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Detection of Biomarkers of Potential Pathogens in Varied Matrices

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Carol Ann Smith
August, 2000

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DEDICATION

This thesis is dedicated

to my parents,

George Pedro Smith, Jr. and Helen Whitesell Smith,

whose love and support

have helped to make this degree possible.

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ABSTRACT

Methods that use signature biomarkers have become increasingly important in the field of environmental microbiology. Signature lipid biomarker (SLB) analysis is a method of analysis for the quantitative definition of viable biomass, community composition, and nutritional status of microbiota isolated from a wide variety of environmental matrices, including air, soil and water. Biomarkers examined in this thesis were glyco- and phospholipids, and dipicolinic acid. Organisms tested were *Cryptosporidium parvum, C. baileyi, C. muris, Eimeria tenella, E. acervulina, E. maxima, Escherichia coli,* and *Bacillus subtilis*.

Three species of *Cryptosporidium* were examined using signature lipid biomarker analysis. This analysis was successfully applied to the species-specific identification of *C. parvum, C. baileyi* and *C. muris.* The glycolipid fraction from the SLB analysis was also examined for *C. parvum*, as the possibility of a unique biomarker (10-hydroxy stearic acid) was present in research done by others. This compound was shown to be an artifact of sample preparation.

SLB analysis was also used to examine the phospholipids of *Eimeria tenella, E. acervulina,* and *E. maxima.* This analysis clearly differentiated these three species from each other.

SLB analysis was also used to examine chlorine-injured *Escherichia coli*. The experiments presented herein ensure the validity of oxirane fatty acids as indicators of sterilization efficiency. The detection of epoxidated fatty acids firmly establishes that the cells have been in contact with hypochlorite, thus providing a chemical marker for hypochlorite exposure and sterility.

For the examination of dipicolinic acid from endospores, a new extraction and methylation technique was devised and GC/MS analysis was employed for the detection of dimethyl dipicolinate. Preliminary studies also evaluated spectrofluorometric analysis using terbium chloride. *Bacillus subtilis* spores were detected using the new in situ SFE extraction and methylation technique and GC/MS with a lower limit of quantification of 3×10^4 spores in a sand mixture, and the lower limit of detection was observed to be 3×10^3 spores.

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PART I.

INTRODUCTION

PART A. BACKGROUND AND RESEARCH OBJECTIVES

The development of detection methods for signature biomarkers to use as indicators of potential pathogens has become increasingly important in many areas that rely on microbiology for detection and identification of health hazards. The risk of exposure to *Cryptosporidium* oocysts and other potentially pathogenic microorganisms as a result of pollution from animals and humans is a problem in the potable water industry. Exposure to potentially pathogenic endospores can result from poor sterility in hospital settings and contamination in the food industry, as well as the risk of exposure through air, water and / or soil contamination due to humans, animals, or biological warfare.

Many different compounds can serve as biomarkers—fatty acids, DNA, and, in the case of endospores, dipicolinic acid. Detection methods for specific biomarkers should be accurate, rapid, highly sensitive, and require minimal sample handling.

This thesis surveys several methods of detection for specific potential biomarkers in different matrices. The goals of these studies were to develop methods of detection and / or to explore the accuracy and usefulness of the following potential biomarkers:

- Phospholipid fatty acid biomarkers, to determine the presence of *Cryptosporidium* and *Eimeria* oocysts;
- 10-Hydroxy stearic acid, for the detection of *Cryptosporidium* oocysts;
- Oxirane fatty acids, to reveal chlorine injury in microorganisms;
- Dipicolinic acid (DPA), for the detection of bacterial endospores.

Matrices covered in this thesis included:

- Chlorinated water, such as might be found in drinking water or recreational water, to determine the usefulness of oxirane fatty acid detection; and
- Sand studies to discover the usefulness of DPA for the detection of bacterial endospores.

PART B. REVIEW OF THE LITERATURE

POTENTIAL BIOMARKERS FOR *CRYPTOSPORIDIUM*

Cryptosporidiosis

Cryptosporidium is a widely distributed and important enteric pathogen of humans and livestock. It is also common in other hosts, in which it is often asymptomatic. Nime *et al.* (1976) and Meisel *et al.* (1976) first described human diarrheal disease due to *Cryptosporidium* in 1976 in immunocompromised hosts. Other early reports of human cryptosporidiosis often reflected a high proportion of immunocompromised patients, particularly those with AIDS, and the immunosuppressed. In the early 1980s, sporadic infections began to be diagnosed in communities, especially in otherwise healthy children (Fayer and Ungar, 1986). In addition, the prevalence of asymptomatic individuals who excrete infectious oocysts is much higher than previously thought (Despommier *et al.*, 1995).

Cryptosporidium infections in normal humans are characterized by acute, self-limiting, gastroenteritis. In the immunocompromised, these infections are persistent and potentially fatal (Current, 1989).

Cryptosporidium parvum appears to be infectious for 79 species of mammals, including humans (O'Donoghue, 1995). Only one case of another species of *Cryptosporidium, C. baileyi,* has been reported as a human infection, that of an immunocompromised patient (Ditrich *et al*., 1991). Natural infections with *C. baileyi* occur in chickens under 11 weeks of age (Goodwin, 1989). *C. baileyi* may also cause severe illness of turkeys, and has resulted in losses of approximately $25 million in North Carolina in 1994 and 1995 (Fayer *et al*., 1997). *C. muris* has been detected in a small range of mammalian hosts, including mice, rats, and cattle. Although no clinical symptoms were reported, chronic infections have been tentatively associated with reduced weight gain in cattle (O'Donoghue, 1995).

There have been five well-documented outbreaks of cryptosporidiosis in drinking water in the U.S. (Rose *et al*., 1997). The largest outbreak, affecting 403,000 persons, was in Milwaukee, WI in 1993 (Leland *et al*., 1993). All five outbreaks were associated with unfiltered water supplies. All source waters were considered pristine, requiring only chlorination to satisfy regulatory criteria. In fact, *Cryptosporidium* oocysts have been found in most surveyed source waters (LeChevallier *et al*., 1991; Rose *et al*., 1991).

Another source of infection from *Cryptosporidium* is recreational water. The first outbreak from this source was in 1988 in Los Angeles.

(Sorville, 1990). Forty-four people became ill as a result of accidental fecal contamination and inoperable filters in a swimming pool. At least four other recreational water outbreaks have been associated with swimming pools. In each outbreak, children were identified as the primary cases and children's pools were identified as a risk factor (Rose *et al*., 1997).

The primary barrier against waterborne cryptosporidiosis is the physical removal of oocysts through coagulation, sedimentation, and filtration (Newman, 1995; O'Donoghue, 1995; Rose *et al*., 1997). Chlorine-based disinfectants, effective against many microorganisms, have had a low level of effectiveness for inactivation of *Cryptosporidium* oocysts (Korich *et al*., 1990; Ransome *et al*., 1993). Since transmission of infection is through ingestion or inhalation of oocysts, most control measures in specific locations such as hospitals consist of managerial practices designed to minimize further host contact with sources of infection and the use of different disinfection procedures to destroy oocysts (O'Donoghue, 1995).

Current laboratory diagnostic tests for *Cryptosporidium* oocysts suggest concentrating the organisms and staining them with monoclonal antibodies tagged with fluorescein (Ortega, 1999). A trained analyst classifies oocysts according to size, shape and internal morphologies using a microscope equipped for epifluorescence and differential interference

contrast. Interference in sample analysis using this indirect fluorescent antibody (IFA) technique consists of algae, which also fluoresce, and turbidity. This method also fails to determine the viability of the oocysts, and it does not differentiate among different species (Newman, 1995; O'Donoghue, 1995).

Lipid analysis and lipid biomarkers

Lipid analysis techniques have been used for a variety of purposes, including determination of community structure and nutritional status of the community (Nichols *et al*., 1989; Ringelberg *et al*., 1989; Smith *et al*., 1989; White *et al*., 1979a). Changes in community structure have been detected using phospholipid fatty acid methyl esters (Bobbie and White, 1980; White, 1988). Lipid biomarkers are cellular components that are either rare or unique to a specific group of organisms. Polar lipid fatty acid (PLFA) biomarkers can be used to separate Type I and Type II methylotrophs (Nichols *et al*., 1985). Hydroxy fatty acids represent another lipid biomarker, and are found in the lipopolysaccharide of Gram-negative bacteria (Edlund *et al*., 1985; Ferguson *et al*., 1972; Nichols *et al*., 1985; Parker *et al*., 1982; Rietschel, 1976).

PLFA phenotypes can be used to detect differences between closely related species and strains (Guckert *et al*., 1991; Nichols *et al*., 1986b).

However, fatty acid composition can vary somewhat with growth conditions such as temperature, light intensity and media content (Al-Hasan *et al*., 1989; Costas and Griffiths, 1984; Lund-Katz and Conner, 1982; Moss and Dees, 1979; Nichols *et al*., 1989; White *et al*., 1979b; Wiebe *et al*., 1989). The identification of fatty acids should include double-bond position and geometry to detect phenotypic differences (Guckert *et al*., 1991; Nichols *et al*., 1986a). Using fatty acid profiles, taxonomic classification of bacteria has been achieved by several groups of researchers (Dowling *et al*., 1986; Drucker and Jenkins, 1989; Guckert *et al*., 1991; Mayberry, 1984a and 1984b; Moss, 1981).

Methods for extraction and analysis of microbial lipids have been well defined by Christie (1982) and Kates (1986). Methods used in this study included the modified Bligh and Dyer (1959) extraction, which uses a chloroform-methanol-buffer solvent system to recover extractable lipids from cells. Lipid classes were separated on silicic acid columns, using solvents of increasing polarity to elute neutral, glyco- and phospholipids. Fractions used in these studies were derivatized using mild alkaline methanolysis, which produces ester-linked fatty acid methyl esters (Ringelberg *et al*., 1989). Derivatized samples were analyzed by gas chromatography / mass spectrometry for peak identification, as well as determination of double-bond position and geometry. Quantitative

analysis was performed using gas chromatography by integration of peak areas.

Phospholipids of Cryptosporidium

Only the basic phospholipid composition of fresh *Cryptosporidium parvum* oocysts has been documented (Mitschler *et al*., 1994; White *et al*., 1997). Mitschler *et al*. (1994) used thin layer chromatography (TLC) for separation of fatty acids, which is an old technique. These researchers found the most abundant fatty acid to be palmitic acid (16:0), followed by linoleic acid (18:2). White *et al*. (1997) used newer and different methods for separation and identification of lipids. That study concentrated on the characterization of fresh oocysts vs. frozen oocysts, in an effort to find differences in the phospholipid patterns. White *et al.* found that the predominant fatty acid in fresh *C. parvum* oocysts was oleic acid (18:1), followed by stearic acid (18:0). These polar lipid fatty acids (PLFA) are quite common in microorganisms; however, a potential biomarker may exist in 10-hydroxy stearic acid.

10-hydroxy stearic acid

10-hydroxy stearic acid (10-OH 18:0) is an 18-carbon fatty acid with a hydroxyl functionality located at the 10 position from the carboxyl end of the molecule. An extremely unusual fatty acid in microorganisms, it is rarely found. It was detected as an ester in the saponifiable lipids of *Mycobacterium tuberculosis* (Polgar and Smith, 1962). It also has been reported to have been formed from exogenously added 18:1ω9 when incubated with five field isolates of *Pseudomonas* and *Xanthomonas* species (Wallen *et al*., 1962). In addition, traces of 10-OH 18:0 were localized at the *sn*-2 position of phosphatidylethanolamine (PE) of *Cryptosporidium* (Schrum *et al*., 1997). Unfortunately, that study did not report results from infectious oocysts of any other species of *Cryptosporidium* besides *C. parvum*. This novel fatty acid was identified by these researchers as 10-OH 18:0 through conventional gas chromatography / mass spectrometry. The rarity of this fatty acid and its novel structure make it a good candidate for a biomarker for the detection of *Cryptosporidium*.

PHOSPHOLIPID FATTY ACID BIOMARKERS FOR *EIMERIA*

Coccidiosis

Coccidiosis in poultry costs the world's commercial chicken producers at least $800 million every year (Williams, 1998). The primary source of infection in all coccidia is the oocyst stage shed in the feces of an infected host (Fayer and Reid, 1982).

Species of *Eimeria* are strongly host-specific. It is rare for one of these parasites to occur naturally in more than one host genus; with few exceptions, they are restricted to closely related species or subspecies. When inoculated into eight species of gallinaceous birds, *E. tenella* developed only in the chicken (Vetterling, 1976). Many species are also tissue-specific. These locations are so characteristic that they are often used as diagnostic features of the individual species. For instance, *E. tenella* is normally found only in the cecum of chickens, while *E. maxima* and *E. acervulina* primarily invade the duodenum and anterior intestine, respectively (Joyner, 1982).

Many of the features of this disease in the host are expressions of the characteristics of individual species of *Eimeria*, and as such are often used as a guide to identification (Joyner, 1982). For example, *E. acervulina*

produces reduction in growth without severe mortality, whereas *E. tenella* causes intestinal or cecal hemorrhage (Joyner and Davies, 1960).

Research on behalf of the poultry, cattle, and other livestock producers has long had the objective to control or prevent economic losses due to coccidiosis. One survey of farms in the Netherlands found that 92% of the farms were positive for *E. acervulina*. *E. tenella* and *E. maxima* were found in 84% and 47% of the flocks surveyed, respectively (Graat *et al*., 1998). Complete elimination of coccidia from either hosts or the environment is considered impractical or uneconomical; however, improvement of current control methods is needed. Control measures commonly used are sanitary management practices, immunological methods and genetic resistance of the host (Fayer and Reid, 1982; Graat *et al*., 1998). Current efforts also include the production of live anticoccidial vaccines, which are currently not suitable for broilers, due to the post-vaccinal setback of weight gain. However, predictions abound for the use of attenuated live vaccines in all classes of floor-reared chickens in the relatively short term (Graat *et al*., 1998; Williams, 1998).

Phospholipids of Eimeria

Only one study examining the lipids of *Eimeria* has been reported (Weppelman *et al*., 1976). In that study, the total lipids extracted were

saponified. They found the predominant fatty acid to be oleic acid (18:1), and palmitic acid (16:0) was the second most abundant fatty acid. Therefore, a more comprehensive analysis of *Eimeria* membrane lipid composition is needed. It is probable that lipid biomarker analysis will enhance detection and differentiation of *Eimeria* species.

OXIRANE FATTY ACIDS AND CHLORINE INJURY

Chlorine treatment of water for disinfection

The earliest proposals to chlorinate water were made before knowledge of water-borne pathogens existed. In 1835, Dunglinson recognized the taste and odor difficulties arising from chlorination. Dr. Dunglinson's discussion suggested that chlorination had been proposed by another as a method to make marsh water potable. Chlorination for water purification was first used in 1908 at the Boonton Reservoir of the Jersey City Water Works (Baker, 1948). Dramatic reductions in typhoid frequently accompanied the introduction of this process (Glaze, 1990; Haas, 1990). It is currently the most widely used form of disinfection of drinking water.

Chlorine is relatively inexpensive, easy to use, and provides residual protection in water distribution systems. After addition to water, a portion of the chlorine reacts with compounds in the water, such as organics. "Free chlorine" is the chlorine that remains available for further reaction (Margolin, 1997).

The ability of chlorine to injure or destroy microorganisms is predominantly due to the ability of hypochlorous acid to oxidize proteins and other structures found on bacteria, viruses and protozoa. It is able to penetrate the organism with relative ease, since it is a neutral compound and has a relatively low molecular weight. Consequently, it has a high germicidal activity. At high pH, the germicidal activity of hypochlorous acid is greatly reduced, probably because hypochlorite ions are the predominant species at alkaline pH values. In this state, the molecule has a negative charge, which may prevent it from coming into close contact with the carboxyl ends of surface proteins (Margolin, 1997).

Stress and injury of bacteria in drinking water

Bacteria, particularly those populations not normally found in aquatic systems, undergo stress from many factors, including a lack of nutrients and sub-optimum temperatures. Using a continuous culture technique, Jannasch (1967) showed that cultures of marine bacteria would

not divide in seawater containing limiting substrate concentrations. He suggested that the cultures were surviving but inactive below certain levels of substrate. Using data from this and other studies, Stevenson (1978) postulated that a significant portion of the bacterial community in most aquatic environments could be described as existing in a physiological state of dormancy—a state of neither activity nor death.

Indicator organisms and enteropathogenic bacteria are usually transient residents in drinking water. These organisms are not well adapted to the physical and chemical conditions in water, as they reproduce within the guts of animals and humans. Consequently, it is not surprising that these bacteria can become physiologically damaged due to aquatic exposure. Injured waterborne indicator bacteria undergo sub-lethal physiological and structural consequences resulting from exposure to injurious factors within aquatic environments. This injury results in the inability of injured cells to reproduce under selective or restrictive conditions that are tolerated by uninjured cells.

Many factors influence the sensitivity of waterborne enteropathogens and indicator organisms to chemical agents such as chlorine. However, studies using similar experimental conditions have reported that *Yersinia enterocolitica, Salmonella typhimurium,* and *Shigella* species were more resistant to the action of chlorine than were the

coliform bacteria (LeChevallier *et al*., 1985; Singh *et al*., 1986; Singh and McFeters, 1987). Higher concentrations of chlorine (0.9 to 1.5 mg of chlorine per liter) were required to induce greater than 90% injury in *Y. Enterocolitica*, *S. typhimurium*, and *Shigella* species, as compared with levels responsible for the same degree of injury in coliform bacteria (0.25 to 0.5 mg of chlorine per liter) (Camper and McFeters, 1979).

As early as 1935, evidence existed that chlorine caused a reversible form of bacterial inactivation (Mudge and Smith, 1935). Later, the reversible inactivation of *E. coli* by chlorine in foods was described (Scheusner *et al*., 1971), followed by similar reports associated with tap water and wastewater (Braswell and Hoadley, 1974; Hoadley and Cheng, 1974). Disinfectants and other biocides used to treat water are probably the major cause of bacterial injury in aquatic systems (Camper and McFeters, 1979; Hoadley and Cheng, 1974; McFeters and Camper, 1983).

The injuries caused by disinfectants and biocides are frequently described as occurring to the cytoplasmic membrane, since the physiological consequences that have been reported have been the loss of functions mediated within the cytoplasmic membrane (Beuchat, 1978; Walsh and Bissonnette, 1983). The percentage of injured bacteria vs. non-injured bacteria in various aquatic systems has been the subject of several studies. Observations of several municipal drinking waters systems have

shown that injured bacteria comprise 43% to 99% of the total bacterial population (Singh and McFeters, 1990). Consequently, injured bacteria may comprise the majority of coliforms in some drinking water systems. These bacteria normally would not be detected using accepted analytical methods.

It is important to note that bacteria injured by chlorination are capable of recovering their ability to grow on selective media (Bissonnette *et al*., 1977; LeChevallier and McFeters, 1984 and 1985; LeChevallier *et al*., 1985). The repair process in enteropathogens has been studied, both in vitro and in the small intestine of experimentally inoculated mice (Singh *et al*., 1986). These researchers found that a substantial portion of the population recovered during the first two hours of intraluminal incubation. The injured cells were able to recover, grow, and cause pathological changes to the mammalian gut.

Lipid biomarkers for stress

Varying environmental conditions induce microorganisms to make membrane lipid composition changes to maintain membrane integrity and stability. Microorganisms grown under stress often exhibit specific lipid markers. For example, as Gram-negative bacteria move from logarithmic to stationary growth conditions, they increasingly convert 16:1ω7c and

18:1ω7c to the cyclopropyl fatty acids cy17:0 and cy19:0, respectively (Guckert *et al*., 1986). This ratio usually falls within the range of 0.05 (log phase) to 2.5 or greater (stationary phase), but varies with the environment and the microorganism (Mikell *et al*., 1987; White, 1983). Gram-negative bacteria also make *trans*-monounsaturated fatty acids, usually as a result of environmental stress factors such as starvation or toxicity. *Trans/cis* ratios greater than 0.1 have been shown to indicate starvation in bacterial isolates (Guckert *et al*., 1986). This value is usually 0.05 or less in non-stressed, healthy populations. The Gram-negative bacterium, *Pseudomonas pudita*, has been shown to convert 16:1ω7c and 18:1ω7c to *trans* fatty acids in the presence of phenol (Heipieper *et al*., 1992).

Oxirane fatty acids

Oxirane fatty acids may be seen as a stress lipid biomarker for bacteria exposed to chlorine. They have not been detected in bacteria that have not been exposed to chlorine. However, a furan ring, 10,13-epoxy-11-methyloctadeca-10,12 dienoic acid, has been found in the cellular lipids of a number of marine bacteria found in the gut of fishes (Shirasaka *et al*., 1995). Oxirane fatty acids are also found in the neutral lipids of oilseeds (Singh *et al*., 1994), *Aeollanthus* (Dellar *et al*., 1996), and rice (Kato et al.,

1993). Oxirane fatty acids are found in the rust fungi (Weete *et al*., 1979), in the pathogen *Pneumocystis carinii* (Kaneshiro, 1998), in marine algae (Bernat *et al*., 1993; Jiang and Gerwick, 1997), and are formed by microbes in adipocere of human cadavers exposed to microaerophilic environments (Takatori, 1996).

Previous experiments in this laboratory have shown that high concentrations of bacteria exposed to 0.24-1.4 ppm of chlorine were damaged sufficiently so that no colony-forming units were found on non-selective and recovery media after the exposure to chlorine. Epoxide fatty acids correlated directly to hypochlorite concentration: the higher the concentration of chlorine, the higher the mole percentage values of oxiranes. These oxiranes were formed at the expense of monoenoics in the bacterial profiles. No epoxides were found in negative control samples (Phiefer, 1998; Smith *et al*., 2000). Therefore, it seems likely that oxirane fatty acids may be lipid biomarkers for bacteria that are stressed from exposure to chlorine.

CHEMICAL ASSAYS FOR DIPICOLINIC ACID AND ENDOSPORES

Endospore-forming bacteria

The endospore-forming bacteria are allocated to 13 validly published genera, comprised of *Alicyclobacillus, Amphibacillus, Bacillus, Clostridium, Desulfotomaculum, Oscillospira, Pasteuria, Sporohalobacter, Sporolactobacillus, Sporosarcinia, Sulfobacillus, Syntrophosphora* and *Thermoactinomycetes* (Berkeley and Ali, 1994). These authors also refer to another group they call genera *incertae sedis*, a group of endospore-formers of uncertain position that have been described but not isolated in pure culture. The genera *Oscillospira* (Gibson, 1986) and *Pasteuria* (Berkeley and Ali, 1994; Sayre and Starr, 1989) have also not been isolated in pure culture, but have been described. Of these 13 genera, two are important in terms of human infection and disease—*Bacillus* and *Clostridium*.

Anthrax, which is caused by *Bacillus anthracis*, is the best-known disease caused by a *Bacillus* species. Although humans are moderately resistant to anthrax in comparison with herbivores, it is a disease about which the public has both some knowledge and great fear. However, despite its reputation, anthrax is not highly contagious (Logan and Turnbull, 1999). *Bacillus cereus* and *B. anthracis* actually belong to the same species, yet remain separate due to their different practical importance

(Berkeley and Ali, 1994). *Bacillus cereus* is second in importance to *B. anthracis* in terms of its pathogenicity for humans and other animals. It is a common causative agent of food-borne illness and opportunistic infections, particularly of the eye. *Bacillus subtilis* and *Bacillus brevis* have occasionally been implicated in food-borne illness (Logan and Turnbull, 1999).

One of the most common bacterial causes of food-borne illnesses in the United States is *Clostridium perfringens* (Shandera *et al*., 1983). It is also the causative agent of gas gangrene, a rapidly progressive, life-threatening condition due to toxin-mediated breakdown of muscle tissue associated with the growth of the organism (Allen *et al*., 1999). Of the *Clostridia*, diseases related to two other species are also well known to the public. *Clostridium botulinum* causes a rare but life-threatening food-borne illness, due to its ability to produce botulinum neurotoxin. *Clostridium tetani* produces a dramatic illness, tetanus, due to the action of its potent neurotoxin, tetanospasmin (Allen *et al*., 1999). Another, less well known to the public but important species in this genus, *Clostridium difficile*, is the major cause of antibiotic-associated diarrhea and pseudomembranous colitis. It is also the most frequently identified cause of hospital-acquired diarrhea (Johnson *et al*., 1990; Kelly *et al*., 1994; Mahony *et al*., 1989).

Both of the above clinically significant genera are ubiquitous in nature, most commonly found in a sporulated state, and are frequently isolated from soil (Baird-Parker, 1969; Logan and Turnbull, 1999; Smith, 1975; Smith and Williams, 1984). This almost guarantees their frequent presence on surfaces exposed to dust contamination, including many food items.

Spore formation as a survival mechanism

Sporulating bacteria exhibit a highly flexible ability to alternate between growth and dormancy. They are capable of responding efficiently to auspicious or foreboding environmental signals. The close linkage of environmental factors and differentiation is manifest in various phenomena of sporulation. Sporulating bacteria are able to control the fraction of the vegetative population that becomes committed to sporulation. They possess the power to limit the degree of dormancy achieved, illustrated by recycling, the spontaneous germination of newly formed spores in the sporulating culture. They are also able to re-sporulate after only a few vegetative divisions. Microcycle sporogenesis (Vinter and Slepecky, 1965), a resporulation of the germinating spore itself to give a functional spore, is another example of the close linkage of environment and differentiation.

Mature spores have no detectable metabolism and can survive for extremely long periods of time in the absence of exogenous nutrients. The process of sporulation is generally initiated when the environment is no longer conducive to survival of an actively metabolizing cell. This usually occurs when one or more nutrients are depleted. The endospore has no detectable metabolism and lacks most common high-energy compounds, including ATP and other nucleoside triphosphates (Setlow, 1992).

Spore structure

Spores are considerably more complex than vegetative cells. The cortex surrounds the germ cell (protoplast) and germ cell wall. The spore coats, of which the outer is denser, surround the cortex. In some spores, a layer (exosporium) beyond the spore coats may be found. In other spores, an exosporium is also present, but surrounds only one dense spore coat. The interior of bacterial spores is essentially the same; thus, the major differences between various species consist of variations in the organization of the number and form of the outer layers (Russell, 1982). Unique chemical components of the bacterial spore are dipicolinic acid (DPA) and spore peptidoglycan.

Dipicolinic acid

Powell (1953) first established the link between dipicolinic acid and spores by noticing that it was secreted by *B. megaterium* during germination. Powell and Strange (1953) had previously determined that it constituted 15% of the spore dry weight. The amount of DPA varies between species, but ranges between 5 and 15% of spore dry weight (Murrell, 1969). When spores are lethally heated, intracellular constituents are released, and there is a progressive loss of DPA and calcium (Rode and Foster, 1960). Woese (1958, 1959) found that the ultraviolet absorption spectra of substances released from autoclaved suspensions of spores of *B. megaterium*, *B. cereus* and *B. mesentericus* showed a peak at 270 nm and that the spectra were those of chelated DPA.

DPA is a strong chelater of divalent cations. As a cell constituent, it is unique to spores, and is always isolated from spores in about a 1:1 mole ratio with Ca^{2+} (Thompson and Leadbetter, 1963). Evidence from ultraviolet absorption spectra (Aoki and Slepecky, 1973), infrared absorption spectra (Norris and Greenstreet, 1958), electron para-magnetic resonance spectra (Johnstone *et al*., 1982; Windle and Sacks, 1963) and laser Raman spectroscopy (Shibata *et al*., 1986) suggests that DPA exists with calcium in some type of chelate in the spore.

Much of the research on the location of DPA inside the spore is indirect. Scherrer and Gerhardt (1972) studied the location of calcium in

B. cereus and *B. megaterium* spores by electron probe X-ray microanalysis. They found that calcium was distributed throughout the spore but was mainly located in the central region corresponding to the spore protoplast. This finding provided indirect evidence for DPA location in the core rather than in the cortex. Germaine and Murrell (1973) have shown that DPA sensitizes spores to ultraviolet light and increases the rate and extent of thymine photoproduct production from DNA. Since DNA is found in the spore protoplast, this finding suggested proximity of DPA to DNA, thus providing further indirect evidence for the presence of at least some of the DPA in the core. Germaine and Murrell (1974) devised a method involving the mechanical breakage of UV-irradiated spores containing radioactive DPA and a subsequent determination of the distribution of "photochemically-bound" radioactivity among the major spore components. This provided evidence for the location of DPA in the core. Beta-attenuation analysis has also indicated that DPA is present in the spore protoplast and not the cortex (Leanz and Gilvarg, 1973). Using high resolution electron probe X-ray, Johnstone *et al.* (1980) have shown that the core of *B. megaterium* spores contains most of the calcium, potassium, magnesium, manganese and phosphorus present in the spore. They point out that to preserve electrical neutrality, a major portion of divalent metal ions must be associated with DPA, which provides additional evidence for

the location of DPA in the core. More recently, several studies which evaluated biocide damage to spore permeability barriers produced leakage of DPA from intact and coat- and cortex-deficient spores treated with oxidizing agents (Bloomfield and Arthur, 1992; Bloomfield and Megid, 1994). They concluded that DPA leakage resulting from damage to spore permeability barriers was not a primary lethal effect but was more closely associated with degradation of the spore cortex. Additionally, they noted that DPA release was not caused by cortex degradation alone, since the total DPA content of normal spores was found to be the same as that of urea / dithiothreitol / sodium lauryl sulfate (UDS) / lysozyme-treated spores (11-11.5 μg / 3 x 10^8 spores).

The roles of DPA in the spore have been the subject of much research. As previously mentioned, DPA preserves electrical neutrality (Johnstone *et al*., 1980). DPA has been found to enhance formation of spore DNA photoproduct in response to ultraviolet irradiation (Setlow and Setlow, 1993). DPA is also a known germinant (Riemann and Ordal, 1961).

Another role commonly ascribed to DPA is its relationship to heat resistance. The controversy surrounding this relationship bears some mention, as there seems to be growing evidence that DPA has little or no role in thermoresistance. The first evidence indicative of this occurred

when Hanson *et al*. (1972) isolated a *B. cereus* T revertant spore that was devoid of DPA and low in calcium content, but was as heat resistant as the parent spore. Isolation of other heat-resistant spores in DPA-minus mutants followed. Beaman *et al.* (1988) showed that fully heat-activated spores were virtually devoid of DPA but retained a high level of heat resistance. Other lines of evidence also exist, reviewed by Gerhardt and Marquis (1989). Perhaps the best evidence on this point is that minerals and DPA can be extracted from spores to yield heat sensitive H-form spores. Then the spores can be re-mineralized with Ca^{2+} in the absence of DPA to regain fully the level of heat resistance of native spores (Gerhardt and Marquis, 1989). They comment that the underlying problem in attempting to correlate DPA content with heat resistance lies in the failure by researchers to separate the effects of DPA from those of calcium and other minerals.

Assays for DPA

A range of techniques has been employed to assay DPA from spores. These include colorimetric techniques (Janssen *et al*., 1958), liquid chromatography (Warth, 1979), gas chromatography (Tabor *et al*., 1976), derivative spectroscopy (Warth, 1983), UV resonance Raman spectroscopy (Ghiamati *et al*., 1992) pyrolysis / mass spectrometry (Beverly *et al*., 1996),

and spectrofluorometric assays (Barela and Sherry, 1976; Hindle and Hall, 1999; Pellegrino *et al*., 1998; Poglazova *et al*., 1996; Rosen *et al*., 1997; Sacks, 1990; Scott and Ellar, 1978). Currently, the most sensitive method in samples is HPLC, reporting 7 x 10^5 spores / gram of salmon (Warth, 1979; Paulus, 1981). The most sensitive method using spore suspensions are spectrofluorometric assays. Rosen *et al.* (1997) reported a lower detection limit of 4.4 x 10^5 *B. subtilis* spores per ml, Pellegrino *et al*. (1998) attained 1.21 x 10^5 *B. globigii* spores per ml, and Hindle and Hall (1999) reported a lower detection limit of 1 x 10^4 *B. subtilis* spores per ml.

Since gas chromatography / mass spectrometry (GC / MS) techniques are more sensitive than spectrofluorometric techniques, the combination of using GC / MS and a rapid extraction method using high pressure and temperature to extract the DPA from spores seems likely to succeed.

PART II.

POTENTIAL BIOMARKERS FOR *CRYPTOSPORIDIUM*

PART A. PHOSPHOLIPID FATTY ACID PROFILES OF THREE SPECIES OF *CRYPTOSPORIDIUM*

INTRODUCTION

A major problem facing the potable water industry is the inability to detect and remove potentially pathogenic microorganisms. Of special concern are those pathogenic microorganisms, such as *Giardia* and *Cryptosporidium*, which are difficult to culture, requiring animal or tissue culture for growth. *Cryptosporidium* is particularly alarming, as it is widespread in surface water samples (LeChevallier *et al*., 1991; Rose *et al*., 1991), has extreme resistance to disinfection by chlorine (Korich *et al*., 1990; Ransome *et al*., 1993), and is potentially fatal to immunocompromised individuals who become infected (Fayer and Ungar, 1986).

Existing methodologies for the detection of *Cryptosporidium parvum* oocysts in drinking water are not satisfactory. Current laboratory diagnostic tests use an indirect fluorescent antibody (IFA) technique, which is difficult to interpret and does not differentiate among different species (Newman, 1995; O'Donoghue, 1995). Successful methods must be

highly sensitive, as the presence of as little as 10-30 oocysts / 100 L of water may indicate that outbreaks are possible (Haas and Rose, 1995). The method must also be highly specific for *Cryptosporidium*, sufficiently rapid so as to be potentially useful to the water industry, and provide an indication of viability / infectivity.

Previous investigators had shown that events that lead to loss in infectivity of oocysts of *Cryptosporidium parvum* towards neonatal Balb / c mice, such as heating or freezing, are reflected in the profile of their polar lipid fatty acids (PLFA) (Fayer *et al*., 1998). Upon loss of infectivity, the total quantity of polar lipid fatty acids was found to decrease, whereas the relative quantities of several common fatty acids such as 18:1ω9 and 18:2ω6 were shown to fluctuate with infectivity (White *et al*., 1997).

Analysis of polar lipid fatty acids is a powerful tool for the chemotaxonomic assignment of microorganisms. Two other studies examining the lipids of fresh *Cryptosporidium* oocysts have been reported, and both included only the lipids typical of *C. parvum* (Mitschler *et al*., 1994; White *et al*, 1997). This study was undertaken to apply signature lipid biomarker (SLB) analysis to purified oocysts from three species of *Cryptosporidium*: *C. parvum*, *C. baileyi*, and *C. muris*. The objectives of this study were to identify possible unique fatty acids in the polar lipid fraction of these three species, and to determine the usefulness of the

relative quantities of fatty acids in this fraction (fatty acid pattern) for confirmation of each species.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade. Authentic standards for fatty acids were purchased from Matreya (Pleasant Gap, PA). All solvents were obtained from Baxter Scientific Products (McGaw Park, IL) and were of gas chromatography grade.

