Role of Race/Ethnicity, Sex, and Age in Surface-Enhanced Raman Spectroscopy- and Infrared Spectroscopy-Based Analysis of Artificial Colorants on Hair

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ABSTRACT: Forensic microscopy has been used in forensic hair analysis to determine the racial origin of hair samples. However, this technique is subjective and often inconclusive. Although, to a large extent, this problem can be solved with the use of DNA analysis, which is capable of identifying the genetic code, biological sex, and racial origin from a strand of hair, this PCR-based analysis of hair is time- and labor-consuming. Infrared (IR) spectroscopy and surface-enhanced Raman spectroscopy (SERS) are emerging analytical techniques that can be used to advance forensic analysis of hair by enabling confirmatory identification of hair colorants. Having said that, it remains unclear whether the race/ethnicity, sex, and age of individuals should be considered upon IR spectroscopy- and SERS-based analysis of hair. Our results showed that both techniques enabled robust and reliable analyses of hair of different races/ethnicities, sexes, and age groups colored using four different permanent and semipermanent colorants. We also found that SERS could be used to identify the race/ethnicity, sex, and age of the individuals via spectroscopic analysis of colored hair, whereas IR spectroscopy was capable of accurately revealing this important anthropological information only from uncolored hair. These results outlined some advantages and limitations of both vibrational techniques in the forensic examination of hair samples.

1. INTRODUCTION

Forensic hair analysis is typically used to establish a connection between a suspect and a crime scene or demonstrate the absence of such connections.[1,2] Therefore, hair samples are collected from a variety of sources, including crime scenes, victims, and suspects, and are examined in order to identify the hair's source, determine whether it is human or animal, and to make other observations about its physical characteristics.[3] Forensic hair analysis is often used in conjunction with other forensic methods, such as DNA analysis and forensic toxicology, in order to build a more complete picture of the events surrounding a crime.[4,5]

Forensic examination of hair can be performed using a variety of techniques, including optical microscopy, chemical, and PCR analyses. Although the use of optical microscopy for hair analysis dates back to the early 20th century,[6] the technique has been refined and improved over time.[7] It can be an important tool in forensic hair analysis, as it allows examiners to closely inspect hair samples and make detailed observations about the physical characteristics of hair. Some of the advantages of optical microscopy in forensic hair analysis include high resolution and versatility. The former is based on the high magnification that can be achieved using optical microscopes, which allows forensic expects to see features that

might not be visible to the naked eye.[6,7] The latter is based on the suitability of optical microscopes to examine a wide range of characteristics, including the shape and size of the hair shaft and the presence or absence of certain pigments, as well as contaminants like dirt or grease.[6,7] At the same time, optical microscopy has some disadvantages. While forensic micros-copy can describe detailed information about the physical characteristics of a hair sample, it cannot provide information about the DNA or other chemical characteristics of the hair such as colorants that are commonly used on hair.[6,8] Microscopic analysis is also time- and labor-consuming, as hair samples must be carefully prepared and mounted on slides.[9,10] Finally, microscopic analysis of hair requires a high level of expertise. Therefore, forensic experts must be extensively trained in order to make reliable conclusions.[6]

These limitations catalyzed the search for alternative techniques that can provide more information during forensic

hair analysis. PCR-based analysis or DNA analysis quickly "took the wheel" of common practice for forensic hair analysis. It not only could identify the genetic code and biological sex of the hair's owner, which appeared as immutable obstacles for forensic microscopy, but also could identify their racial origin with greater accuracy.[11] A growing body of evidence suggests that surface-enhanced Raman spectroscopy (SERS) and Infrared (IR) spectroscopy can be used to detect and identify hair colorants.[9,10,12−14] In SERS, Raman scattering of dyes present on the hair's surface is enhanced a million-fold using noble metal nanostructures (or nanoparticles).[15,16] Using SERS, equipped with a gold nanoparticle solution, Higgins and Kurouski were able to differentiate more than 30 different colorants of different brands and colors.[10] Furthermore, Esparza and co-workers showed that SERS could be used to detect the underlying dyes if the hair was recolored afterward.[12] Several research groups showed that IR spectroscopy also has premise in forensic hair analysis.[17−20] For example, IR spectroscopy could be used to identify the types of amino acids present in hair samples, which can be useful in identifying the source of the hair and in distinguishing human hair from animal hair.[17,18] Recently, Boll and co-workers found that different types and brands of colorants could be identified on hair using IR spectroscopy.[13] It was also demonstrated that IR primarily detected keratin of hair rather than vibrational signatures of the colorants themselves.[14] It should be noted that both SERS and IR spectroscopy can be miniaturized from a large, benchtop-based instruments into handheld tools that can be used directly at a crime scene.[17,21] These spectrometers demonstrated outstanding performance in the identification of plant pathogens,[22] determination of postmortem intervals from the teeth of corpses,[23] and classification of gun powder residue.[24]

A question to ask is whether the race/ethnicity, sex, and age of analyzed hair can alter the accuracy of SERS- and IR spectroscopy-based identification of hair colorants. We hypothesize that, due to the variation in the morphology of hairs (e.g., cuticle thickness and distribution of pigment granules) from different origins, colorants or dyes will bind and stabilize differently respective to each group. To answer this question, we acquired SERS and IR spectra from hairs of different race/ethnicity, sex, and age groups colored with four permanent and semipermanent dyes (Table 1). Next, we utilized partial least-squares discriminant analysis (PLS-DA) to determine the accuracy of SERS- and IR spectroscopy-based identification of hair colorants. We also used PLS-DA to examine whether the race/ethnicity, sex, and age could be predicted based on the colored hair.

