DETECTION LIMITS OF BIOMARKERS BY MICRO-BEAM 532 NM LASER RAMAN SPECTROMETRY (LRS), Jie Wei, Alian Wang, Yanli Lu, Kathryn Connor, Alex Bradley, Dept. of Earth and Planetary Sciences and McDonnell Center for Space Sciences, Washington University in St. Louis, St. Louis, MO, 63130, USA, (jiewei@levee.wustl.edu)

LRS, micro-beam green Raman, and CIRS: Laser Raman Spectroscopy (LRS) provides chemical and structural information of a molecule (organic or inorganic), with much sharper spectral peaks than near- and mid-IR spectroscopy. Raman signal strength is proportional to the covalency of major chemical bonds in molecules (table 1), it thus is extremely sensitive to carbon-carbon bonds in kerogen (100% covalency, but forbidden by IR selection rule) and carbon-(H, N, O) bonds in organic matter (>90% covalency). Aromatic groups have their own characteristic peaks in LRS. For these reasons, micro-beam LRS has been a go-to tool, in the past 30 years, to study carbonaceous materials in extraterrestrial samples (meteorites, stardust, martian meteorites, IDPs) [1-5] and recently, to investigate biomarkers in ancient rocks (to ~ 3.5 billion years) [6-7]. Furthermore, photosynthetic micro-organisms were identified using micro-beam LRS in the interior of halite crusts in the Atacama Desert [8]. Carotenoids were identified in ancient (1.44 Ma) halite brine inclusions from borehole cores [9].

Table 1 Relative strengths of Raman scatterers

Inorganic and organic groups	covalency of major bond	as Raman scatters
carbon, sulfur, O_2, N_2, H_2	100%	strongest
perchlorates, nitrates, carbon-(H, N)	92–95%	strong
sulfates, carbonates	80–81%	strong
chromates, phosphates	75–65%	strong
borates, vanadates	59–62%	medium
silicates	51%	medium
(Mg, Fe, Ca)-O	19–31%	weak

However, Raman scattering is an intrinsically weak process. It requires a carefully crafted optical configuration with high optical efficiency in order to collect decent Raman signals from mineral mixtures of complicated natural materials (rocks and regolith with rough grain surfaces) for a planetary (robotic) surface exploration mission. Through the past twenty years' studies and tests in the laboratory and in natural geological settings, we have developed what we consider the best configuration for a planetary Raman system to satisfy the needs of fine-scale definitive mineralogy and biomarker detection, i.e., a micro-beam Raman unit using the simplest, most mature, and optically most effective techniques, e.g., continuous wave, low power 532 nm laser, visible optics, and ordinary CCD, which is the configuration of the CIRS (Compact Integrated Raman Spectrometer) [11].

CIRS is a non-optical-fiber version of the MMRS (Mars Micro-beam Raman Spectrometer) with augmented capabilities. In 2013, the MMRS was field tested in the Atacama Desert on the Zoe rover in its 50 km traverse, and conducted autonomous core sample analysis where reduced carbon was detected in some subsurface samples [10]. A prototype of CIRS was built in 2014 and is undergoing optimization, supported by MATISSE program [11]. One of the goals of CIRS project is to experimentally find the detection limits of potential biomarkers by this micro-beam green Raman configuration, in comparison with other Raman configurations. Here we report the first set of results.

Biomarkers for life detection: One of the major goals in space exploration is to test for evidence of life beyond Earth. From surface investigations of Mars, it has been concluded that Mars once possessed a habitable fluvio-lacustrine environment [12]. Biomarkers are organic compounds with structures and chemistry that are diagnostic for biological processes, particularly those that are preservable under harsh environmental conditions for long periods of time. Ancient terrestrial biomarkers that have survived extensive diagenesis and catagenesis are suggestive of the compound classes that might be most recalcitrant.

If biomarkers were preserved at the surface and upper subsurface in planetary bodies of the solar system other than Earth, the concentration would be extremely low, although their local enrichment at fine-scale cannot be excluded. In order to look for such "needles in the hay stack", analytical tools for molecular phase identification at a fine scale are essential.

