CHEMISTRY STRATEGIES FOR ORGANIC ^{14}C SAMPLES

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ABSTRACT. Pretreatment of organic samples can be achieved by removal of contaminants, or, alternatively, by isolation of sample-specific components. We discuss the molecular aspects of these two pretreatment types, together with an assessment of their effectiveness in relation to sample type. The main division in sample type is the one between carbohydrates and proteins, leading to opposite chemical strategies for the two sample categories. Recommendations for routine ^{14}C chemistry of organic samples also include the standardization of quality screening procedures using chemical, stable isotope and elemental data that can be collected routinely during the pretreatment of each sample.

INTRODUCTION

The varying effectiveness of sample cleaning is the most important source of erroneous ^{14}C dates on organic samples. Apart from basic comparisons with expected ages, quality control of sample chemistry is not available: other information such as compositional or stable isotope data is not sensitive enough to detect the presence of contaminants. Nonintuitive strategies are required to deal with contamination in a manner that is optimized for the chemical characteristics of each sample type, but often such individualized treatments cannot be applied due to the absence of specific components in the sample material.

Many organic materials that are routinely sampled for ^{14}C dating (*e.g.*, wood, bone collagen, silk, hair, wool, mummified skin) are biopolymers. The traditional chemical cleanup (method type M) for almost all these materials involves removal of potential contaminants by, *e.g.*, acid, alkali, or occasionally an organic solvent treatment. Alternatively, organic molecules can be broken down into their monomers, and these can be isolated by, *e.g.*, solvent extractions or chromatographic means (method type S).

Evidence indicates specific problems with either sampling strategy, problems that will vary according to sample type and will need to be addressed individually.

CHEMICAL PROPERTIES OF MACROMOLECULAR SAMPLE TYPES

Although biological macromolecules are diverse, one major division is that between carbohydrates and proteins. Carbohydrates relevant to dating include the constituents of wood (in particular cellulose and lignine) and their derivatives (plant remains, seeds, linen and cotton, paper artifacts). They are characterized by a monotonous arrangement of glucose molecules linked by β linkages, which provide the strength and relative inertness of cellulose. Because glucose is such a common molecule in nature, the above carbohydrates do not contain characteristic, unique subunits that could be isolated for dating.

In contrast, proteins are assemblages of many different amino acids with a relatively broad spectrum of chemical characteristics, and their structures are highly specific, both in amino acid sequence and in spatial arrangement. They are mostly represented in animal-derived sample types, *e.g.*, bone, ivory, mummy samples, wool and silk. Proteins act as ion exchangers owing to the presence of both anionic and cationic side groups, and thus easily react with charged contaminant molecules.

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CHEMICAL MECHANISMS OF INTERACTION BETWEEN MACROMOLECULES AND EXOGENOUS SUBSTANCES

Some contaminants (especially structural reinforcers that are introduced post-excavation to conserve samples) do not chemically interact, but are present as part of a physical mixture.

All the other types of contaminant do interact with the dated fraction. The structural variability of potential contaminants is immense, and thus the potential complexity of the interactions. No suitable models describe these chemical interactions in a generalized manner, although literature on *diagenetic* alterations exists for some sample types (gastropod shell proteins, bone collagen, bone osteocalcin and other noncollagenous proteins). Attempts have been made to describe chemical changes during bone collagen diagenesis (Collins *et al.* 1996; van Klinken and Hedges 1995), but interaction with exogenous molecules has remained obscure (but for bone see, *e.g.*, Hedges and van Klinken 1993). Broadly speaking, complexing will be the first stage of interactions, often combined with ion-exchange processes; the final stage will be a rearranged macromolecule where parts of exogenous origin are covalently linked to parts of the original macro molecule, becoming indistinguishable from one another within a rearranged molecule. Research into the different stages of interactions is problematic because of difficulties in determining the actual type of linkages that are present: humic substances, which are the main type of exogenous matter, are so variable that they have resisted adequate detailed characterization for many years (Hoering 1973; Schnitzler and Ortiz de Serra 1973; van Klinken and Hedges 1995). In our experience, Fourier transform infrared spectroscopy (FTIR) has not been able to give detailed information about linkages in collagen-humic complexes (van Klinken and Hedges 1995).

Complexation involves weak linkages such as hydrogen bonds. Characteristically, complexation is a fast process (on the order of minutes), and in principle, is reversible. For instance, the complexation of proteins with polyphenolic tannins (to which humic acids bear many resemblances) can be reversed using caffeine (Mejbaum-Katzenellenbogen and Dobryszycka 1962; Mejbaum-Katzenellenbogen *et al.* 1959). We experimented with a humic-collagen complex (see below), and found that a large fraction of the humic acid had apparently irreversibly bound to the collagen, and that none of the M methodologies succeeded in completely removing the humic acid (van Klinken and Hedges 1995). Continuous re-complexation seems to bind any released humic almost immediately.

