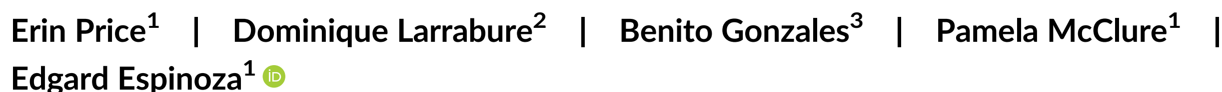
Received: 19 May 2020 | Revised: 14 July 2020 | Accepted: 2 August 2020

DOI: 10.1002/rcm.8916

RESEARCH ARTICLE

Rapid Communications in Mass Spectrometry WILEY

Forensic identification of the keratin fibers of South American camelids by ambient ionization mass spectrometry: Vicuña, alpaca and guanaco



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Funding information
CONICYT, Grant/Award Number: REDI-170208

Rationale: The keratin fleece of the endangered vicuña (*Vicugna vicugna*) commands a high value in international markets, and this trade has caused illegal poaching and a substantial decrease in vicuña populations. Morphological analysis of hairs does not have the resolution to determine the species of origin of camelid natural fibers. In addition, commerce in camelid fleece also includes the legal trade of alpaca (*Vicugna pacos*) and guanaco (*Lama guanicoe*) wool.

Methods: The keratin fiber spectra of vicuña ($n = 19$), guanaco ($n = 20$) and alpaca ($n = 20$) were collected using X-ray fluorescence (XRF) spectrometry, Horizontal attenuated total reflectance Fourier transform infrared (HATR-FTIR) spectroscopy and direct analysis in real time time-of-flight mass spectrometry (DART-TOFMS). Analysis with each technique evaluated the data to determine if the three taxa could be separated using either descriptive or multivariate statistics.

Results: XRF analysis showed that the elements detected and their relative concentrations were similar in all three species, whereas HATR-FTIR analysis could identify alpaca fleece but could not differentiate vicuña from guanaco. Ions detected by ambient ionization using DART-TOFMS, in either positive- or negative-ion mode, gave the best results and showed that each taxonomic group is distinctive. Multivariate analysis of the mass spectra created robust models which resolved each species (LOOCV = 99.9%). The analyses of eight validation samples were correctly assigned to the appropriate species and demonstrated the reliability of DART-TOFMS to infer taxonomic source.

Conclusions: The DART-TOFMS spectra of unmodified keratin fibers infer that the chemotype of each species is heavily influenced by fatty acids, cholesterol and its analogs, and that these ions are useful in separating the fleece of vicuña, alpaca and guanaco. We posit that the etiological source of these chemotype differences is consistent with genetic modulations and is less influenced by diet. Accurate taxonomic identification of fleece is important to identify violations and assists in the protection of rare species.

This article has been contributed to by US Government employees and their work is in the public domain in the USA.

1 | INTRODUCTION

Due to the fine wool they produce, four species of South American camelids are used for the manufacture of clothing.[1] Two wild camelids, vicuña (*Vicugna vicugna*) and guanaco (*Lama guanicoe*), produce exceedingly small diameter wool fibers, a feature that is highly desirable in the production of luxury garments as well as artisan textiles.[2] The commercial value of raw fiber, yarn, or garments from the wool of guanaco and vicuña is significantly higher than for the wool of domestic camelids such as alpaca (*Vicugna pacos*) and llama (*Lama glama*), whose coarser fibers feel rough in comparison.[1]

The vicuña, which inhabits the South American Andean plateau, produces some of the finest wool in the world.[2,3] Each vicuña can produce about 190 g of fleece of fine fibers that have an average diameter of ~13 microns.[3] In the 1950s, because of unsustainable wool harvesting techniques, the vicuña population plummeted from 400,000 individuals to approximately 10,000 by 1967. In 1975 the vicuña was listed in Appendix I of the Convention on International Trade in Endangered Species (CITES).[4] Currently, the shearing of fiber from wild, live vicuñas by local indigenous communities has assisted in an upturn in the population of the species and is an example of sustainable use of wildlife.[5,6] Because of the rate of wool growth, adult camelids can only be shorn once every 2–3 years.[5] Due to its population size and distribution area the vicuña is classified as Least Concern by the IUCN RedList.[7] The guanaco, which inhabits arid areas of South America from sea level to near 5000 m,[8] has a fiber with a diameter somewhat thicker than that of vicuña, between 14 and 19 microns,[9,10] and each individual can produce up to 250 g of undercoat.[11] The harvest of wild guanaco wool is managed in the same way as for that of the vicuña,[12] but there are also a few captive groups on private ranches in Argentinian Patagonia that are harvested.[13] The guanaco, classified as Least Concern by the IUCN RedList due to its increasing population size and wide distribution range,[14] also has an attractive fiber but enjoys a less developed market than that of the vicuña.

