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# Age Estimation and Sex Determination Using Raman Spectra of Human Dentine

# *Procjena starosti i određivanje spola analizom ljudskog dentina s pomoću Ramanova spektra*

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**Abstract**
**Objectives:** This study aimed to determine the sex and estimate the age of individuals by analysing Raman spectra obtained from extracted teeth dentine. **Material and methods:** A total of 25 male and 26 female extracted teeth were collected, disinfected, and fixed in auto acrylate before being cut near the cervical margin. The inclusion criteria were non endodontically treated teeth with the cervical third of the tooth crown preserved. The exposed dentine was recorded using a Raman spectrometer, with 100 scans per dentine site in the spectral range of 3500 to 200 $cm^{-1}$ and a resolution of 4 $cm^{-1}$. Background-corrected, normalized, and decomposed spectra were subjected to principal component analysis using MATLAB 2010 (The MathWorks, Natick, MA, USA). To assess age differences, prominent vibrational bands were observed and statistically analyzed, while sex differentiation employed the T-test on acquired data, exploring vibrational bands with significant intensity differences. **Results:** The obtained results revealed a segmentation of spectra in the male sample group based on the age of teeth at extraction, with an accuracy of age estimation at 7.0048 years. A similar segmentation was observed in the female sample group, with an accuracy of 9.3863 years. **Conclusion:** The recorded spectra were organized into datasets and analyzed with principal component analysis, demonstrating the feasibility of sex determination and age estimation using Raman spectra of teeth. Differences in classification accuracy between sexes may be attributed to hormonally-mediated differences in the biochemical composition of dentine between males and females. The findings of this study can provide valuable insights into methods for forensic and anthropological applications.

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**Author Keywords:** Age estimation; Raman spectroscopy; Forensic odontology; Sex determination; Principal component analysis

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## Introduction

Age estimation through the analysis of mineralized dental tissues, particularly teeth, is a widely employed forensic technique with diverse applications. It proves to be valuable in scenarios such as identifying accident victims, investigating criminal offences, and regulating social benefits. Dental tissues possess distinct advantages for forensic analysis due to their high resistance to postmortem decomposition. The durability of teeth makes them a robust and reliable source of information, contributing significantly to the field of forensic odontology (1). Age estimation in forensic dentistry can be approached through a couple of distinct methods: evaluating morphological characteristics of teeth through visual imaging inspection or radiography, examining histological tooth characteristics using microscopy, and analyzing the biochemical composition of dental tissues through various analytical techniques, such as Raman spectrometry (2).

## Uvod

Pri procjeni dobi na temelju analize mineraliziranih zubnih tkiva korištena je forenzička tehnika s različitim primjenama. Ta je metoda dokazano neprocjenjiva u slučaju identifikacija žrtava nesreća, istraživanje kaznenih djela i regulacije socijalnih prava. Kad je riječ o forenzičkoj analizi, zubna tkiva imaju posebne prednosti zbog visoke otpornosti na postmortalnu razgradnju. Naime, trajnost zuba čini ih snažnim i pouzdanim izvorom informacija, što znatno pridonosi području forenzičke odontologije (1). Dob se u forenzičkoj stomatologiji može procijeniti uporabom nekoliko različitih metoda: procjenom morfoloških karakteristika zuba vizualnim pregledom ili radiografijom, ispitivanjem histoloških karakteristika zuba mikroskopijom i analizom biokemijskog sastava zubnih tkiva s pomoću različitih analitičkih tehnika poput Ramanove spektrometrije (2).

Određivanje spola posebno je važno ako nema podataka o umrloj osobi. U slučaju nesreća, eksplozija, prirodnih ka-

Sex determination is particularly important when no information about the deceased are available. In cases of accidents, explosions, natural disasters, crime investigations and ethnic studies, sex determination is the first priority in the identification of a person by a forensic investigator. Determining sex from skeletal remains poses a major problem for forensic scientists, especially when only fragments of the body are recovered. Forensic odontologists can assist other experts in determining the sex of the remains based on teeth and skull features. Various features of the teeth such as morphology, crown size and root length are characteristics of male and female sexes. There are also differences in the skull pattern and skull features of two sexes (3).The Raman spectrum can distinguish the chemical composition of any mineral, inclusive of human teeth, which makes it an efficient selective method for the study of molecular species (4,5). Contemporary forensic dentistry uses morphology and histology ; however, in the experimental setting, biochemical analyses hold even greater relevance (6). Biochemical methods for estimating age provide significant benefits compared to traditional approaches, making them useful complementary methods (7). For instance, biochemical analyses require very little amounts of mineralized tissue matter and can be applied to severely damaged teeth (8).

The Raman spectrometric analyses of hard tooth tissues have been conducted in various studies, encompassing evaluations such as enamel mineralization (9–11), caries detection (12–14), distinctions in the composition between decidious and permanent teeth (15), and charting the spatial arrangement of organic and inorganic constituents in tooth tissue (16). In addition, Raman spectroscopy has been used to analyse the composition of dental tissues and to study the adhesive dentin-enamel interface (17). A limited number of studies have incorporated Raman spectrometric analysis for age estimation and sex determination purposes (4,18).

The aim of this study was to examine the correlation between chronological age and biological sex with the Raman recorded values. The hypothesis is that the results will correlate strongly.

