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METHODS PAPER

Detection of glass particles on bone lesions using SEM-EDS

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Abstract The problem of identifying the wounding agent in forensic cases is recurrent. Moreover, when several tools are involved, distinguishing the origin of lesions can be difficult. Scanning electron microscopy (SEM)/energy dispersive X-ray analysis (EDS) equipment is increasingly available to the scientific and medical community, and some studies have reported its use in forensic anthropology. However, at our knowledge, no study has reported the use of SEM-EDS in forensic cases involving glass tools, whether in case reports or experiments. We performed an experimental study on human rib fragments, on which we manually created wounds using fragments of window and mirror glass. SEM-EDS was executed on samples without any further preparation on low vacuum mode, then on the same samples after defleshing them completely by boiling them. Window and mirror glass particles were detected on experimental wounds. Both had silica in their spectra, and the opaque side of the mirror contained titanium, allowing for their identification. Boiling and defleshing the bone samples involved a loss of information in terms of the number of wounds detected as positive for glass particles and in the number of glass particles detected, for both window and mirror glass. We suggest the analysis of wounds with suspected glass particles using low vacuum mode and with no defleshment by boiling.

Keywords Forensic anthropology · SEM-EDS · Bone lesion · Glass

Introduction

Scanning electron microscopy (SEM)/energy dispersive X-ray analysis (EDS) has been widely used and described as a useful forensic tool in particle detection since it was first marketed in 1965. Its first forensic application was around 1968, in forensic gunshot residue analyses [1]. The equipment for SEM-EDS is now found in many research laboratories and platforms; so, access to this technology is increasing for physicians and anthropologists. SEM-EDS is now the most commonly used method of analyzing inorganic gunshot residue [2] in forensic laboratories.

Automated systems have been developed [3] and used routinely to detect particles on non-biological samples, but the use of SEM-EDS in biological samples is more recent. In such samples, for wounds from weapons other than gunshots, such as knives, identifying the implement is challenging. A multidisciplinary approach using morphological analysis of toolmarks can be necessary and SEM is considered as the gold standard [4–7]. Despite this, it is still difficult to identify a specific weapon [8]. Bai et al. [9] used SEM-EDS and inductively coupled plasma atomic emission spectrometry experiments to detect particles in wounds made on domestic pigs' skin. Few human cases, where particles of the wounding implement were detected in the wound by SEM-EDS, have been

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reported [10, 11]. Recently, some experimental studies were performed on bone after invasive traumas with metallic tools, and microtraces of metals were found [12, 13]. Alternative material preparation methods and SEM-EDS conditions have been described in the literature (Table 1). Therefore, we tested two sample preparations and two parameters of analysis.

Wounds caused by glass injuries are frequent and the contexts are numerous, such as motor vehicle accidents, domestic or work accidents, or fights. In this latter case, different glasses are found: broken glass bottles, used as a weapon during the assault, and glass windows or mirrors, involuntarily broken during fighting [14–16]. Wounds can be multiple, superficial, or deep, with clean-cut or irregular and abraded margins. The identification of implements used in producing wounds is a challenge in forensic pathology. Rothschild et al. [17] reported three cases of puncture wounds caused by glass being mistaken for stab wounds with a knife. This mistake could be very harmful for following forensic investigations and the understanding of the scenario. In complex cases, replication of the wound in an experimental support and comparison with the 'real wound' could be helpful but are rarely done routinely. Some cases of biomechanical reconstruction of the wound are also reported in the literature [18]. Because SEM-EDS could be a useful tool, we performed an experimental study to assess the potential value of SEM-EDS in the detection of glass particles on bone wounds from a forensic perspective.

Materials

Human rib fragments were harvested from human donors supplied by an anatomy laboratory. Fragments of 2 cm in length were withdrawn from the mean arcs with a vibrating saw. In order to create wounds, we used a fragment of window glass and a fragment of mirror as tools. Analyses were performed using FEI Quanta 250 FEG equipped with a *backscattered electrons and gaseous analytical detector*™, which has an aperture of 500 μm.

