Received: 18 July 2018 | Revised: 13 September 2018 | Accepted: 15 September 2018

DOI: 10.1002/rcm.8285

RESEARCH ARTICLE

WILEY Rapid Communications in Mass Spectrometry

Identification of rhinoceros keratin using direct analysis in real time time-of-flight mass spectrometry and multivariate statistical analysis

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Rationale: Trade in rhinoceros horn is regulated or banned internationally in recognition of its impact on wild populations worldwide. Enforcement of the laws and regulations depends on successfully identifying when violations occur, which is complicated by the presence of alternative/imitation rhinoceros horn keratin (e.g., bovid horn keratin). In this study, we assess the potential for Direct Analysis in Real Time (DART) ionization paired with Time-Of-Flight Mass Spectrometry (DART-TOFMS) to classify different keratin types from four taxonomic groups: rhinoceros, bovid, domestic horse, and pangolin.

Methods: The spectra of 156 keratin samples from all five rhinoceros species (horn keratin), eight genera of bovids (horn keratin), domestic horses (hoof keratin), and all extant species of pangolins (scale keratin) were collected. Fisher ratio analysis identified the most important ions that characterized each class and these ions were used for the training model, which consisted of 143 spectra. Kernel Discriminant Analysis (KDA) was used to classify the different groups.

Results: The spectra collected for each taxonomic group are distinctive. The chemotypes demonstrate that the spectra of rhinoceros, bovids, and domestic horse are similar to each other, whereas the chemotypes of pangolins show a different chemical profile. The model built by KDA resolved each taxonomic group: 95% of samples were correctly assigned using leave-one-out cross validation. The 13 blind samples not used in model development were all correctly classified to taxonomic source.

Conclusions: DART-TOFMS appears to be a reliable approach for taxonomic identification of keratin. This analysis can be carried out with a small sliver of keratin, with minimal sample preparation, inexpensively and quickly, making it a potential valuable tool for identification of rhinoceros horn and other keratin types.

1 | INTRODUCTION

There are five extant species of rhinoceros and all of them face threats to their continued survival as recognized by conservation-related organizations and legislation. For example, the International Union for Conservation of Nature Red List of Threatened Species has identified three species as critically endangered: Sumatran rhinoceros (*Dicerorhinus sumatrensis*), Javan rhinoceros (*Rhinoceros sondaicus*) and Black rhinoceros (*Diceros bicornis*). The White rhinoceros (*Ceratotherium simum*) is classified as near threatened, and the Indian rhinoceros (*Rhinoceros unicornis*) is considered vulnerable.[1-5] All five species are also listed as endangered under the Endangered Species Act of the United States (ESA), which is designed to protect critically imperiled species from extinction.[6] Finally, since 1977, all rhinoceros

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species have been listed as Appendix I under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).[7] Accordingly, all international trade in rhinoceros, including their parts and derivatives, is strictly prohibited, with some exceptions (the South Africa and Swaziland populations of the subspecies *C. simum simum* are listed as CITES Appendix II).[7] Despite protective legislation and CITES regulation, widespread poaching continues in order to satisfy market demands for rhinoceros horn.[8,9]

The demand for rhinoceros horn is driven in large part by its use in Asian medicinal products.[10] Beyond medicinal products, rhinoceros horn is also valued as a raw material for artistic carvings (e.g., libation cups, jewelry, figurines) and, during the last decade, there has been high demand for rhinoceros horn art in some parts of the world.[11] Such items can be valued well over USD 100,000.[11] The prohibition in rhinoceros trade by CITES means that any international trade to meet this demand is illegal (note exception above). While enforcement might appear to be straightforward, it requires knowledge of the taxonomic origin of items, which can be complicated by the presence of imitation products. For example, bovid horn that has been carved to resemble rhinoceros horn has been observed in the trade.[12]

Distinguishing authentic rhinoceros horn from imitation horn can sometimes be accomplished using morphological analysis.[12,13] For example, the solid keratinous horn of rhinoceros differs from the hollow horn sheath of cattle horn.[12] In cases that include fragmentary pieces of keratin or items that have been extensively modified (e.g., carved), morphological analysis may be difficult.