## Material and methods

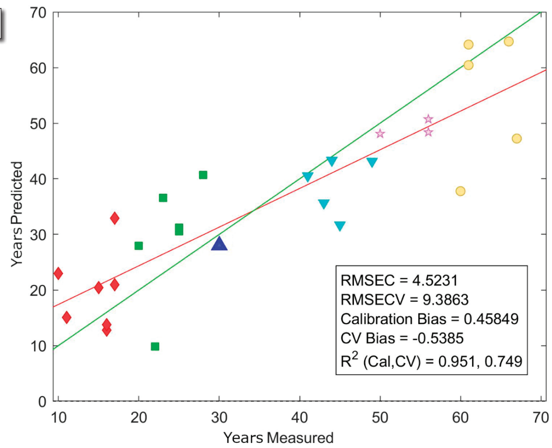
We analyzed a set of 51 extracted human teeth, comprising 6 incisors, 4 canines, 17 premolars, and 24 molars, utilizing Raman spectroscopy. This collection of teeth served as our training set for the regression analysis. The extracted teeth, a total of 25 male and 26 female specimens, were ranged in age from 10 to 67 years before extraction.The tooth samples utilised in this study were sourced from the archives of the Department of Dental Anthropology, School of Dental Medicine, University of Zagreb. The entire process of specimen preparation was done in the Laboratory for hard dental tissues of this Department. The process was funded by the project of the Croatian Science "Tooth Analysis in Forensic and Archeological Research IP-2020-02-9423". The inclusion criteria were as follows: no previously endodontically treated teeth, with the cervical third of the tooth crown preserved. In this way, we tried to avoid any additional extrinsic

tastrofa te istraga kaznenih djela i etničkih sukoba, određivanje spola prvi je prioritet forenzičkog istražitelja u identifikaciji osobe. Utvrđivanje spola iz koštanih ostataka velik je izazov za forenzičke znanstvenike. Forenzički odontolozi mogu pomoći drugim stručnjacima u određivanju spola na temelju zuba i karakteristika lubanje. Različita svojstva zuba poput morfologije, veličine krune i duljine korijena karakteristična su za muški i ženski spol. Između dvaju spolova postoje i razlike u uzorku lubanje i njezinim karakteristikama (3). Ramanovim spektrom može se razlikovati kemijski sastav bilo kojeg minerala, uključujući ljudske zube, što ga čini učinkovitom selektivnom metodom za proučavanje molekularnih vrsta (4, 5). Suvremena forenzička stomatologija koristi se morfologijom i histologijom, no u eksperimentalnom okruženju biokemijske analize imaju još veće značenje (6). Biokemijske metode procjene dobi pružaju značajne prednosti u usporedbi s tradicionalnim pristupima, pa su zato korisne dopunske metode (7). Na primjer, za biokemijsku analizu potrebna je vrlo mala količina mineraliziranoga tkiva i može se primijeniti na teško oštećene zube (8). Ramanovom spektrometrijskom analizom tvrdih zubnih tkiva bavili su se autori raznih studija, obuhvaćajući evaluacije poput mineralizacije cakline (9 – 11), otkrivanja karijesa (12 – 14), razlika u sastavu između mliječnih i trajnih zuba (15) te kartiranje prostornog rasporeda organskih i anorganskih sastojaka u zubnom tkivu (16). Uz to, Ramanova spektroskopija koristi se za analizu sastava zubnih tkiva i proučavanje dentina i cakline (17). Autori nekih studija služili su se Ramanovom spektrometrijskom analizom u svrhu procjene dobi i određivanja spola (4, 18). Cilj ovog istraživanja bio je ispitati povezanost između kronološke dobi i biološkog spola sa zabilježenim vrijednostima u Ramanovu spektru. Hipoteza je da će rezultati biti snažno povezani.

## Materijal i metode

Koristeći se Ramanovom spektroskopijom, analizirali smo skup od 51 ekstrahiranog zuba – 6 sjekutića, 4 očnjaka, 17 pretkutnjaka i 24 kutnjaka. Izvađeni zubi, ukupno 25 muških uzoraka i 26 ženskih, prije vađenja bili su starosti od 10 do 67 godina. Uzorci zuba korišteni u ovom istraživanju potječu iz arhiva Zavoda za dentalnu antropologiju Stomatološkog fakulteta Sveučilišta u Zagrebu. Cijeli proces pripreme obavljen je u Laboratoriju za tvrda dentalna tkiva toga zavoda. Proces je financiran sredstvima iz projekta Hrvatske znanosti *Analiza zuba u forenzičkim i arheološkim istraživanjima* (IP-2020-02-9423).

Kriteriji za uključivanje bili su zubi koji nisu bili endodontski tretirani te s očuvanom cervikalnom trećinom krune. Na taj način pokušali smo izbjeći bilo kakve dodatne vanjske čimbenike koji bi mogli utjecati na dentin. Tijekom prikupljanja uzoraka poštovali smo cijeli proces: izvađeni su, 1




2

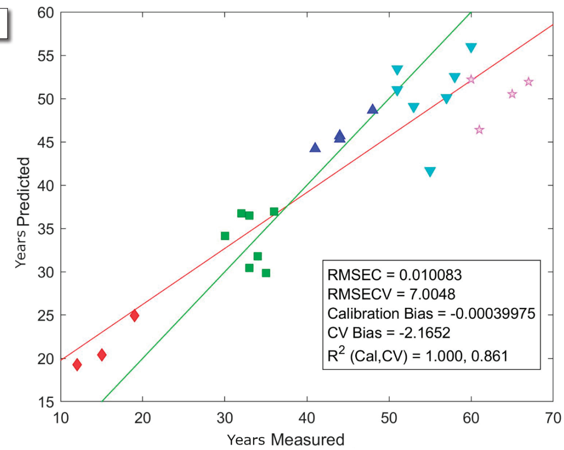



**Figure 1.** Segmentation of spectra based on the age, females (red: 10-19, green: 20-29, dark blue: 30-39, turquoise: 40-49, pink: 50-59, yellow: 60-69) RMSEC- root mean square error of calibration; RMSSECV- root mean square error of cross validation; CV- cross validation; R2- coefficient of determination; (Cal, CV)- (Calibration, Cross Validation); Red line: regression; Green line: ideal predicted values

**Slika 1.** Segmentacija spektara na temelju dobi – žene (crvena: 10 – 19, zelena: 20 – 29, tamnoplava: 30 – 39, tirkizna: 40 – 49, ružičasta: 50 – 59, žuta: 60 – 69) RMSEC – korijenska srednja kvadratna pogreška kalibracije; RMSSECV – korijenska srednja kvadratna pogreška križne validacije; CV – križna validacija; R2 – koeficijent determinacije; (Cal, CV) – (kalibracija, križna validacija); crvena linija: regresija; zelena linija: idealne predviđene vrijednosti

**Figure 2** Segmentation of spectra based on the age, males (red: 10-19, green: 30-39, dark blue: 40-49, turquoise: 50-59, pink: 60-69) RMSEC- root mean square error of calibration; RMSSECV- root mean square error of cross validation; CV- cross validation; R2- coefficient of determination; (Cal, CV)- (Calibration, Cross Validation); Red line: regression; Green line: ideal predicted values

