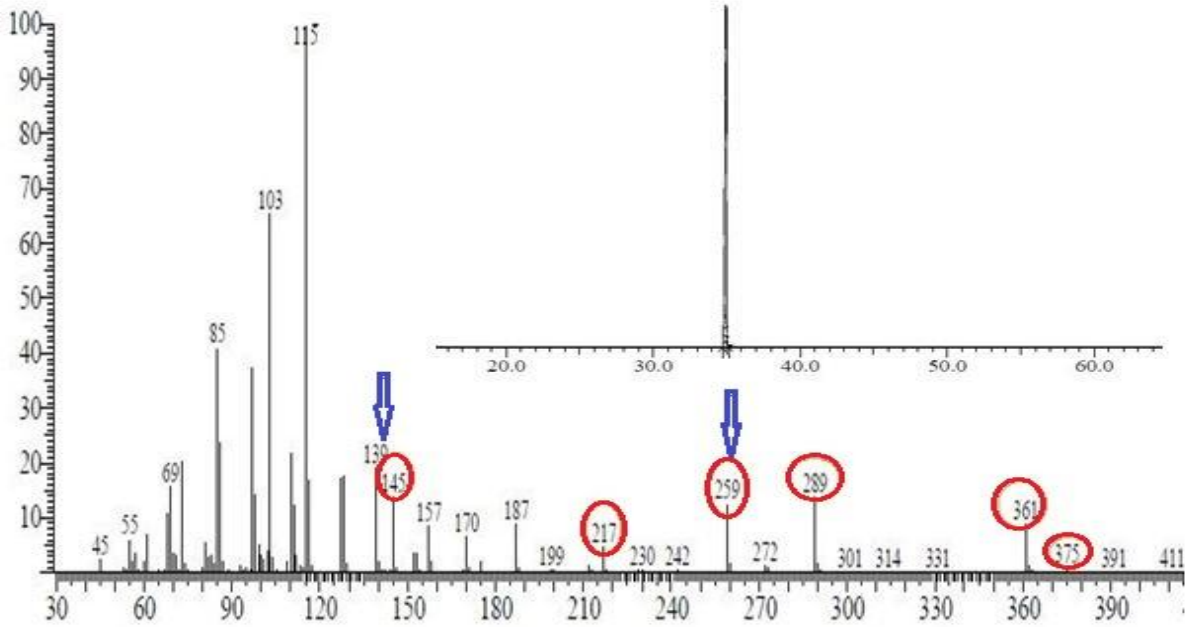

Figure 13: GC-MS of standard mannitol hexaacetate. GC profile shows a peak retention time of ~34.8 and MS spectrum showing m/z of ions produced after fragmentation, significant m/z highlighted in red. Blue arrows indicate m/z 139 and 259 which is higher in intensity for mannitol hexaacetate

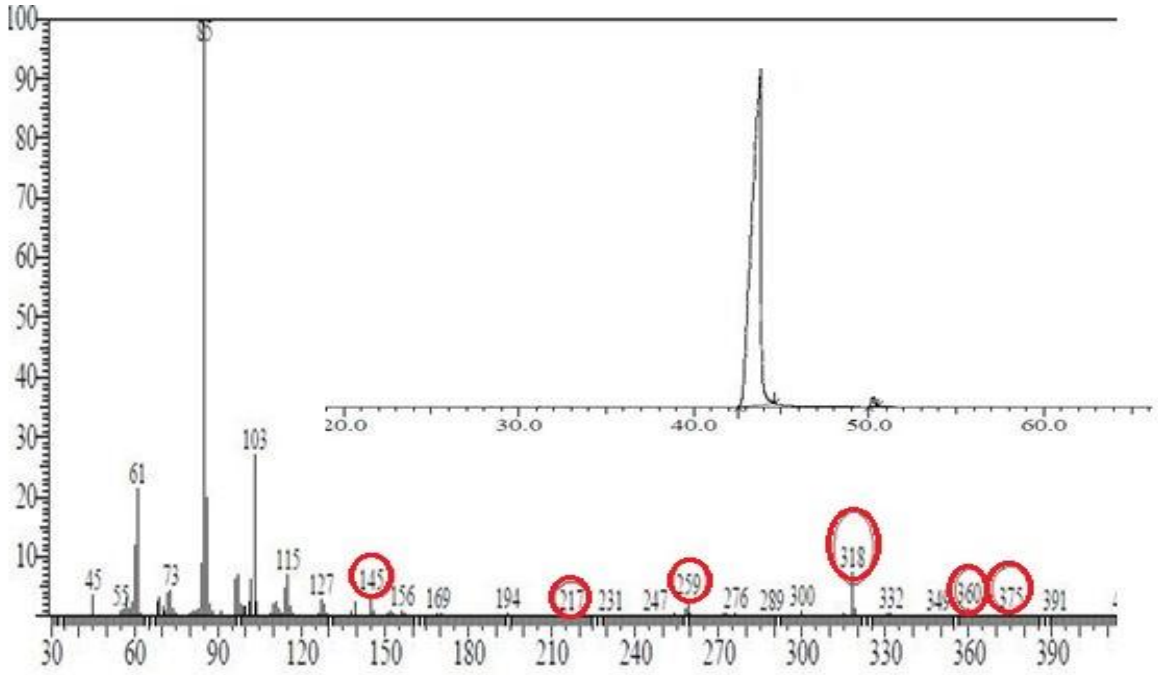

Figure 14: GC-MS of standard N-acetylglucosaminitol hexaacetate. GC profile shows a peak retention time of ~43.6 and MS spectrum showing m/z of ions produced after fragmentation, significant m/z 145, 217,259, 318, 360 and 375 are highlighted in red

Monosaccharide Compositional Analysis Results for β-Glucan

Monosaccharide analysis was conducted on β-glucans isolated from *S. cerevisiae* with multiple extraction conditions. Typically, the concentration of acid and base employed for the extraction was reduced to the point where other cell wall components were observed. The following factors were considered in developing the extraction procedure:

- Employing weaker acids
- Reducing extraction time
- Decreasing concentration of acid and base

Employing Weaker Acids

The results for the compositional analysis of β-glucans isolated with 1 N hydrochloric acid (HCl) versus that isolated using 1 N phosphoric acid (H_3PO_4) is shown in Figure 15. The GC profile for both glucan extracts ehibited a tall peak at 35.4 min which was identified as glucitol hexaacetate as well as a very tiny peak at 34.8 min which was identified as mannitol hexaacetate. The β-glucans isolated with either acid gave a very dominant glucitol hexaacetate peak which was above 99% relative to the mannitol hexaacetate peak.These percentages were found using the integrated peak area from the GC, but the values were not quantitative due to frequent evaporation of solvent in each derivatization step which may have resulted in a loss of alditol acetates as well as the possible insolubility of the intial sample.

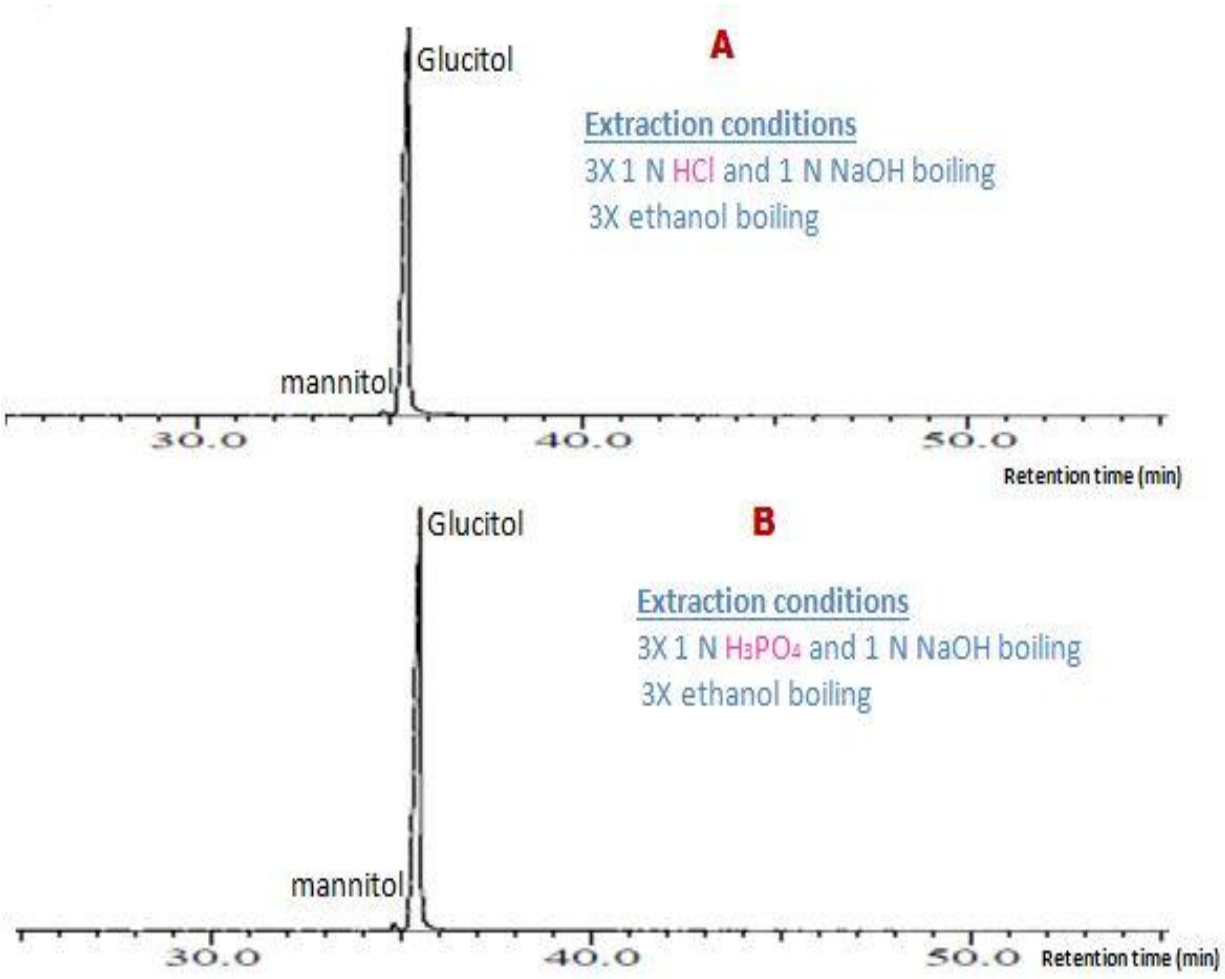

Figure 15: GC profile for β-glucan extracted using a, A) strong acid (HCl) and a B) mild acid (H_3PO_4)

This study indicates that the milder phosphoric acid was as effective as the harsher hydrochloric acid in isolating β-glucans as the product resulted in very little impurities. This work strongly confirms work by Lowman and coworker who employed milder acids to succesfully remove other cell wall components.[42,52] Next, the effect of reducing the time required for each extraction step was investigated.

Reducing Extraction Time

Another disadvantage of the classical procedure involves the multiple sequential extractions in base, acid, and ethanol which consumes large volumes of reagents and results in a 3-5 days process. Therefore, the efficacy of a modified procedure where single extractions were performed in each reagent was investigated. If the efficacy is proved then the modified method will save not only time, but also money and reagents.

The GC profile, Figure 16, for β-glucan isolated through single extractions (i.e. 1X extraction in NaOH, H_3PO_4, and ethanol) and that isolated using the classical method through three extractions (i.e. 3X extraction in NaOH, H_3PO_4, and ethanol) both exhibited one major peak at ~35.4 min and one minor peak at ~34.9 min. The peaks were identified as discussed above (see Determination of Monosaccharide Composition Results) to be glucitol hexaacetate and mannitol hexaacetate, respectively. The novel approach of using a single extraction resulted in a dominant glucitol hexaacetate peak (over 99 %) relative to the minor mannitol hexaacetate peak. The glucans isolated using multiple extractions resulted in smilar results, clearly demonstrating that the milder method is as effective in isolating β-glucan as the classical method. This supports the ^{1}H NMR results conducted in house that one time extraction procedure results in high β-glucan content.

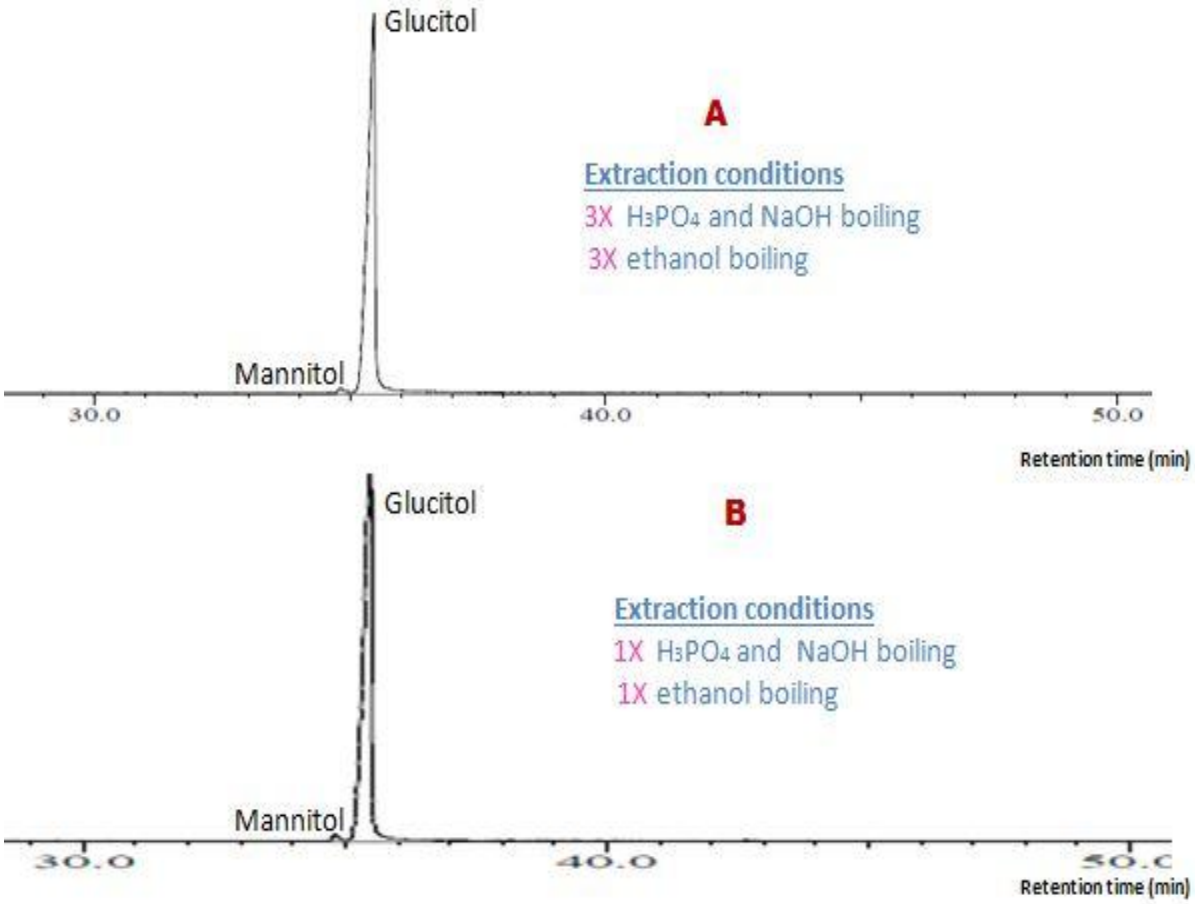

Figure 16: GC profile for compositional analysis of β-glucan isolated using, A) multiple boiling in H_3PO_4, NaOH and ethanol and B) one time boiling in H_3PO_4, NaOH,and ethanol

Decreasing the Concentration of Acid and Base

In order to better optimize the extraction method, the concentration of the base and acid employed was varied. Monosaccharide analysis was conducted for β-glucans which were isolated using concentrations of base and acid from 1N to 0.001N in order to determine the lowest concentrations which were sufficient to remove the other cell wall carbohydrates and proteins. As shown in Figure 17, decreasing the base and acid concentrations from 1 N to 0.1 N resulted in one major peak at ~35.4 min (glucitol hexaacetate) and a minor peak at ~34.8 min (manitol hexaacetate) as in the 1 N extractions. As glucitol hexaacetate was found to be the

major component, decreasing the concentration of phosphoric acid and sodium hydroxide to 0.1N was successful in isolating β-glucans.

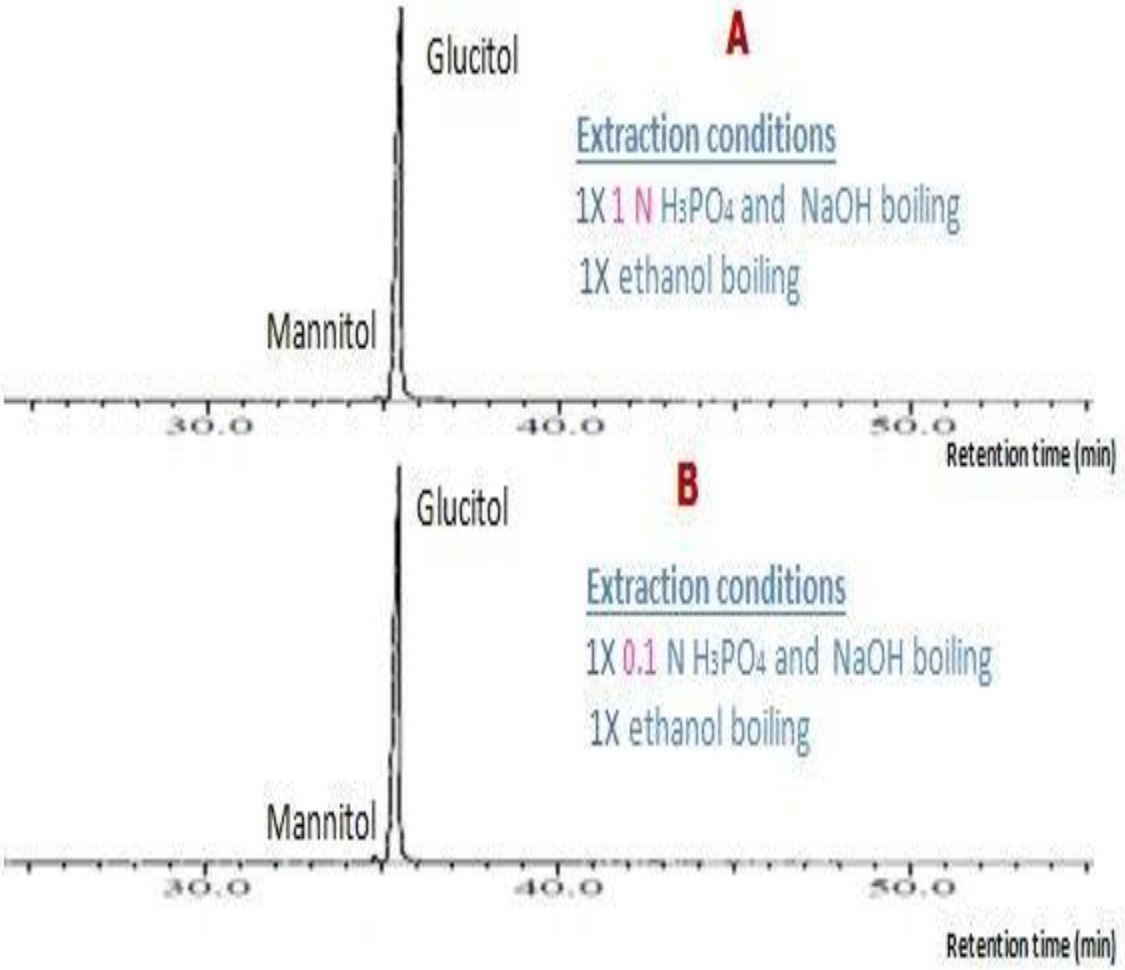

Figure 17: GC profile for β-glucan isolated with different concentrations of phosphoric acid and sodium hydroxide A) 1 N and B) 0.1 N

When the concentrations were decreased further, mannan was found in the extracted β-glucan. The spectra, Figure 18, for β-glucans isolated using base and acid with reduced concentrationss (0.01 N and 0.001 N) resulted in two peaks (~34.8 min and ~35.3 min) of almost identical height. The peaks were identified (per discussed earlier in the chapter) as mannitol and

glucitol hexaacetate respectively.The increase in the height of the mannitol hexaacetate peak for both 0.01 N and 0.001 N extracts show that lowering the base and acid concentrations below 0.1 N results in an unacceptable β-glucan product and therefore, is not an appropriate method.

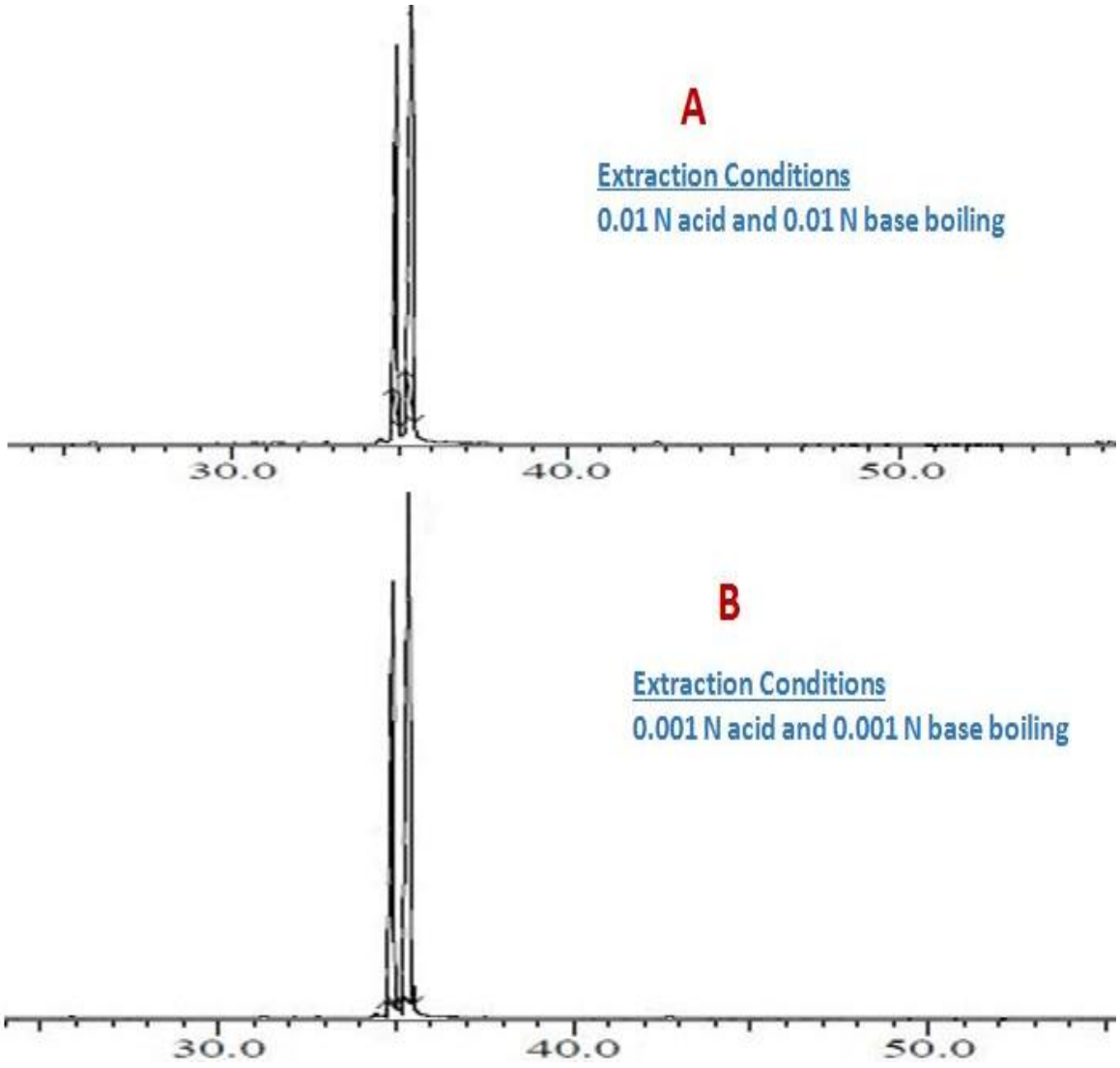

Figure 18: GC profile for β-glucan isolated with different concentrations of phosphoric acid and sodium hydroxide. A) 0.01 N and B) 0.001 N

Table 6 summarizes the major component observed in the GC-MS for yeast samples isolated with varied concentrations of reagents. From the table, using concentrations of 1N and 0.1N resulted in glucitol hexaacetate as the major component of the extracts whereas 0.01 N and

0.001 N resulted in both glucitol and mannitol hexaacetate as major components. From the data, it was clear that the 1N and 0.1 N were the optimum concentrations for the extraction of glucan. As 0.1 N yields equally pure β-glucans, it should be the optimal concentration for isolating β-glucan for structural analysis. The novel method not only resulted in a reasonably pure β-glucan product, but the use of smaller volumes of reagents reduced the cost while the use of a milder acid will also help in waste manangement for industries. Further linkage studies were employed on the 0.1 N extracts in order to confirm our speculations that the milder extraction procedure maintained more of the in situ structure of the β-glucan in the cell wall.

Table 6: Major Component Observed in Chromatogram of Samples Isolated with Different Concentrations of Acid (H_3PO_4) and Base (NaOH).

Concentration (N)	Major Component of Extract
1	Glucitol hexaacetate
0.1	Glucitol hexaacetate
0.01	Glucitol and Mannitol hexaacetate
0.001	Glucitol and Mannitol hexaacetate

Titration Method of Extraction

In order to better understand the role of both the acid and the base, a titration method was employed. First, the extraction procedure was modified by decreasing the sodium hydroxide concentration while keeping the concentration of phosphoric acid constant. The acid was held at 0.1 N while the base was reduced from 0.1 N to 0.01 N. This experiment resulted in two peaks of equal height at ~34.8 min (mannitol hexaacetate) and ~35.4 min (glucitol hexaacetate). Once again, we obseved an increase in height in the mannitol hexaacetate peak as when the concentration of all the reagants were decreased to 0.01 N and 0.001 N discussed above. This indicates that there was a high amount of mannan in this extract. Next, the base was held at 0.1 N

while the acid was reduced from 0.1 N to 0.01 N. This resulted in one minor peak at ~34.8 min (mannitol hexaacetate) and a major peak at ~35.4 min (glucitol hexaacetate). The dominant glucitol hexaacetate peak in this extract indicated that glucan was the main component of this extract.

The GC profile for β-glucan isolated using less sodium hydroxide concentration (0.1 N H_3PO_4 and 0.01 N NaOH) compared to β-glucan isolated using less phosphoric acid concentration (0.01 N acid and 0.1 N base) is shown below in Figure 19. It was observed from the GC profile that reducing the alkali concentration below 0.1 N yield a very significant amount of mannan in the extract.Therefore, it is apparent that the base step of the extraction is required to remove the extra carbohydrates from the cell wall. We speculate that the base extraction step is the most important step in the procedure as it appears to remove the mannans from the extracts. The use of base in the extraction is also known to remove proteins from the β-glucan extract and to hydrolyse lipids to glycerine and fatty acids.[53,54]

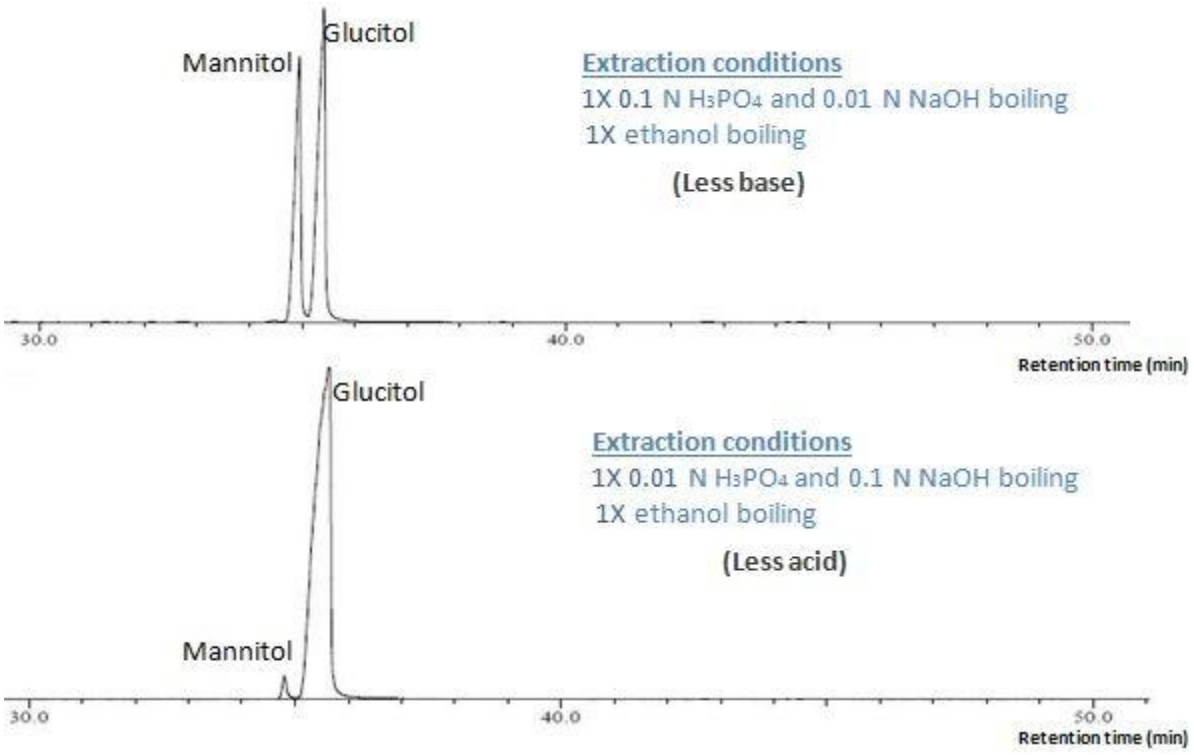

Figure 19: GC profile for low base concentration (0.01 N NaOH and 0.1 N H_3PO_4) extract and low acid (0.01 N H_3PO_4 and 0.1 N NaOH) concentration extract

Monosaccharide Compositional Analysis Results for Mannans

A novel method was developed by another member of the Greene research group to successfully isolate mannans from *C. albicans*. The newly developed method utilized 50 mM sodium hydroxide (NaOH) or phosphoric acid (H_3PO_4) to extract mannan from both the yeast and hyphal morphology of *C. albicans*. To date, mannan had not been successfully extracted from the hyphal form of *C. albicans.* The mannan isolated from both yeast and hyphal form of *C. albicans* was investigated for its monosaccharide content. The sections below discuss the results obtained for mannan isolated with 50 mM base (NaOH) and that isolated using 50 mM acid (H_3PO_4).

Monosaccharide Compositional Analysis Result for Base Extractions

During the monosaccharide compositional analysis of mannan extracts, one major peak and one minor peak were observed for both hyphal and yeast extract in the GC as shown in Figures 20 and 21. The major peak with retention time of ~34.9 min was identified as mannitol hexaacetate due to similar retention time and fragmentation pattern with standard mannitol hexaacetate. The minor peak at ~35.2 min was also identified as glucitol hexaacetate. Interestingly, an additional minor peak was observed in the hyphal mannan extract which was absent in the yeast mannan. The additional peak (red arrow), unique to the hyphal mannan, had a retention time of ~42.9 min, similar to standard N-acetyl glucosamine a component of chitin that is commonly present in the cell wall of fungal species.