As part of the MATISSE-CIRS project, we conducted a series of tests on the detection limits of biosignatures, specifically on two groups that can have long periods of preservation in harsh environments. These include reduced carbon and chemical compounds (biomarkers) that have demonstrated preservation in ancient terrestrial rocks. For this set of tests, we used a laboratory micro-beam 532 nm LRS (Kaiser Hololab 5000, 532 nm, 100 - 4000 cm^{-1}, 10 mW) with performance equivalent to that of the MMRS-CIRS. Biomarker mixtures prepared in these tests will be used for the performance test of CIRS prototype in the fall of 2014.

Similar to all publications from WUSTL Planetary Spectroscopy team that demonstrated the wide planetary applications of laser Raman spectroscopy, the de-

tection limits found in this study are only applicable for *in situ* green Raman system, i.e., MMRS or CIRS type of instrumental configuration.

Detection limit of Reduced Carbon: In living organisms, organic materials typically occur with specific stereochemistry and heteroatom (O, N, S, P) functionalities. Over geological time under harsh environmental conditions, some of these functional groups can get lost and begin to form a complex cross-linked aromatic-carbon dominated moiety called kerogen. Thus the first step to characterize potential biosignatures on Mars is the detection and characterization of kerogen.

The Archean chert sample from West Australia that we used for detection limit testing has a total organic carbon (TOC) of 8 x 10^{-4} w/w [13]. The two characteristic Raman bands of reduced carbon, the G-band at 1581 cm^{-1} and the D-band 1355 cm^{-1} [14], are seen in every LRS spectrum of a 100 point-scan (a line scan without auto-focusing at each point) on the powdered sample (grain sizes < 88 µm).

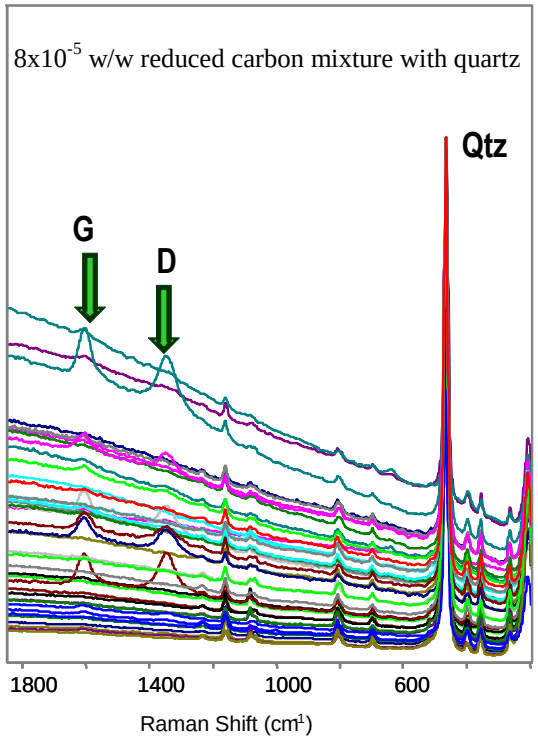


Figure 1 A subset of LRS spectra from a 100-point-scan on a 8x10^{-5} w/w reduced carbon mixture with quartz

We further reduced carbon concentrations by mechanically mixing Archean chert grains with quartz grains of same size range down to 10% and 1% chert concentrations, and then conducted similar 100-point-scans on these mixtures. Figure 1 shows a subset of raw spectra from a 100-spot-scan taken on the mixture of 8 x 10^{-5} w/w of reduced carbon. The two characteristic G- and D-bands of reduced carbons, as marked with green arrows, appear in over 1/6 of LRS spectra of this group. The shape and relative intensity of the two bands reflect the degree of structural disorder and thus the temperatures that these carbon species have experienced. Other peaks in figure 1 are contributed by quartz, with the strongest one at 464 cm^{-1}. On the mixture with the lowest concentration we made, ~ 8 x 10^{-6} w/w, carbon bands appeared in 7 spectra among all the spectra from a 100-spot-scan. These mixing experiments revealed a relatively large Raman cross section of carbon, compared with quartz, and confirmed our estimation of Raman signal strength as shown in table 1.

Table 2 Percentages of LRS spectra showing carbon bands among 100-point-scan on the Archean chert sample and its mixtures with quartz

TOC (w/w)	8x10^{-4}	8x10^{-5}	8x10^{-6}
LRS spectra with carbon G&D bands	100%	14%	**7%**

In comparison, we reported in 2001 a LRS detection of reduced carbon in a South Africa chert sample from Onverwacht subdivision, Swaziland supergroup, Barberton greenstone belt [15]. From an area with an estimated carbon concentration about 50 ppm [16], definitive LRS bands of reduced carbon were observed, which is consistent with current test results.

