Effect of fluoride solutions on enamel erosive wear and validation of methods used for analyses

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Contents

LIST OF PAPERS

The following papers (1-4) have been submitted and published in partial fulfillment of the requirements for the degree Philosophiae Doctor (PhD) at the Faculty of Dentistry, University of Oslo, Norway.

In the thesis they will be referred to as **Study 1, 2, 3** and **4**.

1. Stenhagen KR, Hove LH, Home B, Taxt-Lamolle S, Tveit AB: Comparing different methods to assess erosive lesion depths and progression in vitro. Caries Res 2010; 44: 555-561.

2. Hove LH, Holme B, Stenhagen KR, Tveit AB: Protective Effect of TiF_4 Solutions with Different Concentrations and pH on Development of Erosion-Like Lesions. Caries Res 2011; 45: 64-68.

3. Stenhagen KR, Hove LH, Holme B, Tveit AB: The effect of daily fluoride mouth rinsing on enamel erosive/abrasive wear in situ. Caries Res. 2012 Sep 21; 47(1):2-8. [Epub ahead of print]

4. Stenhagen KR, Hove LH, Home B, Tveit AB: Enamel erosion depths measured on impressions by a White Light Interferometer. Acta Odontol Scand, 2012; Jun 25.[Epub ahead of print]

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INTRODUCTION

Tooth wear can be caused by attrition, abrasion, abfraction, acid erosion or often by combinations of these factors. During the last 15 years there has been an increased focus from clinicians and researchers on pathological tooth wear caused by acid erosion. This was confirmed by data from a recent questionnaire- based study among dentists in Norway in which they reported that a higher numbers of erosive lesions are diagnosed today than 10-15 years ago [Mulic et al., 2012d].

Dental erosion was first simply defined as loss of dental hard tissue without involvement of bacteria [Pindborg, 1970], but was later described as pathological, chronic, localized loss of dental hard tissue that is chemically removed by acid and/or chelation without bacterial involvement [ten Cate and Imfeld, 1996]. Extrinsic acidic challenges to tooth surfaces can be food and drinks [Zero, 1996; Zero and Lussi, 2000], occupational, such as wine tasting [Mulic et al., 2011; Wiegand and Attin, 2007; Wiktorsson et al., 1997] and battery manufacturing [Tuominen et al., 1989; Tuominen et al., 1991] and sports related, such as swimmers exposed to pool chemicals [Geurtsen, 2000]. Intrinsic sources can be gastric acids introduced to the oral cavity when the individual suffers from eating disorders with vomiting [Johansson et al., 2012; Milosevic and Slade, 1989; Rytomaa et al., 1998] or chronic gastric obstructive reflux disease (GORD) [Bartlett et al., 1996; Gudmundsson et al., 1995; Holbrook et al., 2009; Jarvinen et al., 1988; Meurman et al., 1994; Moazzez et al., 2004; Moazzez et al., 2005]. It is assumed that 1-3% of women in UK suffer from bulimia and that 65% of the western population at some time suffers from GORD. Changes in lifestyle in the western countries, leading to high and frequent consumption of acid containing products [PackerC.D., 2009] may have contributed to the increased risk and prevalence of dental erosive wear.

There is some evidence that the prevalence of dental erosive wear has been growing [Ganss et al., 2001; Lussi, 2006]. Data from prevalence studies vary. They are difficult to compare and may be unreliable due to differences in age between the investigated groups, different diagnostic tools like the scoring/gradation systems, and non-reporting of calibration of the observers. Dental erosive wear in the primary dentition has been reported to be from 6% to 52% [Al-Malik et al., 2002; El Aidi et al., 2010; Luo et al., 2005; Millward et al., 1994; Murakami et al., 2011] and in adolescents (12- 18 year olds) from 20% to 58% [Gurgel et al., 2011; Hasselkvist et al., 2010; Margaritis et al., 2011; Mulic et al., 2012b]. There exist few

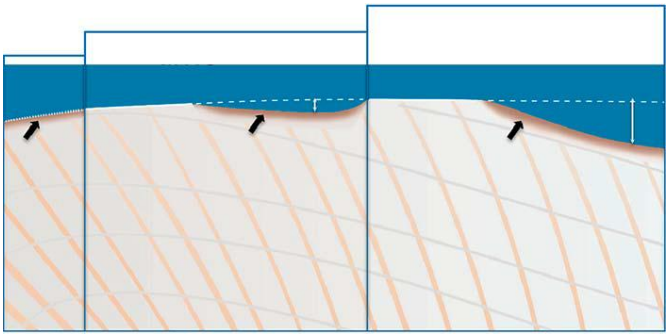
prevalence data from adults [Fares et al., 2009; Lussi et al., 1991; Van't Spijker et al., 2009] and in these individuals it can be difficult to distinguish tooth wear caused solely by erosion from dental erosive wear in combination with abrasion and/or attrition. After all, tooth wear is to some extent a normal physiological result after many years of use and one must distinguish this from pathological tooth wear.

Active erosive lesions on the tooth surfaces will progress when no adequate preventive measures are implemented as indicated in a longitudinal study by [Dugmore and Rock, 2003] where 5% of the investigated 12- yr- olds were diagnosed with deep erosive enamel lesions and 23% two years later. From the patient's point of view, severe dental erosive wear leads to symptoms like hypersensitive teeth, chewing difficulties and poor esthetics, often requiring extensive and expensive restorative treatment as a result [Johansson et al., 2008]. The existing dental treatment philosophy for caries is preventive, non-operative and non-invasive dental therapy [Ekstrand and Christiansen, 2005; Fejerskov, 2004; Hausen et al., 2007; Marthaler, 1965; Raadal et al., 2011] and this concept has guided research into care to prevent tooth substance loss due to erosion.

Dental erosive wear and its progression are influenced by multiple factors. This can be illustrated by the observations that persons suffering from eating disorders with vomiting are at high risk of dental erosion [Bartlett and Coward, 2001; Johansson et al., 2012] but not all bulimic patients have dental erosive wear irrespective of the severity of their disease [Robb et al., 1995]. A similar observation was made in a study by [Mulic et al., 2012b] in which 18-yr- olds reported frequent consumption of acidic drinks, but not all of them were diagnosed with dental erosion. Variations in individual biological factors, like dental enamel and morphology, the protective properties of the acquired pellicle and saliva factors such as flow, buffer capacity and protein or enzyme content, probably influence the occurrence and development of the condition. It is therefore difficult to draw conclusions directly from *in vitro* or *in situ* studies. It is important to test the effect of different preventive procedures and products in clinical studies [Huysmans et al., 2011]. Because of the difficulties in measuring directly on the tooth surfaces *in vivo*, several studies have investigated the possibility of taking impressions of the affected or treated teeth and measuring tooth substance loss directly on the impression surface or on casts [Azzopardi et al., 2001; Bartlett et al., 1997; Schlueter et al., 2005; Sundaram et al., 2007a; Ranjitkar et al., 2009; Holme et al., 2005]. After reviewing a variety of methods and instruments used for assessing tooth substance loss it was

recently suggested that, for *in vivo* studies, surface mapping techniques via replica-based methods would be valuable and should be evaluated further [Schlueter et al., 2011a;Shellis et al., 2011].

An acid attack on the tooth results, firstly, in loss of surface structural integrity and mechanical strength termed "softening" by [Koulourides et al., 1968]. A prolonged erosive challenge will further lead to bulk loss of enamel. In the clinic, it can be difficult to distinguish wear due to erosion from mechanical wear, but in research models this can be controlled. From a methodological point of view, it is considered appropriate to use the two terms "erosion" (chemical) and "erosive tooth wear" (chemical-mechanical) [Huysmans et al., 2011; Shellis et al., 2011].



a b c

Figure 1. Different stages in the continuous erosion process, from left to right: a) softening of the enamel (no material loss) b) partial material loss (erosive tooth wear) and softening of the underlying surface (black arrow) and c) significant loss of material and softening of the underlying surface [Lussi et al., 2011].

In parallel with the increased focus on the prevalence of dental erosive wear and its clinical implications, many studies have been conducted aimed at finding a preventive treatment. Just as the lack of consistency in the design of prevalence studies is frustrating, it has been pointed out, that the results from studies where preventive strategies are investigated are difficult to compare for similar reasons [West et al., 2011]. The methodology varies considerably, regarding experimental design both *in vitro* and *in situ*, the erosive or abrasive challenge and the quantitative and qualitative methods used for characterization and measurement of erosion and erosive tooth wear in enamel or dentine. All methods and instruments have limitations and it is often necessary to include more than one in order to get

information about different aspects of the surface changes. Independently of the instruments used a validation by calculating the accuracy and repeatability (precision) of the measurements is required, so we know what is actually measured and whether it is reliable [Schlueter et al., 2011a]. In the present studies, the experimental erosive challenge (hydrochloric acid) was chosen to imitate gastric acid and we measured quantitative substance loss on the enamel surfaces. This subject is further discussed in the paragraph; Methodological considerations. In previous studies [Hove et al., 2006; Hove et al., 2007a; Hove et al., 2008; Holme et al., 2005] a technique was developed by which a white light interferometer (WLI) was used for measurements of erosive enamel loss. The method and instrument were validated and we wanted to compare the measurements from other instruments often used in erosion studies with those from WLI.

Instruments used for assessment of dental erosion and erosive wear in experimental studies

Atomic absorption spectroscope

Atomic absorption spectroscopy uses absorption of light to measure the concentration of gas-phase atoms. The investigated samples are usually liquids or solids so the actual atoms or ions to be analysed must be vaporised in a flame or graphite furnace. The atoms absorb ultraviolet or visible light and are raised to higher electronic energy levels. The analyst concentration is determined from the amount of absorption. Measurements of the amount of calcium released into the acid, is an indirect way of quantifying changes in surface enamel after acid etch.

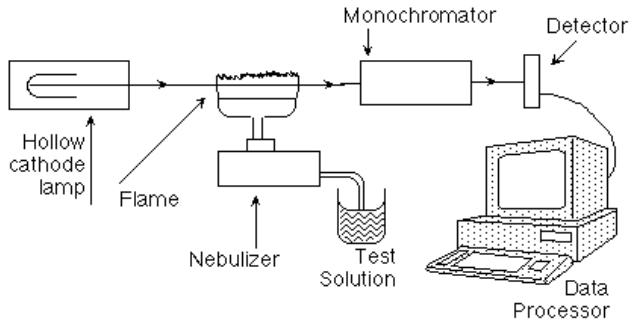


Figure 2. Illustration of an atomic absorption spectroscope [www.chemistry.nmsu.edu, 2012].

This is a reliable and sensitive method for calcium analysis [Trudeau and Freier, 1967; Willis, 1961] and can be used to quantify erosion both on enamel and dentin *in vitro* and *in situ* [Grenby et al., 1990; Hara and Zero, 2008]. It has also been used to analyse calcium release from enamel in an *in vivo* study [Young et al., 2006] and is suitable for longitudinal measurements. One limitation is that the presence of saliva could interfere with the analysis and it does not provide information about mineral gain or physical and morphological changes.

Micro-hardness measurements

Loss of hardness (softening) of the surface of a test specimen is measured by the resistance of the surface to the penetration of an indenter. It is performed by a Knoop (rhomboidal) or Vickers (tetra-pyramidal) indenter. The hardness numbers are calculated from the length of the indentation (on the test surface) and the applied load (Vickers hardness value; $HV = kF/d^2$, where k is a constant relating the contact area to the diagonals, F is the test force, and d is the average indent diagonal and the Knoop hardness number; $KHN = F/A = P/CL^2$).

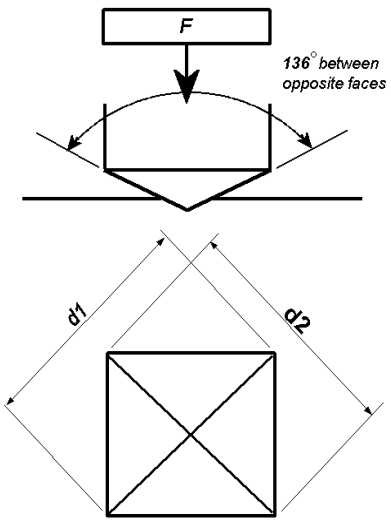


Figure 3. A schematic illustration of the Vickers technique [www.gordonengland.co.uk, 2012].

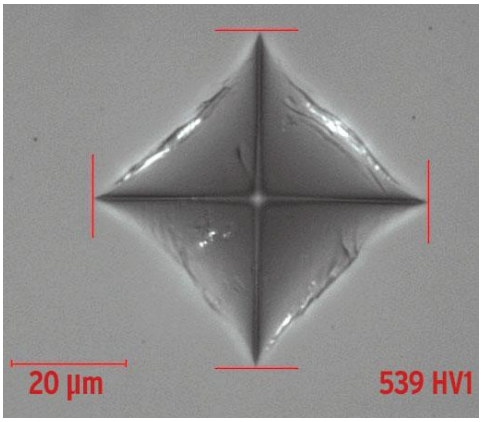


Figure 4. An image of a tetra pyramidal indentation in Vickers technique [www.astm.org, 2012]

It is a simple way to get accurate information about early erosion [Barbour and Rees, 2004]. Tooth surfaces must be flattened in order to obtain optimal accuracy. The Knoop indenter penetrates sound enamel by about 1.5 µm and the Vickers indenter penetrates about 5 µm [Featherstone, 1992], so Knoop hardness seems to be more sensitive to changes in the most superficial layer of an erosive lesion. Changes in micro hardness can be observed after just a few minutes of acid exposure [Hara et al., 2006b; Hara and Zero, 2008]. Limitations of the method are that indentations on highly eroded surfaces or surfaces with precipitates (calcium fluoride and tin- or titanium containing precipitates) are difficult to read and the measurements may not be representative.

Surface profilometry; Contact by mechanical stylus and non-contact by laser light

These methods quantify tissue loss in relation to a non-treated reference area and also provide information about surface roughness [Field et al., 2010]. The surface is scanned to generate a two or three dimensional profile, either by a contact (diamond or steel tip) or a non-contact (white or blue light laser) measuring device.

