The Trouble with Tar: Collagen Preservation in Asphalt Impregnated Bones

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Abstract

The Rancho LaBrea (RLB) tar sand in Los Angeles California is known for its highly preserved megafauna.Researchers have been attempting collagen extraction on RLB faunal assemblages for over 50 years for the purpose of isotope and radiocarbon analyses. There has yet to be extensive research on the applications of Zoo-archaeology by Mass Spectrometry (ZooMS) to these materials. This research fills this gap, as a pilot attempt of ZooMS analysis on LaBrea Tar Sand faunal material. The project aims to answer if ZooMS taxonomic identification is possible on RLB materials. Analysis will be conducted on bone samples which have had tar removed, as well as on untreated bones, aiming to assess the relationship between visible asphalt saturation and total MALDI-TOF spectra peaks. As DNA analysis is costly and has not yet seen success in RLB materials, this research could provide great additional insight to fauna populations of RLB, as well as tar saturated faunal assemblages around the world.

Introduction

The Rancho LaBrea (RLB) tar sand in Los Angeles, California is a site known for its highly preserved megafauna. Archaeologists, paleontologists, and other researchers have been attempting collagen extraction on RLB faunal assemblages for over 50 years for the purpose of isotope and radiocarbon analyses (Wyckoff et al., 1963, Ho et al., 1969). However, no studies have conducted collagen extraction for the purpose of Zooarchaeology by Mass Spectrometry (ZooMS). ZooMS has proven potential to be highly useful in species identification of fragmentary remains, which may be difficult or impossible to identify based on morphological characteristics (Buckley et al., 2009, 2018). This paper seeks to fill this niche, as the first attempt of ZooMS analysis on LaBrea Tar Sand faunal material. This research aims to answer if a ZooMS taxonomic identification on RLB materials is possible.

Analysis will be conducted on bone samples which have had tar removed, as well as on untreated bones, aiming to assess the relationship between visible asphalt saturation and total Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF) spectra peaks. As DNA analysis is costly and has not yet been successful on any RLB materials, this research could provide additional insight to fauna populations of RLB, as well as tar saturated faunal assemblages around the world (Cuba, Ecuador, Trinidad and Tobago etc.).

Background

Rancho LaBrea is a late Pleistocene site, with significant faunal and floral preservation. Millions of remains have been extracted in the last century with over 100 excavations having

occurred (La Brea Tar Pits and Hancock Park, LaBrea Tar Pit, 2022). Biomolecular researchers working on collagen extraction on tar saturated bones such as those at RLB face a complex challenge. Conditions must be carefully controlled in order to avoid destroying surviving collagen during the tar removal processes. If tar removal is not successful, ZooMS spectrum results may be impossible to analyze. Since there is such significant preservation seen at RLB, a good estimate of possible fauna can be drawn from its associated materials.

Animals found to have been preserved within these tar sands include a wide variety of mammals, invertebrates, fish, amphibians, reptiles, and birds (Mammal Collection, LaBrea Tar Pit, 2022). The most plentiful species found in RLB thus far have been Dire wolves, saber tooth cats, and coyotes (La Brea Tar Pits and Hancock Park, LaBrea Tar Pit, 2022). Working on old, tar saturated bones can lead to difficulties reviewing data, as collagen degrades over time and results will be blurred by tar. However, given the size of the bones selected for this analysis (Figure 1), it is possible to reduce anticipated species to those from orders Primate (humans), Xenarthra (ground sloths), Carnivora (dogs, wolves, etc.), Proboscidea (elephantids), Perissodactyla (horses and tapirs), and Artiodactyla (even toed ungulates). This means that despite the potential within this site for significant degradation of collagen that might shift ZooMS spectra results, realistic interpretations of data could still be conducted.

Materials and Methods

Sampling

A total of 11 mammal bones (Fig. 1) were selected from the UBC Lab of Archaeology faunal collection for sampling. Samples are labelled following the UBC ADαPT Laboratory COCR form. Samples are fragmentary and predominantly unidentifiable, though C180 and C181 are each suspected to be a “part of a carnivore rib”, and C178 is a suspected “fragmentary sloth vertebra” (Dr. E. Lindsey, personal communication, September 30, 2022).

