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An in vitro assessment using transverse microradiography of the effect on mineral loss of etching enamel for in situ studies.

P.E. Benson¹
N. Pender²
S.M. Higham²

¹ Department of Oral Health and Development, University of Sheffield, UK.

² Cariology Research Group, Department of Clinical Dental Sciences, University of Liverpool, UK.

Short Title: mineral loss from etched enamel

Key words: orthodontics; demineralisation; enamel; transverse microradiography

Address for correspondence:

Dr Philip Benson
Senior Lecturer/Hon Consultant in Orthodontics,
Department of Oral Health and Development,
School of Clinical Dentistry,
Claremont Crescent,
Sheffield S10 2TA
UK

Tel: 0114 271 7885 (office)

0114 271 7895 (room)

Fax: 0114 271 7843

E Mail p.benson@sheffield.ac.uk

**An in vitro assessment using transverse microradiography
of the effect on mineral loss of etching enamel for in situ
studies.**

Structured Abstract

Objectives – To test the hypothesis that etching enamel with 37% phosphoric acid for 30 seconds does not lead to detectable mineral loss when measured with transverse microradiography.

Design – An in vitro laboratory investigation.

Experimental Variable – 40 bovine incisors were used in the experiment. The crowns of the teeth were covered with acid resistant varnish except for a rectangular area on the labial surface approximately 10mm x 12mm. On the exposed labial surface of 20 teeth an enamel lesion similar to that used in the in situ caries model was induced. Twenty teeth were left without a lesion. The exposed area was divided into 3 areas of equal size. The control area (C) was covered with acid resistant varnish throughout the experiment. The first experimental area (E1) was etched with 37% phosphoric acid for 30 seconds and a simulated bracket was bonded to the surface with composite resin. The second experimental area (E2) was left exposed for the remainder of the experiment. The teeth were placed in a demineralising solution for 24, 48, 72 or 96h to replicate different cariogenic challenges.

Outcome Measure – Mineral loss as measured with transverse microradiography.

Results – There were no significant differences in the mineral loss between etched (E1) and etched (C) areas of enamel. There were significant differences in mineral loss between E1 and E2 for the 48h ($p<0.001$) and 72h ($p=0.001$) exposures without a pre-formed enamel lesion.

Conclusion – There is no detectable mineral loss with TMR when enamel has been etched for 37% phosphoric acid for 30 seconds. The use of in situ enamel specimens with acid etch retained simulated brackets to investigate demineralisation during orthodontics will not significantly affect the outcome compared with unetched specimens.

Introduction

Demineralisation around brackets placed on teeth during orthodontics can be an unwanted sequelae of treatment¹. The in situ caries model system was used as a means of investigating the changes to enamel associated with brackets in the mouths of patients undergoing orthodontic treatment². The enamel samples, quantitated using transverse microradiography³ (TMR) showed both demineralisation and remineralisation of enamel around the bracket base. The change in mineral content of the in situ sample was monitored with a control sample, which was reserved from the experimental environment and was not subjected to any bonding procedures. These bonding procedures include etching of the enamel surface. The etching of specimens has been criticized because the control and experimental samples have been treated differently, i.e. one specimen has been etched and the other not. The results of the experiment might therefore be affected by the etching and not by the conditions in the mouth.

The aim of this study was to measure the mineral loss from enamel after acid etching using TMR. The study was not designed to measure any loss in enamel bulk. The null hypothesis was that there would be no difference in mineral loss as measured by TMR between an area of enamel that had been subjected to the acid-etch technique and an area that had not. Because in situ investigations include enamel samples with a pre-existing enamel lesion⁴, the effect of etching on enamel with a lesion was included in the protocol of this study.

Bovine enamel was used. The main advantage of bovine enamel is that the composition is less variable than human enamel and therefore, hypothetically a more consistent response would be expected⁵. Bovine enamel also has the advantage that it has a large, relatively flat surface and is more porous than human enamel leading to more rapid diffusion and lesion formation. Therefore, bovine enamel should show a significant level of mineral loss more

readily than human enamel⁶ and, for practical purposes, provides a surface large enough to juxtapose a series of sample areas.

