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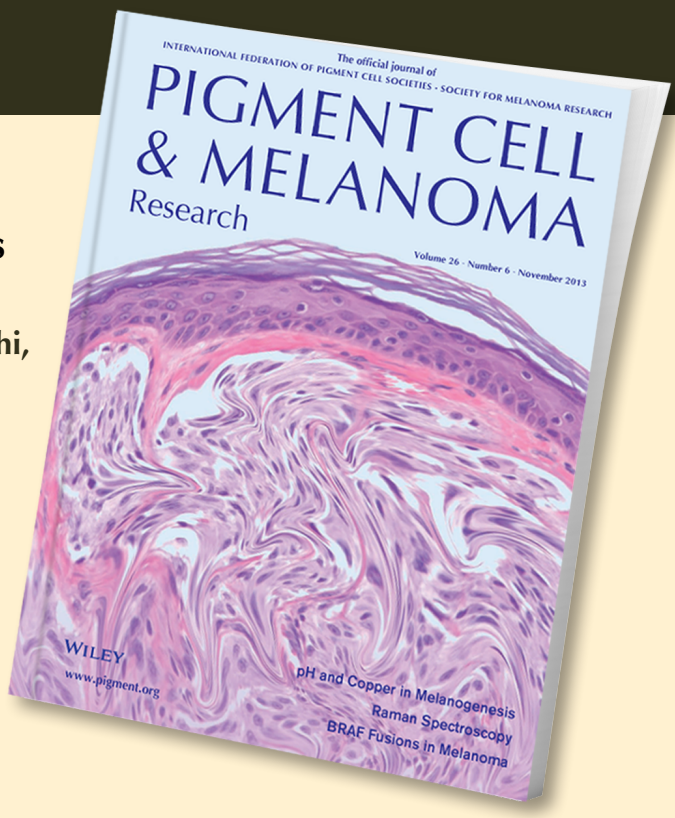

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Raman spectroscopy as a non-invasive technique for the quantification of melanins in feathers and hairs

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Summary

The quantification of melanins is a complex task due to the chemical heterogeneity of the pigments and the difficulty of their isolation. The best accepted procedure currently consists in the chemical cleavage of melanins and the subsequent detection of degradation products by HPLC, which implies the destruction of samples. Here, we show that Raman spectroscopy is a non-invasive technique that can be used to quantify melanins. We made parallel analyses of the characteristics of pheomelanin and eumelanin Raman spectra as measured by confocal Raman microscopy and of degradation products of pheomelanin (4-amino-3-hydroxyphenylalanine, 4-AHP) and eumelanin (pyrrole-2,3,5-tricarboxylic acid, PTCA) as measured by HPLC in feathers of red-legged partridges and hairs of wild boars and humans. We found strong correlations between the spectral Raman characteristics and 4-AHP and PTCA levels, which indicates that the Raman spectra of melanins can be used to determine their content.

Introduction

The development of methods to isolate and quantify melanins in biological tissues and cells has been decisive in the advance of the knowledge about these pigments. This has been a complex task due to the high chemical heterogeneity of melanins, although two general forms are widely recognized: eumelanin, a polymer of 5, 6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) units, and pheomelanin, a polymer of benzothiazine and benzothiazole derivatives (Ito et al., 2011a). The analysis of melanins has also been limited by the difficulty to extract melanins from the tissues where they are embedded because of the almost complete insolubility in all solvents of eumelanin, and because of the lack of simple methods that allow distinguishing eumelanins and pheomelanins (Ito et al., 2011a).

