Heliyon 10 (2024) e30571

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Spectroscopic and dosimetric comparison of tooth enamel separation methods for EPR retrospective dosimetry

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ARTICLE INFO

Keywords:
Tooth enamel
Density separation
Alkaline denaturation
EPR
Retrospective dosimetry

ABSTRACT

Precise estimation of individual radiation dose utilizing biomaterials (fingernail, bone, and tooth) is very challenging due to their complex sample processing. Despite, tooth enamel, the most mineralized tissue of tooth is used for this purpose due to its high radiation sensitivity and ability to produce radiation induced long lived CO_2^- radicals. However, human teeth are not always available, and invasive nature of sample collection adds to the complexity making dose estimation difficult. In such cases, animal teeth (goat, cow, and moose) can be used as a substitute for human teeth due to comparable enamel sensitivity. Moreover, separation of enamel from dentine is a crucial step towards accurate dose estimation from irradiated teeth. In this work, Indian goat teeth were used as it was readily available to us and the comparison of goat enamel sensitivity to radiation was found to be within ~7.4 % that of human. The enamel samples were separated following two chemical methods; (1) density separation using sodium polytungstate, (2) alkaline denaturation using NaOH and the quality was compared based on their purity and radiation sensitivity. Combined results of spectroscopic characterization using X-ray diffraction (XRD), Fourier transform infrared (FTIR), and Raman analysis authenticated the crystallinity and purity of the separated enamel samples. The radiation sensitivity of separated enamel samples was compared by electron paramagnetic resonance (EPR) analysis as a part of dosimetric characterization. The suitability of both the samples for retrospective dosimetry and epidemiological studies was checked by validating the dose estimated from separated enamel samples with standard alanine/EPR dosimeter.

1. Introduction

Electron paramagnetic resonance (EPR) spectroscopy of human tooth enamel has been largely employed for dose reconstruction for victims of several nuclear disasters [1,2], radiation accidents [3,4], bomb survivors [5,6], and medical workers [7,8]. The main advantage of using human tooth is that the individual accumulated dose can be estimated precisely. However, human tooth is not always available, and extraction of healthy tooth from exposed personnel is invasive and may raise ethical concerns. In such cases, animal (especially mammals) tooth can be a good alternative to human tooth to estimate the radiation dose. Few studies are reported on applicability of animal tooth (cow, bovine, mice, goat, etc.) for EPR dose measurements (Table 1). *Toyoda* et al. studied cow and

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https://doi.org/10.1016/j.heliyon.2024.e30571
Received 13 April 2024; Accepted 29 April 2024
Available online 30 April 2024

mice teeth as an alternative to human teeth indicating that the radiation induced signal of cow tooth enamel is very close to human tooth enamel and that of mice tooth enamel is 25 % lower to the human tooth enamel [10]. Comparison of tooth enamel EPR spectra of cows, goats, and humans was studied by *L. Jiao* et al. and suggested that cow and goat teeth samples could be alternative materials for radiation dose estimation [11]. The use of animal tooth is also important in the environmental dose assessment where no human being is involved [18].

Tooth enamel, rich in hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals, is the main constituent of tooth and is considered the most mineralized tissue [19]. During the mineralization process, carbonate (CO_3^{2-}) impurities substitute the hydroxyl (OH^-) or A-sites and phosphate (PO_4^{3-}) or B-sites of hydroxyapatite present in the tooth enamel, which upon irradiation forms radiation induced defects ascribed to carbon dioxide radicals (CO_2^-) [19,20]. These radicals are EPR detectable and their concentration is proportional to the absorbed dose. However, tooth enamel needs to be separated from dentine before dose estimation due to the following reasons;

- To avoid inter tooth variation in EPR signal caused by heterogeneous distribution of dentine [21,22].
- To use pooled enamel samples for dosimetry and epidemiological studies [21].
- To remove larger background from irradiated dentine [19].
- Further lowering detection limit [23,24].
- To enhance radiation induced signal stability [20].
- To get reproducible result [25–28].
- To avoid over estimation of dose due to presence of ingested radionuclides Cs^{137} and Sr^{90} in dentine [3,20,29].

Various mechanical and chemical methods have been developed and reported to separate enamel and dentine from animal teeth, and fossils for retrospective dosimetry and dating (Table 1). Also, the comparison of radiation sensitivity between animal teeth enamel and human teeth enamel was investigated in detail (Table 1). Mechanical separation method utilizes a power wheeled dental drill for the enamel separation, which requires a skilled person for its operation and mainly useful for laboratories associated with medical industries [8]. However, for retrospective dosimetry laboratories of nuclear facilities this method will not be significant. In addition, mechanical separation generates an undesired spurious signal at $g = 2.002$, similar to the dosimetric signal, thus biasing the dose estimation [20,30]. Chemical separation is mainly of two types; application of concentrated alkaline solution (NaOH and KOH) and heavy liquid solutions (sodium polytungstate/metatungstate). Alkaline solution acts as a denaturant, however heavy liquid solution separates enamel and dentine based on the density gradient. The effect of chemical treatment does not generate any EPR interfering chemical by-products unless the time of treatment is exceeded 24 h [31]. Due to the aforementioned complications found as a result of mechanical treatment, mechanical separation of dentine free enamel is not preferable.