Bones were selected based on size, as well as visible tar saturations. A range of 3 visible saturations were targeted, categorized as light tar, moderate tar, and significant tar saturation (Fig. 1, Fig. 2). Bones with light tar saturation have very little visible tar, if any, and are a dark brown colour. Moderate tar describes bones with a visible tar coating (black in colour) without excess sections of tar attached to fragments. Significant tar is described as black bones with excess saturations of tar, including sections of tar attached to fragments. This range of samples was selected to assess the possible relationship between a bone's visible asphalt saturation and the success of collagen extraction. Using bone clippers, 104-308 mg of bone was removed from each sample for detarring treatment (Fig. 3). Between samples, bone clippers were cleaned in order to avoid cross sample contamination. Pre- detarring treatment weight can be seen in figure 2. C172 and C174 were sampled twice, once for ZooMS following de-tar treatment, and the second time to attempt ZooMS without de-tar treatment. As such, these samples have separate numbers, with the secondary sample of C172 labelled C183, and the secondary sample of C174 labelled C185.

Figure 1. Bones pre-sampling

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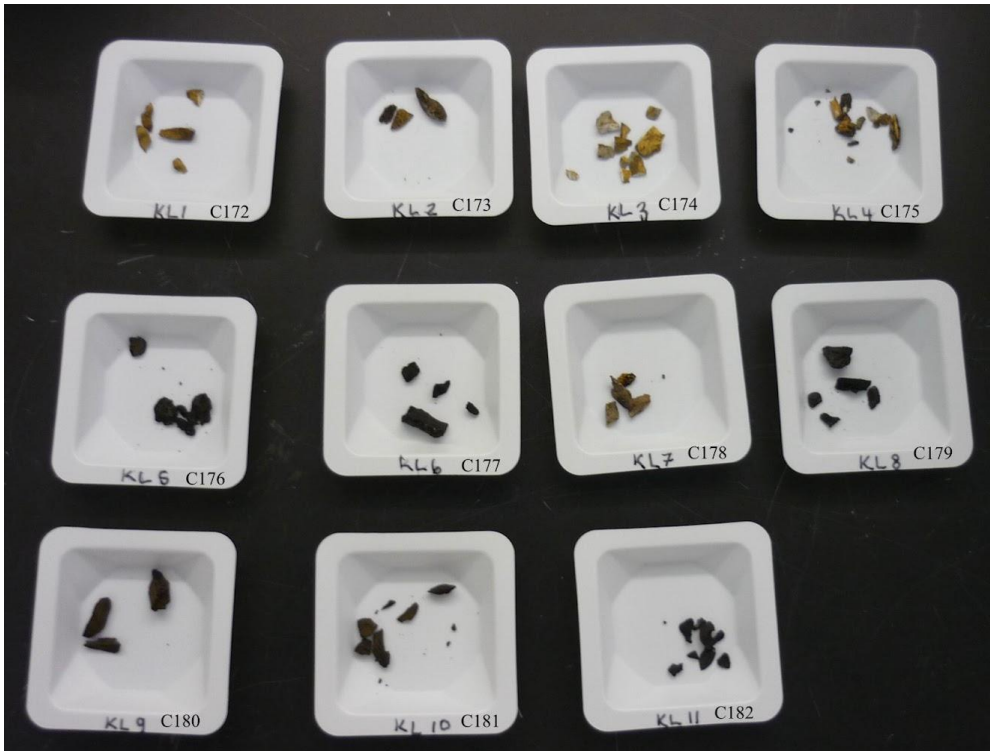
Figure 2. *Tar Saturation, Sampling and Subsampling Weight Table*

ADαPT #	Tar Saturation	Pre-treatment Sample Weight (mg)	Pre-ZooMS Subsample weight (mg)	Possible Morphologic ID
C172	Light	**103**	NA	NA
C173	Light	**127**	**37**	NA
C174	Light	**218**	**27**	NA
C175	Moderate	**111**	**14**	NA
C176	Moderate	**130**	**14**	NA
C177	Moderate	**308**	NA	NA
C178	Moderate	**134**	**35**	Sloth Vertebra
C179	Significant	**166**	**23**	NA
C180	Significant	**280**	N/A	Carnivore Rib

