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ACID TREATMENT EFFECTS ON THE STABLE ISOTOPIC SIGNATURES OF FOSSILS

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Abstract: Prior to geochemical analyses, fossil bones and teeth are often extracted from any surrounding lithified sediments using chemical techniques such as immersion in acid. As stable isotope analysis becomes more commonplace in palaeoecological investigations, it is important to consider what effects these chemical preparation techniques may have on any subsequent isotopic data and to constrain these effects as quantitatively as possible. This study aims to elucidate these effects, as it is vital that variability in a data set should not be introduced as a result of protocols used during sample preparation; in addition, it defines the most effective and viable method of carbonate removal for processing bulk fossil samples without causing alteration of their stable isotopic signatures. Various strengths of two weak acids commonly used during palaeontological preparation were tested to evaluate their effects on the $\delta^{15}N$ and $\delta^{13}C_{org}$ isotopic signatures of the vertebrae of a large Eocene fossil fish. Changes in the isotopic values occurred over time regardless of which acid was used, each causing a variable response in both $\delta^{15}N$ and $\delta^{13}C_{org}$ isotopic values. Without careful monitoring of the acidification process in a controlled environment, any resulting data could therefore confound interpretation. Based on these experiments, it is recommended that 2 M acetic acid be used for the pretreatment of fossils prior to the acquisition of N and C isotope data where carbonate removal is necessary.

Keywords: nitrogen isotopes, carbon isotopes, fossil fish, acetic acid, formic acid.

ISOTOPIC signatures of $\delta^{15}N$ and $\delta^{13}C$ from bones, teeth and scales of fossils are being used increasingly as a means of palaeoecological reconstruction. Stable isotope analyses are used in a wide range of studies and can be a very powerful tool, provided that the isotopic signatures of the fossils have not been chemically altered during diagenesis or sample collection and preparation. In this study, the $\delta^{15}N$ and $\delta^{13}C$ isotopic response of well-preserved Eocene fish bones to treatment with varying strengths of two commonly used acids over a period of up to 32 days was investigated. The isotopic values reported do not necessarily represent the original values of the fish: this is the focus of a future study. However, it was essential that any isotopic data retrieved from the fish would be as reliable as possible. These trials were used to assess the impact of the chemical preparation treatments and to identify 'safe' working limits, to avoid damage to the fossil and any alteration of the isotopic signatures therein. For geochemical analyses, particularly those involving organic carbon, it is vital to remove all traces of inorganic carbonate as this can confound any results. In cases where fossils are found in a fine-grained, carbonate matrix chemical pretreatment is necessary to remove all enclosing calcite that could obscure the results of any isotopic analyses. The calcareous matrix surrounding fossils can often be so fine-grained that mechanical retrieval is only partially successful in removing the attached matrix. Additionally, many fossils are very difficult to extract manually from fine-grained calcareous rocks because of the delicate nature of the fossils themselves. Although chemical extraction is necessary to remove all carbonate in instances such as these, potential etching of the fossils is extremely undesirable for isotopic studies as it may lead to alteration and loss of important information. A suitable method was sought that would meet the requirement of removing any carbonate present without affecting the geochemistry of the fossil itself. Incomplete removal of secondary mineral contaminants is known to affect inorganic carbon and oxygen isotope values (e.g. Koch *et al.* 1997) although little is known of the consequences for nitrogen and organic carbon isotope signatures. In this study, the aim was to identify a method adequate for removing all carbonate present without causing a significant response in the $\delta^{15}N$ and $\delta^{13}C_{org}$ isotopic values. Here we present the optimal extraction parameters necessary for isotopic research involving fossils, as revealed

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through experimentation using fossil fish bones from the Eocene Green River Formation of Wyoming.

