JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, January 2011, Vol. 56, No. 1
doi: 10.1111/j.1556-4029.2010.01567.x
Available online at: interscience.wiley.com

PAPER

CRIMINALISTICS

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Forensic Discrimination of Dyed Hair Color: II. Multivariate Statistical Analysis*,†

ABSTRACT: This research is intended to assess the ability of UV–visible microspectrophotometry to successfully discriminate the color of dyed hair. Fifty-five red hair dyes were analyzed and evaluated using multivariate statistical techniques including agglomerative hierarchical clustering (AHC), principal component analysis (PCA), and discriminant analysis (DA). The spectra were grouped into three classes, which were visually consistent with different shades of red. A two-dimensional PCA observations plot was constructed, describing 78.6% of the overall variance. The wavelength regions associated with the absorbance of hair and dye were highly correlated. Principal components were selected to represent 95% of the overall variance for analysis with DA. A classification accuracy of 89% was observed for the comprehensive dye set, while external validation using 20 of the dyes resulted in a prediction accuracy of 75%. Significant color loss from successive washing of hair samples was estimated to occur within 3 weeks of dye application.

KEYWORDS: forensic science, hair dyes, microspectrophotometry, multivariate statistical analysis, agglomerative hierarchical clustering, principal component analysis, discriminant analysis

Advances in the technology of spectroscopic detectors have aided in the perception of color by the human eye. In addition, the emerging field of chemometrics enables scientists to process complex data sets in an efficient and objective manner. Multivariate analysis of high throughput scientific data allows for the extraction of useful information, effectively reducing the complexity of the data set, thus making computationally demanding analyses more reasonable (1,2). Elucidation of trends inherent in the data set is also enhanced, as even subtle differences are discernable. Adequate sample selection and replicate measurements are crucial to appropriately represent the population under investigation (3).

The application of multivariate statistical analysis spans a broad range of applications and instrumental techniques (2). Pattern recognition and discrimination of data is an integral part of many forensic science applications, where chemometrics would substantially contribute to the field (3). Various forms of trace evidence have been evaluated with multivariate statistical analysis. In this work, UV–visible spectra of dyed hair samples will be analyzed using chemometric methods to assess the discriminating capabilities of the method (4). The statistical analysis scheme chosen for this research, consistent with existing literature, includes agglomerative hierarchical clustering (AHC), principal component analysis (PCA), and discriminant analysis (DA) (5).

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*Funded by the Forensic Sciences Foundation Jan S. Bashinski Criminalistics Graduate Thesis Grant, 2008.
†Presented at the 61st Annual Meeting of the American Academy of Forensic Sciences, February 16–21, 2009, in Denver, CO.

Received 10 Aug. 2009; and in revised form 6 Nov. 2009; accepted 10 Nov. 2009.

Materials and Methods

Information regarding hair samples, dye products, washing and dyeing protocols, instrumental parameters, as well as data pretreatment strategies is summarized in the first paper of this two-part series (4).

Discrimination of Dyed Hair Color

Fifty-five red hair dyes, making up the "comprehensive dye set," were applied to separate, standardized bundles of the hair from the same individual (L602 substrate). Five randomly selected hair samples were set aside for testing. The hair samples were analyzed using MSP over the wavelength range of 200–700 nm. Five scans were collected along the length of each hair. The absorbance values were subjected to a pretreatment procedure consisting of baseline correction and normalization (6). An average of 25 scans (five hairs per dye, five scans per hair) was calculated to represent each dye sample and will be referred to as the "dye average." The dye averages are intended to represent a "known" sample, comprised of several hairs with multiple scans per hair. In addition, the "hair average" of five scans for each hair was also tabulated and utilized to monitor the effects of intra-individual variability (7). Unless otherwise noted, all spectra shown in the figures are baseline corrected, normalized, and depict the average of 25 scans.

