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Development of a matrix-matched standard for the elemental analysis of human hair by LA-ICP-MS

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Human hair as a biological matrix presents a detailed distribution profile of organic and inorganic components in the body. In comparison to blood and urine, hair introduces great advantages as it provides a temporal record with growth and longer detection window of analytes. Elemental analysis by LA-ICP-MS has been studied using different strategies for standards, however there is not a reference material that reproduces the physical and chemical properties of hair. This work demonstrates the development of a matrix-matched calibration standard using a keratin film doped with metals of interest for LA-ICP-MS analysis. The material was synthesized from extracted human hair keratin using the "Shindai method", purified, spiked, and cross-linked to obtain a thin homogenous film. A series of calibration standards were prepared for trace concentrations of Ba, Pb, Mo, As, Zn, Mg, and Cu. Linear calibration models were built with limits of detection as low as 0.43 $\mu\text{g g}^{-1}$ for Pb. The material was characterized by its thickness, homogeneity, and matrix-matching compared to human hair. The calibration materials were cross evaluated with spiked single human hairs for verification. These results provide a new set of standards for LA-ICP-MS to be used in internal medicine, forensic toxicology, and biological anthropology.

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Kaitlyn Bonilla is a fifth-year PhD candidate in the Department of Chemistry at the University of Central Florida. Kaitlyn is under the mentorship of Dr Matthieu Baudelet at the National Center for Forensic Science. She is a recipient of the National Institute of Justice (NIJ) Graduate Research Fellowship for the development of a matrix-matched reference material for the elemental and molecular analysis of hair by laser-ablation. Her

dissertation research focuses on the application of trace elemental hair analysis by laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). Her research addresses the critical challenge in the reference material synthesis for quantification of hair. Kaitlyn's work bridges analytical chemistry, materials science, and forensic toxicology. Her work aims to improve the accuracy and reproducibility for chemical profiling of hair in forensic applications. This research will provide the forensic toxicology community with a new calibration standard for elemental analysis of human hair.

1 Introduction

Hair analysis has a wide variety of applications including medicine, cosmetics, forensic science, and bio-archaeology. Hair as a biological matrix provides a detailed distribution of temporal information in the body. It is composed of a hierarchical organization of subunits consisting of α -keratin protein chains that allow for metals to be contained thanks to a high affinity for the sulfhydryl groups on the amino acids that comprise keratin.¹ Therefore, these metals are easily incorporated into the hair matrix. The growth rate of human hair is approximately 10 mm per month allowing for the concentration of metals in hair to reflect the individual's exposure.

A long-term record of exposure in an individual can be reflected by the metal and metalloid concentrations in the hair matrix. Although urine and blood are commonly studied for analytes of interest in the body, they are only present for days to weeks.² Hair is known to have higher stability of analytes for weeks up to months and can therefore provide a longer record of exposure. Hair also provides greater concentration of analytes,¹ creating a more sensitive analysis. The trace elemental profile in an individual's hair can be separated into two different categories: macro and micro-elements.³⁻⁵ The elements are then further split into either toxic or essential.^{6,7}

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Elements such as Mo, Pb, Ba, Mg, Cu, and Zn are commonly studied for pollution exposure levels,⁸ correlation with disease,⁹ and nutrition.¹⁰ In forensic studies, As is studied to evaluate acute or long-term exposure to toxic elements in populations and individuals.^{11,12} Their typical respective concentrations in human hair are presented in Table S1, although variation in the values exist when considering external contamination at the surface of the hair.^{13,14}

Routine investigation of metals and metalloids within the hair matrix consists of bulk analysis using large amounts of sample, undergoing acid digestion, then analysis by inductively coupled plasma-optical emission spectrometry (ICP-OES), ICP-mass spectrometry (ICP-MS), or electro-thermal vaporization atomic emission spectrometry (ETV-AES).^{6,13,15–17} Bulk analysis is also not demonstrative of variations of growth and external contamination cannot be differentiated from endogenous uptake in the hair. As a solution, laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) allows for direct sampling along the growth, the root, and any portion below the skin surface that is protected from external contamination in a single hair strand.^{1,18}

