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Title: Effect of demineralising agents on organic and inorganic components of dentine

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Abstract (250 words)

There is a requirement to ensure that *in-vitro* studies that use demineralised human dentine models are reliable and clinically relevant. The literature reports several strategies for these studies with a lack of consensus on the mode of action of the different demineralising acids on human dentine.

This *in-vitro* study aims to characterise the effect of clinically-relevant acids on human dentine, using standardised substrates and complementary analytical techniques. The study focuses on an analysis of the mineral content and the integrity of the collagen following partial demineralisation.

Samples of human dentine were exposed to a range of acids commonly encountered in the oral cavity. Characterisation of the mineral content used Vickers micro-hardness, Energy dispersive spectroscopy and X-ray fluorescence. Characterisation of the collagen integrity was undertaken by means of Scanning electron microscopy and Hydroxyproline assay.

The following conclusions were reached: (i) Each demineralising agent tested had a unique effect on the mineral levels; (ii) Chelating agents, strong acids and weak acids affect the mineral and organic phases of dentine in significantly different ways with no correlation between them; (iii) The demineralising agents caused some degree of collagen denaturation, citric acid causing the most damage. Overall, there is no clear link between the type of demineralising agent and the effect on the organic and inorganic dentine. The choice of demineralising agent should be aligned to the experiment objectives so that the selected dentine (caries or erosion) model is fit for purpose.

Introduction

Human dentine is essentially a mineralised (calcium hydroxyapatite) collagen matrix (with non-collagenous proteins), that is arranged in a complex woven matrix around the processes of the odontoblast cells. The calcium hydroxyapatite mineral is subject to demineralisation through different processes that will in turn result in the collapse and degradation of the supporting collagen matrix. Understanding the mode of action, intensity and extent of the demineralisation processes of human dentine in-vivo, will enable the scientific community to establish predictable study models for in-vitro research of these processes and in this way seek effective repair or remineralisation strategies that are more readily translatable as clinical interventions.

In-vivo physiological demineralisation processes are caused by either dental caries or chemical erosion [Herschfeld and Miller, 1978; Nunn et al., 2003]. Both cause catastrophic loss of structure, with caries manifested in the cavitation and collapse of unsupported enamel and erosion in gradual large surface-area tooth surface loss. Whilst the aetiology of these processes is very different, they both cause demineralisation of the mineral scaffold in the hard dental tissues from continuous and/or high-frequency bathing of acids over time. In dental caries, the demineralising lactic acid is derived from the bacterial metabolism of fermentable carbohydrates. The acids responsible for chemical erosion tooth surface loss originate from exogenous sources, either dietary or gastric. That is, the frequent and continuous consumption of dietary acids (e.g.: sipping of soft drink beverages) or the continuous presence of hydrochloric (gastric) acid in the mouth that is associated with regurgitation of food (e.g.: bulimia) or oesophageal reflux (e.g.: gastro-oesophageal reflex disorder - GORD).

In the laboratory, we require reliable and robust demineralised dentine models to undertake studies with predictable clinically-therapeutic relevance. These

models need to be true analogues of the specific demineralisation process that occurs clinically.

The literature reports several strategies for preparing dentine models for remineralisation studies. It is of note, that there appears to be a generalised underlying assumption that any of the recognised demineralisation protocols is suitable for all studies; with a similar result on the dentine. Consequently, it has become standard to use laboratory acids such as formic acid [Eggert and Germain, 1979; Besinis et al., 2014; Kim et al., 2011], acetic and lactic acid [Moron et al. 2012; Lippert et al., 2015] or chelating agents such as ethylenediaminetetraacetic acid (EDTA) [Habelitz et al., 2002; Wang and Spencer 2002; Tartari et al., 2018, Gondolfi et al., 2019] to produce demineralised dentine models for use in in vitro remineralisation studies. Much of the current literature in this topic concentrates on the use of specific acids and chelating agents on root dentine (e.g. 17% EDTA, NaOCl and Citric acid) to simulate the effect of a root-canal treatment clinical procedure [Oh et al., 2015; Ramirez-Bommer et al., 2018, Salas López et al, 2019, Topbaş et al 2019, Unnikrishnan et al, 2019, Gondolfi et al. 2019, Baron et al., 2020]. With consistent findings that varying the concentrations of EDTA and citric acid affected collagen in a dissimilar manner.

In this context, it is important to recall the work by Featherstone and Lussi [2006] in which they suggest two mechanisms by which acids cause demineralisation of the dentine hydroxyapatite; dissociated hydrogen ions and anions binding to calcium. Hydrochloric acid causes demineralisation by dissociation of hydrogen ions whereas EDTA causes demineralisation by mechanism anions biding to calcium. Conversely, dietary citric acid can cause demineralisation by using both mechanisms. In addition to the noted variation in the effect of the acids studied, there is also a significant variation in the types of dentine used, varying between radicular [Oh et al., 2015; Turk et al., 2015, Salas López et al, 2019, Topbaş et al 2019, Unnikrishnan et al, 2019, Gondolfi et al. [2019, Baron et al., 2020] and coronal dentine [Gondolfi et al., 2019] and between human adolescent premolar teeth [Besinis et al., 2016, Unnikrishnan et al, 2019], incisor teeth [Topbaş et al 2019] and third molars [Shellis 2010,

Ramirez-Bommer et al., 2018] and bovine teeth [Moron et al. 2012; Lippert et al., 2015, Tartari et al., 2018, Salas López et al., 2019]. The duration of exposure to the acids also varies significantly between the cited studies.

From the reported literature, there is a lack of consensus on the mode of action of the different clinically relevant demineralising acids on human dentine substrates. This is required to increase the precision and validity of in-vitro studies performed. This in-vitro study aims to characterise the effect of clinically-relevant acids on human dentine, using standardised dentine substrates and appropriate analytical techniques. The study focuses on an analysis of the mineral content and the integrity of the collagen following partial demineralisation.

Materials and Methods

Sample preparation

The study was submitted to the Health Research Authority and received a favourable ethical opinion from a National Health Service (NHS) research ethics committee (reference 12/LO/1189). All experiments in this study involved healthy (non-carious), adult premolars that were extracted for orthodontic purposes. All participants gave full written consent for the use of their teeth in this study. After extraction, the teeth were immediately stored in 20 ml 0.1 % thymol (Alfa Aesar, Massachusetts, USA) at 4°C to prevent any microbial growth and were used within one month of extraction. Dentine blocks (6 x 4 x 2mm) were prepared from coronal dentine by sectioning with a slow speed precision blade saw (Buehler, Dusseldorf, Germany) with a diamond wafering blade, following the removal of pulpal tissue (via root apex) and the enamel. The blocks were placed in a sonicator (Grant Instruments, Cambridge, UK) for 5 minutes to remove any cutting debris.

Prior to demineralisation, dentine blocks were first fixed by immersion in 3 % glutaraldehyde (Sigma Aldrich, Poole, UK) in 0.1 M cacodylate buffer (pH7)

(Sigma Aldrich, Poole, UK) at 4°C for 24 hours. Prior work by the research group had shown that non-fixed specimens did not retain their structure, therefore preventing analysis of mineral content and collagen integrity and is in concordance with prior studies (Unnikrishnan et al, 2019).

After fixation, the specimens were washed with 0.1 M cacodylate buffer (pH7) (2 x 2 minutes) and distilled water (2 x 2 minutes) to remove any remaining fixative. Specimens were blot dried and nail varnish (Max Factor, Cincinnati, USA) was painted onto five surfaces so that only one surface was exposed to 10 ml demineralising solution for up to 14 days (refreshed every 24 hours); the pH was monitored and adjusted as required during after each immersion, in line with previous published studies [Shellis, 2010]. Seven demineralising agents (Sigma Aldrich, Poole, UK) and distilled water as a control were used (Table 1). Demineralisation for all groups was terminated by removing the specimens from the acid solutions and washing in distilled water (2 x 2 minutes) and 0.1 M cacodylate buffer (pH7)(2 x 2 minutes) before being stored in 0.1 M cacodylate buffer (pH 7)at 4°C. Subsequent sample preparation was dependent on the characterisation technique being implemented and is described in the following sections.

Analysis of Mineral Content

(a) Micro-hardness

Micro-hardness was used to measure the surface hardness to give an indication of the mineral content of each of the seven demineralised dentine models at baseline (day 0, before demineralisation) and at day 1, 2, 7 and 14 of demineralisation with measurements taken in triplicate. Sample preparation involved embedding the dentine blocks into Kleer-set FF resin (Metprep, Coventry, UK) and gently grinding the surface until it was level using wet silicon carbide papers, p1200 and p2400, without a lubricant, on a grinder-polisher wheel at 100rpm (Buehler Metaserv, Dusseldorf, Germany). Specimens were stored in cacodylate buffer (pH7) until use.

Micro-hardness analysis of the dentine surface was performed using a Mitutyo HN810 instrument (Hampshire, UK) with a Vickers diamond indenter. A load of 300g was applied to the specimens with a dwell time of 20 seconds. Micro-hardness was expressed in terms of the Vickers Hardness Number (VHN). The VHN of a specimen at each time point was based on the mean of 8 indents with the minimum distance between indentations >100 µm and the outer 0.5 mm of each sample was not tested.

