



An exploration of the utility of TOF-SIMS as a new method for studying the cell surface of *Candida albicans*
by Hong Shi

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Chemical Engineering
Montana State University
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Abstract:

Biomedical implants are becoming increasingly used in the medical field. The most common cause of failure for medical devices is infection, and the pathogenic yeast *Candida albicans* is the third leading cause of these infections. Adhesion is the first step in establishing an infection, the initial adhesion of microorganisms to synthetic polymer surfaces involves physicochemical interactions between molecules present at the polymer surface and those present on the cell surface. These interactions are not well defined, and insight into this area could lead to better material construction to effectively control the infection.

The objective of this thesis is to study the initial adhesion event of *C. albicans* grown in glucose-based medium and galactose-based medium to Fluorinated Ethylene Propylene (FEP) and use Secondary Ion Mass Spectrometry (SIMS) to study the surface structural differences between these two cell surfaces with an attempt to correlate surface functionalities to the adhesion results.

Adhesion experiments were conducted with a laminar flow cell. Yeast cell adhesion was found to be significantly reduced as shear rate at the time of attachment was increased in the flow cell. Glucose-grown cells were more adherent than galactose-grown cells at the same shear rate. Attachment of glucose-grown cells was more affected by shear rate than attachment of galactose-grown cells. Attached cells were tenaciously bound to the surface and unaffected by increased shear rates. This phenomenon was true for both carbohydrate sources.

A freeze-drier with ultimate vacuum less than 1.00×10^{-9} torr was constructed "in house" to freeze-dry cells for SIMS analysis. A filtration method was used to prepare a smooth layer of cells to be freeze-dried. Scanning Electron Microscopy showed that the freeze-dried cells appeared intact. High resolution SIMS spectra were obtained from these freeze-dried cell surfaces and Linear Discriminant Function Analysis combined with Principal Components Analysis were used to analyze the SIMS spectra. The results showed that the surfaces of glucose-grown cells contained more hydrophobic amino acid residues relative to those of galactose-grown cells. These hydrophobic amino acid residues probably promoted the adherence of glucose-grown cells to FEP surface. The results obtained in this study suggest that hydrophobic interactions are important in the initial attachment of *C. albicans* to FEP surface. The SIMS spectra presented in this study were the first report of the freeze-dried *C. albicans*.

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APPROVAL

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Biomedical implants are becoming increasingly used in the medical field. The most common cause of failure for medical devices is infection, and the pathogenic yeast *Candida albicans* is the third leading cause of these infections. Adhesion is the first step in establishing an infection, the initial adhesion of microorganisms to synthetic polymer surfaces involves physicochemical interactions between molecules present at the polymer surface and those present on the cell surface. These interactions are not well defined, and insight into this area could lead to better material construction to effectively control the infection.

The objective of this thesis is to study the initial adhesion event of *C. albicans* grown in glucose-based medium and galactose-based medium to Fluorinated Ethylene Propylene (FEP) and use Secondary Ion Mass Spectrometry (SIMS) to study the surface structural differences between these two cell surfaces with an attempt to correlate surface functionalities to the adhesion results.

Adhesion experiments were conducted with a laminar flow cell. Yeast cell adhesion was found to be significantly reduced as shear rate at the time of attachment was increased in the flow cell. Glucose-grown cells were more adherent than galactose-grown cells at the same shear rate. Attachment of glucose-grown cells was more affected by shear rate than attachment of galactose-grown cells. Attached cells were tenaciously bound to the surface and unaffected by increased shear rates. This phenomenon was true for both carbohydrate sources.

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CHAPTER 1

INTRODUCTION

In recent years, the use of medical implants such as catheters, pacemakers, prosthetic heart valves, and joint replacements has increased dramatically [1]. The global market for these devices is valued at \$86 billion per annum, with a growth of 7% per year [2]. Infections constitute one serious complication of the use of these biomaterials, especially with skin penetrating devices [3]. These devices are easily colonized by microorganisms which form a biofilm consisting of a mono- or multilayer of cells embedded within a matrix of extracellular polymeric material. Release of microorganisms from the biofilm may initiate an acute disseminated infection. Implant-associated infections are difficult to resolve, because biofilm microorganisms are resistant both to host defense mechanisms and antibiotic therapy [1]. Frequently, the implant must be removed. The results are both costly, inconvenient, and in some cases, life-threatening to the patient.

Although the majority of implant infections are caused by gram-positive bacteria, infections due to gram-negative bacteria and fungi tend to be more serious [4]. Fungal infections are most commonly caused by the pathogenic *Candida sp.*. More than 850,000 catheter-related infections occur annually in the U.S. [5], of which 22% are caused by *Candida sp.*, predominantly *Candida albicans* [6]. The attachment of *C. albicans* to various biomaterials and host tissues has been deemed an important step in the initiation of both superficial and deep-seated candidiasis [7].

Because adhesion is the first step in establishing infection, the ideal solution is to prevent the organisms from initially adhering to the surface. To accomplish this, a knowledge of the mechanisms of microbial adhesion to the surface would be valuable. From this knowledge, design strategies for implant devices or well

engineered novel materials used for the construction of these devices can be developed to effectively control the infection.

In the literature, there are only a handful of publications focused on fungal adhesion to biomaterial surfaces, and most of them were focused on *C. albicans* because of its relevance to modern medicine. Previous work on fungal adhesion to polymer surfaces was primarily focused on three aspects. The first of these was to characterize the adhesion differences between different stains, serotype, phenotype or clinical origin of *C. albicans* to polymer surfaces or to characterize the effects of environmental factors such as media, pH, temperature and cation concentration on cell adhesion. The second was to use biochemical methods to characterize the nature of the adhesins. The third was to characterize the contributions of electrostatic and electrodynamic forces to the adhesion, which are theoretically involved in the 'nonspecific' interactions between cells and artificial surfaces. Almost all this work used static adhesion assays which can not accurately portray the adhesion event in vivo situations because cells are usually subjected to the flowing fluids in the human body. Also it is generally impractical to study adhesion kinetics with common static adhesion assays [8].

There have been few studies employing modern methods of surface analysis to study adhesion in the literature. Because the initial adhesion of micro-organisms to synthetic polymer surfaces involves physico-chemical interactions between molecules present on the polymer surfaces and those present on the cell surfaces, highly advanced surface analysis methods including XPS (X-ray Photoelectron Spectroscopy), also known as ESCA (Electron Spectroscopy for Chemical Analysis), and static SIMS (Static Secondary Ion Mass Spectrometry) should be very powerful in this regard. In the outermost 10nm of a surface, XPS can provide: identification of all elements (except H and He) present at concentrations greater than 0.1%; semiquantitative determination of the approximate elemental surface

composition (error $< \pm 10\%$); information about the molecular environment (oxidation state, bonding atoms, etc.) [9]. However, XPS is limited in its ability to provide detailed molecular information. This is particularly true when atoms are in a wide variety of chemical states, as exist in a protein. Also the achievable lateral resolution of XPS is limited to a few micrometers. Hence, it can not analyze smaller spots on the sample surface. Static SIMS is both more surface sensitive (sample depth is within $20\text{\AA}$ of the sample surface) and more chemically selective than XPS. It allows the identification and quantification of all elements, isotopes, and molecular species [10]. Compared with XPS and other widely applied surface analytical techniques, such as Auger spectroscopy, static SIMS offers some unique features: isotope sensitivity; hydrogen sensitivity; direct compound detection by molecular secondary ion emission; extremely high sensitivity for many elements and compounds, very often in the ppm range. As in any MS technique, the quality and reproducibility of the data require that several criteria be met: controlled desorption of atoms and molecular species, efficient ionization of these desorbed particles, and unambiguous identification of the generated ions by their charge/mass ratios. A considerable fraction of molecular surface species should survive these processes without fragmentation. It has been shown that static SIMS meets these criteria and is well suited for elemental and molecular applications [11]. In static SIMS, the primary ion current density is kept very low so that no sample damage will occur during analysis. Nonconductive samples are readily analyzed with charge compensation.

As a surface analytical method, static SIMS is unrivaled in its molecular selectivity, because of its basis in mass spectrometry. Static SIMS has been used to analyze a wide variety of "real" surfaces, ranging from semiconductor materials to complex copolymers. The spectra produced reflect the surface chemistry of the material but, in general, do not contain large ($m/z \geq 500$) molecular ions or

fragments. However, useful information is readily extracted from the fragment ions in this lower mass range. The ability of static SIMS to produce a surface-sensitive mass spectrum gives it great potential as a probe of proteins adsorbed on surfaces [12]. The application of static SIMS for the study of the interaction of polymers with biological molecules and systems has been demonstrated by Mantus, D.S. and co-workers [12]. In their work, a spectral interpretation protocol was established by examining homopolymers of 16 amino acids. This protocol allowed for the assignment of peaks unique to the various amino acids. Consequently, it was used to study proteins adsorbed on polymer surfaces. They also demonstrated that the intensities of peaks in a static SIMS spectrum might be sensitive to the conformation of a macromolecule on a surface. This is a unique characteristics of static SIMS which makes it unrivaled in the study of adhesion between microorganisms and biomaterials, because conformation is very important for the function of a macromolecule such as protein or glycoprotein on a cell surface, and this function may be closely related to the adhesion event.

Freeze-drying is a technique by which biological specimens are frozen, then dehydrated by sublimation, with complete retention of physical detail and form. Sublimation is the process by which the water in cells passes from a frozen to a gaseous state without going through liquid state [13]. No chemical agents are required and freeze-dried cells don't shrink or otherwise become distorted as those dried from non-frozen state. Physical and chemical changes within the cells are reduced to the minimum degree. It is proposed in this study that freeze-drying is the most suitable method for preparing dried samples for surface structure analysis using static SIMS when a cryostage for SIMS is not available.

In this study, a laminar flow cell which has a well-defined hydrodynamic regime was used in the adhesion study of *C. albicans* to a polymer FEP (Fluorinated Ethylene Propylene) which is commonly used in intravenous catheters. Process

and growth parameters were varied to examine their effects on the adhesion kinetics. Subsequently *C. albicans* were freeze-dried using a freeze-drier built "in house" and static TOF-SIMS was used to analyze the surface structure differences between the freeze-died cells cultivated from different media with an attempt to correlate specific functional groups on the cell surfaces to the adhesion results.

CHAPTER 2

BACKGROUND RESEARCH

2.1 *Candida albicans*

2.1.1 A Brief Overview of *Candida albicans*

C. albicans is a dimorphic fungus which may be present in humans either as a commensal or as an agent of infection in the compromised host [14].

The dimorphic yeast *C. albicans* exists as either one of several phenotypes. First, *Candida* cells are capable of growing as a budding yeast form, the hyphal form, or the pseudo-hyphal form. In the budding yeast form, a mature yeast cell forms an evagination which expands into a mature daughter cell. In this growth form, cells are round to ellipsoidal with a diameter of 3-5 microns. In the hyphal form, cells grow as elongated, compartmentalized tubes. Budding cells can be induced to form hyphae and hyphae can be induced to form buds. Hyphae are believed to have evolved primarily as a mechanism for tissue penetration[15]. Another phenotypic variability is white and opaque and *C. albicans* is also able to switch between these two. Two serotypes, serotype A and B, have been defined by the variation in the mannan moiety of surface mannoproteins of *C. albicans*.

C. albicans is also capable of expressing surface hydrophobicity (CSH) when grown in either the yeast or hyphal form. Associated with expression of surface hydrophobicity is enhanced virulence[16]. Several groups of investigators have demonstrated that hydrophobic yeast and hyphal cells attach more than hydrophilic cells to various types of plastics used in prostheses and catheters [17,18,19,20]. Various observations suggest that fungal surface hydrophobicity expression is pivotal to the success of parasitizing *C. albicans* in causing serious infection [16]. The hydrophobic properties of the cell surface of *C. albicans*

appear to exhibit significant variation depending on the strain as well as the growth medium and conditions [21]. *C. albicans* isolates generally appear more hydrophobic when grown to stationary phase at room temperature (23-25°C) than at 37°C [21]. The expression of surface hydrophobicity in vivo may be transient. Hydrophilic cells obtained by growth at 37°C in vitro, exposed hydrophobic surface macromolecules for only a short period when subcultured into fresh medium [22]. The results suggest that antigenic variation occurring during pathogenesis of candidiasis could involve surface hydrophobic molecules in addition to mannoprotein antigens [23,24,25].

One unique aspect of the yeast is its ability to rapidly change its cell surface in response to new environmental conditions. It has been shown that yeasts grown in media promoting hydrophilicity can change to a hydrophobic cell surface within sixty minutes of a change of media [22]. *C. albicans* takes an active role in pathogenesis by producing several virulence factors that may promote the successful colonization or invasion of tissues [26]. Cell wall hydrophobicity and switching of expressed phenotypes have been proposed as attributes contributing to the virulence of this yeast [27,28]. The adhesins of *C. albicans* appear to be diverse, reflecting the ability of the organism to colonize and invade a variety of host cells and tissues [29,30]. Phenotypic and dimorphic states [31,32,33,34, 35,36] as well as environmental factors [37] appear to regulate the expression of *C. adhesins* and it is very likely that multiple adhesins are employed by *C. albicans* in adhering to host cell surfaces [38,39].

For in vitro studies, generally the pH and chemical composition of the growth medium, as well as inoculum size and incubation temperature, determine the growth form. Hyphal formation is generally enhanced in a medium with a pH higher than 6.5 and a non-fermentable carbon source when grown at high temperatures. Growth in an acidic medium using a fermentable carbon source

typically promotes blastospore (budding yeasts) production at low incubation temperature. *C. albicans* reaches stationary phase within approximately 18-24 hours of growth in most media [40].

2.1.2 *Candida albicans* Cell Wall

The cell wall of the organism is essential to its success as a pathogen, since it is required for growth, provides rigidity and protection against osmotic insult, and is the site of contact between the organism and its environment. The outermost layer of the *C. albicans* cell wall plays an important role in pathogenesis, mainly because it possesses macromolecules that adhere to host tissues [41]. Although the synthesis of the cell wall components is dynamically influenced by growth conditions and metabolic states, the literature contains fairly consistent data regarding the chemical composition of the cell wall [42]. The cell wall of *C. albicans* makes up approximately 30% of the dry weight of the cell [43]. A number of studies have shown that the cell wall is composed of mannoproteins (20%-30%), β -1, 3-D-glucans (25%-35%), β -1, 6-D-glucans (35%-45%), a small quantity of chitin (0.6%-2.7%), protein (5%-15%) and lipid (2%-5%) [44]. Observations by electron microscopy reveal at least six layers in the cell wall of *C. albicans* [45]. The architecture of the cell wall includes a fibrillar layer followed, in order, by zones rich in mannoprotein, glucan, glucan-chitin, and mannoprotein, as shown in Figure 1 [42].

Although the appearance of the cell wall suggests a discrete packaging of the various macro-molecules, the layering is more likely a result of differences in the proportions of the components in each layer rather than absolute differences in chemical composition [46].

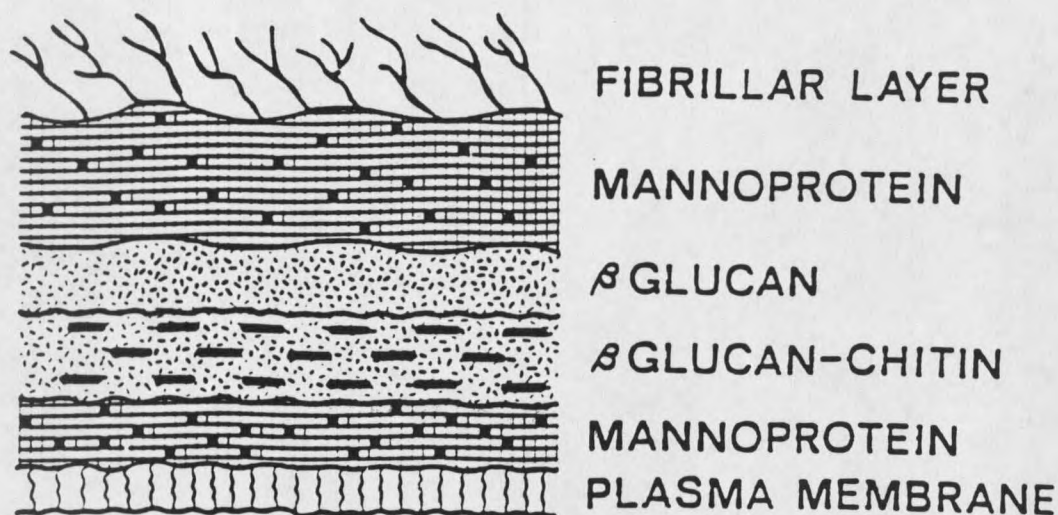


Figure 1. Schematic diagram of the cell wall of *Candida albicans*

The fibrillar layer on the outermost region of the cell wall appears to be composed primarily of mannoproteins [41,42]. The amount of fibrillar material produced depends on the growth medium, is proportional to the concentration of sugar, and increases with the age of the culture [41]. For example, incorporation of galactose or sucrose instead of glucose in the culture medium results in a greater production of the fibrillar outer layer [47,48]. Recently, Yu et al [49] reported that the major structural subunit of the fibrous structure is a glycoprotein which consists of 80%~85% carbohydrate (consisting primarily of D-mannose) and 10%~15% protein. Since this fibrillar outer layer represents the outermost region of the cell, it may play a role in the adhesion of the yeast to host cell or plastic surfaces. Both the roles of protein moiety and oligosaccharides of the mannan moiety in adhesion ability cannot be ruled out [50,51,52,53].

The two outermost and the innermost electron-dense layers that are present on each side of an intermediate electron-lucent layer have been shown to be composed of mannoprotein [45], and are thus called the mannoprotein layer. There

have been various types of mannoprotein consisted of protein and mannan or mannooligosaccharide in different proportions [41]. One of the surface structure mannoproteins of *C. albicans* has been reported to be a 260kDa polymer composed of 1.5% protein and 98.4% hexose [54]. In addition to structural mannoproteins mentioned above, several mannoproteins with distinct adhesion activity which range from 50 to 165kDa have been reported [55,56,57,58], although the mode of the molecular organization of such mannoproteins has not been elucidated. The evidence reported suggests that there are different mannoproteins which contain different protein/peptide groups and possess different functions. Moreover, the wall contains a number of enzymes including N-acetylglucosaminidase, acid phosphatase, proteinase, glucanase and chitinase [45].

2.2 Cellular Attachment to Surfaces

2.2.1 Molecular Mechanisms of Cellular Attachment

Cellular attachment to surfaces is a very complex process, the physico-chemical properties of the organism surface and the substratum surface as well as environmental factors are all important in this process. Two main stages of biological adhesion have been recognized. A reversible initial attachment or primary physical attraction stage followed by a secondary irreversible adhesion stabilization [59]. Initial attachment depends on the general long-range physical characteristics of the organism, the fluid interface, and the substratum [60]. The second stage may be facilitated by a metabolic process or just result from the formation of multiple contacts once the cell is held on the surface. For a cell to become adhered tenaciously to a surface, both of these stages must occur successfully.

Surface free energies must dominate any explanation of the adhesion between different phases which are not mechanically linked [61]. The surface of a substance or phase requires an extra term in the description of its energy which is due to its location at a boundary where there is a sharp change in concentration or properties of the substance. The energy associated with this location is the surface free energy and is described by the energy required to form a new unit area of surface (Eq.1).

$$\frac{dG}{dA} = \gamma \quad (1)$$

where: G= Gibbs free energy

A= area

γ = Surface free energy per unit area

Surface free energy is equivalent to surface tension (the force required to part a unit length of surface) for liquid which is very easy to measure experimentally. But for solid, this is not necessary true. To calculate solid surface energies, Eq.2 is the mostly used empirical equation in the literature.

$$\cos\theta = \frac{(0.015\gamma^{SV} - 2.00)(\gamma^{SV}\gamma^{LV})^{1/2} + \gamma^{LV}}{\gamma^{LV}[0.015(\gamma^{SV}\gamma^{LV})^{1/2} - 1]} \quad (2)$$

Where:

θ = measured contact angle of a sensing liquid on the solid surface.

γ^{SV} = solid vapor interfacial free energy.

γ^{LV} = liquid vapor interfacial free energy (liquid surface tension).

The interfacial free energy between the solid surface and the liquid (r^{SL}) is given by Eq.3

$$\gamma^{SV} = \frac{[(\lambda^{SV})^{1/2} - (\gamma^{LV})^{1/2}]^2}{1 - 0.015(\gamma^{SV}\gamma^{LV})^{1/2}} \quad (3)$$

For adhesion between a fungus and a polymeric surface, the surface free energy of the fungus (r^{FV}) and the interfacial free energy between the fungus and the liquid (r^{FL}) can be obtained from Eq.2 and Eq.3, respectively, by replacing r^{SV} with r^{FV} and r^{SL} with r^{FL} . The change in interfacial free energy which corresponds to the process of adhesion (ΔGa) is given by Eq.4:

$$\Delta Ga = \gamma^{SF} - \gamma^{SL} - \gamma^{FL} \quad (4)$$

where: r^{SF} = interfacial free energy between the solid surface and the fungus.

From thermodynamic point of view, adhesion will occur spontaneously when ΔGa is negative.

From Antonov's equation, r^{SF} is obtained by Eq.5

$$\gamma^{SF} = |\gamma^{SV} - \gamma^{FV}| \quad (5)$$

Hence, by measuring contact angle of a sensing liquid on the polymeric surface and the contact angle of the fungus layer, ΔGa is readily calculated from combination of Eq.2, 3, 4 and 5. These equations are commonly used in the

literature to calculate ΔG_a and surface free energies and hence to explain the adhesion phenomena from thermodynamic point of view.

It should be noted that cells of different species and even cells of different strains of the same species may have widely different surface free energies, and thus the surface free energy of the cells should be measured under conditions close to those which will exist at the time attachment is attempted [61]. Polymeric materials also have a wide range of surface energies. So explaining adhesion events through the surface free energy driving force is case-dependent. It depends on the molecular properties of both the cell and the biomaterial surface.

Surface free energies describe adhesion phenomena once molecular contact has been achieved between the adhering phases. That means surface free energy effects predominate at small separation distances (primary minimum) between the cell and the polymer surface [61]. For a cell to come from a distance into molecular contact with a surface requires consideration of long-range forces which influence approach. These factors include:

- 1) Van der Waals forces
- 2) Hydrophobic forces (or attractive London Van der Waals forces)
- 3) Electrostatic interactions
- 4) Fine surface projections

These factors are expected to play key roles during the initial attachment of a cell to a polymer surface.

The surface of ionizable substances attract ions of opposite charge. The net surface charge of a particle is known as the zeta potential. Clearly, cells will be attracted to surfaces of opposite zeta potential. If cells have the same zeta potential as a surface, attachment is still possible provided the electrostatic barrier can be penetrated by small surface projections [61], or other attractive forces which favor adhesion.

Electrodynamic forces including Van der Waals forces and hydrophobic forces should be considered key elements in adhesion process also. Studies indicate that hydrophobic forces are exerted at distances as great as 15nm, and at 8 to 10nm are 10 to 100 times as great as Van der Waals forces [62]. Van der Waals forces at the secondary minimum (approximately 10nm) can effectively position a particle near the surfaces. Within 1nm or less, it is conceivable that short-range chemical interactions (ionic, hydrogen, and covalent bonding) occur with extracellular moieties [60].

The process of cellular adhesion is a very complex and not a well-defined phenomenon, it consists of the balancing of many different physical and chemical forces [63]. An understanding of the critical aspects of this process would help elucidate the specific interactions between a cell and a surface.

2.2.2 *Candida albicans* Attachment to Surfaces

Of the relatively small amount of data published on yeast adherence to surfaces, the majority has focused on *C. albicans* because of its high relevance to medicine. The surface used for these studies include acrylics, poly (vinyl chloride), polystyrene, teflon, poly (ethyleneterephthalate), poly (methylmerthacrylate) and silicone rubber. Some researchers focused on the effects of environment conditions such as culture media and cations on adherence [64,65]. Others focused on adhesion mechanisms by characterizing contributions of molecular forces such as electrostatic and hydrophobic forces in adhesion process [66,67,68,69,70]. Almost all these researchers used static attachment conditions in which cell suspensions were incubated with synthetic polymers for a period of time and then the adhered cells on the polymer surfaces were counted as adhered cells per unit area. However, in order to closely portray in vivo situations, researchers must use both static assays and dynamic assays, since static fluid

rarely exists in the human body, and yeasts adhesion is usually subjected to circulating fluid in the human body. Also it is generally impractical to study adhesion kinetics with common static assays [8]. Yeast form cells instead of mycelial form cells were used in the majority of these works. That is because the adherence of mycelial form cells is much more complex than that of yeast form cells. Studies using mycelial form cells will be undertaken after an understanding of a simpler system using yeast cells is accomplished.

Douglas, et al. have studied the influence of culture variables on the adherence of *C. albicans* to acrylic surfaces [64]. In their experiments, the adherence of *C. albicans* to acrylic was measured in vitro after growth of the yeast to stationary phase in defined medium containing glucose, sucrose, galactose, fructose, or maltose as the carbon source. They found that incubation of *C. albicans* with high concentrations of sugar enhanced its adherence to acrylic surfaces. Cells grown in 500mM of fructose, glucose, maltose, sucrose or galactose were more adherent than cells grown in 50mM glucose but to a different extent. Cells grown in 500mM galactose promoted adherence to the greatest extent which were eleven times greater than cells grown in 50mM glucose. They also found that addition of divalent cations such as Ca^{2+} , Mg^{2+} or Fe^{2+} promoted adherence of *C. albicans* to acrylic surfaces. Electron microscopy of thin sections of yeasts stained with ruthenium red or polycationic ferritin showed the presence of an outer fibrillar layer on cell surfaces harvested from 500mM sucrose and 500mM galactose, but it was absent or much less evident on yeasts grown in low concentration of glucose (50mM). This structural difference appeared to contribute to the enhanced adherence. They also suggested that this fibrillar component consisted largely of mannoprotein [65]. Also, the most conspicuous change in yeast cell surface composition resulting from growth in medium with a high sugar content was the production of this outer fibrillar-floccular layer which was

visualized with ruthenium red or polycationic ferritin suggesting that it might be rich in acidic polysaccharide [64].

The adherence of yeasts to inert polymeric surfaces is unlikely to be mediated by a receptor-adhesion interaction [66], as that of adherence to mammalian cell surfaces or biological macromolecules, because the nonbiological surfaces cannot be equipped with specific binding sites [67]. They are more likely to rely on nonspecific interactions. Previous studies have indicated that a number of physical forces potentially involved in the "nonspecific" interactions between fungal surfaces and artificial surfaces include electrostatic and electrodynamic forces which include the Van der Waals interactions, and the hydrophobic effect [67] as already discussed in this thesis. Several publications in this regard have focused on characterization of the roles of these molecular forces in the adherence of *C. albicans* to synthetic polymer surfaces [66,67,68,69,70].

The net surface charge of a particle is known as the zeta potential. The zeta potential of a cell surface will be influenced by the pH and ionic strength of the suspending fluid. For example, a protein molecule will possess a negative zeta potential if the pH of the suspending fluid is higher than its isoelectric point. Hence, it will move to the positive electrode if it is subjected to electrophoresis, and vice versa. The zeta potential of a cell depends on the surface - located ionizable moieties and the pH and ionic strength of the suspending fluid. Jones, et al. have studied the role of electrostatic nature of the cell surface of *C. albicans* in adhesion [67]. They suspended *C. albicans* in a series of electrolyte solutions with different electrolyte cation valences, concentrations and pH. These cell suspensions were subjected particle electrophoresis to determine the effects of electrolyte valence, concentration and pH on the electrostatic potential of *C. albicans* cells. The pH of the electrolyte solution was varied between 3.0 and 9.0. Cation valence was varied between +1 and +3. Electrolyte concentration was

varied between 10^{-7} and 10^{-1} M. Under all the conditions studied, the electrostatic potential of the fungal cell surface was found to be negative, but the magnitude of the corresponding electrostatic potentials was found to vary to a large extent over a large range of experimental media. In order to determine whether electrostatic interactions have any bearing on fungal adhesion, in addition to the electrostatic characterization of the fungi, similar determinations of the electrostatic potential were performed on the high index barium titanate glass and White-house Scientific Glass. Under all the conditions studied, the glass surfaces were also electronegative. Subsequently, *C. albicans* were allowed to adhere to the glass surfaces in the same series of media in which the electrostatic potentials of both the yeast and the glass were measured. They used a dynamic assay in which *C. albicans* were subjected to hydrodynamic shear forces during adhesion process. The extent of adhesion was found to be affected by the magnitude of the coulombic repulsion between electronegative fungal surfaces and electronegative glass surfaces. But significant adhesion still occurred, however, even when the coulombic repulsion was a maximum. This was taken to indicate that the fungal surfaces possessed other nonelectrostatic forces which were attractive. So the result indicated that both the electrostatic repulsive and the nonelectrostatic attractive forces were considered to play a role in the adhesion of the fungi to artificial surfaces such as glass. They suggested that this attractive forces were likely to be the so-called electrodynamic attractive forces which were likely to result from the hydrophobic effect. Following the publication of these experiment results, this group published another paper in which they employed another method using fluorescent probe 9-aminoacridine (9AA) to measure the electrostatic properties of the cell surface of *C. albicans* in the similar experiment set-up [68]. The results confirmed their previous results that under all the conditions studied the yeast form of *C. albicans* possessed a net electronegative

surface potential and appeared to behave like simple colloidal particles. They also used neuraminidase to treat the fungi surface. Neuraminidase is known to cleave selectively, the negatively charged moiety, sialic acid. The result was that the neuraminidase-treated fungi possessed a smaller electronegative surface potential, most probably due to the removal of negative charges by the enzymic treatment. This result indicated that sialic acid was present on the surface of *C. albicans* which must account in part for the electronegativity of the *C. albicans* surface [68].

It is of interest to identify the nature of the groups contributing to the surface potential of *C. albicans*. It is likely that the major contributor to this property will be the mannoproteins and charged polysaccharides known to be resident on the fungal surface [68]. A comprehensive study of the contribution made by such proteins has not been reported .

Hydrophobic forces appeared to be very important for the adhesion process of *C. albicans* to plastic surfaces. Klotz, et al. found that there was a nearly linear relationship between the cell number adhering per unit area and the contact angle of the substrate determined with distilled water, i.e., the more hydrophobic the surface, the greater the cell adherence per unit area [66]. To test the supposition that electrostatic interactions contributed to adherence, this group modified the net surface charges of the yeast. The control or untreated yeasts possessed a net negative surface charge in common with all living cells (including yeasts). They found the more positively charged yeasts, i.e., those treated with carbodimide, were considerably more adherent to polystyrene than more negatively charged formalin-treated yeasts. Plastic surfaces possess various degrees of negative net surface charge [71]. Plastic surfaces and yeast cells tended to repel each other since they were both electronegative. But electrical forces were minor to the hydrophobic forces since adherence to a considerable extent still occurred. These

researchers also showed that adherence occurred whenever the sum total of interfacial tensions, i.e., yeast-liquid, yeast-solid, and solid-liquid was reduced, or in thermodynamic terms the change in total free energy was negative [66]. Miyake, et al. found that there was a linear relationship between the number of adherent cells to acrylic surface and cell surface hydrophobicities, i.e., the more hydrophobic the cell surface, the greater the cell adherence per unit area [69]. Their work also confirmed that hydrophobic interaction played an important role in the adherence of *Candida* species to acrylic surfaces.

In addition to hydrophobic forces, others found surface free energy effects were also important in the adherence process. Minagi, et al. showed that increasing surface free energy of denture base resin plates resulted in increasing adherence of *C. albicans*. In their study, linear relationship was also observed between the change of free energy which corresponded to the process of adherence and the adherent-cell counts of *C. albicans* to denture base resin material, i.e., the higher adherence tendency was accompanied by a lower value for the free energy change [70].

Although Klotz, et al. found that *C. albicans* adhered in greater number to more hydrophobic surfaces, Rotrosen, et al. showed that *C. albicans* adhered more extensively to PVC than to teflon, although teflon is more hydrophobic than PVC [72]. These conflicting results may reflect the structure differences of the *C. albicans* cell wall resulting from the different test strains and/or different culture media used.

2.3 FEP (Fluorinated Ethylene Propylene)

FEP is a polymer with the following chemical formula:



It is commonly used as a material to construct intravenous catheters which are most likely to become infected. It is a very hydrophobic material. Limited studies have been performed on the adherence of *C. albicans* to plastics used for intravenous catheters in the literature.

2.4 Freeze-drying

2.4.1 What Is Freeze-drying—An Introduction

Freeze-drying or lyophilization, can be described simply as the dehydration of a frozen aqueous material through the sublimation of ice. The technique is applied almost exclusively to biological materials. In brief, the procedure consists of the following steps. The material is frozen, generally at atmospheric pressure. The specimen is placed into a chamber maintained at a total pressure under which condition ice will sublime directly from the solid phase to the vapor. Heat must be introduced into the specimen to support continued sublimation. By proper balance of heat input and vapor removal from the system either by condensation on a refrigerated surface, adsorption by a desiccant or removal by pumping. Nearly all freeze-drying systems maintain a partial vacuum in the specimen chamber to facilitate the transfer of water vapor away from the specimen [73].

Dehydration by freeze-drying has three principle virtues: the prevention of shrinkage, the minimizing of chemical changes and the maintenance of solubility [73].

Shrinkage is the usual result when flexible hydrated material is dried from the liquid phase. As the total amount of water is reduced, forces exerted by surface tension can become very great. The material will shrink and, on the final drying, will become hard and glassy. When the specimen is frozen first and water is

removed as vapor, surface tension effects are eliminated. In addition, at low temperature, the remaining organic matrix becomes more rigid, further opposing any tendency to shrink or change shape [73].

Chemical change can result from progressive dehydration which produces a concentration of solutes, particularly electrolytes, enzymes and enzyme substrates. Although the initial freezing also produces dehydration and solute concentration, the lowered temperature reduces biochemical reaction rates and permits dehydration under more stable conditions. Once the dehydration is complete and the free water and most of the bound water has been removed, the material can be returned to ambient temperature without rapid degradation [73].

Insolubility following drying from the liquid phase is sometimes due to chemical changes but is more commonly the result of a limited surface area for solvation. When a hydrated material is frozen, ice crystals form through its mass, and when these are removed by sublimation the sites previously occupied by ice crystals remain as empty holes within the specimen, providing an extremely large surface area leading to good solubility on rehydration [73].

These three virtues of freeze-drying justify its use in the preparation and preservation of a variety of biological materials. The maintenance of shape and the prevention of shrinkage have rendered freeze-drying useful for the preservation of pathological and histological materials, and even of whole animals for museum displays. The minimizing of chemical changes during drying and storage has led to widespread application in the preparation of pharmaceuticals and cultures of micro-organisms. To a somewhat cruder degree, it is also the reduction of deleterious chemical changes that has led to the use of freeze-drying for the preparation of dehydrated foods (although the oxidation of fats may be accelerated by freeze-drying). In some cases, materials which are uninjured by air drying are freeze-dried solely to achieve speed and efficiency in rehydration [73].

Freeze-drying has been used in many fields. It has been used for almost a century and has given rise to numerous publications. In the literature, the references relating to freeze-drying of microorganisms were mostly dealing with bacteria, and relatively less work has been done with yeasts. No previous work has been reported using freeze-drying techniques to prepare micro-organisms for cell surface chemical and structure analysis using secondary ion mass spectrometry. The work presented here is the first to report freeze-drying for use in highly advanced surface analytical technique---time of flight secondary ion mass spectrometry (TOF-SIMS) to probe the outermost 20Å of the cell surface of a yeast. The SIMS spectra presented in this thesis are also the first to report freeze-dried *C. albicans*.

2.4.2 Elemental Analysis of the Freeze-drying System

Theoretically, sublimation of ice will occur at temperature below the freezing point of water and the partial pressure of water vapor in the system below the vapor pressure of ice at that temperature. The relationship between vapor pressure of ice and temperature is given by the following equation:

$$\text{Log}(P) = 10.430 - 2688.5/T \quad (6)$$

In which T is absolute temperature (K) and P is pressure given in mmHg. This equation is represented by Figure 2:

From Figure 2, one can see that at temperatures below 0°C, the vapor pressure drops sharply; if water is to be removed efficiently, the system must produce pressures well below the vapor pressure to get high driving force of water vapor removal from the system, which explains why freeze-drying is practical only

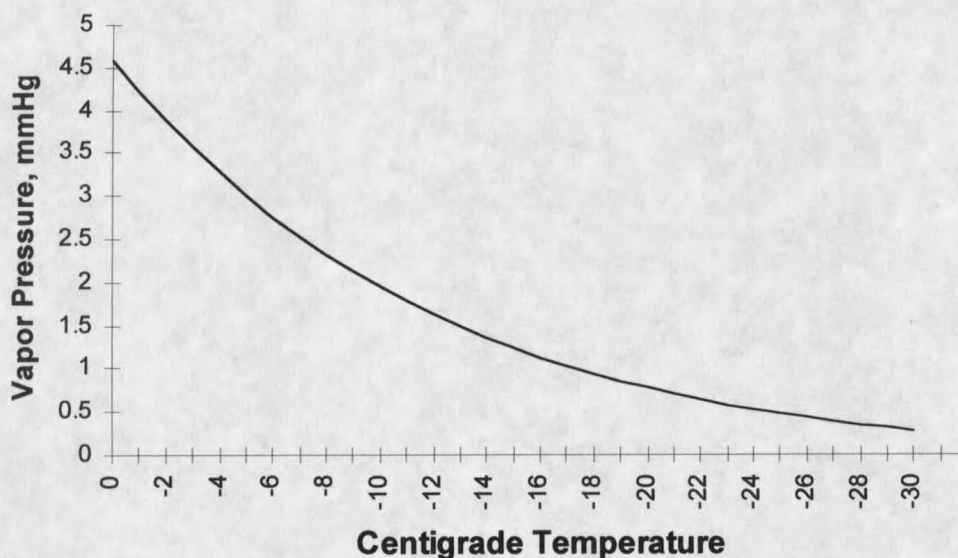


Figure 2. Plot of the vapor pressure as a function of temperature

at low pressures.

The freeze-drying process in its most elementary form can be subdivided into four distinct stages. The first of these is the transfer of heat to the drying boundary. The second is the conversion of ice to water vapor at the surface of the ice crystal. The third stage is the transfer of this water vapor through the already dried shell of the specimen to the specimen surface. The fourth stage is the removal of this water vapor from the specimen surface [73]. This process is schemed in Figure 3:

The water vapor is ultimately removed from the system either by condensation on a refrigerated surface, adsorption by a desiccant or removed by pumping. Nearly all freeze-drying systems maintain a partial vacuum in the specimen chamber to facilitate the transfer of water vapor away from the specimen.

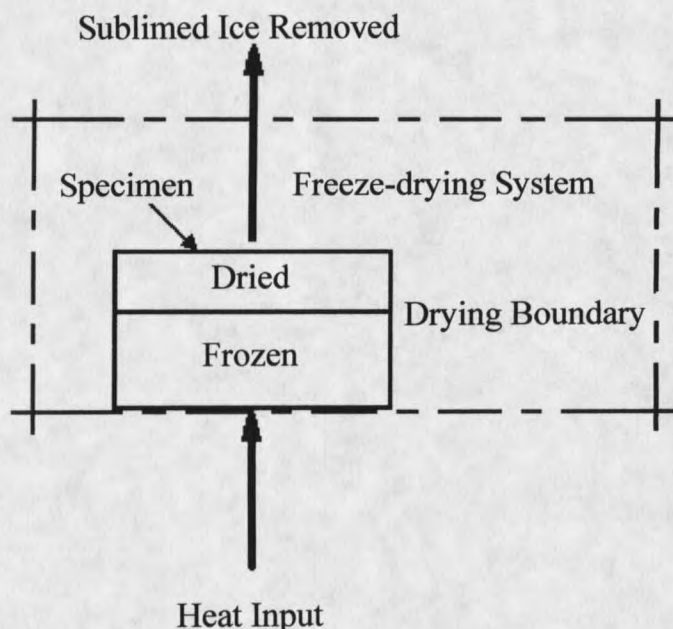


Figure 3. Graph depicting the elementary form of freeze-drying process in a closed system

Heat energy must be introduced into the specimen to support continued sublimation. The latent heat of sublimation of water is approximately 620cal/g which is equal to the combined latent heats of fusion and vaporization (80 and 540 calories respectively) [74] . The heat that is consumed during the sublimation of ice under normal circumstances is drawn from the ice crystal and its immediate environment. This means that if heat is not supplied to the specimen, sublimation will produce a fall in temperature. The reduced temperature will in turn, produce a reduction in the rate of sublimation. The result of such a progression will be a continued fall in temperature and reduction of the sublimation rate until a temperature too low to permit sublimation is reached. If heat is introduced into the system at a fixed rate, an equilibrium temperature will be reached at which the heat consumed by sublimation exactly equals that being introduced. If sublimation takes at a rate more rapid than the supply of heat will support, the temperature will

fall until the sublimation rate is exactly equivalent to that which the heat input can maintain. Conversely, if the specimen temperature is too low, there will be insufficient sublimation to utilize the heat being introduced, and the excess of incoming heat will increase the temperature until sublimation exactly equals that supported by the heat input.

It also should be noted that the rate of sublimation from an ice crystal is a function only of temperature, while the rate of condensation (return of water molecules to the ice crystal surface) is a function of both temperature and vapor pressure. So if the escape of water vapor molecules is impeded and the vapor pressure in the immediate vicinity increases, the amount of water vapor recondensing will increase and the net rate of sublimation will decrease. In order to continue utilizing a fixed rate of heat input, the specimen temperature would have to increase to permit a higher sublimation rate.

In practice, therefore, specimen temperature is determined by the rate of heat input into the specimen and the rate of vapor transfer out of the specimen. Any condition that changes either of these factors will alter specimen temperature [73].

2.4.3 Freeze-drying Microorganisms

Freeze-drying, as a unit operation in chemical engineering, is a complex multistage process which has to be carefully adjusted to each individual case [75]. The choices of the optimal operation parameters depend on the specific specimen and the aim the operator wishes to achieve from the technique. For example, from the point of view of workers dealing primarily with biological specimens, gross histological integrity—structure retention—is the primary consideration, and the factors dealing with the economics of food production are of no consequence [74]. While even with the same aim, but with different specimens, the optimal

operation procedure may be largely different from specimen to specimen. The biochemist practitioner treats lyophilization as a push-button affair, in the belief that the optimization of formulation and protocol must necessarily rely on trial and error, to be established *ab initio* for every new product [76].

In this study, *C. albicans* cells were freeze-dried for TOF-SIMS analysis. In the literature, only the information under the topic of freeze-drying micro-organisms for culture collections and histochemical studies is related to the study here and consequently will be referenced.

Freeze-drying or lyophilization is the most suitable method for preserving bacteria, viruses, yeast and fungi for culture collections, provided that the micro-organisms are able to withstand this procedure [77]. It was found that fungal stains may be kept alive in freeze-drying ampoules for more than 40 years [78]. In principle, the method is based on a process in which cell suspensions are first frozen and then *ca* 95% of the water in the suspension is sublimed. After this, in certain cases, all but the last 1-2% of the residual water is gradually removed under vacuum. This method is very acceptable because most of the clinically interesting micro-organisms may be lyophilized without any significant loss in their viability. Moreover, because lyophilized micro-organisms may be stored for prolonged periods, it costs little to store them, and no special precautions are required for transportation [77]. The long-term viability of the freeze-dried strains is the most concern for culture collections and viability test is uniformly used to optimize the freeze-drying operation procedure.

The freeze-drying of tissues for histological study is one of the earliest recorded applications of freeze-drying. The primary use of histological freeze-drying is for histochemical studies. Conventional methods of fixation and embedding of tissues require the fixation of the tissue and the removal of water through substitution with organic solvents. The majority of enzymes are denatured

or removed by these procedures. Freeze-drying, on the other hand, enables the preparation of good histological sections with a negligible denaturation or displacement of the biochemical components [73]. Under this topic, gross histological integrity—structure retention after lyophilization is the more important concern than the viability of the microorganism after lyophilization. But these two, structure retention and viability, might be closely related to each other under certain circumstances.

For the preservation of good histological detail, drying must be done at temperatures of -30°C or less. At temperature above -30°C , gross histological distortion can be seen, characteristically taking the form of enlarged extracellular spaces with shrinking of the cells [73]. Freezing of tissues for histological studies must be done rapidly in order to produce ice crystals of minimum size, but attempts to achieve rapid freezing with extremely low temperatures, such as would result from total immersion in liquid nitrogen, is almost certain to fracture specimens when the mass is greater than 1 gram. Also as observed by Meryman, when specimens are frozen at extremely low temperatures, the tendency for extracellular crystal formation is greatly reduced, and numerous smaller crystals begin to appear within the cells. This condition slows the drying process greatly [74].

Possible disadvantages of lyophilization may be damage to DNA, to the cell wall or to lipopolysaccharides. Alterations in the membrane permeability are also possible during lyophilization [77].

There is considerable range in the ability of micro-organisms to withstand freeze-drying. In general, resistance to injury is in proportion to the complexity of the organism; many viruses being highly resistant to freezing and drying injury while of higher organisms such a protozoa., only a few will survive drying under special conditions [73].

The factors which have an influence on the survival of micro-organisms during lyophilization are very complex and poorly understood [77,79]. The process of lyophilization may be divided into three steps all of which have an influence on the viability of micro-organism. In the first place, the manner of freezing influences the survival, followed by the actual lyophilization and finally the manner of rehydration of the micro-organisms during the recovery after lyophilization [77].

