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To pretreat, or not to pretreat, that is the question. The value of pretreatment protocols in the stable carbon and nitrogen isotope analysis of archaeobotanical cereal grains from Croatia and Serbia

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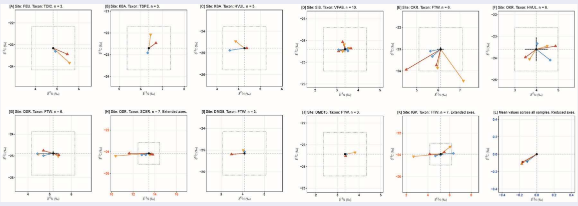
ABSTRACT
Isotopic analysis of archaeological charred plant remains is a useful tool to infer past agricultural practices. However, debate continues over whether charred seeds should be untreated or pretreated before analysis, to counteract any residual contamination and retrieve the "true" isotopic signature of the seed. This paper presents a case study examining whether archaeobotanical remains from Croatia and northern Serbia should be pretreated before isotopic analysis with the aim to provide a pragmatic technique for wider application. A small subset was first examined with an ATR-FTIR and then four different protocols were examined: water rinse only, two different acid-only methods and ABA (acid–base-acid). The results were inconsistent, displaying variability in the effect each protocol had on the isotopic values. Overall, it was concluded that the slight differences between untreated and pretreated sub-samples should not impact the archaeological interpretation, removing the need for pretreatment of the remaining archaeobotanical material.



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Bronze age; Roman; medieval; ATR-FTIR; ABA pretreatment

Introduction

Archaeobotanical stable isotope analysis has grown hugely in popularity over the past two decades (Fiorentino et al. 2015) and is now entering a period of "democratisation" in which the technique is applied broadly, including outside dedicated crop isotope research projects. The popularity of archaeobotanical stable isotope analysis is with good reason, it provides novel information about past agriculture that can significantly enrich the study of the past. These insights expand the boundaries of what we can learn about paleo-economies and enables archaeobotany to continue to present compelling narratives of the past that offer valuable perspectives on present-day issues about adapting to climate change. The use of stable isotopes to infer the nature and variability of husbandry practices and environmental conditions, even at a single site, is a powerful asset that many are keen to explore.

Archaeobotanical stable isotope analysis is on course to becoming "just another tool" for the archaeobotanical researcher. Effective application of the technique is, however, complex – with the real prospect that poor implementation will lead to misleading data. Further patterns in stable isotope results can typically be explained by multiple factors, reflecting the inherent complexity of biological isotope systems. The risk of flawed interpretations is especially acute when the technique is deployed to new geographic regions or chronological horizons that lack experimental ground-truthing data.

In this paper we present a case study on how to approach the ground-truthing of stable isotope analysis in a new study area. The work is based on a new dataset of samples spanning from the Bronze Age to the Middle Ages across a range of archaeological sites in continental Croatia and northern Serbia (Figure 1). This marks the first use of the technique in the region. This paper does not aim to present experiments on known contaminated grains as per

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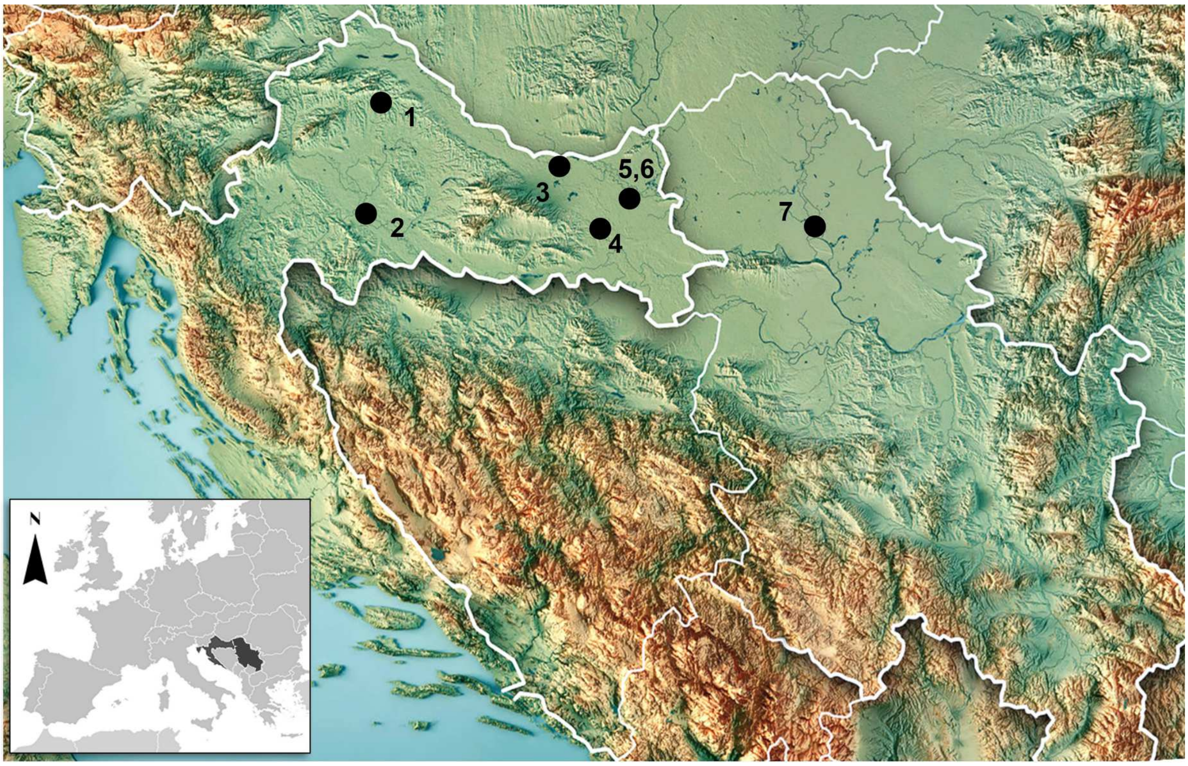

Figure 1. Map of the study sites. (1) Kalnik-Igrišče, (2) Sisak-Pogorelac, (3) Donji Miholjac-Đanovci, (4) Ivanovci Gorjanski – Palanka, (5) Park Kraljice Katarine Kosače, (6) Osijek-Silos, (7) Feudvar.

studies such as Vaiglova et al. (2014). Instead, we present how we identified an effective approach to determining the robustness of the new stable isotope data when contamination is unknown.

Whilst we consider this kind of ground-truthing study to be an essential requirement for the use of stable isotope analysis in a new locale, we also recognize that our resources were limited compared to major research projects that fueled the rise of archaeobotanical stable isotope analysis. Accordingly, we promote a pragmatic approach that seeks to establish a balance between sample size (needed to generate archaeologically informative data) and testing and repetition (needed for data validation). In this we targeted select parts of the assemblage for testing, whilst leaving sufficient resources to include enough samples to make a meaningful contribution to an archaeological study of the region.

This study stays away from dichotomous views on "good" vs. "bad" stable isotope data. The "gold standard" approaches applied by major research projects do produce excellent quality data (see further discussion below). We recognize, however, that the archaeological record is fragmentary and sub-optimal, and there will be occasions when the "gold standard" approaches are not applicable. In these circumstances, we argue that rather than doing no analysis at all, it is better to undertake analysis with a good knowledge of the limitations of the data, which is taken into account in subsequent interpretations. It is hoped the approach outlined here can be a model that informs and inspires other researchers, helping to ensure that during the democratization of archaeobotanical stable isotope analysis the technique continues to be applied with high integrity.

Stable isotope analysis in archaeobotany

Archaeobotanical stable isotope analysis can be used to infer direct evidence of the life history of a plant; the isotopic composition of plant organs produce a predictable record of its growing condition (e.g. Marshall, Brooks, and Lajtha 2007). The technique is also relatively affordable, costing in the region of 10-15% of the cost of a radiocarbon date, at the time of writing. Like radiocarbon dating, the technique is destructive. This presents ethical issues, but these are surmountable – especially when we consider that crop remains are often poorly archived (Flintoft 2023). Methodological advances mean that single grain analysis is entirely feasible, and this in turn facilitates the analysis of even relatively small assemblages that otherwise have limited scope for providing informative results by traditional archaeobotanical techniques. Stable isotope analysis, however, is most informative when applied at a large scale across carefully selected chronological and spatial units (Bogaard, Krause, and Strien 2011) and, further, when paired with radiocarbon dating (Fiorentino et al. 2008) and compared to weed ecology data (Bogaard et al. 2016).

