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Diagenetic effects on pyrolysis fingerprints of extracted collagen in archaeological human bones from NW Spain, as determined by pyrolysis-GC-MS

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ABSTRACT

Ancient collagen is used as archive for multiple *pre-mortem* traits. Testing the quality of the collagen extract is a common concern of those who engage in the reconstruction of ancient diets. The aim of this study is to improve our understanding of the pyrolysis fingerprints of human bone collagen especially in relation with diagenetic alteration. Pyrolysis-GC-MS was applied to 28 collagen samples extracted from archaeological human bone, corresponding to different chronological periods (Bronze Age to post-Medieval period; 1900 BC–1800 AD) and different types of burial environment (acidic and alkaline) from NW Spain. Collagen was extracted following the common methodology used in paleodiet analysis, and a commercial gelatin sample was included for comparison. Data evaluation was based on 58 pyrolysis products using Principal Components Analysis (PCA). Principal component 1 (PC1, 45% of total variance) was related to the relative abundances of pyrolysis products of specific amino acids, with relatively degraded samples having larger proportions of the pyrolysis products of Pro/Hyp, Phe and Ala, while more intact samples showed larger proportions of Tyr, Trp and pyrolysis products of unspecific amino acid origin. PC1 scores were related to the period to which the samples corresponded, which reflects differences in diagenetic impact, probably controlled by a combination of age and burial deposit characteristics. PC2 (15%) probably reflects the well-known effects of disruption of the amino acid sequence (depolymerization), causing a decline in dimerization products (diketopiperazines) upon pyrolysis. This process was more intense in the collagen samples from acidic deposits than in the samples from alkaline deposits (a calcareous cave and coastal sand deposits with biogenic carbonates).

The relationships between the PCA and individual pyrolysis products with known parameters of collagen quality (% C, % N, C/N ratio, % extractable collagen) were generally insignificant or weak. This might be explained by the rather narrow C/N range (3.19–3.36) of the samples, which had to meet the criteria for suitability for paleodiet analysis. Moreover, there was no significant relation between the isotopic composition of the extracted collagen ($\delta^{13}C$, $\delta^{15}N$) and pyrolyzate composition, suggesting that diagenesis has little effect on the isotopic fingerprints used in palaeodietary studies. Finally, no substantial contamination of microbial or exogenous tissue from the deposition environment to the osteological collagen extracts was identified. It is concluded that the $\delta^{13}C$ and $\delta^{15}N$ as proxies of palaeodiet from these diverse necropoleis in NW Spain is sustained.

1. Introduction

Collagen is the most abundant protein in mammals where it is responsible for support, elasticity and strength in connective

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tissues. The collagen biopolymer is characterized by high abundances of the amino acids Gly, Ala, Pro and Hyp (DeNiro and Weiner, 1988; Fratzl and Weinkamer, 2007) (Fig. 1), with roughly every third moiety corresponding to Gly and typical tripeptide sequences Gly–Pro–Hyp and Gly–Pro–Ala (Van Klinken et al., 1994). In bones, collagen constitutes more than 85% or the organic fraction (Termine, 1984), the vast majority of which is type I collagen (Bätge et al., 1990).

It is generally assumed that, in spite of the existence of a debate about the degree of post-mortem chemical alteration of the archaeological collagen (see Dobberstein et al., 2009; Harbeck and Grupe, 2009), diagenesis has little effect on the collagen in extracts used in archaeology and physical anthropology, particularly the stable isotopic ($\delta^{13}C$ and $\delta^{15}N$) fingerprints used as proxies of palaeodiet and ^{14}C content for radiometric dating (Hedges and Law, 1989; Ambrose, 1990; Bocherens et al., 1997). This can be attributed to the sample selection criteria and cleaning and collagen isolation procedures developed. Sample cleaning and collagen isolation remove part of the potential contaminants (carbonates, sediment, humic substances and microscopic plants debris) and bone fractions (bioapatite, lipids and non-collagenous proteins) (Ambrose, 1990), and extract preferentially the hydrolyzable and more intact collagen sequences (Van Klinken, 1999). Furthermore, only samples with a collagen yield (% $Coll_{ext}$) exceeding 0.5–1% of bone weight (Van Klinken, 1999; Pestle and Colvard, 2012) and a C/N ratio of the extracted collagen in the range 2.9–3.6 (DeNiro, 1985; DeNiro and Weiner, 1988; Ambrose, 1990) are considered suitable for stable isotopes and ^{14}C analysis. It has also been shown that collagen degradation becomes more severe when the extraction yield decreases below ca. 0.5–1%, and archaeological human bones, especially from temperate regions in Europe, rarely exhibit such low yields (Beeley and Lunt, 1980; Van Klinken, 1999). However, even though methodological improvements and selection criteria are able to reduce the effects of collagen degradation and contamination on stable isotopic and radiometric compositions, it does not imply that minor effects can still bias the results.