In this work, we separated enamel from dentine and other unwanted organic contents for Indian goat teeth using two widely used chemical methods. The spectroscopic characterization of enamel samples was carried out by XRD, FTIR, and Raman spectroscopy techniques and the results were compared to validate the efficient method for producing high purity enamel. EPR analysis was carried out on separated enamel to compare the radiation sensitivity as a part of dosimetric characterization. The separated enamel was compared with alanine/EPR standard for its use in retrospective dosimetry [32] and validated for accurate and precise dose estimates towards its utilization in epidemiological studies. This work reports a detailed investigation on separated enamel based on purity and radiation sensitivity using chemical methods which was lacking in the reported literatures, to the best of our knowledge. In addition, the EPR spectra of human and goat tooth enamels were compared to check the consistency in sensitivity.

2. Materials and methods

Mandibles of Indian goats aged between ~ 4 and 5 years (to avoid influence of age variation on dose estimates) were collected from a local mutton shop in Kalpakkam, India. As the front deciduous teeth are more prone to sunlight exposure, only molar and premolar

Table 1
Mechanical tools and chemicals used for separation of tooth enamel and dentine for their use in retrospective dosimetry or dating.

Separation method (Tool/chemical used)	Animal species (tooth type)	Radiation sensitivity compared to human	References
Mechanical separation	Bovine/Cow (Incisor)	~1.1 higher	[9]
+ alkaline denaturation (Dental drill + 30 % KOH)		~1	[10]
(Dental drill + 20 % NaOH)	(Molar)	~1	[11]
Alkaline denaturation (5 M NaOH)	Camel (Molar)	30 % lower	[12]
Alkaline denaturation (Supersaturated $KOH_{aq.}$)	Dog canine (Molar and incisor)	~1	[13]
Alkaline denaturation (8 M NaOH)	Goat (Molar and premolar)	~1	[11]
Mechanical separation	Mice (Molar)	25–30 % lower	[10]
+ alkaline denaturation (Dental drill + 8 M NaOH)		~50 % lower	[14]
NA	Moose (Permanent teeth)	~1	[15]
Mechanical separation (Hard alloy dental drill)	Pig (Permanent molar)	NA	[16]
Density separation (Sodium metatungstate)	Japanese Macaque (Molar)	NA	[17]
Mechanical separation	Japanese Wild Boar (Molar and premolar)	NA	[18]
+ alkaline denaturation (Water cooled dental drill +20 % KOH)			

'NA': data not available.

teeth were extracted mechanically from the mandibles and a total of 16 teeth were used in this study. First, the extracted teeth were sterilized with 6 % sodium hypochlorite (NaOCl) diluted 10 times with tap water [19,20] to prevent microbial infection for 24 h. The teeth were rinsed with double distilled water and preserved in distilled water till further separation of enamel. The roots were separated from the crown with the help of a handsaw. The crowns were cleaned with 0.1 M ethylenediamine tetraacetic acid (EDTA) solutions for removal of metal impurities. The cleaned crowns were gently crushed into powder with grain size of 105–150 μm for all the analysis. Two different methods were used for the separation of enamel from dentine. Method 1 is based on heavy liquid density separation [23,33] and method 2 is based on the alkaline denaturation of dentine [21,34]. Anton Paar (BMA 501) density meter was used to achieve the desired density value. A REMI (R-44) make centrifuge was used for better separation of enamel and dentine in method 1. The samples obtained from methods 1 and 2 are named as enamel 1, and enamel 2, respectively.

For tooth enamel sensitivity comparison between human and goat, one human molar tooth collected from DAE Hospital, Kalpakkam, India was used. The enamel from human molar was separated from dentine using alakaline denaturation method in similar manner as done for goat tooth.

2.1. Method 1

In this method, enamel was separated from dentine using high density liquid, sodium polytungstate (SPT) also known as sodium metatungstate (SMT), based on the different relative densities of dentine (2.5 g/cm^3) and enamel (2.9 g/cm^3). The enamel and dentine were separated by gravity separation (sink and swim analysis) method; enamel due to high density will sink and dentine will swim due to its low density value. Five teeth crowns were carefully grounded in an agate mortar to maintain constant pressure throughout the grinding process. After grinding, tooth powder of size range 105–150 μm was obtained using sieves to effectively separate enamel from dentine and to obtain regular shape enamel grains [19]. The tooth grains were immersed in a polypropylene tube (15 mL capacity) containing 10 mL of freshly prepared SPT solution (2.7 g/cm^3) and centrifuged (2000 rpm) for 4 min to get distinct layers between enamel and dentine. The floated (swim) dentine was removed using a micropipette, and settled enamel (sink) was collected by removing SPT solution. The obtained enamel was rinsed with distilled water four times followed by 4 min of centrifugation to clean the traces of SPT, dried at 40 °C overnight and then used for different characterizations and EPR measurements.

2.2. Method 2

This method is based on the denaturation property of dentine in an alkaline solution. Concentrated NaOH solution was utilized in this method over supersaturated KOH due to its corrosive nature and damaging tendency [19,20]. Five teeth crowns were carefully crushed into a few large pieces (4–5 pieces per crown) using agate mortar and pestle. Pieces of the crown were treated with 20 mL of 8 M NaOH solution in a glass beaker (50 mL capacity) for 6 h in an ultrasonic bath at 60 °C temperature. The samples were taken out of the solution and the softened dentine was visually identified from the colour difference (Enamel: transparent white, Dentine: dull white) and removed manually using fingers. The enamel chips were washed several times with distilled water and dried at 40 °C overnight. The separated enamel chips were mixed to get pooled enamel and gently crushed in an agate mortar to obtain 105–150 μm grain size. The powdered enamel was then treated with 20 % acetic acid solution to remove any contamination during the grinding process and dried completely inside a fume hood for further analysis.

