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Original Research

# Forensic characterization of sea turtle oil by ambient ionization mass spectrometry: *Caretta caretta*, *Chelonia mydas*, *Dermochelys coriacea*, *Eretmochelys imbricata*, *Lepidochelys kempii*, and *Lepidochelys olivacea*

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ABSTRACT

Historically the oils from sea turtles have had a high commercial demand, and this trade has contributed to an alarming decrease in sea turtle populations worldwide. Determining the species source of seized animal oils is challenging and this has hindered enforcement of the ban on trade in sea turtle oil. In this work we investigate if the chemotypes obtained from ambient ionization mass spectrometry analysis can assist in making species classifications of oil. Oil was either obtained or yielded from tissue from Green sea turtle (*Chelonia mydas*), Loggerhead sea turtle (*Caretta caretta*), Kemp's ridley sea turtle (*Lepidochelys kempii*), Olive ridley sea turtle (*Lepidochelys olivacea*), Hawksbill sea turtle (*Eretmochelys imbricata*), and Leatherback sea turtle (*Dermochelys coriacea*). Additionally, common oils from vegetable and animal sources were investigated to determine if any other oil sources could be confused with sea turtle oils. The oil samples were ionized both in the positive and negative mode by Direct Analysis in Real Time (DART) and the masses were measured in a time of flight mass spectrometer (AccuTOF by JEOL). Positive and negative mode mass spectra produced species-specific chemotypes and no other oils analyzed in this study produced chemotypes resembling those of sea turtle oils. We conclude that this approach is accurate and very useful for rapidly characterizing neat sea turtle oils without the need for sample derivatization.

## Introduction

All seven species of sea turtles (families *Cheloniidae* and *Dermochelyidae*) have long been used by humans. They were deemed by early mariners to be "so excellent a fishe" because they could turn the turtles plastron-up on the ship's deck, where they lived until needed for one of their many uses, sustaining the sailors for months or years at sea [1]. Turtles have yielded food in the form of eggs, meat, fat, and cartilage for soup (known as "calipée"). Their carapaces and scutes have been used as decorative and utilitarian objects, including taxidermied specimens and tortoiseshell jewelry. Their skin has been tanned for leather, and their fat and oil has been used in food, cosmetics and medicinal preparations [2].

Because sea turtles are long-lived, slow to mature, and particularly vulnerable on nesting beaches, all species have suffered declines due to exploitation, fisheries by-catch, and habitat loss. Six of the seven species have been prohibited in international trade by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1977, with the seventh species, Australian Flatback (*Natator depressa*), joining the other sea turtles on CITES Appendix I in 1981. The six sea turtle species that occur in US waters are protected by the US Endangered Species Act (ESA): Leatherbacks (*Dermochelys coriacea*), Loggerheads (*Caretta caretta*), Greens (*Chelonia mydas*), Hawksbills (*Eretmochelys imbricata*), and Olive Ridley and Kemp's Ridleys (*Lepidochelys olivacea* and L. *kempii*).

Though take and trade of sea turtles in the US is illegal, these threats continue around the world, and the US remains one of the largest consumers of wildlife and wildlife products [3]. Enforcement of laws protecting marine turtles fall under the jurisdictions of the National Oceanic and Atmospheric Administration's National Marine Fisheries Service (NOAA NMFS) when they are at sea, and the US Fish and Wildlife Service (USFWS) when they are on land. Both agencies house forensic laboratories which routinely identify sea turtle products. When such products have been stripped of their identifying morphological characteristics, DNA is used preferentially for species-level identification of sea turtle eggs, meat, and leather (e.g. [4,5],), and spectroscopy is used for identification of tortoise shell items [6]. Sea turtle oils, however, are

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particularly difficult to identify to species, as they are refractory to DNA analysis, and traditional fatty acid analysis of marine oils is time-consuming, data analysis difficult, and species-specific lipid profiles not always apparent (G. Seaborn, pers. communication). The NOAA forensic laboratory performed species identification of marine oils via fatty acid analysis until 2010 [18]. Though sea turtle oils were a minor portion of submitted casework, both the NOAA and USFWS laboratories have continued to receive a steady trickle of requests for analysis of marine oils from vertebrate species. A query of the CITES Trade database for "comparative tabulations" of trade in "Cheloniidae" and "*Dermochelys*" (the only genus in the family Dermochelyidae) between 2010–2019 reveal a low level of trade recorded in oil from Cheloniidae, but none from *Dermochelys*. Among 1321 entries for Cheloniid imports into the US, 13 were categorized as "oil". Additional entries that are ambiguous, but could refer to oil, are "unspecified" (n = 8), "extracts" (n = 3), and "derivatives" (n = 14); (spreadsheet "CITES query.xlsx" available upon request). These represent shipments reported to CITES, and do not represent what is likely a much larger illicit trade. The majority of these instances listed Mexico as the exporter, with Panama and the Dominican Republic also contributing. In all instances, the US was listed as the importer, corroborating the NOAA and USFWS Forensic laboratories' assessment that a method for species identification of these marine oils is still needed.

The common approach to identify animal oils is to characterize the fatty acids, because oils are rich in aliphatic carboxylic acids of the general formula $C_nH_{2n+1}COOH$. The traditional analysis of fatty acids requires a derivatization step which is followed by separating the molecules by using gas or liquid chromatography with flame ionization detection with standard retention time or characterizing the masses of the negative ions using mass spectrometry [18]. In this study, we conducted the analysis without derivatization and analyzed both the positive and negative ions by using a Direct Analysis in Real Time (DART), with the masses of the ions measured in a time of time-of-flight mass spectrometer (TOFMS) (AccuTOF™ by JEOL). Since DART TOFMS has been applied to other challenging forensic evidence such as timber [7], horns [8], and hairs [9] and it offers the advantage of avoiding derivatization, our goal was to explore if this approach would produce useful data for taxonomic classification of sea turtle oil. Analysis was conducted of both the positive and negative ions, but in order to be succinct only the graphs related to the positive ions will be presented since these yielded a greater number of ion/features that were taxonomically relevant to make species classifications (*vide infra*). Data and graphs related to the analysis of the negative ions can be found in the supplemental material.

