INFLUENCE OF STORAGE MEDIA ON THE ELASTIC PROPERTIES OF DENTIN AND TOOTH ENAMEL – A SCANNING ACOUSTIC MICROSCOPY ANALYSIS

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Abstract: The aim of this study was to examine the influence of storage media on the elastic properties of dentin and tooth enamel with respect to the storage period. Several reports suggest that saline and other aqueous solutions may induce chemical reactions and the dissolution of minerals, which in turn may cause alterations of elastic tissue properties. Three non-erupted human wisdom teeth were extracted and divided in three slices. Sections were stored in three different media for a maximum period of 21 days. During this time all sections were inspected by time-resolved 50-MHz scanning acoustic microscopy. Storage in saline solution resulted in a progressive decrease of the acoustic impedance up to 70 % in dentin but not in enamel tissue. Hank's balanced salts solution and artificial saliva appeared to maintain the elastic properties of dentin and enamel during the entire time of storage. The measurements of surface wave velocities did not show significant differences. High resolution (900 MHz) inspection of sections cut perpendicular to the surface exposed to the storage media for 21 days revealed a progressive increase of impedance in dentin up to the initial values at a depth of approximately 300 µm. These findings suggest that quantitative SAM is a suitable tool for assessing surface and sub-superficial elastic properties tooth tissue.

Introduction

Tooth samples prepared for mechanical testing are usually stored in aqueous solutions to maintain hydration. Dentin has a mineral content of 70 wt% and 20 wt% organic substances and 10 wt% water. Enamel consists almost exclusively of mineral, with 95 wt%, mostly hydroxyapatite. The remaining components are 4 wt% organic substances and 1 wt% water. The main function of this mineral layer with a hardness ranging from 300 to 400 HV (hardness vickers) is the protection of the underlying regions against damage [1]. Enamel is built up once and can never be regenerated. It is well accepted that salia has a protective function for teeth. It works as and acid buffer an mineral deliverer. An acid milieu enhances mineral wash-out of teeth and destruction of enamel. On the other hand it is known that ionising irradiation used for cancer treatment in the oral cavity causes considerable deterioration of mechanical stability in teeth. In order to study the effects on the micro elastic properties in enamel and dentin in vitro it is necessary to store the extracted teeth in a physiological solution for a prolonged time.

Habelitz et al. [1] used nanoindentation to compare the elastic modulus after storage in deionised water, calcium chloride buffered saline solution and Hank's balanced salts solution (HBSS). They found that only HBSS did not change elastic properties during a time period of two weeks. Gustafson et al. [2] have shown that storage of bone in calcium dichloride solution protects the tissue against demineralisation and abides the mechanical stability. The reason for demineralisation by storage in deionised water is believed to be the absence of calcium and phosphate ions, which cause a concentration gap between tooth and storage media and enhance the demineralisation. For storage in sodium chloride there are calcium ions, but the concentration is too low to balance the absence of phosphate. Besides this the pH-value is decreased to 5.9 and therefore advances the demineralisation.

Scanning acoustic microscopy (SAM) is a relatively unknown tool for assessing the elastic microstructure of tissues. The contrast arises from the elastic interaction of the mechanical wave with the material under investigation. Complex transmission and reflectance characteristics at interfaces provide a multiplicity of information that can be used for the derivation of elastic tissue parameters. SAM is usually operated in the frequency range from 50 MHz to 2 GHz and therefore reaches a spatial resolution comparable to that of high resolution light microscopes. The measured values like impedance, speed of sound and attenuation are directly linked with mechanical and structural properties like density and elasticity. For example, Maev et al. [3] determined the

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compressional wave velocity c_P in thin sections of human premolar dentin with 50-MHz time-of-flight measurements. In bulk dentin the highest velocities were observed. The velocities in dark mantel dentin was about 8 % lower near the enamel and 15 % lower near the pulp chamber. In cases with destroyed dentin close to carious cavities the decrease was about 17 %.

In this study we have used quantitative scanning acoustic microscopy (SAM) in order to (i) demonstrate its potential in dental research, (ii) assess two-dimensional maps of elastic properties of entire tooth sections, and (iii) to further elucidate the influence of storage media on the elastic properties in enamel and dentin.

