Research Paper

Application of the Mars Organic Analyzer to Nucleobase and Amine Biomarker Detection

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ABSTRACT

The Mars Organic Analyzer (MOA), a portable microfabricated capillary electrophoresis instrument being developed for planetary exploration, is used to analyze a wide variety of fluorescamine-labeled amine-containing biomarker compounds, including amino acids, monoand diaminoalkanes, amino sugars, nucleobases, and nucleobase degradation products. The nucleobases cytosine and adenine, which contain an exocyclic primary amine, were effectively labeled, separated, and detected at concentrations <500 nM. To test the general applicability of the MOA for biomarker detection, amino acids and mono- and diamines were extracted from bacterial cells using both hydrolysis and sublimation followed by analysis. The extrapolated limit of detection provided by the valine biomarker was $\sim 4 \times 10^3$ cells per sample. Products of an NH₄CN polymerization that simulate a prebiotic synthesis were also successfully isolated via sublimation and analyzed. Adenine and alanine/serine were detected with no additional sample cleanup at 120 ± 13 μM and 4.1 ± 1 μM , respectively, corresponding to a reaction yield of 0.04% and 0.0003%, respectively. This study demonstrates that the MOA provides sensitive detection and analysis of low levels of a wide variety of amine-containing organic compounds from both biological and abiotic sources. Key Words: Capillary electrophoresis—Bacterial biomarkers—Prebiotic synthesis—Nucleobase detection—Lab-ona-chip—Microfabrication. Astrobiology 6, 824–837.

INTRODUCTION

RECENT IMAGING and geological and mineralogical observations of Mars by remote and *in situ* instruments, all of which suggest the presence of liquid water, have intensified interest in the possibility of extinct or extant life on the red planet. Photographs from the Mars Orbital Camera revealed gullies possibly formed by liquid water only 1–3 million years ago (Malin and Edgett, 2000, 2003; Reiss *et al.*, 2004). The wet conditions required to form these gullies are thought to have lasted long enough to fill craters and form deltas (Malin and Edgett, 2003; Fassett and Head, 2006), though such standing water may have been episodic and evaporated quickly (Kreslavsky and Head, 2002; Segura *et al.*, 2002). Hydrogen-rich layers, most-likely frozen water, were detected in

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the polar regions by the Gamma Ray Spectrometer (Boynton et al., 2002). In addition, the High Resolution Stereo Camera and the Mars Orbiting Laser Altimeter provided evidence of a frozen sea with an estimated age of \sim 5 million years (Murray et al., 2005). The Mössbauer spectrometer and the Mini-Thermal Emission Spectrometer on the Mars Exploration Rovers detected sulfate minerals that are only formed in aqueous environments (Christensen *et al.*, 2004; Klingelhofer *et al.*, 2004; Squyres *et al.*, 2004b). Finally, the OMEGA imaging spectrometer on Mars Express confirmed the presence of phyllosilicates and sulfates indicative of aqueous alteration (Poulet *et al.*, 2005; Bibring et al., 2006). These observations suggest that, at one time, Mars had the aqueous conditions necessary to support life, and that these locations may be good sites to look for evidence of extinct or extant life (Murray *et al.*, 2005).

The minerals detected at Meridiani Planum by the Mars Exploration Rover Opportunity suggest harsh acidic, saline aqueous conditions that would be challenging for bacterial or other life. However, extremophile bacterial colonies are readily detected in the similar acidic conditions found in the Rio Tinto basin in Spain (Zettler et al., 2002; Fernandez-Remolar et al., 2003, 2005; Bishop et al., 2004). In addition, microorganisms have been detected at low temperatures in ice and permafrost on Earth (Russell, 1990; Thieringer et al., 1998; Rivkina et al., 2000; Junge et al., 2004). The discovery of bacterial colonies that have adapted to these extreme environments on Earth suggests that the harsh conditions on Mars do not preclude past or present microbial life.

In situ and remote sensing instruments have been used to look for biomarker compounds on Mars. The Viking Landers performed analyses on martian soil to look for microbial activity and organic molecules. The negative results are now thought to be due to the highly oxidized and acidic soils (Biemann et al., 1976, 1977; Benner et *al.*, 2000; Glavin *et al.*, 2001; Quinn *et al.*, 2005a,b). More recently, Mars Express detected methane at a global average mixing ratio of 10 parts per billion (ppb) (Formisano et al., 2004). While the activity of subpermafrost microorganisms would be one possible source of this methane, abiotic sources such as volcanic outgassing and destruction of carbonaceous chondrites in meteorites must be considered (Formisano et al., 2004). Thus far attempts to detect biomarker compounds on Mars are ambiguous at best.

