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ORIGINAL ARTICLE

Soft tissue removal by maceration and feeding of *Dermestes* sp.: impact on morphological and biomolecular analyses of dental tissues in forensic medicine

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Abstract Maceration techniques remove soft tissue by the destruction of biomolecules, but the applied techniques may also affect the morphology and the molecular integrity of the hard tissue itself. The impact of seven different techniques for soft tissue removal on morphological and biomolecular parameters of teeth and dental tissues was systematically examined. All methods tested showed significant changes in dental morphology and in the molecular integrity of DNA and the dental proteins, as revealed by aspartic acid racemisation (AAR). In forensic casework this may have severe impacts on the results of morphological methods (e.g. age estimation based on root translucency) and of biomolecular analyses (e.g. age estimation based on AAR and DNA analysis). Therefore, age estimation based on AAR should not be applied to tissue treated in such a manner, and it is recommended that teeth for analysis should be extracted before soft tissue removal. DNA in the hard tissue seems to be less susceptible to soft tissue removal than proteins, and several of the tested maceration techniques appear not to have a damaging effect on DNA. Generally, the indication for soft tissue removal demands a careful case management to avoid methodological collisions.

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Introduction

Whenever adhering soft tissue hinders an exact morphological analysis of bone or teeth, for example, for biomechanical analysis of injuries or for identification of unknown deceased by osteological and odontological analysis, bone maceration is applied. Numerous methods have been described, and common procedures are soft tissue removal by solutions of inorganic and organic chemicals [31, 46] or by feeding insects such as dermestid beetles [43]. Inorganic chemicals used are antiformin [4], ammonium hydroxide [14], sodium hydroxide [11, 15], and other alkaline solutions [32]. Maceration with organic chemicals can be performed with enzymes such as papain or pepsin [12, 24] or with washing powders containing enzymes [2, 19, 20, 44].

Maceration techniques aim to remove soft tissue from bone or teeth, which basically is accomplished by degrading biomolecules, particularly structural proteins. It is possible that the applied techniques may not only affect the adhering soft tissue but also the morphology and the molecular integrity of the hard tissue itself.

Frequently, we are asked to perform morphological analysis of teeth, age estimation based on aspartic acid racemisation (AAR) in dentine, or DNA analysis after jaws have been macerated. In these cases, it is important to understand if the applied maceration techniques have had an effect on the required investigations.

Theoretically, soft tissue removal by any technique may affect morphological and biomolecular findings of the hard tissue. In particular age estimation based on AAR may be

incommensurate with certain maceration techniques. AAR is based on the analysis of the age-dependent increase in the D-aspartic acid content of long-lasting proteins and is mainly applied to dentine for forensic purposes [9, 22, 26, 35, 36, 38], rarely to enamel [29]. Age estimation based on AAR in dentine is one of the most precise methods for age estimation in adults [39, 42]. However, maceration procedures may affect its high precision by provoking an in vitro racemisation, for example, if high temperatures are applied, or by the introduction of artificial degradation of the dentinal proteins.

We systematically examined the impact of seven different techniques for soft tissue removal on morphological and biomolecular features of teeth and dental tissues (e.g. odontological findings, age estimation based on AAR, and DNA analysis). Our data can be used for an optimisation of case management whenever maceration is necessary.

Materials and methods

Teeth were exposed to seven different protocols for soft tissue removal, and morphological examination and biomolecular analyses were performed before and after the procedure. The impact of the tested methods for soft tissue removal on the results was evaluated. In addition, the effect of H_2O_2 treatment on the results of DNA analysis was tested.

Teeth

Teeth were provided from dentists after obtaining informed consent from the patients. In total, 14 teeth with carious lesions and 67 healthy teeth, which were extracted for periodontic and orthodontic reasons, were analysed. The age of the patients ranged from 11 to 97 years.

Methods for soft tissue removal

Six different chemical maceration methods and soft tissue removal by the feeding of *Dermestes* sp. were investigated. Before applying these maceration methods to human material, preliminary tests with jaws from pigs for slaughter were undertaken. All the chosen application conditions resulted in an optimal soft tissue removal. In addition (n=2), teeth were treated with H_2O_2 solution, which is used by dentists as a disinfectant and as a bleaching agent.