Chemometric Methods

XLSTAT®-Pro Version 2008.2.1, a Microsoft Excel® (Redmond, WA) add-in developed by Addinsoft SARL (Paris, France), was utilized for statistical processing of the pretreated data. Clustering techniques, including AHC, illustrate natural groupings of objects as measured by the association between samples. This technique is unsupervised in the sense that no prior knowledge of classes is

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input into the analysis. The samples are successively paired and merged according to similarity, as illustrated in a dendrogram. Corresponding distances are displayed on the *x*-axis and are measures of the degree of dissimilarity between clusters (1,2). A truncation, commonly depicted by a dotted line, strictly represents the threshold defining the statistically significant classes. The statistically derived mean of each class is called the centroid, while the input data closest to the mean is referred to as the central object. AHC utilized Euclidean distances; linking and clustering was achieved using Ward's aggregation criterion (5). The number of classes established in AHC was based exclusively on the dye or substrate average of 25 spectra (five hairs per dye or substrate, five scans per hair) and strict interpretation of the automatic truncation in the dendrogram.

The primary objectives of PCA are to effectively reduce or discover the "true" dimensionality of the data as well as to provide insight into underlying interpretive meaning. A new ordinate system is created, whereby the traditional axes are rotated such that the maximum variability is described. The principal components are not only uncorrelated and orthogonal to one another but also represent the maximum variability starting with the first component and descending with subsequent components. Factor scores are tabulated that depict the locations of each observation in the data set with respect to each of the principal component axes. Factor loadings plots illustrate the contributions of the original variables to the variance described by each principal component (5). The weights associated with each original variable are termed "loadings," while the principal components are sometimes referred to as "factors." Exploration of this plot aids in the discerning chemical trends potentially associated with each component axis (1,2). The spectra representing the dye average were submitted for analysis by PCA to validate the classifications established by AHC, as well as to identify regions of the spectra that contributed significantly to the variance in the data set. Subsequently, a more in-depth analysis of the hair averages was conducted for comparison.

DA is a "supervised" technique, which utilizes a learning ("training") set of known observations to develop canonical variates (CVs) by which new samples are classified. Resembling PCA, DA generates new variables that permit a more compact description of the useful information. In contrast to PCA, the discriminant axes, or CVs, effectively represent the maximum variance between groups and minimum variance within groups, which may not coincide with the factors with greatest variability. The classifications of the samples by DA are often further described by confidence ellipses around the centroid of each group, where 95% probability is commonly depicted. These ellipses are defined by Mahalanobis distances which describe both the variability observed for each sample as well as the relationship of each sample to one another. Based on the models established from the training set, the classification of unknown samples may be predicted, as well as probability of group membership (1–3). The classification accuracy of the discriminant model may be assessed by either evaluating new data (external validation) or submitting existing data into the algorithm and comparing known and predicted classifications (internal validation). Leave-one-out cross-validation provides an internal validation of the learning set whereby each individual sample is removed, the remaining data set is analyzed, and the selected sample is reclassified and compared to the known classification (1,2). An approach for assessing the added discriminating power of chemometrics, called the maximum chance classification, involves calculating the percent contribution of largest class relative to the overall data set. This scheme assumes that all samples would be classified as members of the largest group and allows for comparison between random group assignments relative to statistically derived classifications (3). Classes in DA were assigned based on the AHC classification of the averaged dye spectrum, while the PCA factor scores were utilized to quantitatively represent each of the samples.

Discrimination of Dyed Hair Color (External Validation)

Following the initial evaluation of the comprehensive dye set, new dyed hair samples were selected from each of 20 bundles and analyzed as external validation samples. Three of the samples were the central objects of the AHC classes (see Results and Discussion), with the remainder selected randomly. External validation data were input as supplemental data during PCA analysis of the comprehensive dye set and subsequently analyzed as prediction samples in DA. As supplemental data, PCA factor scores are obtained along with the "training set," but kept separate as a "test set." When submitted to DA, the "training set" is used to construct the discriminate model (comprehensive dye set), while the "test set" consists of new samples that will be classified based on the model (external validation samples). Two trials were conducted, one for the dye averages and the other for hair averages. The accuracy of the predicted identities of the external validation samples was evaluated based on the known identities of the samples along with corresponding AHC classifications.

The Effect of Original Hair Color

For comparison purposes, natural ("undyed') hair samples from 25 different sources were analyzed by MSP and multi-variate techniques. An average of 25 scans (five hairs per source, five scans per hair) was calculated to represent each dye sample and will be referred to as the "substrate average." In addition, the hair average of five scans for each hair was also analyzed. Statistical analysis was also performed on the same hair samples dyed with RCS42 and CHE44.

