Raman spectroscopy and scanning electron microscopy application for physical characterization of horsehairs

Giuseppe Acri,[1] Barbara Testagrossa,[1] Lucia Denaro,[1] Elisabetta Giudice,[2] Giuseppe Piccione,[2] Maria Rizzo,[2] Pietro Pugliatti,[3] Francesca Arfuso,[2] Claudia Giannetto[2]

[1]Department of BIOMORF, University of Messina; [2]Department of Veterinary Sciences, Polo Universitario dell'Annunziata, University of Messina; [3]Department of Clinical and Experimental Medicine, University of Messina, Italy

Abstract

Horsehairs present several common characteristics in their chemical composition and molecular structure. The present study aims to analyze the physical characteristics of horsehairs belonging to different breeds. Morphological analysis of the horsehair fibers was performed using Scanning Electron Microscopy (SEM) and molecular structural characterization using the Raman Spectroscopy (RS) technique. Horse hairs were collected from three different horse groups (group A: mixed-breed; group B: Italian saddle; group C: thoroughbred). Each group was constituted of five horses with a mean body weight of 475±25 kg, aged between 12 and 15 years old. SEM images showed differences in the surface layer (cuticula) and diameter size of horsehairs referred to different breeds. The investigation conducted through RS showed differences in the S – O band, located at 1044 cm^{-1}, where cysteic acid is one of the amino acid constituents of a-keratin; in CH2 bending mode and CH stretching, located at ~1450 cm^{-1} and ~2900 cm^{-1}, respectively. These differences could be attributed to genetic predisposition or metabolism; they could represent the real differentiation among the breeds, detectable by using RS.

Correspondence: Giuseppe Piccione, Department of Veterinary Sciences, Polo Universitario dell'Annunziata, University of Messina, 98168, Messina, Italy.
E-mail: gpiccione@unime.it

Key words: horsehair; Raman spectroscopy; SEM.

Contributions: conceptualization, GA, and CG; methodology, BT; software, LD; formal analysis, GA; investigation, EG, FA; data curation, MR and PP; writing—original draft preparation, GA, and GP; writing—review and editing, CG. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest: the authors declare no conflict of interest.

Ethics approval: not applicable.

Availability of data and materials: all data generated or analyzed during this study are included in this published article.

Received: 18 July 2023.
Accepted: 6 November 2023.
Early view: 21 November 2023.

Publisher's note: all claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article or claim that may be made by its manufacturer is not guaranteed or endorsed by the publisher.

Journal of Biological Research 2023; 96:11591
doi:10.4081/jbr.2023.11591

Introduction

Hair is a natural biopolymer composed of proteins, the most represented of them is keratin. The hair structure has been well investigated, and the molecular structure details and the internal hair organization have been revealed by the X-ray diffraction.[1] Four main regions have been identified, cuticle, cortex, medulla, and cell membrane complex.[2] Starting from the outside, the first region is the cuticle constituted by a superimposition of several layers of amorphous material.[3] Its principal function is to protect from the external agent below regions.[4] Under the cuticle, an elongated fibrous structure parallel to the direction of the fiber, the cortex, gives the hair mechanical strength and elasticity.[5] In the hair central portion, it has been identified an amorphous structure with a vacuolar architecture, the medulla.[6] The main way of diffusion of substances into the interior of the capillary fibers is guaranteed by the cell membrane complex that makes cohesive cuticular and cortical cells.[7]

In the last years, several studies involving hairs, in particular human hair, were conducted; most of them were focused on hair cosmetic treatments, providing information about the characteristics of the fiber and the permeation of different cosmetics.[8-10] In this context, the color, length, and thickness of horsehairs have attracted humans since prehistory, and the artificial selection based on human preference enhanced the variation of these phenotypes.[11] The biological properties of horsehairs were studied for medical and cosmetic applications in humans, like the development of keratin[12,13] and the possibility of using it as a suture material in many surgeries.[14] For this reason, it is important to understand and investigate hair fiber structure from macroscopic to microscopic levels.