BACKGROUND

Considerations during sample collection

As the geochemical analysis of fossils becomes more widespread, chemical preservation and extraction techniques of fossils need to be reviewed. In the past, the damage to fossils during retrieval, preparation and storage was an accepted downfall of the scientific process (McCrae and Potze 2007). However, many modern geochemical analyses require that the fossil material be retrieved and extracted from any surrounding lithified sediment with no alteration to the fossil chemistry and that no evidence be removed, or indeed added, to specimens. During retrieval of vertebrate specimens, adhesives such as cyanoacrylates and acrylic polymers are often used in the field to ensure the integrity of the specimen. Upon arrival in the laboratory, fossil preparators then use physical and chemical treatments to extract the fossil specimens from any enclosing sediments. Furthermore, consolidants and resins are often used to prevent the deterioration of specimens housed in museum collections. If geochemical data representing the original specimen are to be successfully obtained from fossils, then ideally no chemicals should be applied to the material during field collection or storage. Handling should be kept to a minimum and fossils should be stored in unreactive or inert materials that do not shed particulate matter onto the surface of the fossil specimens, such as aluminium foil (although this of course would not be suitable where future analyses of metals are planned). During preparation or extraction from enclosing matrices, mechanical methods are preferable to chemical methods, although it should be noted that the high temperatures reached because of friction with electrical drills and engraving tools may also lead to alteration of fossil geochemistry. In the instance of retrieving macrofossils and some microfossils from calcareous enclosing sediments however, treatment with an aqueous acid or base is often the only viable option. Calcareous sediments are often extremely fine-grained and can remain in small pockets within pitted fossil bone after mechanical preparation. Chemicals are able to access these areas that mechanical tools cannot, and discrimination between matrix and fossil is more subtle and accurate (Lindsay 1987). Acid preparation also has the advantage of gradually revealing fossils hidden below the matrix surface (McCrae and Potze 2007). Chemical preparation techniques of both macro- and microfossils have been widely used since the late 19th century, and the various procedures used are reviewed in texts such as Rixon (1976) and Green (2001). Many of these techniques have been developed principally for the recovery of microscopic fossils from rock samples (see Aldridge 1990 and references therein) and differ according to the rock type and composition of the microfossils. For example, studies investigating microfossils from lithified sediments use a variety of extraction techniques including harsh chemicals such as heated hydrogen peroxide (Pingitore *et al.* 1993) and sodium hypochlorite (Aldridge 1990) to release specimens from any surrounding sediment. These and other procedures can be severe and potentially lead to specimen damage.

Common chemical treatments used in isotope studies and their effects

Many studies using stable isotopes to investigate palaeoenvironment and palaeoecology focus on relatively recent fossil specimens and involve costly and complicated apparatus to extract either the inorganic or organic fraction of bones or teeth (e.g. Tuross *et al.* 1988; Crowson and Showers 1991). These extraction processes usually include chemical treatments to remove the unwanted fraction and isolate the material of interest. A common and long-established chemical treatment used on bioapatites to remove unwanted organic material during preparation for inorganic C and O analyses, for example, involves treatment with sodium hypochlorite, followed by acetic acid to remove diagenetic and secondary carbonates (e.g. Wang *et al.* 1994; Bryant *et al.* 1996; van der Merwe *et al.* 2003). The effects of both of these chemical treatments are well documented (see Zazzo *et al.* 2006 and Balter *et al.* 2002) and can cause isotopic fractionation if not used correctly.

Many studies of Recent vertebrates have investigated N and C isotopes, and although none appear to have encountered samples enclosed in a carbonate matrix, chemical treatments have been used to extract different organic fractions from bone for isotopic analyses. Over 20 years ago, DeNiro and Weiner (1988) obtained $\delta^{15}N$ and $\delta^{13}C$ values from the organic matrix in prehistoric bones using NaOCl to isolate the organic material from within fused aggregates of hydroxyapatite. Similarly, Minami and Nakamura (2005) analysed the N and C isotopic contents of proteins from Recent (>4000 BP) powdered bone fragments. They tested the effects of decalcifying treatments with different concentrations of HCl using modern bone for comparison and found that HCl has a significant effect on N isotope data, which ultimately confounds any palaeoecological interpretations. In trophic level studies of modern ecosystems using $\delta^{15}N$ and $\delta^{13}C$, washing freeze-dried powdered organisms with HCl to remove small amounts of nondietary carbonate has been commonplace for many years (e.g. Rau *et al.* 1983; Fry 1988; Hobson and Welch 1992). The effects of this prac-

tice have been investigated extensively by many other researchers (e.g. Bunn *et al.* 1995; Bosley and Wainright 1999; Pinnegar and Polunin 1999; Jacob *et al.* 2005) and have been reviewed recently in a thorough study by Carabel *et al.* (2006). The acid washing of modern organisms was found by these authors to cause fluctuations in the resulting isotope values, in particular $\delta^{15}N$, highlighting the effects of sample preparation techniques on isotope data and the potential for erroneous interpretations.

Studies using the isotopic signature of calcareous microfossils from Recent unlithified sediments to investigate palaeoclimate also use chemical techniques to clean specimens prior to analysis (e.g. Elderfield *et al.* 2002; Skinner *et al.* 2003). Such techniques often involve mechanical crushing to expose all contaminants within the microfossils, washing with methanol to remove clays, and finally a hot oxidizing treatment using hydrogen peroxide solution to remove unwanted organic matter. Samples are then often polished using a weak nitric acid solution prior to analysis. These methods have also been found to be potentially destructive and can alter the chemistry of the microfossils being studied (Barker *et al.* 2003). These previous investigations highlight the necessity of careful planning prior to the extraction and preparation of any fossil material to be subjected to geochemical analyses.

