2007

Characterization of a black coating overlying rock paintings found in Little Lost River Cave, Idaho using THM-GC-MS

Jamie Brown-Sinha

CHARACTERIZATION OF A BLACK COATING OVERLYING ROCK PAINTINGS FOUND IN LITTLE LOST RIVER CAVE, IDAHO USING THM-GC-MS

by

Jamie Brown-Sinha

Thesis

Submitted to the Department of Chemistry

Eastern Michigan University

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Chemistry

Thesis Committee:

Ruth Ann Armitage, Ph.D., Chair

Heather Holmes, Ph.D.

Ross Nord, Ph.D.

March 13, 2007

Ypsilanti, Michigan

DEDICATION

I am dedicating this thesis to my parents, Raymond and Peggy. The constant support of my parents throughout my entire academic career has been a blessing.

I would also like to dedicate this thesis to my husband, Amitabh, who has changed my life in ways I never imagined.

ACKNOWLEDGEMENTS

I would like to acknowledge my research and thesis committee chair, Dr. Ruth Ann Armitage, for her continued guidance and support. Her encouragement through this entire process has been unrelenting. I would also like to thank my committee members, Dr. Holmes and Dr. Nord, for their time and support. And finally, I would like to acknowledge Dr. Rengan, who has provided many answers to the many questions that have come up during the process.

ABSTRACT

A black coating overlies rock paintings found within Little Lost Rive Cave, Idaho. A calibrated radiocarbon date of 1390-1040 B.C. was obtained by Steelman et al.[1] However, this relies on the assumption that the black coating was formed by some human activity. For further characterization and to verify that the coating is anthropogenic, THM-GC-MS was performed on various samples collected throughout the cave, including soil samples from inside, outside, and above the cave. Humic and fulvic acids, synthetic and natural melanin, and experimentally created cooking residues were also analyzed as standard materials. By comparing the resulting chromatograms and the compounds identified by mass spectrometry, it was determined that the black coating is not a synthetic or natural melanin and bears little resemblance to the cooking residues. The coating bears most resemblance to humic acid standards, indicating an environmental origin.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Introduction to THM-GC-MS

Thermally assisted hydrolysis and methylation (THM) is a sample preparation and derivatization technique utilized in the study of complex molecules by gas chromatography-mass spectrometry (GC-MS). Compounds that are typically nonvolatile, like fatty acids (FAs), are converted to more volatile fatty acid methyl esters (FAMEs) in the presence of a derivatizing agent such as tetramethylammonium hydroxide (TMAH) at elevated temperatures. THM-GC-MS can be used to study complex mixtures of carbohydrates and proteins, as the THM process also decomposes these larger molecules into smaller ones more amenable to identification with simple mass spectrometry methods. Pyrolysis methods, the precursors to THM-GC-MS, use heat alone to decompose polymeric molecules into simpler, yet still characteristic, fragments. The nature and presence of large organic molecules, such as these in archaeological contexts, is important for the application of radiocarbon dating to small and residual materials.

Originally called simultaneous pyrolysis methylation (SPM) when the technique was first utilized in the 1970s, THM-GC-MS became the preferred name in the 1980s, when the reaction mechanism was more clearly understood.[2,3] The hydrolysis and methylation mechanism, shown in Figure 1.1, involves the molecule of interest (A-B) undergoing hydrolysis to form tetraalkylammonium (TAA) salts upon reaction with a tetraalkylammonium hydroxide, such as TMAH. With heating, the TAA salts decompose to form alkyl derivatives of the original molecule.

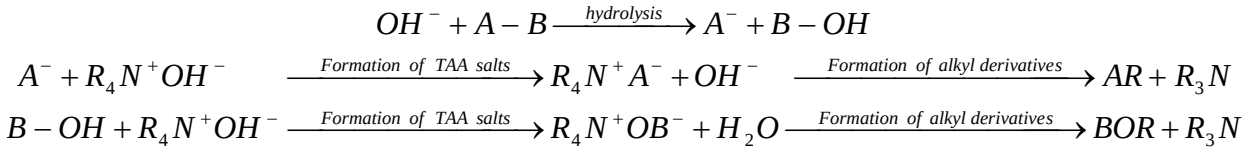

Figure 1.1. Mechanism for methylation by tetraalkylammonium salts at elevated temperatures (after Reference 2).

The TMAH reagent is usually applied to the molecule of interest as a methanolic solution, typically 10-25% by weight. Methanol is the preferred solvent; studies have shown that for THM-GC-MS of aromatic polyesters, methyl esters are the primary product with methanol, whereas ethanol has yielded a significant amount of methyl ethers as well.[4] Pitthard et al. investigated the use of other derivatizing agents at different temperatures to determine the best conditions for THM on fatty acids.[5]

An advantage of using a derivatizing agent such as TMAH is that the reaction can proceed at temperatures as low as 225 ^{o}C but more commonly at 300 ^{o}C. Under typical pyrolytic conditions, temperatures of 500-800 ^{o}C must be reached to completely decompose the nonvolatile compounds of interest.[3,5,6] THM using TMAH has been shown to yield a higher signal-to-noise ratio than pyrolysis.[7]

One disadvantage that accompanies THM-GC-MS, when using TMAH as a derivatizing agent, is that side reactions may lead to unexpected products. For example, TMAH, which has a high pH, may cause the isomerization of polyunsaturated fatty acids, particularly triglycerides.[3] This problem has been investigated with different concentrations of TMAH, which help lower the pH and reduce the base catalyzed side reactions.[8] Ishida et

al. discuss the quantitative disadvantages of using TMAH, wherein the alkali salts present may reduce the conversion of aromatic polyesters into their methyl derivatives.[4]

1.2 Review of the Literature

Minimal sample preparation and small sample size make THM-GC-MS an ideal technique for analysis of archaeological materials. Samples can be analyzed without any pretreatment, and very small amounts of samples can be used, making it possible to perform multiple GC-MS runs on a very small amount of archaeologically precious sample.

THM-GC-MS is widely applied in the characterization of complex organic materials. The technique has been studied for specific compound classes, including carboxylic acids, proteins, phenols, lipids, and humic substances, as well as for specific substances, such as wood, soil, and paints. Challinor provided an extensive review of applications of THM-GC-MS.[3] This review focuses on those which are of primary interest in studies of archaeologically relevant materials.

1.2.1 Resins

Natural resins come from trees such as pine, larch, spruce, and fir and are mainly composed of diterpenoid acids.[9] These natural resins have been used for centuries to help protect paintings from moisture and other damage. Pastorova et al. used THM-GC-MS to detect the presence of various diterpenoid acids, which are characteristic of varnish, on the painting "The Girl with the Pearl Earring" by Vermeer.[9] Three diterpenoid acids were positively identified in this way; the remaining diterpenoids of interest were identified by monitoring their decomposition (via oxidation) with time. Pastorova et al. set a precedent for analyzing paintings using this technique and determined the main components of varnish.

Scalarone and his coauthors studied the effects of aging on two particular diterpenic resins, colophony and Venice turpentine.[10] These compounds were submitted to simulated natural aging (xenon lamp), outdoor aging, and indoor aging (fluorescent light). They concluded that the most significant oxidation occurs early in the aging process. They also assert that this technique is appropriate for detecting diterpenic resins, but some difficulty may be encountered when trying to differentiate between colophony and Venice turpentine, as they have similar mass spectra. Most of the variation occurs in the compounds at lower retention times; therefore, these ion peaks must be used for distinguishing between the various diterpenic resins of interest.

1.2.2 Carboxylic Acids and Phenols

Abraham et al. established in 1985 that THM-GC-MS could be used quantitatively on simple carboxylic acids and phenols.[11] They discussed the advantages of using TMAH derivatization before GC-MS analysis on these polar compounds, such as an increase in the amount of sample recoverable after analysis and shorter analysis time. Their specific goal was to better optimize the experimental parameters surrounding the application of THM-GC-MS to the analysis of compounds with acidic functional groups. This work included an investigation into the effects of parameters such as temperature, volume, and injection rate.

1.2.3 Carbohydrates

Carbohydrates are particularly challenging to characterize using THM-GC-MS because they yield many products. Fabbri et al. were the first to report the products of carbohydrate analysis although they could not explain all of the products found.[12] Methoxy-benzene products and permethylated deoxy aldonic acids were observed in the analysis of

hexose, pentose, and polysaccharide. They suggested that this method was promising for the characterization of saccharides and other complex materials.

Using THM, Schwarzinger attempted to analyze the unidentified products found by Fabbri with some success.[13] As little as 100 mg of carbohydrates yielded a rather complicated mixture of components.[13] He did proffer that saccharinic acids are the originating compounds for carbohydrates and can thus be treated as "markers" for the detection of carbohydrates. He also investigated the influence of temperature on the reaction and concluded that only partial methylation occurs at low temperatures (250 ^{o}C). Performing THM at these low temperatures should therefore be avoided.

Schwarzinger also investigated aldol and retroaldol reactions on carbohydrates during their reaction with TMAH.[14] He reported that under basic conditions, as is the case with TMAH in methanol, retro-aldol cleavages are encountered, followed by aldol reactions that produce high-molecular-weight products. These reactions form 30% of the saccharinic acids found as THM products of carbohydrates and occur closer to the reducing end of the carbohydrate.

1.2.4 Proteins

Hendricker and Voorhees have discussed the drawbacks of applying pyrolysis to proteins, including an array of possible degradation products.[15] They investigated the THM of amino acids and dipeptides to establish their decomposition mechanisms and suggested that THM only produces dimers of amino acids when "simple and non-bulky side chains were analyzed." They recommend this technique for the analysis of oligopeptides, peptides, and proteins, especially when pyrolysis alone fails to yield any identifying fragmentation.

Knicker et al. reported in 2001 the successful use of this technique to compare algal material to albumin.[16] Upon comparison to the use of HCl-hydrolysis to characterize proteinaceous compounds, they found THM-GC-MS to be better suited to protein characterization, as it hydrolyzes insoluble samples more completely. They proposed that TMAH sufficiently penetrates the hydrophobic area of the samples and resultantly allows for better separation and fragmentation of proteins.

Zang et al. reiterated the benefits of using THM-GC-MS for the analysis of proteins and highlighted the advantage of TMAH's first depolymerizing macromolecules and then methylating the resulting, smaller components.[17] For the study, they analyzed bovine serum albumin and humic acid, using THM-GC-MS. They were able to positively identify the amino acids of interest but also encountered many unknown peaks. These peaks were attributed to amino acids that were only partially methylated, as well as to compounds from TMAH itself. These results support those of previous studies that this technique is valid for the analysis of amino, carboxylic, and hydroxyl groups found on amino acids and that this technique is ideal due to its ease of use (in comparison to wet chemistry) and speed of analysis.

1.2.5 Lignin and Humic Acids

In 2001, Martin et al. compared the use of pyrolysis to THM for characterizing humic acids.[18] They found that the two techniques yielded very different structural information about humic acids. THM led to the detection of more aliphatic compounds than did pyrolysis, most probably due to the lower analysis temperature of 250 ^{o}C versus 500 ^{o}C for pyrolysis. They did suggest that THM is sufficient for the detection of humic acids but will not allow for the stoichiometric characterization of the humic acid moiety, thereby limiting

the ability to succinctly identify the various components of the humics. Ikeya et al. recently supported this idea that pyrolysis and THM are complementary techniques for the characterization of humic and fulvic acids.[19] Because humic and fulvic acids are considered contaminants to samples being ^{14}C dated, a technique that detects them would allow researchers to better characterize the archaeologically significant components of the samples. These papers support the use of THM-GC-MS for the detection of humic and fulvic acids.

Page et al. reported the use of THM on dissolved organic matter, including the humus layers, of four reservoirs in Australia.[20] This characterization was a preliminary step to monitoring drinking water, which would provide insight into the reservoir's management and water quality. Although polysaccharides were present in most of the four sources, phenols originating from lignin varied greatly from source to source.

Recently, Klingber et al. investigated the THM-GC-MS parameters for the analysis of lignin, stating that optimal conditions yield intact propane chains on the lignin derivatives.[21] The parameters were tested on milled spruce wood lignin. They concluded that lower temperatures (the tested range was 310-710 ^{o}C) yielded more of the desired products and minimized the undesirable ones, such as the unmethylated lignin monomers. They also suggested using increased TMAH concentrations as well as longer incubation times with TMAH.

