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TECHNICAL NOTE

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Hydroxyproline interference during the gas chromatographic analysis of D/L aspartic acid in human dentine

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Abstract The proportion of D- to L-enantiomers of aspartic acid in metabolically isolated proteins has been used by forensic scientists to estimate age at death. We have demonstrated the interference of a derivative of hydroxyproline (N-TFA isopropyl Hyp ester) with the N-TFA isopropyl L-Aspartic (Asp) acid ester during gas chromatography of amino acids. This has serious implications for the accurate quantification of the D- to L-Asp ratio extracted from collagenous proteins. Having demonstrated the potential for this co-elution in amino acid standards, acid-soluble dentine proteins and non-mineralised collagen, we argue that this problem can be overcome either by high resolution separation or by analysis of the (Hyp-poor) non-collagenous protein fraction.

Key words Hydroxyproline · D/L Aspartic acid · N-TFA isopropyl ester derivatives · Gas chromatography · Collagen

Introduction

The proportion of D- to L-enantiomers of Asp in human dentine has been used since the mid 1970s-seventies (Helfman and Bada 1976) as a method for determination of age at death. The amino acid racemization (AAR) method is becoming increasingly popular for ageing cadavers in forensic cases as it is far less subjective than traditional ageing techniques (Gustafson 1950). Indeed, studies have reported accuracies of up to ± 3 years when applying the method to teeth of unknown age (Ohtani et al. 1988; Ohtani and Yamamoto 1991; Ritz et al. 1990, 1993; Ohtani 1995).

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The value of the racemization method rests upon the accurate quantification of D- and L-Asp enantiomers and this is usually achieved using gas chromatography (GC), employing a chiral capillary column. Most workers who adopt this approach choose to derivatize the amino acids as N-trifluoroacetyl (N-TFA) isopropyl esters. However, Lou et al. (1993) claim that a derivative of hydroxyproline (Hyp) may co-elute with Asp, in which case there is a real danger of inaccurate estimation of the D/L Asp ratios. In order to improve to accuracy of the method we have conducted an investigation of the potential for co-elution of Hyp in the determination of D/L ratios.

Materials and methods

Materials

All amino acid standards were obtained from Sigma (Poole, Dorset). Trifluoroacetic acid anhydride and thionyl chloride were purchased from Fluka (Gillingham, Dorset). Isopropanol was obtained from Fisher. HCl (Aristar grade) and Dowex 50X-W8 cation exchange resin (200–400 mesh) were both obtained from BDH (Poole, Dorset). All other solvents were redistilled from technical grade reagents (Elvet, Durham, UK). Pelt samples (untanned bovine skin) were donated by Ozlem Menderes from the Nene University College Northampton, British School of Leather Technology.

Methods

Preparation of dentine soluble proteins

Dentine from a pooled sample of human teeth was powdered in a ball-mill then separated from enamel using a bromoform acetone gradient. For each sample 150 mg of dentine per sample has demineralised in 0.6 N HCl at 4° C for 4 h with constant stirring. The samples were then centrifuged at 4° C (10 min at 13,000 rpm), the supernatant removed and transferred to another vessel. The pellet was washed twice with cold distilled deionised water (ddw) and the washes were added to the soluble fraction. The soluble fraction was evaporated to dryness in a centrifugal biodrier (GeneVac SF60 VacStop attached to GeneVac CV P100 pump). The residue was resuspended in 0.5 ml of 6 N HCl, transferred to glass tubes, sealed in a flame and heated at 100° C ± 1° C for 6 h. The hydrolysates were dried in centrifugal biodrier, resuspended in 1 ml of ddw and

desalted on Dowex 50X-W8 cation exchange resin. The salts were removed by washing with 10 ml ddw and the amino acids were eluted with 3 M ammonium hydroxide (5 ml) and evaporated to dryness.

Preparation of non-mineralised collagen

In order to obtain a collagen-rich sample, experiments were conducted on fresh cow hide. The hide was washed with 140 l water at 20° C for 45 min. It was then soaked in 140 l water containing 3% NaCl at 20° C for 6 h to remove hyaluronic acid. The pelt was split at the grain-corium junction to remove hair, epidermis and elastin and the corium major was frozen until analysis. Approximately 10 mg was removed and placed in a Pyrex hydrolysis tube, 500 µl of 6 N HCl were added, the tube was sealed and the collagen was hydrolysed for 6 h at 100° C. The hydrolysates were evaporated to dryness in a centrifugal biodrier. Desalting was carried out as for the dentine (see above). Derivatization was carried out in the normal manner to produce N-TFA isopropyl esters and the separation and detection of amino acids was carried out using GC-MS (see below).