Fading of Dyes with Successive Washing

Insight into the ramifications of color loss on the statistical classification of samples is of particular interest, as varying intervals of time exist between the collection of samples from a crime scene and apprehended suspect (8). The natural substrate, L602, was selected as a reference, while dyed samples of L602 (using LCSPF36, LF36, and LP6R dyes) were selected as the representatives of the various tones of dye. The L602 samples were considered a control, as these samples were not dyed and would demonstrate variability within the sample population or process. The control and trial samples were washed, soaked, rinsed, and dried consistently with one another (4). The comprehensive dye set as well as the L602 control samples were included as active members in PCA analysis. To track the fading of samples associated with successive washing, the natural substrate (L602) was included with the comprehensive dye set, and these samples were categorized uniquely as a fourth group in DA. The wash samples from each of the three trials were evaluated as supplemental samples in PCA and as prediction samples in DA, while the results of the L602 control series were derived from the cross-validation confusion matrix.

Results and Discussion

Additional reference UV–visible MSP spectra are provided in the first paper of this two-part series (4).

Discrimination of Dyed Hair Color

Evaluating the ability of UV–visible MSP to differentiate the color of dyed hair is the primary objective of this research. The dendrogram representing the classification of the comprehensive set of 55 red hair dyes is presented in Fig. 1. Spectra corresponding to the central objects are demonstrated in Fig. 2; the L602 natural hair sample is included as a reference. Original dye bundles were arranged according to the groupings established in the AHC dendrogram. The classes were visually consistent with different tones of red, where Class 2 was dark red auburn, Class 1 was medium auburn, and Class 3 was light auburn. Subdivisions were also apparent for each of the three groups; the most substantial deviation was observed for subclasses 1a and 1b. All of the members of subgroup 1b are professional dyes with either extended developing times or a higher developer:base ratio. Consequently, these dyes provided an elevated lift to the natural hair color and thus yield more vivid coloration. Spectra from each of the groups were also visually compared and the groups established in AHC were sensible.