2.3. Characterization techniques

2.3.1. Spectroscopic characterization

X-ray diffraction patterns of tooth and separated enamel samples were recorded using X-ray diffractometer manufactured by GNR Explorer, Italy. The diffraction pattern was obtained by operating the X-ray gun at 40 kV and 30 mA with Cu K_α radiation of 1.5418 Å for 2θ range from 10 to 55° at a step size of 0.05° and an integration time of 2 s at each step. FTIR spectra of extracted samples were recorded using Bruker FTIR spectrometer (Tensor) with 100 scans and 4 cm^{-1} resolution (mid-IR range: 500-4000 cm^{-1}). This technique measures the absorption/transmittance of infrared radiation by the sample versus wave number. In this study, IR transmission has been taken as it is widely used for biological tissues. Pellets of 15 mm diameter were prepared by taking the sample of interest and KBr binder in a weight ratio of 1:100 for quantitative comparisons using FTIR technique. The separated samples were directly used in their native state for Raman characterization without any change. Raman measurements were performed using a Renishaw Invia Reflex micro-Raman spectrometer at normal room temperature with 10 % of 532 nm laser excitation power at the sample, 30 s acquisition time. To reduce the presence of scattered light, measurements were performed confocally with a × 50 lens [35]. A fluorescence spectral baseline correction was applied to obtain a broad luminescence free Raman spectrum.

2.3.2. Dosimetric characterization

The radiation sensitivity property of obtained enamel sample was characterized using EPR spectroscopy. EPR measurements were performed in EPR spectrometer (Bruker make) operating in the X-band and equipped with TE_{102} mode rectangular cavity having a high Q-value (9800 at the time of measurement). The spectrometer parameters used for recording EPR spectra free from distortion, over modulation and saturation were; microwave power (MWP) of 4 mW, modulation amplitude (MA) of 4 G, receiver gain (RG) of 502, modulation frequency of 100 kHz, time constant (TC) of 81.92 ms, conversion time (CT) of 81.92 ms, number of channels 1024, and number of scans 10. The enamel samples were loaded in a quartz sample tube with an internal diameter of 3 mm for all EPR measurements. Four repeated measurements were taken for each sample to minimize the uncertainty and the average of the four was used

as true value. The standard deviation (σ) between measurements was treated as uncertainty in the measurement, represented as error bars in the graphs. A weight of 100 mg sample was used for EPR measurements to obtain reproducible results. Total acquisition time to get one EPR spectrum was 20 min.

2.4. Irradiations

The irradiations of whole tooth, tooth powder, enamel samples and alanine/EPR standard dosimeter were carried out in a gamma chamber supplied by BRIT, Mumbai, India, with ^{60}Co as the gamma energy source. The gamma chamber was calibrated using the Fricke dosimeter prior to irradiation and a dose rate of 0.57 kGy/h was obtained. All the irradiations were performed at room temperature.

3. Results and discussion

3.1. Comparison of human and goat tooth enamel EPR spectra

The enamel samples obtained from human molar and goat molar teeth were utilized for a sensitivity comparison study. Fig. 1 represents the EPR spectra of unirradiated (0 Gy) and irradiated (10 Gy) enamel samples (black line-unirradiated goat enamel; red line-unirradiated human enamel; blue line-irradiated goat enamel; green line-irradiated human enamel). From the figure, it can be inferred that the EPR spectra of both the enamel samples are composed of two components: native (less or no radiation sensitive) signal and radiation induced signal. The EPR signal centred at g = 2.0046 is due to the presence of native radicals in the organic matrix and designated as a native signal (NS). The asymmetry signal with perpendicular component observed at $g_{\perp} = 2.0017$ and parallel component at $g_{\parallel} = 1.9977$ is assigned to axial carbon dioxide (CO_2^-) radicals and termed as dosimetric signal (DS). The line shape of both goat and human enamel EPR signals was found to be similar with the same g-values. The intensity of NSs was measured for both the samples and it was found that human enamel NS is ~60 % higher as compared to goat enamel NS. Similarly, DS intensity measurement showed that the goat enamel sensitivity is within ~7.4 % of human enamel. The low NS and comparable radiation sensitivity of goat enamel with human enamel make the possibility of using goat enamel for retrospective dosimetry study in place of human enamel.

3.2. XRD analysis

Powder XRD patterns have been recorded for the identification of crystalline phases present in tooth and separated enamel samples (Fig. 2). The mineralogical phases in tooth sample are mainly due to the hydroxylapatite (HA) (pdf No.: ICDD 09-0432) and whitlockites (Wtc) ($Ca_9Mg(HPO_4)(PO_4)_6$) (pdf No.: ICDD 70-2064). Among the two phases, HA phase is more crystalline [36] and responsible for stable traps in the mineral matrix of tooth and enamel. It can be observed that the mineral phase of both the separated enamel samples is mainly due to hydroxylaptite. However, the appearance of hydroxylapatite phases is more clear and intense in enamel 2. The XRD pattern of enamel samples show peaks indexed to (hkl) values (002), (210), (211), (300), (202), and (310), which can be attributed to the characteristic peaks of calcium phosphate with apatite phase [36–38]. The peak intensities are higher in the case of enamel 2 and two additional characteristic peaks at (100) and (200) are observed. The absence of some HA phases in enamel 1 sample indicates the inadequate separation of mineralogical phases (Wtc), which may be due to the presence of particles with enamel-dentine interface.