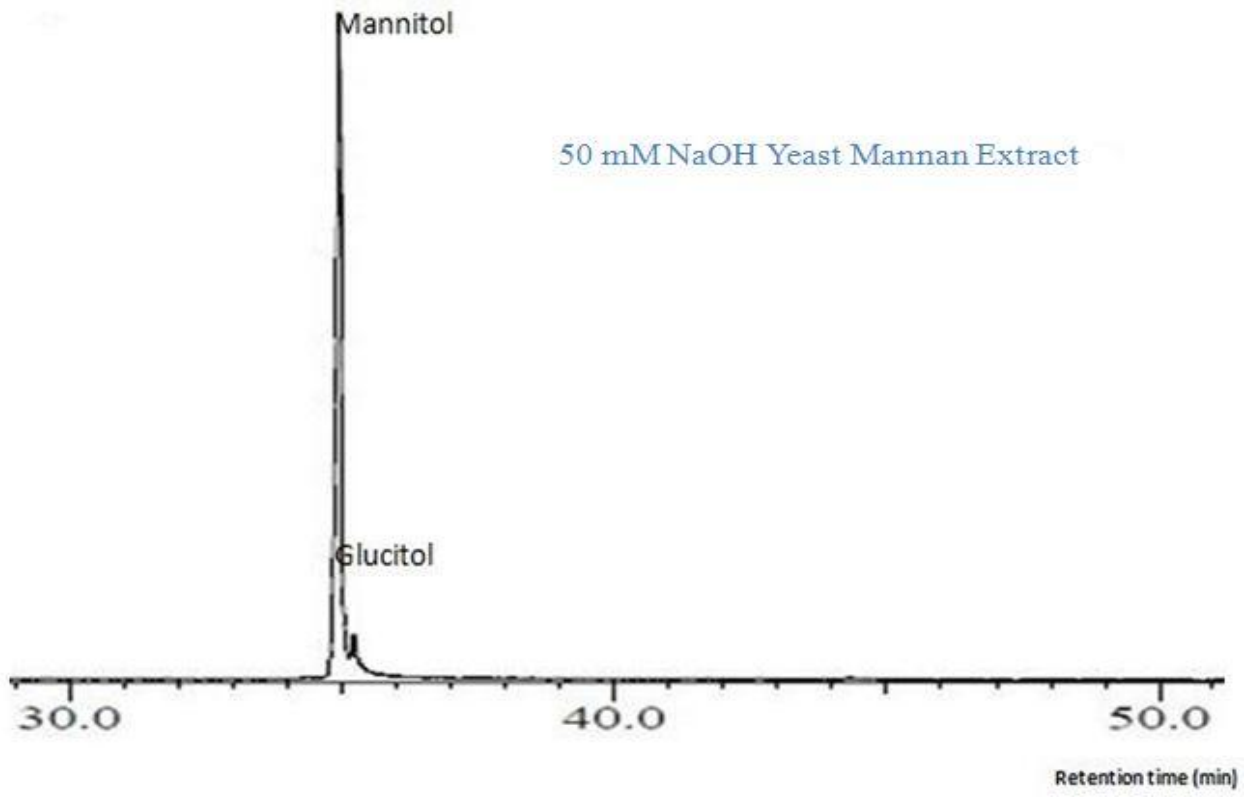

Figure 20: GC profile for yeast mannan extracted with 50 mM base (NaOH)

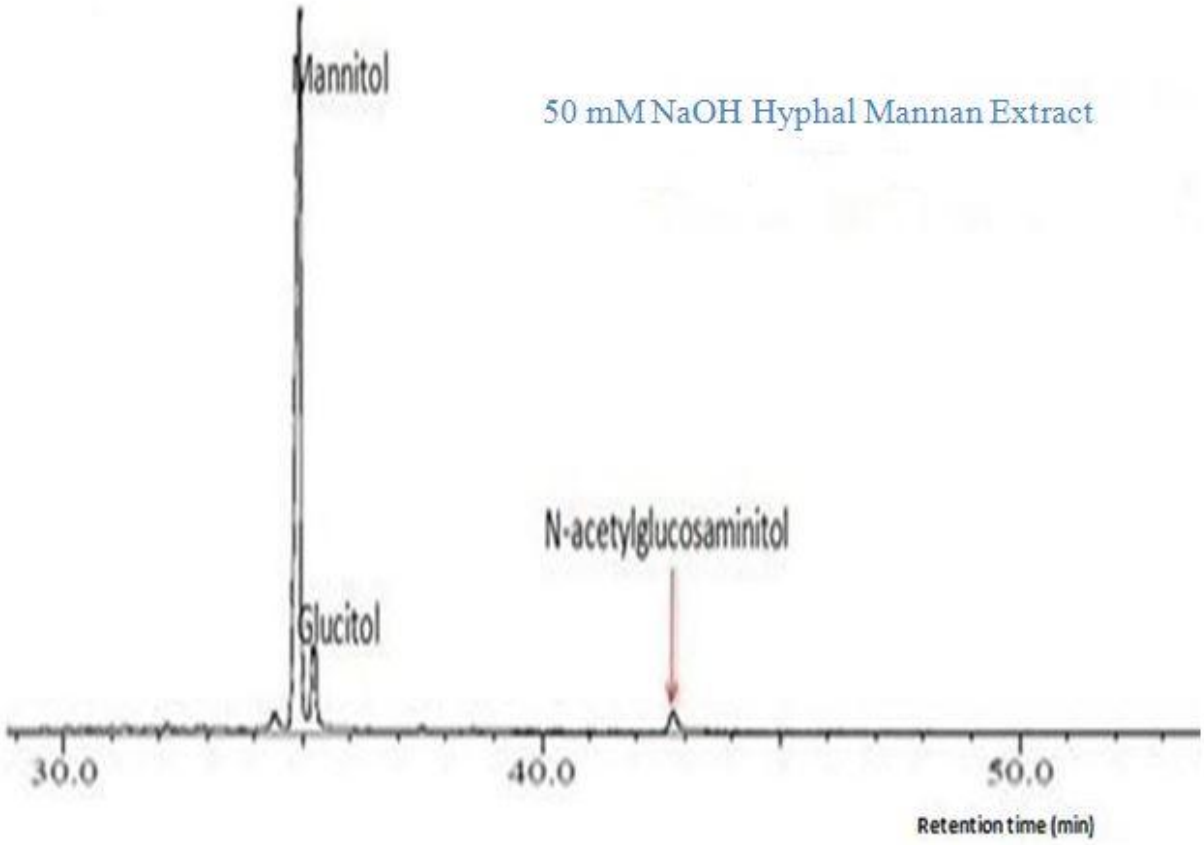

Figure 21: GC profile for hyphal mannan extracted with 50 mM base (NaOH)

To confirm the presence of chitin in the hyphal mannan extract, few milligrams of chitin was added to the hyphal mannan extracts before derivatization. The GC of the spiked mannan extract resulted in three significant peaks: ~34.9 min for mannitol hexaacetate, ~35.2 min for glucitol hexaacetate and ~42.9 min for N-acetyl glucosaminitol hexaacetate. The height of the suspected chitin peak at ~42.9 min increased relative to the mannitol and glucitol peaks as shown in Figure 22. This confirmed the new peak was in fact from chitin and suggested that the hyphal mannan might have considerably more chitin in its cell wall than the yeast mannan. Chitin is known to give considerable support to the cell wall making it difficult for the cell wall to be

penetrated and destroyed by the immune system. This may address why it is so difficult for the immune system to attack hyphal *C. albicans* during an infection.

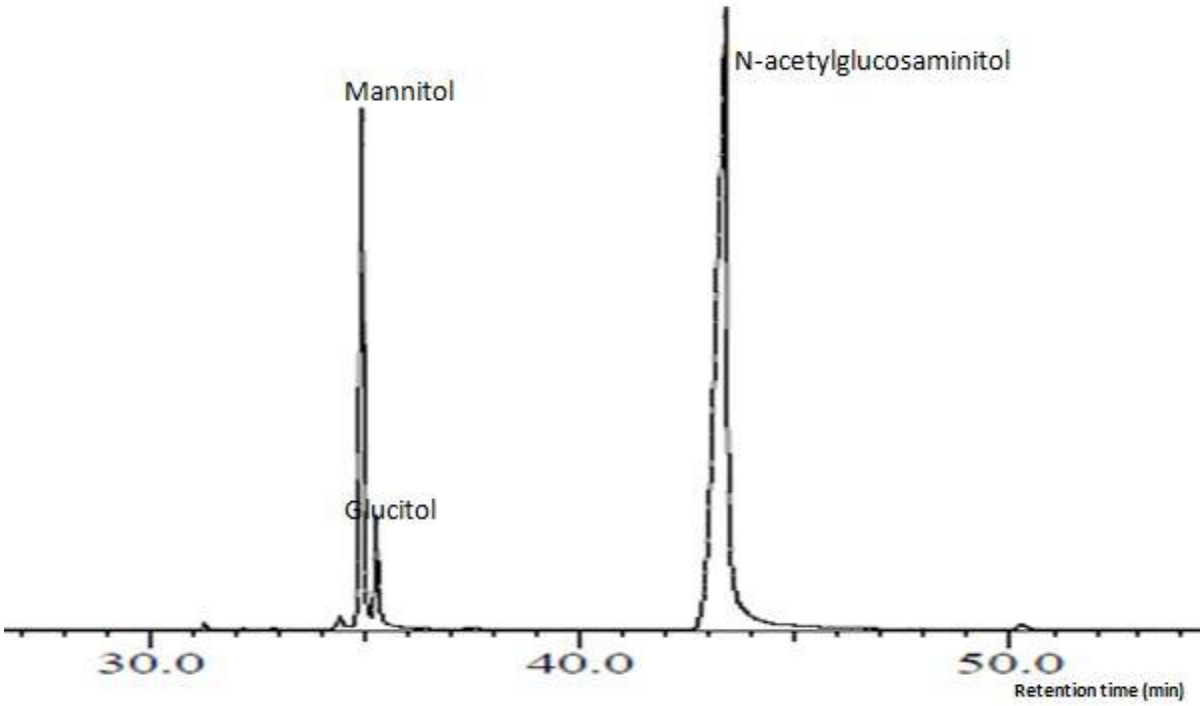

Figure 22: GC profile for 50 mM hyphal mannan extract + standard chitin

Compositional analysis of the hyphal and yeast mannan revealed that both extracts contain a significant amount of mannan due to the dominant mannitol hexaacetate peak observed in their GC profile. This is an indication that the novel base extraction method developed in house was successful in isolating mannan from both the hyphal and yeast form of *C. albicans*. The successful isolation of hyphal mannan is an interesting development as the existing classical method of isolating mannan fails to isolate hyphal mannan.

A small glucitol hexaacetate peak was also observed at 35.2 min for both yeast and hyphal mannan indicating a small amount of glucan in the extracts. While mannan is known to be soluble in aqueous solution, the mannan extracted from the hyphal mannan was only partially soluble. The presence of glucan and chitin explains why the sample is only partially soluble.

Monosaccharide Compositional Analysis Results for Acid Extractions

The monosaccharide content of hyphae and yeast mannan isolated using 50 mM phosphoric acid was also investigated for monosaccharide content. The spectra for both hyphal and yeast morphologies resulted in a dominant mannitol hexaacetate peak at ~34.9 min and a minor second peak for glucitol hexaacetate at ~35.2 min as shown in Figures 23 and 24. The chitin peak at ~42.9 min was again observed for the hyphal mannan. Interestingly, the glucitol hexaacetate peak for both acid extractions was much larger compared to the base extractions. This indicated that the use of acid for the extraction of mannan resulted in an unacceptable amount of glucan in the mannan product. The compositional analysis confirms the ^{1}H NMR and dialysis results conducted on the mannan samples by another member of the research group.

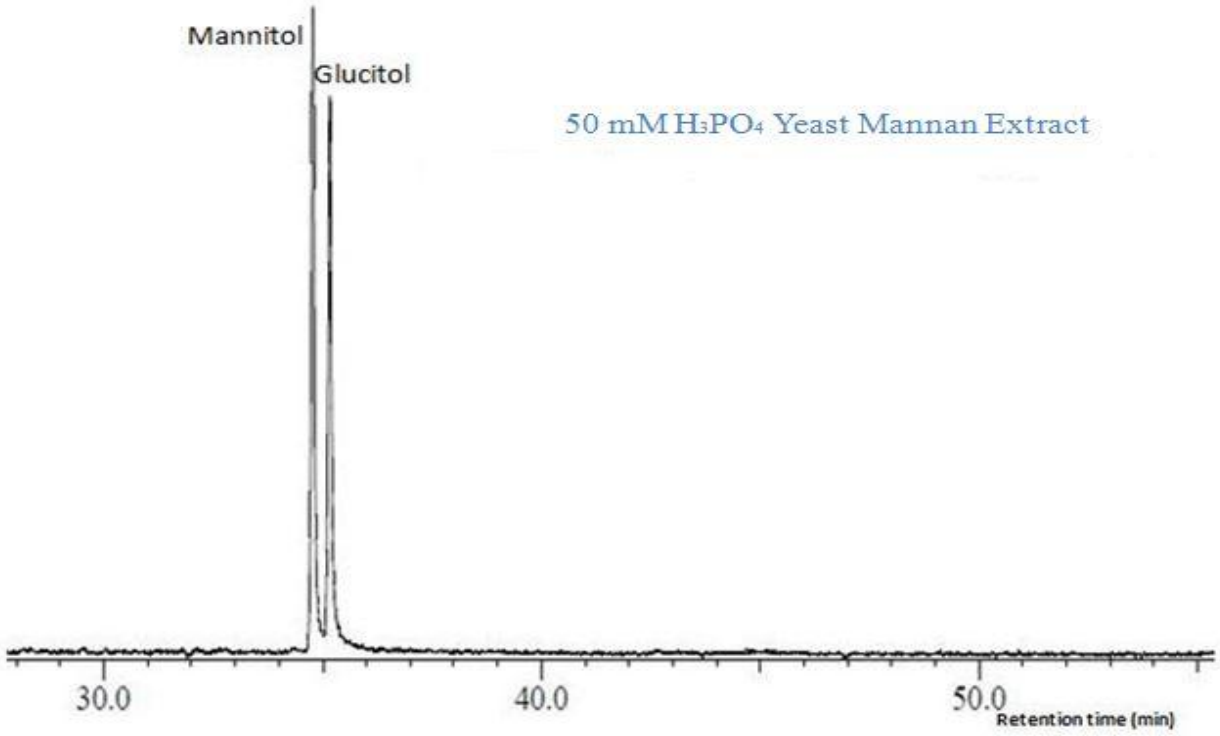

Figure 23: GC profile for yeast mannan extracted with 50 mM of acid (H_3PO_4)

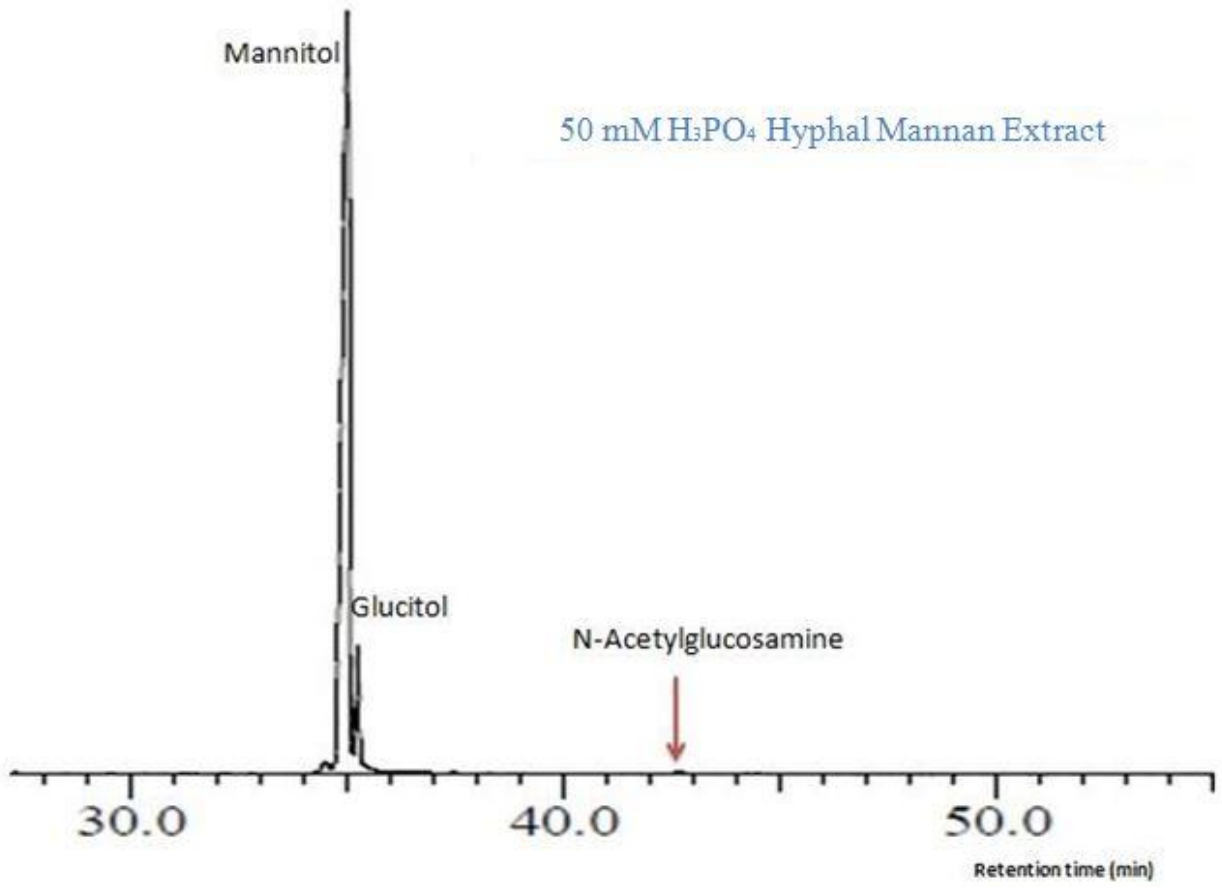

Figure 24: GC profile for hyphal mannan extracted with 50 mM of acid (H_3PO_4)

Methylation (Linkage) Analysis for β-Glucans

After vigorous analysis of the monosaccharide content of β-glucans isolated using the optimized extraction method, linkage analysis was conducted on pure glucan samples (major component being glucose > 90%). Linkage analysis using methylation analysis was conducted on theβ-glucans resulting from the optimized method of single extractions in 0.1 N phosphoric acid, 0.1 N sodium hydroxide, and ethanol and compared to that of β-glucan extracted with the classical method. This was done to better investigate the native structure retained after β-glucan extraction. Linkage information was obtained by methylation of the β-glucan polysaccharide

using the method described by Ciucanu and coworkers, followed by derivatization to a volatile partially methylated alditol acetates that can be detected by GC-MS.[49]

Fourier Transform-Infrared Spectroscopy Results for Permethylated β-Glucans

The permethylation step in the linkage analysis procedure can present a major challenge with high molecular weight and insoluble materials. The low solubility of the glucan extracted from *S. cerevisiae* can yield unreliable results due to undermethylation of the free hydroxyl group on the polysaccharide. Complete methylation was confirmed by FTIR analysis after the methylation step. Each of the β-glucans used in this studies were proven to have undergone complete methylation by the disappearance of broad peak at 3000-3500cm^{-1} (O-H region) in the FTIR spectrum. A strong C-H stretching was also observed in the FTIR spectrum at 2922 cm^{-1} which is characteristic of methyl groups and indicated the replacement of all hydroxyl groups with methyl groups on the polysaccharide. Fiqure 25 below is an example of the IR spectra obtained for the compeletely methylated β-glucan.

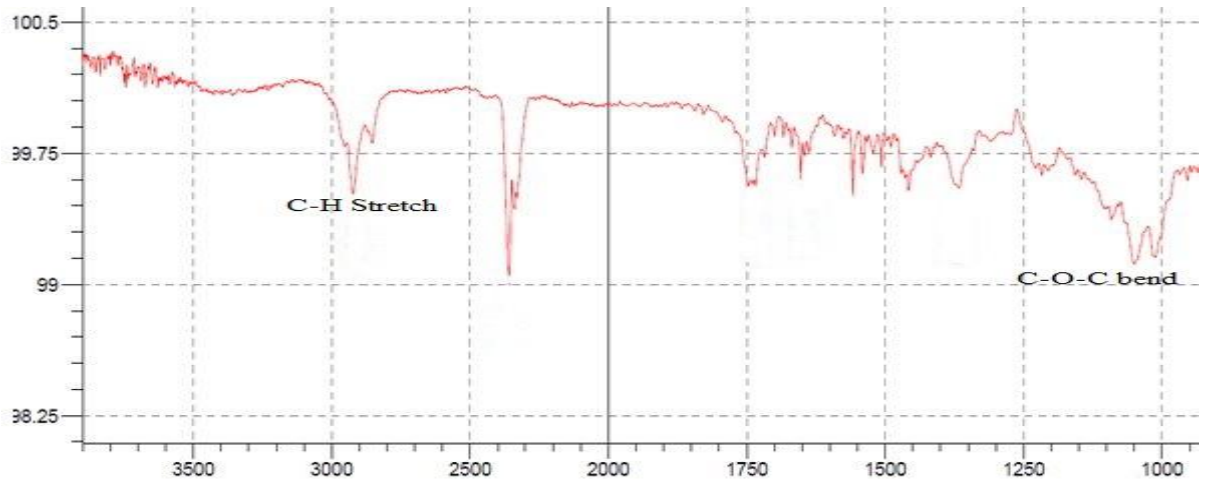

Figure 25: IR spectrum for partially methylated β-glucan with absence of broad peak in the O-H region (3000-3500cm^{-1})

GC-MS Results for Methylation (Linkage) Analysis of β-Glucans

Linkage analysis was conducted on β-glucan extracts isolated using the optimized method as well as the classical method in order to determine the structural linkages that join the individual glucose unit to form the polysaccharide present. Linkage analysis also helps to determine how the isolation procedure affects the structure of glucans in the cell wall. The peaks were identified using their retention times in the chromatogram and their unique fragmentation patterns in the electron impact (EI) mass spectrometer following rules of fragmentation of partially methylated alditol acetates discussed in Chapter 1. The observed ions in the mass spectra were also compared with documented standards of partially methylated alditol acetates in literature.[46]

Methylation (Linkage) Analysis Results for β-Glucans Isolated Using Classical Method

The linkage analysis was first conducted on β-glucans isolated with the classical method as shown in Figure 26 below. The GC profile showed four peaks, one tall and sharp one and three small ones. The fragmentation pattern and its interpretation for the first small peak with a retention time of ~37.6 min is shown in Figure 27. The 278, 262, 146, 129 ions are indicative that C-2, C-3, C-4, and C-6 are methoxylated (i.e. does not partake in linkage). The first peak was thus identified as a 1-linked glucose unit, meaning the glucose unit is joining the other glucose unit with its carbon number 1.

The fragmentation pattern of the second tall peak in Figure 26 with a retention time of ~39.8 min is shown in Figure 28. The 234, 118 and 161 ions are indicative that C-2, C-4 and C-6 positions must be methoxylated (does not form part of the linkage). The second tall peak was thus identified as a 1,3-linkage . The presence of the tall peak identified as a 1,3-β-linkage

indicated that the major linkage type found in β-glucans is the 1,3-linkage; thus, the glucose units that form the linear backbone connect or bond together using their C-1 and C-3. This strongly confirms the linkage mode reported in literature to form the primary backbone in microorganisms such as yeast.[20, 32,55]

The fragmentation pattern of the third small peak in Figure 26 with a retention time of ~ 41.0 min is shown in Figure 29. The 189, 118 and 162 ions were an indication that C-2 and C-3 and C-4 are methoxylated (i.e. not a linkage position). The third peak was identified as a 1,6-linkage which is known to be the linkage found in glucose monomers that form the side chain branching of the β-glucan. Hence, the glucose units that form the side chain are connected together via their C-1 and C-6.

The fragmentation pattern of the final small peak in Figure 26 is shown in Figure 30 below. The 234, 189 and 118 ions mean C-2 and C-4 are methoxylated (not a linkage position), indicative of the existence of a 1, 3, 6-β-linkage. The 1, 3, 6-linkage is the branching point of the glucan polysaccharide. A branching point is the point of attachment where a 1, 6-linked glucan side chain attaches itself to the linear polymer backbone. The glucose monomer in the linear backbone that forms the point of attachment to the 1,6-linked side chain consist of a 1,3,6-linkage. This glucose monomer uses its C-1 and C-3 in bonding to other glucose molecules to form the backbone, and it's C-6 in forming a covalent bond with glucose monomers that form the side chain.

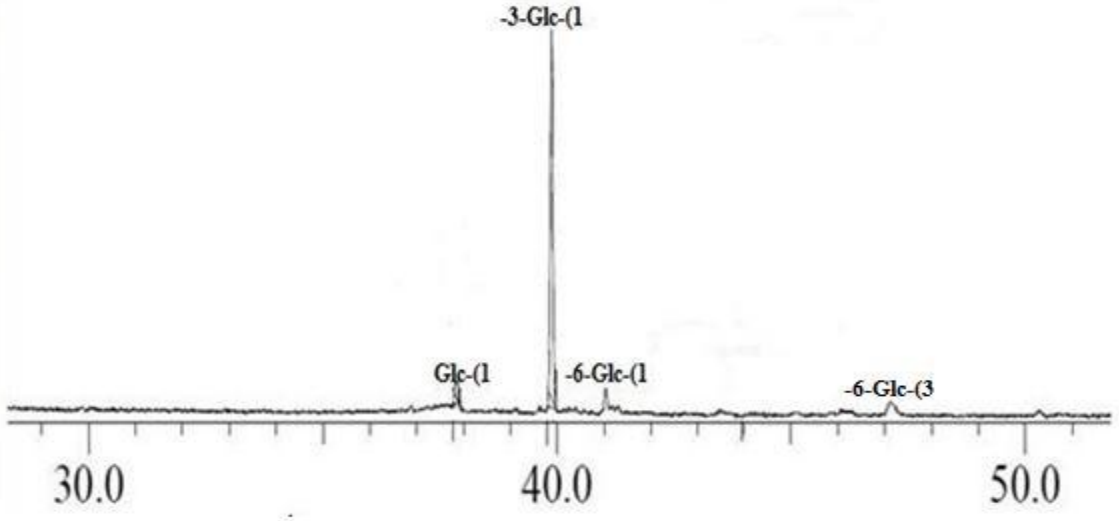

Figure 26: GC profile for linkage studies of β-glucan isolated using the classical method of extraction

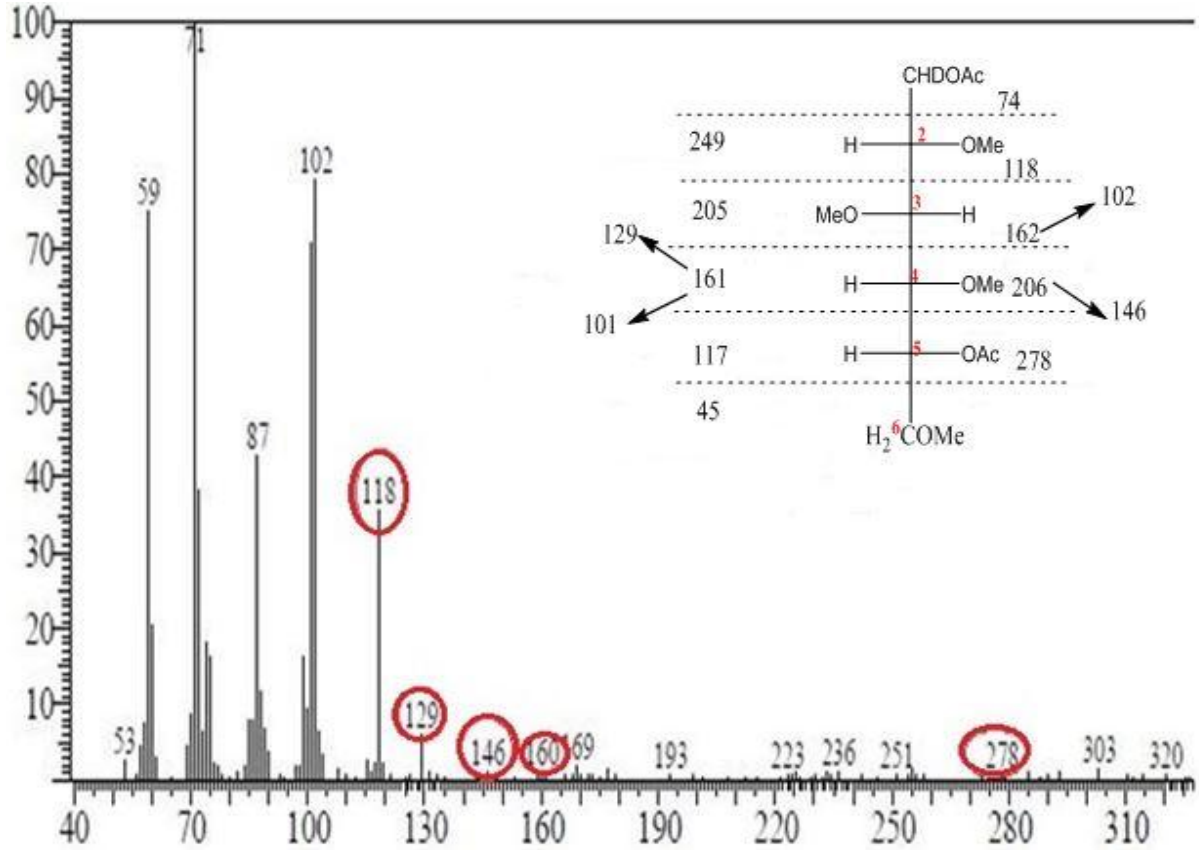

Figure 27: Mass spectrum for terminal linkage in β-glucans. Significant ions are highlighted in red

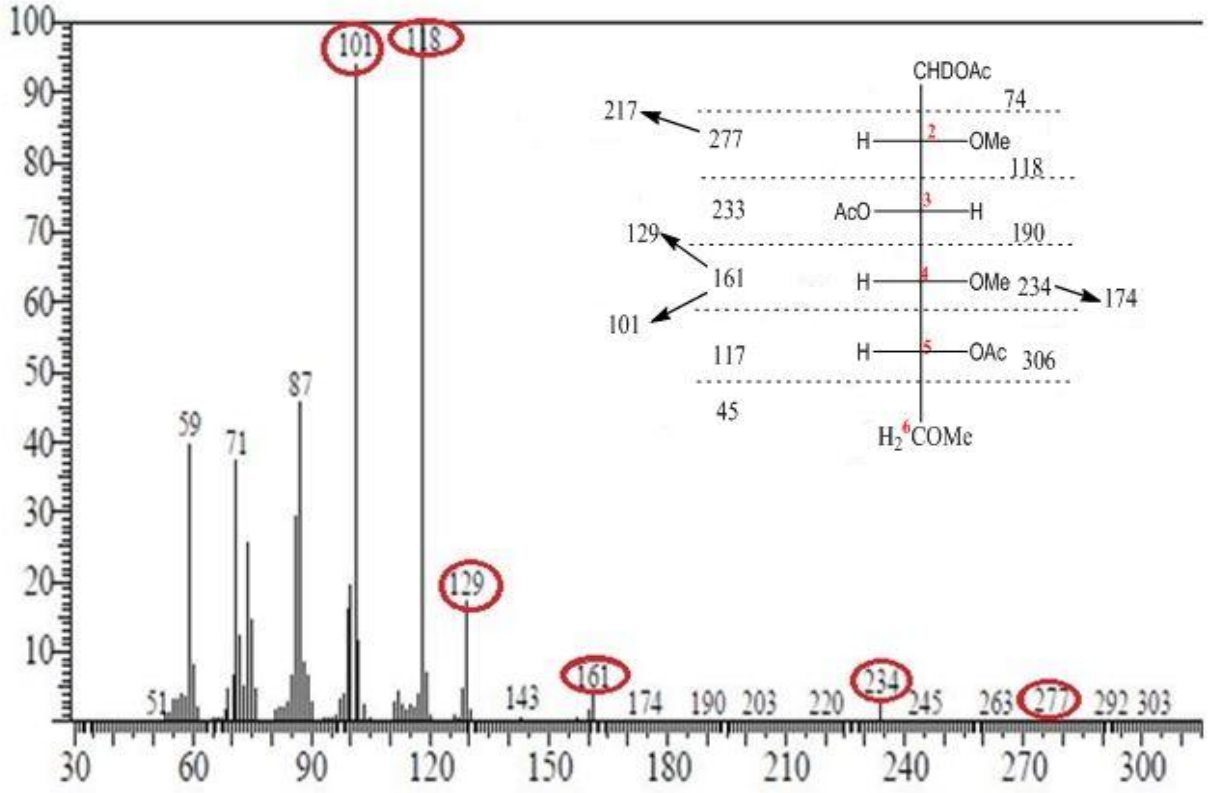

Figure 28: Mass spectrum for 1,3-linkage in β-glucan. Significant ions are highlighted in red

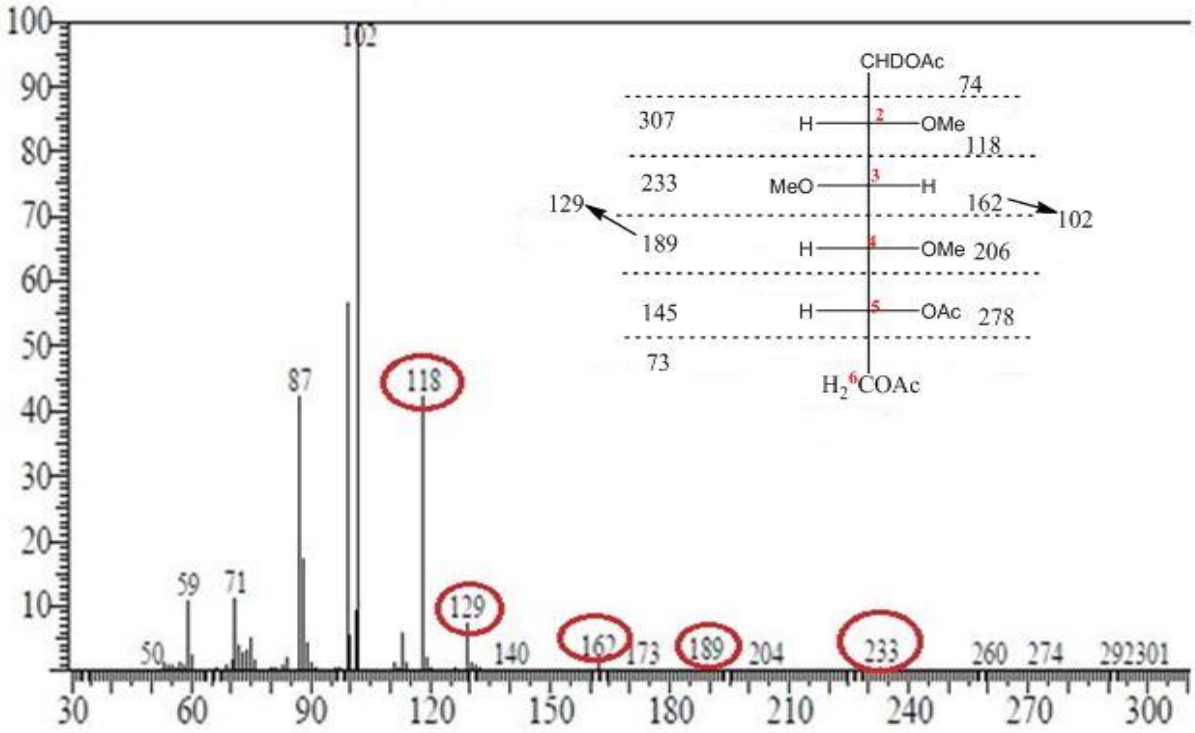

Figure 29: Mass spectrum for 1,6-linkage in β-glucan. Significant ions are highlighted in red

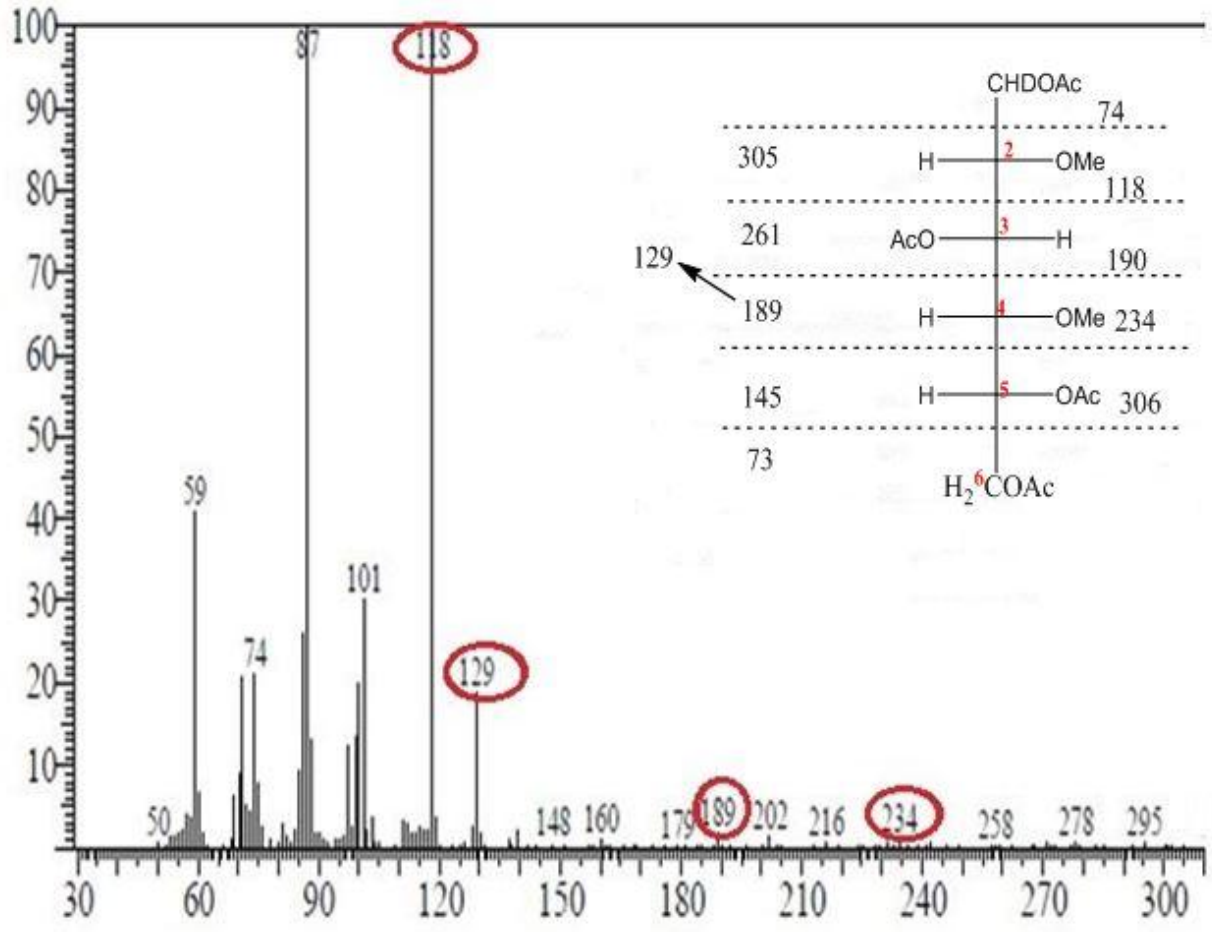

Figure 30: Mass Spectrum for 1, 3, 6-linkage in β-glucan. Significant ions are highlighted in red

Methylation (Linkage) Analysis Result for β-Glucans Isolated Using the Optimized Procedure

The linkage analysis was next conducted on the β-glucans isolated with the optimized method. Interestingly, while the classical method resulted in only four peaks in the GC profile, the optimized method resulted in six peaks as shown in Figure 31 below. The peaks with retention times at ~37.0 min, ~39.9 min, ~41.0 min and ~46.5 min were identified as terminal linkage; 1,3-linkage; 1,6-linkage and 1,3,6-linkage respectively based on their fragmentation patterns in the mass spectrum as already discussed above. The 1, 3-linkage was the dominant

peak in the GC profile for this extract as well, confirming the presence of a major linear backbone component. We observed an increase in height of the 1,6-linkage (side chain) and 1,3,6-linkage (branching point) peak for the optimized procedure compared to that observed for the classical isolation method (see Figure 26 above).

