Applicability of Common Methods for Short Time Erosion Analysis *In Vitro*

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Purpose: Dental erosion can be measured by different methods. The aim of the present study was to check the applicability of common methods to determine initial erosive effects.

Materials and Methods: Enamel surfaces (4.5 mm^2) were eroded *in vitro* by treatment with hydrochloric acid (pH 2, 2.3 and 2.6) for 5 to 60 s or 240 s, respectively. Erosive effects were assayed with three different methods: Knoop's diamond indentation, profilometry and the determination of the dissolved calcium ions (Ca^{2+}) in a colorimetric assay based on the arsenazo-III-reaction.

Results: Erosive mineral loss of > 1 μm are measurable with profilometry. This corresponds to the erosive effects that occur after 60 s or more. Profilometric data yielded variance of up to 50%. Knoop's diamond indentation also showed some limitations: the depth of indentation reached a plateau after 30 to 120 s and the measurements showed variance of up to 85%. With the colorimetric assay, short time erosive effects occurring within 5 s could be assessed precisely and kinetically. The method allowed small amounts of 400 pmol Ca^{2+} per well to be quantified in small volumes with little variability.

Conclusions: For evaluation and quantification of short time erosive effects, the colorimetric method is superior to diamond indentation and profilometry.

Key words: calcium, erosion, microhardness, photometry, profilometry

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The contact of dental hard tissues with acids leads to irreversible mineral loss erosion. The prevalence of erosion in Western societies is increasing due to the consumption of acidic juices and soft drinks (Jaeggi and Lussi, 2006). Chronic gastro-oesophageal reflux disease and bulimia are also of relevance (Bartlett, 2006). Accordingly, the clinician is increasingly faced with the diagnosis and therapy involved in dental erosions (Ganss and Lussi, 2006; Jaeggi and Lussi, 2006). *In vitro* studies are necessary to characterise the erosive potential of different foodstuffs (Attin, 2006).

Erosion of dental hard tissues is linked to two phenomena: the softening and the loss of dental hard tissue, which has to be considered when quantifying and characterising demineralisation processes. Furthermore, *in vivo*, the contact of the dental hard tissues with erosive agents is usually restricted to a few seconds before clearance (Zero and Lussi, 2005; Attin, 2006; Hara et al, 2006). Accordingly, initial erosive lesions are linked with the loss of very few minerals on a nanometre or nanomolar scale and the adopted methods should be of high sensitivity. A few methods have been used for the characterisation and quantification of dental erosions (Barbour and Rees, 2004;

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Attin, 2006). The most established methods are: electron microscopy, microradiography, microindentation, profilometry and chemical analysis (Barbour and Rees, 2004). Electron microscopic techniques require extensive technical equipment, but allow visualisation of dental erosions caused *in vitro* and *in situ* (Hannig and Balz, 1999, 2001; Hannig, et al., 2004b). Microradiography is based on attenuation of x-ray irradiation transmitting dental hard tissue and enables quantification of both mineral loss and demineralisation, but it is not suitable for natural surfaces and quantification of early erosions is limited (Hall et al, 1997; Ganss et al, 2005; Attin, 2006).

According to Vickers or Knoop, common approaches for quantification of erosive effects are also a determination of microhardness. Thereby Knoop's method better reflects alterations of the superficial layer due to the geometry of the indentation diamond (Lussi et al, 2000; Attin, 2006). Another widespread approach for quantification of mineral loss is profilometry. In this case, eroded surfaces are scanned by a laser beam or a contact stylus (Herkstroter et al, 1989, 1991; Attin, 2006). For profilometry and Knoop's method, polished surfaces are necessary for successful assessment of the erosions and the amount of dissolved mineral is not assessed (Attin, 2006).

Chemical analyses based on photometric assays are of high sensitivity (Hattab and Linden, 1984; Morgan et al, 1993; Attin et al, 2005a). Thus, they are already applicable in the initial erosive phase (Hattab and Linden, 1984; Barbour and Rees, 2004; Attin et al, 2005a, b; Hannig et al, 2005, 2007). Examples for photometric approaches are the arsenazo-III-method for detection of calcium or the malachite-green assay for phosphate. (Hattab and Linden, 1984; Morgan et al, 1993; Attin et al, 2005a, b; Hannig et al, 2005). Calcium amounts of 400 pmol/well or phosphate amounts of 240 pmol/well can be detected with these methods. Only very few acids interfere with the photometric assay (Attin et al, 2005a, b; Hannig et al, 2005).

However, comparing these methods seems difficult. There are already papers and reviews dealing with different methods for quantification of erosive effects (Barbour and Rees, 2004; Ganss et al, 2005; Attin, 2006). One investigation directly compared microradiography, profilometry and atomic absorption spectroscopy, but this study considered only effects of 30 min or more (Ganss et al, 2005). Due to the fact that erosive effects occur within a few minutes or even seconds, the quantification of short time erosions is of high significance. Until now, there has been no study investigating and comparing the applicability of current methods for quantification of short time erosions.