Detection limits of potential biomarkers: This study was split into two parts: (1) LRS characterization of a selected set of biomarkers; (2) test of detection sensitivity for each biomarker using a micro-beam green Raman system.

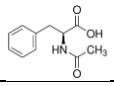
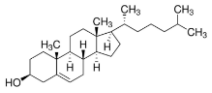
We emphasize that the right selection of biomarkers was the essential basis for this study. Not all organics are generated by bio-activities. Furthermore, many of the biologically generated organic compounds will not survive through long periods of time under harsh environmental conditions. One potentially good set of biomarkers would be those found in ancient terrestrial rocks, but we have to be aware of the difference in environmental conditions on other planetary bodies (e.g., Mars), which may affect the set of biomarkers.

Our selection of biomarkers to be tested has gone through several iterations, table 3 lists four biomarkers that are presented in this study. More candidates are under consideration.

We have reported standard LRS spectra of several studied biomarkers and their LRS peak assignments in another publication [17]. Here we report the results

from a set of experiments to determine their detection limits.

Table 3 Four potential biomarkers in this report

Category	Name	Structure
Amino Acid	N-Acetyl-L-phenylalamine	
Lipids	β-carotene	
	Cholesterol	
Alkanes	Octadecane	$CH_3(CH_2)_{16}CH_3$

In order to determine the lowest concentration of these biomarkers in a mixture that can be sensed by LRS, we made mixtures of these compounds with standard gypsum at 1 mole % first. Gypsum was selected based on several previous studies on biomarker detections [18]. In order to make a homogeneous mixture, we first dissolved a pure biomarker sample into an organic solvent, and then mix the solution with powdered gypsum with particle sizes less than 100 µm. A LRS 100-point line-scan was taken on the mixture, without auto-focusing at each spot. Afterward, we dilute the 1 mole% mixture with the gypsum powder of the same grain size again, to 0.1 mole%, 0.01 mole%, 0.001 mole%, and so on until we obtain no LRS detection of the biomarker in all spectra of a 100-point line scan.

Table 3 Percentages of LRS spectra showing biomarker peaks among 100-point-scan on their mixtures with gypsum

Molar concentration	10^{-2}	10^{-3}	10^{-4}	10^{-5}
N-acetyl-L-phenylalanine	24%	**2-3%**	0	
β-carotene	100%	100%	97%	**10%**
Cholesterol	15%	2-3%	**1%**	0
Octadecane	39%	**2-3%**	0	

Table 3 gives the number of spectra with detectable LRS peaks of each biomarker among the 100 spectra obtained from a linear scan. It shows that octadecane and N-acetyl-L-phenylalanine were detected down to 0.1 mole%, cholesterol was detected down to 0.01 mole%, and carotenoids were detected down to 0.001 mole% due to a Raman resonance effect.

Figure 2 shows a subset of LRS spectra from 100-point scans on mixtures of four biomarkers with gypsum, at the start of the dilution process. These spectra are all unprocessed raw data. The prominent biomarker bands are marked with green arrows. LRS peaks of gypsum in the mixtures occur in two spectral ranges. The two peaks between 3400 and 3600 cm^{-1} are due to the OH stretching vibration of the water moiety in gypsum; and the bands between 400 to 1200 cm^{-1} are due to internal vibrations of the sulfate anion group, of which the strongest one is at 1008 cm^{-1}, the symmetric SO_4 stretching vibration. It is interesting to note that the LRS peaks of biomarker occur in the middle spectral range, aside from the two common LRS spectral ranges that have mineral vibrations. They are also separated into two spectral ranges: C-H stretching vibrations in CH_2 and CH_3 groups dominate the region from 2800 to 3000 cm^{-1}; and the LRS peaks between 1100 and 1650 cm^{-1} are due to CC stretching and CH_2/CH_3 bending or scissoring motions.