In a contact profilometer a stylus is moved vertically in contact with a sample and then moved laterally across the sample for a specified distance and with a specified contact force. The height position of the diamond stylus generates an analogue signal which is converted into a digital signal and stored, analysed and displayed (Figure 5). Contact profilometry can also be used to measure erosion depths on natural surfaces [Ganss et al., 2000], which make

its use possible for *in vivo* studies, but the accuracy is less than for flattened surfaces. A disadvantage of contact profilometry is that a stylus penetrates the eroded surface, which could be partially demineralised in the case of enamel [Ren et al., 2009] or completely demineralised in dentine [Ganss et al., 2009a]. The stylus can damage the enamel surface and lead to an overestimation of early erosion depth.

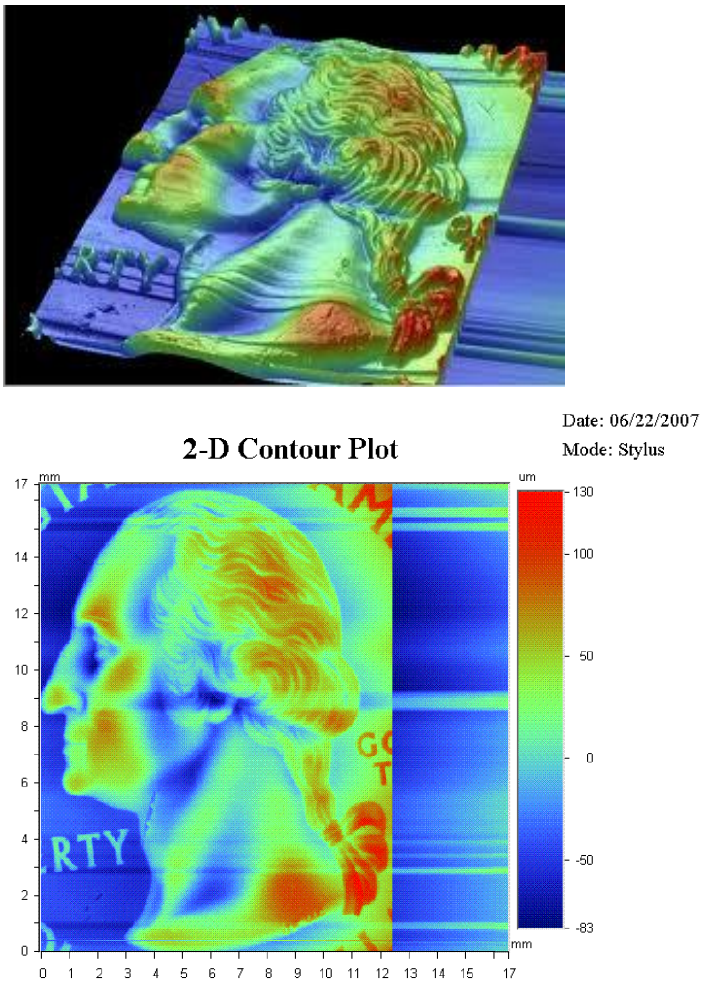


Figure 5. A computerised image (3D and 2D) based on tracings by a steel or diamond tip/stylus of a surface; Dektak 8 Advanced Development Profiler [www.rpi.edu, 2012].

A non-contact confocal laser scanning microscope has a confocal pinhole that rejects out of focus fluorescent light fluorescence. The subject is scanned vertically in steps and every point passes through focus. Only one point (pixel) is observed at a time and the computer builds up the picture (Figure 6). With flattened surfaces erosive lesions around 0.5µm can be consistently detected by white light non- contact profilometry [Hara and Zero, 2008].

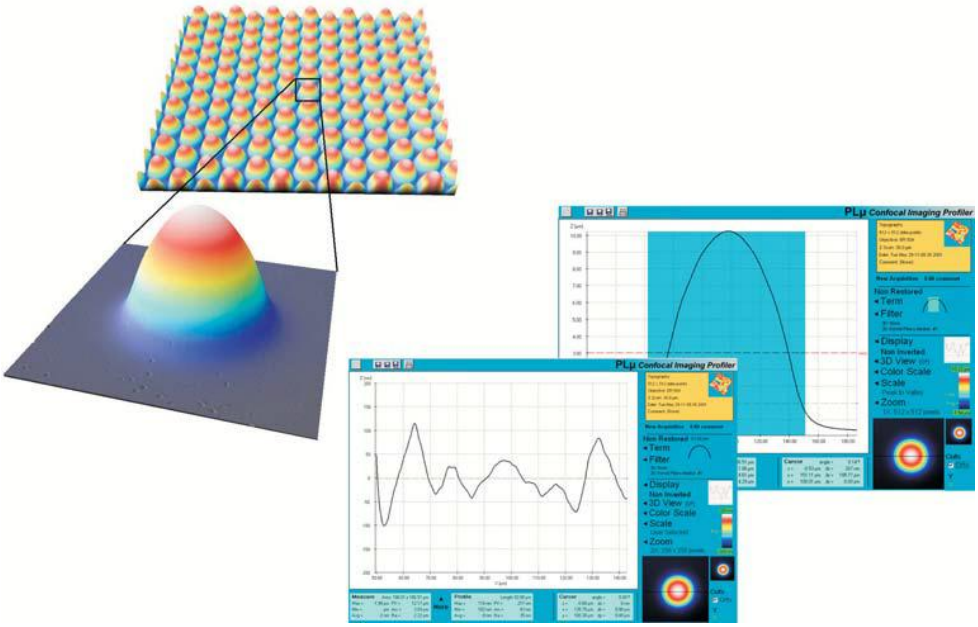


Figure 6. Scanning by a confocal laser scanning microscope; Sensofar Plµ2300 [www.sensofar.com, 2012].

White light interferometry

A white light interferometer uses white light for analyses of surface topography based on a three dimensional evaluation of the whole imaged surface. It has been successfully applied on natural tooth surfaces and also on impressions of natural surfaces in a previous pilot study. This could open up for in vivo studies.

The white light interferometer uses two technologies to measure a wide range of surface heights. In the Phase Shifting Interferometry (PSI) mode the white light beam must be filtered, leaving a more monochromatic light (normally red light at a nominal wavelength of

632 nm) to pass through the interferometer and get to the test surface. A monochromatic light source works best for PSI because it has a longer coherence length than white light (Figures 7 and 8), so high contrast fringes are present through a larger depth of focus. This increases the measurable height range. The maximum height difference between adjacent pixels in PSI is 160 nm, so this mode is limited to generally flat and smooth surfaces.

Vertical Scanning Interferometry (VSI) mode allows measurements on rough surfaces and steps up to several millimeters high. VSI uses white light which has a short coherence length (Figures 7 and 8), thus giving the highest fringe contrast at only best focus (fringe contrast falls off rapidly when translating past focus), which meets the requirement of high modulation.

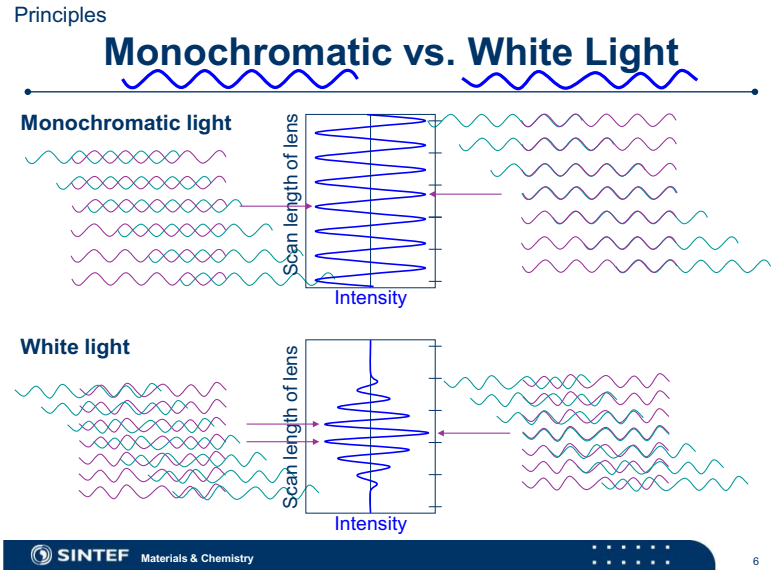


Figure 7.

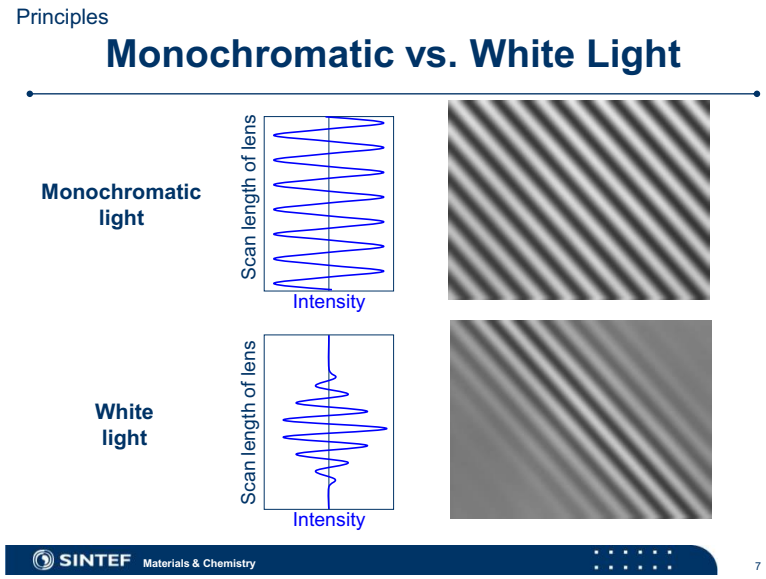


Figure 8.

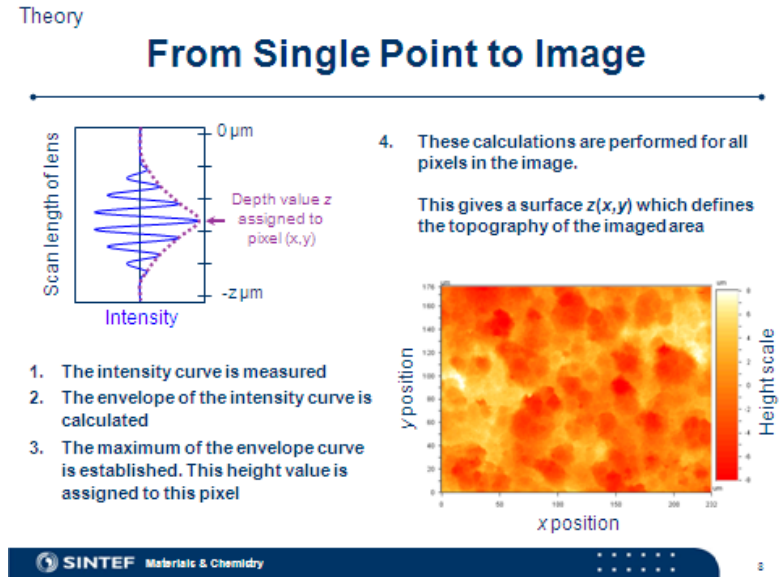


Figure 9. Principles of white light interferometry by Martin Fleissner Sunding, Børge Holme, Joachim Moe Graff, SINTEF Materials & Chemistry.

Fringe modulation is so important for VSI because surface heights will be calculated by processing this modulation data. In VSI, the internal optical assembly and the magnification objective actually move through focus in a controlled manner. As the system scans downward (normally starts at or above focus), an interference signal for each point (pixel) on the surface is recorded. By using a series of advanced computer algorithms, the vertical position corresponding to the peak of the interference signal is extracted for each point (pixel) on the surface, recreating the surface profile (Figure10).

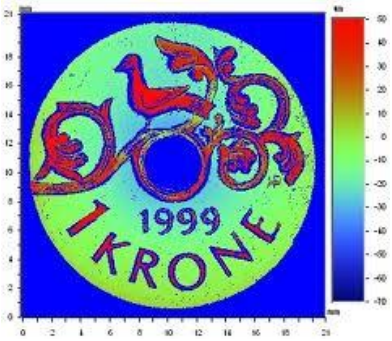


Figure 10. A digital image of a coin made by a white light interferometer. Each pixel represents individual heights as illustrated by the different colors [www.sintef.no, 2012].

The white light interferometer combines an interferometer and microscope into one instrument (Figure11). The light comes from the light source, passes through a filter and comes across a beam splitter after several more lenses. Then half of the light will be reflected from a reference mirror, while the other half is reflected back from the sample surface. The two beams recombine and create bright and dark fringes.

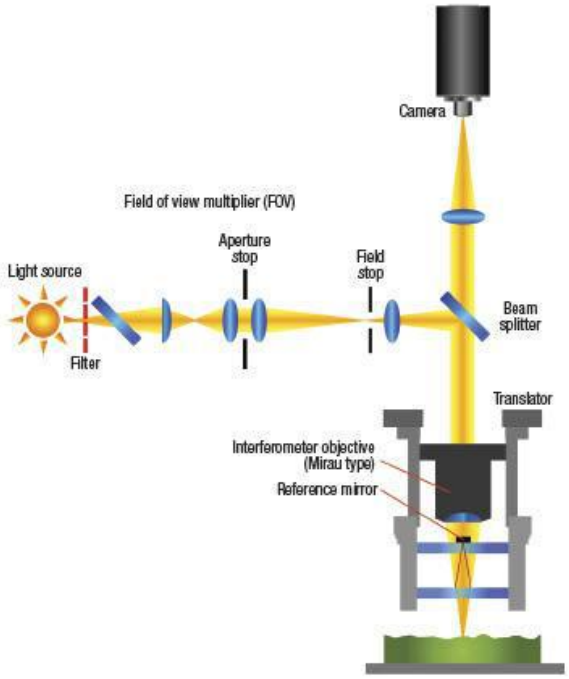


Figure 11. A schematic illustration of a white light interferometer [www.microfacturing.com, 2012].