Stable isotope analysis was introduced to archaeobotany through the pioneering work of two groups that took the principles established in plant physiology (Condon, Richards, and Farquhar 1993; Farquhar and Richards 1984; Farquhar, Leary, and Berry 1982) and the first archaeological applications (DeNiro and Hastorf 1985; Marino and DeNiro 1987), and sought to ground truth their applicability to the archaeobotanical record. The first two decades of stable isotope analysis were subsequently dominated by research from the Araus, Ferrio, Voltas and colleagues group, which tended to focus on south-west Europe (e.g. Araus et al. 1997; Araus et al. 2007; Araus and Buxó 1993; Ferrio et al. 2005; Voltas Velasco et al. 2008) and the Bogaard, Charles, Jones and colleagues group that focused on south-west Asia and central Europe (e.g. Bogaard et al. 2007; Bogaard et al. 2013; Bogaard et al. 2016; Fraser et al. 2011; Wallace et al. 2015). There were of course notable exceptions to this trend (e.g. Flohr et al. 2019; Flohr, Müldner, and Jenkins 2011; Riehl et al. 2014). Through this work literature has emerged to help provide guidance on best practice in archaeobotanical stable isotope analysis. Much of this literature focus on taphonomic processes, especially the impact of preservation by charring and post-deposition contamination (e.g. Aguilera et al. 2008; Araus et al. 1997; DeNiro and Hastorf 1985; Fraser et al. 2013; Vaiglova et al. 2014). Other literature covers variation between species (Lightfoot et al. 2020), sample size selection (Vaiglova et al. 2023), interpretative baselines in isotope values (Bogaard et al. 2013; Wallace et al. 2013) and protocols for reporting results (Szpak, Metcalfe, and Macdonald 2017). When the technique is applied in novel settings, it is critical to consider the robustness of approaches to data collection and data analysis.

Background on pretreatment protocols

The potential for variability during the charring process and from the burial environment (e.g. humidity, pH, temperature and time) to alter the isotope values of archaeobotanical remains has been documented in archaeological applications. Early studies suggested that charring did not bias the isotopic signature (DeNiro and Hastorf 1985), and most subsequent studies have confirmed a minimal isotopic offset in $\delta^{13}C$ (Araus et al. 1997; Fiorentino et al. 2012; Fraser et al. 2013; Marino and DeNiro 1987). For $\delta^{15}N$, results have varied but a small increase due to charring in the temperature range of 200–260°C is typically reported (Aguilera et al. 2008; Fiorentino et al. 2012; Kanstrup et al. 2012; Nitsch, Charles, and Bogaard 2015; Styring et al. 2013). This is also noted for $\delta^{34}S$, where charring has a small but predictable effect (Nitsch et al. 2019). Charring temperatures will of course vary depending on the context, so some studies try to reduce the offset by selecting grains with well-preserved physical characteristics, associated with optimal characterizing, to minimize isotopic variability from poorly preserved grains charred at higher temperatures (Charles et al. 2015; Stroud et al. 2023). Others choose to apply an average offset of up to 1‰ for $\delta^{15}N$ and 0.1‰ for $\delta^{13}C$ values (e.g. Filipović et al. 2019; Gillis et al. 2020; Vaiglova et al. 2020).

As well as the effects of charring on isotopic values, the incorporation of foreign contaminants (e.g. carbonates, humic substances) have also been reported to alter charred plant isotopic signals. Their impact on the stable isotopic composition of buried plant material is potentially extremely complex, but only a handful of studies have begun to examine these issues (e.g. Fraser et al. 2013; Nitsch, Charles, and Bogaard 2015; Styring et al. 2013; Vaiglova et al. 2014). From these studies pre-screening techniques, capable of identifying the presence of contaminants, and protocols that remove contaminants have been developed. Nevertheless, no standard protocol has emerged, with studies using a variety of different solution concentrations, temperatures, and durations. Debate also continues as to whether pretreatment is required at all (Brinkkemper et al. 2018).

One method to identify the presence of carbonate, nitrate, and/or humic contamination is by examining a proportion of the studied grains (usually 10%) with Fourier transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR). ATR-FTIR analysis measures a sample's absorbance of infrared light at various wavelengths to determine the material's molecular composition and structure. Experiments showed that the presence of carbonates in an archaeological sample causes the appearance of peaks at 720 and 870 cm^{-1}, which increase with a higher percentage of contamination (Vaiglova et al. 2014). Nitrate on the other hand is only detectable when contamination is 10% or higher, with peaks at 1085, 1450, 3300 cm^{-1}. Similarly, humic acid contamination is only visible when contamination is 10% or higher, with peaks at 1010, 1080, and 3690 cm^{-1}. The amount of material required for a viable analysis is very small and most analyses can be done relatively quickly with little sample preparation. Nevertheless, few studies seem to implement this step, with only 6 out of 40 studies reviewed here applying this method to identify contaminants. Instead, many assume contamination or aim to prevent the possibility of any by implementing a pretreatment protocol.

Two main types of pretreatment seem to have emerged. First, a version of the acid–base-acid (ABA) protocol was originally developed for radiocarbon dating. Here the acidification of the charred grains removes deposited carbonates while an alkali step removes humic acids. The final acid step is then required to remove any carbonate that may have dissolved from the air during the treatment. Variation exists, although typically,

- Step 1 consists of 0.5 or 1 M of hydrochloric acid (HCl) (aq.) for 30–60 min at either 70 °C or 80 °C.
- Step 2 consists of 0.1, 0.5 or 1 M sodium hydroxide (NaOH) (aq.) at 70 °C or 80 °C for 1–3 h.
- Step 3 involves either 0.5 or 1 M HCl (aq.) for 10–16 h at room temperature or heated at 70 °C or 80 °C, for 25–60 min.
- Finally, it is rinsed three times with distilled water (e.g. Fiorentino et al. 2008; Fraser et al. 2013; Kanstrup et al. 2014; Nitsch et al. 2019; Styring et al. 2013; Varalli et al. 2021).

The effects of applying an ABA pretreatment on the isotopic composition of the archaeological charred plant material has also been explored. Studies have shown that the δ^{13}C offsets between untreated and ABA-treated samples were random but not significant (less than 1‰), while for δ^{15}N values observations showed random (Fraser et al. 2013), elevated (Kanstrup et al. 2014) and lowered values (Vaiglova et al. 2014) with offsets of up to 1.5‰. Thus, the impact of ABA pretreatment is still uncertain. Nonetheless one of the main disadvantages of the ABA protocol is that it causes large mass loss of the samples, especially if powdered, resulting in samples not having enough material to analyse (Kanstrup et al. 2014; Vaiglova et al. 2014).

The second method is an acid-only protocol. Here we see three typical versions with 0.5 or 1 M HCl (aq.) at;

(1) room temperature for up to 24 h (Aguilera et al. 2018; Knipper 2020), although Gillis et al. (2020) soaked samples at room temperature for 30 min or until effervescence ceased;
(2) at 70°C for 30–60 min (Alagich 2018; Filipović et al. 2019; Makhad et al. 2022; Mueller-Bieniek et al. 2019; Styring 2017); or
(3) at 80°C for 30–60 min (Szpak and Chiou 2020; Vaiglova et al. 2020).

All samples were then rinsed with distilled water three times before either freeze-drying or oven-drying.

Several studies have previously experimented with the impacts of pretreatment on charred archaeobotanical remains (Table 1). One of the earliest studies by DeNiro and Hastorf (1985), analysing prehistoric charred plant parts from Peruvian highlands, found both increases (+0.8‰) and decreases (−0.6‰) in δ^{15}N due to chemical pretreatment while changes in the δ^{13}C values were below 0.5‰. This pattern continues to be observed where harsher acid washes are used, however, in general the δ^{15}N values alter by <1‰ (Brinkkemper et al. 2018; Eklund 2019; Kanstrup et al. 2014; Vaiglova et al. 2014). For acid only treatments, studies indicate that no systematic or significant effect on grain δ^{13}C and δ^{15}N signatures occurs (Lightfoot and Stevens 2012; Aguilera et al. 2018). Several of these studies describe inconsistencies and variabilities between and within the samples that have been untreated and pretreated. From these studies it was recommended that gentle scraping of the grains to remove any adhered sediment be conducted in place of pretreatment (e.g. Larsson, Bergman, and Lagerås 2019; Treasure et al. 2019).