Materials and Methods

Specimen Preparation

Forty bovine incisor teeth were used in the experiment. They were extracted from the jaws of freshly culled cattle and stored in water with a few grains of thymol to prevent bacterial contamination. The teeth were assigned to one of two groups, each of 20 teeth.

The allocation and protocol for the procedures carried out on the two groups of teeth is shown diagrammatically in Figure 1. To protect them from demineralisation, the crowns of the teeth were covered with three layers of an acid resistant varnish, except for a large rectangular area three times the size of an orthodontic bracket base on the buccal surface (Stage 1, Figure 1). The teeth in Group I (GI) were not exposed to an initial period of demineralisation. The teeth in Group II (GII) were prepared with a pre-existing enamel subsurface caries-like lesion by being attached to glass rods and placed individually in 10ml of a 40mmol/l acetic acid/potassium hydroxide buffer (pH 4.5) containing 2.2mmol.l⁻¹ of calcium chloride and potassium dihydrogen phosphate and 0.026µmol/l of sodium fluoride. The solution was stirred unchanged at room temperature for 72 hours, after which time the teeth were removed, thoroughly washed in distilled water, dried and a further coat of varnish applied to the buccal surface excepting the rectangular area previously described. Thus, GI had a lesion-free rectangular area and GII a pre-existing lesion in the rectangular area before the intervention protocol was employed.

Both groups of teeth were now treated in the same way (Figure 1). One third of the exposed rectangular area (C) was covered with three layers of acid resistant varnish. The whole of the remaining exposed area was etched with 37% phosphoric acid for 30s, thoroughly washed for 15s and dried with compressed air for a further 15s. A rectangular stainless steel

bracket base incorporating a mesh for bonding purposes (American Orthodontics, 1714 Cambridge Avenue, Sheboygan, WI 53081, USA), was bonded to the left edge of the exposed etched enamel (E1), the centre of the original rectangular area. The base had been previously contoured to the shape of the tooth surface. Bonding was with a no mix composite resin ('Right-on' TP Orthodontics, Inc., La Porte, Indiana, USA). Any excess composite was removed with a Ward's wax carver. The remaining portion of etched exposed enamel was left uncovered (E2).

A second demineralisation step was now carried out (Stage 2, Figure 1). All the teeth were attached individually to glass rods and placed in a fresh preparation of the demineralising solution, pH 4.5 was established at the beginning of this demineralisation phase, the solution was unchanged and continuously stirred. The teeth were stored in the solution, which was stirred at room temperature, and 5 teeth from each group (GI and GII) were removed after periods of 24, 48, 72 and 96 hours respectively. The different time periods were chosen to give increments of demineralising challenge.

After removal from the solution the teeth were washed in distilled water and varnish removed with acetone. The bracket base was carefully separated from the enamel with a sharp excavator, leaving the bonded composite in position.

The crown of each tooth was sectioned from the root with an Isomet saw (Buehler Ltd, Evanston, Illinois, USA). The saw was then used to cut the crown longitudinally on the C side of the bracket base area leaving a margin for analysis (Figure 1). This was done so that the sample could be orientated with regions C, E1, E2, in a set order, once it had been ground. The crown was then cut across the three regions C, E1, E2 into several sections each with a representative from C, E1 and E2. Each section was ground to 100 μ m using hand grinding against a diamond grinding plate and the thickness was checked with a micrometer

(Mitutoyo Corporation, Sakato, Japan), mid-way through the grinding process the sections were reversed in order to grind both sides to obtain a plano-parallel section. Three sections from each tooth were prepared, a maximum of 15 sections for each sub-group and total of 120 sections for the two groups. The sections were placed, in a known but random order, on a specimen holder that also contained an aluminium step-wedge, with 25 μ m steps. Each section was orientated with the flat edge, produced by the orientating saw cut, to the left. A diagram of the plate and the individual sections was produced. A high-resolution radiographic film (Kodak, Rochester, NY, USA) was placed in the specimen holder in a photographic dark room with a photographic safe red light. The specimens were radiographed for 18 min at 25kV and 10mA on Kodak high-resolution plate type 1A. The anode film distance was 30cm.