Validation of analytical methods in general

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use and is an integral part of any good analytical practice. It is evaluated by quantification of parameters like *calibration data, accuracy*, *precision* and *limit of detection/quantification.*

Cross validation is a comparison of validation parameters when two or more analytical methods are used to generate data within the same study. An example of cross-validation would be a situation where an original validated method serves as the reference and another method serves as the comparator [www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf, 2001;www.microfacturing.com, 2012]. This is often the case in medical clinical trials where a well proven treatment or drug is compared with a new one.

Calibration

Instrument calibration is one of the primary processes used to maintain instrument accuracy. Calibration is the process of configuring an instrument to provide a result for a sample within an acceptable range. Eliminating or minimizing factors that may cause inaccurate measurements is a fundamental aspect of instrumentation design.

Although the exact procedure may vary from product to product, the calibration process generally involves using the instrument to test samples of one or more known values called calibrators or standards.

Accuracy

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. Accuracy can also be described as the closeness of agreement between the value that is adopted as a true or accepted reference value, and the value found.

The "true value" for accuracy assessment can be obtained in several ways. One alternative is to compare the results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known.

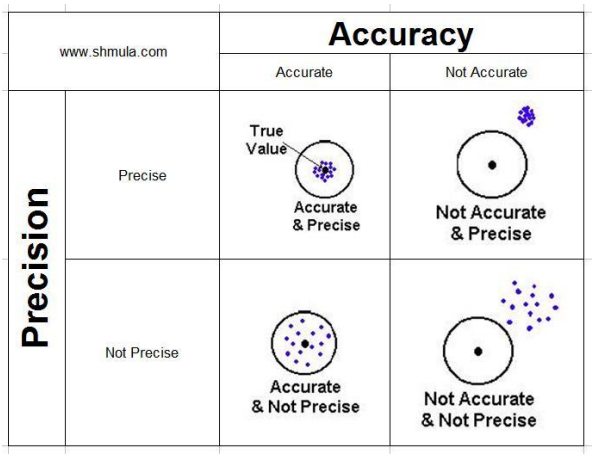
Precision

The precision of a method is the extent to which the individual test results of multiple measurements of a series of standards agree (also referred to as repeatability or reproducibility).

Variations in environmental conditions (temperature, operators, instrument (calibration) could influence the precision. In particular, the risk would increase if the series of measurements were spread over a longer period of time and/or were performed in different locations.

The most important part of any analytical method validation is the precision analysis. The ICH (International Conference on Harmonisation) guidelines [www.ich.org, 2012] break precision into two parts: repeatability (*intra-assay precision)* and *intermediate precision*. Repeatability expresses the precision under the same operating conditions over a short interval of time. Intermediate precision expresses within-laboratory variations: different days, different analysts, different equipment, etc.

[www.fda.gov/downloads/Drugs/.../Guidances/ucm073381.pdf, 1995;www.fda.gov/downloads/Regulator%20yInformation/Guidances/UCM128049.pdf, 1996].



Fig**ure 12.** Visualization of the concepts precision and accuracy.

Limit of detection/quantification

The limit of detection is the point at which a measured value is larger than the uncertainty associated with it. It is determined by a calculation; 3.3(SD/S) where SD= standard deviation of response and S= slope of calibration curve.

The detection limit of an assay is the lowest concentration/ step that can be detected and necessarily quantified; the quantification limit is the lowest concentration/ step that can be quantified with acceptable precision.

Prevention of dental erosions

Preventive strategies against dental erosion and wear include management of GORD (gastro oesophageal reflux disease) and eating disorders and also dietary advice, stimulation of salivary flow, modification of intake of erosive beverages, adequate oral hygiene measures and fluoride treatment. The underlying principle in research regarding the preventive or protective effect of various conventional fluoride compounds against dental erosive wear [Wiegand and Attin, 2003], has been that these agents are successful in caries management [Marinho et al., 2004; ten Cate, 1997] in addition to plaque and diet control.

Erosive demineralization starts with the partial loss of surface mineral causing an increase in roughness [Nekrashevych and Stosser, 2003]. It was shown that consumption of soft drinks for only 20 seconds led to a decrease in surface micro-hardness even with pellicle structures still present on the surface [Hannig et al., 2009]. If the acidic impact continues, bulk mineral loss occurs due to destruction of the apatite crystals, while the remaining surface still exhibits partial demineralization with loss of hardness and vulnerability to physical impacts [Attin et al., 1997; Jaeggi and Lussi, 1999; Voronets et al., 2008]. Eroded enamel appears with a typical etched surface structure as seen in scanning electron microscope [Eisenburger et al., 2004; Meurman and Frank, 1991].

For dissolution of a solid, the critical pH is the pH at which a solution is just saturated with respect to a specified solid, like enamel mineral. If the pH of the solution is less than the critical pH, the solution is under-saturated and can dissolve the solid, while a solution is supersaturated if the pH is above the critical pH and then minerals may be precipitated. The critical pH depends on the solubility of the solid and the concentrations of the relevant mineral constituents of the solution. In the case of tooth mineral these are calcium and phosphate and to a lesser degree fluoride. Due to carbonate impurities in the crystal lattice the acid solubility of dental enamel apatite is greater than for hydroxyapatite and fluorapatite.

The potential anti- erosive effect of conventional fluorides like sodium fluoride and amine fluoride is considered to be by formation of a calcium fluoride (CaF_2) layer which could act as a source of free ions, as a physical barrier protecting enamel and dentin from acid contact or as a mineral reservoir. However, precipitated calcium fluoride is soluble in acids [Ganss et al., 2007b] and apatite deposition does not occur at pH levels below 4.5. So conventional fluorides like sodium fluoride and amine fluoride seems to offer limited protection against dental erosion. The formation of CaF_2 is favoured by high fluoride concentration, ph below 5 and frequent application [Ganss et al., 2007b; Saxegaard and Rolla, 1988]. To obtain high amounts of CaF_2 intensive regimes, where patients should use different forms of preparations such as mouth rinse and gel frequently, would be required. Such recommendations are not suitable for preventive treatment for longer periods of time. The effect of fluoride compounds with polyvalent metal ions like titanium or tin have therefore been investigated and shown promising preventive effects against dental erosion [Wiegand and Attin, 2003]. Erosive mineral losses can be prevented, at least under mild acidic conditions, by stannous fluoride or amine fluoride/stannous fluoride solutions, whereas sodium fluoride or amine

fluoride/sodium fluoride solutions appear to be significantly less effective. This has been shown in studies where different fluoride compounds with the same pH value and same concentration have been compared [Ganss et al., 2008; Schlueter et al., 2007]. Application of titanium fluoride compounds results in deposit of glaze on the tooth surfaces which is resistant to hydrochloric acid [Buyukyilmaz et al., 1997]. In a review by Wiegand et al. [2010b], it was concluded that titanium fluoride seemed promising for preventing dental erosive wear but the results from *in situ* studies showed contradictory results. A 4% titanium fluoride solution showed no effect against enamel loss and softening due to erosion only [Magalhaes et al., 2008], but a 1.5% titanium fluoride solution was even better than NaF and SnF_2 [Hove et al., 2008]. The biocompatibility of the metal fluoride compounds should be taken into account since they could have some adverse effects related to low pH of the titanium tetra fluoride compounds and high concentration of tin [Schlueter et al., 2009c].

Fluoride and dental erosion

Fluoride is the negative ion (cation) of the element fluorine. The fluorine atom has a small radius and its effective surface charge is therefore greater than that of any other element. As a consequence, fluorine is the most electronegative and reactive of all elements. It reacts promptly and is rarely found in the free or elemental state [Banks and Goldwhite, 1966; Glemser, 1986] and is most frequently found as inorganic fluoride.

Fluoride in blood, saliva and tissue fluid will be present as F^- since pKa of HF is 3.4. In the stomach (pH 2.0) fluoride will be in dissociated form, HF. In mineralized tissues some fluoride is incorporated within the crystallites, but it may also be more superficially located on crystal surfaces or in their hydration shells. Conventional fluorides (NaF and AmF) lead to formation of calcium fluoride or other fluoride-rich species on the tooth surface, a process which is enhanced by decreasing the pH and increasing the fluoride concentration in the vehicle [Saxegaard and Rolla, 1988] and frequency of application. Fluoride can greatly increase lattice stability by attracting protons of adjacent apatite hydroxyl ions. The fluoride ion is better aligned with the plane formed by the three adjacent calcium ions and their attraction to the fluoride ions is greater than to hydroxyl. The fluoridated apatite lattices (fluorapatite $Ca_{10}(PO_4)_6F_2$) are more stable and less soluble in acid and will also form more easily because of the low ionic product.

Fluoride which is more superficially located may have little effect on the crystal lattice, but it can affect fluid-crystal equilibrium and thereby affect fluoride concentration in tissue fluids and cells involved in tissue development and mineralization [Arends et al., 1983; Koulourides et al., 1961; ten Cate and Duijsters, 1983]. The mechanisms of the metal- fluorides reaction to the tooth enamel are not fully understood. While NaF (mw 42; Na 23 and F 19) is an inorganic salt, which is readily soluble in water and provides free fluoride into saliva, dental plaque, pellicle and enamel crystallites, SnF_2 (mw 123.9; Sn 156.7 and F 19) forms a tin containing precipitate on the surface ($Sn_3F_3PO_4$, $SnOHPO_4$, Ca $[SnF_3]_2$) at high concentrations [Babcock et al., 1978]. The tin ion hydrolyses and the solution becomes acidic (0.4 % solution pH 3.2, 4 % solution pH 2.1).

The Ti ion has pronounced complex binding ability to fluoride and components in dentin and enamel, thereby enhancing uptake and retention of fluoride [McCann, 1969] after treatment with TiF_4 (mw 123.9; Ti 47.88 and F 19). Dissolution of TiF_4 in water gives a solution of very low pH. This will increase fluoride uptake since a greater part of fluoride will exist as HF which penetrates more easily into the hydroxyl-apatite lattice than the F ion [Hals et al., 1981; Tveit et al., 1985; Ericsson, 1977]. A Ti-rich coating/glaze which is formed on treated tooth surfaces may inhibit caries [Mundorff et al., 1972; Tveit et al., 1985; Wefel and Harless, 1981; Wefel and Harless, 1982; Wei et al., 1976]. The compound is not toxic.

The reported interactions between metal- fluoride solutions and enamel and the promising results from previous *in vitro* and *in situ* studies where the effect of these compounds against dental erosion was investigated, were the background for further testing the present solutions in an *in situ* model (**Study 3)**.

AIMS

The main idea behind this thesis was to investigate the reliability of research techniques and instruments often used in studies where tooth substance loss due to dental erosion is measured and thereafter to study the potentially protective effect of various fluoride compounds against erosive and abrasive enamel wear.

Study 1

To validate instruments and techniques that is commonly used for quantitative measurements of loss of tooth substance in studies and to compare the reliability of the measurements by the respective instruments with those from a white light interferometer. The reliability of the methods was evaluated by calculating the precision and accuracy of repeated measurements.

Study 2

To investigate how lowering the concentration or raising the pH of a 0.5M titanium tetra fluoride (TiF_4) native aqueous solution affects its protective effect against enamel erosive wear *in vitro*.

Study 3

To study whether daily rinsing with sodium fluoride (NaF), stannous fluoride (SnF_2) and titanium tetra fluoride (TiF_4) aqueous solutions (all 0.05M, 0.2% F^-) could protect natural enamel surfaces against erosive or abrasive wear *in situ*.

Study 4

To investigate whether measurements by a white light interferometer on an impression (negative replica) of eroded enamel give reliable values for the etch depths measured directly on enamel.

MATERIALS AND METHODS

Ethical aspects

(Study 1, 2, 3 and 4)

Approval for collection and use of extracted human teeth was given by the Regional Committee for Medical Research Ethics, Norway, and The Norwegian Institute of Public Health's Biobank Register No 2543, project No 6.2008.2058.

(Study 3)

Approval for this in situ study was given by the Regional Committee for Medical Research Ethics (Number 2010/2244-1).

Materials and experimental procedures in study 1, 2, 3 and 4, Table

	Study 1 *In vitro*	**Study 2** *In vitro*	**Study 3** *In situ*	**Study 4** *In vitro*
Teeth/specimens	12/12	8/40	16/64	12/12
Participants			8	
Acid etch: 0.01M HCl pH 2.2	6 + 6 min	2+2+2 min	2 min x 2/day x 9 days	12 min
Fluoride treatment		TiF_4 0.5M F pH 1.2 TiF_4 0.05M F pH 2.1 TiF_4 0.5M F pH 2.1 TiF_4 0.05M F pH 1.2 2 min (1 drop/sec)	0.4% (0.05M F) SnF_2 pH 2.5, 1.2 % (0.05M F)TiF_4 pH 2.1, 0.2% (0.05M F) NaF pH 6.5 2 min (1 drop/sec)/ day x 9 days	
Tooth brushing			30 seconds /day x 9 days Manual brush with water	

Enamel specimens (Study 1, 2, 3 and 4)

Freshly extracted human permanent third molars were kept in containers with Thymol crystals in 100% humidity to prevent bacterial growth until use. The enamel was cleaned with pumice in water, wiped free of debris and rinsed in tap water and kept for at least two weeks in aqueous Thymol solution followed by 30 minutes in 70% ethanol for disinfection according to previously published procedures [Ganss et al., 2007a].

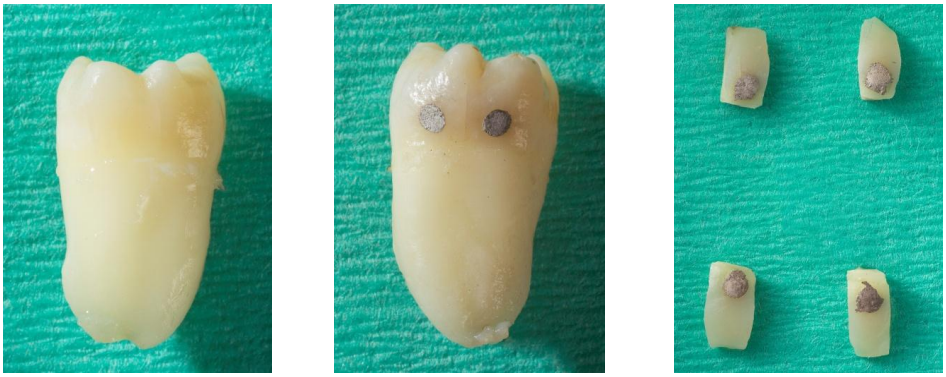


Figure 13. Amalgam fillings (approximately 1 mm in diameter) were made in human third molars about 2 mm above the enamel-cement junction for use as reference surfaces. One specimen included one amalgam filling [Hove, 2008].

