Review Article

Appraisal on potential scope of aspartic acid racemization in human dental remains for precise age estimation: A review

Kulvir Singh [1]*

[1]Formerly UGC SRF, Department of Forensic science, Punjabi University, Patiala.

** Corresponding author: 1.kulvir1@gmail.com*

ABSTRACT

The forensic methods of personal identification have a governing role in the individualization of casualties of mass disaster and criminal acts. Forensic odontology is among such reliable methods for personal identification accompanied by DNA profiling and fingerprints. The morphological and radiological analyses of dental exhibits are less precise and often need rigour expertise. The human dentition composition has been found to contain minute sum of aspartic acid and other amino acids. Consistent reported results had shown the adroitness of aspartic acid racemization (AAR) in precise age estimation of the cadavers. Therefore, aspartic acid racemization can provide a reliable and economic method of age and personal identification of human remains. A probe has been demonstrated for the likely future of AAR in age estimation of cadavers from human dental remains.

Key Words: aspartic acid racemization, age estimation, causalities, forensic identification.

INTRODUCTION:

Forensic or mass disaster investigations often start with the identification of the biological profile. Age, sex, stature, origin, etc, are some of the most investigative problems tackled with utmost wit. The galloping science and technology brings us the panacea of present scientific problems. One of such endowment of science to humans is the determination of age and sex from the human remains. Forensic odontology can play a very crucial role in this context. Age determination from the amino acid racemization from human dental remains is

one of forensic odontology methods with prospective to solve dating problems.

Composition of human dentition:

Human tooth has unique structural components including enamel (hydroxyapatite crystal; hardest tissue containing enamel rods and composite of minerals and organic phase), enamel-dentin junction, dentin, pulp (figure 1). The enamel rod orientation and dental tubule attributed the each zone anisotropic. The dentin, the bulk of the tooth, is considered soft and elastic [1]. The avascular mineralized enamel supported human tooth composed of mainly (by weight) about 70% mineral (carbonate-substituted hydroxylapatite), 20% organic matrix (type 1 collagen, lipids, non-collagenous matrix proteins) and 10% water [2,3]. The inorganics contains carbonates, oxygen, magnesium, sulfur, strontium, zinc, copper, phosphorus, calcium, chloride, potassium, sodium, iron, manganese, lead and metal cations [4]. Importantly, considerable amino acids (AA) are also found in the human dentin composition and mainly includes, arginine, asparagine, alanine, aspartic acid, hydroxyproline, hydroxylysine, histidine, glutamic acid, glycin, methionine, iso-leucine, leucine, phenylalanine, lysine, i-methylhistidine, threonine, serine, proline, valine [5,6]. Amongst these, copious studies have been found on aspartic acid due to its stable role in the age estimation of human dentition.

Aspartic acid:

Mammals naturally synthesize a non-essential amino acid, aspartic acid (letter code ASP/D) or aspartate, which is one of the 20 building-block amino acids. The chiral ASP has two enantiomeric forms, L-aspartic acid and D- aspartic acid (figure 1). L-ASP is used in neurotransmission and in the biosynthesis of proteins.

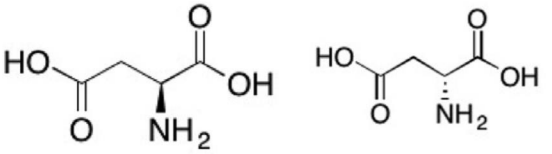

Figure 1. Chemical structure of L- and D-ASP stereoisomers sourced from [7].

Interestingly, ASP was discovered when the asparagine (from the juice of asparagus) boiled in the presence of barium hydrate. The extraterrestrial origin of ASP at lower level was also mentioned in a report [7]. In vivo, the ASP is formed through the transamination of oxaloacetic acid (metabolic product of carbohydrates) with glutamic acid. ASP further metabolized into N-acetylaspartic acid [8]. The probable reported mechanism involved the conversion of primary products of histidine and arginine/proline oxidation, which are asparagine and pyroglutamic acid respectively, into aspartic acid and glutamic acid through acid hydrolysis [9].

Aspartic acid racemization:

Chemically, the conversion of the enantiomeric forms (by heat or chemical reaction) to a 50:50 mixture of enantiomers is known as racemization [10]. It is a natural process, which creates a racemic mixture.

