Anal. Chem. **2000,** *72,* 2428−2432

Electron Monochromator Mass Spectrometry for the Analysis of Whole Bacteria and Bacterial Spores

Michael B. Beverly and Kent J. Voorhees*

Chemistry and Geochemistry Department, Colorado School of Mines, Golden, Colorado 80401

Ted L. Hadfield

Armed Forces Institute of Ptahology, Building 54, CPS-m, Washington D.C. 20306

Robert B. Cody

JEOL USA, Inc., 11 Dearborn Road, Peabody, Massachusetts 01960

Spores from a variety of *Bacillus* species were analyzed with direct probe mass spectrometry using an electron monochromator to select electrons of distinct energies for ionization. Electron energies were chosen to match the electron capture energies of taxonomically important compounds such as dipicolinic acid and fatty acids. Previous negative ion interferences were not observed when the monochromator was used, and the signal-to-noise ratio of targeted compounds was significantly enhanced using this approach. To demonstrate the selectivity of the technique, the monochromator was swept over a range of electron energies while monitoring the masses of compounds with known electron capture energies. Scanning the monochromator while the mass spectrometer was operated in single-ion mode enabled dipicolinic acid to be detected in 10^5 spores. The results presented here demonstrate the utility of the electron monochromator for selectively ionizing compounds directly in bacteria and bacterial spores.

Bacteria from the ubiquitous genera of *Bacillus* and *Clostridia* form protective structures called spores when there is a lack of nutrients.[1] Sporulation enables the bacteria to survive until living conditions become more favorable. Bacterial spores are resistant to solvents and temperatures that would kill nonsporulated bacteria and they are able to remain dormant for extremely long periods of time.[1,2] The robustness of bacterial spores can have significant health consequences. Food contaminated with *Bacillus cereus* spores is a common cause of food poisoning, and *Bacillus anthracis* spores are becoming an increasing concern due to bioterrorism.[3,4] Due to the importance of these bacteria, there is a need for analytical techniques that can identify bacteria and bacterial spores.

Most analytical approaches aimed at the detection and identification of bacteria and bacterial spores are based on the detection of taxonomically important compounds. Bacterial fatty acid profiles have been routinely used to identify specific bacteria and dipicolinic acid (DPA) has been used as a marker for bacterial spores.[5−8] While a commercialized fatty acid profiling system attests to the success of fatty acids in identifying bacteria, DPA has been particularly important as it is specific to sporulating bacteria.[6,8] It has been determined that 7−15% of the bacterial spore's dry weight is made up of DPA, primarily concentrated in the interior of the spore, and it is postulated that DPA serves a role in the heat resistance of bacterial spores.[9,10] Due to its uniqueness, DPA is often used to signal the presence of sporulating bacteria and a variety of analytical techniques have been employed for its detection.[11−16] Most methods of analysis involve extraction of the DPA from the bacterial spore. However, to decrease analysis time, there has been a recent move toward analyzing the bacteria directly without extracting the DPA.[15−17]

* Corresponding author: (Phone) 303-273-3610; (fax) 303-273-3629; (e-mail) kvoorhee@mines.edu.