(b) Energy dispersive spectroscopy (EDS)

The presence of calcium (Ca Kα, 3.692 keV) and Phosphorous (PKα, 2.010 keV) was measured using energy dispersive spectroscopy (EDS), for each of the seven demineralised dentine models and control (n=3 per group). EDS measurements were taken at 4 different parts of the area of analysis on each dentine block, which were prepared as per the micro-hardness samples and then subsequently dehydrated by immersion into graded ethanol solutions and hexamethyldisilazane (HMDS) (Sigma Aldrich, Poole, UK). The specimens were then air-dried in a fume cupboard overnight. Once dehydrated, the blocks were carbon coated (Emitech, Molfetta, Italy) to enable EDS analysis (Jeol 6400, Massachusetts, USA) with an accelerating voltage of 20 kV and spot size 3. Element peak intensities were quantified against a cobalt standard as the base for percentage; x-ray peak Kα, 6.931 keV and density 8.86 g/cm^3.

(c) X-ray fluorescence

X ray fluorescence (XRF) was used to assess the levels of calcium (Ca) and Phosphorous (P) in the dentine specimens. Eight dentine specimens were randomly assigned to a demineralisation group or control group (as outlined above) and were prepared for XRF. Samples were prepared by removing the protective varnish with acetone and grinding (pestle and mortar) the remaining demineralised dentine. The specimen (0.4g ground dentine) was then fused with 8g lithium tetraborate at 1200°C to produce a glass disc. The glass disc was placed into the Phillips PW 2440 XRF instrument for subsequent XRF analysis.

Analysis of Collagen Integrity

(a) Scanning Electron Microscopy

SEM was employed to observe the collagen structure in each of the seven demineralised dentine models and control (n=3 per group). Preparation involved staining the specimens with 0.1 % osmium tetroxide (Sigma Aldrich, Poole, UK) for 1 hour prior to dehydrating as per EDS samples. Specimens were gently air dried in a fume cupboard over a 12-hour period. The blocks were attached to aluminium pin stubs using carbon sticky tabs, coated with silver dag (Agar Scientific, Essex, UK) and then gold spluttered using an EM SCOPE 500 (Quorum Technologies, Sussex, UK). Specimens were analysed using an Inspect F SEM (FEI™, Oregon, USA).

Four areas of each specimen were viewed at a range of magnifications between x1000 and x80000 viewing both the intra- and inter-tubular dentine. When measuring collagen fibre dimensions measurements were taken from 5 areas on 3 high magnification (80000x) images. Measurements were taken with ImageJ software version 1.46 (Open access, NIH, USA).

(b) Hydroxyproline Assay

The hydroxyproline (Hyp) assay was performed on demineralised dentine blocks from each demineralisation group in order to quantify the amount of denatured collagen. The demineralised dentine samples were transferred into 1 ml PBS at pH 7 prior to being incubated at 37 °C with gentle shaking for 24 hours in 20 mg trypsin (treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone, (TPCK)) (Sigma Aldrich, Poole, UK) in 1 ml PBS at pH 8. Dentine blocks were then rinsed with 1 ml PBS pH 8. Each sample solution (100µl) was transferred to a pressure-tight vial and incubated at 120 °C for 3 hours with 100 µl of 12 N hydrochloric acid (Sigma Aldrich, Poole, UK). Activated charcoal (5 mg) (Sigma Aldrich, Poole, UK) was stirred into the solutions before undergoing centrifugation at 13000 x g (Sigma Aldrich, Poole, UK) for 2 minutes. Each supernatant (10µl) was added to a 96 well plate (Greiner BIO-One, Stonehouse, UK) and the quantity of Hyp was measured per mass of sample,

against Hyp standards, using a spectrophotometer at 560nm (Tecan, Switzerland).

Statistical Analysis

All data was checked for normality using the Shapiro–Wilk test. If normally distributed, then a one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test was used to determine the significance between groups ($p < 0.05$). If not normally distributed then a Kruskal-Wallis H Test was used and Dunn's post hoc analysis with Bonferonnii adjustment was performed to determine the significance between groups ($p < 0.05$).
All statistical analysis was performed using the SPSS 20.0.0 statistical software package (IBM, Chicago, USA).

All graphs display standard deviation in the error bars.

Results

Micro-hardness

The Vickers hardness numbers (VHN) were normalised against their baseline before demineralisation (fig.1), showing that the surface hardness of every specimen decreased over the 14-day period. By day one, there was little difference in surface hardness between each specimen (VHN ranged from 41–48) but by day two the hardness values ranged from 33 (lactic acid treated specimen) to 47 (EDTA treated specimen). From day one the specimens began to develop different trends; the surface hardness of the lactic acid specimen declined rapidly whilst the surface hardness of the other specimens appears to have decreased less considerably. After 14 days, the surface hardness of lactic acid specimens had decreased by approximately 80% yet the surface hardness of citric acid specimens had only decreased by approximately 40%, from baseline values.

Figure 2 displays the Vickers hardness values of the specimens after 7 days in the demineralising agents. There was a significant difference ($p< 0.05$) between

lactic acid treated specimens compared to specimens treated with hydrochloric acid, acetic acid and citric acid specimens. There was also significant difference ($p<0.05$) between citric acid treated specimens compared to specimens treated with lactic acid, phosphoric acid, EDTA and formic acid.

Energy Dispersive Spectroscopy and X-ray Fluorescence

Energy dispersive spectroscopy (EDS) and x-ray fluorescence (XRF) analysis was undertaken at day 7 of demineralisation. Despite using a cobalt standard, the EDS results were considered to be qualitative, as the specimen surfaces were not mirror flat. Figures 3 and 4 display the EDS results showing the presence of calcium (Ca) and Phosphorous (P) in the dentine specimens. The levels of Ca and P in the control samples were approximately 50 wt.% and 30 wt.%, respectively and these values were reduced in all demineralised specimens.

There was a significant reduction in Ca levels between the control specimens and all other specimen groups except for the citric acid treated specimens.

The biggest change in P levels was observed for lactic acid, however, there was no significant difference between the lactic acid treated specimens compared with the specimens demineralised with either phosphoric acid, EDTA, hydrochloric acid or acetic acid. Again, citric acid appeared to have the least effect on P levels. However, the citric acid treated specimens only showed a significant difference compared with lactic acid and hydrochloric acid treated specimens. Specimens demineralised with citric acid, formic acid and phosphoric acid all showed no significant difference compared with the control specimen.

Figure 5 displays the XRF results which also indicated a reduction in Ca and P levels following demineralisation. Similarly to EDS, XRF results also appear to demonstrate that citric acid caused the least change in Ca and P content. However, as XRF analysis was only performed once per sample, statistical analysis could not be performed.

Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to observe the collagen structure of the dentine surfaces following a seven-day exposure to the various demineralising agents and non-demineralised control specimens (fig. 6). The effect of specimen dehydration for SEM examination was minimised by using a gentle dehydration protocol that avoided air-drying. In these Figure 6(a) is a low magnification image that shows the geometry and microstructure of the sample surface, images (b) and (c) focus on a dentine tubule to allow for examination of the collagen sub-surface. Findings from the SEM analysis are detailed in Table 2. The collagen fibre widths are presented in Table 3. The average width of the collagen fibres ranged from approximately 20nm to 177nm.

Hydroxyproline Assay

The results from the hydroxyproline (Hyp) (fig.7). The results suggest that all of the acids caused some collagen to be denatured. A one-way ANOVA statistical test with Tukey's post-hoc analysis showed that there was a significant difference between all the groups except between EDTA treated and acetic acid treated specimens. All demineralised specimens differed significantly from the control group.

Discussion

Bacterial acids (e.g. lactic acid), dietary acids (e.g. citric acid, phosphoric acid and acetic acid) and gastric acid (hydrochloric acid) all demineralise the dentine through the processes of caries and acid-erosion. In-vitro remineralisation studies require artificially demineralised dentine models which are often created using laboratory agents, such as formic acid or EDTA, under the presumption that that these have the same effect on the dentine as the erosive and caries related acids.

The aim of this study was to challenge this preconception by characterising and comparing the effects of these acids on the inorganic and organic components of dentine. In this study we used a range of demineralising agents: Bacterial

metabolism (lactic acid), dietary origin (citric acid, phosphoric acid, acetic acid), gastric acid (hydrochloric acid) and laboratory acids (EDTA and formic acid) to create a variety of partially demineralised dentine models. Prior to demineralisation, dentine blocks were first fixed by immersion in 3% glutaraldehyde. There is a recognition that whilst this protocol did not replicate in vivo conditions; but since all samples were demineralised using the same protocol, within study comparisons were able to be made. Prior work in the research group had shown that non-fixed specimens did not retain their structure, therefore preventing analysis of mineral content and collagen integrity. Characterisation techniques were employed to assess and compare the composition and structure of these dentine models to investigate whether the demineralising agents affected dentine in the same way, previously never considered. The pH was kept the same because there is evidence that pH is a determining factor in demineralisation ability. Therefore pH was kept constant to allow comparison of the acids [Featherstone and Lussi, 2006]. This was a proof of principle study to determine the effects of various acids in line with laboratory dentine remineralisation models that will normally use acids in a pure form [Besinis et al., 2014; Kim et al., 2011; Wang and Spencer, 2002; Turk et al., 2015; Oh et al., 2015; Ramirez-Bommer et al, 2018; Tartari et al., 2018; Gondolfi et al., 2019]. As such, the study did not consider other components, including naturally or artificially added buffers, plaque or saliva. The acids were not specifically selected for their pH, but as being representative of common dietary/gastric acids that are encountered in the mouth or that have been used in previous in-vitro studies. The pH was measured for the selected acid to provide a context to the findings from the data analysis.