**Slika 2.** Segmentacija spektara na temelju dob – muškarci (crvena: 10 – 19, zelena: 30 – 39, tamnoplava: 40 – 49, tirkizna: 50 – 59, ružičasta: 60 – 69) RMSEC–- korijenska srednja kvadratna pogreška kalibracije; RMSSECV – korijenska srednja kvadratna pogreška križne validacije; CV – križna validacija; R2 – koeficijent determinacije; (Cal, CV) – (kalibracija, križna validacija); crvena linija: regresija; zelena linija: idealne predviđene vrijednosti

3

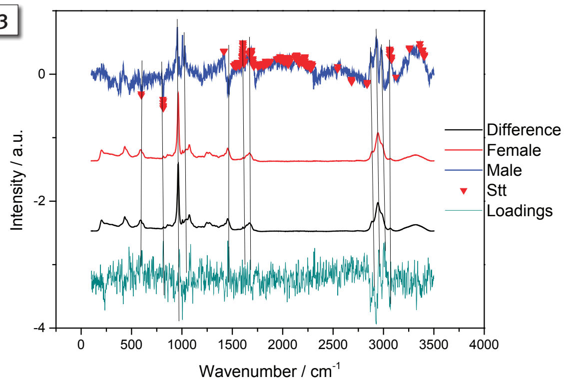
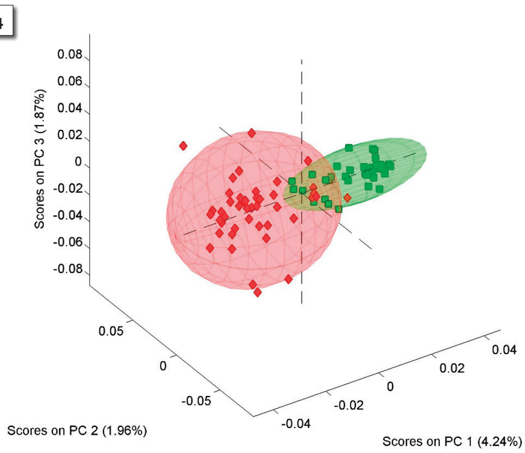



**Figure 3** The mean Raman spectra of male and female teeth, the loadings of the first principal component and the spectrum of the difference between the male and female teeth. A.u. -arbitrary units; Stt- Student t test

**Slika 3.** Srednji Ramanovi spektri muških i ženskih zuba, opterećenja prvoga glavnog komponenta i spektar razlike između muških i ženskih zuba. A.u. – proizvoljne jedinice; Stt – Studentov t-test

4




5

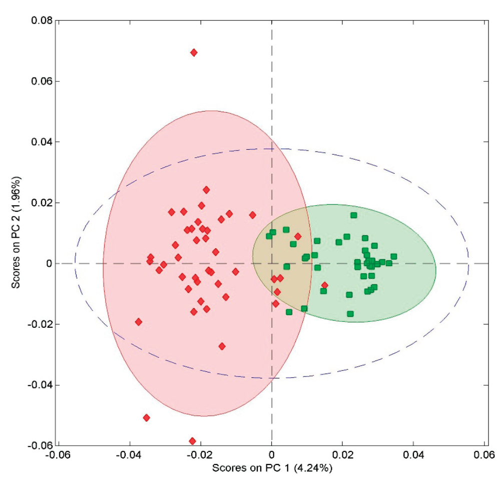



**Figure 4** Schematic differentiation obtained by PCA/PCR processes (Male/Female). PC Principal component
**Slika 4.** Shematska diferencijacija dobivena postupcima PCA/PCR (muško/žensko). PC – glavna komponenta
**Figure 5** Schematic differentiation obtained by PCA/PCR processes (Male/Female). PC Principal component
**Slika 5.** Shematska diferencijacija dobivena postupcima PCA/PCR (muško/žensko). PC – glavna komponenta

factors that may affect tooth dentine. During the collection phase of specimens they underwent a meticulous process: they were extracted, cleansed of blood and soft tissue remnants, and disinfected using a 2% hydrogen peroxide ($H_2O_2$) solution. Following the process of drying at room temperature (Figure 1), the teeth were stored in airtight containers in the archive of the Department.At the time of research analysis the teeth were embedded in fast-setting acrylate (Presi, Eybens, France) (Figures 2) and precision-cut near the cervical margin (Figures 3 and 4), about 1mm towards the apex, using a precision cutter Isomet 1000 (Buehler, Uzwill, Switzerland) (Figure 5). Subsequently, the samples, cut in a horizontal plane, underwent surface polishing with p1200 grit polishing paper (Buehler, Uzwill, Switzerland) and a Phoenix Beta polisher (Buehler, Uzwill, Switzerland) (Figure 6). This process ensured the establishment of a uniformly exposed dentine surface, and it has successfully been proven by several authors (19).

The female samples were categorized into six age groups: 10 to 19, 20 to 29, 30 to 39, 40 to 49, 50 to 59, and 60 to 69 years, sourced from different individuals, representing various teeth. The male group was grouped into five age groups because there were insufficient specimens aged from twenty to twenty-nine years of age. Male specimens were, as well, sourced from different individuals and representing various