(1) Alcamo, I. E. *Fundamentals of Microbiology*, 4th ed.; Benjamin/Cummings: Redwood City, CA, 1994.
(2) Cano, R. J.; Borucki, M. K. *Science* **1995**, *268*, 1060−1064.
(3) Ember, L. R. *Chem. Eng. News* **1999**, *77* (27), 8−17.
(4) Amodio-Cocchieri, R.; Cirillo, T.; Villani, F.; Moschetti, G. *Int. J. Food Sci. Nutr.* **1998**, *49*, 303−308.
(5) Daniels, L.; Hanson, R. S.; Phillips, J. A. In *Methods for General and Molecular Bacteriology*; Murray, R. G. E., Wood, W. A., Krieg, N. R., Eds.; American Society for Microbiology: Washington D. C., 1994.
(6) MIDI, Inc., Sherlock Microbial Identification System, 125 Sandy Drive, Newark, DE 19713.
(7) Odham, G.; Tunlid, A.;Westerdahl, G.; Larsson, L.; Guckert, J. B.; White, D. C. *J. Microbiol. Methods* **1985**, *3*, 331−344.
(8) Powell, J. F. *Biochem. J.* **1953**, *54*, 210−211.
(9) Murrell, W. G. In *The Bacterial Spore*; Gould, G. W., Hurst, A., Eds.; Academic Press: London, 1969; Vol. I, pp 215−273.
(10) Kozuka, S.; Yasuda, Y.; Tochikubo, K. *J. Bacteriol.* **1985**, *162*, 1250−1254.
(11) Janssen, F. W.; Lund, A. J.; Anderson, L. E. *Science* **1958**, *127*, 26−27.
(12) Warth, A. D. *Appl. Environ. Microbiol.* **1979**, *38*, 1029−1033.
(13) Tabor, M. W.; MacGee, J.; Holland, J. W. *Appl. Environ. Microbiol.* **1976**, *31*, 25−28.
(14) Scott, I. R.; Ellar, D. J. *J. Bacteriol.* **1978**, *135*, 133.
(15) Snyder, A. P.; Meuzelaar, H.; Dworzanski, J.; Kim, M.-G. *Proceedings of the 46th ASMS Conference*, Orlando, FA, 1998; p 342.
(16) Beverly, M. B.; Basile, F.; Hadfield, T. L.; Voorhees, K. J. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 455−458.
(17) Goodacre, R.; Shann, B.; Gilbert, R. J.; Timmins, E. M.; McGovern, A. C.; Alsberg, B. K.; Kell, D. B.; Logan, N. A. *Anal. Chem.* **2000**, *72*, 119−127.

10.1021/ac991318g CCC: $19.00
Published on Web 04/25/2000

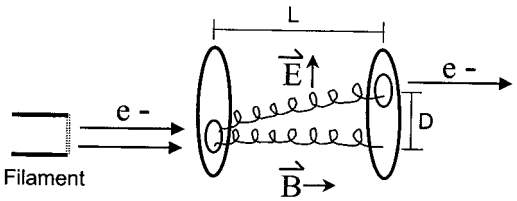

Figure 1. Electron energy selection principle.[21] Electrons (e^-) have different trochoidal paths in the plane parallel to the magnetic field (*B*). The drift velocity of the electron allows control of the electron's energy via the electron's time-of-flight in the electric field (*E*) and magnetic crossed field ($E \times B$) region of length (*L*). The electron drifts in a direction perpendicular to both fields to produce a drift distance of *D*. Electrons of a given energy are selected by having offset entrance and exit openings.

Mass spectral analysis of DPA directly from spores offers a quick and simple method for the detection of spores.

Direct-probe mass spectral analysis with chemical ionization has shown that the $M^{\bullet -}$ ion from DPA is observed from whole spores.[18] The negative ion mass spectra of whole bacteria and bacterial spores are, however, complicated by the presence of extraneous ions and can change with differences in the degree of sporulation in the culture. Spurious ions and ionization reactions in electron capture negative ion mass spectrometry (ECNIMS) are a common problem caused by slight changes in the source pressure and temperature.[19] This problem appears to be exacerbated by the introduction of whole bacteria into the mass spectrometer. To eliminate the pressure effects caused by a moderating gas and remove extraneous ionization reactions, an electron monochromator can be used to supply low-energy electrons for electron capture.

The monochromator operates by focusing electrons from a filament into a region of perpendicular electric and magnetic fields (termed the deflection region) with the electron's path being parallel to the magnetic field. Electrons in crossed magnetic and electric fields follow a trochoidal motion and drift in a direction perpendicular to both fields (Figure 1).[20] A directional drift permits electrons of specific energies to be guided toward exit slits. The drift velocity of the electron is related to the field strength. Control of the field strength and thus the drift velocity of the electron permits electron energy selection. The kinetic energy of the selected electron is determined by the potential difference between the filament and the ion volume.

Control of electron energy enables manipulation of the electron capture reaction and allows compounds to be selectively ionized. Selective ionization by matching the emitted electron energy to the electron capture energy of the analyte increases the specificity of the analysis and adds another dimension to the experiment.[21] The ability of the electron monochromator to significantly enhance ECNIMS has been demonstrated for a variety of chemicals, particularly explosives.[21,22] Reported here is the use of an electron monochromator in the mass spectral analysis of whole bacteria. In an attempt to produce better negative ion mass spectra from whole bacteria, an electron monochromator has been used to supply electrons with energies specific for electron capture by taxonomically important bacterial compounds such as DPA and fatty acids.