The effects on mineral content

The mineral content of partially demineralised dentine was determined by means of energy dispersive spectroscopy (EDS). X-ray fluorescence (XRF) was used to indicate the levels of calcium and Phosphorous following demineralisation and micro-hardness test to compare the levels of mineralisation. There is increasing evidence that remnant mineral is essential for further remineralisation of dentine to occur [Tay and Pashley, 2008; Tay and

Pashley, 2009], highlighting the importance of the need to characterise and assess the remnant mineral in a variety of artificially demineralised dentine models; a study hitherto not undertaken previously.

The analyses of the mineral content of the various demineralised dentine models led to the following findings: (1) each demineralising agent appeared to affect the mineral content differently and (2) the results did not verify which demineralising mechanism was most effective. These points are considered in the following discussion.

(1) Each demineralising agent affected the mineral content differently. Micro-hardness, XRF, EDS and a phosphate assay were all used as an indication of the mineral levels in dentine before and after demineralisation showing that all acids caused a reduction in mineral content resulting in partially demineralised dentine, but did so at differing levels according to the acid used.
In particular, all techniques were consistent in showing that lactic acid caused the most demineralisation, the same conclusion as Moore et al. [1956], who showed that lactic acid, produced by oral bacteria, is the most powerful demineralising agent, causing the greatest loss of mineral. However, in 1981 it was suggested that oral bacteria can also produce some quantities of acetic acid and it was speculated, without verification, to be just as damaging to the tooth surface [Featherstone and Rodgers, 1981]. The results from this current study did not verify whether acetic acid was as damaging to mineral content as lactic acid. Although EDS data (fig. 3 and 4) did not show any significant difference in calcium or Phosphorous levels between specimens treated with the two acids, micro-hardness results (fig. 2) showed significant difference comparing acetic acid and lactic acid, with lactic acid being one of the most effective demineralising agents and acetic acid appeared to be one of the least effective demineralising agents.

The micro-hardness (fig.1 and 2) and EDS (fig. 3) results suggest that citric acid was the least effective demineralising agent because it appears to have caused the least reduction in hardness with no significant difference compared with the control specimen. Table 1 shows that citric acid is a weak acid (pKa 3.13) with

a chelating ability (LogKCa 1.10) which would suggest that it would be effective at removing calcium from the dentine. However, the calcium levels had not reduced significantly (EDS data, Fig. 3). All the other acids resulted in a range of values that were all significantly lower than the control and citric acid treated specimens. The EDS data showing the Phosphorous levels (Fig. 4) also showed no significant difference between the citric acid specimens and the control specimens, suggesting that citric acid was not very effective at removing neither the calcium nor the Phosphorous.

XRF data (fig. 5) showed similar findings to the EDS data, where the acids did not have the same effect as each other and that citric acid appeared to be the least effective.

(2) The results do not confirm which demineralising mechanism is the most effective. There are two mechanisms that cause demineralisation, one is the dissociation of hydrogen ions and the other is the chelation of calcium ions. Part of this investigation was to determine whether one mechanism was more effective than the other.

Hydrochloric acid was the only strong acid investigated. As illustrated in Table 1, because hydrochloric acid is a strong acid it has an extremely low pKa value of -9.30 and fully dissociates into hydrogen ions when in solution. Regarding all of the demineralising agents assessed in this study, hydrochloric acid had the strongest ability to cause demineralisation by this dissociated hydrogen ion mechanism. Furthermore, hydrochloric acid does not have a logKCa value because it does not have the ability to chelate calcium ions. It can only cause demineralisation through its ability to dissociate into hydrogen ions.

On the other hand, EDTA was the only demineralising agent that was investigated which can only cause demineralisation by the chelation mechanism. EDTA has a logKCa value of 10.70 and does not have a pKa value because it does not dissociate into hydrogen ions (Table 1). Regarding all of the demineralising agents used in this study, EDTA had the strongest ability to cause demineralisation via the chelation mechanism because it had the highest

logKCa value. All of the other demineralising agents had the ability to cause demineralisation by both mechanisms.

Due to the way that the different mechanisms work, it could be assumed that a chelating agent (EDTA) would cause the biggest reduction in calcium ions and that a strong acid (hydrochloric acid) would cause the greatest reduction in Phosphorous. However, according to the data analysed from micro-hardness and EDS (fig.1-4), there was no significant difference in calcium or Phosphorous content between specimens treated with EDTA and specimens treated with hydrochloric acid. It was only the phosphate assay (fig. 7) that showed hydrochloric acid causing more phosphate to be released from dentine and EDTA causing the least.

Based on the dissociation of hydrogen ions mechanism, it would be expected that hydrochloric acid (pKa -9.30) would be the most effective followed by phosphoric acid (pKa 2.15) and citric acid (pKa 3.13) but this trend was not observed in any of the experiments. Regarding the chelating mechanism, it would be expected that EDTA (LogKCa 10.70) would be the most effective, followed by lactic acid (LogKCa 3.86) and then phosphoric acid (LogKCa 1.40). The only time this trend may be implied was in the EDS calcium results (fig. 3) where lactic acid and EDTA showed the biggest reduction in calcium. However, this cannot be statistically verified as they were not significantly different to any other variables except citric acid (and the control).

There were five demineralising agents that were all able to cause demineralisation using both mechanisms (Table 1) but there was no evidence to suggest that this allowed greater demineralisation to occur.

The effect on collagen integrity

The second objective, to characterise the integrity of acid-affected collagen in the demineralised dentine models, was carried out by scanning electron microscopy (SEM) to visually characterise the exposed dentine collagen network and a hydroxyproline (Hyp) assay to quantify any collagen that had

denatured. In-vitro remineralisation studies report on the need to have an intact collagen structure to replicate the outer zones of carious lesions which often contain undamaged collagen [Deyhle, 2011; Gower, 2008; Olszta et al., 2003; Forsback et al., 2004; Vollenweider et al., 2007].

The analyses of the collagen integrity of the various demineralised dentine models led to the following findings: (1) Each demineralising agent affected the collagen structure and there were signs of collagen denaturation; (2) There was no obvious correlation between demineralising mechanism and effect on collagen integrity; (3) Citric acid appears to have had a unique effect on collagen integrity. These points are considered in the following discussion.

(1) Each demineralising agent affected the collagen structure and there were signs of collagen denaturation. It is suggested that collagen may be degraded by unspecific bacterial proteases during caries [Kleter et al., 1998; Tjaderhane et al., 1998]. However, it is not clear whether collagen is denatured in the absence of bacteria, e.g. in erosion or artificially demineralised models. Some studies have suggested that acids have an indirect denaturing ability because they can activate matrix metalloproteinases (MMPs) which can lead to collagen degradation. In addition, Zhang et al. [1998] have suggested that a low pH solution may denature proteins but there is little evidence as to whether this applies to collagen, especially because helical collagen has such high rigidity and strength [Ramachan and Sasisekharan, 1961].

SEM analysis showed that collagen fibre banding patterns were present on specimens after treatment with lactic acid or hydrochloric acid. Collagen banding patterns are formed from the assembly of collagen on a macromolecular level, so it can be indicative of native, un-denatured dentine and it is possible that denatured collagen would lose its banding patterns due to breakage of the bonds within the banding pattern. The fact that banding patterns were present on specimens, but the Hyp assay showed signs of denaturation, suggests that the dentine specimens contained a combination of denatured and non-denatured collagen. Another theory is that collagen denaturation may occur within smaller bonds (not within the banding patterns)

and that collagen may be denatured whilst maintaining its cross-links and banding patterns. This could have been facilitated by the use of glutaraldehyde which stabilises the cross-links.

(2) There was no obvious correlation between demineralising mechanism and effect on collagen integrity. Although all of the demineralising agents had a different effect on the collagen, they all caused some degree of denaturation. There appeared to be no correlation between the mechanism of demineralisation and the integrity of the collagen. We did not find that there was evidence to suggest that weak acids, strong acids or chelating agents were more destructive to the collagen than others.

In addition, SEM analysis did not show any relationship between demineralising mechanism and collagen integrity. Only dentine demineralised with lactic acid or hydrochloric acid showed fibres with banding patterns. However, these two acids did not cause demineralisation in the same way, as displayed on Table 1; the properties of the acids differ, lactic acid being a weak acid with chelating ability. The collagen fibre widths, displayed in Table 3, also did not show any relationship between the fibre sizes and type of demineralising agent.

(3) Citric acid appears to have had a unique effect on collagen integrity. The behaviour of citric acid during this study was particularly interesting due to its contrasting effects on mineral content and collagen integrity. The Hyp assay showed high levels of denaturation after citric acid and the SEM images (Fig. 6) showed that the dentine collagen network had completely altered in structure; it was the only demineralising agent that did not show identifiable individual collagen fibres. One theory is that the citric acid did not remove mineral effectively from the collagen surface, making it difficult to identify the collagen network. However, the SEM images of citric acid (fig 6 o and p) specimens appeared different from the control specimens (fig 6 a and b), as the surface appeared smoother and did not resemble a mineral layer. An alternative explanation is that citric acid caused demineralisation, exposed the collagen network to be vulnerable to denaturation, and then precipitated the mineral back onto the dentine surface, creating a surface that was a

combination of denatured collagen and re-precipitated mineral. This may further explain why citric acid resulted in a dentine surface with a high hardness value and preserved high levels of calcium and Phosphorous. However, at this point, there is no scientific evidence to confirm this theory.

