Forensic Science International 238 (2014) 91–95

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Discrimination of human and animal blood traces via Raman spectroscopy

Gregory McLaughlin, Kyle C. Doty, Igor K. Lednev *

Department of Chemistry, University at Albany, State University of New York,, 1400 Washington Avenue, Albany, NY 12222, United States

ARTICLE INFO

Article history:
Received 14 July 2013
Received in revised form 10 February 2014
Accepted 25 February 2014
Available online 12 March 2014

Keywords:
Raman spectroscopy
Chemometrics
Blood
Serology
Human origin

ABSTRACT

The characterization of suspected blood stains is an important aspect of forensic science. In particular, determining the origin of a blood stain is a critical, yet overlooked, step in establishing its relevance to the crime. Currently, assays for determining human origin for blood are time consuming and destructive to the sample. The research presented here demonstrates that Raman spectroscopy can be effectively applied as a non-destructive technique for differentiating human blood from a wide survey of animal blood. A Partial Least Squares-Discriminant Analysis (PLS-DA) model was built from a training set of the near infrared Raman spectra from 11 species. Various performance measures, including a blind test and external validation, confirm the discriminatory performance of the chemometric model. The model demonstrated 100% accuracy in its differentiation between human and nonhuman blood. These findings further demonstrate a great potential of Raman spectroscopy to the field of serology, especially for species identification of a suspected blood stain.

© 2014 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

The identification of a body fluid stain is an important and unavoidable aspect of many forensic investigations. There are a variety of presumptive tests that are currently used for identifying a stain as blood. These tests are most commonly oxidation–reduction assays, which are destructive in nature, using reactants such as leucomalachite green, luminol, phenolphthalein and tetramethylbenzidine [1,2]. A common confirmatory blood test is a microcrystal assay (e.g. Teichmann or Takayama assays). The Ouchterlony or similar immunochromatographic assays can then be employed to determine if the blood is nonhuman [1]. It is ultimately preferable to confirm the presence of blood and the species of origin before forensic DNA profiling, but this can be practically problematic.

Since the amount of suspected blood evidence may be miniscule, it needs to be preserved and analyzed efficiently. Identification testing schemes need to be minimally destructive to preserve the sample for DNA analysis [3]. Since both presumptive and confirmatory tests use chemicals that are destructive, their use consumes a portion of the sample. To minimize this effect, a modified testing scheme is employed. Typically, after a stain has been presumptively identified as blood, further characterization is not carried out [4]. If a DNA profile is not extracted from the sample, then the suspected blood is presumed to be of animal origin [4]. This is a problematic testing scheme primarily because there is a lack of confidence that the sample is of human origin and in fact blood. This uncertainty is based on the fact that presumptive tests have known occurrences of false positives. This streamlined testing approach could also be detrimental for crime labs since time and money would be wasted on nonhuman or non-blood samples. Therefore, a non-destructive screening technique to efficiently identify human blood would be highly valuable. Raman spectroscopy is a technique that has the potential both as a non-destructive confirmatory identification for blood [5,6] and as a species of origin assay [7].

Raman spectroscopy has been proven as an effective and versatile analytical technique for a variety of forensic applications [8,9], including identification of lipsticks [10], drugs [11], condom lubricant [12] and fibers [13]. There is often no sample preparation needed for Raman analysis and the sample size can be on the order of picograms. More importantly, Raman spectroscopy is typically non-destructive to the sample which is crucial for forensic applications. This technique is based on the detection of light that is inelastically scattered by a sample upon irradiation from a monochromatic light source. A Raman spectrum contains numerous distinctive bands which correspond to specific molecular vibrational modes [14]. The Raman spectrum of blood in particular

* Corresponding author. Tel.: +1 518 591 8863; fax: +1 518 442 3462.
E-mail address: ilednev@albany.edu (I.K. Lednev).

http://dx.doi.org/10.1016/j.forsciint.2014.02.027
0379-0738/© 2014 Elsevier Ireland Ltd. All rights reserved.

provides rich detail and has been targeted previously for forensic studies [15,16]. The popularity of Raman spectroscopy has been growing in forensic laboratories especially due to reduced cost of instrumentation and its numerous possible applications.