Genetic analyses of rhinoceros horn have proven successful for taxonomic identification,[14,15] and even individualization.[16] Such methods can be costly and time-consuming, particularly when species- and individual-level identifications are unnecessary.

Recently, several studies have used mass spectrometry for taxonomic identification of keratin. For example, peptide mass fingerprinting has been shown to distinguish common mammal keratins at the genus level,[17] and this same method has been used for species identification of keratinous baleen in whales.[18] Chemical odor profiles of African rhinoceros species appear to differ based on gas chromatography and time-of-flight mass spectrometry.[19] Taken together, mass spectrometry appears to be a promising tool that could be useful for forensic identification of keratinous items. Moreover, there have been recent advancements in mass spectrometry methods that allow analysis of items under ambient conditions (Direct Analysis in Real Time Time-Of-Flight Mass Spectrometry – DART-TOFMS),[20] which could provide rapid and cost-effective analyses to aid law enforcement.

Here we evaluate the use of DART-TOFMS for determining the taxonomic source of keratin tissue. DART-TOFMS has been successful in the identification of solid wood samples to determine species source.[21-23] This ambient ionization technique allows for exact mass measurements of ions from a solid matrix without the need to modify or derivatize the sample. Sample preparation is simple, requiring only a sliver of sample for analysis, which results in minimal destruction to the original item. The specific goal of this study is to assess if the chemotypes obtained from DART-TOFMS analysis can differentiate among common sources of keratin that have been observed in the wildlife trade, specifically rhinoceros horn and potential rhinoceros horn look-alikes (bovid horn, domestic horse hoof). We also include pangolin scale keratin, which, although unlikely to be confused with rhinoceros horn, is also prohibited in international commercial trade.

2 | EXPERIMENTAL

2.1 | Materials

Samples of rhinoceros horn, bovid horn, and domestic horse hoof were collected from reference specimens within the U.S. National Fish & Wildlife Forensics Laboratory (Ashland, OR, USA), museum specimens, and live animals (e.g., zoo and farrier donations). Pangolin scale samples were obtained from collections at the American Museum of Natural History (New York, NY, USA). The samples included all extant species of rhinoceros: Black rhinoceros (*Diceros bicornis*, $n = 17$ individuals), White rhinoceros (*Ceratotherium simum*, $n = 18$ individuals), Sumatran rhinoceros (*Dicerorhinus sumatrensis*, $n = 5$ individuals), Indian rhinoceros (*Rhinoceros unicornis*, $n = 8$ individuals), and Javan rhinoceros (*Rhinoceros sondaicus*, $n = 3$ individuals); 34 individual bovids (Family Bovidae) representing 20 genera (horn keratin); 36 domestic horses, *Equus caballus* (hoof keratin); and 35 individual pangolins (Family Manidae) representing all eight extant species (scale keratin). The total sample size was 156 individuals.

Sample collection techniques were designed to avoid the potential influence of surface contaminants on the spectrum. Horse hoof samples were collected by first removing a section of the surface to expose a fresh keratin surface. Wood-working tools were used to cut a sliver of keratin from the new keratin surface. Bovid horn keratin was sampled using a power drill fitted with a 1/8-inch drill bit to produce a spiral keratin sample from an internal location. Rhinoceros horn samples were obtained as spiral samples, as for the bovid samples, or shavings. Pangolin keratin was sampled using a standard fingernail clipper to cut slivers from the scales. Because the pangolin samples included surface keratin, each sliver was washed by sonication in water for 10 min followed by sonication in methanol for 10 min. Analysis was performed after the samples had dried.

Reference standards of cholesterol, heptadecanoic acid, linolenic acid, oleic acid, and palmitic acid were purchased from Sigma-Aldrich (St Louis, MO, USA), and were used to validate selected mass spectral assignments.