Table 1. *Bacillus* Spores Analyzed by Direct-Probe MS

B. anthracis Sterne	*B. cereus* ATCC 14579
B. thuringensis ATCC 10792	*B. globigii* var. Niger
B. lichenformis ATCC 14580	*B. subtilis* ATCC 6051

EXPERIMENTAL SECTION

All monochromator spectra were collected on a JEOL 505AX magnetic sector instrument. Standard negative ion mass spectra were collected on a JEOL MStation using isobutane at 10^{-3} Pa as the moderating gas. The coupling of the electron monochromator to the magnetic sector instrument has been previously described.[23] Electrons of energies between 0.03 and 30 eV with a bandwidth of ±0.3 eV (fwhm) could be selected by the monochromator. The electron energy resolution and tuning of electron energy was carried out with hexafluorobenzene. Hexafluorobenzene has known electron capture energies of 0.03 eV for $M^{\bullet -}$ and 4.5 and 8.3 eV for the $C_6F_5^-$ fragment.[20] The electron energy resolution was determined by measuring the peak width for the $M^{\bullet -}$ ion at 0.03 eV. The near-zero electronvolt resonances are very narrow, so the measured width for this resonance is attributed to the electron energy resolution of the monochromator.

In both instruments, samples were introduced with a direct solids probe. The probe was heated from 30 to 400 °C at 256 °C/min for 10 min to thermally desorb the samples. Roughly 50 μg of lyophilized bacteria or spores was placed in glass capillary tubes that were inserted into the direct solids probe. Standards of dipicolinic acid, palmitic acid, nitrobenzene, and hexafluorobenzene were obtained from Aldrich (St. Louis, MO).

Test Organisms. The *Bacillus* samples analyzed were obtained from the Armed Forces Institute of Pathology (Table 1). The samples of *Bacillus globigii* were from Dugway Proving Grounds, Dugway UT, and were not an ATCC designated strain. *Bacillus* spores were grown in a shaker incubator at 200 rpm and 35 °C for two to three weeks (or however long it took for them to sporulate). The degree of sporulation in a culture was assessed with microscopy, and spore concentration was determined with a cell counting chamber and by plate counts. Once sporulated, cultures were centrifuged at 7000 rpm for 10 min, the supernatant was poured off, and the remaining pellet was washed with saline. This procedure was repeated twice. Bacterial samples were live except for samples of *Bacillus anthracis*, which were killed by γ irradiation and then lyophilized. Samples of *Escherichia coli* (K12) were purchased from Aldrich and *Pseudomonas fluorescens* were obtained from the American Type Culture Collection (Rockville, MD).

RESULTS

The monochromator was first tuned to the electron capture energy of dipicolinic acid. A standard ECNIMS mass spectrum

(18) Beverly, M. B.; Basile, F.; Voorhees, K. J.; Hadfield, T. L. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 747−758.
(19) Stockl, D.; Budzikiewicz, H. *Org. Mass. Spectrom.* **1982**, *17*, 470−474.
(20) Stamatovic, A. a. S., G. J. *Rev. Sci. Instrum.* **1968**, *39*, 1752−1753.
(21) Laramee, J. A.; Mazurkiewicz, P.; Berkout, V.; Deinzer, M. L. *Mass Spectrom. Rev.* **1996**, *15*, 15−42.
(22) Laramee, J. A.; Berkout, V.; Phillips, E.; Deinzer, M. L. *Proceedings of the 46th ASMS Conference*, Orlando, FA, 1998; p 345.
(23) Laramee, J. A.; Cody, R. B.; Hermannsfeldt, W. B.; Deinzer, M. L. *Proceedings of the 44th ASMS Conference*, Portland, OR, 1996; p 71.

