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Comparison of Calcium Analysis, Longitudinal Microradiography and Profilometry for the Quantitative Assessment of Erosion in Dentine

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Key Words
Calcium analysis · Erosion, dentine · Longitudinal microradiography · Profilometry

Abstract
Erosion of dentine causes mineral dissolution, while the organic compounds remain at the surface. Therefore, a determination of tissue loss is complicated. Established quantitative methods for the evaluation of enamel have also been used for dentine, but the suitability of these techniques in this field has not been systematically determined. Therefore, this study aimed to compare longitudinal microradiography (LMR), contacting (cPM) and non-contacting profilometry (ncPM), and analysis of dissolved calcium (Ca analysis) in the erosion solution. Results are discussed in the light of the histology of dentine erosion. Erosion was performed with 0.05 M citric acid (pH 2.5) for 30, 60, 90 or 120 min, and erosive loss was determined by each method. LMR, cPM and ncPM were performed before and after collagenase digestion of the demineralised organic surface layer, with an emphasis on moisture control. Scanning electron microscopy was performed on randomly selected specimens. All measurements were converted into micrometres. Profilometry was not suitable to adequately quantify mineral loss prior to collagenase digestion. After 120 min of erosion, values of 5.4 ± 1.9 μm (ncPM) and 27.8 ± 4.6 μm (cPM) were determined. Ca analysis revealed a mineral loss of 55.4 ± 11.5 μm. The values for profilometry after matrix digestion were 43.0 ± 5.5 μm (ncPM) and 46.9 ± 6.2 (cPM). Relative and proportional biases were detected for all method comparisons. The mineral loss values were below the detection limit for LMR. The study revealed gross differences between methods, particularly when demineralised organic surface tissue was present. These results indicate that the choice of method is critical and depends on the parameter under study.

Various methods have been used for the quantification of erosive mineral loss [Barbour and Rees, 2004; Attin, 2006], and these have different strengths and weaknesses. A widely used procedure is profilometry, which mirrors the bulk tissue loss occurring after erosive impacts and is the most illustrative method when the clinical appearance of erosive defects is considered. Profilometry is easy to perform and can assess both initial surface changes and advanced tissue loss. This method is appropriate for the evaluation of enamel since the subsurface changes in mineral content are relatively small. In contrast, dentine erosion has a complex histology that is characterised by centripetal mineral loss beginning in the peritubular areas and followed by intertubular demineralisation. Finally, a zone of fully demineralised dentine develops that can be relatively thick and is stable while hydrated [Kinney et al., 1995]. This histological fea-

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ture indicates that there is no bulk tissue loss. Instead, there is a demineralised organic surface layer, on the outer aspect continuous with the original surface and on the inner aspect separated from the underlying fully mineralised tissue by a thin partly demineralised band. Therefore, profilometry is difficult to utilize for the quantification of the mineral status due to the presence of this organic material on the specimen surface and the inability to detect mineral loss or uptake in the tubular structure of the tissue. Longitudinal microradiography (LMR) [de Josselin de Jong et al., 1988] may be suitable as it is not subject to these limitations. However, this method was primarily established and validated for enamel and has rarely been used for dentine. In principle, microradiography reflects the change in the X-ray absorption properties of the tissue under study and yields information on the total mineral content of a given specimen. With LMR, a defined specimen area can be repeatedly and non-destructively analysed to determine the total mineral content per unit tooth area, and the mineral loss or gain can be compared with baseline values. However, LMR gives no information about the mineral content as a function of depth, which could be of particular importance in dentine. Modifications of transversal microradiography have been applied for the analysis of erosion [Hall et al., 1997; Amaechi et al., 1998], but both approaches require relatively thin sections that are not suitable for combined erosion/abrasion experiments and necessitate prolonged radiation times. A further established method is the quantification of mineral loss by analysis of calcium or phosphate released into the dissolving solution. Like LMR, this method yields no information regarding the structure of the remaining tissue, but has the advantage of being highly sensitive.