With laser-based analytical techniques, matrix-matched standards are necessary for quantification to reduce sample matrix effects. Reference materials for elemental hair analysis exist as hair powder to validate the elemental concentration. Previously studied reference materials include “No. 13 Human hair” from National Institute for Environmental Studies (NIES) in Japan, and “ERM-DB001” from the Joint Research Center (JRC) in Belgium. However, these materials are not matrix-matched because of their granularity. For LA-ICP-MS hair analysis, commercially available certified or standard reference materials (CRM or SRM) have been studied by Noël *et al.*^{19,20} They analyzed grizzly bear hairs by LA-ICP-MS using dogfish liver powder (CRM DOLT 2) pressed into a pellet. However, it has been evaluated compact powder samples show a different laser ablation behavior in comparison to solid structured materials.²¹ Consequently, this approach of a pressed powder pellet provides challenges for quantification of a single hair. Another strategy for matrix-matched hair standards includes soaking of single hair strands in solution with a known concentration of elements of interest.²² This approach has been studied by Dressler *et al.*²² concluding high sample throughput and low limits of detection (LOD) from ng g⁻¹ to µg g⁻¹. This procedure provides versatility as it can be applied to a variety of hair types including human or animal. However, this approach can provide limitations as the samples are not homogenous and the concentrations of trace elements already present in the hair induce a natural bias in the referenced hair strand.

An innovative study by Cheajesadagul *et al.*²³ incorporated the use of keratin films doped with Pb to use as a matrix-matched standard for LA-ICP-MS analysis. In this study, the use of Pb doped keratin films were compared to soaked human hair strands in solution. The keratin was extracted from human hair using the “Shindai method”, producing a protein-rich solution. The film was then formed by self-assembly, self-aggregation, and cross-linking of the keratin proteins with

trichloroacetic acid (TCA) and calcium chloride (CaCl₂). The doped keratin films showed better linearity and Pb retention than the soaked hair approach. Due to the limited surface area on a hair strand, they could not retain higher concentrations. However, keratin films provide a larger surface area allowing for a higher quantity of analytes. The quantification of Pb using this approach provided a LOD of 0.082 µg g⁻¹. This LOD is very low, thanks to the hair samples used for synthesis of this material being Pb-free. The authors encouraged the expansion to other trace elements for quantification. While innovative, aspects of the film preparation were not optimal for a representative hair calibration standard. The dimensions of the sample were not controlled as only pipetted on a square slide of 2.5 × 2.5 cm². This may have led to non-reproducible contours for the material if not covering the whole slide. If the material were square, this may have increased the heterogeneity of material distribution due to the lack of symmetry. There was also no mention of the thickness of the samples, especially in comparison to hairs.

This present work presents the synthesis of keratin films spiked with trace metals to be used as matrix-matched calibration standard for LA-ICP-MS analysis. This material was designed as a single-layered keratin sample of 100 µm to reproduce hair thickness. To increase homogeneity, the material was prepared in a circular mold, providing physical constraint to enhance the reproducibility of its synthesis. This study presents the morphology, characterization and evaluation of the keratin film for quantitative elemental analysis of hair.

2 Materials and methods

2.1 Instrumentation

The standards and samples were analyzed by LA-ICP-MS using a J200 Tandem LA/LIBS system (Applied Spectra Inc, West Sacramento, CA) coupled with a PlasmaQuant MS Elite (Analytik Jena, Beverly, MA). The optimized conditions of the LA and ICP-MS system are shown in Table 1. These parameters were optimized with a keratin film and hair samples using 10 laser

Table 1 LA-ICP-MS instrument parameters

Laser ablation	J200 (Applied Spectra Inc.)
Laser wavelength	266 nm
Laser repetition rate	10 Hz
Laser spot size (diameter)	40 µm
He flow	2.0 L min ⁻¹
Ar flow	0.5 L min ⁻¹
Laser fluence	3.3 J cm ⁻²
ICP MS	PlasmaQuant Elite (Analytik Jena)
RF power	1380 W
Plasma gas flow	9 L min ⁻¹
Auxiliary gas flow	1.2 L min ⁻¹
Dwell time	10 ms



pulses per sampling location. Fluence was adjusted to ablate approximately 75% of the hair diameter without breakage. Gas flows (He and Ar) were optimized with the keratin film as the amount to transport the maximum mass from the ablation cell to the ICP torch.

