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dissertation entitled

APPLICATIONS OF TRIPLE QUADRUPOLE MASS SPECTROMETRY TO THE DIRECT DETERMINATION OF MICROBIAL BIOMARKERS AND THE RAPID DETECTION AND IDENTIFICATION OF MICROORGANISMS

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Analytical Chemistry

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Major professor

Date December 5th, 1990

APPLICATIONS OF TRIPLE QUADRUPOLE MASS SPECTROMETRY TO THE DIRECT DETERMINATION OF MICROBIAL BIOMARKERS AND THE RAPID DETECTION AND IDENTIFICATION OF MICROORGANISMS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1990

ABSTRACT

APPLICATIONS OF TRIPLE QUADRUPOLE MASS SPECTROMETRY TO THE DIRECT DETERMINATION OF MICROBIAL BIOMARKERS AND THE RAPID DETECTION AND IDENTIFICATION OF MICROORGANISMS

By

Mark James Cole

Methodology has been developed that takes full advantage of the triple quadrupole mass spectrometer for detecting and identifying microbial biomarkers. Complete tandem mass spectrometry (MS/MS) data were acquired on the pyrolysis products obtained from bacteria. These data showed that very little additional information could be obtained from pyrolysis using tandem mass spectrometry over that using conventional mass spectrometry. A rapid, sensitive, and automated method was developed for the direct determination of phospholipid structures, from crude lipid extracts, using fast atom bombardment/MS/MS. This method takes advantage of neutral loss, parent ion, and daughter ion scan modes to provide independent diagnostic mass spectra for each of many specific phospholipid classes, and the compositions and positions of the fatty acid constituents on each phospholipid. A rapid extraction procedure combined with the automated instrument control program produces this information in a few minutes from a single sample. Examples of

similar data were also obtained, using this same methodology, from glycolipids. This technique has been applied, in this work, to the detection and identification of microorganisms - including bacteria, fungi, and amoebae. Applications in urinary tract infections and food-borne contaminants are presented The need for, and general structure of, a searchable database of the detected biomarkers in a large variety of microorganisms is discussed.

The ability of the triple quadrupole mass spectrometer to perform ion-molecule reactions was exploited for differentiating phospholipid classes through reactions with ethyl vinyl ether in the second quadrupole. The protonated molecules and reaction product ions observed permitted the differentiation of various phospholipid classes. Particular neutral gain scans are specific for each phospholipid class. Ion dissociation products can be observed in the same scan as the ion reaction products to provide data on the fatty acid composition and position on the glycerophosphate core, along with the phospholipid class.

A preliminary investigation shows great promise for the application of tandem mass spectrometry in the detection and identification of sterols in crude lipid extracts. Particular parent scans are shown to be specific for particular classes of sterols. The identification of sterols may be especially valuable for fungal identification.

"At least I knew who I was
when I got up this morning,
but I think I must have been changed
several times since then."

Alice, in *Alice's Adventures in Wonderland.*

"What kind of data did you *expect* to get
after using a hand-grenade for sampling?"

Dr. David C. White, upon hearing of our pyrolysis results.

ACKNOWLEDGEMENTS

Foremost, I would like to thank my advisor, Chris Enke. Through his intellect, insight, sensitivity, and creativity, he has provided me an outstanding role-model on which to base my scientific career. I am forever grateful to him for allowing me the freedom to pursue my research interests. Joining his research group was one of the best decisions I ever made. I would also like to thank the members of my graduate committee, Dr. Jack Watson, Dr. George Leroi, and Dr. James Tiedje for their advise and assistance.

I would like to thank Eric Hemenway and Kate Noon for their help in co-organizing the 1st Annual Conference for Users of the Enke Group's TSQ-70B in Microbial Analysis (UEGTMA). This small, but fanatical, users-group meets on Lake Huron once a year (or more, depending on the wind) to discuss important stuff. I would also like to thank Kevin Hart, Jon Wahl, Ron Lopshire, and the rest of the Enke group for their help and friendship.

Three important friends deserve special mention. Tom Wiese has been the greatest friend a person could have. He is intelligent, compassionate, knows how to sail, and can sneak me in skiing for free. I owe much of my sanity, along with much of my insanity, to him. Secondly, I would like to thank Mary Puzycki. She has always made my world a little more surrealistic, and I'm probably the better for it. Our "discussions" always motivated me to spend long hours working alone in the lab. I am grateful for her love and friendship. Of all the

friends I have made during my stay at MSU, Kris "Mel-Mauler" Kurtz is one who truly knows how to let loose. She alone is responsible for the rest of my insanity. I am grateful to her for making the last five years anything but boring.

Dr. Dave White has played an important role in my career. His friendship and to-the-point advice has made an impact on me. If I ever get rich, he deserves the credit. Also deserving mention are Dr. James Tiedje, Dr. Larry Forney, and Dr. Mike Klug. These three have been a great help to me and my research.

My parents have provided me with love and support throughout my life. I am also grateful to my grandparents for their moral and financial support through difficult times.

My boat, *Fetish*, has provided me with more fun than any graduate student deserves. She has never failed to be there when I needed her; she is always gentle, and never complains. I feel very fortunate to have such an escape from frustration. I have overcome many problems in my research while aboard her.

Last, I would like to thank those who financially supported my research: the National Science Foundation's Center for Microbial Ecology at Michigan State University, the United States Department of Agriculture, the State of Michigan's Research Excellence Fund, the MSU Biomedical Research Support Grant, and the Yates Research Fellowship.

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CHAPTER 1

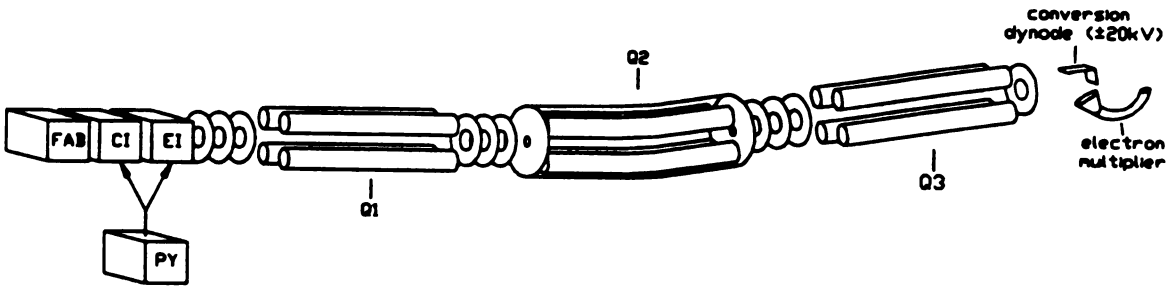
Introduction

Introduction

Microbiologists have long held the desire to possess a simple method for the rapid, reliable, and sensitive detection and identification of microorganisms without prior cultivation. Traditional microbial methodology was developed many years ago and has remained relatively unchanged since then; much of its roots are imbedded in the very beginnings of microbiology. While these methods are currently used to differentiate and identify thousands of microbial species and strains, they are time-consuming, prone to errors, and often rely on numerous subjective decisions during the course of an identification. All of these methods require prior isolation and cultivation of the organism - precluding identification of organisms that will not grow outside their natural environment. More current methods employ highly developed routines that use sophisticated morphological, serological and biochemical procedures. Some of them are so specific that they have no general applicability and can only be used when the identification is nearly complete. Thus, an unfulfilled need exists in microbiology for new methodology which can achieve most of the requirements of cultivation, speed, objectivity, and applicability.

Because it meets these requirements, mass spectrometry is finding increased applications in microbiology. Tandem mass spectrometry becomes an even more powerful tool for the detection of microbial biomarkers by providing the high sensitivity characteristic of mass spectrometry along with a great improvement in selectivity. This selectivity allows for rapid and complete characterization of different components of a microbe without their prior separation. In addition, tandem mass spectrometry has the ability to detect trace quantities of these components in complex mixtures and matrices.

The triple quadrupole mass spectrometer (TQMS) provides many advantages for detecting and profiling biomarkers. The TQMS provides high selectivity due to unit resolution for both mass analyzers, high sensitivity due to the high efficiency of the collisional dissociation process, a full array of ionization methods, and the ability to be highly automated - allowing for rapid scan rates and easy implementation of the MS/MS operating modes. A schematic of a TQMS, along with the different scan modes possible, are shown in Figure 1.1. The daughter scan produces a mass spectrum of all the fragment ions formed from a selected parent ion. This is accomplished by setting the first quadrupole to pass ion current at a particular parent m/z value and scanning the third quadrupole. Daughter spectra are useful for structure determination of selected ions. The parent scan produces a mass spectrum of all the parent ion masses that dissociate to form a particular daughter ion. This is obtained by scanning the first quadrupole, while the third quadrupole is set for the desired daughter m/z value. Parent spectra provide compound class



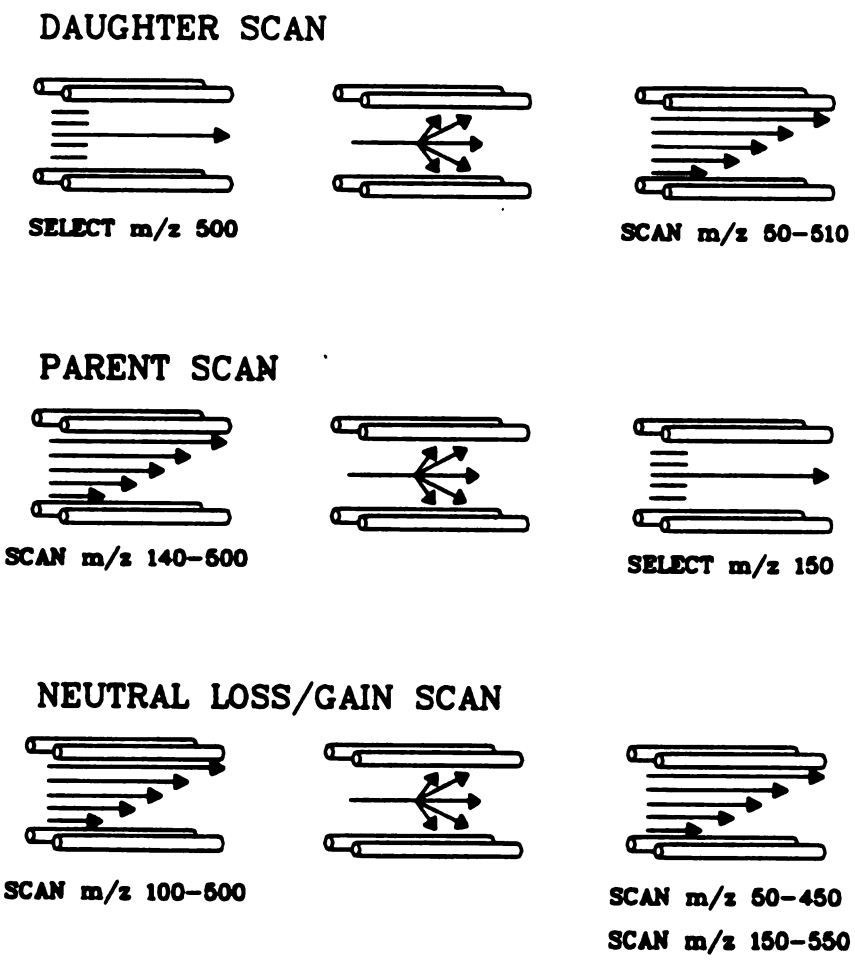


Figure 1.1 Schematic of a triple quadrupole mass spectrometer along with the different scan modes possible.

determination. By choosing a daughter m/z value that corresponds to a fragment characteristic of a particular compound class, a mass spectrum is obtained containing peaks representing only those ions formed in the source that belong to that class. The neutral loss and neutral gain scans also provide compound class determination. A neutral loss spectrum contains peaks representing all of the parent ion masses that dissociate to lose a particular neutral mass, while a neutral gain spectrum contains peaks representing all of the parent ion masses that react with a reactant gas to form product ions of a particular mass gain. This is implemented by scanning the first and third quadrupoles concurrently, but with a mass offset equal to the selected neutral loss or neutral gain. By choosing a neutral loss characteristic of a particular compound class, a mass spectrum is obtained containing peaks representing only those ions that belong to that class. Neutral gain scans provide even greater selectivity of compound class due to their reliance on a particular chemistry of the chosen class of compounds. Only those ions that have the correct chemistry to react in a selected manner will be detected. By choosing a reaction unique to the compound class of interest, a mass spectrum is obtained containing peaks representing only ions that can chemically undergo the particular reaction.

Research Goals

This research is concerned with taking full advantage of the triple quadrupole mass spectrometer in detecting and identifying microbial biomarkers. Microorganisms contain a multitude of different

biomolecules that are needed to perform the functions required for each microbial species to fill, and survive in, its niche in nature. The natural selective forces imposed on different microorganisms have required microbes to adapt and optimize specific sets of biomolecules for survival in specific environments. Examples of these biomolecules include the lipids needed for membrane function and integrity, the respiratory quinones needed for metabolism, the polysaccharides needed for protection from the environment, and other molecules required for survival. The collection of the molecules needed by each microbial species is unique to that species. In fact, individual subsets of this complete collection of biomolecules are often unique to a particular species. Identification of these unique subsets can provide biomarkers for identification of microorganisms. This research is centered around developing tandem mass spectrometry methodology for identifying these biomarkers and applying it to the rapid detection and identification of microorganisms. Special attention is given to speed and sensitivity, as these are areas where current techniques fall far short of being truly useful.

Background

Microbial identification techniques have undergone an evolution that has taken them from early visual observations, through highly sophisticated molecular biology, and into very sensitive instrumental techniques. Putting this evolution in perspective is important in recognizing where this research fits into the scheme of things. Three areas in particular are important in the development of different

technologies: 1) traditional and non-instrumental methods of analysis, 2) instrumental analysis of whole cells, and 3) instrumental analysis of specific biomarkers.

Traditional and Non-Instrumental Methods of Analysis

Numerous methods exist for monitoring the presence of microbes in biological samples. These methods involve the direct detection of the organism, or some part of the organism, or the indirect detection of a characteristic metabolite of the organism. The most widely used method is the selective agar pure culture assay. The microorganism in question is grown on a selective medium in which the suspect microbe is known to react in a consistently predictable manner. Examples of typical reactions include the change of agar or solution color, evidence of motility, the evolution of gas, and the production of characteristic metabolites. A chart showing a few of these morphological and biochemical tests for the identification of bacterial genera is shown in Figure 1.2. In this example, bacteria are grouped according to their responses to these tests. This chart is only for identification of bacterial genera, species determination involves even more tests and can be much more difficult. While widely used, these techniques are time-consuming; many require 2 to 4 days to obtain presumptive results and 5 to 7 days to obtain complete results. Furthermore, confidence in these tests may be compromised by reliance on subjective judgments such as a change of color or production of gas. Three of these tests along with their negative results, ideal positive results, and real-life positive results are listed in Table 1.1. The positive results most often obtained in real working

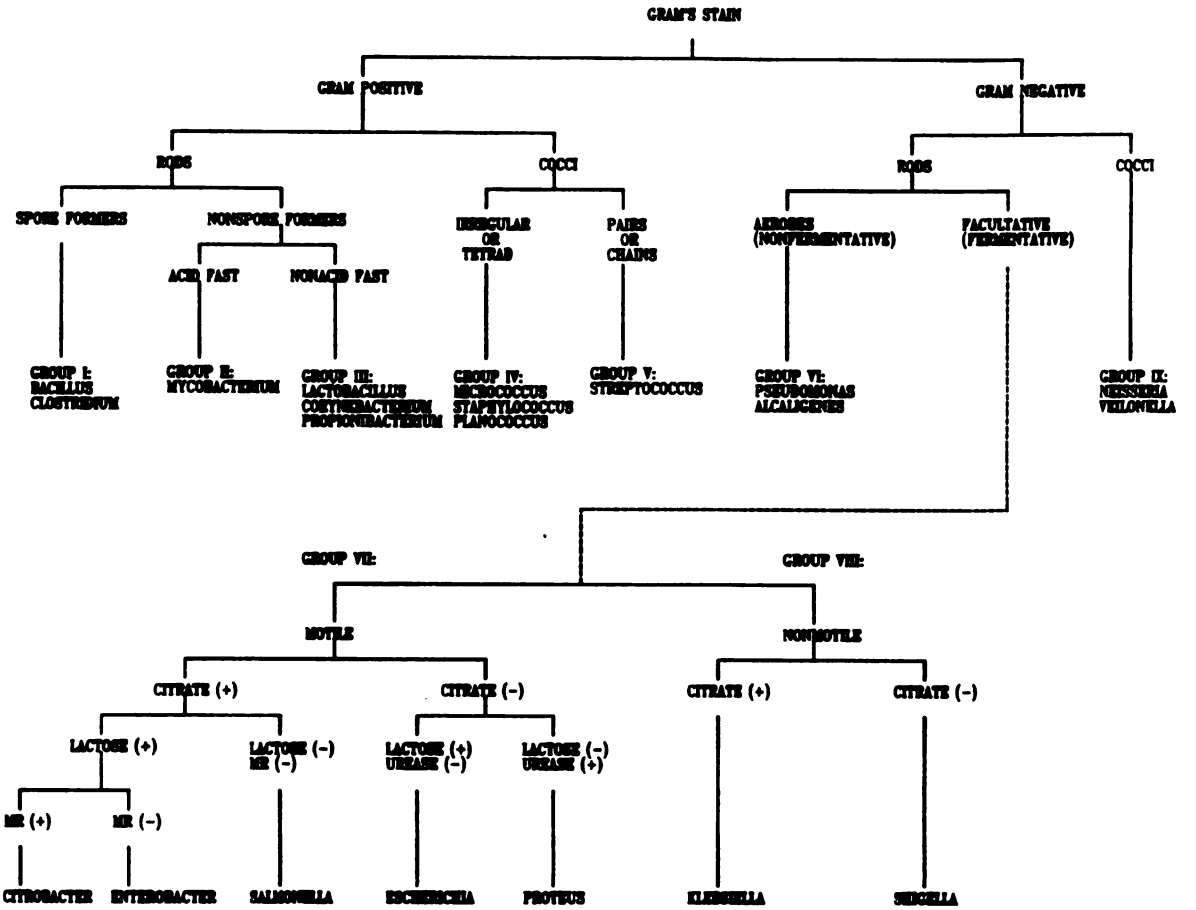


Figure 1.2 Chart of a few of the morphological and biochemical tests used for the identification of bacterial genera.

Table 1.1 Three of the biochemical tests used for bacterial genera determination

TEST	POSITIVE RESULT	NEGATIVE RESULT	REAL-LIFE POSITIVE RESULT
CARBOHYDRATE FERMENTATION	YELLOW, WITH GAS BUBBLES FORMED	RED, NO GAS	REDDISH-YELLOW, POSSIBLE GAS FORMED
METHYL RED MIXED ACID FERMENTATION	RED*	YELLOW	REDDISH-YELLOW
CITRATE UTILIZATION	BLUE	GREEN	BLUE-GREEN

* Requires a 5-day incubation

conditions require a subjective judgment in interpreting the results; a mistake can easily be made on a color test, and many bacteria do not produce gas readily enough for a positive identification.

Another widely used set of techniques is the immunoassays; the three most common being enzyme immunoassay (EIA), radioimmunoassay (RIA), and immunofluorescence assay (IFA). Each method consists of using a labeled antibody specific for an antigen associated with the suspect organism. Enzyme immunoassay uses an enzyme-labeled antibody, RIA uses an isotopically-labeled antibody, and IFA uses a fluorescently-labeled antibody. False-positive results, due to non-specific binding, cross-reactions, and interferences of other components in the sample are common [1,2]. While more rapid than the pure culture assay, these techniques still require up to 27 hours and numerous steps [2,3], although newer developments have facilitated less cumbersome assays which can be completed in an 8 hour working day [4].

More recently, genetic methods of detection and identification by DNA colony hybridization have been studied [5]. Detection is accomplished by the hybridization of isotopically-labeled DNA probe segments, that are specific for the selected organism, with the complementary strands of the DNA from the selected organism. While this method has a 95 percent accuracy [5], it is expensive and requires many elaborate and technically difficult procedures. Also, the DNA probe used must be unique and specific to some function of the

microbe, requiring that the microbe be well characterized. This initial characterization can be very time and labor intensive.

Indirect methods of detection depend on the ability to detect metabolites produced by the microorganism. Chromatographic and colorimetric techniques are often used. These methods must be specific for the particular microbe of interest and some require elaborate sample workup. Chromatographic identification is based on retention values that can lead to greatly decreased confidence in the results when an interfering matrix is introduced.

Instrumental Analysis of Whole Cells

The first efforts toward instrumental analysis of bacteria were performed using analytical pyrolysis with gas chromatography, and later mass spectrometry, on whole microbial cells. The use of whole cells presented a problem to gas chromatographs and mass spectrometers in that they were not volatile. Therefore pyrolysis was used to break the whole cells up into volatile components which are more amenable to the instrumental analysis. Pyrolysis is defined as a chemical transformation through the application of energy in the form of heat alone, resulting in a series of more volatile fragment molecules (pyrolyzate) [6]. More simply, a non-volatile sample is rapidly heated in an inert environment. No net oxidation can occur, so molecules in the sample cleave at their weakest bonds resulting in the formation of more volatile components from the sample. Since the molecules fragment at specific points, the final mixture of low molecular weight

products is expected to contain information characteristic of the original sample [7].

The first application of analytical pyrolysis to the detection of microorganisms was by Wilson and Oyama in conjunction with the Viking mission to Mars [8]. Gas chromatography was used as the means of detection, and this work triggered an extensive series of studies by other researchers [9,10,11]. Recent work in Py/GC has been reviewed by Bayer [12] and Fox [13]. Despite the rapid development of the technique, its use for microbe characterization remains limited due to poor standardization and the lack of inter-laboratory reproducibility [14]. This is due to several practical gas chromatographic problems, such as insufficient column resolution, gradual deterioration of column performance, or sudden differences caused by column replacement. These problems pose constraints on the establishment of a library of standard fingerprints, thereby restricting this technique to a single laboratory for successful completion of a given project.

The advances occurring in mass spectrometry instrumentation and computing soon had researchers turning toward pyrolysis-mass spectrometry for the study of microbes. In an attempt to overcome those problems inherent in pyrolysis-gas chromatography, Meuzelaar and coworkers developed techniques for obtaining reproducible mass spectra of bacteria [15]. They were able to differentiate the few species studied at the genus, species, and subspecies level without gas chromatography. Recent work on the application of PY/MS in

microbiology has been reviewed by Wieten, *et al.* [16]. So far, analyses by pyrolysis/mass spectrometry of over 35 bacterial strains have been reported [17]. However, spectral reproducibility was found to vary between different instruments and laboratories [18].

Instrumental Analysis of Specific Microbial Biomarkers

The complexity of the data obtained with pyrolysis techniques presented problems with interpretation. Involved mathematical and computer tools were developed to aid in this interpretation. However, it became apparent to some researchers that most of the data contained in pyrolysis is not very useful for microbial identification because microbes shared many common biomolecules. These researchers started looking for specific sets of biomarkers that would vary among microbial species and thus be useful for identification.

By far the most successful technique in this area is the profiling of cellular fatty acids by gas chromatography [19] and gas chromatography/mass spectrometry [20]. In this method, fatty acids are extracted from whole microbial cells and derivatized to their methyl esters. The fatty acid methyl esters are analyzed by GC or GC/MS, and a quantitative profile is obtained for the microbe studied. The cellular fatty acids are found to be good biomarkers for microbial identification, as their content and abundance remain unique and predictable under strict growth conditions. The problems with this technique lie in the need for a fairly large number of cells, the large amount of sample handling and preparation in the extraction and derivatization, and the general reproducibility problems associated

with GC. The use of GC/MS has greatly improved the sensitivity of this technique [21], but the other problems still remain.

A recent twist on this method is the use of pyrolysis to obtain fatty acid information. One approach uses a methanolic solution applied directly to the whole cells contained on the pyrolysis filament [22,23]. Pyrolysis produces fatty acid methyl esters which are analyzed by GC. Fatty acid profiles are obtained which are nearly identical to the previously described method. The other approach uses pyrolysis to directly produce fatty acid fragments from whole cells, with subsequent analysis by mass spectrometry [24]. However, since only the nominal masses of the fatty acids are obtained, this method suffers from an inability to identify fatty acid isomers, branched chain fatty acids, and hydroxy fatty acids. Therefore, much useful information is lost.

Research has recently concentrated on the use of microbial phospholipids for identification. The phospholipids contain all of the fatty acid information as well as the information regarding the presence of their classes and species within each microorganism. Much mass spectrometry work has been performed toward elucidating phospholipid structure, but only a few researchers are attempting to use phospholipids for microbial identification. Some of the first work involved fast atom bombardment, plasma desorption, and laser desorption of phospholipids directly from lysed cells [25,26]. This work demonstrated the usefulness of cation and anion spectra for obtaining phospholipid information from bacteria. The sensitivity was

good, and little sample preparation was required. In further work, the anion spectra were used with a linear regression analysis for library-matching of unknown spectra [27]. Pramanik *et al.* applied this method to other bacterial species [28].

This is the current extent of the evolution of analytical microbial identification techniques. Research must now focus on increased sensitivity, speed of analysis, new biomarkers, and improvements in analysis methodology. The research presented here directly addresses these issues and is a logical next step in the continuing development of analytical techniques for microbial identification.

Thesis Organization

Chapter two contains the details of an attempt to develop a method for microbial identification based on pyrolysis and tandem mass spectrometry. Previous work with pyrolysis and conventional mass spectrometry showed promising results for identifying specific portions of the data obtained as unique to specific bacterial species. Because pyrolysis is such a harsh fragmentation technique, the assumption made at the time was that the spectra obtained from pyrolysis would contain peaks representing many isomass ions, and that unique components could be found by resolving these spectra. This work set out to resolve and identify the differences between spectra by taking complete tandem mass spectrometric "maps" of the entire pyrolysis data space. The design, construction, and operation of a slow-leak pyrolyzate containment inlet system is described. This

system was necessary to keep the pyrolyzate around long enough to collect complete MS/MS maps of the sample. The results obtained and reasons for abandonment of this system for other methods are discussed.

A rapid, sensitive, and automated method for the direct determination of phospholipid structures in bacteria is presented in Chapter three. The pyrolysis work showed the need for identifying biomarkers which could be used for microbial identification. Previous work with phospholipids showed their usefulness for this purpose. Full advantage was taken of the triple quadrupole mass spectrometer operating modes to develop a general scheme that characterizes the phospholipid content of crude lipid extracts using fast atom bombardment ionization. Neutral loss and parent scans provide independent diagnostic mass spectra for each of many specific phospholipid classes, while daughter scans provide the composition and positions of the fatty acid constituents on each phospholipid. An automated MS/MS instrument performs an extensive phospholipid screening on a single sample. A useful mass profile of the phosphatidylethanolamine species present in a one-picogram sample of mixed phospholipids (equivalent to ten *E. coli* cells) has been obtained. The spectra are reproducible and proportional to concentration over at least the 5-log range of cell concentration studied. A rapid extraction procedure combined with the automated instrument control program produces profiles of the phospholipid classes along with fatty acid composition and position information on selected phospholipid species in a few minutes from a single sample.

Although applied here to bacterial phospholipids, this method is a general one for any sample containing phospholipids.

In Chapter four, the phospholipid work is extended through the use of some interesting chemistry. The ability of the triple quadrupole mass spectrometer to perform ion-molecule reactions was exploited for differentiating phospholipid classes through reactions with ethyl vinyl ether in the second quadrupole. The protonated molecules and reaction product ions observed permit the differentiation of various phospholipid classes. The pattern of addition reaction products formed is shown to depend solely on the functionality of the lipid polar head group and not on the fatty acyl constituents. Neutral gain scans are performed which are specific for each phospholipid class. Ion dissociation products are observed in the same scan as the ion reaction products to provide data on the fatty acid composition and position on the glycerophosphate core along with the phospholipid class. Although this method is less sensitive than the method described in Chapter 3 for most phospholipid classes, it can identify phospholipids that do not readily lose their head group, and detect phospholipids in mixtures containing species that give interfering neutral losses.

