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Flash detection/identification of pathogens, bacterial spores and bioterrorism agent biomarkers from clinical and environmental matrices☆

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Abstract

We propose to develop an integrated rapid, semiportable, prototype point microbial detection/identification system for clinical specimens that is also capable of differentiating microbial bioterrorism attacks from threats or hoaxes by defining the pathogen. The system utilizes ''flash'' extraction/analytical system capable of detection/identification of microbes from environmental and clinical matrices. The system couples demonstrated technologies to provide quantitative analysis of lipid biomarkers of microbes including spores in a system with near-single cell (amol/μl) sensitivity. Tandem mass spectrometry increases specificity by providing the molecular structure of neutral lipids, phospholipids, and derivatized spore-specific bacterial biomarker, 2,6-dipicolinic acid (DPA) as well as the lipopolysaccharide-amide-linked hydroxy-fatty acids (LPS-ALHFA) of Gram-negative bacteria. The extraction should take about an hour for each sample but multiple samples can be processed simultaneously.

Keywords: Flash extraction; Pathogen; Lipid biomarkers

1. The system

This system is based on sequential extraction of neutral lipids, polar lipids, and specific spore and lipopolysaccharide (LPS) biomarkers. The sequential extraction/concentration renders subsequent biomarker structural analysis essentially independent of the environmental matrix. The system focuses on detection of key neutral lipids, polar lipids, bacterial spore, and LPS biomarkers. The sequential extraction also provides fractionation before the rapid delivery of biomarkers to tandem mass spectrometry for ultrasensitive structural determination. The tandem mass

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spectral detection system has been shown to be sensitive to between 10 and 100 bacteria and 3000 spores and may be improved to detection levels of 20–50 spores.

The proposed system includes integration of a specially designed sequential ''flash'' supercritical carbon dioxide ($SFECO_2$) extraction, followed by enhanced solvent extraction (ESE), followed by a third $SFECO_2$ extraction. This process will provide three extracts of each sample for subsequent high-performance liquid chromatography/electrospray ionization/tandem mass spectrometry (HPLC/ESI/MS/MS) analysis. (1) A neutral lipid fraction containing the respiratory quinones, sterols, diglycerides, and DNA. (The $SFECO_2$ extraction lyses the microbes facilitating the recovery of DNA that can be recovered with aqueous buffer extraction of the residue of the neutral lipid extraction for analysis with a different apparatus.) (2) Next in the sequence is an ESE of the neutral-lipid-extracted residue for recovery of polar lipids with a one-phase chloroform/methanol/buffer solvent. (3) The twice-extracted residue ($SFECO_2$ + ESE) is then treated with a derivatizing agent and the 2,6-dipicolinic acid (DPA) of bacterial spores and/or the amide-linked hydroxy-fatty acids of the lipopolysaccharide (LPS-ALHFA) are recovered with $SFECO_2$. A methanol wash is used between each sequential extraction of the residue. The process is potentially automatable.

2. The extraction

$SFECO_2$, particularly with polar alcohol enhancers, allows for rapid extraction and recovery of organic pollutants from environmental samples (Hawthorne, 1990). The great advantage of $SFECO_2$ extraction is that with release of pressure, the solvent (CO_2) disappears as a gas and the extractants can be recovered in a tiny volume of organic solvent like hexane. Nivens and Applegate (1997) working in the Center for Environmental Biotechnology have shown that when rendered supercritical at temperatures greater than 31.1 °C and pressures greater than 72.85 atm, CO_2 enabled essentially quantitative extraction of neutral lipids (steroids and respiratory quinones), pollutants/contaminants such as polynuclear aromatic hydrocarbons, pesticides, and petroleum hydrocarbons. $SFECO_2$ extraction also facilitates release of nucleic acids from a number of microorganisms including *Escherichia coli*, *Pseudomonas fluorescens*, *Sphingomonas paucimobilis*, *Mycobacterium* spp. and *Rhodococcus* spp. with extraction using aqueous buffer. Before $SFECO_2$ exposure, the bacteria were collected on 25-mm glass fiber filters. The recovery of PCR-quality DNA was obtained through a 1-min contact with supercritical CO_2 (80 °C and 400 atm) with a flow rate of 1.5 ml min through the restrictor. Lysis efficiency was determined by monitoring the decrease in acridine orange direct counts that ranged between 70% and 90% compared to the untreated samples. Increased recovery of neutral lipids like sterols from yeast and polynuclear aromatic hydrocarbons and pesticides from sediments was seen with increases in the $SFECO_2$ temperature and pressure up to 100 °C and 400 atm (the highest pressure tested). Yields of *Mycobacterium* and *Sphingomonas* DNA increased when polar amendments were included in the ISCO $SFECO_2$ systems.

