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Recommendations for stable isotope analysis of charred archaeological crop remains

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Stable isotope analysis of plant remains recovered from archaeological sites is becoming more routine. There remains a lack of consensus, however, on how to appropriately select archaeological plant remains for isotopic analysis, how to account for differences in preservation and the effect of potential contamination, and how to interpret the measured isotope values in terms of the conditions in which the plants grew. In this paper, we outline the main issues to be considered when planning and conducting an isotopic study of archaeobotanical remains. These include: (1) setting out the research question(s) that will be answerable using available analytical approaches, (2) considering the archaeological context from which plant remains derive, (3) determining appropriate sample size through consideration of estimate precision, (4) establishing the conditions in which plant remains have been preserved and potential effects on their isotope values, and (5) accounting for possible contamination during deposition. With these issues in mind, we propose some recommendations for researchers to consider when planning and conducting an isotopic study of archaeobotanical remains.

KEYWORDS

carbon, nitrogen, $\delta^{13}C$, $\delta^{15}N$, agriculture, archaeobotany

1 Introduction

Stable isotope values of plants reflect their growing conditions, which can be influenced by both natural environmental conditions and anthropogenic activities. In the last 10–15 years, there has been an increasing number of studies that have analyzed the stable carbon and nitrogen isotope values of charred crop remains recovered from archaeological sites to provide insights into past agricultural practices (e.g., Araus et al., 2014; Bogaard et al., 2013; Li et al., 2022; Riehl et al., 2008) and serve as an isotopic reference for reconstructing human and animal diets (e.g., Fraser et al., 2013b; Isaakidou et al., 2022; Knipper et al., 2020; Styring et al., 2018). Specifically, the stable carbon isotope ($\delta^{13}C$) values of crops can provide information about their water status (Ehleringer et al., 1993), which can help to identify watering practices such as irrigation in otherwise dry environments (e.g., Araus et al., 1997). Stable nitrogen isotope ($\delta^{15}N$) values of crops reflect the source and cycling of soil nitrogen, so can be affected by the addition of fertilizers such as animal manure, as well as environmental conditions such as waterlogging and aridity (e.g., Högberg, 1997).

TABLE 1 Examples of common research questions and case studies that have employed stable isotope analysis of archaeobotanical remains.

Research question	Examples
Comparison of cultivation practices between crop species and assemblages and their potential variability over time and space	Araus et al., 2014
	Bishop et al., 2022
	Kanstrup et al., 2014
Determining a mean crop isotope value for comparison with human/faunal isotope values to reconstruct dietary inputs, potentially within an integrated isotopic mixing model	Knipper et al., 2020
	Styring et al., 2018
	Tao et al., 2022
Identifying agricultural intensification and extensification and labor budgets in agro-pastoral systems at variable scales and periods in different parts of the world	Bogaard et al., 2013
	Styring et al., 2022
	Tian et al., 2022
Establishing long-term trajectories of environmental change and soil health	Gron et al., 2021

When interpreting stable isotope values of archaeological crop remains in terms of past agricultural practices, it is important to consider the theory, method and practice underlying the disciplinary approach. One of the main principles underlying this approach is the extrapolation of experimental observations of modern crop experiments to the archaeobotanical record. This is a key theoretical pillar of experimental archaeology (Coles, 1979), but it is important to recognize that the modern observations are based on simulation experiments, not direct replication of the unknowable combination of soil, climate, plant physiology and societal factors for any one field or cultivation plot in the human past (Outram, 2008; Reynolds, 1999). As such, studies that outline the variability of modern crop isotope values in different soil and climatic conditions can be used as a quantitative guide and *framework* for interpretation, but caution must be used when directly quantitatively comparing the modern to the ancient isotopic values, a key theoretical consideration discussed in the wider experimental archaeology literature (Lin et al., 2018; Outram, 2008).

These theoretical considerations implicitly and explicitly underpin the types of research questions that are commonly set when analyzing stable isotope values in archaeological crop remains. There is a wide range of research establishing the modern baseline isotopic variability for different crop, climatic and soil amendment conditions across different parts of the world (e.g., Araus et al., 1997; Blanz et al., 2019; Dong et al., 2022; Fraser et al., 2011; Wallace et al., 2013) and an increasing number of taphonomic studies addressing issues of isotopic fractionation through preservation processes, such as carbonization, and how to identify, record and mitigate for these factors from experimental and archaeological crop remains (e.g., Nitsch et al., 2015; Stroud et al., 2023a; Teira-Brión et al., 2024; Varalli et al., 2023). Examples of common research questions, alongside some case studies that have addressed these using stable isotope analysis of archaeobotanical remains, are in Table 1.

Despite the increasing number of studies employing stable isotope analysis of archaeological crop remains, there remains a lack of consensus on how best to plan and conduct this type of research. Complications associated with the effect of charring, contamination, and post-depositional alteration on plant isotope values, as well as the interpretation of crop isotope values in terms of palaeoenvironment, agrarian practices, and geographic origin were discussed in Fiorentino et al. (2015). But as with any scientific application, the method reconfigures when research interests evolve. The last decade has seen considerable development in new research questions using stable isotope measurements of plants in an interdisciplinary domain and it is now timely to revisit and expand upon these methodological issues. Here, we set out a series of recommendations on how to plan and conduct stable isotopic investigation of archaeobotanical remains to encourage synergies among studies in different environmental settings and to inspire new research questions. In doing so, we reiterate some of the pressing points made in Vaiglova et al. (2022), which was a broader guide to best practice in archaeological isotope studies, but we expand on the issues that are particular to plant remains recovered from archaeological sites. In this paper we focus on stable isotope analysis of charred large-grained cereal grains (e.g., wheats and barley) and pulses using the C_3 photosynthetic pathway because these have been the primary focus of research development so far, but we consider points relevant to C_4 crops, including smaller grained cereals like millets and sorghum, where pertinent.

2 Challenges associated with stable isotope analysis of archaeological crop remains

There are several issues to be taken into consideration when planning and conducting isotopic analysis of archaeobotanical remains. These can influence the sampling protocol, the preparation method, the instrumental set-up and can ultimately determine whether the analyses are justified and interpretations are robust. We provide an overview of these issues as a background to suggesting steps that can be taken to ensure that studies try to address these as satisfactorily as possible.

2.1 Analytical difficulties of determining stable isotope values of plant material

Depending on the sensitivity of the isotope ratio mass spectrometer (IRMS) used, there is a minimum mass of C and N required for accurate and precise isotope measurements. Uncharred and charred cereal grains comprise relatively high %C and relatively low %N, giving rise to relatively high C:N atomic ratios (Table 2). This means that it can be difficult to simultaneously determine the $\delta^{13}C$ and $\delta^{15}N$ values of a cereal grain without either having too little N or overloading the IRMS with carbon dioxide. Even in elemental analyser (EA)-IRMS instruments where it is possible to dilute the carbon dioxide peak relative to the nitrogen gas peak, there is still a minimum quantity of plant material required to yield sufficient N for an accurate and precise $\delta^{15}N$ value. As a result, single grain analysis of small-grained crops such as millets and pseudocereals is not yet practical due to their low mass.

