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DO WEAK OR STRONG ACIDS REMOVE CARBONATE CONTAMINATION FROM ANCIENT TOOTH ENAMEL MORE EFFECTIVELY? THE EFFECT OF ACID PRETREATMENT ON RADIOCARBON AND $\delta^{13}C$ ANALYSES

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ABSTRACT. In hot environments, collagen, which is normally targeted when radiocarbon (^{14}C) dating bone, rapidly degrades. With little other skeletal material suitable for ^{14}C dating, it can be impossible to obtain dates directly on skeletal materials. A small amount of carbonate occurs in hydroxyapatite, the mineral phase of bone and tooth enamel, and has been used as an alternative to collagen. Unfortunately, the mineral phase is often heavily contaminated with exogenous carbonate causing ^{14}C dates to underestimate the true age of a sample. Although tooth enamel, with its larger, more stable crystals and lower porosity, is likely to be more robust to diagenesis than bone, little work has been undertaken to investigate how exogenous carbonate can be effectively removed prior to ^{14}C dating. Typically, acid is used to dissolve calcite and etch the surface of the enamel, but it is unclear which acid is most effective. This study repeats and extends earlier work using a wider range of samples and acids and chelating agents (hydrochloric, lactic, acetic and propionic acids, and EDTA). We find that weaker acids remove carbonate contaminants more effectively than stronger acids, and acetic acid is the most effective. However, accurate dates cannot always be obtained.

KEYWORDS: pretreatment, radiocarbon dating, stable isotopes, tooth enamel.

INTRODUCTION

Radiocarbon (^{14}C) dating of skeletal material is severely hindered by the poor survival of protein in the hot environments that are common between latitudes of 40°N and 40°S (roughly between the Mediterranean and southern Australia). Even with screening protocols to identify bones which may contain marginal levels of collagen (Brock et al. 2012; Jacob et al. 2018), many sites contain no datable bones, or too few to produce a precise Bayesian chronology (Storm et al. 2013; Wood et al. 2013; Calo et al. 2015).

Several researchers have attempted to produce accurate dates on carbonate within the hydroxyapatite phase in unburnt bone, which out-survives the organic phase in all but the most acidic of environments, but with little success outside of arid regions (Haynes 1968; Zazzo 2014; Zazzo and Saliège 2011). Carbonate in the mineral phase of tooth enamel may provide an alternative material, but again, attempts to ^{14}C date the material have met with limited success outside of arid regions (Hedges et al. 1995; Zazzo 2014; Wood et al. 2016) despite the application of a range of pretreatment methods (Hedges et al. 1995; Surovell 2000; Cherkinsky 2009; Hopkins et al. 2016). With no pretreatment, more than 10% of the carbonate in tooth enamel from karstic environments in Vietnam was found to be a contaminant (Wood et al. 2016), and in some cases tooth enamel appears to contain more carbonate contamination than bone apatite (Zazzo 2014). This is somewhat surprising given that, in comparison with bone, enamel porosity is low (Hedges et al. 1995; Millard and Hedges 1996), and enamel crystallites are more stable as they are larger (26.3 × 100–1000 nm vs. 5 × 100 nm) (Bottero et al. 1992; Cui and Ge 2007) and contain less carbonate (3.5 wt% vs. 6 wt%) (Elliott 2002), but may be related to protection offered by

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the close relationship between the mineral and protein components in well preserved bone (Zazzo 2014).

Carbonate contaminants can range in age, but they are likely to have a younger ^{14}C age than the buried tooth. Although some of the dissolved carbonate in water percolating sediments in limestone caves may derive from the bedrock and be ^{14}C free, most is likely to derive from processes occurring within the soil above the cave, such as plant and soil respiration (Cerling 1984; Fohlmeister et al. 2020), and thus contain young carbon. As a consequence, dates on enamel are usually found to be erroneously young (Zazzo 2014).

Improvement to ^{14}C dating methods for enamel is hindered by the limited knowledge of how tooth enamel degrades, and where the exogenous carbon sits within the hierarchical structure of enamel. Tooth enamel consists of crystallites separated by a high magnesium amorphous phosphate (Gordon et al. 2015; La Fontaine et al. 2016), grouped into prisms which are woven into the enamel fabric (Simmelink and Piesco 2001). Diagenesis mechanisms which can affect carbonate isotope signatures are thought to include:

1. the deposition of secondary carbonates on the surface and in cracks
2. diffusion and adsorption of carbonate ions into pores between both the prisms and the crystallites
3. isotopic exchange during dissolution/precipitation of hydroxyapatite

(Lee-Thorp and van der Merwe 1991; Zazzo et al. 2004; Zazzo and Saliège 2011; Wood et al. 2016). These require water to be present, meaning that teeth from arid regions are often found to be less affected by contaminants (Zazzo 2014).

Enamel is rarely ^{14}C dated outside of forensic applications, with only occasional use in arid and tropical regions. However, it is regularly analyzed for carbon and oxygen stable isotopes which are less sensitive to small amounts of contamination (Lee-Thorp 1989; Makarewicz and Sealy 2015; Roberts et al. 2017). Despite this, Pellegrini and Snoeck (2016) have called for a consistent protocol to be adopted as results appear to differ depending on pretreatment, suggesting that the endogenous isotopic value of tooth enamel is changed by pretreatment and/or that carbonate contaminants may affect the stable isotope signature of tooth enamel.

Although a variety of methods have been proposed, both ^{14}C and stable isotope fields typically use an acetic acid leach to remove secondary carbonates prior to analysis (Haynes 1968; Lee-Thorp et al. 1989; Cherkinsky 2009; Zazzo and Saliège 2011; Ventresca Miller et al. 2018). It is recommended that enamel is finely powdered before leaching to allow removal of carbonate contaminants in the pores between the prisms and crystallites (Lee-Thorp et al. 1997; Wood et al. 2016). Whilst the surfaces exposed to acid will dissolve rapidly, the acid leach will also preferentially remove the more soluble endogenous phases of tooth enamel such as the high magnesium amorphous phosphate (Gordon et al. 2015) and crystallite cores which contain the highest concentration of carbonate (Simmelink and Piesco 2001).

Two studies have investigated whether the type of acid used to leach enamel affects the ^{14}C date obtained (Table 1). Hedges et al. (1995) found that two out of three Pleistocene-aged samples from Kents Cavern, UK, produced older ages after a leach in acetic acid than HCl, and concluded that a leach with acetic acid may produce better, though still inaccurate, dates. In contrast, Hopkins et al. (2016) recommended the use of 0.05M HCl to leach tooth enamel after examining one Pleistocene-aged sample from Sutton Courtenay, UK, where

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Table 1 Published ^{14}C dates comparing age after leaching in HCl and acetic acid. Data from Kent's Cavern is from Hedges et al. (1995) and data from Stanton Harcourt is from Hopkins et al. (2016). Data referring to dates on collagen is given in italics. Further associated data (for example yield, stable isotope, and FTIR analysis) is included in the original publication.