Figure 14. The enamel specimens with amalgam reference surfaces were mounted in epoxy resin blocks (Epofix ®, Struers, Denmark) (**Study 1, 2** and **4).** The specimens were ground flat in **Study 1 and 4**, but kept natural in **Study 2.**

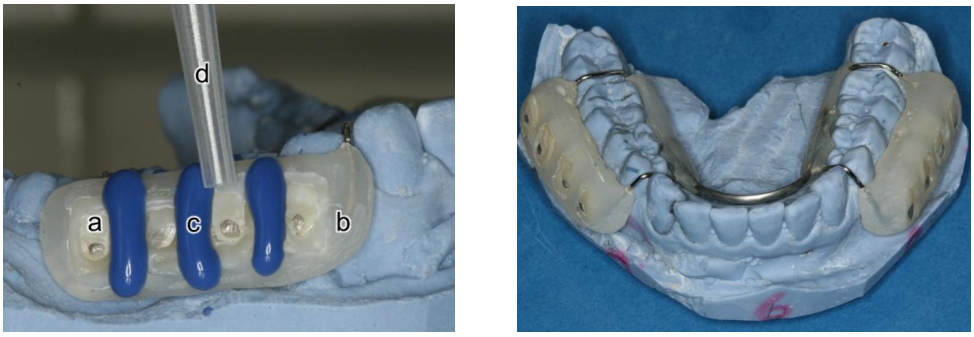


Figure15. Left photo: A mandibular appliance (**Study 3**) with a) 4 enamel specimens (natural surfaces) from the same tooth mounted on the same side and randomly assigned to 4 treatment groups b)Triad acrylic, c) Permadyne to avoid contamination during application of fluoride solutions with a pipette(d) mimicking a mouth rinse.

Right photo: Plaster replica of the lower jaw of participant 6. The appliance (has tooth number11 on the right side and number 12 on the left side).

Analytical techniques

White Light Interferometry (WLI) (Study 1, 2, 3 and 4)

The specimens were analysed by a White Light Interferometer (WYKO® NT2000, **Study 1 and 4** and WYKO® NT 9800 **Study 2 and 3,** Veeco, USA,) which is a computerized optical interference microscope. It was operating in the vertical scanning interferometry mode (VSI) and produced topographic images of the surface under study. The sample images made by WLI contained about 1/3 amalgam and 2/3 enamel. A baseline image was made of the enamel surface of every specimen before treatment and etch (Figure 16 a). This baseline image was subtracted from the image taken after acid exposure for every separate specimen (Figure 16 b), thus creating the respective "difference images" (Figure 16 c). This topography image contained a step which showed the enamel loss in the sampling area. A 400-800 µm band in **Study 1** and **4** and a 100-200 µm wide band in **Study 3** (Figure 16 c) along the amalgam-enamel border was excluded from the analysis areas. The purpose was to avoid errors from artefacts like: 1) Fractures or fissures between the amalgam and enamel. 2) Over-etched regions in the cases where such fissures had allowed retention of acid. 3) Build-up of plaque (**Study 3** *in situ*) in specimens where the amalgam was a bit higher (Figure 16a) than the enamel. The width of this area was narrower in **Study 2** and **3**, since it was observed that these artefacts were minimal and we wanted to avoid losing good data. The step height provided an estimate of the depth of the lesion. A computer program performed automatic alignment of the images before subtraction. For this program to work optimally, any region in the amalgam that had undergone changes from the beginning to the end of the experiment were manually excluded by the operator, e.g. parts of amalgam that had broken off, or deep pits that had been filled with plaque. The program calculated the depth distribution of pixels in amalgam and enamel separately, to get a measure of the goodness- of- fit and the lateral variability of the etch rate or wear rate, respectively. The average lesion depth in each image was calculated from the average height of the amalgam region to the average height of the enamel region. The technique has previously been described [Holme et al., 2005; Hove et al., 2006], but has been further developed and improved throughout the present studies.

The objective had 10 X magnification and the pixel size was 3.13 µm by 3.65 µm of the rectangular pixels (NT2000 in **Study 1** and **4**). The image resolution was 368 x 240 pixels. For NT9800 used in **Study 2** and **3**, the pixel size was 3.53 µm squared, and the image resolution 320 by 240 pixels. In **Study 4** a topography image corresponding to that of the

specimen surface was obtained by inverting the image of the impression. The inverted impression images were analysed in the same way as the images of the specimen surfaces in order to measure the step height.

Analyses by WLI were performed at baseline, and after each etch in **Studies 1, 2** and **4**. In **Study 3** WLI analyses were performed at baseline and at the end of the experimental period (9 days).

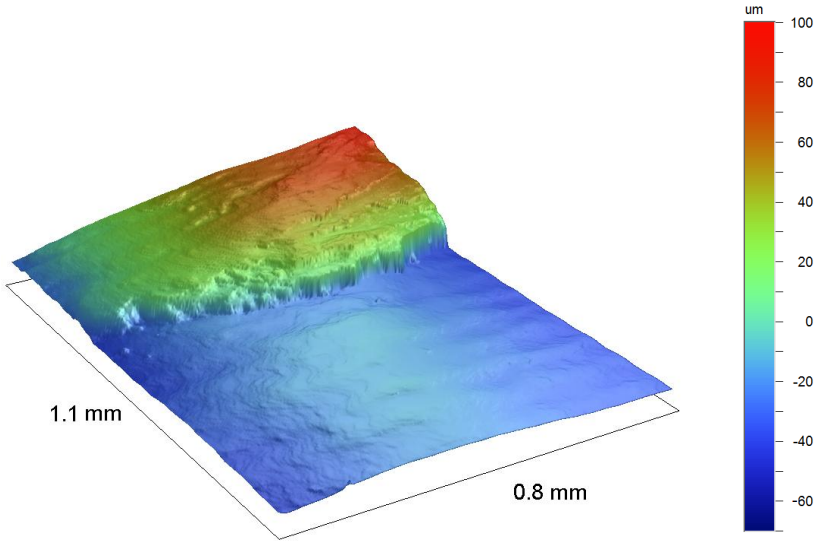


Figure 16 a. A baseline image (before treatment and wear processes) of one natural specimen surface from tooth number 8, participant D in **Study 3** *in situ*. The amalgam reference surface can be seen to the left and is above the level of the enamel.

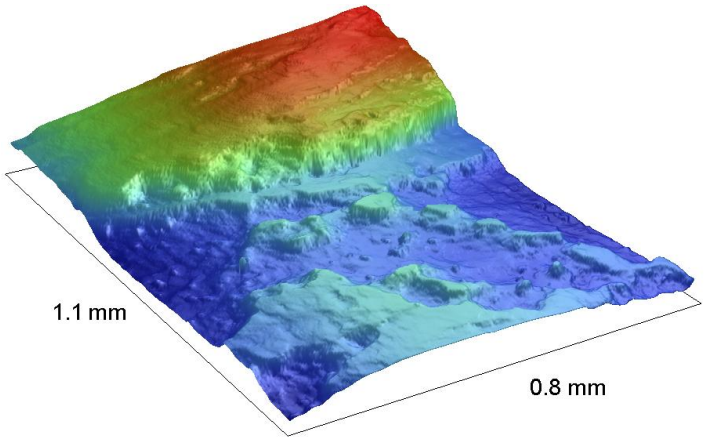


Figure 16 b. An image of the same specimen (treated with TiF_4) imaged after completion of **Study 3** *in situ*. The enamel shows areas with and without glaze.

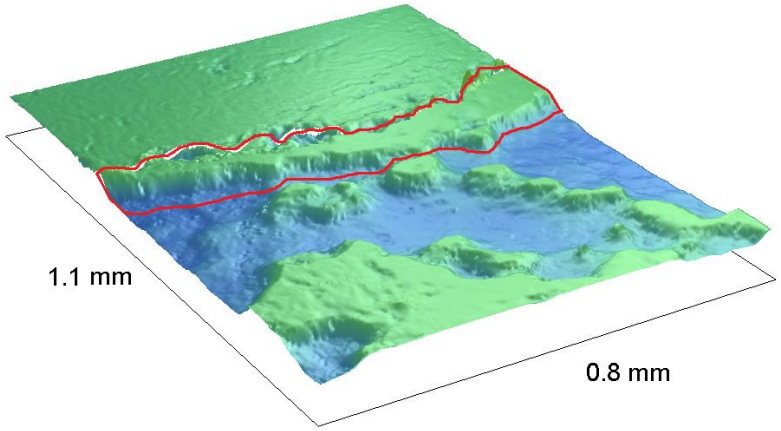


Figure 16 c. The difference image of the same specimen where the baseline image is subtracted from the end of experiment image. A 100µm area close to the amalgam surface with artefacts was masked. The enamel areas with glaze show no substance loss whereas the areas without coating show do.

Surface micro- hardness (SMH)
The surface micro- hardness (**Study 1**) was analysed by a Vickers® FM-700 Micro hardness tester (Future Tech.Corp., Japan). This instrument utilizes a 136° diamond pyramid indenter that forms a square indent in the surface. The indentations were made by HV 03 i.e. with a 300 g load applied for 10 seconds.

The size of the indent was determined optically by measuring the two diagonals of the square indent. The Vickers hardness number is a function of the test force divided by the surface area of the indent. The average of the two diagonals is used in the following formula to calculate the Vickers hardness value; HV= kF/d^2, (k is a constant relating the contact area to the diagonals, F is the test force and d is the average indent diagonal. This measurement was performed in an area of each specimen that did not interfere with the other analyses and was performed at baseline and after the first and second acid etch.

Optical profilometry (OP)
The specimen surfaces (**Study 1**) were analyzed by an optical profilometer (Sensofar® Plμ 2300 (Terrassa, Spain).The instrument used a LED blue light beam of wavelength 470 nm which was reflected from the specimen surface and back into the instrument. The confocal objective used was Nikon 20XEPI, 0.45NA LU plan fluor for 3D profiling and the field of view was (625 x 468 pixels). A spatial sampling of 0.83 μm was given for the 20X objective but it was adjusted to 1.66 μm (2 x 0.81 μm) in the z-plane to match the speed of WLI and SP for one image/map.

Sampling areas were 1142 x 827μm^2 and the resolution was 358 x 251 pixels. The pixel size was 10.5 μm^2. Baseline images made by WLI were used as orientation maps, to ensure measurements of exactly the same areas as by the WLI, and the analyses were performed after each acid etch. The measurements were analysed by the software SensoMap Plus 4.1 (Sensofar-Tech. S. L., Terrassa, Spain).

Stylus profilometry (SP)
Following analysis in the WLI and the OP (**Study 1**), the specimen surfaces were analyzed with a mechanical profilometer (Dektak® 8 Stylus Profiler, Veeco, USA) after each acid etch. Baseline images from WLI were used as orientation maps, to ensure measurements of exactly the same areas as for WLI and OP. The instrument stylus was moved laterally across the surface of the specimens with a weight of 5 mg and a scan length of 3000 μm during 50

seconds. Three parallel scans 100 µm apart were made on each sample and the etching depth was determined by measuring the average step height from the amalgam to the average of the profile 400-800 µm from the amalgam edge.

Calcium analysis

The calcium content in ppm (mg/L) of the acid samples (Study 1) was measured by a Model 3300 Atomic Absorption Spectrophotometer; Perkin Elmer Analytical Instruments (Norwalk, CT, USA) directly after each acid etch. Lanthanum chloride ($LaCl_3$) (VWR International, Fontenay sous Bois, France) was added to the final 0.5% concentration to suppress phosphate interference with the calcium determination. Lesion depth was calculated by means of the calcium loss, according to the conversion formula described by [Ganss et al., 2005; Dijkman, 1982; ten Cate, 1979]. Erosive loss (µm) = mineral loss/ (density of HA x experimental area). The density of HA = 3.15 g/cm^3.

Calibration of instruments

In order to get information on the performance of these specific instruments, they were calibrated by measurements on ideal standards given by their respective manufacturers.

The calibration of the NT2000 white light interferometer (**Study 1 and 4**) was verified at the beginning and end of every measurement series by checking against a 10.07 µm glass step standard with an absolute uncertainty in the height of 0.04 µm. If the measured step height was off by more than 0.5 % (0.05 µm), a calibration factor was adjusted until the instrument correctly measured the step to within 0.01 µm. Each calibration check was based on ten consecutive step measurements. This allowed the precision and the standard deviation of the ten measurements to be determined. The average precision was 0.008 ±0.001 µm (average ± one standard error of the mean) for a series of six calibration verifications (60 measurements). With constant room temperature, the NT2000's accuracy could be as good as 0.01 µm immediately after calibration for step heights up to 10 µm [Holme et al., 2005]. Thus, the uncertainty in the ideal step standard's height value (0.04 µm from the certificate) limited the absolute accuracy in this case.

The newer NT9800 instrument, used for **Study 2 and 3**, has a built-in laser interferometer which performs an automatic calibration upon start-up and ensures that the instrument stays accurate at all times (further described in Methodological considerations). Calibration verifications for NT9800 before and after dental measurements in **Study 2** and **3** showed that

the accuracy (difference from the glass standard value of 10.07 µm) after the six calibration runs was 0.007 ±0.002 µm. The very high stability of the NT9800 therefore gave accuracies and precisions better than the certified uncertainty in the height of the glass step standard and the overall accuracy of the WLI measurements for ideal stepped surfaces was in this case limited by the step uncertainty of 0.04 µm, or 0.4 %.
The precision of the glass step measurements was 0.008 ± 0.001 µm, which for the 10.07 µm step becomes 0.08 %.

The stylus profilometer (**Study 1**) calibration was checked once by measuring a silicon step standard of 0.200 µm height 3 times. The accuracy was 0.004 µm and the precision was 0.003 µm, giving an accuracy and precision of about 2 % based on the calibration data.

