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The use of micro-Raman spectroscopy to differentiate between sound and eroded primary enamel

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Objective. The aim of this study was to investigate differences in phosphate group concentration between areas of sound and eroded enamel in primary teeth using micro-Raman spectroscopy (MRS).

Methods. The Raman spectroscopic technique enables researchers to obtain information about molecules by analysing scattered light caused by monochromatic laser excitation. Ten extracted anterior primary teeth with areas of sound and eroded enamel were used. Three, 10-s scans were carried out at three points along a 3-μm linear area in both sound and eroded primary enamel, and Raman spectra were obtained with the LabRam 300 fitted with an Olympus BX40 microscope. Scanning electron microscopy was used to confirm the presence of sound and eroded enamel on each tooth.

Results. The phosphate ν_1 band was located between 958.5 and 967.0 cm^{-1}. There was no statistically significant difference between the area under the phosphate ν_1 band values for the eroded and sound areas ($P = 0.7302$). Scanning electron microscopy confirmed the presence of sound and eroded areas on all specimens.

Conclusion. In this study, there was no statistically significant difference found between phosphate group concentration in eroded and sound primary enamel when analysed by MRS.

Introduction

Erosion is the loss of dental hard tissue by a chemical process not involving bacteria[1], and this is a problem that has clinical significance. Data collected for the Children's Dental Health in the United Kingdom 2003 survey showed that 53% of 5-year-olds had erosion affecting the lingual surfaces of their upper anterior teeth[2]. A study carried out on a cluster-randomized sample in Birmingham, UK[3], showed that 51% of 14-year-olds had moderate erosion and that there was significantly more erosion in those from lower socioeconomic groups. Clinical measurement of erosion is difficult, however, as is comparison between studies, since many scales and indices have been used. The true prevalence is, therefore, difficult to ascertain.

Most *in vitro* investigations of erosion have examined permanent enamel. A number of studies using a variety of analytical methods, however, have looked at the erosive process in primary teeth. These include surfometry[4], microhardness[5] and atomic force spectroscopy combined with nanoindentation[6]. The surface layer of teeth needs to be polished prior to indentation and microhardness testing to ensure a flat surface, and because the surface layer is more highly mineralized and contains a higher concentration of fluoride, its removal may compromise results. Amaechi *et al*. used microradiography since this technique is sensitive to changes in mineral content of enamel[7]. Grando used scanning electron microscopy (SEM) to assess the damage caused during the erosive process by different beverages[8]. It was concluded that permanent enamel is harder than primary enamel[5,6]. Amaechi and Hunter *et al*. found that primary enamel was more susceptible to erosion than permanent enamel, although Lippert *et al*. found that there was no statistically significant difference between the two types of enamel[4,6,7].

Unlike most other analytical methods, micro-Raman spectroscopy (MRS) is a nondestructive technique, where samples require no physical preparation prior to analysis, and therefore, it has potential to be a useful tool in the study of the erosive process.

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When light is scattered by any form of matter, the energy of the majority of photons remains unchanged. Approximately one in a million photons will gain or lose energy that corresponds to the vibrational energy of the scattering molecules. These can be seen as additional peaks on the scattered light spectrum and is known as Raman or inelastic scattering. These additional peaks are specific to the molecules within the compound under investigation, and therefore, produce a 'fingerprint' for that particular compound. The intensity of these peaks can give information about the concentration of particular groups within the hydroxyapatite molecule. For example, hydroxyapatite has a higher concentration in enamel than in dentine, and this can be seen on the Raman spectrum.

Micro-Raman spectroscopy is a very useful tool for analysing the chemical composition of both synthetic and biological materials and has the potential to provide information regarding low-level biochemical changes in tissue. The technique has become commonly used in recent years as a result of the development of lasers and charge-coupled device (CCD) detectors, which increase the speed with which spectra can be obtained. It has been used in the field of medicine to study hard tissues, DNA and white blood cells *in vitro*[9–12]. More recently, MRS has also been used *in vivo* to study pre-malignant lesions in breast and atherosclerotic plaques[13,14].

