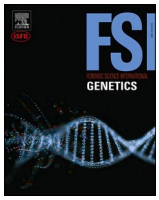
Forensic Science International: Genetics 73 (2024) 103113

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsigen



Transfer and recovery of DNA and metal particles: A proof-of-concept application of a parallel strategy by DNA and environmental scanning electron microscopy analysis

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ARTICLE INFO

Keywords:
Forensic genetics
DNA transfer
Touch DNA
ESEM
Metallic residues
Copper

ABSTRACT

According to the principle of Locard "*Every contact leaves a trace*", when touching a surface, a bi-directional transfer of self and non-self-DNA residing on the hands and touched objects can occur. Metals are commonly encountered in forensic evidence and, during hand contact with these surfaces, a transfer of metal particles could occur together with the transfer of human DNA. This study proposes a proof-concept approach for the original detection of metal particles and touch DNA to track the activity performed by a donor and particularly to assess the metallic substrate touched before the contact with a subsequent surface. To this scope, a scenario of contact events was simulated by three volunteers, who participated in fingerprint deposition firstly on copper and then on plastic and glass surfaces. Twenty-four stubs were collected on the hands of volunteers and the secondary surfaces and then analyzed by environmental scanning electron microscopy (ESEM). DNA was quantified only from copper and plastic surfaces. Ten additional volunteers followed the same protocol of deposition on copper and then on plastic surfaces to evaluate DNA transfer only. On 20 touch DNA samples, the copper surface yielded significantly lower DNA amounts, ranging from 0.001 to 0.129 ng/μl, compared to the secondary touched plastic surface, ranging from 0.007 to 0.362 ng/μl. ESEM-EDS analysis showed that copper particles could be abundantly detected on the hands of the volunteers after contact with the copper surface. Particles containing silicates with copper were shown on plastic, while they were only found in 1/3 of samples on glass. Our proof-of-concept study has shown that ESEM-EDS analysis has the potential to detect copper particles transferred to the hands of volunteers during contact with a copper metallic surface and deposited on secondarily touched items. The results suggest that this original ESEM-DNA parallel approach could potentially allow the tracking of DNA transfer and metal particles at a crime scene, although this represents only a first step and further research on a wider casuistry could help to address the interpretation of results given activity level propositions.

1. Introduction

The increased sensitivity of DNA profiling analyses has allowed, for several years now, the generation of short-tandem repeat (STR) profiles for personal identification from decreasing quantities of DNA, even from trace samples left on touched objects. Indeed, when touching a surface with bare hands, a so-called "primary" transfer of a person's DNA material to the handled object takes place, and this was first described as "touch DNA" by van Oorschot et al. [1]. According to the principle of Locard "Every contact leaves a trace", traces are left and might also be taken away [1,2] so that a bi-directional transfer of self and non-self DNA residing on the hands and on touched objects can occur [3].

Trace DNA samples have been increasingly used to assist investigations of criminal activity, especially in the identification of the person responsible for criminal offences [4]. The amount of DNA deposited by the hands upon physical contact is dependent on several factors, including the donor's ability to shed their own DNA, the time since hand washing, the manner and time of contact, the composition of substrates, and the frequency, duration, and type of surfaces contacted previously [3,5,6]. However, more research is required to provide data

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https://doi.org/10.1016/j.fsigen.2024.103113
Received 22 March 2024; Received in revised form 18 June 2024; Accepted 30 July 2024
Available online 3 August 2024

on the impact of variables, including substrates on DNA transfer, persistence, prevalence, and recovery (DNA-TPPR) [1].

Metals and alloys are frequently encountered as forensic evidence, being components of built environments (e.g., doors and doorknobs), firearms, ammunition, cables, and wearable items, and some studies have highlighted the challenges in the recovery of touch DNA from metal surfaces, including a recent review [7–9]. Interaction between negatively charged nucleic acids and metal ions, particularly Copper (Cu^{2+}), as well as the generation of reactive oxygen species (ROS), could hamper the ability to obtain and amplify DNA from such surfaces [7]. Copper has been linked to PCR inhibition and allelic dropout, although in a recent study on bloodstains collected from metal surfaces, the increased copper percentage was not associated with increased allelic dropout [10,11]. Moreover, copper has recently been shown to have the lowest DNA persistence compared to other metals [12].

The "transfer mechanism" of exogenous particles by contact, penetration, or dislocation [13,14], can be studied by the scanning electron microscope (SEM) coupled to Energy-dispersive X-ray spectroscopy (EDS or EDX), which has been increasingly applied in forensic pathology, allowing to combine a morphological analysis on a sub-micron scale with an elemental composition analysis.

SEM has also been used to study fingermarks, although the coating step and the high-vacuum chamber permanence inevitably lead to uncontrolled changes in the marks [15].

Environmental SEM (ESEM), not requiring particular sample preparation and operating at a lower vacuum, is more suitable for the analysis on biological and metal surfaces and has been applied to fingermarks, showing detectable skin cells, confirmed by the detection of carbon at EDS [15].

Our experimental hypothesis is that, beside a direct transfer of biological material, metal ions and particles could contextually be picked up upon contact on the hands, acting as vectors, and later transported on secondarily touched surfaces together with DNA.