Sample	Pretreatment	OxA-	Age (BP)
Kent's Cavern	*Collagen*	*3403*	*39630 ± 1420*
Rhino, 25	None	4271	16250 ± 200
	None	4275	18540 ± 200
	Bleach, HCl (1M, 5 min)	4273	24570 ± 310
	Bleach, Acetic acid (1M, overnight)	4274	19760 ± 200
Kent's Cavern	*Collagen*	*4829*	*40600 ± 1700*
Rhino, 4/3470	Bleach, HCl (1M, 5 min)	4823	17530 ± 140
	Bleach, Acetic acid (1M, overnight)	4821	25400 ± 280
Kent's Cavern	*Collagen*	*4831*	*27640 ± 400*
Horse, '2/3536	Bleach, HCl (1M, 5 min)	4827	10920 ± 100
	Bleach, Acetic acid (1M, overnight)	4824	21040 ± 240
Stanton Harcourt,	*Collagen*	*20989*	*39200 ± 800*
wooly rhino	None	X-2529-7	15940 ± 75
	0.01M HCl 2 hr	X-2529-8	17920 ± 100
	0.01M HCl 4 hr	X-2529-9	17700 ± 90
	0.05M HCl 2 hr	X-2529-10	20070 ± 150
	2M Acetic acid 4 hr	X-2529-11	19390 ± 170

they found treatment with acetic acid produced a younger age than treatment with more concentrated HCl. The difference was small at just 680 ± 230 ^{14}C years. Neither study reacted the sample under vacuum. Neither study was able to propose a reason why the two acids could give different results, although Hedges et al. (1995) surmised that "Presumably, the dissolution of hydroxyapatite by HCl is less discriminating."

Dissolution of teeth is caused by reaction with the hydrogen ion from acids and by chelating agents (anions which can bind or complex calcium) (Featherstone and Lussi 2006). Both play a role in enamel erosion and the formation of caries, and have been studied within dentistry. When a whole tooth is exposed to erosion, the two types of reactions preferentially attack different regions of the enamel (Simmelink et al. 1974). When human enamel is exposed to acid the prism centers are preferentially lost, but when exposed to a chelator, the peripheral regions of the prisms are lost probably because of the larger pore sizes in this area. However, EDTA and acid have the same effect on the dissolution of separated crystals, removing the surface and the core. Despite these early observations, there has been little work on how the different types of acid may attack different parts of the enamel and in particular, the crystallite structure.

To examine whether different acids remove carbonate contaminants from tooth enamel to different extents, this study examined a larger number of teeth from both Holocene and Pleistocene sites with a wider range of acids than Hedges et al. (1995) and Hopkins et al. (2016) alongside the strong chelating agent EDTA.

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METHODS

Samples

In total 10 teeth split into three groups have been studied. The first group contains three teeth from beyond the limit of ^{14}C, providing a sensitive indicator for modern carbon contamination. Five teeth from a late Holocene site demonstrate the effectiveness of the protocol on young materials from a very different depositional environment, and two modern teeth act as standards. All samples are *Sus scrofa* third molars. Pigs are abundant in South East Asian Pleistocene and Holocene assemblages and their third molars are large, with enough material for multiple dates.

The three Pleistocene teeth (DU795, 798 and 801) are from the site of Duoi U'Oi (Man Duc village, ca. 85 km SW Hanoi), a cave in tower karst in humid subtropical northern Vietnam where an assemblage of faunal remains was recovered from a breccia in 2003 (Bacon et al. 2008, 2015). A speleothem found towards the top of the bone bearing breccia has been dated by U-series to 66 ± 3 ka (Bacon et al. 2008), and a U-series date on DU795 suggests an age of at least 100 ka (Wood et al. 2016). As the teeth date beyond the limit of ^{14}C, any ^{14}C present must be a contaminant.

Five teeth from Lo Gach (Table SOM 1), in southern Vietnam provide an indication of the success of pretreatment from a younger and very different depositional environment. Lo Gach is a late-Neolithic to Early Metal Age site on the banks of the Vam Co Tay river. A ca. 1 m cultural deposit was excavated in 2014 by a collaborative team from Long An Provincial Museum, the Southern Institute for Archaeological Research, Ho Chi Minh City, and the School of Archaeology and Anthropology, Australian National University (Piper personal communication 2019). The archaeological site consisted of a series of external deposits, probably associated with a nearby dwelling, that had built up through various processes including the deliberate laying of surfaces, dumping of organic and inorganic refuse and detritus from agricultural (rice processing) and the stalling of animals (Castillo 2019). Together, these processes produced a complex sequence of depositional events and multiple thin strata. No collagen is preserved in the tooth dentine, and comparative ages are not available.

One modern sample (FP) was from a road kill in Corsica, collected defleshed in 2014. The other (BB9) was taken from a pig farm near Byron Bay, Australia, in 2015 and was again collected defleshed. Both died a few years prior to collection and are in the early phases of diagenesis having been weathered on the surface and/or partially buried.

Pretreatment

Following Wood et al. (2016) the dentine, surface and material within cracks was carefully removed from enamel with a Dremel™ drill with a tungsten carbide drill bit and diamond cutting disk. Dust was removed by repeatedly ultrasonicating in MilliQ™ water, before the enamel was crushed by hand in an agate pestle and mortar under MilliQ water. The powder was then ground under MilliQ water in a McCrone™ microniser with agate beads for 30 min (in 5-min intervals to avoid overheating) and freeze-dried.

Initially, two acid leaching protocols similar to Hedges et al. (1995) and Hopkins et al. (2016) were applied, comparing the effectiveness of acetic acid (VWR HiPerSolv Chromanorm™) and HCl (VWR AnalR Normapur™) in teeth from Lo Gach and DU801. Subsequently,

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Table 2 Summary of pretreatment conditions used in this study. When the pKa of a weak acid equals the pH, 50% of the acid is dissociated. The higher the pKa, the weaker the acid. $LogK_{Ca}$ refers to the stability constant with calcium ion (thermodynamic values for 25°C). Higher values indicate stronger binding. pKa and $LogK_{Ca}$ values are from Featherstone and Lussi (2006).

Protocol	Acid type	pKa	$LogK_{Ca}$	Concentration	Volume, per 100 mg enamel
1	HCl	<1		0.1 M	4.2 mL
2	Lactic acid	3.86	1.45	0.1 M	2.8 mL
3	Acetic acid	4.74	1.18	1 M	2 mL
4	Propionic acid	4.87		0.5 M	2 mL
5	EDTA/acetic acid		10.7 EDTA, 1.18 (acetic acid)	0.05 M EDTA, 1M acetic acid	2 mL EDTA/ 1.5 mL acetic acid

DU798 was also pretreated with propionic (Sigma Aldrich™) and lactic (Alfa Aesar™) acids to examine a potential link between contaminant removal and pKa. Acid concentration and volume was designed to remove approximately 30% of the enamel, and conditions are listed in Table 2. Most samples were reacted for 20 hr at room temperature under a weak vacuum in a Vacutainer™. To achieve a weak vacuum, the Vacutainer was evacuated to $<10^{-1}$ Torr prior to the addition of acid. After 1 hr, the acid was frozen in a dry-ice/ethanol slush to protect the vacuum pump, and evacuated to $<10^{-1}$ Torr. Samples leached in HCl from Lo Gach, were leached for 1 hr after which time the reaction was complete.