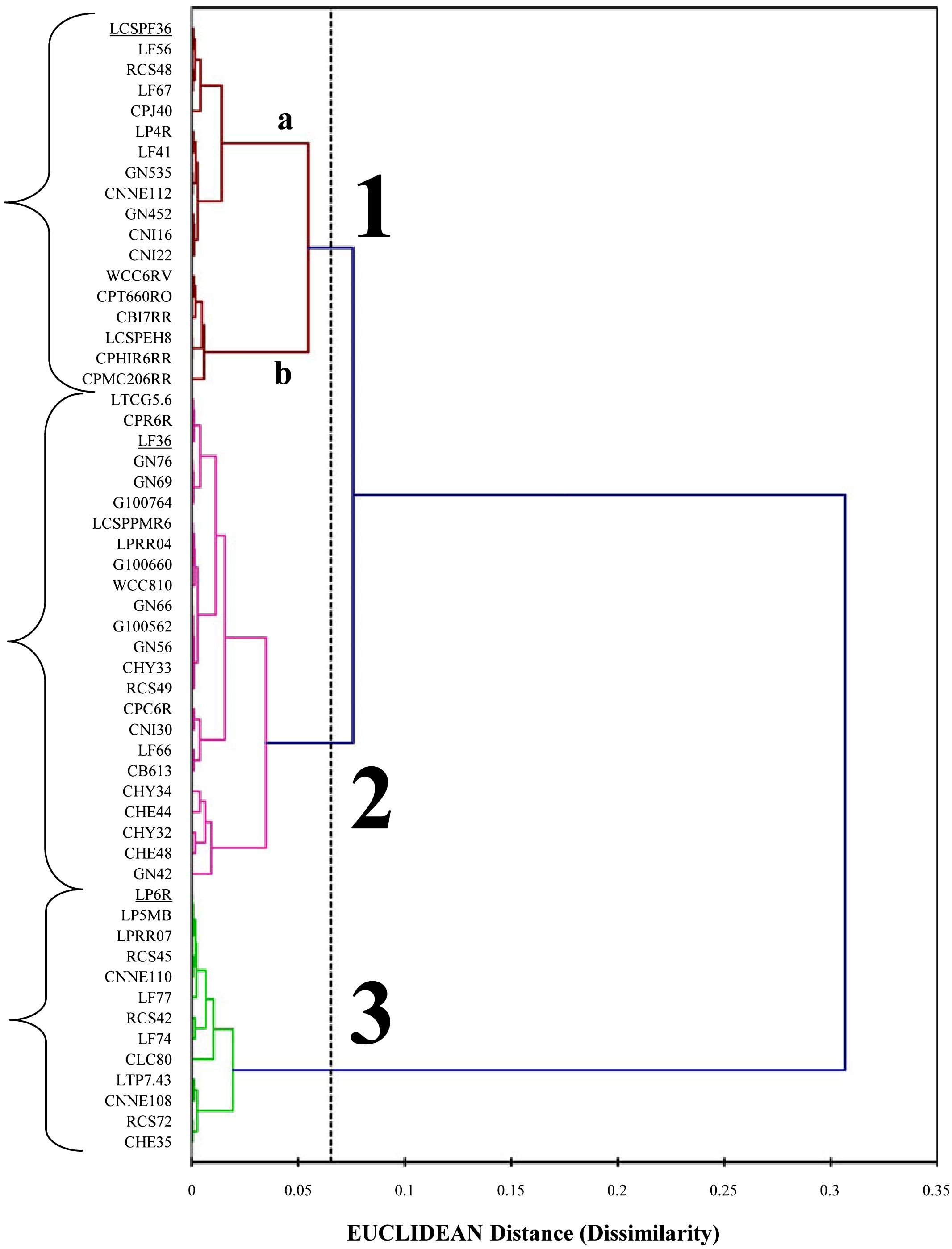

FIG. 1—*Dendrogram resulting from agglomerative hierarchical clustering of UV–visible MSP spectra of L602 hair dyed with 55 red hair dyes. The automatic truncation, illustrated as the dotted line, defines three major classes, each of which has two subsets. Three entries are underlined which correspond to the object closest to the mean of the class, which is referred to as the central object.*

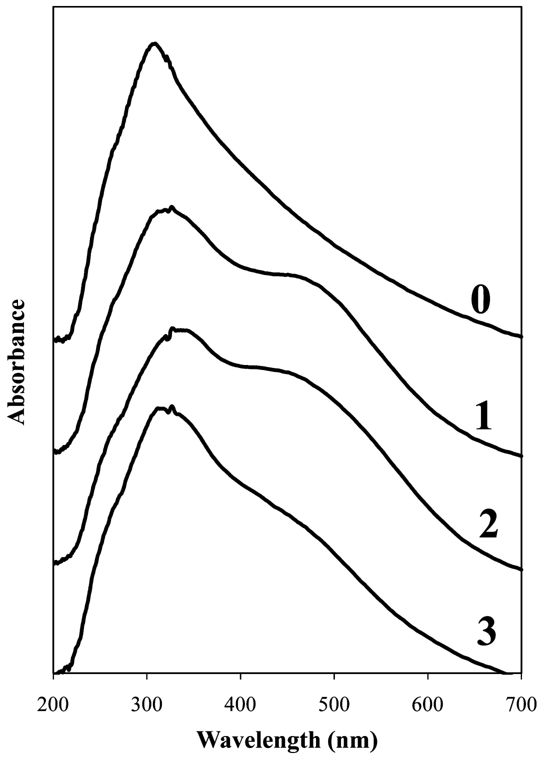

FIG. 2—*UV–visible MSP spectra of the central objects, as identified by agglomerative hierarchical clustering, plotted relative to the undyed substrate. (0) L602 undyed hair sample and (1) Class 1, LCSPF36; (2) Class 2, LF36; (3) Class 3, LP6R dyed hair samples.*

