

Raman studies on amino acids profiles at the dentin-enamel-junction in human and ancient/recent animal teeth

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Abstract

Raman microscopy enables the delimitation of the dentin-enamel junction (DEJ) in human and animal teeth, by using, e.g., the ν_1 oscillations of PO_4^{3-} (960 cm^{-1}) for the boundary with the enamel and of stretching modes for C-H_2 ($\sim 2935\text{ cm}^{-1}$) for the boundary with the dentin and it can be strictly compared with the optical image of DEJ. In this way, one can observe the distribution of some elements of collagen fibrils crossing DEJ, by using the oscillations for proline ($\nu(\text{C-C})$ of ring at 855 and 921 cm^{-1}), for hydroxyproline ($\nu(\text{C-C})$ of ring at 876 cm^{-1}), and for phenylalanine ($\nu(\text{C-C})$ of ring $\sim 1003\text{ cm}^{-1}$). The systematic although not identical drop in the concentrations of these amino acids was observed on passing from the dentin to enamel side. Several other oscillations were traced as well (for organic chain fragments, amide groups). The observation was widened on the zones adhering on the enamel side (up to $15\text{ }\mu\text{m}$) and on the dentin side ($15\text{ }\mu\text{m}$ outside the boundary) to the DEJ. The investigations suggest the reorganization of the organic matter in these zones.

Keywords: raman microscopy; dentin-enamel junction; amino acids profiles

Introduction

The dental-enamel-junction (DEJ) has proved to be surprisingly significant although small in size as part of the teeth. It joins two essential components of the tooth – the mesoderm-born dentin and the ectoderm-born enamel, which are very dissimilar materials. Although made of the same inorganic materials – hydroxyapatites, in greater (over 20% for the dentin) or smaller (2% - 20%) for the enamel) quantities, the two parts of the tooth play different roles. The enamel protects the whole tooth body against mechanical and chemical actions. This part is more mechanically stable, harder, more chemically resistant and more mineral in character. It has to cooperate with the softer and more elastic dentin. The phase boundary is a zone which is narrow (up to 30 microns) and ensures the immediate coupling between the parts, in addition to delivering some elasticity to the contact area. This elasticity is extremely important for spreading and potentially stopping cracks which travel from the tooth surface inside the bulk. This kind of efficient and interesting coupling of two dissimilar phases has inspired the biomimetic syntheses of similar materials (1).

One of the more important discoveries was that bundles of collagen I fibrils penetrate the DEJ zone, going from the dentin side and finishing their presence in the first thin layer of the enamel (2). Collagen I is the main organic component of the dentin and it is present within the bundles mentioned inside DEJ. The amino acid composition of the collagen I is well known (the dominance of proline, hydroxyproline and glycine), although there are the controversies as to whether so simple a composition is present in the dentin (3). Nevertheless, the presence of proline, hydroxyproline and possibly phenylalanine gives a unique possibility to trace the collagen components by Raman spectroscopy (4-7), since the amino acids mentioned belong to a small group of amino acids that can be specifically identified. Unfortunately, it has not been undertaken for the DEJ zone. Although many descriptions of DEJ zone have been proposed in recent years (2,8-10), they focused only on optical images in the part concerning the collagen bundles and not their spectrometric determination. Our contribution aims in:

- carrying out the analyses of those amino acids that are possibly present in the collagen fibrils and can be individually observed in the Raman spectra;
- reconstruction of the individual amino acids profiles inside the Dentin-Enamel Junction (DEJ);
- comparison of the presence of amino acids in human and animal teeth both ancient and recent.

Materials and methods

Samples of adult human molars were selected for the study. Thin slices (up to 1 mm wide) were carefully cut out with a diamond saw along the longitudinal cross section of the teeth, in parallel to the jaw direction. After cutting out the upper cover of the slice in the first operation of cutting, the raw

surface was polished with the fine diamond polishing target, then the lower cover was cut out and not polished. The whole slice was washed shortly in distilled water and cleaned with an ultrasonic device to remove the powdered particles from the surface. The samples were photographed and later they were analyzed with a Raman microscope with a movable and computer controlled sample holder.

The same procedure was used for the teeth of contemporary African buffalo from the Republic of South Africa (to provide a comparison with teeth of another mammal) and of extinct shark *Squalicorax pristodontus* from Morocco, from the Thanetian/Ypresian Period (58-48 Mya), for the estimation of archaic nature of that kind of inter-phase junction.

