Quantifying the Demineralisation of Enamel Using a Hyperspectral Camera Measuring Fluorescence Loss

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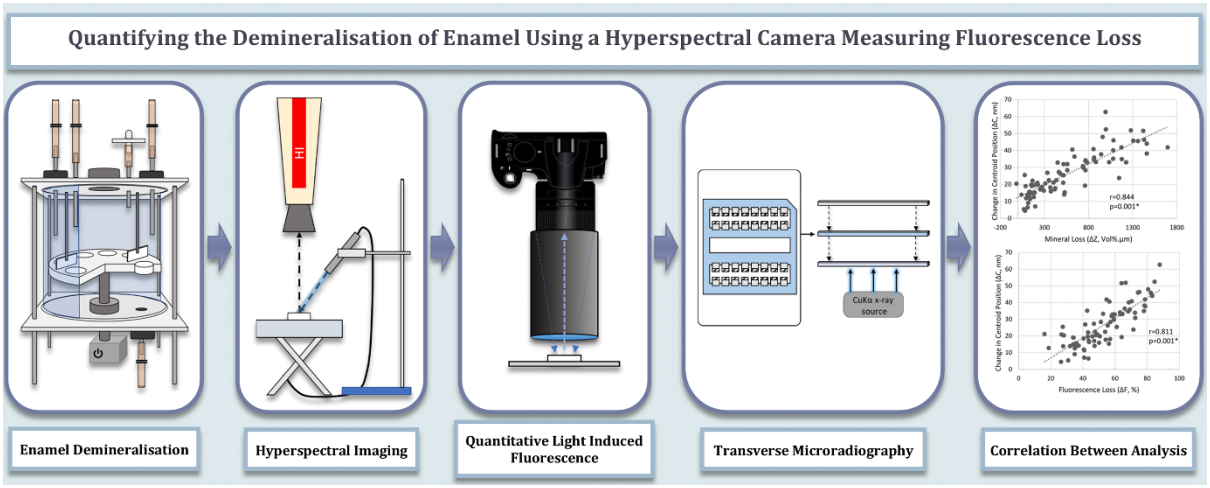

Abstract

Background: The gold standard for quantifying mineral loss of enamel is transverse microradiography (TMR) and is complimented by the non-destructive quantitative light induced fluorescence (QLF) which measures changes in autofluorescence. Fluorescence loss has been shown to correlate with mineral loss. Building upon the established method, the use of hyperspectral fluorescence imaging (HI) allows the capture of a broader range of wavelengths to quantify fluorescence changes more accurately.

Method: Bovine Enamel was demineralised within the dual constant depth film fermenter over 14 days and analysed using TMR, QLF and HI. The mineral change values were compared using Pearson's Correlation Coefficient.

Results: The analysis showed a statistically significant correlation that was equal between TMR and HI (r=0.844) and TMR and QLF (r=0.844), but weaker between QLF and HI (r=0.811).

Conclusions: The correlations indicate that HI is a promising valid non-destructive method for quantifying mineral loss from bovine enamel that is as accurate as QLF and complements TMR.

Key Words

Enamel – Fluorescence – Demineralisation – Biofilm – Hyperspectral Fluorescence Imaging

Introduction

Demineralisation of enamel is currently quantified using the gold standard method of transverse microradiography (TMR). It has become accepted as the best method available to determine mineral loss with the ability to calculate the actual mineral lost (Vol%) and the depth of the lesion (µm). The mineral loss value over the lesion depth value gives the integrated mineral loss value, ΔZ (Vol%.µm) that is used to comparatively evaluate the degree of mineral loss [1]. Complimenting the TMR method, demineralisation can also be assessed by measuring the loss in autofluorescence using the quantitative light induced fluorescence (QLF) method [2]. An area of demineralisation appears darker under QLF light conditions than sound enamel as the penetration depth of the light is less than for the

sound enamel (figure 1). The light becomes increasingly scattered as the lesion progresses and is less able to reach the dentine. As the light is not reaching the dentine and enamel dentine junction (DEJ), there is less chance of hitting a fluorophore and exciting it. The excitation of the fluorophores within the enamel, dentine and DEJ releases fluorescent photons in all directions. Some of the photons leaving via the enamel are captured using the QLF camera and a value assigned [3].