Simple techniques of melanin analysis include the measurement of absorbance at 500 nm (A500) of a solution of melanins, Soluene-350, and water, which can be used to qualify the total combined amount of eu- and pheomelanins, and the ratio A650/A500 is also useful in

Significance

To date, all analytical methods to quantify pheomelanin and eumelanin required the chemical degradation of samples. Raman spectroscopy (RS) provides different spectra for pheomelanin and eumelanin that can be obtained without pigment isolation, and the characteristics of the spectra correlate with pheomelanin and eumelanin contents as measured by HPLC. Therefore, RS represents a rapid and non-destructive technique for melanin quantification, particularly suitable for the analysis of valuable and in vivo samples. This opens new perspectives for the analysis of pigmentation in a wide range of fields from evolutionary biology to medicine.

estimating the eumelanin/total melanin ratio (Ozeki et al., 1996). The spectrophotometric method thus provides a convenient way to qualitatively characterize eu- and pheomelanins (Ozeki et al., 1996). A similar method that measures A450 and uses NaOH and hydrogen peroxide has been used to quantify melanins (Galván et al., 2010). It is also possible to distinguish melanins from carotenoids given the shape of their reflectance spectra, but it is difficult to distinguish between pheomelanin and other pigments with similar reflectance profiles such as iron oxide and porphyrins (Toral et al., 2008). However, the difficulty in the isolation of melanins has made that the most widely accepted analytical method consists in the quantification of specific marker degradation products of the pigments. Thus, eumelanin is oxidized with alkaline hydrogen peroxide and then the resulting products pyrrole-2,3,5-tricarboxylic acid (PTCA) and pyrrole-2,3-dicarboxylic acid (PDCA) are estimated by HPLC (Ito et al., 2011b), while pheomelanin is hydrolyzed with HI to form aminohydroxyphenylalanine (AHP) isomers that are then estimated by HPLC (Wakamatsu et al., 2002). PTCA, PDCA, and AHP are specific markers for eumelanin and pheomelanin, so these degradation methods are currently the most commonly used techniques to characterize and quantify melanins. Panzella et al. (2007) developed a method that combined the advantages of the alkaline hydrogen peroxide degradation used in the aforementioned methods with a procedure that does not require fractionation steps and is not affected by products derived from tissue protein hydrolysis. Therefore, all analytical methods that allow distinguishing eumelanin and pheomelanin available to date require chemical treatments of different complexity, which necessitates time consuming and costly procedures in which at least part of the sample is destroyed.

Here, we make parallel analyses with confocal Raman microscopy and HPLC to show that Raman spectroscopy (RS hereafter) represents the only non-destructive technique so far capable of distinguishing and quantifying eumelanin and pheomelanin in feathers and hairs without the need of pigment isolation and thus maintaining the integrity of the samples. RS consists in the detection of the inelastic scattering produced when a molecule is excited with light from a ground state to a virtual energy state, so that the change in frequency of the molecule between its initial state and the state after excitation (Stokes shift) is specific to the bonds nature or the molecule structure (Colthub et al., 1990). It has recently been shown that eumelanin and pheomelanin have different Raman spectra and can therefore be distinguished on the basis of their vibrational state (Galván et al., 2013). The Raman spectra of pheomelanin have a characteristic three 'humps' shape caused by three wide bands at about 500 per cm (band 1), 1490 per cm (band 2), and 2000 per cm (band 3) (Galván et al., 2013) (Figure 1). Pheomelanin's bands 1 and 2 are probably caused, respectively, by the out-of-plane deformation and the stretching vibration of the phenyl rings in the molecule structure, while band 3 is probably due to overtone or combination bands (Galván et al., 2013). The spectra of eumelanin, on the other hand, have a band at 500 per cm (band 1), two close bands at 1380 per cm (band 2), and 1580 per cm (band 3) resembling the D and G bands characteristic of disordered graphite, and absence of signal in the 1750–2500 per cm region (Galván et al., 2013; Huang et al., 2004) (Figure 1). Eumelanin's band 2 is caused by the stretching of the hexagonal carbon rings in the molecule structure, and band 3 is caused by the stretching of three of the six C-C bonds within the rings, while band 1 is probably caused by the out-of-plane deformation of phenyl rings (Galván et al., 2013). We show that the characteristics of these bands of the Raman spectra of eumelanin and pheomelanin can be used to quantify these pigments in feathers and hairs, as the spectral characteristics are associated with the results obtained by HPLC detection of degradation products from the same samples.