De Wael et al. was the first to apply vibrational spectroscopy to the problem of species identification of blood samples [17]. They reported the inability to differentiate between blood particles originating from human, cat and dog samples through their infrared and Raman spectra. The infrared spectrum of human and sheep blood has also been reported to be visually indistinguishable [18]. However, a recent study proves that discrimination can be enhanced with chemometric modeling of Raman spectroscopic data [7]. Virkler et al. reported the ability to distinguish blood samples from human, cat and dog using a Principal Component Analysis (PCA) model. Separation between these classes within the PCA model exceeded a 99% confidence interval. Even though there is agreement that the spectra obtained from the three classes appeared very similar, chemometric models are designed to exaggerate minor variations in the data. This method of data analysis enhances the selectivity of Raman spectroscopy and has been used extensively to build similar classification models [19,20].

To expand upon the proof of concept work of Virkler et al., we analyzed blood from a wider survey of species (12 in total). Bearing in mind forensic purposes, the animal (nonhuman) samples were selected to represent three groups: animals that are bred for domestication (cat, dog, horse and cow), those that are consumed as food (chicken, cow, pig, and rabbit), and those that are integrated with human existence (mouse, rat, opossum and raccoon). Analyzing a wider variety of animals enhances the forensic practicality of the study and adds more certainty to model predictions. To account for the increased complexity of the dataset, a Partial Least Squares-Discriminant Analysis (PLS-DA) classification model was used. These models have more class discrimination power than those of PCA and provide automatic soft prediction of classes [21]. A PLS-DA model was used to differentiate a large training dataset of human and animal blood spectra and to classify a set of unknown samples. As an external validation step, predictions were performed on cow spectra which were excluded from the training dataset. The constructed model demonstrates a great ability to discriminate human from animal blood. We demonstrate a more comprehensive and robust method for screening a suspected blood stain to identify human origin. Our analytical approach aims to be used for the rapid and non-destructive identification and characterization of a blood stain at a crime scene.

2. Methods and materials

2.1. Raman microscope and blood samples

A Renishaw inVia confocal Raman spectrometer and a Renishaw PRIOR automatic stage were used for data collection for all blood species experiments. The instrument was calibrated with a silicon standard before all measurements. Spectra were accumulated with a 20× long range objective with 785 nm excitation in the spectral range of 250–1800 cm^{-1}. Laser power at the sample was approximately 4.0 mW. A Raman map consisting of 35 spectra was collected from each of the samples. WiRE software version 3.2 was used to operate the instrument.

Animal (nonhuman) blood from cow, cat, dog, horse, pig, mouse, opossum, raccoon, rabbit, rat and chicken were obtained from Bioreclamation, LLC. For each animal species class, 2 mL of blood were procured from 10 individual donors. Animal blood was collected randomly from both genders to ensure donor diversity. Human blood was also obtained from Bioreclamation, LLC with consideration to gender, race and age diversity. Blood samples were kept frozen until sample preparation. For each blood sample, approximately 30 μL was placed on an aluminum covered microscope slide and allowed to dry for at least 60 min. Raman measurements were obtained within 48 h of initial sample preparation.

2.2. Data preparation and statistical treatment

All data preparation and construction of statistical models were performed with the PLS Toolbox 7.0.3 (Eigenvector Research Inc.) operating in MATLAB version R2010b. The spectra were truncated to the data range 252–1709 cm^{-1}. For each sample, the 35 spectra were baseline corrected with a sixth-order polynomial and normalized by area. These 35 spectra were averaged to form a single spectrum representing one sample. These averaged spectra were imported into a data matrix. The dataset was finally mean centered before models were calculated. The model was cross-validated using the Venetian Blinds method [22].