Various techniques have been used to obtain isotopic data from much older fossil material with some success. The majority of studies involving pre-Quaternary fossilized vertebrate organisms have focussed on the analysis of oxygen isotopes from biogenic phosphates for palaeoclimate and physiological reconstruction (e.g. Kolodny *et al.* 1983; Barrick and Showers 1994; Fricke *et al.* 1998; Showers *et al.* 2002) and were subject to some of the pretreatments outlined above (e.g. Crowson and Showers 1991). The $\delta^{15}N$ and $\delta^{13}C_{org}$ isotopic composition of bones and teeth from deep time appears to have been overlooked however, perhaps because of assumptions that sample fidelity will have been degraded by decay and diagenetic change, although it has long been known that remnant organic matter within crystalline aggregates of hydroxyapatite can survive (DeNiro and Weiner 1988). Two studies utilizing $\delta^{15}N$ and $\delta^{13}C_{org}$ values to interpret ancient food webs that include vertebrate organisms have recently been reported (Schweizer *et al.* 2006, 2007) and involved no chemical pretreatment techniques for the majority of samples. Both focus on German Tertiary fossil lagerstätten from lacustrine ecosystems and conclude that the $\delta^{15}N$ and $\delta^{13}C$ values are valid and can be used to reconstruct trophic structure. As palaeontologists continue to apply techniques used in modern ecology such as these to reconstruct past ecosystems and feeding strategies, both the effects of the aforementioned chemical preparation protocols must be considered, as well as any prior treatments used to remove adhering rock matrices. When extracting fossils from an enclosing fine-grained matrix, it is important to now consider the effect that any chemical procedures may have on future isotopic analyses. Simple acid treatments are often used for the processing of bulk samples to liberate them from enclosing rock, and these must now be reviewed.

Selection of suitable acids for experimentation

Despite the demonstrable effects of acid washing and pretreatments on stable isotopic values, it is still necessary to remove enclosing carbonates from fossils such as the Green River fish prior to any isotopic analyses if accurate values are to be retrieved. For this end, a suitable acid was sought, of a strength that would have minimal impact on isotopic values while readily removing inorganic carbonate from the study specimens. An economically viable, simple procedure was required, where bulk samples could be processed in a timely and efficient manner. One molar HCl is routinely used by field geologists as a standard for determining effervescence class and readily dissolves carbonate, with 10% HCl being reported as a method for removing carbonate from Oligocene reptile teeth prior to $\delta^{15}N$ and $\delta^{13}C$ analyses (Schweizer *et al.* 2007). However, as HCl is a strong acid it was considered to have a high potential for causing textural damage to the fossils, hence other weaker acids were considered for use in this study. Two weak acids commonly used in palaeontological preparation techniques are acetic (CH_3COOH) and formic (HCOOH) acid, and these were thought to be the most suitable for our purposes. Both of these organic acids are highly miscible with water, relatively safe and easy to use and economic when processing a large sample set. However, when used for the extraction of phosphatic conodonts from lithified sediments, as detailed in Jeppsson *et al.* (1985, 1999; Jeppsson and Anehus 1995), these acids were identified as being potentially destructive/harmful to the fossils and therefore need careful monitoring in a controlled environment to prevent dissolution of any phosphate. These acids have also recently been identified as having the ability to modify the oxygen isotopic signatures of conodonts when used to break down host rocks using standard procedures (Wheeley *et al.* in preparation). In our investigation, both buffered and unbuffered weak acids were tested to find the optimal technique for removing inorganic carbonate in preparation for isotope analysis. Prior to these experiments, 1 M acetic acid had been used on Green River fish samples as part of a pilot study and was found to leave some inorganic carbonate residue. This caused the organic carbon values obtained to be much more positive than expected, exhibiting an average shift of 10‰, although it had little effect on the nitrogen isotope values

obtained. One molar formic acid was also tested on these pilot samples and considerably depleted the amount of fossil specimen present, leading to the conclusion that fluorapatite dissolution was taking place and therefore the potential alteration of isotopic signatures. For comparison, 1 M acetic and 1 M formic acids were included in the experiments detailed herein. The main objective was to identify a maximum exposure time for fossils in acid of a certain pH before any alteration occurred.