The laser Raman microscope in Via Reflex (produced by Renishaw, UK) was utilized in making the spectral analyses. The instrument was installed at the Chemistry Department of Antwerp University, Belgium. A Leica stereoscopy microscope was attached, enabling a precise optical focus on a selected point of the sample. A laser diode emitting radiation in the IR region (785 nm) was applied for the excitation. The laser power was set on 300 mW. A Renishaw SynchroScan mode from 400 to 3200 cm^{-1} with a spectral resolution of about 2 cm^{-1} was used for making scans. The radiation from the laser was focused on the $\sim 1 \mu\text{m}$ wide locations on the sample. The data were treated initially using the Wire2 computer program. The observation of the sample with an optical Eclipse E400 microscope coupled with a digital camera (a Coolpix 950 by Nikon Europe B.V.) was the basis for the selection of the scan direction. The parallel collection of the image from microscope and relevant chemical data enabled correlation of substances with the topographical details.

Results and discussion

Figure 1 shows consecutively the spatial distributions of proline ($\nu(\text{C-C})$ of ring at 853 and 921 cm^{-1}) (a); hydroxyproline ($\nu(\text{C-C})$ of ring at 879 cm^{-1}) (b) and phenylalanine (~ 1003 and 1032 cm^{-1}) (c). It is showed on a background, formed with the use of ν_1 oscillations of PO_4^{3-} (960 cm^{-1}) and stretching modes for C-H_2 ($\sim 2935 \text{ cm}^{-1}$). The background oscillations delimit the boundaries of DEJ. It is clearly observed that all the amino acid profiles are similar. Still, the hydroxyproline and proline distributions are much more similar to the CH_2 profile inside the DEJ than the phenylalanine distribution is. The CH_2 profile generally reflects the distribution of organic substances (mainly collagen) in the dentin. Hydroxyproline and proline profiles deviate somewhat the CH_2 profile in the bulk dentin, outside the zone of the DEJ. They then deviate alternately. The phenylalanine profile is strictly the same as the organic matter in bulk dentin. Thus, the collagen I inside the DEJ zone is probably enriched in the hydroxyproline and the proline, while the collagen I in the bulk is enriched in the phenylalanine. Figure 1d shows the radical jump in the ratio of proline against hydroxyproline after crossing the wall-like first zone of the enamel.

Figure 2 concerns the analogous distributions of proline (a), hydroxyproline (b) and phenylalanine (c)

for the African buffalo. Both proline and hydroxyproline profiles show the same similarity towards the general distribution of organic matter (collagen) inside DEJ and deviate a little outside. But here, even the deviations, especially in the dentin zone are in pairs. The profile of the phenylalanine is different and more similar to the CH₂ profile outside the zone than inside. Figure 2d shows two jumps in the proline/hydroxyproline ratio, close to the first wall-like layer of the enamel.

For the extinct shark, it was impossible to detect the amino acids mentioned. Nevertheless, not the whole organic structure was not lost. We could detect both stretching modes for C-H₂ (~2935 cm⁻¹), supplementing PO₄³⁻ line 960 cm⁻¹ in delimitation of DEJ and some lines showing the secondary peptide structure – amide I and amide III (as in human and buffalo teeth.)

Figure 3 shows the amide III profile, prepared using the limited zone 1270-1250 cm⁻¹ (corresponding to random coils) and 1250-1220 cm⁻¹ (corresponding to beta-sheets) selected from whole amide III spectral band (1350-1200 cm⁻¹). We avoided using the stronger amide I band due to its similarity with the water vibrational bands (11). The profile is superimposed of the DEJ structure, delimited by PO₄³⁻ and CH₂ profiles. The amide III profiles for human (Figure 3a), buffalo (b) and extinct shark (c) are all similar.