Therefore, the aim of the present *in vitro* study was to compare the more recent colorimetric approach for quantification of erosive effects with the widely used profilometry and the indentation of Knoop's diamond with respect to their applicability for investigation of initial erosive effects. Thereby, the validity, reliability and precision of the three methods were tested. This was also done to facilitate comparison of different studies based on the named methods.

MATERIALS AND METHODS

Samples

Cylindrical enamel specimens of bovine incisors from 2-year old cattle were obtained from slaughter houses in Paderborn and Freiburg, Germany and were ground plane-parallel. All specimens were BSE-negative and the use of these bovine enamel specimens for *in vitro* and *in situ* studies on dental erosions was approved by the Ethics committees in Freiburg and Göttingen. Irrespective of the adopted method, all enamel surfaces were polished by wet grinding with abrasive paper (400 to 4000 grit). In the standardised grinding procedure, around 200 μm of enamel was removed. This was controlled with a micrometre measurement device (HHW, Hommel, Schwenningen, Germany) (Hannig et al, 2005). Before use in the experiments, the samples were ultra-sonicated for 5 min in double distilled water.

Acid-induced erosion

Erosion was conducted with HCl of pH 2.0 (10 mmol/l), 2.3 (5 mmol/l) and 2.6 (2.5 mmol/l), respectively. Usually, 100 μl or 50 μl acid was adopted if not stated differently.

The acid was continuously moved using a piston pipette. Additional colorimetric experiments were carried out without movement of the acid.

There were two incubation modes: short time erosion and long time erosion.

During short time erosion for 5 to 60 s, every 5 s a sample of 5 μl was taken to analyse the dissolved calcium.

In the long time experiment, the acid was applied for 240 s. Every 15 s, the acid was analysed and replaced with fresh acid without rinsing the enamel samples.

During application of the acid, enamel samples were covered with adhesive tape (Tesafilm, Beiersdorf,

Hamburg, Germany) leaving a window of 4.5 mm^2. The standardised windows were cut with a ticket punch usually adopted for rubber dam. Uneroded parts of the specimens covered with tape during erosions served as a reference.

Profilometry

Profilometry was carried out with a stylus profilometer (Perthometer, Mahr, Göttingen, Germany, vertical resolution ±250 μm). Prior to the experiments, basic surface profiles from all specimens were taken to obtain reference surfaces for calculating enamel loss. Five profile measurements were performed in the centre of each specimen at intervals of 500 μm and averaged. The length of the profile measurements amounted to 4 mm, with recording of measure points every 0.69 μm. With specially designed software (Mahr Perthometer Concept version 7.0, Mahr, Göttingen, Germany) the average depth of the surface area of the specimens relative to the basic surface profiles was calculated (Wiegand et al, 2006).

A number of six enamel specimens were evaluated per pH value in each profilometric assay.

Mineral loss of the enamel samples was calculated from the mean depth of the eroded enamel as measured profilometrically. Based on the mean depth of the eroded area, the known surface area of eroded enamel (4.5 mm^2) and the mean density of enamel (2.95 g/cm^3), the mineral loss (g) was estimated (Attin et al, 2005a, b).

Microhardness/indentation of Knoop's diamond

The surface indentation of Knoop's diamond was adopted for characterisation of the erosive effects (Knoop-instrument, Leitz, Wetzlar, DIN 52333). Application of Knoop's diamond results in a rhomboid indentation of a certain length, which allows calculation of the impression depth. The indentation was performed with 1.96 N. Impression of the diamond was determined before application of the acid (as a reference value) and after the erosion took place. The difference in these values is given in the results. Accordingly, the values given for untreated samples were 0. The depth of Knoop's diamond impression was calculated from the length of the impression according to the geometry of the diamond. The indentation of Knoop's diamond amounts to 1/30 of the length.

Ten enamel samples were investigated per pH value in each experiment, one indentation was carried out per sample and each indentation was measured twice.

Colorimetric determination of calcium dissolution

Release of calcium was measured using the arsenazo-III-method (Fluitest®, Ca-A-II, Analyticon, Lichtenfels, Germany) as described previously (Attin et al, 2005a; Hannig et al, 2005). Arsenazo-III reacts with calcium in an acid solution to form a blue–purple complex. The intensity developed is proportional to the calcium concentration. Absorption can be determined at λ = 650 nm. The reagent used for determination was composed of 100 mmol/l imidazole buffer (pH 6.5) and 0.12 mmol/l arsenazo-III. Individual standard curves were obtained for each pH with standardised calcium solutions for determination of the calcium concentrations. Precision of the measurements was validated with the standard solutions. Twelve enamel samples were investigated per pH value in each experiment in repeat determination.

Mineral loss of the enamel samples was estimated from the calcium loss. In brief, the calcium loss (in mg) was calculated from the calcium concentrations. About 35 wt% of the enamel is calcium (Elliott, 1997). This allowed determination of the enamel loss (in mg) (Patel et al, 1987). Based on the calculated enamel loss, the known surface area of eroded enamel (4.5 mm^2) and the mean density of enamel (2.95 g/cm^3), the depth of the substance loss was estimated (Attin et al, 2005a, b).

