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Pre-analytical processing of archaeological mammal enamel apatite carbonates for stable isotope investigations: A comparative analysis of the effect of acid treatment on samples from Northwest Australia

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Abstract

Stable isotopic analysis of palaeontological and archaeological biogenic apatite carbonates from herbivorous mammalian species represents an important tool for worldwide palaeoecological research. Tooth enamel carbonates are more resistant to taphonomic processes than bone or dentine carbonates but are not invulnerable to diagenesis. As such, they require careful pre-analytical processing that considers depositional environment and age. An established part of this process includes a weak acid treatment to remove soluble exogenous carbonates; however, published treatment times for isotopic studies of archaeological tooth enamel are variable and range from fifteen minutes to over eight hours. This study tests three different pre-treatment protocols on modern and Pleistocene age archaeological kangaroo teeth (dating from contemporary to 46000 BP) to assess the effect of acid treatment time on isotopic integrity. The results indicate that treatment time is a critical parameter for producing consistency across results and shorter pre-treatments of four hours or less are preferable for removing diagenetic carbonates while minimising alteration of the biological signal.

1. Introduction

Stable isotopic analyses of preserved animal tissues (including collagen, dentine, and tooth-enamel) is a well-established method for investigating paleoecological relationships and past environments in global archaeological and palaeontological research (Arnold et al. 2013; Ayliffe and Chivas 1990; Balassae et al. 2003; Chrisholm et al. 1882; Deith 1983; DeNiro and Epstein 1978; Disspain et al. 2011; Eerkens et al. 2013; Fisher and Valentine 2013; Fraser et al. 2008). This is because the isotopic composition of herbivorous mammal tissue is primarily a function of diet and water intake and therefore allows for important aspects of paleo environments (including relative humidity and vegetation structure) to be inferred (Ambrose 1991; Ben-David and Flaherty 2012; Bryant and Froelich 1995; Cerling and Harris 1999; Heaton et al. 1986; Shackleton 1973; Shoeninger and de Niro 1984; Sullivan and Kruger 1983; van de Merwe 1982). Importantly, tooth enamel bioapatite, when compared with bone collagen or bone mineral, is generally considered to be the most reliable biogenic material due to its high mineral content and corresponding low susceptibility to diagenetic alteration (Balasse 2002; Hedges 2002; Koch 2007; Shin and Hedges 2012). Bioapatite phosphate is more resistant to diagenetic change than bioapatite carbonate; however, due to procedural and cost efficiencies, enamel

carbonates are more often favoured for analytical studies (Clementz 2012; Sharma et al. 2004; Skrzypek et al. 2011).

Despite the benefits and relative stability of enamel carbonates, the potential for taphonomic impacts on bioapatite isotopic signatures is not insignificant (Kohn and Cerling 2002; Lee-Thorpe and van der Merwe 1991). Indeed, shifts in isotopic signal due to the absorption of intrusive diagenetic carbonates may potentially mimic the level of variation expected for archaeologically important shifts in climate, vegetation structure, and animal diet (Beasley et al. 2014; Beshah et al. 1990; Bocherens 1994; Keenan et al. 2016; Koch et al. 2007; Kruger 1991; Norman Wilson 2013; Sponheimer and Lee-Thorp 1999). It is hence critical that effective and consistent pre-analytical protocols be developed and implemented. As a result, there is a substantial corpus of research aiming to: (i) model diagenetic processes (Nielson-Marsh and Hedges 2000a; Wang and Cerling 1994; Zazzo et al. 2004); (ii) assess the integrity of biogenic signal in carbonate samples (Lebon et al. 2014; Shin and Hedges 2012; Zazzo 2014); and (iii) determine optimum chemical pre-treatment protocols for removing post-depositional contaminants (Balasse et al. 2002; Crowley and Wheatley 2014; Garvie-Lok et al. 2004 Koch et al. 2007; Nielson-Marsh and Hedges 2000b; Norman-Wilson 2013; Snoeck and Pellegrini 2015; Pellegrini and Snoeck 2016). Although considerable work has been undertaken, there is significant variation in the range of reported methods used by researchers and no clear consensus on a preferred global procedure.

This paper presents a pre-treatment protocol for mammalian tooth enamel carbonates excavated from archaeological cave deposits. The procedure is based on a review of current methods and new experimental work that utilises kangaroo tooth enamel from the Northwest Australian arid zone to optimise acid treatment times for bioapatite. This research is timely for improving the accessibility of isotopic methods and providing a foundational process for the generation of comparable and consistent results. The following description of the carbonate component of tooth enamel, and existing methods for pre-treating teeth, provides the necessary background for the development, testing, and presentation of a best practice procedure.

1.2 Carbonate component of enamel apatite

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), also known as bioapatite, is the major constituent of tooth enamel (>96% by weight) (Brudevold and Soremark 1967; Trautz 1967; Wang and Cerling 1994). The carbonate component in mammal bioapatite is frequently used for investigating ecological relationships (Ericson et al. 2008; Fricke

and O'Neil 1996; Hoppe et al. 2004; Kohn et al. 1998; Zazzo et al. 2010). This is on the basis that: (i) the carbon isotope ratios ($\delta^{13}C$) reflect relative intake of different vegetation types (DeNiro and Epstein 1987; van der Merwe 1982; Sullivan and Kruger 1983) and; (ii) the oxygen isotope ratios ($\delta^{18}O$) are influenced by ingested and metabolic water (Bryant and Froelich 1995; Fry 2006; Podlesak et al. 2008). Carbonate ions (CO_3^{2-}) in bioapatite can be either structural or labile (Shin and Hedges 2012). Structural carbonate ions are substituted into the crystal lattice at the phosphate ion (PO_3^{4-}) and hydroxyl (OH^-) positions (Kohn and Cerling 2002). Labile carbonate, also known as absorbed or exogenous carbonate, refers to the more soluble, non-lattice bound ions (Crowley and Wheatley 2014). Given that intrusive diagenetic carbonates from soil and ground water in the depositional environment can be incorporated into archaeological tooth enamel, an acid pre-treatment is generally undertaken to remove any labile carbonates (Shin and Hedges 2012). This is in addition to a chemical pre-treatment of hyrdrogen peroxide or sodium hypochlorite to remove organic matter (Crowley and Wheatley 2014; Snoeck and Pellegrini 2015).