TABLE 2 C and N content and C:N atomic ratios of uncharred cereal grains (barley, bread wheat, einkorn, emmer, oat, rye, spelt) and pulses (lentil, pea) and cereal grains and pulses charred at between 215 and 300°C for between 4 and 24 h (from Stroud et al., 2023b: Dataset_1).

Material	%C	95% confidence interval (CI)	%N	95% CI	C:N atomic ratio	95% CI	Number of analyses
Uncharred cereal grains	40.0	(39.9, 40.9)	1.6	(1.5, 1.7)	30.6	(28.3, 33.0)	33
Charred cereal grains	61.8	(61.0, 62.6)	3.3	(3.1, 3.4)	24.0	(23.2, 24.8)	273
Uncharred pulses	40.9	(40.1, 41.7)	3.7	(3.4, 4.0)	13.0	(12.0, 14.0)	6
Charred pulses	60.2	(58.9, 61.5)	6.2	(6.0, 6.4)	11.5	(11.2, 11.8)	69

2.2 Intra-plant and intra-plot variability in crop stable isotope values

Most studies that have analyzed crop stable isotope values in modern experimental and farm plots homogenized multiple grains randomly sampled from across the growing plot to average out isotopic variation among plants (e.g., Blanz et al., 2019; Bogaard et al., 2007; Christensen et al., 2022; Fraser et al., 2011; Kanstrup et al., 2011; Styring et al., 2019; Treasure et al., 2016; Wallace et al., 2013). If single archaeological grains are to be analyzed individually, however, a good grasp of the isotopic variability among individual grains growing in the same conditions is needed in order to know whether differences in isotope values are representative of meaningful differences in growing conditions and not just a result of isotopic variability within a single plant/plot.

Relatively few studies have determined the isotopic variability among individual grains/seeds within a single cereal ear/pulse pod, but those that have been conducted have combined standard deviations below 0.4‰ (Table 3). The pooled standard deviation of δ^{15}N values of 21 individual cereal ears is 0.36‰, while the pooled standard deviation of δ^{13}C values of 11 individual cereal ears is 0.25‰. The pooled standard deviation of δ^{15}N values of 8 pulse pods is 0.22‰. This variability is similar to the analytical uncertainty of stable isotope measurements, which is generally ~0.2‰—0.3‰, and therefore random sampling of grains/pulses from different parts of a cereal ear or pulse pod is unlikely to contribute significantly to inter-grain/pulse isotopic variability. However, more research is needed to expand the taxa investigated and to consider seasonal variations in flowering and/or grain filling conditions of a single plant/plot.

Even fewer studies have determined the isotopic variability among individual grains/pulses within a cultivation plot. Those that have been conducted show that the pooled standard deviation in δ^{15}N from 7 plots is 0.76‰ and the pooled standard deviation in δ^{13}C from 11 plots is 0.30‰ (Table 4). There are indications that there is greater isotopic variability among cereal grains grown in manured plots because of spatial variability in manure application (Larsson et al., 2019). For this reason, nitrogen isotopic variability within plots is likely constrained by the isotopic difference between the applied soil amendment and endogenous soil nitrogen. Similarly, the intra-plot variability in plant δ^{13}C values is likely constrained by surface water availability and light intensity, which will vary more in plots with diverse topographies, and presence of or proximity to water bodies and/or overhanging vegetation. These values of intra-plot isotopic variability should therefore be treated as rough guidelines that will likely vary by context.

2.3 Environmental variability in crop stable isotope values

While the main focus of isotopic studies of archaeological crop remains has been to provide insights into past agricultural practices such as watering and soil amendment, there are environmental and physiological factors which also affect plant δ^{13}C and δ^{15}N values. Stable δ^{13}C values of plants are influenced by the ratio of leaf intercellular (c_i) to ambient (c_a) carbon dioxide concentrations (Farquhar et al., 1982b). In C_3 plants, this ratio is strongly affected by stomatal conductance and photosynthetic activity, which are in turn affected by water availability, soil salinity, and light intensity (Farquhar et al., 1989). When soil moisture levels decrease, stomatal conductance decreases, decreasing the c_i and resulting in less negative δ^{13}C values. The δ^{13}C values of C_3 plants are therefore negatively correlated with mean annual precipitation (e.g., Kohn, 2010) and water inputs in general—which could include irrigation—but other factors such as soil texture and organic matter content can affect soil water retention and also contribute to variability in crop δ^{13}C values (Hudson, 1994). There is no straightforward relationship between water availability and the δ^{13}C values of C_4 plants (Cernusak et al., 2013), but a number of studies have observed that the δ^{13}C values of C_4 plants increase slightly with increased water availability (i.e., along a rainfall gradient; Schulze et al., 1996; Tieszen and Boutton, 1989), the opposite trend to that observed in C_3 plants. High soil salinity reduces the ability of plants to take up water, thereby increasing the δ^{13}C values of plant tissues of both halophytic and non-halophytic C_3 species (Farquhar et al., 1982a), but decreasing the δ^{13}C values of C_4 plants slightly (Omoto et al., 2012).

Light intensity is an additional factor that has been found to influence plant δ^{13}C values, with plants growing in lower light levels having lower rates of photosynthesis, leading to higher c_i that results in lower δ^{13}C values (Ehleringer et al., 1986, 1987). While most crops would be expected to have been cultivated in open plots and so would have been unaffected by low light intensity, if plots were situated in woodland clearings, there could be shading from surrounding vegetation for part of the day. This effect could be particularly apparent in tropical or semi-tropical

TABLE 3 Intra-ear/pod variability in $\delta^{13}C$ and $\delta^{15}N$ values of single grains/pulses (full dataset in Supplementary Table 1).

Study	Taxon	Pooled standard deviation	Number of analyses
Bogaard et al. (2007)	*Triticum aestivum*	$\delta^{15}N$: 0.48‰	4 ears (between 17 and 23 single grains per ear)
Heaton et al. (2009)	*Triticum aestivum*	$\delta^{13}C$: 0.50‰	2 ears (8 and 10 single grains per ear)
Larsson et al. (2019)	*Hordeum vulgare* ssp. *vulgare*	$\delta^{15}N$: 0.44‰	8 ears (6 single grains per ear)
McKerracher et al. (2023)	*Triticum aestivum*	$\delta^{13}C$: 0.16‰	9 ears (10 single grains per hear)
		$\delta^{15}N$: 0.10‰	
Combined	**All cereals**	**$\delta^{13}C$: 0.25‰**	**11 ears**
		$\delta^{15}N$: 0.36‰	**21 ears**
Fraser et al. (2013a: Supplementary Table 4)	*Vicia faba*	$\delta^{15}N$: 0.2‰	6 pulses from a single pod
Treasure et al. (2016)	*Vicia faba*	$\delta^{13}C$: 0.21‰	5 pods (between 3 and 4 pulses per pod)
		$\delta^{15}N$: 0.21‰	
Szpak et al. (2014)	*Phaseolus vulgaris*	$\delta^{15}N$: 0.27‰	2 pods (3 pulses per pod)
Combined	**All pulses**	**$\delta^{15}N$: 0.22‰**	**8 pods**

Combined variability is highlighted in bold.