Results

Pheomelanin analysis

A partial least squares regression (PLSR) model for pheomelanin Raman spectra of 38 partridge feathers generated a factor that explained 46.5% of the variation in

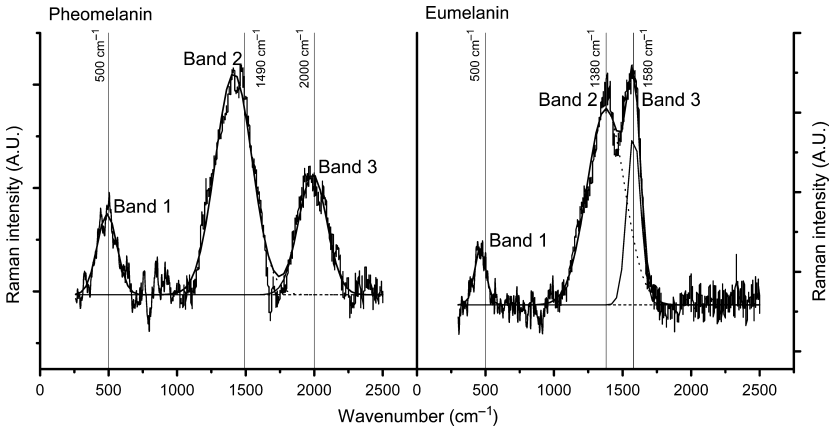

Figure 1. Raman spectra of pheomelanin and eumelanin showing the three characteristic bands used in the study and the results of fitting Gaussian deconvolution functions to the curves. Both spectra are from red-legged partridge feathers. A.U.: arbitrary units.

Table 1. Predictor weights of five partial least squares regression (PLSR) analyses explaining the correlation between the formation (ng/mg) of degradation products of melanins (pheomelanin: 4-AHP; eumelanin: PTCA) measured by HPLC in feathers and hairs (response variables) and the characteristics (X: position, A: area, W: width, Y: intensity) of three diagnostic bands of the Raman spectra of pheomelanin and eumelanin obtained from the same subjects (predictor variables)

	4-AHP				PTCA					
	Partridge feathers		Wild boar hairs		Partridge feathers		Wild boar hairs		Human hairs	
	Weight	*r*	Weight	*r*	Weight	*r*	Weight	*r*	Weight	*r*
X1	0.10	0.69	**0.30**	0.33	**0.36**	0.50	−0.19	0.06	**−0.25**	0.00
X2	−0.08	0.27	**−0.48**	0.18	**−0.35**	0.45	**0.39**	0.47	0.13	0.11
X3	−0.00	0.00	−0.13	0.48	**−0.34**	0.67	**0.62**	0.00	−0.07	0.00
A1	−0.01	0.18	0.16	0.33	0.17	0.29	**0.31**	0.00	**0.40**	0.38
A2	**0.41**	0.91	**−0.40**	0.53	**0.33**	0.65	0.05	0.03	**0.42**	0.55
A3	−0.07	0.29	−0.22	0.00	**0.36**	0.73	−0.11	0.00	0.12	0.85
W1	**−0.29**	0.12	**0.41**	0.40	−0.03	0.00	−0.09	0.08	−0.09	0.00
W2	0.04	0.08	−0.22	0.06	−0.09	0.20	**0.31**	0.12	0.16	0.00
W3	**−0.34**	0.37	0.11	0.34	0.16	0.10	**−0.41**	0.36	−0.11	0.00
Y1	**0.43**	0.79	0.01	0.32	**0.31**	0.59	−0.17	0.00	**0.42**	0.74
Y2	**0.46**	0.92	**−0.34**	0.50	**0.35**	0.72	−0.08	0.00	**0.41**	0.64
Y3	**0.44**	0.67	**−0.28**	0.04	**0.33**	0.69	0.03	0.00	**0.41**	0.67
% Variance accounted for	46.5		43.1		51.0		40.3		89.3	