Table 1. Hair Owners' (Subjects') Information

subject	age	race/ethnicity	sex
1	20	Caucasian	male
2	43	Caucasian	male
3	22	Hispanic	male
4	25	Asian	male
5	32	Indian	female
6	20	Asian	female
7	30	Indian	female
8	22	Caucasian	female
9	36	Indian	male
10	21	Hispanic	female

2. MATERIALS AND METHODS

2.1. Hair Collection and Treatment. The hair used for this research was undyed virgin hair collected from hairbrushes, combs, and hair stylists' capes. Hair was dyed using Ion brand hair dye either of Ion Jet Black (permanent black), Ion Sapphire (permanent blue), Ion Blackest Black (semipermanent black), or Ion Sapphire (semipermanent blue). For the purposes of this experiment, permanent black and blue are denoted as PBA and PBU, respectively, and semipermanent black and blue, SBA and SBU, respectively. A clean beaker was used to mix permanent hair dye and an activator, and a clean graduated cylinder was used to pour equal portions of each hair colorant onto each batch of hair. The colorant was then gently rubbed with each batch until all hair strands in each batch were completely coated. After the elapsed time indicated by the brand of dye on the box (since they varied) passed, the hair was rinsed off under low-pressure deionized water within a small stainless-steel strainer until the water running off was clear, after which the hair was left to air dry.

In parallel, undyed, virgin hair originating from a Caucasian female was used for Fourier transform infrared (FT-IR) analysis of hair colored with a multitude of colorants. Permanent is denoted as PM, semipermanent as SP, and demipermanent as DP colorants. The following colorants and their chosen colors were used: Ion Blackest Black, SP (color: black); Ion Burgundy Brown, SP (auburn); Ion Medium Warm Brown, SP (brown); Ion Sapphire, SP (blue); Ion Radiant Orchid, SP (purple); Ion Magenta, SP (pink); Ion Garnet, SP (red); Ion Jet Black, PM (black); Ion Medium Golden Brown, PM (brown); Ion Medium Burgundy Brown, PM (auburn); Ion Tanzanite, PM (blue); Ion Radiant Orchid, PM (purple); Ion Magenta, PM (pink); Ion Garnet, PM (red); Wella Dark Sand, DP (auburn); Wella Medium Natural Brown, DP (brown); Wella Black, DP (black); Wella Blue, SP (blue); Wella Wild Orchid, SP (purple); Wella Raspberry, SP (pink); Wella Red, SP (red); Wella Dark Auburn, PM (auburn); Wella Medium Natural Warm Brown, PM (brown); Wella Black, PM (black); L'Oréal Fresh Ink Blue, PM (blue); L'Oréal Majestic Violet, PM (purple); L'Oréal Chroma Ruby, PM (red); Clairol Light Reddish Brown, SP (auburn); Clairol Medium Warm Brown, SP (brown); Clairol Jet Black, SP (black); Clairol Light Neutral Brown, PM (brown); and Clairol Ultra Cool Black, PM (black).

2.2. Forensic Microscopy. Undyed hair was utilized for forensic microscopy as colorants have negligible change in size and granule distribution of hair.[6] Ten strands per subject were analyzed using a 20× (for diameter and pigmentation) and 4× (for undulation) objective of a TE-2000U Nikon inverted confocal microscope and calibrated using a 10× graticule eyepiece (micrometer) and a 1 mm/0.01 mm division stage micrometer (Graticules Optics Ltd., Tonbridge, U.K.). Subject hairs were identified as the most likely racial origin using available keys.[6,8] According to the keys, Caucasian and Hispanic derive from Caucasoid and Asian and Indian fall under Mongoloid.

2.3. Raman Spectroscopy. The nanoparticle solution, excitation wavelength laser light, equipment, and power were chosen based on published methods from Esparza et al. and Higgins and Kurouski. SERS spectra were collected using a TE-2000U Nikon inverted confocal microscope, equipped with a 20× objective. A solid-state laser generated 785 nm light, while power through each sample was kept at 1.8 mW.
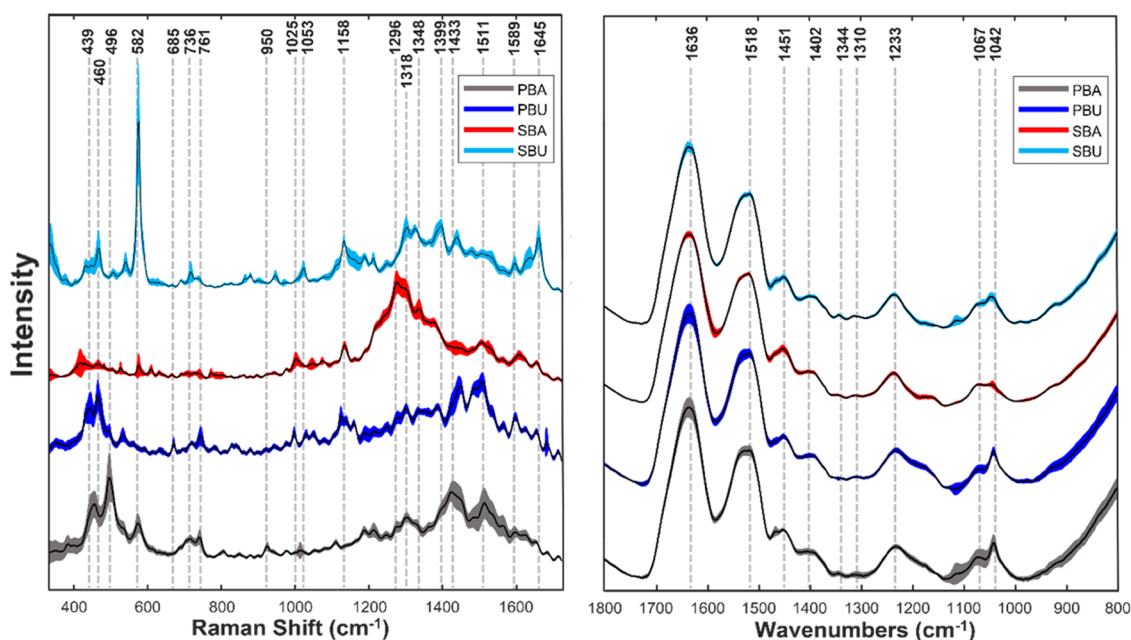
Figure 1. Mean (solid black line) and standard deviations (color-filled areas) of averaged spectra from SERS (left) and FT-IR spectroscopy (right) of each colorant.