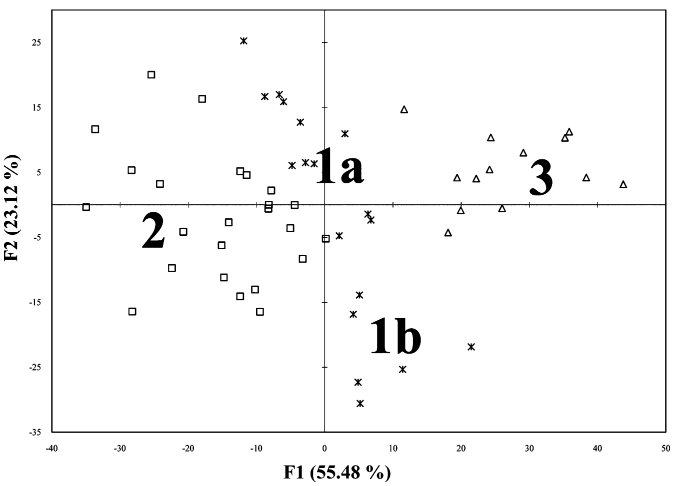

FIG. 3—*Principal component analysis observations plot for dye averages, describing 78.6% of the overall variance in the first two axes. Symbols represent members of the following classes: (*) Class 1, (□) Class 2, and (Δ) Class 3.*

For the comprehensive dye set, a two-dimensional PCA plot, representing 78.6% of the overall variance, is demonstrated in Fig. 3. A graph of the factor loadings for the first two principal components is shown in Fig. 4. The first component (F1) relates the ratio of the intensity of the hair (<380 nm)/dye (>400 nm) contribution, where positive values indicate a larger ratio. The ratio of the peak intensity between the hair and dye peaks (~375 nm) relative to the dye peak (~475 nm) is described by the second

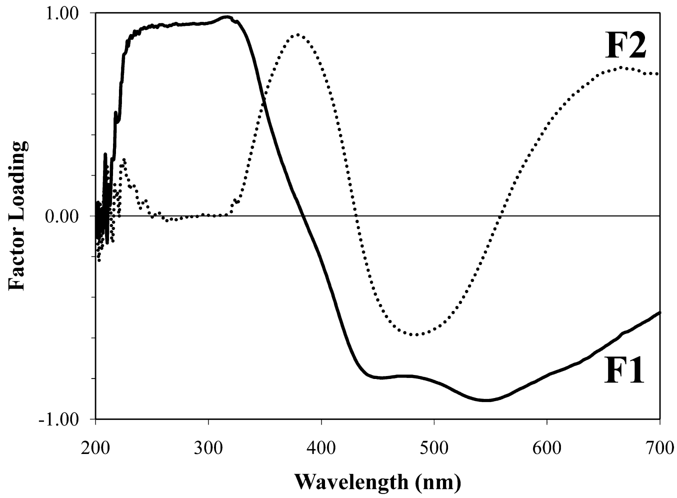

FIG. 4—*Principal component analysis factor loadings plot for dye averages. Factor 1 (F1), representing 55.5% of the overall variance, is represented as a solid line. Factor 2 (F2) is designated by a dotted line and accounts for 23.1% of the overall variance.*

component (F2). Members from the three previously defined classes in AHC (refer to Fig. 1) were clustered together in the PCA plot. Spanning the F1 axis, Class 2 members were located in the negative region, Class 1 was clustered around the origin, and Class 3 samples were in the positive region of axis. The second axis generally described more subtle trends, with the exception of the subgroup 1b members that were segregated from other Class 1 members along the negative vertical axis. The formulations of the commercial and professional base colorants were reviewed to discern any relationships between dye components and PCA factor scores (4). Trends were not readily observed, as many of the red hair dyes have very similar primary intermediates and couplers, applied with variable vehicles. The distinction in tones relies on the reactivity and relative amounts of these components, dye conditions, along with the complex interaction between the substrate and reactive dye system. Additional insight into relationships regarding the chemical composition of residual dye components may be pursued more effectively with a suitable orthogonal technique.

Four techniques were evaluated for determining the appropriate number of significant principal components to retain for discriminant analysis: 95% variance, Scree, Kaiser, and *F*-test (1,2,6). Generally, while the Scree, Kaiser, and *F*-test methods incorporated more PCs and hence more variance, the classification accuracy tended to decrease, respectively. Ultimately, the 95% variance method was selected for consistency and ease of use, as well as for relative accuracy for this data set. Five significant components were retained for the dye average set, while six components were kept for the hair average set.

