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Determination of materials present in skeletonized human remains and the associated DNA: Development of a GC/MS protocol

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ABSTRACT

DNA testing of skeletonized human remains is considered to be challenging, especially when the remains have been exposed to inhibitory materials during decomposition. Inhibitors affect the processing of DNA, either by preventing efficient extraction or interfering with down-stream PCR-based processes. Limited studies have been performed on real-world samples that have been exposed to such inhibitors. This paper presents the development of a gas chromatography/mass spectrometry (GC/MS) protocol for the evaluation of materials present in skeletonized human remains recovered from the field, as well as the DNA extracted from the same materials. Twenty-one bone samples and seventeen DNA extracts were evaluated across three solvents and multiple GC/MS parameters to determine the optimal conditions for the recovery of trace materials present. The aim of this work is to provide a technique that can determine the presence of inhibitors prior to DNA extraction, allowing analysts to optimize removal of inhibitory materials.

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1. Introduction

Retrieval of DNA from osseous materials continues to be a challenging aspect of the process of human identification. In cases of mass fatality, where there is a high degree of commingling, efficiency in DNA extraction is key to a timely resolution of the identification process. Downstream processing of the DNA recovered from skeletonized remains has continued to increase in sensitivity. STR kits have been optimized for smaller sized loci (i.e., MiniFiler) and increased sensitivity (Modified Y-filer: [1,2]; and current Next Generation Sequencing (NGS, aka MPS) protocols are able to recover DNA from chemically compromised and aged remains that were previously considered untestable [3,4]. Efforts have been made to improve DNA extraction protocols; however, these have largely focused on improvements in the release of DNA from the complex skeletal matrix. Complete demineralization of bone in extraction protocols [5–8] has reduced the required volume of skeletal materials, and increased overall success of testing [9].

Concurrent with the need to increase DNA yield from skeletal materials, is a need to remove inhibitors that may be present in the bone. Full demineralization releases a large amount of calcium and other minerals into the extraction buffer that need to be removed or there is a risk that downstream processing will be inhibited. Other inhibitory materials are commonly found in the soil and the environment surrounding the remains during decomposition. Humic acid [10,11], heme [12], and indigo [13] are among the most common chemicals encountered; however, in mass fatality events other compounds may be encountered, such as fuel oil [14,15] or metals [16]. Remains being transported or preserved for long term storage may also be treated with compounds, such as formalin, that prevent efficient recovery of DNA from biological materials [17].

In the optimization of extraction protocols, efforts have been made to not only recover as much DNA as possible but to strip away any inhibitors that might carry-over to the purified DNA and thus impede downstream processes. Unfortunately, by making a broad-scale attempt to remove any and all inhibitors, the loss of associated DNA can be extensive. When performing a DNA extraction using an organic purification, a large amount of DNA is lost during the post-purification washes [18]. The same can be said for protocols using inorganic purifications, in which 20–60% of the DNA present may

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be lost during the purification process [19,20].

Optimization of DNA extraction protocols to remove inhibitors known to be present in the skeletal materials would be desirable as this could decrease wash steps and increase the amount of DNA recovered in an extraction event. There is no need to remove every potential inhibitor if a specific subset could be targeted. For example, if a set of remains is known to have been buried in soil with an elevated presence of humic acid, an extraction pathway could be chosen that removes humic acid and not necessarily all other possible inhibitors. In effect, this is similar to the difference in choosing an organic purification versus an inorganic purification when the remains have been subject to saponification. Organic purification methods are more effective at the removal of fats and proteins than an inorganic purification [9,21,22].

The first stage of developing an inhibitor-specific extraction protocol is to determine the materials present in the remains. For modern mass disasters, it may be easily apparent what chemicals the remains have been exposed to. With remains recovered with an unknown provenience, assumptions can be made about inhibitor exposure based on the soil or historical accounts of the event, which may or may not prove to be correct. Many studies have focused on animal bones (seal ribs: [23]; salmonid bones [24]: and synthetic bones spiked with known quantities of inhibitory materials [25]. There have been some small scale real-world studies of human remains [26] as well as staged sets of remains (Mundorff and Davoren 2014); however, few studies have sought to analyze remains recovered across a broad range of burial conditions and environments.

Gas chromatography/mass spectrometry (GC/MS) is widely used in forensics for the detection of trace amounts of materials, particularly in toxicology [27] and arson investigations [28]. GC/MS relies on the solubility of desired materials in organic solvents. Molecules of compounds may be detected with optimized instrument parameters [29]. recently used DART (Direct Analysis in Real Time) ionization with mass spectrometry to analyze DNA recovered from blood samples spiked with multiple known PCR inhibitors. DART relies on direct ionization of a solid phase. As inhibitory materials may be found in low-concentrations throughout a skeletal element, and therefore require concentration before detection is possible, DART was not selected for use in this study. GC/MS workflow provides the ability to remove materials from a substrate through exposure to solvents and a subsequent concentration by volatilization prior to loading on the instrument. This paper describes the development of a protocol that can be used to detect both the presence of inhibitory compounds within osseous materials, as well as those that may carry through into the extracted DNA.

2. Methods and materials

2.1. Collection of skeletal materials

Osseous materials were submitted to the Armed Forces Medical Examiner – Armed Forces DNA Identification Laboratory (AFMES-AFDIL, aka AFDIL) from the Defense POW/MIA Accounting Agency (DPAA) as part of regular casework submissions. Samples used in this study were chosen randomly from casework samples submitted between May 2016 and August 2016. Skeletal materials have post-mortem intervals of 45–80 years, and have been recovered from a variety of burial conditions (e.g., surface, shallow, curated, coffin, preserved). The peri-mortem conditions were also widely variant and included ground losses, high-impact plane crashes, and sunken ships.

During standard casework processing, the bone is cleaned by removing the exterior removed using a Dremel® tool (Bosch, Stuttgart, Germany) fitted with an aluminum oxide sanding bit. The detritus generated during this process is typically discarded as medical waste. However, for the purposes of this study, the 'powder' was collected and stored in 15 mL polypropylene tubes (Sarstedt, Nűmbrect, Germany) at −20 °C until needed. A total of 439 samples were collected and anonymized with a code number so as to eliminate bias or expectations during analysis. The code number was randomly assigned. The first number represents the two-week period during which the sample was collected and the second number being the order in which the sample was randomly pulled from that grouping. For example sample 3–1 was the first sample pulled from the third collection set.

2.2. Extraction of DNA

The DNA extraction protocol used is described at length in [8]. It is briefly summarized here. DNA was extracted from the cleaned bones using a complete demineralization protocol coupled with an organic phenol:chloroform:isoamyl alcohol purification or a complete demineralization coupled with an inorganic purification using QIAquick PCR purification Kit (QIAGEN, Hilden, Germany). In both instances, samples are further concentrated using Amicon Ultra-4/30K centrifugal filter units (Millipore, Billerica, MA) and brought to a final volume of 50–200 μL with TE^{-4} (10 mM Tris, 0.1 mM EDTA; pH 7.5).

2.3. Selection of samples for GC/MS testing

For the purposes of this proof-of-concept study, 21 osseous material samples were chosen from the 439 collected (Table 1). These samples were selected based on having approximately 1.0 g of powder or more available, which would allow for a variety of solvents to be tested. The samples also had to be completed through regular casework processing so the associated DNA extracts could be used without the possibility of being needed for additional testing. DNA extracts were chosen from 17 of the associated samples (Table 2).

Five of these proof-of-concept samples were taken from remains recovered from the USS *Oklahoma*. These were specifically selected due to the known fuel contamination immediately post-mortem. The osseous elements themselves retained an odor of fuel and the surface materials removed were black and somewhat sticky (Fig. 1a). There was an expectation that these samples would provide a result with almost any solvent, which would allow for a possible benchmark from which other testing could be based. Other samples, such as those from the Korean War (Fig. 1b), were very powdery and lacking in coloration.

2.4. Testing parameters for osseous materials

Three different solvents were used to extract materials from the osseous detritus: methanol, acetonitrile, and dichloromethane (HPLC, LC/MS grade). Variations in the solubility of the various compounds in these solvents will affect the chromatography of the samples. Eleven different GC/MS and solvent combinations were used. These are summarized in Table 3, but described in more detail below. Some samples were tested multiple times simply due to the quantities available. The testing strategies progressed systematically towards increasing sensitivity and generating readable data.

Unless otherwise indicated, 1.5 mL of the solvent being evaluated was added to approximately 0.1 g of bone powder contained within a 2.0 mL polypropylene tube. Samples were vortexed and

Table 1
Samples used for testing. An attempt was made to select samples from a variety of locations; however, the general deciding factor for sample selection was whether an adequate amount of osseous detritus had been generated during the cleaning process.