The following techniques and conditions were applied:

Maceration with sodium hydroxide (NaOH) Maceration was performed according to the method described by Piechokie and Altner [31] at 40°C in 5% NaOH with 20 g/l NaCl for 3.5 h (20 ml solution/1 g tissue); samples were washed with distilled water until pH 6 was achieved.

Maceration with washing powder, Persil megaperls [44] Samples were macerated in a 1% Persil solution with 2 % NaCl at pH 10 (20 ml solution/1 g tissue) for 57 h at 60°C [25].

Maceration with Biozym SE [2] A solution of 2 g Biozym, 20 g NaCl and 1,000 ml distilled water at pH 8 was used for maceration at 60°C for 56 h (20 ml solution/1 g tissue).

Maceration with papain (P3250, Sigma Aldrich Chemie GmbH, Steinheim, Germany) [24] After boiling the samples for 5 min in distilled water, maceration was performed in a solution of 0.5 g papain, 20 g NaCl, and 1,000 ml distilled water at pH 8 at 40°C for 56 h (20 ml solution/1 g tissue).

Maceration with Enzyrim OSS [24] Samples were macerated in a solution of 20 ml Enzyrim and 980 ml distilled water with 20 g/l NaCl at pH 8 at 60°C for 20 h (20 ml solution/1 g tissue).

Maceration with Enzyrim OSS combined with ultrasound cleaning [12] Maceration with Enzyrim (see above) was performed at 60°C under ultrasound cleaning for 3 h (5 ml solution/1 g tissue).

Soft tissue removal by feeding of dermestid beetles [43] Samples were exposed for 24 h in the 25°C infrared-light-heated terrarium.

Treatment with 3% H_2O_2 at 21°C for 24 h
(only for DNA analyses)

The pH of the solutions was controlled and adjusted with sodium carbonate twice a day.

Morphological analysis

In 14 teeth with carious lesions a thorough odontological examination and photographic documentation was performed before and after treatment with the tested methods for soft tissue removal. The findings before and after treatment were evaluated regarding the following questions:

- Changes in the appearance of carious lesions?
- Brightening of dental colour?
- Removal of calcified or pigmented dental plaques?
- Changes in root transparency?

Determination of AAR

Preparation of the dentine samples

Cementum from the surface of the dental roots and pulps was abraded under constant water cooling. The roots of the teeth were separated from the crowns using a dental handpiece and tungsten carbide burs under constant water cooling. The quality of the preparations was controlled by exposure to UV light (dentine exhibits fluorescence under UV light at wavelength 366 nm, cementum does not). The prepared root dentine was washed for 1 h in a 15% NaCl solution (w/w), followed by 15 min in ethanol/ether (3:1) and for 1 h in 2 % sodium dodecyl sulfate (SDS; w/w). All washing steps were conducted at 4°C. After washing, samples were freeze-dried and pulverised.

Analysis

AAR was determined as described previously [35, 37], taking into account defined quality standards [38]. Briefly, the dried samples were hydrolysed in 6 N HCl at 100°C for 6 h; thereafter, hydrochloric acid and water were removed in a vacuum. The hydrolysate was esterified with isopropanol/sulphuric acid (10:1) for 1 h at 110°C. After alkaline extraction with dichloromethane, acetylation was performed with trifluoroacetic anhydride (TFAA) at 60°C for 15 min. These amino acid derivatives (TFA-isopropylesters) were analysed by gas chromatography (Shimadzu GC-9, Split 1:50) using a flame ionisation detector and hydrogen as carrier gas on a chiral capillary column (Chirasil-L-Val, Varian, Middelburg, The Netherlands). The ratio of D-aspartic acid to L-aspartic acid was determined.

Interpretation

AAR values were used for age estimation according to Ritz et al. [35] and Ritz-Timme et al. [38], and the calculated ages were compared with the actual ages.

DNA analysis

Roots and crowns were separated under water cooling with a dental hand piece and tungsten carbide burs at the enamel cementum junction. The samples were cleaned for 2.5 h in 99% ethanol, for 30 min in 70% ethanol, for 15 min in water, freeze-dried overnight, and pulverised.