Materials and methods

In order to explore pretreatment protocols, this study devised a series of tests based on previous isotopic studies to determine whether the carbonized botanical remains selected from Croatia and Serbia have evidence of contaminants, and what effects the different pretreatments have on the stable isotope ratios. This was important to determine the methods to be used for the final analyses of the material.

Seven settlement sites were selected for this study, ranging from the Bronze Age to the Middle Ages (Figure 1). Reed and Wallace (2023) provide a summary of the sites, as well as additional supplementary data relevant to this paper (see Supplementary Information at https://doi.org/10.17605/OSF.IO/67C25). The sites selected from Croatia and Serbia represent archaeobotanical collections with relatively high densities of remains. The sites of Sisak (SIS), Feudvar (FEU), Kalnik-Igrišče (KBA) and Osijek-Silos (OSR) represent primary in-situ burning (Karavanić and Kudelić 2019; Kroll 1990; Kroll 1998; Kroll and Reed 2016; Marekovic et al. 2015; Reed 2020; Reed et al. 2019; 2021). Park Kraljice Katarine Kosače (OKR) and Ivanovci Gorjanski – Palanka (IGP) represent potentially secondary deposition, as there was no evidence of in-situ burning, however, they were both interpreted as being deposited over short periods, possibly as one depositional act (Reed et al. 2022a; 2022b). The remains from Donji Miholjac-Đanovci (DMD) are also likely to be secondary depositions, however, the densities are relatively low compared to the other sites and the depositional practices are less secure (Reed et al. 2022b). Thus, it is likely that some of these cereal remains from DMD derived from different harvests and/or arable plots.

All the carbonized archaeobotanical material used in this study was processed either through bucket flotation or using a flotation machine. Due to the limited number of cereal taxa preserved, a minimum of four grains were selected for each major cereal crop identified at the eight study sites. Crop taxa tested include free-threshing wheat (*Triticum aestivum/durum*), barley (*Hordeum vulgare*), emmer (*Triticum dicoccum*), spelt (*Triticum spelta*), new glume wheat (*Triticum* cf. *timopheevii*), and rye (*Secale cereale*). Preparation of the seeds included gentle removal of any visible surface contaminants, such as adhering sediments or plant roots using a scalpel.

Table 1. Summary of previous studies examining the isotope values of untreated and pretreated charred archaeobotanical remains.

Study	Method	Pretreatment protocol	Conclusion
Lightfoot and Stevens 2012	• 2 pits • 37 charred grains • ~ 50% of grains subjected to acid only pretreatment	6 M HCl for 24 h at room temperature and then rinsing	For $\delta^{13}C$ and $\delta^{15}N$ no significant difference between pretreated grains and untreated grains.
Kanstrup et al. (2014)	• 31 charred grains • Bulk samples (10 homogenized grains) tested with ABA and untreated.	1) 1 M HCl for 1 h at 80°C, 2) 1 M NaOH at 80°C for 3 h after discarding the HCl. 3) 1M HCl for ~16 h at room temperature (~ 20°C), 4) rinsing 5) drying at 80°C	Average increase in $\delta^{15}N$ of 0.7‰ for samples treated with ABA (excluding five outliers), but the effect on $\delta^{13}C$, apart from a few outliers, was minimal.
Vaiglova et al. 2014	• 42 archaeological batch samples (~ 10 grains per sample) and 12 modern charred batch samples	ABA-full gentle, ABA-neutrality, A-only gentle, ABA-full harsh, A-only harsh	Taken individually, none of the treatments had a consistently significant effect. However, overall $\delta^{15}N$ values decreased with the use of harsher acid treatments (by ca 1.1‰) and with ultrasonication in Milli-U water (by ca 1.0‰)
Wallace et al. 2015	• 105 samples from 8 sites • Subset of samples were pretreated with ABA. • $\delta^{13}C$ values were only examined	ABA protocol followed Fraser et al. 2013	The $\delta^{13}C$ values tended to be slightly higher after pretreatment (mean effect of pretreatment = + 0.18‰, $n = 96$) but were not significant.
Brinkkemper et al. 2018	• 22 samples • 645 charred grains & seeds • Grains amalgamated per sample and were untreated, acid-only and ABA treated.	Acid only: 1.0 M HCl at 85°C for 30 min, rinsing. ABA: 1.0 M HCl at 85°C for 30 min, rinsing, 1.0 M NaOH at 85°C for 60 min, rinsing, 1.0 M HCl at 85°C for 30 min, rinsing	None of the $\delta^{13}C$ offset values exceeded 1‰ and the vast majority did not exceed 0.5‰. All the $\delta^{15}N$ values are within 1‰, except for Acid only in sample 17 (−1.93‰).
Aguilera et al. 2018	• 3 sites, 80 bulk samples (~10 grains per sample) • 795 charred grains • Grains were pretreated with 2 acid-only methods either whole or powered and amalgamated per sample.	1 and 6 M HCl on entire and powdered grains. HCl for 24 h at room temperature, soaked in distilled water three times (24 h-12 h-6 h), oven-dried at 60°C for 48 h.	For $\delta^{15}N$ (NS, $P = 0.32$) and $\delta^{13}C$ (NS, $P = 0.881$) values no significant difference existed between 1 and 6 M HCl concentrations. Further no significant effect of using pretreatments to remove contamination from entire or powdered grains.
Eklund 2019	• 1 site • 30 charred grains • Grains amalgamated (10 per species) and were untreated, acid-only and ABA treated.	Acid only: 1.0 M HCl at 85°C for 30 min, rinsing. ABA: 1.0 M HCl at 85°C for 30 min, rinsing, 1.0 M NaOH at 85°C for 30 min, rinsing, 1.0 M HCl at 85°C for 30 min, rinsing	Lower $\delta^{13}C$ values for ABA-treated material, but never more than 0.5‰. The $\delta^{15}N$ values fluctuate up to 1‰ but less consistently with differences noted between wheat and barley.
Halvorsen, Mørkved, and Hjelle 2023	• 16 sites • 76 charred single grains and 22 modern charred grains • ~ 50% of grains subjected to ABA pretreatment	ABA protocol described in Fraser et al. (2013b),	For $\delta^{15}N$ no significant difference between pretreated grains and non-pretreated grains.

Over the last decade seed samples have either been isotopically analysed in bulk (i.e. several seeds from an individual archaeological context) or as individual single-seed samples (Vaiglova et al. 2023). The choice usually depends on the research aims, the quantity of grain available per context, or if the amount of nitrogen (%N) of small samples is too low for reliable analysis of stable nitrogen isotope values ($\delta^{15}N$). Bulk sampling mixes grains that are assumed to derive from primary contexts and is useful when inter-plant variability is not needed. For this study single grains were analysed as the wider study wished to investigate inter-plant variability. In addition, some of the contexts selected had limited numbers of viable grains and it was unclear if they were from the same harvest. Subsequently the testing was conducted on single grains.

ATR-FTIR analysis

Initially a small percentage of the seeds from SIS, OSR, OKR, FEU, and IGP (Table 2) were screened to determine carbonate, nitrate, and/or humic contamination based on the observations by Vaiglova et al. (2014). The samples were analysed at the University of Bath using Fourier Transform Infrared Spectroscopy (ATR-FTIR): a Perkin-Elmer Frontier with a diamond Attenuated Total Reflectance (ATR) head. The seeds were ground until they passed through a 75 µm Sieve. Spectra were collected over a range of 4000-450 cm^{-1} using a resolution of 2 cm^{-1} and 5 scans per spectrum. Corrections were made for ATR and background using Perkin-Elmer Spectrum software. See Reed and Wallace (2023) for the raw ATR-FTIR data.

Table 2. Seeds selected for ATR-FTIR screening.

Site	Taxa	Quantity	Testing ref no.
Sisak (SIS)	*Vicia Faba*	1	SIS_1
Osijek-Silos (OSR)	*Triticum aestivum/durum*	1	OSR_3
Osijek-Silos (OSR)	*Secale cereale*	1	OSR_4
Park Kraljice Katarine Kosače (OKR)	*Triticum aestivum/durum*	1	OKR_5
Feudvar (FEU)	*Triticum dicoccum*	1	FEU_6
Ivanovci Gorjanski – Palanka (IGP)	*Triticum aestivum/durum*	1	IGP_8

Pretreatment protocols

To verify the presence of contaminants, and determine the influence on stable isotope results, untreated and pretreated samples were analysed. The δ^{15}N and δ^{13}C values were determined using a Thermo Finnigan Delta plus XP IRMS at the Stable Isotope Laboratory in the School of Environmental Sciences, East Anglia. Measurement uncertainty was monitored using in-house casein and collagen standards with well-characterized isotopic compositions tested every 10–15 samples. The δ^{13}C values are expressed relative to VPDB and the δ^{15}N values relative to AIR. Precision (u(Rw)) was determined to be ±0.3 for δ^{13}C and ±0.4 for δ^{15}N based on repeated measurements of calibration standards and check standards. Accuracy (u (bias)) was determined to be ±0.15 for δ^{13}C and ±0.4 for δ^{15}N based on the difference between the observed and known δ values of the check standards and the long-term standard deviations of the check standards.

