Lipid extraction has tissue-dependent effects on isotopic values ($\delta^{34}$S, $\delta^{13}$C, and $\delta^{15}$N) from different marine predators

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Rationale: The use of sulfur isotopes to study trophic ecology in marine ecosystems has increased in the past decade. Unlike other commonly used isotopes (e.g., carbon), sulfur can better discriminate benthic and pelagic productivity. However, how lipid extraction affects sulfur isotopic values has not been assessed, despite its frequent use to remove lipid effects on $\delta^{13}$C values.

Methods: We used white muscle and liver samples from two species of sharks and skin samples from two species of pinnipeds (sea lion and fur seal) to assess the effects of lipid extraction on stable isotope values for $\delta^{34}$S, $\delta^{13}$C, and $\delta^{15}$N. Isotopic values were determined using a continuous flow-isotope ratio mass spectrometer coupled to an elemental analyzer.

Results: Lipid extraction significantly decreased $\delta^{34}$S values in shark tissues, more so for liver than muscle ($-4.6 \pm 0.9\%$ vs $-0.8 \pm 0.3\%$, average change), with nearly no change in their standard deviations. Lipid extraction did not affect $\delta^{34}$S values from pinniped skin samples ($0.2 \pm 0.8\%$, average change). After lipid extraction, consistent increases in $\delta^{13}$C values ($0.2\%$–$7.3\%$) were detected as expected, especially in tissue with high lipid content (C:N >4). After lipid extraction, significant increases in $\delta^{15}$N values ($0.5\%$–$1.4\%$) were found in shark muscle and liver tissues. For pinniped skin samples, $\delta^{15}$N values were not significantly lower after lipid extraction ($-0.4\%$ to $-0.1\%$).

Conclusions: Lipid extraction did not have a strong impact on $\delta^{34}$S values of shark muscle and pinniped skin ($\leq1\%$). However, our results suggest it is essential to consider the effects of lipid extraction when interpreting results from $\delta^{34}$S values of shark liver tissue, as they significantly depleted values relative to bulk tissue ($\sim5\%$). This may reflect selective removal of sulfolipids and glutathione present in higher concentrations in the liver than in muscle and skin and requires further investigation.

1 | INTRODUCTION

Stable isotope analysis is one of the most widely used techniques for understanding spatial and trophic relationships in both ancient and contemporary ecosystems. Trophic studies of aquatic ecosystems often use this approach due to the elusive nature of many aquatic organisms and the biases resulting from traditional methods (e.g., direct observation and stomach content analysis). Carbon and nitrogen are the most commonly used elements in stable isotope analysis because these are among the most abundant elements across...
all biological materials, and their role in biological cycles and environmental gradients is well known. The use of different elements allows us to understand the aspects of consumer ecology. The stable isotope ratio of nitrogen (δ15N) is a proxy of the trophic level of an organism, and δ13C broadly indicates habitat use by identifying the source of primary energy (coastal vs oceanic, benthic vs pelagic). With improvements and refinements in instrument sensitivity, other elements (e.g., sulfur, oxygen, and hydrogen) have been incorporated into stable isotope studies, providing complementary or novel information on food webs and animal movements. The integration of a third element can improve ecological assessments by providing higher levels of resolution and greater discrimination power between different components of a trophic network. For example, the combined use of the stable isotope ratios of sulfur (δ34S) and δ13C has higher resolution capability for the identification of primary producers in an environment than carbon and nitrogen alone, even in estuarine and coastal environments with several potential sources of organic matter.

Stable isotope ratios of sulfur have become increasingly important in trophic studies since the 2000s. This expansion is a consequence of the recent improvements in mass spectrometry that have simplified this complex analytical technique allowing it to be performed relatively routinely, reducing both cost and the total amount of sample required for measurement. As sulfur has low trophic fractionation between consumers and their diet (~1.0‰), δ34S allows a researcher to identify the contribution of assimilated prey, with sulfur originating from different sources of primary production (benthic vs pelagic, terrestrial vs marine). Consequently, δ34S has helped reveal complex trophic networks where carbon has failed to identify some of the trophic chain elements and provide higher resolution in ecological niche occupancy. In addition, δ34S can improve diet quantification estimates from mixing models, with smaller confidence intervals around mean estimates of consumers’ potential prey groups. Although the use of sulfur in trophic ecology studies is increasing, there remain some methodological considerations, for example, the effect of lipid extraction, that need to be undertaken to ensure repeatability and comparability between samples and studies.

