RAPID EXTRACTION AND FRACTIONATION OF PARTICULATE BW AGENTS FOR DETECTION OF SIGNATURE BIOMARKERS BY MASS SPECTROMETRY

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Cells and enveloped virus BW agents in aerosols can be detected by the presence of membrane phospholipids. Phospholipids in bacteria are sufficiently labile to be indicators of "viable" cells in the environment. GC/MS analysis of patterns of phospholipid ester-linked fatty acids (PLFA) have been utilized to detect specific bacteria from complex matrices such as soils, sediments and infections. Present technology with PLFA requires complex and tedious manual purifications and derivatizations that must be automated and accelerated to be useful in the development of a field BW detection system. Initial experiments show rapid extraction of bacteria by solvents and supercritical fluids with subsequent fractionation between lipid classes are possible first steps in an automated MS detection system.

INTRODUCTION

The need to rapidly identify bacterial warfare agents has lead to the development of the SEFOLD strategy - Sampling, Extraction, Fractionation, and On-Line Detection. This takes maximum advantage of the information content of biological agents by delivering different classes of biomolecules to the mass spectrometer sequentially. The chemical agents such as nerve gasses, blister agents, and trichothecenes are also sensitively detected. Experiments have shown the feasibility of this method for the detection of chemical and biological agents.

Our approach is based upon three considerations: the biological characteristics of the bacterial and viral agents, the superior performance of supercritical fluids as extractants, and the limited number of different molecules a mass spectrometer can sensitively detect at one time.

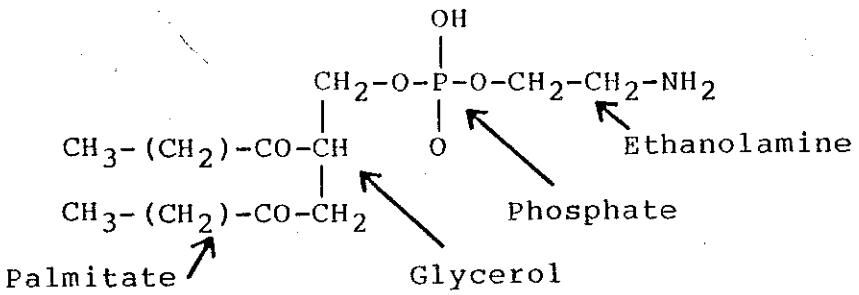
Of the millions of microbial species, very few are pathogenic to man, and of these only a handful are useful biological warfare

agents. The problem is to distinguish those from the natural background of bacteria. Just as their virulence is a biological characteristic so are their constituent molecules. Cellular lipids are distinctive at the species and subspecies level[2]. Lipids that are useful include cholesterol (which indicates an enveloped virus rather than bacterium), triglycerides, lipopolysaccarides, and quinones. The benefits of analyzing for lipids rather than proteins, sugars, or nucleic acids include ease of extraction and handling and their sensitive and selective detection. The detector commonly employed is the same as is used for the chemical agents, blister agents, and trichothecenes: the mass spectrometer.

The bacterial lipids most commonly used as biomarkers are the phospholipid fatty acids. Approximately 200 different fatty acids are found in the cell membranes of bacteria.

FIGURE 1.

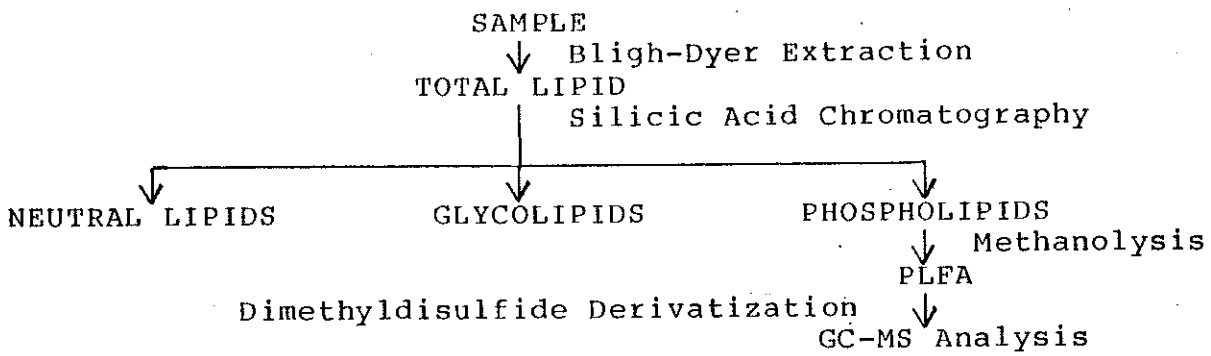
Dipalmityl phosphatidylethanolamine, a Representative Phospholipid.



