Application of amino acid racemization in enamel to the estimation of the age of human teeth.

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ABSTRACT

Estimation of age-at-death for skeletonised forensic remains is one of the most significant problems in forensic anthropology. The majority of existing morphological and histological techniques are highly inaccurate, and show a bias towards underestimating the age of older individuals. One technique which has been successful in forensic age estimation is amino acid racemization in dentine. However, this method cannot be used on remains where the post-mortem interval is greater than 20 years. An alternative approach is to measure amino acid racemization in dental enamel, which is believed to be more resistant to change post-mortem. The extent of amino acid racemization in the acid soluble fraction of the enamel proteins was determined for modern known age teeth. A strong correlation was observed between the age of the tooth and the extent of racemization. No systematic bias in the direction of age estimation errors was detected. For the majority of teeth analyzed, the presence of dental caries did not affect the results obtained. In a minority of cases, carious teeth showed a higher level of racemization than would be expected given the age of the individual. These results indicate that amino acid racemization in enamel has the potential to be used in age estimation of skeletal remains.

Keywords

aspartic acid, serine, age estimation, enamel

INTRODUCTION

Knowing the age at death of an individual is a central concern to archaeologists and forensic anthropologists alike. However, age estimation is also well known to be one of the most problematic fields within forensic anthropology and palaeodemography. At present the majority of age estimation techniques are believed to be highly inaccurate [e.g. 1, 2, 3]. Most existing age estimation methods in adults rely on measuring degenerative changes in the skeleton, which are strongly affected by the lifestyle, health and nutrition of the individual. Many of these traits also show a wide range of natural variability in the timing of their appearance between individuals [2]. Thus, there is a pressing need for the development of methods for age estimation whose timing is more consistent between individuals.

One age estimation method used in forensic work, which has the potential to meet these criteria, is amino acid racemization. Amino acids are the building blocks from which proteins are formed, and they exist in two forms – L and D – which are non-superimposable mirror images of each other. When proteins are formed in the body, all the amino acids are in the L form, but during the life of the protein they gradually convert to a mix of L and D forms by a process called racemization. This reaction is strongly temperature dependent. In temperate climates, the temperature experienced by the body post-mortem is often significantly lower than body temperature, slowing the racemization reaction significantly (40 fold at a burial temperature of 10ºC; Ea 106 kJ mol^{-1}). Thus, the levels of D and L amino acids in proteins which formed early in life should reflect the age at death of individuals buried in cooler climates [4]. In warmer climates, the rate of racemization has been shown to show little change post-mortem, due to the higher temperature experienced by the body post-mortem [5]. Thus, it is important to know the

conditions of the burial, particularly the temperatures to which the body has been exposed, prior to applying amino acid racemization to the age estimation of forensic remains.

Aspartic acid is the most commonly used amino acid for age estimation purposes, due to its fast racemization at body temperature. Asx D/L is used to denote the combined racemization of both the aspartic acid and asparagine residues in the original protein, due to the interconversion of aspartic acid and asparagine during the sample preparation process and therefore the inability to distinguish them analytically [6]. Glx is used to denote glutamic acid and glutamine for similar reasons.

Aspartic acid racemization in dentine is a well-established method for age estimation in forensic research [e.g. 7; 8]. Dentine has been the tissue of choice, as it forms early in the life of the individual and undergoes little biochemical turnover during life. However, the application of this method to remains with a post-mortem interval of greater than 20 years has to date proven problematic. Gillard *et al.* [9] measured the extent of amino acid racemization in dentine extracted from the teeth of the Spitalfields Collection of known age at death archaeological individuals. Whilst there appeared to be some tendency for racemization to increase with age, the correlation of the extent of racemization with age at death was poor. Collins *et al.* [10] speculated that this was because the principal protein in dentine is collagen, and racemization of Asx residues in collagen is controlled by its highly ordered molecular structure. Succinimide formation, the pathway for peptide bound Asx racemization, is effectively prevented in the extended polyproline backbone of collagen, thereby restricting sites of racemization to those Asx residues in the short flexible telopeptides at either end of the molecule [11]. The telopeptides

contain only 6% of the total Asx residues in Type I collagen. These telopeptide Asx residues take approximately a century to undergo complete racemization at 37 ºC, and therefore Asx racemization in dentine is ideal for estimating age at death in recently deceased individuals. However, prolonged burial will differentially affect younger and older individuals, as the former still have many telopeptide Asx residues which can racemize during burial, whilst older individuals do not. The net effect is to observe a gradual move to the same narrow range of Asx D/L values (of approximately 0.036) in all individuals, as observed at Spitalfields [9].

