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Dentistry — External tooth bleaching products

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Contents

Page

Foreword	iv
Introduction	v
1 Scope	1
2 Normative references	1
3 Terms and definitions	2
4 Classification	2
4.1 General.....	2
4.2 Products for professional application.....	2
4.3 Products for consumer application.....	2
5 Requirements	3
5.1 Concentration of active ingredients for bleaching.....	3
5.2 Surface microhardness.....	3
5.3 Surface erosion.....	3
6 Measurements and test methods	3
6.1 Preparation of tooth specimens.....	3
6.2 Preparation and application of tooth bleaching product.....	3
6.3 Surface microhardness.....	3
7 Packaging, marking and information to be supplied by the manufacturer	3
7.1 General.....	3
7.2 Packaging.....	4
7.3 Marking and instructions for use.....	4
Annex A (informative) Test method for the measurement of hydrogen peroxide concentration	5
Annex B (informative) Light microscopy method for measuring erosion of enamel and dentine caused by external tooth bleaching products	6
Annex C (informative) Test method for laboratory assessment of tooth bleaching efficacy	19
Bibliography	22

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 106, *Dentistry*, Subcommittee SC 7, *Oral care products*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 55, *Dentistry*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 28399:2011), which has been technically revised. The main changes compared to the previous edition are as follows:

- a light microscopy method is recommended as an example for the measurement of erosion of enamel and dentine caused by external tooth bleaching products;
- a visual assessment with shade guide has been revised.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

External tooth bleaching products are used in dentistry for changing the colour of natural teeth towards a lighter or whiter shade. They are applied in the oral cavity directly on the outer surfaces of teeth. This document includes requirements and test methods for products intended for external bleaching of natural teeth by chemical means.

Specific qualitative and quantitative requirements for freedom from biological hazard are not included in this document. It is recommended, that reference should be made to ISO 10993-1 and ISO 7405 when assessing possible biological or toxicological hazards.

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Dentistry — External tooth bleaching products

1 Scope

This document specifies requirements and test methods for external tooth bleaching products. These products are intended for use in the oral cavity, either by professional application (in-office tooth bleaching products) or consumer application (professional or non-professional home use of tooth bleaching products), or both. It also specifies requirements for their packaging, labelling and manufacturer's instructions for use.

This document is not applicable to tooth bleaching products:

- specified in ISO 11609;
- intended to change colour perception of natural teeth by mechanical methods (e.g. stain removal) or using restorative approaches, such as veneers or crowns;
- auxiliary or supplementary materials (e.g. tray materials) and instruments or devices (e.g. lights) that are used in conjunction with the bleaching products.

This document does not specify biological safety aspects of tooth bleaching products.

NOTE Maximum concentration of a bleaching agent for professional or non-professional use is subject to each country's regulatory body.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 1042, *Laboratory glassware — One-mark volumetric flasks*

ISO 1942, *Dentistry — Vocabulary*

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 6344-1, *Coated abrasives — Grain size analysis — Part 1: Grain size distribution test*

ISO 7405, *Dentistry — Evaluation of biocompatibility of medical devices used in dentistry*

ISO 8601-1, *Date and time — Representations for information interchange — Part 1: Basic rules*

ISO 8601-2, *Date and time — Representations for information interchange — Part 2: Extensions*

ISO 10993-1, *Biological evaluation of medical devices — Part 1: Evaluation and testing within a risk management process*

ISO 11664-1:2007, *Colorimetry — Part 1: CIE standard colorimetric observers*

ISO 11664-2:2007, *Colorimetry — Part 2: CIE standard illuminants*

ANSI/ADA Specification No. 41: *Recommended Standard Practices for Biological Evaluation of Dental Materials*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 1942 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1 bleaching

<natural teeth> removal of intrinsic or acquired discolorations of natural teeth, or changing their colour towards a lighter or whiter shade using chemicals, sometimes in combination with the application of auxiliary means

Note 1 to entry: Changing the colour of natural teeth towards a lighter or whiter shade is not limited to a discoloration.

Note 2 to entry: Auxiliary means for bleaching other than the application of external energy are also conceivable.

[SOURCE: ISO 1942:2009, 2.28, modified — Note 1 and Note 2 to entry have been added.]

3.2 professional home use

<of a product> use prescribed by a professional and for use at home under the repeated supervision of the dentist

3.3 erosion

<of tooth surface> progressive loss of calcified tissue by chemical processes that do not involve bacterial action

[SOURCE: ISO 1942:2009, 2.292, modified — terms have been modified and a domain has been added.]

4 Classification

4.1 General

External tooth bleaching products can be classified for either:

- a) professional application; or
- b) consumer application.

NOTE External tooth bleaching products can be used alone or in conjunction with auxiliary means of application.

4.2 Products for professional application

These products are tooth bleaching products that are intended by the manufacturer to be applied only by dental professionals (in office tooth bleaching products).

4.3 Products for consumer application

These products are tooth bleaching products that are intended by the manufacturer to be applied by the consumer (for professional home use or for non-professional home use).

NOTE Such external bleaching products can be prescribed by a dental professional or directly available to consumers.

5 Requirements

5.1 Concentration of active ingredients for bleaching

The concentration of active ingredients for bleaching (equivalent to hydrogen peroxide) delivered by the unexpired product according to manufacturer's instructions for use shall be within the range of +10 % and -30 % of the original concentration stated by the manufacturer for the unopened product. [Annex A](#) or other equivalent method can be used for testing.

5.2 Surface microhardness

The reduction in the Knoop hardness (KHN) or Vickers hardness (VHN) after bleaching shall not exceed 10 %, when tested in accordance with [6.3](#).