Variation in lipid content between tissues and organisms can affect stable isotopic values and lead to misleading results and, therefore, potentially erroneous ecological interpretations. Lipids are depleted approximately 8‰–8‰ in 13C relative to pure protein; therefore, analyses of tissues with higher lipid content lead to lower δ13C values. Chemical lipid extraction before undertaking δ13C measurements avoids this problem and has been recommended for standardizing δ13C for samples with high lipid content or when comparing across taxonomic groups that may have different lipid tissue content. However, lipid extraction can also alter δ15N by washing out nitrogenous compounds, causing an increase in the δ15N values relative to non-extracted replicates. Analyzing δ15C in samples with lipid extraction and δ15N in non-lipid extracted replicates overcomes this issue but increases the costs, time of processing, amount of sample needed, and use of hazardous chemicals such as chloroform and methanol. A second option is to use mathematical corrections for isotopic ratios that consider the effects of lipid extraction on the isotopic values of the different elements (e.g., references). However, knowledge of how lipid extraction affects the isotopic values for different tissue types and species is required to generate these mathematical corrections. Sulfur is an essential compound, primarily found in proteins with cysteine and methionine amino acids and sulfolipids. Therefore, the presence of sulfur in lipids could lead to biased sulfur isotopic values in samples with high lipid content, as outlined earlier with carbon. However, despite the increasing use of sulfur in ecological studies, the potential influence of lipid extraction on sulfur isotopic values has been tested only on eggs of a few species of seabirds. Compared to the replicates without lipid extraction, the extracted egg yolks showed significant differences in δ34S values with variable magnitude (~0.1‰ to 2.3‰) depending on the species analyzed. Despite this evidence, the effect of lipid extraction on commonly used tissues (e.g., muscle and liver) of nonmarine marine predators has not been tested.

In this study, we compared the effects of two treatments, nonlipid extracted versus lipid extracted, on three different commonly used tissues of four marine predator species: muscle and liver of two species of sharks (Carcharias taurus and Notorynchus cepedianus) and skin samples from two species of pinnipeds (Arctocephalus australis and Otaria byronia). We (a) assessed the effect of lipid extraction on δ34S, δ13C, and δ15N values and compared this with previously published information and (b) provided correction factors for these species and tissues where required.

2 | EXPERIMENTAL

2.1 | Sample collection

Four species of coastal marine predators that occur off the Atlantic coast of Uruguay were included in this study. In January 2020, we collected muscle and liver samples from two shark species targeted by local artisanal fisheries: gray nurse shark (C. taurus, n = 15) and sevengill shark (N. cepedianus, n = 15). Total length (TL) varied between 133.5 and 259 cm in gray nurse and between 157 and 239 cm in sevengill sharks. Shark samples were collected in collaboration with the artisanal and recreational fisheries monitoring programs of the National Directorate of Aquatic Resources (DINARA, acronym in Spanish). Between 2018 and 2020, we collected skin samples from fresh, stranded carcasses of two species of pinnipeds, the South American fur seal (A. australis, n = 15, TL = 70–175 cm) and the southern sea lion (O. byronia, n = 16, TL = 105–255 cm). All samples were frozen at −20°C until further analysis. Fieldwork and sample collection were conducted under permit 252/2018 issued by DINARA.
2.2 | Sample treatment

Skin samples from pinnipeds were dissected from hair and blubber using tweezers, keeping the epidermis and dermis layers for analysis. All samples (skin, muscle, and liver) were rinsed with deionized water to eliminate any residue that could affect the isotopic signal and oven-dried at 60°C for 72 h. Dried samples were ground to a fine powder using an A11 Basic Analytical Mill (IKA-Werke GmbH & Co. KG, Staufen, Germany) for liver and muscle samples and an MM200 ball mill (Retsch GmbH, Haan, Germany) for skin samples. Each sample was split into two subsamples for different treatments, one for analysis without lipid extraction (referred to as bulk samples) and the other for analysis after lipid extraction (referred to as lipid extracted [LE] samples).

Lipid extraction of ~1 g of tissue was conducted using chloroform-methanol (2:1) solution adapted from Folch et al. Shark liver typically has a high lipid content (~50% in gray nurse and sevengill sharks). Therefore, the process was repeated until the supernatant liquids were clear, indicating that lipids have been successfully removed. The samples were dried for 48 h or until the solvent completely evaporated to remove the remaining solvent. The retention of urea and trimethylamine N-oxide (TMAO) in the tissues of elasmobranchs allows them to sustain osmotic balance and may influence the stable isotope values of δ¹⁵N and δ¹³C, leading to misleading interpretation of the data. We conducted urea extraction in shark tissues to study the effect of lipid extraction avoiding any biases produced by urea and TMAO, making our results comparable among species. All shark samples (including bulk and LE samples) were urea extracted following an adaptation of Kim and Koch protocol. Each sample was rinsed with 5 mL of deionized water, allowing a reaction time of 10 min, and vortexed for 1 min. The samples were centrifuged, and the supernatant was discarded. This procedure was repeated thrice consecutively. The samples were oven-dried for 24 h at 60°C or until the sample was dried (usually not more than 48 h). Finally, the dried samples were weighed as 2–2.5 mg pellets and placed into tin containers and sent for analysis.