Sample	Conflict	Location Recovered	Approximate PMI (years)	Element
1–1	Southeast Asia	Cambodia	47	Thoracic Vertebra
1–2	Southeast Asia	Cambodia	47	Temporal
2–1	Southeast Asia	Laos	47	Femur
2–2	WWII	Philippines	75	Occipital
2–5	Southeast Asia	Laos	47	Frontal
2–12	WWII	Papua New Guinea	75	Lumbar Vertebra
3–1	Korean War	South Korea	67	Temporal
3–7	WWII	Hawaii	77	Lumbar Vertebra
3–8	Southeast Asia	Vietnam	47	Cranium
3–9	Southeast Asia	Vietnam	47	Cranium
3–12	WWII	Solomon Islands	75	Os Coxa
3–13	Korean War	South Korea	67	Ulna
3–14	Korean War	South Korea	67	Humerus
4–2	Southeast Asia	Laos	47	Tibia
4–3	Korean War	Namjong-gu	67	Temporal
5–2	WWII	Solomon Islands	75	Patella
5–7	WWII	Tarawa	75	Occipital
6–1	WWII	Hawaii	77	Occipital
10–5	WWII	Hawaii	77	Vertebra
10–6	WWII	Hawaii	77	Tibia
10–9	WWII	Hawaii	77	Ulna

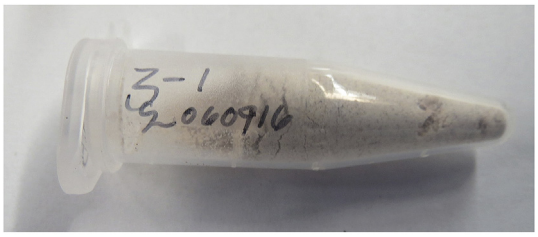
Table 2
DNA Samples used in the testing strategies. DNA extracts were generated during the course of regular casework. Samples selected for the GC/MS testing had been completely through the casework process and limited extract was available; therefore, there are skeletal samples tested that do not have associated DNA.

Sample	Conflict	Location Recovered	Extraction Protocol
1–1	Southeast Asia	Cambodia	Inorganic
1–2	Southeast Asia	Cambodia	Inorganic
2–1	Southeast Asia	Laos	Inorganic
2–2	WWII	Philippines	Inorganic
2–4	Southeast Asia	Vietnam	Inorganic
2–5	Southeast Asia	Laos	Inorganic
2–6	WWII	Kiribati	Inorganic
2–12	WWII	Papua New Guinea	Inorganic
3–1	Korean War	South Korea	Inorganic
3–7	WWII	Hawaii	Organic
3–8	Southeast Asia	Vietnam	Inorganic
3–9	Southeast Asia	Vietnam	Inorganic
3–12	WWII	Solomon Islands	Inorganic
3–13	Korean War	South Korea	Inorganic
3–14	Korean War	South Korea	Inorganic
4–3	Korean War	Namjong-gu	Inorganic
10–5	WWII	Hawaii	Organic

(a)

Fig. 1a. Surface materials removed from a lumbar vertebra recovered from the USS *Oklahoma*. The outer surface of the osseous samples clumps upon removal and can form a waxy coating on the sanding bit.

placed on a rotating shaker for 1–17 h at room temperature. There are modifications to this strategy as noted in the more detailed descriptions below.



(b)

Fig. 1b. Surface materials removed from a temporal bone recovered from South Korea. The materials removed were very powdery and talc-like.

For preparation of each sample, 9 mm glass vials with screw caps (Thermo Fisher, Walther, MA, USA) were used. Analysis was done using an Agilent 7890A/5875C GC/MS System with a 20 m column (Agilent, Santa Clara, CA) with a full scan and no subtraction of possible known elements. This was deliberately done, as it was unknown as to what would be detected, if anything, from the osseous detritus. All injections were split except where noted. A splitless injection, in which all of the analyte is passed to the column for detection, was used for only the final few methods described below.

At least one blank was used for each run. The blanks consisted of methanol within 9 mm glass vials with screw caps.

Analysis was performed using ChemStation (Agilent) and comparison to the NIST 2005 Spectral Library using the Mass Hunter software. In the event that peaks were not automatically called by the software, analysis was performed by the analyst with a comparison of the spectra generated to those determined to be the closest possible matches by the software.

2.4.1. Sample method #1 (SM#1)

Sample 10–5 was selected for testing. Approximately 0.1 g of detritus was placed in a 2.0 mL polypropylene tube with 1.5 mL methanol (≥99.9%, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA). Sample was vortexed and incubated for 2 h with periodic agitation. At the end of incubation, the sample was spun down to reduce the particulates and 550 μL was removed to the loading vial. The oven temperature was set at 200 °C for a run of 20 min.

Table 3
Parameters Tested. Description of tests performed on skeletal material (SM) in order. All injections were split, with the exception of SM#9 and SM#9a. "SM" is the abbreviation of "Sample Method".

Method	Solvent	# of samples	Treatment	GC/MS Parameters
SM #1	Methanol	1	Incubation in solvent & direct injection	200 °C oven. Hold for 20min.
SM #2	Methanol	2	Incubation in solvent & direct injection	200 °C oven. Ramp to 300 °C at 20 °C/min. Hold for 20min.
SM #3	Methanol	1	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	200 °C oven. Ramp to 300 °C at 20 °C/min. Hold for 20min.
SM #4	Methanol	7	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	150 °C oven. Ramp to 300 °C at 20 °C/min. Hold for 20min.
SM #5a	Methanol	4	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	150 °C oven. Ramp to 300 °C at 20 °C/min. Hold for 30min.
SM #5b	Acetonitrile	4	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	150 °C oven. Ramp to 300 °C at 20 °C/min. Hold for 30min.
SM #6	Dichloromethane	3	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	150 °C oven. Ramp to 300 °C at 20 °C/min. Hold for 30min.
SM #7	Dichloromethane	3	Same portions as tested in SM#6	150 °C oven with a hold for 20min. Ramp to 250 °C at 20 °C/min. Hold for 30min.
SM #8	Dichloromethane	5	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	150 °C oven with a hold for 20min. Ramp to 250 °C at 20 °C/min. Hold for 30min.
SM #9	Dichloromethane	5	Same fractions as from SM #8	150 °C oven with a hold for 20min. Ramp to 250 °C at 20 °C/min. Hold for 30min. Splitless injection.
SM #9a	Dichloromethane	1	Fraction of sample 3–7 was concentrated overnight and resuspended in meOH	150 °C oven with a hold for 20min. Ramp to 250 °C at 20 °C/min. Hold for 30min. Splitless injection.

2.4.2. Sample method #2 (SM#2)

Samples 3–13 and 10–5 were used for testing. Approximately 0.1 g of detritus from 3-13 was placed in a 2.0 mL polypropylene tube with 1.5 mL methanol. Sample was vortexed and incubated overnight at room temperature. At the completion of incubation, each sample was spun down to pellet the particulate and 700 μL placed in the glass loading vial. A fraction of the 10-5 sample that had been prepared for SM#1 was removed to a glass loading vial. The loading program for the instrument was modified to a starting oven temperature of 200 °C, with ramp to 300 °C at a rate of 20 °C/min, followed by a hold at the final temperature for 20 min.

2.4.3. Sample method #3 (SM#3)

The remaining methanol fraction of 10–5 from the initial preparation was removed from the detritus and placed in a clean glass beaker. The beaker was placed in a chemical fume hood and the sample was allowed to volatilize overnight at room temperature. The concentrated material was resuspended in 700 μL of methanol, of which 500 μL was placed in a glass loading vial. The program on the instrument was the same as in SM #2.

2.4.4. Sample method #4 (SM#4)

Seven new samples were selected for testing (1-1, 1–2, 2-2, 2–12, 3–13, 4–2, 10–6). For each sample, approximately 0.1 g of osseous material was placed into each of three 2.0 mL tubes. Methanol (1.5 mL) was added to each tube. Samples were vortexed and placed on a nutator at room temperature for 1 h. Samples were spun down to pellet particles. The solvent extract was removed to clean watch glasses. The three solvent extracts for each sample were combined on a single watch glass. Samples were allowed to dry completely before being recovered off the watch glass with 1.0 mL methanol. The loading program of the instrument was modified to a starting temperature of 150 °C. The run progressed with a ramp to 300 °C at a rate of 20 °C/min, followed by a hold at the final temperature for 20 min.

2.4.5. Sample method #5 (SM#5a and SM#5b)

A new solvent was added in this method. Four samples (3–8, 4–3, 5–2, 10–9) were selected for incubation in methanol and four (3–1, 3–9, 5–7, 6–1) were selected for incubation in acetonitrile (Sigma-Aldrich). As with SM#4, 0.1 g of sample was placed into each of three 2.0 mL tubes. To each tube was added 1.5 mL of the designated solvent. Samples were placed on the nutator and allowed to incubate for an hour at room temperature. After incubation, the tubes were spun down to pellet the materials and the solvent poured off into a clean watch glass. The three aliquots for each sample were placed in a single watch glass. Volatilization continued until the samples were dry and the dried material was resuspended in 1.0 mL of the respective solvent. The instrument protocol started with an oven temperature of 150 °C, followed with a ramp to 300 °C at a rate of 20 °C/min, and a hold at the final temperature for 30 min.