2.1. ESE extraction of polar lipids

The second of the sequential extractions will utilize ESE of the residue after SFE cell lysis and neutral lipid extraction. The yield will be compared to classical Bligh and Dyer one-phase chloroform/methanol/buffer solvent extraction at room temperature and pressure as published previously (White et al., 1996; Ringelberg et al., 1988). The lipid patterns and concentrations are well known, having been determined using the classical methods developed in this laboratory (White et al., 1996; Ringelberg et al., 1988). Initial studies have shown that using the Dionex ASE-200 Accelerated Extraction system with two cycles, 80 °C and 1200 psi enabled recovery of 3-fold more phospholipid fatty acid (PLFA) from *Bacillus* sp. spores and 2-fold more PLFA from *Aspergillus niger* spores than did the standard one-phase extraction system (Macnaughton et al., 1997). Preliminary studies showed that increases in pressure to 8000 psi at 80 °C in a hand-operated ISCO SFX apparatus (Hawthorne et al., 1992) further increased the recovery of lipids from *Bacillus* spores.

The 'flash'' sequential extraction system is a modification of the Applied Separations (Allentown, PA) ''fast PSE system'' which sequentially extracts the six

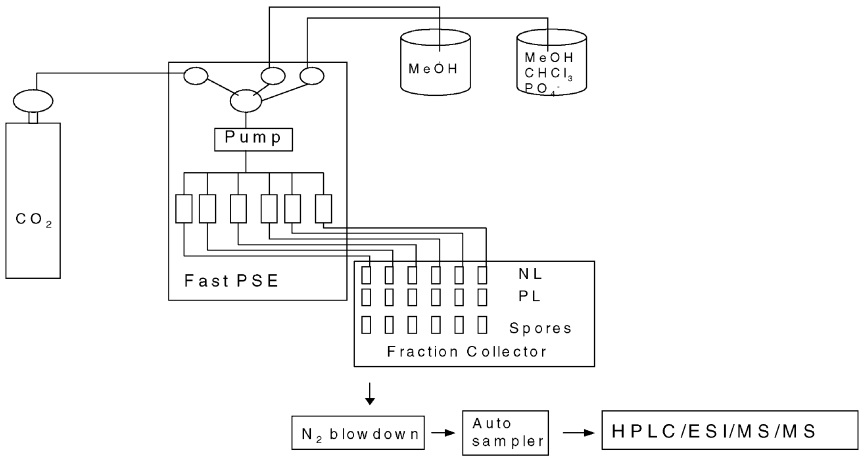

Fig. 1. Schematic of the extraction apparatus.

different samples in parallel. The extractor achieves pressures to 10,000 psi and temperatures to 150 °C. Each extraction is delivered to an ISCO System Si-10x fraction collector.

Analytes are then concentrated in the nitrogen ''blow-down'' and the samples transferred in sequence to the HPLC autosampler that is coupled to the ESI/MS/MS. Fig. 1 diagrams the system.

Analysis of the neutral and polar lipid fractions are particularly important in the absolute identification of bacterial spores from which DNA is difficult to recover. The proposed system could be integrated into systems being developed by other agencies with agent concentrators for aerosols and water, and could be adapted to soils, tissues, biofilms, food, skin tape lifts, or other systems.

3. Lipids as target biomarkers

Lipid biomarkers were selected as targets as they can be extracted and concentrated rapidly in an automated system. The sequential extraction/concentration makes further structural analysis essentially independent of the complexity of the environmental matrix. Lipids are generally regarded as the most comprehensive and quantitative means for the rapid assessment of the presence of viable pathogenic agents. In addition, lipid analysis provides insight into the nutritional/physiological status of the pathogens as microbial communities can give indications of infectivity in some instances. Examples of the relationship of infectivity to physiological status indicated by lipid structure are the shift from culturability to nonculturability (but with retained infectivity) of the cholera bacillus, *Vibrio cholerae* (Guckert et al., 1986), and the shifts in patterns of micocerosic acids and secondary alcohols derived from the surface waxes which correlated with drug resistance in *Mycobacterium tuberculosis* (Almeida et al., 1995). Polar lipid structure modifications can also correlate with culturability. The presence of oxyrane fatty acids in the phospholipids of Gram-negative bacteria on exposure to oxidative biocides indicates unculturability (Smith et al., 2000).