For an optimum recovery, cryoprotective agents may need to be added to the medium before the micro-organisms are frozen. The protection that these additives offer depends on their exclusion from the solution in the immediate vicinity of the membrane proteins. In the liquid phase and during freezing the thermodynamic conditions are thus unfavorable for the denaturation of membrane proteins. A wide variety of chemical compounds such as proteins, saccharides and inorganic salts can be used as cryoprotective agents [77].

The inactivation of micro-organisms during lyophilization depends on the denaturation of membrane proteins by withdrawal of water and is, therefore, fundamentally different from inactivation during freezing. The hydrogen bonds between membrane proteins and water are split during lyophilization. Thus, for a good recovery of micro-organisms after lyophilization, the cryoprotective agents in the medium must adopt the role of water and re-establish the hydrogen bonds with the membrane proteins. Thus, for any given micro-organism, there are only one or two suitable cryoprotective agents [77].

In addition to the cryoprotective agents, the cooling rate of the cells during the cooling phase is a critical factor in the freeze-drying process [78]. The optimum cooling rate results from two opposite effects: Concentration of intra- and extra-cellular solutes is responsible for cell injury when the cooling rate is lower than optimum. At very slow cooling rates, extracellular and intracellular

osmolalities can remain in equilibrium. As water is frozen out of solution, there is a progressive concentration of intra- and extra-cellular solutes, leading to cell damage and shrinkage. At higher cooling rates, the cell doesn't have time to lose water when the suspension is freezing, and ice nucleates in the cells leading to an important loss of viability. The optimum cooling rate appears to be that one at which the cell doesn't lose its water and reaches the eutectic point frozen in an amorphous state [78]. Each cell type has an optimal cooling rate that depends on the water permeability of the cell and its surface to volume ratio [80].

The optimal cooling rate for yeast cells as shown by Mazur was between $-1^{\circ}\text{C}/\text{min}$ and $-10^{\circ}\text{C}/\text{min}$ [81]. Mazur, et al.; Hwang, et al.; Grout and Morris also have shown that the survival rate of frozen fungal cells increased considerably when cells were cooled at a rate of $-1^{\circ}\text{C}/\text{min}$ [82].

Berny, J.F. and Hennebert, G.L. found that the viability rate of the fungal cells of *Saccharomyces cerevisiae* was increased from 30% to 96-98% by using an appropriate protecting medium containing 10% skim milk with 2 compounds among honey, sodium glutamate, trehalose or raffinose in the freeze-drying process carried out at a $-3^{\circ}\text{C}/\text{min}$ cooling rate [78]. Theunissen, J.J.H. et al. studied the effects of medium and rate of freezing on the survival of *Chlamydia*s after lyophilization. They found that the survival was higher after freezing at a rate of $-1^{\circ}\text{C}/\text{min}$ and lyophilization than that after rapid freezing at -70°C or -196°C . The recovery (5%) was higher when fetal calf serum containing glucose, saccharose or lactose were used as lyophilization media than that (0.5-3%) when yolk-sac, skimmed milk or phosphate buffer containing sucrose, glutamine and 10% fetal calf serum were used [77]. Owen, R.J. et al. studied the effect of cooling rate, freeze-drying suspending fluid and culture age on the preservation of *Campylobacter pylori*, and found that the largest loss in viability occurred at the primary drying stage and losses resulting from freezing and secondary drying were

less marked and the rate of freezing had only a marginal effect on recovery. They also found that other factors, such as age of culture and number of viable bacteria in the before-dry suspension, did not have a significant effect on survival. They concluded that the degree of recovery of *C. pylori* was apparently largely strain-dependent [79] .

Tan, C.S. et al. have studied the influence of the cooling rate on the survival of conidia after lyophilization. In their experiment, eleven organisms with different size and wall thickness of conidia were used. They found that survival of the conidia in relation to the cooling rate was dependent on the size and the wall thickness of the conidia. The viability of large and/or thick-walled conidia was significantly influenced by the cooling rate and survival was optimal when conidia were cooled at $-1^{\circ}\text{C}/\text{min}$ prior to drying. Small thin-walled conidia were not influenced significantly by the cooling rate. Large thick-walled conidia were less susceptible to damage caused by cooling and freeze-drying than the large thin-walled conidia. The cell wall of the thick-walled conidia is impregnated with melanin pigments which probably protected cells against damage caused by freezing and dehydration as was also observed by Tan, et al. [80].

The yeast *Saccharomyces cerevisiae*, has been shown by Mazur [83] to survive freezing in a gelatin-saline solution down to -13°C where a rapid fall in recovery occurred which he attributed to intracellular freezing. Greaves[84] found that when this organism was dried at progressively lower temperature, the survival from freeze-drying fell in coincidence with Mazur's curve of freezing injury. Greaves also reported that the rapid injury at -13°C could be prevented by the addition of glucose, or PVP, or both to the drying medium. These observations are consistent with the assumption that protective additives prevent water from freezing, maintaining a liquid phase in which electrolytes can remain in solution. Thus, instead of a eutectic solution which crystallizes, a non-freezable solution

exists which slowly solidifies into a glass with decreasing temperature. These observation suggests one manner by which additives may function to give protection during the initial freezing [73].

The another important factor which influences the survival of micro-organism after lyophilization is the drying temperature during lyophilization. Drying should be done from the solid state, which is the basic concept of freeze-drying, so the specimen temperature must be maintained below the minimum temperature of incipient melting which is usually the minimum of all eutectics of major salts in the cell. There is no question but that drying organism at a temperature above the eutectic of a major salt can often be injurious, presumably on the basis that a liquid phase is necessary for continued chemical reaction. The presence of liquid eutectic solution thus can be deleterious. It is also a source of cell shrinkage. Muggleton and Greaves have reported a marked increase in the recovery of living organisms when the drying temperature was lowered from -20°C to -28°C . A further reduction in drying temperature to -35°C increased the survival to 60% . From this point of view, the conventional type of laboratory freeze-dryer in which material in ampoules, previously frozen, is dried on a manifold exposed to room air, can not be depended upon to maintain temperatures below -30°C throughout the drying cycle. For example, cultures initially frozen in dry ice slush at -70°C , will undergo negligible drying until the temperature rises to about -40°C after they are placed on the manifold. For a brief period of time, the rate of sublimation will be sufficient to keep specimen temperature low but as the dried shell increases in thickness, the obstruction to vapor flow will increase. Since the rate of heat input is constant and uncontrolled, being derived from radiation and conduction from the surrounding environment, specimen temperature will rise in order to maintain the rate of sublimation equivalent to the rate of heat input. During the later portion of the drying cycle, therefore, specimen temperatures may

not only rise well above -30°C , but may approach the melting point. It is probable that many failures to attain high recoveries of organisms following freeze-drying can be attributed to inadequate control of drying temperatures [73] .

When the drying temperature is above the eutectic zone, different organism can stand drying in different degree. Burlacu-E, et al. found that employing the same protective medium, *E. coli* cultures proved to be more easily affected by lyophilization and cryo-desiccation above eutectic zone as compared to *S. aureus* cultures, fact that may be due to the differences between the wall structures of G- bacteria and G+ bacteria, respectively [85] .

From the above discussion, one can see that freeze-drying is a multistage process and should be carefully adjusted to each individual case in order to get beneficial result.

2.5 Thin Film Analysis Methods

2.5.1 Scanning Electron Microscopy

In our rapidly expanding technology, scientists and engineers are often required to observe and correctly explain phenomena occurring on a micrometer and submicrometer scale. The scanning electron microscopy (SEM) is a relatively new and powerful instrument which permits the characterization of heterogeneous materials and surfaces on such a fine scale. Among the major types of information obtained are compositional information of both a qualitative and quantitative nature, and topographic information contained in images obtained at relatively high resolution. The SEM, with its large depth of field and easily interpreted images of samples that often require little or no sample preparation for viewing, is capable of providing significant information about rough samples at magnifications ranging from 50X to 100,000X [86]. This range overlaps considerably with the light microscope at the low end, and with the electron

microscope at the high end. A magnification of 100,000X with a spatial resolution better than 100Å can be obtained with SEM, while for optical microscopes, these figures are 1,000X and 10,000Å, respectively. SEM can give a depth of field 2-4µm at 10,000X magnification and 0.2-0.4mm at 100X magnification which are much deeper than those of optical microscopes.

The two major drawbacks of SEM are that a conducting sample is needed, and the experiment is conducted in a high vacuum meaning that hydrated samples cannot be observed. The first restriction can be overcome by gold sputtering of the sample to help eliminate problems of surface charging [87]. The second problem, can be solved by using a cryostage or a field-emission SEM.

In SEM, the specimen is irradiated by a finely-focused electron beam. This releases secondary electrons, backscattered electrons, characteristic X-rays, and several other types of radiation from a small part of the specimen. They are obtained from specimen emission volumes within the sample and are used to measure many characteristics of the sample (composition, surface topography, crystallography, etc.). This process is illustrated in Figure 4 [88].

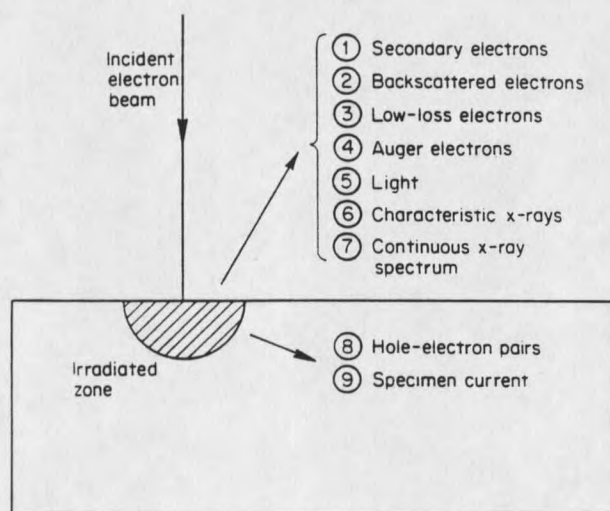


Figure 4. Signals from specimen in a SEM instrument

The intensity of these signals will depend in some way on the shape, chemical composition, and crystal orientation of the irradiated volume. There are two ways in which these signals can be used. Either the beam remains stationary at a point to give, for example, an X-ray analysis or an Auger electron analysis at that point, or it is scanned in a television raster to obtain information over an area [88].

A schematic diagram of a SEM instrument is illustrated in Figure 5 [88].

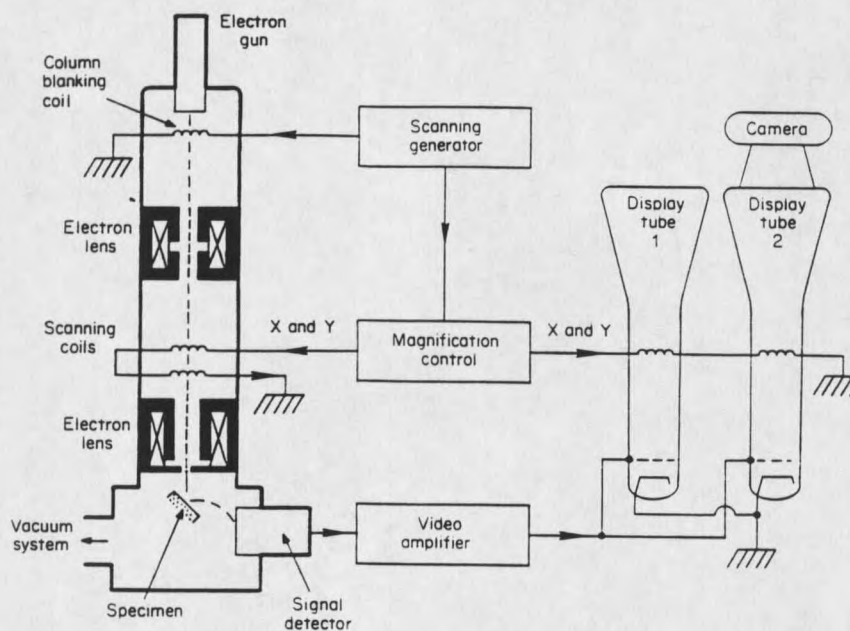


Figure 5. Schematic diagram of a SEM instrument

The electron column is on the left, and the scanning and display circuits are in the center and on the right. The electron column consists of an electron gun, demagnifying lenses, beam-defining apertures, and scanning coils [88].

The operation of SEM must be done in a vacuum. Because the operation of SEM required that the electron beam has unimpeded passage down the column, through the lenses and apertures, to collide with the specimen. It is not possible to

maintain a coherent electron beam down the column if a significant number of gas molecules are present. In addition, a poor vacuum results in a large number of gas molecules in the area of the electron-emitting cathode which may cause failure of the cathode.

The electron gun is the source of the electron beam. Electrons are drawn from a negative cathode and accelerated to an energy of 2 to 40keV toward an anode at ground potential. Almost all electron microscopes use one of three types of electron guns: (1) electron gun with thermionic emission from a tungsten cathode, (2) electron gun with thermionic emission from a lanthanum hexaboride (LaB_6) cathode, or (3) electron gun with field emission from a tungsten cathode [89].

Commercial SEMS can have either two or three lenses. These form a fine beam of electrons that can be moved over the surface of the specimen. A detector that is sensitive to the chosen output signal from the specimen is connected through a video amplifier to the grid of a cathode-ray tube that is scanned in synchronism with the beam on the specimen. In other words, the brightness at any point on the screen will depend on the strength of the signal from the corresponding point on the specimen. In this way, an image of the specimen surface is built up on the cathode-ray-tube screen point by point.

Generally, in the surface SEM, secondary electrons are collected to form the image. The secondary electron imaging works on the principle that the electron beam generates a "splash" of electrons with kinetic energies much lower than the primary incident electrons, called secondary electrons. Because of their low energies and low penetration depth, the detection of secondary electrons as a function of primary beam position makes it possible to attain high magnification and high resolution for imaging the areas of interest [90]. The secondary electron

image is very similar in appearance to a light optical image, except that there is no color and both the resolution and the depth of focus are greatly improved.

In the microanalyzer, characteristic X-rays are collected which can be related to the specimen composition.

2.5.2 X-ray Photoelectron Spectroscopy

Of all the contemporary surface characterization methods, X-ray photoelectron spectroscopy (XPS) is perhaps the most widely used. XPS is also called electron spectroscopy for chemical analysis (ESCA). The popularity of XPS as a surface analysis technique is attributed to its high information content, its sound theoretical basis and its easiness to use.

Surface analysis by XPS involves irradiating a solid in a ultra high vacuum with monoenergetic soft X-rays and analyzing the emitted electrons by energy. Because the mean free path of electrons in solids is very small, the detected electrons originate from only the top few atomic layers, making XPS a surface-sensitive technique. XPS is an information-rich method. In the outermost 10nm of a surface, XPS can identify all elements (except H and He) present at concentrations not less than 0.1%. It can semiquantitatively determine the approximate elemental surface composition (error $< \pm 10\%$). It can provide information about the molecular environment (oxidation state, bonding atoms, etc.) [9]. It can also analyze hydrated (frozen) surfaces with a cryostage. XPS is a soft analysis method. Sample damage usually won't occur during analysis. Hence XPS can be used to analyze surfaces of delicate powder material, polymers and organic coatings. Insulating samples are readily analyzed with charge compensation.

The drawback of XPS is that it is limited in its ability to provide detailed molecular information, because identification of unknown complex molecules by chemical shift is not possible. This is particularly true when atoms are in a wide

variety of chemical states, as exist in a protein. Also, the achievable lateral resolution is limited to a few micrometers. Static Secondary Ion Mass Spectrometry(SIMS) is both more surface sensitive ($\sim 20\text{\AA}$) and more chemically selective than XPS.

2.5.2.1 Principles of the Technique

The basic XPS experiment is illustrated in Figure 6. using carbon atom as an example.

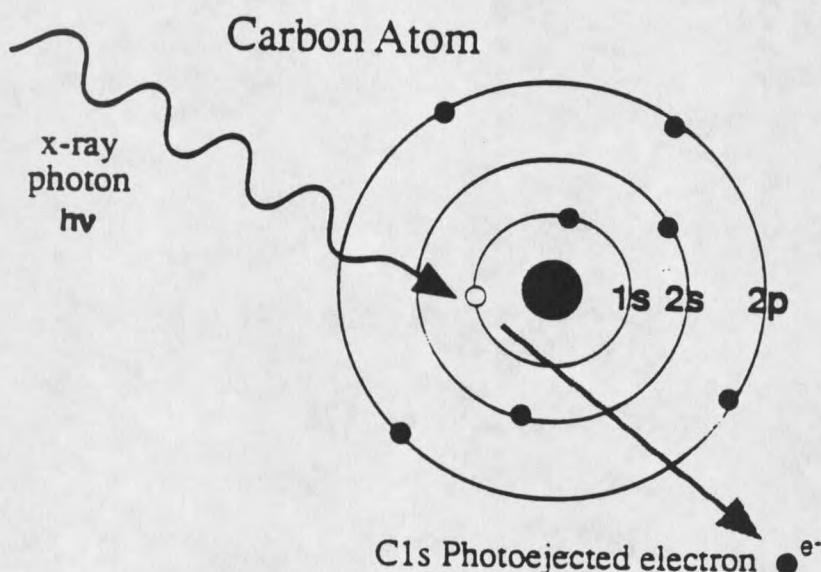


Figure 6. The physical basis of XPS

The surface to be analyzed is first placed in a vacuum environment and then irradiated with photons. For XPS, the photon source is in the X-ray energy range. The atoms comprising the surface emit electrons (photoelectrons) after direct transfer of energy from the photon to the core-level electron. These emitted electrons are subsequently separated according to kinetic energy and counted. The energy of the photoelectrons is related to the atomic and molecular environment

from which they originated. The number of electrons emitted is related to the concentration of the emitting atom in the sample. The basic physics of this process can be described by the Einstein equation.

$$E_B = h\nu - KE - \phi \quad (7)$$

In which E_B is the binding energy of the electron in the atom (a function of the type of atom and its environment), $h\nu$ is the energy of the X-ray source (a known value), and KE is kinetic energy of the emitted electron that is measured in the XPS spectrometer. ϕ is the work function of the XPS system which is corrected by the calibration procedure. From Eq.7, the binding energy, the quantity that provides us with valuable information about the photoemitting atom is easily obtained. Only electrons whose binding energy is less than the energy of the incident photon are emitted by photoemission effect.

The concept of binding energy of an electron in an atom requires elaboration. A negatively charged electron will be bound to the atom by the positively charged nucleus. The closer the electron is to the nucleus, the more tightly we can expect it to be bound. Binding energy will vary with the type of atom (i.e. a change in nuclear charge) and the addition of other atoms bound to that atom (bound atoms will alter the electron distribution on the atom of interest). Different isotopes of a given element have different numbers of neutrons in the nucleus, but the same nuclear charge. Changing the isotope will not appreciably affect the binding energy. Weak interactions between atoms such as those associated with crystallization or hydrogen bonding will not alter the electron distribution sufficiently to measurably change the binding energy. Therefore, the variations we see in the binding energy that provide us with the chemical information content of XPS are associated with covalent or ionic bonds between

atoms. These changes in binding energy are called binding energy shifts or chemical shifts. Typical C_{1s} binding energies are listed in Table 1 [9].

Table1. Typical C_{1s} Binding Energies

Functional group		Binding energy (eV)
hydrocarbon	$\underline{C}-H, \underline{C}-C$	285.0
amine	$\underline{C}-N$	286.0
alcohol, ether	$\underline{C}-O-H, \underline{C}-O-C$	286.5
Cl bound to carbon	$\underline{C}-Cl$	286.5
F bound to carbon	$\underline{C}-F$	287.8
carbonyl	$\underline{C}=O$	288.0
amide	$N-\underline{C}=O$	288.2
acid, ester	$O-\underline{C}=O$	289.0
urea	$\begin{array}{c} O \\ \\ N-\underline{C}-N \end{array}$	289.2
carbamate	$\begin{array}{c} O \\ \\ O-\underline{C}-N \end{array}$	289.9
carbonate	$\begin{array}{c} O \\ \\ O-\underline{C}-O \end{array}$	290.3
2 F bound to carbon	$-\underline{CH}_2-\underline{CF}_2-$	290.6
carbon in PTFE	$-\underline{CF}_2-\underline{CF}_2-$	292.0
3 F bound to carbon	$-\underline{CF}_3$	293-294

An XPS peak of an element may actually be combination of several gaussian type peaks each specific to a certain binding environment for that element. So upon resolving the peak of a given element, it is possible to locate the different binding states of the element in question. This is important when trying to determine the chemical structure of an unknown surface.

2.5.2.2 Instrumentation

A schematic drawing of a contemporary XPS instrument is shown in Figure 7 [9].

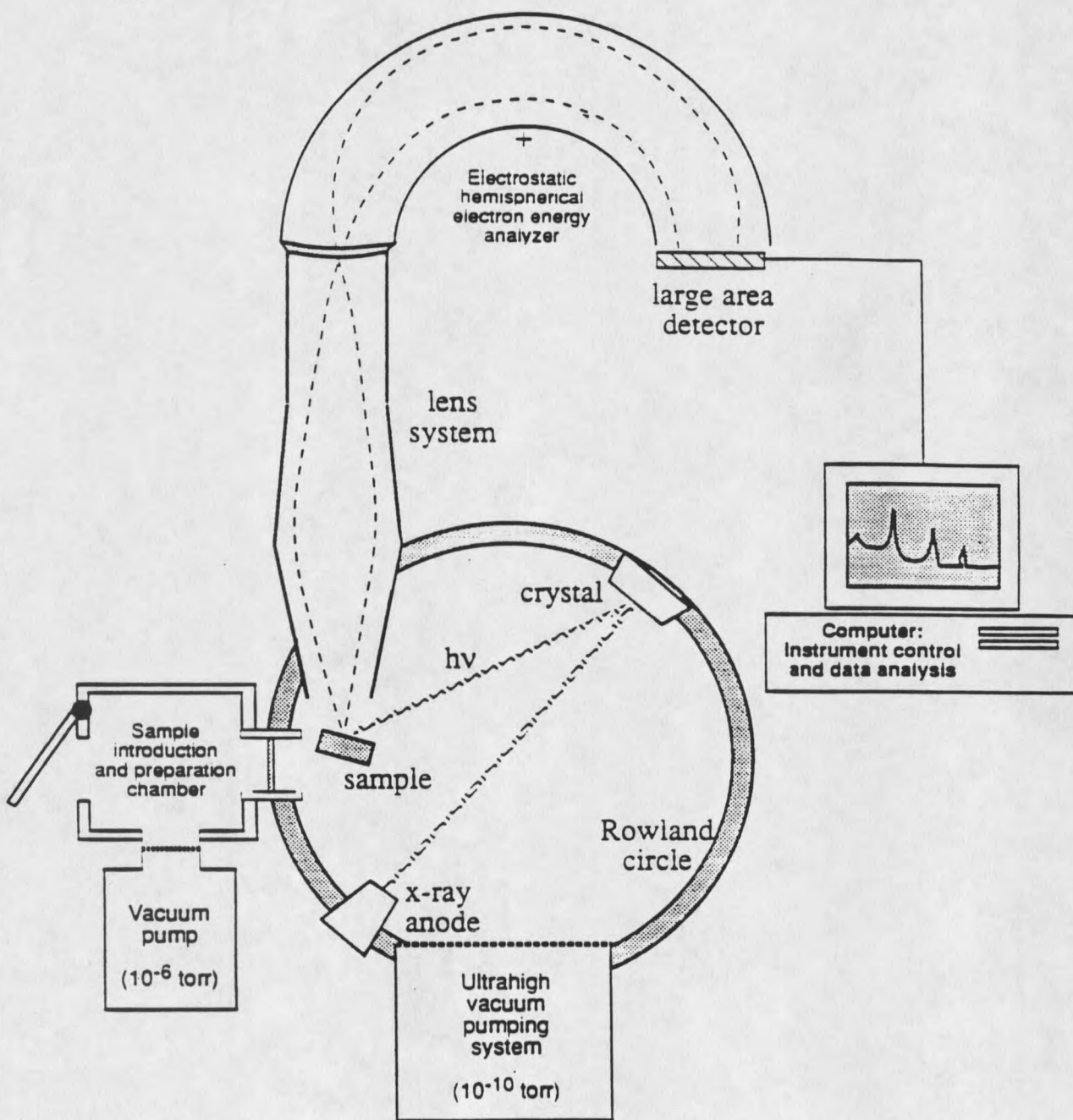


Figure 7. Schematic diagram of an XPS instrument

The basic components of the XPS instrument include vacuum systems, X-ray sources, analyzers, data systems and accessories.

The heart of the XPS instrument is the main vacuum chamber where the sample is analyzed. The XPS experiment must be done under vacuum for three reasons. First, the emitted photoelectrons must be able to travel from the sample through the analyzer to the detector without colliding with gas phase particles. Second, some components such as the X-ray source require vacuum conditions to remain operational. Third, the surface composition of the sample under investigation must not change during the XPS experiment. For most applications a vacuum of 10^{-10} torr is adequate.

X-rays for an ESCA experiment are usually produced by impinging a high-energy electron beam on a target. Core holes are created in the target material or anode, which in turn emits X-rays and electrons. These X-rays are used in the XPS experiment. Common anodes are Al and Mg which emit either Al K α or Mg K α X-rays respectively. High resolution spectra are obtained using monochromatized Al K α or Mg K α X-ray source.

The electron energy analyzer is the most crucial part of any XPS instrument. The function of the energy analyzer is to measure the number of photoejected electrons as a function of their energy and to disperse them across a detector or detector array. The most common type of energy analyzer is the electrostatic hemispherical analyzer.

Modern computers provide a powerful means for both controlling instrument operation and performing data analysis such as automatic peak fitting and quantification, depth profiling, and chemical state imaging, etc.. In general, as the speed and power of computer systems increase, so do the capabilities for XPS data acquisition and analysis.

In addition to the basic requirements discussed above, most modern XPS spectrometers will also be equipped with almost limitless accessories which depend on the applications that are planned for the system. Common accessories include ion guns, electron guns, gas dosers, and quadrupole mass spectrometers. For monochromatized XPS systems, the most important accessory is the low energy electron flood gun, which is required to obtain high-quality spectra from insulating material [9]. A more detailed discussion of instrumentation can be found elsewhere [91, 92].

2.5.3 Static Secondary Ion Mass Spectrometry

Secondary ion mass spectrometry (SIMS) is a technique used to determine the molecules present on a surface. It is the mass spectrometry of ionized particles which are emitted when a surface, usually a solid is bombarded by energetic primary particles. The primary particles may be electrons, ions, neutrals, or photons. The emitted (so-called secondary) particles will be electrons; neutral species, atoms, and molecules; atomic and cluster ions. It is the secondary ions which are detected and analyzed by a mass spectrometer and separated by their mass to charge ratio which constitute the mass spectrum characteristic of the chemical composition and structure of the analyzed surface. SIMS is a surface analytical technique having all the advantages of mass spectrometry.

SIMS can be operated in static mode as well as dynamic mode. In static SIMS (SSIMS) it is the intention to get information on the composition of the uppermost monolayer, virtually without disturbing its composition and structure. This is achieved by very low primary ion current densities, so that secondary ions are emitted from areas not previously damaged, and the surface monolayer lifetime is in the order of some hours, well in excess of the time required for analysis. Because secondary ion is emitted from only the top few atomic layers of a surface,

SSIMS is very surface sensitive. The information depth of SSIMS is $\sim 20\text{\AA}$ within the sample surface. Insulating samples can be analyzed with charge compensation.

Compared with other widely applied surface analytical techniques, such as Auger spectroscopy, static SIMS offers some unique features which are isotope sensitivity; hydrogen sensitivity; direct compound detection by molecular secondary ion emission; extremely high sensitivity for many elements and compounds, very often in the ppm range [93].

2.5.3.1 Principles of The Technique

Figure 8 schematically outlines the mechanism of SIMS as it is presently understood [94].

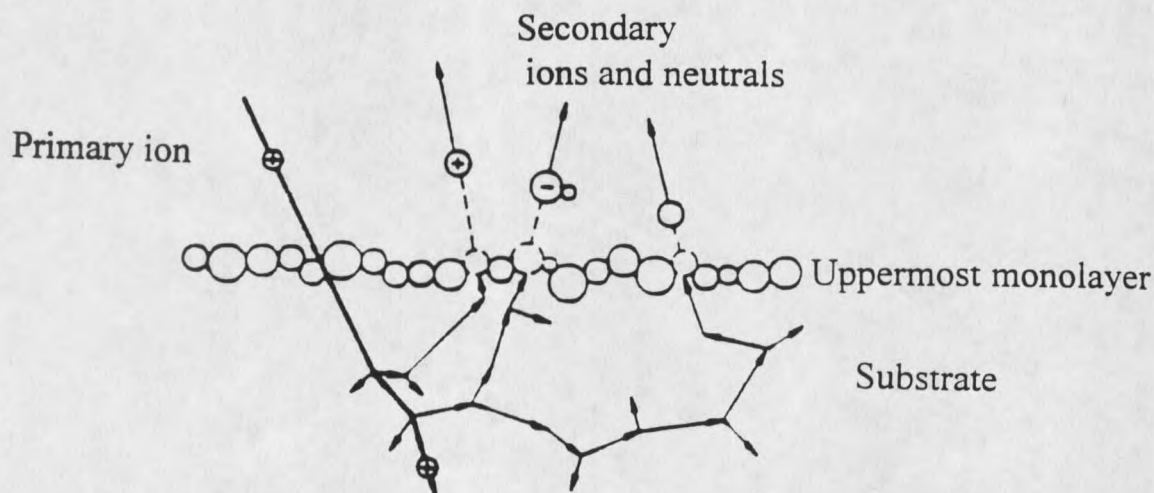


Figure 8. Diagram of the SIMS phenomenon indicating the collision of the primary particles with a solid surface and the emission of secondary particles.

In the case of ion bombardment the energy of the primary ion beam, usually Xe^+ or Ar^+ ion beams is transferred to the atoms in the solid by a collision, a

billiard-ball-type process. A cascade of collisions occurs between the atoms in the solid; some collisions return to the surface and result in the emission of atoms and atom clusters (a process known as sputtering), some of which are ionized in the course of leaving the surface and can be analyzed with a mass spectrometer.

Most recoil particles have low energies and only cascade particles from near-surface regions-with momenta directed toward the surface- can overcome the surface binding energy and thus leave the target (sputtered particles). SIMS, therefore, is a very surface-sensitive analytical technique (information depth is <3 monolayers).

The drawback of SIMS is lack of quantitation because the stability of ions is a major factor affecting peak intensities. Cyclic ions, ions with aromaticity, and ions with electron-withdrawing groups or heteroatoms, all exhibit relative intensities greater than what would be expected from simple stoichiometry. Also the charge state of the emitted atomic or molecular particles depends strongly on the chemical environment of the sputtered species. By changing this chemical environment, going from a pure metal to, for example, an oxide, the ionization probability of the same species, for example, a metal atom may be changed by several orders of magnitude. So these make quantification of atomic concentration directly from peak intensities impossible, although the analog signal from the detector produces peaks whose intensities are proportional to the abundance of the ions. Quantification can be achieved by semiempirical ionization models or by the additional application of analytical techniques such as XPS, AES (Auger electron spectroscopy) or RBS (Rutherford backscattering spectroscopy). Especially in static SIMS the combination of a number of such complementary surface analytical techniques is considered as an optimum approach [93].

Mass to charge ratio, m/z , is the ratio of the mass number (m) of a given particle to the number (z) of electrostatic charge units (e) carried by the particle.

Thus, m/z is a dimensionless ratio that is the parameter measured by the mass analyzer. The molecular ion results from ionization of the analyte molecule. This ion represents the intact molecule and is the ultimate precursor of all the fragment ions composing the mass spectrum of the analyte molecule. The molecular-ion peak appears at an m/z value numerically equal to the nominal molecular weight of the compound. The molecular-ion together with its fragment ions constitute the mass spectrum of the analyte molecule, hence represent the fingerprint of the analyte which can be used to identify it in a mixture. Doubly charged ions are insignificant in the mass spectra of most compounds. However, for those molecules that can produce stable, doubly charged ions, the corresponding peaks in the mass spectrum can be used to advantage in data interpretation. To interpret the spectra and elucidate the fragmentation process, nitrogen rule, isotope patterns, rings plus double bonds rule, and fragmentation rules such as homolytic or heterolytic cleavage; σ -bond cleavage; rearrangement that are known from electron impact mass spectrometry can be applied [95].

The analysis of insulating surfaces using SIMS is more difficult than conducting surfaces because of the charging of the sample during analysis. In common with all methods of surface analysis involving either incoming or outgoing charged particles, surface charging of insulators can be a problem. The SIMS process usually involves bombardment with primary positive ions, which gives rise to the emission of secondary ions and neutrals and large numbers of secondary electrons. The predominant species will usually be electrons and neutrals, thus with incoming positive ions insulating surfaces usually charge positive. The main effect of this charging is that positive ions are given further energy, accelerating them beyond the acceptance energy of the analyzer. The mass resolution of the spectra may be effectively decreased because of serious charging. In the case of negative secondary ions positive surface potential will inhibit or

totally suppress their emission. Because of this, the analysis of polymer surfaces is more difficult than conducting surfaces, since most polymers are insulators. To correct the charging problem, the polymer sample must be flooded by a negatively-charged beam such as a low energy electron beam to equalize the charge on the sample. And also the sample surface must be as smooth as possible to let the built-up positive charges dissipate effectively.

2.5.3.2 Instrumentation

1) Vacuum systems

SIMS experiments are performed in high vacuum for two separate reasons: first, to avoid scattering of the primary and secondary beams; second, to prevent interfering adsorption of gases on the surface under investigation.

2) Ion guns

For static SIMS, generally a broad beam source is used, unless analysis of specific features is required. A beam diameter of 1 mm - 1 cm is common (larger areas present problems in secondary ion collection), and uniform current density is desirable. Beam currents of 10^{-10} ~ 10^{-8} A are used giving monolayer lifetimes > 100s. The beam energy lies between 500eV and 5keV. The lower values simplify construction of the gun and power supplies and cause less disruption in the surface and sub-surface of the sample. Less disruption gives more confidence that the analysis is representative of the original surface, but also means a low sputter rate. High beam accelerating voltages give higher sputter rates, and also give higher beam currents from a given ion source. Higher values of beam energy therefore contribute to a more rapid analysis. The beam species must also be sufficiently heavy ($m > 30$), to produce efficient sputtering, and the inert gasses argon and xenon are often chosen to preclude chemical modification of the surface [96].

The primary ion source is typically Ga^+ , Cs^+ , or Xe^+ . All of these ion sources have applications which are well suited to their specific characterizations. Cesium and Xenon are very heavy ions which cause a large collision cascade when they impact the surface. Gallium is a lighter ion, but it can be finely focused, so it is a very good choice when imaging applications are desired.

3) Mass spectrometer

The other essential part of a SIMS system is a mass spectrometer to measure the mass of the secondary ions sputtered from the sample. The two typical types of mass analyzer used with SIMS application are quadrupole mass analyzer and time of flight mass analyzer. The quadrupole mass analyzer uses DC and RF voltages to selectively allow only ions of a certain mass to be transmitted from the ionization chamber to the detector. By increasing the DC and RF fields whilst maintaining a constant ratio between them, this resonant condition is satisfied for ions of each ascending mass in turn, allowing the collection of a complete mass spectrum. the drawback of this mass spectrometer is low transmission rate (1%), hence low sensitivity. The mass resolution can only achieve unit resolution. This means that different compounds with the same nominal mass cannot be distinguished from each other.

The second type of mass analyzer is the time of flight (TOF) mass analyzer. The working principle of this mass filter is that when ions are accelerated to a given potential so that they have the same kinetic energy then ions of different mass/charge ratio will have different velocities. If these ions pass through a region of field-free drift space, they will spread out in time, with the higher mass ions arriving later. A time-sensitive detection system is then all that is needed to produce a mass spectrum. Because all ions are transmitted from the ion source to the detector, the sensitivity is higher than that of quadrupole mass analyzer

(e.g. 10^5 over a quadrupole). Mass range is theoretically unlimited, but at the expense of long flight times, which means slow pulsing frequencies and hence long analysis times [96]. In practice, the resolution limit of a time of flight instrument is about 10,000.

Figure 9 is a schematic drawing of a TOF-SIMS instrument.

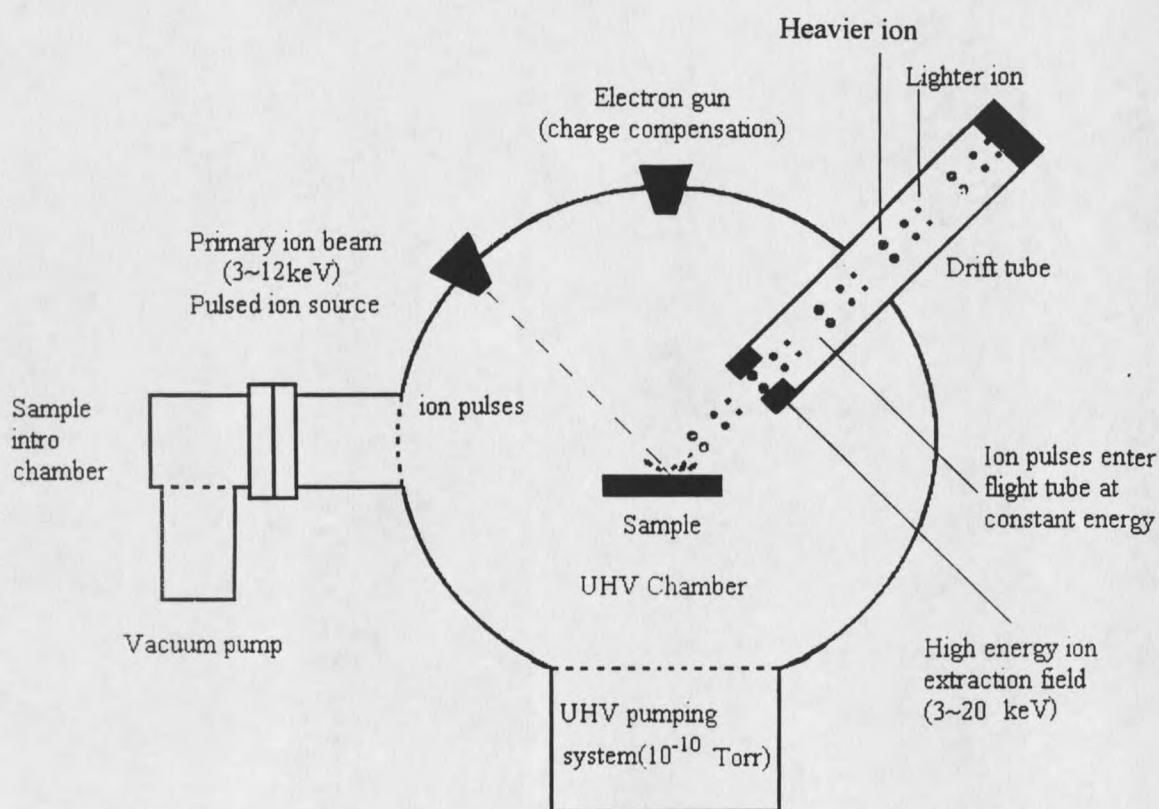


Figure 9. Schematic diagram of a TOF-SIMS instrument

2.5.3.3 Applications

There are two basic areas of application of the static SIMS technique. As a mass spectrometric method, static SIMS can be used to desorb and ionize biomolecules from specially prepared surfaces. Benninghoven et al. pioneered this use of static SIMS, and the method has been widespread use for the analysis of thermally labile molecules.

As a surface analysis method, static SIMS is unrivaled in its molecular selectivity, because of its basis in mass spectrometry. Static SIMS has been used to analyze a wide variety of 'real' surfaces, ranging from semiconductor material to complex copolymers. The spectra produced reflect the surface chemistry of the material but, in general, do not contain large ($m/z > 500$) molecule ions or fragments. However, useful information is readily extracted from the fragment ions in this lower mass range [12]. Static SIMS has been widely applied to structural characterization of polymers by using fingerprint spectra or fragments characteristic of the backbone and pendant groups. This includes the characterization of the surface chemistry of polymers of biomedical importance [97,98]. The ability of static SIMS to produce a surface-sensitive mass spectrum gives it great potential as a probe of proteins adsorbed on surfaces [12]. The application of SIMS for the study of the interaction of polymers with biological molecules and systems has been demonstrated by Mantus, D.S. and co-workers. In their work, a spectral interpretation protocol was established by examining homopolymers of 16 amino acids. Table 2 is a summary of the poly (amino acid) and Table 3 shows the major positive ions in the SIMS spectra of these amino acid [12]. This protocol allowed for assignment of peaks unique to the various amino acids. Consequently, it was used to study proteins adsorbed on polymer surfaces. They also demonstrated that the intensities of peaks in a static SIMS spectrum might be sensitive to the conformation of a macromolecule on a surface, because

of the surface sensitive characteristics of static SIMS. This advantage makes static SIMS an unrivaled surface analytical technique to study macromolecules on a surface such as cell surface of a microorganism. The conformation of a macromolecule such as a protein or glycoprotein may be closely related to its specific function such as adhesion. Some peak assignments in the results and discussion parts of this study were based on Mantus's work. The poly (amino acid) they used have the general structure $(-\text{NH}-\text{CHR}-\text{CO}-)$. Corresponding R groups, abbreviations and the m/z of the R group are listed for each amino acid in Table 2.

Table 2. A summary of the poly (amino acids)

amino acid	abbrev	R	m/z of R
glycine	Gly, G	-CH	1
alanine	Ala, A	-CH ₃	15
valine	Val, V	-CH(CH ₃) ₂	43
leucine	Leu, L	-CH ₂ CH(CH ₃) ₂	57
serine	Ser, S	-CH ₂ OH	31
methionine	Met, M	-CH ₂ CH ₂ SCH ₃	75
glutamic acid	Glu, E	-CH ₂ CH ₂ COOH	73
aspartic acid	Asp, D	-CH ₂ COOH	59
lysine	Lys, K	-(CH ₂) ₄ -NH ₂	72
ornithine	Orn, O	-(CH ₂) ₃ -NH ₂	58
arginine	Arg, R	-(CH ₂) ₃ -NHC(NH ₂)NH	100
proline	Pro, P		42
phenylalanine	Phe, F		91
tyrosine	Tyr, Y		107
tryptophan	Trp, W		130
histidine	His, H		81

Table 3. Summary of major positive ions in the SIMS spectra of homopolymers of 16 Amino Acids*

AA	I	I-H ₂	<u>other</u>						
Gly	30(100)	28(2)							
Ala	44(100)	42(12)							
Val	72(100)	70(6)							
Leu	86(100)	84(10)	44(32)	30(33)					
Pro	70(100)	68(24)							
Phe	120(43)	118(6)	91(100)	77(36)	58(47)	56(26)	44(35)	42(50)	30(28)
Tyr	136(40)	134(8)	107(100)	91(83)	77(72)	44(36)	30(930)		
Trp			130(56)	77(100)	44(62)	30(46)			
His	110(91)	108(9)	82(62)	81(100)	44(8)	30(15)			
Ser	60(78)	58(24)	44(100)	30(46)					
Met	104(18)	102(11)	91(23)	61(100)	56(53)	44(16)	42(17)	30(15)	
Lys			84(18)	68(3)	56(24)	44(9)	30(100)		
Arg	129(3)	127(4)	128(4)	97(25)	73(32)	70(100)	60(25)	44(48)	30(53)
Orn			70(100)	44(17)	30(95)				
Asp	88(80)	86(12)	72(100)	62(69)	44(19)	30(18)			
Glu	102(15)		84(64)	56(100)	44(86)	30(69)			

*Relative intensities are given in parentheses. Peaks indicative of hydrocarbons, residual solvent, and sodium have been excluded from this summary. I= immonium ion. These summaries include only ions that are believed to contain single amino acid residue information.

2.6 Statistical Analysis Methods

2.6.1 Principal Components Analysis

Today's laboratory instruments produce a plethora of data. It is not uncommon for processes to have hundreds or thousands of measured variables. Some analytical instruments measure tens of thousands of variables. For instance, in a typical FTIR spectrometer the absorbance is measured at over 10,000 frequencies. Chemical processes are becoming more heavily instrumented and the data is recorded more frequently. This is creating a data overload, and the result is that a good deal of the data is "wasted", i.e., no useful information is obtained

from it, The problem is one of both compression and extraction. Generally, there is a great deal of correlated or redundant information in laboratory and process measurements. This information must be compressed in a manner that retains the essential information and is more easily displayed than each of the variables individually. Also, often essential information lies not in any individual variable but in how the variables change with respect to one another, i.e., how they co-vary. In this case the information must be extracted from the data.

Principal Components Analysis (PCA) is a favorite tool for data compression and information extraction. PCA finds combinations of variables, or factors, that describe major trends in the data. Mathematically, PCA relies upon an eigenvector decomposition of the covariance or correlation matrix of the process variables. Principal components are nothing more than the eigenvectors of a variance-covariance or a correlation matrix. Principal Components Analysis is shown graphically as in two-dimensional cases in Figure 10. Shown here are the values of two variables measured on a collection of samples. It is apparent that the samples can be enclosed by an ellipse. It is also apparent that the samples vary more along one axis of the ellipse than along the other. The first PC describes the direction of the greatest variation in the data set, which is the major axis of the ellipse. The second PC describes the direction of second greatest variation, which is the minor axis of the ellipse. In this case, a PCA model with two principal components adequately describes all the variation in the measurements. The ellipse is defined by the variance-covariance matrix calculated from the original data set. So the inherent correlations between the original data can be better viewed along the dimensions defined by principal component axes than along the original axes. Principal components are the eigenvectors of the variance-covariance matrix of the original data set. Each original observation can be converted to what is called a principal component score by projecting it onto the principal axes. The elements of

the eigenvectors that are used to compute the scores of observations are called principal component loadings. Actually the scores are the new locations in the dimensions defined by principal axes. More detailed description of the Principal Components Analysis can be found elsewhere [99].