The optical profilometer (**Study** 1) was calibrated by ten measurements on a calibration standard of 30.09 ±0.01 µm height and repeated 5 times. The accuracy was 0.8 ±0.4 µm and the precision was 0.2 ±0.1 µm. Thus the estimated accuracy was 3 % and the precision 0.6 %.

The performance of the surface micro hardness instrument (**Study 1**) was checked by 5 measurements on two steel standards with hardness (HV 03) of 804 and 833 kp/mm^2. The accuracy was estimated to be 4 % while the precision was 1 %.

The calibration of the atomic absorption spectroscope (**Study** 1) was performed against a known standard according to the instructions from the manufacturer.

Accuracy and precision of step height measurements on enamel

Study 1 and 2

The *accuracy* is expressed by the deviation from the true value and includes all systematic errors. Lacking an absolute measurement of the etch depths on the amalgam/enamel samples, the technique from which the calibration had shown the best relative accuracy and precision was considered closest to the actual value. Thus the data obtained by SP and OP were compared with the individual measurements from WLI. From the slope of the respective regression lines (100 % (1-slope)), we could estimate the accuracy of SP and OP for measurements of enamel etch depths.

The area to be exposed to acid on each specimen was defined using a light cured resin (Filtek ® flow 3M Espe) and imaged by a low magnification optical microscope for measurement.

The total size of these twelve areas was needed only for the calcium analysis since all specimens were etched at the same time (mounted together in one epoxy block) and we had to calculate a mean value for conversion to lesion depth. Only the value of the total Ca-loss from all 12 specimens was available so an average calcium loss was calculated and compared with WLI to estimate the accuracy of the indirect Ca-loss method.

The *precision* of the measurements is the ability to obtain identical results by repeated measures and was calculated as the average standard deviation (SD) of the three step measurements made of each specimen after each acid etch. The relative precision was calculated as the SD divided by the mean lesion depth for each specimen and then expressed as the average of the relative precision of the twelve specimens.

Study 3

Analysis of the 2 images made for each sample before any treatment showed that for these etch depth measurements (range from 0.45 µm -10.21µm) by WLI on naturally curved enamel, the accuracy was 0.05 µm, the precision was 0.1 µm and the detection limit was 0.3 µm.

Study 4

In order to obtain an estimate of the precision of the technique on these natural enamel samples, two images of each specimen were made after the in situ experiment. The precision was 0.2 µm. The accuracy of the WLI instrument was verified by measuring a 10 µm high ideal reference step before and after running each analysis and was better than 50 nm in all cases.

Experimental procedures

In **Study 1** and **4** all specimens (one from each tooth) were etched in the same way (Table 1).

In **Study 2** all specimens from one tooth (8 teeth) were randomly assigned to 5 experimental groups: (1) TiF_4 (0.5 M F, pH 1.2), (2) TiF_4 (0.05 M F, pH 2.1), (3) TiF_4 (0.5 M F buffered to the same pH as 0.05 M F with NaOH) or (4) TiF_4 (0.05 M F buffered to the same pH as 0.5 M F with HCl) and 5) control.

In **Study 3** in situ, the participants wore the appliances day and night, except while eating and tooth brushing. During these extra-oral periods, the appliances were kept at 100 % humidity.

The participants used non-fluoride toothpaste (Solidox® without fluoride, Lilleborg, Norway) during the experimental period. The specimens were brushed in the morning, separated by Permadyne and fluoride solutions were applied (Figure 15, left**).** The appliances were then kept in the mouth (>2 hours) until midday when the specimens were etched. The same procedure was repeated in the afternoon in order to simulate two gastric reflux/vomiting episodes per day. All treatments and analyses were performed extra-orally.

Statistical analysis

Statistical procedures were performed with the Statistical Package for Social Sciences Inc., Chicago, Ill., USA (SPSS 16.0) for Windows.

Study 1

The precision of the measurements from each method, performed after the first and second 6 minutes of acid exposure, was calculated by reliability analysis and expressed by single measures Intra Class Correlation (ICC), a two-way mixed effects model that measures the reliability of repeated measurements by one instrument. The paired samples *t*-test was used with a 5% significance level when comparing the relative precisions of WLI/ SP and WLI/ OP.

Study 2

Paired t- tests were used to assess the significance of differences between the various treatments and the control. For multiple comparisons, p- values were adjusted using the Bonferroni method. The level of significance was set at 0.05.

Study 3

The significance of differences between the treatment groups was assessed by paired t-test ($p \leq 0.05$). For each tooth, one out of four specimens (all in the same mouth) was treated with NaF, one with SnF2, one with TiF4 and one served as control, i.e. 16 tooth specimens were used for each regimen. When comparing each pair of regimens on the outcome data (6 comparisons: 1: control/NaF, 2: control/ SnF2, 3: control/ TiF4, 4: NaF/SnF2, 5: NaF/ TiF4 and 6: SnF2/ TiF4), we used a two-sided paired t-test, with a significance level of 0.9% (5%/ 6). This significance level was chosen in order to keep the probability of at least one false significant result below 5% (The Bonferroni adjustment method for multiple comparisons).

The distributions of surface loss differences between pairs of regimens were checked by visual inspection of empirical distributions (histogram) and no outliers were found. Mixed Model Analysis was applied when comparing simultaneously the 4 regimens on the outcome data ($p<0.001$). Positive "etch depths" (coating/ build up) were adjusted to zero.

Study 4

A Bland Altman plot of mean etch depth for repeated measurements on enamel and impressions versus the difference in etch depth measured on enamel and impressions was made with mean deviation from zero (95% Confidence Interval) of the differences, as well as the ordinary least square regression of the scatter plot. The *p*-values related to this difference and to the slope were calculated. The precision of the measurements from each method after 12 minutes acid exposure was calculated by reliability analysis and expressed by single measures Intra Class Correlation (ICC). The correlation of measurements by WLI on enamel surfaces versus on impressions after 12 min etch was calculated by regression analysis. The paired samples *t*-test was used with a 5 % significance level when comparing the precision of repeated measurements on enamel versus on impressions.

METHODOLOGICAL CONSIDERATIONS

The white light interferometer

Calibration

When the NT2000 was calibrated on a standard a calibration factor (a screw) was adjusted manually if the measured step height was off by more than 0.05 µm. This may occur as a result of changes in room temperature or vibration of the instrument board.

The newer NT9800 instrument, used for **Study 2 and 3**, has a built-in laser interferometer which performs an automatic calibration upon start-up and ensures that the instrument stays accurate at all times. The absolute accuracy of the NT9800 has been checked on a 1 mm step standard which had been measured independently by a separate white light interferometer built in the quantum optics lab at the University of Oslo [Holme et al., 2012].
For the 1 mm step, the deviation of the NT9800 was less than 0.5 µm, or 0.05 %. On the 10 µm glass step standard used for calibration verification, the accuracy is then better than 0.005 µm if we assume a linear relationship. More than 100 repeated measurements with the NT9800 of the 10 µm glass step over three years, indicates that its height – at the particular position that is always used for the calibration checks – is 10.028±0.001 µm. We may therefore take 10.028±0.005 µm as an updated step height value at the position used for the calibration checks in the present studies. This is a much more precise height determination than the 10.07±0.04 µm given on the step standard's certificate.

For step height measurements based on difference images, particularly of naturally curved enamel (**Study 2 and 3)**, the main source of inaccuracies lie in the alignment of the two images before subtraction. Significant efforts were made to ensure that the appliances could be put back with the same orientation after the in situ experiment as when the baseline images were made. However, some tilting was difficult to avoid. Therefore, the analysis software compared the amalgam part of the two images and performed the required lateral shifts and angular rotations before the images were subtracted. This method was, however, not perfect, mostly due to small changes in the amalgam surfaces. The repeatability of a step height measurement, based on comparing the results of two different images made after etching, turned out to be 0.2 µm for the "worst case" data of **Study 2**.

Based on these findings it is clear that the accuracy and precision of the WLI instrument in itself is far better than the reproducibility of the full step height measurements. The variation

in enamel loss between different teeth is even much larger than this, typically 5 µm for a 30 µm step height. So neither the WLI instrument itself, nor the analysis method with difference images give any significant contributions to the variability of the step heights observed in the data presented here. The spread in enamel loss is dominated by the different resistance to wear of different tooth samples, the in- homogeneities within one tooth (Studies 1, 2, 3 and 4) or in variation in biological conditions in the oral cavity among the participants (Study 3).

Disinfection of human teeth for *in situ* or *in vivo* experiments

The chosen procedure for disinfection of teeth (bovine or human) used in studies is not always reported. However, transmission of microorganisms through human teeth especially with regard to prion disease raises ethical problems when conducting *in situ* or *in vivo* studies. Therefore sterilisation of the tooth specimens is mandatory [West et al., 2011]. It is also important to know whether the chosen disinfection procedures will alter the tooth surfaces and thereby influence the results of the studies.

The type of disinfection in the *in situ* study **(study 4)**, where aqueous thymol solution and ethanol were used, was based on previously reported disinfection procedures for human teeth [Ganss et al., 2004; Ganss et al., 2007b; Ganss et al., 2007a; Ganss et al., 2010; Schlueter et al., 2011b]. It has even been used for disinfection of bovine teeth [Hara et al., 2009b]. However, it is not known whether it is fully effective against prions.
Ethanol is often used in addition to thymol solution for disinfection of teeth for use *in situ*, but there is scarce information in the literature regarding a probable effect of thymol plus alcohol on the enamel surface integrity that would influence the experiments and the outcome. It has been speculated, based on findings in pilot studies, that dehydration of the enamel after pre treatment with ethanol could enhance the uptake of water containing fluoride solutions. However, this was not confirmed in a later study [Wegehaupt et al., 2009].
Thymol is a phenolic compound found in thyme oil and ajowan seed and is also produced synthetically today. Thymol has antimicrobial mechanism whereby it disrupts the bacterial cell wall and causes cytoplasm to leak out and it also has antioxidant and anti-inflammatory properties in humans. As discussed by Ingram et al. [1997] and Amaechi et al. [1998], "enamel slabs for intra-oral application may not constitute a source of cross-infection as long as they are stored in 0.1% thymol and handled aseptically thereafter".

Formaldehyde (2 % pH 7, for 30 days) has also been used to disinfect teeth [Rios et al., 2006], but there is concern regarding the toxicity of aldehydes *in vivo* and they are known to stabilise prions [West et al., 2011]. In a study by Bjorvatn and Tveit [1981], it was found that pretreatment with formalin solutions influenced the chemical reactions on the tooth specimens by leading to increased fluoride uptake in enamel and reduced uptake in dentin.

γ -irradiation has been used for optimal sterilisation without effect on the structural integrity of the tooth surface [Amaechi et al., 1999a], but this technique is not recommended for prion sterilisation [World Health Organization:WHO, 1999]. Ethylene oxide sterilisation may be used [Hara et al., 2006b;Vieira et al., 2007], but the technique is not available in all countries.

Sodium hypochlorite (NaOCl) is listed in the WHO guidelines as an agent that can deactivate prions and 30 minutes immersion in a sodium hypochlorite solution with >16.500 ppm chlorine is recommended. NaOCl does not seem to affect enamel hardness but could extract the organic matrix and alter the mineral phase of dentin [Sakae et al., 1988].

Erosive challenge

In studies into dental erosion/erosive wear, the chosen acid challenge should be relevant for one of the main clinical problems; like consumption of soft drinks or reflux/ bulimia [Shellis et al., 2011].

For modeling gastric acid the use of hydrochloric acid (HCl) of appropriate concentration and pH is most practical [Young and Tenuta, 2011]. Gastric acid presents with a pH 0.9-1.5 range, but the pH in the oral cavity is seldom below 1.5 due to dilution and buffering by saliva. In bulimia patients the vomit has a pH of 2.9-5.0 [Milosevic et al., 1997]. HCl solution at 0.01 M (pH 2.2) was therefore chosen in **study 1, 2, 3 and 4**, based on previous protocols [Holme et al., 2005; Hove et al., 2006; Hove et al., 2007a; Hove et al., 2007b; Hove et al., 2008]. It is prepared using 0.833 ml of 37% HCl, density 1.175-1.188kg, 12M, mw 36.46 with 999.17 ml H_2O. The solution has a titratable acidity of 1.73ml, measured as the volume of 0.1 M solution of sodium hydroxide (NaOH) required to raise 20 ml of the experimental solution to pH 7.0 by adding increasing volume of sodium hydroxide solution followed by agitation and equilibrium for two minutes until the pH reaches 7.0. In the *in situ* study (**study 4**) the mouth appliances with the tooth specimens were kept intra-orally for at

least 2 hours before each acid etch ("reflux or vomiting episode") in order to allow pellicle formation. Optimum protection appears to be achieved after two hours of pellicle formation or probably less [Amaechi et al., 1999b; Wetton et al., 2006].

In a recent study by Schlueter et al. [2012b] erosive-abrasive wear of dentin under simulated bulimic conditions was investigated. The authors pointed out that after reflux episodes or vomiting, proteolytic digestive enzymes (pepsin and trypsin) can reach the oral cavity and a hydro- chloric- pepsin solution was used as erosive agent. The HCl (pH 1.6) was adjusted to a pH normally found in the stomach. Gastric acid contains about 750 mg/ml pepsin but after a meal it can be up to 2000 mg/ml so a concentration of 1500 mg/ml was used. Trypsin is normally present in the duodenum (2000 BAEE units/ml) as well, and in that study it was dissolved in a separate mineral salt solution since it requires calcium.

Tooth- brush abrasion

The brushing procedures and RDA (Relative Dentin Abrasivity) of the tooth pastes used vary considerably in studies as pointed out by Wiegand and Attin [2011]. The impact of the tooth-brushing per se has been considered to be lower than the impact of the toothpaste [Hara et al., 2009a; Wiegand et al., 2008c; Wiegand et al., 2009a], so one might argue that the abrasive effect may have been reduced with the use of water instead of tooth- paste slurry in Study 3. On the other hand, the brushing time of 30 sec (one stroke per second) on each specimen surface is quite long in contrast to a general recommendation of two minutes for all teeth. The procedure is in line with recommendations (10-15 sec, 2x/ day) by Wiegand and Attin [2011] that focused on standardization and clinical relevance in study design.

