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Optimizing Methods for Extraction of Organic Compounds from Molluscan Shells

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Optimizing Methods for Extraction of
Organic Compounds from Molluscan Shells

by

Kaydee Jo West

A thesis submitted in partial fulfillment
Of the requirements for the degree of
Master of Science
School of Geosciences
College of Arts and Sciences
University of South Florida

Major Professor: Gregory S. Herbert, Ph.D.
Gregory S. Ellis, Ph.D.
Jonathan Wynn, Ph.D.

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Keywords: conservation paleobiology, demineralization, shell organic matrix, shell demineralization methods

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DEDICATION

My father was tremendously proud that I was working towards a master’s degree, although he never understood exactly what I was studying and asked me frequently why I had decided to study snails. My sister summed it up best when she said he would “try to explain it to anyone and everyone who would listen.” I wish he had lived to see me complete this work, but it is with great pride and affection that I dedicate this thesis to my father. This one’s for you, Dad.

Roger Dale West

(July 19, 1955—April 21, 2016)
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Finally, I thank my mother and sister, two of the strongest women I know for their tremendous support of me, particularly in these last few difficult months.
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ABSTRACT

Mollusk shells contain proteins within and between the crystals of calcium carbonate. These organic molecules play an important role in biomineralization and shell function, and their stable isotope ratios of carbon and nitrogen are also thought to record important ecological information about the animal's diet and nutrient sources. These proteins can be preserved for millions of years, offering potential insight into pre-anthropogenic ecological conditions. However, shell organics in older shells are typically recovered in reduced abundances due to leaching and remaining organics are often converted from insoluble proteins to soluble, free amino acids, making them difficult to detect and recover. Therefore, demineralization and organics-capture methods must be optimized for yield to extract much-needed ecological information from older shells. This project compared insoluble and soluble organic yields of modern gastropod *Strombus alatus* shells demineralized with acids of varying concentrations and temperatures. Results suggested that demineralizing shell fragments with 0.1 M HCl at a ratio of 0.9 L HCl/g of shell was optimal. Average percent organic yields ~0.2% for modern and ~0.06% for fossil *Strombus spp*. Future applications of this work include using this refined method to reconstruct food webs across broad temporal scales.
INTRODUCTION

The biosphere is changing in response to human activities, including rapid loss of species richness (Ceballos et al., 2015), shifts in trophic dynamics (Estes et al., 2011), and depletion of viable habitat in marine environments (Deutsch et al., 2015). However, the extent of change is, in most cases, unknown. Ecology, a relatively young discipline, has yet to establish robust data sets extending beyond decadal scales (Jackson, 1997), and the fact that coastal ecosystems and coral reefs were not “pristine” went largely unrecognized by scientists until the end of the twentieth century (Jackson, 2001). The magnitude of change is further obscured by the relatively short time scale of a human generation compared to the length of time humans have altered the planet. This phenomenon, termed “shifting baseline syndrome,” is the tendency for each new generation of scientists, policy makers, and the public to characterize the environments they first encountered early in their careers or as children as “pristine,” even if those environments were, in fact, already quite deteriorated (Pauly, 1995; Jackson, 1997). Available records suggest that ecosystems have deteriorated significantly over historical time scales (Jackson, 1997; McClenachan, 2009; Blight et al., 2015). However, the extent of damage over longer time scales is even less frequently studied and more poorly understood.
To address this critical gap in knowledge, scientists have begun to expand their use of compound-specific isotope analysis (CSIA; Popp et al., 2007; Larsen et al., 2009), an emerging tool in ecology that can extract information about food webs and nutrient sources over temporal scales ranging from decades (Ruiz-Cooley et al., 2014) to millennia (McMahon et al., 2015). CSIA provides an advantage over bulk stable isotope analysis, especially since when apex predators are used, a snapshot of the food web can be obtained from a single specimen (McClelland & Montoya, 2002). CSIA analyzes isotopic ratios of amino acids (AAs), which record ecological information and are found in soft tissues and skeletal hard parts like bone (Jim et al., 2006), teeth (Martin et al., 2011), and shell (Abelson, 1954). For example, nitrogen isotope ratios of “source” AAs (e.g., phenylalanine, lysine, threonine, glycine, and serine; Popp, et al., 2007) can be used to determine information about nutrient sources at the base of the food web (Ellis, Herbert & Hollander, 2014) since there is virtually no enrichment of these AAs from diet to consumer (Popp, et al., 2007). Conversely, “trophic” AAs (e.g., glutamic acid, aspartic acid, alanine, isoleucine, leucine, proline, and valine) do exhibit significant enrichment from diet to consumer (Popp, et al., 2007). Since trophic enrichment factors (TEFs), or δ^{15}N enrichment between diet and consumer, have been determined for a broad range of taxa (Chikaraishi et al., 2009; Lorrain et al., 2009; Dale et al., 2011; Germain et al., 2013; Hoen et al., 2014; McMahon et al., 2015; Nielsen, Popp & Winder, 2015), δ^{15}N of source and trophic AAs can be used in combination to determine trophic position (McClelland & Montoya, 2002). Thus, whenever proteinaceous tissues or
skeletal parts containing indigenous AAs are available, CSIA can be used to investigate trophic structure (Bowes & Thorp, 2015) and nutrient sources (Larsen, et al., 2009) within the ecosystem and may provide insight into shifts in trophic dynamics, response to climate change, and critical thresholds that previously led to ecosystem collapse and recovery.