Chapter five presents the application of the phospholipid analysis technique described in Chapter 3 to the detection and identification of microorganisms. This technique has been applied to bacteria, fungi, and amoebae; a complete listing of the number and types of samples analyzed is tabulated. Of particular interest is the

application in the detection and identification of urinary tract infections and food-borne contaminants. Actual clinical and industrial samples were analyzed with promising preliminary results. The need for, and general structure of, a searchable database is discussed.

The thesis is concluded in Chapter six with the application of other lipid biomarker analyses to the detection and identification of microorganisms. Lipid biomarkers, other than phospholipids, that have been investigated in this research include glycolipids and sterols. Other potential lipid biomarkers are discussed along with their amenability to mass spectrometry and possible applications.

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CHAPTER 2

Use of Pyrolysis / Triple Quadrupole Mass Spectrometry for Microbial Identification

Introduction

Every species of microorganism contains unique chemical components and/or combinations of components which could, in principle, be distinguished from those of all other species. The ability to measure the quantities required to make this distinction is another story. The differences in chemical composition that make microbes unique among each other are related to very important and specialized functions required for the survival of each particular microorganism. Because of this, these distinguishing components are often so highly incorporated into the cell's biochemical machinery that their extraction from the cell is difficult, or they present physical or chemical difficulties, such as high molecular weights or complex structures that differ in only minor details. Analytical instrumentation has only recently been able to overcome some of these difficulties. Although recent advances in mass spectrometry instrumentation have made increasingly less volatile substances amenable to analysis, whole microbial cells, and many of their components, still remain out of reach. Methods must be used that either break the cells down into smaller fragments or extract the components that are more amenable to mass spectrometric analysis.

The former method was the first method chosen to achieve the overall goal of microbial detection and identification.

The key to producing useful fragments from whole cells is in generating fragments that still retain some of the uniqueness and characteristics of the original distinguishing molecules. Important to a rapid and easy analysis is the production of these fragments with a minimum of sample preparation and workup. Pyrolysis satisfies these needs by producing fragmentation from whole cells within seconds, and requiring no sample preparation other than loading the sample onto the pyrolysis wire. As mentioned in Chapter 1, the pyrolyzate obtained is expected to contain information characteristic of the original sample. Working on the hypothesis that the pyrolyzate is composed of many components that overlap in the conventional spectrum, tandem mass spectrometry was employed in an attempt to resolve these overlays. Identification could then be made by wading through the large tandem mass spectrometry data space obtained and identifying the unique components. The thrust of the research presented in this chapter is an attempt to find those portions of the MS/MS data space that could be used for microbial detection and identification.

Background

Pyrolysis

Three common methods exist for pyrolysis: laser pyrolysis, heated filament or "pyroprobe", and Curie-point pyrolysis. Of these three

techniques, Curie-point pyrolysis has been shown to be the most reliable for reproducibility of results [1,2], and is the method that was chosen for this work.

Curie-point pyrolysis, first described by Giacobbo and Simon [3], utilizes a high frequency coil to inductively heat a ferromagnetic wire to its Curie-point: the temperature at which the wire becomes paramagnetic and ceases to absorb rf energy. A schematic diagram of a Curie-point pyrolyzer is shown in Figure 2.1. A small amount of sample is placed directly on the ferromagnetic wire, and the wire is placed inside a quartz reaction tube. This whole assembly is inserted into a high-frequency rf coil and the wire is heated inductively. As the temperature of the wire reaches its Curie-point, the wire changes from ferromagnetic to paramagnetic and stops absorbing energy. Below the Curie-point temperature, the wire becomes ferromagnetic and again starts to heat. As long as the high-voltage rf field is applied, the wire stays at its Curie-point temperature. The final temperature is therefore determined by the wire's Curie-point temperature which, in turn, is a function of the metal alloy composition of the ferromagnetic wire. Various mixtures of iron, nickel, chromium, and colbalt are generally used. A range of temperatures between 358^{0} and 980^{0} C are available from different alloys, as shown in Table 2.1. These temperatures are extremely reproducible, with temperature rise times of 20-30 milliseconds from room temperature to the Curie-point. Curie-point pyrolysis offers the steadiest pyrolysis conditions of all the pyrolysis techniques. This has been found to be the most important factor for obtaining good spectral reproducibility [4].

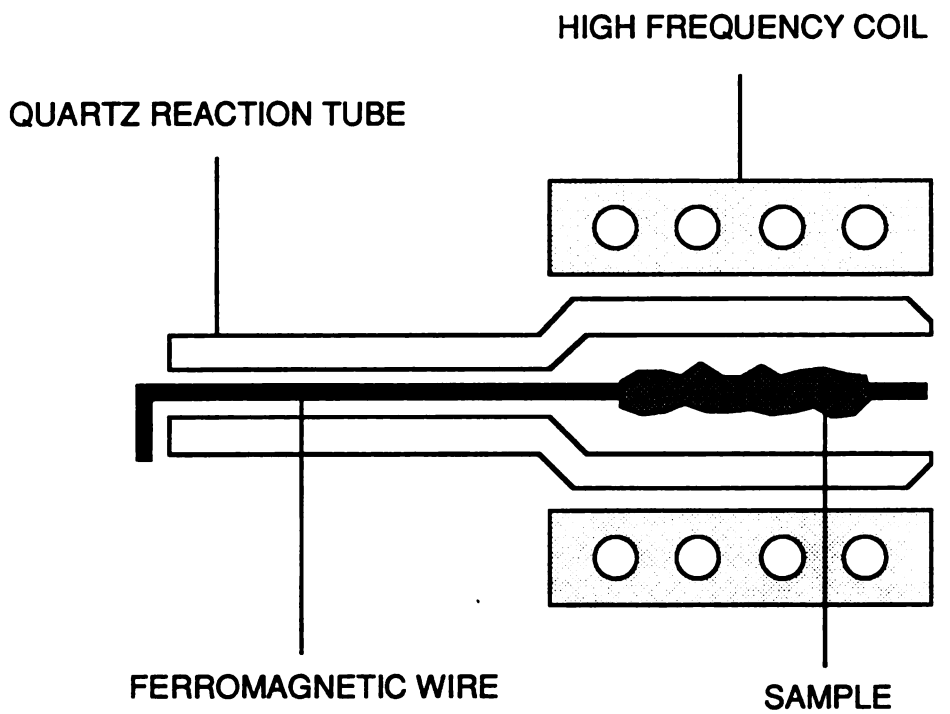

Figure 2.1 Schematic diagram of a Curie-point pyrolyzer

Table 2.1 Curie-Point Temperature as a Function of Composition

WIRE COMPOSITON	CURIE-POINT TEMPERATURE (°C)
100 of Ni	358
48 of Fe:51 of Ni:1 of Cr	440
49 of Fe:51 of Ni	510
40 of Fe:60 of Ni	590
30 of Fe:70 of Ni	610
67 of Ni:33 of Co	660
100 of Fe	770
40 of Ni:60 of Co	900
50 of Fe:50 of Co	980

Pyrolysis/Mass Spectrometry

The chemical complexity of bacteria leads to pyrograms that have a peak at almost every m/z value up to around m/z 500. A pyrolysis/chemical ionization mass spectrum of *Corynebacterium nephridii* is shown in Figure 2.2. Because of the large amount of data, the original method of trying to visually identify pyrolysis mass spectra of whole bacteria has been mostly abandoned. Present means of interpretation now center around two approaches: the identification of chemical components specific to a species [5], and the mathematical deconvolution of whole spectra to identify those areas where they differ [6]. Unfortunately, both of these approaches suffer from serious disadvantages.

In order to detect a component which is specific for a particular organism, that component must be found, identified, and its pyrolysis pattern characterized. While this procedure has the potential for detection of microbes in mixtures, the process of finding and identifying the pyrolysis characteristics of components specific to each particular species is time consuming. Also, much sample clean-up is required for the detection of these components. In the other approach, identification is based on finding minor spectral differences between species by the mathematical deconvolution of whole pyrolysis mass spectra. Other investigators have put much effort into developing programs toward this purpose; some of the specialized tools developed include applications of factor and discriminant analysis, linear regression, graphical rotation, variance diagrams, and various mapping techniques, among others [7,8,9]. Although there is

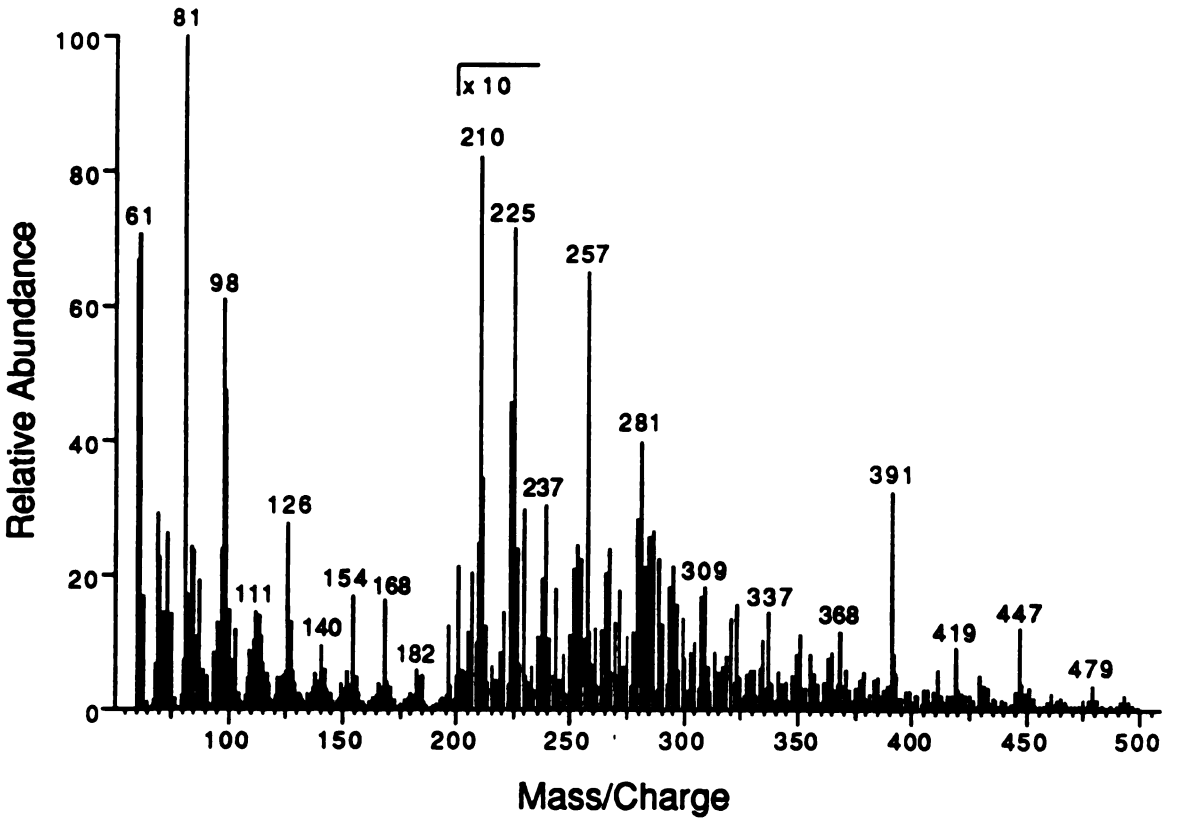

Figure 2.2 Pyrolysis/chemical ionization mass spectrum of *Corynebacterium nephridii.*

very little sample clean-up needed, these approaches depend on high-ordered mathematics that do not work well for minor components of a mixture or data sets that exceed 20 samples [10].

Pyrolysis/Tandem Mass Spectrometry

Tandem mass spectrometry achieves a dramatic increase in available information over that from conventional mass spectrometry by providing not just the mass, but an entire mass spectrum for the ions of each m/z value in the conventional mass spectrum. This added dimension of data allows for the determination of trace components and the resolution of overlaying contributors to a particular ion peak in the primary spectrum. There have been isolated reports on the use of tandem mass spectrometry to examine the structures of the ions from conventional pyrolysis mass spectra [11,12,13], but they have shown limited success.

Only two reports exist on the use of the full capabilities of pyrolysis/tandem mass spectrometry for bacterial differentiation [14,15]. These experiments were exploratory in nature, and made limited use of daughter, parent, and neutral loss scan modes for the differentiation of three bacterial species. An interesting scan mode labeled "mixed daughter scans" was employed, in conjunction with factor analysis and graphical rotation, in an effort to determine which daughter ions would be useful ones for which to collect parent ion scans [14]. Mixed daughter scans are total daughter ion scans in which the first quadrupole is operated in an rf-only mode, collision gas is added to the second quadrupole, and the third quadrupole is

scanned [15]. The resulting spectrum contains all the daughters of all the parents in the pyrolyzate. The use of parent scans for 3 different daughter ions showed limited success. By far the biggest achievement of these experiments was the demonstration of the usefulness of tandem mass spectrometry for reduction of chemical noise and identification of small portions of characteristic data from large uncharacteristic data sets.

These experiments raise the possibility of finding unique parent-daughter ion-pairs and neutral losses, in the complete MS/MS pyrolyzate data space, that would be useful for microbial identification. To find these unique transitions, methodology for collecting and analyzing complete MS/MS maps needed to be developed. This was the goal of the research presented in this chapter.

Experimental

All experiments were performed using a Finnigan (San Jose, CA) TSQ-70 triple stage quadrupole instrument equipped with a specially - designed pyrolysis inlet system. The pyrolysis inlet is described in detail later in this chapter. Pyrolysis was accomplished using a Varian Aerograph/Fischer (Walnut Creek, CA) model 9425 Curie-point pyrolyzer power supply (1.5 kW, 1.1 MHz) and a Curie-point pyrolysis unit [16] modified to fit the pyrolysis inlet system. The pyrolysis unit was obtained from Dr. Kent Voorhees at the Colorado School of Mines. Curie-point wires rated at 510^{0} C were used for analysis. Spectra were

acquired and processed using the Finnigan TSQ-70 data system and software.

All bacteria were obtained from Dr. Jim Tiedje at Michigan State University. Cultures were grown on trypticase soy agar plates at 37° C for twenty four hours prior to analysis. For analysis, the bacteria were scraped off the plates and coated onto the Curie-point wires.

The samples were pyrolyzed at 510° C for 4 seconds. The pyrolysis inlet system was maintained at 200° C. Positive ion chemical ionization spectra were collected using methane as the reagent gas. Daughter spectra were obtained using argon as a collision gas. The collision gas pressure was 0.5 mtorr and the collision energy was between 30 and 40 eV.

Results and Discussion

Pyrolysis Inlet System

In order to find those combinations of the few parent, daughter, and neutral loss values that are characteristic of an organism, a daughter spectrum of every ion in the conventional spectrum needed to be collected. This required the pyrolyzate to be presented to the ion source long enough for the instrument to perform all the necessary daughter scans. Because of this time requirement, a slow-leak, pyrolyzate-containment inlet system was designed and interfaced with the TSQ-70 to provide a constant source of pyrolyzate to the mass spectrometer.

A basic schematic of the final design is shown in Figure 2.3. This design consists of the pyrolysis unit connected to a quartz expansion chamber via a viton O-ring and clamp (not shown). The expansion chamber fits through the GC inlet Cajun fitting on the TSQ-70 and mates with the ion source via a vespel ferrule. The expansion chamber is double-walled: the inner wall (actual chamber) is wrapped with NiChrome wire, and the outer wall provides the seal with the Cajun fitting. The NiChrome wire allows the chamber to be heated to a controlled temperature by connection to a Variac. The end of the chamber connecting to the mass spectrometer source has a small leak hole that allows pyrolyzate to slowly leak into the source.

The TSQ-70 needs about 100 seconds to collect a complete MS/MS map of the ions obtained from the pyrolysis of whole bacteria. Therefore, the leak hole needed to be adjusted to optimize the flow of pyrolyzate out of the chamber. If the pyrolyzate is allowed to stay inside the chamber at higher pressures and longer times, secondary reactions will occur and the spectra will change as a function of the storage time. Also, secondary reactions will cause the spectra to be irreproducible between samples. A compromise was sought: keep the pyrolyzate around only as long as needed for the instrument to collect data. The pyrolyzate profiles from the different versions of the chamber are shown in Figure 2.4. Reconstructed ion chromatograms were obtained for the total ion current produced after pyrolysis. The profile in Figure 2.4a shows a leak-hole that is too large - the pyrolyzate does not stay around long enough to permit all needed data

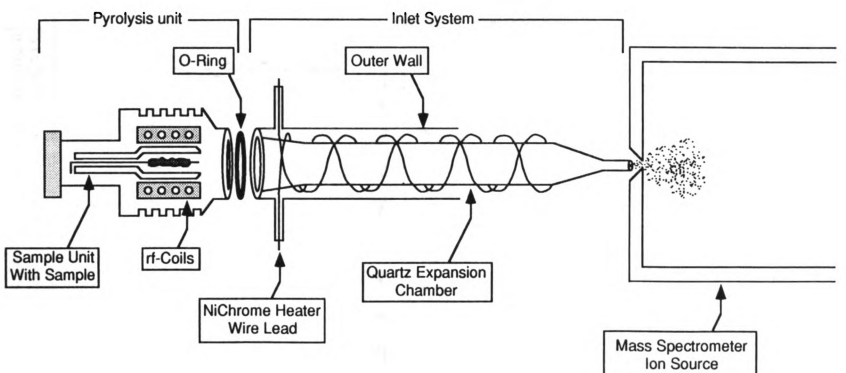

Figure 2.3 Schematic of the slow-leak, pyrolyzate containment, inlet system.

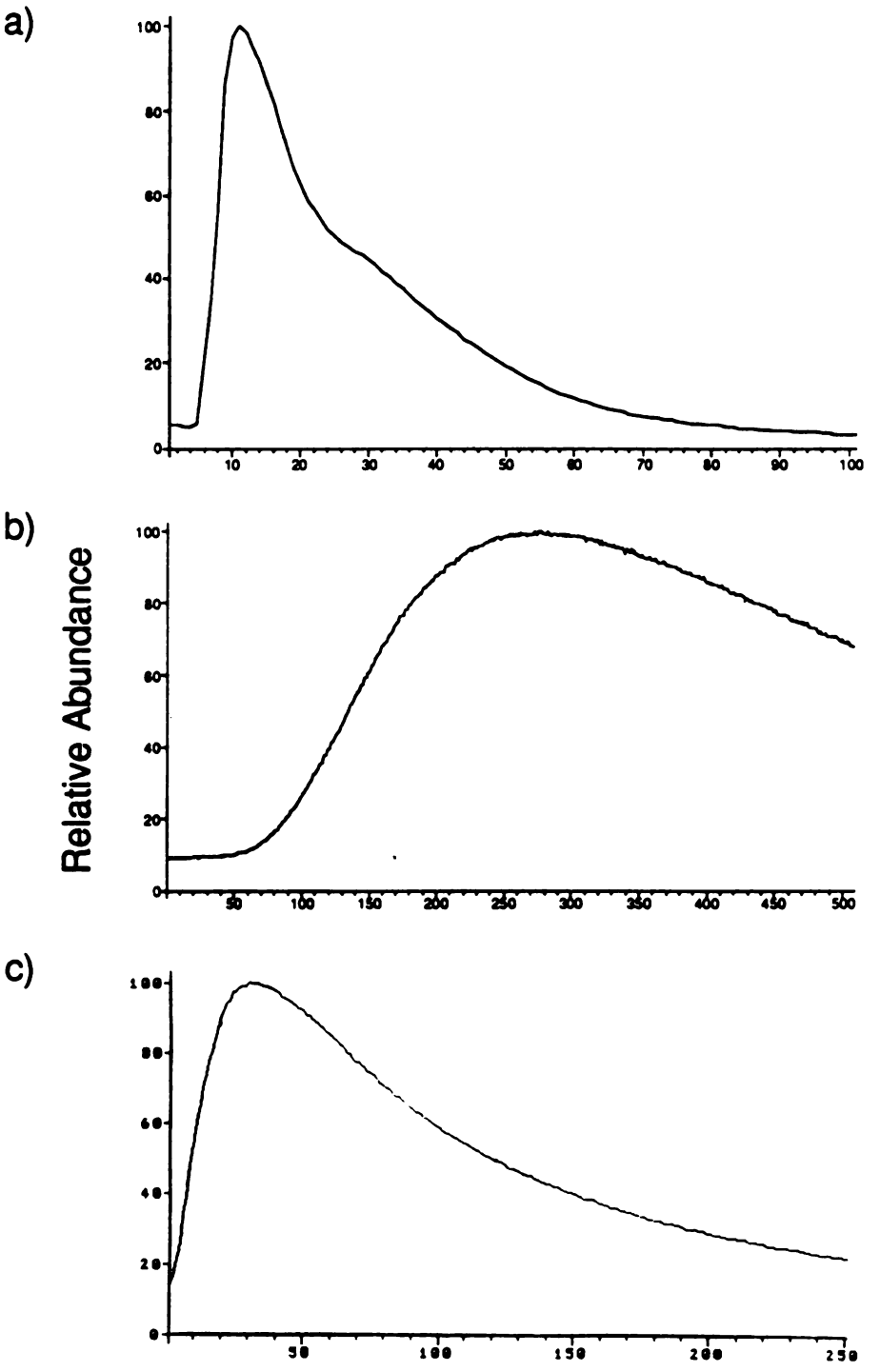

Figure 2.4 Pyrolyzate profiles from different versions of the pyrolyzate inlet system: a) leak hole too large, b) leak hole too small, c) leak hole correct size.

to be collected. After changing the hole size, the hole became too small, as shown by the results in Figure 2.4b. In this configuration, secondary reactions were occurring, and a full minute was needed before spectra acquisition could even be started. After a number of tries, the correct configuration was obtained. This is shown in Figure 2.4c. Fifty percent of the pyrolyzate has been evacuated from the chamber by the time the instrument has finished collecting data, and almost none of the pyrolyzate remains after 4 minutes.

Collection of Complete Pyrolysis/MS/MS Data Fields

Most of the pyrolysis/MS work done so far has used electron impact ionization. Only one use of chemical ionization has been reported [15]. Chemical ionization was chosen in this study for two reasons: 1) to retain as much of the high-mass information as possible, and 2) to increase the sensitivity of the technique. Pyrolysis/CI mass spectra for three different bacteria are shown in Figure 2.5. These three bacteria provide a good test group: *Escherichia coli* and *Pseudomonas aeruginosa* are similar organisms in that they are both gram-negative rods, while *Corynebacterium nephridii* is a gram-positive club-shaped bacilli which, taxonomically, is significantly different from the other two. These spectra are quite similar to each other; they contain a peak at almost every m/z value, and differ only in the relative intensities of these peaks. As expected, the spectra from *E. coli* and *P. aeruginosa* were similar, while the *C. nephridii* spectrum showed the most contrasts among the three spectra.

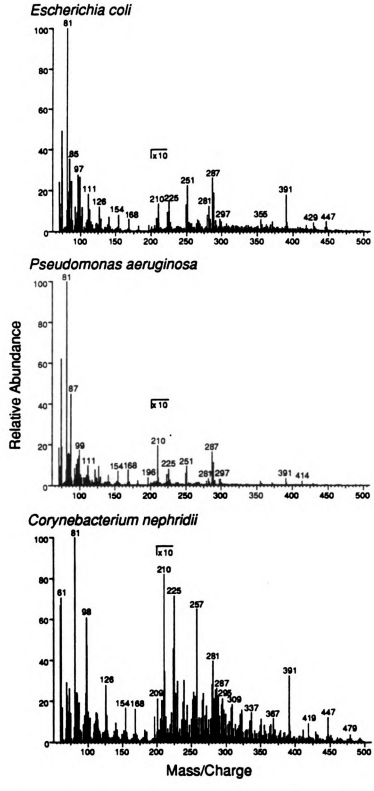

Figure 2.5 Pyrolysis/chemical ionization mass spectra for three different bacteria.

Next, daughter spectra were taken of all the ions present in these conventional spectra. In contrast to results obtained by Voorhees *et al.* [14], good fragmentation was observed from most of the parent ions, and rich daughter spectra were obtained. The complete pyrolysis/MS/MS data space obtained from *E. coli* is shown in Figure 2.6. The parent ions occur along the front axis and the daughter ions occur along the trailing axis. Examples of typical daughter spectra of m/z 196 obtained from the three bacteria are shown in Figure 2.7. Taking the noise level into account, these three spectra contain the same daughter ions. A visual search of all the daughter spectra showed that very few differences existed between these three bacteria. Some of the differences that were found in the daughter spectra are shown in Figures 2.8 and 2.9. The ions that formed peaks at m/z 209 and m/z 225 appear to be different for *E. coli* and *C. nephridii*. Daughter spectra of these ions contain unique daughter ion masses, along with having some daughter ion masses in common. This observation supports the original hypothesis that the pyrolyzate contains isomass fragments that would be overlayed in the conventional mass spectrum. Unfortunately, very few of these differences were found. Those differences that were found existed only in minor components of the pyrolyzate.

Conclusions

The original hypothesis that was made appears to be sound; differences due to overlapping components do exist in the conventional spectrum and can be resolved using tandem mass

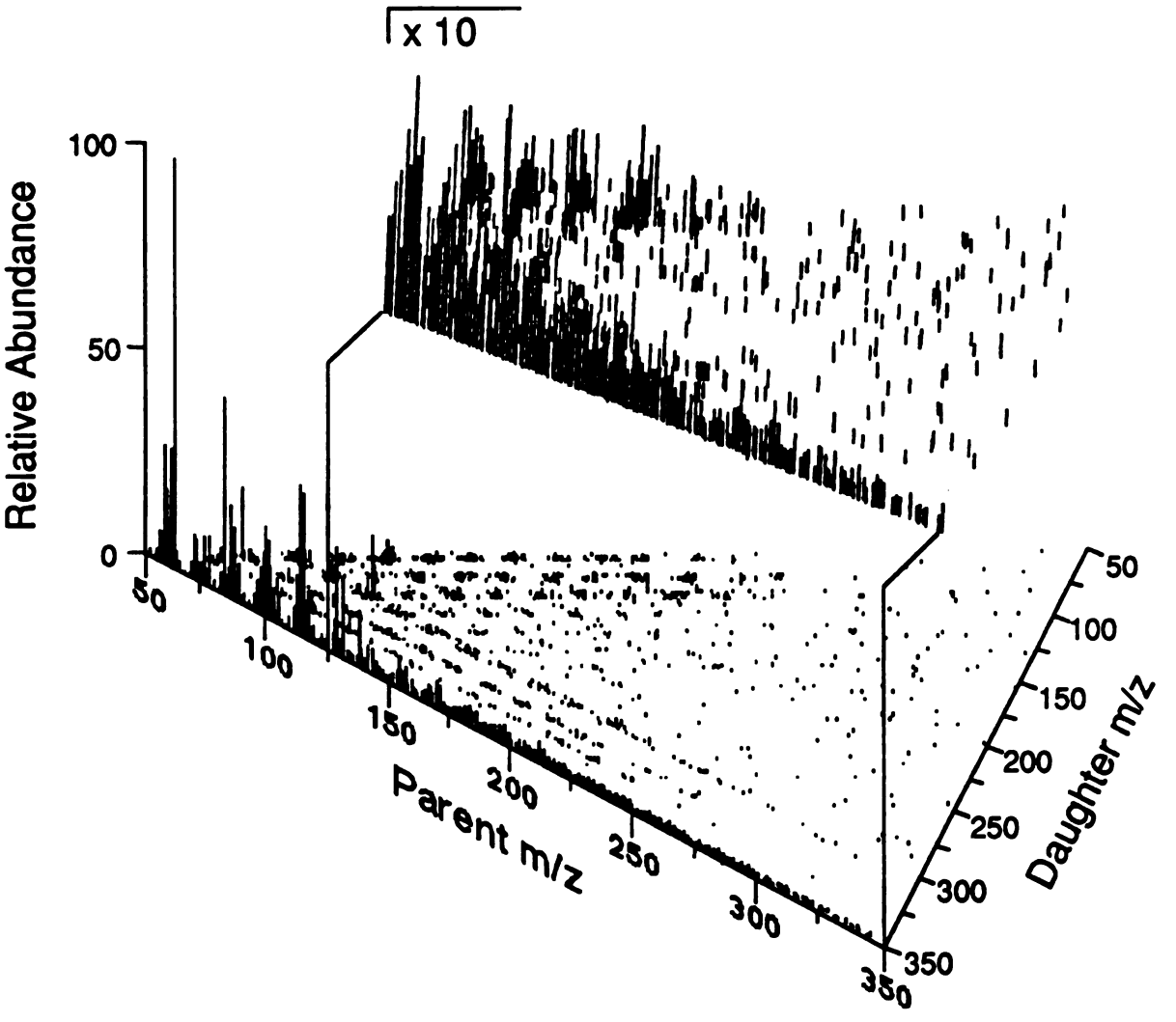

Figure 2.6 Complete pyrolysis/MS/MS data space obtained from *E. coli*.