5.3 Surface erosion

Surface erosion of the teeth tested shall be less than the level which is caused by the standard reference solution. The method described in [Annex B](#) or other equivalent methods can be used.

6 Measurements and test methods

6.1 Preparation of tooth specimens

Prepare enamel and dentine specimens taken from a consistent location on extracted human or bovine teeth, that have been stored in a neutralized solution that disinfects but does not alter the physical properties. Grind the specimen surface under a constant flow of water in accordance with ISO 3696 starting at P400 and sequentially to a minimum of P1200 silicon carbide paper in accordance with ISO 6344-1. Then polish the surface using a slurry or paste of 0,3 µm mean particle size aluminium oxide. Ensure a minimum of 1 mm thickness of enamel or dentine tissue for the test specimen. Prevent dehydration of test specimens during the preparation procedure.

6.2 Preparation and application of tooth bleaching product

The dispensing, processing and application of the tooth bleaching product used in tests (see [Annex C](#)) shall follow the manufacturer's instructions for use. The method of bleach application shall simulate the clinical procedure in quantity, frequency and duration of the application. Between bleaching intervals, and for 24 h after the last bleach application prior to testing, specimens shall be stored at 37 °C in an artificial saliva solution similar to that described in the ANSI/ADA Specification No. 41^[4].

6.3 Surface microhardness

Evaluate enamel surface microhardness before and after bleaching treatment.

Determine KHN or VHN surface microhardness by applying a load of 0,49 N (equivalent to a 50 g load) for 15 s. Evaluate a minimum of 10 specimens for each group, with three indentations for each specimen. Prevent dehydration of test specimens during the specimen preparation procedure.

7 Packaging, marking and information to be supplied by the manufacturer

7.1 General

Additional information may be included at the discretion of the manufacturer or as required by regulation in accordance with ISO 22727.

7.2 Packaging

The components of the material shall be supplied in properly sealed containers which adequately protect the contents and do not adversely affect the product quality.

7.3 Marking and instructions for use

For each package, the following applies.

- a) Information shall be clearly marked on the outermost package or containers appropriate to the product, as indicated in [Table 1](#).
- b) Instructions shall accompany each package of the product and shall include the information appropriate to the product, as indicated in [Table 1](#).

Table 1 — Requirements for marking and instructions for use

No.	Information	Outermost package	Container	Manufacturer's instructions for use
1	Name of the product	M	M	M
2	Identification or name of the manufacturer	M	M	M
3	Address of the manufacturer or the agent responsible for sale	M	—	M
4	Recommended conditions of storage	M	—	M
5	Manufacturer's lot number	M	M	—
6	Expiry date given in accordance with ISO 8601-1 or ISO 8601-2	M	M	—
7	Classification of the external tooth bleaching products (Clause 4)	M	—	M
8	Clinical application of the external tooth bleaching products (Clause 4)	—	—	M
9	Number of containers	M	—	—
10	Net mass of product in each container	M	M	—
11	Chemical name of active ingredient(s)	M	—	M
12	Concentration of active ingredient(s)	M	M	M
13	Concentration equivalent to hydrogen peroxide	M	M	M
14	Instructions for use	—	—	M
15	Recommended auxiliary device(s), exposure times and any special instructions for use of the equipment (for the materials requiring an auxiliary device only)	—	—	M
16	Specific contra-indication(s) and/or warning(s), such as 'irritation', 'avoid contact with eyes', as necessary	—	—	M
17	Statement equivalent to 'It is recommended that you consult with your dental professional before using this product.'	—	—	M
18	Date of issue or latest revision, if applicable	—	—	M
Key M: mandatory information —: non-mandatory information				

For single use containers, instructions may be labelled on the secondary packaging. The minimum information on single use containers required are: manufacturer name, trade name, lot number, and expiry date.

Annex A (informative)

Test method for the measurement of hydrogen peroxide concentration

A.1 Principle

The content of hydrogen peroxide (H₂O₂) in tooth bleaching products is determined using a modified thiosulfate titration method.

A.2 Test condition

Perform the test at (23 ± 2) °C.

A.3 Procedure (modified thiosulfate titration method, USP^[5])

Equivalent methods can also be used.

Use analytic grade sulfuric acid, potassium iodide, ammonium molybdate, sodium thiosulfate, starch and hydrogen peroxide. Conduct a titration calibration curve using a series of freshly prepared H₂O₂ solutions at concentrations that include the highest possible H₂O₂ concentration in the test product. Add approximately 1,0 g (weighing precision to 0,001 g) of test product or an amount appropriate to the test, with rapid stirring, to 400 ml distilled water that contains 10 ml of sulfuric acid (25 %), 25 ml potassium iodide (10 %), and 4 drops of ammonium molybdate solution (5 %). Use starch as the indicator, and perform the titration using 0,1 N (normality) sodium thiosulfate.

Determine the H₂O₂ content using the titration calibration curve.

When using standardized titrants (e.g. USP standard grade), construction of a calibration curve is not necessary. Calculate the mass concentration of H₂O₂ using [Formula \(A.1\)](#):

$$C = (1,701\ 18 \times V/m) \times Y/0,1 \times 100 \quad (\text{A.1})$$

where

C is the mass concentration of H₂O₂, expressed as a percentage;

V is the titre of 0,1 N sodium thiosulfate, expressed in millilitres;

m is the mass of the test product dispensed, expressed in milligrams;

Y is the accurate concentration of sodium thiosulfate, expressed in mol/litre.

Repeat the measurement five times ($n = 5$) and calculate the mean H₂O₂ concentration.