2.2 | Spectral data acquisition and processing

For each keratin sample and reference standard, mass spectra were collected on a DART-standardized voltage and pressure ion source (DART-SVP) (IonSense, Saugus, MA, USA) combined with a JEOL AccuTOF 4G LC Plus time-of-flight mass spectrometer (JEOL USA, Peabody, MA, USA) and operated in positive ion mode. Settings for the DART-TOFMS instrument were as follows: electrode 1 voltage, 150 V; electrode 2 voltage, 250 V; helium gas heated to 250°C. The optimum temperature setting for keratin analysis at the DART ion source was determined by using the IonRocket temperature gradient system (BioChromato, Inc., Fujisawa, Japan). The mass spectrometer settings included: ring lens voltage, 5 V; orifice 1 voltage, 20 V; orifice 2 voltage, 5 V; cone temperature, 120°C; peak voltage, 600 V; bias

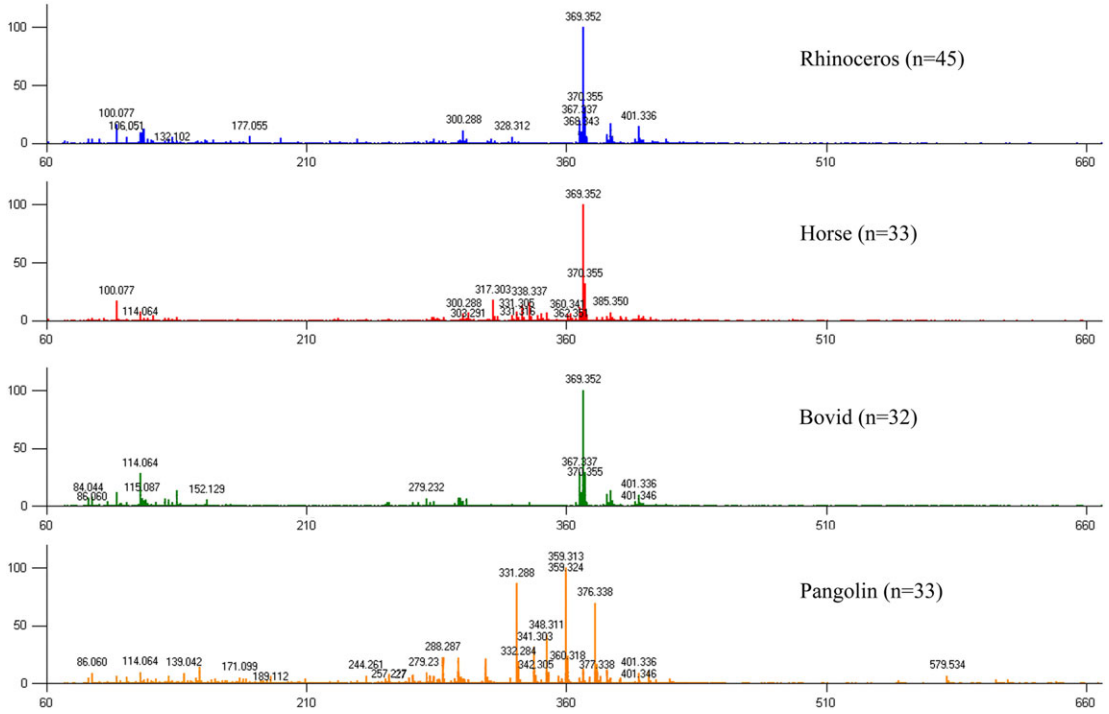


FIGURE 1 Average spectra of individual samples for each taxonomic keratin group: rhinoceros horn ($n = 45$); domestic horse hoof ($n = 33$); bovid horn ($n = 32$); pangolin scales ($n = 33$) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Mass-to-charge ratios of select ions that discriminate among the keratin of rhinoceros, bovids, horse, and pangolins and their assignments, some of which are provisional. Box shading indicates presence within the taxa [Color table can be viewed at wileyonlinelibrary.com]