Differences between sub-samples under different pretreatment regimes allow differences in stable isotope values to be quantified. During this stage of the study, it was decided to include the sites Kalnik-Igrišče (KBA) and Donji Miholjac-Đanovci (DMD). For all seven sites, stable isotope values were taken for a subset of the grains (Table 3). Grains were individually crushed using a mini pestle before treatment. A singular crushed grain was then divided into four to allow for the following tests.

(1) No treatment.
(2) Rinsed three times with ultra-pure water then freeze dried.
(3) Acidifying in 10 ml of 0.5 M HCl @ room temp for 30 minutes, rinsed three times with ultra-pure water then freeze dried.
(4) Acidifying in 10 ml of 0.5 M HCl, heating for 30 minutes at 80 °C, rinsed three times with ultra-pure water then freeze dried.

A further subset was then examined comparing ABA pretreatment to no pretreatment (Table 4). The following protocol was used for ABA:

(1) 10 ml of 0.5 M HCl, heating for 60 minutes at 70°C, then rinsed three times with ultra-pure water.
(2) 10 ml of 0.1 M NaOH, heating for 60 minutes at 70°C, then rinsed in ultra-pure water until the solution is clear and the pH is neutral, with a minimum of three rinses.
(3) 10 ml of 0.5M HCl, heating for 25 minutes at 70°C, then rinsed three times with ultra-pure water (as above), then freeze dried.

Statistical analyses

All data were subjected to analysis of variance (ANOVA) to ascertain the effect of chemical treatments on stable isotope values. Unless otherwise stated, differences were considered statistically significant when $P < 0.05$.

Results

ATR-FTIR analysis

To identify possible contamination, the ATR-FTIR spectra from the study sites was compared with ATR-FTIR spectra created by Vaiglova et al. (2014), who artificially contaminated their samples with carbonates, nitrates and humic acid (Figure 2). Before starting its important to note that variability in peak height can occur due to differences in particle size or the amount of material used (Reed 2023), so peak height should be viewed relative to other peaks. Peaks that may indicate nitrate contamination were not detected at 1540 and 3300 cm^{-1}, with only a small peak seen at 1085 cm^{-1} for SIS_1. For carbonates, no peaks are seen at 720 cm^{-1}, however, FEU_6 and OSR_4 both show

Table 3. List of taxa treated per site. Four treatments were performed per grain: untreated, rinse only, ambient HCl and hot HCl.

Site	Taxa	Quantity	Testing ref no.
Sisak (SIS)	*Vicia Faba (VFAB)*	1	1
Osijek-Silos (OSR)	*Triticum aestivum/durum (FTW)*	1	3
Osijek-Silos (OSR)	*Secale cereale (SCER)*	1	4
Feudvar (FEU)	*Triticum dicoccum (TDIC)*	1	6
Ivanovci Gorjanski – Palanka (IGP)	*Triticum aestivum/durum (FTW)*	1	8
Park Kraljice Katarine Kosače (OKR)	*Triticum aestivum/durum (FTW)*	1	5
Park Kraljice Katarine Kosače (OKR)	*Hordeum vulgare (HVUL)*	2	13
Kalnik-Igrišče (KBA)	*Hordeum vulgare (HVUL)*	1	11
Kalnik-Igrišče (KBA)	*Triticum spelta (TSPE)*	1	12
Donji Miholjac-Đanovci (DMD8)	*Triticum aestivum/durum (FTW)*	1	14
Donji Miholjac-Đanovci (DMD15)	*Triticum aestivum/durum (FTW)*	1	15

Table 4. List of taxa treated per site. Two treatments were performed per grain: untreated and acid-base-acid sequence.

Site	Taxa	Quantity	Testing ref no.
Osijek-Silos (OSR)	*Hordeum vulgare (HVUL)*	2	20a, b
Osijek-Silos (OSR)	*Triticum aestivum/durum (FTW)*	1	22a
Kalnik-Igrišče (KBA)	*Triticum aestivum/durum (FTW)*	1	32a
Kalnik-Igrišče (KBA)	*Triticum dicoccum (TDIC)*	1	33b
Kalnik-Igrišče (KBA)	*Triticum spelta (TSPE)*	1	34g
Donji Miholjac-Đanovci (DMD15)	*Triticum aestivum/durum (FTW)*	2	29g, 30b
Ivanovci Gorjanski – Palanka (IGP)	*Triticum aestivum/durum (FTW)*	2	38h, j
Park Kraljice Katarine Kosače (OKR)	*Triticum aestivum/durum (FTW)*	1	40a
Park Kraljice Katarine Kosače (OKR)	*Hordeum vulgare (HVUL)*	2	41a, b

peaks at 870 cm^{-1}. Evidence of humic acid contamination is potentially seen at 1010 cm^{-1}, where all the samples show a peak; OSR_4 and FEU_6 have the highest peaks. Although No peaks are noted at 3690 cm^{-1} and only SIS_1 has a small peak at 1080 cm^{-1} possibly indicating little to no humic acid contamination.

Rinse and HCl sequences

In the following results, untreated values are represented as a mean of all the isotope determinations for the untreated sub-sample (variation between these replicates is shown in Reed and Wallace (2023); Supplementary Data_ Stable isotopes). For analyses of pretreated samples, each replicate sample is shown individually to demonstrate the level of variability between replicates.

A total of 57 individual pretreated sample combinations (including duplicates) were examined. The results of testing the rinse and ambient and hot HCl sequences are presented in Figure 3. The results are presented for each individual sample normalized to the mean isotope value of the untreated control sample. The difference between the $\delta^{13}C$ of untreated control samples and their pretreated counterpart was overall small, resulting in an average decrease of 0.09‰. For $\delta^{15}N$ pretreated samples were, on average, 0.15‰ lower than untreated controls.

The rinse-only pretreatment sequence resulted in the smallest differences in $\delta^{13}C$ and $\delta^{15}N$. $\delta^{13}C$ values tended to be higher after the rinse, by an average of 0.08‰. In some cases, $\delta^{13}C$ was lower after pretreatment, and the absolute (i.e. magnitude) change was on average 0.11‰. The range of differences spanned