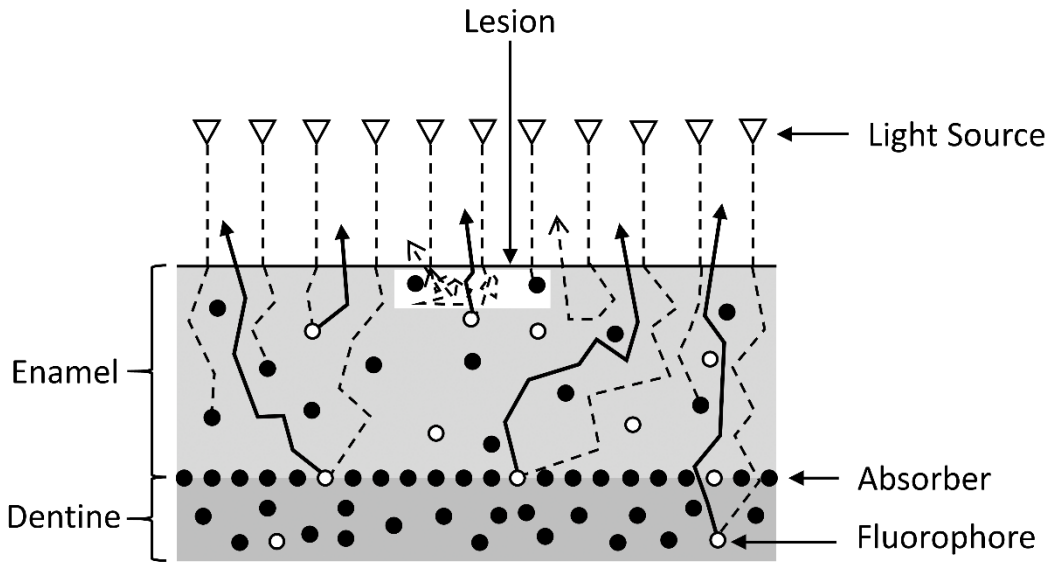

Figure 1 : The reflection and absorption of light during QLF analysis: *An overview of the light scattering, and absorption produced by enamel and dentine compared with a carious lesion. The light penetrates less into the lesion and is scattered by a greater amount than in sound enamel affecting the fluorescence value produced, the light is not able to reach the dentine. Figure adapted from van der Veen & de Josselin de Jong 2000.*

Hyperspectral fluorescence imaging (HI) is a novel method to measure fluorescence loss using a hyperspectral camera. The principal is similar to QLF, a 405nm laser excites fluorophores within the tooth structure which is captured using a camera. However, the HI camera captures a broad spectrum of wavelengths which may give it a distinct advantage over the QLF system, QLF captures 520 nm whereas HI captures throughout 420 nm to 720 nm. This may enable an increased sensitivity to fluorescence changes and produce a more accurate measurement of demineralisation. As enamel

demineralises there is a darkening of the enamel and a shift in wavelength spectra towards red light [4,5].

The aim of this study therefore is to assess the hyperspectral camera as a method of measuring autofluorescence loss and how it compares with the established QLF and TMR methods of analysis.

Materials and Methods

Demineralisation of Bovine Enamel

Polished enamel discs, 5 mm in diameter, were produced from mandibular bovine incisors and used as the substrate for this study. The mature lower incisors were extracted at an abattoir (ABP Food Group, Shrewsbury, UK) from cows under 36 months of age. The enamel was polished using three grades of sandpaper (1200, 4000 and 7000) and each disc was imaged using QLF and HI to capture baseline readings. The discs were then recessed to a depth of 200 µm within Polytetrafluoroethylene (PTFE) pans, each holding five bovine enamel discs. The disc loaded pans were sterilised using 4000 Gy gamma radiation [6].

An 8 L volume of artificial saliva [7,8] was prepared with the following composition; 1 $g.L^{-1}$ Lab Lemco Powder (Thermo Scientific, Leicestershire, UK), 2 $g.L^{-1}$ Yeast Extract (Sigma-Aldrich Ltd., Poole, UK), 5 $g.L^{-1}$ Proteose Peptone (Sigma-Aldrich Ltd., Poole, UK), 2.5 $g.L^{-1}$ Mucin from Porcine Stomach (Sigma-Aldrich Ltd., Poole, UK), 0.2 $g.L^{-1}$ NaCl (Sigma-Aldrich Ltd., Poole, UK), 0.2 $g.L^{-1}$ KCl (Sigma-Aldrich Ltd., Poole, UK) and 0.05 ppm fluoride (F-, as NaF, Sigma-Aldrich Ltd., Poole, UK). This was autoclaved at 121°C and 2200 mBar for 15 minutes. A smaller 500 mL volume of artificial saliva minus F- was also prepared and sterilised. The two F- sources were prepared to 228 ppm F- using NaF or SnF_2. Sodium gluconate (2.08 %) was added to SnF_2 to act as a stabiliser [9]. Neutralising the solution resulted in SnF_2 precipitating out of solution, so was left at pH 4.5. Sodium gluconate (2.08 %) was also added to the 228 ppm NaF solution and adjusted to pH 4.5 to match the SnF_2 solution. The F- solutions and all

other equipment including the CDFF units and silicon pipes were also autoclaved at 121°C. A 2 L volume of 2 % sucrose was prepared and autoclaved at a lower temperature of 116°C and 1900 mBar to prevent any sugar caramelisation. The disc containing pans were introduced to the sterile CDFF units aseptically under laminar flow.

First, a pellicle was let to be formed atop the bovine enamel discs over a 3.5-hour period using the 8 L artificial saliva source at a rate of 0.38 $ml.min^{-1}$, controlled by peristaltic pumps (101U/R MK2; Watson Marlow, Falmouth, UK). This was switched off and inoculation of the dCDFF units was performed. A microcosm of oral bacteria was used for inoculation from saliva collected from 23 volunteers (ethical approval, University of Liverpool; Ref: RETH001026) who were dentate, had not taken antibiotics within 2 months and were over 18 years old [10]. Participants were informed with an information sheet outlying the purpose of the study and how their saliva was to be used. Participants who wished to continue and provide a sample gave written permission for collection of their saliva. A 1 ml aliquot of the pooled saliva was added to the 500 ml artificial saliva and pumped into the CDFF units at a rate of 0.3 $ml.min^{-1}$ over a 16-hour period and fully depleted. Once complete the 8 L artificial saliva source was restarted and the timed sources of sucrose and starch began. The carbohydrate sources were pulsed into the appropriate CDFF unit eight times daily for 30-minute intervals at a rate of 0.38 $ml.min^{-1}$. This was performed over 16-hours of a 24-hour cycle. 30 minutes before and after the sucrose pulses, the sources of F- were pulsed in at a rate of 0.38 $ml.min^{-1}$ for 2 minutes. The artificial saliva source continued throughout the additional media pulses. Discs were taken from the CDFF units on days 3, 7, 10 and 14.