Some potential threat agents have sufficiently specific lipids that they can be identified from the PLFA analysis as currently performed on total polar lipids [particularly intercellular pathogens like *Coxsiella*, *Francisella* (Nichols et al., 1985), *Legionella* (Walker et al., 1993), *Mycobacterium* (various species; Almeida et al., 1995)], and some with class-specific lipids (*Staphylococcus* spp., *Sphingomonas* spp., *Pseudomonas* spp., *Clostridia* spp. (White et al., 1996). In this proposal, we will not only increase the speed and facility of lipid biomarker analysis (LBA) but will increase the specificity by examination of position and structure of the fatty acids in each major individual polar lipid. This will greatly increase the specificity of the polar phospholipid analysis.

Defining the head-groups and isoprenologue patterns of the respiratory quinones will further increase this specificity.

Bacterial spores are a special problem as they are especially effective infectious agents and can be difficult to rapidly detect in complex environmental matrices. Recently, we have developed a proprietary rapid extraction of 2,6-DPA from bacterial spores (Smith et al., 1998) that is proposed for incorporation into this sequential extraction system for the rapid detection of bacterial spores.

Use of lipid biomarkers in detection of pathogens from environmental matrices has not been exploited in the past as the technique required expertise in the field of analytical chemistry, a long and complex series of manipulations, and knowledge of the complexities and nuances in the interpretation of lipid patterns before the generation of useful results. These requirements are compounded for detection of infectious agents at very low levels. Conventional wet chemical techniques require a minimum of 12 h for extraction and are usually followed by lipid fractionation, derivatization, and chromatography before analysis by gas chromatography/mass spectrometry (GC/MS). The GC/MS requires about 10^4 cells (pmol/μl) to be reproducibly detected.

4. Lipid biomarker analysis

Fig. 2 illustrates the analytical sequence for LBA.

The sequential "flash" extraction begins with (1) the $SFECO_2$ extraction of the neutral lipids. The respiratory ubiquinones (UQ) are analyzed directly and the diglycerides and sterols derivatized before analysis. (2) ESE is used to recover the polar lipids. A portion of the intact lipids would be separated by capillary HPLC for ESI/MS/MS analysis. A second portion of the polar lipid would be transesterified and the methyl esters of the PLFA analyzed by GC/MS which is used as a control for reference to the classical techniques of LBA. (3) The extracted residue is derivatized in situ in $SFECO_2$ and the 2,6-DPA recovered for analysis of bacterial spores, and the amide-linked 2- and 3-hydroxyl fatty acids from the LPSs analyzed by HPLC/ESI/MS/MS.

5. Analysis of neutral lipids

5.1. Ubiquinones

Gram-negative bacteria form respiratory ubiquinones when the terminal electron acceptor is oxygen