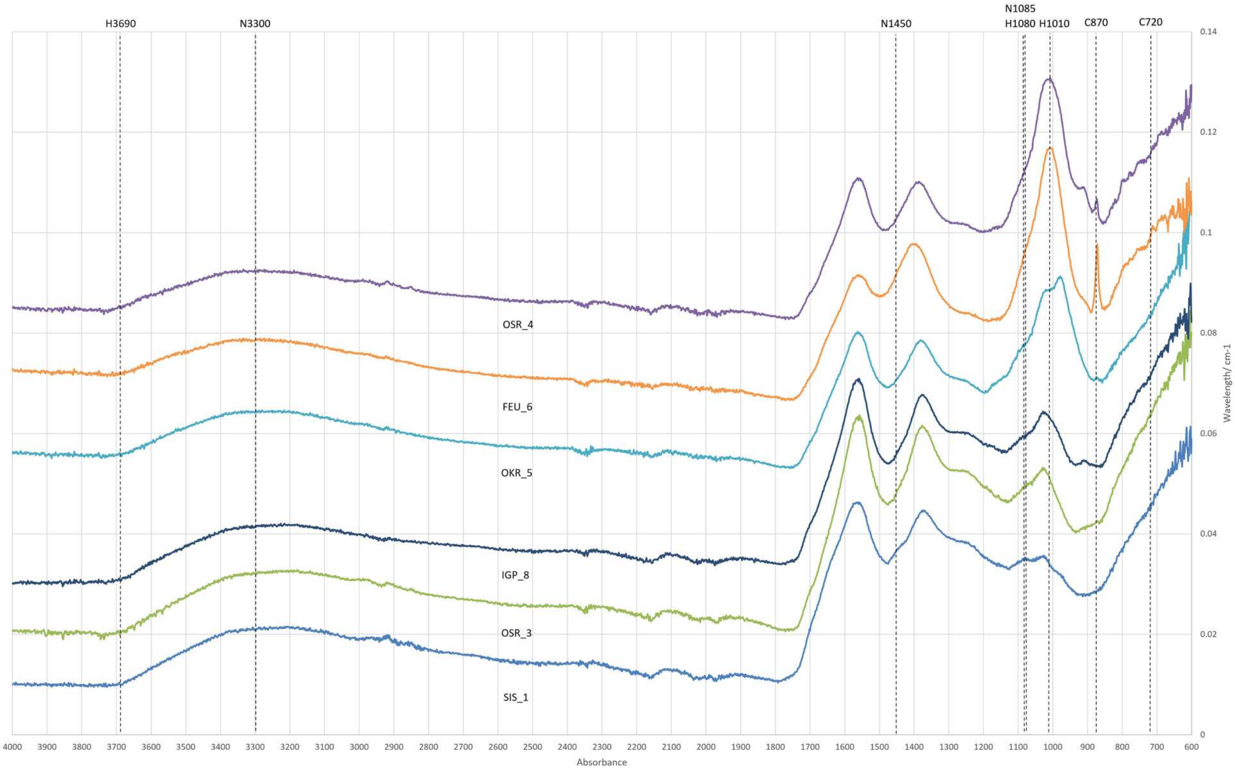

Figure 2. ATR–FTIR spectra of the seeds analysed as part of this study. H[no.] = wavelengths of humic acid peaks, N[no.] = wavelengths of nitrite peaks, C[no.] = wavelengths of carbonate peaks, all contaminant wavelengths according to Vaiglova et al. (2014). The x–axis – or horizontal axis – represents the wavenumber, while the y–axis – or vertical axis – represents the amount of infrared light absorbed or transmitted by the material being analysed. The peaks, which are also called absorbance bands, correspond with the various vibrations of the sample's atoms when exposed to the infrared region of the electromagnetic spectrum.

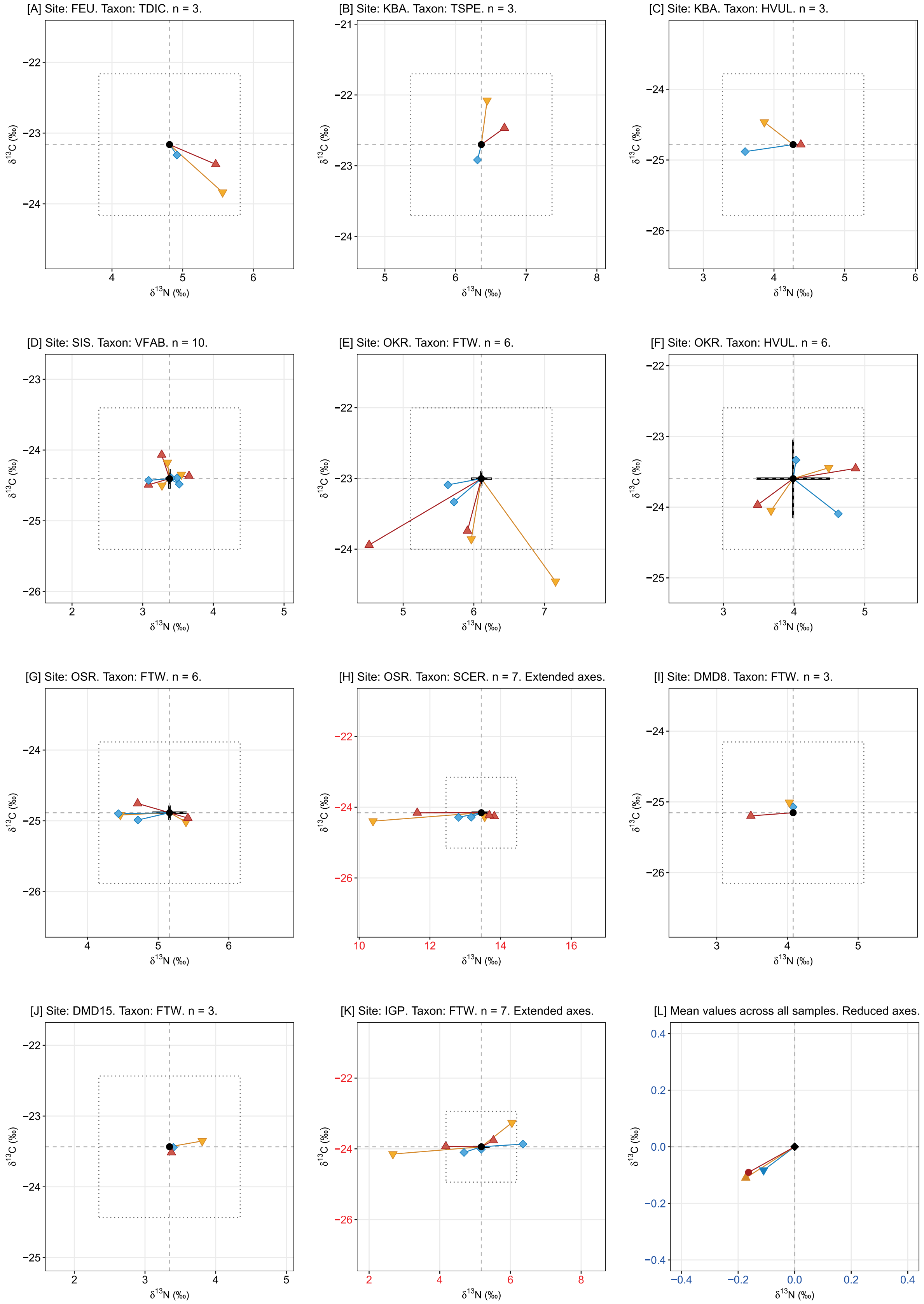

Figure 3. Changes in δ^{13}C and δ^{15}N for each sample subjected to a rinse-only or HCl pretreatment sequences. Each panel represents a single sample, identified by Site Code and Taxon Code (see below). Black marker and error bars show the mean and standard deviation for untreated sub-samples. Coloured markers represent individual pretreated sub-samples, as follows: blue = rinse-only, orange = ambient HCl, and red = hot HCl. The axis of each panel is centered on the mean of untreated sub-samples. Panels with black axis text have a range of 1.6 on both axes, red text indicates doubled range (3.2), and blue text indicates halved range (0.8). The dotted box always indicates 1‰ boundary around the untreated mean. Site Codes: FEU = Feudvar, KBA = Kalnik-Igrišče, SIS = Sisak, OKR = Park Kraljice Katarine Kosače, OSR = Osijek-Silos, DMD8 = Donji Miholjac-Đanovci (8th century), DMD15 = Donji Miholjac-Đanovci (fifteenth century), and IGP = Ivanovci Gorjanski – Palanka. Taxon Codes: TDIC = *Triticum dicoccum*, TSPE = *T. spelta*, HVUL = *Hordeum vulgare*, VFAB = *Vicia faba*, FTW = *T. aestivum/durum*, and SCER = *Secale cereale*.

−0.26‰ to +0.50‰ (standard deviation = 0.16‰). The δ^{13}C difference between untreated and rinsed samples was statistically significant (paired sample *t*-test: $t = 2.36$, df = 19, $p = 0.03$). δ^{15}N also tended to increase, by an average of 0.11‰. δ^{15}N differences were more varied, however, ranging from − 1.18‰ to +0.72‰ (sd = 0.46‰). The magnitude of change was 0.32‰, and the variation in results meant the difference in rinse-only sub-samples from their untreated control samples was not statistically significant (t = 1.07, df = 19, $p = 0.30$).

Ambient HCl pretreatment produced a similar pattern of results to that of rinse-only but with greater levels of variation. Δ^{13}C for pretreated samples were on average 0.11‰ higher, ranging from −0.68‰ to +1.45‰ (sd = 0.51‰), with an average magnitude of 0.36‰. Paired sample *t*-test indicates that the difference between untreated and ambient HCl pretreated δ^{13}C values were statistically non-significant ($t =$ 0.91, df = 18, $p = 0.38$). Pretreatment δ^{15}N values were 0.17‰ higher, ranging from −1.05‰ to +3.06‰ (sd = 1.05‰). The average magnitude of change was 0.84‰, and the differences were non-significant ($t = 0.70$, df = 17, $p = 0.50$).

