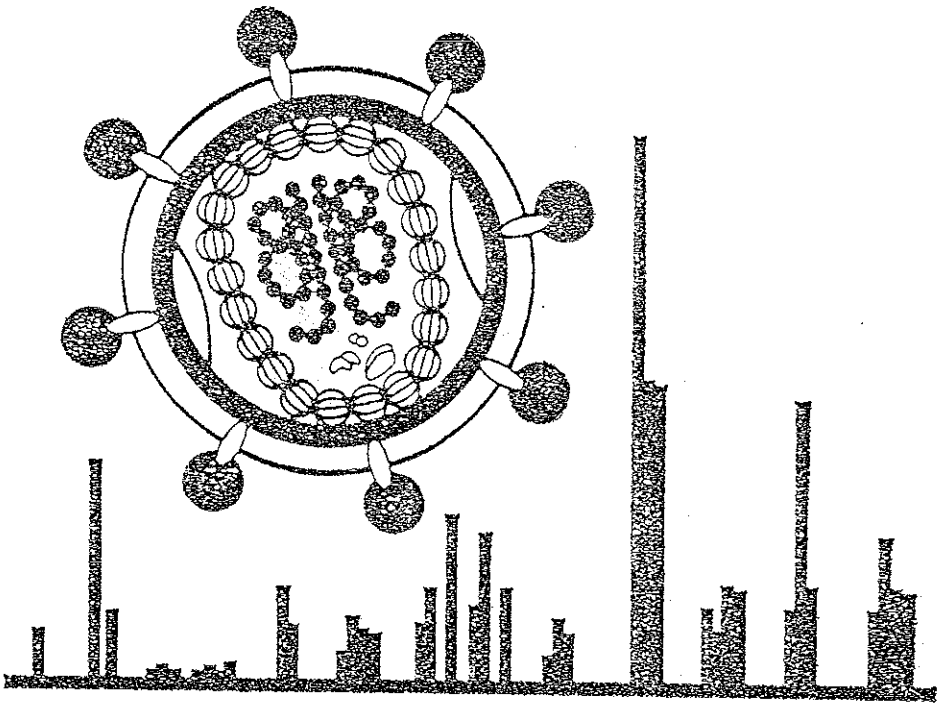
323

Mass Spectrometry for the Characterization of Microorganisms



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323

Chapter 2

Rapid Identification of Microbes from Clinical and Environmental Matrices

Characterization of Signature Lipids

D. C. White[1,2], D. B. Ringelberg[1], D. B. Hedrick[1], and D. E. Nivens[1]

[1]Center for Environmental Biotechnology, University of Tennessee, Knoxville, TN 37932–2567
[2]Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, and Department of Microbiology, University of Tennessee, Knoxville, TN 37996–0845

Lipid biomarkers can be recovered from isolates, clinical specimens, and environmental samples by a single-phase chloroform/methanol extraction, fractionation of the lipids on silicic acid, and derivatization prior to analysis by capillary gas-liquid chromatography/mass spectrometry. Polar lipid fatty acid profiles are a quantitative measure of bacterial and eukaryotic viable biomass and community structure, while sterols are used to measure the eukaryotes and isoprenoid ether lipids the archaea. Changes in a microbe's environment are reflected in the adaptive responses of its lipids, and can be used to delineate details of the microbe's metabolic status and environmental conditions. Rapid methods of sample preparation such as supercritical fluid extraction will extend the range of application of lipid biomarker analysis.

Since every cell has a lipid membrane, lipid composition varies with species, and cellular lipid modification is necessary to adapt to environmental conditions, microbial lipids are indicative of microbial biomass, community structure, and metabolic status. Rapid methods are being developed which will expand the range of applicability of lipid biomarker analysis by reducing time, expense, and waste production per sample analyzed, as well as (in some applications) increasing analytical sensitivity (*1*). Mass spectroscopy (MS) is the analytical detection system of choice for many of these applications due to its high sensitivity and selectivity.

In this review, specific classes of lipid biomarkers and examples of their application, approaches to their rapid analysis, and some areas of potential application will be described. Mass spectral analysis of intact phospholipids, glycolipids, and lipopolysaccharides, as well as pyrolysis MS are considered in other chapters of this symposium.