2.4.6. Sample method #6 (SM#6)

A new solvent was used in this method. Three samples (3–1, 5–7, 6–1) were incubated in dichloromethane (HPLC grade; Pharmco AAPER, Brookfield, CT, USA). Approximately 0.1 g of osseous material was incubated in 1.6 mL solvent for 1 h on a nutator. Multiple tubes of substrate were not available for this reaction, as the detritus had been exhausted by previous testes. After 1 h, the tubes were spun down and the solvent removed to individual watch glasses for volatilization. Once the samples were completely dry, they were resuspended in 550 μL of methanol and placed in the glass loading vials. The program was not modified from SM#5.

2.4.7. Sample method #7 (SM#7)

The same fractions of solvent extract generated in SM#6 were used in this testing strategy. Modifications were made to the injection program. The starting oven temperature remained at 150 °C, but remained at that temperature for a 20 min hold before ramping at 20 °C/min to 250 °C for an additional 30 min hold.

2.4.8. Sample method #8 (SM#8)

A new set of five samples was selected for testing using SM#7. Approximately 0.1 g of osseous detritus was incubated in 1.5 mL dichloromethane on a nutator for 1 h at room temperature. Samples were centrifuged to pellet any particulates, and the solvent fraction removed to a watch glass. Complete volatilization occurred in approximately 1 h. Samples were resuspended in 500 μL of methanol and placed in the glass loading vials. The run parameters were the same as described in SM#7.

Table 4
Parameters Tested for DNA Extractions. Description of tests performed on DNA extracts in order. All injections were split with the exception of DNA#5.

Method	Solvent	# of samples	Treatment	GC/MS Parameters
DNA #1	Methanol	3	10 μL DNA added to 500 μL MeOH	200 °C oven. Ramp to 300 °C at 20 °C/min. Hold for 20min.
DNA #2	Methanol	5	10 μL DNA added to 500 μL MeOH	150 °C oven. Ramp to 300 °C at 20 °C/min. Hold for 20min.
DNA #3	Acetonitrile	4	10 μL DNA added to 500 μL Acetonitrile	150 °C oven. Ramp to 300 °C at 20 °C/min. Hold for 30min.
DNA #4	Dichloromethane	5	10 μL DNA added to 500 μL Dichloromethane	150 °C oven with a hold for 20min. Ramp to 250 °C at 20 °C/min. Hold for 30min.
DNA #5	Dichloromethane	5	Same fraction as from DNA #4	150 °C oven with a hold for 20min. Ramp to 250 °C at 20 °C/min. Hold for 30min. Splitless Injection.

2.4.9. Sample methods #9 and #9a (SM#9 and SM#9a)

The five samples used for SM#9 were the same as those used in SM#8. Rather than modify the solvent, the instrument parameters were adjusted to a splitless injection. Otherwise, the run module remained the same as in SM#7.

SM#9a contained only one sample: a concentrated version of sample 3–7. The watch glass containing the concentrated solvent fraction had remained at room temperature overnight. The concentrated residue was black and tarry. Using 1.85 mL of methanol, the sample was recovered from the watch glass for injection as SM#9a.

2.5. Testing parameters for extracted DNA

Three different solvents were used to suspend DNA extracted from skeletal materials received in the course of regular casework. Samples were extracted using either an organic inorganic purification method (Table 2) and suspended in TE^{-4} (10mM Tris, 0.1mM EDTA; pH 7.5). DNA extracts had been stored in 1.7 mL polypropylene tubes (Costar, Corning, NY, USA). The demineralization buffer used to decalcify the osseous materials contains 1% *N*-Lauroylsarcosine, a detergent, so there was some initial concern that this might cause bubbles during injection on the instrument. As with the osseous materials, an aliquot of DNA was combined with the indicated solvent in 9 mm glass vials with screw caps. Testing strategies are summarized in Table 4 and described in more detail below.

2.5.1. DNA method #1 (DNA #1)

Ten microliters of three DNA extracts (2–12, 3–13, 10–5) were

Table 5
Summary of Skeletal Materials Tested. Samples were randomly assigned a number based on the date of testing in order to prevent cognitive bias during analysis. Some samples were tested multiple times under different parameters, due to the large amount of detritus available for testing. Compounds detected are summarized. Most peaks were not over the analytical threshold set by the instrumentation; however, they were well defined and manually analyzed. Only the analysis of the primary peaks detected is listed. Refer to Table 3 for the testing parameters. Samples are listed in the order in which they were tested.

Sample	Conflict	Location Recovered	SM Test	# Peaks Detected	Compounds Detected
10–5	WWII	Hawaii	1	None	None
3–13	Korean War	South Korea	2	None	None
10–5	WWII	Hawaii	2	None	None
10–5	WWII	Hawaii	3	1	Cocaine
1–1	Southeast Asia	Cambodia	4	4	Phthalic acid; a broad-leaf herbicide; an analgesic
1–2	Southeast Asia	Cambodia	4	3	Phthalic acid; by-products of decomposition
2–2	WWII	Philippines	4	3	Cyclopentane; siloxane; variant of a compound used in a broad spectrum sunscreen.
2–12	WWII	Papua New Guinea	4	2	Fatty acids; quinine or a derivative
3–13	Korean War	South Korea	4	1	Glycerol
4–2	Southeast Asia	Laos	4	2	Glycerol; an alkaloid associated with plant materials
10–6	WWII	Hawaii	4	3	Fatty acids
3–1	Korean War	South Korea	5b	1	By-product of decomposition
3–8	Southeast Asia	Vietnam	5a	None	None
3–9	Southeast Asia	Vietnam	5b	None	None
4–3	Korean War	Namjong-gu	5a	None	None
5–2	WWII	Solomon Islands	5a	None	None
5–7	WWII	Tarawa	5b	None	None
6–1	WWII	Hawaii	5b	Numerous	Anthracene; aromatic hydrocarbons; cholestan
10–9	WWII	Hawaii	5a	Numerous	Plastic precursors; fatty acids; cyclohexane
3–1	Korean War	South Korea	6	Numerous	Broad-spectrum fungicide; benzoic acid; by-products of decomposition
5–7	WWII	Tarawa	6	Numerous	By-products of decomposition; benzoic acid
6–1	WWII	Hawaii	6	Numerous	Accelerant cluster; by-products of decomposition; benzoic acid
3–1	Korean War	South Korea	7	3	By-product of decomposition; benzoic acid; Allylamine
5–7	WWII	Tarawa	7	2	Benzoic acid
6–1	WWII	Hawaii	7	Numerous	By-product of decomposition; benzoic acid; traces of non-specific fuels
2–1	Southeast Asia	Laos	8	Numerous	Fatty acids; Tetraoxane; phthalic acid;
2–5	Southeast Asia	Laos	8	Numerous	Benzoic acid; phthalic acid; Benzamide; by-products of decomposition
3–7	WWII	Hawaii	8	Numerous	Accelerants; Boric acid; by-products of decomposition
3–12	WWII	Solomon Islands	8	2	By-products of decomposition; Mevalonic acid
3–14	Korean War	South Korea	8	Numerous	Sulfameter; by-products of decay; preservatives; herbicide
2–1	Southeast Asia	Laos	9	Numerous	Siloxane; phthalic acid; methyl palmate; possible fuel additive
2–5	Southeast Asia	Laos	9	7	Siloxane; phthalic acid; methyl palmate; possible fuel additive
3–7	WWII	Hawaii	9	Numerous	Accelerant cluster; dodecane; triphenylene
3–7	WWII	Hawaii	9a	Numerous	Accelerant cluster (less resolution than SM #9)
3–12	WWII	Solomon Islands	9	Numerous	By-products of decomposition; sugars; medication
3–14	Korean War	South Korea	9	Numerous	By-products of decomposition; plastics precursor

Table 6
Summary of DNA Extracts Tested. Sample numbering corresponds to the skeletal sample tested. Some samples were tested more than once. In most cases, the same fraction was used.

Sample	Conflict	Location Recovered	DNA Test Number	# Peaks Detected	Compounds Detected
2–12	WWII	Papua New Guinea	1	None	None
3–13	Korean War	South Korea	1	None	None
10–5	WWII	Hawaii	1	None	None
1–1	Southeast Asia	Cambodia	2	None	None
1–2	Southeast Asia	Cambodia	2	None	None
2–2	WWII	Philippines	2	None	None
2–4	Southeast Asia	Vietnam	2	None	None
2–6	WWII	Kiribati	2	None	None
3–1	Korean War	South Korea	3	None	None
3–8	Southeast Asia	Vietnam	3	None	None
3–9	Southeast Asia	Vietnam	3	None	None
4–3	Korean War	Namjong-gu	3	None	None
2–1	Southeast Asia	Laos	4	2	Dipeptides
2–5	Southeast Asia	Laos	4	2	Benzene or Oxazine
3–7	WWII	Hawaii	4	None	None
3–12	WWII	Solomon Islands	4	None	None
3–14	Korean War	South Korea	4	1	Benzene or Oxazine
3–7	WWII	Hawaii	5	Numerous	Sugars; accelerant complex; by-products of decomposition
2–1	Southeast Asia	Laos	5	Numerous	Siloxane
2–5	Southeast Asia	Laos	5	Numerous	Siloxane; by-products of decomposition
3–12	WWII	Solomon Islands	5	Numerous	Sugars; by-products of decomposition
3–14	Korean War	South Korea	5	Numerous	Sugars; by-products of decomposition