In order to use racemization for age at death estimation on remains with a prolonged post-mortem interval, an alternative protein pool was required. Dental enamel also forms early in the life of an individual and undergoes little biochemical turnover. However, unlike dentine, enamel contains virtually no collagen, so the specific problems with dentine illustrated in the earlier Spitalfields study could be avoided. In addition, the proteins of mature enamel are processed during enamel maturation by proteolytic enzymes to form low molecular weight peptides, which presumably lack secondary and higher order structure that would restrict succinimide formation [12]. Furthermore, Sykes *et al.* [13] and Collins & Riley [14] have argued that predictable racemization could only be derived from intra-crystalline proteins. During enamel formation the enamel proteins become entrapped in the growing crystalline structure of the enamel. As a result, enamel provides a much better source of intra-crystalline proteins than dentine. Moreover, enamel is much more resistant to change in the burial environment [15].

Enamel racemization has not been widely used in forensic age estimation, as the levels of protein are so low that it presents a real analytical challenge [16, 17], and the correlation between

racemization and age is not as strong as in dentine [8]. Previous work has required large amounts of enamel, sometimes requiring destruction of an entire tooth for a single analysis. However, the introduction of reverse phase – high pressure liquid chromatography (RP-HPLC) with fluorescence detection has made it feasible to determine the extent of racemization of much smaller quantities of amino acids than had previously been possible. RP-HPLC can detect amino acids at quantities as low as 0.9 pg [18]. One aspect of this investigation was to therefore use the sensitivity offered by RP-HPLC to develop an extraction method that was minimally destructive. Using an analytical technique (such as RP-HPLC) with a lower detection limit allows analysis of enamel samples with much smaller quantities of amino acids. The use of RP-HPLC in this study has meant that the amount of enamel required for each analysis was smaller than in previous studies of enamel racemization [16, 17]. Such an approach is particularly valuable when working with scarce or irreplaceable materials, such as forensic remains. This paper presents the application of amino acid racemization in enamel to the age estimation of healthy modern teeth.

MATERIALS AND METHODS

The modern teeth used in this study were provided by the Edinburgh Dental Institute and the Clockhouse Dental Surgery, York. Thirty one healthy teeth with known age of extraction were selected for analysis by amino acid racemization, chosen to provide as broad a range of ages as possible. Thirteen teeth with dental caries were also selected to examine the potential effect of caries on racemization. All the teeth were rinsed with bleach after extraction and transported to the BioArch laboratory either dry (for samples from the Edinburgh Dental Institute) or in bleach (for samples from the Clockhouse Dental Surgery).

To minimize the amount of destruction to each tooth, enamel was removed from the surface of the tooth by dissolution in acid, rather than by sectioning the tooth. In order to remove any foreign proteins from the oral environment attached to the tooth surface, the surfaces of each tooth were cleaned by applying a 0.2 ml PCR tube filled with 6M HCl (total volume approximately 230 μl) to each of the labial and lingual sides of each tooth for one minute, then rinsing the tooth surfaces with HPLC grade methanol. The teeth were then placed in 12% wt/vol sodium hypochlorite for two days in order to remove contaminating proteins from handling of the teeth or from the burial environment, and then rinsed in ultrapure water and HPLC grade methanol. Samples were taken by applying two 0.2 ml PCR tubes filled with 6M HCl (total volume of each tube approximately 230 μl) to each of the labial and lingual surfaces for two consecutive time intervals of one minute. This method removes approximately 9±3 mg of enamel apatite.

All samples obtained were hydrolyzed at 110°C for six hours in an N_2 enriched atmosphere. Although the use of acid in the sampling methodology may induce racemization, as the samples were either hydrolysed shortly after collection or stored in a freezer until they were hydrolysed, the extent of induced racemization is expected to be minimal. The hydrolysis temperature applied here is also slightly higher than that used by some previous researchers [16, 17]. While this will result in a greater amount of induced racemization due to the hydrolysis procedure, as the same hydrolysis procedure was used for all the samples included in this study, the amount of induced racemization will be roughly identical for all the samples. Thus, the Asx D/L values obtained here would be expected to be higher than those obtained by previous studies, but to show a similar relationship between Asx D/L and age.