Aspartic acid primarily found in L-form *in vivo*. Once the L-form isolated from the biochemical processes the optically active amino acid converted into optically inactive. Such conditions materialized the racemization process in which reversible conversion of L-form into D-form initiated until it approached to equal (one) D/L ratio [11]. The temperature dependent rate of conversion of L- to D- form is slow therefore this conversion ratio can be calculated to approximate the age of the protein. The ASP frequently used in dating due to its comparatively faster rate of conversion than other amino acids [12].

It has been expected that total amino acids of bodily tissues in L-form would be transformed by racemization to the D-form in around one thousand hundred years. The non-enzymatic aspartate racemization causes rapid buildup of D-aspartic acid with the growing age in sufficiently measureable quantity as compared to other slowly accumulated D-amino acids [13,14]. Since the aspartic acid is more stable amino acid among the rest, it can be used efficiently to age the the birth or death of the cadaver. The morphological and radiological analyses of dental exhibits for age estimation are less precise and often need rigour expertise [15,23].

Aspartic acid racemization and forensic dating:

The post-translational modification of protein structures caused the age-allied buildup of abnormal enzymatic forms. The age-allied modifications in proteins incorporate oxidation of side chains of amino acids, asparaginyl and glutaminyl deamidation, isomerization and racemization of aspartyl and asparaginyl residues [16,17]. The buildup of these biologically inactive stereoisomers had been suggested as one of the possible mechanism of ageing. Alternatively, this ageing process can also help the forensic scientists to estimate the chronological age of the diseased. The ASP is only the metabolically stable amino acid and has longer half-life for L to D- ASP racemization. Racemization of ASP occurs at a rate of 0.1 percent per year in human tooth enamel [14]. Temperature has a major influential part in the rate of racemization (L- to D-ASP). The rate D-ASP buildup found to be 0.14 percent per year in human eye nucleus [18]. Studies on the human brain showed the D-ASP buildup from the birth until the age 50 and further remained constant for the rest of life [19].

The other factors effecting rate of racemization are humidity and pH. It has been found that D-ASP buildup in the organs with low metabolic rate *i.e.,* vessel free or bradytrophic tissues. Therefore, human tooth is the best organ for forensic dating studies through ASP racemizaton [20]. However different rate of ASP racemization have been found among enamel, dentin and root components of the teeth [21,22]. The age estimation from crown dentin ASP racemization has been found to be more accurate than the enamel [13,14] and other studies had also reported similar findings with inconsistent accuracy level [20,23].

Methodology:

Sample preparation and retrieval:

Almost all types [24] (molars [21,25,26], premolars [27,28], incisors [22,29,30,31]) and different regions (horizontal sections, vertical sections, lingual regions, labial regions, longitudinal sections, transverse sections, crown region, root region, primary dentin, and secondary dentin) of teeth can be used to study the ASP racemization for the dating purposes [21,23,26,27,32,33,34,35]. Teeth can be obtained from the children [36], dentists (periodontal/orthodontics treatment [37]), cadavers during autopsy [13,14,21] and cemetery [38]. Likewise, different authors had adopted different cleaning protocols and sample retrieval strategies due to the lack of standard collection and after collection protocols. The divergent recommendations are briefed in table 1 and table 2 respectively.

Table 1. The divergent recommendations of teeth cleaning protocols.

Cleaning protocol	**Reference**
Cleaned off adhering soft tissues and stored in desiccator.	13, 14, 37
Cleaned with distilled deionized water (DDI) by a toothbrush (dil. HNO_3-washed); stored in papain solution (2% w/v) in a plastic micro beaker; then rinsed in sequence with DDI; 3% (v/v) hydrogen peroxide; DDI, dried overnight at 85°C in oven.	36
Washed and stored in distilled water (DW).	4
Washed in water to remove attached tissues; one hour immersion in sodium hypochlorite (12% free Cl) and rinsing with water and stored at -18^{o}C.	39

Washed under running tap water and air-dried; deep-froze at -21°C.	40
Preserved in a dry state without any medium.	41
Cleaned in 10% formalin (at room temperature) for 24 hrs; air-dried.	42, 32
Stored at 4^{o}C in 0.9% phosphate saline buffer (pH-7.4) doped with 0.002% sodium azide.	3
Decontaminated by bleach treatment (12% hypochlorite solution)	43, 46
Brushed using toothpaste; rinsed with DW; air dried (room temperature); stored at freezing temperature.	44
Cleaned with a sand blaster.	45
A dental hand-piece and a silicone point were employed to remove any adhering tissues or blood from the teeth samples.	47
Adhering tissues were cleaned; washed with hydrogen peroxide; air-dried.	48
Water cleaned; dried and stored (-20^{o}C).	49

Table 2. The common divergent recommendations of sample retrieval protocols.

