Application of Raman spectroscopy for the identification of organic inclusions in minerals for the field of exobiology

Využití Ramanovy spektroskopie pro identifikaci organických inkluzí minerálů pro účely exobiologie

Ph.D. thesis

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Abstract

The multidisciplinary field of astrobiology has grown rapidly in recent years. The major goals of research in the field have been the search for habitable environments both within and outside our solar system, the search for evidence of prebiotic chemistry and life on Mars and other bodies in our solar system, laboratory and field research into the origins and early evolution of life on Earth, and studies of the potential for life to adapt to challenges on Earth and in space. NASA and ESA are heavily focused on a number of upcoming exploratory missions (e.g., the Mars Science Laboratory, with its planned launch in the fall 2011; ExoMars 2018; and the follow-up Mars Sample Return missions beyond 2020). A Raman spectrometer is now being miniaturized for the ExoMars Rover Instrument Suite. This Raman instrument is expected to be used to identify organic compounds and mineral products that could be related to signatures of life, as well as provide a general mineralogical overview, especially those minerals produced by water-related processes. This thesis describes the results of laboratory investigation into the feasibility of Raman spectroscopy to detect different types of biomarkers (pigments, carboxylic acids, and aminoacids) first mixed in the mineral matrices and then covered by UV-transparent crystals of different thicknesses. Experiments were performed using near infrared 785 nm and visible 514 nm excitation wavelengths sources. Another goal of this thesis has been to grow model crystals containing organic compounds in different concentration levels embedded within fluid inclusions, thereby developing "mineralogical standards" suitable for testing via non-destructive micro-Raman spectroscopy. Raman spectroscopy has proven able to detect different biomolecules, not only those which are dispersed in mineral matrices and but also those which are dissolved and embedded in fluid inclusions non-destructively, and furthermore without any sample preparation, in the submicrometer range, in short measurement times, and in relatively low concentrations.

Abstrakt

Astrobiologie je multidisciplinární vědní obor, který zaznamenává v současné době prudký rozvoj. Mezi hlavní cíle současného výzkumu patří: vyhledávání obyvatelných zón, a to jak v naší sluneční soustavě, tak mimo ni, hledání důkazů prebiotické chemie a života na Marsu a jiných tělesech v naší sluneční soustavě, laboratorní i terénní výzkum mapující vznik a raný vývoj života na Zemi a studium možností živých organismů přizpůsobit se jak terestrickým nepříznivým podmínkám, tak i podmínkám ve vesmíru. Pozornost vesmírných agentur, NASA a ESA, se nyní obrací na chystané výzkumné mise (zejména na Mars Science Laboratory, která odstartuje na podzim 2011; ExoMars, který je v plánu v roce 2018; a následné mise „Mars Sample Return" po roce 2020). Ramanův spektrometr je momentálně zmenšován pro využití na palubě mise ExoMars. Od Ramanova spektrometru se očekává, že identifikuje případné organické sloučeniny a biominerály a podá informace o základní mineralogii, zejména o minerálech, které vznikají v přítomnosti vody. Tato disertační práce shrnuje výsledky laboratorního výzkumu zaměřeného na využitelnost Ramanovy spektrometrie pro identifikaci biomarkerů (pigmentů, karboxylových kyselin a aminokyselin) ve směsích s minerálními prášky a při simulaci pevných inkluzí v minerálech pomocí UV-transparentních krystalů různé tloušťky. Jako excitační zdroje v této studii byly použity lasery v infračervené (785 nm) a viditelné oblasti (514,5 nm). Dalším záměrem bylo vypěstovat modelové krystaly, které budou obsahovat ve svých fluidních inkluzích rozpuštěné biomarkery v různých koncentracích. Tímto způsobem vytvořené minerální standardy byly dále podrobeny studiu pomocí Ramanovy spektrometrie. Ramanova spektrometrie prokázala schopnost detekovat zmíněné biomarkery v práškách i v inkluzích nedestruktivně, bez jakékoliv přípravy vzorků, v krátkém časovém úseku, i v inkluzích o rozměrech několika mikrometrů, a v neposlední řadě v relativně nízkých koncentracích.

Preface

This dissertation has been written on the basis of experiments conducted from 2006 to 2011 at the Institute of Geochemistry, Mineralogy and Mineral Resources, Faculty of Science, Charles University in Prague in the scientific group of Prof. Jan Jehlička. The thesis focuses on testing of the feasibility of Raman spectroscopy to detect molecular biosignatures embedded in inclusions of minerals of astrobiological interest. This work includes scientific background information, a summary and a discussion of the results, followed by an outlook and four papers included as appendices:

Paper I — Osterrothová K., Jehlička J. (2009) Raman spectroscopic identification of usnic acid in hydrothermal minerals as a potential Martian analogue, Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 73, 576–580. *Reprinted with permission from Elsevier.*

Paper II — Osterrothová K., Jehlička J. (2010) Raman spectroscopic identification of phthalic and mellitic acids in mineral matrices, Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 77, 1092–1098. *Reprinted with permission from Elsevier.*

Paper III — Osterrothová K., Jehlička J. (2011) Feasibility of Raman microspectroscopic identification of biomarkers through gypsum crystals, Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, doi:10.1016/j.saa.2010.12.085. *Reprinted with permission from Elsevier.*

Paper IV — Osterrothová K., Jehlička J., Investigation of biomolecules trapped in fluid inclusions inside halite crystals by Raman spectroscopy. *Submitted to Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy.*

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Hearty thanks also go out to Adam Culka and Petr Vítek, my fellow Ph.D. students at the Raman spectroscopy laboratory, for the insightful discussions we had during the hours spent at our adjacent workstations. Of course, those discussions would not have been as fruitful without their sharp wit and unparalleled senses of humor. In particular, I am grateful to Adam for the work he did on our common grant project no. 133107 for the Grant Agency of Charles University.

Although these three deserve the biggest gratitude, others at the Geology Institute helped me along the way. To all of them I say: "Thank you!"

I would also like to thank Madeleine Albright, who in her autobiography describes how it took her 13 years to finish her dissertation, mainly because she was caring for her three young children. Her experiences stayed in my mind throughout the long process of completing my dissertation, reminding me that I was not alone in trying to balance two lives, personal and academic, and helping me to stay sane.

My eternal gratitude also goes out to my parents for their endless support during my academic career and to my grandmother, who has always been my biggest fan.

Special thanks go to my husband, Jeffrey, for all he's done over the past five years to encourage and support me. Thanks for always being there when I needed it and for being my best friend out there in the universe! Last but not least, I would like to acknowledge my daughter Sara and son Samuel for their patience with me while I was working on my project and our dog Larry for our brain-clearing walks.

Declaration

I hereby declare that no part of this thesis has been previously submitted to this or any other university as part of the requirement for a higher degree. The work described herein was conducted solely by the undersigned except for those colleagues and other workers acknowledged in the text.

Prague, June 2011

Kateřina Osterrothová

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Chapter 1. Introduction

Life as we know it requires the availability of liquid water, a source of energy and an adequate supply of the organic molecules. Water is in its gaseous and solid forms omnipresent in the most distant galaxies, among the stars, in the Sun, on its planets and their satellites and ring systems, and in comets. NASA even initially chose the motto ‘‘Follow the Water” for its Mars exploration program, which was recently updated to ‘‘Seeking Signs of Life.” In living organisms, water serves as a solvent, a temperature buffer, a metabolite, a living environment and a lubricant (Hanslmeier, 2011). Life on Earth obtains its energy either by harvesting light or by using chemical energy via respiration or through a fermentation process.

There are basically two theories about the origin of life on Earth: Either a) it formed spontaneously from chemical precursors, or b) it came from elsewhere (panspermia) in the form of a microbial organism, probably equipped with our current genetic code or a similar precursor (Tepfer, 2008). The theory of spontaneous generation in a lukewarm marine origin is supported by experiments conducted by Miller (1953) and Miller and Urey (1959). By simulating prebiotic electric discharges and producing amino acids, Sagan and Chyba (1997) proposed that particulates of organic polymers (tholins) were produced by ultraviolet (UV) light high in a primitive Earth atmosphere with $CO_2/CH_4 < 1$. A benthic thermophilic origin of life on Earth near hydrothermal vents has been supported by the demonstration that peptide bonds can form by the activation of amino acids with CO on (NiFe)S surfaces at high temperatures (Huber and Wächtershäuser, 1998). An ice-water origin is supported by Trinks *et al.* (2005), who proposed that sea ice may have provided the optimal conditions for early replication of nucleic acids, and they supported the concept by using cyclic temperature changes to produce polyadenylic acid from adenylic acid imidazolides directed by polyuridylic acid in artificial sea ice.

Life on Earth can thrive in almost every ecological niche. Extremophiles such as Bacteria, Eucarya, and, most of all, Archaea not only survive harsh conditions but even prosper in extreme environments. Several papers and books cover this theme (for example, Rothschild and Mancinelli, 2001, Cavicchioli, 2002 and Horikoshi, 2011). Knowledge of extremophile habitats, such as environments with high salinity and acidity, with low temperatures, aridity, and high radiation and with oxidizing soils, can help us prepare instruments for future exploratory missions and identify the best locations and approaches for searching for biological signatures.

However, the answer to the question how life emerged on our planet still remains puzzling. We can conclude that the search for the origin and evolution of life on Earth can help generalize from this case to a broader range of possibilities in outer space. While searching for life in the universe, the detection of biosignatures and their strong assignment as biological evidence are key goals. Selection of these molecular targets and their further research is crucial for upcoming exploratory missions.

This thesis focuses on the detection of such biosignatures by means of Raman microspectroscopy in laboratory conditions. Raman spectroscopy is a powerful characterization method based on the inelastic scattering of incident laser light by molecules of the sample. This technique is rapid, and chemical information can be obtained without any extraction procedure or sample preparation. The Raman effect is highly sensitive even to minimal differences in chemical structure. The system offers multiple laser excitations, so fluorescence of the sample can be efficiently avoided. Minerals often contain inclusions (solid, liquid, or gaseous) that were trapped during mineral precipitation and provide information about conditions existing during the time of entrapment. Their sizes range from submicrometers to several millimeters. A Raman system coupled with a confocal microscope provides information with a high spatial

resolution (<1μm) and analyzes inclusions nondestructively in positions below the surface. An outline of the thesis is given below.

Chapter 2 provides a scientific background of the thesis. Its first part covers the brief introduction to the multidisciplinary field of astrobiology and introduces the planet Mars as a prime candidate for surface exploration and the search for life. The second part of the chapter summarizes the phenomenon called biosignatures, features that only living systems can leave behind and whose presence, or absence, indicates life. The next part of the chapter discusses organic inclusions and their astrobiological implications. This is followed by a review of the Raman spectroscopy technique and its application to the study of inclusions.

Chapter 3 deals with the methodology used in the framework of the thesis.

Chapter 4 provides a summary of papers included as appendices and referred to as I–IV.

Chapter 5 discusses and concludes the topics covered in this thesis. The need for further work within the topic is argued and recommendations are made.

Chapter 2. Background

This chapter's opening section will offer a brief overview of the multidisciplinary field of astrobiology and introduce the planet Mars as a prime candidate for surface exploration and the search for life. The second part of the chapter will summarize the phenomenon called biosignatures, features that only living systems can leave behind and whose presence, or absence, indicates life. In the chapter's third section, organic inclusions and their astrobiological implications will be discussed, and in the chapter's concluding section, one will find a review of the Raman spectroscopy technique and its application to the study of inclusions.

2.1 Astrobiology: Exploring the Living Universe

Astrobiology is a modern science that studies the origin, evolution, distribution, and future of life in the universe. It engages the sciences of biology, chemistry, paleontology, geology, planetary physics, and astronomy, among others. Three fundamental questions that need to be answered by the transdisciplinary science field astrobiology are (1) What is life? (2) What is the course of life? and (3) Are we alone in the universe? Astrobiologists optimistically believe that life has had a fair chance to evolve elsewhere and, if that be the case, we should be able to detect it. The search for extraterrestrial life now includes *in situ* exploration, spectroscopy of solar and extra-solar planetary atmospheres, and the search for extraterrestrial intelligence.

The term astrobiology was first coined by Laurence J. Lafleur (1941) and later by Otto Struve (1955). While NASA officially adopted the term in 1996 while establishing the Astrobiology Program, its studies in the field of exobiology—a predecessor to astrobiology—date back to the beginning of the U.S. space program. NASA funded its first exobiology project in 1959 and established an Exobiology Program in 1960. Exobiology research is now an element of the Astrobiology Program and focuses on research into the origin and early

evolution of life, the potential of life to adapt to different environments, and the implications for life elsewhere. Although used in different times for different things, the terms exobiology and astrobiology actually mean the same thing.

Several recent discoveries made the possibility of existence of extraterrestrial life, especially microbial life, more plausible:

(1) the discoveries made by the two Mars Exploration Rovers, *Spirit and Opportunity*, which have greatly expanded our knowledge of the history and the current status of liquid water on Mars (Squyres *et al.*, 2004);
(2) the finding that terrestrial *extremophiles*, organisms adapted to live in extreme conditions, can demonstrate that life is far more resilient and therefore much more common than previously believed (Pikuta *et al.*, 2007);
(3) the announcement that the meteorite ALH 84001, found in the Allan Hills region of Antarctica in 1984, may contain Mars fossils (McKay *et al.*, 1996);
(4) the discovery of more than 551 extrasolar planets (as of May 2011), which indicates that our solar system is not unique after all (http://exoplanet.eu);
(5) the discoveries of super-Earth planets—the Kepler space observatory mission recently released a list of around 300 new super-Earth candidates (http://kepler.nasa.gov);
(6) the confirmation by the Cassini/Huygens mission of evidence of organic compounds and water geysers in the southern polar region of Saturn's moon Enceladus and Titan's rich organic chemistry (Kieffer *et al.*, 2006; Shemansky *et al.*, 2005);
(7) the discovery of various organic molecules and compounds in space, including ethyl formate and n-propyl cyanide (Belloche *et al.*, 2009); and

(8) the identification of glycine of extraterrestrial origin in samples brought back by NASA's Stardust spacecraft (Elsila *et al*., 2009).