1.3 Development of contemporary pre-analytical procedures for removing diagenetic carbonates from enamel

To be considered optimal, a chemical pre-treatment will remove diagenetic contaminants without altering the isotopic integrity of a biogenic material (Crowley and Wheatley 2014). Contemporary acid pre-treatment procedures for removing intrusive carbonates from bone and tooth enamel are derived from foundational experimental work published by Lee-Thorpe and van der Merwe (1991). In this research, infrared spectroscopy and isotopic results demonstrated that treatment with acetic acid effectively removes intrusive carbonates from fossil tooth enamel specimens. However, after prolonged acid treatment, isotopic shifts were also evident in modern specimens. Given that fresh tissue should not contain non-lattice bound carbonate, these shifts were interpreted to be the result of recrystallization of dissolved carbonates to a secondary mineral such as brushite. Since this initial research, concern regarding the impact of chemical treatments on bone and enamel isotope ratios has continued to grow. Indeed, subsequent studies have often focused on introducing subtle modifications to acid concentration and treatment times in order to minimise undesirable isotopic offsets (e.g. Balasse et al. 2002; Garvie-Lok et al. 2004; Koch et al. 1997; Yoder and Bartelink 2010). While it is now well established that treatment with weaker acid is preferable, treatment time is more contentious (Balasse et al. 2002; Garvie-Lok et al. 2004; Koch et al. 1997).

In order to effectively evaluate the efficacy of a pre-treatment process, it is necessary for researchers to distinguish between isotopic shifts resulting from the intended removal of contaminants and changes arising from the unintended alteration of the primary biological signal. Unintended isotopic effects may be the result of partial dissolutions and recrystallization as suggested by Lee-Thorpe and van der Merwe (1991) or, alternatively, could be the outcome of incomplete reactions or the production and absorption of secondary materials during treatments (Crowley and Wheatley 2014; Garvie-Lok et al. 2004). In general, researchers have aimed to identity the occurrence of these undesirable processes in four main ways: (i) tracking changes in crystalline structure using infrared spectroscopy; (ii) examining variations in carbonate content; (iii) quantifying acid dissolution rates; and (iv) analysing fluctuations in isotopic signals (including considering scale and direction of change) (Balasse et al 2002; Crowley and Wheatley 2014; Garvie-Lok et al. 2004; Koch et al. 1997). Importantly, dissolution rates and isotopic signal are frequently focused on particularly in archaeological studies (Balasse et al 2002; Garvie-Lok et al. 2004).

Tracking changes in acid dissolution rate and volume is a simple but effective means for identifying potential changes and underlying reactions. Diagenetic non-lattice bound carbonates should be more soluble than structural carbonates and represent only a small proportion of a powdered sample (Garvie-Lok et al. 2004). Hence, in theory, acid treatment should result in a dissolution profile in which there is an initial sharp drop representing the removal of secondary carbonate. Experimental work by both Balasse et al. (2002) and Garvie-Lok et al. (2004) suggests that this process concludes within the first four hours of treatment. More specifically, Garvie-Lok et al. (2004) noted a plateau in dissolution after four hours while Balasse et al (2002) highlighted that overall sample loss after eight hours results in unacceptable sample loss. That is, based on overall percentage loss, it is evident that structural carbonates have been impacted. Interestingly, acid dissolution has not been examined in detail for treatments times of less than four hours.

Analysis of the impact of acid treatments on isotope ratios can also shed light on the effectiveness of the pre-treatment processes. In theory, modern samples should not contain diagenetic material and hence minimal isotopic change should be observed; however, it has been demonstrated that exposure to acid will generally result in lower $\delta^{13}C$ values and higher $\delta^{18}O$ after prolonger treatment times (>four hours) (Garvie-Lok et al. 2004; Koch et al. 1997). Hence, assuming remnant organic material has been successfully removed from modern samples prior to acid treatment, an effective process can be defined as a protocol that results in negligible

isotopic offsets. However, the same is not necessarily true for archaeological samples.

While it is expected that an initial change should represent the removal of diagenetic material, ongoing disparate variances (such as a reversal in the direction of change and irregular variation in signal magnitude) may be indicative of complex underlying processes. This is because partially dissolved and recrystallised secondary minerals are likely to be isotopically distinct from diagenetic and structural carbonates (Crowley and Wheatley 2014). Furthermore, the impact of pre-treatments is likely to differ relative to the age of the sample and the depositional environment from which it was recovered. For example, structural carbonate in fossilized samples may be more robust to prolonged acid treatment time than recent material (Lee-Thorpe and van der Merwe 1991). Overall, it is likely more important for a pre-treatment protocol to produce consistent results across all samples, rather than produce a particular outcome such as a minimal isotopic offset in archaeological material. Hence, identifying an acid treatment time that results in consistent isotopic change is a key focus for the research presented here.

2. Materials and Methods

2.1 Sampling and pre-analytical processing

In order to test the impacts of acid treatment time on isotopic results, six archaeological and four modern tooth samples from large kangaroo species (*Osphranter* spp.) were collected from modern and archaeological contexts in Northwest Australia (Figure 1 and Table 1). While the data set is small due to the relative scarcity of teeth available from archaeological contexts, the sample size is larger than the majority of published pre-treatments studies (Balasse et al. 2002; Garvie-Lok et al. 2004; Lee-Thorpe and van der Merwe 1991). Given that zooarchaeological assemblages are highly vulnerable to degradation and often poorly preserved in the archaeological record, it is critical that experimental research minimise sample size in order to limit impacts to unreplaceable assemblages. In general, the represented kangaroos are preferential grazers, non-obligate drinkers, and well adapted to arid and semi-arid habitats (Strahan 1995). Archaeological teeth were selected from three northwest sites (Juukan-2, Boodie Cave, and PIL_3160) to represent preservation conditions from early Pleistocene occupation (46000 years ago) through to more recent Holocene (1000 ka) deposits (Figure 2). More detailed analyses of archaeological material excavated from these sites are reported elsewhere (Ditchfield et al. 2018; Veth et al. 2017; Ward et al. 2017; Slack et al. 2018)

Prior to analysis, enamel condition was noted and the exterior surface of each tooth was cleaned of debris (i.e. organic material, dirt, and plaque) using a diamond drill bit. Samples were washed in an ultra-sonicator using demineralized water and allowed to air dry at room temperature. Teeth were then sectioned using a diamond edge blade and the enamel was separated from the internal dentine.

Enamel samples were ground to a fine powder using a mortar and pestle and then enamel for each tooth was separated into four 35 mg aliquots. All powdered tooth enamel samples were treated overnight with 3% hydrogen peroxide to remove organic matter (40 µL per 1.0 mg of enamel). This was followed by treatment with 0.1M acetic acid to remove diagenetic and absorbed carbonate (40 µL per 1.0 mg of enamel). Aliquots for each tooth were subject to acid treatment times of 0, 0.25, 4, and 8 hours. These treatment times were chosen to reflect the range of times used in prior isotopic studies of archaeological kangaroos (Table 2). All pre-treatments were conducted at room temperature. After treatment, samples were rinsed with demineralized water, centrifuged four times, and dried in a vacuum oven.

