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Electrospray Ionization Mass Spectrometric Analysis of Blood for Differentiation of Species

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The National Fish and Wildlife Forensic Laboratory is responsible for the determination of species of birds, reptiles, and mammals from the United States, as well as international species falling under the protection of CITES treaties. We have recently found electrospray ionization mass spectrometry to be an effective means of rapidly analyzing blood samples for species identification. Nearly 1000 individuals were analyzed which comprised 62 species represented by birds, mammals, and reptiles. Whole blood and dried blood samples were analyzed without purification to provide simultaneous molecular weights from the α- and β-proteins present in each sample's hemoglobin. The combination of the two molecular weights for the hemoglobin proteins (i.e., α/β-pairs) was used as species determining markers. In all, 133 distinctive α/β-pairs were observed from the individuals analyzed. Despite the variability in the hemoglobins evaluated, 86% of these α/β-pairs were found to be diagnostic for a particular species to the exclusion of all other species studied. No other single protein system studied by a single analytical technique can so effectively resolve species from a wide range of taxa as can the hemoglobin system when analyzed by electrospray ionization mass spectrometry. © 1999 Academic Press

The National Fish and Wildlife Forensics Laboratory is a federal wildlife crime laboratory composed of biologists and chemists and is called upon to employ interdisciplinary approaches in performing species identifications on a wide variety of mammals, birds, and reptiles. These identifications are performed in conjunction with the enforcement of state, federal, and international wildlife laws. Species identifications have historically been the domain of biologists, arising from morphological characteristics of the organism in question, or from identifications based upon either DNA or diagnostic protein markers isolated from blood and tissue samples. Our approaches for making positive identifications may utilize several different techniques, and most of the techniques can generally be considered to fall into these two areas of species determination depending upon the items being analyzed. For example, morphological samples such as bones, hairs, hides, and feathers provide species-specific indicators while serological samples such as blood and tissue yield species-specific information through DNA and protein markers.

One area of chemistry that has contributed greatly to the understanding of biological systems is that of structure elucidation of macromolecules. The techniques of nuclear magnetic resonance spectroscopy and mass spectrometry are routinely used to unravel complex molecular structures to provide a deeper understanding of the function of biological systems. While chemists are not normally involved with species determinations, we became interested in applying the chemical technique of electrospray ionization mass spectrometry (ESIMS)[1] to the characterization of hemoglobin in blood samples from different species as a means of differentiating one species from another. The technique readily affords the molecular weights of proteins allowing for the independent characterization of the α- and β-protein chains present in hemoglobin based upon their primary amino acid sequences. It has already been established that electrospray ionization mass spectrometry effectively differentiates hemoglobins from several different North American mammals via the differing molecular weights of their α- and β-chains (1). The samples analyzed in these instances,

[1] Abbreviations used: ESIMS, electrospray ionization mass spectrometry.

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however, were all purchased through Sigma (St. Louis, MO) and consequently represented single individuals of each species which were "clinically pure" samples. We were interested in analyzing "real world samples" and determining whether the molecular weight markers of the α- and β-chains in hemoglobin would be sufficiently discriminating for individual species with the understanding that intraspecies hemoglobin variability can be considerable in some instances (2, 3). The larger the variability within species, the greater the likelihood for finding overlapping markers between species, thus causing a diminished ability of the technique to differentiate one species from another with a great degree of reliability. Therefore, we wanted to begin to ascertain the degree of intraspecies hemoglobin variability for a wide range of species, as well as determine the extent of interspecies overlap of the α- and β-chain molecular weight markers that might preclude species identifications.

EXPERIMENTAL

ESIMS spectra were acquired using a Perkin–Elmer Sciex API*1* single quadrupole mass spectrometer employing an ionspray voltage of 4500 V and an orifice potential of 85 V. Molecular weights of the α- and β-chains from each sample's hemoglobin (i.e., α/β-pair) were obtained through the BioMultiView deconvolution software provided with the spectrometer. Sample introduction was through direct infusion employing a syringe pump (BAS, West Lafayett, IN) operating at a flow rate of 5 μL/min. Both dried and whole blood samples were analyzed. Whole blood samples were prepared by dissolving 2 μL of whole blood in 1 mL of water. One hundred microliters of this solution was dissolved in 900 μL of a protonating solution. (The protonating solution consisted of a 1:1 mixture of CH_3CN and 0.1% aqueous acetic acid). Dried blood samples were prepared from samples dried on cotton gauze. A small piece of the gauze (approx 0.5 cm^2) was removed and placed in 1 mL of water. One hundred microliters of this solution was removed and added to 900 μL of the protonating solution.