It has also been used in dentistry to study synthetic minerals[15], calcium fluoride formation in enamel[9], the orientational Raman characteristics of enamel, i.e. the direction in which the hydroxyapatite crystals lie in surface enamel[10], identification of mineral phases in dental calculus[16], and the resin–dentine interface in restored teeth[17,18].

Previous studies of the dental hard tissues[19,20] have used the phosphate ν_1 band, located at around 960 cm^{-1}, which is indicative of the P-O stretch associated with hydroxyapatite. This represents the vibrational energy associated with the bond between phosphate and oxygen in the hydroxyapatite molecule, and cannot be altered. Therefore, this can be used to analyse changes in the phosphate group concentration within the hydroxyapatite molecule.

In order to compare samples, the area under the band is used to compare the bands produced by different specimens[19–21]. A background spectrum, resulting from stray light and dark current produced by the detector, is present during spectral collection. By using software to calculate the area under the band, however, this background spectrum is eliminated from the final calculation, resulting in an accurate comparison of bands produced from different samples.

The aim of this study was to investigate the differences in the phosphate group concentration between areas of sound and eroded enamel in primary teeth using MRS.

Materials and methods

Consent was given by the parents of the children involved for the extracted teeth to be used for research purposes. Ten extracted, eroded upper anterior primary teeth from patients living in an area with no water fluoridation were cleaned of blood and debris immediately after extraction using surface disinfection wipes containing 25 g ethanol and 35 g 1-propanol/100 g of solution (Unowipes, Unodent, Withim, UK). They were then stored in distilled water in a sealed container at room temperature for less than 3 months, and were not allowed to become dehydrated at any time.

Prior to examination under MRS, the palatal bulbosity of each tooth was reduced using diamond burs in a high-speed hand-piece with water coolant. This provided a flat surface to facilitate mounting of the specimen and assist focusing.

Calibration of the micro-Raman equipment was carried out prior to every session. The CCD (Spectrum One, Horiba Jobin Yvon, Stanmore, Middlesex, UK) was initially cooled to 228 °K prior to calibration and maintained at this temperature throughout. The CCD had a resolution of 1024×256 with a 16-bit dynamic range and a pixel size of 27 μm. A silicon specimen, with a characteristic band at 520.8 cm, was used to calibrate the computer software program linked to the LabRam 300 (Horiba Jobin Yvon). An Olympus BX40 microscope (Olympus, Southall, Middlesex, UK) was incorporated into the system.

Eroded and sound areas of enamel on each tooth were identified visually without magnification, and photographs were taken using the camera incorporated in the Laboratory Ram 300 at × 100 magnification. These images were stored on a computer hard drive (Dell Optiplex Gn, Dell Inc., Austin, TX, USA) using the Microsoft Windows 95 operating system (Microsoft Corp., Redmond, WA, USA). The eroded areas were generally located more incisally than the sound areas, although there was individual variation between teeth, and in some cases, the only sound area identified was located interproximally.

Spectra were obtained from all specimens using a 20-mW red laser (HeNe) with a wavelength of 632.817 nm, combined with a slit of 150 μm, a confocal hole of 250 μm and a 1800 mm g^{-1} holographic grating.

A 3-μm linear region was selected in each of the sound and eroded areas by utilizing the video camera function and the LabSpec, Version 4.08, computer software program (Horiba Jobin Yvon). One scan was obtained over 10 s at three, 1-μm steps along the linear areas, resulting in a total of three spectra for each eroded and sound area. The software program then combined the three spectra to produce a mean spectrum for analysis.

Measurements taken were the wave number (cm) and the band width of the phosphate ν_1 band at half maximum height (FWHM). These measurements were used to confirm that the band was, in fact, a true representation of the phosphate group associated with hydroxyapatite, and also to ensure that the equipment could produce the expected bands.