Glassware

All glassware was washed in a 10% (vol / vol) Micro cleaner solution (Baxter Diagnostics, Deerfield, IL), rinsed ten times with tap water and then ten times in deionized water. The glassware was then heated overnight in a muffle furnace at 450°C to remove any carbon contamination.

Parasites

Purified oocysts of *Cryptosporidium* were kindly provided by Ronald Fayer (USDA, Beltsville, MD). Purification procedures are detailed in Fayer (1995). Purity was checked upon receipt by microscopic examination.

Lipid extraction

Purified oocysts loaded on glass filters were extracted at room temperature and atmospheric pressure using a modified Bligh / Dyer technique (Bligh and Dyer, 1959; Guckert *et al*., 1985; White *et al*., 1979b). This method extracts cellular lipids using a one-phase chloroform / methanol / phosphate buffer (50 mM, pH 7.4, 1:2:0.8 by volume) solvent system for 4 hours before adding chloroform and deionized water to form two phases (final solvent ratios: chloroform / methanol / phosphate buffer / water, 1:1:0.4:0.5 by volume). Then the organic phase was collected and dried under a stream of nitrogen. The lipids were stored at -20°C until purification.

Purification of lipids

The total lipid extracted was dissolved in 0.5 ml chloroform and separated using silicic acid columns (10-cm column length, 0.5-cm inner

diameter, 100-200 mesh particle size). Each sample was loaded onto a column in 100-200 μl chloroform and then eluted in batches with 5 ml chloroform, 5 ml acetone, and 10 ml methanol so as to elute neutral, glyco- and polar lipids, respectively (Guckert *et al*., 1985). Bulk elution of the neutral lipids, including sterols and diglycerides, with chloroform was followed by recovery of the free fatty acids in the acetone, or glycolipid, fraction. Phospholipids were recovered after the glycolipids by elution in methanol. For each fraction, the solvent was removed under a stream of nitrogen. The lipid fractions were stored at -20°C until methylation.

Mild alkaline methanolysis

A mild alkaline methanolysis procedure (Guckert *et al.*, 1985) was utilized to prepare methyl esters of the ester-linked fatty acids of the polar lipids. The polar lipid fatty acid methyl esters (FAMEs) were dried under a stream of nitrogen and then were stored at -20°C until analysis.

Dimethyl disulfide (DMDS) adducts

A dimethyl disulfide derivatization procedure (Nichols *et al*., 1986a) was used for verification of monounsaturated fatty acid double bond position and geometry.

GC analysis

A Hewlett-Packard 5890 series II gas chromatograph coupled to a flame ionization detector (FID) provided quantitative analysis of FAME components. Chromatographic separations were carried out on a HP-1 fused-silica capillary column [50 m by 0.2 mm (inner diameter)] of 0.11 μm film thickness. The column was kept at an initial temperature of 60°C for 2 minutes, and the temperature was programmed to increase from 60 to 150°C at 10°C / min, then to 312°C at 3°C / min (Ringelberg *et al*., 1989). The injector and detector were maintained at 290°C and 300°C, respectively. Splitless injections were performed with a Hewlett-Packard model 7673 autosampler. The split valve was opened 1 minute after injection. Hydrogen was the carrier gas. Preliminary peak identification was performed by comparison of retention times with synthetically prepared standards (Matreya, Inc., Pleasant Gap, PA). FAMEs were quantified by integration of peak areas, comparing the peak response to methyl nonadecanoic acid (19:0) added to the sample as a recovery standard just prior to injection (Christie, 1989; Guckert *et al*., 1985). Peak areas were determined using a programmable laboratory data system, ChromPerfect version 3.5 (Chromatography Data Systems, Palo Alto).

GC/MS analysis

A Hewlett-Packard 5971 mass selective detector provided for the qualitative assignment of lipid structure. The column, temperature program, and injection procedures were as described above for GC analysis. The carrier gas, helium, was maintained at an inlet pressure of 195 kPa to establish a carrier gas flow rate of approximately 1ml/min (Ringelberg *et al*., 1989). Fatty acid methyl esters were identified based on their retention indices (RI) as well as their mass spectra in comparison to synthetically prepared standards (Matreya, Inc., Pleasant Gap, PA), and to a mass spectral library of FAME standards (Christie, 1989; Guckert *et al*., 1985). Peak areas were determined using a programmable laboratory data system, Enhanced ChemStation (Hewlett-Packard, Palo Alto).

Phylogenetic analysis of 18S sequences

Partial 18S sequences were retrieved from GenBank *via* the National Institute for Biotechnology Information (NCIB) internet node using the Entrez facility (Schuler *et al*., 1996), with accession numbers as follows: AF093495 (*Cryptosporidium baileyi*), X64342 (*Cryptosporidium muris*), U67115 (*Eimeria acervulina*), U67117 (*Eimeria maxima*), U40263 (*Eimeria nieschulzi*), and U40264 (*Eimeria tenella*). They were manually

aligned to the 18S sequence data from the Ribosomal Database Project (RDP) (Larsen *et al*., 1993).

Phylogenetic trees were constructed by applying the Felsenstein correction, DNA-DIST and the neighbor-joining method, using the ARB program (Strunk and Ludwig, 1996). The tree was pruned from a larger analysis containing all the representative 18S sequences compiled by RDP.

Fatty acid nomenclature

Fatty acids are abbreviated by the number of carbon atoms, a colon, degree of unsaturation, which is then followed by an omega symbol (ω) indicating the number of carbons from the methyl end of the molecule to the position of the first, and sometimes only, double bond. The prefix "cy" represents the cyclopropyl isomer of a fatty acid, and "i" and "a" represent *iso*-branched and *anteiso*-branched isomers, respectively. The number preceding the abbreviation "Me" indicates the position of a methyl group relative to the carbonyl carbon followed by the number of carbons in the fatty acid chain. Suffixes "c" and "t" represent *cis* and *trans* geometric isomers of the unsaturation, respectively. An ambiguity in this naming convention is that the number of carbons given for *iso*-, *anteiso*-, unknown branched, and cyclopropyl fatty acids include all of the carbons in the molecule except the esterified methyl group. Therefore, 10Me18:0

indicates a methyl group attached to an 18-carbon side chain at the 10 position from the carbonyl (Kates, 1986).

Statistical analysis

FAMEs are expressed in pmol/g or as mole percentages. If mole percent values are used for statistical analysis, arcsine transformation was applied to the data to remove the percentage bias.

STATISTICA software (StatSoft, Tulsa, OK) was utilized for cluster analysis. Cluster analysis was performed using complete linkage rules and Euclidean distances. Similarity between species can be measured by the linkage distances, which could range between 0 (identical) and 100 (no similarity).

RESULTS

The phospholipid FAME compositions of *Cryptosporidium parvum*, *Cryptosporidium muris*, and *Cryptosporidium baileyi* are shown in Table IIA-1. Straight chain saturated FAMEs (42-57%), terminally branched saturated FAMEs (0.7-25%), and monounsaturated FAMEs (24-37%) were the major lipid classes detected. Both *C. parvum* and *C. baileyi*

Table IIA-1. Relative proportions (mole percentages) of phospholipid fatty acids from three *Cryptosporidium* species (n = 1). Unusual FAMEs appear in bold print.

Fatty acid	*C. parvum*	*C. muris*	*C. baileyi*
Straight chain saturates			
14:0	1.41	1.39	2.64
15:0	0.13	---	---
16:0	32.20	27.16	28.40
17:0	0.60	2.80	1.04
18:0	13.97	9.67	18.04
20:0	---	0.46	5.30
22:0	---	0.68	1.99
Total straight chain saturates	**48.31**	**42.15**	**57.42**
Terminally branched saturates			
a13:0	---	0.51	---
i15:0	---	3.42	0.21
a15:0	0.24	10.63	0.34
i16:0	---	2.24	0.70
i17:0	0.18	3.86	---
a17:0	0.27	4.41	---
Total terminally branched saturates	**0.69**	**25.06**	**1.25**
Monounsaturates			
16:1ω10	---	**2.11**	---
16:1ω9c	0.16	---	---
16:1ω7c	0.37	5.89	2.85
18:1ω9c	29.49	11.44	12.91
18:1ω7c	5.52	3.18	8.25

Table IIA-1 (Continued).

Fatty acid	*C. parvum*	*C. muris*	*C. baileyi*
18:1ω7t	0.39	1.34	0.78
18:1ω5c	0.28	---	---
19:1ω12	0.23	---	1.02
20:1ω9c	0.35	---	0.44
Total monounsaturates	**36.78**	**23.95**	**26.24**
Polyenoics			
18:2ω6	9.91	5.71	9.54
20:2ω6	0.36	---	---
20:4ω6	0.61	---	---
Total polyenoics	**10.88**	**5.71**	**9.54**
Miscellaneous			
cy17:0	---	---	2.48
cyclohexyl 17:0	---	**1.32**	---
cyclohexenyl 18:0	**0.75**	---	---
cy19:0	---	---	3.07
membr20:1	0.90	---	---
Total miscellaneous	**1.65**	**1.32**	**5.55**
Unknowns			
unk 1	---	0.99	---
unk 2	---	0.81	---
unk 3	1.08	---	---
unk 4	0.19	---	---
unk 5	0.20	---	---
unk 6	0.22	---	---
Total unknowns	**1.69**	**1.81**	**0.00**

had high amounts of straight chain saturates, followed by monounsaturated fatty acids, and produced relatively minor amounts of terminally branched saturates. Although *C. muris* also produced large amounts of straight chain saturates, followed by monounsaturated fatty acids, a much higher percentage of its profile consisted of terminally branched saturates.

Hexadecanoic acid (16:0) was the most abundant FAME in all three species. In *C. parvum*, 18:1ω9c was second, followed by stearic acid (18:0). These were reversed in *C. baileyi*, which had a greater percentage of 18:0, followed by 18:1ω9c. Although *C. muris* contained 18:1ω9c as its second most abundant fatty acid, its profile had an almost equal amount of *anteiso*-pentadecanoic acid (a15:0), raising its percentage of terminally branched saturates to a much higher proportion than in either *C. parvum* or *C. baileyi*.

Minor amounts of three unusual fatty acids (in bold in Table IIA-1) were found in two of the three profiles. *C. parvum* contained cyclohexenyl 18:0, whereas cyclohexyl 17:0 and the monounsaturated fatty acid 16:1ω10 were both found in *C. muris*.

A hierarchical tree diagram of the data is presented in Figure IIA-1. This analysis shows that the relationship between *C. muris* and *C. baileyi*

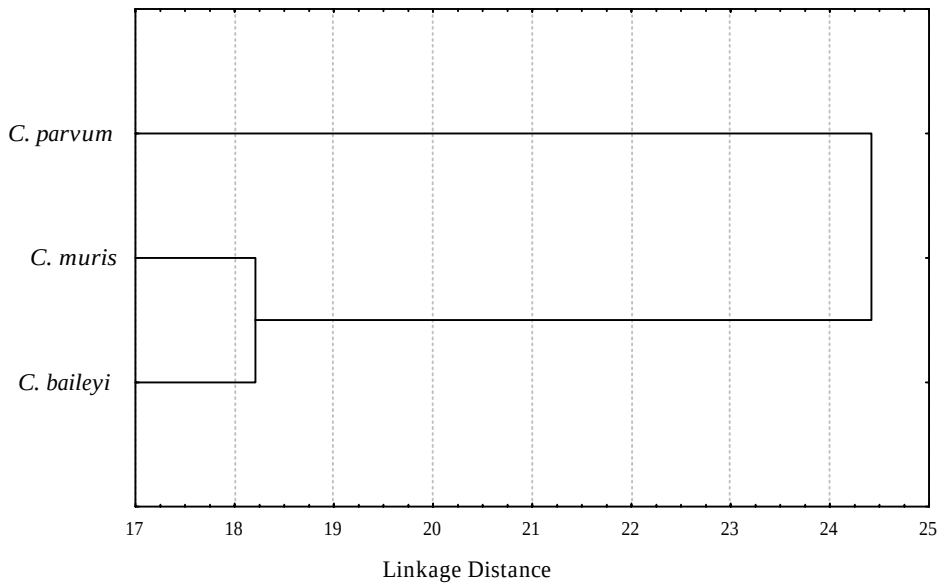


Figure IIA-1. Hierarchical tree diagram of three *Cryptosporidium* species. This diagram includes data from Table IIA-1. Cluster analysis was performed using complete linkage rules and Euclidean distances. Respective arms of the dendrogram are identified for each species of *Cryptosporidium*. Similarity between species can be measured by the linkage distances, with a distance of 17 being more similar than a distance of 25.

is much closer than the relationship of either of these species to *C. parvum*, as evidenced by the linkage distances between the three species.

A phylogenetic tree (Figure IIA-2) was constructed from partial 18S DNA sequences retrieved from GenBank. The sequences were manually aligned to the 18S sequence data from the Ribosomal Database Project (RDP) (Larsen *et al*., 1993). After construction, the tree was pruned from a larger analysis containing all the representative 18S sequences compiled by RDP. This tree shows that *C. baileyi* and *C. muris* are more closely related to each other than to *C. parvum*. However, all three species are more closely related to each other than to other species in the data bank, including other oocyst-producing species such as *Eimeria.*

DISCUSSION

Only the basic phospholipid composition of fresh *Cryptosporidium parvum* oocysts has been documented (Mitschler *et al*., 1994; White *et al.*, 1997). The current study was undertaken to characterize the phospholipids of *Cryptosporidium parvum* more thoroughly than past studies, and to compare the profile obtained for *C. parvum* to the profiles

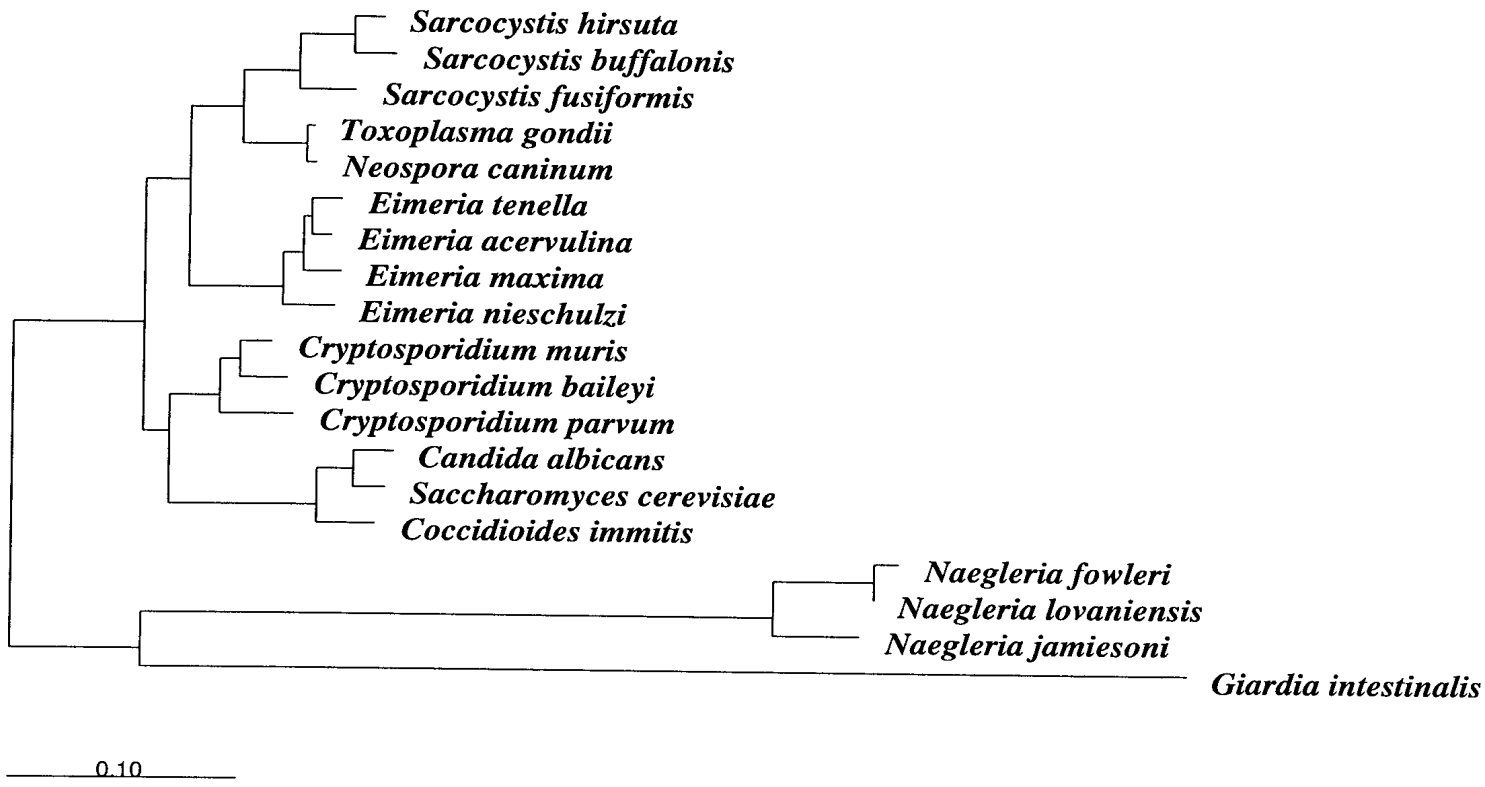


Figure IIA-2. Phylogenetic tree using 18S DNA sequences. Partial 18S sequences were retrieved from GenBank, with accession numbers as follows: AF093495 (*C. baileyi*), X64342 (*C. muris*), U67115 (*E. acervulina*), U67117 (*E. maxima*), U40263 (*E. nieschulzi*), and U40264 (*E. tenella*). They were manually aligned to the 18S sequence data from RDP (Larsen *et al.*, 1993).

of *C. muris* and *C. baileyi*. Such a comparison might identify fatty acids unique to *Cryptosporidium*.

As Table IIA-1 demonstrates, analysis of polar lipid fatty acids clearly differentiates these three species of *Cryptosporidium* from each other. The differences most likely are due to the relative quantities of the three most abundant fatty acids in the profiles, as well as the appearance of minor amounts of three unusual fatty acids found in two of the three species.

The results shown in Table IIA-1 for *Cryptosporidium parvum* correlate well with those previously published by Mitschler *et al*. (1994). That study also found the predominant fatty acid to be palmitic acid (16:0), at approximately the same mole percentage found in this study (Table IIA-2). The major discrepancy between the two profiles lies in the difference in the relative proportions of 18:1 and 18:2. This difference can be explained by the fact that 18:1ω9 is a precursor for 18:2ω6 (Anderson, 1987). Therefore, growth conditions of the oocysts could account for the presence of more 18:2 than 18:1 in Mitschler's study, in comparison to the profile from this study.

The techniques used in the Mitschler study probably account for the smaller variety of fatty acids found when compared to the variety identified in this study. Some of the fatty acids found in minor amounts

Table IIA-2. Comparison of relative proportions (mole percentages) of phospholipid fatty acids from *Cryptosporidium parvum* in this study (Table IIA-1) and results obtained by Mitschler *et al*. (1994).

Fatty acid	*C. parvum*[a]	*C. parvum*[b]
Straight chain saturates		
14:0	1.41	--- [c]
15:0	0.13	---
16:0	32.20	31.1
17:0	0.60	---
18:0	13.97	15.6
20:0	---	trace
22:0	---	---
Total straight chain saturates	**48.31**	**46.7**
Terminally branched saturates		
a13:0	---	---
i15:0	---	---
a15:0	0.24	---
i16:0	---	---
i17:0	0.18	---
a17:0	0.27	---
Total terminslly branched saturates	**0.69**	**0.0**
Monounsaturates		
16:1	0.53	trace
18:1	35.68	22.4
19:1	0.23	---
20:1	0.35	---
Total monounsaturates	**36.79**	**22.4**

Table IIA-2 (Continued).

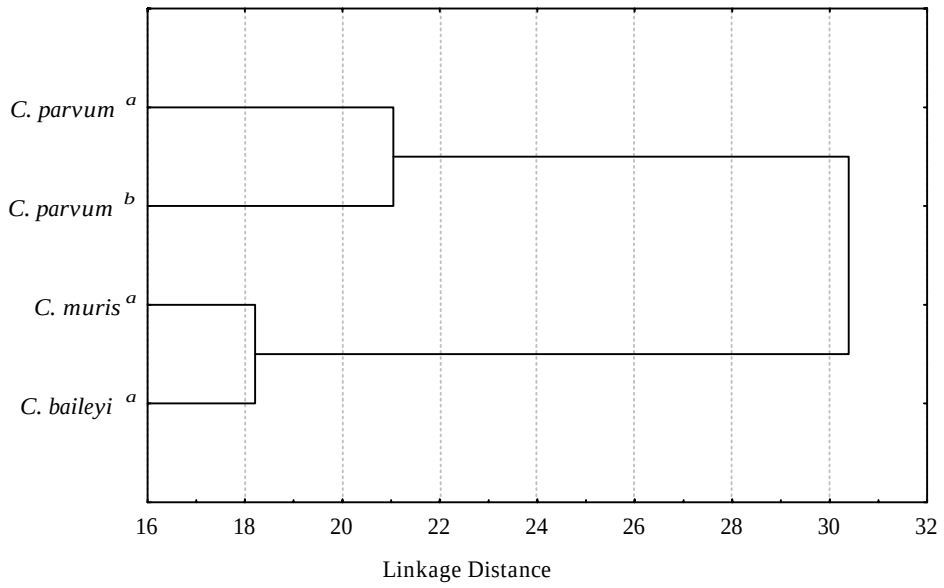
Fatty acid	*C. parvum*[a]	*C. parvum*[b]
Polyenoics		
18:2	9.91	28.6
20:2	0.36	---
20:4	0.61	2.3
Total polyenoics	**10.88**	**30.9**
Miscellaneous		
cy17:0	---	---
cyclohexyl 17:0	---	---
cyclohexenyl 18:0	0.75	---
cy19:0	---	---
membr20:1	0.90	---
Total miscellaneous	**1.65**	**0.0**
Unknowns		
unk 1	---	---
unk 2	---	---
unk 3	1.08	---
unk 4	0.19	---
unk 5	0.20	---
unk 6	0.22	---
Total unknowns	**1.69**	**0.0**

[a]Data from this study (Table IIA-1)
[b]Data from Mitschler *et al*. (1994)
[c]Dash (--) indicates the fatty acid was not detectable

in this study were not detected by Mitschler *et al* (1994). That group used thin layer chromatography (TLC) for separation of fatty acids, modified from the method of Gilfillan *et al.* (1983). Lipids were visualized with iodine vapor, and plates were then scraped for transesterification and analysis. This is an old separation procedure, and some loss of sample is possible when plates are scraped. Further, the visual identification of the location of lipids on the TLC plates with iodine vapor may be more difficult for small quantities of lipids.

A hierarchical tree diagram, which includes both data from this study and from the study by Mitschler et al., is presented in Figure IIA-3. This cluster analysis confirms that Mitschler's profile is most similar to the *C. parvum* profile in this study, and that significant differences are found between Mitschler's data for *C. parvum* and the data in this study for *C. muris* and *C. baileyi*.

Other researchers found that the majority of the PLFA in *C. parvum* consisted of 18:1ω9c (53.1 %), followed by 18:0 (32.3%). The third and fourth most abundant fatty acids were 16:0 (17.0%) and 18:2 (12.6%), respectively (White *et al*., 1997). The differences in these data are difficult to interpret, but could be due to differences in sample holding time and storage, as these parameters were not provided (Amy *et al*., 1994; White *et al*., 1979b).



[a]Data from Table IIA-1
[b]Data from Mitschler *et al*. (1994)

Figure IIA-3. Hierarchical tree diagram of three species of *Cryptosporidium*, combining data from Tables IIA-1 and IIA-2. The FAME profiles used for this cluster analysis included only those fatty acids from Table IIA-1 comprising ≥ 1 % of the total. Cluster analysis was performed using complete linkage rules and Euclidean distances. Respective arms of the dendrogram are identified for each species of *Cryptosporidium*, along with the study from which the data was drawn. Similarity between species can be measured by the linkage distances, with a distance of 16 being more similar than a distance of 32.

To determine the similarity of the hierarchical tree diagram constructed with PLFA data (Figure IIA-1) to DNA phylogeny, a phylogenetic tree using 18S DNA analysis (Figure IIA-2) was also constructed. A comparison of these two figures shows that the PLFA hierarchical tree compares favorably to the phylogenetic tree, as *C. baileyi* and *C. muris* are closer to each other on both trees, and *C. parvum* is more distantly related to the other two.

The Signature Lipid Biomarker (SLB) method of analysis has been successfully applied to the species-specific identification of *C. parvum*, *C. muris* and *C. baileyi*. It should be noted that the relatively minor amounts of 16:1ω10, cyclohexyl 17:0 and cyclohexenyl 18:0 may or may not be reproducible, due to the small quantities of these fatty acids in the profiles. However, the differences in 16:0, 18:0, and 18:1ω9c (in combination with 18:2ω6), should give a clear identification for the differentiation between these three species. The usefulness of SLB analysis removes one of the pitfalls of indirect fluorescent antibody (IFA) analysis--errors associated with the interpretation of IFA data. A lack of replicates across the studies suggests that additional work on the PLFA profiles of *Cryptosporidium* would be beneficial to the signature lipid biomarker distinctions of the genus. Increasing the number of replicates would allow for the use of other statistical methods, such as principal

component analysis and discriminant analysis. Sterol analysis might also be helpful, as these are commonly found in microeukaryotes (Nichols *et al*., 1987).

Since purified oocysts were used in this study, studies that include other matrices would also be beneficial. In addition, the results of this study also suggest that it might be necessary to concentrate the oocysts by a method such as filtration of large quantities of drinking water or recreational water to be able to detect the small quantities of unusual fatty acids by SLB analysis. However, the application of SLB analysis should be useful for the identification of *Cryptosporidium*, particularly the human pathogen *C. parvum*, in these matrices. It also provides a way to easily distinguish *C. parvum* from other species of *Cryptosporidium*, thus indicating the presence or absence of the potential threat to human health in drinking water and recreational water.

PART B. 10-HYDROXY STEARIC ACID AND *CRYPTOSPORIDIUM*

INTRODUCTION

The detection, identification, and determination of the presence of infectious protozoan parasites are a major problem in the potable water industry. Cryptosporidium is of special concern as it is widespread in surface water samples, has extreme resistance to disinfection by chlorine, completes its infectious cycle only in human or mammalian hosts (Bird and Smith, 1980), and is potentially fatal to immunocompromised individuals who become infected (Fayer et al., 1997). Most clinical infections in humans and mammalian livestock are due to C. parvum, although infection with a C. baileyi-like organism in a severely immunocompromised individual has been reported (Ditrich et al., 1991). C. muris infections in humans have not been substantiated, although infections have been reported in animals other than mice (Casemore et al., 1997).

Existing methodologies for the detection of C. parvum oocysts in drinking water are not satisfactory (Newman, 1995). Successful methods

for detection of Cryptosporidium must be highly sensitive, as the presence of as few as 10-30 C. parvum oocysts per liter of finished water may initiate infections (Haas and Rose, 1995). This method must be highly specific to Cryptosporidium, sufficiently rapid so as to be potentially useful to the water industry, and also provide an indication of viability and/or infectivity.

Studies have shown that freezing *C. parvum* oocysts at -70°C not only rendered them noninfectious to neonatal BALB/c mice but also induced shifts in membrane lipid composition (Fayer and Nerad, 1996; White *et al*., 1997). The shifts included a change in the polar lipid/neutral lipid ratio, a shift in fatty acid composition, a decrease in cholesterol, and loss of an unusual fatty acid, 10-hydroxy stearic acid (10-OH 18:0) (Schrum *et al*., 1997), which was proposed as a potentially quantitative biomarker (White *et al*., 1997). Identification of this fatty acid as unique to the genus *Cryptosporidium* could provide a signature lipid biomarker (SLB) for use in complex environmental matrices, such as water distribution system biofilms.

The objective of this study was to determine the uniqueness of 10-hydroxy stearic acid as a signature lipid biomarker for the genus *Cryptosporidium*.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade. The standards for hydroxy fatty acids (2-hydroxy eicosenoic acid, 12-hydroxy stearic acid, and 6-hydroxy stearic acid) were purchased from Matreya (Pleasant Gap, PA). All solvents were obtained from Baxter Scientific Products (McGaw Park, IL) and were of gas chromatography grade. N/O-*bis*(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce Chemical Company (Rockford, IL).

Glassware

All glassware was washed in a 10% (vol/vol) Micro cleaner solution (Baxter Diagnostics, Deerfield, IL), rinsed ten times with tap water and then ten times in deionized water. The glassware was then heated overnight in a muffle furnace at 450°C to remove any carbon contamination.

Rubber bulbs

Amber rubber bulbs (2-ml, catalog number 14065B, Fisher Scientific Co., Pittsburgh, PA) were utilized with glass pipettes to transfer solutions, except where noted.

Parasites

Oocysts of *Cryptosporidium parvum* were purified from calf feces and kindly provided by Ronald Fayer (USDA, Beltsville, MD). Purification procedures are detailed in Fayer (1995). Purity was checked upon receipt by microscopic examination.

Lipid extraction

Purified oocysts loaded on glass filters were extracted at room temperature and atmospheric pressure using a modified Bligh / Dyer technique (Bligh and Dyer, 1959; Guckert *et al*., 1985; White *et al*., 1979b). This method extracts cellular lipids using a one-phase chloroform / methanol / phosphate buffer (50 mM, pH 7.4, 1:2:0.8 by volume) solvent system for 4 hours before adding chloroform and deionized water to form two phases (final solvent ratios: chloroform / methanol / phosphate buffer / water, 1:1:0.4:0.5 by volume).

Then the organic phase was collected and dried under a stream of nitrogen. The lipids were stored at -20°C until purification.

Purification of lipids

The total lipid extracted was dissolved in 0.5 ml chloroform and separated using silicic acid columns (10-cm column length, 0.5-cm inner diameter, 100-200 mesh particle size). Each sample was loaded onto a column in 100-200 μl chloroform and then eluted in batches with 5 ml chloroform, 5 ml acetone, and 10 ml methanol so as to elute neutral, glyco- and polar lipids, respectively (Guckert *et al*., 1985). Bulk elution of the neutral lipids, including sterols and diglycerides, with chloroform was followed by recovery of the free fatty acids in the acetone, or glycolipid, fraction. For each fraction, the solvent was removed under a stream of nitrogen. The lipid fractions were stored at -20°C until methylation.

HPLC/ESI/MS analysis

Reverse-phase high-performance liquid chromatography (C-18 Alltech Altima 5 μm particle size, 150 mm in length, 1 mm diameter column; Deerfield, IL) with a mobile phase consisting of methanol / ultrapure water / aqueous ammonia (100:6:1) at a flow rate of 50 μl / minute was used to separate hydroxylated free fatty acids from

phosphatidylethanolamine (PE) (Burkhalter *et al*., 1998). Those components eluting with a retention time typical of free fatty acids (5-14 minutes) were nebulized into the electrospray ionization (ESI) inlet of either the VG (Micromass, Inc., Beverly, MA) Quattro II triple quadrupole mass spectrometer or the Micromass Platform II single quadrupole mass spectrometer. Analyses were performed in the negative mode of ionization using elevated cone voltages first, and later using low orifice voltages. Tuning was performed such that maximum sensitivity was attained while achieving a minimum of unit resolution at a *m/z* value of 300 atomic mass units (a.m.u.) (resolution = 300). Resolution values of between 750 and 1,000 satisfied this criterion. Narrow mass scans in the continuum mode of data acquisition were performed over a mass range spanning 320-270 a.m.u. at a scan rate of 0.4 s with an interscan delay of 0.1 s. In addition, selected ion monitoring of mass 299 was utilized to provide increased sensitivity.

Mass spectral optimization studies found no significant gain in sensitivity upon the addition of organic bases. This was advantageous, since reverse-phase columns are not generally stable at pH values greater than 9. Addition of water to methanol resulted in a slight loss in sensitivity with model synthetic compounds. The initial mobile phase of

methanol / water / aqueous ammonia was utilized so as to resolve free fatty acids from PE.

After discovery that the novel biomarker was present as a free fatty acid and no PE species were present in the glycolipid fraction (see Results section), the chromatographic conditions were altered for a more efficient separation. Using a mobile phase of 100% methanol reduced the analysis time with a slight gain in sensitivity. The voltage applied across the capillary was –2.13 kV, the high voltage lens was set as 0.2 kV, and the initial cone voltage was set at –22 V so as to promote "soft" ionization. The nitrogen drying gas and nebulizing gas flow rates were set at 300 and 25 L / h, respectively. The temperature of the ESI source was set at 120°C. The photomultiplier detector was set to its maximum gain value.

Extracted ion chromatograms at a *m/z* value of 299 were monitored. Ion currents for 299 in the 11-14 minute range were diagnostic of 10-OH 18:0 in the free fatty acid form. These were averaged with background subtraction, resulting in a diagnostic mass spectrum.

Mild alkaline methanolysis

A mild alkaline methanolysis procedure (Guckert *et al.*, 1985) was utilized to prepare methyl esters of the ester-linked fatty acids of the

glycolipids. The glycolipid fatty acid methyl esters were dried under a stream of nitrogen and then were stored at -20°C until analysis.

Preparation of trimethylsilyl ether adducts

Immediately before GC / MS analysis, 100 µl BSTFA was added to the glycolipid methyl esters and the samples were heated for 30 minutes at 60°C (Nichols *et al*., 1983). The samples were then dried under a stream of nitrogen, resuspended in hexane and analyzed.

GC/MS analysis

Verification of fatty acid structure was achieved using mass spectrometry performed on a Hewlett Packard 5971 mass selective detector coupled to a Hewlett-Packard model 5890 series II gas chromatograph. Chromatographic separations were carried out on a HP-1 fused-silica capillary column [50 m by 0.2 mm (inner diameter)] of 0.11 µm film thickness. The column was kept at an initial temperature of 60°C for 2 minutes, and the temperature was programmed to increase from 60 to 150°C at 10°C / min, then to 312°C at 3°C / min. The injector and detector were maintained at 290°C and 300°C, respectively. Splitless injections were performed with a Hewlett-Packard model 7673 autosampler. The split valve was opened 1 min after injection. The carrier gas, helium, was

maintained at an inlet pressure of 195 kPa to establish a carrier gas flow rate of approximately 1ml/min (Ringelberg *et al*., 1989). Fatty acid methyl esters were identified through comparison of their mass spectra and retention behavior with synthetically prepared standards (Matreya, Inc., Pleasant Gap, PA), and to a mass spectral library of FAME standards (Christie, 1989; Guckert *et al*., 1989). Peak areas were determined using a programmable laboratory data system, Enhanced ChemStation (Hewlett-Packard, Palo Alto).