Total DNA for each sample was extracted out of 0.5 g of tooth powder using the Invisorb forensic kit I (Invitek, Berlin, Germany) according to the manufacturer's recommendations.

DNA quality and quantity were tested by separating 10 µl of DNA solution for each ethidium bromide containing 1% agarose gel and use of the Gel doc imaging system (Biorad, Munich, Germany).

To check for the presence of minute DNA amounts, which are not visible on agarose gels, and to test the amplifiability of present DNA, a duplex polymerase chain reaction (PCR) was performed to amplify a 164-bp nuclear-specific and a 260-bp mitochondrial-specific DNA fragment [47].

PCR products were analysed using an ABI Prism310 automated sequencer system: 1 µl of each duplex PCR amplification product and 0.2 µl of an internal standard (Genescan-500 ROX) were added to 11.8 µl formamide (Applied Biosystems, Weiterstadt, Germany). The mix was analysed without prior denaturation. Genescan Analysis 2.1 software was employed to determine fragment sizes and the relative amount of PCR products by taking the area under the curves.

Results

Impact of maceration techniques on tooth morphology

All the methods for soft tissue removal tested had an effect on the morphological findings, albeit to a different extent with each technique (Table 1, example in Fig. 1a,b). Caries lesions were visibly altered concerning colour and size by all methods. All but the Biozym SE technique affected the dental colour (brightening). The NaOH, Persil megaperls, and Enzyrim OSS methods had the potential to remove mineralised dental plaques (Fig. 1a,b). All but the papain and the *Dermestes* techniques removed pigmented dental plaques. An increase in the root translucency was provoked by all methods apart from Biozym SE and *Dermestes*. The NaOH method exhibited the strongest effect on tooth morphology, with changes observed in both samples in all five criteria (Table 1).

The impact of maceration techniques on the AAR in dentine

Figure 2 shows the deviations of the AAR-based estimated ages from the actual ages. In 17 (35%) of 49 cases deviations of more than 5 years between the estimated and the actual ages were observed; the maximum deviation was 23 years (NaOH technique). In most cases where a deviation was observed, false high ages were estimated (71%).

Deviations between the calculated and actual ages of more than 5 years were observed in nearly all the methods tested, albeit in different frequencies and to different extents (Fig. 3). Such deviations were produced most often (six of seven cases) and extensively (range of −9 to +23) by the

Table 1 The impact of maceration techniques on the morphological examination of teeth: morphological findings before and after exposition to seven methods for soft tissue removal were compared (two teeth per method)

Method of tissue removal	Sample	Change in the appearance of the carious lesions	Brightening of dental colour	Removal of calcified dental plaques	Removal of pigmented dental plaques	Changes in root transparency
NaOH	1	+	+	+	+	+
	2	+	+	+	+	+
Persil	3	+	+	+	+	−
megaperls	4	+	+	−	+	+
Biozym SE	5	+	−	−	+	−
	6	+	−	−	+	−
Papain	7	+	+	−	−	+
	8	+	−	−	−	+
Enzyrim	9	+	+	+	−	−
	10	+	+	−	+	+
Enzyrim +	11	+	+	−	−	+
ultrasound	12	+	+	−	+	+
Dermestid	13	−	−	−	−	−
beetles	14	+	+	−	−	−