These methodology issues have not been systematically evaluated for dentine. In particular, the role of the organic portion of the tissue has not been adequately studied.

The present study sought to compare the quantitative results of tissue loss after various erosive challenges in dentine obtained by the following methods: (1) profilometry with both a contacting (cPM) and a non-contacting (ncPM) device, (2) LMR and (3) the chemical analysis of calcium in the dissolving solution. Profilometry and microradiography were performed before and after the enzymic removal of the demineralised organic surface layer. Scanning electron microscopy (SEM) was performed in randomly selected specimens in order to visualise the histological structures and to verify that the organic matrix was completely removed by enzymic digestion.

Materials and Methods

Preparation of Dentine Specimens

From previously impacted human third molars, plane-parallel longitudinal slices of coronal dentine were prepared (thickness: 800 μm; Exact Trennschleifsystem and Exact Mikroschleifsystem, Exakt-Apparatebau, Norderstedt, Germany) and polished with P4000 (nominal grain diameter: 5 μm; Leco, St. Joseph, Mich., USA). All grinding and polishing procedures were performed with a water-cooled grinding machine (Exact Mikroschleifsystem; Exakt-Apparatebau). The experimental area was covered with a square piece of tape (2 × 2 mm), and the 4 edges of this area were marked with small holes (diamond bur ISO No. 806 314 540534009; Komet, Lemgo, Germany) for identification in the microradiography system. The specimens were then completely covered with a light curing acrylate (Technovit 7230 VLC; Kulzer-Exakt, Wehrheim, Germany). Afterwards, the tape was carefully removed with a scalpel to achieve precise margins of the experimental area, which was inspected with a stereomicroscope (magnification: ×10; SMZ-1 stereomicroscope; Nikon, Düsseldorf, Germany) to ensure that the surface was clean. The acrylic protected the reference area during erosion and was completely removed with a scalpel after the erosion treatment. Finally, the experimental and reference areas were thoroughly checked with a stereomicroscope for any remnants. The specimens were maintained at 100% humidity and room temperature until they were submitted to further measurement procedures.

Erosive Demineralisation

Each specimen was immersed in 10 ml of 0.05 M citric acid (pH 2.5), in 20-ml plastic scintillation vials (Massanalytische Glas- und Laborgeräte Vertriebsgesellschaft, Rabenau, Germany) at 37°C and under constant agitation on a shaker (horizontal movement; frequency: 35/min). Erosion times were 30, 60, 90 or 120 min, and the group size was 25 each (total n = 100). Immediately after immersion in acid, the plastic tubes were thoroughly sealed and stored at room temperature until chemical analysis. In the following, the erosive loss of each individual specimen was quantified by each of the 4 methods. Four groups of 5 additional specimens (total n = 20) were eroded for time periods of 30, 60, 90 and 120 min, respectively, for SEM.

Longitudinal Microradiography

LMR was performed as described by de Josselin de Jong et al. [1988]. X-ray projections (CuKα X-ray radiation operated at 20 kV and 50 mA; exposure time: 2.5 min) were performed on a high-resolution film (high-speed holographic film, Fuji positive, purchased via Inspector Research Systems BV, Amsterdam, The Netherlands) at baseline and after erosive demineralisation. The films were developed under standard conditions, in accordance with the recommendations of the manufacturer. From the resulting images, the mineral content was calculated using a computer-controlled microdensitometer (scan area: 1 × 1 mm; Leitz MPV Compact Ortholux II; Leitz, Wetzlar, Germany). The holes, which could be easily identified on the images, assured that the scan area was situated within the experimental area. The erosive mineral loss was automatically calculated by the LMR software and expressed in micrometres. A repeated analysis of X-ray projections of the same specimen on different films (n = 15) yielded a standard deviation of 11.4 μm.