Methods

The parameters of EDS were set with a spot at 6.0 and a high voltage at 20 kV. Each tool to be used to create wounds—window glass and mirror fragments—was analyzed individually to get reference spectra for EDS.

Preparation of fresh bones

On each fresh bone, we partially defleshed bones manually, retaining the periosteum. For each type of glass tested, ten clear one-way wounds were made manually on the bones by the same operator. Defleshed bone without wounds was also

Table 1 Main publications on SEM-EDS in forensic context

Publication	Type	MEB/EDS	Parameters	Sample(s)	Weapon(s)	Sample preparation	Comment(s)
Vermeij, 2011	Concrete cases	Quanta 400 FEI	Low vacuum 0.1–0.4 mbar water vapor Si(Li) detector High voltage: 20 kV Measure: 60 live sec	Bone (human)	Bicycle lock, hammer, knife, rock	70° in ultraclean water, soft tissue removing, dried at 40 °C	Recommendations on cleaning, contamination, recognition
Bai, 2007	Experimental	Model 3200C (Army Instruments Inc.,USA)	Low vacuum 100–200 mTorr Si(Li) detector High voltage: 25 kV	Skin (pigs)	Knife	No preparation	
Muccino, 2015	Concrete case	Cambridge Stereoscan 360 EDX Oxford Link Pentafet	Data not shown	Scalp, eyelid margins, left eye, optic nerve	Ceramic statue	Fixed by 10% buffered formalin then dehydrated in alcohol	
Pechnikova, 2012	Experimental	Cambridge Stereoscan 360 EDX Oxford Link Pentafet	Low vacuum High voltage: 20 kV	Bone (metatarsal bovine)	Blunt force injury	Soft tissue cleaning with plastic knife; graphite coated	
Gibelli, 2012	Experimental	Cambridge Stereoscan 360 EDX Oxford Link Pentafet	Data not shown	Bone (radius, human)	Sharp force injury	Data not shown	
Kinoshita, 2004	Concrete case	Hitachi S-3000 N	VP-SEM 30 Pa High voltage: 15 kV Room temperature: −20 °C	Skin (human)	Electrocution	Formalin-fixed tissues and dried at room temperature	

tested as a negative control. Because the chamber size of SEM-EDS was limited, we cut the bones with a mechanical saw in segments of 3.04 cm (±0.58 cm). The wound was located at about 1.24 cm (±0.5) from the nearest bone extremity.

Identification of glass particles on fresh bones

First, we performed experiments to establish criteria to identify glass particles in fresh bones. We tested two modes of EDS measurement, the spot mode in which the volume analyzed is 1 μm^3 and the full frame mode, where the entire field is analyzed. After spotting the wound, we identified and counted glass particles.

Analysis of wounds on fresh bones

The samples were analyzed without any further preparation in low vacuum mode (with chamber pressure set at 100 Pa) as previously described [9].

Preparation of boiled bones

Because boiled bones are frequently studied in the literature, we completed further defleshment of the same bones by boiling them and removing the soft tissues, taking care not to alter the wounds. Bones were dried in ambient air for at least 2 days [6].

Identification of glass particles on boiled bones

As with fresh bones, glass particles were identified and counted.

Analysis of wounds on boiled bones

The complete defleshed samples were analyzed in a low vacuum by putting them in a vacuum chamber in order to release gas and dry them completely. Because it is considered that high vacuum is a better mode than low vacuum for morphology analysis, the experiment was performed in high vacuum mode, at pressure in the order of 10^{-3} Pa.

