Applications of Raman and Infrared Spectroscopies to the research and conservation of subterranean cultural heritage

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ABSTRACT: Here we report on the applicability of infrared and Raman vibrational spectroscopies as useful techniques in the scientific research of the subterranean cultural heritage (such as wall paintings and archaeological objects found in prehistoric caves, mural paintings in churches and ancient catacombs, mummified bodies and human remains in ancient burials). We will also pay attention to the use of vibrational spectroscopies techniques in the investigation of the underground cultural heritage biodegradation caused by fungi and bacteria.

1 INTRODUCTION

Nowadays, it is well-known that microorganisms play a key role in the deterioration of cultural heritage, as exemplified by the Altamira and Lascaux caves or the Tutankhamun's tomb in Luxor's famous Valley of the Kings, which were visibly and irreparably damaged over decades by the damp breath of large numbers of visitors flocking to see the boy king's burial chamber or the often called "Sistine Chapel of Prehistoric Art" (Allemand & Bahn 2005, Saiz-Jimenez et al. 2011). Until they were completely closed to the public, and carefully-crafted replicas were built nearby.

Over the last years, infrared absorption and Raman scattering spectroscopies have become two of the main analytical techniques applied to items of cultural heritage due to their versatility and ability to provide information on the chemical nature of both organic and inorganic compounds (Bersani et al. 2008, Cappitelli et al. 2005, Egel and Simon 2013, Lindgren et al. 2011, Manso & Carvalho 2009, Mysak et al. 2011, Naumann et al. 2005, Shahack-Gross et al. 2014, Spring et al. 2008, Tomasini et al. 2012). In particular, micro-IR and micro-Raman are both non-destructive surface techniques that require no sample preparation, which have significantly improved the potential of vibrational spectroscopy and allow for the fast and reliable identification of mixtures of compounds. Thus, the constituent materials of a heterogeneous sample give rise to a specific spectral fingerprint, which can be properly interpreted, from an analytical chemistry point of view, with the help of appropriate spectral libraries or from the infrared/Raman spectra collected for reference compounds.

The Raman spectrum of a sample may be often obscured by the strong fluorescence emission of the own specimen in the visible region of the electromagnetic spectrum, particularly when using laser excitations in the blue and green regions (400-520 nm). This problem can be overcome in many cases by using a near-infrared (NIR) excitation wavelength (typically upon laser excitation at 785 or 1064 nm).

When coupled to a microscope, a Raman instrument with a high spatial resolution (of even less than 1 μm) is available. Raman microscopes exhibit a high signal-to-noise ratio and are ideal for characterizing in detail, in the laboratory, weak scattering samples, specific regions within a heterogeneous sample or object, the scientific study of artworks or archaeological tools, or the nondestructive analytical characterization of ancient materials showing signs of being degraded in their natural environments. All of this with the additional and major advantage that no chemical or mechanical pretreatment of the sample is necessary for the presentation of the specimen to the micro-Raman spectrometer (Adar et al. 2007, Casadio et al. 2010, Ernst 2010, Mahmoud 2013, Pelosi et al. 2013). Of particular relevance to the scientific research of biodegradation of artwork is the ability to obtain Raman spectra directly from specimens in their natural state of hydration, without further desiccation, provided that the weak Raman scatterings from glass and water will not strongly affect the observation of the Raman spectrum of the sample under study (Schiavon et al. 2013). This ability also makes Raman spectroscopy an useful analytical technique in Biology, Biochemistry, Medicine, Geology, Astrobiology, Paleontology and in many other fields of research (Amini et al. 2014, Chen et al. 2007, Edwards et al. 2012, Keating & Byrne 2013, Keiner et al. 2013, Kong et al. 2013, Steele et al. 2007, Stetten et al. 2008, Thomas et al. 2013, 2014).