Derivatization

From each standard amino acid (DL Asp, *trans*-4-hydroxy-L-proline and *cis*-4-hydroxy-L-proline) 2 µmoles were dried onto glass vials. The derivatization method was essentially that of Goodfriend (1991) where 3 M thionyl chloride in isopropanol (250 µl) were added to dry samples which were heated for 15, 60 and 240 min at 100° C in a heating block (Stuart Scientific test tube heater SHT 2D). The isopropyl esters were cooled to room temperature then the reagents were evaporated under a slow stream of nitrogen. Dichloromethane (DCM): trifluoroacetic anhydride (3:1) was added and the samples were heated for 5, 20 or 80 min at 100° C. The vials were cooled then the reagents were evaporated and the derivatives dissolved in 1 ml DCM.

The samples were passed down solid phase extraction Isolute silica preparatory columns (diol phase, 100 mg/1ml,) from Jones Chromatography Ltd., Mid-Glamorgan, Wales), to remove any remaining contaminants or underivatized material; previous experience has shown that GC column life can be prolonged by this clean-up and it did not alter the D/L ratios. The columns were conditioned with 2 volumes of DCM, the samples were passed down and collected immediately, then washed with 10 volumes of DCM, which was then evaporated to dryness in a stream of nitrogen. The derivatives were normally stored dry (i.e. no DCM) at –70° C, except where storage at room temperature was being used to explore the degradation of the samples over time. The samples were redissolved in DCM for chromatography when required.

Gas chromatography

The separations were carried out using a Carlo-Erba HRGG 5300 gas chromatograph equipped with a Grob split/splitless injector and a Flame Ionisation detector. The split ratio was set at 20 ml/min. The carrier gas was high purity hydrogen at 50 kPa fitted with a gas-clean oxygen trap (Chrompack, Netherlands) on the carrier gas line. The column was a Chirasil-L-Val capillary column (Chrompack), 25 m × 0.39 mm o.d., 0.25 mm i.d. with a film thickness of 0.12 µm. Temperature programming was as follows: 50° C for 1 min, 4° C/min to 195° C, hold 195° C for 20 min, then cool. The injector temperature was 250° C and the detector was at 270° C. Acquisition was controlled and peak areas were quantified using automated peak area integration (X-Chrom, Labsystems, Altrincham, Cheshire).

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry was performed with a Fisons 8060 gas chromatograph and a Fisons Trio 1000 mass spectrometer (electron voltage 70 eV, filament current 4.2 A, source current 1000 µA, source temperature 250° C, multiplier voltage

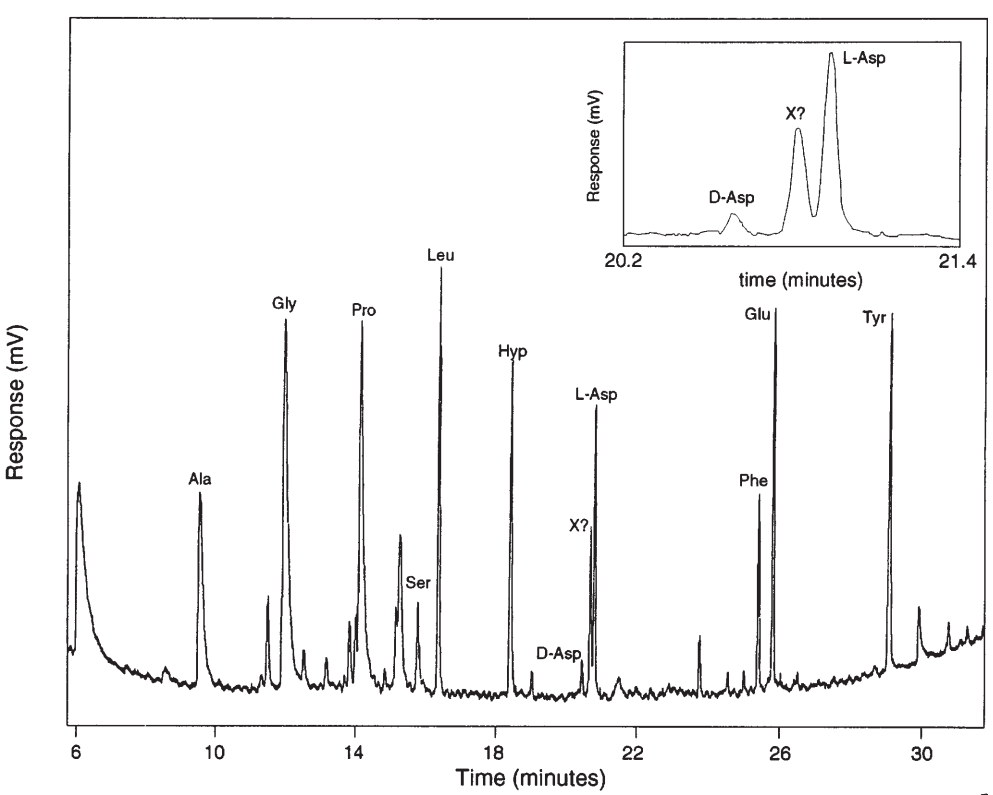

Fig. 1 GC trace of soluble dentine protein extracted as described in methods, with expanded scale (inset) for 20.2–21.4 min. Note the presence of an unidentified peak (peak X) eluting between D- and L-aspartic acid