The aim of the present proof-of-concept is to evaluate whether an innovative parallel strategy including DNA and ESEM-EDS analysis might allow not only to generate a DNA profile, useful for personal identification of the donor from fingerprint deposited on a metal surface, but also to detect metallic residues from a metallic surface that previously came in contact with the hands. The transfer of DNA and of metal particles acting as a tracer of the activities performed by a crime offender at a crime scene has never been explored to the best of the authors' knowledge.

2. Materials and methods

2.1. Experimental design

The study was conducted in compliance with ethical standards and was approved by the Bioethical Committee of the University of Bologna (Prot. n. 0201147 approved on July 20th, 2023). Volunteers aged 25–50 were recruited and asked to complete and sign the informed consent form.

The research involved the transfer of both metal particles and DNA following the protocol depicted in Fig. 1 by three volunteers, two women, and a man.

2.2. Substrates and definitions

As the three substrates for the fingerprint deposition a flat copper bar, microscope glass slides (50 mm × 75 mm) and plastic sheets were used. Copper was used as *primary substrate* for a direct or primary DNA transfer, according to the definition of van Oorschot [1], and as a source of metal particles to be transferred on the hands of subjects.

Glass and plastic were defined as *secondary substrates* concerning the indirect transfer of metal particles.

To remove any potential background extraneous DNA, each substrate was first cleaned with a 3 % bleach solution, followed by rinsing with bi-distilled water and absolute ethanol. Prior to use, substrates

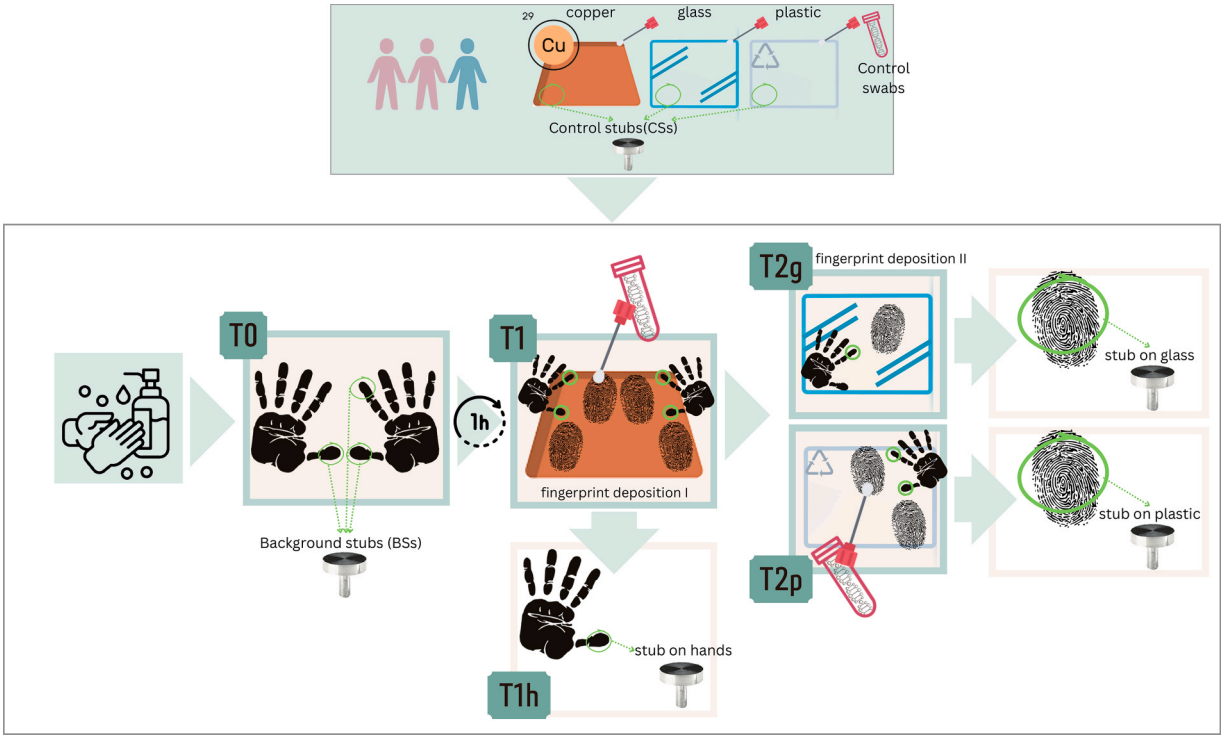

Fig. 1. Diagram of the experimental design, showing on the upper part the three volunteers and the three surfaces for deposition (from left to right: copper, glass and plastic) as well as the control stubs and swabs. The protocol for fingerprint deposition and collected stubs and swabs are shown in the lower part. 1.5 column fitting image.

were then irradiated overnight under ultraviolet (UV) light to ensure the absence of any contaminating DNA.

2.3. Fingerprint deposition protocol

T0. Volunteers were asked to wash their hands with hand soap and water, dry them in air, and then continue their daily routine for 1 hour, refraining from rewashing their hands.

T1, copper. One hour after hand washing, volunteers placed the first two fingers of the dominant and non-dominant hand on the copper bar's primary substrate. This was supposed to realize a direct transfer of DNA to the primary surface and metal ions and particles from the primary substrate to the hands.