očišćeni od krvi i ostataka mekog tkiva te dezinficirani otopinom vodikova peroksida ($H_2O_2$) u koncentraciji od 2 %. Nakon sušenja na sobnoj temperaturi (slika 1.) pohranjeni su u hermetičkim posudama u arhivi zavoda. Pri analizi istraživanja zubi su ugrađeni u brzo stvrdnuti akrilat (Presi, Eybens, Francuska) (slika 2.) i precizno rezani reznim alatom Isomet 1000 (Buehler, Uzwill, Švicarska) blizu cervikalnog ruba (slike. 3 i 4.) otprilike 1 mm prema vrhu (slika 5,). Zatim su uzorci rezani horizontalno i polirana im je površina brusnim papirom p1200 (Buehler, Uzwill, Švicarska) i Phoenix Beta (Buehler, Uzwill, Švicarska) (slika 6.). Taj postupak osigurao je uspostavu ravnomjerno izložene površine dentina, što je uspješno dokazalo nekoliko autora (19). Različiti ženski uzorci kategorizirani su u šest dobnih skupina: 10 do 19 godina, 20 do 29, 30 do 39, 40 do 49, 50 do 59 i 60 do 69 godina, a dobiveni su od različitih pojedinki. Muška skupina bila je grupirana u pet dobnih skupina jer nije bilo dovoljno uzoraka za dob od 20 do 29 godina. Muški uzorci također su različiti i potječu od različitih pojedinaca. Detaljne karakteristike svih uzoraka navedene su u tablicama 1.1. i 1.2. Analiza Ramanove spektrometrije obavljena je na Katedri za fiziku Medicinskog fakulteta Sveučilišta u Zagrebu. Nakon toga uzorci su analizirani u Ramanovu spektrometru FT-Raman sa spektrometarskim dodatkom Spectrum GX (Perkin Elmer, Waltham, MA, SAD) koji sadržava neodimijski doped alu-

**Table 1.1** Characteristics of samples, females.
**Tablica 1.1.** Karakteristike uzoraka – žene

| Specimen code • Specifikacijski kod | Year of Birth • Godina rođenja | Year of extraction • Godina ekstrakcije | Tooth type* • Vrsta zuba* | Age at extraction • Dob pri ekstrakciji |
|---|---|---|---|---|
| LB160219Z.sp | 2002 | 2019 | 3 | 17 |
| LB20920Z.sp | 2009 | 2020 | 3 | 11 |
| LB210521Z.sp | 2005 | 2021 | 3 | 16 |
| LB60520Z.sp | 2005 | 2020 | 3 | 15 |
| LB209620Z.sp | 1996 | 2020 | 4 | 24 |
| LB219520Z.sp | 1995 | 2020 | 4 | 25 |
| LB229120Z.sp | 1991 | 2020 | 4 | 29 |
| LB389620ZV2.sp | 1996 | 2020 | 4 | 24 |
| LB99520Z.sp | 1995 | 2020 | 4 | 25 |
| LB139020Z.sp | 1990 | 2020 | 4 | 30 |
| LB81187Z.sp | 1981 | 2018 | 4 | 37 |
| LB832020Z.sp | 1983 | 2020 | 3 | 37 |
| LB83205Z.sp | 1983 | 2020 | 3 | 37 |
| LB862112Z.sp | 1986 | 2021 | 4 | 35 |
| LB167120Z.sp | 1971 | 2020 | 4 | 49 |
| LB177621Z.sp | 1976 | 2021 | 1 | 45 |
| LB317620Z.sp | 1976 | 2020 | 4 | 44 |
| LB3046420Z.sp | 1964 | 2020 | 4 | 56 |
| LB317020Z.sp | 1970 | 2020 | 2 | 50 |
| LB62205Z.sp | 1962 | 2020 | 1 | 58 |
| LB664189Z.sp | 1964 | 2018 | 3 | 54 |
| LB125819Z.sp | 1958 | 2019 | 2 | 61 |
| LB236020Z.sp | 1960 | 2020 | 4 | 60 |
| LB35420Z.sp | 1954 | 2020 | 4 | 66 |
| LB45518Z.sp | 1955 | 2018 | 1 | 63 |
| LB55819Z.sp | 1958 | 2019 | 2 | 61 |

* Incisor 1, Canine 2, Premolar 3, Molar 4. • * sjekutić 1, očnjak 2, pretkutnjak 3, kutnjak 4

teeth. Detailed characteristics of all samples are provided in Tables 1.1 and 1.2.

Raman spectrometry analysis has been conducted in the Department of Physics, School of Medicine, University of Zagreb. Subsequently, the samples underwent analysis in the Raman spectrometer FT-Raman accessory of the Spectrum GX spectrometer (Perkin Elmer, Waltham, MA, USA), featuring a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser with a wavelength of 1064 nm (Figure 7). The red laser pointer integrated into the Raman spectrometer was employed to meticulously identify the location of exposed dentine, ensuring that only dentine tissue was within the sampling area. To facilitate this process, a specialized fixation tool was utilized, and sampling was conducted using Raman spectrometry (Figure 8).

Each spectrum was precisely recorded by averaging 100 scans within the spectral range from 3500 to 200 $cm^{-1}$, with a spectral resolution of 4 $cm^{-1}$. Subsequently, all spectra underwent baseline correction and normalization, utilizing the peak at 960 $cm^{-1}$ (symmetric phosphate ion [$PO_4$] stretching) to eliminate potential variations arising from recording conditions. The entire Raman spectra (3500 to 200 $cm^{-1}$) were employed for principal component analysis (PCA) and principal component regression (PCR), with three principal components.

To assess the efficacy of PCA in segregating spectra based on the age of the tooth donor, the spectra were categorized

minijev granat (Nd: YAG), laser s valnom duljinom od 1064 nm (slika 7.). Crveni laserski pokazivač integriran u Ramanov spektrometar koristio se za pažljivo određivanje položaja izloženog dentina i osiguravao je da se dentinsko tkivo nalazi unutar područja uzorkovanja. Da bi se olakšao taj postupak, upotrijebljen je specijalni alat za fiksiranje, a uzorci su obrađeni s pomoću Ramanove spektrometrije (slika 8.). Svaki spektar precizno je snimljen – obavljeno je prosječno 100 skeniranja unutar spektralnog raspona od 3500 do 200 $cm^{-1}$, sa spektralnom razlučivošću od 4 $cm^{-1}$. Zatim su svi spektri podvrgnuti korekciji pozadine i normalizaciji, koristeći vrh na 960 $cm^{-1}$ [simetrično istezanje fosfatnog iona (PO4)] da bi se eliminirale potencijalne varijacije koje nastaju zbog uvjeta snimanja. Cijeli Ramanov spektar (3500 do 200 $cm^{-1}$) korišten je za analizu glavnih komponenti (PCA) i regresiju glavnih komponenti (PCR) s trima glavnim komponentama. Da bi se procijenila učinkovitost PCA-e u segregaciji spektara na temelju dobi donatora zuba, spektri su kategorizirani u pet dobnih skupina. Ta kategorizacija koristila se za bojenje rezultata PCR-a u grafikonima, pružajući vizualnu reprezentaciju odvajanja spektara na temelju dobi donatora. PCR je zatim korišten za uspostavljanje odnosa između zabilježenih spektara i dobi donatora. Cijeli proces PCA-e i PCR-a proveden je s pomoću modela stvorenoga unutar MATLAB-a 2010 (The MathWorks, Natick, MA, SAD) zajedno s njegovim dodatkom PLS_Toolbox (Eigenvector Research, Wenatchee, WA, SAD). Napredna tehnika pretproce-