After hydrolysis, the samples were dried on a rotary evaporator overnight, and then rehydrated in 50 μl of 0.003 M HCl, 0.01 mM L-homo-arginine (as an internal standard) and 0.77 mM sodium azide, with a pH of 2. The sample vials were vortexed to aid dissolution, centrifuged and the supernatant collected and analyzed on the HPLC. Samples were separated on a Hypersil BDS column, by reverse phase HPLC using a modification of the method of Kaufman and Manley [19]. Two analyses were conducted for each sample: one using the chromatographic method outlined in Kaufman and Manley [19], and the second using an adapted version of the Kaufman and Manley method, with the concentration gradient halved, in order to enhance separation of the L- and D- Asx peaks. Absolute concentrations of L- and D- amino acids were calculated from the integrated area under each peak, normalized to the peak area of the internal standard, L-homo-arginine.

Chromatographs were carefully checked for evidence of contamination. One important source of contaminant proteins is bacteria from the oral or burial environment. This contamination was detected by examining the D/L values for Glx. Glx racemizes very slowly at body temperature and levels of D-Glx in enamel proteins are normally low. However, bacteria contain high levels of D-Glx, as glutamic acid is found in peptidoglycan within the bacterial cell wall [20, 21, 22]. Samples with abnormally high values of D-Glx were assumed to be contaminated and were not analyzed further. While it is possible that samples with lower values of D-Glx may also contain small amounts of contamination, at such low levels the contamination is unlikely to significantly affect the Asx and Ser D/L values obtained.

RESULTS AND DISCUSSION

Amino acid racemization in the enamel of healthy teeth

The method applied in this study to the extraction and analysis of amino acids is able to successfully separate and measure L- and D- Asx, Glx, serine (Ser), and L-threonine (Thr), L-arginine (Arg), and L-alanine (Ala), as well as glycine (Figure 1). At least one sample from each tooth analyzed was found to have a low level of D-Glx and thus to be free of contamination and able to be analyzed further. Comparison of the proportions of these amino acids with those found in previous work on the acid soluble fractions of enamel and dentine confirmed that enamel proteins had been extracted, and not proteins from dentine [23, 24]. A good correlation (R^2 = 0.92) between racemization and age of extraction was observed for the modern healthy teeth studied (Figure 2). Using the regression line shown, it should be possible to determine the age at death of an individual to ± 8.7 years with 95% confidence. Determination of age is more accurate in teeth younger than 35 years, where the error in age estimation is ± 6.2 years. The slope of the correlation line is similar to that observed by other researchers working on racemization in enamel [17, 25], and thus it seems that the proteins extracted by etching behave in a similar age dependent way to those obtained by the analysis of the total enamel. The D/L values obtained using acid etching are higher than those determined using the total enamel, as this technique only extracts the acid soluble fraction of the enamel proteins rather than analyzing the total enamel protein content.

No systematic variation was observed between the values determined for the labial and lingual sides of each tooth. This does not fit well with the findings of Ohtani [26] in dentine, who found variations in the Asx D/L values from the labial and lingual parts of the dentine from the

tooth crown [26]. Furthermore, the results of this study contradict those of Ohtani *et al.* [27] on the total protein content of the enamel, which indicated that racemization occurs at different rates at different parts of the mouth due to variations in temperature within the mouth. However, a range of tooth types were used in the present study, from various positions within the mouth, yet no systematic variation in the rate of racemization in teeth from different parts of the mouth was observed other than that which is due to the different times of formation of the tooth types. Therefore, the same equation for calculating age from Asx D/L values was used for all tooth types. It is possible that the acid soluble fraction of the enamel examined here is less susceptible to variations in the rate of racemization between locations within the mouth than the total enamel protein content.

The Y-intercept of the relationship between Asx D/L and age is also higher than that observed in previous research, and represents a higher level of racemization than is likely to be induced during the hydrolysis step of the sample preparation process. It is likely that the high intercept is due to the probable non-linearity of the racemization curve at very young ages. Non-linear racemization has been suggested to occur in tooth dentine by Waite and Collins [28], and this proposal is strongly supported by the very fast racemization observed in the teeth of young rats, where much younger teeth were able to be analysed than is possible when studying human populations [29]. Non-linear trends in the relationship between Asx D/L and age have also been observed to occur in older samples [30]. As few teeth were analysed with a tooth age over 40 years, it is unclear whether the trend observed here in younger individuals will become non-linear at greater ages. Analysis of further healthy teeth of known age covering a wider age range should help clarify this situation.

The correlation observed in modern teeth suggests that amino acid racemization has the potential to provide more accurate age estimates than most morphological methods. A correlation of age indicator with known age of $R^2 > 0.9$ has been cited as necessary for an age estimation method to be useful [31]. Few age estimation methods meet these criteria, but preliminary data on aspartic acid racemization in enamel indicate that this method does.