Statistics

Linearity of the erosive degeneration measured with the colorimetric method and with profilometry as well as comparison of the methods was determined by Pearson correlations (Excel, Microsoft Office 2000).

RESULTS

Profilometry

Fig 1 gives an impression of typical diagrams gained with profilometry. The waviness of the surface amounted to 0.2 μm for polished surfaces (Fig 1a), up to 1 μm for eroded surfaces and the roughness was about 0.04 μm (Fig 1b). This limited the sensitivity of the method. Thus, short time erosive effects could not be monitored precisely. Even with strong acids, for example HCl (pH 2), only erosive demineralisation occurring after 60 s or more could be differentiated from the innate waviness of the eroded surfaces, despite the fact that the degradation showed a linear

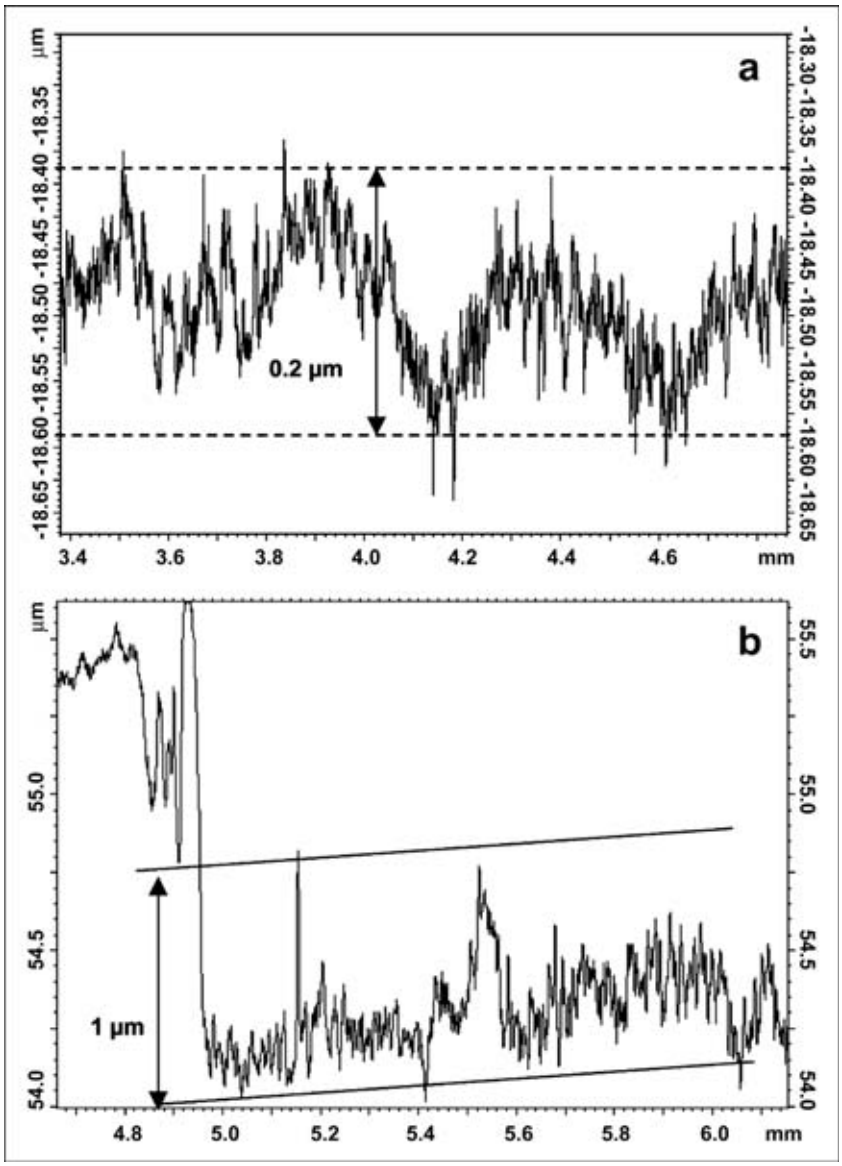

Fig 1 Stylus profilometry of a polished enamel surface (a) and of an eroded enamel surface (b). The innate waviness of the polished surface amounted to 0.2 µm, the waviness of the eroded surface was up to 1 µm. In (b), the eroded area of the specimen can be differentiated from the part of the sample covered by adhesive tape during the acid treatment.

course (Fig 2). The enamel loss showed a strong correlation with the acid contact time (pH 2: r = 0.999; pH 2.3: r = 0.998; pH 2.6: r = 0.938).

The depth of the erosion should amount to at least 1 µm when profilometry is used. With HCl of a higher pH, an erosion depth of 1 µm is reached only after 150 s (pH 2.3) or after 175 s (pH 2.6). Even after this time, variance of up to 50% was observed.

Colorimetric determination of calcium dissolution

Colorimetric determination of calcium loss was performed under different conditions.