Scanning Electron Microscopy (SEM) found its application in

many industrial, commercial, and research fields; in the last years, it has been also applied in human hair research.[15,16] Another technique useful for structural analysis is Raman Spectroscopy (RS). This technique is based on the inelastic diffusion capacity of laser light.[17,18] The resulting spectrum reveals the structural characteristics of the sample under examination, providing a fingerprint.[19,20] RS providing essential information about the sample finds application in chemistry, physics, biology, and material sciences, and is also used as a diagnostic tool in medicine.[21-23] Analytical studies were conducted on human hair by using RS,[24,25] and spectroscopic studies have been reported on archeological and ancient hair stored in museums.[26,27]

To our knowledge, no studies concerning SEM and RS analysis on horsehairs are available in the literature. In this context, this study aims to characterize different horsehairs coming from different horse breeds by using these techniques.

Materials and Methods

Sample collection and preparation

Fifteen bay horses of different breeds, divided into three equal groups (group A: mixed-breed; group B: Italian saddle; group C: thoroughbred), 7 females and 8 males, aged between 12 and 15 years old, with a mean body weight of 475±25 kg, and not belonging to the same family tree were included in this study. None of the females were pregnant.

For each horse, from 50 to 100 horsehairs were plucked from the horse's forelock, by using medical gloves, and placed in plastic bags identified with the id of the horse. No chemical or physical treatment was performed on the samples before the analysis.

SEM analysis

The SEM measurements were conducted with a Jeol JMC-6000 (Jeol Co., Akishima, Tokyo, Japan). Before the SEM procedure, an adhesive black carbon tab was mounted on the sample holder. The "high vacuum mode" (HV) was used. An electron beam generated by the electron gun scanned the sample surface in the X-Y direction. The SEM measurements were conducted at 15 kV and a magnification of 300× was used. All images were digitized and stored as Tagged Image File Format (.tiff) files in the microscope computer and were not elaborated in any way.

Diameters of ten different hairs of each breed were measured using the SEM Software. For each analyzed sample, three different measurements were performed.

The obtained results are reported as the mean value of the different measurements effectuated on every target sample.

Raman Spectroscopy analysis

A DXR-SmartRaman Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for Raman measurements. A diode laser with an excitation wavelength of 785 nm was applied. All Raman spectra were acquired over the wavenumber range of 3300–400 cm^{-1}, with a resolution of about 2 cm^{-1}, and irradiated with a laser power of 20 mW, coming out from a 50 µm spot. Before measurements, a standard sample of known wavenumber furnished by the manufacturer was used for calibrating the DXR Raman Spectrometer. After that, samples, constituted by the 30 – 40 hairs of the same horse, were acquired for 30.0 s and averaged over 16 acquisitions (total acquisition time: 8 minutes). For each horse, 3 Raman spectra were acquired and for each spectrum, different hairs from the same horse have been analyzed. A spline baseline correction was performed, to remove the noise due to fluorescence background and scattering, and the obtained spectrum was normalized to the phenylalanine peak (center: about 1003 cm^{-1}). The average spectrum was stored in SPA format for further analysis. For each breed, no statistical differences were observed among the average spectra. In the end, for the A, B, and C groups, we have computed the average spectrum and the Standard Deviation (SD), starting from the average spectrum of each horse of the same breed.

Results

SEM

The morphological structure of the horsehair samples was identified by using SEM technique. In Figure 1 the SEM images of the natural horsehair for a representative animal of each group are shown.

By the image analysis, it is possible to observe no damage in the horsehair structure. Among the three groups differences in cuticular structure were recorded. The sizes of horsehairs performed by the SEM software are reported in Table 1. Figure 2 shows the size measurements of the natural horsehair for a representative animal of each group.

Raman Spectroscopy

Samples were obtained to characterize the proteins' sec'ndary structure of the horsehairs by the application of RS vibrational information on the individual groups and bonds.