RESULTS AND DISCUSSION

During the course of our study we examined 980 individuals representing 62 species. Table 1 summarizes the data collected for the species analyzed. The values observed for the hemoglobin proteins represent the average values obtained from the individuals examined within a species that afforded a particular α/β-pairing. These observed values also reflect the primary signals displayed in the mass spectra for the α- and β-chains after performing the deconvolution of the original mass spectral data collected. In many instances, more than one signal appears in the deconvoluted mass spectral data centered around the regions associated with the molecular weight values for hemoglobin α- and β-chains. Figures 1 and 2 compare the varied types of hemoglobin spectra acquired in the compilation of Table 1; Fig. 1 illustrates a simple spectrum which displays only primary α- and β-chain signals, whereas Fig. 2 illustrates a more complex spectrum displaying several secondary signals in addition to the primary signals for the α- and β-chains. The secondary signals observed in many of the spectra collected might be attributed to the presence of multiple forms of hemoglobin within individuals (2, 3) since masses from secondary signals in one spectrum often match the masses given by primary signals in other spectra for a particular species. However, secondary signals in deconvoluted spectra also appear which do not coincide with mass values from primary signals in other spectra obtained for a given species, implying that degradation and/or other blood proteins may contribute to the appearance of these secondary signals in the mass spectral data.

The spectrum of the cougar (*Puma concolor*) shown in Fig. 2 displays both types of secondary signals and is one of the more complex spectra observed for the individuals reported in Table 1. The primary α/β-pair displayed in the spectrum shown in Fig. 2 is 15302/15926. This particular cougar's blood spectrum corresponds to one of the 19 individuals represented by the cougar 15303/15926 α/β-pair molecular weight markers depicted in Table 1. When compared to cougar α/β-pair values in Table 1, the molecular weight values for the secondary signals found in the Fig. 2 spectrum show that some of these values correspond to molecular weights for α- and β-chains expressed in other cougars as the primary α/β-pair signals. However, not all of the signals found in the spectrum shown in Fig. 2 are expressed as primary signals in other cougar spectra (i.e., the signals corresponding to molecular weight values of 15114, 15422, and 15957 were never displayed as primary signals in the spectra of the other 23 cougar samples analyzed). While the identity of some or all of these secondary signals may prove to be useful for species identification in the future, we have chosen to classify individual blood types based upon the primary hemoglobin signals (i.e., most abundant) displayed by an individual's blood.

A quick glance at Table 1 shows that most species do not display a single hemoglobin type. Twenty-three of the 62 species examined expressed a single α/β-pair. Hemoglobin variants are well documented for many species (2–4) and, considering that 10 of these 23 single α/β-pair species had less than 10 individuals serving as representatives of the species, the likelihood for uncovering hemoglobin variants within species is quite great upon the examination of more individuals. We believe that, typically, species will display 2–5 differ-

TABLE 1

Hemoglobin α- and β-Chain Molecular Weights (M_r) Obtained from ESIMS Data for Various Species

