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TOOTH REPLICATION TECHNIQUES, SEM IMAGING AND MICROWEAR ANALYSIS IN PRIMATES: METHODOLOGICAL OBSTACLES

ABSTRACT: Dental microwear analyses are among the most significant techniques though which a researcher can make dietary and ecological inferences from primate fossil specimens. Hard particles, such as plant phytoliths or silica-base sands, can scratch tooth enamel surfaces during food mastication producing a dietary specific pattern of microwear on the enamel surface. The density, axis length and orientation of microwear features, either striations or pits, are highly informative of dietary habits in both extant and fossil primates. The analysis of tooth enamel surfaces requires the use of scanning electron microscopy (SEM) techniques, because of its high resolution power, including gold-coating of teeth for observation. Problems arise when specimens to be analysed are unique and there is no possibility of a direct observation with an environmental microscope. Negative moulds must then be made and silicone-base components are indicated for high quality replication of enamel surfaces. A positive cast needs to be obtained, and epoxy-base resins are frequently used for their good quality and durability. However, successive silicon and epoxy replications result in the loosing of surface detail and precision. Surface observation errors can also be caused be the SEM technology itself, especially if back-scattered electrons are used instead of secondary electrons for maximizing the topographical information of enamel images. This paper reviews the most commonly used methodological approaches to tooth moulding and casting, comparing SEM micrographs of casts with actual tooth surfaces, and contrasting the reliability of SEM images for dietary interpretation of tooth microwear in both extant and fossil primates.

KEYWORDS: Scanning Electron Microscopy – Epoxy resin – Tooth cast – Polyvinylsiloxane impression – Microwear – Primates

INTRODUCTION

Museums holding osteological and paleontological collections of primate bones and teeth are storing highly valuable, and seldom unique, original samples. Wild-caught primate collections constitute an irreplaceable evidence of primate ecology and adaptations, with significant scientific value for systematics, functional anatomy, and evolutionary studies of primates (Albrecht 1982). The *Primate Specialist Group* of the *Species Survival Commission* of the *World Conservation Union* estimated that half of the world's 250 species of primates were of serious conservation concern, 96 of them nowadays considered to be critically endangered (Chapman, Peres 2001). A great effort in the conservation of these worthy specimens is needed. Sometimes, collections of skulls and skins of wild-caught primates are enormous (Tappen 1969), being these of great interest to researchers since large samples of skeletal materials can be gathered (Almquist 1973). Primate specimens brought-up in captivity are also frequent in museum collections, but are of lesser value because of possible captivity drawbacks or simple lack of information about their provenance (Albrecht 1982). Conservation of fossil hominid specimens is also of great concern, and repeated handling of remains by specialists is among the main causes of their deterioration. Curators tend to prevent this by not providing access to specimens. This is of special concern for teeth collections, since teeth are the most abundant remain in the human osteological record and many researchers focus their investigations on their evolutionary history. Availability of high quality tooth moulds at museums or research units (Galbany *et al.* 2004) would reduce the handling problem without reducing access to such valuable remains.

Tooth casts are the main source for Scanning Electron Microscopy (SEM) research since tooth observation frequently requires sample metallization and chamber vacuum. However, reliability of casts needs to be ascertained in terms of surface and feature measurements precision compared to the original tooth. In addition, SEM technology shows some other limitations in microwear research that need to be considered. In this paper we analyse the accuracy of various tooth moulding techniques in enamel microwear research and consider the difficulties of obtaining good, high quality digitalized SEM images of enamel surfaces for microwear analyses.