The area under the phosphate ν_1 band is delineated as described by Heigl *et al.* (Fig. 1), which is then calculated by the software[22]. The measurements obtained by analysis of the area under the phosphate ν_1 band were recorded on three separate occasions, approximately 30 min apart, and a mean was produced for statistical analysis.

Following analysis with the micro-Raman spectrograph, images were taken of all specimens to confirm the extent of the erosion using SEM (SEM 505, Philips/FEI, Eindhoven, the Netherlands) with a spot size of 50 nm. The enamel specimens were dehydrated by taking them through graded concentrations of acetone (70–100%). Each specimen was then critical-point dried (E3000 SII CPD, Quorum Technologies, Newhaven, East Sussex, UK) using liquid carbon dioxide at 31.1 °C and 1075 p.s.i. Specimens were then sputter coated (Emscope SC500, Emitech, Ashford, Kent, UK) with a 20-nm coating of gold/palladium (60:40).

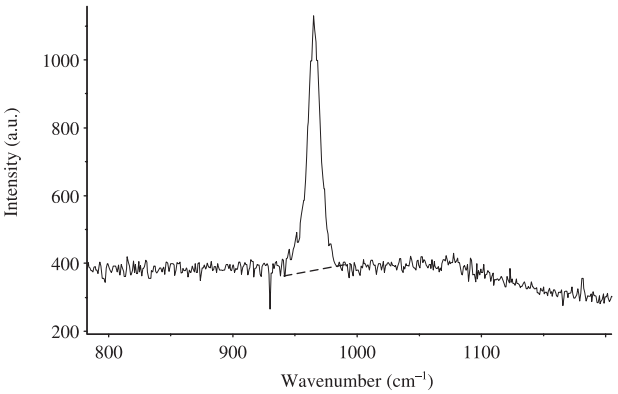

Fig. 1. Measurement of the area under the phosphate band is delineated as described by Heigl *et al.*[22]

Statistical analysis

The measurements obtained for the area under the phosphate ν_1 band were analysed using the Instat computer software program (GraphPad Software Inc., San Diego, CA, USA). An unpaired *t*-test was applied to the data collected.

Results

Scanning electron microscopy and photographs showed that all teeth had regions that appeared to be eroded and regions that appeared to be sound (Fig. 2). The areas identified as sound and eroded were easily identified at low magnification.

The phosphate ν_1 band, a characteristic of the ν_1 symmetric stretching mode of the tetrahedral phosphate (PO_4^{3-}), was seen clearly in all specimens between 958.5 and 967.0 cm^{-1}. A representative spectrum is shown in Fig. 3.

The range (median) of the width of the phosphate band at half maximum height was 10.34–11.98 cm^{-1} (11.16 cm^{-1}) in sound areas and 9.25–11.98 cm^{-1} (11.03 cm^{-1}) in eroded

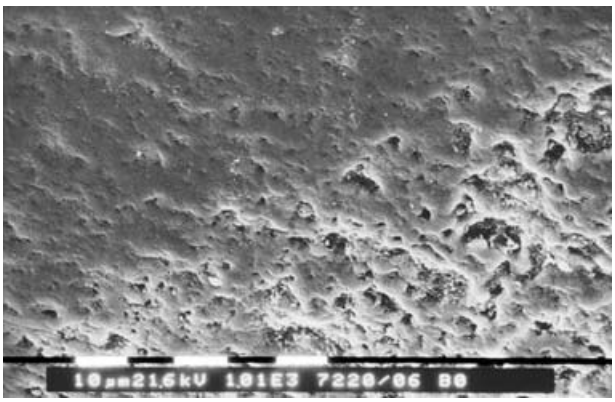

Fig. 2. Scanning electron micrograph of specimen 1, showing eroded enamel next to an area more sound enamel.