Determining the most likely target analytes for detection of extinct or extant life on Mars is challenging. Observation of present microbial activity through respiration and cellular growth would confirm the existence of life, but this approach does not work for past activity. Since biopolymers rapidly degrade over geological time spans, especially in the oxidizing and harsh conditions on Mars, the detection of RNA, DNA, and proteins requires the presence of viable organisms. In contrast, detection of monomeric biomarkers such as amino acids and nucleobases is possible from both extant and extinct organisms because of their longer lifetimes. Furthermore, a wide variety of bioamines should also be targeted because amino acids and nucleobases both degrade to mono- and diamines (Glavin et al., 2001; Saccani *et al.*, 2005).

Organic molecules on Mars might also originate from in situ prebiotic reactions or from meteoritic in-fall. Prebiotic reactions that involve the polymerization of NH₄CN or HCN can produce a wide variety of products (Miller, 1953; Oró and Kamat, 1961; Oró and Kimball, 1961; Sanchez et *al.*, 1967; Wolman *et al.*, 1971; Borquez *et al.*, 2005). Simple polymerizations produce dimers and trimers that can lead to formamide, methyl- and ethylamine, diamines, and even racemic α -amino acids (Miller, 1953). More complex reactions can produce cyclic, aromatic amines, including purines such as adenine (Borquez et al., 2005). Meteoritic in-fall may include simple organic amines such as amino acids, nucleobases, or more complex polycyclic aromatic hydrocarbons (Mc-Sween, 1994; McKay et al., 1996; Becker et al., 1997; Glavin et al., 1999; Sephton et al., 2002; Stephan et al., 2003; Meierhenrich et al., 2004). Techniques and instrumentation that target a wide range of organic amine-containing molecules are important for Mars *in situ* measurements because this ensures that molecules indicative of extant organisms, extinct organisms, in-fall, or prebiotic chemistry will be observable.

We previously developed a portable microchip capillary electrophoresis (CE) instrument called the Mars Organic Analyzer (MOA) and characterized its performance for composition and chirality analysis of amino acid biomarkers. Amino acids were labeled with fluorescamine and analyzed with ppb to parts per trillion sensitivities from a 1-g soil sample (Skelley *et al.,* 2005). This portable instrument was also extensively field-tested at two Mars analog sites: the Panoche Valley, CA (Skelley *et al.*, 2005) and the Atacama Desert, Chile (see http:// astrobiology.berkeley.edu) (A.M. Skelley *et al.*, manuscript in preparation). We report here results that expand the utility of the MOA, which include the development of labeling techniques and CE analysis conditions for an expanded list of bioamines as well as nucleobases. The system is then used to analyze biomarker amines extracted from bacterial cells and from a prebiotic synthesis.

MATERIALS AND METHODS

Analysis of fluorescamine-labeled bioamines

All samples were analyzed by CE using microfabricated devices of the type pictured in Fig. 1A (Skelley et al., 2005). These microfluidic devices integrate all-glass CE separation channels and microfabricated polydimethylsiloxane (PDMS) membrane valves and pumps (Grover *et al.*, 2003) in a multilayer format. The separation channels are 21.4 cm long and contain 0.6-cm-long cross injection channels. The four CE reservoirs (sample, waste, cathode, and anode) are accessed by separate fluidic bus structures for delivering buffer and sample to the channel as well as for rinsing the separation channel between runs. The separation and fluidic layers are integrated into one multilayer device structure, which has been extensively described (Skelley et al., 2005).

CE separations were performed on a portable instrument (Fig. 1B) that contains the electronics and power supplies for CE separations, pneumatics to control the PDMS valves and pumps, and optics for confocal laser-induced fluorescence detection (Skelley *et al.*, 2005). The $10- \times 25$ - \times 31-cm instrument interfaces with the microfluidic device using an external manifold that carries the four electrophoresis electrical contacts as well as 16 pneumatic lines to supply vacuum or pressure for valve control. Integrated power supplies drive the electrophoresis potentials, 404-nm diode laser, confocal fluorescence detection optics, and photomultiplier tube. Active temperature control during the separation, when necessary, is available via a thermoelectric cooler and fan, also shown in Fig. 1B.

The separations were performed at room temperature in 10 m*M* Na₂CO₃ buffer, pH as specified. Two different injection techniques were used to analyze the samples. A regular injection (injecting the sample across to the waste reservoir for 10 s) was used for high-resolution separations. A 300-ms direct injection (a 10-s regular injection to waste followed by injecting sample directly towards the cathode for 300 ms) was used to inject a larger plug on the column, which increases the signal while still maintaining good resolution (Skelley *et al.*, 2005).



FIG. 1. CE device and portable MOA instrument. (A) The microfabricated device integrates glass separation channels (red) with PDMS membrane valves and pumps for fluid manipulation in the multilayer device. **(B)** The instrument contains the electronics, pneumatics, and optics necessary for CE separation and fluorescence detection. PMT, photomultiplier tube; TEC, thermoelectric cooler. Adapted from Skelley *et al.* (2005).