During long-time experiments with supplement of fresh acid, linear kinetics of calcium loss were observed (Fig 3). The calcium loss showed a high correlation with the acid contact time (r > 0.999).

Also short time erosive effects occurring within 5 to 60 s could be quantified (Fig 4). In the present study, minimum calcium amounts of 0.4 nmol/well were quantified successfully. The dynamics of the acid-induced demineralisation had considerable impact on the amount of calcium dissolved. If the acid was moved by a piston pipette, linear dissolution was observed until the acid was consumed after 40 s, as no fresh acid was supplemented. There was a high correlation of the calcium loss with the duration of the erosive attack (pH 2: r = 0.996; pH 2.3: r = 0.993; pH 2.6: r = 0.994). If the acid was not moved continuously using the piston pipette, only half the amount of eroded calcium was detected

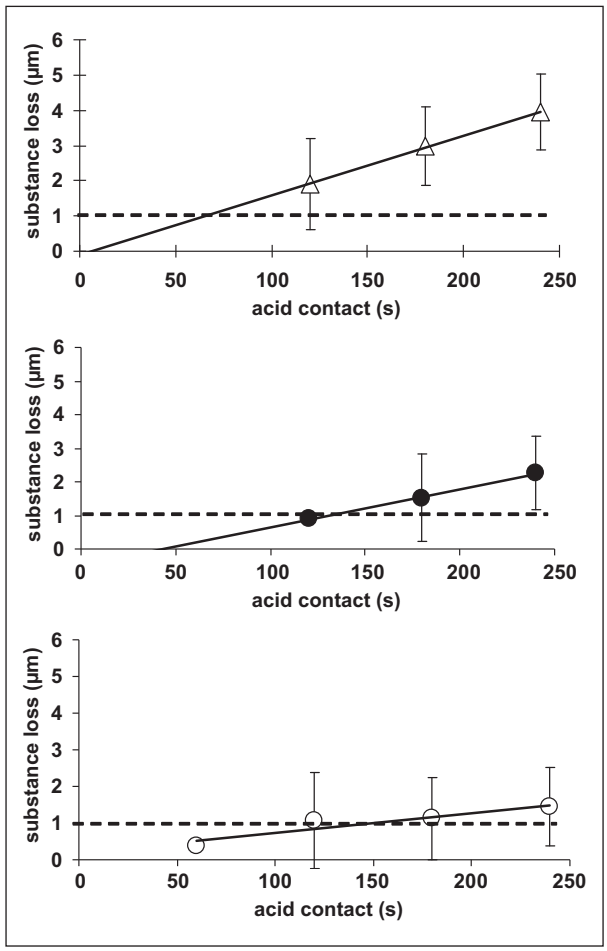

Fig 2 Stylus profilometry (MV ± SD), enamel loss after treatment with HCl of differing pHs. The reference level for determination of the mineral loss was the untreated enamel surface of the sample. The discontinuous line marks the lower limit for determination of erosive mineral loss with profilometry predetermined by the waviness of the eroded areas (compare with Fig 1). Please note the high standard deviation (n = 6). Δ: pH 2.0; ●: pH 2.3; ○: pH 2.6.

and the kinetics showed no linear course. Note the low standard deviation of the measurements. The maximum variance amounted to 33%, observed only with very small amounts of calcium (Fig 4b).

Knoop's diamond indentation

The indentation depth of Knoop's diamond is given as the difference between the value after use of the acid and the reference value of the untreated sample. Irrespective of the pH, the depth of the Knoop's diamond impression reached a plateau after 50 to 120 s (Fig 5), despite the fact that the erosive degradation of the enamel surface

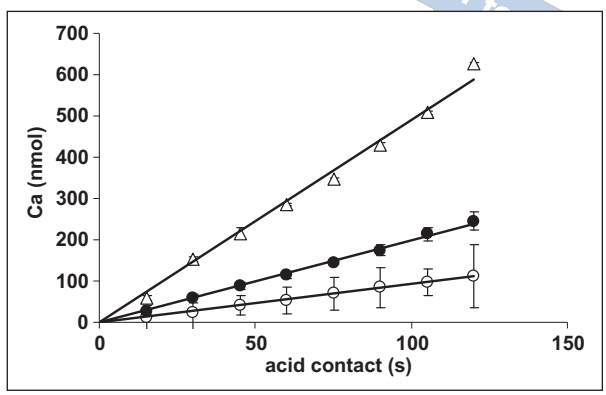

Fig 3 Calcium loss (MV ± SD, n = 10) determined colorimetrically parallel to the indentation of Knoop's diamond (n = 10). One hundred microlitres of the acid was applied on 4.5 mm^2 enamel for 15 s each. The calcium loss during the different acid applications was cumulated. Please note the low standard deviation as compared with Fig 4. Δ: pH 2.0; ●: pH 2.3; ○: pH 2.6.