Shell organic matrix is a particularly promising biomineralized tissue for CSIA applications to past ecosystems. The organic matrix within the shell regulates shell growth, and during biomineralization, some proteins and their constituent AAs are trapped within and between the crystals (Penkman et al., 2008). Once incorporated into the shell matrix, AAs can remain preserved for millions of years (Abelson, 1954; Gregoire, 1959; Weiner, Lowenstam & Hood, 1976; Nance et al., 2015). Proteins can be isolated from fossils in sufficient quantities for analysis, and methods optimization could extend the utility of CSIA to deeper time scales where fossil material is available.

Although applications of CSIA to shell organics are promising, the challenge remains that, even in modern shells, organics make up at most 5% of total shell weight (Marin & Luquet, 2004) with some shell types consisting of as little as 0.01-1% organics (Hare & Abelson, 1965). In fossils, these abundances are further depleted by diagenetic processes including post-burial heating, bacteria, or contact with water, which break peptide bonds and separate proteins into individual AAs (Mitterer, 1993) that are difficult to isolate due to their small size.
Since shell organics were first characterized over half a century ago (Abelson, 1954), several approaches have been used to extract them from the shell (Table 1). Traditionally, either HCl or a chelating agent, such as ethylenediaminetetraacetic acid (EDTA), is used to remove CaCO$_3$ and isolate shell organics. Chelating agents form multiple bonds with metal ions. In the case of mollusk shells, EDTA should theoretically bind to calcium while leaving the remainder of the shell intact. However, EDTA has been shown to form complexes with organic compounds, potentially altering $\delta^{15}$N and $\delta^{13}$C values, and it is difficult to remove, even with "exhaustive" dialysis (Meenakshi, Hare & Wilbur, 1971; El-Daoushy, Olsson & Oro, 1978; Wheeler et al., 1987). Other variations in techniques include altering reaction speed by changing temperature or acid concentration. Although some reports suggest that organics constitute 1-5% of the shell by weight (Marin & Luquet, 2004; Su, Zhang & Heuer, 2004), percent organics for crossed-lamellar shell microstructure ranges from 0.01-1% (Hare & Abelson, 1965), and the majority of studies report combined (carbohydrates, proteins, lipids, and lipid-derivatives such as pigments) organic yields of ~0.4% or less (Table 1). Therefore, methods optimization is essential if shell organics, specifically proteins, are to be reliably retrieved in quantities sufficient for isotopic analysis.