MATERIALS AND METHODS

Moulding of teeth

The main reason for using tooth replication techniques in paleontology and anthropology is because the original specimens are too valuable to be studied directly by SEM (Beynon 1987), and the replicas, which are copies of the original teeth obtained with various reproduction procedures, allow in turn to visualize inaccessible areas on the original specimen (Beynon 1987). Silicon-base replication procedures are frequently used to obtain negative casts from the original teeth by applying hydrophobic polyvinylsiloxane silicones. Polyvinylsiloxane *President microSystem™* (*Coltène®*), usually *Regular Body*, impression material is widely used for odontological practice and dental microwear research (Ungar 1996, Ungar, Spencer 1999) because it reproduces features with resolutions to a fraction of a micron (Teaford, Oyen 1989a) and maintains the resolution for many years (Beynon 1987). *Coltène Light Body* resin provides a more faithful cast, but *Regular Body* shows enough resolution to analysis by SEM at high magnification, whereas *Coltène Heavy Body* shows somewhat less resolution. In the present study, SEM images of enamel surfaces were obtained using all three *Light*, *Regular*, and *Heavy Body* impression materials to test their fidelity against the original enamel surface. Prior to the moulding procedure, all tooth enamel surfaces were cleaned with pure acetone, to remove chemical preservatives, and then rinsed with 70% ethanol, by gently rubbing the enamel surface with a cotton ear-cube. For image analysis, teeth with non-preserved enamel or presence of tartar deposits or enamel defects were discarded. The polyvinylsiloxane was applied with a thin tip (provided by the manufacturer) to reduce the chance of bubble forming in contact to the enamel surface of the studied tooth. Right before the negative casts had completely dried, conserving their flexibility, they were pulled-out from the teeth and then kept in labelled plastic bags, away from dust. This first reproduction of the enamel surface was used to obtain a high resolution positive cast of the tooth, dimensionally precise and capable of resolving fine surface details (Beynon 1987).

This two-stage technique is advantageous because the primary putty impression closely adapts to the specimen surface, and the second stage, a low viscosity pseudo-plastic impression material, is subjected to high shear forces or to centrifugation, being forced into inaccessible areas which may not be replicated by a single stage impression technique (Beynon 1987). All the positive moulds were obtained using epoxy resin Epo-Tek #301 (parts A and B are mixed in a 1:4 weight ratio), yielding faithful replicas with excellent detail for scientific research (Rose 1983) or biological specimens (Benevius, Hultenby 1991), despite Teaford and Oyen (1989a) indicate that the resolution of some impressions/epoxy moulds is not as good as that obtained with other techniques. Still, epoxies are generally the easiest materials to use because they offer the best combination of working time, setting time, viscosity, resolution of detail, and dimensional stability, remaining the material of choice for most high-resolution casting purposes, not only for casting primate or hominid teeth (Teaford, Oyen 1989a) but also for other zoological groups. Epoxy resins have been used to mould teeth from different sources: reptiles – Cuban crocodile *Cocodrylus rhombifer* (Maas 1994), carnivores – Viverridae (Taylor, Hannam 1987), marsupials – American opossum *Didelphis marsupialis* (Kay, Covert 1983), koala *Phascolarctos cinereus* (Young, Robson 1987), or Artiodactyls, wild moose *Alces alces* (Young, Marty 1986), or sheep *Ovis aries* (Maas 1994). However, most applications have focused on Primates, such as Strepsirrhini – brown lemur *Lemur fulvus* (Maas 1994), New World monkeys (Teaford, Walker 1984, Teaford 1985, Teaford, Robinson 1989; Teaford, Glanger 1991, Teaford, Runestad 1992), Old World monkeys (Ryan 1979a, Teaford, Oyen 1989b, Hojo 1991, Ungar, Teaford 1996), *Hominoidea* (Gordon 1984, 1992, Flynn Zuccotti *et al.* 1998), fossil primates (Ryan 1979b, Teaford, Walker 1984, Ungar, Grine 1991, 1996, Teaford *et al.* 1996, Ungar, Teaford 1996, Flynn Zuccotti *et al.* 1998, King *et al.* 1999), fossil hominids (Grine 1986, Beymon 1987, Ungar, Grine 1991, Ungar *et al.* 2001) or *Homo sapiens sapiens* (Peters 1982, Benevius, Hultenby 1991, Maas 1994, Ungar, Spencer 1999, Grine *et al.* 2001, Göhring *et al.* 2002, Hojo 2002).

Before pouring the epoxy resin into the casts, the two components (A+B) were thoroughly stirred, in order to mix them, and centrifuged during 1 minute at 3,000 rpm (Orto-Alresa Digicen centrifuge) to eliminate air bubbles from the mixture. Before casting it is usually necessary to build a wall around the mould, particularly if the mould is very flat or irregular, to prevent epoxy from seeping out of