At the time of this research, both species of wild camelids are included in the CITES Appendices for the international trade of their products. The entire guanaco populations of Peru, Bolivia, Paraguay, Argentina and Chile are found in Appendix II of CITES.[14] The vicuña populations of Ecuador, Peru, Bolivia, Argentina and part of Chile are also listed in Appendix II, but a small population located in the geographic regions of Antofagasta and Atacama in Chile remains in Appendix I of CITES.[15] Despite legal protection, poaching and trafficking of vicuñas has persisted because of the high value of their fibers and the garments that those fibers produce. It has been reported that since 2008 more than 5000 vicuñas have been illegally hunted in the Andean plateau of Peru, Bolivia, Chile and Argentina.[8] After the animals are killed, they are skinned and the pelts are trafficked; fiber is generally removed from the pelt and eventually reaches local markets.[16] It is speculated that illegal fiber trade evades customs controls and is trafficked through remote international routes, which may eventually reach markets outside the species range.

Once the wool has been removed from the animal it can be difficult to differentiate the species source, especially between vicuña and guanaco. A fast and reliable method is necessary to discriminate fiber samples and clothing made from South American camelids. Traditional fiber characterization techniques, although accessible and fast,[17] cannot be used reliably to differentiate the taxonomic source of these species based on fiber diameter alone (González, personal observation).[13] Genetic analysis from fresh samples of tissue, blood, or feces can easily differentiate the four species of camelids[18] and even identify population provenance[19,20,21,22]; these techniques have been used in forensic cases.[23,24] However, this precision is not attainable through the traditional analysis of keratin from hair or undercoat.[25] The genetic markers that persist in the fibers would be those of mitochondrial DNA, which at this time is only capable of determining the genera *Lama* or *Vicugna*.[23]

As early as 1965, Satlow described the anatomical features of the hair of selected taxa, including alpaca, in an effort to develop a technique to determine taxonomic source of these fibers.[26] Chemical analysis and the determination of amino acids of keratin fibers were reported by Tucker et al,[27] Rivett et al,[28] and Logan et al[29] with limited speciation success. One of the chemical techniques that has shown promise for the analysis of solid samples is ambient ionization using direct analysis in real time time-of-flight mass spectrometry (DART-TOFMS). DART-TOFMS can capture the small molecule profile of a sample, reflecting both similarities and differences according to chemotype.[30] DART-TOFMS has had extensive use in forensic science including drug analysis[31] and identification of trafficked species of flora and fauna.[30,32,33] Recently, DART-TOFMS was successfully used to distinguish rhinoceros horn from the hoofs of horse and cattle, which are fraudulently found in trade,[33] offering optimism that DART-TOFMS could be used to classify the fleece fibers of South American camelids.

Another chemical technique used extensively to study the chemical components in hair is Fourier-transform infrared (FTIR) spectroscopy.[34] It has been useful for differentiating amino acids,[35] which could potentially allow for the classification of taxa through analysis of secondary amino acid structures.[36] A third analytical tool, X-ray fluorescence (XRF) spectrometry, has shown promise in the classification of keratin analysis and was recently used to differentiate captive-bred from wild-caught short beaked echidnas through analysis of their keratin quills.[37] While XRF spectrometry is new to the field of keratin research, it shows clear potential for a wider range of keratinous materials.

Due to the commercial importance of the main textile species of South American camelids, and in view of the constant threat of poaching and trafficking of fine fibers from wild species, the objective of this work is to characterize the chemical compounds of the vicuña, guanaco and alpaca using XRF, HATR-FTIR and DART-TOFMS, in order to discriminate fibers at the species level and thus support forensic investigation in the fight against poaching and illegal trafficking of vicuña and guanaco products.

2 | METHODS

2.1 | Sample preparation

The underfur and guard hairs from field-found carcasses were collected by sampling 3 × 3 cm fleece samples cut from the center of the last rib of vicuña (n = 19) and guanaco (n = 23). Reference alpaca (n = 22) samples were obtained from domestic animals whose wool was sheared. Three guanaco and two alpaca samples were reserved for "blind" verification testing. For vicuña, two spools of commercial grade spun fibers were used for model verification. Origin information for all samples can be found in Table S1 (supporting information). Samples were not cleaned or treated prior to analysis.

2.2 | X-ray fluorescence (XRF) spectroscopy

Approximately 10 loose fibers from the training set were wrapped in X-ray polypropylene film (Chemplex Industries Inc., Tuckahoe, NY, USA) and analyzed under vacuum with an Orbis Micro-XRF analyzer (EDAX, Mahwah, NJ, USA). The background of each spectrum was subtracted and underwent peak deconvolution using Orbis Vision software (version 2.1; EDAX Inc.). Descriptive statistics of the XRF results were obtained using Excel software (Microsoft Corp., version 2010).