In this study, we have taken a comprehensive approach to characterise acid-affected partially demineralised dentine, using a range of complementary analytical techniques. Notwithstanding, prior to establishing any conclusions, we should consider the impact of the methodological approaches that we adopted on the results obtained.

Recognising the highly heterogeneous structure of dentine; in this project the dentine samples were obtained from permanent premolars, extracted from individuals of an adolescent age (12 to 18 years). In this way, reducing the variance in dentine structure between different age groups, different teeth and between primary and permanent dentition. All surface analysis was undertaken on the occlusal surface of the dentine blocks to reduce the variability from the orientation of the tubules and between crown and root dentine.

Generally, micro-hardness is associated with mineral content levels but with dentine, this can sometimes lead to non-linear relationships between micro hardness and mineral content. Microhardness assessment of partly or fully demineralised dentine structures is challenging and somewhat inconclusive as the indentation depth is affected by the microstructure, tubule density, hydration state, fixing treatments and storage conditions [Craig and Peyton, 1958; Craig et al., 1959; Pashley et al., 1985; Miyauchi et al., 1978; Landis, 1995; Marshall et al., 2001; Kinney et al., 2003; Pugach et al., 2009]. To limit these effects, an effort was made to measure as many areas of the dentine surface as possible, as advised by Pashley et al., [1987]. The micro-hardness of all specimens had a baseline hardness between 46 and 52 VHN (average 49 VHN), which is indicative of sound dentine, which has a hardness value of 50 VHN [Gutiérrez-Salazar and Reyes-Gasga, 2003].

The use of a fixative is essential before analysis with an SEM or EDS but is not essential before analysis using micro-hardness, for example. To remain consistent and to reduce the already high levels of variance between specimens, all specimens were chemically fixed prior to the demineralisation stage, as was the case with a 2002 study looking at the effects of a fluoride varnish [Schmit et al., 2002]. Glutaraldehyde is a highly effective fixative and it is possible that it could help to resist the effect of the demineralising agents by cross linking the collagen and preventing degradation [Unnikrishnan et al, 2019].

From the assessment of acid-affected dentine in this study and with due consideration to the limitations outlined above, our premise to challenge the hypothesis that all acids demineralise dentine in the same/similar manner is valid. This is based on the following conclusions reached through this investigation.

- Each of the demineralising agents (chelating agents, strong and weak acids) tested had a unique effect on the mineral levels and organic phases.

- Demineralisation led to the partial loss of hydroxyapatite crystallinity (size and order) with more lattice distortion and crystals present in lower numbers plus a related absence of other calcium phosphate phases. The collagen structure was also affected by all agents with signs of denaturation, with citric acid causing the most damage. There is no clear link between the type of demineralising agent and the effect on the organic and inorganic phases of dentine.

- The assumption that higher degrees of demineralisation result in higher levels of collagen denaturation was dismissed; each acid affected the collagen structure and the mineral composition in a different way.

The results from this study indicate that investigators should exercise care to match the choice of demineralising agent to the experiment objectives so that the selected dentine (caries or erosion) model is fit for purpose. Demineralised

dentine models should be carefully characterised, in terms of organic and inorganic components, before being used in remineralisation studies.

References

Barón M, Morales V, Fuentes V, Linares M, Escribano N, Ceballos L. The influence of irrigation solutions in the inorganic and organic radicular dentine composition. Aust Endod J 2020;56:217-225.

Besinis, A, van Noort, R. Martin, N: Remineralization potential of fully demineralized dentin infiltrated with silica and hydroxyapatite nanoparticles. Dent Mater J 2014;30:249-262.

Besinis A, van Noort R, Martin N: The use of acetone to enhance the infiltration of HA nanoparticles into a demineralized dentin collagen matrix. Dent Mater J 2016:32;385-393.

Craig, RG, Peyton FA 1958: The microhardness of enamel and dentin: J Dent Res 1958;37;661-668.

Craig, RG, Gehring, PE, Peyton, FA 1959: Relation of structure to the micro-hardness of human dentin. J Dent Res 1959;38:624-630.

Deyhle, H, Bunk, O, Mueller B: Nanostructure of healthy and caries-affected human teeth. Nanomedicine 2011;7:694-701.

Egami T, Billinge SJL: Underneath the Bragg Peaks: Structural Analysis of Complex Materials. Pergamon Materials Series. Oxford, U.K., Elsevier; 2003, p.35.

Eggert, FM, Germain, J: Rapid demineralization in acidic buffers. Histochemistry 1979:59;215-224.

Featherstone JDB, Lussi A: Understanding the chemistry of dental erosion. Dental Erosion: from Diagnosis to Therapy. Monogr Oral Sci. 2006;20:66-76.

Featherstone, JDB, Rodgers BE: Effect of acetic, lactic and other organic-acids on the formation of artificial carious lesions. Caries Res 1981:15;377-385.

Forsback, AP, Areva S, Salonen JI. 2004: Mineralization of dentin induced by treatment with bioactive glass S53P4 in vitro. Acta Odontol Scand 2004:62;14-20.

Gandolfi M, Taddei P, Pondrelli A , Zamparini F, Prati C, Spagnuolo G. Demineralization, Collagen Modification and Remineralization Degree of Human Dentin after EDTA and Citric Acid Treatments. Materials (Basel):2019:12;E25. doi: 10.3390/ma12010025

Gower LB: Biomimetic Model Systems for Investigating the Amorphous Precursor Pathway and Its Role in Biomineralization. Chem Rev 2008:108;4551-4627.

Gutiérrez-Salazar, MDP, Reyes-Gasga J: Microhardness and chemical composition of human tooth. Mat Res 2003:6;367-373.

Habelitz, S, Balooch, M, Marshall, SJ, Balooch G. & Marshall, GW: In situ atomic force microscopy of partially demineralized human dentin collagen fibres. J Struct Biol 2002;138:227-236.

Hershfeld JJ, Miller WD: The ‘chemico-parasitic’ theory of dental caries. Bull Hist Dent 1978:26;11-20.

Karlinsey RL, Mackey AC, Walker ER, Frederick KE: Preparation, characterization and in vitro efficacy of an acid-modified beta-TCP material for dental hard-tissue remineralization. Acta Biomater 2010: 6; 969-978.

Kim, DS, Kim J, Choi KK, Kim, SY: The influence of chlorhexidine on the remineralization of demineralized dentine. J Dent 2011:39;855-862.

Kinney, JH, Habelitz S, Marshall SJ, Marshall, GW: The importance of intrafibrillar mineralization of collagen on the mechanical properties of dentin. Journal of Dental Research 2003:82;957-961 .

Kleter, GA, Damen, JJM, Buijs, MJ, Ten Cate, JM: Modification of amino acid residues in carious dentin matrix. J Dent Res 1998:77;488-495.

Landis, WJ: The strength of a calcified tissue depends in part on the molecular-structure and organization of its constituent mineral crystals in their organic matrix. Bone 1995;16:533-544.

Lindenmeyer, PH, Hosemann, R: Application of the Theory of Paracrystals to the Crystal Structure Analysis of Polyacrylonitrile. Int J Appl Phys 1963:34;42–45.

Lippert F, Churchley D, Lynch R. Effect of lesion baseline severity and mineral distribution on remineralization and progression of human and bovine dentin caries lesions. Caries Res 2015;49:467-476.

Liu DM, Yang QZ, Troczynski T, Tseng WJJ: Structural evolution of sol-gel-derived hydroxyapatite. Biomaterials 2002:23;1679-1687.

Marshall GW, Habelitz S, Gallagher R, Balooch M, Balooch, G, Marshall SJ: Nanomechanical properties of hydrated carious human dentin. J Dent Res 2001: 80; 1768-1771.

Miyauchi, H, Iwaku, M, Fusayama, T: Physiological re calcification of carious dentin. Bull Tokyo Med Dent Uni 1978:25;169-179.

Moore BW, Carter, WJ, Dunn JK, Fosdick, LS: The formation of lactic acid in dental plaques. I. Caries-active individuals. J Dental Res 1956:35:778-785.

Nunn JH, Gordon PH, Morris AJ, Pince CA, Walker A. Dental erosion - Changing prevalence? A review of British national children's surveys. Int J Paed Dent 2003;13:98-105.

Oh S, Perinpanayagam H, Lee Y, Kum JW, Yoo YJ, Lim SM, et al.: Effect of acidic solutions on the microhardness of dentin and set OrthoMTA and their cytotoxicity on murine macrophage. Restor Dent Endod 2015. http://dx.doi.org/10.5395/rde.2016.41.1.12.

Olszta, MJ, Odom, DJ, Douglas EP, Gower, LB: A new paradigm for biomineral formation: Mineralization via an amorphous liquid-phase precursor. Connect Tissue Res 2003:44;326-334.

Panda, RN., Hsieh, MF, Chung RJ, Chin, TS: FTIR, XRD, SEM and solid state NMR investigations of carbonate-containing hydroxyapatite nano-particles synthesized by hydroxide-gel technique. J Phys Chem Solids 2003:64;193-199.

Patterson, AL: The Scherrer Formula for X-Ray Particle Size Determination. Phys Rev 1939:56;978–982.

Pashley D, Okabe A. Parham, P: The relationship between dentin microhardness and tubule density. Endod Dent Traumatol 1985:1;176-179.

Pashley DH, Andringa, HJ, Derkson, GD, Derkson, ME, Kalathoor, SR: Regional variability in the permeability of human-dentin. Arch Oral Biol 1987:32;519-523.