TABLE 4 Intra-plot variability in $\delta^{13}C$ and $\delta^{15}N$ values of single cereal grains (full dataset in Supplementary Table 2).

Study	Taxon	Pooled standard deviation	Number of analyses
Heaton et al. (2009)	*Triticum aestivum*	$\delta^{13}C$: 0.51‰	6 plots (6 randomly sampled single grains per plot)
Larsson et al. (2019)	*Hordeum vulgare* ssp. *vulgare*	Plot 1.A0 (manured) $\delta^{15}N$: 1.64‰	6 single grains from 4 different plants per plot
		Plot 2.A0 (unmanured) $\delta^{15}N$: 0.56‰	
		Combined $\delta^{15}N$: 1.23‰	
McKerracher et al. (2023)	*Triticum aestivum* (low level manuring)	$\delta^{13}C$: 0.23‰	5 quadrants from a large plot (90 randomly sampled single grains from one quadrant, 10 randomly sampled single grains from the remaining 4 quadrants)
		$\delta^{15}N$: 0.49‰	
Combined	**All cereals**	**$\delta^{13}C$: 0.30‰**	**11 plots**
		$\delta^{15}N$: 0.76‰	**7 plots**

Combined variability is highlighted in bold.

environments with horticultural traditions and where rainforests can form particularly dense canopies. Furthermore, the grains of different plant taxa grown in the same environmental conditions have been observed to have different $\delta^{13}C$ values, which is likely due to different timing of grain filling resulting in the $\delta^{13}C$ values reflecting water availability at different times of the year (e.g., Araus et al., 1997; Wallace et al., 2013).

The $\delta^{15}N$ values of plants are affected by the N isotopic composition of the soil in which they grow, which is influenced by the isotopic composition of any N inputs and by the loss of any N as a result of N cycling processes (e.g., Högberg, 1997). N can be added in the form of human/animal manure, bird guano, and/or composted organic matter, all of which tend to have relatively high $\delta^{15}N$ values but can vary according to their source (see Szpak, 2014 for a compilation of the d^{15}N values of different soil amendments). Soil properties may also moderate the isotopic effect of soil amendments, with an experimental study finding that similar inputs of biogas residues as fertilizer at two locations with different soil types resulted in very different $\delta^{15}N$ values of cereal grains (Larsson et al., 2024). However, any process that favors the volatilisation of ^{15}N-depleted N also drives an increase in the $\delta^{15}N$ value of soil and plants (Handley et al., 1999). Such processes include aridity (e.g., Hartman and Danin, 2010), waterlogging (Finlay and Kendall, 2008), recent forest clearance by burning (Ehrmann et al., 2014), and high levels of organic N relative to plant demand (Craine et al., 2009). The presence and type of mycorrhizal associations also affect plant $\delta^{15}N$ values (e.g., Craine et al., 2009) and plants growing in saline soils tend to have higher $\delta^{15}N$ values than those growing in non-saline soils (Heaton, 1987). Each of these factors can result in isotopic variability that

can confound interpretation of plant isotope values in terms of agricultural practice.

2.4 Preservation

The majority of plant remains in temperate contexts are preserved by charring (Zohary et al., 2012, p. 10). Charring results in changes to crop seed $\delta^{13}C$ and $\delta^{15}N$ values, the degree of which varies with the crop species and the temperature and duration of heating (Aguilera et al., 2008; Fraser et al., 2013a; Hart and Feranec, 2020; Hartman et al., 2020; Nitsch et al., 2015; Poole et al., 2002; Stroud et al., 2023a; Styring et al., 2019; Varalli et al., 2023). While it is not possible to determine the exact conditions in which crop remains were charred from morphological or chemical investigation, it has been shown that heating between 230 and 300°C results in well preserved cereal grains that are identifiable to species, although grains heated above 260°C tend to have a less dense matrix with voids that can become filled with soil (Stroud et al., 2023a). The average isotopic offset associated with heating cereal grains (barley, oat, pearl millet, rye, sorghum, wheat) between 230 and 300°C has been calculated to be ∼0.2‰ for $\delta^{13}C$ values and ∼0.3‰ for $\delta^{15}N$ values (Nitsch et al., 2015; Stroud et al., 2023a; Styring et al., 2019; Varalli et al., 2023).

While it has long been posited that the isotope values of desiccated plant remains are unreliable due to the effect of diagenesis (DeNiro and Hastorf, 1985), more recent studies have demonstrated that they can in fact yield reliable isotope values (Metcalfe and Mead, 2018; Szpak and Chiou, 2019). Szpak and Chiou (2019) propose plotting the C:N atomic ratios of archaeological plant remains against their $\delta^{15}N$ values, and if a strong positive correlation is observed this could indicate the preferential loss of ^{14}N during degradation. No investigation of the effect of waterlogging on the isotope values of uncharred plant remains has yet been carried out.

2.5 Contamination

Plant remains that have been buried in the soil are susceptible to contamination. The main potential contaminants are carbonates (with $\delta^{13}C$ values of ∼0‰; Schidlowski, 2001), nitrates (with $\delta^{15}N$ values that have been found to vary between −2 and +8‰; Rock et al., 2011), and soil-derived humic acids (with $\delta^{13}C$ and $\delta^{15}N$ values similar to those of plants because this is what soil organic matter is derived from; Schnitzer and Khan, 1975). Vaiglova et al. (2014) used Fourier Transform-Infrared Spectroscopy (FT-IR) to detect these contaminants in charred plant material down to 10 % by dry weight of the charred plant material analyzed (down to 5 % for carbonate; Figure 1). Figure 2 shows the change in the $\delta^{13}C$ and $\delta^{15}N$ values of a powdered sample of charred peas ($\delta^{13}C = -22.9‰$, $\delta^{15}N = 6.4‰$) from an archaeological site contaminated with different proportions of carbonate ($\delta^{13}C = 2.5‰$), nitrate fertilizer ($\delta^{13}C = 43.8‰$, $\delta^{15}N = -1.8‰$), and a commercially purchased humic acid sodium salt ($\delta^{13}C = -25.9‰$, $\delta^{15}N = 2.7‰$) (data from Vaiglova et al., 2014: Table 4). With the exception of the nitrate fertilizer, 10 % by dry weight contamination of the archaeological pea sample resulted in changes in its $\delta^{13}C$ or $\delta^{15}N$ value of < 0.5‰.