continued as shown by profilometry and colorimetric determination of calcium loss. The difference of the indentation depth after reaching the plateau was about 200 nm, indicating the loss of hardness due to erosion of the enamel. The impression depth of the Knoop's diamond showed coefficients of variance up to 85% after 15 s of acid contact. Some aspects inherent to the method are to be considered in connection with the high variability of the measured values. The optical resolution of the Knoop instrument amounted to 500 nm and impression lengths of about 60 µm were recorded. Due to the fact that both ends of the rhomboid impression have to be read out precisely, the inaccuracy may be enhanced. Under optimal conditions, the standard deviation of the length, just owing to the optical resolution amounts to 1000 nm, meaning a SD of 33 nm for the depth (the impression depth is 1/30 of the length). However, the surfaces altered by erosion and the outlines of the indentations were often diffuse. This hampered precise estimation of the impressions. Reference surfaces that were not eroded featured an impression depth of about 2200 nm and eroded enamel of 2400 nm. The difference in these two measurements is the extent of the erosive destruction. Accordingly, the measured value is less than 10% of the background or reference value and errors of the measurement cause extensive variance in the final value. If the basic measurement of the impression depth (2200 nm) has a variance of only 5% meaning a standard deviation of 110 nm, the difference value in the range of 200 nm has a standard deviation of 110 nm, that is, a variance of 55%.

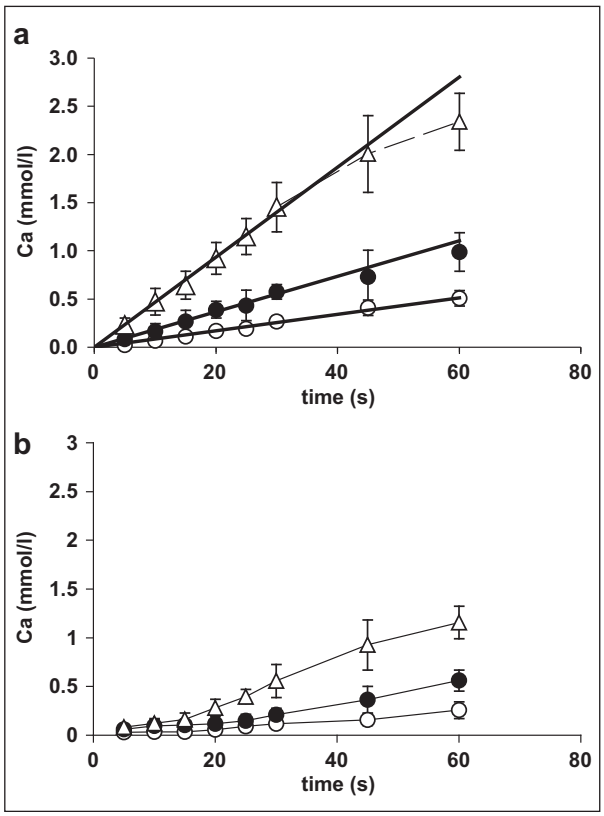

Fig 4 Calcium release (MV ± SD) from enamel at different time points with (a) and without (b) movement of the applied acid (n = 12 specimens/pH). Δ: pH 2.0; ●: pH 2.3; ○: pH 2.6.

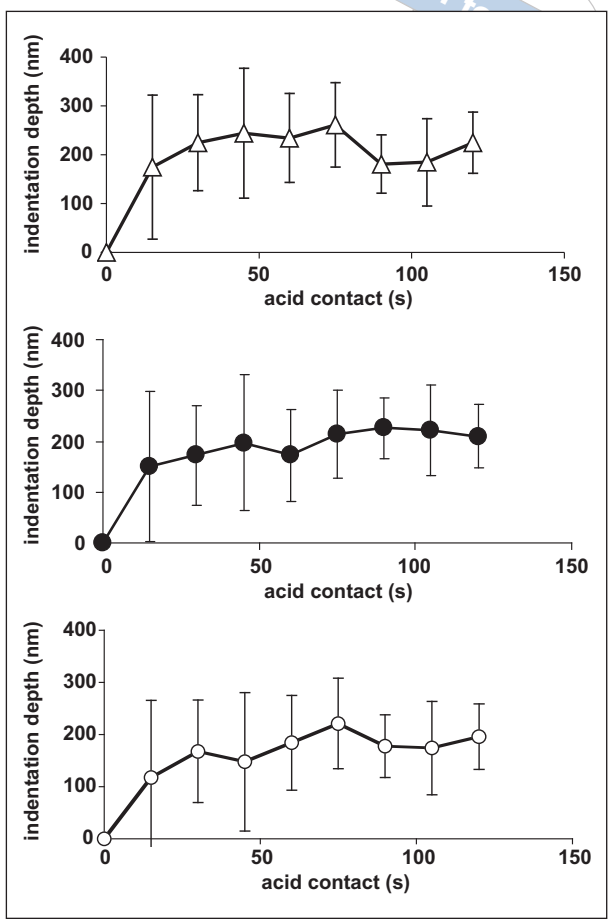

Fig 5 Depth of Knoop's indentations (MV ± SD) at different time points after treatment of enamel with HCl of differing pHs using 100 µl HCl at each time point. The impression depth is given as difference of eroded samples and untreated references. Please note the high standard deviation (n = 10). Δ: pH 2.0; ●: pH 2.3; ○: pH 2.6.