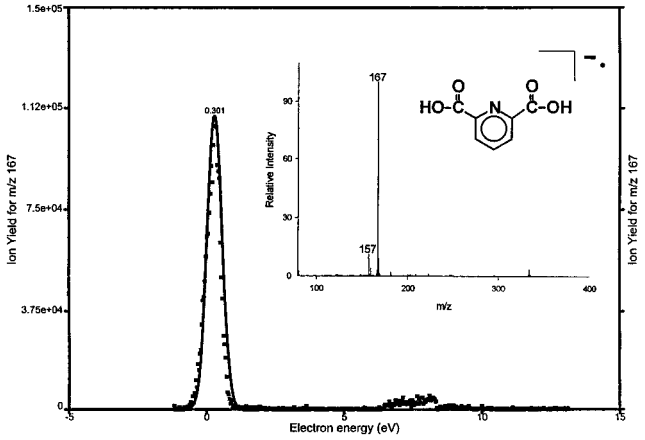

Figure 2. Electron energy scan of dipicolinic acid. The electron capture energy (eV) for dipicolinic acid that produces the greatest intensity of $M^{\bullet -}$ ions at *m*/*z* 167 occurs at 0.301 eV. The inset shows the ECNIMS spectra of a standard of dipicolinic acid with the $M^{\bullet -}$ ion at *m*/*z* 167.

of DPA shows a strong $M^{\bullet -}$ peak at *m*/*z* 167 (Figure 2 inset). The maximum ion intensity of *m*/*z* 167 occurred at 0.3 eV (Figure 2). The electron capture energy of dipicolinic acid has not been established by traditional methods.

The ECNI mass spectra of *Bacillus cereus* spores are shown in Figure 3. The $M^{\bullet -}$ ion of dipicolinic acid at *m*/*z* 167 is clearly visible in the spectra; however, as the degree of sporulation decreased, so did the relative intensity of the ion at *m*/*z* 167. The presence of interfering peaks is most pronounced in cultures that are 1% sporulated. The 1% sporulated *B. cereus* sample in Figure 3 was then reanalyzed with the monochromator tuned to the electron capture energy of DPA. Using the monochromator, the $M^{\bullet -}$ ion of DPA at *m*/*z* 167 became the base peak in the spectrum and the signal-to-noise ratio of the peak was significantly improved (Figure 4). The signal-to-noise ratio for *m*/*z* 167 in the 1% sporulated sample (Figure 3) is 4.5 whereas when analyzed with the monochromator the signal-to-noise ratio is improved to 25. Selection of the electron capture energy for DPA eliminated most of the ions that were present in the standard negative ion chemical ionization spectrum as those ions did not capture electrons of the same energy as DPA. When analyzed by the monochromator, spectra of the 60% and 100% sporulated samples of *B. cereus* were identical to the 1% sample. The bacteria in Table 1 also produced spectra similar to that of *B. cereus* with *m*/*z* 167 as the base peak.

Electron Energy Scans. The electron monochromator can also be scanned over a range of electron energies. Scanning through electron energies while performing the mass analysis produces a mass spectrum specific to each electron energy. This type of experiment allows an analyte to be identified by both its *m*/*z* ratio and its known electron capture energy. For this experiment, a selected ion monitoring (SIM) scan was conducted for *m*/*z* 167 while electron energies from 0 to 10 eV were repeatedly scanned. An internal electron energy calibration compound, nitrobenzene, was used in the analyses to track when 0 eV electrons were produced. Nitrobenzene has an electron capture energy of 0.0 eV, close to DPA, but produces an ion at *m*/*z* 123. By monitoring the ions at *m*/*z* 167 and 123 while scanning the monochromator, the electron energy could be internally calibrated.