m/z	Formula	Assignment	Rhinoceros	Horse	Bovid	Pangolin
90.055	$C_3H_7N_1O_2 + H$	Alanine	■			
100.077	$C_5H_{11}N_1O_2 + H - H_2O$	Valine	■	■	■	
106.051	$C_3H_7N_1O_3 + H$	Serine	■			
114.064	$C_6H_{13}NO_2 + H - H_2O$	Leucine or isoleucine	■	■	■	■
115.087	$C_6H_{10}O_2 + H$	C 6:1	■			
116.071	$C_5H_9N_1O_2 + H$	Proline	■			
128.080	$C_6H_{14}N_2O_2 - H_2O$	Lysine			■	
137.045	$C_6H_9N_3O_2 - H_2O$	Histidine	■			
139.111	$C_9H_{16}O_2 + H - H_2O$	C 9:1				■
152.129	$C_{10}H_{18}O_2 - H_2O$	10:1 Fatty acid	■		■	
156.105	$C_6H_{14}N_4O_2 - H_2O$	Arginine	■			
163.040	$C_9H_{11}N_1O_3 - H_2O$	Tyrosine	■			
205.087	$C_{11}H_{12}N_2O_2 + H$	Tryptophan	■			
257.240	$C_{16}H_{32}O_2 + H$	Palmitic acid (16:0)	■			
279.232	$C_{18}H_{30}O_2 + H$	Linolenic acid (18:3)	■		■	■
283.266	$C_{18}H_{34}O_2 + H$	Oleic acid (18:1)	■			
288.266	unknown	unknown	■			■
297.232	$C_{18}H_{34}O_3 - H$	18:1 OH				■
313.265	$C_{22}H_{34}O_2 + H - H_2O$	22:5 Fatty acid				■
359.313	unknown	unknown				■
367.337	$C_{27}H_{44} - H$	Cholesta-3,5-diene	■	■	■	
369.352	$C_{27}H_{46}O$ M + H − H_2O	Cholesterol	■	■	■	■
376.338	unknown	unknown				■
401.336	$C_{27}H_{44}O_2 + H$	5-Cholesten-3-ol-7-one	■		■	■

voltage, 28 V; focus voltage, −120 V; reflectron voltage, 870 V; pusher voltage, 778 V; pulling voltage, −778 V; suppression voltage, 0.00 V; flight tube voltage, −7000 V; and detector voltage, 2300 V. Spectra were obtained over the mass range of *m/z* 60 to 1000 at one scan per second. The helium flow rate for the DART source was 2.0 mL/s. The resolving power of the mass spectrometer, as stated by the manufacturer, is 6000 at full width half maximum.[20]

Keratin was analyzed by holding the sample in the heated helium gas stream of the DART ion source. A mass calibration spectrum of the standard was obtained at the beginning of the sequence, after every fifth sample, and at the end of the sample sequence, by dipping a capillary tube into a container of neat poly(ethylene glycol) 600 (Ultra Scientific, Kingstown, RI, USA), and then holding the dipped capillary tube in the gas stream of the DART-TOFMS instrument. Reference standards were also analyzed using a capillary tube that had been dipped into the reference solution.

Once the sample spectra had been acquired, the data were processed using TSS Unity Universal Reporting software (version 1.06; Shrader Analytical Labs, Detroit, MI, USA). This software produces text files of calibrated mass spectra. Assignment of selected peaks was possible due to the analysis of reference standards, the high resolution of the mass spectrum and agreement with the literature. All statistical analyses were conducted with Mass Mountaineer software (RBC Software, Peabody, MA, USA).

2.3 | Taxonomic differentiation of keratin

A training set using 143 spectra (rhinoceros horn: $n = 45$; bovid horn: $n = 32$; domestic horse hoof: $n = 33$; pangolin scales: $n = 33$) was created by assigning each sample within a dataset to a class using Mass Mountaineer (RBC Software). A heat-map of the training set was constructed to visualize the differences among the keratin classes. Given that the mass spectrometer data contained more than 1000 ions (variables), Fisher ratio analysis[24,25] was used to select ions that had the most discriminating power to separate the classes. Fisher ratio analysis identifies features that maximize the difference between classes while minimizing differences within each class.[26] A total of 227 ions were selected based on the Fishers discriminant results.

Mass Mountaineer© software was used to evaluate if the four taxonomic groups could be differentiated using Kernel Discriminant Analysis (KDA), a supervised classification algorithm. The mass tolerance was set at 10 milli *m/z* units with a standard deviation of 100; the KDA graphical plots were produced using K Means Clustering. The robustness of the model was evaluated using leave-one-out cross-validation (LOOCV), which is an algorithmic process that treats each sample from the dataset as an unknown. Each sample is removed from the training set and then assigned to a class from the training model; this is repeated sequentially until all samples have been assigned to a group and results totaled. A LOOCV of 100% indicates that every sample had been assigned to the correct class. In addition, a blind test was performed using the 13 spectra not included in the training set (rhinoceros horn: $n = 6$; bovid horn: $n = 2$; domestic horse hoof: $n = 3$; pangolin scales: $n = 2$). These samples were treated as "unknowns" and assigned to a taxonomic group, with an associated probability, based on the training model.