Comparison of the methods

Regarding the precision of the three methods, colorimetric assays yielded the lowest coefficients of variance (Figs 3 and 4).

The profilometric method allows calculation of the mean mineral loss in nm/min. This amounted to 1167 nm/min for pH 2.0, 679 nm/min for pH 2.3 and 317 nm/min for pH 2.6, respectively. From the calcium loss recorded in the same experiments, the thickness of the lost enamel was estimated based on the percentage of calcium in enamel (35%) and on the density of enamel.

The calculated mineral loss was 966 nm/min for pH 2.0, 678 nm/min for pH 2.3 and 342 nm/min for pH 2.6, respectively. The measured or calculated results of both methods were in good accordance (r = 0.972, Fig 6).

Calcium release was also measured, along with determination of Knoop-hardness (Fig 3). In contrast to the impression depth of Knoop's diamond, showing a high coefficient of variance and reaching a plateau (Fig 5), continuous linear release of calcium was detected. The standard deviation of the calcium assay was very low and a strong correlation of the calcium loss with the residence time of the acid was recorded (pH 2: r = 0.994; pH 2.3: r = 0.997; pH 2.6: r = 0.997).

The comparison of Knoop's diamond indentation and profilometry was depicted for pH 2.3 (Fig 7). The erosive substance loss, as indicated by profilometry, increases linearly, whereas the softening of the

surface as indicated by the indentation depth of Knoop's diamond reached a plateau after 1 min. The combination of Knoop's diamond indentation and profilometry is depicted schematically in Fig 8. The depth of Knoop's diamond indentation reached a plateau, but the erosion proceeded and substance was lost as indicated by the line of stylus profilometry. This indicated that after a certain time of erosive surface softening, portions of the enamel got lost and the demineralisation front moved forward (Fig 8).

DISCUSSION

The present study aimed to compare the applicability of different methods for evaluation of initial erosions under standardised conditions in an *in vitro* approach. As in previous studies, bovine enamel was chosen for the experiments, because large samples of homogeneous quality can be easily gained (Hannig et al, 2004a, b, 2005). Furthermore, the chemical composition of human and bovine enamel is quite similar (Nakamichi et al, 1983). Despite the fact that erosions are caused mainly by organic acids and HCl is only relevant clinically in the context of bulimia or regurgitation, this acid was chosen because it is nearly completely dissociated. An increase in pH means direct consumption of the acid occurs as no further acid molecules can dissociate. This offers well-defined and reproducible experimental conditions. Furthermore, in contrast to organic acids, no chemisorption is shown on enamel surfaces (Fu et al, 2004, 2005). Some organic acids, such as citric acid, chelate calcium ions and are not appropriate for validation of different methods (Hannig et al, 2005; Featherstone and Lussi, 2006).

The guidelines for bioanalytical methods demand for intra-assay and inter-assay precision (Shah et al, 1991, 2000; Attin, 2006). Furthermore, it is necessary to know the lower limit of quantification, which has to be determined before use of the assay for a certain subject. Only those readings that are higher than the value of detection limit plus five times the standard deviation should be considered in the analysis (Shah et al, 1991, 2000; Attin, 2006). Under the given conditions, depth of Knoop's diamond indentation seems to be an inappropriate approach when quantifying initial erosive mineral loss. The data showed a high coefficient of variance and reached a plateau after 40 to 60 s of erosion. Eroded dental hard tissues are softened inhomogeneously, causing the high standard deviation. If erosion proceeds, some parts of the eroded substrate get lost irreversibly, but the impression of Knoop's diamond remains identical (Fig 8).

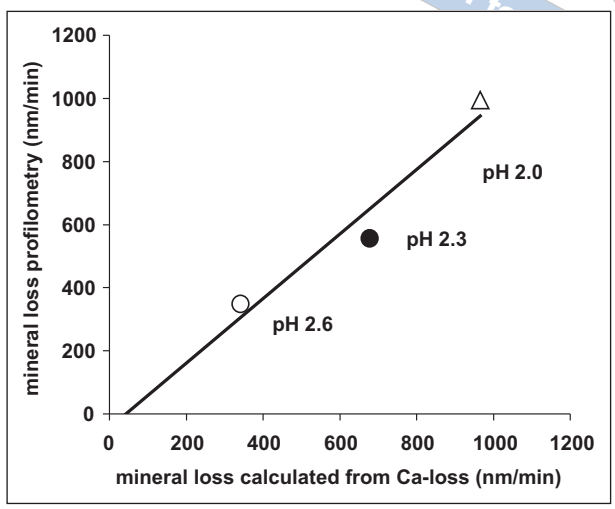

Fig 6 Substance loss (nm/min) as measured by profilometry and calculation of the enamel loss according to recorded calcium loss at pH 2 (Δ), 2.3 (●) and 2.6 (○). One hundred microlitres of the acid was applied for 60 s each (n = 6 samples/method, pH and mean values).