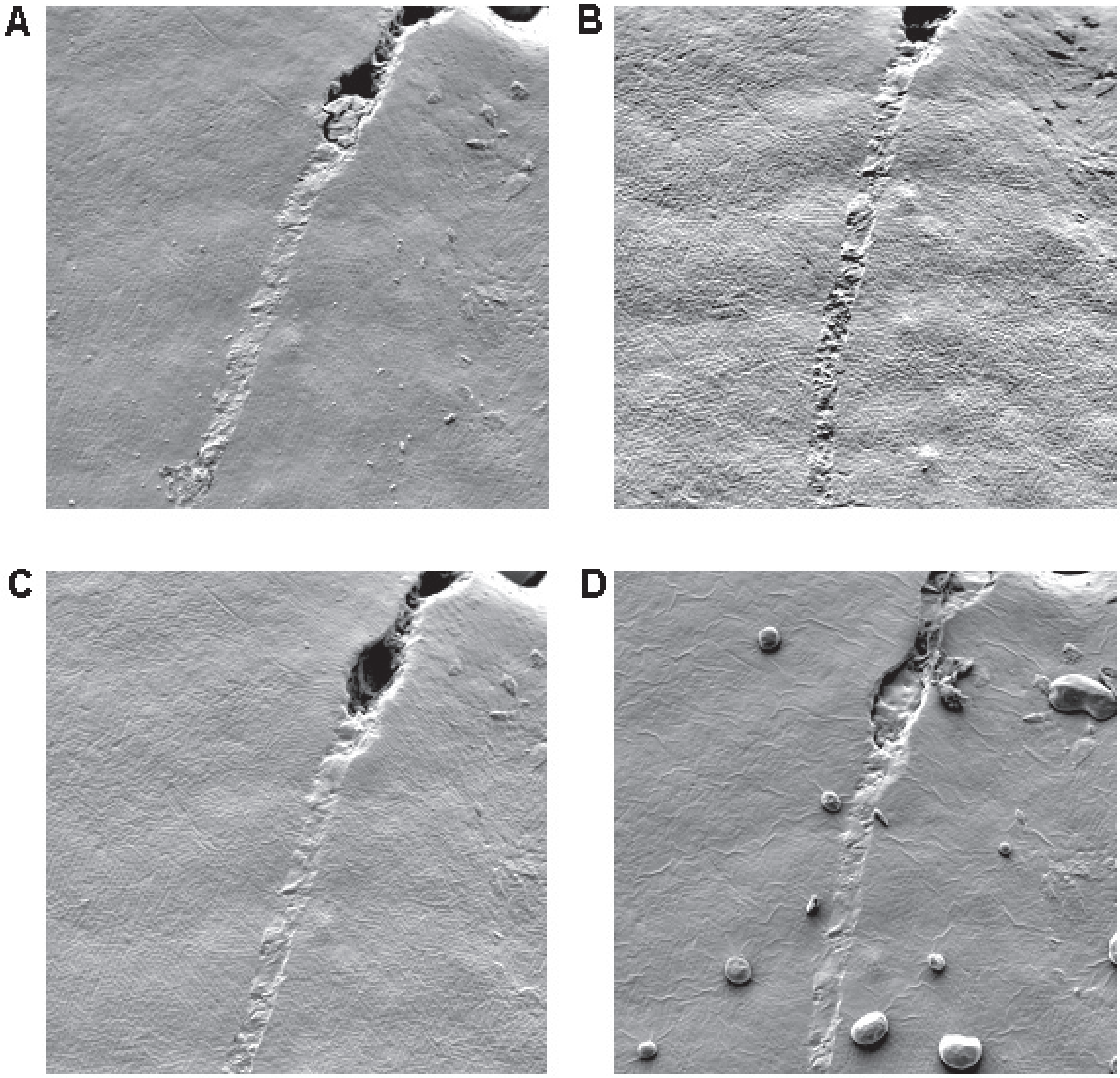

FIGURE 1. Four images of the same enamel surface (lingual surface of the lower left first molar belonging to a modern *Homo sapiens* from La Olmeda, Spain ca XII–XV BC), moulded with different *President microSystem*™ (*Coltène*®) polyvinylsiloxane viscosities. A: original tooth surface, B: *Light Body*, C: *Regular Body* and D: *Heavy Body*. All images are at 100× magnification.