3 | RESULTS

Figure 1 shows the average spectrum for each taxonomic keratin class (rhinoceros horn $n = 45$; bovid horn $n = 32$; domestic horse hoof $n = 33$; pangolin scales $n = 33$). The rhinoceros, bovid, and horse spectra are dominated by the base peak at *m/z* 369.352, which is the $[M + H - H_2O]^+$ ion of cholesterol ($C_{27}H_{45}O^+$). The average spectrum for pangolin scales is different and shows a base peak at *m/z* 359.313 from an unidentified compound. The *m/z* values of the spectra for each taxonomic class suggest that the predominant compounds include amino acids, fatty acids, cholesterol and steroidal metabolites. A list of ions and their assignments is shown in Table 1. Analysis of the reference standards (cholesterol, heptadecanoic acid, linolenic acid, oleic acid, and palmitic acid) revealed that the *m/z* values are in agreement with some of the molecules detected in the keratin samples, and thus corroborate their provisional assignments. These results demonstrate that there is no single diagnostic ion for each taxonomic keratin group, but they do suggest that fatty acids may play an important role in separating the keratins analyzed in this study.

A heat map is a two-dimensional graphical matrix in which the data are represented as colors. A heat map graphical representation of all the samples analyzed is shown in Figure 2. The X-coordinate is the *m/z* value (mass-to-charge ratio) of the ion obtained from a

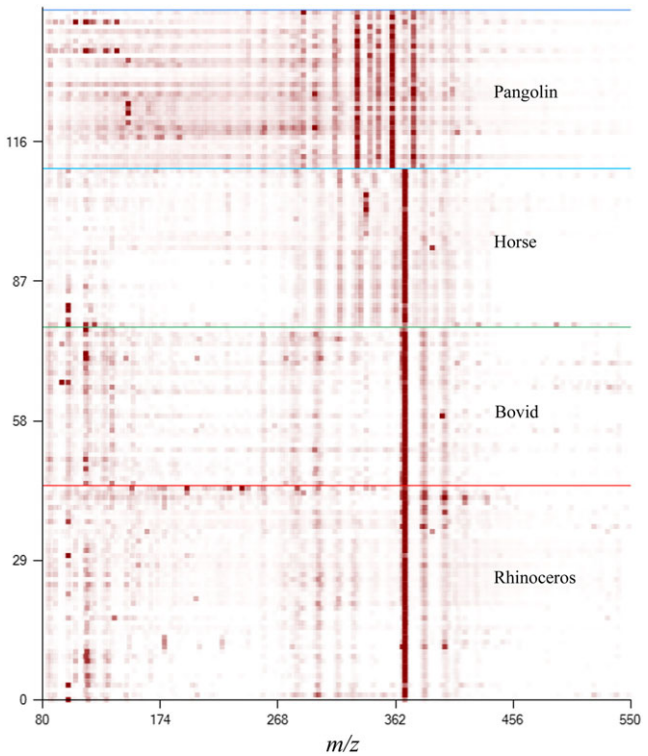


FIGURE 2 Heat map of the ions present in the samples of rhinoceros and bovid horns, domestic horse hooves, and pangolin scales. The X-axis displays the ions present in a sample, and the Y-axis represents each individual sample. The intensity in color of each ion correlates to the relative quantity of that molecule present in the corresponding sample [Color figure can be viewed at wileyonlinelibrary.com]

molecule and the Y-coordinate represents the sample analyzed. Therefore, each row is indicative of all the ions found in that specific sample. The color intensity is an indication of the relative amount of the detected ion present in each specimen. A visual examination of the heat map also shows that the chemotypes associated with pangolin scales are different from those of rhinoceros and bovid horn and horse hooves. Cholesterol (*m/z* 369.351) appears to be the most intense ion in the rhinoceros, bovid and horse spectra, but it is only detected at low levels in the pangolin scale spectra. Conversely, pangolins have intense ions at *m/z* 359.298 and 376.362 (Table 1, Figure 1); the compounds producing these ions are absent from the other three taxa. The identities of these compounds have not been determined.