1.3 Applications to Materials of Art and Archaeological Interest

THM-GC-MS has been used for the characterization of various artifacts, including pottery and binding material found in paints. By characterizing residues found in pottery, it

is possible to determine the previous use of the vessel and provide archaeologists with insight into a past society, including information on food consumption as well as trade routes.

Paintings require a slightly different chemical characterization because of their complexity. To ensure a bond between the paint pigment (typically inorganic) and the surface upon which it is being applied, a painter must use some sort of binding medium. Typically, an organic material, such as blood, eggs, saliva, etc., would be used to allow the paint to stay on the surface. Of most interest to chemists, when characterizing paint samples, is the organic binder used by the painter because it provides insight into the time at which the paint was applied to the surface. This organic matter is radiocarbon dated with the assumption that the carbon is associated with the time at which the painting was placed on the surface.[22]

Another difficulty in characterizing paintings is their constant exposure to the environment. In typical analyses, such as GC, sufficient pretreatment must first be performed to ensure removal of environmental contaminants, including but not limited to fungi, algae, soil-soluble organic matter, and insect deposits.[22]

1.3.1 Binding Media

Chiavari and his coworkers used THM-GC-MS to characterize the binding media found in ancient painting media.[23] They suggested that THM is a tool desirable for its ability to detect low-molecular-weight fatty acids that are markers for egg yolk, siccative oils, and linseed oil, traditional binding media employed by artists of the Renaissance and later. They also discussed the advantage of using small amounts of sample as well as the technique's minimal sample-preparation needs.

Cappitelli investigated the binding media of two modern paintings, *Yellow Island* by Jackson Pollock and *Break Point* by Fiona Banner, using THM-GC-MS and infrared spectroscopy.[24] He recommended this technique for the characterization of oils, alkyd resins, and acrylic resins, which are commonly found in 20th-century paintings. He used the technique specifically to monitor the palmitic/stearic acid ratios attributable to various oils.

1.3.2 Pottery

To investigate wine residues found in ancient pottery, Garnier et al. used THM-GC-MS.[22] Specifically, they were looking at the preservation of tannins in this pottery because ceramics are often used to establish trade routes. They determined that using at little as 0.1 mg of ceramic, it is possible to detect the presence of wine and therefore provide information regarding wine trade routes from ancient times.

1.4 Significance of Project

The purpose of this project was to provide further insight into the origin of a black coating found in Little Lost River Cave, Idaho. We analyzed newly collected samples, using THM to illustrate geographic and physical differences between black coating samples found within the cave. With understanding the nature of the coating, we will be able to determine whether or not the radiocarbon date obtained by Steelman et al. should be considered diagnostic of human activity or, rather, nondiagnostic and therefore irrelevant.[1]

1.5 Objectives

1. To compare the molecular composition of the black coating to that of other materials from the cave – including biological residues and soils – to ascertain the likely origin of the coating.
2. To clarify whether the origin of the carbon that was dated by Steelman et al. is anthropogenic and therefore diagnostic of human activity or geologic and therefore irrelevant.

CHAPTER 2

BACKGROUND

2.1 Background on Little Lost River Cave

The first reported exploration of Little Lost River Cave was conducted in 1954 by Albert Whiting.[25] It is a solution cave, created by the dissolution of dolomitic limestone. Located in Butte County, Idaho, the site carries the Smithsonian designation 10BT1 (Figure 2.1). The cave is approximately 16 meters long and 1.5 meters tall.[25, 26]

Figure 2.1. Map of Idaho illustrating approximate location of Little Lost River Cave (10BT1).

The cave is of particular interest to archaeologists because it contains red and yellow pictographs on its walls. Overlying these pictographs is a black coating (Figure 2.2) that was first documented during the 1955 excavation performed by Idaho State College participants.[25] It was originally thought that if both chemical and radiocarbon analyses were performed on the less precious black coating, some insight regarding the age of the paintings could be obtained while still preserving the paintings.

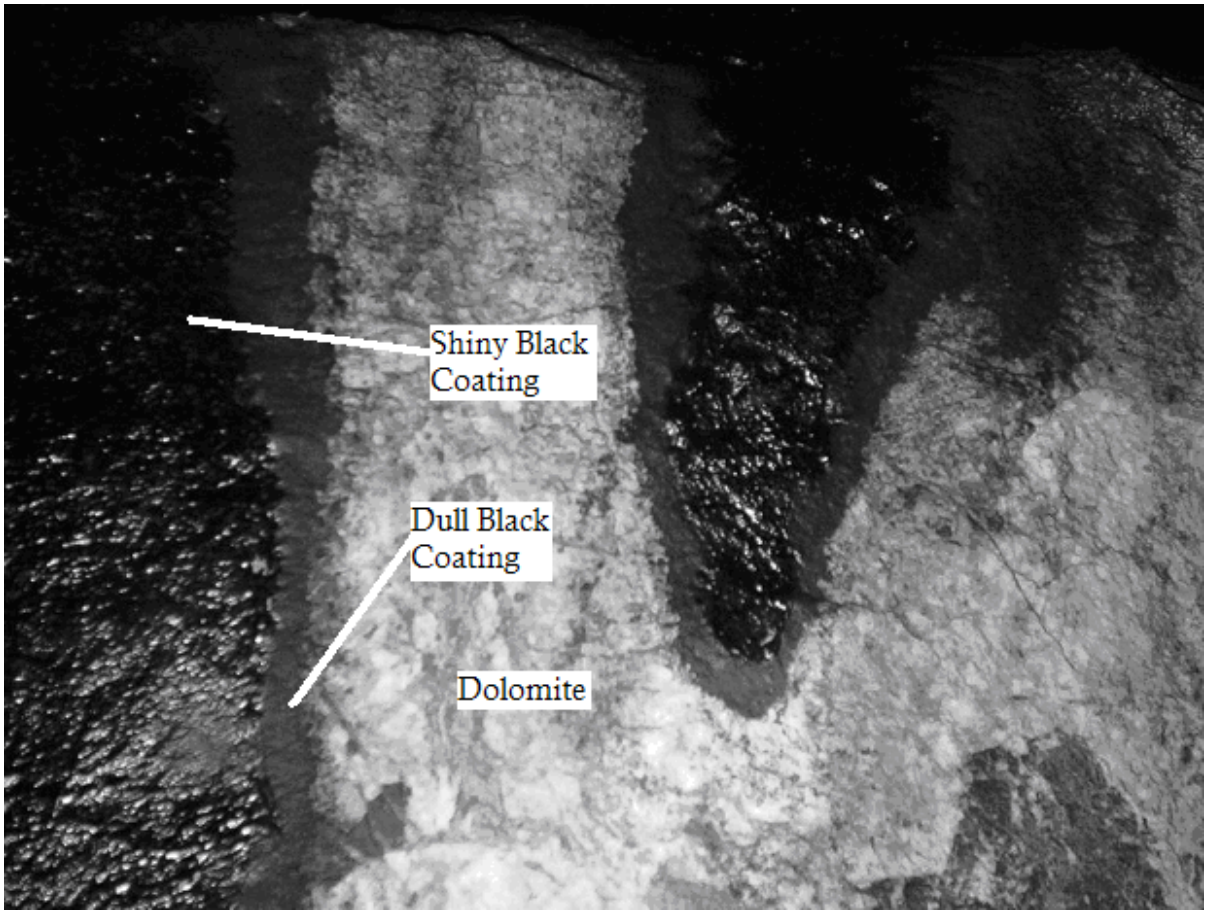

Figure 2.2. A picture of the black coating, illustrating the shiny black coating, dull black coating and dolomite.

2.2 Background on the Black Coating

Dr. Marvin Rowe and his research students at Texas A&M University conducted radiocarbon dating on the black coating, using plasma chemical oxidation-accelerator mass spectrometry (PCO-AMS). This technique is selective for surface organic carbon in the presence of oxalates and carbonates.[27] A calibrated radiocarbon age of 1390-1040 B.C. was obtained by Steelman et al.[1]

Radiocarbon dating in archaeological contexts relies on the assumption that the carbon being dated was formed by one event and that this one event is characteristic of the desired human activity. In this case, the activity to be dated was the creation of the paintings, but because the coating was sampled instead, a minimum age for the paintings was sought. If the carbon being dated is from an environmental event instead of being anthropogenic, its connection to the object of interest is unknown. Therefore, there is no connection between the carbon being dated and the object being dated. For example, this investigation focuses on a black coating of unknown origin. If the coating is a cooking or smoke residue, it would have been formed by one human event and would therefore be characteristic of the time at which the underlying rock paintings were placed on the cave walls. Instead, if the coating is a humic or fulvic acid, the carbon will bear no temporal connection to the underlying paintings. The humic acids from the soil above the cave can contain carbon that is both older and younger than the paintings inside the cave, and therefore, a radiocarbon date of this material would provide no insight into the actual age of the underlying paintings. On the other hand, an event such as a wild fire might deposit an environmental coating that would

exclusively postdate the painting activity. Thus, understanding the origin of the material is important for understanding the date obtained.

Stable isotope analysis was performed to further characterize the black coating.[1] The material was found to be "animal like" in origin and thought to be possibly a cooking residue.[1] Steelman et al. noted when polishing a section of the coating that the material was water soluble.

To characterize the black coating on a molecular level and to help determine whether the source of carbon was environmental or anthropogenic, THM-GC-MS and pyrolysis-GC-MS were initially performed.[28] Fezzey and Armitage concluded that the substance was most similar to a standard humic acid; however, it was not identical, and further investigation of the soil in and around Little Lost River Cave was needed. This investigation has since been expanded to include comparisons between the substance and possible environmental sources (humic and fulvic acids and melanin) as well as possible anthropogenic sources (cooking residues).

Humic acids are produced through the decay of organic matter in soils and are indicative of fertile soil.[29] These substances contribute to the soil's ability to retain water and promote plant growth and have many other chemical properties, such as acting as pH buffers, redox catalysts, etc.[29] Humic substances are complex materials, having chemical compositions that reflect those of their original materials. Humic and fulvic acids are water-soluble humic materials having different molar weight ranges. Predominant in soluble soil organic matter, humic acids could be transported into a cave with moving ground water. Little Lost River Cave is an active wet cave, so it may be that the coating is derived from

redeposited soil organics moved by the slow precipitation of ground water through the cave ceiling and walls.

Melanins are generally very dark colored, almost black, and are biological macromolecules produced by microbes, animals, protozoans, and plants.[30] Much like humic acids, the exact structure of microbial melanins is still unknown; however, they are composed of various phenolic and indolic monomers usually associated with protein.[30] If the black coating was formed by bacterial growth, its composition should be similar to that of bacterial melanins.

CHAPTER 3

EXPERIMENTAL

3.1 Instrumentation and Experimental Parameters

A Varian, Inc. GC-3800 gas chromatograph, coupled with a Saturn 2200 ion trap mass spectrometer, was used for all of these investigations. The experimental parameters used are in Table 3.1. Split ratios for the GC were increased for the analysis of the experimental cooking residues only, as large signals were observed for these materials.

Table 3.1. Experimental Parameters for All THM-GC-MS Experiments

GC	Column Type	VF-5ms
	Column Dimensions	30 m long, 0.25 mm id, 0.25 mm film thickness
	Column Flow Rate	40 psi for 1.10 min 9.3 psi for 47 min
	Carrier Gas	99.999% Helium
	Temperature Program (oven)	40°C for 5 min 250°C at 6.5°C/min for 10 min
	Injector (Chromatoprobe)	40°C for 0.10min – 100:1 Split 84°C at 200°C/min for 1.00min – 100:1 Split 300°C at 200°C/min for 10 min – 50:1 Split
MS	Ionization	Electron Impact
	Ion Range	35-650 m/z
	Solvent Delay	8 min
	Pressure	<40 μTorr
	Trap Temperature	150 °C
	Manifold Temp	35 °C
	Transfer Line Temp	260 °C

Pyrolysis conditions require a high rate of heating, typically referred to as “ballistic heating”, reaching up to 20,000 °C/s. These conditions require specialized – and expensive – equipment. Modified GC sample injection ports can be used.[5] The Varian 1079 injector can

be heated ballistically although only at a rate of 200 ^{o}C/min and with a maximum temperature of 425 ^{o}C. The Chromatoprobe injection system allows solid or liquid samples to be introduced into the GC system in a manner similar to the sample introduction with a pyrolysis instrument. Figure 3.1 (a) shows the Chromatoprobe injector; the solid or liquid sample is placed in a small glass vial (b), which is then placed upright in the Chromatoprobe. The entire probe is placed into the 1079 injection port. A separate temperature program is applied to the injector port, volatilizing the sample in the vial. The gaseous products are then swept onto the GC column for separation and identification by MS.