NOTE Formation of iodine can be indicative of the presence of sodium hypochlorite, which is relevant when the method is used for testing an unknown active ingredient.

Annex B (informative)

Light microscopy method for measuring erosion of enamel and dentine caused by external tooth bleaching products

B.1 Principle

The depth of erosion of enamel and dentine caused by external tooth bleaching products is determined using a light microscope.

Test summary: sound enamel and dentine slabs of approximately $4 \times 4 \times 1,4$ mm are cut from caries and erosion free molars or incisors of human or bovine tooth. These slabs are mounted on a plastic rod and the natural surface of the slab is removed by sanding to expose fresh enamel or dentine. The surface is protected with fingernail polish leaving a 1,0 mm unprotected stripe down the centre of the fresh enamel or dentine surfaces. The specimens are exposed to the potentially erosive solution/gel following manufacturer's instructions for use or for standard solutions, 1 h while stirring. The specimens are rinsed with distilled water (D-H₂O), cleansed in mild detergent solution, rinsed with ethanol, and rinsed again with D-H₂O and visually inspected to assure that all product residues have been removed. The specimens are then sliced perpendicular to the exposed stripe to yield 3 or 4 cross-section slices from each specimen slab. The cross-section slices are mounted on a glass slide, and a digital image is obtained under microscope. A digital image of a reference scale such as a TEM grid or mm ruler is also obtained at the same magnification. The reference scale and the cross-section can be in the same digital image. The digital image for each cross-section is evaluated for surface loss using software capable of assessing gray scale values of pixels¹⁾. The surface loss for each cross-section from a specimen slab is averaged to give the erosion loss for each specimen.

B.2 Test Condition

Perform the test at (23 ± 2) °C, i.e. ambient temperature.

B.3 Apparatus and materials

B.3.1 Apparatus

B.3.1.1 Stereo microscope with digital camera, see [Figure B.1 b](#)).

B.3.1.2 Slow speed saw, see [Figure B.1 a](#)).

B.3.1.3 Sandpaper, 600 carbide grit (1 200 grit EU standards).

B.3.1.4 Disposable beakers, 50 ml.

B.3.1.5 Glass beakers, 100 ml.

¹⁾ ImageJ software methods are described in B.10 as an example. ImageJ software is freely available and in the public domain and downloadable from <https://imagej.nih.gov/ij/>. ImageJ bundled with Java 1.8.0_112 (64-bit Java) is available for Windows system and ImageJ bundled with Java 1.8.0_172 is available for Mac OS X and follow the installation instructions. This information is given for the convenience of users of this document and does not constitute endorsement by ISO of this software.

B.3.1.6 Volumetric flask, 100 ml, in accordance with ISO 1042, Class A.

B.3.1.7 Magnetic stir plate and magnetic stir bars.

B.3.1.8 Plastic rods (poly methyl methacrylate), 6 mm diameter × 15 cm length.

B.3.1.9 Grid, of known dimensions or mm scale to 0,5 mm.

B.3.1.10 pH/mV electrometer (pH meter), with a sensitivity of + 0,1 mV.

B.3.2 Materials

B.3.2.1 Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$).

B.3.2.2 Sodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$).

B.3.2.3 Distilled water, in accordance with ISO 3696, Grade 2.

B.3.2.4 Potassium hydroxide (KOH) at 0,1 mol/l.

B.3.2.5 Hydrochloric acid (HCl) at 0,1 mol/l.

B.3.2.6 Ethanol.

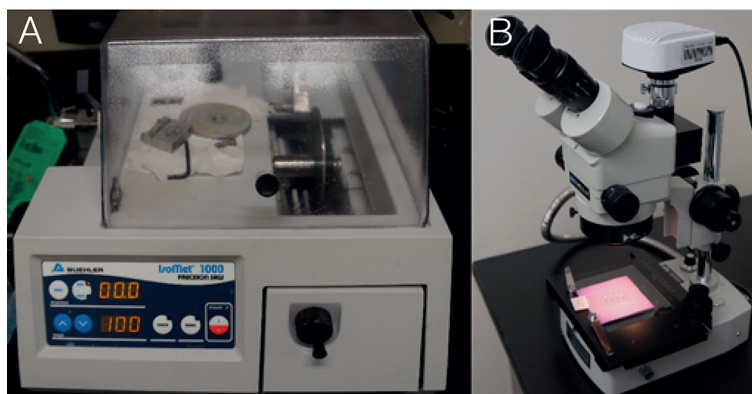
B.3.2.7 Teeth (human or bovine; erupted only or unerupted only, if known) with caries free and erosion free surfaces, sufficient amounts of teeth for collecting six ($4 \times 4 \times 1,4$) mm specimens for each group, disinfected in storage solution.

B.3.2.8 Fingernail polish, pigmented.

B.3.2.9 Fingernail polish, clear.

B.3.2.10 Cyanoacrylate glue.

B.3.2.11 Detergent solution, e.g. 1 % sodium lauryl sulfate solution.



Key

- A slow speed saw
B stereo microscope with digital camera

NOTE For A), 1 000, Buehler or other equivalent slow speed saws can be used. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Figure B.1 — Apparatus for preparing and measuring specimen

B.4 Standard reference erosion controls

B.4.1 Negative control

Grade 2 distilled water ([B.3.2.3](#)) as the negative control.

B.4.2 Positive control and standard reference solution

B.4.2.1 Prepare buffer solutions in a 100 ml glass beaker ([B.3.1.5](#)) or other suitable container.