When acetic acid is applied to carbonate sediments, the result is consumption of acid and limestone, production of calcium acetate and some evaporation of water and acetic acid (Jeppsson *et al.* 1999) as shown in Equation 1. This reaction results in an increase in pH and cessation of dissolution of the carbonate as the acid becomes neutralized and was thought to be the reason that 1 M acetic acid used in preliminary studies left behind some carbonate residue. In these experiments, two higher concentrations of acetic acid were tested to see whether the increased concentration of hydrogen ions would dissolve all of the carbonate while still leaving the fluorapatite unaffected. Conversely, the 1 M formic acid used in preliminary studies had proved too aggressive, although by buffering formic acid a higher pH can be achieved; the solution can then resist changes in pH, and dissolution effects on the fossils should be minimized. Buffering has been found to decrease the risk of dissolution of phosphatic fossil material when kept at a pH above 3.6 (see Jeppsson and Anehus 1995). While formic acid can be destructive in some instances, it has the advantage over acetic acid for some other rock matrices in that it can break down dolomite, although no dolomite was present in the Green River fossil fish samples. Equation 2 shows the consumption of formic acid and limestone to produce calcium formate, carbon dioxide and water. To constrain the optimal pH and time necessary to remove all carbonate with no acid attack on the specimens, a variety of concentrations of both acetic and formic acids were tested over a time range from as little as 1.5 h up to a maximum of 32 days.

$$2CH_3COOH_{(l)} + CaCO_{3(s)} \rightarrow (CH_3COO)_2Ca_{(l)} + CO_{2(g)} + H_2O_{(l)} \quad (1)$$

$$2HCOOH_{(l)} + CaCO_{3(s)} \rightarrow Ca(CHO_2)_{(l)} + CO_{2(g)} + H_2O_{(l)} \quad (2)$$

MATERIALS AND METHODS

Five vertebrae of a large *Phareodus testis* Cope, 1877, specimen from the Eocene Green River Formation of Wyoming, USA, were selected for this study. The fish was found in Fossil Lake, facies F-1 (as denoted by Grande and Buchheim 1994), collected without the use of chemicals and was stored in aluminium foil. The vertebrae were assumed to have a fairly uniform isotopic signature and were ground together into one, homogenized sample, so that each subaliquot used in these experiments would be equal in all aspects and have the same isotopic composition at the outset. Prior to acid testing, all sample powders were subject to XRD to identify mineral composition and to confirm that there was no phase variation between the homogenized vertebrae portions. This was performed using a Phillips PW1720 with a Phillips PW1050/80 goniometer and a Phillips PW3313/20 Cu k-alpha anode tube run under standard conditions of 40 kV and 20 mA. All samples were found to be comprised of fluorapatite and calcite. XRD is not quantitative, but the percentages of these two mineral phases were assumed to be similar in all samples, based on visual observations.

All chemicals used in these experiments were of reagent grade, to prevent the addition of any impurities, and all acid solutions were prepared using deionized water (DIW). This experiment used three concentrations of unbuffered acetic acid and three concentrations of unbuffered and buffered formic acid in aqueous solution (see Table 1). The acid dissociation constant or pK_a value for formic acid is given as 3.75 in tables in Beynon and Easterby (1996). Buffers give effective pH control when within about one pH unit on either side of the pK_a value; therefore, pH 3 and 3.7 were chosen for these experiments. One molar NaOH solution was added to 1 M formic acid until the desired starting pH was achieved. The pH was measured using a Hanna pHep® 4 waterproof pH/temperature tester, which was calibrated immediately prior to

TABLE 1. The six different acid solutions tested in this experiment.

Description	Label	Base added	Initial pH
Unbuffered acetic acid, 1 M	A1	None	2.2
Unbuffered acetic acid, 2 M	A2	None	1.9
Unbuffered acetic acid, 3 M	A3	None	1.9
Unbuffered formic acid, 1 M	F1	None	1.6
Buffered formic acid, 1 M	FB3	Sodium hydroxide (NaOH)	3.0
Buffered formic acid, 1 M	FB4	Sodium hydroxide (NaOH)	3.7