Predictor weights represent the contribution of each predictor variable to the PLSR factor as they are computed so that each of them maximizes the covariance between the response variables and the corresponding factor scores. Predictor weights that retain > 5% of the information content of the PLSR factor (i.e., most important predictors) are shown in bold type. Repeatability values (r) for the Raman spectral characteristics are also shown. A zero repeatability indicates that variance within the same samples (i.e., individual feathers or hairs) was higher than between different samples.

the 4-AHP levels of feathers measured by HPLC (Table 1). 4-AHP levels were significantly correlated with this factor (Figure 2A). This PLSR factor was positively related to A2 and Y1-3, and negatively to W1 and W3 (Table 1), meaning that 4-AHP levels increased with increasing the area (particularly that of band 2) and intensity and with decreasing the width (particularly that of bands 1 and 3) of pheomelanin Raman bands. Two of these predictors (A2 and Y2) were highly repeatable between different feathers of the same birds ($r > 0.9$, Table 1), and the mean repeatability of the most important predictors (0.63) was higher than the repeatability of 4-AHP measurements by HPLC (0.54).

The PLSR model for 12 wild boar hairs explained 43.1% of the variation in 4-AHP levels, which were significantly correlated with this factor (Figure 2B). This PLSR factor was negatively related to A2, positively related to W1, and negatively related to Y2-3, thus coinciding with the predictors of 4-AHP levels in feathers (Table 1). X1 and X2 were additional predictors in this model. The highest repeatability values ($r \sim 0.5$) were for the same predictors than in the model for feathers: A2 and Y2 (Table 1).

Eumelanin analysis

A PLSR model with information on eumelanin Raman spectra of 37 partridge feathers explained 51% of the variation in PTCA levels, which were significantly correlated with this factor (Figure 2C). This PLSR factor was positively related to X1, A2-3 and Y1-3, and negatively related to X2-3 (Table 1). The highest repeatability values were obtained for X3 and A3 and Y2, with moderate repeatability between feathers ($r \sim 0.7$, Table 1). These repeatability values were lower than the repeatability of PTCA measurements by HPLC (0.85).

The PLSR model with information on eumelanin Raman spectra of 15 wild boar hairs explained 40.3% of the variation in PTCA levels, which were significantly correlated with this factor (Figure 2D). The most important predictors (i.e., highest predictor weights) in this model were X2 and X3, which were also predictors of PTCA levels in feathers. X2 also accounted for the highest repeatability in this model ($r \sim 0.5$). Additional predictors were A1, W2, and W3 (Table 1).

In the case of human hairs, and despite the small sample size (10 hairs), the model explained 89.3% of the variation in PTCA levels, which were strongly correlated with this factor (Figure 2E). The predictors were X1, A2, and Y1-3 (all these were also predictors of PTCA levels in feathers but not in wild boar hairs), in addition to A1 (which was predictor in wild boar hairs but not in feathers). The highest repeatability between hairs was for the strongest among these predictors (Y1, $r > 0.7$; Table 1).

Discussion

Our results show that the melanin contents measured by HPLC in feathers and hairs are associated with the