B.4.2.2 Weigh powdered citric acid monohydrate ([B.3.2.1](#)) and sodium citrate dihydrate ([B.3.2.2](#)) in separate weighing dishes; combine crystals in a 100 ml volumetric flask ([B.3.1.6](#)) and add distilled water ([B.3.2.3](#)) until the meniscus nears the graduation line. See [Table B.1](#).

B.4.2.3 Determine the pH of these solutions using a suitably calibrated pH/mV electrometer (pH meter) ([B.3.1.10](#)) and meter while agitating using a magnetic stirrer ([B.3.1.7](#)).

B.4.2.4 If the pH is more than $\pm 0,05$ units away from the expected pH then adjust the pH accordingly with 0,1 mol/l potassium hydroxide ([B.3.2.4](#)) or 0,1 mol/l hydrochloric acid ([B.3.2.5](#)) to the expected value.

B.4.2.5 Add distilled water ([B.3.2.3](#)) to make up to the final volume of 100 ml.

Table B.1 — Positive controls and standard reference solution

Solution	% Citric acid mass fraction %	Sodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) Mass (g)	Citric acid monohydrate ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) Mass (g)	Expected pH
Positive control	1,00	0,451	0,766	3,60

Table B.1 (continued)

Solution	% Citric acid mass fraction %	Sodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) Mass (g)	Citric acid monohydrate ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) Mass (g)	Expected pH
Standard reference solution	0,25	0,114	0,193	3,68

B.5 Specimen preparation

B.5.1 General

Samples of enamel or dentine of 4 mm × 4 mm × 1,4 mm dimension (see [Figure B.2](#)) are recommended for this procedure. Sound enamel specimen may be obtained from caries and erosion free molars or incisors of human or bovine tooth. Dentine specimens may be obtained from incisor, premolar or molar roots (just below the dentino-enamel junction). Sample preparation details are presented below organized by the specimen source.

B.5.2 Cutting an enamel slab from molar teeth (see [Figure B.2](#))

B.5.2.1 Mount tooth ([B.3.2.7](#)) in a clamp such that the tooth is horizontal in the diamond saw (see [Figure B.2 A](#))

B.5.2.2 Using water cooled diamond wafering blade remove the cusps of the tooth.

B.5.2.3 Set the saw micrometre to zero.

B.5.2.4 Advance the holder toward the dentino-enamel junction 4,1 mm and cut the top of the tooth off of the root (see [Figure B.3 E](#)).

B.5.2.5 Retrieve the 4 mm section of tooth and mount in the clamp such that a smooth surface of caries free enamel can be sliced off the tooth (see [Figure B.2 B](#)).

B.5.2.6 Align the sample such that the outer edge of the tooth just touches the wafering blade, set the micrometre to zero.

B.5.2.7 Advance the sample 1,4 mm.

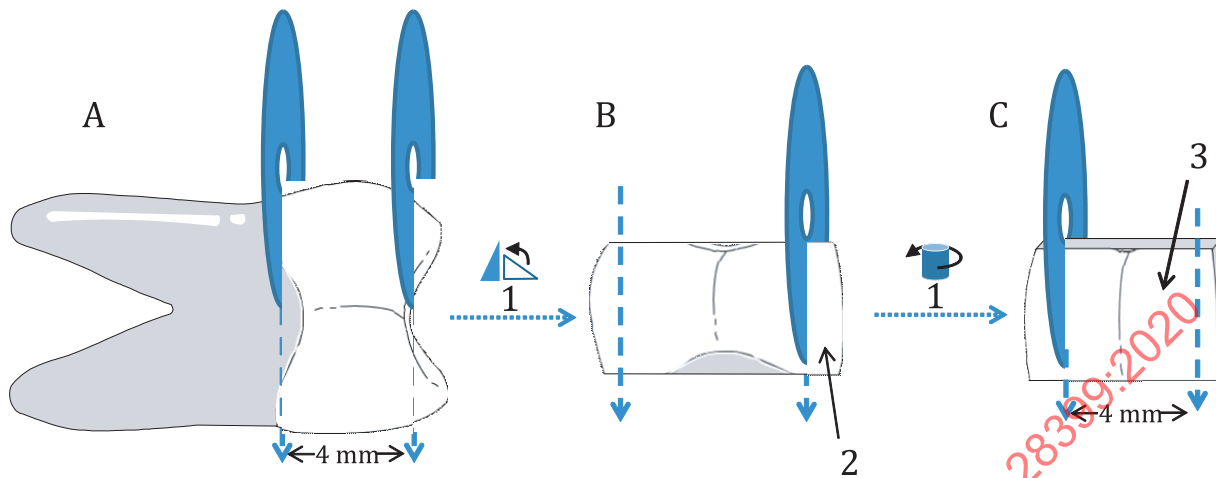
B.5.2.8 Cut the enamel away from the tooth, preserving the removed enamel slice.

B.5.2.9 Carefully mount the enamel slice in the clamp (avoiding breaking the sample).

B.5.2.10 Align the sample to remove the tapered edge of the slice and remove the taper (see [Figure B.2 C](#)). Reset the micrometre to zero.

B.5.2.11 Advance the micrometre 4 mm and cut the enamel slab off of the slice.

B.5.2.12 Retrieve the 4 mm × 4 mm × 1,4 mm slab and with a pencil place an 'x' mark on the inner cut side of the slab.