Region/ section of tooth	**Retrieval protocol**	**Reference**
Enamel	Enamel was removed from a desiccated tooth using a pestle mortar; carious/discolored fragments were discarded; dentine removed (fluorescing under UV); enamel fragments (non-fluorescing) powdered using pestle mortar; flotated fractionation using tetrabromoethane (87.8%) and acetone (12.2%), enamel sinked while dentine floated; three times acetone washed; three times double-DW washed; hydrolyzed in 6M double-distilled HCl in sealed tubes for 6 hrs at 100^{o}C; HCl evaporated; resuspended in DDW; desalted.	13
Crown, coronal dentin	Crown and root regions were dissociated using discs and burs; cooled in liquid nitrogen (LN2); the coronal dentin was separated from enamel [13]; dentin fragments were ultrasonicated in DW; followed by three times ultrasonicated in 0.2M HCl and DW; dried and powdered in mortar; shifted into pyrex glass tubes; untrasonicated (3x) in DW;	32

	dried, added 1mL 6M HCl; glass tubes sealed under nitrogen temperature; hydrolyzed for 6 hrs at 100^{o}C ± 1^{o}C; hydrolyzate evaporated under a stream of nitrogen; resuspended in 1 ml of 0.06M HCl.	
Crown, dentin	After separation; dentine located close to pulp; purity inspected under UV light; ultrasonicated once in DW and two times in 0.7N HCl; hydrolyzed for 6 hrs in 6N HC1 at 100 ^{o}C; dried in a vacuum.	31
Root, radicular dentine	Cementum and crown were removed using dental bur; pulp was removed using endodontic file; radicular dentin fragments (0.20g) were washed; dried and hydrolyzed in 6M HCl for 24 hrs at 110°C.	6
Root and crown, root dentin and coronal dentin	Mechanical removal of enamel-cementum junction; cementum and pulp; root dentin was retrieved; root dentin was washed in 15% NaCl solution at 4^{o}C for 1 h; washed in ethanol:ether (3:1) for 15 min; washed in 2% sodium dodecyl sulphate for 1h and water rinsed; overnight freeze-dried; pulverized. Coronal dentin [31]	21
whole tooth, horizontal slice at crown/root junction	Dried in vacuum desiccator over phosphorus pentoxide; transferred into universal sample tube with 1M HCl (20 mL); sealed (tube) and placed on a roller; demineralized at room temperature for 36 hrs with constant rolling; tooth was removed and washed with water (HPLC grade); horizontal sectioned with microtome blade; transferred into tube with 20ml fresh 1M HCl; sealed and placed on roller for 12 hrs; remove section; water washed; dried in vacuum desiccators; hydrolyzed in Pyrex test tube with 500 εL 6M HCl; froze in solid CO_2 and alcohol mixture; thawed to remove dissolved oxygen; repeated freeze and thaw; heated on heating block at 110^{o}C at 110^{o}C for 9 hrs; vacuum desiccators dried over NaOH pellets and phosphorus pentoxide; resuspended in 500 εL HPLC grade water; centrifuged and filtered; stored at 4^{o}C.	39
Root, Longitudinal section	Roots were separated using low speed diamond saw (water cooled); enamel, stained sections and dentin were removed carbide burr and dental air turbine (water cooled); soft tissue surrounding surface scraped, frozen in LN2; crushed with a nipper; pulp was removed	43, 50