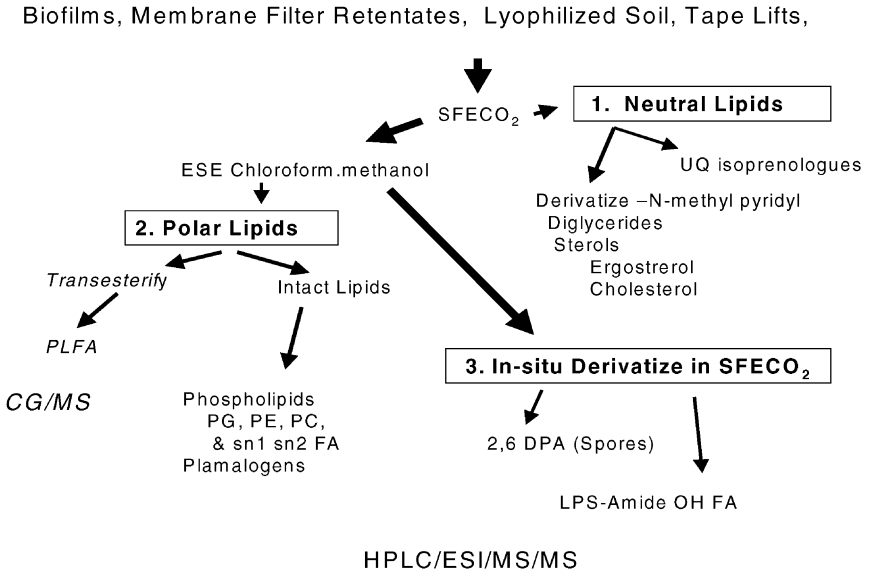

Fig. 2. Schematic for the "flash" sequential extraction LBA.

(Hedrick and White, 1986). Under anaerobic growth conditions, Gram-negative bacteria may form respiratory naphthoquinones or no quinones. Gram-positive bacteria form respiratory naphthoquinones when grown aerobically. Respiratory ubiquinones are found in concentrations about 200 times less than the PLFA or about 0.5 μmol/g dry weight (Hollander et al., 1977). Knowing the isoprenoid side chain length has important taxonomic implications. Eukaryote mitochondria contain UQ-10 (80-carbon side chain). Gram-negative bacteria contain isoprenologues from UQ-4 to UQ-14 (Collins and Jones, 1981). An HPLC method for the separation of ubiquinone isoprenologues with a 95.5:0.5 v/v methanol containing 1 mM ammonium acetate/H_2O in an isocratic mode does not suppress ESI (Fig. 3). Limit of detection (LOD) of 75 fmol/μl of UQ-7 (LOQ 225 fmol/μl) using a positive ion of m/z = 197. The LOD with this single quadrupole MS system represents about 150 bacteria the size of *E. coli* growing aerobically. With the new capillary HPLC system, the sensitivity can be increased 50 times by decreasing the HPLC flow rate to 1 μl/min from 50 μl/min, decreasing the sensitivity to a few cells.

5.2. Diglycerides and sterols

Two other important neutral lipids require derivatization of their primary alcohol moieties to be charged and thus are good candidates for ESI. The ESI requires coulombic repulsion to generate the microdroplets containing a single molecular species and thus requires a charge to be present or is induced on the analyte molecule. Primary alcohols have been particularly difficult to successfully electrospray. Two alcohols, sterols and diglycerides, are especially valuable indicators of the microbial community composition and physiological status. Sterols are valuable in assessing microeukaryotes. In the work done in this laboratory, analysis of sterols allowed the diets of filter-feeding marine invertebrates to be assessed (Canuel et al., 1995). With cell death, the bacterial phospholipids rapidly form diglycerides by the action of endogenous and exogenous phospholipases (White et al., 1979). The ratio of diglyceride/PLFA is a valuable indicator of conditions promoting cell lysis in the subsurface (Ringelberg et al., 1997). Recently, a derivatizing agent was developed for primary alcohols based on the formation of ferrocenoyl carbamates from ferrocenoyl azide that have subfemtomolar sensitivities in ESI/MS (Van Berkel et al., 1998). Fig. 4 illustrates the mass spectrum of the ferrocenyl derivative of cholesterol after HPLC/ESI/MS.

The primary alcohol of the 1,2 diglycerides can be derivatized with another charge-promoting moiety. This derivatization of uncharged alcohols is based on the formation of ESI-active *N*-methylpyridyl alco-

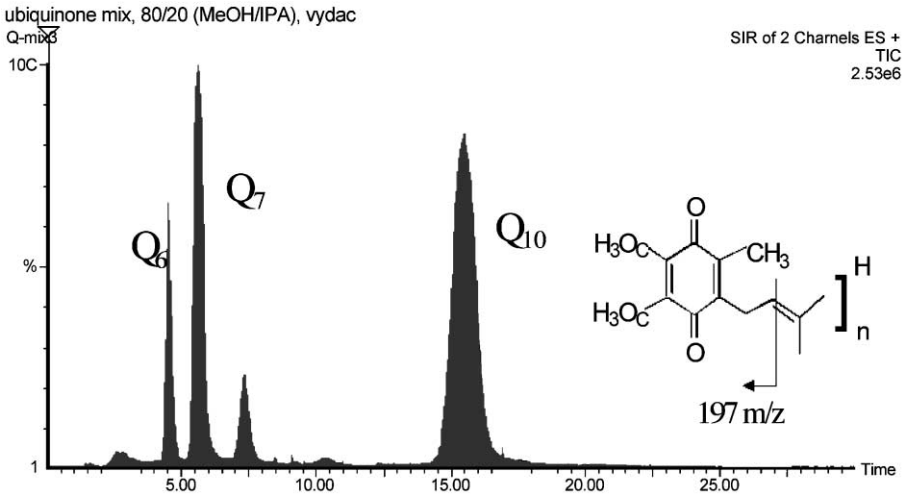

Fig. 3. Separation of ubiquinone isoprenologues.