Results

Reference spectra

The spectra of window glass and mirror fragments were established using EDS, either in low or high vacuum using spot and full frame modes. We found six significant peaks for the window glass, corresponding to the elements: oxygen (O), sodium (Na), magnesium (Mg), silica (Si), calcium (Ca), and aluminum (Al) (Fig. 1a). We considered this small peak of Al to be a false positive due to the presence of aluminum on the platform supporting the sample, as shown in Fig. 1. EDS was executed on both sides of the mirror fragment. One side had the same spectrum as the window glass, so hereafter, we call this the glass side. The other side, which we hereafter call the opaque side, was positive for O, Ca, and titanium (Ti) (Fig. 1b, c). Due to the fact that Ca and O are present in the bones and would therefore appear in a bone EDS spectrum, the elements that can be used to identify glass are Si, Mg, and Na, while only Ti allows us to distinguish mirror from window glass. No difference was found between low and high vacuum modes.

Spectra of defleshed bone without wounds

On bones without wounds, we did not find any particles with glass spectra.

Aspects of wounds

Glass is a sharp-edged material that produces an incised wound. The lesion is clear cut with a regular edge. As expected in this kind of lesion, the wound is deeper at the beginning and shallow at the end. Glass particles are particularly numerous at the tailing compared to the beginning. Some are superficial and others deeply inserted in the wall of the lesion. Almost all wounds concerned only cortical bone without deeply reaching spongious bone. No morphological difference was found between mirror and glass wounds (suppl Fig. 1).

Identifying glass particles on fresh bones

For each particle discovered on fresh bones, we performed EDS to confirm its identity. We found that glass fragments had a smooth aspect with acute angles forming an irregular shape. Mirror fragments had the same morphological characteristics. Fragment sizes ranged from 5 to 350 μm for window glass and 5–250 μm for mirror fragments. Identifying these particles only on the basis of morphological specifications is therefore difficult, because bone fragments can take many forms, including the sizes and shapes exhibited here by the glass fragments. In the EDS analysis, the full frame mode showed interferences from bone elements, so the peaks of interest were unclear. As a result, we used only spot mode EDS to identify particles (Fig. 2).

Semi-quantification of glass particles on fresh bones

To compare the methods of using fresh bones and boiled bones, and to evaluate the loss of particles during boiling, we counted the number of glass particles detected for each wound. We set up three groups: one with no glass particles detected, one with 1–9 particles, and one with 10 or more