Analysis of amines, nucleobases, and amino sugars

Aqueous solutions of bioamines were prepared in water and mixed with the Mars 7 Amino Acid Standard [1 mM total amino acid concentration, containing 133 μM valine, alanine, serine, glutamic acid, and aspartic acid, 66 μM glycine, and 266 μM aminoisobutyric acid (AIB)] for calibration and quantitation. Aliquots (10 μ l) of the sample mixture were combined with 10 μ l of 10 mM Na₂CO₃ (pH 9.78) (pH 9.78 buffer) followed by 20 μ l of 20 mM fluorescamine in acetone for labeling (Skelley and Mathies, 2003; Skelley et al., 2005). After 10 min of reaction at room temperature, the acetone was removed under house vacuum (3 min). The solutions were diluted from 1:4 to 1:20 (initial volume:final volume) in 10 mM Na₂CO₃ buffer at the specified pH for analysis.

Analysis of adenine and cytosine

After labeling, all nucleobase samples were diluted 1:4 with pH 9.78 buffer before injection. Triplicate injections from the same samples were performed, though data from the first injection only were used because the signals were higher. Peak heights from the baseline were determined and divided by twice the baseline standard deviation to determine a signal-to-noise ratio (S/N). The limit of detection (LOD) was set at S/N = 3.

Analysis of Escherichia coli cells

E. coli bacteria were grown and processed as described previously (Glavin *et al.*, 2004). Briefly, *E. coli* (strain MG1655) cells were grown overnight in glass tubes at 37°C in a water bath. The optical density of a 1:10 dilution was measured at 460 nm using an HP 8452A diode array spectrophotometer (Hewlett Packard, Palo Alto, CA). The concentrations of *E. coli* cells in solution were determined using an apparent extinction coefficient of 10^8 cells per absorbance unit (Glavin *et al.*, 2004). The samples were then diluted in double distilled water (ddH₂O) as desired.

E. coli cells (in dilutions ranging from 4.8×10^8 cells/ml to 4.8×10^3 cells/ml) were processed using three sets of conditions: (1) A 200-µl suspension of cells in water was dried in a sublimation apparatus (Glavin *et al.*, 2004) and then sublimed in an oven preheated to 450°C, 0.5 mm Hg, for 5 min to a cold finger held at liquid N₂ temperature

(Glavin *et al.*, 2002). The cold finger was rinsed with 1 ml of ddH₂O, and this wash was transferred to an Eppendorf tube and dried under vacuum. (2) A 200- μ l suspension of cells was dried in a test tube, and 1 ml of 95% formic acid (Aldrich, Milwaukee, WI) was added. The tube was sealed and heated at 100°C for 24 h. The formic acid was removed under vacuum. The residue was then sublimed as described above. (3) A 200- μ l suspension of cells was acid-hydrolyzed and dried as described above. The residue was transferred with two 200- μ l aliquots of ddH₂O to an Eppendorf tube and dried for direct analysis.

The samples were suspended in 20 μ l of pH 9.78 buffer and split into two aliquots: buffer (10 μ l) was added to one aliquot, and standard (10 μ l; 125 μ M Mars 7 Standard, 2.8 mM cytosine, 2.8 mM adenine, 205 μ M cadaverine, and 50 μ M glucosamine in pH 9.78 buffer) was added to the other aliquot. The samples were labeled with 20 mM fluorescamine as above and diluted 1:4 in pH 9.78 buffer for analysis.

*NH*₄*CN* polymerization

Aqueous NH₄CN, prepared from 1 M NH₄Cl and 1 M NaCN, was placed in a sealed tube and heated at 100°C on a covered heating block for 5 days. A 200-µl aliquot was removed, dried, transferred to the quartz sublimation apparatus, and then held in a tube furnace (preheated to 1,100°C) for 30 s. This sublimation protocol was previously characterized to yield sample temperatures of 500°C for at least 30 s (Glavin *et al.*, 2001, 2004). The sample was rinsed off the cold finger using 1 ml of ddH_2O , and then 240 μ l of the wash was dried under vacuum. The sample was brought up in 20 μ l of pH 9.78 buffer, and $10-\mu$ l aliquots were removed and combined with 10 μ l of pH 9.78 buffer or 10 μ l of standard (580 μ M adenine, 560 μ M cytosine, 100 μM Mars 7 Standard, ~100 μM methylamine, and $\sim 100 \ \mu M$ ethylamine) and derivatized with fluorescamine as described above. The sample was diluted 1:4 with pH 9.78 buffer prior to injection.