T2, glass and plastic. Immediately after the fingerprint deposition on copper, volunteers placed the second finger of the non-dominant hand on the glass slide (T2g) and the first two fingers of the dominant hand on the plastic sheet (T2p), realizing direct DNA transfer and indirect transfer of metal ions and particles on the secondary surfaces.

As performed in previous experiments [16], the volunteers made contact with substrates by pressing the palm down the fingertips of the hands for 15 seconds, exerting pressure but without rubbing.

2.4. Microtraces sampling procedures for ESEM-EDS analysis

Microtraces were sampled using a metallic stub covered by double-sided graphite tape. The tape was directly pressed against the fingertips or the surfaces to be analyzed and then positioned on metallic stubs using sterile plastic tools.

Control stubs (CSs) were obtained from the three substrates (copper, glass, and plastic) before any fingerprint deposition, for a total of 3 CSs. Background stubs (BSs) were obtained from the first two fingers of the volunteers' dominant and non-dominant hands immediately after hand washing and air drying, amounting to 12 stubs (T0).

After the fingerprint deposition according to the protocol in Fig. 1, the following stubs were performed: on the first fingertip of the non-dominant hand, immediately after the fingerprint deposition on copper (T1h); on the glass and plastic surfaces, immediately after the fingerprint deposition on these substrates (T2g and T2p), amounting to 9 stubs, for a total number of 24 collected samples. No stub was performed on the copper bar after fingerprint deposition.

After sampling, stubs were immediately placed in sterile tubes for storage until ESEM-EDS analysis.

2.5. DNA sampling procedure

Fingerprints deposited by the second finger of the non-dominant hand on copper (T1) and by the second finger of the dominant hand on plastic (T2p) were collected. No fingerprint was collected from the glass surface for DNA analysis. Since it was not feasible to use a single fingerprint deposit for both ESEM and DNA analyses, fingerprints were deposited simultaneously, but separately processed and the protocol was optimized by duplicating the deposition step on plastic, allowing one fingerprint to be analyzed by ESEM and one by DNA-based method.

No swab was performed on the fingertip of the volunteer after contact with the copper bar.

In total, six touch DNA samples deposited following the protocol depicted in Fig. 1 were collected, two from each volunteer.

Ten additional volunteers, five women, and five men, different from the previously employed ones, were recruited only to study the TPPR of touch DNA. These volunteers placed the first finger of the dominant hand on the copper bar and immediately placed a deposit on a plastic sheet, yielding additional 20 touch DNA samples. Fingertips were deposited 1 hour after washing the hands, by pressing the palm down the fingertips of the hands for 15 seconds, exerting pressure but without rubbing, according to the deposition procedure followed by the previous volunteers.

Within 5 minutes from deposition, the sampling was carried out with flocked nylon swabs 4N6FLOQSwabs™ Crime Scene (Copan Italia S.p.A., Italy). The swab was lightly moistened in RNase-/DNase-free water to rehydrate the cells on the surface and facilitate the recovery of cellular material. Negative background controls from the used surfaces, i.e., areas of the copper bar and the plastic sheet that had not been touched, were swabbed and analyzed for background DNA.

Buccal swabs from the ten volunteers were collected with a sterile dry cotton swab (Copan Italia S.p.A., Italy) as reference samples.

After DNA collection, the swabs were stored at −20 °C until further processing.

2.6. DNA extraction, quantification and profiling

DNA from samples collected on copper and plastic sheets was isolated using a manual silica-based DNA extraction system, the QIAmp DNA Investigator Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol "Isolation of Total DNA from Surface and Buccal Swabs", with a final elution volume of 30 µL.

Buccal swabs were submitted to the Chelex extraction method [17], using the ReadyAMP™ Genomic DNA Purification System Kit (Promega). For each extraction session, a negative control was used.

DNA quantification was performed using the PowerQuant System (Promega) on the QuantStudio 5 Real-Time PCR System for Human Identification (Applied Biosystems), following the manufacturer's protocol. Of the additional 20 touch DNA samples deposited to study only DNA- TPPR, the three samples showing the highest DNA quantification values on both copper and plastic were chosen to be amplified by multiplex PCR.

Touch DNA samples were amplified by GlobalFiler IQC PCR Amplification Kit (Thermo Fisher Scientific) in accordance with the manufacturer's recommendations, using the standard 29 cycles on the VeritiPro Thermal Cycler (Applied Biosystems) instrument. Amplified products were separated and detected on the SeqStudio™ Genetic Analyzer (Applied Biosystems). Data collection and fragments analysis were conducted using GeneMapper® ID-X v 1.6 (Thermo Fisher Scientific) with an analytical threshold set to 100 relative fluorescence units (RFU).

2.7. ESEM analysis

ESEM analyses were performed with a FEI Quanta 200 FEG (FEI, Hillsboro, OR, USA) coupled with an Energy-Dispersive Spectrometer (EDS) (EDAX Inc., Mahwah, NJ, USA).

Observations and semiquantitative elemental measurements were conducted under High Vacuum Mode (10^{-5}- 10^{-6} mbar) in the area of the electron beam and Low Vacuum Mode (0.8 – 0.9 mbar) in the sample chamber.