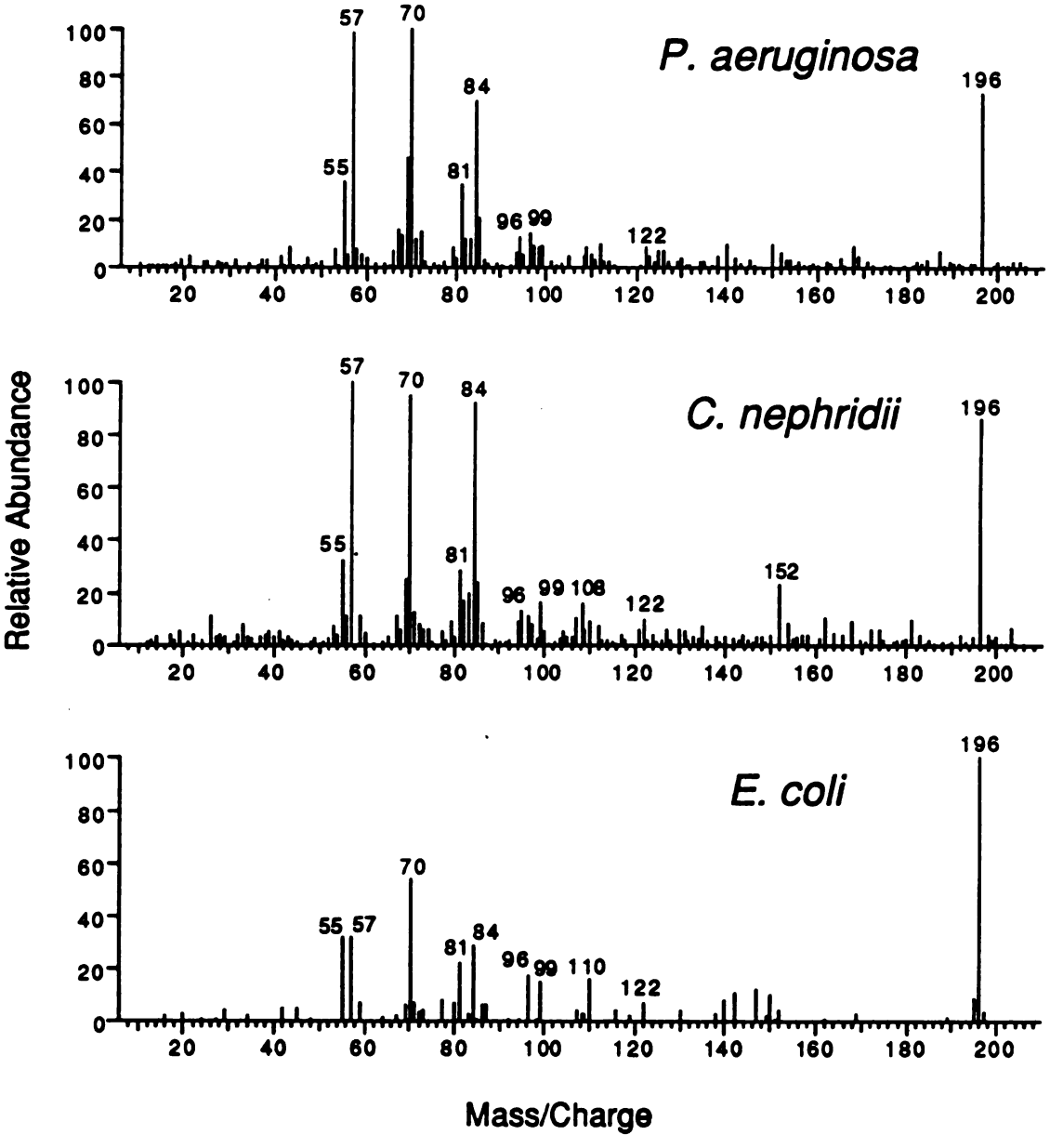

Figure 2.7 Daughter spectra of m/z 196 obtained from the pyrolyzate of three different bacteria.

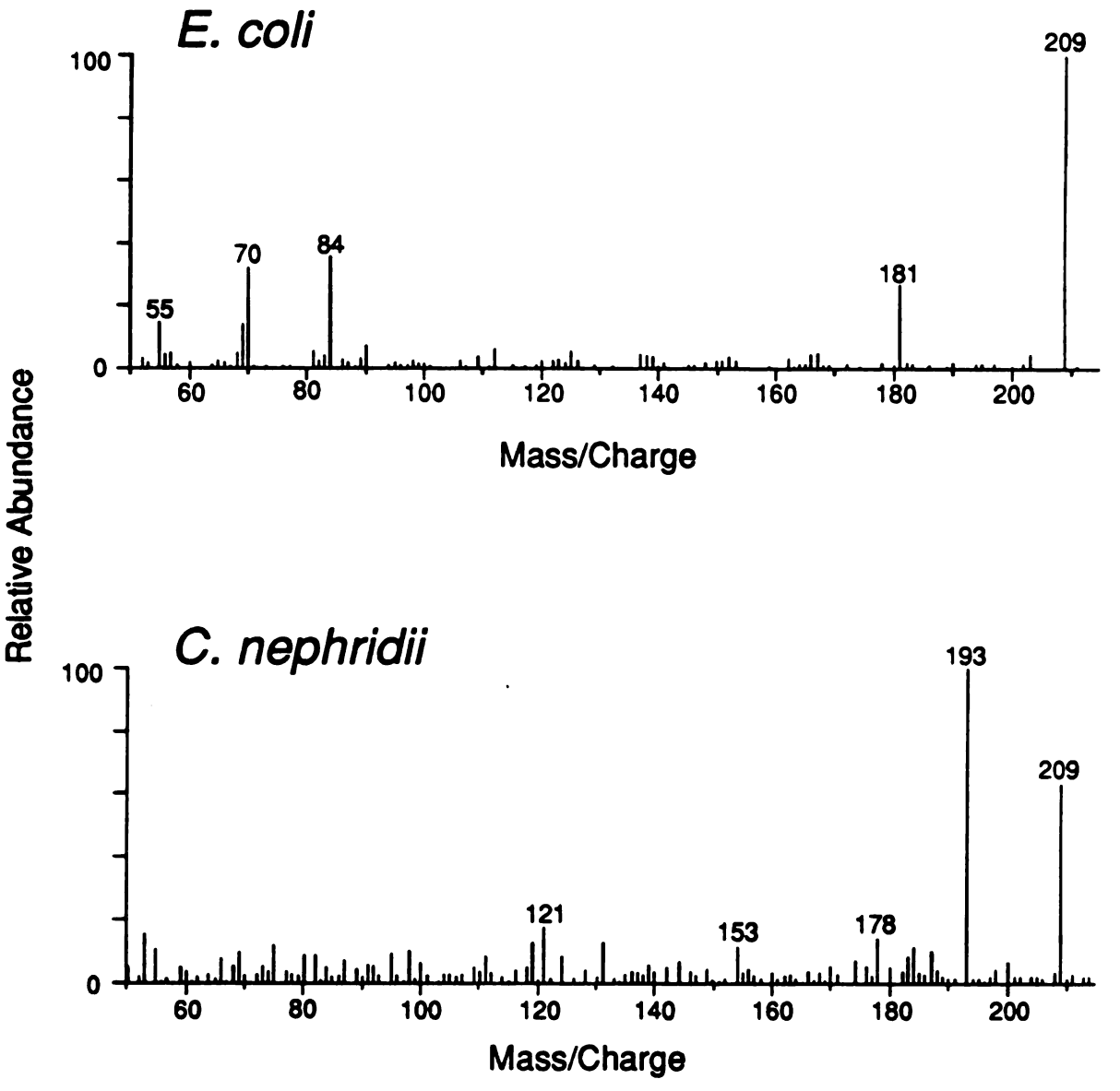

Figure 2.8 Daughter spectra of m/z 209 obtained from the pyrolyzate of three different bacteria.

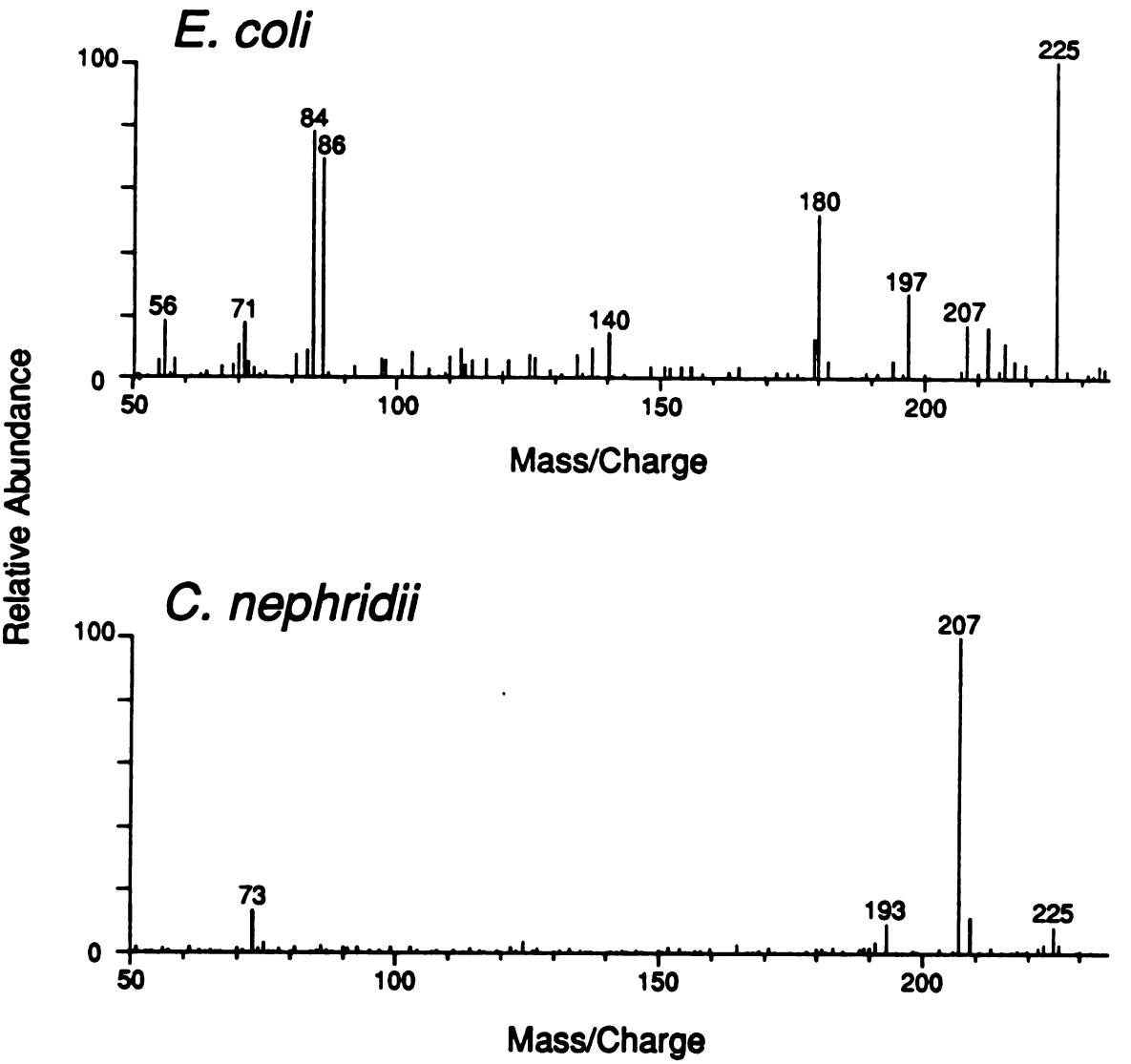

Figure 2.9 Daughter spectra of m/z 225 obtained from the pyrolyzate of three different bacteria.

spectrometry. However, only a few differences were found among the limited number of bacteria used for this study. These differences will probably not provide enough information to be useful for microbial identification on a larger scale. The probability of absolute uniqueness in the tandem mass spectrometry data space of every organism in a large grouping seems low. Due to this reason, pyrolysis was abandoned as an identification technique. Instead, methods for selective analysis of known classes of biomarkers were investigated for their usefulness in microbial detection and identification.

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CHAPTER 3

Direct Determination of Phospholipid Structures in Bacteria by Fast Atom Bombardment / Triple Quadrupole Mass Spectrometry

Introduction

Pyrolysis proved to be too harsh a technique for accessing the unique components of microorganisms. Classes of molecules useful as biomarkers were fragmented to such an extent as to lose their identity and uniqueness. The results of the pyrolysis experiments underlined the importance of identifying and selectively obtaining specific information on whole biomarkers. The need for selective analysis of known classes of biomarkers led to an exploration of microbial lipids.

The diversity of lipids in microorganisms signifies a diversity of functions. Lipids, either directly or indirectly, play a significant role in the specialized functions each species maintains to survive in its particular environmental habitat. Lipids carry out many responsibilities including storage, membrane structure and function, photosynthetic processes, and most energy-generating processes. The types and distribution of lipids contained in microorganisms can be very species specific [1-3].

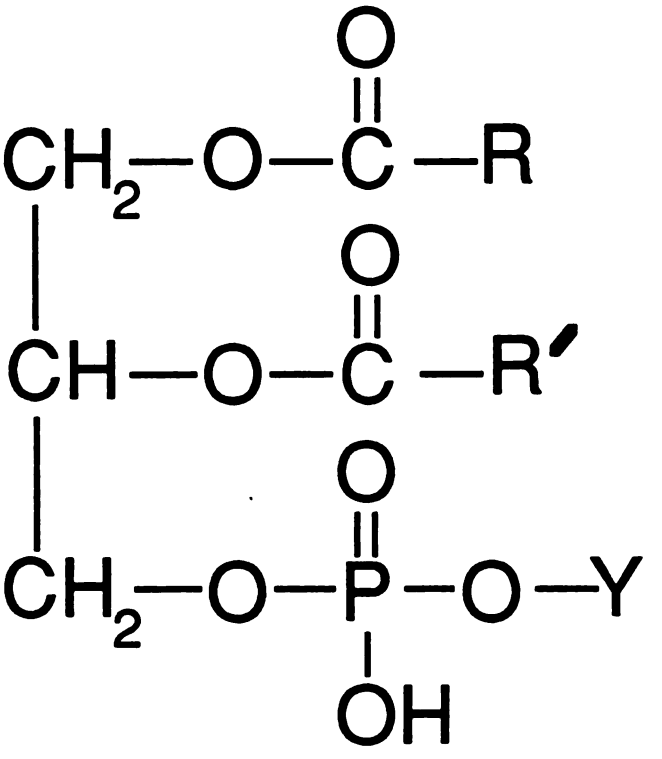
Not all of the cellular lipids are easily accessed. Phospholipids are responsible for the structure of the cell membrane, and thus, ultimately its function. In addition, these membrane phospholipids are easily accessed through simple extractions and provide a useful and abundant biomarker for microbial detection and identification. Our interest in phospholipids stems from these reasons. This chapter describes a general method for rapidly characterizing the phospholipid content and structures in crude lipid extracts, with the ultimate goal toward application of this information to microbial detection and identification.

Background

The resurgence of interest in the mass spectrometry of phospholipids is due primarily to the development of fast atom bombardment (FAB) ionization and tandem mass spectrometry (MS/MS). Fast atom bombardment ionization allows the nonvolatile phospholipids to be analyzed without prior derivatization, and MS/MS allows phospholipid mixtures to be fully characterized without prior separation. Phospholipids are found in abundance in the cell membranes of living organisms. They have been used as biomarkers for bacteria [4-6] and algae [7], for detection of the generation of platelet activating factor [8-10], and for the determination of general membrane function in living matter.

The phospholipids in which this research is concerned are the glycerophospholipids (Structure 3.1). Glycerophospholipids consist of four primary functional groups: a glycerol-3-phosphate core on which two fatty acids (R, R') have been esterified to the two free hydroxyl groups in the sn-1 and sn-2 positions, and a second alcohol (Y) is esterified to the phosphate group in the sn-3 position (except for phosphatidic acid, which contains a protonated phosphate group). This head group (Y) is the functional group which defines the specific class to which the phospholipid belongs, while the fatty acids distinguish the individual phospholipid molecular species within each class. Examples of different phospholipid classes are shown in Figure 3.1.

For a method to be useful in obtaining a profile of bacterial phospholipids in natural samples, it must have high sensitivity as well as high selectivity. While most of the phospholipid information is contained in the conventional FAB/MS spectrum [4,6], the data are difficult to interpret; signals from lipid species at low concentrations are often buried in the high chemical background associated with FAB/MS, and FAB/MS does not work well for mixtures of phospholipids. Fast atom bombardment / tandem mass spectrometric analysis of daughter ions produced by collisionally induced dissociation (CID) of molecular ion precursors has proven useful for phospholipid structure analysis [11-17]. While this method is useful for obtaining information on the content, structures, and relative positions of the fatty acids present on individual phospholipids, screening a mixture of phospholipids for the classes present would be a complex procedure



Structure 3.1 Basic glycerophospholipid structure.

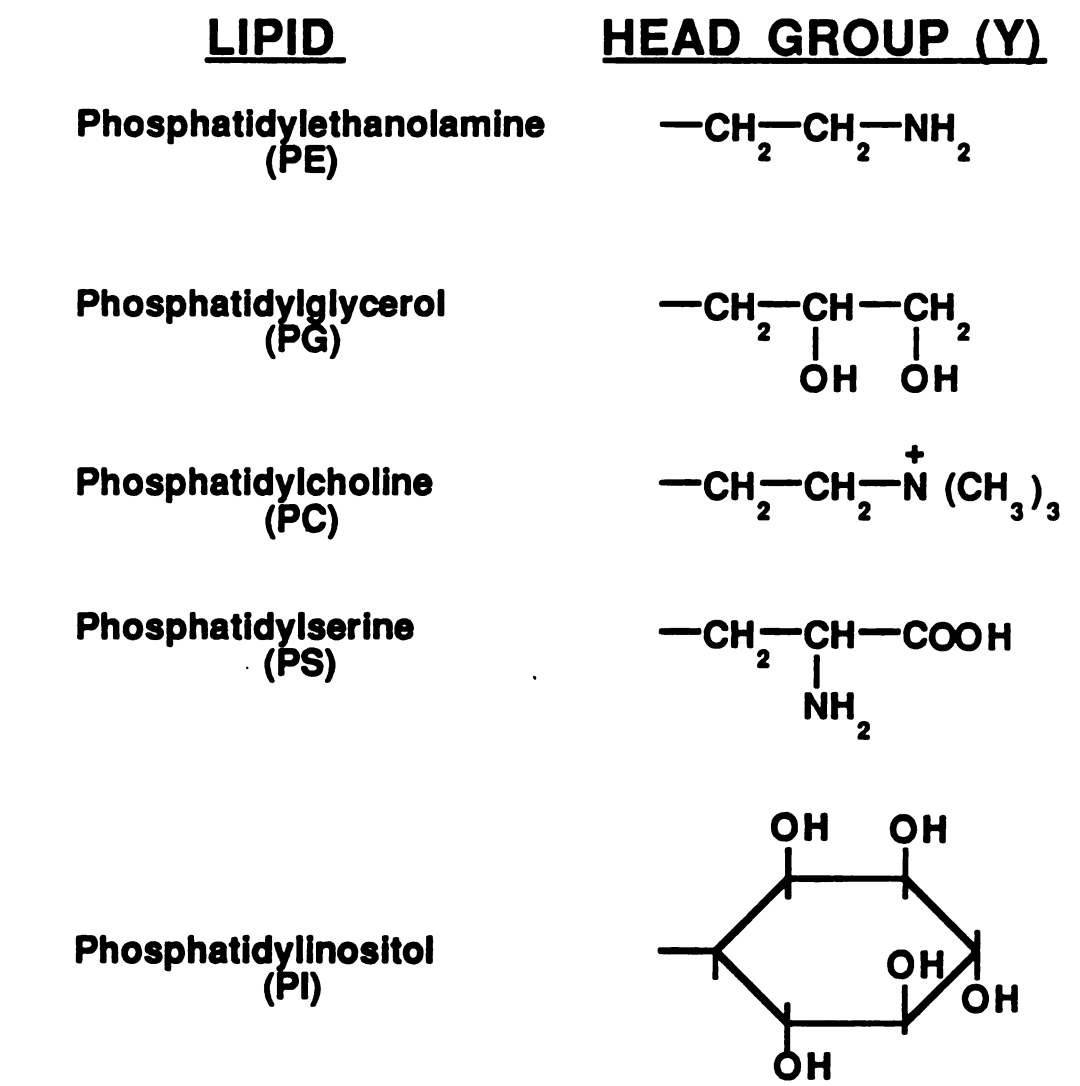


Figure 3.1 Examples of the head groups from five phospholipid classes.

requiring a mathematical analysis of the data set composed of the daughter spectrum from the ion current at every mass. Constant neutral loss scanning for polar head functional groups has been shown to be very useful for detection and differentiation of phospholipid classes in complex matrices and mixtures [13,18].

When phospholipid ions undergo low energy CID, only a few different fragment ion masses are formed. However, the fragment ions produced are the result of cleavages at specific points which are common to all classes of phospholipids. These cleavages occur around the phospholipid functional groups and, thus, provide significant structural information about the parent ion. The low energy CID fragmentation of phospholipids for both positive and negative ions is shown in Figure 3.2. In the positive ion mode, the major reaction occurring is cleavage of the phosphate/glycerol bond resulting in the loss of the polar head group as a neutral, while the rest of the ion retains the charge. In the negative ion mode, one or the other fatty acid is cleaved and retains the charge while the rest of the ion is lost as a neutral. Examples of some of the possible positive ion neutral losses and fatty acid anions are given in Table 3.1. This research has taken advantage of the fact that phospholipids always cleave around their functional groups to develop a general scheme for the rapid characterization of phospholipid classes present in bacteria, including information on the fatty acids present on the species within each class. This scheme is a sequence of positive ion neutral loss and parent scans, and negative ion daughter scans.

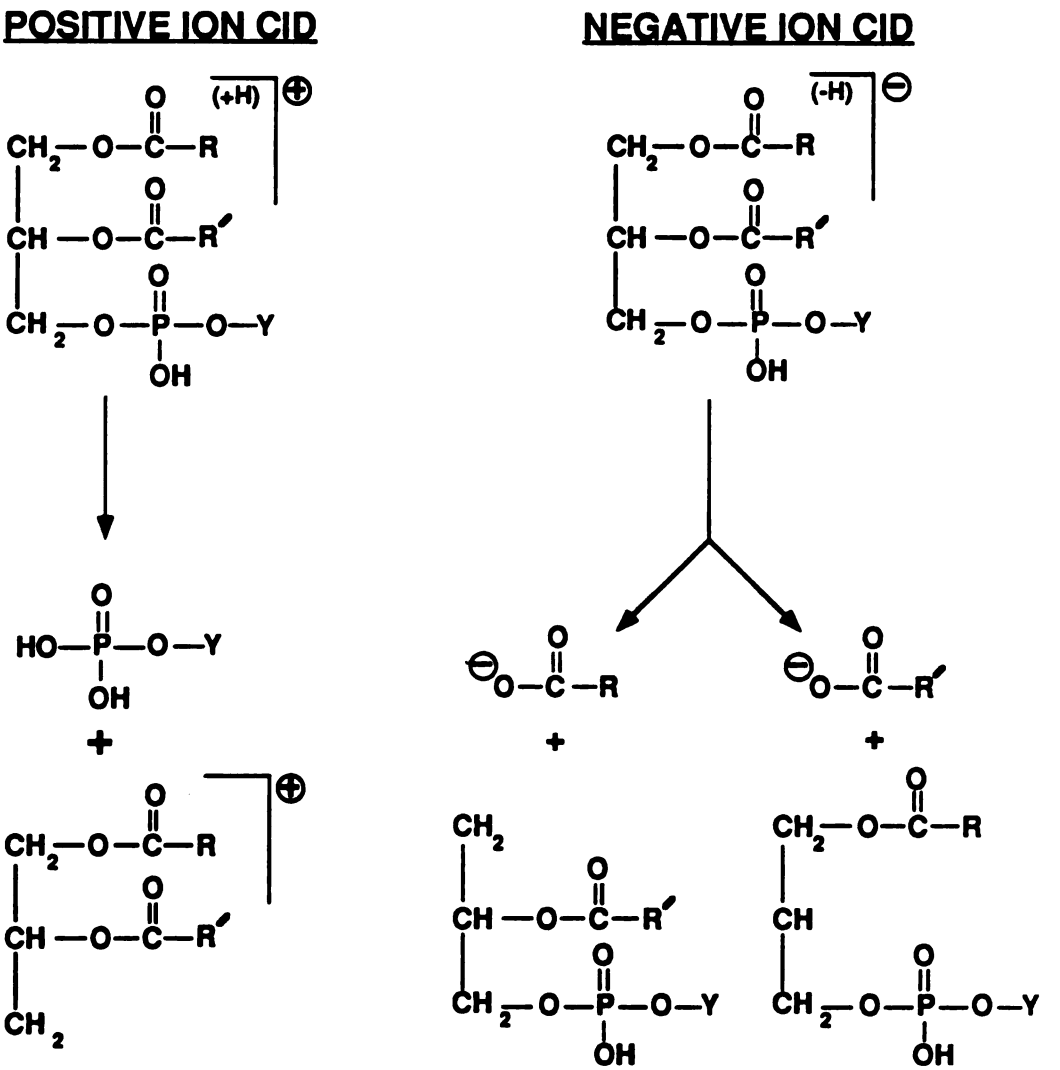


Figure 3.2 Low-energy CID fragmentation of phospholipids for positive and negative ions.

Table 3.1 Characteristic Positive Ion Neutral Losses for Various Head Groups and Fatty Acid Anions in the Daughter Ion Spectra

NEUTRAL LOSSES:[a]	FATTY ACID ANIONS:[b]
PE of 141 u	C14:0 at m/z 228
PG of 172 u	C14:1 at m/z 226
PS of 185 u	C15:0 at m/z 241
PI of 260 u	C15:1 at m/z 239
PC of 183 u	C16:0 at m/z 255
PA of 98 u	C16:1 at m/z 253
LPG of 300 u	C17:0 at m/z 269
PMME of 155 u	C18:0 at m/z 283
PDME of 169 u	C18:1 at m/z 281

[a] PE=phosphatidylethanolamine, PG=phosphatidylglycerol PMME = phosphatidylmonomethylethanolamine, PDME = phosphatidyldimethylethanolamine, PS = phosphatidylserine, PI=phosphatidylinositol, PC= phosphatidylcholine, PA=phosphatidic acid, LPG=lysylphosphatidylglycerol.

[b] C15:0 is a 15-carbon fatty acid with no unsaturation, C15:1 is a 15-carbon fatty acid with one point of unsaturation, etc.

EXPERIMENTAL

All experiments were performed using a Finnigan (San Jose, CA) TSQ-70B triple stage quadrupole instrument equipped with a standard Finnigan FAB source and a JEOL (Boston, MA) MS-009 charge-transfer FAB gun and power supply that has been modified to fit and operate on the TSQ-70B. Spectra were acquired and processed using the Finnigan TSQ-70B data system and software.