## Methods

### *Species confirmation of sea turtle reference tissues*

Sea turtle reference tissue samples used in the study were from a variety of sources, including strandings (collection authorized under NMFS permit, 22435) and law enforcement seizures. When sufficient morphology was present, as in stranded animals, it was used to confirm species identity. Species of origin for tissues from law enforcement seizures were confirmed with DNA sequencing. Species of label for oils could not be confirmed with DNA or morphology, but were analyzed alongside confirmed reference materials.

DNA extraction of sea turtle muscle tissue was completed using the Qiagen® DNeasy kit on a Qiagen® QIAcube HT robotic platform according to the manufacturer's tissue protocol. Negative extraction blank controls were included in each extraction run. A universal primer set was used to amplify an ~345 base pair segment of mitochondrial DNA that codes for the Cytochrome *b* gene [H15149 + L14841] (Kocher et al. 1989). PCR reactions contained 1−10 ng DNA template and final reagent concentrations of 125 μM dNTPs, 1X PCR buffer, 2.0 mM $MgCl_2$, 1.5U Taq DNA polymerase (Promega, United States), 0.16 μg BSA, and 175 nM of each primer. Annealing temperature was 55 °C. DNA sequencing was performed on purified PCR amplicons by dideoxy-nucleotide sequencing (Sanger et al. 1977) with the Life Technologies BigDye Terminator v3.1® sequencing kit. Resulting sequences were analyzed by capillary electrophoresis on an ABI 3500xl Genetic Analyzer (Thermofisher). Positive and negative controls were run with all PCRs.

Sequences were imported into the Geneious R6 software package (Biomatters, Ltd.) for editing and primer end-trimming prior to comparative alignment to sequences in the National Fish and Wildlife Forensic Laboratory (NFWFL) sea turtle sequence database which includes all seven sea turtle species. The NFWFL database is composed of publicly available (e.g., GenBank) sequences, sequences from in-house vouchered reference materials, and sequences from adjudicated forensic casework samples. Species origin was determined by comparing sequences from unknown samples with the database sequences.

### *Sample sources*

The sea turtle reference specimens used in this study consisted of six fatty tissue and two oil specimens of *C. mydas,* 3 oil specimens of *D. coriacea*, six tissue specimens of *E. imbricata*, two tissue specimens of *C. caretta,* four tissue specimens of L. *kempii* and two oil specimens of L. *olivacea.* Supplemental Table 1 lists the sources of these samples and the number of spectra replicates collected given the unequal sample sizes per taxon. Additional replicate samples were used for validation purposes (see Supplemental Table 2).

For tissue samples, oil was extracted from approximately 200 mg of tissue by finely cutting tissue into small cubes, which were simmered on a propane stove at low temperature. The tissue yielded from 1 to 25 mls of oil, which was transferred to a glass tube pending analysis. The oil rendered from the tissue had a yellow color and became solid at room temperature; this observation is consistent with descriptions of Thomssen [10] that stated that sea turtle oil "is solid at room temperature, with mild, distinctive odour and on melting, a deep yellow colour" [20].

In order to determine if the sea turtle oil chemotypes could be confounded with common oils, commercial animal and vegetable oils were procured. Supplemental Table 2 shows the commercial oils analyzed and their provenance. For these, species origin of label was assumed to be correct. Confirmation of the mass spectrum assignments included the analysis of the following reference fatty acids purchased from MilliporeSigma (https://www.sigmaaldrich.com): arachidonic acid, docosahexaenoic acid, linoleic acid, linolenic acid, oleic acid and palmitoleic acid.

### *Sample preparation for DART-TOFMS*

Mass spectra measurements were collected on a time-of-flight mass spectrometer (AccuTOF, JEOL, USA, INC., Peabody, MA. USA) fitted with a DART ion source (DART-SVP, IonSense Inc., Saugus, MA. USA). The resolving power, as stated by the manufacturer, is 6000 FWHM. The DART was operated in positive and negative-ion modes from a range of 50 *m/z* to 1000 *m/z*. Orifice 1 had a voltage of 30 V, orifice 2 had a voltage of 5 V, and the ring lens was set to 5 V.

Fifty μl of oil was diluted in 0.5 mL of 2-propanol. Samples were vigorously vortexed for 10 s and extracted for 30 min. Before analysis, each extraction was vortexed again. Positive-ion and negative-ion spectra were sampled by dipping the sealed end of a glass capillary tube (Pyrex #9530−4) into the extract and placing it in front of the DART heated protonated helium stream (350 °C). Positive mode spectra were calibrated every fifth sample with poly(ethylene glycol) 600 (Ultra Scientific, Kingstown, RI, USA) whereas negative mode spectra were calibrated with Fomblin®Y (Aldrich, St. Louis, MO, USA).