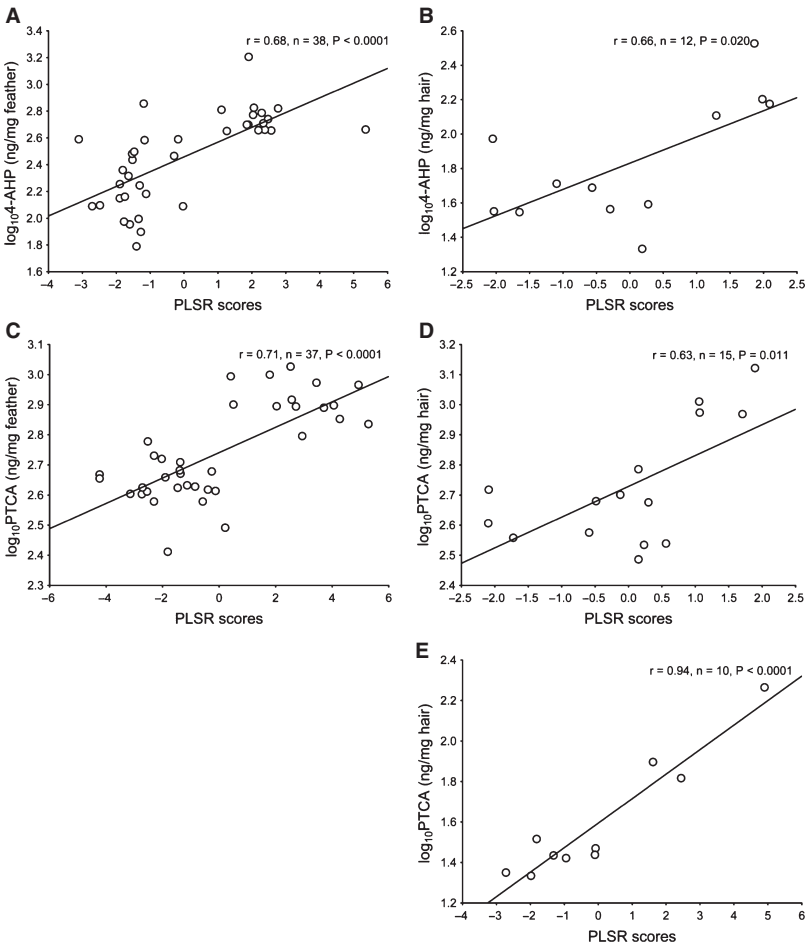

Figure 2. Relationship between pheomelanin and eumelanin levels measured by HPLC and the scores of PLSR factors collecting information on characteristics of the Raman spectra of pheomelanin (when 4-AHP is response variable) and eumelanin (when PTCA is response variable). A and C: red-legged partridges. B and D: wild boar hairs. E: human hairs. Regression lines and Pearson correlation tests are shown.

spectral Raman characteristics of melanins, which can therefore be used as predictors of melanin content in unaltered biological samples. Thus, RS represents a rapid and non-destructive tool to quantify melanins.

It is noticeable that the predicting capacity of the spectral Raman characteristics was marked even with very small sample sizes as in the case of human hairs (spectral predictors of eumelanin were able to explain almost 90% of variation in PTCA levels) and that most predictors of 4-AHP and PTCA levels, especially the former, coincided for feathers and hairs of different species, which indicates a general validity of RS as a quantification tool of melanins. It must be noted that the sign of the association of particular spectral Raman characteristics with 4-AHP and PTCA levels was opposite for feathers and hairs (Table 1), although the overall association between 4-AHP and PTCA levels and all spectral predictors was always positive and significant (Figure 2). We therefore suggest the use of the whole set of spectral Raman characteristics included here to predict melanin contents even though some predictors are more important than others (Table 1). In this case, we have collected the information of the whole sets of spectral Raman characteristics into single PLSR factors (X-axes in Figure 2). As the intended use of RS is to quantify melanin content without necessarily performing previous HPLC analysis, future users will not have response variables as we have in this study (i.e., 4-AHP and PTCA levels), so that only the synthesis of the predictor variables (i.e., spectral Raman characteristics) into one or more factors that collect the information content of these predictors will be necessary to get estimates of melanin content. In these cases, statistical techniques that reduce the multidimensionality in the predictor variables such as principal component analysis (PCA; Carrascal et al., 2009) should be used to calculate melanin concentrations. If there are limitations to use several predictor variables in the analyses because of low ratios of number of cases to number of variables, those that are more consistent between different feathers or hairs should be used (A2 and I2 were clearly the most consistent spectral characteristics of pheomelanin and tended to be so of eumelanin too).