	using forceps; retrieved fragments were sequentially washed in NaCl (15%), bathed in protease inhibitor solution (with overnight stirring at 4^{o}C), rinsed in ethanol:ether (3:1) mixture (7 min); sonicated for 1 hr at room temperature in sodium dodecyl sulfate solution (2%); cold distilled water washed; extraction with 0.6N HCl at 4^{o}C for 4 days; undissolved matrix was separated using filter funnel; extraction (20x) with 1M HCl NaCl; extraction with 0.05M Tris/HCl (pH 7.6) at 4^{o}C for 48 hrs, former both extractions were dialyzed against DW; both extracts were lyophilized; pulverized; hydrolysed for 6 hrs in 6N HC1 at 100 ^{o}C.	
Enamel, longitudinal section	Enamel was separated using a cutter; ultrasonic sequential cleaning with DW, ethanol and ether for 5 min each; dried.	51
Whole tooth, buccolingual longitudinal section	Outer layer of enamel and cementum were removed with a high speed diamond burr (water cooled); fragments were powdered using a vibratory mill for 15 sec; demineralization in 0.5 M NA_2EDTA (2 mL, pH 7.4 and adjusted with 2M NaOH, 0.05 mM NaN_3 was used to satblized the reagent) solution in centrifuge tubes; Intensive shake (2 hrs); centrifugation at 5000 rpm for 5 min; supernatant was discarded; sediment was water washed and centrifuged (3x) at at 5000 rpm for 5 min (this step ensured the removal of Ca- and Mg-EDTA and free EDTA residues); 1 mg sediment in a hydrolyzing tube was hydrolyzed with 6M HCl (600 µl); sealed the tube on flames and heated at 100^{o}C for 6 hrs; vacuum dired at 70^{o}C.	24, 41
Whole tooth, median longitudinal section	Tooth was cut at median longitudinal section with a low speed cutter; dentine was retrieved; sequential ultrasonic bathed in 0.2 M HCl, in DW (3x), in ethanol, and ethyl ether for 5 min. each; dentin fragments were pulverized using a pestle mortar.	15, 20
Whole tooth and Crown neck	Pulverized the cleaned tooth using a freezer mill (LN_2 cooled); retrieval of dentin powder by drilling the crown neck; dentin powder was sonicated in 2N HCl (1 mL); resuspended in phosphate-buffer saline (5 mL); dialyzed by dialysis membrane (24 hrs); hydrolyzed	45

dentin	with 6M HCl for 20 hrs at 100^{o}C under N_2 atmosphere; desalted using conc. HF.	
Labial and lingual sections of teeth	The sections in PCR tube, were cleaned with 6M HCl for 1 min; surface rinsing with HPLC grade methanol; two days immersion in sodium hypochlorite (12% w/v) solution; rinsed with methanol and ultrapure water; hydrolyzed at 110^{o}C for 6 hrs under under N_2 atmosphere.	38, 46, 52
Whole tooth, dentin	Removal of cement by rotating dental tools; dental pulp was drilled and separated from the crown; quality checked by UV florescence (only dentine fluoresce); retrieved dentin was washed with 14 ml of 15% NaCl solution for 1 h; washed for 15 min in ethanol:ether (3:1); rinsed with 2% SDS (1h) at 4^{o}C; rinsed with DW; lyophilized; pulverized by a hydraulic press.	44
Whole tooth, midline longitudinal section	Midline longitudinal sections cut with a low-speed saw (water cooled); dentin fragments were ultrasonicated in 0.2M HCl, pure water (3x), ethanol and ether for 5 min each; dried; pulverized by a grinder (74ï 297 µm particle size); hydrolyzed in 6M HCl at 100^{o}C for 6 hrs.	37
Whole tooth, dentin	Dissection of teeth using a low speed saw; enamel, cementum, and pulp were separated and removed; dentin was washed for 5 min in 0.2M HCl (5 mL); rinsed (3x) with DI-water (5 mL); ultrasonicated in ethanol and ether (5 mL each); air-dried; pulverized by a multi-beads shocker; hydrolyzed in 6N HCl (5 mL) for 6 hrs at 100^{o}C in a dry bath incubator.	47
Longitudinal section, dentin	longitudinal section by a diamond disc cutter; extracted dentin; dentin was washed in 5 mL of 0.2N HCl and in (5 mL) ultrapure water (3x) in ultrasonicator; again ultrasonicated in ethanol and diethyl ether (5 mL each); (3000 rpm for 30-60 sec); stored at 4^{o}C; hydrolyzed in 6N HCl for 6 hrs at 100^{o}C; hydrolyzate was desiccated in a evaporator.	48
Longitudinal section, dentin	Longitudinal sections were made; dentin was drilled by a diamond sinter conical drill and powder was collected.	53