Fatty acid nomenclature

Fatty acids are abbreviated by the number of carbon atoms, a colon, degree of unsaturation, which is then followed by an omega symbol (ω) indicating the number of carbons from the methyl end of the molecule to the position of the first, and sometimes only, double bond. The prefix "cy" represents the cyclopropyl isomer of a fatty acid, and "i" and "a" represent *iso*-branched and *anteiso*-branched isomers, respectively. The number preceding the abbreviation "Me" indicates the position of a methyl group relative to the carbonyl carbon followed by the number of carbons in the fatty acid chain. Suffixes "c" and "t" represent *cis* and *trans* geometric isomers of the unsaturation, respectively. An ambiguity in this naming convention is that the number of carbons given for *iso*-, *anteiso*-,

unknown branched, and cyclopropyl fatty acids include all of the carbons in the molecule except the esterified methyl group. Therefore, 10Me18:0 indicates a methyl group attached to an 18-carbon side chain at the 10 position from the carbonyl (Kates, 1986).

RESULTS

Identification of 10-OH 18:0 as a free fatty acid

Retention time

The acid 10-OH 18:0 migrates in reverse-phase high performance liquid chromatography under the conditions described in the Materials and Methods with a retention time of 5-14 minutes (Figure IIB-1, higher left peak). This is characteristic of hydroxylated free fatty acids (2-OH 20:0) (Figure IIB-2), whereas phosphatydylethanolamine (PE) elutes at 20-22 minutes under the same conditions (Figure IIB-1, right peak, and Figure IIB-3).

Examination of ion current

Analyses were initially performed in the negative mode of ionization so as to promote fragmentation ensuing from cleavage of

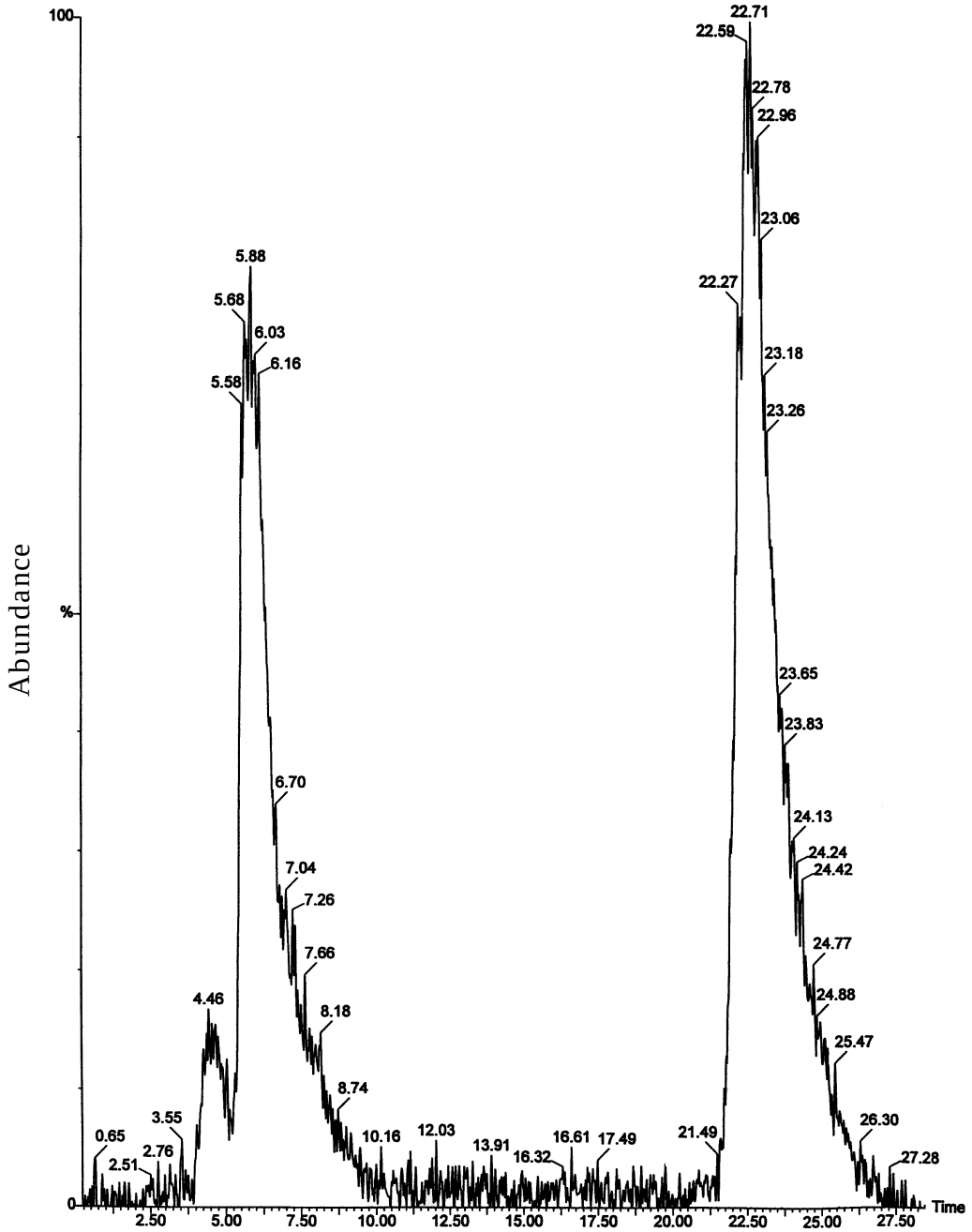


Figure IIB-1. Chromatogram of 10-OH 18:0 (left peak) and phosphatidylethanolamine (right peak) using reverse-phase high performance liquid chromatography.

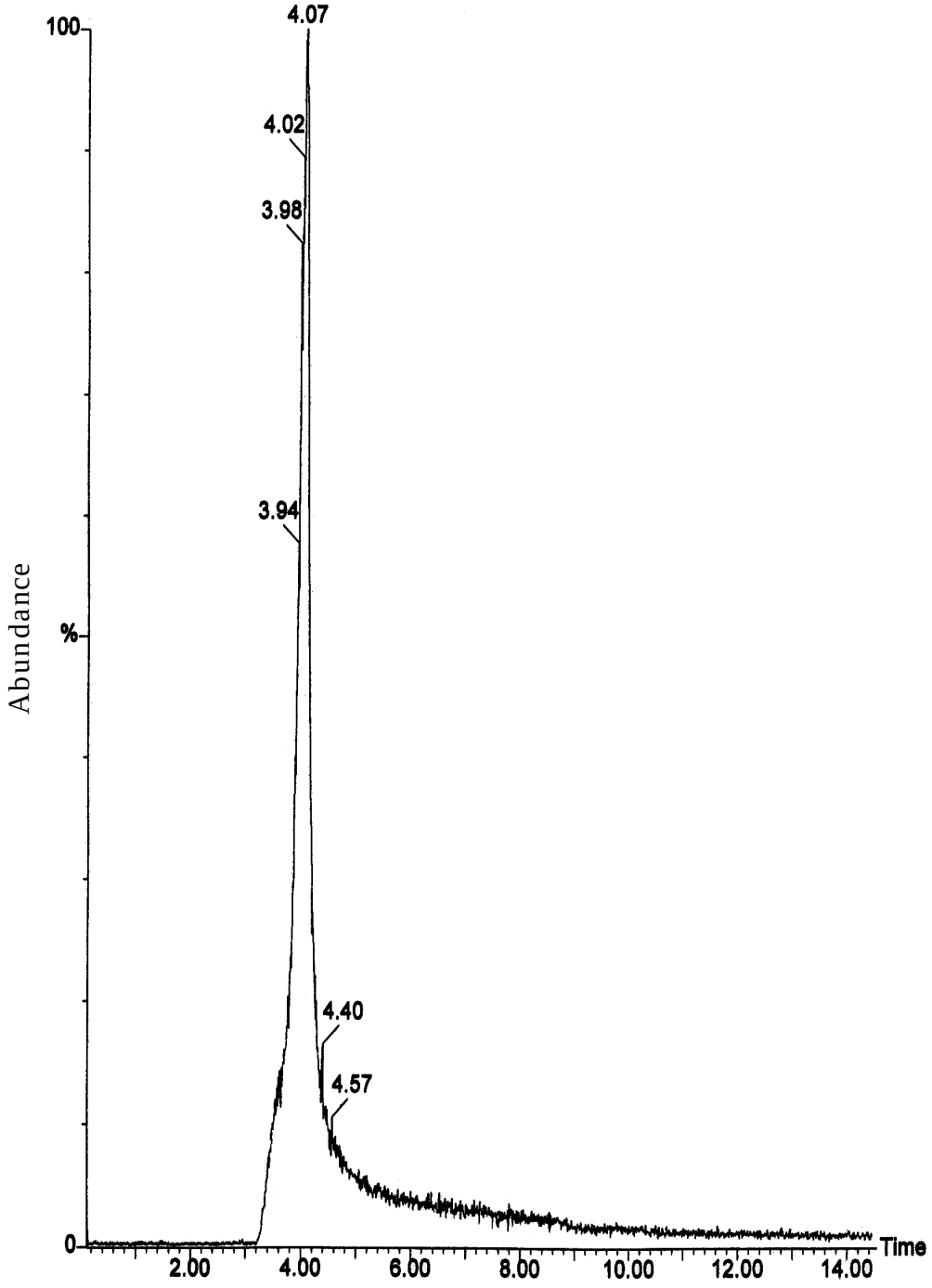


Figure IIB-2. Chromatogram of 2-OH 20:0 using reverse-phase high performance liquid chromatography.

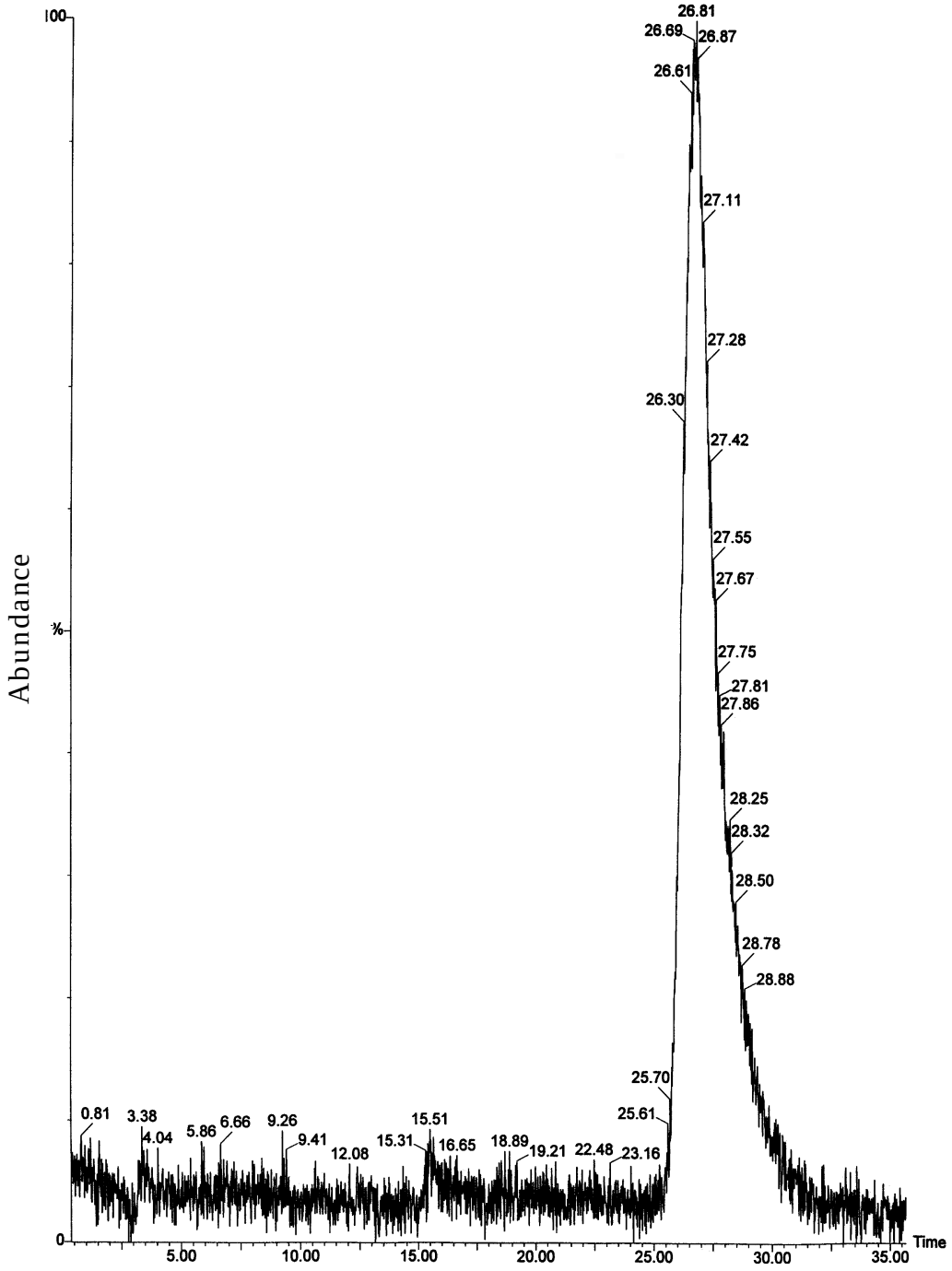


Figure IIB-3. Chromatogram of phosphatidylethanolamine using reverse-phase high performance liquid chromatography.

the two-acyl functionalities with charge retention on the free fatty acids formed through acyl cleavage. Figure IIB-4 illustrates the fragmentation behavior of phospholipids under negative ion electrospray ionization. Electrospray ionization provides information regarding the endogenous phospholipid (deprotonated molecular ion), class of phospholipid (fragment 1—polar head group fragment), as well as the nature of the fatty acid constituents (fragments 2 and 3) acylated to a known phospholipid. Fragmentation at the *sn*-2 position (fragment 2) is generally, but not always, favored over fragmentation at the *sn*-1 (fragment 3) position.

Examination of the ion current for a *m/z* (mass / charge) value of 299 at both high and low orifice voltages supplied additional evidence as to the nature of the 10-OH 18:0. The ion current for 10-OH 18:0 was over two orders of magnitude less at high orifice voltages than at low orifice voltages (8.53×10^5 peak height units for –200 V, compared to 1.63×10^8 peak height units at –22 V). The cone voltage of –200 V was found to maximize the dissociation of the acyl groups of synthetic phospholipids, resulting in a maximal ion current for the fatty acyl constituents at maximal orifice voltage settings. Lower cone voltages (-22 V) resulted in

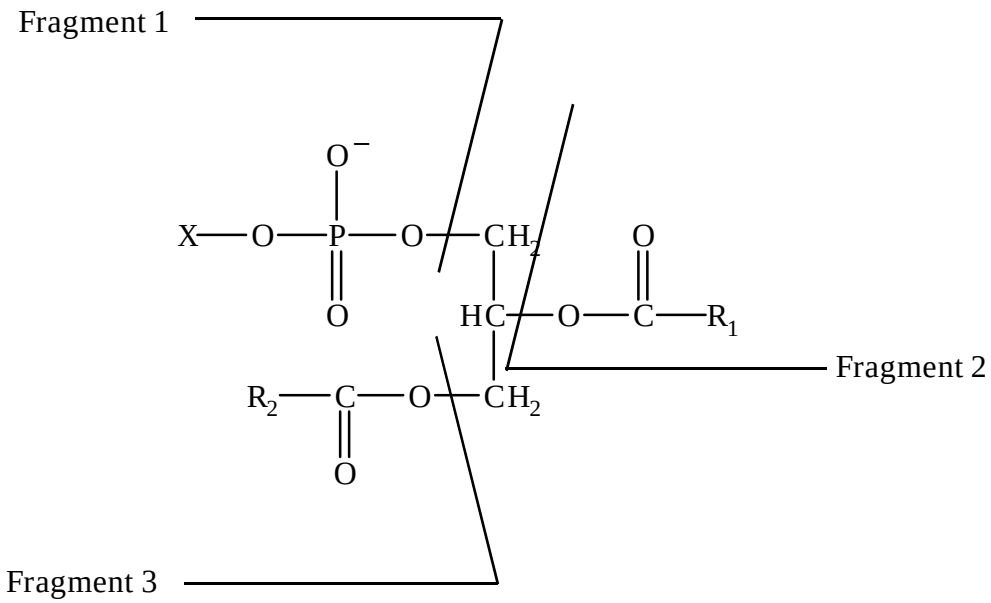


Figure IIB-4. Fragmentation behavior of phospholipids under negative ion electrospray ionization. ESI provides information regarding the endogenous phospholipid (deprotonated molecular ion), class of phospholipid (fragment 1—polar head group fragment), as well as the nature of the fatty acid constituents (fragments 2 and 3) acylated to a known phospholipid. Fragmentation at the *sn*-2 position (fragment 2) is generally, but not always, favored over fragmentation at the *sn*-1 (fragment 3) position.

maximal ion currents for the intact molecular species. Therefore, this observation provided further evidence that 10-OH 18:0 existed as an intact molecular species and was not acylated to any phospholipid.

Parent ion mass spectra

Previous investigators found the 10-OH 18:0 localized at the *sn*-2 position of PE (Schrum *et al*., 1997). However, this was not supported during utilization of the derivative ion scanning of specific parent ion capabilities of the triple quadrupole mass spectrometer in the negative ionization mode. Examination of those parent ions that result in a fragment ion corresponding to 10-OH 18:0 revealed no parents which could correspond to PE or any possible phospholipid.

Constant neutral mass loss scans

Constant neutral mass loss scans for a neutral loss of 141 in the acetone fraction in the positive mode of ionization revealed that no detectable quantities of phosphatidylethanolamine (PE) species were present. PE was found to be present in the methanol (polar lipid) fraction through neutral loss mass scans.

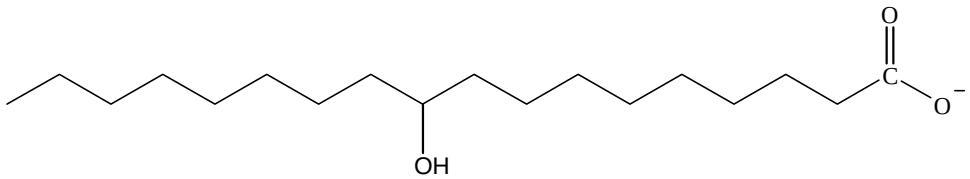
Identification of the free fatty acid as an eighteen-carbon fatty acid with hydroxyl functionality localized at the 10 position

Daughter ion mass spectra

Several fatty acids theoretically could produce a *m/z* value of 299 in the negative mode of ionization. Any monohydroxylated 18-carbon fatty acid with the hydroxyl group at position 2 through 17 would yield a deprotonated molecular ion at 299. A 17-carbon monoenoic fatty acid with a sulfhydryl (-SH) substituent is another possibility, along with the polyenoic fatty acid 20:6n-3. However, comparison of the parent / derivative ion mass spectrum of 10-OH 18:0 with synthetically available standards (6-OH 18:0 and 12-OH 18:0) showed that the free fatty acid was indeed 10-OH 18:0.

The first quadrupole acted as the mass filter, allowing only ions with a *m/z* value of 299 to pass into the second mass analyzer. Collision-induced dissociation (CID) of the parent molecule was accomplished in the hexapole collision chamber between the first and third quadrupoles in the presence of 0.5 Torr of argon at a collision energy of 30 eV. Scanning the third quadrupole mass analyzer monitored diagnostic fragments.

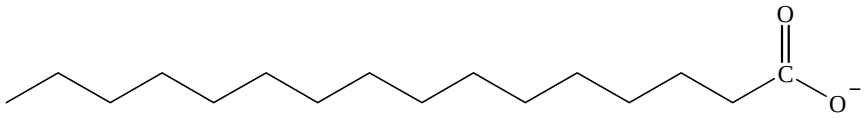
Figure IIB-5 shows the ions that result during low energy CID fragmentation which are indicative of hydroxy-substituted



10-hydroxy stearic acid

- H_2O → *m/z* = 281

-HCOOH → *m/z* = 253



palmitic acid

↓

No fragmentation observed under low energy CID conditions

Figure IIB-5. Ions resulting from low energy collision induced dissociation (CID) fragmentation which are indicative of hydroxy-substituted fatty acids as a class, contrasted with non-hydroxylated free fatty acids which do not undergo any significant fragmentation.

fatty acids as a class at a *m/z* value of 281 (loss of H_2O from the hydroxyl moiety) and at a *m/z* value of 253 (loss of formic acid). Under similar conditions, non-hydroxylated free fatty acids (*i.e.*, palmitic acid) do not undergo any significant fragmentation.

The site of the hydroxy functionality was confirmed to be in the 10 position through the examination of two charge-remote fragment ions that overlapped at a *m/z* value of 141 for 10-OH 18:0. Figure IIB-6 illustrates the expected fragment ions.

The daughter ion spectra for the *m/z* value of 299 in *Cryptosporidium parvum* are shown in Figure IIB-7. The peaks which are indicative of hydroxy-substituted fatty acids as a class (*m/z* values of 281 and 253) are present, as are the peaks which are indicative of site specific fragmentation for 10-OH 18:0 (*m/z* values of 141 and 155).

The two charge-remote fragmentation processes were confirmed through inspection of the daughter ion spectra of two other mid-chain, branched hydroxylated fatty acids, 6-OH 18:0 and 12-OH 18:0, under identical conditions. Analogous peaks expected (Figure IIB-8) and observed (Figure IIB-9) for 6-OH 18:0 were at *m/z* values of 197 and 85. Fragment ions for 12-OH 18:0 at *m/z*

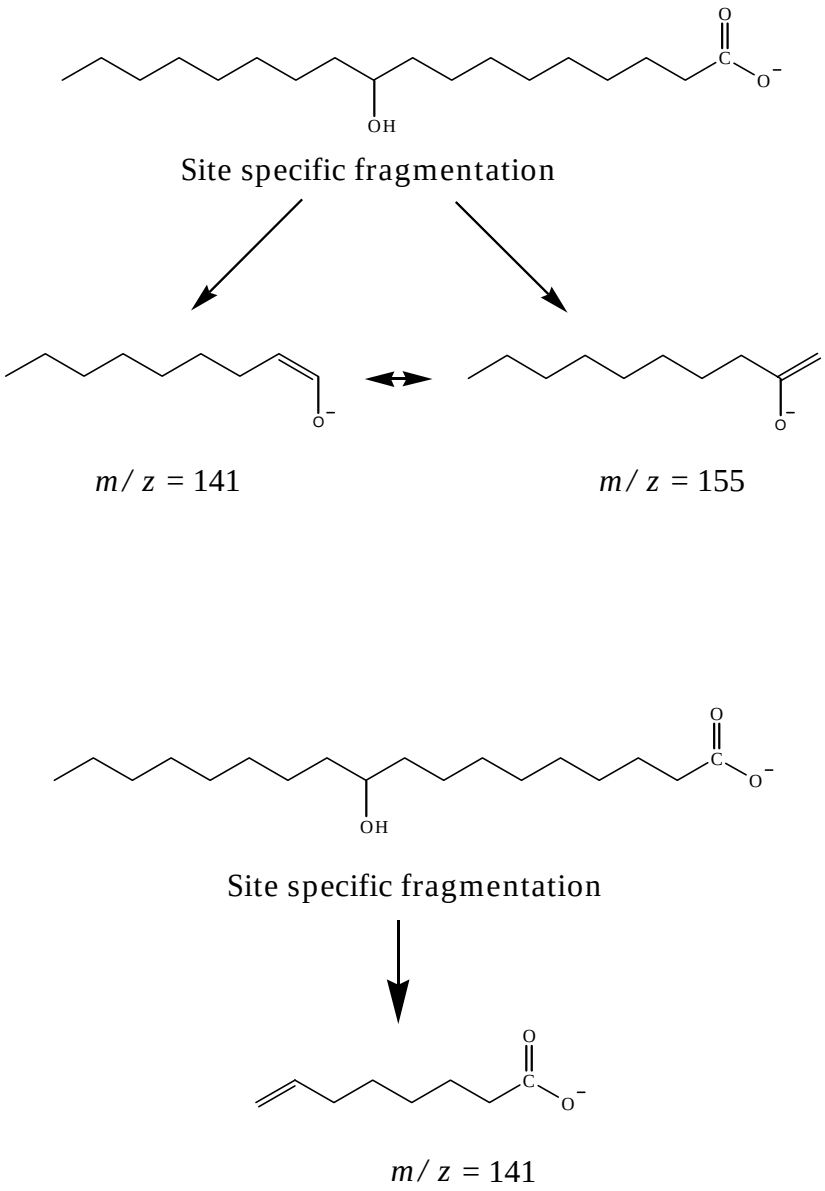


Figure IIB-6. Illustration of the two charge-remote fragment ions that overlap at a *m/z* value of 141 for 10-OH 18:0.

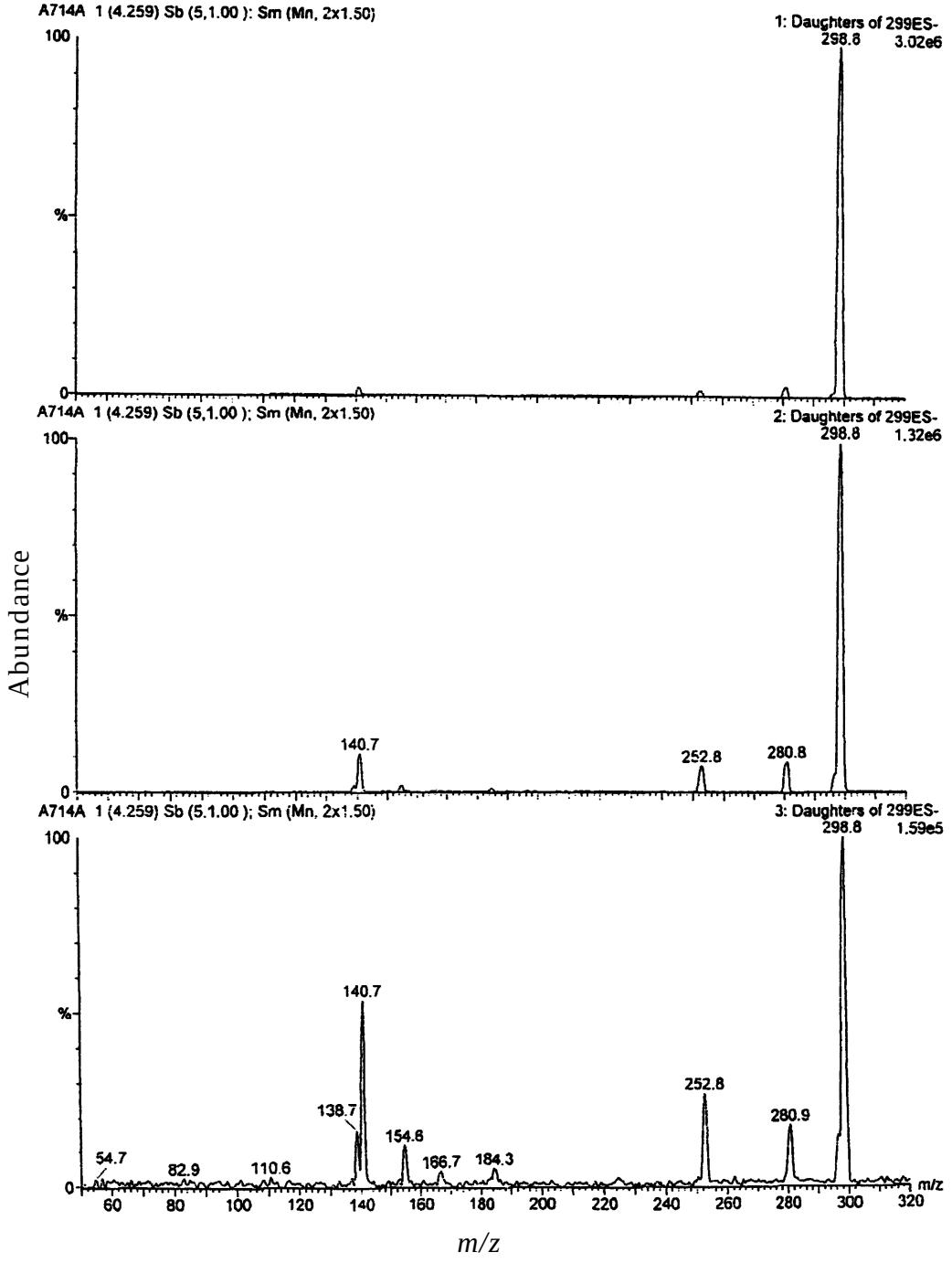


Figure IIB-7. Daughter ion spectra for the *m/z* value of 299 in *Cryptosporidium parvum*.

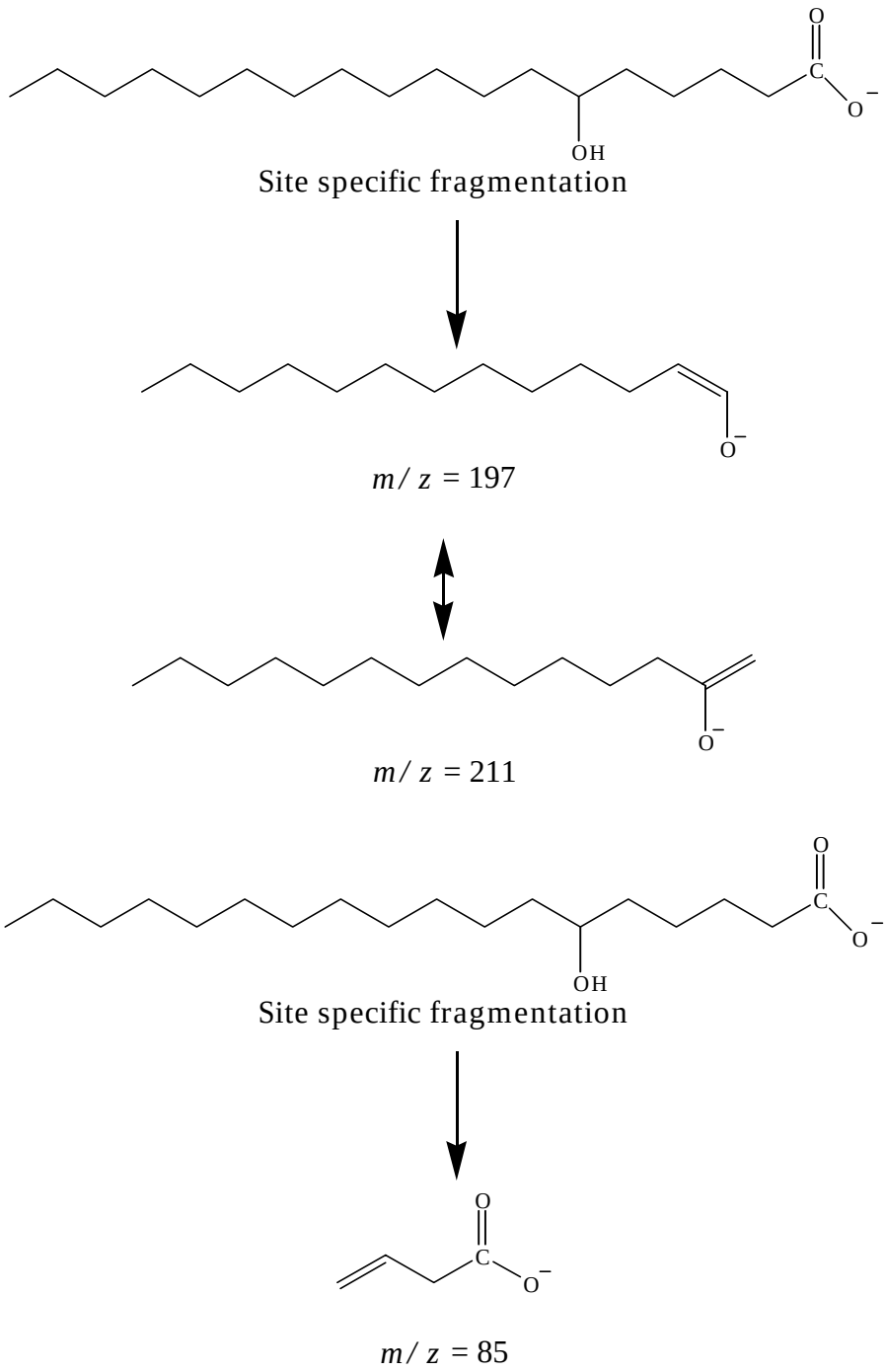


Figure IIB-8. Illustration of the two charge-remote fragment ions expected for 6-OH 18:0.

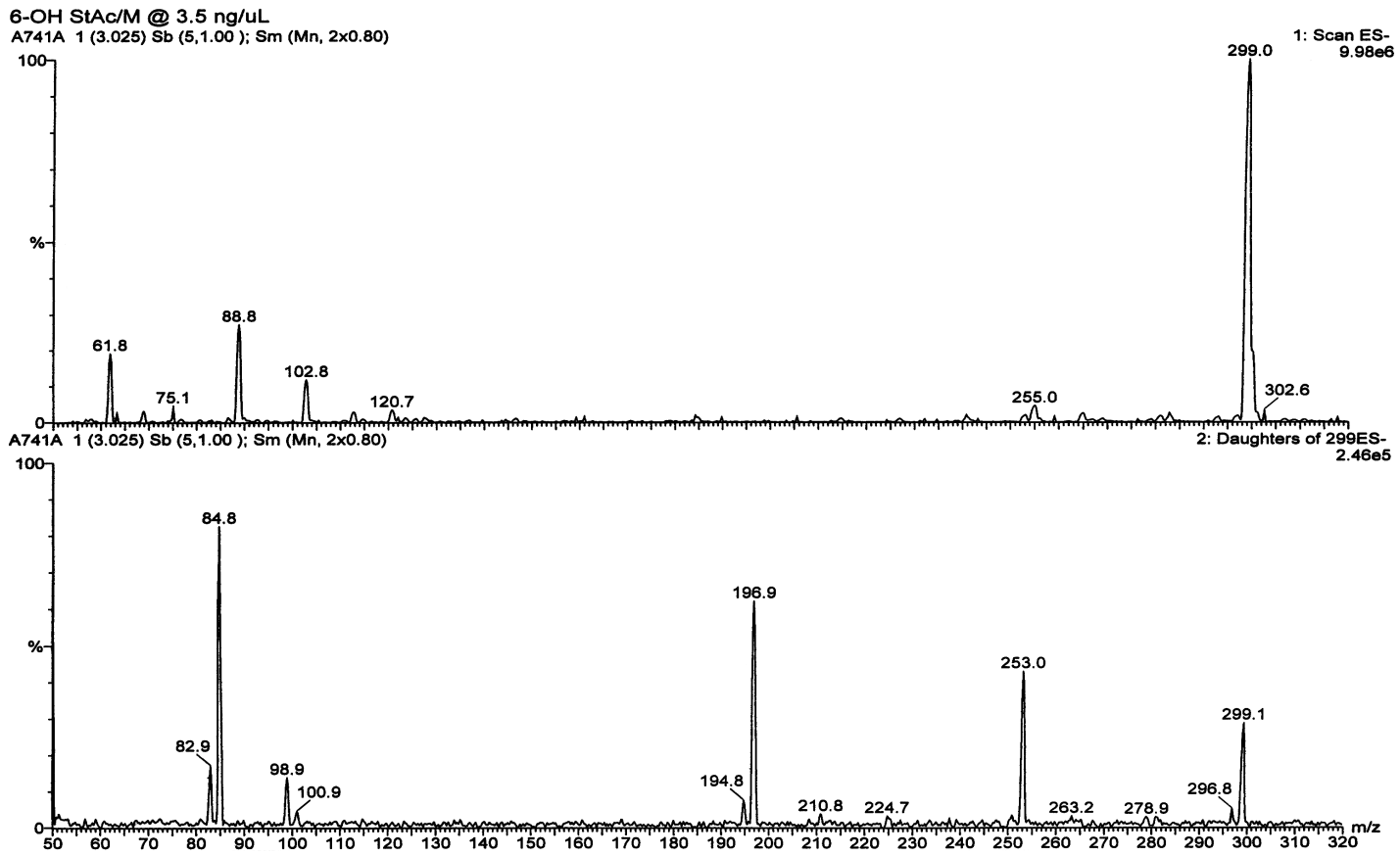


Figure IIB-9. Daughter ion spectra for the *m/z* values of 299 in 6-OH 18:0.

values of 113 and 169 were expected (Figure IIB-10) and observed (Figure IIB-11).