Reaction under vacuum was used to speed the rate of reaction. Acid leaching of enamel removes both carbonates (e.g. calcite) and bioapatite producing CO_2 gas and dissolved phosphates. The latter can precipitate under some conditions as secondary phosphates, such as brushite. Therefore, reaction under a vacuum may also avoid the inclusion of young carbon from the atmosphere or evolved from the sample, which could be incorporated as trace contaminants in such secondary phosphates.

To test the effect of a strong chelating agent, EDTA was used to clean two teeth, DU795 and DU801. The ability of a molecule to act as a chelating agent is related to the stability constant (K) for the anion-calcium interaction. This is a measure of the strength of interaction, and the stronger the interaction the more likely the anion is to pull the calcium ion from the apatite surface and into solution. EDTA (log K_{Ca} 10.7) is a well-known chelating agent that can demineralize bone and enamel at neutral pH (Tuross et al. 1988; Korlević et al. 2018). Some acids can act in both ways, continuing to demineralize enamel in a neutral pH. For example, lactic acid (log K_{Ca} 1.45), can demineralize enamel in a neutral pH more readily than acetic acid (log K_{Ca} 1.18) (Featherstone and Lussi 2006).

Samples were ultrasonicated in 1 mL of MilliQ water for 1 hr to break aggregates, and 1 mL/ 100 mg enamel of 0.1 M EDTA (Sigma Aldrich™, ACS) (at pH9) was added, resulting in 2 mL of 0.05 M EDTA/100 mg enamel. Samples were reacted for 10 min, before being rinsed in MilliQ water. As CO_2 is soluble in a basic solution, the samples were subsequently treated with acetic acid under vacuum for 20 hr. Again, the pretreatment conditions were designed

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to remove around 30% of the initial enamel weight. In an experiment on modern enamel, the EDTA treatment removed around 10% of the sample and the acetic acid around 20%.

Radiocarbon Dating

The routine S-ANU laboratory protocol for carbonates includes freeze-drying of the cleaned sample, before reaction with phosphoric acid (85%, 80ºC, 2 hr) in a Vacutainer^{TM} previously evacuated to less than 3 x 10^{-3} Torr. The CO_2 generated is cryogenically collected and reacted with hydrogen over an iron catalyst to produce graphite, prior to measurement in an NEC Single Stage AMS (Fallon et al. 2010). Dates are calculated according to Stuiver and Polach (1977) using an AMS derived $\delta^{13}C$.

However, we have noticed that it often takes more than 6 hr to graphitize the CO_2 generated from reaction of enamel with phosphoric acid. This is presumably due to the presence of sulphur dioxide, although it is unclear why this should be present in enamel. We therefore followed protocols common when dating cremated bone which can contain high concentrations of sulphur dioxide (Naysmith et al. 2007). After reaction with phosphoric acid, the CO_2 was cryogenically collected into a quartz ampule evacuated to less than 3 × 10^{-3} Torr, and heated to 900ºC for 6 hr in the presence of silver foil and copper oxide wire prior to graphitsation. Dates on enamel from S-ANU 54306 onwards have been treated with this acidification-combustion method. This extra step introduces slightly more laboratory derived CO_2 than the routine protocol for carbonates, and the background calculation is described in the supplementary online material (SOM 2.1). Dates on enamel with S-ANU 54305 or earlier have had the routine laboratory background subtracted based on the long-term average of dates on IAEA-C1 graphitized without the precombustion step.

Given the exceptionally large surface area of the micronized samples, it was thought prudent to check whether enough atmospheric CO_2 could adsorb to the enamel surface between freeze-drying and reaction with phosphoric acid to affect the ^{14}C age. DU798 was exposed to air for up to 3 weeks, but contamination from atmospheric CO_2 was not observed (SOM 2.2). As a precaution, acid cleaned samples were exposed to atmosphere for ≤1 hr before graphitization in this study. Where samples were not exposed to atmosphere, yield information is not available.

FTIR

FTIR was undertaken to assess preservation of the original enamel structure and identify secondary phosphates and carbonates. Methodology details are given in the supplementary online material (SOM 3.1). As a likely carbonate contaminant, calcite presence was assessed using a band at 711 cm^{-1} (Lee-Thorp and van der Merwe 1991). When enamel is left in a weakly acidic solution, brushite ($CaHPO_42H_2O$) can form, with amounts increasing as reaction time is extended (Lee-Thorp and van der Merwe 1991). Reaction was carried out under a weak vacuum and brushite will only contain carbon as an impurity, meaning the ^{14}C dates and stable isotope analyses should not be greatly affected. However, %C measurements could be affected. Brushite was primarily identified by a band at –525 cm^{-1} resulting from a $\nu 4$ O-P-O deformation. A sharp band at –1645 cm^{-1} resulting from the $\nu 2$ water H-O-H deformation was seen in samples containing the most brushite, and a band at –792 cm^{-1} resulting from a γ_{OH} out of plane P-O-H deformation was used to identify smaller quantities of brushite (Berry and Baddiel 1967; Petrov et al. 1967; Lee-Thorp and van der Merwe 1991).

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A variety of indices derived from FTIR spectra are widely used amongst the stable isotope community to assess enamel preservation and thus argue for the integrity of the stable isotope data (Table SOM 3) (Roche et al. 2010; Roberts et al. 2018). The Infra Red Splitting Factor (IRSF) gives information about crystal order, a combination of crystal size and level of impurities. BPI and API give information on the amount of carbonate in the B (substituting for the phosphate ion) and A (substituting for the hydroxyl ion) positions in the hydroxyapatite lattice.

Bandwidth increases with increasing grain size (Ruppin and Englman 1970), strongly affecting indices (Surovell and Stiner 2001) making it difficult to compare the untreated samples which were hand-ground with the micronized and leached samples. It is however possible to compare within the micronized samples. One sample (LGa923HG) has been analyzed 3 times. Uncertainties are <0.1, <0.02, and <0.01 for IRSF, BPI and API respectively for indices derived from both the grinding curve and individual KBr pellet methods.

IRMS

%C, $\delta^{13}C$, and $\delta^{18}O$ analyses were undertaken either on a Gas Bench connected to a Sercon 20-22 isotope ratio mass spectrometer operating in continuous flow mode, or on a Thermofisher Delta Advantage mass spectrometer coupled to a Kiel IV carbonate device.