The microradiograph films were developed using a standard method. Briefly, Kodak HR developer (Kodak House, Hemel Hempstead, Herts, UK) and Kodak Unifix were used before a final 30min wash. The cassette was unloaded in 100% humidity. Both the film and diagram were re-coded by one investigator to allow for blind analysis by another investigator, who carried out all the assessments. The measurement of mineral loss (ΔZ) from each section, was carried out on a computerised image analysis system (TMRW program version 1.22) using an algorithm developed by de Josselin de Jong⁷.

Three regions were measured on each section designated (Figure 1):

- C Control - the area next to the orientating flat surface under the acid resistant varnish during the second stage of the experiment. This had not been exposed to the acid-etch technique.
- E1 Experimental Area 1 - the area under the orthodontic bracket base bonded to etched enamel.

E2 Experimental Area 2 - the area that remained exposed after etching throughout the experiment.

Samples were rejected if the composite, which represented the boundary between regions, could not be visualised. Three readings of each area were taken and these were averaged to obtain one reading representative of the whole area of each sample. The readings were made at a site distant from the edge of the area to avoid possible crossover of effects between treatments given to each area.

Three films, containing a total of 29 sections were randomly chosen for an error analysis. They were re-coded by one investigator, to allow a second blind assessment by a second investigator, at a time interval of at least two weeks after the first set of measurements.

Statistical Analysis

Statistical analysis was carried out using SPSS for Windows version 10 (SPSS Inc., 444 Michigan Avenue, Chicago, IL, USA). The data were checked for normality using frequency histograms of the differences between the groups, Normal Q-Q plots and the Shapiro-Wilk test. These data were considered to be normally distributed and therefore parametric statistics were applied.

The experiment was a mixed design with both within-sample and between-sample factors. The within-sample factor included the areas C, E1 and E2 that had been subjected to different conditions. The between-sample factors were firstly, whether there was a pre-existing lesion at the end of stage 1 or not and secondly, the length of time the exposed etch area was subjected to the demineralising solution. To avoid confounding the intra- and inter-sample variation, the data from replicate sections were averaged to give a mean mineral loss value for each tooth (N=5). A three-factor mixed analysis of variance was carried out. The results of this analysis showed that the within-sample analysis of mineral loss was highly significant ($p=0.001$), therefore a one-factor within-sample analysis of variance was performed for each group and exposure time to test the difference in mineral loss between

areas C, E1 and E2. The dependent variable was the mean mineral loss for each tooth. The independent variable of interest was the within-sample factor. Following the analysis of variance, pairwise comparisons of significant results were carried out using a paired t test with Bonferroni correction.

To assess reproducibility a one-sample t test was used to monitor any systematic error and the intraclass correlation coefficient of reliability between replicates was calculated to assess random error⁸.

Results

The error analysis of 29 sections shows the reproducibility of the method with no systematic error and a low random error (Table 1).

Many of the specimens subjected to 96h exposure to demineralising solution showed evidence of cavitation, which made measurement of mineral loss using TMR difficult. Because of this cavitation only the results from the 24h, 48h and 72h exposures were analysed. The means and 95% confidence intervals for the two groups, GI and GII, are shown graphically in Figure 2 and Figure 3 respectively. Mean mineral loss was greater for GII after two periods of demineralisation. Mineral loss in GII also showed greater variability. Table 2 shows the results of the one-factor within-subjects analysis of variance. This table shows that, within each exposure time sub-group, there were no significant differences between C, E1 and E2 for any of the demineralisation times in GII, with a pre-existing lesion and 24h GI without a pre-existing lesion.

Table 3 displays pairwise comparisons for the significant results from the within-subjects analysis of variance. The 48h and 72h exposures in GI gave a highly significant difference between the mineral loss from the exposed etched enamel (E2) and both the enamel under the acid resistant varnish (C) and etched enamel under the bracket base (E1). There was,

however, no significant difference between the enamel under the acid resistant varnish (C) and the etched enamel under the bracket base (E1).