Hyperspectral Fluorescence Imaging

HI images were captured using a hyperspectral camera (Cri Nuance Multispectral Imaging system; Perkin Elmer, MA, USA) with an EF-S 60 mm macro lens attached (Canon, Tokyo, Japan). The camera was placed on a tripod above the samples to ensure the distance between lens and sample was the same for every image and a laser set to 405 nm was used to induce fluorescence. Images were

captured of the discs using the proprietary software included (Nuance Software Version 3.0; Perkin Elmer, MA, USA). The enamel discs were imaged before being placed in PTFE pans and irradiated as described before acting as baseline images. Once completely air dried after exposure to the CDFF, the enamel discs were once again imaged. The discs were tracked throughout the experiment to ensure the same disc is compared with the correct baseline image. For each disc the spectra captured was between 420 nm and 720 nm in 5 nm steps ensuring a broad range was captured. The exposure was set to 15 ms, Gain to 4 and Bit-depth to 12 with an image resolution of 1392x1040 px. The generated spectra outputs for both baseline and post CDFF exposure were opened in Excel (Microsoft, WA, USA) and the spectra normalised to 520 nm for analysis. 520 nm was selected to match the cut-off of the QLF system, as this is the wavelength at which the QLF system begins capture of excited yellow fluorescence [11].

To quantify the shift of the spectra from the baseline images to the post CDFF images the movement of the centroid position was calculated for each spectra output. The movement of this position was calculated from difference between the centroid of the baseline capture and post CDFF capture (ΔC, nm). This value was used to determine whether the spectra changes between the two conditions was significantly significant.

Quantitative Light Induced Fluorescence

QLF Images were captured using the QLF Biluminator system (Biluminator; Inspektor Pro Research Systems, Amsterdam, Netherlands) attached to an SLR camera (Canon 660D; Canon, Tokyo, Japan) with an EF-S 60 mm *f*/2.8 macro lens (Canon, Tokyo, Japan). The Biluminator contained 8 blue LEDs (405 nm) and 4 white LEDs (6500 k). A >520 nm yellow cut-off filter was placed between the lens and the Biluminator. Images were captured in a dark room with standardised settings for blue and white light images; blue light images: 2592x1728 px, 1/40 s shutter speed, 8.0 aperture, daylight white balance, ISO 1600; white light images: 2592x1728 px, 1/40 s shutter speed, 11.0 aperture, manual white balance, ISO 160. The height between sample and camera was kept consistent for all samples.

The captures were made using the proprietary software (QLF Capture Software C3 v1.26; Inspektor Pro Research Systems, Amsterdam, Netherlands). As with the HI captures, the enamel discs were imaged before being placed in PTFE pans and irradiated acting as baseline images, and after they were completely air-dried post exposure to the CDFF. Using photo editing software (Paintshop Pro X9; Corel Corporation, Ottawa, Canada) before and after pictures were combined, no other edits were made to the images.

The baseline and post-experiment combined disc images were analysed using proprietary software (QLF Analysis Software QA2 v 1.26; Inspektor Pro Research Systems, Amsterdam, Netherlands) and used to produce a fluorescence loss value, ΔF (%), to correspond to mineral changes in the enamel. The difference in fluorescence between the baseline image and post CDFF exposure image was used for ΔF, the whole baseline disc acting as the sound region of enamel and the whole post experiment disc as the non-sound region.

Transverse Microradiography

The discs were cut into 4-5 sections of ~1.2 mm thickness using a precision diamond wire saw (Model 3241; Well Diamantrahtsagen GmbH., Mannheim, Germany). The sections were then mounted atop 11 mm custom made brass anvils and polished using a diamond encrusted grinding disc (Custom-made containing 15 µm sized particles; Buehler, Illinois, USA) to an 80 µm thickness. The sections were mounted onto an acetate template with the sound enamel sitting on the acetate and the lesion over empty space. The sections were covered with a thin X-ray film membrane to protect them. A window in the middle of the template allowed step wedge positioning for later calibration. The section covered template was positioned atop a 12-step aluminium step wedge, with the section side touching a high-resolution x-ray film plate (HTA Enterprises Microchrome Tech Products, CA, USA) and the emulsion layer facing the x-ray source. This was exposed to a CuKα X-ray source operating at 20 mA and 20 kV for a 12-minute exposure time. The plates were then developed and fixed in solutions according to

158 manufacturer instructions and dried before reading (Developer; EMS replacement for Kodak
159 Developer D- 19, EMS, PA, USA. Fixer; Ilford Rapid Fixer, Harman Technologies Ltd, UK). The
160 radiographic slides were examined using an optical microscope (BX51, Olympus, Tokyo, Japan) with a
161 DSLR camera fitted (EOS 550D, Canon, Tokyo, Japan) through the TMR 2000 Software (version
162 4.0.0.23, Inspektor Research Systems BV., Amsterdam). The images were analysed using the TMR 2006
163 Software (version 3.0.0.17, Inspektor Research Systems BV., Amsterdam) this provided values for
164 mineral loss (Vol%), lesion depth (µm) and integrated mineral loss (ΔZ; Vol%.µm).