the mould before it sets (Rose 1983). *President microSystem* was used because of its easy and clean application. Immediately after, the resin was poured carefully into the negative casts by using a Pasteur pipette and centrifuged again during 3 minutes at 2,500 rpm (Meditronic Selecta centrifuge), in order to remove any possible air bubbles in contact with the enamel surfaces. The centrifugation reduces the number of bubbles trapped at the mould/cast interface and ensures a good cast (Waters, Savage 1971, Rose 1983). When the epoxy is fully cured, its surface is not tacky to the touch, usually in 6 to 8 hours (Rose 1983), although in our experience epoxy replicas do not completely dry until at least 48 hours. Speeding this step by oven ‘cooking' is not advisable. Finally, the tooth replicas were mounted with term fusible gum in either a brass disc or on aluminum stubs. In any case, a colloidal argent belt (Electrodag 1415M-Acheson Colloiden) solution was applied for electron dispersal in the plastic cast, to prevent accumulation of electrostatic charges during SEM observation (Rose 1983). Finally, the sample was sputtered coated with a 400 Å gold layer to allow observation into the SEM and kept clean, dry and dark, preferably inside a storage case, to maintain them dust-free.

In addition to the extended use of epoxies for enamel microwear or abrasion analyses, other materials have also been used for the same purpose. Epoxy resins provide high quality casts but lack some advantages that other materials have. In our initial studies we used Triafol (Balzers Union BU 008 002-T) dissolved in chloroform, that provided very good quality one-step casts (Lalueza, Pérez-Pérez 1993, Lalueza *et al.* 1993, Pérez-Pérez *et al.* 1994, Lalueza *et al.* 1996), which could be directly observed under SEM. Despite it was a fast and easy method, it could only be applied to small enamel surfaces. In the present study we