use in these experiments. Temperature was not one of the monitored variables; the pH values of both acetic and formic acids are relatively unaffected by changes in temperature (Beynon and Easterby 1996). The effect of ionic strength was not considered to be significant here and no neutral salts were added. To prevent any airborne contamination affecting the samples, all acid treatments were carried out in a specially designed clean over-pressured glove box. Laboratory equipment was washed in a solution of Decon 90® surface active cleaning agent where possible and rinsed thoroughly with DIW. Additionally, all glassware was soaked in 10% HCl overnight and rinsed five times in DIW before use. One hundred milligram subsamples of the powdered fossil *P. testis* vertebrae were weighed into each of 60 labelled beakers using a high-precision microbalance (Sartorius MC5, ±2 μg). Replicate samples were also run to monitor reproducibility. Ten time periods on a logarithmic scale were chosen for this study, from 1.5 h up to 768 h or 32 days (1.5, 3, 6, 12, 24, 48, 96, 192, 384, 768 h). Forty millilitres of the relevant acid was added to each beaker, and the pH and time (t_0) were noted. All beakers were stirred midway through each time increment ($t_{½}$) and the pH noted. After each time increment had elapsed (t_1), the pH was measured for a final time, the acid drawn off and each powdered fossil subsample rinsed twice with DIW before being left to dry at room temperature. The dried subsamples were then accurately weighed before being re-examined using XRD to identify any overall changes in mineral composition. For the analysis of $\delta^{15}N$ and $\delta^{13}C_{org}$ content, portions of each powdered fossil subsample (1.5–16 mg) were sealed in tin capsules and measured using an in-house procedure on a Thermo Delta^{plus} continuous flow isotope ratio mass spectrometer (CF-IRMS) with a CE Instruments Flash EA 1112 Elemental Analyzer™ (EA). Samples were analysed on the same day in a random order to prevent any systematic bias in the results and corrected to international standards using an L-Alanine ($C_3H_7NO_2$) working standard. Experimental precision based on these replicates was ±7.4% RSD for $\delta^{15}N$ and ±3.3% RSD for $\delta^{13}C$. All results are presented using standard delta (δ) notation expressed as a deviation from the ratio in an international standard where δ (‰) = $((R_{sample}-R_{standard})/R_{standard}) \times 1000$. Data for all samples left in acid for a total of 192 h were discounted because of suspected contamination during DIW rinses.

GREEN RIVER FISH RESULTS

Variations in pH

Although quite low amounts of carbonate were thought to be present within the samples, there was an immediate effect on the pH of all four unbuffered acids at t_0 when they were added to each relevant aliquot of ground *P. testis* vertebrae. For acid A1, an average change of 0.65 pH units was observed, for A2 an average change of 0.75 pH units was observed and for acid A3 a change of 0.6 pH units was observed in each beaker at t_0. Similarly, a change of 0.5 pH units for the unbuffered formic acid F1 was observed at t_0, whereas the buffered formic acids remained constant. Further pH measurements were taken at $t_{½}$ and t_1, and it was found that this trend towards more basic pH values continued with time (Fig. 1A). Those samples remaining in the lower molarity acetic acid for the longest time showed the most marked changes in the pH of the resultant solution. Surprisingly, the solutions containing samples in buffered formic acid also showed some changes in pH over time. Both FB3 and FB4 were stable for at least 48 h after being added to the fossil samples, but by 768 h after the experiment began, FB3 had increased by 0.5 pH units and FB4 had increased by 0.6 pH units.

Variations in weight

Each beaker contained 100 mg of powdered *P. testis* vertebrae at the outset (t_0). After each time increment had lapsed (t_1), the dried powder remaining was weighed (Fig. 1B). The unbuffered formic acid (F1) had the most marked effect, decreasing the sample weight by 90% within 24 h, confirming the qualitative visual assessment of pilot studies. Buffering was found to considerably reduce this dissolution of fluorapatite and gave similar results to the acetic acid treatments. For the samples treated with the acetic acids and buffered formic acid FB3, at least 20% weight loss occurred in the first 1.5 h of the experiment. This may indicate that around 20% of the samples from *P. testis* vertebrae were composed of readily dissolved $CaCO_3$, or perhaps that fluorapatite dissolution was also taking place. Within the same time frame, only 10% of the sample in acid FB4 had dissolved, indicating that the highly buffered acid perhaps lacked the capacity to dissolve $CaCO_3$ successfully or indeed that fluorapatite was not being dissolved in this instance. In most cases, following an overall steady decrease, the weight of the remaining sample then increased after a treatment period of between 8 and 16 days. XRD analyses were unable to detect any new mineral phases accumulating within any of the samples of *P. testis* treated with the various acids. The XRD traces for all samples indicate that although no change in the overall mineral composition was observed after the acid treatments were applied for 768 h, the presence of calcite had decreased substantially. Weights of N and