Whole tooth	Teeth were sectioned with a diamond saw (water cooled), enamel and dentine was removed; hand-milled with mortar, larger fragments (size >100 µm) were grounded by a disc mill, dried at 60^{o}C for 24 hrs in a furnace; again powdered using hand-mill and disc mill; pelletized.	4
Root, dentin	Retrieval of root dentin by a dental drill under constant cooling; at the enamel-dentine junction, lower third of roots were separated from the crown; cementum and pulp tissue was removed, inspected in UV-light; rinsed in DW; washed in NaCl (15 % solution) for 1 h; washed in ethanol:diethylether (3:1) for 15 min; washed in sodium lauryl sulfate (2%) for 1 h; lyophilized; stored at -20°C; pulverized; hydrolyzed in 6N HCl for 6 hrs at 100^{o}C; vacuum desiccators dried.	49
Longitudinal buccal-lingual section, dentin	Longitudinal sections were made by a low speed saw; enamel, and cementum were separated and removed by a dental hand piece (water cooled high speed diamond bur); bathed in an ultrasonicator with 0.2M HCl (5 mL); ultrapure water (3x) and ethyl alcohol for 5 min each, air-dried; powdered in a stainless steel mill (15 s); intensive shake, and demineralized in in 4 mL 0.5 M Na_2EDTA (4 ml, 7.4 pH, 2 hrs); washed in ultra-pure water.	54
Dentin	Intra-enamel guiding punch was made with a round diamond bur on the buccal surface (at a right angle to the longitudinal axis, midway between the occlusion plane and the cement-enamel junction); dentin retrieved using hand-piece (attached microtrephine); followed [20].	40
Dentin	Labiolingual and buccolingual sections using a low-speed cross-section saw; washed (ultrasonically) sequentially with 0.2M HCl, DW (3x); ethanol and ether for 5 min each; powdered.	55
Root and crown dentin	Water cooled diamond bur was used for the removal of enamel (tooth crown) and cementum layer (root); cementoenamel junction region was dissected by a diamond disc; dried; washed sequentially with 0.2M (0.7%) HCl and DDW (3x) each in separate vessels; washed in ethanol (96%) and diethyl ether (99.5%) for 5 min each; pulverized in an agate mortar, powdered.	42

Isolation of ASP fraction:

As evident from the table 1 and 2, various authors had adopted different sample retrieval strategies and similar tendency had been observed in the isolation of the ASP. Consider the following table 3 reports.

Table 3. The recommendations of ASP isolation protocols (from hydrolyzate/powder).

ASP isolation protocol	**Reference**
Automatic amino-acid analyzer was used to separate the ASP from other amino acids. The consequent eluate was desalted (on unused Dowex 50W-X8 resin). The collected eluate was cleaned for incomplete broken peptides by ion-exchange chromatography (Dowex 50W-X8), and subsequently treated with L-leucine-N-carboxyanhydride (which converted the eluate into diastereomeric dipeptides). The dipeptides such as L-leucyl-L-aspartic acid and L-leucyl-D-aspartic acid were further isolated by amino acid analyzer and analyzed.	13,14,56
The collected hydrolyzate was processed through Bio-Rad AG 50-X8, (50-100 mesh) cation exchange pre washed column. 2M NH4OH was used to elute amino acids at the solvent front. The eluate in screwed glass vials were dried under a stream of nitrogen. Next, esterified with isopropyl alcohol for 3 hrs at 100^{o}C. Excess of isopropyl alcohol was evaporated and added (2 mL) dichloromethane with 0.2 mL trifluoroacetic anhydride to the residue. After 2 hrs stand at room temperature, the solution was dried under N_2 stream, resuspended in 0.2 mL dichloromethane, and injected into gas chromatograph instruments (Chirasil-Val and SP-2100, fused silica capillary coulumn in Hewlett Packard 5880 A and Shimadzu W-7 A, both with flame ionization detector). The retention times and co-injected standards were studied for the identification of enantiomers.	32
Esterification of hydrolyzate with isopropanol/HCl at 110^{o}C for 1 h. Next, acetylation with trifluoroacetic anhydride at 110^{o}C for 15 min. Subsequently, quantified and analyzed by GC (permabond L-chirasil-Val fused silica chiral capillary column, 50 m length, 0.32 m inner diameter, Macherey-Nagel, Duren, FRG, carrier gas- H_2, flame ionization detector).	21, 31
Neutralization of hydrolyzates with neutralizer and citrate buffer (1:2:2); neutralized hydrolyzate was filtered (Millex-GS 0.22 µm) and redissolved in citrate buffer (1:1); The amino acids in replica were processed though ion-exchange amino acid	6