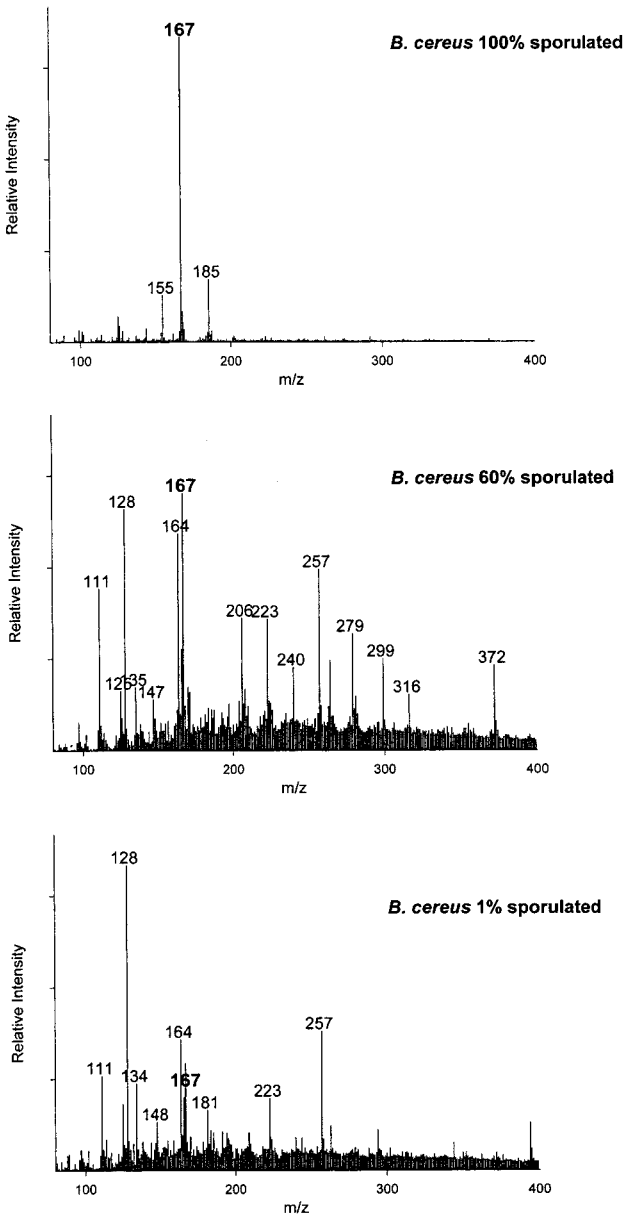

Figure 3. Standard ECNIMS spectra of samples of *B. cereus* at various degrees of sporulation.

A sample of *P. fluorescens*, which does not form spores, was used as the blank. No signal was observed at *m*/*z* 167 and 0.0 eV for *P. fluorescens* (Figure 5) compared to a sample of *B. globigii* spores (Figure 6). In both samples, there is a noticeable ion current for *m*/*z* 167 at electron energies above 0 eV. These ions however, can be ruled out as being from DPA as DPA shows no electron capture signal for electrons with energies above 0.3 eV (Figure 2). Using this SIM approach, 5 μL of a 1×10^7 spores/mL solution of 100% sporulated *B. globigii* was analyzed (50 000 spores, S/N = 2), which is currently the detection limit for this technique on this instrument.

Fatty Acids. Other bacterial compounds of interest can also be selectively analyzed using the monochromator. Bacterial fatty acids roughly reflect Gram-type with Gram-positive *B. cereus* containing more C15:0 (where 15 represents the number of carbons and 0 represents the number of unsaturations) acid and less palmitic, C16:0, than the Gram-negative *E. coli*.[24,25] Selection

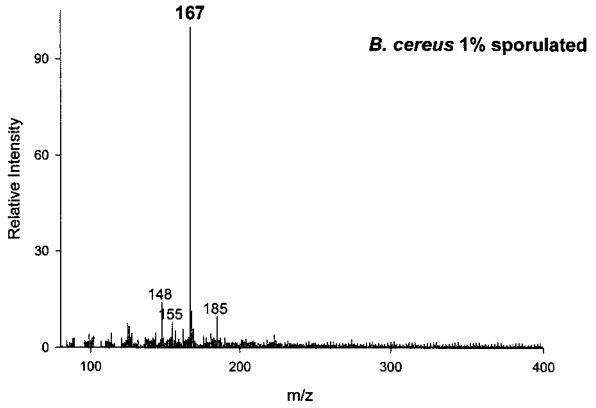

Figure 4. The 1% sporulated sample shown in Figure 3 analyzed with the monochromator tuned to the electron capture energy of dipicolinic acid, 0.301 eV.