Scattered light was collected using the same magnification and directed using a 50/50 beam splitter into an IsoPlane-320 spectrometer (Princeton Instruments) equipped with a 600 groove/mm grating. Prior to entering the spectrometer, elastically scattered photons were cut off with a long-pass filter (Semrock, LP03-785RS-25). Inelastically scattered photons were collected using a PIX-400BR CCD (Princeton Instruments). Fifty spectra from each colorant group of each subject were collected by placing each hair on a glass cover slide and applying 5 μL of a homemade gold nanorod solution according to the steps listed by Esparza and co-workers.[12] The strand of hair was coated by the 5 μL drop of gold nanorod solution by moving the hair around the slide until the nanorod solution outlined ~10 mm in length (incidental of whether the strand was longer or shorter than 10 mm) of the strand of hair. The laser light was positioned on the hair proximally, as had the most consistently intense peaks for bands of interest. Overall acquisition times ranged from 18 to 30 s.

2.4. Infrared Spectroscopy. Three IR spectra were collected from three different hair strands of each group using a Spectrum 100 IR spectrometer (PerkinElmer, MA). Raw data were processed using attenuated total reflectance (ATR) correction and display in absorbance by PerkinElmer spectrum express.

2.5. Data Analysis. All spectra were baseline-corrected and normalized before analysis using MATLAB. Chemometric analysis of acquired spectra was done in MATLAB equipped with PLS_Toolbox 9.0 (Eigenvector Research, Inc., Manson, WA). For PLS discriminant analysis (PLS-DA), cross-validations from 100% calibration models were employed. Preprocessing of each model was done using MSC (Mean) filtering and 1st-derivative smoothing (n = 2, fl = 15 pt.). Latent Variables (LV) were selected based on the "suggested" models in MATLAB and are listed in their corresponding tables.

Partial least-squares discriminant analysis (PLS-DA) was chosen over other methods such as the support vector machine (SVM), soft independent modeling of class analogy (SIMCA),

and principal component analysis (PCA) due to its ability to handle complex data sets with high multicollinearity and noise.[25,26] PLS-DA combines the regression and classification methods and considers the relationship between the spectral data and the sample classes. Unlike SVM and SIMCA, which are binary classifiers, PLS-DA can classify samples into multiple classes, making it more suitable for multiclass classification problems.[27] Furthermore, unlike PCA, which only extracts the most significant components in the data, PLS-DA extracts latent variables that maximize the correlation between the spectral variables and the class variables.[28] This makes PLS-DA more efficient for data sets with complex class structures and a small number of samples. Therefore, PLS-DA was chosen as the most appropriate method for the analysis of Raman and Infrared spectroscopy data in this study.[29]

3. RESULTS AND DISCUSSION

We first investigated whether SERS and IR spectroscopy could be used to identify colorants on hair of different racial origins. In the SERS spectra we acquired from PBA-colored hair, we detected peaks at 496, 582, 736, 761, 950, 1318, 1433, 1511, and 1589 cm^{-1}; see Figure 1 and Table 2. We found 100% accuracy using SERS to differentiate between PBA spectra and other colorants indicated by a 1.00 true positive rate (TPR) generated through PLS-DA; see Table 3. Comparatively, we found that IR spectra also gave 100% accuracy in PBA identification among the other colorants; see Table 4. These

Table 2. Vibrational Bands Present in the SERS Spectra of Each Colorant-Specific Dyed Hair That Can Be Used for Hair Colorant Identification

colorant	corresponding vibrational bands present in SERS spectra (cm^{-1})
PBA	496, 582, 736, 761, 950, 1318, 1433, 1511, 1589
PBU	439, 460, 685, 736, 761, 1025, 1158, 1318, 1511, 1589, 1645
SBA	460, 582, 685, 1025, 1158, 1296, 1348, 1511, 1645
SBU	460, 582, 736, 1053, 1158, 1318, 1399, 1589, 1645

Table 3. PLS-DA Cross-Validation Results from the SERS-Based Analysis of Four Colorants on Hair of Different Races/ethnicities, Sexes, and Age Groups

