Effect of Demineralizing Agents on Organic and Inorganic Components of Dentine

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Abstract
There is a requirement to ensure that in vitro studies that use demineralized 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 demineralizing acids on human dentine. This in vitro study aims to characterize the effect of clinically relevant acids on human dentine, using standardized substrates and complementary analytical techniques. The study focuses on an analysis of the mineral content and the integrity of the collagen following partial demineralization. Samples of human dentine were exposed to a range of acids commonly encountered in the oral cavity. Characterization of the mineral content used Vickers micro-hardness, energy-dispersive spectroscopy, and X-ray fluorescence. Characterization of the collagen integrity was undertaken by means of scanning electron microscopy and hydroxyproline assay. The following conclusions were reached: (i) each demineralizing 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; and (iii) the demineralizing agents caused some degree of collagen denaturation, citric acid causing the most damage. Overall, there is no clear link between the type of demineralizing agent and the effect on the organic and inorganic dentine. The choice of demineralizing agent should be aligned to the experiment objectives so that the selected dentine (caries or erosion) model is fit for the purpose.

Introduction
Human dentine is essentially a mineralized (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 demineralization 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 demineralization 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 remineralization strategies that are more readily translatable as clinical interventions.

In vivo physiological demineralization processes are caused by either dental caries or chemical erosion [Hershfeld 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. While the aetiology of these processes is very different, they both cause demineralization of the mineral scaffold in the hard dental tissues from continuous and/or high-frequency bathing of acids over time. In dental caries, the demineralizing 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 the regurgitation of food (e.g., bulimia) or oesophageal reflux (e.g., gastro-oesophageal reflex disorder).

In the laboratory, we require reliable and robust demineralized dentine models to undertake studies with predictable clinically therapeutic relevance. These models need to be true analogues of the specific demineralization process that occurs clinically.

The literature reports several strategies for preparing dentine models for remineralization studies. It is of note that there appears to be a generalized underlying assumption that any of the recognized demineralization 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 [Patterson, 1939; Lindemeyer and Hosemann, 1963; Eggert and Germain, 1979; Liu et al., 2002; Egami and Billinge, 2003; Panda et al., 2003; Karlinsky et al., 2010; Kim et al., 2011; Besinis et al., 2014], acetic and lactic acids [Moron et al., 2013; Lippert et al., 2015], or chelating agents such as ethylenediaminetetraacetic acid (EDTA) [Habelitz et al., 2002; Wang and Spencer 2002; Tartari et al., 2018; Gandolfi et al., 2019] to produce demineralized dentine models for use in in vitro remineralization 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; Gandolfi et al., 2019; Salas López et al., 2019; Topbaş et al., 2019; Unnikrishnan et al., 2019; Barón 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 2 mechanisms by which acids cause demineralization of the dentine hydroxyapatite: dissociated hydrogen ions and anions binding to calcium. Hydrochloric acid causes demineralization by dissociation of hydrogen ions, whereas EDTA causes demineralization by mechanism anions binding to calcium. Conversely, dietary citric acid can cause demineralization 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; Gandolfi et al., 2019; Salas López et al., 2019; Topbaş et al., 2019; Unnikrishnan et al., 2019; Barón et al., 2020] and coronal dentine [Gandolfi 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., 2013; 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 demineralizing 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 characterize the effect of clinically relevant acids on human dentine, using standardized dentine substrates and appropriate analytical techniques. The study focuses on an analysis of the mineral content and the integrity of the collagen following partial demineralization.

**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, Haverhill, MA, USA) at 4°C to prevent any microbial growth and were used within 1 month of extraction. Dentine blocks (6 × 4 × 2 mm) were prepared from coronal dentine.
Demineralization of dentine using a Mitutoyo HN810 instrument (Hampshire, UK) with a Vickers polisher wheel at 100 rpm (Buehler Metaserv, Düsseldorf, 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 min to remove any cutting debris.

Prior to demineralization, dentine blocks were first fixed by immersion in 3% glutaraldehyde (Sigma Aldrich, Poole, UK) in 0.1 M cacodylate buffer (pH 7) (Sigma Aldrich, Poole, UK) at 4°C for 24 h. 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 in concurrence with prior studies [Unnikrishnan et al., 2019].