It must be stressed that, although the amount of explained variation in 4-AHP and PTCA levels was acceptable (ranging between 40 and 89%), it was probably not larger because we detected Raman signal in untreated biological samples (i.e., undegraded melanins; Galván et al., 2013) where 4-AHP and PTCA, albeit specific markers, are present at small amounts (Ito et al.,

2011a). This in turn leads to another advantage of RS as a melanin quantification technique, as it can provide concentration values of pure melanins instead of degradation products if calibration curves are made with different concentrations of synthetic pheomelanin (Galván et al., 2013) and commercial *Sepia* eumelanin as standards for pheomelanin and eumelanin, respectively.

The proposal of this novel technique for the analysis of melanins is particularly useful for analyzing pigmented samples of high value or that cannot be brought to a laboratory, as RS does not require destroying the samples as HPLC degradative analysis does (Ito et al., 2011b; Wakamatsu et al., 2002), and there are portable and handheld Raman spectrometers commercially available. This also means that RS is especially suitable for measuring melanin content in in vivo samples from animal subjects including humans (Zhao et al., 2010). There are no a priori reasons to think that this technology is not valid for any other pigmented structures such as skin (see Scarmo et al., 2013 for an example of RS applied to the quantification of skin carotenoids) and melanocyte cultures. The potential of RS as a non-invasive analytical tool for melanin quantification in medicine should be thus explored by future studies.

It must be mentioned that the Raman spectra of pheomelanin and eumelanin were not always easily distinguishable in hairs, as some spectra showed characteristics of both pigments (we discarded these spectra from the analyses), indicating that the Raman beam was detecting a mixture of pheomelanin and eumelanin. Thus, the performance of RS in melanin analysis is likely to depend on the spatial segregation and size of melanin polymers in relation to the Raman spatial resolution, which in turn highlights another advantage of RS in addition to the simplicity of the analyses and its non-destructive nature, namely the high spatial resolution (1 μm) of RS used with confocal Raman microscopy. This allowed us to obtain Raman signal of melanins in very small structures such as individual barbules of feathers or different parts of individual hairs, thus avoiding the problems derived from small quantities of material that make difficult the analysis by HPLC of, for example, individual hairs. This property opens the excellent possibility of using confocal Raman microscopy to explore the fine-tuned distribution of melanins along pigmented tissues.

The main limitation of RS to quantify melanins is that the Raman signal of melanins from untreated biological samples is often weak, especially in the case of hair pheomelanins. Indeed, we had to discard some Raman spectra in which the deconvolution function used to obtain the spectral parameters did not fit the curves because of low signal-to-noise ratio (SNR). In these cases where Raman signal is weak, results may be improved using longer laser excitation wavelengths in the infrared region (ca. 1000 nm), as this uses to allow for a larger efficient observation of Raman bands (e.g., Antunes et al., 2006). Alternatively, the near-infrared fluorescence properties of melanins (Huang et al., 2006) may serve as a complement to detect and quantify melanins when Raman spectra are weak, although fluorescence spectra that are specific to eumelanin and pheomelanin remain to be reported. Additionally, we did not detect Raman signal of both pheomelanin and eumelanin in some samples in which both 4-AHP and PTCA were actually quantified, although this problem may be solved by increasing the number of Raman measurements per sample. Indeed, it is expected that more Raman spectra than those obtained here for each sample increase the amount of variation explained in 4-AHP and PTCA levels.

Methods

Samples

We collected 40 dorsal feathers from 10 adult red-legged partridges *Alectoris rufa* (four feathers/bird) kept at captivity at the IREC-CSIC (Ciudad Real, Spain), 40 facial hairs from 20 wild boars *Sus scrofa* (two hairs/wild boar) in Doñana National Park (southwestern Spain), and blond, medium and red human hairs from 11 American subjects and one black hair from one Colombian female (one hair/subject) (Galván et al., 2013).