**Table 1.2** Characteristics of samples, males.
**Tablica 1.2.** Karakteristike uzoraka – muškarci

| Specimen code • Specifikacijski kod | Year of Birth • Godina rođenja | Year of extraction • Godina ekstrakcije | Tooth type* • Vrsta zuba* | Age at extraction • Dob pri ekstrakciji |
|---|---|---|---|---|
| LB150318M.sp | 2003 | 2018 | 3 | 15 |
| LB80820M.sp | 2008 | 2020 | 3 | 12 |
| LB002017M.sp | 2000 | 2020 | 3 | 20 |
| LB942032M.sp | 1994 | 2020 | 3 | 26 |
| LB992134M.sp | 1999 | 2021 | 4 | 22 |
| LB168420M.sp | 1984 | 2020 | 4 | 36 |
| LB38720M.sp | 1987 | 2020 | 4 | 33 |
| LB68220M.sp | 1982 | 2020 | 4 | 38 |
| LB98518M.sp | 1985 | 2018 | 4 | 33 |
| LB127620M.sp | 1976 | 2020 | 4 | 44 |
| LB257721M.sp | 1977 | 2021 | 4 | 44 |
| LB298121M.sp | 1981 | 2021 | 1 | 40 |
| LB67119M.sp | 1971 | 2019 | 3 | 48 |
| LB116518M.sp | 1965 | 2018 | 3 | 53 |
| LB236121M.sp | 1961 | 2021 | 4 | 60 |
| LB286320M.sp | 1963 | 2020 | 4 | 57 |
| LB329420M.sp | 1995 | 2020 | 4 | 25 |
| LB377021M.sp | 1970 | 2021 | 1 | 51 |
| LB641917M.sp | 1964 | 2019 | 1 | 55 |
| LB672012M.sp | 1967 | 2020 | 3 | 53 |
| LB682015M.sp | 1968 | 2020 | 3 | 52 |
| LB702135M.sp | 1970 | 2021 | 3 | 51 |
| LB16020M.sp | 1960 | 2020 | 2 | 60 |
| LB215920M.sp | 1959 | 2020 | 4 | 61 |
| LB25419M.sp | 1954 | 2019 | 3 | 65 |

* Incisor 1, Canine 2, Premolar 3, Molar 4. • * sjekutić 1, očnjak 2, pretkutnjak 3, kutnjak 4

into five age groups. This categorization was utilized to colour-code the data in PCR score plots, providing a visual representation of the separation of spectra based on the donor's age. PCR was then employed to establish a relationship between the recorded spectra and the donor's age.

The entire process of PCA and PCR was performed using a model created within MATLAB 2010 (The MathWorks, Natick, MA, USA) along with its PLS_Toolbox add-on (Eigenvector Research, Wenatchee, WA, USA). An advanced preprocessing technique, generalized least squares weighting, was applied before the PCA modelling to eliminate interference from compounds without sacrificing relevant ageing-related variability within the data (20–23).

Distinct PCA/PCR models were constructed based on the spectra collection site and the donor's sex, resulting in combinations: dentin male and dentin female. All models underwent a cross-validation using the Venetian blind method.

An examination of the entire spectrum was conducted in the dentine area of the tooth. As a control measure, an analysis of the acrylate spectrum was carried out to mitigate the possibility of contamination in the recordings.

siranja, ponderiranje generaliziranim najmanjim kvadratima, primijenjena je prije modeliranja PCA-e kako bi se eliminiralo ometanje spojeva bez žrtvovanja relevantne varijabilnosti vezane za starenje unutar podataka (20 – 23). Izrađeni su posebni modeli PCA/PCR-a na temelju mjesta prikupljanja spektara i spola donatora, rezultirajući kombinacijama: dentin muškarci i dentin žene. Svim modelima provjerena je valjanost metodom Venetian blind. Obavljeno je ispitivanje cijelog spektra u području dentina zuba. Kao kontrolna mjera poslužila je analiza spektra akrilata kako bi se umanjila mogućnost kontaminacije snimki.

## Results

In the female sample group, a clear segmentation of spectra based on the age of teeth at the time of extraction is apparent, with an accuracy of age estimation at 9.3863 years (R2: 0.749). (Figure 1) Similarly, in the male sample group, the segmentation of spectra based on the age of teeth at the time of extraction is visible, with better accuracy of age estimation at 7.0048 years (R2: 0.861) (Figure 2). Furthermore, a notable distinction of biological sex was observed when comparing the spectra of both male and female samples after analysing the spectrum differences.

Figure 3 shows the mean Raman spectra of male and female teeth, the loadings of the first principal component, and the spectrum of the difference between the male and female mean Raman spectra. The main differences can be found at vibrational bands: 964 $cm^{-1}$, 1006 $cm^{-1}$, 1453 $cm^{-1}$, 1730 $cm^{-1}$, 2881 $cm^{-1}$, 2925 $cm^{-1}$, 2977 $cm^{-1}$, 597 $cm^{-1}$ and 3063 $cm^{-1}$. Table 2 lists the assignments of vibrational bands that can be found in the Raman spectra of teeth that have been assigned to changes in collagen (24). Figures 4 and 5 show the schematic differentiation obtained by PCA/PCR processes. While this cannot offer a numeric accuracy, separation of the scores belonging to the spectra of male and female teeth clearly show there is a correlation between the teeth and biological sex.