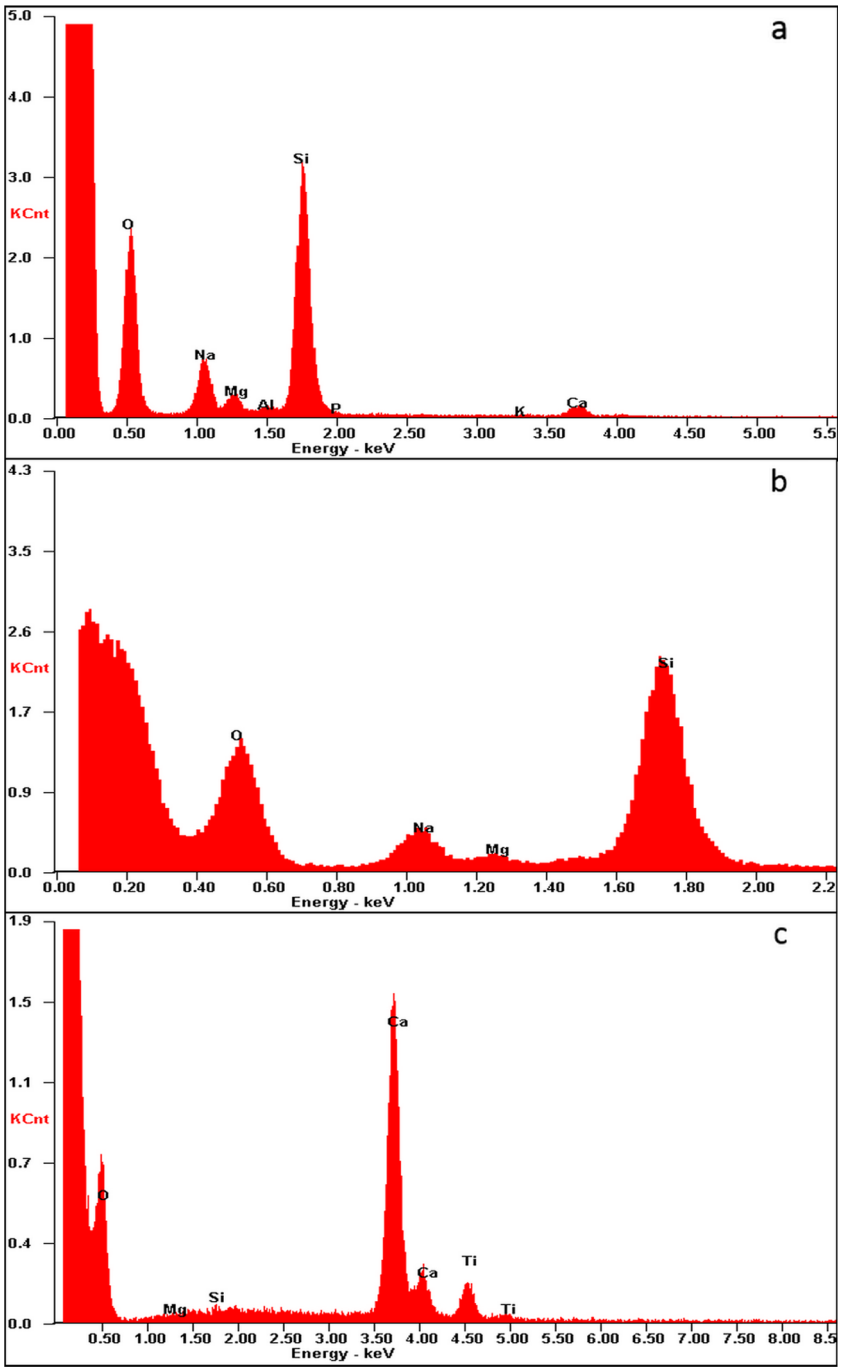

Fig. 1 Spectra produced using SEM-EDS of **a** a window glass fragment, **b** the glass side of a mirror fragment, and **c** the opaque side of a mirror fragment

particles (Tables 2 and 3). For window glass, three wounds had more than 10 particles and the others ranged between 1 and 3 particles. For mirror fragments, five wounds were found with more than 10 particles, and the rest ranged between 6 and 8 particles (Table 3).

Analysis of wounds on fresh bones

Of ten window glass wounds examined on fresh bones, nine tested positively for glass in low vacuum mode (Fig. 2). Glass fragments were distributed either on the wall, floor or surface of the bone. Some particles were aggregated, suggesting that a larger glass fragment had been broken into pieces during the injury. We found particles placed on the bone surface, on and in soft tissues, and also inlaid on the bone.

Lesions made by mirror fragments exhibited the same morphological specifications as glass fragments in terms of distribution and contamination. Eight lesions were positive for particles with the spectrum described for window glass, and only one lesion was positive for Ti. These Ti-positive particles had