SERS (LV = 3)		actual colorant			
predicted colorant	accuracy, %	PBA (n = 500)	PBU (n = 500)	SBA (n = 500)	SBU (n = 500)
PBA	100	500	1	0	0
PBU	99.8	0	499	0	0
SBA	99.6	0	0	498	0
SBU	100	0	0	2	500

Table 4. Morphological Features of Subjects' Hair Described Using Forensic Microscopy ("dist." Is Distribution of Granules)

subject	actual race/ethnicity	hair thickness (μm)	expected thickness (μm)	pigmentation	expected pigmentation	cuticle	expected cuticle	undulation	expected undulation	predicted racial origin(s)
1	Caucasian	68.0−93.8	70−100	even dist. & brown	even dist.	thick	medium	absent	absent	Caucasoid
2	Caucasian	62.5−76.8	70−100	even dist. & gray/brown	even dist.	medium	medium	absent	absent	Caucasoid
3	Hispanic	84.6−95.7	70−100	even dist. & black	even dist.	thick	medium	absent	absent	Caucasoid
4	Asian	72.3−105.1	90−120	even dist. & auburn	dense auburn	thick	thick	absent	absent	Mongoloid
5	Indian	68.6−85.4	90−120	even dist. & black/auburn	dense auburn	thick	thick	absent	absent	Mongoloid/Caucasoid
6	Asian	58.1−84.4	90−120	even dist. & auburn	dense auburn	medium	thick	absent	absent	Mongoloid/Caucasoid
7	Indian	58.1−81.9	90−120	even dist. & black/auburn	dense auburn	thick	thick	absent	absent	Mongoloid/Caucasoid
8	Caucasian	76.6−84.4	70−100	even dist. & light brown	even dist.	medium	medium	absent	absent	Caucasoid
9	Indian	57.8−73.8	90−120	even dist. & black	dense auburn	medium	thick	absent	absent	Caucasoid
10	Hispanic	43.2−50.4	70−100	moderately dense	even dist.	thin	medium	present	absent	Negroid

Table 5. PLS-DA Cross-Validation Results from the IR-Based Analysis of Four Colorants on Hair of Different Races/Ethnicities, Sexes, and Age Groups

FT-IR (LV = 8)		actual colorant			
predicted colorant	accuracy, %	PBA (n = 30)	PBU (n = 30)	SBA (n = 30)	SBU (n = 30)
PBA	100	30	2	0	0
PBU	90.0	0	27	0	0
SBA	96.7	0	1	29	0
SBU	100	0	0	1	30

results showed that PBA dye could be accurately detected and identified on hair of different races/ethnicities using both SERS and IR spectroscopy.

In the SERS spectra we acquired from PBU-colored hair, we detected peaks at 439, 460, 685, 736, 761, 1025, 1158, 1318, 1511, 1589, and 1645 cm^{-1}; see Figure 1 and Table 2. We found 99.8% accuracy using SERS to differentiate between PBU acquired spectra and other colorants using PLS-DA; see Table 3. On the other hand, we found that IR spectra only gave 90% accuracy of identifying PBU-colored hair among other colorants; see Table 4. These results showed that although PBU dye could be correctly identified on hair of different races/ethnicities using both SERS and IR spectroscopy, SERS enabled a higher accuracy of the dye identification compared to IR spectroscopy.

In the SERS spectra from SBA-colored hair, we detected peaks at 460, 582, 685, 1025, 1158, 1296, 1348, 1511, and 1645 cm^{-1}; see Figure 1 and Table 2. SERS-collected spectra from SBA-colored hair yielded a 99.6% accuracy to differentiate it among the other colorants (Table 3), whereas IR spectra of SBA-colored hair yielded only 96.7% accuracy; see

Table 4. These results showed that SBA dye identification and detection using SERS and IR spectroscopy was not significantly affected by different types of hairs the colorant is applied to; however, SERS was found to have a higher accuracy of identification.

Finally, in the SERS spectra of SBU-colored hair, we detected peaks at 460, 582, 736, 1053, 1158, 1318, 1399, 1589, and 1645 cm^{-1}; see Figure 1 and Table 2. Both PLS-DA results of SBU-colored hair spectra from SERS and IR yielded 100% accuracies at identifying SBU-colored spectra as SBU among other colorants. These results showed that SBU dye could be accurately detected and identified on hair of different races/ethnicities using both SERS and IR spectroscopy.

Next, we investigated whether SERS and IR could be used to reveal information about the race/ethnicity of individuals that donated hair, as well as the accuracy that both techniques provide relative to conventional optical microscopy. We found that optical microscopy was able to determine the correct racial origin for only half of the analyzed subjects (subjects 1, 2, 3, 4, and 8); see Table 5. The other half were either deemed undecided (subjects 5, 6, and 7) due to having equal amounts