I empirically investigated the effects of demineralization techniques (i.e., acid concentration and reaction speed) on yield of organics isolated from modern and fossil specimens of the *Strombus alatus* species group, a marine gastropod that is common in the Neogene fossil record of Florida and today. Species belonging to the genus *Strombus*
exhibit a crossed-lamellar shell microstructure and have some of the lowest percent organic content (0.01-1%) of any molluscan shell (Hare & Abelson, 1965), which makes extraction of organics difficult. Thus, optimization of methods for extracting organics from *Strombus* will be useful in adapting the technique more broadly to other molluscan taxa, most of which have a higher percent organic content (Su, Zhang & Heuer, 2004).
MATERIALS

Modern *Strombus alatus* Gmelin, 1791 shells were obtained from live animals collected via capetown dredge from the Gulf of Mexico depths of ~9 m (26°08'59.0" N, 81°59'52.0" W). Fossil *Strombus spp.* shells were collected from the Quality Aggregates quarry (27°22'50.9" N, 82°22'58.9" W) exposures that are part of the Tamiami Formation (Late Pliocene; 3—3.5 MYA) and Williams Pit (26°59'06.5" N, 81°44'55.9" W) units that are part of the Caloosahatchee Formation (Early Pleistocene; 1.8—2.5 MYA).
METHODS

*Strombus alatus* shells (n=20; each ~40 g, dry weight) were soaked in 13% sodium hypochlorite (CAS #: 7681-52-9) for 1 h, sonicated in deionized (DI) water for 30 minutes, and scrubbed with small brushes to remove contaminants. Sonication and scrubbing steps were repeated, and sand paper (coarse 60 grit) was used to remove any remaining periostracum.

Shells were crushed and homogenized using a Shatterbox 8500. Powdered shell was pooled, sieved to <500 microns, and divided into six treatment groups, each consisting of ten ~3 g, dry-weight aliquots. Reactions were run at two temperatures and three acid strengths to test the effects of reaction speed and acid strength on yield. Temperature treatments were ~25 °C (ambient room temperature) and 5 °C, while acid strengths were 0.025 M, 0.05 M (Ellis, *et al.*, 2014), and 0.1 M HCl. The reaction of calcium carbonate with HCl is as follows:

\[ 2 \text{HCl} + \text{CaCO}_3 \rightarrow \text{CaCl}_2 + \text{CO}_2 + \text{H}_2\text{O} \]

Each aliquot of powdered shell was placed in dialysis tubing (ThermoScientific SnakeSkin® dialysis tubing; 3500 molecular weight cut-off) and submerged in an acid bath (1 L of acid/aliquot of shell powder) (Figure 1A—C). Acid baths were replaced every 48 h until no visible carbonate remained (Figure 1D). Following demineralization, the samples were dialyzed against 10 L of DI water for 48 h with water bath changes
every 12 hr. Shell organics were rinsed from the dialysis tubing with DI water and separated via centrifugation following Ellis et al. (2014). The supernatant, which contained soluble organics, was pipetted off and placed in an oven at 100 °C until completely dehydrated. The remaining insoluble materials contained a mixture of brown, organic powder and a small amount of silicate grains that were possibly incorporated into the shell during biomineralization or when using sandpaper during the cleaning procedure (Figure 1E).