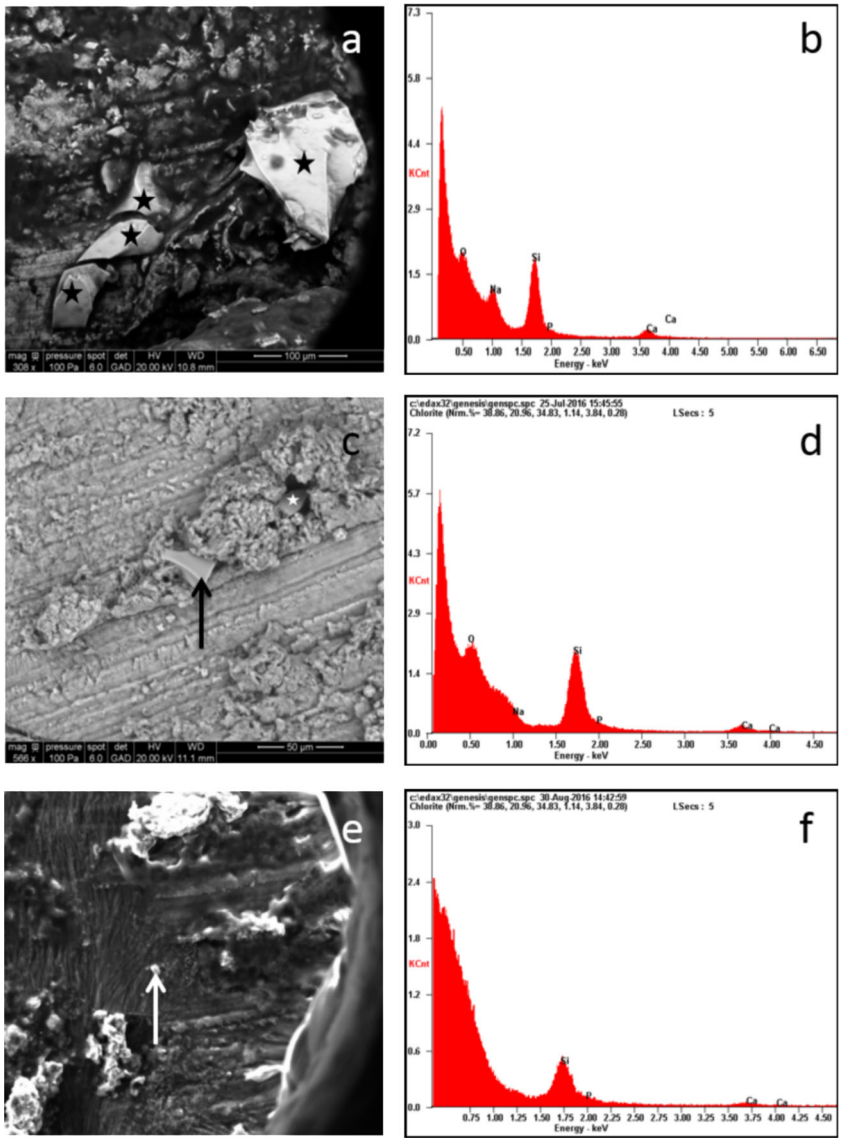

Fig. 2 Capture of particles detected as glass (*black* or *white stars*, *white* or *black arrows*) with associated spectra for the lesions caused by mirror fragments in **a**, **b** low vacuum conditions on fresh bone samples, in **c**, **d** low vacuum conditions on boiled bone samples, and in **e**, **f** high vacuum conditions on boiled bone samples

no specific morphological characteristics to distinguish them from glass ones (Fig. 2).

Experiments in low vacuum mode on boiled bones

Experiments conducted after boiling the bones and removing the soft tissues returned five positive lesions in the window glass group. As expected, glass particles exhibited no modification of their morphological specifications due to boiling. The removal of soft tissues revealed more of the bone surface, which had a very irregular aspect. This made it difficult to spot the particles of interest. We noticed alteration of the bone surface in the form of a round crater, which we assumed was the result of the ejection of gas confined inside the bone when

Table 2 Number of lesions in term of particle number detected in window glass group

Method	Number of particles		
	0	<10	≥10
Fresh/low vacuum	1	6	3
Defleshed/low vacuum	5	4	1
Defleshed/high vacuum	6	4	0

Table 3 Number of lesions in terms of particle number detected in mirror group

Method	Number of particles		
	0	<10	≥10
Fresh/low vacuum	2	3	5
Defleshed/low vacuum	6	3	1
Defleshed/high vacuum	9	1	0

it was subjected to vacuum conditions. We observed some cracks on the bone that were also present on the negative controls, so we classified them as artifacts. One positive lesion on the boiled bones was found to have more than ten particles, while only one particle was detected on the other wounds (Table 2). In the mirror lesion group, five lesions were positive for glass particle spectra, but none contained Ti. One positive lesion had more than ten particles and the rest contained 1–6 particles.