## Rezultati

U skupini uzorka uzetih od ženskih osoba, jasna je segmentacija spektara temeljena na dobi zuba u vrijeme vađenja s točnošću procjene starosti od 9,3863 godina (R2: 0,749) (slika 1.). Slično tomu, u skupini uzorka uzetih od muških osoba, vidljiva segmentacija spektara temeljena je na dobi zuba u vrijeme vađenja, s boljom točnošću procjene starosti od 7,0048 godina (R2: 0,861) (slika 2.). Nadalje, značajna je razlika u biološkom spolu pri usporedbi spektara muških i ženskih uzoraka poslije analize razlika u spektru.

Na slici 3. su srednje vrijednosti Ramanova spektara muških i ženskih zuba, opterećenja prvoga glavnog komponenta i spektar razlike između srednjih vrijednosti muških i ženskih zuba. Glavne razlike mogu se pronaći na vibracijskim trakama: 964 $cm^{-1}$, 1006 $cm^{-1}$, 1453 $cm^{-1}$, 1730 $cm^{-1}$, 2881 $cm^{-1}$, 2925 $cm^{-1}$, 2977 $cm^{-1}$, 597 $cm^{-1}$ i 3063 $cm^{-1}$. U tablici 2. navedene su dodijeljene vibracijske trake koje se mogu pronaći u Ramanovu spektru zuba, a povezane su s promjenama u kolagenu (24). Na slikama 4. i 5. nalaze se shematske diferencijacije dobivene postupcima PCA/PCR-a. Iako to ne može pružiti numeričku točnost, razdvajanje bodova koji pripadaju spektru muških i ženskih zuba jasno pokazuje da postoji korelacija između zuba i biološkog spola.

**Table 2** Assignments of vibrational bands (25).
**Tablica 2.** Pridruživanje vibracijskim trakama (25)

| Raman Band ($cm^{-1}$) • Raman traka ($cm^{-1}$) | Assignment • Pridruživanje |
|---|---|
| 817, 853, 920 | Collagen • Kolagen |
| 960 | v1 of $PO_4^{3-}$ |
| 1005 | Collagen (Phe) • Kolagen (Phe) |
| 1070 | v1 of $CO_3^{2-}$ |
| 1248, 1273 | Collagen (amide III) • Kolagen (amid III) |
| 1450 | Collagen ($CH_2$) • Kolagen ($CH_2$) |
| 1670 | Collagen (amide I) • Kolagen (amid I) |

## Discussion

Raman spectrometry has confirmed its value in the field of forensic dental medicine. It is a very convenient method of chemical analysis of tissues as it can be done both on live and "ex vivo" specimens (26). Namely, Raman spectroscopy can collect information on specimens in their natural state before any additional chemical pre-treatment. The only mechanical manipulation of the specimens is cutting in order to expose the dentin area that has been preserved from extrinsic factors. On the other hand, it has been proven that blood contamination can influence the results, therefore this is a needed prerequisite (27). According to Gremlich et al. the samples that have been prepared for different examination methods, i.e. optical microscopy and are protected using coverslips from glass, are suitable as well (28). This serves in favor of the claimed variability of the method. Our specimens are also bound to be re-purposed in further research that will examine cement thickness using optical magnification and digital measurement. Since we are dealing with human remains, it is an imperative that in our dental research we take into consideration that every sample we use is handled in a minimally invasive manner and, whenever possible, we strive to achieve the ability to repurpose samples. The importance of this as well as some guidelines in sample management has been reviewed by Nawrocka and Lukomska-Symanska in 2019 (29).

Tramini et al. utilized Raman spectrometry, examining different positions of longitudinally cut teeth to establish a correlation between age and specific wavelengths. Furthermore, they recorded multiple areas of dentine (4). To streamline the age estimation process and enhance its applicability within the broader field, in this research, the number of readings from a single horizontally cut dentine location was increased using a precision saw.

Kumari et al. examined permanent extracted tooth samples (40) ranging from 12 to 74 years old. The teeth were collected, sectioned longitudinally, and subjected to Raman spectroscopy analysis across various dentine and cementum areas. The search for correlation coefficient ratios exceeding 0.40 resulted in the selection of ratios specifically for dentine areas, thus enabling the development of a regression formula (18).

In this study, the analysis of dentin by Raman spectroscopy showed a correlation between the developmental stages of dentin with increasing age and the variability of Raman spectra. These results are in agreement with the results of Tramini et al. for the dentin analysis part. Tramini et al. additionally analysed cementum and it was found that cementum spectra from incremental lines of cementum did not act as a good predictor of age estimation (4).

Consistent with this approach, our focus has been directed exclusively toward dentine areas, which have proven to be the optimal sites in hard dental tissues for Raman spectrometry analysis. Notably, tooth cementum was identified by Birimisa et al. in 2021 as the tooth structure of utmost importance in age estimation, particularly through tissue thickness measurement (30). However, it was based on a different age estimation method, leading us to avoid choosing cement over dentine. As mentioned previously in this paper while se-

## Rasprava

Ramanova spektrometrija potvrdila je svoju vrijednost u području forenzičke dentalne medicine. To je vrlo prikladna metoda kemijske analize tkiva zato što se može primijeniti kako na živim tako i na uzorcima *ex vivo* (26). Naime, Ramanovom spektroskopijom mogu se prikupiti informacije o uzorcima u njihovu prirodnom stanju prije bilo kakvog dodatnog kemijskog tretmana. Jedina mehanička manipulacija s uzorcima jest rezanje radi izlaganja područja dentina koje je sačuvano od utjecaja vanjskih čimbenika. S druge strane, dokazano je da kontaminacija krvi može utjecati na rezultate, pa je to nužan preduvjet (27). Prema Gremlichu i suradnicima, uzorci pripremljeni za različite metode ispitivanja, na primjer za optičku mikroskopiju, i zaštićeni pokrovima također su prikladni (28). To ide u prilog tvrdnji o varijabilnosti metode. Naši uzorci također su predviđeni za ponovnu upotrebu u daljnjim istraživanjima kada će se proučavati debljina cementa s pomoću optičkog povećanja i digitalnog mjerenja. S obzirom na to da se bavimo ljudskim ostatcima, imperativ je u dentalnom istraživanju uzeti u obzir da se svaki uzorak kojim se koristimo obradi minimalno invazivno, stoga, kad god je moguće, nastojimo da se može ponovno upotrijebiti. Važnost toga, kao i neke smjernice u upravljanju uzorcima, pregledale su 2019. godine Nawrocka i Lukomska-Symanska (29).