In place of ethanolamine, other phospholipids may have for example glycerol, serine, choline, or glucose. The fatty acid groups range from 10- to 26-carbon atoms long and may include methyl branches at various positions, cis or trans unsaturations, hydroxyl groups, and/or cyclopropyl moeities.

FIGURE 2.

Analytical Scheme Used in this Laboratory For the Routine Analysis of Marker Lipids.

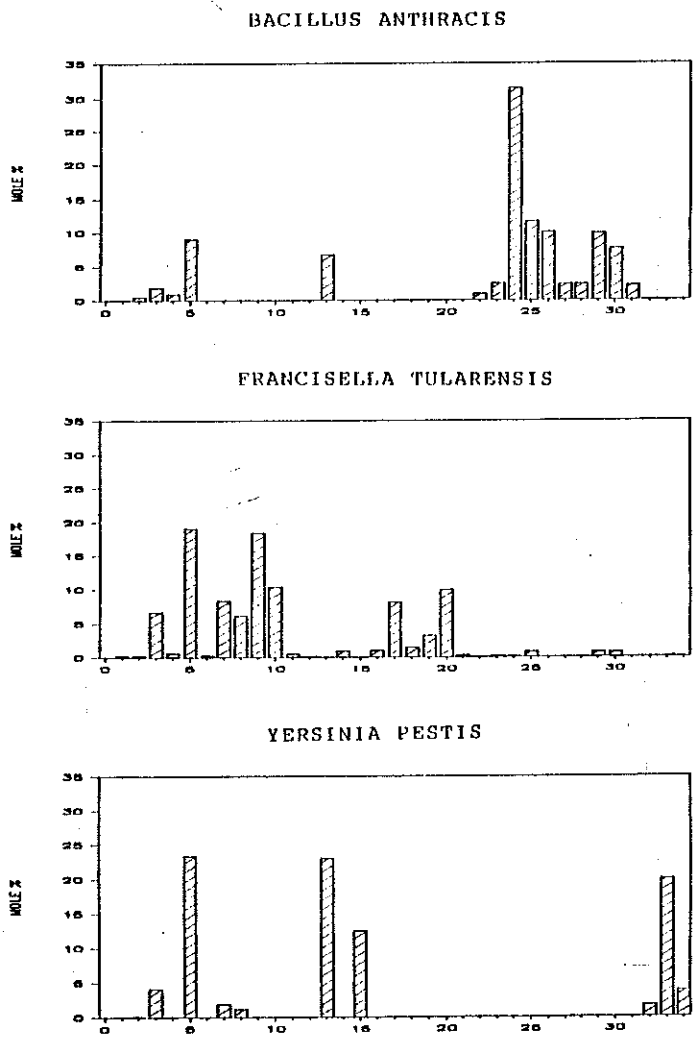


The scheme routinely used in this laboratory for the analysis of phospholipid fatty acids consists of the extraction of the total lipids with chloroform/methanol/water. The lipid is then fractionated into neutral lipids, glycolipids, and phospholipids by silicic acid column chromatography. The phospholipid fatty acids are cleaved from the glycerol backbone and derivatized by a mild alkaline methanolysis and purified by thin layer chromatography before capillary gas chromatography. The identities of individual fatty acids are verified by mass spectroscopy. Further derivatization with dimethyldisulfide can be used to determine the position and conformation of double bonds.

The many applications of phospholipid fatty acids include routine clinical analysis, diseases of plants, and detecting the effects of pollutants. An example of of using the phospholipid fatty acids

FIGURE 3.