Analysis of the fatty acids in the negative mode was accomplished by the voltage setting of the mass spectrometer. Analyzing the fatty acids, steroidal and di- & triglyceride components in the positive mode consisted of changing the instrumental voltage parameters and repeating the process described. It was surprising to note that the number of features

**Table 1**
Number of ions detected in positive and negative modes for each species analyzed.

| Ions | *C. caretta* | *C. mydas* | *D. coriacea* | *E. imbricata* | *L. kempii* | *L. olivacea* |
|---|---|---|---|---|---|---|
| Positive | 365 | 220 | 269 | 166 | 724 | 283 |
| Negative | 132 | 79 | 78 | 113 | 710 | 39 |

(i.e., ions) detected in the positive mode were three times or greater than the number of negative ions detected (see Table 1). This can be explained by the fact that negative ions analysis only detected fatty acids whereas positive ambient ionization detected selected fatty acids (*m/z* range of ~100−330 amu), steroidal ions (*m/z* range of ~ 327–415 amu) and di- & triglyceride molecules (*m/z* range ~ > 340 amu). These results encouraged us to report the results of the positive ions, but we have included the results of all the negative ion analysis in the supplemental data.

Spectra were acquired, calibrated, averaged and background subtracted using msAxel (version 1.0.5.2, JEOL Ltd.) and centroided mass spectra were exported as text files for chemometric analysis with Mass Mountaineer software (RBC Software, Peabody, MA, USA).

Multivariate analysis used the correlation algorithm for Principal Component Analysis (PCA) model. PCA is an unsupervised algorithm that measures the variation of samples within a group. Using a discriminant PCA (Discriminant Analysis of the Principal Components; DAPC) model identifies between-group variations. The ions used for multivariate analysis were generated from the heat maps by selecting the most abundant *m/z* values for analysis of variance (ANOVA). Ions whose values were $p \leq 0.05$ were retained as classifying features.

The accuracy and robustness of the model was calculated by challenging the algorithm with 30 spectra from 17 samples not used to develop the model and by using Leave-One-Out Cross Validation (LOOCV), wherein a value of 100 % indicates that all samples were assigned to their correct class.

## Results

### *Confirmation of species*

For reference specimens used in this study, Supplemental Table 1 indicates which specimens were confirmed by DNA and which specimens' taxonomic origins were accepted solely based on the authority of the collector and/or the label on the sample.

### *DART-TOFMS*

For each species, the mass spectra from each sample were averaged (*C. caretta* = 14; *C. mydas* = 16; *D. coriacea* = 15; *E. imbricata* = 15; L. *kempii* = 17; L. *olivacea* = 10), and the averaged spectrum for the positive ion measurements is shown in Fig. 1, whereas the averaged spectrum for the negative ion measurements is shown in Supplemental Fig. 1. Not all the ions detected by the mass spectrometer could be characterized, but the tentative assignments of the most intense ions are listed in Table 2. Molecular assignments were made by comparing the high-resolution masses detected by DART TOFMS against the Lipidomics database curated by LIPID MAPS (https://www.lipidmaps.org/).

The analysis of the negative ion measurements is consistent with the expected fatty acid profiles (Supplemental Fig. 1) and the spectrum of all

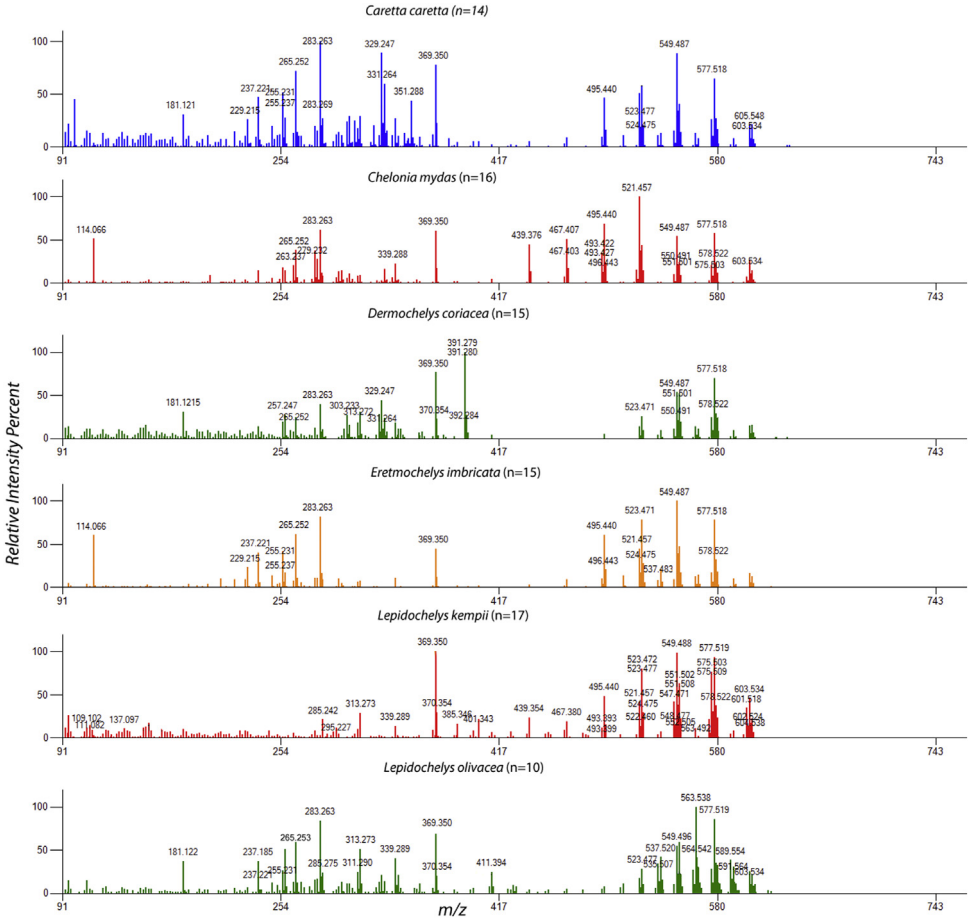



**Fig. 1.** Average spectra of positive-ion DART-MS spectra of Loggerhead (n = 14), Green (n = 16), Leatherback (n = 15), Hawksbill (n = 15), Kemp's ridley (n = 17), and Olive ridley (n = 10) sea turtles. Base peaks are cholesterol, sterols, di- and triglycerides.