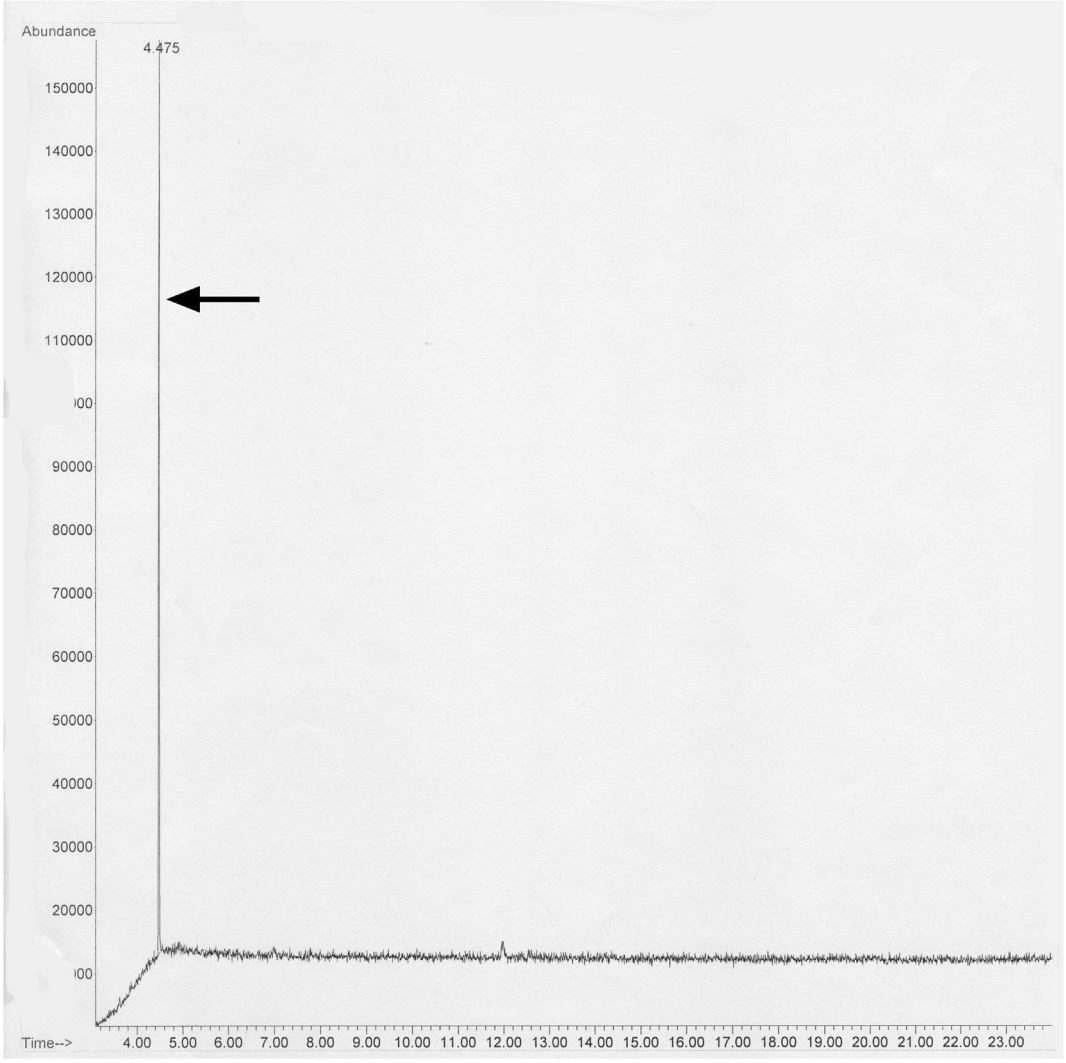


Fig. 2. The trace image generated by the GC/MS analysis of sample 10-5 subjected to a methanol extraction and SM#3. The peak indicated by the arrow was called by the instrument software as cocaine. The parent osseous element was recovered from the USS *Oklahoma*.

added to 500 μL of methanol in the glass loading vials. Samples were run at the same parameters as SM#3.

2.5.2. DNA method #2 (DNA #2)

Ten microliters of five DNA extracts (1-1, 1–2, 2-2, 2–4, 2–6) were added to 500 μL of methanol in the glass loading vials. Samples were run at the same parameters as SM#4.

2.5.3. DNA method #3 (DNA #3)

Ten microliters of four DNA extracts (3–1, 3–8, 3–9, 4–3) were added to 500 μL of acetonitrile in the glass loading vials. Samples were run at the same parameters as SM#5.

2.5.4. DNA method #4 (DNA #4)

Ten microliters of five DNA extracts (2–1, 2–5, 3–7, 3–12, 3–14) were added to 550 μL of dicholoromethane in the glass loading vials. Samples were injected onto the instrument with the same parameters as SM#7.

2.5.5. DNA method #5 (DNA #5)

The same fractions used in DNA #4 were used. The run parameters from SM#7 were used on the instrument, with the exception of the injection being splitless.

3. Results

The testing strategies had varying degrees of success. The results are summarized briefly in Tables 5 and 6 and described more fully below.

3.1. Sample method results

3.1.1. Sample methods #1 and #2

No detectable peaks were generated.

3.1.2. Sample method #3

A single peak was detected (Fig. 2). This peak was called by the Mass Hunter software as cocaine. A fraction of the sample was re-run to confirm, but no detectable peaks were recovered.

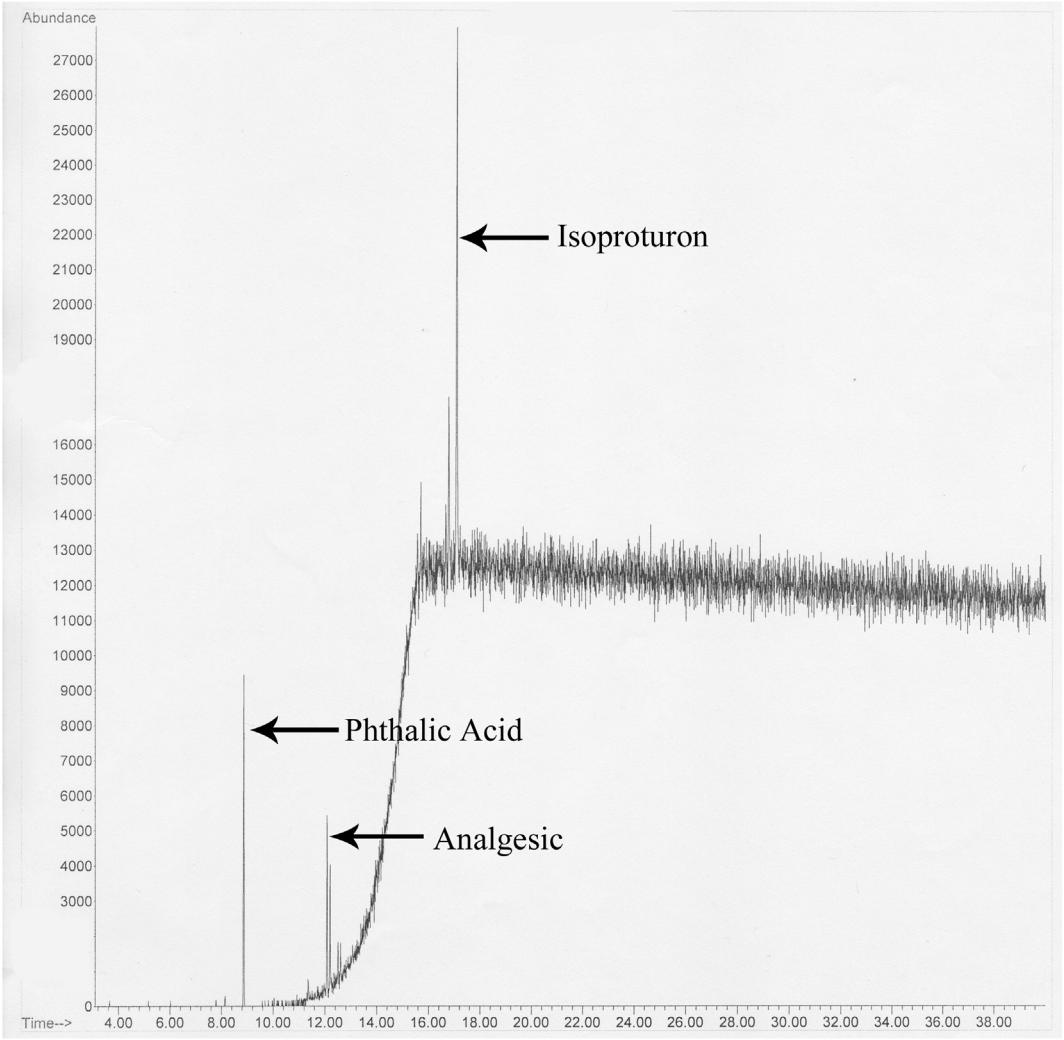


Fig. 3. The trace image generated by the GC/MS analysis of sample 1-1 subjected to a methanol extraction and SM#4. Sample 1-1 was recovered from Cambodia and was deposited during the US conflict in Southeast Asia. While the trace shows some compression, there are analyzable peaks. The three most distinctive peaks are indicated by arrows and labeled according to the most likely material as indicated by Mass Hunter. The analgesic was determined to most likely be phenacetin, which was banned in the United States in 1983.