3 Ways of addressing the challenges associated with stable isotope analysis of archaeological crop remains

3.1 Instrumental analysis of crop remains

Approximately 2 mg of charred cereal grain will yield ∼1,200 μg C and 60 μg N (assuming 60% C and 3% N content). This is at the lower end of most IRMS limits for accurate and precise $\delta^{15}N$ value measurements. It is therefore useful to analyse reference materials comprising plant material alongside samples to get a reasonable idea of the accuracy and precision of the isotopic measurements carried out. Wheat flour and sorghum flour IRMS reference materials are available from Elemental Microanalysis and millet flour (USGS90) and rice flour (USGS91) are available from the Reston Stable Isotope Laboratory or Arndt Schimmelmann, Indiana University. These reference materials have a lower N content (∼1.5% N) than charred cereals, however, meaning that they are even more challenging to analyse by EA-IRMS and require 2–4 mg per analysis, which proves costly when performing a large number of analyses.

The development of in-house reference materials with isotopic and elemental compositions approximating those of the measured samples will likely be necessary for any researchers performing large quantities of isotopic analyses of plant remains; commercially-produced flours, similar to the USGS standards mentioned above, are frequently homogenous enough to produce consistent internal reference materials with stable isotope values measured over many years.

The carbon dioxide peak will always need to be diluted relative to the nitrogen gas peak (or diverted) in plant samples to avoid carryover to the nitrogen gas peak. If dilution is not possible or can only be applied to some limited extent, the $\delta^{13}C$ and $\delta^{15}N$ values could be measured on a small (<1 mg) aliquot of the sample. This analysis will produce a reliable $\delta^{13}C$ value as well as a reliable %N (but not $\delta^{15}N$) value. Based on this %N value, the analyst can then produce a second aliquot weighed specifically to produce precisely the appropriate amount of nitrogen gas required, while removing any of the produced CO_2. The mass required for accurate and precise $\delta^{15}N$ value measurements is higher for uncharred material (which tends to have lower %N) and lower for pulses that have higher %N (see Table 2). Given that preparation of samples (at a minimum involving crushing and weighing into tin capsules) invariably results in loss of material, it is advisable to weigh all potential samples before proceeding with any further preparative work to ensure that they are a minimum of 5–10 mg.

3.2 Determining optimal sample sizes using precision for planning

The method of "precision for planning" (also called Accuracy in Parameter Estimation, AIPE) is a powerful tool for determining

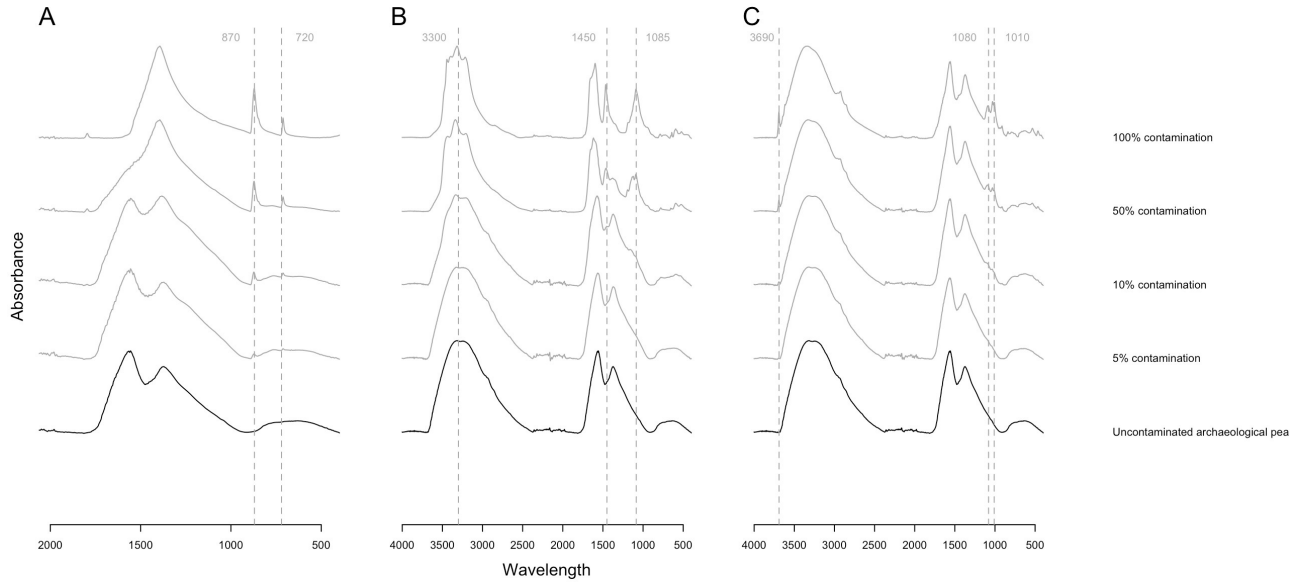

FIGURE 1
FTIR spectra of archaeological charred pea contaminated with varying percentages by dry mass of **(A)** carbonate, **(B)** nitrate fertilizer, and **(C)** humic acid sodium salt. Numbers next to dotted vertical lines refer to the wavelength of contaminant peaks identified by Vaiglova et al. (2014). Data from Vaiglova et al. (2014).