2.2 Isotopic analyses

Macropod tooth enamel has a carbonate content of approximately 5%. Therefore, approximately 6 mg samples of treated enamel (0.3 mg of carbonate) were analyzed for $\delta^{13}C$ and $\delta^{18}O$ using a GasBench II coupled with a Delta XL Mass Spectrometer (Thermo-Fisher Scientific) at the West Australian Biochemistry Centre, School of Plant Biology, The University of Western Australia (Paul and Skrzypek 2007). The isotope results were standardized to the Vienna PeeDee Belemnite (VPDB) and are given in per mil (‰). Three-point normalization was used in order to reduce raw values to the international scale (Skrzypek 2013) and normalization was performed based on international standards provided by IAEA: L-SVEC, NBS19 and NBS18. The external error of $\delta^{13}C$ analyses is <0.10‰ and $\delta^{18}O$ is <0.10‰ (1 st dev). Values of international standards for carbon ($\delta^{13}C$) were based on Coplen et al. (2006).

3. Results

3.1 Dissolution in acid

Sample loss due to acid treatment is expressed here as the percentage loss of dried sample calculated relative to the percentage loss at the end of the preceding interval (Figure 3). As such, a negative percentage loss indicates that the weight of the sample increased during that interval. The rate of sample loss averaged 0.39% per minute in the first fifteen minutes. During this interval, the average total sample loss

was 7.39% for modern samples and 4.74% for archaeological samples. During the next treatment interval (15 minutes to four hours), the rate of sample loss slowed from 0.39% per minute to 0.03% per minute. That is, the slope of the mass loss line decreased. Interestingly, Sample 10 recorded a marginal increase in mass (0.30%) during this interval. This increase may be due to recrystallization but is more likely the result of measurement error. Again, the average total loss was slightly higher for the modern samples (7.27%) compared to the archaeological samples (5.89%). During the four hour to eight hour interval, five out of the ten samples increased in mass while the remaining five decreased in mass.

3.2 Carbon isotopes

The acetic acid treatment is shown to have an impact on $\delta^{13}C$ values for both modern and archaeological samples at all treatment time intervals (Figures 4 and 5). For the majority of archaeological samples, the fifteen-minute acetic acid treatment resulted in $\delta^{13}C$ signals that were more positive than those recorded for the untreated samples. In particular, four out of the six samples recorded an increase in $\delta^{13}C$. This trend is reversed in the modern samples with three out of four showing a decrease in $\delta^{13}C$. There is an observable difference in means between the treated and untreated archaeological specimens. The untreated group registered a mean $\delta^{13}C$ of -1.45‰ while the mean $\delta^{13}C$ for treated specimens was -1.94‰. This is a change of 0.49‰ with an effect size of 0.29. Note that effect size was calculated using Cohen's *d*. Conversely, the modern samples were much more similar with a change of only 0.04‰ (effect size = 0.026) between the treated and untreated groups.

After four hours the measured $\delta^{13}C$ increased relative to the untreated samples for all modern samples and three out of six archaeological teeth. These increases ranged from 0.07‰ to 0.72‰ (mean =0.39‰). Decreases for the archaeological enamel were much smaller ranging from -0.03‰ to -0.25‰ (mean -0.12‰). By four hours the difference in means between the treated and untreated specimens is 0.36‰ (effect size = 0.21) for the archaeological specimens and 0.41‰ (effect size =0.24) for the modern specimens. By the eight-hour mark, $\delta^{13}C$ in nine out of the ten samples had become more positive (range = 0.02‰. to 1.29‰; mean =0.54‰.). The remaining sample became more negative by 0.04‰. At eight hours, the difference in means between the treated and untreated specimens was 0.008‰ (effect size = 0.005) for the archaeological specimens and 0.55‰ for the modern specimens (effect size =0.32). Overall, the average variance in means between the treated and untreated groups is relatively marginal for both archaeological and modern specimens. In particular, the maximum change does not exceed 0.55‰ at any time.

However, this variance is within the range that may be expected for changes in mammal feeding ecology and therefore should not be dismissed.

3.3 Oxygen isotopes

As was the case for $\delta^{13}C$, the acetic acid treatment influenced $\delta^{18}O$ signatures, but the registered shifts were not necessarily substantial (Figures 6 and 7). As a result of the fifteen-minute treatment, $\delta^{18}O$ increased for six samples, decreased for three samples, and remained stable for one specimen. Increases ranged from 0.02‰ to 0.54‰ (mean =0.20‰), and decreases ranged from -0.08‰ to -0.18‰ (mean =-0.12‰). The difference in means between the treated and untreated groups for archaeological specimens was 0.09‰ (effect size = 0.045), while the difference for modern specimens was 0.11‰ (effect size = 0.041). After four hours the difference in the mean values between the treated and untreated group continued to change. Isotope measurements for six samples decreased (range = - 0.01‰ to -1.18‰; mean = -0.34‰) while ratios for four sample increased (range = 0.12‰ to 0.33‰; mean = 0.21‰). The difference in means between the treated and untreated specimens is 0.0017‰ (effect size = 0.0009) for the archaeological specimens and 0.25‰ for the modern specimens (effect size =0.093).

The mean difference between treated and untreated specimens also changed for both modern and archaeological samples during the prolonged eight-hour treatment. The $\delta^{18}O$ values decreased for six samples (range = -0.12‰ to -0.34‰; mean = -0.395‰) and increased for four samples (range = 0.34‰ to 0.57 ‰; mean = 0.344‰). The difference in means between the treated and untreated specimens is 0.158‰ (effect size = 0.07608) for the archaeological specimens and 0.0725‰ for the modern specimens (effect size = 0.02772). Although isotopic shifts for longer treatment times were still marginal, changes in signal were of a magnitude large enough to potentially influence interpretations of relative humidity.

4. Discussion

4.1 Relationship between treatment times and dissolution in acid

Prior research examining bone carbonate dissolution in acetic acid has indicated that a plateau reflecting saturation of the solution is typically reached after four hours (Garvie-Lok et al. 2004). In this study, analysis was conducted at the fifteen-minute mark to examine reactivity prior to potential saturation. The average rate of sample loss due to acetic acid treatment is highest in the first fifteen minutes for both modern and archaeological samples. The rate of dissolution for modern (0.49% per minute)

and archaeological material (0.31% per minute) is not substantially different and the mean percentage loss was only 2.65% higher for modern tooth enamel. Although the average rate of dissolution slows considerably in the next four hours, the similarity between the modern (0.03% per minute) and archaeological (0.02% per minute) material is still evident. Given that the modern tooth enamel samples are unlikely to contain significant proportions of absorbed carbonates, it is logical to deduce that structural carbonates are being dissolved even during short treatment times.

After eight hours of treatment, three of the six archaeological samples and two of the four modern samples show an increase in mass. This likely reflects the recrystallization of dissolved carbonates following the saturation of the acetic acid solution. Overall this aligns well with existing literature suggesting that prolonged treatment times should be avoided (Balasse et al. 2002; Garvie-Lok et al. 2004; Lee-Thorpe and van der Merwe 1991).