analyzer (Beckman 6300, 25 cm lithium column and a four-buffer system); Quantified and Chromatographed on a Hewlett-Packard 3390A integrator.	
Isolation of amino acids by the use of derivatizing reagents such as O-phthaldialdehyde and N-isobtyryl-L-/D-cysteine and a gradient mobile phase system (acetonitrile/methanol and sodium acetate buffer) Separation through RP-HPLC.	39
Rehydration of hydrolyzates in 50 µL of internal standard solution (1-homo-arginine, 0.01 mM, 0.003M HCl, 0.77 mM sodium azide, pH-2). The sample was vortexed and then centrifuged. The retrieved supernatant was analyzed by HPLC (Agilent technology, Hypersil BDS column (Hypersil, Runcorn, UK), reverse phase HPLC).	38, 46, 52, 57
Hydrolyzate was dried (using an evaporator). Rehydrated with pure water and passed through a highly acidic ion-exchange resin. Eluate was dissolved in 2N ammonia (aq) and dried. Then esterified using isopropyl alcohol and acetyl chloride and later cooled by N2 aeration. Esters were acetylate with anhydrous trifluoroacetic acid and methylene chloride and dried under N2 aeration. The solution was added with ethyl acetate and was analyzed in gas chromatography with hydrogen flame ionization detector (GC-9A Shimadzu, capillary column (length 25 m, 0.3 mm inner diameter), chirasil-val coated).	15, 20, 43, 48, 49, 51, 55, 58
Desaltation of hydrolyzate with HF (conc.). Sequential derivatization using esterification (firstly with pure thyonil chloride in isopropanol and secondly with N-trifluoroacetilation with pure trifluoroacetic acid anhydride. The derivatized products were processed through GC (Hewlett-Packard 5890, Chirasil L-Val column, NPD detector at 300^{o}C).	45, 53
Hydrolyzate was dissolved in 0.1M HCl. To the hydrolyzate solution, ophthaldialdehyde (5.5 mg) in methanol (420 µL) and N-acetyl-L-cysteine (13.4 mg) were added. After 5 min., 0.3M sodium phosphate buffer (pH 7.5, 200 µL) was added and left for 5 min. The obtained derivative (225 µL) was analyzed through HPLC (Thermo separations, C8, 5 mm column (Waters, Milford), 15 cm length, 3.9 mm ID).	24, 41, 54
HCl was removed from the hydrolyzate using a centrifugal concentrator. Rehydrated in DI water and was processed through a strong acid cation-exchange	42, 47

resin column. and rehydrated in deionized water. Then followed the similar protocol [20, 43, 51, 55, 58]. After drying in centrifugal concentrator, resuspended in ethyl acetate (100 µL) and injected into GC-MS column ()optical active capillary column 5975C, Agilent Technolgies, CA).	
Hydrolyzate was derivatized by 3M thionyl chloride solution in a hot air oven at 100°C for 2 h. to the cooled residue (1 mL) mixture of dichloromethane:trifluoroacetic anhydride (3:1) was added and heated in a hot air oven at 100°C for 20 min. The cooled sample was vortexed and subjected to GC/MS analysis (electron ionization, CP-Chirasil-L-Val, 25 × 0.25, carrier gas ï He, flow (constant mode)- 1.0 mL/min., Oven initial temperature 80oC for 2 min., Ramp 1ï 4 deg/min 190°C hold for 2 min).	40

Follow-up:

Commonality:

Various authors had adopted a general outlined strategy of age resolution via ASP racemization. The following numbered hierarchy has been observed from the given studies (table 1, 2, 3).

1. Selection of type of teeth
2. Number of teeth samples
3. Selection of region/section of the teeth
4. Pre-cleaning of whole teeth
5. Sectioning/cutting of teeth
6. Post-washing of sectioned teeth
7. Drying of sections and quality check
8. Pulverization
9. Mineralization
10. Hydrolysis
11. Pre-separation of AA fractions (ion-exchange)
12. Determination of D/L ratio of ASP using instruments (automatic amino-acid analyzer, GC, GC-MS, HPLC).

Various authors had used either water (DW, DDW, DDI) or chemical (sodium hypochlorite, hydrogen peroxide) for the pre-cleaning of teeth samples. The common tools used for sectioning of teeth are dental discs, burs, and low-speed saw. For pulverization, hand mill (pestle mortar) and disc mill were used to powder the teeth sections. The D/L ASP ratio was calculate using statistical principles.