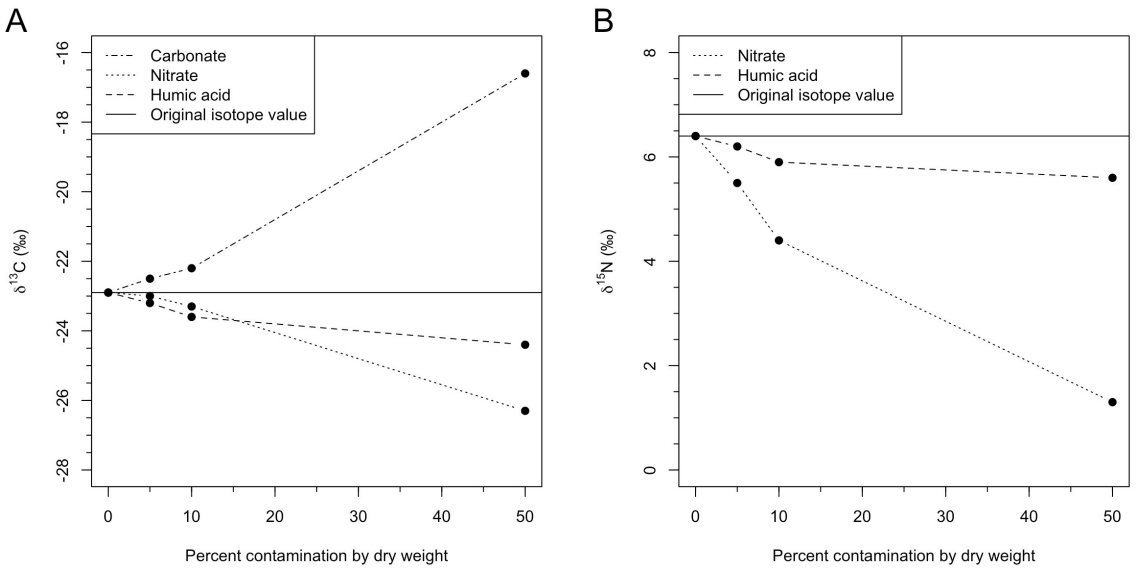

FIGURE 2
The effect of contaminating a powdered sample of charred archaeological peas ($\delta^{13}C = -22.9‰$, $\delta^{15}N = 6.4‰$; solid line in plots) with differing percentages by dry weight of carbonate ($\delta^{13}C = 2.5‰$), nitrate fertilizer ($\delta^{13}C = -43.8‰$, $\delta^{15}N = -1.8‰$), and a commercially purchased humic acid sodium salt ($\delta^{13}C = -25.9‰$, $\delta^{15}N = 2.7‰$) on **(A)** its $\delta^{13}C$ values and **(B)** $\delta^{15}N$ values (data from Vaiglova et al., 2014: Table 4).

optimal number of samples by specifying a desired statistical precision and calculating how many samples it would take to reach that precision. Tools such as the one provided on thenewstatistics.com > esci web > precision for planning (https://esci.thenewstatistics.com/esci-precision.html#tab-1) enable us to set the target precision on the x-axis and read the optimal sample size off the y-axis. Here, the margin of error (MoE)—which is one half of a confidence interval around a mean (i.e., the distance between the mean and the lower or upper limits of its confidence interval)—is used to determine the target precision. MoE can be standardized by dividing by the standard deviation, which makes it possible to work with samples that come from populations with different variabilities.

Here, we use precision for planning instead of statistical power because of the strong concerns that have been raised by the statistical community over null hypothesis significance testing (e.g., Amrhein et al., 2019; Byrd, 2007; Cohen, 1990; Cumming, 2014; Fidler et al., 2004; Goodman, 2008; Greenland et al., 2016; Ioannidis, 2005). In analyzing plant stable isotope datasets, we recommend the use of estimation science (i.e., focusing on describing what the actual difference between groups is) over declaring whether or not a test statistic passes an arbitrary threshold such as $p = 0.01$ or $p = 0.05$. Cumming and Calin-Jagerman (2024) provide many useful discussions and tools for applying estimation and inferential statistics in research in place of null hypothesis significance testing.

A common rule of thumb is to choose a MoE that is half of the expected effect size (i.e., the difference between the groups of interest). Let's consider a hypothetical archaeological scenario to illustrate how this works. Suppose we have archaeobotanical remains from a European site dating to three occupation phases: Neolithic, Bronze Age and Iron Age. The aim of our study is to determine whether barley was grown in different soil conditions during the three periods. Based on the differences in cereal grain δ^{15}N values between low, medium and high levels of manuring established by Bogaard et al. (2013), we set our expected effect size to 3.0‰. In other words, we predict that if there is a true difference in soil management conditions, the mean δ^{15}N values would differ by 3.0‰ or more. This would make the target MoE 1.5‰ (half of 3.0‰). Using the pooled standard deviation in δ^{15}N values of individual grains/pulses from single cultivation plots of 0.76‰ (Section 2.2; Table 4), the standardized MoE would be $1.5/0.76 = 2$ in standard deviation units.

Figure 3 demonstrates how the online esci web > precision for planning tool can be used to calculate the optimal number of samples. Figure 3A shows that setting the MoE to 2 (by toggling the slider on the *x*-axis) results in four barley grains required per phase. However, this precision will be reached only 51% of the times, since 49% of the distribution curve (in orange) lies above MoE = 2.0‰. To determine sample size at 99% assurance, the corresponding checkbox can be clicked in the panel on the left-hand side. When that is done (Figure 3B), the distribution curve (in orange) shifts so that 99% of the area falls under MoE = 2.0 and the optimal sample size changes to 6. However, because archaeobotanical grains rarely come from the same cultivation plot (or even the same temporal unit within an occupation phase), it should be expected that the real standard deviation is larger, in which case more conservative estimates should be made (e.g., MoE = 1.5/1 = 1.5, yielding a minimum of nine grains per period at 99% assurance).

It is useful during project preparation and grant application to consider what sample sizes would be required to detect realistic effect sizes under a range of expected standard deviations. Table 5 shows sample sizes required for capturing an effect size of 1.0‰ (Figure 3A) and an effect size of 3.0‰ (Figure 3B) with expected standard deviations between 0.2 and 1.0‰. As expected, fewer samples are needed to identify larger effect sizes. For example, only seven samples per group are needed to detect a difference of 3.0‰ with 99% assurance when SD = 0.8‰, but 30 samples per group are needed to detect a difference of 1.0‰ with 99% assurance when SD = 0.8%. However, even though large target MoE can in theory be achieved with very small sample sizes, we need to be particularly careful about using very small n (such as < 4). In replication studies, when the standard deviation of the underlying population is not actually known (as is the case in plant stable isotope studies), the width of the confidence intervals can bounce around substantially, so distinct sets of four samples can produce very different results, and the outcomes need to be treated with caution.

When performing precision for planning, it is best to be conservative about the expected standard deviation, especially given the difficulties with understanding variability in archaeological datasets (Section 2.2). If it is underestimated, the results could risk missing the detection of meaningful differences. Additionally, it is important to remember that this method only works under the assumption that the samples in each group come from the same population. Thus, six samples each of wheat and barley may detect a 3.0‰ difference in δ^{15}N values between a plot of wheat and a plot of barley, but not across several fields from distinct time periods. If chronological change is of interest, six samples should be obtained of each species from each period. If contexts are primary storage deposits (which are often the richest contexts in terms of crop remains), it may be that all items in that deposit came from the same population (i.e., same cultivation plot), but they could alternatively represent grain sourced and pooled from several fields. If primary contexts are unavailable, and the optimal sample sizes are unachievable, a 3.0‰ difference might go undetected. Further details on using precision for planning using different research designs can be found in Cumming and Calin-Jagerman (2024: Chapter 10).

Lastly, small sample sizes lead to large confidence intervals (i.e., low precision) around the estimates of interest. In some cases, this may result in a meaningful difference being obscured and undetectable. In other cases, large confidence intervals may make it impossible to identify the direction of an effect. For example, if a study aims to identify a linear correlation between δ^{15}N and grain width and measures a Pearson's r of 0.4 using 10 grains, when a confidence interval (CI) is attached to the correlation coefficient, the result is $r = 0.4$, 95% CI (−0.3, 0.8). This means that the true relationship could be anywhere from weakly negative (−0.3) to strongly positive (0.8). If the isotopic difference between wheat ($n = 3$) and barley ($n = 4$) is 0.3‰, 95% CI (−1.0, 1.6), it means that the true difference could be anywhere from −1.0‰ to 1.6‰. When small sample sizes are unavoidable, it is imperative to restrict interpretations to descriptive statistics, without making unrealistic predictions about what the trends imply for the underlying population.