Analysis of CE traces

All traces were analyzed using Grams 32 (Thermo, San Jose, CA). The analyte peak areas in the sample run and the spiked sample-plusstandard run were used to determine the concentrations of the analytes of interest. The two traces were normalized using a common unspiked peak area as an internal standard. Subtraction of the area in the sample run from the spiked run revealed the area to be due solely to the standard, enabling calibration.

RESULTS

In addition to amino acids (Skelley et al., 2005), amine-containing molecules such as nucleobases, amino sugars, and mono- and diamines are important tracers of biological activity. The separation of fluorescamine-labeled adenine and cytosine as a function of pH is presented in Fig. 2. At $pH \le 8.67$ both of these labeled analytes had net -1 charge (from the fluorescamine carboxylic acid) and eluted in the monoamine region. The amino acids in the Mars 7 Standard eluted later, either as a result of the additional carboxylic acid on the amino acid backbone, which would have given a net -2 charge (valine, AIB, alanine/serine, and glycine), or as a result of the additional carboxylic acid on the side chain, which would have given a net -3 charge (glutamic and aspartic acid). At intermediate pH values, adenine and cytosine shifted towards the -2 charged analytes. This shift to longer migration times is indicative of an increase in average negative charge due to deprotonation of the ring amines $[pK_a \text{ values of } 9.8 \text{ and }$ 12.2 for adenine and cytosine (Lide, 2005)], and has been used previously to resolve nucleobase mixtures (Suzuki *et al.*, 2000). At $pH \ge 10.15$, the nucleobases eluted with the -2 charge group. A pH of 9.78 was chosen for subsequent separations because the nucleobases migrated between the -1charged amines and the -2 charged amino acids.

The LOD of cytosine and adenine was determined using two different injection methods (Fig. 3). The regular cross injection procedure could detect cytosine down to 2.1 μM and adenine down to 2.0 μM . Both cytosine and adenine exhibited linear relationships between injected concentration and S/N. Using a 300-ms direct injection time, the LOD was extended to 470 nM for cytosine and 480 nM for adenine. The higher LOD compared with the low nanomolar values obtained for amino acids (Skelley et al., 2005) was likely due to the different labeling efficiency of these exocyclic amine analytes. This decrease in labeling efficiency may be due to the increased steric hindrance, the decrease in nucleophilicity of the amine group in the nucleobases, or a combination of both effects.

The ability to label, detect, and analyze nucleobase derivatives and degradation products is also potentially important for detecting traces of extinct life. Figure 4 presents results on fluorescamine-labeled nucleobases (adenine and cytosine), nucleosides (adenosine), and nucleotides [adenosine 3'-monophosphate (AMP) and cytidine 3'-monophosphate (CMP)] at pH 9.78.



FIG. 2. Analysis of the pH-dependent mobility of fluorescamine-labeled adenine and cytosine. At pH 8.67 (top trace), the nucleobases coeluted with the amines. As the pH was increased, adenine (A) and cytosine (C) shifted to slower migration times, which indicated an increase in net negative charge with increasing pH. A pH of 9.78 was chosen to position adenine and cytosine between the amine and amino acid peaks. Injected concentrations were 145 μ M A, 140 μ M C, 30 μ M methylamine (MEA), and 25 μ M Mars 7 Standard.



FIG. 3. Plot of the LOD of cytosine (A) and adenine (B) using different injection techniques. A regular cross injection has a detection limit of 2.1 μ *M* cytosine and 2.0 μ *M* adenine (S/N = 3); the 300-ms direct injection can detect down to 470 n*M* cytosine and 480 n*M* adenine. *R*² values for the concentration dependence of S/N for regular injection were 0.9948 for cytosine and 0.9558 for adenine and for 300-ms direct injection were 0.9853 for cytosine and 0.9965 for adenine. Presented data are from the first injection for each sample.

Adenosine eluted in the -1 charge region, possibly because the sugar residue that resides on the ring amine prevented it from participating in acid-base equilibria. The nucleotides eluted between the -2 and -3 charged amino acids; this was presumably because of the -2 charge supplied by the phosphate group, which would yield a net -3 charge on the relatively large molecule. AMP eluted before CMP because of the larger size of the purine base compared with the pyrimidine base.

Purine nucleobases and nucleotides such as AMP may be broken down within organisms or degraded by oxidants to smaller, more highly charged analytes (Benner *et al.*, 2000) via the pathway shown by Voet and Voet (1995):



This degradation should still be detected by the MOA as long as significant reactive amino groups remain. To test this hypothesis, the molecules in the AMP degradation pathway were labeled with fluorescamine and separated on the MOA system. Labeling allantoin (Fig. 4B, top) produced one intense peak that migrated close to alanine/serine. At pH 9.8, the ring structure of allantoin must support a negative charge, which would give the labeled molecule a net -2 charge. Allantoic acid (Fig. 4B, middle) produced two parent peaks: one that co-eluted with valine and perturbed the peak shape and a second that eluted in the same region as allantoin, which suggests the presence of two species of the same -2charge but slightly different mass. These peaks are likely due to the full allantoic acid molecule (assuming a single fluorescamine label) and allantoic acid minus a urea group (ureido-oxalate). In addition, a strong peak appeared in the net -1region and was assigned as urea. After incubation in solution for >24 h, the parent peaks were reduced in area relative to the urea peak, which indicated further degradation of allantoic acid to urea. Urea (Fig. 4B, bottom) produced a single peak in the -1 region, and the migration time was consistent with a single fluorescamine label.