In **Study 3** we did not include a group with erosion only, so in order to get an estimate of the impact of the brushing, we compared mean enamel loss (32.3µm) after erosion/abrasion in the control group (**Study 3**) with mean enamel loss (18.1µm) for the controls after just erosion in a previous in situ study by Hove et al.[2008], where the erosion protocol was exactly the same. Since the difference in enamel loss was 14.2 µm (78 %) one may assume that this was mainly caused by the tooth brushing; the only "new" element in the procedures for the controls. However, a direct comparison is not entirely correct since both biological conditions among the individuals and the tooth specimens could differ between these two studies.

All brushing procedures were performed manually by the same operator with individual tooth brushes (for each volunteer and treatment) in order to avoid contamination. A brushing force of 2-3 N has often been used in automatic brushing machines in vitro, which is similar to the mean load applied on manual tooth brushes [Ganss et al., 2009b]. In **Study** 3, the load was not measured but held constant by the use of just one operator as previously suggested [Ganss et al., 2009b; Hooper et al., 2003; Vieira et al., 2007]. It has been shown that powered toothbrushes may be more aggressive to eroded dental tissue than manual brushes at the same force [Wiegand et al., 2006] which also is influenced by the type of brush end filament stiffness [Wiegand et al., 2008c; Wiegand et al., 2009a]. This must be kept in mind when comparing results from different studies. Still the impact of the toothbrush may be lower than the impact of the toothpaste [Hara et al., 2009a; Wiegand et al., 2008c; Wiegand et al., 2009a]. We tried to imitate a situation (**Study 3**) where a person brushed their teeth in the morning, used a fluoride rinse and then later during the day had two gastric reflux/ vomiting episodes. These episodes usually occur after a meal (lunch or dinner). There has been a focus on whether the patient should be advised to brush their teeth before or after an acidic impact without consistent results. Ganss et al. [2007a] concluded that brushing before erosion decreased enamel loss only by 12% *in situ* and that waiting for two hours after erosion had no protective effect. In contrast, Wiegand et al. [2008a] concluded that patients should brush their teeth prior to rather than after an acidic challenge to minimize wear. However, the experimental design is very different in these studies and may explain the different conclusions. The waiting period before brushing was 2 hours in the former and 5 minutes in the latter. This is essential since during pellicle formation the initial adsorption of proteins to a tooth surface takes only a couple of minutes [Hannig, 2006], but the full thickness is not achieved until after 30-90 minutes [Kuboki et al., 1987; Skjorland et al., 1995; Sønju et al., 1974]. Optimum protection from pellicle appears to be achieved after 2 hours formation or probably less [Amaechi et al., 1999b; Wetton et al., 2006], but some protective effect has also been found after 3 minutes [Hannig et al., 2004]. The aggressiveness of the wear processes differed as well, since the outcome data was in the range 41-81µm in the former and 2.3-6.4 µm in the latter study.

RESULTS AND DISCUSSION

Comparing different methods to assess erosive lesion depths and progression *in vitro* (Study 1)

Studies (*in vitro* and *in situ*) into prevention of dental erosion have escalated during the last 10-15 years and a variety of techniques are in use for assessment of tooth substance loss. In general, the most important parameter to consider in relation to relevant study outcome is the chosen method or measuring device and its reliability. Methods for assessing erosion and/or tooth wear have been reviewed repeatedly [Barbour and Rees, 2004; Field et al., 2010; Schlueter et al., 2011a; Attin, 2006].

A new technique for measuring loss of enamel by use of a white light interferometer (WLI) was developed and validated in our research group in collaboration with a physicist with expertise on the particular instrument [Holme et al., 2005; Hove et al., 2006]. The use of amalgam (not affected dimensionally by acid) as a suitable reference surface was evaluated, in addition to software especially designed for calculation of etch depths. Since the etch depth data are calculated from difference images, both negative and positive values can be presented. The latter is important, since in particular application of metal fluorides can give precipitates on the enamel surfaces, which could lead to a glaze or coating on the surface (positive "etch depth" or build-up). In a pilot study, promising results were obtained regarding measurements on naturally curved surfaces (not ground flat) and on impressions (negative replica) of eroded enamel [Holme et al., 2005].

In **Study 1** the measurements of enamel loss after acid exposure were performed on enamel specimens that were ground flat, since both hardness indenters and contact- and non-contact profilometers give more accurate measurements on flat than on curved surfaces [Ganss et al., 2000; Schlueter et al., 2011a]. Also the time for each analysis (on one specimen) and the size of the analysed area (~0.91mm^2) was the same for all instruments, with the purpose to keep differences in the experimental setting that could affect the outcome from the five different instruments, at a minimum. For comparison between instruments, the measurements by WLI were considered as the "true value" and were 5.1 ±1.1 µm (after 6 min etch) and 10.4 ±1.9 µm (after 12 min etch).The respective etch depths measured by the contact profilometer (SP) were 5.1 ±1.1 µm and 10.4 ±1.9 µm and by the non-contact profilometer (OP) they were 6.0 ±1.1 µm and 11.7±1.8 µm. From these data it is clear that all three instruments could detect

progression of the etch depths caused by repeated acid attacks; but OP presented approximately 15% higher etch depths both after the first and second etch. The standard deviations reflect the relative variability between twelve teeth and were the same for these three methods.

When evaluating the accuracy of outcome from one instrument, method or treatment, it is usually evaluated against a gold standard [www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf, 2001]. In **Study 1,** where etch depths on enamel were measured, a possible gold standard to give the actual etch depth would be by transversal micro-radiography, but this is a destructive technique since it requires cutting of the specimens. In **Study 1** it was important to perform repeated measurements on the same surfaces by all five techniques, so WLI was used as "reference" or "true value". This is referred to as a cross-validation in the literature; a situation where an original validated method serves as the reference and another method serves as the comparator[www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf, 2001]. The accuracy of the WLI was calculated from calibration runs of a glass standard with step height 10.07 μm (with an absolute uncertainty in the height of 0.04 μm) and was 0.007±0.002 μm (based on 6 calibrations x 10 step measurements). Therefore, the overall accuracy of the WLI measurements for ideal stepped surfaces was in this case limited by the step uncertainty of 0.04 μm, or 0.4 %. The absolute uncertainty in the height of 0.04 μm had been determined by an independent check of the 10.07 μm glass step standard, by measuring the step repeatedly with a different WLI instrument, which again had been calibrated against a step measured by an interferometry setup based on caesium radiation, where the wavelength is known to be 10^{-17} m. This procedure further justified that measurements by WLI worked as "true values". In comparison with WLI the accuracies for SP and OP were calculated from the slope of the respective regression lines (100 % (1-slope), and were 0.7 % and 12 % respectively.

The precision was calculated as the average standard deviation (SD) of three step measurements on each specimen after each acid etches. The relative precision was calculated as the SD divided by the mean lesion depth for each specimen and then expressed as the average of the relative precision of the twelve specimens. In **Study 1** the WLI precision (repeated measurements) was not as good on enamel (0.5 %) as on the ideal glass standard (0.008 ±0.001 μm =0.08 %), since the rough etched enamel surface gives more "noise" in the

data than the smooth glass surface. The respective precisions for SP and OP were 4.7 % and 1.4 %.

These results from **Study 1** show that measurements by the SP and OP were not as accurate and precise as those by WLI as already seen from the calibration data for measurements of the respective ideal standards. For SP, a silicon standard (height 0.200 µm) was measured and the accuracy (0.004 µm) and precision (0.003 µm) was 2 %. The calibration of OP was performed on a metal standard (30.09 µm) and the accuracy was 3 % (0.8 ±0.4 µm) and the precision was 0.6 % (0.2 ±0.1 µm). From these data, it can be assumed that this particular SP under the present experimental conditions, performed measurements close to the "true value" (WLI) and etch depths down to 3 µm were detected with good accuracy. This is in accordance with results from previous studies where different contact profilometers using mechanical stylus have been confirmed to be sensitive instruments and able to detect mineral loss well under 1µm [Ganss et al., 2000; Ganss et al., 2005], from 0.1 µm [Attin et al., 2009], above 1 µm [Hannig et al., 2008] and above 2 µm [Schlueter et al., 2005]. The precision of repeated measurements for SP in **Study 1** was not as good as for WLI. SP performs tracings on the surface creating topography profiles. To obtain good precision it is important to repeat the measurements on the same area. The baseline images from WLI were used as maps to enable this, but still the three 100 µm tracings could deviate from the baseline tracings or not be as representative for the whole area as the topography images from the WLI analyses that were based on information from each pixel. Factors like the load, number of tracings and custom-made jigs for exact repositioning in repeated measurements could also influence on the precision of SP [Attin et al., 2009]. In **Study 1** a repositioning jig was used, but only three tracing were made, which could explain the minor precision (0.33 µm) compared with 0.031 µm in the study by Attin et al. [2009], where a large number of tracings were made. The reason for choosing three tracings in **Study 1** was to spend equal time for each analysis for all methods.

For the OP instrument the precision of repeated measurements in **Study 1** equalled those by WLI, but the ICC values showed that the measurements by OP after the second 6 minutes etch were less precise than after the first 6 minutes etch. One interpretation of that finding may be that OP is less suited for measuring depths from 7-13 µm. When relating to reports from other studies where non-contact profilometry has been used, erosive lesions around 0.5µm deep can be detected consistently [Hara and Zero, 2008]. The precision has been

reported to be 0.06 µm for repeated measurements of erosion depths on enamel in the range 0.5-3.5 µm [Steiner-Oliveira et al., 2010]. The accuracy of measurements by OP in **Study 1** was minor as could be expected when considering the calibration data. In addition to background noise in the instrument, external factors that could have influenced the data were vibrations of the instrument and variation in room temperature. We used an objective with 20X magnification (spot size (field of view) =625x469 pixels) and therefore we had to stitch four such images together with 20 % overlap in order to obtain data from the same area as for WLI and SP. This may also have influenced the accuracy of the data. A spatial sampling (step over distance) of 0.83 µm was given for the 20X objective but it was adjusted to 1.66 µm (2x0.81 µm) in the z-plane to match the speed of WLI and SP for one image/map. If the step-over distance is too large, some information may be lost. However, the imaged surfaces in the present study were relatively smooth and the 2x step- over distance allowed measurements of these etch depths. The operator factor was under control since the physicist was the same for all analyses and certified for operating this instrument.

Regarding the atomic absorption spectroscope (AAS) the results in **Study 1** are difficult to compare directly to those from WLI, SP and OP, since the instrument detects released calcium from the enamel specimens into the acid and this value was converted into tissue loss in µm based on the density of hydroxyapatite in enamel. The reported etch depth was also an average value, since the whole epoxy block was stirred in the acid and calcium loss from all twelve specimens was calculated. These two aspects could have influenced the accuracy of 17% of the reported "etch depth" (8.81 µm AAS and 10.4 µm WLI). Other studies have concluded that quantification of calcium and phosphate release by an acid solution is a well established method for assessing dental erosion [Grenby, 1996] and that atomic absorption spectroscopy is a reliable and sensitive method for calcium analysis and can be used to quantify erosion both on enamel and dentin [Grenby et al., 1990; Hara and Zero, 2008]. The lower AAS etch depth compared with WLI in **Study 1** is in accordance with results from a study by Vieira et al. [2005], who found significantly lower etch depths from measurements by AAS than for white light confocal microscopy. On the other hand, [Ganss et al., 2005] reported opposite results with 20 % lower values for substance loss measured by a mechanical stylus than by chemical analysis.

The Vickers hardness tester (**Study 1)** was calibrated on steel standards and the accuracy was estimated to be 4 % and the precision 1 %. The precision of hardness testing on the enamel

surfaces was 5 %. Since the calculation of hardness values is based on a reading of the length of the diagonal of the indentation, it is important that the indentation is distinct. Several factors could influence these analyses on etched enamel; it is not always possible to make the indentations on the same spot of the specimen, the surface changes on the eroded enamel could vary in different locations of the experimental area and the indentations could be difficult to distinguish due to the rough eroded surface. The present hardness values (**Study 1)** showed no consistent changes related to the two stages of acid etches. This is not unexpected when the histo-pathological process occurring on the enamel during aggressive and repeated acid attacks is considered. During an initial or mild acid attack with free protons present, mineral from the enamel surface is dissolved due to the unsaturated acid with respect to tooth mineral (Ca^{2+} and PO_4^{2-}). The reduced mineral density leads to a reduction in surface hardness of enamel (softening) and the thickness of this layer (Figure 1) was reported to be 0.2-3.0 µm [Amaechi and Higham, 2001; Eisenburger et al., 2001; Voronets and Lussi, 2010; Wiegand et al., 2007; Cheng et al., 2009]. If the acid attacks are aggressive and frequent, the hydroxyl apatite crystals will dissolve and the result is bulk loss of enamel. However, the outermost layer after bulk loss is also demineralised and softened (Figure 1). This demineralised layer may be re-mineralised by salivary minerals and/or fluoride agents, but the bulk loss cannot be reversed. Therefore, surface hardness measurements seem to be most suitable for evaluation of de- and re-mineralisation processes related to mild acidic impacts and initial softening of enamel [Hara and Zero, 2008; Jaeggi and Lussi, 1999; Schlueter et al., 2011a; Shellis et al., 2011].

Taking into consideration that the results from **Study 1** verified that WLI is a reliable instrument for measuring enamel bulk loss, in line with mechanical and optical profilometry techniques, we felt confident to continue to use and develop this technique in further studies.