C181	Significant	**131**	N/A	Carnivore Rib
C182	Moderate	**108**	**30**	NA
C183	Light	N/A	**23**	NA
C185	Light	N/A	**30**	NA

Figure 3. *Samples in weigh boats prior to de-tarring*



Pre-treatment De-tarring

Due to the high asphalt saturations seen in materials from RLB, detarring treatment is required prior to collagen extraction. As tar is a hydrocarbon, biodiesel and alcohols are adequate solvents for its removal. For this project, 2:1 toluene/methanol solution was utilized as methods adapted from Fuller et al. (2014). Since these methods were formulated for the purpose of isotopic analyses, and not for ZooMS, ZooMS collagen extraction methods from Richter et al., (2020) and Buckley et al., (2009) will replace those within Fuller et al., (2014). De-tarring steps used are as follows:

1. Crush bones to 1 mm fragments
2. Sonicate bone fragments repeatedly in 2:1 toluene: methanol solution until supernatant runs clear; or until 5 toluene: methanol rinses have been run.
3. Sonicate in methanol (1h)
4. Sonicate in Milli-Q water (1h)

Bone samples were crushed to 1 mm fragments using a mortar and pestle, and weighed in labelled weigh boats. Crushed bones were then poured into 12x75mm borosilicate glass vials. Approximately 2.5mL of 2:1 toluene: methanol solution (hereafter referred to as T:M) was added to each glass vial using clean glass Pasteur pipettes. Both glass vials and Pasteur pipettes were individually labelled to associate with dedicated sample numbers. Sonication was conducted a total of 7 rounds.

Sonication 1: Supernatant became quite dark following the initial addition of T:M all samples, as seen in figure 4. c). All samples (C172-C182) were then sonicated 60 minutes in T:M. After the first round of sonication, samples displayed varying degrees of tar concentration within T:M solution.
Sonication 2: All samples (C172-C182) supernatant were removed using pipettes dedicated to waste removal. Before the second round of sonication, some variation in colouration was seen, the range of which can be seen in figure 5. C172-C175 supernatant ran clear, as seen in figure 4. a).
Sonication 3: Supernatant was removed from C172-C175, and ~2.5mL of methanol was added under the principle that 'like dissolves like' to dissolve remaining toluene. Samples C176-C182 repeated step 1, while C172-C175 were sonicated in methanol for 1 hour.
Sonication 4: Methanol was removed from C172-C175 and MilliQ water added to cover the sample, then sonicated for 1 hour. C176-C182 had varying stages of opacity, none of which were clear, and were run through another round of step 1. Following sonication 4, all samples' supernatants were removed, replaced with MilliQ water and left in a fume hood overnight due to time constraints.
Sonication 5: MilliQ water in samples C172-C175 was replaced with new MilliQ water, and samples C176-C182 water was replaced by T:M. C172 dissolved almost completely overnight. Sample C181 dissolved completely overnight. C172-C175 developed a cloudy substance above bone pellets as seen in figure 4. b). This cloud is suspected to be fat, though there is low certainty regarding this, and no data of this occurring in Fuller et al. (2014). A fifth round of T:M sonication was run on C176-C182, while C173-C175 remained in MilliQ water. The majority of samples softened marginally in colour but remained dark, as seen in figure 4. d). Some samples' supernatant ran clear but with large, suspected fat distributions resting above bone pellets as seen in figure 4. b) and figure 4. e). These suspected fat clouds resting above the pellets were easily removed by pipettes, and difficult to avoid removing.
Sonication 6: Samples C176-C180 and C182 had varying levels of supernatant colouring, due to residual asphalt contamination not having been fully removed by the solvent, none of which ran clear. Despite this, all remaining samples (C176-C180 and C182) proceeded to step 2 in methanol in order to limit further sample loss.
Sonication 7: Methanol was removed from C176-C180 and C182, and MilliQ water was added to cover bone pellets. Samples C173-C180 and C182 were placed into labelled 1.5mL Eppendorf tubes using glass Pasteur pipettes with water in order to facilitate transfer.