The graphical results of the KDA of the rhinoceros, bovid, horse and pangolin are shown in Figure 3. All five species of rhinoceros cluster together to the exclusion of the other taxa. Similarly, the 20 genera of bovids cluster together and are differentiated from the other taxa. Leave-one-out cross validation (LOOCV) is one way of evaluating model accuracy, and the LOOCV of the KDA model was 95.8%, meaning that of the 143 spectra used to create the model, only six spectra were misclassified. The blind analysis using 13 "unknown" samples (i.e., not included in the training model) resulted in correct assignment of each sample to the appropriate taxon; the associated probabilities are shown in Table 2.

4 | DISCUSSION

Determining the taxonomic source of keratin objects found in the wildlife trade is important for enforcing laws and regulations associated with the industry. The results of our study suggest that analyses using spectra obtained with DART-TOFMS can discriminate among some taxonomic keratin groups with a high degree of accuracy (>95%): rhinoceros horn, bovid horn, domestic horse hoof, and pangolin scales. Accordingly, our approach suggests that keratin source can be readily distinguished among four mammalian families (rhinocerotids, bovids, equids, and manids). These results are in line with previous studies suggesting that chemical profiles of keratin differ among taxonomic groups.[17,18] In this regard, we acknowledge that only two of our sample groups include individuals representing all extant species: rhinocerotids and manids. Data for equids are represented by a single species (domestic horse: *Equus caballus*). Our bovid sample was more taxonomically diverse (20 genera), but it

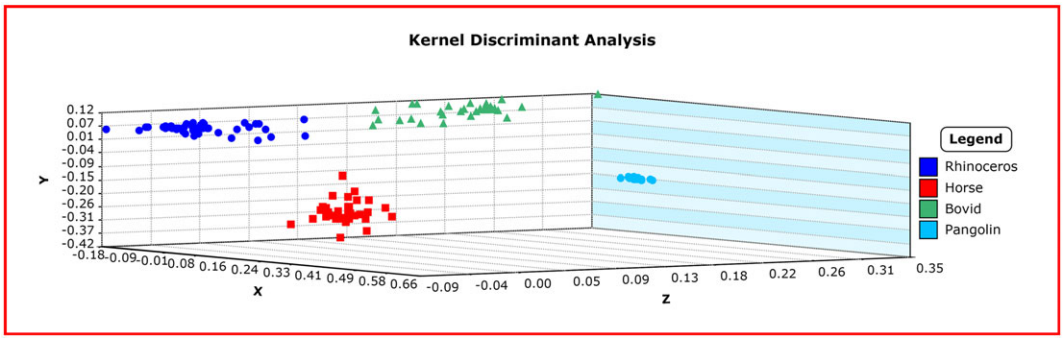


FIGURE 3 Graphical representation of the Kernel Discriminant Analysis (KDA) based on 227 ions from 143 samples of rhinoceros, bovid, horse, and pangolin keratin. The leave-one-out cross-validation of the KDA model was calculated to be 95.8% [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Results of blind sample assignments based on the KDA model

Family	Species	Sample	Classified as:	Assignment probability
Bovidae	*Bos gaurus*	MN 1285	Bovid	99.9%
Bovidae	*Connochaetes taurinus*	MN 2165	Bovid	99.9%
Equidae	*Equus caballus*	MN 2659	Horse	99.9%
Equidae	*Equus caballus*	MN 2662	Horse	99.9%
Equidae	*Equus caballus*	MN 2664	Horse	99.9%
Manidae	*Manis gigantea*	M-53855	Pangolin	99.9%
Manidae	*Manis gigantea*	M-53857	Pangolin	99.9%
Rhinocerotidae	*Ceratotherium simum*	MN 38	Rhinoceros	99.9%
Rhinocerotidae	*Ceratotherium simum*	MN 712	Rhinoceros	99.6%
Rhinocerotidae	*Ceratotherium simum*	MN 2686	Rhinoceros	99.9%
Rhinocerotidae	*Diceros bicornis*	MN 2413	Rhinoceros	99.9%
Rhinocerotidae	*Diceros bicornis*	MN 2572	Rhinoceros	95.7%
Rhinocerotidae	*Diceros bicornis*	MN 2577	Rhinoceros	99.9%

represents only a subset of the large taxonomic breadth recognized within the family.[27] Future research should incorporate samples representing alternative species within these groups, as well as other keratin types (e.g., bovid hoof).