The software calculates under the assumption that the specimen consists of 100% mineral and that the mineral consists of stoichiometric hydroxyapatite (HA). To convert this value to spatial tissue loss based on the portion of mineral in dentine, the thickness of all individual specimens (d_{micro}) was determined with a micrometre screw (Mitutoyo, Tokyo, Japan) and related to the value of specimen thickness obtained by the LMR software (d_{LMR}). The mean d_{micro}/d_{LMR} ratio was 1.98 ± 0.06 indicating a mineral content of 50.6 ± 1.6 vol%. This value corresponds with values given in the literature [Nikiforuk, 1985; Frank and Nalbandian, 1989].

Contacting and Non-Contacting Profilometry

The profilometric procedure utilized was previously described [Ganss et al., 2005]. A Perthometer S8P (Perthen-Mahr, Göttingen, Germany) was equipped with a contacting (FRW-750) or non-contacting (Focodyn, Rodenstock, Germany) pick-up. Three tracings were performed in the following standardised manner: the stylus was first inserted into the hole, marking the edge of the experimental area, then positioned 0.7 mm away from the hole in the reference area and finally moved 0.75 mm along the lesion border. Three tracings, 250 μm apart, were performed. The specimen surface was covered with distilled water for 30 s prior to each tracing. The water was removed with absorbent tissue without contacting the experimental area, and the surfaces were immediately traced. The tracings were interpreted with special software (Perthometer Concept 4.0; Perthen-Mahr). The vertical distance of the regression lines constructed in the reference and experimental areas was defined as tissue loss (in micrometres), and the loss of a given specimen was reported as the mean of the 3 tracings. For a specimen with a loss in the order of 20 μm, 10 repeated analyses with the non-contacting device yielded a standard deviation of 0.8 μm, and with the contacting device of 0.6 μm. The respective values for a specimen with a loss in the order of 40 μm were 0.7 and 1.1 μm. With the organic matrix present, the standard deviation was 1.1 μm for the non-contacting, and 1.9 μm for the contacting device.

Chemical Analysis

The calcium concentration in the erosion solution was determined by atomic absorption spectroscopy (Instrumentation Laboratory AA/AE Spectrophotometer IL 157), performed in the presence of lanthanum (0.1%) to suppress interference by phosphate. Repeated measurements yielded a standard deviation of 2%. Mineral loss was calculated from the calcium concentration under the assumption that dentine mineral consists of stoichiometric HA:

$$\text{Mineral loss } (\mu g) = \frac{\text{Ca concentration (mmol/l)} \times \text{volume of erosion solution} \times \text{relative atomic mass}}{\text{Mass fraction of Ca in HA}}$$

where volume of erosion solution = 10 ml and mass fraction of calcium in HA = 0.399 [Nikiforuk, 1985]. Erosive loss was then determined as:

$$\text{Erosive loss } (\mu m) = \frac{\text{Mineral loss } (\mu g)}{\text{Density of dentine } (g/cm^3) \times \text{Experimental area } (mm^2)}$$

where density of dentine = 2.35 g/cm^3 [Nikiforuk, 1985]. The experimental area was measured with a microscope (Leica M420; Leitz) connected to a measuring device (Mitutoyo).

Removal of Organic Matrix

Collagenase obtained from *Clostridium histolyticum* type VII (100 units/ml; Sigma Aldrich, St. Louis, Mo., USA), with a collagen digestion activity of 1,680 units/μg solid at 25°C and a pH of 7.5 in the presence of calcium ions, was dissolved in a mineral solution consisting of 4.08 mM H_3PO_4, 20.10 mM KCl, 11.90 mM Na_2CO_3 and 1.98 mM $CaCl_2$ (chemicals from Merck) with a pH of 6.7 [Gerrard and Winter, 1986]. Specimens were immersed in the solution for 96 h at 37°C under constant agitation.