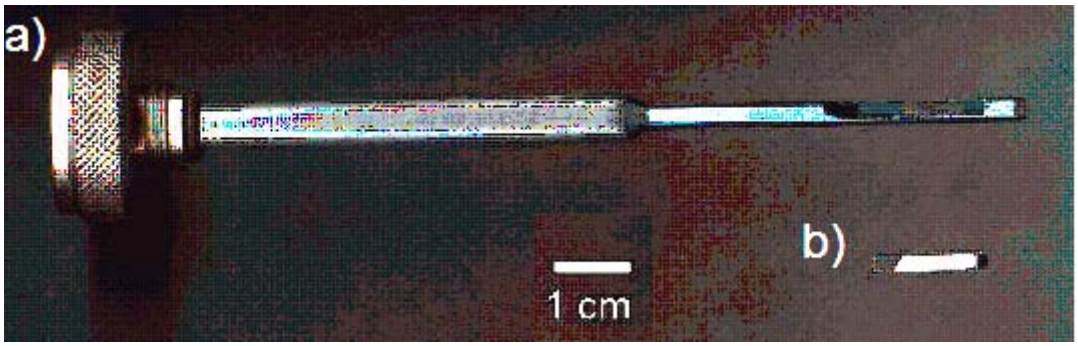

Figure 3.1. The injector used for all THM-GC-MS experiments, a Chromatoprobe, is pictured on the top (a). (b) is a sample vial, used in conjunction with the Chromatoprobe.

3.2 Materials

All samples were collected by Dr. Ruth Ann Armitage in May 2005, under the guidance of Carolynne Merrell and Richard D. Hill, U.S. Bureau of Land Management archaeologists. Dr. Armitage used a sterile scalpel blade to scrape materials from the walls and ceilings and then wrapped them in clean aluminum foil and stored them in individual

plastic bags. Samples were collected from various locations (see Figure 3.2) throughout the cave. Coating samples 1-8 differed in appearance, and three soil samples were collected for comparison. Close inspection of the coating showed that a shiny layer seemed to overlay a black, sooty material underneath. The shiny, yellow substance collected (samples 3 and 7) is believed to be the top layer, and the sooty black coating (sample 1) appears to be the lower layer. Sample descriptions and identifiers are tabulated in Table 3.2.

Table 3.2. Physical Descriptions of Samples Analyzed Using THM-GC-MS

Sample Number	Description
1	Sooty, black coating
2	Sooty, black coating, not sampled for GC-MS
3	Shiny. Contained separated portions of black and yellow coating
4	Sticky, black coating
5A, 5B	Shiny, black coating
6	Flakes of black coating, not sampled for GC-MS
7	Shiny, yellow coating
8A, 8B	Wet, grayish plant material, collected at cave entrance
D1	Dolomite used for background, collected from roof fall inside
D2	Dolomite used for background, collected from outside
S1	Soil collected from cave interior, light brown
S2	Soil collected from cave exterior
S3	Soil collected from above cave, dark brown

Soil humic and fulvic acid standards were purchased from the International Humic Substances Society. Synthetic and natural melanins were purchased from MP Biomedicals (CAS # 8049-97-6). Experimental cooking residues were produced as needed. Amberat, resinous urine excreted by packrats, was collected from nearby Jackknife Cave (10BT46); this comparative material was collected by C. Merrell in 2004 in the same manner as were

the samples. Tetramethylammonium hydroxide (TMAH) was purchased from Alfa Aesar as a 25% (w/w) solution in methanol (CAS # 75-59-2).

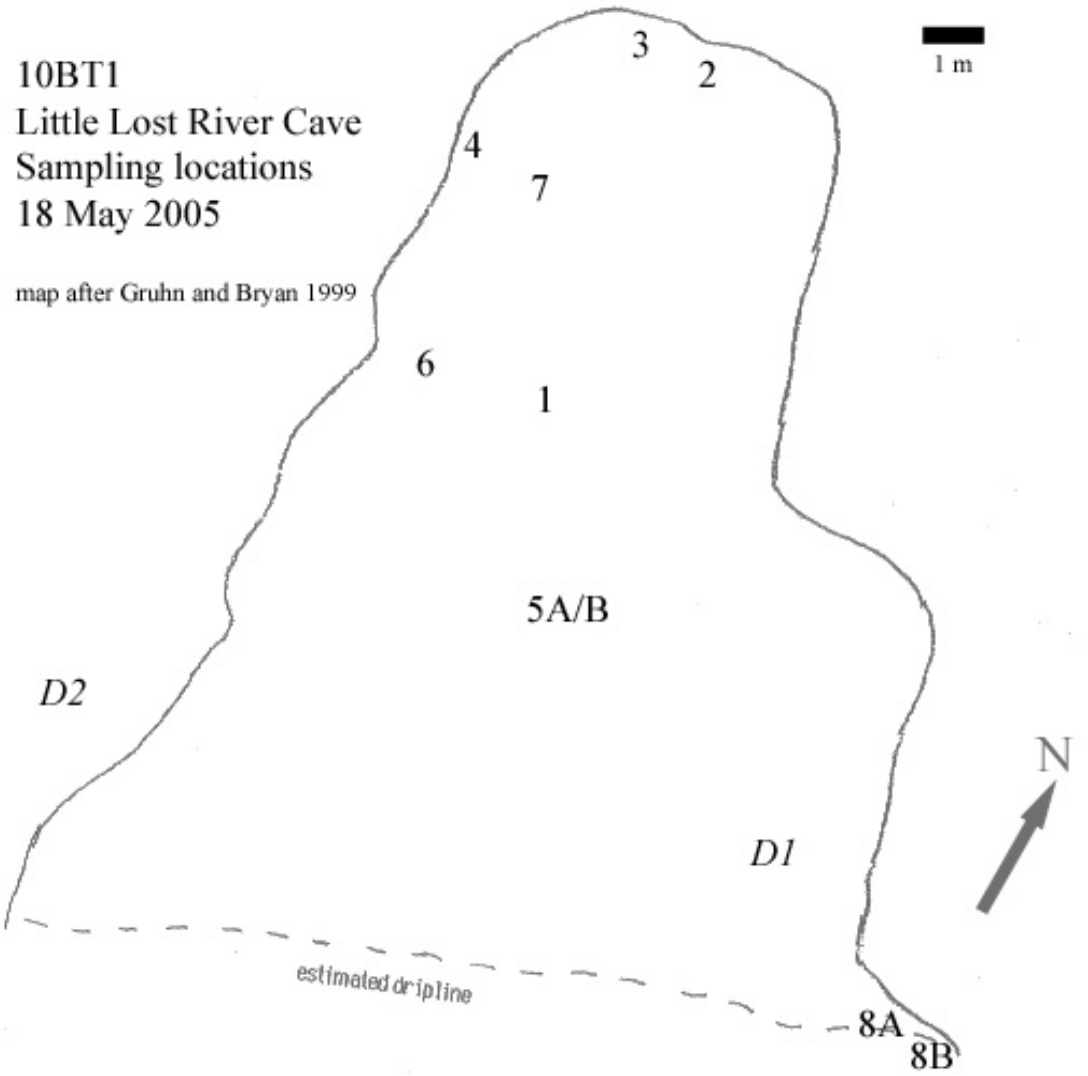

Figure 3.2. Map showing the locations of the samples collected.

3.3 Sample Treatment – Extraction Procedure

Researchers ground samples using a cleaned (by baking at 500 °C for several hours) mortar and pestle, sonicated with about 10 drops of distilled deionized water for 20 minutes, and then centrifuged for 10 minutes. The water fraction was collected on a glass slide and allowed to dry in an oven at approximately 100 ^{o}C for 30 min, yielding a substance which varied from very light yellow to light brown. The remaining water-insoluble fraction was gray to black. This sonication and centrifugation procedure was repeated to ensure that all water-soluble compounds were collected. If the coating was caused by ground water's percolating into the cave, carrying water-soluble environmental contaminants with it, the water-soluble compounds had to be analyzed separately from water insoluble compounds in order to compare them to standard environmental sources, like humic acids.

3.4 Experimental Conditions for THM

For analysis using the GC-MS, the sample vials pictured in Figure 3.1 were filled with just enough sample to be visible, and then, 0.5 μL of TMAH (25% in methanol) was added. The researchers used THM-GC-MS to examine the water-soluble fraction, water-insoluble fraction, and whole materials.

The Chromatoprobe inlet had an initial temperature of 40 ^{o}C, which was held for 0.1 minutes and then ramped to 84 ^{o}C at a rate of 200 ^{o}C/min and held for 1.00 min to evaporate any excess methanol. After 1.32 min, the temperature was ramped to 300 ^{o}C at a rate of 200 ^{o}C/min; this is the temperature at which THM occurred. The instrument utilizes cryogenic cooling to gradually reduce the temperature of the Chromatoprobe, minimizing the time between runs (250 ^{o}C held for 24.66 min, followed by 100 ^{o}C for 9.45 min). The GC oven

was held at 40 ^{o}C for five minutes and then ramped to 250 ^{o}C at a rate of 6.5 ^{o}C/min, at which it was held for 5 minutes. The flow rate of the helium carrier gas was programmed to 40 psi for the first 1.10 min and then to 9.3 psi for the remaining 46.13 min to facilitate removal of the methanol solvent from the samples. A solvent delay of 5 min was utilized, and masses ranging from 40 to 650 were collected. There was a split of 100:1 for 1.32 min to facilitate removal of excess methanol solvent and then one of 50:1 for the remainder of the analysis time. Table 3.1 shows the full experimental parameters.

3.5 Identification of Compounds

Researchers used MS Data Review (v. 6.8, service pack 1) software provided by Varian, Inc. to identify compounds on the basis of their mass spectra. The software is supported by the NIST Mass Spectral Search Program, version 2.0a, from July 2002. An additional database, *Mass Spectra of Geochemicals, Petrochemicals and Biomarkers* (authored by J. W. DeLeeuw, ISBN 0471647985, published by Wiley-VCH) was added for additional clarification of some of the breakdown products from humic and fulvic acids.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Reproducibility

4.1.1 Reproducibility of the Experimental Procedure

Demonstrating the reproducibility of results is important if we are to make relevant comparisons of the chromatograms obtained from the materials over time. Because the THM-GC-MS method involves many steps (derivatization, separation, fragmentation, and identification), each step must occur reproducibly throughout the entire course of the investigation. If the same sample is analyzed twice, chromatograms and mass spectra should reasonably be expected to not differ significantly.

To evaluate the reproducibility of the technique, two samples of the IHSS humic acid standard were run one day apart. The resulting chromatograms are shown in Figure 4.1. The compound list is given in Table 4.1.

The same compounds were observed in each of the chromatograms in Figure 4.1. This indicates that all the processes were significantly reproducible: the sample was derivatized in a reproducible manner, the compounds were separated by GC in the same order with very similar retention times, the mass spectrometer fragmented the compounds reproducibly, and the MS software identified all eluting compounds in the same way. This comparison allows a researcher to have confidence when comparing samples of unknown composition to other samples of unknown composition or to other standards.