2.3. Blind test

To evaluate the performance of the classification model, a set of ten unknown samples were prepared from the available blood samples. Five human and five animal samples were arbitrarily chosen in a single-blind fashion (where the analysts were not aware of the identity) and assigned as unknown #1–10.

3. Results and discussion

The main objective of this study is to develop a method to differentiate human blood samples from a wide variety of animal blood samples through their Raman spectra. The experimental design, including laser power, excitation wavelength, data acquisition, data processing, and model selection were made based upon previous studies of body fluid traces in our laboratory. A PLS-DA classification model was built using a training dataset containing Raman spectra from human and selected animal samples (excluding cow). The number of components was selected by choosing a local minimum of total data variance captured using a scree plot (not shown). The PLS-DA model was constructed in a binary manner by classifying each spectrum as either human or animal. The model was tested by attempting to classify ten unknown samples and ten known samples (cow) which were outside of the training dataset.

3.1. Spectral analysis of training dataset

For each blood sample, a Raman spectral map of 35 points was collected. The spectra within each map were preprocessed by baseline treatment, normalized by area and averaged. The training dataset consisted of ten mean spectra from every species considered (excluding the ten cow samples) for a total of 110 mean spectra. The preprocessed training dataset spectra are shown in Fig. 1A. The prominent features in a Raman spectrum of blood correspond to specific vibrational modes related to hemoglobin [23]. From visual inspection, all spectra look to be identical in terms of the number of spectral features and their location. There are variations visible in the relative intensity of many Raman bands which are confirmed by the standard deviation of this dataset (Fig. 1B). A comparison of the animal and human class mean spectra is shown in Fig. 1C. These spectra are the total averaged data for their respective classes after preprocessing. The largest difference between the animal and human spectra is primarily in the shape and intensity variations within the 1220–1300 cm^{-1} spectral range, corresponding to stretching vibrational modes of hemoglobin [23].

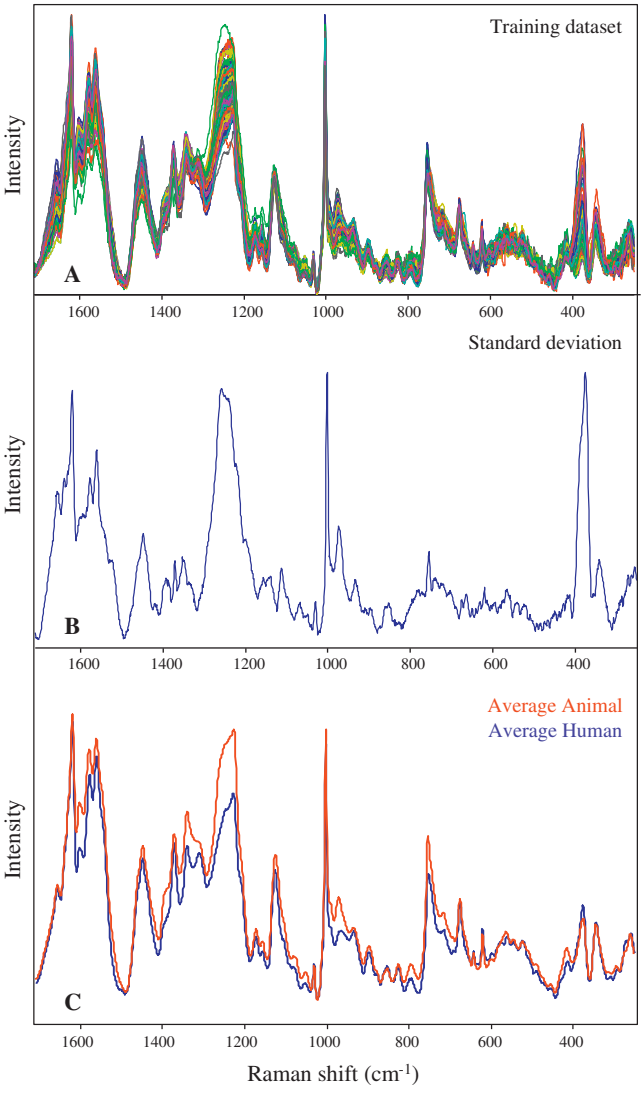

Fig. 1. (A) Comparison of the preprocessed Raman spectra from 11 species and (B) the standard deviation of this dataset. (C) Average spectrum of all human (n = 10) spectra and all animal (n = 100) spectra.