4.1 Impact of treatment times on isotopic signals

The $\delta^{13}C$ results here indicate that acetic acid treatment, in addition to removing secondary carbonates, does influence isotopic integrity of the primary enamel. More specifically, over the course of treatment from zero to eight hours, measured $\delta^{13}C$ for both archaeological and modern specimens tended to increase. Critically, modern samples are not expected to contain secondary carbonates and thus the isotopic signal is predicted to remain stable regardless of treatment time (Koch 1997). However, isotopic shifts are clear in the experimental data and increase with protracted reaction times. This aligns strongly with earlier research (e.g. Garvie-Lok et al. 2004; Lee-Thorpe and van der Merwe 1991; Pellegrini and Snoeck 2016; Wheatley and Crowley 2014) and clearly indicates that shorter treatment times are preferable for minimising biological isotopic offsets.

It is important to note that the overall trend towards increasing $\delta^{13}C$ is least evident in the first fifteen minutes and is not unilateral across all treatment times. In fact, identifiable reversals in the direction of change are evident between the four-hour and eight-hour treatments. This mirrors experimental results presented by Crowley and Wheatly (2014) that showed: (i) $\delta^{13}C$ for modern samples will initially increase after 15 minutes and then decrease again at protracted treatment times; and i) $\delta^{13}C$ for fossil samples initially decreases after 15 minutes and then will eventually increase again. While differences in the direction of change between modern and archaeological specimens have been identified elsewhere in the literature (e.g. Garvie-Lok et al. 2004; Lee-Thorpe and van der Merwe 1991), this is the first time

that an association between multiple reversals and prolonged treatment times has been highlighted. It is not clear what process has resulted in this disparity. However, given that results are most consistent (in terms of both direction of change) during the fifteen-minute interval, it is clear that the impact of the acetic acid on the primary carbonate signal is at its lowest during this time. Interestingly, the difference in mean $\delta^{13}C$ between the treated and untreated samples is also largest in the first fifteen minutes and decreases over time. This may be indicative of the initial dissolution of absorbed carbonate followed by the slower modification of the primary carbonate through dissolution and recrystallization. Overall, the $\delta^{13}C$ signal does not necessarily change substantially. However, the potential for recrystallization after four hours, as demonstrated by increased mineral yields, suggests shorter treatment times are preferable. This finding is considered relevant to pre-treatment protocols for isotopic analyses of teeth from archaeological sites globally.

As was the case for $\delta^{13}C$ values, experimental data indicates that $\delta^{18}O$ signal in both archaeological and modern samples is also impacted by the acetic acid treatment during all time intervals. Again, the modern samples should not contain diagenetic minerals, and therefore these impacts cannot simply be linked to the removal of adsorbed carbonates. Modern samples may be less crystalline than archaeological teeth and therefore less resistant to acid treatment and recrystallization processes (Lee Thorpe and van der Merwe 1991). However, given that the dissolution rates for modern samples were relatively restricted compared to the archaeological samples, it is unlikely that recrystallization processes alone account for the observed changes. Instead, the isotopic shifts likely arise, at least in part, as a result of the modification of the primary crystalline apatite. Importantly, the fifteen minute treatment resulted in an increase in $\delta^{18}O$ for the majority of modern and archaeological specimens. This is consistent with other available data for bone samples (Garvie-Lok et al. 2004). However, this trend is no longer evident after four hours. Similar unpredictable variability in the direction and magnitude of $\delta^{18}O$ changes has been demonstrated elsewhere (Wheatley and Crowley 2014; Garvie-Lok et al. 2004; Pellegrini and Snoeck 2016) and likely reflects complex underlying processes including partial dissolution of biological apatite and recrystallization of isotopically distinct secondary minerals (Lee Thorpe and van der Merwe 1991; Nielsen-Marsh and Hedges 1997).

Although changes in isotopic values between sample treatment times are relatively small, a key concern with both the $\delta^{13}C$ and $\delta^{18}O$ values is the evident reversals in the direction of change across treatment times for numerous specimens. That is, the isotopic values for some samples increase and decrease back and forth over the course of eight hours. In some instances, changes are very small and thus

effectively negligible (e.g. 0.01%); however, these changes do occur and tend to increase with prolonged time. Importantly, it is the inconsistency in these changes that strongly suggest that they are linked to interrelated and overlapping reaction processes that likely effect the primary carbonate signal. Overall, this suggests that shorter treatment times are preferable and raises the possibility that a treatment time of even less than fifteen minutes may be superior. Interestingly, the identified reversals in the direction of change are not replicated between the $\delta^{13}C$ and $\delta^{18}O$ measurements and, as such, there is no discernible connection between the ways in which $\delta^{13}C$ and $\delta^{18}O$ values respond to the acidic treatment. This replicates the differences in the acid response profiles for bone $\delta^{13}C$ and $\delta^{18}O$ values reported by Garvie-Lok et al. (2004).

Changes in $\delta^{13}C$ and $\delta^{18}O$ across all time intervals were relatively marginal but were lower for shorter treatment times. Although changes were generally small, it is important to note that shifts are within the range expected for minor to moderate alterations in climate or animal diet. Hence, it is critical that consistency in pre-analytical protocols (including treatment time, powder fineness, and ratio of solution to sample) be prioritized. Given that offsets become more substantial and unpredictable after eight hours, it is suggested that treatment times should not exceed four hours. Although there is not a considerable difference in results between the fifteen minute and four hour intervals, the increasing variability in the predictability of the direction of change (particularly for $\delta^{18}O$) indicates that treatment times of as little as 15 minutes may be favourable. This is supported by the rapid rate of dissolution during this period. Overall, the results of this study align with the conclusions presented by Pellegrini and Snoeck (2016) that acetic acid pre-treatments can have a detrimental effect on structural carbonates and therefore acid treatment times should be limited.

5. Conclusion

We conclude that, given the potential for isotopic analyses to significantly contribute to the investigation of paleoecological relationships in archaeological contexts, there is a substantial and current impetus to optimise methods for conducting research; particularly protocols for pre-analytical processing. More specifically, secondary intrusive carbonates have the potential to materially alter the integrity of the biogenic signal in archaeological tooth enamel carbonates and require careful removal. While it is well established that the treatment of samples with weak acid is effective at removing more labile intrusive carbonates, the standardization of pre-treatment

parameters (specifically acid treatment time) is critical for the production of replicable, consistent and comparable results.

The research presented here specifically investigates the impact of variable acid pre-treatment treatment times on the isotopic integrity of both modern and archaeological kangaroo teeth (dating from contemporary to 46000 BP) from Northwest Australia. The experimental results show that isotopic integrity is substantially impacted during prolonged acid treatment times and indicates that reaction times should not exceed four hours. Given the rapid dissolution of samples during the fifteen minute interval, and the variation in the direction of change for measured isotope ratios in some samples after this time period, it is likely that very short treatment times of fifteen minutes or less are preferable for archaeological mammalian taxa. Future research should focus on verifying this outcome by using FTIR to investigate changes in crystallinity during very short treatment times.