165 ## Statistical Analysis

166 Statistical analyses were performed using SPSS for Windows Version 25.0 and Version 27.0 (SPSS UK
167 Limited, Woking, UK). Calculations for mean and standard error were performed in Excel (Office 365
168 Versions 1901 and 2109; Microsoft Corporation, Redmond, WA, USA). For statistical tests and
169 correlations, a 95 % certainty was applied, therefore the threshold for significance was $P \leq 0.05$. The
170 T-test was applied to find significance between the two conditions and Pearson's correlation
171 coefficient was used to compare the 3 analytical methods.

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Results

Hyperspectral Fluorescence Imaging Spectra Output

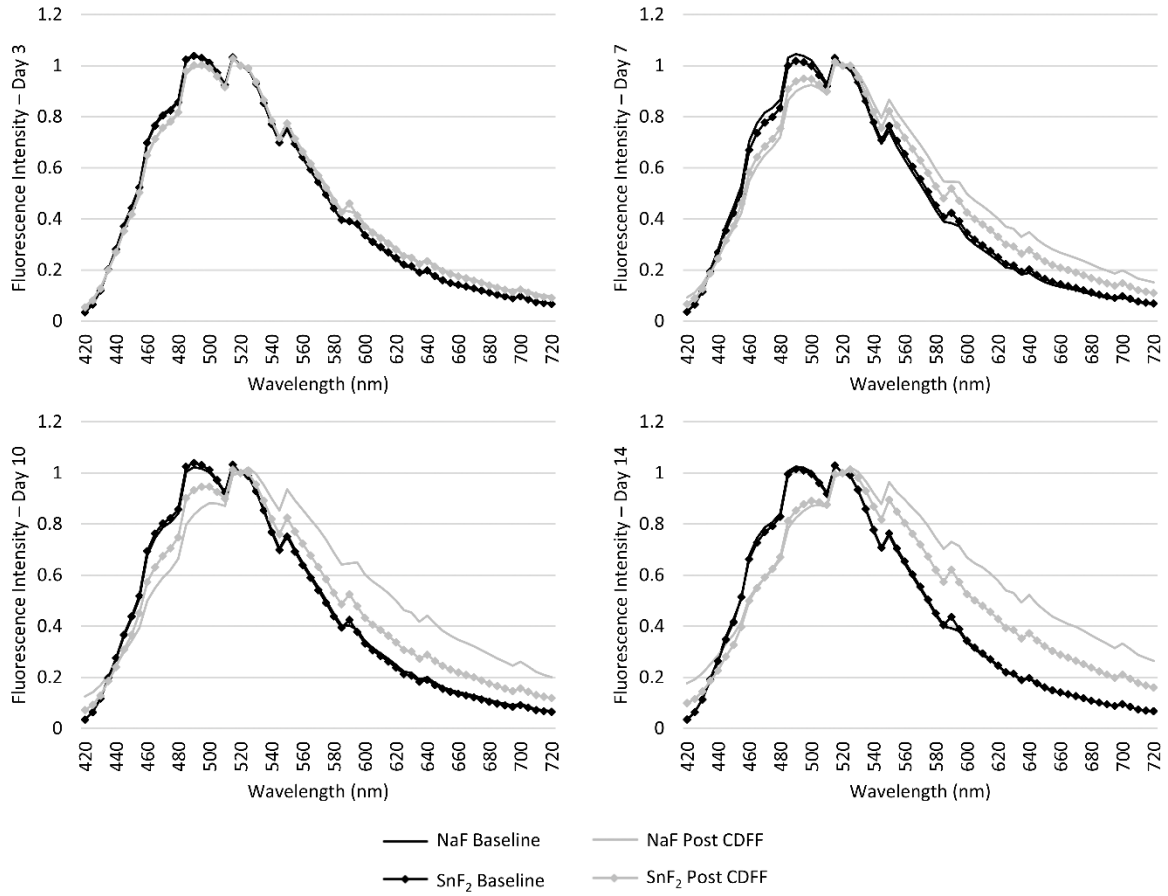


Figure 2. Spectra normalised to 520 nm was produced using hyperspectral fluorescence imaging of the discs before the CDFF run and then after CDFF exposure. Discs were exposed to either 228 ppm F- in the form of SnF_2 or NaF.

Images of the discs were taken before exposure to the CDFF to provide baseline readings (n = 40), the spectra for these (figure 2) aligned showing little difference between the discs placed into either the NaF or SnF_2 containing CDFFs. Each spectrum produced was normalised to 520 nm for comparison. The spectra graphs show a red shift of the spectra over the 14 days under both NaF and SnF_2 exposure (n = 10). The enamel exposed to NaF has a greater spectral shift to the right above 520 nm from the baseline capture to post CDFF exposure than SnF_2 exposed enamel

Mineral and Fluorescence Loss

	ΔF - NaF	ΔF – SnF_2	ΔF - P Val	ΔZ - NaF	ΔZ – SnF_2	ΔZ - P Val	ΔC - NaF	ΔC – SnF_2	ΔC - P Val
Day 3	35.54	35.51	0.993	133.00	104.67	0.263	11.78	15.05	0.16
Day 7	58.42	41.22	0.01*	595.17	315.33	0.0001*	27.90	19.57	0.031*
Day 10	67.80	48.99	0.001*	991.67	439.83	0.0001*	37.51	23.56	0.002*
Day 14	79.39	59.05	0.0001*	1277.67	833.33	0.0001*	45.02	33.70	0.001*

Table 1. Summary of mineral changes within enamel exposed to either NaF or SnF_2 over 14 days, analysed by HI (ΔC), QLF(ΔF) and TMR (ΔZ).