500 V, interface temperature 300° C). The acquisition was controlled by a TVM 486 computer (Masslab software), in full scan mode (50–550 amu/s). In the case of the unmineralised collagen samples the main ions of N-TFA Hyp isopropyl ester (m/z = 182) and N-TFA L-Asp isopropyl ester (m/z = 184) were selectively monitored. The chromatographic conditions were exactly the same as for GC except that helium was the carrier gas (flow 1 ml/min, pressure of 50 kPa, split at 20 ml/min).

Storage of derivatives

Derivatives were routinely kept at –70° C until required for analysis. It has been observed that the interfering peak "disappears" upon drying and storage at room temperature. This phenomenon was investigated further by performing GC and GC-MS on samples stored for varying times at room temperature.

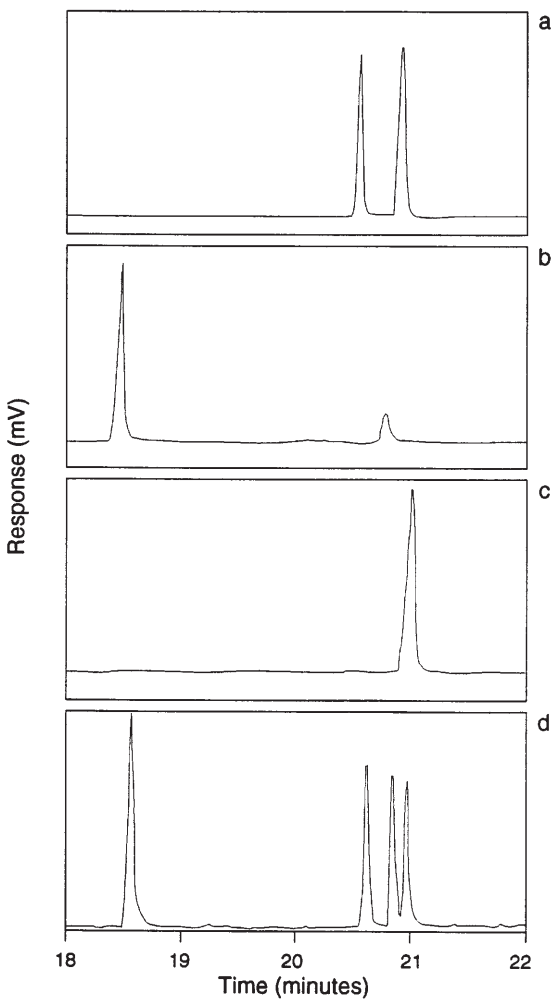

Fig. 2 a–d GC traces of amino acid standards **a** DL aspartic acid, **b** *trans*-4-hydroxy-L-proline, **c** *cis*-4-hydroxy-L-proline, **d** DL aspartic acid and *trans*-4-hydroxy-L-proline. Note the presence of the peaks at 21 min for *trans*-4-hydroxy-L-proline and *cis*-4-hydroxy-L-proline eluting very near L-Asp. *Ttrans*-4-hydroxy-L-proline has another peak at 18.5 min, and *cis*-4-hydroxy-L-proline at 23 min (not shown)

Molecular mechanics analysis

Molecular mechanics calculations were performed on *cis*- and *trans*-4-Hyp and their singly and doubly acylated esterified analogues. In these calculations the AMBER force field was used (Cornell et al. 1995) in conjunction with the Delphi molecular mechanics program (van de Graaf and Baas 1984). The charge distribution in the molecules was calculated using the EEM-approach (Mortier et al. 1986) combined with EEM-parameters optimised to reproduce the charge distribution for the amino acid fragments used in the AMBER force field (van Duin and Collins 1998). A conformational search was performed for each compound to identify its global energy minimum conformation. This conformational search involved generation of various conformations for each compound by rotating around 4 (diacylated and esterified compounds), 3 (monoacylated and esterified) or 1 (*cis*- and *trans*-hydroxyproline) torsion angle(s) and subsequent energy minimisation of these conformations. The thus identified lowest energy conformations were checked to reflect energy minima by the presence of six zero-eigenvalues in the force constant matrix (van de Graaf and Baas 1984; Baas et al. 1978).