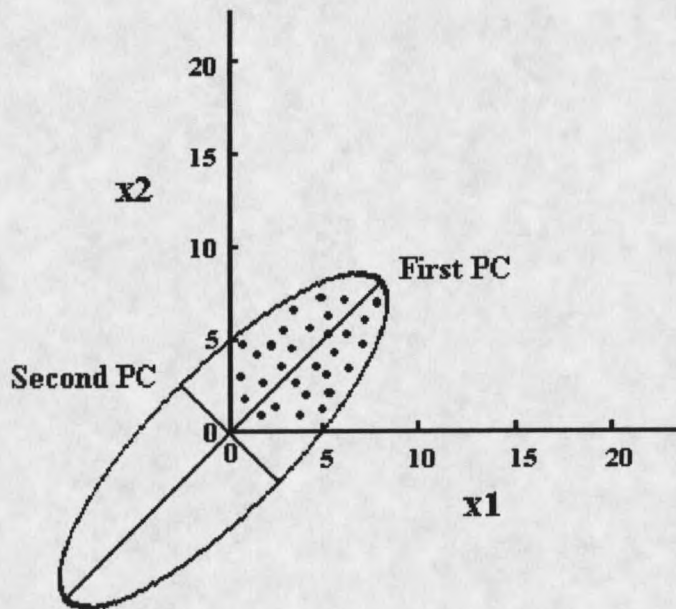


Figure 10. Graphical representation of Principal components Analysis shown for two-dimensional cases. Original data can be enclosed in a ellipse. The first PC accounts for most of the total variance, the second PC for the rest variance of the data.

2.6.2 Linear Discriminant Function Analysis

Discriminant Function Analysis consists of finding a transform which gives the minimum ratio of the difference between a pair of group multivariate means to the multivariate variance within the two groups. If the two groups are regarded as consisting of two clusters of points in multivariate space, Discriminant Function Analysis will search for the one orientation along which the two clusters have the greatest separation while simultaneously each cluster has the least inflation. A

linear discriminant function transforms an original set of measurements on a sample into a single discriminant score. That score, or transformed variable, represents the sample's position along a line defined by the linear discriminant function. The discriminant function can therefore be thought of as a way of collapsing a multivariate problem down into a problem which involves only one variable. This can be graphically shown for two-dimensional cases, as in Figure 11 [99]. An adequate separation between group A and B cannot be made using either variable X_1 , or X_2 . However, it is possible to find an orientation along which the two clusters are separated the most and inflated the least. The coordinates of this axis of orientation are the linear discriminant function [99].

To find the set of coefficients of the discriminant function, the following equation can be solved:

$$[\lambda] = [S_p^{-1}] [D] \quad (8)$$

in which the elements of the column vector $[\lambda]$ are coefficients of the discriminant function, $[S_p^{-1}]$ is the inversion matrix of an $m \times m$ matrix of pooled variances and covariances of the m variables. $[D]$ is the column vector of m differences between the means of the two groups. The set of λ coefficients are entries in the discriminant function equation of the form:

$$R = \lambda_1 \psi_1 + \lambda_2 \psi_2 + \dots + \lambda_m \psi_m \quad (9)$$

This is a linear function; that is, all the terms are added together to yield a single number, the discriminant score. Every observation in the analysis can be entered into the equation by substitution of ψ to get its discriminant score and its position along the discriminant function will be located according to its

discriminant score. More detailed description of the Linear Discriminant Function Analysis can be found elsewhere [99].

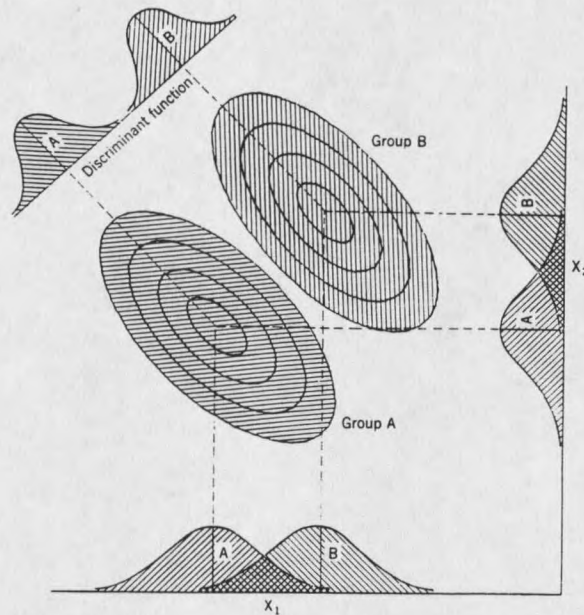


Figure 11. Plot of two bivariate distributions, showing overlap between groups A and B along both variables x_1 , and x_2 . Groups can be distinguished by projecting members of the two groups onto the discriminant function line.

CHAPTER 3

MATERIALS AND METHODS

3.1 Method for Cleaning Glassware

All glassware used in these studies was cleaned by the following processes.

A solution named "base bath" was used for a basic solution to clean the glassware. Base bath was made by the following recipe. Potassium hydroxide pellets were dissolved in 500ml nanopure water. The pellets were added until the solution was saturated. When a saturated solution of potassium hydroxide/water was obtained, it was added to 2 liters of ethanol until the color of the mixture turned from clear to orange and no more water would dissolve into the ethanol. That means the saturation point of the ethanol/water/Potassium hydroxide solution was reached. After 1 or 2 days, this solution would turn dark brown or even black. This solution was then used as base bath. Caution should be taken when preparing this solution because all mixing processes are exothermic. All mixing processes should be done slowly to let heat dissipate efficiently. Also, base bath is very corrosive and care should be taken during handling to avoid being hurt.

A no-chromix/sulfuric acid solution was used as acidic solution to clean the glassware. No-chromix is an inorganic oxidizer and when mixed with sulfuric acid is a metal-free cleaning solution.

Glassware was then cleaned by soaking in base bath for at least 3 hours followed by being rinsed with nanopure water. The glassware was then placed into the no-chromix/sulfuric acid solution for at least 3 hours. Then the glassware was thoroughly rinsed with nanopure water, covered with tinfoil, and then placed into a 110 ~120°C oven to be baked dry.

3.2 Yeast Culture and Harvest

C. albicans strain1 (CA1) was a clinical isolate received from the collection of Dr. Diane Brawner of Montana State University. It is a serotype B organism [100]. Suspensions of yeast cells in glycerol were prepared and placed into -70°C freezer for usage as freezer stock. A loopful of the freezer stock was removed once a month and streaked onto Sabaroud-dextrose agar (SDA) plates. The plates were incubated for 48 hours at 35°C . Isolated colonies were removed from the plates and streaked onto SDA slopes. The slopes were incubated for 48 hours at 35°C . The slopes were then stored at 4°C in the refrigerator for one month and used as refrigerator stock.

Liquid media were prepared as follows. Yeast extract at 0.6% (w/v), peptone at 1% (w/v), and a carbon source of either glucose or galactose at 5% (w/v) were dissolved in nanopure water and thoroughly mixed. 100 milliliters of the solution was placed into 250ml Erlenmeyer flasks and autoclaved for exactly 15 minutes. Sterilized media were stored at room temperature until usage. Media were not stored for a period exceeding three weeks.

Yeast inoculation into the liquid medium was achieved by aseptically removing a loopful of yeast from the refrigerator stock slope. The inoculated medium was then incubated for 24 hours in a shaking incubator set at 35°C and 160rpm. After 24 hours of growth, 0.3ml of cell/medium suspension was removed aseptically and transferred into fresh medium. This flask was then incubated again for 24 hours at 35°C and 160rpm. At the end of the second incubation period, the yeast cells were harvested.

Yeast cells were harvested by removing 4ml of yeast/medium suspension and placing it into a glass culture tube. The suspension was centrifuged for 2 minutes to pellet the yeast cells. The supernatant was discarded, and 2ml of ice-

cold PBS (phosphate-buffered saline, PH 7.2, 0.01M) was added to the culture tube containing yeast pellets. The tube was then spun for 1 minute to wash cells in PBS and then the tube was centrifuged for 2 minutes to pellet yeast cells. The supernatant was discarded. This wash step was repeated three times. At the end of the third wash, the supernatant was discarded, and the cells were suspended in 5 ml ice-cold PBS for freeze-drying experiments, or 2ml in ice-cold PBS for flow cell experiments. This cell/buffer suspension was kept cold by ice or in the 4°C refrigerator during every experiment.

For flow cell experiments, 200 μ l of the cell/buffer suspension was removed from the 2ml cell/buffer suspension, diluted 1:500 with PBS, and counted with the use of a hemacytometer and a microscope (450x). Cell counts were recorded, and the concentration of the 2ml cell suspension was calculated. Before making 100ml cell/buffer suspension with concentration of 10^6 cells/ml, 99ml cold PBS from refrigerator stock was placed into a 250ml Erlenmeyer flask and degassed by a vacuum pump (Sargent-Welch Co., model 1400). This was done by connecting the flask and the pump inlet by a rubber tubing and running the pump for 15 minutes at room temperature. After 15 minutes, the flask was still cold. An amount of the concentrated yeast/buffer suspension from original 2ml suspension in the culture tube to achieve 100ml of the desired concentration (10^6 cells/ml) was added to the 99ml cold degassed buffer. Then an amount of cold buffer was added to this cell/buffer suspension to achieve the final 100ml of the total volume. So the cell/buffer suspension to be used in the flow cell experiments was 100ml cell/buffer suspension with concentration of 10^6 cells/ml (For high flow rate experiments, 100ml is not sufficient to finish the experiments, the same method was used to make cell/buffer suspension with concentration of 10^6 cells/ml and desired volume). The flask was then placed into a 600ml beaker containing an ice/water slurry to keep the suspension at 4°C and ready for flow cell experiments.

3.3 Method for Making Dry Ice-Acetone Slush

Dry ice-acetone slush used for freeze-drying experiments was prepared by the following recipe.

Two pounds of dry ice was finely crushed. This was done by placing the solid mass of dry ice in a sack and crushing it with a hammer. The dry ice should be finely divided because the rate of cooling is proportional to the surface, not to the mass, of the ice. The only purpose of acetone is to transfer heat promptly and efficiently. Then the dry ice was transferred into a metal pan. Approximately 400ml acetone was then added slowly and carefully to the pan. There would be an initially violent ebullition of CO_2 , and acetone vapor as the mixture cooled down, and there might be some splashing of the mixture over the top of the pan. This was the point at which care was required. The early, violent bubbling would soon cease as the mixture became increasingly cold.

The bath was ready when the contents appeared slightly syrupy in consistency, and when the rate of bubbling was very low. The bath was now at a temperature of -75°C , and ready to use.

3.4 Preparation of FEP Samples

FEP (Teflon) samples were prepared from thin film obtained from Dr. Timothy Minton of Montana State University. The film was cut into 1cm square pieces. The square pieces were cleaned by rinsing with nanopure water, then placing into HPLC grade methanol (Fisher Scientific) in a clean beaker. The beaker was placed into a ultrasonic bath and the FEP samples were ultrasonically cleaned in methanol for 30 minutes. Then the methanol in the beaker was removed by using a clean pipet connected to a rubber aspirator. Then the samples were taken out, put into fresh methanol in another clean beaker and cleaned

ultrasonically again for 1 hour. After 1 hour, the methanol was removed by using a clean pipet connected to a rubber aspirator. The samples in the beaker were then dried overnight before usage. Eight samples were prepared at one time, and they were used within one week of their preparation. If the samples had been stored for more than one week, they were cleaned again.

The FEP samples prepared by this method were examined by XPS to check their cleanness, as well as surface chemistry of the FEP film.

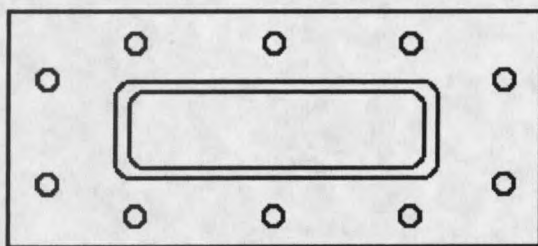
3.5 Flow Cell Experiments

Flow cell experiments were conducted with the use of a Teflon flow cell designed by Kevin J. Siedlecki, a previous graduate student of Montana State University [100]. A diagram of the flow cell is shown in Figure 12. The base of the flow cell was machined from polytetrafluoroethylene (PTFE, Teflon).

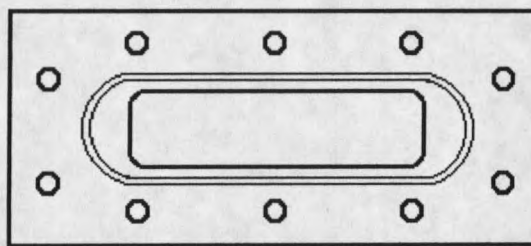
A flow channel with dimensions of 48mm long, 12mm wide, and 0.6mm deep was machined in the PTFE base. Two 1cm x 1cm squares were recessed 1mm into the floor of the flow channel to allow placement of the FEP samples. To mount FEP samples, the following procedure was used.

Glass microscope slides (Clay Adams) were cut into 1cm x 1cm squares using a diamond tip pencil. The thickness of these slides was 0.95~1.05mm. FEP squares were mounted on the glass squares with 3M double-sided adhesive tape. Then the glass squares with FEP samples were placed into the two recessed wells of the flow cell. These samples fit snugly into the wells and were even with the floor of the flow cell.

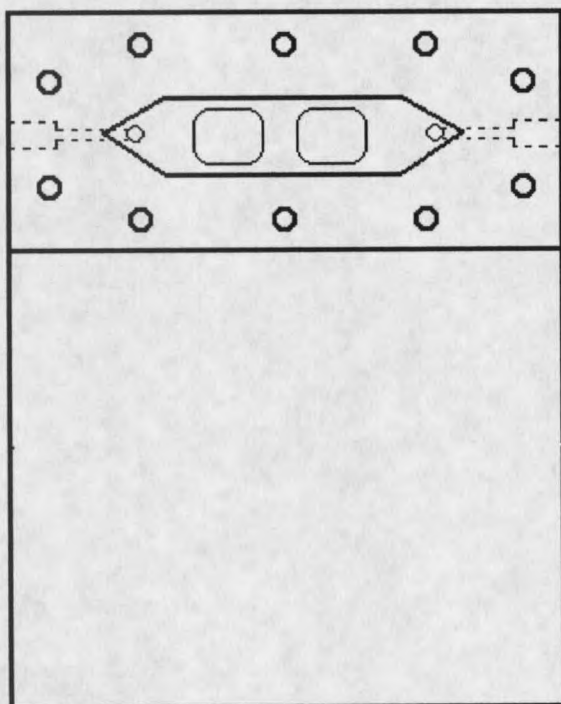
The top of the flow channel was completed by placing a #2 glass cover slip of size 24 x 60mm on top of the recessed channel. An aluminum top plate was



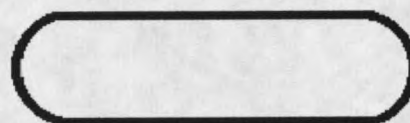
Aluminum Top Plate w/Viewing Port



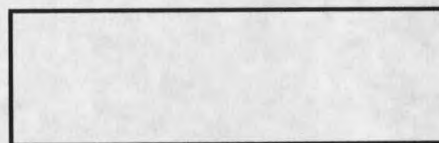
Aluminum Top Plate (Underside)



PTFE Base Plate

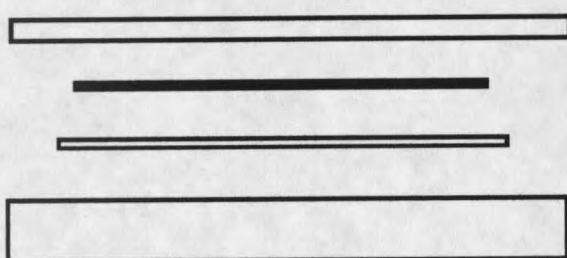


Viton O-ring



Glass Cover Slip

Figure 12a. Top view diagram of the flow cell constructed.



Aluminum Top Plate

Viton O-ring

Glass Cover Slip

PTFE Base Plate

Figure 12b. Side view of the flow cell as assembled.

then screwed onto the flow cell with the use of bolts coming through the top of the plate, and nuts placed into recessed grooves on the underside of the flow cell. The aluminum top plate had a rectangular viewing port with beveled edges to allow insertion of a microscope objective lens into the port for real time observation of the adhesion process. A recessed ovular groove on the inside of the aluminum cover plate allowed placement of an Viton O-ring to securely and evenly press the glass cover slip onto the PTFE base. For experiments done under high shear rates, the top of the flow cell was modified by adding a Poly (Vinyl Chloride) cover slip of similar dimensions on top of the glass covers slip. This allowed high shear rates to be achieved with minimal leakage in the system. Flow entered the flow channel through two 1/16" circular tubes machined in each end of the flow channel which connected to Swagelok fittings. The flow system was completed as illustrated in Figure 13.

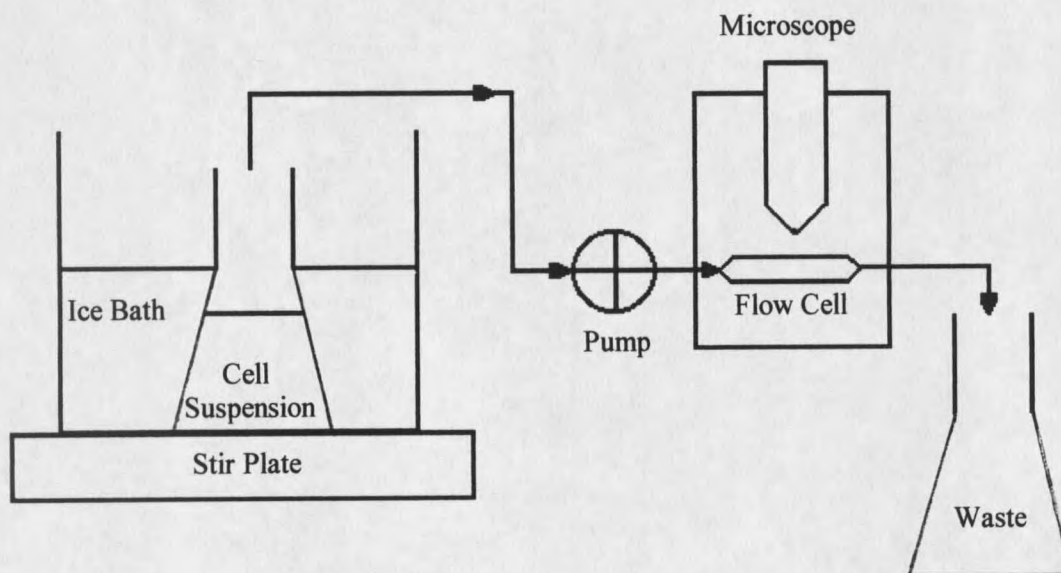


Figure 13. Schematic drawing of the flow cell experiment system

A Masterflex peristaltic pump with a 1-100 drive rating in combination with a three roller, #14 pump head was used. A 8 in. length of Tygon grade size 14 tubing was used in the pump. The rest tubing of the system was FEP Teflon tubing (1/16" OD). The Teflon tubing was slid into the Tygon tubing and held firmly by placing four cable ties around the Tygon tubing to connect the two tubing together. The Teflon tubing was connected to the flow cell using stainless steel Swagelok fittings.

The flow cell, the two glass squares, a stir bar and the glassware used in flow cell experiments were base/acid cleaned and baked dry as described previously and then stored in the 4°C refrigerator before each experiment. All Teflon tubing was cleaned with base bath, rinsed with nanopure water, and placed in the 4°C refrigerator before each experiment. The used Tygon tubing was discarded and changed to a new one after each experiment. It was connected to the cleaned Teflon tubing by cable ties and also placed in the 4°C refrigerator before each experiment. The Swagelok fittings were cleaned in base bath, rinsed with nanopure water, and cooled to 4°C before usage.

Flow dynamics in the system was analyzed and a flow regime similar to plug flow was observed which correlated well to the low Reynolds number in the system (1.5 to 43.5) [100]. The velocity regime of a laminar flow in a channel is described by Eq.10 and Figure 14.

$$V = V_{\max}[1-(x/\sigma)^2] \quad (10)$$

where x equals the distance from the channel centerline, and σ is one-half the channel thickness. Shear rate is defined as the local velocity gradient, therefore at the surface where the yeast cells adhere, it is the local velocity gradient at the wall. The velocity gradient is calculated by taking the derivative of Eq.10 and

using the relationship for laminar flow that the average velocity is one half of the maximum velocity. At the wall, the position x equals one half of the channel thickness. These relationships yield the equation:

$$\text{Shear rate} = \left. \frac{dV}{dX} \right|_{x=\sigma} = 4 \frac{V_{avg}}{\sigma} = \frac{2Q}{W\sigma^2} \quad (11)$$

In which Q is the volume flow rate in the flow channel and W is the width of the channel.

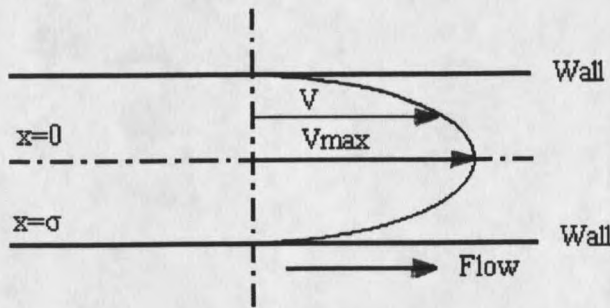


Figure 14. Schematic drawing of flow regime of a laminar flow in a channel

Shear rate has the units of inverse seconds. Shear rate is proportional to the shear force on the adherent yeast cells. Experiments were performed at shear rates of 28, 46, 71, 120, and 350sec⁻¹.

At the beginning of each experiment, the ice-cold cell-free PBS was pumped through the flow cell for about 5 minutes. This allowed for adjusting the flow rate of the system, elimination of any air bubbles in the system and focusing of the microscope. The yeast/buffer suspension previously described was then allowed to flow through the system for 30 minutes. Microscopic observations were made using an Olympus Microscope (Model BH-2) operating in reflected light mode. A lens system of a 20x lens coupled with a 1.5x multiplier as well as a

10x eyepiece was used yielding a total magnification of 300x. Polarized filters were used to enhance image contrast. Cell counts were taken using an ocular grid located in the eyepiece. The size of this grid at 300x was calibrated to be 0.18mm x 0.18mm yielding a total area inside the grid of 0.0324mm^2 . For adhesion kinetic studies, cell counts were taken at various times throughout the 30 minutes. A minimum of 4 areas were observed and the average was taken. Adhesion experiments were performed at shear rates of 28, 46, 71, 120 and 350sec^{-1} with either 5% glucose-grown cells or 5% galactose-grown cells.

After 30 minutes of flow, detachment studies were performed to explore the tenacity of the adhered yeast cells in response to increasing shear rates. This was done by flushing the system using cold cell-free PBS with increasing flow rates. The flow cell was flushed with cold buffer for four minutes at the same flow rate as the attachment portion of the experiment. At the end of four minutes, the flow was increased one pump setting. It was allowed to stay there for one minute and was then decreased for one minute to allow for counting of the still adherent cells. The flow was then increased one pump setting over the previous highest value and again allowed to remain there for one minute. This procedure was repeated until either significant leakage in the flow cell occurred, or maximum throughput (flow rate: 27.9ml/min , shear rate: 861sec^{-1}) of the pump had been achieved. In most cases, maximum throughput was achieved before significant leakage occurred. After experiments, the flow cell was disassembled and cleaned.

3.6 Freeze-drying Experiments

3.6.1 Background of the Freeze-drying Experiments

In this study, glucose-grown and galactose-grown *C. albicans* cells were freeze-dried using an instrument designed and built "in house". To design and build the freeze-drier was the part of the study. Freeze-dried samples were used

for surface structure analysis using SSIMS. There are three reasons for constructing this freeze-drying system in this study. The first comes from the flow cell experiment results obtained from this study which has shown distinct adhesion behavior of cells grown from media containing different carbohydrate sources to FEP. Kevin J. Siedlecki who used the same flow cell experiment set-up except different polymer sample (PVC, Poly (Vinyl Chloride)) has obtained the similar results [100]. These results indicate that surface structure difference between glucose-grown cells and galactose-grown cells exists, which has contributed to their different adhesion behavior to polymer surfaces. Knowledge of this structure difference should be valuable for studying the adhesion between *C. albicans* and polymer surfaces. The second reason is that a cryostage for SIMS which allows for analyzing hydrated samples in vacuum is not currently available in ICAL (Imaging and Chemical Analysis Lab) of Montana State University. The third reason is that all available freeze-driers in campus are dirty and not suitable for preparing clean samples for surface analysis. Because these freeze-driers are usually pumped by mechanical pumps and tend to have the problem of backstreaming of mechanical pump oils to the freeze-drying chamber which will contaminate samples being freeze-dried. Turbomolecular pumps don't backstream hydrocarbons and help build a clean vacuum. Also these commercial freeze-driers are built for biochemical or biological studies in which a big volume of cell suspension in a flask is pre-frozen first in a cold bath and then plugged onto the freeze-drier to be dehydrated in vacuum at room temperature. Powder-like freeze-dried cell masses obtained from these freeze-driers are not useful for SIMS analysis because cell powders must be pressed into indium foil to get a flat surface which is favorable for TOF-SIMS analysis. The design of the spectrometer of TOF-SIMS is that it requires a flat and relatively smooth surface for analysis. This is because the electric fields in the vicinity of the surface must

be precisely shaped, and large sample irregularities or curvature will distort the field, alter the ion trajectories, and hence cause poor spectra resolutions. Cell structure will probably be destroyed when being pressed into the indium foil. Also contamination is likely to occur in those freeze-driers because of the freeze-drying procedure and the low vacuum. Hence special sample preparation methods should be explored in this study in order to get useful SSIMS spectra .

3.6.2 The Freeze-drying System

A schematic diagram of the freeze-drying system which has been designed and built is given in Figure 15. The main parts of the system are: N₂ gas line, freeze-drying chamber, freeze-drying chamber isolation valve, pumping line, and Dewar flask and universal platform.

1) N₂ gas line

A stream of dry and hydrocarbon-free N₂ gas was used to break the vacuum and bring the system to atmospheric pressure after the freeze-drying operation was finished. The N₂ gas was from compressed nitrogen tank (General Distributing Co., UN1066) and was filtered by a R&D moisture trap (model MT200-4) and a R&D hydrocarbon trap (model HT200-4) obtained from R&D Separations Co. before going into the freeze-drying chamber.

2) Freeze-drying chamber

The freeze-drying chamber was made of a CF3-3/8in. stainless steel six-way cross. One flange of the six-way cross was connected to a CF3-3/8in. stainless steel blank flange which acted as the "door" of the chamber. One flange was

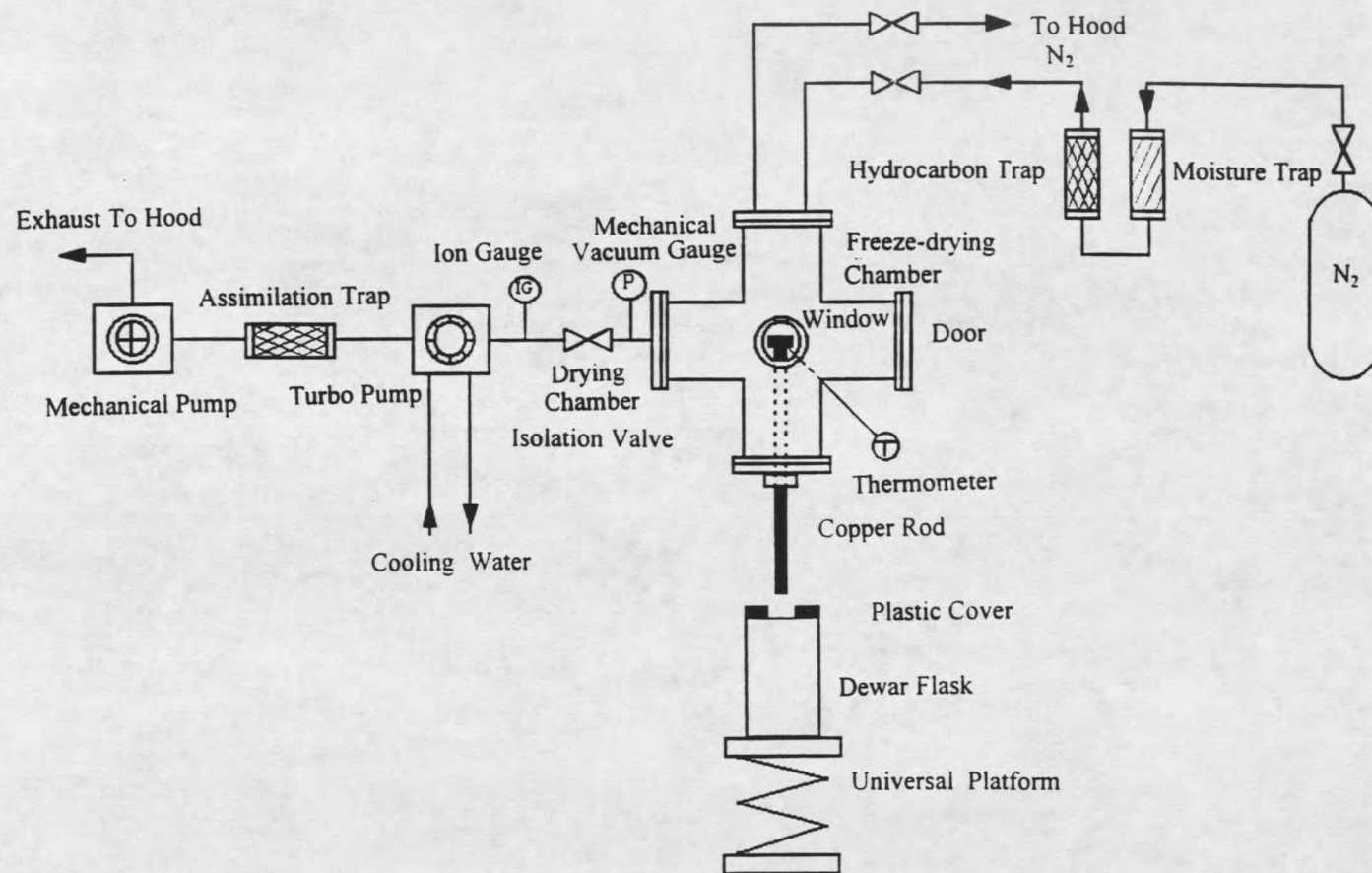


Figure 15. Schematic drawing of freeze-drying system

connected to a CF3-3/8 in. stainless steel blank flange which had a welded-in Ashcroft mechanical vacuum gauge (30 to 0 in. Hg vac, E-68022-00, Cole-Parmer, Instrument Co.). The gauge can measure low vacuum to atmospheric pressures in terms of pressures relative to 760mmHg. The main purpose of this mechanical gauge was to read the chamber pressure when N₂ gas was purged to the chamber to bring it to the atmospheric pressure. A flange of the six-way cross was connected to the N₂ gas line through a stainless steel shutoff valve. The remaining three flanges of the six-way cross were used as follows. One was connected to the pumping line through a stainless steel gate valve. The gate valve was used to isolate the freeze-drying chamber from the pumping line. One was connected to a CF 3-3/8in. stainless steel blank flange which had a welded-in copper rod with about 13 inches in length and 0.6 inches in diameter. The copper rod came through the blank flange with about 5 inches in the chamber (the six-way cross) and about 8 inches outside of the chamber. The part in the chamber formed a sample holder which allowed a copper sample stub (1in. in diameter, 0.4in. in height) with sample on it to sit in the sample holder. The longer part of the copper rod out of the freeze-drying chamber was used to chill the sample when it was immersed totally in LN₂ (-195⁰C) or dry ice-acetone slush (-75⁰C). So the role of the copper rod was to transfer heat into or out of the sample being freeze-dried. Copper was chosen because of its high thermal conductivity (3.98W/cm-⁰c at 25⁰C). The last flange of the six-way cross (freeze-drying chamber) was connected to another CF 3-3/8in. stainless steel blank flange which had a thermal couple probe welded through it. The thermal couple probe (Type T, -250⁰C to 343⁰C, E-08505-63) was purchased from Cole-Parmer Instrument Co. The sensing end-point of the thermal couple probe was touched with the edge of the copper sample holder in the freeze-drying chamber. The thermal couple plug (out of the freeze-

drying chamber) was plugged into a Digi-Sense dual J-T-E-K thermometer (E-91100-40, Cole-Parmer Instrument Co.). From the thermometer, the temperature of the copper sample holder, hence the temperature of the sample could be monitored.

3) Freeze-drying chamber isolation valve

A stainless steel high vacuum gate valve from MDC, Inc. was used to isolate the freeze-drying chamber from the high vacuum side of the pumping line when it was completely closed.

4) Pumping line

The pumping of the freeze-drying chamber to high vacuum was achieved by the pumping line. The pumping line included a high vacuum side and a foreline. The high vacuum side included an ion gauge and a turbomolecular pump with a CF 6 in. inlet flange. The ion gauge in combination with an ion gauge controller was used to measure high vacuum pressures. The ion gauge controller (model TIC-100) was from K. H. Frederick Co., Inc.. Its reading range is from 1.00×10^{-9} torr to 1.0×10^{-3} torr in terms of absolute pressures. The turbomolecular pump (Turbovac 150) was purchased from Leybold Inc.. The pumping speed (volume flow rate) is 145 l/sec for N_2 , 135 for He and 115 for H_2 . The compression ratio is $>1 \times 10^9$ for N_2 , 2×10^4 for He and 850 for H_2 . The ultimate pressure of this pump is $<10^{-9}$ mbar. Its rotational speed is 50,000rpm. Its start-up time is 2 minutes. The operation of the turbomolecular pump was controlled by a turbomolecular pump frequency converter (Model NT150/360, Leybold Inc.), such as start, stop, protection and normal operation of the pump. The pumping mechanism of a turbomolecular pump is that a turbomolecular pump is actually a bladed molecular turbine that compresses gas by momentum transfer from the high-speed rotating

blades to the gas molecules. The relative velocity between the alternate slotted rotating blades and slotted stator blades makes it probable that a gas molecule will be transported from the pump inlet to the pump outlet. The pumps operate at rotor speeds ranging from 24,000 to 60,000rpm and are driven by solid state power supplies or motor-generator sets. The blades impart momentum to the gas molecules most efficiently in the molecular flow region; therefore this pump, like a diffusion pump must be backed by a rough mechanical pump [101]. Turbovac 150 uses ceramic bearings and it has built-in life-time oil supply which is used to lubricate and cool the bearings. The lubrication oil was cooled by the tap water supply when the pump was running in the experiments.

The foreline (low vacuum side) included a foreline trap and a rough mechanical pump. The rough mechanical pump was a dual-stage rotary vane pump(SD-300) from Franklin Electric. It was used to back up the turbomolecular pump to achieve the high vacuum of the system. The pump was operated with Inland 19 vacuum pump fluid. A assimilation trap using copper wool was placed between the turbomolecular pump and the rough pump to stop the backstreaming of the rough pump oil to the high vacuum side upon start and stop of the pumps or inadvertent power shutoff.

The ultimate vacuum of the whole freeze-drying system was less than 1.00×10^{-9} torr (out of the reading range of the ion gauge). High vacuum was desired in this study, which would help increase the pumping speed of water vapor removal and also help produce samples with high cleanness.

5) Dewar flask and universal platform

A 665ml cylindrical Dewar flask (Fisher Scientific, 10-195A) was used to hold LN₂ and dry ice-acetone slush. The wall of the Dewar flask is vacuum-sealed to keep the content heat-insulating.

The universal platform was used to raise or lower the Dewar flask during the freeze-drying operation.

All the parts excluding the N_2 gasline of the freeze-drying system were made of stainless steel. All the stainless steel parts in the freeze-drying system whose cleanness was in doubt before assembly were cleaned by base bath, rinsed by distilled water and dried in a clean hood before usage. The copper parts whose cleanness was in doubt were cleaned by ultrasonication in methanol before assembly.

3.6.3 Experimental Procedure

3.6.3.1 Sample Preparation

It has been mentioned previously in this thesis that the design of the spectrometer is that it requires a flat and relatively smooth surface for analysis. This is because the electric fields in the vicinity of the surface must be precisely shaped. Samples which have a rough or curved surface are difficult, if not impossible to analyze since the uneven surface will distort the electric fields locally, which will greatly impact the ability to get useful information from the sample. For insulating samples, a flat sample surface is more desirable than for conducting samples, because of charging of the insulating samples during analysis. A flat surface can facilitate charge compensation (neutralization) of the insulating sample.

So a method to produce a smooth layer of cells for analysis was explored in this study-a filtration method.

A schematic drawing of the filtration operation is shown in Figure 16.

The filtration unit included a Pyrex glass filter funnel (15ml, ASTM 10-15) with pore size of $15\mu m$, a rubber funnel holder coupled with the filter funnel purchased from Chemical & Lab stores of Montana State University, and a 250ml

diameter of 13mm (Anodisc 13) purchased from VWR Scientific was placed on the bottom of the funnel. The purpose of the funnel was just to hold the membrane filter. Anodisc 13 membrane filters are made of a high purity alumina matrix, with a precise nondeformable honeycomb pore structure. The unique capillary pore structure, uniform pore size and flat surface capture virtually 100% of particulates larger than rated pore size. They contain no monomers, plasticizers, adhesives, surfactants or wetting agents for minimal sample interference. For biochemical applications, membranes exhibit extremely low protein binding. Also alumina has a high thermal conductivity, so Anodisc 13 membrane filters were chosen in this study. These filters were used as received.

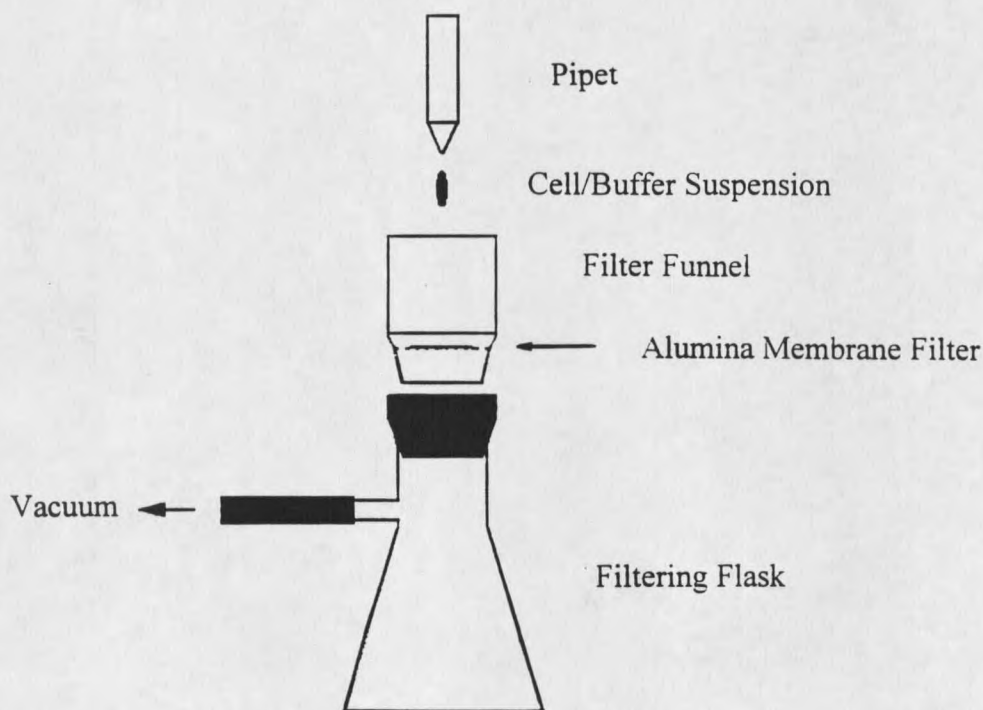


Figure 16. Schematic drawing of the filtration operation. The filter funnel and the filter funnel holder are separated to facilitate viewing.

The filter funnel was cleaned using base bath and acid and baked dry as described previously and stored in the 4°C refrigerator before each experiment. The membrane filters were also stored in the 4°C refrigerator before each experiment.

Filtration began by opening the vacuum switch, then pipetting 40 μ l of ice-cold cell/buffer suspension from the 5ml cell/buffer suspension in the culture tube obtained by procedures described previously onto the middle of the alumina membrane filter. After three minutes, almost all PBS was filtered into the filtering flask and then a 40 μ l ice-cold nanopure water was pipetted onto the middle of the circular area covered by cells. The nanopure water would spread over the whole circular area of cells and wash off the residual PBS. After three minutes, another drop of 40 μ l ice-cold nanopure water was pipetted onto the middle of the circular area covered by cells. The nanopure water would again spread over the whole circular area of cells and wash the residual PBS off. After three minutes, the circular area would look dry and the vacuum switch was turned off. Then the membrane filter was removed from the funnel carefully and placed onto a cold copper sample stub. The copper sample stub was cleaned by methanol, placed into a clean glass petri dish and stored in the 4°C refrigerator before usage. The membrane filter was stuck onto the copper sample stub by two small pieces of 3M adhesive tape. Then the sample stub was ready to go into the freeze-drier.

The reason that PBS should be washed off from the sample is due to the so-called "matrix effect". The matrix which comprises or supports an organic material has a very significant influence on the identity and yield of cluster ions. Yet because the matrix not only absorbs the primary excitation, but is also the source of the desorbing species, and mediates the ionization process, it is extremely difficult to unravel the processes involved. Enhanced ionization can occur through

cationization or anionization. Sodium or potassium can play a role in ionization by providing cations (C^+) which complex with sputtered neutral molecules to yield the observed $(C+M)^+$ ions. These peaks together with the strong Na^+ and K^+ peaks in the spectra will make the subsequent spectra interpretation and analysis using statistical method in this study very difficult. Hence, salt concentration in the sample must be diluted to a low level so that matrix effect is eliminated or negligible.

3.6.3.2 Freeze-drying *Candida albicans*

In this study, galactose-grown and glucose-grown *C. albicans* cells were freeze-dried using the freeze-drying system built "in house".

Before experiments, the freeze-drying chamber was at atmospheric pressure with its door closed. Experiments began by initiating the cooling water flow of the turbo pump and starting the rough pump and the turbo pump simultaneously with the freeze-drying chamber isolation valve closed. Then sample was prepared by the filtration method described previously. When the sample was ready, the door of the freeze-drying chamber was opened and the copper sample stub with sample on it was quickly placed into the copper sample holder in the chamber with the sample surface facing up. Then the door was quickly closed and shut tightly. The outside part of the copper rod was then immersed totally in a full Dewar flask of LN_2 , poured from a big Dewar used to store LN_2 . The temperature of the sample was read from the thermometer and recorded with time. These data were used to determine the cooling rate of the sample. During the time when the sample was chilled down, dry ice-acetone slush was made as described previously. After the temperature of the sample ceased to decrease for 8 minutes, the Dewar flask was lowered from the copper rod and removed. The remaining LN_2 in the Dewar was poured back into the big Dewar used to store LN_2 , and dry ice-acetone slush was

placed into the empty Dewar flask. Then the copper rod was immersed totally in the dry ice-acetone slush. At this time, the reading of the ion gauge would be $1\sim 2 \times 10^{-9}$ torr and the freeze-drying chamber was still at atmospheric pressure. At this point, the freeze-drying chamber isolation valve was opened and the sublimation of ice from the frozen sample began. When the reading of the ion gauge reached a pressure less than 1.00×10^{-9} torr (the lowest limit of the gauge) and stayed at pressures less than 10^{-9} torr for 8 minutes, the sample was considered completely dry. Then the Dewar flask was removed and the sample was warming-up at room temperature. During the warm-up, the pressure of the chamber stayed lower than 1.00×10^{-9} torr, which confirmed that the sample was already dry before the Dewar flask was removed. After the sample temperature reached room temperature, the turbo pump and the mechanical pump were stopped simultaneously and a dry and clean N_2 gas was purged into the system to bring it to the atmospheric pressure. The sample stub was then taken out and the door was shut tightly to keep the system clean. During the drying process, the temperature of the sample and the pressure of the chamber were recorded with time. The sample stub was then placed into a clean glass petri dish, placed into the $4^\circ C$ refrigerator and ready for SEM, XPS or SSIMS analysis. In SEM and XPS, the sample was analyzed on the copper sample stub. For SSIMS, the filter membrane was removed from the copper sample stub carefully and mounted in the SSIMS sample holder and analyzed.

3.7 Thin Film Analysis

3.7.1 Scanning Electron Microscopy

The freeze-dried *C. albicans* cells were examined by Scanning Electron Microscope located at the Imaging and Chemical Analysis Laboratory (ICAL) at Montana State University. A JEOL 6100 system with a LaB_6 electron source

operating at 9.0kV was used. The samples were first sputter-coated with gold/palladium to eliminate the surface charging problems of insulating materials. SEM pictures were taken using Polaroid 665 instant films. The system has a spatial resolution of 40Å at best. The pressure in the analysis chamber during analysis was typically near 10^{-6} torr.

3.7.2 X-ray Photoelectron Spectroscopy

XPS analysis was conducted with a PHI 5600 XPS system in the ICAL at Montana State University. A monochromatized aluminum 2mm source operated at 1486.6eV and 300W was used as the X-ray source. An aperture setting of 4 corresponding to an 800µm spot size of analysis was used. Charge compensation was achieved using an electron flood gun since the FEP and freeze-dried *C. albicans* are all nonconductive samples. Pressure in the analytical chamber was maintained about 10^{-9} torr. The system has a spatial resolution of 30µm at best. The survey spectra of all the samples were usually taken at 93.9eV pass energy and the elemental binding energy curves were taken at pass energy of 58.7eV to get better resolution. Analysis times were held less than 10 minutes in all cases.