Table 1 summarises the changes to the enamel using the 3 analysis methods, TMR, QLF and HI. The mineral loss (ΔZ, n = 10) and fluorescence loss (ΔF, n = 10) data showed greater mineral changes under NaF exposure than SnF_2 for all 4 time points, however significant differences were only observed on days 7, 10 and 14. The shift in spectra (ΔC, n = 10) however showed a greater centroid shift under SnF_2 at day 3 but was not significant. This reversed from day 7 with significantly greater centroid shifts under NaF exposure.

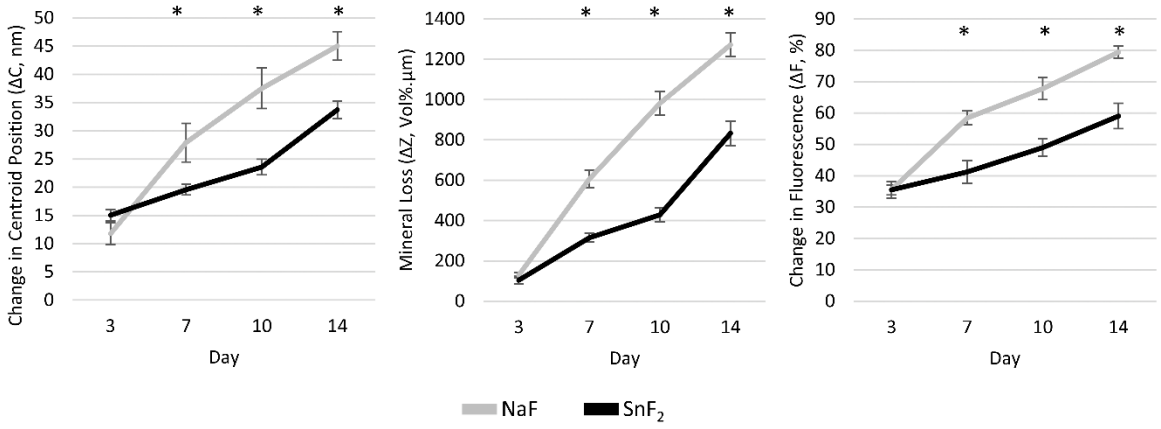


Figure 3: ΔC, ΔZ and ΔF changes over 14-days of exposure to 288 ppm F- delivered as either SnF_2 or NaF twice daily. Error bars represent standard error of the sample set (n = 10). An asterisk (*) denotes significant difference between the two conditions (P ≤ 0.05).

Correlations Between Methods

A statistically significant linear relationship (P = 0.001) between ΔF and ΔZ was observed (figure 4). The direction of the relationship showed the fluorescence loss and mineral loss are positively correlated with r = 0.844. Comparing TMR and HI (figure 5) also presented a statistically significant relationship (P = 0.001) and an overall strength of the relationship as strong (r = 0.844). Comparing QLF and HI (figure 6) also gave a significant relationship (P = 0.001) that is overall strong correlation (r = 0.811).

