Miniaturized Raman instrumentation detects carotenoids in Mars-analogue rocks from the Mojave and Atacama deserts

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This study is primarily focused on proving the potential of miniaturized Raman systems to detect any biomolecular and mineral signal in natural geobiological samples that are relevant for future application of the technique within astrobiologically aimed missions on Mars. A series of evaporites of varying composition and origin from two extremely dry deserts were studied, namely Atacama and Mojave. The samples represent both dry evaporitic deposits and recent evaporitic efflorescences from hypersaline brines. The samples comprise halite and different types of sulfates and carbonates. The samples were analysed in two different ways: (i) directly as untreated rocks and (ii) as homogenized powders. Two excitation wavelengths of miniaturized Raman spectrometers were compared: 532 and 785 nm. The potential to detect carotenoids as biomarkers on Mars compared with the potential detection of carbonaceous matter using miniaturized instrumentation is discussed.
1. Introduction

(a) Evaporitic environments and microbial life

Evaporitic deposits within extreme desert environments were found to represent important habitats for microorganisms in conditions of extreme dryness and high solar flux, including UV. According to Warren [1], evaporites are defined as salt rocks that were originally precipitated from a saturated surface or subsurface brine by solar evaporation, comprising a wide range of chemically precipitated salts, including alkaline earth carbonates (for comprehensive information related to evaporites, see [1]). In general, when speaking about extremophilic biota from the evaporitic environment, two main types of such an environment may be described. The first one, which can be called ‘wet’, comprises brines with freshly precipitated salts (solar salterns, salt lakes). The second type is represented by dry salts from extremely arid areas, where the crucial source of water for photosynthesis is airborne moisture absorbed within the salt crust. In this study, miniaturized Raman systems were tested on samples from both types of environment.

Brines and salt lakes are often inhabited by halophilic organisms, even at a salt concentration of halite saturation. It is evident from the salt concentration limits for particular metabolic types presented by Oren [2,3] that just a few metabolic types occur at halite saturation—namely, oxygenic photosynthesis, anoxygenic photosynthesis, aerobic respiration, denitrification (based on laboratory studies on pure cultures) and aerobic methane oxidation (based on study of natural communities). The archaea Halobacterium salinarum is a typical representative of these extremely halophilic microorganisms. This archaea is pink red mainly owing to a C-50 carotenoid pigment, α-bacterioruberin (and its derivatives) [4]. Other pigments responsible for coloration of photoactive haloarchaea are rhodopsins, which act as a light-driven proton pump and enable facultative autotrophic activity under low oxygen conditions. Gypsum is a suitable substrate forming within solar saltern brines of salt concentration varying around 200 g l$^{-1}$. It was found to be colonized by endolithic microbial communities due to optical properties of the mineral which enable the photosynthetically active radiation to penetrate through the matrix and simultaneously protects against harmful UV irradiation [5–8].

The green alga Dunaliella is a typical primary producer in hypersaline environments (see [9]). Certain Dunaliella strains produce extremely high amounts of β-carotene (up to 12%, as a response to a particular dose of irradiation). Cyanobacteria, both unicellular and filamentous, are representatives of prokaryotic phototrophs found within hypersaline systems throughout the world [10–12]. More recently, extremely halophilic bacteria have been also recognized within salt lakes approaching NaCl saturation. The rod-shaped bacteria are now known as Salinibacter ruber [13]. The carotenoid pigments are produced as protective biomolecules under conditions where high dose of irradiation—harmful for organisms—is present.

Even precipitated halite can provide a refuge for microbes in the liquid inclusions within the crystal and can possess conditions for long-term preservation [14–16]. The first isolates of viable microorganisms were described in Permian salt deposits in the 1960s by Reiser & Tasch [17] and Dombrowski [18]. Dombrowski’s results have been confirmed by Bibo et al. [19] under highly controlled conditions to avoid contamination. They isolated extreme halophiles from Zechstein salt cores of Permian age, described as Gram-positive cocci and rod-shaped sporeformers (see the mini review by McGenity et al. [20]). More recently, Vreeland et al. [21] isolated viable halohalobacteria using cellulose from a Permian (250 Ma old) Salado formation (USA). Viable haloarchaea including new species have been reported by Radax et al. [22] and Stan-Lotter et al. [23] in the Zechstein deposits dated Late Permian. Interestingly, spore-forming bacteria (Bacillus) have been extracted and reactivated from inclusions in halite crystals from Permian salt deposits in New Mexico [24]. Although the claimed age of 250 Ma has been questioned [25,26], the real possibility that dormant or even active microbes or their remnants may be preserved in ancient halite rocks is very distinct and has important implications for astrobiology.

Different, more recent scenarios of life adaptations to highly saline conditions can be found in hot desert areas throughout the world. Ancient prokaryotes have been observed microscopically...
by Schubert et al. [27] in ancient halite inclusions from a core drilled 90 m deep in up to 100 kyr old evaporite deposits in Death Valley. Although the microbial preservation was found to be rare, the presence of eukaryotic *Dunaliella* cells within these fluid inclusions in halite was recently confirmed as well as halophilic archaea. Some of the inclusions still contained intact pigment molecules such as chlorophyll and carotenoids [28]. β-Carotene is sensitive to light, oxygen and temperature and easily degrades under their influence. However, it did not show any evidence of degradation in inclusions in up to 34 000-year-old halite crystals studied by Schubert et al. [28], which was interpreted as being a result of the low oxygen environment of a fluid inclusion and dark conditions as no sunlight reached the buried halite deposits. More recently, carotenoid pigments dated from 21 ka to 1.44 Ma were detected using Raman spectroscopy within inclusions in halite from drill cores in Death Valley, Saline Valley and Searles Lake, Mojave Desert, CA, USA [29].