On the Sercon 20-22 instrument, approximately 22 mg enamel was reacted with 0.5 mL 99% H_3PO_4 at 80ºC to generate CO_2. Samples were measured against an in-house calcium carbonate standard and normalized to Vienna Peedee Belemnite scale (VPDB) using NBS18, LSVEC and an in-house standard P3. %C was calculated from beam area.

On the Thermofisher instrument results have been normalized with the NBS-19 and NBS-18 carbonate standards. The composition of the reference gas used during analysis is $\delta^{13}C$ –20.620‰ VPDB and $\delta^{18}O$ –3.450‰ VPDB. Samples were reacted with 105% H_3PO_4 at 75ºC to produce CO_2. The pressure of the purified CO_2 gas in the Kiel device (MV1) is related to the weight of the carbonate in the sample/standard in a linear fashion. This was used to calculate an estimate of the %C in the samples by feeding their MV1 into the linear correlation obtained using the weight and MV1 pressure of the carbonate standards (from the same and previous day's run). Since the correlation is not perfectly linear and can vary with the conditions of the run, the systematic error associated to this calculation can be up to 20% in $\%CaCO_3$ and therefore %C is used as an estimate.

1-sigma random uncertainties, calculated as the standard deviation of repeat measurements of tooth enamel, were typically 0.01%, 0.1‰, and 0.2‰ for %C, $\delta^{13}C$, and $\delta^{18}O$, respectively on the Sercon 20-22 instrument, and 0.01%, <0.1‰ and 0.2‰ for %C, $\delta^{13}C$, and $\delta^{18}O$, respectively on the Thermofisher instrument.

The low number of samples available for each tooth limits the statistical analysis possible, so only broad trends have been identified within the data.

RESULTS AND DISCUSSION

Preservation of Tooth Enamel

FTIR analysis of the enamel shows that calcite is not present in any sample (Table 3). Although the hydroxyapatite appears relatively well preserved when compared with modern pigs, small

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Table 3 Results of the ^{14}C, stable isotope and FTIR analyses on samples pretreated with different acid protocols. 1 σ random uncertainties are listed. Given potential large systematic uncertainties in %C, these values should only be used as estimates. Protocol 0 represents no treatment, 1. hydrochloric acid, 2. lactic acid, 3. acetic acid, 4. propionic acid, and 5. acetic acid and EDTA (Table 2). BPI refers to the B-carbonate on phosphate index, and API the A-carbonate on phosphate index (Table SOM 3).

Sample	Protocol	% yield	S-ANU#	$F^{14}C$	^{14}C age (BP)	Brushite	%C ± <0.02	$\delta^{13}C$	$\delta^{18}O$	IRSF ± <0.1	BPI ± <0.02	API ± <0.01	API/BPI
DU801	0*	NA	51712	0.1418 ± 0.0013	15693 ± 76	NA	0.85	–11.8 ± 0.2	–5.58 ± 0.03	4.00	0.352	0.141	0.40
	1	72.9	51716	0.0846 ± 0.0009	19841 ± 88	s	0.70	–13.0 ± 0.2	–6.58 ± 0.03	4.99	0.270	0.075	0.28
	1	72.4	51719	0.0854 ± 0.0009	19762 ± 93	s	0.67	–13.1 ± 0.2	–7.20 ± 0.03	4.71	0.258	0.069	0.27
	3	67.7	51717	0.0333 ± 0.0007	27323 ± 177	s	0.62	–13.8 ± 0.2	–6.79 ± 0.03	5.31	0.255	0.070	0.27
	3	68.3	51720	0.0329 ± 0.0007	27424 ± 183	s	0.60	–13.9 ± 0.2	–6.93 ± 0.03	5.19	0.257	0.071	0.28
	5	62.7	61321	0.0332 ± 0.0009	27345 ± 212	s	0.48	–14.26 ± 0.02	–6.19 ± 0.2	5.34	0.254	0.071	0.28
DU798	0*	NA	NA	NA	NA	NA	NA	NA	NA	3.96	0.330	0.134	0.41
	1	NA	54316	0.0749 ± 0.0013	20817 ± 139	y	0.66	–12.8 ± 0.1	–5.81 ± 0.1	5.19	0.270	0.063	0.23
	2	NA	54317	0.0542 ± 0.0012	23410 ± 177	N	0.63	–12.8 ± 0.1	–6.15 ± 0.1	5.34	0.278	0.059	0.21
	3	68.4	54311	0.0359 ± 0.0011	26725 ± 246	s	0.64	–13.1 ± 0.1	–5.98 ± 0.1	4.83	0.257	0.076	0.29
	4	NA	54318	0.0409 ± 0.0012	25672 ± 236	N	0.70	–13.1 ± 0.1	–6.3 ± 0.1	5.14	0.269	0.065	0.24
DU795	0*	NA	44709	0.1262 ± 0.0010	16627 ± 68	NA	0.74	–12.2 ± 0.1	–8.2 ± 0.2	3.96	0.330	0.134	0.41
	3	68.3	44721	0.0214 ± 0.0008	30878 ± 298	n	0.61	–13.6 ± 0.1	–7.48 ± 0.2	4.03	0.305	0.134	0.44
	4	60.0	61320	0.0288 ± 0.0008	28506 ± 240	s	0.60	–13.64 ± 0.02	–7.6 ± 0.2	5.23	0.242	0.065	0.27
14LGa786	0*	NA	50735	0.7503 ± 0.0021	2308 ± 27	NA	0.70	–13.1 ± 0.1	–5.7 ± 0.2	3.99	0.274	0.118	0.43
	1	70.9	50717	0.7338 ± 0.0019	2487 ± 26	y	0.60	–13.5 ± 0.1	–5.5 ± 0.2	4.35	0.242	0.094	0.39
	3	62.6	50716	0.7346 ± 0.0019	2478 ± 26	s	0.65	–13.6 ± 0.1	–5.1 ± 0.2	4.39	0.246	0.100	0.41
14LGa921	0*	NA	50736	0.7389 ± 0.0019	2431 ± 26	NA	0.73	–11.2 ± 0.1	–4.9 ± 0.2	3.96	0.291	0.121	0.41
	1	73.3	50720	0.7335 ± 0.0020	2489 ± 27	y	0.55	–11.7 ± 0.1	–4.6 ± 0.2	5.25	0.234	0.068	0.29
	3	64.0	50719	0.7290 ± 0.0020	2539 ± 28	s	0.62	–11.7 ± 0.1	–4.0 ± 0.2	4.24	0.232	0.105	0.45
14LGa923	0*	NA	50931	0.7424 ± 0.0024	2393 ± 32	NA	0.69	–12.0 ± 0.1	–6.9 ± 0.2	3.83	0.282	0.132	0.47
	1	72.4	50725	0.7366 ± 0.0022	2456 ± 29	y	0.53	–12.5 ± 0.1	–7.0 ± 0.2	5.15	0.226	0.069	0.30
	3	67.1	50724	0.7319 ± 0.0020	2507 ± 27	y	0.56	–12.5 ± 0.1	–6.9 ± 0.2	5.57	0.221	0.063	0.29