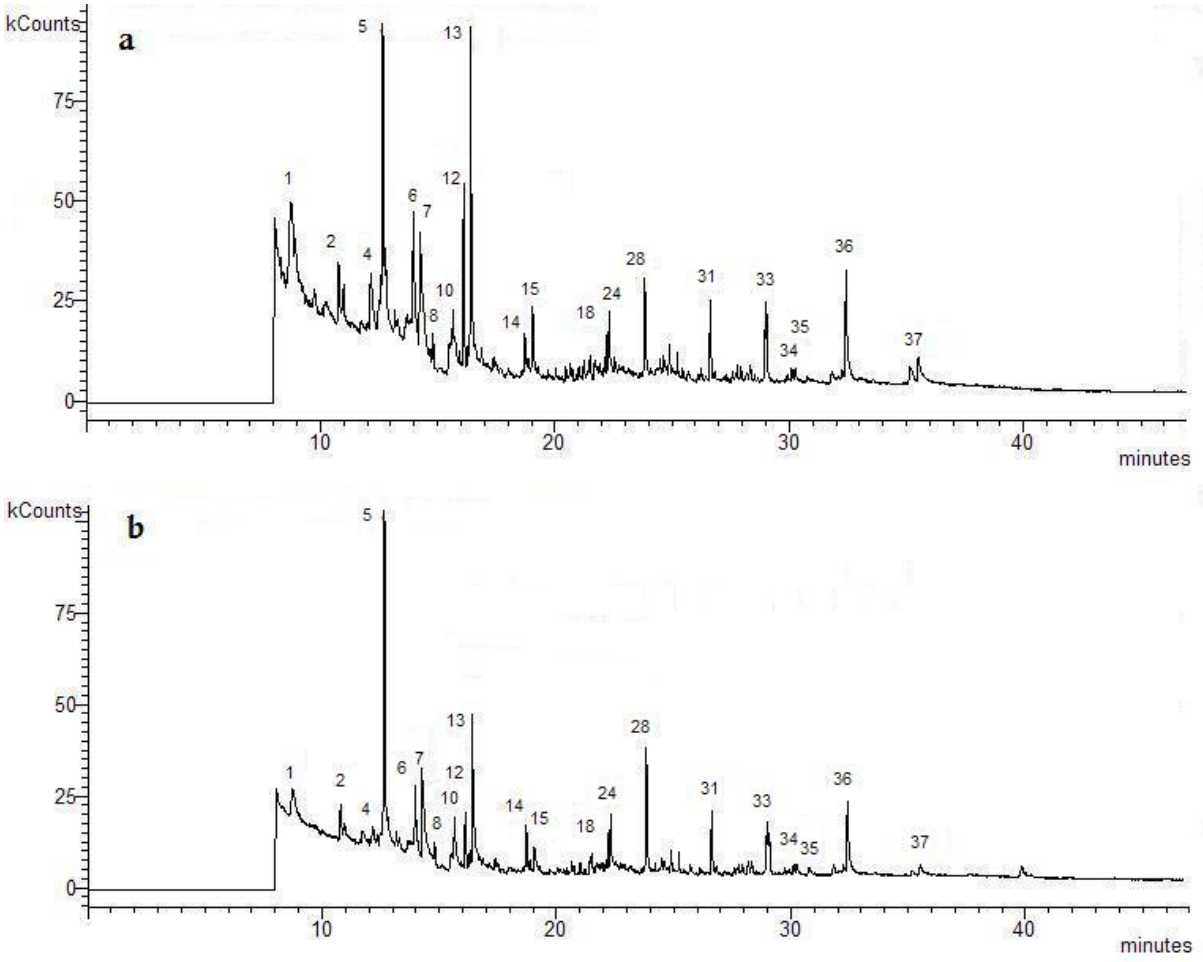

Figure 4.1. Chromatograms of IHSS-humic acid. Standards were analyzed on (a) February 8, 2006 and (b) February 9, 2006. The numbered peaks correspond to compound identities, listed in Table 4.1.

4.1.2 Reproducibility of THM-GC-MS Performed on Different Instruments

Previous THM-GC-MS analysis was performed by Fezzey and Armitage on black coating samples collected from Little Lost River Cave.[28] Fezzey and Armitage used a GC-MS instrument with pyrolysis for sample introduction to perform these analyses; samples were pyrolyzed at 300 ^{o}C for 10 s. The Chromatoprobe and the pyrolysis systems differ in

heating rate (200 ^{o}C/min vs. 20,000 ^{o}C/s, respectively). A slight shift in retention times is expected in comparing results from these two methods.

Fezzey and Armitage analyzed samples collected from 10BT1 by C. Merrell in 2004, whereas the current project focused on samples collected in 2005. Humic acid standards differed in the two projects; Fezzey used a generic humic acid for which the source was not known, whereas this project used a soil humic acid of known geographic origin. Both projects analyzed packrat urine, also known as amberat, collected from Jacknife Cave. A metabolite found in amberat, hippuric acid, is considered a marker for positive identification of amberat.[28] As this compound was absent in all of the Little Lost River Cave samples analyzed, it was conclusively determined that amberat was not the origin of the black coating.

To compare instrumental reproducibility, the results for the amberat from both of these studies are shown in Table 4.1. The chromatograms are illustrated in Figure 4.2. Fezzey used an MS with a quadrupole mass analyzer, whereas the current project used a slightly more sensitive ion trap. The ion trap also allowed for the collection of higher mass ions (650 m/z versus the 425 m/z maximum for the quadrupole). A 50:1 split ratio was employed for the ion trap GC-MS, as signals obtained under splitless conditions were too high.

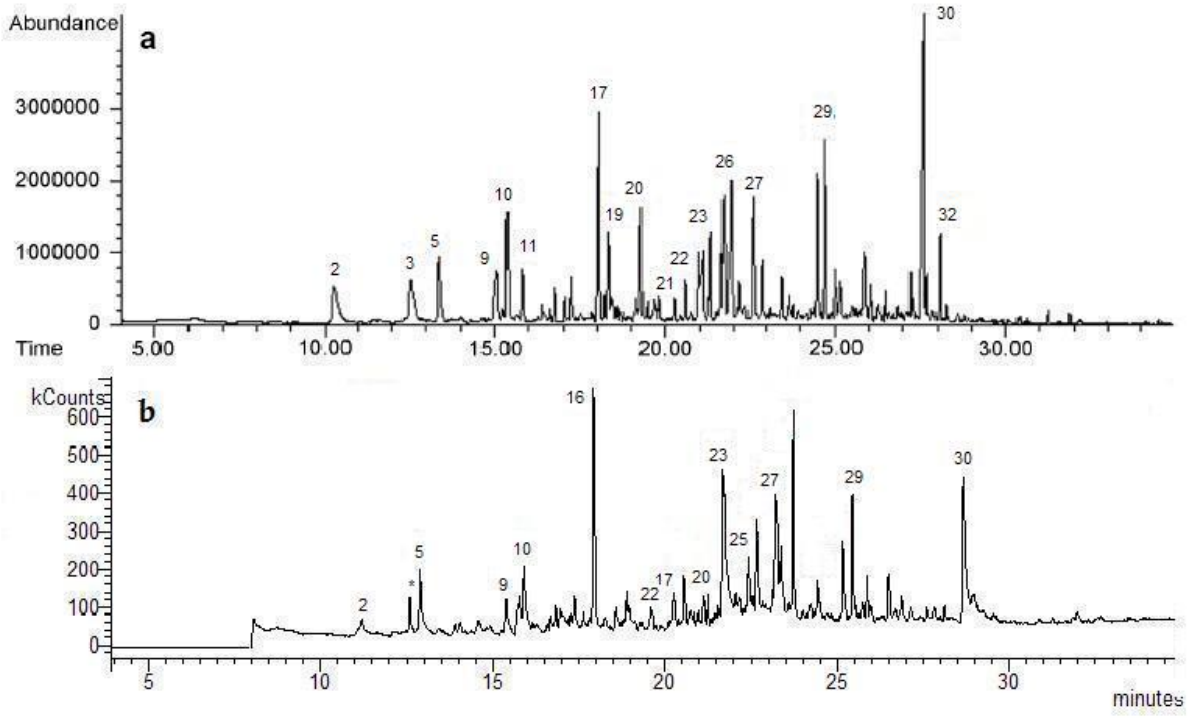

Figure 4.2. Comparison of an amberat sample analyzed by researchers using (a) a pyrolysis sample introduction method and (b) the Chromatoprobe sample introduction method.

Table 4.1. Compound List for Peaks Found in Figures 4.1 and 4.2

Peak #	Compound Identity	4.1 - Humic		4.2 - Amberat	
		a	b	a	b
1	3-Furaldehyde	X	X		
2	Methoxy benzene	X	X	X	
3	Phenol			X	X
4	2-Furancarboxaldehyde, 5-methyl-	X	X		
5	Benzene, (methoxymethyl)-	X	X	X	X
6	Butanedioic acid, DME[a]	X	X		
7	Benzenemethanamine, N,N-dimethyl	X	X		
8	Butanedioic acid, methyl-, DME[a]	X	X		
9	Methyl phenol			X	X
10	Benzoic acid, ME[b]	X	X	X	X
11	Ethyl methyl phenol			X	
12	Levoglucosenone	X	X		
13	1.3-Benzenedimethanamine, N,N,N',N'-tetramethyl-	X	X		
14	5-Hydroxy-2-methylthiopyrimidine	X	X		
15	Benzothiazole	X	X		
16	Borneol				X
17	Dimethoxy toluene			X	X
18	1,3,5-Triazine-2,4,6(1H,3H,5H)-trione, 1,3,5-trimethyl-	X	X		
19	Ethyl phenol			X	
20	Methoxy methyl phenol			X	X
21	Contaminant			X	
22	Propyl phenol			X	
23	Benzamide			X	X
24	Benzoic acid, 3-methoxy-, ME[b]	X	X		
25	Benzamide, N,N-dimethyl-				X
26	Trimethoxy benzene			X	
27	Methoxy benzeneacetic acid, ME[b]			X	X
28	2,4(1H,3H)-Pyrimidinedione, 1,3,5-trimethyl-	X	X		
29	Anisyl propionate			X	X
30	Hippuric acid, ME[b]			X	X
31	Benzoic acid, 3,4-dimethoxy-, ME[b]	X	X		
32	Dimethoxy benzene propanoic acid			X	
33	Tridecanoic acid, 12-methyl-, ME (C14:0)[b]	X	X		
34	Tetradecanoic acid, 9-methyl, ME (C15:0)[b]	X	X		
35	Pentadecanoic acid, ME (C15:0)[b]	X	X		
36	Pentadecanoic acid, 14-methyl-, ME (C16:0)[b]	X	X		
37	Octadecanoic acid, ME (C18:0)[b]	X	X		
*	Contaminant				X

(a) DME = Dimethyl Ester
(b) ME = Methyl Ester

The results of the THM-GC-MS analysis of amberat using the pyrolysis inlet differed from those obtained by using the Chromatoprobe. Because of the many instrumental differences (i.e., pyrolysis vs. Chromatoprobe inlet), this is not surprising. Faster heating of the sample will cause a slight shift in retention time, with a greater shift occurring at higher retention times. Using differing mass analyzers may have also caused variations in major peak identification. The peaks identified in Table 4.1 are the major peaks found in the chromatograms (Figure 4.2). Using two different mass analyzers may have caused minor peaks that appeared in the quadrupole instrument to appear as major peaks in the ion trap instrument. And finally, using a split of 50:1 for the ion trap instrument versus splitless for the quadrupole instrument may also have introduced some variance. When performing under split conditions, the instrument sweeps away compounds found in the headspace of the injector port. It is possible that more of the lighter compounds are swept away under split conditions, whereas the heavier compounds are lying in the bottom of the injector port. Therefore, a greater number of heavier compounds would be analyzed by the ion trap instrument.

Many peaks were identified in both samples (5, 9, 10, 17, 20, 23, 27, 29, and 30). Of most importance is hippuric acid (peak 30). This is considered a major component of amberat and is characteristic of the metabolism of packrats. It has therefore been used as a marker for the presence of amberat in unknown substances. Its presence in both samples analyzed (Figure 4.2 a and b) is crucial to the comparison between instruments.

4.2 Analysis of Background Materials

To verify that the compounds being identified are attributable only to the black coating of interest, control samples were analyzed for comparison (Table 4.2 and Figure 4.3). The derivatizing agent, TMAH, alone provided a kind of method blank, indicating what compounds could be formed when TMAH reacted at elevated temperatures. Uncoated portions of dolomite rock were collected from the cave to serve as control samples as well. Because the dolomite samples in the study were exposed to the environment of the cave, they represent the surface contamination that should be present from wind-blown soil and any microbiological growth that might be present.

From the appearance of the chromatograms, peak 5 (methoxymethyl benzene; see Table 4.2) is common to all background materials. There is also a series of peaks toward higher retention times that is common to all dolomite fractions; these peaks can only be identified as long-chain hydrocarbons. These materials appear in many of the chromatograms obtained in this study; they are not reproducible and often occur in blank GC runs (where neither sample nor derivatizing agent is present). These hydrocarbons then are considered to be contamination and not attributable to the material under analysis.

Contaminants can be identified on the basis of their presence in the chromatograms from these background materials. For example, peak 5 (methoxymethyl benzene) appears in all of the background analyses, indicating that is a contaminant from the method. The compounds found in the TMAH background run are characteristic of the chemical itself.