Results

Extraction of the soluble protein fraction of dentine revealed an unknown peak (peak X) eluting between the D- and L-enantiomers of Asp (Fig. 1), which from preliminary mass-spectral analysis was thought to be a derivative of hydroxyproline. Chromatography of Hyp standards revealed two peaks for *trans*-4-hydroxy-L-proline one of

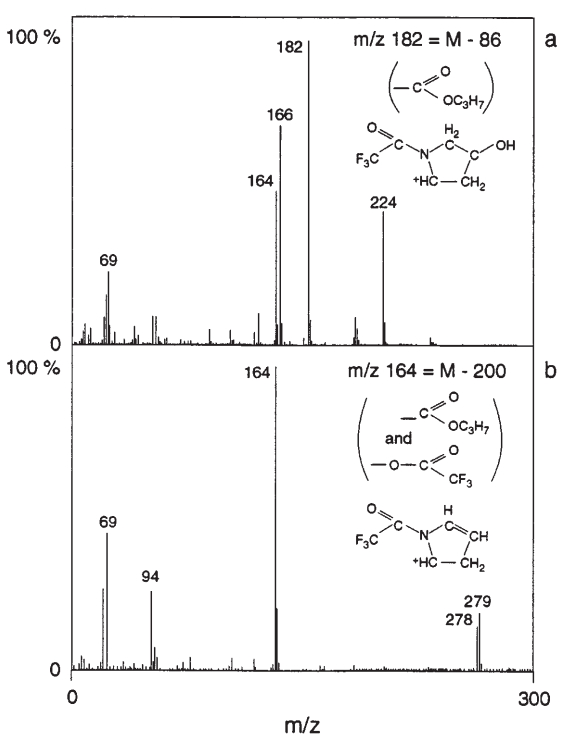

Fig. 3 a, b Mass spectral analysis of peak X (**a**) and the N,O-TFA isopropyl ester of *trans*-4-hydroxy-L-proline (**b**). The main ions from the N,O-TFA isopropyl ester are m/z 164 and 279, in contrast with the spectrum for peak X, the N-TFA isopropyl ester whose main ion is m/z 182 followed by 166. Structures of the main ions are shown

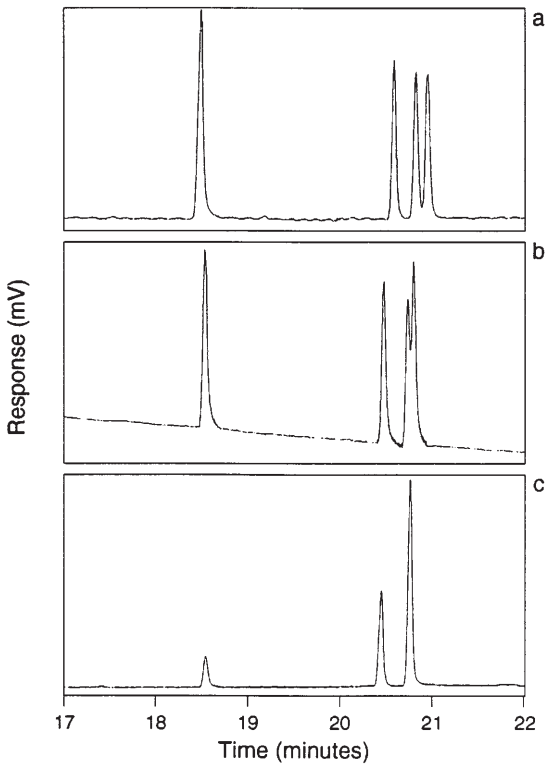

Fig. 4 a–c Effect of storage at room temperature upon the relative proportions of singly and doubly acylated derivatives of *trans*-4-hydroxy-L-proline. Following derivatization there is almost complete separation of N-TFA Hyp and N-TFA L-Asp (**a**), but following 7 h storage at room temperature, a decrease in the area of the earlier eluting N,O-TFA isopropyl ester is observed and at the same time an apparent increase in retention time of the singly acylated derivative (**b**). After 24 h storage at room temperature co-elution of N-TFA Hyp and L-Asp occurs, thereby altering the measured D/L ratio from 1 to 0.48 (**c**)

which has a retention time between that of D- and L-Asp (Fig. 2). The major peak which eluted earlier was suspected to be the N,O-TFA isopropyl ester (i.e. doubly- or di-acylated derivative, from now on called the d-form), the later eluting fraction (peak X) another derivative. This was confirmed by mass spectral analysis of the two peaks (Fig. 3), peak X being identified as the singly- or mono-acylated form (m-form).

Storage of the derivatives at room temperature leads to changes in both the relative proportions of the two derivatives (Fig. 4) and more significantly changes the retention time of N-TFA Hyp. The latter has serious consequences for AAR as the singly acylated derivative begins to co-elute with N-TFA L-Asp (Fig. 4 b, c). Co-elution is confirmed by GCMS of the combined peak (Fig. 5) which now includes a mixture of the L-Asp derivative and N-TFA isopropyl Hyp ester. Integration of such samples, without appreciating the contribution to N-TFA L-Asp from degraded N-TFA Hyp, would lead to serious errors in the estimation of D/L ratios.