Table 6. Collective PLS-DA Models for All SERS and FT-IR Spectra, Calibrated by Race/Ethnicity for PBA (A), PBU (B), SBA (C), and SBU (D)

	predicted race/ ethnicity	SERS accuracy, %	actual race/ethnicity Asian (n = 100)	Caucasian (n = 150)	Hispanic (n = 100)	Indian (n = 150)	FT-IR accuracy,%	actual race/ethnicity Asian (n = 6)	Caucasian (n = 9)	Hispanic (n = 6)	Indian (n = 9)
A	Asian	100	100	1	0	1	50.0	3	1	2	0
	Caucasian	99.3	0	149	0	2	33.3	1	3	2	3
	Hispanic	100	0	0	100	0	0	0	0	0	1
	Indian	98.0	0	0	0	147	55.6	2	5	2	5
B	Asian	100	100	0	0	0	16.7	1	1	3	0
	Caucasian	98.7	0	148	0	0	77.8	0	7	0	6
	Hispanic	99.0	0	1	99	3	50.0	1	0	3	0
	Indian	98.0	0	1	1	147	33.3	4	1	0	3
C	Asian	100	100	0	1	0	16.7	1	2	2	2
	Caucasian	97.3	0	146	1	1	44.4	1	4	1	3
	Hispanic	98.0	0	0	98	3	50.0	3	1	3	0
	Indian	97.3	0	4	0	146	44.4	1	2	0	4
D	Asian	89.0	89	0	3	9	16.7	1	1	1	2
	Caucasian	100	0	150	0	0	55.6	0	5	3	4
	Hispanic	97.0	0	0	97	0	0	2	2	0	0
	Indian	94.0	11	0	0	141	33.3	3	1	2	3

Table 7. Collective PLS-DA Models for All SERS and FT-IR Spectra, Calibrated by the Age Group for PBA (A), PBU (B), SBA (C), and SBU (D)

	predicted age groups	SERS accuracy, %	actual age group 20−25 (n = 300)	30−36 (n = 150)	43 (n = 50)	FT-IR accuracy, %	actual age group 20−25 (n = 18)	30−36 (n = 9)	43 (n = 3)
A	20−25	100	300	2	0	66.7	12	1	1
	30−36	98.0	0	147	0	88.9	2	8	0
	43	100	0	1	50	66.7	4	0	2
B	20−25	95.3	286	2	0	66.7	12	1	0
	30−36	98.7	14	148	0	88.9	5	8	0
	43	100	0	0	50	100	1	0	3
C	20−25	96.0	288	5	0	61.1	11	3	1
	30−36	96.7	12	145	1	44.4	3	4	0
	43	98.0	0	0	49	66.7	4	2	2
D	20−25	96.3	289	5	0	55.6	10	1	0
	30−36	96.7	11	145	1	88.9	7	8	0
	43	98.0	0	0	49	100	1	0	3

of characteristics for two separate racial origins or they were misidentified (subjects 9 and 10). According to Bisbing, at the time, Hispanics fell under Caucasoid due to genetic drift (in the Americas), but the existence of genetic drift can allow Hispanics to show more Caucasoid, Mongoloid, or Negroid characteristics, as shown by subject 10's misclassification.[6]

Our results showed that SERS analysis of PBA-colored hair could be used to identify Asians with 100% accuracy, Caucasians with 99.3% accuracy, Hispanics with 100% accuracy, and Indians with 98% accuracy; see Table 6. In contrast, we found that IR could only differentiate between the hairs of Asians with 50% accuracy, Caucasians with 33.3% accuracy, Hispanics with 0% accuracy, and Indians with 55.6% accuracy. SERS analysis of PBU-colored hair could be used to identify Asians with 100% accuracy, Caucasians with 98.7% accuracy, Hispanics with 99% accuracy, and Indians with 98% accuracy. However, IR spectroscopy could give positive identification between the hairs of Asians with 16.7% accuracy, Caucasians with 77.8% accuracy, Hispanics with 50% accuracy, and Indians with 33.3% accuracy. We also found that SERS analysis of SBA-colored hair could be used to identify Asians with 100% accuracy, Caucasians with 99.3% accuracy,

Hispanics with 100% accuracy, and Indians with 98% accuracy. IR spectroscopy-collected spectra gave positive identification between the hairs of Asians with only 16.7% accuracy, Caucasians and Indians with 44.4% accuracy, and Hispanics with 50% accuracy. Finally, SERS analysis of PBU-colored hair could be used to identify Asians with 89% accuracy, Caucasians with 100% accuracy, Hispanics with 97% accuracy, and Indians with 94% accuracy. It should be noted that all spectra that were misidentified for Asians were identified as Indians and vice versa. This is interesting since Indians are considered Southern Asians and both are classified as Mongoloids. IR spectroscopy could only identify the hairs between Asians with 16.7% accuracy, Caucasians with 55.6% accuracy, Hispanics with 0% accuracy, and Indians with 33.3% accuracy. These results show that SERS spectra contain information that are highly specific to different races/ethnicities of colored hair, which cannot be probed using IR spectroscopy. Further reasoning for the importance that the colorants play in the analysis by Raman can be found in a study by Cappa de Oliveira et al. where Raman spectroscopy was utilized to produce spectra from bleached, heat-treated, undyed hair of Caucasian and Afro ethnic origins; the results showed very minimal differences in

Table 8. Collective PLS-DA Models for All SERS and FT-IR Spectra, Calibrated by Sex for PBA (A), PBU (B), SBA (C), and SBU (D)[a]

	predicted sex	SERS accuracy, %	actual sex female (n = 250)	male (n = 250)	FT-IR accuracy, %	actual sex female (n = 15)	male (n = 15)
A	female	97.2	243	2	86.7	13	2
	male	99.2	7	248	86.7	2	13
	MCC = 0.964				MCC = 0.733		
B	female	98.8	247	3	86.7	13	3
	male	98.8	3	247	80.0	2	12
	MCC = 0.976				MCC = 0.668		
C	female	95.2	238	12	80.0	12	1
	male	95.2	12	238	93.3	3	14
	MCC = 0.904				MCC = 0.740		
D	female	97.6	244	11	93.3	14	1
	male	95.6	6	239	93.3	1	14
	MCC = 0.932				MCC = 0.867		