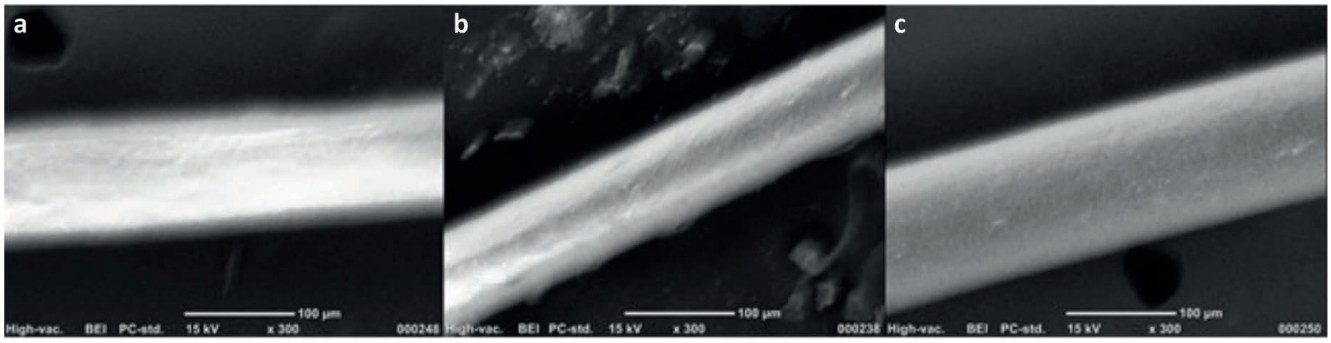

Figure 1. SEM images of a representative animal of group A: mixed-breed (a); group B, Italian saddle (b); group C: thoroughbred (c).

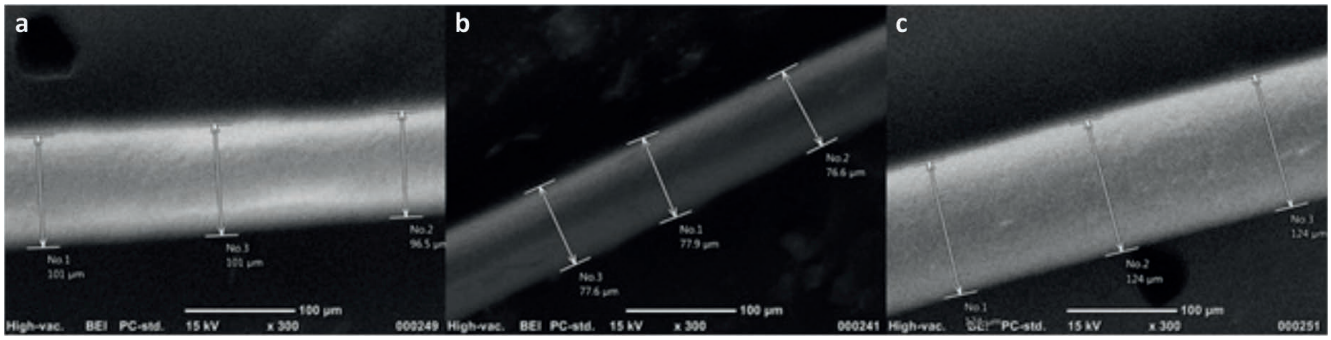

Figure 2. Size measurements of the natural horsehair for a representative animal of each group. group A: mixed-breed (a); group B, Italian saddle (b); group C: thoroughbred (c).

Figure 3 shows the similarities among Raman spectra collected on different hairs from the same horse, in the three groups. In Figure 4, the average Raman spectra and SD obtained from the different horsehair breeds are shown. The spectra exhibit the main typical vibrational modes. The interpretation and characterization of the spectral data were made based on literature[28-30] and are reported in Table 2.

Discussion

SEM technique is one of the instruments used to evaluate hair microstructure.[31] The SEM images showed that the microstructure of horsehairs is irregular and rough. In particular, Figure 1 shows the superficial layer of the three different horsehair breeds. In mixed-breed the cuticle seems compact and packed; in Italian saddle horsehair appears steaked and empty spaces are evident between cuticle cells; however, it is worth noting that no perpendi-

Table 1. Results of dimension measurements related to each horsehair analyzed, coming from different breeds.