3.7.3 Static Secondary Ion Mass Spectrometry

Secondary Ion Mass Spectrometry was used to analyze the freeze-dried *C. albicans* cells. It was conducted using the Charles Evans TRIFT system in ICAL at Montana State University. The Gallium source at an accelerating voltage of 15kV was used. Charge compensation was achieved by using an electron flood gun and a thin uniform stainless steel grid mounted in the sample holder in front of the sample to help maintain a uniform electric field in the analytical region and to facilitate charge compensation of the sample. Pressure in the analytical chamber was maintained at 10^{-8} to 10^{-9} torr during analysis. Spectra were typically collected

from 0-1000amu. Analysis times were held under 5 minutes to insure static conditions. An analysis area of 67 μ m was typically used for positive ion spectra, and 100 μ m was typically used for negative ion spectra.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Teflon Flow Cell Results

The following set of data was obtained using the Teflon flow cell system previously described. The purpose was to look at the effect of culture and process variables on yeast cell adhesion.

4.1.1 Adhesion Kinetic Studies

Adhesion kinetic studies were completed by taking cell counts for at least four locations every three minutes. Cell counts were collected and the averages were plotted versus time. A representative graph of the kinetic process is shown in Figure 17.

This graph was obtained using data from two different experiments run under identical conditions. One interesting feature of the graph is the reproducibility found between experiments, typical for all data sets. The most significant aspect of this graph is the linearity of the adhesion process at least to a time period of 30 minutes. This linearity was found for galactose-grown cells at all shear rates studied as Figure 18 depicted, but this linearity was not found for glucose-grown cells when shear rate was greater than 71sec^{-1} , as Figure 19, 20 depicted.

In Figure 17, data for the cells grown in galactose can be accurately fit with a straight line ($R^2=0.986$). In Figure 19, a linear regression to the data for the cells grown in glucose gives an R^2 value of only 0.858. Comparison of the replicate experiments shows a statistically significant lack of fit for the linear model. These data are better fit by Eq.12:

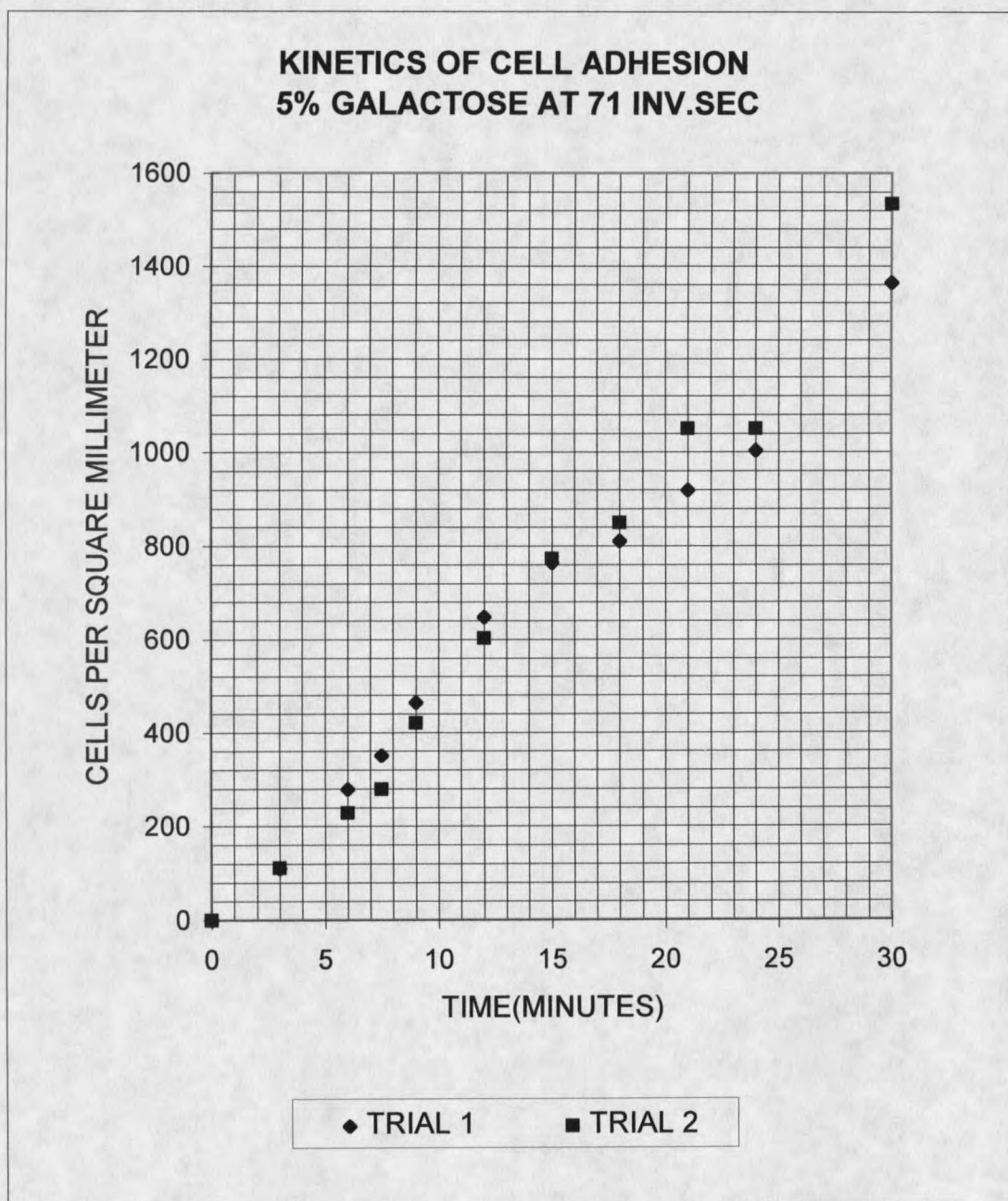


Figure 17. Graph depicting the kinetics of cell adhesion for the first 30 minutes of experiment time. The cells were grown in 5% galactose and the shear rate during attachment was 71sec^{-1} .

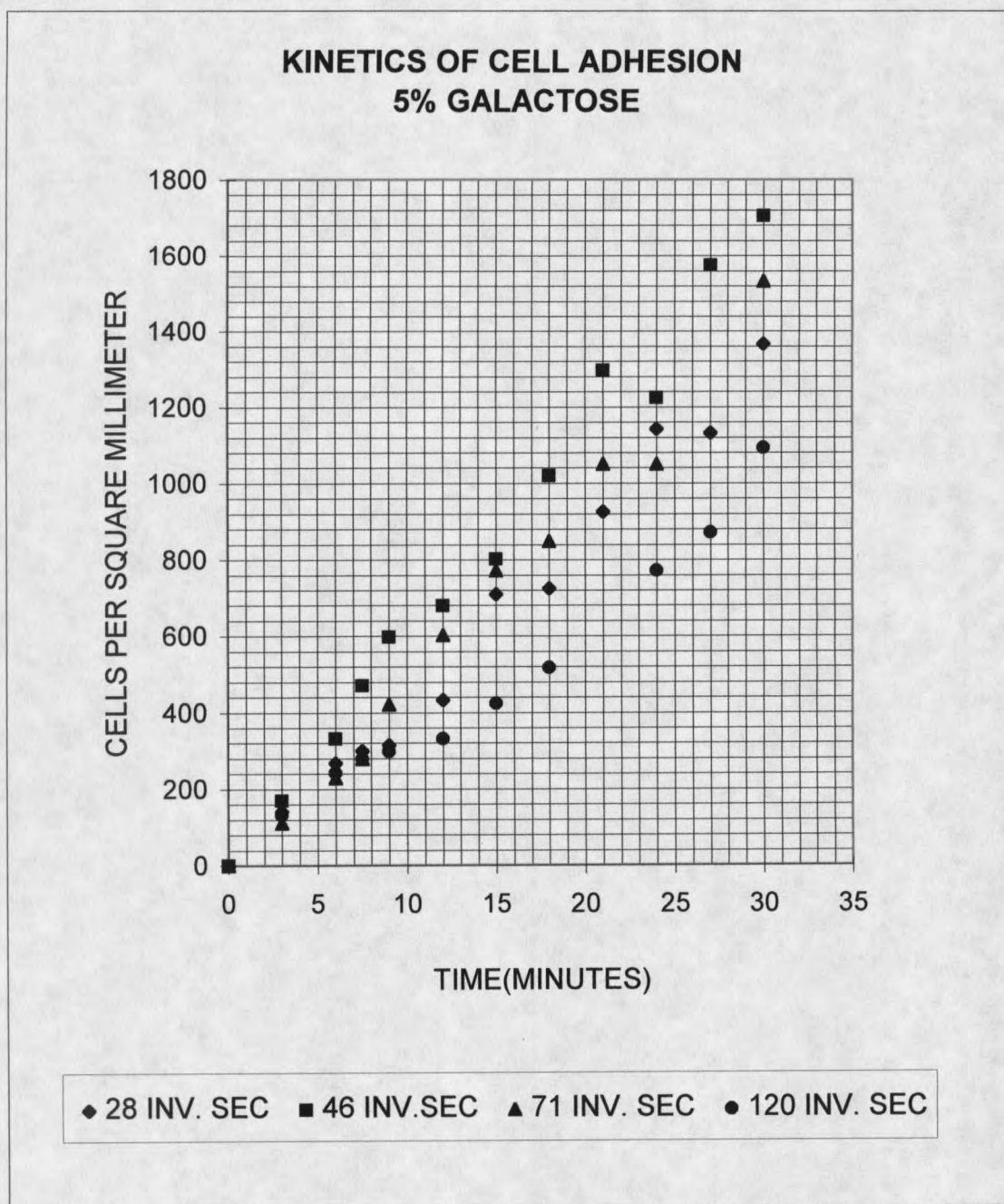


Figure 18. Graph depicting the kinetics of cell adhesion for the first 30 minutes of experiment time. The cells were grown in 5% galactose and the shear rate during attachment was 28,46,71 and 120sec^{-1} . The linearity was observed for all data sets.

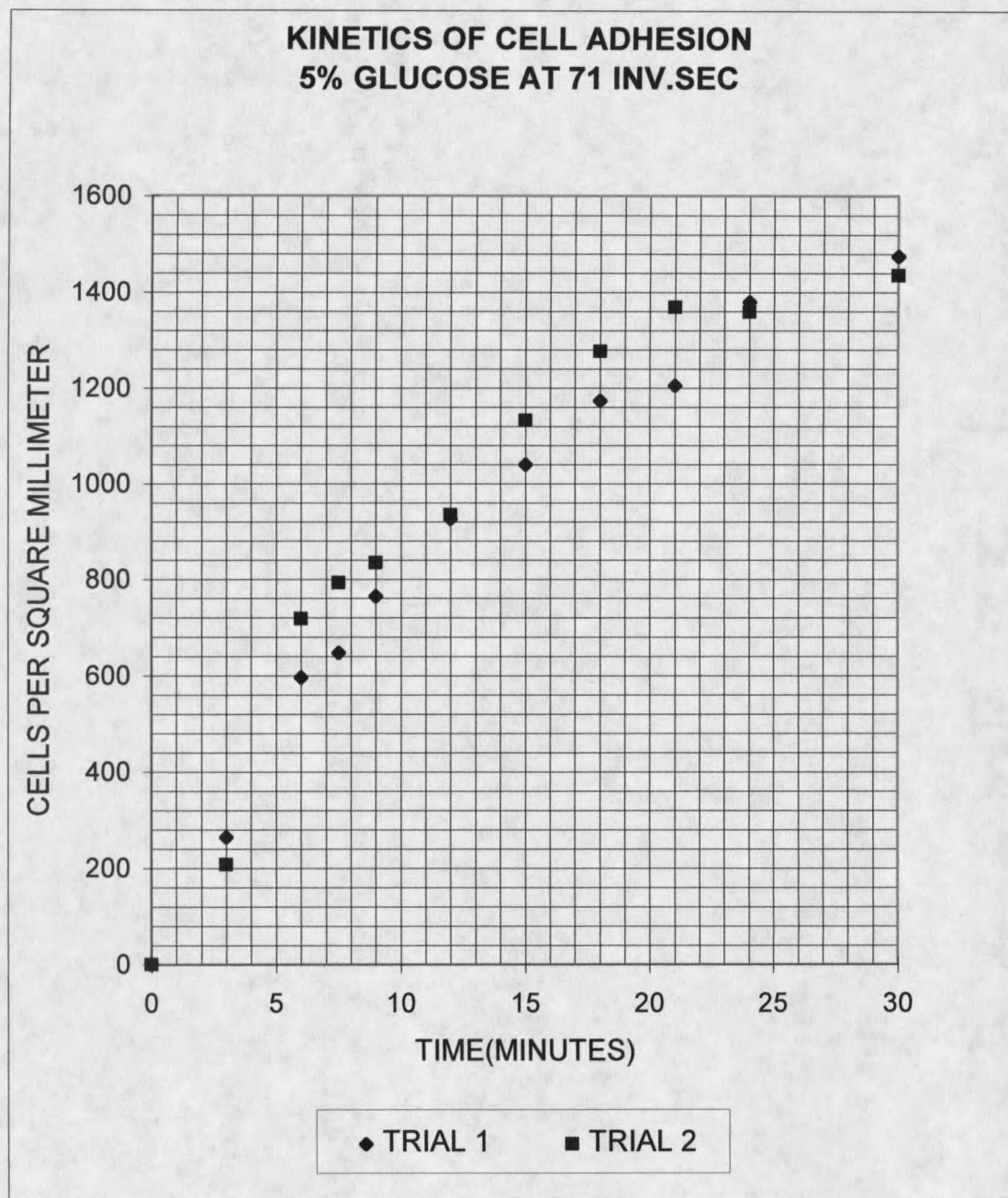


Figure 19. Graph depicting the non-linearity of the cell adhesion process at higher shear rates for glucose-grown cells. Notice that a doubling is not observed in cell counts from 15 to 30 minutes.

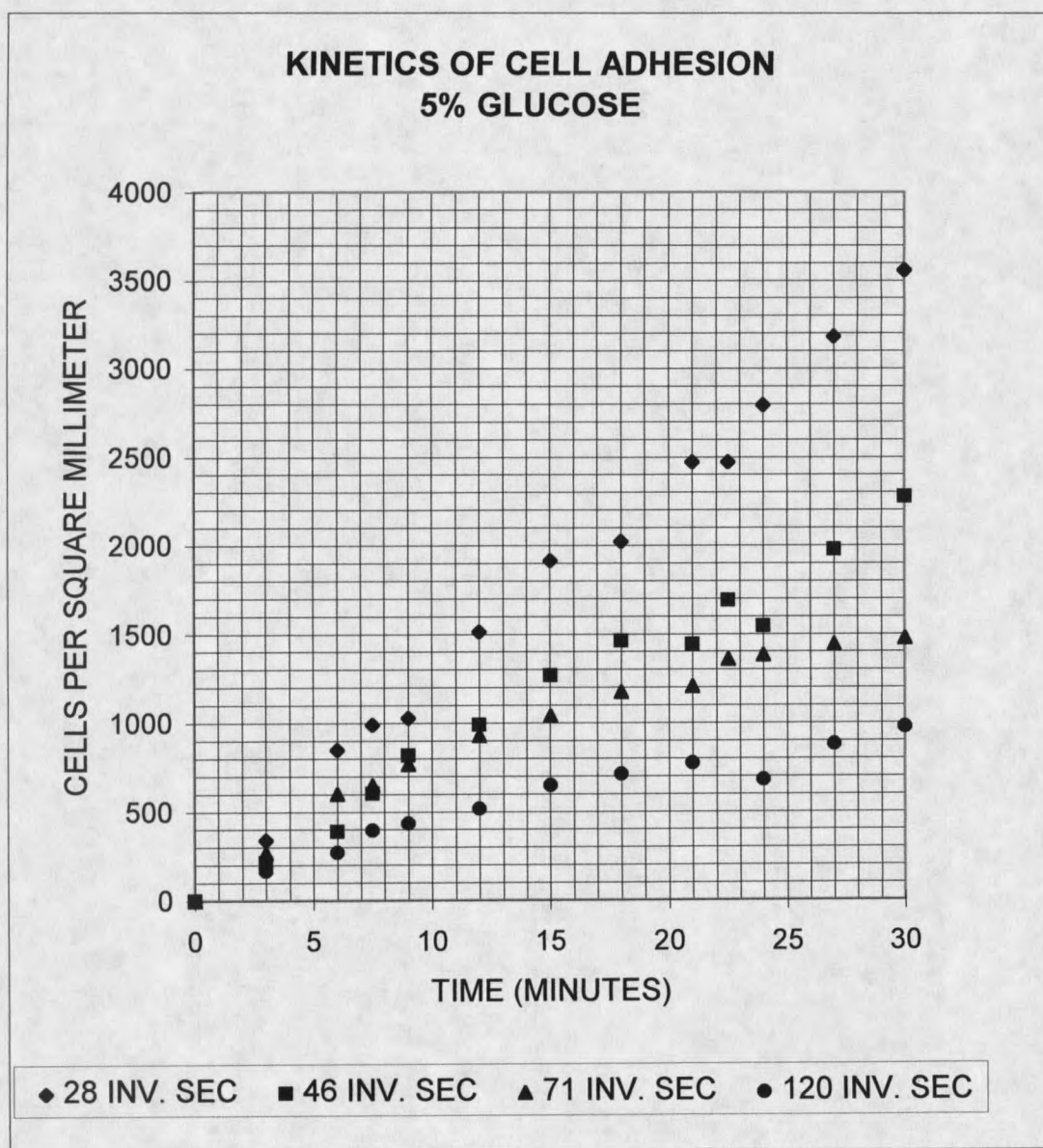


Figure 20. Graph depicting the kinetics of cell adhesion for the first 30 minutes of experiment time. The cells were grown in 5% glucose and the shear rate during attachment was 28, 46, 71 and 120sec^{-1} . The linearity was observed at low shear rates. But at shear rate greater than 71sec^{-1} , non-linearity was observed.

$$\#cells = Q(1 - e^{-Kt}) \quad (12)$$

where Q represents a saturation level achieved after a long period of time and t is a first order rate constant. At short times, Eq.12 will approximate a straight line with slope QK . It is expected that after longer times, the surface will become saturated under all conditions. In adhesion experiments, measurements made at short times reflect the initial adhesion rate (QK) and measurement made at very long times reflect the saturation level. Unless measurements are taken as a function of time, it is impossible to tell which of these values is being measured and therefore impossible to make meaningful comparison between experiments. In these studies, the saturation level can't be accurately estimated because of the short experiment time. These saturation levels may not follow the same trends seen at 30 minutes. For example, at 71sec^{-1} shear rate, the number of adherent glucose cells is starting to level off after 30 minutes while the number of adherent galactose cells is continuing to increase linearly. It is possible that after longer times the number of galactose-grown cells adhered to the surface may exceed the number of glucose grown cells adhered to the surface.

4.1.2 Attachment Studies

The influence of the shear rate on cell attachment to FEP surfaces was studied by conducting a number of experiments under identical growth and adherence conditions, with shear rate being the only variable between experiments. Shear rate effects were observed for both 5% glucose-grown and 5% galactose-grown cells. Figure 21 shows a plot of numbers of adherent cells versus shear rate after 30 minutes of experiment time. The data are the average of at least three

repeated experiments and the standard deviation was calculated and shown as the error bars. The corresponding data are summarized in Table 4.

Table 4. Adherent cell counts for FEP after 30 minutes of experiment time

Shear rate Inv. sec	5% Glucose		5% Galactose	
	Cells per mm ²	Std. dev.	Cells per mm ²	Std. Dev.
28	3161	318	1582	223
46	2034	212	1450	217
71	1476	101	1367	134
120	955	68	940	108
350	90	14	89	16

From Figure 21, one can see that the rate of adhesion to FEP decreases with increasing shear rate for both galactose and glucose-grown cells. The rate of attachment of glucose-grown cells is more strongly affected by shear rate than the rate of attachment of galactose-grown cells. When the shear is increased from 28sec^{-1} to 120sec^{-1} , the number of adherent glucose-grown cells decreased by 70%, while the number of adherent galactose-grown cells decreased only by 41%. At 28sec^{-1} shear rate, glucose-grown cells are twice as adherent as galactose-grown cells. The rate of adhesion for glucose and galactose-grown cells approach the same value at higher shear rates. There is no statistically significant difference between glucose and galactose-grown cells at shear rates above 120sec^{-1} .

From Figure 21, it is apparent that shear rate effects play a major role in the adherence process. The rate of adhesion decreases with increasing shear rate, and the mechanism used by galactose-grown cells to adhere is less affected by shear rate than glucose-grown cells.

THE EFFECTS OF SHEAR RATE ON ATTACHMENT 5% CARBON SOURCE

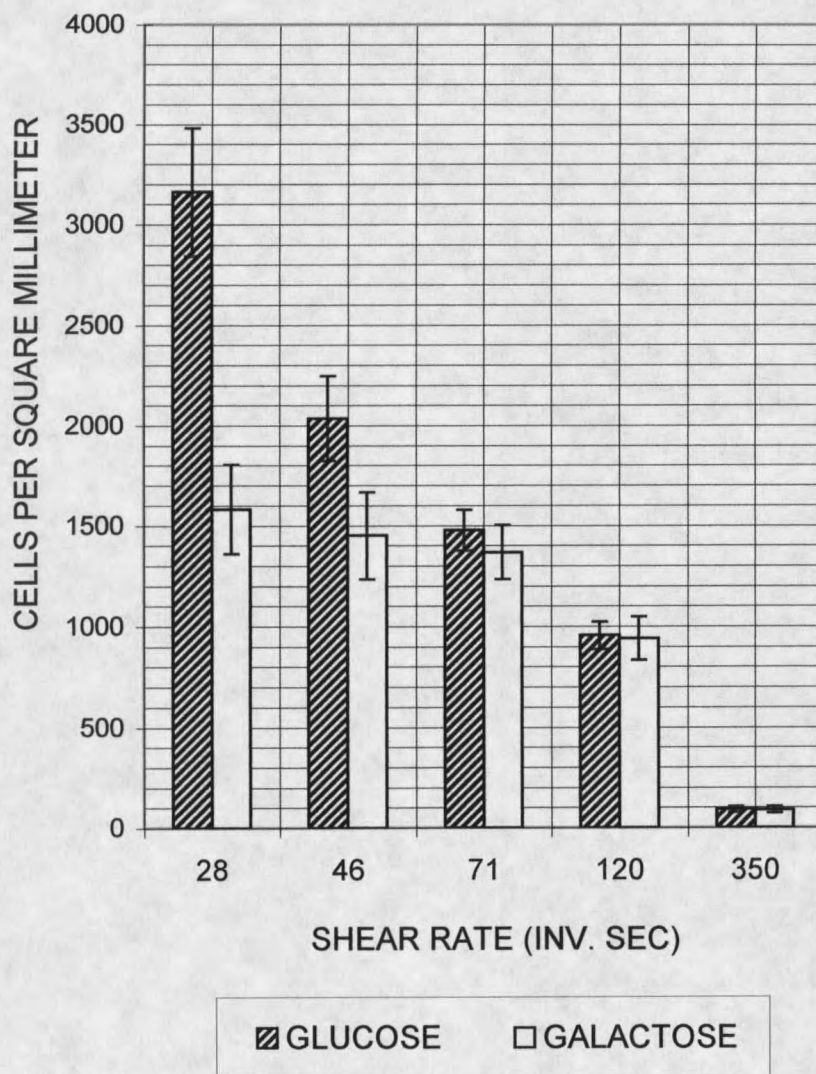


Figure 21. Graph depicting effects of the shear rate on attachment after 30 minutes of experiment time. The cells were grown in either 5% glucose or 5% galactose.

4.1.3 Detachment Studies

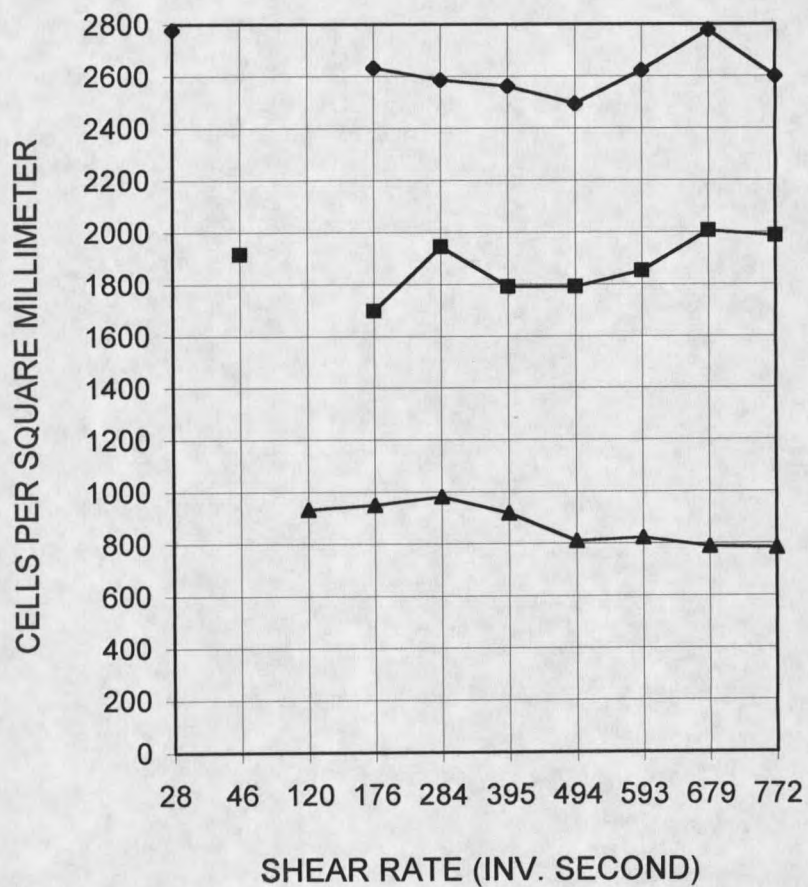
One possible explanation for the shear effects shown above might be that many of the cells which adhere at low shear rates form only a weak bond with the surface which is unable to withstand greater shear forces. To rule out this possibility, detachment studies were performed to observe the effect of increased shear rate on already adherent cells. Detachment studies were conducted as described previously in this thesis. A 4°C cell/buffer suspension was pumped through the system to allow cells to adhere to the FEP sample surfaces, and after 30 minutes of time, a 4°C cell-free PBS was pumped through the system and the shear rate was incrementally increased to determine its effect on adherence. Repeated experiments were conducted, and similar results were found throughout. A plot of the results is shown in Figure 22.

As is shown by Figure 22, the strength of attachment appears to be independent of the shear rate during attachment. Yeasts attached at lower shear rates (28sec^{-1}) seem to be bound as tenaciously to the surface as cells adhered at higher shear rates (120sec^{-1}). No statistically significant change in the number of adherent cells was observed in any of the experiments. Even when cells initially adhered at the lowest shear rate (28sec^{-1}), there was no change in the number of adherent cells when shear rate was increased to 772sec^{-1} . This result was observed for both glucose and galactose-grown cells.

4.2 Discussion of the Flow Cell Results

In this study, a laminar flow cell with well-defined hydrodynamic regime was used to study the initial adhesion event of *C. albicans* cells to FEP surfaces. The results show that shear rate is a critical factor in this adhesion process. As

THE EFFECTS OF SHEAR RATE ON DETACHMENT 5% GLUCOSE



—◆— ADHERED AT 28 INV. SEC —■— ADHERED AT 46 INV. SEC
—▲— ADHERED AT 120 INV. SEC

Figure 22. Graph depicting the effect of increased shear rate upon already adherent cells. Note that the cells are adhered to the surface very tenaciously regardless of the shear rate during attachment.

shear rate increased, a significant decrease in cell adhesion was noticed. However, cells which attached even at the lowest shear rate (28sec^{-1}) were tenaciously bound to the surface and did not detach from the surface when subjected to shear rates up to 772sec^{-1} . Growth conditions also influence the adhesion rate of *C. albicans* adherence to FEP surface. The initial adhesion rate of cells grown in 5% glucose-containing media is greater than for cells grown in 5% galactose-containing media. But this difference diminishes with increasing shear rate, and there is no statistically significant difference between glucose and galactose-grown cells at shear rates above 120sec^{-1} . Although the results are contrary to the results of J. McCourtie, et al. who found that 9% galactose-grown cells to be four times as adherent to acrylics as 9% glucose-grown cells, for adhesion of *C. albicans* on dental acrylics [64], the difference may be attributed to differences in the experimental assays or to different cell surfaces because of different growth media or different strains of *C. albicans* used. They grew *C. albicans* in yeast nitrogen base medium containing 9% glucose or 9% galactose, while in this study, yeast extract and Bacto-Peptide (YEP) together with 5% glucose or 5% galactose were used. Also they used a static adhesion assay with poorly defined hydrodynamics. Because they did not obtain kinetic data, it is impossible to tell whether the values they reported represent initial adhesion rates or surface saturation values. These difference in experiment design prevent direct comparisons between their results and the results presented here.

The results in this study also show that glucose-grown cells are more sensitive to shear rate effect than galactose-grown cells. It suggests that there may be a structural difference in the cell wall moieties that contributes to the shear-sensitive behavior of glucose-grown cells and promotes the adhesion of glucose-grown cells to FEP at low shear rates. Glucose-grown cells seem to exhibit an "adhesion advantage" in comparison to galactose-grown cells, but this advantage is

not strong enough to overcome the shear rate effect because this advantage diminishes with the increasing shear rate.

The cell transport from the bulk fluid to the FEP surface is not the limiting step in the adhesion process in this study. At higher shear rates, increased cell transport from the bulk fluid to the FEP surface should increase the adherence of cells to the FEP surface if the cell transport from the bulk fluid to the FEP surface is the limiting step, but cell adherence was found to be reduced with the increasing shear rate in this study. Also the cell sedimentation velocity is negligible in this study as already shown by Kevin J. Siedlecki who studied the adherence of *C. albicans* to Poly (Vinyl Chloride) instead of FEP[18], which means that cell size won't contribute to the cell adherence to the polymer surface.

The adherence of yeasts to inert polymeric surfaces is unlikely to be mediated by a receptor-adhesion interaction [66], like that of adherence to mammalian cell surfaces or biological macromolecules. This is because the nonbiological surfaces cannot be equipped with specific binding sites [67]. They more likely rely on nonspecific interactions. These nonspecific interactions include a multitude of molecular forces. Electrodynamic forces including Van der Waals forces and hydrophobic forces should be considered key elements in the adhesion process. Studies indicate that hydrophobic forces are exerted at distances as great as 15nm, and at 8 to 20nm are 10 to 100 times as great as Van der Waals forces. Electrostatic forces also play a role in mediating the adherence process. Two main stages of biological adhesion have been recognized in previous work-a reversible initial attachment or primary physical attraction stage followed by a secondary irreversible adhesion stabilization [59]. Initial attachment depends on the general long-range physical characteristics of the organism, the fluid interface, and the substratum [60]. The second stage may be facilitated by a metabolic process or just result from the formation of multiple contacts once the cell is held on the surface.

For a cell to become adhered tenaciously to a surface, both of these stages must occur successfully. This process is depicted in Figure 23.

Three theories can be proposed to explain the different adhesion rate between glucose-grown cells and galactose-grown cells with the influence of shear rate. The first comes from the previous work done by other researchers [66, 67, 68, 69, 70]. The adherence of *C. albicans* to FEP as to other plastic surfaces may be predominantly controlled by what have been collectively called hydrophobic forces, but are more properly designated as attractive London Van der Waals forces. FEP may possess a negative net surface charge under experiment conditions as other plastic surfaces which possess various degrees of negative net

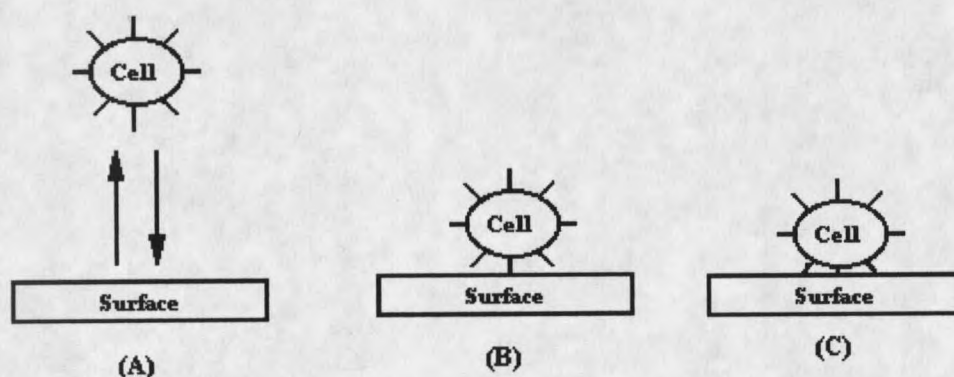


Figure 23. Cartoon depicting the process of cell adhering to a polymer surface. (A) Cell is in primary physical attraction stage. (B) Cell forms a weak bond with the surface which is easily broken. (C) Cell is tenaciously bound to the surface by secondary stabilization.

surface charge. And *C. albicans* cells may also possess a net negative surface charge which is common for all living cells (including yeasts). Hence, the electrostatic forces between the *C. albicans* cell and FEP are repulsive. But significant adherence still occurred even in the presence of this repulsive force, that may be because the repulsive electrical forces are minor to the hydrophobic forces. The surfaces of galactose-grown cells may be more electronegative than those of glucose-grown cells under the experiment conditions in this study, so the repulsive forces between galactose-grown cells and FEP surface may be stronger than those between glucose-grown cells and FEP surface. This may explain the low adhesion rate of galactose-grown cells compared with that of glucose-grown cells in lower shear rate region (under 120sec^{-1}). With the increasing shear rate, the adhesion rates of glucose- and galactose-grown cells approach the same value because the electrostatic potential difference between these two cell surfaces is overshadowed by shear rate effects.

It is likely that the major contributor to surface potential of *C. albicans* will be the mannoproteins and charged polysaccharides known to be resident on the fungal surface [68]. Acidic amino acids and acidic polysaccharides are likely to contribute to the electronegativity of the cell surface.

The second theory is that surface free energy effects may be important in the adherence process. The free energy driving force is dependent on the structure of the cell wall, which varies with carbohydrate source[100]. This may explain different adhesion rates of glucose and galactose-grown cells.

The third theory is that glucose-grown cells may have some adhesins on their outer surfaces which are not resident or so augmented on the outer surfaces of galactose-grown cells. These adhesins are supposed to promote cell adhesion to

FEP. These adhesins give an advantage to glucose-grown cells over galactose-grown cells to bind to the FEP surface during the initial attachment. However the initial attachment by these adhesins may be weak or easily broken. This can explain the increased adhesion rate of glucose-grown cells at low shear rates with the help of these adhesins. Once the attachment by this adhesin succeeds and withstands the shear force during attachment, the cell will quickly form stable strong adhesive complexes regardless of the shear force present. Increasing the shear forces on the cells decreases the ability of glucose-grown cells to attach to the FEP surface with these adhesins. Hence they can be called "shear-sensitive" adhesins.

Ultimately, all of the above theories are the consequence of the structural difference between cells grown from different carbohydrate sources. Understanding this structural difference will help understand this adhesion process. Hence *C. albicans* cells were freeze-dried in these studies and high resolution SIMS spectra were collected from these freeze-dried cells to look at the structural difference between these two cell surfaces.

4.3 Freeze-drying Experiment Results

4.3.1 Cooling Rate Determination

The preparation of the sample for freeze-drying experiments was conducted as described previously. After the sample was placed into the copper sample holder in the freeze-drying chamber and the door of the chamber was shut, the copper rod was immersed totally in a full Dewar flask of LN₂. Then the temperature of the sample was read from the thermometer and recorded with time. This cooling curve is shown in Figure 24.

From Figure 24, one can see that at temperatures lower than -61°C, there is a little change in temperature with time, so the cooling rate is largely dependent on

the linear portion of the curve. Linear regression of the data which are greater than -61°C , yielded a line with slope of -5 with R^2 of 0.96 . Hence the cooling rate is $-5^{\circ}\text{C}/\text{min}$ which is within the range of the optional cooling rate for yeast cells observed previously by Mazur [81] which is $-1^{\circ}\text{C}/\text{min} \sim -10^{\circ}\text{C}/\text{min}$. Similar results were found throughout the experiments.

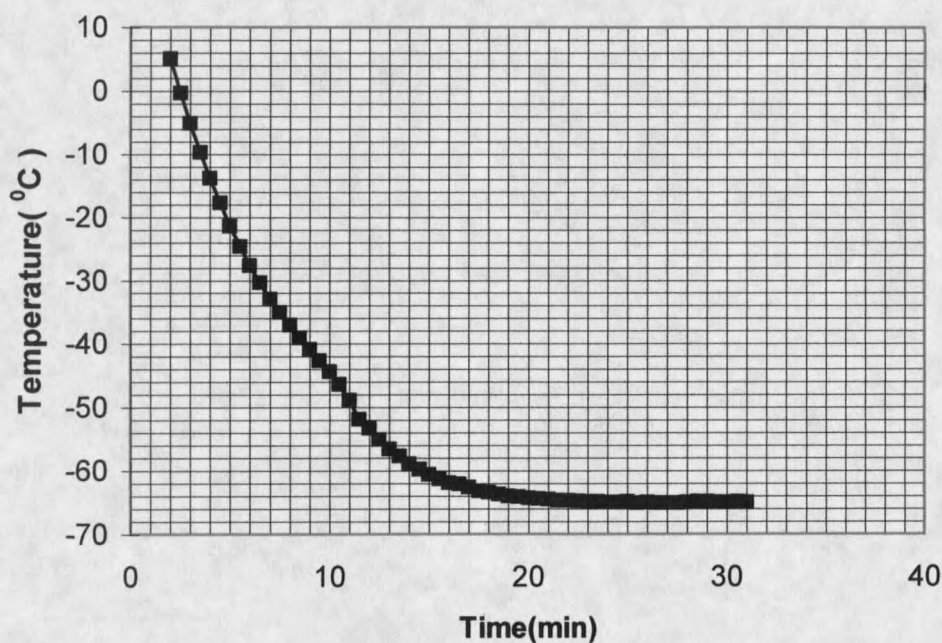


Figure 24. The change of temperature with time during freezing of *Candida albicans* cells.

4.3.2 Freeze-drying *Candida albicans*

Freeze-drying *C. albicans* cells was conducted as described previously. Cells were frozen first in the drying chamber at atmospheric pressure by chilling the copper rod in a full Dewar of LN_2 . After the lowest temperature was achieved and without changing for 8 minutes, LN_2 was changed with dry ice-acetone slush. Then the freeze-drying chamber isolation valve was opened and the frozen sample

was exposed to the high vacuum. When the reading of the ion gauge reached a pressure less than 1.00×10^{-9} torr (the lowest limit of the gauge) and stayed at pressures less than 1.00×10^{-9} torr for 8 minutes, the sample was considered completely dry. Then the Dewar flask was removed and the sample was warmed up at room temperature. During the warm-up, the pressure of the chamber stayed lower than 1.00×10^{-9} torr, which confirmed that the sample was already dry before the Dewar flask was removed. After the temperature reached room temperature, the sample was taken out and put with SEM or SIMS. During the drying process, the temperature of the sample and the pressure of the chamber was recorded with time with the start time set at the point when freeze-drying chamber isolation valve was opened. A representative recording of this process is shown in Table 5.

The content of Table 5 needs some explanation. At the start time, the temperature of the sample was -64°C , and the chamber at atmospheric pressure. The reading of the ion gauge at this point was 2×10^{-9} torr. At this point, the chamber isolation valve was opened completely and the sample was exposed to high vacuum. In the beginning of the drying, the temperature of the sample suffered a drop because of the high sublimation rate of the ice under the high pumping speed for water vapor removal. The sublimation rate was greater than that of the heat input could supply, so sublimation produced a fall in the sample temperature. As described previously and shown in Figure 3, heat must be introduced into the specimen to support continued sublimation, and the drying boundary is actually a heating surface. This reduced temperature in turn produced a reduction in the rate of sublimation and an increase of the sample temperature because of heat input as shown in Table 5 in which temperature increased from -80.9 to -48.6°C . The increased temperature then produced an increase in sublimation and a reduction in temperature again until the system was in equilibrium with a constant temperature and pressure as shown in Table 5 in which

Table 5. The recording of process variables with time during drying

Time(min)	T(°C)	P(Torr,10 ⁻⁵)	Operation
0	-64	atm	Open chamber isolation valve
5.5	-80.9	0.0005	
7	-78.4	0.006	
11.5	-67.6	0.008	
14	-63.3		
21	-55.6		
27	-51.1	0.00044	
29	-50.7	0.00048	
31	-50.7	0.0006	
33	-50.3	0.00096	
34	-50.1	0.0015	
36	-49.6	0.0033	
38	-49.6	0.014	
43	-49.1	2.7	
44	-49.1	3.1	
47	-48.9	4.2	
50	-48.6	3.6	
53	-48.6	0.0012	
55	-48.6	0.00056	
57	-48.6	0.0004	
59	-50.8	0.00034	
62	-51.4	0.00028	
65	-51.6	0.00025	
67	-51.6	0.00024	
100	-51.5	0.00028	
124	-51.3	0.00024	
164	-50.7	0.00021	
216	-50.5	0.00018	
316	-50.4	0.00018	
364	-49.9	0.00015	
410	-48.6	0.00012	
430	-48	<0.000100	Take Dewar off
438	-47.7	<0.000100	
452	-38.3	<0.000100	
480	5.9	<0.000100	Take sample out
497	11.8	<0.000100	

the temperature stayed between -50.8°C and -50.4°C and the corresponding pressure around $2\sim 3\times 10^{-9}\text{ torr}$. After 430 minutes, the temperature of the sample was -48°C and the pressure was lower than $1.00\times 10^{-9}\text{ torr}$. After 8 minutes, the Dewar flask was taken off and the sample was warmed up at room temperature. During the warming-up, the pressure of the chamber stayed lower than $1.00\times 10^{-9}\text{ torr}$, which confirmed that the sample was already dry before the Dewar flask was removed. When the sample reached 11.8°C , the sample was taken out. So the sample was freeze-dried under -48°C as Table 5 described.

Identical operation was performed for every experiment and similar results were found throughout. Samples were typically frozen until $-62 \sim -64^{\circ}\text{C}$ and freeze-dried under -40°C . The drying time was typically 7~8 hours excluding the warming-up time.

4.4 Analytical Results

4.4.1 Scanning Electron Microscopy

In order to confirm if the freeze-dried cells were in good shape and were not distorted resulting from shrinking or exploding after being freeze-dried, scanning electron microscope (SEM) was used to examine the cells routinely after freeze-drying experiments in this study. It was mentioned previously in this thesis that the design of the spectrometer of TOF-SIMS is that it requires a flat and relatively smooth surface for analysis. For insulating samples, flat sample surface is more desired than for conducting samples, because of the charging problem during SIMS analysis. A flat surface can facilitate charge compensation (neutralization) of the sample. A filtration method described previously was used to prepare samples for freeze-drying. The use of SEM also aimed at examining if the filtration method could prepare a smooth layer of cells for SIMS analysis.

As described previously, in the filtration operation, a drop of cold cell/buffer suspension was deposited on the alumina membrane filter to filtrate PBS off and then 2 drops of cold nanopure water were deposited onto the cells on the membrane filter. There were two purposes of using nanopure water to flush the cells. One was to dilute PBS in order to eliminate matrix effect caused by the salts from PBS as explained previously. The other one was to produce a smooth layer of cells on the membrane surface. As described previously, the nanopure water drop was deposited onto the middle of the circular area of cells, so cells were pushed away from the middle and redeposited. This would form a smooth layer with good coverage on the membrane filter as shown in Figure 25 and Figure 26.

From Figure 25 and Figure 26, one can see that cells appear to be intact. They are plump and ovoid in shape. Galactose-grown cells are smaller than glucose-grown cells. Both cells have smooth surface at this magnification. Some cells are connected by cell wall indicative of budding phase yeast cells. The another feature of the picture is that cells stand side by side and form a smooth layer. This situation is very favorable for SIMS analysis since a smooth surface with good coverage is desired. If, in the filtration operation, the cells weren't flushed with water and then freeze-dried, the corresponding result is shown in Figure 27. As shown in Figure 27, the cells pile up in clusters. This situation is not favorable for SIMS analysis since the sample surface is not smooth and the large background from the membrane filter will decrease the quality of the SIMS spectra. This kind of sample was not used for any XPS or SIMS analysis in this study. Only samples flushed with water and obtained as described previously were used in the subsequent XPS and SIMS analysis.