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statistical analysis by providing PLFA and ether lipid data on the same sample rather than on parallel samples. The combination of PLFA and archaeal ether lipid analysis has been applied to methanogenic bioreactors (*27, 28*) and deep-sea hydrothermal vent ecosystems (*29*), and fresh-water sediment communities (*30*). Other archaeal ether lipid analyses include methanogens in submarine hypersaline basins (*31*) and in fresh water sediments (*32*)

Using this method (*29*), a transect of 4 samples through the metal sulfides of a deep-sea hydrothermal vent spanning from the 2°C ambient seawater to the ~350°C hydrothermal vent fluids was analyzed. The highest bacterial biomass was detected in the sample adjacent to the 2°C seawater where archaea were undetectable. Archaeal biomass was higher than bacterial in the next sample of the transect, comparable to bacterial biomass in the seawater-exposed sample. Both measures of biomass decreased greatly in the next sample, becoming nearly undetectable in the hydrothermal vent fluid-adjacent sample.

Sterols. Microeukaryotes have polar lipids that are generally not as structurally diverse as bacteria, so their PLFA are not as useful for identification. Fungi and protozoa can be classified by their membrane sterols (*33*). The use of sterols as a measure of microeukaryote biomass has been tested using estuarine sediment in the laboratory (*34*). In one treatment eukaryotic growth was stimulated by addition of sucrose and nutrient broth and bacterial growth was inhibited by the addition of penicillin and streptomycin. In the second treatment bacterial growth was stimulated by phosphate and glutamine while and eukaryotic growth was inhibited with cycloheximide. The ratio of sterols to lipid phosphate was much greater in the first treatment than the second, as predicted by the physiological requirements of fungi versus bacteria, and as shown by microscopic examination.

Measures of Microbial Community Structure. A microbial community's structure is the proportions its component viable organism populations. The pattern of individual fatty acids in the PLFA profile is representative of the microbial community contributing to the profile. The ability to identify the position and conformation of the double bond in monoenoic PLFA has greatly increased the specificity of the signature biomarker analytical system (*35*). Eukaryotes predominantly synthesize ω9 cis monounsaturated PLFA (where ω9 indicates the unsaturation is 9 carbons from the methyl end of the fatty acid). Bacteria most often make the ω7 isomers. Bacteria also form monoenoic PLFA with other positions of monounsaturation such as ω5 and ω8, which are characteristic of specific groups of microbes (*36-38*).

Identification of Specific Organisms. Bacterial species are routinely identified in pure culture by their PLFA (*1*). In certain clinical samples (blood, urine, or cerebrospinal fluid) any bacterium is a suspect pathogen. Larsson et al. (*39*) have successfully utilized the detection of the PLFA tuberculostearic acid (10-methyloctadecanoate) in sputum for the diagnosis of miliary tuberculosis. The pentafluorobenzyl ester of tuberculosteric acid was formed after saponifying the sputum, and was detected by GC-negative ion chemical ionization-MS (methane). The correct 7 sputum samples out of 23 coded samples were determined to contain *Mycobacterium*. This is a significant advance given the unreliability of direct

Triglycerides. Neutral lipid triglycerides are accumulated as carbon and energy storage compounds by eukaryotes. There is evidence that in some species, triglycerides are accumulated preparatory to cell division (*50*). The triglyceride content of samples is quantified as the neutral lipid glycerol or ester-linked fatty acids by GC (*51*), or as intact triglycerides by supercritical fluid chromatography (*52*) or high temperature GC (*53*).

Rapid Analysis of Lipid Biomarkers

MIDI System. One approach to the rapid analysis of bacterial lipids is the commercially available MIDI system (*54*). Under standardized conditions, bacteria are cultured and harvested, and their whole cell fatty acids are quantified by saponification, methylation, and GC-MS with an autosampling system. Since the culturing step requires 24 to 48 hours, and the procedure requires a pure culture to begin with, the time required to obtain an identification is from two to four days. The system comes with a library of bacterial fatty acid profiles and the software to calculate similarities between profiles and determine identifications. This system has been used in clinical applications (*55*), identification of plant diseases (*56*), and microbial ecology (*57*).