Salt-crusted pans and playas (locally known as salars) occur in depressions in the semiarid to hyperarid regions of the central Andes and the Atacama Desert. These are formed by massive halite crusts (deepest parts of the crust in Salar Grande, Chile are more than 160 m thick) [1,30]. Location between two mountain ranges prevents the moisture from the coast from approaching the central regions of the Atacama Desert resulting in extreme dryness in this area. Wierzchos et al. [31] have shown that halite can provide a suitable habitat for cyanobacteria of the genus closely related to the *Halothecae* [32] in the hyperarid core of the Atacama Desert, which is one of the driest areas on Earth with mean annual rainfall less than 2 mm yr$^{-1}$ [33]. Endolithic (endoevaporitic) colonies have been observed in pore spaces within the halite crust by a variety of microscopic techniques. Soils in this hyperarid region contain almost no bacteria, as documented by DNA amplification as well as examination of culturable bacteria [34]. Even hypolithic (living below the surface rocks) organisms are rare and exist in small isolated patches in this region [35]—so why is there abundant life in halite? The reason lies in great part in halite deliquescence—the hygroscopic properties of halite enable water to condense within the pores during periods when the relative humidity exceeds 75%. Consequently, liquid water becomes available for endolithic microbial communities owing to the deliquescence process [36] and owing to capillary condensation within the nanopore network present in halite [37]. Raman spectroscopic analysis was performed on these halite-inhabiting cyanobacterial colonies [38] confirming the presence of scytonemin and photosynthetic pigments. Gypsum has also been found to harbour life within the hyperarid zone of the Atacama Desert [39].

The above-described environments represent extreme environments on Earth and can be in some ways important analogues for Martian scenarios. Although these modern terrestrial habitats are more or less wetter and warmer than present-day Mars where photosynthetic life is unlikely, the evaporite deposits on modern Mars are thought to have arisen during a warmer, wetter periods in its past, when halophilic phototrophs could have occurred. The possibility of preservation of halophilic organisms throughout geological times strengthens probability of the hypothesis about microbial life in evaporitic environment as a model system for the search of life on Mars [40].

Deposits of evaporitic minerals (sulfates, chlorides) have been widely observed to date within the Martian surface by both orbit-based measurements [41–44] and by *in situ* analyses by robotic missions (see the references below). Sulfate minerals have been identified in outcrops as well as soils on Mars, analysed within the Mars exploration rover missions Spirit and Opportunity in two distinct areas on Mars—Gusev crater and Meridiani Planum, respectively [45,46]. Mg, Fe and Ca sulfates, including jarosite, occur within the studied outcrops at different localities studied by Opportunity (see [47,48]). The later findings of sulfate deposits on Mars have been recently supported by Curiosity rover detecting sulfate salts within bedforms in Gale crater [49].

(b) Raman spectroscopy of microbial pigments in evaporites

There are different approaches to detecting microbes in the frame of unknown samples of rocks, and the review of these is not a purpose of this paper. The presence of pigments of different
origin can be followed in the frame of native colonizations or cultivated microorganisms with advantage using Raman spectroscopy (see mini review by Jehlička et al. [50]). The advantage consists of the fast and direct mode of analysis of the biomass or stone colonization. In addition, resonance Raman signal enhancement is encountered in the case of carotenoids when using approximately 500 nm excitation. Raman spectra of bacterioruberin, the typical carotenoid of *Halolarchaea*, were obtained on different members of the group [51,52]. In another case of salinixanthin from *S. ruber* Raman spectra indicate that there are few structural differences [52]. Other studies also demonstrated in laboratory conditions that benchtop instrumentation is able to detect biomolecules associated with the extremophilic endolithic colonization of rocks [38,53–60].

Such geobiological systems provide model terrestrial environments as analogues for those that may have been present on Mars. Raman spectroscopy is one of the analytical techniques to be employed in the ExoMars mission now scheduled to depart in 2018. This will have a specially constructed miniaturized Raman system for *in situ* robotic measurements on Mars. It is therefore important and relevant for future space missions to evaluate miniaturized Raman spectrometers within Earth scenarios for the detection of molecular traces of life.

A few studies have been published dealing with portable Raman systems in the frame of detection of biomarkers associated with extremophiles. Dickensheets et al. [61] presented some preliminary results that were obtained with a miniaturized Raman system with 852 nm excitation for the detection of biomolecules associated with the yellow Antarctic lichen *Acarospora chlorophana*. Vítek et al. [62] applied 1064 nm excitation in a miniaturized system for the detection of biosignatures of the same epilithic lichen species and for a preliminary survey of endolithic microbial communities in halite pinnacles from the Atacama Desert. Recently, lichens from desert environment were studied by a miniaturized Raman system by Miralles et al. [63].

The saltern ponds near Eilat (Israel) were used for such kind of study [64]. Bottom gypsum sediments of some of the crystallizer ponds contain coloured layers which are zones of colonization by halophiles. A portable Raman spectrometer with a 532 nm excitation laser was used for fast screening of pigments in the field; this light handheld instrument provided acceptable quality spectra to be obtained quickly outdoors. Raman bands of carotenoids were obtained and interpreted as echinenone- and spirilloxanthin-like carotenoids.