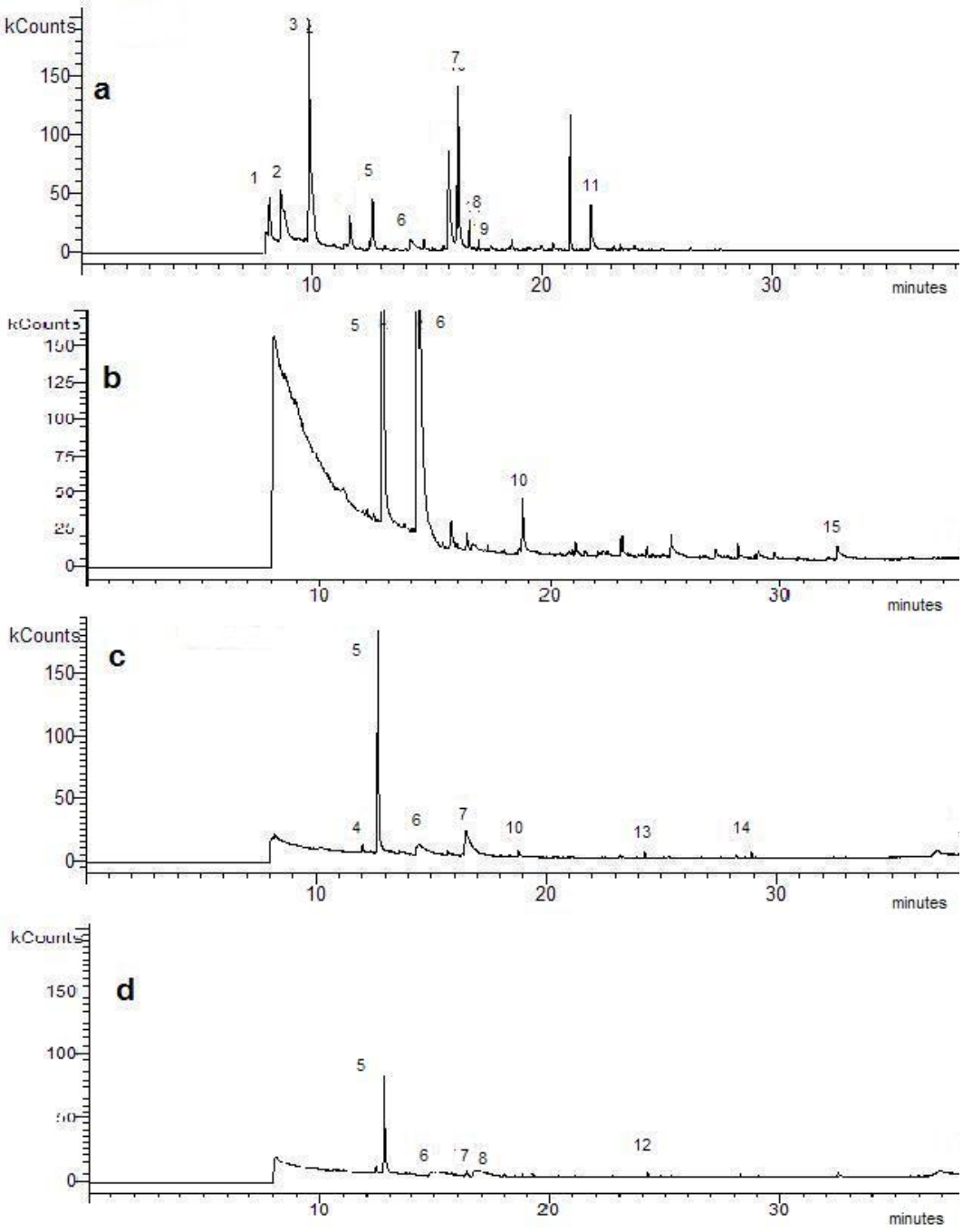

Figure 4.3. Chromatograms of control and background materials. (a) TMAH, (b) water-soluble fraction of dolomite, (c) whole dolomite sample and (d) insoluble fraction of dolomite.

Table 4.2. Compound List for Chromatograms in Figure 4.3. (a) TMAH, (b) water-soluble fraction of dolomite, (c) whole dolomite sample and (d) insoluble fraction of dolomite

Peak #	Compound Identity	TMAH a	Dol-Sol b	Dol-Whole c	Dol-Insol d
1	Methyl dimethylcarbamate	X			
2	N,N-Dimethyl-2-ethoxyethylamine	X			
3	[2-(N,N-Dimethyl)]-1,2-propanediamine	X			
4	Octane, 4-chloro-			X	
5	Benzene, (methoxymethyl)-	X	X	X	X
6	Benzenemethanamine, N,N-dimethyl-	X	X	X	X
7	1,3-Benzenedimethanamine, N,N,N',N'-tetramethyl-	X		X	X
8	cis-2-Methyl-2-butenedioic acid, DME[a]	X			X
9	Cyclopropane-1,2-dicarboxylic acid, 1-methyl-, DME[a]	X			
10	Nonanoic acid, ME[b]		X	X	
11	4-Imidazolidinone, 2-thioxo-	X			
12	Benzoic acid, 2-ethyl-6-hydroxy-, ME[b]				X
13	Decane, 1-chloro-			X	
14	1-Phenoxycarbonylaminoanthraquinone			X	
15	Pentadecanoic acid, 14-methyl-, ME[b]		X		

(a) DME = Dimethyl Ester
(b) ME = Methyl Ester

4.3 Comparison Between Samples

Preliminary analyses were carried out on a sample of the coating obtained in 2004 by K. Steelman. According to C. Merrell, the consulting archaeologist who provided the second sample of the coating, the black residue was consistent in appearance throughout the cave. However, Armitage reported that the coating was actually quite variable in appearance and could be described variously as sooty, shiny, black, yellow, and sticky. The variation may be the cause of inconsistencies found by Fezzey in the preliminary work. By classifying the samples on the basis of their appearance and location, we were able to compare the composition of the various coatings.

Upon examination of the chromatograms for the whole samples, found in Figure 4.4 and identified in Table 4.3, it is evident that each sample has a different composition.

Sample 5 (Figure 4.4 c) contains very few compounds comparatively. Sample 4 (Figure 4.4 b) has more compounds at higher retention times, and sample 1 (Figure 4.4 a) has more compounds at lower retention times. Sample 7 (Figure 4.4 d) compounds elute throughout most of the collection time. Samples 1 and 7 are the most similar, whereas 4 and 5 are significantly different. This is somewhat surprising, as 4 and 5 were most similar in appearance, whereas 1 and 7 were strikingly different. However, this may explain why Fezzey found the original samples to be irreproducible: if Merrell had combined material from locations 4 and 5, the resulting chromatograms would be confusing at best.

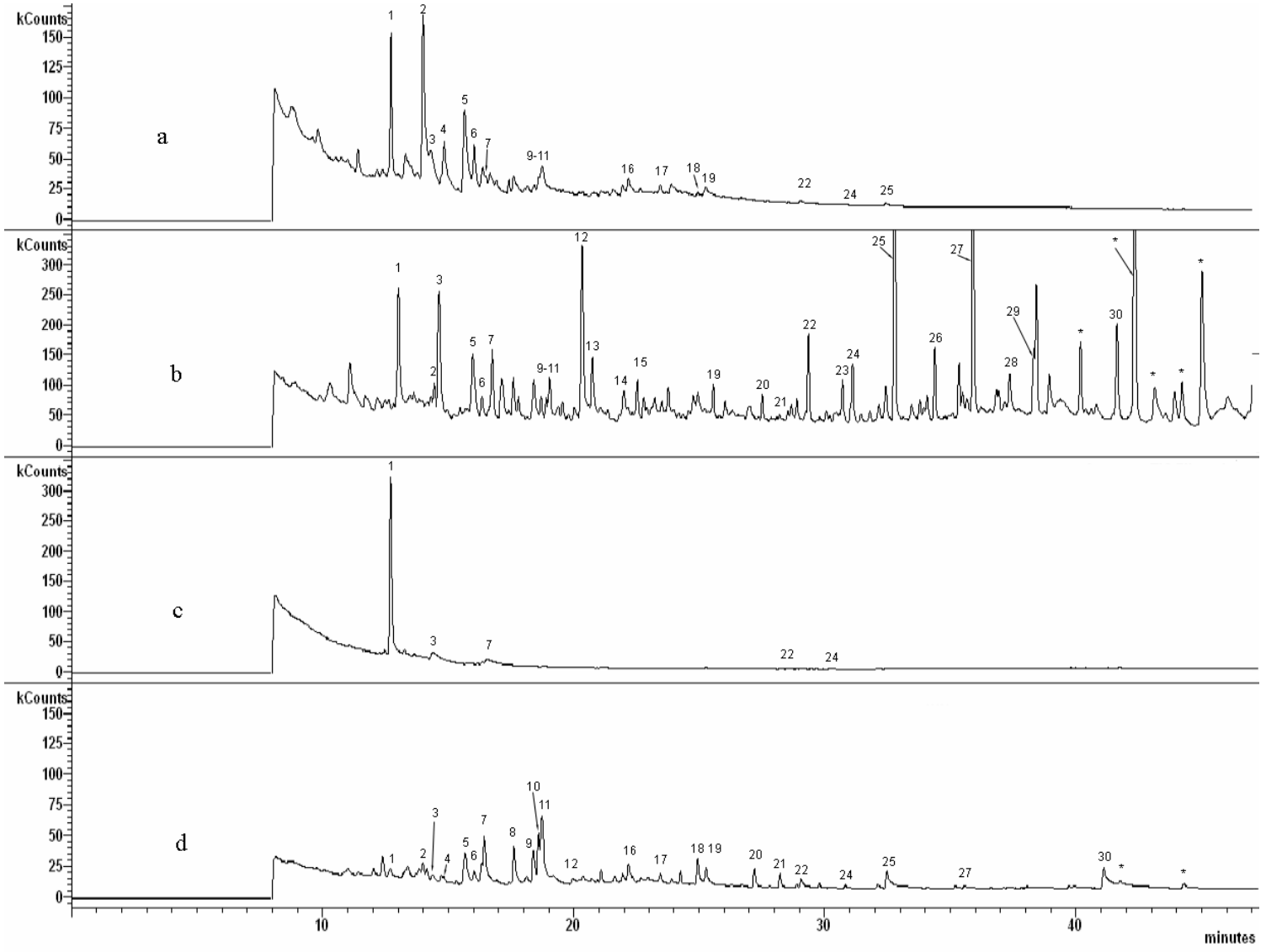

Figure 4.4. Chromatograms for 10BT1 materials, analyzed whole: (a) sample #1; (b) sample #4a; (c) sample #5a; (d) sample #7.

Table 4.3. Compound List for Chromatograms in Figure 4.4. Whole samples (a) 1, (b) 3, (c) 4, (d) 5, (e) 7

		Whole Samples			
Peak #	Peak Identity	1	4	5	7
1	Benzene, (methoxymethyl)-	X	X	X	X
2	Butanedioic acid, DME[a]	X			X
3	Benzenemethanamine, N,N, dimethyl	X	X		X
4	Butanedioic acid, methyl-, DME[a]	X			
5	Piperidine-2,5-dione	X			X
6	2,3,4-Trimethyl-isoxazol-5(2H)-one	X			X
7	1,3-Benzenedimethanamine, N,N,N',N'-tetramethyl-		X	X	X
8	Unknown, BP=117[b], M+=144[c]	X			X
9	2,4(1H,3H)-Pyrimidinedione, dihydro-3-methyl-	X			X
10	2,4(1H,3H)-pyrimidinedione, 1,3-dimethyl	X	X		X
11	Unknown, BP=42, 127, 142[b]	X	X		X
12	3-hydroxymethyl-1-methyl piperidine		X		X
13	Phenol, 2-ethyl-4,5-dimethyl		X		
14	Methylimidizolidinedione		X		
15	Trimethyltriazinetrione		X		
16	1,2,4-trimethoxybenzene	X			X
17	1H-isoindol-1,3 (2H)dione, 2-methyl	X	X		X
18	1,4-Benzenedicarboxylic acid, DME[a]	X			X
19	Dodecanoic acid, ME[d]	X	X		X
20	Benzeothiazole, 2-(methylthio)-		X		X
21	Phenol, 4-(1-methyl-1-phenylethyl)-		X		X
22	12-methyltridecanoic acid, ME (C14:0)[d]	X	X	X	X
23	Hexadecanenitrile		X		
24	Pentadecanoic acid, ME (C15:0)[d]	X	X	X	X
25	Hexadecenoic acid, ME (C16:0)[d]	X	X		X
26	Heptadecanoic acid, ME (C17:0)[d]		X		
27	Octadecenoic acid, ME (C18:0)[d]	X	X		X
28	Nonadecanoic acid, ME (C19:0)[d]		X		
29	Eicosanoic acid, ME (C21:0)[d]		X		
30	4,4'-(4,4'-Bipheylylenedioxy)dianiline		X		X
*	Hydrocarbon contaminants		X		X

(a) DME= Dimethyl Ester
(b) BP = Base Peak
(c) M+ = Molecular Ion Peak
(d) ME = Methyl Ester

4.3 Comparison between Samples and Standards

4.4.1 Whole Samples and Standards

Humic and fulvic acids are decomposition products of organic matter and thus are found in soils. Because they are also water soluble, soil humics were investigated as a possible source of a black coating inside a Spanish cave.[31] Saiz-Jimenez and Hermosin concluded that the ground water percolating through the soil above the cave was carrying humic acids into the cave and depositing them onto the walls, yielding a black coating on the cave walls. The researchers compared soil samples from above the cave to the black coating samples to illustrate the humic connection. On the basis of the results of this similar study, humic and fulvic acids as well as soil samples collected above, near, and inside the Little Lost River Cave were used as comparative standards and samples for this investigation.