3.1.3. Sample method #4

All samples generated at least one readable peak. Samples 1–2, 2–12, 3–13, 4–2, and 10-6 showed evidence of fatty acids and metabolic materials (e.g., glycerol). Sample 4-2 had a peak consistent with a plant alkaloid, possibly nantenine. Sample 1-1 contained a peak consistent with Isoproturon, a broad-leaf herbicide (Fig. 3). Samples 1-1 and 1–2, both recovered from sites in Southeast Asia, contained phthalic acid, which can be derived from naphthalene.

3.1.4. Sample methods #5a and #5b

Five of the eight samples tested using these parameters failed to generate any readable peaks. Sample 3–1 (Korea), incubated in acetonitrile, showed evidence of by-products of decomposition. Two other samples, 6–1 and 10–9, both recovered from the USS *Oklahoma*, showed evidence of possible accelerants and fats. Sample 10–9 (Fig. 4a), incubated in methanol, mainly showed peaks of fatty acids and sugars with a peak that is characteristic of flammable materials, but lacking an accelerant arc. This arc is present in sample 6–1 (Fig. 4b), which was incubated in acetonitrile.

3.1.5. Sample method #6

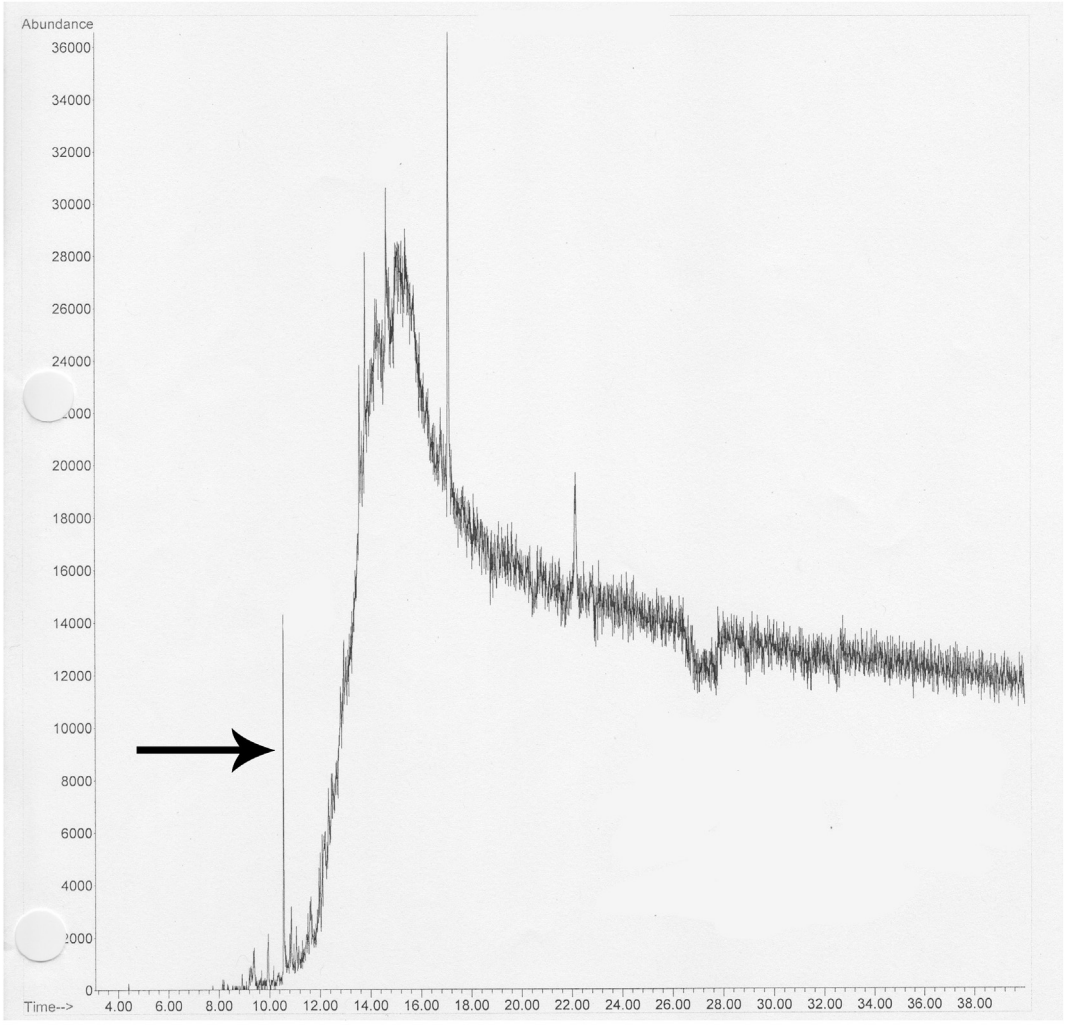
All three samples produced detectable results. Sample 3–1 (Korea) showed data similar to that recovered previously with products of decomposition. However, there was also possible evidence of a broad-spectrum fungicide. Sample 5–7 (Tarawa), which had previously generated no results under SM#5b, now showed a series of peaks mostly related to materials of human decomposition (Fig. 5). Sample 6–1 (USS *Oklahoma*) showed an accelerant arc that is difficult to characterize, due to the interaction between the fats and the fuel present.

3.1.6. Sample method #7

The number of peaks generated reduced in samples 3–1 and 5–7. Both retained some of the original compounds, but at different retention times due to the change in protocols. Sample 6-1 maintained a profile showing an accelerant trace and by-products of decomposition.

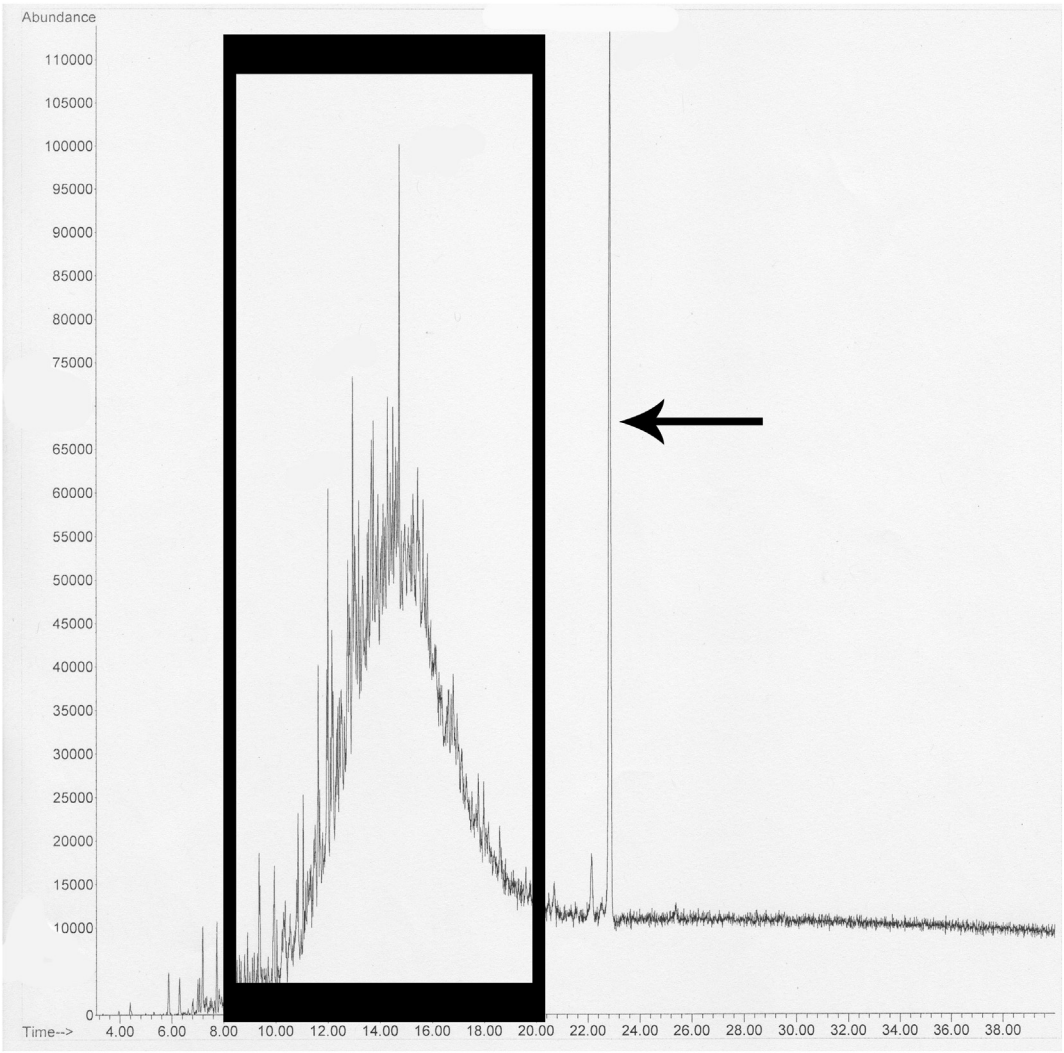
3.1.7. Sample method #8

Sample 3–12 (WWII) produced the least number of analyzable



(a)

Fig. 4a. The trace image generated by the GC/MS analysis of sample 10-9 subjected to a methanol extraction and SM#5. While the osseous sample was recovered from the USS *Oklahoma*, the visible peaks are mainly those of fatty acids. The peak indicated by the arrow is from a flammable liquid.



