Journal of Experimental Marine Biology and Ecology 474 (2016) 54–57

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Lipid extraction techniques for stable isotope analysis of bird eggs: Chloroform–methanol leads to more enriched ^{13}C values than extraction via petroleum ether

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ARTICLE INFO

Article history:
Received 23 June 2015
Received in revised form 25 September 2015
Accepted 26 September 2015
Available online 8 October 2015

Keywords:
Ancient murrelet
Leach's storm-petrel
Lipid extraction
Pelagic cormorant
Rhinoceros auklet
Stable isotope analysis

ABSTRACT

Stable isotope analysis is often used to determine dietary origin in ecological studies. Lipids are depleted in ^{13}C compared with protein and, so, variation in lipid content can confound interpretations of diet. To avoid this issue, lipids can be extracted from samples prior to stable isotope analysis. The most common solvent used for lipid extraction, chloroform–methanol, is toxic and cannot be used in some laboratories. Here, the use of chloroform–methanol as a solvent was compared with an alternative method that uses petroleum ether as a solvent ($N = 32$ eggs from seven species). The $\delta^{13}C$ values using both methods were highly correlated ($R^2 = 0.99$) but values derived from samples that were lipid-extracted using chloroform–methanol were enriched in ^{13}C by $0.90 \pm 0.07‰$ compared with samples that were lipid-extracted using petroleum ether. The C:N ratio was 3.60 ± 0.02 (s.e.) for chloroform–methanol and 4.25 ± 0.03 for petroleum ether implying that chloroform–methanol (which is more polar) more completely extracted lipids than petroleum ether. Furthermore, $\delta^{15}N$ values derived from samples that were lipid-extracted using chloroform–methanol were enriched in ^{15}N by $0.41 \pm 0.05‰$ compared with samples that were lipid-extracted using petroleum ether. In conclusion, lipid extraction by chloroform–methanol more thoroughly removes lipids than lipid extraction by petroleum ether and consequently $\delta^{13}C$ is higher after lipid extraction by chloroform–methanol than after lipid extraction by petroleum ether. An algebraic correction formula for each method is provided.

1. Introduction

Stable isotopes can be used as tracers for the dietary origin of tissue in wild animals (Rubenstein and Hobson, 2004; Inger and Bearhop, 2008). In particular, stable isotope ratios are often used to disentangle variation in contaminant levels associated with diet from variation associated with environmental factors (Elliott and Elliott, 2013; Braune et al., 2014, 2015; Miller et al., 2014, 2015). However, lipids are depleted in ^{13}C compared with protein and variation in lipid content can confound interpretation of diet (De Niro and Epstein, 1977; Post et al., 2007; Ricca et al., 2007; Oppel et al., 2010). It is therefore usually necessary to chemically extract lipids from samples used to measure stable isotope ratios, or to algebraically account for such effects (Sotiropoulos et al., 2004; Sweeting et al., 2004, 2006; Post et al., 2007; Bodin et al., 2007; Kojadinovic et al., 2008; Mintenbeck et al., 2008; Kaufman et al., 2014).

Several methods have been used to chemically extract lipids from tissues. The most common method uses chloroform–methanol as a solvent (Folch et al., 1957; Bligh and Dyer, 1959). However, because chloroform is toxic, those methods cannot be used in some laboratories, and petroleum ether, hexane and ethyl acetate/alcohol are used as alternative solvents (Chambellant et al., 2008; Elliott et al., 2014). As both chloroform–methanol and ethyl acetate are more polar than petroleum ether or hexane, those compounds extract a greater proportion of polar compounds, including proteins, than petroleum ether or hexane (Dobush et al., 1985). As a result, stable isotope values on tissue extracted with polar solvents tend to be more enriched in ^{13}C and ^{15}N than tissue extracted with non-polar solvents (Logan and Lutcavage, 2008). However, some studies have found no difference between techniques (Schlechtriem et al., 2003; Chambellant et al., 2008). To be able to compare among studies, it is important to understand whether different chemical extraction methods affect stable isotope values. To the best of our knowledge, no study has compared different lipid extraction techniques on lipid-rich tissue, such as avian egg tissue, which would be expected to be particularly susceptible to such bias.