Previous pyrolysis-GC-MS research on the coating from Little Lost River Cave showed the presence of compounds, particularly acetamide, indicative of bacteria.[32] Bacteria can produce melanin, which can in turn form black coatings on surfaces over time. The presence of bacterial melanins on stone monuments has been shown to degrade the material and to mar its appearance; THM-GC-MS has been used to identify bacterial melanins on such surfaces.[33] For comparison, both synthetic and natural melanin were also used as standards.

There were few compositional similarities between the two melanins and the coating samples (Figure 4.5). The peak identities for the natural melanin are included in Table 4.4. The melanin standard is very different from the samples in that it contains only five major components. Of those, two are found in blanks and control samples (peaks 4 and 13). The remaining three are fatty acid methyl esters, which are also found in humic and fulvic acid

standards. The natural melanin has fewer compounds in common with the whole coating samples than do humic and fulvic acids; therefore, the humic and fulvic acids are a better match for the composition of the black coating than are the melanins.

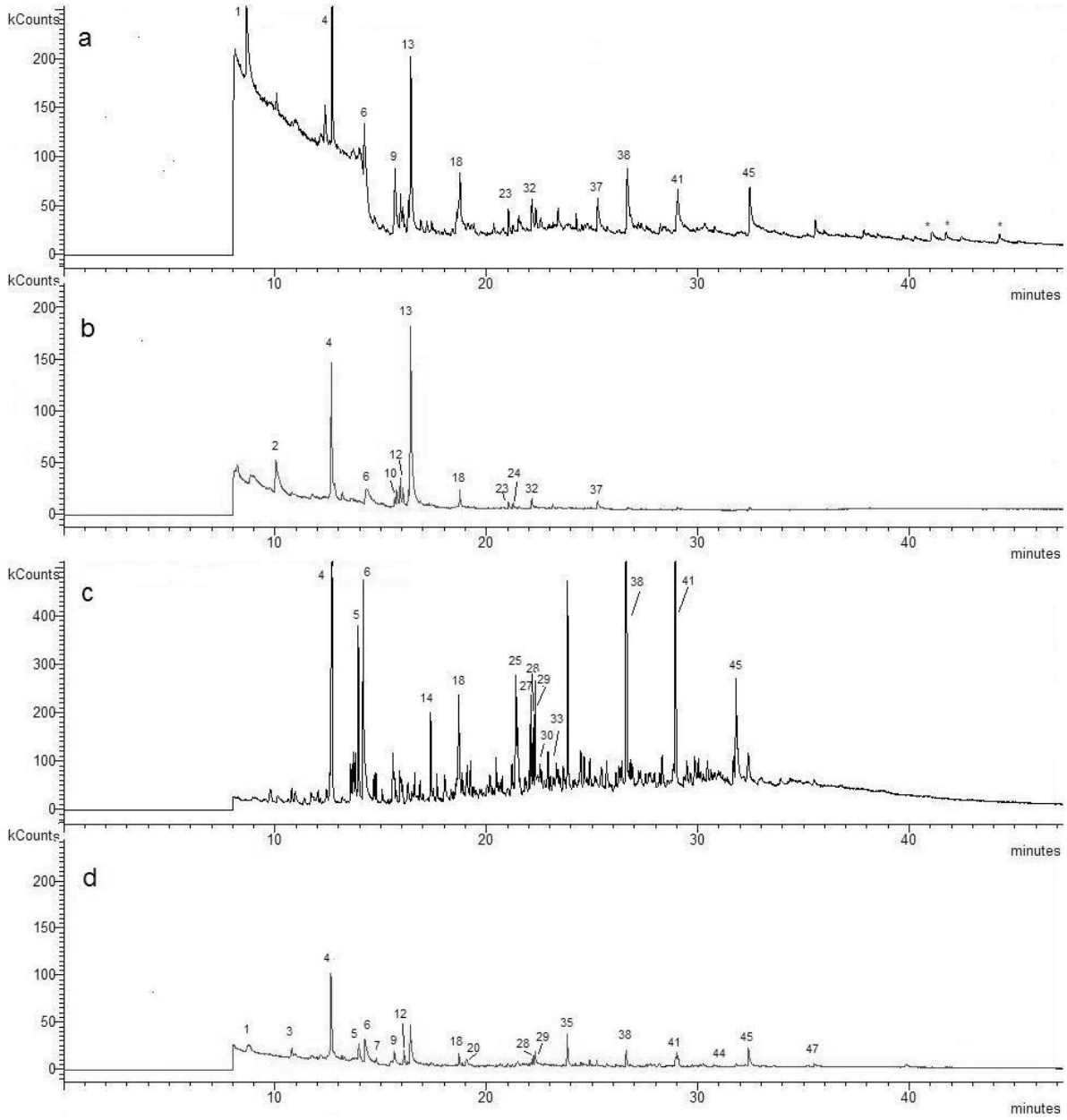

Figure 4.5. Chromatograms of standard materials: (a) soil collected from inside the cave, (b) soil collected from outside the cave entrance, (c) fulvic acid standard, and (d) humic acid standard. See Table 4.4 for peak identities.

Table 4.4. Peak Identities for Figure 4.5, Including Natural Melanins

		Whole Samples				Standards			Soils	
Peak #	Peak Identity	1	4	5	7	Humic	Fulvic	Natural Melanin	S1	S2
1	3-Furaldehyde					X			X	
2	N-(2-Methoxyethyl)isopropylamine									X
3	Benzene, methoxy-					X				
4	Benzene, (methoxymethyl)-	X	X	X	X	X	X	X	X	X
5	Butanedioic acid, DME	X			X	X	X			
6	Benzenemethanamine, N,N, dimethyl	X	X		X	X	X		X	X
7	Butanedioic acid, methyl-, DME	X				X				
8	Piperidine-2,5-dione	X			X					
9	Benzoic acid, ME					X			X	
10	Butanoic acid, 2-methyl-2-(1-methylethyl)-3-oxo-, EE									X
11	2,3,4-Trimethyl-isoxazol-5(2H)-one	X			X					
12	Octanoic acid, ME					X				X
13	1,3-Benzenedimethanamine, N,N,N',N'-tetramethyl-		X	X	X	X		X	X	X
14	Benzene, 1,4-dimethoxy-						X			
15	Unknown, BP=117, M+=144	X			X					
16	2,4(1H,3H)-Pyrimidinedione, dihydro-3-methyl-	X			X					
17	2,4(1H,3H)-Pyrimidinedione, 1,3-dimethyl	X	X		X					
18	Nonanoic acid, ME					X	X		X	X
19	Unknown, BP=42, 127, 142	X	X		X					
20	Benzothiazole					X				
21	3-hydroxymethyl-1-methyl piperidine		X		X					
22	Phenol, 2-ethyl-4,5-dimethyl		X							
23	Decanoic acid, ME								X	X
24	4-Imidazolidinone, 2-thioxo-									X
25	Pyrrolid-2-one-5-carboxylic acid, N-methyl-, EE						X			
26	Methylimidizolidinedione		X							
27	1,2,4-Trimethoxybenzene						X			
28	1,3,5-Triazine-2,4,6(1H,3H,5H)-trione, 1,3,5-trimethy-					X	X			
29	Benzoic acid, 3-methoxy-, ME					X	X			
30	2-Propenoic acid, 3-phenyl-, ME						X			
31	Trimethyltriazinetrione		X							
32	1,2,4-Trimethoxybenzene	X			X				X	X
33	Trimethyl 1,2,3-propanetricarboxylate						X			
34	1H-isoindol-1,3 (2H)dione, 2-methyl	X	X		X					
35	2,4(1H,3H)-Pyrimidinedione, 1,3,5-trimethyl-					X				
36	1,4-Benzenedicarboxylic acid, DME	X			X					
37	Dodecanoic acid, ME	X	X		X				X	X
38	Benzoic acid, 3,4-dimethoxy-, ME					X	X		X	
39	Benzeothiazole, 2-(methylthio)-		X		X					
40	Phenol, 4-(1-methyl-1-phenylethyl)-		X		X					
41	Tridecanoic acid, 12-methyl-, ME (C14:0)					X	X	X	X	
42	12-methyltridecanoic acid, ME (C14:0)	X	X	X	X					
43	Hexadecanenitrile		X							
44	Pentadecanoic acid, ME (C15:0)	X	X	X	X	X				
45	Hexadecenoic acid, ME (C16:0)	X	X		X	X	X	X	X	
46	Heptadecanoic acid, ME (C17:0)		X							
47	Octadecenoic acid, ME (C18:0)	X	X		X	X		X		
48	Nonadecanoic acid, ME (C19:0)		X							
49	Eicosanoic acid, ME (C21:0)		X							
50	4,4'-(4,4'-Bipheylylenedioxy)dianiline		X		X					
*	Hydrocarbon contaminants		X		X				X	

The characterization of humic acids as part of a complex mixture can be somewhat difficult because of their origin: they can be derived from forests, litter, grasslands, etc. Additionally, they are composed of several moieties including polyphenols, lignin, lipids,

polysaccharides, and amino acids [29] and can range in size from several hundred to several hundred thousand Daltons.[34] Unlike amberat, which contains a marker (hippuric acid) for the positive identification of its presence in an unknown substance, humic acids do not have one particular marker because of their complex nature. In the absence of marker compounds, positively identifying an unknown material is quite difficult, and one must instead use significant differences between knowns and unknowns for drawing conclusions. Previous researchers, such as Saiz-Jimenez, discussed below, have used comparative materials collected from the location of interest to aid in identifying unknown black coatings.

Saiz-Jimenez and Hermosin suggested that the black coating found in a Spanish cave (Cueva del Encajero) was humic-like in nature and derived from the decomposition of olives from an olive grove above the cave.[31] This was concluded after comparing the black coating with humic acid-like fractions isolated from waste waters collected from above the cave. Although the substances were not identical in composition, olives from above the cave were still considered the cause of the coating. It was concluded that the differences (unsaturated fatty acids and dicarboxylic acids) were attributable to the decomposition of the olives in the soil.[31]

THM-GC-MS characterization of humic acids has established that benzoic acid derivatives and phenols are indicative of the polysaccharide and lignin moieties within the larger humic acid structure.[34] Other products of THM on humic acids include benzenecarboxylic acids and C_{12} to C_{26} fatty acids, with even-numbered chains most prominent and C_{16} and C_{18} the most observed.[34] It has also been noted that α,ω-dimethylesters (ranging from C_8 to C_{26}) were products of the THM of humic fractions.[31, 35]

The humic standard shares many peaks with the black coating samples (peaks 4, 5, 6, 7, 8, 13, 44, 45, and 47; see Table 4.4). The presence of 3-furaldehyde (peak 1) found in the soil from inside the cave and in the humic standard (Figure 4.5 a and d, respectively) indicates the presence of polysaccharides. THM yielded methoxy benzene, 1,4-dimethoxy-benzene, 3,4-dimethoxy-phenol, and 4-(1-methyl-1-phenylethyl)-phenol as derivatives of aromatic compounds (Table 4.4).