Astrobiological research is not the domain of one particular country or another. In fact, several countries undertaking, or planning to undertake, human and robotic space exploration programs while at the same time developing the *in situ* instruments that will target the Moon, Mars and its moons and near-Earth objects (NEOs) (Ansdell *et al.*, 2011). Each targeted solar body tells us different things about life. Investigations of the Moon, for example, provide us with unique information about the earliest periods of our solar system. On the other hand, the prime target in our solar system for discovering evidence of extinct life and possibly extant biosignatures is Mars, which has been extensively investigated for water and its mineralogy in the past; any scientific breakthroughs regarding the search for life on Mars will have a strong impact on all future exploration missions. Finally, the investigation of small bodies such as comets and asteroids provides us with important insights into the original composition of the solar nebula from which the planets formed.

NASA's lunar missions in the not-too-distant future include Gravity Recovery and Interior Laboratory (GRAIL), to be launch in September 2011, and the Lunar Atmosphere and Dust Environment Explorer (LADEE), with its launch date in May 2013. More distant but just as promising, Mars is another main target of US space exploration. The Mars Science Laboratory (MSL), to be launched in November 2011, will try to determine whether the particular landing area of the craft ever had, or still has today, the environmental conditions favorable to microbial life. The research will not end there. Two years later, in November 2013, the Mars Atmosphere and Volatile Evolution (MAVEN) spacecraft will be ready to launch. MAVEN will explore the red planet's upper atmosphere and ionosphere, not to mention its interactions with the sun and solar wind. This will be just the beginning. The long-term

cooperation between ESA and NASA regarding the exploration of Mars will start in 2016 with the ESA-led and NASA-launched Trace Gas Orbiter plus an Entry Descent and Landing Demonstrator Module (EDM). The Orbiter will perform remote observations of the Martian atmosphere, searching for evidence of gases of possible biological importance, such as methane and its degradation products, while the EDM will provide information about the technology for landing on the surface of Mars with a controlled-landing orientation and touchdown velocity.

Cooperation between Europe and the United States should continue far into the future. In particular, the 2018 NASA-led mission will include two rovers: the European Exomars and American MAX-C (Fig. 1). Both rovers will be integrated in the same aero shell and will be delivered to the same site on Mars. After landing on the surface of Mars, each rover will proceed with its mission objectives: ExoMars's main focus will be the subsurface, meaning it will penetrate the Martian soil, collect samples, and analyze molecules in order to study the Martian geology and mineralogy and search for biosignatures. The MAX-C rover, on the other hand, will characterize the Martian geology by examining the surface and will collect selected samples for caching for an eventual return to Earth. The Mars Sample Return mission will follow these missions sometime after 2020 and will place further emphasis on finding detailed answers to the questions about habitability and life.

ESA, which the Czech Republic joined as the eighteenth member state on November 12, 2008, is also planning to launch the ESMO, or European Student Moon Orbiter, in late 2013 or early 2014 and is preparing the first European Moonlander, which will for the first time visit the South Polar Region of the Moon.

Fig. 2.1 An artist's impression of the American rover, known as MAX-C (left), and the European ExoMars Rover. *Credit: ESA, NASA/JPL*

The Europeans and the Americans, however, are not the only nations involved in the search for signs of life in space. Russia, for one, is particularly active. The Russian space agency, Roscosmos, recently announced the launch in October 2011 of the Phobos-Grunt spacecraft, which will delivery samples of Mars' satellite Phobos to Earth and provide the opportunity for remote studies of Mars. This will be followed by the Luna-Glob mission, to be launched after 2015, which will study the internal structure of the Moon and, in particular, its crater Aitken on the South Pole, explore natural sources, and study the influence of incoming corpuscular fluxes and electromagnetic emissions on the Moon. Finally, the Venera–D mission to Venus, scheduled for the year 2016, will measure the chemical composition this planet's atmosphere; estimate the mineral composition of its surface layer; measure the temperature and pressure, radiant fluxes, and characteristics of the aerosol environment; and gather data on seismic activity on this planet.

Not to be left behind, three Asian nations are also very active.

- The highest priority of Japan's Jaxa mission will be the investigation of the Moon, and its Selene 2 and 3 moon orbiters are also planned for the near future.
- The Chinese space agency, CNSA, recently announced the next phase in this country's moon exploration. The Chang'e-3 lander and rover, which will be launched in 2013, will include a robot to detect, collect, and analyze samples. The Chinese Yinghou-1 (YH-1) Mars orbiter will piggyback on the Russian Phobos-Grunt mission in late 2011 and conduct space-environment, atmospheric, gravity, and surface-imaging studies of Mars (Zheng *et al.*, 2011).
- The Indian Space Research Organization (ISRO) is planning a moon mission, Chandrayaan (orbiter and rover), in 2013.

Aside from the various space-exploration missions described above, there are also numerous Earth-based field research programs (currently summarized by Ansdell *et al.*, 2011) which are exploring the subject. These programs analyze various extreme terrestrial environments that represent places on Earth whose geological or environmental conditions are similar to those found on extraterrestrial bodies; in this way, they provide analogues to landing and operation sites on the Moon, Mars, and beyond. Analogue studies enable the development and validation of biosignatures and detection techniques. Analogue environments have four general functions: (1) to learn about the planetary processes on Earth and elsewhere; (2) to test technologies, methodologies, and protocols; (3) to train highly-qualified personnel, as well as science and operations teams, and (4) to engage the public, space agencies, media, and educators (Léveillé, 2009).

Several organic host analogues, sites on Earth that mimic the putatively low organic content of Mars, are currently being reviewed by Marlow *et al.* (2011). Low temperatures (Antarctic ice; Arctic permafrost; Sea ice), aridity (Mojave Desert, California; Atacama Desert, Chile), and high radiation and oxidizing soils (Atacama Desert, Chile; Antarctic dry valleys) characterize modern-day

Mars, and acid-saline waters represent the planet's warmer and wetter past (the acid-saline lakes of Western Australia and Rio Tinto, Spain). While these Earth-based research programs certainly provide valuable insight into the necessary conditions for the formation of life, describing their individual contributions would be far too time-consuming for this work and therefore will not be undertaken here.

2.1.1 Destination Mars

There are several promising places after Earth where life might have emerged or persisted. For example, Jupiter's moon Europa and possibly the moons of Saturn, Titan or Enceladus, are important targets for detailed exploration of extraterrestrial life, traces of life, and biomolecules or their precursors. However, the prime candidate to search for extraterrestrial life is undoubtedly Mars. This planet is also one of the easiest targets to reach with space missions, with launch windows every 26 months. Although the surface of Mars may currently be uninhabitable by indigenous life, regions in the subsurface may still harbor life or remnants of past life.

Before entering into our discussion of explorations of the Martian surface, it would be beneficial to remind ourselves of Mars' general characteristics. Mars is the fourth planet from the Sun and has equatorial radius of 3,396.2 km, or approximately half of that of the Earth. Mars's maximum distance from Earth is 401.3 million kilometers and its minimum is 55.7 million kilometers. The average temperature on the surface is 210 K (-63 °C), and temperatures can vary between 140 K (-133 °C) at the poles in winter to 300 K (+27 °C) around the equator on the day side in summer. At present, the ground is frozen to an average depth of several kilometers, which has led to the formation of a thick cryosphere in which any water at the present time would be frozen. A Martian day (called a "sol") lasts 24 hours and 37 minutes, and a Martian year lasts 687 Earth days (667 sols). The tilt of the Martian rotational axis (25°) is nearly equal to that of the Earth's; thus, Mars experiences similar seasonal variations

to those on the Earth. Mars has a thin atmosphere with a surface pressure at a mean radius of 6.36 mbar (variable from 4.0 to 8.7 mbar depending on the season). The atmospheric composition is 95% CO_2, 2.7% N_2, 1.6% Ar, 0.13% O_2, and 0.08% CO, with trace amounts of H_2O, NO, Ne, HDO, Kr, and Xe. Because the atmosphere is so thin, the Sun's ultraviolet radiation passes through it almost undisturbed to the surface. Mars has two satellites, Phobos and Deimos.

A total of 39 missions to Mars have been carried out to date; many of them have failed, but the success of the others provides us with valuable information. Four spacecrafts are currently operating on or around Mars: The Orbiter Mars Odyssey, which was launched in 2001, has collected more than 100,000 images and continues to send information back to Earth about the Martian geology, climate, and mineralogy. The main objective of the Mars Express mission, launched in 2003, is to search for sub-surface water from orbit. The Mars Exploration Rovers Spirit and Opportunity were also launched in 2003, and after Spirit fell silent in March 2010, Opportunity continues to traverse the Martin surface, conducting field geology and making atmospheric observations. The Mars Reconnaissance Orbiter, launched in 2005, is still seeking out the history of water on Mars.

A comprehensive summary of information from several exploratory missions about the geologic evolution of Mars was recently published by Carr and Head (2010), Fairén (2010), and Fasset and Head (2011). The findings of the instruments from the most recent missions confirm that the Martian environment was moister, and probably warmer, during the first few hundred million years following its formation. It would benefit the discussion to provide some brief highlights at this moment. Major time periods of Martian history could be divided into three sections based on type localities: the Noachian, Hesperian, and Amazonian (Scott and Carr, 1978; Scott and Tanaka, 1986). The timeframe for the end of Noachian period was estimated to be around 3.7 Gyr

ago and for the Hesperian period around 2.9–3.3 Gyr ago (Hartmann and Neukum, 2001), as can be seen in **Fig.1.**

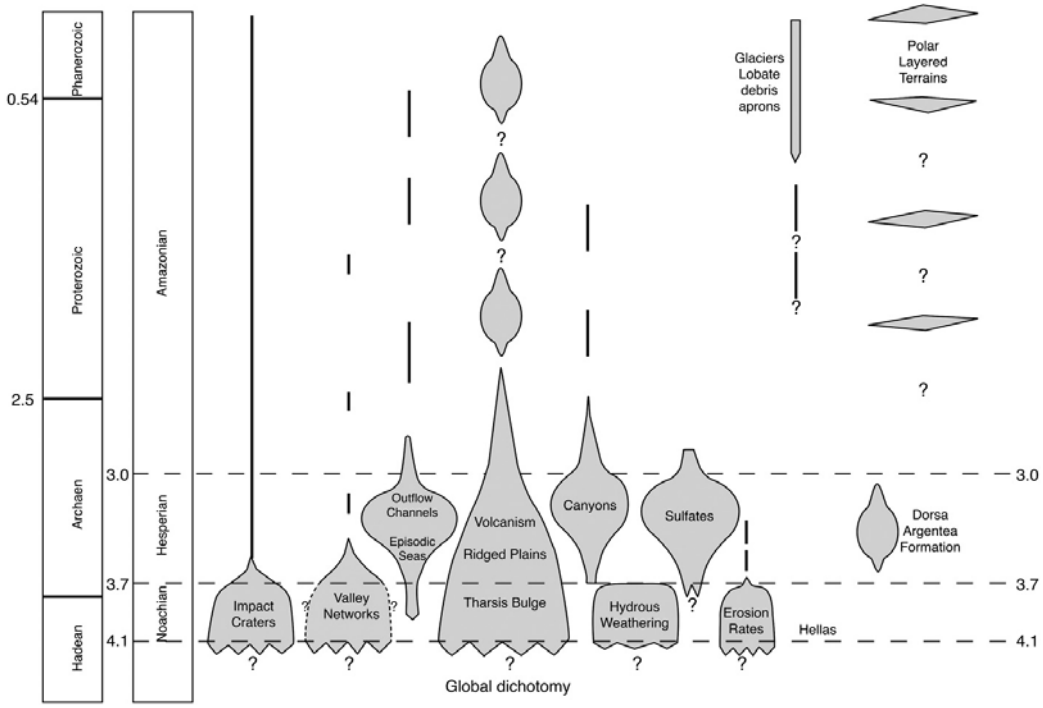


Fig. 2.2 Geological activity as a function of time on Mars. Shown are the relative importance of different processes (impact cratering, volcanism), the time and relative rates of formation of various features and units (valley networks, Dorsa Argentea Formation), and types and rates of weathering, as a function of time. The approximate boundaries of the major time periods of Mars history are shown (Hartmann and Neukum, 2001), and are compared to similar major time subdivisions in Earth's history (Head, 2006), reprinted with permission from Carr and Head (2010).

The pre-Noachian period extends from the time of formation of the planet 4.5 Gyr ago to the time of formation of Hellas, a large circular impact basin located in the southern hemisphere of the planet. Mars accreted and differentiated into the core, mantle, and crust within approximately 50 million years of the formation of the solar system (Solomon *et al.*, 2005). The planet had a global magnetic field (Acuña *et al.*, 1999) and Tharsis may have started to accumulate before the era was over.

Mars has two very different hemispheres—the heavily cratered highlands in the southern hemisphere and the relatively smooth lowland plains in the northern hemisphere—which is a phenomenon called "global dichotomy." This global dichotomy is expressed in three ways: in differences in elevations, in crustal thickness, and in crater densities (Fig. 2.3). Despite the new data regarding the origin of the hemispheric dichotomy, whether this may have set the course for most of the subsequent geologic evolution of Mars, including the Tharsis volcanic and tectonic province, remains unclear (Watters, 2007).