Class	Family	Genus species	Common name	n^a	Observed α M_r^b	Reported α M_r^c	Observed β M_r^b	Reported β M_r^c
Aves	Accipitridae	*Aquila chrysaetos*	Golden eagle	5	**15472**	15457 (10)	**16277**	16277 (10)
			Golden eagle	7	15472		16338	
			Golden eagle	4	15476		16282	
			Golden eagle	3	15532		16338	
Aves	Accipitridae	*Buteo jamaicensis*	Red-tailed hawk	7	**15415**		**16252**	
			Red-tailed hawk	1	15415		16278	
Aves	Accipitridae	*Haliaeetus leucocephalus*	Bald eagle	7	15411		16311	
			Bald eagle	3	15413		16288	
			Bald eagle	25	**15414**		**16252**	
			Bald eagle	1	15794		16312	
Aves	Accipitridae	*Harpia harpyja*	Harpy eagle	17	15532		16236	
Aves	Anatidae	*Aix sponsa*	Wood duck	2	15293		16307	
			Wood duck	3	**15305**		**16308**	
			Wood duck	1	15426		16308	
			Wood duck	1	15427		16431	
Aves	Anatidae	*Anas acuta*	Northern pintail duck	14	**15251**		**16322**	
Aves	Anatidae	*Anas americana*	American wigeon	5	**15251**		**16322**	
			American wigeon	1	**15370**		**16361**	
			American wigeon	1	15370		16440	
			American wigeon	1	**15371**		**16320**	
Aves	Anatidae	*Anas crecca*	Green-winged teal	6	**15251**		**16322**	
			Green-winged teal	1	**15370**		**16322**	
Aves	Anatidae	*Anas platyrhynchos*	Mallard	1	**15249**	15248 (11)	**16365**	
			Mallard	27	**15251**		**16322**	16319 (12)
			Mallard	1	**15373**		**16324**	16329 (11)
			Mallard	1	15556		16322	
Aves	Anatidae	*Anas strepera*	Gadwall	1	15200		16365	
			Gadwall	1	**15249**		**16320**	
			Gadwall	3	**15250**		**16365**	
			Gadwall	2	**15370**		**16365**	
			Gadwall	1	15555		16365	
Aves	Anatidae	*Branta canadensis*	Canada goose	1	**15249**		**15996**	
			Canada goose	24	15292	15289 (13)	16293	16291 (13)
			Canada goose	2	15307		16295	
			Canada goose	5	15411		16294	
			Canada goose	1	15411		16414	
Aves	Anatidae	*Cygnus columbianus*	Tundra swan	6	15260		16293	
Aves	Anatidae	*Mergus cucullatus*	Hooded merganser	3	15064		16308	
			Hooded merganser	7	15264		16308	
Aves	Columbidae	*Zenaida macroura*	Mourning dove	12	15115		16136	
			Mourning dove	1	15139		15967	
Aves	Meleagrididae	*Meleagris gallopavo*	Turkey	1	15155		16310	
			Turkey	2	15274		16310	
			Turkey	1	15274		16429	
			Turkey	22	**15310**	15310 (14)	**16309**	
			Turkey	11	**15340**		**16309**	
Aves	Phasianidae	*Phasianus colchicus*	Ring-necked pheasant	2	15330	15344 (15)	16309	
			Ring-necked pheasant	2	15449		16309	
			Ring-necked pheasant	4	15636		16310	
Aves	Psittacidae	*Ara ararauna*	Blue-and-yellow macaw	4	15049		16118	
			Blue-and-yellow macaw	1	**15109**	15105 (16)	**16181**	16216 (16)
Aves	Psittacidae	*Ara aulicollis*	Yellow-collared macaw	7	**15106**		**16178**	
Aves	Psittacidae	*Ara chloroptera*	Green-winged macaw	7	15167		16179	
Aves	Psittacidae	*Ara severa*	Chestnut-fronted macaw	7	**15124**		**16178**	
Aves	Psittacidae	*Aratinga guarouba*	Golden conure	5	**15123**		**16181**	