Hot HCl pretreatment results were comparable to those for ambient HCl. Δ^{13}C values were 0.09‰ higher on average, ranging from −0.34‰ to +0.94‰ (sd = 0.31‰), and with an average magnitude of 0.20‰. Differences between hot HCl pretreated and untreated samples were not significant ($t = 1.26$, df = 18, $p = 0.22$). δ^{15}N values were 0.16‰ higher, −0.88‰ to +1.81‰ (sd = 0.71), and the average magnitude in the differences was 0.53‰. Differences were not significant (t = 1.00, df = 18, $p = 0.33$).

Across the 57 analyses, only one resulted in a difference in $\delta^{13}C \geq 1$‰ (−1.45‰ for ambient HCl analysis of OKR_FTW, Figure 3(E)), sufficient to meaningfully influence interpretation. A separate ambient HCl analysis from the same sample produced a smaller difference, albeit in the same direction (−0.86‰). For δ^{15}N, the number of analyses that resulted in a difference ≥1‰ was seven (from OSR_SCR, OKR_FTW and IGP_FTW). All three pretreatment types produced large differences. For OSR_SCR, both ambient and hot HCl sequences led to lower δ^{15}N values yet replicates of both sequences produced far smaller differences (Figure 3(H)). For OKR_FTW, the two analyses with differences of ≥1‰ were in opposite directions: −1.58‰ for an ambient HCl analysis and +1.05‰ for hot HCl (Figure 3(E)). Likewise, for IGP_FTW, differences occurred in opposite directions; −2.50‰ for ambient HCl to +1.18‰ for rinse-only (Figure 3(K)).

In only one sample, FEU_TDIC, did the different pretreatment regimes produce consistent results. For this sample all pretreatment regimes resulted in decreased δ^{13}C and increased δ^{15}N (Figure 3(A)). The magnitude of these changes was greater for HCl-based pretreatments (ambient: δ^{13}C −0.68‰, δ^{15}N +0.75‰; hot: δ^{13}C −0.28‰, δ^{15}N −0.65‰) than rinse-only pretreatment (δ^{13}C −0.15‰, δ^{15}N −0.11‰). Nevertheless, despite the consistency in the differences between pretreated and untreated samples, the total impact is minor.

Though there were occasional results for pretreated samples that were substantially different from their untreated counterparts, these outliers were never repeatable within a sample. Differences in both δ^{13}C and δ^{15}N varied within and between sub-samples, except for FEU_TDIC – although the difference was small (Figure 3(A)). The net effect of these results is that pretreated isotope results tend to be slightly lower for both δ^{13}C (by −0.09‰) and δ^{15}N (by −0.15‰). The net difference is statistically significant in the case of δ^{13}C (t = 2.047, $p = 0.045$), but not for δ^{15}N (t = 1.474, $p = 0.146$), but both are too small to have any meaningful bearing on archaeological interpretations.

ABA sequences

A subset of 12 samples from 5 sites were pretreated using the ABA protocol (Table 3). One barley grain from OKR (sample 41a) had a very low weight and produced very different values compared to the barley from sample 41b (retrieved from the same context) and so was removed from the analysis as no replicates were possible. The ABA results are presented in Figure 4. Overall, the ABA treatment reduced the δ^{13}C values by 0.27‰ of the untreated mean (−23.4‰, SD = 0.27‰) and δ^{15}N reduced by 3.6‰ from the untreated mean (11.2‰, SD = 0.35‰). The average effect of ABA pretreatment was to increase δ^{13}C by +0.37‰ and decrease δ^{15}N by −0.57‰. For 4 of the 12 samples, the change in δ^{13}C after ABA pretreatment was >0.5‰ (all increases), and for 6 of the 12 samples the change in δ^{15}N was >0.5‰ (all decreases).

Site-wise results

At the Bronze Age settlement of Feudvar (FEU), one grain was pretreated using rinse-only and ambient and hot HCl regimes (Figure 3(A)). For both δ^{13}C and δ^{15}N, all three pretreatments resulted in differences <1‰. All three pretreatment regimes lowered δ^{13}C (mean = −0.37‰) and increased δ^{15}N (+0.50‰). The greatest difference between the untreated and pretreated results was for the ambient HCl treatment, where δ^{13}C produced a difference >0.5‰. The small magnitude of these differences means that whether untreated or pretreated samples were analysed, the archaeological interpretation would likely be similar.

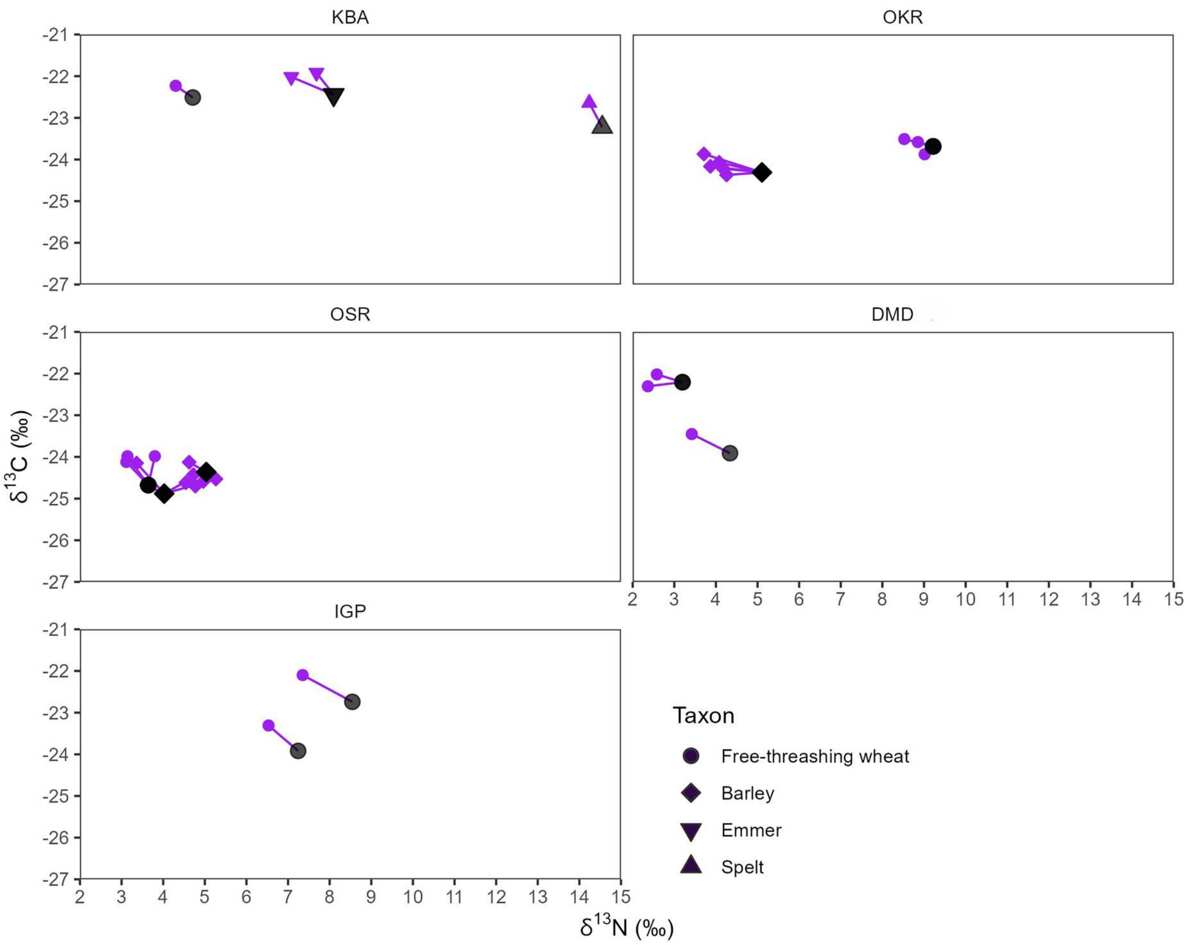

Figure 4. Changes in δ^{13}C and δ^{15}N for each sample subjected to the ABA pretreatment sequences. Each panel represents all samples of all taxa (see below) from an individual site, identified by Site Code (see below). Black markers and error bars (all within symbol) show the mean and standard deviation for untreated sub-samples. Purple markers represent individual pretreated sub-samples. Site Codes: KBA = Kalnik-+65xz Igrišče, OKR = Park Kraljice Katarine Kosače, OSR = Osijek-Silos, DMD = Donji Miholjac-Đanovci (8th and 15th century), and IGP = Ivanovci Gorjanski – Palanka. Taxon Symbols: downward-triangle = *Triticum dicoccum*, upward-triangle = *T. spelta*, circle = *T. aestivum/durum*, and diamond = *Hordeum vulgare*.