Pugach, MK, Strother J., Darling, CL, Fried D, Gansky, SA, Marshall, SJ, et al.: Dentin Caries Zones: Mineral, Structure, and Properties. J Dent Res 2009:88;71-76.

Ramachan G, Sasisekharan V. Structure of collagen. Nature 1961:190:1004-1005.

Ramırez-Bomme C, Gulabivala K, Ng Y, Young A: Estimated depth of apatite and collagen degradation in human dentine by sequential exposure to sodium hypochlorite and EDTA: a quantitative FTIR study. Int Endo J 2018:51;469–478.

Salas López MM, Puca Castronuevo MM, De La Casa ML, López ME. Presence of Proteins in 17% Edta after Contact with the Bovine Dentine. J Clin Med Res. 2019;1:1-8.

Schmit, JL, Staley, RN, Wefel, JS, Kanellis M, Jakobsen, JR, Keenan PJ: Effect of fluoride varnish on demineralization adjacent to brackets bonded with RMGI cement. Am J Orthod Dentofacial Orthop 2002:122;125-134.

Shellis R. Formation of caries-like lesions in vitro on the root surfaces of human teeth in solutions simulating plaque fluid. Caries Res 2010:44;380-389.

Sui T, Salvati E, Harper R.A., Zhang H, Shelton R.M, Landini G, et al.: In situ monitoring and analysis of enamel demineralisation using synchrotron X-ray scattering. Acta Biomater 2018:77;333-341.

Tartari L, Bachmann L, Zancan RF, Vivan RR, Duarte M, Bramante CM: Analysis of the effects of several decalcifying agents alone and in combination with sodium hypochlorite on the chemical composition of dentine. Int Endo J 2018:51; e42–e54

Tay FR, Pashley DH: Guided tissue remineralisation of partially demineralised human dentine. Biomaterials 2008:29;1127-1137.

Tay FR, Pashley DH: Biomimetic remineralization of resin-bonded acid-etched Dentin. J Dent Res 2009: 88: 719-724.

Tjaderhane, L, Larjava, H, Sorsa, T, Uitto, VJ, Larmas, M, Salo, T: The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. J Dent Res 1998:77;1622-1629.

Topbaş C, Adıgüzel Ö, Çölgeçen Ö. Investigation of the effects of different chelating solutions on the microhardness and surface roughness of root canal dentin. Int Dent Res 2019:9;22-29

Turk T, Kaval ME, Şen B: Evaluation of the smear layer removal and erosive capacity of EDTA, boric acid, citric acid and desy clean solutions: an in vitro study. BMC Oral Health 2015:15;104. doi: 10.1186/s12903-015-0090-y

Unnikrishnan M, Mathai V, Sadasiva K, Santhakumari RS, Girish S, Shailaja Kumari AK. The evaluation of dentin microhardness after use of 17% EDTA, 17% EGTA, 10% citric acid, MTAD used as chelating agents combined with 2.5% sodium hypochlorite after rotary instrumentation: An in vitro SEM study. J Pharm Bioall Sci 2019;11:S156-163.

Vollenweider M., Brunner, TJ, Knecht S., Grass, RN, Zehnder M., Imfeld T, et al.: Remineralization of human dentin using ultrafine bioactive glass particles. Acta Biomater 2007:3;936-943.

Wang Y, Spencer, P: Analysis of acid-treated dentin smear debris and smear layers using confocal Raman microspectroscopy. J Biomed Mater Res 2002:60;300-308.

Zhang Y, Agee K, Nor J, Carvalho R, Sachar, B, Russell, C, et al.: Effects of acid-etching on the tensile properties of demineralized dentin matrix. Dent Mater J 1998:14:222-228.

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Disclosure Statement

The authors have no conflicts of interest

Statement of Ethics

This study received ethical approval from the UK National Health Service (NHS) Health Research Authority (HRA) for the collection of human teeth (STH16442) and NHS Research Ethical Committee (Reference 12/L0/1189) was given. All participants gave full written consent for the use of their teeth in this research.

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Author Contributions

N.M and C.A.M conceived the idea. E.M.A., C.D., N.M and C.A.M. planned the experiments. E.M.A carried out the experiments. C.A.M., E.M.A., C.D., N.M and R.D.M. contributed to the interpretation of the results. E.M.A., C.D., N.M., R.D.M., C.A.M. and L.M.M.E.S. wrote the manuscript. All authors provided critical feedback and helped shape the manuscript.

List of Tables

Table 1: Key properties of the 7 demineralising agents, including their pKa values and logKCa values.

Demineralising agents	Molecular formula	Type of acid	pH	Concentration	pKa	logKCa	Mechanisms of demineralisation
Lactic acid	$C_3H_6O_3$	Weak	2	0.1 M	3.86	1.45	Hydrogen ions & Anions
Hydrochloric acid	HCl	Strong	2	0.01 M	-9.30	N/A	Hydrogen ions
Citric acid	$C_6H_8O_7$	Weak	2	0.1 M	3.13	1.10	Hydrogen ions & Anions
Acetic acid	$C_2H_4O_2$	Weak	2	4 M	4.76	1.18	Hydrogen ions & Anions
Phosphoric acid	H_3PO_4	Weak	2	0.02 M	2.15	1.40	Hydrogen ions & Anions
Formic acid	CH_2O_2	Weak	2	0.5 M	3.75	0.53	Hydrogen ions & Anions
EDTA	$C_{10}H_{16}N_2O_8$	Weak	7	0.5 M	N/A	10.70	Anions

Table 2: Qualitative interpretation of the SEM images reported in Figure 6 a - p

Fig. 6	**Dentine treatment**	**Qualitative findings**
a and b	Non-demineralised (control)	Tubules appear exposed but with no individual fibres visible as the collagen fibre network seems masked with mineral.
c and d	Demineralised with formic acid	The intra-tubular dentine appears to be demineralised with exposed collagen fibres. The collagen network within the tubules appears to be collapsed. There seems to be some residual mineral left within the inter-tubular dentine.
e and f	Demineralised with EDTA	The intra-tubular dentine appears demineralised and the collagen fibres appear exposed. The collagen network seems to have maintained its 3D structure.
g and h	Demineralised with phosphoric acid	The intra-tubular collagen network appears to be exposed, but the matrix appears condensed. Collagen fibres appear exposed in the inter-tubular regions.
i and j	Demineralised with lactic acid	The collagen network appears to be condensed. There seems to be residual mineral on the inter-tubular dentine surface. The banding patterns on the individual collagen fibres are apparent.
k and l	Demineralised with hydrochloric acid	The inter-tubular collagen network is partly exposed. Collagen banding patterns are apparent.
m and n	Demineralised with acetic acid	The inter-tubular collagen shows areas of residual mineral. The intra-tubular dentine appears to be exposed.
o and p	Demineralised with citric acid	Citric acid seems to have a unique effect on dentine collagen. There appears to be a smear layer of mineral on the surface. The tubules seem to be exposed but the inter-tubular collagen and individual fibres are not visible

Table 3: Measurements taken from SEM images of dentine at 80000x magnification (n = 15). Measurements could not be taken from the citric acid sample because individual collagen fibres could not be identified. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc analysis*.

Specimen type:	Collagen fibre width (mean average)	Standard deviation	Significant difference ($p < 0.05$) with:
Formic acid	54.21	20.40	EDTA, hydrochloric acid
EDTA	102.30	38.21	formic acid, acetic acid, phosphoric acid
Phosphoric acid	68.60	15.20	EDTA, hydrochloric acid
Lactic acid	77.00	18.09	Acetic acid
Hydrochloric acid	104.44	20.98	formic acid, acetic acid and phosphoric acid
Acetic acid	45.70	10.78	EDTA, hydrochloric acid, lactic acid
Citric acid	N/A	N/A	

** There is significant difference with EDTA results compared with formic acid, acetic acid and phosphoric acid specimens. There is significant difference with hydrochloric acid specimens*

List of Figures

demineralised with lactic acid pH 2 taken at 80000x; (k) demineralised with hydrochloric acid pH 2 for taken at 4000x; (l) demineralised with hydrochloric acid pH 2 for taken at 80000x; (m) demineralised with acetic acid pH 2 taken at 4000x; (n) demineralised with acetic acid pH 2 taken at 80000x; (o) demineralised with citric acid pH 2 taken at 4000x; (p) demineralised with citric acid pH 2 taken at 8000.

Fig 7: Hyp assay showing quantity of denatured collagen from dentine specimens following 7 days in each demineralising agent. Statistical analysis using one-way ANOVA with Tukey's post hoc analysis; unless specified, all group combinations showed significant difference ($p < 0.05$). ns = no significance.