+ Changes were observed; − no changes were detected

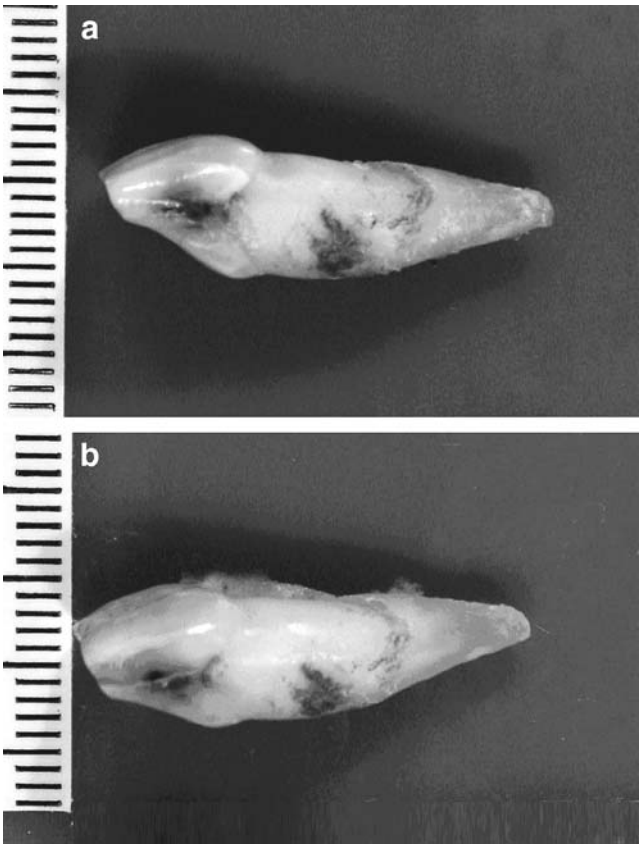


Fig. 1 **a** Tooth (sample 11) before maceration with Enzyrim OSS + ultrasound cleaning. **b** Tooth (sample 11) after maceration with Enzyrim OSS + ultrasound cleaning. In this sample, a change in the appearance of the carious lesion, a brightening of the dental colour, and an increase in the translucency of the root tip can be observed. The reproduction of the degree of translucency is restricted by the photograph's quality

NaOH method, followed by the mainly enzymatic techniques, which were papain (three of seven cases), Enzyrim OSS + ultrasound cleaning (three of seven cases), Biozym SE (two of seven cases), and Enzyrim OSS (one of seven cases). The Persil megaperls technique resulted in deviations of up to 6 years.

The impact of maceration techniques on DNA analysis

Agarose gel electrophoresis revealed different impacts of the described maceration techniques on DNA integrity (Fig. 4). The use of H_2O_2 led to the most obvious degradation of total DNA. Although some samples still contained high molecular weight DNA (e.g. in lanes 11 and 13 in Fig. 4), a large amount of highly degraded DNA was also visible. The worst results concerning DNA quality and quantity were observed after treatment with NaOH (lanes 8 and 31). Treatment with

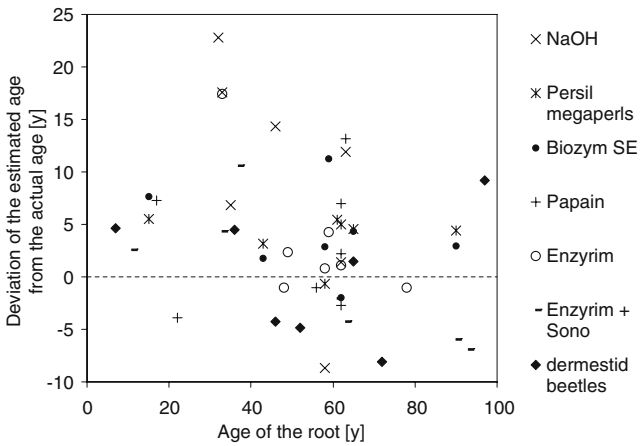


Fig. 2 Deviations of the AAR-based estimated ages from the actual ages, depicted with regard to the analysed age groups

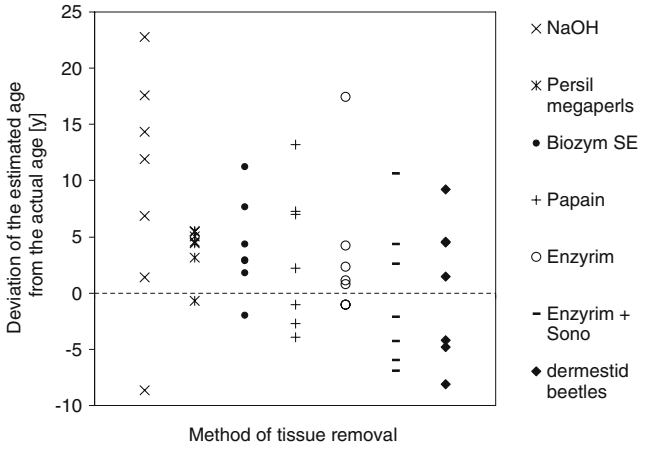


Fig. 3 Deviations of the AAR-based estimated ages from the actual ages, depicted with regard to the analysed methods for soft tissue removal

Persil megaperls resulted in much less destruction (lanes 24 and 25). Regarding all treatments, the best DNA extraction results were obtained after maceration with Enzyrim regardless of the use of ultrasound cleaning during Enzyrim treatment (lanes 9, 26, 27, and 30).