Major technical advances over the last few years have resulted in smaller and easier to use infrared and Raman lightweight field-portable miniaturized spectrometers and interferometers of sufficient quality, opening up much wider application of infrared and Raman spectroscopy, particularly for adoption into field usage, than usual laboratory bench-top FTIR or Raman instruments. Nonetheless, portable or handheld Raman spectrometers produce data that usually are much noisier than what a high-quality bench-top spectrometer produces due to the shorter spectral acquisition time and lower number of averaged scans (Capel-Ferrón et al. 2014, Hernández et al. 2012, Jorge-Villar et al. 2011, Vandenabeele et al. 2014).

Figure 1. Raman spectroscopic analysis performed by means of a portable instrument of (a) a prehistoric stone lamp and (b) a lithic instrument with traces of pigments. c. Portable Raman spectrometer equipped with an optical fiber and a head probe. d. Raman spectrum performed with a portable spectrometer.

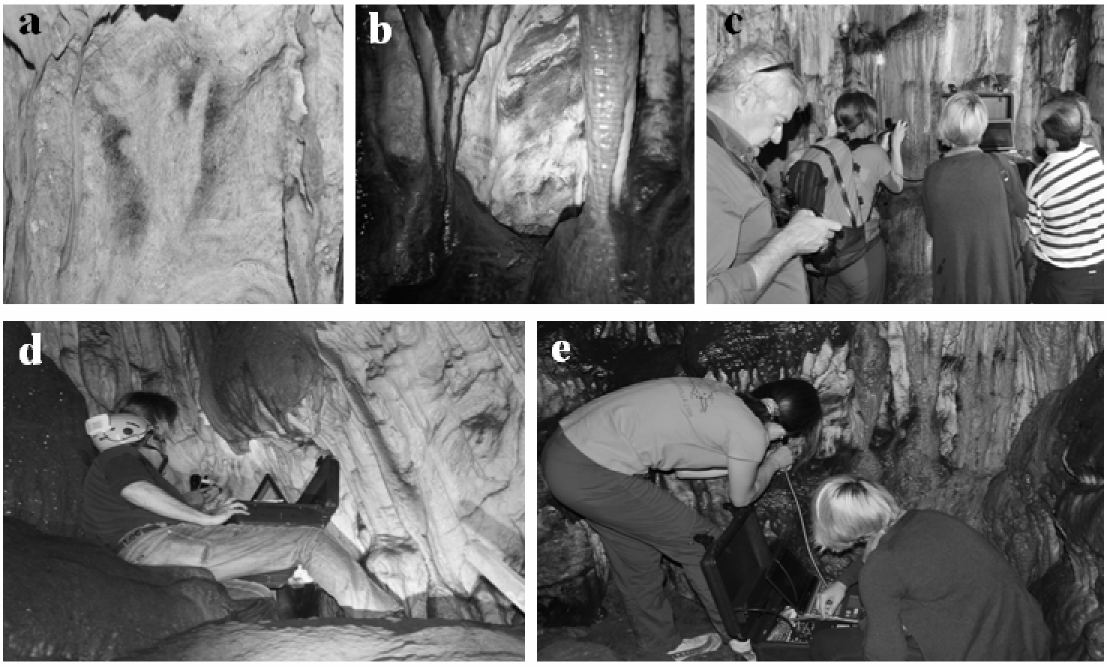

Figure 2. a and b. The well-known negative hand imprint and one of the several prehistoric finger paintings documented in many sites of the Ardales Cave (Guadalteba County, Málaga, Spain). c. In situ Raman analysis of a hind head. d. Raman analysis of the negative hand imprint (this rock painting is located near five meters above the ground floor). e. Two of us performing Raman measurements of the mineral pinkish pigment used to draw a finger painting in a wet wall of the Ardales Cave.