2.2 Reagents and samples

Metal standard solutions were prepared from 1000 $\mu\text{g g}^{-1}$ standard solution of Zn, Cu, Mg, Ba, Pb, Mo, and As (SCP SCIENCE, USA). All dilutions were prepared with ultra purified deionized water (Smart2Pure, Thermo Scientific, USA). The keratin film and hair samples were digested with 67% nitric acid (TraceMetals grade, Fisher Chemical, USA) using a 5000 Multiwave microwave digestion system (Anton Paar, Ashland, VA). The digested samples were prepared with 2% nitric acid for ICP-MS analysis.

2.3 Keratin film standards

The overview of the keratin film synthesis is shown in Fig. 1. The synthesis began by keratin extraction from a variety of human hairs donated from anonymous individuals at a local hair salon (Great Clips, Orlando, FL). The hair strands were washed by sonication in distilled water for 30 minutes. The hair samples were then submerged in a methanol–chloroform solution (2 : 1 v/v) for 24 h and rinsed for 30 minutes with distilled water by sonication for removal of external lipids. The cleaned hairs (5 g) were mixed with 100 mL of Shindai solution,^{23–25} which contains 25 mM Tris–HCl (pH 8.5), 2.6 M thiourea, 5 M Urea, and 5% 2-mercaptoethanol (2-ME) at 50 °C for 72 h. The keratin mixture was then filtered at room temperature. For protein isolation, the solution was dialyzed by centrifugal filtration for 4 h (4 °C) at 5000 rpm (Macrosep 10 K, Cytiva, USA). The purified protein solution was analyzed by fluorometric quantification (Qubit 4, Thermo Fisher, USA) protein assay ensuring a concentration of 40–60 $\mu\text{g }\mu\text{L}^{-1}$ for appropriate cross-linking. The dialyzed keratin solution was then directly pipetted into 40 mM CaCl_2 (for Ba, Pb, Mo, Zn, Mg, Cu standards) or 5% TCA (for As standard) in a 10 mm round cavity silicone mold (Electron Microscopy Sciences, USA) forming a film from a single layer of keratin. The protein aggregate was formed immediately by self-assembly, self-aggregation, and cross-linking. The films were cross-linked for 1 h at room temperature then were washed twice with ultra purified deionized water and left to dry at room temperature overnight.

The keratin films were spiked in multi-element solutions with three groupings consisting of {Ba, Pb, Mo}, {Zn, Mg, Cu}, and {As} to minimize interferences. The multi-elemental solutions consisted of a 0.075 M EDTA buffer brought to pH 9. The purified keratin was then mixed with the multi-element buffered solutions and cross-linked to fabricate the film. Two groupings of standards were prepared with nominal concentrations of {0, 2, 10, 20, 40 $\mu\text{g g}^{-1}$ } for Ba, Pb, Mo, As and {0, 20, 100, 200, 400 $\mu\text{g g}^{-1}$ } for Zn, Mg, Cu to represent the ranges of concentrations in hair. The nominal concentrations are defined as S1, S2, S3, S4, S5.

The spiked keratin film concentrations were verified by nebulization ICP-MS. The entire keratin film (~10 mg) was digested with 2 mL 67% nitric acid then diluted to fit in the operational ICP-MS calibration curve.

2.4 Homogeneity

The homogeneity of the keratin film calibration materials was measured by a $4.5 \times 4.5 \text{ mm}^2$ grid of 100 spots across the surface. Each location was separated by 500 μm to cover the entirety of the calibration material. The keratin films were secured between two cardstock and fixed to a glass microscope slide for LA-ICP-MS analysis. The LA-ICP-MS conditions for homogeneity determination of the reference materials are displayed in Table 1.