After fixation, the specimens were washed with 0.1 M cacodylate buffer (pH 7) (2 × 2 min) and distilled water (2 × 2 min) to remove any remaining fixative. Specimens were blot dried and nail varnish (Max Factor, Cincinnati, OH, USA) was painted onto 5 mm × 5 mm × 1.5 mm sections of dentine blocks into Kleer-set FF resin (MetPrep, Coventry, UK) for 1 h prior to dehydrating as per EDS samples. Specimens were then air-dried in a fume cupboard overnight. Once dehydrated, the blocks were carbon coated (Emitech, Molfetta, Italy) and gently air dried in a fume cupboard over a 12-h period. The specimens were then air-dried in a fume cupboard overnight. Once dehydrated, the blocks were carbon coated (Emitech, Molfetta, Italy) and gently air dried in a fume cupboard over a 12-h period. The minimum distance between indentations > 100 µm, and the outer 0.5 mm of each sample was not tested.

**Energy-Dispersive Spectroscopy**

The presence of calcium (Ca Kα, 3.692 keV) and phosphorous (P Kα, 2.010 keV) was measured using energy-dispersive spectroscopy (EDS), for each of the 7 demineralized 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 subsequently dehydrated by immersion into graded ethanol solutions and hexamethyldisilazane (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 a spot size of 3. Element peak intensities were quantified against a cobalt standard as the base for percentage: X-ray peak Ka 6.931 keV and density 8.86 g/cm³.

**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 demineralization group or a 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 demineralized dentine. The specimen (0.4 g ground dentine) was then fused with 8 g lithium tetraborate at 1,200°C to produce a glass disc. The glass disc was placed into the Phillips PW 2440 XRF instrument for subsequent XRF analysis.

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

| Demineralizing agents | Molecular formula | Type of acid | pH | Concentration (M) | pKa | logKCa | Mechanisms of demineralization |
|-----------------------|-------------------|--------------|----|------------------|-----|--------|-------------------------------|
| Lactic acid           | C₃H₆O₃            | Weak         | 2  | 0.1 M            | 3.86| 1.45   | Hydrogen ions and anions      |
| Hydrochloric acid     | HCl               | Strong       | 2  | 0.01 M           | −9.30| N/A    | Hydrogen ions                 |
| Citric acid           | C₆H₈O₇            | Weak         | 2  | 0.1 M            | 3.13| 1.10   | Hydrogen ions and anions      |
| Acetic acid           | C₂H₄O₂            | Weak         | 2  | 4 M              | 4.76| 1.18   | Hydrogen ions and anions      |
| Phosphoric acid       | H₃PO₄            | Weak         | 2  | 0.02 M           | 2.15| 1.40   | Hydrogen ions and anions      |
| Formic acid           | CH₂O₂            | Weak         | 2  | 0.5 M            | 3.75| 0.53   | Hydrogen ions and anions      |
| EDTA                  | C₁₀H₁₆N₂O₈        | Weak         | 7  | 0.5 M            | N/A | 10.70  | Anions                        |

EDTA, ethylenediaminetetraacetic acid.
blocks were attached to aluminium pin stubs using carbon sticky tabs, coated with silver dag (Agar Scientific, Essex, UK) and then gold sputtered 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 ×1,000 and ×80,000 viewing both the intra- and inter-tubular dentines. When measuring collagen fibre dimensions, measurements were taken from 5 areas on 3 high magnification (×80,000) images. Measurements were taken with ImageJ software version 1.46 (Open access, NIH, Bethesda, MD, USA).

Hydroxyproline Assay

The hydroxyproline (Hyp) assay was performed on demineralized dentine blocks from each demineralization group in order to quantify the amount of denatured collagen. The demineralized dentine samples were transferred into 1 mL PBS at pH 7 prior to being incubated at 37°C with gentle shaking for 24 h in 20 mg tryptosin (treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone) (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 (100 μL) was transferred to a pressure-tight vial and incubated at 120°C for 3 h 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 13,000 g (Sigma Aldrich, Poole, UK) for 2 min. Each supernatant (10 μL) 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 560 nm (Tecan, Männedorf, Switzerland).

Statistical Analysis

All data were 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 Bonferroni 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, IL, USA). All graphs display standard deviation in the error bars.