2 PREHISTORIC ROCK ART, ETRUSCAN CATACOMBS AND FRESCOES

Studying of wall paintings or frescoes in churches, historical buildings or ancient catacombs, in which sampling is not allowed, constitutes an evident example in which there is a need to bring the scientific Raman instrumentation to the cultural heritage site, in order to perform *in situ* noninvasive vibrational spectroscopic measurements, often in combination with other analytical techniques, such as XRF analysis. Examples of this approach are the *in situ* Raman spectroscopic study of San rock art in South Africa (Tournié et al. 2010); the analysis of antique Egyptian wall paintings in the tomb of Menna (Vandenabeele et al. 2009) and the Theban tomb TT277 near Luxor (Mahmoud 2013); the *in situ* noninvasive Raman microspectroscopic investigation of polychrome plasterworks in the Alhambra of Granada, Spain (Dominguez-Vidal et al. 2012); the *in situ* non-destructive analysis, by making use of a suite of three different portable instruments, for carbon screening before sampling for dating prehistoric rock paintings in the Rouffignac-Saint-Cernin and Villars caves, both of them located in Dordogne, France (Beck et al. 2013, Lahlil et al. 2011); the archaeometric study of medieval wall paintings in a large group of rock hewn churches in Cappadocia, Turkey, dating back to a period between the sixth and ninth centuries (Pelosi et al. 2013); the micro-Raman spectroscopic and GC/MS analysis of wall paintings of the 19th century iconographer Dicho Zograph, in churches from Republic of Macedonia (Cukovska et al. 2012); or the 16th century wall paintings in the church of Agios Sozomenos in Galata, Cyprus (Nevin et al. 2008).

Raman spectroscopy, in combination with other analytical techniques, has been also used to study the Etruscan art of wall painting, such as in the “Tomba della Quadriga Infernale” (4th before BC), in Sarteano, Siena, Italy (Pallecchi et al. 2009); on the Tomba dell’Orco in the Etruscan necropolis of Tarquinia, Lazio, Italy (Sodo et al. 2008): on Etruscan polychromes on architectural terracotta panels at present on display at the Villa Giulia Etruscan Museum in Rome (Bordignon et al. 2007); or on black powders found in three different types of bronze vessels at the Pompeii archaeological site (Canevali et al. 2011); as well as on powdered pigments found in bowls from the Pompeii archaeological site and some wall-painting fragments from the Vesuvian area, conserved in the National Archaeological Museum of Naples (Aliatis et al. 2010). Piovesan et al. (2011) also performed Raman spectroscopy, among a wide range of analytical methods, on 57 fragments of wall painting excavated from the Temple of Venus, Pompeii.

Several scientific papers have been published so far on the Raman characterization of prehistoric rock art, and most of them by combining both on-site investigations with further analyses of micro-samples in the laboratory (de Faria et al. 2011, Gomes et al. 2013, Goodall et al. 2009, Hernanz et al. 2008, 2010, 2012, Iriarte et al. 2013, Jezequel et al. 2011, Lofrumento et al. 2011, Mas et al. 2013, Ravindran et al. 2013). However, the requirements for the portable Raman instrumentation when dealing with prehistoric rock art are even more stringent that the analysis of wall paintings in churches, ancient buildings or catacombs, provided that constraints of weight and access to electrical power are typically more larger and difficult in the former case. Moreover, inside the prehistoric caves, the ground surface is not usual flat, and the use of a tripod, to mount the headprobe of the mobile Raman spectrometer, is not so easy in this situation. In addition, the sample of interest may be located on a rock face several meters above the ground, thus creating additional problems not only related to the carriage of the Raman instrumentation from the “parking area” into the prehistoric cave, but also to bringing the headprobe of the mobile Raman spectrometer into near contact with the specimen for exact positioning and fine focusing, and quite often lacking of the desired help of a sufficiently stable scaffolding.