2.3 | Horizontal attenuated total reflectance Fourier transform infrared spectroscopy (HATR-FTIR)

Fiber pellets were prepared by placing the unwashed underfur in a 13 mm FTIR pellet die (Specac Ltd, Orpington, UK) and pressed with 8 tons of pressure using a laboratory press (Fred Carver Inc., Wabash, IN, USA) for 15 s. The resulting pellets weighed approximately 0.1353 g and resembled felt disks. Each underfur pellet was analyzed using a Nicolet iS50 FTIR spectrometer with a built-in Smart iTR (HATR) accessory (Thermo Scientific, Waltham, MA, USA). Collection parameters have already been reported.[38] Spectra were collected by placing the sample disk on the diamond crystal surface, and isopropyl alcohol was used to clean the crystal between samples. A background spectrum was collected every 60 min. Samples were scanned 80 times per spectrum over a range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} and converted into log (1/R) vs wavenumber (cm^{-1}). The training set spectra were each assigned to their respective classes using TQ Analyst 9 v.9.11.728 software (TQ Analyst, 1996–2019, Thermo Scientific). A single spectrum from each class was selected randomly for validation, and discriminant analysis was carried out in the fingerprint region (1788–413 cm^{-1}). The discriminant analysis method used a constant path length, mean centering, and 10 principal components covering 98.35% of the variability. The class average was subtracted from each spectrum in the class, creating unique distributions, and the resulting variances were utilized for differentiation (TQ Analyst, 2011). The method's accuracy was determined by the performance index, which calculates the similarity of each validation spectrum in the training set to its actual class; a value of 100% indicates that all samples are correctly assigned to their class.

2.4 | Direct analysis in real time time-of-flight mass spectrometry (DART-TOFMS)

Mass spectra of the training and test samples were recorded with a time-of-flight mass spectrometer (AccuTOF; JEOL, USA, Inc., Peabody, MA, USA) fitted with a DART ion source (DART-SVP; IonSense Inc., Saugus, MA. USA). The resolving power, as stated by the manufacturer, is 6000 FWHM (full width at half maximum). The DART-TOFMS instrument was operated in positive- and negative-ion mode from a range of m/z 50 to 1000. Orifice 1 had a voltage of 20 V, orifice 2 had a voltage of 5 V, and the ring lens voltage was set to 5 V.

A quarter of each fiber pellet was cut and placed in a test tube with 1 mL of MeOH; each quarter weighed approximately 41 mg. Samples were vortexed for 10 s and extracted for 30 min. Before analysis, each extraction was vortexed again. Positive- and negative-ion spectra were acquired by dipping the sealed end of a glass capillary tube (Pyrex #9530–4) into the extract and placing it in front of the DART heated protonated helium stream (250°C). Positive mode spectra were calibrated with poly(ethylene glycol) 600 (Ultra Scientific, Kingstown, RI, USA) and negative mode spectra were calibrated with Fomblin®Y (Aldrich, St Louis, MO, USA).

Spectra were acquired, calibrated, averaged and background subtracted using msAxel (version 1.0.5.2; JEOL Ltd) and centroided mass spectra were exported as text files for chemometric analysis with Mass Mountaineer software (RBC Software, Peabody, MA, USA).

To compensate for sampling intensity differences, the spectra were normalized by dividing the relative abundance of each feature m/z by the sum of the relative abundances. Multivariate analysis used the correlation algorithm for Principal Components Analysis (PCA) model. PCA is an unsupervised algorithm that measures the variation of samples within a group. The use of a discriminant PCA (DAPC) model finds between-group variations.[39] The ions used for multivariate analysis were generated from the heat maps by selecting the most abundant m/z values for analysis of variance (ANOVA). Ions whose values were $p \leq 0.05$ were retained as classifying features.

The robustness of the model was calculated using Leave-One-Out Cross Validation (LOOCV), wherein a value of 100% indicates that all samples were assigned to their correct class. Accuracy was determined by analyzing blind samples that had not been used to develop the DAPC.

3 | RESULTS

3.1 | XRF

The major elements detected by XRF spectroscopy were silicon, sulfur, potassium, calcium, iron, copper, and zinc. The results are

reported as percent concentration. Descriptive statistics are shown in Table S2 (supporting information). A box-and-whisker plot (Figure S1, supporting information) of the results shows that the elements detected and their relative abundance by XRF analysis are not helpful for determining the taxonomic origin of the fleece. It is interesting to note that the elemental profile and their relative concentration did not vary between the specimens collected in South America (vicuña and guanaco) versus North America (alpaca).

3.2 | HATR-FTIR

Analysis of the spectra provided no visible differences between the three species used in this study (Figure S2, supporting information). The spectra were dominated by the expected amide stretches as reported in previous research.[38,40,41] Discriminate analysis was applied to the training spectra using 10 PCs. Alpaca showed a clear separation from the other taxa, while vicuña and guanaco samples consistently grouped together, signifying that the two species are challenging to separate by HATR-FTIR (Figure S3, supporting information).