Table 3 (*Continued*)

Sample	Protocol	% yield	S-ANU#	$F^{14}C$	^{14}C age (BP)	Brushite	%C ± <0.02	$\delta^{13}C$	$\delta^{18}O$	IRSF ± <0.1	BPI ± <0.02	API ± <0.01	API/BPI
14LGa932	0*	NA	50932	0.7601 ± 0.0025	2204 ± 31	NA	0.80	–12.3 ± 0.1	–6.5 ± 0.2	3.77	0.335	0.154	0.46
	1	71.4	50727	0.7432 ± 0.0020	2384 ± 26	y	0.53	–12.8 ± 0.1	–7.3 ± 0.2	4.24	0.245	0.100	0.41
	3	70.9	50726	0.7373 ± 0.0020	2448 ± 27	s	0.65	–13.0 ± 0.1	–6.3 ± 0.2	4.84	0.240	0.084	0.35
14LGa935	0*	NA	50933	0.7867 ± 0.0035	1927 ± 40	NA	0.78	–10.2 ± 0.1	–3.8 ± 0.2	4.01	0.387	0.182	0.47
	1	77.2	50730	0.7498 ± 0.0021	2314 ± 27	y	0.60	–12.4 ± 0.1	–6.1 ± 0.2	5.25	0.278	0.102	0.37
	3	60.8	50729	0.7401 ± 0.0021	2418 ± 27	y	0.66	–12.7 ± 0.1	–5.8 ± 0.2	4.29	0.244	0.098	0.40
BB9	0*	NA	NA	NA	NA	n	0.72	–8.0 ± 0.03	–4.0 ± 0.3	4.08	0.284	0.112	0.40
	1	66.2	NA	NA	NA	n	0.69	–8.1 ± 0.03	–4.7 ± 0.3	4.41	0.252	0.093	0.37
	3	61.2	NA	NA	NA	n	0.69	–7.8 ± 0.03	–4.6 ± 0.3	4.59	0.258	0.090	0.35
	5	48.8	NA	NA	NA	n	0.56	–8.14 ± 0.02	–3.6 ± 0.2	5.26	0.241	0.077	0.32
FP	0	NA	NA	NA	NA	NA	0.67	–14.85 ± 0.1	–4.04 ± 0.2	4.34	0.296	0.099	0.33
	1	70.4	NA	NA	NA	n	0.63	–14.8 ± 0.1	–4.16 ± 0.2	4.74	0.254	0.068	0.27
	2	70.8	NA	NA	NA	n	0.64	–14.6 ± 0.1	–5.71 ± 0.2	4.58	0.281	0.077	0.27
	3	NA	NA	NA	NA	y	0.57	–15.0 ± 0.1	–5.22 ± 0.2	5.00	0.245	0.070	0.29
	4	59.9	NA	NA	NA	y	0.52	–14.9 ± 0.1	–5.01 ±0.2	4.82	0.239	0.064	0.27

s – Small peak around –792 cm^{-1}, but no shoulder or band at –525 cm^{-1}. y – Shoulder or band at –525 cm^{-1}. May also have a sharp band at –1645 cm^{-1}. n – No brushite observed. * – FTIR indices are derived from a grinding curve.

differences are seen between the modern and ancient pig teeth in this study. Unexpectedly, the IRSF of the ancient enamel (ranging between 3.8–4.0) is lower than the modern pigs (ranging between 4.1–4.3) suggesting lower crystal order. The reverse is expected from a recrystallization process which is likely to result in larger crystals with less carbonate (Asscher et al. 2011). %C is generally higher in the ancient teeth (ranging 0.69–0.85 %C) than in the modern teeth (ranging 0.67–0.72 %C), perhaps in part explaining the lower crystal order of the ancient samples.

API and BPI are both generally higher in the ancient samples (0.12–0.18 and 0.27–0.39 respectively) than the modern teeth (0.10–0.11 and 0.28–0.30). This contrasts to Roche et al. (2010) and Roberts et al. (2017) who both found that BPI did not increase during burial, whilst API did. However, the ratio between these (API/BPI) is slightly higher in the ancient teeth (ranging 0.40–0.47) than the modern teeth (0.33–0.40) suggesting a larger amount of A-type carbon relative to the B-type carbon, in line with Roche et al. (2010) and Roberts et al. (2017).

Currently, it is unclear why the ancient samples contain more carbonate within the apatite lattice, and why the location of this carbonate within the crystal structure seems to vary in comparison with previous studies. Whilst likely diagenetic, it may also reflect the small sample size and substantial natural variation. For example, carbonate content decreases during tooth formation (Sydney-Zax et al. 1991), between permanent and deciduous teeth (Sønju Clasen and Ruyter 1997) and across the enamel thickness (Xu et al. 2012).

Pretreatment

% yield ranged between 60 and 80%, with an average of 66 ± 3% surviving the acetic acid leach, and 73 ± 2% surviving the HCl treatment. Brushite was sporadically observed in the pretreated apatite, and was caused by both weak and strong acids (Table 3).

Effect of Leaching on Radiocarbon Dating

The Pleistocene Duoi U'Oi samples are heavily contaminated, as previously found (Wood et al. 2016) (Table 3). If we assume that the contaminant is modern in age, nearly 15% of the carbon in the carbonate fraction of both DU795 and DU798 must be a contaminant. The actual contaminant load is likely to be much higher as the contaminant is probably several thousand ^{14}C years old due to dissolved limestone in the breccia water.

All Duoi U'Oi samples produced markedly different results between HCl (protocol 1) and acetic acid (protocol 3) treatments (Figure 1), and the effect of pretreatment was very consistent when replicated on DU801, despite the large contaminant load. After HCl treatment, nearly 8% modern carbon remained, but this was more than halved when acetic acid was used. In terms of BP, ages improved from around 20 kBP to 27 kBP between HCl and acetic acid treatments. Our HCl acid leach removed slightly less material than the acetic acid (27± 2% and 34 ± 3% respectively). Unfortunately, the enamel samples were small and did not allow for a repeat of the experiment. However, the small difference in yield between the two pretreatments is unlikely to have caused such a large difference in $F^{14}C$. Brushite was not observed in the enamel leached with HCl from Duoi U'Oi and could not have affected the results.