Discussion

This study has shown no difference in mineral loss between etched and unetched bovine enamel, with or without a pre-formed caries lesion. This suggests that etching does not produce detectable mineral loss measured using TMR, a recognised method of quantifying mineral loss. This is important in the use of in situ modelling systems that incorporate etching of attachments onto enamel slabs². Studies designed to assess remineralisation of in situ models^{9,10} do not use etched attachments. However, longitudinal clinical trials of orthodontic iatrogenics around enamel slabs will need to incorporate etching in the protocol to be clinically relevant.

The overall mineral loss from enamel without a pre-existing lesion was generally less and with smaller variability than was the case in the presence of the pre-existing lesion. It may be, for this reason, that the sample used in this study with a pre-existing lesion did not demonstrate more mineral loss from the exposed etched enamel. The increased confidence limits for the mean mineral loss from the specimens with the pre-existing enamel lesion ensures that these specimens showed reduced sensitivity to further mineral loss after being placed in the demineralising solution. These results agree with Mellberg⁵, who considers that the choice of whether to use a sample with or without a pre-existing enamel lesion will differ according to whether the study is investigating the factors affecting lesion formation or is investigating the effects of treatment on remineralisation. In the oral environment, there is potential for both the remineralisation and the further demineralisation of enamel that bears a pre-existing lesion. The potential for remineralisation is greater if the enamel presented to the remineralising oral environment is initially more demineralised. Indeed, in vitro remineralisation of etched human enamel gives a greater reduction in lesion depth compared with non-etched enamel¹¹. The rationale for placing an in situ model with a lesion

present is that intact enamel may fail to demonstrate any detectable mineralising change, which would render the assessment of an experimental protocol inconclusive.

The techniques used in the present study were etching with 37% phosphoric acid for 30 seconds, which is the normal clinical method, and transverse microradiography, which has a limited ability to measure mineral loss in outer micrometers of a specimen. Therefore, it is well suited to the measurement of early lesions that is the major concern for orthodontic patients. Recently, transverse microradiography has detected acid erosion with 37% ortho-phosphoric acid at pH 3.0, which is a lower pH than the clinical method, for 15-minute exposures up to one hour on human enamel specimens that had been cut into 100 to 150 μ m sections¹². Mineral loss was detected with an exposure time of 30 minutes.

The sample size would appear to be small, however it was not possible to perform a sample size calculation prior to carrying out the experiment, because of the lack of available data. It is possible to employ data from the study to calculate the power of this study. Using the standard deviation of the unetched enamel from GI as an estimate of the standard deviation of the differences we can show that this study had a power of 80 percent to detect a difference of 200 vol%. μ m, which is acceptable.

The present study did not set out to examine the loss of enamel bulk, rather loss of mineral as measured by microradiography. It is notable that the in vitro exposure of the bovine enamel for 96h had the effect of destroying the structure to the point of cavitation such that microradiography was not possible. Several studies have shown loss of enamel thickness due to the process of acid etching, placement and removal of brackets and this is of relevance to the clinical practice of etching and bonding. Fitzpatrick¹³ found a loss due to 55.6 μ m as result of etching, bracket placement, bracket removal and clean up, attributing a mean loss of 9.9 μ m enamel due to the etching. Brown¹⁴ found loss of 3.0 μ m with etching

when comparing this with polishing alone with zirconium silicate and water on a rotating bristle brush for 10 to 15 seconds, which removed 26µm. It is pointed out that although this is a small amount it does eliminate the fluoride rich layer, which declines rapidly in the first 20µm. Both of these latter studies used the longer etching time of 90 seconds.

In conclusion the results of this study show that, using the technique of transverse microradiography, no significant additional detectable mineral loss was found when enamel was etched prior to bonding orthodontic brackets. Therefore the use of in situ enamel specimens with acid etch retained simulated brackets to investigate demineralisation during orthodontics will not significantly affect the outcome compared with unetched specimens.

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Tables

Table 1

Tables showing the mean difference (vol%. μm), standard deviation and confidence intervals for the difference between the repeat readings of the specimens (N=29). Also shown is a one sample t test to assess systematic error and the intraclass correlation coefficient of reliability to assess random error.