Key

- 1 rotate
- 2 1,4 mm thick enamel slabs
- 3 4 mm × 4 mm × 1,4 mm thick enamel slabs
- A alignment of tooth to diamond saw
- B slicing off enamel
- C retrieval of enamel slab

Figure B.2 — Molar enamel slab preparation

B.5.3 Cutting an enamel slab from incisor teeth (see [Figure B.3](#))

B.5.3.1 Mount the tooth in a clamp such that the tooth is perpendicular in the diamond saw (see [Figure B.3 A](#))

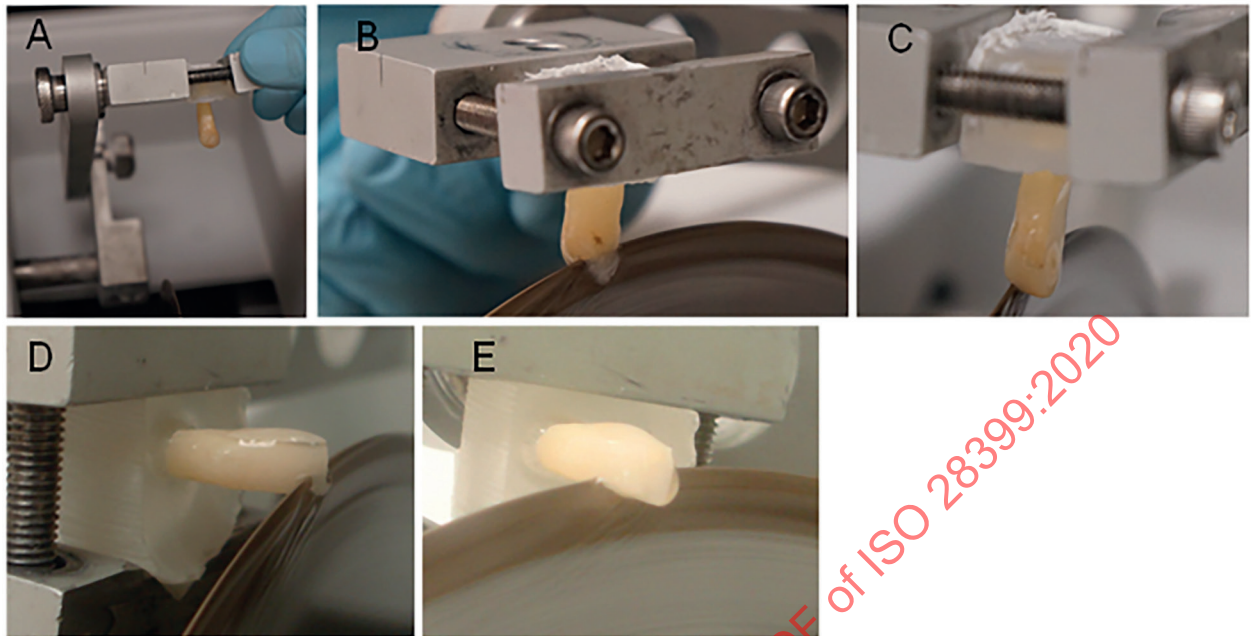
B.5.3.2 Align the saw blade 2,1 mm to the right of the centre of the incisor and using water cooled diamond wafering blade make a cut through the enamel into the root (see [Figure B.3 B](#)).

B.5.3.3 Set the saw micrometre to zero, advance the saw blade 4,1 mm to the left of the zero position and using the water-cooled diamond wafering blade make a cut through the enamel into the root ([Figure B.3 C](#)).

B.5.3.4 Rotate the incisor such that the tooth is horizontal to the diamond saw blade and remove the incisal edge of the tooth (see [Figure B.3 D](#)). Set the micrometre to zero.

B.5.3.5 Advance the holder latterly 4,1 mm and cut the enamel off of the root (see [Figure B.3 E](#)).

B.5.3.6 Retrieve the 4 mm × 4 mm slab and with a pencil place an 'x' mark on the inner side of the slab.



Key

- A mounting the tooth
- B aligning the saw
- C cutting through the enamel
- D cutting of incisal edge
- E removal of coronal portion at CEJ

Figure B.3 — Incisor enamel specimen preparation

B.5.4 Cutting the dentine slab from incisor, premolar teeth or molar teeth

B.5.4.1 Mount the tooth in a clamp such that the tooth is perpendicular in the diamond saw (see [Figure B.3 A](#)).

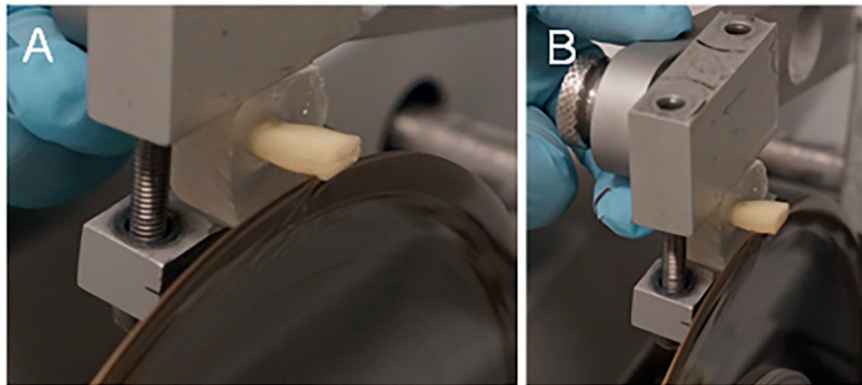
B.5.4.2 Align the saw blade 2,1 mm to the right of the centre of the tooth. Using water cooled diamond wafering blade make a cut through the enamel into the root (see [Figure B.3 B](#)).

B.5.4.3 Set the saw micrometre to zero, advance the saw blade 4,1 mm to the left of the zero position and using the water-cooled diamond wafering blade make a cut through the enamel into the root (see [Figure B.3 C](#)).