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7. Supporting information

All isotopic data available as supplementary tables.

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Table 1. Summary of archaeological and modern kangaroo samples.

Sample	Type	Site/Locality	Age (years)	Condition and Appearance
1	Archaeological	Juukan-2/Brock 21, Pilbara	17000 cal BP	Moderate (intact enamel with some discolouration)
2	Archaeological	Juukan-2/Brock 21, Pilbara	10000 cal BP	Good (well preserved enamel)
3	Archaeological	Juukan-2/Brock 21, Pilbara	14000 cal BP	Good (well preserved enamel)
4	Archaeological	Juukan-2/ Brock 21, Pilbara	21000 cal BP	Moderate (intact enamel with some discoloration)
5	Archaeological	PIL_3160, Pilbara	1000 cal BP	Good (well preserved enamel)
6	Archaeological	Boodie Cave, Barrow Island	46200 cal BP	Good (well preserved enamel)
7	Modern	Barrow Island	0	Excellent
8	Modern	Barrow Island	0	Excellent
9	Modern	Inland Pilbara	0	Excellent

10	Modern	Inland Pilbara	0	Excellent

Table 2. Summary of published acid pre-treatment protocols for fossil, archaeological, and modern kangaroo and wallaby species (species based on Jackson and Groves 2015).

Locality	Species	Age	Sample type	Acid concentration	Acid to sample ratio (micro L/mg)	Treatment time	Reference
61 Collection Sites, Eastern Australia	*Macropus fuliginosus* *Osphranter rufus* *Macropus giganteus* *Osphranter robustus*	Modern	Powdered	No treatment listed	No ratio listed	No time listed	Fraser 2005
793 Collection Sites, Australia	*Notamacropus agilis* *Osphranter antilopinus* *Macropus fuliginosus* *Macropus giganteus* *Osphranter robustus* *Notamacropus rufogriseus* *Osphranter rufus*	Modern	Powdered	No treatment listed	No ratio listed	No time listed	Murphy et al. 2007a; Muphy et al. 2007b
Thylacoleo	*Osphranter spp.*	Modern; Fossil	Powdered	0.1 M acetic	No ratio	15 minutes	Prideaux et

Caves, Nullabor Plain	*Procoptodon spp.* *Bohra spp.* *Baringa spp.* *Sthenurus spp.* *Metasthenurus sp.* *Congruus sp.*	(approx. >780 kya - 100 kya)		acid	listed		al. 2007
South-eastern Australia	*Osphranter spp.* *Procoptodon sp.*	Modern; Fossil (Pleistocene)	Powdered	0.1 M acetic acid	No ratio listed	15 minutes	Prideaux et al. 2009
Megafaua Site, Kangaroo Island, South Australia	*Macropus fuliginosus* *Notamacropus eugeneii*	Modern; Fossil (aprox. 100 ka BP - 50 ka BP)	Powdered	1 M acetate buffer acetic acid	40 µL/mg	>8 hours (overnight)	Forbes et al. 2010
Transect stretching south from the Flinders Ranges, and south-southeast from Woomera, South Australia	*Macropus fuliginosus* *Osphranter rufus* *Macropus giganteus* *Osphranter robustus*	Modern	Powdered	0.1 M acetic acid	0.1 mL/mg	4 hours	Brookman and Ambrose 2012; Brookman and Ambrose 2013
Boodie Cave,	*Lagorchestes conspicillatus*	Modern;	Powdered	0.1 M acetic	40 µL/mg	4 hours	Skippington

Barrow Island, Western Australia	*Osphranter robustus*	Archaeological (appox. 51 kya – 7 kya)		acid			et al. 2018

Figure legend

Figure 1. Example of macropod teeth used for isotopic analysis. (a) Sample 1. Molar of *Osphranter spp.;* (b) Sample 2. Maxilla fragment of *Osphranter spp. (only second molar analysed)*; (c) Sample 3. Molar of *Osphranter spp.*; (d) Sample 5. Maxilla fragment of *Osphranter spp. (only second molar analysed)*.

Figure 2. Percentage of sample remaining by treatment time.

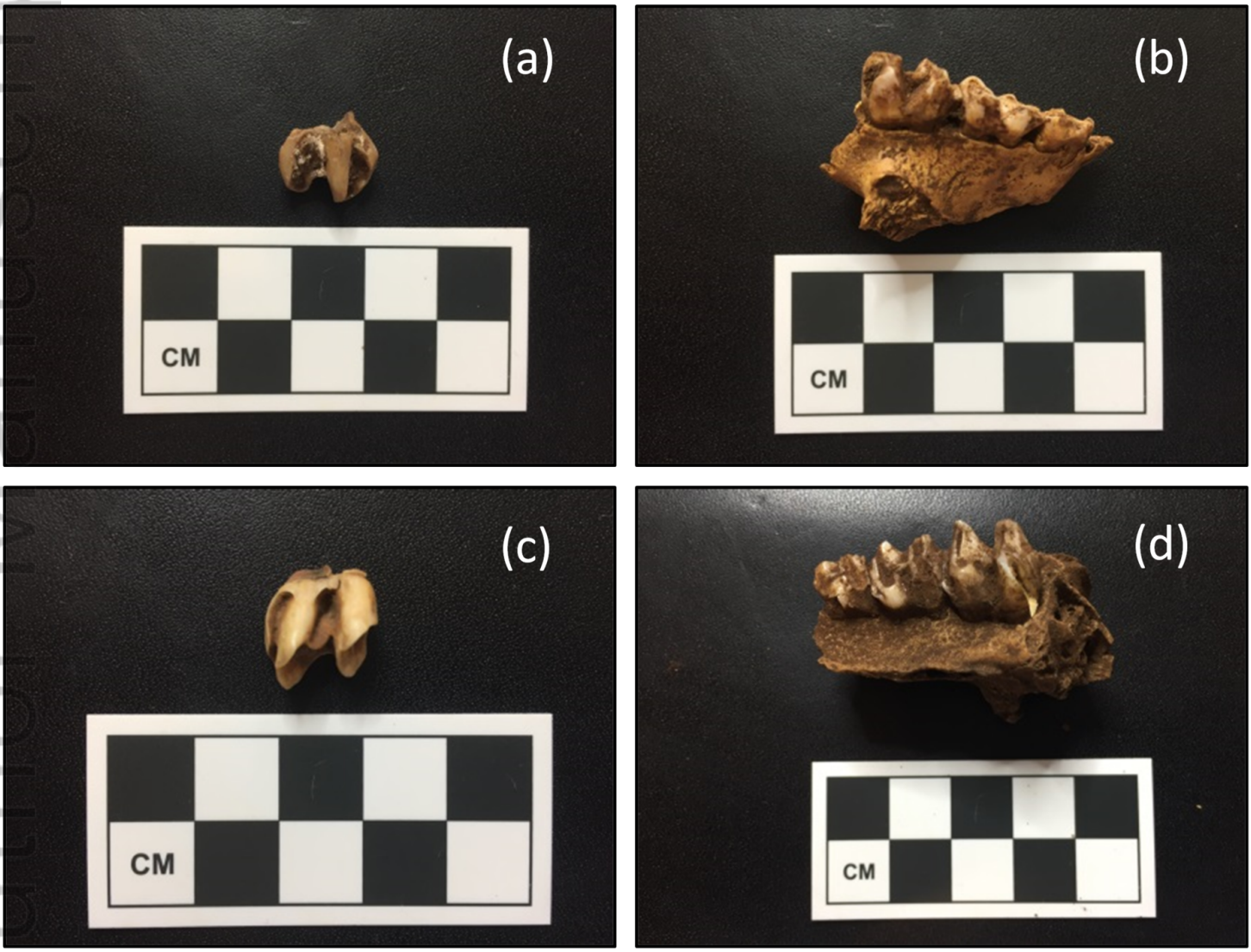
Figure 3. Boxplot percentage of sample loss by treatment time.