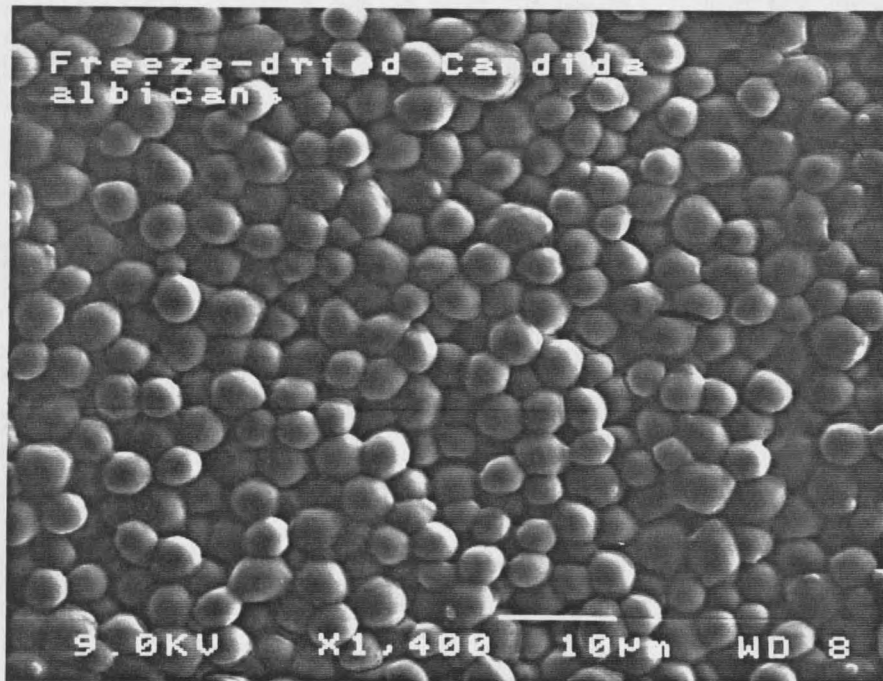


Figure 25. SEM micrograph of freeze-dried glucose-grown cells

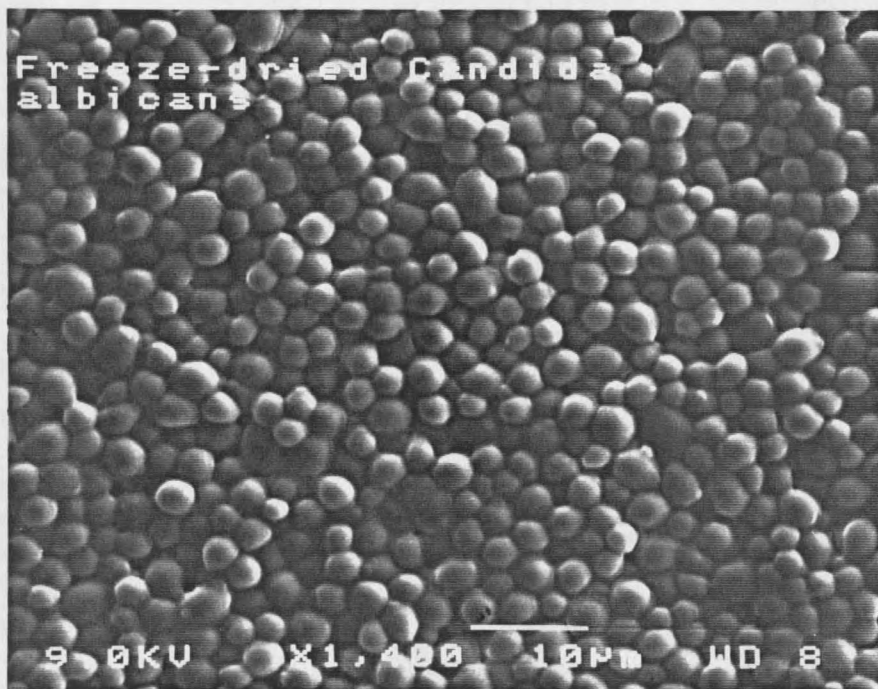


Figure 26. SEM micrograph of freeze-dried galactose-grown cells

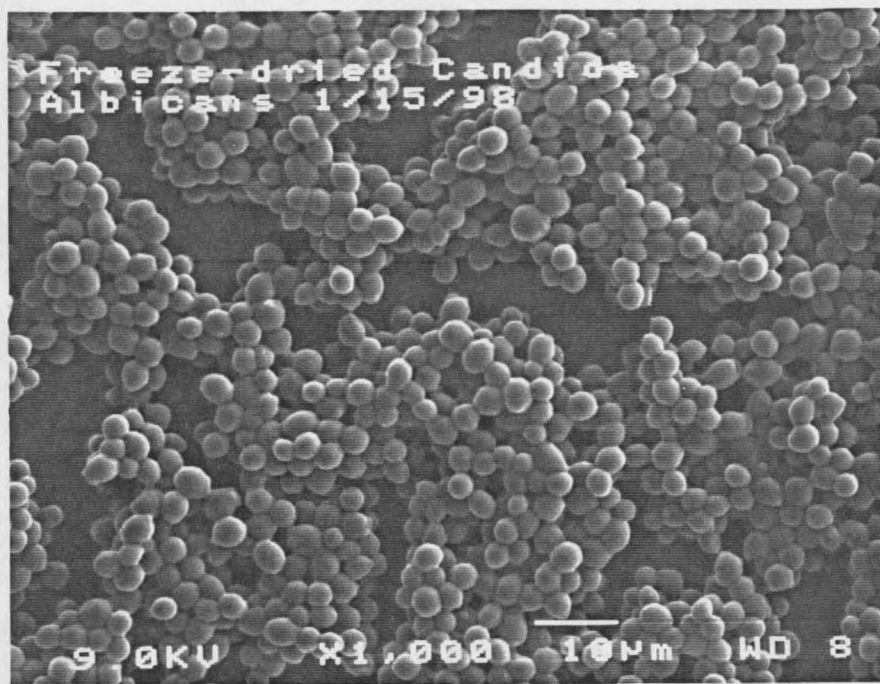


Figure 27. SEM micrograph of freeze-dried glucose-grown cells without being flushed with water before freeze-drying

4.4.2 X-ray Photoelectron Spectroscopy

X-ray Photoelectron Spectroscopy (XPS) was used to determine the surface chemistry of FEP sample used in the flow cell experiment. The sampling depth of the XPS technique is generally regarded to be 50~100Å.. It can identify all elements (except H and He) present at concentrations not less than 0.1%. It can determine the approximate elemental surface composition (error $\leq\pm 10\%$). It can also provide information about the molecular environment. FEP samples were cleaned by the procedure described previously and then analyzed by XPS. A representative survey spectra of the FEP sample is shown in Figure 28. The C1s

and F1s binding energy curves of clean FEP sample are shown in Figure 29 and Figure 30 respectively.

From Figure 28, one can see that the spectra contains only carbon and fluorine as expected. From the molecular formula of FEP, the atomic ratio of these two elements should be 2:1 fluorine: carbon. The actual values obtained for a number of polymer surfaces were $32.1 \pm 0.8\%$ in carbon and $67.9 \pm 0.8\%$ in fluorine. Other elements indicative of contaminants were not detected. This can be seen from the C1s binding energy curve, since no other functional groups with carbon were detected except the functional groups of fluorine with carbon. The common contaminants are hydrocarbons, but hydrocarbon peak at binding energy of 285.0eV was not detected in the C1s binding energy curve, which indicated the cleanness of the FEP sample. Hence the FEP samples used in the flow cell experiments were clean and free of contaminants.

XPS was also used to examine the salt concentration from the contribution of PBS on the freeze-dried samples. Survey spectra were taken from areas without aluminum (from the alumina membrane filter) indicated from the spectra. Spectra which showed aluminum were discarded. A representative survey spectrum taken from glucose-grown cells is shown in Figure 31.

From Figure 31, just carbon, nitrogen and oxygen were detected, which showed the elemental composition of the cell wall of *C. albicans*. No sodium and phosphorus were detected which meant that their atomic concentration was lower than 0.1% which is the detection limit of XPS. Under this low salt concentration, matrix effect was negligible. Spectra were taken from three spots without aluminum from the sample and similar results were found. Freeze-dried galactose-grown cell samples were not examined to check the salt concentration, because it was expected to be at the same level as that of glucose-grown cells since the sample preparation procedure was the same and also SIMS spectra taken from

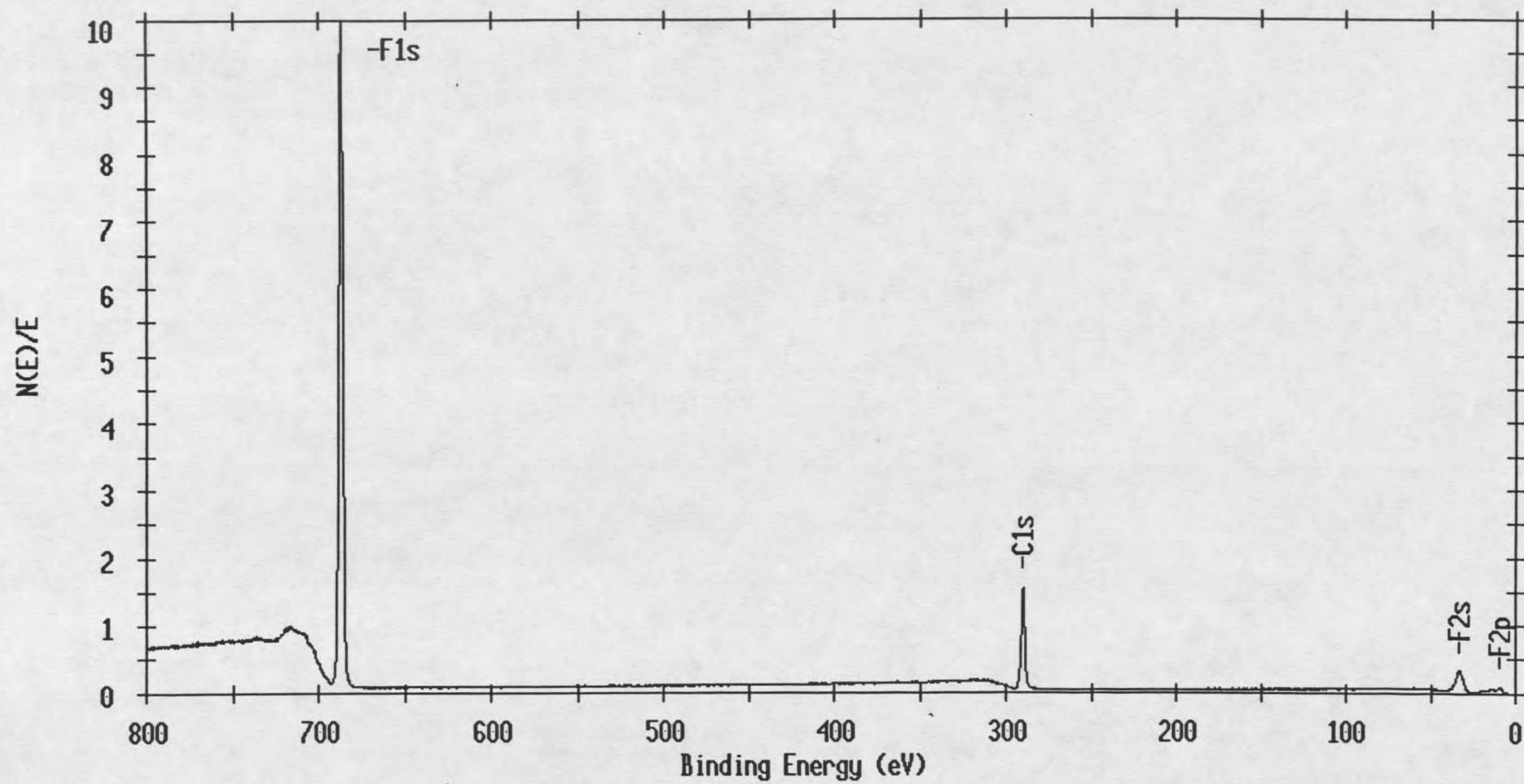


Figure 28. XPS survey spectrum of clean FEP sample

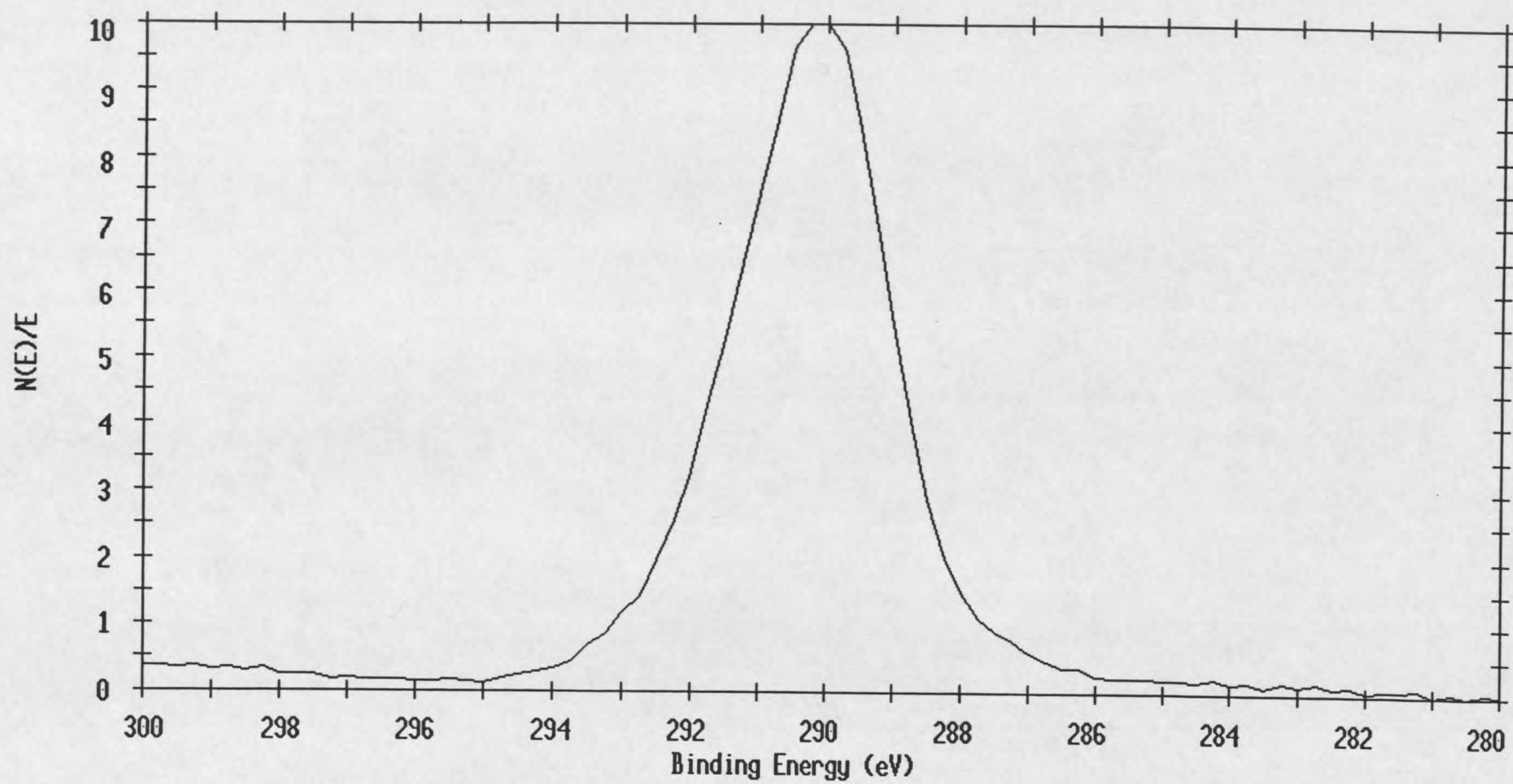


Figure 29. XPS C1s binding energy curve for clean FEP sample

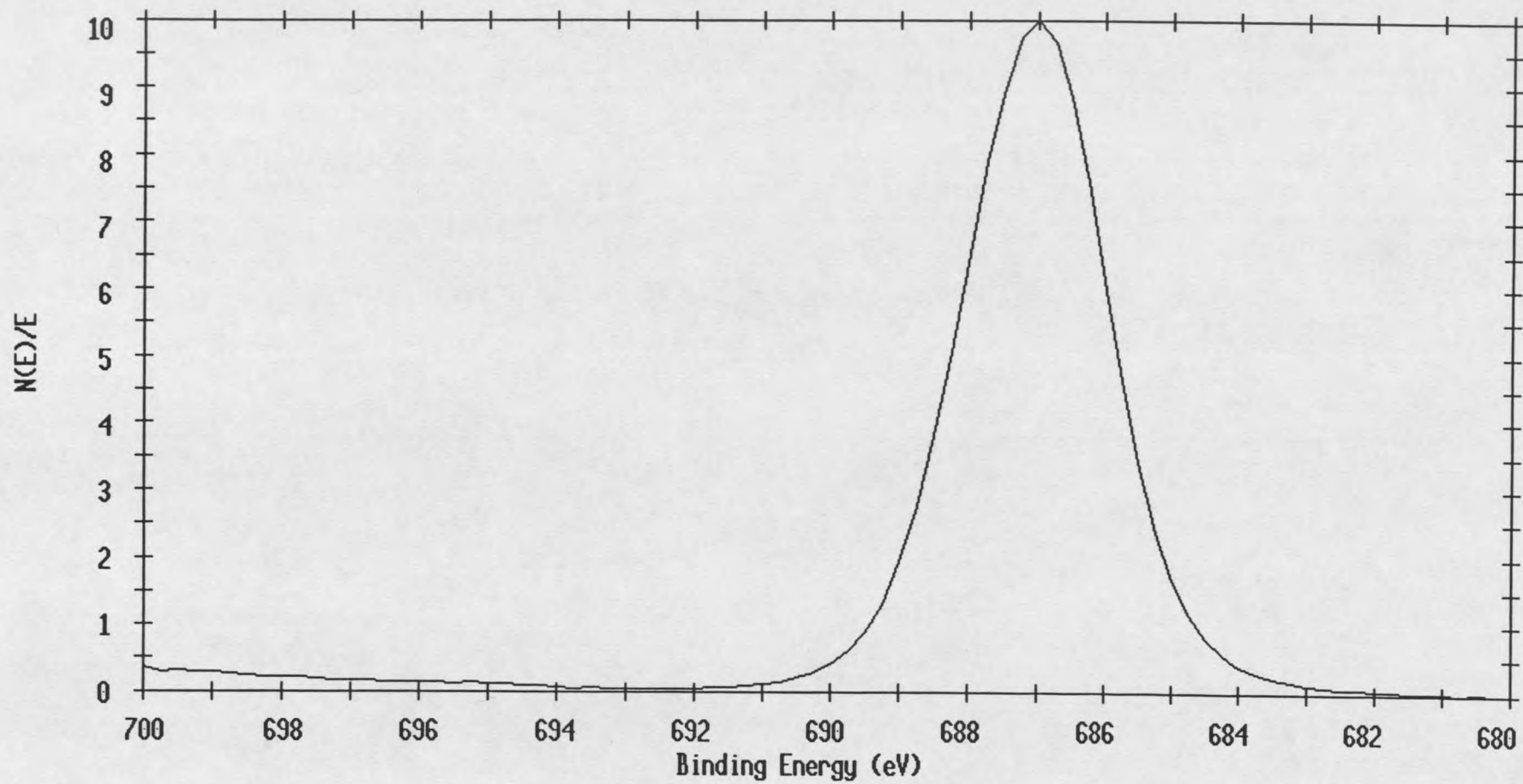


Figure 30. XPS F1s binding energy curve for clean FEP sample

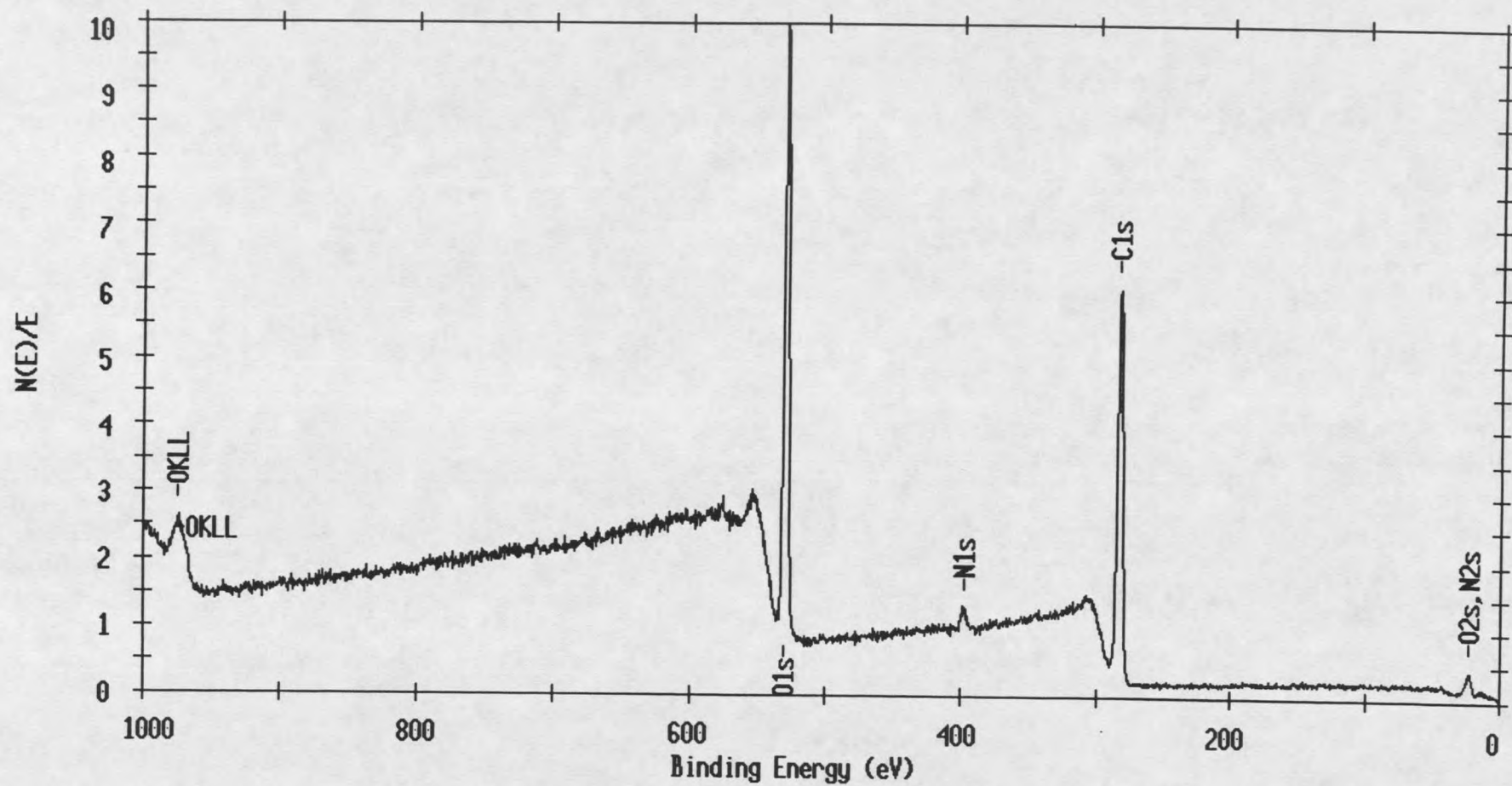


Figure 31. XPS survey spectrum of freeze-dried glucose-grown *Candida albicans* cells on alumina membrane filter

from these two samples have indicated that their salt concentration was at the same level. This can be seen in the SIMS spectra in the following section of this thesis.

4.4.3 Static Secondary Ion Mass Spectrometry and Statistical Analysis

Static Secondary Ion Mass Spectrometry (SSIMS) was used to analyze the surface structural difference between galactose-grown cells and glucose-grown cells with an attempt to correlate specific functional groups on the cell surface to the relative different adhesion results obtained from flow cell experiments. The sampling depth of SSIMS is $\sim 20\text{\AA}$, so SSIMS is very surface sensitive. Freeze-dried samples were made as described previously. Spectra were taken from areas without aluminum (from alumina membrane filter). Three samples of galactose-grown and three samples of glucose-grown cells were made for SSIMS, and spectra were taken from three spots of each sample. Both positive and negative ion spectra were taken. The freeze-dried glucose-grown cells SSIMS spectra are shown in Figure 32 to Figure 43. Figure 32 to Figure 43 are negative ion spectra. The freeze-dried galactose-grown cells SSIMS spectra are shown in Figure 44 to Figure 55. Figure 44 to Figure 52 are positive ion spectra and Figure 52 to Figure 55 are negative ion spectra. Each positive ion spectrum is shown with mass range from 0 to 550amu. Each negative ion spectrum is just shown with mass range from 0 to 200amu.

In positive ion spectra, the peak at m/z 23 is Na^+ from PBS. This ion may have different relative intensity for each spectra which is the result of the washing operation. But it won't influence spectra comparison and interpretation since this peak was subtracted from the data before statistical analysis. Also from the XPS result, salt concentration was lower than 0.1%, and matrix effect was negligible in this study. Positive ion spectra show good reproducibility and high mass resolution. The mass resolution of SSIMS is calculated by dividing the mass by the

separable mass limit. This means that for mass resolution of 10,000, a mass of 10,000amu can be distinguished from a mass of 10,001amu. This also means that a mass of 10,000 can be distinguished from a mass of 10.001amu. This allows molecules of the same nominal mass to be distinguished from each other. Positive ion spectra yield valuable information, but negative ion spectra show very few peaks and poor information content. Only the positive ion spectra were analyzed by statistical analysis method subsequently.

Principal Components Analysis and Discriminant Function Analysis were used in this study to analyze the SSIMS positive ion spectra data shown in Figure 32 to Figure 40 and Figure 44 to Figure 52. In summary, there were 18 spectra (or samples), 9 for glucose-grown cells and 9 for galactose-grown cells. Statistical analysis was done with MATLAB software. Areas of peaks with the same nominal mass were added together in each spectrum. Peaks with nominal mass greater than 500amu were discarded since all important information were contained in m/z lower than 500amu. The peak intensities of all the peaks in each spectrum were normalized to that of peak with m/z 55, since just relative peak intensities can be used to compare between different spectra and obtain useful information. Then the 18 samples with normalized peak intensities were put together with each variable (m/z) being a column and each sample (peak intensity) a row. This matrix was the original data matrix and used subsequently for statistical analysis. It was a 18 x 499 matrix. SSIMS was used to look at the surface structural difference of glucose-grown cells and galactose-grown cells. Linear Discriminant Analysis is a favorite tool to analyze the SSIMS data. As described previously, Discriminant Function Analysis consists of finding a transform which gives the minimum ratio of the difference between a pair of group multivariate means to the multivariate variance within the two groups. If the two groups are regarded as consisting of two clusters of points in multivariate space, Discriminant Function Analysis will search for the

one orientation along which the two clusters have the greatest separation while simultaneously each cluster has the least inflation as Figure 11 depicted. In this study, two groups of data were collected. One was glucose-grown cells positive ion SIMS spectra and the other was galactose-grown cells positive ion SIMS spectra. Linear Discriminant Function Analysis can find the peaks (variables) which contribute the separation (difference) of these two groups, which is desired in this study. From Eq.8 in order to use Linear Discriminant Function Analysis, $[sp^2]$ must be a $m \times m$ matrix of pooled variance and covariance of m variables. That means at least 499 samples must be collected in order to analyze the original data matrix with 499 variables (m/z). In this study, 18 samples (spectra) were collected, so Discriminant Function Analysis can not be used directly in this study. As described previously Principal Components Analysis (PCA) is a tool of data compression and information extraction. So PCA was used first to compress the original data matrix with 499 variables (m/z) to 7 variables (principle component). PCA obtained 17 eigenvectors of the variance-covariance matrix of the original data matrix. The first eigenvectors were used to transform the original 18 observations to the 7 principal component axes to get the scores of the 18 observations since the first 7 eigenvalues captured 98.94% of the total variance of the original data matrix and hence contained all important information of the original data set. So the original data matrix with dimension of 18×499 was transformed by PCA to a matrix with dimension of 18×7 . This 18×7 data matrix was used in the subsequent Linear Discriminant Function Analysis. It was done in MATLAB. The calculation procedure is described simply as the following. In Eq.8, $[sp^2]^{-1}$ was the inversion matrix of the 7×7 matrix of pooled variance and covariance matrix of the 18×7 data matrix obtained from PCA. $[D]$ was the column vector of 7 difference between the means of the two groups (The 18×7 matrix was divided to two 9×7 matrices with observations from glucose-grown

cells in one group and observations from galactose-grown cells in another group). Hence the coefficients of the discriminant function $[\lambda]$ were obtained. These coefficients are shown in Figure 56. In Figure 56, one can see that there are 499 variables (mass to charge ratios) instead of 7. That is because the 7 variables were transformed back to the original variables to facilitate the subsequent data interpretation.

The scores of the original 18 observations along the linear discriminant function are shown in Figure 57. From Figure 56, the mass to charge ratios which have corresponding large positive or negative values of coefficients should contribute the separation of the two groups. These mass to charge ratios are summarized in Table 6.

Table 6. Summary of the mass to charge ratios which have large positive or negative linear discriminant function coefficients.

Coefficients	m/z
Positive	28, 30, 44, 69, 72, 86
Negative	41, 43, 57, 71, 149

From Figure 57, one can see that samples of galactose-grown cells have relatively negative scores compared with those of glucose-grown cells, which means that functional groups corresponding to the peaks (m/z) with large negative coefficients (m/z=41, 43, 47, 71, 149) in Table 6 are more on galactose-grown cells than glucose-grown cells, and functional groups corresponding to the peaks (m/z) with large positive coefficients (m/z=28, 30, 44, 69, 72, 86) are more on glucose-grown cells than on galactose-grown cells.

The high resolution SSIMS peaks listed in Table 6 are shown in Figure 58 to Figure 68. The corresponding functional group assignments to these peaks are summarized in Table 7.

Table 7. Summary of functional group assignments to peaks in Table 6.

	Normalized m/z	Peak Position (amu)	Peak Assignment		Chemicals
			Function Group	Mass (amu)	
Peaks more on glucose- grown cells	28	28.0192	CH_2N^+	28.0187	Glycine
	30	30.0345	CH_4N^+	30.0344	
	44	44.053	$\text{C}_2\text{H}_6\text{N}^+$	44.05	Alanine
	69	69.0721	C_5H_9^+	69.0704	Valine
	72	72.0833	$\text{C}_4\text{H}_{10}\text{N}^+$	72.0813	
	86	86.098	$\text{C}_5\text{H}_{12}\text{N}^+$	86.097	Leucine
Peaks more on galactose- grown cells	41	41.0395	C_3H_5^+	41.0391	Polysaccharides
	43	43.0547	C_3H_7^+	43.0548	
		43.0196	$\text{C}_2\text{H}_3\text{O}^+$	43.0184	
	57	57.0713	C_4H_9^+	57.0704	
		57.0346	$\text{C}_3\text{H}_5\text{O}^+$	57.034	
	71	71.0126	$\text{C}_3\text{H}_3\text{O}_2^+$	71.0133	
		71.0914	$\text{C}_5\text{H}_{11}^+$	71.0861	
	149	149.0239	$\text{C}_8\text{H}_5\text{O}_3^+$	149.0239	

The functional groups corresponding to these peaks were found on both freeze-dried glucose-grown cell surfaces and galactose-grown cell surfaces by examining all the spectra used for the statistical analysis. So the difference is only their relative abundance on the two different cell surfaces. By comparing Table 7 with Table 3, one can easily see that peaks at $m/z = 29, 30, 44, 72$, and 86 are immonium ions of some neutral nonpolar amino acid residues which are glycine, alanine, valine and leucine respectively. From Table 2, one can see that all of these amino acids have neutral nonpolar aliphatic hydrocarbon R groups which are $-\text{H}$, $-\text{CH}_3$, $-\text{CH}(\text{CH}_3)_2$ and $-\text{CH}_2\text{CH}(\text{CH}_3)_2$, respectively. Neutral R groups don't bear positive or negative charges and interact poorly with water, so glycine, alanine,

valine, leucine are all hydrophobic amino acids. Statistical analysis has shown that these hydrophobic amino acid residues are more abundant within 20Å of the outer surface of glucose-grown cells than within 20Å of the outer surface of galactose-grown cells.

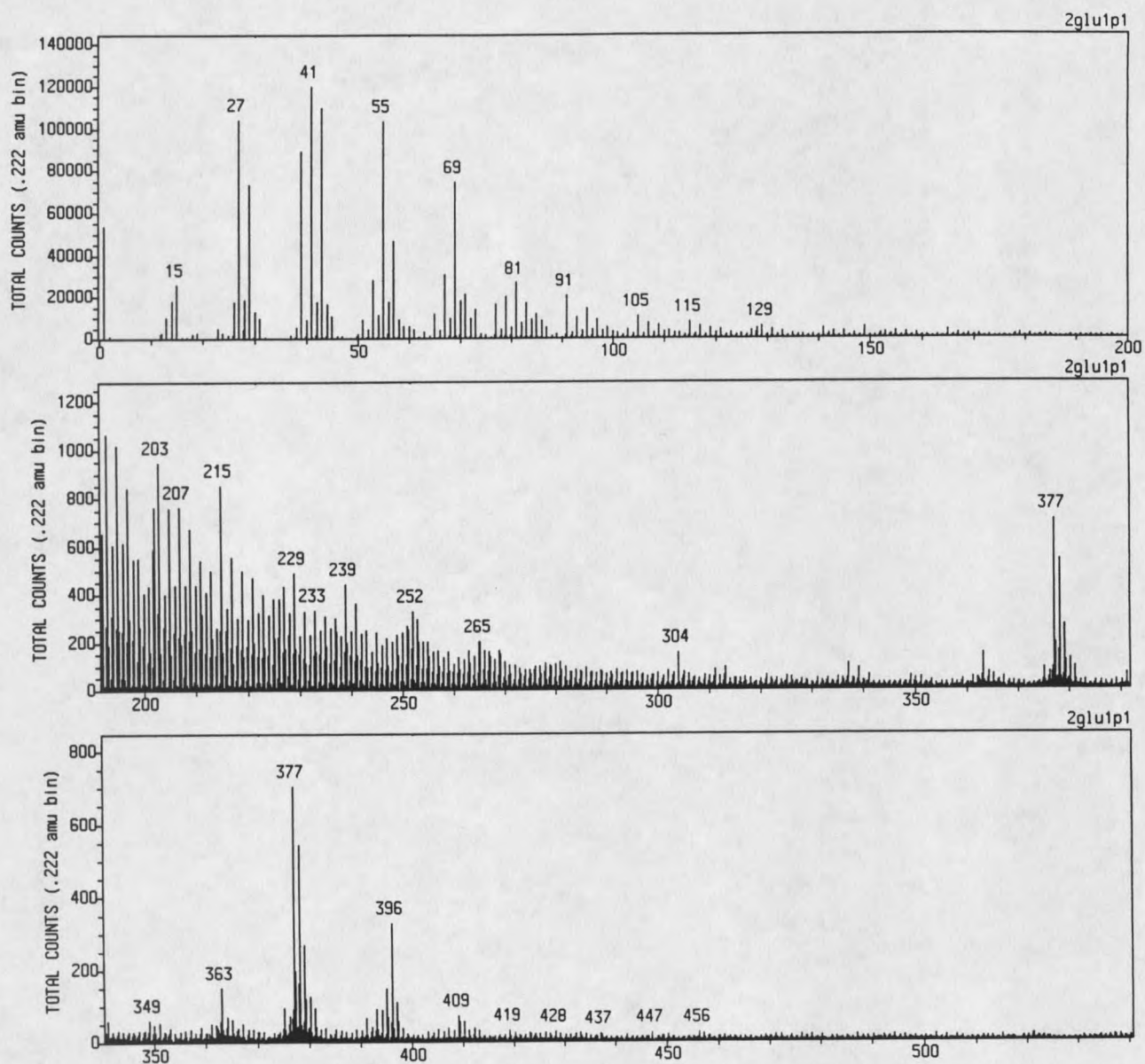
Also from Table 7, peaks at $m/z = 43, 57, 71$ are most likely due to the fragmentation of polysaccharides present on the cell surface [102], which means that polysaccharide moieties are more abundant on the surfaces of galactose-grown cells than on those of glucose-grown cells.

The above results are not unexpected. Flow cell results in this study have shown that glucose-grown cells have greater tendency to adhere to FEP and are more shear-sensitive than galactose-grown cells suggesting that there may be structural differences in the cell wall moieties that contribute this different adhesion behavior. SIMS results show that the surface of glucose-grown cells has more hydrophobic amino acid residues than that of glucose-grown cells. These hydrophobic amino acid residues are probably from protein, mannoprotein or glycoprotein on the outer cell surface. Previous studies have shown that hydrophobic forces are very important for the adhesion of *C. albicans* to plastic surfaces [66,67,69]. FEP is a very hydrophobic surface and that the surfaces of glucose-grown cells have more hydrophobic amino acid residues probably gives them advantages during the initial attachment of these cells to FEP surface. These hydrophobic adhesins may also help glucose-grown cells to form more multiple contacts with FEP surfaces than galactose-grown cells if these adhesins are not as abundant on galactose-grown cell surfaces as on glucose-grown cell surfaces. But the initial attachment formed by these hydrophobic adhesins may be weak and not strong enough to withstand greater shear forces or the multiple contacts by these adhesins may be harder to form at high shear fields, which may explain why at

high shear fields, the adherence of glucose-grown cells and galactose-grown cells to FEP approached the same value.

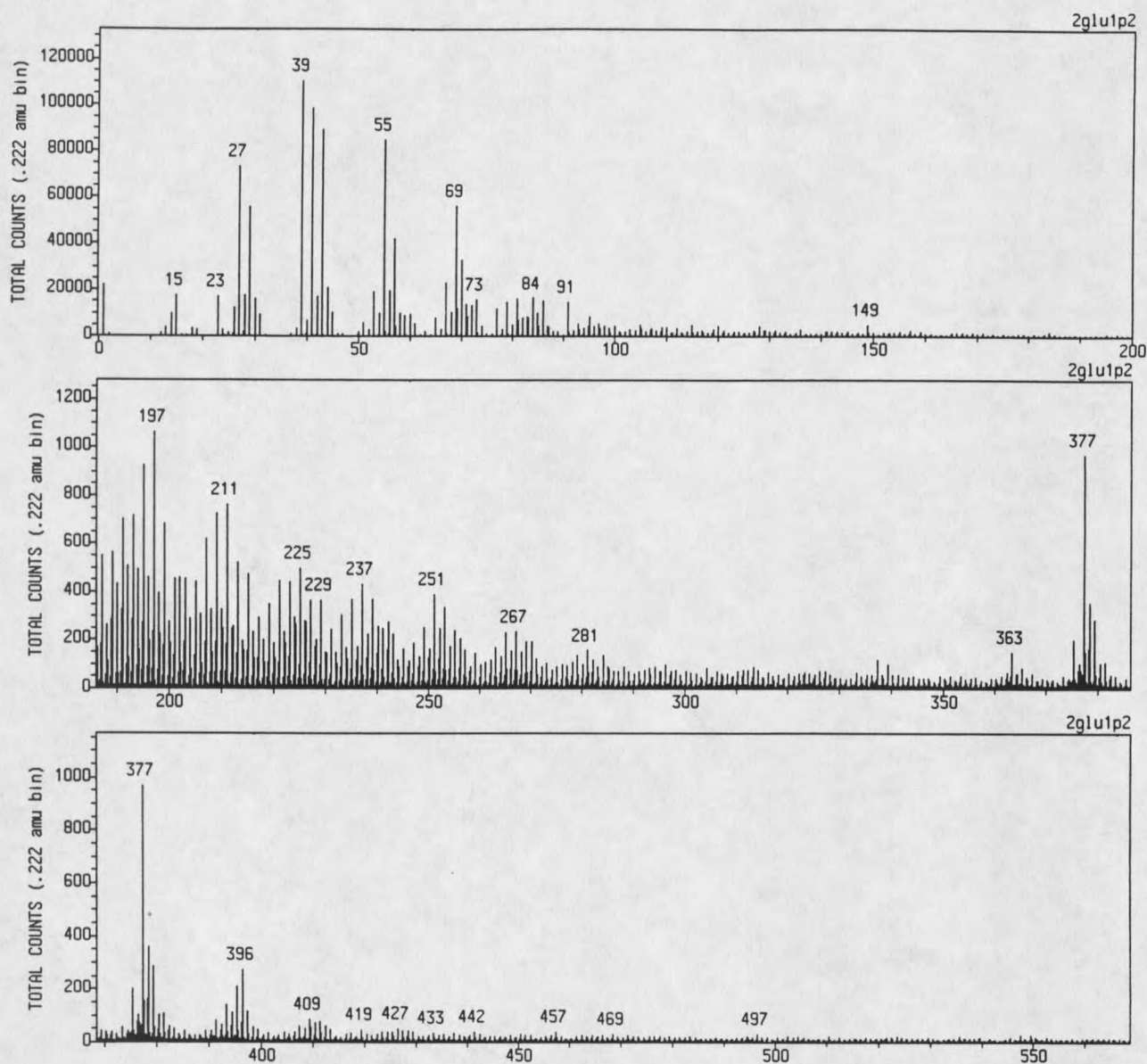
It should be noted that although it appears that hydrophobic interactions are important in the initial attachment of *C. albicans* cells to FEP, it is possible that other factors may also contributed to the different adhesion behavior of glucose-grown and galactose-grown cells. Flow cell experiments have also shown that galactose-grown cells are more resilient to shear rate effects than glucose-grown cells, which may indicate another mechanism is also used in the adherence of galactose-grown cells to FEP. In this regard, electrostatic interactions should be considered as suggested by other researchers [64,67,68] who found that electrostatic interactions were involved in the adherence of *C. albicans* to polymeric surfaces. For example, Douglas, et al found that the addition of divalent cations such as Ca^{2+} , Mg^{2+} , and Fe^{2+} promoted adherence of *C. albicans* to acrylic surfaces. Divalent cations promote adherence by reducing the forces of electrostatic repulsion which exist between the microorganism and its negatively charged substratum [64]. The involvement of electrostatic interactions in the adherence process of *C. albicans* cells to FEP is also suggested by the SIMS result that galactose-grown cell surfaces have more polysaccharide moieties than glucose-grown cell surfaces. Previous studies have indicated that charged polysaccharides and sialic acid [68] are resident on the fungal surface and are the contributors to the surface potential of *C. albicans*. Acidic polysaccharides and sialic acid contribute to the electronegativity of the cell surface of *C. albicans*. It is possible that in this study, galactose-grown cell surfaces are more electronegative than glucose-grown cell surfaces because of the enhanced contributions of acidic polysaccharides which are more abundant on the surfaces of galactose-grown cells than on the surfaces of glucose-grown cells. The FEP surface may also have electronegative surface potential under the experimental conditions.

Electronegative repulsion forces may mediate the adhesion process, and may explain the reduced adhesion of galactose-grown cells and their resilience to shear rate effects compared with glucose-grown cells. However, this theory should be subjected to the confirmation by further research results, because the sources of the peaks indicative of polysaccharides are not certain in this study due to the lack of model compound spectra to compare. It is not certain if they are fragments of acidic polysaccharides or something else. Further research should involve collecting more model compound spectra of acidic polysaccharides in order to compare the SIMS results obtained in this study with the model compound spectra to estimate the degree of the contribution of electrostatic interactions to the adhesion process.



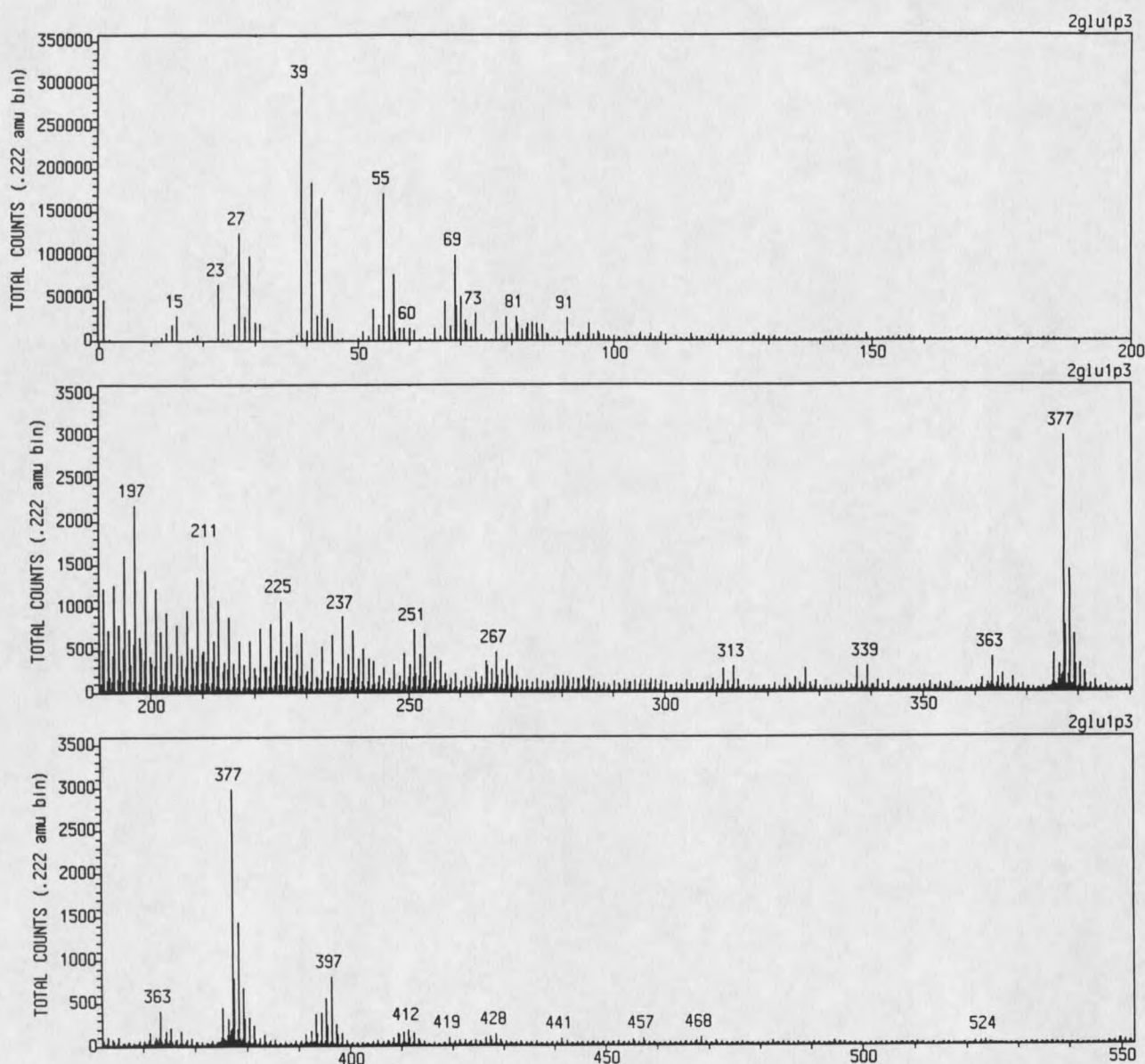
FILE NAME: 2glu1p1 DATE : 16 Apr 98 17:48 ACQUISITION TIME: 4.7 MIN. SPECTRUM INTEGRAL : 1797971
 glucose grown cells, flushed 2 times with H₂O;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 32. SIMS positive ion spectrum of freeze-dried glucose-grown cells, sample1, spot1.