A beam voltage intensity (acceleration voltage) at 25 kV was applied, working at magnifications between 100x and 300,000x. All images were acquired with no preliminary treatment of samples, using the signal obtained from backscattered electrons (BSE), which offers compositional contrast.

The particles of heavy atoms present on the surface of the specimen were identified by visually mapping the entire surface of the specimen at magnifications ranging from 1000X to 10,000X.

In BSE mode, the particulate is very bright (suggestive of a high atomic number), becoming a region of interest for acquiring images and data in EDS.

EDS was equipped with an X-ray ECON detector (Edax Carbon Oxygen Nitrogen) 6 UTW and a Genesis software analysis.

Each individual particle of the sample was analyzed in EDS with a spectrum acquisition time of 100 seconds, a detector saturation time (Amp Time) of 51 seconds, an incident electron beam current of 290 µA, and a spot size ranging between conventional values of 3.7 and 3.9 (about 33 pA of beam current at an acceleration voltage of 25 kV).

To obtain semiquantitative data in graphical representation, three measurements under the same conditions were performed for each sub-sample of particulate element, in accordance with the ENFSI guidelines [18], by focalizing the incident electron beam on the central area of the particle of interest. Considering equivalent areas on different sub-samples, the identified elements were classified as abundant when more than 15 particles were detected and scarce when particles were <15. When particles were morphologically detected by ESEM, the EDS semiquantitative ratio, and the relative percentages of chemical elements composing them, allowed to define the presence of silicates with metals. The classification was performed by two blinded independent raters, with the involvement of a third one in case of contrast.

2.8. Data interpretation and statistical analysis

DNA profiles were classified into single source, mixed or inconclusive profiles when considered not suitable for comparison, with less than ten typed loci [16]. The DNA profiles were compared to the reference samples from the donors, counting loci and alleles dropout (the DNA profile with more than ten typed loci, but characterized by allele or locus dropout after comparison with the reference one was classified as a partial profile). The biostatistical evaluation for the LR assessment was performed using LRmix Studio software v. 2.1.5, after estimating the dropout probability [19].

In the present study, single source profiles and mixed profiles providing a value of LR $\geq 10^6$ were deemed informative profiles to identify the donors.

The parametric or non-parametric distribution of quantification data was assessed by Stata sktest, that takes into evaluation skewness and kurtosis [20].

Since p was < 0.05, the hypothesis of a normal distribution was rejected. Therefore, descriptive statistics of the quantification data was performed to describe the median and interquartile range (IQ). A comparison of DNA content recovered from copper (T1) and plastic surface (T2p) was made using a non-parametric paired t-test (Wilcoxon signed rank test). Wilcoxon signed rank test was also used to test differences in their degradation index.

In all statistical analyses, the significance level was set at < 0.05.

The results of the ESEM-EDS analysis were evaluated only by considering abundant material and calculating the prevalence of each abundant material among the analyzed samples.

Statistical analysis and graphs were obtained using Stata/MP 15.1 and GraphPad Prism version 8.2.1.

3. Results

3.1. DNA analysis

When considering the touch DNA samples deposited by three volunteers following the protocol depicted in Fig. 1, the samples obtained after fingerprint deposition on the copper bar (T1) and plastic (T2p) yielded a very small DNA amount of 0.000–0.002 ng/µl. Background control samples did not show quantifiable DNA (data not shown). Due to the low amount of DNA, amplification of these samples was not carried out.

When considering the additional samples deposited by ten volunteers, not coupled to ESEM analysis, the median value for the DNA amount recovered from copper and plastic was 0.016 ng/µl (IQ = 0.004–0.036) and 0.038 ng/µl (IQ = 0.018–0.048), respectively. The difference between copper and plastic tested statistically significant (p = 0.0284). Results are shown in Fig. 2. The data from the qPCR of the extracted samples exhibited limited signs of degradation, with a degradation index ranging from 1.2 to 3.5 for copper (5 samples above 2.5) and from 1.7 to 3.5 for plastic (5 samples above 2.5). No statistically significant difference was shown (p = 0.5556).

IPC shift values obtained using the PowerQuant Analysis Software were less than the value specified in the sample assessment tab (≥ 0.3) for all samples except for two samples recovered from the plastic surface at T2p (IPC shift value of 0.73 and 0.40, respectively).