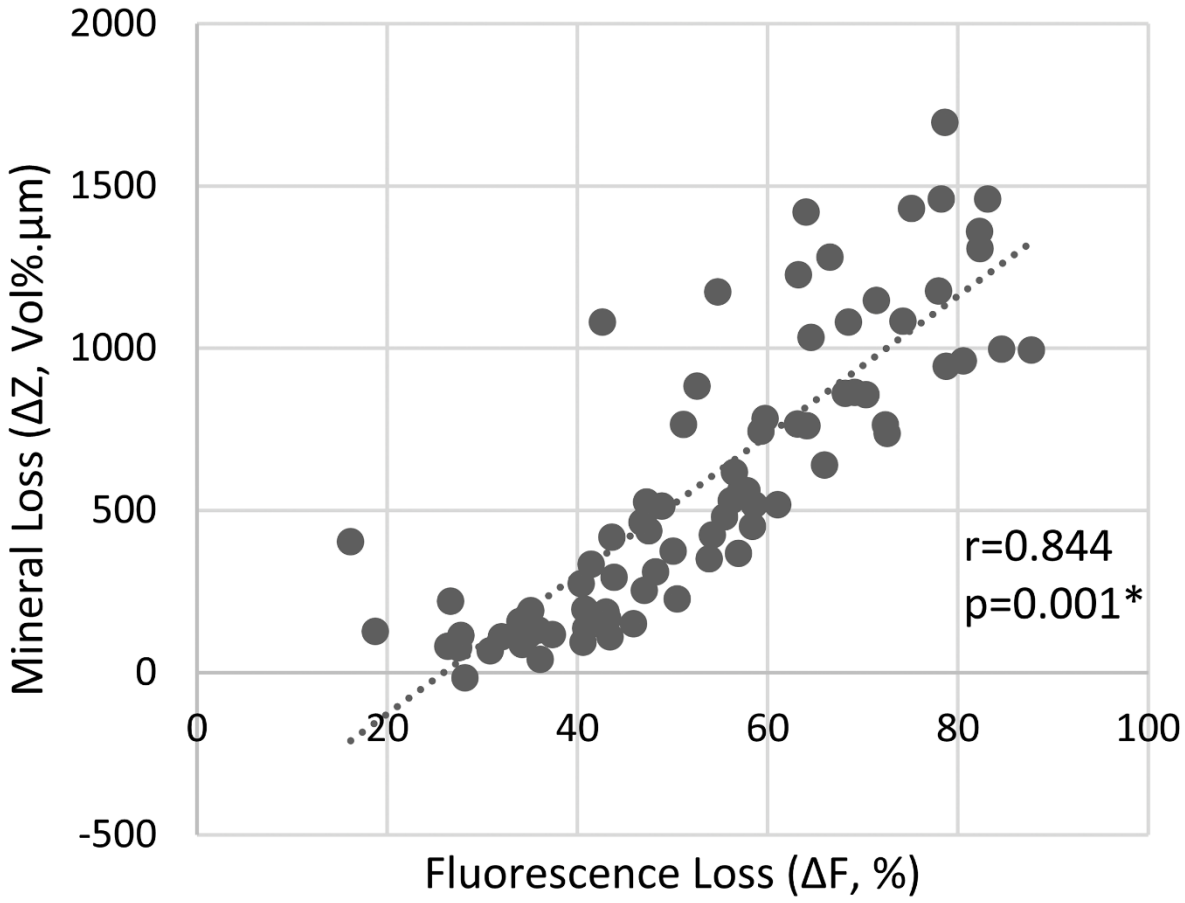
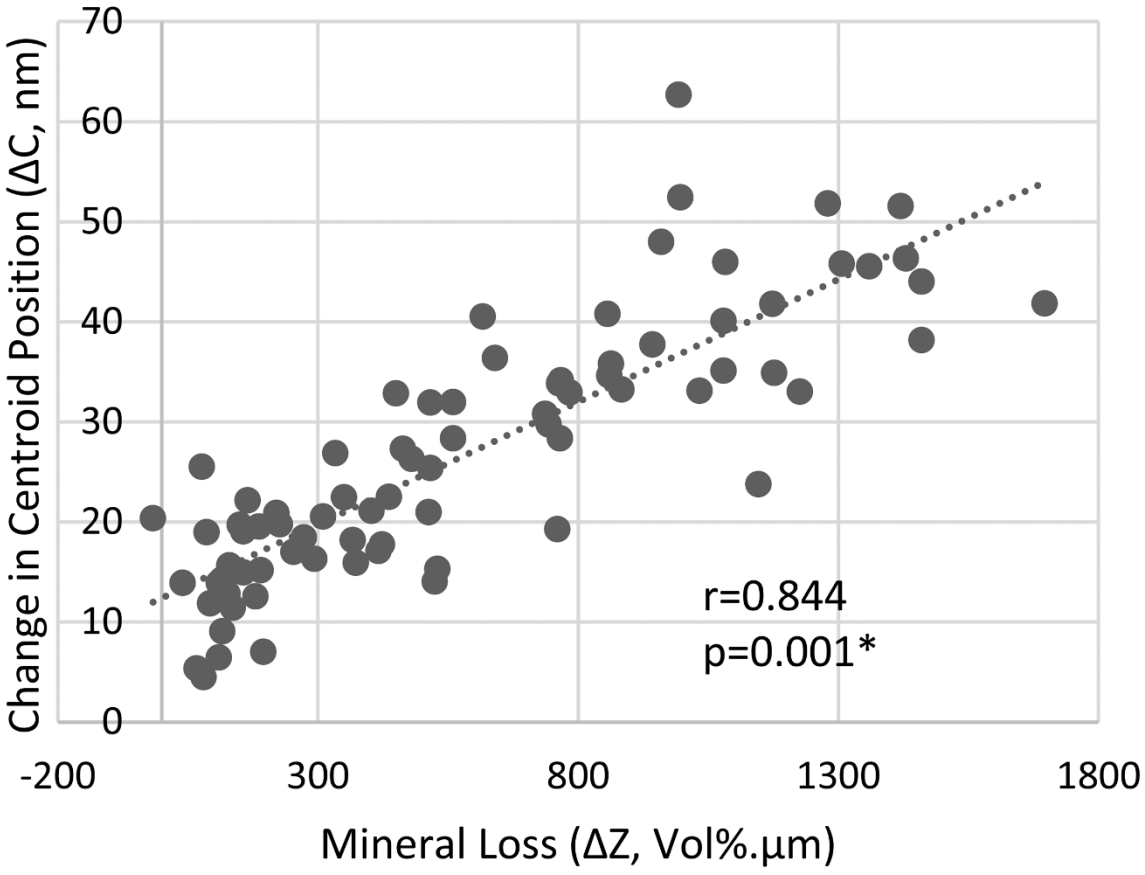


Figure 4: Correlation of data from the mineral loss (ΔZ) produced from TMR analysis and the change in fluorescence (ΔF) produced from QLF analysis, of bovine enamel demineralised within the dCDFF.