Stable isotopic values of δ¹³C, δ¹⁵N, and δ³⁴S of pinniped samples were determined using a continuous flow-isotope ratio mass spectrometer Hydra 20-22 (Sercon Ltd., Cheshire, UK) coupled with a Europa EA-GSL Elemental analyzer (Europa Scientific Inc., Cincinnati, OH, USA) at Griffith University Stable Isotope Laboratory, Queensland, Australia. Stable isotopic ratios were measured in part per mille (%) deviation from international standards (for ¹⁵N: IAEA-N1 and IAEA-N2, for ¹³C: IAEA-CH-6, and for ³⁴S: IAEA-S1, IAEA-S2, and IAEA-S3). The standard deviation (SD) for measurements of known standards (bovine liver, Glycine NBS127, Glycine LSU 1 Delta, Hi Max, and Low Mix) was δ¹⁵N = 0.0‰±0.1‰, δ¹³C = 0.0‰±0.1‰, and δ³⁴S = 0.1‰±0.3‰. Shark samples were analyzed at the Stable Isotope Laboratory at the University of Hong Kong, Hong Kong, using a continuous flow-isotope ratio mass spectrometer EA IsoLink IRMS System (Thermo Fisher Scientific Inc., Waltham, MA, USA). The following international standards were used for data normalization: USGS-40 and USGS-41a for ¹⁵N and ¹²C and IAEA-S-1 and IAEA-S-2 for ³⁴S. Analytical accuracy was evaluated using the reference material USGS-42 (δ¹⁵N = 0.1‰±0.3‰, δ¹³C = 0.0‰±0.1‰, and δ³⁴S = 0.1‰±0.7‰).

2.3 | Statistical analyses

Paired t-tests were used to test for differences between the bulk and lipiddextracted samples. The significance level was set at 0.05. The difference between the paired observations was checked for normal distribution before analysis using the Shapiro–Wilk normality test. We used the SD within non-lipid and lipid extracted samples to assess changes in the dispersion of the data sets. The carbon-to-nitrogen (C:N) ratio of bulk tissue was used as a proxy for the lipid content of the tissue. The general trend is that higher C:N values indicate higher lipid content, with some possible exceptions (see references 26 and 44). To assess whether the C:N ratio (as a proxy of lipid content) accounts for the differences between δ¹³C and δ³⁴S before and after lipid extraction, we studied these relationships visually using an adjusted model whenever possible. The final models were inspected for a normal residual distribution. All statistical analyses were performed in R software version 4.1.0 (R Core Team, 2021).

3 | RESULTS AND DISCUSSION

Lipid extraction in shark tissues led to significant differences in δ³⁴S, δ¹³C, and δ¹⁵N values. In contrast, pinniped skin samples showed significant differences only in δ¹³C values. Shark muscle and liver were the only tissues that showed significant decreases in δ³⁴S values due to lipid removal. As predicted, tissues with higher lipid content showed the greatest differences in δ³⁴S and δ¹³C values due to lipid extraction.

The mean and SD of isotopic ratios (δ³⁴S, δ¹³C, and δ¹⁵N); C:N ratios; and carbon (%C), nitrogen (%N), and sulfur content (%S) for each species, tissue, and treatment are presented in Table 1. Boxplots for each compound before and after lipid extraction are shown in Figures S1–S6 (supporting information).

Lipids were successfully removed after lipid extraction, even in shark liver samples with high lipid content. The C:N ratios for liver from gray nurse sharks decreased from 13.6 ± 2.2 to 3.6 ± 0.2 after lipid extraction and from 17.8 ± 3.7 to 3.8 ± 0.2 in sevengill sharks. In contrast, shark muscle samples with low lipid content (usually <1%) (e.g., references 45–48) had low variation in C:N ratios after lipid removal, from 2.8 ± 0.1 to 3.2 ± 0.0 in gray nurse sharks and from 2.7 ± 0.1 to 3.1 ± 0.0 in sevengill sharks. Skin from pinnipeds also had low variation in C:N ratios after lipid extraction (from 3.2 ± 0.3 to 3.0 ± 0.2 in southern sea lions and from 3.8 ± 0.8 to 3.2 ± 0.1 in South American fur seals), probably due to low lipid content in their skin.
TABLE 1  Mean values and standard deviation (SD) of $\delta^{334}S$, $\delta^{15}N$, and $\delta^{13}C$ ($‰$); sulfur, carbon, and nitrogen content (%S, %C, and %N); and C:N ratios for bulk (non-lipid extracted) skin samples from *Otaria byronia* and *Arctocephalus australis* and for muscle and liver samples from *Notorynchus cepedianus* and *Carcharias taurus*