Breed	Diameter size (mean value±SD) µm
Mixed Breed	98.1±2.6
Italian Saddle	79.3±0.2
Thoroughbred	122.8±3.4

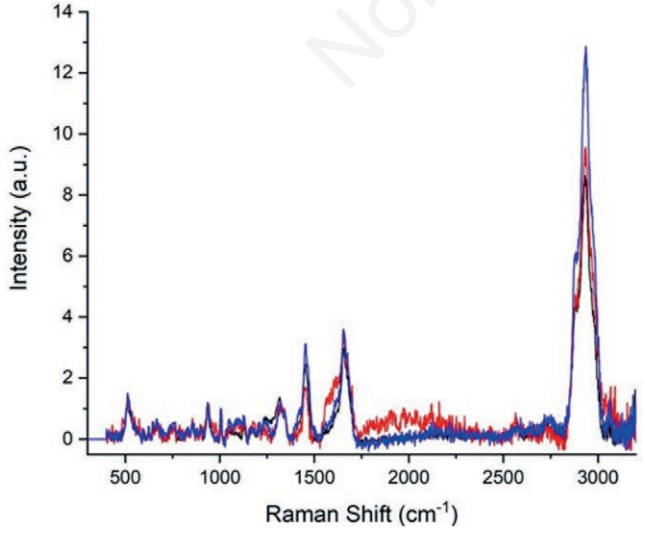

Figure 3. Raman spectra collected on different hairs from the same horse belonging to the three different breeds.

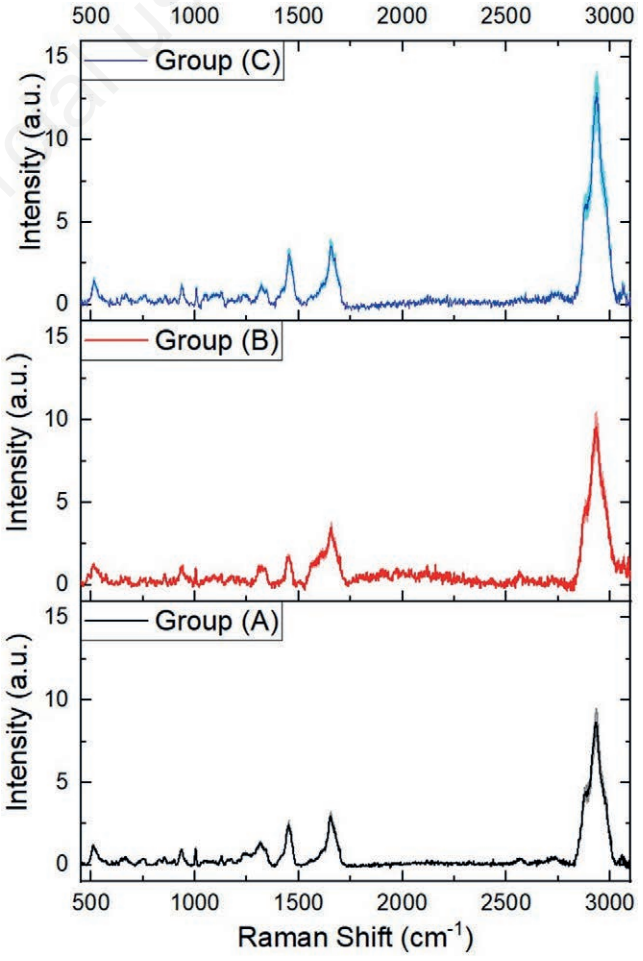

Figure 4. Average Raman spectra and SD of group A: mixed-breed (black line), group B: Italian saddle (red line) and group C: thoroughbred (blue line) breeds.

cular cracks are visible. In thoroughbred, cuticle cells are arranged around the cortex like roof tiles. In addition, the dimension size of the horsehairs was measured and the average diameter size, calculated from 30 measurements (10 for each horsehairs breeds) highlighted thoroughbred has the larger diameter followed by mixed bred and Italian saddle breeds.