Furthermore, the scatter around the regression line does not show an age dependent pattern. Many age estimation methods display a bias in the direction of age estimation errors [32, 33]. Our preliminary data indicate that this is not true for amino acid racemization. This suggests that amino acid racemization could make a useful contribution to multifactorial methods for age estimation. One of the frequent criticisms of multifactorial methods is their use of numerous age estimation techniques, which display the same bias in their age estimates. The use of multiple age indicators that show the same inaccuracies in their age estimates has been argued to result in little improvement in the quality of the final age produced [e.g. 34, 35]. As the inaccuracies in the age estimation method presented here are different to those observed in other age estimation techniques, it could be a valuable addition to existing multifactorial age estimation methods.

Effect of caries on amino acid racemization

One potential complicating factor in the use of amino acid racemization is the impact of caries upon the protein content of the acid soluble fraction of the enamel, and hence potentially also on racemization. The amino acid composition of carious teeth showed a small but statistically significant difference to that of healthy teeth (Table 1). Carious teeth were found to

have lower concentrations of Glx, Ser, Thr and Arg in comparison to Gly. Little research has been done to date on the effect of caries on the amino acid composition of the enamel. Researchers examining the amino acid composition of carious lesions found little difference between the composition of the lesion and that of the enamel adjacent to it [36]. However, previous work by Hess and Lee [37] found that there was a significant difference in the amino acid composition of healthy enamel from carious and healthy teeth, with a decrease in the concentration of Glx, Ser, Thr and Ala in enamel from carious teeth. The results of the present study appear to support these findings, with the exception of the decrease in Arg rather than Ala. It is unclear why the presence of caries produces such changes in the content of the enamel. Nevertheless, it is apparent that the presence of a carious lesion in a tooth produces alterations in the biochemical content of the enamel, which would be expected to produce differences in the relationship between Asx D/L and age.

This discovery of a change in the biochemistry of enamel after exposure to caries was supported by the variable impact of caries on Asx D/L in the teeth included in this study (Figure 3). Many of the teeth showed only a slight change in Asx D/L, while some showed a distinctly higher or lower Asx D/L than would be expected for healthy teeth of the same age. An increase in Asx D/L would be consistent with the observations of Ritz-Timme (pers. comm.), who has noted an increase in Asx D/L in the enamel of carious teeth, regardless of the site sampled. Such an increase may be due to either catalysis of the racemization reaction by the caries bacteria [see 38] or contamination of the enamel by bacterial proteins, which are enriched in certain D-amino acids [20, 21, 22]. However, the majority of the carious teeth analysed here do not show an increase in Asx D/L. In part, this variability in the impact of caries on enamel biochemistry may be due to

the different severity of carious lesions on the teeth, although visual inspection of the teeth after analysis did not indicate a link between caries severity and deviation from the expected Asx D/L value. This suggests that the effects of caries on the rate of racemization are more complicated than was initially expected.

Of most concern are the two teeth which showed a decrease in Asx D/L. Examination of the amino acid content of these teeth suggests that they may have been contaminated, perhaps by collagen from the tooth dentine or environmental amino acids. As most sources of contamination contain recently formed proteins, which will be less highly racemized than those in the enamel, the presence of contamination would be expected to decrease Asx D/L. The presence of a similar decrease in Ser D/L lends further support to this explanation.

The presence of caries in the teeth analysed appears to also have a varying effect on Ser D/L (Figure 4). However, the majority of the Ser D/L values obtained are closer to those expected from healthy teeth than was seen with the Asx D/L values, with the exception of one tooth which displays an abnormally high Ser D/L. The Asx D/L value for this tooth does not differ greatly from that predicted for healthy teeth. Although this could be due to selective catalysis of Ser in preference to Asx in this tooth, this seems unlikely. The reason for this deviation is unclear at present.

Ritz-Timme has suggested that even a small, localised carious lesion will affect the Asx D/L throughout the tooth (pers. comm.). However, the results presented here do not support this idea. The main effect of caries on Asx D/L seen in this study is an increase in the variability in

the results obtained. While some teeth show an increase in Asx D/L, many show little change from the value expected in healthy teeth. Furthermore, the majority of the teeth analysed showed little alteration in Ser D/L. This suggests that it may be possible to use carious teeth to estimate age using amino acid racemization in enamel, but that larger error margins would need to be applied to the results obtained to take into account the heightened variability. A greater emphasis on Ser D/L may also improve the age estimates produced, given the much smaller effect of caries on this amino acid. Analysis of further carious teeth would be necessary to quantify the extent to which the errors inherent in amino acid racemization age estimation would be increased by the analysis of teeth with caries.