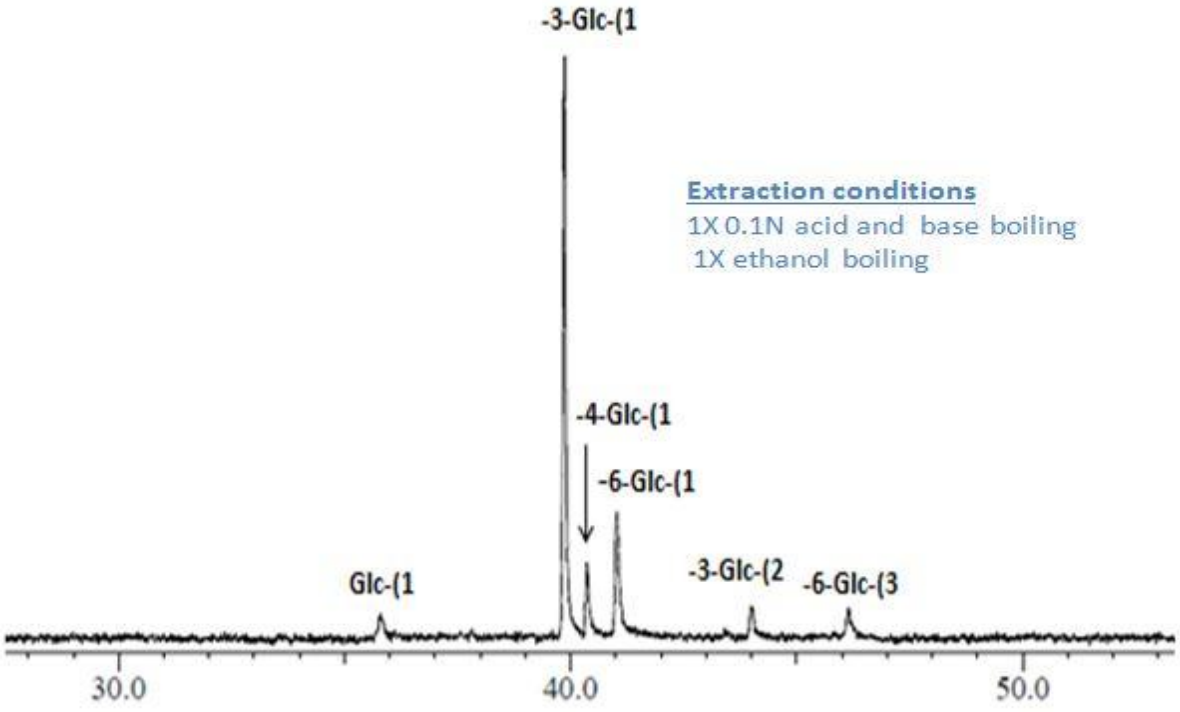

Figure 31: GC profile for β-glucan isolated using the optimized method of extraction

The Figures 32 and 33 shows an MS spectrum with fragmentations observed for the peaks with retention times of ~40.4 min and ~44.2 min respectively unique to the optimized method. In Figure 32, presence of 233, 162 and 118 ions confirmed that C- 2, C-3 and C-6 are methoxylated (not a linkage position). The peak at ~40.4 min was thus identified as a 1,4-linkage. In Figure 33, the 262, 202, 161 and 129 ions tells that C-4 and C-6 are methoxylated (not linkage positions)

indicative of the presence of a 1, 2, 3-β-linkage. This helped identify the peak at ~44.2 min to be a 1,2,3-linkage.

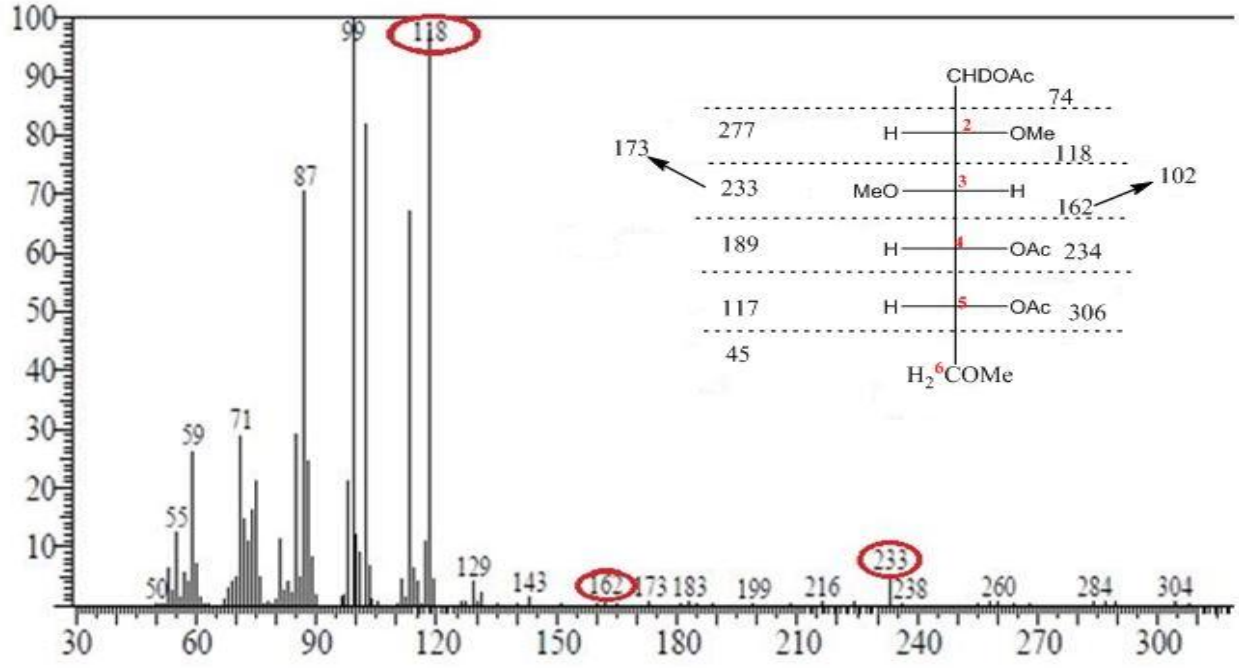

Figure 32: Mass spectrum for 1, 4-linkage in β-glucan. Significant ions are highlighted in red

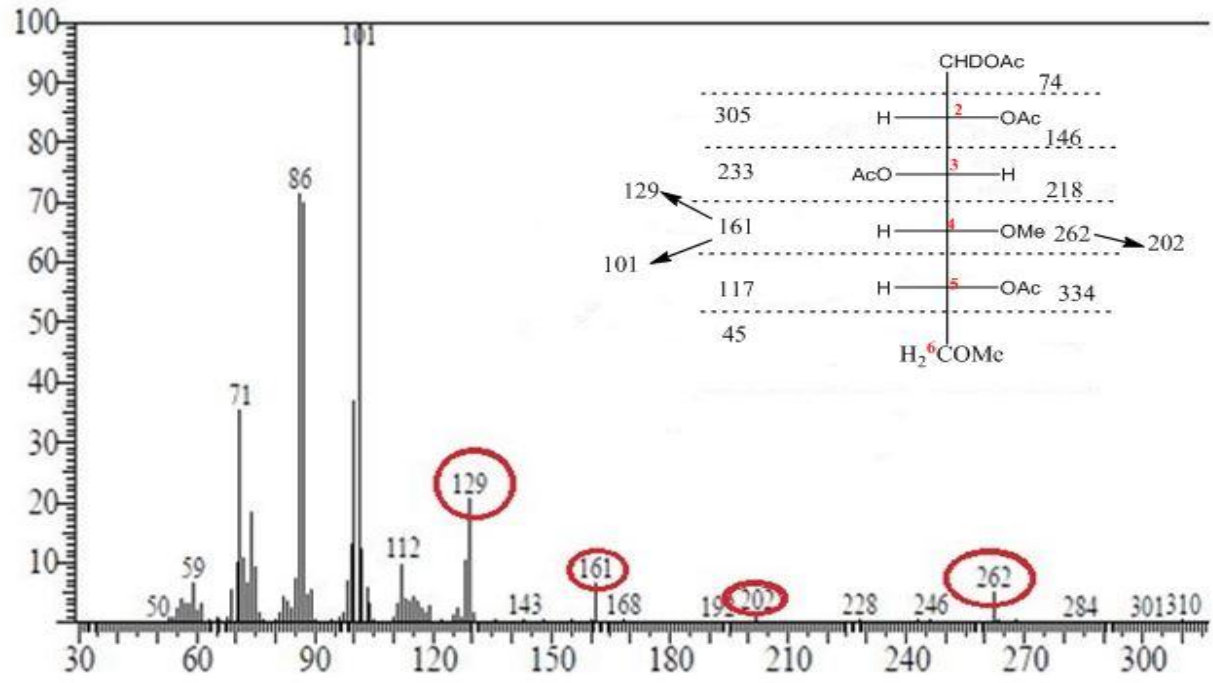

Figure 33: Mass Spectrum for 1, 2, 3,-linkage in β-glucan. Significant ions are highlighted in red

In the case of both the classical and optimized method, the 1,3; 1,6, and 1,3,6 linkages were expected as they have been observed previously.[20, 55] The diminishing of the 1,6-linkage peak observed in the GC profile for the classical method is an indication of degradation of the highly branched structure of the β-glucan. Glucose monomers that form the side chain in a glucan polymer are joined covalently by the 1,6-linkage; therefore, a diminishing 1,6-linked peak suggest that the polymeric side chain was cut off or reduced during the extraction process. The sharp increase in the 1,6-linkage peak observed in the GC profile for the optimized method relative to the classical method indicates an increase in the side chain length of the glucan polysaccharide thereby better retaining the native structure.

The presence of 1,3,6-linkage indicates that the 1,6-linked side chain glucans are attached to the carbon number 6 of a backbone 1,3-glucose unit. The observed increase in the height of

the 1,3,6-linkage in the optimized procedure indicate more branching point or branches formed along the linear backbone. The 1,2,3-linkage observed in the optimized method is novel to *S. cerevisiae*. The 1,2,3 linkage has previously only been observed in the hyphal morphology of *C. albicans* and thus far has been considered to be unique to this species.[40] To date the purpose of the 1, 2, 3 linkage is unknown. It is however believed to be a possible attachment sight of other cell wall component to the glucan polysaccharide. The interesting observation of this novel branching in β-glucans isolated from *S. cerevisiae* led to the speculation that the C-2 position may be a site for interconnection of fungal cell wall β-glucan with other cell wall components. This might explain how the cell wall of fungal species is structured. It might also help to resolve the problem of how the components of the cell wall are interconnected. The newly observed 1,2,3-linkage could possibly help also in understanding the heavy cross-linking between cell wall components in the fungal cell wall.

The 1, 4-linkage was also unexpected. It is possible be a point of attachment of chitin to the β-glucan.[6] However, due to the absence of chitin in the extracted β-glucans, it is difficult to conclude on this hypothesis. The presence of a 1,4-linkage in *S. cerevisiae* has long been believed by researchers to be due to an alkali soluble "yeast glycogen" which are physically adsorbed.[56,57,58] Arvindekar and Patil suggested an explanation to the presence of the 1,4-linkage as α-glucan covalently bonded to the 1,3-β-glucan through the 1,6-side chain.[59] This finding was later confirmed by Kwiatkowski and coworkers, they also explained the absent of this linkage in other reported literature for S. cerevisiae to be as a result of the method used in extractions.[60] Hence, an interesting explanation to the observed 1,4-linkage could be the existence of a mixed glucan in which (1→4)-α-glucan is connected to (1→3)-β-D-glucan through a (1→6)-β-glucan linkage.

The presence of other linkage positions in β-glucans isolated using the optimized method is an interesting confirmation of the hypothesis that a milder isolation procedure could yield a structure of the β-glucan which is a true identity of the β-glucan in the fungal cell wall. This result strongly agrees with ^{1}H NMR results and goes to confirm the work done by Lowman and coworkers that using mild extraction conditions increases the degree of polymerization to give a better representation of the native β-glucan structure in the cell wall. Also, the linkage information obtained in this study will facilitate insights to how β-glucans are linked to the other cell wall components in the cell and the purpose of this linkage in its interaction with the immune system.

CHAPTER 4

CONCLUSION AND FUTURE WORK

Monosaccharide Compositional Analysis of β-Glucans

The monosaccharide compositional analysis conducted on β-glucans was successful in confirming the effectiveness of the classical isolation procedure as well as the newly optimized isolation method. In conducting the compositional analysis of the β-glucan extracts isolated using the optimized procedure, we determined that the use of a milder approach yielded a good β-glucan product with little contaminant. In terms of reducing the strength of the acid (i.e. switching from a strong acid to a weak acid) employed in the extraction, as well as single sequential boiling in the reagents, it was determined that the extracts contain glucose as its major monosaccharide component indicating that the isolation method produces β-glucan product free of other carbohydrates. Therefore, a mild acid is sufficient to cleave the linkages between the glucan and the other cell wall components. Additionally, a single extraction by each reagent is sufficient to dissolve other cell wall components.

When the concentrations of the phosphoric acid and sodium hydroxide employed in the isolation of β-glucans were reduced from 1N to 0.1N, compositional analysis revealed a similar low level of contaminants with glucose being the major component in the extract. Reduction of the concentration further to 0.01N and 0.001 N resulted in an increase in contaminant. It was found that an unacceptable amount of mannan is present in the product when the concentration was lowered. Comparison of the sugar content in 0.1 N and 1 N extracts proved that reducing the concentration of acid and base from 1 N to 0.1 N did not affect the efficacy of the isolation procedure.

Further modification of the isolation method where the sodium hydroxide was held at 0.1 N and the phosphoric acid concentration was reduced to 0.01 N, resulted in glucose as the major component. When the concentration of the acid was held at 0.1 N and the base was reduced to 0.01 N concentrations, the major carbohydrates were both mannose and glucose. This indicates that the base extraction step is essential to remove all other cell wall components for β-glucan isolation. The optimum concentrations which were found to be effective in isolating pure glucans were 0.1N or 0.01 N phosphoric acid with 0.1 N sodium hydroxide.

Monosaccharide Compositional Analysis of Hyphal and Yeast Mannans

Monosaccharide analysis of hyphal and yeast mannan isolated with 50 mM NaOH revealed mannose as the main component in the extract. Unfortunately, isolation with 50 mM phosphoric acid revealed the presence of a significant amount of glucans in both the hyphal and yeast mannan samples. Therefore, the use of base in the newly developed mannan isolation procedure is successful for the isolation of mannan from both the hyphal and yeast form of *C. albicans*. An interesting observation was the partial solubility of the hyphal mannan in aqueous solution which was also found to be due to the presence of chitin in the hyphal mannans. Therefore, the hyphal form of *C. albicans* must have more chitin in its cell wall than the yeast form.

Methylation (Linkage) Analysis for β-Glucans

Linkage analysis conducted on β-glucans isolated using the classical extraction method demonstrated a terminal linkage; 1,3-linkage,1,6-linked branching, and a 1,3,6-linkage. In contrast, β-glucans isolated using the optimized method showed a terminal linkage, 1,3-linkage,1,6-linkage,1,3,6-linkage, 1,4- linkage, and a novel 1,2,3-linkage. In addition to the

absence of the 1,4 and 1,2,3-linkage in extracts isolated with the classical method, the height of the 1,6 and 1,3,6-linkage peaks decreased in comparison to extracts isolated using the optimized method. Thus, it was determined that while the classical method is successful in isolating β-glucan, it significantly degrades the glucan structure leading to less linkage information.

Linkage analysis of pure β-glucan isolated using single sequential boiling of ethanol and 0.1N phosphoric acid-sodium hydroxide concentration in the optimized procedure revealed more linkage information. The introduction of a new 1,2,3-linkage and the increase in the 1,6 and 1,3,6-linkage peak in the optimized method demonstrates that much of the highly branched polymeric structure of the β-glucan was retained. We speculate that the 1,2,3-linkage could be a point of attachment of other cell wall components. The observation of a 1,4-linkage also confirms the presence of a mixed (1,4)-α-(1,3)-β-glucan reported in literature to be present in glucans from *S. cerevisiae*. Linkage analysis proved that, using the optimized condition is an excellent method to successfully extract pure β-glucans whiles retaining much of the highly branched native structure of the complex in the fungal cell wall.

Future Work

The use of the linkage information using the optimized method to learn exactly what purpose these linkages serve in the fungal cell wall is a potential area to explore in future studies. Furthermore, additional linkage studies on mannans are also recommended to find any structural difference between hyphal and yeast mannan.

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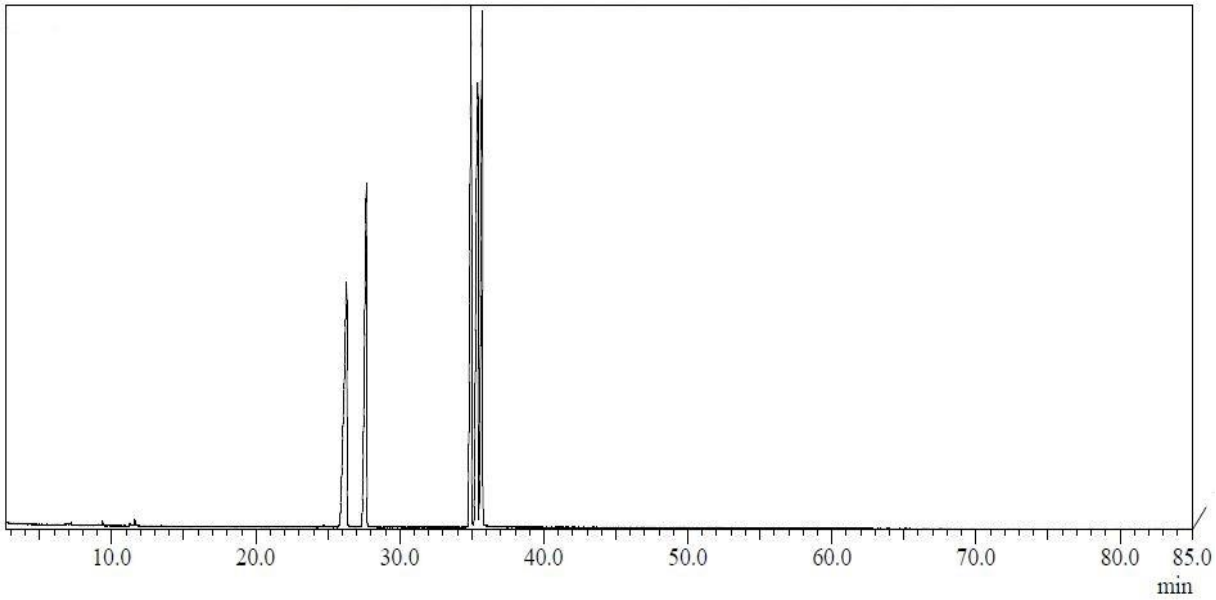
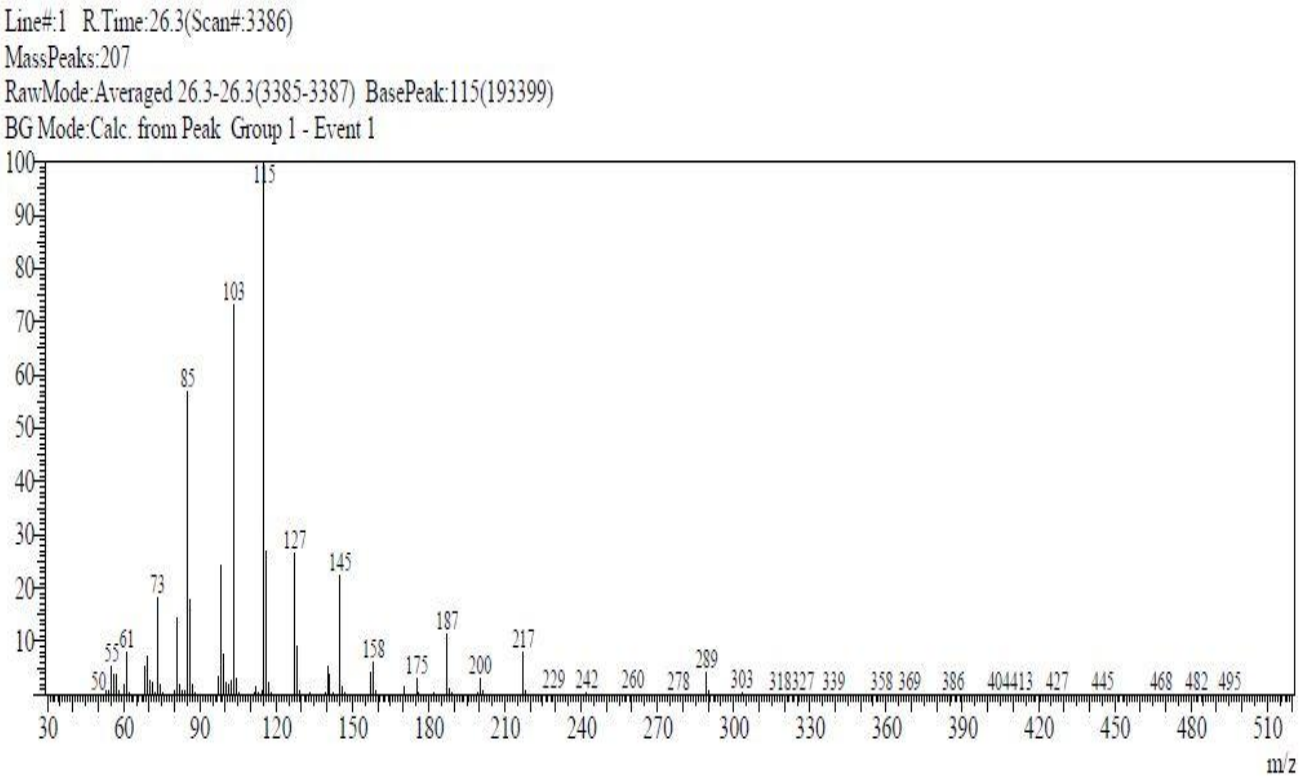
APPENDICES

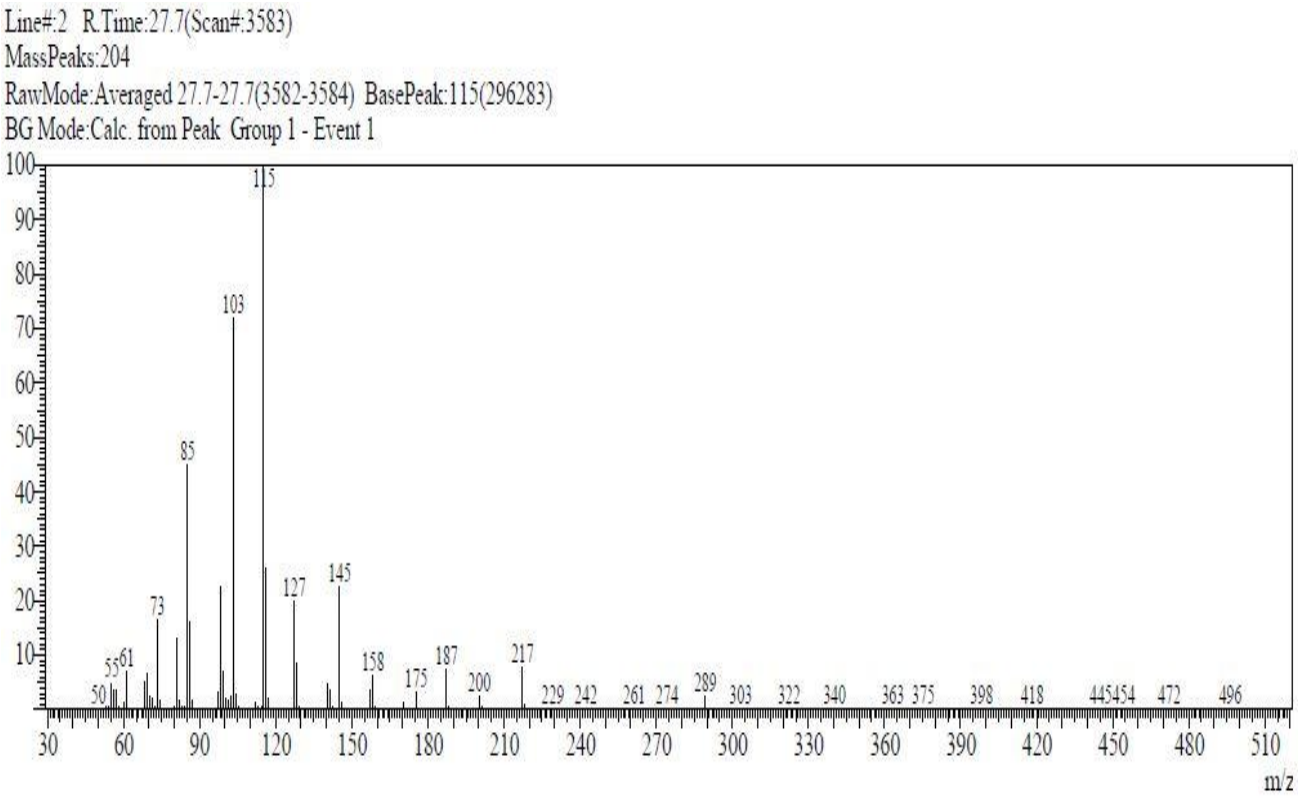
APPENDIX A: GC-MS Spectra for Five Standard Alditol Acetates