B.5.4.4 Rotate the tooth such that it is horizontal to the diamond saw blade and remove the coronal portion the tooth at the dentino-enamel junction (see [Figure B.4 A](#)). Set the micrometre to zero.

B.5.4.5 Advance the holder latterly 4 mm and cut through the root to obtain a 4 × 4 mm section (see [Figure B.4 B](#)).

NOTE The root portion of incisor teeth that were sectioned for enamel specimens ([B.5.3.5](#)) will be used for dentine specimens.



Key

- A rotation of tooth
- B cutting through the root

Figure B.4 — Dentine specimen preparation

B.5.5 Mounting the specimens and exposing fresh enamel or dentine surfaces

B.5.5.1 Attach the enamel/dentine specimen to the end of a plastic rod (B.3.1.8) with a small amount of cyanoacrylate glue (B.3.2.10) such that the natural surface is facing up.

B.5.5.2 Completely cover the sample with fingernail polish, pigmented (B.3.2.8) (see Figure B.5 A). Place the sample in cold water to set the fingernail polish (or let it air dry for several hours).

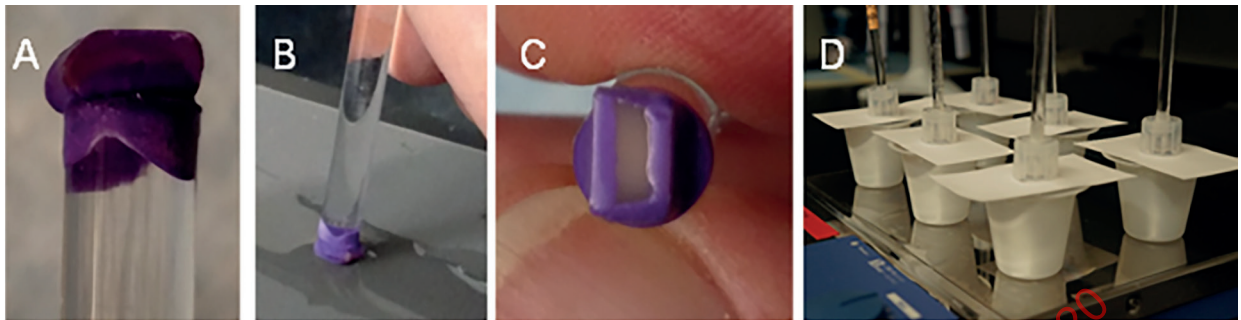
B.5.5.3 Hold the outside surface directly against wet sandpaper (B.3.1.3) and sand to remove the fingernail polish and expose a fresh enamel/dentine surface (see Figure B.5 B). It is important to make this exposed surface flat.

B.5.5.4 Blot dry the exposed surface with a paper towel and carefully paint the freshly exposed enamel/dentine surface with fingernail polish leaving a straight 1 mm ($\pm$ 0,2 mm) stripe unprotected across the slab (see Figure B.5 C).

B.5.5.5 Place the sample in cold water to set the fingernail polish (or let it air dry for several hours).

B.5.5.6 Place the sample in distilled water overnight to fully hydrate.

B.5.5.7 Allocate six enamel and six dentine specimens to each test product and the negative and positive control and standard reference solution.



Key

- A covering the sample with fingernail polish
- B sanding the outside surface of sample
- C straight 1 mm stripe
- D hydration in distilled water

Figure B.5 — Specimen mounting, polishing, preparation and erosive challenge

B.6 Erosion of enamel and dentine

B.6.1 Application of bleaching products

Apply the bleaching product to the exposed area of the specimens according to the manufacturer's instructions for use in terms of preparation of the formulation, application time and number of applications.

B.6.2 Controls and standard reference solution treatments

B.6.2.1 Positive control and standard reference solution treatments

Suspend the sample in 25 ml positive control (1,0 % citric acid), described in [B.4.2](#), with magnetic stirring for 4 h (see [Figure B.5 D](#)).

Suspend the sample in 25 ml standard reference solution (0,25 % citric acid), described in [B.4.2](#), with magnetic stirring for 4 h (see [Figure B.5 D](#)).

B.6.2.2 Negative control treatments

Treat the specimens in distilled water ([B.3.2.3](#)) with magnetic stirring for 60 min.

B.7 Specimen cleaning after the erosion challenge is completed

B.7.1 Cleansing steps

Clean the specimens with the following sequential rinse: a 15 s spray of water, a 10 min soak in warm detergent solution ([B.3.2.11](#)), a 15 s spray of water, a 15 s spray of ethanol, finishing with a 15 s spray of water to remove any residue. Inspect the specimen under a stereo microscope to assure that all product residue has been removed. Repeat cleansing steps if necessary.

B.7.2 Coating the exposed surface

Blot the exposed surface dry with a paper towel and coat the exposed surface with fingernail polish, clear ([B.3.2.9](#)).

NOTE This protects the surface from chipping while making cross-sections.

Dip the sample in ice cold water for a few minutes to set the fingernail polish (or let it air dry for several hours).

B.8 Cutting specimen cross sections

B.8.1 Mounting the specimen in the clamp

Mount the specimen, while still on the plastic rod ([B.3.1.8](#)), in the clamp such that the exposed enamel is perpendicular to the wafering blade (see [Figure B.6 A](#)).

B.8.2 Positioning the specimen

Position the sample over the wafering blade 1,3 mm from the outer edge of the slab.

B.8.3 Cutting the specimen

Slowly (gently) cut through the enamel slab just into the plastic rod. Carefully avoid chipping or damaging the sample while cutting.