2.5 Hair samples

Validation of the standard material was performed on 60 single human hairs. The samples were scalp hair, cut close to the scalp, from one individual. The samples were cleaned following the methanol–chloroform procedure as the synthesis of the keratin film in Section 2.3. The single hairs were doped by soaking in a 0.075 M EDTA buffer solution for 72 h in three groupings {Ba, Pb, Mo}, {Zn, Mg}, and {As} to minimize interferences. The hairs were doped at nominal concentrations of 20 $\mu\text{g g}^{-1}$ (Ba, Pb, Mo, As) and 200 $\mu\text{g g}^{-1}$ (Zn, Mg). The hair cuttings (1 cm) were fixed on a glass microscope slide using copper double-sided adhesive tape for LA-ICP-MS analysis, justifying the exclusion of Cu from the list of tested elements. Copper double-sided adhesive tape was nonetheless preferred to plastic double-sided tape for its smoothness and flatness. LA-ICP-MS analysis of each hair was measured at 25 locations along the length. The LA-ICP-MS conditions for the elemental determination of the spiked hairs are the same as for the calibration materials (Table 1).

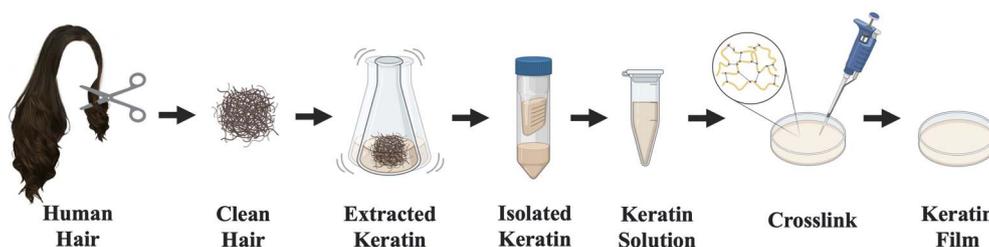


Fig. 1 Synthesis overview of reference material. This figure was created with BioRender.



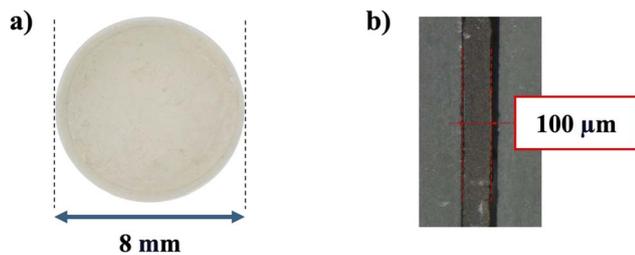


Fig. 2 Dimensions (a) and thickness (b) of the keratin film.

2.6 Microscopic analysis

Microscopic 3-D depth profiles were acquired to characterize the morphology and laser ablation of both the reference material and hair samples using digital microscopy (VHX 6000, Keyence, Itasca, IL, USA).

3 Results and discussion

3.1 Material characterization

3.1.1 Dimensions of material. The dimensions of the standard material are shown in Fig. 2, measured by digital microscopy.

The diameter is approximately 8 mm, with a thickness of 100 μm . The thickness was measured by cutting the film in half with

a utility knife and placed between holders to measure the thickness transversally. The standard shows similar thickness in comparison to human hair ($\sim 80\text{--}100\ \mu\text{m}$).¹²

3.1.2 Laser ablation characterization. The keratin films were evaluated for their ablation behavior in comparison to single human hairs. The reference materials and hairs were ablated using the same conditions (40 μm laser spot size and 1.86 μJ laser energy). The crater size shows comparable ablation behavior to human hairs, as shown in Fig. 3. The depth profile of the reference material was measured by digital microscopy after 10 laser pulses resulting in a crater width of 85 μm and 25 μm in depth, respectively. The similarity of the dimensions of the laser ablation sampling demonstrates the matrix-matching between the reference material and human hair for laser-ablation.

3.1.3 Elemental spiking yield. The elemental yield of the spiked reference materials were evaluated by nebulization ICP-MS (results shown in Table 2). The reference material blank (S1) contained low concentrations of metals, except for Mg, Zn, and Cu. The presence of these trace elements in the blank keratin matrix is possibly due to their intrinsic presence within human hair.²⁶ Although the protein solution was purified prior to cross-linking, these essential elements were retained, introducing a bias. For calibration, the trace metals were paired in several elemental groupings according to their physical properties. The spiking of the metals within the keratin films yielded concentrations between 14 and 100%,