Materials and Methods

Three freshly extracted non-erupted third molars were sectioned longitudinally in three slices (one for each storage medium). Each section was fixed with Technovit® on special sample holders. The surfaces were then grinded and polished with successively decreasing grain size. Acoustic inspection was conducted immediately after preparation (day 0) and 1, 3, 7, 10, 14 and 21 days after preparation. On each tooth slice three measurement regions were selected within dentin and enamel, so that for every storage medium 27 measurements were performed. The media were replaced after every three days and after each measurement. Between the measurements the samples were stored at 5°C.

The sections were stored in physiological saline solution (NaCl, pH: 6.3), Hank's balanced salts solution (HBSS, pH: 7.6), and artificial saliva (AS, pH: 7.0). The composition of HBSS according to [4] is: 400 mg/l KCl, 60 mg/l KH_2PO_4, 8000 mg/l NaCl, 1000 mg/l glucose, 90 mg/l $Na_2HPO_4 \cdot 7H_2O$, 350 mg/l $NaHCO_3$, 140 mg/l $CaCl_2$, 100 mg/l $MgSO_4 \cdot 7H_2O$, and 100 mg/l $MgCl_2 \cdot 6H_2O$.

The preparation of artificial saliva is more complex and has been taken from [5]. Briefly, it consists of 0.375 g/l $CaCl_2 \cdot 2H_2O$, 0.125 g/l $MgCl_2 \cdot 6H_2O$, 1.2 g/l KCl, 0,85 g/l NaCl, 2.5 g/l $NaHPO_4 \cdot 12H_2O$, 1 g/l sorbine acid, 5 g/l carboxymethylcellulose sodium, and 43 g/l sorbitol solution (70%, non cristalline).

Acoustic inspection

Acoustic impedance Z, surface skimming compression wave velocity (SSCW) c_P and Rayleigh wave velocity c_R were determined using a custom time-resolved scanning acoustic microscope SAM200Ex. It consists of a three-axis high-precision scanning stage, a 200-MHz pulser/ receiver (Panametrics 5900PR, Waltham, MA), and a 500 MS/s A/D-card (Gage 8500). All components are controlled by a custom software (SAMEx; Q-BAM, Halle, Germany). For the measurements a 50-MHz broadband transducer (V605 Valpey Fisher, Hopkinton, MA) with an aperture angle of 60° and a confocal spatial resolution of 23 µm was used. The samples were completely immersed in a temperature controlled tank filled with physiological saline solution at 25°C. For a precise definition of the measurement regions the samples were scanned in the C-mode first (Figure 1). The locations for the impedance and SAW velocity measurements were then selected manually. Consecutive pulse-echo sequences *V(z,t)* were acquired in A(z)-mode, that is, the transducer is scanned towards the sample surface. A defocus range from approximately + 100 µm to -1200 µm relative to the confocal position was scanned with an increment of 4 µm. The result is a two-dimensional envelope picture, named *V(z,t)* image (Figure 2).

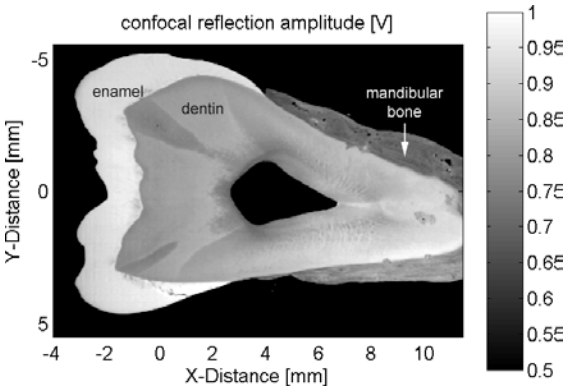

Figure 1: Overview of C-scan (tooth #1 at day 0). The gray level corresponds to the confocal reflection amplitude. Different properties in tooth enamel, dentin and mandibular tissue as well as the heterogeneity of dentin can clearly be resolved.

The confocal reflection amplitude is proportional to the reflection coefficient R and can be determined by calibration with the reflection amplitude of reference materials, as detailed in [6-9]. The acoustic impedance is the determined from:

$$R = \frac{Z_2 - Z_1}{Z_2 + Z_1}, \qquad (1)$$

where Z_2 and Z_1 are the impedances of the sample and the coupling fluid, respectively.