It has also been demonstrated that the miniaturized Raman instrumentation is capable of detecting clear carotenoid signals in natural halite from extremely dry desert areas, namely endolithic cyanobacterial colonies from the Atacama Desert. Carotenoids were identified using the 532 nm laser for excitation in both untreated halite sample and powdered ‘colonized’ zone of the halite crust [65]. Moreover, the 785 nm excitation wavelength on the handheld portable Raman spectrometer was proved to be capable of detecting scytonemin within the same type of samples, although here the time-consuming (of the order of minutes) positioning of the system was necessary to achieve some spectral features of this cyanobacterial UV-screening compound.

In fact, carotenoids are exceptional pigments in Raman spectroscopic analysis of microbial communities because they (i) have an extremely strong Raman signal even in a non-resonant mode and (ii) are widespread in microbial communities and are present in all phototrophic microorganisms. The strong coloration of carotenoids (from yellow to black) is due to the high electric dipole transition moment of the $\pi-\pi^*$ electronic transition resulting in absorption in the visible range of the electromagnetic spectrum. The colours are dependent on the number of the double bonds in the polyene chain—more conjugated C=C double bonds cause darker coloration [66]. The Raman signal of carotenoids can be significantly enhanced by using the proper excitation wavelength which coincides with the absorption band of an allowed $\pi-\pi^*$ electronic transition, resulting in the resonance Raman effect [51,66–68].

Owing to the resonance Raman effect, generally, the most favourable excitation wavelength for the identification of carotenoids lies around 500 nm (e.g. 488, 514.5 nm) [51,66]. However, for identification of a broader range of organic molecules, 785 nm is an ideal compromise excitation wavelength [69,70] with still relatively high sensitivity towards carotenoids [71].
Carotenoids have two strong Raman bands owing to in-phase $\nu_1$(C=C) and $\nu_2$(C−C) stretching vibrations of the polyene chain \([67,68]\). The Raman spectrum of $\beta$-carotene—a typical carotenoid—is characterized by the $\nu_1$ band located at 1515 cm\(^{-1}\) and $\nu_2$ band at 1157 cm\(^{-1}\). A feature of medium intensity occurs at 1008 cm\(^{-1}\), corresponding to the in-plane rocking modes of the CH\(_3\) groups attached to the polyene chain. The wavenumber positions of both $\nu_1$ and $\nu_2$ bands are dependent on the length of the polyene chain (number of conjugated double bonds). The shift in the band position is much more pronounced in the case of the $\nu_1$ band. A longer polyene chain causes a shift in the $\nu_1$ band to lower wavenumber positions and vice versa.

As described by de Oliveira et al. \([72]\), carotenoid bonding within the biomass which affects the main polyene chain can cause a significant shift of the $\nu_1$ band position owing to the change in electronic delocalization. This means that unambiguous identification of particular carotenoids in complex organic material on the basis of Raman spectra alone may not be possible. Nevertheless, although careful interpretation is necessary, information about the carotenoid band positions can still be useful for investigation of rock-inhabiting phototrophic organisms. Other factors affecting the band shifts in carotenoid Raman spectra are substitution related to molecular termination (which results in a very small wavenumber change), isomerism and molecular conformation in solid and liquid state, respectively (see \([66,69]\) and discussion in \([72]\)). Moreover, the $\nu_1$ band position of carotenoids depends on the laser wavelength used for excitation \([73,74]\).

Here, two miniature Raman spectrometers have been tested to detect biomolecules associated within the native specimens of different types of evaporitic rocks from desert environments. Two excitation wavelengths were used, namely 532 nm, which is that adopted for the laser used in the ExoMars RLS Raman spectrometer, and 785 nm which was used with good performance of the portable counterparts for identification of inorganic mineral phases as shown for example in Jehlička et al. \([75]\) or Vítek et al. \([76]\).

2. Material and methods

(a) Samples and sites

Samples from localities in two different deserts were analysed, namely the Mojave Desert and the Atacama Desert. The samples contained distinct forms of microbial colonization, which is in some cases known from earlier studies and some of them not (figure 1). In such a case, a basic documentation was undertaken using optical microscopy. X-ray diffraction analysis was performed in the cases where the mineralogical composition was not possible to unequivocally recognize visually or by Raman spectroscopy.

(i) Mojave Desert

Deep Springs Lake. The area where the composition of the evaporitic crust from surface to shallow subsurface was studied was Deep Springs Lake, a small sulfate–carbonate-rich playa, about 13 km\(^2\) in area (37°16.450’ N; 118°02.764’ W), which is situated in Deep Springs Valley, east of Owens Valley. The groundwater is fed by many springs surrounding the playa, some of which are artesian. Seasonal lake formation occurs within the wet period of the year, drying in summer to form salt crusts with horizontal zonation from playa margins to the centre: calcite/aragonite–dolomite–gaylussite–thenardite–burkeite; and vertical zonation from lower to upper layers: carbonate muds–nahcolite–thenardite–burkeite–trona–halite + sylvite \([77,78]\).