3.3 | DART-TOFMS

The within-species spectra of the three distinct classes were remarkably reproducible. In all three taxa the positive-ion mode mass spectra were dominated by cholesterol and cholesterol analogs.[42] Figure 1 shows the average positive-ion spectra. Table 1 shows the tentative assignment of the most intense peaks. The base peaks in Figure 1 are tentatively assigned to cholesterol (369.352 *m/z*; $[C_{27}H_{46}O - OH]$). This observation is in agreement with Rivett et al[28] and Logan et al[29] who have reported that cholesterol and its analogs were abundant in the fine fibers of sheep. Assignments of other masses are shown in Table 1.

The negative-ion mode analysis for all three camelids was dominated by fatty acids. Figure 2 shows the averaged negative-ion mode spectra. Table 1 shows the tentative assignment of the most intense peaks. The base peak at *m/z* 283.265 in Figure 2 is tentatively assigned to fatty acids (16:0) and this observation is in agreement with Rivett et al[28] and Logan et al[29] who reported that 16:0 was the most abundant fatty acid detected in alpaca. The assignment of other masses is shown in Table 1.

A positive-ion mode heat map was generated to visually demonstrate the interspecies reproducibility and intraspecies chemotype differences for the taxa tested (Figure 3). It is visually apparent that alpaca has a series of molecules (above *m/z* 450) which are detected in vicuña but not in guanaco. These molecules, which were not identified, add weight to the value of chemotaxonomy since they were only observed in the *Vicugna* genera but not in the genus *Lama*. The negative-ion heat map (Figure S4, supporting information) shows the fatty acid profiles for each taxa. Alpaca showed the most distinctive fatty acid profiles.

The positive-ion spectra were analyzed using a correlation matrix PCA using 150 ions. The first principal component accounted for 24.8% of the variability of the data whereas PC2 and PC3 accounted

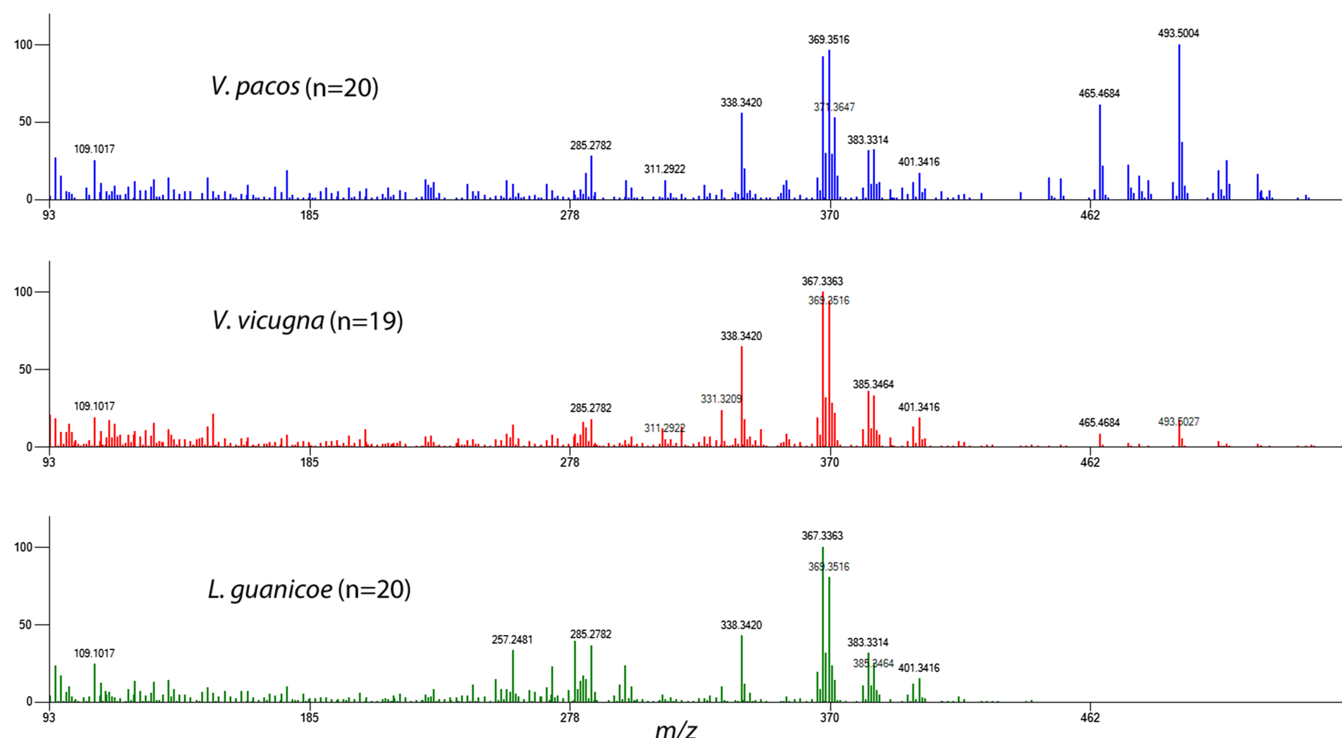

FIGURE 1 Positive-ion DART-MS spectra of alpaca, vicuña, and guanaco fibers. Base peaks are cholesterol and cholesterol analogs [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 The dominant ions found using DART-MS in both positive- (+) and negative-ion (–) mode with their molecular assignments