3.3 Accounting for environmental variability in crop isotope values

It is often not possible to distinguish between the effect(s) of environmental variability and agricultural practice on crop isotope values, but the potential of environmental factors to contribute to any differences or changes in crop isotope values should be considered and discussed for each case. For this reason, care must be taken when applying the "manuring bands" and water availability frameworks established by modern studies in particular regions (Bogaard et al., 2013; Wallace et al., 2013)

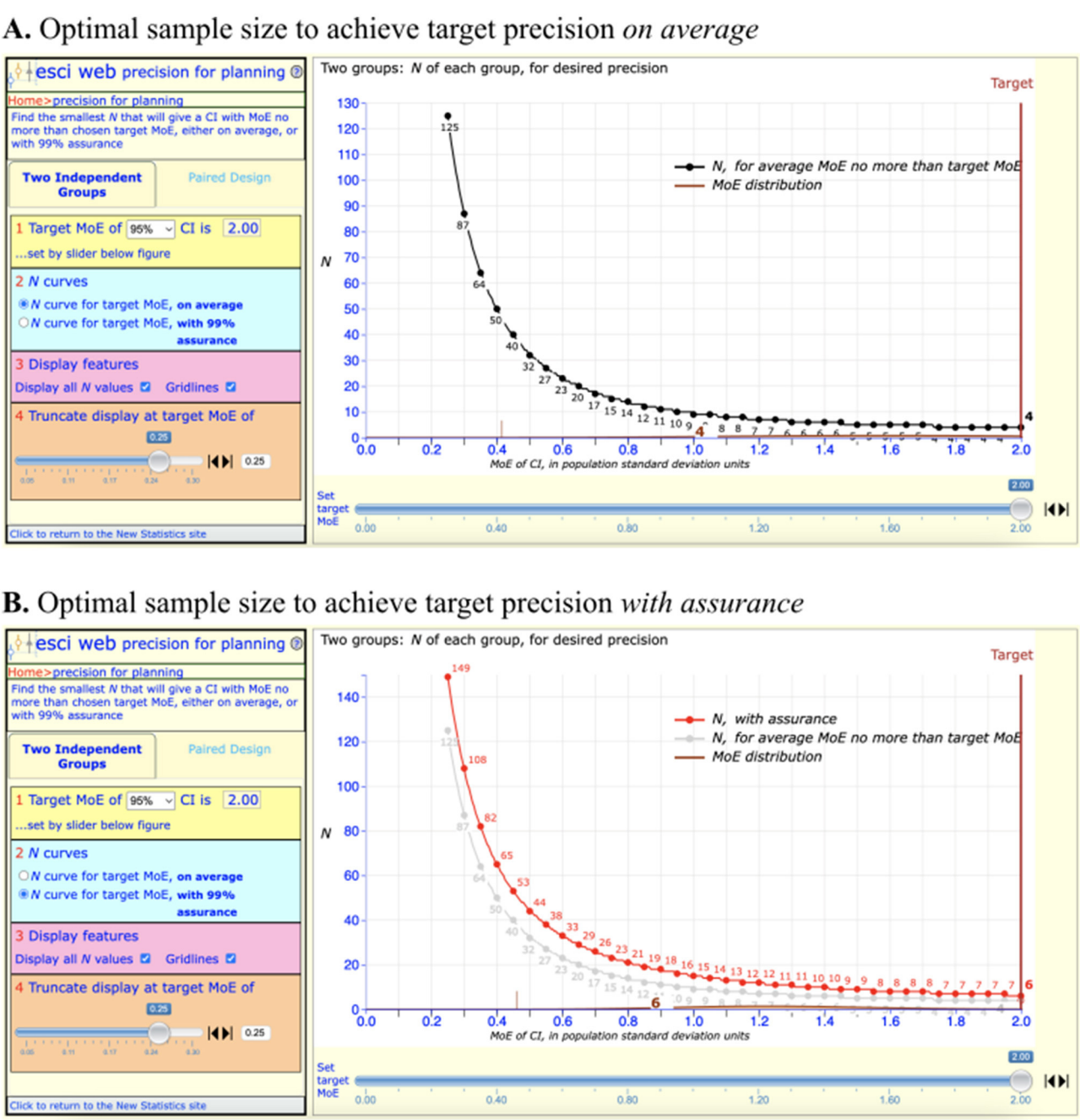

FIGURE 3
Demonstration of using "precision for planning" to determine optimal sample sizes. The example uses an online tool from thenewstatistics.com > esci web > precision for planning (https://esci.thenewstatistics.com/esci-precision.html#tab-1) (Cumming and Calin-Jagerman, 2024). **(A)** Using a target margin of error of 1.5‰ and a prediction that SD will be 0.76, the optimal number of samples for detecting a difference of 3.0‰ (on average) is 4 samples per group. **(B)** Using a target margin of error of 1.5‰ and a prediction that SD will be 0.76, the optimal number of samples for detecting a difference of 3.0% (at 99% assurance) is 6 samples per group.

to others with very different climate and soil types. Previous studies comparing crop isotope values from sites located in areas with differing annual rainfall levels have attempted to account for the effect of aridity on crop δ^{15}N values and to normalize crop δ^{15}N values between sites using linear regression in order to avoid incorrect interpretation of high crop δ^{15}N values as solely due to manuring (Styring et al., 2016, 2017a, 2022). More experimental studies investigating the interplay of environmental factors and agricultural practices on crop isotope values will only improve these attempts at disentangling such confounding variables.

Another approach has been to estimate a "base-interval" of isotope values to be expected for unmanaged plants, by subtracting the mean offset between bone collagen and diet (~4‰ for δ^{15}N; e.g., Steele and Daniel, 1978) from the isotope values of preserved wild herbivore bone collagen from the same archaeological sites

TABLE 5 Hypothetical combinations of expected standard deviations and effect sizes, and their effect on optimal sample sizes calculated using precision for planning.

Standard deviation (SD, 1σ)	Target MoE (in original units,‰)	Target MoE (in population SD units)	*N* (on average)	*N* (with 99% assurance)
Expected effect size = 1.0‰				
0.30	0.50	1.67	5	8
0.40	0.50	1.25	7	11
0.50	0.50	1.00	9	15
0.60	0.50	0.83	13	20
0.70	0.50	0.71	17	25
0.80	0.50	0.63	21	30
0.90	0.50	0.56	26	37
1.00	0.50	0.50	32	44
Expected effect size = 3.0‰				
0.80	1.50	1.88	4	7
0.90	1.50	1.67	5	8
1.00	1.50	1.50	5	9

Top: scenario for an expected effect size of 1.0‰ (MoE of 0.5‰). Bottom: scenario for an expected effect size of 3.0‰ (MoE of 1.5‰).

and contexts as crop remains (e.g., Aguilera et al., 2018; Bogaard et al., 2013; Styring et al., 2017b). This relies, however, on the presence of wild herbivore bones and on the assumption that the plants consumed by wild herbivores were growing in similar environments to crops but that their isotope values were unaffected by human management. Domestic herbivore bone collagen isotope values are not used because they are more likely to reflect isotope values of plants that *have* been affected by human management.