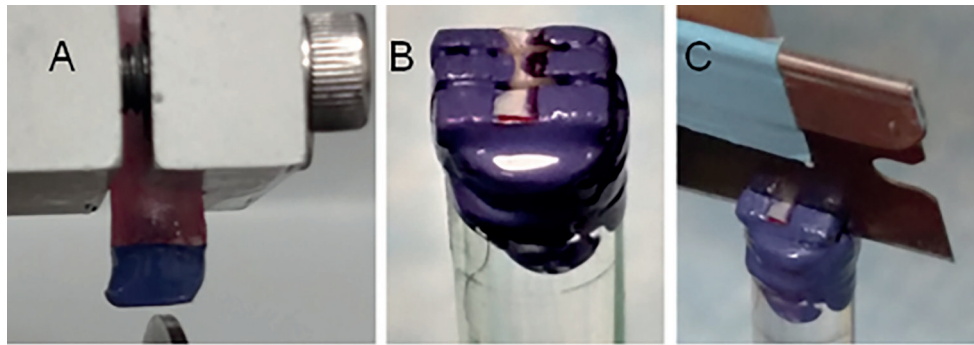
Advance the sample 1,3 mm and cut again. Repeat these steps until 3 or 4 sections have been cut (see [Figure B.6 B](#)).

B.8.4 Removing the sections

With a razor blade (safety blade) remove the sections from the plastic rod (see [Figure B.6 C](#)). Alternatively, the samples may be separated from the plastic rod by rotating the rod such that the diamond wafering saw is aligned with the bottom of the sample and the sample sections can be cut from the plastic holder.

B.8.5 Attaching the sections on the glass slide

Position all sections from a specimen on a glass slide and attach with a small amount of cyanoacrylate glue ([B.3.2.10](#)) on the back side of each section. There should be 3 or 4 cross-sections for each specimen. Keep the slide with the sample sections in a humid container until digital photographs are taken.



Key

- A mounting of specimen
- B cutting of 3 or 4 sections
- C removal of sections from rod

Figure B.6 — Preparation of cross-sections from specimen

B.9 Microphotography measurement method

B.9.1 Procedure

Place the slide with enamel cross-sections on a dark background under the stereo microscope and adjust the position and magnification of the sample such that the eroded area and the protected edges are visible (see [Figure B.7 A](#)).

B.9.1.1 Use incident lighting (light ring) and minimize shadows.

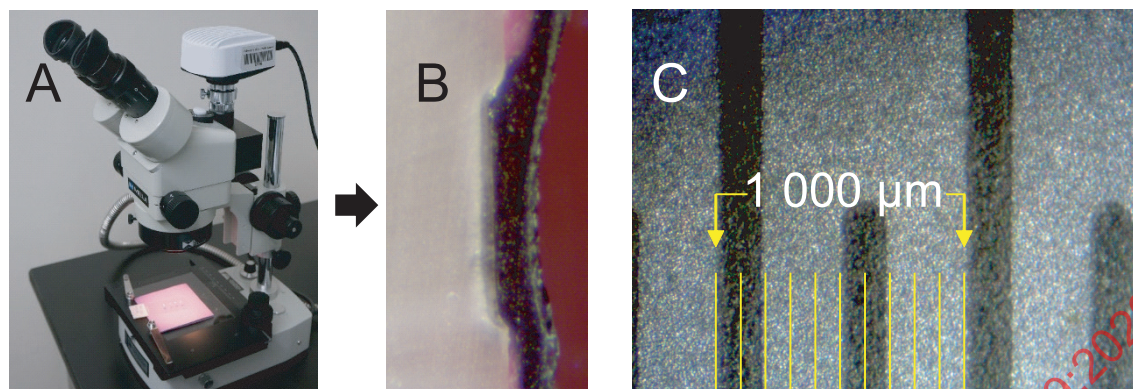
B.9.1.2 Focus on the top edge of the cross-section.

B.9.1.3 Capture a digital image of the cross-section with the greatest resolution possible (see [Figure B.7 B](#)).

B.9.1.4 Capture an image for each of the cross-sections at the same magnification.

B.9.1.5 Record the magnification, TIFF images are preferred.

B.9.1.6 Capture a digital image of the TEM grid or the mm ruler at the same magnification (see [Figure B.7 C](#)).



Key

- A stereo microscope
- B digital image of cross-section
- C digital image of mm ruler

Figure B.7 — Sample imaging

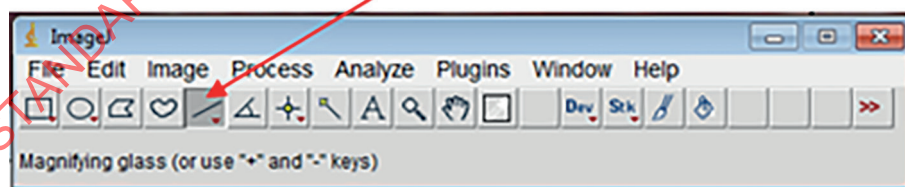
B.10 Evaluation using digital imaging software

B.10.1 Set measurement scale

B.10.1.1 Open and load the image of the scale (mm ruler or TEM grid) that is at the same magnification as the samples.

B.10.1.2 Draw a line across the increments of known distance (i.e. tips of the mm marks on the ruler image). Start on the left side of the first mark and end on the left side of the last mark for greatest accuracy. See [Figure B.8](#) for example.

NOTE The line is usually set across a ruler scale of 1 mm (1 000 μm).



NOTE The red arrow in the figure indicates the button of 'straight line segment' command to draw a line.

Figure B.8 — Example of the 'straight line segment' command button in Main ImageJ window

B.10.1.3 Input the known distance for the length of the line you drew. Set the parameter of unit of measurement (or length) as μm .