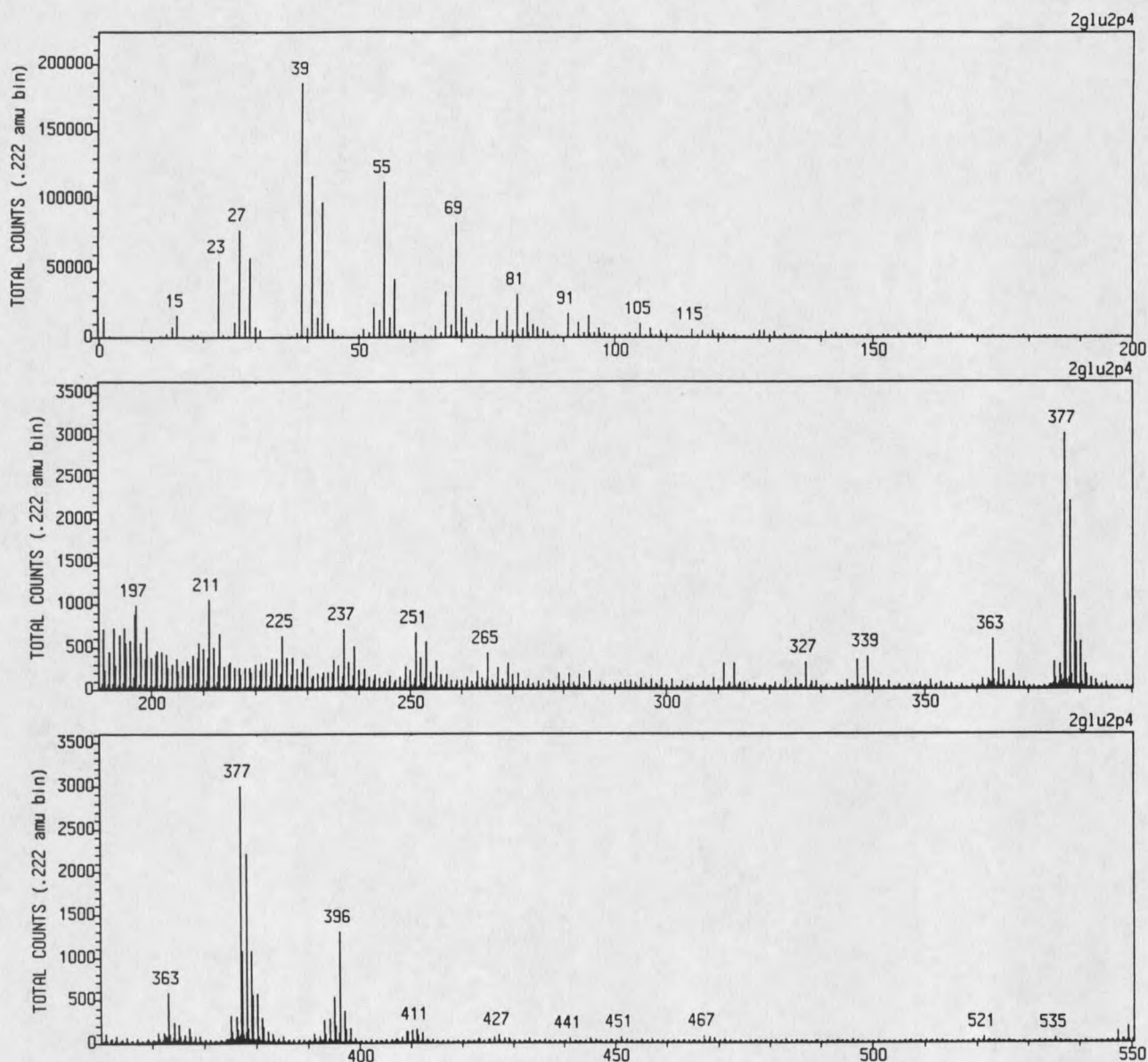
FILE NAME: 2glu1p2 DATE : 16 Apr 98 18:02 ACQUISITION TIME: 1.6 MIN. SPECTRUM INTEGRAL : 1582846
 glucose grown cells, flushed 2 times with H₂O;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 33. SIMS positive ion spectrum of freeze-dried glucose-grown cells, sample1, spot2.



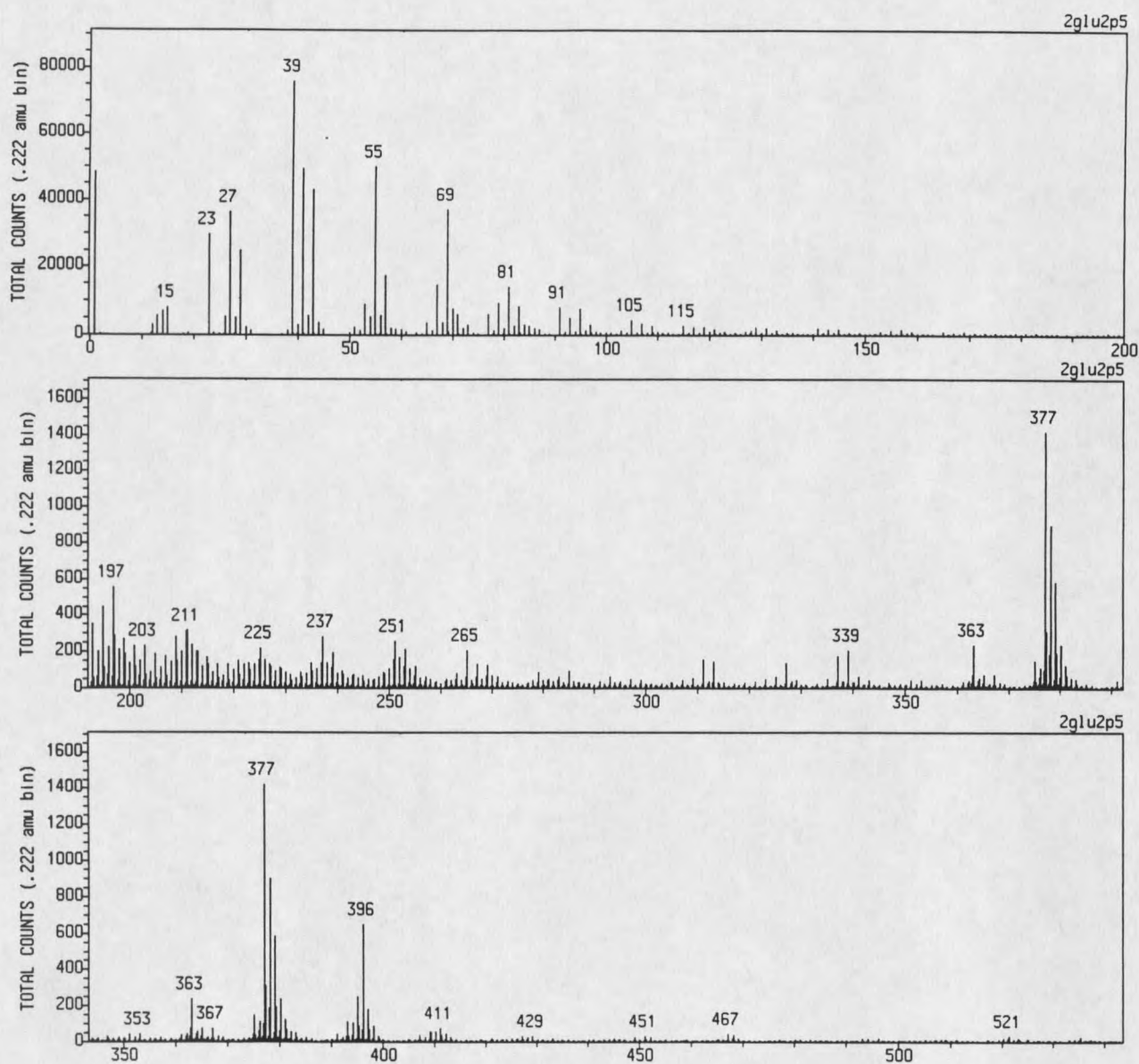
FILE NAME: 2glu1p3 DATE : 16 Apr 98 18:05 ACQUISITION TIME: 5.0 MIN. SPECTRUM INTEGRAL : 2999711
 glucose grown cells, flushed 2 times with H₂O;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 34. SIMS positive ion spectrum of freeze-dried glucose-grown cells, sample1, spot3.



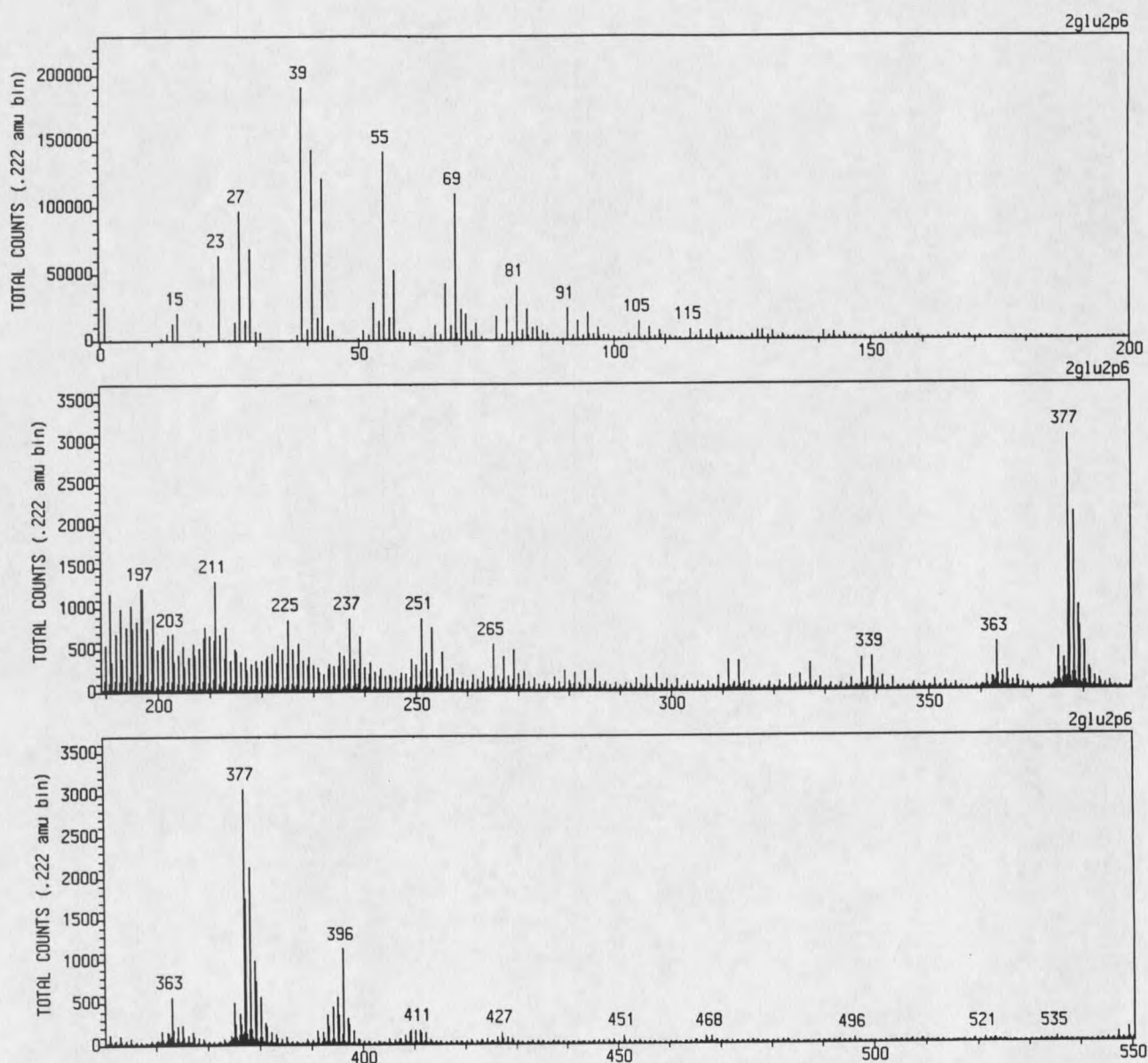
FILE NAME: 2glu2p4 DATE : 16 Apr 98 11:41 ACQUISITION TIME: 5.0 MIN. SPECTRUM INTEGRAL : 1672568
 Freeze-dried glucose-grown cells, flushed by water 2 times;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 0 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 35. SIMS positive ion spectrum of freeze-dried glucose-grown cells, sample2, spot1.



FILE NAME: -2g1u2p5 DATE: 16 Apr 98 12:13 ACQUISITION TIME: 9.0 MIN. SPECTRUM INTEGRAL: 772325
 Freeze-dried glucose-grown cells, flushed by water 2 times;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 0 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 36. SIMS positive ion spectrum of freeze-dried glucose-grown cells, sample2, spot2.



FILE NAME: 2glu2p6 DATE : 16 Apr 98 12:53 ACQUISITION TIME: 3.7 MIN. SPECTRUM INTEGRAL : 2091100
 Freeze-dried glucose-grown cells, flushed by water 2 times;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 0 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 37. SIMS positive ion spectrum of freeze-dried glucose-grown cells, sample2, spot3.

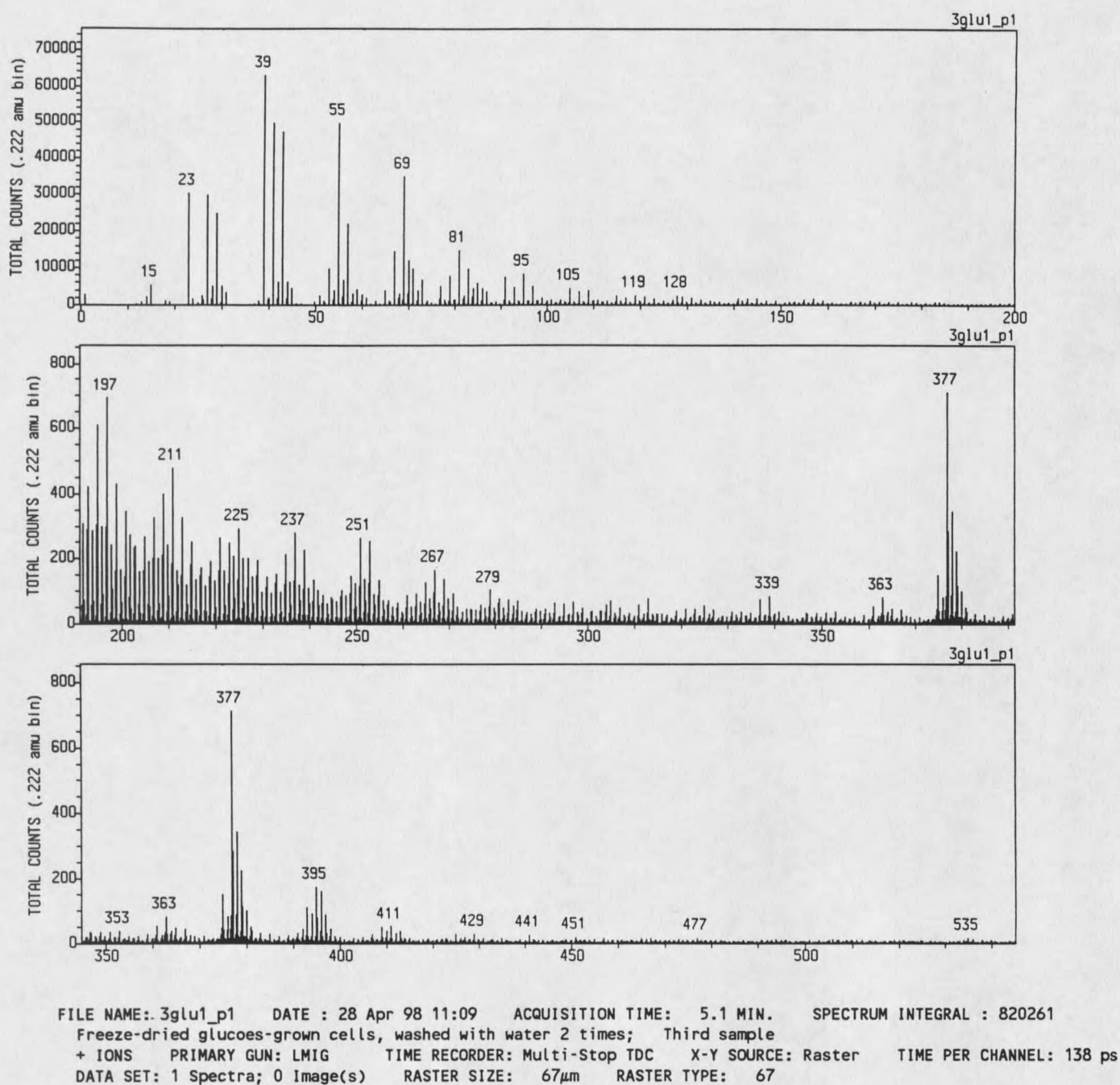
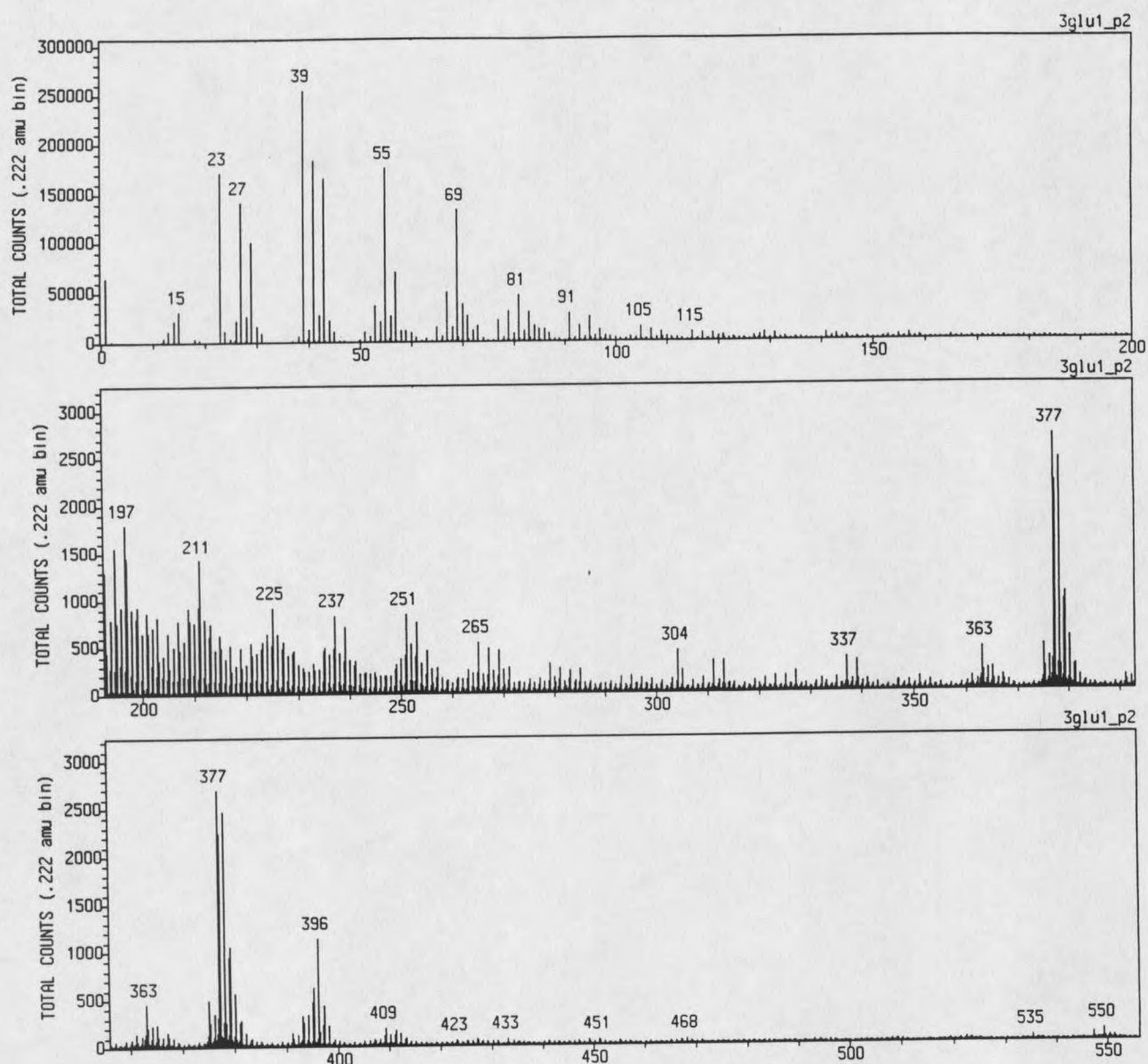
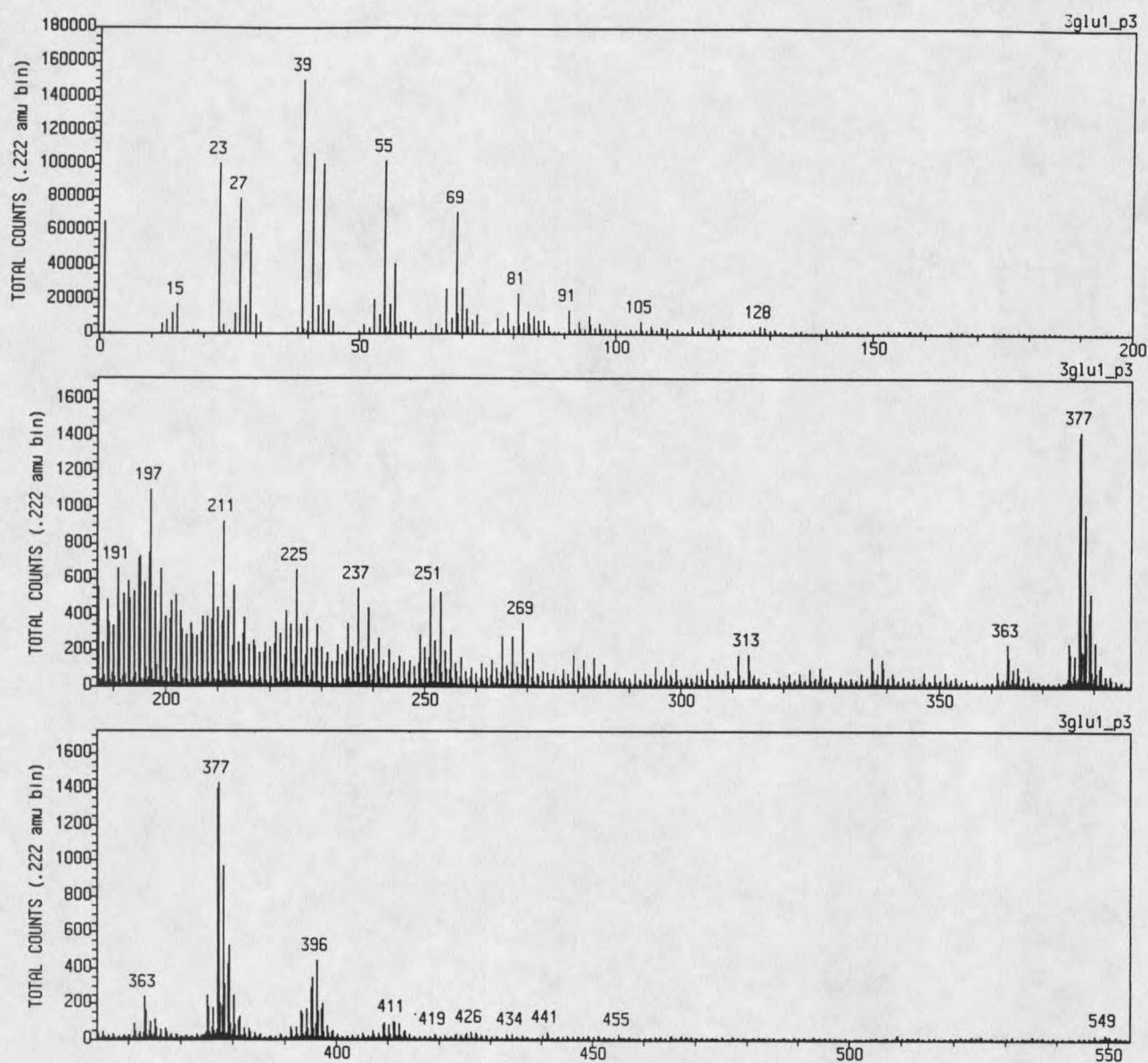


Figure 38. SIMS positive ion spectrum of freeze-dried glucose-grown cells, sample3, spot1.



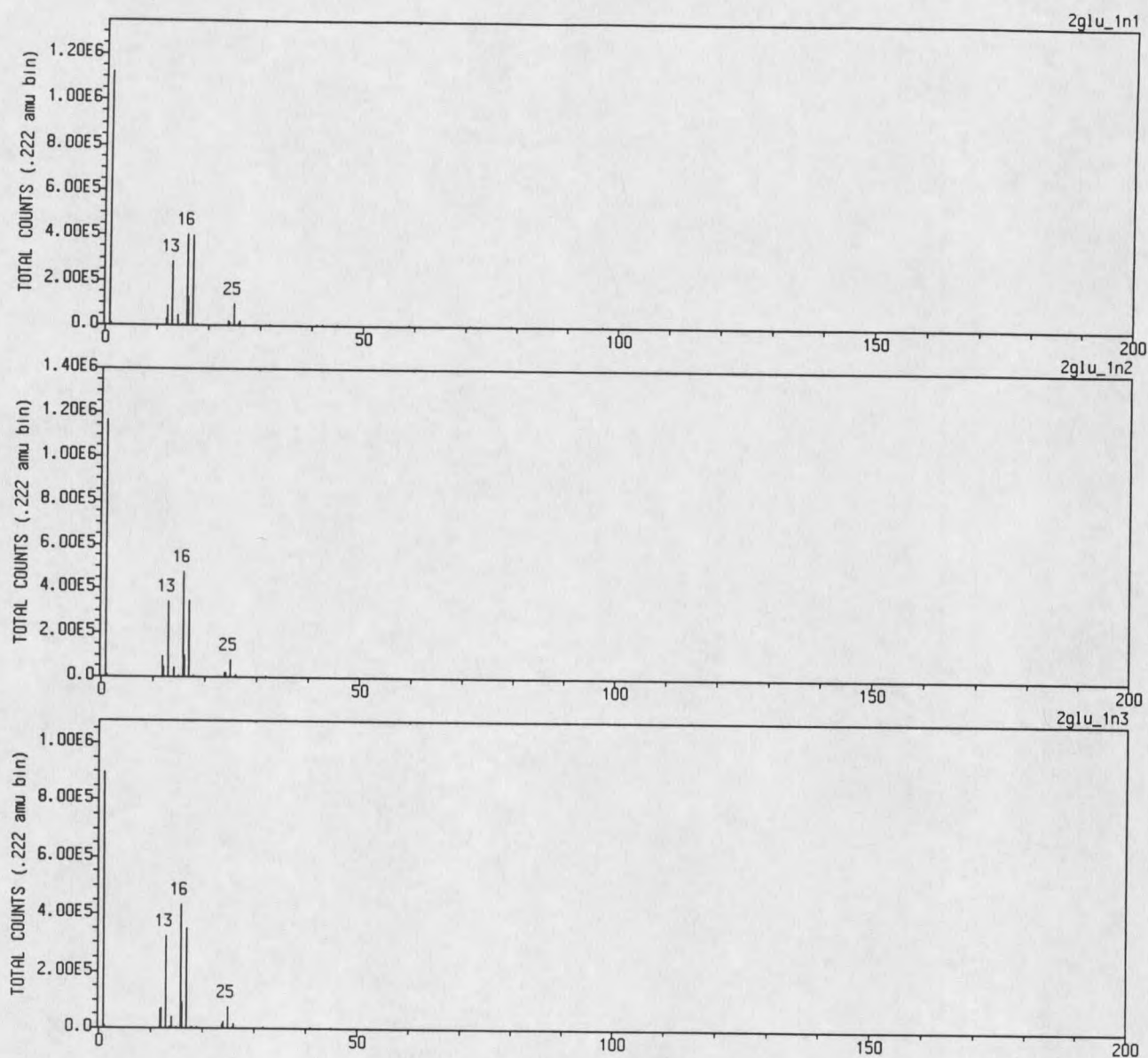
FILE NAME: 3glu1_p2 DATE : 28 Apr 98 11:26 ACQUISITION TIME: 5.2 MIN. SPECTRUM INTEGRAL : 2905917
 Freeze-dried glucoses-grown cells, washed with water 2 times; Third sample
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 39. SIMS positive ion spectrum of freeze-dried glucose-grown cells, sample3, spot2.



FILE NAME: 3glu1_p3 DATE : 28 Apr 98 11:57 ACQUISITION TIME: 4.1 MIN. SPECTRUM INTEGRAL : 1765863
 Freeze-dried glucoses-grown cells, washed with water 2 times; Third sample
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 40. SIMS positive ion spectrum of freeze-dried glucose-grown cells, sample3, spot3

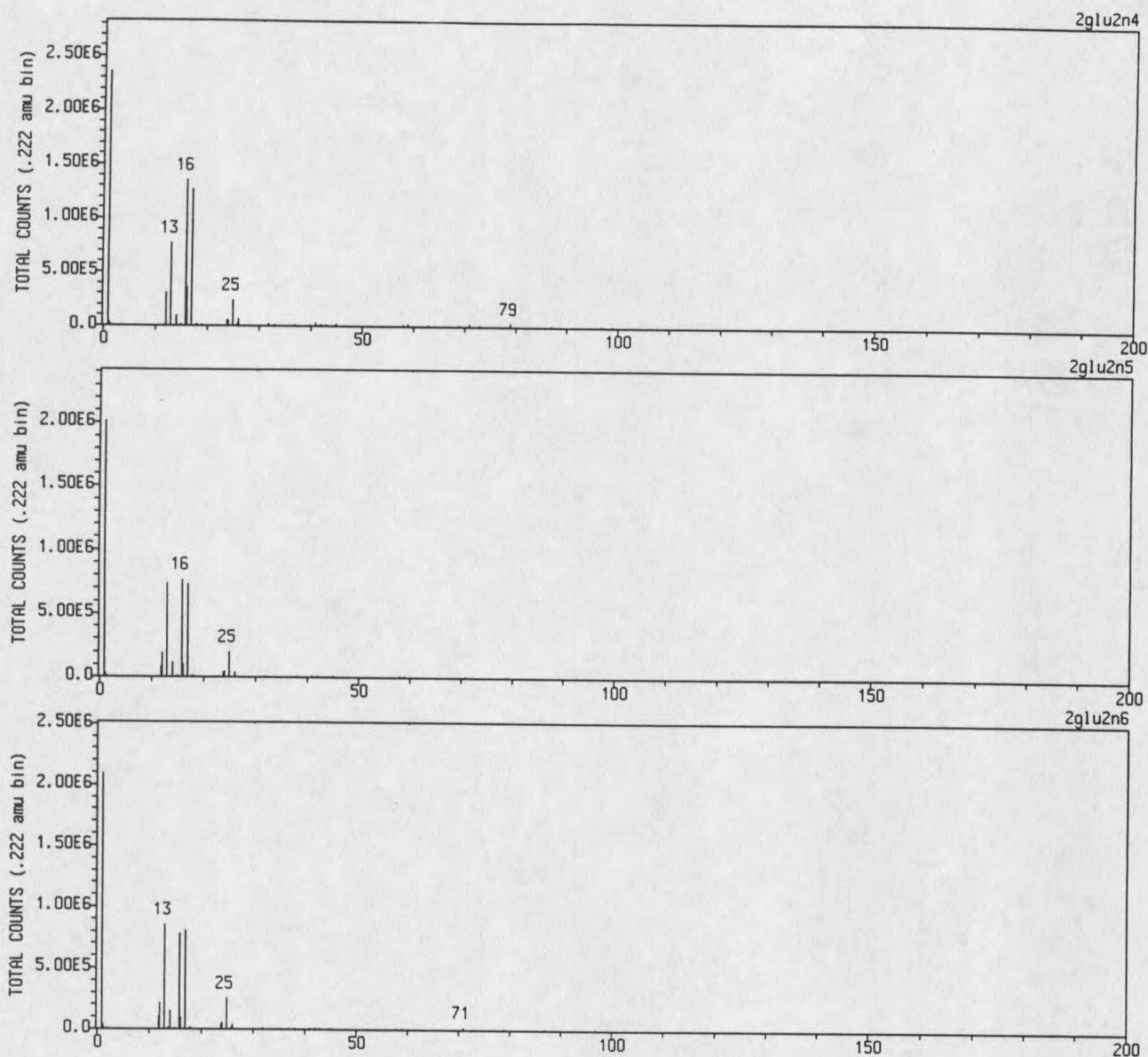


FILE NAME: 2glu_1n1 DATE : 17 Apr 98 13:14 ACQUISITION TIME: 5.1 MIN. SPECTRUM INTEGRAL : 2831572
 freeze dried galactose grown cells, flush 2 times with H₂O;
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 100 μ m RASTER TYPE: 100

FILE NAME: 2glu_1n2 DATE : 17 Apr 98 13:34 ACQUISITION TIME: 7.1 MIN. SPECTRUM INTEGRAL : 2830086
 freeze dried galactose grown cells, flush 2 times with H₂O;
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

FILE NAME: 2glu_1n3 DATE : 17 Apr 98 14:04 ACQUISITION TIME: 2.3 MIN. SPECTRUM INTEGRAL : 2501511
 freeze dried galactose grown cells, flush 2 times with H₂O;
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 2 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 41. SIMS negative ion spectra of freeze-dried glucose-grown cells, sample 1. The top one is spot1. The middle one is spot 2. The bottom one is spot3.

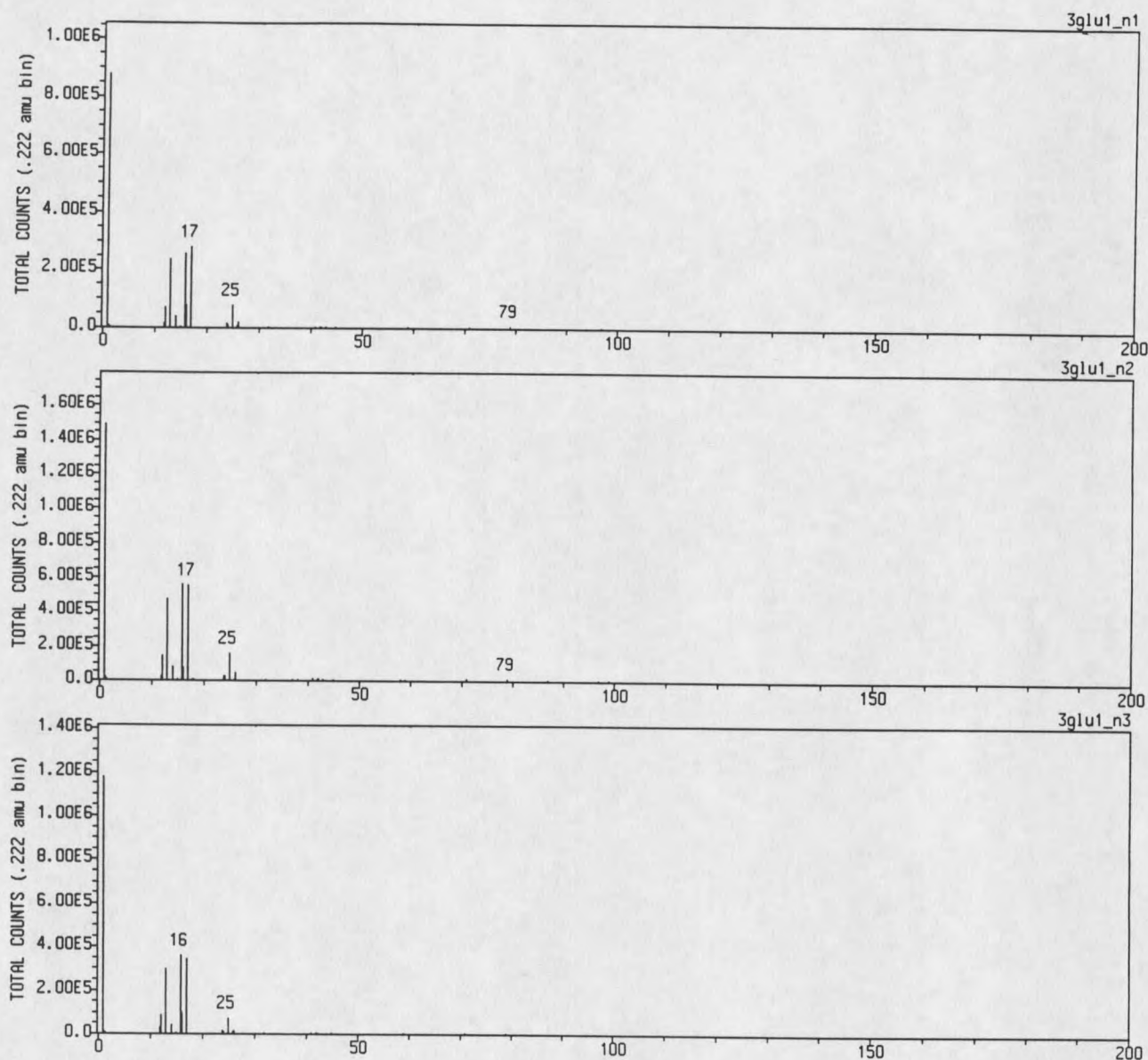


FILE NAME: 2glu2n4 DATE : 16 Apr 98 12:03 ACQUISITION TIME: 7.5 MIN. SPECTRUM INTEGRAL : 7544776
 Freeze-dried glucose-grown cells, flushed by water 2 times;
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

FILE NAME: 2glu2n5 DATE : 16 Apr 98 12:39 ACQUISITION TIME: 6.2 MIN. SPECTRUM INTEGRAL : 5346669
 Freeze-dried glucose-grown cells, flushed by water 2 times;
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

FILE NAME: 2glu2n6 DATE : 16 Apr 98 13:00 ACQUISITION TIME: 6.7 MIN. SPECTRUM INTEGRAL : 5914218
 Freeze-dried glucose-grown cells, flushed by water 2 times;
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 42. SIMS negative ion spectra of freeze-dried glucose-grown cells, sample 2. The top one is spot1. The middle one is spot 2. The bottom one is spot3.

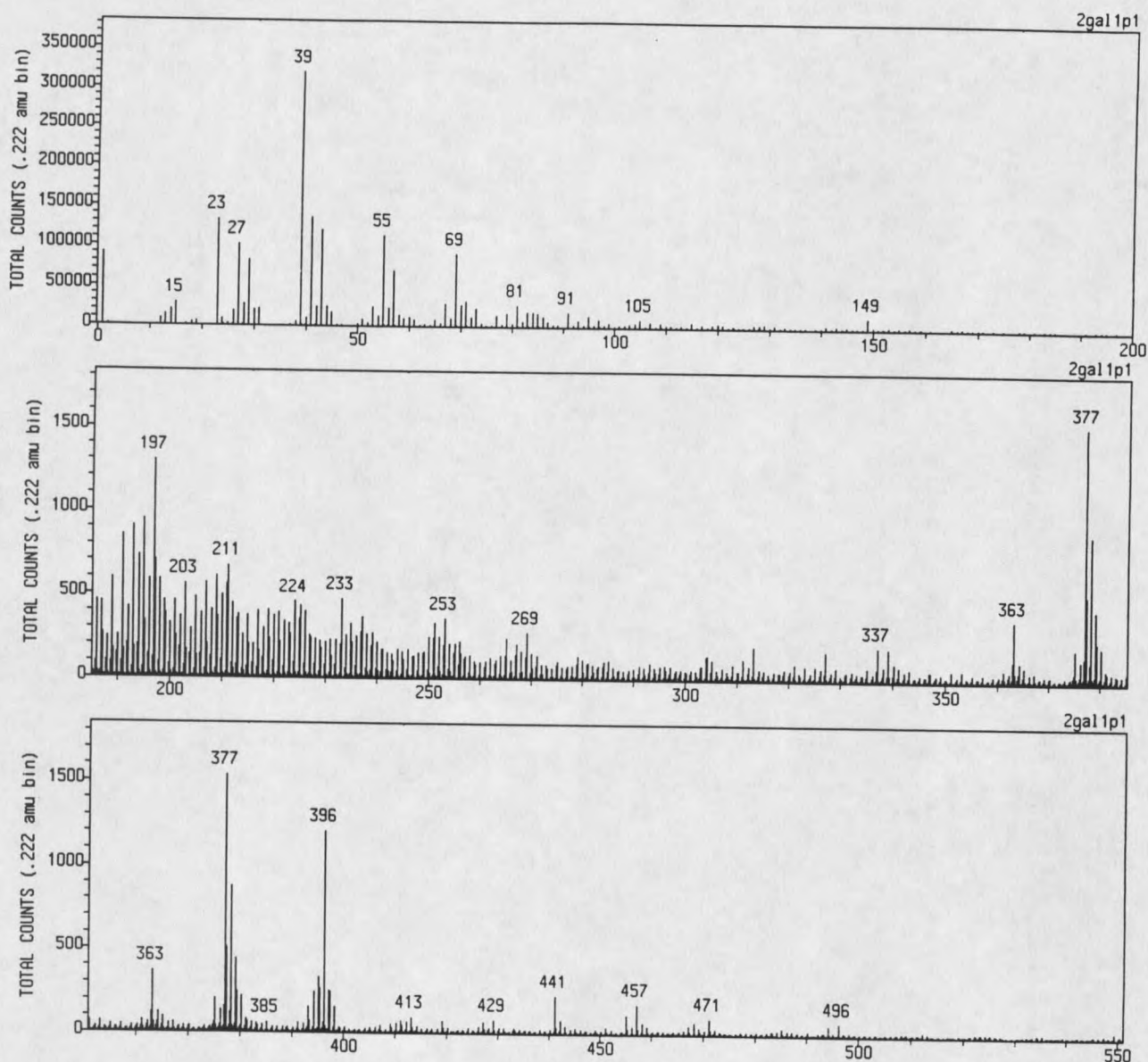


FILE NAME: 3glu1_n1 DATE : 28 Apr 98 11:35 ACQUISITION TIME: 1.8 MIN. SPECTRUM INTEGRAL : 2149962
 Freeze-dried glucoses-grown cells, washed with water 2 times; Third sample
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 100 μ m RASTER TYPE: 100

FILE NAME: 3glu1_n2 DATE : 28 Apr 98 11:39 ACQUISITION TIME: 4.4 MIN. SPECTRUM INTEGRAL : 4011356
 Freeze-dried glucoses-grown cells, washed with water 2 times; Third sample
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 100 μ m RASTER TYPE: 100

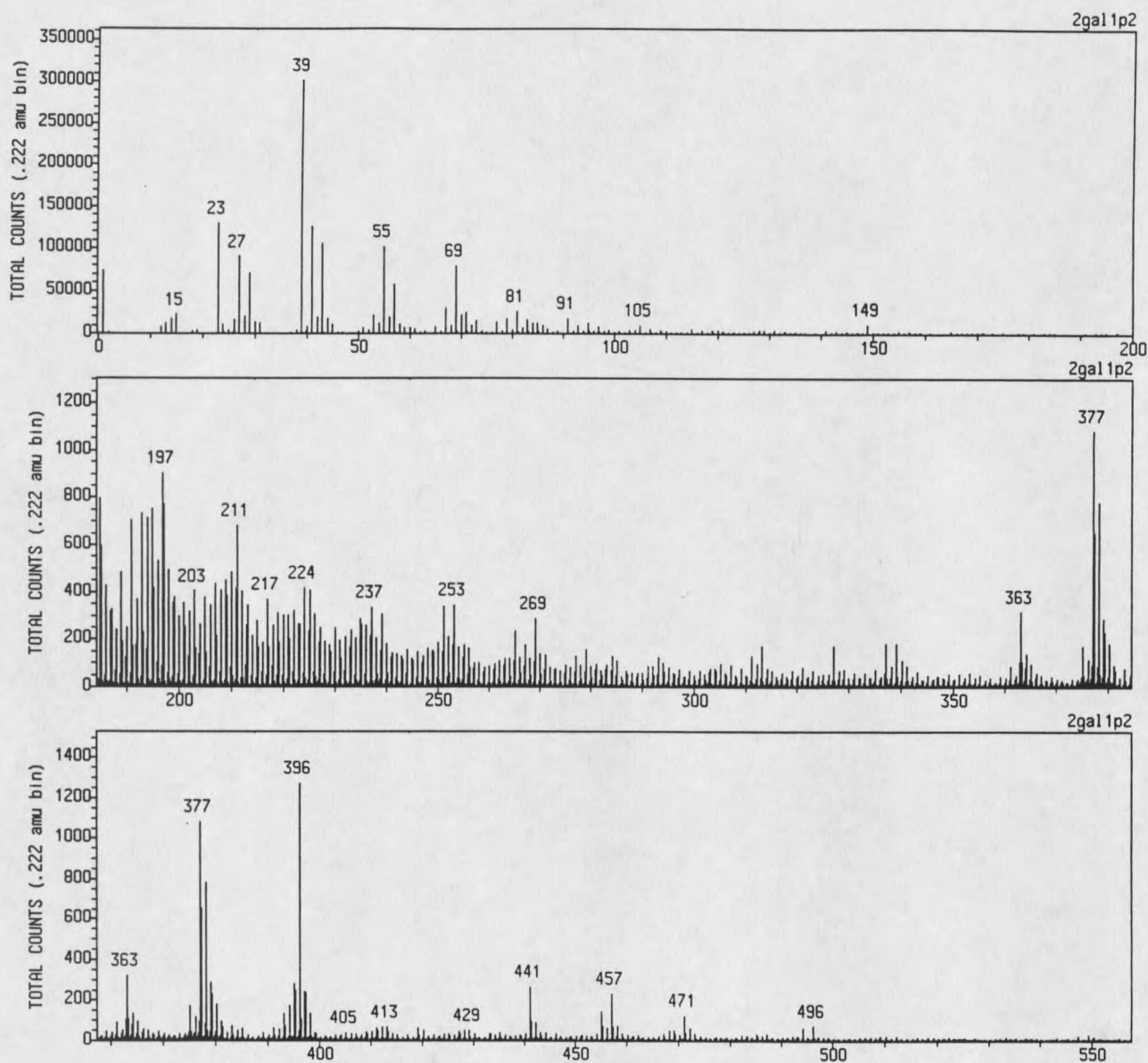
FILE NAME: 3glu1_n3 DATE : 28 Apr 98 12:30 ACQUISITION TIME: 4.0 MIN. SPECTRUM INTEGRAL : 2716368
 Freeze-dried glucoses-grown cells, washed with water 2 times; Third sample
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 100 μ m RASTER TYPE: 100

Figure 43. SIMS negative ion spectra of freeze-dried glucose-grown cells, sample 3. The top one is spot1. The middle one is spot 2. The bottom one is spot3.