The Noachian period, the earliest Martian geological epoch (4.1–3.7 Gyr ago), was characterized mainly by cratering, erosion, and valley formation. Most of the Tharsis, a volcanic plateau centered near the equator in Mars's western hemisphere, was formed and widespread production of hydrous weathering products, such as phyllosilicates (nontronite, Fe-rich chlorites, saponite and montmorillonite), occurred (Murchie *et al.*, 2008). Sulfate deposits accumulated late in the era and in the subsequent Hesperian period. The warmer and wetter conditions, which are needed for fluvial activity, probably occurred only occasionally in the late Noachian period, forming hemispheric oceans of water and ice in the northern lowlands (Clifford and Parker, 2001; Fairén *et al.*, 2003) and lakes within impact craters (such as Argyre or Hellas) in the southern highlands (Wilson *et al.*, 2007). Concentration due to evaporation and freezing left ponds of chloride deposits globally widespread over the highlands, mostly in Noachian terrains (Osterloo *et al.*, 2008). The loss of atmosphere at the end of the Noachian period and the subsequent drop in temperature formed widespread depositions of salt minerals (Fairén, 2010).

The Hesperian period, ranging from 3.7 to 3 Gyr ago, saw the end of high rates of impacts, valley formations, weathering, and erosion, although volcanism continued to form the extensive lava plains (Carr and Head, 2010). Temperatures were arguably higher than those today, but it is not likely that they rose to levels comparable to those achieved in the Noachian period

(Fairén, 2010). The amount of surface water in liquid form was massively reduced in comparison to the Noachian. However, large water floods formed episodically, possibly leaving behind small seas in the northern lowlands (Fairén *et al.*, 2003). Canyons, such as the Valles Marineris, formed during this period. Sulfate rich deposits (mainly hydrated Mg and Ca sulfates), namely in Columbia Hills, Meridiani, several locations in the western hemisphere, but also around the north pole, are present and glaciers (The Dorsa Argentea Formation) developed (Head *et al.*, 2004).

The Amazonian period dates from some 3 billion years ago to the present day. Geological activity in the Amazonian period has declined rapidly. The climate could be characterized as very cold with low atmospheric pressure and with a limited planetary budget of water (Fairén, 2010). The formation of features like polar layered deposits, glacial deposits on volcanoes, ice-rich veneers at high latitudes, lobate debris aprons, lineated valley fill, and concentric crater fill and gullies is typical for this time (Carr and Head, 2010).

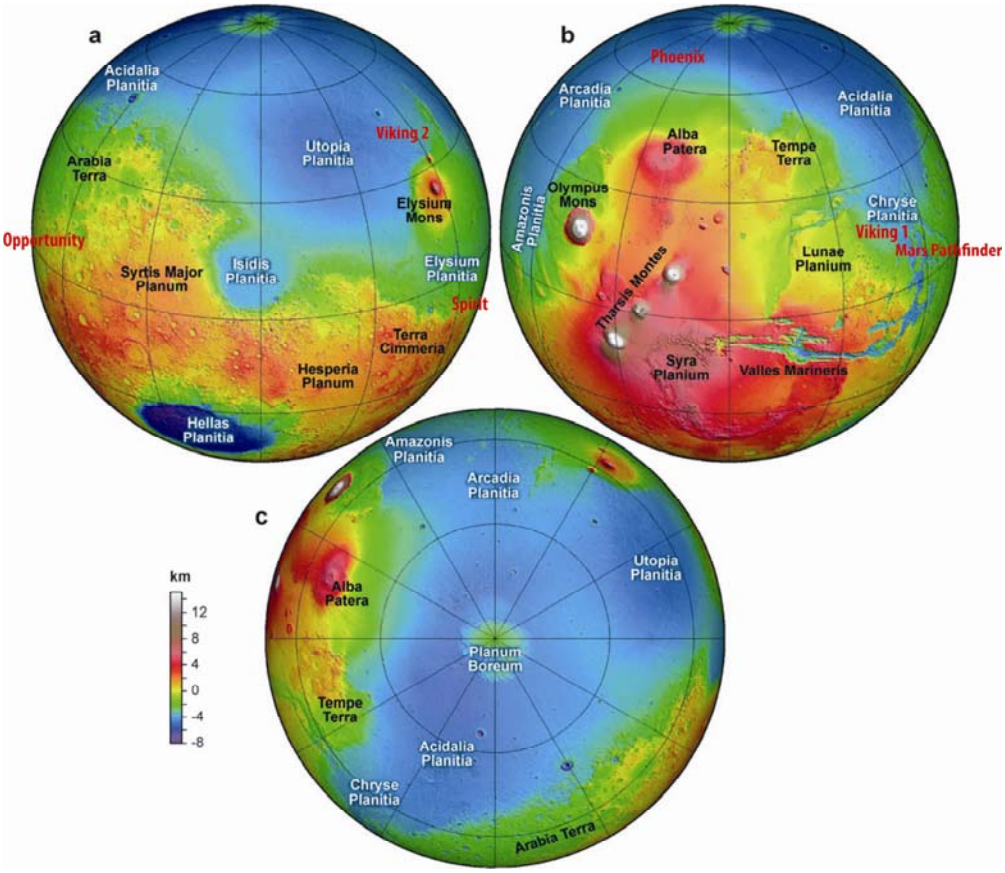


Fig. 2.3 Topographic maps of Mars. (*a*) The eastern hemisphere centered at 20◦N, 90◦E; (*b*) the western hemisphere centered at 20◦N, 270◦E; and (*c*) the northern hemisphere centered at 90◦N. The topographic maps were derived from MOLA gridded data (Smith *et al.* 2001). The Martian crustal dichotomy is seen through the change in elevation between the highlands of the southern hemisphere and the lowlands of the northern hemisphere. In red text are the landing sites of various missions to Mars (adapted from Watters, 2007).

2.2 Signatures of Life

The Viking missions to Mars were the first life-detecting missions, yet they failed to detect any organic molecules on the Martian surface (Biemann *et al.*, 1976, 1977, and Biemann, 2007). This was surprising due to the relatively high amounts of reduced carbon estimated to be delivered to Martian surface each year by carbonaceous meteorites (Flynn, 1996). Harsh Martian conditions, such as intense UV radiation and highly oxidizing conditions on the surface of

Mars, may have contributed to the destruction of organic compounds (Benner *et al.*, 2000). Nevertheless, the future life-detection mission ExoMars will search for signs of life, but this time in the subsurface of Mars, where organic molecules, if present, might be better preserved.

One of ExoMars's primary goals will be to search for biosignatures – features which only living systems can leave behind and whose presence or absence tells us whether or not life has ever existed. Biosignatures are classified into three groups: morphological (such as cells, colonies, biofilms/mats, extracellular polymeric substances), chemical (organic, elemental – Ni, Cu, Mn, Co, Mo, Se, V, Fe, and/or mineral–magnetite, microcrystalline calcite, aragonite, dolomite), and isotopic traces (C, O, S, N, Fe) of organisms preserved in minerals, sediments, and rocks. (For more details on these groups, see Westall and Cavalazzi, 2011.)

Biosignatures can be detected either directly by simple detection of living organisms or indirectly through the detection of chemical compounds or physical structures that their metabolism has left on the environment. Obviously, the Earth is for the moment our only laboratory available in which we can search for and define biosignatures for further planetary exploration. Thus, these biosignatures are defined on the basis of our particular understanding of life that terrestrial life is based on carbon chemistry and operates in an aqueous environment. All living matter, as we know it, basically consists of four types of molecules and their polymers and combinations: carbohydrates, lipids, nucleo-bases, and amino acids. Biomarkers should fulfill these characteristics that suggest their biological origin taken after Summons *et al.* (2008): (1) enantiomeric excess (chirality), (2) diastereoisomeric preference, (3) structural isomer preference, (4) repeating constitutional subunits or atomic ratios, (5) systematic isotopic ordering at molecular and intramolecular levels, and (6) uneven distribution patterns or clusters (C-number, concentration, or $\delta^{13}C$) of structurally related compounds.

The preservation potential of biosignatures is an important issue for the detection of life on other solar-system bodies (Fig. 2.4). Several factors contribute to the degradation of biological matter, and certain conditions exist which avoid or slow degradation (taken from Parro *et al*., 2008). Factors contributing to the degradation of the biological matter include (1) enzymatic and microbial activities, (2) radiation (UV and others), (3) oxidation, (4) metal attacks, (5) Maillard reactions (condensation of the carbonyl group of reducing sugars and primary amino group of amino acids), and (6) high temperatures or extreme pHs. Conditions that avoid or slow such degradation are (1) low temperatures, which means lower metabolic rates and catalytic activities; (2) a rapid burial to favor anoxic environments, which confines and protects against UV radiation and oxidation; (3) inclusions in salt crystals and polymerized resins like amber (hypersaline solutions slow the catalytic degradation and favor desiccation); (4) precipitation on the surface of colloid biominerals; (5) anoxic environments rather aerobic ones (the former have lower metabolic rates); (6) rapid dehydration that severely restricts the catalytic activities; (7) mild pH values (extreme pH severely affects molecule preservation favoring depurinization of DNA at low pH or RNA degradation at alkaline one); (8) absence of degradative metal ions (some metal ions or radicals severely affect the biomolecules structure).

Parnell *et al.* (2007) suggests a list of over sixty biosignatures representing extinct life, extant life, abiotic chemistry (of meteoritic origin), and mission-borne Earth contamination, which are further prioritized in A, B, and C categories (in decreasing priority). An important target of extant biota on Mars would be findings of energy and storage compounds, such as ATP (results of experiments indicate that ATP is likely to have relatively long residence times on Mars). Long residence times for ATP under Martian conditions suggest that prelaunch cleaning protocols may need to be strengthened to militate against possible ATP contamination of life-detection experiments on Mars landers (Schuerger *et al.*, 2008). Informational biopolymers, such as DNA, RNA and

generic nucleobases, would be proof of biology, but their survival rate is highly debated (Wayne *et al.*, 1999; Pääbo *et al.*, 2004).

Another significant target would be porphyrins, degradation products of light-harvesting pigments such as chlorophyll, and bacteriochlorophyll (Xiong, 2006). Porphyrins are extremely durable molecules and have been identified essentially unaltered in sediments dating back at least 340–280 Ma (Izmailova *et al.*, 1996). Archaeal lipids, as phytane and pristane, the isoprenoid component of archaeal membranes, are amongst the most widely found biomarkers in the geosphere (Volkman and Maxwell, 1986; Rontani and Bonin, 2011). Phytane is also a membrane component of methanogenic archea (Woese *et al.,* 1990), which could be a possible source for the methane observed in the atmosphere of Mars (Mumma *et al.,* 2009). All 20 proteinogenic L-amino acids and modified aminoacids should serve as extinct biomarkers, although they can degrade easily under Martian conditions (Ten Kate *et al.,* 2005, 2006; Garry *et al.,* 2006), but they can survive preserved in sulphate minerals (Aubrey *et al.,* 2006). Carotenoids and their diagenetically altered products are excellent targets for further investigation as biosignatures (Marshall and Marshall, 2010); their preservation potential in sediments is dependent on the type of molecule and on the preservation potential of the site (Reuss *et al.*, 2005, and references therein). Hopanoids and steroids, with their polycyclic structures, appear to be very resistant to postdepositional degradation (Allen *et al.*, 2010). Hopanes are derived from lipids (bacteriohopanepolyols) present in cell membranes of primarily Bacteria; on the other hand, steranes come from lipids (sterols) in cell membranes of mainly Eukaryota. Steroids, which come mainly from Eucaryota, have lower priority than hopanoids as biosignatures suitable for detection on Mars.

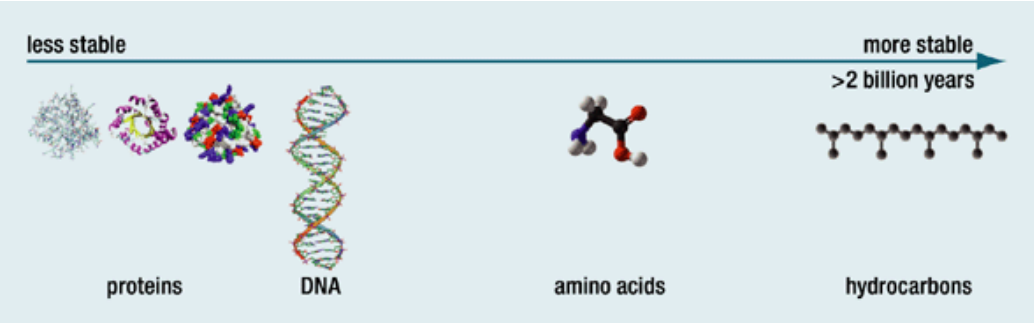


Fig. 2.4 Degradation rates of different biomarkers under Martian conditions. Less stable biomarkers include DNA, RNA, and proteins, while the most stable biomarkers includes isoprenoids. (Reprinted with permission from Martins, 2011).

The estimated mass of meteoritic material reaching the surface of Mars should be between 1.63×10^{-6} and 7.36×10^{-8} kg $m^{-2}yr^{-1}$ (Flynn and McKay, 1990), meaning that abiotic extraterrestrial organic compounds may be expected on Mars. Compound classes of interest include amino acids, carboxylic acids, sugar-related compounds, nucleobases, and polyaromatic hydrocarbons.

To conclude, the challenge for scientists seeking evidence of present or past life elsewhere in the universe is to exclusively differentiate between products generated by biotic and abiotic processes. There is often a significant overlap between them. A further complication is that physical and chemical processes over time can transform both biological and non-biological products in ways that obscure differences that were originally present when they formed. Therefore, only those products which can be demonstrated to form exclusively through biological processes and which could not plausibly form by any non-biological processes can truly constitute biosignatures (McCollom, 2011).

2.3 Organic Inclusions: Implications for Astrobiology

The study of organic inclusions in minerals allows us characterize the potential and stability of different environments on Earth while understanding some of the processes that might be important in the search for life on Mars and beyond. Indeed, if Mars ever really experienced a warm and wet climate early

in its history, it may have more similarities to Earth than we realize. Robotic *in situ* missions are able to detect areas with water ice, characterize mineralogy and petrology, and drill in order to detect organic molecules below the oxidizing surface. But since there can be no advanced sample preparation or heavy instrumentation, a detailed investigation of samples is difficult to perform. Thus, in order to improve the chances of finding extant or extinct biota, robotic sample return missions will probably be necessary. Sample return missions could obviously offer higher sensitivity and accuracy, not to mention a greater scope, than is possible with *in situ* instrumentation. However, sample return missions have one major disadvantage: the potential contamination of Martian samples with terrestrial organics and microorganisms. Such contamination must be avoided, and nondestructive analysis of inclusions in Martian samples could be one solution.