There is another approach for quantification of mineral loss based on Knoop's diamond indentation: impressions of the diamond are placed on samples after acid-induced erosion and measured with and without brushing abrasion. The reduced length or depth of the impression is used to calculate mineral loss of softened enamel (Jaeggi and Lussi, 1999; Joiner et al, 2004; Attin et al, 2005c). This option was not carried out in the present study, but due to the observed high standard deviations, the precision of this approach might be limited when investigating short time erosive effects.

Profilometry yielded high standard deviations, and waviness of eroded enamel limits the applicability of profilometry on initial erosive effects occurring within the first 120 s. Depending on the pH of the acid, only the erosive mineral loss after 60 s or more can be quantified sufficiently. Notwithstanding these limitations with profilometry, linear erosive degradation of the surface was observed as has been previously demonstrated during long time experiments of 30 min or more (Ganss et al, 2005).

The lower limit of quantification for conventional stylus profilometry amounts to 1 μm as confirmed in the present study (Barbour and Rees 2004; Attin 2006). The dissolution of dental hard tissues may yield a surface roughening up to 1 μm. However, one investigation distinguished different abrasiveness of dentifrices inducing hard tissue loss of 0.5 μm (Hooper et al, 2003; Attin 2006).

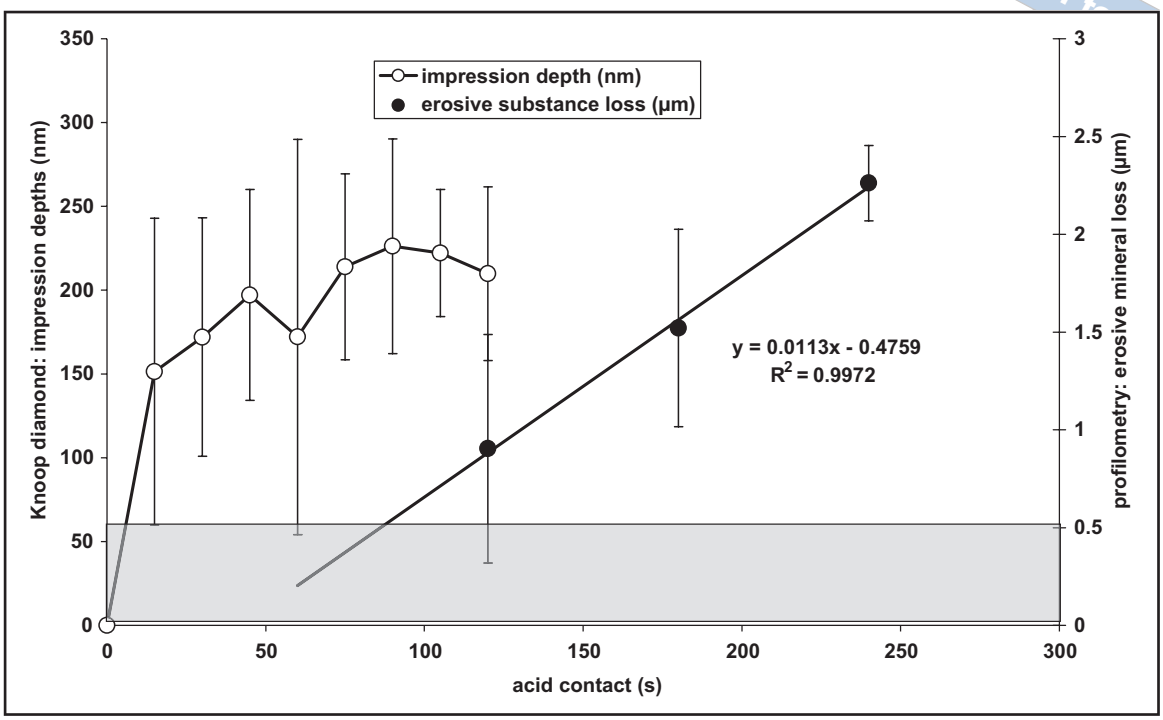

Fig 7 Comparison of profilometry (right) and depth of Knoop's diamond indentation (left). Incubation with HCl of pH 2.3.