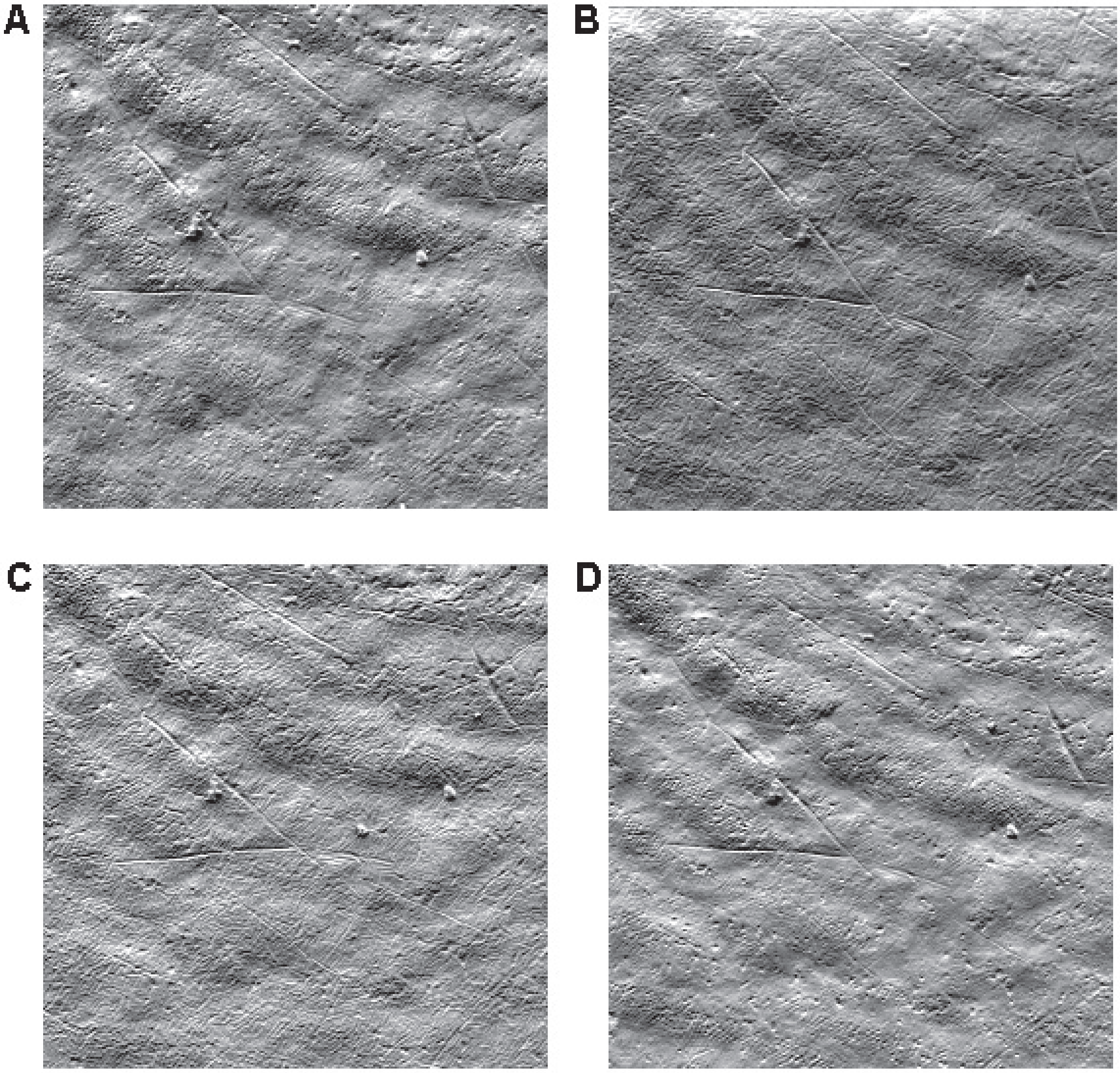

FIGURE 2. Four images of the same enamel surface (buccal surface of lower right second molar belonging to a modern *Homo sapiens* from La Olmeda, Spain ca XII–XV BC), moulded with different *President microSystem*^{TM} (*Coltène*®) polyvinylsiloxane viscosities. A: original tooth surface, B: *Light Body*, C: *Regular Body* and D: *Heavy Body*. All images are at 100x magnification.

have also used Feropur PR-55 (Feroca SL), a fast, bicomponent polyurethane that hardens at room temperature in only five minutes. It has excellent fluidity, it is clean and easy to work with, and only takes some minutes to harden completely. The two components have to be mixed at equal proportions (1:1 in volume) before pouring it inside the negative cast. Centrifugation is recommended for removing air bubbles from the negative mould surfaces. Feropur replicas do not differ in size and form from the original teeth and the cost per cast is significantly lower compared to the epoxy ones.

SEM imaging of enamel surfaces

Before SEM imaging, all teeth were observed at 40× magnification with a VMT Zeys binocular magnifying glass in order to detect well-preserved enamel surfaces on each tooth. Although initially all of the teeth seemed to be in good condition, some of them proved not to be useful because their enamel had extensive microscopic damage. SEM pictures were obtained with two different Scanning Electron Microscopes, a Hitachi 2300 and a Cambridge Stereoscan 120, at the *Serveis Cientifico-Tècnics* (SCT) of the University of Barcelona. In all cases, casts were placed in a horizontal position, with zero degrees of tilt. SEM pictures were taken at 100× magnification on the medial surface of the buccal surface of the tooth, avoiding both the occlusal and cervical thirds of the tooth. This is the standard procedure that we are widely using for microwear analysis on the buccal surface of teeth to infer dietary habits because it covers a significantly broad patch of enamel, where striations of various lengths are the main feature that can be observed (Pérez-Pérez *et al.* 1994). Gordon

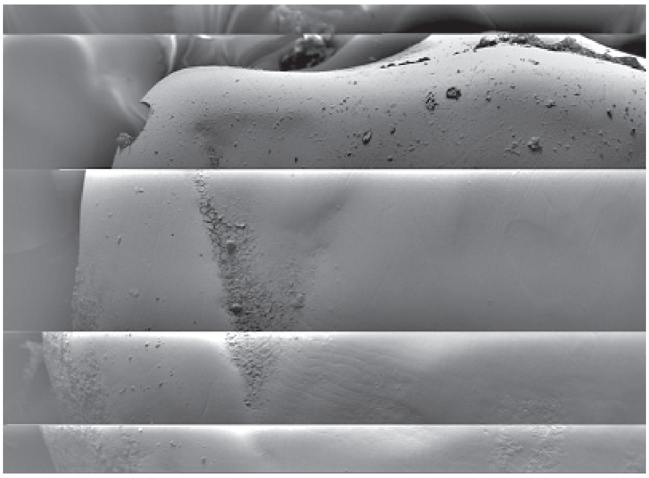

FIGURE 3. SEM image affected by high electrostatic charge caused by an incorrect colloidal argent belt between the stub and the mould, necessary for electron dispersal.