An inclusion can be defined as a material trapped within the body of a crystal which is different from the primary elements of the host crystal. Inclusions are generally other minerals (solid inclusions), or they can be based on pure water, brines of various salinity, gas or gas-bearing liquids, petroleum, silicate, sulfide, or carbonate melts (fluid and melt inclusions). In some cases they are organic in nature. There are basically three types of inclusions:

1) Protogenetic inclusions are those which were already present before the host mineral was formed – the host mineral grew around them, and therefore they are older than the host crystal.

2) Syngenetic inclusions are those which were formed at the same time as the host mineral. These inclusions can be solids, liquids, or gases, or combinations of any of the three forms of matter.

3) Epigenetic inclusions are inclusions which were formed after the host crystal was formed. These inclusions are usually either formed by exsolution or from the recrystallization of a fracture in a host mineral.

They may also be liquid, solid, or gaseous, and they are younger than the host crystal.

Especially interesting to astrobiologists are inclusions in minerals that have been precipitated in low temperatures and in the presence of microorganisms (Parnell *et al*., 2002). Fluid inclusions act as sealed microchambers and might preserve fluids in regions where water is now absent, such as the Martian surface. The size of most inclusions vary from sub-micrometers to (very rarely) millimeters, and typical mass content ranges from nano- to femtograms; however, with techniques such as Raman spectroscopy (Pasteris *et al.*, 1988, Keir *et al.*, 2002), ToF-SIMS (Siljeström *et al.*, 2009, 2010), and off-line and on-line crushing followed by GC–MS analysis (Dutkiewicz *et al.*, 2006; George *et al.*, 2008), it is still possible to obtain data from these fluids, including biosignatures and physical remains of life.

The earliest evidence of life on Earth comes from carbonaceous inclusions in apatite within >3.83 Gyr marine sediments from West Greenland that were found by ion microprobe analysis to contain isotopically light carbon, however the actual age of the carbon is subject to debate (see Mojzsis *et al.,* 1996; Lepland *et al.*, 2005; McKeegan *et al*., 2007; and Nutman and Friend, 2008). The oldest oil in the world was found in oil-bearing fluid inclusions in sandstone dating back ~3 Ga (Dutkiewicz *et al.*, 1998). The fluid inclusions in this case acted as inert pressure vessels that protected the oil from subsequent degradation, and such inclusions can yield valuable information about the size and diversity of the early biosphere. Hence, Rasmussen and Buick (2000) reported that the oil from fluid inclusions within hydrothermal barite, the Pilbara craton of Australia, dated from the Early Archean period (~3,235 Ga). The results demonstrated that sub-seafloor hydrothermal petroleum generation was providing an energy and carbon source for a subsurface microbiota metabolizing hydrothermal sulfur species. Steranes, hopanes, and isoprenoids have been detected in oil-bearing fluid inclusions from a ~2.45 Ga

fluvial metaconglomerate of the Matinenda Formation at Elliot Lake, Canada. The presence of abundant biomarkers for cyanobacteria and eukaryotes in rocks deposited before the Great Oxidation Event is consistent with an earlier evolution of oxygenic photosynthesis. It also implies that eukaryotes survived several extreme climatic events, including the Paleoproterozoic "snowball Earth" glaciations (Dutkiewicz *et al.*, 2006; George *et al.*, 2008).

Meteorites from Mars and elsewhere also contain fluid inclusions. Bodnar (1999) reported the occurrence of carbon dioxide fluid inclusions in thin sections of SNC meteorites – Nakhla (NSNM 5891-3) and ALH 84001. The carbon dioxide was migrating through these igneous rocks for some time or after their formation on Mars. Fluid inclusions in halite (NaCl) crystals were found in the matrix of two freshly-fallen brecciated H chondrite: Monahans (H5) and Zag (H3-6) (Zolensky *et al.*, 1999; Bridges and Grady, 2000). The halites were dated by K-Ar, Rb-Sr, and I-Xe systematics to be 4.5 billion years old and some (but not all) of the meteoritic halite crystals from Zag fluoresce under long-wave UV radiation, which might indicate the presence of potential organics (Zolensky *et al.*, 2010). These findings confirm that fluid inclusions could be a valuable source of information in planetary exploration. The preservation of terrestrial fluid inclusions of the Archean age provides encouragement that early rocks on Mars may preserve samples of inclusion fluids from a time when water was more abundant at the surface (Parnell *et al.,* 2002).

The detection of organic material, including amino acids and their amine degradation products, preserved in a matrix of ancient terrestrial sulfate minerals was reported by Aubrey *et al.* (2006). Inclusions in halite can easily trap microorganisms and organic debris. Reiser and Tasch (1960) and Tasch (1960) reported diplococcus-like forms in crushed salt samples and interpreted them as dead Permian bacteria. Dombrowski (1963, 1966) isolated bacterial strains from Permian and even pre-Cambrian salt deposits. Vreeland

et al. (2000, 2007) isolated bacteria from fluid inclusions in Permian halite (~250 Ma) and halophilic archaea from Cretaceous-age (121–112 Ma) halite. Mormile *et al.* (2003) isolated *Halobacterium salinarum* from fluid inclusion in a 100,000-year-old halite crystal from Death Valley, California. Halophilic Archaea were cultured from ancient fluid inclusions in a 90-m-long (0- to 100,000-year-old) salt core from Death Valley, California.

Results demonstrating the survival of halophilic prokaryotes in ancient fluid inclusions are rare, but they are possible in subsurface halite for up to 34,000 years (Schubert *et al.* 2009, 2010a). Cells of the algal genus Dunaliella co-trapped with prokaryote cells in fluid inclusions in halite (Fig. 2.5) were reported from the same salt deposit. Dunaliella cells hypothetically provided glycerol, the carbon source needed by halophilic Archaea for survival over periods of tens of thousands of years (Schubert *et al*., 2010b). Halophilic organisms were also collected from the Mg-sulfate Basque Lakes (playas) of British Columbia, Canada. Bacterial pigments (carotenoids), cells, and other soluble organic constituents were found trapped within fluid inclusions or fluid-filled void spaces between intergrown crystals (Foster *et al*., 2010). The cellulose macromolecules were recovered from fluid inclusions in halite collected from 650 m below the surface of the Late Permian Salado Formation in southeastern New Mexico (USA). These cellulose microfibers represent the oldest native biological macromolecules to have been directly isolated, and it makes cellulose an ideal macromolecular target in the search for life on other planets in our solar system (Griffith *et al*., 2008). Although the age of the halite crystals and fluid inclusion were sometimes challenged, these studies conclude that microorganisms can survive in evaporates for periods of thousands to hundreds of millions of years. Such conclusions have implications for the long-term survival of biota on Earth and elsewhere in the solar system.

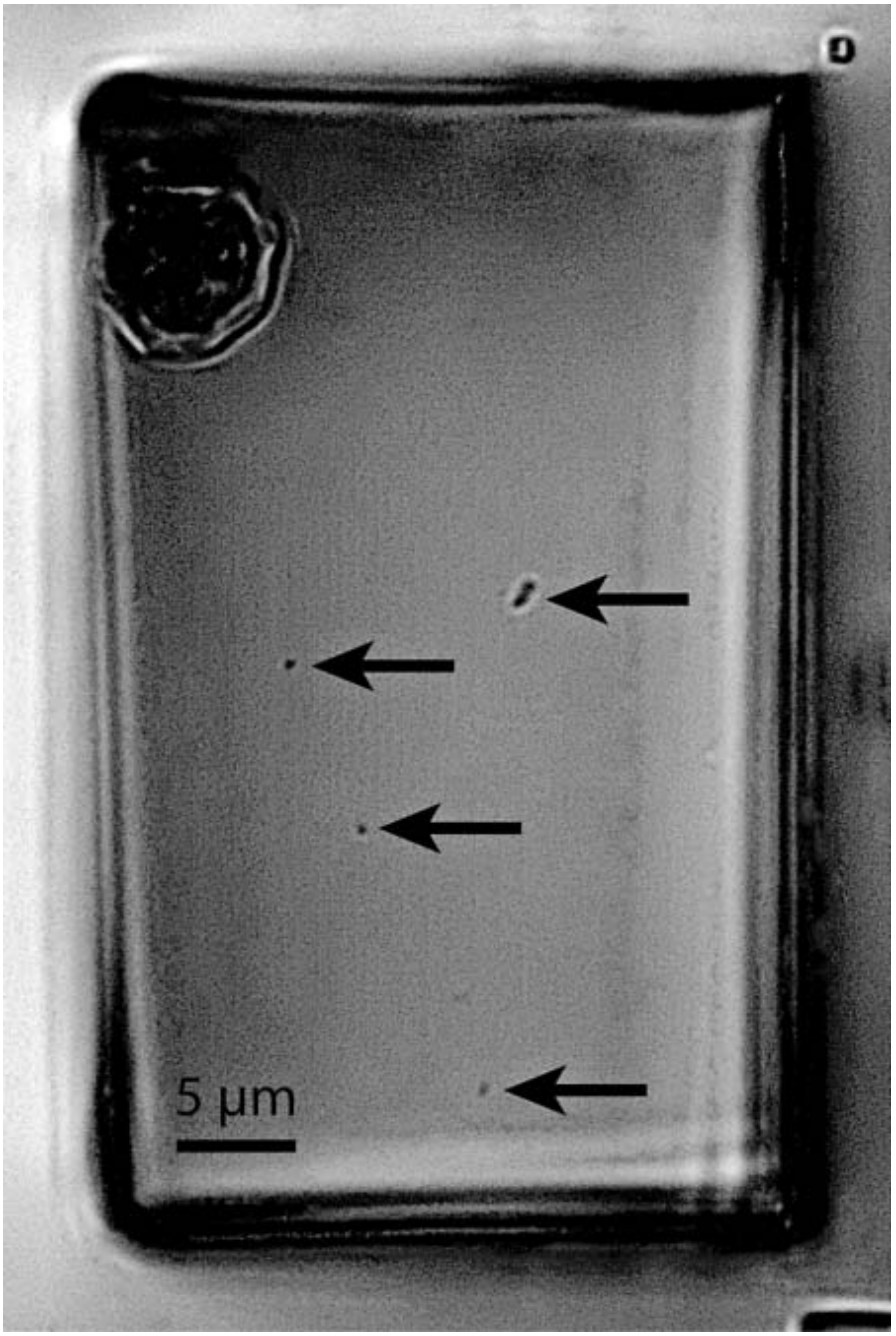


Fig. 2.5 Black and white photomicrograph of a *Dunaliella* cell and several prokaryote cells in a fluid inclusion in halite from 8.7 m (12,000 years old) in the Death Valley core. Arrows point to one short (0.5 × 1.5 μm), rod-shaped prokaryote cell and three small (~0.5 µm diameter), coccoid-shaped prokaryote cells. Reprinted with permission from (Schubert, 2010b).

2.4 Raman Spectroscopy and Inclusions

The Indian physicist, Chandrasekhara Venkata Raman, and his collaborators discovered the radiation effect that bears his name on February, 28, 1928 (Raman and Krishnan, 1928), and two years later, Professor Raman was awarded the 1930 Nobel Prize in Physics. However, the Raman spectroscopy wasn't much considered for applications to geology during the first 40 years after its discovery because of the weakness of the Raman signal. The development of high-power, visible-gas lasers in the 1960s and, more recently, the introduction of near-IR, solid-state lasers and the use of micro-Raman techniques (Delhaye and Dhamelincourt 1975; Rosasco *et al.*, 1975) have led to a renaissance of the Raman field for mineralogists, geologists, and even for planetary exploration. Among the first planetary applications was the use of Raman spectroscopy for the investigation of lunar samples brought back by the Apollo 11, 12, and 14 missions (Perry *et al.*, 1972).

The first Raman microspectrometer was the MOLE by Jobin-Yvon, followed later by the Ramanor U-1000. Both instruments had a photomultiplier as a detector, and the analysis of a single fluid inclusion was extremely slow (Dubessy *et al.*, 1982; Bény *et al.*, 1982). The first Raman microspectrometer with a multichannel detector was the Microdil-28 of Dilor (Burke and Lustenhouwe, 1987). In the 1990s, significant improvement in the quality of data obtained from inclusions came with the LABRAM or the system 1000/2000/3000 Renishaw. These new generation microspectrometers are equipped with holographic notch filters for Rayleigh-line blocking; confocal configuration to perform spatial- and depth-resolved measurements with a resolution in the micrometer scale and with decreased fluorescence; a thermoelectrically cooled CCD detector; an air-cooled laser; and software that is able to recognize cosmic rays and automatically remove them from the spectrum.

Further technological developments have moved the Raman spectroscope from mainly being a research instrument in academic laboratories to being an instrument that can be used *in situ*, even in the space. Miniaturized Raman instruments that are small enough to fit in a human hand have recently been developed and have wide applications in number of areas (chemical research, material sciences, forensics, geological sciences and planetary exploration, art applications, law enforcement, the pharmaceutical industry, government agencies, and the military). However, laboratory equipment offers better results concerning spectral and spatial resolution (objectives with magnification of ×100 allow spatial resolution of ~1 μm) and acquisition times. These advantages, not to mention the confocality of the laboratory micro-Raman spectrometer, enable scientists to analyze fluid and solid inclusions while at the same time minimizing the signal of the host matrix. The choice of different excitation wavelengths in laboratory systems also successfully help to minimize the eventual fluorescence and employ the Raman resonance phenomena (Bersani and Lottici, 2010).