Some authors have even designed, constructed and developed their own specific instrumentation for the scientific diagnosis of art objects, such as a mobile Raman-XRF apparatus developed by Andrikopoulos et al. (2006) with the aim of its non-destructive application to old master paintings, providing both Raman and XRF spectra from the same spot on the surface of the specimen under study. The experimental validation of such a mobile Raman-XRF instrument was performed on an experimental icon painted with traditional Byzantine techniques, as well as on minute samples from a post-Byzantine icon. Or the helium jet aimed directly at the laser spot of a 785 nm portable Raman spectrometer, developed by Ruvalcaba-Sil et al. (2013) to ensure the safe study of sensitive works of art and other types of fragile materials. The system, denoted as HERAS (Helium Raman System), simply consisted of a pinhole collimator, coupled to a helium line and a gas mass flux control, being finally tested on an original XVI painting of Baltasar de Echave Orio at the National Museum of Art in Mexico City. As in the preliminary tests on pyroxylin, vermilion and ochre paint references and some pigment samples, the small burns that can be caused by the 785 nm laser beam on the surface of the specimen under analysis without such a helium jet, were fully avoided in all cases.

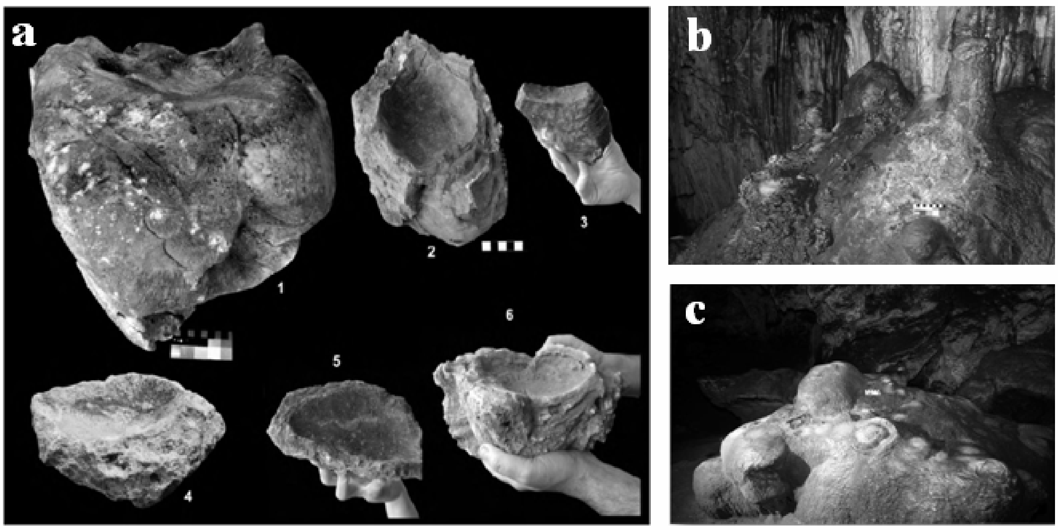

Figure 3. Prehistoric mobile (a) and fixed lamps (b and c) found in the Ardales Cave (Guadalteba County, Málaga, Spain).