Figure 4. *Samples at varying stages of detarring treatment*

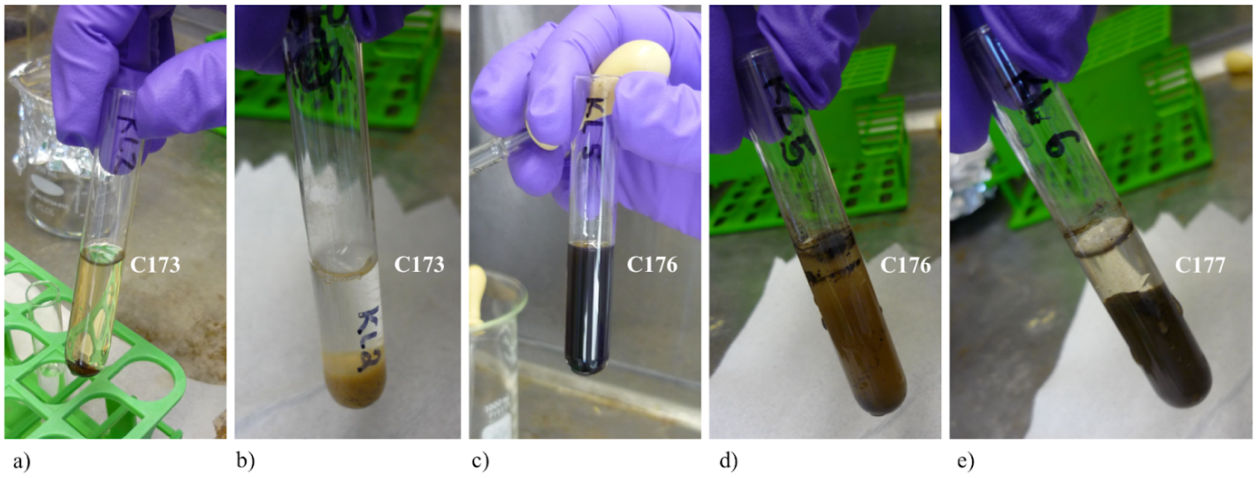


Figure 5. *Test tubes between sonication baths 2 and 3*

ZooMS

ZooMS procedures according to Buckley et al. (2009) were followed. De-tarred samples were placed into a centrifuge for 1 minute before excess water was removed from Eppendorf tubes using a micropipette. In samples C172, C177 and C180, the suspected fat did not condense to the bottom of the Eppendorf tube, and as a result the entirety of the tube's contents was removed by micropipette, with no bone fragments remaining at the bottom of the tube. C173-176, C178 and C182 were centrifuged, and the supernatant was removed. Ionized water was added to the samples. Samples were centrifuged again and the supernatant removed. Using a clean, metal spatula, 14-37 mg of bone was subsampled, with the pellet placed into a new labelled 1.5mL Eppendorf tube, as well as the addition of an extraction blank. Individual

sample weight can be seen in figure 2. These samples included both gritty pellet fractions as well as fine powdered bone.

For bones C183 and C185, which were not de-tarred, 500μL of hydrogen peroxide was added to degrease samples. Samples were left for 1 hour at room temperature. Hydrogen peroxide was removed and an additional 500 μL of hydrogen peroxide was added to each and left at room temperature for ~30 mins. The supernatant was removed, and samples were rinsed 5x with ionized water. Samples C183 and C185 had 250 μL of HCl added to them and were left in the fridge overnight. Subsequently, C183, C185, and extraction blank eBK1 were checked with a metal needle, removed from acid, and placed into water. Then, 250 μL of NaOH was added to C183 and C185, which were then vortexed and centrifuged. The supernatant was discarded.

Remaining samples (C173-C176, C178, C179, C182, C183 and C185) were rinsed three times with 200 μL AmBic. Samples were then placed into a heating block at 65℃ for 1 hour. Following this, 50 μL was removed from second extraction and placed into EXT-tubes, to each of which 1 μL of trypsin was added and left in the heating block overnight at 37℃. After, samples were placed into the centrifuge and 1 μL 5% TFA was added to deactivate trypsin. Sample collagen was purified using C18S ZipTips. Samples were then spotted onto a MALDI plate using 1 μL of sample and 1 μL of α-Cyano-4-hydroxycinnamic acid. The plate was run using MALDI-TOF-MS at the University of York, UK. Results were averaged, peak picked using mMass software (Niedermeyer and Strohalm, 2012), and analyzed using the ADαPT facility ZooMS reference database.