Not surprisingly, Raman microspectroscopy is nowadays frequently applied to the analysis of samples and even contents of inclusions of astrobiological interest. Several important studies have been performed in this area. McKeegan *et al.* (2007) analyzed by means of 3-D Raman imagery the same sample as that from which apatite-hosted isotopically light graphitic inclusions were reported by Mojzsis *et al.* (1996) and demonstrated the occurrence of graphitic inclusions within apatite grains of Akilia rocks. The occurrence of apatite-hosted graphite was previously challenged by Lepland *et al.* (2005), who failed to find any such occurrences by means of optical and scanning electron microscopy. The Raman results suggest that moderately disordered graphite is similar to the biological carbonaceous matter that composes ancient fossil microorganisms (Schopf and Kudryavtsev, 2005). Zolensky *et al.* (2011) has been using Raman microscopy to study aqueous fluid inclusions embedded in halite (NaCl) and sylvite (KCl) in different types of chondrites,

and by this method has been trying to answer questions about the earliest history of water in the solar system. Raman microspectroscopy was also employed to identify some chemical characteristics of the "hairy blobs" – unusual bodies which consist of tiny sulfate crystals and organic matter that may represent microbial remains and which are present as solid inclusions or associated with fluid inclusions in acid saline-lake evaporites. The Raman spectra of the hairy blobs were dominated by a wide, usually double peak that should be interpreted as disordered graphite, and the authors predict organic origin. Results of this study help us to understand the role of microorganisms in the acidic extreme environment (Benison *et al*., 2008). Fendrihan *et al*. (2009) used Raman spectroscopy for the detection of nine different extremely halophilic archaeal strains which had been previously embedded mostly within fluid inclusions of laboratory-grown halite crystals. Haloarchaeal C_{50} carotenoid compounds (mainly bacterioruberins) features due to peptide bonds (amide I, amide III) and nucleic acids were distinguishable in the Raman spectra. Results from this project will contribute to a growing database of Raman spectra of terrestrial microorganisms from extreme environments.

Chapter 3. Methodology

The Raman microspectrometric analyses were carried out at the Institute of Geochemistry, Mineralogy and Mineral Resources, Faculty of Science, Charles University in Prague using a multichannel Renishaw InVia Reflex microspectrometer. The Raman system is equipped with a 785nm line of a diode laser with a maximum output of 300mW and the 514.5nm Ar-ion laser has a maximum output of 25 mW, a Leica microscope, and a charge-coupled detector (CCD). The spectral data were acquired using Wire 2.0 spectral software. Raman spectra were then exported into the Galactic *.SPC format and analyzed using GRAMS AI (version 8.0, Thermo Electron, Waltham, MA, USA).

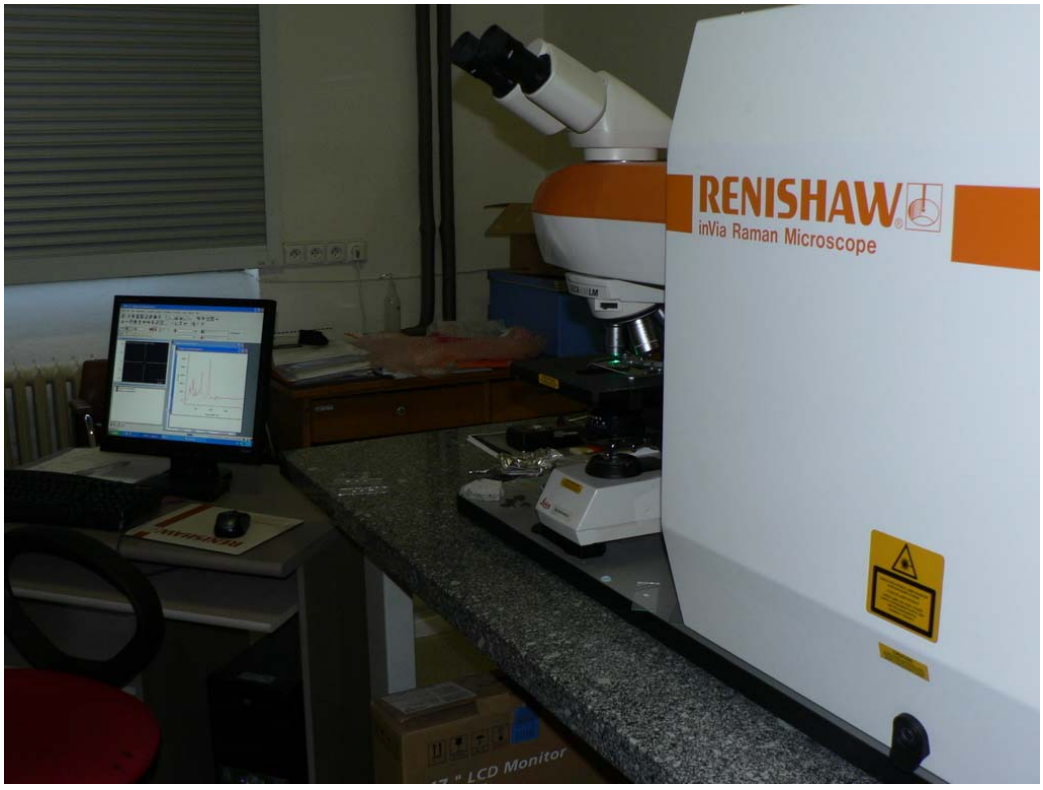


Fig. 3.1 The Raman microspectrometer at the Institute of Geochemistry, Mineralogy and Mineral Resources, Faculty of Science, Charles University in Prague (Photo: Adam Culka)

For a detailed description of the methodology used within the different experiments conducted in the framework of this thesis, see the papers that are included as appendices.

Chapter 4. Results

The following chapters provide a summary of papers included as appendices and referred to as I–IV. The experiments could be divided into two parts:

Papers I, II, and III:

Investigation of the feasibility of Raman microspectrocopy to detect selected biosignatures (pigments, carboxylic acids, and aminoacids) mixed in different concentration levels within artificially prepared evaporitic matrices, evaluating the choice of suitable excitation wavelength (785 nm or 514 nm) and analyzing also the ability of Raman spectroscopy as a depth-resolving method via simulating organic inclusions entrapped in minerals of astrobiological interest by analyzing previously mentioned mixtures through transparent mineral plates of different thicknesses.

Paper IV:

The development of mineralogical standards (halite crystals with biosignatures entrapped inside fluid inclusions in different concentration levels) and applying Raman microspectroscopy as a nondestructive method for detecting these biosignatures, which could be suitable for experiments on samples brought back from Mars on account of the low risk of contamination.

4.1 Paper I — Raman Spectroscopic Identification of Usnic Acid in Hydrothermal Minerals as a Potential Martian Analogue.

In this study we show the feasibility of Raman spectroscopy to detect usnic acid, secondary metabolite, which serves as the UV screening pigment, uniquely found in lichens (especially abundant in genera such as Alectoria, Cladonia, Usnea, Lecanora, Ramalina, and Evernia). Lichens are symbiotic organisms of fungi (mycobiont) and algae or cyanobacteria (photobionts), and

they seems to be able to thrive in a harsh environments that would kill most (if not all) complex life forms. When exposed to unpleasant conditions (like open space) the lichens can revert to a dormant state and stop metabolizing until they are again able to encounter more favorable conditions. Raman microspectroscopic study of usnic acid (Fig. 4.1 and 4.2) in experimentally prepared mixtures with calcite and gypsum was performed in order to evaluate the potential of Raman spectroscopy to detect this biomarker in low contents in hydrothermal minerals. Hydrothermal minerals were chosen because they require water to form and because they provide possible niches for some early organisms on Earth. Both calcite and gypsum have been found on Mars (Boynton *et al.*, 2009; Langevin *et al.*, 2005).

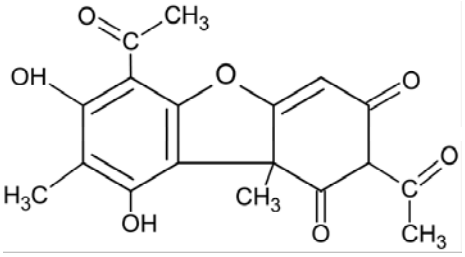


Fig. 4.1 Usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzofurandione); $C_{18}H_{16}O_7$

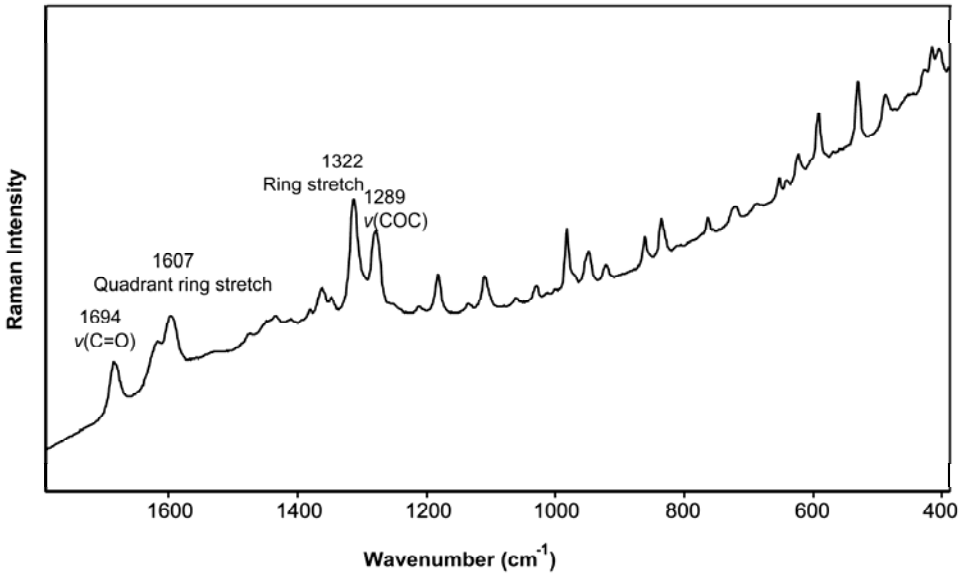


Fig. 4.2 Raman spectra of usnic acid with four selected key features, wavenumber region 1800–400 cm^{-1}.

Samples prepared by mixing usnic acid with powdered gypsum and calcite were investigated using both 514 nm and 785 nm excitation wavelengths, however 514 nm excitation laser was later proved to be inapplicable. Various concentrations of usnic acid in the matrix (250, 100, 10, 5, and 1 g kg^{-1}) were investigated to determine the detection limits of this biomarker under the conditions of this experiment. Survival strategies for lichens involve the biogeological modification of their habitats. In the most extreme environments, endolithic growth within translucent rocks can represent the ultimate protection. Hence, we situated usnic acid mixed with gypsum (respectively, calcite) in the frame of the UV-transparent crystal of gypsum ($CaSO_4 \cdot 2H_2O$) (approximately 2mm thick), thereby simulating organic inclusions within the rocks.

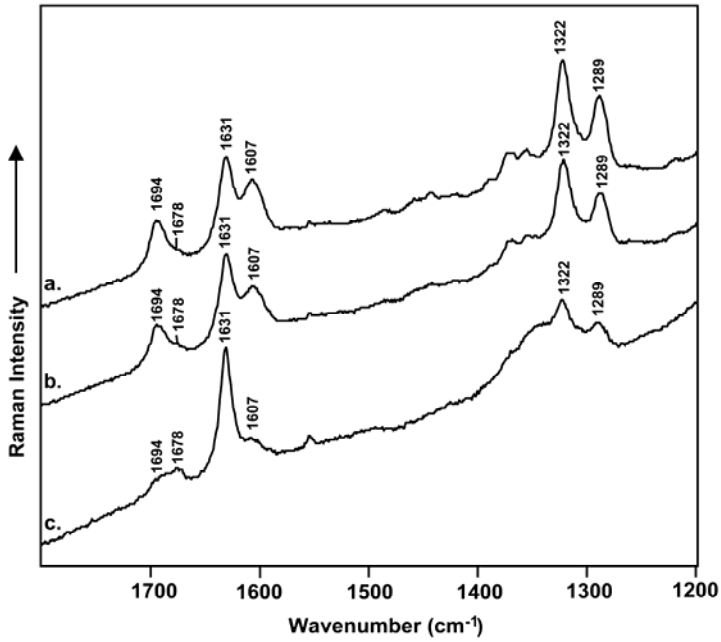


Fig.4.3 Raman spectra of usnic acid in the mixture with gypsum: (a) 10 g kg^{-1}, (b) 5gkg^{-1}, and (c) 1 g kg^{-1} usnic acid, wavenumber region 1800–1200 cm^{-1}.

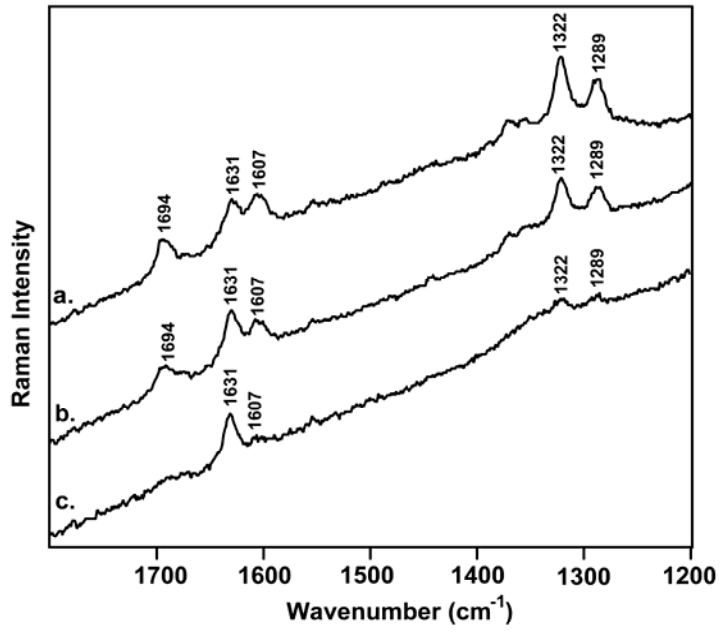


Fig. 4.4 Raman spectra of usnic acid in gypsum matrix through gypsum crystal: (a) 10 g kg^{-1}, (b) 5 g kg^{-1}, and (c) 1 g kg^{-1} usnic acid, wavenumber region 1800–1200 cm^{-1}.