Scanning Electron Microscopy

From all groups, 5 specimens were randomly selected after matrix digestion, and the 5 additional specimens that had been submitted to 30, 60, 90 and 120 min of erosion, each without matrix digestion, were used for SEM. Specimens were fractured into halves, critical-point dried (Critical Point Dryer CPD 030; Baltec, Witten, Germany) and lightly gold sputtered. Cross-sections were inspected by the use of a SEM (type XL20; Philips Electron Optics, Eindhoven, The Netherlands) equipped with an LaB_6 cathode. The acceleration voltage was set to 10 kV. Images were recorded using a secondary electron detector, with the voltage of the collector grid biased to +300 V in order to improve the signal-to-noise ratio and to reveal optimal topographical contrast. The settings of the SEM for tilt angle, spot size and scanning mode were constant. The magnification was set at 500-fold.

Statistical Methods

Statistical procedures were performed with SPSS 17.0 statistics software (SPSS GmbH, Munich, Germany). The Shapiro-Wilk test revealed no significant deviation from the gaussian distribution. Differences between erosion times within the methods were analysed with ANOVA. Tamhane's post hoc test was used since there was a significant deviation from homogeneity of variance (Levene's test). The methods were compared with Bland-Altman plots, in which the x-axis represents the mean of the results obtained by the methods on comparison, and the y-axis represents the absolute difference between the 2 methods [Bland and Altman, 2003]. A relative bias is indicated when the difference of the means (δ) significantly differs from 0 by the one-sample t test, a proportional bias is indicated when the ordinary least squares regression line fitted to the plot has a slope that differs significantly from 0 [Ludbrook, 2002]. In all analyses, the significance level was set at $p = 0.05$.

Results

For the evaluation of tissue loss over time (table 1; fig. 1e), all methods revealed significantly increasing tissue loss from 30 to 60 and 60 to 90 min of erosion time ($p \leq 0.001$ for all methods), with the exception of LMR and ncPM before matrix digestion. Importantly, there was no further increase from 90 to 120 min of erosion time ($p > 0.05$ for all methods, except LMR before matrix di-