The registered profiles of proline, hydroxyproline and phenylalanine were determined for the collagen fibrils penetrating the DEJ zone. The profiles of proline and hydroxyproline are to a great extent similar to the profile of the organic matter as delivered from the stretching modes for C-H₂ (~2935 cm⁻¹), as it is commonly used in such type of studies. The profile of phenylalanine somewhat deviates from the previously mentioned ones. Moreover, the clear signals from the amino acids mentioned can be traced well inside the first, wall-like layer of the enamel. It is surprising, since in some papers it is announced that the collagen fibrils terminate inside the layer such thin as below 1 µm wide (12). We estimate that the layer is at least 10-15 times wider. In the very end of that layer on the enamel side, the sudden growth in the ratio of the amino acids occurs, with the excess of proline and phenylalanine and deficit of hydroxyproline. Perhaps, it is the chemical reorganization of the collagen fibrils leading to their termination.

Figure 1 DEJ zone in human molar, delimited by the PO_4^{3-} 960 cm^{-1} oscillation (enamel side – solid line with open squares) and C-H 2935 cm^{-1} oscillation (dentin side – solid line with crosses) with superimposed

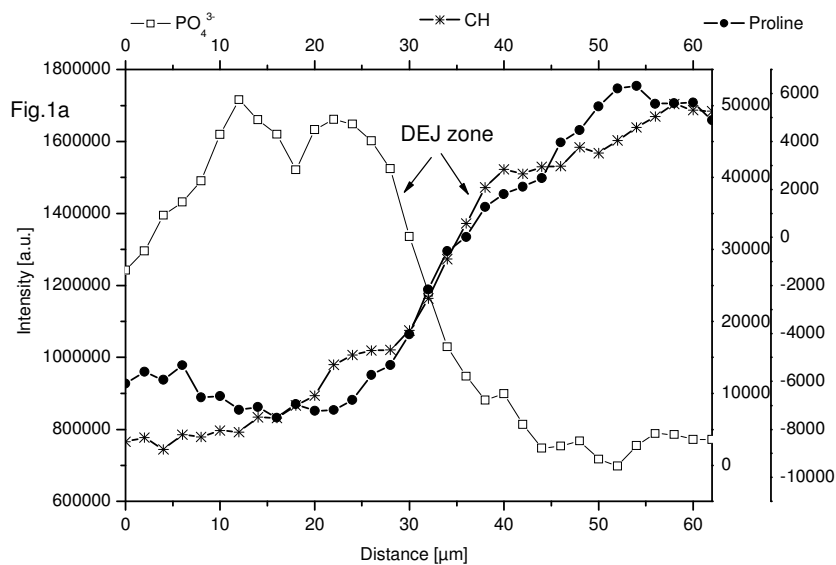


Figure 1A proline attributed oscillation $\nu(\text{C-C})$ of ring at 853 cm^{-1} – solid line with full squares

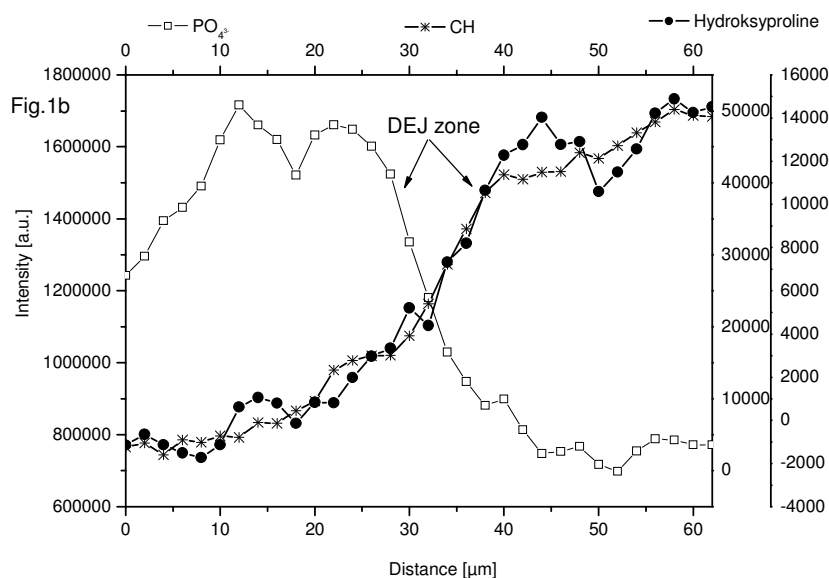


Figure 1B hydroxyproline oscillation $\nu(\text{C-C})$ of ring at 879 cm^{-1} – solid line with full squares