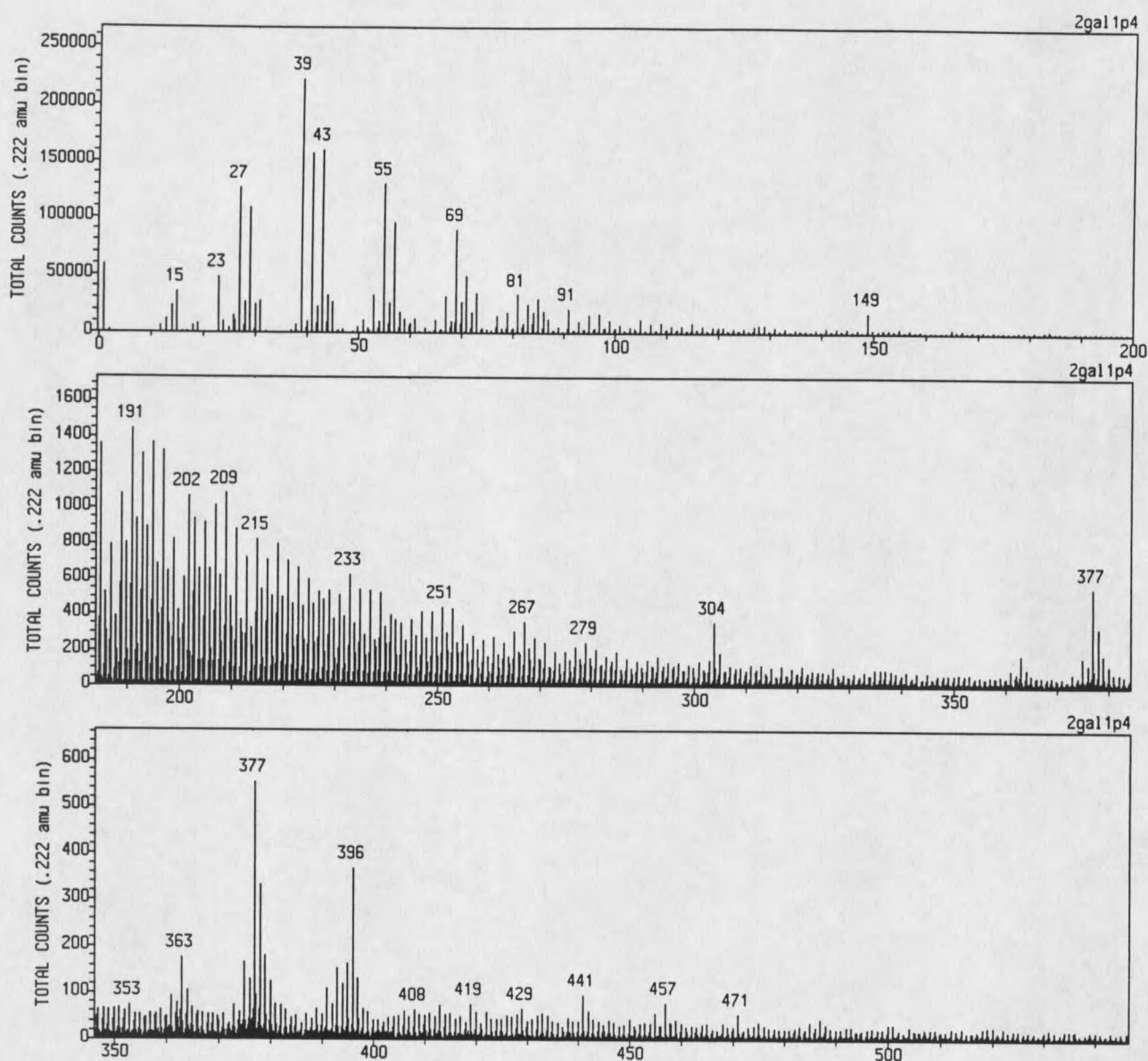
FILE NAME: 2gal1p1 DATE : 16 Apr 98 15:50 ACQUISITION TIME: 4.1 MIN. SPECTRUM INTEGRAL : 2384210
 galactose grown cells, flushed 2 times with H₂O;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 44. SIMS positive ion spectrum of freeze-dried galactose-grown cells, sample1, spot1.



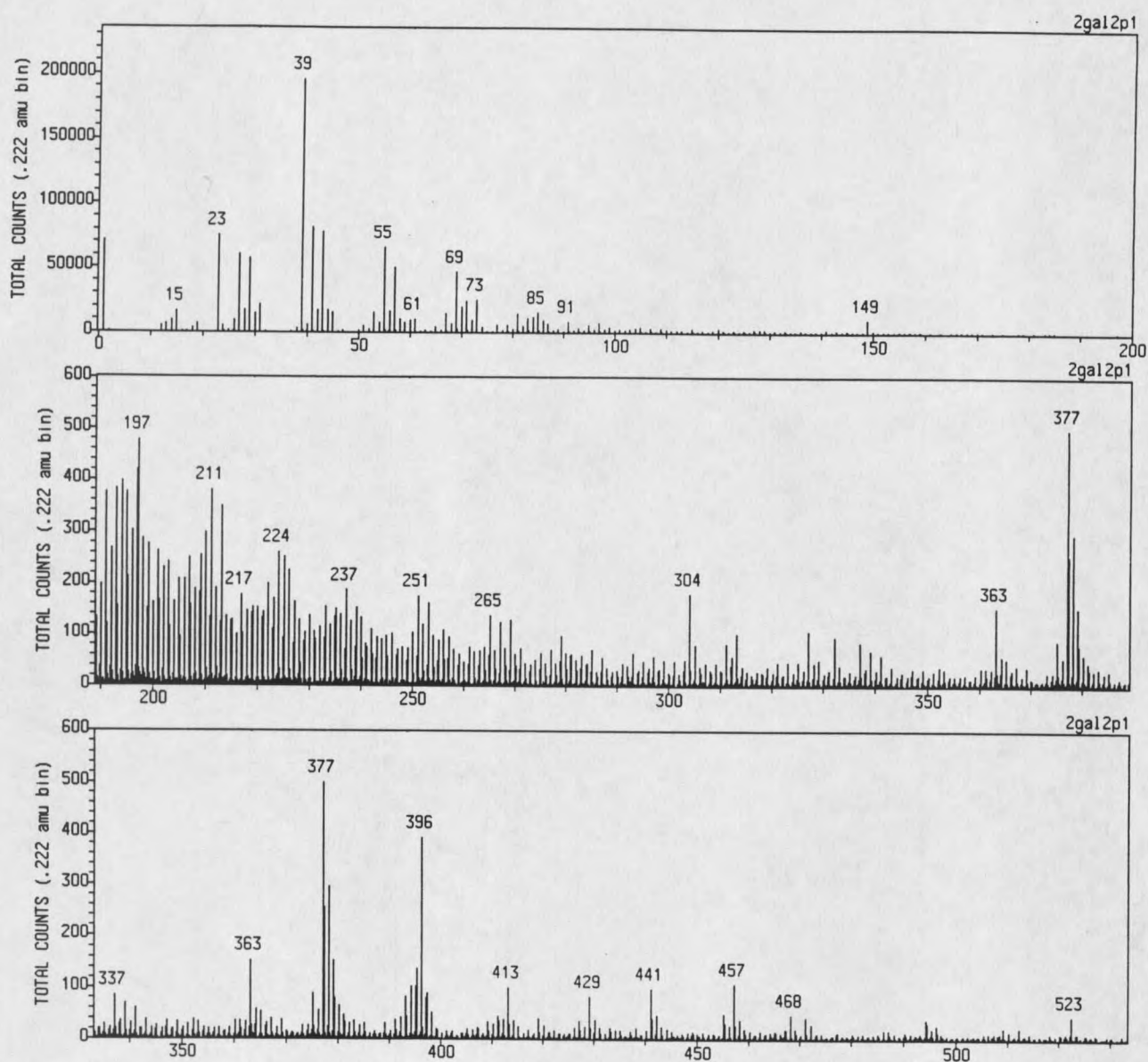
FILE NAME: 2gal1p2 DATE : 16 Apr 98 15:56 ACQUISITION TIME: 3.5 MIN. SPECTRUM INTEGRAL : 2080973
 galactose grown cells, flushed 2 times with H₂O;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 45. SIMS positive ion spectrum of freeze-dried galactose-grown cells, sample1, spot2.



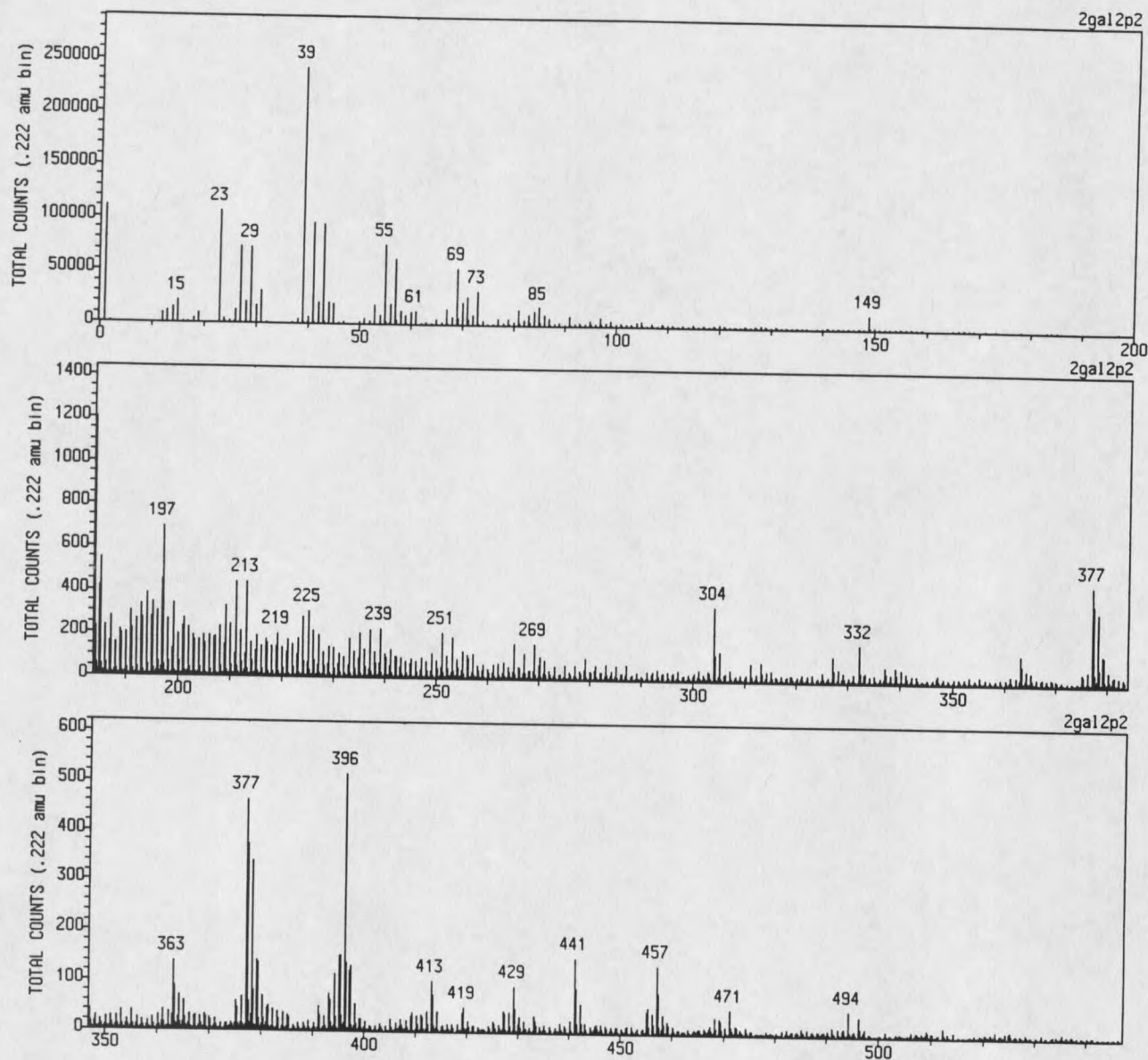
FILE NAME: 2gal1p4 DATE : 16 Apr 98 17:12 ACQUISITION TIME: 5.0 MIN. SPECTRUM INTEGRAL : 2756626
 galactose grown cells, flushed 2 times with H₂O;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 46. SIMS positive ion spectrum of freeze-dried galactose-grown cells, sample1, spot3.



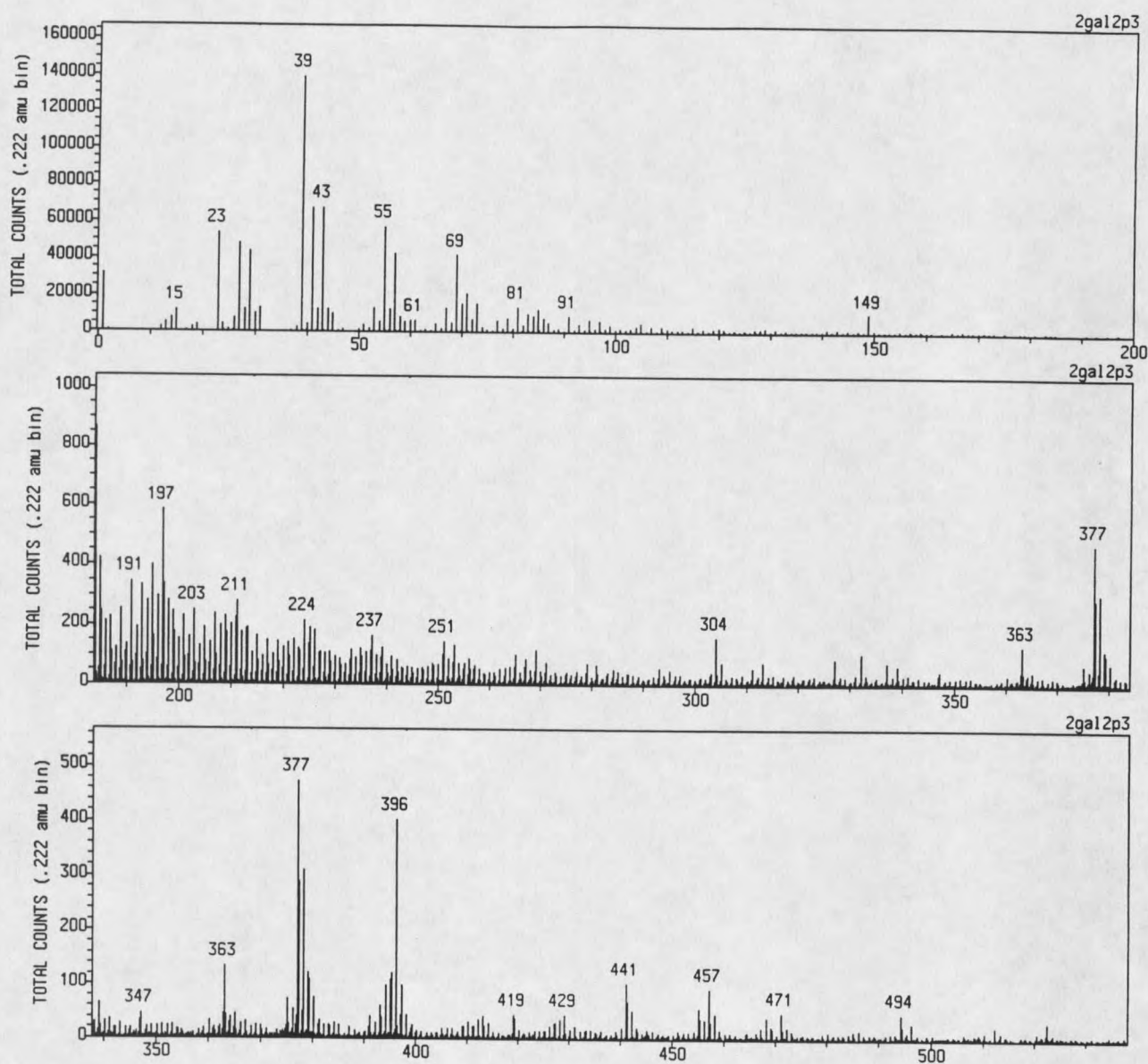
FILE NAME: 2gal2p1 DATE : 16 Apr 98 13:41 ACQUISITION TIME: 7.3 MIN. SPECTRUM INTEGRAL : 1526647
 galactose grown cells, flushed 2 times with H₂O;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 2 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 47. SIMS positive ion spectrum of freeze-dried galactose-grown cells, sample2, spot1.



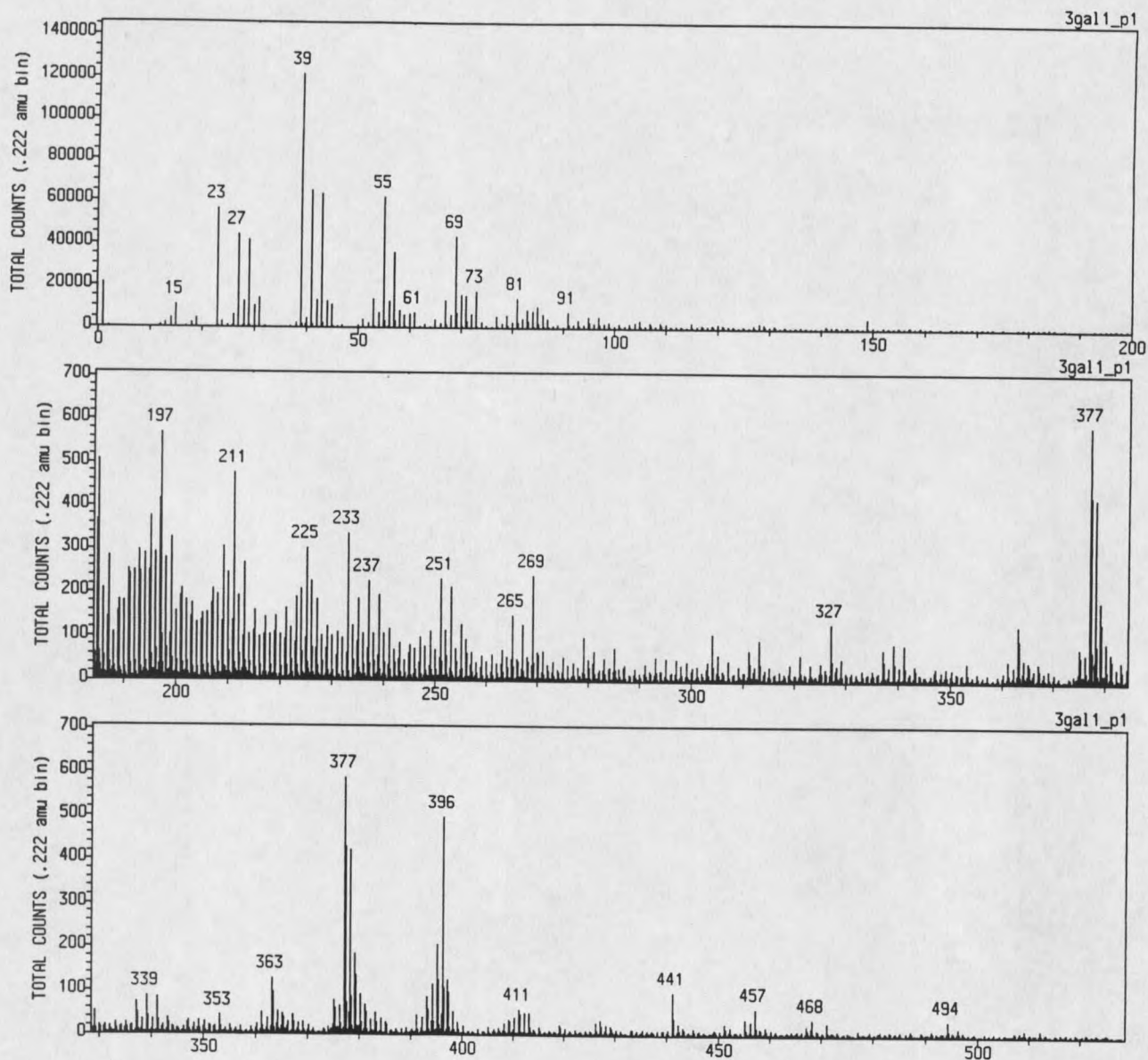
FILE NAME: 2gal2p2 DATE : 16 Apr 98 15:15 ACQUISITION TIME: 4.1 MIN. SPECTRUM INTEGRAL : 1875737
 galactose grown cells, flushed 2 times with H₂O;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 48. SIMS positive ion spectrum of freeze-dried galactose-grown cells, sample2, spot2.



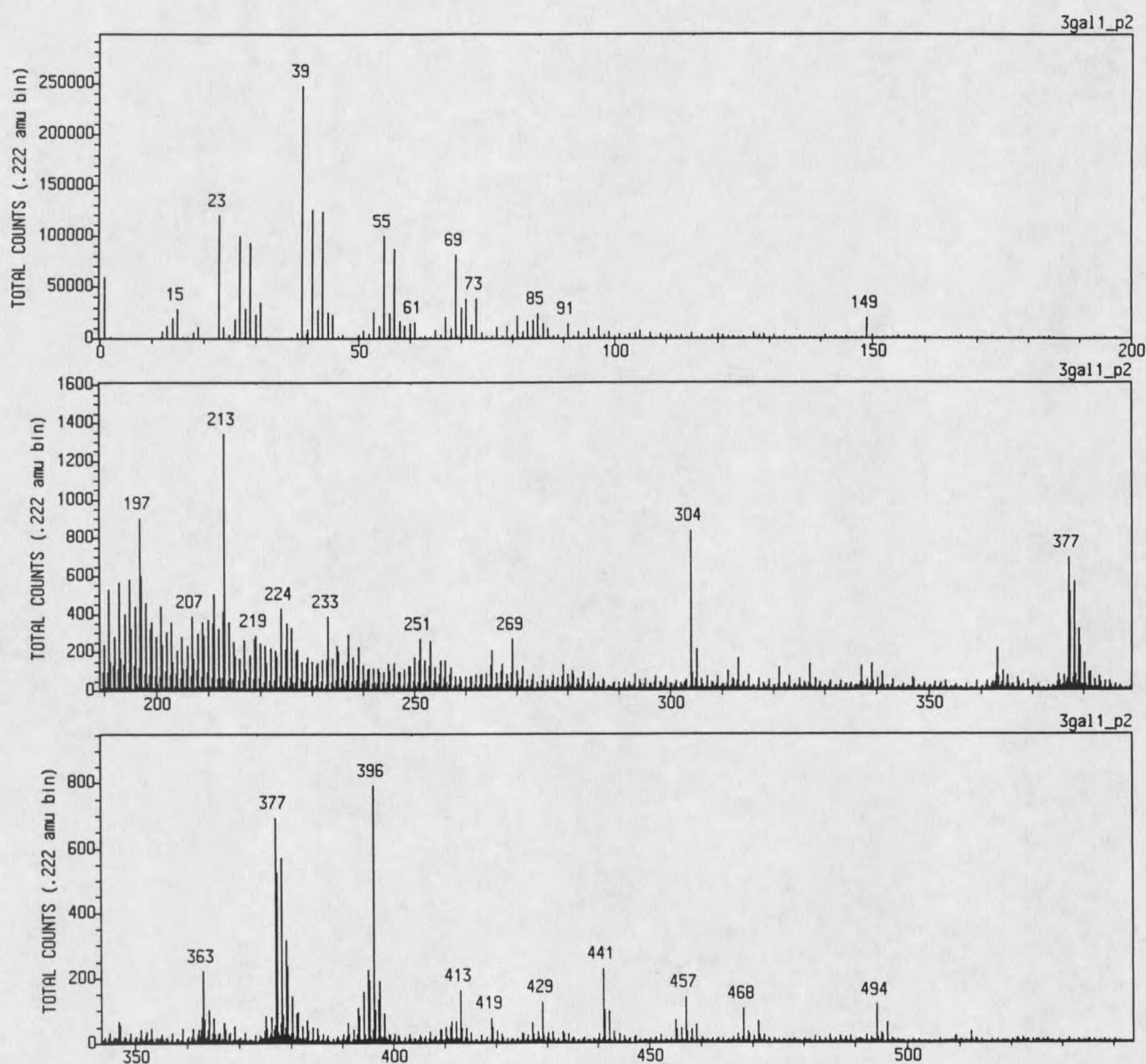
FILE NAME: 2gal2p3 DATE : 16 Apr 98 17:21 ACQUISITION TIME: 5.0 MIN. SPECTRUM INTEGRAL : 1169652
 galactose grown cells, flushed 2 times with H₂O;
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 49. SIMS positive ion spectrum of freeze-dried galactose-grown cells, sample2, spot3.



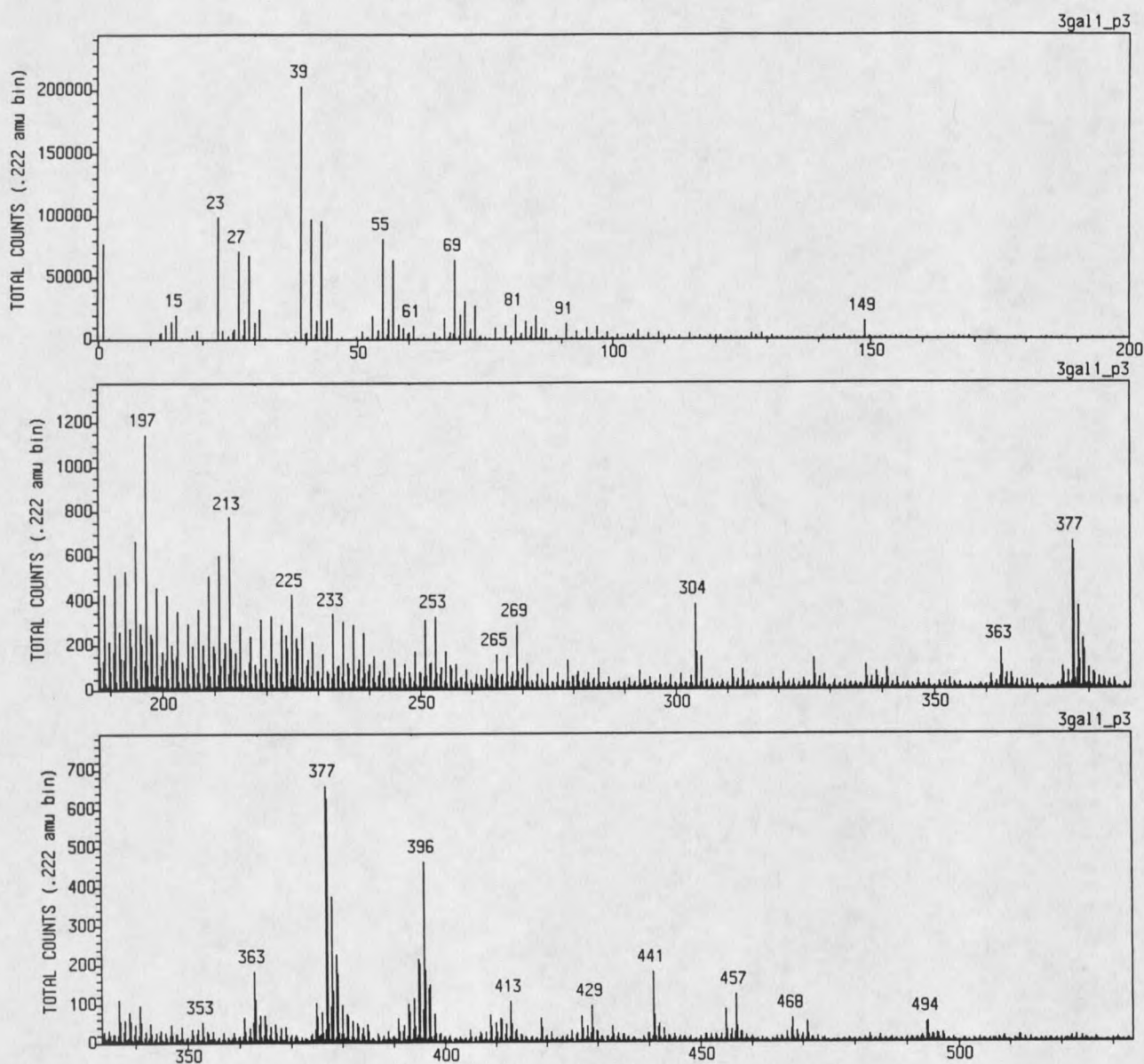
FILE NAME: 3gal1_p1 DATE : 28 Apr 98 12:40 ACQUISITION TIME: 4.7 MIN. SPECTRUM INTEGRAL : 1157596
 Freeze-dried galactose-grown cells, washed with water 2 times; Third sample
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 50. SIMS positive ion spectrum of freeze-dried galactose-grown cells, sample3, spot1.



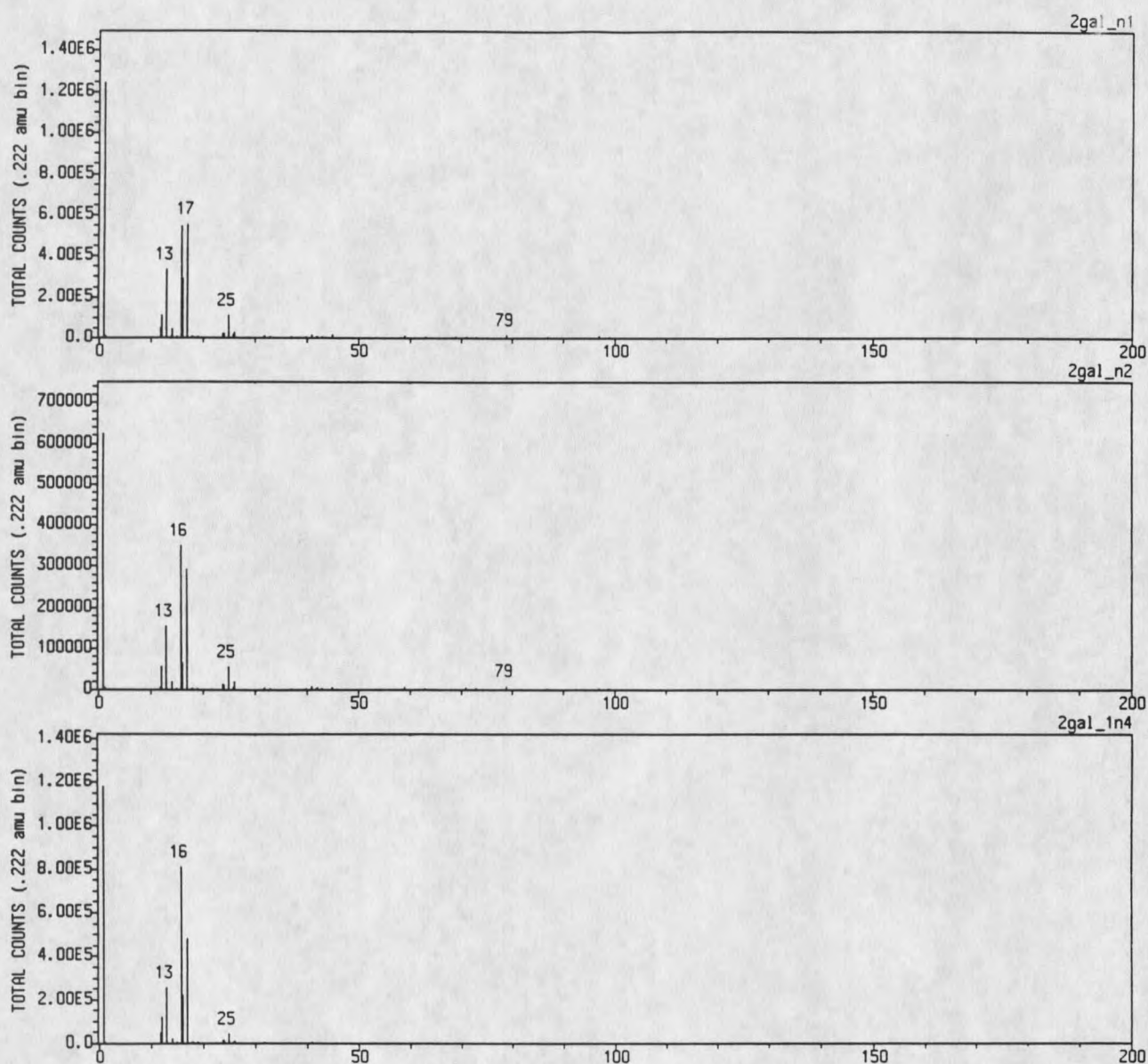
FILE NAME: 3gal1_p2 DATE : 28 Apr 98 13:26 ACQUISITION TIME: 4.7 MIN. SPECTRUM INTEGRAL : 2294311
 Freeze-dried galactose-grown cells, washed with water 2 times; Third sample
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 2 Image(s) RASTER SIZE: 100 μ m RASTER TYPE: 100

Figure 51. SIMS positive ion spectrum of freeze-dried galactose-grown cells, sample3, spot2.



FILE NAME: 3gal1_p3 DATE : 28 Apr 98 13:35 ACQUISITION TIME: 4.1 MIN. SPECTRUM INTEGRAL : 1813262
 Freeze-dried galactose-grown cells, washed with water 2 times; Third sample
 + IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 2 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

Figure 52. SIMS positive ion spectrum of freeze-dried galactose-grown cells, sample3, spot3.



FILE NAME: 2gal_n1 DATE : 17 Apr 98 11:14 ACQUISITION TIME: 5.1 MIN. SPECTRUM INTEGRAL : 3686917

- IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 100 μ m RASTER TYPE: 100

FILE NAME: 2gal_n2 DATE : 17 Apr 98 11:33 ACQUISITION TIME: 2.7 MIN. SPECTRUM INTEGRAL : 1832976

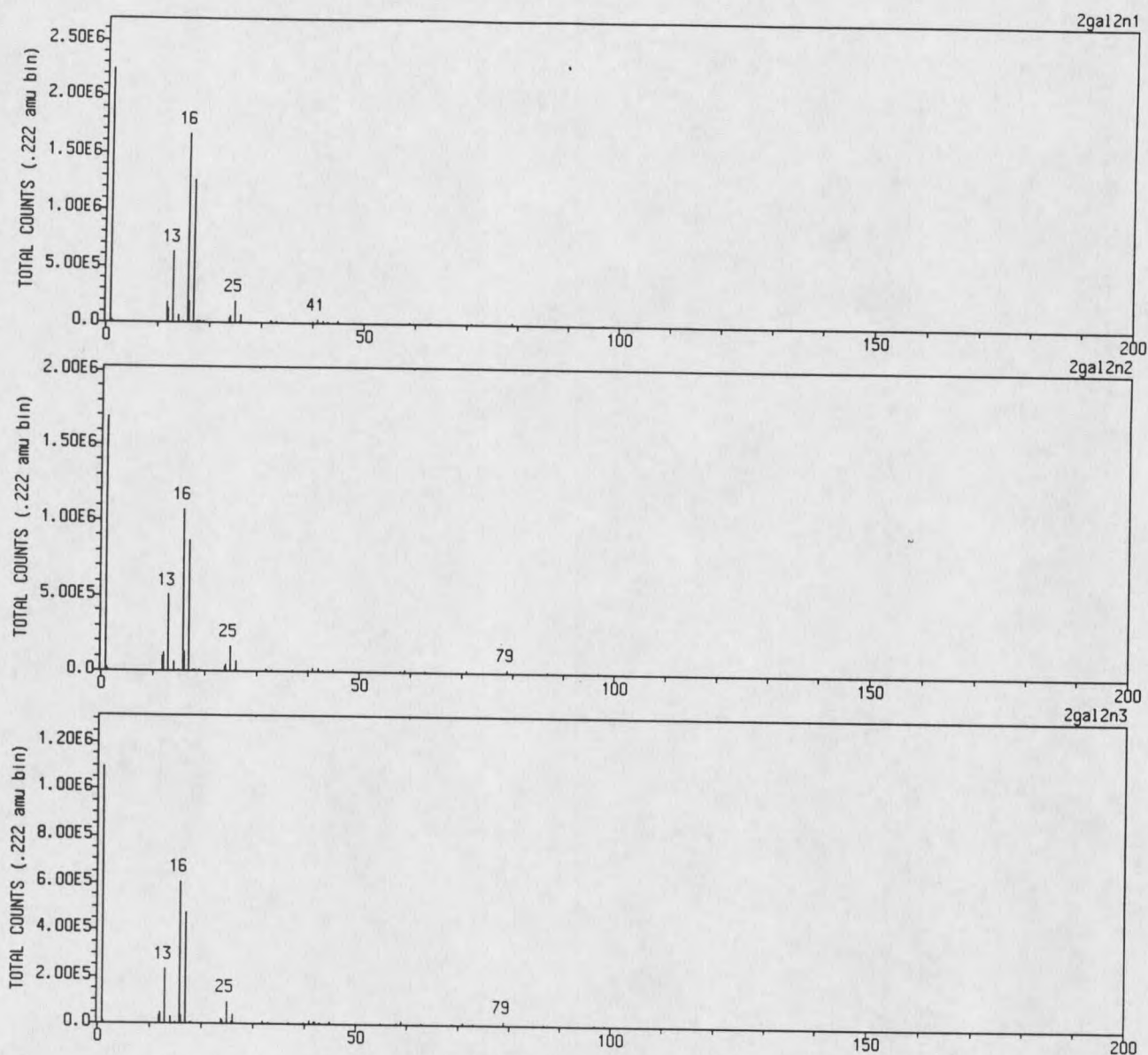
- IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

FILE NAME: 2gal_n4 DATE : 17 Apr 98 11:53 ACQUISITION TIME: 5.4 MIN. SPECTRUM INTEGRAL : 3322963

freeze dried galactose grown cells, flush 2 times with H₂O;

- IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 100 μ m RASTER TYPE: 100

Figure 53. SIMS negative ion spectra of freeze-dried galactose-grown cells, sample 1. The top one is spot1. The middle one is spot 2. The bottom one is spot3.

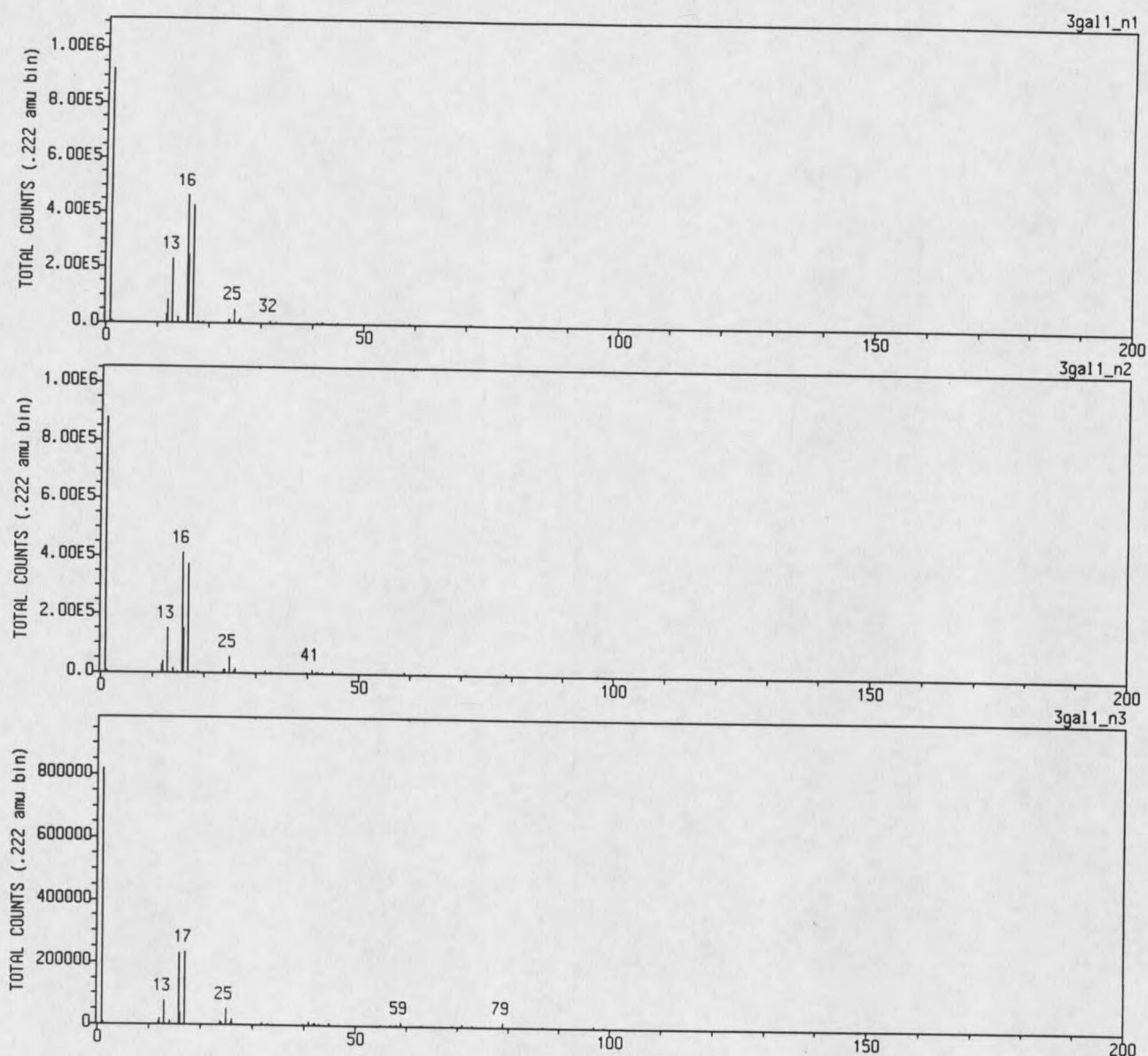


FILE NAME: 2gal2n1 DATE : 16 Apr 98 13:59 ACQUISITION TIME: 7.3 MIN. SPECTRUM INTEGRAL : 7183910
 galactose grown cells, flushed 2 times with H₂O;
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

FILE NAME: 2gal2n2 DATE : 16 Apr 98 15:26 ACQUISITION TIME: 5.1 MIN. SPECTRUM INTEGRAL : 5299020
 galactose grown cells, flushed 2 times with H₂O;
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 67 μ m RASTER TYPE: 67

FILE NAME: 2gal2n3 DATE : 16 Apr 98 17:32 ACQUISITION TIME: 5.0 MIN. SPECTRUM INTEGRAL : 3016341
 galactose grown cells, flushed 2 times with H₂O;
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 143 μ m RASTER TYPE: 143

Figure 54. SIMS negative ion spectra of freeze-dried galactose-grown cells, sample 2. The top one is spot1. The middle one is spot 2. The bottom one is spot3.



FILE NAME: 3gal1_n1 DATE : 28 Apr 98 13:45 ACQUISITION TIME: 4.2 MIN. SPECTRUM INTEGRAL : 2652374
 Freeze-dried galactose-grown cells, washed with water 2 times; Third sample
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 100 μ m RASTER TYPE: 100

FILE NAME: 3gal1_n2 DATE : 28 Apr 98 13:51 ACQUISITION TIME: 4.1 MIN. SPECTRUM INTEGRAL : 2314882
 Freeze-dried galactose-grown cells, washed with water 2 times; Third sample
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 100 μ m RASTER TYPE: 100

FILE NAME: 3gal1_n3 DATE : 28 Apr 98 13:58 ACQUISITION TIME: 5.3 MIN. SPECTRUM INTEGRAL : 1719043
 Freeze-dried galactose-grown cells, washed with water 2 times; Third sample
 - IONS PRIMARY GUN: LMIG TIME RECORDER: Multi-Stop TDC X-Y SOURCE: Raster TIME PER CHANNEL: 138 ps
 DATA SET: 1 Spectra; 1 Image(s) RASTER SIZE: 100 μ m RASTER TYPE: 100

Figure 55. SIMS negative ion spectra of freeze-dried galactose-grown cells, sample 3. The top one is spot1. The middle one is spot 2. The bottom one is spot3.