Tramini i suradnici koristili su se Ramanovom spektrometrijom pri ispitivanju različitih položaja uzdužno rezanih zuba da bi uspostavili korelaciju između dobi i određenih valnih duljina. Uz to, zabilježili su više područja dentina (4). Kako bismo pojednostavili postupak procjene dobi i poboljšali njegovu primjenjivost u širem području, u ovom istraživanju povećan je broj mjerenja s jednoga horizontalno rezanog mjesta dentina koristeći se preciznom pilom.

Kumari i suradnici ispitivali su uzorke izvađenih zuba (40) od osoba u dobi od 12 do 74 godine. Zubi su prikupljeni, rezani uzdužno i podvrgnuti analizi Ramanovom spektroskopijom na različitim mjestima dentina i cementa. Pretraživanje omjera koeficijenata korelacije većih od 0,40 rezultiralo je odabirom omjera specifično za područja dentina, omogućujući razvoj regresijske formule (18).

U ovom istraživanju analiza dentina Ramanovom spektroskopijom pokazala je korelaciju između razvojnih stadija dentina s povećanjem dobi i varijabilnosti Ramanova spektra. Ti rezultati slažu se s rezultatima Traminija i suradnika za dio analize dentina. Taj tim znanstvenika dodatno je analizirao i cement te zaključio da spektri cementa iz inkrementalnih linija cementa nisu dobar prediktor procjene dobi (4).

U skladu s tim pristupom, usredotočili smo se isključivo na područja dentina koja su se pokazala optimalnim lokacijama u tvrdim dentalnim tkivima za analizu Ramanovom spektrometrijom. Istaknimo da su zubni cement 2021. godine identificirali Birimise i suradnici kao najvažniju strukturu zuba za procjenu dobi, posebno mjerenjem debljine tkiva (30), ali temeljila se na drukčijoj metodi procjene dobi, što nas je navelo na to da ne odaberemo cement umjesto dentina. Kao što je već navedeno u ovom radu, pri odabiru područja za snimanje spektralnih valnih duljina koristili smo se cervikalnim dijelom korijena. To smo učinili da bismo izbjegli eventualne lecting the area for spectroscopic wavelength recording, we used a cervical part of the root. This was done to avoid possible extrinsic factors from possible endodontic treatment of the tooth, as certain authors have found it to be relevant. Amonkar et al. analysed the dentin after usage of intracanal medicines and concluded that certain medications significantly affect the microhardness and fracture resistance of radicular dentin (31). Furthermore, Çiftçioğlu et al. performed the laser-assisted dentin conditioning and observed some changes in Push bond strength in endodontic canals, which is different from solely using Ethylenediaminetetraacetic acid (EDTA) and Sodium hypochlorite (NaOCl) (32). This led us to the conclusion that endodontic treatment would affect the spectrometric footprint of our samples, hence all available specimens of endodontically treated teeth were avoided.

The application of mathematical and statistical techniques to better understand chemical information is called chemometrics. It aids to correlate physical properties with data from analytical instruments. Numerous chemometric analyses are available for processing spectroscopy data such as partial least square regression (PLS), pattern recognition, principle component analysis (PCA), and clustering (27). The hydroxyapatite crystals of enamel and dentin were studied by Raman spectroscopy (33). A change in the intensity of the phosphate band ($PO_4^{3-}$) is linked to the dentin type, its anatomical placement, and the individual's age. Although there are many studies on age estimation using teeth, spectroscopic analysis of dentin and cementum for age estimation has not been widely researched (34–36).

Interestingly enough, Kumari K (17) et al. discovered that age group specimens from 10 to 12 years and from 20 to 29 years predicted age more accurately compared with the 60- to 69-year-old age group.

Finally, in this study, we observed some differences in accuracy between male and female specimens.

Distinctive traits in teeth, such as morphology, crown dimensions, and root lengths, exhibit characteristic differences between males and females (37). Sex identification in dental profiling relies on assessing tooth size, as well as congenital and acquired morphological features of the teeth and their placement. It is established that men on average exhibit larger teeth in larger jaws, particularly noticeable in the second molar and canine. Additionally, men tend to have a minimal difference in mesiodistal diameters between the central and lateral incisors. Women generally have smaller teeth in smaller jaws, featuring prominent second molars and canines, and a substantial difference in mesiodistal diameters between central and lateral incisors (38). These variations may stem from general physiological differences between men and women, including variations in hormones, lifestyle habits, and other factors. Such distinctions can also influence differences in dentin composition (39). This study offers valuable insights into methods for forensic and anthropological applications, expanding our understanding of the influence of physiological factors on spectral analysis in these contexts. Further expansion of the number of specimens may offer an even more precise insight into how to estimate both chronological age and biological sex from Raman spectra recorded from preserved dentine sites of extracted teeth.

vanjske čimbenike koji proizlaze iz mogućega endodontskog liječenja zuba, kako su to neki autori smatrali relevantnim. Amonkar i suradnici analizirali su dentin poslije primjene intrakanalnih lijekova i zaključili da određeni lijekovi znatno utječu na mikrotvrdoću i otpornost na lom korijenskog dentina (31). Osim toga, Çiftçioğlu i suradnici proveli su laserski potpomognuto kondicioniranje dentina i uočili promjene u snazi povezivanja *push*-testa u endodontskim kanalima, za razliku od korištenja samo etilendiamintetraoctene kiseline (EDTA) i natrijeva hipoklorita (NaOCl) (32). Na temelju toga zaključili smo da bi endodontski tretman utjecao na spektrometrijski otisak naših uzoraka pa smo izbjegavali sve dostupne primjerke endodontski tretiranih zuba.