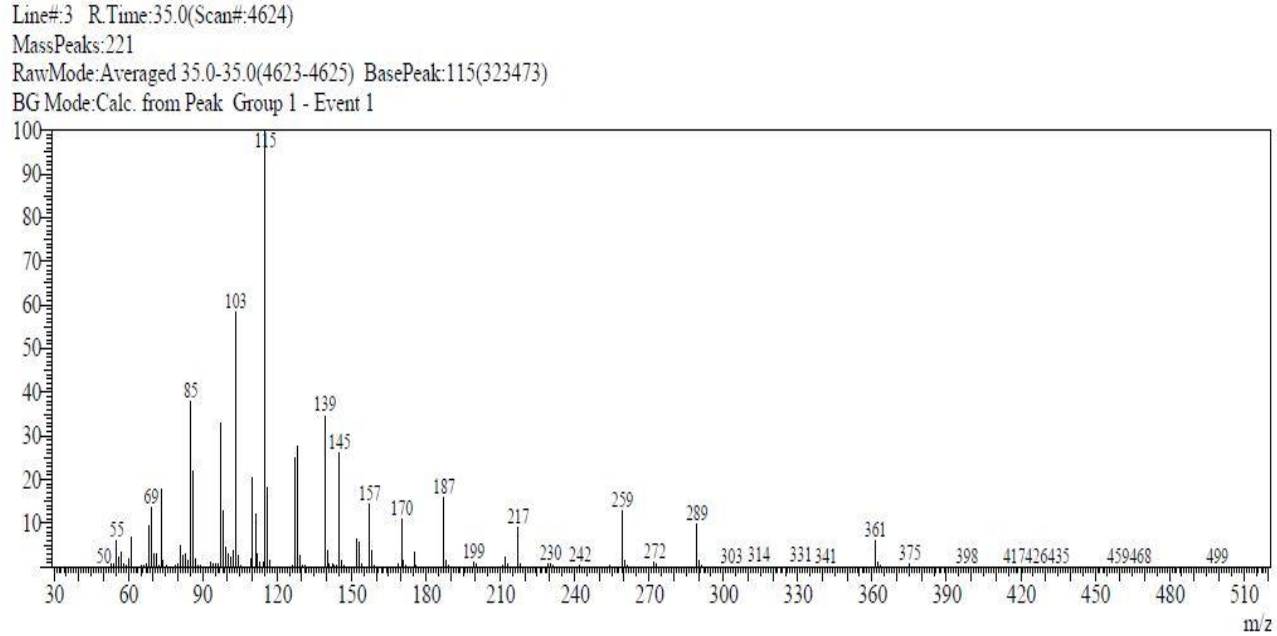
GC profile



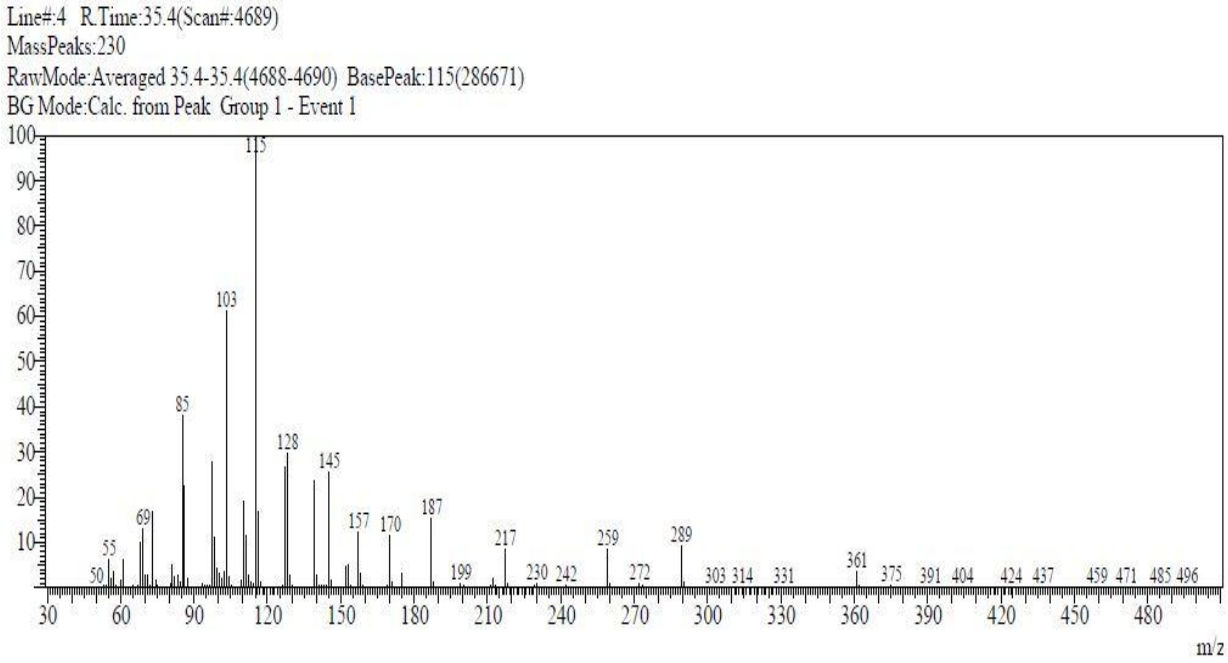
MS for Xylitol pentaacetate



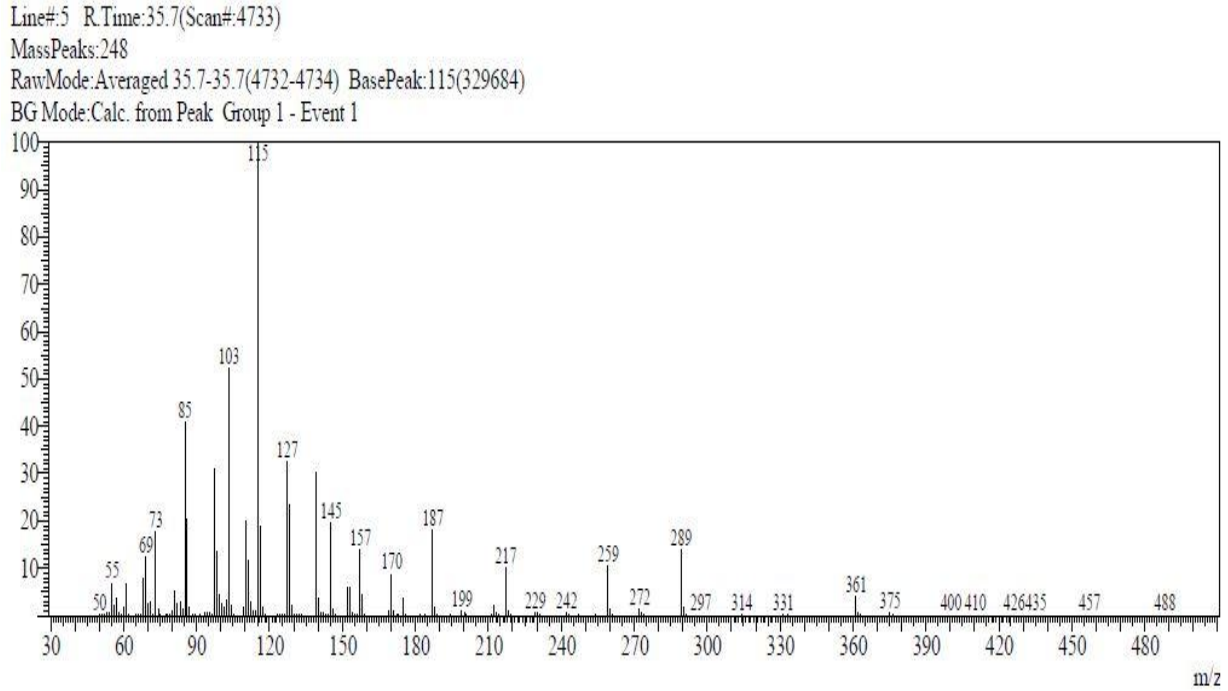
MS for Arabinitol pentaacetate



MS for Mannitol hexaacetate

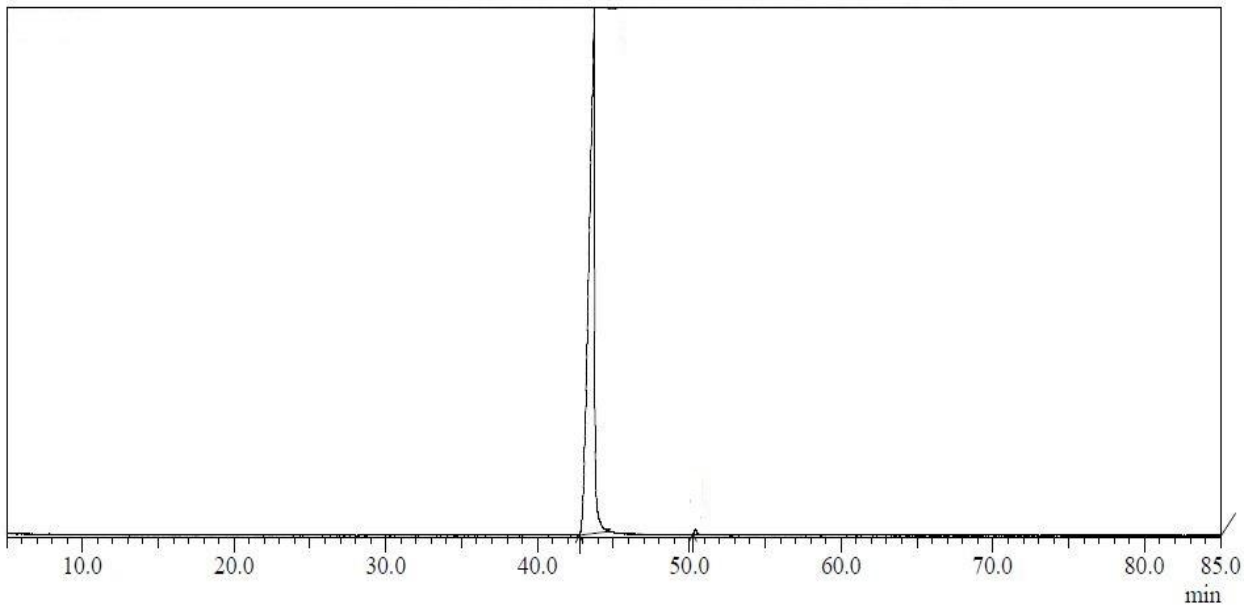


MS for Glucitol hexaacetate

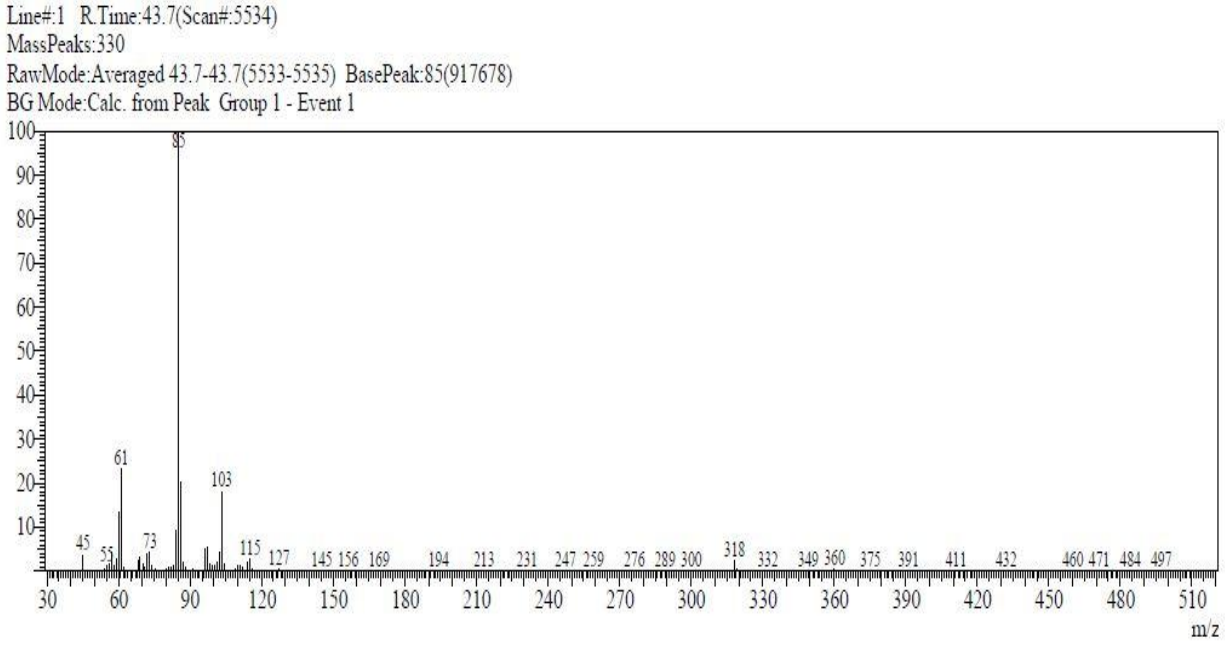


MS for Galacitol hexaacetate

APPENDIX B: GC-MS Spectra for N-Acetylglucosaminitol Hexaacetate

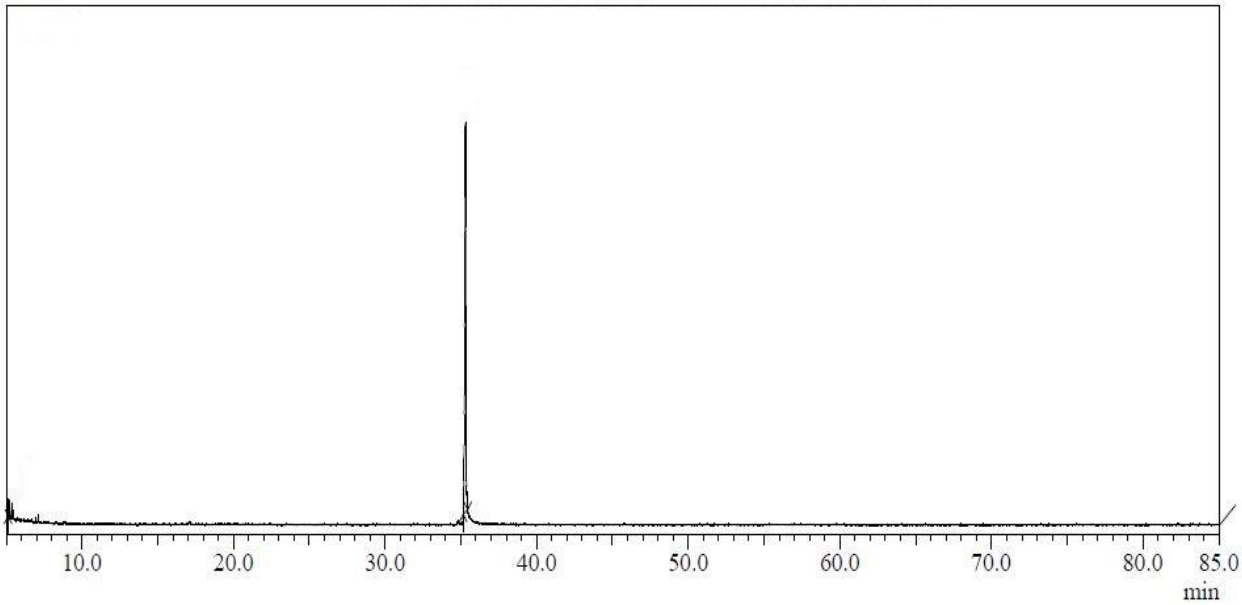


GC profile

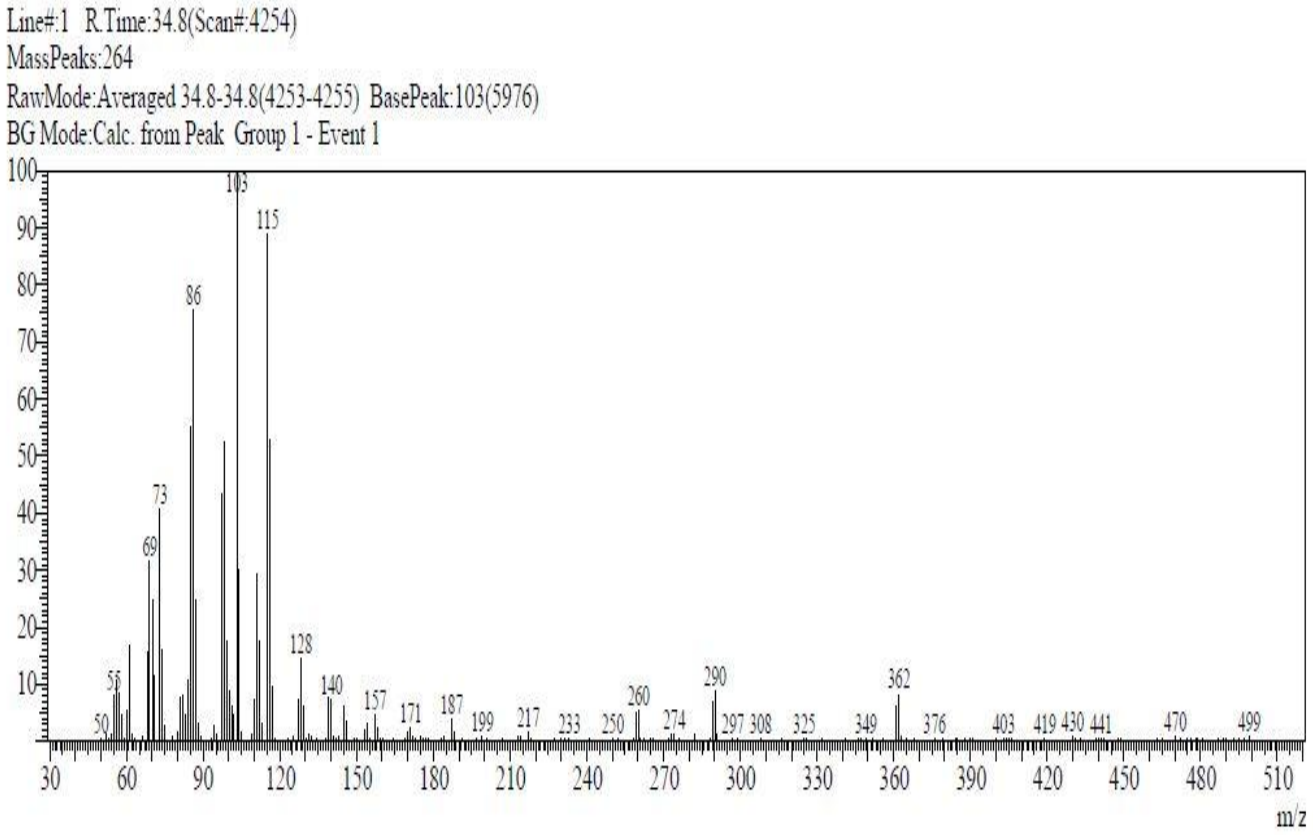


MS for N-acetylglucosaminitol hexaacetate

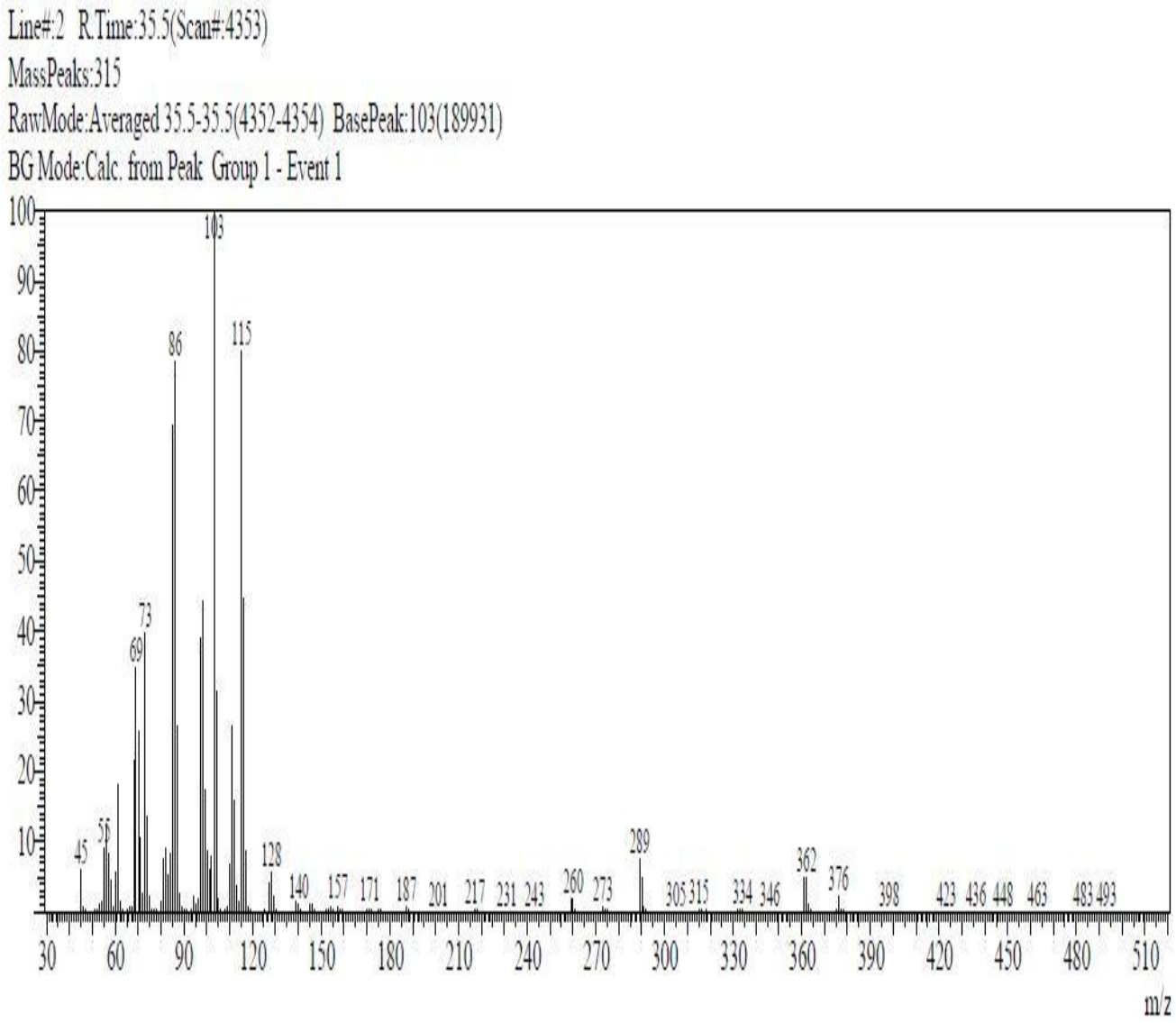
APPENDIX C: GC-MS Spectra for β-Glucan Isolated Using Hydrochloric Acid



GC profile

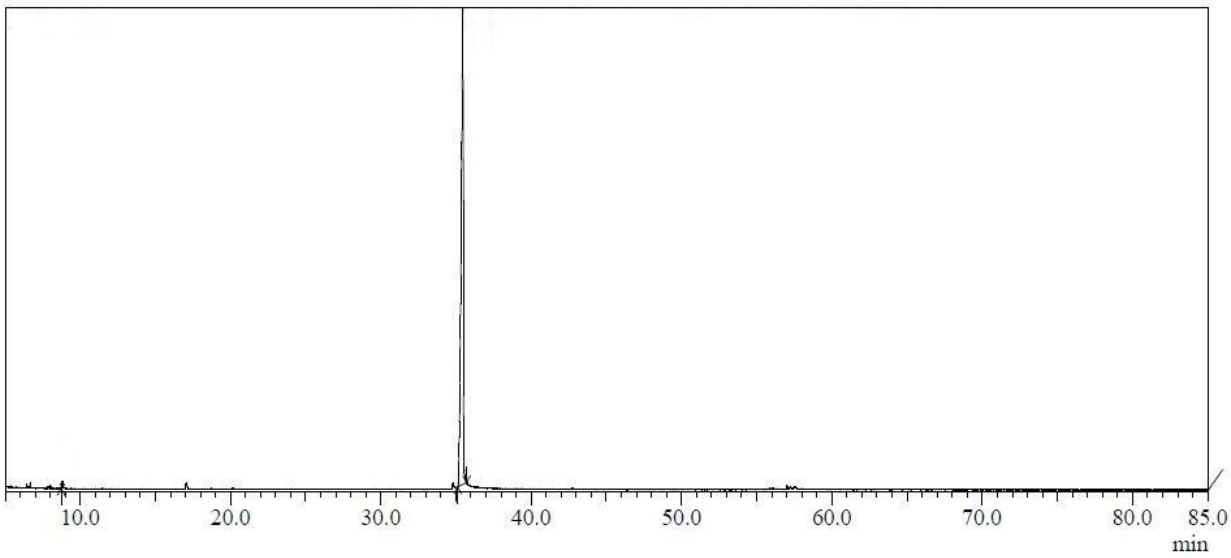


MS for Mannitol hexaacetate

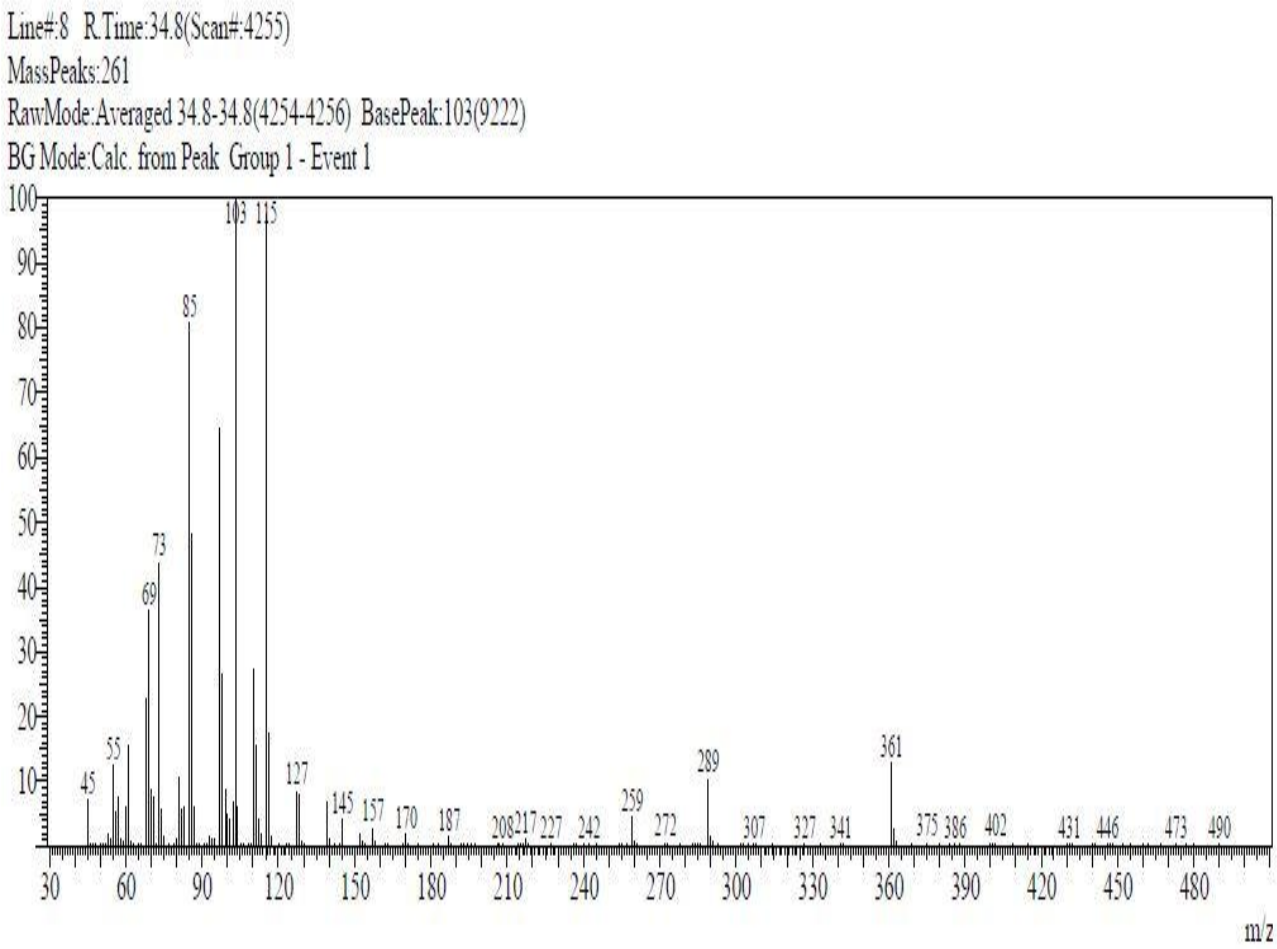


MS for Glucitol hexaacetate

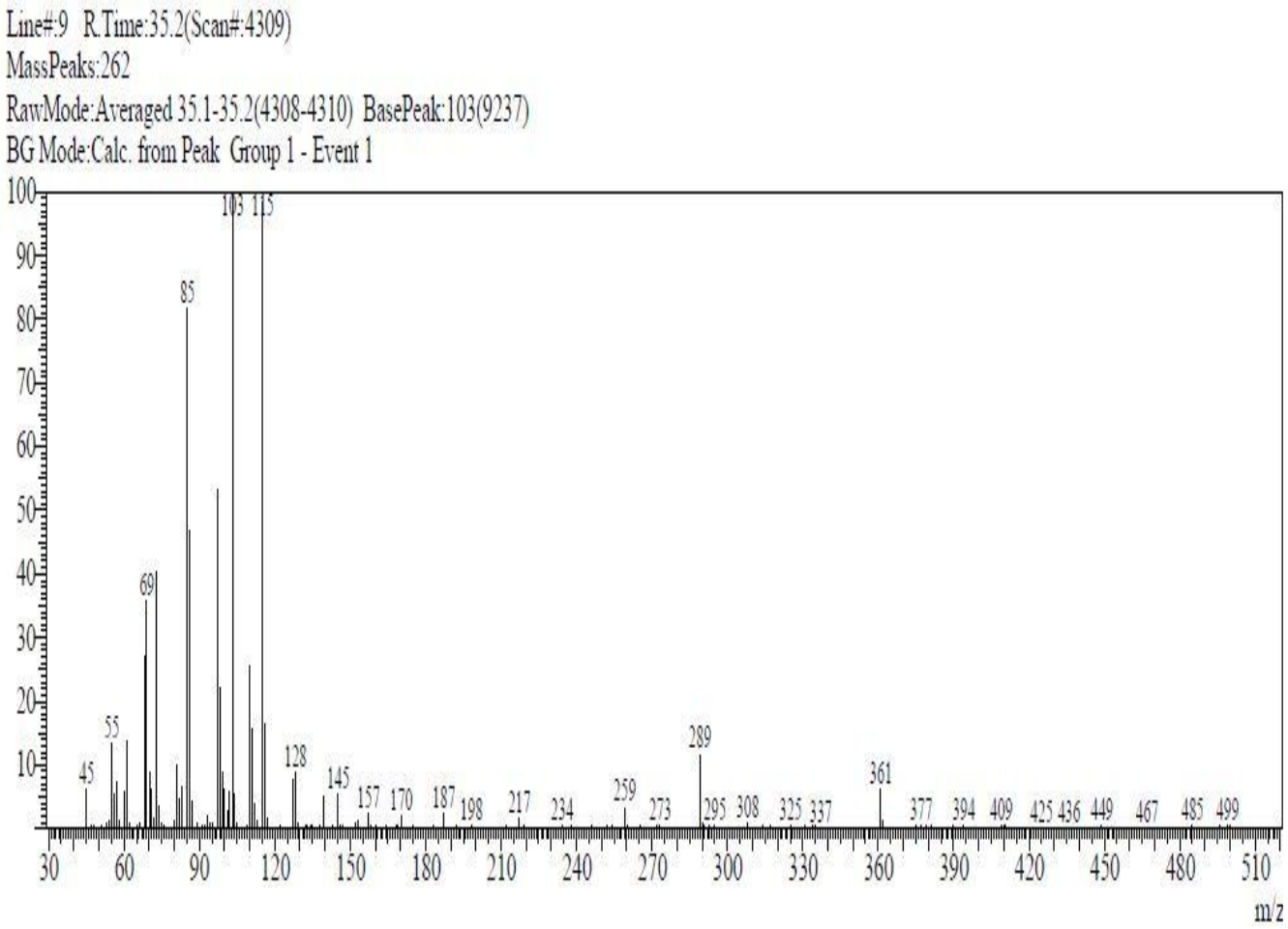
APPENDIX D: GC-MS Spectra for β-Glucan Isolated Using Phosphoric Acid



GC profile

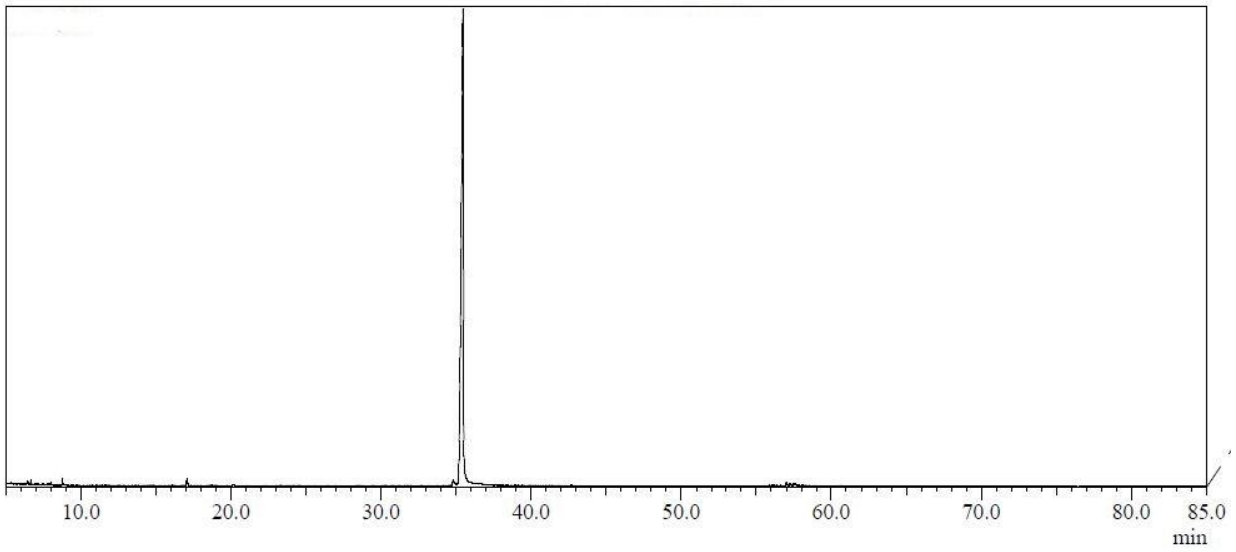


MS for Mannitol hexaacetate

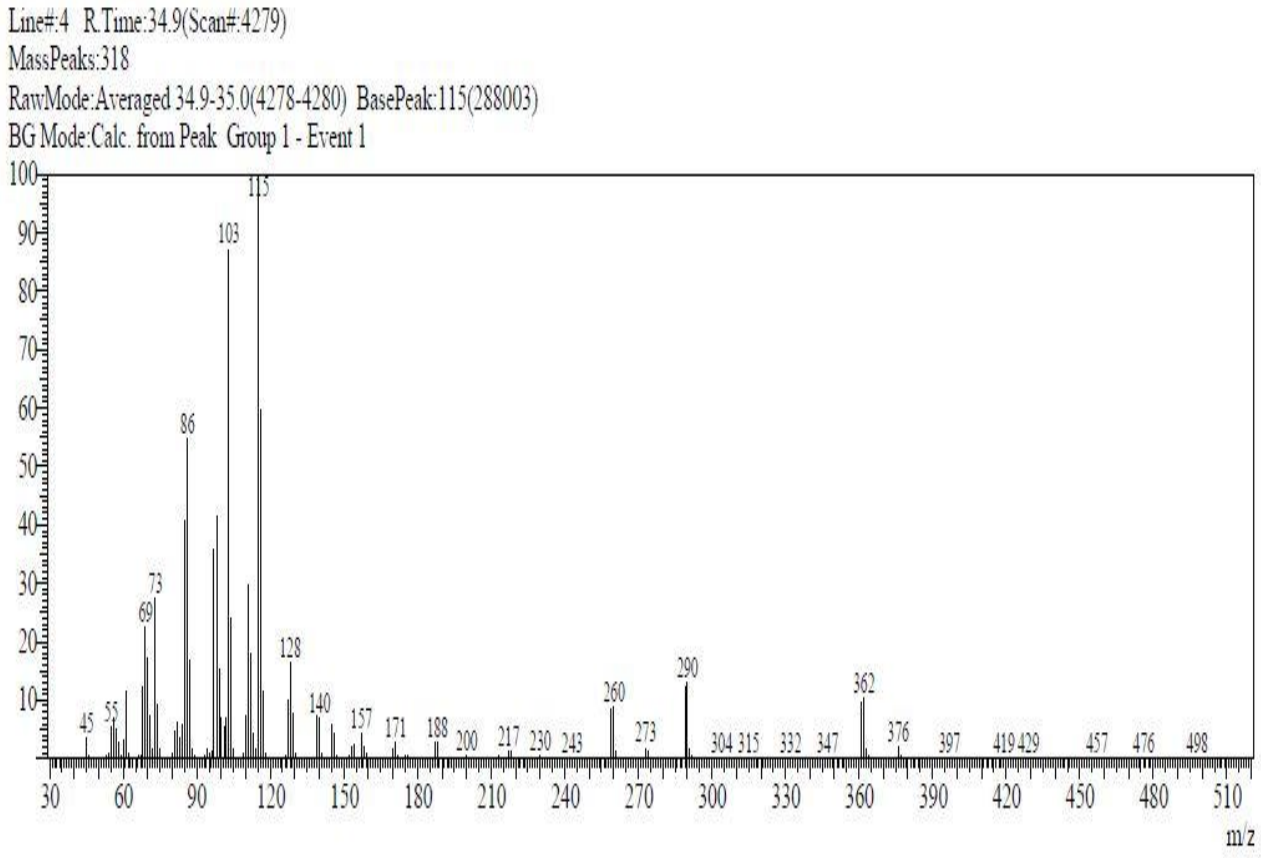


MS for Glucitol hexaacetate

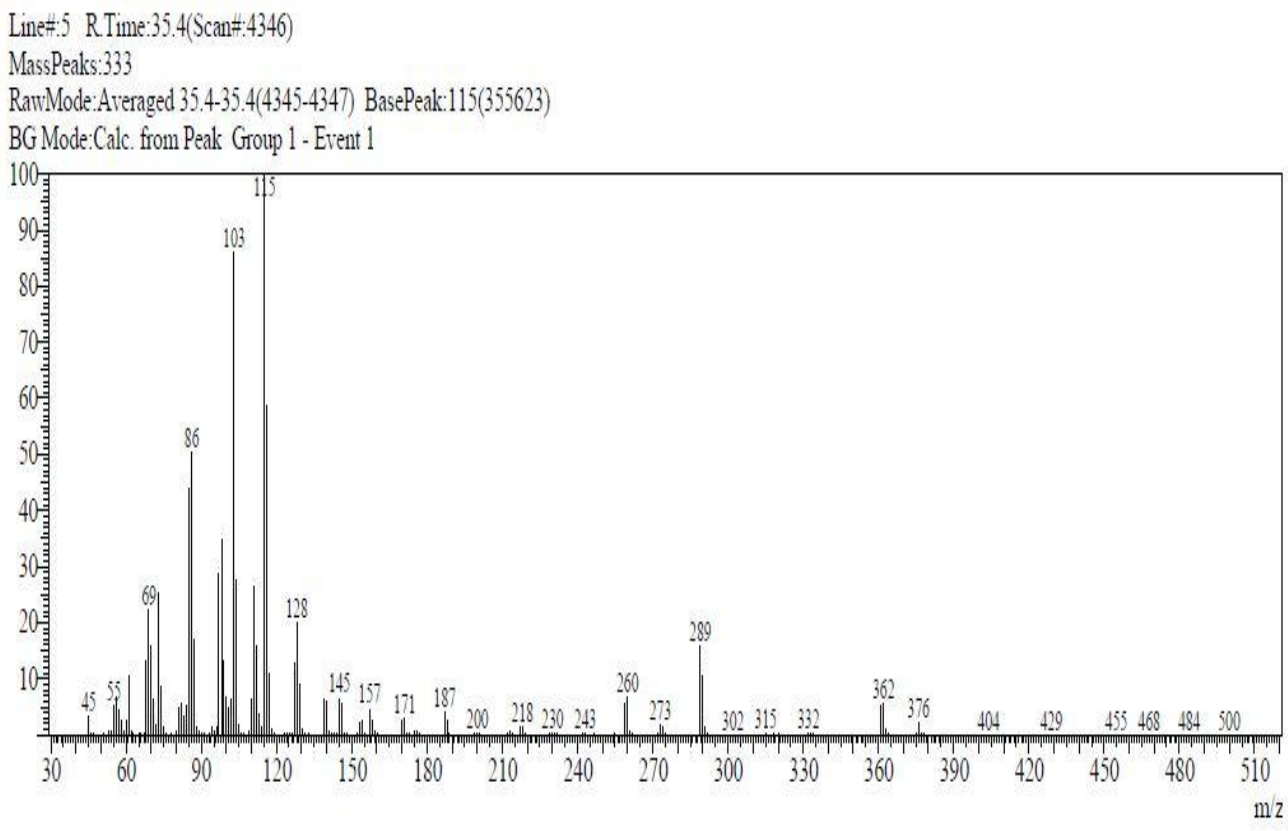
APPENDIX E: GC-MS Spectra for β-Glucan Isolated Using 3 X Extractions