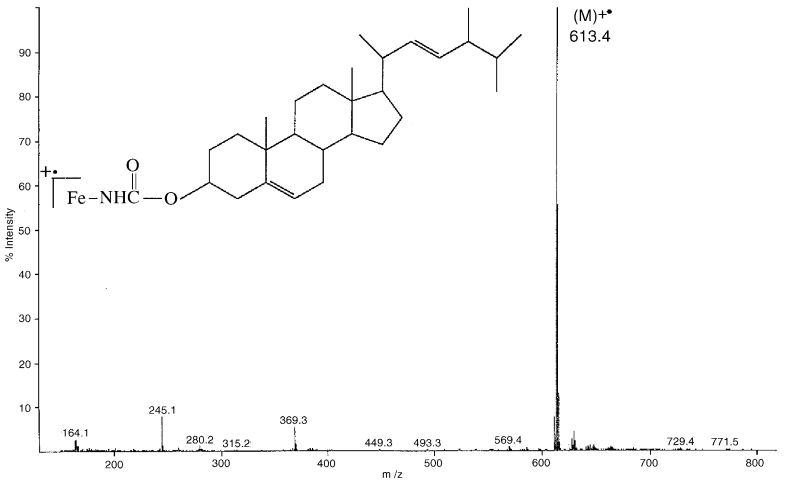

Fig. 4. The mass spectrum of the ferrocenyl derivative of cholesterol after HPLC/ESI/MS.

hol salt. The *N*-methylpyridyl salt is formed from the diglyceride by direct reaction with 2-fluoro-1-methylpyridinium *p*-toluenesulfonate (Quirke et al., 1994). The *N*-methylpyridyl ether salt will give an $(M+92)^+$ (where *M* is the mass of the diglyceride or sterol) (Quirke et al., 1994). Fig. 5 illustrates the mass spectrum of the derivatized synthetic diglyceride dipalmityldiglyceride.

Note that the LOD of these two derivatized alcohols is in the attomoles per microliter range.

6. Analysis of polar phospholipids

6.1. HPLC

Our laboratory developed a reverse-phase HPLC with a resistant C-18 column material for separation of intact polar lipids in a methanol solvent with 0.002% piperidine as base, with a postcolumn addition of 0.2% piperidine in methanol that allows the detection of phospholipids as negative ions with no

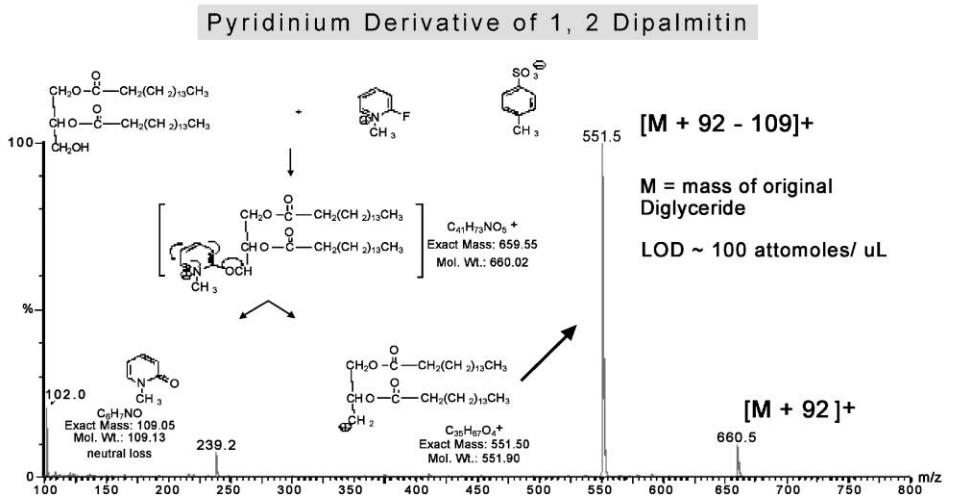

Fig. 5. ESI/MS detection of dipalmityldiglyceride as *N*-methylpyridyl ether salt.

compromise to the ESI (Lytle et al., in press). This has proved a significant advance in providing rapid (>8 min) separation of intact polar lipids with ESI-compatible solvents isocratically with no re-equilibration and made possible the rapid analysis of phospholipids.