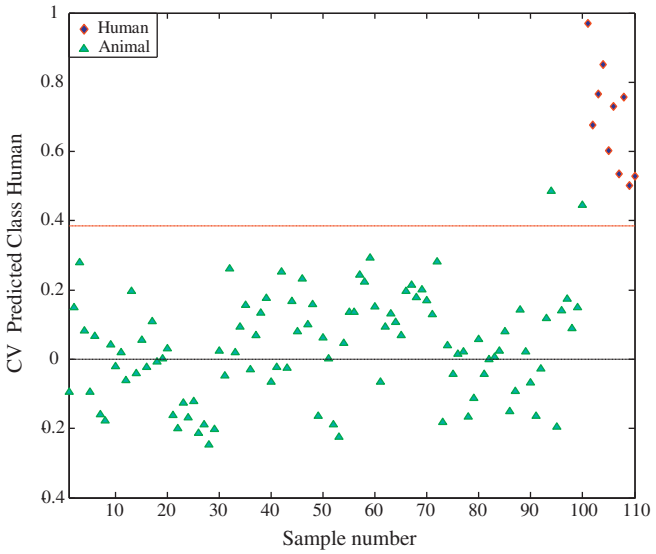

Fig. 3. Cross-validated prediction scores for human class using the binary PLS-DA model. Red line represents the default classification threshold. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Construction of a binary model

A PLS-DA model was constructed using the training dataset containing 110 spectra classified as either human or animal. The model was built with four latent variables. A scores plot showing the distribution of the spectra on the first three latent variables is shown in Fig. 2. From this scores plot, there is almost complete separation of the human class from the animal class. The cross-validated (CV) prediction results for the human class are shown in Fig. 3. This plot displays the scores for each spectrum on a selected class metric. This illustrates the classification ability of the model for every spectrum in the training dataset. However, there are two animal spectra which score very close to human with cross-validation. These spectra score above the classification threshold, which generate the two misclassifications (false positives). Both of these misclassified spectra originate from different chicken samples. The internal classification results of the model under CV, with respect to human classification ability, are 98 and 100