GC profile

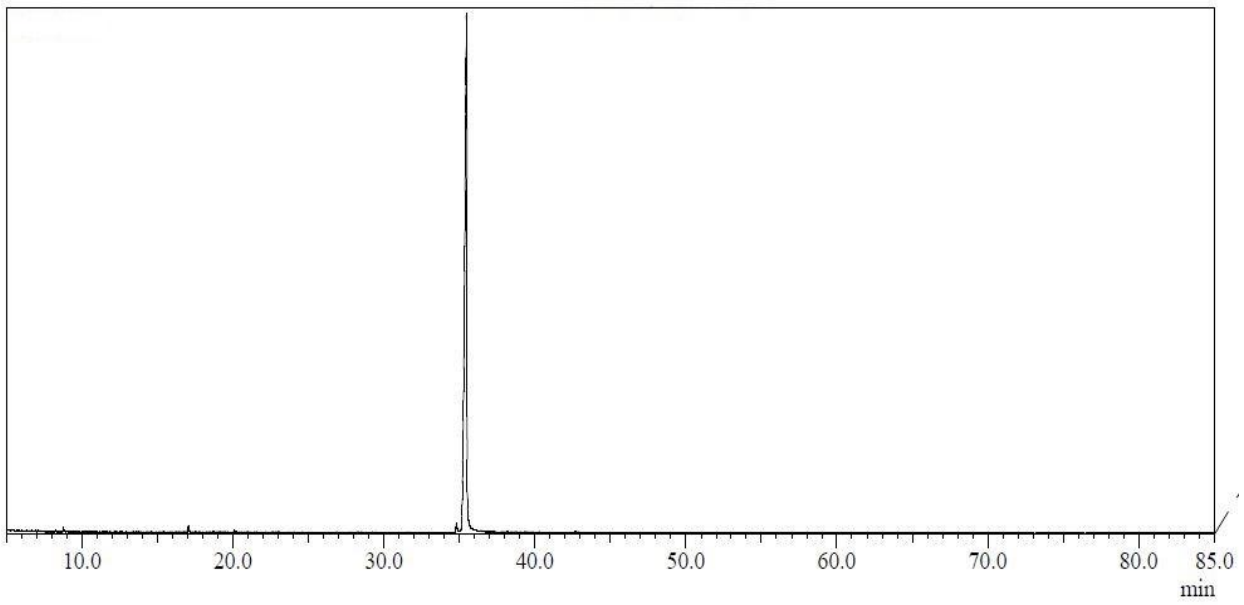


MS for Mannitol hexaacetate

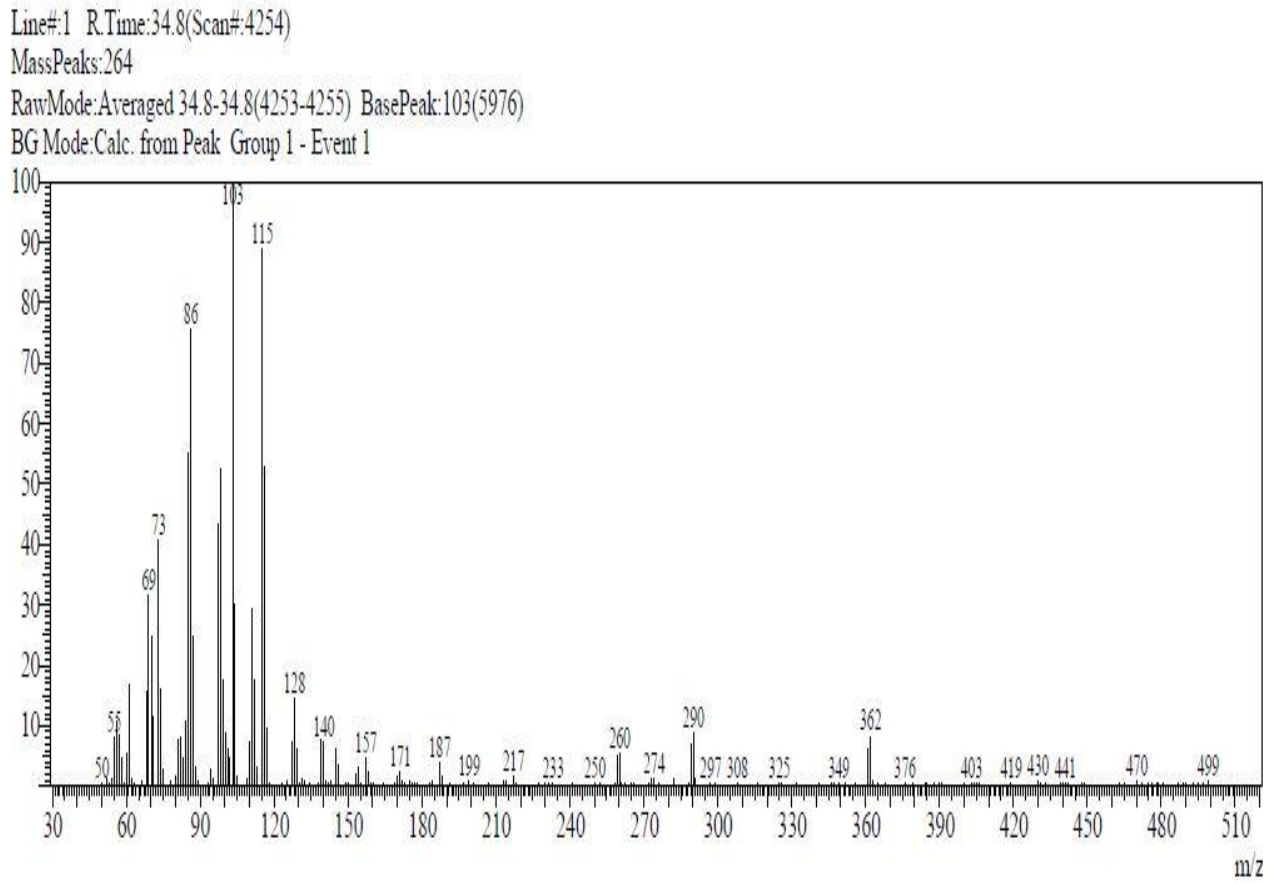


MS for Glucitol hexaacetate

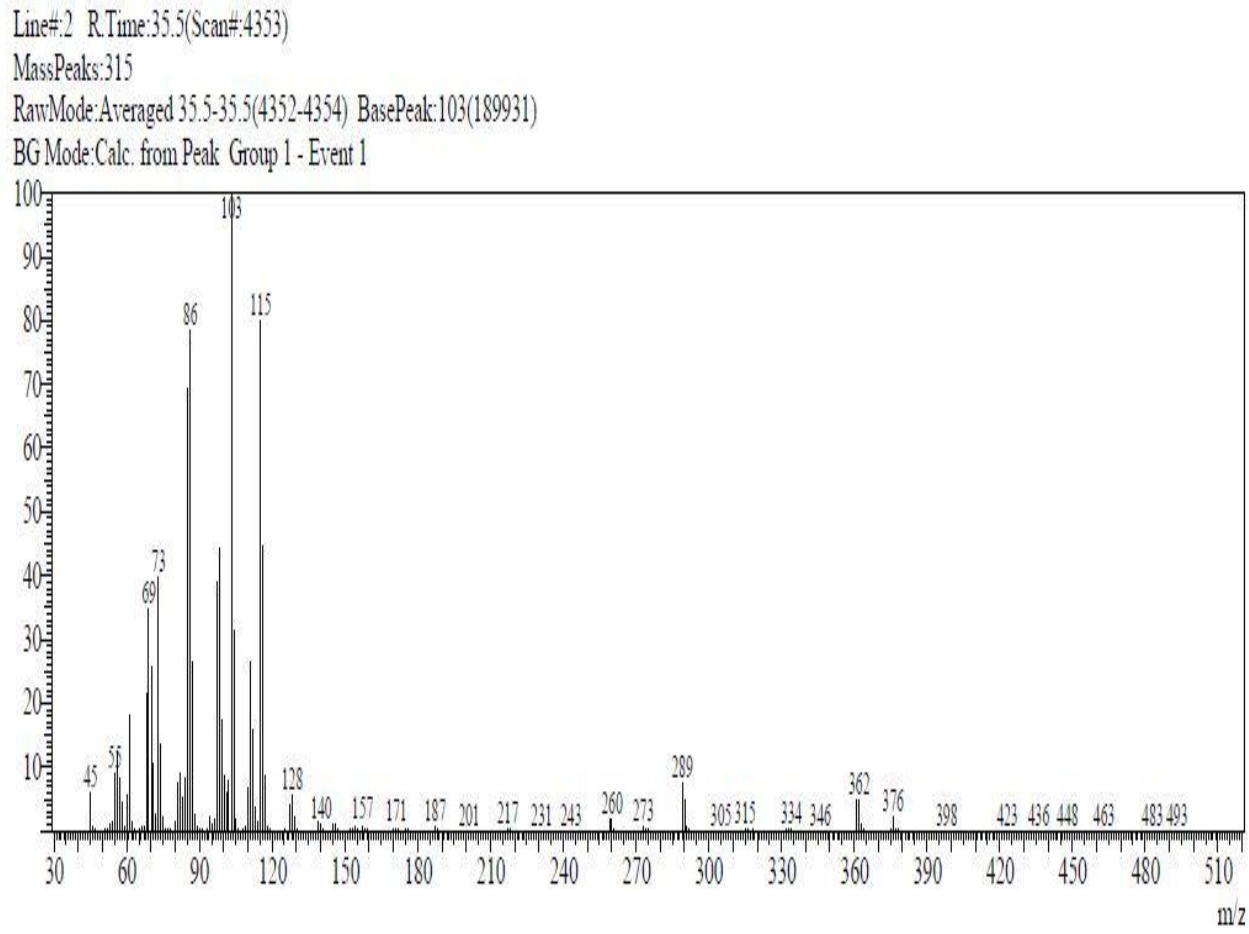
APPENDIX F: GC-MS Spectra for β-Glucan Isolated Using 1x Extraction



GC profile

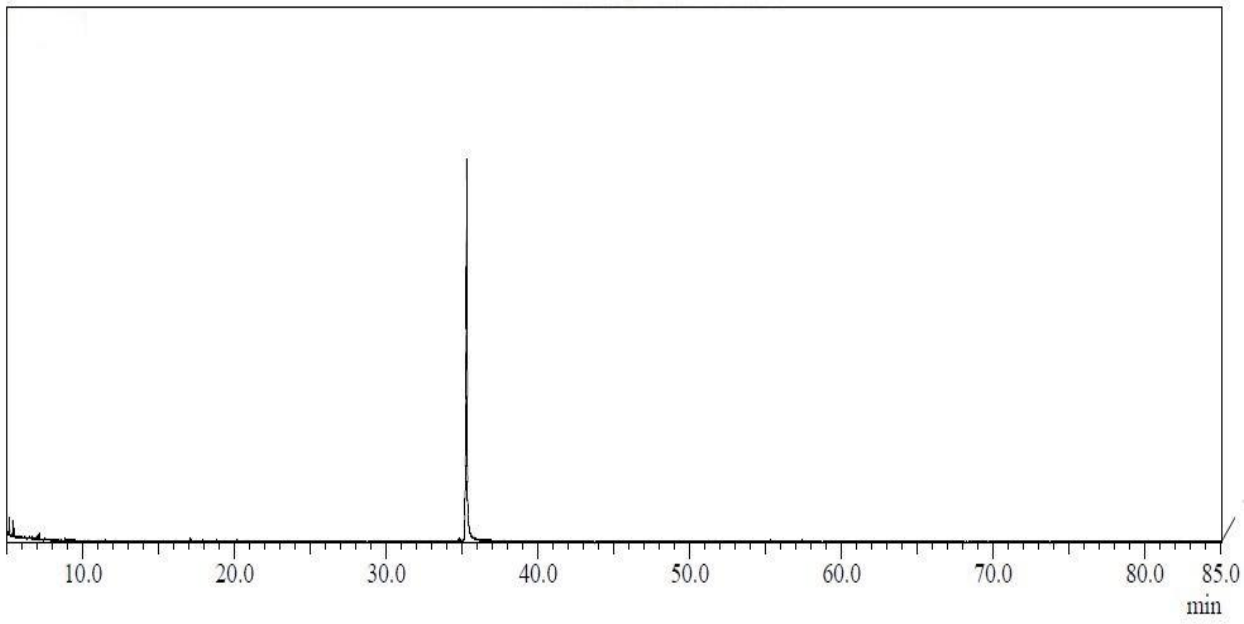


MS for Mannitol hexaacetate

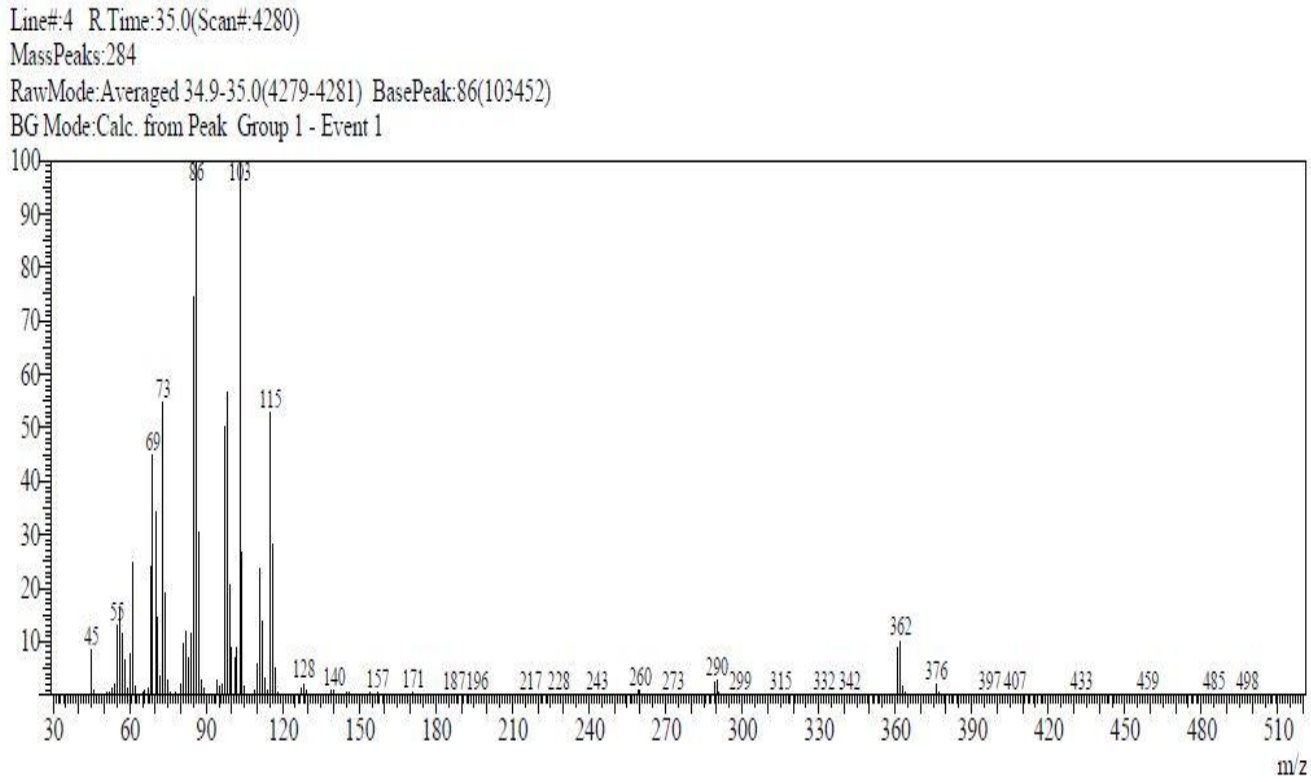


MS for Glucitol hexaacetate

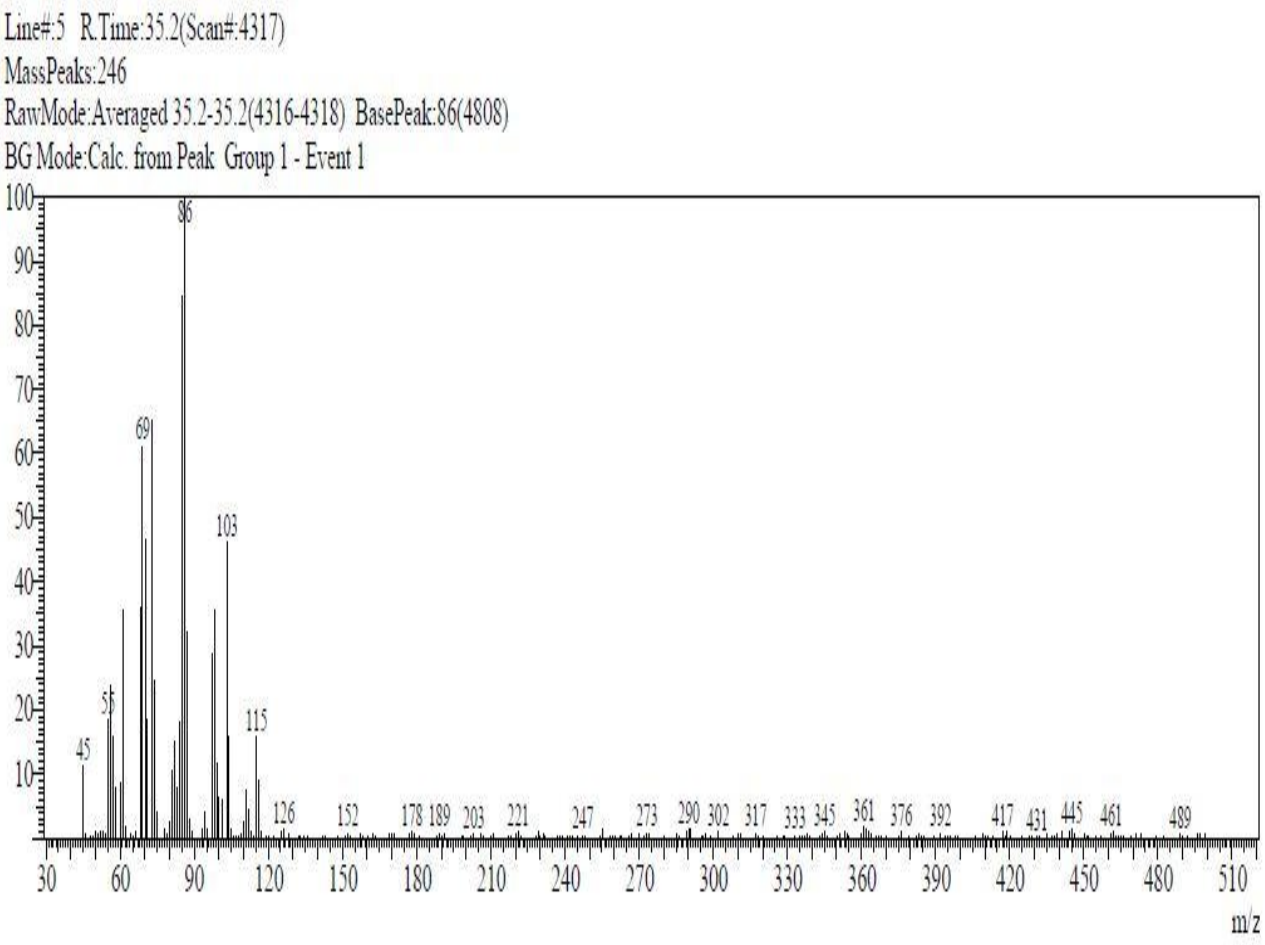
APPENDIX G: GC-MS Spectra for β-Glucan Isolated Using 1 N Concentration of H_3PO_4 and NaOH



GC profile

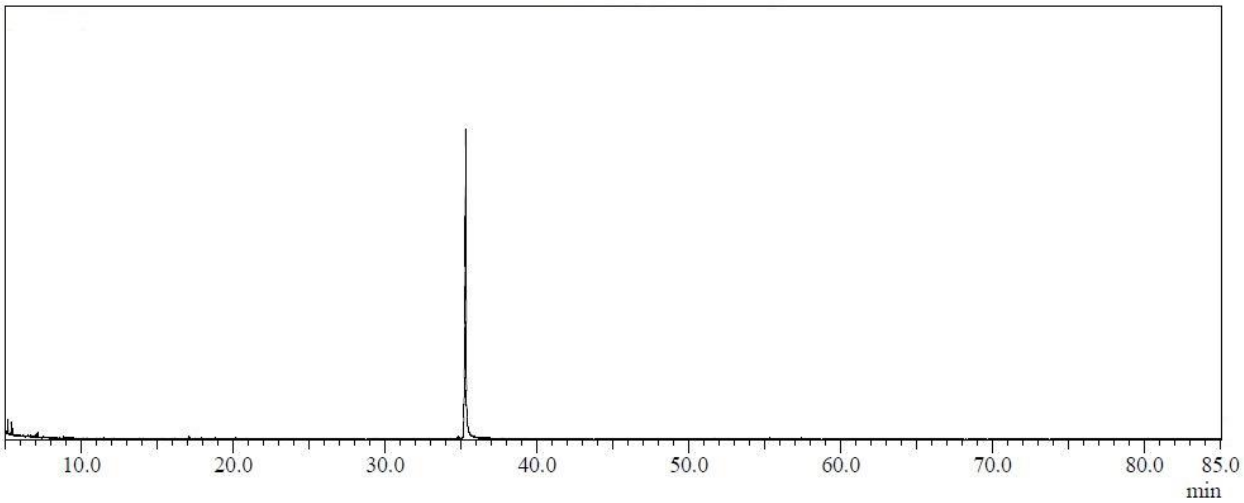


MS for Mannitol hexaacetate

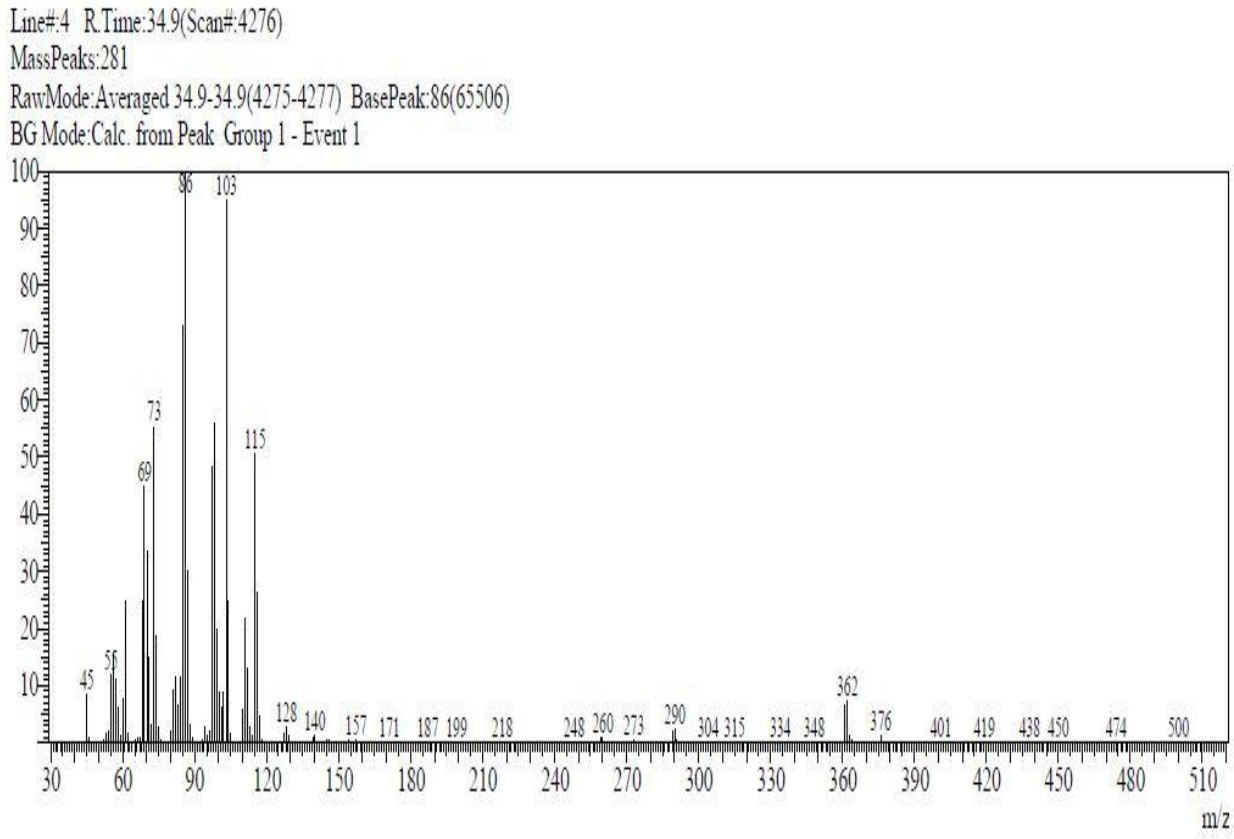


MS for Glucitol hexaacetate

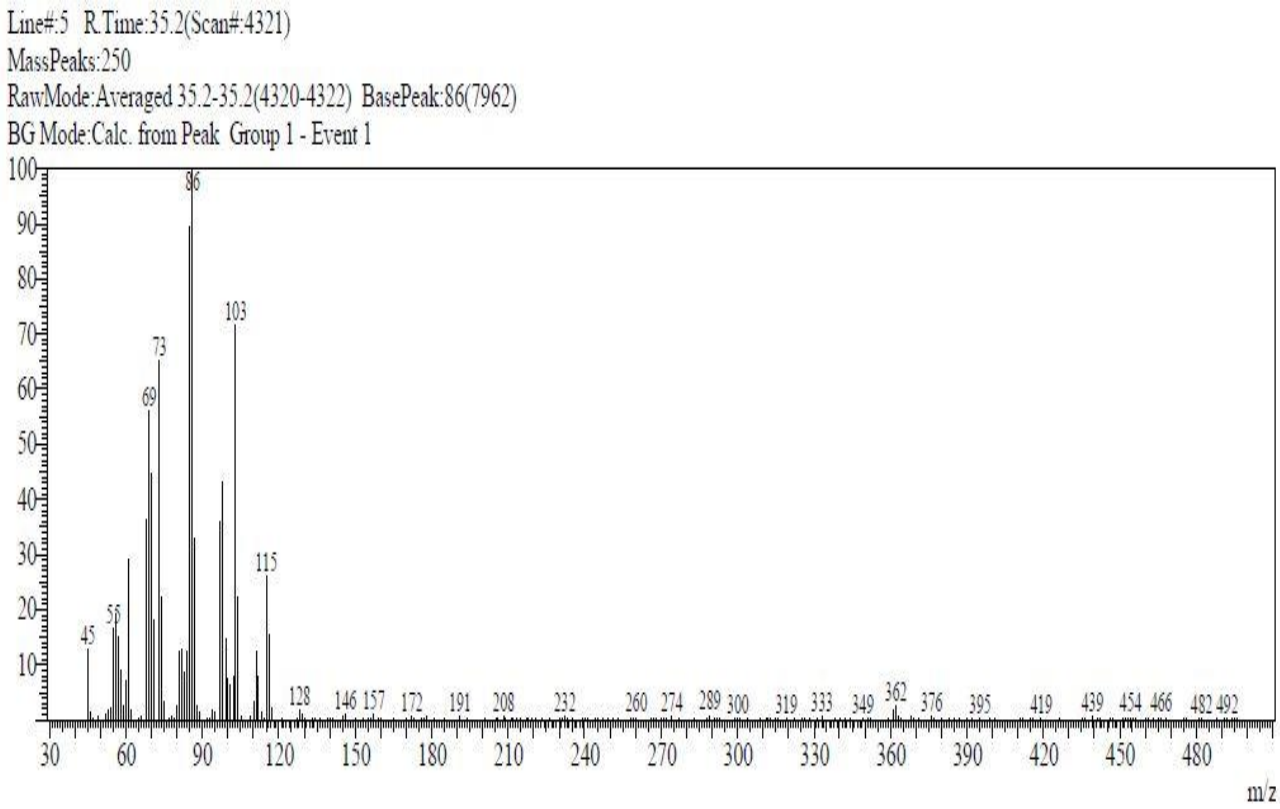
APPENDIX H: GC-MS Spectra for β-Glucan Isolated Using 0.1 N Concentrations of H_3PO_4 and NaOH