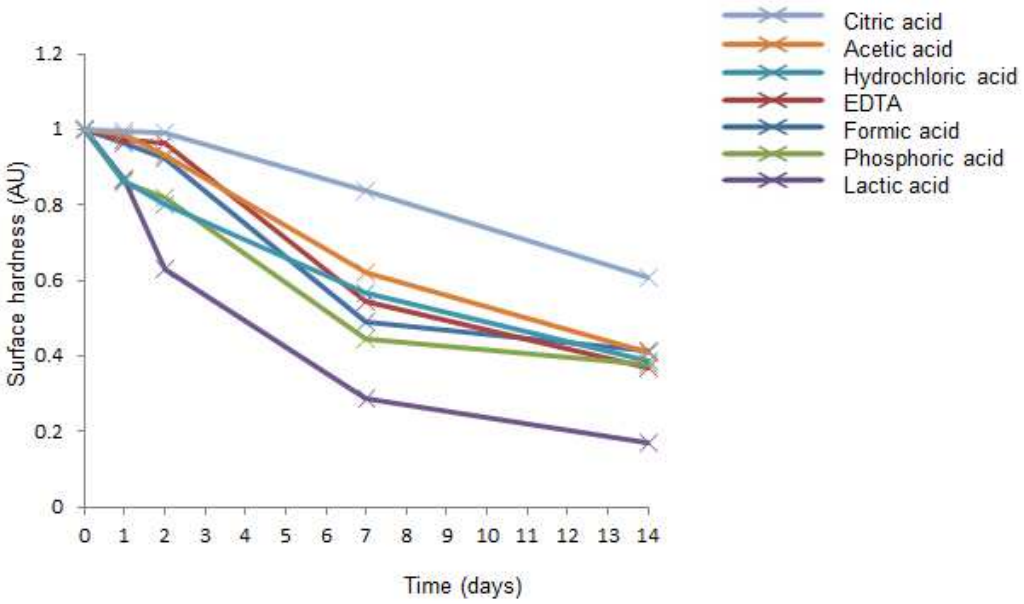


Fig. 1: Normalised values for micro-hardness of dentine specimens following demineralisation with various acids from day 0-14.

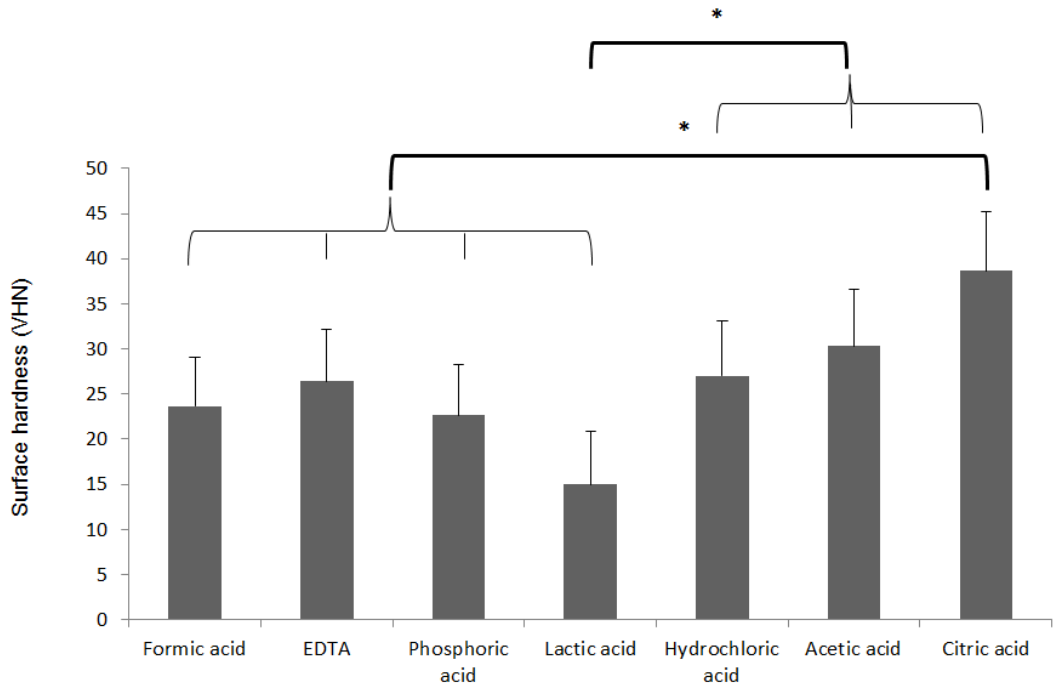


Fig. 2: Vickers micro-hardness (VHN) after 7 days in the demineralising agents. There are no significant differences between variables unless indicated. * = Significant differences ($p < 0.05$) using one-way ANOVA and Tukey post-hoc analysis. The bold brackets compare the variables that are significantly different from one another. The thin braces group variables together where they are all significantly different from another variable. For example, hydrochloric acid, acetic acid and citric acid are not significantly different from one another but are significantly different from lactic acid.

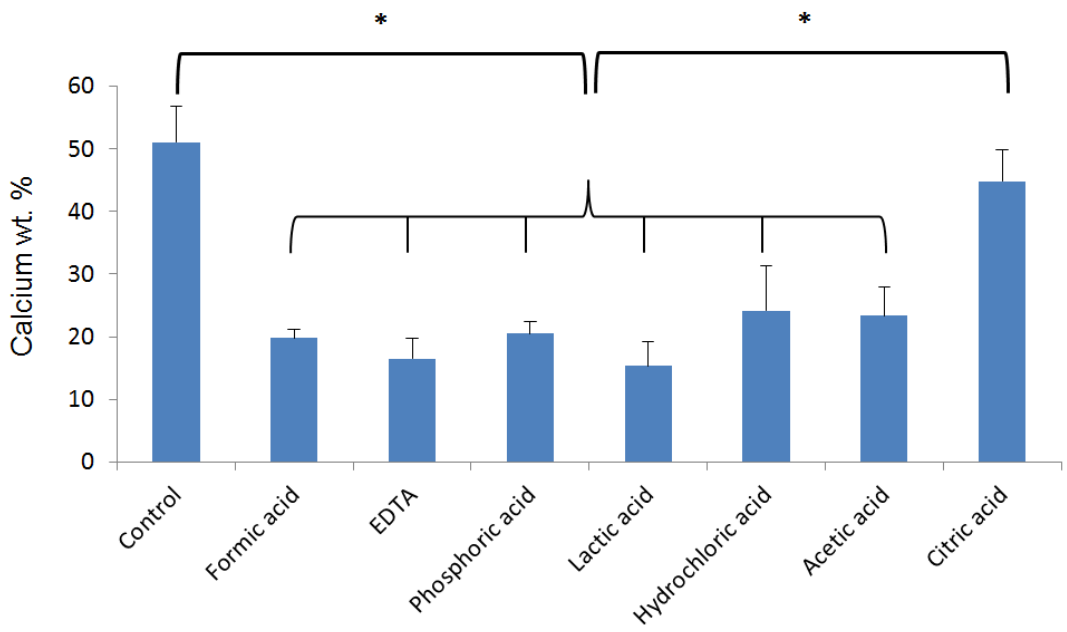


Fig. 3: Energy dispersive spectroscopy (EDS) results showing the levels of calcium Kα (3.692 keV) (wt. %) after 7 days in the demineralising agents.

* = Significant differences ($p < 0.05$) using one-way ANOVA and Tukey post-hoc analysis. There are no significant differences between variables unless indicated. The bold brackets compare the variables that are significantly different from one another. The thin braces group variables together where they are all significantly different from another variable. For example, formic acid, EDTA, phosphoric acid, lactic acid, hydrochloric acid and acetic acid are not significantly different from one another but are all significantly different from the control and the citric acid variable.

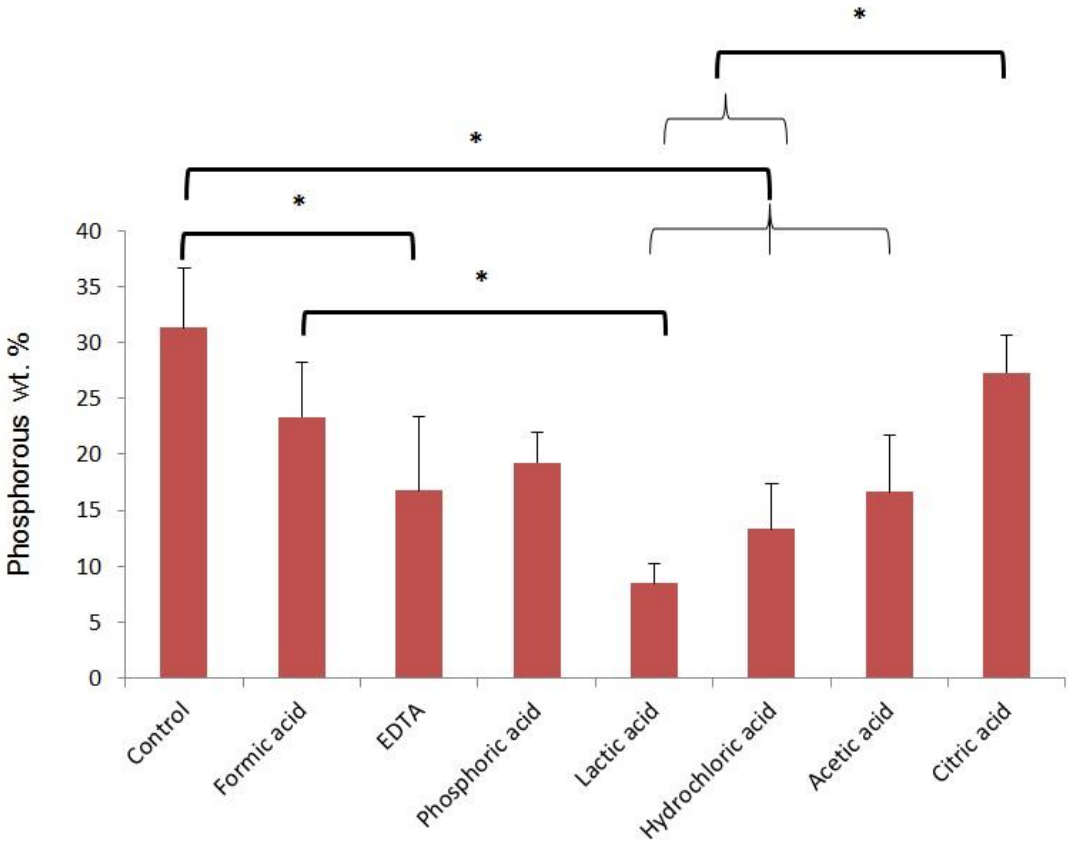


Fig 4: Energy dispersive spectroscopy (EDS) results showing the levels of phosphorous Kα (2.1010 keV) (wt. %) after 7 days in the demineralising agents. There are no significant differences between variables unless indicated.