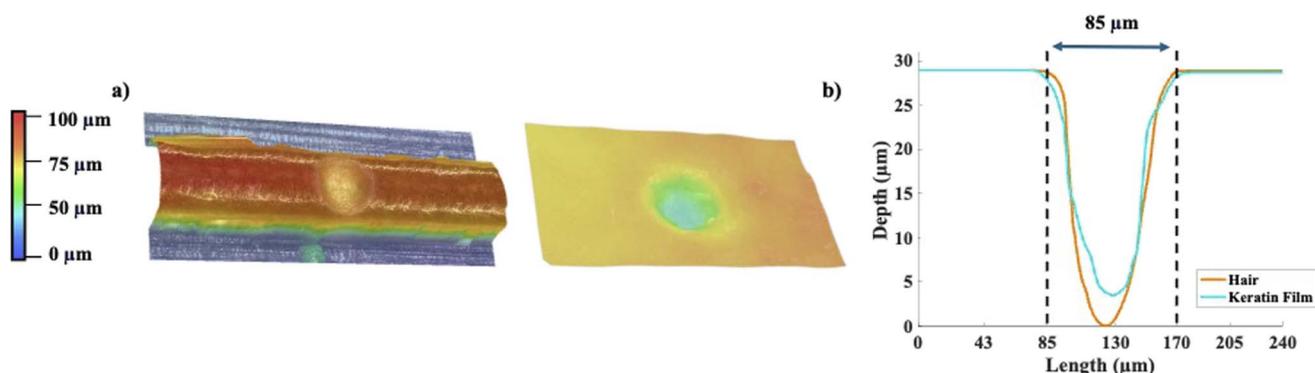


Fig. 3 (a) Comparison of laser-ablation crater of human hair (left) and reference material (right). (b) A cross-section of the depth profile is measured along the xz-plane.

Table 2 Metal concentration of keratin films obtained by ICP-MS

Element	ICP-MS concentration ($\mu\text{g g}^{-1}$)				
	S1	S2	S3	S4	S5
Nominal ($\mu\text{g g}^{-1}$)	0	2	10	20	40
⁷⁵ As	0.35 \pm 0.09	0.80 \pm 0.35	1.39 \pm 0.68	6.63 \pm 0.36	14.6 \pm 0.84
⁹⁸ Mo	0.69 \pm 0.07	1.06 \pm 0.09	2.64 \pm 0.33	4.51 \pm 0.19	7.78 \pm 0.31
¹³⁸ Ba	0.29 \pm 0.03	0.86 \pm 0.04	2.72 \pm 0.06	4.09 \pm 0.07	5.57 \pm 0.32
²⁰⁸ Pb	0.38 \pm 0.03	1.07 \pm 0.12	4.32 \pm 0.27	7.80 \pm 0.39	11.9 \pm 0.35
Nominal ($\mu\text{g g}^{-1}$)	0	20	100	200	400
⁶⁵ Cu	6.41 \pm 0.50	15.2 \pm 0.60	47.4 \pm 1.63	75.4 \pm 1.43	153 \pm 1.14
²⁴ Mg	15.9 \pm 0.33	20.1 \pm 0.32	45.3 \pm 0.14	67.6 \pm 0.71	90.9 \pm 0.69
⁶⁶ Zn	8.61 \pm 0.10	14.8 \pm 1.13	52.8 \pm 1.09	109 \pm 1.99	228 \pm 1.51



due to their binding interactions with the EDTA chelating agent.²⁷ The decreasing yield for larger nominal concentrations is possibly due to the saturation and the natural

presence of these elements embedded within the keratin matrix. The metals could have also yielded lower concentrations due to loss during the washing process.