Measured *m/z*	Assignment	Composition and adduct	Ionization mode	Vicuña % abundance	Alpaca % abundance	Guanaco % abundance
199.170	12:0	$C_{12}H_{24}O_2$ –H	–	7.3	–	–
227.202	14:0	$C_{14}H_{28}O_2$–H	–	18.2	–	24.9
241.218	15:0	$C_{15}H_{30}O_2$–H	–	21.4	–	42.7
255.234	16:0	$C_{16}H_{32}O_2$–H	–	82.9	49.5	100.0
269.250	17:0	$C_{17}H_{34}O_2$–H	–	59.9	28.9	87.3
283.265	18:0	$C_{18}H_{36}O_2$–H	–	100.0	100.0	70.8
311.295	20:0	$C_{20}H_{40}O_2$–H	–	25.7	10.7	–
367.336	Cholesta-3,5-diene	$C_{27}H_{44}$–H	+	100.0	92.0	100.0
369.352	Cholesterol	$C_{27}H_{46}O$–OH	+	94.3	96.3	80.3
383.331	5-Cholesten-3β-ol-7-one	$C_{27}H_{44}O_2$–OH	+	35.7	31.4	31.4
385.346	Hydroxycholesterol or isomers	$C_{27}H_{46}O_2$–OH	+	32.8	32.4	24.9
401.342	Hydroxycholesterol or isomers	$C_{27}H_{46}O_2$–H	+	18.4	17.0	14.8
493.498	Undetermined	$C_{33}H_{66}O_2$–H	+	16.8	100.0	–