Identification of the source of 10-OH 18:0

The true origin of the 10-OH 18:0 in the *Cryptosporidium* samples was discovered after many repetitions of this assay. Wild fluctuations in the quantities of 10-OH 18:0 were observed. Occasionally no 10-OH 18:0 was present in an oocyst profile. This was attributed to handling of the oocysts, e.g., glass fibers, or clumping of oocysts. The random appearance in one of the sample blanks was the first indication that the problem was generated by the sample processing procedure itself. This was thought to have originated from an interfering contaminant. However, formation of the trimethylsilyl ether adduct of the methyl ester in the sample blank produced an analyte that possessed the chromatographic behavior and a mass spectrum of the analyte described by Schrum *et al.* (1997) as 10-OH 18:0.

This result motivated a re-examination of the sample handling procedure to locate the possible source of the 10-OH 18:0. All organic solvents (methanol, chloroform, and acetone) used in the assay were concentrated from an initial volume of 100 ml to a final volume of 100 µl

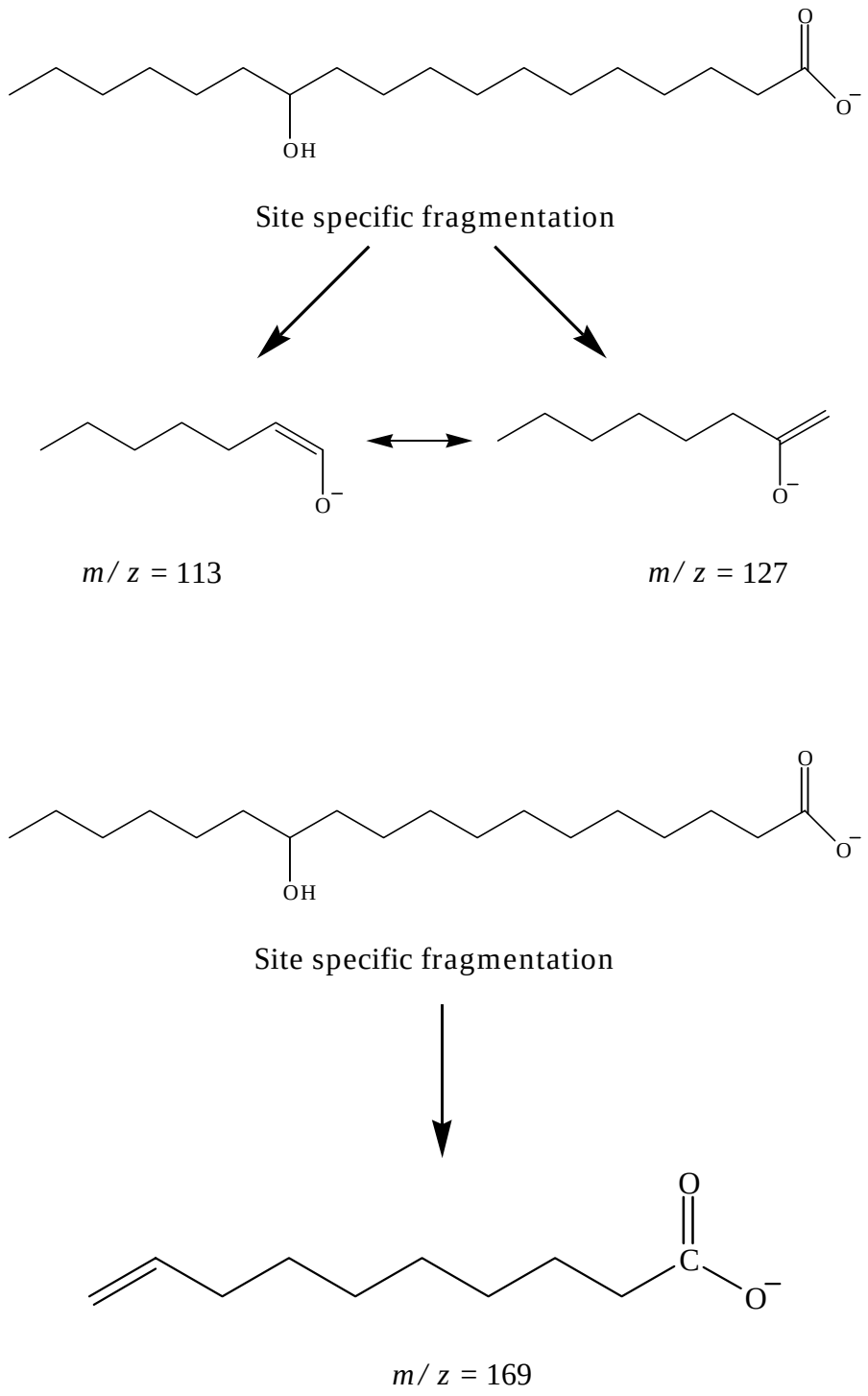


Figure IIB-10. Illustration of the two charge-remote fragment ions expected 12-OH 18:0.

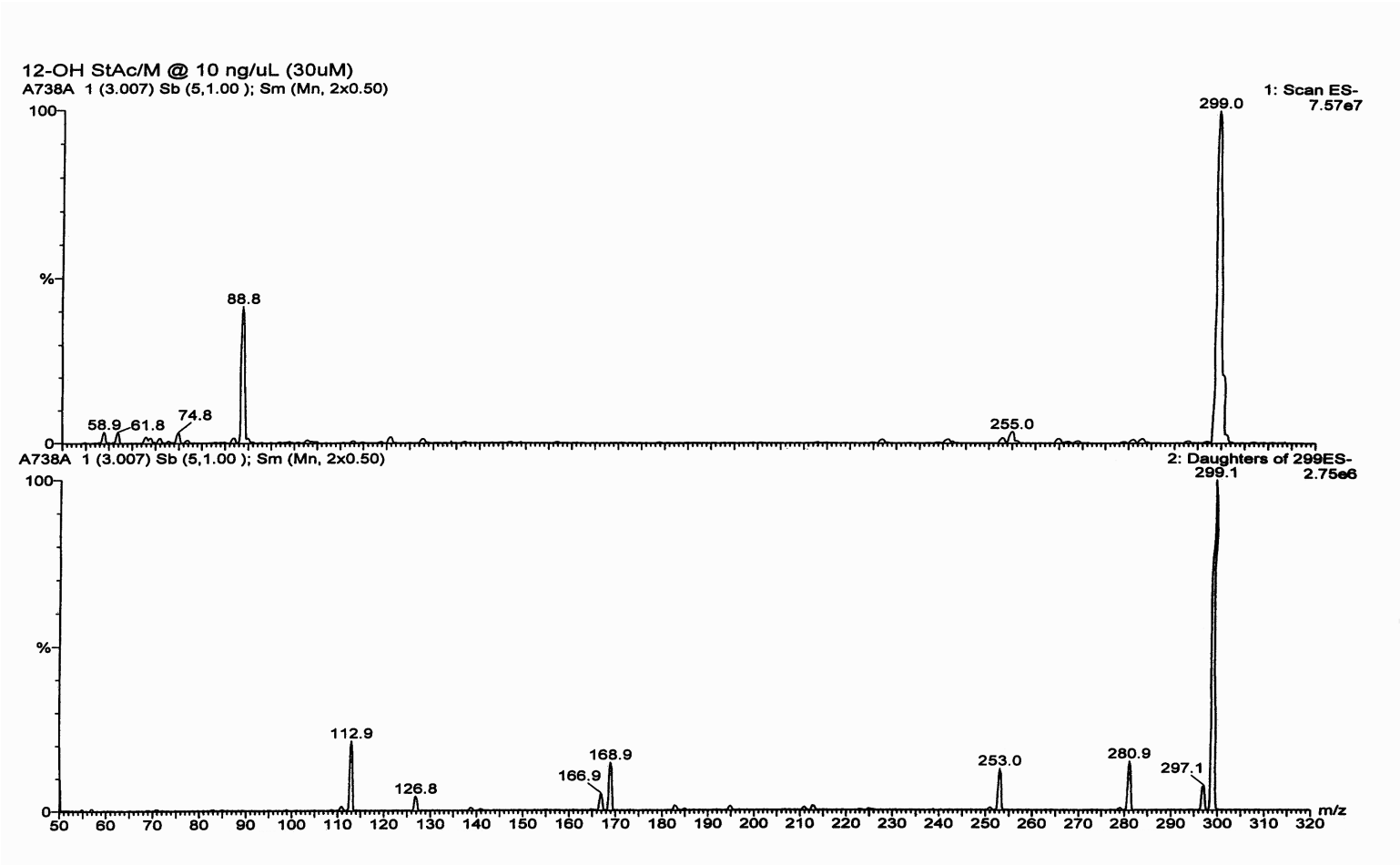


Figure IIB-11. Daughter ion spectra for the *m/z* value of 299 in 12-OH 18:0.

and injected onto the HPLC/ESI/MS system. None of the organic solvents were found to contain any 10-OH 18:0.

Then extractions were performed on the soaps and sterilization agents utilized to clean glassware: 1 ml of Fisherbrand solvent-free concentrator, 1 ml of Fisherbrand vesphenne II, and 1 ml of a 1:1 (vol/vol) mixture of the two were extracted using the modified Bligh/Dyer method (Bligh and Dyer, 1959; Guckert *et al*., 1985; White *et al*., 1979b). No 10-OH 18:0 was found in any of these three samples.

Subsequently, a series of blanks were carried through the extraction procedure. These blanks used all other possible variables, including the phosphate buffers, Millipore (Milford, MA) water, and rubber bulbs (Table IIB-1). Of the eight blanks, only blanks #2 and #5 contained 10-OH 18:0. Figure IIB-12 illustrates the fragmentation pattern of this analyte. The GC/MS spectrum of the trimethylsilyl ether adduct of this analyte revealed ions with *m/z* values of 59, 73, 215 and 273. Fragmentation patterns for 6-OH 18:0 (Figure IIB-13) and 12-OH 18:0 (Figure IIB-14) are included for purposes of comparison. The GC/MS spectrum of 6-OH 18:0 showed ions with *m/z* values of 217 and 271, while the spectrum of 12-OH 18:0 revealed ions with *m/z* values of 203 and 317. Both of these compounds also showed the characteristic ions at *m/z* values of 59 and 73.

Table IIB-1. Experimental design using blanks to locate the source of 10-OH 18:0.

Sample Name	Buffer	Repipetter	Bulb	Cap
Blank 1	#1[a]	Small[c]	No[e]	Good[g]
Blank 2	#1	Small	Yes[f]	Good
Blank 3	#1	Large[d]	No	Good
Blank 4	#2[b]	Large	No	Good
Blank 5	#2	Large	Yes	Good
Blank 6	#2	Small	No	Good
Blank 7	#1	Large	No	Bad[h]
Blank 8	#2	Small	No	Bad

[a]Buffer #1—in refrigerator, no preparation date on label
[b]Buffer #2—prepared 2/18/98
[c]Small—solvents used from small set of repipetters
[d]Large—solvents used from large set of repipetters
[e]No—no bulb in blank
[f]Yes—inserted bulb in blank
[g]Bad—caps had line through them or were badly discolored
[h]Good—caps were not discolored

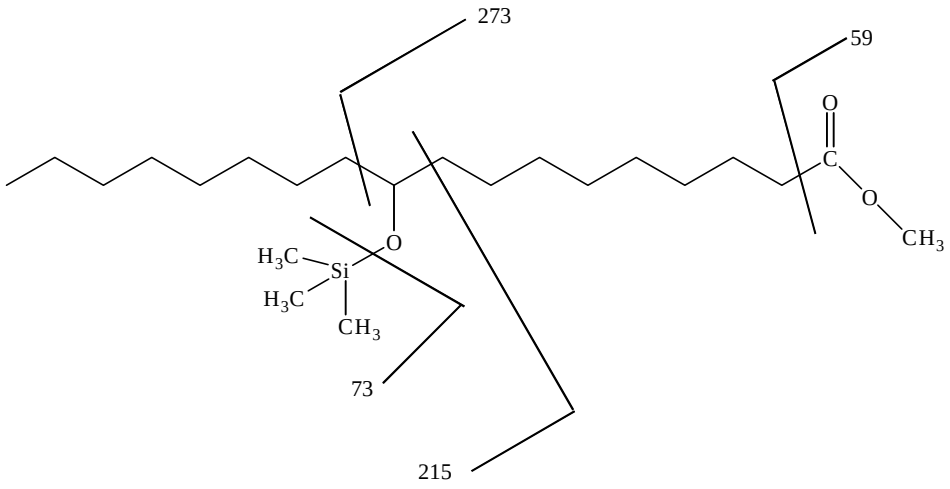


Figure IIB-12. Fragmentation pattern for the trimethylsilyl ether adduct of 10-OH 18:0 methyl ester.

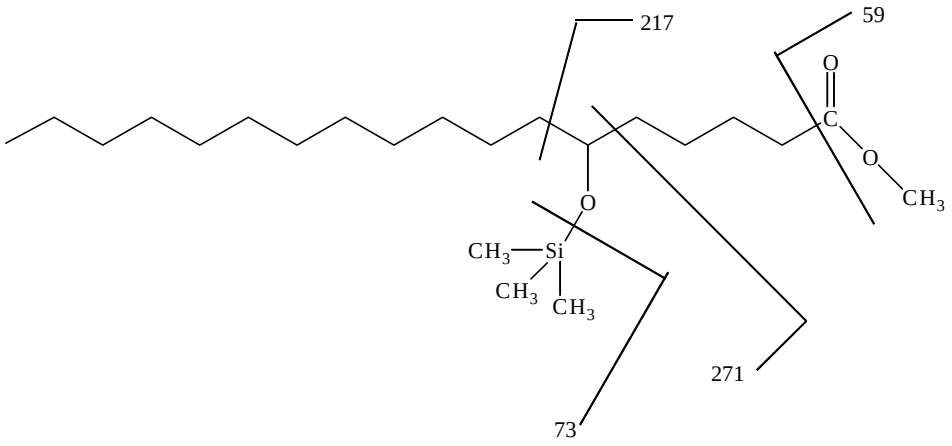


Figure IIB-13. Fragmentation pattern for the trimethylsilyl ether adduct of 6-OH 18:0 methyl ester.

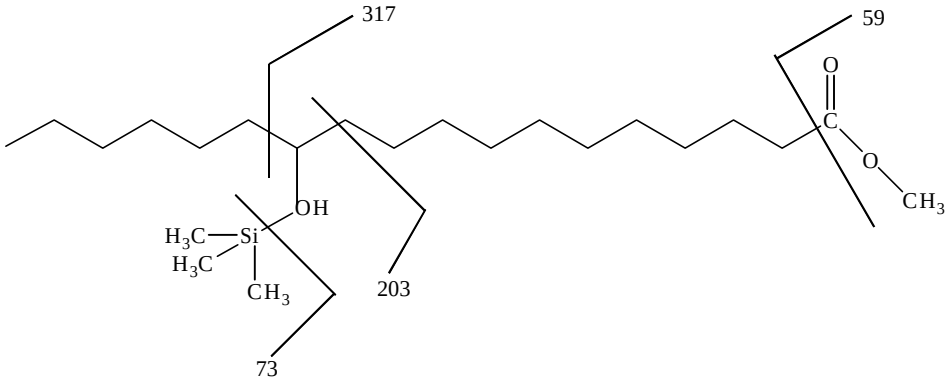


Figure IIB-14. Fragmentation pattern for the trimethylsilyl ether adduct of 12-OH 18:0.

This result incriminated the disposable amber rubber bulbs. Consequently, disposable amber rubber pipette bulbs that were not powdered and a sample of the powder used in the bulbs were obtained from Fisher Scientific Company (Pittsburgh, PA). A set of five rubber bulbs was extracted, and separate extractions were performed in triplicate using the powder sample. Although no 10-OH 18:0 appeared in the powder sample extractions, all five of the rubber bulbs were found to contain authentic 10-OH 18:0 as a free acid. This was confirmed through both HPLC/ESI/MS analysis and GC/MS separation and identification of the trimethylsilyl ether adducts of the methyl ester of the samples.

Finally, an extraction of *Cryptosporidium parvum* oocysts was performed without using any rubber bulbs to eliminate the possibility that 10-OH 18:0 was a lipid component of *Cryptosporidium* as well as a component of the rubber bulbs. Two samples of 1×10^8 oocysts of *Cryptosporidium* previously found to contain 10-OH 18:0 were extracted. Neither set of oocysts was found to contain any detectable quantity of 10-OH 18:0 through HPLC/ESI/MS analysis (Figure IIB-15) and GC/MS analysis when disposable rubber bulbs were not used in the sample preparation. This established that the target analyte was not an interfering contaminant, but was in fact an artifact of the sample preparation procedure.

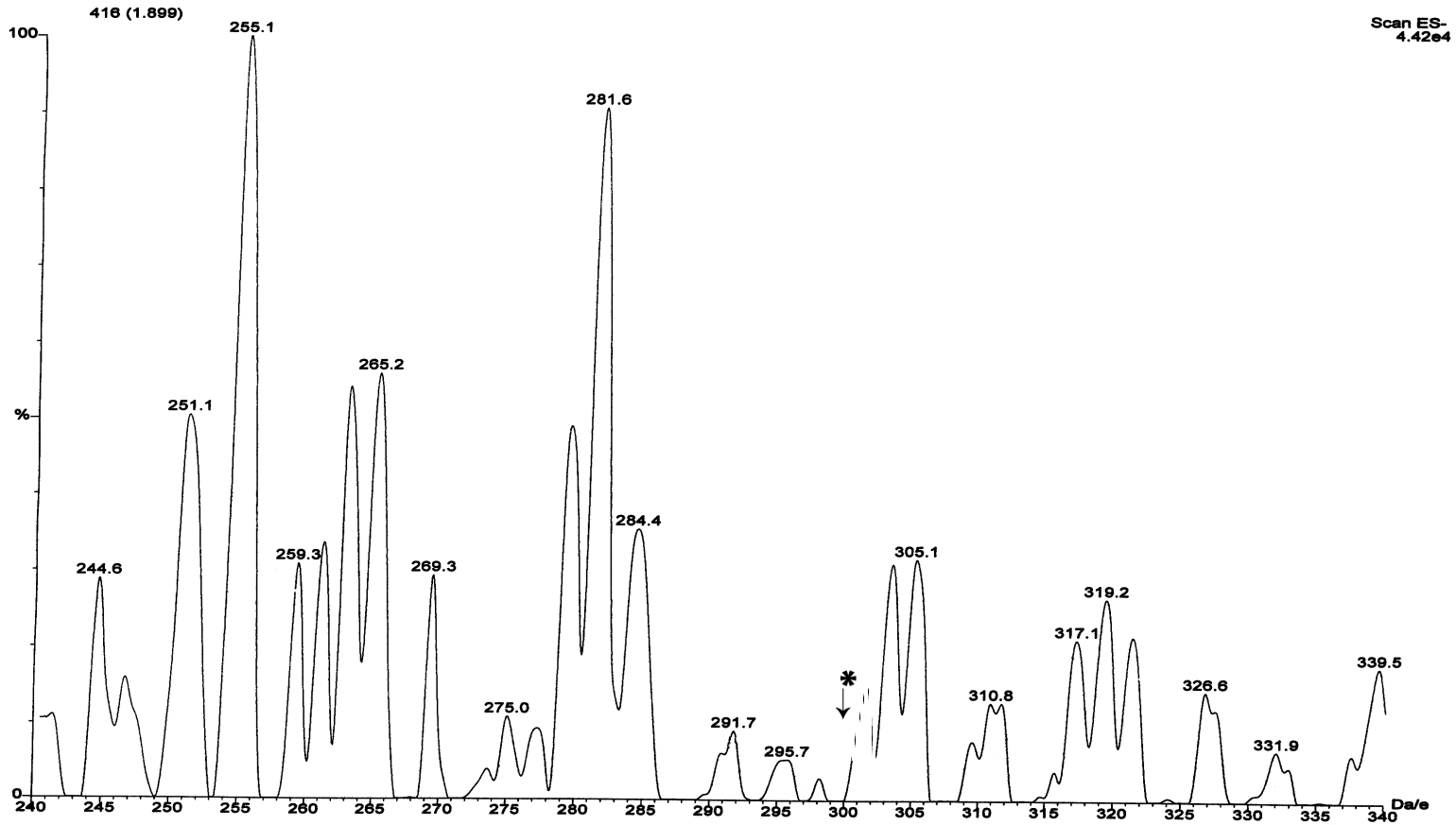


Figure IIB-15. ESI/MS spectrum of the glycolipid fraction of *Cryptosporidium parvum* cells without HPLC column. No rubber bulbs were used in this sample preparation. A star (*) shows the location of m/z 299, which is absent in this chromatogram.

DISCUSSION

The putative occurrence of such an unusual component as 10-OH 18:0 in *Cryptosporidium* (Schrum *et al.*, 1997; White *et al.*, 1997) prompted the investigation of the complex native form of this fatty acid in *C. parvum*. Adapting the identification of this analyte to HPLC/ESI/MS would provide the benefit of decreasing sample handling, as samples would not have to be methylated. This should result in a much quicker assay with less risk of sample contamination.

The putative signature became even more unique when HPLC/ESI/MS techniques revealed that 10-OH 18:0 was a free fatty acid. Free fatty acids are extremely unusual in nature, and the potential of 10-OH 18:0 as a unique "signature" for *Cryptosporidium* appeared to be excellent. Efforts were focused on a more complete characterization of the target analyte of interest and the establishment of lower limits of detection. Soon a major problem arose while attempting to produce a calibration curve for oocyst numbers vs. content of 10-OH 18:0. The amount of 10-OH 18:0 in *C. parvum* oocyst samples fluctuated wildly, and occasionally was not seen at all. Microscopic examination of the preparations revealed the oocysts were clumped together and attached to the glass fibers used to purify the fecal preparations. The clumping

increased with storage at 4°C. Consequently, difficulty in dispersing the oocysts reproducibly seemed to be the reason for the fluctuation in the amount of 10-OH 18:0 in the samples. Unfortunately, the random nature of the appearance of the 10-OH 18:0 prevented its appearance in a sample blank until many repetitions were performed. Clearly, when examining for traces of specific analytes such as might be seen in 10-30 oocysts, comprehensive blank analyses must be rigorously performed for each step of the procedure. The results of this study formed the basis for a paper which disclosed the origin of 10-OH stearic acid in *Cryptosporidium* samples in previously published results (Burkhalter *et al*., 1998). Fortunately, the extraction procedures are simple and straightforward, which eased the efforts to localize the source of contamination once 10-OH 18:0 was realized to be an artifact of sample isolation and preparation.

PART III.

PHOSPHOLIPID FATTY ACID BIOMARKERS FOR *EIMERIA*

INTRODUCTION

Coccidiosis in poultry costs the world's commercial chicken producers at least $800 million every year (Williams, 1998). Many of the features of this disease in the host are expressions of the characteristics of individual species of *Eimeria*, and as such are often used as a guide to identification. For example, *E. acervulina* produces reduction in growth without severe mortality, while *E. tenella* causes intestinal or cecal hemorrhage (Joyner and Davies, 1960).

Analysis of polar lipid fatty acids, a powerful tool for the chemotaxonomic assignment of microorganisms was applied to the analysis of purified oocysts from three species of *Eimeria: Eimeria tenella, Eimeria acervulina,* and *Eimeria maxima.* Only one other study of the lipids of *Eimeria* has been reported, and it includes only the lipids of *E. tenella* (Weppelman *et al*., 1976). Therefore, technological advances might be beneficial to the study of *Eimeria* by the application of signature lipid biomarker (SLB) analysis. Toward this end, a study was undertaken to identify possible unique fatty acids in the polar lipid fraction of these three species and to determine the usefulness of the relative quantities of fatty acids in this fraction (fatty acid pattern) for discrimination of each species.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade. Authentic standards for fatty acids were purchased from Matreya (Pleasant Gap, PA). All solvents were obtained from Baxter Scientific Products (McGaw Park, IL) and were of gas chromatography grade.

Glassware

All glassware was washed in a 10% (vol / vol) Micro cleaner solution (Baxter Diagnostics, Deerfield, IL), rinsed ten times with tap water and then ten times in deionized water. The glassware was then heated overnight in a muffle furnace at 450°C to remove any carbon contamination.

Parasites

Purified oocysts of *E. tenella, E. acervulina,* and *E. maxima* were kindly provided by the National Institutes of Health (Bethesda, MD). Purity was checked upon receipt by microscopic examination.

Lipid extraction

Purified oocysts were extracted at room temperature and atmospheric pressure using a modified Bligh / Dyer technique (Bligh and Dyer, 1959; Guckert *et al*., 1985; White *et al*., 1979b). This method extracts cellular lipids using a one-phase chloroform / methanol / phosphate buffer (50 mM, pH 7.4, 1:2:0.8 by volume) solvent system for 4 hours before adding chloroform and deionized water to form two phases (final solvent ratios: chloroform / methanol / phosphate buffer / water, 1:1:0.4:0.5 by volume). Then the organic phase was collected and dried under a stream of nitrogen. The lipids were stored at -20°C until purification.

Purification of lipids

The total lipid extracted was dissolved in 0.5 ml chloroform and separated using silicic acid columns (10-cm column length, 0.5-cm inner diameter, 100-200 mesh particle size). Each sample was loaded onto a column in 100-200 µl chloroform and then eluted in batches with 5 ml chloroform, 5 ml acetone, and 10 ml methanol so as to elute neutral, glyco- and polar lipids, respectively (Guckert *et al*., 1985). Bulk elution of the neutral lipids, including sterols and diglycerides, with chloroform was followed by recovery of the free fatty acids in the acetone, or glycolipid, fraction. Phospholipids were recovered after the glycolipids by elution in

methanol. For each fraction, the solvent was removed under a stream of nitrogen. The lipid fractions were stored at -20°C until methylation.

Mild alkaline methanolysis

A mild alkaline methanolysis procedure (Guckert *et al.*, 1985) was utilized to prepare methyl esters of the ester-linked fatty acids of the polar lipids. The polar lipid fatty acid methyl esters (FAMEs) were dried under a stream of nitrogen and then were stored at -20°C until analysis.

Dimethyl disulfide (DMDS) adducts

A dimethyl disulfide derivatization procedure (Nichols *et al.*, 1986a) was used for verification of monounsaturated fatty acid double bond position and geometry.

GC analysis

A Hewlett-Packard 5890 series II gas chromatograph coupled to a flame ionization detector (FID) provided quantitative analysis of FAME components. Chromatographic separations were carried out on a HP-1 fused-silica capillary column [50 m by 0.2 mm (inner diameter)] of 0.11 μm film thickness. The column was kept at an initial temperature of 60°C for 2 minutes, and the temperature was programmed to increase from 60 to

150°C at 10°C / min, then to 312°C at 3°C / min (Ringelberg *et al*., 1989). The injector and detector were maintained at 290°C and 300°C, respectively. Splitless injections were performed with a Hewlett-Packard model 7673 autosampler. The split valve was opened 1 minute after injection. Hydrogen was the carrier gas. Preliminary peak identification was performed by comparison of retention times with synthetically prepared standards (Matreya, Inc., Pleasant Gap, PA). FAMEs were quantified by integration of peak areas, comparing the peak response to methyl nonadecanoic acid (19:0) added to the sample as a recovery standard just prior to injection (Christie, 1989; Guckert *et al*., 1985). Peak areas were determined using a programmable laboratory data system, ChromPerfect version 3.5 (Chromatography Data Systems, Palo Alto).

GC/MS analysis

A Hewlett-Packard 5971 mass selective detector provided for the qualitative assignment of lipid structure. The column, temperature program, and injection procedures were as described above for GC analysis. The carrier gas, helium, was maintained at an inlet pressure of 195 kPa to establish a carrier gas flow rate of approximately 1ml / min (Ringelberg *et al*., 1989). Fatty acid methyl esters were identified based on their retention indices (RI) as well as their mass spectra in comparison to

synthetically prepared standards (Matreya, Inc., Pleasant Gap, PA), and to a mass spectral library of FAME standards (Guckert *et al*., 1985; Christie, 1989). Peak areas were determined using a programmable laboratory data system, Enhanced ChemStation (Hewlett-Packard, Palo Alto).

Phylogenetic analysis of 18S sequences

Partial 18S sequences were retrieved from GenBank *via* the National Institute for Biotechnology Information (NCIB) internet node using the Entrez facility (Schuler *et al*., 1996), with accession numbers as follows: AF093495 (*Cryptosporidium baileyi*), X64342 (*Cryptosporidium muris*), U67115 (*Eimeria acervulina*), U67117 (*Eimeria maxima*), U40263 (*Eimeria nieschulzi*), and U40264 (*Eimeria tenella*). They were manually aligned to the 18S sequence data from the Ribosomal Database Project (RDP) (Larsen *et al*., 1993).

Phylogenetic trees were constructed by applying the Felsenstein correction, DNA-DIST and the neighbor-joining method, using the ARB program (Strunk and Ludwig, 1996). The tree was pruned from a larger analysis containing all the representative 18S sequences compiled by RDP.

Fatty acid nomenclature

Fatty acids are abbreviated by the number of carbon atoms, a colon, degree of unsaturation, which is then followed by an omega symbol (ω) indicating the number of carbons from the methyl end of the molecule to the position of the first, and sometimes only, double bond. The prefix "cy" represents the cyclopropyl isomer of a fatty acid, and "i" and "a" represent *iso*-branched and *anteiso*-branched isomers, respectively. The number preceding the abbreviation "Me" indicates the position of a methyl group relative to the carbonyl carbon followed by the number of carbons in the fatty acid chain. Suffixes "c" and "t" represent *cis* and *trans* geometric isomers of the unsaturation, respectively. An ambiguity in this naming convention is that the number of carbons given for *iso-, anteiso-,* unknown branched, and cyclopropyl fatty acids include all of the carbons in the molecule except the esterified methyl group. Therefore, 10Me18:0 indicates a methyl group attached to an 18-carbon side chain at the 10 position from the carbonyl (Kates, 1986).

Statistical analysis

FAMEs are expressed in pmol/g or as mole percentages. If mole percent values are used for statistical analysis, arcsine transformation is applied to the data to remove the percent bias.

STATISTICA software (StatSoft, Tulsa, OK) was utilized for cluster analysis. Cluster analysis was performed using complete linkage rules and Euclidean distances. Similarity between species can be measured by the linkage distances, which could range between 0 (identical) and 100 (no similarity). Fatty acids comprising less than 1% of the total profiles were eliminated from this analysis.

RESULTS

The FAME compositions of *Eimeria tenella*, *Eimeria acervulina*, and *Eimeria maxima* are shown in Table III-1. Straight chain saturated FAMEs (38-51%), monounsaturated FAMEs (30-53%) and polyenoics (2-12%) were the major lipid classes detected. *E. tenella* produced large amounts of monounsaturated fatty acids, followed by straight chain saturates, with relatively minor amounts of polyenoics. *E. acervulina* also produced large amounts of monounsaturated fatty acids, followed by straight chain saturates of an almost equal value. A higher percentage of its profile consisted of polyenoics, when compared to *E. tenella*. In contrast, *E. maxima* produced a greater amount of straight chain saturates, followed

Table III-1. Relative proportions (mole percentages) of phospholipid fatty acids from three *Eimeria* species (n = 1). Unusual FAMEs appear in bold print.

Fatty acid	*E. tenella*	*E. acervulina*	*E. maxima*
Straight chain saturates			
14:0	10.62	4.83	4.85
15:0	0.49	0.47	1.36
16:0	19.59	23.99	29.34
17:0	---	0.62	0.74
18:0	7.16	8.89	15.59
20:0	---	0.65	0.89
22:0	---	---	---
Total straight chain saturatess	**37.86**	**38.98**	**51.41**
Terminally branched saturates			
a13:0	---	---	---
i15:0	---	0.28	1.52
a15:0	---	0.31	1.12
i16:0	---	0.22	0.48
i17:0	---	---	0.16
a17:0	---	0.35	0.35
Total terminally branched saturates	**0.00**	**1.16**	**3.12**
Monounsaturates			
16:1ω9c	1.56	0.19	0.16
16:1ω7c	3.20	4.50	2.42
18:1ω9c	44.81	36.72	24.32

Table III-1 (Continued).

Fatty acid	*E. tenella*	*E. acervulina*	*E. maxima*
18:1ω7c	2.95	2.74	2.37
18:1ω7t	0.94	0.36	1.07
20:1ω9c	---	0.19	0.16
Total monounsaturates	**53.46**	**44.32**	**30.34**
Polyenoics			
18:2ω6	2.00	11.48	8.74
18:3ω3	---	0.39	0.33
20:2ω6	---	---	---
20:3ω3	---	0.21	0.22
20:4ω6	---	0.66	0.66
Total polyenoics	**2.00**	**11.87**	**9.07**
Miscellaneous			
7,8-epoxide 16:0	**0.48**	**---**	**---**
cy19:0	---	1.10	2.07
7,8-epoxide 18:0	**0.74**	**---**	**---**
9,10-epoxide 18:0	**5.41**	**---**	**---**
mebr20:1	---	0.62	0.72
Total miscellaneous	**6.63**	**1.72**	**2.79**

by monounsaturated fatty acids. It produced approximately the same mole percentage of polyenoics as *E. acervulina.*

The most abundant FAME in both *E. tenella* and *E. acervulina* was 18:1ω9c, followed by hexadecanoic acid (16:0). These are reversed in *Eimeria maxima*, as 16:0 is the most abundant fatty acid followed by 18:1ω9c. The third most abundant fatty acid was different in each species. In *E. tenella* and *E. maxima*, the third most abundant fatty acids were straight chain saturates, myristic acid (14:0) in *E. tenella* and stearic acid (18:0) in *E. maxima*. *E. acervulina*, on the other hand, had the polyenoic fatty acid 18:2ω6 as its third most abundant fatty acid. Minor amounts of three unusual epoxide fatty acids were found only in *E. tenella* (bold print in Table III-1). The profile of this species contained 7,8-epoxide 16:0, 7,8-epoxide 18:0, and 9,10-epoxide 18:0.