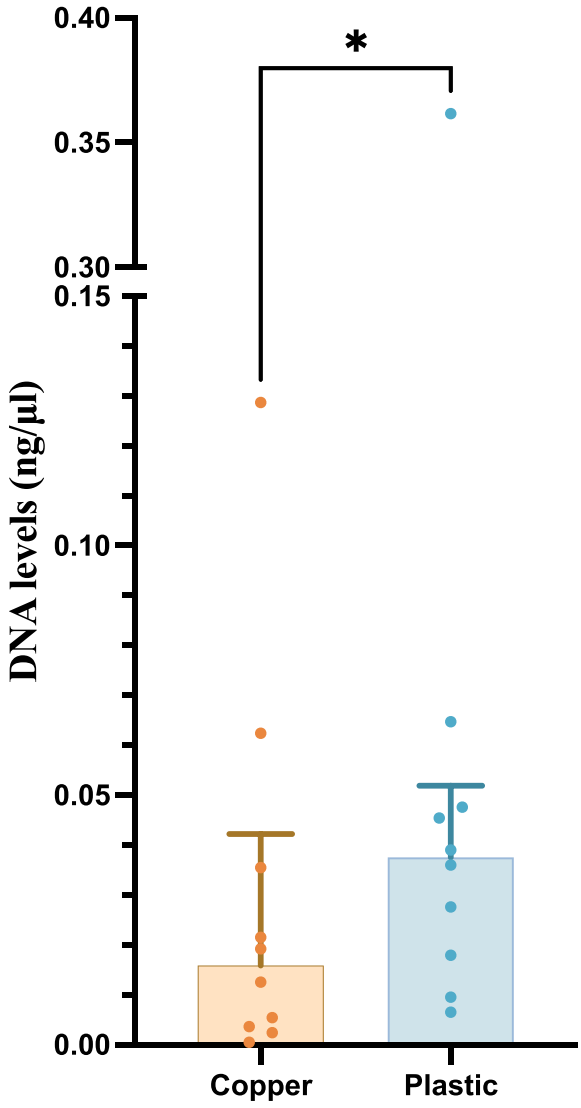

Fig. 2. Touch DNA samples deposited by ten volunteers to study DNA-TPPR only. Single column fitting image.

Of the 20 samples, those yielding the highest DNA values (from the copper bar at T1: 0.062, 0.036 and 0.129 ng/µl; from plastic at T2p: 0.045, 0.039 and 0.362 ng/µl) were selected for amplification. Of the six amplified samples 4 out of 6 profiles generated from fingerprints deposited on both substrates (copper and plastic) appeared as single source partial profiles, due to the presence of allele and locus dropout. Only 2 profiles, one per substrate, were single source and complete. Calculated LR values from the three samples swabbed on copper were always above 10^{14}, from 2.19×10^{14} to 3.38×10^{33}. On the plastic material, LR ranged from 1.00×10^{16} to 1.10×10^{24}.

3.2. ESEM-EDS analysis

ESEM analysis coupled with EDS allowed the visualization of the morphological data of all samples, and the mapping of the elemental composition. Results are reported in Table 1, considering only abundant material and displaying the prevalence of each material among the analyzed samples. A graphical representation is given in Fig. 3. The high levels of C and O identified on most graphical representations are due to

Table 1
Results of the ESEM-EDS analysis involving samples deposited by three volunteers, as in Fig. 1. The detection of abundant material and its prevalence (n. of positive samples) among the analyzed samples is reported. N.: number; *borderline as abundant or scarce.

Sample	Abundant material	N. positive samples	Epithelial cells and notes
Control stub (CS) on copper	Copper, copper phosphide	1/1	No epithelial cells
Control stub (CS) on glass	Iron, aluminum, gold, silver, silicates*	1/1	No epithelial cells, scarce particles
Control stub (CS) on plastic	Iron, silicates with magnesium, silicates with iron*	1/1	No epithelial cells, scarce particles
Background stubs (BSs)	Iron and silicates with iron	10–12/12	Epithelial cells ranging from
	Sulfur, barium	6–8/12	absent to
	Silicon, zirconium, sodium, aurum	3–4/12	abundant
	Bismuth, chromium, nickel	2–3/12	
	Aluminum, magnesium, copper, calcium	1/12	
T1 stub on hands (T1h)	Copper, iron	3/3	Abundant epithelial cells
	Silicates with iron, silicon	2/3	
	Sulfur, calcium, sodium	1/3	
T2 stub on glass (T2g)	Silicates with iron, zirconium, silicates with calcium and magnesium, calcium	2/3	No epithelial cells
	Copper, silicates with iron and copper	1/3	
T2 stub on plastic (T2p)	Silicates and oxides with copper, zirconium, silicates with calcium and calcium	3/3	No epithelial cells
	Iron and silicates with iron	2/3	
	Copper	1/3	

the adhesive substrate background.

CSs performed on copper, plastic and glass appeared relatively clean, with no epithelial cells. The copper bar showed aggregate particles, consisting mainly of copper and copper phosphide (Fig. 3, A, B). The glass and plastic surfaces demonstrated scarce particles, including iron, silicates, gold, silver, and aluminum, but copper was always absent (Fig. 3, C, D).

BSs on the hands of volunteers demonstrated a relatively clean surface, containing three types of structures: larger aggregates containing carbon; aggregates containing sodium, chlorine, and potassium; round/oval formation, showing silicon (Si) (Fig. 3, F, I). Varied and abundant metals, especially iron, were detected at ESEM-EDS analysis of particles, except for copper, which was non-abundant (Table 1 and Fig. 3, E, H).

At T1, the stubs on the fingertips of volunteers after contact with the copper bar (T1h) demonstrated abundant copper and iron (Fig. 3 J, K, L, M), as well as particles with sodium, chlorine, and potassium, similarly to BSs.

At T2, stubs performed on the glass surface (T2g), after the fingerprint deposition, demonstrated a very clean surface, except for some round/oval formations containing Si and again sodium, chlorine, and potassium, similarly to BSs.

Stubs performed on the plastic surface (T2p) demonstrated a more heterogenous surface with more particles compared to the glass, and said particles contained particularly copper. Abundant calcium and silicates with calcium were also found.