The particle sizes of separated enamel samples were determined using Scherrer formula represented by equation (1) to check the

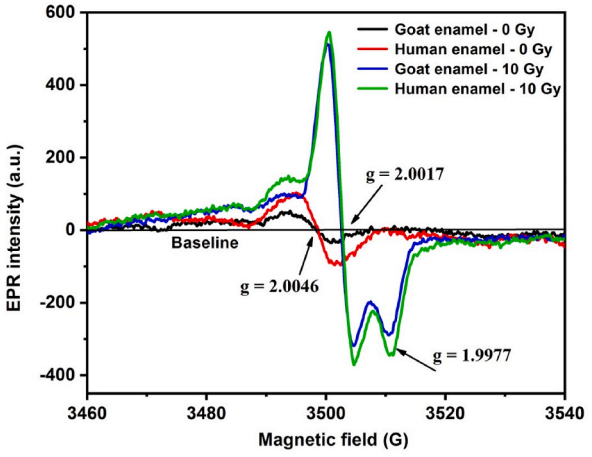

Fig. 1. EPR spectra of unirradiated (0 Gy) and irradiated (10 Gy) goat and human tooth enamel samples.

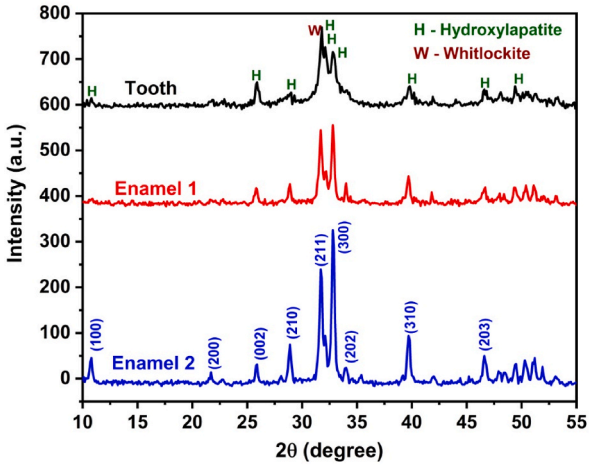

Fig. 2. XRD patterns of tooth and enamel samples obtained from two different methods.

crystallinity.

$$\text{Particle size (nm)} = \frac{0.9 \times \lambda}{b \cos \theta} \quad (1)$$

where, $\lambda = 0.154$ nm for copper K_α, b = FWHM (full width at half maximum), and θ = diffraction angle and 0.9 is the shape factor [38]. The particle sizes were calculated for the corresponding HA peaks of extracted samples and are presented in Table 2. The average particle size of enamel 1 is 19.8 nm and enamel 2 is 22.8 nm which means enamel 2 is more crystalline in nature. From XRD results we can conclude that method 2 separates enamel with more crystallinity than method 1.

3.3. FTIR analysis

A detailed FTIR spectroscopic investigation was carried out to identify the functional groups and check the purity of separated enamels. FTIR spectra of tooth and separated enamel samples are presented in the 500-4000 cm^{-1} range (Fig. 3).

In the case of tooth, the characteristic IR peaks were observed for hydroxyl (OH^-) radicals; one broad asymmetric peak in the range of 3182–3679 cm^{-1} due to mixture of bending and stretching modes of structural water (strongly bound to the enamel tissue) and another at 1648 cm^{-1} due to bending vibration of adsorbed water (weakly bound to the enamel tissue) [39], phosphate radicals (PO_4^{3-}); one prominent broad peak at 1033 cm^{-1} due to P – O stretching modes, two IR bands at 604 cm^{-1} and 560 cm^{-1} due to O – P – O bending modes, and carbonate radicals (CO_3^{2-}); two peaks at 1414 cm^{-1} and 873.5 cm^{-1} assigned to stretching and bending modes of carbonate ions, of hydroxyapatite crystals present in the mineral matrix of tooth sample [40,41].

Further, a detailed investigation was carried out to determine the content of structural water in enamel samples, which governs the degree of crystallinity and contributes to the exceptional stability of radiation induced radicals. The hydroxyl groups of structural water in the tooth sample exhibited a broad peak in the range 3679–3182 cm^{-1}. However the peak shape became asymmetric with increased peak area and shift towards lower band for separated enamel samples, which indicates the dominance of structural hydroxyl group that are associated with high degree of crystallinity [41] (the peak ranges are given in the inset of Fig. 3). Also, the increased asymmetry behaviour and peak area of enamel 2 compared to enamel 1 show that method 2 effectively separates the enamel with higher crystallinity than method 1. Besides, the low intense peaks in 3000–2800 cm^{-1} range due to C – H stretching vibrations of

Table 2
Determination of particle size of enamel samples obtained from different methods.