CONCLUSIONS

Amino acid racemization in acid soluble enamel proteins is a promising revival of an old age estimation technique, which displays a higher level of accuracy in modern remains than many existing age estimation methods. The data shown here indicate that racemization in modern human enamel shows a steady increase in racemization with age, which could be used to estimate age at death in remains that have been buried for only a short period of time. The presence of dental caries in the tooth analyzed only has a small effect on the racemization of the tooth, and thus even carious teeth could be used in age estimation, albeit with slightly larger confidence intervals. Amino acid racemization in enamel could thus find use as an age estimation method in forensic work, in cases where minimal sample destruction is required.

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REFERENCES

[1] M. Jackes. Building the bases for paleodemographic analysis: adult age estimation, in: M.A. Katzenberg and S.R. Saunders (Eds.) Biological Anthropology of the Human Skeleton. Wiley-Liss, New York, 2000, pp. 417-466.

[2] A. Kemkes-Grottenthaler, Aging through the ages: historical perspectives on age indicator methods, in: R.D. Hoppa and J.W. Vaupel (Eds.) Paleodemography: Age Distributions from Skeletal Samples, Cambridge University Press, Cambridge, 2002, pp. 48-72.

[3] S. Ritz-Timme, C. Cattaneo, M.J. Collins, E.R. Waite, H.W. Schutz, H.J. Kaatsch and H.I.M. Borrman, Age estimation: The state of the art in relation to the specific demands of forensic practise. International Journal of Legal Medicine 113 (2000) 129-136.

[4] B.J. Johnson and G.H. Miller, Archaeological Applications of Amino Acid Racemization. Archaeometry 39 (1997) 265-287.

[5] S.I. Al-Qattan, Age at Death and Gender Determination of Kuwaiti Individuals From Dentine, PhD thesis, University of Edinburgh, 2001.

[6] R.L. Hill, Hydrolysis of proteins. Advances in Protein Chemistry 20 (1965) 37-107.

[7] S. Ritz-Timme, G. Rochholz, H.W. Schultz, M.J. Collins, E.R. Waite, C. Cattaneo and H-H.J. Kaatsch, Quality assurance in age estimation based on aspartic acid racemization. International Journal of Legal Medicine 114 (2000) 83-86.

[8] S. Ohtani and T. Yamamoto, Strategy for the estimation of chronological age using the aspartic acid racemization method with special reference to coefficient of correlation between D/L ratios and ages. Journal of Forensic Sciences 50 (2005) 1020-1027

[9] R.D. Gillard, A.M. Pollard, P.A. Sutton and D.K. Whittaker, An improved method for age at death determination from the measurement of D-aspartic acid in dental collagen. Archaeometry 32 (1992) 61-70.

[10] M.J. Collins, E.R. Waite and A.C.T. van Duin, Predicting protein decomposition: the case of aspartic-acid racemization kinetics. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 354 (1999) 51-64.

[11] E. Gineyts, P.A.C. Cloos, O. Borel, L. Grimaud, P.D. Delmas and P. Garnero, Racemization and isomerization of type I collagen C- telopeptides in human bone and soft tissues: assessment of tissue turnover. Biochemical Journal 345 (2000) 481-485.

[12] M. Xie and R.L. Schowen, Secondary structure and protein deamidation. Journal of Pharmaceutical Sciences 8 (1999) 8-13.

[13] G.A. Sykes, M.J. Collins and D.I. Walton, The significance of a geochemically isolated (intra-crystalline) organic fraction within biominerals. Organic Geochemistry 23 (1995) 1059-1066.

[14] M.J. Collins and M. Riley, Amino acid racemization in biominerals: the impact of protein degradation and loss, in: G.A. Goodfriend, M.J. Collins, M.L. Fogel, S.A. Macko and J.F. Wehmiller (Eds.) Perspectives in Amino Acid and Protein Geochemistry, Oxford University Press, Oxford, 2000, pp. 120-142.

[15] P. Budd, J. Montgomery, B. Barreiro and R.G. Thomas, Differential diagenesis of strontium in archaeological human dental tissues. Applied Geochemistry 15 (2000) 687-694.

[16] P.M. Helfman, and J.L. Bada, Aspartic acid racemization in dentine as a measure of ageing. Nature 262 (1976) 279-281.