Figure 1: Photographs of shell powder (3 g) during demineralization and remaining insoluble organics after demineralization is complete. All dialysis tubing was cut to lengths of ~10 cm prior to use. A) Sample at start of demineralization; B) Sample after 2 days of demineralization; C) Sample after 5 days of demineralization; D) Completely demineralized sample; E) Remaining insoluble organics (scale bar = 5 mm)

Yields of soluble organics were determined by dissolving them with 30 µL of nanopure water, pipetting the liquid containing organics into previously-weighed 5 x 3.5 mm tin capsules, evaporating off the water at 100 °C, allowing the tin capsules to cool to room temperature, and re-weighing them. Weights of soluble organics were determined by subtracting the empty capsule weight from the capsule weight after drying. Precision of the scale, calculated as absolute average deviation for 10 empty tin capsules weighed 10 times each, was ~0.08 mg.
Insoluble organics were dried in an oven at 30 °C for 12 h and weighed. Because insoluble residue included a small amount of silicate grains, insoluble organics were then oxidized with 5 mL 13% sodium hypochlorite. The supernatant was pipetted off, the remaining silicate grains were dried and weighed, and the mass of silicate was subtracted from the total mass of insoluble organics plus silicate to determine the mass of insoluble organics. ANOVA is relatively robust to departures from normality (Khan & Rayner, 2003; Schmider et al., 2010), and normality testing has relatively low power when n<50 (Ruxton, Wilkinson & Neuhäuser, 2015). Therefore, differences in organic yields for each treatment group were evaluated with ANOVA using the “stats” package in the R programming environment (R Core Team, 2015). Tukey’s post-hoc test was run using the “multcomp” package (Torsten Hothorn, 2016).

Following the initial yield experiment, two exploratory experiments using alternative methods from the existing literature were conducted to further optimize yield. In the first experiment, six 2 g aliquots of shell powder from remaining modern samples were prepared as previously described. Half were demineralized using 3.25 M HCl at a ratio of 30 mL of HCl/g of shell as described in Nance et al. (2015). The remaining samples were demineralized using 0.1 M HCl at a ratio of 0.9 L of HCl/g of shell, which is stoichiometrically equivalent to Nance et al.’s methods.

In the second experiment, one modern Strombus shell from the same locality as the rest was cleaned as previously described. The shell was broken into fragments with
a hammer. Six samples (~2 g each) were obtained. The fragments were mixed and either crushed into powder using a mortar and pestle (n=3) or left as fragments (<1 cm/1 cm; n=3). All samples were demineralized with twice the volume of 0.5 M HCl stoichiometrically necessary to completely dissolve CaCO₃.

To determine the percent organic content available in fossils, shell fragments from 5 Strombus shells was used from each of two localities. With the exception that sandpaper was not used since periostracum was not present in fossils, the shells were cleaned using the methods described above. Fragments of shell (<2 cm x <2 cm) were removed starting from the lip until the target weight of 5 g was acquired. Fragments were demineralized at room temperature with 0.1 M HCl at a ratio of 0.9 L/g of shell. Because insoluble residue included visible minerals including iron oxides, insoluble organics were then oxidized with 5 mL 13% sodium hypochlorite. The supernatant was pipetted off, the remaining minerals were dried and weighed, and the mass of the minerals was subtracted from the total mass of insoluble organics plus minerals to determine the mass of insoluble organics. Weights of soluble organics were determined as previously described.
RESULTS

In the modern yield experiments, all samples were recovered from each treatment group with the exception of the 0.1 M HCl at 5 °C treatment, in which 2 samples were lost. Average percent total (soluble + insoluble) organic yields for modern samples ranged from 0.19—0.27% among the treatments groups (Figure 2), with the insoluble portion constituting an average of 93.6—95.6% of the recovered organics. Yields for insoluble organics ranged from 1.8—2.5 mg/g of shell, while yield for soluble organics ranged from 0.07—0.13 mg/g of shell (Figure 3; error on the mass balance was ~0.08 mg).

![Figure 2: Mean percent total organic weights (calculated as a percentage of shell powder weight less quartz) for all treatment groups. Ambient temperature treatments are denoted with circles. Chilled treatments are denoted with squares. Error bars represent standard deviation.](image)
The total organic yields from each of the treatment groups are shown via boxplot (Figure 4). To determine statistical significance of the differences observed, an ANOVA was used, and homogeneity of variance was verified using the non-parametric Fligner-Killeen test ($p=0.08693$). For the modern yield experiments, temperature had a
marginally insignificant effect (F=3.934, df=1, p=0.05250), acid concentration had a highly significant effect (F=7.686, df=1, p=0.00766), and the interaction between temperature and acid concentration was significant (F=6.127, df=1, p=0.01655).