The subsequent steps are even less well understood, and involve rearrangement reactions in which molecules gradually merge into large, covalently linked, often aromatic, humic-like substances. Discussion of these processes is outside the scope of this paper, but it needs stressing that through these *in-situ* processes contaminant carbon becomes indistinguishable from the sample carbon.

SAMPLE TREATMENT PROCEDURES

Cleaning Macromolecular Samples (M Methods)

This type of sample pretreatment is the most common for purposes of ^{14}C dating. In essence, all these methods are implicitly based on the assumed presence of a "core" of original, intact material, with contaminant molecules attached during their depositional history that can be washed off. All the conventional pretreatments employ a sequence of acid, base, bleach and solvent steps, accompanied by rinse and centrifuge steps.

When will this approach be likely to work? Three causes for success can be identified: 1) when diagenetic effects are still relatively small; 2) when substances have been applied (*e.g.*, for conservation purposes) that are sufficiently chemically different from the indigenous macromolecule and

are only present as a mixture, not chemically bonded to the macromolecule, and easily separable in a physical manner; and 3) when contaminants are present with approximately the same age as the sample. In this last case, the extent of the chemical interactions between sample and contaminant does not matter. However, when diagenesis or other chemical processes have led to an intimate arrangement of both, likely to involve covalent bonds, it becomes probable that the treatment will not be successful.

Isolating Sample-Specific Parts (S Methods)

The isolation of specific subsections of macromolecules can involve the isolation of monomers, or, alternatively, of parts of monomers. An example of the latter is the isolation of carboxylic CO_2 from proteins, released from peptide-bonded carboxylic groups by the ninhydrin method (Nelson 1991). This approach effectively deals with all nonproteinaceous contaminants, and because proteinaceous contaminants in bone are generally recycled from collagen (which is used by bacteria growing within bone), the effect of bacterial contamination on ^{14}C dating will be negligible. The main drawback we found is the lack of reliability (mainly variable yields) that make the process less suitable for routine use. *Isolation of monomers* can involve the hydrolysis of proteins, followed by the isolation of the amino-acid mixture; in the case of carbohydrate-type polymers such as chitin or wood cellulose, it would mean the isolation of N-acetyl glucosamine, or glucose, respectively. The *isolation of specific single or specific sequences of amino acids* means an increasing sample specificity. Both Stafford (Stafford *et al.* 1991, 1987) and van Klinken (van Klinken 1991; van Klinken and Mook 1990) have shown that bone dates can be improved by the isolation of hydroxyproline (Hyp), although a drawback is that Hyp is not completely unique to collagen (van Klinken, Bowles and Hedges 1994). The isolation of the specific tripeptides GlyProHyp/GlyProAla has proved successful in treating contaminated bone samples in our laboratory: theoretically, these peptides represent the highest achievable specificity, and practically all bone samples can be successfully treated (with the *caveat* at the end of this section).

An effort is underway to employ extracts of specific lipid fractions isolated from ancient pottery (at the Oxford Radiocarbon Accelerator Unit, with R. P. Evershed from Bristol University). The lipids originate from the cooked foodstuffs, and are isolated by preparative gas chromatography.

Our own main experience is with the isolation of single amino acids (especially Hyp), and with enzymatic collagen digestion plus isolation of specific tripeptides by high-performance liquid chromatography (HPLC). Both methods are labor-intensive and, for that reason, impractical to apply to all bone samples that are submitted for dating. Of the two, single amino-acid extractions are the least reliable and useful in a dating context. We feel that when there are indications of contamination, and sufficient sample is available, the tripeptide approach is the most reliable, theoretically and practically. In all S methods it is accurate to say that covalently bonded, contaminant carbon is simply discarded, in that sense these methods are absolute chemical strategies when it can be demonstrated that no contaminant molecule identical to the sample-specific molecule is present in the sample.

Another practical consideration for ^{14}C dating is the difference in theoretical yield between the different types of S approaches: very high yields can be expected for the isolation of monomers from, *e.g.*, carbohydrates, because the macromolecule consists almost completely of these monomers; much lower yields are achievable in the case of carboxylic carbon extraction, specific amino acids or peptides, requiring much larger sample sizes (a minimum of 50 mg collagen is needed). This excludes some small samples from being treated with this methodology. Also, the financial demands of these types of extractions (the investment in equipment that is needed, and the high actual costs per sample due to labor-intensive procedures) are not negligible for most laboratories.

But even a theoretically perfect strategy does not always work. When the collagenase method was applied to bog body tissue and bone, very little GlyProHyp could be extracted, although the enzyme digestion still showed a considerable release of that tripeptide. It turned out that simultaneous with the breakdown of the bog body collagen, the GlyProHyp (a small molecule) instantaneously complexed with the humic substances that were released by the enzymatic reaction. It was now part of a complex with the humic acids as the *smaller* molecule and thus co-migrated with these during the chromatographic separation. If this re-complexation could be inhibited, the GlyProHyp could be isolated. Unfortunately, we have so far been unable to achieve this.