GC profile

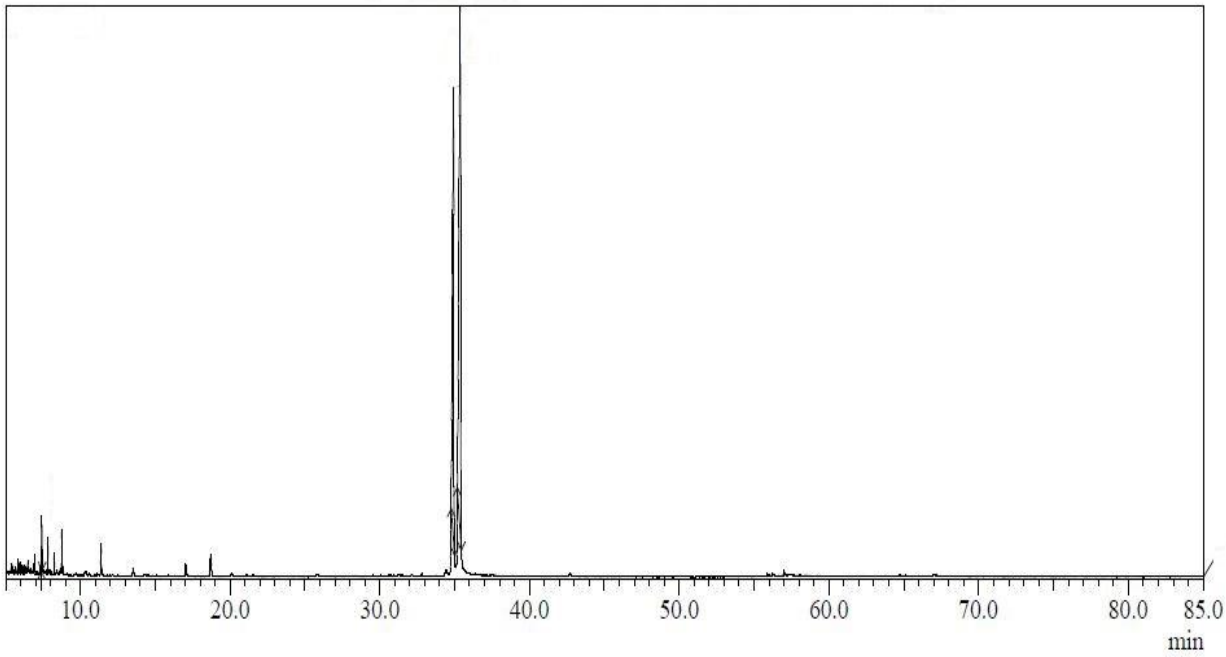


MS for Mannitol hexaacetate

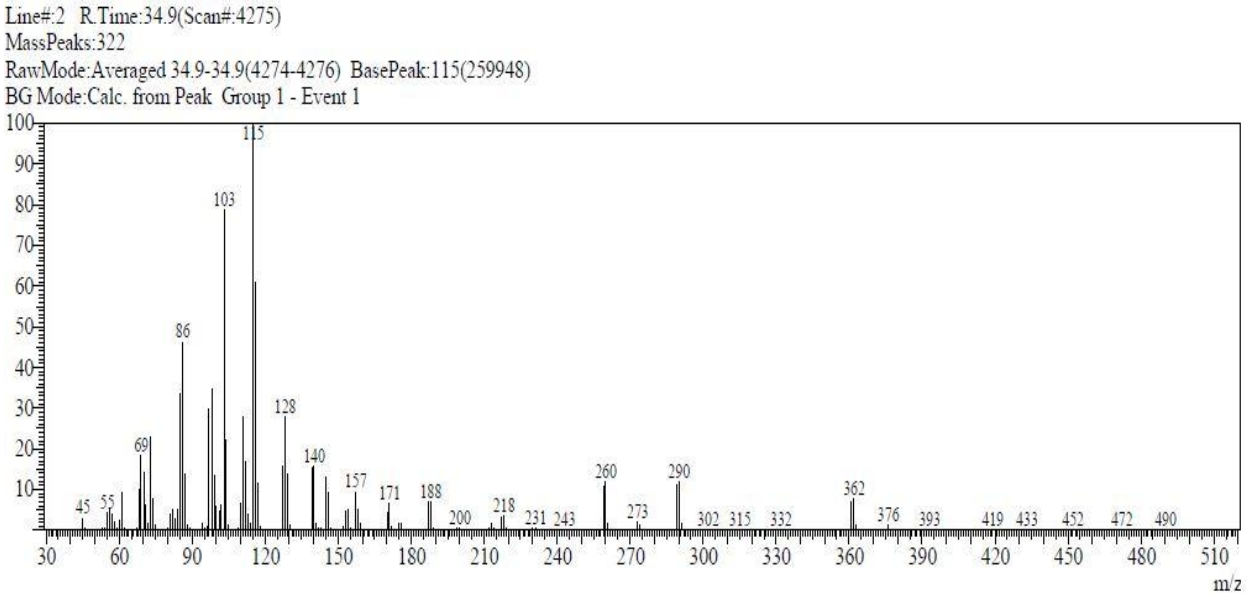


MS for Glucitol hexaacetate

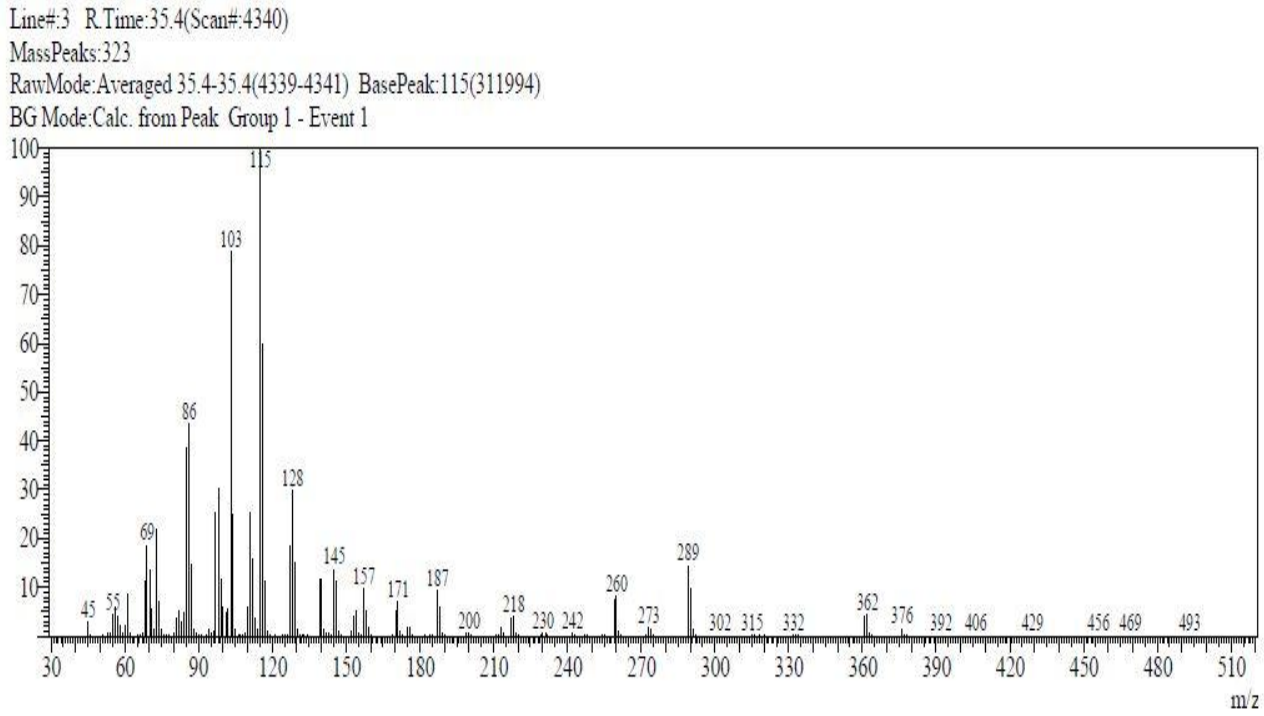
APPENDIX I: GC-MS Spectra for β-Glucan Isolated Using 0.01 N Concentrations of H_3PO_4 and NaOH



GC profile

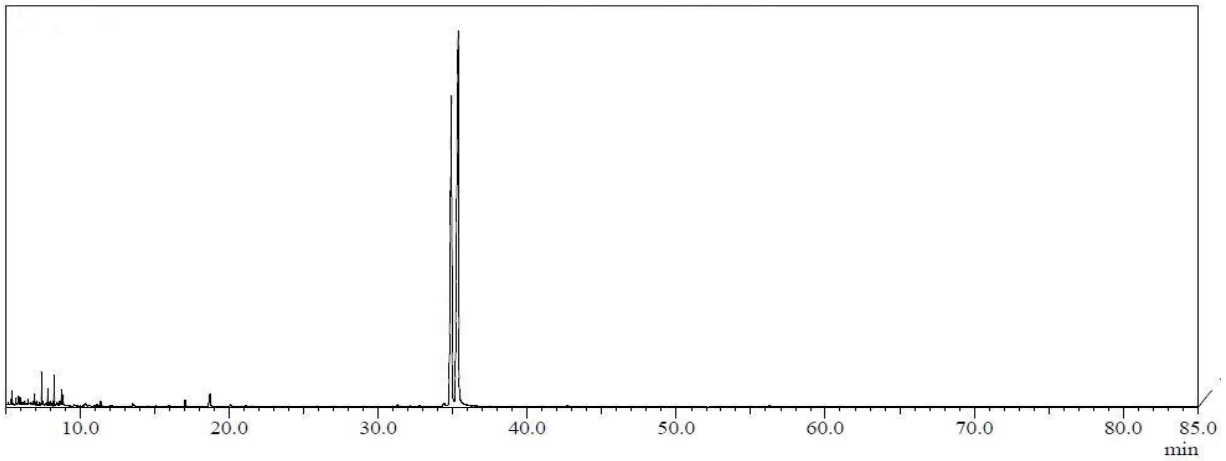


MS for Mannitol hexaacetate

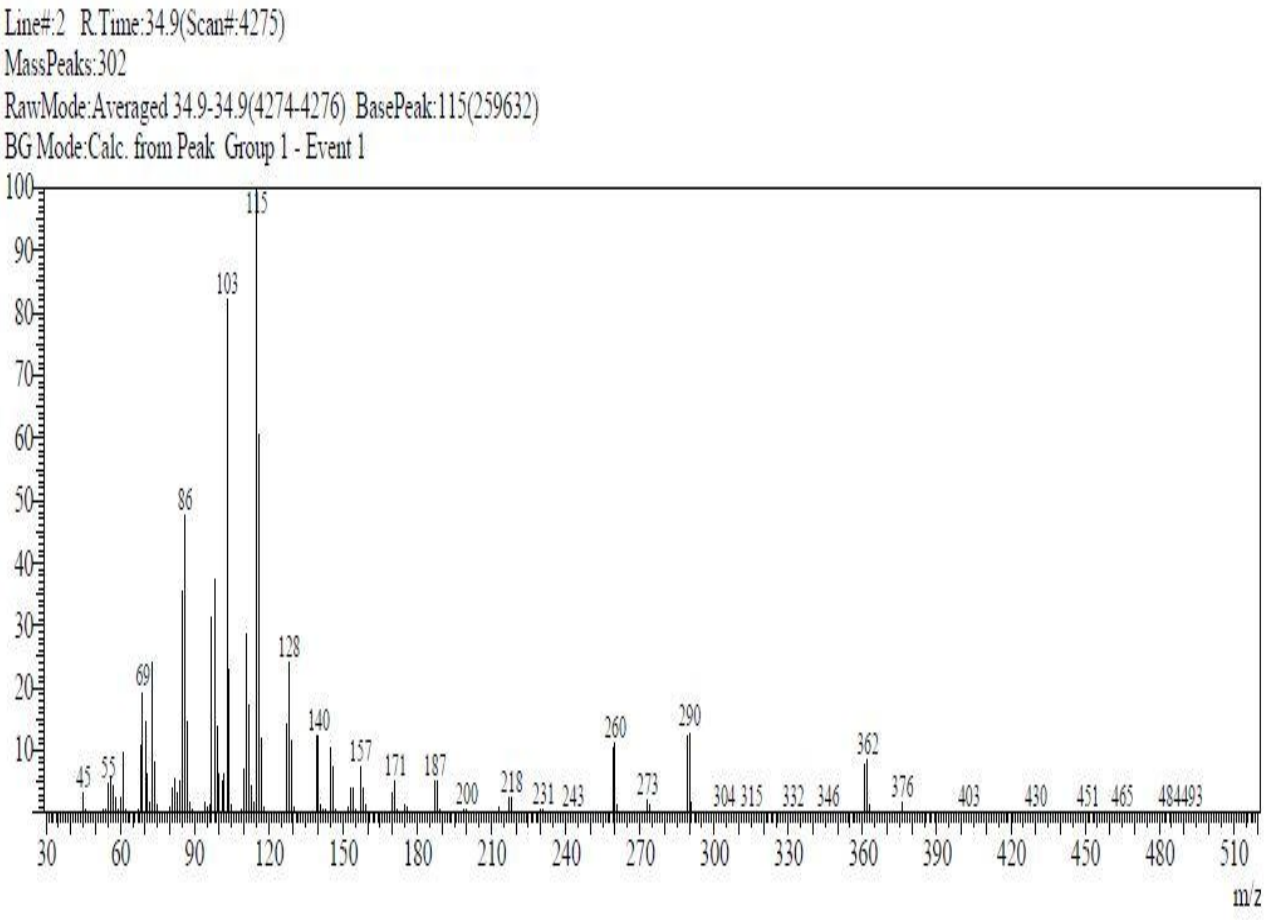


MS for Glucitol hexaacetate

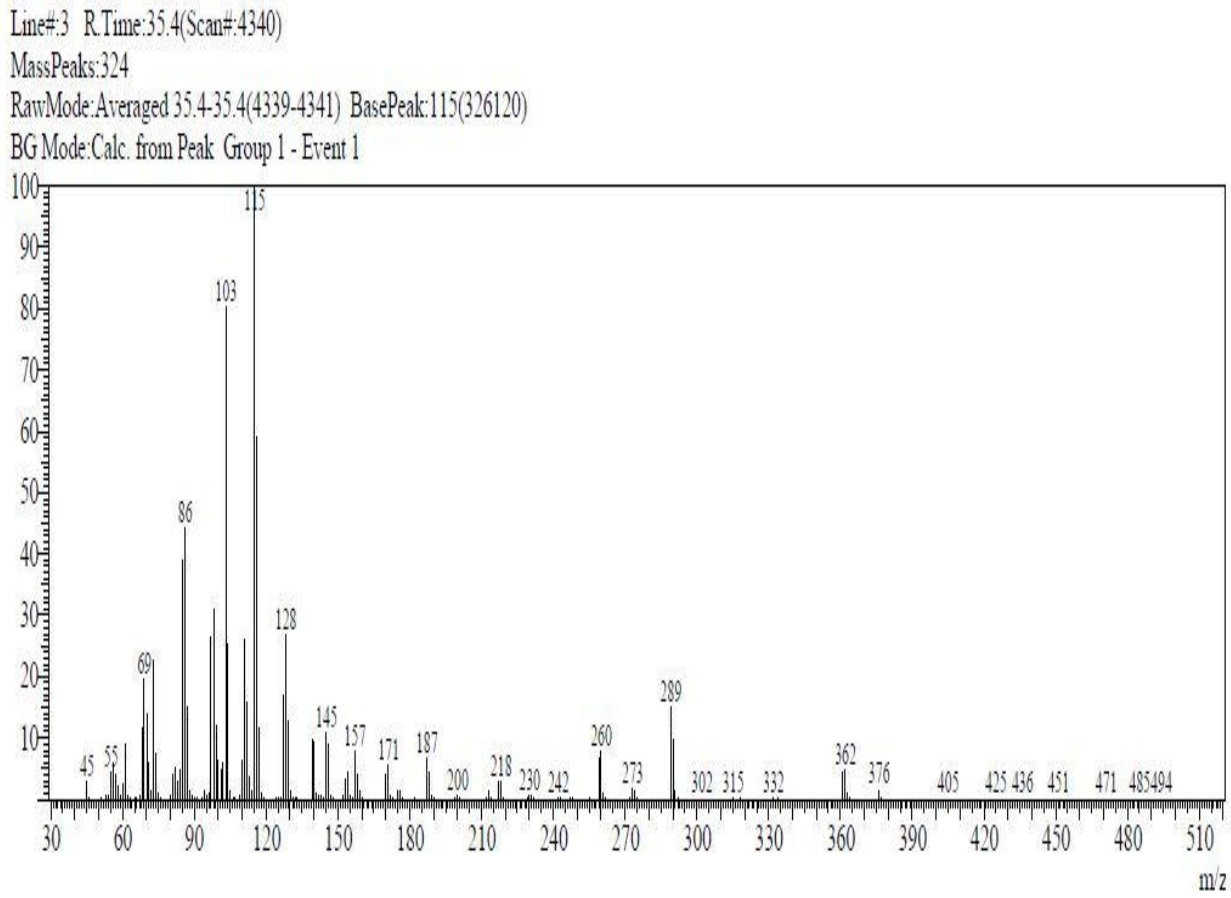
APPENDIX J: GC-MS Spectra for β-Glucan Isolated Using 0.001 N Concentrations of H_3PO_4 and NaOH



GC profile

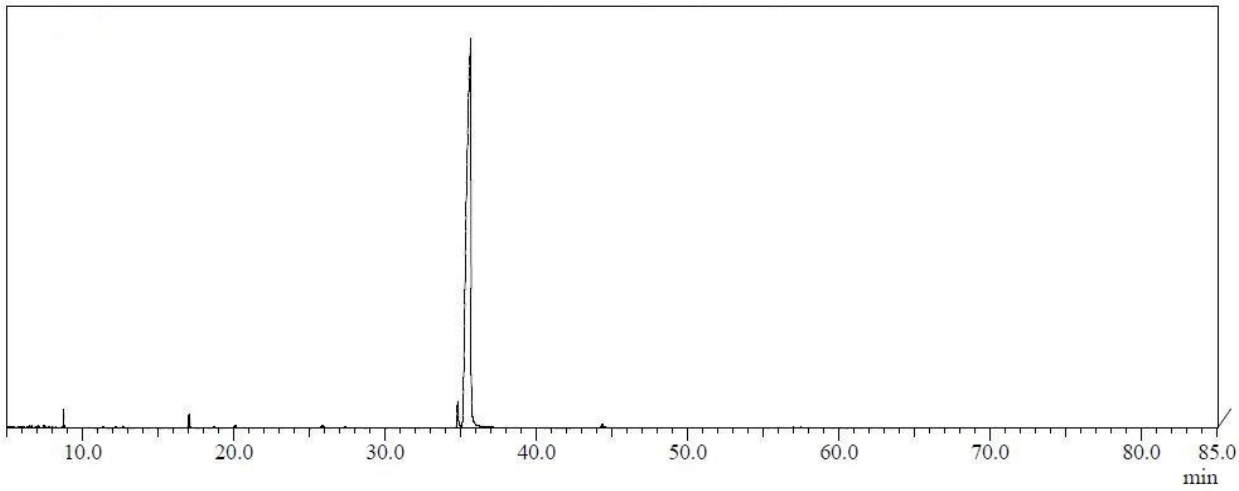


MS for Mannitol hexaacetate

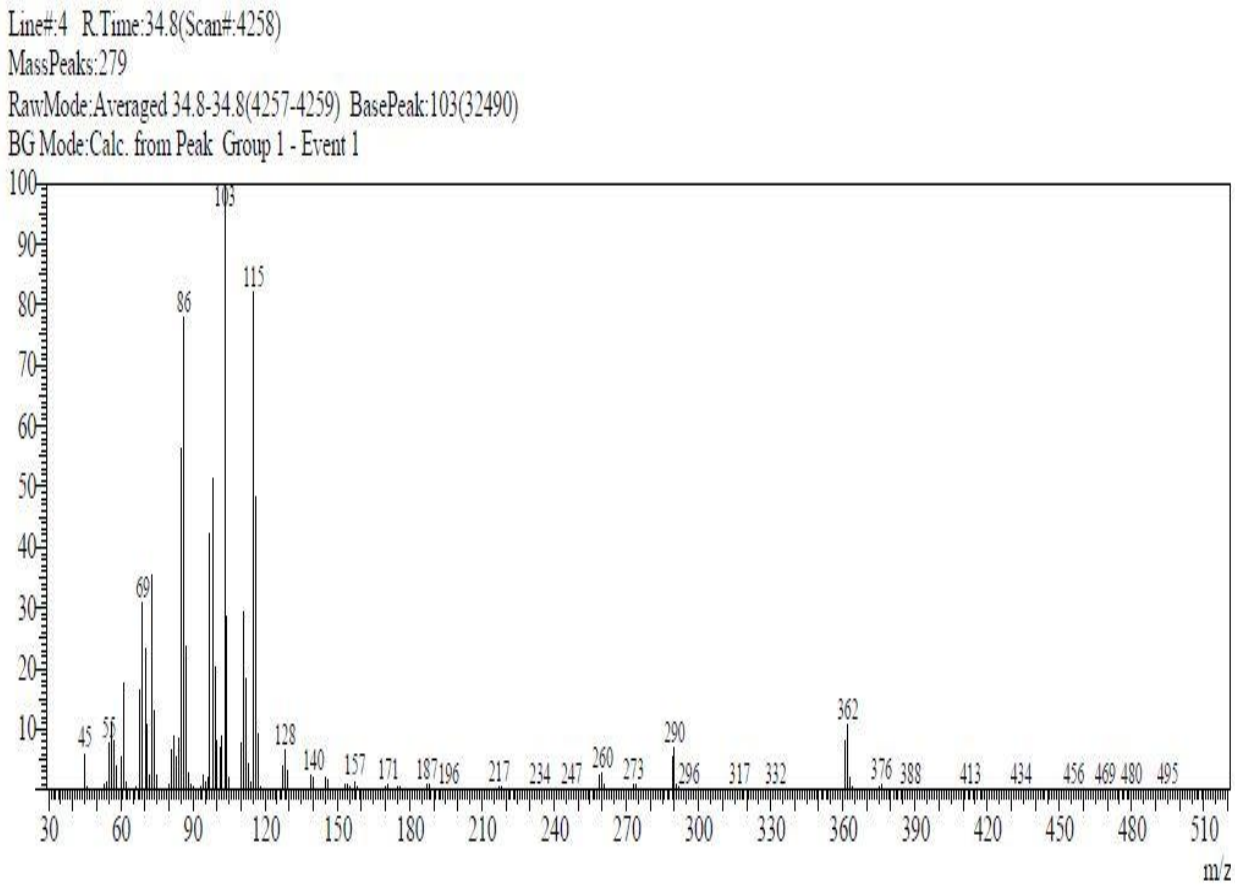


MS for Glucitol hexaacetate

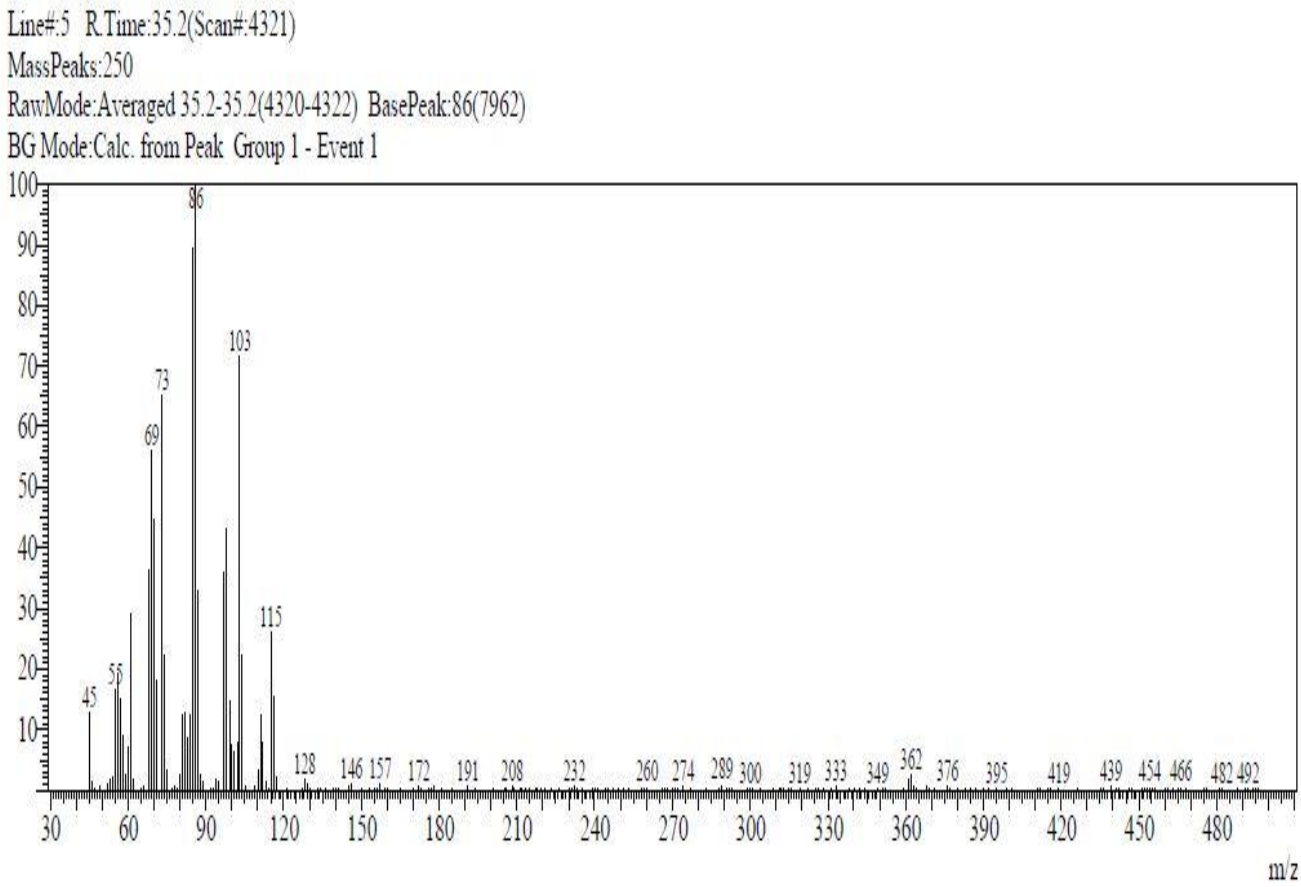
APPENDIX K: GC-MS Spectra for β-Glucan Isolated Using 0.01 N H_3PO_4 and 0.1 N NaOH



GC profile

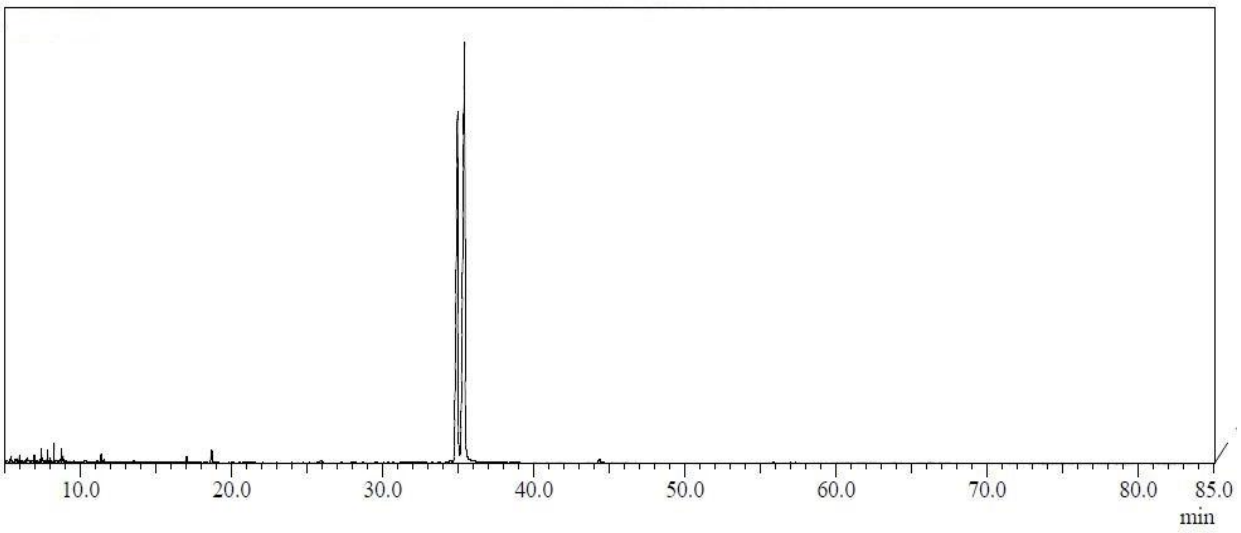


MS for Mannitol hexaacetate

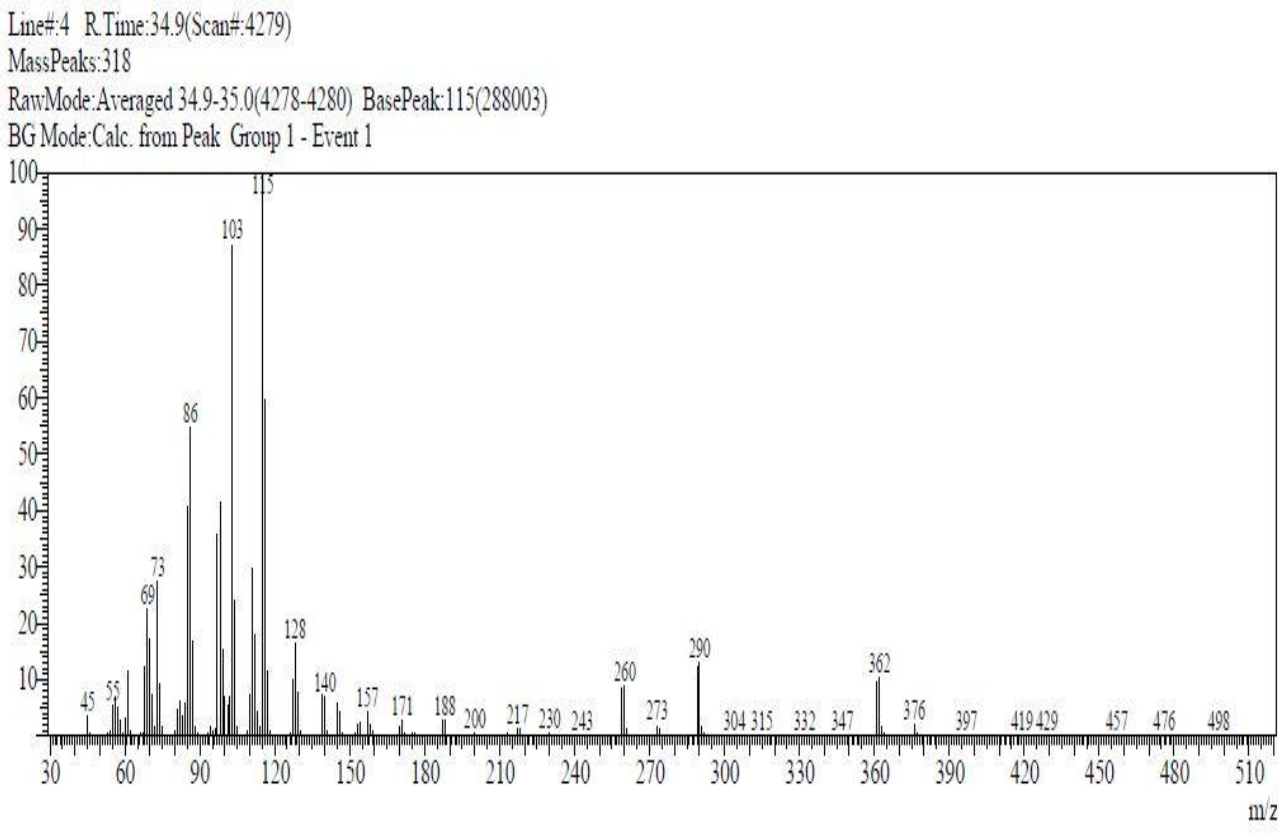


MS for Glucitol hexaacetate

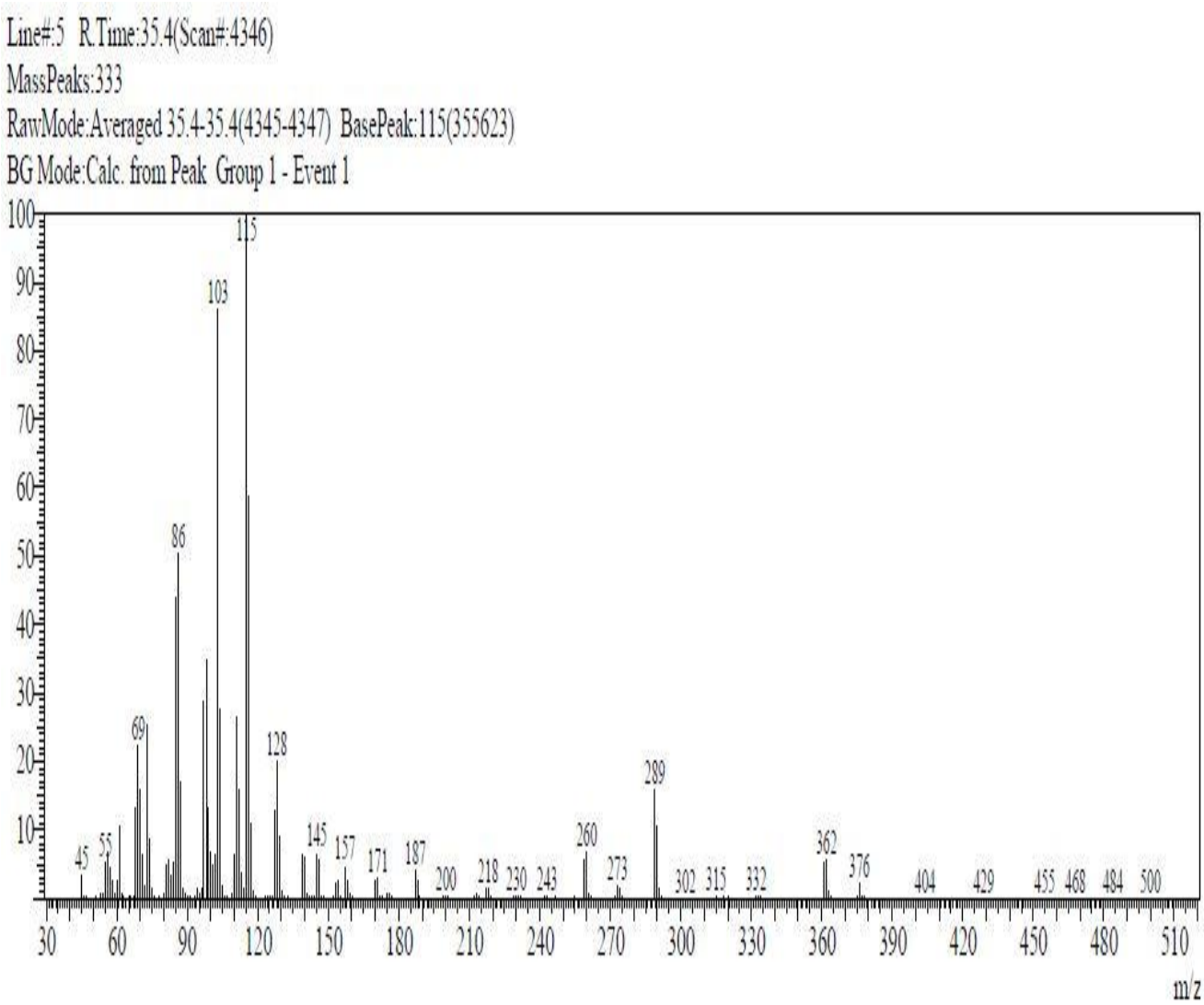
APPENDIX L: GC-MS Spectra for β-Glucan Isolated Using 0.1 N H_3PO_4 and 0.01 N NaOH