Results

ZooMS

Level of success between samples varied (Figure 6, Figure 7). A total of four samples (C172, C177, C180, C181) were lost during pre-ZooMS de-tar treatment due to bone dissolution in supernatant. Three samples (C175, C183, C185) that underwent ZooMS yielded 0 peaks. Four samples which underwent ZooMS yielded unidentifiable peaks, with C174, C176, and C179 peak values being lower than any in the database, while C173 peak values were higher but did not align with any markers. The extraction blank had no peaks corresponding with any markers, confirming contamination was not a factor within these results. Samples C182 and C178 had potentially positive identifications.

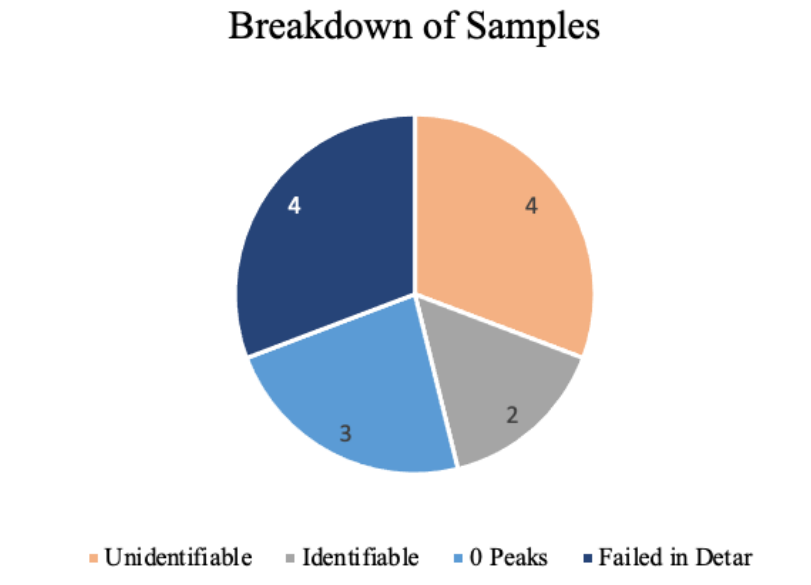
Sample C182 (Figure 8) had a total of 6 peaks, 1 of which (2807.45) could align with marker E_ α2 454 (COL1α2 454 - 483) at value 2808.4 (Buckley, Larkin and Collins 2011; Buckley and Collins 2011; Welker et al 2016; Buckley, Harvey and Chamberlain 2017). Due to sample degradation, a degree of latitude regarding association of results to markers must be taken. This would suggest, based on the context of this site, the possibility of sample C182 being of the family Elephantidae or Mammutidae. Although, because this is the only peak marker remotely comparable to other markers, it is hard to say with any certainty.

Sample C178 had 26 peaks, significantly more than the other samples. Two of these peaks could indicate this bone as a carnivore. The peaks seen in Sample C178 (Figure 9) are 1107, which could align with P1 _ α1 508 (COL1α1 508 - 519) at marker 1105; as well as 1453, which matches to B _ α2 484 (COL1α2 484 - 498) marker 1453.7 (Buckley, Larkin and Collins 2011; Buckley and Collins 2011; Welker et al 2016; Buckley, Harvey and Chamberlain 2017; Kirby et al 2013). These two markers, based on site context, suggest this bone to be a possible Carnivora or Elephantidae, but again, not enough markers were present for a preferred degree of certainty.