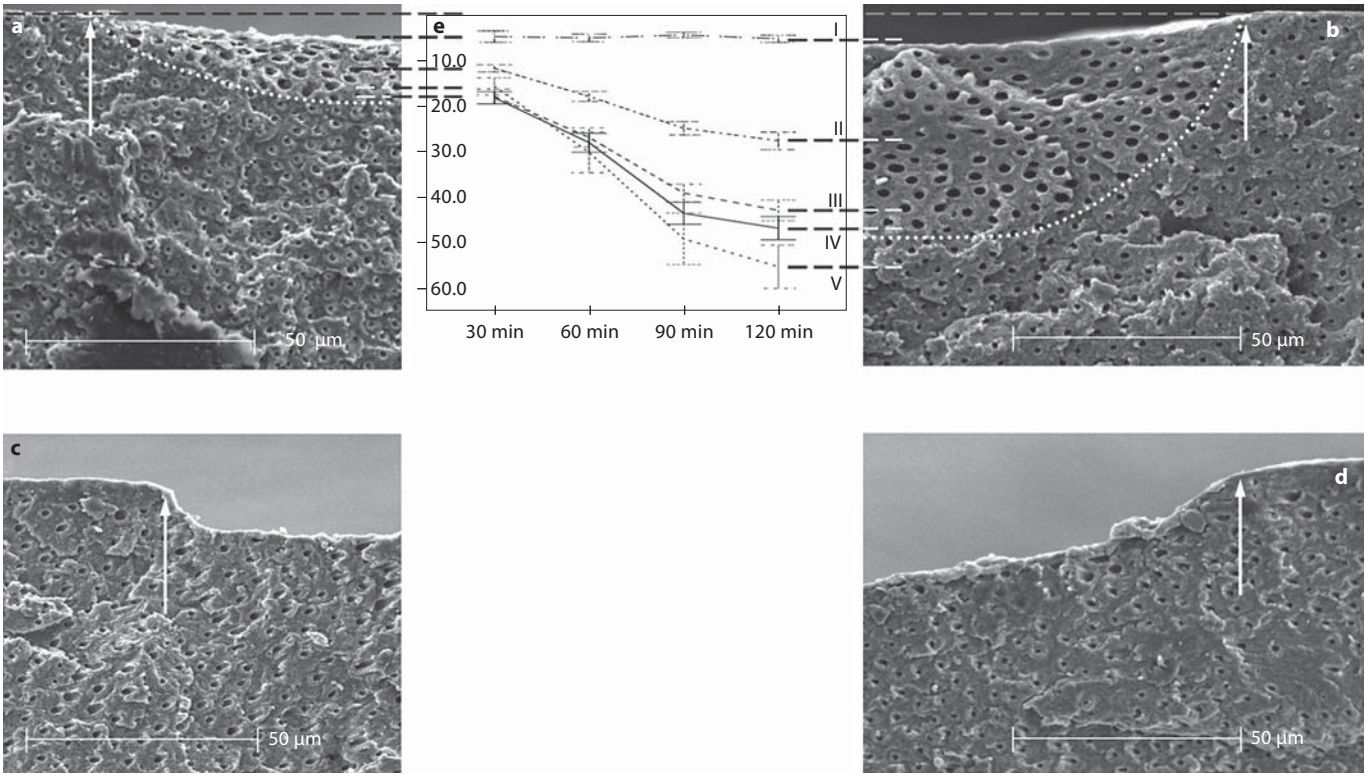

Fig. 1. SEM images of specimens after erosion for 30 min (**a**, **c**) and 120 min (**b**, **d**) before (**a**, **b**) and after (**c**, **d**) removal of the demineralised surface layer by digestion with collagenase. Dotted white lines in **a** and **b** indicate the boundary between demineralised organic material and sound tissue. Arrows in **a–d** indicate the boundary between reference and experimental area. **e** Results obtained by ncPM before (I) and after (III) collagenase, by cPM before (II) and after (IV) collagenase and by Ca analysis (V) over time. All loss values are shown in micrometres as mean and 95% confidence interval. Hatched lines connecting the graph (**e**) and the SEM pictures (**a**, **b**) represent the means of I–V and demonstrate the relation of the loss values after 30 and 120 min erosion time to the histological structure of the respective specimens. The scale of the graph corresponds to that of the SEM pictures.

Table 1. Erosive loss values obtained by ncPM, cPM, LMR and Ca analysis

Erosion time	Before matrix digestion, µm			After matrix digestion, µm			Ca analysis, µm
	ncPM	cPM	LMR	ncPM	cPM	LMR	
30 min	5.0 ± 3.0[a]	11.9 ± 1.9[a]	29.8 ± 7.5[a, b]	18.1 ± 3.9[a]	18.3 ± 3.2[a]	27.8 ± 14.2[a]	15.9 ± 4.6[a]
60 min	5.3 ± 2.0[a]	18.1 ± 2.7[b]	27.0 ± 12.4[a]	27.1 ± 5.0[b]	28.2 ± 5.0[b]	31.0 ± 20.6[a]	30.3 ± 10.8[b]
90 min	4.6 ± 5.8[a]	25.1 ± 3.5[c]	26.8 ± 14.0[a]	39.2 ± 4.7[c]	43.7 ± 5.8[c]	36.7 ± 15.2[a]	49.3 ± 13.7[c]
120 min	5.4 ± 1.9[a]	27.8 ± 4.6[c]	38.4 ± 13.3[b]	43.0 ± 5.5[c]	46.9 ± 6.2[c]	36.5 ± 12.2[a]	55.4 ± 11.5[c]

Values denote means ± SD. Within columns (between time points), means sharing the same superscript letter are not significantly different.

gestion). LMR did not detect changes in mineral content over time and ncPM prior to matrix digestion did not reveal a noticeable spatial tissue loss.