[17] S. Ohtani and K. Yamamoto, Estimation of Age from a Tooth by Means of Racemization of an Amino-Acid, Especially Aspartic-Acid - Comparison of Enamel and Dentin. Journal of Forensic Sciences 37 (1992) 1061-1067.
[18] T. Benesova, A. Honzatko, A. Pilin, J. Votruba and M. Flieger, A modified HPLC method for the determination of aspartic acid racemization in collagen from human dentin and its comparison with GC. Journal of Separation Science 27 (2004) 330-334.
[19] D.S. Kaufman and W.F. Manley, A new procedure for determining DL amino acid ratios in fossils using reverse phase liquid chromatography. Quaternary Science Reviews 17 (1998) 987-1000.
[20] K.H. Schleife and O. Kandler, Peptidoglycan types of bacterial cell-walls and their taxonomic implications. Bacteriological Reviews 36 (1972) 407-477.
[21] H.J. Rogers, Bacterial Cell Wall Structure, Van Nostrand Reinhold, Wokingham, 1983.
[22] D. Voet and J.G. Voet, Biochemistry, Wiley, Chichester, 2004.
[23] S.M. Weidmann and D.R. Eyre, The protein of mature and foetal enamel, in R.W. Fearnhead and M.V. Stack (Eds.) Tooth enamel II, John Wright and Sons, Bristol, 1971, pp. 72-78.
[24] C. Robinson, J.A. Weatherell and A.S. Hallsworth, Amino acid composition, distribution and origin of 'tuft' protein in human and bovine dental enamel. Archives of Oral Biology 20 (1975) 29-42.
[25] P.M. Helfman and J.L. Bada, Aspartic acid racemization in tooth enamel from living humans. Proceedings of the National Academy of Sciences of the USA 72 (1975) 2891-2894.
[26] S. Ohtani, Different racemization ratios in dentin from different locations within a tooth. Growth, Development and Aging 61 (1997) 93-99.
[27] S. Ohtani, R. Ito, S. Arany and T. Yamamoto, Racemization in enamel among different types of teeth from the same individual. International Journal of Legal Medicine 119 (2005) 66-69.
[28] E. Waite and M. Collins, The interpretation of aspartic acid racemization of dentine proteins, in G.A. Goodfriend, M.J. Collins, M.L. Fogel, S.A. Macko and J.F. Wehmiller (Eds.) Perspectives in amino acid and protein geochemistry, Oxford University Press, New York, 2000, pp. 182-194.
[29] S. Ohtani, Y. Matsushima, H. Ohhira and A. Watanabe, Age-related changes in D-aspartic acid of rat teeth. Growth, Development and Aging 59 (1995) 55-61.
[30] G.A. Goodfriend and P.E. Hare, Reply to comment by K.L.F. Brinton and J.L. Bada on "Aspartic acid racemization and protein diagenesis in corals over the last 350 years". Geochimica et Cosmochimica Acta 59 (1995) 417-418.
[31] J. P. Bocquet-Appel and C. Masset, Farewell to Paleodemography. Journal of Human Evolution 11 (1982) 321-333.
[32] R.G. Aykroyd, D. Lucy, A.M. Pollard and C.A. Roberts, Nasty, brutish, but not necessarily short: A reconsideration of the statistical methods used to calculate age at death from adult human skeletal and dental age indicators. American Antiquity 64 (1999) 55-70.
[33] T. Molleson and M. Cox, The Spitalfields Project. Volume 2: The Anthropology, Council for British Archaeology, York, 1993.
[34] J.L. Boldsen, G.R. Milner, L.W. Konigsberg and J.W. Wood, Transition analysis: a new method for estimating age from skeletons, in: R.D. Hoppa and J.W. Vaupel (Eds.) Paleodemography: Age Distributions from Skeletal Samples, Cambridge University Press, Cambridge, 2002, pp. 73-106.

[35] A. Schmitt, P. Murail, E. Cunha, and D. Rouge, Variability of the pattern of aging on the human skeleton: evidence from bone indicators and implications on age at death estimation. Journal of Forensic Sciences 47 (2002) 1203-1209.
[36] C. Robinson, J.A. Weatherell and A.S. Hallsworth, Changes in the Amino-Acid-Composition of Enamel During Carious Attack. Caries Research 15 (1981) 189-190.
[37] W.C. Hess and D.C. Lee, The amino acid composition of proteins isolated from the healthy enamel and dentin of carious teeth. Journal of Dental Research (1954) 62-64.
[38] A.M. Child, R.D. Gillard, and A.M. Pollard, Microbially induced promotion of amino acid racemization in bone: isolation of the micro-organisms and detection of their enzymes. Journal of Archaeological Science 20 (1993) 159-168.

Table 1. Amino acid concentrations [aa], calculated as a percentage of [Glycine], and average total amino acid concentration (sum of Asx, Glx, Ser, Glycine and Ala; in pmol/μl) of carious and healthy teeth. T- test values that are statistically significant are indicated in bold print.