Figure 6. *Success of Samples Table*

ADαPT #	# of Spectra Peaks	Identifiable?	Possible ZooMS ID
C173	2	No	Unknown
C174	6	No, peaks <910	NA
C175	0	NA	NA
C176	3	No, peaks <820	NA
C178	26	Yes?	Carnivora (Sloth?)
C179	7	No, peaks <1100	NA
C182	6	Yes?	Elephantid
C183	0	NA	NA
C185	0	NA	NA

Figure 7. *Breakdown of Results*



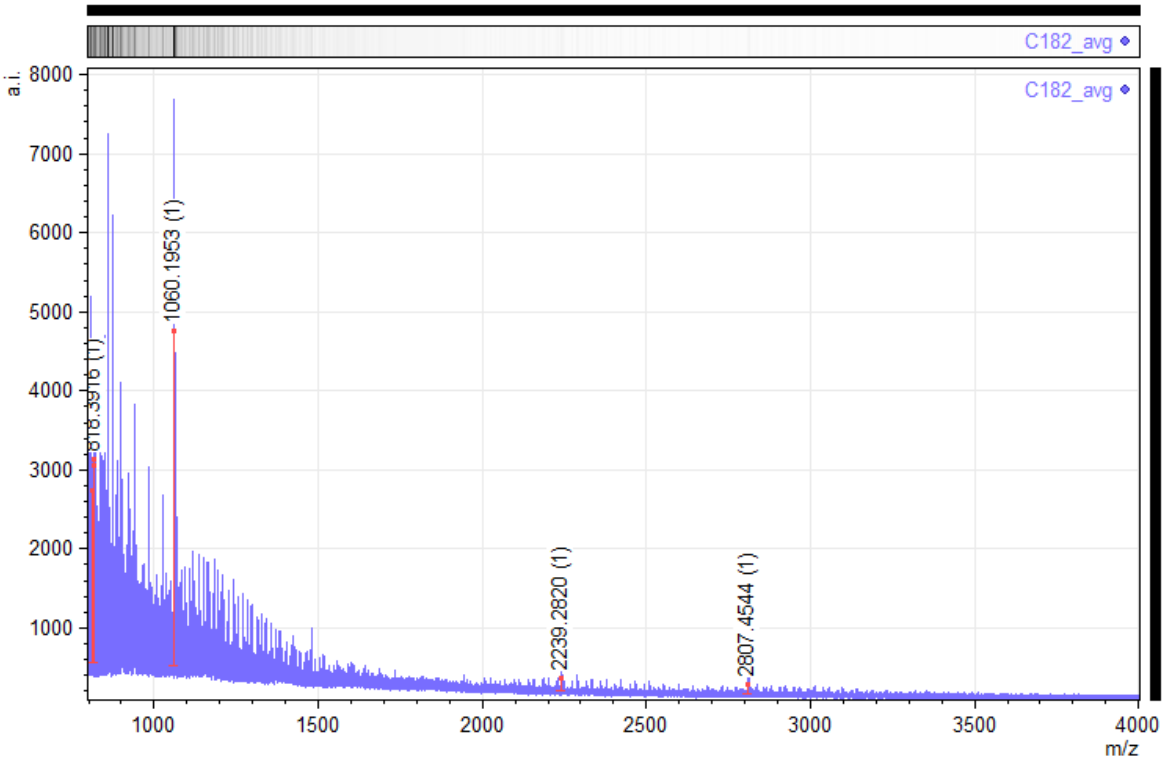
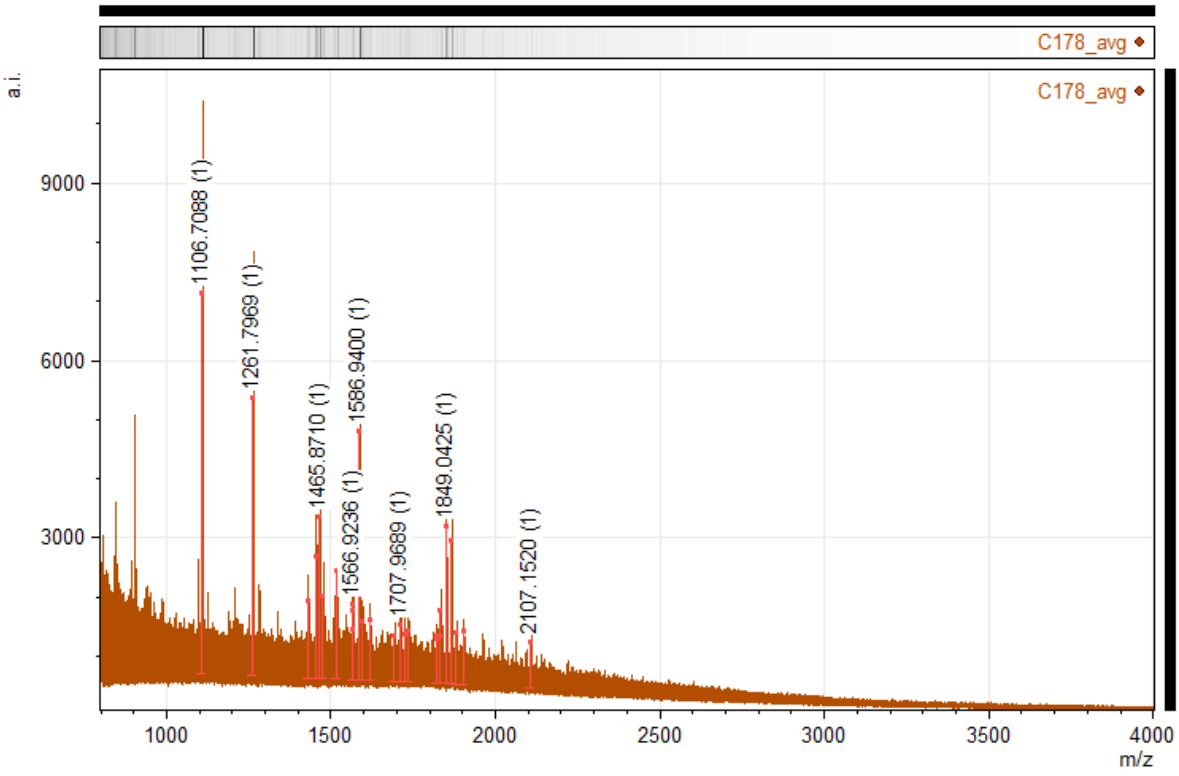