GC profile

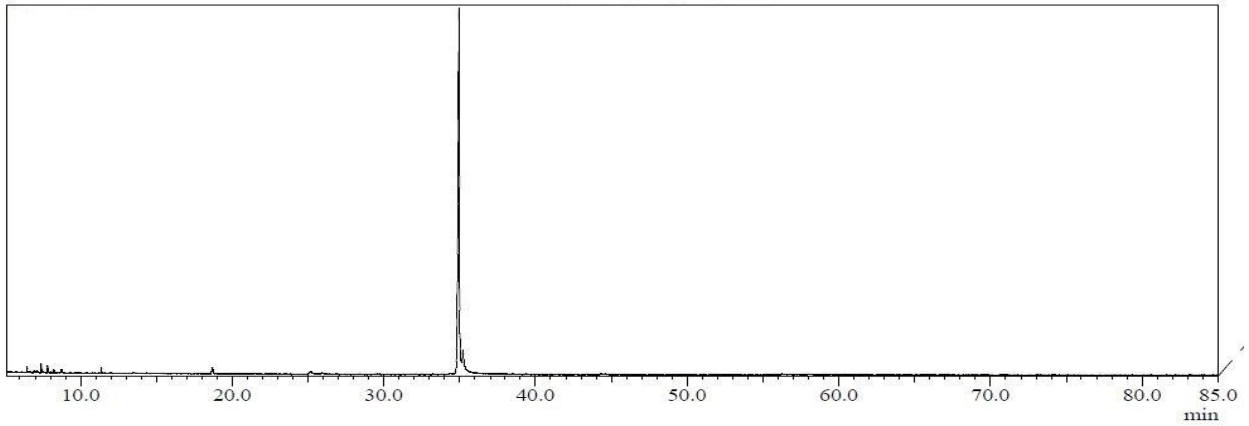


MS for Mannitol hexaacetate

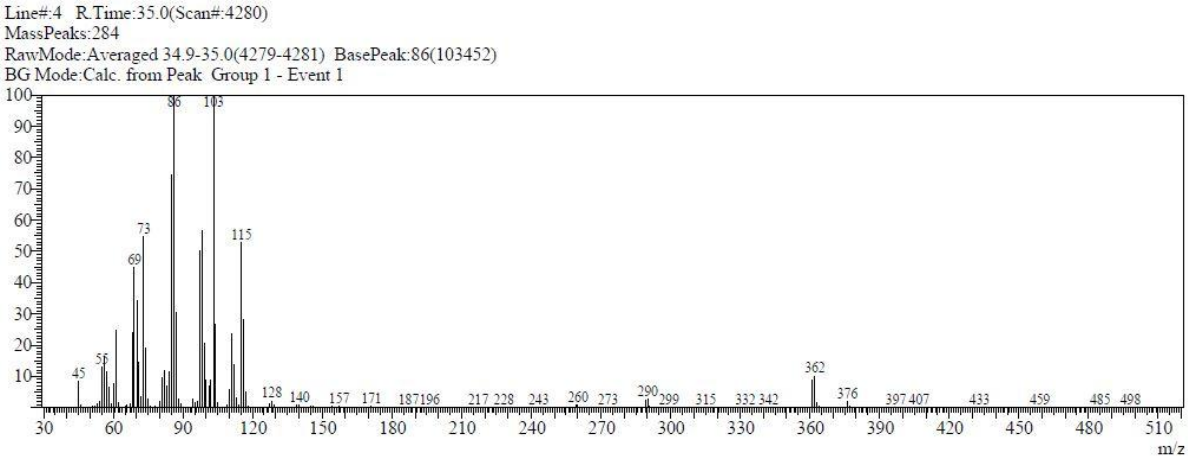


MS for Glucitol hexaacetate

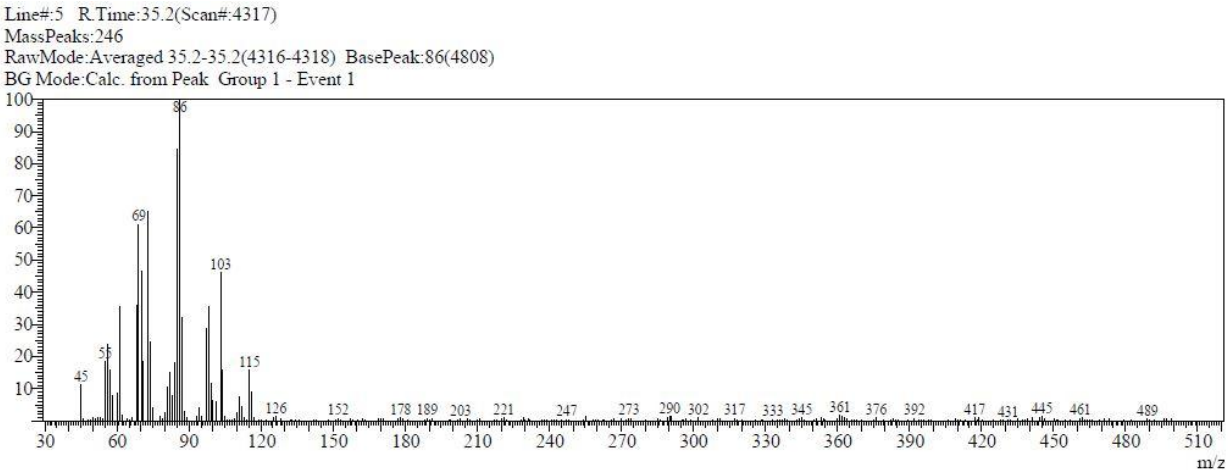
APPENDIX M: GC-MS Spectra for Yeast Mannan Isolated Using 50 mM NaOH



GC

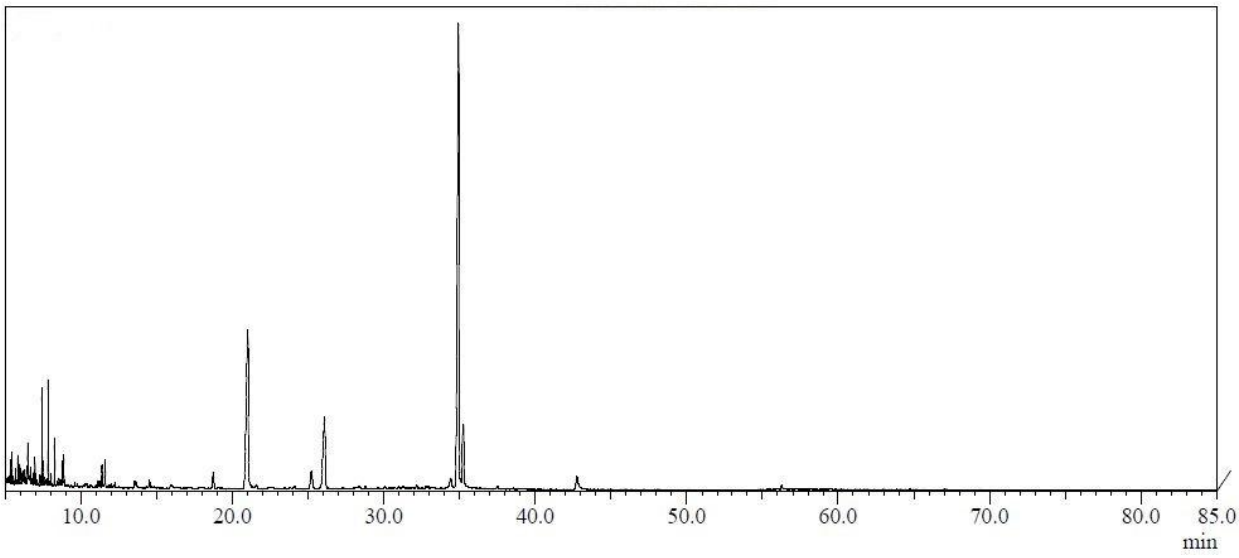


MS for Mannitol hexaacetate

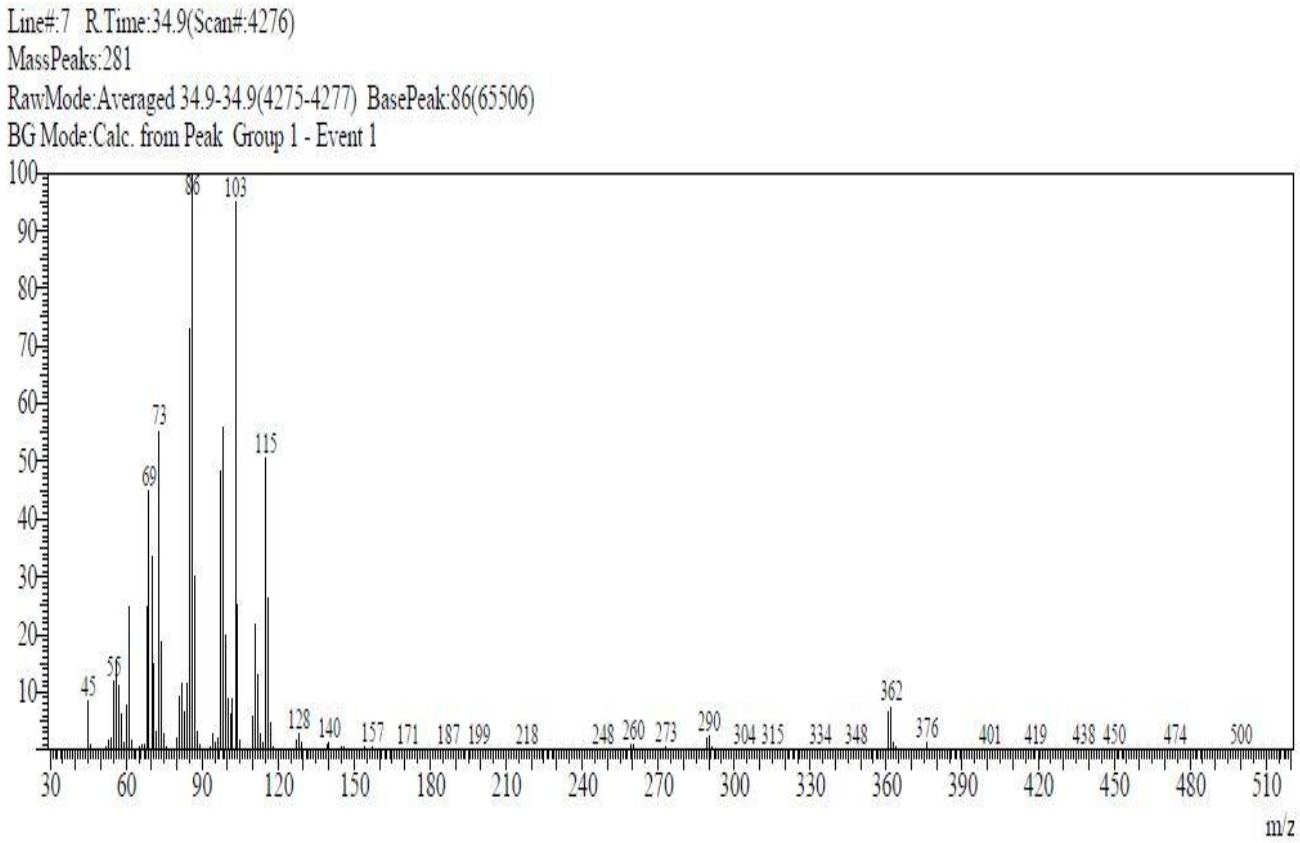


MS for Glucitol hexaacetate

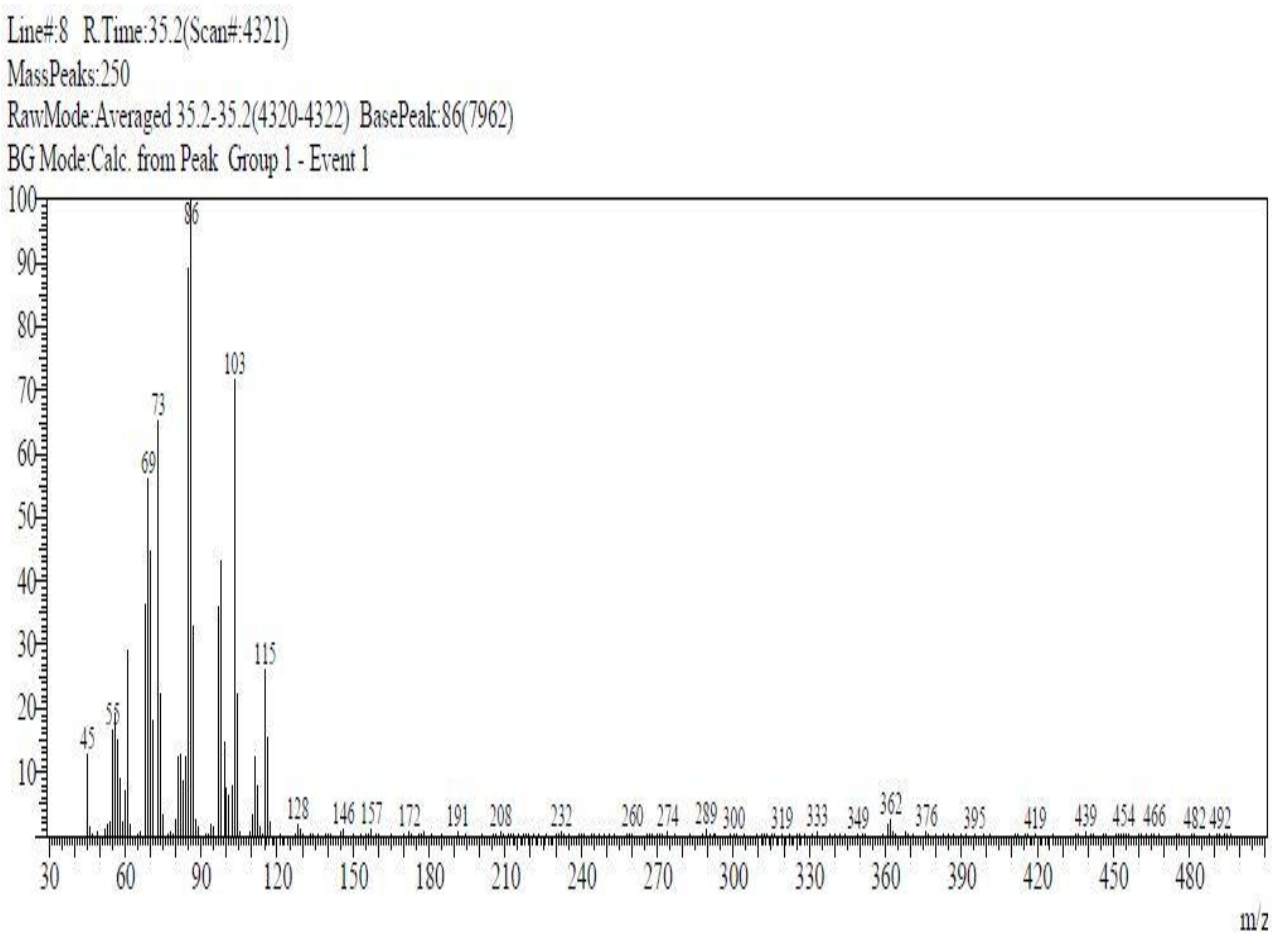
APPENDIX N: GC-MS Spectra for Hyphal Mannan Isolated Using 50 mM NaOH



GC profile

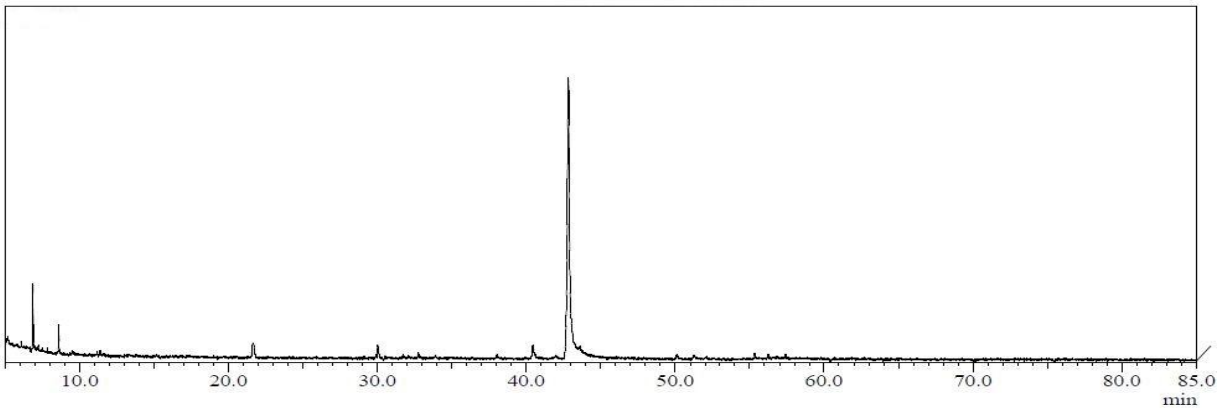


MS for mannitol hexaacetate

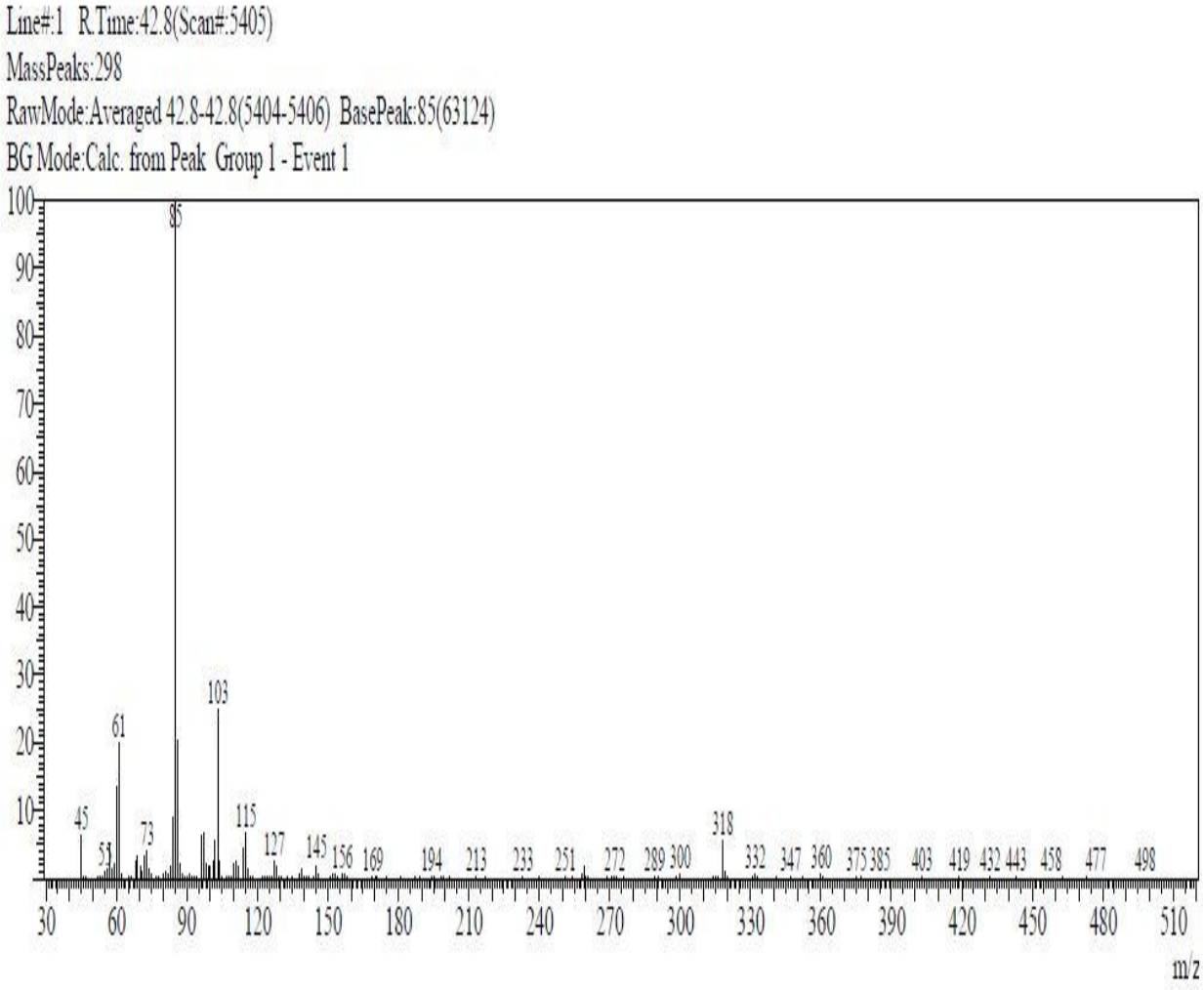


MS for glucitol hexaacetate

APPENDIX O: GC-MS Spectra for Standard Chitin

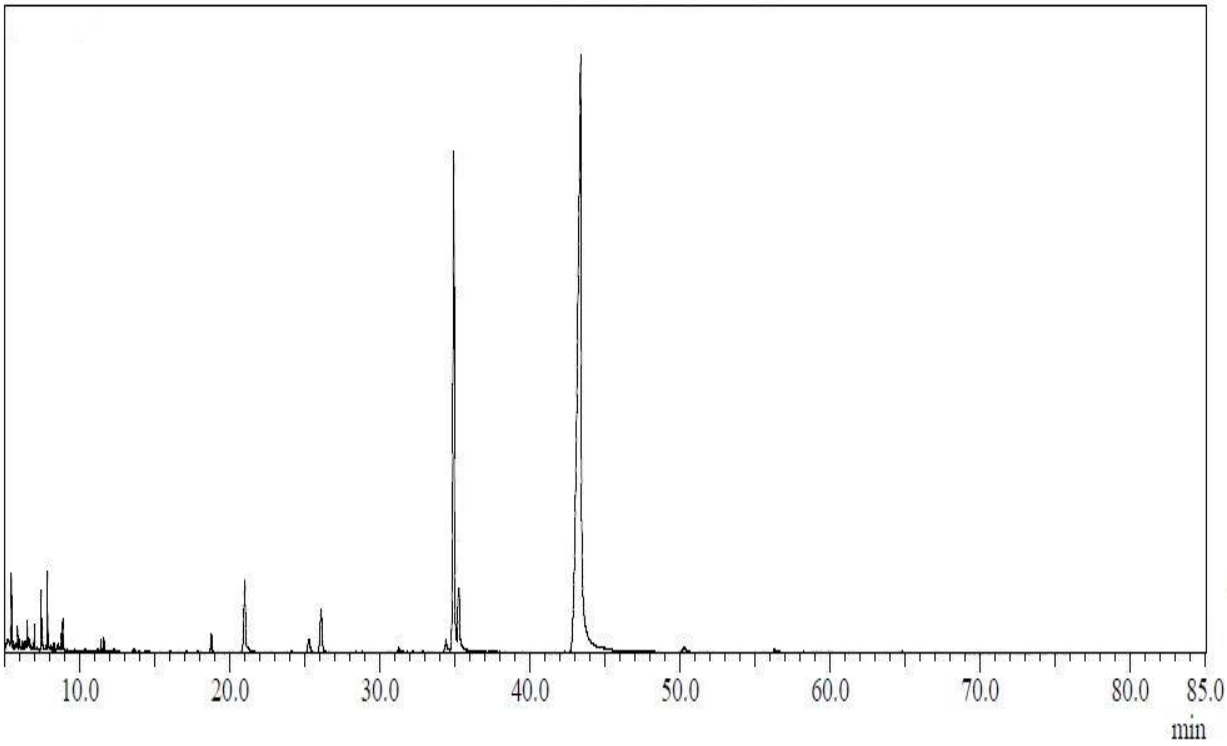


GC profile



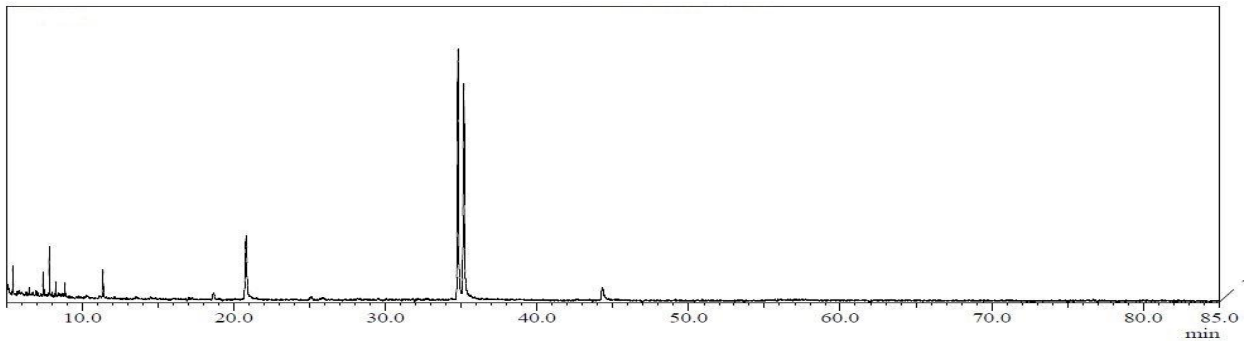
MS for standard chitin

APPENDIX P: Gas Chromatogram for Hyphal Mannan Isolated Using 50 mM NaOH + Standard Chitin

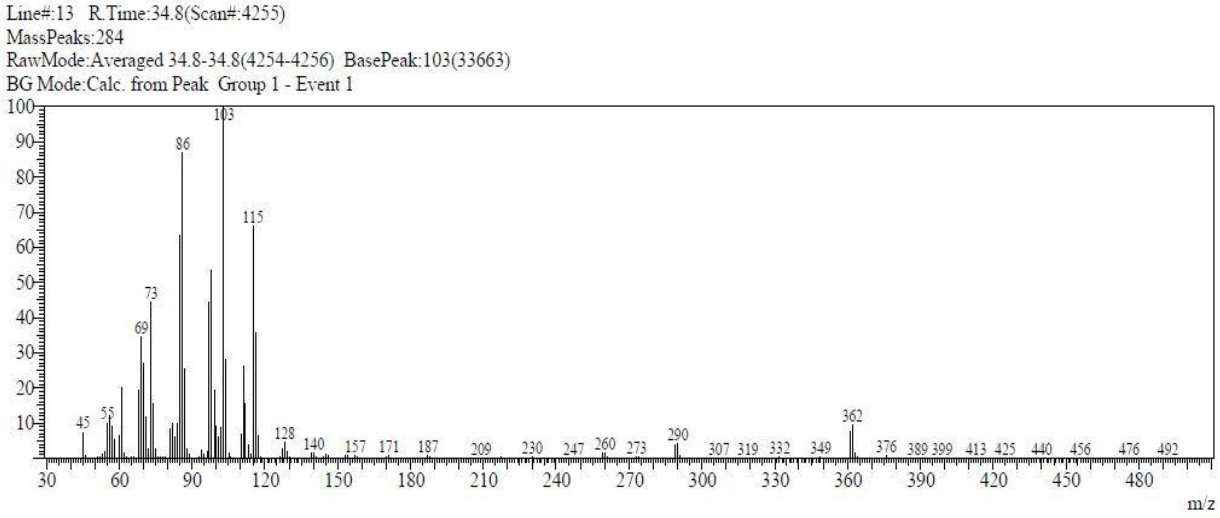


GC profile

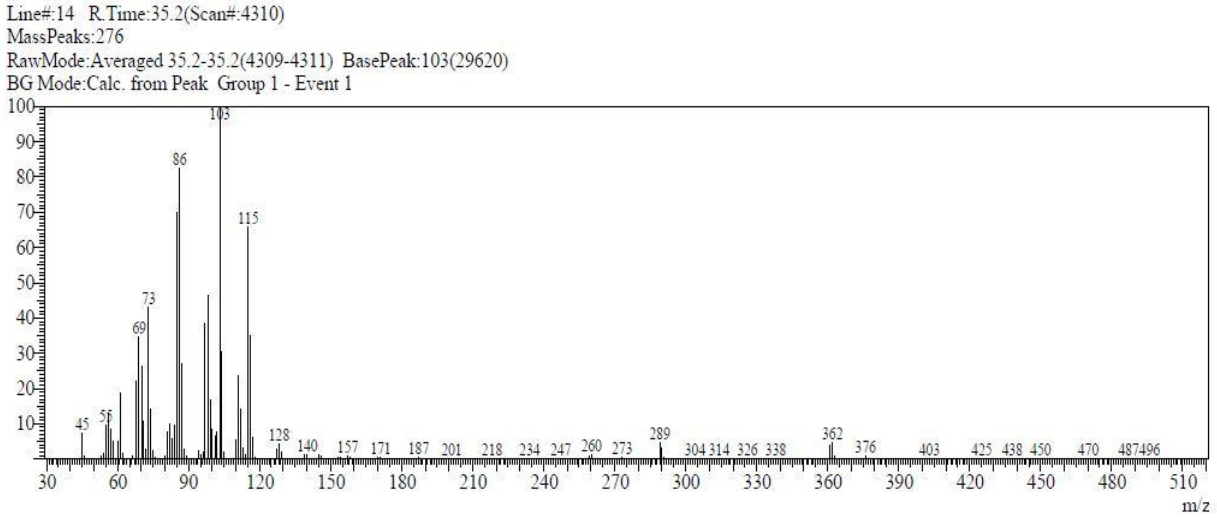
APPENDIX Q: GC-MS Spectra for Yeast Mannan Isolated Using 50 mM H_3PO_4