		Asx	Glx	Ser	Thr	His	L-Arg	Ala	Total
Carious (n=13)	[aa]	35	54	31	18	12	9	19	63
	S.D.	6	8	4	3	4	2	5	17
Healthy (n=31)	[aa]	37	64	39	25	12	12	21	59
	S.D.	8	13	8	7	3	3	5	15
T test p value		0.18	**0.00054**	**3.0×10^{-6}**	**4.2×10^{-6}**	0.49	**1.2×10^{-5}**	0.090	0.26

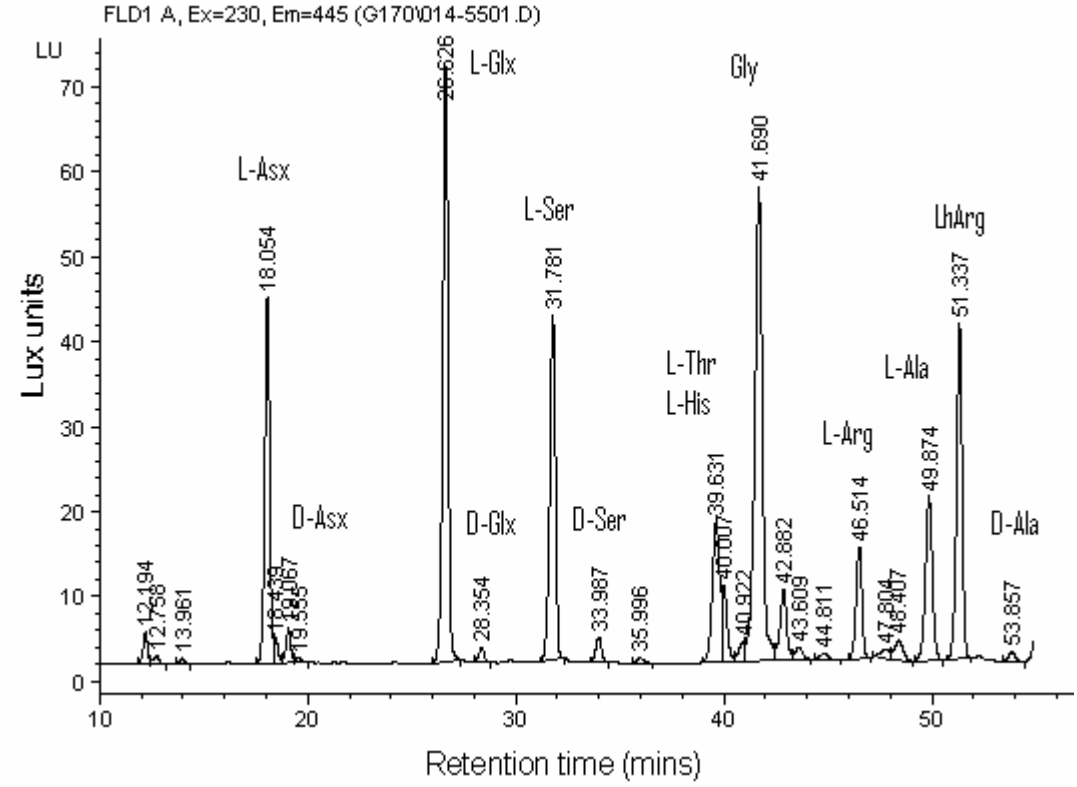

Figure 1. Chromatogram of a typical healthy tooth sample

Figure 2. ln ((1 + Asx D/L)/(1 - Asx D/L)) by age for modern healthy (non-carious) teeth of known age. Error bars represent 1st standard deviation about the mean for multiple measurements from the same tooth.