* = Significant differences ($p < 0.05$) using one-way ANOVA and Tukey post-hoc analysis. The bold brackets compare the variables that are significantly different from one another. The thin braces group variables together where they are all significantly different from another variable. For example, lactic acid, hydrochloric acid and acetic acid are not significantly different from one another but are all significantly different from the control, and the control is also significantly different from the EDTA variable.

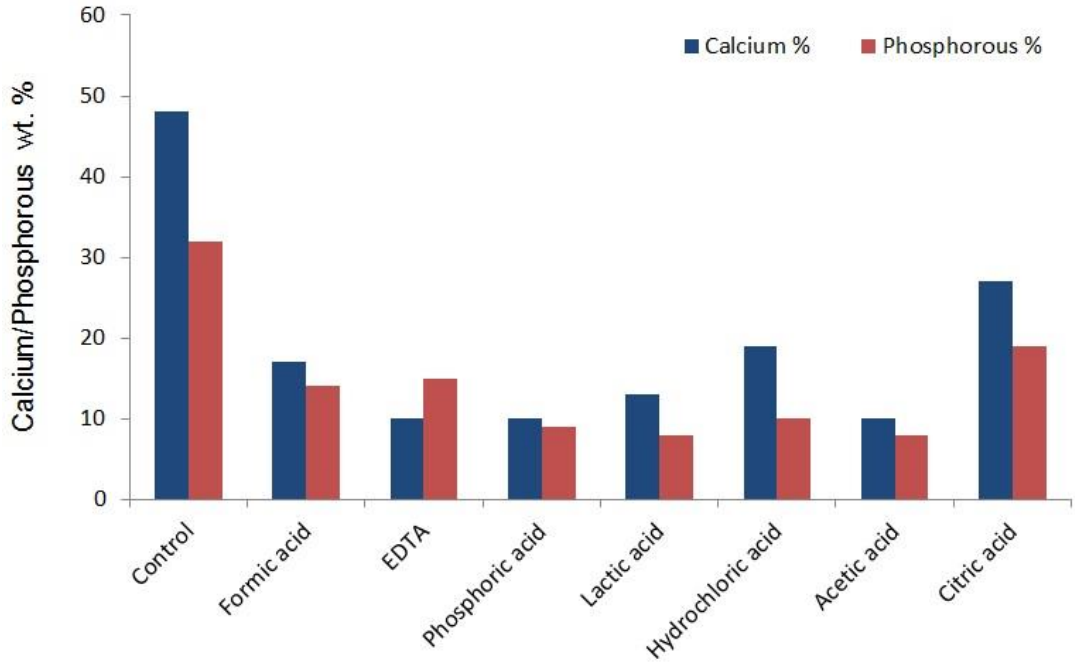
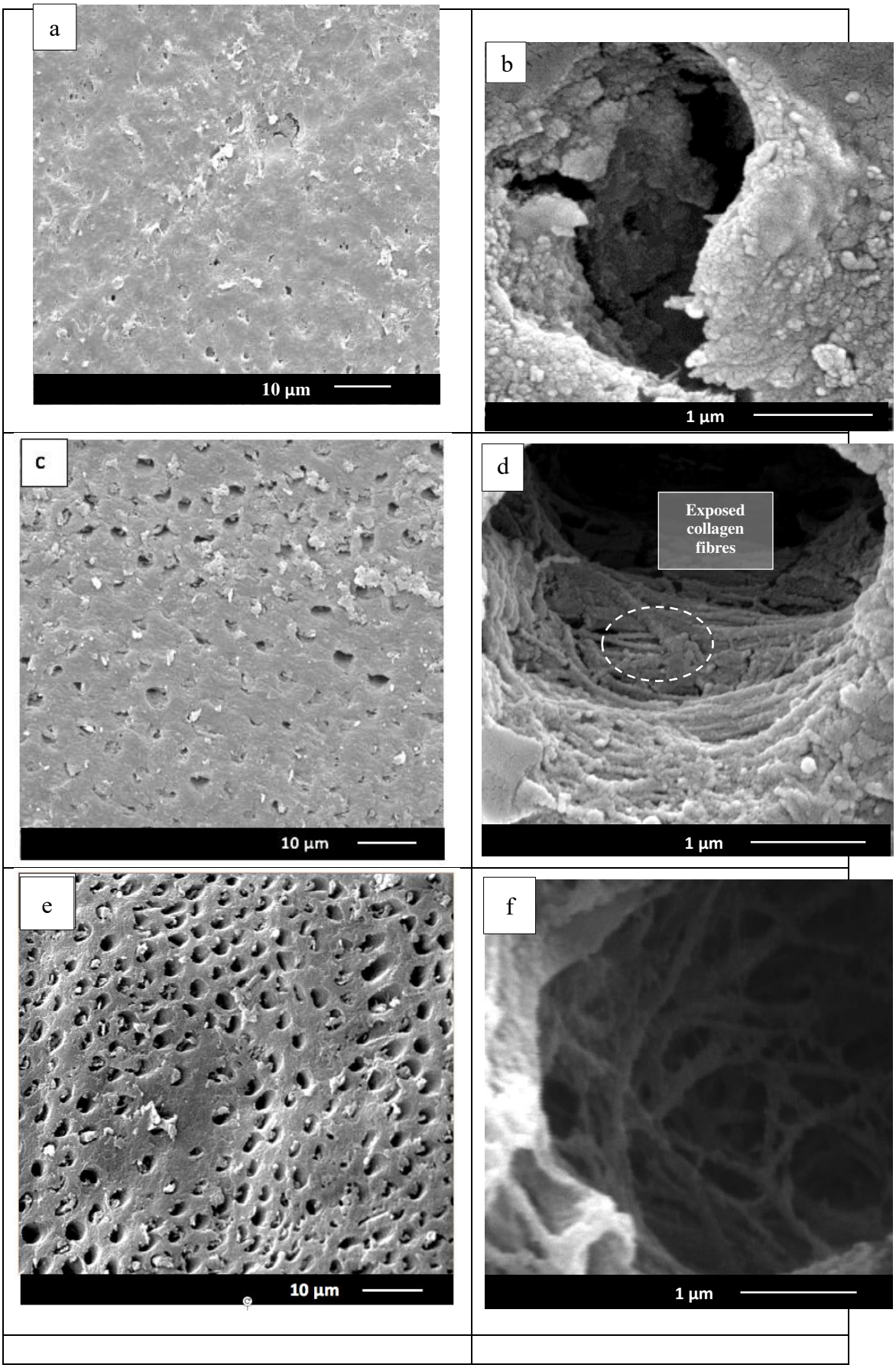
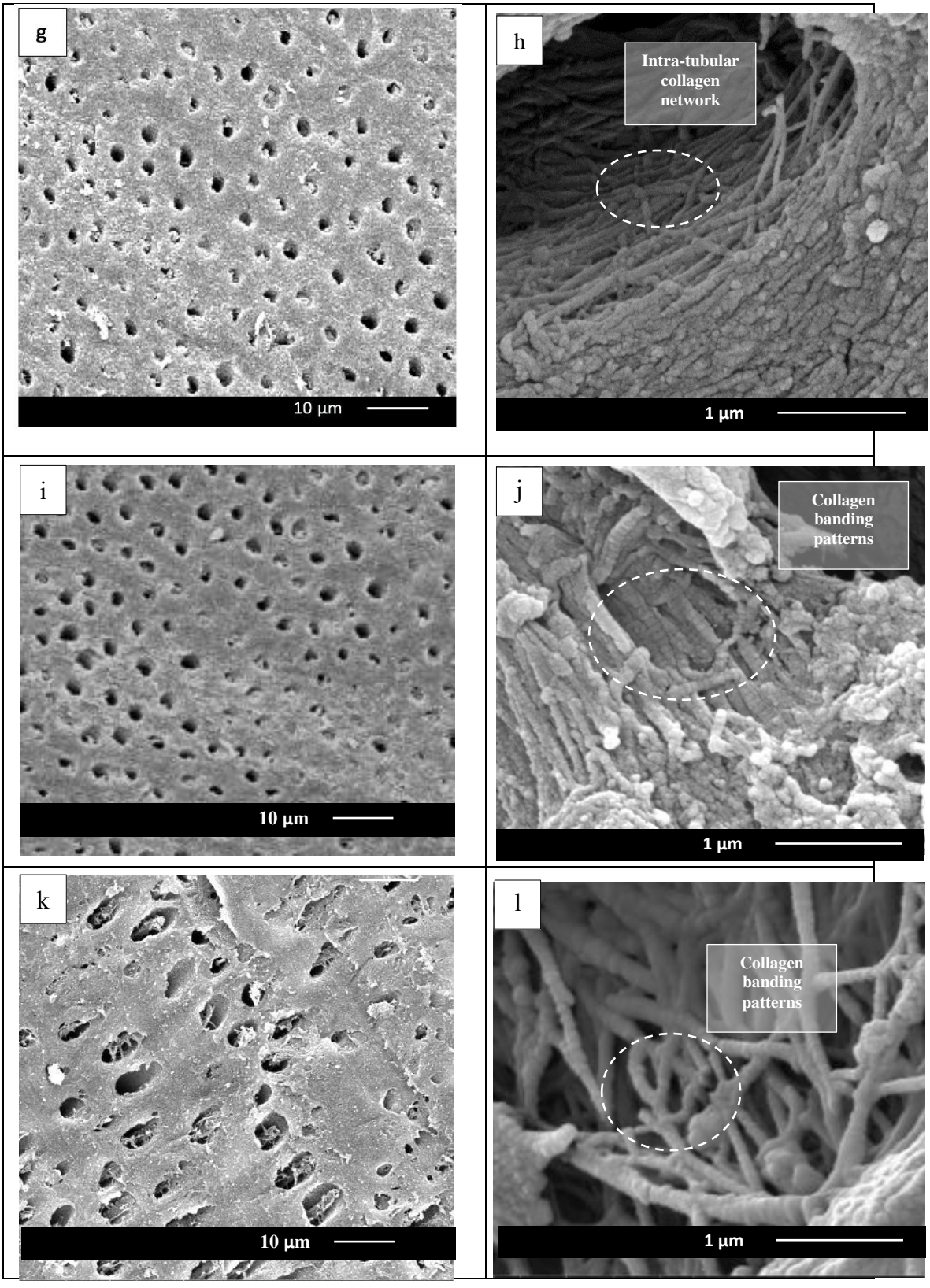