Histograms of the phospholipid fatty acids of Francisella tularensis, Bacillus anthracis, and Yersinia pestis, expressed as mole percent of each fatty acid.



to identify bacterial species is shown in Figure 3. Numbers 1 through 11 are straight chain saturated fatty acids, 12 through 21 are unsaturated, 22 through 31 iso- and anteiso-branched, 32 is 3-hydroxy-hexadecanoate, and 33 and 34 are cyclopropyl 17 and 19. As these histograms show, Francisella tularensis, Bacillus anthracis, and Yersinia pestis are easily distinguished.

In order to ensure rapid and complete extraction of cellular lipids and chemical agents, a supercritical fluid is used in the initial stages. The viscosity and diffusion coeficient of a supercritical fluid are more similar to a gas than a liquid, which means that it can penetrate the sample matrix very rapidly. (See Table 1.)

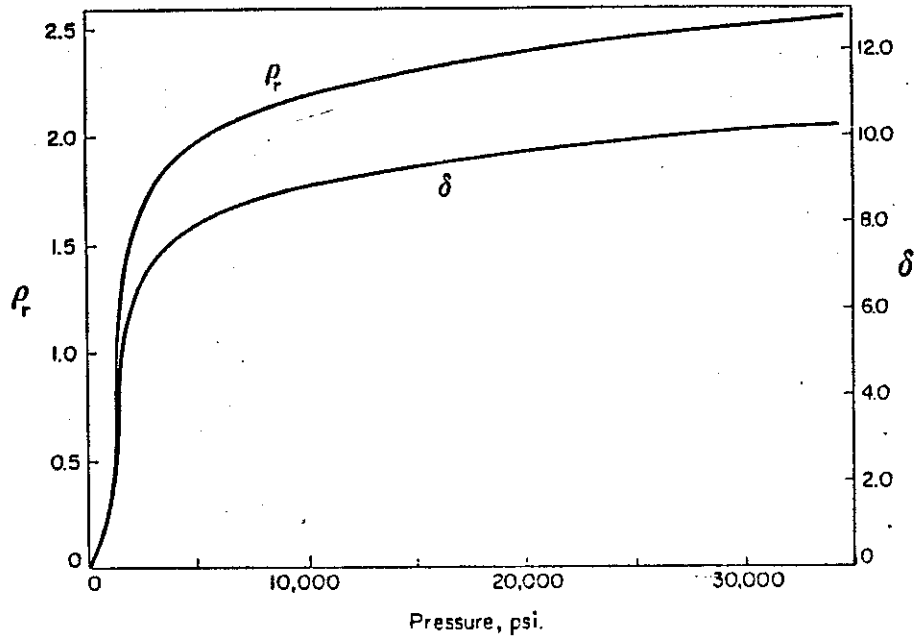
TABLE 1.

Physical Parameters of Supercritical Fluids Compared with Those of Liquids and Gasses. After reference 3.

	Gas	Supercritical Fluid	Liquid
Diffusivity (cm^2/sec)	10^{-1}	10^{-3}	10^{-6}
Viscocity (g/cm x sec)	10^{-4}	10^{-4}	10^{-2}
Density (g/cm^3)	10^{-3}	0.3	1

FIGURE 4.

Influence of Pressure on Reduced Density (rho) and the Solubility Parameter (delta) of CO_2 at 40°C. After reference 4.



Conversely its density is more similar to that of a liquid making it a good solvent. Another useful characteristic of supercritcal fluids is that the solvent power changes very rapidly as a function of pressure. (See Figure 4.)

Carbon dioxide is a superior solvent for this application for many reasons: it is non-toxic, non-flammable, and cheap. It does not require high temperatures and pressures to reach supercritical conditions. It is not so reactive a molecule as to dominate the spectrum or react with sample molecules if introduced into the source of a mass spectrometer. Not only are the contaminants water and oil, the chemical agents, the trichothecenes, and the neutral cellular lipids soluble in supercritical carbon dioxide, but by varying the pressure they may be extracted at different densities of fluid and delivered to the detector at different times.