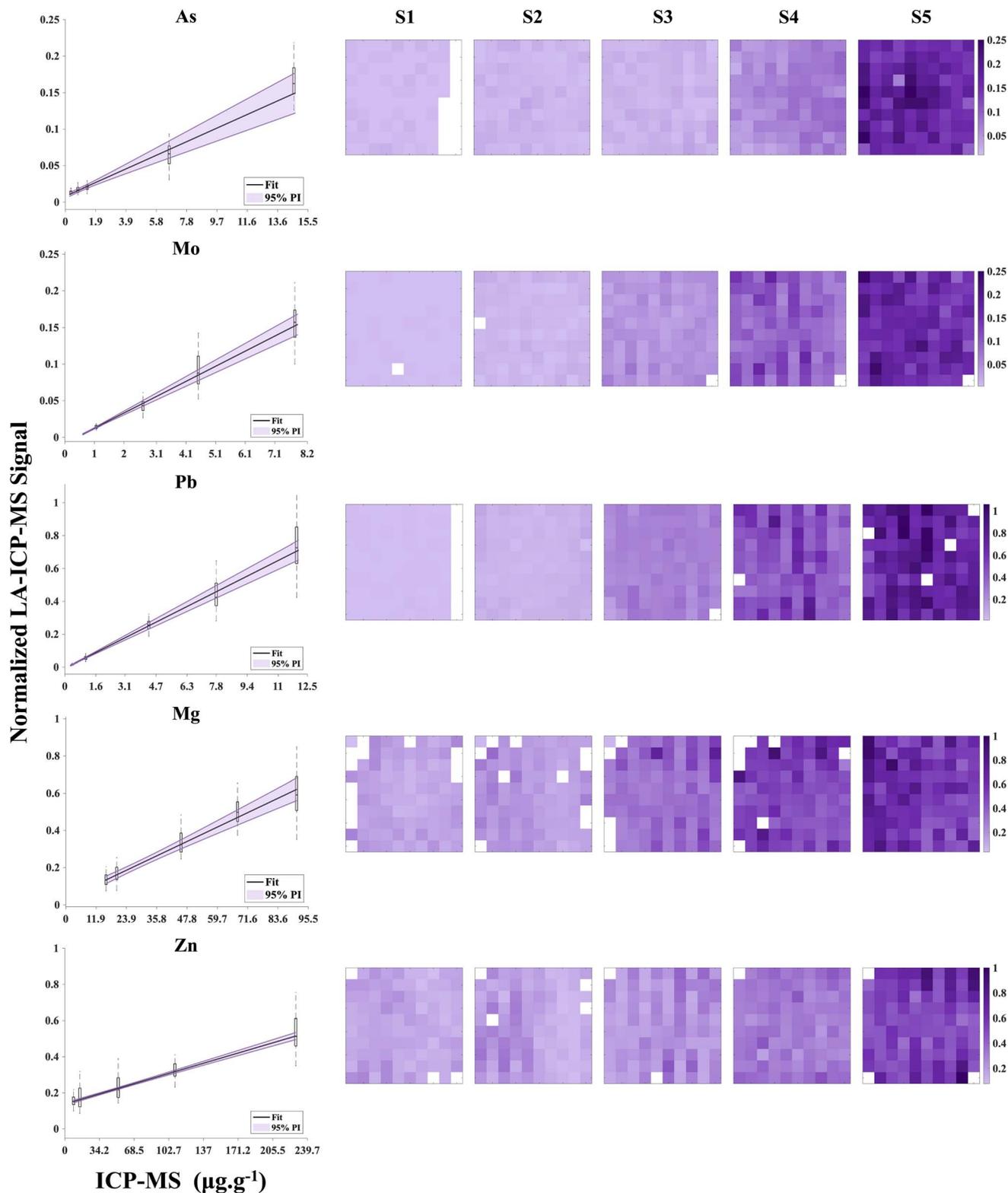


Fig. 4 (Left) LA-ICP-MS calibration curves for As, Mo, Pb, Mg, and Zn. The black line represents the linear fit and the purple shaded area is the 95% prediction interval (PI). (Right) Maps of LA-ICP-MS signal for each reference material (normalized to ^{34}S signal). Calibrations for Ba and Cu are available in Fig. S1.



Table 3 Analytical figures of merit for the keratin film calibration curves (R^2 , LOD, and LOQ in $\mu\text{g g}^{-1}$)

Element	R^2	LOD	LOQ
As	0.9877	0.62	0.88
Mo	0.9973	0.74	0.77
Ba	0.9915	0.45	0.60
Pb	0.9978	0.43	0.48
Cu	0.9918	8.67	10.9
Mg	0.9943	19.0	22.1
Zn	0.9988	13.0	17.1

3.1.4 Homogeneity of material. The homogeneity of the spiked reference materials (S1–S5) was characterized by mapping the spatially resolved LA-ICP-MS signal of 100 locations distributed on a 10×10 grid (Fig. 4, right section). The element of interest in each film was characterized by its median and its interquartile range (IQR) between the 25th and 75th percentile of the 100 values. Any value outside of the IQR was considered an outlier adding to any heterogeneity of the film for this element. As shown in Fig. 3, these outliers mostly occurred on the outer region of the film (due to the coffee ring effect²⁸), and for the S1 films where the intrinsic bias may be inhomogeneous in the keratin structure of the films.