[a]Matthew's correlation coefficient (MCC) indicates the level of reliability between binary classifications.
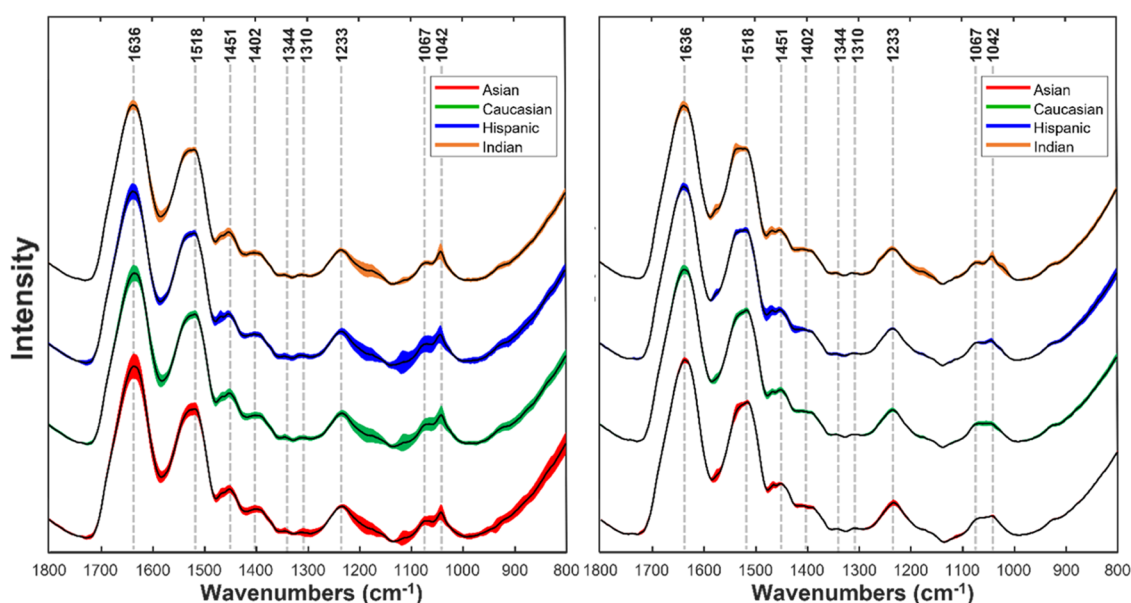
Figure 2. Average FT-IR spectra (solid black line) with corresponding standard deviations (color-filled areas) acquired form dyed (left) and undyed (right) hair of individuals of different races/ethnicities.

the spectra between this ethnic groups of hair before and after exposure to these treatments, unlike what we see in our results after dye treatments.[30]

We also investigated whether SERS and IR spectroscopy could be used to predict age groups. We found that SERS could differentiate between PBA-colored hair of ages 20−25 and 43 with 100% accuracy and 0−36 with 98% accuracy; see Table 7. IR spectra of PBA-colored hair could be used to differentiate between ages 20−25 and 43 with 66.7% accuracy and 30−36 with 88.9% accuracy. The SERS spectra of PBU-colored hair could differentiate between ages 20−25 with 95.3% accuracy, 30−36 with 98.7% accuracy, and 43 with 100% accuracy. IR spectra of PBU-colored hair could differentiate between ages 20−25 with 66.7% accuracy, 30−36 with 88.9% accuracy, and 43 with 100% accuracy. SERS spectra from SBA-colored hair could differentiate between ages 20−25 with 96% accuracy, 30−36 with 96.7% accuracy, and 43 with 98% accuracy. IR spectra of SBA-colored hair gave differentiations between ages 20−25 with 61.1% accuracy, 30−36 with 44.4% accuracy, and 43 with 66.7% accuracy. SERS

spectra from SBU-colored hair gave differentiations for ages 20−25 with 96.3% accuracy, 30−36 with 96.7% accuracy, and 43 with 98% accuracy. IR spectra of the same colorant gave differentiations for ages 20−25 with 55.6% accuracy, 30−36 with 88.9% accuracy, and 43 with 100% accuracy. These results suggest SERS is more reliable than IR spectroscopy at generating spectra capable of sex differentiation of hair collected and dyed from different owners.