This simplified view of the biomarker-mineral matrix system proved the ability of Raman spectroscopy to detect organic compounds and minerals in the mixture. In a previous work, Vítek *et al.* (2009a, b) investigated beta-carotene as a biomarker-mineral matrix system and obtained a beta-carotene signal at the lowest concentration of 1 mg kg^{-1} using a 785 nm excitation wavelength and even 0.1 mg kg^{-1} when the resonance Raman mode was employed by using a 514.5 nm excitation laser. The detection limit for beta-carotene when analyzed through a 2 mm gypsum crystal was at a concentration of about 1–10 mg kg^{-1} (depending on the excitation wavelength). In this work, we found that the concentrations of usnic acid in the same systems were far higher than those of beta-carotene, 1 g kg^{-1} for the matrix (Fig. 4.3) and 5 g kg^{-1} for the crystal (Fig. 4.4), which means that the sensitivity of Raman spectroscopy toward pigments in a non-resonant mode is limited. The obtained results have significant implications for planned *in situ* measurements on Mars or elsewhere.

4.2 Paper II — Raman spectroscopic identification of phthalic and mellitic acids in mineral matrices.

The 1976 Viking missions failed to detect organic molecules on the Martian surface, even those expected from meteoritic bombardment. Since then, it is believed that the Martian regolith is highly oxidative and would convert all organic molecules to metastable intermediates, which might be embedded in soils and rocks. These results suggest that, in order to detect organic molecules that may have arisen from life on Mars or may have been delivered to Mars via meteorites, it is necessary to dig deep below the Martian surface. The miniaturized Raman spectrometer is considered to be a candidate instrument for the Pasteur payload planned for the ExoMars rover, the ESA-NASA mission, to be launched in 2018. ExoMars will, for the first time, combine mobility and access to subsurface locations (down to a depth of 2m) where organic molecules, when present, may have better chance to stay preserved. Several

types of organic compounds are known to have come to Mars via meteorites (alkanes, alkylbenzenes, naphthalene and higher polycyclic aromatic hydrocarbons, kerogen, amino acids, hydroxyacids) (Mullie and Reisse, 1987). It has been proven that naphthalene, phenanthrene, and anthracene all convert to phthalic acid in the generic oxidation process (Bunce *et al.*, 1997; Barbas *et al.*, 1996 and Theurich *et al.*, 1997), and higher polycyclic aromatic hydrocarbons and kerogen transform into benzenecarboxylic acid products (e.g., mellitic acid) during oxidation (Juettner, 1937). Phthalic and mellitic acids (Fig. 4.5) could therefore be very appropriate organic molecular targets for future detection of signatures on the Martian surface or near subsurface.

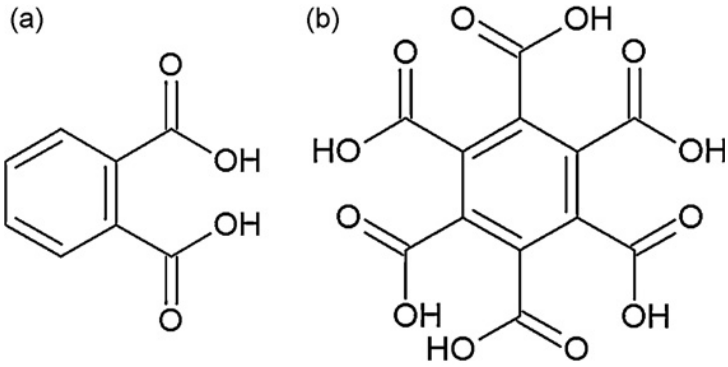


Fig. 4.5 (a) Structure of phthalic acid. (b) Structure of mellitic acid.

This work was carried out to evaluate the potential of Raman spectroscopy to detect key molecular features with high relevance to exobiological studies and also to compare data from 785 nm and 514 nm excitation wavelengths of Raman spectroscopy. The choice of Raman excitation wavelength is a key issue for exobiological studies on Mars and potentially elsewhere. A major weakness of Raman spectroscopy when analyzing organics is fluorescence, which can be avoided by selecting the appropriate wavelength. Samples consisting of carboxylic acid (phthalic and mellitic acids) mixed one by one with powdered minerals (gypsum and halite) were examined. Various concentrations of carboxylic acids (250, 100, 50, and 10 gkg^{-1}) in the mineral matrices were

prepared to determine the detection limits of Raman spectroscopy for the detection of these biomarkers. Carboxylic acids mixed with mineral powders were then covered by a UV-transparent crystal of the same minerals (gypsum crystal approximately 2mm, and halite crystal approximately 5mm thick); thereby simulating the possibility that such organic material can be trapped inside investigated minerals.

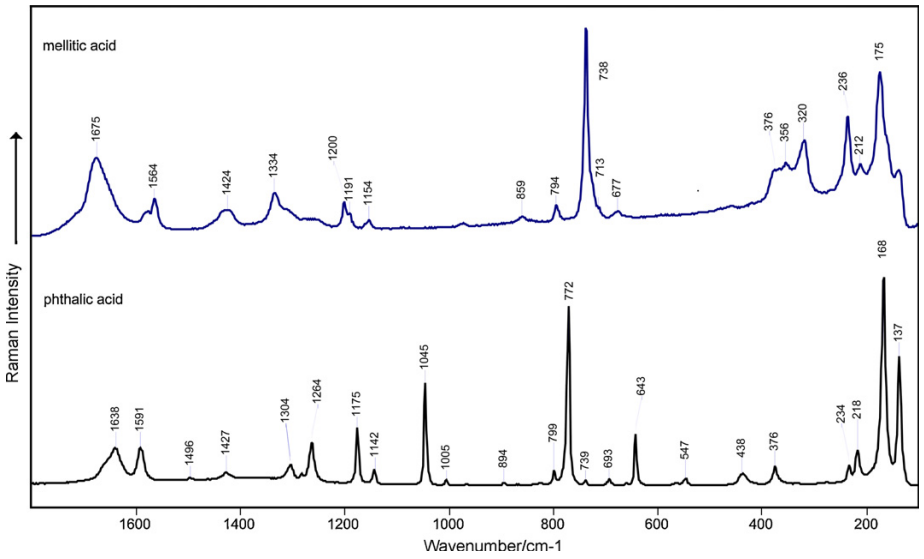


Fig. 4. 6 Raman spectra for phthalic and mellitic acids used as biosignatures.

In 2000, Benner concluded that 2 kg of meteoric-derived mellitic acid may have been generated per square meter of the Martian surface over 3 billion years and that if mixed in the regolith to a depth of 1m, we can expect 500 $mgkg^{-1}$ by weight. In addition, if gardening mixes the material to a depth of 1 km, benzenecarboxylates will be present at concentration of 0.5 $mgkg^{-1}$ (Benner *et al.*, 2000). However, we were not able to reach a concentration of 0.5 gkg^{-1}, which Benner predicted is present in the Martian regolith. The detection limit of phthalic acid mixed in mineral matrices and analyzed through crystals was 10 gkg^{-1}, using both excitation wavelengths. A Raman mellitic acid signal was obtained at a concentration as low as 10 gkg^{-1} in a halite matrix, and at a concentration of 50 gkg^{-1} when analyzed through a halite

crystal. In the case of mellitic acid mixed with gypsum and analyzed through a gypsum crystal, the detection limit is 50 gkg^{-1} using both excitation wavelengths. Our study shows that both excitation wavelengths could be successfully used to determine these carboxylic acids in mineral matrices and through the transparent crystals of minerals of astrobiological interest. However, the sensitivity toward selected carboxylic acids for Raman spectroscopy is relatively low, similar to those reported by Culka and Jehlička (2010) on the study of urea in calcite and gypsum powder matrices, 10 gkg^{-1} of urea in both the calcite and the gypsum mineral matrices and 50 gkg^{-1} when measured through appropriate transparent crystals (approx. 2 mm thick). On the other hand, halite crystals provided great potential for Raman spectroscopy, because the crystal itself has no Raman active modes, so there is no risk of masking the results by the surrounding matrix. We have shown that even in a depth of 5 mm it is still possible to register good quality spectra (Fig. 4.7), and further research on halite crystals with embedded organics as solid or fluid inclusions from an astrobiological point of view seems to be promising.

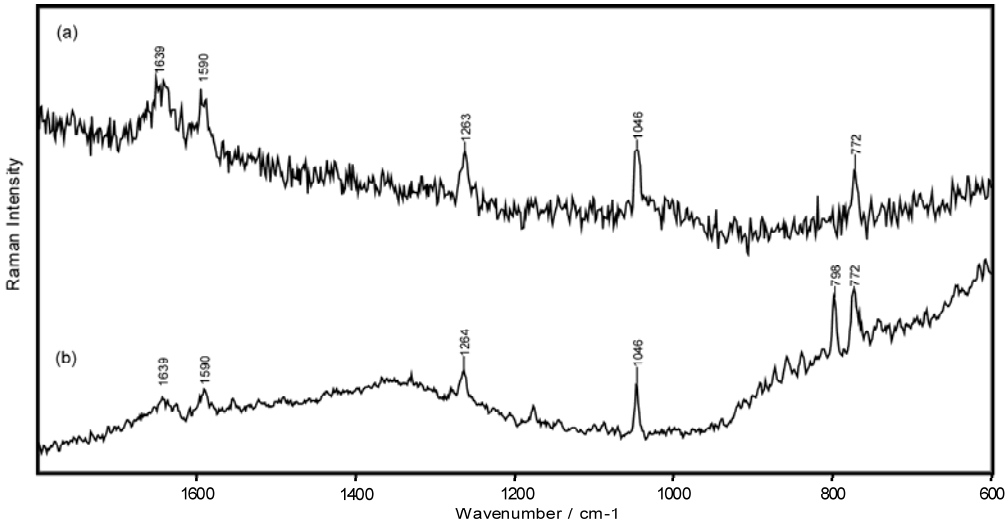


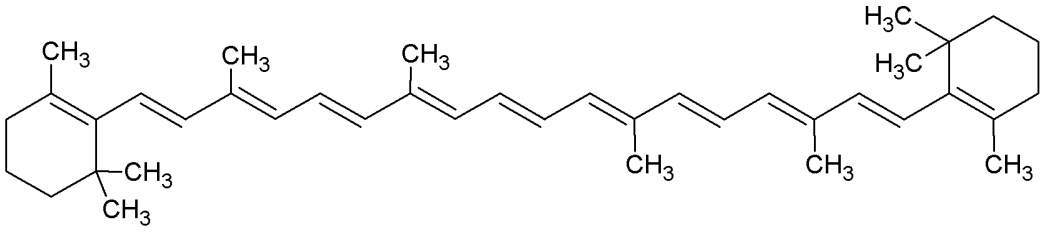
Fig. 4.7 Raman spectrum of phthalic acid in a halite matrix through a 5 mm thick halite crystal, (a) 10gkg^{-1}, excitation wavelength 514 nm, (b) 10gkg^{-1}, excitation wavelength 785 nm, wavenumber region 1800–600cm^{-1}.

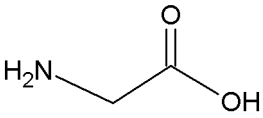
4.3 Paper III — Feasibility of Raman microspectroscopic identification of biomarkers through gypsum crystals

The search for and possible detection of life on Mars or on other promising solar system bodies is a great challenge for future space missions. If life ever arose on Mars, the gradual deterioration of surface environmental conditions might have forced the emerging biota to retreat to protective oases (Horneck, 2000). Evaporitic crystals are among the potential protected habitats that have been postulated. Organic material could be also entrapped within fluid inclusions, which are usually abundant in minerals such as halite, gypsum, or epsomite, all of which have been discovered on Mars by the NASA rovers Spirit and Opportunity and which form in the presence of liquid water. These inclusions could therefore contain organic compounds as we know from terrestrial samples such as aminoacids, carboxylic acids, humic acids, and hydrocarbons (Thurman, 1985), which are related to breakdown products of microbial life.

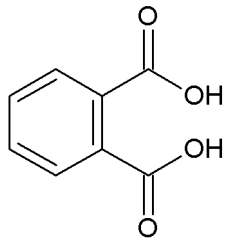
Beta-carotene, glycine, and phthalic acid (Fig. 4.8) were chosen as model biosignatures for the present study. The primary goal of this research was to evaluate the ability of Raman spectroscopy to non-destructively detect key molecular features of selected biosignatures first buried in different concentrations (500, 100, and 10 gkg^{-1}) in powdered evaporites and then analyzed through transparent gypsum plates of different thicknesses. This gypsum plates (3.3mm, 5.2mm, and 8.5mm thick) were prepared by cutting the natural crystal using perfect cleavage in the (0 1 0) direction.

(a)





(b)



(c)

Fig. 4.8 (a) Structure of beta-carotene (b) glycine, and (c) phthalic acid.