TABLE 1—*Continued*

Class	Family	Genus species	Common name	n^a	Observed αM_r^b	Reported αM_r^c	Observed βM_r^b	Reported βM_r^c
Aves	Rallidae	*Fulica americana*	American coot	5	15351		16280	
			American coot	1	**15469**		**16279**	
Aves	Strigidae	*Bubo virginianus*	Great horned owl	1	15153		16327	
			Great horned owl	7	15308		16326	
			Great horned owl	1	15614		16327	
Aves	Strigidae	*Strix varia*	Barred owl	2	15280		16282	
			Barred owl	1	15338		16339	
			Barred owl	2	15399		16282	
Aves	Tetraonidae	*Bonasa umbellus*	Ruffed grouse	12	**15343**		**16310**	
			Ruffed grouse	1	15462		16310	
			Ruffed grouse	1	15649		16311	
Mammal	Antilocapridae	*Antilocapra americana*	Pronghorn	18	15012		15934	
Mammal	Bovidae	*Aepyceros melampus*	Impala	4	15194		15980	
			Impala	1	15194		15957	
			Impala	1	15247		15957	
			Impala	2	15272		15979	
Mammal	Bovidae	*Bison bison*	Bison	21	15037		15977	
Mammal	Bovidae	*Bos taurus*	Cow	12	15054	15053 (17)	15956	15954 (18)
			Cow	3	15055		15972	15972 (7)
			Cow	8	15055		15981	15981 (7)
			Cow	1	15055		16003	16002 (7)
Mammal	Bovidae	*Capra aegagrus*	Cretian goat	1	15037		15609	
			Cretian goat	7	15037		16038	
Mammal	Bovidae	*Capra hircus*	Domestic goat	8	15036	15033 (19)	16024	16021 (20)
			Domestic goat	5	15036		16047	16043 (7)
			Domestic goat	1	15035		16052	16049 (7)
Mammal	Bovidae	*Oreamnos americanus*	Mountain goat	7	15065		16113	
			Mountain goat	19	15041		16115	
Mammal	Bovidae	*Ovis aries*	Sheep	6	15049		16075	16073 (21)
Mammal	Bovidae	*Ovis canadensis*	Big horn	16	15073		16076	
Mammal	Bovidae	*Ovis dalli*	Dall's sheep	10	15075		16105	
Mammal	Bovidae	*Tragelaph strepsiceros*	Greater kudu	6	15052	14949 (22)	16057	16053 (22)
Mammal	Canidae	*Canis familiaris*	Dog	16	**15248**	15247 (23)	**15996**	15996 (23)
Mammal	Canidae	*Canis lupus*	Wolf	1	15221		16000	
			Wolf	23	**15248**		**15997**	
Mammal	Cervidae	*Alces alces*	Moose	8	15116	15115 (24)	16223	16223 (24)
Mammal	Cervidae	*Axis axis*	Axis deer	1	**15091**		**16076**	
			Axis deer	1	15092		16069	
			Axis deer	1	15092		16081	
			Axis deer	2	15129		16068	
Mammal	Cervidae	*Cervus elaphus*	Elk	12	**15113**		**15939**	
			Elk	39	**15113**		**16011**	
			Elk	1	15113		16043	
			Elk	1	15231		16011	
			Elk	1	15231		16040	
Mammal	Cervidae	*Cervus nippon*	Sika deer	2	**15114**		**15940**	
			Sika deer	3	15115		15912	
			Sika deer	5	15143		15939	
			Sika deer	7	15230		15910	
			Sika deer	19	15230		15940	
			Sika deer	2	15231		15949	
Mammal	Cervidae	*Odocoileus hemionus*	Black-tailed deer	19	15158		15869	
			Black-tailed deer	2	15161		16108	
Mammal	Cervidae	*O. hemionus*	Mule deer	24	15159		16107	
Mammal	Cervidae	*Odocoileus virginianus*	White-tailed deer	31	15113	15109 (7)	15883	15824 (7)
			White-tailed deer	1	15114		16092	
			White-tailed deer	4	15129	15125 (7)	15883	
			White-tailed deer	2	15129		16094	
			White-tailed deer	1	15130		16108	