(b)

Fig. 4b. The trace image generated by the GC/MS analysis of sample 6-1 subjected to an acetonitrile extraction and SM#5. The area surrounded by the rectangle is a series of peaks characteristic of an accelerant cluster. Even though the fuel is known to have come from the USS *Oklahoma*, the fuel cannot be accurately characterized using GC/MS as the presence of lipids is obscuring the profile generated by the fuel oils. The peak indicated by the arrow is a form of cholestan, a cholesterol derivative.

peaks, both of which appear to be by-products of metabolic pathways. Sample 3–7 (USS *Oklahoma*) produced an accelerant arc, as well as some evidence of by-products of decomposition. Sample 3–14 (Korea) produced peaks consistent with by-products of decomposition, but also evidence of sulfameter, which is a long acting sulfonamide (Fig. 6).

3.1.8. Sample methods #9 and #9a

All samples generated analyzable peaks. Samples 3–12 and 3–13 both generated peaks consistent with sugars and by-products of decomposition. Samples 2–1 and 2–5, both recovered from Laos, showed a similar series of peaks (Fig. 7a and b), containing fats and a possible fuel additive. Sample 3–7 produced the now expected accelerant cluster. The concentrated version of Sample 3–7 generated a similar cluster, but the peaks lacked resolution and the overall trace image lacked resolution.

3.2. DNA method results

3.2.1. DNA methods #1, #2, and #3

No detectable peaks were generated.

3.2.2. DNA method #4

Samples 3–7 and 3–12 generated no detectable peaks. All three showed a peak that was characterized by the Mass Hunter as analyzable, but unresolvable as a specific compound. The most likely result was determined to be a benzene or oxazine (which can be derived from benzene).

3.2.3. DNA method #5

All five samples generated analyzable peaks. Each sample appeared to contain some degree of by-products of decomposition, including sugars. There is some detection of siloxane, which may be from the column itself. Sample 3–7 generated a profile most similar to that of the associated skeletal material (Fig. 8a and b), and

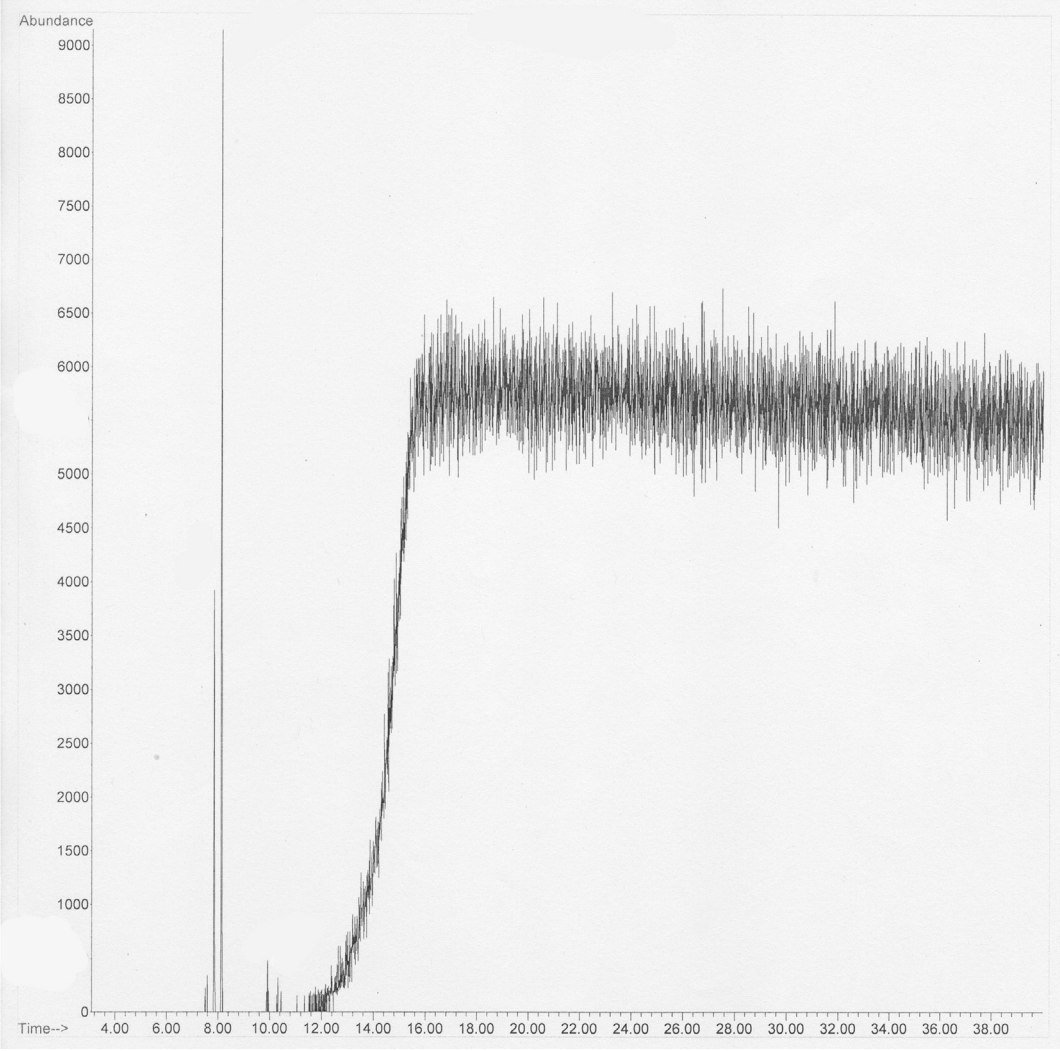


Fig. 5. The trace image generated by the GC/MS analysis of sample 5–7 subjected to an acetonitrile extraction and SM#6. The parent osseous sample was recovered from the Tarawa Atoll. The signal noise past 12 min is indicative of the solvent front and no detectable materials. All other peaks present are indicative of biological materials that are by-products of human decomposition.

showed a limited accelerant trace. The 3–7 DNA was immediately after the blank, so carry-over from a previous run was not possible.

4. Discussion

4.1. Detection of materials in the osseous samples

A large variety of materials were detected in the osseous samples tested. The use of a less stringent solvent (methanol), failed to generate a great deal of data, and it was initially expected that the amount of data that could be recovered from skeletal materials would be very low. Prior to the start of this testing series, it was thought that samples would need to be extensively concentrated, using strategies similar to those used in arson investigations. This has been shown not be true. Using more stringent solvents, such as acetonitrile and dichloromethane, data can be generated from samples at least as old as 77 years post mortem.

The results appear to be slightly inconsistent in terms of specific items, such as the fungicide present in sample 3–1 for SM#6 and not in SM#7. However, as the parameters for the injections were constantly being changed, this was an expected result. As further work is done and more replicates of data are gathered using the same set of solvents and parameters, the results should be repeatable.

4.2. Detection of modern materials

A possible confounding factor of this testing strategy is the detection of modern materials. Sample 2-2, tested using SM#4, showed a peak representative of a component found in a broad-spectrum sunscreen. This sunscreen is thought not to be authentic to the skeletal materials themselves, as it is unlikely both that the compound would persist for the over 70 years post-mortem and that the particular sunscreen was manufactured during World War II. It is known that field teams often handle the skeletal materials without gloves; therefore, modern compounds may be transferred to the skeletal elements. Testing of additional samples from the same recovery site would be necessary to determine if the compound was conveyed throughout the remainder of the samples.