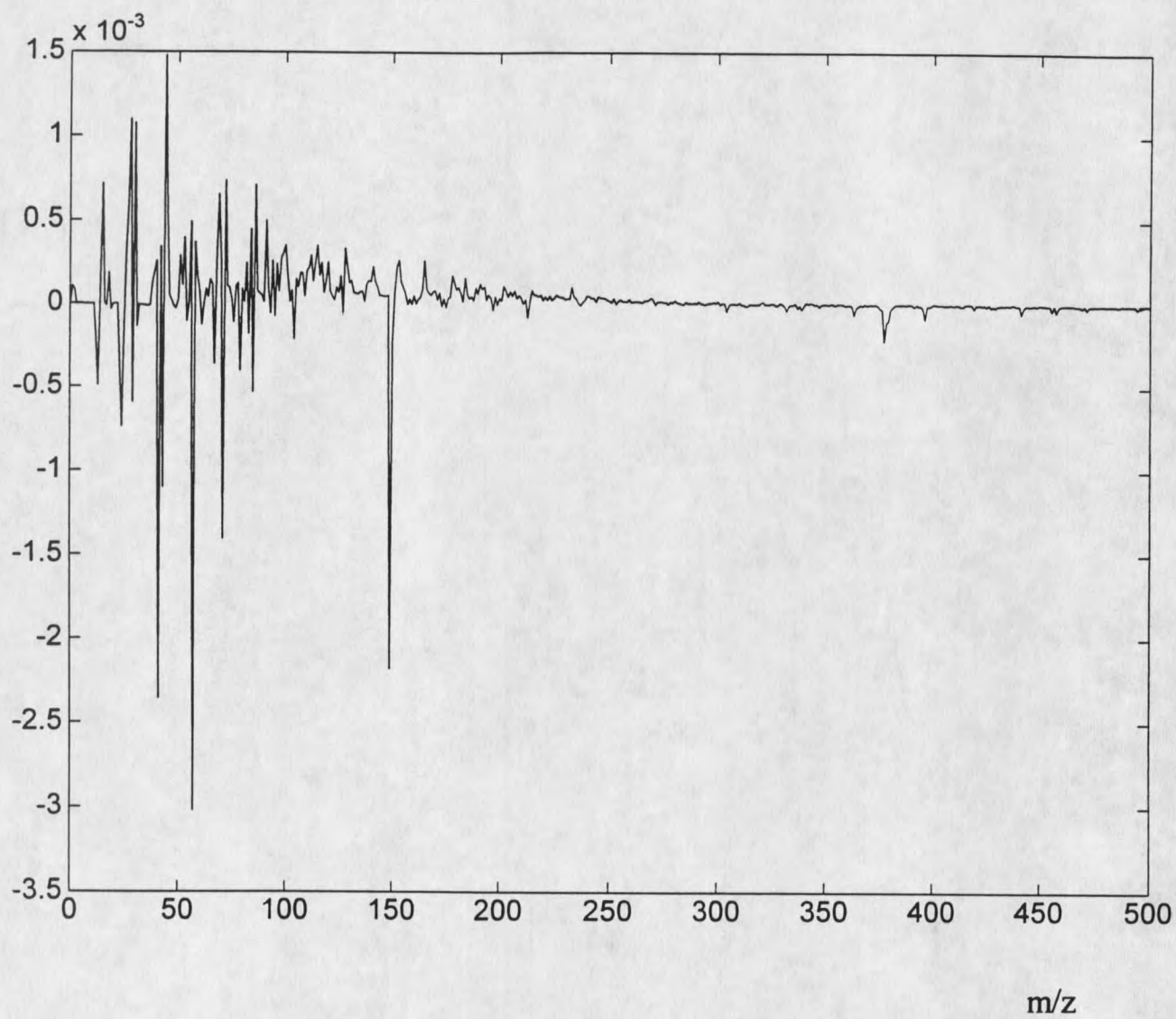


Figure 56. Coefficients of the linear discriminant function with corresponding mass to charge ratios.

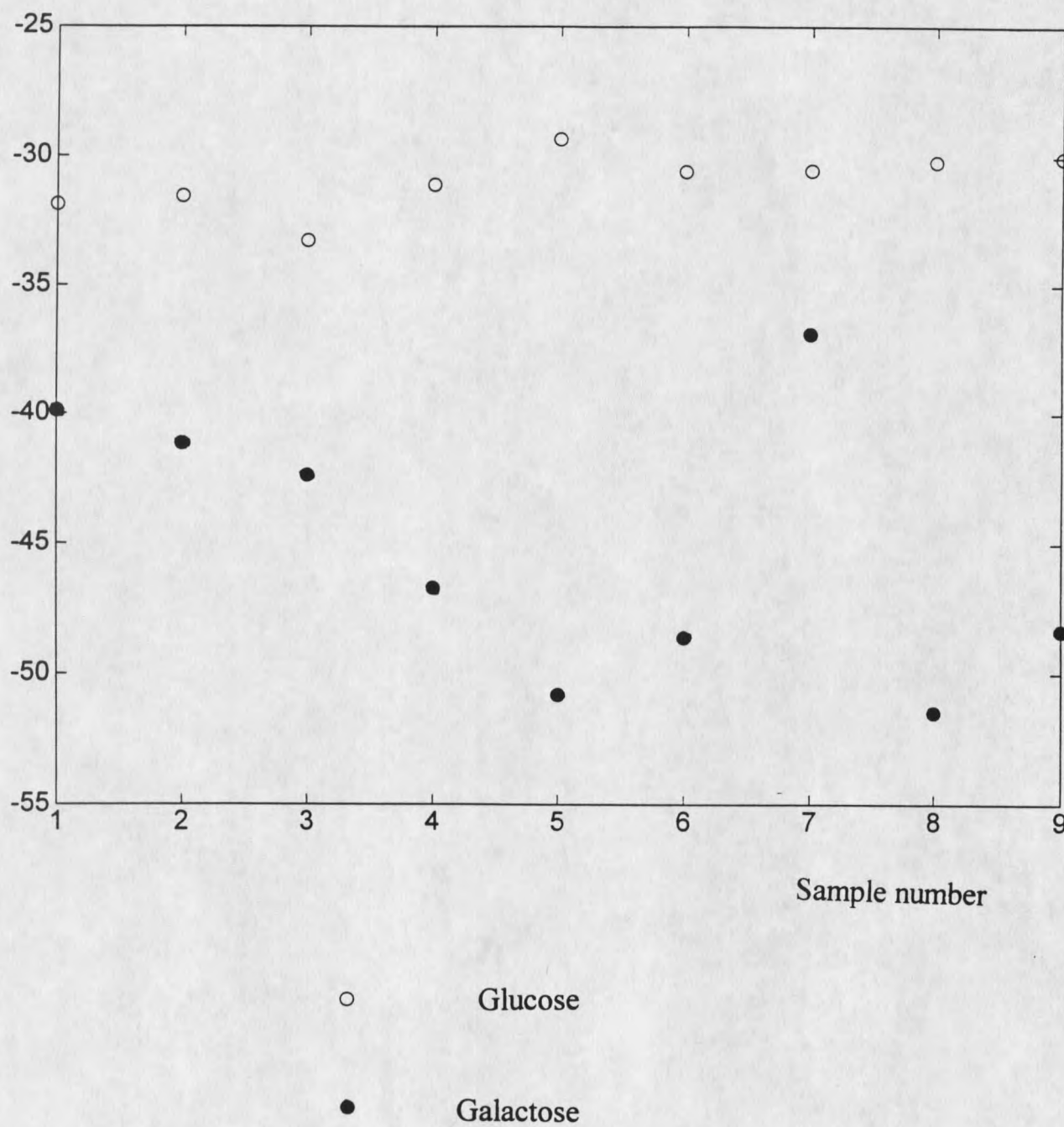


Figure 57. Scores of the freeze-dried samples along the linear discriminant function.

2GAL1P2.SUR: galactose grown cells, flushed 2 times with H ₂ O				Company Name
Acq: 98 Apr 16	+ToFSIMS LMIG	Cal: yes	AcqTime: 3.54 min	Bin: 0.552 ns
Integral: 2080973		Max: 40293	Approx Mn/Mw: 28.0/28.0	

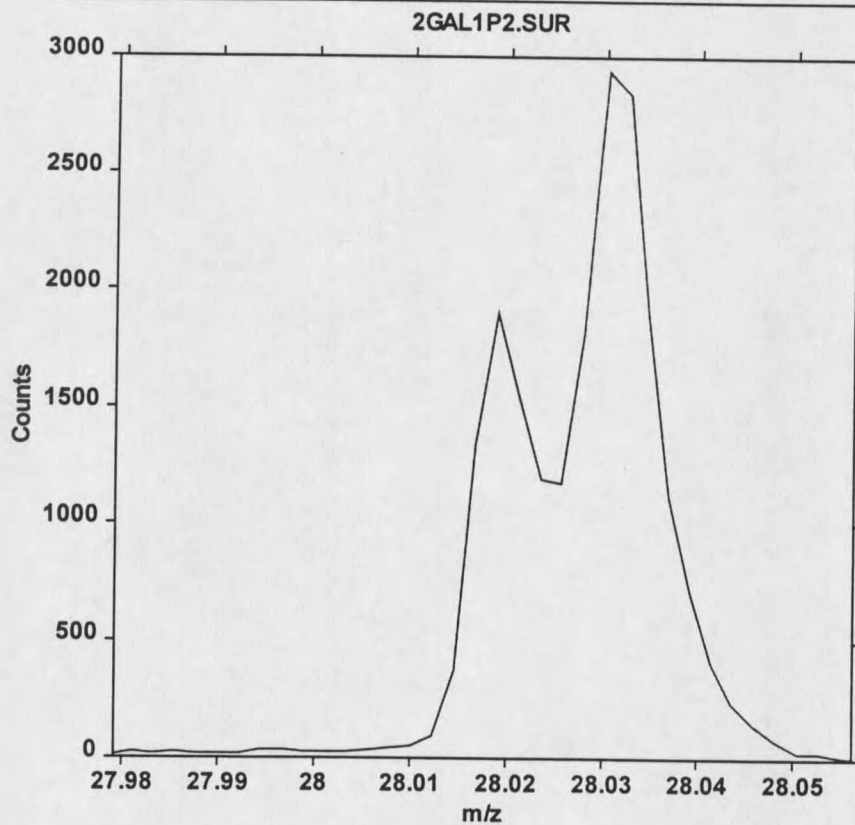


Figure 58. SIMS high resolution peak of freeze-dried *Candida albicans* at m/z 28.

2GLU1P1.SUR: glucose grown cells, flushed 2 times with H ₂ O				Company Name	
Acq: 98 Apr 16	+ToF/SIMS LMIG	Cal: yes	AcqTime: 4.68 min	Bin: 0.552 ns	
Integral: 1797971		Max: 26183	Approx Mn/Mw: 30.0/30.0		

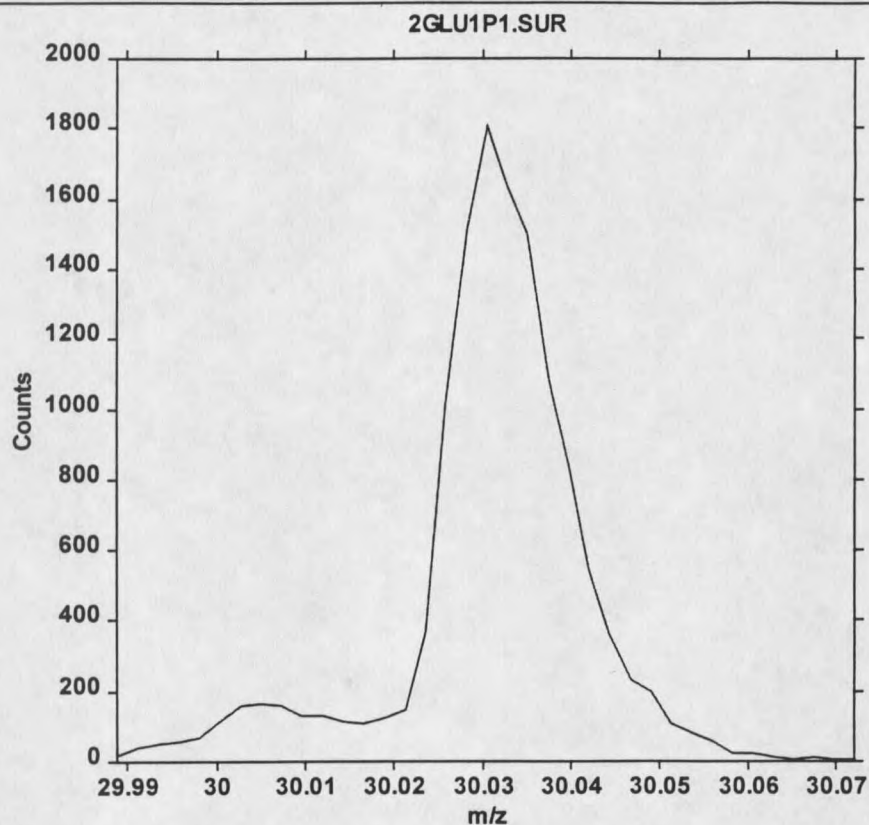


Figure 59. SIMS high resolution peak of freeze-dried *Candida albicans* at m/z 30.

2GLU1P1.SUR: glucose grown cells, flushed 2 times with H ₂ O				Company Name
Acq: 98 Apr 16	+ToF/SIMS LMIG	Cal: yes	AcqTime: 4.68 min	Bin: 0.552 ns
Integral: 1797971		Max: 26183	Approx Mn/Mw: 44.0/44.0	

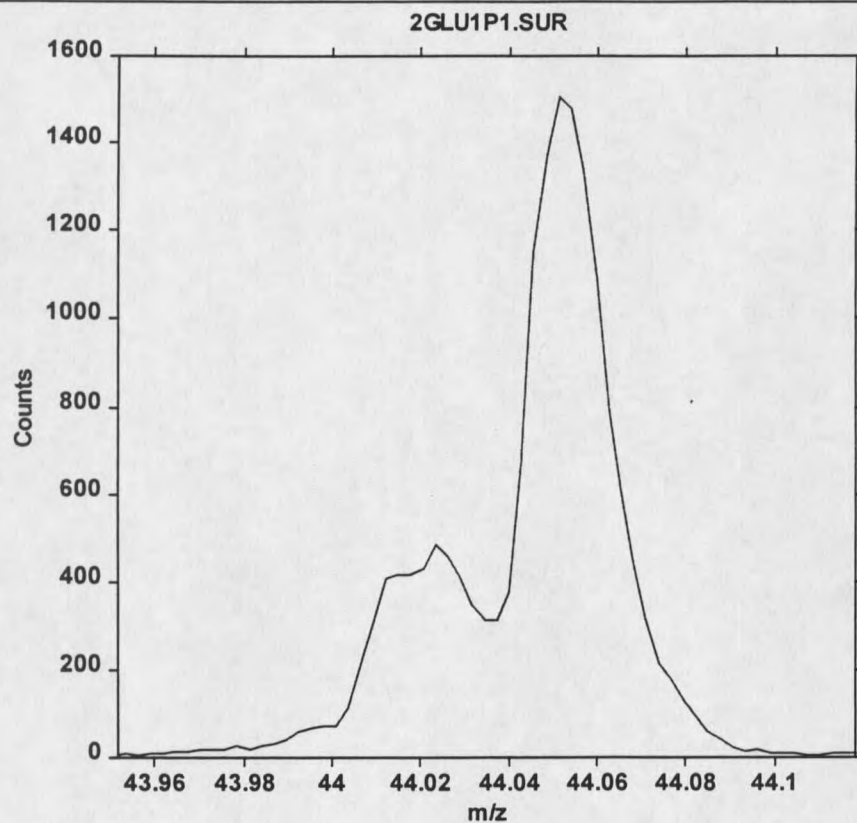


Figure 60. SIMS high resolution peak of freeze-dried *Candida albicans* at m/z 44.

2GLU1P1.SUR: glucose grown cells, flushed 2 times with H2O					Company Name
Acq: 98 Apr 16	+ToFSIMS LMIG	Cal: yes	AcqTime: 4.68 min	Bin: 0.552 ns	
Integral: 1797971		Max: 26183	Approx Mn/Mw: 69.1/69.1		

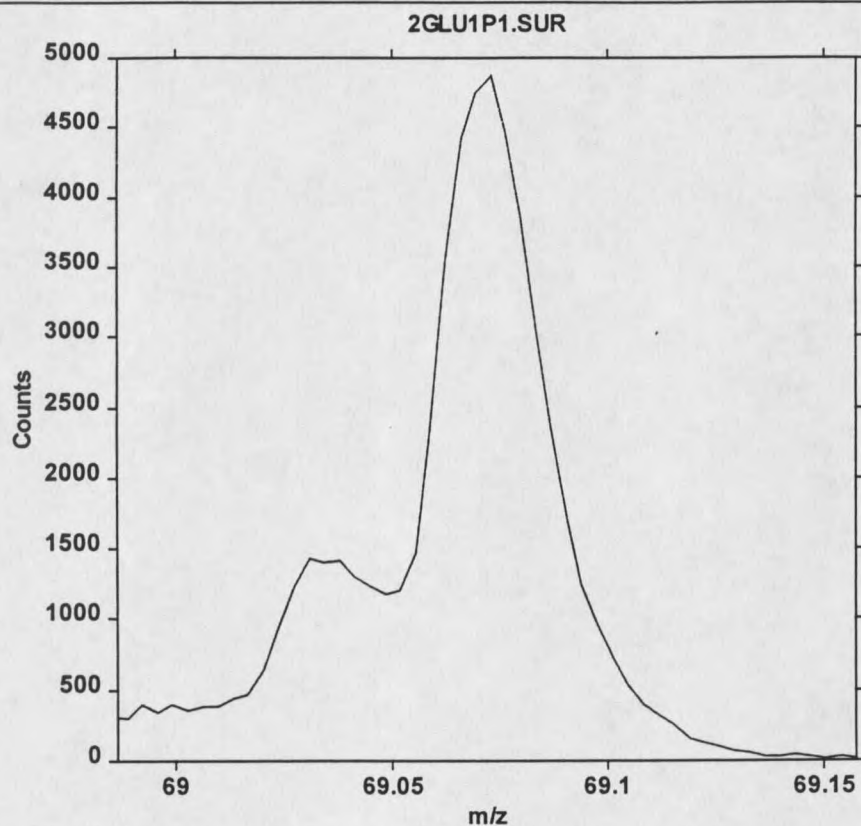


Figure 61. SIMS high resolution peak of freeze-dried *Candida albicans* at m/z 69.

2GLU1P1.SUR: glucose grown cells, flushed 2 times with H2O				Company Name
Acq: 98 Apr 16	+ToF/SIMS LMIG	Cal: yes	AcqTime: 4.68 min	Bin: 0.552 ns
	Integral: 1797971	Max: 26183	Approx Mn/Mw: 72.1/72.1	

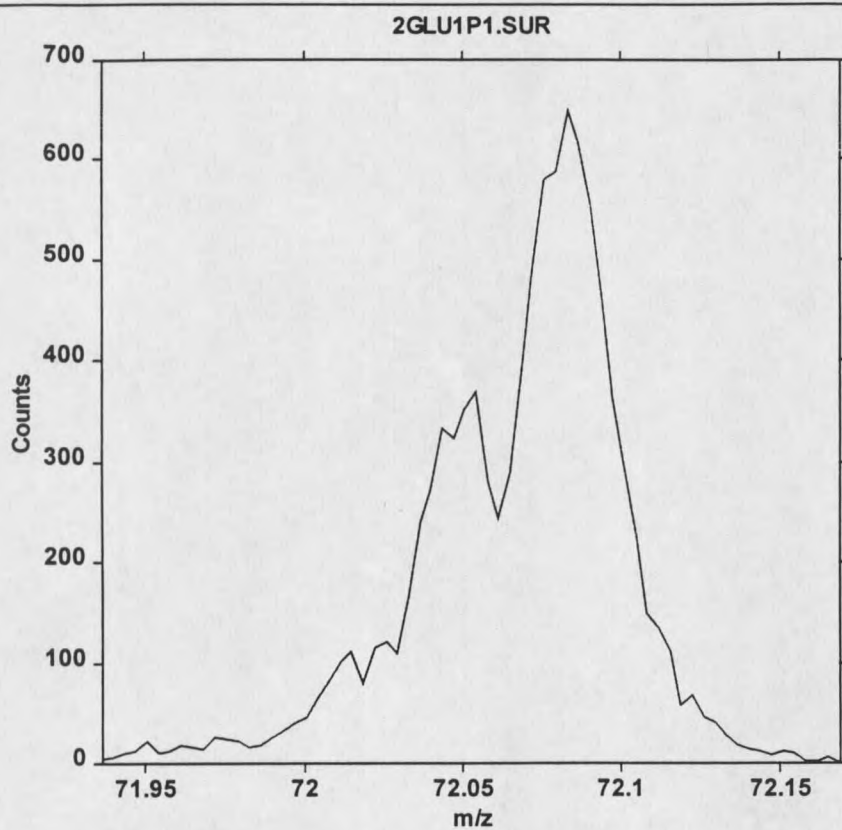


Figure 62. SIMS high resolution peak of freeze-dried *Candida albicans* at m/z 72.

2GAL1P1.SUR: galactose grown cells, flushed 2 times with H ₂ O				Company Name
Acq: 98 Apr 16	+ToF SIMS LMIG	Cal: yes	AcqTime: 4.15 min	Bin: 0.552 ns
Integral: 2384210		Max: 54520	Approx Mn/Mw: 86.1/86.1	

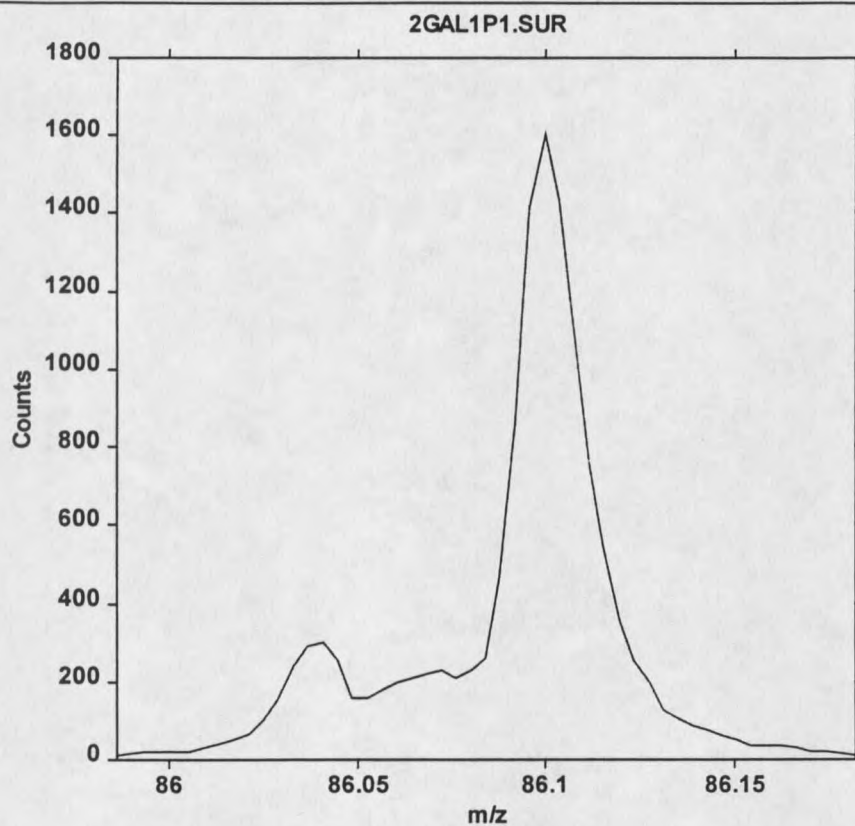


Figure 63. SIMS high resolution peak of freeze-dried *Candida albicans* at m/z 86.

2GAL1P1.SUR: galactose grown cells, flushed 2 times with H2O				Company Name
Acq: 98 Apr 16	+ToF SIMS LMIG	Cal: yes	AcqTime: 4.15 min	Bin: 0.552 ns
Integral: 2384210		Max: 54520	Approx Mn/Mw: 41.0/41.0	

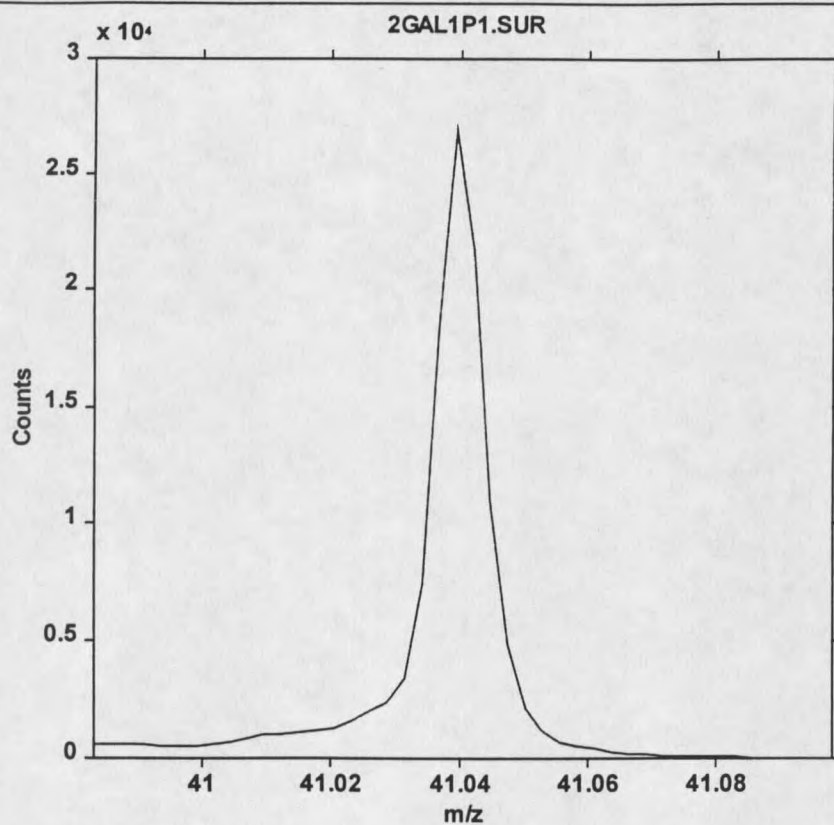


Figure 64. SIMS high resolution peak of freeze-dried *Candida albicans* at m/z 41.

2GAL1P1.SUR: galactose grown cells, flushed 2 times with H ₂ O				Company Name
Acq: 98 Apr 16	+ToF/SIMS LMIG	Cal: yes	AcqTime: 4.15 min	Bin: 0.552 ns
Integral: 2384210		Max: 54520	Approx Mn/Mw: 43.0/43.0	

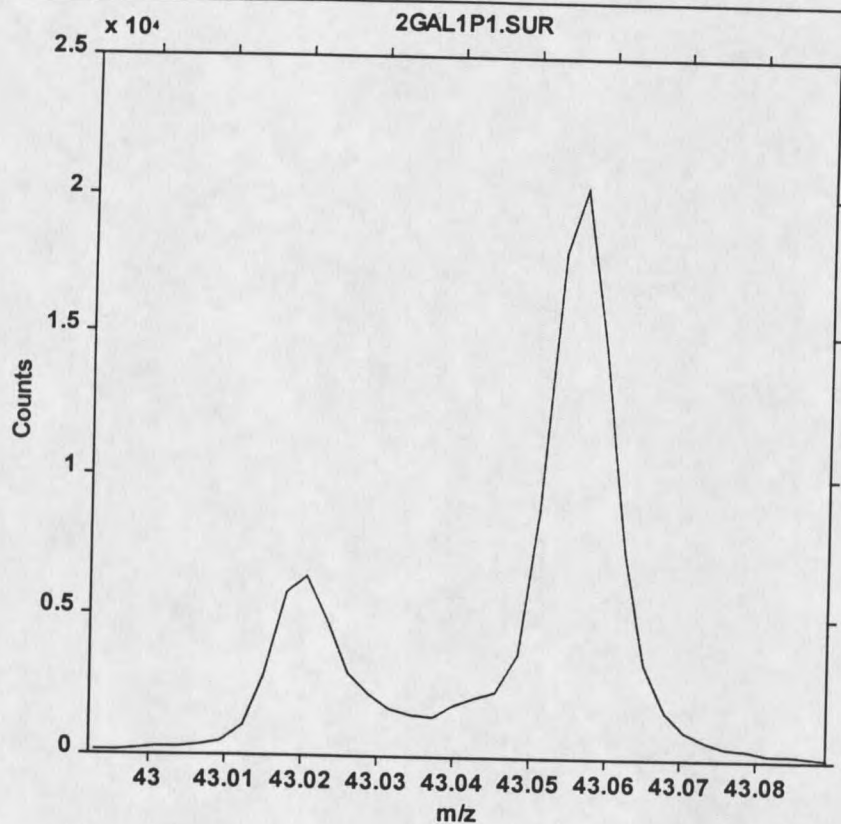


Figure 65. SIMS high resolution peak of freeze-dried *Candida albicans* at m/z 43.

2GAL1P1.SUR: galactose grown cells, flushed 2 times with H ₂ O				Company Name
Acq: 98 Apr 16	+ToFSIMS LMIG	Cal: yes	AcqTime: 4.15 min	Bin: 0.552 ns
Integral: 2384210		Max: 54520	Approx Mn/Mw: 57.1/57.1	

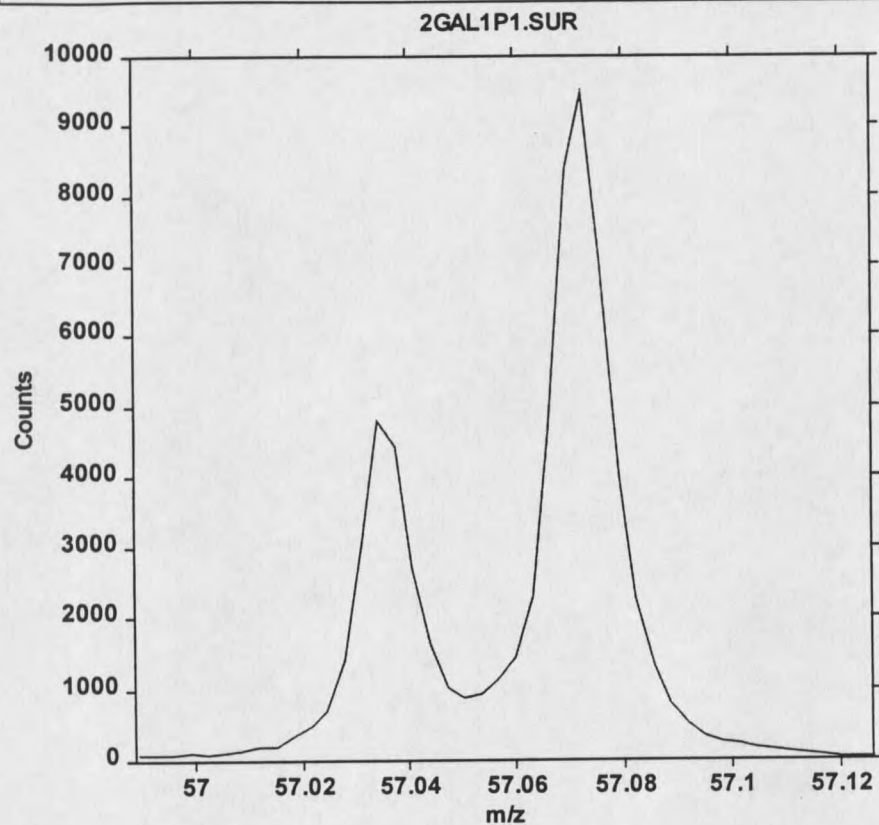


Figure 66. SIMS high resolution peak of freeze-dried *Candida albicans* at m/z 57.

2GAL1P1.SUR: galactose grown cells, flushed 2 times with H₂O
Acq: 98 Apr 16 +ToFSIMS LMIG Cal: yes AcqTime: 4.15 min Bin: 0.552 ns Company Name
Integral: 2384210 Max: 54520 Approx Mn/Mw: 71.1/71.1

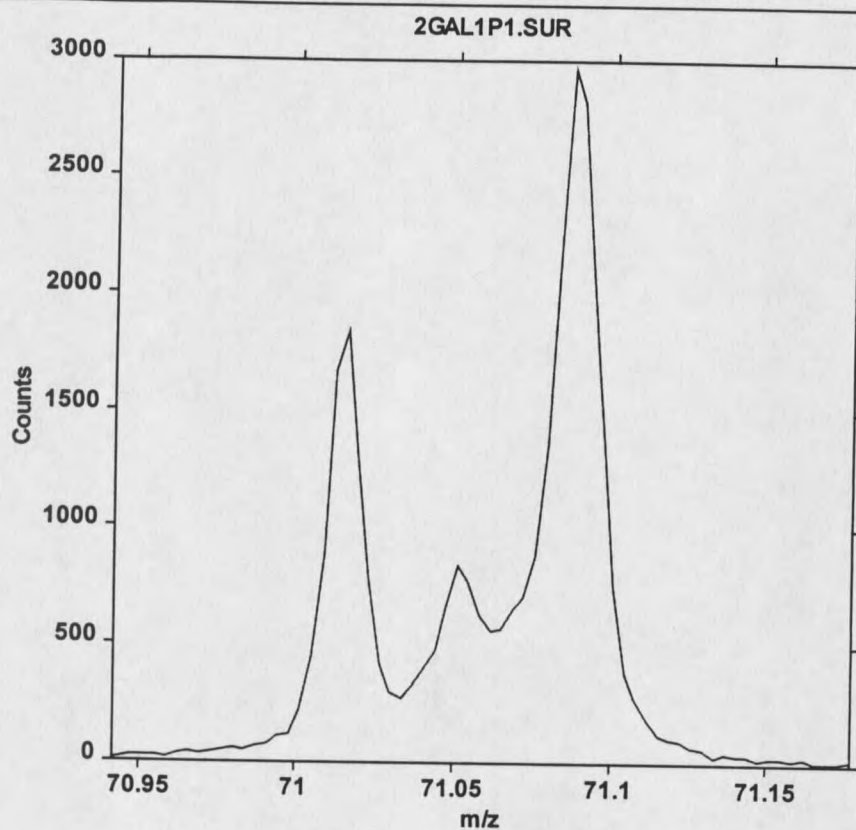


Figure 67. SIMS high resolution peak of freeze-dried *Candida albicans* at m/z 71.

2GAL1P1.SUR: galactose grown cells, flushed 2 times with H2O				Company Name	
Acq: 98 Apr 16	+ToFSIMS LMIG	Cal: yes	AcqTime: 4.15 min	Bin: 0.552 ns	
Integral: 2384210		Max: 54520	Approx Mn/Mw: 149.0/149.0		

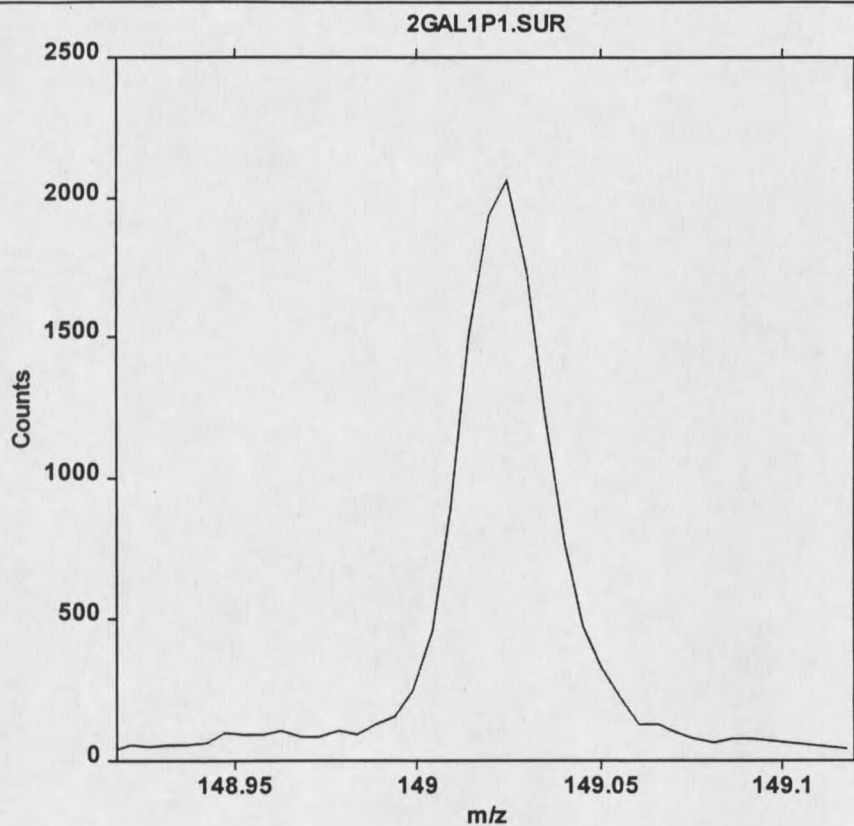


Figure 68. SIMS high resolution peak of freeze-dried *Candida albicans* at m/z 149.

CHAPTER 5

SUMMARY

This thesis presents results which are focused on the characterization of the cell surface differences of *C. albicans* grown in glucose-based medium and galactose-based medium using SSIMS with an attempt to correlate these differences to their adhesion results to FEP.

The initial attachment events of *C. albicans* to FEP were studied by using a laminar flow cell with a well-defined hydrodynamic regime. Experiments were performed at shear rates of 28, 46, 71, 120 and 350sec^{-1} . Media effects and shear rate effects were significant on yeast cell adherence. Yeast cells grown in glucose-based medium were found to adhere in greater numbers to FEP than those grown in galactose-based medium. At 28sec^{-1} shear rate, glucose-grown cells were twice as adherent as galactose-grown cells. Shear rate was also a critical factor in the adherence process. As shear rate increased, a significant decrease in numbers of adherent cells was noticed for both glucose-grown and galactose-grown cells. Glucose-grown cells were more sensitive to shear rate effects than galactose-grown cells. As shear rate was increased from 28sec^{-1} to 120sec^{-1} , the number of adherent glucose-grown cells decreased by 70%, while the number of adherent galactose-grown cells decreased only by 41%. The rate of adhesion for glucose-grown and galactose-grown cells approached the same value at higher shear rates. There was no statistically significant difference between the adherence of glucose-grown cells and galactose-grown cells at shear rate above 120sec^{-1} . Detachment studies showed that the strength of attachment appeared to be independent of the shear rate during attachment. Yeasts attached at lower shear rates were bound to

the surface as tenaciously as cells adhered at higher shear rates. At shear rates up to 772sec^{-1} , no significant amount of detachment was observed for both glucose-grown and galactose-grown cells. Adherence kinetics were linear for both carbohydrate sources except at shear rates greater than 71sec^{-1} for glucose-grown cells. At higher shear rates, glucose-grown cells appeared to be saturating the surface with first order kinetics. Surface saturation may be reached for one of two reasons: cells adhering to the surface may interfere sterically with attachment of new cells or the surface may be passivated by cells that collide with the surface but do not adhere.

Three theories have been proposed for the understanding of the different adhesion behavior of glucose-grown and galactose-grown cells in this study. The first comes from the previous work done by other researchers [66,67,68,69,70]. The adherence of *C. albicans* to FEP, as to other plastic surfaces, may be predominantly controlled by hydrophobic forces. The surfaces of *C. albicans* cells may possess a net negative surface charge which is common for all living cells, and the FEP surface may also possess a negative net surface charge under experiment conditions as other plastic surfaces which possess various degrees of negative net surface charge. Hydrophobic forces and electrostatic forces tend to be repulsive and attractive, respectively. Electrostatic forces tend to mediate instead of predominating the adherence process since a considerable amount of adhesion still occurred even in the presence of this repulsive force. The surfaces of galactose-grown cells may be more electronegative than those of glucose-grown cells under the experiment conditions in this study, which can explain the reduced adherence of galactose-grown cells compared with glucose-grown cells. The second theory is that surface free energy effects are important in the adherence process. The free energy driving force is dependent on the structure of the cell wall, which varies with carbohydrate source in the culture media. The third theory

is that glucose-grown cells have some adhesins on their outer surfaces which are not resident or so augmented on the outer surfaces of galactose-grown cells. These adhesins may promote cell adhesion to FEP providing an advantage to glucose-grown cells over galactose-grown cells to bind to the FEP surface during the initial attachment. However the initial attachment by these adhesins may be weak or easily broken. This can also explain the enhanced adherence of glucose-grown cells to FEP at lower shear rates. At higher shear rate, this advantage is overshadowed by the shear rate effects. Ultimately, all of the above theories are the consequence of the structural differences between cells grown in different carbohydrate sources. Knowledge of the structural differences between these two cells will help understand the adhesion process.

A freeze-drier was designed and constructed "in house" in this study to allow the preparation of freeze-dried cells for SIMS analysis. The ultimate vacuum of the freeze-drier is less than 1.00×10^{-9} torr. A filtration method was explored and used to prepare a smooth layer of cells to be freeze-dried. Scanning Electron Microscopy (SEM) showed that this method produced a uniform layer of cells with good coverage on the membrane filter. SEM also showed that the freeze-dried cells were plump and in very good shape. Glucose-grown cells were found to be roughly 1 μ m larger in diameter than galactose-grown cells and both cells had very smooth surfaces. The cooling rate of the freeze-drying experiments was found to be approximately $-5^{\circ}\text{C}/\text{min}$ which is within the range of the optimum cooling rate for yeast cells observed previously [81]. Cells were typically frozen until $-62 \sim -64^{\circ}\text{C}$ and freeze-dried under -40°C . XPS was used to estimate the concentration of the remaining PBS on the freeze-dried samples. Survey spectra were taken from areas without background contribution from the alumina membrane filter. No sodium or phosphorous was detected in XPS technique which meant that the concentration of the remaining PBS was less than 0.1% at which level the "matrix

effect" was negligible in SIMS analysis. Freeze-dried cells were then subjected to SSIMS analysis. Spectra were taken from areas without background contribution from the alumina membrane filter. The resolution of the spectra was several thousands. Discriminant Function Analysis combined with Principal Components Analysis were used to analyze SIMS spectra obtained from freeze-dried cells. Statistical results yielded that the functional groups corresponding to the peaks at $m/z = 28, 30, 44, 72, 86$ were more abundant on the surface of glucose-grown cells than on the surface of galactose-grown cells. These peaks are corresponding to the immonium ions of hydrophobic amino acids, glycine, alanine, valine and leucine. These hydrophobic amino acid fragments are probably from protein, mannoprotein or glycoprotein on the outer cell surface. These results support the theory that hydrophobic interactions are important in the adherence of *C. albicans* to FEP surface. These hydrophobic adhesins probably give glucose-grown cells advantages during the initial attachment of these cells to FEP surface. These hydrophobic adhesins may also help glucose-grown cells to form more multiple contacts with FEP surface than galactose-grown cells if these adhesins are more abundant on glucose-grown cell surfaces than on galactose-grown cell surfaces, which can explain the increased adherence of glucose-grown cells than galactose-grown cells. But the initial attachment formed by these hydrophobic adhesins may be weak and not strong enough to withstand greater shear rate or harder to form at high shear rate fields, which can explain that under high shear fields, the adherence of glucose-grown cells and galactose-grown cells to FEP approached the same value.

Electrostatic interactions may also be involved in the adhesion process of *C. albicans* to FEP since SIMS and statistical analysis results showed that galactose-grown cells were rich in polysaccharide moieties relative to glucose-grown cells. These polysaccharide fragments may come from acidic

polysaccharides resident on the cell surface which make the cell surface of galactose-grown cells more electronegative than that of glucose-grown cells. Hence the repulsive forces between galactose-grown cells and FEP may be stronger than those between glucose-grown cells and FEP, which can also explain the reduced adherence of galactose-grown cells and their retardation to the shear rate effects relative to glucose-grown cells. But this theory should be subjected to the confirmation by further experiments because the sources of the peaks indicative of polysaccharide moieties are not certain in this study. It is not certain if they are fragments of acidic polysaccharides or anything else. Further studies should involve collecting more model compound SIMS spectra of acidic polysaccharides in order to compare the SIMS results obtained in this study with the model compound spectra to estimate the degree of the contribution of the electrostatic interactions to the adhesion process. Further experiments may also involve measuring the surface electrostatic potential of these two cells using particle electrophoresis. Further studies may also attempt to modify the net surface charges of the yeast with carbodimide and formalin and then look at the adhesion using flow cell experiment.

Further studies should also involve XPS analysis of the cell surface using cryostage and using Transmission Electron Microscopy (TEM) to look at the cell wall structure to verify that cell surfaces have not changed after freeze-drying.

SSIMS is a valuable tool to study the cell surface structural differences as shown by the results obtained in this study. While more further work need to be done to prove that the differences of SSIMS results are directly related to the adhesion differences. Also more model compound SIMS spectra need to be collected in order to obtain detailed information of the cell surface moieties and functional groups. A nontrivial experiment which would attempt to fractionate the cell wall into its various components and look at the mass fragmentation pattern of

these chemicals would be helpful in this regard. Growth conditions should also be varied with an attempt to correlate cell surface characteristics to adhesion results.

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