Sample	(*hkl*)	2θ (in °)	b (FWHM) (in °)	b (in radian)	Particle size (nm)	Average particle size (nm)
Enamel 1	(002)	25.84	0.434	0.007575	17.8	19.8
	(210)	28.944	0.391	0.006842	19.6	
	(211)	31.746	0.356	0.006213	21.5	
	(300)	32.776	0.382	0.006667	19.9	
	(310)	39.724	0.372	0.006493	20.1	
Enamel 2	(002)	25.84	0.343	0.005986	22.6	
	(210)	28.944	0.332	0.005794	23.2	
	(211)	31.746	0.342	0.005969	22.3	22.8
	(300)	32.776	0.299	0.005219	25.5	
	(310)	39.724	0.366	0.006388	20.4	

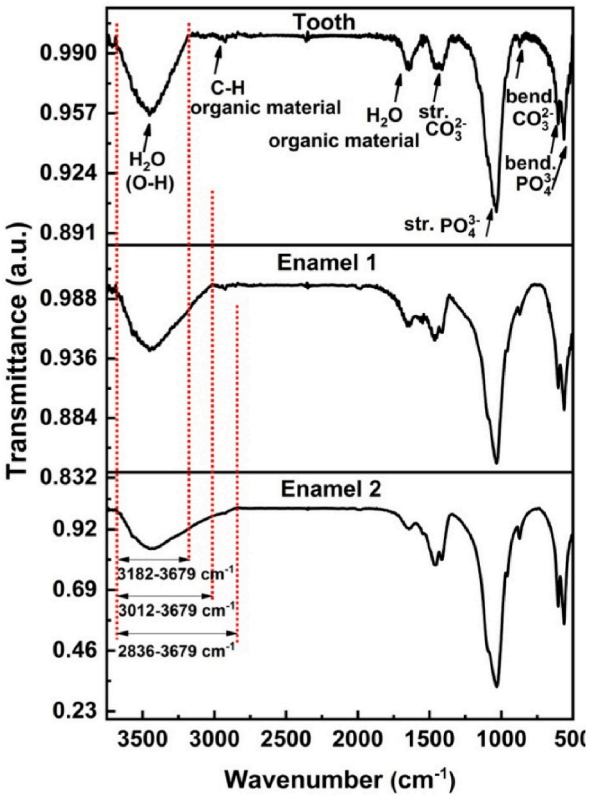

Fig. 3. Baseline corrected and mass normalized transmission spectra of tooth and enamel samples separated by two methods recorded in 500–4000 cm^{-1} range.

organic impurities were observed in tooth and enamel 1 and completely absent in enamel 2, indicating higher purity of enamel 2. The presence of organic impurity in enamel 1 sample is obvious because SPT solution does not exhibit denaturation property.

FTIR spectra of tooth and separated enamel samples are further explored for identification of different vibrational modes by plotting in the 500 to 1800 cm^{-1} range (Fig. 4). The list of functional groups assigned to different vibrational modes and IR band positions identified for the enamel samples separated from two methods are presented in Table 3.

The IR bands of CO_3^{2-} radicals and PO_4^{3-} radicals are known to exhibit four vibrational modes, ν_1 to ν_4, in tooth enamel [42]. Among these, ν_1 (CO_3^{2-}), ν_4 (CO_3^{2-}), and ν_2 (PO_4^{3-}) vibrational modes are not active in mid-IR range spectra and can be observed in Raman spectra (see section 3.3) [43]. The IR band of ν_2 (CO_3^{2-}) at 879 cm^{-1} corresponding to O − C − O bending vibrations and ν_3 (CO_3^{2-}) at

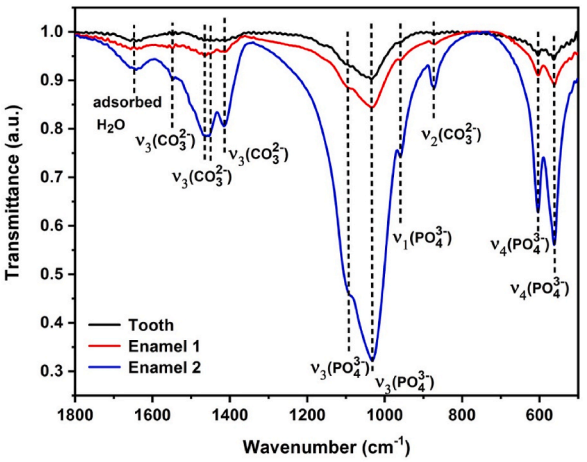

Fig. 4. Baseline corrected and mass normalized FTIR spectra of tooth and enamel samples separated by two methods in 500–1800 cm^{-1} range.

1414 cm^{-1}, 1449 cm^{-1}, 1465 cm^{-1}, 1547 cm^{-1} due to antisymmetric C − O stretching vibrations of carbonate radicals were observed for both the sample, but the intensity is more for enamel 2 sample. Of four vibrational modes of phosphate radicals, ν_1 (PO_4^{3-}) at 960.5 cm^{-1} corresponding to symmetric stretching vibrations of P − O and two peaks of antisymmetric stretching vibrations of P − O at 1033 cm^{-1} and 1094 cm^{-1} that is ν_3 (PO_4^{3-}), were observed in the separated enamel samples and were more pronounced in enamel 2 compared to enamel 1. It was also observed that for the same amount of enamel samples, the characteristic IR peaks (PO_4^{3-}, CO_3^{2-}, and OH^- of structural water) of enamel 2 is roughly 4.1 to 4.4 times more intense than enamel 1 (Fig. 4). These findings confirm that though both the methods separate enamel from dentine, the efficient separation was achieved by method 2. A similar observation was found in XRD analysis.