| Species         | Tissue | n  | Parameter | Mean ± SD | $\Delta$(LE - bulk) | Paired t-test | t-Value |
|-----------------|--------|----|-----------|-----------|----------------------|---------------|----------|
| *O. byronia*    | Skin   | 16 | C:N       | 3.2 ± 0.3 | −0.3 ± 0.2           | <0.05         | −4.0     |
|                 |        |    | $\delta^{34}S$ | 15.8 ± 0.8 | 0.2 ± 0.7            | 0.34           | 1.0      |
|                 |        |    | %S        | 0.4 ± 0.1 | 0.0 ± 0.1            | 0.86           | −0.2     |
|                 |        |    | $\delta^{13}C$ | −14.6 ± 0.7 | 0.6 ± 0.4          | <0.05         | 6.4      |
|                 |        |    | %C        | 46.6 ± 2.0 | −2.3 ± 2.0           | <0.05         | −4.7     |
|                 |        |    | $\delta^{15}N$ | 22.1 ± 0.8 | −0.1 ± 0.7          | 0.70           | −0.4     |
|                 |        |    | %N        | 14.4 ± 0.6 | 0.4 ± 0.6            | <0.05         | 2.8      |
| *A. australis*  | Skin   | 15 | C:N       | 3.8 ± 0.8 | −0.6 ± 0.8           | <0.05         | −3.3     |
|                 |        |    | $\delta^{34}S$ | 16.8 ± 0.6 | 0.2 ± 0.9            | 0.37           | 0.9      |
|                 |        |    | %S        | 0.5 ± 0.1 | 0.0 ± 0.1            | 0.36           | 1.0      |
|                 |        |    | $\delta^{13}C$ | −16.1 ± 1.2 | 1.1 ± 1.2          | <0.05         | 3.7      |
|                 |        |    | %C        | 49.4 ± 3.1 | −4.9 ± 3.4           | <0.05         | −5.6     |
|                 |        |    | $\delta^{15}N$ | 20.3 ± 1.1 | −0.4 ± 1.2          | 0.20           | −1.3     |
|                 |        |    | %N        | 13.3 ± 1.5 | 0.8 ± 1.5            | <0.05         | 2.2      |
| *N. cepedianus* | Muscle | 15 | C:N       | 2.7 ± 0.8 | −0.4 ± 0.8           | <0.05         | 22.6     |
|                 |        |    | $\delta^{34}S$ | 18.5 ± 0.3 | −0.8 ± 0.3           | <0.05         | −9.9     |
|                 |        |    | %S        | 0.8 ± 0.0 | 0.2 ± 0.1            | <0.05         | 14.1     |
|                 |        |    | $\delta^{13}C$ | −15.6 ± 0.3 | 0.5 ± 0.2          | <0.05         | 11.3     |
|                 |        |    | %C        | 45.0 ± 1.8 | 4.7 ± 2.5            | <0.05         | 7.4      |
|                 |        |    | $\delta^{15}N$ | 19.7 ± 0.7 | 1.4 ± 0.6          | <0.05         | 8.7      |
|                 |        |    | %N        | 16.6 ± 0.5 | −0.8 ± 0.6           | <0.05         | −4.9     |
| *N. cepedianus* | Liver  | 15 | C:N       | 17.8 ± 3.7 | −14.0 ± 3.6         | <0.05         | −15.2    |
|                 |        |    | $\delta^{34}S$ | 23.1 ± 0.6 | −5.2 ± 0.6           | <0.05         | −35.2    |
|                 |        |    | %S        | 0.3 ± 0.1 | 0.8 ± 0.1            | <0.05         | 50.2     |
|                 |        |    | $\delta^{13}C$ | −22.3 ± 0.6 | 7.3 ± 0.5          | <0.05         | 55.8     |
|                 |        |    | %C        | 71.9 ± 3.0 | −22.7 ± 3.8         | <0.05         | −23.1    |
|                 |        |    | $\delta^{15}N$ | 18.5 ± 0.5 | 1.4 ± 0.3          | <0.05         | 18.0     |
|                 |        |    | %N        | 4.2 ± 0.7 | 9.0 ± 0.6            | <0.05         | 58.8     |
| *C. taurus*     | Muscle | 15 | C:N       | 2.8 ± 0.1 | 0.4 ± 0.1            | <0.05         | 24.8     |
|                 |        |    | $\delta^{34}S$ | 19.0 ± 0.3 | −0.7 ± 0.3           | <0.05         | −10.6    |
|                 |        |    | %S        | 1.0 ± 0.1 | 0.0 ± 0.0            | 0.90           | −0.1     |
|                 |        |    | $\delta^{13}C$ | −15.0 ± 0.2 | 0.2 ± 0.1          | <0.05         | 7.9      |
|                 |        |    | %C        | 45.5 ± 1.0 | 6.4 ± 1.1            | <0.05         | 23.4     |
|                 |        |    | $\delta^{15}N$ | 18.9 ± 0.6 | 0.5 ± 0.1          | <0.05         | 11.8     |
|                 |        |    | %N        | 16.4 ± 0.4 | 0.0 ± 0.4            | 0.98           | 0.0      |
| *C. taurus*     | Liver  | 15 | C:N       | 13.6 ± 2.2 | −10.0 ± 2.2         | <0.05         | −17.6    |
|                 |        |    | $\delta^{34}S$ | 22.1 ± 0.7 | −4.0 ± 0.7           | <0.05         | −22.9    |
|                 |        |    | %S        | 0.4 ± 0.0 | 0.7 ± 0.1            | <0.05         | 27.2     |
|                 |        |    | $\delta^{13}C$ | −21.0 ± 0.5 | 5.9 ± 0.4          | <0.05         | 52.9     |
|                 |        |    | %C        | 68.0 ± 3.9 | −18.7 ± 6.1         | <0.05         | −11.9    |
|                 |        |    | $\delta^{15}N$ | 16.8 ± 0.9 | 0.9 ± 0.3          | <0.05         | 13.1     |
|                 |        |    | %N        | 5.1 ± 0.6 | 8.5 ± 1.3            | <0.05         | 25.9     |