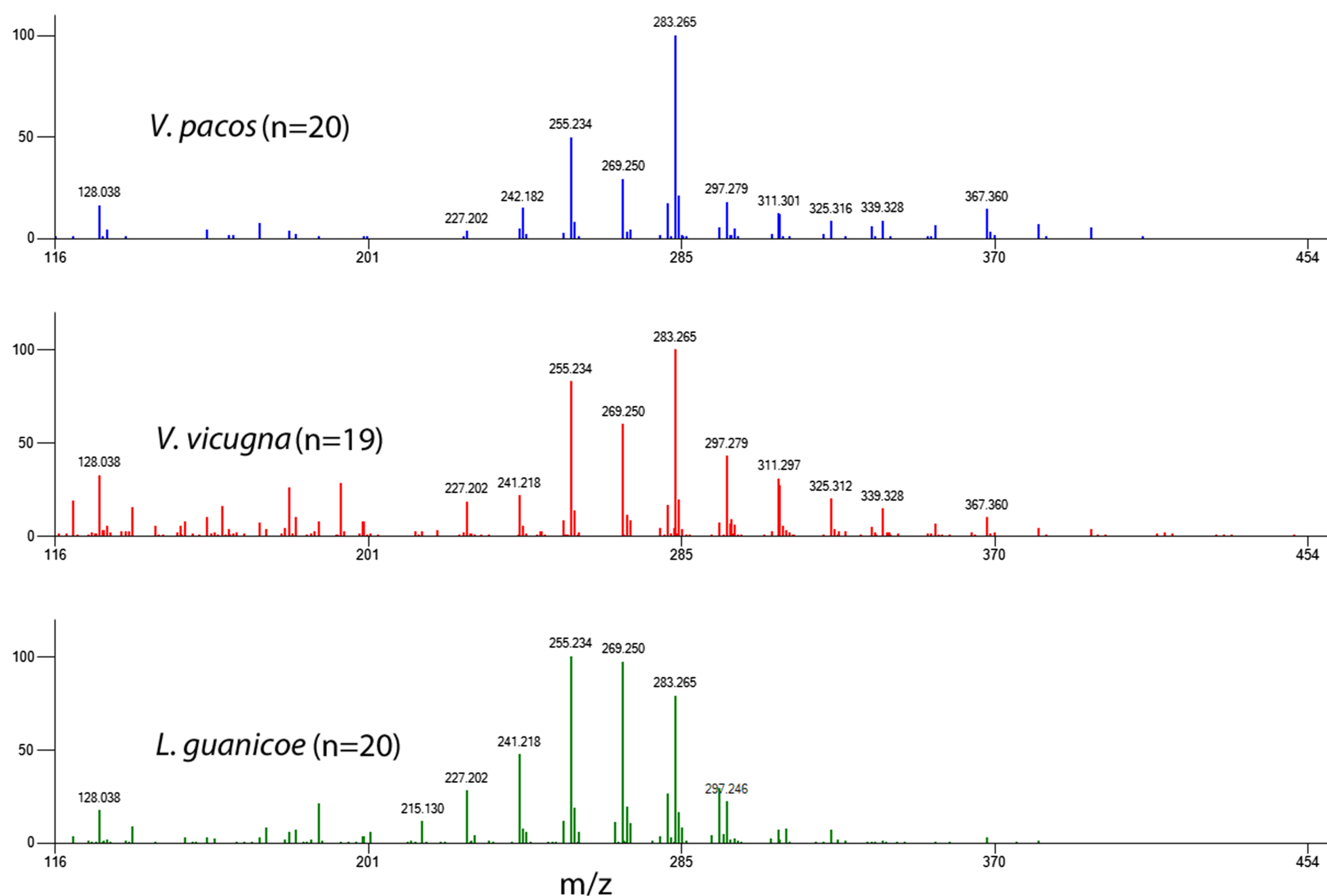

FIGURE 2 Negative-ion DART-MS spectra of alpaca, vicuña, and guanaco fibers. Base peaks are fatty acids

for 13.9% and 8.6%, respectively (which accounted for 47.4% of the variability). The classification accuracy for the PCA using LOOCV was 87.9% (graph not shown). Discriminant Analysis of Principal Components (DAPC) using 24 principal components produced a graph (Figure 4) that showed each taxon to be well differentiated with a calculated LOOCV of 99.9%, indicating that none of the 59 samples

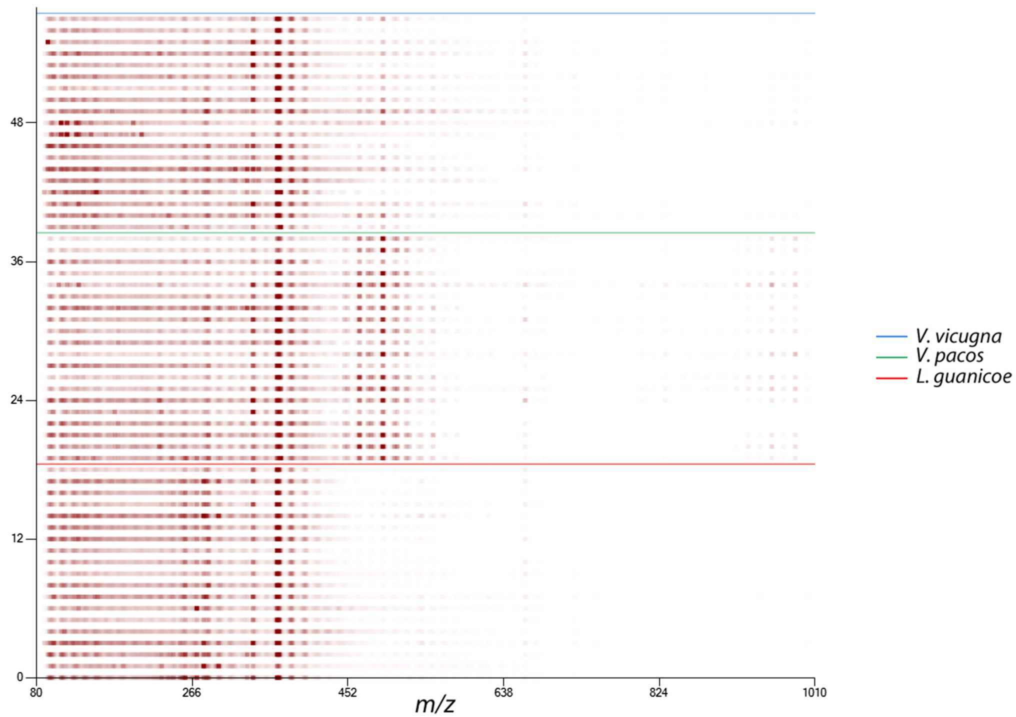

FIGURE 3 A positive-ion heat map containing the camelid training set. The y-axis contains all spectra, and the x-axis portrays each ion within a spectrum; the intensity of the color is an indication of the relative intensity of each ion