At Lo Gach, substantial amounts of brushite were present in all HCl leached samples. Whilst one sample, LGa786, sees little difference in age between the two treatments (an increase of just

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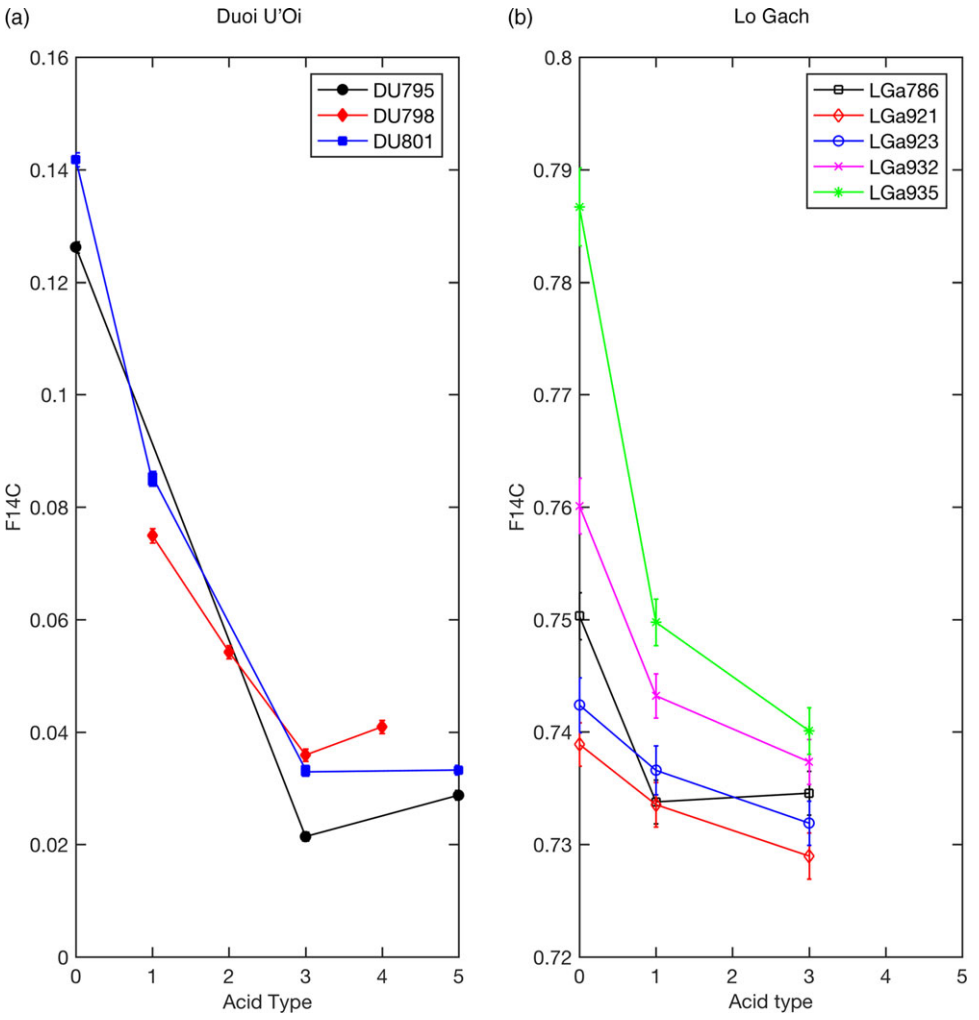

Figure 1 The effect of the type of acid used in pretreatment on $F^{14}C$ for (a) samples known to date beyond the limit of ^{14}C from Duoi U'Oi and (b) samples of ca. 2000 cal BC from Lo Gach. 0 represents no treatment, 1. hydrochloric acid, 2. lactic acid, 3. acetic acid, 4. propionic acid, and 5. acetic acid and EDTA.

9 ± 37 ^{14}C years between acetic acid and HCl), the remaining four samples saw the age increase after treatment with acetic acid in comparison with hydrochloric acid. However, the increase was only significant for LGa935 at 95 % probability (X^2, df 1, T=7.4 (5% 3.8)). This sample also has the greatest reduction in $F^{14}C$ between unleached and leached samples, suggesting it is the most contaminated and therefore the most likely to be affected by different leaching protocols. Indeed, it would not be possible to distinguish between samples similar in age to those at Lo Gach with 6% and 4% modern carbon contamination. It is likely that the less pronounced trends at Lo Gach reflect the reduced sensitivity to contamination in younger periods. However, the consistency of the decrease in $F^{14}C$ between HCl and acetic acid leaches at Lo Gach supports the pattern seen in the much older and more sensitive samples from Duoi U'Oi.

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To examine why these differences occurred, one Pleistocene sample was also subjected to lactic acid (protocol 2) and propionic acid (protocol 4) to cover a range of pKa's. With increasing pKa the age appears to increase, with lactic acid producing an age mid-way between the sample treated with HCl and the sample treated with acetic acid. Propionic acid produced a similar, though slightly younger age, to acetic acid. This implies a link between acid strength and removal of carbonate contaminant.

The combined acetic acid-EDTA treatment (protocol 5) gave an age indistinguishable from that produced by the acetic acid treatment for tooth DU801 and slightly younger for tooth DU795. It is likely that EDTA does not remove more contamination than the acetic acid alone.

Effect of Acid Leaching on Stable Isotopes

Phases that are enriched in carbonate are preferentially removed by all pretreatments, as %C tends to decrease during pretreatment (Table 3, Figure 2a–c). These carbon rich phases also contain a higher contaminant load, as at Duoi U'Oi %C increases with $F^{14}C$ in all three teeth (Figure 3a). The presence of large amounts of brushite in the teeth from Lo Gach leached in HCl hinders interpretation of %C.

$\delta^{13}C$ also appears related to $F^{14}C$ in enamel from both Duoi U'Oi and Lo Gach (Figure 3c–d), with offsets between the untreated and leached samples being more than 2‰ in DU801 and LGa935. Correlation analyses for samples with just 3 data points are difficult to analyze statistically, and so a relationship is implied by all teeth showing increasing $\delta^{13}C$ with $F^{14}C$, rather than assessed with e.g. Pearsons Correlation Coefficient. Treatments that are more efficient at removing ^{14}C contamination, also remove carbonate higher in $\delta^{13}C$.

Although several studies have noticed changes in the $\delta^{13}C$ of modern enamel leached in acetic acid (Koch et al. 1997; Pellegrini and Snoeck 2016), this change is often much smaller. For example, Koch et al. (1997) found a decrease of just 0.27 ± 0.09‰ between untreated enamel and enamel leached in 1M acetic acid after a bleaching step. There is also only a little variation in the two modern pig molars examined here, with FP values varying by 0.3‰ (with an analytical uncertainty of <0.1‰) and BB9 by 0.4‰ (with an analytical uncertainty of 0.1‰) (Figure 2f). Moreover, this variation is not systematic with some treatments giving values more enriched in ^{13}C and others giving values depleted in ^{13}C compared to the untreated sample, and variation may just imply inhomogeneity within the sample.

$\delta^{18}O$ shows little systematic change between leached and unleached enamel and does not appear related to $F^{14}C$ (Figures 2g–i and 3e–f). This is probably due to fractionation during the long acid leach (Balter et al. 2002).