Note: Differences between stable isotope values of lipid extracted (LE) and bulk samples are presented as $\Delta$(LE - bulk) ($‰$). The results of the paired t-test ($p$-value and t-value) comparing the LE and bulk samples of the stable isotope ratio values ($\delta^{34}S$, $\delta^{15}N$, and $\delta^{13}C$) and isotopic content are presented for all tissue types and species. Significance level is based on $\alpha = 0.05$. n, sample size.
3.1 | Sulfur

Muscle and liver from shark species showed significant decreases in $\delta^{34}$S values after lipid extraction ($p < 0.05$, Table 1; Figure 1A). Liver samples of gray nurse shark and sevengill shark had the greatest decrease in $\delta^{34}$S values ($-4.0 \pm 0.7\%$ and $-5.2 \pm 0.6\%$, respectively), differences that could have an impact on the ecological interpretation of these types of data. For example, $\delta^{34}$S isotopic gradients in marine ecosystems typically range from $\sim 20\%$ (pelagic) to $\sim 1\%$ (benthic).\textsuperscript{2,14,49,50} Distinct trophic groups in marine fishes can be differentiated by mean $\delta^{34}$S values between 2% and 3%,\textsuperscript{51} a difference smaller than those in $\delta^{34}$S values resulting from lipid extraction. Therefore, care must be taken when comparing data from shark liver samples using different chemical treatments, especially when the analysis of the liver, given its relatively fast turnover rate,\textsuperscript{52} is critical to ecological studies of sharks revealing novel insights into their trophic ecology (e.g., references 16, 52, and 53). Although shark muscle samples also showed a significant decrease in $\delta^{34}$S values, these differences were less than 1% in both gray nurse sharks ($-0.7 \pm 0.3\%$) and sevengill sharks ($-0.8 \pm 0.3\%$), approximating the analytical accuracy of the instruments. Lipid extraction caused a significant but small increase in relative sulfur content in gray nurse shark liver ($0.7 \pm 0.1\%$) and muscle and liver samples of sevengill shark ($0.2 \pm 0.1\%$ and $0.8 \pm 0.1\%$, respectively). Previous works on tissue with high lipid content (egg yolk) of four species of seabirds\textsuperscript{37,38} showed significant effects on $\delta^{34}$S values after lipid removal, with