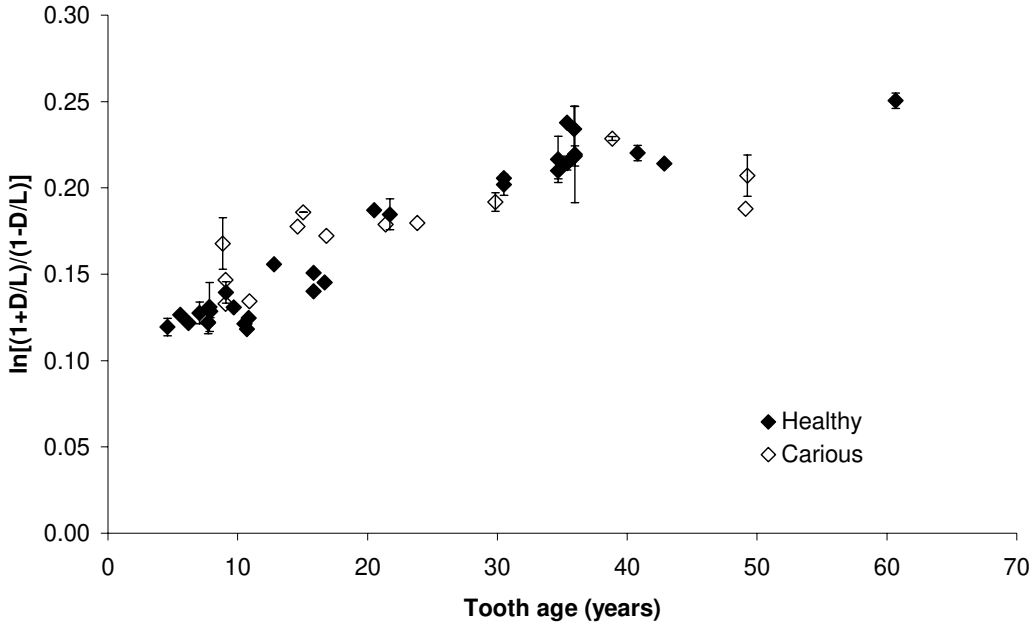

Figure 3. ln ((1 + Asx D/L)/(1 - Asx D/L)) by age for caries-free enamel from carious teeth and healthy teeth. Error bars represent 1st standard deviation about the mean for multiple measurements from the same tooth.

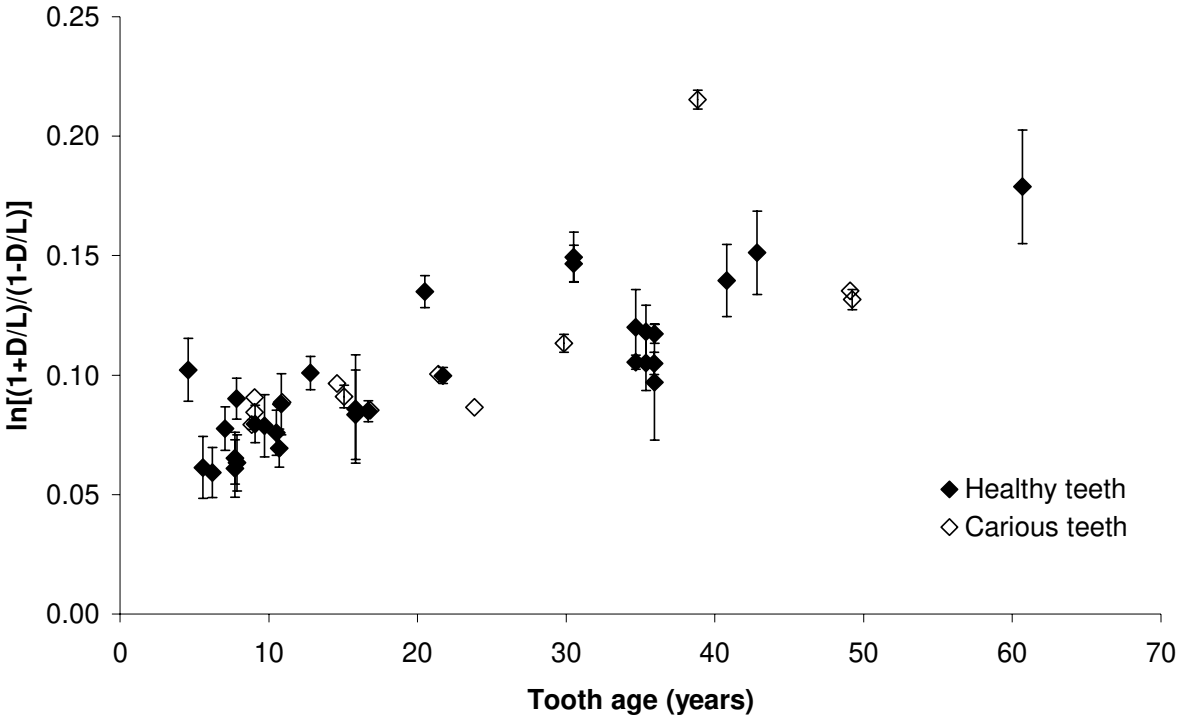

Figure 4. ln ((1 + Ser D/L)/(1 - Ser D/L)) by age for caries-free enamel from carious teeth and healthy teeth. Error bars represent 1st standard deviation about the mean for multiple measurements from the same tooth.