Interestingly, while all four taxonomic groups in our study classified separately, the general keratin chemotypes of rhinoceros horn, bovid horn, and domestic horse hoof were similar (Figure 1). Pangolin keratin, on the other hand, was different. Understanding why pangolin scale keratin is different is an area of ongoing research in our lab. Our data do suggest that the ions responsible for differentiating the taxonomic groups are characteristic of amino acids and fatty acids, but the most intense ions observed in pangolin scale keratin could not be identified.

The ability to identify taxonomic sources of keratin using mass spectral analysis could provide an additional or alternative method for wildlife forensic analyses. Identification of intact horns can usually be accomplished by morphological analysis if the item has not been modified.[12] Determining the origin of carved keratin objects can be challenging when the diagnostic morphological characters are absent. Although genetic analyses may be used under such circumstances,[14-16] they can be time-consuming and can potentially yield inconclusive results if DNA quality is low. Mass spectral analysis could be a welcome addition to a forensic toolkit in cases where traditional methods fail. In addition, because our method has so far been used to distinguish among broad taxonomic keratin groups, it could be a useful screening tool for identifying objects that may require additional forensic analyses (e.g., species-level identification using DNA analysis).

The analysis of keratin using DART-TOFMS also has several advantages. For example, solid materials require no sample preparation, and the cost of analysis per sample is inexpensive. Moreover, analysis using DART-TOFMS is rapid with results obtained within seconds. Given that trade in some keratin items, such as rhinoceros horns and pangolin scales, is either regulated or banned, rapid and accurate identification of taxonomic source of keratin objects is important for identifying violations of international statutes that regulate wildlife trade (e.g., CITES convention), as well as facilitating and expediting legal trade.

5 | CONCLUSIONS

DART-TOFMS analysis of keratin sources that may be found in the wildlife trade, specifically rhinoceros horn, potential rhinoceros horn look-alikes (bovid horn and horse hoof), and pangolin scales, is a reliable method for taxonomic identification. This approach requires a small sample (e.g., sliver of keratin) with no sample preparation. The technique is rapid, efficient, and could be a useful addition to wildlife forensic science in helping combat the illegal wildlife trade.

LEGAL NOTE

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U. S. Fish and Wildlife Service.

ACKNOWLEDGEMENTS

We could not have accomplished this research without the help of several agencies and people that graciously provided reference samples for this study. They include Daniel Kringberg Johansson, Eline Lorenzen and Kristian Murphy Gregersen of the Natural History Museum of Denmark, University of Copenhagen Zoological Museum, Jeff Bradly of the Burke Museum Mammalogy, University of Washington, Darrin P. Lunde of the Smithsonian Institution National Museum of Natural History, Jim Dines of the National History Museum of LA County, Ned Gilmore of The Academy of Natural Sciences, Department of Vertebrate Zoology, April Gorow of the San Diego Zoo, Wildlife Disease Laboratory, Stephanie Earhart of Zoo Atlanta, Donie Sprague of the U.S. Fish and Wildlife Service National Eagle and Wildlife Property Repository, Neil Duncan of the American Museum of Natural History, farrier Rock Earnheart for collecting and providing most of the *Equus caballus* samples, and, again, the American Museum of Natural History, especially Neil Duncan, Eileen Westwig and Brian O'Toole, for allowing us to sample their collection of pangolin scales. We would also like to thank Doina Voin, U.S. National Fish and Wildlife Forensics Laboratory, for her tireless pursuit of rhinoceros reference samples.

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How to cite this article: Price ER, McClure PJ, Jacobs RL, Espinoza EO. Identification of rhinoceros keratin using direct analysis in real time time-of-flight mass spectrometry and multivariate statistical analysis. *Rapid Commun Mass Spectrom*. 2018;32:2106-2112. https://doi.org/10.1002/rcm.8285