**Table 2**
Tentative assignments of selected ions detected.

| Tentative assignment (or Isomers) | Composition | Measured *m/z* | *C. caretta* | *C. mydas* | *D. coriacea* | *E. imbricata* | *L. kempii* | *L. olivacea* |
|---|---|---|---|---|---|---|---|---|
| **Negative mode analysis** | | | | | | | | |
| 12:0 Lauric acid | $C_{12}H_{24}O_2$ -H | 199.173 | Y | Y | | | | |
| 14:0 Myristic acid | $C_{14}H_{28}O_2$ -H | 227.205 | Y | Y | Y | Y | Y | Y |
| 16:2 | $C_{16}H_{28}O_2$ -H | 253.215 | | Y | Y | Y | | Y |
| 16:1 Palmitoleic acid | $C_{16}H_{30}O_2$ -H | 253.220 | Y | Y | Y | Y | Y | Y |
| 16:0 Palmitic acid | $C_{16}H_{32}O_2$ -H | 255.236 | Y | Y | Y | Y | Y | Y |
| 18:2 (n−6) Linoleic acid (LA) | $C_{18}H_{32}O_2$ -H | 279.236 | Y | Y | | | Y | |
| 18:1 Oleic acid | $C_{18}H_{34}O_2$ -H | 281.247 | Y | Y | Y | Y | Y | Y |
| 18:0 Stearic acid | $C_{18}H_{36}O_2$ -H | 283.265 | Y | Y | | Y | Y | Y |
| 18:1 OH Ricinoleic acid | $C_{18}H_{34}O_3$ -H | 297.247 | | Y | Y | Y | | Y |
| 20:5 (n-3) Eicosapentaenoic acid (EPA) | $C_{20}H_{30}O_2$ -H | 301.218 | Y | Y | Y | | Y | |
| 20:4 Arachidonic acid | $C_{20}H_{32}O_2$ -H | 303.232 | Y | Y | Y | | | |
| 22:6 (n-3) Docosahexaenoic acid (DHA) | $C_{22}H_{32}O_2$ -H | 327.232 | | Y | Y | | | |
| 22:5 (n-5) Docosapentaenoic acid (DPA) | $C_{22}H_{34}O_2$ -H | 329.252 | Y | Y | Y | | | |
| **Positive mode analysis** | | | | | | | | |
| MG 16:0/0:0/0:0 | $C_{19}H_{38}O_4$ -OH | 313.273 | Y | | Y | | Y | Y |
| C21 Sterol analogue or derivative | $C_{22}H_{32}O_3$ -OH | 327.231 | | | Y | | | |
| C22 Sterol analogue or derivative | $C_{22}H_{34}O_3$ -OH | 329.247 | Y | | Y | | | Y |
| MG 16:0/0:0 or C22 Sterol analogue or derivative | $C_{22}H_{36}O_3$ -OH | 331.264 | Y | | Y | | | |
| Oleylglycerol | $C_{21}H_{40}O_4$ -OH | 339.288 | | Y | Y | Y | | Y |
| MG 18:0/0:0/0:0 | $C_{21}H_{42}O_4$ -OH | 341.303 | Y | | Y | | | Y |
| Cholesterol | $C_{27}H_{46}O$ -OH | 369.350 | Y | Y | Y | Y | Y | Y |
| Cholic acid | $C_{24}H_{40}O_5$ -OH | 391.284 | | | Y | | | |
| C24 Sterol analogue or derivative | $C_{30}H_{52}O$ -OH | 411.398 | | | | | Y | Y |
| DG 12:0/ 12:0 | $C_{27}H_{52}O_5$ -OH | 439.376 | | Y | | | | |
| DG 13:0/ 13:0 | $C_{29}H_{56}O_5$ -OH | 467.407 | | Y | | | | |
| DG 12:0/ 16:1 | $C_{31}H_{58}O_5$ -OH | 493.425 | | Y | | Y | | |
| DG 12:0/ 16:0 | $C_{31}H_{60}O_5$ -OH | 495.439 | Y | Y | | Y | Y | |
| DG 12:0/18:1 | $C_{33}H_{62}O_5$ -OH | 521.454 | | Y | | Y | | Y |
| DG 12:0/ 18:0 | $C_{33}H_{64}O_5$ -OH | 523.468 | | Y | | Y | | Y |
| DG 12:0/18:0/0:0 | $C_{33}H_{64}O_5$ -OH | 523.472 | | | | Y | | |
| DG 14:0/17:0/0:0 | $C_{34}H_{66}O_5$ -OH | 537.487 | Y | | | Y | | Y |
| DG 14:0/ 18:1 | $C_{35}H_{66}O_5$ -OH | 549.486 | | Y | Y | Y | | Y |
| DG 13:0/ 19:0 | $C_{35}H_{68}O_5$ -OH | 551.500 | Y | Y | Y | Y | Y | Y |
| DG O-16:0/18:1 | $C_{37}H_{72}O_4$ -OH | 563.538 | | | | | | Y |
| DG 13:0/20:0/0:0 | $C_{36}H_{70}O_5$ -OH | 565.516 | Y | | | Y | | Y |
| DG 14:0/20:1 | $C_{37}H_{70}O_5$ -OH | 577.517 | Y | Y | Y | Y | Y | Y |
| DG 14:0/20:0/0:0 | $C_{37}H_{72}O_5$ -OH | 579.535 | | | Y | | | Y |
| DG 19:1/17:1/0:0 | $C_{39}H_{72}O_5$ -OH | 603.536 | Y | Y | | | Y | Y |
| DG 19:1/17:0/0:0 | $C_{39}H_{74}O_5$ -OH | 605.551 | | Y | | | | Y |