The degree of collagen alteration, and preservation, is affected by several factors, such as the time-temperature and moisture regimes during burial and characteristics of the deposition environment, particularly its acidity, in addition to chemical and/or physical protection by the mineral (bioapatite) matrix (Child, 1995; Collins et al., 2002; Pestle and Colvard, 2012). Regarding the effects of deterioration on the quality of collagen and collagen extracts, a distinction has to be made between contamination from the deposition environment and actual chemical alteration and breakdown of the collagen itself (Van Klinken, 1999). In case of the former, there are many examples of incorporation of humic substances into archaeological human bone (Stafford et al., 1988; Van Klinken et al., 1994; Van Klinken and Hedges, 1995; Wang et al., 2010). Many suggestions have been made for removing these and other contaminations, prior to collagen solubilization, as cleaning steps in collagen extraction procedures, particularly demineralization by HCl, extraction of humic substances in NaOH and ultrafiltration techniques. The possibility of such contaminations being incorporated by covalent bonds into the collagen structure, e.g. through Maillard reactions (Van Klinken and Hedges, 1995), implies that one should be cautious of such contaminations. Chemical alteration of the collagen structure itself, on the other hand, involves depolymerization (hydrolysis) of the biopolymer and subsequent leaching of the released building blocks (Grupe and Turban-Just, 1998; Tuross, 2002). This is also known as the collagen transition to gelatin followed by leaching (Collins et al., 2002). As the loss rate may be different for the diverse amino acids, witnessed in some studies of collagen extracts (Grupe and Turban-Just, 1998), this process may affect $\delta^{13}C$ and $\delta^{15}N$ because different amino acids have disparate C/N ratios and isotopic compositions (e.g. Tuross et al., 1988). It has been shown that these changes in amino acid composition can also affect $\delta^{13}C$ and $\delta^{15}N$ within the range of "suitable collagen extracts" defined from the molar C/N ratio, by laboratory degradation experiments of bone collagen (Grupe et al., 2000; Harbeck and Grupe, 2009). However, whether this diagenetic effect on collagen's amino acid composition may be strong enough to affect archaeological collagen samples, remains unclear, and is likely to be influenced by site-specific conditions. It may be anticipated that the different deposition environments will not only differ in the intensity of collagen degradation, but also on the nature of the process itself. Nonetheless, our current knowledge on collagen degradation on the molecular level is very limited.

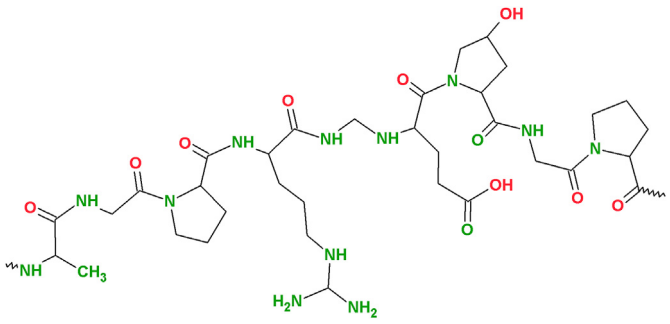

Fig. 1. A typical amino acid sequence in collagen (Ala–Gly–Pro–Arg–Gly–Glu–Hyp–Gly–Pro). Reproduced from Martin Chaplin (London South Bank University), with permission.

Common techniques applied for the assessment of collagen composition are elemental analysis, amino acid analysis, spectroscopic techniques such as Raman, FTIR and NMR, and analytical pyrolysis. Pyrolysis in combination with gas chromatography and mass spectrometry (PY-GC-MS) is a rapid screening tool for macromolecular organic substances (Wampler, 1999). Pyrolysis yields low molecular weight products that are separated by GC and identified using MS. It has demonstrated value for many types of natural organic matter from different types of deposits, including archaeological ones (e.g. Shedrinsky et al., 1989; Colombini and Modugno, 2009). Early pyrolytic studies of individual and oligomeric amino acids (Tsuge and Matsubara, 1985; Boon and de Leeuw, 1987; Smith et al., 1988; Chiavari and Galletti, 1992) provided the framework that enables unravelling complex protein pyrolysis fingerprints. Most amino acids yield characteristic pyrolysis products, with the exception of low molecular weight amino acids such as Gly, which is almost completely converted into tar and undiagnostic small volatiles, at least when analysed as a pure compound (Chiavari and Galletti, 1992). Whereas the identification potential of PY-GC-MS on proteins and degraded proteins is limited, because many proteins have a similar amino acid composition, collagen may be considered an exception to this rule as it has a very distinctive amino acid profile (Evershed and Tuross, 1996). Several studies assessed the composition of collagen, and collagen derivatives from artwork materials (gelatin, animal glue) by PY-GC-MS. Stankiewicz et al. (1997) analyzed human tissue from peat bog bodies and found that skin collagen was impregnated with tannins from the deposition environment. The pyrolysis fingerprints of animal glue analysed by Chiavari and Prati (2003) and Chiavari et al. (2006) were dominated by pyrrole and C_1-pyrroles, and diketodipyrrole allegedly from Hyp–Hyp linkages (Van Bergen et al., 1998; Chiavari and Prati, 2003; Chiavari et al., 2003). Recently, Fabbri et al. (2012) demonstrated the usefulness of diketopiperazines as markers of amino acid sequences in various proteins including collagen, and