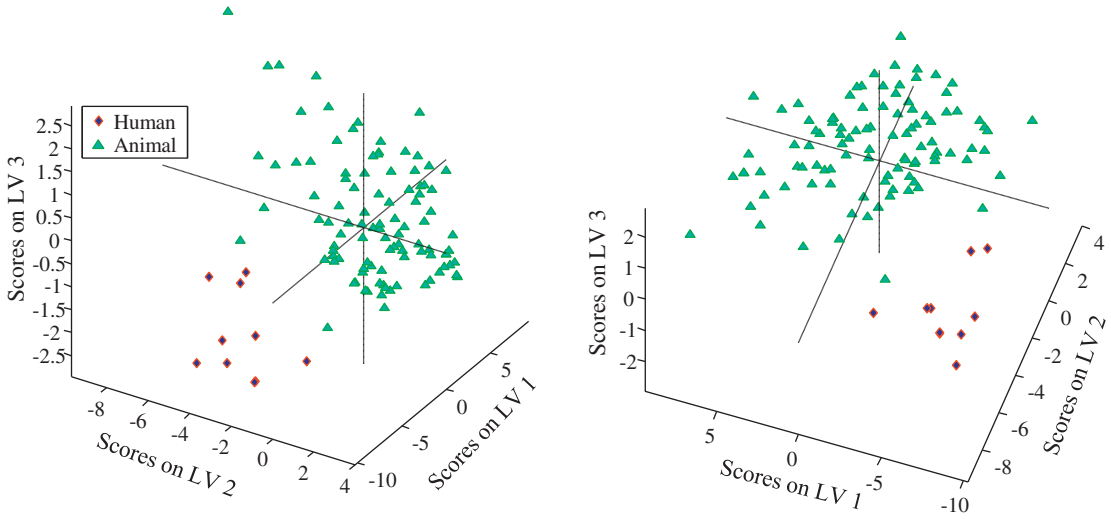

Fig. 2. Three-dimensional scores plot of the PLS-DA binary model. Both figures are the same scores plot rotated slightly for perspective. *Note*: there is a fourth dimension to the model that is not represented in these figures.

(rate of true negative and rate of true positive, respectively). These values differ slightly from the calibrated model performance values where the true negative and true positive rates are both 100.

3.3. Blind test and external validation

To confirm the performance of the model, predictions were calculated for twenty spectra – ten spectra originating from ten cow blood samples and ten spectra originating from ten human and/or animal samples chosen in a single-blind fashion (Section 2.3). Spectra accumulated from cow blood were excluded from the training data. The cow spectra were excluded as an external validation step to test how the model would classify samples outside of the training dataset. This is an important consideration since it is practically impossible to compile training data for every animal species. The prediction results for these twenty spectra for the model are displayed in Table 1.

The model performed with 100% accuracy for unknown class predictions. Human and animal spectra were correctly assigned for the ten unknown samples and the ten cow samples. The cow spectra scored extremely low for the human class prediction in the binary model (Fig. 4). This demonstrates that it would be very unlikely for a cow spectrum to be misidentified as human. However, this result does not preclude the possibility of some unconsidered species misclassifying as human. The prediction score of unknown #5 fell slightly below other human samples in the training dataset. However, this is corrected when more latent variables are added (data not shown), a sign that the current model is somewhat underfitted [21]. Training dataset misclassifications under cross validation are eliminated with additional latent variables. A blind test with unknown samples that were not used to build the training dataset would assist with determining precisely the number of latent variables to use for this model. The described tests confirm the model's performance but more stringent validation steps are necessary and ongoing.

The model characteristics are defined by the shape of each component loading variable. The four loading variables for the model are provided in Supplemental Fig. 1. Although the largest visual spectroscopic differences exist within the range of 1220–1300 cm^{-1}, the discriminating factors for the model are based on numerous peaks throughout the fingerprint region of Raman spectra. This indicates that there is not one characteristic peak within the Raman spectrum of blood that can be used to distinguish the classes from each other. The differentiation demonstrated in this

Table 1
Summary of model predictions from blind and external validation test spectra.

Spectrum	Binary	Actual identity
Unknown 1	Animal	Chicken
Unknown 2	Animal	Horse
Unknown 3	Human	Human
Unknown 4	Animal	Rabbit
Unknown 5	Human	Human
Unknown 6	Animal	Mouse
Unknown 7	Animal	Raccoon
Unknown 8	Human	Human
Unknown 9	Human	Human
Unknown 10	Human	Human
Cow 1	Animal	Cow
Cow 2	Animal	Cow
Cow 3	Animal	Cow
Cow 4	Animal	Cow
Cow 5	Animal	Cow
Cow 6	Animal	Cow
Cow 7	Animal	Cow
Cow 8	Animal	Cow
Cow 9	Animal	Cow
Cow 10	Animal	Cow