4.4.2 Comparison Between Whole Samples and Cooking Residues

Isotopic ratios for carbon and nitrogen found in the coating were consistent with the hypothesis that the coating formed through condensation of smoke and cooking residues formed when animals were "barbecued" inside the cave.[1] Faunal evidence of charred bone and the presence of two hearth features also were indicative that cooking had occurred at the site. To compare the molecular composition of such residues to the 10BT1 coating, samples were prepared by cooking meat (beef and pork) over hardwood fires. The residue from cooking meat in such a way over a long period of time was also collected from a smoker barbecue. The researchers used THM-GC-MS to analyze these materials.

The cooking residues varied greatly depending on the meat used and the location from which the material was collected (e.g., directly above the food versus the end of the cooking chamber). For example, most of the compounds observed in the residue that were formed by cooking beef over hardwood charcoal eluted at low or high retention times, whereas the residue from the end of the cooking chamber showed compounds eluting primarily in the middle retention times. For simplification purposes, only three cooking residues have been compared to the whole samples. These cooking residues (the residue

collected from the end of the barbeque, the residue collected from the barbeque lid, and the residue collected from the smoke box, which is representative of smoke alone) were found to bear the most similarities in appearance to the black coating samples being analyzed.

Few compounds were found to be common to both the whole samples and cooking residues (Figure 4.6). Piperidine-2,5-dione (peak 7; see Table 4.5) and octadecenoic acid, methyl ester (peak 45) were found in both the whole residues and the experimental cooking residues. The remaining compounds, primarily aldehydes, were found only in the cooking residues.

Although some similarities exist between the coating and the experimentally created cooking residues, the black coating is water soluble, but the cooking residues are not. Also, many different compounds were identified in the coating versus the cooking residues, and the materials appeared different. These differences led to the conclusion that the black coating is not primarily a cooking residue. However, on the basis if the faunal and charcoal evidence in the cave, we know that fires did take place there, so the black, sooty portions of the coating may have a component of actual cooking-fire soot, which may make up a small proportion of the organic material present in the coating.

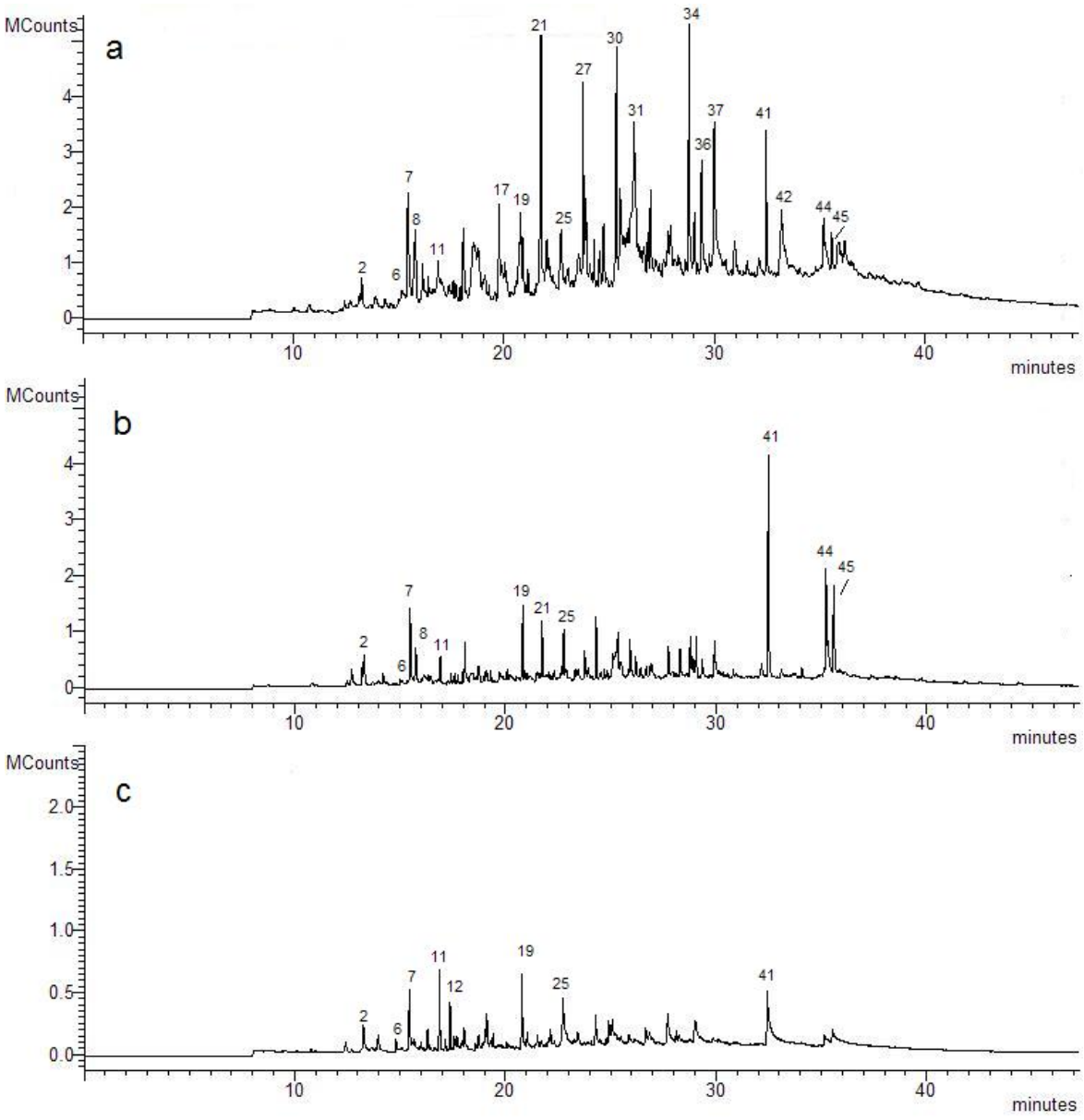

Figure 4.6 Chromatograms of experimental cooking residues: (a) end residue, (b) lid residue, and (c) smoke box residue. Peak identities can be found in Table 4.6 with compounds for whole samples.

Table 4.5. Compound List for the Three Experimental Cooking Residues as They Compare to the Whole Coating Samples

		Whole Samples				Cooking Residues		
Peak #	Peak Identity	1	4	5	7	End Residue	Lid Res	Smoke Box
1	Benzene, (methoxymethyl)-	X	X	X	X			
2	Unknown, BP=71					X	X	X
3	Butanedioic acid, DME	X			X			
4	Benzenemethanamine, N,N, dimethyl	X	X		X			
5	Butanedioic acid, methyl-, DME	X						
6	Phenol, 2-methoxy-					X	X	X
7	Piperidine-2,5-dione	X			X	X	X	X
8	d-Ribose, 2-deoxy-bis(thiononyl)-dithioacetal					X	X	
9	2,3,4-Trimethyl-isoxazol-5(2H)-one	X			X			
10	1,3-Benzenedimethanamine, N,N,N',N'-tetramethyl-		X	X	X			
11	Benzene, 1,2-dimethoxy-					X	X	X
12	Benzene, 1,4-dimethoxy-							X
13	Unknown, BP=117, M+=144	X			X			
14	2,4(1H,3H)-Pyrimidinedione, dihydro-3-methyl-	X			X			
15	2,4(1H,3H)-pyrimidinedione, 1,3-dimethyl	X	X		X			
16	Unknown, BP=42, 127, 142	X	X		X			
17	3,5-Dihydroxyanisole					X		
18	3-hydroxymethyl-1-methyl piperidine		X		X			
19	1,2,3-Trimethoxybenzene					X	X	X
20	Phenol, 2-ethyl-4,5-dimethyl		X					
21	2,4,6-Trihydroxybenzaldehyde					X	X	
22	Methylimidizolidinedione		X					
23	Trimethyltriazinetrione		X					
24	1,2,4-Trimethoxybenzene	X			X			
25	3,4-Dimethoxy-5-hydroxybenzaldehyde					X	X	X
26	1H-isoindol-1,3 (2H)dione, 2-methyl	X	X		X			
27	1,2,3-Trimethoxybenzene					X		
28	1,4-Benzenedicarboxylic acid, DME	X			X			
29	Dodecanoic acid, ME	X	X		X			
30	3.4-Dimethoxy-5-hydroxybenzaldehyde					X		
31	Unknown, BP=43, 180					X		
32	Benzeothiazole, 2-(methylthio)-		X		X			
33	Phenol, 4-(1-methyl-1-phenylethyl)-		X		X			
34	Acridine, 9,10-dihydro-9,9-dimethyl					X		
35	12-methyltridecanoic acid, ME (C14:0)	X	X	X	X			
36	Benzaldehyde, 3,4,5-trimethoxy-					X		
37	Anisole, p-styryl-					X		
38	Hexadecanenitrile		X					
39	Pentadecanoic acid, ME (C15:0)	X	X	X	X			
40	Hexadecenoic acid, ME (C16:0)	X	X		X			
41	Hexadecanoic acid, ME (C16:0)					X	X	X
42	Gibberelic acid					X		
43	Heptadecanoic acid, ME (C17:0)		X					
44	Octadecanoic acid, ME (C18:0)					X	X	
45	Octadecenoic acid, ME (C18:0)	X	X		X	X	X	
46	Nonadecanoic acid, ME (C19:0)		X					
47	Eicosanoic acid, ME (C21:0)		X					
48	4,4'-(4,4'-Bipheylylenedioxy)dianiline		X		X			
*	Hydrocarbon contaminants		X		X			

4.4.3 Water-Soluble Fractions and Soil Comparison

During an attempt by previous researchers to section the black coating, it was noticed that the material was water soluble.[36] This suggested a possible connection between water-soluble soil organic matter and the coating. Soil organic matter is predominantly made up of humic substances; those humic substances that are water soluble, such as fulvic acids and, to a lesser extent, humic acids, may be borne into the cave through percolating ground water, precipitating onto the surface. To investigate this possibility, water-soluble fractions of the black coating were analyzed and compared to the water-soluble fraction of the soil collected from above the cave.

When the researchers compared the coating samples to the water-soluble fraction of the soil collected from above the cave (S3), it was noticed that S3 was most similar to the yellow coating samples collected (3yellow and 7). Therefore, a separate comparison between the soil and the yellow coatings was performed (see Table 4.6). This may indicate that water-soluble compounds entered the cave in ground water from above and created a coating on the walls but that that is not the only source of chemicals in the coating. Changes caused by drying and oxidation, as well as through any action of bacteria, would yield additional products beyond those expected from the source material. This has been observed by others studying water-deposited soil organic matter in caves, as described above.[31] Saiz-Jimenez noted that there are differences between the humic-like fractions of waste waters and the black coating in Cueva del Encajero; the most important difference is the absence of unsaturated fatty acids, accompanied by the appearance of aliphatic dicarboxylic acids. This is attributed to the oxidation and/or microbial degradation of lipids. The conclusion reached by Saiz-Jimenez et al. on the basis of the similarities between the humic-like fraction of the

waste waters and that particular black coating was that the compounds found in the soil above the cave were percolating through the soil and redepositing inside the cave.