**FIGURE 1** The effects of lipid extraction on isotopic ratios of sulfur ($\delta^{34}$S, A), carbon ($\delta^{13}$C, C), and nitrogen ($\delta^{15}$N, E) and their respective sulfur (%S, B), carbon (%C, D), and nitrogen content (%N, F) in skin samples from pinnipeds (Otaria byronia and Arctocephalus australis) and muscle and liver samples from sharks (Carcharias taurus and Notorynchus cepedianus). Positive values denote a higher concentration of the isotopic compound or higher values of isotopic ratios due to lipid extraction. Asterisks (*) indicate significant paired Student’s t-test ($p < 0.05$). Whiskers represent the standard deviation (SD) of the mean for each parameter. LE: lipid extracted, Ob: Otaria byronia, Aa: Arctocephalus australis, Nc: Notorynchus cepedianus, Ct: Carcharias taurus [Color figure can be viewed at wileyonlinelibrary.com]
variations in magnitude between species, ranging from $-0.1 \pm 0.9\%$ to $2.3 \pm 1.1\%$. Oppel et al.\(^{28}\) suggested that the altered $\delta^{34}S$ values could result from an incidental loss of sulfur-bearing amino acids in proteins associated with polar structural lipids or sulfolipids. A decrease in $\delta^{34}S$ values after lipid extraction occurred in conjunction with an increase in sulfur content in both shark tissue types (Table 1; Figures 1A and 1B). The increase in sulfur percentage suggests that lipids found in liver tissue are sulfur poor, and by extracting lipids (33%–57\%\(^{30}\) and 48%\(^{31}\) in gray nurse sharks and sevengill sharks, respectively), the relative proportion of sulfur increases in the extracted replicate. In contrast, pinniped skin samples showed no significant differences in $\delta^{34}S$ and sulfur content values between the bulk and LE samples (Table 1; Figures 1A and 1B). Lipid extraction did not cause significant changes in skin $\delta^{34}S$ values in either pinniped species (southern sea lion: $\rho = 0.34$, South American fur seal: $\rho = 0.37$). Variation in sulfur content after lipid extraction was negligible for both pinniped species (Table 1; Figure 1B).

The decrease in sulfur isotopic values in muscle and liver could be due to the removal of $^{34}S$-enriched sulfur-containing lipids (sulfolipids) during lipid extraction. Sulfolipids have been reported in the liver and muscle of different terrestrial mammals (dogs, rabbits, and humans).\(^{54}\) Differences in the magnitude of the decrease between tissues could be explained by differences in the mean bulk C:N ratio (a proxy of lipid content). Sharks are characterized by high lipid content in the liver,\(^{30}\) which stores energy and provides buoyancy control, compared to muscle tissue in which lipid levels can be relatively low.\(^{28,55}\) Therefore, we expected that higher C:N values would be related to higher differences in $\delta^{34}S$ due to lipid extraction. However, we found the relationship between bulk C:N ratio and $\Delta \delta^{34}S = (\delta^{34}S_{LE} - \delta^{34}S_{ Bulk})$ was unclear, suggesting that only lipid content does not explain the magnitude of decrease in $\delta^{34}S$ after lipid extraction (Figure 2B). This may be explained by the very small proportion of sulfolipids compared to total lipids.\(^{56,57}\) Moreover, our results suggest that lipid extraction will affect $\delta^{34}S$ values differentially depending on the tissue and the species analyzed. These results support previous studies conducted on seabird egg tissue.\(^{57}\)

The decrease in $\delta^{34}S$ values after lipid extraction may also be associated with the selective removal of another S-containing compound. Glutathione (GSH, C\(_{10}\)H\(_{17}\)N\(_{3}\)O\(_{6}\)S) is an antioxidant derived from the free amino acid cysteine, one of the few sulfur-bearing amino acids.\(^{58}\) This molecule is synthesized mainly in the liver, where it is found in higher concentrations than in the rest of the body.\(^{59}\) Results from several terrestrial mammal studies showed that GSH is present at high levels in the liver, whereas muscle has lower reserves.\(^{60}\) GSH can be obtained directly through diet, although its origin is mainly endogenous and its main precursor, cysteine, is derived from the breakdown of dietary protein.\(^{59,61}\) However, we found no information on its factionation relative to dietary sulfur when it is synthesized in the body. Finally, GSH is extracted effectively with methanol,\(^{62}\) the same solvent discarded during our lipid extraction protocol. If the distribution of GSH is the same in shark muscle and liver as in terrestrial mammals, this may explain the differential decrease in sulfur between the two tissues. The removal of GSH by lipid extraction further explains the increase in %S as $\delta^{34}S$ decreases. Because of the elemental composition of GSH, the removal of GSH from the sample leads to an increase in %S relative to the total sample. This is because it loses 10 times more carbon and 3 times more nitrogen than sulfur. In turn, this means that the decrease in %C of lipid extracted shark liver samples using chloroform–methanol protocols is caused not only by lipid removal but probably also by GSH removal. In addition, $\delta^{34}S$ values in the non-lipid extracted liver of gray nurse sharks ($22.1 \pm 0.6\%$) and sevengill sharks ($23.1 \pm 0.7\%$) exceeded the typical values of a fully pelagic consumer ($\sim 20\%\). Given that these species do not feed exclusively on pelagic prey, their values should be in accordance with benthic and demersal diets.\(^{53,64}\) Therefore, this suggests that $\delta^{34}S$ values from lipid extracted liver samples may not exclusively reflect the diet of the consumer. Unfortunately, we could not obtain information to confirm