Mean Difference (vol%. μm)	-14.0
Sd	199.0
Confidence Intervals (vol%. μm)	-57.6 – 29.6
One sample t test (t)	-0.6
P value for t test	0.531
Intraclass correlation coefficient of reliability	0.911

Table 2

Table showing the results of the one-factor within-subjects analysis of variance to assess the differences between within-sample factors (the regions) for Group I (GI) and Group II (GII) and for the different exposure times to the demineralising solution (N = 5 teeth in each group).

Exposure Times (hours)	GI	GII
	P	P
24	0.131	0.214
48	0.001	0.421
72	0.034 ⁺	0.301 ⁺

⁺ more conservative statistic applied as heterogeneity of covariance detected.

Table 3

Results of the pairwise comparisons between the groups of teeth (* highly significant), which showed a significant difference in mineral loss (vol%. μm) for the within-sample factors (where C = control under the acid resistant varnish, E1 = under the orthodontic bracket base and E2 = exposed throughout the experiment).

Group (see Figure 1)	Regions Compared	Mean Difference	Sd	Confidence Intervals	P
GI48	C – E1	60.3	169.8	-33.7 – 154.3	0.191
	C – E2	-323.5	306.4	-493.2 – -153.8	0.001*
	E1 – E2	-383.8	304.1	-552.2 – -215.3	<0.001*
GI72	C – E1	-30.7	97.8	-87.2 – 25.8	0.261
	C – E2	-1422.5	1176.5	-2133.5 – -711.6	0.001*
	E1 – E2	-1412.1	1201.5	-2138.2 – -686.1	0.001*

Figures

Figure 1

Flow diagram showing the design of the experiment with the two main groups, Group I (GI) without a pre-existing caries lesion and Group II (GII) with a pre-existing caries lesion. There are four subgroups with exposure times to the demineralising solution (demin soln) of 24, 48, 72 and 96 hrs. A total of 120 sections were produced. The crown was covered with acid resistant varnish except for a rectangular window on the buccal surface. One experimental region, E1= [diagonal lines] was covered with an orthodontic bracket base. A second experimental region, E2= [white] was left exposed. A control region, C= [black] was coated with acid resistant varnish either at the start (G1) or after an initial period of demineralisation.