6.2. ESI/MS/MS

Tandem mass spectrometry greatly increases the sensitivity and specificity of this environmental analysis system without any increase in the analysis time (Smith et al., 1995; Le Quere, 1993; Cole and Enke, 1991). Neutral loss scans are particularly useful for the rapid screening of targeted lipid ions as demonstrated by Cole and Enke (1991) who showed that phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) could be readily detected using a neutral loss of m/z = 141 and 154, respectively. In the negative ion mode, it proved possible to detect the position of each ester-linked fatty acid, that is, the fatty acid component at the 1 position had 20% of the abundance of the fatty acid at the 2 position of the glycerol. Tandem mass spectrometry, ESI/MS/MS, provides great advantages in the structural analysis of phospholipids. Product ion spectra and multiple reaction monitoring (MRM) were used to investigate PG containing a 16:0 fatty acid at the *sn*-1 position and an 18:0 fatty acid at the *sn*-2 position. Fig. 6A shows the ESI spectrum for 1 ppm PG when scanning from m/z 110 to 900; Fig. 6B shows the product ion spectra for m/z 747 as the progenitor ion selected in the first quadrupole with collisionally induced dissociation (CID) in the second quadrupole in the presence of 3.7×10^{-3} mbar Ar yielding the product ions that were analyzed by scanning between m/z 110 and 900 in the third quadrupole. MS/MS decreases chemical noise in the product ion spectra thereby increasing the signal-to-noise (s/n) ratio and the resulting sensitivity. Comparing the *s*/*n* ratio of the up-front CID of the *sn*-1 and *sn*-2 fatty acids in the upper panel of Fig. 6A to the product ion spectra from CAD in the second quadrupole, the product ion spectrum for PG is more sensitive by a factor of 50 (Fig. 6B, lower panel). By scanning over a narrower range, the sensitivity can be increased. Acquiring product ion spectra by scanning the dissociated fragments of the progenitor ion, the LOD and LOQ were experimentally determined to be 446 amol/μl and 1.3 fmol/μl, respectively. If the third quadrupole is scanned for m/z 281 product of m/z 745, a roughly 5-fold additional gain in sensitivity is achieved. This is MRM, m/z 747 → m/z 281 and represents the most sensitive application of this HPLC/ESI/MS/MS.

We have demonstrated LOD of 90 amol/μl using a single molecular species of phosphatidyl glycerol (*sn*-1 palmitic acid m/z = 255, *sn*-2 oleic acid 18:1 m/z = 284, parent ion m/z = 747) as the parent ion selected for CID by monitoring m/z = 284 negative ions. That is essentially equivalent to the total phospholipid in a single *E. coli* cell! Again with a capillary HPLC system, the flow rate can be decreased from 50 to 1 μl/min.

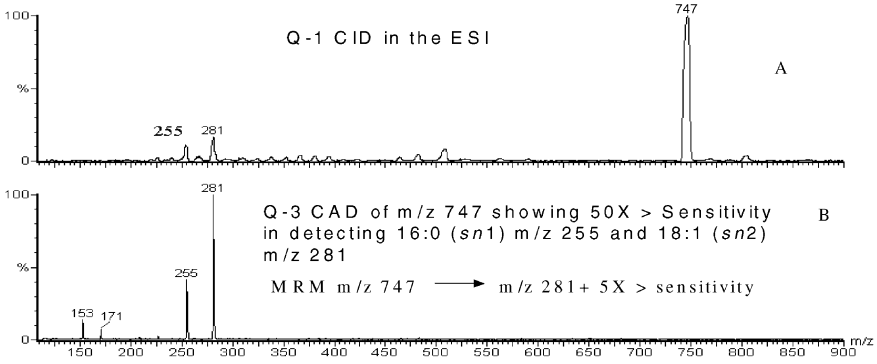

Fig. 6. (A) ESI spectra for 1 ppm PG 16:0/18:1 when scanning from m/z 110 to 900. (B) ESI product ion spectra for m/z 747 of 1 ppm PG 16:0/18:1 when scanning from m/z 110 to 900.