As the transducer is defocused the phase difference $\Delta\theta$ between the surface reflection and leaking surface waves is successively increasing:

$$\frac{\Delta\theta}{\Delta z} = 2k\left(1 - \cos\theta_{SAW}\right). \qquad (2)$$

$k=2\pi/\lambda$ is the wave number and θ_{SAW} is the critical angle for the generation of a surface wave [10]. The resulting spatial oscillation frequency due to this interference in a monochromatic system, e.g. a horizontal line in the *S(z,f)* image, is:

$$\frac{1}{\Delta z} = \frac{2 \cdot f}{c_0}\left[1 - \cos\theta_{SAW}\right], \qquad (3)$$

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whereas f is the acoustic frequency and v_0 is the speed of sound in the coupling fluid.

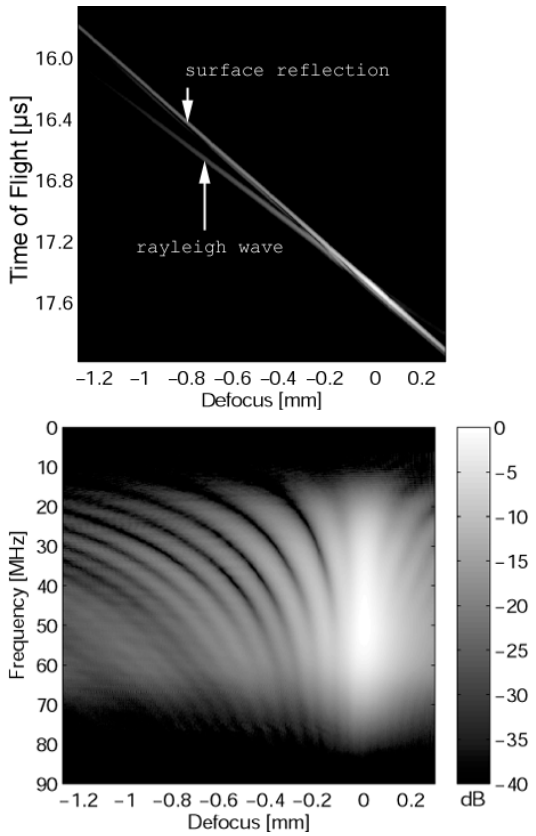

Figure 2: Hilbert-transformed *V(z,t)*-image (top) of the broad-band measurement in tooth enamel. The acoustic impedance is determined from the confocal (z = 0 mm) reflection amplitude. The evident separation of the Rayleigh wave from the surface reflection with increasing defocus causes a typical oscillation pattern in the frequency domain representation *S(z,f)* of the sequence (bottom).

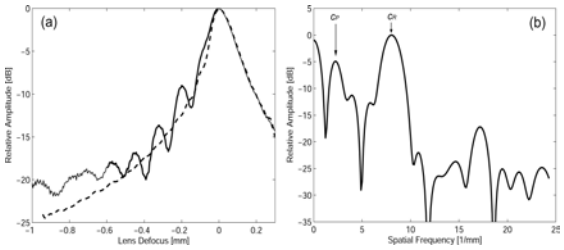

Figure 3: SAW speed evaluation in tooth enamel 50 MHz (a). The dashed line is the V(z) signal of teflon. The bold section was used for FFT analysis after subtraction of the teflon signal. The two peaks in the spatial frequency spectrum in (b) correspond to oscillations caused by SSCW (c_P) and Rayleigh (c_R) waves.

The phase velocity of the surface wave is obtained using Snell's law:

$$c_{SAW(ph)} = \frac{c_0}{\sin\theta_{SAW}} \,. \qquad (4)$$

In many cases two or more SAW waves are generated. For a reliable estimation of the spatial oscillation frequencies another Fourier transformation has to be applied (Figure 3). The use of broadband pulses allows to determine the phase velocities as a function of frequency [11]. The group velocity is given by:

$$c_{SAW(gr)} = \frac{\delta\omega}{\delta k} \,, \qquad (5)$$

where $\omega = 2\pi f$ is the angular frequency. For the determination of the phase and group velocities a special software was developed. The spatial oscillation frequencies were analysed within a