Duplex PCR showed that all samples still contained nuclear and mitochondrial fragments of about 200 bp (Fig. 5, 164 bp for nuclear and 260 bp for mitochondrial DNA). The strongest signals can be amplified after the Enzyrim treatment.

Discussion

Impact of maceration techniques on tooth morphology

All the methods tested for soft tissue removal exhibited effects on the morphological findings (Table 1) such as changes in the morphology of caries lesions, dental colour, and the removal of dental plaques. The increase in root translucency, caused by all but the Biozym SE and the *Dermestes* methods, is of importance for age estimation based on the extent of root translucency because it may lead to false high results and, therefore, overestimations of age [7, 13, 16, 18, 21, 23, 30, 33, 45].

Influence of maceration techniques on AAR in dentine and impact on age estimation based on AAR

Basically, age estimation by AAR in dentine uses the age-dependent accumulation of D-aspartic acid in dentinal proteins, where there is a very close relation between AAR and age. Deviations of more than 5 years between estimated and actual ages are very unusual and can indicate the influence of external factors [26–28, 37].

The kinetics of AAR mainly depend on the protein structure, namely, on the nature of the adjacent residues [6, 17, 40], the local environment of the residue within the protein [10], and physical constraints related to higher-order structures [8, 41]. Therefore, a constant protein composition of the samples is an essential prerequisite for precise results in age estimation based on AAR in dentine. This is ensured by a high standardisation of sampling and of sample treatment [38]. Protein degradation induced by the removal of soft tissue, most notably by NaOH treatment or enzymes (papain, Biozym SE, Enzyrim OSS, perhaps also in the case of feeding of *Dermestes* sp.), may result in a critical increase in small peptide fragments with concomitant rapid AAR in the sample and, therefore, in false high AAR values.

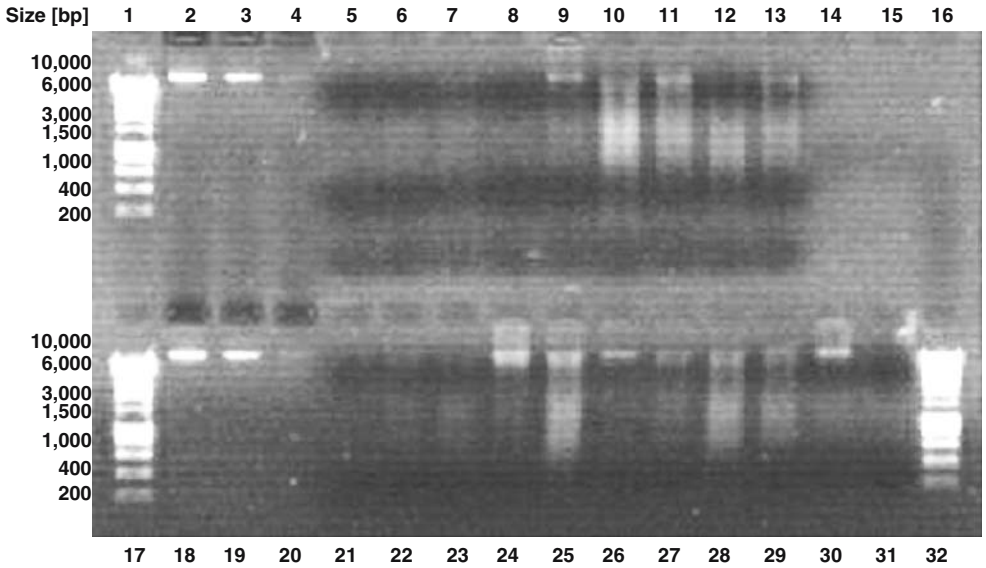
The kinetics of AAR may also be influenced by the physical surroundings (e.g. salt concentration, pH, and temperature) [3, 5]. These values are largely constant in vivo, but during maceration, samples are exposed to non-physiological temperatures and pH values. High temperatures and extreme pH values have been found to accelerate AAR [27]. The most frequent and extensive deviations between estimated and actual ages were found with the NaOH method (Fig. 3), most likely due to the extreme alkaline conditions leading to excessive protein degradation.