six sea turtle oils shows high intensity (i.e., concentration) for the peaks at a mass-to-charge ratio (*m/z*) of 281.247 and 255.236, characteristic of oleic acid ($C_{18}H_{34}O_2$ -H) and palmitic acid ($C_{16}H_{32}O_2$ -H) and their corresponding isotopes. Seaborn et al. [11] and Ackman et al. [12] reported the presence of 22:6 (n-3) docosahexaenoic acid (DHA) in *C. mydas,* and this molecule is detected by us as $C_{22}H_{32}O_2$ -H at *m/z* 327.232 (Supplemental Fig. 1). The common fatty acids 12:0 and 14:0 (lauric and myristic acids) reported by Joseph et al. [13], Ackman et al. [12], and Guitart et al. [14] were detected in their de-protonated form ($C_{12}H_{24}O_2$ -H & $C_{14}H_{28}O_2$ -H) at *m/z* of 199.173 and 227.205, correspondingly. Additional assignments of selected negative ions are listed in Table 2, and in agreement with the findings of other authors [12–14,11].

The analysis of the positive ions is consistent with the expected steroid isomers reported by Weldon et al. [15], and mono-, di-, and tri-glycerides and their corresponding isotopes are in agreement with Price et al. [16] (Fig. 1). All taxa show the presence of cholesterol at *m/z* 369.35 ($C_{27}H_{46}O$

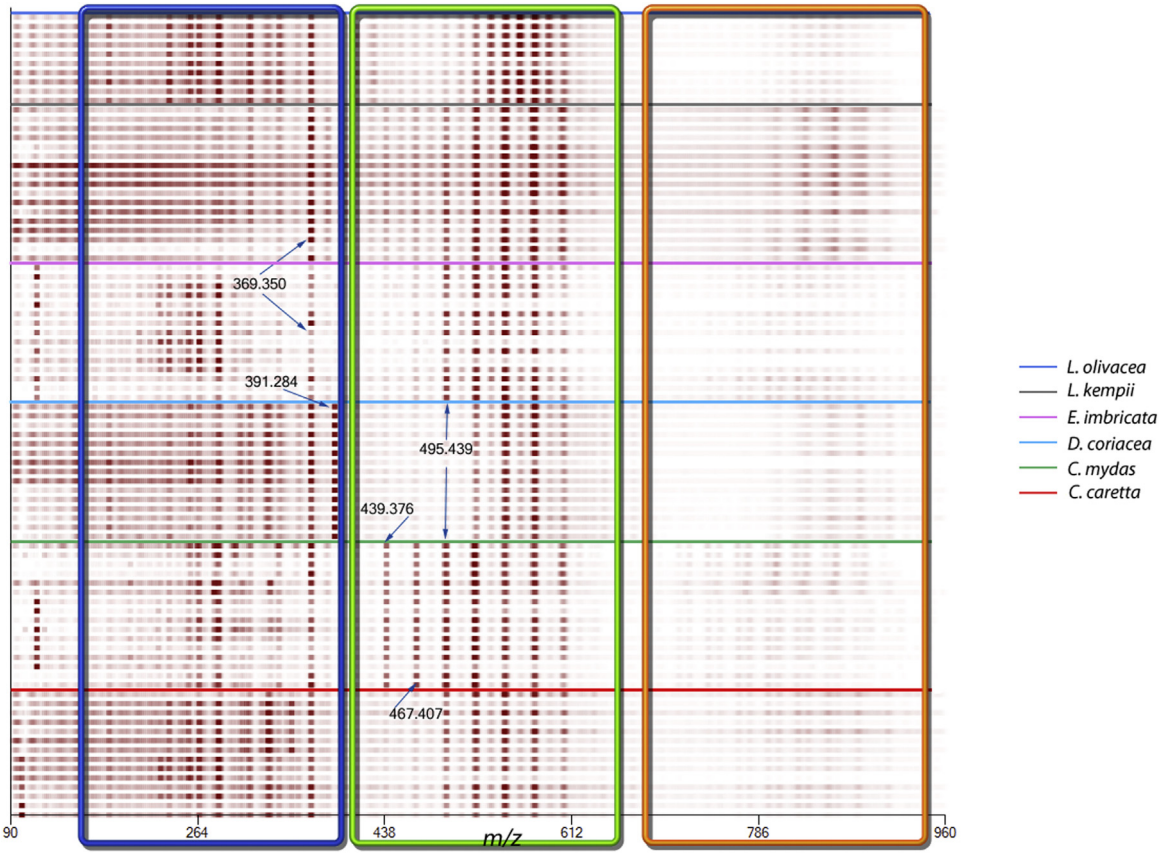



**Fig. 2.** Heatmap of the positive-ion DART-MS spectra of Loggerhead (n = 14), Green (n = 16), Leatherback (n = 15), Hawksbill (n = 15), Kemp's ridley (n = 17), and Olive ridley (n = 10) sea turtles. The blue inset box highlights the mass range of the fatty acids, the green inset box shows the mass range of the di-glycerides and the orange inset box shows the tri-glyceride masses. Each horizontal row represents a unique positive-ion spectrum.