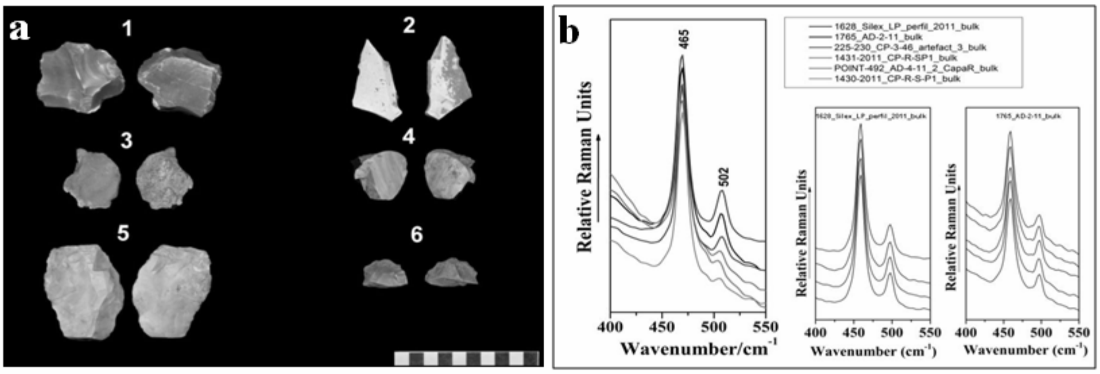

Figure 4. a. Photographs of the six lithic tools exhumed along 2011 in two karst sites of the Guadalteba County (Málaga, Spain) : (1) 1628-Silex-LP-perfil-2011-bulk; (2) 1765-AD-2-11-bulk; (3) 225-230-CP-3-46-artefact-3- bulk; (4) 1431-2011-CP-R-S-P1-bulk; (5) Point-492-AD-4-11-2-CapaR-bulk; (6) 1430-2011-CP-R-S-P1-bulk. b. FT-Raman spectra of the whole series of lithic tools subject of study and comparison between the FT-Raman profiles collected for the 1628- Silex-LP-perfil-2011-bulk and 1765-AD-2-11-bulk specimens at different spots on their surface.

3 FUNERARY ARTEFACTS, MUMMIFIED BODIES AND ANCIENT BURIALS

FTIR spectroscopy has been applied to the analysis of pathological and non-pathological human remains for determining burial duration of skeletal remains, which is one the most important aspects of forensic anthropology, using the crystallinity index and carbonate-phosphate index as a means of distinction between recent and archaeological, anthropological bone samples. Tuberculosis and syphilis infected human bones from different burial environments were also analyzed by means of the same FTIR-based method to see if changes in crystallinity interfere with the process of burial dating (Nagy 2008). In a similar way, Raman microspectroscopy with 785 nm laser excitation has been also used to study dynamic chemical changes in turkey bone tissues during short burial intervals, between 12 and 62 days. The results of this Raman spectroscopic study indicated that chemical changes upon burial of turkey bone fragments, due to soil bacteria, are time-dependent in a scale of days, and mainly reflect the collagen structural conformational changes taking place in the so-called “amide I spectral region”. Thus illustrating the potential use of Raman spectroscopy as a fast, non-destructive and reliable method for estimating the burial duration of bones for forensic purposes (McLaughlin & Lednev 2011). Later on, the same authors published a second article in which they again made use of Raman spectroscopy to discriminate bone samples originating from four different species (bovine, porcine, turkey and chicken). The treatment of the collected spectral data, using partial least squares discriminate analysis (PLS-DA) with leave-one-out cross-validation, resulted in the successful discrimination of bones from various animal species, without no overlap between groups (McLaughlin & Lednev 2012).

Reiche et al. (2004) reported on the *in situ* Raman spectroscopic investigation of the adorning gemstones on the reliquary *Heinrich’s Cross* from the treasury of Basel Cathedral. On the other hand, a collaboration between the Getty Conservation Institute Museum Research Laboratory and the Getty Museum Antiquity Conservation Department has been recently carried out on the J. Paul Getty Museum’s mummy 91.AP.6 dating to the first century (and known by the name “Herakleides” from a painted inscription on the feet of its shroud), to answer questions about the traditions surrounding Romano-Egyptian mummification, about the preparation and use of Roman pigments, and about the long term preservation of this object and other like it. Preliminar results of this study have revealed considerable information regarding the materials, fabrication, and rituals used to create and preserve this mummy. Herakleides belongs to a small group of similar Romano-Egyptian mummies known collectively as “red-shroud mummies” due to their comparable decorative schemes and because they are painted from head to toe with the pigment red lead, Pb_3O_4, as the major phase, and a minor phase,

Pb_2SnO_4, of lead tin oxide. Lead isotopes ratios were found to match the mixed lead sources typically associated with Rio Tinto, Spain (a site extensively mined for silver during the first century AD). Lead tin oxides does not occur naturally, and its incidental occurrence within the sample indicated that the material was heated under oxidative conditions at temperatures in excess of 650 ºC (Svoboda & Walton 2007, Walton & Trentelman 2009).