Confocal Raman microscopy

Feather and hair samples were analyzed in a Thermo Fisher DXR confocal dispersive Raman microscope (Thermo Fisher Scientific, Madison, WI, USA) operating in the Museo Nacional de Ciencias Naturales (MNCN-CSIC, Madrid, Spain) with a point-and-shoot Raman capability of 1-μm spatial resolution and using an excitation laser source at 780 nm of 20 mW (red-legged partridge and human samples) or 8 mW (wild boar samples) power. The single spectra were obtained using a 100x confocal objective, a slit aperture of 50 μm, and a grating of 400 lines/mm. These conditions produced an average spectral resolution of 2.2–4.4 per cm in the wavenumber range of 100–2500 per cm. An integration time of 3 s × 16 accumulations (red-legged partridge and human samples) or 3 s × 12 accumulations (wild boar samples) allowed getting an acceptable SNR (red-legged partridge feathers: 2.13, wild boar hairs: 132.96, human hairs: 51.15; average SNR values calculated as the ratio of the peak signal to the root mean square (rms) of the noise voltage) and a photobleaching time of 30 s. Laser power and integration time values were chosen to optimize SNR values in each type of sample while avoiding damage to them (breakage of the focal feather barbs and barbules and hairs), which the confocal microscope allowed us to determine at real time. The system was operated with Thermo Fisher OMNIC 8.1 software. Calibration and aligning of the spectrograph were checked using pure polystyrene.

The Raman beam was focused at four barbs and four barbules chosen at random of each feather, and at three points approximately equally separated (proximal, middle, and distal parts) of each hair. A single spectrum was obtained from each of these structures, which was either pheomelanin-based or eumelanin-based. We thus obtained 320 Raman spectra of partridge feathers, 120 spectra of wild boar hairs, and 32 spectra of human hairs. We used the diagnostic bands (bands 1-3; Figure 1) to assign the Raman spectra of feathers and hairs to either pheomelanin or eumelanin. Pheomelanin also has a characteristic Raman band at about 1150 per cm (Galván et al., 2013), but we did not include this band in the analyses (see below) because of its small relative area. The Raman spectrum of trichochromes is similar to that of eumelanin, but it does not have

a band about 500 per cm as the spectra of natural eumelanin (Galván et al., 2013), so it is very unlikely that we assigned spectra of eumelanin to trichochromes. Furthermore, only small amounts of trichochromes are produced during pheomelanogenesis (Ito et al., 2011a), so any confounding effect would be irrelevant.

After discarding the region of very high Raman intensities at the first part of the curve below 300 per cm, we smoothed it by the adjacent averaging technique. We then fitted a Gaussian function to the curve (e.g., Filik et al., 2003) to obtain spectral parameters derived from each spectrum, considering the three peaks for pheomelanin and eumelanin described above (Figure 1). For each spectrum, we obtained the area (A1, A2, and A3 for bands 1, 2, and 3, respectively), the width (W1, W2, and W3), the spectral position (X1, X2, and X3), and the Raman intensity (Y1, Y2, and Y3) of the three bands. The analyses of the Raman spectra were made with ORIGIN v.7 software (OriginLab Corporation, Northampton, MA, USA).