Results

Micro-Hardness

The VHNs were normalized against their baseline before demineralization (Fig. 1), showing that the surface hardness of every specimen decreased over the 14-day period. By day 1, there was a little difference in surface hardness between each specimen (VHN ranged from 41 to 48), but by day 2, the hardness values ranged from 33 (lactic acid-treated specimen) to 47 (EDTA-treated specimen). From day 1, the specimens began to develop different trends; the surface hardness of the lactic acid specimen declined rapidly, while 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 demineralizing 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 a significant difference (p < 0.05) between citric acid-treated specimens compared to specimens treated with lactic acid, phosphoric acid, EDTA, and formic acid.

EDS and XRF

EDS and XRF analyses were undertaken at day 7 of demineralization. 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 phosphorus (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 demineralized 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.
Fig. 2. VHN after 7 days in the demineralizing agents. There are no significant differences between variables unless indicated. *Significant differences (p < 0.05) using one-way ANOVA and Tukey’s 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. ANOVA, analysis of variance; VHN, Vickers micro-hardness; EDTA, ethylenediaminetetraacetic acid.

Fig. 3. EDS results showing the levels of calcium Kα (3.692 keV) (wt%) after 7 days in the demineralizing agents. *Significant differences (p < 0.05) using one-way ANOVA and Tukey’s 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. EDTA, ethylenediaminetetraacetic acid; ANOVA, analysis of variance; EDS, energy-dispersive spectroscopy.

Fig. 4. EDS results showing the levels of phosphorous Kα (2.1010 keV) (wt%) after 7 days in the demineralizing agents. There are no significant differences between variables unless indicated. *Significant differences (p < 0.05) using one-way ANOVA and Tukey’s 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. EDTA, ethylenediaminetetraacetic acid; ANOVA, analysis of variance; EDS, energy-dispersive spectroscopy.
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 demineralized with 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 demineralized 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 demineralization. Similar to EDS, XRF results also appear to demonstrate that citric acid caused the least change in Ca and P contents. However, as XRF analysis was only performed once per sample, statistical analysis could not be performed.

Table 2. Qualitative interpretation of the SEM images reported in Figure 6a–p

| Figure 6 | Dentine treatment                        | Qualitative findings                                                                 |
|----------|------------------------------------------|---------------------------------------------------------------------------------------|
| a and b  | Non-demineralized (control)              | Tubules appear exposed but with no individual fibres visible as the collagen fibre network seems masked with mineral. |
| c and d  | Demineralized with formic acid           | The intra-tubular dentine appears to be demineralized 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  | Demineralized with EDTA                  | The intra-tubular dentine appears demineralized and the collagen fibres appear exposed. The collagen network seems to have maintained its 3D structure. |
| g and h  | Demineralized 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  | Demineralized 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  | Demineralized with hydrochloric acid     | The inter-tubular collagen network is partly exposed. Collagen banding patterns are apparent. |
| m and n  | Demineralized with acetic acid           | The inter-tubular collagen shows areas of residual mineral. The intra-tubular dentine appears to be exposed. |
| o and p  | Demineralized 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. |

EDTA, ethylenediaminetetraacetic acid; SEM, scanning electron microscopy.

Table 3. Measurements taken from SEM images of dentine at ×80,000 magnification (n = 15)

| Specimen type          | Collagen fibre width (mean average) | Standard deviation | Significant difference (p < 0.05) with                      |
|------------------------|-------------------------------------|--------------------|-------------------------------------------------------------|
| Formic acid            | 54.21                               | 20.40              | EDTA and hydrochloric acid                                  |
| EDTA                   | 102.30                              | 38.21              | Formic acid, acetic acid, and phosphoric acid               |
| Phosphoric acid        | 68.60                               | 15.20              | EDTA and 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, and lactic acid                    |
| Citric acid            | N/A                                 | N/A                |                                                             |

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. (There is a significant difference with EDTA results compared with formic acid, acetic acid, and phosphoric acid specimens. There is a significant difference with hydrochloric acid specimens.) EDTA, ethylenediaminetetraacetic acid; SEM, scanning electron microscopy.