The effect of derivatization conditions on the proportion of double to single acylated derivatives was investigated (see Table 1). As production of a singly acylated *trans*-Hyp derivative is undesirable, it would appear that 60 min esterification and 20 min acylation produce the optimum results. However, the results obtained with standards were not reproducible and changed depending upon the mixture of amino acids present. The ratio of monoacylated : diacylated (m : d) product is greatest when standard amino acids were used (Hyp only present – see Fig. 2), slightly less when the dentine soluble fraction was analysed (see Fig. 1) and the lowest abundances of the m-form were observed for skin collagen (Fig. 6). An attempt

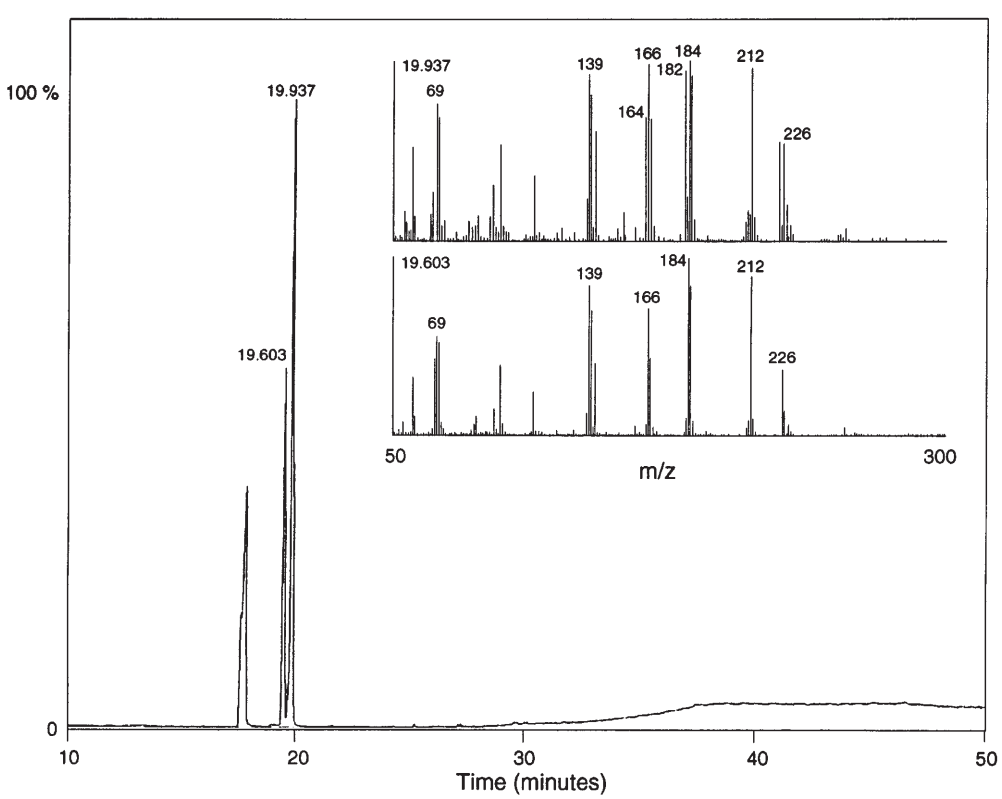

Fig. 5 GC-MS analysis of DL aspartic acid and *trans*-4-Hyp. The total ion chromatogram shows co-elution of N-TFA L-Asp isopropyl ester and N-TFA Hyp isopropyl ester at 19.937 min. The mass spectrum of this peak (*top inset*) confirms that it comprises ions of the two compounds combined. (The spectrum of Asp is included for reference, *lower inset*)

Table 1 Percentage of singly acylated derivative of *trans*-4-Hyp as proportion of the total (mono-plus di-acylated) Hyp

Time of acylation (min)	Time of esterification (min)		
	15	60	240
5	39%	32%	62%
20	42%	28%	55%
80	51%	33%	50%