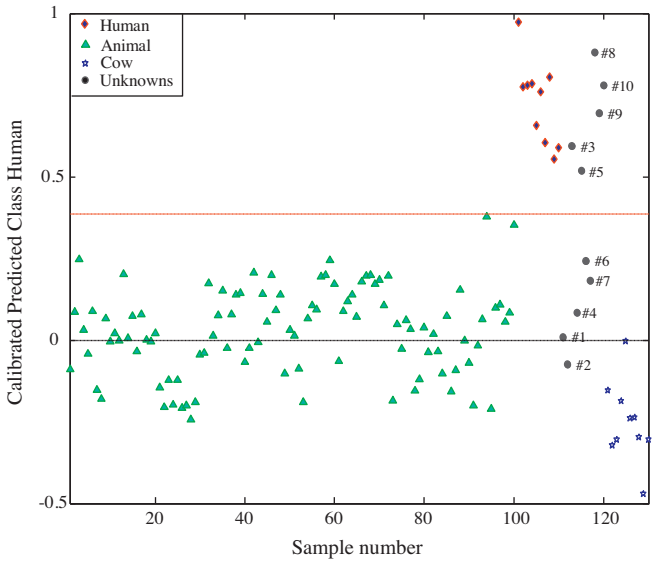

Fig. 4. Prediction scores for human class using the binary PLS-DA model with cow spectra (blue stars) and unknowns (black circles) loaded as predictions. Red line represents the default classification threshold. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

study is likely not due to any particular biomarker or component in blood. According to the literature, the concentration and chemical characteristics of some blood components (e.g. red blood cell count or total leucocytes) are different for animals and humans [24]. For example, there are 18 amino acid residue mutations (total of 36) in both the alpha and beta chains of hemoglobin between horse and human [25]. This could alter the vibrational spectroscopic properties resulting in differences in the Raman spectrum. Following this hypothesis, Raman spectra of human and gorilla blood should be most similar since the amino acid profile of hemoglobin differs in only three residues total. Collectively, these biochemical interspecies differences in human and animal blood could account for the spectroscopic variances observed. The influence of these factors on the Raman spectrum of blood has not been extensively investigated and is beyond the scope of this study.

These results illustrate the classification ability of the method (model) to identify human blood. The remarkable classification performance and the non-destructive nature of Raman spectroscopy make this approach well-suited for the forensic species discrimination of an unknown blood sample. This is especially important in cases where the sample is limited and destructive techniques of species identification cannot be afforded. Furthermore, the increasing capabilities of on-field Raman analysis [26] supports the idea that rapid species identification is feasible.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.forsciint.2014.02.027.

4. Conclusions

The combination of Raman spectroscopy and chemometric modeling was demonstrated to be a powerful tool toward the human identification of a blood sample. A binary PLS-DA model was constructed using a training dataset formed from Raman spectral data collected from human blood and a wide variety of animal species' blood. The constructed model demonstrated very good internal classification ability with zero occurrences of false negative assignments of the human class. Furthermore, the model performed well under two performance measures – a blind test of internal samples and an external validation test of animal spectra

(cow) which was excluded from the training dataset. This has potential applications in forensic science since it is advantageous over the current methodology, primarily due to the non-destructive nature of analysis. The method described conforms well to other Raman methods in the field, in that the same spectral data used to identify the sample as blood can then be used for a deeper determination of the species of origin. Further investigation is ongoing to determine if the specific species class can be determined in a similar fashion.

Acknowledgements

This project was supported by Award No. 2011-DN-BX-K551 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice (I.K.L.). The opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect those of the U.S. Department of Justice.