Gniadecka et al. (1997) carried out a Raman spectroscopic study on 500-year-old mummified skin samples obtained from four mummies found in Qilakitsoq in Greenland, dating from AD 1475 (± 50 years) and being the oldest preserved bodies in the Artic region. In this case, the analysis was performed by means of a bench-top FT-Raman spectrometer, with NIR laser excitation at 1064 nm. The spectra of the different mummified skin samples were all very similar, but distinctly different from those of fresh and freeze-dried contemporary skin specimens. Particularly in the Raman spectra of the ancient skin, the *amide I* (1640-1680 cm^{-1}) and *amide III* (1220-1290 cm^{-1}) bands displayed a very low intensity, indicating loss of protein and/or changes in the secondary protein structure. Previously, Williams et al. (1995) performed Raman spectroscopy of the 5300-year-old skin of the late Neolithic man (the Alpine 'Iceman', also known as Ötzi) whose body was preserved by freeze-drying in a glacial field on the border of Italy and Austria. The Raman data suggested that the keratin component of stratum corneum was degraded while the conformation of the lipids was still intact. The results of these two Raman studies were also in agreement with the Raman study performed on 1000-year-old skin samples from mummified bodies of the Chiribaya culture at the Southern Peruvian desert, what implied that most changes in the molecular structure of the human skin (represented mainly by collagen) take place in a relatively short time interval during the natural mummification process. Although the Raman spectra of the Peruvian mummies suggested an increased lipid content in lightly pigmented skin compared to contemporary skin and the skin of the mummies preserved in ice, likely due to embalming, by which means a better preservation is achieved (Gniadecka et al. 1999).

Howell Edwards, Professor in Molecular Spectroscopy at the University of Bradford, has been very active in the applications of Raman spectroscopy to chemical problems in diverse areas of art history and archaeology, forensic science, extremophiles and astrobiology (Edwards 2004). For instance, he and his collaborators performed a comprehensive Raman spectroscopic study of several pigments from ancient Egyptian funerary artefacts dating from the 17th Dynasty to the Graeco-Roman period, representing some 2000 years of Egyptian history, and using different laser excitation wavelengths. The artefacts included sarcophagi, coffin lids, shroud covers and mummy face-masks (Edwards et al. 2004). Edwards et al. (2007a) performed Raman spectroscopy on *natron*, a naturally occurring *evaporitic* mineral deposit from the Wadi Natrun, used in the mummification ritual in ancient Egypt, which involved the evisceration of the corpse and its desiccation using *natron*. Edwards and collaborators also applied Raman spectroscopy, with near-infrared laser excitation at 1064 and 785 nm, on specimens from human remains exhibiting unusual preservation excavated from a seventh century stone cist burial at Towyn y Capel in Anglesey, UK (Edwards et al. 2007b), and on an intact Bronze Age log coffin found in 1834 in a tumulus at Gristhorpe, North Yorkshire, UK, fashioned from the hollowed-out trunk of an oak tree, which was found to contain a well-preserved skeleton stained black from the oak tannins, wrapped in an animal skin and buried with a range of grave artefacts, including a bronze dagger, flints and a bark vessel (Edwards et al. 2010, Melton et al. 2010).