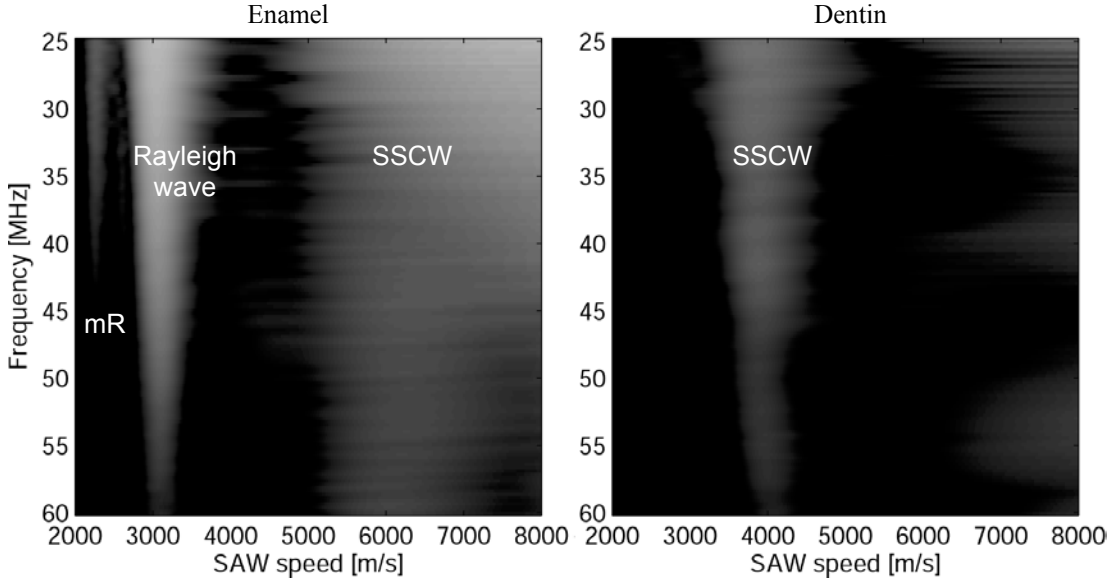

Figure 4: SAW speed image of enamel (left) and dentin (right). SSCW waves (vertical lines) can be seen in both tissues. A Rayleigh wave is only generated in enamel. Moreover a multiple reflection of the Rayleigh (mR) wave can be seen.

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defocus range from 0 to -800 µm. The phase velocities were determined in the frequency range from 30 MHz to 50 MHz and within this frequency range the group velocities were derived from the linear slope of $\omega(k)$ according to Eq. (5).

Results

Figure 4 shows examples of phase velocity images measured in enamel and dentin. It can be seen that in enamel two peaks corresponding to the SSCW and Rayleigh waves occur, while in dentin only one peak that corresponds to the SCCW is visible. The accuracy of the velocity estimation is inversely proportional to the velocity value. This results in a considerable broadening of the SCCW peak in enamel. Therefore only the Rayleigh wave velocity was analysed.

The course of impedance values with respect to storage time is shown in Figure 5. The samples stored in saline solution exhibited significantly reduced values in dentin and enamel starting from days 1 and 10, respectively (ANOVA, $p < 0.05$). The maximum reduction of impedance at day 21 was approximately 8 % in enamel and between 30 % and 70 % in dentin. It should be noted that the increasing standard deviations of the dentin values indicate different slopes of impedance loss with time for the different samples (see Table 1).

The impedance values of dentin of the samples stored in HBSS and artificial saliva did not change during the entire time of examination. The values in enamel were slightly reduced from day 10 to 21 compared to baseline. The impedance decrease was comparable to that observed in saline solution.

In contrast to the dependency of the acoustic impedance on storage medium and time no significant variations were observed for the sound velocities. The results of impedance and speed of sound measurements at days 0 and 21 are summarized in Table 1.

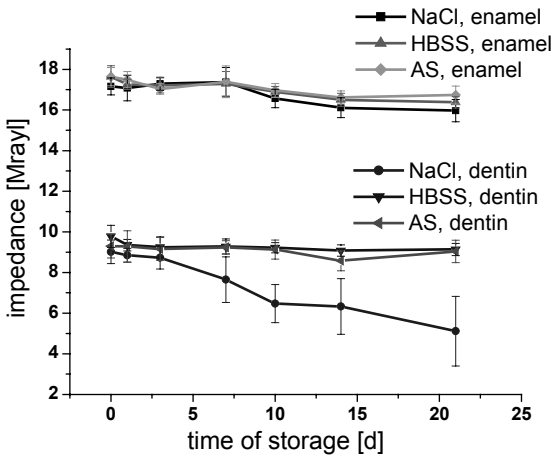

Figure 5: Acoustic impedance versus storage time in NaCl, HBSS, and artificial saliva (AS) solution (error bars are standard deviations).