Using a long–working distance objective, all studied concentrations of biomarkers mixed in gypsum powder were easily detected. The characteristic Raman bands were observable for a 100 gkg^{-1} mixture of all chosen biomarkers through a 3.3mm, and even through a 5.2mm, gypsum plate. It was possible to detect a Raman signal of 10 gkg^{-1} phthalic acid/gypsum mixture and 10 gkg^{-1} beta- carotene/gypsum mixture even through a 5.2mm gypsum plate. 10 gkg^{-1} beta-carotene/gypsum mixture was still clearly distinguishable through an 8.5mm gypsum crystal due to the known resonance Raman effect of the molecule. This study shows that Raman microspectroscopy can be successfully used for the non-destructive detection of potential biosignatures buried in crystals of astrobiological interest. Because microscopic fossils have been discovered in gypsum deposits on Earth (Schopf *et al*., 2010) and may also exist in similar deposits on Mars, it is important to further investigate sulphate minerals in terrestrial conditions. Furthermore, such research complements the previous investigations of our group in which we have analyzed the lowest content levels of various biomarkers dispersed in powdered mineral matrices using bench and handheld Raman instruments. Culka *et al.* (2011) reported the detection of the mixture of nucleobases (thymine and adenine) at concentrations of 10 gkg^{-1} in a gypsum host mineral and the mixture of aminoacids (glycine and L-alanine) at the concentration 100 gkg^{-1} under outdoor winter Alpine conditions (including heavy snowfall), and using a

portable Raman instrument (Ahura First Defender XL equipped with a 785 nm diode laser and a fixed frontal probe).

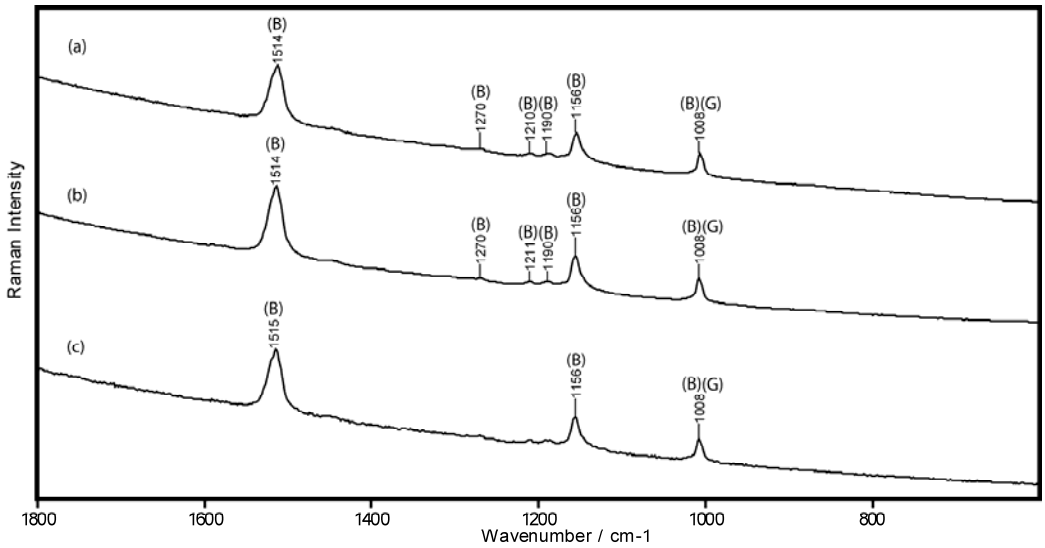


Fig. 4. 9 Raman spectrum of 10 gkg^{-1} betacarotene in a gypsum matrix through a gypsum crystal, (a)3.3mm, (b)5.2mm, (c)8.5mm, excitation wavelength 514.5 nm, wavenumber region 1800–600cm^{-1}. (B) represents the beta-carotene band and (G) represents the gypsum band.

4.4 Paper IV — Investigation of biomolecules trapped in fluid inclusions inside halite crystals by Raman spectroscopy

If life truly existed on Mars, it is reasonable to look for it in the areas which may be suitable for the preservation of biosignatures. From studies of fossil records on Earth, we can conclude that these areas usually consist of sedimentary rocks, mainly evaporates (halite [NaCl], gypsum, [$CaSO_4.2H_2O$], and anhydrite [$CaSO_4$]) (Mancinelli *et al.*, 2004). Evaporitic sedimentary deposits have been observed in numerous areas on Mars (Squyres *et al.*, 2004; Osterloo *et al.*, 2008). For this reason, halite crystals are among the important targets for astrobiological investigation, and model crystals are suitable for testing the abilities of Raman spectroscopy and other techniques.

The major significance of this study lies in its exploration of limits and possibilities of Raman microspectroscopy to be successfully used for the nondestructive detection of biosignatures trapped in the frame fluid inclusions inside laboratory-grown halite crystals. Hence, we provided laboratory-grown halite crystals (Fig. 4.10), which contained organic compounds in different concentration levels embedded within their fluid inclusions (Fig. 4.11). A series of aminoacids were chosen as biosignatures, because aminoacids are abundant in every terrestrial environment, and their use as biosignatures is strengthened by their chirality, which serves as an indicator of biotic origin. Raman microspectroscopy was employed to detect glycine, L-alanine, β-alanine, L-serine, and γ-aminobutyric acid trapped prepared in concentrations of 0.5M, 0.1M, and 0.05M inside fluid inclusions of laboratory-grown halite crystals.

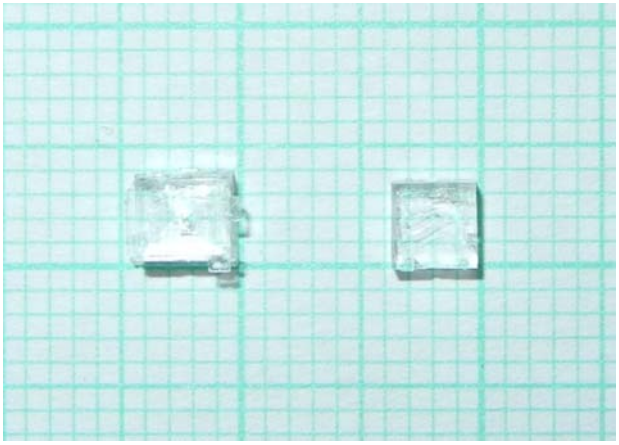


Fig. 4. 10 Laboratory-grown halite model crystals.

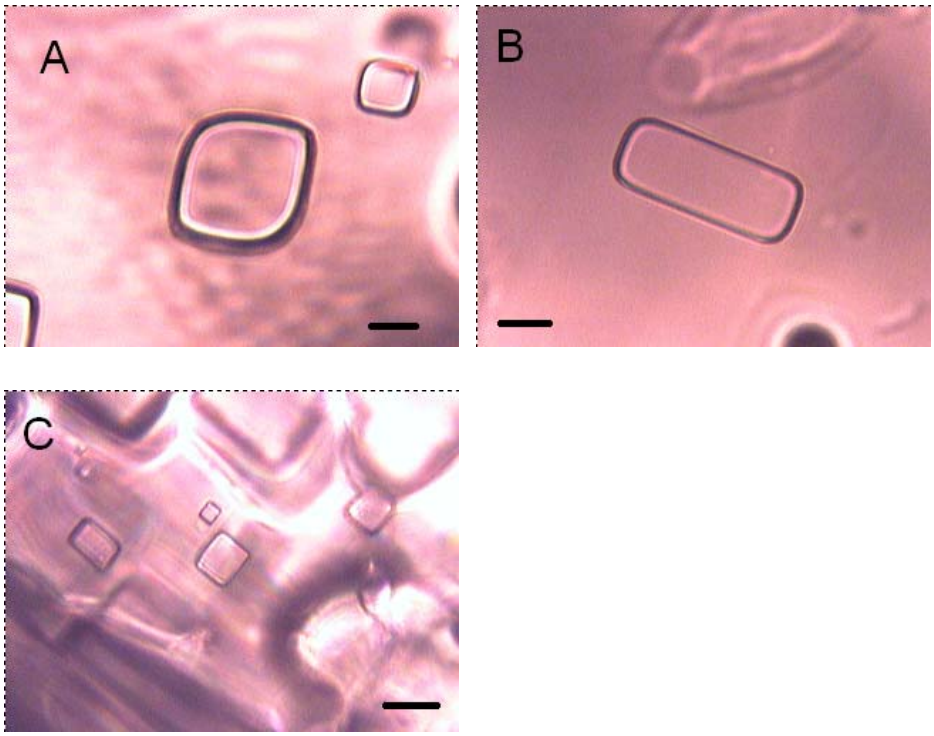


Fig. 4. 11 Photomicrographs of fluid inclusions, which usually had square or rectangular shapes in halite crystals. The scale bar is 20 μm.

The investigated biomolecules represent important targets for future astrobiological missions, especially to Mars. A Raman spectrometer is currently being miniaturized for the future ESA and NASA joint mission (ExoMars) to be launched in 2018. Such identification of organic molecules might be crucial in the search for life on Mars and other solar bodies.

We know from terrestrial conditions that organic molecules and microorganisms can be sealed within fluid inclusions and survive intact for even hundreds of millions of years. With the planned sample return missions from outer space, the contamination of Martian samples with terrestrial organics and microorganisms is a potential problem that must be avoided. Therefore, we opted for the novel approach of nondestructive analysis of fluid inclusions, which could be one way to explore future Martian samples.

Raman spectroscopy has proven to be able to detect such biomolecules nondestructively without any sample preparation, in the submicrometer range, in short measurement times, and in relatively low concentrations. The Raman spectra of these amino acids were successfully obtained and could easily be distinguished. The number of registered peaks and their intensity clearly correlate with the concentration of the given biomolecules within the inclusions. We can conclude that under the given circumstances the obtained detection limit of glycine (Fig. 4.12a, b) and L-alanine trapped inside fluid inclusions is as low as 0.05M, while the limits for β-alanine and L-serine were 0.1M and that of γ-aminobutyric acid only 0.5M.

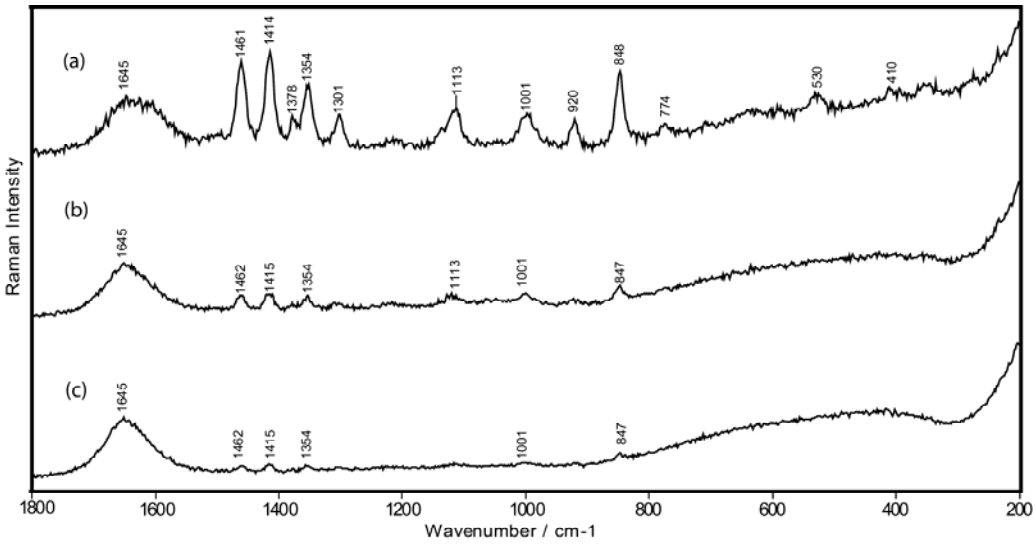


Fig. 4.12a Raman spectrum (1800–200 cm^{-1} range) of L-alanine measured in fluid inclusion: (a) 0.5M aqueous solution of L-alanine, (b) 0.1M aqueous solution of L-alanine, (c) 0.05M aqueous solution of L-alanine

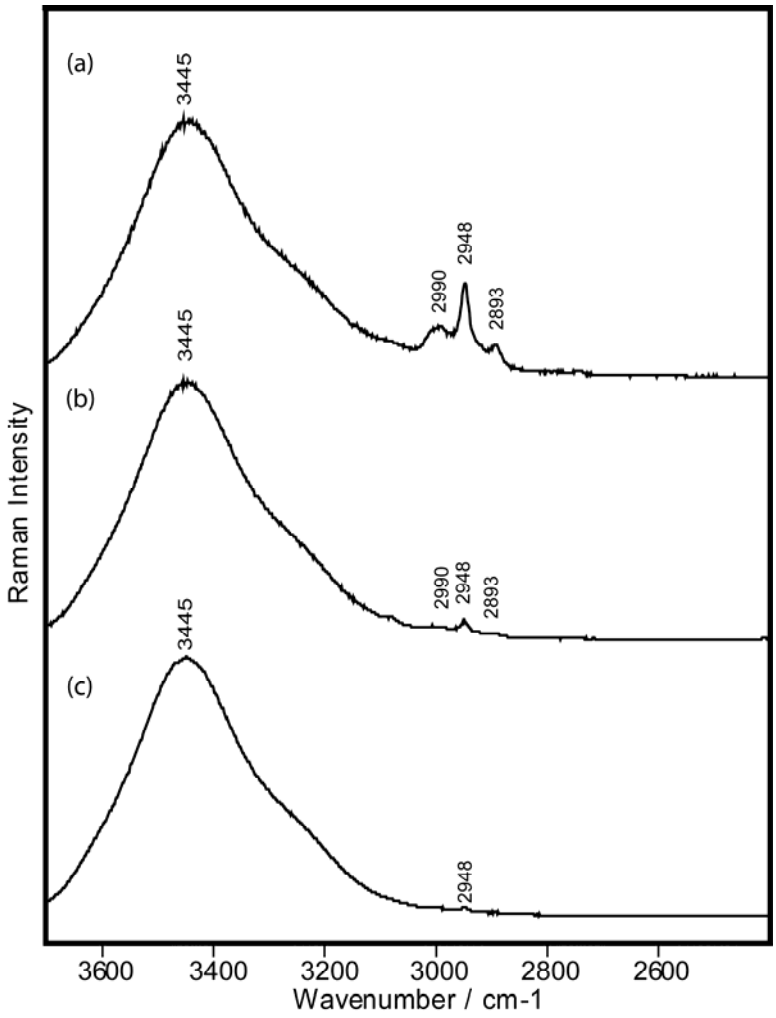


Fig. 4.12b Raman spectrum (3700–2400 cm^{-1} range) of L-alanine measured in fluid inclusion: (a) 0.5M aqueous solution of L-alanine, (b) 0.1M aqueous solution of L-alanine, (c) 0.05M aqueous solution of L-alanine

Chapter 5. Discussion, Conclusions and Outlook

With the upcoming NASA and ESA *in situ* rover exploration missions (the Mars Science Laboratory, with its planned launch in the fall of 2011; ExoMars 2018; and the follow-up Mars Sample Return missions beyond 2020) (Chapter 2.1), the question where to begin to look for the prebiotic and possibly biotic (and postbiotic) history of Mars becomes a serious issue. The success of future life-detection missions is highly dependent on carefully selected, potentially habitable geological targets as landing sites, which could enable both the concentration and the preservation of biological and nonbiological organics. A candidate site should be evaluated by its context, diversity, habitability, and preservation potential of organics. Because the surface of Mars is so inhospitable (Chapter 2.1.1), it is necessary to target physically protected areas that offer protection from galactic cosmic rays, such as evaporites (halite [NaCl], gypsum, [$CaSO_4.2H_2O$], and anhydrite [$CaSO_4$]), which could preserve organic molecules, as has been concluded from terrestrial studies on fossil records (Chapter 2.3). The large reservoirs of evaporites detected via remote sensing and in situ studies on Mars make them primary targets in the search for organic compounds, because they represent places where liquid water might still exist today or might have existed in the past.