Because mono- and diamines are potential biodegradation products, mixtures of fluorescamine-labeled amines and diamines were analyzed as shown in Fig. 5A. Monoamines (methylamine, ethylamine, isopropylamine, and isoamylamine) were all resolved from each other, even though they only differed by one or two methyl mass units. In Fig. 5B, the separation of a mixture of biologically relevant monoamines and diamines is presented. Cadaverine (1,5-diaminopentane) exhibited distinct peaks due to the singly labeled (cadaverine-1) and doubly labeled (cadaverine-2) products. Glucosamine exhibited two peaks due to the α - and β -anomers



FIG. 4. CE analysis of fluorescamine (FA)-labeled nucleobase derivatives and degradation products with the MOA. (A) CE separation of nucleobases, nucleosides, and nucleotides along with the Mars 7 Standard in 10 mM Na₂CO₃ buffer, pH 9.78. Injected concentrations were 6.25 μ M Mars 7 Standard, 73.5 μ M adenine, 70 μ M cytosine, 12.8 μ M CMP, 22.5 μ M AMP, and 30.5 μ M adenosine. (B) CE analysis of the degradation products of AMP along with the Mars 7 Standard in 10 mM Na₂CO₃, pH 9.8. Saturated solutions at room temperature of allantoin, allantoic acid, and urea were labeled and diluted 1:5 before injection. All traces were aligned in time using the peaks of the Mars 7 Standard.

(Skelley and Mathies, 2006) that were resolved from the amines and diamines.

Direct analysis of bacterial samples

E. coli cells were subjected to different extraction techniques followed by fluorescamine labeling and analysis. The three different extraction techniques are compared in Fig. 6 for 9.6×10^7

cells. Subliming the samples (Fig. 6A) effectively isolated amines and amino acids $(1-2 \mu M)$ in the 200- μ l reaction volume) but with low overall efficiency. Hydrolyzing the samples prior to sublimation (Fig. 6B) yielded slightly higher concentrations of amino acids (2–6 μM for valine, alanine/serine, and glycine) and lower concentrations of amines. Using acid hydrolysis alone (Fig. 6C), the overall signal intensities increased by approximately five- to 10-fold, and peaks in the neutral amino acid region of this sample overloaded the photomultiplier tube. The high amine



FIG. 5. CE analysis of fluorescamine-labeled amines and diamines with the MOA. (A) CE separation of fluorescamine-labeled monoamines along with the Mars 7 Standard in pH 9.78 buffer. Injected concentrations were 21.5 μ M isoamylamine, 113 μ M isopropylamine, 30 μ M ethylamine, 30 μ M methylamine, and 25 μ M Mars 7 Standard. (B) CE analysis of complex amines and diamines along with the Mars 7 Standard, pH 9.78 buffer. Both singly and doubly labeled cadaverine peaks are resolved, along with the α - and β -anomers of glucosamine. Injected concentrations were 51 μ M cadaverine, 5 μ M glucosamine, 30 μ M methylamine, 30 μ M ethylamine, and 25 μ M Mars 7 Standard.



FIG. 6. Analysis of fluorescamine-labeled amine-containing compounds extracted from *E. coli* cells using different extraction techniques. (A) *E. coli* cells were sublimed, and the biomolecules collected on the cold finger were eluted and then labeled with fluorescamine. (B) *E. coli* cells were hydrolyzed and then sublimed before labeling with fluorescamine. (C) *E. coli* cells were hydrolyzed and then directly labeled with fluorescamine. Analytes were identified and quantified by spiking the sample with the standard prior to labeling and CE analysis. Traces were aligned in time, though relative intensities were not rescaled.

and amino acid concentrations reduced the injection efficiency so the actual concentrations were even greater, as determined relative to an internal standard. The valine concentrations detected for the three extraction techniques are summarized in Table 1. The concentrations of valine extracted from the sublimed and sublimed/hydrolyzed cells were similar, but the hydrolyzed and directly detected cells yielded ~30–100-fold higher concentrations. Identical trends were observed for alanine/serine and glycine concentrations.