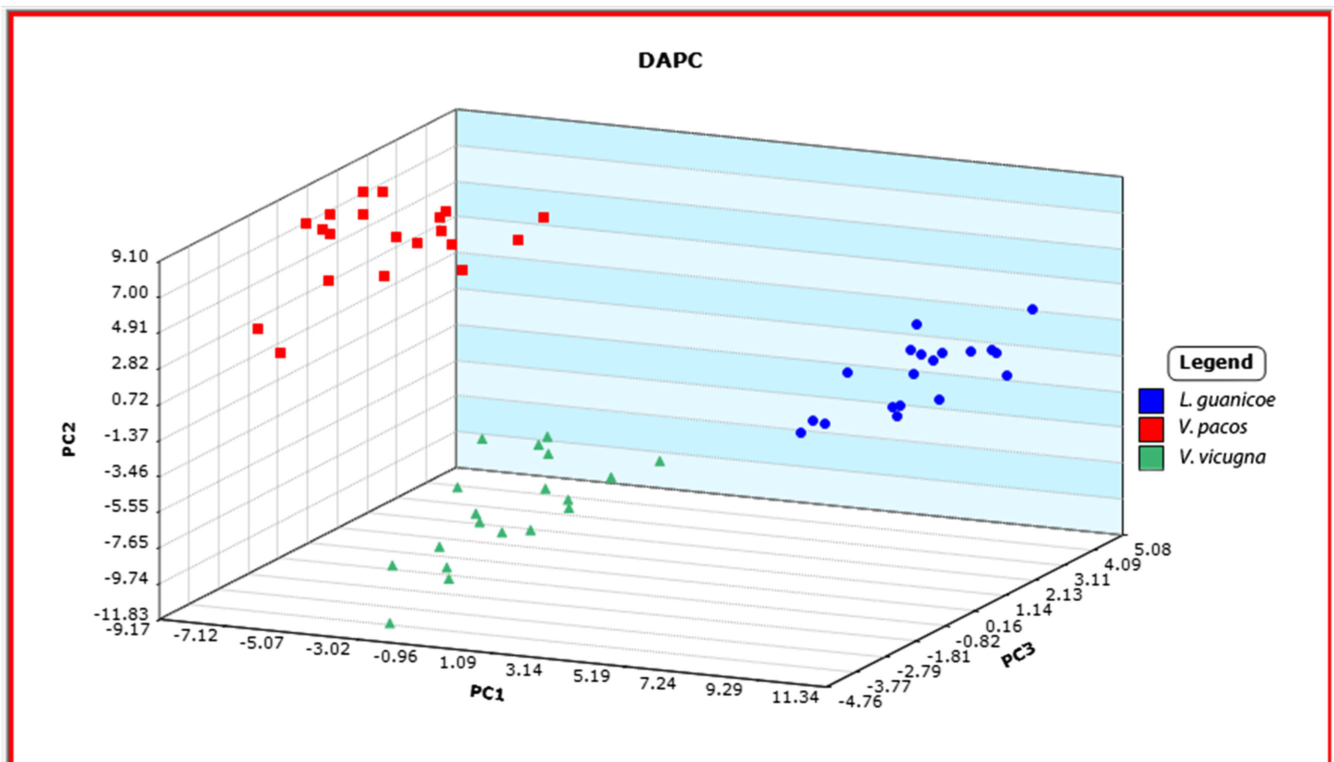

FIGURE 4 DAPC analysis of guanaco, vicuña, and alpaca. LOOCV of 99.9% indicates that each sample was correctly assigned to its corresponding class

TABLE 2 Results of the assignment of the validation samples from the DAPC of positive-ion spectra

Validation specimen	Assignment probability	Spectra classified as:
*Vicugnapacos*_VAL-1.Txt	99.71%	*V. pacos*
*Vicugnapacos*_VAL-2.Txt	98.22%	*V. pacos*
*Vicugnavicugna*_ST834.81–1.Txt	100%	*V. vicugna*
*Vicugnavicugna*_ST834.81–2.Txt	100%	*V. vicugna*
*Lamaguanicoe*_MAM1918_H0003.Txt	99.68%	*L. guanicoe*
*Lamaguanicoe*_MAM1956_I0009.Txt	100%	*L. guanicoe*
*Lamaguanicoe*_MAM1990_P0008.Txt	100%	*L. guanicoe*

from the training set were misidentified. This supports the concept that the model is effective for making species assignments. The DAPC for the ions selected in the negative-ion mode spectra (Figure S5, supporting information) yielded similar results. Sixteen principal components were used in a correlation DAPC and the calculated LOOCV value was 93.4%, indicating that four samples from the training set were misclassified.

In order to test the model accuracy seven specimens that had not been used to train the DAPC model were analyzed as unknowns. This included three specimens of guanaco, and two samples of alpaca and vicuña samples. All spectra were correctly classified with an assignment probability that exceeded 98%. Table 2 shows the results of the validation samples.

4 | DISCUSSION

Determining the taxonomic origin of keratin from the fleece of closely related species is important to enforce national and international laws and regulations of wildlife trade. Two of these camelids in this study are wild, vicuña and guanaco, and their fibers have a high commercial value.[1] The third species, alpaca, is domesticated.

The XRF and HATR-FTIR analyses were not effective in discriminating the three species. Elemental analysis of the underfur of the camelids by XRF did not reveal significant differences in elements present or in their abundance. The elemental profiles in vicuña, alpaca and guanaco yielded similar results to the analysis of alpaca found by Mucha and Janeczek.[43] A more sensitive technique than XRF, such as inductively coupled plasma (ICP)-MS, may be more effective in showing differences among these taxa. HATR-FTIR, a powerful tool for protein characterization,[44] lacked the resolving power to separate the three species. The HATR-FTIR spectra were dominated by the amide group, and small but significant stretching frequencies were observed in the fingerprint region which hinted at species identification. The similarity of the spectra in the fingerprint region could be explained by the relatively high levels of cystine and cysteic acid as reported by Tucker et al.[27] While discriminant analysis showed that alpaca specimens clustered as a distinct group, the spectra of vicuña and guanaco clustered together and were not differentiated (Figure S3, supporting information). This makes sense because vicuña and guanaco are both wild forms while alpaca is domesticated.