The effect of different fluoride solutions on enamel erosion and erosive/abrasive wear *in vitro* and *in situ*
(Study 2 and 3)

Previous studies performed by our research group have investigated the protective effect of different types of fluoride solutions against dental erosion both *in vitro* and *in situ* [Hove et al., 2006; Hove et al., 2007a; Hove et al., 2007b; Hove et al., 2008]. The types of fluoride solutions studied were sodium fluoride (NaF), stannous fluoride (SnF_2) and titanium tetra fluoride (TiF_4) at high concentration (0.5M F) and native pH. From these studies it was

concluded that 1.5 % (0.5 M) TiF4 solution (pH1.3) showed best protection against enamel erosion. A 3.9 % (0.5 M) SnF_2 solution (pH 2.6) protected the enamel significantly *in vitro* and even better *in situ*. A 2.1(0.5 M) % NaF solution (pH 8.0) showed significant protection *in vitro* but not *in situ*. However, there were some concerns about the bio-compatibility of the TiF_4 and SnF_3 solutions due to the high fluoride and stannous concentrations and the low pH of the solutions. The toxic effect of 1% TiF_4 solution on fibroblasts has been evaluated and the toxicity was dependant of the duration of the exposure [Sen et al., 1998]. Adverse effects of tin containing agents (1900 ppm tin), like a dull feeling on the tooth surface, astringent sensation on oral mucosa and discoloration of teeth have also been reported [Schlueter et al., 2009c; Schlueter et al., 2011b]. These solutions would also have to be applied by dental professionals in the clinic due to regulations regarding fluoride concentration for products for self administration which is 0.15 % F. In Norway there are no specific regulations regarding the pH of such oral care products. From the patient's point of view, it would be more convenient with a mouth-rinse that could be administered daily at home.

It was speculated if the concentration of the TiF_4 solution could be reduced or the pH increased and still retains the excellent protective effect. It had been demonstrated that good protection was obtained by concentrations of TiF_4 solution from 1 to 4%) in many *in vitro* studies [Buyukyilmaz et al., 1997; Hove et al., 2006; Hove et al., 2007a; Hove et al., 2007b; Schlueter et al., 2007; van et al., 2003; Wiegand et al., 2008b; Wiegand et al., 2009b], but the results from *in situ* studies were contradictory [Magalhaes et al., 2008; Hove et al., 2008] . The results from **Study 2** indicated that lowering the concentration and adjusting the pH of the TiF_4 solution decreased the protective effect. However, the native TiF_4 solution (pH 2.1) with lower concentration (0.05M F versus 0.5M F) offered significant protection after two minutes etch, although this effect was lost after further acid challenges. Results from a study by Wiegand et al. [2009b] showed that only TiF_4 solution at pH 1.2 and not at pH 3.5 (buffered) reduced enamel surface loss significantly. This is in accordance with the findings in **Study 2**, where TiF_4 0.5 M F buffered to pH 2.1 gave no significant protection of the enamel compared with the native solution with the same concentration. In a study by [Tveit et al., 1983], it was suggested that a TiF_4 solution at 0.5 M F with its native pH promotes the optimal complex binding ability of Ti to phosphate groups in the enamel.

This possibility is consistent with the findings in **Study 2**, where both native solutions showed an erosion-protective effect while pH adjustment of native solutions reduced the

erosion-protective effect. In the study by Wiegand et al. [2009b], the authors concluded that the efficacy of TiF_4 to prevent erosive surface loss is related to the glaze layer at native pH, since this surface layer was absent after application of the solution with pH 3.5, and these considerations are in accordance with those by Tveit et al. [1983] and with the findings in **Study 2**.

It has been shown that after extensive acid exposure, enamel surfaces treated with TiF_4 solutions are not etched homogeneously [Hove et al., 2007a; Wiegand et al., 2009b]. The surface often shows un- etched regions that are not affected by the acidic challenge due to the presence of a glaze/ coating, and areas where the coating is disrupted by cracks. A similar etch pattern was also found in **Study 2** and **3**. In the cracks, the enamel was etched in nearly the same way as the control enamel, with a predictable step distance from the baseline. Wiegand et al. [2009b] suggested that the glaze-like layer presents some in-homogeneities or micro-cracks which allow for the penetration of acid into the subsurface enamel layer.

Based on these considerations, it was suggested that further studies ought to be performed as close to a clinical situation as possible [Wiegand and Attin, 2011], where the fluoride applications are frequently repeated and tooth brushing included.

In **Study 3**, the concentration of the fluoride solutions was lowered and native 0.15 % titanium tetra fluoride (pH 2.1), 0.4% stannous fluoride (pH 2.5) and 0.2 % sodium fluoride solutions (pH 6.5), all at 0.05 M F, were applied every day to imitate daily mouth rinse. This *in situ* study went on for nine days. The results are very promising since titanium tetra fluoride and stannous fluoride solution reduced enamel loss by 90 % and 94% respectively, despite quite aggressive wear processes with two episodes of hydrochloric acid etch every day (mimicking two vomiting episodes) and tooth brushing once per day. Other studies have shown 55% to 82% reduction in enamel loss after use of SnF_2 solutions *in situ* [Ganss et al., 2010; Schlueter et al., 2009c; Schlueter et al., 2011b], but the specimens in those studies were not subjected to tooth brushing. In addition, the concentrations, compositions and pH of the SnF_2 solutions that were applied once a day in those studies had been modified to avoid adverse/side effects. In contrast to the results in **Study 3**, TiF_4 showed better effect than SnF_2 against erosion in a previous *in situ* study where abrasion was not included [Hove et al., 2008]. In an *in situ* study by Wiegand et al. [2010a], a single application of TiF_4 solution (0.5M F) reduced enamel loss significantly compared to the controls and the protective

potential was only slightly decreased by abrasion. At this concentration a precipitation/glaze is expected to be formed on the surfaces after application and the results indicate that the glaze was not weakened by the abrasion. However, when looking at the enamel loss outcome in that study, it seems obvious that the wear processes must have been less aggressive than in **Study 3**, since the enamel loss on the controls at the end of experiment was ~1.3µm, in contrast to 32.3 µm in **Study 3.** It is therefore difficult to make a direct comparison of the outcome.

In general, when **Study 3** was initiated, very few *in situ* studies [Wiegand et al., 2010a] into the efficacy of metal fluorides against erosive tooth wear had been performed and especially where tooth brushing was a part of the experimental design. It is highly relevant to evaluate the impact of tooth brushing, since the mode of action of these two fluoride agents is mainly by formation of precipitates like a coating or glaze on the tooth surfaces. Little was known about their durability when challenged by mechanical forces, like tooth brush and/or tooth paste abrasion. The enamel specimens in **Study 3** were brushed with ordinary individual tooth brushes and water. The load, brushing time and strokes per minute, are in accordance with recommendations given in the paper of Wiegand and Attin [2011]. It can be argued that brushing the tooth surfaces with tooth paste instead of water could have weakened the glaze/coating further. However, in order to better understand the independent influence of each of these two factors on the erosive tooth wear process, we opted for using water instead of toothpaste slurry in **Study 3**. Even without the abrasive effects of the toothpaste, relatively higher mean wear values were observed for the eroded and brushed control specimens in the present study (32.3µm), compared to the eroded-only controls (18.1µm) from the *in situ* study by Hove et al. [2008]. The abrasive impacts of tooth brushing and/or tooth paste is further discussed in the paragraph “Methodological considerations”.

Even the sodium fluoride solution reduced enamel loss (18 %) compared to the control in **Study 3**. Other studies have also shown effect of daily application of NaF solution with low fluoride concentration on moderate and severe acidic impacts [Ganss et al., 2010; Schlueter et al., 2009c], but in these studies, the solutions were acidified to improve the effect. One may speculate that the frequent applications and relatively high concentration (0.2 %) of the neutral NaF solution in **Study 3** could have contributed to the significant effect by formation of calcium fluoride as supported by previous studies [Saxegaard and Rolla, 1988].

The software that was used for calculating enamel loss based on the difference image from WLI as described in **Study** 1, was further developed in **Study 2** and **Study 3** where enamel areas with and without surface glaze/coating were differentiated. In **Study 3,** specimens treated with stannous fluoride solution showed a mean "etch depth" of 2.6 µm on areas without coating and 18.0 µm on not-coated areas on surfaces treated with titanium tetra fluoride solution. These findings are interesting and lead to speculations about the interactions between these two metal fluoride solutions and the enamel. All samples that were rinsed with the metal fluoride solutions showed areas with intact coating, coating with cracks or uncovered enamel (to varying extent respectively) as illustrated in Figures 16 b and c. Some specimens showed average positive "etch depth" (build- up) at the end of the experiment and were almost completely covered by glaze/coating (Figure 17).

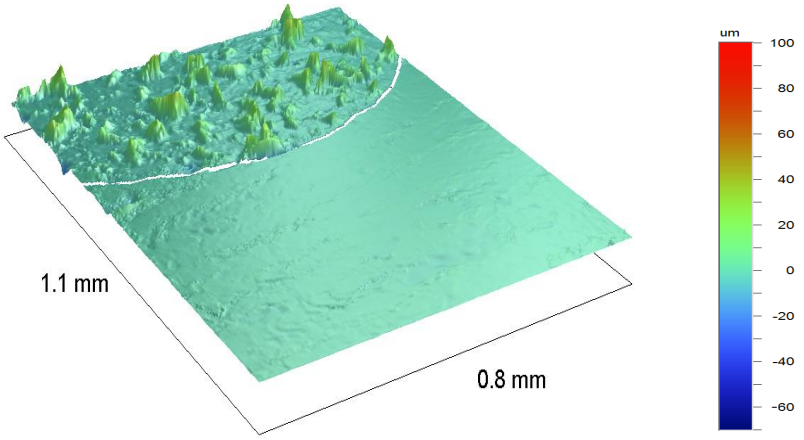


Figure 17.A difference image (at the end of **Study 3** *in situ*) of one specimen from tooth number 10, participant E treated with TiF_4 solution showing a perfect glaze on the enamel.

Even though areas without coating/glaze were demonstrated (**Study 3)**, the stannous fluoride and titanium tetra-fluoride applications resulted in significant reduction in enamel loss. Different explanations are possible. Firstly, the break-up of the coating/glaze may have taken place at a late stage of the experimental period, allowing only a shallow etch of the exposed enamel. Secondly, a patch of broken glaze, where the enamel had been etched, may later have

been covered by a new layer of coating/glaze as a result of repeated treatment. Thirdly, a continuous incorporation of tin, titanium and fluoride ions into the enamel surface from repeated applications could have resulted in a lower etching rate than in non-treated enamel [Ganss et al., 2008; Ganss et al., 2010; Ribeiro et al., 2006; Schlueter et al., 2009a; Schlueter et al., 2009b; Schlueter et al., 2011b].

An estimate of the glaze/ coating thicknesses after nine days experimental protocol was made in **Study 3** and it was 1.0 µm (range 0.1-2.4 µm) on the specimens treated with SnF_2 and 0.6 µm (range 0.2-1.2 µm) on those treated with TiF_4 solution. So the former was thicker and could thereby be more resistant against abrasion by the tooth brush. As far as we know, the thickness of these precipitate layers has not been reported previously. The thickness was estimated by measuring the change in height difference between the enamel and amalgam reference surface on the topographic difference image. A positive etch depth/enamel wear value means a net build-up of material on the enamel, relative to the amalgam. Without independent measurements of the coating/ glaze thickness by sputtering or cross-section microscopy, the actual coating/ glaze thickness cannot be known exactly.

Enamel erosion depths measured on impressions by a White Light Interferometer (Study 4)

It has often been stated that there is a need for clinical studies in the field of aetiology and mechanisms behind tooth wear processes and their prevention. The reason for these considerations is that tooth wear is a multi-factorial condition (Figure 18).

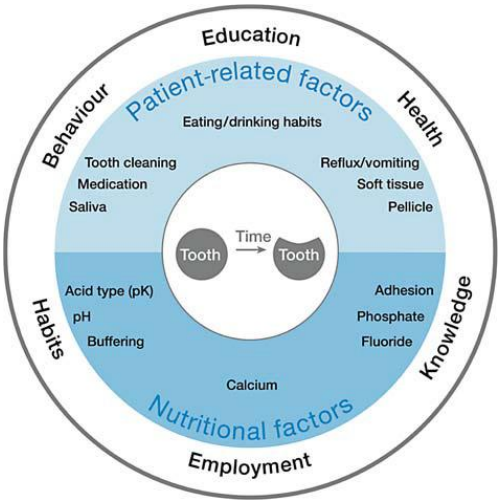


Figure 18. Patient-related and nutritional factors affecting the development of erosion [Lussi et al., 2011].

Individual variations in biological factors like the enamel quality, salivary parameters (flow rate, buffering capacity and enzymes), formation and properties of the acquired pellicle and enzymes from oesophagus/stomach influence the process and the effect of preventive fluoride treatment [Lussi et al., 2011]. Regarding enamel, disturbances in main proteins involved in enamel formation; ameloblastin, amelogenin, enamelin and tuftelin, could alter enamel crystallite structure and mineral content and thereby influence the susceptibility for dental erosion. Such connections have been suggested for individual caries susceptibility in humans [Deeley et al., 2008; Patir et al., 2008] and susceptibility to dental fluorosis in animal studies [Everett, 2011]. The pellicle protects dental hard tissue against mechanical and erosive impacts [Joiner et al., 2008; Hara et al., 2006a] and acts as a barrier and buffer against erosive attacks. However, its thickness is reduced by acid and the protective effect is therefore dependant on time and pH [Hannig and Balz, 2001] and with ongoing frequent acid attacks it will be destroyed [Nekrashevych et al., 2004]. The pellicle could possibly be altered by proteolytic enzymes in saliva and influence the progression of erosion lesions [Schlueter et al., 2012a]. There have been reported observations which support that variations in biological factors may influence the individual susceptibility to erosive wear. For instance in bulimic patients reporting many vomiting episodes per day for years, some suffer from tooth wear and some not [Robb et al., 1995]. The same phenomenon has also been reported for other potential risk groups like wine tasters [Mulic et al., 2011] and persons with frequent intake of

soft drinks [Hooper et al., 2007; Hughes et al., 1999; Mulic et al., 2012a] and frequent high intensity physical exercise [Mulic et al., 2012c].

Type of lifestyle and habits are also very important factors to consider during risk assessment, since moderating or eliminating identified risk factor is valuable for prevention. These background factors can vary between western and non-western countries, socio-economic groups and with gender [Arnadottir et al., 2010; Hooper et al., 2007; Mulic et al., 2012a]. Examples of important lifestyle factors related to tooth wear could be; frequent consumption of soft drinks, high intensity physical exercise resulting in periodically reduced salivary flow in combination with intake of sports drinks, healthy diets consisting mainly of fruits, salads with acetic dressing, exaggerated oral hygiene habits and occupational risks (wine tasting, acidic fumes).