3.4. Raman analysis

Fig. 5 illustrates the vibrational Raman spectra recorded for tooth, and separated enamel samples from two different methods. The peak shifts for the dominant bands with their molecular assignment is reported in Table 4; ν_1, ν_2, ν_3, and ν_4 corresponds to the type of vibrations used for FTIR spectra analysis (Table 3). In the case of tooth, two less intense peaks were observed; one at 1072.7 cm^{-1} due to phosphate ions (PO_4^{3-}) of inorganic mineral matrix and the other one at 2944 cm^{-1} due to C − H vibrations of organic matrix [35,44,45]. However, the Raman spectra of enamel samples showed transitions due to phosphate (PO_4^{3-}), carbonate (CO_3^{2-}) and hydroxide (O − H) ions of apatite present in the mineral matrix of the enamel samples. All the four vibrational modes of phosphate ions and ν_1 vibrational mode of carbonate ions are present in both the separated enamel samples (Fig. 5). The intensity of characteristic peaks could not facilitate quantitative information for determining a better enamel separation technique as it is based on surface analysis of the samples. However, the absence of ν_1 vibrational mode of structural O − H (dominant in enamel tissue) and presence of ν_1 mode of free O − H in the enamel 1 spectra indicated the effective separation of enamel in method 2 compared to method 1. Also the presence of organic impurity (C − H vibrations) in enamel 1 shows the partial separation of enamel from dentine. This is because SPT solution cannot remove organic impurity from the surface; it only separates the enamel and dentine based on difference in density. From the Raman analysis, it can be concluded that though method 1 separated enamel from dentine, it has not adequately removed the organic impurity which may mislead the dose information. Hence in order to avoid this ambiguity, method 2 could be preferred over method 1 for getting reliable accuracy in dose estimation. These findings are also in agreement with the FTIR and XRD results.

3.5. EPR analysis

3.5.1. Analysis of native EPR signal

The presence of low background signal is one of the important dosimetric characteristics to be satisfied by the material used as retrospective dosimeter. Also, from dosimetric point of view, less background of the sample lowers the minimum detection dose limit. In order to understand this property, the EPR spectra of unirradiated tooth and separated enamel samples were recorded and are shown in Fig. 6. The EPR spectra of tooth and enamel samples are symmetric in origin and consist of a single peak centred at g = 2.0046 that can be attributed to the native radicals present in the organic matrix of tooth (~30 % due to dentine) and enamel (~1 % due to protein binder amelogenin). The obtained g-value matches with the reported literatures [10,11,19,20]. The intensity (peak-to-peak height) of native radicals in enamel 1 is reduced by 28 %; however, in enamel 2, the reduction of background intensity is approximately 57 %. This indicates that method 2 effectively separated enamel by complete denaturation of organic proteins present in dentine.

The less reduction of native radical concentration in enamel separated by method 1 shows incomplete removal of dentine. EPR analysis confirmed the presence of some amount of dentine in enamel 1 as enamel-dentine interface grains and method 2 effectively separated dentine free enamel. Similar finding was also observed from XRD, FTIR, and Raman analysis.

Table 3
The list of functional groups (along with the modes of vibration and the band positions) identified in the enamel samples separated from two different methods.

Functional group	Vibration modes	Wave number (cm^{-1})
H_2O adsorbed water	ν_2 (symmetric bending)	1648
H_2O structural water	ν_1 (symmetric stretching)	2836–3679
	$2\nu_2$ (symmetric bending)	3575
CO_3^{2-} carbonate radical	ν_2 (symmetric bending)	879
	ν_3 (antisymmetric stretching)	1414
		1449
		1465
		1547
PO_4^{3-} phosphate radical	ν_1 (symmetric stretching)	960.5
	ν_3 (antisymmetric stretching)	1033
		1094
	ν_4 (antisymmetric bending)	560
		604

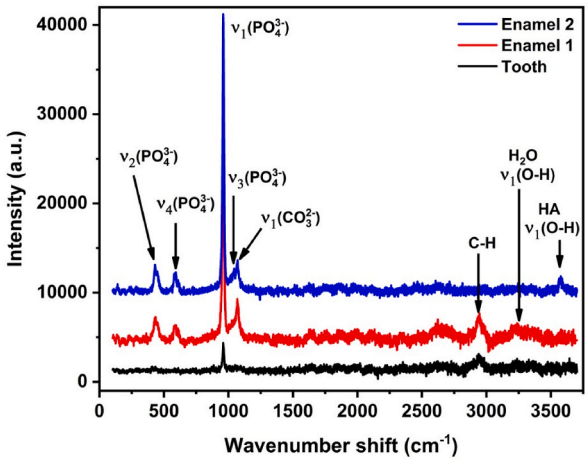

Fig. 5. Raman measurements of extracted tooth and separated enamel samples of Indian goat. The spectra were corrected with fluorescence background and presented with Y-offset. The most intense peak shifts were identified.

Table 4
The assignment of identified molecules to Raman shift for separated enamel samples from two different methods.