In both the glass and mirror groups, we were unable to detect two wounds on the bone surface. We assumed that lesions performed with tools did not indent the periosteum enough to leave a significant mark on the bone that would be recognizable as a toolmark, and not an artifact described above, after boiling.

Experiments in high vacuum mode on boiled bones

We analyzed both groups (window glass and mirror fragment wounds) in high vacuum mode, returning four wounds that were positive for glass particles. However, in the mirror group, we could not obtain a sufficient vacuum in seven samples. In the remaining three, only one was positive for glass particles, and none of these particles were positive for Ti. Furthermore, we observed the movement of fragments on the bone's surface, probably due to the high depression. This could be responsible for the movement of glass particles, interfering with their detection. Only one particle was detected in each positive lesion.

Contamination

We also found particles of a different shape, with significant peaks of Al and Si revealed by EDS, which is compatible with metallic residue. We suspect that these residues were due to contamination from the saw we used to cut the bones before analyzing them with EDS. Thus, we analyzed the saw blade (Suppl Fig. 2). As expected, we found Al-Si, Cr, and Fe peaks, confirming the hypothesis.

Yeasts and filamentous fungi were identified on unprepared samples, probably due to the extended length of storage time of the samples.

Discussion

Our study demonstrates for the first time that it is possible to use EDS to detect glass residue on bones, either from a window glass or mirror fragment. But, morphological aspects are not sufficient to identify glass fragments: EDS is necessary to differentiate them from bone and identifying silica peak (Si).

SEM is a non-destructive method generating high-resolution images of shapes. Usually, for morphological analysis, the SEM analysis is carried out under high vacuum, particularly for conductive elements, because the resolution of images is increased. If elements are not conductive, the surface could be coated with gold, platinum, carbon, or graphite to improve the conductivity. This treatment is not advisable in forensic investigation if EDS-X is required, because it generates X-ray peaks that interfere with particle-generated peaks in the EDS spectrum. The low vacuum mode admits little air into the SEM room. Even if the resolution of the image is reduced, this mode is used for nonconductive or hydrated samples, and so preferentially for EDS analysis [19]. Environmental mode could also be used. It allows imaging the specimen surface in a gaseous environment (in water vapor), but the resolution of the images could be low. We first analyzed our samples in a low vacuum mode because glass is a non-conductive material. Glass particles were found easily in hydrated bones and with difficulty in boiled bones. In high vacuum mode, we did not detect more particles, and their detection was not easier. To find the chemical composition of particles, EDS analysis was performed down to a spot size, which measured the signal on 1 μm^3 volume.

Some issues can be highlighted as differential diagnoses. Glass particle spectrum identification relies on Si as a major element and Na, Ca, Mg, and Al as minor elements. Two materials with a major Si peak have been described in the literature: ceramic and some minerals. Muccino et al. [11] reported a patricide with a ceramic statue, in which the superficial layer revealed Si as a major element but was associated with several minor elements, such as iron (Fe), Ti, chromium (Cr), or Al; the particle morphology also seemed different. Vermeij et al. [10] described particle detection on a wound from minerals. Quartz, pyroxene, and ilmenite minerals were reported on a wound, and the particle morphology looked like the glass particles we described. Si was the major element for both quartz and pyroxene. Pyroxene particle spectra revealed Al, Mg, and Ca as minor elements, and no minor element was identified in quartz particle spectrum; so, the absence of Na could guide identification. This illustrates that implementing class identification could be difficult between glass particles and certain minerals. However, these mineral particles have been described in wounds resulting from blunt injury, and the glass particles we described were produced by sharp force injury.