TABLE 1—*Continued*

Class	Family	Genus species	Common name	n^a	Observed α M_r[b]	Reported α M_r[c]	Observed β M_r[b]	Reported β M_r[c]
			White-tailed deer	1	15130		16160	
			White-tailed deer	1	15143	15139 (7)	15883	
			White-tailed deer	1	15143		15897	
			White-tailed deer	1	15144		15793	
			White-tailed deer	1	15144		15870	
			White-tailed deer	1	15144		16162	
			White-tailed deer	10	15159	15155 (7)	15883	
			White-tailed deer	4	15159		16161	
			White-tailed deer	1	15160		15901	
			White-tailed deer	1	15160		15957	
			White-tailed deer	1	15161		16095	
Mammal	Cervidae	*Rangifer tarandus*	Caribou	10	15103	15101 (25)	16122	16166 (25)
Mammal	Equidae	*Equus caballus*	Horse	4	**15090**		**16079**	
			Horse	1	**15115**	15114 (26)	**16008**	16008 (27)
Mammal	Felidae	*Acinonyx jubatus*	Cheetah	12	**15303**		**15928**	
Mammal	Felidae	*Felis bengalensis*	Leopard cat	4	**15317**		**15916**	
			Leopard cat	14	**15317**		**16017**	
			Leopard cat	1	**15460**		**16031**	
Mammal	Felidae	*Felis catus*	Domestic cat	2	15306	15305 (6)	15973	15926 (6)
			Domestic cat	6	15307		15946	15956 (7)
			Domestic cat	2	15308		16002	15912 (7)
Mammal	Felidae	*Felis serval*	Serval	6	15360		16003	
Mammal	Felidae	*Felis temmincki*	Asian golden cat	1	15318		16094	
			Asian golden cat	7	15320		15997	
			Asian golden cat	1	15320		16066	
Mammal	Felidae	*Felis viverrinus*	Fishing cat	6	**15317**		**15916**	
			Fishing cat	2	**15317**		**16016**	
Mammal	Felidae	*Lynx lynx*	Lynx	7	15393		15926	
Mammal	Felidae	*Lynx rufus*	Bobcat	3	15286		15913	
			Bobcat	2	15286		15985	
			Bobcat	1	15316		15986	
Mammal	Felidae	*Neofelis nebulosa*	Clouded leopard	17	15459		16047	
			Clouded leopard	2	15522		16048	
Mammal	Felidae	*Panthera tigris*	Tiger	12	**15459**	15455 (28)	**16031**	15986 (28)
Mammal	Felidae	*Panthera uncia*	Snow leopard	13	15490		16031	
Mammal	Felidae	*Puma concolor*	Cougar	3	15144		15926	
			Cougar	1	15272		15926	
			Cougar	19	**15303**		**15926**	
			Cougar	1	15305		16049	
Mammal	Ursidae	*Ursus americanus*	Black bear	16	**15112**		**16009**	
			Black bear	23	15112		16024	
Mammal	Ursidae	*Ursus arctos*	Grizzly	26	**15112**		**16008**	
Mammal	Ursidae	*Ursus maritimus*	Polar bear	13	**15112**	15110 (29)	**16008**	16009 (29)
Reptiles	Cheloniidea	*Caretta caretta*	Logger head sea turtle	7	15691	15687 (30)	16653	16602 (30)
			Logger head sea turtle	1	15692		16613	

Note. Boldface values represent interspecies overlapping of the α/β-pair's molecular weights. Values are considered to be overlapping at ± 4 amu.

[a] The total number of individuals (n) within a species providing a particular α/β-pair.

[b] Average values from the individuals (n) analyzed. No species' observed α- or β-chain M_r values deviated more than 4 amu over the number of individuals (n) expressing a given α/β-pair.

[c] Literature references are given in parentheses. Not every reported polymorphic α- or β-chain M_r is given for species where values could be found. In those instances where there is not agreement between the observed and reported values, the reported values for the α- and β-M_r appear in the first row of the species' entry.