Primjena matematičkih i statističkih tehnika za bolje razumijevanje kemijskih informacija naziva se kemometrija. Pomaže u povezivanju fizikalnih svojstava s podatcima iz analitičkih instrumenata. Postoji mnogo kemometrijskih analiza za obradu podataka spektroskopije, na primjer, parcijalna regresija najmanjih kvadrata (PLS), prepoznavanje obrazaca, analiza glavnih komponenti (PCA) i klasteriranje (27). Kristali hidroksiapatita cakline i dentina proučavani su Ramanovom spektroskopijom (33). Promjena u intenzitetu fosfatne vrpce ($PO_4^{3-}$) povezana je s vrstom dentina, njegovim anatomskim položajem i dobi osobe. Iako postoji mnogo studija o procjeni dobi s pomoću zuba, spektroskopska analiza dentina i cementa za procjenu dobi nije detaljno istražena (34 – 36).

Zanimljivo je da su Kumari (17) i suradnici otkrili da se uzorcima iz dobne skupine od 10 do 12 godina i od 20 do 29 godina preciznije određuje dob u usporedbi s dobnom skupinom od 60 do 69 godina.

Na kraju, u ovom istraživanju uočili smo razlike u točnosti između muških i ženskih uzoraka. Karakteristična svojstva zuba, poput morfologije, dimenzija krune i duljine korijena, pokazuju razlike između muškaraca i žena (37). Identifikacija spola u dentalnom profiliranju temelji se na procjeni veličine zuba i prirođenih i stečenih morfoloških obilježja te njihova položaja. Ustanovljeno je da muškarci u prosjeku imaju veće zube u većim čeljustima, a posebno je to primjetno kod drugog kutnjaka i očnjaka. Uz to, muškarci imaju minimalnu razliku u meziodistalnim promjerima između središnjih i bočnih sjekutića. Žene općenito imaju manje zube u manjim čeljustima, ističu se prominentnim drugim kutnjacima i očnjacima, te značajnom razlikom u meziodistalnim promjerima između središnjih i bočnih sjekutića (38). Te razlike mogu nastati zbog općih fizioloških razlika između muškaraca i žena, uključujući varijacije u hormonima, navikama i drugim čimbenicima. Također mogu utjecati na razlike u sastavu dentina (39). Ovo istraživanje omogućuje vrijedne uvide u metode za forenzičke i antropološke primjene te proširuje naše razumijevanje utjecaja fizioloških čimbenika na spektralnu analizu u tim kontekstima. Daljnje povećanje broja uzoraka, snimljenih na očuvanim mjestima dentina ekstrahiranih zuba, može zahvaljujući Ramanovu spektru pružiti još precizniji uvid u procjenu kako kronološke dobi, tako i biološkog spola.

## Conclusions

The organized datasets of recorded spectra were subjected to principal component analysis, revealing the potential of Raman spectra from the teeth for sex determination and age estimation. The correlation between Raman spectra readings and the chronological age of subjects was high, which confirmed our hypothesis. Also, a correlation between Raman spectra readings and biological sex is present. Differences in classification accuracy between biological sexes were attributed to hormonally-mediated differences in the biochemical composition of dentine between males and females. In order to achieve more precise results, a larger amount of specimens is needed. The samples should be distributed evenly in all age groups, tooth types, and they should include both sexes.

## Zaključak

Organizirani skupovi podataka snimljenih spektara podvrgnuti su analizi glavnih komponenti, otkrivajući potencijal Ramanova spektra zuba za određivanje spola i procjenu dobi. Zabilježena je korelacija između očitanja Ramanova spektra i kronološke dobi ispitanika. Također je zabilježena korelacija između očitanja Ramanova spektra i biološkog spola. Razlike u točnosti klasifikacije između bioloških spolova pripisane su hormonski posredovanim razlikama u biokemijskom sastavu dentina između muškaraca i žena.

## Conflict of interest

Authors declare no conflict of interest.

## Sukob interesa

Autori nisu bili u sukoba interesa.

## Institutional Review Board Statement:

The study was conducted in accordance with the Declaration of Helsinki. This research was approved by the Ethics Committee of the School of Dental Medicine, the University of Zagreb, at the 18th regular session held on June 4th 2020, decision number 05-PA-30-XVIII-6/2020.

## Izjava institucionalnog odbora za etiku

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**Author's contribution:** **L.B.** and **O.G.** – conceived the study and were in charge of overall direction and planning; performed the experiment and analyzed the data; **O.G.** – supervised the work; **L.B.** – wrote the manuscript; **L.B.** and **M.B.** contributed to the collection of specimens. The final manuscript underwent revisions and received approval from all authors.

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**Sažetak**
**Ciljevi:** Ramanovim spektrom željelo se odrediti spol i procijeniti starost pojedinaca analizom dentina iz izvađenih ljudskih zuba. **Materijal i metode:** Prikupljeno je ukupno 25 izvađenih zuba muškaraca i 26 od žena te su dezinficirani i fiksirani u autoakrilatu prije rezanja blizu cervikalnog ruba. Kriteriji za uključivanje bili su zubi koji nisu endodontski tretirani i s očuvanom cervikalnom trećinom krune. Izloženi dentin snimljen je Ramanovim spektrometrom – učinjeno je 100 skeniranja po mjestu dentina u spektralnom rasponu od 3500 do 200 $cm^{-1}$ i razlučivošću od 4 $cm^{-1}$. Spektri s korekcijom pozadine podvrgnuti su analizi glavnih komponenti s pomoću MATLAB-a 2010 (The MathWorks, Natick, MA, SAD). Da bi se procijenile razlike u dobi, promatrane su i statistički analizirane vibracijske trake, a za diferencijaciju spola koristio se t-test na prikupljenim podatcima, istražujući vibracijske trake sa značajnim razlikama u intenzitetu. **Rezultati:** Rezultati su pokazali segmentaciju spektara u skupini s uzorcima zuba muškaraca na temelju dobi pri vađenju, s točnošću procjene starosti od 7,0048 godina. Slična segmentacija uočena je u skupini s uzorcima zuba žena, s točnošću od 9,3863 godina. **Zaključak:** Organizirani skupovi podataka snimljenih spektara podvrgnuti su analizi glavnih komponenti. Zabilježena je korelacija između očitanja Ramanova spektra i kronološke dobi ispitanika. Također je zabilježena korelacija između očitanja Ramanova spektra i biološkog spola. Razlike u točnosti klasifikacije između bioloških spolova pripisane su hormonski posredovanim razlikama u biokemijskom sastavu dentina između muškaraca i žena.

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