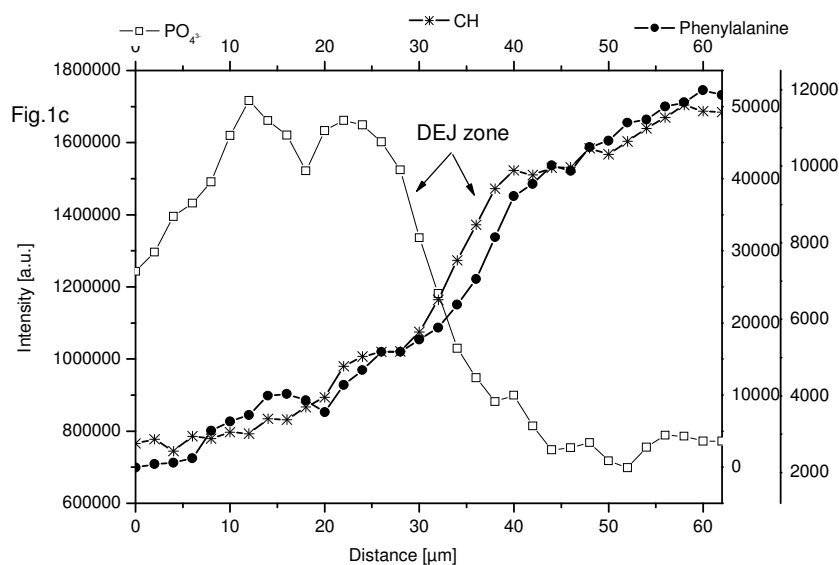


Figure 1C phenylalanine oscillation ($\sim 1003\text{ cm}^{-1}$) - solid line with full squares

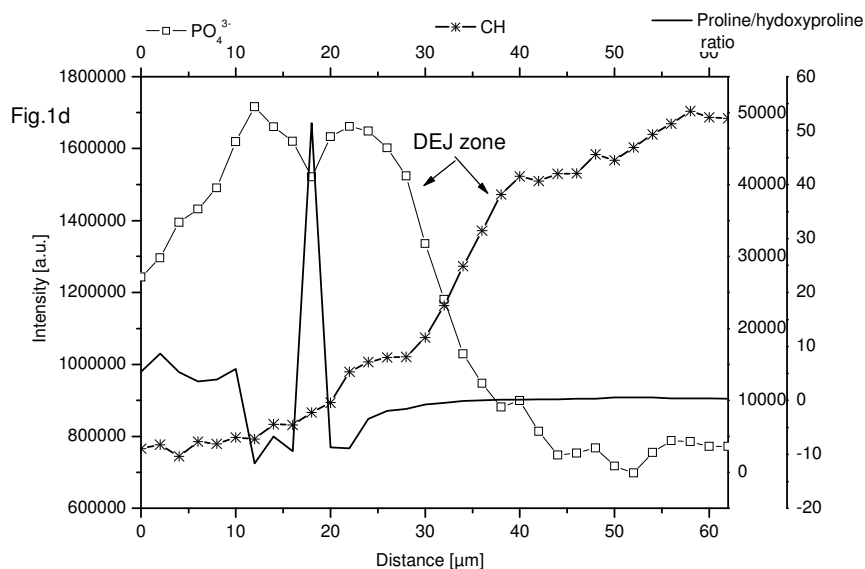


Figure 1D intensity ratio proline/hydroxyproline – dotted line

Figure 2 DEJ zone in African buffalo teeth, delimited by the PO_4^{3-} 960 cm^{-1} oscillation (enamel side – solid line with open squares) and C-H_2 2935 cm^{-1} oscillation (dentin side – solid line with crosses) with superimposed