Raman shift (cm^{-1})	Molecular assignment	Associated vibration
440	$\nu_2(PO_4^{3-})$	O – P – O bending
590	$\nu_4(PO_4^{3-})$	O – P – O bending
960.5	$\nu_1(PO_4^{3-})$	P – O stretching
1043	$\nu_3(PO_4^{3-})$	P – O stretching
1072.7	$\nu_1(CO_3^{2-})$	C – O stretching
2944	C – H	C – H bending
3253.2	ν_1(O – H)of H_2O	O – H stretching
3579	ν_1(O – H) of HA	O – H stretching

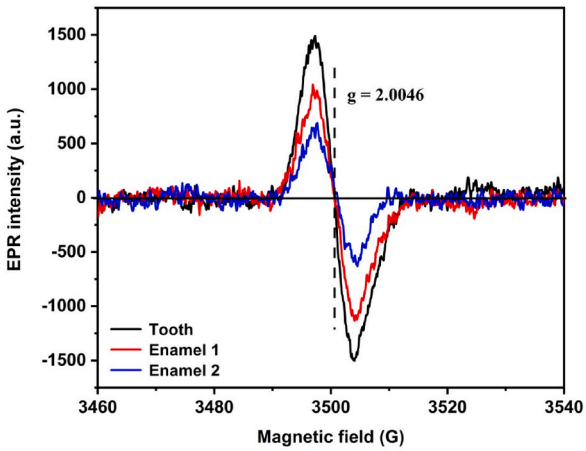

Fig. 6. EPR spectra of unirradiated tooth and enamel samples obtained from two different methods. The black horizontal line is the base line.

3.5.2. Analysis of dosimetric EPR signal

Recognizing the nature of radicals contributing to the EPR spectrum and radiation sensitivity property is crucial for each material in retrospective dosimetry. The radiation sensitivity of tooth and separated enamel samples was compared by recording EPR spectra of tooth and enamel samples irradiated to a gamma dose of 5 Gy (Fig. 7).

The EPR spectrum of tooth consists of at least two components, native (less or no radiation sensitive) signal and radiation induced signal. The EPR signal centred at g = 2.0046 is attributed to the native radicals present in the organic matrix and termed as native signal (NS) [11,19,20]. The asymmetric signal with perpendicular component at $g_{\perp} = 2.0017$ and parallel component at $g_{\parallel} = 1.9977$ is

assigned to axial carbon dioxide (CO_2^-) radicals and termed as dosimetric signal (DS) [11,19]. As can be seen from Fig. 6, same EPR signals (line shape and width) are observed in both the separated enamel samples irradiated to 5 Gy gamma dose but with a variation in dosimetric signal amplitude (peak-to-peak height of DS). The amplitude of NS at g = 2.0046 is less for enamel 2 compared to tooth and enamel 1 samples. Also, the amplitude of DS is higher in the case of enamel 2, indicating the effective removal of dentine and other undesired organic impurities from enamel in method 2 compared to method 1.

3.6. Validation of separated enamel with EPR standard for retrospective dosimetry

In order to validate the separated enamels by two different methods for retrospective dosimetry, three whole teeth were irradiated to three different gamma doses (2, 5 and 30 Gy) along with tissue equivalent alanine/EPR standard dosimeters. Each tooth was cut into two halves, one half is used for enamel separation by method 1 and the other one is utilized for method 2 of enamel separation. The separated enamel samples were used for dose reconstruction using single aliquot additive dose (SAAD) method [20].

The alanine/EPR doses were estimated using the standard calibration procedures. The reconstructed doses from irradiated enamel samples and alanine/EPR dosimeter are presented in Fig. 8. The dose estimates of enamel 1 were within ±11 % of the delivered dose, and the dose estimated using alanine/EPR standard dosimeters. However, the estimated doses from enamel 2 were within ±4 % of the delivered doses and the alanine/EPR doses. It can be concluded from the study that both the methods for enamel separation can be used for retrospective dose estimation as the variation is ±11 % which is within the acceptance criteria of IEC standard [46], however, enamel 2 could be preferred due to less variation in dose estimates.

3.7. Validation of enamel separation methods for accurate dose estimation

The study on accurate dose estimation from retrospective dosimeters is essential for epidemiological studies to understand the chronology of biological effects of radiation. For this study, three numbers of whole teeth were irradiated directly in a gamma chamber to a dose of 3 Gy and each tooth was cut into two equal halves. One half is used for dose estimation from enamel separated by method 1 and the other one is utilized in method 2 separation for dose estimation. The SAAD technique was used for the estimation of dose from both the enamel samples, and the results are presented in Table 5. The mean dose was calculated by taking the average of three doses of enamel samples 1 and 2. The standard deviation σ was determined between the three estimated dose values to check how precisely the delivered dose (3 Gy in this case) can be reconstructed. From the results we can observe that dose estimated from enamel 2 is accurate (mean dose ~ 3.02 Gy) with good precision (σ ~ 0.03 Gy) compared to that of enamel 1; mean dose ~ 2.71 Gy and σ ~ 0.10 Gy suggesting that method 1 could produce enamel leading to high uncertainty in dose value than method 2. Thus, for accurate measurement of retrospective dose from animal tooth, method 2 of enamel separation would be more efficient.

3.8. Summary of the comparative study

A comparative study of enamel separation by two widely used chemical methods disclosed that the results of spectroscopic characterization highly complemented the dosimetric properties. XRD analysis revealed that enamel 2 is more crystalline than enamel 1 which is because enamel 1 contains whitlockites mineral phases corresponding to dentine as impurities. The combined results of FTIR and Raman analysis indicated the inadequate removal of organic contamination from enamel sample separated by method 1. Additionally, presence of enamel-dentine grains affects the EPR background of enamel and gives errors in dose estimation. High purity enamel results in EPR signal with maximum dosimetric signal intensity as shown by enamel 2. Therefore, method 2 of chemical separation is more preferable for retrospective dosimetry and epidemiological studies.