Three different types of samples were collected in July 2012, namely two crusts of thenardite of different origin—one from the northwestern edge of the lake forming an approximately 0.5 cm thin surface cover (DSL1), the second originates from the central parts of the playa from an approximately 20 cm thick massive subsurface layer under a saline mud and surface crust (DSL3h). The third sample comes from the same part of the lake, but rather from the surface crust, dominated by burkeite (DSL3).
Owens Lake. Surface and shallow subsurface (approx. 15 cm deep) evaporitic efflorescence deposits of Owens Lake were also studied (36°21.935′N; 117°57.757′W). The lake is located in the southern part of Owens Valley, eastern Sierra Nevada, California. Until 1924, the lake had a permanent body of water. However, since that time much of the Owens River was diverted to the Los Angeles Aqueduct resulting in lake desiccation. Today, restoration efforts have slightly enhanced the water inflow into the lake, which now contains small brine ponds. The lake deposits are formed by a sequence of evaporitic minerals rich in Na and Ca (magnesium salts are very rare), interbedded fine-grained sands, silts, clays and lacustrine limestone. Roughly, the brines dominate in Na–Ca cations and Cl–HCO₃ anions, with minor to moderate sulfates also resulting in precipitation of the following most common evaporitic minerals: halite, natron, thenardite, mirabilite, trona, burkeyite, nahcolite, gaylussite and pirssonite [79]. The lake is divided into individual ponds by artificial boundaries and is affected by water level manipulation. This results in different conditions (actual salinity, chemical composition of the brines) occurring within individual ponds. Recently precipitated salts from different ponds were collected in July 2012 (samples OWL2, OWL4, OWL6, OWL7, OWL8). Some of the brines are rich in Dunaliella sp., causing green to orange coloration; the deep purple layers are supposed to be dominated by anoxyogenic phototrophs.
Searles Lake. Searles Lake in Searles Valley is located south of Owens Valley in the Mojave at approximately 35°45'35.28''N; 117°20'7.08''W. It is an alkaline lake with brines approaching halite saturation (see [80]). The samples of halite were collected from the surface parts of the lake in 2012 (samples SL1 and SL4). The samples have been obtained from Arzell Hale (Searles Valley Minerals Co., Trona, CA, USA) and Ray Ramirez (Los Angeles Department of Water and Power, Keeler, CA, USA).

(ii) Atacama Desert

Gypsum crusts. Samples of Ca–sulfate crusts were collected in May 2011 from the Atacama Desert from a sampling site designated JH with site coordinates of 20°43'56.9''S; 69°58'30.2''W. This location lies within the Central Depression in the hyperarid core of the Atacama Desert and is separated by the Coastal Cordillera from the Pacific coast. More detailed information about the microenvironmetal parameters can be found in Wierzchos et al. [39]. The crusts were mainly composed of gypsum with some anhydrite [39] and were randomly distributed through the surface terrain. They form small features about 10 cm in diameter and 0.5–5 cm in thickness with smooth upper and irregular lower surfaces. The irregular topography of the bottom surface and concave shapes of the gypsum crust features mean that the gypsum crusts are not in direct contact with the underlying soil, hence allowing light penetration in some cases to the lower surfaces of the crust. The gypsum contains algal and cyanobacterial endolithic and hypoendolithic colonization layers [39]. Detailed Raman data obtained by Raman microspectrometry are presented in [60].

The second sample of gypsum comes from a location tagged BH (24°05.252' S; 69°59.674' W) and according to former microscopic and Raman studies was considered to be lacking in an autotrophic microbial colonization. Hence, this sample was considered as a blind sample.

Salar Llamara ponds. The Salar Llamara is located within the Central Depression in northern Chile (21°13'00''S; 69°40'00''W). The ponds there are representatives of perennial water bodies. We have studied gypsum precipitated in the form of monocrystals approximately 5 cm in length, collected in May 2011 (sample A-LG). The crystals contain entrapped microorganisms as inclusions and also degraded biomass inside the crystal and also on the crystal surface. According to the works of Escudero et al. [81] and Demergasso et al. [82], these represent unicellular cyanobacteria. The microscopic observation showed a relatively ‘patchy’ distribution of cells inside the gypsum. Standard point Raman microanalysis and Raman imaging has shown signatures of pigments related to photosynthetic apparatus and within some of the aggregates also the presence of scytonemin, a UV protective pigment (P. Vítek et al. 2012, unpublished data).

(b) Sample preparation

In general, two different types of samples have been studied regarding the distribution pattern of microbes inside the mineral matrix. The first of these is a spatially well-defined colonization layer formed by phototrophic microorganisms occupying pore spaces near the sample surface, which is typical for the studied gypsum crust from the Atacama Desert. In this case, the analysis was focused on the colonization layer or the piece of rock containing the colonization layer (powdered samples). The samples from the wetter and dynamically evolving evaporitic systems from the Mojave Desert, on the other hand, contain a significantly different pattern of microbe distribution. In this case, they are typically entrapped within crystals of evaporites forming spatially more pronounced pigmented zones. In such a case, a typical zone with average pigmentation was selected for analysis within the particular sample. First, these three zones were analysed directly on the sample, without any further pre-treatment. Second, the parts of the mineral matrix that contained the colonization zone were separated. The separated parts were crushed, powdered and homogenized in an agate mortar. The flattened surface of the powder was then measured at randomly selected positions in three replications under optimized settings of the spectrometer. The sample crushing and powdering is a part of the
sampling protocol that is going to be applied within the ExoMars mission, where the Raman spectroscopic analysis will be a first-pass interrogation of powdered/crushed rock or drill core samples.