![Figure 2](https://example.com/figure2.png)  
**Figure 2** Relationship between C:N ratios from non-lipid extracted samples and the observed change in (A), $\delta^{34}S$ ($\delta^{34}S_{\text{lipid-precursor}} - \delta^{34}S_{\text{non-lipid extracted}}$) and (B), $\delta^{13}C$ ($\delta^{13}C_{\text{lipid-precursor}} - \delta^{13}C_{\text{non-lipid extracted}}$), due to lipid extraction in each tissue type. Liver and muscle samples from sharks and skin samples from pinnipeds are represented. Solid lines represent the corresponding adjusted linear and logarithmic models that were significant, with shaded areas representing the 95% confidence intervals. LE: Lipid extracted, Ct (●): *Charcharias taurus*, Ct (♦): *Notorynchus cepedianus*, Ac (■): *Arctocephalus australis*, Ob (●): *Otaria byronia* [Color figure can be viewed at wileyonlinelibrary.com]
that GSH is enriched in $^{34}$S. Future work will be necessary to confirm our hypothesis. Studies that provide information on the biochemical composition of the extracts obtained by lipid extraction will allow us to elucidate which compounds are removed through lipid extraction.

3.2 | Carbon and C:N ratios

Lipid extraction led to increases in $^{13}$C values, and carbon content, for all species and tissue types ($p < 0.05$) (Table 1; Figures 1C and 1D). The smaller increases were in pinniped skin samples (0.6%–1.1% mean values) and shark muscle samples (0.2%–0.5%o) (Table 1; Figure 1C). Previously reported differences between lipid extracted and non-lipid extracted replicates in muscle $^{13}$C values in sevengill sharks (0.7 ± 0.6%) align with our results (0.5 ± 0.2%). The same is true for values previously reported for the gray nurse shark (−0.4%, cf. 0.2 ± 0.1%o—this study). Shark liver samples showed the highest increase in $^{13}$C values (5.9%–7.3%) (Table 1; Figure 1C). This is consistent with reports of extracts from the livers of marine mammals (e.g., references 66–68) and sharks (e.g., reference 33). Lipid extraction resulted in a significant decrease ($t$-test, $p < 0.05$) in the amount of carbon detected in skin and liver samples, with the lowest reduction shown in pinniped skin samples (−2.3% to −4.9%) and the highest in shark liver samples (−18.7% to −22.7%) (Table 1; Figure 1D). However, shark muscle samples showed the opposite trend, and lipid extracted samples increased their amount of carbon ($t$-test, $p < 0.05$) between 4.7% and 6.4% compared to their non-lipid extracted replicates (Table 1; Figure 1D). C:N ratios from bulk tissue showed a nonlinear relationship with the observed change in $^{13}$C due to lipid extraction (Figure 2A), in accordance with previous work (e.g., references 26, 34, and 69). After lipid extraction, all tissue types showed significant differences in C:N values (paired $t$-test, $p < 0.05$). Differences between C:N values of lipid extracted and non-lipid extracted replicates of shark liver samples (tissue known for its high lipid content) decreased between −10.0 ± 2.2% (gray nurse sharks) and −14.0 ± 3.6% (sevengill sharks) (Table 1). For shark muscle samples (tissue known for its low lipid content), a 0.4 ± 0.1%o difference in C:N values occurred, which is consistent with a mean difference of 0.5 ± 0.1%o previously found in muscle samples of pelagic shark species.33 The C:N ratios of non-lipid extracted muscle samples for both species of shark was ~2.7 ± 0.1%, also consistent with previous studies of sharks, that is, 3.1 ± 0.3% in bull sharks, *Carcharhinus leucas*, and <3.0% in sandbar sharks, *Carcharhinus plumbeus*.70