Tukey's post-hoc test showed statistically significant differences when comparing the treatment using 0.025 M HCl at ambient temperature to all other treatments. Differences for 0.05 M HCl at ambient temperature and 0.1 M HCl at 5 °C were significant (p=0.02030 and p=0.01017, respectively) while differences for 0.05 M HCl at 5 °C (p=0.00811), 0.025 M HCl at 5 °C (p=0.00684), and 0.1 M HCl at ambient temperature (p=0.00158) were highly significant. At the α=0.05 level, there were no statistically significant differences between percent yields of the remaining treatments and each other (p>0.97 in all cases).
Demineralization times for treatment groups ranged from 8-28 days (Figure 5). The least concentrated HCl used (0.025 M) at 5 °C took the longest to demineralize the CaCO$_3$, and 0.1 M HCl at ambient temperature took the least amount of time.

![Figure 5: Time (in days) for complete dissolution of CaCO$_3$ to occur at each acid concentration. Ambient temperature treatments are denoted with black circles. Chilled treatments are denoted with blue squares.](image)

In the exploratory experiments, average insoluble organic yields for shell powder treated with Nance et al.’s (2015) methods were 0.275% (2.75 mg/g shell powder; range=2.5-3 mg/g; SD=0.25; n=3) when demineralized with 3.25 M HCl and 0.31% (3.1 mg/g shell powder; range=2.95-3.3 mg/g; SD=0.18; n=3) when demineralized with 0.1 M HCl, respectively. When treated with 0.5 M HCl, yields for shell powder averaged 0.15% (1.48 mg/g of shell; range=1.25-1.65 mg/g of shell; SD=0.21, n=3) while yields for shell fragments were 0.19% (1.87 mg/g of shell; range=0.7-3.1 mg/g; SD=1.20, n=3).
Organic yield of fossil shells (Figure 6) from the Caloosahatchee Formation averaged 0.01% (0.08 mg/g; shell powder; range=0.04—0.12 mg/g; SD=0.03; n=5) for soluble organics and 0.05% (0.48 mg/g of shell; range=0.18—0.66 mg/g; SD=0.20; n=5) for insoluble organics. Shells from the Tamiami Formation (Figure 6) yielded an average of 0.01% soluble organics (0.13 mg/g of shell; range=0.10—0.18 mg/g; SD=0.04; n=5) and insoluble organic weights averaging 0.05% (0.45 mg/g of shell; range=0.1—1.0 mg/g; SD=0.38; n=5) Average percent total organic yield was 0.06% for shells from both the Caloosahatchee and Tamiami Formations (Figure 7).

Figure 6: Soluble (black diamond) and insoluble (green triangle) weights for all fossil shells.
Figure 7: Mean percent total organic weights (calculated as a percentage of shell powder weight) for all fossils. Error bars represent standard deviation.
DISCUSSION

I hypothesized that slower reaction speeds (as regulated by acid temperature and concentration) would result in higher organic yield since the slower reactions should be less destructive to large organic molecules. Demineralization times varied as expected with more concentrated acid at ambient temperature requiring the least time and the least concentrated acid at 5 °C requiring the most. The 0.025 M HCl treatment at ambient temperature resulted in the highest yield, which supports the hypothesis. However, the range of variation within this group was high, which may have been the result of experimental error or an effect of the treatment. The samples in this group were submerged in room-temperature acid for 26 days (twice as long as any other 25°C treatment), and this length of time increased the likelihood of contamination. However, since increased demineralization times did not result in increased yield for other treatment groups, additional experiments across a broader range of acid concentrations and temperatures are needed to determine whether the outlier was a result of experimental conditions or experimental error. The yields of the remaining treatment groups were not discernibly different from one another, regardless of temperature or acid concentration.