References

[1] L. Kobilinsky, Forensic Chemistry Handbook, Wiley, Hoboken, 2011.
[2] K. Virkler, I.K. Lednev, Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene, Forensic Sci. Int. 188 (2009) 1–17.
[3] E. Gebel, Species in a snap: Raman analysis of blood, Anal. Chem. 81 (2009) 7862.
[4] M. Houck, J. Siegel, Fundamentals of Forensic Science, Elsevier Limited, Burlington, 2006.
[5] K. Virkler, I.K. Lednev, Raman spectroscopy offers great potential for the non-destructive confirmatory identification of body fluids, Forensic Sci. Int. 181 (2008) e1–e5.
[6] K. Virkler, I.K. Lednev, Raman spectroscopic signature of blood and its potential application to forensic body fluid identification, Anal. Bioanal. Chem. 396 (2010) 525–534.
[7] K. Virkler, I.K. Lednev, Blood species identification for forensic purposes using Raman spectroscopy combined with advanced statistical analysis, Anal. Chem. 81 (2009) 7773–7777.
[8] J.M. Chalmers, H.G.M. Edwards, M.D. Hargreaves, Infrared and Raman Spectroscopy in Forensic Science, John Wiley & Sons Ltd., West Sussex, 2012.
[9] A.H. Kuptsov, Applications of Fourier-transform Raman spectroscopy in forensic science, J. Forensic Sci. 39 (1994) 305–318.
[10] C. Rodger, D. Broughton, The *in-situ* analysis of lipsticks by surface enhanced resonance Raman scattering, Analyst 123 (1998) 1823–1826.
[11] C.M. Hodges, J. Akhavan, The use of Fourier transform Raman spectroscopy in the forensic identification of illicit drugs and explosives, Spectrochim, Acta Mol. Biomol. Spectrosc. 46 (1990) 303–307.
[12] T. Coyle, N. Anwar, A novel approach to condom lubricant analysis: *in-situ* analysis of swabs by FT-Raman spectroscopy and its effects on DNA analysis, Sci. Justice 49 (2009) 32–40.
[13] J.V. Miller, E.G. Bartick, Forensic analysis of single fibers by Raman spectroscopy, Appl. Spectrosc. 55 (2001) 1729–1732.
[14] L.A. Nafie, Theory of Raman scattering, in: I.R. Lewis, H.G.M. Edwards (Eds.), Handbook of Raman Spectroscopy, Marcel Dekker Inc., New York, 2001.
[15] S. Boyd, M.F. Bertino, S.J. Seashols, Raman spectroscopy of blood samples for forensic applications, Forensic Sci. Int. 208 (2011) 124–128.
[16] S. Boyd, M.F. Bertino, D. Ye, L.S. White, S.J. Seashols, Highly sensitive detection of blood by surface enhanced Raman scattering, J. Forensic Sci. 58 (2013) 753–756.
[17] K. De Wael, L. Lepot, F. Gason, B. Gilbert, In search of blood—detection of minute particles using spectroscopic methods, Forensic Sci. Int. 180 (2008) 37–42.
[18] K.M. Elkins, Rapid presumptive fingerprinting of body fluids and materials by ATR FT-IR spectroscopy, J. Forensic Sci. 56 (2011) 1580–1587.
[19] G. McLaughlin, I.K. Lednev, Spectroscopic discrimination of bone samples from various species, Am. J. Anal. Chem. 3 (2012) 161–167.
[20] V. Sikirzhytski, K. Virkler, I.K. Lednev, Discriminant analysis of Raman spectra for body fluid identification for forensic purposes, Sensors 10 (2010) 2869–2884.
[21] K. Varmuza, P. Filzmoser, Introduction to Multivariate Statistical Analysis in Chemometrics, Taylor & Francis, Boca Raton, 2009.
[22] B.M. Wise, N.B. Gallagher, R. Bro, J.M. Shaver, W. Windig, R.S. Koch, PLS_Toolbox 3.5 for Use with MATLAB, Eigenvector Research Inc., Wenatchee, WA, 2005.
[23] W.R. Premasiri, J.C. Lee, L.D. Ziegler, Surface-enhanced Raman scattering of whole human blood, blood plasma, and red blood cells: cellular processes and bioanalytical sensing, J. Phys. Chem. B 116 (2012) 9376–9386.
[24] P.L. Altman, Blood, Other Body Fluids, Federation of American Societies for Experimental Biology, Washington, DC, 1961.
[25] L.C. Pauling, B. Kamb, L.P. Kamb, P.J. Pauling, A. Kamb, Linus Pauling: Biomolecular Sciences, World Scientific Publishing Co. Pte, River Edge, 2001.
[26] E.L. Izake, Forensic and homeland security applications of modern portable Raman spectroscopy, Forensic Sci. Int. 202 (2010) 1–8.