An alternative to chemical extraction is algebraic correction whereby the effect of lipids is accounted for algebraically based on the ratio of carbon to nitrogen in the sample (Post et al., 2007; Logan et al., 2008; Lesage et al., 2010). Although such corrections are up to five times less precise (Ehrich et al., 2010; Tarroux et al., 2010; Yurkowski et al., 2015), algebraic corrections are common in the literature because

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http://dx.doi.org/10.1016/j.jembe.2015.09.017
0022-0981/

of the time, cost and logistics required for chemical lipid extraction, and because chemical extraction alters nitrogen and sulfur stable isotope ratios (reviewed for avian egg tissue in Elliott et al., 2014) necessitating two sets of analyses. Furthermore, for analyses of lipophilic contaminants, that are principally stored within lipids, the dietary origin of lipids may be more important than the dietary origin of proteins. Algebraic correction methods may be more useful for such datasets (Elliott et al., 2014). Regardless, if different extraction methods influence stable isotope ratios, then different algebraic correction formulae will be needed for each method and the comparability of the different equations needs to be considered (Logan and Lutcavage, 2008).

The current manuscript compares the carbon and nitrogen stable isotope values derived from two methods of chemical extraction: chloroform–methanol and petroleum ether. Values in lipid-rich eggs were measured from the suite of aquatic birds used as indicator species for monitoring of environmental contamination by Environment Canada on the Pacific coast of Canada (Elliott et al., 1989; Elliott et al., 1992). As chloroform–methanol is more polar than petroleum ether, it was hypothesized that chloroform–methanol would extract more lipids while at the same time removing a higher proportion of proteins, such as membrane-bound proteins and proteins with a high proportion of hydrophobic amino acids. As such, it was predicted that chloroform–methanol would lead to enriched ^{13}C and ^{15}N and lower C:N ratio relative to petroleum ether.

2. Methods

Egg homogenates were obtained from the Environment Canada's Specimen Bank for seven species of aquatic birds: osprey (*Pandion haliaetus*, N = 1), double-crested cormorant (*Phalacrocorax auritus*, N = 5), Leach's storm-petrel (*Oceanodroma leucorhoa*, N = 9), rhinoceros auklet (*Cerorhinca monocerata*, N = 8), pelagic cormorant (*Phalacrocorax pelagicus*, N = 4), great blue heron (*Ardea herodias*, N = 3) and ancient murrelet (*Synthliboramphus antiquus*, N = 2). The suite of species included species that foraged over a range of habitats, from off the continental shelf (storm-petrels) to freshwater lakes (ospreys). As part of Environment Canada's contaminant monitoring program, many samples were lipid-extracted via petroleum ether in the past (petroleum ether was the primary option available at the Pacific Wildlife Research Centre, Ottawa, Canada, due to health and safety concerns with chloroform) while more recent samples were processed at the National Wildlife Research Centre using chloroform-methanol.

In each case, eggs were collected in the field, homogenized in the lab, frozen, thawed for the current analyses, freeze-dried, lipid-extracted, dried again and encapsulated for stable isotope analyses. One sub-sample was left non-lipid-extracted, one sub-sample was lipid-extracted using a Soxhlet apparatus with petroleum ether as the solvent, and one sub-sample was lipid-extracted using 2:1 chloroform:methanol. For the Soxhlet extractor, a thimble filled with dried sample was placed in the extractor and washed with petroleum ether at 94°C for 8 h. For the chloroform–methanol, approximately 2 mL of 2:1 chloroform:methanol was added to the samples and removed until the supernatant was clear, indicating that lipids had been extracted (Bligh and Dyer, 1959). The solvent was then distilled off and the residue dried for 60 min in a drying oven. Stable isotope analysis on encapsulated samples occurred at the Hatch laboratory (University of Ottawa) via combustion on a Cube elemental analyzer (Elementar, Hanau, Germany) followed by "trap and purge" separation and on-line analysis by continuous-flow with a DeltaPlus Advantage isotope ratio mass spectrometer coupled with a ConFlo III (both from Thermo Fisher Scientific, Waltham, USA). Internal standards covering the natural range of carbon and nitrogen isotope ratios were ($\delta^{15}N$, $\delta^{13}C$): C-51 Nicotiamide (0.07‰, −22.95‰), C-52 mix of ammonium sulfate + sucrose (16.58‰, −11.94‰), C-54 caffeine (−16.61‰, −34.46‰) and blind standard C-55: glutamic acid (−3.98‰, −28.53‰). All $\delta^{15}N$ values are reported relative to air and normalized to internal standards calibrated to international standards IAEA-N1 (+0.4‰), IAEA-N2 (+20.3‰), USGS-40 (−4.52‰) and USGS-41 (47.57‰). All $\delta^{13}C$ values are reported relative to Pee Dee Belemnite and normalized to internal standards calibrated to international standards IAEA-CH-6 (−10.4‰), NBS-22 (−29.91‰), USGS-40 (−26.24‰) and USGS-41 (37.76‰). The blind C-55 standards (N = 3), not used for calibration, had an average value of $\delta^{15}N = -3.97$ (expected value of −3.89) and $\delta^{13}C = -28.57$ (expected value of 28.50). The analytical precision on the standards was 0.05 (s.d.) for $\delta^{13}C$ and 0.10 for $\delta^{15}N$ and on egg duplicates was 0.11 for $\delta^{13}C$ and 0.15 for $\delta^{15}N$. Samples were included in the same set of eggs used by Guigueno et al. (2012) and Elliott et al. (2014), and more details are described therein. All of the stable isotope raw data used in this manuscript are in the Data Appendix, which also provides the location of collection.