Effect of Acid Leaching on Enamel Structure; FTIR

Detailed analysis of FTIR parameters is complicated by the variable bandwidth between the handground unleached samples and the micronized leached samples. For example, IRSF tends to be higher in the leached samples (Figure SOM 2a–c), although this trend would be expected based on the smaller grain size of the leached samples. Overall, there seems little relationship between $F^{14}C$ and IRSF within the leached samples (Figure SOM 3a–b).

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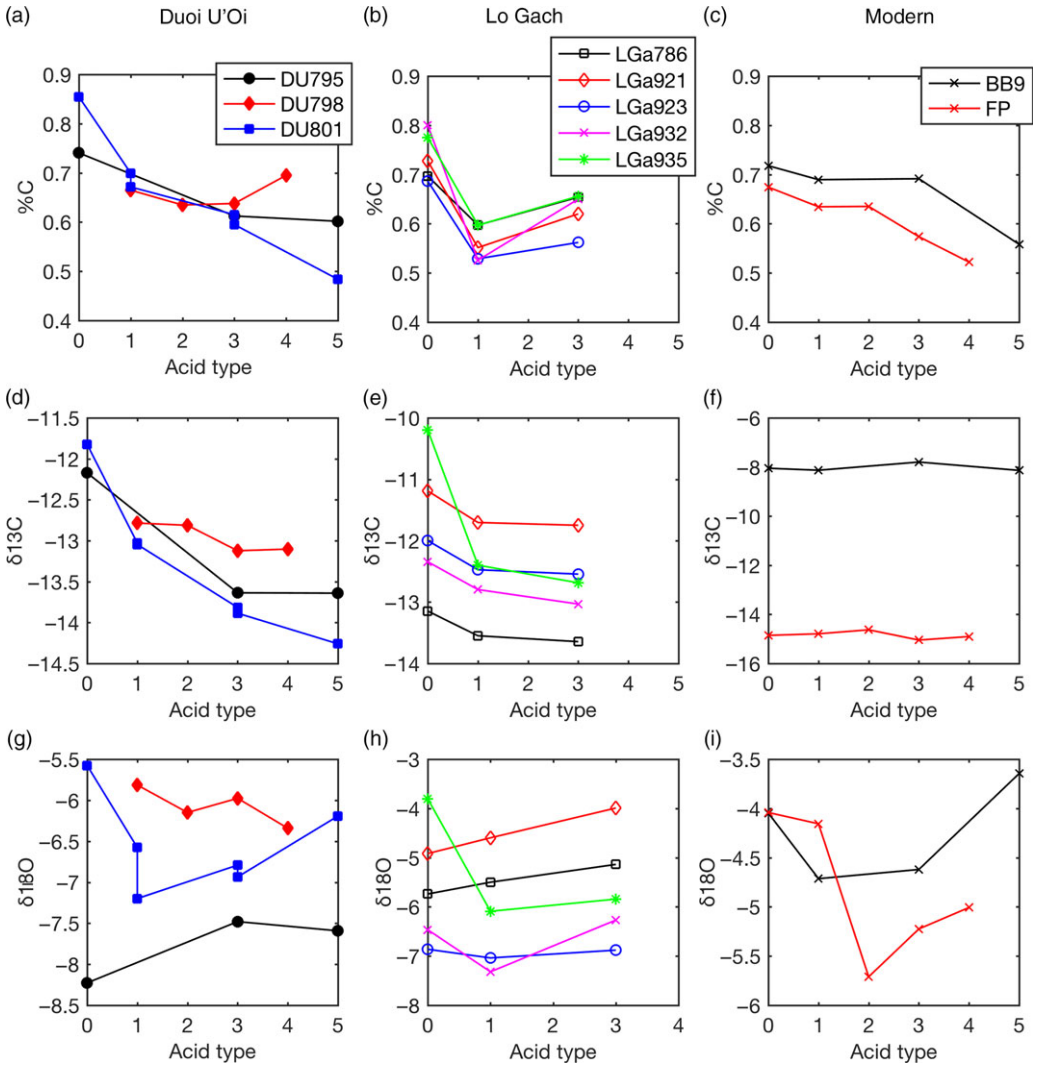

Figure 2 The effect of the type of acid used in pretreatment on (a–c) %C, (d–f) $\delta^{13}C$, (g–i) $\delta^{18}O$, for teeth from Duoi U'Oi (a, d, g), Lo Gach (b, e, h) and modern teeth (c, f, i). 0 represents no treatment, 1. hydrochloric acid, 2. lactic acid, 3. acetic acid, 4. propionic acid, and 5. acetic acid and EDTA.

As may be expected from the reduction in %C after pretreatment both BPI and API decrease during pretreatment (Figure SOM 2d–i), and a positive relationship between $F^{14}C$ and BPI is particularly clear for the teeth from Lo Gach (Figure SOM 3d). Indeed, the relationship between BPI or API and $F^{14}C$ is clearer than for %C at this site, probably because of the presence of brushite in the enamel leached with HCl. Both BPI and API indices were elevated in the untreated ancient teeth in comparison to the modern teeth (Figure SOM 2g–l), and both decrease to approximately the same value as the modern teeth treated in the same way. However, although API/BPI was also elevated in the ancient untreated teeth (Figure SOM j–l), pretreatment does not consistently reduce the ratio towards the values seen in the modern teeth, and there seems little relationship between API/BPI and $F^{14}C$ at