3.2 Calibration standards

The keratin films were evaluated for their analytical performance as calibration standards by measuring analytical figures of merit including linearity (R^2), limit of detection (LOD), and limit of quantification (LOQ). Calibration curves were built by correlating the LA-ICP-MS signal for 100 points (minus the outliers as described in Section 3.1.3) per sample (normalized to ^{34}S) with the resulting ICP-MS concentration ($\mu\text{g g}^{-1}$) in the spiked keratin films (Fig. 4, left section). The boxplots illustrate the LA-ICP-MS signal distribution by highlighting the median and IQR. As shown in Table 3, the doped elements displayed good linearity (0.98 or above), presenting strong correlation between the ablated signal response and target concentration in the keratin film. The LOD and LOQ were determined using the 95% prediction intervals of the linear model, as described by J. M. Mermet.²⁹ The LOD and LOQ range between 0.43 and $0.48 \mu\text{g g}^{-1}$ for Pb to 19.0 and $22.1 \mu\text{g g}^{-1}$ for Mg. Due to their presence in hair, further investigation should be performed to decrease the signal dispersion on the higher concentration standards for Cu, Mg, Zn to reduce the LOD and LOQ values.

Table 4 Comparison between ICP-MS and LA-ICP-MS concentrations on soaked hair samples ($\mu\text{g g}^{-1}$)

Element	Nominal	ICP-MS	LA-ICP-MS
Zn	200	88.2 ± 1.8	83.4
Mg	200	81.3 ± 1.0	95.0
As	20	4.82 ± 0.45	3.33
Pb	20	0.25 ± 0.06	0.29
Mo	20	1.17 ± 0.04	0.82
Ba	20	1.39 ± 0.07	Not detected

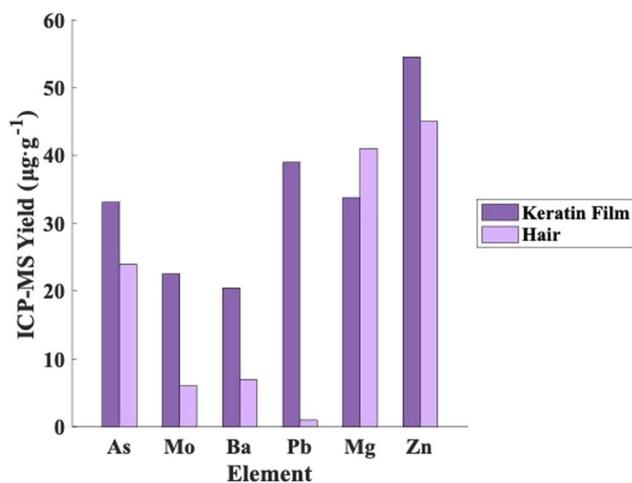


Fig. 5 Comparison of ICP-MS yield of keratin film S4 and soaked hairs.

3.3 Elemental analysis of hair

To evaluate the analytical performance of the calibration material, sixty hair samples from one individual were studied under the same LA-ICP-MS conditions as the keratin films. The single hairs were doped with a multi-elemental solution using an EDTA buffer as described in the methods Section 2.5. Table 4 lists the nominal concentration of the doping solution, median LA-ICP-MS concentration, and their corresponding bulk ICP-MS values in the spiked hairs. Only As, Mo, Mg, and Zn showed concentrations above LOD for LA-ICP-MS. The concentration results for Pb and Ba were below LOD due to their low