Scanning Electron Microscopy

SEM was used to observe the collagen structure of the dentine surfaces following a 7-day exposure to the various demineralizing agents and non-demineralized control specimens (Fig. 6). The effect of specimen dehydration for SEM examination was minimized by using a gentle dehydration protocol that avoided air-drying. In these, Figure 6a is a low magnification image that shows the ge-
ometry and microstructure of the sample surface, and Figure 6b and c focuses on a dentine tubule to allow for the examination of the collagen subsurface. 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 20 nm to 177 nm.

**Hyp Assay**

The results from the Hyp are shown in 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 demineralized 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 acids (hydrochloric acid) all demineralize the dentine through the processes of caries and acid erosion. In vitro remineralization studies require artificially demineralized dentine models, which are often created using laboratory agents, such as formic acid or EDTA, under the presumption 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 characterizing and comparing the effects of these acids on the inorganic and organic components of dentine. In this study, we used a range of demineralizing agents: bacterial metabolism (lactic acid), dietary origin (citric acid, phosphoric acid, and acetic acid), gastric acid (hydrochloric acid), and laboratory acids (EDTA and formic acid) to create a variety of partially demineralized dentine models. Prior to demineralization, dentine blocks were first fixed by immersion in 3% glutaraldehyde. There is a recognition that this protocol did not replicate in vivo conditions; but since all samples were demineralized 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, thereby preventing the analysis of mineral content and collagen integrity. Characterization techniques were employed to assess and compare the composition and structure of these dentine models to investigate whether the demineralizing 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 demineralization ability. Therefore, pH was kept constant to allow the 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 remineralization models that will normally use acids in a pure form [Wang and Spencer, 2002; Kim et al., 2011; Besinis et al., 2014; Oh et al., 2015; Turk et al., 2015; Ramirez-Bommer et al., 2018; Tartari et al., 2018; Gandolfi 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 representa-
tive 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 pro-
vide a context to the findings from the data analysis.

The Effects on Mineral Content

The mineral content of partially demineralized den-
tine was determined by means of EDS. XRF was used to
indicate the levels of calcium and phosphorous following
demineralization and micro-hardness test to compare the
levels of mineralization. There is increasing evidence that
remnant mineral is essential for further remineralization
dentine to occur [Tay and Pashley, 2008; Tay and Pash-
ley, 2009], highlighting the importance of the need to
characterize and assess the remnant mineral in a variety
of artificially demineralized dentine models, a study hith-
terto not undertaken previously.

The analyses of the mineral content of the various de-
mineralized dentine models led to the following findings:
(1) each demineralizing agent appeared to affect the min-
eral content differently and (2) the results did not verify
which demineralizing mechanism was most effective.
These points are considered in the following discussion.
1. Each demineralizing agent affected the mineral con-
tent differently. Micro-hardness, XRF, EDS, and a
phosphate assay were all used as an indication of the
mineral levels in dentine before and after demineral-
ization, showing that all acids caused a reduction in
mineral content resulting in partially demineralized
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 demineralization, the
same conclusion as Moore et al. [1956], who showed that
lactic acid, produced by oral bacteria, is the most powerful
demineralizing 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 ace-
tic acid was as damaging to mineral content as lactic acid.
Although EDS data (Fig. 3, 4) did not show any significant
difference in calcium or phosphorous levels between spec-
imens treated with the 2 acids, micro-hardness results
(Fig. 2) showed a significant difference comparing acetic
acid and lactic acid, with lactic acid being one of the most
effective demineralizing agents and acetic acid appeared
to be one of the least effective demineralizing agents.

The micro-hardness (Fig. 1, 2) and EDS (Fig. 3) results
suggest that citric acid was the least effective demineral-
izing agent because it appears to have caused the least re-
duction in hardness with no significant difference com-
pared with the control specimen. Table 1 shows that citric
acid is a weak acid (pKₐ 3.13) with a chelating ability (log-
KCa 1.10), which would suggest that it would be effective
at removing calcium from the dentine. However, the cal-
cium 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 ac-
id-treated specimens. The EDS data showing the phos-
phorous levels (Fig. 4) also showed no significant differ-
eence between the citric acid specimens and the control
specimens, suggesting that citric acid was not very effec-
tive at removing neither the calcium nor the phospho-
rus. 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 ef-
effective.