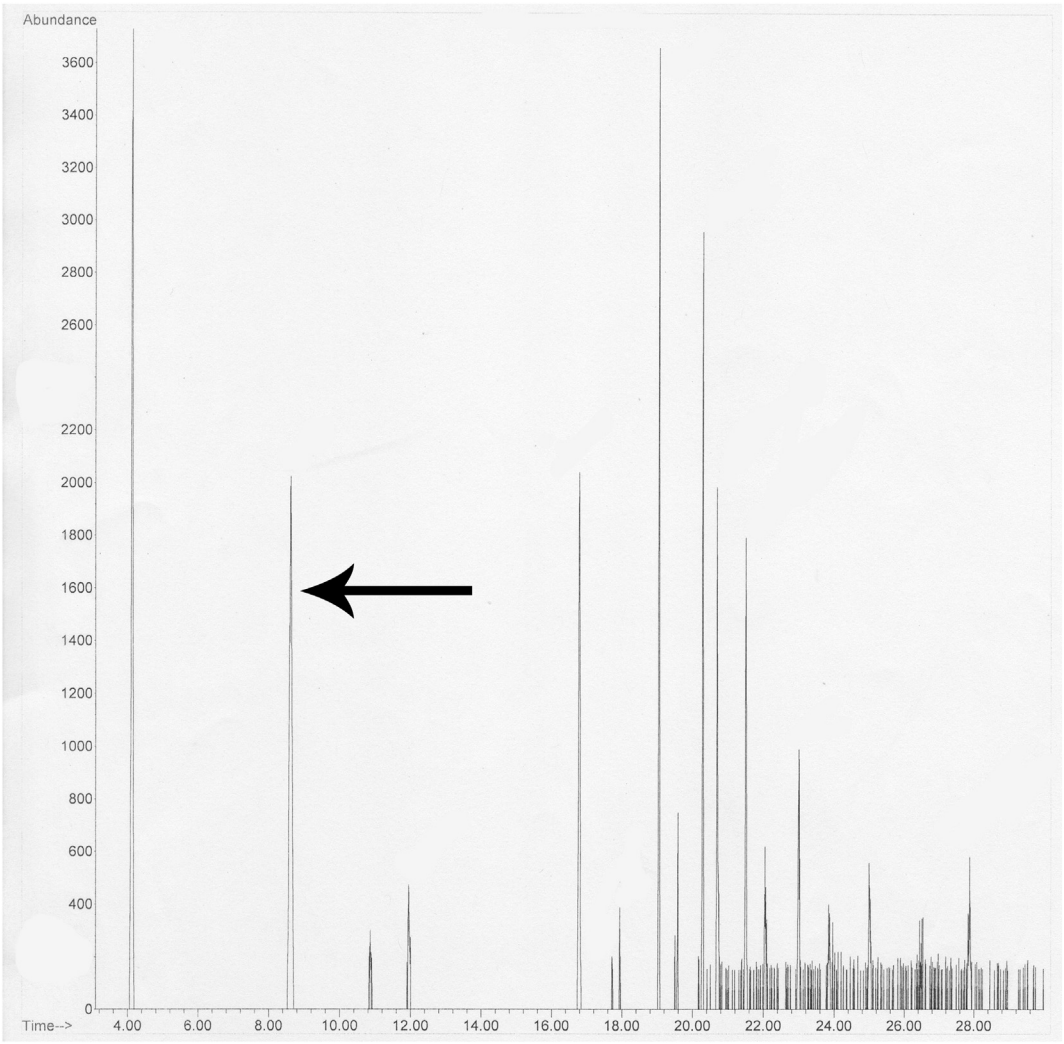


Fig. 6. The trace image generated by the GC/MS analysis of sample 3–14 subjected to a dichloromethane extraction and SM#8. The peak indicated by the arrow is sulfameter, which is a long acting sulfonamide used to treat infections. All other peaks are by-products of human decomposition or siloxanes.

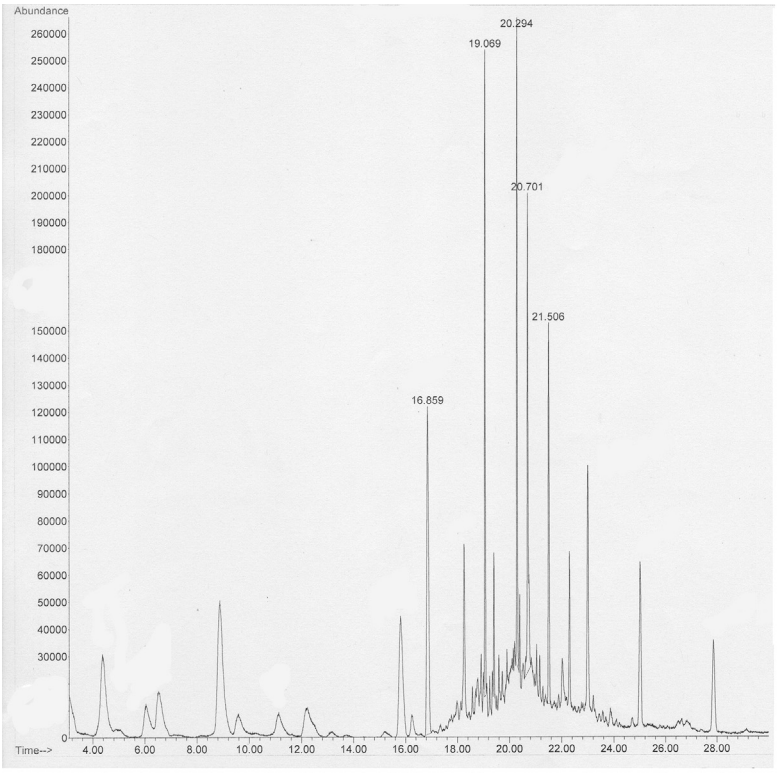
In addition, there was some detection of plastics and plastic precursors in some of the samples. These could be conveyed to the samples themselves from the polypropylene tubes the samples were stored in prior to testing. While this is a possibility, a result of "plastics" was not consistent between samples.

4.3. Detection of materials in the DNA

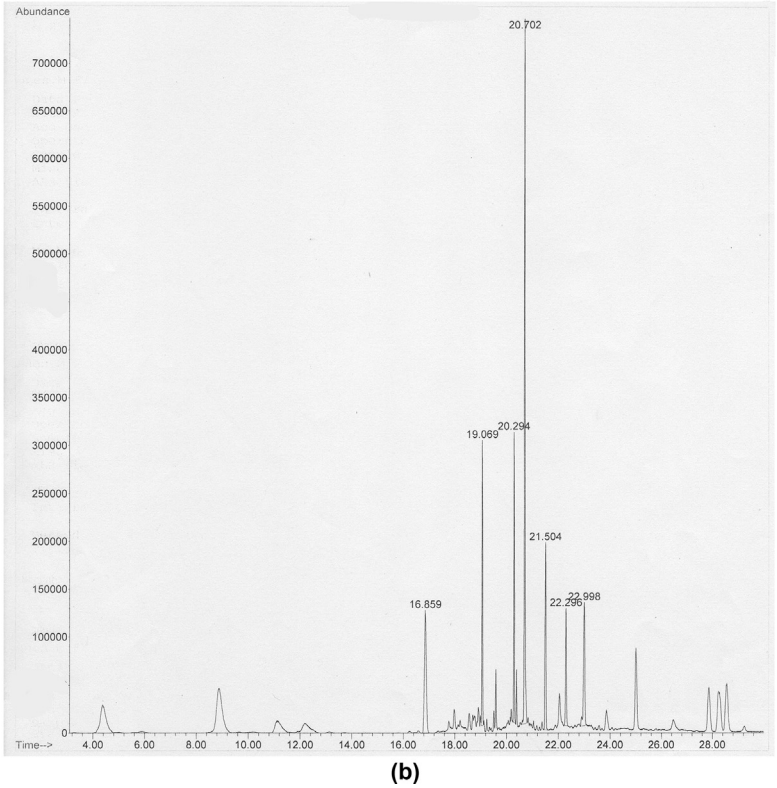
Unlike [29]; very little carry over from the extraction procedure to the DNA was found. No phenol or guanidinium was detected in the recovered DNA. Rather, expected materials were recovered, such as sugars or other products of human decomposition. In only one instance was there marked carry-over from the bone sample to the DNA extract (sample 3–7, DNA Method #5). This DNA extract was from a USS *Oklahoma* sample that generated a similar series of peaks related to accelerants. One other sample from the same incident was also tested in this series of experiments, yet failed to generate any evidence of carry-over (10–5, DNA Method #1). It is tempting to make a conclusion that this DNA extract was free from many impurities; however, DNA sample 3–7 also failed to show evidence of carry-over when tested with different parameters (DNA Method #4). It is most likely that the sensitivity of the testing was not sufficient until the final test series, at which point, the DNA extracts had been exhausted.

4.4. Sample 10–5 and the unexpected result

The detection of cocaine for sample 10–5 in SM#3 was wholly unexpected. A fraction of the DNA sample plus solvent was run through a spectrophotometer. The spectrum indicated the presence of DNA, in addition to environmental materials. It is not uncommon that the parent peak of a chemical would be detected without any of the associated metabolite peaks; however, given the previous results of (i.e., no detectable peaks), it seemed unlikely that only a drug peak would be present. This particular sample was taken from osseous materials recovered from the USS *Oklahoma,* which had been soaked in fuel oil within the ship for approximately two years prior to being recovered and buried in a cemetery on the island of Oahu. It would be more likely that components of fuel and fats would be detected, and indeed this is what was seen in other USS



(a)



(b)

Fig. 7. a and b. The trace images generated by GC/MS analysis of two samples subjected to a dichloromethane extraction and SM#9. Sample 2–1 (Fig. 7a) and Sample 2–5 (Fig. 7b) were ostensibly recovered from the same location in Laos, and potentially the same individual.