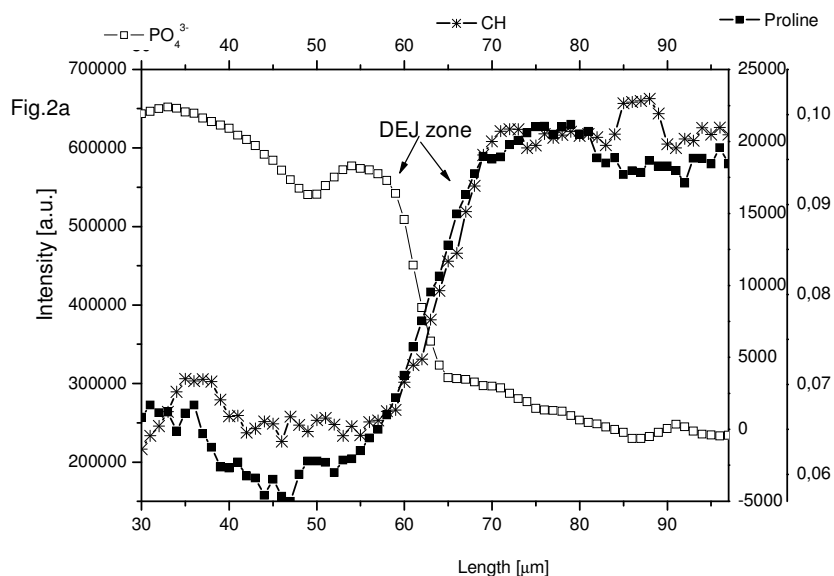


Figure 2 A proline oscillation $\nu(\text{C-C})$ of ring at 853 cm^{-1} – solid line with full squares

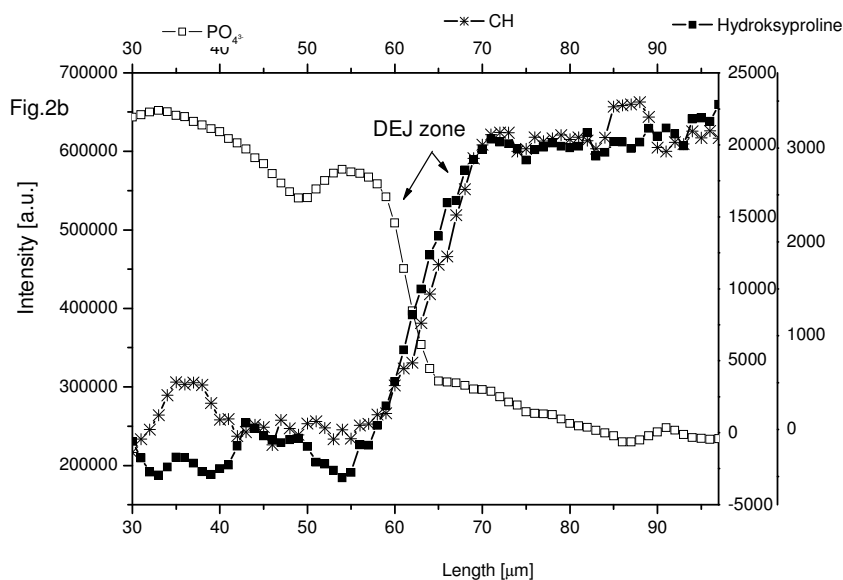


Figure 2 B hydroxypoline oscillation $\nu(\text{C-C})$ of ring at 879 cm^{-1}

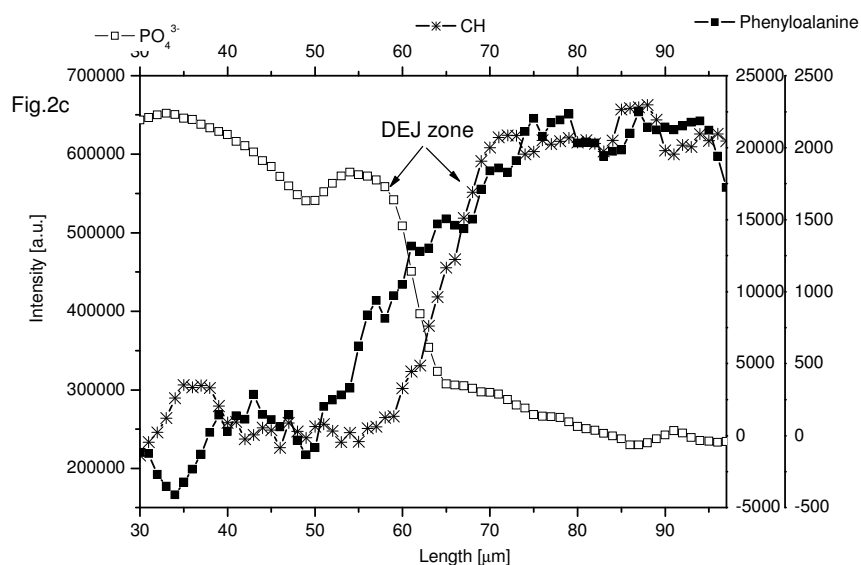


Figure 2 C phenylalanine oscillation ($\sim 1003 \text{ cm}^{-1}$)