We discarded some spectra (96 from red-legged partridges, 55 from wild boars, and four from humans) where the function did not fit due to low SNRs and, in the case of some hairs, that could not be assigned to either pheomelanin or eumelanin because there were spectral characteristics of both pigments. However, as we analyzed different feathers or hairs from each subject, the removal of the above-mentioned spectra only implied the removal of one individual red-legged partridge and one individual wild boar from the analyses. For the rest of subjects, we could analyze Raman spectra from at least one feather or hair. It is necessary to note that the fact that we removed spectra with characteristics of both eumelanin and pheomelanin does not mean that we removed samples where both pheomelanin and eumelanin were present. Indeed, both pigments were present in all feather and hair samples as shown by the HPLC analyses and the Raman spectra, but each Raman spectrum corresponded to either pheomelanin or eumelanin (i.e., the laser beam detected eumelanin or pheomelanin granules) so that, for each sample, we had Raman spectra for pheomelanin and Raman spectra for eumelanin. For each subject, we calculated the mean of the spectral parameters from the Raman spectra of pheomelanin and eumelanin that were obtained in the different feathers or hairs. In the case of human hair, we detected Raman signal of pheomelanin in hairs from only five subjects, so we did not include human hair in the analysis of pheomelanin due to the small sample size.

HPLC analyses

Feather and hair samples were exposed to an oxidation by alkaline H_2O_2 to form the degradation products PTCA and PDCA specific to eumelanin and to a hydriodic acid (HI) reductive hydrolysis to measure the degradation product 4-amino-3-hydroxyphenylalanine (4-AHP) specific to pheomelanin. The degradation products were analyzed with HPLC (Ito et al., 2011b; Wakamatsu et al., 2002). We took 4-AHP and PTCA levels as indicative of pheomelanin and eumelanin levels, respectively, in feathers and hairs.

Each individual feather was analyzed by HPLC, but there was not enough material in each individual wild boar hair to analyze both pheomelanin and eumelanin by HPLC because this technique requires a minimum amount of material (> 1 mg of feathers or hairs) that is not obtained with single hairs, which can however be analyzed by RS. We thus had to include wild boar hairs different from those that were analyzed by RS to reach the minimum necessary material to make the HPLC analyses, although each analysis always contained samples from the same wild boars. Therefore, Raman and HPLC analyses were made on the same samples in feathers, but in the case of wild boar hairs, the HPLC analyses included additional samples to those that were analyzed by RS. In the case of human hairs, Raman and HPLC analyses were made on completely different samples from the same subjects.

Statistical analyses

We analyzed the relationships between 4-AHP and PTCA levels (response variables) and Raman spectral characteristics (position, area, width, and intensity of bands; all them as predictor variables) of feathers and hairs by means of partial least squares regressions (PLSR; Carrascal et al., 2009), using the individual subject (partridge, wild boar or human) as the sample unit. This statistical tool, which is widely used in analytical chemistry and particularly in quantification by RS (e.g., Glenn et al., 2007), is an extension of multiple regression analysis where associations are established with factors extracted from predictor variables that maximize the explained variance in the dependent variable. These factors are defined as a linear combination of independent variables, so the original multidimensionality is reduced to a lower number of orthogonal factors to detect structure in the relationships between predictor variables and between these factors and the response variable. The extracted factors account for successively lower proportions of original variance. The relative contribution of each variable to the derived factors can be estimated by means of the square of the predictor weight. Each predictor weight maximizes the covariance between the response variables and the corresponding factor scores. Therefore, PLSR analyses can identify the best Raman spectral predictors of melanin content while avoiding the problems derived from multicolinearity and small number of observations in comparison with the number of predictor variables (Carrascal et al., 2009). We only considered the first extracted factor. We introduced all variables $\log_{10}$-transformed in the PLSR models. PLSR models were run with STATISTICA 8.0 software (StatSoft, Inc., Tulsa, OK, USA).

We tested for the correlation between the content of melanins measured by HPLC (4-AHP and PTCA levels) and the PLSR factors generated consisting of spectral Raman predictors by means of Pearson correlation tests. We also measured the repeatability of Raman spectral characteristics and 4-AHP and PTCA levels between feathers or hairs of the same subjects following Lessells and Boag (1987). It must be noted that this repeatability test does not measure the performance of the Raman or HPLC measurements, just how consistent are Raman spectral characteristics and HPLC measurements between different structures (feathers or hairs) chosen at random of the same subjects.

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