The most distinct differences between the methods were observed prior to matrix digestion. Losses obtained by ncPM were much lower than those by cPM, and neither method reached the range of values obtained by Ca analysis. After collagenase treatment, the loss values for both cPM and ncPM increased. The values from both profilometric procedures were similar. In particular, the

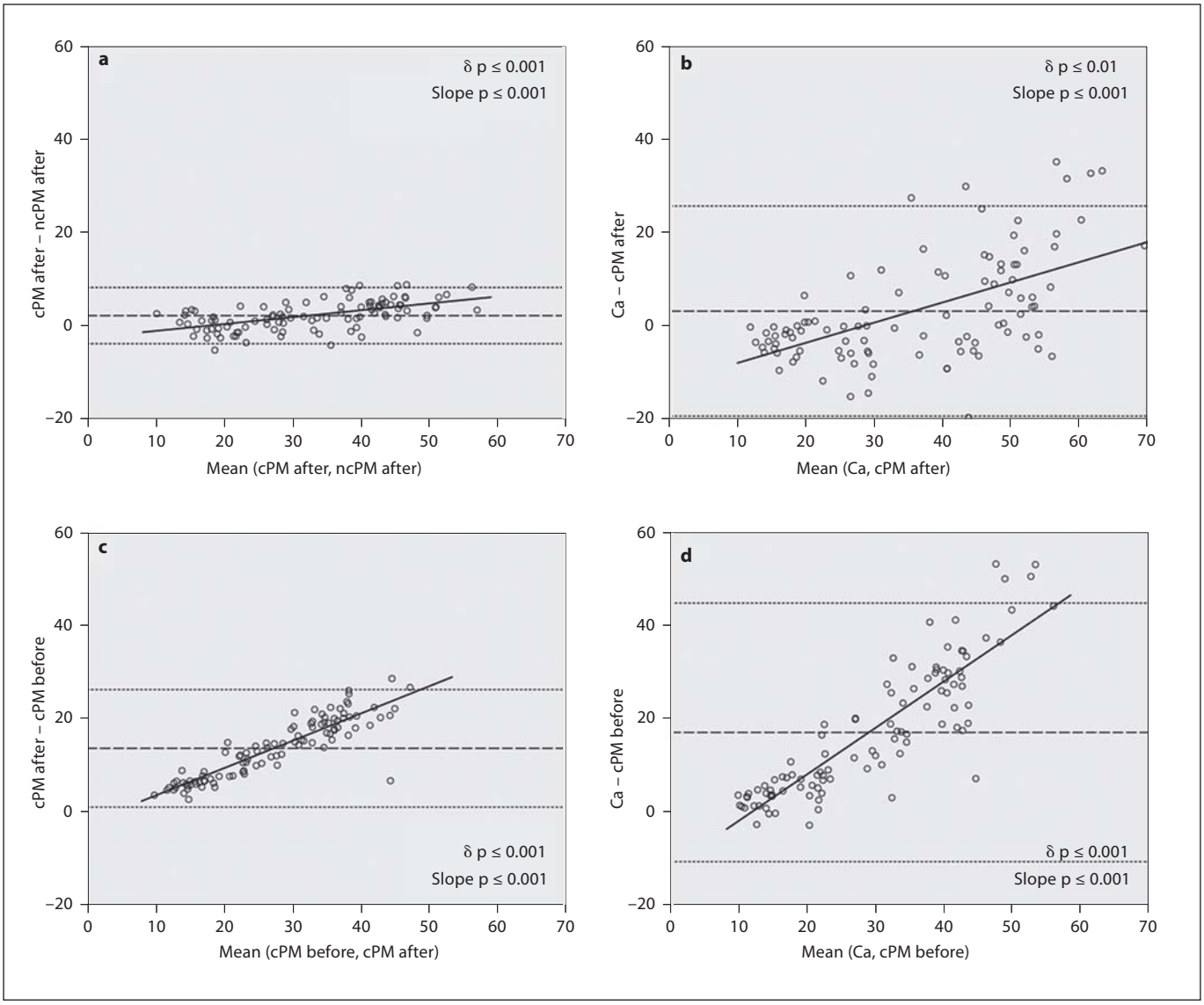

Fig. 2. Bland-Altman plots. Differences between ncPM and cPM (**a**), Ca analysis (Ca) and cPM after matrix digestion (cPM after) (**b**), cPM before matrix digestion (cPM before) and cPM after (**c**), and Ca and cPM before (**d**). Dashed lines = mean difference (δ); dotted lines = 95% confidence interval; solid lines = ordinary least squares regressions.

values from cPM were in accordance with those obtained by Ca analysis. The loss values obtained by ncPM were somewhat lower than those obtained by Ca analysis following 90 and 120 min of erosion.