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213 *Figure 5: Correlation of data from the mineral loss (ΔZ) produced from TMR analysis and the change in fluorescence spectra*
214 *(change in centroid position, ΔC) from HI analysis of bovine enamel demineralised within the dCDFF.*

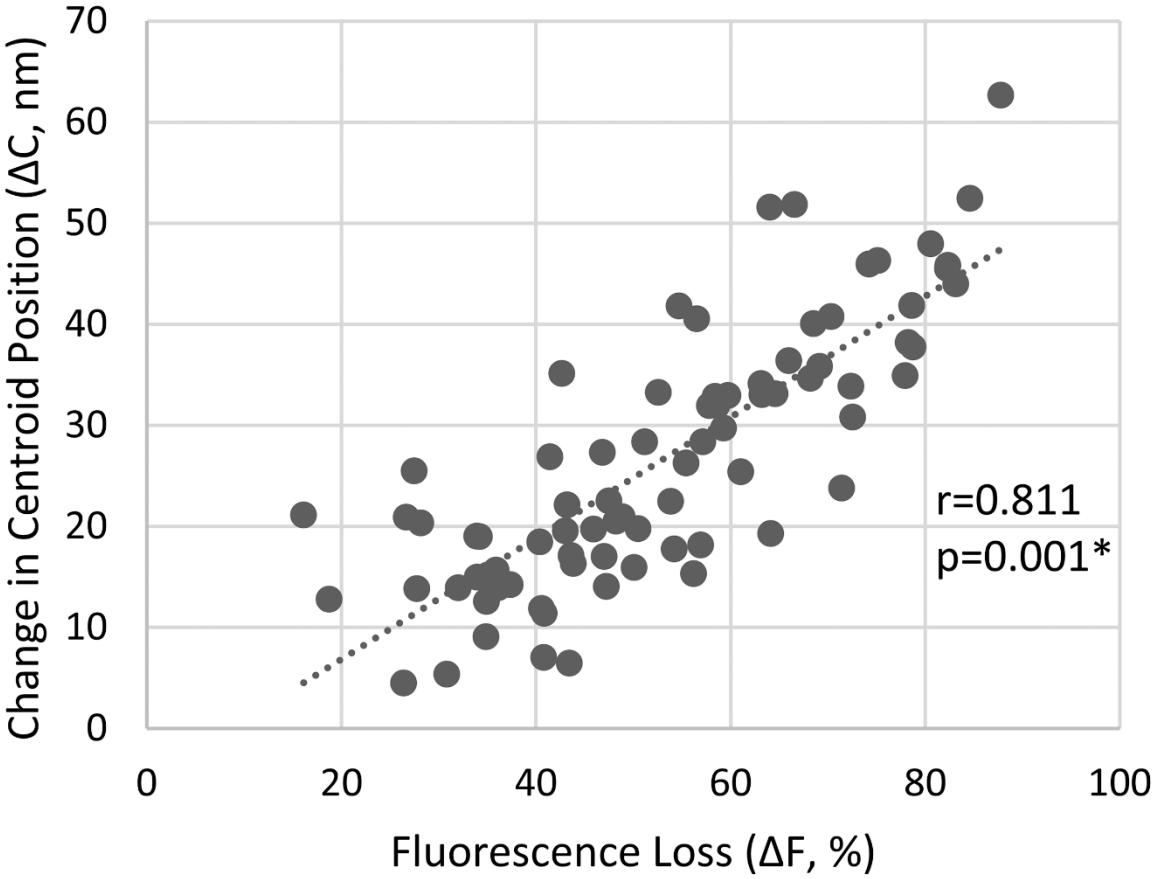

Figure 6: Correlation of data from the two methods of measuring change in fluorescence, HI (change in centroid position, ΔC) and QLF (ΔF), of bovine enamel demineralised within the dCDFF.

Discussion

It has previously been shown that there is a correlation between mineral loss from enamel and loss of autofluorescence. A previous study [2] analysed demineralised enamel using TMR and QLF and showed the relationship between the two methods was a strong linear correlation that was highly significant (P = 0.001, r = 0.84). In this current study the relationship between TMR and QLF was similar, with a statistically significant strong linear correlation (P = 0.001, r = 0.844) which further validates the link between these two established methods.

As previously stated it has been shown that mineral loss contributes to the red shift of the spectra [4,5]. The movement of the spectra towards higher wavelengths, red shift, indicates a lower energy

of the reflected light than at the lower wavelengths. This red shift by the spectra was used to calculate the change in fluorescence within the enamel. The spectral change was quantified by calculating the shift in the centroid position of the baseline spectra and the post dCDFF spectra, ΔC.

The change in red shift may not solely be due to structural changes of the enamel. For example, a previous study described a red shift of proteins containing tryptophans, due to the structure of the proteins being changed by folding and unfolding [12]. This may be occurring with other proteins present within the enamel that fluoresce within the 420 nm to 720 nm captured by HI. One of the main causes of protein denaturation is an increase or decrease in pH [13], so the acids produced by the bacteria within the CDFF may have been affecting the surface proteins.

HI has not been used to analyse enamel in a way similar to this study, but the strong relationship to more established methods shows potential as a valid *in vitro* mineral loss analysis method. HI overlaps with QLF methods as both quantify fluorescence changes within the enamel, so it was expected that there would be a strong relationship between the two. This study shows there was a significant relationship between the two ($P = 0.001$) that was positively linear ($r = 0.811$). This demonstrates that HI is at least as sensitive as QLF and is able to complement or replace the QLF method.