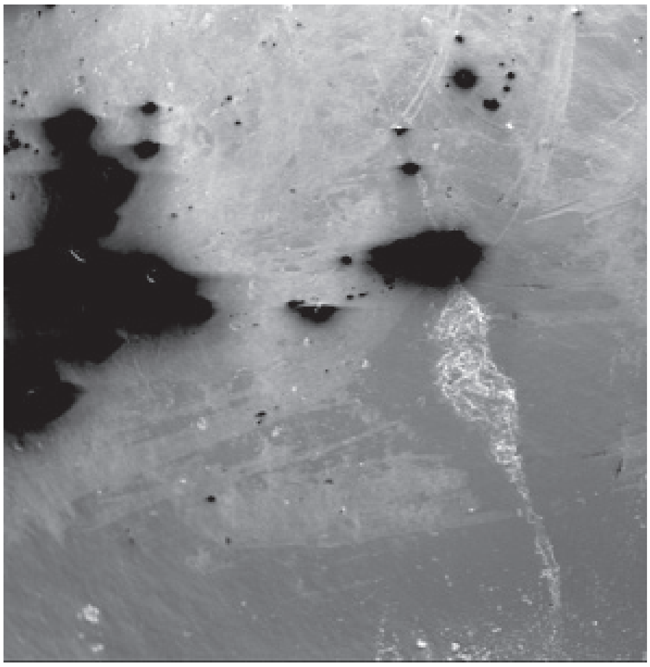

FIGURE 4. The dark areas on the enamel surface are formed by the accumulation of dust on the mould, not allowing for electron dispersal inside the SEM.

(1988) indicated that 120–130× magnification is a good compromise between the covered area and clarity of image. At higher magnifications (i.e. 500×) on the buccal surface of teeth, only a few fragmented fine striations can be distinguished underneath more recent scratches, and the covered area is too small to yield meaningful results (Pérez-Pérez *et al.* 1994). Electron acceleration used was relatively low, 10–15 KV, and working distance (WD) ranged between 15–25 mm, depending on the size of the tooth. SEM pictures were digitalized with SEM Image Slave software, obtaining a 1024×832 pixels image in both the H-2300 and S-120 SEM microscopes used.

Both secondary and back-scattered electrons were used. Back-scattered SEM electrons provide a topographic image in which surface relief is maximized and, therefore, this may be the choice for some morphological studies. Recently, the use of back-scattered electrons has also been advised for SEM microwear analyses on enamel surfaces, since teeth generally show flat surfaces with low relief of microwear features, such as striations, pits, furrows, perikymata, or enamel defects. However, several authors have shown that the use of other than secondary electrons introduces a great methodological error because extinction of linear features occurs when feature orientation parallels that of the SEM electron detectors (Pérez-Pérez *et al.* 2001). An experimental analysis was designed to provide evidence of such feature extinction in two different SEM microscopes (JEOL-840 and Cambridge Stereoscan-120). A metallic disc was gently scratched in a single direction with abrasive glass paper, producing strictly parallel

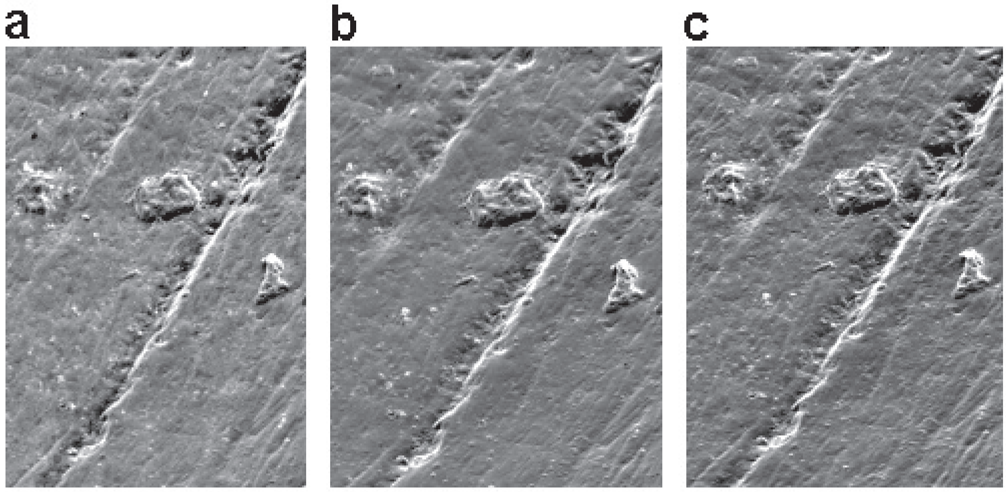

FIGURE 5. Three images of the same enamel surfaces obtained with the original teeth (a), the Epo Tek #301 cast (b), and the Feropur PR-55 cast (c). All images were obtained at 500× magnification.