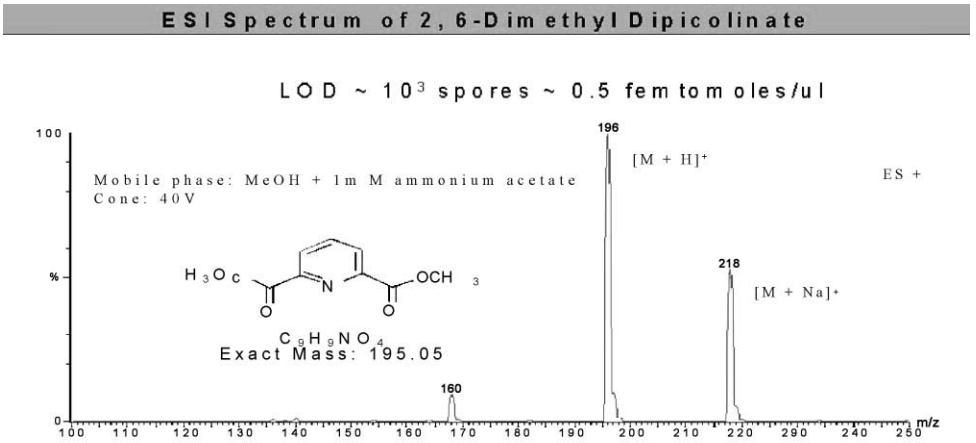

Fig. 7. ESI/MS spectra of dimethyl dipicolinate.

6.3. Recovery of DPA and LPS-ALHFA after in situ derivatization

The chelator 2,6-DPA is specific to all known bacterial spores and is primarily responsible for their remarkable resistance to heat and dryness (Ellar, 1978). A combined derivatization/extraction procedure that quantitatively forms the dimethyl ester of spore 2,6-DPA in situ and allows extraction with $SFECO_2$ has been developed (Smith et al., 1998). The resulting dimethyl DPA separates well on a reverse-phase HPLC column in a solvent of methanol with 1 mM ammonium acetate. ESI/MS results in the formation of the protonated molecular ion corresponding to DPA in the positive ion spectrum. In-source CID produces a fragment ion at m/z = 168 of the molecular ions at m/z 196 $(M+H)^+$ and 218 $(M+Na)^+$. The LOD is 100 fg/μl or 2.7×10^3 *B. subtilis* spores, $s/n = 2.6$. The assay is linear over three orders of magnitude $R^2 = 0.9997$, $y = 0.5525$, and takes less than 1 h. Fig. 7 shows the ESI/MS of dimethyl DPA.

Capillary HPLC will deliver the analytes at 1 μl/min as opposed to the 50 μl/min system that has been utilized. This should increase the sensitivity to 2 fg/μl or ~50 spores. This system allows detection of all spores (whether inducible to grow or not) in about an hour as compared to the current clinical standard culture that requires 4 days and measures only the viable (cultivable) spores.

The same in situ hydrolysis will be used to release the LPS-ALHFA of Gram-negative bacteria (Parker et al., 1982). Water biofilm organisms, such as *Pseudomonas*, have 3-OH 10:0 and 3-OH 12:0 as ALHFA in contrast to enteric pathogens, such as *Salmonella* and *E. coli*, which contain 3-OH 14:0 (White et al., 1996). Water pathogens, such as *Sphingomonas* species, have amide-linked 2-OH fatty acids. The ALHFA analysis on the in situ-derivatized residue extracted with $SFECO_2$ which can yield confounding hydroxy-fatty acids from tannins in peaty soils is used so the detection is generally restricted to clinical or biofilm samples.

7. Conclusions

The "flash" sequential extraction of a variety of environmental and clinical samples with subsequent analysis by HPLC/ESI/MS/MS provides a rapid, potentially automatable, semiportable, analytical system for detection/identification of pathogens and spores at levels of a few bacterial cells. Specific lipid components can also provide insight into the viability and potential infectivity of the pathogens detected in the samples.

Acknowledgements

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