Figure 4. *Measured $\delta^{13}C$ by treatment time for archaeological samples. The external error of δ13C analyses is <0.10‰ (1 st dev).*

Figure 5. *Measured $\delta^{13}C$ by treatment time for modern samples. The external error of δ13C analyses is <0.10‰ (1 st dev).*

Figure 6. Measured δ18O by treatment time for archaeological samples. The external error of δ18O is <0.10‰ (1 st dev).

Figure 7. Measured $\delta^{18}O$ by treatment time for modern samples. The external error of δ18O is <0.10‰ (1 st dev).



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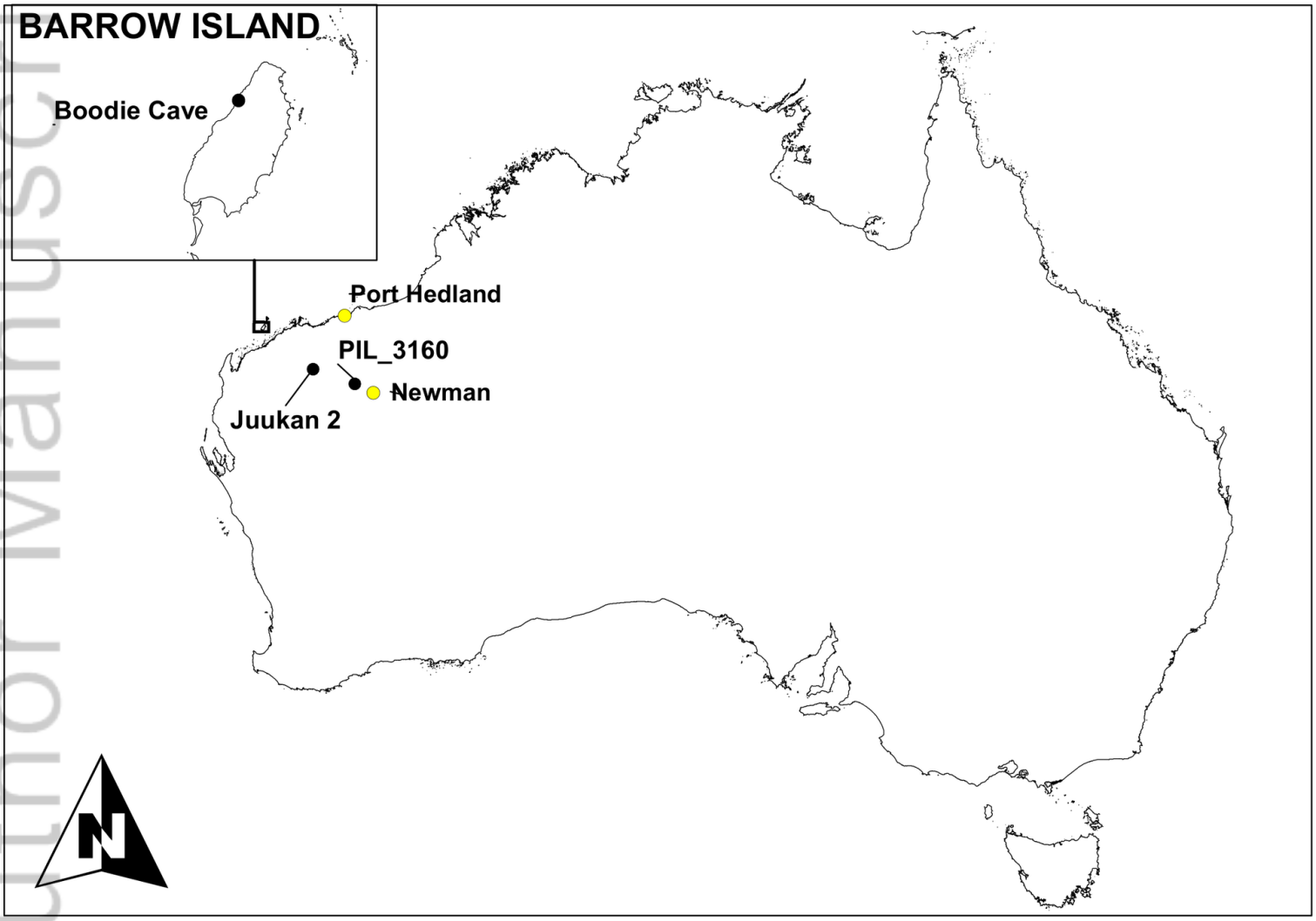