EXAMPLE When ImageJ software is used, click 'Analyze' and select 'Set Scale...' to open the dialog box for 'Set Scale'. Input the numerical values of the known distance and unit of length in the dialog box and check 'Global', then click 'OK'.

NOTE The upper left corner of the image will show the dimensions in mm.

B.10.2 Sample measurement

B.10.2.1 Open and load the image to be used.

B.10.2.2 Retain the calibration for the images.

B.10.2.3 Rotate the image to vertical, if the image of the erosion is not vertical. Input the degrees of the angle expected to position the image (i.e. 90°) to adjust the degrees needed to make the image vertical.

B.10.2.4 Draw a rectangle on the image according to [Figure B.9](#) (it can only be a vertical shape) that spans the erosion zone and is aligned with the protected surface of the tooth on the right and the bottom of the erosion zone on the left and measure the depth of the erosion zone.

B.10.2.5 Record the loss (in μm) for each section.

B.10.2.6 Erosion for the specimen is the average of the erosion loss (in μm) of the cross-sections for each specimen.

NOTE When the dentine specimen is measured, it demands to label the demineralized area correctly apart from the protein loss area (i.e. not eroded) by the reviewer.

EXAMPLE An example of procedures of sample measurement by using ImageJ software is as follows: Click 'File' and choose 'Open...' from the scrolling list. Select the file to be used then click 'Open' to load the image. The first time an image is opened after setting the calibration a dialog box will appear asking about the global calibration. In this case, uncheck the 'Disable Global Calibration', check 'Disable These Messages' and click 'OK' to retain the calibration. Click 'Image' and move the cursor to 'Transform' and select 'Rotate...' to rotate the image to vertical such that the erosion zone is vertical as shown in [Figure B.9 A](#). Enter 'Angle (degrees)' of the dialog box to position the image (i.e. 90) without touching the "enter key". Adjust the degrees needed to make the image vertical then click 'OK'. Click the 'rectangle shape' button in the second line of command bar to draw a rectangle on the image (see yellow box in [Figure B.9 A](#)). Click 'Analyze' then choose 'Plot Profile' to generate the graph of the gray scale of the selected area (see [Figure B.9 B](#)). Click 'List' on the window of the graph. Scroll to the bottom of the list and record the last X-value in the list. In [Figure B.9 C](#), the value is 51,916 3 (51,9 μm). This is the number of microns from left to right of the yellow box and the depth of the erosion.

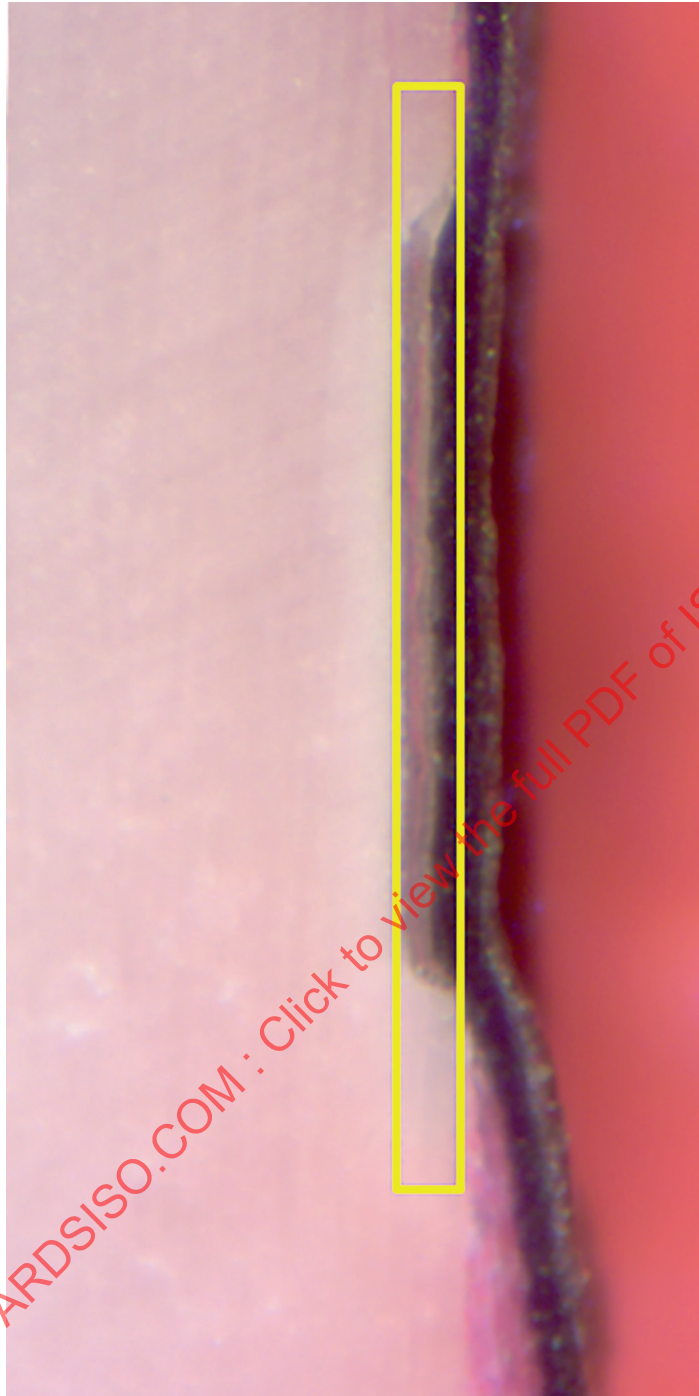


Figure B.9 — Example of usage of the ImageJ software