4 ÖTZI, THE "TYROLEAN ICEMAN": AN UNPRECEDENTED ARCHAEOLOGICAL DISCOVERY

Ötzi, also referred to as the "Alpine Iceman", is the well-preserved natural mummy of a man who lived about 5300 years ago, and who was discovered in late summer 1991 by two German hikers in the Schnalstal glacier, Ötztal Alps, on the border between Austria and Italy. He is Europe's oldest natural human mummy found until now, and the exhaustive multidisciplinary research performed on his body and belongings, which are displayed in the South Tyrol Museum Archeology in Bolzano, has attracted widespread attention and offered a new picture of Chalcolithic Europeans (Bahn & Everett 1993, Barfield 1994). The exceptional preservation of this prehistoric corpse was possible thanks to its location in an almost horizontal gully in which it remaining motionless, frozen to the ground in cold ice, after his violent death at an age of approximately 40-50 years. Ötzi was endowed with a unique archaeological collection of several exceptionally preserved items of clothing and equipment, and his finding constitutes one of the most sensational archaeological discoveries ever and is a real breakthrough for the archaeological and paleoanthropological sciences. After numerous radiological studies during ten years, an arrowhead lodged within the mummy's left shoulder region was finally discovered (Gostner et al. 2002). Researchers were even able to obtain, by means of multislice CT scan technology, detailed images of the damage caused to the blood vessel by the arrowhead (Pernter et al. 2007). The re-appraisal of the former radiological examinations of the Tyrolean Iceman using modern instrumentation finally allowed for the exact identification of the stomach, and to the finding that it was not empty, as initially thought, but well-filled, thus shedding new light on the scenario leading to his violent death (Gostner et al. 2011). Geneticists also sequenced his DNA, finding that modern inhabitants of the Mediterranean's Corsica have the most similar sequences (Keller et al. 2012).

Mummified skin is extremely resistant to decomposition. External influences or the action of microorganisms, however, can degrade the connective tissue and lay the subjacent tissue open. To determine the degree of tissue preservation in mummified human skin and, in particular, the reason for its durability, Janko et al. (2010) extracted skin samples from three sites of Ötzi, in order to investigate the structural integrity of its main protein, type I collagen, by means of atomic force microscopy and Raman spectroscopy. Both methods indicated that the ultrastructure and molecular structure of the 5300-year-old glacier mummified collagen were preserved extremely well. Raman spectroscopy revealed spectra that were characteristic of type I collagen, and the *amide I* (1667 cm^{-1}) and *amide III* (1245-1270 cm^{-1}) Raman features indicated that the collagen molecules retained their helical conformation. The loss of interstitial water resulted in a more densely packed structure of the fibrils and the generation of additional cross-links within the collagen. No evidence for collagen degradation was found that could have been caused by freeze-thaw cycles, microorganisms or other biological influences. The AFM/Raman results also supported the theory that the Tyrolean Iceman was covered by snow and ice immediately after his violent death.

Later on, the same scientists discovered what appeared to be intact red blood cells (RBCs) in tissue samples from a wound that one Ötzi's hand suffered, as well as the arrow wound to the chest (Janko et al. 2012). The same authors wrote in their scientific report: "It was initially assumed that the blood had disintegrated owing to autolysis within the corpse". Autolysis is the process where oxygen, which is especially abundant within oxygen-carrying red blood cells, reacts with and fragments the proteins that comprise cells. The morphological and molecular composition of the blood corpuscle was verified by atomic force microscopy and Raman spectroscopy. The cell size and shape approximated those of healthy, dried, recent RBCs. Raman spectra of the ancient corpuscle revealed bands that are characteristic of haemoglobin. Additional vibrational modes typical for other proteinaceous fragments, possibly fibrin, suggested the formation of a blood cot. The Raman band intensities, however, were approximately an order of magnitude weaker than those of recent RBCs. This fact pointed to a decrease in the RBC-specific metalloprotein haemoglobin and, thus, to a degradation of the cells. Together, the AFM and Raman data confirmed that Ötzi's red blood cells were preserved for more than 5300 years and gave the first insights into their degradation (Janko et al. 2012).

5 BIODEGRADATION OF SUBTERRANEAN CULTURAL HERITAGE

The paintings from *Tomba della Scimmia* and *Tomba del Colle* (or *Tomba Casuccini*), both in Tuscany, are representative of the heavy bacterial colonization experienced in most Etruscan necropolises (Diaz-Herraiz et al. 2013, 2014). Nugari et al. (2009) have also reported on the biodeterioration of mural paintings in rocky habitats like the Crypt of the Original Sin, Matera, Italy (Nugari 2009). Herrera et al. (2009) reported on the combined use of molecular biology and physico-chemical techniques for surface analysis and materials characterization in regards to the study of biodeterioration and weathering effects on cultural property.