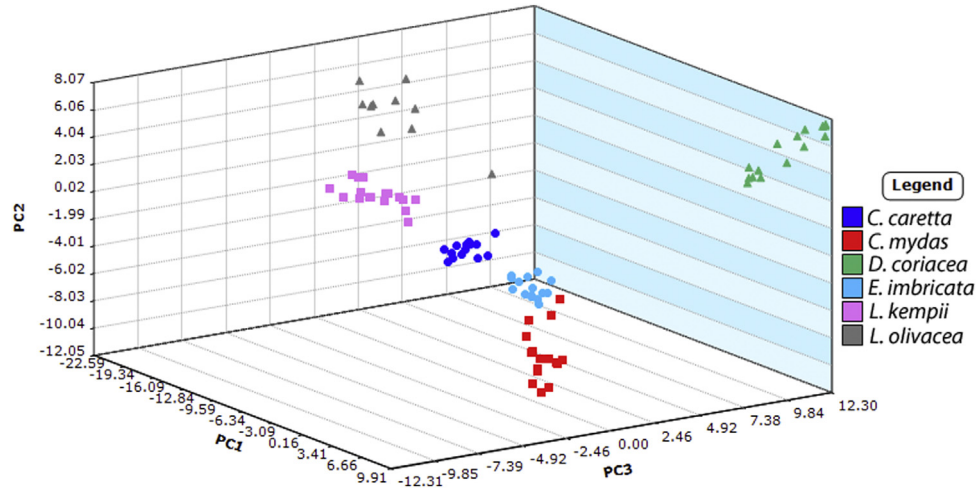



**Fig. 3.** Graphical representation of the cholesterol, sterols, di- and triglycerides (positive ions) DAPC analysis. LOOCV = .98.8 %.

−OH), but each taxon exhibits a distinct di- and tri-glyceride profile. The assignment of ions shown in Fig. 1 clearly shows patterns of ions characteristic to each of these species.

A heat map is a clever way of visualizing all the spectra collected for a particular species to determine if the chemotypes are reproducible within species (i.e., reproducibility of a pattern) and also assists in determining if each species has a distinct and unique pattern (i.e., chemotype). Fig. 2 is the heat map of the positive ion spectra, in which the color intensity is correlated to the abundance of a particular ion; color intensity correlated to concentration, where a dark color implies an abundant ion in the spectra. For example, the spectra (Fig. 1) and the positive ion heat map (Fig. 2) shows that all the specimens in each species had an intense ion at *m/z* 369.350 which is assigned to cholesterol. Additionally, *C. mydas* samples exhibit the presence of diglycerides 12:0/12:0 and 13:0/13:0 at approximate *m/z* of 439.376 and 467.407 which is absent or in lower concentrations in the other taxa. Conversely, L. *olivacea* and *D. coriacea* do not exhibit the ion (*m/z* 495.439) assigned to diglycerides 12:0/16:0. The intense ion found at *m/z* 391.284 in *D. coriacea* is assigned as one of several protonated isotopes of phthalates which are plasticizer contaminants from the disposable tubes (https://en.wikipedia.org/wiki/

Phthalate); the other specimens in this study were wrapped in aluminum foil, and were free of plasticizer contamination. The negative mode analysis (Supplemental Figs. 1 and 2) show that only *C. mydas* has an abundant ion at *m/z* 199.173 which is assigned as lauric acid (12:0), while *C. caretta* has a higher concentration of docosahexaenoic acid (22:6 (n-3) @ *m/z* 327.232) than the other taxa in this study. These differences in the presence or absence of specific ions, or in the intensity of ions in the taxa facilitate developing predictive models for the analysis of unknown oil.

Fig. 3 shows the graphical representation of the Discriminant Analysis of the Principal Components (DAPC) of the positive ion (Negative mode DAPC is shown in Supplemental Fig. 3). In both graphs, each spectrum correctly clusters within the corresponding species and groups away from the other taxa. One validation scheme of the DAPC model was to use the cross validation algorithm of LOOCV, and both DAPC models were calculated to be 98.8 % correct, with only one spectrum from the 86 used to build the model incorrectly classified; the cross validation analysis of the models (LOOCV) showed that in both the negative and positive analysis, a *C. mydas* specimen was incorrectly classified as *E. imbricata*, and an examination of the DAPC models (Figs. 3 and Supplemental Fig. 3) shows that these two taxa clustered close to each other causing the misclassification. A second validation was conducted by analyzing the spectra of 30 samples that were not used to create the DAPC model and included spectra from *C. mydas* (n = 15), *E. imbricata* (n = 7), and L. *kempii* (n = 8). The positive mode validation had two spectra mis-classified (93.3 % accuracy) whereas the negative ion DAPC model had three misclassified spectra (90.0 % accuracy). All misclassified spectra stemmed from one individual Green turtle (results are shown in Supplemental Table 3).

Since the heatmap suggests that each species has a unique chemotype, we analyzed a subset of the data using Ward's method of hierarchical cluster analysis (100 bootstrap) and the resulting radial dendrogram is shown in Fig. 4. This analysis was done with a subset of the data in order to have equivalent sample sizes for each taxa (Green sea turtle n = 12, Leatherback sea turtle n = 12, Hawksbill sea turtle n = 12, Kemp's ridley n = 12, and Olive ridley sea turtle n = 9). This unsupervised analysis reveals that there is group structure to each species and similarity within species, with only one of 69 spectra clustered incorrectly: an *E. imbricata* specimen grouped within the *C. caretta* group. We can infer from this that the chemotypes have the robustness needed for making classifications of unknown evidentiary oils.

In order to determine if sea turtle oils could be confused with oils from other sources, analysis was conducted on animal oils (n = 8) and commercial vegetable oils (n = 10); the species analyzed, and their source are shown in Supplemental Table 2. Fig. 5 shows the results of a positive-ion heatmap of the animal oils (negative-ion heatmap is Supplemental Fig. 4). Fig. 6 shows a positive-ion heatmap of the results of the vegetable oils (negative-ion heatmap is Supplemental Fig. 5). These heat maps demonstrate that the chemotypes of sea turtle oils could not be confused with oils from other common sources.