For the average dye data, the cross-validation accuracy was 89.1%. An observations plot for DA is presented in Fig. 5. The misclassification errors indicated that members of Class 1 were correctly classified most frequently and that no misclassifications took place between Classes 2 and 3, which exhibit the greatest variability. Three members of Class 3 (CLC80, LF74, and RCS42) were misclassified as members of Class 1. Three members of Class 2 (G100660, CPC6R, and WCC810) were also misclassified as members of Class 1. When comparing these results to the DA observations plot, it is evident that many of these samples fall in regions of overlapping confidence ellipses and have reduced probabilities. Cross-validation results for hair-by-hair analysis resulted in

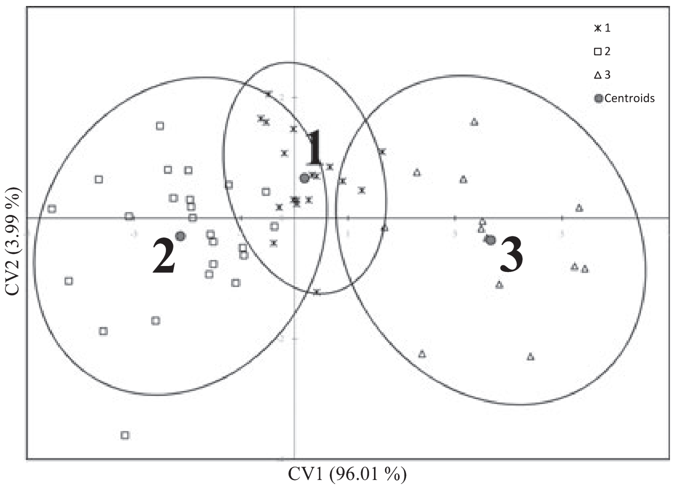

FIG. 5—*Discriminant analysis observations plot for dye averages, describing 100% of the overall variance in the first two axes. Symbols represent members of the following classes: (*) Class 1, (□) Class 2, and (Δ) Class 3. Class centroids, calculated class means, are denoted by (•); confidence ellipses represent the 95% boundary of each of the three uniquely defined classes.*

an accuracy of 81.45%. These results are similar to those observed for the dye averages, where the majority of the confusion was apparent for Class 1 with the adjacent two classes, near overlapping regions. Overall, the confusion between Class 2 and Class 3 represented <0.5% of the data set. The classification accuracies for average dye (89%) and individual hair analyses (81%) are substantially improved from the maximum chance probability of 44% for this data set. (Class 2 is the largest class, representing 24 dyes. Of the 55 total dyes, members from Class 2 represent 44%.)

Individualization of evidence is highly desirable for forensic associative evidence. Given the nature of supervised classification, it is possible to define classes at the level of individual natural or dyed hair samples. However, based on several DA calculations, it is evident that this technique is not able to successfully discriminate the 25 natural hair samples from one another (25 classes, one for each hair sample, classification accuracy of 22%) nor can it reliably individualize the 55 hair dyes (55 classes, one for each dye, classification accuracy of 56%). Finally, cross-validation of the discrimination of commercial versus professional dyes yielded an accuracy of 83%; however, it should be noted that the commercial group represents nearly 75% of the overall data set. As a result of the underrepresentation of professional dyes and bias toward vibrant tones, it is believed that this cross-validation accuracy assessment is overestimated. This data suggest that this technique cannot discriminate between commercial and professional dyes.

Discrimination of Dyed Hair Color (External Validation)

When external samples were used to test the classification, the cross-validation accuracy for the average dye samples is 75%. Five of the 20 samples were misclassified, including CBI7RR, GN56, CPC6R, CNNE110, and RCS42. All of these samples are found in the overlapping central region, where heightened confusion is not unfounded. The percent error associated with each class is slightly distorted as each error is magnified because of the smaller size of the sample set. Of the five averaged dye samples that were misclassified in the validation set, CPC6R and RCS42 were also misclassified in the training set. The other three samples are distant from the original input samples. Although within overlapping regions in the initial subset, CBI7RR, GN56, and CNNE110 were drawn closer to the alternative class assignment. The prediction results for the representative hair samples were comparable with that of the dye averages, yielding an accuracy of 76%. Ten of the validation dyes possessed at least one problematic hair sample, with four of the representative dyes exhibiting problems with three or more of the five hairs. The most problematic samples were consistent with the dyes described for the average dye data.