Vibrational, rotational, and other low-frequency transition information are provided by Raman spectroscopy. Therefore, when horsehairs of different breeds are compared by the application of Raman spectroscopy, adequate information on biochemical changes due to breed is expected. Nucleic acids, lipids, carbohydrates, and complex biological systems are identified by Raman Spectroscopy[32,33] giving a spectral fingerprint of each sample.[34] For this reason, differences in peak position, width, and intensity between two or more spectra are used to differentiate the samples under investigation.[35]

As can be seen from an inspection of Figure 3, no differences are evident in the SS stretching region (510 cm^{-1}), in which the concentration of cystine in the cuticular layers is higher when compared to the cortical area and medulla;[8] in the C-S stretching region (665 cm^{-1}); in the amino acid tyrosine region (852 cm^{-1} and 828 cm^{-1}); in the region of the α-helix conformation of the protein (932 cm^{-1}); in the Amide III band (1245 cm^{-1}); in the 1320 cm^{-1} band (CH2 bend) and 1655 cm^{-1} band, attributed to Amide I (α-helix and β-sheet).

The spectral analysis shows the main differences in the S-O band, located at 1044 cm^{-1}, where cysteic acid is one of the constituents' amino acids of α-keratin; in particular, it is possible to observe a lower intensity only in the mixed breed. More evident differences are located at 1450 cm^{-1} band and ~2900 cm^{-1} band. The two bands are related to CH2 bending mode and CH stretching, respectively. The differences in intensity could potentially be explained by several mechanisms. They could be due to a change in the coordination structure of the carboxylate group and/or could be related to lipid disorganization. In particular, in the band at 1450 cm^{-1}, the thoroughbred has the major intensity, followed by mixed-breed and Italian saddle. In the ~2900 cm^{-1} band, always the thoroughbred has a major intensity, but, in this case, it is followed by the Italian saddle and then by the mixed-breed one.

These differences are not joined to the hair color; in fact, the main ones responsible for hair color are the melanin pigments eumelanin (EM) and pheomelanin (PM). Melanin exhibits a characteristic Raman spectrum. The bands are located at 1220 cm^{-1} (C – OH stretch), 1340 cm^{-1} (C-N stretch), 1390 cm^{-1} (C = C aromatic structure), 1562 cm^{-1} and 1598 cm^{-1} related to C=C vibrations and E2g mode, respectively.[36,37]

Hair analysis represents an important research field, for human hair. It has been used in forensic investigations, medical fields, and cosmetic industries.[38] In these research fields the main instrumentation used is the microscope for the comparison of hair found.[39] Alternative analytical methods to examine hair are liquid chromatography and mass spectrometry used to detect warfare agents and numerous abused drugs.[40,41] The above techniques are destructive and time-consuming; for this reason, in the last decade, Raman spectroscopy has proven itself an excellent tool for the identification of samples.

Table 2. Tentative vibrational assignment for the peaks/bands observed in Raman spectra of natural horsehairs of different breeds.

Raman Shift (cm^{-1})	Assignment
510	S-S stretching (cystine)
650	Tyrosine
747	Tryptophan
852	Ring of breathing mode of tyrosine
893	Tryptophan
936	Symmetric C-C stretching band (α helix of protein)
1002	Symmetric ring breathing oh phenylalanine
1045	S-O band (cysteic acid)
1125	C-N stretching
1177	Tyrosine
1245	Amide III band
1320	CH2 bend
1450	CH2 bending mode
1614	Tyrosine and Tryptophan
1655	C=O Amide I
2879	CH stretching (CH_2 and CH_3)
2935	CH stretching (CH_2 and CH_3)

Conclusions

The present study aimed to physically characterize horsehairs from different breeds to observe possible differences from the morphological and vibrational analysis. The characterization was made by using SEM and RS techniques, since, to our knowledge, no studies of such type are available on these specimens in the literature. SEM images showed differences in cuticular surface and diameter dimensions. RS analysis of horsehairs, coming from different breeds, showed differences in the bands located at ~1044 cm^{-1}, ~1450 cm^{-1}, and ~2900 cm^{-1}, which can be attributed to cysteic acid, CH2 bending mode, and CH stretching, respectively. These differences could be attributed to intrinsic or extrinsic factors, for example, their genetic predisposition or their metabolism, and they could represent the real differentiation among the breeds.