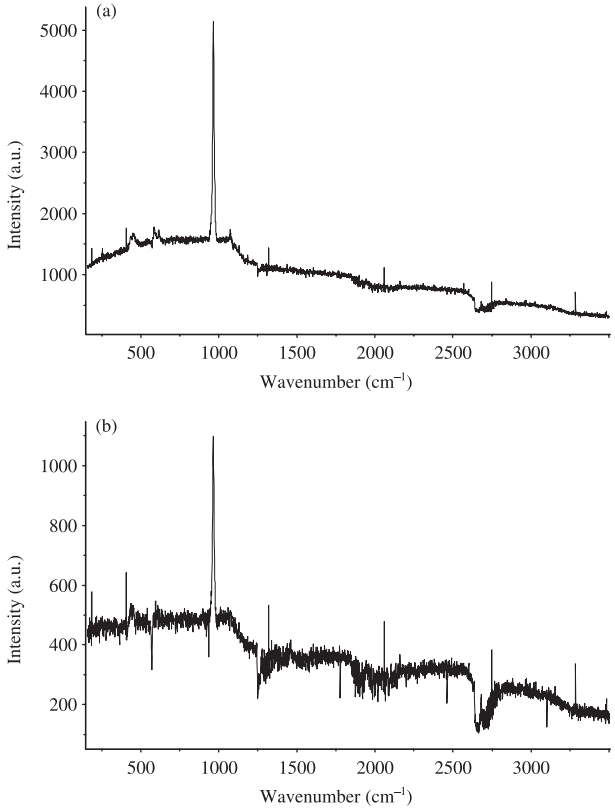

Fig. 3. (a) Representative spectrum obtained from a sound area of primary enamel showing the characteristic band associated with the phosphate ν_1 band. (b) Representative spectrum obtained from an eroded area of primary enamel showing the characteristic band associated with the phosphate ν_1 band.

areas. Descriptive statistics for all measurements are shown in Table 1.

When analysing the area under the phosphate ν_1 band in sound regions, the area ranged (median) from 4473 a.u. to 59 625 a.u. (12 057 a.u.), with a mean (SD) of 16 421.6 a.u. (15 764 a.u.).

Table 1. Descriptive statistics for the area under the phosphate ν_1 band in primary enamel shown in arbitrary units (a.u.). This represents the integrated intensity of the band.

Variable	Eroded area (a.u.)	Sound area (a.u.)
Mean	12 290.80	16 421.60
Standard deviation	7 313.90	15 764.00
Lower 95% confidence limit	7 059.10	5 145.40
Upper 95% confidence limit	17 522.00	27 698.00
Minimum	1 320.00	4 473.00
Median (fiftieth percentile)	11 485.00	12 057.00
Maximum	25 888.00	59 625.00

For eroded regions, the range (median) was 1320–80 989 a.u. (13 501 a.u.), with a mean (SD) of 19 490.8 a.u. (22 783 a.u.).

An unpaired *t*-test was carried out that revealed that this was not statistically significant ($P = 0.7302$).

Discussion

The primary mineral content of dental enamel is hydroxyapatite. The analysis of the Raman spectrum of dental tissues can provide information about the concentration of the phosphate group associated with the hydroxyapatite molecule. Dissolution of enamel in acid media occurs as follows:

$$Ca_{10}(PO_4)_6(OH)_2 + 8H^+ = 10Ca^{2+} + 6HPO_4^{2-} + 2H_2O$$

Therefore, analysis of the concentration of phosphate within the enamel is a good indicator of the degree of mineralization[23,24]. It would be expected that phosphate would be released in both the erosive and caries process, thus resulting in a decrease in the intensity of the band related to this group.

Confocal MRS allows molecular analysis of the dental tissues. The output information is provided in the form of a spectrum. The bands represent the intensity of the signal according to frequency, and the mathematical exploitation of this permits comparative and quantitative analysis.