All bacteria, except *E. coli* were obtained from researchers in the National Science Foundation's Center for Microbial Ecology at Michigan State University. Cultures were grown on trypticase soy agar plates at 37° C for twenty four hours prior to harvesting. *E. coli* was obtained lypholyzed from Sigma Chemical Co. (St. Louis, MO).

Suspensions of *E. coli* were made by weighing out a known quantity of lypholyzed cells and suspending them in enough distilled/deionized water (DDI) to make a $1x10^9$ cells/ml concentration using the conversion factor $5.9x10^{12}$ cells/gram dry weight [19]. Dilutions of this suspension were made to obtain solutions of different cell concentrations.

Crude lipid extracts were obtained from the bacteria by a modified Bligh-Dyer procedure [20]. One ml of a cell suspension, or a transfer loop full of bacteria, was placed in a clean, sterile 25-ml sample vial to which 15 ml of a 2:1 methanol:chloroform mixture was added. After the lipids were extracted, enough DDI water was added

to separate the chloroform from the $MeOH/H_2O$. The chloroform layer, containing the lipids, was drawn off for analysis. For those experiments requiring a known cell concentration on the probe tip, the chloroform was blown off, using a stream of nitrogen, until just enough solution remained to be placed on the probe tip for analysis (about 5 μl).

For the analysis, 3-5 μl of the chloroform solution was placed on the probe tip and dissolved in a drop of nitrobenzyl alcohol. Xenon was used as the FAB gas and the FAB gun was operated with a filament current of 10 mA and a xenon beam energy of 8 keV.

Neutral loss and parent ion spectra were obtained in the positive ion mode with no CID gas added to the center quadrupole other than the residual gas remaining in the analyzer manifold. The fragmentation observed may be predominantly the result of metastable decomposition. Daughter spectra were obtained in the negative ion mode using argon as a collision gas. The collision gas pressure was 0.5 mtorr and the collision energy was 30 eV.

RESULTS AND DISCUSSIONS

Phospholipid data are obtained by this technique on two levels. The first level is referred to as the *(class) mass profiles* which are the separate mass spectra for each phospholipid class. From these data, information is obtained regarding the phospholipid classes present, the masses of individual phospholipid species within each class, and

the relative intensities of the species within each class. The second data level consists of the daughter spectra of individual phospholipid species and is referred to as *(class)(fatty acid) formula data.* These data can be interpreted to provide empirical formula information on the fatty acids and structural information on each phospholipid species. The data levels and their interrelation are shown as a data space diagram in Figure 3.3. In this space, each phospholipid class forms its own data plane with the mass profile of its species contained along one dimension of the plane and the fatty acid masses for each of its species along the other dimension. As discussed later, low-energy CID does not yet allow the complete structure of the fatty acid groups to be obtained. The method described here collects *(class)(mass) profiles* and *(class)(fatty acid) formula data.* Research is underway toward obtaining complete fatty acid structural information.

General Profiling Scheme

The first step in the general scheme is to obtain the positive ion neutral loss scans for loss of the masses of the different phospholipid polar head groups. The instrument is programmed to scan a series of possible polar head group neutral losses, thereby obtaining *(class) mass profiles* of all the phospholipid classes scanned. These profiles approximate the relative abundances of all species within each phospholipid class. The resulting spectra are free of any non-phospholipid peaks, and many peaks of interest which might have been hidden previously in the chemical background of the conventional mass spectrum show up clearly. An example of the use of neutral loss scans for collecting *(class) mass profiles* of two of the

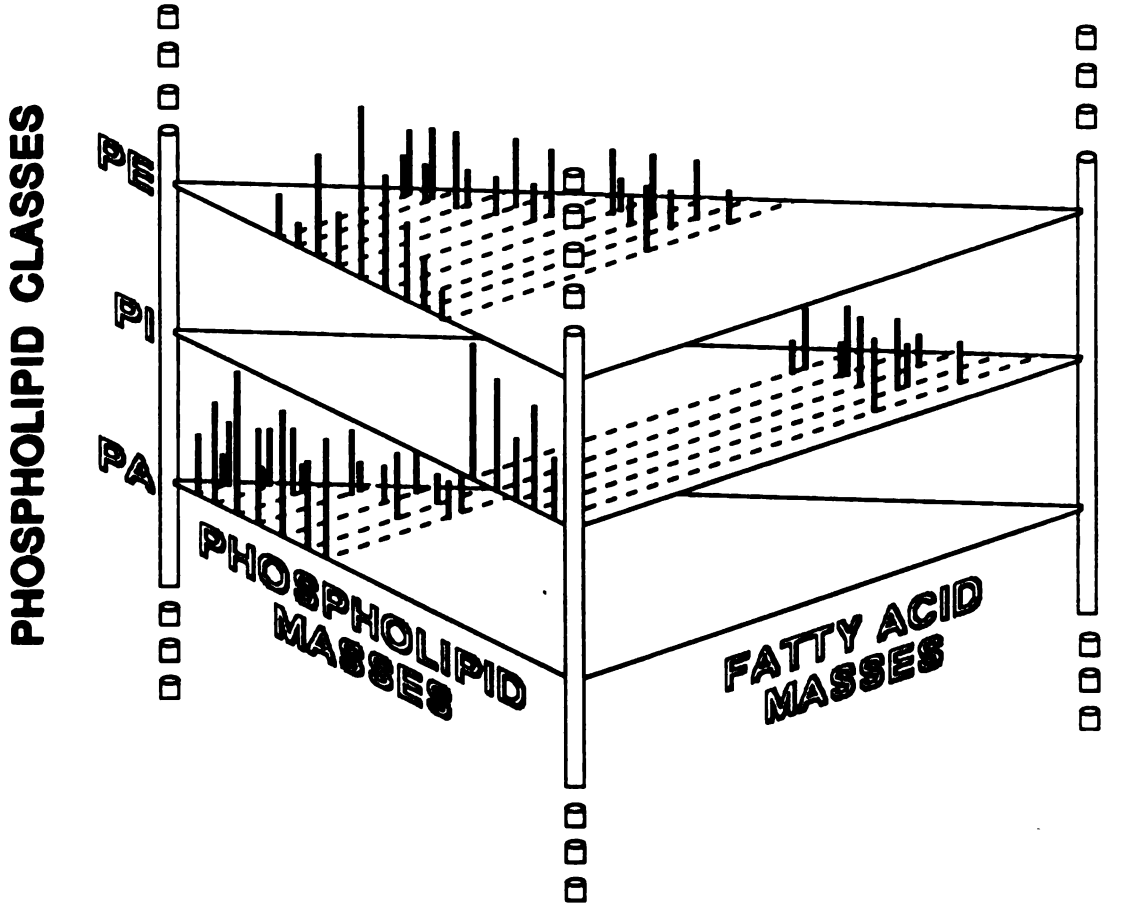


Figure 3.3 The FAB/MS/MS data space obtained by this technique. The masses of the phospholipids in each class are obtained by neutral loss or parent ion scans specific for each class. The daughter spectrum of each phospholipid species provides the masses and positions of the fatty acids.

phospholipid classes contained in *Proteus vulgaris* is shown in Figure 3.4. Note that the peaks due to phosphatidylglycerol (neutral loss 172) are completely buried in the chemical background of the conventional spectrum. This added detectability is a direct result of the additional selectivity afforded through tandem mass analyzers.

The relative intensities of the phospholipid species obtained in each *(class) mass profile* reflects the relative intensities of the species in the conventional mass spectrum. Apparent differences in intensities between the conventional spectrum and the neutral loss spectra are seen when more than one phospholipid class contributes to the abundance of a particular peak in the conventional mass spectrum. These overlaying peaks are resolved by the neutral loss spectra. The only exception that was found to using neutral loss scans is in the characterization of phosphatidylcholine. Phosphatidylcholine fragments so that the head group usually retains the charge. While the neutral loss scan does work for phosphatidylcholine, greater sensitivity was obtained through the use of a parent scan for the head group ion. A parent scan for m/z 184 produces the *(class) mass profile* for phosphatidylcholine.

The two remaining pieces of information needed for a complete phospholipid profile are the composition, structure, and relative positions of the fatty acids on the glycerophosphate core. Much of this information can be directly obtained from the negative ion daughter spectra of the individual phospholipid species detected in the series of *(class) mass profiles* obtained from the neutral loss scans previously

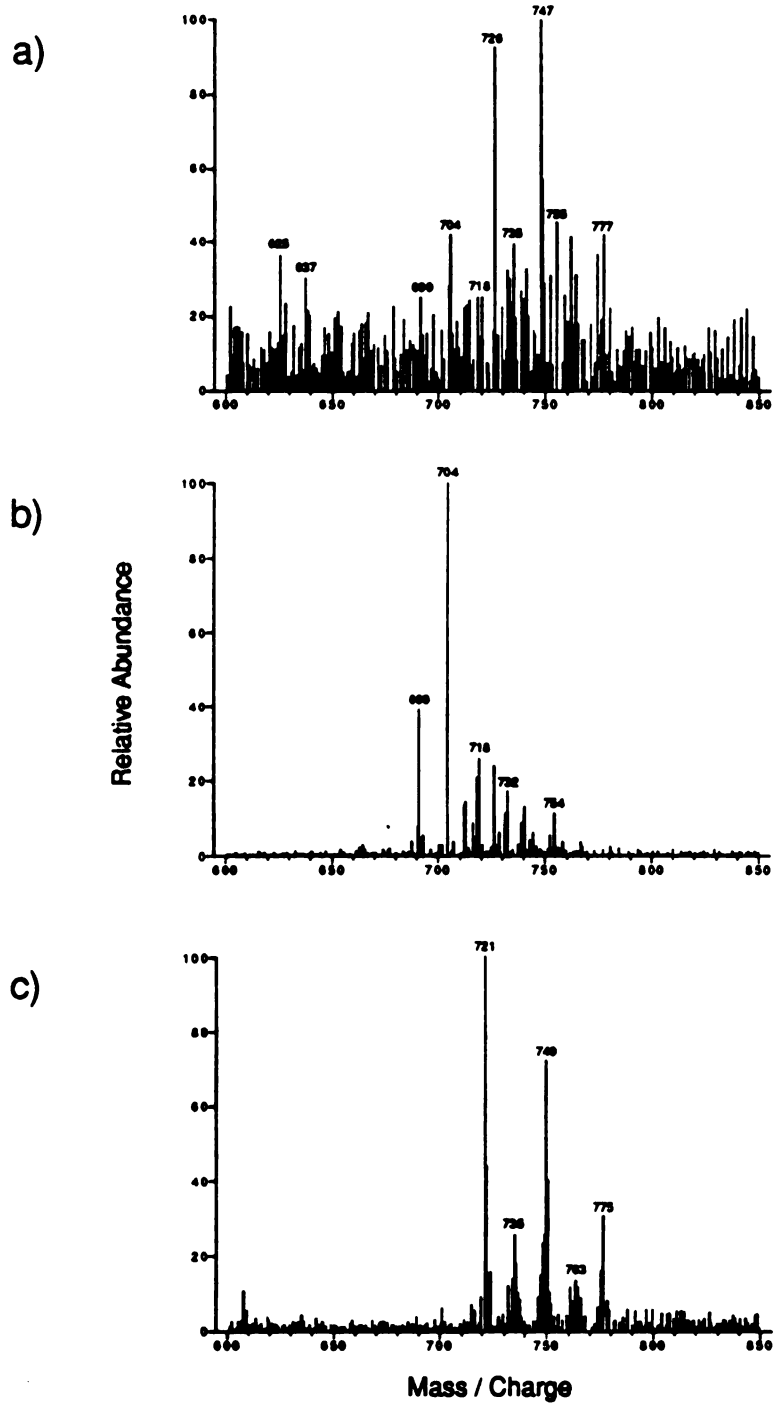


Figure 3.4 *a)* Conventional mass spectrum of a *Proteus vulgaris* extract. *b)* Neutral loss spectrum of 141 u; specific for PE. *c)* Neutral loss spectrum of 172 u; specific for PG.

performed. As shown in Figure 3.2 for $[M-H]^-$ ion dissociation, the two major fragments present in a negative ion daughter spectrum are the peaks due to the two fatty acids contained on the phospholipid ion. The mass/charge value of the fragment ion corresponds to the molecular weight of the free fatty acid, less one hydrogen, as shown in Table 3.1. In this manner, the number of carbons contained in the fatty acid may be determined, along with the degree of unsaturation. It is important to note that from these spectra, only the empirical formulae of the fatty acids may be determined with certainty. At the present time, low-energy daughter spectra cannot determine the location of double bonds on a fatty acid, differentiate between a point of unsaturation and a cyclic (cyclopropyl) structure, or differentiate normal from branched fatty acids. Research using low-energy methods to solve these problems has begun in this laboratory.

The negative ion daughter spectra also can provide information on the relative positions of the two fatty acids on the phospholipid. Zirrolli, *et al.* [9] have postulated preferential formation of the carboxylate anion from the fatty acid at the sn-2 position (closest to the head group) over that of the fatty acid at the sn-1 position. When this is true, the daughter ion peak with the greater intensity is due to the fatty acid from the sn-2 position. While this general rule apparently does not apply to phospholipids containing highly unsaturated fatty acids or when large differences in the chain lengths of the two fatty acids occur [21], it does hold for phospholipids containing fatty acids with fewer than three points of unsaturation and with chain lengths differing less than 10 carbons from each other.

Because fatty acids which fit the exceptions to this rule are extremely rare in bacterial phospholipids, this rule is being used to obtain positional information about the fatty acids present in our samples. The exceptional fatty acids are easily diagnosed and the data treated accordingly. Figure 3.5 is an example of the use of negative daughter ions for *Salmonella abaetetuba.* The peak at m/z 733 represents the $[M-H]^-$ ion of a phosphatidylglycerol species. The two daughter ion peaks obtained at m/z 255 and m/z 267 correspond to a C16:0 fatty acid and a C17:cyc fatty acid, respectively. As shown by the difference in peak intensities, the C17:cyc fatty acid occurs at the sn-1 position on the phospholipid.

An automated instrument control procedure has been written that performs the necessary neutral loss or parent scans, in the positive ion mode, for all 9 phospholipid classes in which this research is interested, switches the instrument into the negative daughter ion mode, and collects the individual daughter spectra from the 10 major peaks present in each of the phospholipid classes previously detected. The resulting data file contains up to the 9 possible *(class) mass profiles* and the daughter spectra of up to 90 phospholipid species. The automated procedure can collect this information from a sample in under ten minutes. A small portion of the complete data set used to obtain a phospholipid profile for *E. coli* is shown in Figure 3.6.

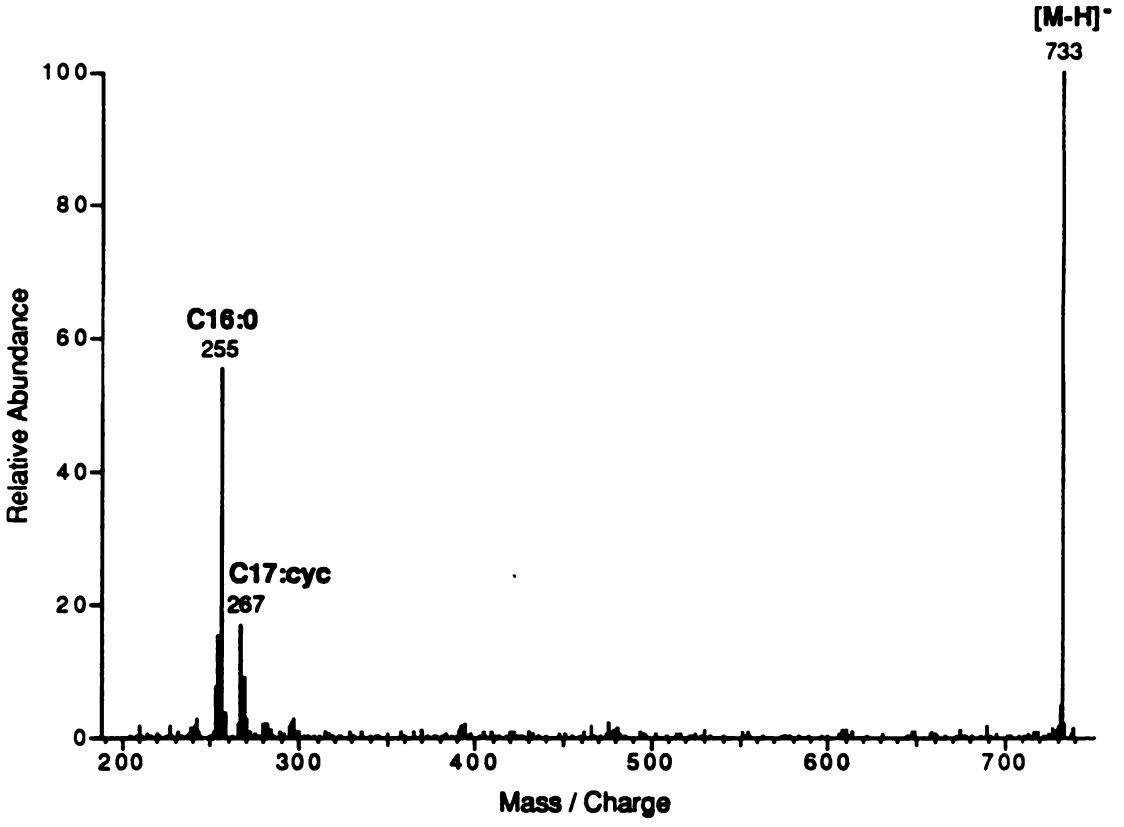


Figure 3.5 Negative ion daughter spectrum of a phosphatidylglycerol species at m/z 733 showing the two fatty acid ions and their relative intensities.

C16:0 is a 16-carbon fatty acid with no unsaturation; C17:cyc is a 17-carbon propyl fatty acid.

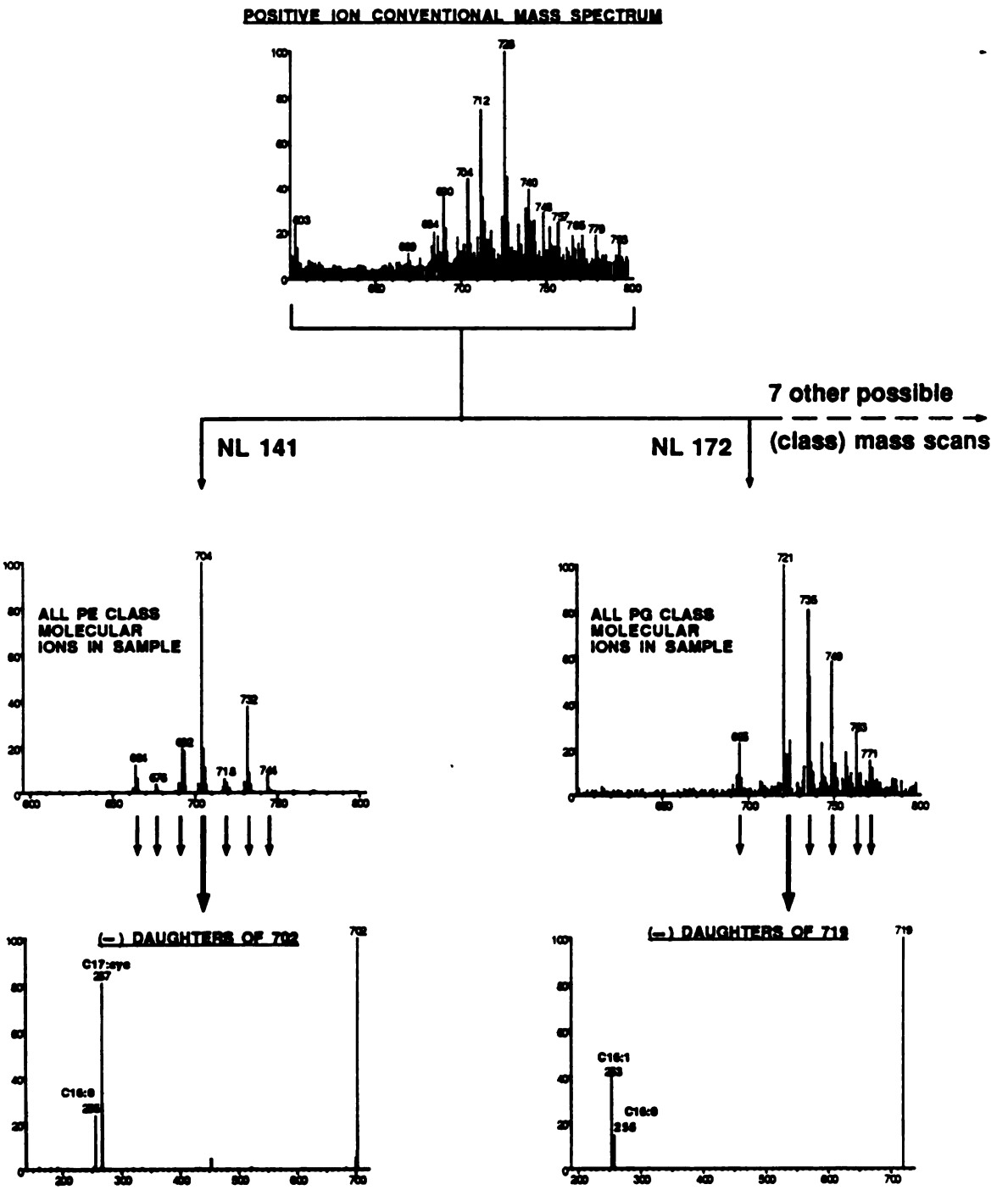


Figure 3.6 An example of the use of neutral loss and daughter spectra for obtaining a partial profile of *E. coli.* The neutral loss scans obtain the *(class) mass profiles* while the daughter scans obtain the *(class)(fatty acid) formula data.* As indicated, seven other neutral loss scans are normally performed for a "complete" profile. Daughter spectra are obtained for the major peaks in each *(class) mass* scan.

Sensitivity

The increase in selectivity due to the use of tandem mass spectrometry gives outstanding detection limits which are unattainable for these samples in conventional mass spectrometry. The top spectrum in Figure 3.7 is a neutral loss scan for phosphatidylethanolamine (neutral loss of 141 u) of one picogram of a mixture of phospholipids which are mostly phosphatidylethanolamine species extracted from *E. coli*. This is roughly the phospholipid content of ten *E. coli* cells. For comparison, the bottom spectrum shows the same scan of 1 microgram of the same mixture; equivalent to 10^7 cells. All of the phosphatidylethanolamine species present in the 1-microgram mixture have been detected in the one picogram spectrum. This extremely low sample size requirement allows for the analysis of phospholipids at bacterial levels found in natural samples without prior cultivation of the organisms.

Precision

For this technique to be useful in bacterial phospholipid profiling, the spectra must be reproducible though varying concentrations of bacterial cells extracted. If the spectra changed with varying cell concentrations, the information regarding the classes, individual species, and fatty acid content of the phospholipids would be retained, but all information pertaining to the relative abundances of the individual species within each class would be dependent on the total quantity of phospholipid present. The degree of spectral variance obtained on our instrument for various cell concentrations is shown in Figure 3.8. Using neutral loss scans, the percent of the total

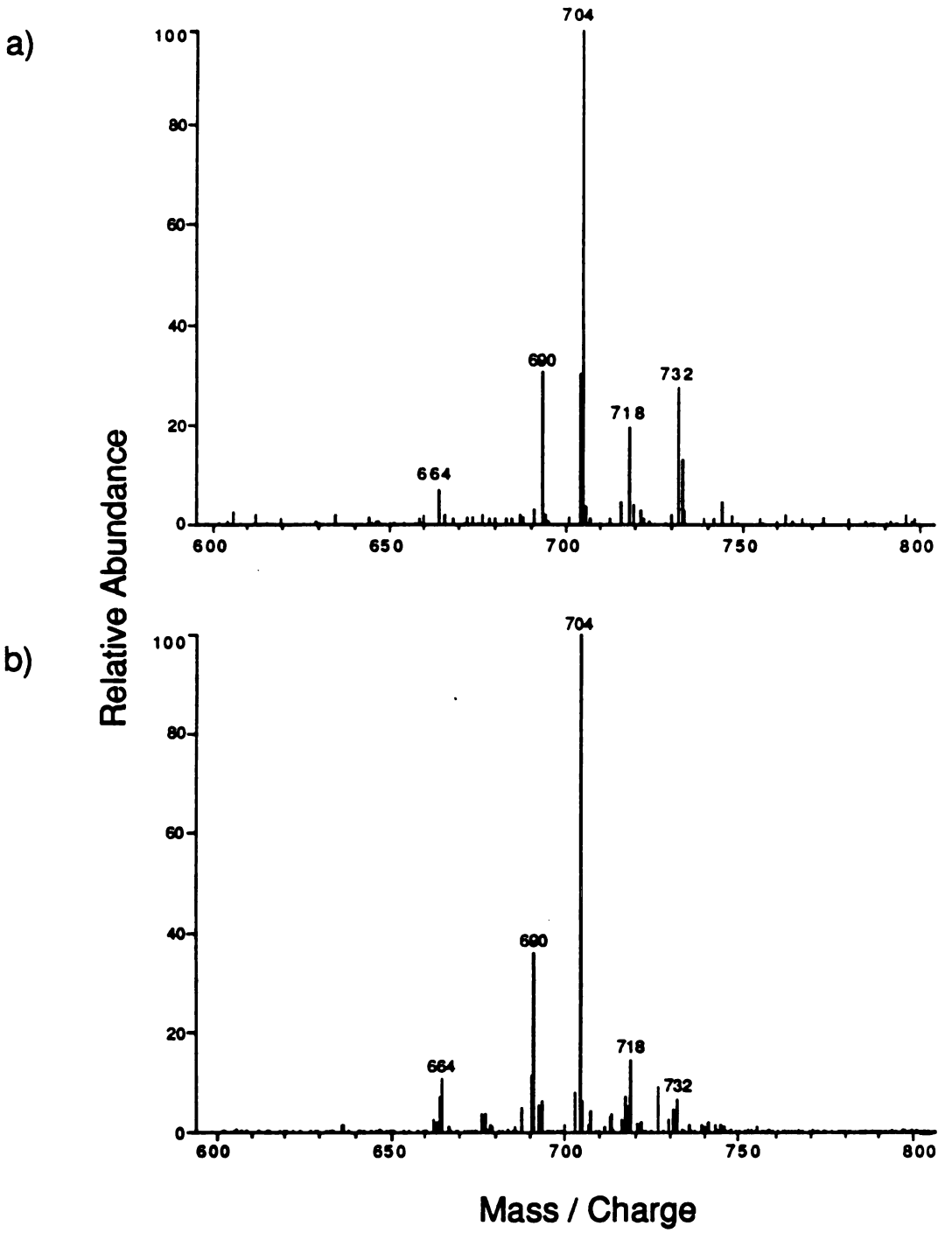


Figure 3.7 *a)* PE mass profile of 1 picogram PE extracted from *E. coli*; equivalent to 10^1 cells. *b)* PE mass profile of 1 microgram PE extracted from *E. coli*; equivalent to 10^7 cells.