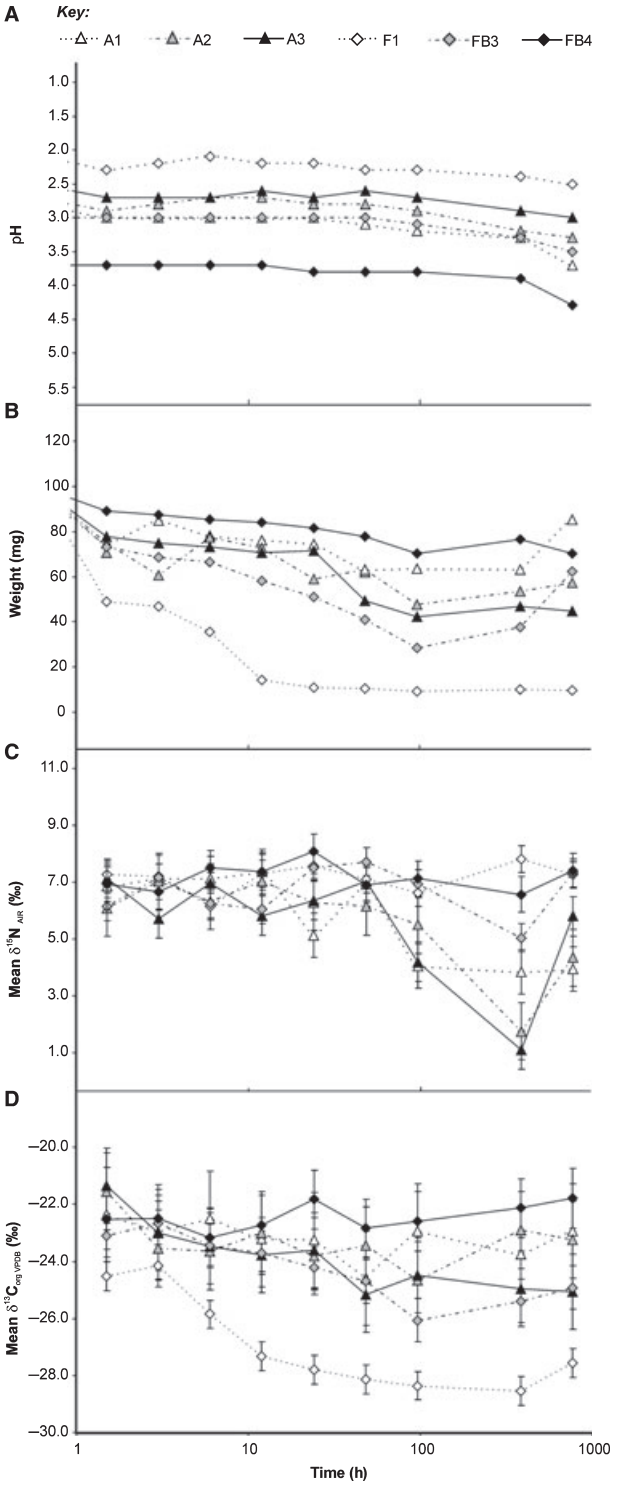

FIG. 1. Results at t_1 for all six acids at nine time intervals from 1.5 to 768 h (time shown on logarithmic scale). Samples were treated using the following: A1, 1 M acetic acid; A2, 2 M acetic acid; A3, 3 M acetic acid; F1, 1 M formic acid; FB3, 1 M formic acid buffered to pH 3; and FB4, 1 M formic acid buffered to pH 3.7. A, pH data at t_1; B, weight data at t_1; C, mean $\delta^{15}N$ isotope ratios at t_1 ($n \geq 3$); D, mean $\delta^{13}C_{org}$ isotope ratios at t_1 ($n \geq 3$).

C in mg (calculated from CF-IRMS %N and %C data) varied slightly between beakers but did not show any significant trends over time with any of the acid treatments.

Variations in $\delta^{15}N$

The $\delta^{15}N$ isotope values of the *P. testis* vertebrae samples treated with the six different acids show increasing fluctuations with increasing time, particularly those treated with the three different molarities of acetic acid (Fig. 1C). The samples treated with the three formic acids all show different trends, with the unbuffered 1 M acid (F1) samples showing a fluctuating profile over time with a general positive trend and the profile of the samples treated with buffered FB3 acid being similar to the acetic acid trends. The samples treated with FB4 acid show a general increase to the 24 h mark before decreasing to a minimum at 384 h followed by a return to higher values. The $\delta^{15}N$ isotope values of the samples treated with F1 and FB4 show less dramatic (<2‰) fluctuations overall, staying between 6 and 8‰ at all times. The $\delta^{15}N$ isotope values for all samples, regardless of acid strength, appear to be most stable within the first 48 h of the experiment, with larger variations occurring after this time.