At Late Bronze Age Kalnik-Igrišče (KBA) the results were more varied. Five grains were analysed (two received rinse-only and HCl pretreatments, and three were ABA pretreated). All pretreatments that involved an acid step (i.e. excluding rinse-only) resulted in higher δ^{13}C after pretreatment, and in some cases with a difference δ^{13}C > 1.0‰. For δ^{15}N, both increases and decreases occurred. The decreases were of a greater magnitude than the increases, but in almost all cases the difference from untreated material was <0.5‰. Notably all three ABA pretreated samples had lower δ^{15}N than their pretreated counterparts.

At Iron Age Sisak (SIS) only one broad bean was analysed, with three replicates for the rinse-only and HCl pretreatment regimes (Figure 3(D)). For all pretreated sub-samples the difference from the untreated sample was <0.5‰. Pretreated δ^{13}C results were above and below that of the untreated sample, with the absolute average difference being +0.04‰ and the mean magnitude was 0.10‰. δ^{15}N results were similar with the absolute difference averaging at −0.01‰ and the magnitude 0.16‰. There was no trend between the different pretreatment regimes.

At Park Kraljice Katarine Kosače (OKR), the five grains analysed (excluding 41a) showed no consistent trend due to pretreatment. δ^{13}C values tended to be lower by a small amount following rinse-only and HCL regimes (mean = −0.43‰), whilst ABA tended to increase δ^{13}C (mean = + 0.12‰). The magnitude of δ^{13}C varied but was occasionally large – especially for HCl regimes on OKR_FTW (Figure 3(E), mean = 1.00‰). All pretreatment regimes tended to lower δ^{15}N by around 1‰, and this was especially the case for ABA pretreatment on the barley grain (sample 41b, mean = −1.09‰).

The other Roman site, Osijek-Silos (OSR) had five grains analysed (two received rinse-only and HCl pretreatments, and three were ABA pretreated). The δ^{13}C values for three of the samples tended to be minimal (Figure 3(G,H) and Figure 4, HVUL), with no differences from untreated values <0.5‰. For one FTW ABA sample (22a), however, pretreated samples δ^{13}C was >0.5‰ higher than the untreated material. In contrast, the $\delta^{15}N$ values for pretreatment tended to be slightly lower, around 0.5‰, for all five samples.

The site of Donji Miholjac-Đanovci (DMD) has samples from an 8th and fifteenth century AD settlement. The former is represented by one grain that was subjected to the rinse-only and HCl regimes and one subjected to ABA pretreatment. These pretreatments resulted in minimal changes in both $\delta^{13}C$ and $\delta^{15}N$ values, except for one hot HCl replicate that resulted in $\delta^{15}N$ decreasing by around 0.5‰ (Figure 3(I)). For the fifteenth century samples three grains were analysed, including one subjected to ABA pretreatment. The sample subjected to rinse and HCl pretreatment produced similar results to the earlier period with minimal change, except one ambient HCl replicate that increased $\delta^{15}N$ by around 0.5‰ (Figure 3(J)). The ABA results did, however, result in some differences with untreated samples. One sample (30b) saw an increase in $\delta^{13}C$ around 0.5‰, whilst $\delta^{15}N$ was reduced in both samples after pretreatment by around 0.5‰ to 1.0‰.

The final site is late medieval Ivanovci Gorjanski – Palanka (IGP) where three grains were analysed (one received rinse-only and HCl pretreatments, and two were ABA pretreated). Here, pretreatment either resulted in a minimal difference or a slight increase of around 0.5‰ for $\delta^{13}C$. These increases were detected in two pretreatment regimes (ambient HCl and ABA). $\delta^{15}N$ results after pretreatment were highly varied, and it is difficult to ascertain a consistent pattern. Increases and decreases were apparent, both by >1‰ change. Decreases were most common amongst the replicates, however, and the largest increase was following rinse-only pretreatment, which for other sites resulted in the smallest change.

Discussion

Using ATR-FTIR to determine contamination of carbonized macro-remains

Interpretation of the ATR-FTIR spectra of carbonized macro-remains can be complex, with only a handful of studies providing experimental data for comparison. The work by Vaiglova et al. (2014) focuses on a visual inspection of the ATR-FTIR spectra to detect prominent peaks at certain wavenumbers that indicate different contaminants that could ultimately affect stable isotope data. Although it is suggested that high peaks equal high contamination, it is unclear how those peaks relate to other non-diagnostic peaks or whether you need to have all the diagnostic peaks or just one to indicate contamination. This may result in misinterpretation when assigning meaning to the peaks within the spectra.

Archaeobotanical material will also be subjected to different charring and depositional processes (Styring et al. 2013), as well as residing in different soil conditions that in themselves display a complex and broad range of soluble substances such as humic materials, decomposed animals and/or plants as well as micro-organisms. How these variables affect and are identified in archaeobotanical material using ATR-FTIR is as yet largely unexplored.

The ATR-FTIR results here exhibit only one diagnostic peak for carbonate, nitrate and humic contamination as outlined by Vaiglova et al. (2014). The peaks around 1000 cm^{-1} are also relatively consistent, considering the sites under study are located in different locations, while OSR_4 and OSR_5 are from the same sample/deposit. Whether the height of the peak is comparable with the contamination peak heights identified by Vaiglova et al. (2014) is also unclear, especially as particle size and the amount of material used can affect the peak height (Reed 2023). Furthermore, Vaiglova et al. (2014) used only one type of humic salt and other forms could react with charred material and with pretreatment methods in different ways. Other studies indicate humic and fulvic acid peaks between 850 and 1100 cm^{-1} (Lebon et al. 2016; Mylotte et al. 2015), while phosphates (PO_4^{3-} group) can also form intensive IR absorption bands at 560 and 600 cm^{-1} and at 1000–1100 cm^{-1} (Berzina-Cimdina and Borodajenko 2012; Coates 2000).

The ambiguity around the ATR-FTIR results is also supported by the isotopic values generated in this study from the untreated, rinse and ambient and hot HCl test sequences (Table 5). Experiments by Vaiglova et al. (2014) showed that the $\delta^{13}C$ values for carbonate contaminated samples increase with higher calcite content. For humic acid, Vaiglova et al. (2014) showed that the presence of 10% and 50% contamination also caused larger shifts in the $\delta^{13}C$ values than in the $\delta^{15}N$ values. So, for one archaeological sample contaminated with humic acid the $\delta^{13}C$ value was ca 1.0‰ lower than that of the uncontaminated sample (−22.9‰). Pure humic acid treated with base-acid (BA) also yielded a more negative $\delta^{13}C$ value (BA treated: −27.6‰; untreated: −25.9‰). For the samples in this study, we should therefore see an increase in $\delta^{13}C$ as humic acid is removed, while for carbonate contamination a decrease in $\delta^{13}C$ should be seen. However, this pattern is not really seen, with only OKR_FTW (Figure 3(E)) and FEU_TDIC (Figure 3(A)) showing a decrease and IGP_FTW (Figure 3(K)) showing both a decrease and increase depending on the pretreatment. Overall, we see no clear correlation between potential contaminants identified through ATR-FTIR and the untreated and treated isotopic values. Further, the small differences noted here in stable isotope values before and after pretreatment are unlikely to change the archaeological interpretation: although it must be noted that this is an extremely small sample of only six grains.

Table 5. Interpretation of the ATR-FTIR spectra for peaks indicating nitrate, carbonate and humic acid contamination and summary of the impact that the rinse and ambient and hot HCl sequences had on isotopic values.

ATR-FTIR Reference	Nitrate contamination	Carbonate contamination	Humic acid contamination	Isotope Reference	Pretreatment impact on $\delta^{13}C$	Pretreatment impact on $\delta^{15}N$
SIS_1	Low	None	Low	SIS_VFAB	Nominal	Nominal
OSR_3	None	None	Low	OSR_FTW	Nominal	Decrease
OSR_4	None	Low	High	OSR_SCER	Nominal	Decrease
OKR_5	None	None	High	OKR_FTW	Decrease	Decrease/increase
FEU_6	None	Medium	High	FEU_TDIC	Decrease	Increase
IGP_8	None	None	Low	IGP_FTW	Decrease/increase	Decrease/increase

Comparing untreated and pretreated samples to inform future site-specific interpretations

An alternative approach to identifying the presence of contaminants is through the comparison of untreated and pretreated sub-samples of the same sample material. This constitutes a "black box" approach in that the cause of a difference in isotope values between untreated and pretreated counterparts is not identified, as it is in ATR-FTIR. In cases where the isotope values for untreated and pretreated sample material are similar, however, it can be supposed that no contaminants exist at a sufficient level to influence isotope values. In cases where the isotope values differ, the magnitude of the effect on isotope values of the contaminant (or other cause of variation) can be assessed.