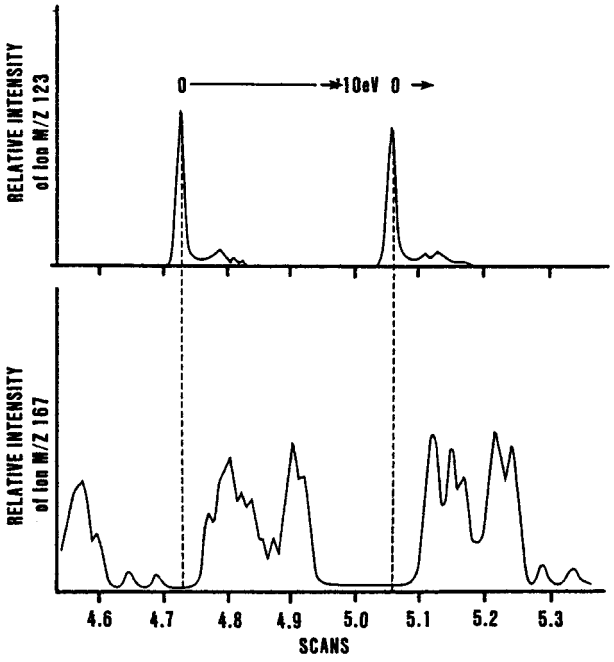

Figure 5. Electron energy scan of *P. fluorescens* for *m*/*z* 167. A SIM experiment was conducted while the monochromator was repeatedly scanned from 0 to 10 eV. The *y* axis shows ion current for *m*/*z* 167, the lower *x* axis marks mass spectrometer scans, and the upper *x* axis indicates the cycling of electron energy. Upper trace is a SIM scan of the 0 eV internal energy calibrant nitrobenzene at *m*/*z* 123. Lower trace is a SIM scan of *m*/*z* 167 for DPA. Dotted line marks the 0 eV energy. There is no ion signal for *m*/*z* 167 at 0 eV in the Gram-negative bacteria.

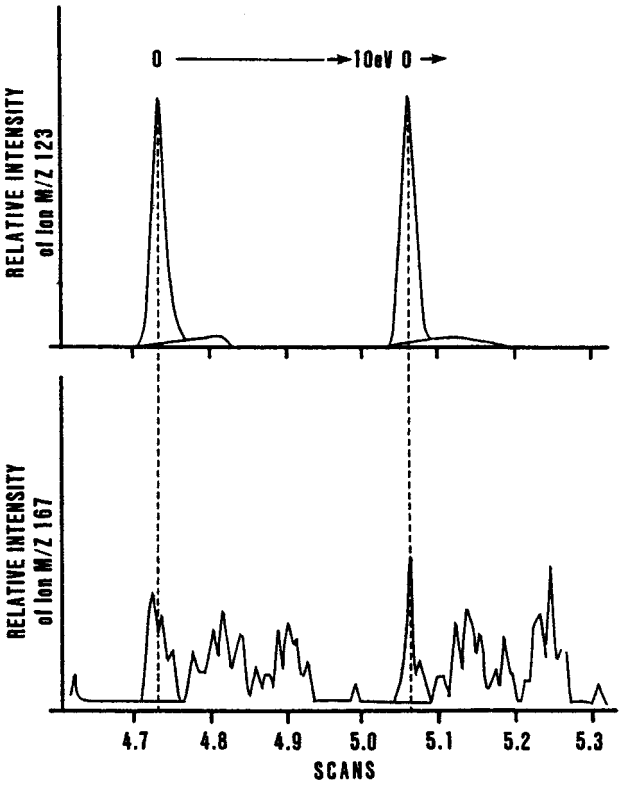

Figure 6. Electron energy scan of *B. cereus* spores for *m*/*z* 167 as described in Figure 5. Upper trace is a SIM scan of the 0 eV internal energy calibrant nitrobenzene at *m*/*z* 123. Lower trace is a SIM scan of *m*/*z* 167 for DPA. Dotted line marks the 0 eV energy.