(c) Artificially prepared mixtures for testing the limits of detection of the miniaturized equipment

Samples were prepared by mixing separately weighed amounts of synthetic β-carotene (Sigma–Aldrich, approx. 95%) with a commercially obtained crystalline powder of halite (NaCl; Merck). The mixtures were pulverized and homogenized in an agate mortar. Powders of four different concentrations of β-carotene were prepared —0.1 g kg⁻¹, 1 mg kg⁻¹, 0.1 mg kg⁻¹ and 0.05 mg kg⁻¹. The flattened surface of each powdered sample was analysed soon after preparation to avoid β-carotene decomposition. Measurements were performed at different spots to ensure reproducibility. Graphite and shungite were mixed separately with gypsum in a similar manner in weight concentrations of 100, 10, 1 and 0.1 g kg⁻¹. The measurements using the miniaturized Raman system with a 532 nm laser for excitation (see below) were undertaken in order to compare limits of detection for carotenoids and carbonaceous matter.

(d) Raman instrumentation

The portable Raman spectrometers Advantage and Rock Hound by DeltaNu (Laramie, WY, USA) were employed for this study. The Rock Hound is a handheld portable instrument (1.9 kg) equipped with a 785 nm diode laser for excitation (with a maximum output power of 120 mW) and a thermoelectrically cooled charge-coupled device detector (with an operational wavenumber range of 200–2000 cm⁻¹ and spectral resolution of 8 cm⁻¹). The Advantage 532 is a compact Raman spectrometer that measures 12" × 8" × 4" in size and weighs 9 kg. Although miniaturized, the Advantage 532 is not a handheld unit as is the 785 nm system. It is equipped with a 532 nm frequency doubled Nd:YAG laser that has a maximum laser power of 100 mW and a thermoelectrically cooled charge-coupled device detector, with an operational wavenumber range of 200–3400 cm⁻¹. The spectral resolution of the instrument is 10 cm⁻¹, which is slightly lower than that planned for the ExoMars Raman instrument (6–8 cm⁻¹). Both instruments are equipped with NuSPEC software, which permits control of spectrometer functions, namely laser power level (five steps), exposure time, the number of spectral accumulations acquired and the spectral resolution. The exposure time (up to 30 s) and laser power were optimized for each individual sample to obtain optimal signal-to-noise (S/N) ratio and simultaneously avoiding sample destruction and oversaturation of the detector. Spectra were recorded as single scans. All spectra are baseline corrected, but no other spectral treatments were undertaken.

3. Results

Raman signals from biomarkers have been detected within five different evaporitic mineral types: halite (NaCl), gypsum (CaSO₄ · 2H₂O), thenardite (Na₂SO₄), burkeite (Na₆(CO₃)(SO₄)₂), trona (Na₃(CO₃)(HCO₃) · 2H₂O) (table 1). The organic Raman bands were detected in 14 of 16 samples, 12 of them showed signal stable through replications and for both analytical approaches—direct measurement on the rock and the analysis of powdered, homogenized samples. The unique biomarkers detected were carotenoids, except the gypsum sample JH, where weak signs of chlorophyll together with carotenoids were recognized by the 785 nm laser (see below). Typical Raman bands of carotenoids owing to the ν₁(C=C) and ν₂(C=C) stretching vibrations were registered at wavenumber positions 1504–1523 cm⁻¹ and 1148–1154 cm⁻¹, respectively (figure 2). The detection of these two bands allows the unequivocal detection of a carotenoid molecule. A corroborative feature of weak-to-medium intensity around 1004 cm⁻¹, δ(C=CH₃), was observed.
in nine of 12 measurements, where other carotenoid bands were detected. In some cases, even weaker carotenoid bands were observed (table 1).

A blind sample was also analysed, represented by gypsum from the Atacama Desert (BH) where no pigmented microbial colonization was observed by optical microscopy and no biomarker signatures were detected by laboratory Raman microspectrometry, either by nucleic acids detection using specific staining and fluorescence microscopy (J. Wierzchos et al. 2011, unpublished data). No biomolecular Raman signal was registered during replicated measurements of this sample both powdered and rock.

(a) 532 versus 785 nm excitation

Important differences have been registered between the two spectrometers equipped with 532 nm and 785 nm excitation sources, respectively. The above-described carotenoid Raman signal was reliably detected using the spectrometer with 532 nm laser line. By contrast, the 785 nm handheld portable instrument did not successfully detect any biomolecular Raman signal, except gypsum.
<table>
<thead>
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<th>locality</th>
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<th>pigmentation</th>
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<td></td>
<td></td>
<td></td>
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<td>Searles Lake</td>
<td>SL1</td>
<td>halite</td>
<td>purple</td>
<td>P: 1504s, 1284w, 1192vw, 1148s, 1004m, 957vw</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: 1506s, 1286w, 1148s, 1002m</td>
</tr>
<tr>
<td></td>
<td>SL4</td>
<td>halite</td>
<td>purple&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P: 1505s, 1278vw, 1148s, 1003m</td>
</tr>
<tr>
<td></td>
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<td>R: 1506s, 1286w, 1148s, 1003m</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>green&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P: 1512m, 1151m, 1005vw</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: 1510w, 1152vw</td>
</tr>
<tr>
<td>Atacama</td>
<td>JH site</td>
<td>JH</td>
<td>green</td>
<td>P: 1523w, 1153w</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>R: 1517s, 1153s</td>
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<td>Llamara pond</td>
<td>A-LG</td>
<td>gypsum</td>
<td>brown</td>
<td>P: n.a.</td>
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<td>R: 1514s, 1154s, 1006mw</td>
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<tr>
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<td>BH</td>
<td>gypsum</td>
<td>no</td>
<td>P: n.a.</td>
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<td>P: n.a.</td>
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</table>

<sup>a</sup>The mineral identification is based on optical recognition where possible and Raman analysis, supplemented with X-ray diffraction in the case of ambiguous Raman data.