The interpretation of stable isotope values derived from archaeobotanical remains is equivocal. We assume that the pretreated value is a "truer" representation of the original plant material's stable isotope values. This is on the basis that the pretreatment has removed contaminants that influence the stable isotope values. It should be noted, however, that we do not know what the chemical processes that occur during pretreatment do to the original charred plant material. Additional factors could also cause variation in stable isotope values. These factors include local atmospheric (for carbon isotopes) and soil (for nitrogen isotopes) conditions, a myriad of environmental factors, human action (the main target of investigation), plant biology, preservation processes and the post-depositional environment. Despite the manifold of influencing factors, we know that interpretative meaning can be derived from isotope values. We must, however, remain cautious in "over-interpreting" what is inherently noisy data.

A cornerstone of robust interpretation is to avoid assigning importance to small fluctuations in stable isotope values. Accordingly, slight differences between untreated and pretreated sub-samples should not unduly influence archaeological interpretation. There is no specific value at which a difference becomes meaningful and will depend on sample size. Indeed, high resolution sampling of large sample sets could be used to interpret slight variations in stable isotope values. Yet, such high quality datasets are, sadly, still rare in the fragmentary archaeological record. We therefore propose three arbitrary thresholds as an *indication* of the level of impact on archaeological interpretation the difference between untreated and pretreated samples may have. Here, we interpret a difference in carbon or nitrogen stable isotopes values up to ±0.5‰ to have *minor* importance for archaeological interpretations, for differences greater than 0.5‰ but less than ±1.0‰ to have *moderate* importance, differences up to ±1.5‰ to have *major* significance, and any value greater to have *extreme* significance.

Most of the sites examined in this study exhibited a small trend in the difference of $\delta^{13}C$ values between untreated and pretreated counterparts (Table 6). There is no apparent chronological or geographic trend as to which sites showed differences. The greatest variation was at Park Kraljice Katarine Kosače (OKR), where a Roman pit containing large amounts of organic matter was sampled. Here the pretreated samples were above and below the untreated $\delta^{13}C$

Table 6. Summary of the direction (boas) and magnitude (accuracy) of the difference between stable isotope values before and after pretreatment.

		Pretreatment impact on $\delta^{13}C$ values		Pretreatment impact on $\delta^{15}N$ values	
	No. of grains	Direction	Magnitude	Direction	Magnitude
Feudvar (Bronze Age)	1	Decrease	Minor (∼0.5‰)	Increase	Moderate (∼1.0‰)
Kalnik-Igrišče (Late Bronze Age)	5	Increase	Minor (∼0.5‰)	Decrease	Minor (∼0.5‰)
Sisak (Early Iron Age)	1	Equivalent	–	Equivalent	–
Park Kraljice Katarine Kosače (Early Roman)	6	Decrease	Moderate (∼1.0‰)	Decrease	Moderate (∼1‰)
Osijek-Silos (Mid/Late Roman)	5	Increase	Minor (∼0.5‰)	Decrease	Minor (∼0.5‰)
Donji Miholjac-Đanovci (8th c. AD)	2	Increase	Minor (∼0.5‰)	Decrease	Moderate (∼1.0‰)
Donji Miholjac-Đanovci (15th c. AD)	2	Equivalent	–	Decrease	Moderate (∼1.0‰)
Ivanovci Gorjanski – Palanka (Late Medieval)	3	Increase	Minor (∼0.5‰)	Decrease	Major (∼1.5‰)

For direction, increase indicates pretreated samples had higher values than their untreated counterparts, vice-versa for decrease, whilst equivalent indicates that there was either little difference between untreated and pretreated samples or that both increases and decreases were observed. Magnitude indicates the scale of the difference between untreated and pretreated samples, regardless of whether pretreated values are higher or lower than their untreated counterparts.

value, though on average the values tended to be lower by no more than a moderate amount (≤1‰). The sites of Kalnik-Igrišče (KBA, a house storage context) and Ivanovci Gorjanski – Palanka (IGP, a pit) both had somewhat varied results, with values above and below that for untreated samples. In both cases the average change was near zero, but there was a greater tendency for $\delta^{13}C$ values to be higher by a minor amount (≤0.5‰). Results from Feudvar (FEU, settlement contexts) were consistently lower after pretreatment but by a minor amount (≤0.5‰). The least variation was from the two phases from Donji Miholjac-Đanovci (DMD, pits), where $\delta^{13}C$ value after pretreatment were very similar to the values for untreated samples.

For stable nitrogen isotope analysis, the results per site tended to show greater variation and a higher magnitude of difference with the untreated counterparts. Interestingly, however, all but one site exhibited a decreased in $\delta^{15}N$ following pretreatment. Three sites – Osijek-Silos (OSR), Park Kraljice Katarine Kosače (OKR) and Ivanovci Gorjanski – Palanka (IGP) – exhibited considerable variation, with some extreme outliers and increases as well as decreases in $\delta^{15}N$. In these cases, however, the results tended to show on average a decrease in $\delta^{15}N$, the magnitude of which, however, varies. The remaining sites, Kalnik-Igrišče (KBA) and Donji Miholjac-Đanov (DMD), showed a more consistent decrease in $\delta^{15}N$, usually by a minor amount.

Conclusion

Archaeobotanical stable isotope analysis can, and should, become a routine element of the study of past agriculture. The design, implementation and interpretation of stable isotope programmes can be achieved by archaeobotanists that do not have a geochemistry background. There are substantial pitfalls in the application of stable isotopes, however, and it would be undesirable for the benefits of democratization to be outweighed by the proliferation of low-quality studies. Accordingly, what we have sought to do here is present an example of how issues of contamination and the confidence of interpretations can be considered in an accessible manner.

Our extension of archaeobotanical stable isotope studies to continental Croatia and northern Serbia involved testing several different pretreatment methods for ancient charred plant material with the aim to determine how robust subsequent interpretations of crop $\delta^{13}C$ and $\delta^{15}N$ values could be. We hope that beyond the study's specific regional significance, the approach we have taken can be used as a model showcasing that archaeobotanical stable isotopes analysis is a technique that can be utilized across the archaeobotanical research community.

The use of the ATR-FTIR to identify contaminants indicated possible humic, carbonate and nitrate contamination on some of the grains. The big benefit of using ATR-FTIR is that it is quick, allowing you to scan a range of grains easily and is useful to initially rule out contamination. However, this method proved inconclusive in this study once isotopic tests were conducted, suggesting that contamination was minimal and unlikely to influence the isotopic values (<1.0‰). Thus, if contamination peaks are identified in the ATR-FTIR spectra pretreatment testing is recommended, especially as interpretation of the spectra is still unclear. More experimental studies are needed to help determine the causal effects of contamination and pretreatment and how this can be identified using just ATR-FTIR. Research would also benefit from characterizing the molecular compounds that are removed during pretreatment (via a form of chromatography/spectrometry) to better understand the contamination, including its sources (with implications relating to depositional environment, etc).

Overall, the pretreatment sequences, rinse, acid-only and ABA, produced minor to moderate (~1.0‰) impact on the isotopic values. In the few cases where the differences are sufficient to influence interpretation, the difference is not repeated across the analyses of the same sample. Further, the $\delta^{13}C$ and $\delta^{15}N$ values change erratically with pretreatments, resulting in both increases and decreases in no particular pattern. Thus, the slight differences between untreated and pretreated sub-samples should not impact the archaeological interpretation, removing the need for pretreatment of the remaining archaeobotanical material. This method produced, in our minds, the most robust outcomes to determine that pre-treatment was not needed for the rest of the samples.

Our approach of qualifying the extent of variation expected based on pretreatment results means that even with a small dataset it is possible to determine an appropriate level of confidence with which to interpret stable isotope values when contamination is unknown. The efficiency of this approach ensures that the costs and resources for analysis can be spent effectively. In smaller projects, testing and pretreatment of all or many samples may result in the number of samples being analysed to be too few to discern patterns of archaeological significance. The combination of low-cost visual inspection and washing, tactical use of pretreatment and thoughtful consideration of confidence levels means that high quality archaeobotanical stable isotope studies can be universally achieved.

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Disclosure statement

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