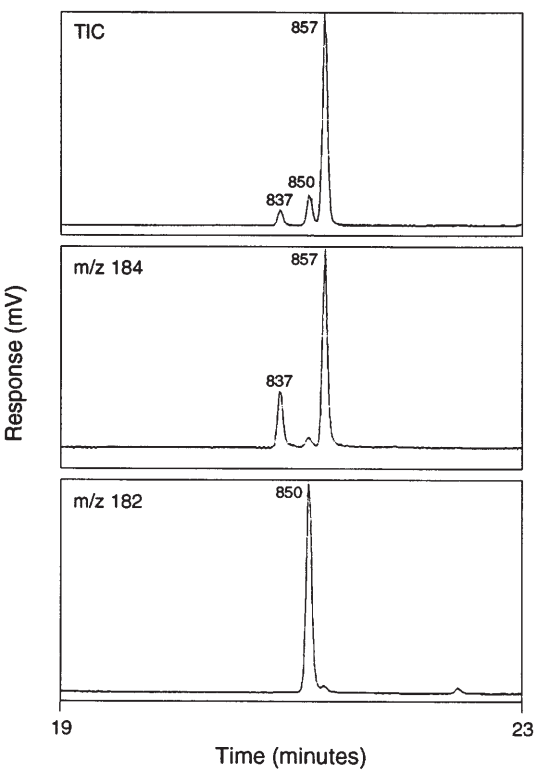

Fig. 6 GC-MS analysis of non-mineralised collagen showing TIC, and selected ion monitoring for m/z 182 and m/z 184, the main characteristic ions for N-TFA Hyp and N-TFA L-Asp, with scan numbers included to aid comparison. (New capillary column gives slightly later retention times)

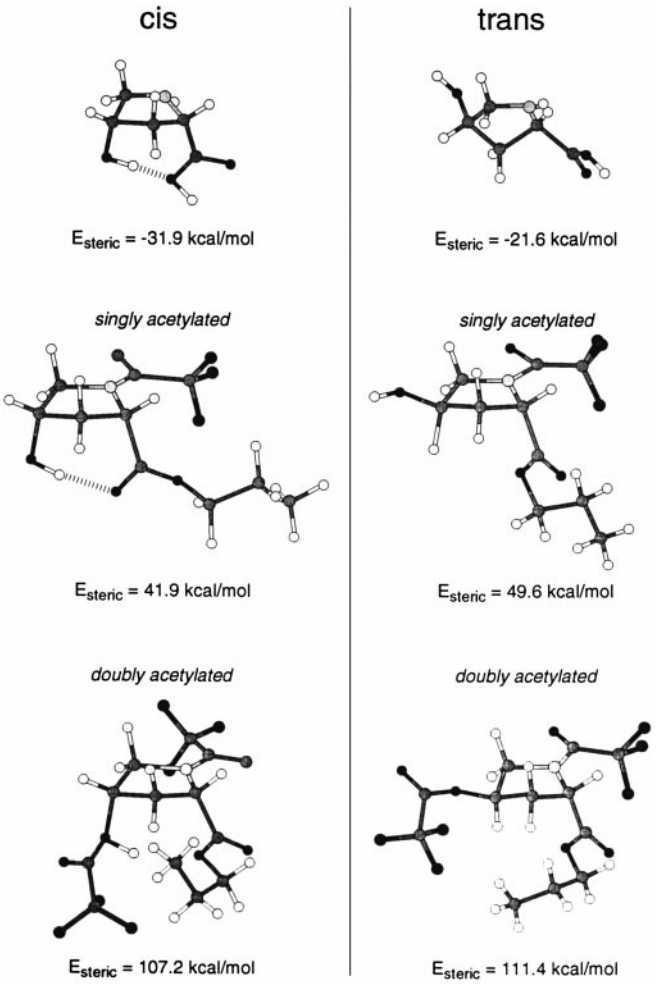

Fig. 7 Structures and stabilities of *trans*- and *cis*-4 hydroxprolines and their doubly and singly acetylated derivatives generated from molecular mechanics calculations. Note the possible hydrogen bonding between the -OH and -COOH group for the *cis*-form

was made to explain the different degrees of acylation using molecular mechanics (MM) analysis of the amino acids and their derivatives in the gas phase. The results of these calculations can be seen in Fig. 7.

Discussion

When using gas chromatography to determine Asp D/L ratios, the amino acids are usually derivatized as N-trifluoroacetyl (N-TFA) isopropyl esters. This is a two-step process, in the first step the isopropyl esters are formed, followed by the acylation of the amino group (see Fig. 8 a). As Cruikshank and Sheehan (1964) noted, free hydroxyl groups can also be acylated and thus (as in the case of Hyp) the derivatization protocol can lead to the formation of more than one product (Fig. 8 b). Chromatography of Hyp-containing tissues is further complicated by the presence of several different forms of Hyp, including *cis*- and *trans*-isomers, and even different sites of hydroxylation itself. In most cases this complication may not prove problematic, but for collagen-rich tissues such as bones and teeth, where Hyp is very common, the diversity of products has the potential to cause problems.