Analytical precision on the mass spectrometer requires a target amount of 2—5 mg of organics per sample with a minimum acceptable weight of 1 mg when running
CSIA (G. Ellis, personal communication, Jan. 5, 2016). Percent organic yield is useful in estimating how much total shell material is required to obtain these sample weights. For example, assuming ~0.2% yield of modern *Strombus* shell as reported here, a starting weight of ~1.5 g of shell powder should yield sufficient total organics (~3 mg) for analysis. Fossil *Strombus* from the Pliocene (Tamiami Formation) and Pleistocene (Caloosahatchee) yielded ~0.6% organics, suggesting that a starting weight of ~5 g should be sufficient for CSIA of total shell organics.

Although amount of total organics recovered from shell decreased with age, the amount of soluble organics remained comparable (~0.01%) across the temporal range studied. Proteins hydrolyze into individual AAs over time (Mitterer, 1993), and previous work has suggested that the soluble portion of shell organic matrix corresponds to intracrystalline organics (Meenakshi, Hare & Wilbur, 1971). Intracrystalline organics are trapped within the CaCO₃ minerals and shielded from diagenetic processes (Penkman *et al.*, 2008). Thus, recovery of soluble organics will be essential for application of CSIA to fossils.

Recommendations for shell sampling vary depending on the age, condition, and microstructure of the shell. In cases where particularly low yields are expected (i.e., old or small shells), pooling multiple whole shells may be the only viable approach (Goodfriend, 1988). However, isotopic homogeneity between individual animals, even from the same ecosystem, cannot be assured. Conversely, in cases where expectations
for yield are higher, such as in recent or very large shells, it may be possible to subsample the shell to obtain a record of trophic level throughout ontogeny.

Based on the results presented here, general recommendations for extracting organics from shells include using 0.1 M HCl for demineralization and using fragments of shell instead of powder. The sample size for comparing shell powder to fragments was low (n=3) and variability between samples was high, although the average yield for samples demineralized as fragments was higher than those demineralized as powder. Since shell growth is regulated by proteins and occurs along the edge of the shell (Su, Zhang & Heuer, 2004), it is possible that protein abundances are more concentrated along the growing edge. Future work should investigate the source of this variability since it is unknown whether the amount of organic matrix within the shell is homogenous throughout.
Table 1: Synopsis of methods from the literature.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Acid Type and Concentration</th>
<th>Shell Sample Type</th>
<th>Temperature</th>
<th>Starting Shell Weight</th>
<th>Total Organic Yield</th>
<th>Soluble Yield</th>
<th>Insoluble Yield</th>
<th>Fossil or Modern</th>
<th>Taxon</th>
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<tr>
<td>Meenakshi, Hare &amp; Wilbur (1971)</td>
<td>5% EDTA and 0.01 M HCl</td>
<td>Fragment</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Gastropoda (Nassarius obsoletus, Littorina irrorata, Polinices duplicatus, Thais floridana, Campeloma decisum, Pila virens)</td>
</tr>
<tr>
<td>Crenshaw (1972)</td>
<td>0.5 M EDTA</td>
<td>Powder</td>
<td>—</td>
<td>100 g</td>
<td>0.403%</td>
<td>0.054% ±0.004%</td>
<td>0.349% ±0.058%</td>
<td>Modern</td>
<td>Bivalvia (Mercenaria mercenaria)</td>
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<tr>
<td>Weiner et al. (1979)</td>
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<td>Fragment</td>
<td>—</td>
<td>5 g</td>
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<td>—</td>
<td>—</td>
<td>Fossil</td>
<td>Cephalopoda (Baculites inornatu)</td>
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<td>Powder</td>
<td>18-21°C</td>
<td>50 g</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Modern</td>
<td>Bivalvia (Crassostrea virginica and Mercenaria mercenaria)</td>
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### Table 1 (Continued)