In this study, the area under the phosphate ν_1 band was chosen for analysis because this

has been shown to be an accurate method of comparison[22]. It excludes the background spectrum produced by incident light and dark current from the CCD detector, and therefore, measures only the signal produced by the excitation of the phosphate molecule.

The results demonstrated that, using MRS, there was no statistically significant difference between the phosphate ν_1 band in sound and eroded areas of the primary enamel, as measured by the area under the band. This author used a larger sample than a previous study[20], That study also used intact enamel; however, no numerical information was given to support the results obtained from the enamel specimens. For these reasons, it was not possible to conduct a power calculation prior to commencing this study. A *post hoc* power calculation was undertaken to inform future studies, however. The power calculation revealed that a sample of 81 teeth would have been required to attain a 0.80 power at a significance level of 0.05. Therefore, this study, together with previous research, would appear to be significantly underpowered.

The results of this research demonstrate that the spectra produced are comparable with those described in previous studies[19,20]. The bandwidths at half maximum height of the phosphate ν_1 band, 9.24–11.98 cm^{-1} compared well with those found by Tsuda and Arends, and Tramini[19,20]. Cosmic rays may also be incorporated within the spectrum. These can appear at any wave number, but are narrow and sharp, in contrast to those produced by stretches within the molecule. Therefore, it is important to guarantee that these are not included in any analysis. The bandwidths at half maximum height confirmed that the correct band had been identified and that cosmic rays were not included in the analysis. The elimination of these was also aided by carrying out multiple scans and producing a mean spectrum for analysis, thus reducing the frequency with which these were included in the final spectrum. When compared with the background spectrum, the peak intensity was adequate to allow the authors to identify this as the band associated with the phosphate group and enable them to analyse it.

Because there was an area of sound enamel as well as an area that was eroded on each tooth, each sample acted as its own control. Although all the eroded areas examined were within the enamel, there may have been variation in the extent of erosion, since the process had taken place *in vivo* and not under standardized laboratory conditions. Therefore, the areas designated as sound may also have experienced the early stages of erosion, although these had not experienced the tissue loss seen in the eroded areas.

Scanning electron microscopy images were used in combination with × 100 magnification to ensure that the areas examined were indeed eroded and sound. The SEM photographs clearly showed the presence of sound areas adjacent to those with destruction of the enamel prisms, indicating that chemical changes had occurred.

Previous studies have found that phosphate is indeed lost when enamel is placed in an erosive substance[24,25]. Since the teeth in this study were eroded within the oral environment, however, the affected areas may have been remineralized, and therefore, there was no difference in the surface chemistry detected. Nevertheless, it is interesting that the SEM images clearly showed an alteration of the structure of the surface enamel, but no difference in the phosphate group chemistry was detected. Arends and ten Bosch stated that the analysis of the phosphate released into solution can be inaccurate because it is impossible to measure this with an accuracy of better than 1%[26]. Furthermore, since the volumes involved were so small, there could be an overestimation of the amount of phosphate released from the apatite crystal. This could offer another explanation for noting no difference in the phosphate chemistry.

Further study is required to fully analyse the spectra obtained from sound primary enamel along with that of sound permanent enamel and so compare the differences. While statistically significant differences in the phosphate ν_1 band were not noted, future investigation of the entire spectrum may reveal further information regarding the chemical process involved in erosion of primary enamel. In order to standardize conditions, it may be desirable to first demineralize the enamel *in vitro*.

What this paper adds
- This paper describes a novel technique for analysing mineral concentration in teeth.
- A post hoc power calculation is presented to inform further studies of this kind.
- Further work may show this to be a useful technique for measuring erosion.

Why this paper is important to paediatric dentists
- This paper has explored erosion in primary teeth, an area where there is little knowledge compared to secondary teeth.
- The results of this study suggest over understanding of the chemical processes involved in erosion are incomplete.

Conclusion

In this study, MRS found no statistically significant difference between the phosphate group concentration in eroded and sound primary enamel.

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