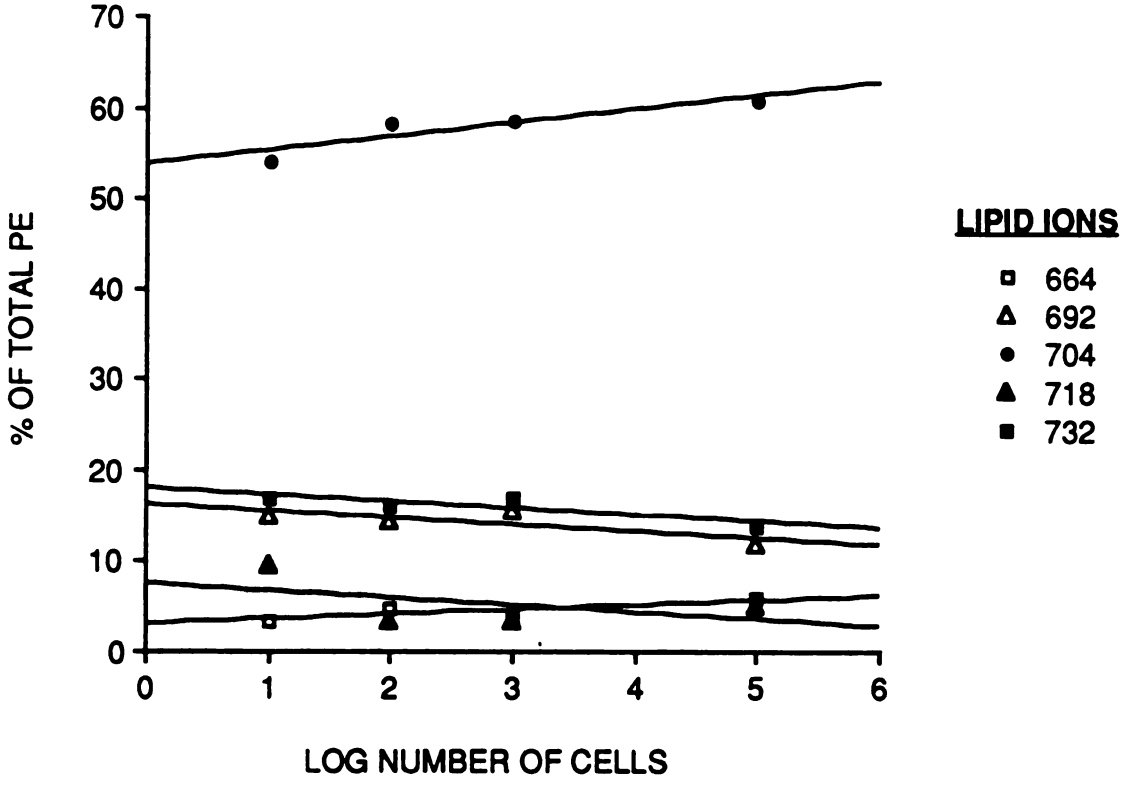


Figure 3.8 Percent of total PE as a function of the log number of cells for the major species present in *E. coli*.

phosphatidylethanolamine ion current was plotted as a function of the log number of cells extracted for the major species present in *E. coli*. High precision was obtained, with the relative intensities of the masses within the spectra varying no more than 5 percent over at least a 5-log range of bacterial cell concentration.

Extraction Process

Related to the problem of spectral precision as a function of sample size is the problem of phospholipid recovery as a function of cells extracted. The recovery of this method was checked by analyzing cell extracts of *E. coli* at various concentrations. The cells were extracted for two hours before sampling. Neutral loss scans were performed for phosphatidylethanolamine and phosphatidylglycerol, the two most abundant phospholipids in *E. coli*; the total ion current obtained was used as a measure of phospholipid abundance. Table 3.2 shows the log of the total phospholipid ion intensity obtained for several values of the log number of cells extracted. These data follow a linear fit with a correlation coefficient of 0.997, showing that the amount of phospholipid recovered in an extraction, relative to the number of cells extracted, remains constant.

With an automated instrument control procedure that can produce a phospholipid profile in under ten minutes, the time required for extraction can become the limiting step with regards to total analysis time. Typically, the samples are extracted for 24 hours [22], with reports of more rapid extractions of 2 hours [23]. In an effort to decrease the total analysis time for a sample, the dependence

Table 3.2 Phospholipid Recovery for Various Values of Log Number of *E. coli* Cells Extracted

Log Number of Cells	Log Lipid Ion Current
5.0	5.58
6.0	6.66
7.0	7.70
8.0	8.54

Correlation Coefficient = 0.997

of phospholipid recovery on the total extraction time was determined. Neutral loss scans were performed on a series of five *E. coli* samples which had been extracted for 1 minute, 10 minutes, 1 hour, 2 hours, and 24 hours, respectively. The total phospholipid ion intensities were used as a measure of the phospholipid recovery. Table 3.3 shows the phospholipid recovery, relative to the recovery obtained from the 24-hour extraction, for various extraction times. These data suggest that little recovery advantage is gained after the first 10 minutes of extraction. In fact, a 1-minute extraction yielded greater than 80% phospholipid recovery. These rapid extractions significantly shorten the total analysis time and open up the possibility of performing on-line extractions coupled with a technique such as continuous-flow FAB.

CONCLUSIONS

The use of neutral loss, parent, and daughter scans in combination with rapid extractions provides a quick and sensitive method for profiling the phospholipid content and distribution in bacteria. Since bacteria maintain a characteristic and rather constant membrane phospholipid content, this method has a great potential for detecting and identifying bacteria based on phospholipid profiling. Research in this area is described in detail in Chapter 5.

This technique is not restricted to use in bacterial phospholipid profiling; it can be used as a general method for any sample containing phospholipids. We are using this same method to characterize the phospholipid content of plants, fungi and amoebas. This research is

Table 3.3 Phospholipid Recovery from *E. coli* for Various Extraction Times

Extraction Time (minutes)	Number of Samples	Average Phospholipid RIC*	Relative Standard Deviation
1	3	3,867,690	23.4%
10	3	4,544,030	23.3%
60	3	4,487,700	28.2%
120	3	4,765,410	28.9%
1440	3	4,791,830	23.9%

* RIC = Reconstructed Ion Current

also described in Chapter 5. However, the caveat about fatty acid position data must be taken into account when working with samples which might contain fatty acids included in the exceptions mentioned earlier in this chapter. Many plant species contain highly unsaturated fatty acids and/or large differences in the chain lengths of the two fatty acids present on the phospholipids.

Finally, the basic concepts used in this approach also can be used to profile types of lipids other than glycerophospholipids. These concepts are being applied to the analysis of the content and distribution of glycolipids in bacteria, and sterols in fungi. This is discussed in greater detail in Chapter 6. Applications to phosphonolipids, ether lipids, and other types of lipids are certainly possible, and also are discussed in Chapter 6.

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CHAPTER 4

Fast Atom Bombardment / Ion-Molecule Reactions for the Differentiation of Phospholipid Classes

Introduction and Background

Constant neutral loss and parent scanning for polar head functional groups is a very useful technique for differentiation of phospholipid classes and analysis of phospholipids in complex matrices and mixtures. However, in low-energy CID, some classes of phospholipids do not readily lose their polar head group while others fragment to give a non-unique neutral loss [1,2]. Therefore, it is not always possible to differentiate phospholipid classes using low-energy CID. Reactions occurring in the second quadrupole between protonated phospholipids and ethyl vinyl ether were found to be useful for differentiating phospholipid classes. In this chapter, these ion-molecule reactions are explored as an alternative to low-energy CID methods for the differentiation of phospholipid classes.

Experimental

All experiments were performed using a Finnigan (San Jose, CA) TSQ-70 triple stage quadrupole instrument equipped with a standard Finnigan FAB source and an Ion Tech (Teddington, UK) saddle field

gun. Spectra were acquired and processed using the Finnigan TSQ-70 data system and software.

All phospholipids were obtained from Sigma Chemical Co. (St. Louis, MO). Ethyl vinyl ether and glycerol were obtained from Aldrich Chemicals (Milwaukee, WI).

The phospholipid samples were dissolved in the glycerol matrix and 1-3 μl of this mixture was placed on the probe tip for analysis. Xenon was used as the FAB gas and the FAB gun was operated with a current flow of 2.4 mA and a beam energy of 7.5 keV. The individual protonated lipids formed in the ion source were selected by the first quadrupole to pass through to the second quadrupole (rf-only) for reaction. Ethyl vinyl ether was introduced into the second quadrupole to a pressure of 3.5 - 4.0 mtorr as indicated by the instrument's convectron gauge, which is connected to the reaction chamber. The reaction and dissociation products were analyzed by scanning the third quadrupole over a wide mass range above and below the mass of the selected parent ion.

The formation and abundance of the reaction products depends on the instrumental parameters. The most important of these parameters are the DC offset on Q2 and the voltages on the lenses immediately before and immediately after Q2. These potentials control the kinetic energy of the reactant ion being passed by Q1. The instrumental parameters were optimized by adjusting them to maximize the abundance of a product ion of m/z 101 produced in Q2

when protonated glycerol ions react with ethyl vinyl ether. This ion is due to the loss of acetaldehyde from the complex of protonated and neutral ethyl vinyl ether molecules [3]. The optimum DC offset on Q2 was always found to be between +2.5 V and +4.0 V; the lens immediately preceding Q2 was found to optimize between +5.0 V and +6.0 V; the optimum voltage for the lens immediately following Q2 was found to be between -1.0 V and -5.0 V. These conditions are optimum for the ethyl vinyl ether reactions used in this study.

Results and Discussion

Unique Reaction Products

Upon reaction with ethyl vinyl ether, each phospholipid class gave a unique pattern of reaction products which permitted the differentiation of the various phospholipid classes studied. Reaction product spectra from representative members of four different classes of phospholipids are shown in Figure 4.1. The selected reactant ion is the lowest mass peak in the spectrum. The masses of the neutral additions are given in parenthesis above each product ion peak in the mass spectrum. Each phospholipid class except phosphatidylcholine gave at least one unique reaction product with ethyl vinyl ether. Phosphatidylcholine gave a non-unique reaction product, but it was the only class to give a single reaction product. The mechanisms of the reactions that are occurring are not yet known. However, the most probable compositions of product species in the observed addition reactions are listed in Table 4.1.

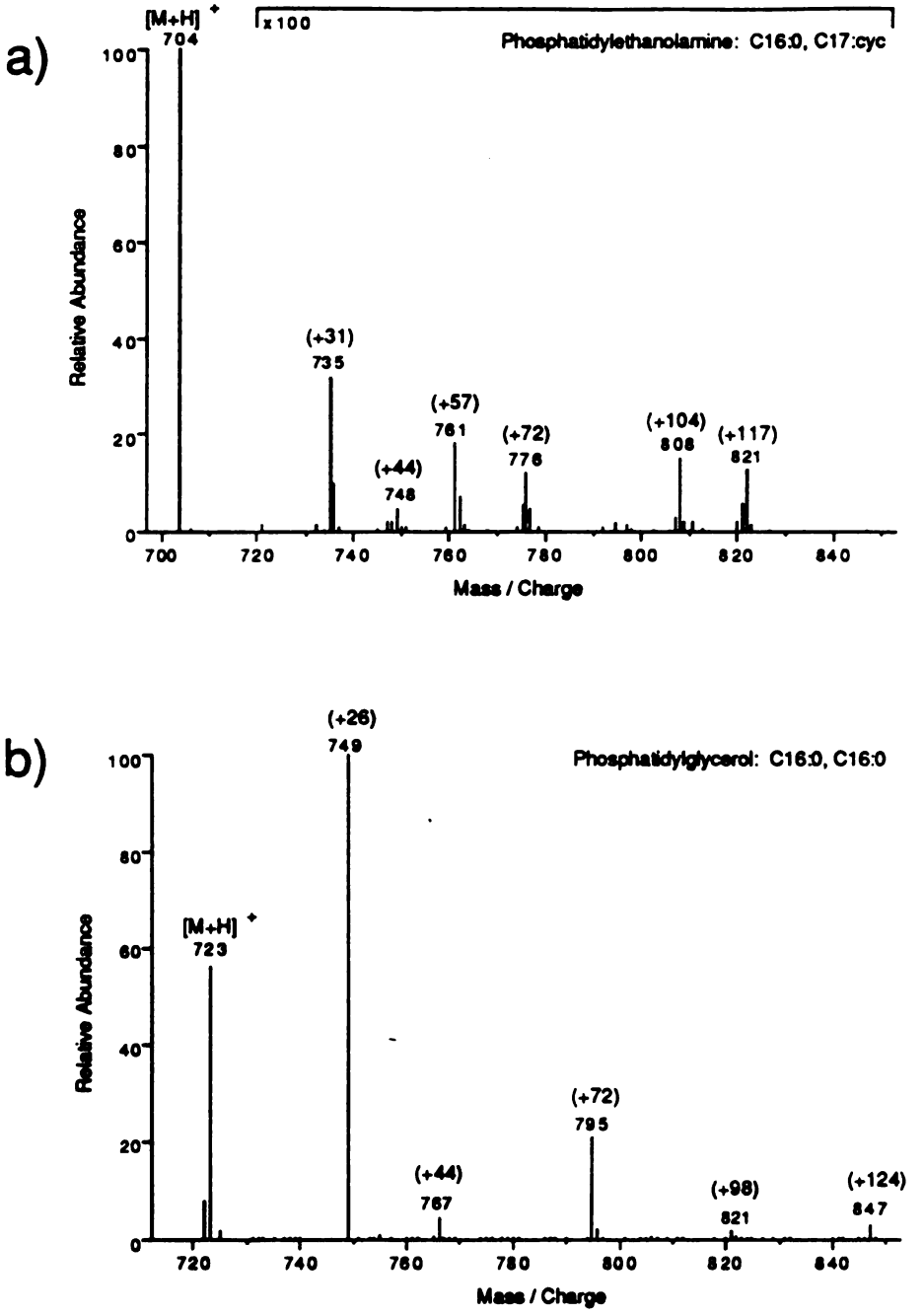

Figure 4.1 Mass spectra of the reactions of four phospholipid classes: *a)* PE C16:0, C17:cyc *b)* PG C16:0, C16:0 *c)* PC C18:0, C18:2 *d)* PI C15:0, C12:0.
C16:0 is a 16-carbon fatty acid with no unsaturation, C17:cyc is a 17-carbon propyl fatty acid, C18:2 is an 18-carbon fatty acid with 2 points of unsaturation, etc. The order in which the fatty acids are listed correspond to their positions on the glycerol core.

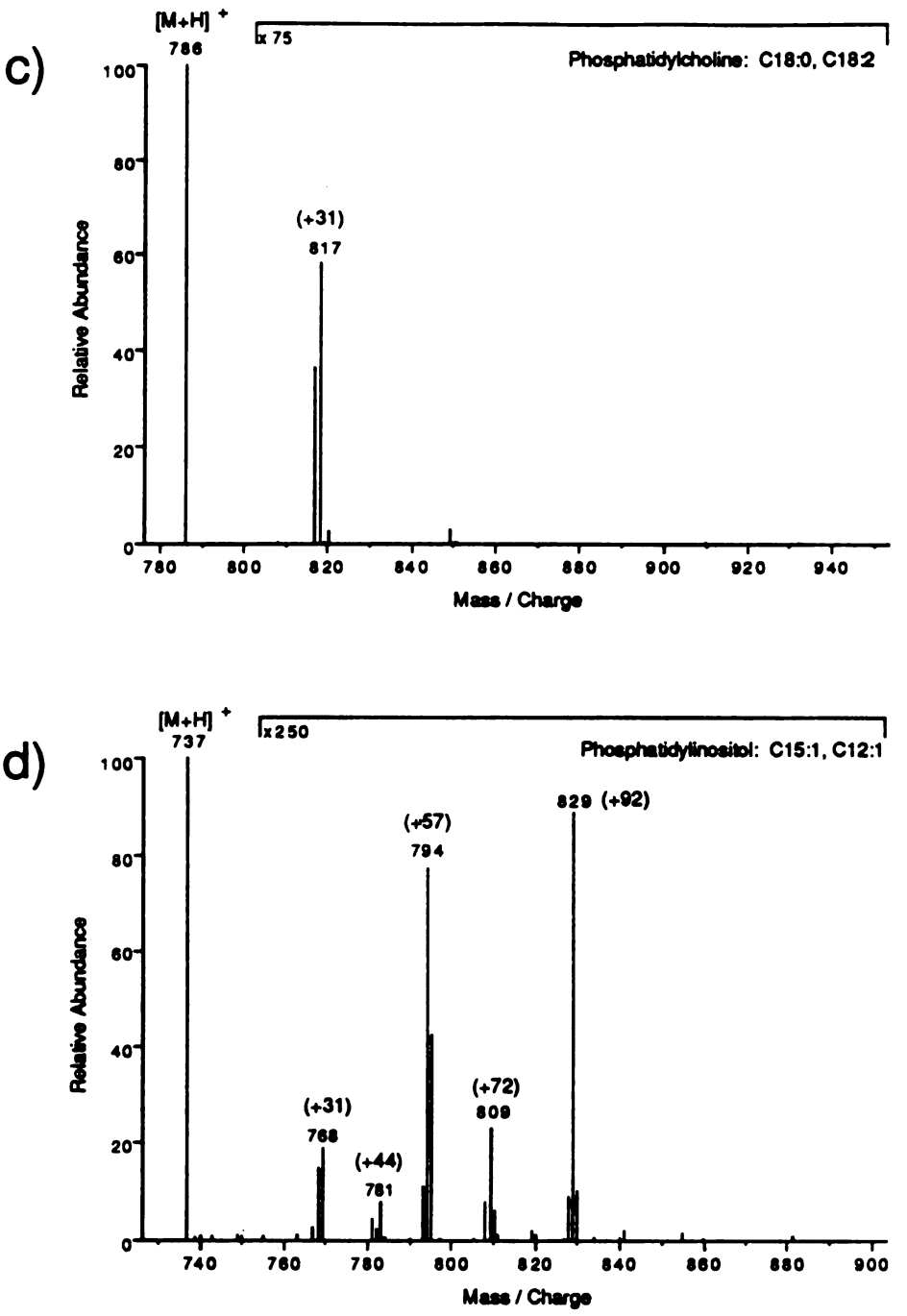

Figure 4.1 cont.

Table 4.1 Compositions Of Addition Species

ethyl vinyl ether (Neu): CH_2= CH-O-CH_2CH_3

MASS	SPECIES	MASS	SPECIES
26	C_2H_2	92	2Neu - C_4H_8
31	CH_3O	98	Neu + C_2H_2
44	C_2H_4O	104	Neu + CH_3OH
57	C_3H_5O	117	Neu + C_2H_5O
72	C_4H_8O (Neu)	124	Neu + C_4H_8

Where The Reactions Occur

For application in phospholipid class differentiation, only the reactions of ethyl vinyl ether with the polar head group will be useful. If the differences in reaction products were dependent on the differences in fatty acyl groups, then different reaction products would be obtained within the same class. The spectra of two phosphatidylethanolamine analogues which differ only in their fatty acyl content are shown in Figure 4.2. The same reaction products (relative to the reactant ion) were obtained in each spectrum. These data demonstrate that the reactions are not occurring on the fatty acyl moieties of the phospholipid, but are occurring on the polar head group. When fragment ions which have lost their polar head group are reacted, all classes of phospholipids form the same reaction products. This is further evidence that the reactions are occurring on the polar head group and are thus indicative of the phospholipid class.

Collisionally Induced Dissociation Data

A full daughter scan of m/z values above and below the mass value of the protonated molecule, can provide both ion-molecule reaction data and collisionally induced dissociation data. A full daughter scan of a selected phosphatidylethanolamine parent ion that contains a 16-carbon saturated fatty acid on the first position of the glycerophosphate core, and a 17-carbon propyl fatty acid on the second position is shown in Figure 4.3. Phospholipids dissociate to form ions that contain information on the fatty acid composition. These ions appear as the glycerol core with a loss of the head group and one or the other of the fatty acids. The characteristic dissociation

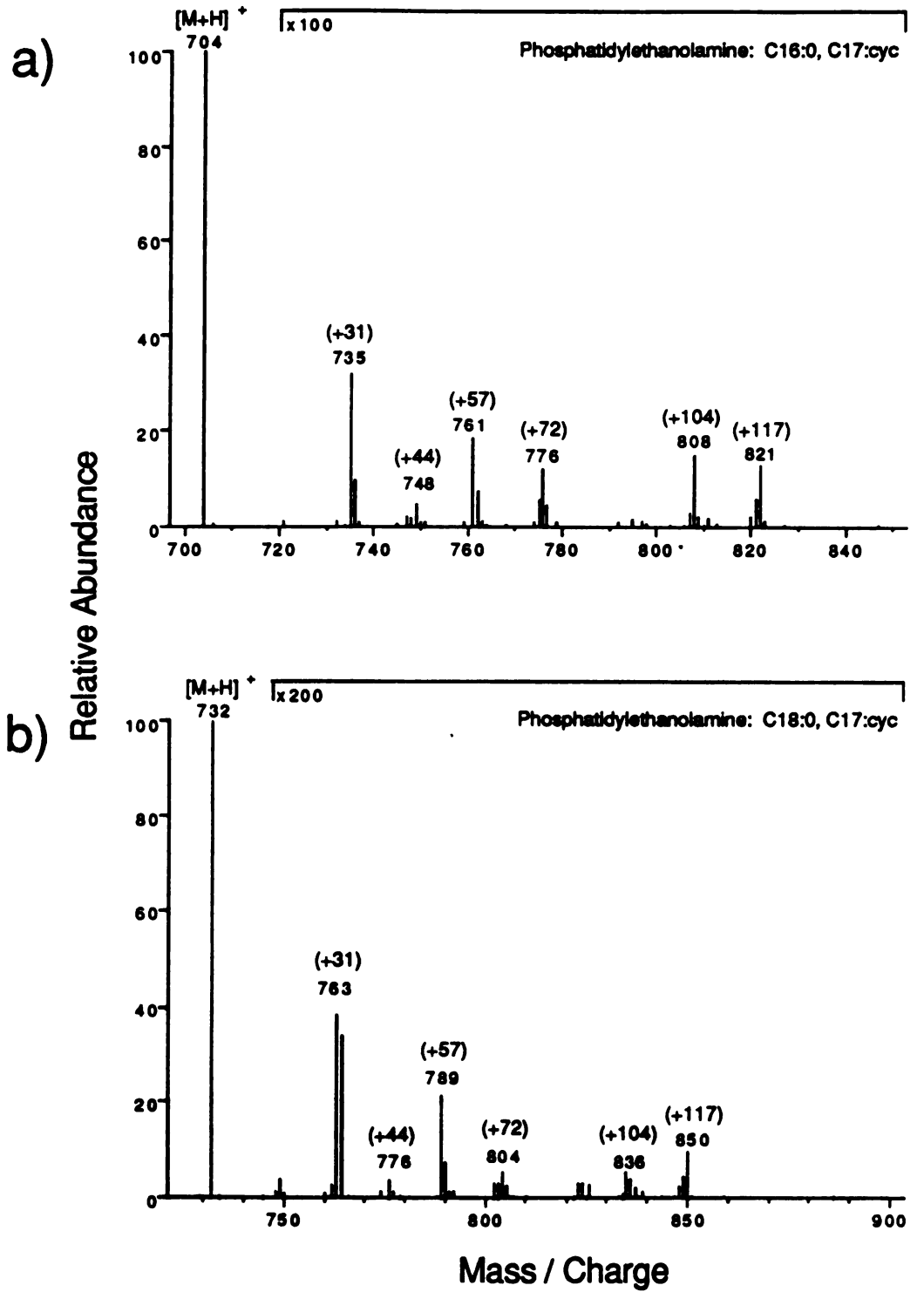

Figure 4.2 Mass spectra of two phosphatidylethanolamine analogues. These lipids are of the same class but differ in their fatty acid content. *a)* PE C16:0,C17:cyc *b)* PE C18:0,C17:cyc

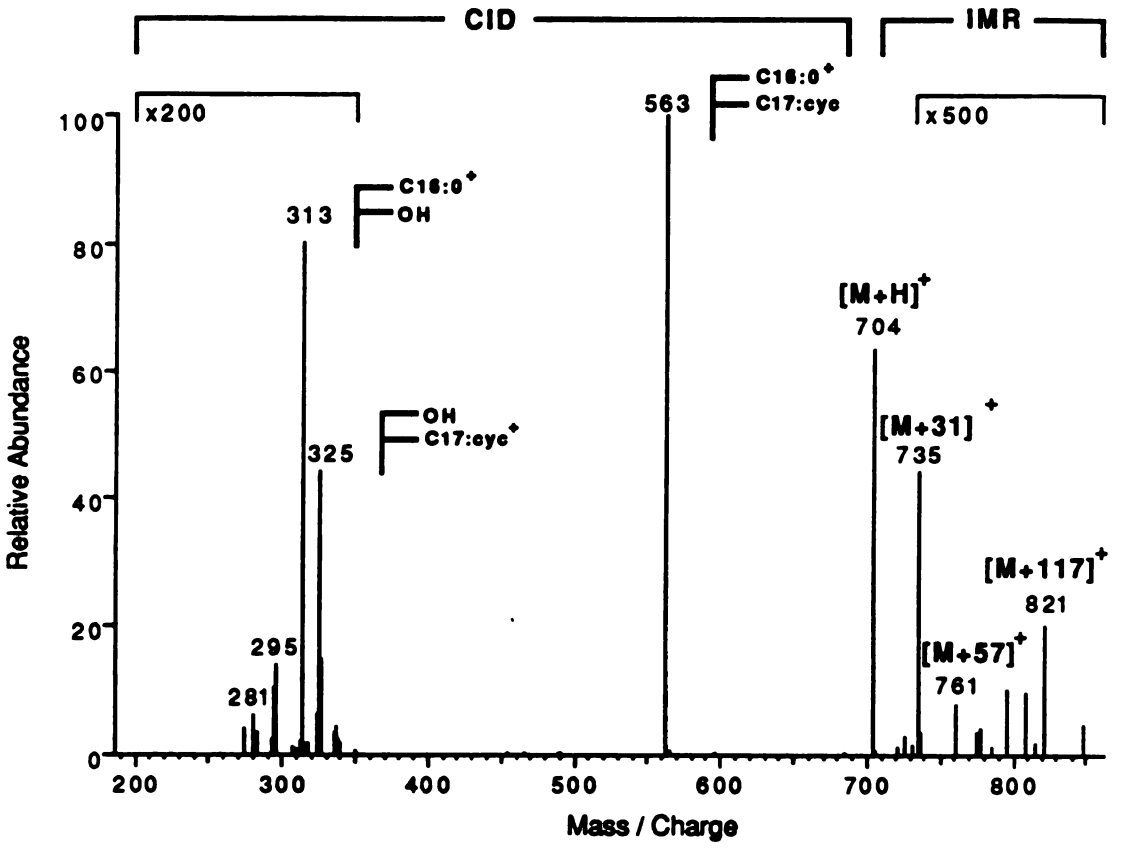

Figure 4.3 Full daughter scan of a selected phosphatidylethanolamine parent showing CID fragments as well as the characteristic reaction products.

fragments shown in Figure 4.3 appear along with the characteristic reaction products. In the positive ion mode, the peak corresponding to a loss of the fatty acid from the sn-2 position is more intense than the peak corresponding to a loss of the fatty acid from the sn-1 position. This conclusion is drawn from observations of the fragmentation patterns of different phospholipid classes containing various fatty acids. The same pattern is also observed in the conventional FAB mass spectrum of each phospholipid. These relative intensities observed in the positive ion spectra are reversed from those found by Zirrolli *et al.* in the negative ion spectra [4], where the relative daughter ion peak intensity for the fatty acid in the sn-2 position is greater than the daughter ion peak intensity for the fatty acid in the sn-1 position. Huang *et al.* have shown that this general rule apparently does not apply to phospholipids containing highly unsaturated fatty acids or when large differences in the chain lengths of the two fatty acids occur [5]. It is not yet known if these exceptions also occur in the positive ion CID spectra; this should be kept in mind when analyzing these types of data. However, for phospholipids which do not fall under these exceptions, a single scan can structurally characterize a phospholipid by giving the class of the phospholipid, the fatty acids contained on the phospholipid, and the relative positions of the fatty acids on the phospholipid.

Neutral Gain Scans

In addition to identifying classes of phospholipids through the unique reaction products of selected individual peaks in the mass spectrum, specific neutral gain scans can be performed that are selective for

each particular class. In this way, all peaks in a mass spectrum that belong to a particular class are identified in the same scan. An example of a neutral gain scan is shown in Figure 4.4. The top spectrum is the conventional FAB mass spectrum of a crude lipid extract containing phosphatidylglycerol. The bottom spectrum is the neutral gain scan for the addition of 26 u, an ethyne moiety. This addition is specific for phosphatidylglycerol. The neutral gain scan selected only that peak in the conventional mass spectrum resulting from the protonated molecule of the one phosphatidylglycerol species present. The neutral gain scan greatly increases specificity and signal-to-background ratio, thereby improving the detection limit. This technique is similar to neutral loss scanning with the advantage that those ions that do not fragment easily to lose their head group as a neutral loss, do readily react to add a neutral gain.