Figure 9. *C178 Spectra*



Discussion

De-Tarring

Though there was no direct correlation seen here between visible tar saturation and quantity of MALDI peaks, both samples C183 and C185, which were analyzed without a de-tar treatment, yielded 0 peaks. This supports the necessity for de-tarring procedures even on these bones with minimal visible tar saturation. De-tar treatment necessity on less saturated bones could further be explored using a larger sample size.

Samples C172, C177, C180 and C181 were lost prior to ZooMS due to the toluene and methanol solution, despite methanol and MilliQ sonication baths. More thorough rinsing procedures following T:M could prevent extended bone reactions within this solution, which was seen in Figure 4. b., and reduce bone loss in the de-tarring process. Future testing on the cause of suspected fat deposits, which developed during T:M rinse, may also assist in the reduction of sample loss.

There is a high possibility that the samples which had not been run in methanol yet to dissolve toluene, which were left overnight in MilliQ water, faced extended toluene exposure. This is likely what caused the damage of both the bone pellets and the collagen, explaining the loss of samples. The application of a centrifuge between sonication baths may also reduce this suspected fat condensation to a smaller pellet, also allowing for reduction of sample loss.

Database

The ZooMS database is a partially limiting factor to this research, particularly in relation to sample C178, the suspected sloth vertebra. The RLB fauna record contains the Harlan's ground sloth, Jefferson's ground sloth, and Shasta ground sloth (Mammal Collection, LaBrea Tar Pit, 2022). Since no ZooMS data has been published using sloths, or any closely related animals at the point of this project, sample C178 cannot be positively or negatively identified as such. The sample is then limited to an identification of a possible carnivore based on 2 of 26 peaks. The addition of a sloth database could impact the results seen within this paper. This lack of spectra also leaves room for consideration regarding non-identifiable peaks of sample C173, which could align with a species similar to these ground sloths which are not yet recorded in the ZooMS database.

Conclusion

The Rancho LaBrea Tar Pits are a complicated location to undertake any kind of biomolecular research at, which is evident in the results of this paper. However, the limited positive sample identification aligns with suspected animals in this area, which affirms the potential for ZooMS applications on this site, and other tar saturated faunal assemblages. Overall, procedures utilized in this research could also be refined with modified processing of suspected fat saturated supernatant, in order to preserve greater bone mass for further ZooMS analyses.

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