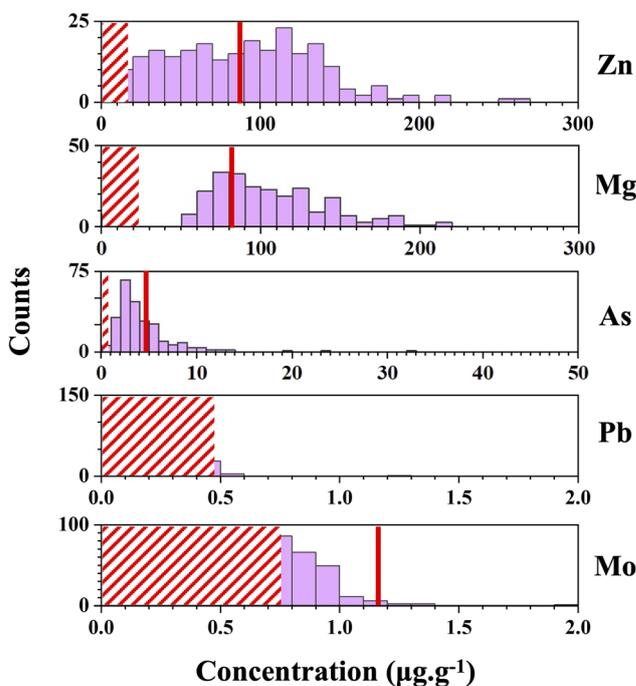


Fig. 6 LA-ICP-MS distribution of soaked hairs in comparison with the corresponding bulk ICP-MS value (red line). The red dashed section represents the LA-ICP-MS concentrations below LOQ.



concentrations within the hair, despite the soaking. Although the use of soaked hair strands is a well-established technique for reference material development,²² it resulted in a lower doping yield compared to the doped keratin films (shown in Fig. 5), despite both being prepared using the same nominal concentration. Mg is an exception, most likely because of its intrinsic presence in hair. Using the calibration curves presented in Fig. 4, the elemental analysis of 10 of the 60 hairs, picked at random, was performed for each element listed in Table 4. The distribution of concentration values obtained by LA-ICP-MS is presented in Fig. 6, in comparison with the corresponding bulk ICP-MS concentration in red. The ICP-MS results of the doped hairs in comparison to the median LA-ICP-MS concentrations are comparable. A deviation between the concentration values occurred mostly for elements present at low concentrations, such as Mo, Pb and Ba, with the LA-ICP-MS values being below the nebulization ICP-MS values. This may be due to the heterogeneity of the distribution of elements at lower concentrations in hair.³⁰

4 Conclusion

Multi-elemental doped keratin films were successfully synthesized and used as calibration standards for LA-ICP-MS determination of As, Mo, Pb, Mg, and Zn. This reference material behaved similarly to human hairs in its ablation and demonstrated reproducibility in its spiking. The calibration curves developed using the standard materials demonstrated good linearity and acceptable limits of detection and quantification. Although it was not the goal of this study, further analysis should include lowering the LOD and LOQ levels, opening new doors to detect lower concentrations in hair. These matrix-matched standards allow for the use of analysis of single hairs from a single individual. These results are promising for the further use of the high spatial resolution of LA-ICP-MS, allowing for temporally-resolved elemental profile along the hair. To strengthen the reliability of the reference materials, future studies should investigate comparison to real-world hair samples and interlaboratory testing to promote standardization. These keratin films pave the way for a new set of reference materials for quantitative LA-ICP-MS analysis for a variety of disciplines including medicine, forensic toxicology, and biological archaeology.

Ethical statement

This study was reviewed by the ethics committee at University of Central Florida and determined not to involve human subjects as defined by DHHS, FDA, and 28 CFS Part 46. All procedures were conducted in accordance with institutional ethical standards and the guidelines at the National Institute of Justice, U.S. Department of Justice.

Author contributions

Kaitlyn Bonilla: conceptualization, methodology, investigation, data curation, formal analysis, visualization, funding

acquisition, writing – original draft. Ashley Fox: methodology, investigation, data curation. Chloe Phillips: methodology, validation, investigation. Matthieu Baudelet: validation, supervision, project administration, resources, writing – original draft.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data will be available through the National Archive of Criminal Justice Data (NACJD) upon completion of the project in accordance with the National Institute of Justice.

Supplemental information is available showing a summary of elemental concentration reference values in hair from the literature, and additional calibration data for barium and copper. See DOI: <https://doi.org/10.1039/d5ja00242g>.

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