Supercritical Fluid Extraction. Supercritical fluid extraction (SFE) can replace lipid extraction procedures using chloroform/methanol (*10*) in lipid biomarker analysis. Increasing the speed, reducing the expense, and eliminating most of the waste solvents would make lipid biomarker analysis more attractive in many areas, and could open up totally new applications.

SFE was used to replace soxhlet extraction of free fatty acids, sterols, sterol esters, and triglycerides from hamster feces in a nutritional study (*13*). Analysis of extracts was by supercritical fluid chromatography. Where soxhlet extraction required 3 days and 500 mL of solvent per sample, SFE required 1 hour and 2 mL of solvent. All components recovered by soxhlet extraction were found in SFE extracts, at from 9.2% to 17.2% higher levels, depending upon the SFE conditions used.

Derivatization/Supercritical Fluid Extraction. The membrane lipids found in the glycolipid and polar lipid fractions are not soluble in supercritical carbon dioxide or sulfur hexafluoride. Derivatization/supercritical fluid extraction (D/SFE) is the derivatization of a chemical moiety to a less polar form which can be extracted by SFE. Lyophilized *Escherichia coli* cell mass was treated with the reagent trimethylphenyl ammonium hydroxide to derivatize polar lipid fatty acids to their methyl esters prior to extraction by SFE (*58*). Figure 1 is a comparison of the PLFA profiles generated by D/SFE with the solvent extraction, silicic acid fractionation, and derivatization method (*59*). The reduction in the amount of time required for sample preparation by this method is considerable - by supercritical fluid chromatography 8 samples can be prepared and analyzed by GC in one day, while by solvent extraction the same samples would require about 3 days to prepare.

Applications of Rapid Lipid Biomarker Analysis

Rapid Diagnosis of Acute Infections. The rapid and accurate diagnosis of infectious disease is critical to appropriate patient management (*60*) as well as epidemiologic surveillance (*61*). Isolation and culturing of infectious agents was essential in the definition of the etiology of specific infectious diseases and still represent the definitive diagnostic criteria. The problem with this approach is that the procedures require time to isolate the organism and then to grow it on specific media. The media must be chosen judiciously based on the clinical situation since many pathogens require specific growth conditions and nutrients (*62*). There is also an increasing recognition that there are infections with agents that are "viable but not culturable" (*63*).

There has been great progress in rapid diagnosis of specific microorganisms that is based on DNA probes (*64, 65*), rRNA probes (*5*), and polyclonal or monoclonal antibodies (*66*). In each case, however, the nature of the pathogen(s) must be suspected to choose the proper probes. Some of the probes are too specific and miss different strains of the pathogen and in some cases the probes are too general.

Examples of potential marker lipids and their associated disease organisms include: tuberculostearic acid from *Mycobacterium tuberculosis* (*vide supra, 39*), ergosterol from the yeast *Candida* spp. (*33*), and the distinctive PLFA and quinones of *Legionella pnuemophila* (*67*).

Monitoring and Predicting Bioremediation Strategies. Signature lipid biomarker analysis can be utilized for monitoring and control of bioremediation systems. For example, aerobic trichloroethylene biodegradation is a co-metabolic process of either methanotrophs consuming methane, or alkanotrophs consuming propane. Subsurface sediment bioremediation has been found to have the highest rate when the bacteria are nutritionally stressed, as indicated by a high ratio of poly-ß-hydroxyalkanoates to PLFA (*68*). PLFA profiles have also been used to monitor the populations of methane (*69*) or propane (*70*) oxidizing bacteria in bioreactors and subsurface sediments.

Environmental Toxicity Assessment. Prediction of the effects of industrial or municipal effluents on natural systems is complicated by the mixture of components in any real waste stream and the multitude of organisms composing most ecosystems. Methods proposed or applied to monitoring the effects of putative toxins on natural environments include quantification of the toxic compounds (*71*), and physiologic functions such as reproductive success (*72*). Application of PLFA analysis to stream periphyton communities (*73*) at 3 sites with sequentially less organic and metals contamination showed that the microbial community structure had been strongly impacted by effluents entering the stream, in agreement with fish liver function biomarkers, and algal periphyton density and physiological stress. However, little evidence was found for acute or chronic toxicity using either the *Ceriodaphnia* or fathead minnow bioassays.

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