3.3 | Nitrogen

$^{15}$N values from muscle and liver samples of both species of shark significantly increased after lipid and urea extraction (paired $t$-test, $p < 0.05$) (Table 1; Figure 1E). However, the magnitude of the increase was relatively small, from 0.5 ± 0.1%o in muscle samples of gray nurse sharks to 1.4 ± 0.6%o in muscle samples of sevengill sharks. Significant effects of lipid extraction in $^{15}$N values of muscle samples from sevengill sharks have been reported previously and provided a comparable difference between lipid extracted and non-lipid extracted replicates of 1.51 ± 0.61%o. Hussey et al. reported a smaller increase in $^{15}$N values (−0.2%) after lipid extraction in muscle samples from gray nurse sharks. Significant increases in $^{15}$N values due to lipid extraction in muscle and liver have been found in other elasmobranch species70 and several marine and freshwater bony fishes.30,32,71,72 However, it has been reported that lipid extraction does not change $^{15}$N values in several species of coastal elasmobranchs.73,74 In contrast to shark tissues, we found no significant effect of lipid extraction on $^{15}$N values on skin samples in either species of pinniped (southern sea lion: $p = 0.70$, South American fur seal: $p = 0.20$) (Table 1). This is consistent with reports of no changes produced on $^{15}$N by lipid extraction in skin samples from other groups of marine mammals (i.e., cetaceans,34,69 odobenids,47 and Sirenia34). We found strong evidence that lipid extraction increased $^{15}$N values for our sharks but not our pinniped samples, supporting growing evidence that such effects can vary between species and tissue types.26,29,34,75 Our data showed that we could perform stable isotope analysis of $^{15}$N and $^{13}$C on skin samples of South American fur seals and southern sea lions, without the need to separately analyze the elements to avoid biases in $^{15}$N due to lipid extraction.

Lipid extracted skin samples from pinnipeds were slightly higher in nitrogen but by less than 1% for both species compared to non-lipid extracted samples (Table 1; Figure 1F). An increase in %N was found in the LE liver samples, which showed around 9% more nitrogen than the non-lipid extracted replicate. Urea and TMAO extraction led to an increase in %N, as reported in some shark species.76 As urea and TMAO are synthesized in shark liver,77 its presence could lead to a greater increase in %N in this tissue. Variation in nitrogen content between muscle replicates of gray nurse shark was negligible and not significantly different ($p = 0.98$). Muscle samples from sevengill sharks were the only tissue that showed a significant reduction in its nitrogen content (~1%, paired $t$-test: $p < 0.05$) along with an increase in $^{15}$N values after lipid extraction (Table 1; Figure 1F).

There are three possible explanations for the increase in $^{15}$N values after lipid and urea extraction from liver samples. The effect of urea and TMAO extraction is more obvious, as both compounds are depleted in $^{15}$N, their removal can lead to an increase in $^{15}$N values. However, it has been reported that lipid extraction can differ in isotopic composition (e.g., references 79 and 80). It may be that the amino acid protein extracted in association with polar lipids is isotopically lighter than specific tissue proteins, thus increasing $^{15}$N values. Alternatively, the consistent increase in $^{15}$N values could be the
result of removing nitrogenous wastes resulting from cellular respiration (ammonia [NH₃] and ammonium [NH₄⁺]) soluble in lipids and organic solvents being removed in lipid extraction.⁸¹ We suggest that neither of these processes on their own explains the increase in δ¹⁵N values, but rather either a combination of these or an alternative hypothesis. The different patterns of δ¹⁵N and %N values found after lipid and urea extraction support the hypothesis that structural and physiological differences between tissues produce these divergent patterns. The consistency of the effects of lipid extraction in δ¹⁵N values in muscle of gray nurse shark and sevengill shark between studies supports this hypothesis. Our results highlight the need for conducting new experimental studies to understand these biochemical processes and how they differ between tissue types.

4 | CONCLUSIONS AND RECOMMENDATIONS

Our work is the first study to assess the effect of lipid extraction on sulfur isotopic ratios of sharks and pinnipeds. Both species of pinnipeds showed a negligible increase in skin δ³⁴S after lipid extraction. Sharks similarly showed negligible changes in δ³⁴S values from muscle tissues. However, shark liver samples have high levels of enrichment of δ³⁴S resulting from lipid and urea extraction treatments. We provide a general correction factor of −4.6 ± 0.9 to adjust for δ³⁴S values of shark liver samples that undergo lipid and urea extraction. Until the origin of variations in δ³⁴S values from shark liver samples can be determined (i.e., removal of sulfolipids and/or glutathione), we suggest caution when interpreting results. The effects of lipid extraction treatments on δ³⁴S values vary between tissue type and species of marine predators, similar to δ¹⁵N. Consequently, in future research, if no information is available on how δ³⁴S values react to lipid and urea extractions for the tissues of interest, we recommend a priori evaluation of the composition of the sample to detect and therefore account for possible effects of lipid and urea extractions. Our findings also demonstrated that stable isotope analysis of δ¹⁵N and δ¹³C on skin samples from southern sea lions and South American fur seals could be performed without the requirement to separately analyze elements to minimize lipid extraction-induced biases in δ¹⁵N.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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