4. Discussion

In this study, we simulated a scenario including contact events to analyze the bidirectional transfer of both human DNA and metal particles between fingertips and touched surfaces. Among different possible metals, our proof-of-concept study particularly focused on transfer involving copper, given its presence in wires and environmental components that could be encountered in forensics as well as its uniform composition compared to other metals [8,9,12].

According to the literature, in similar contact events situations, in order to assess the probability of detecting DNA, the possible variables impacting each transfer event should be considered [1].

The first feature of the present study was the total yield amount of DNA obtained from the copper substrate. The contact of the fingertips of the hands of the three volunteers with both the copper bar, the primary surface, and the plastic surface, the secondary substrate, provided very small amounts of DNA. This could be due to a low propensity of the 3 volunteers to deposit DNA by touch – as described for light or poor shedders [21]. Moreover, a little contact time (15 seconds) was employed in our study, while longer times could be necessary to mimic most criminal acts and intervals of 30 seconds to a few minutes are experimentally used for handling doorknobs and weapons [22].

The present conditions were chosen to simulate a “worst case scenario”, but bearing in mind that a longer contact time could provide more DNA amounts.

To better assess the impact of the metal surfaces on the DNA-TPPR, fingerprints were deposited on copper and plastic by ten other volunteers for the DNA quantification and typing only, considering that in our study ESEM and DNA analysis were performed in parallel on fingerprints deposited simultaneously, but separately processed. After DNA measurement with qPCR, the results demonstrated a wide inter-individual variability in the amount of deposited material, further confirming the individual differences on the ability to shed DNA [21], to the point that some DNA transfer on copper yielded DNA quantity in the lower picogram range. This was partially expected, since it was shown that more DNA is transferred and recovered from porous (e.g. wood and fabric) compared to glass, metal and plastic surfaces [5,6] and poor recovery from copper surfaces has been particularly reported [7]. According to these studies, a statistically lower amount of DNA was recovered from copper than from plastic in our research.

The results might partially be explained by the sampling medium. The rinse/soak with a chelating agent or tape-lifting might represent an alternative approach to collect “touch DNA” from copper-containing metal surfaces, allowing better recovery [7,9,23]. On the other hand, Martin B et al. demonstrated that tapelifting on average produced less donor alleles than swabbing from different surfaces [24] and several limitations have been reported for both the tapelifting, the stickiness of which might complicate the DNA extraction procedure, and soaking, due to its destructive nature and possible impact on nucleic acid integrity [7].

The hypothesis that the low levels of DNA recovered from copper are due to low persistence seems unlikely, as the DNA samples were taken within 5 minutes of deposition. [12].

Metal substrates are also known to interfere with DNA profiling. However, limited degradation due to the presence of copper was shown, similarly to Patterson et al. [10] and no inhibition was observed for samples recovered from the copper surface.

As demonstrated by the swabs performed on plastic after the second fingertip deposition (T2p), DNA was again transferred in amounts that allowed to identify the donor with LR $> 1.00\times10^{16}$. This outcome was once more expected, given the primary or direct nature, from a DNA perspective, of the contact with the secondary surface.

When selecting the three samples yielding the highest amounts of DNA from both copper and plastic, these allowed to generate STR profiles always matching the donor, with no inconclusive STR profiles produced from copper-containing metal substrates.

Regarding SEM and ESEM, the crucial role of such technology in detecting metallic residues left by weapons has already been widely highlighted [13,14,25,26]. However, to the best of the authors' knowledge, no experimental research has been performed so far to