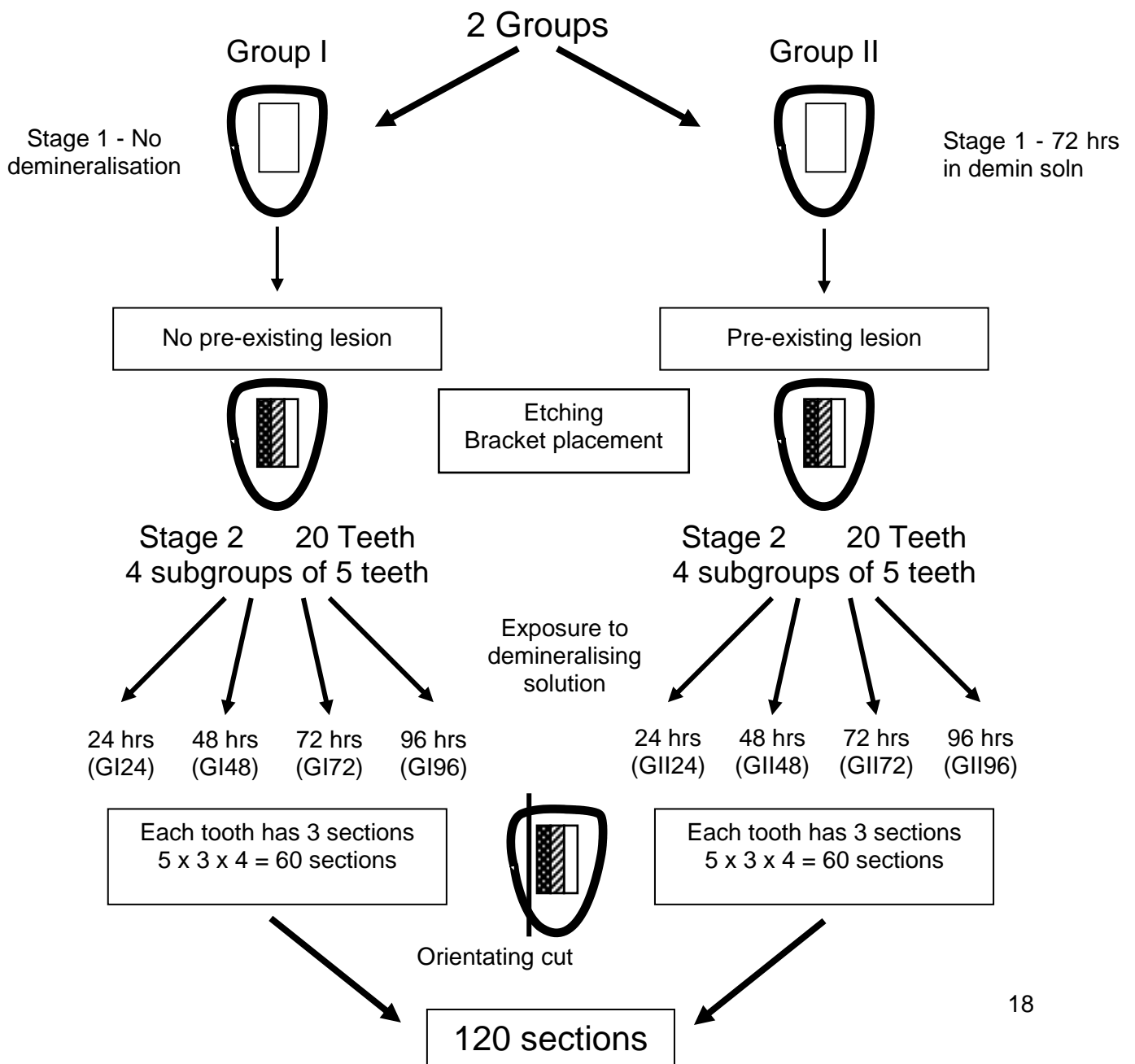


Figure 2

Mean mineral loss (vol%.μm) and 95% confidence intervals for the mean mineral loss in the samples without a pre-existing enamel lesion (Group I).