A range of cell concentrations, from 9.6×10^7 to 9.6×10^2 cells in a 200-µl volume, were analyzed to determine the LODs for acid-hydrolyzed cells (Fig. 7). At 9.6×10^5 cells, all peaks were within



FIG. 7. Analysis of amines released from hydrolyzed *E. coli* cells. Aliquots (200 μ l) containing the indicated number of cells were hydrolyzed for 24 h with formic acid, dried under vacuum, and then directly labeled with fluorescamine for analysis. All samples were analyzed at identical dilution factors. The signal initially decreased with the cell count from 10⁷ to 10⁴ cells, and then the trend was obscured by uncontrolled systematic variation in the background amino acid and amine levels. The blank (200 μ l of ddH₂O, hydrolyzed and dried) exhibited only weak amine and amino acid signals.

| Cells/200 µl | Concentration (μM) of valine extracted | | |
|---------------------|---|-------------------------|-------------------|
| | Sublimed | Hydrolyzed and sublimed | Hydrolyzed |
| 9.6×10^{7} | 1.5 | 3.6 | ~100 ^a |
| $9.6 	imes 10^{6}$ | 0.70 ± 0.3 | 0.15 ± 0.06 | 15 |
| $9.6 	imes 10^{5}$ | ND | 0.08^{b} | 1.1 |
| $9.6 	imes 10^{4}$ | 0.22 ^b | ND | 0.15 ± 0.02 |
| $9.6 	imes 10^{3}$ | ND | 0.1 ± 0.1 | 0.6 ± 0.1 |
| $9.6 	imes 10^{2}$ | ND | 0.07 ^b | 0.88 ± 0.03 |

 TABLE 1.
 CONCENTRATIONS OF VALINE EXTRACTED FROM E. COLI CELLS

Concentrations are not blank corrected. Values with error are average of cross and 300ms direct injections. Single-point values were determined by cross injection only unless otherwise noted. ND, not detected.

^aBased on relative signal height.

^bConcentration could only be determined by 300-ms direct injection.

the range of the detector, excellent peak shapes and resolutions were observed, and amino acids, monoamines, and diamines were detected and easily quantified. Further diluting the cells by 10and 100-fold lowered the peak heights by a factor of 10-100 as expected, and amino acids and amines were still detectable from 9.6×10^4 cells. For the more dilute samples, an uncontrolled systematic variation in the background level of amines and amino acids was consistently observed. For example, the peak intensities for 9.6×10^2 cells (data not shown) strongly resembled the intensities from 9.6×10^3 cells (Fig. 7). A similar variability was observed for the other extraction techniques at the two lowest cell concentrations. Dilutions below 10⁵ total cells were not considered reliable.

The valine S/N detected from the hydrolyzed cells using a cross injection is shown in Fig. 8A. From 10^8 to 10^5 cells, a linear relationship between S/N and cell count was observed. The background noise for all runs varied by only \sim 10%, so this decrease in S/N was directly related to the observed signal. The slope of the line was less than 1, which indicated incomplete hydrolysis, labeling, or lower injection efficiency for the higher concentration samples. A similar decrease in signal with dilution was observed for the other amino acids. Figure 8B shows that the observed trends are not unique to valine. Cell counts $\leq 10^4$ per sample were not included in the linear fits because of the background problems mentioned previously. All samples were also analyzed using a 300-ms direct injection. Similar dilution profiles were observed with signals approximately eightfold higher than those observed with the cross injection (data not shown). The LOD was not improved with the 300-ms direct injection because the limiting factor was the background contamination.

Analysis of prebiotic synthesis products

Organic amines produced from a prebiotic reaction of 1 M NH₄CN were labeled with fluorescamine and analyzed. The sample was reacted at 100°C for 5 days, dried down, and sublimed before labeling and analysis (Fig. 9). Adenine and alanine/serine, in addition to methylamine, ethylamine, and large monoamines, were identified. Adenine was present at $120 \pm 13 \mu M$, and alanine/serine was present at 4.7 \pm 1 μ M in the injected sample. Using a starting concentration of 1 *M* NH₄CN, the percent yield of adenine and alanine/serine was calculated (moles produced per initial moles of reactant). Adenine was produced at $0.040 \pm 0.004\%$, while alanine/serine was produced at $0.00035 \pm 0.0001\%$. These results agree well with the yields reported in the literature (Oró and Kamat, 1961; Oró and Kimball, 1961; Sanchez et al., 1967).