<sup>b</sup>Visible zonation with layers of different pigmentation.

<sup>c</sup>Data obtained using 532 nm laser; P, bands obtained on powders; R, bands obtained on rocks; s, strong; m, medium; w, weak; vw, very weak; vvw, very very weak; corroborative bands of carotenoids in italics.
sample JH. This sample represents an endolithic colonization from extreme dry Atacama Desert in the form of condensed green layer close to the sample surface. In that case, bands of carotenoid (1527, 1154 cm$^{-1}$) and also chlorophyll (1326, 915, 747 cm$^{-1}$) were registered. Raman features of pigments were detected only after positioning and focusing on parts with high density of algal colonization. In fact, this was the only case in this study when organic Raman bands were registered also using the 785 nm laser. No bands owing to organics were detected within the powdered JH sample. On the other hand, the 785 nm laser showed better S/N ratio for the detection of sulfate/carbonate mineral matrix in the cases when the fluorescence background was at reasonable level (figure 2).

A strong fluorescence background was registered when employing the 785 nm laser for analysis of pigmented crusts from the Mojave salt lakes. In fact, this is considered to be the main reason for no carotenoid Raman bands being detected within these samples using this laser wavelength. The spectra achieved by the spectrometer equipped with the 532 nm excitation source also have fluorescence background (see the comparison of the raw and baseline-corrected spectra for the both laser lines in figure 3). Nevertheless, its intensity is lower compared with that observed at 785 nm, which together with the attendant resonance Raman enhancement of the carotenoid Raman signal allowed the detection of carotenoids to be accomplished successfully in the majority of the samples. The strong fluorescence background is responsible for the noisy spectra without any Raman features depicted in figure 2 (785 nm). During these measurements, very short acquisition times had to be used to avoid oversaturation of the detector by a fluorescent background.
Figure 4. Raman bands of carotenoids (grey bands) detected in burkeyite from Deep Springs Lake (a) and halite from Searles Lake (b). Three replications are depicted as obtained on powder (upper three spectra) and directly on the rock (lower three spectra).

(b) Rocks versus powders

The Raman features of carotenoids were typically detected stable within at least three replications when applying both analytical approaches—namely the measurement performed on the powdered sample and the direct measurement on the rock (without any treatment) (figure 4). The exceptional sample from this point of view is a gypsum monocrystal from the Llamara ponds (see below). However, the registered S/N ratio in the observed spectra varied significantly in some other cases between the two analytical approaches.

In figure 5, the Raman spectroscopic signal as obtained from massive thenardite from Deep Springs Lake can be observed. The thenardite was sampled from approximately 30 cm depth beneath the saline mud and thin surface evaporitic crust. The pale purple pigmentation, varying in intensity, can be observed within the sample. The Raman features obtained from the typical pigmented zone reveal the presence of carotenoids (1510 and 1151 cm$^{-1}$). The carotenoid signal is stable through the replications and was observed in both powdered and hard rock samples, but the S/N ratio is higher in the case of the hard rock. The same situation was observed in the case of gypsum crust from the HJ site in the Atacama Desert (figure 6a) and is also confirmed by the measurement of the same gypsum using the 785 nm laser.

The gypsum sample from the Llamara ponds in the Atacama Desert has a different origin compared with the gypsum crust at JH. In Llamara, the gypsum is crystallized from a brine within the ponds. Gypsum crystals of length about 5 cm were formerly subjected to fluorescence microscopy and Raman microspectrometry. Autofluorescing pigments have been observed and also Raman spectroscopic measurements revealed the presence of carotenoids and other pigments entrapped in the gypsum crystal. However, as documented by the fluorescence microscopy, the presence of microbial cells is not ubiquitous. They are very scarce and ‘patchy’. We assume that this is the reason why we have obtained inconsistent Raman signals using the miniaturized...
Figure 5. Raman bands of carotenoids (grey bands, C) as detected in thenardite from Deep Springs Lake. Three replications are depicted as obtained on powder (upper three spectra and) directly on the rock (lower three spectra). The discriminations of the carotenoid Raman signal from the bands of thenardite are shown on the right.

Figure 6. Carotenoid signal (grey bands) from two distinct gypsum samples from the Atacama Desert—gypsum crust (JH) (a) and brine precipitated monocrystal from Llama ponds (b). The role of distribution pattern of organic matter in the rock is shown on these samples and is discussed in the text.
instrument. No Raman spectroscopic signals owing to pigments have been observed in the case of powdered material. For the direct analysis on the crystal, the miniaturized Raman spectrometer detected clear carotenoid Raman features only in one of the three replications (figure 6b).