2. The results do not confirm which demineralizing
mechanism is the most effective. There are 2 mecha-
nisms that cause demineralization, one is the dissocia-
tion of hydrogen ions and the other is the chelation of
calcium ions. Part of this investigation was to deter-
mine whether one mechanism was more effective than
the other.

Hydrochloric acid was the only strong acid investi-
gated. 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 demineralizing agents as-
sessed in this study, hydrochloric acid had the strongest
ability to cause demineralization by this dissociated hy-
drogen 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 demin-
eralization through its ability to dissociate into hydrogen
ions.

On the other hand, EDTA was the only demineralizing
agent that was investigated which can only cause demineral-
ization by the chelation mechanism. EDTA has a log-
KCa value of 10.70 and does not have a pKₐ value because
it does not dissociate into hydrogen ions (Table 1). Re-
garding all of the demineralizing agents used in this study,
EDTA had the strongest ability to cause demineralization
via the chelation mechanism because it had the highest
logKCa value. All of the other demineralizing agents had
the ability to cause demineralization by both mecha-
nisms.

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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 ion mechanisms, 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 have been 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 5 demineralizing agents that were all able to cause demineralization using both mechanisms (Table 1), but there was no evidence to suggest that this allowed greater demineralization to occur.

The Effect on Collagen Integrity

The second objective, to characterize the integrity of acid-affected collagen in the demineralized dentine models, was carried out by SEM to visually characterize the exposed dentine collagen network and an Hyp assay to quantify any collagen that had denatured. In vitro remineralization studies report on the need to have an intact collagen structure to replicate the outer zones of carious lesions which often contain undamaged collagen [Olsztar et al., 2003; Forsback et al., 2004; Vollenweider et al., 2007; Gower, 2008; Deyhle et al., 2011].

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

1. Each demineralizing 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, for example, in erosion or artificially demineralized models. Some studies have suggested that acids have an indirect denaturing ability because they can activate matrix metalloproteinases, 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 [Ramachand and Sasishekar, 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, undenatured 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 while maintaining its cross-links and banding patterns. This could have been facilitated by the use of glutaraldehyde, which stabilizes the cross-links.

2. There was no obvious correlation between demineralizing mechanism and effect on collagen integrity. Although all of the demineralizing 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 demineralization 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 demineralizing mechanism and collagen integrity. Only dentine demineralized with lactic acid or hydrochloric acid showed fibres with banding patterns. However, these 2 acids did not cause demineralization in the same way, as displayed on Table 1; the properties of the acids differ, lactic acid being a weak acid with chelat-
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 demineralizing 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, p) specimens appeared different from the control specimens (Fig. 6a, b), as the surface appeared smoother and did not resemble a mineral layer. An alternative explanation is that citric acid caused demineralization, 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 characterize acid-affected partially demineralized 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.

Recognizing the highly heterogeneous structure of dentine, in this project the dentine samples were obtained from permanent premolars, extracted from individuals of an adolescent age (12–18 years). In this way, the variance in the dentine structure between different age groups, between different teeth, and between primary and permanent dentition is reduced. 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. Micro-hardness assessment of partly or fully demineralized 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; Miyauchi et al., 1978; Pashley et al., 1985; 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 VHNs (average 49 VHNs), which is indicative of sound dentine, which has a hardness value of 50 VHNs [Gutiérrez-Salazar and Reyes-Gasga, 2003].

The use of a fixative is essential before analysis with an SEM or an 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 demineralization 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 demineralizing 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 demineralize dentine in the same/similar manner is valid. This is based on the following conclusions reached through this investigation.

- Each of the demineralizing agents (chelating agents, strong and weak acids) tested had a unique effect on the mineral levels and organic phases.
- Demineralization 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 demineralizing agent and the effect on the organic and inorganic phases of dentine.
- The assumption that higher degrees of demineralization 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 demineralizing agent to the experiment objectives so that the selected...
dentine (caries or erosion) model is fit for purpose. De-
mineralized dentine models should be carefully charac-
terized, in terms of organic and inorganic components,
before being used in remineralization studies.

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Statement of Ethics

This study received ethical approval from the UK National Health
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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.

Conflict of Interest Statement

The authors have no conflicts of interest.

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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 criti-
cal feedback and helped shape the manuscript.

Data Availability Statement

The data that support the findings of this study are available
from the corresponding author upon reasonable request.

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