Fig 5: XRF results displaying the levels of calcium and phosphorous in the various demineralised dentine specimens. There is no statistical analysis because n = 1


a
10 µm
b
1 µm
c
10 µm
d
Exposed collagen fibres
1 µm
e
10 µm
f
1 µm


g
10 µm
h
Intra-tubular collagen network
1 µm
i
10 µm
j
Collagen banding patterns
1 µm
k
10 µm
l
Collagen banding patterns
1 µm

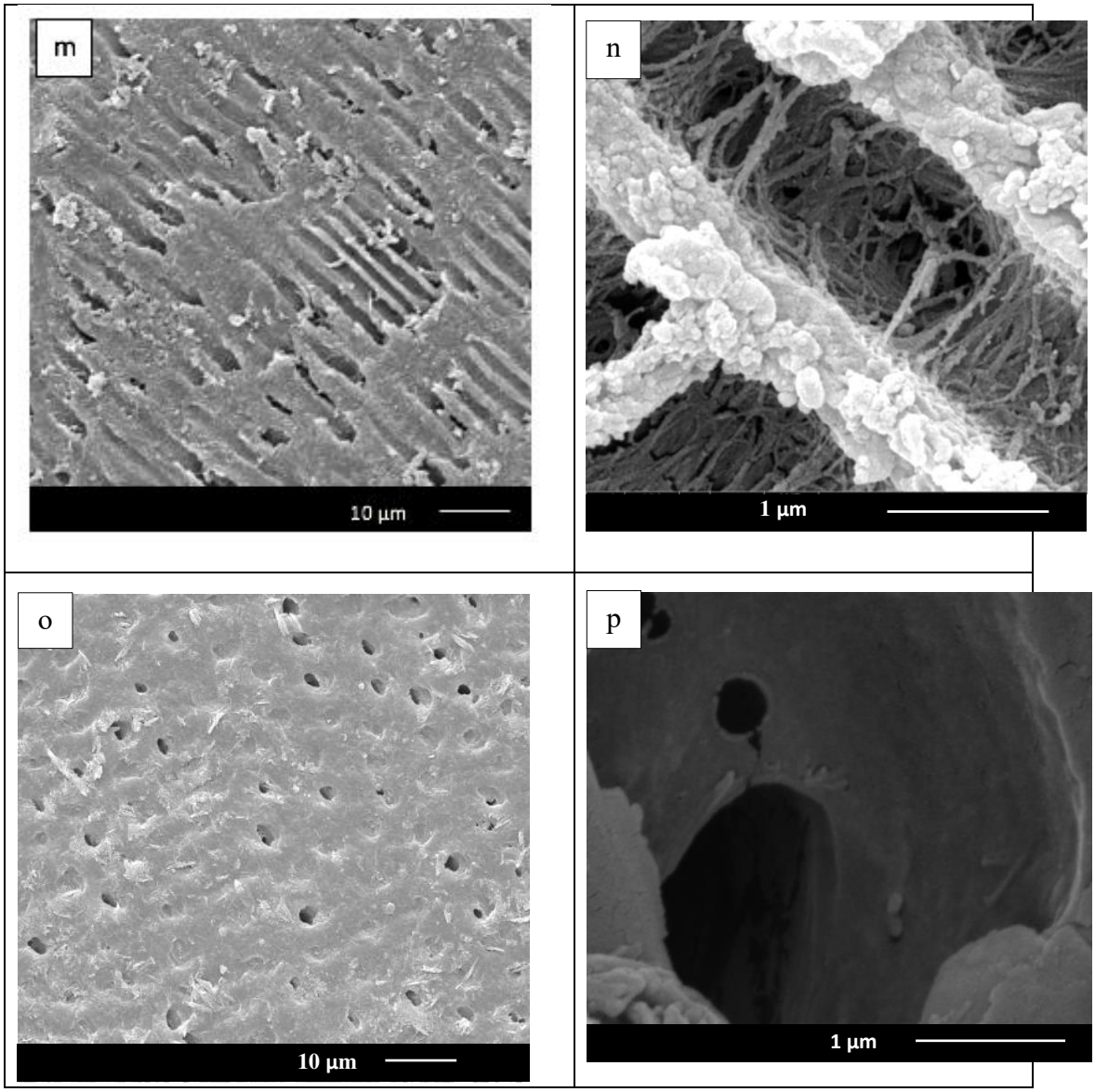


Fig 6: SEM micrographs of dentine control and demineralised for 7 days as follows (a) dentine specimen not demineralised taken at 4000x; (b) dentine specimen not demineralised taken at 80000x; (c) demineralised with formic acid pH 2 taken at 4000x; (d) r demineralised with formic acid pH 2 taken at 80000x; (e) **demineralised with EDTA** pH 7 taken at 4000x; (f) **demineralised with EDTA** pH 7 taken at 80000x; (g) demineralised with phosphoric acid pH 2 taken at 4000x; (h) demineralised with phosphoric acid pH 2 taken at 80000x; (i) demineralised with lactic acid pH 2 taken at 4000x; (j) demineralised with lactic acid pH 2 taken at 80000x; (k) demineralised with hydrochloric acid pH 2 for taken at 4000x; (l) demineralised with hydrochloric acid pH 2 for taken at 80000x; (m) demineralised with acetic acid pH 2 taken at 4000x; (n) demineralised with acetic acid pH 2 taken at 80000x; (o) demineralised with citric acid pH 2 taken at 4000x; (p) demineralised with citric acid pH 2 taken at 8000.

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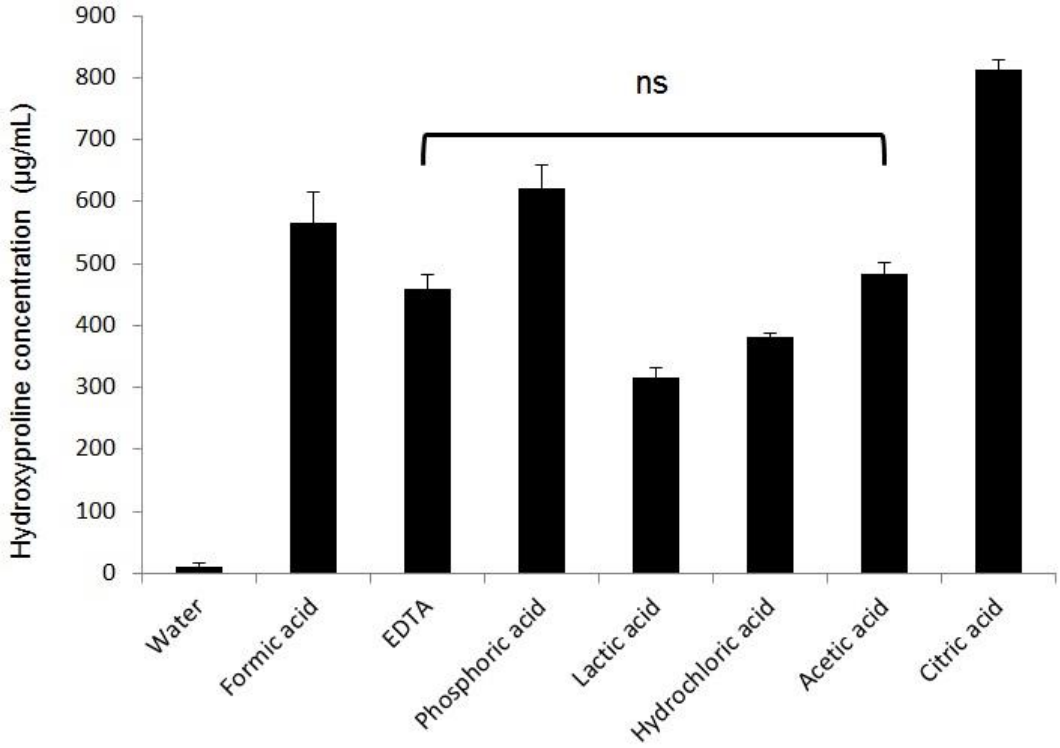

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