(c) Spectral discrimination of different carotenoids

The spectral differences between different carotenoids are most significantly reflected by the wavenumber position of the band corresponding to the \( \nu(C=\!C) \) vibration in the region between 1480 and 1550 cm\(^{-1}\).

This carotenoid feature varied in measurements from 1504 to 1523 cm\(^{-1}\), and the position was stable through replications with differences up to 2 cm\(^{-1}\) in the majority of the spectra possessing strong corroborative bands. By contrast, relatively larger variations in wavenumber position of the band within identical samples might be observed in the case of weak or very weak band intensities.

(d) Limits of detection—carotenoids versus carbonaceous matter

The limits of detection have been studied using the same analytical approach for \( \beta \)-carotene, as an example of an important biomarker as selected in this study. To compare Raman spectroscopic detection of a carotenoid (intact biomolecule) and eventual altered biomass which has undergone metamorphic processes, carbonaceous matter represented by graphite and shungite were analysed in the same manner. The lowest concentration level of 0.1 mg kg\(^{-1}\) was successfully detected by observation of \( \nu(C=\!C) \) band of \( \beta \)-carotene in the gypsum matrix using the instrument with 532 nm excitation (figure 7a). By contrast, the lowest concentration allowing the detection of the graphite G-band and shungite G- and D-bands was 1 g kg\(^{-1}\) (figure 7b,c).

4. Discussion

This study follows our earlier results, where halite from the hyperarid zone of the Atacama Desert was studied using an identical analytical approach [65].

The fact that 785 nm excitation used with the miniaturized Raman system is good for the detection of evaporitic minerals, but not favourable for detection of carotenoids compared with 532 nm system confirms our previous experience [65,76].

Higher levels of fluorescence background observed sometimes during the analysis of powders compared with hard rocks were noted. We interpret this observation as a possible result of dispersion of the fluorescent agent, which is not normally associated with the cells in the powdered mixture. It resulted in the lower exposure times for the case of powders in contrast to hard rock samples. This difference of exposure time was required to avoid oversaturation of the detector during higher exposure times.

There is another possible reason for the higher S/N ratios observed in some cases of direct measurement on the rock which is the role of the distribution pattern of microbial colonization inside the rock. In the case of the gypsum crust from the Atacama Desert (sample JH), the colonization (mostly algal) forms a thin layer just beneath the sample surface (see [39,60]). In such a case, the measurement performed directly on the condensed colonization zone may be beneficial rather than in contrast to its homogenization together with the surrounding mineral matrix when powdered sampling has been undertaken. This is true for both excitation wavelengths.

It is now being realized that the assignment of an exact carotenoid to the observed Raman spectrum is hard to achieve owing to different parameters affecting the positions of the bands, especially the \( \nu(C=\!C) \) band [72]. Nevertheless, the precision of the \( \nu(C=\!C) \) band position obtained in this study was high enough, when reasonable band intensity is achieved, to permit reasonable suggestions about the types of carotenoids to be made at least in terms of the number of conjugated double bonds in the main polyene chain.
Figure 7. Spectra at various concentration levels pointing to limits of detection for β-carotene in gypsum (a), graphite in gypsum (b) and shungite in gypsum (c) as obtained by miniaturized Raman spectrometer equipped with 532 nm laser.

(a) Carotenoids versus diagenetically altered organic matter

The studied samples represent rocks with more or less recent microbial colonization. Carotenoids in those samples are biomolecules that did not undergo any alteration process. From Earth, we know that carotenoids are vulnerable towards oxidation and photodecomposition during senescence (see [83]). However, they may be still preserved in dark conditions with no input of oxygen (see [28,29]). The oldest intact carotenoids were identified in sediments of the Miocene age, whereas diagenetically altered carotenoids are reported from much older sediments and petroleum (see review by Sinninghe Damsté & Koopmans [84] and references therein).

The quantification of carotenoid pigments in native rock samples detectable by miniaturized Raman systems remains a task for future research. However, from our earlier studies [71,85], it is evident that Raman spectroscopy is very sensitive towards carotenoids even using a laser wavelength that is out of resonance (785 nm) and even much more when the resonance Raman effect plays a role (e.g. using excitation wavelengths near 500 nm). In that case, the $\nu_1(C=C)$ carotenoid band was detectable at 0.1 ppm concentration level; however, the signal was not stable through the replication measurements using laboratory Raman microspectrometer. It was also found that portable Raman systems may benefit from a relatively large laser spot-size, enhancing the measured sample volume [86] and that such a setting may be advantageous when fine-grained powders are analysed. Here, this phenomenon is confirmed using a portable Raman system with 532 nm laser for excitation. The lowest concentration level of 0.1 mg kg\(^{-1}\) of β-carotene in gypsum matrix (figure 7) was achieved with the signal being stable through replications. Hence, this performance is even slightly better than obtained with a benchtop Raman microspectrometer [85]. For a detailed discussion about detection limits in Raman spectroscopy of solid mixtures, see Vandenabeele et al. [87].