Figure 4.5 is a comparison of a neutral loss spectrum and a neutral gain spectrum. The top spectrum is the neutral gain of 57 u, which is specific for phosphatidylinositol, while the bottom spectrum is the neutral loss of 260 u for the same sample. This neutral loss value also is specific for phosphatidylinositol. The actual intensity axis has been left on each spectrum for comparison. All of the phosphatidylinositol species present in the sample were detected by both scan modes. For phosphatidylinositol, the neutral loss spectrum is an order of magnitude more intense than the neutral gain spectrum. This fact is a function of the individual phospholipid class; the relative sensitivities of the two methods depend upon the facility with which each phospholipid class fragments to lose its polar head group as a

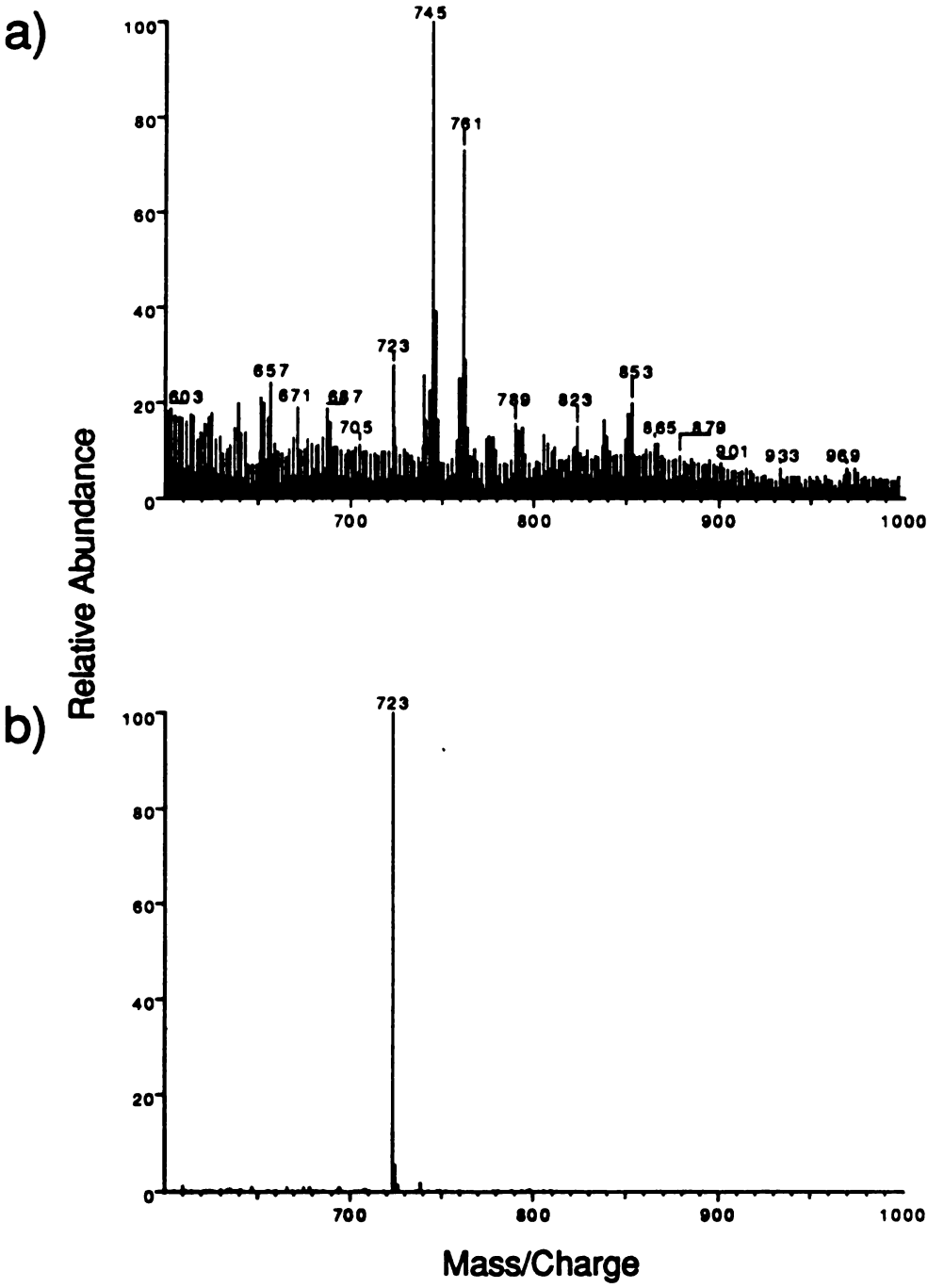

Figure 4.4 *a)* Conventional mass spectrum of a sample containing phosphatidylglycerol. *b)* Neutral gain scan for the addition of 26 u. This addition is specific for phosphatidylglycerol.

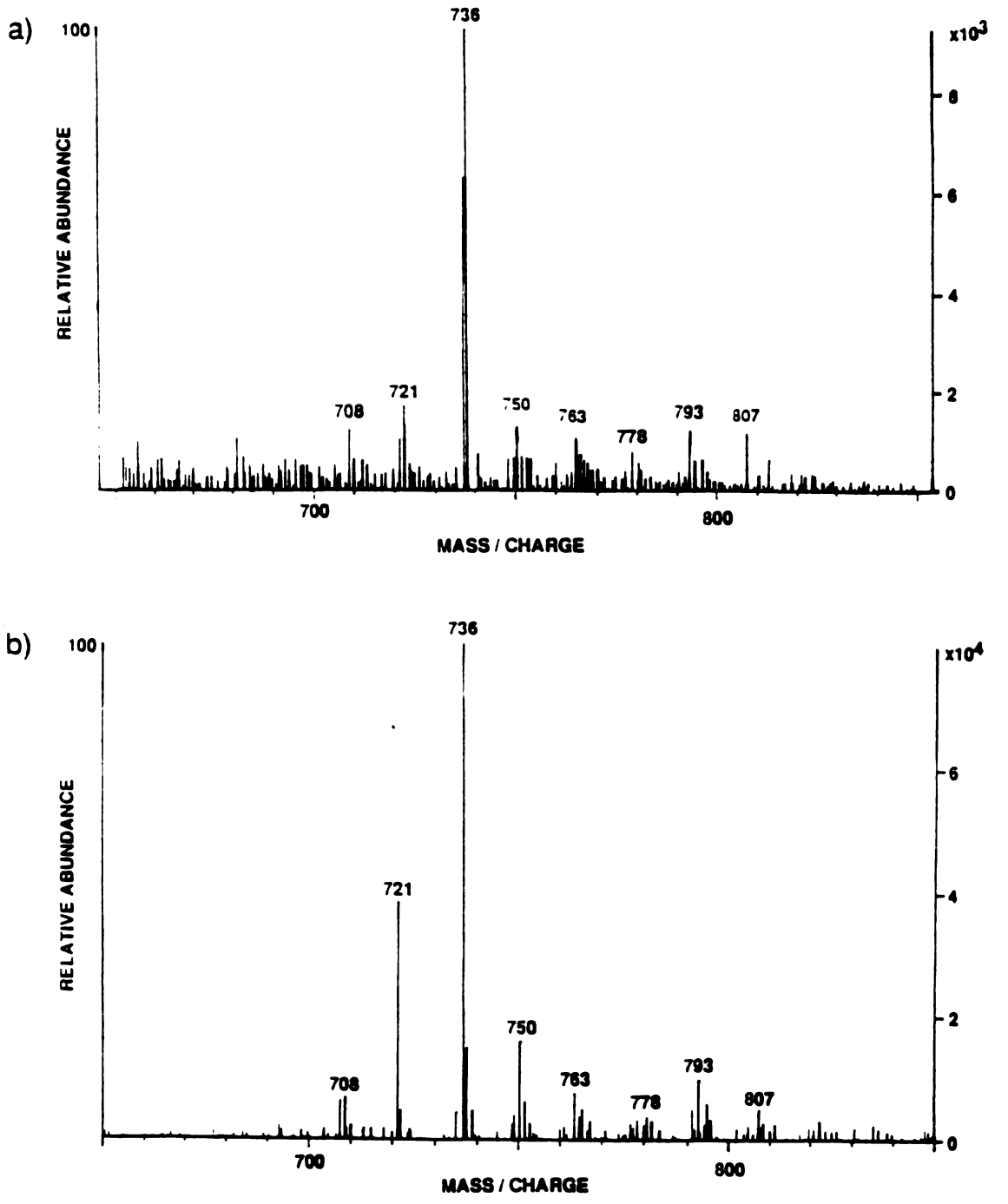

Figure 4.5 *a)* Neutral gain of 57 u; specific for PI. *b)* Neutral loss of 260 also specific for PI.

neutral loss. These IMR neutral gain scans do not provide information different from that obtained from neutral loss spectra, but they are highly advantageous in situations where neutral loss data are ambiguous or unavailable.

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CHAPTER 5

Applications of Phospholipid Analysis to the Detection and Identification of Microorganisms

Introduction and Background

The sensitivity of mass spectrometry - from the standpoint of the uniqueness of specific phospholipid patterns to individual microorgansims - should lead to its widespread use for microbial detection, characterization, and identification. In fact, this has not been the case. While many researchers have used mass spectrometry to analyze phospholipid species [1-4], only one other use of phospholipids for the general identification of bacteria has been reported [5]. This method relies on linear regression of patterns in conventional spectra of phospholipid extracts from bacteria. Although accurate identifications were made from the limited library used, this method is highly dependent on spectral reproducibility. Also, difficulties will arise when the number of cells in the sample becomes so low that the high chemical background associated with fast atom bombardment becomes a major obstacle.

The low chemical background inherent in tandem mass spectrometry makes the method described in Chapter 3 a powerful means of identifying microorgansims. Almost all of the phospholipid information can be obtained, including the classes and number of

species present, the empirical formulae of the fatty acids present on each species, and the positions of the fatty acids present on each species. This combined information forms a unique and detailed fingerprint which can be used to identify microorganisms.

This chapter describes the application of the general phospholipid analysis technique from Chapter 3 to the detection and identification of microorganisms. Fingerprint profiles have been collected from a number of bacteria, fungi, and amoebae; a listing is given of microorganisms analyzed up to this point in the research. Results from two of the countless potential clinical and industrial applications are described. In the clinical application, the causative agents of urinary tract infections are profiled. These profiles are then used to identify bacteria present in infected urine samples. Sensitivity and speed are exemplified. The industrial application described is in the context of the food industry. Three bacteria important in food-borne contamination are differentiated. Here, the focus is on the general ability of the technique obtain the unique data. The three bacteria studied are very difficult to differentiate using the common known methodologies. This chapter concludes with a discussion on the need for, and general structure of, a database/library containing microbial phospholipid profiles.

Experimental

All experiments were performed using a Finnigan (San Jose, CA) TSQ-70B triple stage quadrupole mass spectrometer equipped with a standard Finnigan FAB source and either an Ion-Tech (Teddington, UK) saddle field FAB gun or a JEOL (Boston, MA) MS-009 charge-transfer FAB gun and power supply that has been modified to fit and operate on the TSQ-70B. Spectra were acquired and processed using the Finnigan TSQ-70B data system and software.

Infected urine samples were obtained from Dr. John Dyke, Sparrow Hospital, Lansing, Michigan. *Borrelia burgdorferi* strains and *Leptospira interrogans icterohemorrhagiae* were obtained from Dr. Michael Kron, Department of Pathology and Medicine, Michigan State University. The gram positive bacteria were obtained from Dr. Harold Sadoff, Department of Microbiology, Michigan State University. The bacteria responsible for food-borne contamination were obtained from Dr. Edward Richter, Silliker Laboratories of Ohio, Inc. *Legionella pneumophila knox*, *Naegleria 5049*, and the 7 deep-probe subsurface isolates were obtained from Dr. David C. White, Instituted for Applied Microbiology, University of Tennessee. All other microorganisms were obtained from researchers in the National Science Foundation's Center for Microbial Ecology at Michigan State University.

Crude lipid extracts were obtained from the microorganisms by a modified Bligh-Dyer procedure [6]. One ml of a cell suspension, or a

transfer loop full of sample, was placed in a clean, sterile 25-ml sample vial to which 15 ml of a 2:1 methanol:chloroform mixture was added. After the lipids were extracted, enough distilled/deionized water was added to separate the chloroform from the $MeOH/H_2O$. The chloroform layer, containing the lipids, was drawn off, evaporated to dryness, and the remaining lipids reconstituted in 1 ml of chloroform.

For the analysis, 3-5 μl of the chloroform solution was placed on the probe tip and dissolved in a drop of nitrobenzyl alcohol. Xenon was used as the FAB gas and the FAB gun was operated with a filament current of 10 mA and a xenon beam energy of 8 keV.

Neutral loss and parent ion spectra were obtained in the positive ion mode with no CID gas added to the center quadrupole other than the residual gas remaining in the analyzer manifold. The fragmentation observed may be predominantly the result of metastable decomposition. Daughter spectra were obtained in the negative ion mode using argon as a collision gas. The collision gas pressure was 0.5 mtorr and the collision energy was 30 eV.

All other experimental procedures, equipment, and conditions are given in the specific sections in which they are discussed in this chapter.

Comparing the Data

The differentiation of the spectra presented in this chapter is based on an acquired familiarity with the similarities and differences that occur among the patterns that characterize different microorganisms. An appreciation of which differences constitute the normal variance for a single organism, and which are valuable in distinguishing organisms, has been obtained through the observation of many spectra. Before this chapter proceeds, it is important to discuss the inferred significance of the observed features and differences and the rationale behind these conclusions.

Differences among the spectra obtained from replicates of the same microbial species can be due to a number of factors. The foremost causes of spectral variance are small differences in the age and growing conditions of the cultures. Since microbes present a dynamic living system, small variances in their chemical composition can be expected between replicate cultures. Small spectral variances also can occur in the day to day operation of the instrument. As long as these spectral variances remain within certain predescribed limits, they can be taken into account when matching spectra.

Now it becomes important to decide in what ways, and to what extent, spectra may vary and still be considered identical. Observations of the spectra obtained from the microorganisms used in this research have led to some conclusions on how these spectra differ

among organisms. First, many organisms differ in the classes of phospholipid they contain. This leads to an unambiguous identification regardless of how much data they have in common. Likewise, many organisms differ in their content of individual phospholipid species within each class. This also leads to an unambiguous identification. The gray area arises when two or more microorganisms contain the same phospholipid classes and the same phospholipid species within each class. Here, identification is based solely on differences in intensities of the phospholipid species. In this research, the base peak(s) is considered most important and, as such, is considered invariant for a given species. The overall pattern of the phospholipid peak intensities is the next important criterion. If the pattern of relative intensities differs to a large extent, the spectra are considered different. Next, individual peaks may vary within predescribed "zones". These zones are defined as major peaks (75% to 90% of the base peak), intermediate peaks (20% to 75% of the base peak), and minor peaks (less than 20% of the base peak).

At this time, pattern recognition is done crudely; all matching is performed by eye on limited sets of data. However, this work clearly shows that distinct differences in phospholipid content and distribution do exist among the microorganisms studied so far. Recognition of these differences, and identification of the similarities, by the "human pattern recognition method" has not presented any difficulties, or even near-ambiguities, for the limited data set available. Given the large number of distinguishing parameters available in the complete phospholipid profiles, it is reasonable to assume that

computer techniques can be developed to recognize these patterns on a broader scale.

For an automated pattern recognition method to succeed, it will have to determine those criteria that are essential for judging spectra. In addition, such a method will need to identify and quantitate the variations that can be tolerated for a correct match. The database acquired so far is a limited one; this technique needs to be applied to many more samples to determine which features and differences are most important for taxonomic distinction. In this way, the pattern recognition can be refined and optimized.

Microorganisms Analyzed

To date, about 50 separate microbial species have been characterized using this technique. A general listing of these microorganisms is given in Table 5.1. Where known, the strain(s) of the particular species is also given. The cricket gut isolates, Savannah River isolates and fungi isolates are presently unidentified species and form the basis of three ongoing projects to compare different analysis techniques. The Savannah River isolates were obtained from a deep-well drilling probe at the Savannah River nuclear site and are part of a collaborative study with Dr. David C. White at the Institute of Applied Microbiology, University of Tennessee and Dr. David Stahl at the University of Illinois. The cricket gut and fungi isolates are part of collaborative studies with Dr. Mike Klug and Dr. Pete Stahl at the Kellogg Biological Station, Michigan State University.

Table 5.1 Listing of Microorganisms Characterized by this Technique

BACTERIA

Gram (-) Bacteria

Pseudomonas fluorescens ARC 33512 Biotype II

Pseudomonas fluorescens Aruna AK-15

Pseudomonas fluorescens Janet B8-1

Pseudomonas aeruginosa - many strains

Pseudomonas aureofaciens

Escherichia coli - HB101, and many other strains

Alcaligenes faecalis ATCC 15554

Alcaligenes eutrophus - many strains

Citrobacter freundii - many strains

Salmonella abaetetuba

Klebsiella pneumophila - many strains

Klebsiella oxytoca - many strains

Morganella morganii

Proteus vulgaris

Borrelia Burgdorferi - many strains

Leptospira interrogans ecterohemorrhagiae

Legionella pneumophila knox

2 Cytophagus species

Gram (+) Bacteria

Corynebacterium nephridii ATCC 11425

Bacillus subtilis

Bacillus megaterium

Bacillus cereus

Mycobacterium phlie

Leuconostoc mesenteroides

Streptococcus faecalis

Table 5.1 cont.

5 Cricket Gut Bacterial Isolates from Kellogg Biological Station

Raff-4	Possible Shigella sp.
M/L-3	Possible Citrobacter sp.
AD-25	Unknown
Bac-4	Possible Citrobacter sp.
LBG-2	Possible Shigella sp.

7 Bacterial Isolates from Deep-Well Drilling at Savannah River

C484	C564
C553	C678
C570	C651
C528	

FUNGI

8 Isolates from Kellogg Biological Station

101-4 B&D	101-3	101-6
112-1 B&D	113-2	113-2 B&D
138-1	106-1	

AMOEBAE

Naegleria 5049

Detection and Identification of Urinary Tract Infections

The urinary tract is the most common site of infection during hospitalization, and is considered infected when bacterial counts of $1x10^5$ cells/ml are obtained from properly collected urine specimens [7]. Most infected urine contains only a single species of bacteria; at most, only two or three species may be present. In the uninfected state, urine is sterile and contains no microbes. For these reasons, urinary tract infections presented the least complex samples and were chosen as the first real-life clinical problem in which to apply this technique.

The normal procedure in identifying a urinary tract infectant is the use of the selective agar pure culture assay described in Chapter 1. This assay requires 48 hours for isolation and incubation of the infecting microbe, and is vulnerable to the inaccuracies and uncertainties inherent to these tests. While urinary tract infections may not of themselves be life threatening, in their advanced stages they can be quite painful and serve as a source of invasion of the bloodstream. This can lead to renal damage, infection of other tissues, and endotoxic shock. A rapid and sensitive identification procedure would help to avoid such problems by quickly detecting the presence, and identifying the causative agent, of an infection in its earliest stages.

The experimental procedure developed for extracting the urine samples is rapid and simple. The urine samples are centrifuged at 1800 rpm for 3 minutes to spin the cells down to the bottom of the

sample vial used to collect the urine. The urine is then carefully drawn off from the top until about 1 ml of urine remains in the vial. This remaining 1 ml contains most of the cells from the 5 ml of urine originally present in the sample vial. The sample is then extracted using the procedure described in the experimental section of this chapter.

The first attempts in analyzing urine samples met with failure. In these experiments, American Type Culture Collection (ATCC) cultures of bacteria were used as the standards with which to compare the bacteria obtained from the urine samples. The first problem encountered involved the urine samples themselves. Infected urine can contain many more cells from sources other than just those from the bacteria causing the infection. In addition to the bacteria, cells from blood and urethra sloughage may also be present. These additional cells can comprise the majority of the cells present in the urine and lead to very intense interferences in the analysis. As a result, none of the urine spectra were similar to the standard spectra.

This problem was overcome through the use of 10 μm Acrodisc syringe filters obtained from Gelman Sciences (Ann Arbor, MI). Since the bacteria responsible for urinary tract infections have cell dimensions smaller than 10 μm, and the eukaryotic cells associated with the human host have cell dimensions greater than 10 μm, filtering the urine through 10-μm filters prior to extraction removed all of the non-infectant cell matter from the urine. The resulting samples were then centrifuged and extracted as previously described.

Filtering the samples worked so well it uncovered a second problem hidden in these experiments. A hospital environment presents to bacteria many selective pressures that can cause the occurrence of new strains that overcome these pressures. Specifically, the widespread use of antibiotics in a hospital often results in bacterial strains that are resistant to those antibiotics that were previously effective. Therefore, the specific strain of bacteria responsible for a urinary tract infection in a particular hospital is not necessarily the same as the ATCC strain used as a standard. The experiments described here clearly indicate the importance of the standards upon which the identification is based.

The spectra obtained after filtering the urine were still not identifiable as any of the ATCC species used as standards. Since this problem was still thought to be a result of the sampling procedure, bacteria, from urine samples previously identified by the hospital, were cultured on trypticase soy agar (TSA) plates at 37^{o} C for 24 hours in an attempt to discover the differences due to the sampling problem. Single colonies were removed from these plates, transferred to a second series of TSA plates, and grown at 37^{o} C for another 24 hours. These bacteria were harvested, extracted, and analyzed. The spectra obtained compared reasonably well with those spectra obtained from the initial filtered urine extract. Examples of phosphatidylethanolamine mass profiles obtained from an ATCC strain of *Pseudomonas aeruginosa*, a filtered urine sample infected with *Pseudomonas aeruginosa*, and a culture from the same urine sample

after two passes of growing on TSA are shown in Figure 5.1. The noteworthy points in these spectra are the increased intensities of the peaks at m/z 704 and m/z 744, in the bottom two spectra, over those in the top spectrum. Also, a peak at m/z 732 occurs only in the bottom two spectra. The large differences in the spectra between the ATCC strain and the strain grown on two passes through TSA lends strong evidence to the existence in the hospital of a different strain of *Pseudomonas aeruginosa*. The similarity between the filtered urine spectra and the spectra obtained from the bacteria grown on TSA is corroboration that the sampling procedure is sound; the filters are indeed filtering away interfering cell matter. The differences seen in these two spectra could be the result of differences in the sample histories. The growth phase and conditions of the bacteria collected straight from the urine are different than the rich conditions offered those isolated and cultured on TSA. Just how much these spectra will be allowed to vary, and still be considered to belong to the same species of organism, requires further research on many more samples.

The next step in developing these experiments was to use bacteria obtained from actual infected urine samples - ones in which the infecting bacteria had been previously identified by the hospital - as the known standards with which to compare the unknowns. This approach has the advantage of using the same strains of bacteria for the standards as those occurring in the urinary tract infections. Examples of phosphatidylethanolamine mass profiles for *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Citrobacter freundii*, and *Morganella Morganii* are shown in

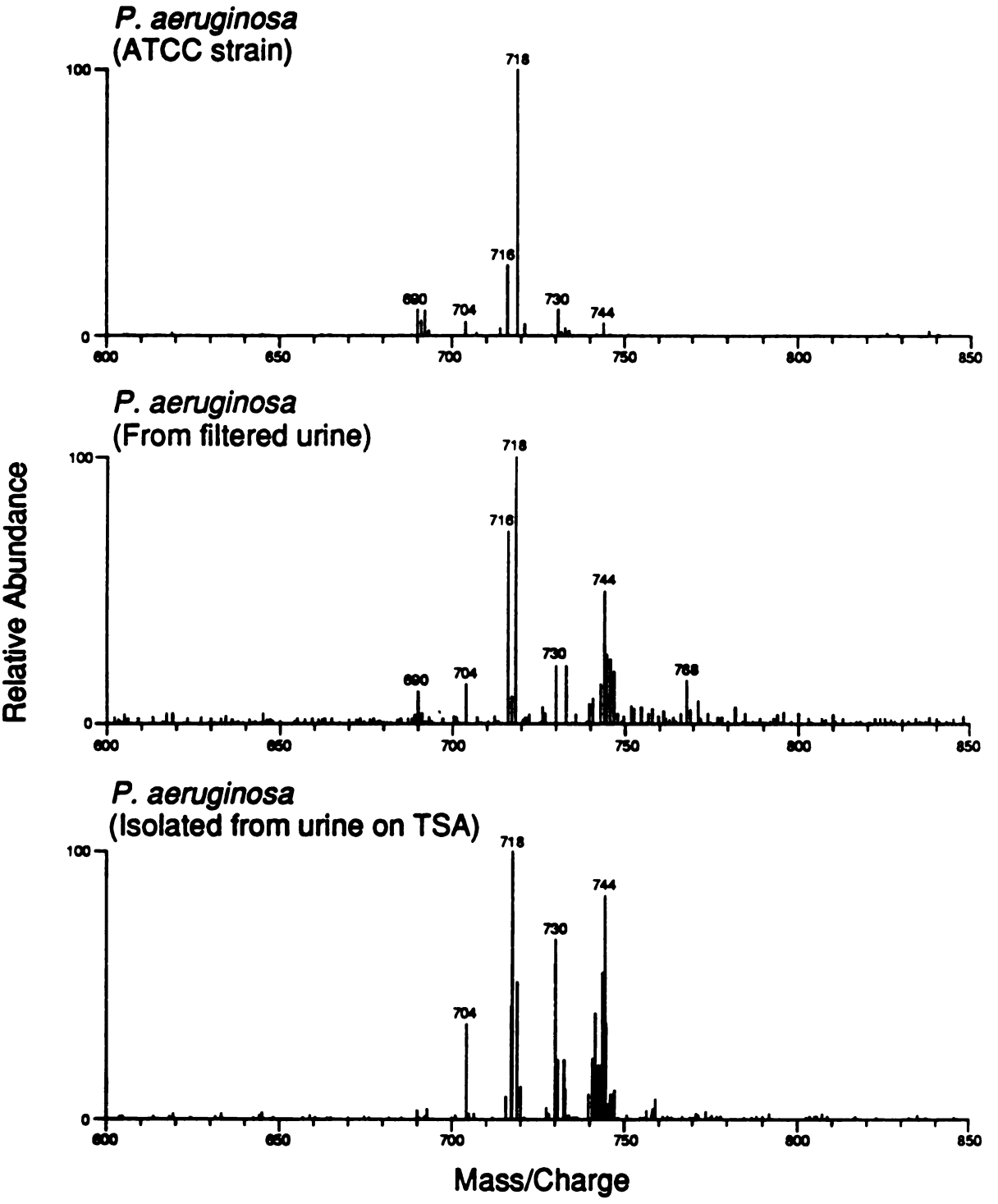

Figure 5.1 Phosphatidylethanolamine mass profiles from three different isolates of *Pseudomonas aeruginosa*.

Figure 5.2. These small pieces of the complete profiles only represent some of the differences among the bacteria; the complete information for these six bacteria takes up considerable space and for this reason has been excluded from this thesis.