Mass spectrometry analysis showed that the high resolution of DART-TOFMS could distinguish the species source of the fibers with a high level of accuracy (>99%) without sample derivatization. Table 1 show a series of selected ions and their abundances which were detected in each species, but statistical classification was contingent on the entire metabolome rather than single diagnostic compounds. Analysis of the positive-ion metabolome produced robust models. The negative-ion metabolome was dominated by fatty acids, but these chemotypes were not as discriminatory as the positive-ion metabolome.

Surprisingly, this data demonstrates that the keratin chemotypes can be used to differentiate species within a closely related taxonomic group, although we recognize that this phenomenon may not occur within other taxonomic groups.[33] Given the nature of hair and fleece keratin, we speculate that the species distinguishing signal is either environmental (diet), genetic, or both. McGregor and Tucker [45] reported that there is a direct relationship between nutrition and the chemical composition of fiber. The differences include an overall reduction in the percentage concentration of amino acids found in fibers for animals that had a reduced protein diet.[46] McGregor and Umar[47] reported that supplementing sheep diets showed a direct increase in the rate of fiber production as well as fiber diameter, whereas Russel and Redden[48] noted that supplementing the diets of alpacas gave a proportional increase in fiber length. These results indicate that dietary differences influence the chemical composition of fiber. Vicuña and guanaco are wild animals that subsist on a diet of wild grasses and shrubs,[49,50] making a stark contrast to the diet of farmed alpaca which predominantly feed on grasses but are regularly provided with supplemental protein, vitamins, and minerals.[51]

It is clear that diet alone could not explain why these closely related taxa can be taxonomically differentiated using their keratin chemotypes. Chamut et al[52] hypothesized that eccrine sudoriferous glands, which surround the hair follicles, play an important role in intraspecific chemical communication of vicuña and it is feasible that some of the ions detected in this study were extruded from these glands. In this study the positive- and negative-ion spectra of guanaco did not reveal significant differences although the specimens were collected from a range that exceeded 6000 miles apart and from sea level to over 5000 m. In addition, the samples of alpaca were all obtained from domestic sources in the USA, but their positive- and negative-ion spectra were more similar to those of vicuña, a wild species that lives in an extremely harsh environment over 3000 m altitude. Alpaca is currently recognized as the domestic form of the vicuña, making the results of the study consistent with this relationship.[23] Given these observations, one is led to the conclusion that results of the positive and negative chemotypes are probably modulated primarily by genetic control and to a lesser extent by diet.

Current methods for DART-TOFMS do not allow for single fiber analyses due to the difficulty of maintaining a single fiber in the gas stream for enough time to acquire a comprehensive spectrum. In order to accomplish separation of blended materials that could

potentially consist of more than a single species, an additional tool for keeping a single fiber in the gas stream of the instrument would have to be developed.

The successful separation of *Vicugna vicugna* (vicuña), *Vicugna pacos* (alpaca) and *Lama guanicoe* (guanaco) fleeces through chemometric analysis using either positive- or negative-ion DART-TOFMS shows that a chemotaxonomic approach is capable of separating closely related species of fibers. Accurate and rapid identification of the taxonomic source of specimens containing fleece is important to identify violations of international wildlife trade agreements, such as CITES and Vicuña Conventions, as well as to favor and support legal trade. Future research will include specimens from wild alpacas (which also live in extreme conditions in the Andean plateau), and will add fiber samples of llama (*Lama glama*), the domestic evolutionary descendent of guanaco.

ACKNOWLEDGEMENTS

The authors thank Cristobal Barros (DOI ITAP, Chile) and Christina Kish (DOI ITAP, Washington DC, USA) for their logistical and financial support in facilitating many aspects of this project and to Barry Baker for his careful editorial comments. The authors also wish to thank Renate and Richard Gyuro, owners of Alpacas at Lone Ranch, for their generous contribution of alpaca wool samples. B.A.G. was partially funded by CONICYT (REDI-170208) when this manuscript was written. 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. Vicuña and guanaco fiber specimens were imported into the USA with a CITES COSE permit N° CL005.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/rcm.8916.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Price E, Larrabure D, Gonzales B, McClure P, Espinoza E. Forensic identification of the keratin fibers of South American camelids by ambient ionization mass spectrometry: Vicuña, alpaca and guanaco. *Rapid Commun Mass Spectrom*. 2020;34:e8916. https://doi.org/10.1002/rcm.8916