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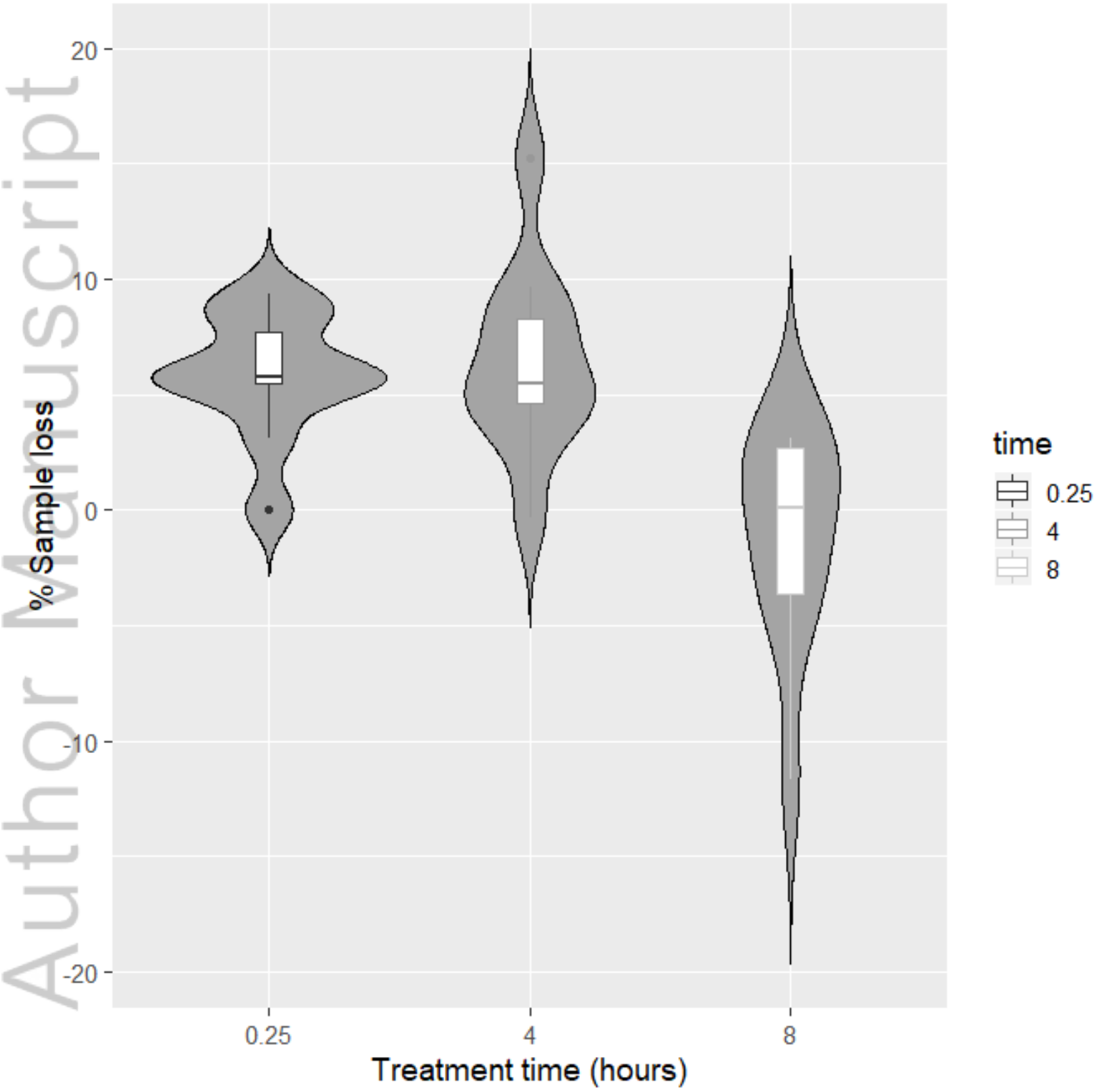

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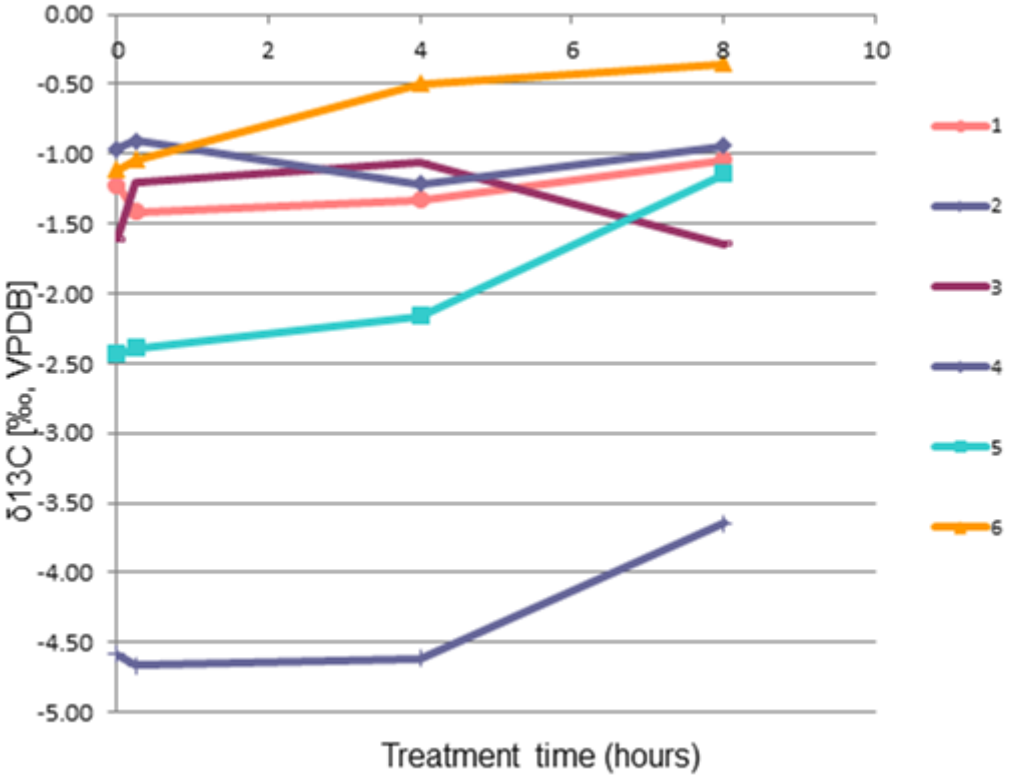

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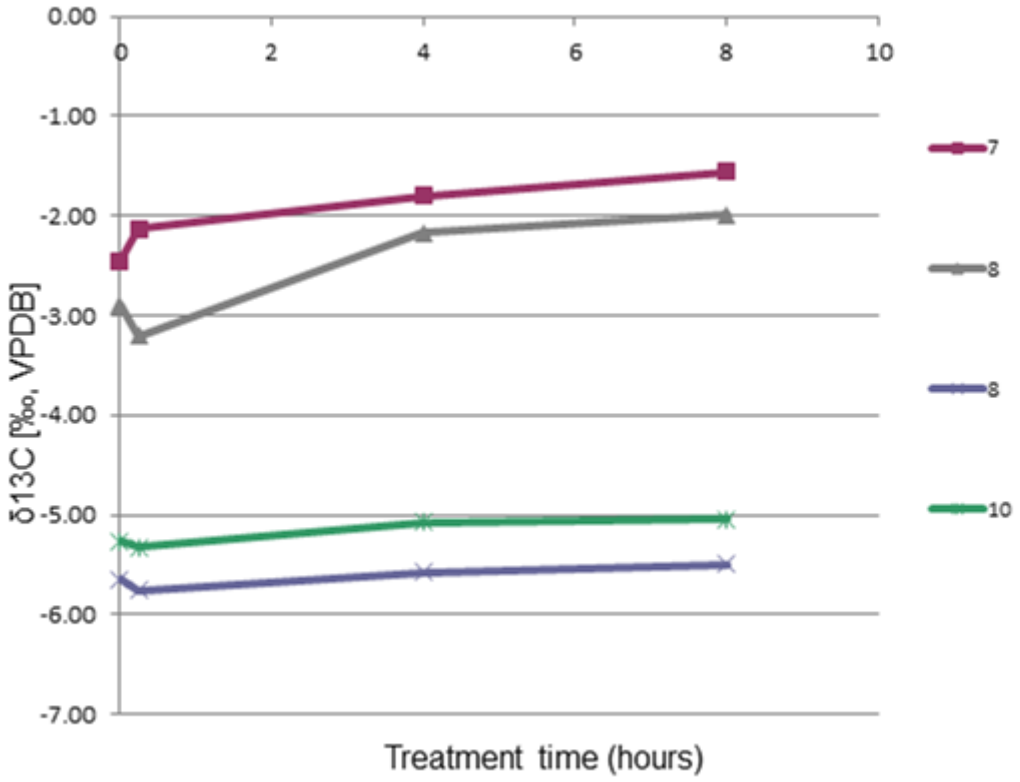