The nature of black stains in Lascaux Cave, France, has been studied by means of surface-enhanced Raman spectroscopy (Martin-Sanchez et al. 2012). Raman spectroscopy was also performed in Marcus Lucretius House, Pompeii, to investigate the nature and distribution of carotenoids in brown patinas from a deteriorated wall painting (Maguregi et al. 2012). μ-Raman mapping also allowed to unequivocally identify, at high spatial resolution and in cross-sections, the two common forms of the calcium oxalate fims, namely whewellite and weddellite, which are often observed on the surfaces of several ancient monuments (Conti et al. 2012). Oxalate film formation is a pathology that frequently also occurs in mural paintings, and which may result from the concomitant action of microorganisms and environmental conditions (Rosado et al. 2013).An innovative Raman gas spectrometry was introduced in 2013 for the rapid and nonconsumptive online quantification of CO_2 and O_2 in situ in the headspace of the bacterial culture, aiming at investigating the respiratory activity of carbonate-precipitating *Anthrobacter sulfonivorans*, isolated from the recently discovered Herrenberg Cave in the Thuringian Forest, Germany, which offers a unique opportunity to study an undisturbed and unexplored microbial population, which developed in the total absence of light (Keiner et al. 2013, Rusznyák et al. 2012).

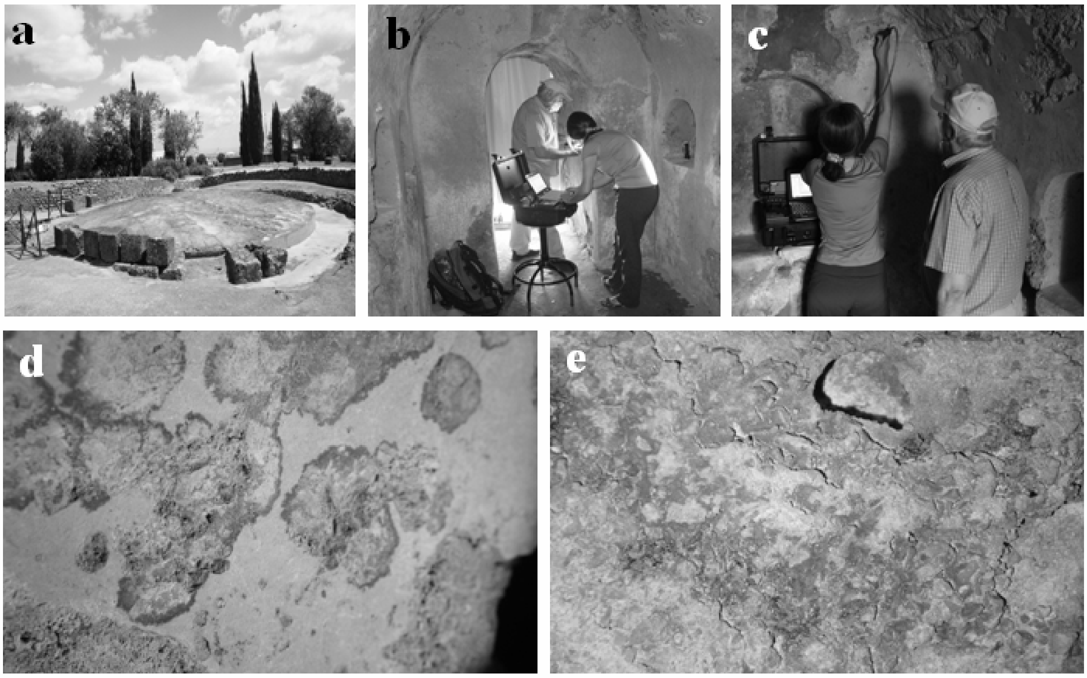

Figure 5. a. Outside overall view of the Circular Mausoleum in Necropolis of Carmona (Seville, Spain). b and c. In situ Raman analysis in the Necropolis of Carmona. d and e. Cyanobacterial/algal biofilms causing biodegradation in the Circular Mausoleum.

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