DISCUSSION

Extinct or extant life on other planets may consist of bacteria analogous to extremophiles found on Earth (*e.g.*, Glavin *et al.*, 2004). Since bacteria are 55% amino acids and 24% nucleosides/nucleotides by dry weight (Glavin *et al.*, 2001), the direct analysis of these building blocks of biological polymers is a potentially powerful approach for life detection. The MOA has already been demonstrated to provide high-sensitivity amino acid composition and chirality analysis (Skelley *et al.*, 2005). The MOA has also been field-



FIG. 8. Log-log dilution plot for the detection of amino acids from hydrolyzed E. coli cells using a cross injection method. (A) Though a linear decrease in valine S/N from 10^8 to 10^5 cells was observed, the signal from the 10^4 and 10³ cell samples was overwhelmed by background contamination. Extrapolating the linear trend to the presumed LOD $[S/N = 3, \log (S/N) = 0.5]$ predicts that valine from $\sim 10^4$ hydrolyzed cells should be detectable using a cross injection. (B) Plot of the relative peak signals for all other identified amino acids. Each amino acid signal set was normalized relative to the signals from 10^7 cells. Only data points that corresponded to an S/N = 3were plotted. A similar linear trend was observed for all amino acids at high cell concentrations; at low concentrations contamination was observed, and the highest contamination levels were recorded for glycine. In both plots, data points from the second analysis of 10⁶ cells were not plotted as amino acid peaks were not observed.

tested in the Panoche Valley, CA, and the Atacama Desert, Chile, where it successfully detected amino acids from evaporite soils that included jarosite at parts per trillion levels. Here we demonstrate that our fluorescamine labeling and CE separation method can also be easily and sensitively extended to cover a larger class of aminecontaining analytes that include nucleobases.

The key to the facile extension of the MOA to additional analytes was the use of the fluorescamine dye reagent to label nucleobases, nucleosides, nucleotides, and nucleobase degradation products followed by CE analysis. The total analysis time was only 12 min (10 min for labeling and 2 min for separation), and all analytes were easily resolved using a single separation condition. Conventional CE separations of nucleobases, nucleotides, and nucleosides and of allantoin have been reported, though such separations are generally not as effective or convenient. Conventional CE separation of purine bases and nucleosides resulted in an LOD of 0.5 μM for an absorbance detector (Grune et al., 1993). The use of an ultraviolet detector reduced the analysis time of allantoin and arginine to 5 min (Zhang et al., 2004). Elisabeth et al. (1998) used micellar electrokinetic capillary chromatography to resolve $\sim 5 \text{ mM}$ nucleotides in under 10 min. Deoxynucleotides labeled with nearinfrared dyes were detected with 5 pM sensitivity; however, the derivatization requires high-performance liquid chromatography purification, and the adenine and cytosine-containing analytes were not resolved (Li et al., 2003). The significantly faster separations and improved LODs presented here are attractive features for space flight applications.

Amino sugars form polymers such as chitin and peptidoglycan, which are structurally important in the shells of crustaceans, arthropods, and prokaryotic cell walls. Amino sugars also



FIG. 9. Analysis of the products of $1 M \text{ NH}_4 \text{CN}$ reacted at 100°C for 5 days. The sample was dried and sublimed prior to labeling with fluorescamine and analysis with the MOA. Amines, nucleobases, and amino acids were identified and quantified by spiking the sample with a standard prior to labeling and analysis.

contribute to both the carbon and nitrogen in dissolved and particulate organic matter in oceans, and are therefore important biomarkers (Benner and Kaiser, 2003). Simple amines, such as methyland ethylamine, may be produced from the decarboxylation of amino acids and are produced when natural products are sublimed (Glavin and Bada, 1998). Diamines are also common biodegradation products (Saccani et al., 2005). After labeling with fluorescamine, the resolution between the simple linear amines (methylamine to ethylamine) was better than the resolution between linear and branched amines (ethylamine and isopropylamine). Our CE separations are exquisitely sensitive to the side chain size and shape. In addition, both the singly and doubly labeled products of cadaverine and the α - and β anomers of glucosamine were detected and resolved from the monoamines with approximately nanomolar sensitivity. In the case of highly degraded amine-based biopolymers or primitive prebiotic products, small, charged bioamines may be the only biomarkers present on Mars. The demonstrated ability of the MOA to obtain highly sensitive analyses of such compounds is, therefore, of utmost importance.

Biomarkers from E. coli and prebiotic synthesis

Sublimation has been used to extract nucleobases from both standards as well as from bacterial cells in a serpentine soil analogue with extraction efficiencies that range from 99% for pure standards to ~10% for *E. coli* used to spike the specimen (Glavin *et al.*, 2001, 2002, 2004). Adenine isolated via sublimation has been used to estimate cells counts in natural samples over the range from 10^9 to 10^5 cells/g (Glavin *et al.*, 2004). However, only trace levels of nucleobases (~0.05 nmol/g of cells) have been detected using high-performance liquid chromatography at 10^5 cells/g and below.