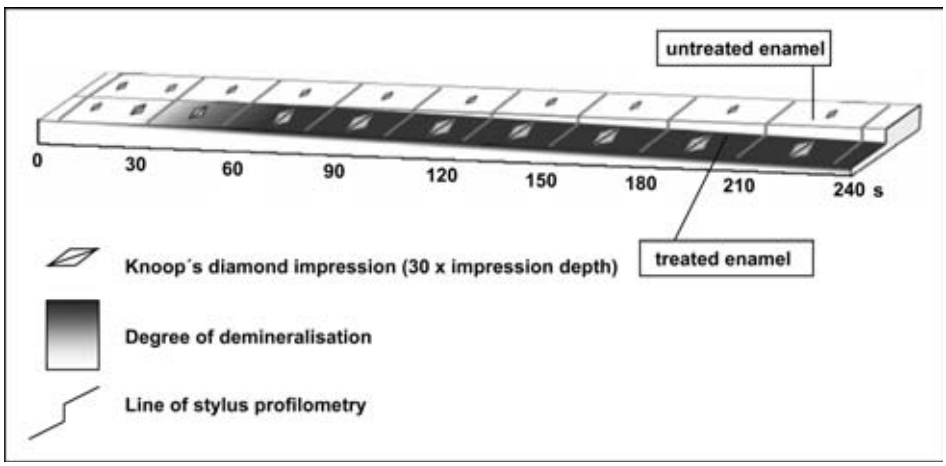

Fig 8 Schematic drawing of erosive demineralisation on a time scale up to 240 s with a smooth transition from softening to enamel loss. The impression of Knoop's diamond increases to reach a plateau according to the superficial degree of demineralisation. The profilometry indicates continuing loss of completely eroded enamel.

The outer layer of eroded enamel is extremely vulnerable and fragile, therefore, the stylus of a conventional profilometer may penetrate and destroy this structure (Attin, 2006). If a laser beam is used instead, a higher resolution is achieved in a non-destructive manner. However, the laser stylus may cause overshoots at the sharp edges at the bottom of the grooves resulting in artefacts (Whitehead et al, 1999; Attin, 2006). A more recent method for precise surface exploration is nano-indentation. The technique yields data on surface structure and surface properties such as plastic and elastic energy (Finke et al, 2001; Attin, 2006). Disadvantages of this promising technique are the costs and the time-consuming measurements (Attin, 2006).

Determination of calcium release was the most sensitive assay in the present study, thereby yielding the lowest coefficient of variation as demanded in

recent guidelines for biometric assays (Shah et al, 1991, 2000). Mineral loss was linear with time, and initial calcium loss that occurred within the first few seconds could be measured precisely. The photometric method allows quantification of very low amounts of calcium (400 pmol) in a kinetic manner (depending on the tested acid) in small sample volumes of 1 to 20 µl (Attin et al, 2005a, b; Hannig et al, 2005). The method has proved successful *in situ* and *in vitro* (Hannig et al, 2005, 2007). In contrast to physical methods, a main advantage of the photometric approach is that the fragile softened enamel is not touched. No conclusion can be drawn from the structural modifications of the eroded dental hard tissues. Accordingly, it is recommended that these colorimetric techniques be combined with another technique (Hannig et al, 2007).

The present study shows the colorimetric and profilometric data is transferable. Despite the high standard deviation of the profilometry, the data corresponded well with the substance loss per min as calculated from the calcium loss measured colorimetrically. Accordingly, conversion of results from different studies for purpose of comparison is possible if the experimental conditions are similar. However, this is not possible with Knoop's diamond impression.

Another study has already compared different methods for quantification of erosions including profilometry and atomic absorption spectroscopy-based calcium and phosphate measurement. The profilometric data and the calcium and phosphate release yielded a high correlation of r = 0.946 (Ganss et al, 2005). However, this study only considered erosion effects of 30 min or more (Ganss et al, 2005).

A further approach to evaluate the erosive potential of drinks is to add hydroxyapatite powder to the beverages and to record the pH. Based on these data, the amount of apatite lost per second can be calculated. Despite the fact that hydroxyapatite is used instead of enamel, the method allows characterisation of the erosive potential yielded by variable beverages in a standardised manner (Jensdottir et al, 2006).

The authors' study focused on the comparison of different established and recent methods with respect to their application for quantification of initial erosive demineralisation. Accordingly, factors modulating the erosive demineralisation in the oral cavity were excluded, such as type of acid, saliva or the acquired pellicle (Hannig and Balz, 1999, 2001; Hannig et al., 2003, 2004b, 2005). The acquired pellicle, a mainly proteinaceous layer, may serve as a semi-permeable barrier and buffer, thereby exposing some protective properties. The colorimetric method has already proved to be a success in an *ex vivo/in vitro* study considering pellicle coated samples (Hannig et al, 2007). Further studies based on the colorimetric method combined with imaging techniques such as electron microscopic approaches would be desirable so as to elucidate short time erosive effects. One parameter influencing the kinetics of dental erosions was examined in the present *in vitro* approach, namely the movement of the acid. It was shown clearly that the flow agitation of the acidic solution has a strong impact on the extent of the erosion (Lussi et al, 1993; Wiegand et al, 2007). Also *in vivo*, erosions take place in a dynamic manner and not in a steady state, supplying fresh acid at the tooth surface continuously. In general, the present study confirmed the relevance of the pH in the extent of erosive mineral loss (Hannig et al, 2005).

CONCLUSIONS

Due to the high sensitivity and the high precision, colorimetric determination of calcium loss is superior to other methods for quantification of initial erosive demineralisation. The application of profilometry and Knoop's diamond indentation for evaluation of initial erosive effects is limited. However, for in-depth characterisation of dental erosions, combination of two or more methods is recommended.

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