STRATEGIES FOR SAMPLE-SPECIFIC OPTIMIZATION OF CHEMISTRY

What are the Criteria for an Effective Chemical Strategy?

1. The strategy needs to be useful for >5–10% of samples. This number is rather arbitrary, obviously, but reasonable when we want to curb "overkill" on one hand (*e.g.*, tripeptide extraction of *all* bone samples is very costly and time-consuming if only a small percentage of bone samples is contaminated and would gain from such a treatment), and ineffectiveness on the other (samples come through the chemical cleanup stages insufficiently cleaned).
2. An adequate mechanism to detect problematic samples needs to be implemented. Non-^{14}C parameters are not sensitive enough to detect low-level contamination. However, in certain cases, a particular parameter, or especially combinations of parameters, can be quite effective: C/N ratios for proteinaceous samples, seeds, charred seeds, and chitin; combined % collagen yield, %C content of collagen, C/N ratio, and $\delta^{13}C$ for bone samples; $\delta^{13}C$ and %C for wood and charcoal samples can detect most of the contaminated samples. All this data is routinely generated during sample combustion in a CN analyzer–mass spectrometer combination, and does not require extra efforts above the ones needed for straightforward sample preparation. Moreover, the Oxford ^{14}C database is optimized to routinely generate quality control details for each sample after chemistry and combustion.
3. A distinction needs to be made between the Gaussian distribution of repeat dating results that is expected as a result of chance effects, and the effects of contamination, but in many cases that distinction is not easy to make. Contamination resulting in shifts $<1\ \sigma$ between measurements on chemically differently treated aliquots of the same sample is indistinguishable from measurement uncertainties. When shifts resulting from improved cleanup amount to $3\ \sigma$ or more the presence of contamination is obvious. Unfortunately, a "gray area" exists between the two categories, and this is the hardest category to deal with. A given shift of $2\ \sigma$ between two duplicates can be caused by contamination, but can also be a chance effect. In other words, if a true duplicate measurement had been made, similar discrepancies could have occurred. Investigations such as these need more than just a few measurements to find the causes for ^{14}C discrepancies.

Which Methods are Suitable for Which Samples?

We now return to considering the initial divide we made between the different sample types. Carbohydrates generally do not contain components that are sufficiently specific to be isolated for dating. Only chitin derived from insect, crab and other arthropod exoskeletons consists of arrays of molecules that are themselves sufficiently specific. This means that wood, and similar sample materials such as paper, plant remains, cotton and linen—in short, all samples that rely on the dating of some form of cellulose—are not suitable to be treated with S methodology. This is also the case for charcoal and other charred plant remains, but here there is no remaining specific molecule present in the amorphous carbon of these samples, and only M methodology is possible.

Proteins are mainly derived from animal and human remains, and are the most suitable class for S methodology, using a whole range of approaches from the isolation of specific amino acids to the isolation of large (peptide) subunits. Enzymatic cleavage can be carried out with many different enzymes that are currently available on a commercial basis. The problem with many non-bone (non-collagenous) samples is that they are usually not supplied in sufficient numbers to warrant the developmental effort. Plant seeds might contain either sufficient protein or specific lipids to isolate specific fractions from; specific lipids are also an option for some plant-derived materials, and also for burnt bone.

Quality Control of Chemical Pretreatment

In our opinion, most plant-derived materials can be treated only with M methods, the effectiveness of which may differ from case to case. In addition, a sample quality screening procedure based on chemical, stable isotope, and other combustion data cannot detect contamination with sufficient sensitivity to an absolute level. However, the development at ORAU of such a screening procedure is proving quite successful in earmarking suspect samples, especially when a *combination* of indicative parameters can be employed. The procedure is incorporated into the functioning of our laboratory database, and functions in a semi-automatic manner.

Conclusion

1. Conventional chemistry (M) methods are not capable of completely removing contamination. When diagenetic condensation reactions have advanced to such an extent that there is no clear chemical divide between macromolecule and contaminant within the rearranged molecule, interactions become irreversible. These conditions might occur very locally within macromolecules.
2. Isolation of specific components (S methods) leaves damaged parts of the sample molecule behind. In many cases, the cleanup is absolute, depending on how sample-specific the isolated fraction is (there is no theoretical chance of contaminants ending up in the dated fraction).
3. Carbohydrate sample types are much less amenable to isolation of sample-specific components than are proteinaceous samples.
4. As a workable alternative, for each individual sample type, a suite of quality control criteria can be developed that will be capable of indicating the presence of contaminants in most but not all cases. These procedures are especially effective when a combination of criteria can be employed, and can be incorporated into a laboratory data management system and thus automated.
5. The detection of contamination through the use of a repeat ^{14}C measurement (for instance, after an extra cleanup step is added to test its effect) is capable of detecting only the serious cases (>3 σ). It is impossible to distinguish between chance and the presence of contamination when the discrepancy between two duplicate measurements becomes <2 σ.

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