A hierarchical tree diagram of the data is presented in Figure III-1. This analysis shows that the relationship between *E. tenella* and *E. acervulina* is much closer than the relationship of either of these species to *E. maxima*, as evidenced by the linkage distances between the three species.

A phylogenetic tree (Figure III-2) was constructed from partial 18S DNA sequences retrieved from GenBank. The sequences were manually aligned to the 18S sequence data from the Ribosomal Database Project

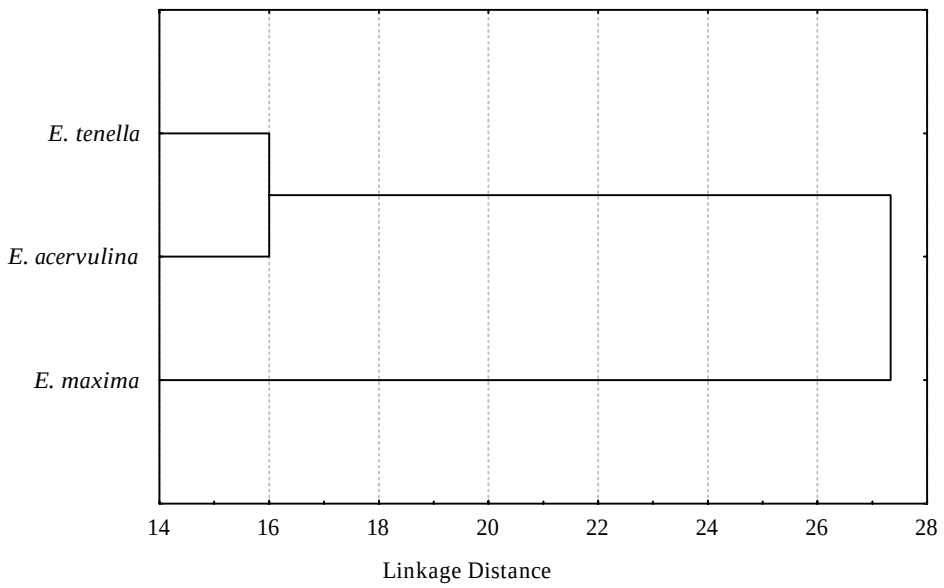


Figure III-1. Hierarchical tree diagram of three *Eimeria* species (data from Table III-1). Cluster analysis was performed using complete linkage rules and Euclidean distances. Profiles were simplified to eliminate fatty acids comprising less than 1% of the total profiles. Respective arms of the dendrogram are identified for each species of *Eimeria*. Similarity between species can be measured by the linkage distances, with a distance of 14 being more similar than a distance of 28.

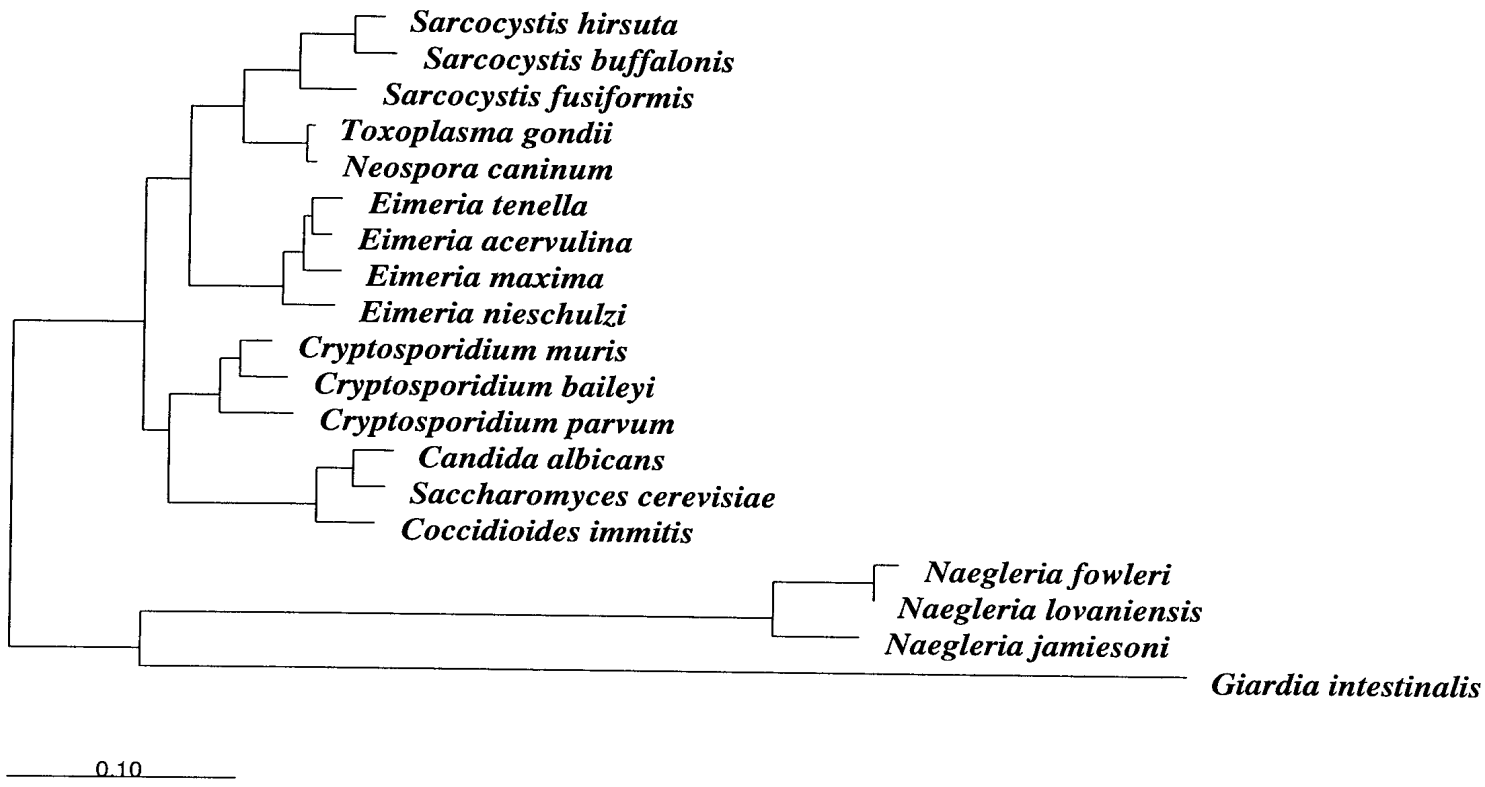


Figure III-2. Phylogenetic tree using 18S DNA sequences. Partial 18S sequences were retrieved from GenBank, with accession numbers as follows: AF093495 (*C. baileyi*), X64342 (*C. muris*), U67115 (*E. acervulina*), U67117 (*E. maxima*), U40263 (*E. nieschulzi*), and U40264 (*E. tenella*). They were manually aligned to the 18S sequence data from RDP (Larsen *et al.*, 1993).

(RDP) (Larsen *et al*., 1993). After construction, the tree was pruned from a larger analysis containing all the representative 18S sequences compiled by RDP. This tree shows that *Eimeria tenella* and *Eimeria acervulina* are more closely related to each other than to *Eimeria maxima.* However, all three species are more closely related to each other than to other species in the data bank, including other oocyst-producing species such as *Cryptosporidium*.

DISCUSSION

The current study was undertaken to characterize and compare the phospholipids of *Eimeria tenella, Eimeria acervulina* and *Eimeria maxima.* Analysis of polar lipid fatty acids clearly differentiates these three species of *Eimeria* from each other, as Table III-1 demonstrates. The differences probably are due to the relative quantities of the three most abundant fatty acids in the profiles, as well as the appearance of minor amounts of three unusual fatty acids found only in *Eimeria tenella.*

Only one other study of the lipids found in *E. tenella* has been reported (Weppelman *et al*., 1976). In that study, the total lipids extracted were saponified, whereas this study is comprised only of the polar lipids.

A comparison of the relative proportions of fatty acids found in this study and those found by Weppelman *et al.* is presented in Table III-2. Weppelman *et al.* also found the predominant fatty acid to be 18:1 (75%), and 16:0 was the second most abundant fatty acid (12%). That study included the glycolipids and neutral lipids, which were removed during the fractionation step in the silicic acid procedure used in this study. This probably accounts for the variation in the relative mole percentages between these two studies.

To determine the similarity of the hierarchical tree diagram constructed with PLFA data (Figure III-1) to DNA phylogeny, a phylogenetic tree using 18S DNA analysis (Figure III-2) was also constructed. A comparison of these two figures shows that the PLFA hierarchical tree compares favorably to the phylogenetic tree, as *E. tenella* and *E. acervulina* are closer to each other on both trees, and *E. maxima* is more distantly related to the other two.

The signature lipid biomarker (SLB) method of analysis has been successfully applied to the species-specific identification of *E. tenella*, *E. maxima* and *E. acervulina*. It should be noted that the relatively minor amounts of 7,8-epoxide 16:0 and 7,8-epoxide 18:0 might or might not be reproducible, due to the small quantities of these fatty acids in the profiles. The higher quantity of 9,10-epoxide 18:0 should ensure its

Table III-2. Comparison of relative proportions (mole percentages) of phospholipid fatty acids from *Eimeria tenella* in this study and results obtained by Weppelman *et al* (1976).

Fatty acid	*E. tenella*[a]	*E. tenella*[b]
Straight chain saturates		
14:0	10.62	1
15:0	0.49	---
16:0	19.59	12
18:0	7.16	10
Total straight chain saturates	**37.86**	**23**
Monounsaturates		
16:1	4.76	1
18:1	48.70	75
Total monounsaturates	**53.46**	**76**
Polyenoics		
18:2	2.00	Trace
Total polyenoics	**2.00**	**Trace**
Miscellaneous		
7,8-epoxide 16:0	0.48	---
7,8-epoxide 18:0	0.74	---
9,10-epoxide 18:0	5.41	---
Total miscellaneous	**6.63**	---

[a]Data from this study
[b]Data from Weppelman *et al*., 1976
Dash (--) indicates the fatty acid was not detectable

presence in the profile of *E. tenella.* Also, the differences in 14:0, 18:0 and 18:1ω9c should be helpful for a clear identification for the differentiation between these three species. A lack of replicates across the studies suggests that additional work on the PLFA profiles of *Eimeria* would be beneficial to the signature lipid biomarker discrimination of the genus. Increasing the number of replicates would allow for the use of other statistical methods, such as principal component analysis and discriminant analysis. Sterol analysis might be helpful, as these are commonly found in microeukaryotes (Nichols *et al*., 1987). Since purified oocysts were used in this study, studies that include other matrices would also be beneficial. However, the application of SLB analysis should be useful for the detection of these species of *Eimeria* in farm runoff water and water used for livestock.

PART IV.

OXIRANE FATTY ACIDS AND CHLORINE INJURY

INTRODUCTION

The final stage of all water treatment processes involves the treatment of the water with a chemical agent to assure the destruction of bacteria. One or more disinfectants are often applied by municipal facilities during the water treatment process. For reasons of efficiency and convenience, chlorine is used most frequently in the United States.

Injured waterborne indicator bacteria undergo sub-lethal physiological and structural consequences resulting from exposure to injurious factors within aquatic environments. This injury results in the inability of injured cells to reproduce under selective or restrictive conditions that are tolerated by uninjured cells.

The percentage of injured bacteria vs. non-injured bacteria in various aquatic systems has been the subject of several studies. Observations of injured bacteria in municipal drinking waters systems have ranged from 43% to 99% (Singh and McFeters, 1990). The indications are that injured bacteria may comprise the majority of coliforms in some drinking water systems (McFeters, 1990). These bacteria normally would not be detected using accepted analytical methods. The repair process in enteropathogens has been studied also, both in vitro and in the small intestine of experimentally inoculated mice (Singh *et al*., 1986). These

researchers found that a substantial portion of the population recovered during the first two hours of intraluminal incubation. The injured cells were able to recover, grow, and cause pathological changes to the mammalian gut.

In an earlier experiment in this laboratory, the community composition of biofilms, collected on glass beads exposed to drinking water containing 0.24-1.4 parts per million (ppm) of free chlorine, was examined. Surprisingly, high levels of 16 and 18 carbon oxirane (epoxidated) fatty acids were recovered (Phiefer, 1998; Smith *et al*., 2000). It was postulated that epoxidated fatty acids might result from direct oxidation of formerly monoenoic fatty acids, a predominant cellular membrane component in Gram-negative bacteria.

The objectives of this study were to determine whether or not this relationship also occurs with organisms at concentrations closer to those found in drinking water and how much time is necessary for the reaction to take place.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade. Authentic standards were purchased from Matreya (Pleasant Gap, PA). All solvents were obtained from Baxter Scientific Products (McGaw Park, IL) and were of gas chromatography grade.

Glassware

All glassware was washed in a 10% (vol/vol) Micro cleaner solution (Baxter Diagnostics, Deerfield, IL), rinsed ten times with tap water and then ten times with deionized water. The glassware was then heated overnight in a muffle furnace at 450°C to remove any carbon contamination.

Preparation of synthetic epoxidated (oxirane) fatty acids

Since epoxidated or oxirane fatty acids were not commercially available, it was necessary to prepare these standards synthetically. Epoxidated analogs of formerly monounsaturated fatty acids (16:1ω5c, 16:1ω7c, 16:1ω9c, 18:1ω7c, 18:1ω9c, and 18:1ω12c) were prepared as follows: just prior to epoxidation, the highly reactive and unstable peroxy

acid of formic acid, performic acid, was prepared by mixing stoichiometric quantities of acetic acid and 30% (vol/vol) hydrogen peroxide. A 10-fold excess of this reagent was then added directly to a dried quantity of the monoenoic fatty acid methyl ester precursor and heated to 60°C for 48 hrs. This produces an electrophilic addition reaction. Being a concerted reaction, the formation of an epoxidated fatty acid methyl ester is stereoselective (Loudon, 1988). Thus, *cis* and *trans* fatty acids yield *cis* and *trans* epoxidated (oxirane) analogs, respectively. An example of this reaction is shown in Figure IV-1.

Bacterial strain and initial growth conditions

Escherichia coli ATCC 75922 was used for all experiments. All media reagents were purchased from Difco Laboratories (Detroit, MI). *E. coli* was grown overnight in 1:1000 nutrient broth.

Acridine orange direct counts (AODC)

Filtered suspensions were stained with acridine orange (Murray *et al*., 1994) and examined under epifluorescent illumination to determine total cell counts.

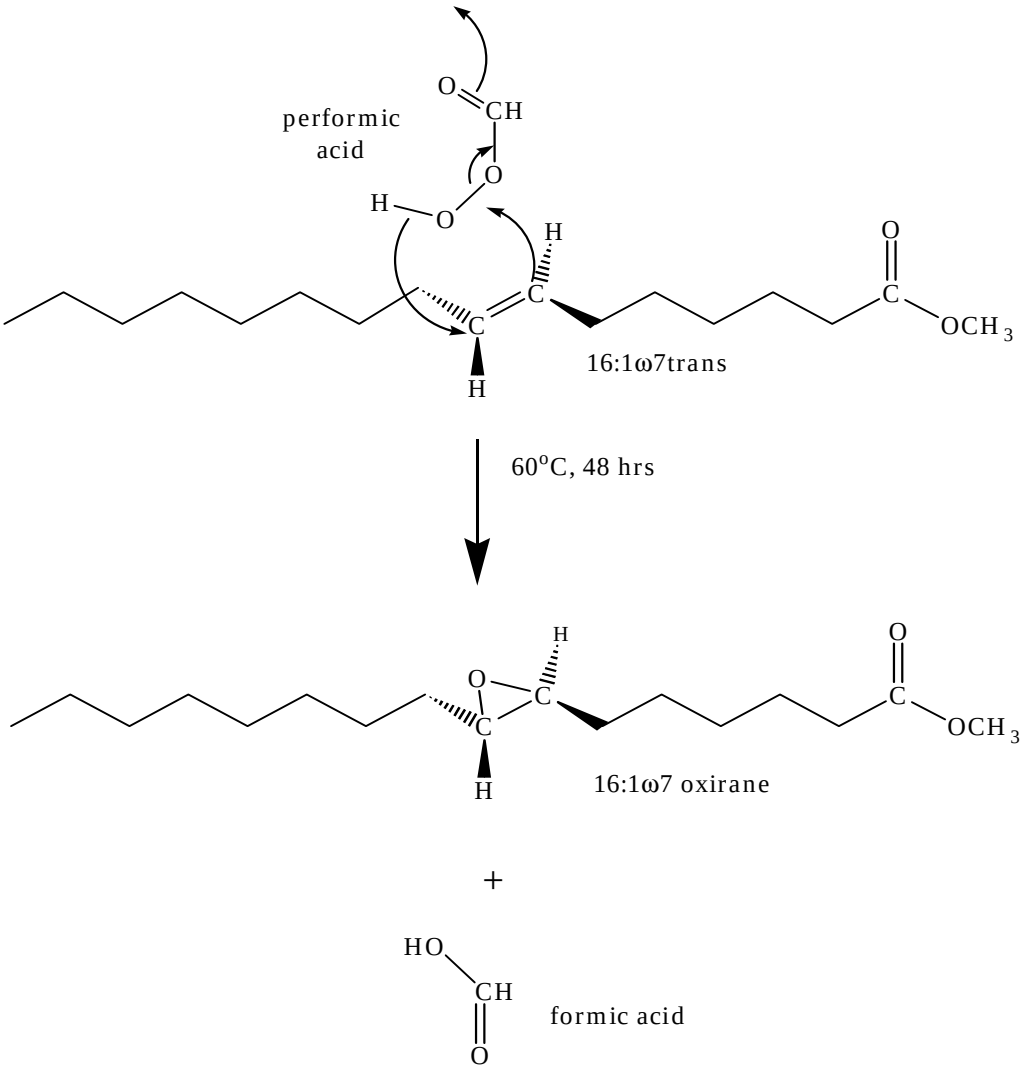


Figure IV-1. Formation of an epoxidated fatty acid methyl ester. The concerted reaction is stereoselective (Loudon, 1988). As a result, a *trans* oxirane analog is obtained from a *trans* monoenoic precursor.

Free chlorine determination

The concentration of free chlorine was determined by the N,N-diethyl-*p*-phenylenediamine (DPD) colorimetric method (Cooper *et al.*, 1982), using a Hach Pocket Colorimeter (Leveland, CO).

Growth and chlorine exposure of E. coli

Experiment one

Approximately 10 ml of an overnight culture of *E. coli* was used to inoculate one liter samples of filter-sterilized Milli-Q water, giving a final dilution of 10^5 cells / 100 ml, determined by acridine orange direct counts. This culture was then exposed to 0.5 or 2.0 ppm free chlorine (hypochlorite) at 25°C. Cultures that were not exposed to free chlorine were treated in an identical manner, while excluding the chlorine addition step. At 0, 1, 5, 7.5, 24 and 48-hour time points, 125 ml samples were removed, with 100 ml used for PLFA analysis and 25 ml used for AODC and spread-plate assays.

Experiment two

Approximately 0.1 ml of an overnight culture of *E. coli* was used to inoculate 100 ml samples of filter-sterilized Milli-Q water for a final dilution of 10^9 cells per sample, determined by AODC.

This culture was then exposed to 2.0 ± 0.4 ppm free chlorine (hypochlorite) at 25°C. Cultures that were not exposed to free chlorine were treated in an identical manner, while excluding the chlorine addition step. Cultures were sacrificed at 0 and 24 hours and analyzed for PLFA.

Spread-plate enumeration

Suspensions were spread-plated onto nutrient agar and R2A agar and incubated at 25°C for 24 hours, after which colony forming units (CFU) were counted. Negative plates were incubated and rechecked for growth after 48 and 72 hours.

Recovery of the lipids

Each 100 ml sample was filtered through a Whatman (Maidstone, England) Anodisc filter (0.2 μm, 47 mm). The filter was extracted with the modified Bligh / Dyer solvent system (Bligh and Dyer, 1959;Guckert *et al*., 1985; White *et al*., 1979b).

Purification of lipids

The total lipid extracted was dissolved in 0.5 ml chloroform and separated using silicic acid columns (10-cm column length, 0.5-cm inner

diameter, 100-200 mesh particle size). Each sample was loaded onto a column in 100-200 μl chloroform and then eluted in batches with 5 ml chloroform, 5 ml acetone, and 10 ml methanol so as to elute neutral, glyco- and polar lipids, respectively (Guckert *et al*., 1985). Bulk elution of the neutral lipids, including sterols and diglycerides, with chloroform was followed by recovery of the free fatty acids in the acetone, or glycolipid, fraction. Phospholipids were recovered after the glycolipids by elution in methanol. For each fraction, the solvent was removed under a stream of nitrogen. The lipid fractions were stored at -20°C until methylation.

HPLC/ESI/MS Analysis

The polar lipid fraction was resuspended in a minimum of 100 μl of a 1:1 (vol / vol) mixture of methanol and acetonitrile and passed through a 0.2 μm filter. Five μl of the polar lipid fraction, consisting primarily of glycerophospholipids, was injected onto a Microtech reversed phase high performance liquid chromatographic system consisting of a Columbus (250 mm × 2mm) 5 μm particle size octadecylsilyl (ODS or C18) column (Phenomenex, Torrence, CA) at a flow rate of 150 μl / min. The mobile phase consisted of 1:1 methanol / acetonitrile containing 0.5% by volume of concentrated (27-30%) ammonium hydroxide (Miller, 1988). The flow rate was 150 μl / min. The mobile phase effluent was nebulized into a VG

Platform II mass spectrometer. Nebulization was assisted by a 300 ml/min flow rate of nitrogen into an electrospray ionization ion source operated in the negative mode of ionization. The voltage applied across the capillary was -2.67 kV and the cone voltage value was –80 V. Preliminary experiments performed with synthetic standards of phospholipids had revealed these conditions to be at or near optimum for the production of stable ion currents attributable to fatty acyl anions cleaved from the glycerol backbone, as well as for negative ions which might be attributed to the nature of the polar head group.

Mild alkaline methanolysis

A mild alkaline methanolysis procedure (Guckert *et al.*, 1985) was utilized to prepare methyl esters of the ester-linked fatty acids of the remaining 95% of the polar lipids. The polar lipid fatty acid methyl esters were dried under a stream of nitrogen and then were stored at -20°C until analysis.

GC/MS analysis

Verification of fatty acid structure was achieved using mass spectrometry performed on a Hewlett Packard 5971 mass selective detector coupled to a Hewlett-Packard model 5890 series II gas

chromatograph. Chromatographic separations were carried out on a HP-1 fused-silica capillary column [50 m by 0.2 mm (inner diameter)] of 0.11 μm film thickness. The column was kept at an initial temperature of 60°C for 2 minutes, and the temperature was programmed to increase from 60 to 150°C at 10°C / min, then to 312°C at 3°C / min. The injector and detector were maintained at 290°C and 300°C, respectively. Splitless injections were performed with a Hewlett-Packard model 7673 autosampler. The split valve was opened 1 min after injection. The carrier gas, helium, was maintained at an inlet pressure of 195 kPa to establish a carrier gas flow rate of approximately 1ml / min (Ringelberg *et al*., 1989). Fatty acid methyl esters were identified through comparison of their mass spectra and retention behavior with synthetically prepared standards (Matreya, Inc., Pleasant Gap, PA), and to a mass spectral library of FAME standards (Christie, 1989; Guckert *et al*., 1985). Peak areas were determined using a programmable laboratory data system, Enhanced ChemStation (Hewlett-Packard, Palo Alto).

Fatty acid nomenclature

Fatty acids are abbreviated by the number of carbon atoms, a colon, degree of unsaturation, which is then followed by an omega symbol (ω) indicating the number of carbons from the methyl end of the molecule to

the position of the first, and sometimes only, double bond. The prefix "cy" represents the cyclopropyl isomer of a fatty acid, and "i" and "a" represent *iso*-branched and *anteiso*-branched isomers, respectively. The number preceding the abbreviation "Me" indicates the position of a methyl group relative to the carbonyl carbon followed by the number of carbons in the fatty acid chain. Suffixes "c" and "t" represent *cis* and *trans* geometric isomers of the unsaturation, respectively. An ambiguity in this naming convention is that the number of carbons given for *iso-*, *anteiso-*, unknown branched, and cyclopropyl fatty acids include all of the carbons in the molecule except the esterified methyl group. Therefore, 10Me18:0 indicates a methyl group attached to an 18-carbon side chain at the 10 position from the carbonyl (Kates, 1986).

RESULTS

Viability of E. coli

Plate counts showed that only *E. coli* exposed to chlorine in both sets of experiments were not viable, as no growth was observed on any spread-plates. All control cultures containing *E. coli* grew after 24 hours of incubation on both nutrient agar and R2A agar plates.

LC/MS analysis of polar lipids

Figure IV-2 displays the HPLC chromatogram of the polar lipids endogenous to cultures of *E. coli* prior to chlorine exposure (i.e. time zero). Selected ion monitoring (SIM) of mass to charge (m / z) ratios associated with 16 and 18 carbon monoenoic fatty acids was utilized to generate the upper chromatographic trace. The lower trace was generated through SIM monitoring of m / z values that might be generated by the respective 16 and 18 carbon reaction products. The time zero experiments revealed a large quantity of phospholipids containing acyl linked monoenoic fatty acids and no phospholipids containing possible oxirane fatty acids. Further LC / MS experiments revealed the phospholipids observed in the upper chromatographic trace belonged to phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) or phosphatidylethanolamine (PE) class of phospholipids, typical of Gram-negative bacteria (Harwood and Russell, 1984).

Oxiranes began to appear in the five-hour samples, and in all subsequent time point samples, from experiment 1. Figure IV-3 displays the HPLC chromatogram of the polar lipids endogenous to cultures of *E. coli* after 24 hours of exposure to free chlorine levels of 2 ± 0.4 ppm (experiment 2). Monitoring the ion current that might be associated with 16 and 18 carbon monoenoic fatty acids generated the upper

Figure IV-2. HPLC chromatographic separation of glycerophospholipds of *Escherichia coli* prior to exposure to chlorine treatment (0 hours). The upper chromatographic trace is generated by selected ion monitoring of ions attributable to 16 and 18 carbon acyl-linked monounsaturated fatty acids (*m/z* values of 253 and 281). The lower chromatographic trace is generated by selected ion monitoring of ions attributable to 16 and 18-carbon acyl-linked oxirane fatty acids (*m/z* values of 269 and 297).

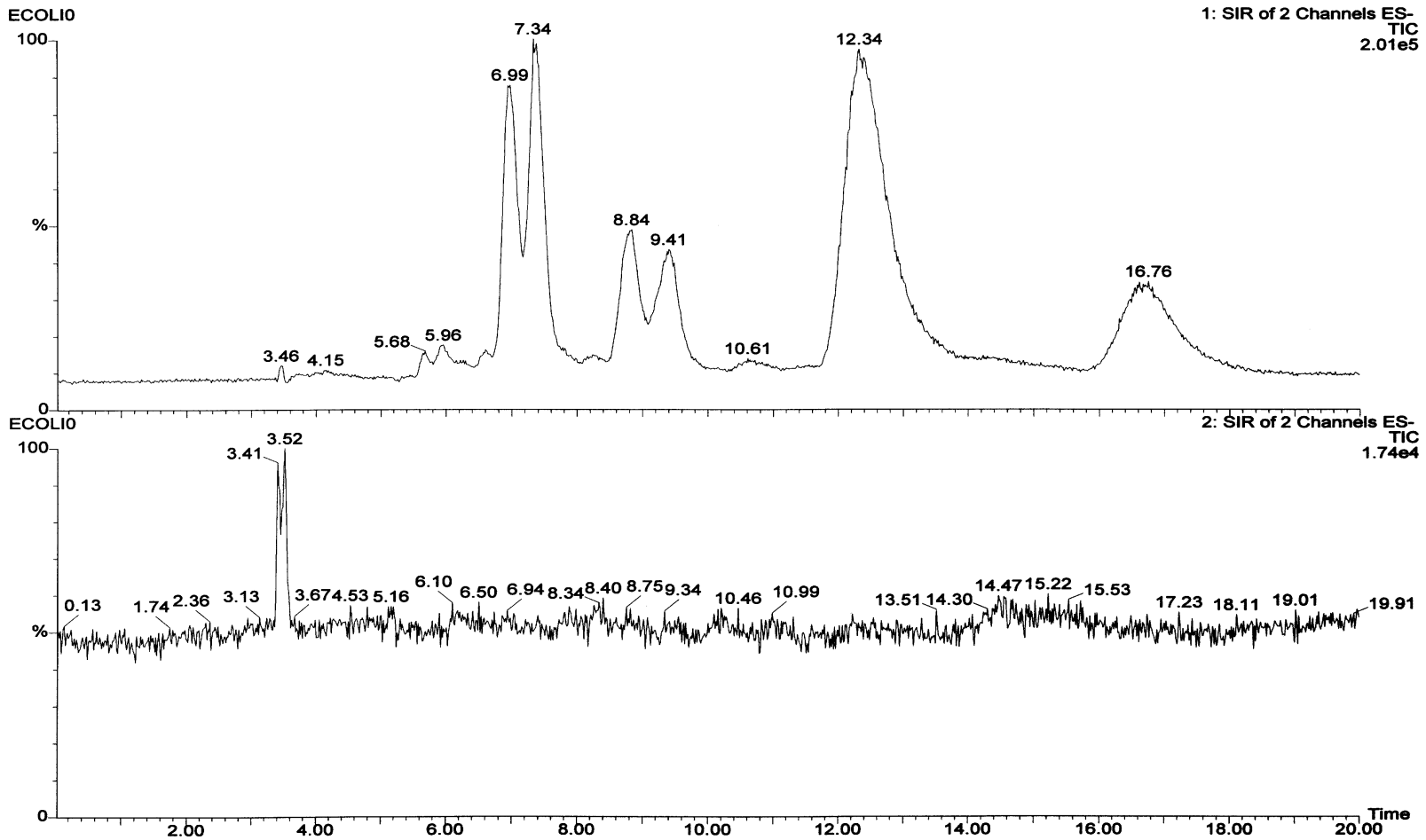
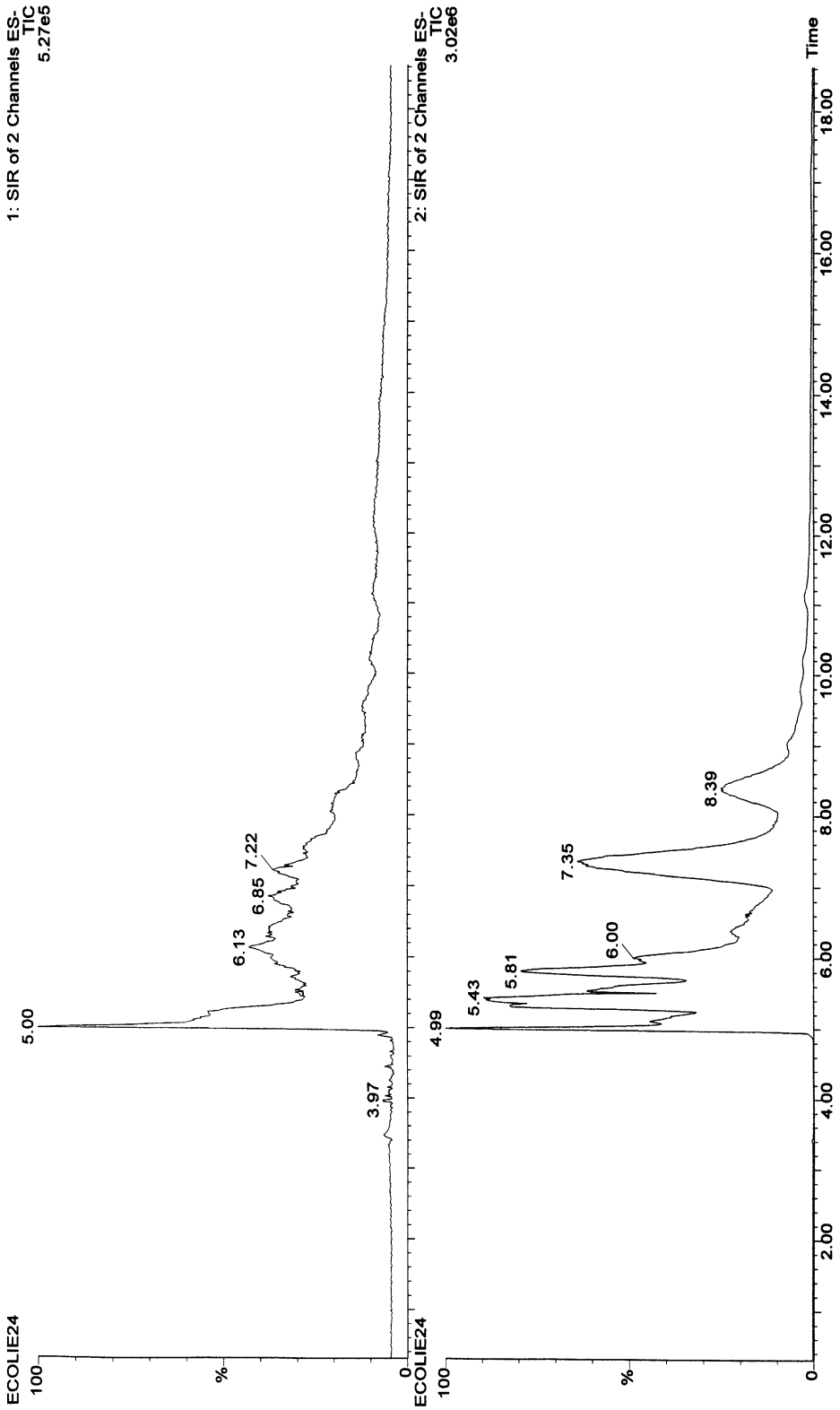

ECOLI0
1: SIR of 2 Channels ES-
TIC
2.01e5
100
%
0
3.46
4.15
5.68
5.96
6.99
7.34
8.84
9.41
10.61
12.34
16.76
ECOLI0
2: SIR of 2 Channels ES-
TIC
1.74e4
100
%
0
0.13
1.74
2.36
3.13
3.41
3.52
3.67
4.53
5.16
6.10
6.50
6.94
8.34
8.40
8.75
9.34
10.46
10.99
13.51
14.30
14.47
15.22
15.53
17.23
18.11
19.01
19.91
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Time

Figure IV-3. HPLC chromatographic separation of glycerophospholipds of *Escherichia coli* after 24 hours of exposure to chlorine treatment. The upper chromatographic trace is generated by selected ion monitoring of ions attributable to 16 and 18 carbon acyl-linked monounsaturated fatty acids (*m/z* values of 253 and 281). The lower chromatographic trace is generated by selected ion monitoring of ions attributable to 16 and 18-carbon acyl-linked oxirane fatty acids (*m/z* values of 269 and 297).