In order to elucidate this unexpected result the

Table 1: Impedances and SAW velocities at days 0 and 21 in dentin and enamel. The sections of #1 and #2 that were stored in saline solution were grinded, polished and measured again after day 21 (approximately a layer of 400 µm thickness was removed). The number of measurements is written in parenthesis behind the sample number.

	dentin		enamel	
	Z [Mrayl]	c_P [m/s]	Z [Mrayl]	c_R [m/s]
day 0				
#1 (9)	9.27 ± 0.03	3909 ± 181	17.08 ± 0.39	3064 ± 33
#2 (9)	9.52 ± 0.07	3864 ± 97	17.43 ± 0.37	3036 ± 57
#3 (9)	9.40 ± 0.92	3816 ± 51	17.97 ± 0.33	3030 ± 20
day 21				
$\#1_{NaCl}$ (3)	6.02 ± 1.23	3783 ± 106	15.94 ± 0.47	3067 ± 37
$\#2_{NaCl}$ (3)	3.09 ± 0.53	3981 ± 70	16.49 ± 0.12	3043 ± 15
$\#3_{NaCl}$ (3)	6.21 ± 0.87	3894 ± 223	15.48 ± 0.44	3041 ± 16
$\#1_{HBSS}$ (3)	9.14 ± 0.27	3815 ± 131	16.45 ± 0.31	3070 ± 27
$\#2_{HBSS}$ (3)	8.97 ± 0.35	3895 ± 28	16.56 ± 0.18	3020 ± 59
$\#3_{HBSS}$ (3)	9.33 ± 0.25	3841 ± 6	16.13 ± 0.18	3045 ± 36
$\#1_{AS}$ (3)	9.03 ± 0.22	3800 ± 38	16.43 ± 0.36	3044 ± 35
$\#2_{AS}$ (3)	9.11 ± 0.60	3843 ± 85	17.20 ± 0.12	3025 ± 55
$\#3_{AS}$ (3)	8.98 ± 0.89	3862 ± 37	16.60 ± 0.36	3048 ± 24
after regrinding				
$\#1_{NaCl}$ (3)	8.83 ± 0.41	3873 ± 19	16.67 ± 0.26	3054 ± 20
$\#2_{NaCl}$ (3)	9.16 ± 0.09	3825 ± 38	16.54 ± 0.27	3050 ± 18

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section of tooth #3 that was stored in saline solution was then cut perpendicular to the previously inspected surface. The depth dependence of the acoustic impedance was qualitatively assessed using high resolution SAM measurements (SAM2000, Krämer Scientific Instruments, Herborn, Germany; frequency: 900 MHz; spatial resolution: 2 µm) on this section plane. Figure 6 shows the mean reflection amplitude of dentin with respect to the distance from the surface that was exposed to the storage medium. It can be seen that the voltage is gradually increasing and approaches an equilibrium at a depth of approximately 300 µm. The other two sections ($\#1_{NaCl}$ and $\#2_{NaCl}$) were then grinded and polished again to remove a 400-µm tissue layer. Another acoustic inspection revealed that the initial impedance values and SAW velocities were recovered (Table 1).

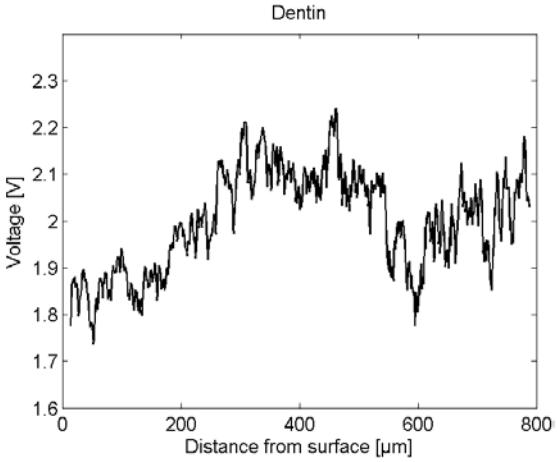

Figure 6: Reflection amplitude as a function of distance from the surface that was exposed to saline solution storage medium. The voltage gradually increases and approaches and equilibrium at a depth of approximately 300 µm.