During the two-step derivatization procedure, both *trans*-4-L-Hyp and *cis*-4-L-Hyp produce doubly and singly acylated derivatives in variable proportions, depending on the sample and conditions during esterification. These four derivatives all elute at different times during GC using a chiral column: diacylated *trans*-4-L-Hyp, monoacylated *trans*-4-L-Hyp, diacylated *cis*-4-L-Hyp and monoacylated *cis*-4-L-Hyp in that order. Unfortunately, monoacylated *trans*-4-L-Hyp and diacylated *cis*-4-L-Hyp are both likely to interfere with the L-Asp derivative peak and thus prevent accurate determination of the D/L ratio. The potential co-elution of monoacylated *trans*-4-Hyp has been noted in previous chromatographic studies (Lou et

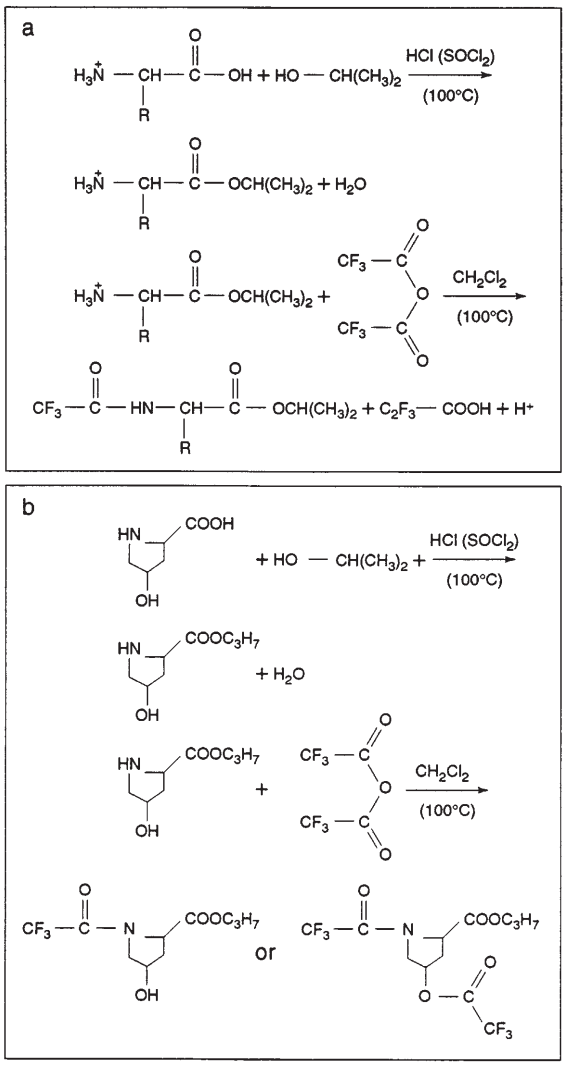

Fig. 8 a, b Scheme for derivatization of (**a**) generalised amino acid and (**b**) hydroxproline to their N-TFA or N,O-TFA isopropyl esters

al. 1993; Tredget et al. 1993) but not reported by those conducting racemization analysis. During racemization analysis steps must be taken to try to exclude the interfering peak of N-TFA Hyp isopropyl ester as it can potentially lead to errors in the D/L ratio and thus produce incorrect age estimations. The accuracy and reproducibility of studies from well-established research groups (e.g. Ohtani et al. 1988; Ohtani and Yamamoto 1991; Ritz et al. 1990, 1993) would suggest that they have overcome the Hyp co-elution problem. Our derivatization conditions are slightly different from those of these two groups, which may explain the discrepancy between the studies. Inexperienced users should be aware of the potential problem it poses for D/L Asp determinations and should check for the presence of an interfering peak.

In Type I collagen, the naturally occurring form of Hyp is *trans*-4-hydroxy-L-proline. *Cis*-4 Hyp occurs in some organisms, but the most likely source of this isomer in mammalian collagen is from the epimerization of the *trans*-isomer to *cis*-4-hydroxy-D-proline. This epimerization would be expected to be slow for native collagen (c.f. van Duin and Collins 1998) or free amino acids, but might be expected to proceed more rapidly in hydrolysis mixtures of peptides. Indeed, upon hydrolysis of collagen, the amount of *cis*-epimer formed is related to the time of heating in concentrated HCl (Dziewiatkowski et al. 1972).

Based upon the data given in Dziewiatowski et al. (1972) we estimate the amount of *cis*-epimer induced during hydrolysis (6 h, 100° C) to be 0.7% (k = 0.0012 h^{-1} $mole^{-1}$). The main problem with coelution would appear to be from the monoacylated *trans*-4-L-Hyp derivative. The amount of this species produced under different conditions seems to be bewilderingly variable and unpredictable. It is present in huge amounts for the Hyp standard alone but small amounts when a mixture of amino acids is analysed. The contribution of a coeluting *trans*-4-L-Hyp derivative to D/L ratio estimation should not be underestimated; even in the collagen samples where little of the m-form is produced, it can be sufficient to invalidate L-Asp peak integration.