## Discussion

Determining the taxonomic origin of oil from closely related species is important for enforcing domestic and international laws and regulations

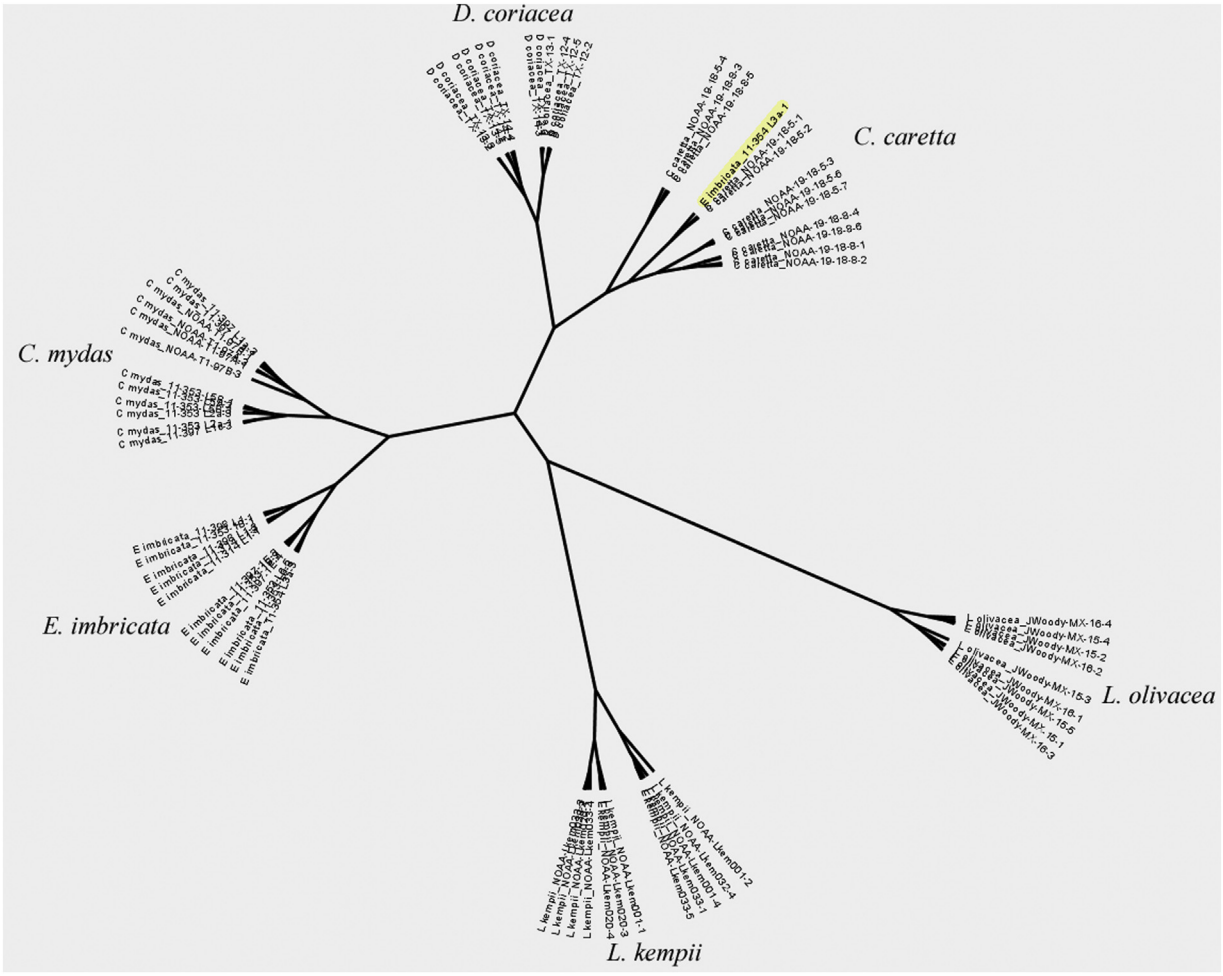



**Fig. 4.** Radial dendrogram of a hierarchical cluster analysis (Ward's algorithm) of Loggerhead (n = 14), Green (n = 12), Leatherback(n = 12), Hawksbill (n = 12), Kemp's Ridley (n = 12), and Olive Ridley(n = 9). The mis-classified spectrum of Hawksbill sea turtle is highlighted in yellow.