3.4 Assessing the preservation of archaeological crop remains

There exists a corpus of images of cereal grains and pulse seeds experimentally heated for between 215 and 260°C for 4–24 h (Stroud et al., 2023b) that can be compared with the external morphology of archaeological crop remains to identify those that are likely to have been charred in similar conditions and thus minimize any isotopic offset due to charring. These are generally crop remains that can be identified to species and conform to preservation grades P2 or P3 (after Hubbard and al Azm, 1990). It is possible that even those crop remains whose external morphology suggests that they are well-preserved have voids in their cross-section indicating that they were heated to a higher temperature. These voids can also have infilling of soil, making contamination a concern. Cereal grains/pulses can therefore be cut in half using a scalpel to examine the internal morphology of the cross-section (also pictured in Stroud et al., 2023a,b) and discarded if large voids or soil contamination are present.

3.5 Addressing contamination of archaeological crop remains

Vaiglova et al. (2014) investigated the effectiveness of various pre-treatment methods in removing the most likely forms of contamination from soil: carbonates, nitrates and humic acids. They found that carbonate contamination was removed using a gentle acid wash (0.5 M HCl at 80°C for 30 min), nitrate was removed with three rinses in deionised water, and humic acid was removed with a gentle base-acid wash (0.1 M NaOH and 0.5 M HCl at 80°C, 60 min base treatment, 30 min acid treatment). This and various other studies have observed relatively small (<1.0‰ for $\delta^{13}C$ and <1.5‰ for $\delta^{15}N$ in 96% of cases; Brinkkemper et al., 2018) but variable changes in the isotope values of charred archaeological plant remains after varying combinations and concentrations of acid and base washes (Brinkkemper et al., 2018; Fraser et al., 2013a; Kanstrup et al., 2014; Vaiglova et al., 2014). It is very difficult to determine whether these changes in isotopic composition are due to removal of contaminants, or due to preferential removal of certain parts of the plant material that have different isotope values. Moreover, multiple washes of plant material results in its loss, with sample weight losses of 22 ± 9% with acid-only treatment, 37 ± 22% for base-acid treatment and 50 ± 17% for acid-base-acid treatment having been observed (Brinkkemper et al., 2018). In samples with low starting mass (such as single grains/seeds) pre-treatment could result in insufficient material remaining for isotopic analysis.

Given the uncertainties associated with the isotopic effect of pre-treatment methods on uncontaminated charred grains and seeds, and the relatively small changes that contaminants (and particularly humic acids) have on crop $\delta^{13}C$ and $\delta^{15}N$ values (Figure 2), three strategies have been proposed: (1) perform chemical pre-treatment on all samples, (2) screen a portion of archaeological crop samples from a range of context types and levels of preservation/visible dirtiness for potential contamination and only apply pre-treatment protocols if contamination is observed to be present, or (3) perform no chemical pre-treatment but undertake the physical removal of visible sediment, and report larger isotope value uncertainties to account for the unknown effect of contamination (cf. Brinkkemper et al., 2018). FT-IR spectroscopy

is a good screening method as it uses minimal material, is very quick, and the spectra of contaminated crop material have been published (see Vaiglova et al., 2014; Figure 1). Researchers could also use their knowledge of the geology of an archaeological site to judge the likelihood of carbonate contamination, in particular. The only sites where carbonates have been detected in archaeological crop remains thus far are located on limestone geologies (e.g., Greece, northern Syria, south-east England, southern France; Alagich et al., 2018; Stroud, 2022; Styring et al., 2022; Vaiglova et al., 2020). Ultimately, more experimental studies on samples with known levels of contamination would be valuable to more conclusively determine how to effectively remove contamination in a wider range of situations.

4 Recommendations for planning and conducting stable isotope analysis of archaeological crop remains

The aim of this paper is to set out the challenges associated with crop stable isotope analysis and present potential strategies to mitigate against them. It is the intention that this format allows the reader to engage critically with the issues rather than follow a set protocol, and that this is the start rather than culmination of a discussion regarding best practice in stable isotope analysis of archaeological crop remains. In this regard, we propose the following recommendations for researchers planning to conduct an isotopic study of archaeological crop remains.

4.1 Set out the research question

The most successful isotopic studies are those that have a clear hypothesis to test, involving the comparison of two or more groups of interest or exploring the relationship between isotope values and an independent variable such as date. In archaeological studies, we are generally interested in how human actions have affected the isotope values of plants, to gain insights into the type and intensity of agricultural management. Given the multitude of environmental factors (e.g., soil type, climate, topography), aside from agricultural practices such as watering and fertilization, that can influence plant $\delta^{13}C$ and $\delta^{15}N$ values, comparisons of plant isotope values *within* sites are likely to be most robust because it can generally be assumed that these environmental factors are more consistent across the site. The isotope values of different crops recovered from the same archaeological contexts can be compared to investigate differences in management practices among crops; the isotope values of one or more crops can be determined through time to detect changing agricultural practices; and the isotope values of crops can be compared between/among discrete periods of occupation.

When comparing crop isotope values among sites, there is a greater chance that the isotope values will vary according to environmental differences rather than management practices *per se*. It is therefore useful to have a point of reference unlikely to be influenced by human management to act as an isotopic "base-interval" (cf. Vaiglova et al., 2022) for comparison with the crop isotope values (see Section 3.3).

4.2 Select appropriate samples

If plant remains derive from a secondary context (e.g., floor, midden), it cannot be assumed that multiple seeds come from the same growing condition and therefore isotopic analysis of single seeds will conserve the true variation among contexts. If plant remains derive from primary storage contexts (e.g., storage bins, pots), it might be the case that all items came from the same cultivation plot. In this case, isotopic analysis of a "bulk sample" of multiple grains will average out the inherent isotopic variability within the growing condition, providing a more precise estimate of the mean isotope value for that growing condition when budget and time are limited. Large primary deposits (e.g., large pits, granaries), however, could comprise grains sourced and pooled from multiple fields, or even from multiple settlements, meaning that analysis of single grains is actually more appropriate. Regardless, it is important that archaeological context is recorded for each sample (e.g., in Supplementary information) so that the isotope values of samples (whether individual grains or "bulk samples" of multiple grains) can be interpreted correctly in terms of expected variability and context. This information could include the total number of grains/pulses recovered from an archaeological context (but not necessarily isotopically analyzed), since this would indicate whether a deposit was large and therefore more likely to have derived from multiple sources.