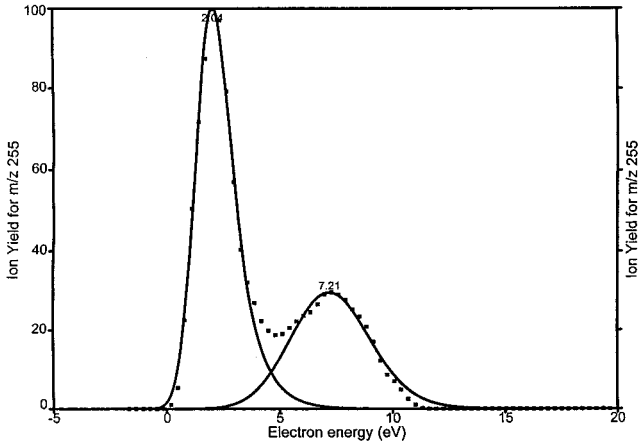

Figure 7. Electron energy scan of palmitic acid. The electron energy of 2.04 eV produces the greatest intensity of the $[M - H]^-$ ion at *m*/*z* 255.

of electron energies to maximize ionization by fatty acids permits collection of a negative ion mass spectrum specific for fatty acids. Palmitic acid was found to have a maximum ion intensity for the $[M - H]^-$ ion at an electron energy of 2 eV (Figure 7). A mass spectrum of *E. coli* recorded at this energy reveals a base peak at *m*/*z* 255, which is the most prominent fatty acid in this Gram-negative bacteria (Figure 8). Although the electron energy was set to maximize ionization by palmitic acid, the electron capture energies of straight-chain fatty acids have been found to overlap one another.[26] This is not surprising as the fatty acid carbon chain may change in length but the site of ionization, the carboxylic acid, remains the same. Because of similar electron capture energies, there are ions present in the spectrum that correspond to fatty acids other than palmitic acid. The prominent peak at *m*/*z* 267 appears to be the $[M - H]^-$ ion from a cyclopropyl C17:0 fatty acid. The peaks at *m*/*z* 281 and 295 are probably the $[M - H]^-$ ions from C18:1 and C19:1 fatty acids. Similarly, the peaks at *m*/*z* 199 and 227 could be the $[M - H]^-$ ions from C12:0 and C14:0 fatty acids or, as is most likely, fragments from C15:0.

(24) Kaneda, T. *J. Bacteriol.* **1967**, *93*, 894−903.
(25) Kaneda T. *Microbiol. Rev.* **1991**, *55*, 288−302.

(26) Cody, R. B., personal communication.