GC profile

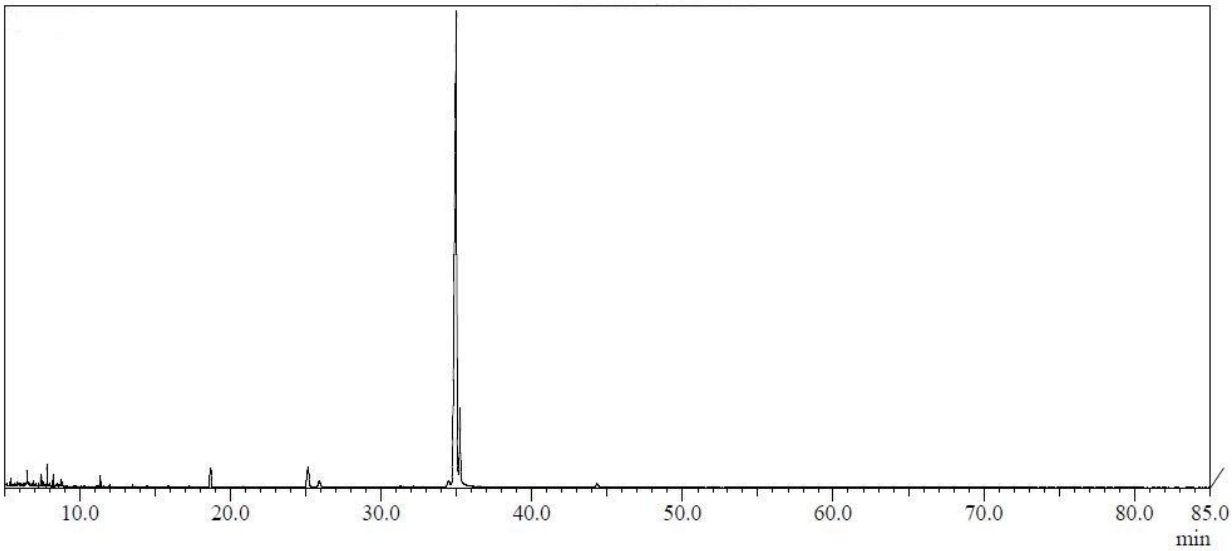


MS for Mannitol hexaacetate

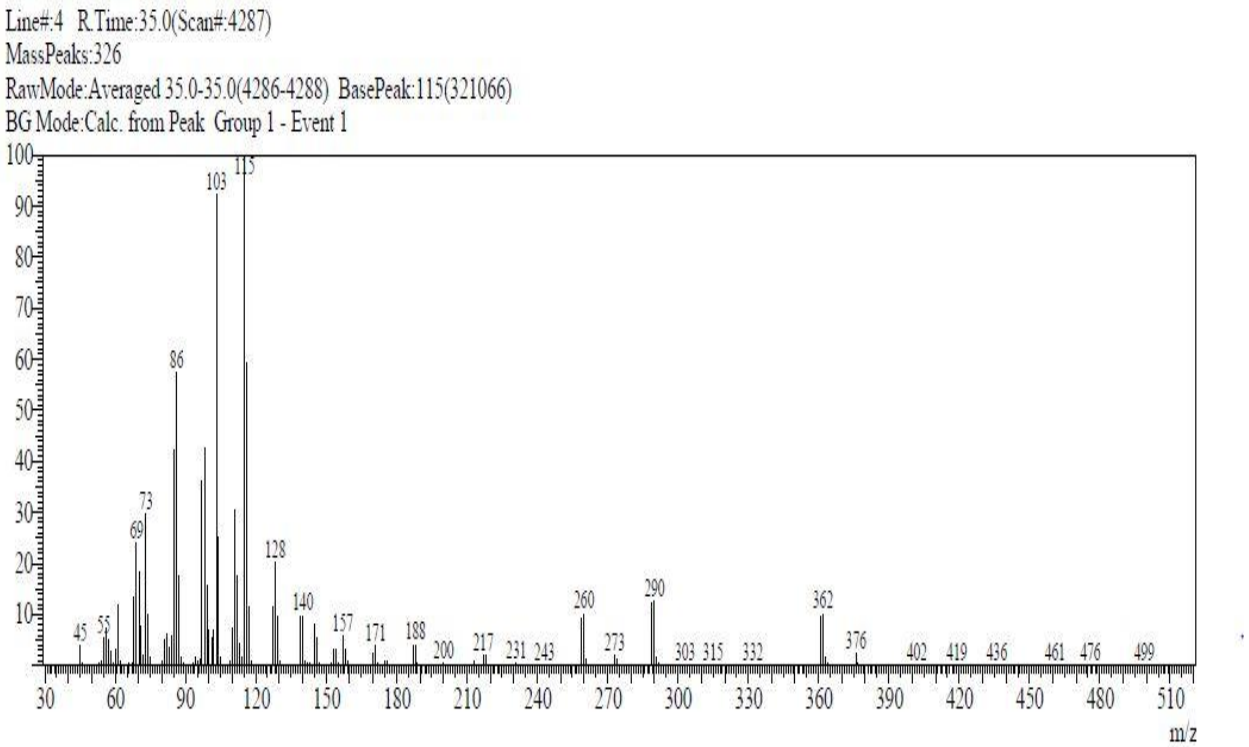


MS for Glucitol hexaacetate

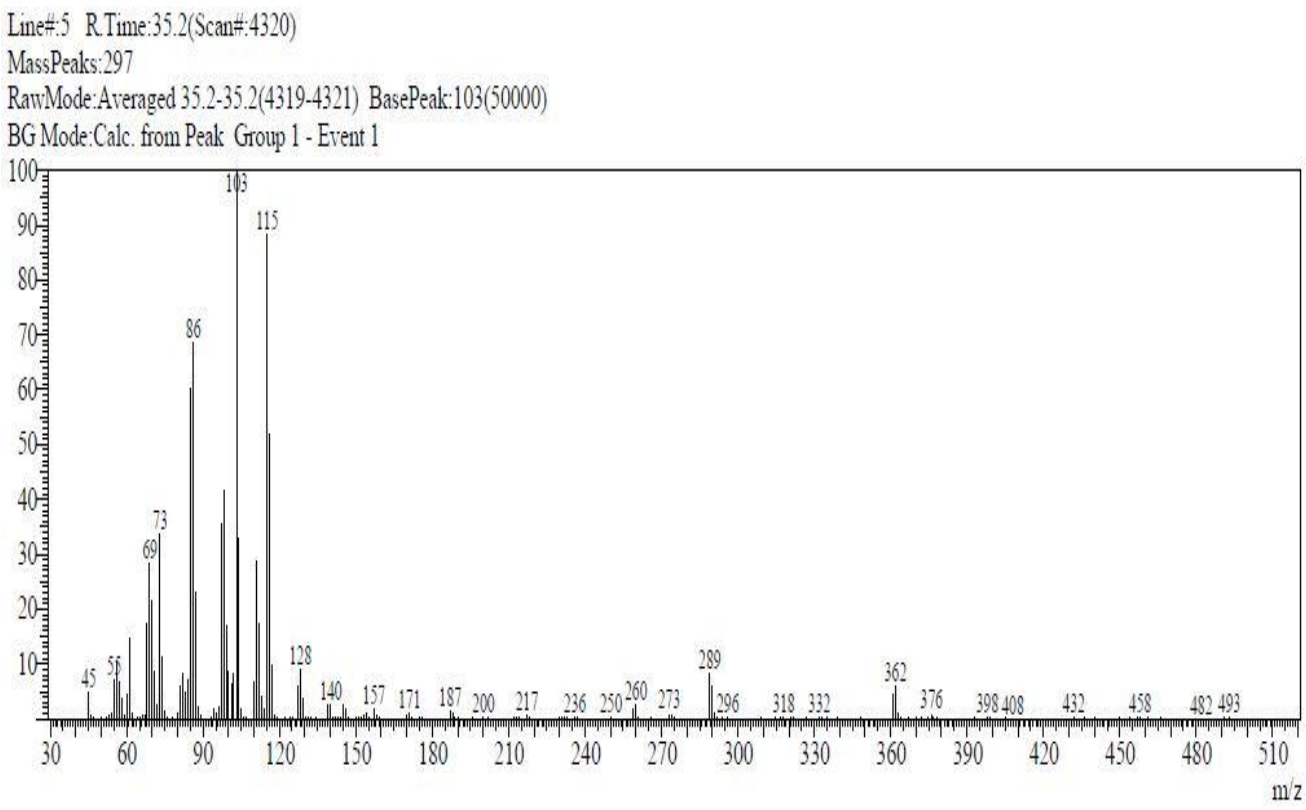
APPENDIX R: GC-MS Spectra for Hyphal Mannan Isolated Using 50 mM H_3PO_4



GC profile

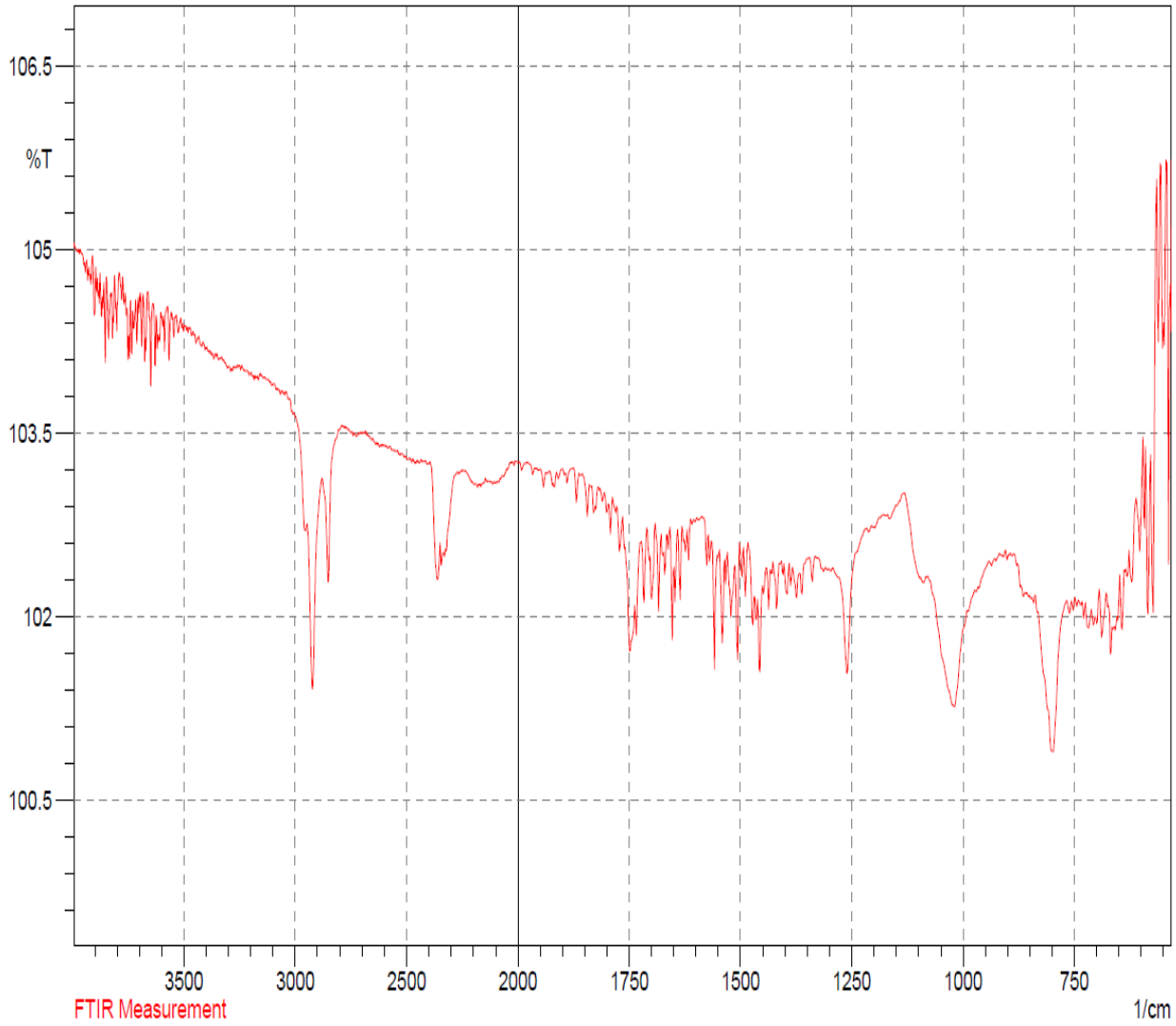


MS for Mannitol hexaacetate

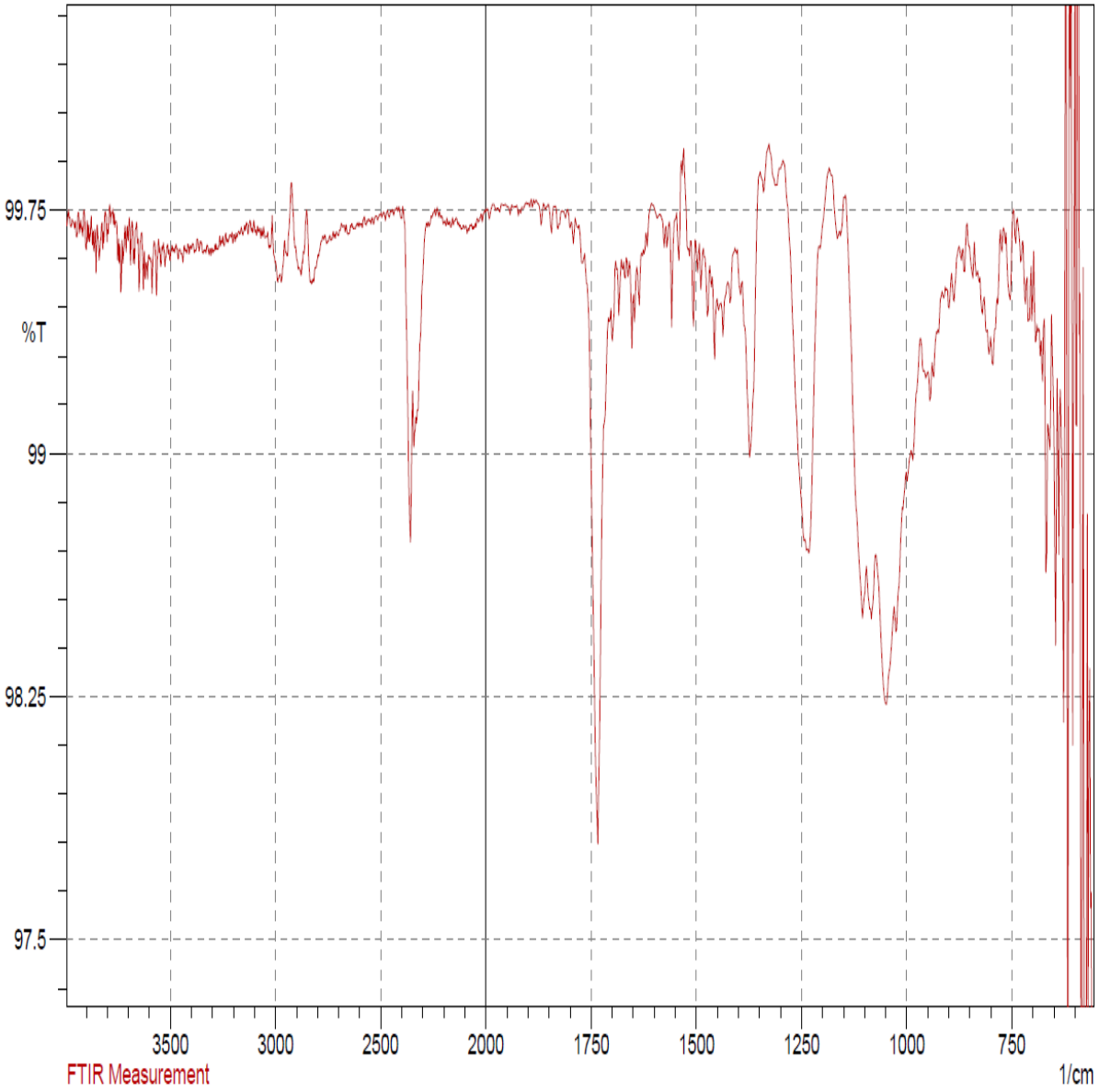


MS for Glucitol hexaacetate

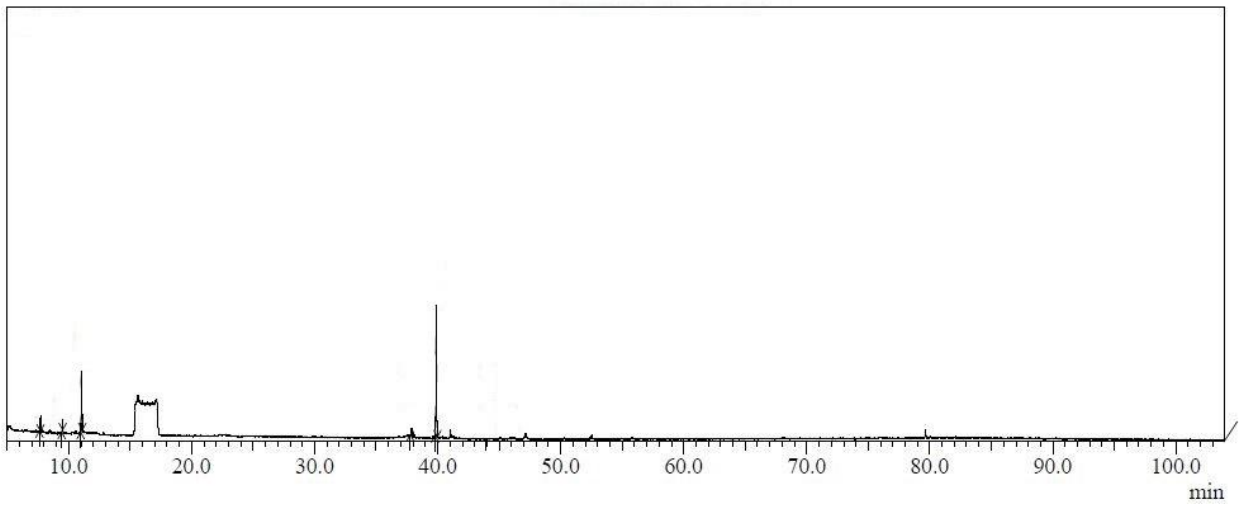
APPENDIX S: FTIR Spectrum of Partially Methylated β-Glucan (Classical method)



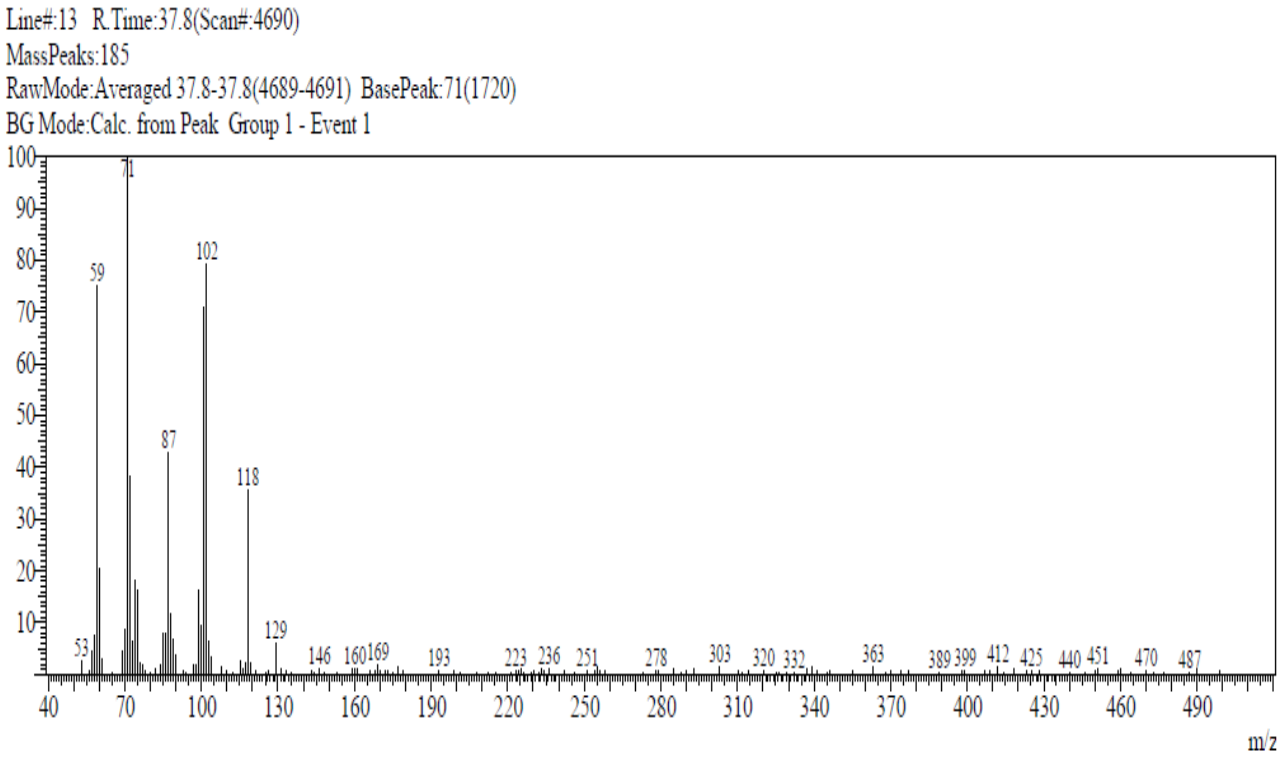
APPENDIX T: FTIR Spectrum of Partially Methylated β-Glucan Isolated Using Optimized Method (1X 0.1 N H_3PO_4, NaOH and ethanol extraction)



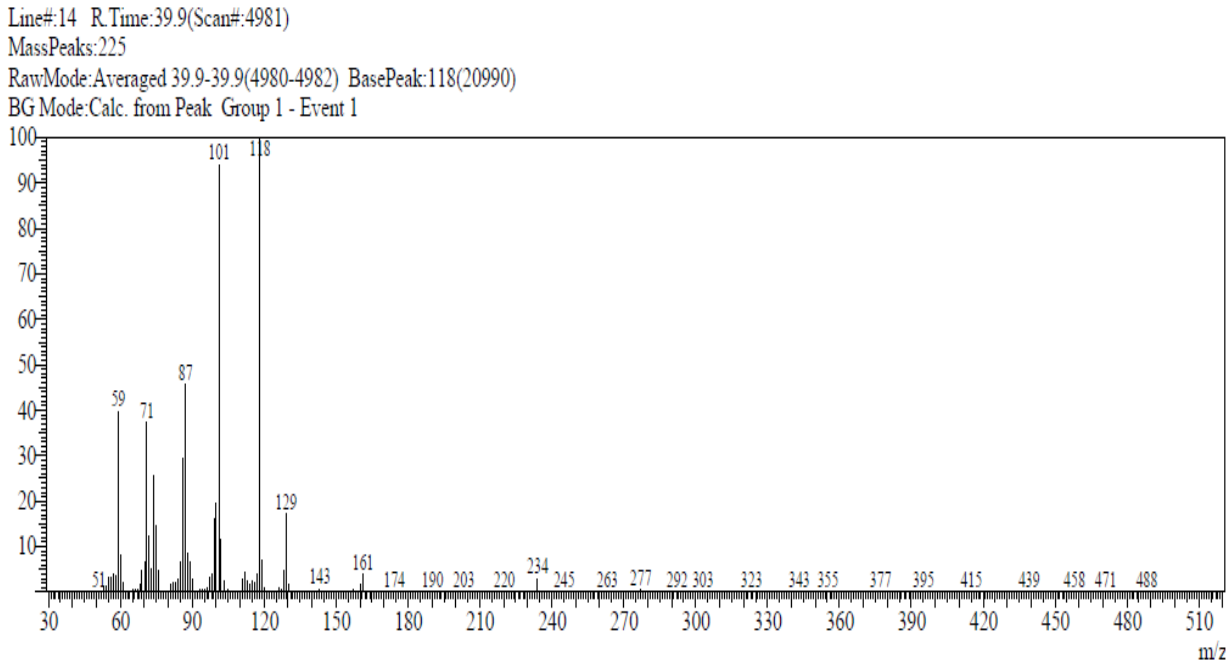
APPENDIX U: GC-MS Spectra for Linkage Analysis of β-Glucan Isolated Using Classical Method



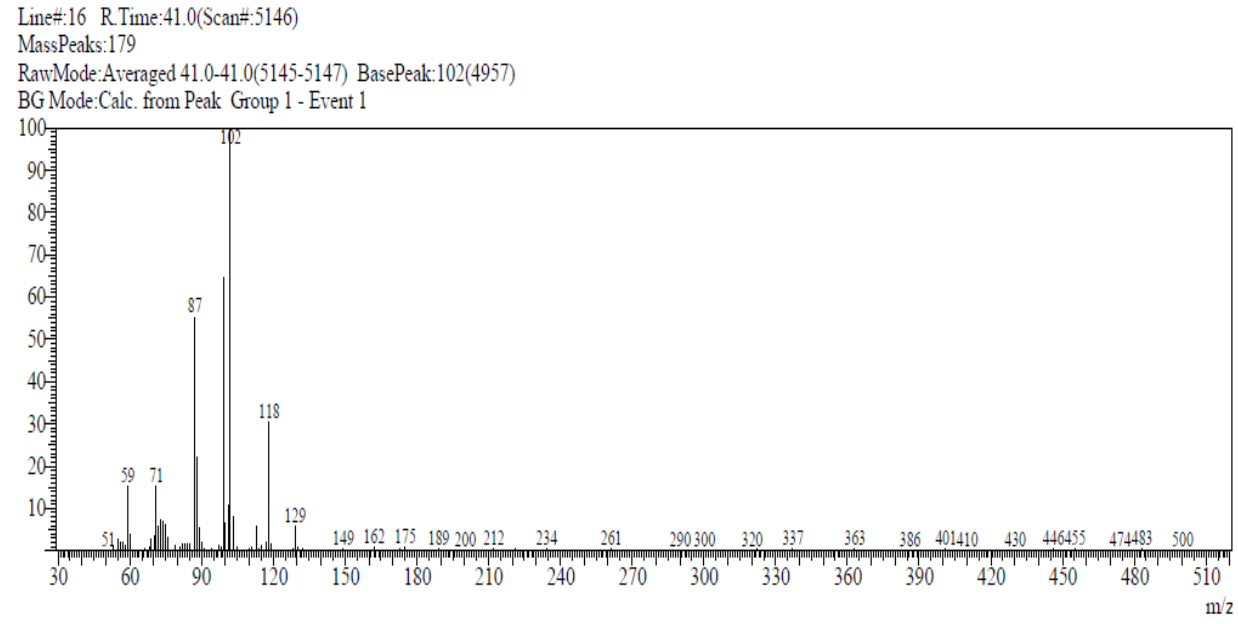
GC profile



MS for 1-linked-β-glucan

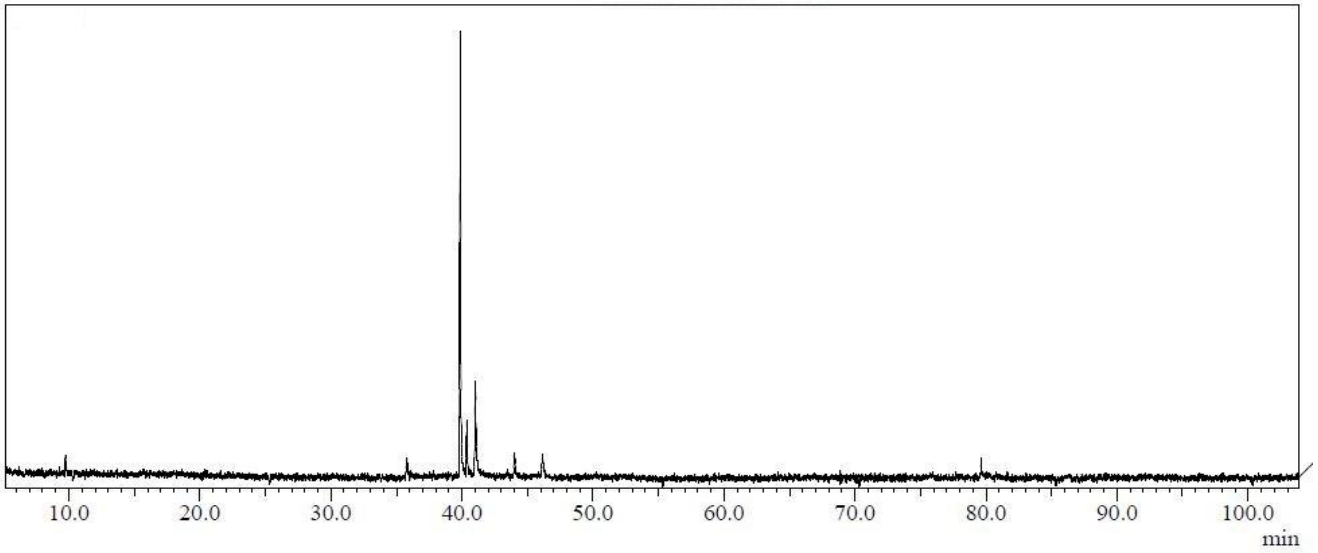


MS for 1,3-linked-β-glucan

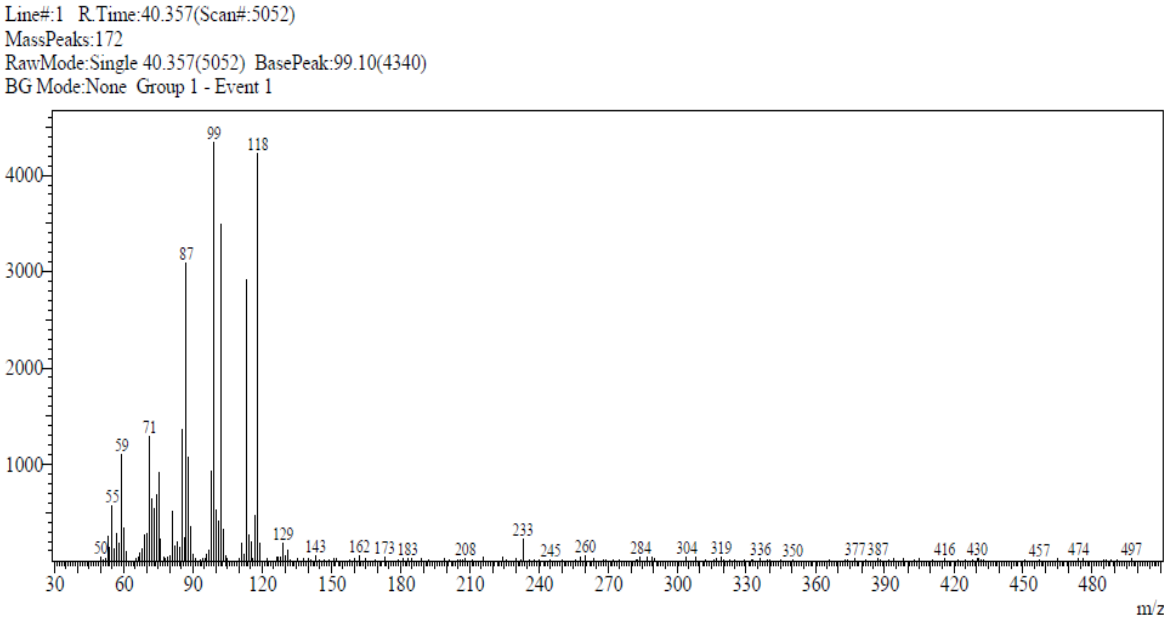


MS for 1, 6-linked-β-glucan

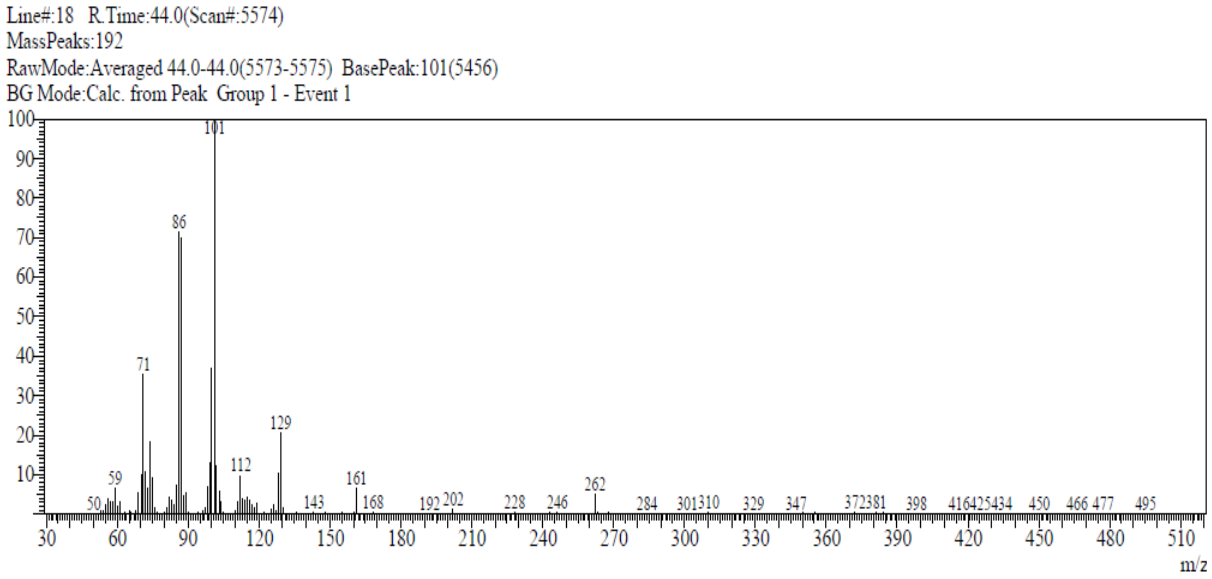
APPENDIX V: GC-MS Spectra for Linkage Analysis of β-Glucan Isolated Using Optimized Method



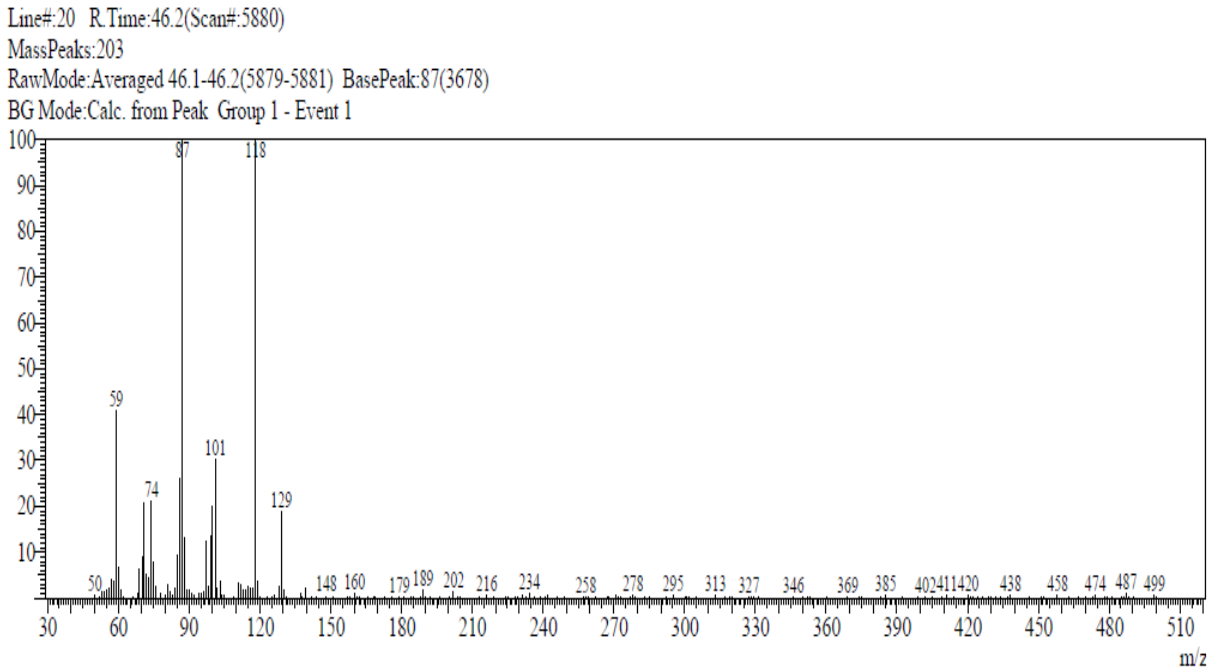
GC profile



MS for 1, 4-linked-β-glucan



MS for 1, 2, 3-linked-β-glucan



MS for 1, 3, 6-linked-β-glucan

VITA

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