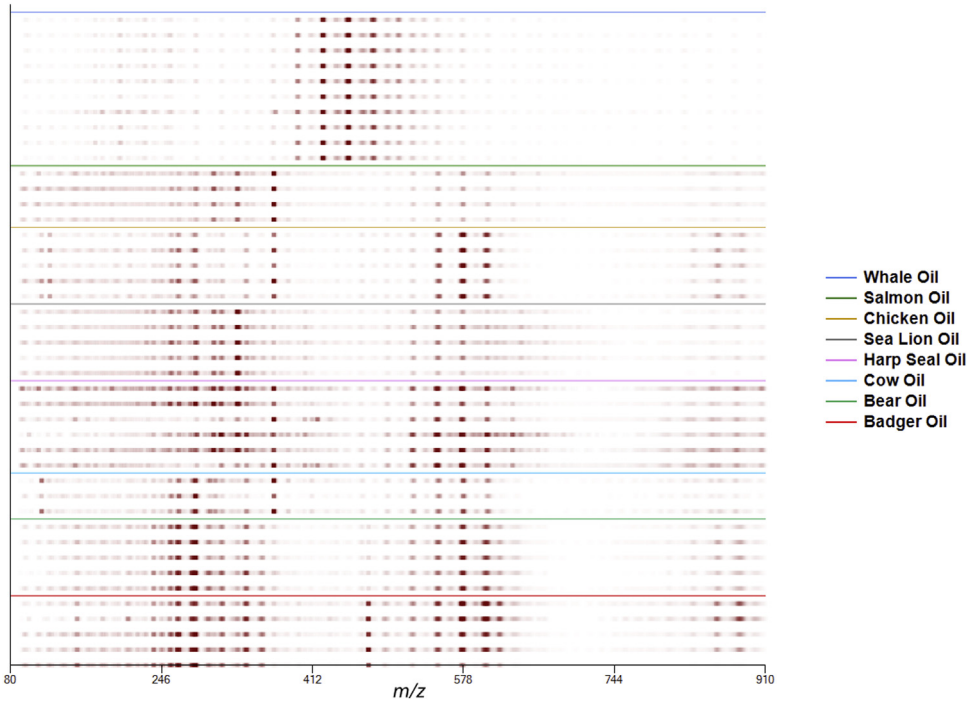



**Fig. 5.** Positive ion heatmap of diverse animal oils demonstrating that the chemotypes do not resemble the profile of sea turtle oils. Each horizontal row represents a unique positive-ion spectrum.

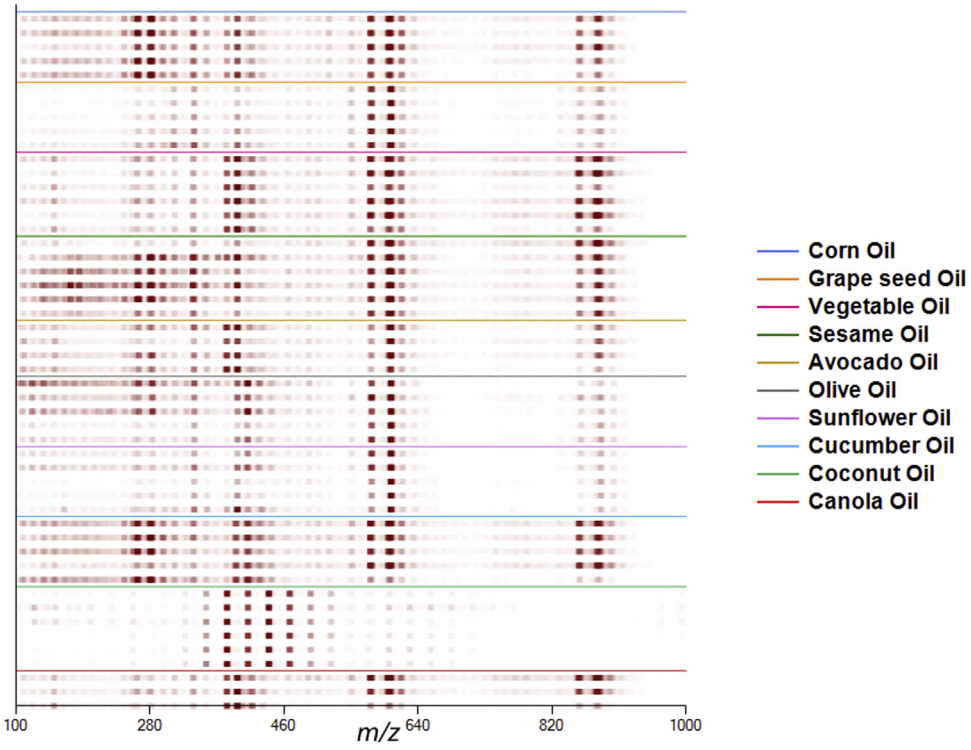



**Fig. 6.** Positive ion heatmap of diverse vegetable oils demonstrating that the chemotypes do not resemble the profile of sea turtle oils. Each horizontal row represents a unique positive-ion spectrum.

governing wildlife trade. In this study, we compared oils rendered from six of the seven extant sea turtle species. Green turtles, so named for their green fat, are preferred for the high quality of their meat and oil [17], and are likely the most often targeted for oil production. Leatherbacks are known to produce up to 40 L of oil per animal and are used in medicinal preparations in Africa [2]. Though Loggerheads, Hawksbills, and Olive ridleys are not known to be targeted for oil, it is likely a byproduct of their primary uses for meat, tortoiseshell, and leather, respectively. Kemp's ridleys are unlikely to appear in trade, due to their extremely restricted breeding range and heavy protection resulting from their critically endangered status, but we thought it important to include them here as close relatives of the more populous Olive ridley. Australian flatbacks are

not analyzed here, but are the least likely to appear in international trade, having the most restricted geographic range of all species; indeed, they were the least common species in the CITES trade database query, and have never been seen in casework at the NOAA and USFWS Forensic Laboratories.

Mass spectrometry analysis showed that the high resolution of DART-TOFMS could distinguish the species source of the oils with a high level of accuracy (> 98 %) without sample derivatization. Table 1 shows a series of selected ions and their abundance, which were detected in each species, but statistical classification was contingent on the chemotype detected rather than single diagnostic compounds. Analysis of the positive ion metabolome produced robust models. The negative-ion metabolome was dominated by fatty acids, but these chemotypes were not as discriminatory as the positive-ion metabolome.

## Declaration of Competing Interest

The authors report no declarations of interest.

## CRediT authorship contribution statement

**Edgard O. Espinoza:** Supervision, Writing - original draft, Conceptualization, Methodology, Formal analysis, Investigation, Validation. **M. Katherine Moore:** Writing - original draft, Resources, Writing - review & editing. **Brian C. Hamlin:** Writing - original draft, Methodology, Investigation, Writing - review & editing. **Barry W. Baker:** Writing - original draft, Writing - review & editing. **Aiden J. Espinoza:** Resources, Methodology.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fsiae.2021.100008.

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