Preservation of intact biomarkers known from Mars analogues from Earth is one of the possible scenarios taken into account when considering the search for life in Martian rocks. However, if carotenoids have ever been biosynthesized by some form of microorganism on Mars, then one should target relevant rocks from positions where such molecules could have survived. Moreover, on Mars, galactic cosmic rays and solar cosmic rays play an important role in the degradation of organic molecules near the planetary surface. Owing to the dosage of the cosmic irradiation, the models by Pavlov et al. [88] suggested that organic molecules with masses greater than 100 amu would be effectively destroyed in less than one billion years in the top 5 cm of the Martian rocks. In addition, cosmic rays may produce oxidative radicals which may further increase the rate of
degradation of organic molecules. Therefore, to search for traces of life on Mars in the form of intact organic molecules, the sampling depth should be at least 2 m, or the rock should be sampled from a fresh outcrop [89].

Another scenario for possible presence of remnants of former microbial life is represented by microfossils in the form of carbonaceous matter. On Earth, putative microfossils have been discovered from cherts as old as 3.5 to 3.0 Ga in Pilbara, Australia [90–92], and the penecontemporaneous Swaziland Supergroup, South Africa [93–95]. The biomolecules associated with the original biota have been thermally degraded during metamorphism of these rocks which spans to prehnite–pumpellyite to greenschist facies.

The biogenic character of the precursor is difficult to be documented unambiguously in archean rocks using Raman spectroscopy, although such an attempt has been made by some authors. Others consider Raman spectroscopy as a technique which does not allow the direct unambiguous determination of the biological or abiological nature of the precursor of a carbonaceous residue (see [96,97]). However, the discussion about biogenicity of these structures is beyond the scope of this paper and has already been comprehensively described by Marshall et al. [96] with emphasis on Raman spectroscopic analysis. Here, we point out the potential of detection of such carbonaceous matter, whatever biogenic or abiogenic origin.

More recently, new results confirmed the potential of portable Raman systems to detect traces of fossilized carbonaceous matter dispersed in silica-rich stromatolites. In the dark laminae of a stromatolite of Ordovician age, Olcott Marshall & Marshall [98] recorded Raman spectra of carbonaceous matter. They compared the 532 and 785 nm lasers for excitation and conclude that the near infrared laser wavelength could be recommended owing to the possibility to record spectra with low fluorescence.

The limits of detection achieved in our study here simulating the analytical protocol planned for the ExoMars mission (e.g. analysis of powdered and homogenized samples) for graphite and shungite in gypsum using the miniaturized Raman spectrometer with 532 nm laser (1 g kg\(^{-1}\)) are four orders of magnitude higher for both materials compared with \(\beta\)-carotene. Although the limitations have to be taken into account about the possible preservation potential of relatively unstable molecules, like carotenoids on Mars, the polyene structure causes a unique sensitivity of Raman spectroscopy towards these pigments. This allows their detection in significantly lower concentration in the bulk rock matrix compared with carbonaceous matter.

5. Conclusion

A miniaturized Raman system has been used to successfully detect molecular traces of life (biomarkers) in the geological materials from extreme Earth desert environments. These rocks, namely evaporites (sulfates, carbonates and halite), are highly relevant for Martian scenarios.

Carotenoids have been the only biomarkers ubiquitously detected within this study using a 532 nm laser for excitation. This laser wavelength proved to be successful in the detection of these carotenoids in native desert samples. No organics have been detected using a 785 nm laser within this study, except very rich parts of condensed colonization layer in gypsum from the Atacama Desert (sample JH).

The distribution pattern of organics in rock plays an important role during Raman spectroscopic analysis. Hand in hand, the analysed sample volume controlled mainly by the laser spot-size is crucial.

The results obtained here have important implications for the analytical potential of future robotic Raman equipment which is going to be used on Mars. The following points are especially important regarding the possible detection of molecular traces of life on Mars:

— Carotenoids, examples of extremely good Raman scatterers, can be detected in evaporites from desert environments containing microbial colonization which can be recognized macroscopically through visible pigmentation.
— A broad variety of evaporitic rocks contained microbial life with its carotenoid compounds detectable by miniaturized Raman spectroscopy.
— The detection of such carotenoid signals in these natural samples may be achieved without any special handling and proved to be stable through replicated measurements. This is true especially for the powdered and homogenized samples and also for the direct analyses on the rocks containing relatively homogeneous spatial distribution of microbial cells.
— On the other hand, the original spatial distribution of microbes within the rocks is lost through homogenization and this may cause a decrease in the intensity of the Raman signal compared with the direct measurement of the colonized zone in the case of rocks with spatially well-defined zones of colonization (layers).
— It is important to note that remotely selected samples without known microbial colonization represent much more challenging scenarios which should be taken into account during future testing experiments.
— Flight-like prototypes which are going to be on board future robotic Mars rovers should be tested in a similar way taking into account the limitations of the prescribed and defined analytical protocol, including the simulation of the selection of the samples for analysis at the landing site.

Acknowledgements. We thank Petr Drahota from the Institute of Geochemistry, Mineralogy and Mineral Resources for his X-ray diffraction analysis of some of the samples. We thank Linda Nedbalová for her help during microscopic documentation of the samples from Mojave Desert. We kindly acknowledge Ray Ramirez from the Los Angeles Department of Water and Power in Keeler for his help during the fieldwork. We thank also Arzell Hale from the Searles Valley Minerals Co. in Trona who provided some of the samples from Searles Lake.

Funding statement. This work has been supported by the Czech Science Foundation (project nos. 210/10/0467 and P210/12/P330). H.G.M.E. and I.H. acknowledge support from the UK Space Agency and J.W. and C.A. from Spanish Ministry of Economy and Competitiveness (projects CGL2010-16004 and CGL2013-42509)

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