The Effect of Original Hair Color

Although the focus of this work is on discriminating dyed hair color, the original hair color is an important factor (4,9). In this study, 25 samples that represented seven different shades of hair ranging from dark brown to light blonde were acquired. The classification of the spectra obtained from undyed hair using AHC resulted in four main classes, which were generally consistent with the shade. It was also observed that a "leveling effect" occurred upon dyeing. For example, in AHC, the distance that joined all observations into a single cluster for the natural substrates occurred at a Euclidean distance of *c.* 1.4, while the distances observed after applying the RCS42 and CHE44 dyes were 0.25 and 0.18, respectively. As predicted, the substrate influence was more substantial for the RCS42 dye, which exhibits minimal absorbance. Analysis of each of these subsets with PCA validated the previously described trends.

The ability of MSP to differentiate natural hair color was evaluated with DA. The accuracy with which DA classified samples into the four groups as determined by AHC using an average substrate spectrum (25 scans/sample) was good (84%). As expected, the accuracy decreased (71%) when the average hair spectra (for five scans/sample) were considered as there was more opportunity for confusion between hairs of the same shade. Consistent with the leveling effect discussed above, the ability of DA to correctly discern the substrate color decreased upon application of the RCS42 dye (64% classification accuracy of substrate average data) and even further upon application of the CHE44 dye (56% classification accuracy of substrate average data). The reduction of the variability between hairs exhibiting different pigmentation is one of the desirable characteristics of dye products, which draws consumers to this cosmetic application, primarily to conceal or blend gray hair.

Fading of Dyes with Successive Washing

The effect of color loss from successive washing on classification can also be estimated. Migration in the positive direction of both the F_1 and F_2 principal component axes was observed with repetitive washing of the dyed hair samples. This effect was also monitored in a DA observations plot, where the only sample to hold true to its original class designation was LP6R. Notably, none of the dyes reverted back to the region predominated by the L602 natural hair samples and remain differentiated in DA. Trends in the classification provided insight into the detrimental effect of washing on the successful classification of the sample to the original, freshly dyed hair.

To track the progression of color loss observed for successive washing on classification result, the following system was devised and is illustrated in Fig. 6. A classification score for each hair sample was calculated based on the membership probabilities (Pr.) from the DA prediction results, whereby the relative amount of red character represented by each class is Class #2 > Class #1 > Class #3 > Control Class. The theoretically pure level of each class,

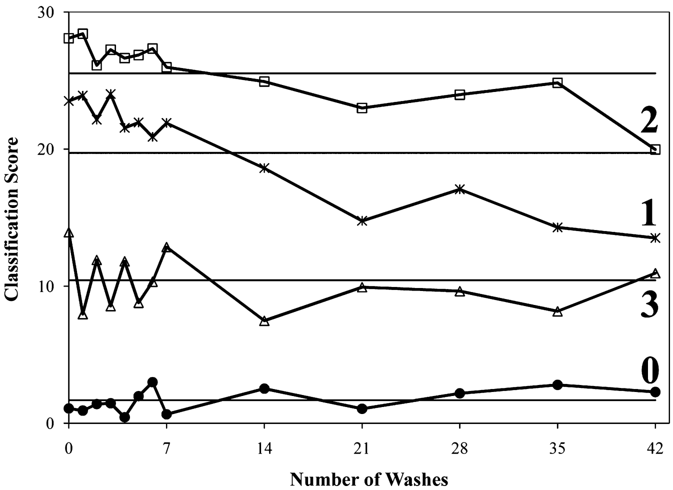

FIG. 6—*Estimated fading of dyes with successive washing. The average classification score for each representative class was tabulated and plotted as a function of the number of washes corresponding to the data point. (•, 0) L602 undyed control samples; (*, 1) Class 1, LCSPF36; (□, 2) Class 2, LF36; (Δ, 3) Class 3, LP6R. The means from the representative classes are denoted by a solid line in the figure.*

yielding a probability of 100%, is assigned a value ten units separated from adjacent classes, with the L602 natural substrate defined as zero. Equation (1) was utilized to define the arbitrary classification scores utilized for graphically demonstrating the observed classification trends, where Pr.x is the probability associated into class x (where x = 2,1,3, and control).