Finally, we investigated whether SERS and IR spectroscopy could be used to predict biological sex. We found that SERS spectra of PBA-colored hair could differentiate between females with 97.2% accuracy and males with 99.2% accuracy; see Table 8. IR spectra of PBA-colored hair yielded differentiation between females and males with 86.7% accuracy. Within SERS spectra of PBU-colored hair, we could differentiate between females and males with 98.8% accuracy. IR spectra of PBU-colored hair could differentiate between females with 86.7% accuracy and males with 80% accuracy. Acquired SERS spectra of SBA-colored hair gave differentiations between females and males with 95.2%
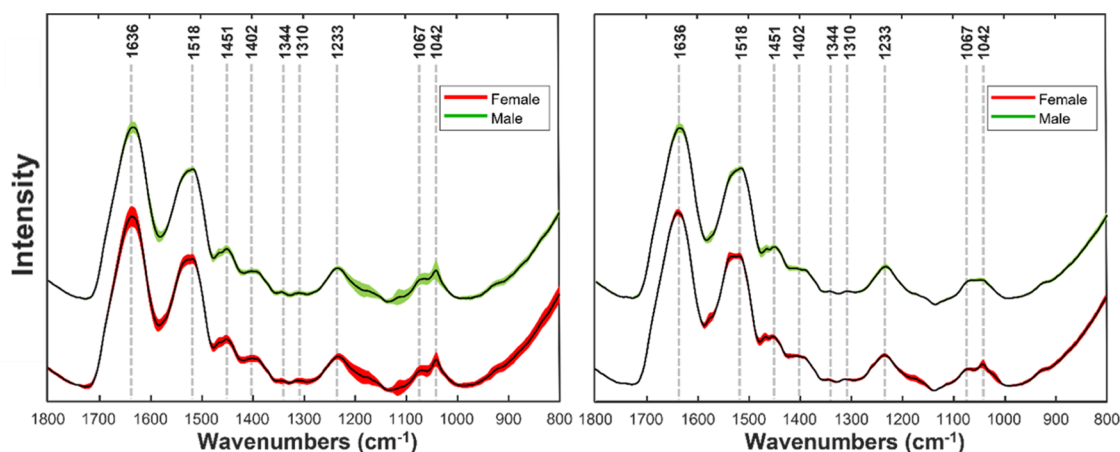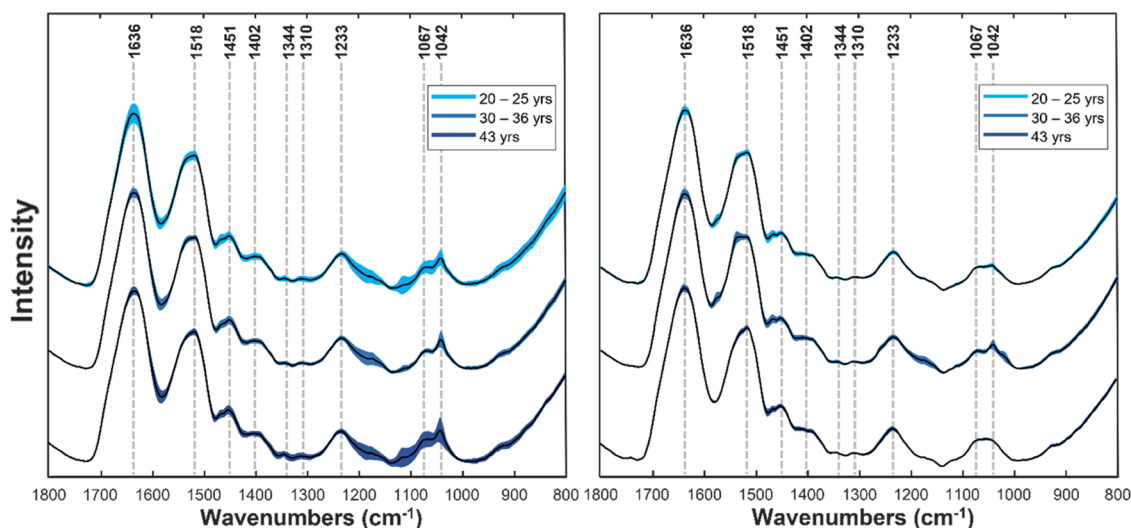
Figure 3. Average FT-IR spectra (solid black line) with corresponding standard deviations (color-filled areas) acquired form dyed (left) and undyed (right) hair of both male and female individuals.

Table 9. PLS-DA Models for Calibration by the Race/Ethnicity of Dyed Hair (DH) and Undyed Hair (UH) FT-IR Spectra

DH FT-IR		actual race/ethnicity				UH FT-IR	actual race/ethnicity			
predicted race/ethnicity	accuracy, %	Asian ($n = 24$)	Caucasian ($n = 36$)	Hispanic ($n = 24$)	Indian ($n = 36$)	accuracy, %	Asian ($n = 24$)	Caucasian ($n = 36$)	Hispanic ($n = 24$)	Indian ($n = 36$)
Asian	25.0	6	5	3	6	91.7	22	1	0	0
Caucasian	41.7	6	15	4	2	88.9	2	32	2	4
Hispanic	54.2	6	7	13	6	79.2	0	2	19	8
Indian	61.1	6	9	4	22	66.7	0	1	3	24

Table 10. PLS-DA Models for Calibration by the Sex of Dyed Hair (DH) and Undyed Hair (UH) FT-IR Spectra[a]

DH FT-IR		actual sex		UH FT-IR	actual sex	
predicted sex	accuracy	female ($n = 60$)	male ($n = 60$)	accuracy	female ($n = 60$)	male ($n = 60$)
female	91.7%	55	4	91.7%	55	2
male	93.3%	5	56	96.7%	5	58
MCC = 0.850				MCC = 0.884		

[a]Matthew's correlation coefficient (MCC) indicates the level of reliability between binary classifications.