The HI results show an equally strong relationship with TMR ($r = 0.844$) as the relationship between QLF and TMR ($r = 0.844$), this shows the HI method is a valid means of measuring mineral changes and is an equal complement to TMR as QLF. The TMR and QLF results both show that SnF_2 had a greater efficacy than NaF for the reduction of mineral loss under an acidic environment. The significantly higher red shift under NaF correlates with this further, indicating the link between a shift in spectra and a change in the mineral structure and composition of enamel. One difference between HI and the other two methods was seen on day 3 (table 1), there was a greater shift for SnF_2, whereas all other results showed more mineral loss for NaF. Although this figure is not significant it may indicate that HI has a different sensitivity during early caries formation when the primary mineral loss is closest to the

surface. This however would need further investigation to compare the methods within the 0-to-3-day period.

Conclusion

Hyperspectral fluorescence imaging is an exciting novel method for analysing mineral loss from enamel due to its accuracy and non-destructive nature. The correlation between HI and TMR was equal to that of the already established QLF and TMR, with potential indication of a higher sensitivity during early caries formation. This is significant as it provides another complementary method to assessing mineral loss when there are few methods currently available. By using both QLF and HI the destructive nature of TMR may be avoidable, however the HI method like QLF does not provide depth data as given by TMR. Future work using HI should focus on surface changes such as erosion and early caries formation.

References

[1] M.-C.D.N.J.M. Huysmans, C. Longbottom, The challenges of validating diagnostic methods and selecting appropriate gold standards., J. Dent. Res. 83 (2004) C48-52. https://doi.org/10.1177/154405910408301s10.

[2] S. Al-Khateeb, J.M. ten Cate, B. Angmar-Månsson, E. de Josselin de Jong, G. Sundström, R.A.M. Exterkate, A. Oliveby, Quantification of Formation and Remineralization of Artificial Enamel Lesions with a New Portable Fluorescence Device, Adv. Dent. Res. 11 (1997) 502–506. https://doi.org/10.1177/08959374970110041801.

[3] M.H. van der Veen, E. de Josselin de Jong, Application of Quantitative Light-Induced Fluorescence for Assessing Early Caries Lesions, in: R. V Faller (Ed.), Assess. Oral Heal., Karger, Basel, 2000: pp. 144–162. https://doi.org/10.1159/000061639.

[4] R. Hibst, R. Paulus, New approach on fluorescence spectroscopy for caries detection, in: J.D.B. Featherstone, P. Rechmann, D. Fried (Eds.), Lasers Dent. V, 1999: p. 141. https://doi.org/10.1117/12.348341.

[5] R. Alfano, W. Lam, H. Zarrabi, M. Alfano, J. Cordero, Human teeth with and without caries studied by laser scattering, fluorescence, and absorption spectroscopy, IEEE J. Quantum Electron. 20 (1984) 1512–1516. https://doi.org/10.1109/JQE.1984.1072351.

[6] B.T. Amaechi, S.M. Higham, W.M. Edgar, Efficacy of Sterilisation Methods and Their Effect on Enamel Demineralisation, Caries Res. 32 (1998) 441–446. https://doi.org/10.1159/000016485.

[7] S.L. Kinniment, J.W.T. Wimpenny, D. Adams, P.D. Marsh, Development of a steady-state oral

microbial biofilm community using the constant-depth film fermenter., Microbiology. 142 (1996) 631–638. https://doi.org/10.1099/13500872-142-3-631.

[8] J. Pratten, Growing Oral Biofilms in a Constant Depth Film Fermentor (CDFF), Curr. Protoc. Microbiol. (2007) 1B.5.1-1B.5.18. https://doi.org/10.1002/9780471729259.mc01b05s6.

[9] B.B. Beiswanger, P.M. Doyle, R.D. Jackson, M.E. Mallatt, M. s Mau, B.W. Bollmer, M.M. Crisanti, C.B. Guay, A.C. Lanzalaco, M.F. Lukacovic, The clinical effect of dentifrices containing stabilized stannous fluoride on plaque formation and gingivitis--a six-month study with ad libitum brushing., J. Clin. Dent. 6 Spec No (1995) 46–53.

[10] C.K. Hope, K. Bakht, G. Burnside, G.C. Martin, G. Burnett, E. de Josselin de Jong, S.M. Higham, Reducing the variability between constant-depth film fermenter experiments when modelling oral biofilm, J. Appl. Microbiol. 113 (2012) 601–608. https://doi.org/10.1111/j.1365-2672.2012.05368.x.

[11] B. Angmar-Månsson, J.J. ten Bosch, Quantitative light-induced fluorescence (QLF): A method for assessment of incipient caries lesions, Dentomaxillofacial Radiol. 30 (2001) 298–307. https://doi.org/10.1038/sj.dmfr.4600644.

[12] J.T. Vivian, P.R. Callis, Mechanisms of tryptophan fluorescence shifts in proteins, Biophys. J. 80 (2001) 2093–2109. https://doi.org/10.1016/S0006-3495(01)76183-8.

[13] P. Hammarström, B.-H. Jonsson, Protein Denaturation and the Denatured State, in: Encycl. Life Sci., John Wiley & Sons, Ltd, Chichester, UK, 2005. https://doi.org/10.1038/npg.els.0003003.