EDS clearly contributes in implementing identification class involved in a wound in addition to particle morphology on SEM. X-ray detection is still possible with EDS on non-planar surfaces such as wounds on bone or skin. Suzuki et al. [14] demonstrated the benefit of trace element detection in the identification of unique bottle glass using a combination of inductively coupled plasma mass spectrometry and refractive index measurement in an experimental study. However, EDS detection limit and discriminating power do not permit, in these conditions, trace element detection, and only major

and minor elements are detected. For this reason, EDS cannot confirm that a weapon, found on a crime scene, for example, is responsible for the wound but can only determine the implement class involved.

Wavelength-dispersive X-ray spectrometers (WDS) are known to have a better detection limit and resolution power, allowing good qualitative identification of trace elements. However, this technique needs a planar surface, as an irregular surface shape can alter the qualitative identification of trace element peaks. Bai et al. [9] reported an experimental study with inductively coupled plasma atomic emission showing promising results in deducing different implements in the same implement category on wounds.

Bone preparation is a key of EDS analysis success and a critical stage. Unfortunately, in the studies we found in the literature, the criteria for the selection of sample preparation are not explained (Table 1). That is why we tested fresh bone analysis and boiled bone analysis.

Boiled bones revealed less information than fresh bones for both glass tools used. We hypothesize that some of the loss was due to particles being located in soft tissues, such that they were extracted during the process of defleshment, or even due to the washing by boiling treatment. Therefore, it is important to observe the wound without any treatment to localize particles of interest. This could be carried out with SEM in addition to stereomicroscopy.

Additionally, some particles may have been missed by the analysis because of the large quantity of bone fragments interfering in the visual detection of potential glass particles, as illustrated by the difference in the number of positive lesions between the high vacuum group and the low vacuum one. Moreover, we assume that, for some wounds we performed, we did not cross the periosteum, partly explaining the negative results.

We therefore recommend using bones with soft tissues in low vacuum mode to investigate glass wounds. Even with soft tissues, we were able to get a suitable pressure in the SEM chamber. This is consistent with previous methods [12, 13], where bones were analyzed with flesh but using a graphite coating. As graphite coating was not available in our laboratory, we did not coat samples. However, most of the time we could obtain a correct image and we were not hampered by an eventual excess electron load on the sample surface. Other preparation procedures in the literature in a forensic context include pre-fixation of soft tissues with 10% buffered formalin and dehydration with alcohol [11], and formalin fixation and using SEM-EDS equipped with a freeze-drying sample preparation system [15]. These procedures represent a way to further explore the analysis of glass wounds.

Contamination is a major concern in identifying the tools used to create a lesion. Assessing contamination experimentally is not easy, because producing a model in which the environment can contaminate a lesion, such as a person passing through a pane of glass after being beaten, seems impossible. Nevertheless, regarding our observations, we assume that glass particles inlaid on the edge of bone lesions show specifically that the lesion was created by glass. Indeed, energy is needed to obtain this configuration in comparison to particles placed on the sample surface. We found contamination with metallic residues that we could assign to the sampling procedure, as we used a vibrating saw to obtain the bone samples. This contamination proves that to cut at about 1 cm of wound is not adequate. Therefore, we recommend cutting further, but the suitable distance is unknown. More research is needed to evaluate whether some procedures can eliminate or reduce this contamination, for example by using a material to protect the lesion during harvesting, if this does not interfere with the residue detection. Particularly, non-experimental studies are needed, namely using forensic cases.

Conclusion

We report our experience on the feasibility of glass particle detection on bone lesions using SEM-EDS and confirm that EDS is a helpful tool. Because glass residues are often located on the surface of the wound, we recommend analyzing fresh bone without boiling it. The sample size that the SEM chamber can accept is limited. If it is necessary to cut the bone before analyzing it, it is crucial to protect the wound because of the risk of contamination. Further studies are needed with forensic case report series to determine the predictive values of EDS.

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