Variations in $\delta^{13}C$

Figure 1D shows the organic carbon isotope signatures for samples at t_1 after treatment with each of the six acids, with time in hours plotted on a logarithmic scale. The three profiles for samples treated with different molarities of acetic acid are similar, showing an initial drop, then fluctuating between approximately −25 and −23‰. The results for samples treated with formic acid FB4 show a varying profile, with fluctuations limited to between −23.2 and −21.8‰. Samples treated with F1 and FB3 formic acids show relatively smooth curves, with an overall more negative trend over the first 96 h and a slight return to more positive values towards the end of the experiment. Both acids caused changes of around 4‰ in the organic carbon isotope value of the *P. testis* vertebrae overall. However, acid F1 seems to cause the most marked changes in the $\delta^{13}C$ isotope values after just 3 h, when compared with the isotope results for all samples treated with the other acids. The $\delta^{13}C$ isotope values for *P. testis* samples treated with all other acids appear to be most stable within the first 48 h of the experiment, with larger variations occurring after this time.

DISCUSSION

Effects of pH

All unbuffered solutions showed an immediate change in pH on addition to samples of homogenized *P. testis* vertebrae. This was attributed to $CaCO_3$ reacting with the acid upon contact, thereby making the resultant solutions more basic. In the solutions treated with acetic acid, this trend continued and there was an inverse relationship between the overall pH change and the molarity of acid used over time; larger final changes in pH were observed at t_1 in those samples treated with lower molarity acids. The fewer acid ions available to react with the relatively constant amount of $CaCO_3$ present in each beaker meant that the pH quickly became more basic. Conversely, the higher-molarity acetic acids allowed a less marked change because available ions were still present and the reaction shown in Equation 1 did not go to completion. Weaker acids with fewer ions available for reaction with $CaCO_3$ led to the total consumption of acetic acid and the production of calcium acetate, with excess $CaCO_3$ remaining in the sample. For samples containing more carbonate than those herein, unbuffered acetic acid should be changed frequently to prevent the solution becoming saturated with calcium acetate and unable to dissolve any more $CaCO_3$. Based on the findings in this study, the treatment of fossil samples should be adjusted according to the amount of $CaCO_3$ present. The powdered fossil vertebrae used here were relatively unaltered over a certain amount of time in the acetic acid; despite having very little $CaCO_3$ present in the samples compared to the volume of acid used, little dissolution of the fossil powders took place. It is presumed from these findings that fossil samples containing a much higher proportion of $CaCO_3$ would have the increased ability to force Equation 1 to the right, with all available acid being used at a much faster rate. This indicates that the addition of acetic acid to a sample with higher $CaCO_3$ would show a much more marked increase in initial pH. Therefore, the relevant acid should be replaced at frequent intervals, within the time frame identified here as 'safe' in terms of fluorapatite dissolution, until only an increase in pH of *c.* 0.6 occurs after the addition of fresh acetic acid, as noted here at t_0. Once this initial pH is achieved, the fossil sample can be safely left in the acid for less than 48 h.

The 1 M unbuffered formic acid used in these experiments showed the smallest pH change of all the unbuffered acids, just 0.4 pH units between t_0 to t_1. This indicates that more ions were available for reaction with the $CaCO_3$ in the formic acid than in any of the acetic acid solutions, probably due to its higher ionization constant. However, the dramatic decrease in sample weight to just 10% of its initial mass after 24 h shows that this method is wholly unsuitable when dealing with small quantities of fossil material during preparation for isotope geochemistry. The most marked changes in $\delta^{13}C_{org}$ of 4.39‰ were also observed when using this acid, further demonstrating the unsuitability of this acid for isotope geochemistry preparation. When buffered to pH 3 using NaOH, 1 M formic acid FB3 shows similar qualities, but less pronounced. Although changes in pH were negligible throughout these experiments, a decrease in sample weight to *c.* 50% of the initial mass occurred during the first 24 h, indicating that dissolution of the fluorapatite was not halted by the buffered pH. Similarly, large changes (up to 2.96‰) were observed in the $\delta^{13}C_{org}$ values, indicating that 1 M formic acid buffered to pH 3 is also unsuitable for preparation of fossil material for isotopic analyses. Jeppsson and Anehus (1995) observed that buffering decreased the risk of dissolution of phosphatic microfossils when kept at a pH above 3.6. Here, 1 M formic acid was buffered to pH 3.7 but almost 35% of the original sample still dissolved within 192 h of adding this acid to the powdered vertebrae. The pH remained almost constant for the majority of these experiments however, and very few changes were observed in the trends of either $\delta^{15}N$ or $\delta^{13}C_{org}$. The main drawback of using this acid for fossil preparation specifically to remove $CaCO_3$ is that very little carbonate dissolution appears to take place on the timescale used here. Visual observations indicate that this highly buffered acid has a very low capacity for consumption of excess $CaCO_3$, with uptake inhibited by the volume of NaOH added. Based on these observations, it is suggested that fossil samples containing a much higher proportion of $CaCO_3$ than the vertebrae of *P. testis* could take a matter of months to prepare using this buffered acid.