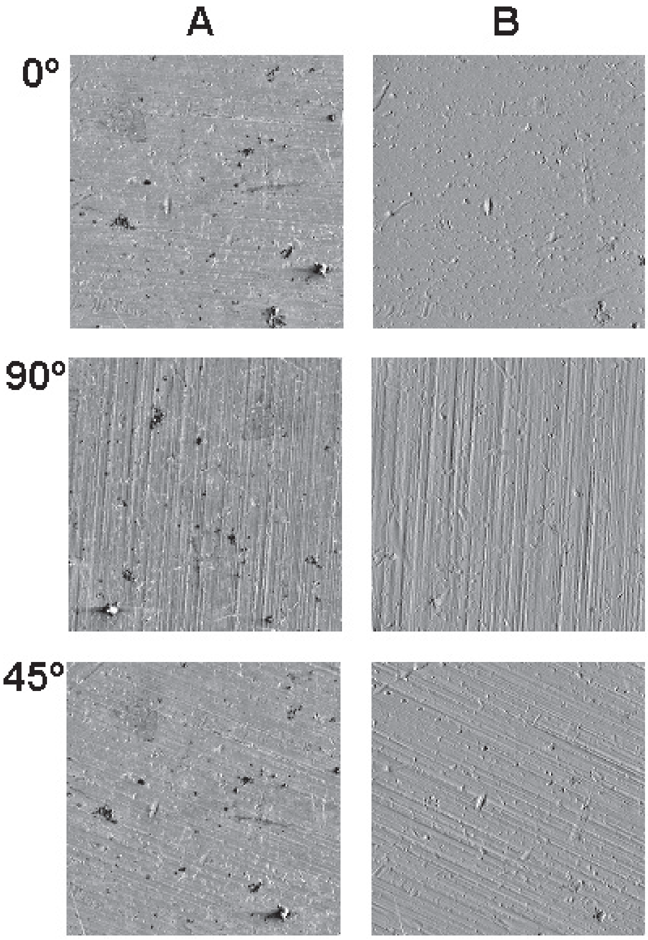

FIGURE 6. Feature extinction experiments made with an artificially parallel-scratched disc. Images were obtained with SEM (JEOL-840) using secondary (A) and back-scattered (B) electrons at different orientation angles (0º, 45º, and 90º) with regard to the electron detector alignment.

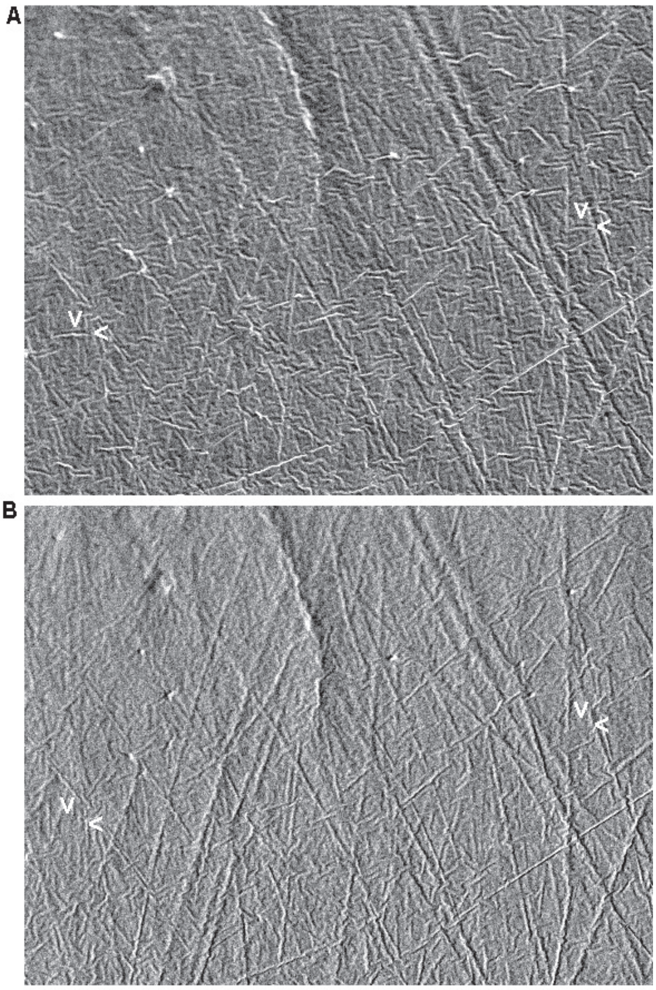

FIGURE 7. SEM images of the same enamel surface obtained with secondary (A) and back-scattered (B) electrons. Some horizontal features are not present in the back-scattered electron image.