Raman spectroscopy will hopefully have the opportunity to accompany the ExoMars (2018) exploration missions, because of its non-destructive nature and ability to unambiguously detect C-C and C-H bonds, carbonates, sulfates, chloride minerals, and most generally organics (Chapter 4). A miniaturized version with its low mass makes it appropriate for small payloads. The ExoMars mission will access the subsurface for the first time. The Rover subsurface sampling device will drill to the required depth (but a maximum of 2 m) while investigating the borehole wall mineralogy and collecting samples. The samples will then be crushed into a fine powder, which will be investigated by means of Raman spectroscopy (see our spectroscopic analysis

on powders in Chapters 4.1–4.3) and other instruments. When sample return missions become a reality beyond 2020, it will be essential to minimize the contamination risk, perhaps via nondestructive analysis of solid and fluid inclusions in minerals. Possible approaches to the high-resolution detection of biosignatures inside inclusions of minerals should include, among others, Raman spectroscopy (Chapter 4.4).

In the framework of this thesis, it has been proven that Raman spectroscopy: (1) is applicable for detection of biosignatures in solid states as well as dispersed in aqueous solutions, and (2) is sensitive to both organic and inorganic compounds. The analytical resolution is unfortunately low compared to some other techniques, but Raman spectroscopy is nevertheless a very useful tool for specific purposes that have been studied recently in our scientific group: for example, analysis of endolithic colonies (Vítek *et al.*, 2010); analysis of biosignatures and rocks in situ even in harsh conditions by means of hand-held instruments with no need for any sample preparation (Culka *et al.*, 2011; Jehlička *et al.*, 2011; Culka *et al.*, 2010; Jehlička *et al.*, 2010, Vítek *et al.*, 2011); and the study of biosignatures embedded in fluid inclusions beneath the surface of translucent minerals using spatial resolution down to the micron-level (Osterrothová and Jehlička, Appendix IV).

Several suggestions can be made with respect to the search for biosignatures on Mars based on the studies conducted in this thesis:

(1) Many lichen species are considered to be extremophiles in terms of temperature, radiation, and desiccation survival. Therefore, lichens have been proposed, together with unicellular algae and bacteria, as the living systems most likely to resist the extreme conditions of outer space (Sancho *et al.*, 2008). Extremophile organisms like lichen support the Panspermia theory, speculation about the possibility of life transfer between Earth and other planets. Substances found in lichens that absorb harsh UV radiation (parietine and carrotenoids in Xanthoria

elegans, and usnic acid in Rhizocarpon geographicum) are thought to be responsible for the organism's resistance to high doses of UV radiation (Wynn-Williams *et al.*, 2002a; Wynn-Williams and Edwards, 2002b; Edwards *et al.*, 2003a). The results of the usnic acid study by means of Raman microspectroscopy indicate that a near-infrared laser at an excitation wavelength of 785 nm provides the clearest and the most informative spectra due to the reduction of fluorescence emission, as opposed to the 514.5 nm excitation wavelength, which proved to be inadequate. Edwards *et al.* (2003b) also successfully conducted spectroscopic study on usnic acid using a 1064 nm excitation from an Nd3+/YAG laser. However, the excitation wavelength currently being considered for the Raman spectrometer on board ExoMars should be in the visible region (532 nm), which is probably less sensitive to the eventual detection of such a pigment.

The concentrations of usnic acid dispersed in the mineral matrices were far higher than those of beta-carotene: 1 g kg^{-1} for the matrix and 5 g kg^{-1} when mixtures were analyzed through the gypsum crystal, which means that the sensitivity of Raman spectroscopy toward pigments in the non-resonant mode is limited. The laser spot size (using a 50× objective lens resulting in a laser spot of approximately 2 μm in diameter) likely plays a major role when analyzing the low contents of biosignatures dispersed in mixtures where the homogeneity of the sample is crucial. ExoMars's Raman prototype currently counts on a laser spot of around 50 μm on the target (to be compatible with the other analytical tools of the mission), which might solve the problem with little inhomogeneity of the powder samples.

On the other hand, Raman spectroscopy has proven the ability of the depth-resolving method, analyses through the appropriate crystals show low noise and good quality. (Chapter 4.1, Appendix 1)

(2) The estimated mass of meteoritic material reaching the surface of Mars should be between 1.63 × 10-6 and 7.36 × 10-8 kg $m^{-2}yr^{-1}$ (Flynn and McKay, 1990), meaning that abiotic extraterrestrial organic compounds may be expected on Mars. Compound classes of interest also include carboxylic acids. Raman spectroscopy demonstrated the capability for identifying phthalic and mellitic acids, carboxylic acids of high relevance to astrobiological studies, in admixtures and when analyzed buried inside gypsum and halite crystals. The detection limit of phthalic acid mixed in mineral matrices and analyzed through crystals was 10 gkg^{-1}, approximately ten times higher than for previously studied usnic acid and far higher (10,000 times; the detection limit was proposed to be 1 $mgkg^{-1}$) than for beta-carotene, using both 514.5 and 785 nm excitation wavelengths. A Raman mellitic acid signal was obtained at a concentration as low as 10 gkg^{-1} in a halite matrix and at a concentration of 50 gkg^{-1} when analyzed through a halite crystal. In the case of mellitic acid mixed with gypsum and analyzed through a gypsum crystal, the detection limit is 50 gkg^{-1} using both excitation wavelengths. These results are similar to those reported by Culka and Jehlička (2010a) on the study of urea in calcite and gypsum powder matrices: 10gkg^{-1} of urea in both the calcite and the gypsum mineral matrices and 50gkg^{-1} when measured through the appropriate transparent crystals (approx. 2 mm thick).

Our study shows that both excitation wavelengths could be successfully used to determine these carboxylic acids in mineral matrices and analyzed through crystals, but Raman microspectroscopy probably cannot offer detection limits sensitive enough to the concentrations of carboxylic acid expected to be found, for example, in the Martian regolith. To achieve better detection limits, incorporation of the ultra-sensitive surface enhanced Raman scattering (SERS) for trace detection will be necessary and the samples will need further

manipulation and pretreatment. On the other hand, the Raman system coupled with a confocal microscope continues to be able to collect high-resolution spectra of carboxylic acids even through a 5 mm halite crystal by means of adjusting the pinhole and in this way regulating the signal of the matrix. From an astrobiological point of view, halite crystals provide especially great potential for Raman spectroscopy, because the crystal itself has no Raman active modes and therefore there is no risk of obscuring the results, although sometimes background noise can disvalue the collected spectra (Chapters 4.2, Appendix 2).

(3) Evidence for evaporitic sulfate minerals, such as gypsum and jarosite, has recently been reported on Mars (Squyres *et al.*, 2004; Langevin *et al.*, 2005; Gendrin *et al.*, 2005). Microscopic fossils have been discovered in gypsum deposits on Earth (Schopf *et al.*, 2010). The detection of organic material, including amino acids and their amine degradation products, in ancient terrestrial sulfate minerals have also been reported (Aubrey *et al.*, 2006). Amino acids and amines appear to be preserved for geologically long periods in sulfate minerals, and a few millimeters of gypsum on Mars should be able to shield potential organic molecules from UV penetration and determination. These data prove that sulfate minerals should be among the prime targets in the search for organic compounds, including those of biological origin, on Mars.

Detection of beta-carotene, glycine, and phthalic acid buried in different concentrations (500, 100, and 10 gkg^{-1}) in powdered evaporites and then analyzed through transparent gypsum plates of different thicknesses (3.3mm, 5.2mm, and 8.5mm thick) complement our previous studies and prove that if biosignatures are concentrated and embedded even in great depth in minerals of interests as solid

inclusions it is possible to obtain chemical information without any extraction and sample preparation. The detection limit is again probably not sensitive enough for biomolecules in a non-resonant mode or to be used in planetary exploration. The characteristic Raman bands were observable for a 100 gkg^{-1} mixture of all chosen biomarkers through a 3.3mm, and even through a 5.2mm, gypsum plate. It was possible to detect a Raman signal of 10 gkg^{-1} phthalic acid/gypsum mixture and 10 gkg^{-1} beta-carotene/gypsum mixture even through a 5.2mm gypsum plate. The 10 gkg^{-1} beta-carotene/gypsum mixture was still very clearly distinguishable through an 8.5mm gypsum crystal due to the known resonance Raman effect of the molecule. While excited with visible 514.5 nm Argon laser, no fluorescence or residual spectral background was observed for the studied molecules. Raman microspectroscopy has thus proven to be an appropriate instrument for non-destructive sample-return missions (Chapters 4.3, Appendix 3).

(4) Especially interesting to astrobiologists are inclusions in minerals that have been precipitated in low temperatures and in the presence of microorganisms (Parnell *et al.*, 2002). It has been argued that inclusions in halite can easily trap microorganisms and organic debris. Although the age of the halite crystals and fluid inclusion are sometimes challenged, these studies conclude that microorganisms can survive in evaporates for periods of thousands to hundreds of millions of years. Such conclusions have implications for the long-term survival of biota on Earth and elsewhere in the solar system. In our study, Raman spectroscopy was tested for the identification of biomolecules of astrobiological interest (glycine, L-alanine, β-alanine, L-serine, and γ-aminobutyric acid) trapped in fluid inclusions inside halite model crystals. We conclude that under the given circumstances the obtained detection limit of glycine and L-alanine trapped inside fluid inclusions

is as low as 0.05M, while the limits for β-alanine and L-serine were 0.1M and that of γ-aminobutyric acid only 0.5M. Edwards *et al.* (2011) investigated the liquid samples of ergosterol-dichlormethane mixtures and obtained a detection limit that was a bit lower: 0.015 M. The liquid-sample mixtures resolved the problem with sample homogeneity. Raman microspectroscopy has proven to be able to detect investigated aminoacids trapped in fluid inclusions nondestructively and without any sample preparation, in short measurement times, and in relatively low concentrations. The number of registered Raman bands of investigated aminoacids and their intensity clearly correlate with the given concentration of biomolecules within fluid inclusions. The depth of inclusions under the surface was up to 100 μm. All measured Raman spectra remained identical in this setting condition for individual biomolecules embedded in fluid inclusions of model halite crystals; however, the spectra collected from deeper inclusions show bigger noise. The sizes of the inclusions studied in these experiments were in the range of 20–100 μm, and the size in this region did not have a significant influence on the quality of the given spectrum. As was already mentioned above, halite crystals with no Raman active modes, and therefore with no risk of obscuring the results, are among important targets for astrobiological purposes and for study by Raman microspectroscopy. Other techniques would provide better analytical resolution when analyzing biosignatures trapped in fluid inclusions, but the nondestructive behavior, coupled with the fact that there is no need to prepare samples, are the main advantages when analyzing inclusions for astrobiological purposes (Chapters 4.4, Appendix 4).

This Ph.D. thesis has sought to answer several questions concerning the feasibility of Raman spectroscopy for the identification of organic inclusions in minerals for the field of exobiology. However, the work beckons follow-up research in certain areas:

(1) Raman imaging based on chemical composition distribution in heterogeneous samples can probably offer significant advantages in the field, especially in the study of the distribution of different phases within the inclusions.

(2) Achieving lower detection limits, preferably ppm to ppb, for organic molecules from natural samples without exaction or any preparation would be beneficial.

(3) Further analysis of natural samples with embedded microorganisms and organic debris is needed in the study of the capability of Raman microspectroscopy.

To conclude, Raman spectroscopy has proven to be a valuable method due to its ability to identify organic compounds, even embedded in inclusions in minerals. However, the small laser spot size (2 to 10 μm diameter, depending on the use of the objective lens) is both an advantage and likely also a handicap of the Raman microspectroscopy. When analyzing biosignatures in mineral powders, homogeneity on a micro scale is almost impossible to achieve. Multiple measurements are needed in order to reproduce the data. Hence, the proposed laser spot size (around 50 μm) in the Raman instrument on board ExoMars might be good choice. On the other hand, high spatial resolution is favorable when analyzing inclusions in minerals. The choice of excitation wavelength remains a major issue for the proposed Raman studies on Mars. An extension of the database of biosignatures that reflect the fundamental and universal characteristics of life and their detection limits by different methods is needed for future life-detecting missions. Further research activities are necessary that would a) improve the exploration methodology and instrumentation capabilities, b) increase the chances of astrobiological discoveries, and c) maximize the input of the Mars sample return.

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Appendices