Discussion

Quantitative time-resolved scanning acoustic microscopy was employed to provide two-dimensional maps of the acoustic impedance and to measure phase and group velocities of two types of surface acoustic waves in tooth enamel and dentin. The spatial resolution of the impedance measurement is approximately 23 µm. For the SAW velocity estimation the transducer has to be defocused. Therefore the diameter of the contributing surface area was approximately 1 mm [10]. In principle all parameters can be considered to be equally sensitive to anisotropic elastic tissue properties. For example, Raum et al. [12] have shown that the impedance is directly correlated ($R^2 = 0.99$) with the elastic coefficient c_{ii} in bone tissue. Jorgensen and Kundu [13] used the SSCW and Rayleigh wave velocities to derive both Young's and shear modulus in trabecular bone. However, alterations of the surface elastic properties due to the storage in saline solution did affect the acoustic impedance, but not the SAW velocities. High resolution measurements of the depth dependence of the acoustic reflectivity support the hypothesis that chemical dissolution of the mineral phase cause a gradual softening of the surface layer that is exposed to the storage medium. The depth of the affected layer after 21 days is approximately 300 µm. For comparison the wavelengths of the SSCW wave in dentin and the Rayleigh wave in enamel are ~100 µm and ~76 µm, respectively. The requirement to generate a SAW wave is that the maximum angle of the incident wave exceeds the critical angle. However, if the superficial layer is softened this condition is not fulfilled anymore. Therefore it is likely that the incident waves are refracted at the soft surface and propagate parallel to the surface in a sub-superficial layer that is not affected by chemical dissolution (Figure 7).

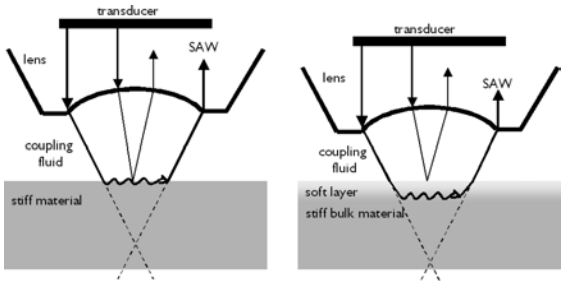

Figure 7: In fresh tissue the SAW propagate along the surface (left). If the superficial layer is gradually softened due to chemical dissolution of the mineral phase the SAW's is assumed to propagate in a deeper tissue layer that is not affected by the chemical dissolution. Therefore only the surface reflection amplitude is reduced.

On the other hand the acoustic impedance derived from the surface reflection appeared to be very sensitive to elastic surface variations. The interaction depth is in the order of the compressional wavelength, e.g. for dentin $\lambda_P \sim 80$ µm at 50 MHz.

We have shown that the elastic surface properties are remarkably altered during storage in physiological saline solution. A rapid wash-out of the mineral phase was observed particularly in dentin. In agreement with findings of Habelitz et al. [1] storage in HBSS did not induce remarkable elastic alterations in dentin and enamel. In contrast to their nanoindentation study we observed a slight decrease of the acoustic impedance in enamel after 10 days. However, there was no further decrease up to day 21 and the decrease was only slightly above the significance level. Artificial saliva had a similar potential to preserve elastic properties compared to that of HBSS.

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Conclusions

The choice of storage medium is important to preserve micro elastic tissue properties. Minerals are rapidly dissolved in dentin when it is stored in saline solution. The wash-out occurs gradually and affects only a thin superficial layer. The depth sensitivity is different for the acoustic impedance and the SAW velocity measurement. While Z is very sensitive to elastic variations of the surface properties, the SAW velocities reflect the elastic properties of the underlying bulk tissue. The particular mechanisms of the depth sensitivity need to be addressed in future studies.

HBSS and artificial saliva are suitable storage media if micro-elastic properties of tooth tissues are of concern. Moreover, SAM can be provide invaluable information in dental research, e.g. for a non-invasive monitoring of tissue de- and remineralization, depth profiling of the demineralisation zone, or to study the effects of ionising radiation on the elastic tissue properties.

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