In the present study, hydrolysis and sublimation were performed both separately and together to extract analytes from bacterial cell samples. The ability to label and detect analytes from all three extraction techniques is significant. A comparison of the different extraction protocols revealed that acid hydrolysis yielded signals \sim 100fold higher than those obtained by the other extraction techniques. In the absence of a highsalt background, direct hydrolysis followed by labeling was thus a viable and sensitive analysis technique. Amino acids, monoamines, and diamines were detected from samples that contained from 10^8 to 10^5 cells. Valine was selected as the best test biomarker because high signals that were well resolved from the other amines and amino acids were observed. Extrapolating from the valine S/N values for 10^5 cells and assuming an LOD of S/N = 3 revealed that approximately 3×10^4 cells would be detectable with a cross injection and $\sim 4 \times 10^3$ cells would be detectable using a 300-ms direct injection.

Biomarker detection sensitivity is perhaps the most critical issue for Mars exploration. While the Viking Gas Chromatograph-Mass Spectrometer (GCMS) was designed for higher sensitivity, its true sensitivity is now thought to have been $3 \times$ 10^7 cells/g of martian soil (Glavin *et al.*, 2001). Benner et al. (2000) suggested that the highly oxidizing soils on Mars produced smaller charged molecules such as mellitic acid that are both nonvolatile and highly polar, which would have prevented biomarker detection by the GCMS instrument (Glavin et al., 2001). In our experiments, the variable terrestrial background contamination prevented a direct determination of our ultimate LOD. However, we have demonstrated an LOD of 10⁵ cells per sample, and the available data suggested an LOD of $\sim 4 \times 10^3$ cells per sample—an \sim 10,000-fold improvement over the Viking GCMS. This extrapolated LOD is expected to be attainable in more sterile environments, such as might be encountered on Mars, where the biomarker backgrounds should be much lower.

The detection of products from abiotic sources, such as meteoritic in-fall and prebiotic synthesis, is also critical for in situ detection of carbon-bearing compounds on Mars. In our analysis of the organic products of a prebiotic synthesis, adenine and alanine/serine were identified, and the percent yields observed were consistent with previously reported results for prebiotic reactions (Oró and Kamat, 1961; Oró and Kimball, 1961; Sanchez et al., 1967). The extraction protocols used were compatible with the field-tested version of the Mars Organic Detector (Kminek et al., 2000). Translating the results observed for adenine and alanine/serine into soil concentrations (assuming a 0.2 g mass), the concentrations detected would be 54 \pm 6 and 1.5 \pm 0.3 parts per million, respectively, assuming a 100% sublimation efficiency. Previous work by Glavin et al. (2002) indicated that the calculated percent yield is within a factor of 10 of the true yield of the synthesis. Detection of the products from a prebiotic synthesis has not been performed previously using a portable instrument designed for Mars exploration.

While we have successfully detected organic biomarkers from biological and abiological sources, the impact of the sulfate, oxidant, and acidic characteristics of the soils recently found on Mars on our detection sensitivity must be considered (Klingelhofer et al., 2004; Squyres et al., 2004a,b; Poulet et al., 2005; Bibring et al., 2006). Fortunately, several Mars simulant soils, such as jarosite from the Panoche Valley, CA, and oxidizing soils from the Atacama Desert in Chile, have been identified. Recent analyses of these soil samples have shown that organic molecules such as amino acids are preserved even in a high-salt matrix (Skelley et al., 2005; Aubrey et al., 2006). Methodologies, such as sublimation and subcritical water extraction, have been developed to provide differential extraction of organic molecules from these soil matrices. These extraction instruments, in combination with MOA analysis, have been successfully demonstrated on both the jarosite and Atacama samples (Skelley et al., 2005; X. Amashukeli and F.J. Grunthaner, manuscript in preparation; A.M. Skelley et al., manuscript in preparation). If biotic, abiotic, or prebiotic amines are present on Mars at ppb levels or better, they should therefore be detectable using the MOA system.

Within the next decade, both NASA and ESA plan to send rovers to Mars to search for organic molecules and signs of life. It is crucial that the instrumentation chosen be capable of sensitively detecting a wide range of relevant biomarker molecules, including those likely to be found as a result of extinct or extant bacterial cells, generated by prebiotic reactions, or deposited by meteorite infall. The results presented here dramatically broaden the classes of target analytes that can be successfully detected with high sensitivity by the MOA. Our analyzer is part of the Urey Instrument package that has been selected for the ESA ExoMars Opportunity (Bada *et al.*, 2005; http://astrobiology.berkley.edu).

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ABBREVIATIONS

AIB, aminoisobutyric acid; AMP, adenosine 3'monophosphate; CE, capillary electrophoresis; CMP, cytidine 3'-monophosphate; ddH₂O, double distilled water; GCMS, Gas Chromatograph-Mass Spectrometer; LOD, limit of detection; MOA, Mars Organic Analyzer; PDMS, polydimethylsiloxane; ppb, parts per billion; S/N, signal-to-noise ratio.

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