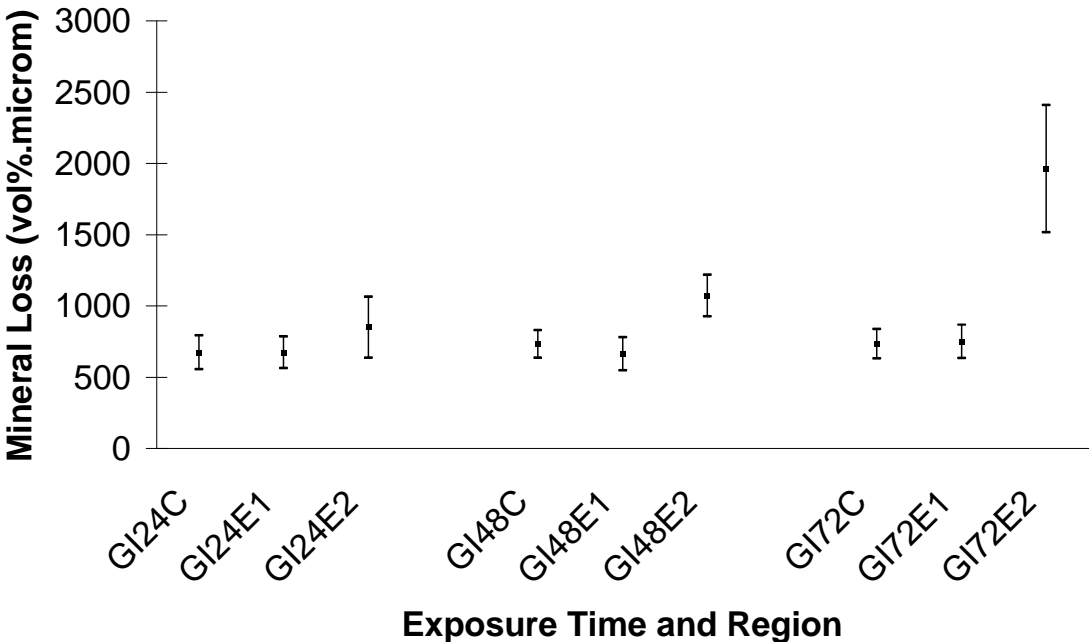
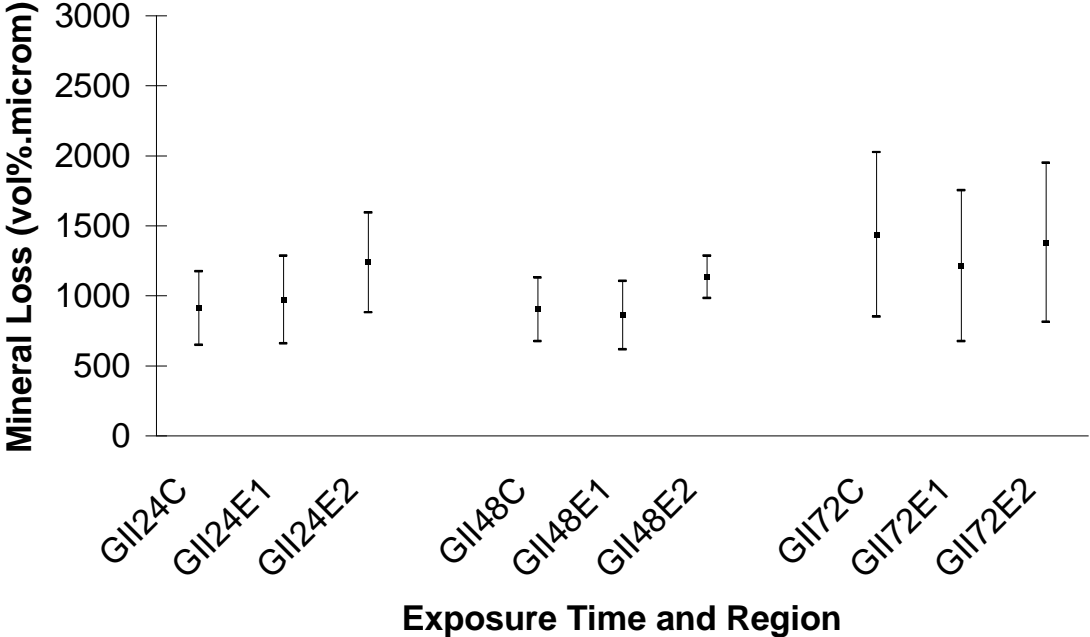


Figure 3

Mean mineral loss (vol%.μm) and 95% confidence intervals for the mean mineral loss in the samples with a pre-existing enamel lesion (Group II).



Legends

Tables

Table 1

Tables showing the mean difference (vol%. μm), standard deviation and confidence intervals for the difference between the repeat readings of the specimens (N=29). Also shown is a one sample t test to assess systematic error and the intraclass correlation coefficient of reliability to assess random error.

Table 2

Table showing the results of the one-factor within-subjects analysis of variance to assess the differences between within-sample factors (the regions) for Group I (GI) and Group II (GII) and for the different exposure times to the demineralising solution (N = 5 teeth in each group).

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Figures

Figure 1





Flow diagram showing the design of the experiment with the two main groups, Group I (GI) without a pre-existing caries lesion and Group II (GII) with a pre-existing caries lesion. There are four subgroups with exposure times to the demineralising solution (demin soln) of 24, 48, 72 and 96 hrs. A total of 120 sections were produced. The crown was covered with acid resistant varnish  except for a rectangular window on the buccal surface. One experimental region, E1=  was covered with an orthodontic bracket base. A second experimental region, E2=  was left exposed. A control region, C=  was coated with acid resistant varnish either at the start (G1) or after an initial period of demineralisation.

Figure 2

Mean mineral loss (vol%. μm) and 95% confidence intervals for the mean mineral loss in the samples without a pre-existing enamel lesion (Group I).

Figure 3

Mean mineral loss (vol%. μm) and 95% confidence intervals for the mean mineral loss in the samples with a pre-existing enamel lesion (Group II).