A phosphatidylethanolamine mass profile from an infected urine sample in which the causative bacteria was unknown is shown in Figure 5.3. Comparing this part of the data space with the corresponding part for the six bacteria in Figure 5.2, the unknown spectrum most closely resembled that from the standard of *P. aeruginosa*. The identification was based on the occurrence of the peaks at m/z 716 and m/z 718 as the two most intense peaks in the spectrum, coupled with the peak at m/z 744 as the next highest in intensity. Either of the peaks at m/z 716 or m/z 718 could be the base peak, but the other should be within 75% of the base peak. In addition, the lack of a peak at m/z 664 was unique to the spectrum obtained from the *P. aeruginosa* standard. *P. aeruginosa* was the only standard whose phosphatidylethanolamine *(class)mass profile* contained this pattern of peaks. This was, in fact, the organism responsible for the patient's urinary tract infection. It must be pointed out that the possibility exists that individual strains could differ from one hospital to the next. In routine application, standards would need to be run that were specific for each particular hospital. In addition, these standards would need to be checked on a routine basis to assure their integrity against the occurrence of new strains. The data presented here are the results of a preliminary study. Many replicates of each species need to be performed so that the

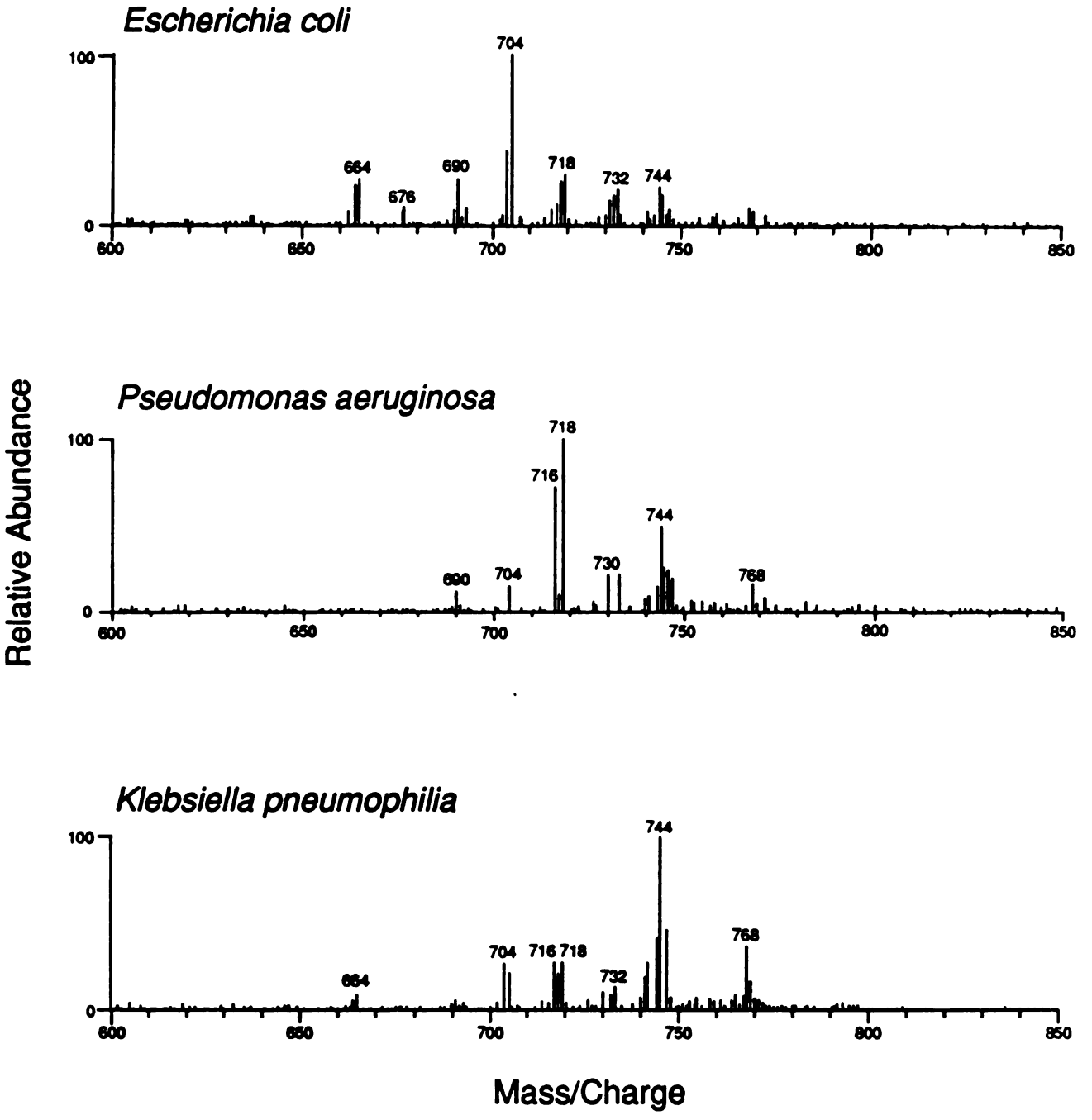

Figure 5.2 Phosphatidylethanolamine mass profiles from six bacterial species known to cause urinary tract infections.

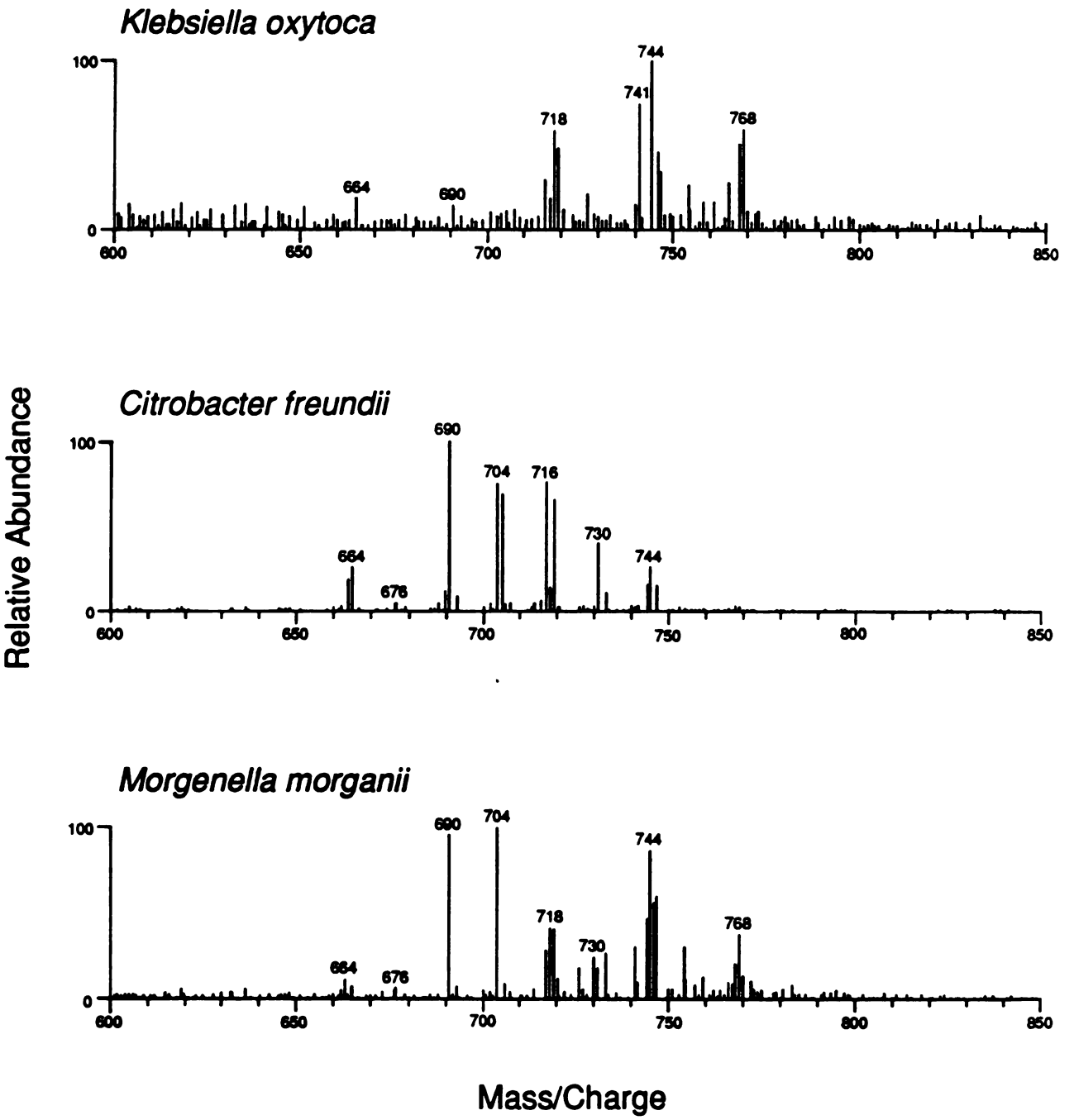

Figure 5.2 cont.

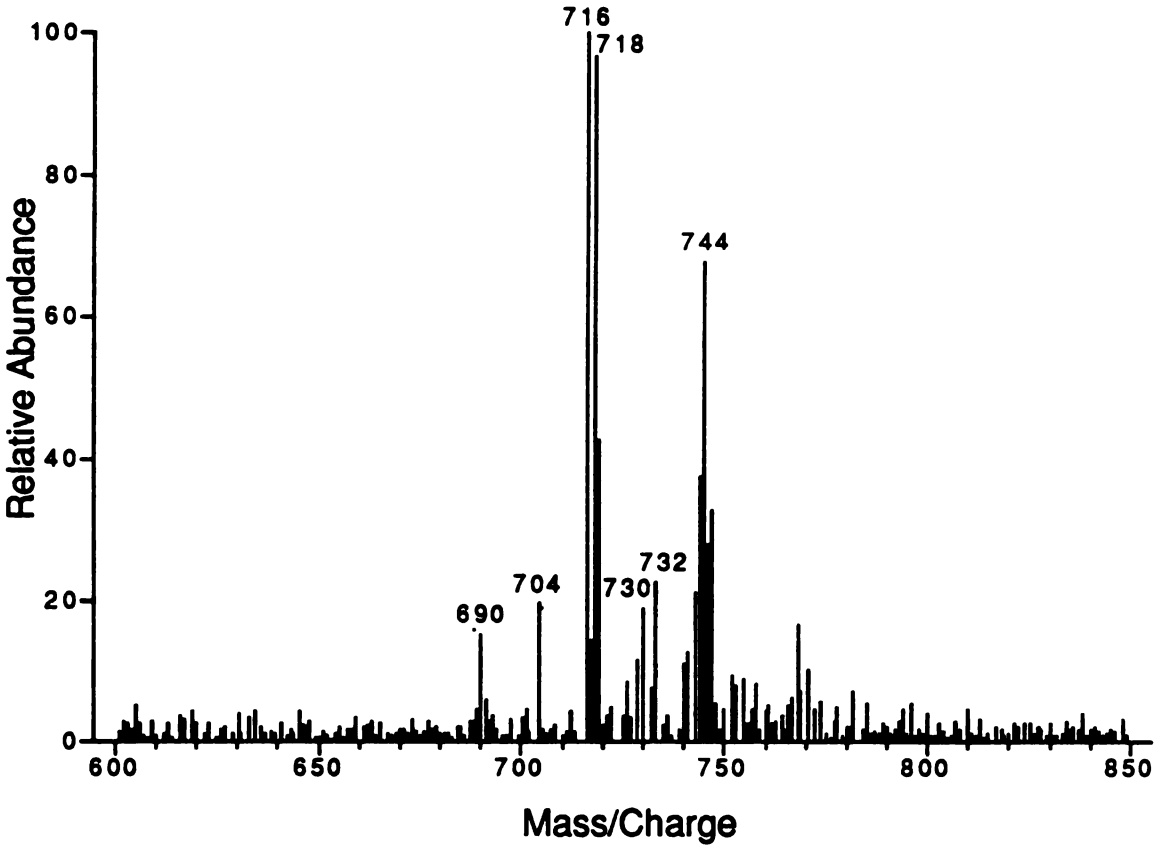

Figure 5.3 Phosphatidylethanolamine mass profile from an infected urine sample.

occurrence of different strains can be monitored. In addition, mathematical data analysis techniques need to be developed for the identification of co-infecting bacteria.

Differentiation of Food-Borne Contaminants

Salmonella species are very difficult to correctly differentiate from *Escherichia* species using common microbial techniques. Escherichia and Salmonella have up to 50 percent of their DNA sequences in common [8]. In the food testing industry, this presents a formidable problem. Since *Escherichia* are rarely pathogenic to human beings, government regulations will allow food products to contain some *Escherichia*. In contrast, *Salmonella* are usually pathogenic to human beings; government regulations do not allow the presence of *Salmonella* in foods. The methodology currently used to differentiate *Salmonella* from *Escherichia* involves complex immunological assays that take up to 7 days for results. This presents other problems: the cost of storage and the spoilage incurred can be very expensive. Also, the implications of time and expense in discovering a *Salmonella* infection 7 days downstream of a production process could be enormous. The food industry is very interested in shortening the time period needed to obtain results. Research has begun in this laboratory toward a solution to this problem.

The *Salmonella abaetetuba*, *Escherichia coli*, and *Citrobacter freundii* used in this study were grown at 37^{0} C for 48 hours in both tetrathionate broth, containing potassium iodide and brilliant green, and trypticase soy agar plates. Tetrathionate broth is partially

selective for Salmonella, and is the medium used for this type of testing in industry. Other bacteria, such as the *E. coli* and *C. freundii* used in this study, also can grow in this medium, but they do not grow as easily or as fast as *Salmonella*. *Citrobacter freundii* is another microbe of importance to the food industry. Like *E. coli*, it also is usually non-pathogenic to humans and can be difficult to differentiate from *Salmonella*.

The phosphatidylethanolamine mass profiles for the three bacteria grown in the tetrathionate broth are presented in Figure 5.4. As shown by the patterns obtained, the three bacteria are easily differentiated from each other. Again, for the sake of brevity only enough information needed to make this point is presented. The total data space provides the unique and characteristic information set for each microorganism. A vial of previously sterile tetrathionate broth was received that had become contaminated with one of the organisms. This contaminant was readily identified as *Salmonella abaetetuba* as shown by the phosphatidylethanolamine mass profiles in Figure 5.5. Comparing the spectra, the spectrum from the unknown most closely resembles that from *S. abaetetuba*, and differs greatly from those of *E. coli* and *C. freundii*

The results obtained from the organisms grown on TSA plates were very interesting. The phosphatidylethanolamine mass profiles are shown in Figure 5.6. While *C. freundii* is easily differentiated from the other two bacteria, the phospholipid *(class)mass profiles* of *E. coli* and *S. abaetetuba* are nearly identical. These results illustrate the

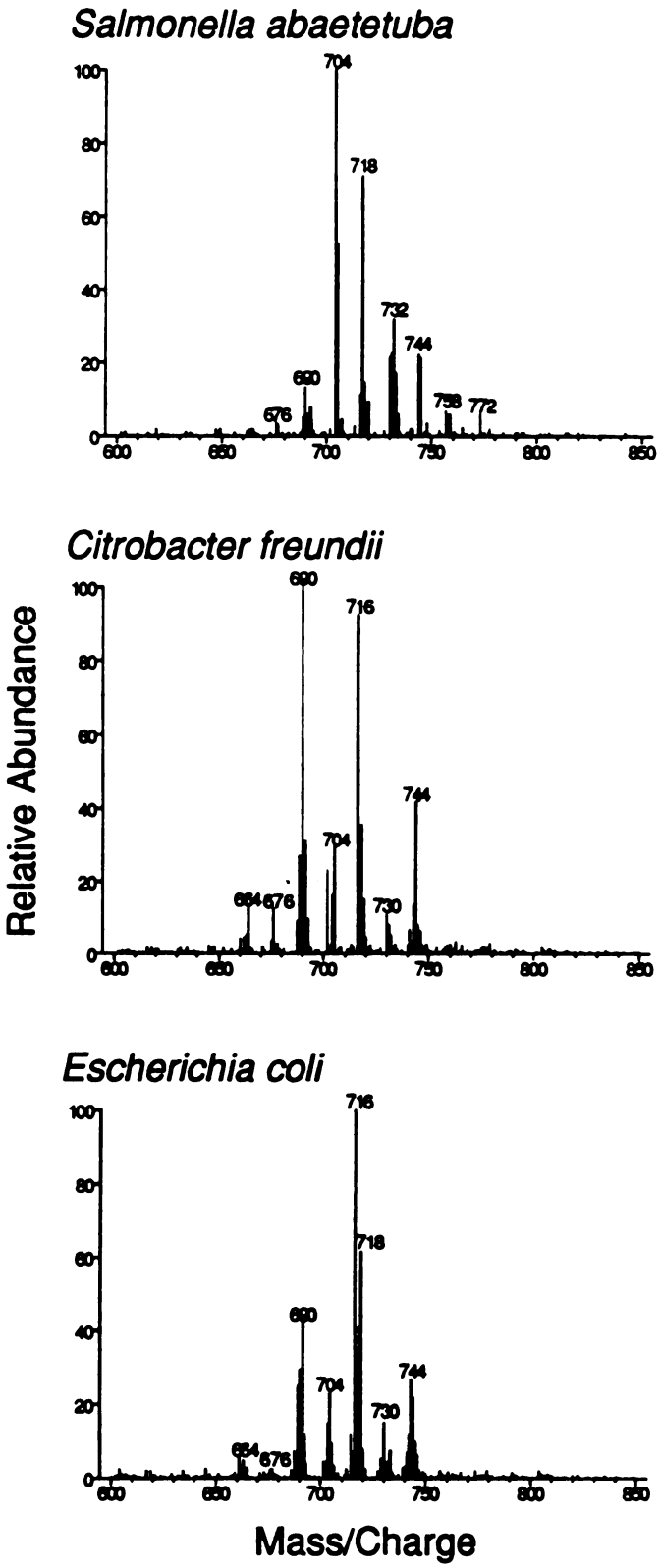

Figure 5.4 Phosphatidylethanolamine mass profiles from three bacteria, grown in tetrathionate broth, important to the food industry.

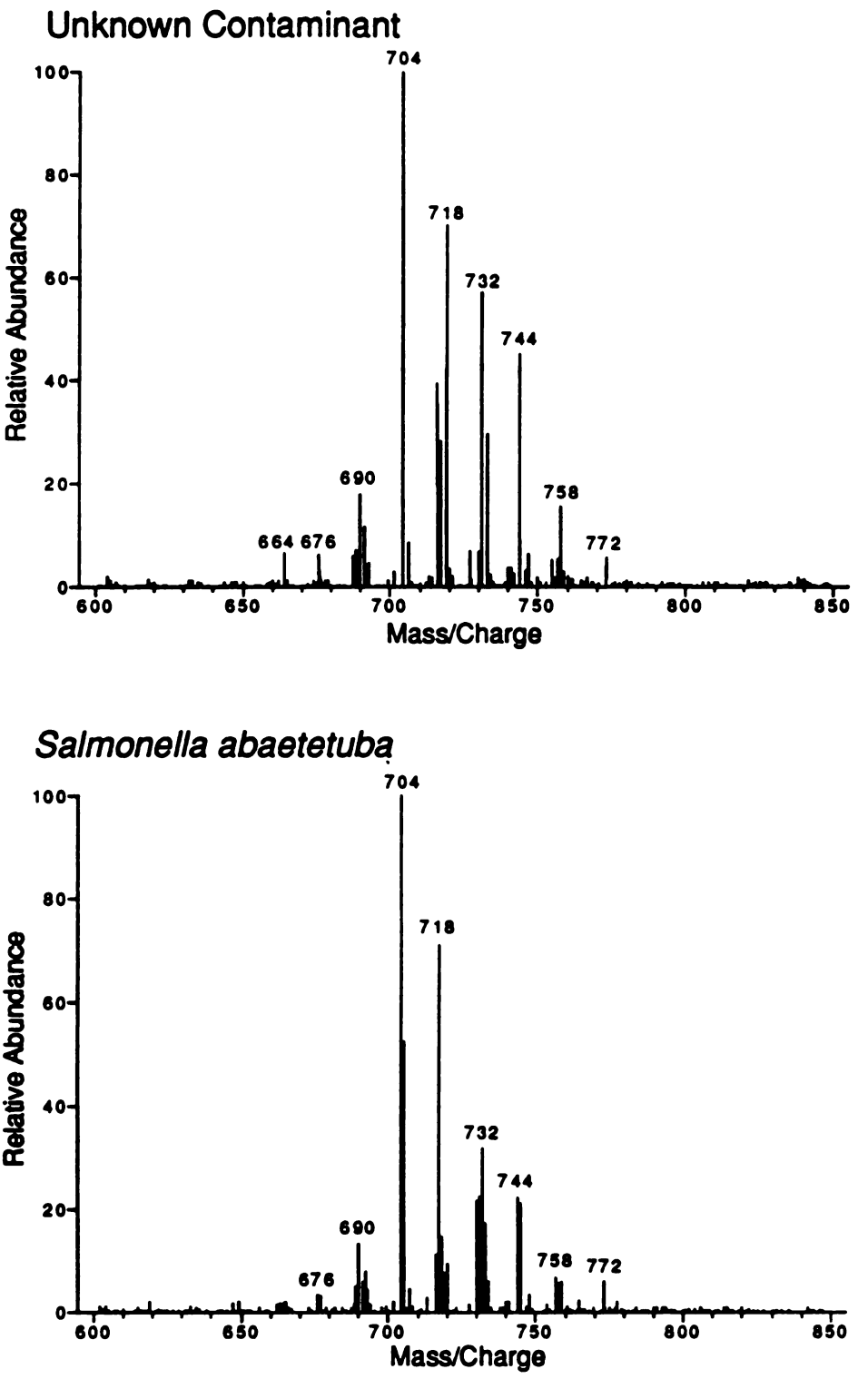

Figure 5.5 Phosphatidylethanolamine mass profiles from an unknown contaminant and *Salmonella abaetetuba*.

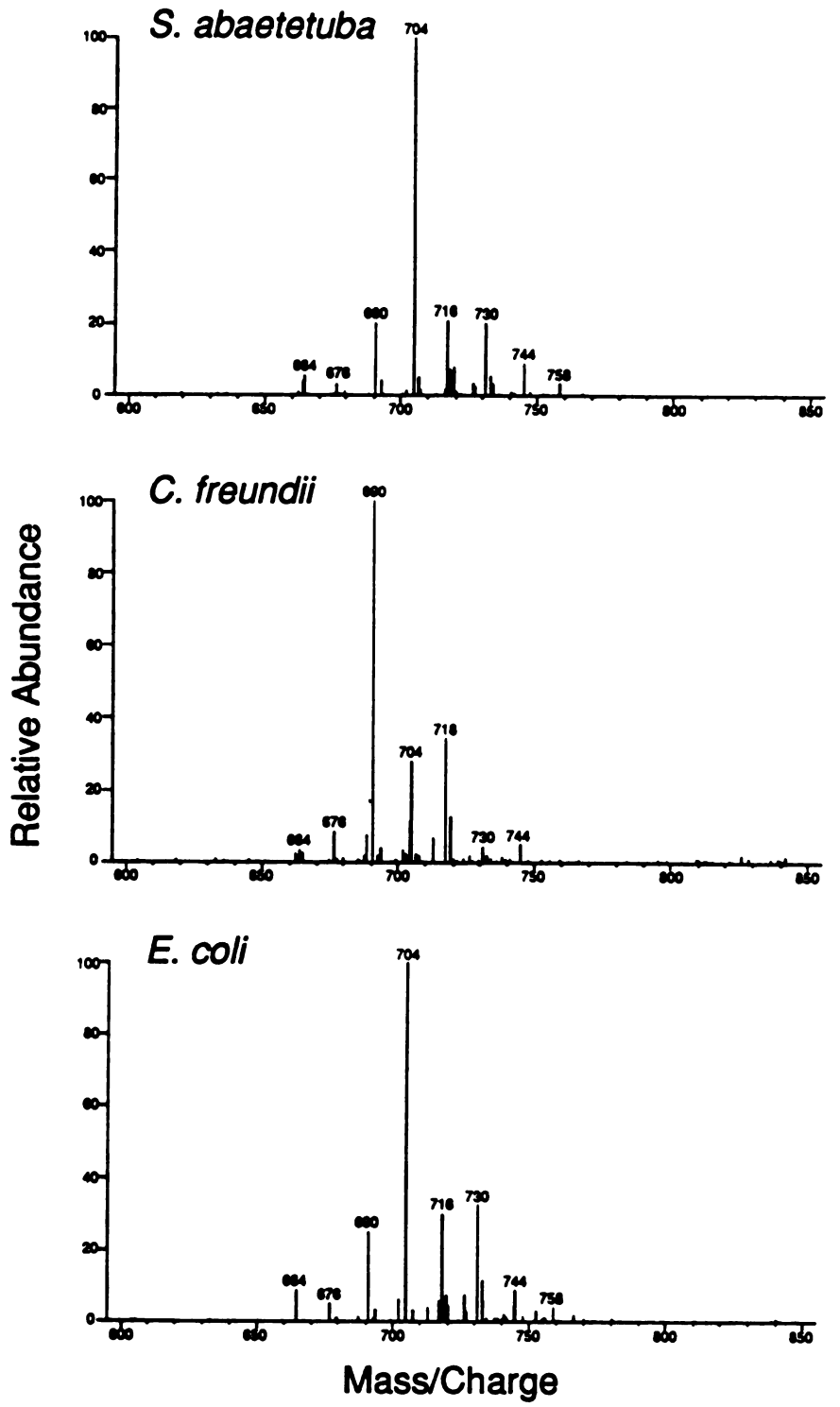

Figure 5.6 Phosphatidylethanolamine mass profiles from three bacteria, grown on trypticase soy agar, important to the food industry.

importance of the role the growth medium can play. Tetrathionate broth puts a selective pressure on *Escherichia* and *Citrobacter*, while *Salmonella* grows freely. This may account for the differences in the membrane phospholipid content. In contrast, all three organisms grow well on TSA. Comparing Figure 5.6 to Figure 5.5, *Salmonella* shows only small differences between the two growth media. These differences remain small throughout the entire data space. On the other hand, *E. coli* and *C. freundii* give quite different *(class)mass profiles* between the two media. No new phospholipid classes occur with the different media, rather the variations are in the form of differences between the relative abundances of phospholipid species within particular classes. Experiments of this type may be a potential means of exploring how microorganisms respond to stress. Also, the possibility is raised of subjecting very similar organisms to stressful growth conditions in order to obtain more distinctive phospholipid profiles.

Database / Library of Microbial Profiles

On a limited scale, comparison and matching of spectra among a small number of microorganisms can be done using the human brain for pattern recognition. For this technique to be useful on a large scale, however, computerized methods must be developed for storing these data, and for searching the resulting database. Figure 3.3 in Chapter 3 is a visual representation of how this database might look in its simplest form.

The top level of the database would contain the phospholipid class quantitative data. These data consist of the relative abundances of the phospholipid classes contained in each microorganism. The next level contains the phospholipid *(class)mass profiles* segregated by class. The third level would contain the *(class)(fatty acid) formula data*. Because the complete data set may not always be needed for an identification, the search would be performed from the top level down and continue until a positive match had been made. At the *(class)mass profile* level, some type of intelligence will be needed to accommodate small differences in the relative abundances of individual phospholipid species between the unknown profile and those contained in the database. As discussed earlier in this chapter, these differences can be due to many factors, including instrumental parameters and small differences in the age and growing conditions of the microbes. Identification based on matching of the *(class)(fatty acid) formula data* should be relatively straight forward, and not require any additional intelligence.

This data base structure will need to be flexible and relational among a variety of spectral and microbial characteristics. In some cases, certain characteristics might correlate to a subset of the total number of microorganisms whose profiles are contained in the database. These characteristics could include the presence or absence of a particular phospholipid class, the occurrence of a certain combination of phospholipid classes, the presence of a particular phospholipid species, or any combination of the above. This, by no

means, is a complete list of all possible correlating characteristics that might be found in such a database.

Each of these characteristics would point to the subset of microorganisms that contains them. Searching for these characteristics first would limit the complete phospholipid profile search to a subset of microorganisms that share correlations. These subsets could be as broad as a whole phylum, or as narrow as a subset that contains different strains of the same species. In this manner, portions of the data space that might have had limited usefulness for identification because they were shared by many microorganisms in the total database, will become more useful through limiting the search to a particular subset of microorganisms. As these correlations are discovered, the database must be able to easily accommodate their use. In addition, routines should be developed to search the database for correlations that are not immediately obvious.