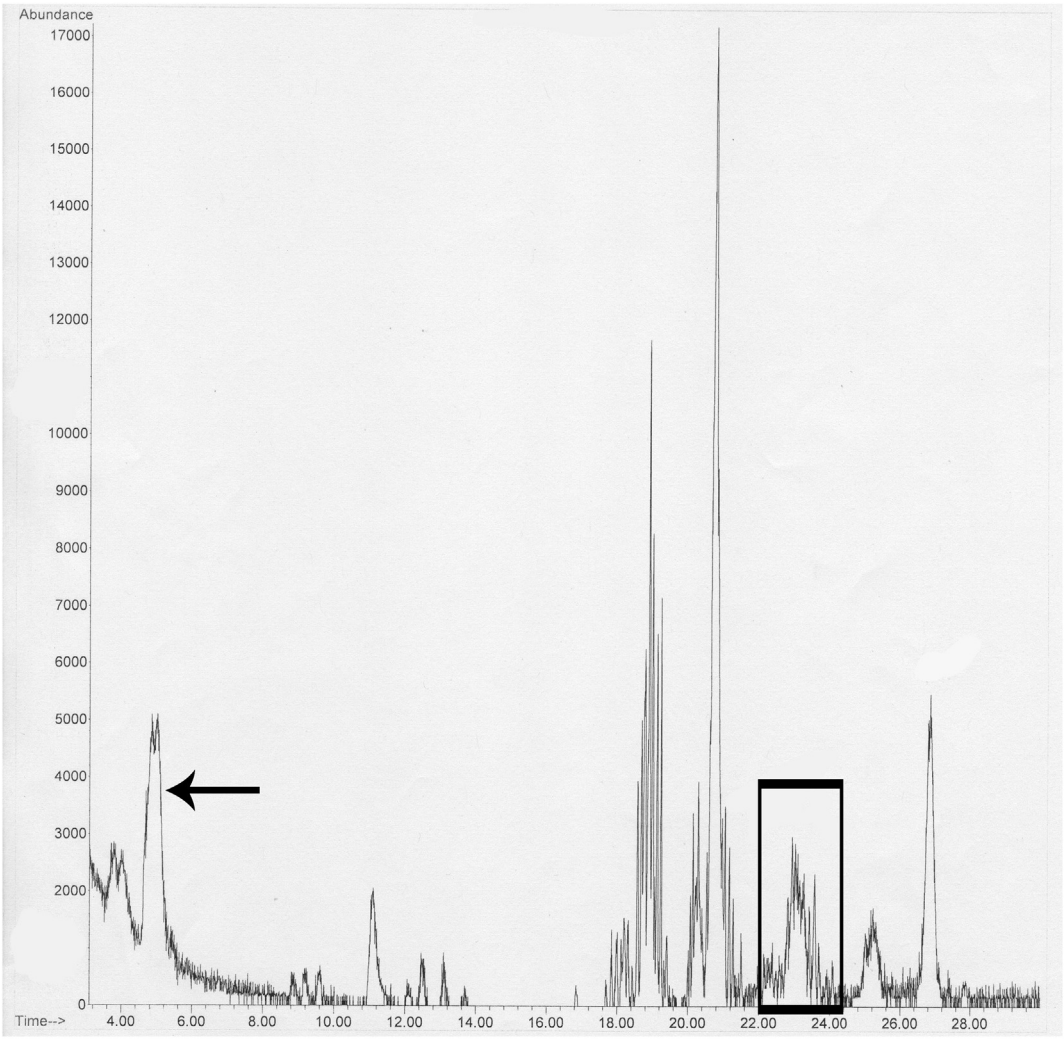


Fig. 8a. The trace images generated by GC/MS analysis of DNA from osseous sample 3–7. The extracted DNA was diluted with methanol and injected onto the instrument using DNA#5 parameters. There is a cluster of sugars between 22 and 24 min, indicated by the square. The peak indicated by the arrow is phenol, most likely a carry-over from the extraction.

Oklahoma samples that were tested (6–1, 10–9, and 3–7). The source of the cocaine was not determined during trouble-shooting. The laboratory in which the experiments were run does not have a license for the handling or testing of Schedule II controlled substances.

5. Conclusions

GC/MS has been shown to be potentially a very useful tool of the identification of biological and environmental compounds present in osseous remains. This is particularly useful when remains have been stored for extended periods of time and the provenience not known. Typically extraction protocols in a forensics laboratory are designated as a single pathway; however, detection of certain materials might allow for the analyst to consider alternative methods prior to extraction. The presence of fats or waxes in skeletal samples could point the DNA analyst to using an extraction protocol that would be more efficient in the removal of such materials. Detection of fuels or accelerants might indicate a different extraction pathway would be necessary.

It is clear that there are a plethora of biological and chemical materials that would need to be removed from the skeletal material during an efficient extraction of DNA. Additional studies are being undertaken to determine if the DNA extraction procedure is efficient at producing a purified extract, free from potential PCR inhibitors. The protocol developed here (SM#9), coupled with acetonitrile and dichloromethane extractions, has been successfully used on the remaining 439 osseous fractions and the associated DNA extracts. The data is currently being analyzed, and the hope is to be able to provide the forensic community with not only information regarding the carry-over of potential inhibitors and other materials, but a pathway by which GC/MS could be used to determine the optimal DNA extraction protocol for use on sets of osseous remains.

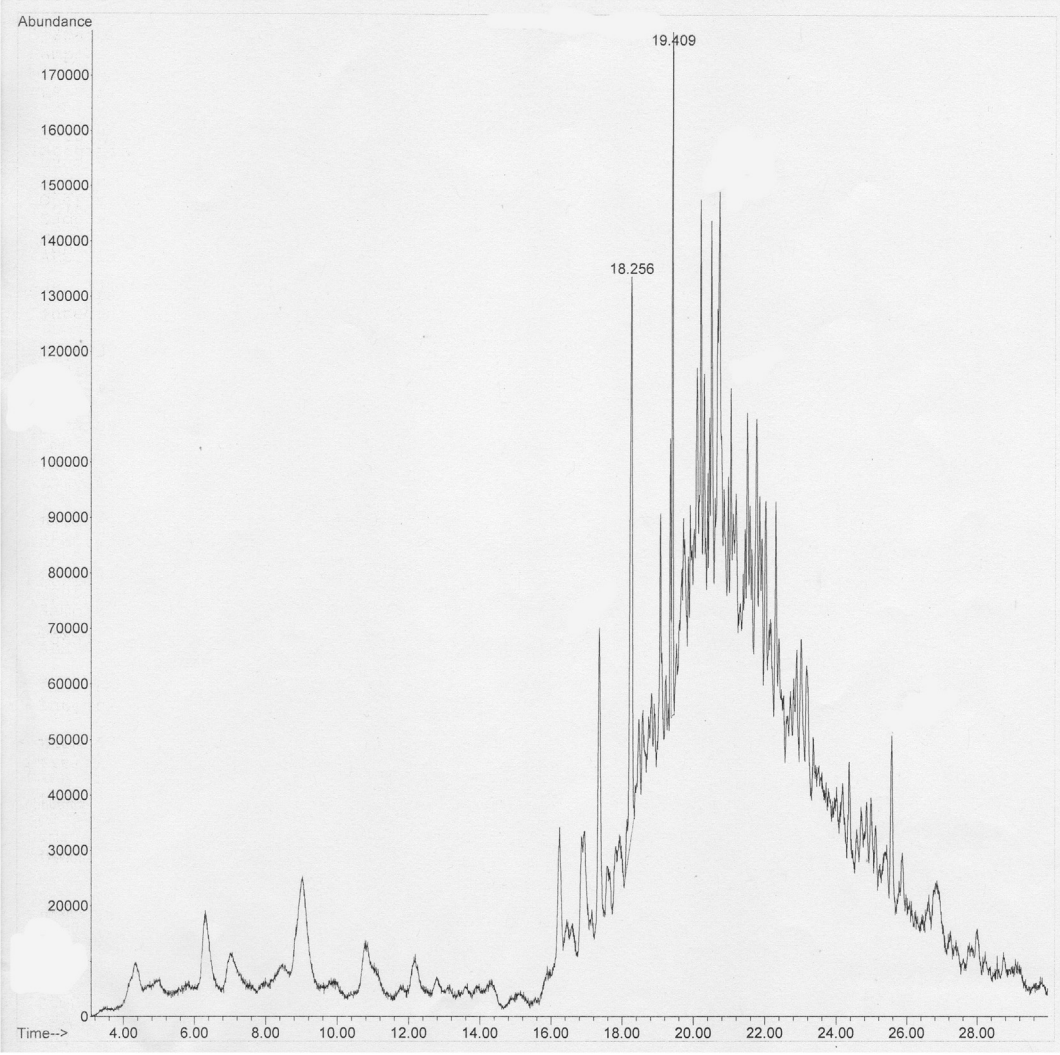


Fig. 8b. The trace image generated by GC/MS analysis of sample 3–7 subjected to a dichloromethane extraction and SM#9. The series of dominant series of peaks is fuel oil and fats. There is some carry-over of these materials to the DNA.

Disclaimer

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Declaration of competing interest

Authors declare there is no conflict of interest.

CRediT authorship contribution statement

Suni M. Edson: Conceptualization, Formal analysis, Project administration, Investigation, Writing - original draft. **Marcel Roberts:** Methodology, Writing - review & editing.

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