Significant relative and proportional biases were found for all methods when agreement was estimated by Bland-Altman plots (fig. 2a–d), particularly when cPM before matrix digestion was compared to cPM after matrix digestion and to Ca analysis (fig. 2c, d). By cPM before matrix digestion, 95% of all measurements were between 10.6 units higher and 44.6 units lower than those obtained by Ca analysis, and 95% of all measurements were between 1.0 and 26.1 units lower than those obtained by cPM after matrix digestion.

After the removal of the matrix, cPM and ncPM revealed only a small, albeit significant, relative bias. However, a significant proportional bias was present, indicating lower values for ncPM at higher loss values (fig. 2a). A small relative bias was also detected when cPM was compared to Ca analysis (fig. 2b), but with a greater con-

fidence interval; 95% of the values from cPM would be 19.2 units higher and 25.9 units lower than those obtained by Ca analysis. The proportional bias indicated that Ca analysis yielded higher values than cPM at higher loss values.

Scanning Electron Microscopy

A distinct zone of demineralisation was found in all specimens prior to matrix digestion, which was demarcated from the sound tissue by slight surface shrinkage and by increased tubule diameter, and increased in thickness with increasing erosion times. The surface level of the demineralised tissue almost reached the level of the reference area (fig. 1a, b). After matrix digestion, a clear step was visible between the untreated reference area and the experimental area (fig. 1c, d). The demineralised tissue was completely removed. Minor signs of the demineralisation of the remaining mineralised tissue were present, and small remnants of organic material persisted on the surface of some specimens.

Discussion

The quantification of dentine erosion is challenging since these lesions have a complex histology that is primarily characterised by a completely demineralised surface zone, which can be of significant thickness and is resistant to physicochemical impacts [Ganss et al., 2009]. The presence of this layer not only interferes with the determination of the underlying mineralised tissue, but also indicates a considerable susceptibility to dimensional changes under ambient air [Ruben and Arends, 1993; Ganss et al., 2007].

The present study aimed to address both these aspects by analysing the specimens in the stage in which these characteristics appear after the erosive demineralisation, both with the demineralised organic material remaining on the specimen surface and after the enzymic digestion of these structures to expose the demineralisation front. The collagenase used for this purpose consists of at least seven distinct collagenases, which differ in their mode of attack on native collagen. Therefore, these collective enzymes degrade collagen non-specifically. Previous studies have demonstrated that collagenase digestion selectively removes the demineralised tissue [Ganss et al., 2007]. In the present study, this result was confirmed by SEM visualisation. Further, particular attention was paid to moisture control. The specimens were permanently stored in humidity and were re-wetted during profilometry prior to each tracing. Earlier experiments have shown that a re-wetting time of 30 s is sufficient to achieve a complete recovery of dehydrated demineralised surface structures [Ganss et al., 2007]. The erosion times were chosen to display 4 progressive stages of erosion to cover a range of commonly used experimental settings, according to the literature, and to permit comparison with the results of other publications [Ganss et al., 2005; Hara et al., 2005].

For the purpose of comparison, the results of calcium analysis and LMR were converted to a loss of mineralised dentine tissue in micrometres. When doing so, for instance, assumptions about density or mineral content, on which varying data exist, are necessary, and the results should be interpreted accordingly. When using these methodologies for describing a study, it is of course more appropriate to use the inherent units.