1: SIR of 2 Channels ES-
TIC
5.27e5
ECOLIE24
100
%
0
5.00
3.97
6.13
6.85
7.22
2: SIR of 2 Channels ES-
TIC
3.02e6
ECOLIE24
100
%
0
4.99
5.43
5.81
6.00
7.35
8.39
Time
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00

chromatographic trace. The bottom trace was generated through selective ion monitoring of the ion current that might be generated through formation of the 16 and 18 carbon oxirane fatty acid biomarkers.

A comparison of the upper traces (monoenoic precursors) of Figures IV-2 and IV-3 reveals that the phospholipid composition has clearly been modified. The monoenoic precursors endogenous to *E. coli* that are evident in the time 0 trace have been depleted after 24 hours of chlorine exposure. The disappearance of monoenoics may be attributed to the formation of 16- and 18-carbon oxirane products. This increase is evident from a comparison of the lower traces (oxirane products trace) in these two figures.

It is also evident that the lower trace in Figure IV-3 (oxirane product trace) is similar in pattern to the upper trace in Figure IV-2 (monoenoic precursor trace). However, in all instances, the retention times have shifted to shorter values, which would be expected for the generation of more polar, oxirane products. Further LC/MS experiments revealed again that the phospholipids observed in the lower chromatographic trace of Figure IV-3 were PG, DPG, or PE, respectively, in order of increasing retention time.

GC/MS analysis of authentic standards

GC/MS analysis of polar lipid FAMEs provided the additional information of *cis/trans* configuration of double bonds and the site of methyl branching. It also provided the ability to easily resolve and differentiate oxirane fatty acids from odd-chain saturated fatty acids based on retention behavior and fragmentation patterns obtained under standard electron impact conditions. To gain a better understanding of the retention behavior and fragmentation patterns of oxirane fatty acids, a series of oxirane standards were synthesized as described earlier. Figures IV-4, IV-5 and IV-6 display the electron impact mass spectra of three 18 carbon fatty acids prepared synthetically from 18:1ω12*c*, 18:1ω9*c*, 18:1ω7*c*, respectively. Mass spectra of oxiranes formed from monounsaturated fatty acid methyl esters give clearly recognizable fragments due to cleavage alpha to the epoxide group. The expected ions resulting from such cleavage for 18:1ω12, 18:1ω9 and 18:1ω7 are shown in Figure IV-7. The fragments 155 and 199, for example, from the cleavage of the oxirane product formed from 18:1ω9 (Figure IV-7) can be clearly seen in Figure IV-5. In fact, for a significant period of time, epoxidation has been a standard derivatization method for the localization of double bonds in fatty acid methyl esters (Minnikin, 1978). Low mass fragment ions of the following m/z value and relative abundance (m/z (rel. ab.)) may be considered

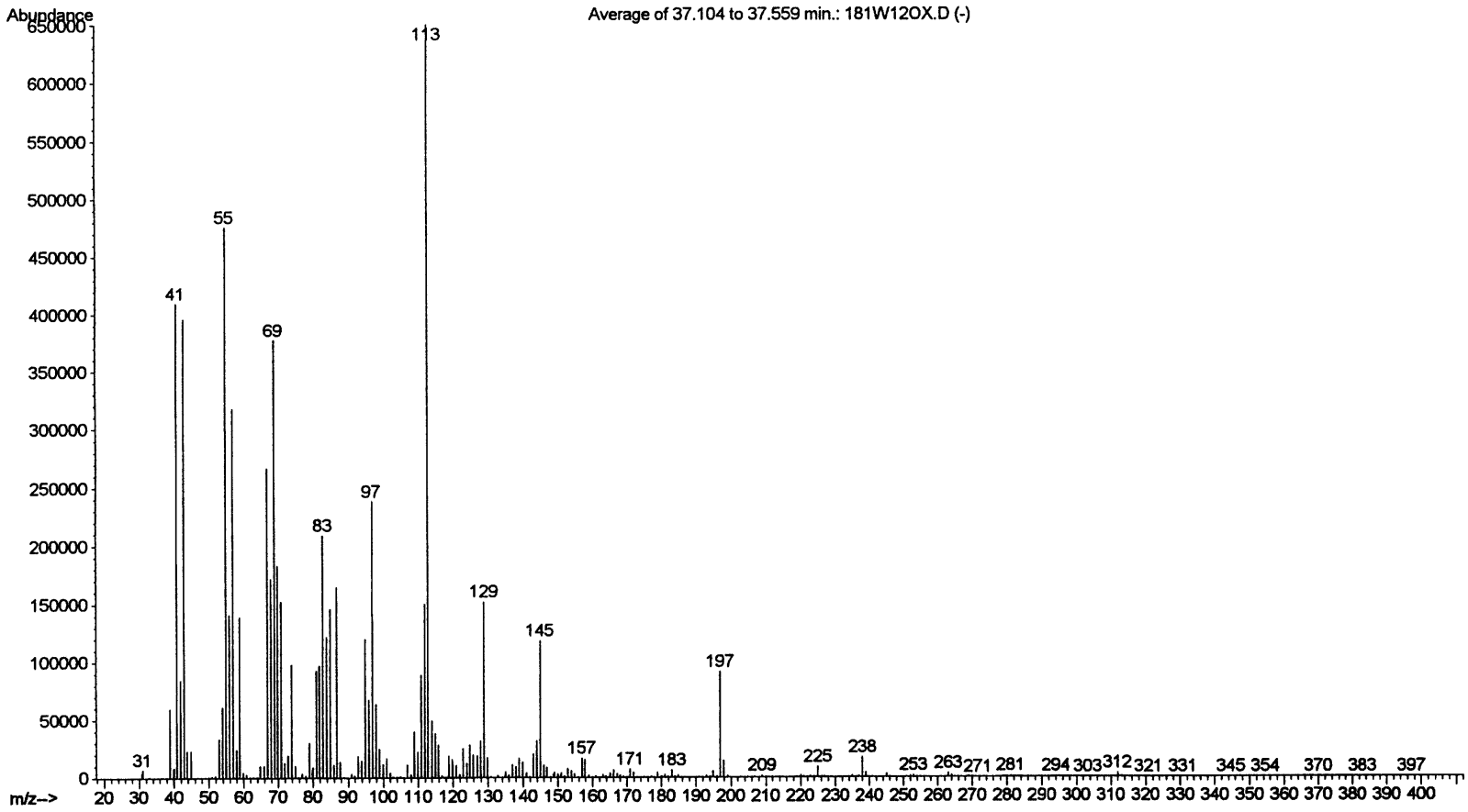


Figure IV-4. GC / MS spectrum of the epoxidated analog of 18:1ω12c.

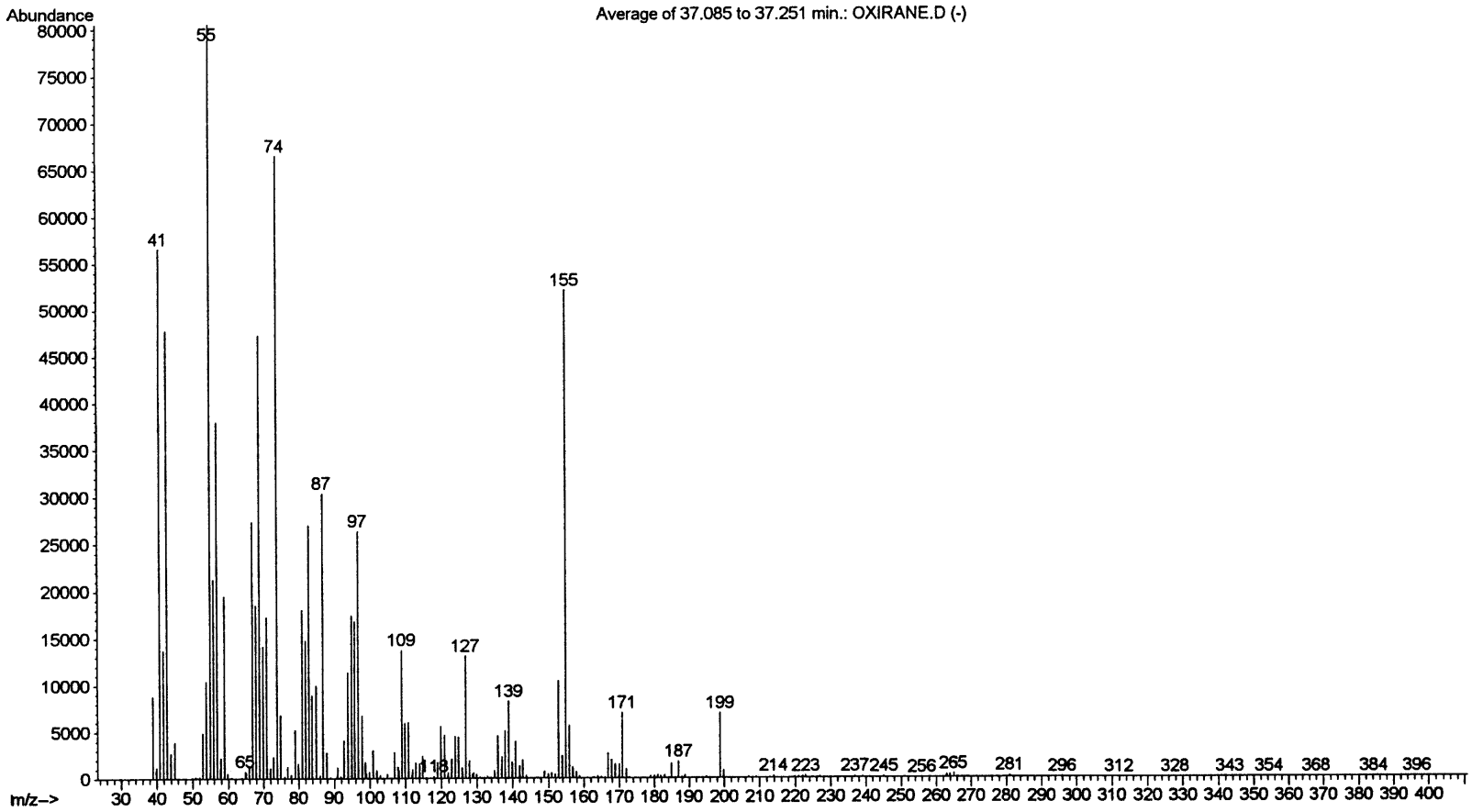


Figure IV-5. GC/MS spectrum of the epoxidated analog of 18:1ω9c.

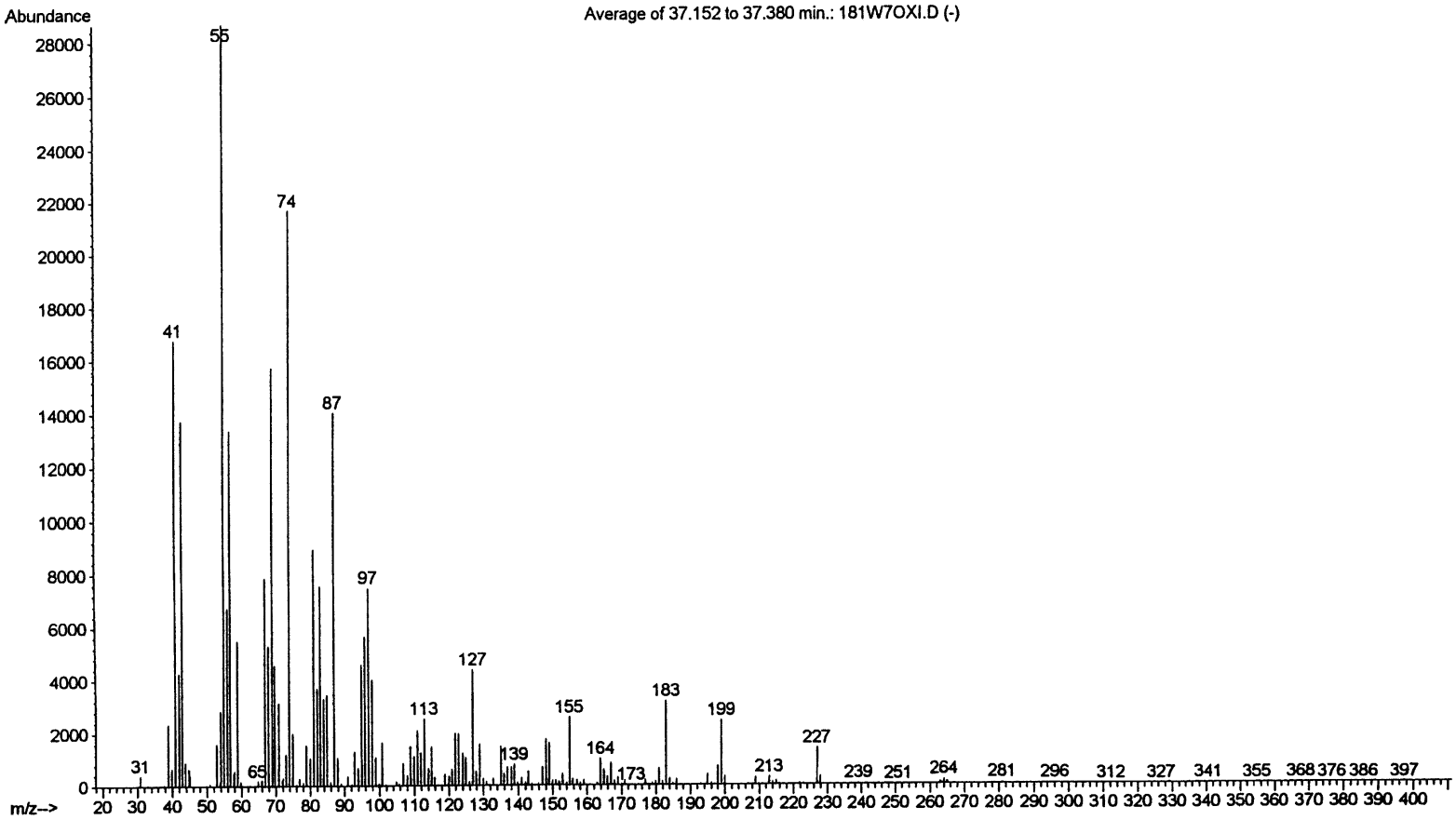


Figure IV-6. GC/MS spectrum of the epoxidated analog of 18:1ω7c.

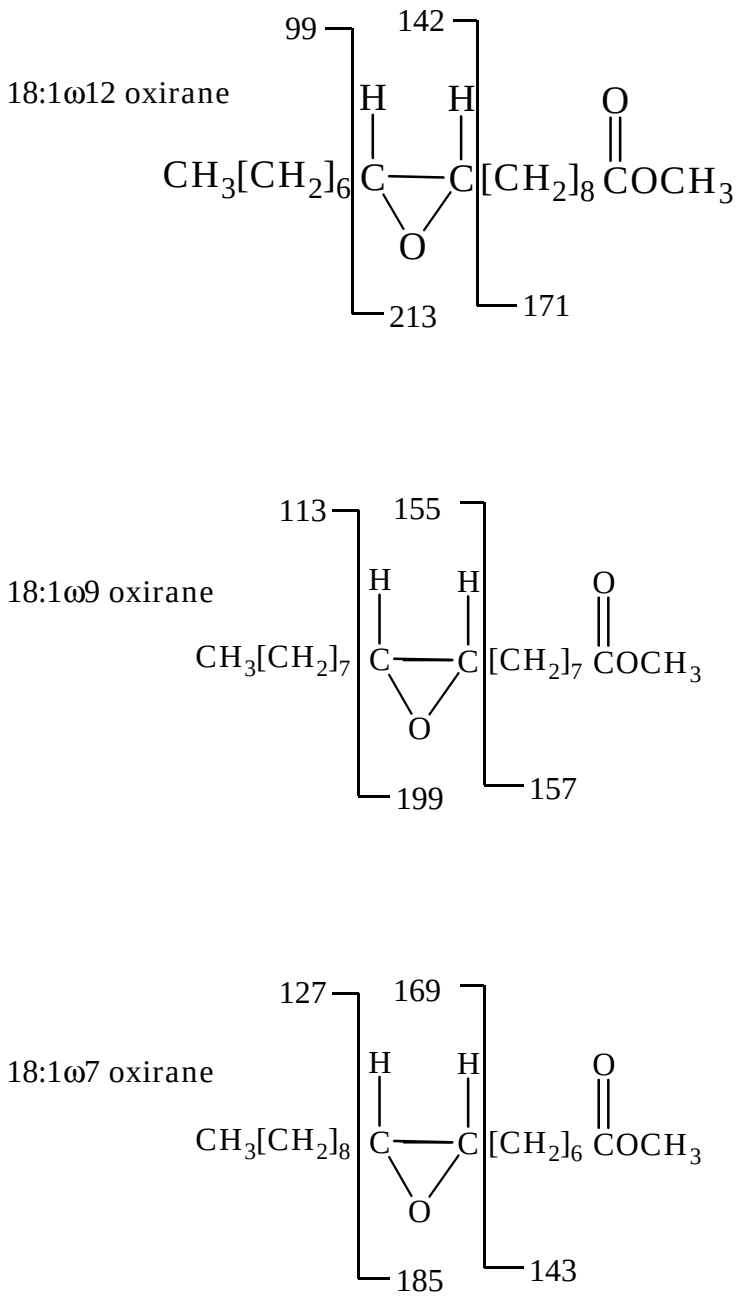


Figure IV-7. Higher mass fragment ions due to cleavage alpha to the epoxide groups.

diagnostic of epoxidated or oxirane fatty acids as a class: 55 (100), 74 (73), 41 (61), 69 (52), 87 (51), 43 (46). Higher mass α-cleavage products are diagnostic of the site of epoxidation (Figure IV-7). GC retention behavior, i.e. a capacity factor, was tabulated for each synthetic standard.

GC/MS analysis of polar lipid FAMEs from E. coli

Figure IV-8 shows the chromatogram obtained from *E. coli* prior to exposure to chlorine (time 0). The monoenoic precursors 16:1ω7 (26.44 minutes) and 18:1ω7 (32.28 minutes) are clearly evident in the FAME profile. The chromatogram obtained from *E. coli* after 24 hours of exposure to chlorine treatment is displayed in Figure IV-9. The peaks for the monoenoic precursors are not apparent in this chromatogram. Instead, peaks for the oxirane products of 16:1ω7 (31.46 minutes) and 18:1ω7 (37.29 minutes) are strongly evident. The spectrum of the peak at 31.46 minutes (Figure IV-10) confirms its identity as 7,8-epoxide 16:0, as the spectrum of the peak at 37.29 minutes (Figure IV-11) confirms the identification of the compound as 7,8-epoxide 18:0. Using GC/MS, the retention times for the epoxides is longer than for their monoenoic precursors, in contrast to their retention behavior using HPLC.

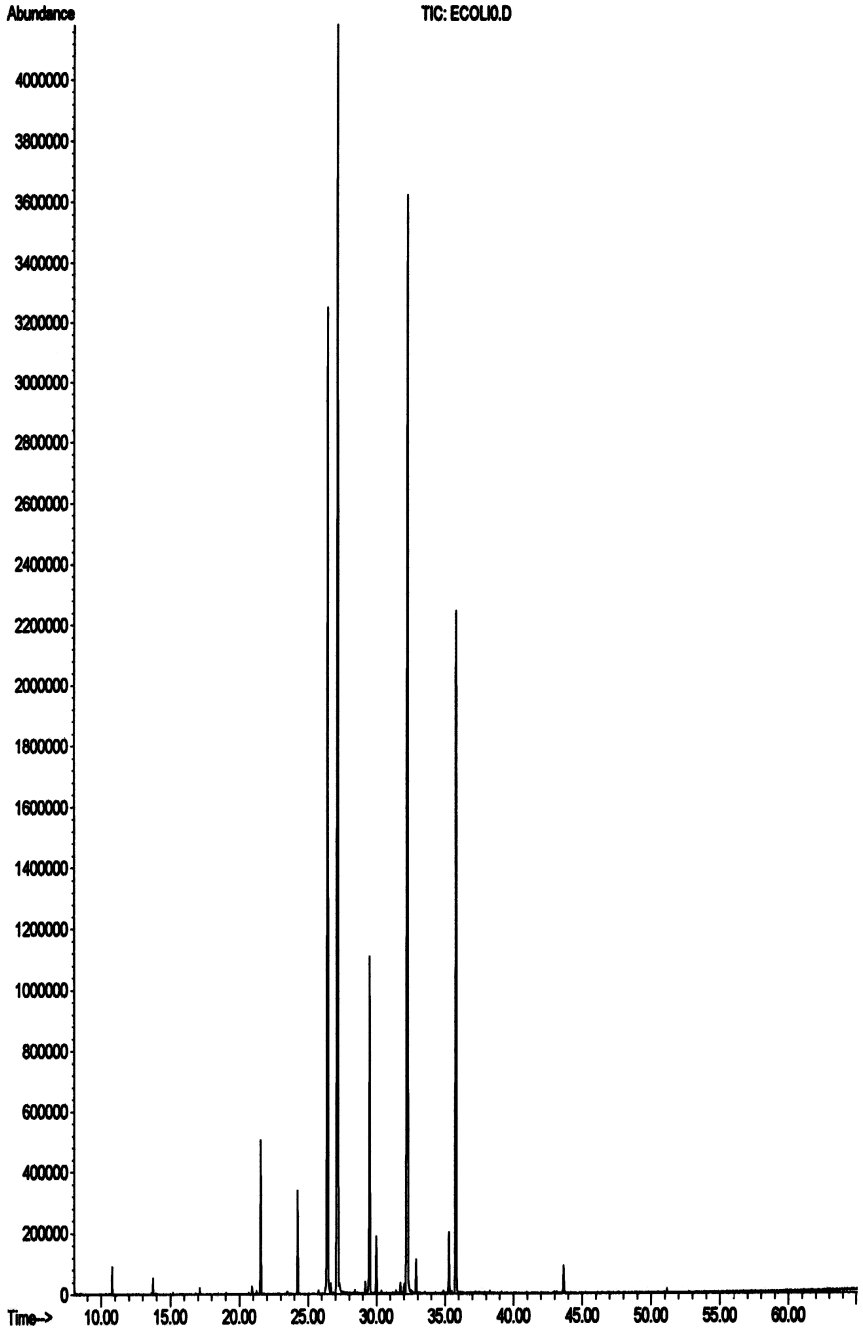


Figure IV-8. GC / MS chromatogram of *Escherichia coli* prior to exposure to chlorine. Monoenoic precursors 16:1ω7c and 18:1ω7c appear at 26.44 minutes and 32.28 minutes, respectively.

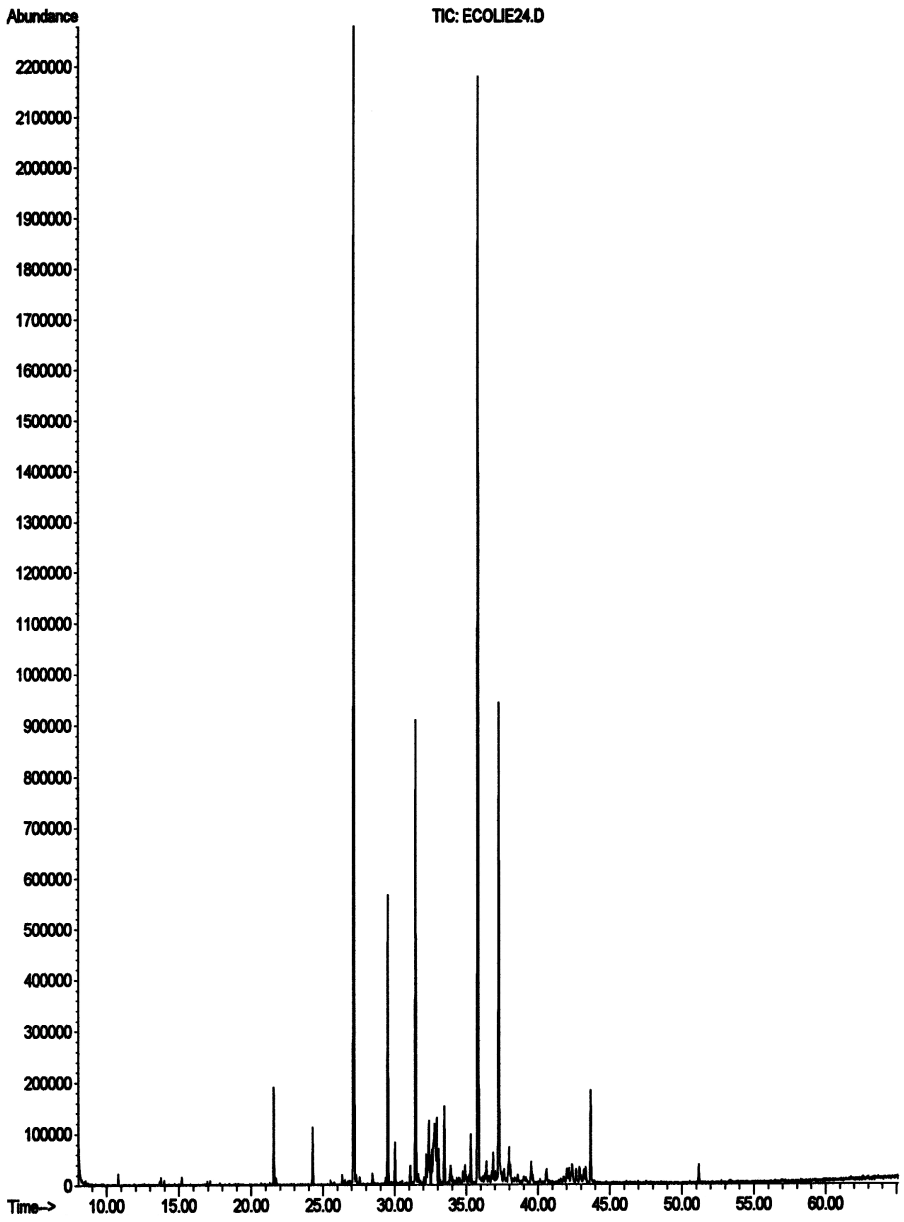


Figure IV-9. GC / MS chromatogram of *Escherichia coli* after 24 hours exposure to chlorine treatment. Peaks for monoenoic precursors 16:1ω7c (26.44 minutes) and 18:1ω7c (32.28 minutes) are not evident. Instead, peaks for the oxirane products of 16:1ω7 and 18:1ω7 are evident at 31.46 minutes and 37.29 minutes, respectively.

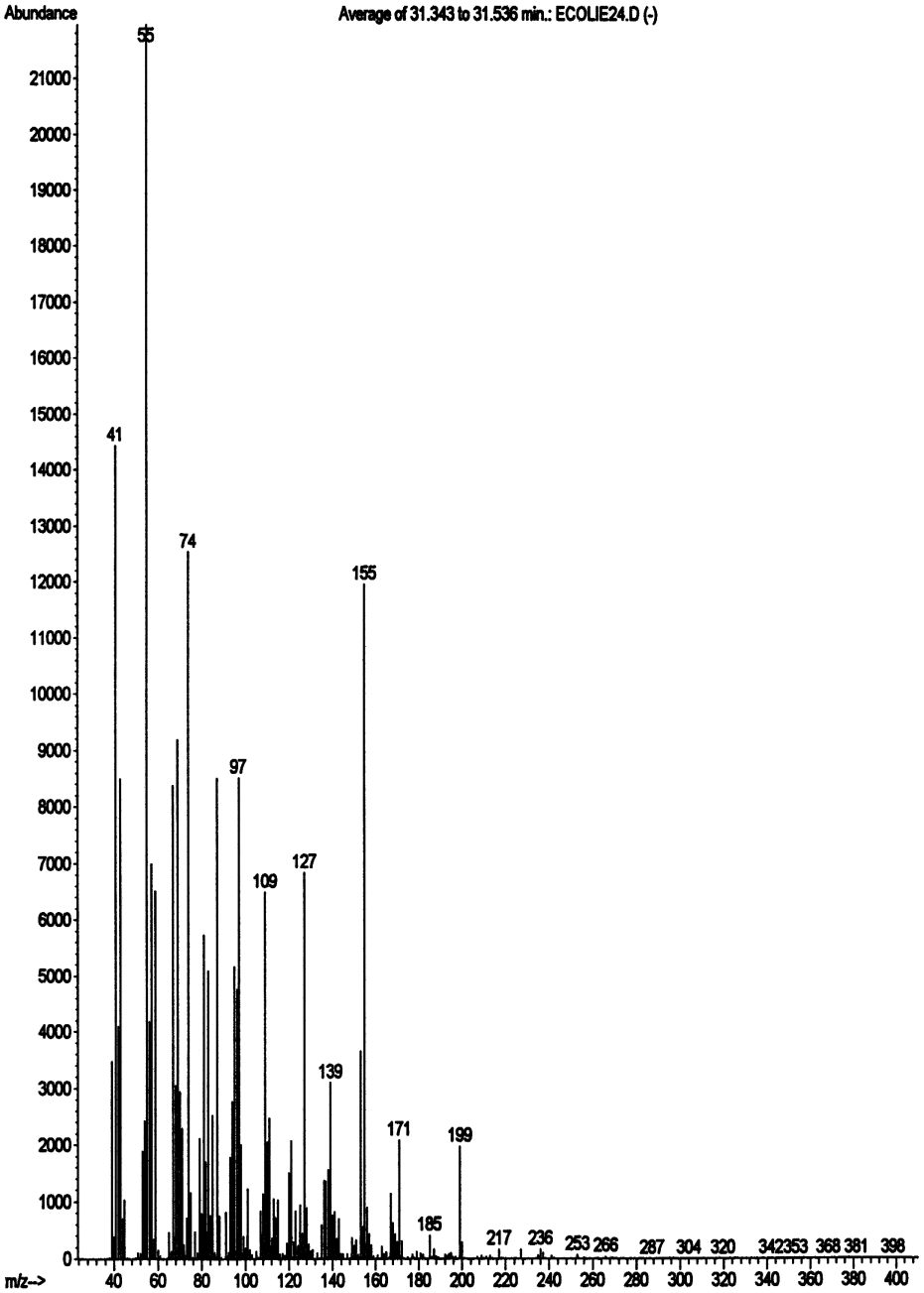


Figure IV-10. Spectrum of 7,8-epoxide 16:0. This spectrum was obtained by averaging the peak between 31.343 to 31.536 minutes in the chromatogram for *E. coli* after 24 hours exposure to chlorine. The chromatogram is shown in Figure IV-9.

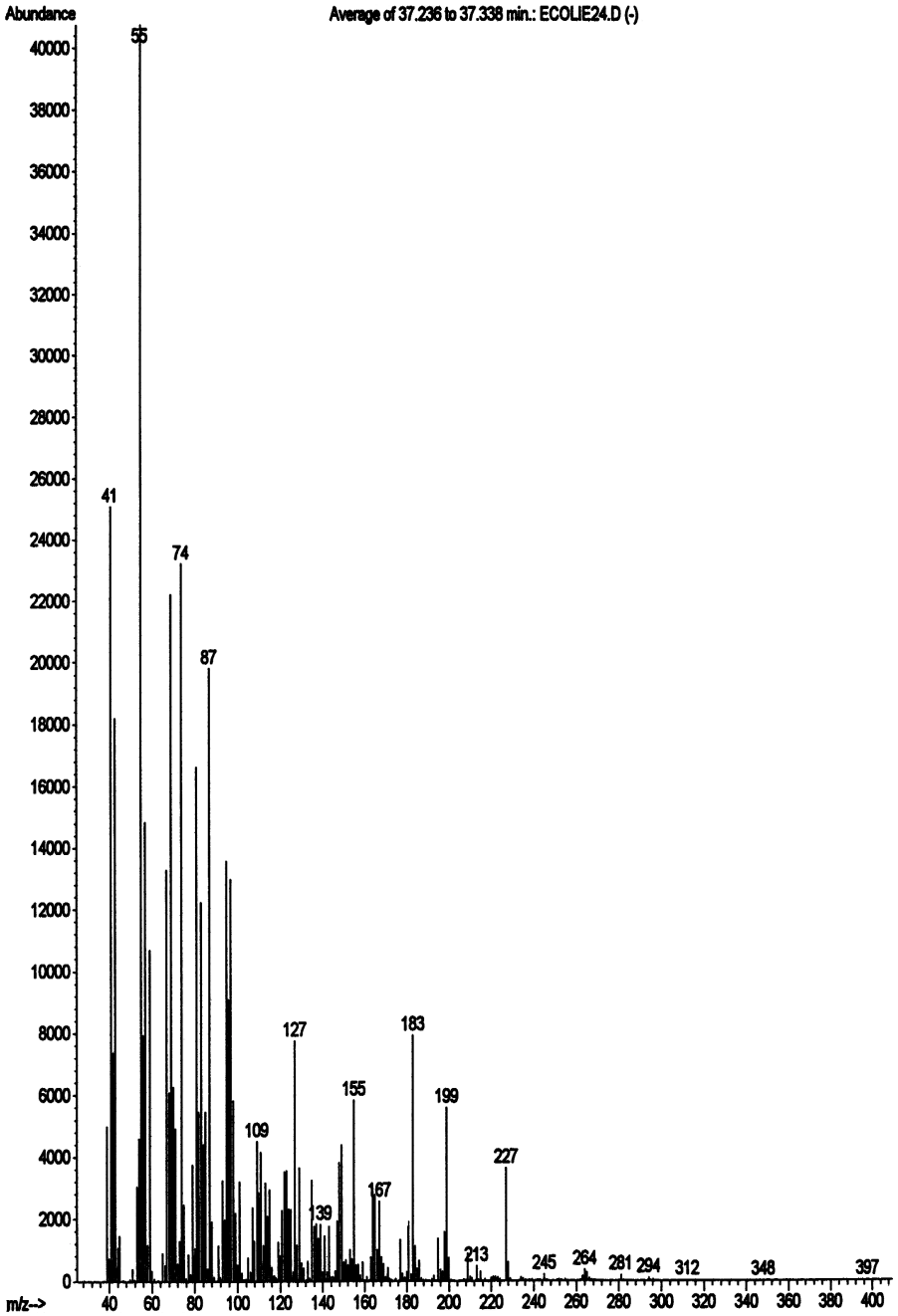


Figure IV-11. Spectrum of 7,8-expoxide 18:0. This spectrum was obtained by averaging the peak between 37.236 to 37.338 minutes in the chromatogram for *E. coli* after 24 hours exposure to chlorine. The chromatogram is shown in Figure IV-9.