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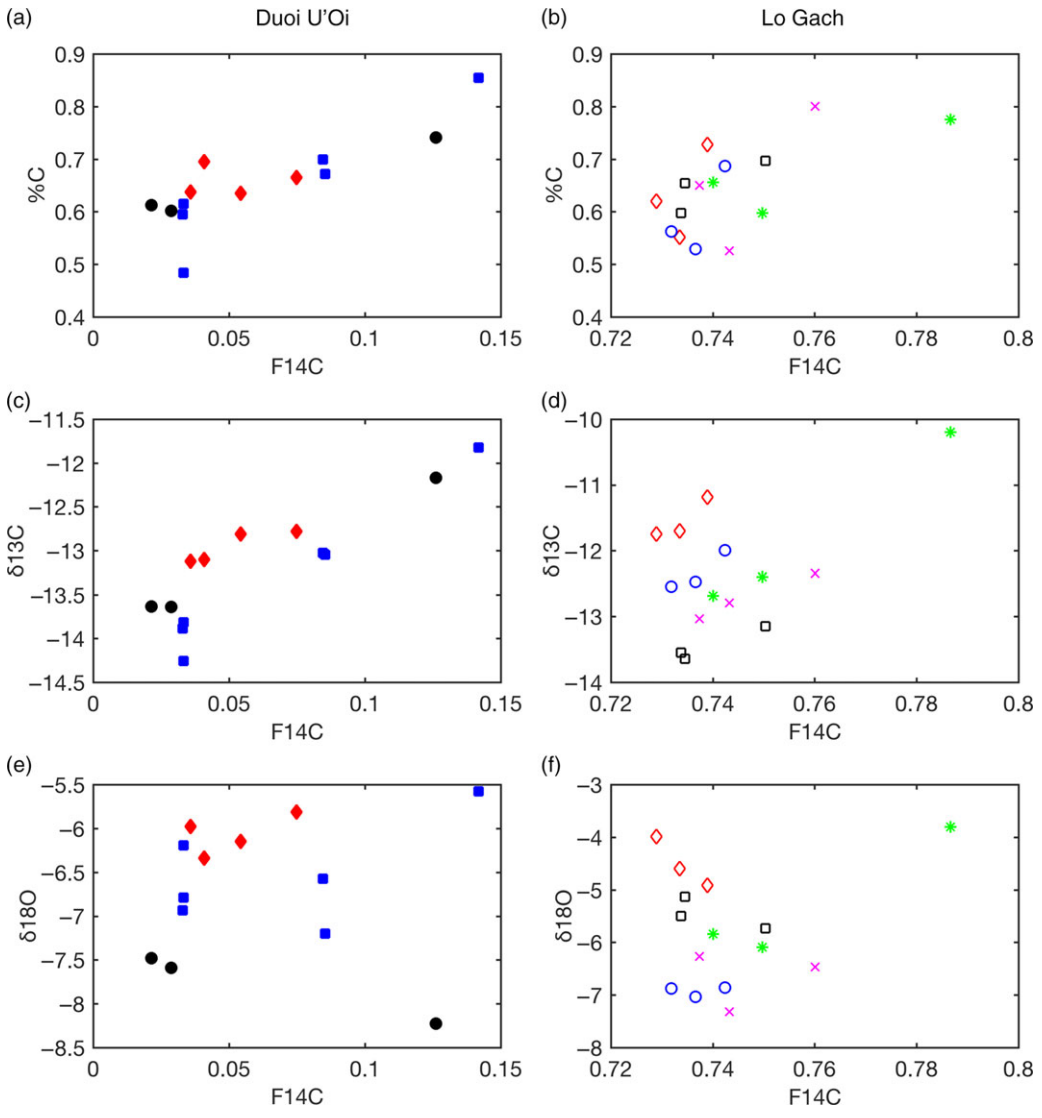

Figure 3 Comparison of (a–b) %C, (c–d) $\delta^{13}C$, (e–f) $\delta^{18}O$ with $F^{14}C$ for teeth from Duoi U'Oi (a, c, e) and Lo Gach (b, d, f). For legend, please refer to Figure 2.

either Duoi U'Oi or Lo Gach (Figure SOM 2g–h). Therefore, acids with a higher pKa generally remove more carbon from ancient teeth than those with a lower pKa, but this does not seem to be specific to either B or A type carbonate environments.

CONCLUSION

The type of acid used to remove contamination from tooth enamel appears to have a significant effect on the ^{14}C date obtained. In contrast to Hopkins et al. (2016), but in agreement with Hedges et al (1995), we find that weaker acids (with higher pKa's) remove more contamination than stronger acids. This effect is consistent across two sites containing teeth

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likely to have had different diagenetic histories. Brushite is sporadically formed during leaching in both weak and strong acids. Unexpectedly, HCl treatment appeared to cause larger quantities of brushite to form more regularly than acetic acid.

Despite the presence of brushite in some samples and not others, the relationship between $\delta^{13}C$ and $F^{14}C$ is maintained in all teeth presumably because brushite only contains a small amount carbonate as an impurity. This relationship either implies that contamination causes both the $\delta^{13}C$ and $F^{14}C$ to be shifted to higher values, or that contaminants are located in the same location in the enamel structure as endogenous carbonate with higher $\delta^{13}C$ values (Koch et al. 1997), and both are removed at the same rate by different acid leaching treatments. These possibilities are not exclusive of each other. However, it is clear from this study (alongside August et al. in prep.) that young carbonate contaminants are also present in the ancient teeth and it is likely that these have a different isotopic signature to those of the enamel. It would seem implausible that the shift seen in the $\delta^{13}C$ of ancient tooth enamel is not related to contamination to some degree.

The reason weaker acids remove more contaminants than stronger acids is unclear from the rather coarse FTIR data obtained here. A number of non-exclusive possibilities exist.

1. The nanoparticles produced by mechanical grinding are electrostatic and prone to form aggregates. This could hinder contaminant removal as surfaces of crystals at the center of the aggregate may be protected during acid leaching. Although the aggregates are not easily broken apart by vortex mixing or ultrasonication, they can be disrupted by chelating agents which bond strongly to the surface of the crystal (Corrêa de Araujo et al. 2010). Few visible aggregates were observed when EDTA was used in this study, but we see no improvement between the EDTA and acetic acid treatments. Therefore, formation of aggregates may not play a major role in the unsuccessful removal of contamination.
2. It is possible that physical or chemical mechanisms control which part of the enamel structure and what kind of carbonate the acid reacts with. If it is assumed the majority of contamination is located at the surface of the prisms and crystallites and/or in the amorphous phase (Wood et al. 2016), the large bubble generated by exceptionally vigorous and fast reaction of the strong acids may hinder access of the hydrogen ion into the pores. The slower and continuous reaction of the weaker acids over the full 20 hr may allow a more even reaction over the surface and some reaction with the surface of the pores.
3. Different acids may preferentially react with different phases within the tooth enamel. Acid preferentially attacks apatite rich in carbonate with %C, API and BPI all decreasing during acid leaching and with $F^{14}C$. However, it is not clear that enamel richer in the A type carbonate, which Roche et al. (2010) and Roberts et al. (2017) propose may be diagenetic, is attacked preferentially. Alternatively, the weaker acids may preferentially react with the more soluble amorphous phase which is present between the hydroxyapatite crystallites (Gordon et al. 2015), whilst the stronger acids are able to rapidly react with the hydroxyapatite. The standard FTIR indices only examine the relative proportion of carbonate within the A and B positions in apatite, and it is unlikely that this would be affected by such a mechanism. However, we would expect a difference in carbonate in surface positions or within the amorphous phase. So called "labile" carbonate has been observed in FTIR spectra of bone and enamel, for example at around 866 cm^{-1} (Rey et al. 1989, 1991). Unfortunately, we were unable to produce

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consistent results by peak deconvolution in the very low intensity carbonate band in this study.

Although the reason for the differences between the acids remains uncertain, it does appear from this dataset that hydrochloric acid should not be used to leach tooth enamel for either ^{14}C or stable carbon isotope analysis, and acetic acid should be favored, as normally employed. Regardless of these pretreatment protocols, significant contamination still resides in ancient tooth enamel from sub-tropical environments resulting in an underestimate of the age of the tooth.

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SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit https://doi.org/10.1017/RDC.2021.32

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