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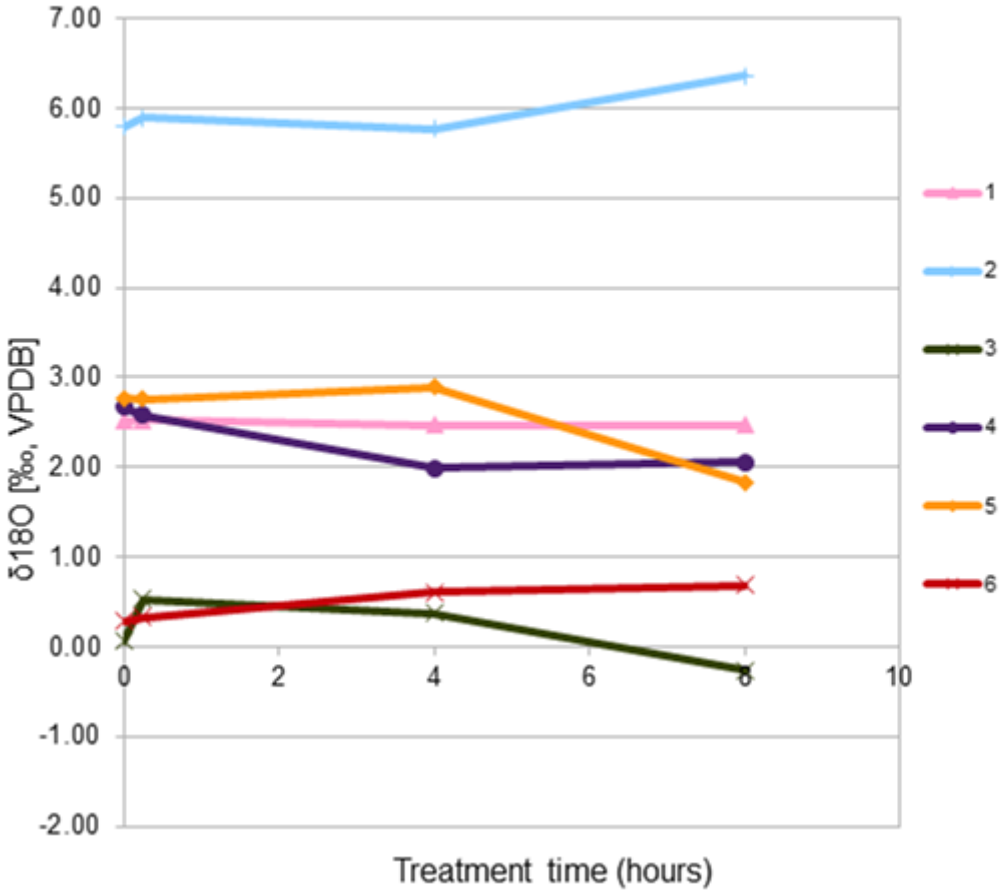

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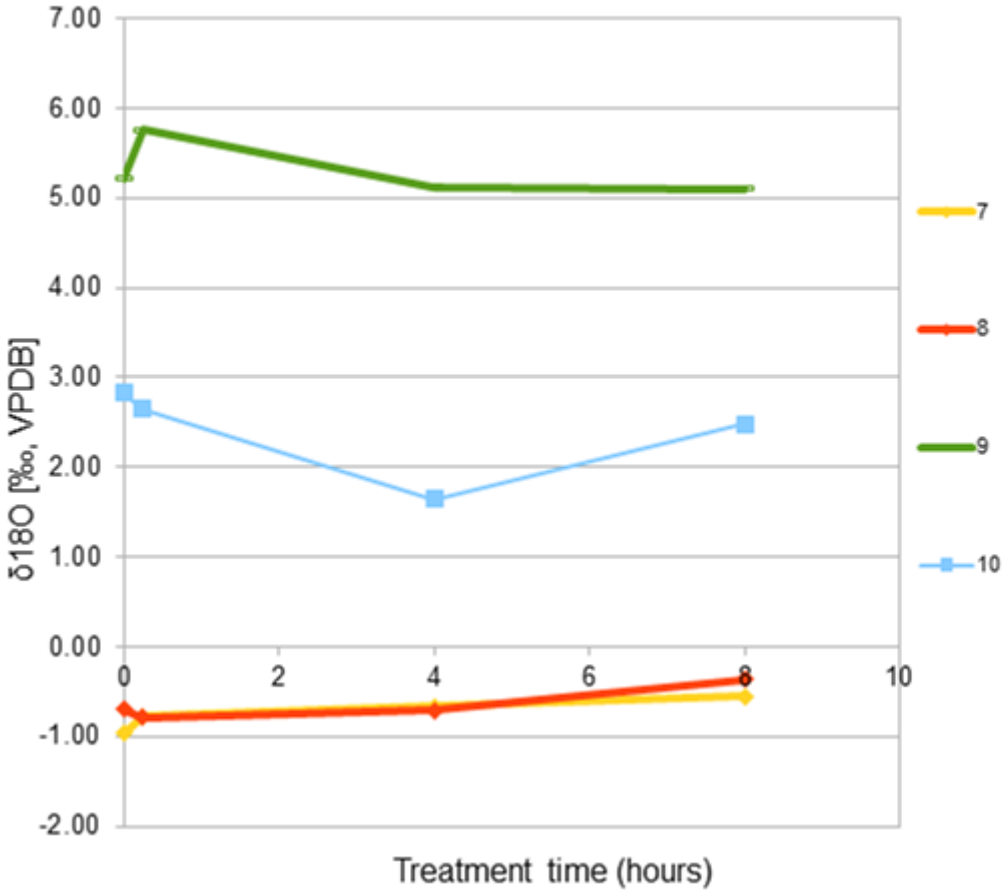

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