When analysing eroded dentine, the question under study must be carefully regarded. Considering its histology, tissue loss is not easy to be defined because both mineral and spatial tissue loss are possible target variables. The latter would include the demineralised organic part of the tissue. There is only little knowledge about the relevance of these structures, for instance, with respect to their capability to remineralise [Klont and ten Cate, 1991a; Hara et al., 2008]. Further, it is unknown to which extent such a matrix layer would occur in vivo. Therefore, and also when the focus of the current literature is considered, it appears reasonable to define dentine loss as the loss of mineralised tissue, ignoring the persisting organic fraction. The methods are discussed in the following, under the assumption that loss of mineral is the parameter under study.

Under the erosive conditions chosen here, a loss of mineral over time occurred, but LMR was not able to detect changes in mineral status either before or after treatment with collagenase. Dentine is less mineralised than enamel. Loss values of up to 50 μm in the present study would mean a mineral equivalent of 25 μm, under the assumption that the mineral fraction in dentine is 50 vol%. Given that mineral loss in enamel in the order of 20 μm was not reliably detected by LMR [Ganss et al., 2005], the mineral loss obtained under the experimental conditions chosen here was most likely below the detection limit of the method. This finding leads to the conclusion that LMR has limited application in studies of dentine erosion.

ncPM was also unable to detect changes in mineral status unless the organic matrix was removed. This result can be explained by the hydration of the matrix under the

chosen conditions, which thus nearly represents the original specimen surface (fig. 1e). The small values for loss are most likely due to dimensional changes in the matrix during the time needed for focusing the system and performing the tracing. cPM revealed significantly higher values than the non-contact approach. Importantly, these values did not reach the order of values obtained after collagenase treatment, which indicates that the mechanical stylus had not penetrated the matrix deeply enough to reach the demineralisation front even by the shortest erosion time (fig. 1a, b, e). The Bland-Altman plot clearly demonstrated that there was a distinct bias towards lower loss values with increasing mineral loss for values obtained before matrix digestion. The stylus most likely carves into the matrix to a certain extent, regardless of its thickness. The recession of the mineral front is therefore not proportionally mirrored, which could be particularly important for experiments with wide ranges of mineral loss.

Both ncPM and cPM revealed comparable results after matrix digestion even if a small significant relative bias was detected. It must, however, be considered that, particularly in the presence of a proportional bias, the method of plotting differences against means, as with the Bland-Altman plot, is at risk of overdiagnosing relative bias [Ludbrook, 2002], and also that the conclusion that methods agree sufficiently is not a statistical decision. In addition to the small relative bias, there was a tendency towards relatively lower values at higher mineral loss for the non-contacting device. This finding is most likely due to small remnants of organic material retained on the specimen surface, which occur more often with a thicker matrix. These remnants are pushed aside by the mechanical stylus, resulting in a more valid value for the mineral loss.

The results of both ncPM and cPM for erosion times of 30 and 60 min were in good accordance with the values obtained by calcium analysis. For more advanced stages of mineral loss, the loss values obtained by calcium analysis were somewhat higher than those obtained by profilometry. The calcium in the demineralisation solution also includes mineral dissolved by acid diffusion along the tubular structures of the tissue. This is supported by the SEM findings; in figure 1d, enlarged tubules are visible near the surface, indicating peritubular demineralisation, which was not determined by profilometry.

As mentioned above, information regarding the demineralised organic matrix could also be of interest. The difference between the values obtained by ncPM before and cPM after matrix digestion could be calculated to determine the thickness of the demineralised layer. In the present experiment, the thickness of this layer would have been estimated as 13, 23, 39 and 42 μm after 30, 60, 90 and 120 min of erosion, respectively.

The determination of the demineralised tissue thickness could be important, for instance, in studies on the effects of intraoral proteolytic enzymes on eroding dentine. Until now, enzyme effects have mostly been verified by measuring the degradation products in the enzyme solution [Klont and ten Cate, 1991b; Schlueter et al., 2007], which provides information regarding the amount of collagen digested, but not about the demineralised structures remaining in the specimen.

In conclusion, gross differences between microradiography, profilometry and chemical analyses of a dissolved mineral were observed, particularly in the presence of demineralised surface tissue. Therefore, an adequate selection and operation of methods according to the parameter under study is essential.

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