References

1. James V. The importance of good images in using hair to screen for breast cancer. J Med Gen 2001;38:e16
2. Robbins CR. Chemical and Physical Behavior of Human Hair. 4th ed. New York: Springer; 2001.
3. Wagner RCC, Kiyohara PK, Silveira M, Joekes I. Electron microscopic observations of human hair medulla. J Microscopy 2007;226:54–63.
4. Chen N, Bhushan B. Morphological, nanomechanical and cellular structural characterization of human hair and conditioner distribution using torsional resonance mode with an atomic force microscope. J Microscopy 2005;220:96–112.
5. Feughelman M, Lyman DJ, Willis BK. The parallel helices of the intermediate filaments of alpha-keratin. Int J Biol Macromol 2002;30:95-96.
6. Sandt C, Borondics F. Super-resolution infrared microspectroscopy reveals heterogeneous distribution of photosensitive lipids in human hair medulla. Talanta 2023;254.
7. Chen N, Bhushan B. Morphological, nanomechanical and cellular structural characterization of human hair and conditioner distribution using torsional resonance mode with an atomic force microscope. J Microscopy 2005;220:96–112.

8. Dias Santos J, Pinto PF, Edwards HGM, Cappa de Oliveira LF. Characterization by Raman and infrared spectroscopy and fluorescence microscopy of human hair treated with cosmetic products. Spectrochim Acta A Mol Biomol Spectrosc 2022;280:121577.
9. Essendoubi M, Meunier M, Scandolera A, et al. Conformation changes in human hair keratin observed using confocal Raman spectroscopy after active ingredient application. Int J Cosmet Sci 2019;41:203-12.
10. Kuzuhara A. Internal structural changes in keratin fibres resulting from combined hair waving and stress relaxation treatments: a Raman spectroscopic investigation. Int J Cosmet Sci 2016;38:201-9.
11. Wutke S, Benecke N, Sandoval-Castellanos E, et al. Spotted phenotypes in horses lost attractiveness in the Middle Ages. Sci Rep 2016;6:1-9.
12. Rouse JG, Van Dyke ME. A review of keratin-based biomaterials for biomedical applications. Materials 2010;3:999-1014.
13. Yang W, Yu Y, Ritchie RO, Meyers MA. On the strength of hair across species. Matter 2020;2:136-49.
14. Yedke SR, Raut SY, Jangde C. Experimental evaluation of horse hair as a nonabsorbable monofilament suture. J Ayurveda Integr Med 2013;4:206-10.
15. Meduri A, Severo AA, De Maria A, et al. PMMA intraocular lenses changes after treatment with Nd:Yag Laser: A scanning electron microscopy and X-ray spectrometry study. Appl Sci 2020;10:6321.
16. Kaliyadan F, Gosai BB, Al Melhim WN, et al. Scanning electron microscopy study of hair shaft damage secondary to cosmetic treatments of the hair. Int J Trichology 2016;8:94-8.
17. Acri G, Sansotta C, Salmeri FM, et al. Use of Raman Spectroscopy, Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy in a multi-technique approach for physical characterization of purple urine bag syndrome. Appl Sci 2022;12:4034.
18. Austin LA, Osseiran S, Evans CL. Raman technologies in cancer diagnostics. Analyst 2016;141:476–503.
19. Ye K, Li K, Lu Y, et al. An overview of advanced methods for the characterization of oxygen vacancies in materials. TrAC Trends Anal Chem 2019;116:102–8.
20. Acri G, Falcone A, Giannetto C, et al. Preliminary study for the application of Raman spectroscopy for the identification of *Leishmania* infected dogs. Sci Rep 2022;6;12:7489.
21. Acri G, Testagrossa B, Faenza P, Caridi F. Spectroscopic analysis of pigments of the Antonello Gagini annunciation's sculptural marble group, church of st. Theodore martyr (Bagaladi, Reggio Calabria, Italy): Case study. Mediterr Archaeol Archaeom 2020;20:1–5.