It remains unclear why some treated samples exhibited large deviations between estimated and actual ages, and yet other samples after an identical treatment did not. One example is the Enzyrim OSS method, where only one of seven samples exhibited a deviation (of 16 years!). Obviously the underlying processes are very complex and depend on many factors that may vary from sample to sample.

Even if such extensive deviations are observed only in single cases, the presented data (Figs. 2 and 3) show the risk of introducing errors into age estimation based on AAR by methods of soft tissue removal.

The Persil megaperls technique did not produce deviations of more than 5 years. Whilst this is encouraging, errors cannot be ruled out even after maceration with this method. Persil megaperls is a commercially available washing powder and, amongst other chemicals, contains proteases [25] with an unpublished formula, which may be modified at any time without notification. Thus, this method cannot be standardised, and so as with the other methods, it bears the risk of providing false results in age estimation based on AAR.

In conclusion, age estimation based on AAR should not be performed after soft tissue removal by maceration or feeding of *Dermestes* sp. If it is necessary to remove the soft tissues from the jaws, at least one tooth should be extracted before soft tissue removal to enable age estimation based on AAR.



Lane	Sample	Lane	Sample
1	Bioline HyperLadder I	17	Bioline HyperLadder I
2	100 ng control DNA	18	100 ng control DNA
3	50 ng control DNA	19	50 ng control DNA
4	10 ng control DNA	20	10 ng control DNA
5	Aqua bidest.	21	Aqua bidest.
6	Biozym SE	22	Papain
7	Biozym SE	23	Papain
8	NaOH	24	Persil megaperls
9	Enzyrim	25	Persil megaperls
10	H_2O_2	26	Enzyrim + ultrasonic cleaning
11	H_2O_2	27	Enzyrim + ultrasonic cleaning
12	H_2O_2	28	dermestid beetles
13	H_2O_2	29	dermestid beetles
14	Empty	30	Enzyrim
15	Empty	31	NaOH
16	Empty	32	Bioline HyperLadder I

Fig. 4 DNA retrieval from teeth after application of different maceration techniques, agarose gel electrophoresis

Impact of maceration techniques on DNA analysis

Since DNA analysis is increasingly used for identification of skeletal remains, it is crucial to know how different preparation techniques might influence subsequent genetic investigations. In this study, the DNA quality and quantity was first analysed on agarose gels containing ethidium bromide. This method allows to roughly estimate the DNA quantity based on the known amount of a high molecular weight standard DNA on the gel. It also shows the level of degradation but gives no information on the amount of total DNA (high molecular weight and degraded) in a sample. However, the different methods tested already showed striking differences on agarose gels (Fig. 4). There are better methods for DNA quantification, for example, real-time PCR [1, 48], but for this experimental design, they were not essential. As only one tooth had to be used for every maceration experiment, it must be taken into account that the DNA yield differs from tooth to tooth and also from individual to individual. Thus, it was thought to be more important to look at DNA quality and amplifiability.

To also check for the presence of PCR inhibitors, a duplex PCR (Fig. 5) was performed, which was a valuable indicator before short-tandem repeat system (STR) typing [47].

Some of the chemicals used can potentially alter DNA integrity or influence subsequent PCR analysis negatively when they are coextracted during DNA isolation. However, several maceration techniques exist that apparently do not damage DNA [34]. In our experiments the use of Enzyrim gave the best results.