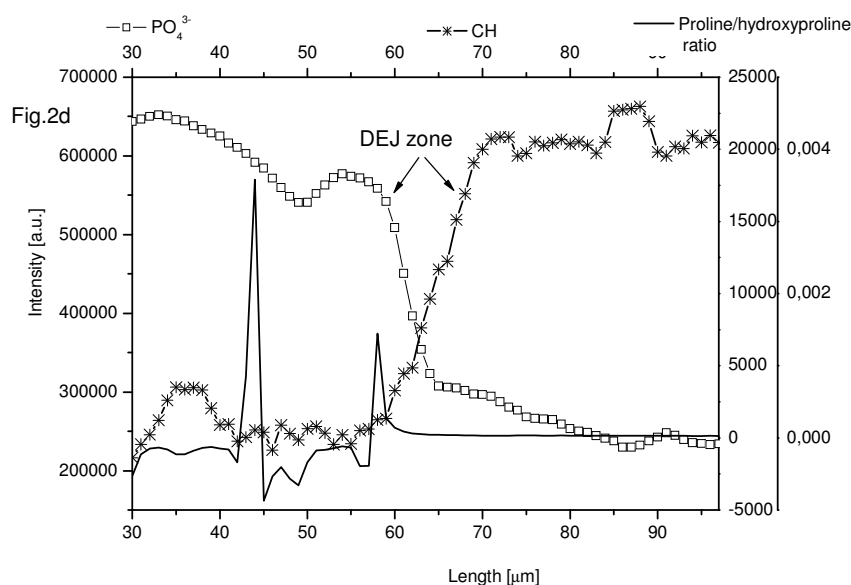


Figure 2 D intensity ratio proline/hydroxyproline dotted line

Figure 3 Profiles of amide III (extracted using bands 1270-1220 cm^{-1}) – solid lines - superimposed on the outline of DEJ zone for a) human molar; b) African buffalo; c) extinct shark *Squalicorax Pristodontus*