22. Acri G, Romano C, Costa S, Caridi F. Raman spectroscopy technique: a non-invasive tool in celiac disease diagnosis. Diagnostics 2021;11:1277.
23. Khan MSI, Oh SW, Kim YJ. Power of scanning electron microscopy and energy dispersive x-ray analysis in rapid microbial detection and identification at the single cell level. Sci Rep 2020;10:2368
24. Kuzuhara A. A Raman spectroscopic investigation of the mechanism of the reduction in hair with thioglycerol and the accompanying disulphide conformational changes, Int J Cosmet Sci 2018;40:34–43.
25. Zhou AJ, Liu HL, Du ZQ. Secondary structure estimation and properties analysis of stretched Asian and Caucasian hair. Skin Res Technol 2015;21:119–28.
26. Wilson AS, Edwards HGM, Farwell DW, Janaway RC. Fourier transform Raman spectroscopy: evaluation as a non-destructive technique for studying the degradation of human hair from archaeological and forensic environments. J Raman Spectrosc 1999;30:367–73.
27. Edwards HGM, Hassan NFN, Wilson AS. Raman spectroscopic analyses of preserved historical specimens of human hair attributed to Robert Stephenson and Sir Isaac Newton. Analyst 2004;129:956–62
28. Robbins CR. Chemical and Physical Behavior of Human Hair, 4th ed., Springer, New York, 2001.
29. Kuzuhara A. Internal structural changes in keratin fibres resulting from combined hair waving and stress relaxation treatments: a Raman spectroscopic investigation. Int J Cosmet Sci 2016;38:201-9.
30. Essendoubi M, Meunier M, Scandolera A, et al. Conformation changes in human hair keratin observed using confocal Raman spectroscopy after active ingredient application. Int J Cosmet Sci 2019;41:203-12.
31. Mucha A, Janeczek M. Morphological and elemental analysis of alpaca hair using scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM – EDX). Med Weter 2018;74:295-300.
32. Acri G, Testagrossa B, Giudice E, et al. Application of Raman spectroscopy for the evaluation of metabolomic dynamic analysis in athletic horses. J Equine Vet Sci 2021;96:103319.
33. Huang L, Sun H, Sun L. et al. Rapid, label-free histopathological diagnosis of liver cancer based on Raman spectroscopy and deep learning. Nat Commun 2023;14:48
34. Ripanti F, Fasolato C, Mazzarda F, et al. Advanced Raman spectroscopy detection of oxidative damage in nucleic acid bases: probing chemical changes and intermolecular interactions in guanosine at ultralow concentration. Anal Chem 2021;93:10825-33.
35. Giannetto C, Acri G, Giudice E, et al. Quantifying serum total lipids and tryptophan concentrations by raman spectroscopy during standardized obstacle course in horses. J Equine Vet Sci 2022;108:103820.
36. Wakamatsu K, Ito S. Recent advances in characterization of melanin pigments in biological samples. Int J Mol Sci 2023;24:8305.
37. Eliato TR, Smith JT, Tian Z, et al. Melanin pigments extracted from horsehair as antibacterial agents. J Mater Chem B 2021;9:1536-45
38. Kurouski D, Van Duyne RP. In situ detection and identification of hair dyes using surface-enhanced Raman spectroscopy (SERS). Anal Chem 2015;87:2901-6.
39. Robertson J. In Forensic Examination of Hair; Taylor and Francis: London, 1999; pp 79-154.
40. Jakobsson G, Kronstrand R. Segmental analysis of amphetamines in hair using a sensitive UHPLC-MS/MS method. Drug Test Anal 2014;6:22-9.
41. Tzatzarakis MN, Barbounis EG, Kavvalakis MP, et al. Rapid method for the simultaneous determination of DDTs and PCBs in hair of children by headspace solid phase microextraction and gas chromatography-mass spectrometry (HSSPME/GC-MS). Drug Test Anal 2013;6:85-92.