Lipid profile of E. coli as a function of chlorine exposure

Table IV-1 illustrates the effect that chlorine exposure has upon the signature lipid profile of *E. coli*. This is a simplified PLFA profile, including only those FAMEs representing greater than 1 % of the total polar lipid profile. The profile was simplified to more clearly illustrate the effect of free chlorine on the monounsaturated fatty acids. Several points can be observed from this table. First, oxirane fatty acids were not detected in cultures of *E. coli* prior to chlorine exposure. Secondly, monounsaturated fatty acids were not observed in cultures of *E. coli* after 24 hours of exposure to disinfecting levels of free chlorine (2 ppm). Finally, oxirane fatty acids are formed at the expense of their monoenoic precursors. The conversion of monounsaturated fatty acids to oxirane analogs was not quantitative, as other oxidative products were also found. Nine and 11 carbon dioic acids were detected at low levels and probably resulted from further oxidation at the site of epoxidation in 16:1ω7 and 18:1ω7, respectively. Other more polar derivatives of monounsaturated fatty acids were detected, but the thermally labile nature of these products prevented formation of an intact molecular ion, which prevented further identification. Finally, one other point concerning the oxirane products observed in the 24-hour column of Table IV-1 should be noted. Both the 16:1ω7 oxirane and the 18:1ω7 oxirane fatty acids detected were of the

Table IV-1. Polar lipid fatty acid methyl esters (FAMEs) profiles of *Escherichia coli* prior to (0 hours) and after 24 hours of exposure to 2 ± .04 ppm free chlorine.

Fatty acid	0 hours	24 hours
14:0	2.27	2.38
15:0	1.64	1.48
16:1ω7c	23.15	---
16:0	34.04	38.71
cy 17:0	6.29	8.30
16:1ω7 oxirane	---	14.42
18:1ω7c	29.96	---
18:0	<1	2.1
cy 19:0	1.07	1.5
18:1ω7 oxirane	---	16.64

trans configuration. This is in contrast to the time zero experiment in which essentially all of the monounsaturated fatty acid precursors were of the *cis* configuration. *Cis/trans* isomers were determined through calculation of a capacity factor as well as through the method of standard addition with synthetically prepared standards. This suggests that the initial step in the transformation of monounsaturated fatty acids into their epoxidated (oxirane) analog in an environment high in free chlorine is the isomerization of *cis* monoenoics to *trans* monoenoics as a reaction to stress caused by the elevated levels of disinfectant present (Guckert et al, 1986). *Cis* to *trans* isomerization is immediately followed by the direct epoxidation of the alkene bond to form the *trans* epoxidated fatty acid (Figure IV-1). Finally, it must be noted that cultures of *E. coli* exposed to levels of free chlorine sufficient to produce oxirane fatty acids were rendered non-culturable.

DISCUSSION

Signature lipid biomarker analysis can be used to detect potential pathogens that may not routinely be detected by classical plate-counting methods. These experiments ensure the validity of oxirane fatty acids as

indicators of sterilization efficiency. The detection of epoxidated fatty acids firmly establishes that cells have been in contact with hypochlorite, thus providing a chemical marker for hypochlorite exposure and sterility. At free-chlorine levels sufficient to render the organisms non-culturable, conversion of monoenoic fatty acid precursors to their epoxidated product analogs was observed. Oxirane fatty acids were not detected in cultures of *E. coli* that had not been exposed to hypochlorite.

LC/MS analysis with a single quadrupole mass analyzer is capable of providing a great deal of information regarding the nature of cellular membrane components, which may act as biomarker signatures of a microorganism's presence. LC/MS analysis with a single quadrupole provides information regarding the m/z value of acyl linked fatty acids and the nature of the polar head group, as well as information regarding the nature of the intact molecular species. However, with low-resolution single quadrupole mass analyzers, the potential exists for ambiguity in the information attained. Consequently, in these analyses with the VG Platform II system described earlier, 16- and 18-carbon oxirane fatty acid ion currents are indistinguishable from 17- and 19-carbon saturated fatty acids. Odd-chain saturated fatty acids do not represent a significant portion of the fatty acid profile in Gram-negative bacteria such as *E. coli* and, thus, did not pose a problem in the application of this technology for

this project. However, the application of this technique to a complex water distribution system biofilm would be limited due to the presence of Gram-positive bacteria and the odd-chain saturated fatty acids attributable to them. This ambiguity may be overcome through the use of more expensive, but more structurally informative tandem mass analyzers. Unfortunately, a tandem mass spectrometer is not currently available in this laboratory. Therefore, it was necessary to utilize the complementary technique of gas chromatography-mass spectrometry so as to add a higher degree of specificity to this analysis.

In this study, LC/MS provided additional information in that the epoxidated fatty acids were formed *in vivo* as oxidative products of phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylethanolamine. The application of GC/MS provides additional specificity to the analysis resulting in information of greater reliability. The detection and identification of oxirane fatty acids through these two techniques should provide a rapid, routine, and sensitive method for ensuring the safety of water consumers through careful monitoring of the water distribution system effluent.

This study did not make a determination as to whether the bacteria exposed to chlorine were “dead or alive”. Further studies would be necessary to discover the status of these bacteria, through recovery and

enrichment studies. It is probable that the bacteria exposed to chlorine are disintegrating, and the formation of oxiranes is simply a chemical reaction of the lipids left from their demise, based on the amount of time it takes for the oxiranes to appear in the lipid profile. It would be interesting to perform this experiment using some of the more chlorine-resistant species of pathogens.

PART V.

CHEMICAL ASSAYS FOR DIPICOLINIC ACID AND ENDOSPORES

INTRODUCTION

One of the most important organisms that cannot be detected with a sensitive and rapid assay is the bacterial endospore. Certain endospore-forming bacteria are pathogenic to humans and are the causative agents for serious diseases. The species most toxic to humans are *Bacillus anthracis, Clostridium botulinum, Clostridium perfringens,* and *Clostridium tetani*. Media coverage over the past several years has brought some of these bacteria into the public eye. For instance, *Bacillus anthracis* has been reported in several incidents as a biological threat in potential terrorist attacks. These reports have brought to light the inadequacy of the methodology currently employed to rapidly assay for the presence of spores in the environment. Other less fatal but regular causative agents of food poisoning, *Bacillus cereus* and *Bacillus subtilis*, are of particular interest to the food industry. The sensitivity of an analytical assay for spores is somewhat a function of its application; however, a recent study has proposed that a reasonable goal would be the detection of less than 10^4 spores per ml (Hindle and Hall, 1999).

The target analyte for endospore detection using chemical methods has been dipicolinic acid (DPA). The reason for its choice lies in the uniqueness of DPA as a constituent of spores. It is synthesized during the

final stages of sporulation, is released when the spore germinates and is not found as a constituent of vegetative cells. Various methods have been developed for detection of DPA from spores. Currently, the most successful using real samples are high performance liquid chromatography (HPLC) methods, reporting 7 x 10^5 spores per gram of salmon (Paulus, 1981; Warth, 1979). The most sensitive method using spore suspensions are spectrofluorometric methods, which report a lower limit of detection of 1 x 10^4 spores per ml (Hindle and Hall, 1999).

The objective of this study was to develop a sensitive, quantitative assay for DPA that can be used in various environmental matrices to determine the total endospore population in a sample. Since gas chromatography / mass spectrometry (GC / MS) techniques are considerably more sensitive than spectrofluorometric techniques, a combination using GC / MS and a rapid extraction method seemed a likely possibility. Using supercritical fluid and a methylating reagent in situ seemed likely to be an easier and faster solution to determine the presence or absence of endospores in environmental samples.

MATERIALS AND METHODS

Note: The supercritical fluid extraction method has been summarized, due to patent disclosure filed with the University of Tennessee.

Chemicals

All reagents were of analytical grade. Authentic standards were purchased from Matreya (Pleasant Gap, PA). All solvents were obtained from Baxter Scientific Products (McGaw Park, IL) and were of gas chromatography grade. The methylating reagent and sand were purchased from Sigma Chemical Co (St. Louis). Diatomaceous earth was obtained from VWR Scientific Products (Brisbane, CA).

Glassware

All glassware was washed in a 10% (vol / vol) Micro cleaner solution (Baxter Diagnostics, Deerfield, IL), rinsed ten times with tap water and then ten times with deionized water. The glassware was then heated overnight in a muffle furnace at 450°C to remove any carbon contamination.

Spectrofluorometric analysis with authentic standard

Aqueous serial dilutions of dipicolinic acid (DPA) were prepared and compared to solutions without DPA added. A 100 mM terbium chloride solution (Johnson Matthey, Ward Hill, MA) was added to each dilution. Excitation was at either 280 or 270 nm, and emission spectra were measured at 542 or 546 nm using a UV-vis spectrofluorometer (SPEX 0.22 m Double Spectrometer, Edison, NJ).

Bacterial spores

A stock spore suspension of *Bacillus subtilis* ATCC 6633 was purchased from Difco Laboratories (Detroit). The concentration of spores in this suspension was approximately 3×10^8, determined by AODC counts. Serial dilutions were made by centrifugation of the spore suspension and re-suspension in methanol.

Sand and diatomaceous earth

Sand and diatomaceous earth were heated separately overnight in a muffle furnace at 450°C to remove any carbon contamination.

Preparation of methyl esters of authentic standards

Preliminary experiments with 2,6-, 2,4-, 2,3- and 3,5-pyridinedicarboxylic acids used a bench-top acid methanolysis technique (Parker *et al*., 1982). Solutions of authentic standards were made in methanol. Dilutions were dried down under nitrogen and then methylated.

Sample preparation for supercritical fluid extraction

Eight-ml cartridges for the Isco SFX 2-10 Extractor (Lincoln, NE) were rinsed in methanol and then partially filled with diatomaceous earth. A spore suspension or sand mixed with spores was then added to the cartridge with a methylating reagent and acid mixture and methanol. Any remaining void volume was filled with diatomaceous earth.

Spore extraction in supercritical fluid and purification of DPA methyl esters

Filled cartridges were immediately placed in the SFX 2-10. A combination of static and dynamic extractions was performed with CO_2. The sample was collected in methanol. Then the sample was dried under a stream of nitrogen. If necessary, the sample was stored overnight at -20°C. The organic layer was dried under a stream of nitrogen and resuspended in hexane:diethyl ether (1:1, vol/vol) for GC/MS analysis.

GC/MS analysis

Verification of DPA methyl esters was achieved using mass spectrometry performed on a Hewlett Packard 5971 mass selective detector coupled to a Hewlett-Packard model 5890 series II gas chromatograph. Chromatographic separations were carried out on a HP-1 fused-silica capillary column [50 m by 0.2 mm (inner diameter)] of 0.11 μm film thickness. The column was kept at an initial temperature of 40°C for 2 minutes, and the temperature was programmed to increase from 40 to 120°C at 10°C / min, then to 160°C at 3°C / min, then to 312°C at 10°C / min. The injector and detector were maintained at 290°C and 300°C, respectively. Splitless injections were performed with a Hewlett-Packard model 7673 autosampler. The split valve was opened 1 min after injection. The carrier gas, helium, was maintained at an inlet pressure of 195 kPa to establish a carrier gas flow rate of approximately 1ml / min (Ringelberg *et al*., 1989). DPA methyl esters were identified through comparison of its mass spectrum and retention behavior with a synthetically prepared standard (Matreya, Inc., Pleasant Gap, PA). Peak areas were determined using a programmable laboratory data system, Enhanced ChemStation (Hewlett-Packard, Palo Alto).

RESULTS

Spectrofluorometric analysis

The calibration curve for DPA using excitation at 270 nm and emission spectra measured at 546 nm is shown in Figure V-1. The curve obtained was linear between concentrations of 33 ng / ml and 10 mg / ml. The lower limit of detection (LOD) of 33 ng / ml is equivalent to approximately 9 x 10^5 spores (Bloomfield and Arthur, 1994). The results obtained with the same dilution series, using excitation at 280 nm and emission spectra measured at 542 nm, are shown in Figure V-2. The calibration curve obtained was linear between concentrations of 10 ng / ml and 10 mg / ml, and the lower limit of detection (LOD) was 10 ng / ml, which is equivalent to approximately 2.7 x 10^5 spores (Bloomfield and Arthur, 1994). The R^2 for the curve using excitation of 270 nm was somewhat better than for the curve using excitation of 280 nm, but the curve in Figure V-2 was linear for lower dilutions of DPA.

GC/MS analysis

GC / MS analysis of authentic standards revealed differences in the fragmentation patterns of the isomers. Figure V-3 shows the fragmentation pattern of dimethyl dipicolinate (2,6-pyridinedicarboxylic

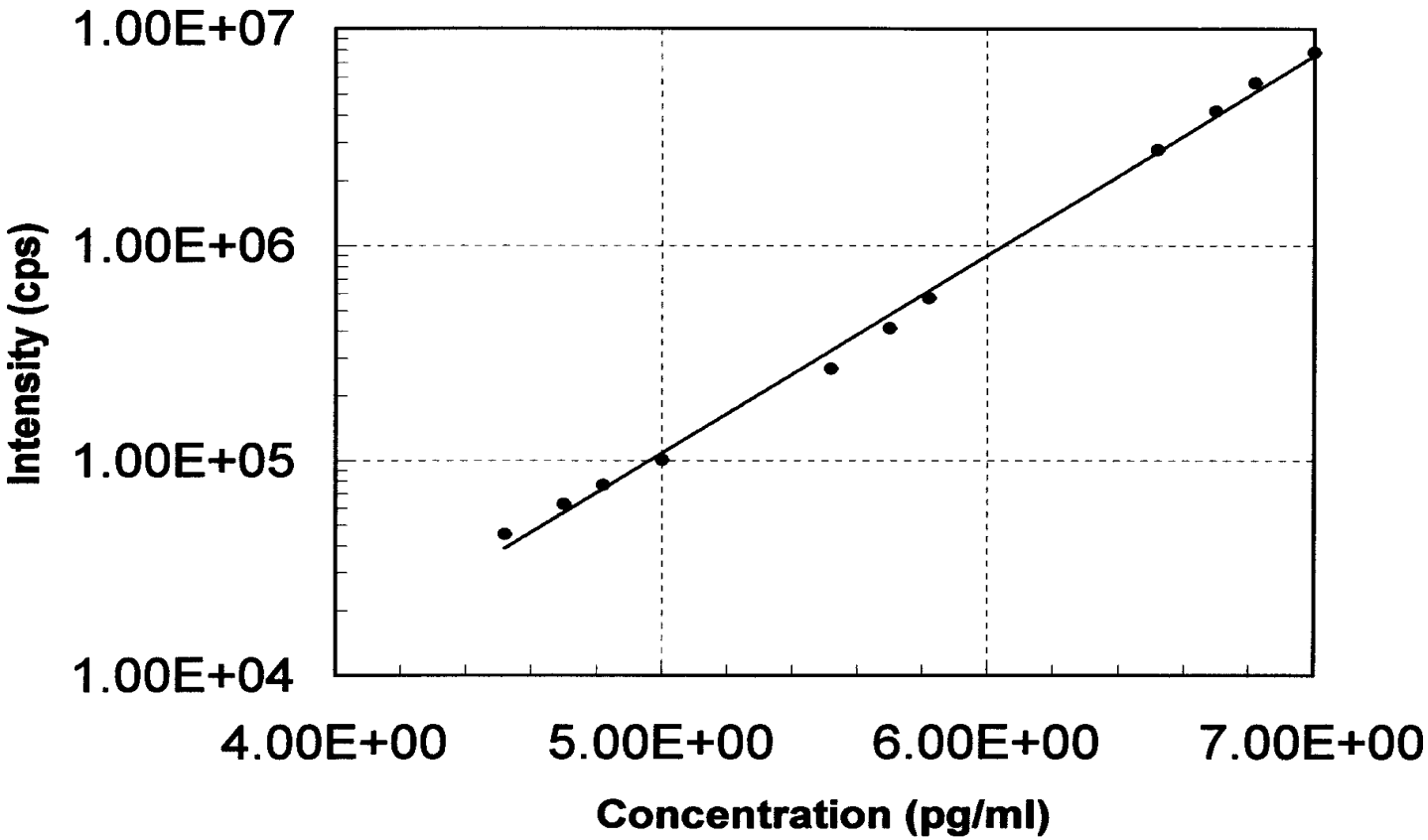


Figure V-1. Calibration curve for DPA using excitation at 270 nm and emission spectra measured at 546 nm (n=3). R^2 = 0.787.

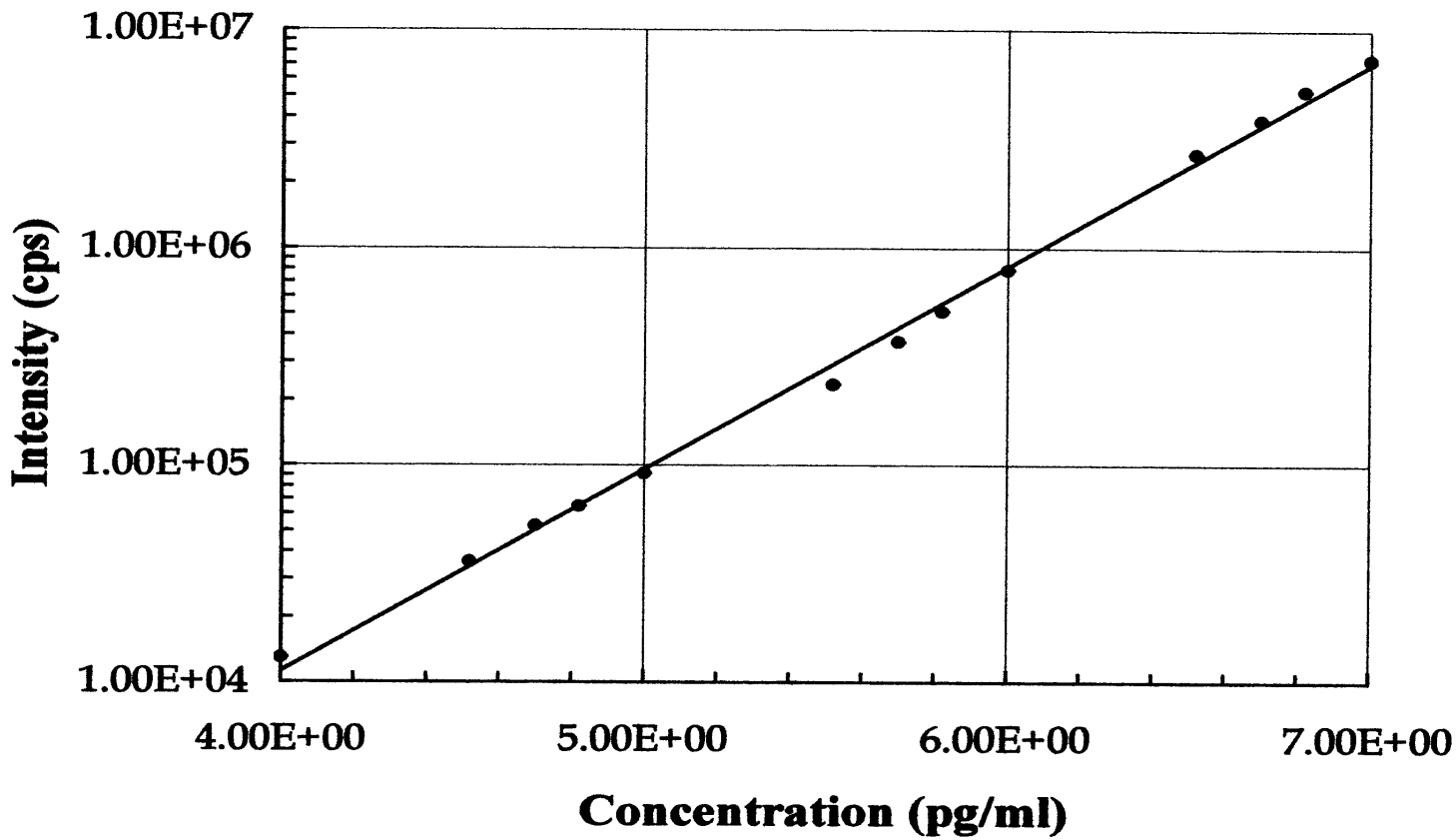
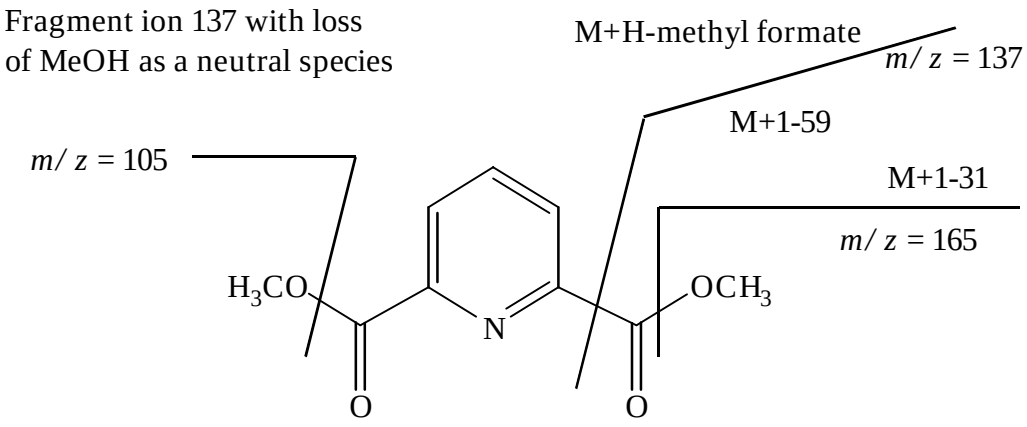


Figure V-2. Calibration curve for DPA using excitation at 280 nm and emission spectra measured at 542 nm (n=3). R^2 = 0.734.



Dimethyl Dipicolinate
MW = 195

Figure V-3. GC / MS fragmentation pattern of dimethyl dipicolinate (2,6-pyridine dicarboxylic acid dimethyl ester, or DPA dimethyl ester).

acid dimethyl ester, or DPA dimethyl ester). The chromatographic separation of the four isomers is shown in Figure V-4. The peaks are 2,3- (27.530 min), 3,5- (27.972 min), 2,4- (29.188 min) and 2,6- (29.790 min) pyridinedicarboxylic acid dimethyl esters, in order of increasing retention time on the column.

The mass spectra of these four peaks are shown in Figures V-5 through V-8. An examination of these four spectra reveals that the relative abundance ratios of only a few ions clearly differentiate one isomer from another. For instance, both 2,3- and 3,5-pyridinedicarboxylic acid dimethyl esters have a greater abundance of *m/z* values 164 and 136 (Figures V-5 and V-6), while the 2,4- and 2,6- isomers have an *m/z* value of 137 as the most abundant ion in their spectra (Figures V-7 and V-8). The higher relative abundance of 105 (*m/z*) in the 2,6- isomer (dimethyl dipicolinate) distinguishes it from the 2,4- isomer, which has a higher relative abundance of 108 (*m/z*). Consequently, the 2,6- isomer (dimethyl dipicolinate), the biomarker of interest, can be easily distinguished from the other isomers by selected ion monitoring (SIM) of three major ion fragments, 105 (62), 137 (100) and 165 (12) (*m/z* (relative abundance)).

A GC/MS chromatogram of SFE-extracted 3 x 10^8 spores is shown in Figure V-9. The chromatogram depicts MS scanning between 50 and 250 a.m. u. The mass spectrum of the peak located at 21.99 min shows

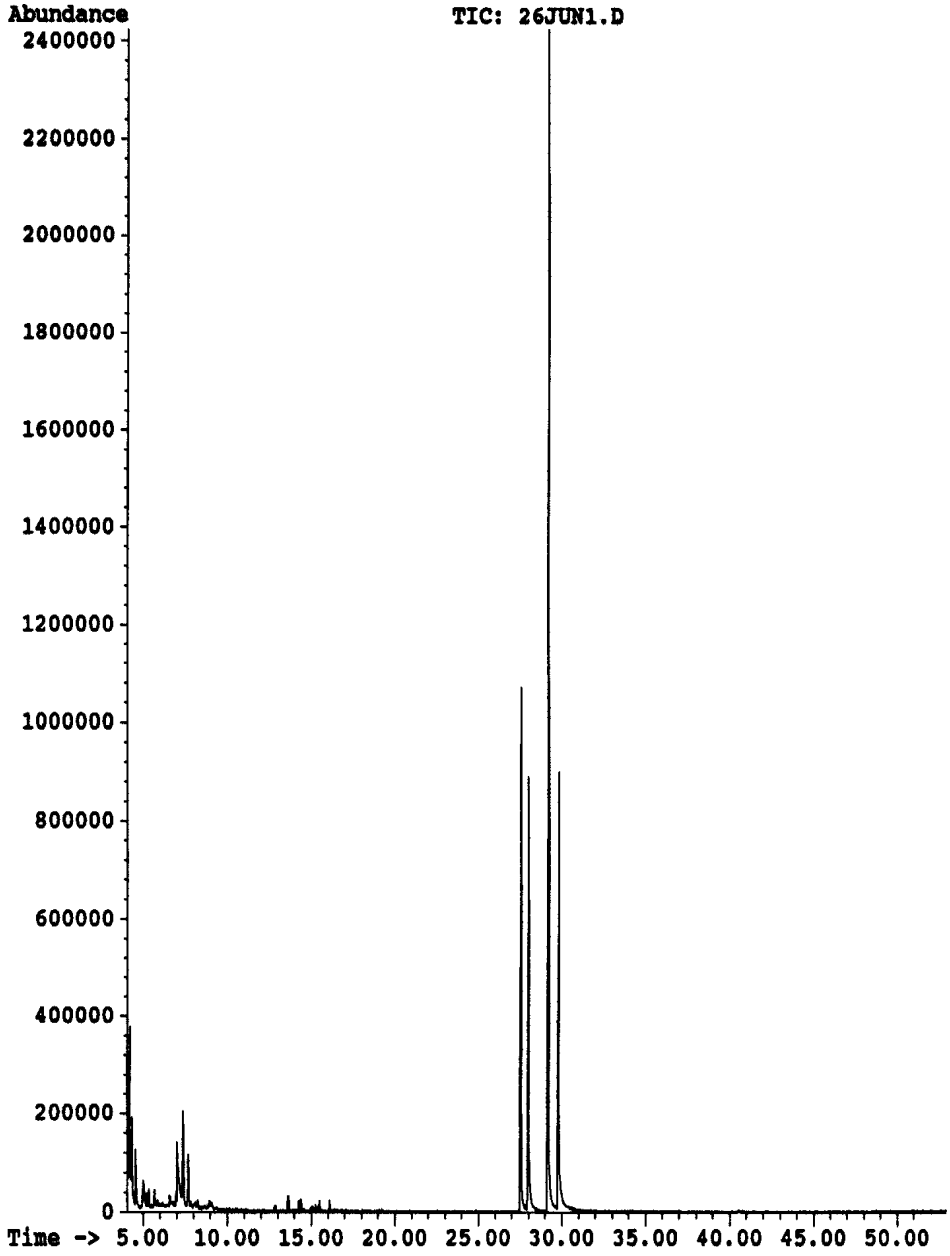


Figure V-4. GC / MS chromatogram of dimethyl esters of authentic standards of pyridine dicarboxylic acid isomers. The peaks are 2,3-pyridine dicarboxylic acid (27.538 min), 3,5-pyridine dicarboxylic acid (27.972 min), 2,4-pyridine dicarboxylic acid (29.188) and 2,6-pyridine dicarboxylic acid (29.790 min), dimethyl esters.

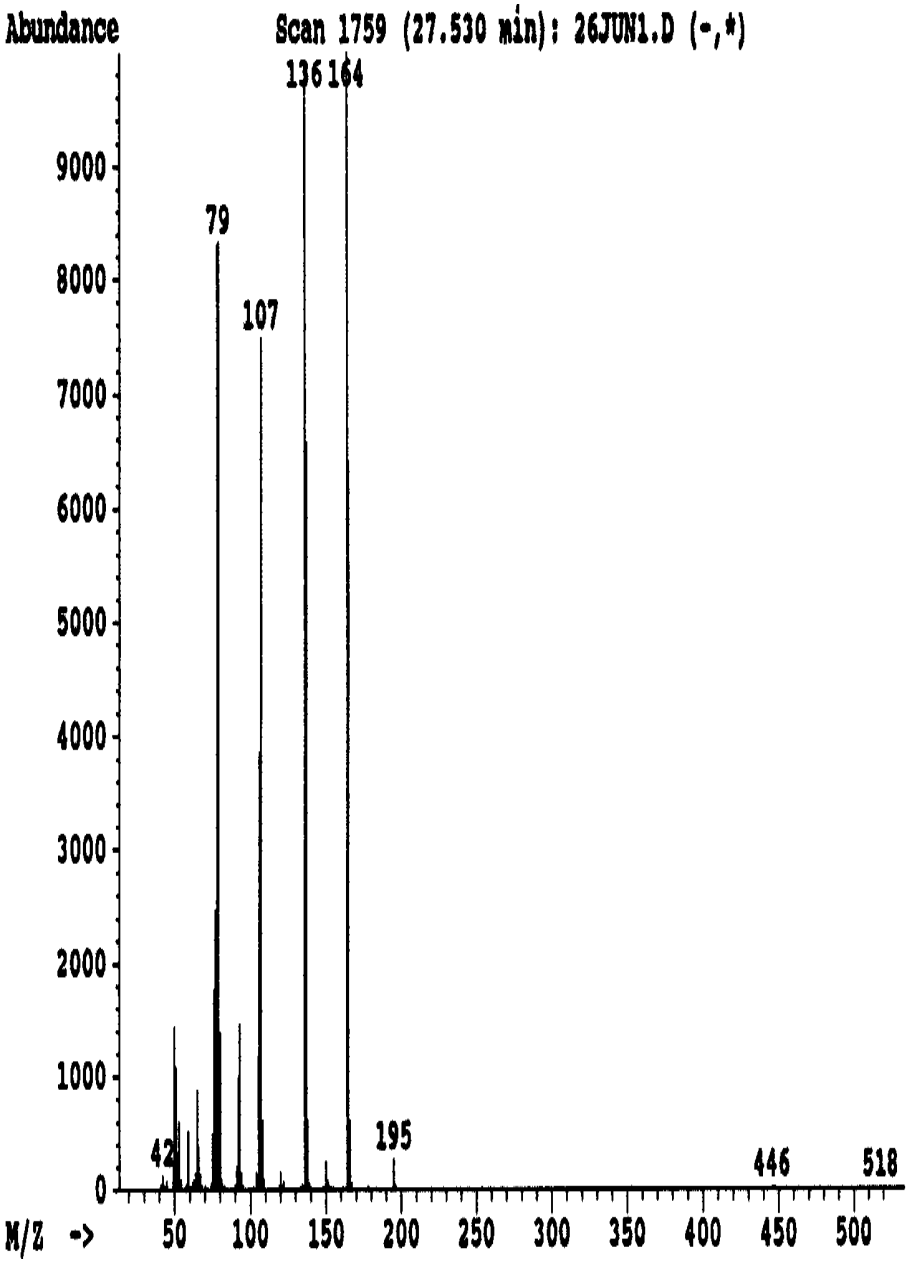


Figure V-5. Mass spectrum of 2,3-pyridine dicarboxylic acid dimethyl ester. This spectrum is from the 27.530 min peak in the chromatogram in Figure V-4. Note the most abundant ions are 164 and 136, with a high abundance of ion 107 also apparent. The molecular ion is 195.

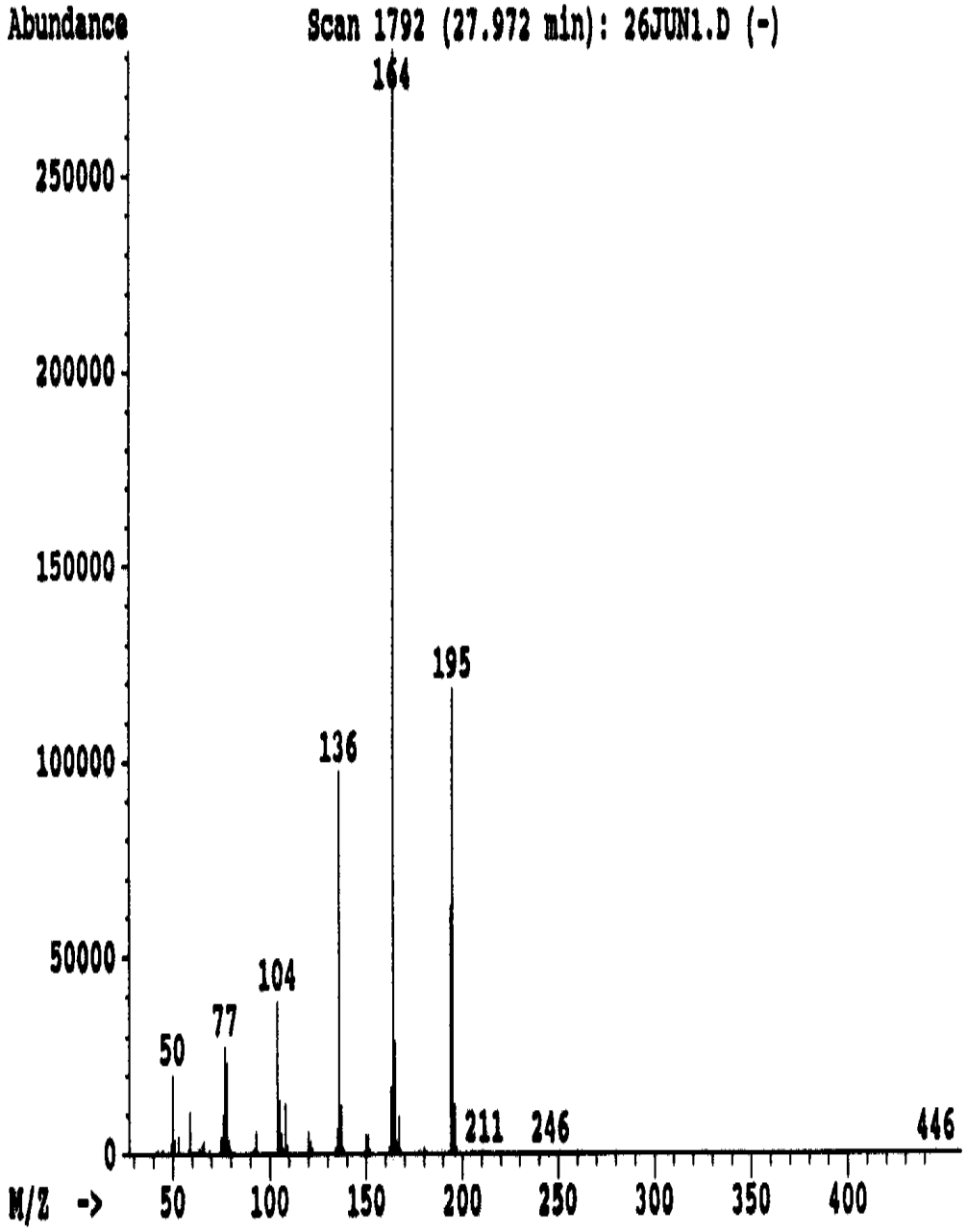


Figure V-6. Mass spectrum of 3,5-pyridine dicarboxylic acid dimethyl ester. This spectrum is from the 27.972 min peak in the chromatogram in Figure V-4. Note the most abundant ions are 164, with a lesser amount of ion 136. Ion 104 is also apparent in its profile. The molecular ion is 195.