The relative ability to generate m- and d-acyl forms of the *cis*- and *trans*-isomers was tested using MM. Analysis of the predicted structure of the two isomers indicates the presence of an internal hydrogen bond between the free -OH and the carbonyl of the adjacent carbon in the *cis*-form, but this is not present in the *trans*-isomer in which the hydroxyl projects in the opposite plane (see Fig. 7). Esterification of the carboxyl group and single acylation of only the amino group does not remove the hydrogen bond; the energy difference between the mono-acylated *cis*- and *trans*-isomers is similar to the difference for the non-derivatized amino acids. The internal hydrogen bond should limit the extent of acylation of the hydroxyl group in the *cis*-isomer but this does not appear to be the case under the conditions used. Double acylation removes the H-bond which leads to a smaller difference in stability of the two isomers.

There are two outstanding problems remaining with the interpretation of our experimental data. Firstly the unexpected acylation of the hydroxyl group for *cis*-Hyp, and secondly the production of more m-form for pure compounds than mixtures and the variability in the amount of m : d forms. It is very difficult to explain why so much of the m-form is produced from pure Hyp standard alone; it seems counter-intuitive that less m is produced when more amino acids are present to compete for the derivatizing agent. A second concern is that the ratio of m:d varies so much between samples (cf. Figs. 1, 2 b, 2 d, 6). Small variations in the conditions of derivatization (e.g. the acidity of the isopropanol) could explain the different yields of the two species, although these are kept as constant as possible; esterification time has been found to strongly influence the quantitative recovery of amino acid derivatives (Cruikshank and Sheehan 1964; MacKenzie and Tenaschuk 1979; Molnar-Perl et al. 1987). Our findings (Table 1) support those of the above authors who concluded that for optimal esterification of all amino acids it was necessary to heat the reactants for 60 min.

The conditions of the subsequent step of acylation appear to be less critical (Felker and Bandurski 1975).

Hydroxyproline, although a relatively uncommon amino acid, is present in both bone and dentine comprising approximately one-eighth of the residues in Type I collagen (Ramachandran and Ramakrishnan 1976). Therefore the vast bulk of Hyp in dentine proteins derives from collagen. To avoid the interference from the N-TFA Hyp isopropyl ester derivative during chromatography, it would be advisable to analyse only the (collagen poor) soluble proteins from dentine. We have recently demonstrated that contamination of the soluble proteins with collagen can occur as a result of sample preparation, notably the use of grinding or powdering of the tissue (Collins and Galley [in press]).

An alternative approach to this problem is to use the different characteristic mass spectra which two derivatives of Hyp give when subjected to GC-MS. Single ion monitoring of the characteristic ions of Hyp (as demonstrated by Tredget et al. 1990, 1993) and of the ions of Asp (as in this paper Fig. 6) could differentiate the interfering derivative from L-Asp, although quantification of the two co-eluting species would still be problematic. For accurate quantification a standard calibration curve of varying amounts of m-Hyp and L-Asp and their resulting response factors in relation to an internal standard would need to be constructed (see Tredget et al. 1990). Ultimately, a different system of analysis may be necessary, such as alternative derivatives for GC (e.g. Abe et al. 1997) or even the use of HPLC. However, the method described here does seem to be the method of choice for researchers using GC, which is nearly always preferred over HPLC in the forensic science literature (and therefore is perhaps the "standard" accepted method). We have found that separation of enantiomers varies considerably between different manufacturers' columns, and that column life may be as long as 500 injections. However, the very similar retention times of the m-form of Hyp and L-Asp mean that in samples containing Hyp a column can only provide accurate D/L Asp ratios before m-Hyp and L-Asp begin to co-elute. Running the GC programme isothermally at the temperature at which the peaks of interest elute improves their separation (data not shown), but once the column resolution has begun to deteriorate co-elution cannot be avoided.

It has been observed that if the derivatized amino acids are left to dry at room temperature, the amount of the doubly acylated Hyp derivative diminishes with time (Tredget et al. 1990; H.-W. Schütz 1997 personal communication). Tredgett et al. (1990) suggested that there is conversion from the d- to the m-Hyp form, reporting that this change is slow and unpredictable. Indeed, we have demonstrated a reduction in the amount of N,O-TFA Hyp isopropyl ester which is accompanied by an increase in the singly acylated form. However, during storage the peak from the singly acylated form begins to migrate into the L-Asp peak (Fig. 4b) and eventually co-elutes with the L-Asp peak (Fig. 4c); storage at room temperature is therefore not recommended.

In conclusion, during the analysis of dentine proteins we have demonstrated that the interference of a singly acylated derivative of *trans*-Hyp (and potentially also a doubly acylated derivative of *cis*-Hyp) presents problems for D/L Asp ratio determination and therefore will hinder accurate age estimation. If collagen, and thus Hyp cannot be completely excluded from an extract then either high resolution (i.e. new) GC columns must be used or the ions characteristic for Asp could be monitored specifically using GC-MS-SIM. In the latter case care should be taken to establish relative response factors of the derivatives.

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