striations. The disc was then photographed at 100× magnification at three different angles of orientation (0º, 45º and 90º) with respect to the electron detector plane in the two SEM microscopes, both using secondary and back-scattered electrons. The digitalized images were enhanced with Adobe Photoshop v.5. First of all, the selected area was cropped to include an enamel surface area of 0.56 mm^2. Next, a high-pass filter at 50 pixels was used to remove shade effects in the image and an automatic adjustment of grey levels was applied to increase image contrast. This procedure provides a great contrast for microwear feature measuring. This is usually done with a semi-automatic *software*, usually Microwear 4.0 by P. Ungar or a standard package such as SigmaScan Pro 5.0 by SPSS. The Microwear program automatically discriminates between pits and scratches using a 4:1 length/width ratio. Various length-to-width ratios have been proposed. Teaford and Walker (1984) considered as scratches features above the 10:1 ratio, considering objects with smaller ratio as pits or relatively broad scratches. It is clear that pits and scratches are found at opposite ends of a continuum of surface wear phenomena, and the decision about where to divide the continuum is always arbitrary (Gordon 1988), although pits are generally considered as features having approximately equal length and breath, from 1:1 to 2:1, with no discernible axis of orientation (Gordon 1988).

RESULTS AND DISCUSSION

The comparison of the different polyvinylsiloxane moulds has shown differences in fidelity and replicability of enamel features. SEM images of the original tooth surfaces were always of highest quality (*Figures 1A* and *2A*). Some moulds made with *Light Body* polyvinylsiloxane seemed to more accurately replicate small features, such as enamel prisms (*Figure 1B*). *Regular Body* polyvinylsiloxane proved to be highly reliable, accurately replicating all microwear striations on the enamel surfaces (*Figures 1C* and *2C*). The *Heavy Body* polyvinylsiloxane cast showed some degree of feature obliteration (*Figure 1D*). The samples were cleaned before starting a SEM session in order to prevent dust accumulation or a lack of metallic contact between the mould and the stub (*Figures 3* and *4*). Additional colloidal argent and gold coating was applied whenever necessary and an air duster was always used prior to SEM observation. No differences in surface fidelity were observed between the original tooth surface observed at 500× and the surfaces replicated with epoxy Epo-Tek #301 (*Figure 5B*) or Feropur PR55 (*Figure 5C*), both made from the *Regular Body* negative. The hardening speed and the reduced cost of Feropur make this material a good alternative to Epoxy resins. For this reason, in order to obtain a quality result, negative moulds should always be taken at least with a polyvinylsiloxane material similar to *President microSystem™ regular body* (*Coltène®*) and then positive moulds be made with a high quality resin, such as Epoxy resins or Feropur PR-55.

The sample was properly oriented inside the SEM, standardizing SEM parameters throughout the whole analysis. 100× magnification is suitable for buccal surface analyses, whereas 200× and 500× are frequently applied to occlusal facets. Scanning Electron Microscopes provide a wide set of image composition possibilities. The most common is the use of secondary electrons, although back-scattered electrons can also be used. The experimental analyses showed that total feature extinction is of major concern for the back-scattered electrons images whenever the major orientation axis of the striations and that of the SEM detector were coincident (*Figure 6*). Less extinction appeared in the secondary electron images, though a somewhat reduced image relief was observed. Some degree of feature extinction was also observed in back-scattered mode for some non-experimental enamel tooth surfaces (*Figure 7*). This effect was, however, less significant since tooth enamel shows more randomly oriented striations, and only those scratch segments with parallel orientations to the detector were affected. Therefore, the use of back-scattered electrons should be avoided in SEM enamel tooth microwear analyses where feature density and length by categories of orientations are to be measured for intra- or inter-population comparisons, despite secondary electron images show somewhat less surface relief.

In future research the use of tooth crown moulds collections (Galbany *et al.* 2004) will increasingly be the first choice since original fossil specimens need to be preserved from handling damage. Accuracy of replication and conservation of moulds will also be required. In addition, methodological standardization of SEM research should also be considered.

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