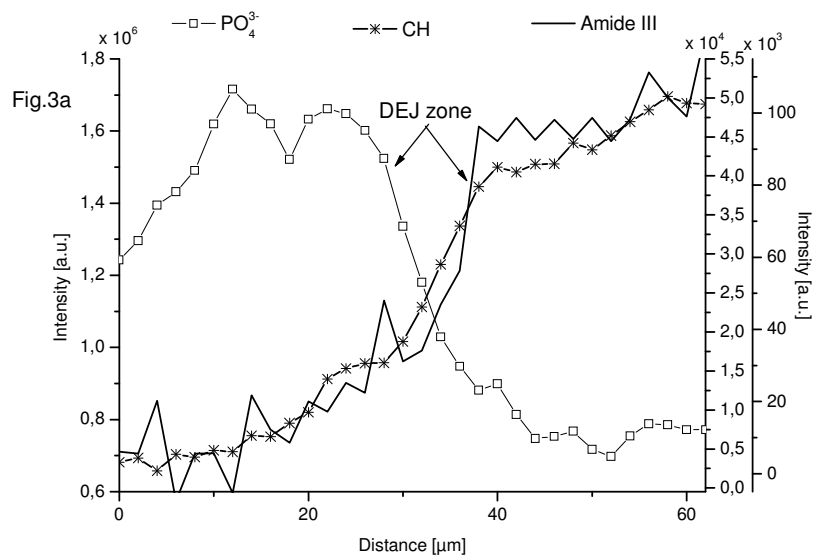


Figure 3 A human molar

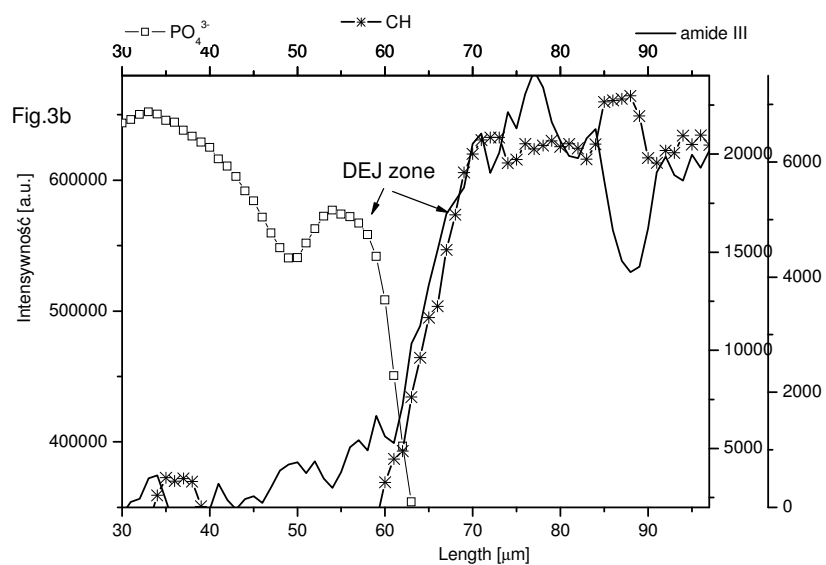


Figure 3 B African buffalo

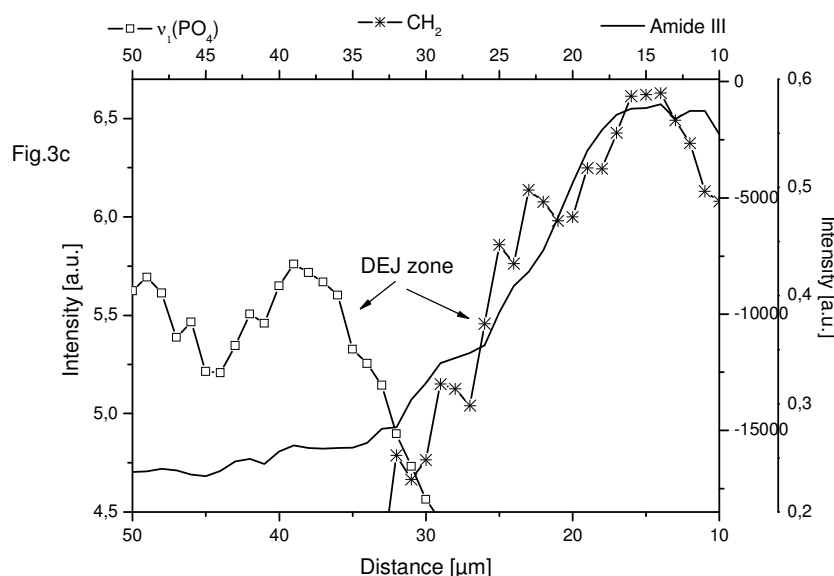


Figure 3 C extinct shark *Squalicorax Pristodontus*

Conclusion

Proline, hydroxyproline, phenylalanine and tyrosine could be detected separately with the Raman microscopy. The possibilities of clear identification of other amino acids with the Raman spectroscopy were limited, if possible at all. The systematic drop in concentration of the detectable amino acids was observed, starting with the dentin towards the enamel location. The drop starts at the same point where the drop in the CH_2 oscillation starts (rather trivial) and stops where the oscillation of PO_4^{3-} terminates in the location of its first maximum on the enamel side. In that way, amino acids from the collagen enter some 10-15 μm inside enamel before their signals become undistinguishable from the background. The slope of the drop is similar in the case of proline and hydroxyproline while less rapid for phenylalanine. Inside the dentine zone, the small differences in amino acids levels can be observed.

The results were repeated for the tooth of the African buffalo, with very similar output. It testifies about the universality of the chemical changes inside DEJ for mammals, apart from whole clear 3D topographic differences between teeth of different species.

The trial of the detection of amino acids inside the DEJ of extinct shark *Squalicorax Pristodontus* failed. This was due to the lack of intensity of the detected amino acid lines. At the same time, it managed to register the CH_2 and amide III lines for all 3 studied species and the results obtained from this were very similar. Probably, in the further studies with a more sensitive Raman probe, longer measurement time and using better background subtraction methods it will be possible to detect the amino acids studied under recent scrutiny also in the shark teeth. It would then confirm the archaic nature of the dental-enamel junction.

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