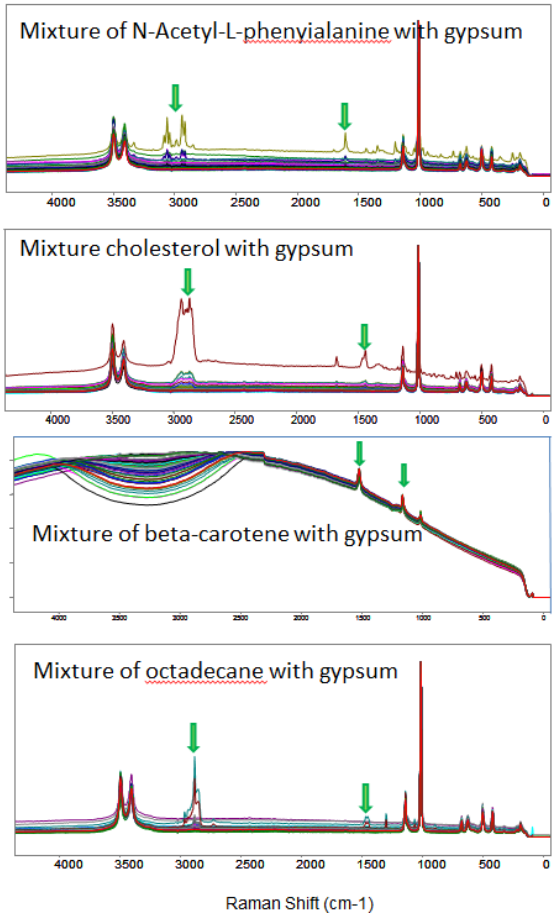


Figure 2. Micro-beam 532 nm Raman spectra of 1 mole% mixtures of 4 potential biomarkers with gypsum. Prominent biomarker peaks are marked with green arrows. Other peaks are mainly due to gypsum.

N-acetyl-L-phenylalanine is one of the potential amino acid biomarkers, which has been detected in meteorites. The N-H stretching produces the peak at the highest Raman shift of 3329 cm^{-1}, which is present on the shoulder of the OH stretching bands of gypsum. A benzene structure is contained in this compound, which produces intense LRS peaks. The peak at 1604 cm^{-1} can be attributed to the ring stretching [19]. The peaks between 3000 to 3100 cm^{-1} are characteristic of aromatic C-H stretching modes, which is higher than the C-H stretching modes in aliphatic CH_2 and CH_3 groups.

Cholesterol belongs to the lipid group that has demonstrated long-period preservation in ancient rocks on Earth. Its Raman spectrum has characteristic peaks at 1672 and 1438 cm^{-1} due to C=C stretching and CH_2 and CH_3 bending vibrations, respectively [19]. CH stretching vibration causes the strong band centered at 2900 cm^{-1}.

Carotenoids are p-electron-conjugated chain molecules. The presence of both intact and diagenetically altered carotenoids in sediments dating back as far as the Miocene has been indicated by GC/MS analysis [8]. The peaks in the Raman spectrum of β-carotene at 1515 cm^{-1} and 1155 cm^{-1} are due to C=C and C-C stretching vibrations [8] .

Alkane-containing molecules are used in biological membranes. As a saturated alkane, octadecane is found very stable in terrestrial ancient rocks. The Raman peak at 1133 cm^{-1} is due to C-C stretching vibration; the peak at 1295 cm^{-1} is CH_2 out-of-plane bending (twisting); and the band at 1458 cm^{-1} is due to CH_2 and CH_3 bending or scissoring motions [20].

Conclusion: Overall, this set of experiments show the detection sensitivity of reduced carbon at 8 x 10^{-6} w/w in chert-quartz mixtures, and of four biomarkers at mole concentrations from 10^{-3} to 10^{-5} mixed with gypsum, by a micro-beam green Raman system.

It is worth noting that a reported detection sensitivity of reduced carbon at $<10^{-3}$ w/w, and of aromatic and aliphatic organics from $<10^{-2}$ to $< 10^{-4}$ w/w by a UV-Raman system [21, 22]. In the detection of reduced carbon and biomarkers, *in-situ* green Raman compares favorably to deep UV-Raman due to the generally lower signal strength of the latter. We have observed over 100 times lower Raman signals from the UV-Raman channel during tests on two commercial Raman systems (Horiba and Renishaw), both have equally optimized UV- and Vis-Raman channels. A commonly accepted reason in the Raman spectroscopy community for the relatively poor Raman signal strength under UV-excitation is the shallow penetration depth of UV radiation into a solid sample, as well as the lower efficiency of many optical components in the UV-spectral range.

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