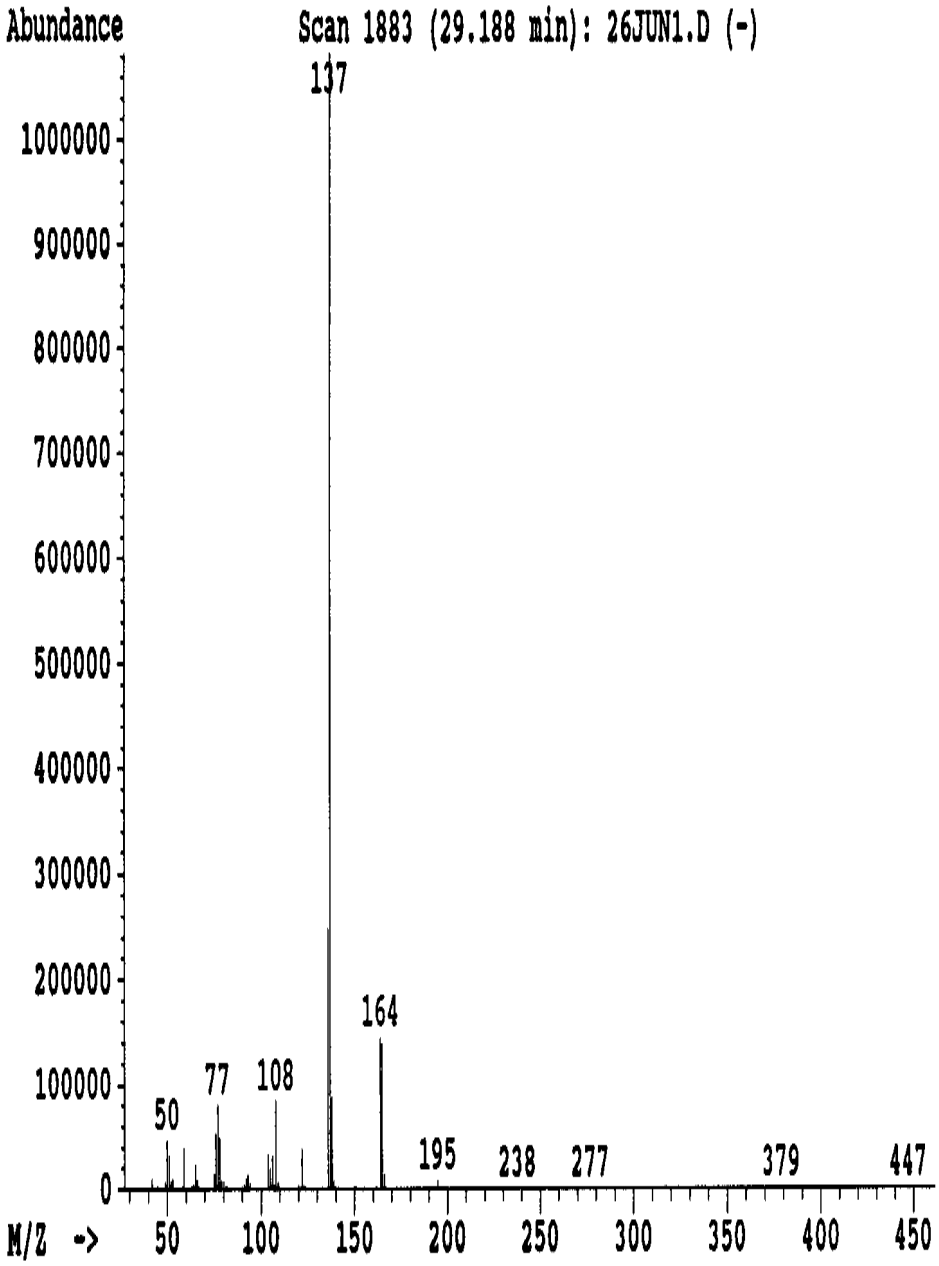


Figure V-7. Mass spectrum of 2,4-pyridine dicarboxylic acid dimethyl ester. This spectrum is from the 29.188 min peak in the chromatogram in Figure V-4. Note the most abundant ion is 137, with a lesser amount of ion 136. Ion 164 is also apparent in its profile, as well as ion 108. The molecular ion is 195.

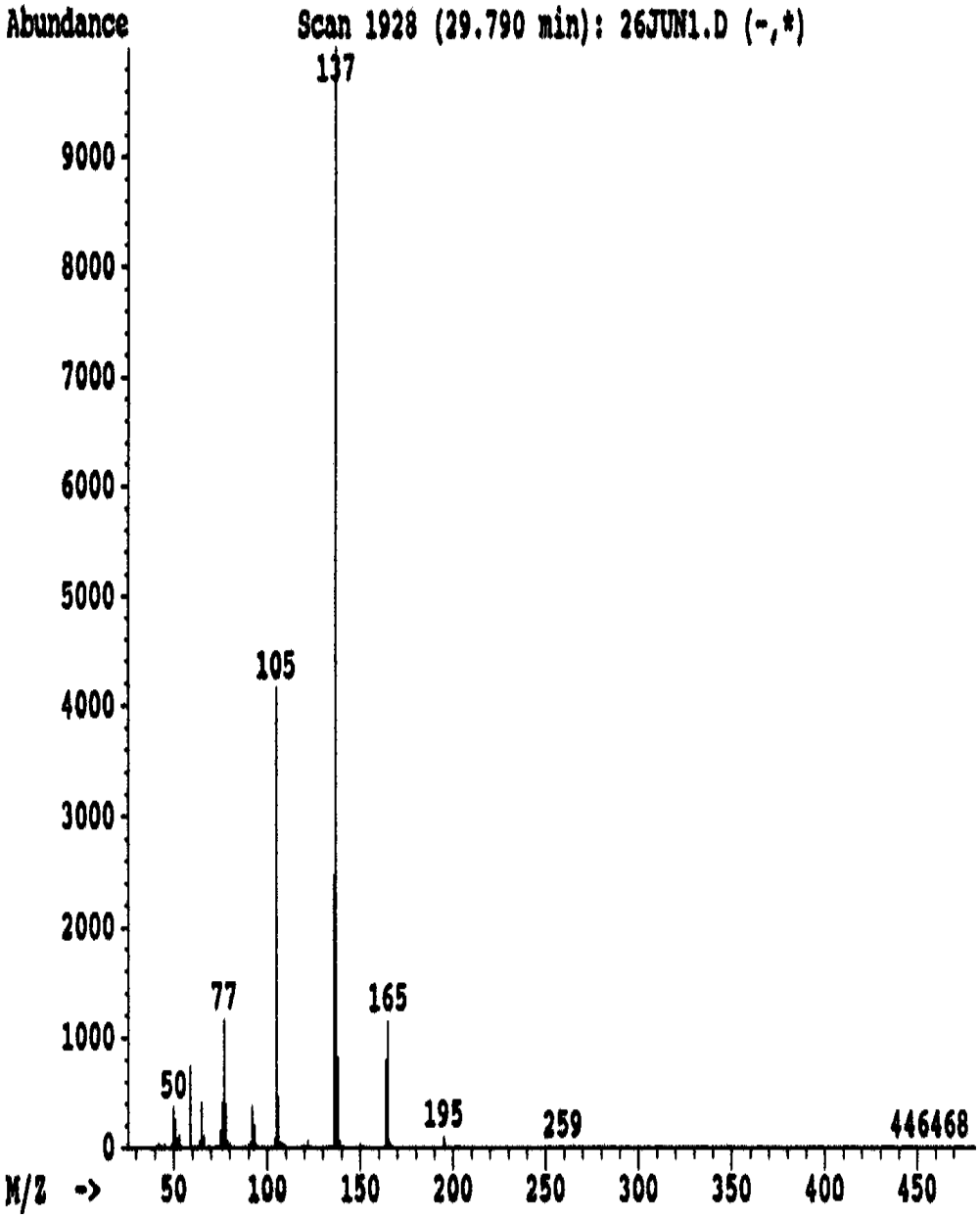


Figure V-8. Mass spectrum of 2,6-pyridine dicarboxylic acid dimethyl ester (DPA). This spectrum is from the 29.790 min peak in the chromatogram in Figure V-4. Note the most abundant ion is 165, with a lesser amounts of ion 105 and 165. The molecular ion of the dimethyl ester is 195.

```
File        : E:\SPORE16.D
Operator    : Cas
Acquired    :  6 Jan 99  12:13     using AcqMethod DPAAUTO
Instrument  :   GC/MS Ins
Sample Name: 3 e8 spores in sand with sfe magic mix
Misc Info   :
Vial Number: 2
```

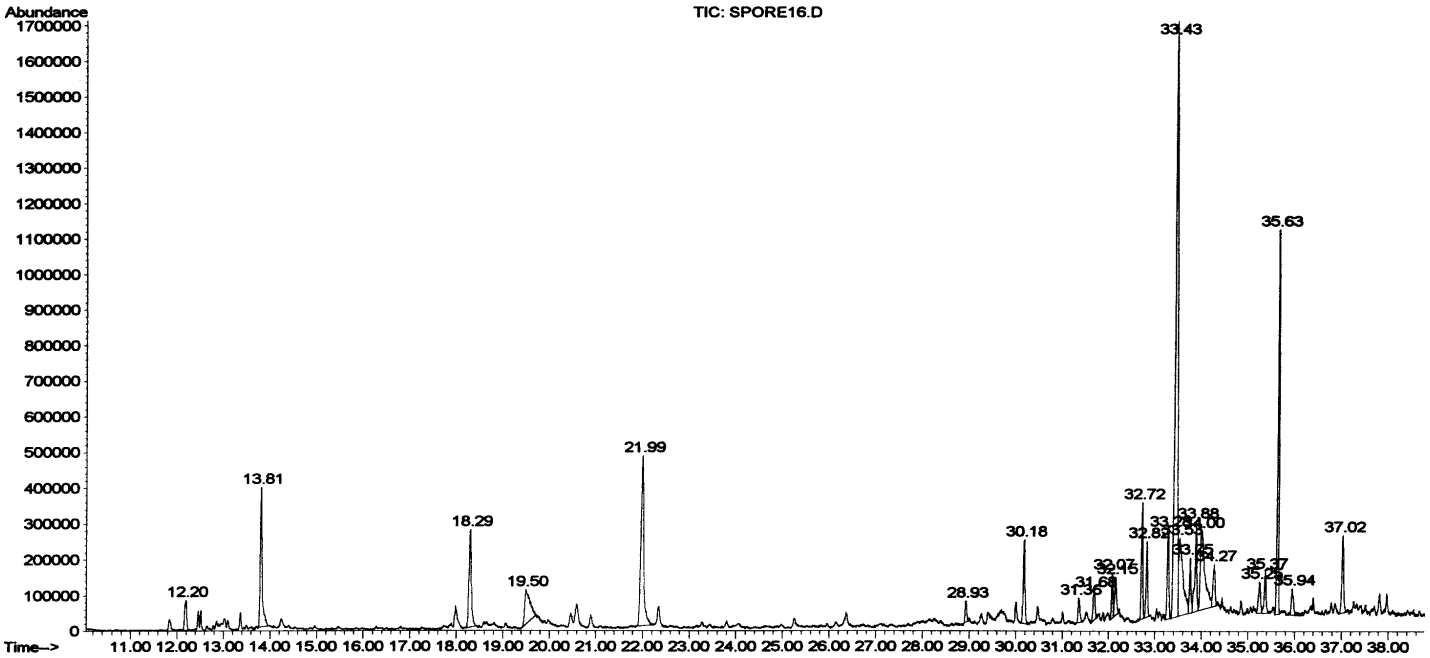


Figure V-9. GC/MS chromatogram of 3 x 10^8 spores. The peak located at 21.99 min is dimethyl dipicolinate. Other peaks consist of hydrocarbons at the far end of the run time and some column bleed at the beginning of the run. Other fatty acids, if not degraded by the method, would have greater retention times than the DPA peak.

that it is dimethyl dipicolinate (Figure V-10). Ion extraction is often used to locate specific peaks of interest in a full-scan mass chromatogram. When ions 105, 137, and 165 are extracted from the full-scan chromatogram, the resulting extracted chromatogram shows no significant peaks other than the peak at 21.99 min (Figure V-11).

Preliminary experiments with dilutions made from a 3 x 10^4 spore sample in hexane:diethyl ether showed that the DPA dimethyl ester could be seen with a 1:4 dilution in 100 µl, and a 1:10 dilution was observed in 20 µl, using a SIM program monitoring for ions 105, 137, and 165. These peaks were not quantifiable, but nevertheless were detectable.

Figure V-12 shows a graph of the results obtained with spore/sand mixtures. Each point represents a separate extraction of spore/sand samples. Ions 105, 108, 136, 137, 164 and 165 were monitored using a SIM program. The lower limit of detection (LOD) of the GC/MS in this experiment was 3 x 10^4 spores in sand, and the lower limit of quantification (LOQ) was 6 x 10^4 spores. At levels lower than this, the spectrum of the analyte did not exhibit the expected relative abundance ratios, due to the limitations of the mass spectrometer used.

```
File        : E:\SPORE16.D
Operator    : Cas
Acquired    :  6 Jan 99  12:13     using AcqMethod DPAAUTO
Instrument :   GC/MS Ins
Sample Name: 3 e8 spores in sand with sfe magic mix
Misc Info  :
Vial Number: 2
```

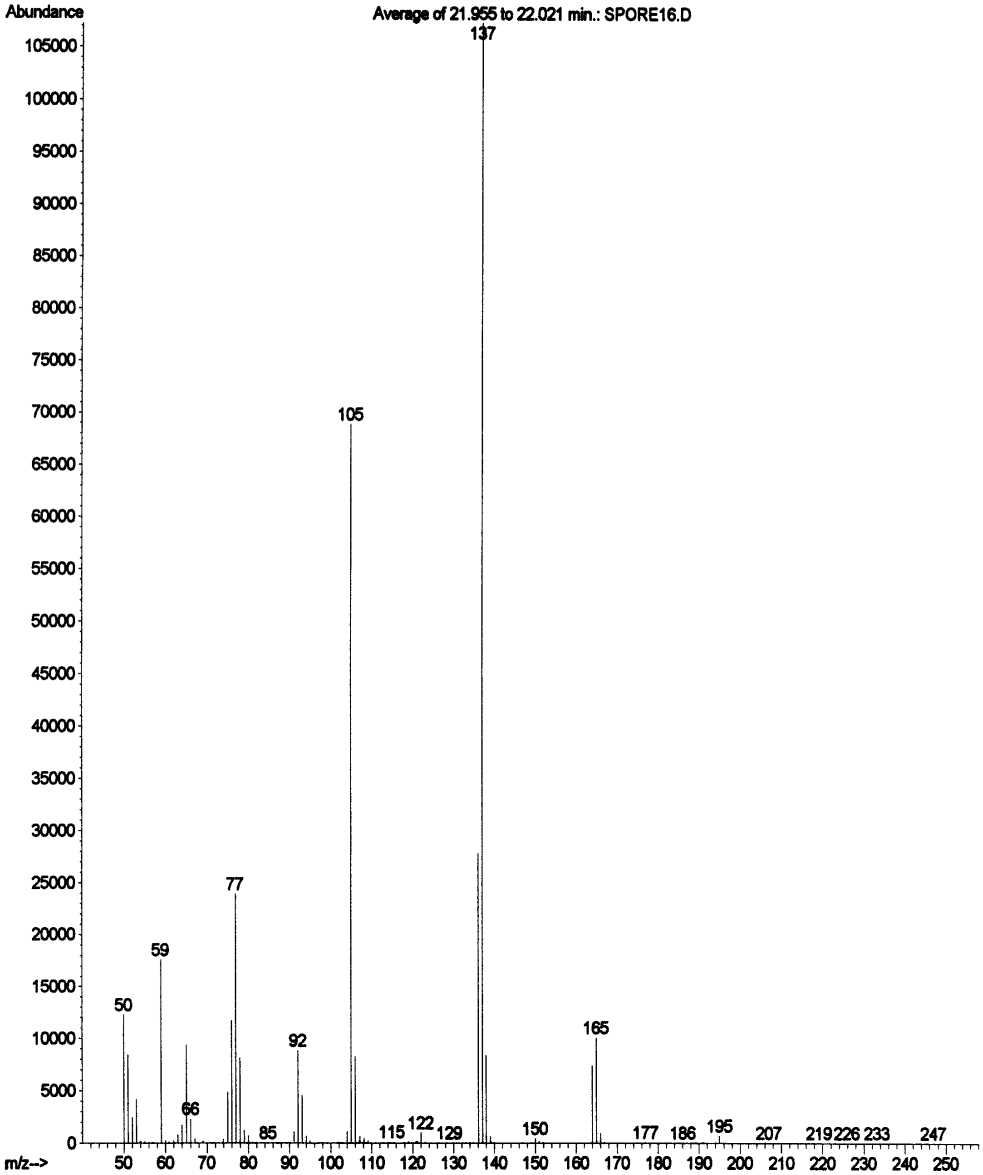


Figure V-10. GC/MS spectrum of DPA dimethyl ester peak at 21.99 min in Figure V-9. This spectrum shows the major ions of dimethyl dipicolinate: the most abundant ion is 137, followed by 105 and a smaller abundance of 165.

```
File        : E:\SPORE16.D
Operator    : Cas
Acquired    :  6 Jan 99  12:13     using AcqMethod DPAAUTO
Instrument  :   GC/MS Ins
Sample Name: 3 e8 spores in sand with sfe magic mix
Misc Info   :
Vial Number: 2
```

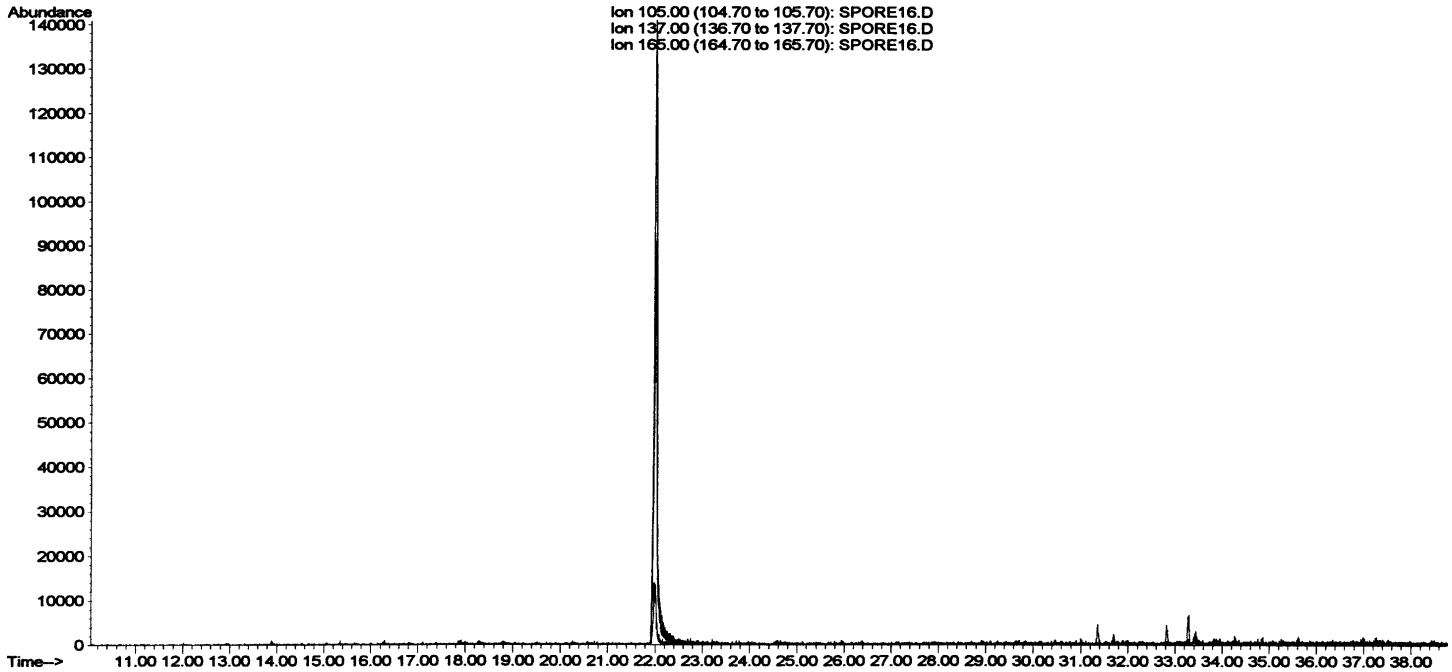


Figure V-11. Extracted ion GC / MS chromatogram of Figure V-9. This chromatogram is only of the extracted ions 105, 137, and 165. The peak of DPA dimethyl ester is clearly the only significant peak in the chromatogram with these three ions.

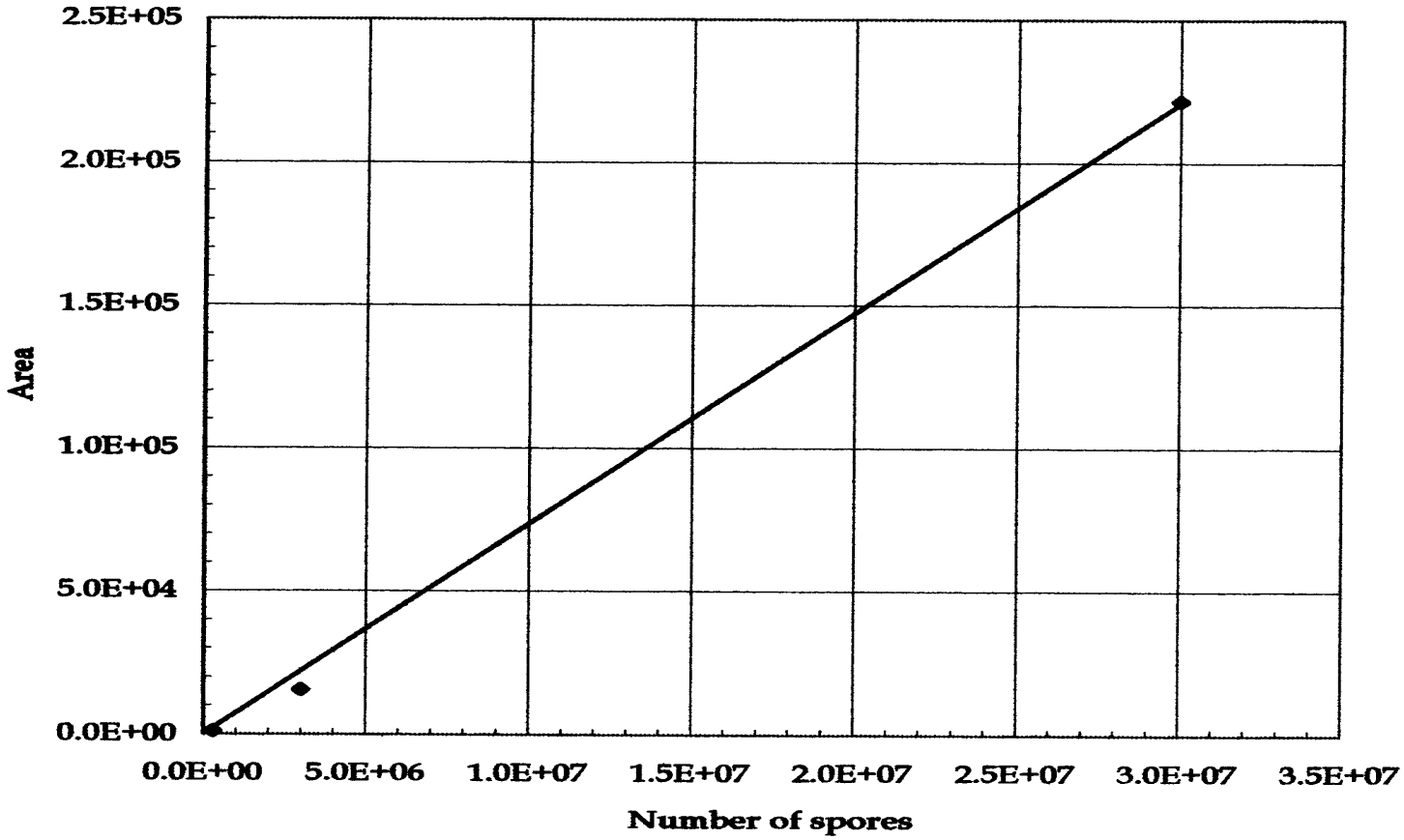


Figure V-12. Results of supercritical fluid extraction of *B. subtilis* spore dilutions and sand (n = 1; R^2 = 0.9986.

DISCUSSION

The quantification of terbium chloride with dipicolinic acid is an old and well-known method that has been used in many investigations (Barela *et al*., 1976; Richardson and Reihl, 1977; Richardson, 1982). It seemed possible that this might be reversible, and that dipicolinic acid could be quantified with terbium chloride. Consequently, spectrofluorometric studies were performed to determine the usefulness of such a procedure for possible application to bacterial endospores. Previous investigations using *Bacillus megaterium* spores had shown that wavelengths of 270 and 280 were most likely to provide the quantification sought (Powell, 1953).

Preliminary spectrofluorometric studies were completed in the search for a sensitive and accurate assay for bacterial spores. The calibration curves were found to be linear with concentration, confirming the results of studies done by others (Barela *et al*., 1976; Richardson and Reihl, 1977; Richardson, 1982). However, since the LOD obtained in this lab with authentic standards was the equivalent of approximately 3×10^5 spores, this method was discarded as not being as sensitive as would be necessary for some practical applications.

A few months later, as further preliminary experiments using GC / MS methods were in progress, Rosen *et al.* (1997) published results that seemed to confirm the LOD of the preliminary spectrofluorometric studies presented herein. Their results showed a detection limit of 4.4 x 10^5 CFU / ml. Rosen *et al.* performed their tests without lysis of the spore, although filtration of the terbium-treated spore suspension was necessary. This was considered to be advantageous by the authors. The following year, Pellegrino *et al.* (1998) published further studies using this method that extended their lower the limit of detection 3.6-fold, to 1.21 x 10^5 CFU / ml. However, these follow-up studies showed some problems with their method, as some freshly prepared spore suspensions, including *B. subtilis*, gave no signal at all.

The current lowest detection limit reported using terbium chloride, or any other published method, is 1 x 10^4 *B. subtilis* spores (Hindle and Hall, 1999). Their technique included lysis of fresh spores by heat shock before addition of terbium chloride. Their data also showed that ungerminated spores gave only a low fluorescence with terbium, which seemed to be one of the problems with the studies done by Rosen *et al.* (1997) and Pellegrino *et al*. (1998). In the opinion of Hindle and Hall, the inconsistent results obtained by Rosen *et al*. were due to the fact that they were looking solely at DPA, which had either leached from the spores

during storage, or DPA that was present in the spore coat. However, researchers have not substantiated Hindle and Hall's speculation that the spore coat also contains DPA. Comments made by Hindle and Hall refer to the necessity for dedicated instrumentation, and allude to the possibility of lower detection limits through concentration of the sample.

GC/MS analysis of dimethyl esters of DPA has been reported by several groups of researchers. The best results have been obtained by Beverly *et al.* (1996) using a Curie-point pyrolysis/triple quadrupole mass spectrometer. DPA in whole cells of sporulated *Bacillus anthracis* was reacted in situ during pyrolysis with tetramethylammonium hydroxide (TMAH) to form dimethyl dipicolinate. The concentration of spores used by these researchers was 2.2×10^7 spores per ml.

GC/MS is considerably more sensitive than spectrofluorometric methods. The in situ supercritical extraction of spores presented herein represents the most sensitive method of spore detection developed for GC/MS. It has been shown to be useful in matrices such as sand. It should be readily adaptable to other matrices as well. For example, air and aqueous environmental samples should be easily amenable to this method, if collected on appropriate matrices. The detection of DPA in complex matrices is currently possible, as these studies show. The characteristic ions of the analyte should be unmistakable, even in a mixed

culture. Unfortunately, the limits of sensitivity of this method using GC/MS have not been fully explored, as this lab currently does not have a tandem MS or triple quadrupole MS. The use of this type of instrument should lower the limit of detection considerably, as an ion trap would allow for the selection of specific ions for quantitative purposes. Another alternative is the use of HPLC/ESI/MS, but preliminary results (not presented herein) had shown that the likelihood of lowering the quantitation of dimethyl dipicolinate was not greatly enhanced by the use of such an analysis, since it employed only an MS. Further studies are needed to optimize this method. More sensitive equipment is needed, *i.e.*, a tandem MS or triple quadrupole MS. Further studies using equipment that is more sensitive are needed to optimize the SFE extraction method, also. Presently, the SFE equipment is no longer available in this lab. With the availability of these types of equipment, the optimization would surely reduce extraction time and increase limits of quantitation and limits of detection.

SUMMARY AND CONCLUSIONS OF PARTS II-V

SUMMARY AND CONCLUSIONS

Methods that use signature biomarkers have become increasingly important in the field of environmental microbiology. Signature lipid biomarker (SLB) analysis is a method of analysis for the quantitative definition of viable biomass, community composition, and nutritional status of microbiota isolated from a wide variety of environmental matrices, including air, soil and water. In many cases, unique molecules specific to microorganisms that could be useful in the detection, identification and determination of the infectious potential of life-threatening microbial pathogens have been selected and biomarkers tested. Microorganisms can be concentrated from air or water as aerosol or aqueous filter retentates, or recovered directly from environmental matrices such as sediments, soils, vectors and clinical specimens. These organisms can be detected and identified from analysis of their components.

The analysis of biomarkers involves concentration of the microorganisms or their components from environmental matrices, extraction of the biomarkers from the organisms, separation of the biomarkers from other components and detection of the biomarkers. Biomarkers examined in this thesis were glyco- and phospholipids, and

dipicolinic acid. Organisms tested were *Cryptosporidium parvum, C. baileyi, C. muris, Eimeria tenella, E. acervulina, E. maxima, Escherichia coli,* and *Bacillus subtilis*.

Three species of *Cryptosporidium* were examined using signature lipid biomarker analysis. This analysis was successfully applied to the species-specific identification of *C. parvum, C. baileyi* and *C. muris.* Three unusual fatty acids were found in minor amount in two of the profiles: cyclohexenyl 18:0 in *C. parvum*, 16:1ω10 and cyclohexyl 17:0 in *C. muris*. The lipid profiles also exhibited differences in the relative abundance of 16:0, 18:0, and 18:1ω9c which should give a clear identification for the differentiation between these three species. The glycolipid fraction from the SLB analysis was also examined for *C. parvum*, as the possibility of a unique biomarker (10-hydroxy stearic acid) was present in research done by others. This compound was shown to be an artifact of sample preparation.

SLB analysis was also used to examine the phospholipids of *Eimeria tenella, E. acervulina,* and *E. maxima.* This analysis clearly differentiated these three species from each other. Minor amounts of three unusual fatty acids, 7,8-epoxide 16:0, 7,8-epoxide 18:0 and 9,10-epoxide 18:0, were found only in the polar lipid profile of *Eimeria tenella.* Differences in the relative

abundance of 14:0, 18:0 and 18:1ω9c should also be helpful for distinguishing between these three species.

SLB analysis was also used to examine chlorine-injured *Escherichia coli*. The experiments presented herein ensure the validity of oxirane fatty acids as indicators of sterilization efficiency. The detection of epoxidated fatty acids firmly establishes that the cells have been in contact with hypochlorite, thus providing a chemical marker for hypochlorite exposure and sterility. At free-chlorine levels sufficient to render the organisms non-culturable, essentially quantitative conversion of monoenoic fatty acid precursors to their epoxidated product analogs was observed. Oxirane fatty acids were not detected in cultures of *E. coli* that had not been exposed to chlorine. This study provides a rapid, routine, and sensitive method for ensuring the safety of water consumers through careful monitoring of the water distribution system effluent.

For the examination of dipicolinic acid from endospores, a new extraction and methylation technique was devised and GC/MS analysis was employed for the detection of dimethyl dipicolinate. Preliminary studies also evaluated spectrofluorometric analysis using terbium chloride. The lower limit of detection using spectrofluorometric analysis was 10 ng DPA/ml, approximately equivalent to 2.7 x 10^5 *Bacillus subtilis* spores. *Bacillus subtilis* spores were detected using the new in situ SFE

extraction and methylation technique and GC/MS with a lower limit of quantification of 3 x 10^4 spores in a sand mixture, and the lower limit of detection was observed to be 3 x 10^3 spores.

Of these biomarkers, some studies could be expanded. The phospholipid analyses of *Cryptosporidium* and *Eimeria* would benefit from analysis of more replicates of each species. Sterol analysis might also be helpful, as these are commonly found in microeukaryotes. Since purified oocysts were used in these studies, further studies that include other matrices would also be beneficial. However, the application of SLB analysis should be useful for the detection of *Cryptosporidium* in drinking water and recreational water. SLB analysis should also be useful for the detection of *Eimeria* in farm runoff water and water used for livestock.

This study did not make a determination as to the viable status of bacteria exposed to chlorine. Further studies would be necessary to determine whether the bacteria produced oxiranes before death or if the formation of oxiranes is simply a chemical reaction of the lipids left from the disintegration of the bacteria. Recovery and enrichment studies would be necessary to make this determination. Examination of some of the more chlorine-resistant species of human pathogens would also be interesting.

The new method presented herein for the detection of DPA from bacterial endospores could also be improved and expanded. Further work is needed to optimize the SFE extraction and methylation method. The addition of a recovery standard to the samples, such as 2,4-pyridinedicarboxylic acid, would also improve analysis. Unfortunately, the limitations of the equipment used in this study reduced efforts to determine a lower limit of detection for the extraction and methylation technique. With better equipment, this extraction method may prove to be much more sensitive than any other analysis currently in use.

The work presented in this thesis can be applied currently to increase knowledge of stress and locations of microorganisms. The result of the use of these techniques can better identify the presence of potential pathogenic organisms in the environment. This will also result in a better understanding of microbial ecology.

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Carol Ann Smith was born in Charlottesville, Virginia and was raised in Oak Ridge, Tennessee and Knoxville, Tennessee. She graduated from The University of Tennessee, Knoxville, with a Bachelor of Science degree in microbiology and a minor in biochemistry in 1972. She the worked for the laboratory division of the Tennessee Department of Public Health in the enteric bacteriology section. While working full-time as a microbiologist, she attended The University of Tennessee, Nashville, at night in 1976 and 1977, taking accounting and other business courses to allow her to sit for the Certified Public Accountant examination. She began a career in accounting in 1978 with Daniel, Henderson & Associates in Hermitage, Tennessee, and became a CPA in 1980. She worked in public accounting with several public accounting firms (Main Hurdman in Texas, Cannon and Company in Tennessee and Arthur Andersen in Florida) until 1991, rising to the position of Tax Manager and specializing in tax accounting and employee benefit plans. In 1992, she taught tax courses for Internal Revenue Service agents in several IRS districts. In 1993, she returned to the University of Tennessee, Knoxville, where she entered as a non-degree student, taking courses in microbiology and mathematics. In 1996, she became a graduate student and also worked as

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