Table 4.6. Compound List Identifying Peaks in the Chromatograms from the Water-Soluble Fraction of the Soil Collected from Above the Cave (S3), the Yellow Portion of Sample 3, and Sample 7 (a Piece of Shiny Yellow Coating)

Peak #	Peak identity	Soil	3Yellow	7
1	Methoxymethylbenzene	X	X	X
2	N,N-dimethylbenzenemethanamine	X	X	X
3	1-methyl-2,5-pyrrolindinedione		X	X
4	Benzoic acid, ME (C6:0)	X	X	X
5	Octanoic acid, ME (C8:0)	X	X	X
6	N,N,4-trimethylbenzenemethanamine	X	X	X
7	2-methoxy-4-methylphenol	X	X	X
8	1,4-dimethoxybenzene	X	X	X
9	3-acetoxy-3-hydroxy-2methylpropionic acid, ME		X	X
10	2,4 (1H, 3H)-pyrimidinedione, dihydro-3-methyl	X	X	X
11	Isopropylimidazole-2-thione	X	X	X
12	Nonanoic acid, ME (C9:0)	X	X	X
13	Indole	X	X	
14	Decanoic acid, ME (C10:0)	X	X	X
15	3-methoxybenzoic acid	X	X	X
16	1,2,4-trimethoxybenzene	X	X	X
17	Trimethyltriazinetrione		X	X
18	3-phenyl-2-propenoic acid, ME	X	X	
19	1-chlorodecane	X	X	X
20	1,4-benzendicarboxylic acid, DME	X	X	X
21	Dodecanoic acid, ME (C12:0)	X	X	X
22	Nonanedioic acid, DME	Tr[a]	X	Tr[a]
23	2-(methylthio)-benzothiazole		X	X
24	5-(4H)oxalolone, 2-methyl-4-(phenylmethylene)		X	
25	4-(1-methyl-1-phenylethyl)-phenol		X	X
26	Tetradecanoic acid, ME (C14:0)	X	X	X
27	Hexadecenoic acid, ME (C16:1)		X	X
28	Hexadecanoic acid, ME (C16:0)	X	X	X
29	Octadecenoic acid, ME (C18:1)		X	X
30	Octadecanoic acid, ME (C18:0)	X	X	X

(a) Tr = Trace Amounts

As suggested by Saiz-Jimenez, the presence of dicarboxylic acids in the coating within the cave could be indicative of decomposition. The only dicarboxylic acids present in any of the three samples was 1,4 benzenedicarboxylic acid, dimethyl ester (peak 20), and nonanedioic, dimethyl ester (peak 22), and they were present in all three samples. This indicates that little or no decomposition occurred during the redeposition of the humic substances. This is also supported by the appearance of two unsaturated fatty acids (peaks 27 and 29).

There are substantial similarities between the water-soluble fraction of the soil collected from above the cave (S3) and the two yellow coating samples (3 yellow and 7). Only 8 of the 30 compounds were absent from the soil but found in either yellow coating, indicating remarkable similarity between the water-soluble compounds found in the soil and the compounds present in the yellow coating. Additionally, several peaks identified in the yellow coating of Little Lost Rive Cave were also identified by Saiz-Jimenez in Cueva del Encajero (peaks 1, 12, 17, 20, 21, 22, 26, 28, 29, and 30).[31] The similar compounds were primarily carboxylic acids with the exception of trimethyltriazinetrione. The chromatograms from the water-soluble fractions of samples 4 and 5 were also compared to that of the soil from above the cave and again showed marked similarities. All of the major components (peaks 1, 2, 5, 6, 10, 11, 12, 13, 14, 15, 17, 18, 20, 21, 26, 28, and 30 from Table 4.6) were identified in the chromatograms of the water-soluble fractions of samples 4 and 5. The identified compounds were all found in the soil from above the cave.

The similarities between the overlying soil and the water-soluble and yellow fractions of the coating strongly indicate that water is likely percolating through the soil above the cave and the water-soluble compounds found in the soil are then redeposited onto the walls

of the cave in the form of a shiny, yellow coating. Where this overlies dark soot or mineral deposits, the coating appears black. Because the yellow material has a chemical composition different from that of the underlying sooty coating, it appears that they likely have different origins. The two fractions should be separated prior to radiocarbon analysis, as they appear to be independent.

CHAPTER 5

CONCLUSIONS

5.1 Conclusions

THM-GC-MS results show that the black coating found within Little Lost River Cave is not consistent with the black coatings deposited by bacterial growth, evidenced by differences between the coating and natural melanin standards. Upon comparison with experimental cooking residues, the coating bears few similarities to these cooking residues; only two compounds were common to the coating and cooking residues (piperidine-2,5-dione and octadecenoic acid methyl ester). Also, because the coating is also largely water soluble, the black coating found in Little Lost River Cave differs fundamentally from a residue formed by cooking meat. Many similarities have been found among soil humic acid standards, the soil collected from above the cave, and the black coating. Although the presence of compounds such as aldehydes and derivatives of aromatic compounds provides a connection between the black coating and humic acids, because there is no chemical marker for humic acids, it is difficult to positively identify the black coating as a humic acid.

It has also been shown that the black coating is not a homogeneous substance; its chemical composition varies by its spatial location within the cave, as well as its physical description. Previous analyses performed by Fezzey and Armitage showed irreproducibility within the black coating samples.[28] For the current investigation, samples were collected by their location, labeled on a map, and given a specific physical description. This allowed for specific comparison between the black coating samples, illustrating that the coating did indeed vary in composition throughout the cave.

On the basis of the THM-GC-MS characterization of multiple samples of the black coating found in Little Lost River Cave, the coating appears to have a geologic or biogeochemical origin. The similarities between the yellow portions of the coatings and the water-soluble fraction from the soil above the cave indicate that the coating was caused by water's percolating through the soil above the cave and redepositing water-soluble chemicals onto the walls of the cave. Therefore, the radiocarbon age of this coating is probably not a reliable minimum age for the underlying paintings.[1] If the coating formed, as originally hypothesized, through human activity – anthropogenic fires and cooking – within the cave after the paintings had been executed, then the radiocarbon age of that material *would* be relevant. Humic substances borne into the cave as dissolved soil organic matter, on the other hand, may both pre- and postdate the creation of the paintings. The THM-GC-MS analysis indicates similarities between soil humics, the overlying soil, and samples of the coating and significant differences between the coating and either amberat or cooking and smoke residues. Therefore, the radiocarbon date obtained for the black coating should not be interpreted as an indicator of the age of the rock paintings.

5.2 Future Analysis

All molecular chemical analyses performed so far on the black coating from Little Lost Rive Cave have been on a qualitative scale. THM-GC-MS is an effective technique for providing a general idea of the characteristic compounds in complex substances like humic acids. It has also been shown to be an instrumental tool in characterizing mixtures.

A quantitative approach would use the GC-MS to compare ratios of specific fatty acids, such as C_{16} and C_{18}; other possible comparisons include the C_{15}:C_{16} ratio, as proposed

by Durand et al.[37] This quantitative method has been previously used to determine the identity of food residues remaining in archaeological ceramics[38] and to compare the concentrations of lipids in soil and potsherds.[39] This approach would clarify the similarities and differences between the fatty acids observed in the black coating and those in the soil and cooking residue samples.

REFERENCES

(1) Steelman, K.; Rowe, M.; Boutton, T.; Southon, J.; Merrell, C.; Hill, R. *J. Arch. Sci.* **2002**, *29*, 1189-1198.

(2) Challinor, J. M. *J. Anal. Appl. Pyrolysis* **1989**, *16*, 323-333.

(3) Challinor, J. M. *J. Anal. App. Pyrolysis* **2001**, *61*, 3-34.

(4) Ishida, Y.; Ohtani, H.; Tsuge, S. *J. Anal. Appl. Pyrolysis* **1995**, *33*, 167-180.

(5) Pitthard, V.; Finch, P.; Bayerova, T. *J. Sep. Sci.* **2004**, *27*, 200-208.

(6) Stankiewicz, B. A.; van Bergen, P. F.; Smith, M. B.; Carter, J. F.; Briggs, D. E. G.; Evershed, R. P. *J. Anal. Appl. Pyrolysis* **1998**, *45*, 133-151.

(7) Martin, F.; Gonzalez-Vila, F. J.; del Rio, J. C.; Verdejo, T. *J. Anal. Appl. Pyrolysis* **1994**, *28*, 71-80.

(8) Challinor, J. M. *J. Anal. Appl. Pyrolysis* **1996**, *37*, 185-197.

(9) Pastorova, I.; van der Berg, K. J.; Voon, J. J.; Verhoeven, J. W. *J. Anal. Appl. Pyrolysis* **1997**, *43*, 41-57.

(10) Scalarone, D.; Lazzari, M.; Chiantore, O. *J. Anal. Appl. Pyrolysis* **2002**, *64*, 345-361.

(11) Abraham, S. J.; Criddle, W. J. *J. Anal. Appl. Pyrolysis* **1985**, *9*, 53-64.

(12) Fabbri, D.; Helleur, R. *J. Anal. Appl. Pyrolysis* **1999**, *49*, 277-293.

(13) Schwarzinger, C. *J. Anal. Appl. Pyrolysis* **2004**, *71*, 501-514.

(14) Schwarzinger, C. *J. Anal. Appl. Pyrolysis* **2003**, *68-69*, 137-149.

(15) Hendricker, A.; Voorhees, K. *J. Anal. Appl. Pyrolysis* **1998**, *48*, 17-33.

(16) Knicker, H.; del Rio, J. C.; Hatcher, P.; Minard, R. *Org. Geochem.* **2001**, *32*, 397-409.

(17) Zang, X.; Brown, J.; van Heemst, Jasper D. H.; Palumbo, A.; Hatcher, P. *J. Anal. Appl. Pyrolysis* **2001**, *61*, 181-193.

(18) Martin, F.; Almendros, G.; Gonzalez-Vila, F. J.; Vedejo, T. *J. Anal. Appl. Pyrolysis* **2001**, *61*, 133-145.

(19) Ikeya, K.; Ishida, Y.; Ohtani, H.; Watanabe, A. *J. Anal. Appl. Pyrolysis* **2006**, *75*, 174-180.

(20) Page, D. W.; van Leeuwen, J. A.; Spark, K. M.; Mulcahy, D. E. *J. Anal. Appl. Pyrolysis* **2002**, *65*, 269-285.

(21) Klingber, A.; Odermatt, J.; Meier, D. *J. Anal. Appl. Pyrolysis* **2005**, *74*, 104-109.

(22) Hedges, R.; Ramsey, C.; Klinken, G.; Pettitt, P.; Nielsen-Marsh, C.; Etchegoyen, A.; Niello, J.; Boschin, M.; Llamazares, A. *Radiocarbon* **1998**, *40*, 35-44.

(23) Chiavari, G.; Galletti, G.; Lanterna, G.; Mazzeo, R. *J. Anal. Appl. Pyrolysis* **1993**, *24*, 227-242.

(24) Cappitelli, F. *J. Anal. Appl. Pyrolysis* **2004**, *71*, 405-415.

(25) Fichter, E.; Hopkins, M.; Isotoff, A.; Liljeblad, S.; Lyman, R. A.; Strawn, M.; Taylor, E. Idaho State College, Pocatello, ID., *Exploratory Excavations in Little Lost River Cave; No. 1, A Progress Report.* Unpublished work, **1954**.

(26) Gruhn, R.; Bryan, A. *Report on a Test Excavation at Little Lost River Cave No. 1 (10BT1) in 1990;* Report on Cultural Resource Permit ID-I-27727; Bureau of Land Management: Idaho Falls, ID, October **1990**.

(27) Russ, J.; Hyman, M.; Shafer, H. J.; Rowe, M. W. *Nature* **1990**, *348*, 710-711.

(28) Fezzey, S.; Armitage, R. A. *J. Anal. Appl. Pyrolysis* **2006**, *77*, 102-110.

(29) Davies, G.; Ghabbour, E.; Steelink, C. *J. Chem. Ed.* **2001**, *78*, 1609-1614.

(30) Butler, M.; Day, A. *Can. J. Microbiol.* **1998**, *44*, 1115-1136.

(31) Saiz-Jimenez, C.; Hermosin, B. *J. Anal. Appl. Pyrolysis* **1999**, *49*, 349-357.

(32) Fezzey, S. Characterization of black deposit associated with rock paintings in Little Lost River Cave, Idaho using Pyrolysis GC/MS. MS. Thesis, Eastern Michigan University, Ypsilanti, MI, 2005.

(33) Saiz-Jimenez, C. *Sci. Total Environ.* **1995**, *167*, 273-286.

(34) Saiz-Jimenez, C.; Hermosin, B.; Trubetskaya, O.; Reznikova, O.; Afanas'eva, G.; Trubetskoj, O. *Geoderma* **2006**, *131*, 22-32.

(35) Grasset, L.; Ambles, A. *J. Anal. Appl. Pyrolysis* **1998**, *47*, 1-12.

(36) Steelman, K.; Rowe, M.; Guillemette, R.; Merrell, C.; Hill, R. *American Indian Rock Art* **2002**, *28*, 111-120.

(37) Durand, C.; Rubban, V.; Ambles, A. *J. Anal. Appl. Pyrolysis* **2005**, *73*, 12.

(38) Eerkens, J. W. *Archaeometry* **2005**, *47*, 83-102.

(39) Heron, C.; Evershed, R.; Goad, L. *J. Arch. Sci.* **1991**, *18*, 641-659.