Linear regressions (R 3.0.3) were used to correlate lipid-extracted values of $\delta^{13}C$ and $\delta^{15}N$ using petroleum ether as a solvent with lipid-extracted values using chloroform-methanol as a solvent. Pairwise t-tests were used to test for significant differences between the lipid-extraction methods at an α-value of 0.05. All values shown are mean ± SE.

3. Results

Carbon isotope values ($\delta^{13}C$) for petroleum ether-extracted tissue were highly correlated with values for chloroform-methanol extracted tissue (linear regression: $t_{30} = 54.85$, $P < 0.0001$, $R^2 = 0.99$). Nitrogen isotope values ($\delta^{15}N$) petroleum ether-extracted tissue were also highly correlated with values for chloroform-methanol extracted tissue (linear regression: $t_{30} = 29.12$, $P < 0.0001$, $R^2 = 0.97$). However, the $\delta^{13}C$ values for petroleum ether-extracted tissues were significantly lower than $\delta^{13}C$ values for chloroform/methanol-extracted tissues by 0.90 ± 0.07‰ for $\delta^{13}C$ (pairwise $t_{30} = 12.92$, $P < 0.0001$) and 0.41 ± 0.05‰ for $\delta^{15}N$ (pairwise $t_{30} = 8.91$, $P < 0.0001$; Fig. 1). The C:N ratio was higher for petroleum ether-extracted tissue (4.26 ± 0.03) than chloroform–methanol extracted tissue (3.61 ± 0.02; pairwise $t_{30} = 15.60$, $P = 0.04$; Table 1). There was no relationship between C:N ratio after lipid-extraction and the change in $\delta^{13}C$ (petroleum ether: $t_{31} = -0.06$, $P = 0.95$, $R^2 = 0.00$; chloroform–methanol: $t_{31} = -1.46$, $P = 0.15$, $R^2 = 0.09$) implying that lipids were uniformly extracted.

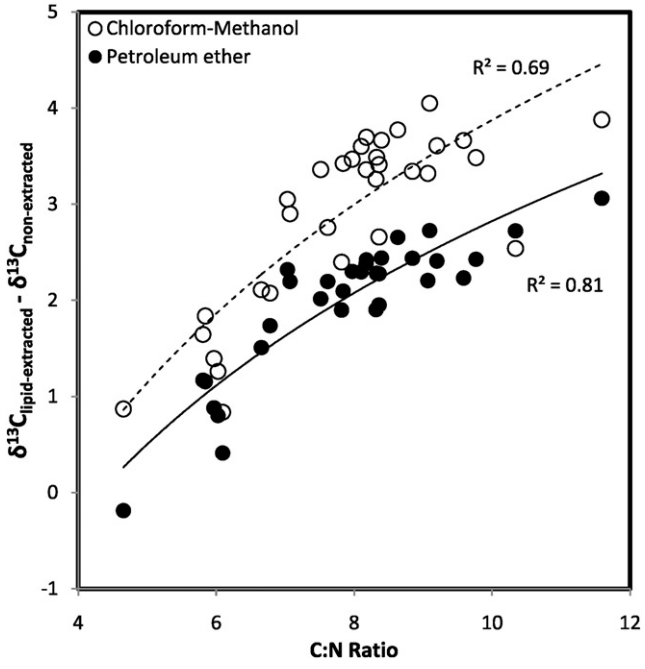

Fig. 1. The effect of lipid extraction method on carbon stable isotope ratios as a function of the C:N ratio. For petroleum ether (solid line): $\delta^{13}C_{lipid\text{-}extracted} = \delta^{13}C_{non\text{-}extracted} + 3.35 * Ln(C:N\ Ratio) - 4.89$. For chloroform-methanol (dashed line): $\delta^{13}C_{lipid\text{-}extracted} = \delta^{13}C_{non\text{-}extracted} + 3.94 * Ln(C:N\ Ratio) - 5.21$.