Given the variability in individual cereal grain isotope values within a cultivation plot (Table 4), it is necessary that sufficient samples are analyzed in order to robustly identify any meaningful differences in isotope values among the groups of interest that cannot be accounted for by this inherent variability. Determining the number of samples necessary to determine these differences depends on the research question, the estimated effect size of a management regime of interest and the expected variability within a management regime. Although it is notoriously difficult to estimate effect sizes and variability, the availability of isotope data from modern experimental farming sites means that we can make a reasonable attempt at estimating the number of samples required, following the approach in Section 3.2. Care should be taken to ensure comparability between the groups of interest, so if, for example, the research question is whether there is a difference in manuring practice between the Neolithic and Bronze Age at a particular site, the crop species sampled should be consistent for both time periods.

4.3 Assess the suitability of samples for isotopic analysis

Well-preserved grains/seeds should be selected for isotopic analysis to minimize the isotopic offset resulting from charring and to minimize the risk of internal contamination with soil. Photographs of the dorsal and lateral views of intact grains provide

a record of their external morphology that can be used for future geometric morphometric analysis (e.g., Bonhomme et al., 2017; Portillo et al., 2019; Wallace et al., 2019), and a photograph of the cross-section of each grain/pulse provides a record of the internal morphology which could be used to cross-reference to reference material charred under different conditions. It is possible that future research will enable better constraint of the isotopic changes associated with different preservation conditions and so having a visual record of the crop remains that have been analyzed (and ultimately destroyed) could be useful for refining interpretations.

Archaeobotanical remains could be screened for potential contamination and the spectra reported alongside isotopic data. FT-IR spectroscopy is a good method for this and we recommend that at least 10% of samples are screened in this way. This represents a balance between screening all samples (with the risk of sample loss that will preclude subsequent isotopic analysis) and screening enough to capture any potential contamination. If screening a subset of samples, it is advisable to screen samples from a range of context types and levels of preservation/visible dirtiness so that they are as representative as possible.

4.4 Prepare samples for isotopic analysis

There is a lack of consensus on whether to pre-treat all samples, regardless of contamination, pre-treat only those with evidence of contamination, or not to pre-treat at all. We leave it up to the reader to judge whether the potential level of contamination is likely to affect the isotope values more than pre-treatment to remove any potential contamination. Nevertheless, we recommend in all cases to scrape any visible dirt from the surface of grains/seeds using a scalpel under a low-power microscope or magnifying glass and to rinse grains/seeds in deionised water prior to any analyses to remove substantial amounts of soil and any surface debris. Samples should then be dried in a low temperature oven (~40°C) or lyophilised. Samples can then be crushed using an agate mortar and pestle and the resulting powder stored wrapped in aluminum foil inside microcentrifuge tubes to reduce the effect of static that can disperse the sample.

4.5 Conduct isotope measurements on archaeobotanical remains

Approximately 2 mg of charred cereal grain will yield enough C and N for accurate and precise $\delta^{13}C$ and $\delta^{15}N$ value measurements, although this depends on the EA-IRMS. The carbon dioxide peak will likely need to be diluted relative to the nitrogen gas peak if $\delta^{13}C$ and $\delta^{15}N$ values are to be determined simultaneously on the same sample. Best practice in terms of two-point normalization of isotopic data has been covered comprehensively in other publications (see Szpak et al., 2017). Wheat flour and sorghum flour IRMS reference materials from Elemental Microanalysis and millet flour (USGS90) and rice flour (USGS91) from Arndt Schimmelmann, Indiana University, are useful "check" standards as they are matrix-matched to plants, but other in-house reference materials could be used.

4.6 Report isotopic measurements of archaeobotanical remains

Again, best practice as to the reporting of isotope values has been discussed in other publications (e.g., Roberts et al., 2018; Vaiglova et al., 2022), but we recommend that the following information is published alongside the isotopic data when analyzing archaeobotanical remains: (1) archaeological context information for each sample, including the number of grains/pulses represented by each sample and the total number of that species recovered from the context, (2) photographs of external and internal morphology of each grain/seed, (3) FT-IR spectra where available, (4) detailed information of any pre-treatment of samples, and (5) elemental and isotopic data for all samples and reference materials included alongside these, including mass of plant sample analyzed.

5 Conclusion and future directions

In the last four decades or so, stable isotope analysis in archaeological research has moved from a technological innovation to an intellectual revolution. Many new research questions have been asked and addressed, and new interdisciplinary collaborations have been forged. Stable isotopic studies are situated naturally between numerous subfields of modern archaeology, including zooarchaeology, bioarchaeology, geoarchaeology, archaeobotany and genetic research, bringing great synergies to these related communities. The more recent application of stable isotope analysis to archaeobotanical macro-remains is one example of such collaboration—between archaeobotanists and isotope communities—that has enabled and continues to enable novel research questions to be addressed, in turn allowing us to develop new, compelling and relevant narratives.

While initial investigations have focused on large-grained cereals and pulses that originated in southwest Asia and spread to Europe, there has been considerable momentum in understanding ancient farming systems in regions beyond western Eurasia using these methods. One such example is the recent development in understanding the carbon and nitrogen isotopic compositions and the growing conditions of millet crops (e.g., Lightfoot et al., 2016; Reid et al., 2018; Sanborn et al., 2021; Styring et al., 2019). This new development has the potential to expand plant isotope research into regions historically less investigated, such as east, south and central Asia and sub-Saharan Africa, where more than twenty taxa of small-grained and ecologically hardy crops collectively known as millets were domesticated and sustained ancient populations. Moreover, stable sulfur isotope ($\delta^{34}S$) values of archaeological plant remains have the potential to track anaerobic conditions in waterlogged soil (Lamb et al., 2023; Nitsch et al., 2019) and even identify non-local plants whose $\delta^{34}S$ values don't align with baseline $\delta^{34}S$ variability. With such future developments in mind, this paper outlines the main issues to be considered when planning and conducting an isotopic study of archaeobotanical remains, in the hope of facilitating the proliferation of future research efforts in this field.

Author contributions

AS: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. PV: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. AB: Writing – review & editing. MC: Writing – original draft, Writing – review & editing. DG: Writing – review & editing. ML: Writing – review & editing. XL: Writing – review & editing. ES: Conceptualization, Writing – review & editing. PS: Writing – review & editing. MW: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fearc.2024.1470375/full#supplementary-material

SUPPLEMENTARY TABLE 1
Summary data on intra-ear/pod variability in $\delta^{13}C$ and $\delta^{15}N$ values of single cereal grains/pulses collated from Bogaard et al. (2007), Heaton et al. (2009), Larsson et al. (2019), McKerracher et al. (2023), Fraser et al. (2013a), Treasure et al. (2016) and Szpak et al. (2014). Data was used to calculate pooled variability shown in Table 3.

SUPPLEMENTARY TABLE 2
Summary data on intra-plot variability in $\delta^{13}C$ and $\delta^{15}N$ values of single cereal grains collated from Heaton et al. (2009), Larsson et al. (2019) and McKerracher et al. (2023). Data was used to calculate pooled variability shown in Table 4.

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