Taking these factors into consideration it is important to go a bit further in research into the field of dental erosion and erosive tooth wear and perform clinical studies as pointed out by Huysmans et al. [2011]. The author suggested that in epidemiology there are three population groups in which clinical studies could be conducted; **a)** healthy volunteers**, b)** populations with erosive wear and **c)** populations with aggressive tooth wear. For ethical reasons, **a)** healthy individuals may only be suitable for erosion studies on changes in mineral loss or surface characteristics with an initial softening of the enamel that could be reversed by remineralisation. In **b)** populations with erosive wear, the main focus for clinical research should be on products modulating the effect of the erosive process (both qualitatively and quantitatively). In **c)** groups with aggressive tooth wear (patients with GORD or bulimia) the assessment of beneficial effects of new products and approaches will be complicated by parallel behavioural modifications and medical interventions.

The Workshop on Methodology in Erosion research in Zürich, 2010 was summarized by Shellis et al. [2011] and the discussion focused on the feasibility of clinical trials. It was suggested that the clinical measure of erosion should be an index rather than a measurement technique such as profilometry, since the latter has not been fully validated [Schlueter et al., 2011a]. Most instruments (like WLI, SMH, OP and SP in **Study 1)** that are used for assessment of tooth substance loss and surface changes in relation to progression of tooth wear and/or the effect of preventive actions cannot be used directly in the oral cavity. To make impressions/replicas **(Study 4)** of the tooth surfaces under study could therefore be an

appropriate technique for clinical studies [Schlueter et al., 2011a] and has been investigated previously [Azzopardi et al., 2001; Bartlett et al., 2011; Bartlett et al., 1997; Mitchell et al., 2003; Ranjitkar et al., 2009; Rodriguez et al., 2009; Schlueter et al., 2005; Sundaram et al., 2007a; Sundaram et al., 2007b]. However, the authors have pointed out some shortcomings related to the methods.The possibility to measure enamel loss on impressions of tooth specimens by the use of WLI as described by Holme et al. [2005], was further investigated in **Study 4**. The results showed that in an etch depth range from 7-14 µm measured on enamel; repeated measurements on impressions of the same etch depths were precise (0.11 %), but the accuracy of the measurements was 7 %. This means that the etch depths measured on impressions were systematically 7% higher than those measured directly on enamel ("true value"), also verified by the mean difference 0.71 (±0.42) µm from zero (95% CI:0.45-0.98).

The technique needs further development, since the specimen surfaces were ground flat as will not be the case in vivo. Profilometry has been used to measure erosions on natural surfaces *in vitro*, but the accuracy is less than for flat surfaces [Ganss et al., 2000]. With the WLI technique, a topography image of the original surface is subtracted from a topography image of exactly the same area after an experimental period. This topographic difference image contains a step from the amalgam region to the enamel region, regardless of the original curvature of the sample. By the use of specially designed software the surface loss may be calculated and also information about local changes in the topography can be registered. This is an advantage with the WLI technique compared to mechanical and optical profilometers that perform best on flat ground tooth surfaces. Measurements by WLI on pellicle covered naturally curved enamel surfaces before treatment were validated in an *in situ* study and the accuracy was 0.05 µm and the detection limit 0.15 µm [Hove et al., 2008] and in **Study 2** *in vitro*, the precision was 0.1µm and the detection limit 0.3 µm. There is reason to believe that the accuracy of measurements on impressions of naturally curved surfaces would be equally good, based on the findings in **Study 4**. An appropriate type of reference device must be evaluated, since for monitoring etch depths it is important to identify the reference and thereby re-measure on exactly the same area against a reference unaffected by the wear processes. For the WLI technique an adjusted orthodontic bracket or tooth jewellery could work as reference devices, but these options have to be tested in trials. Also the type of impression material must be evaluated, since the silicon polymer (Microset®) that was used in **Study 4**, is used for industrial purpose and not specifically approved for

intraoral use. The accuracy of the replica material has been evaluated using WLI (WYKO NT9100 and Vision software) and for step heights beyond 1µm less than 10 % error is expected and for in vivo trials it was suggested to go for a study design with 10 µm step heights or more [Ying and Holme, 2012]. The Microset (silicone polymer) impression material was also compared with Permadyne (polyether) dental impression material and the latter gives a more brittle and rougher surface than Microset, so on that wavy surface small steps could not be seen (Figure 19).

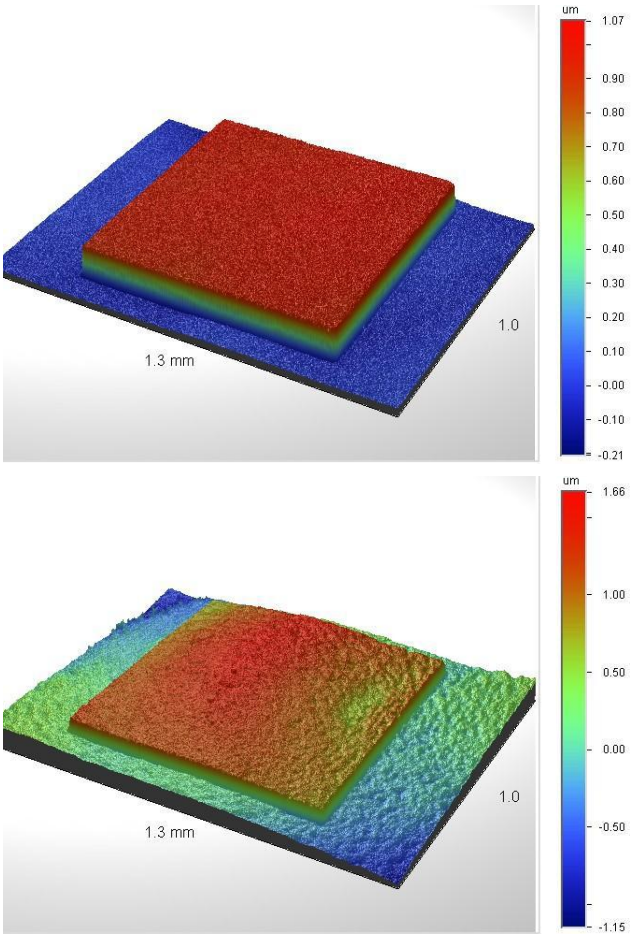


Figure 19. Subject with step height of 980±20 nm on Microset (upper) and Permadyne (below) [Ying and Holme, 2012]

The replica technique has been used in other studies and depending on the type of reference area, the detection limit was reported to be 15-20 μm [Bartlett et al., 1997; Lambrechts et al., 1989; Schlueter et al., 2005] and for surface mapping systems the precision was reported to be 15 μm and the detection limit 50 μm [Mitchell et al., 2003]. Indirect profilometry was used in a clinical trial where the effect of bonding agent application on the wear of exposed dentine was evaluated [Sundaram et al., 2007b]. A split mouth design was used and the study lasted 20 months, but all metal reference discs were lost at that point of time. An evaluation of measurements of tooth wear using a non contact laser profilometer and surface matching software was recently published [Rodriguez et al., 2012a]. Scanning and superimposing the same model introduced mean error of 2.7μm, whereas scanning and superimposing separate casts from repeated maxillary impressions introduced error of 14.8μm. This technique was used in an *in vivo* study for measuring progression of dentine tooth wear over 12 months [Rodriguez et al., 2012b]. The results revealed that median wear on tooth level was less than the measurement error (15μm). Scanning of the tooth or surface using the CAD-CAM technique also represent a way of measuring tooth wear *in vivo*, but a significant of work is required before these systems can be adapted for this purpose [Huysmans et al., 2011].

SUMMARY AND CONCLUSIONS

Study 1

Four instruments often used for assessment of tooth substance loss in erosion and abrasion studies (a white light interferometer, stylus profilometer, optical profilometer and atomic absorption spectroscope) were validated. They could measure progression of etch depth on enamel surfaces in the range of 3-14 μm, related to successive acid exposures on the same enamel surfaces. In the validation process, measurements by the white light interferometer were considered as "true values". The white light interferometer was superior to the stylus profilometer and optical profilometer; both regarding precision (repeatability) and accuracy of the measurements of enamel etch depths. This emphasizes the value of this instrument in further research in this field.

The surface micro hardness tester gave information about changes in surface hardness, but the changes gave no information related to the different stages of lesion depth. It may be argued that surface micro hardness tester should only be used for studying initial demineralisation of the tooth surfaces after mild acid challenges to enamel, where no quantitative substance loss is suspected.

The chemical analyses by the atomic absorption spectroscope were precise. The accuracy of the instrument was low since it was used for indirect assessment of etch depths on enamel surfaces; measured calcium loss into the acid was converted to etch depths values.

Study 2

There have been some concerns about the biocompatibility of metal fluoride solutions with high concentration and low pH. This study investigated the possibility of achieving a protective effect against enamel erosion to the same extent as previously found for native titanium tetra fluoride solution (0.5 M F), by lowering the fluoride concentration or by adjusting (buffering) the pH the solution. It can be concluded that reducing the concentration of a native aqueous titanium tetra fluoride solution (from 0.5 M F to 0.05M F) and increasing the pH of the solution (from pH 1.2 to ph 2.1) decrease the erosion-inhibiting effect. A glaze/precipitation was formed on the enamel surfaces after fluoride application, except for the 0.05M solution with adjusted pH. The low concentrated native titanium tetra fluoride solution (0.05M F) gave significant protection after 2 minutes etch, but this effect was reduced after successive acid etches. These findings led to the idea of investigating further if

the protective effect of the native low concentrated titanium tetra fluoride solution could be improved by frequently repeated applications.

Study 3

The protective effect of fluoride solutions at low concentration against enamel erosion and tooth brush abrasion was further investigated *in situ*. The results showed that daily application of either, 0.4 % stannous fluoride (pH 2.5), 0.15 % titanium tetra fluoride (pH 2.1) or 0.2 % sodium fluoride (pH 6.5 solutions (all with 0.05 M F) significantly reduced enamel loss from erosion plus abrasion *in situ*. Both the stannous fluoride and titanium tetra fluoride %) solutions resulted in significantly better effect than the sodium fluoride solution (94 %, 90 % and 18 % respectively). This superior protective effect against erosive and abrasive enamel wear as a result of daily rinse with either stannous or titanium fluoride solutions is promising. However, the clinical applicability of the stannous fluoride and titanium tetra fluoride solutions needs further investigation with a focus on possible adverse effects caused by the low pH and the relatively high concentrations of metal ions.

Study 4

With focus on possible techniques that could make clinical studies possible, it was evaluated if the white light interferometer could give reliable measurements of enamel etch depths on impressions of the affected tooth surfaces. The white light interferometer gave reliable repeated measurements of etch depths in the range from 7-14 μm on impressions of eroded ground enamel surfaces with a 7 % overestimation (0.7μm) compared to the etch depths measured directly on enamel. This was the first step for the validation of a method that could make *in vivo* studies possible. However, there is a need for further adjustment of the method before it could be adopted in the clinic, with focus on type of impression material and reference surface and measurements on natural curved tooth surfaces.

FURTHER RESEARCH

Based on the findings from **study 1, 2, 3** and **4**, there are several relevant fields for further research.

The clinical applicability of products containing stannous- or titanium tetra fluoride for protection against dental erosive wear needs further investigation with emphasize on optimal concentration and pH for obtaining best effect and biocompatibility.

Studies into the effect of various types of agents like tooth paste, rinse, gel or combinations of these, are relevant. The use of tooth pastes for instance, is an integrated part of daily oral hygiene habits for most people in western countries. Altering the active ingredients in toothpastes, like type of fluoride, could improve the effect against dental erosive wear. Substitution of conventional sodium fluoride in toothpastes with a metal fluoride which has shown to give better protective effect against tooth wear used in solutions and gel could be a new approach. Several studies by other research groups on this topic have been performed already, mainly focusing on stannous fluoride as active ingredient.

Related to observations of variations in susceptibility to erosive wear and response to preventive agents among individuals, identifying such factors is a field where there is need of more research.

Performing *in vivo* and clinical studies related to pathological dental erosive wear would be a huge step forward. Since measurements of tooth wear on impressions of tooth surfaces by a white light interferometer have been validated and proved to give reliable outcome data, the use of this method could be a way into clinical studies. There is a lack of both longitudinal studies on progression of tooth wear and intervention studies where treatment outcome is studied preferably in risk groups.

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Errata

Page 8. First paragraph: …will lead to further bulk loss of enamel (Fig.1).

Page 9. Second paragraph:… atomic absorption spectroscopy (Fig.2) uses …

The analysed concentration…

Page 10. Second paragraph: …or Vickers (tetra-pyramidal indenter) (Fig.3 and 4).

Page 13. First paragraph: …focus fluorescence light.

Pages 14 and 15. Figure legends:

Fig.7. Schematic illustration of long coherence length for monochromatic light versus short for white light.

Fig.8. The short coherence length of white light gives the highest fringe contrast only at best focus.

Page 18. Third paragraph: …method and the true value agree (Fig.12).

Fifth paragraph:…The precision(Fig.12) of a method…

Page 20. Third paragraph: …sodium fluoride and amin-fluoride seem to offer…

Page 22. First paragraph: SnF_2 (mw 156.7; Sn 118.7 and F 19)

Page 23. Second paragraph: To validate instruments and techniques that are…

Page 24. Legend:

Table 1. Materials and experimental procedures in study 1, 2, 3 and 4.

Fluoride treatments in study 2: TiF_4 0.5M F pH 1.2, TiF_4 0.05M F pH 2.1, TiF_4 0.5M F pH 2.1, TiF_4 0.05M F pH 1.2

Page 26. Figure 15. Triad acrylic, Dentsply, Permadyne, 3M Espe

Right photo: The appliance has tooth number 11 on the right side and number 12 on the left side.

Page 29. Figure 16c. The enamel areas with glaze show no substance loss whereas the areas without coating do.

Page 30. Second paragraph:…indent diagonal).

Page 37. First paragraph:…the inhomogeneities within one tooth…

Page 66. Mulic et al 2012a. Epub. Ahead of print.

I

II

III

IV