The limitation of supercritical carbon dioxide as a solvent is that it can not extract very polar molecules such as the phospholipids. Two different strategies have been applied to this problem. Either a different solvent is used which will dissolve the phospholipids and carry them to the mass spectrometer, or they are chemically cleaved to produce the methyl esters of the fatty acids which are then analyzed. Both of these approaches have been demonstrated in our laboratory. Which one is eventually applied depends upon many variables such as the amount of data available from each form, whether a chromatographic step can be included between extraction and the mass spectrometer, and whether the mass spectrometer can tolerate the reagents.

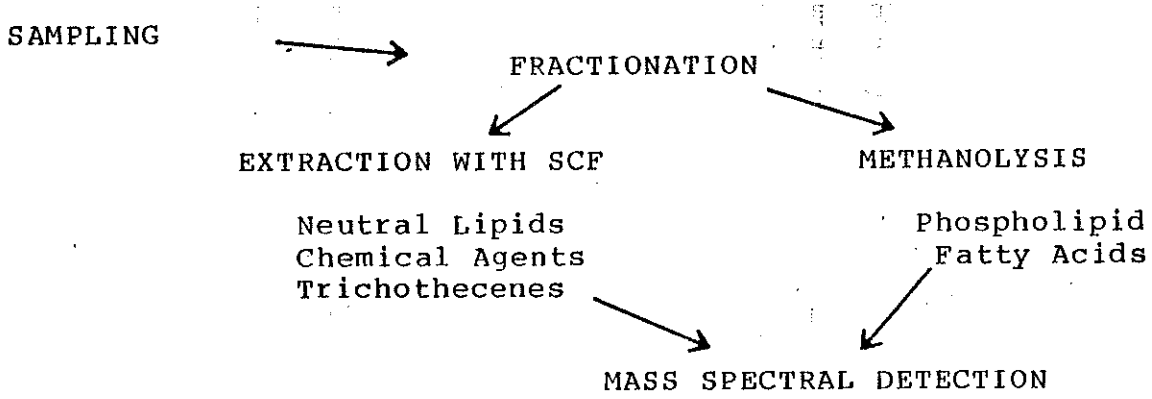
The sensitivity of a mass spectrometer is roughly inversely proportional to the number of masses at which it is collecting data. By extracting the sample at a series of increasing fluid densities, different classes of molecules are delivered to the mass spectral source at different times and the mass spectrometer need only monitor the masses of interest. The second way that this approach increases the sensitivity of the mass spectrometer is by separating the contaminants from the molecules of interest, increasing the signal to noise ratio.

PROPOSED APPARATUS

The aerosols are sampled by pulling the suspect atmosphere through a filtering material. (See Figure 5.) Volatile compounds can be trapped on a polymeric coating on the filter for concentration and analysis. An optional sample chamber may be included to extract solid samples. The sample is extracted with carbon dioxide at a series of increasing densities, fractionating the compounds present into predictable and reproducible chemical classes. The chemical agents, and the neutral lipids are removed from the sample. These compounds are swept into the source of a tandem quadrupole MS/MS. The separation of when different compounds reach the mass spectrometer means it only needs to monitor the relevant masses - its sensitivity is greatly increased. Once the sample is simplified by the removal of the neutral compounds, it is treated with alkaline methanol which cleaves the bacterial phospholipids. The resulting fatty acid methyl esters are much more soluble and their mass spectra more informative than the entire phospholipids.

FIGURE 5.

Flow Diagram of the SEFOLD Apparatus: Sampling, Extraction, Fractionation, On-Line Detection.



While this is a novel approach, it is basically a new combination and application of existing technologies. The use of phospholipid fatty acids to classify bacteria is well established. Supercritical fluid extraction and fractionation are finding wide application in chemical engineering.

CONCLUSIONS

Bacterial lipids, especially the phospholipid fatty acids, can be used to distinguish bacteria from background contamination and individual bacterial species. Both the biological and chemical weapons will be identified by the proposed SEFOLD apparatus. The combination of rapid extraction and on-line analysis will produce enough high quality information to respond to the CBW threat.

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