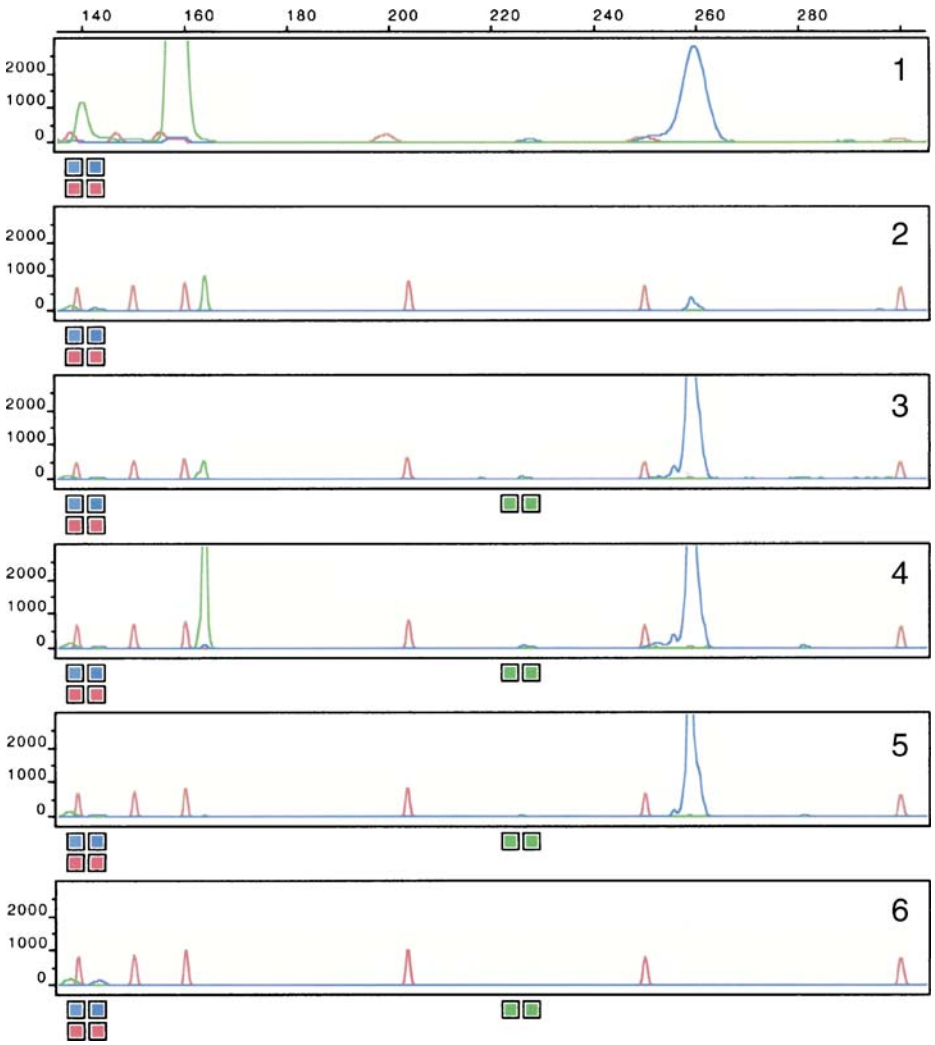


Fig. 5 Influence of different maceration techniques on DNA amplifiability. Electropherogram after duplex PCR. Shown is the amplification of a 164-bp fragment of nuclear DNA (*beta globin* gene) and a 260-bp mitochondrial specific fragment of the *ND1* gene. The *first panel* shows amplification of the positive control (1 ng total DNA, Promega); the other *panels* represent typical results after different maceration procedures: *2*, treatment with H_2O_2; *3*, Persil megaperls; *4*, Enzyrim; *5*, NaOH. The *sixth panel* displays the negative control (only PCR reagents and H_2O bidest)

Conclusions

The tested methods for soft tissue removal all led to changes in dental morphology and in the molecular integrity of DNA and the dental proteins, as revealed by the AAR data. In forensic case work, this may have negative consequences for the applicability of morphological methods (e.g. age estimation based on root translucency) and of biomolecular methods (e.g. age estimation based on AAR and DNA analysis). Age estimation based on AAR should not be performed after soft tissue removal, and teeth for analysis should be removed beforehand. DNA seems to be less susceptible to damage, and several maceration techniques exist that apparently do not destroy DNA. Generally, the requirement and approach for soft tissue removal demands careful case management to avoid methodological collisions.

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