Figure 4. Average FT-IR spectra (solid black line) with corresponding standard deviations (color-filled areas) acquired form dyed (left) and undyed (right) hair of individuals belonging to different age groups.

accuracy, as opposed to IR spectra, which gave 80% and 93.3% accuracies for females and males, respectively. SERS spectra from SBU-colored hair could differentiate between females with 97.6% accuracy and males with 95.6% accuracy. IR spectra of the same colorant gave 93.3% accuracies for both females and males. These results show that PLS-DA obtains higher accuracies for sex differentiation when using SERS spectra compared to IR spectra of dyed hair. Averaged SERS

Table 11. PLS-DA Models for Calibration by the Age Group of Dyed Hair (DH) and Undyed Hair (UH) FT-IR Spectra

DH FT-IR		actual age group			UH FT-IR	actual age group		
predicted age group	accuracy, %	20−25 ($n = 72$)	30−35 ($n = 36$)	43 ($n = 12$)	accuracy, %	20−25 ($n = 72$)	30−35 ($n = 36$)	43 ($n = 12$)
20−25	63.9	46	6	3	95.8	69	3	0
30−36	75.0	17	27	3	88.9	3	32	0
43	50.0	9	3	6	100	0	1	12

spectra acquired from all groups discussed above demonstrate that spectral discrimination is likely to be due to the colorant signals, which suggests that interactions between colorants and hair depend on the ethnicity, age, or sex.

Such a poor performance of IR spectroscopy in the identification of race/ethnicity, sex, and age is rather unexpected, primarily because experimental results reported by our and other research groups showed that IR spectroscopy probed the bulk volume of hair samples, which is dominated by keratin.[13,14] Chemical modifications of keratin, which are taken place upon hair bleaching, enabled 100% differentiation between bleached and unbleached hair.[14] One could expect that race/ethnicity-, sex-, and age-related changes in keratin should be detected by IR spectroscopy. To address this concern, we acquired IR spectra from uncolored hair from the discussed groups (Figure 2 and Table 1). We found that IR analysis of uncolored hair could be used to identify races/ethnicities of analyzed individuals with ~82% accuracy, whereas spectroscopic analysis of colored hair revealed this important anthropological information only with 45.5% accuracy (Figure 3 and Table 9). Although the sex of analyzed individuals could be predicted relatively accurately (92.5%) based on the IR spectra acquired from colored hair, spectroscopic analysis of uncolored hair enables much more accurate identification (94.2%) of the sex of hair donors (Figure 3 and Table 10). Finally, we found that the age of hair donors could be identified with ~95% accuracy upon the IR analysis of uncolored hair, whereas colored hair could be used for only 62.9%, on average, age identification (Figure 4 and Table 11). These results demonstrated that the presence of colorants on hair substantially complicated IR spectroscopy-based analysis of the unique structural differences in keratin molecules present in hair of individuals of different races/ethnicities, sexes, and ages. This results in poor predictions of these important anthropological and biological differences between hair of different individuals.

To further examine the potential of IR spectroscopy in the identification of hair colorants, we acquired FT-IR spectra from exactly the same samples that were analyzed by Higgins and Kurouski.[10] Our results show that the PLS-DA model allows for an all-around 0% positive identification rate (accuracy) between different hair colorants (Figure S1 and Tables S1−S4). Of the four brands, it identified Clairol with 77.8% accuracy, Ion with 47.6% accuracy, L'Oréal with 55.6% accuracy, and Wella with 50% accuracy. Of the seven colors, it identified Auburn and Black with 27.8% accuracy, Red with 16.7% accuracy, and all other colors with 0% accuracy. When, instead, differentiating between PM and SP, it gave 89.6 and 90.5% accuracies, respectively (Figure S1 and Tables S1−S4). DP only obtained a 44.4% positive identification, with a majority of its misclassified spectra being identified as SP (Tables S1−S4). These results demonstrate that IR cannot accurately differentiate between a large number of different colors and brands, but it can differentiate between semi-permanent and permanent hair dyes, which is in a good agreement with the experimental results reported by Boll and co-workers.[13]

4. CONCLUSIONS

Our results, overall, indicate a superiority of SERS to yield spectra that reliably differentiate between the colorant, racial origin, age group, and biological sex of artificially dyed hairs from different persons than both IR spectrosopy and forensic microscopy. SERS spectra yielded high accuracies of 99 to 100% to differentiate between four colorants, 89 to 100% to differentiate between four races/ethnicities, 95−100% to differentiate between different age groups, and 95.2 to 99.2% to differentiate between males and females. We also found that IR spectroscopy could not be used to accurately identify different colors and brands of different artificial dyes on hair. Due to the inability of IR spectroscopy to differentiate between different colorants with high accuracy, we expect that SERS has shown that these dyes bind and stabilize somewhat specifically to its classified origin (race, sex, etc.). Using SERS, identifications can be conducted a lot quicker than DNA analysis. With this information, SERS should require more focus and study for its applications in forensics and other sciences, especially in forensic hair analysis. It should be noted that, to make our claims stronger, a larger number of individuals of different races, age groups, and different sexes must be involved in the experiments reported in this study. Such large-group research is a subject for a separate study.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c01241.

PLS-DA confusion matrix of the calibration (100% cross-validation) model (LV = 1) for differentiation of all colorants using FT-IR spectra of dyed hair; PLS-DA (LV = 4) confusion matrix of the calibration model for brands of colorants using FT-IR; PLS-DA (LV = 5) confusion matrix of the calibration model for dye permanence of colorants using FT-IR; PLS-DA (LV = 2) confusion matrix of the calibration model for each color using FT-IR; PBA mean spectra from SERS and FT-IR of different races; PBA mean spectra from SERS and FT-IR of different races; PBA mean spectra from SERS; and FT-IR for the different sexes (PDF)

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