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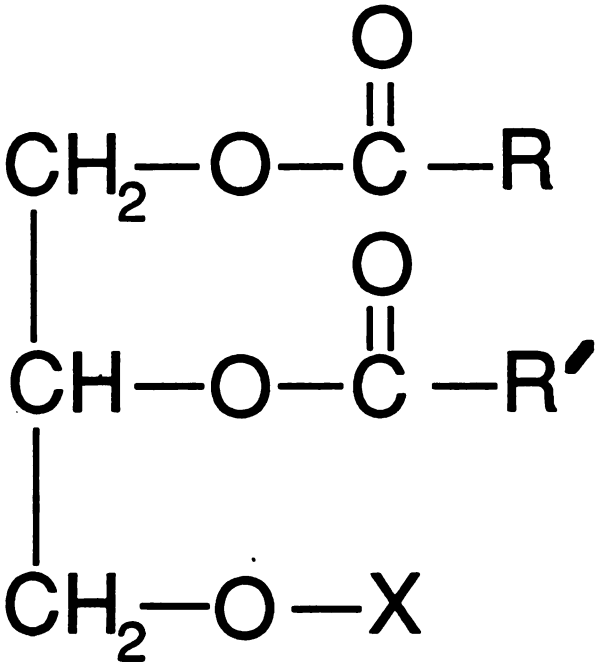
CHAPTER 6

Applications of Other Biomarker Analyses to the Detection and Identification of Microorganisms

Introduction and Background

In the previous chapters, the focus of the research presented in this thesis has been on microbial phospholipids. However, many other types of lipids exist that are useful biomarkers for detection and identification of microorganisms. In combination with the phospholipids, these lipids can provide an even greater level of distinction on which to base a microbial identification. A preliminary exploration of two other classes of lipids, glycolipids and sterols, is presented here. Glycolipids contribute substantially to the total lipid content of algae and Gram-positive bacteria [1], while sterols can be used for fungal detection [1] and identification [2,3].

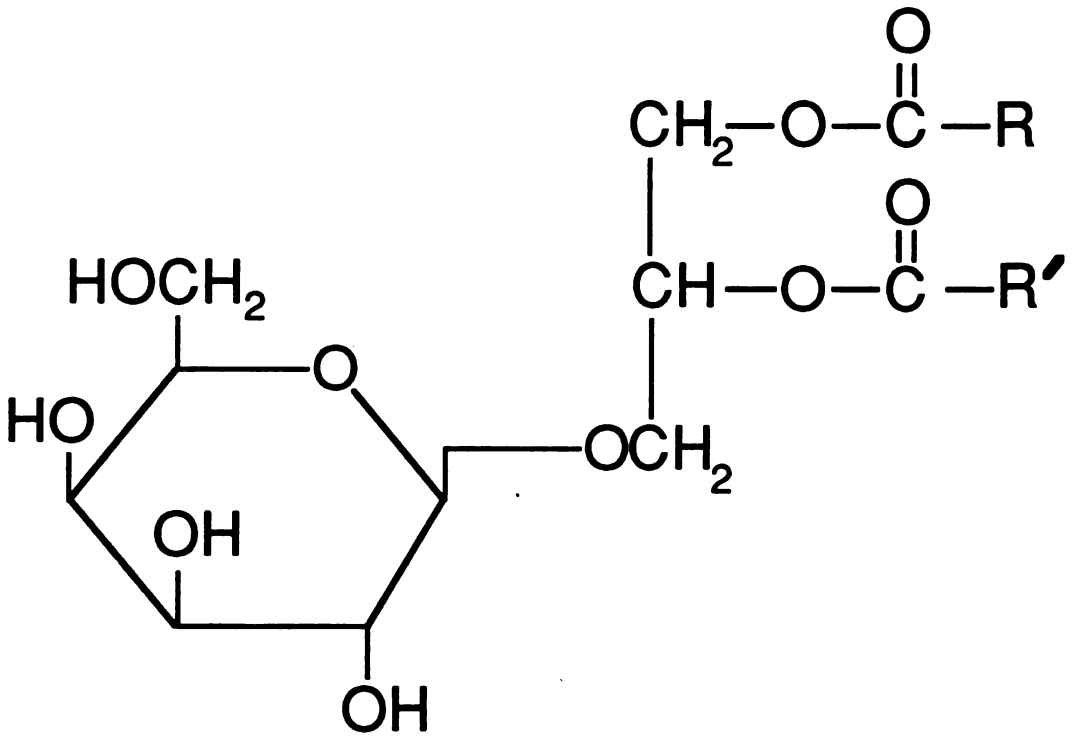
The glycolipids investigated in this research are the glycosyldiacylglycerols (Structure 6.1). The general term "glycolipid" will be used in this chapter when referring to glycosyldiacylglycerols. Like the phospholipids, glycosyldiacylglycerols consist of four primary functional groups: a glycerol-3-phosphate core on which two fatty acids (R, R') have been esterified to the two free hydroxyl groups in the sn-1 and sn-2 positions, and a sugar moiety (X) attached to the



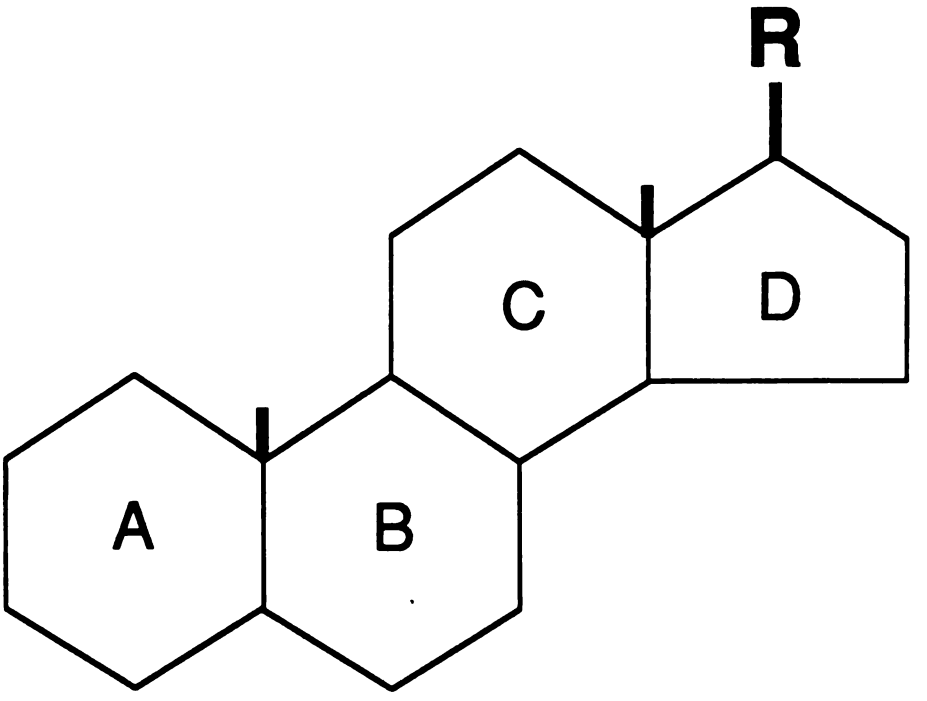
Structure 6.1 General glycosyldiacylglycerol structure.

sn-3 position. An example of the glycolipid monogalactosyldiacylglycerol is shown in Structure 6.2. The sterols studied in this research have the general structure shown in Structure 6.3. This structure consists of 4 rings (A, B, C, D), with an alkyl group (R) attached to ring "D". Many different configurations of sterols are possible through unsaturation, hydroxylation, and methylation of the ring system, and differences in the attached alkyl groups. An example of the most common fungal sterol, ergosterol, is given in Structure 6.4.

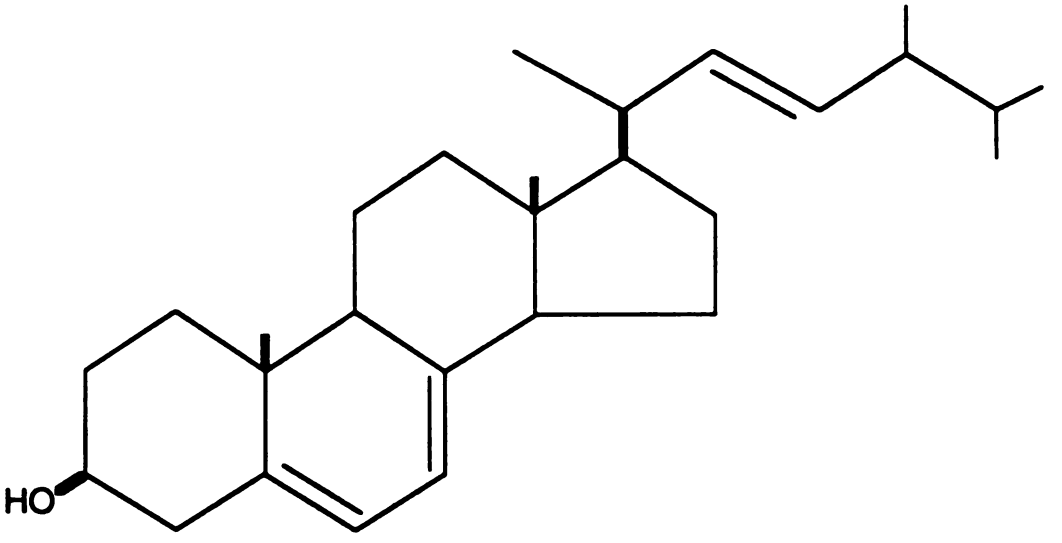
For glycolipids, structural information is obtained using the same methodology as for the phospholipids. This information can be obtained with the same sample, and in the same analysis, as the phospholipids. The sterol information is obtained by electron impact ionization, off a heated direct exposure probe (DEP), using parent ion scans. Sterols also are contained in the same extract as that for the phospholipids. This chapter describes the tandem mass spectrometry methodology developed, along with the type of information obtained, for characterizing the content and structures of glycolipids and sterols in crude microbial extracts. This information will, ultimately, be used in combination with the phospholipid information toward the detection and identification of microorganisms. In this chapter, the thesis is concluded with a discussion on other potential lipid biomarkers and their possible applications.



Structure 6.2 Monogalactosyldiacylglycerol



Structure 6.3 General sterol structure.



Structure 6.4 Ergosterol

Experimental

All experiments were performed using a Finnigan (San Jose, CA) TSQ-70B triple stage quadrupole instrument equipped with a standard Finnigan EI/FAB source, a Finnigan direct exposure probe and a JEOL (Boston, MA) MS-009 charge-transfer FAB gun and power supply that has been modified to fit and operate on the TSQ-70B. Spectra were acquired and processed using the Finnigan TSQ-70B data system and software.

All bacteria and fungi, except *Legionella pneumophila knox*, were obtained from researchers in the National Science Foundation's Center for Microbial Ecology at Michigan State University. *Legionella pneumophila knox* was obtained from Dr. David C. White at the Institute for Applied Microbiology, University of Tennessee. Cultures were grown on trypticase soy agar plates at 37° C for twenty four hours prior to harvesting.

Crude lipid extracts, containing both the glycolipids and sterols, were obtained from the bacteria by a modified Bligh-Dyer procedure [4]. One ml of a cell suspension, or a transfer loop full of bacteria, was placed in a clean, sterile 25-ml sample vial to which 15 ml of a 2:1 methanol:chloroform mixture was added. After the lipids were extracted, enough DDI water was added to separate the chloroform from the MeOH/H_2O. The chloroform layer, containing the lipids, was drawn off and evaporated to dryness. The samples were then reconstituted in 1 ml of chloroform for analysis.

For the FAB analysis of glycolipids, 3 to 5 μl of the chloroform solution was placed on the probe tip and dissolved in a drop of nitrobenzyl alcohol. Xenon was used as the FAB gas and the FAB gun was operated with a filament current of 10 mA and a xenon beam energy of 8 keV. For sterol analysis using the DEP, 3 to 5 μl of the chloroform solution was placed on the probe filament, and the chloroform was allowed to evaporate. For analysis, the DEP filament was ballistically ramped to 250 mA, which corresponds to 250° C, and held at that temperature for the length of the analysis. Electron impact ionization was performed with a source filament current of 200 mA and an electron energy of 70 eV.

For the glycolipid analysis, neutral loss spectra were obtained in the positive ion mode with no CID gas added to the center quadrupole other than the residual gas remaining in the analyzer manifold. The fragmentation observed may be predominantly the result of metastable decomposition. Daughter spectra were obtained in the negative ion mode using argon as a collision gas. The collision gas pressure was 0.5 mtorr and the collision energy was 30 eV. For the sterol analysis, parent spectra were obtained in the positive ion mode using argon as a collision gas. Collision gas pressure was 0.5 mtorr and collision energy was 10 eV.

Glycolipids

In addition to sharing structural similarities with the phospholipids, glycolipids also undergo collisionally induced dissociation to fragment in a manner similar to the phospholipids. During CID of the protonated molecule, glycolipids fragment to lose their sugar moiety as a neutral loss, while the rest of the ion retains the charge. In the negative ion mode, one or the other fatty acid is cleaved from the molecular anion and retains the charge, while the rest of the ion is lost as a neutral.

Because these fragmentation patterns are identical to those for the phospholipids, the same characterization scheme used for the phospholipids can be applied to the glycolipids. Positive ion neutral loss scans, specific for the loss of each possible sugar moiety, are first performed. These scans identify the classes of glycolipids present in the sample. An example of a neutral loss scan of 252 u, specific for monogalactosyldiacylglycerol, from *Legionella pneumophila knox* is shown in Figure 6.1. To obtain the fatty acid information, the negative ion daughter spectra can be collected for each of the glycolipid species detected in the neutral loss scans. The daughter ions present in these scans would represent the fatty acids contained on the glycolipids. The neutral loss and daughter scans can be added, along side those for the phospholipids, to the automated instrument control procedure that collects the data. In this manner, both the glycolipid

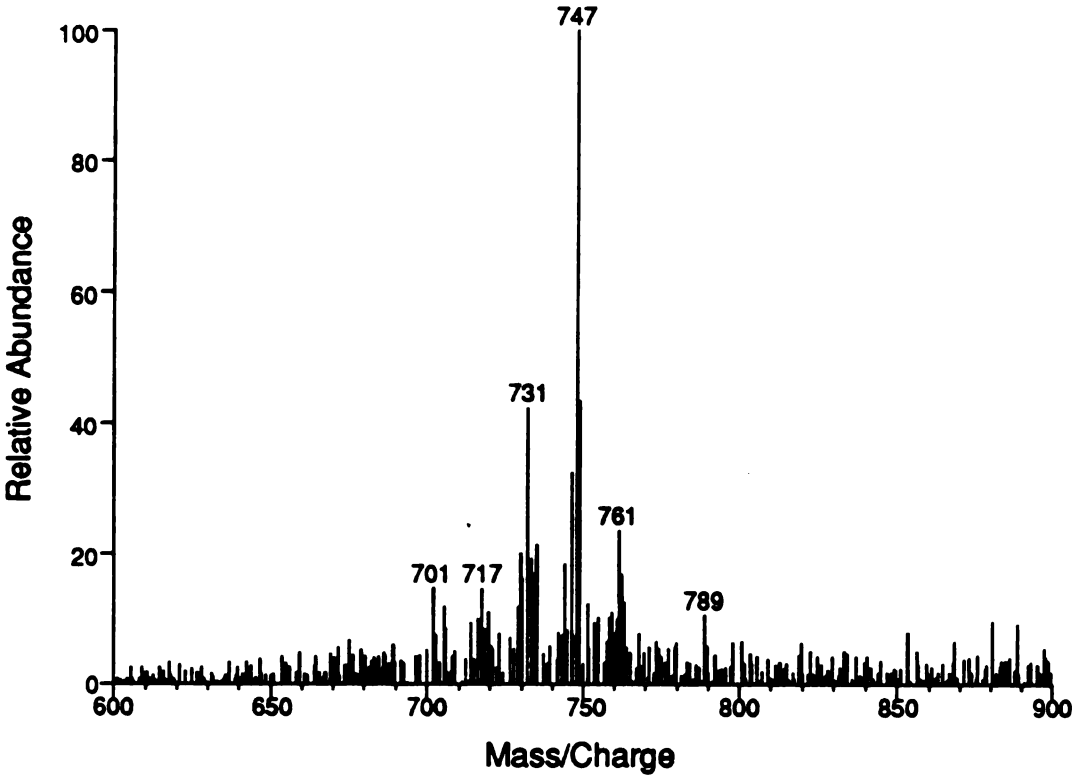


Figure 6.1 Neutral loss of 252 u, specific for monogalactosyl-diacylglycerol, from *Legionella pneumophila knox*.

and phospholipid data may be collected from the same sample, and in the same analysis.

Sterols

Sterols are major lipid components of fungi and can be used for detection and identification of fungal species. Ergosterol is present in almost all species of fungi, and is not present in other microorganisms [1]. Therefore, the presence of ergosterol can be used as a biomarker for the detection of fungi in a sample. The content and distribution of the other sterols have been found to be unique to each particular species [2,3], and can be used in much the same way as phospholipids for species identification. This provides much needed information for fungi, since this research has found that the phospholipid and fatty acid content does not vary as widely among fungal species as it does among other species of microorganisms.

The volatility of sterols is relatively high compared to that of phospholipids, and is well within the range of a heated inlet probe. Since FAB produces a considerable amount of chemical background, which can interfere with an analysis, the heated direct exposure probe was chosen, along with electron impact ionization. This configuration has the advantage of producing cleaner spectra, but it also has the disadvantage of a more rapid loss of sample from the probe. Electron impact ionization (EI) was initially chosen to study ergosterol. Ergosterol contains conjugated double bonds in the B ring that cause the $M^{+\bullet}$ radical cation to predominate over the $[M+H]^+$ protonated

molecule in the FAB spectrum [5]. Therefore, the assumption was made that chemical ionization (CI) would not offer any advantages over EI for this form of sterol. However, the usefulness of CI for other forms of sterols has yet to be investigated.

Under collisionally induced dissociation, the sterols produced fragmentation patterns that were identical for all of the common sterols. The most characteristic fragmentation was the neutral loss of the hydrocarbon chain attached to ring D (Structure 6.2). Taking advantage of this fragmentation, parent scans are performed that monitor the formation of the fragment ion corresponding to the loss of the alkyl chain from the ring system. The resulting spectrum contains peaks that represent the molecular weights of the sterols present in the original sample.

Since only a few different ring systems are used for the majority of the sterols, parent ion scans can be performed for those fragment ions corresponding to the ring systems; these parent ion scans identify most of the sterols present in the sample. In addition, the sterols are all expected to have the same relative responses to ionization. Therefore, the relative abundances of the peaks present in a spectrum should correlate well with the relative abundances of the sterols in the sample.

Isomers present a problem to this method. Some of the sterols differ only in the position of the double bond within their ring system. These sterols have identical molecular weights, and produce isomass

parent ions. Therefore, a peak in the parent ion spectrum might represent several sterol isomers. The possibility exists for differentiation of these isomers using daughter spectra. One of the daughter ions formed from sterols is a result of the opening of ring A, with subsequent cleavage of a portion of the ring. Since this fragmentation might be controlled through the position of the double bond(s) within ring B, different daughter spectra might be obtained from different isomers. Research has begun in this laboratory toward solving this problem.

A parent scan for m/z 271 from fungal sample 138-1, an unknown fungal isolate from the Kellogg Biological Station, is shown in Figure 6.2. This particular parent ion value (m/z 271) represents a fragmentation from ergosterol (nominal molecular weight 396) in which an ion is formed from the ring structure. This ion may not be unique to ergosterol; any sterol containing a ring structure of this mass would be detected. However, the limited information available regarding fungal sterol content does not contain the presence of these sterols in fungi [1]. As mentioned earlier in this chapter, ergosterol is a sterol contained only in fungi. Therefore, this parent scan can be used as a quick screening method for detecting the presence of fungi in a sample.

A parent scan for m/z 273 from the same sample is shown in Figure 6.3. This value represents a fragmentation from a multitude of different sterols whose basic ring structure contains one less unsaturation than that shown in Structure 6.4. Each of the peaks

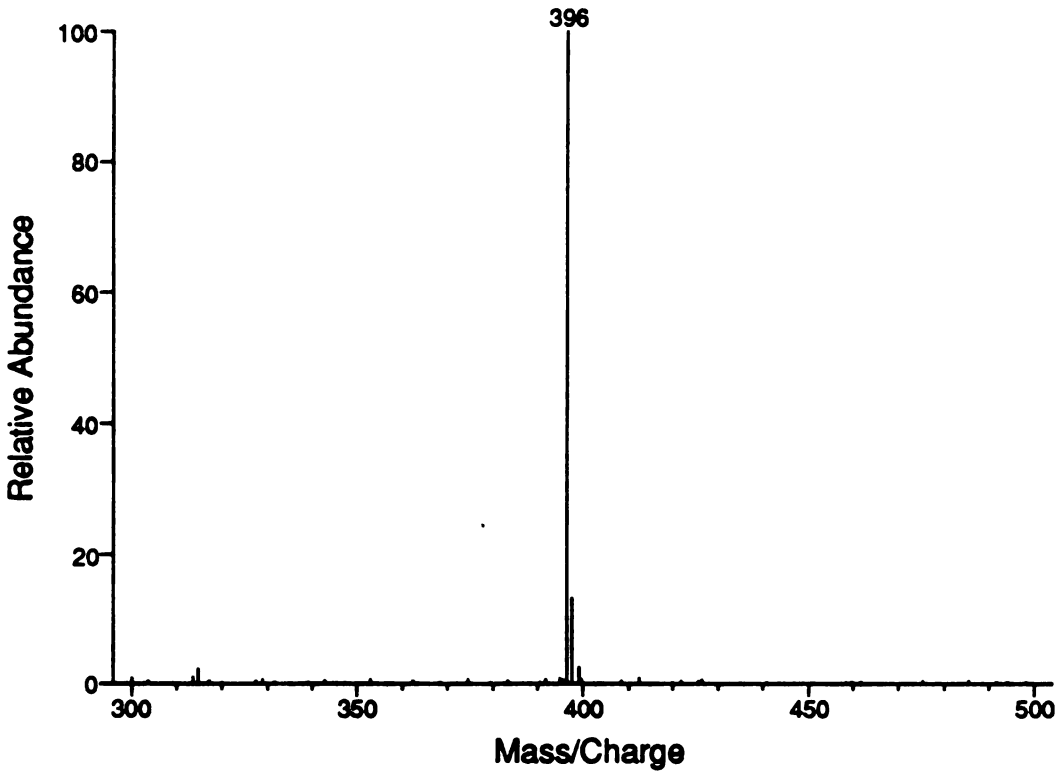


Figure 6.2 Parent scan for m/z 271 from fungal sample 138-1.

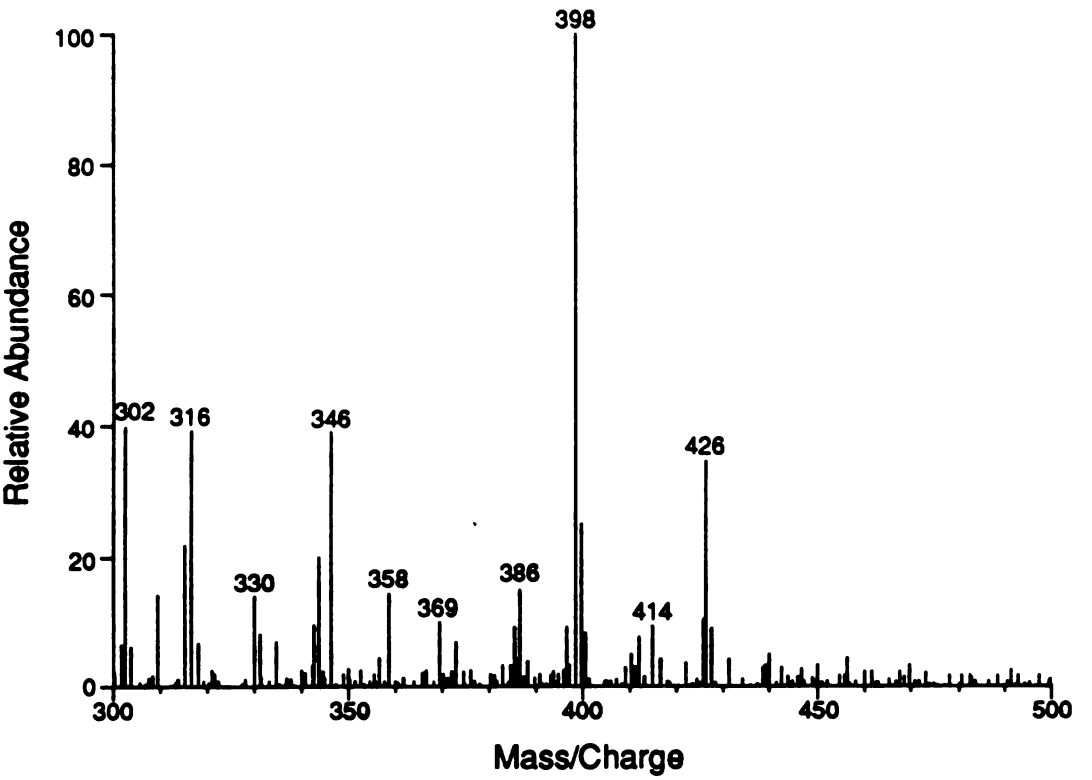


Figure 6.3 Parent scan for m/z 273 from fungal sample 138-1.

present in this spectrum are due to one or more sterol species. Four of the peaks have been tentatively identified: the peak at m/z 386 is cholesterol; the peak at m/z 398 could be episterol, fecosterol, brassicasterol, or a combination of these; the peak at m/z 414 is sitosterol; and the peak at m/z 426 could be stigmasterol, fucosterol, or a combination of the two. The remainder of the peaks have not yet been identified, but their patterns in the mass spectrum can still be used for identification. A series of different parent ion scans can be used that cover a broad range of sterol species. Research is underway in this laboratory in using these sterol profiles for detection and identification of fungi.

Other Potential Lipid Biomarkers

The research presented in this thesis has focused on three lipid biomarkers: phospholipids, glycolipids, and sterols. These are the lipids most common to microorganisms, and have the broadest applications. However, many other types of lipids exist that could be very useful for microbial detection and identification. These lipids range from being useful for a relatively wide range of organisms, to being specific for a particular group, or even a particular species, of microorganisms. Three of these lipids, the isoprenoid quinones, capnoids, and isopranyl ethers, are of particular interest to this research due to their applicability in identifying microorganisms. Developing the methodologies for their detection is the next goal in this research.

Isoprenoid quinones have shown potential value as chemotaxonomic markers for bacterial classification [6], and as such, they are of interest to this research. The isoprenoid quinones are involved with electron-transport and oxidative phosphorylation; thus, they are associated with the plasma membranes of bacteria and the photosynthetic membranes of phototrophic organisms. Because quinones are widely distributed throughout all microorganisms, they could be used for general identification. Both the classes of quinones, and the different structural details contained within each class, vary among microorganisms and can provide significant information for microbial identification. In addition, certain types of quinones can be specific for individual species of microorganisms. These could be used as biomarkers for the detection of specific organisms in complex communities containing many microbial species.

The capnoids, a sub-group of the sulphonolipids, occur only in gliding bacteria, and can serve as biomarkers for the presence of these bacteria [7]. As mentioned in Chapter 5, biomarkers specific to small groups of organisms can be very useful when searching a database by narrowing the focus of the search. In addition, these lipids have many variations and can be used in the identification of the microorganisms that contain them.

Isopranyl ether is a general term for any of the acylglycerols, such as phospholipids or glycolipids, in which the fatty acids are attached to the glycerol core through ether-linkages. Like the capnoids, they occur in a defined group of bacteria - in this case the

archaebacteria - and can be used for detection and identification of this group of bacteria [1]. The archaebacteria occupy environments of extreme conditions, such as high temperatures, high salt content, or high acid content. Because their major lipids consist of isopranyl ether lipids, these biomarkers will be very important when attempting to identify this type of organism. The information regarding the classes of isopranyl ethers present in a sample can be obtained in the same way as that for the phospholipids and glycolipids, since they share the same head groups and general structures. However, obtaining the structural information regarding the ether-linked fatty acids might present a problem to low-energy CID methods. This requires further investigation.

The biomarkers mentioned in this thesis are by no means a complete list. Many other biomarkers exist that can be useful, at some level, for the detection and identification of microorganisms. Tandem mass spectrometry is emerging as a promising technique for accessing this information. The outlook for this technique in the field of microbial taxonomy is very bright indeed. Many newly discovered microorganisms do not fit into any of the taxonomic catagories that exist today. New data obtained on the molecular and genetic levels are forcing many microorganism out of their old categories. New taxonomic systems, based on chemical, molecular, and genetic similarities and differences, are needed. Tandem mass spectrometry promises to be at the forefront of this field.

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