The natural logarithmic expression [58] of D/L ASP ratio was calculated by incorporating the obtained data in the following formula: ln[(1+D/L)/(1-D/L)] [13,14,15,22,25,41,42,55,58,etc.]. The D-ASP composition was correlated and expressed to the content of L-ASP [15]. On x-axis, the chronological age was plotted

and on y-axis, the racemization ratio; then the linear regression equation was derived by the least square method as follow:

$\ln[(1+D/L)/(1-D/L)]_t = 2kt + \ln[(1+D/L/(1-D/L)]_{t=0}$

where, ln[(1+D/L)/(1-D/L)] = log transformed racemization ratio, t = chronological age and k = racemization rate constant. For the estimation of chronological age, the linear regression equation was derived and followed. The age was plotted on y-axis and racemization ratio on the x-axis.

$t = \{\ln[(1+D/L)/(1-D/L)]_t$ ï $\ln[(1+D/L)/(1-D/L)]_{t=0}\}/2kt$

By substituting the D/L ratio in this equation, the age was calculated.

Variation:

Variations among major procedural steps have been observed as evident from the given reports (table 1, 2, 3). Variations have been observed in the selection of type, region and section of teeth samples; in the pre-concentration, mineralization, hydrolysis, and instrumentation of retrieved dentin samples. The different rate of ASP racemization in human dentin had been found among various studies. It had been suggested that different results are due to the different adopted methodologies [23].

Need of standardization:

The ASP racemization technique had been applied on different kinds of tissues in order to determine the chronological age. The tissues other than teeth such as human lens [61], human sclera [62], white matter of brain [63], rib cartilage [64], elastin [65], bone [66-68], intervertebral discs [69], skin [70], and epiglottis [71] had been studied but inconsistent relationship between age and ASP racemization had been found. Therefore, teeth could be better and more reliable alternative for age estimation studies.

Arguably, the best tissue for the age determination is entire dentin of central longitudinal sections [21,28]. It had been reported that rate of ASP racemization varied with the type of tooth. It is highest for the second molar and least for the canine (first molar > second premolar > central incisor > first premolar > lateral incisor > canine) [20,37,40,59]. Though, some authors recommended premolar teeth [24,27,40]. When D/L ratios had been compared among region of a tooth such as enamel, cementum, dentin; and the dentin had been found best for age estimation. This might be due to the higher surrounding temperature with its root membranes and due to its diverse protein composition [20,29,58,59]. Same rate of racemization had been reported for the homonymous teeth of same jaw because of same time of dentin formation. Racemization among the parts of dentin such as lingual and labial also varied and found to be higher D/L ratio

in lingual region. Further, crown part of the lingual dentin had higher D/L ratio than the labial region of the crown dentin. Crown part of dentin had comparatively higher D/L ratio than the root dentin [60]. Formative studies backed the recent findings and indicated that dentin is more reliable because of its higher composition than enamel and importantly, it suffers less alternation and contamination due to attrition [13,14]. The whole tooth dentin had shown the higher rate of racemization when compared with fraction dentin. The correlation coefficient was lower and standard deviation was higher. Thus entire dentin of central longitudinal sections had been recommended [28,29,47]. The dental ailments, temperature, humidity, pH appeared to effect this first order chemical reaction [49,52]. Therefore, estimation of age of human remains via racemization could be effected under such conditions and should be considered during the interpretation of D/L ratio data.

Further, the sensitivity and specificity of modern instrumentation resulted in the higher resolution of D/L ï ASP. However, authors are subjective in selection of particular instrumentation. Several studies recommended GC as evident from the table 3, along with HPLC analysis. Some recommendation had been given to standardize the current inconsistent methodologies [23] so that current limited applicability of ASP racemization can be broadened widely [62].

Future perspective

Age estimation of human body remains (proximally near to the chronological) is still a challenge for forensic scientists. The methods based on the morphological changes such as closure of cranial sutures, pubic symphysis, and ribs end modifications are of high error rate methods and that could not precisely estimate the age [15,23,72]. The dentin AAR has been found to be more precise, reliable, and possibly approximate the chronological age within ±3 years [28]. Therefore, AAR from teeth can be analyzed for more accurate calculation of the chronological age. However, teeth from the human remains should be of sufficiently perseverance to be used for AAR analysis [73]. Hence, AAR from teeth will require evaluation, validation, and standardization. After such standardizations, this technique can be applied as a universal method of chronological age estimation from human remains.

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