Furthermore, time taken for enamel separation and available quantity of tooth sample play significant role in rapid dose assessment. In both the circumstances, method 1 is more suitable over method 2 as method 1 requires 30 min and method 2 takes 6 h for separation excluding pre and post sample treatments. Also, method 1 utilizes grains in micron range for its separation hence is capable of giving enamel even for a small sized animal tooth but method 2 could not as it needs chip size samples.

As enamel purity and crystallinity are two main factors affecting EPR signal sensitivity and stability, therefore, pure and crystalline enamel makes method 2 potential for dose estimation; however, method 1 can be utilized for rapid dose estimation by compromising the under estimation in reconstructed dose.

4. Conclusion

In this work, we compared two widely used chemical methods (density separation by SPT treatment and dentine denaturation by NaOH treatment) for separation of enamel from goat teeth based on their spectroscopic and dosimetric characteristics. The diffraction peaks and crystallite size obtained from XRD analysis for enamel samples showed that treatment of NaOH solution effectively removed the unwanted mineralogical phases affecting the crystallinity compared to SPT treatment. FTIR and Raman analysis confirmed the presence of organic impurities in SPT treated sample and dominance of structural O − H responsible for high degree of crystallinity with no organic impurities in NaOH treated sample resulting more pure enamel. The decrease in background EPR signal intensity and increase in dosimetric signal intensity in NaOH treated sample was mainly due to the complete removal of dentine and other undesired organic contents. Additionally, the validation study with alanine/EPR standard dosimeter showed that the enamel obtained from SPT treatment adds error in dose values resulting in under estimation profile. Therefore, the enamel separation based on dentine dena-

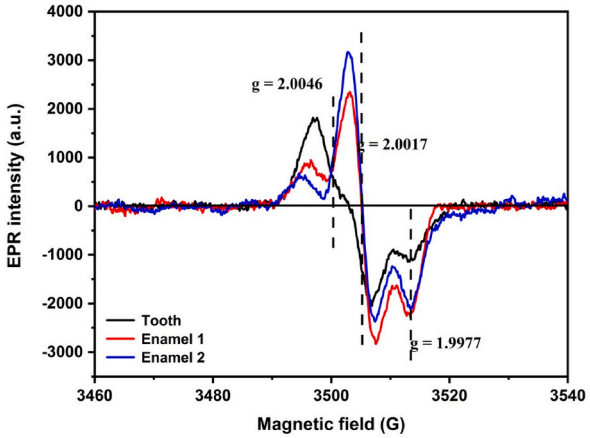

Fig. 7. EPR spectra of irradiated (5 Gy) tooth and enamel samples obtained from two different methods. The EPR spectra were mass normalized for comparison.

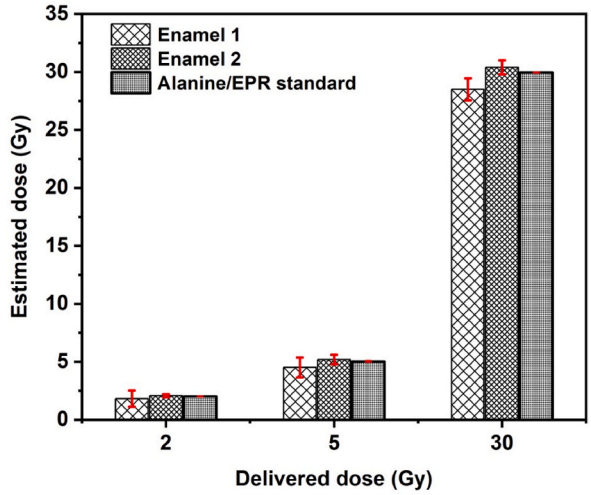

Fig. 8. Comparison of reconstructed dose from irradiated enamel samples with delivered dose and the standard alanine/EPR dosimeter. The error bar is the standard deviation of three different measurements of the same sample.

Table 5
Estimated dose from enamel samples separated from each half of irradiated (3 Gy) teeth by method 1 and 2.

Tooth sample	Estimated dose using EPR (Gy)			
	Method 1	Mean	Method 2	Mean
1	2.69		3.05	
2	2.82	2.71	3.03	3.02
3	2.63		2.99	
Dose	Mean ± σ	2.71 ± 0.10		3.02 ± 0.03

turation by NaOH solution will be a suitable method for retrospective dosimetry and epidemiological studies. However, the density separation method of enamel by SPT solution can be used for rapid assessment of radiation dose as it is less time consuming and utilizes micron sized tooth grains.

CRediT authorship contribution statement

Madhusmita Panda: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Shailesh Joshi:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **O. Annalakshmi:**

Formal analysis, Methodology, Validation, Writing – review & editing. **Venkata Srinivas C:** Resources, Supervision. **B. Venkatraman:** Resources, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors would like to thank the members of Regional Calibration Facility, IGCAR for gamma irradiation. The authors would like to acknowledge Mr. Sanjit Parida, MCMFCG, IGCAR for XRD analysis, Mr. Kishore Kumar, MSG, IGCAR for Raman analysis, and Mrs. Suja Subramanian, EAD, IGCAR for FTIR analysis.

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