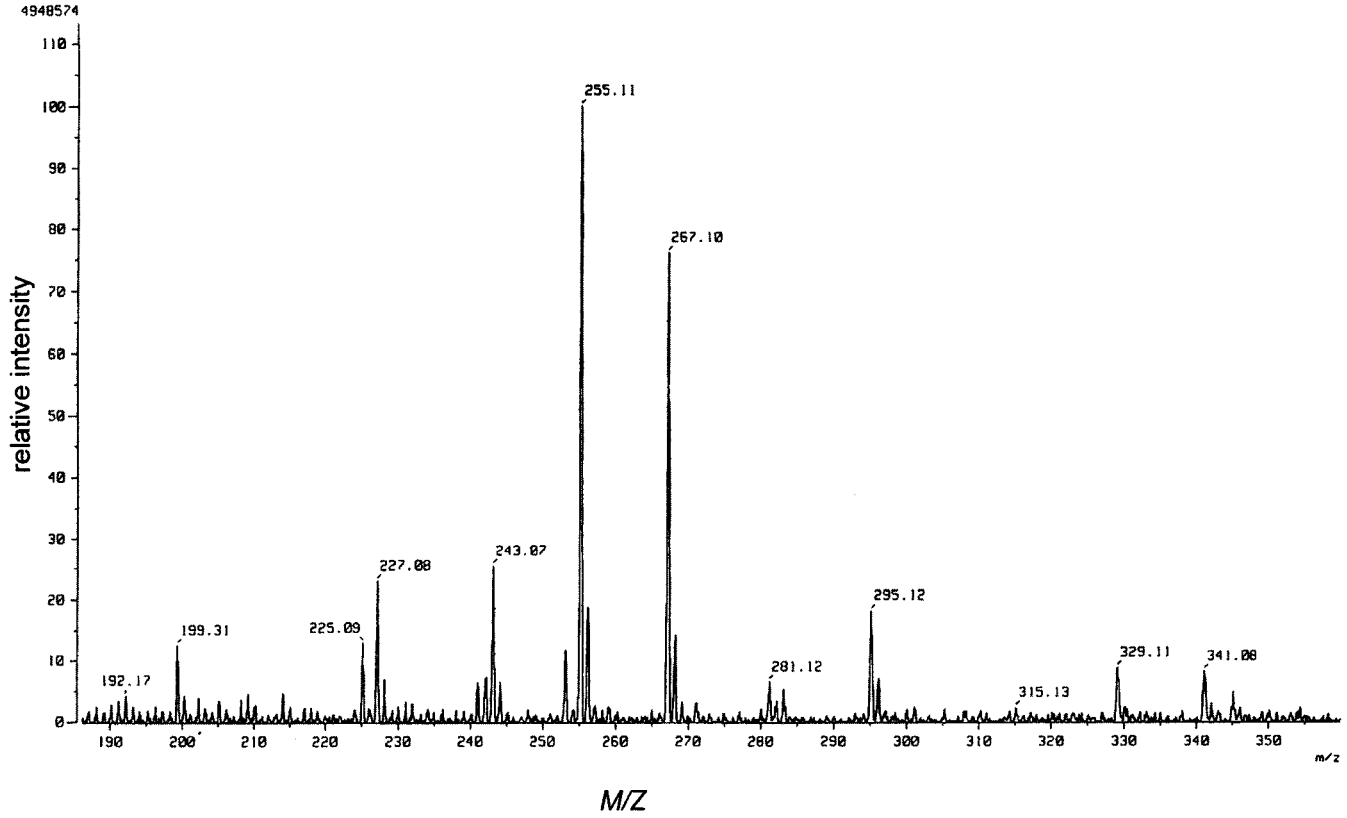

Figure 8. Mass spectrum of *E. coli* when the electron monochromator is tuned to the electron capture energy of palmitic acid. The base peak at *m*/*z* 255 is the $[M - H]^-$ ion from palmitic acid.

However, MS/MS was not conducted to confirm the identity of these assignments.

CONCLUSION

The electron monochromator significantly improved the signal-to-noise ratio of the negative ion spectra of target compounds in whole bacteria. Selecting electrons of energies specific for DPA virtually eliminated any background ions in the spectrum and allowed DPA to be the base peak in the spectrum regardless of the degree of sporulation. Scanning of the electron monochromator while monitoring selected masses added another dimension of specificity to the SIMS analysis of DPA. Although only a SIM scan is shown here, it is also feasible to carry out a full mass scan while scanning the electron energy. Doing this provides a complete mass spectrum for each electron energy scanned. In addition to DPA, palmitic acid in *E. coli* was also selectively ionized with the monochromator to produce a negative fatty acid spectrum in which palmitic acid was the base peak. Due to some overlap in the electron capture energies of fatty acid, peaks associated with fatty acids other than palmitic appeared in the spectrum. By scanning the monochromator through the electron capture energies of different fatty acids, it should be possible to obtain a negative ion fatty acid profile from whole bacteria. The specificity of this approach however, will be dependent on the amount of overlap in electron capture energies between fatty acids.

While the benefit of this technique to eliminate interferences by unwanted ionization has been demonstrated, the effect on detection limits has not been thoroughly studied. Currently, the detection limit of this technique for DPA in spores versus ECNIMS is the same. However, this was not a direct comparison as these experiments were carried out on two different instruments. The ECNIMS experiments were conducted on a JEOL Mstation, which has much greater ion transmission than the older JEOL 505AX.[26] To obtain a valid comparison of ECNIMS and the monochromator, it is important that certain factors are equalized. For example, the electron current in the monochromator is ~300 μA, which is much less than typical filament currents in ECNIMS. This could be expected to decrease the amount of sample that is ionized and thus increase the limits of detection. However, lower limits of detection are anticipated with the monochromator as unwanted ions that can participate in neutralization reactions and increase noise levels are removed.

Future work will focus on using the monochromator to enhance the negative ion spectra of other taxonomically important compounds such as polyhydroxybutyrate and muramic acid. With the selectivity and sensitivity of negative ion mass spectrometry, the electron monochromator has the potential to greatly aid in the analysis of whole bacteria.

ACKNOWLEDGMENT

The authors thank the Colorado School of Mines and JEOL USA, Inc. for financial support of this study.

Received for review November 17, 1999. Accepted March 2, 2000.

AC991318G