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<tr>
<th>Authors</th>
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<th>Sample Type</th>
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<th>Temperature</th>
<th>Mass (g)</th>
<th>Nitrogen (%)</th>
<th>Oxygen (%)</th>
<th>Sulphur (%)</th>
<th>Fossil/Marine Type</th>
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<tr>
<td>Goodfriend (1988)</td>
<td>6 M HCl</td>
<td>Whole shell</td>
<td>—</td>
<td>15–25 shells at 10–15 g each</td>
<td>0.003%</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Fossil (Trachioidea seetzeni)</td>
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<td>Serban, Engel &amp; Macko (1988)</td>
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<td>Powder</td>
<td>—</td>
<td>18 g</td>
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<td>—</td>
<td>—</td>
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<td>Fossil (Mercenaria sp.)</td>
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<td>Ambient</td>
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<td>0.35%</td>
<td>Modern (Gastropoda (Halioptis rufescens))</td>
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<tr>
<td>Walton &amp; Curry (1994)</td>
<td>2 M HCl</td>
<td>Powder</td>
<td>—</td>
<td>—</td>
<td>0.00007%</td>
<td>—</td>
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<td>Fossil (Brachiopoda (Terebratella sanguinea))</td>
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<td>2–4 g</td>
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<td>—</td>
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<td>Modern (Gastropoda (Helix pomatia))</td>
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<td>Sykes, Collins &amp; Walton (1995)</td>
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<td>Powder</td>
<td>110°C</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Modern (Bivalvia (Arctica islandica and Cepaea sp.))</td>
</tr>
<tr>
<td>Marxen &amp; Becker (1997)</td>
<td>0.00001 M HCl</td>
<td>Powder</td>
<td>4°C</td>
<td>100 g</td>
<td>0.864%</td>
<td>0.856% ±0.18%</td>
<td>0.0338% ±0.0083%</td>
<td>Modern (Gastropoda (Biomphalaria glabrata))</td>
<td></td>
</tr>
<tr>
<td>Dauphin (2001)</td>
<td>50% acetic acid</td>
<td>Powder</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Modern (Bivalvia (Dosina ponderosa))</td>
</tr>
</tbody>
</table>
Table 1 (Continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Acid Strength</th>
<th>Sample Type</th>
<th>Temperature</th>
<th>Modern:</th>
<th>Fossil:</th>
<th>Both</th>
<th>Fossil Organism(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O’Donnell, Macko &amp; Wehmiller (2007)</td>
<td>6 M HCl</td>
<td>Fragment</td>
<td>Ambient</td>
<td>100 mg</td>
<td>200–400 mg</td>
<td>Both</td>
<td>Bivalvia (Mercenaria sp.)</td>
</tr>
<tr>
<td>Penkman et al. (2008)</td>
<td>2 or 7 M HCl</td>
<td>Powder</td>
<td>110°C</td>
<td>10–40 mg</td>
<td>–</td>
<td>Both</td>
<td>Bivalvia (Corbicula fluminalis, Margar-itifera falcata, Bithynia tentaculata and Valvata piscinalis)</td>
</tr>
<tr>
<td>Ellis, Herbert &amp; Hollander (2014)</td>
<td>0.05 M HCl</td>
<td>Powder</td>
<td>Ambient</td>
<td>1 g</td>
<td>–</td>
<td>Modern</td>
<td>Bivalvia (Crassostrea virginica)</td>
</tr>
<tr>
<td>Nance et al. (2015)</td>
<td>3.25 M HCl</td>
<td>Fragment</td>
<td>–</td>
<td>2 g</td>
<td>–</td>
<td>Fossil</td>
<td>Gastropod (Ecphora sp.)</td>
</tr>
</tbody>
</table>

“—” = Not specified
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