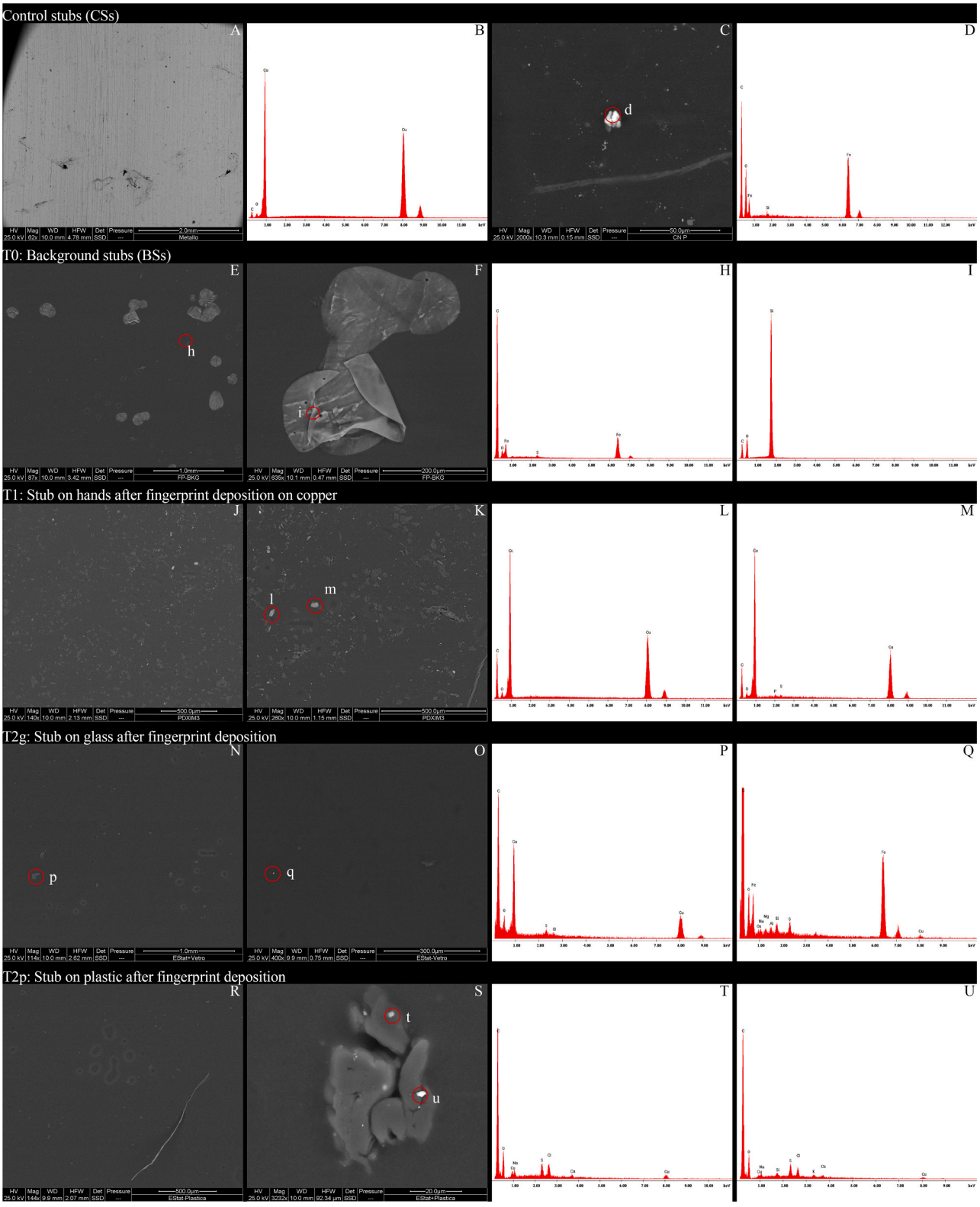

Fig. 3. ESEM topographical/morphological images and Energy-dispersive x-ray spectrum with compositional analysis of different exogenous residues. A-D represent Control stubs (CSs); in A, the morphology of the copper bar; in B, the typical spectrum consistent with a copper (Cu); in C, an example of a spectrum obtained from glass; in D, an example of a spectrum obtained from plastic. E-I show Background Stubs (BSs); in E and F, a few epithelial cells are visible, as well as round/oval formation; in H and I, the spectra of the particles marked with the respective small letter in E and F are displayed, showing iron (Fe) and silicon (Si). J-M show the results of the stubs performed on the first non-dominant finger of the volunteers after fingerprint deposition on copper; in J and K, abundant particles; in L and M, the spectra of the particles chosen in J and Km, named "l" and "m", showing mostly copper (Cu). In N-Q, results of the stubs performed on glass after the fingerprint deposition at T2p; in N and O, very clean surface; in P and Q, the spectra of the few elements visible in N and O, named "p" and "q", shows varied elements, including copper. In R-U, stubs performed on plastic after the fingerprint deposition at T2p; in R and S: morphological examination with clean surface, except for some particles; in T and U, the spectra obtained from particles "t" and "u" showing varied elements, including copper. 2-column fitting image.

evaluate the possibility of detecting metallic residues left during a contact event.

The results of the present proof-of-concept showed the possibility of detecting metal residues on the hands of the volunteers, acting as vectors, as well as on secondary touched surfaces, with constituent elements matching the touched surface in copper. As shown by our results, during the contact event with copper, while DNA was transferred from the hands to the substrate, copper particles were picked up onto the fingertips, leading to positive ESEM-EDS detection at T1h, with abundant copper on all samples. The result is consistent with other transfer mechanisms previously described in forensic pathology on cadaveric skin, e.g. during electrocution or sharp force trauma [13]. In the latter case, it has been shown that the detection of metallic microtraces on body injuries might allow to link the wounds to the murder weapon [14].

ESEM-EDS analysis also allowed to detect metal particles that were transferred along with DNA material, being deposited on secondary surfaces in glass and plastic during the second contact events.

Secondary surfaces were both relatively poor of particles, particularly the glass surface, where copper was detected by ESEM-EDS analysis only in 1/3 of the samples. Plastic on the other hand contained more particles, including aggregates with abundant copper, shown as silicates and oxides with copper, allowing to suggest that plastic could be more suitable as a secondary surface to collect metal particles.

On both secondary touched surfaces and BSs, some round-oval formations, containing Si, were also detected. It is known that contaminants could be found on fingermarks analyzed by ESEM-EDS analysis, due to food, dust, cosmetics, nicotine and so on [27]. In our study, such formations were considered consistent, from a morphological and elemental point of view, with the previous use of soap.

No epithelial cells were found on secondary surfaces by ESEM-EDS analysis, and their presence was rather inconstant also on BSs. This might be explained by the inter-individual variability in the skin hydration, since larger uplifting desquamating layers of the stratum corneum have been observed on dry compared to normal skin. Cosmetics or medications as well, such as the use of hand creams, might impact the visibility of epithelial cells sloughed from the outermost layer of the skin surface [28]. Moisture could also impact the deposits of DNA from the hands, but it has to be reminded that skin-derived DNA is represented by a combination of sources, including cell-free DNA [1].