Effects on isotopic signature

Samples of *P. testis* vertebrae immersed in any of the six aqueous acid solutions showed demonstrable changes in their isotopic signatures and weights over time. The overall magnitude of the trends shown in Figure 1C is several times the $\delta^{15}N$ value used to represent one dietary isotopic shift between fish species (see Sweeting *et al.* 2007), when in fact all of the data here represent the same fish specimen. This kind of shift in the data would confound any interpretation of palaeoecology and trophic structure such as that discussed by Schweizer *et al.* (2006, 2007). It is postulated that as the fluorapatite breaks down and goes into solution, as reflected in the overall weight decrease in the samples, so must some of the organic matter contained within its mineral matrix. Generally, more negative nitrogen isotope values were observed with increasing time, particularly after treatment with the acetic acids that appeared to selectively leach the heavier isotope, predominantly after 48 h had lapsed. Similarly, a general decrease

in the $\delta^{13}C$ values was observed with increasing time. This suggests that the heavier isotopes are preferentially released and taken into solution with the acid, or in the case of C, are used in the production of calcium acetate, calcium formate or carbon dioxide. As shown in Equations 1 and 2, there are numerous pathways for the released organic carbon atoms to take during the dissolution reactions. The heavier isotope of carbon may form stronger bonds within the new chemical species formed during these reactions, thereby remaining in solution. Alternatively, the lighter isotope of carbon may preferentially remain in the organic matter within the fluorapatite lattice, or its uptake into new chemical species could be inhibited by the stronger reactivity of the heavier isotope. The net effect of these processes would be reflected in the decreased isotopic values shown in Figure 1C and D.

The more positive upturn in both N and C isotopic signatures after 192 h had lapsed cannot easily be explained. However, this does coincide with an increase in weight in the majority of the samples immersed in the different aqueous acid solutions. If a new precipitate or solid is beginning to form or settle out of suspension at this time, then perhaps the heavier isotopes are preferentially incorporated into this new mineral species. The appearance of this proposed solid in the solutions after 192 h is unusual in that it appears to form in both the samples treated with acetic acid and those treated with formic acid. All isotopic data for these experiments were acquired on the same day, and samples were run in a random order to prevent any systematic bias in the results, so these unexplained trends appear to be real. This may be due to calcium acetate and calcium formate precipitation within each solution. These are not evident in the XRD traces obtained; however, they may be present in quantities that are below the detection limits of the apparatus or may not be crystalline. Only fluorapatite and calcite are shown to be present using XRD, but it is unlikely that any weight increase could be due to the re-precipitation of either mineral. In order for the reactions to operate in reverse, the solutions would have had to approach a pH of 9 to enter the carbonate stability field (see Zeebe and Wolf-Gladrow 2001). The most likely explanation is the formation of calcium salts, as observed during the acid treatment of vertebrate fossil bone samples by previous authors (see McCrae and Potze 2007 and references therein), thus causing the observed weight increase in the dry powder residue post-treatment.

CONCLUSIONS

While formic acid is very effective for removing any carbonate matrix from palaeontological samples, care should be taken if geochemical data are to be retrieved. A pH of approximately 3.7, achieved by adding an appropriate base, can counteract these adverse effects. However, the higher pH means that the acid has less ability to consume $CaCO_3$ in a timely manner. By consistently replenishing the buffered formic acid, it is thought that $CaCO_3$ will eventually dissolve completely over an extended time period, although this may not prove financially viable. When using a preparation treatment prior to geochemical analysis of fossils, consideration should always be given to the time period involved if the effects on the isotope geochemistry are to be negligible. The results shown here suggest a time frame of <24 h is most suitable when only small amounts of carbonate are present; longer leach periods lead to significant artefacts in the resulting isotopic data. When larger amounts of calcite are to be dissolved, replacing the acid frequently until such time as only a small increase in pH of around 0.6 occurs is recommended prior to this <24 h leach period. If all samples are treated using the exact same method and for an equal, short amount of time, then the resulting data should be robust, making direct comparison within the data set possible. A weak acid was sought for use that would produce the least effect on the isotopic values and weights of the fossil fish being tested, balanced with being the most able to dissolve $CaCO_3$. Based on the findings herein, 2 M acetic acid, when used for no more than 24 h, has the least significant effect on the N and C isotopic values, pH and weight of the fluorapatite fossils, coupled with the highest potential for dissolving $CaCO_3$ when compared to the other acids used in this investigation. Therefore, 2 M acetic acid is recommended for future pretreatment of fossils prior to the acquisition of N and C stable isotope data.

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