$$\text{Classification Score} = (30 \times \text{Pr.2}) + (20 \times \text{Pr.1}) + (10 \times \text{Pr.3}) + (0 \times \text{Pr.Control}) \quad (1)$$

The average classification score for each representative class was tabulated and plotted as a function of the number of washes corresponding to the data point, thus illustrating the fading behavior for three class representatives as well as the variability associated with the L602 control samples. Class means were calculated based on the probabilities of each of the dyes within the class. The corresponding values were 25.5, 19.7, 10.4, and 1.7 for Class 2, 1, 3, and the control, respectively.

Scatter in the data may be attributed to intra-individual variability between the hairs, as different hairs were tested in each stage. Both LF36 and LCSPF36 samples faded substantially over the course of the study, as indicated upon macroscopic visual inspection, spectral review, as well as PCA and DA analyses. Over the span of the simulated 6-week testing period, both LF36 and LCSPF36 samples diminished by approximately one classification level, corresponding to a classification score approximately ten points lower than the initial sample. Fitting a trendline to the data, both of these trials exhibited a negative slope with correlation coefficients (R^2 values) of 0.78 and 0.90 for LF36 and LCSPF36, respectively. Additionally, a *t*-test was performed for the initial and final values (10). The null hypothesis was rejected in both circumstances, statistically verifying that the initial and final values are different. Although the LP6R sample is not static, the classification is oscillating about the initial classification. Upon the completion of the study, none of the dyed hair samples converged with the natural hair samples. The L602 control sample is relatively consistent, with scores ranging from 1–2.5 points. For both the LP6R and L602 samples, no slope was observed, and the null hypothesis for the *t*-test of the initial and final values was not rejected. The point corresponding to the decline of approximately half of a classification level for LF36 and LCSPF36 is observed at 3 weeks. At this point, both samples drop below the threshold of the initial classification and are weighted toward being classified as a member of another class. After three simulated weeks of washing, the null hypothesis of the *t*-test was rejected for both LF36 and LCSPF36, indicating that the values are statistically different; while, after 2 weeks, the null hypothesis failed to be rejected in both cases. Consequently, it is roughly approximated that reasonably reliable sample classification accuracy is achieved only within the first 3 weeks following the initial application of dye. Brown and black dyes are projected to be retained in the cuticle for a longer duration of time, relative to red hair dyes, because the coupled dye molecules are larger (11).

Conclusions

A comprehensive set of 55 red hair dyes was analyzed by UV–visible MSP and evaluated with multivariate analyses. Three primary groups were identified and logically supported with macroscopic visual inspection of the dyed hair samples. A cross-validation accuracy assessment of 89% was obtained for averaged spectra representing each dye and an accuracy of 81% was obtained for hair-by-hair analysis of the samples. Twenty dyes were selected randomly for an external validation. New data were collected for each of these dyes and analyzed as prediction samples in the analysis, with an accuracy of 75%. This technique is not capable of individualizing natural hair samples nor dyed hair samples. In general, commercial and professional dyed hair samples were not distinguishable. Significant fading associated with successive washing was estimated to occur after three weeks of daily washing.

Overall, the results obtained from chemometric analysis of UV–visible MSP data of red hair dyes support future research focused on validating the method and optimizing it for implementation in routine analysis of dyed hair samples in forensic crime laboratories. The discriminating capabilities of the proposed UV–visible MSP methodology would be also enhanced with an orthogonal technique capable of providing more insight into the chemical compositions of residual dye components.

Acknowledgments

Without the generosity of the following organizations, this research would not have been possible. Intellectual and technical support was provided by Paul Martin and associates of CRAIC Technologies. Hair samples were donated by Aveda Fredric's Institute and Honors Beauty College in Indianapolis, Indiana.

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