Since the DNA samples submitted to STR typing could not be inspected by ESEM, a direct connection between cellular material observed by ESEM and DNA typing results is lacking. To visualize cellular material and to inform on the abundance of recovered epithelial cells, fluorescent dyes could be successfully applied especially on non-porous surfaces [29], and could be explored in a future study.

Nevertheless, it has to be stressed that ESEM and DNA analysis cannot be applied to the same sample, necessitating a parallel approach with separate fingerprints for each analysis in this proof of concept study, not excluding however that for future application on criminal cases the substrate of interest could permit both analyses. Clearly, the usefulness of the approach for the donor identification is contingent on the yield of sufficient DNA amounts, which in turns is dependent on several variables impacting DNA TPPR, for example higher DNA could be yielded if the contact time is longer or if the hands of the perpetrator are contaminated by body fluids rich of self-DNA.

Applied to a crime scene, these results highlight how stubs performed on the hands of a suspected perpetrator or on secondarily touched surfaces, especially on plastic, might allow to trace the metal touched before committing the crime, i.e. a copper door handle or weapon. This method is qualitative, requires a comparison with background stubs on the involved surfaces, but could be used to discriminate between different touched surfaces or objects and for DNA activity level assessment.

Moreover, some considerations connected to the background of the hands should be further discussed. Indeed, BSs performed on the hands of the volunteers prior to the contact with the primary surface revealed abundant and heterogenous non-metal and metal material. The presence of carbon confirmed the skin origin and proved the reliability of our method [15]. The observed particles containing sodium, chlorine and potassium were considered as signs of sweat, since it is known that inorganic compounds of eccrine origin could be quantified in fingermarks, including sodium, potassium, chlorine, ammonia, calcium, sulphur, magnesium [27,30], and this has been shown also by ESEM-EDS analysis [15]. On the other hand, the above mentioned round-oval formations, likely due to the use of soap, underlines that handwashing could not only remove exogenous material [13], washing out metallic residues and nullifying the analysis, but also result in additional contaminants, further complicating the interpretation. The high sensitivity of ESEM-EDS analysis, demonstrating particles likely deposited with the washing procedure itself, required in our study a careful analysis with classification into abundant and scarce material. This could suggest that, translated into an authentic forensic case, only abundant material shown on repeated samples could be used as evidence and background samples are essential.

In addition to that, the elemental composition of BSs samples also revealed several and variable metal particles on the hands of volunteers. Metal particles can be identified in fingerprints, particularly iron which originates from apocrine glands [19], and this was consistent with our results. The varied material recovered on the hands of volunteers, despite a previous handwashing, that should have removed exogenous material, certainly represents a drawback of the here proposed approach. The results further highlight the need for background samples to be collected from the involved surfaces, from the hands of a suspected perpetrator, on different areas, as well as the meaningfulness of a detailed framework of circumstances for assessment under activity level proposition.

To avoid metals too often detected in fingerprints and to prevent heterogeneity in the composition as seen with metallic alloys, in this study copper was chosen to study the transfer of metallic particles, and, as expected, copper was detected only in scarce amounts in the BSs. More complex patterns are expected from heterogeneous materials, such as steel.

Lastly, a major limitation of the present study resides in the low number of volunteers involved in the fingerprint for ESEM-EDS analysis, although substantial efforts were invested, and results seem promising. Nevertheless, the present work was intended as a proof-of-concept to highlight the transferability of metal particles, as well as the feasibility and potential usefulness of a parallel ESEM-DNA approach to assess this transfer.

An example of relevant propositions at the activity level could be the following:

a) The suspect touched a copper doorknob before strangling the victim b) The suspect had social contact with an unknown person who touched the copper doorknob and strangled the victim.

In both scenarios involving transfer material, it would be important to select the variables, graphically nodes, to describe the Bayesian network structure and to facilitate the elicitation of probabilities.

Clearly, this preliminary study does not aim to provide probability data that can be used in the context of a Bayesian network, but only to suggest the incorporation of variables, particularly corresponding to the transfer event and the background presence, within the network. Future studies on a wider casuistry are needed to assess the probability of transfer and recovery of metal particles and DNA, depending on a framework of circumstances, to address activity levels proposition.

5. Conclusion

Our proof-of-concept study has shown that the detection strategy based on ESEM analysis of metal particles transferred to the hands of volunteers during contact with a copper metallic surface and deposited on secondarily touched items could potentially allow for tracking the

DNA transfer together with metal particles at a crime scene. The ESEM-DNA parallel approach here presented is a preliminary and necessary step highlighting how further research on a wider casuistry could help to address the interpretation of results given activity level propositions.

Ethics

The present study has been carried out in accordance with the Declaration of Helsinki

and has been approved by the Bioethical Committee of the University of Bologna (Prot. n. 0201147 approved on July 20th, 2023).

Funding source

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Giulia Fazio: Formal analysis. **Laura Valentini:** Formal analysis. **Carla Bini:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Sara Amurri:** Formal analysis. **Pietro Gobbi:** Methodology. **Susi Pelotti:** Supervision, Conceptualization. **Arianna Giorgetti:** Writing – original draft, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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