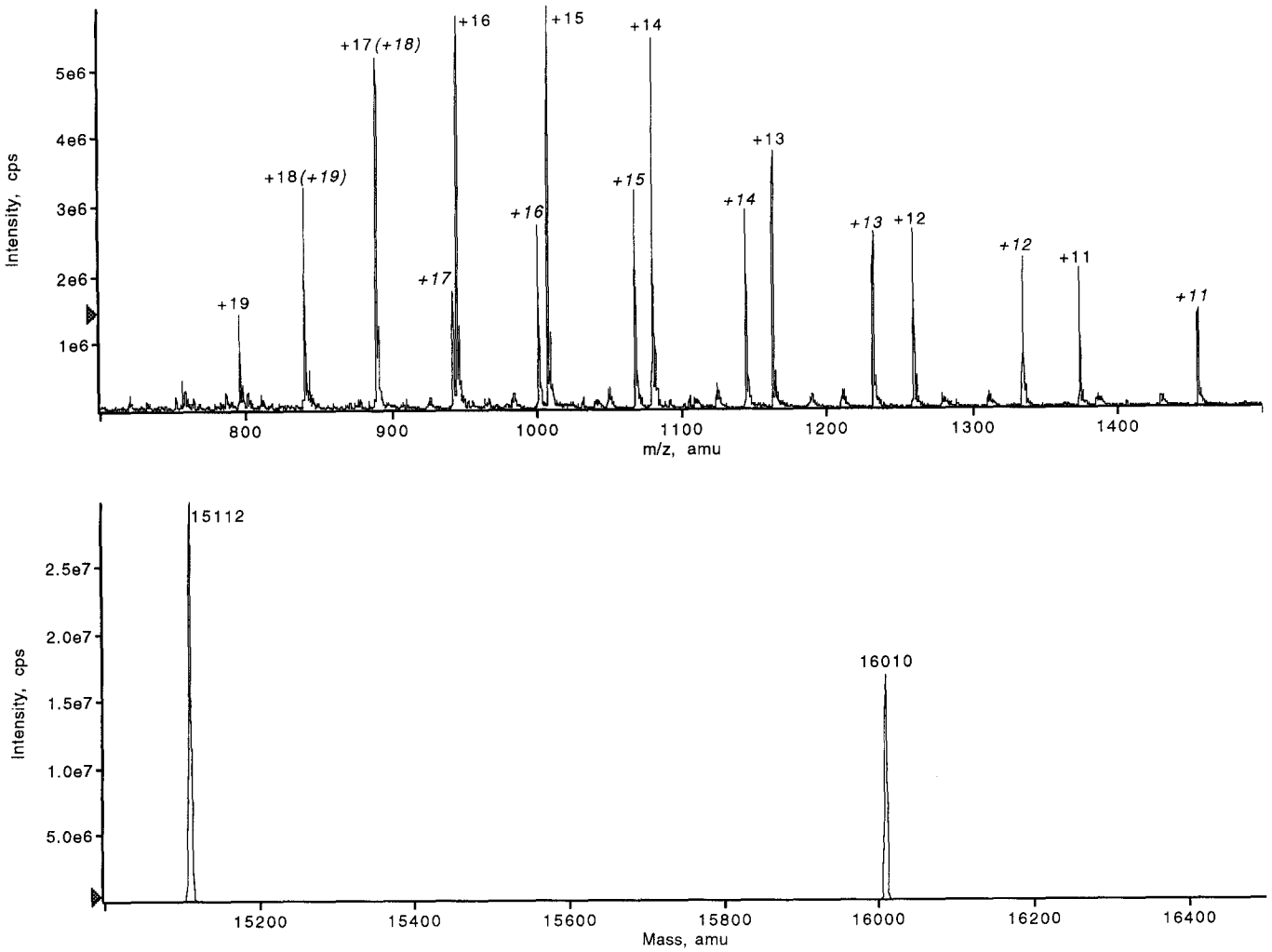


FIG. 1. Originally acquired spectrum (top) and deconvoluted spectrum (bottom) of *Ursus americanus*. Charge states of the α-chain and β-chain (shown in italics) responsible for the signals in the original spectrum are shown, while the mass values derived from the deconvolution of the original spectrum are shown in the lower spectrum.

ent hemoglobin types as greater numbers of individuals are examined within each species based upon the data compiled in Table 1. It should be noted that only the individuals from one of the 23 species which displayed a single hemoglobin type were sampled from a single location. These were the dog samples (*Canis familiaris*) which were taken from different breeds of dogs from a local animal shelter. In all other instances for the list of species found in Table 1, the data reflect samples gathered from as diverse a geographic area as possible.

Despite the hemoglobin variability expressed by many of the species found in Table 1, identification of species occurs quite nicely. The α/β-pairs appearing in boldface type in Table 1 represent those values that have some interspecies overlap (i.e., species identification from these α/β-pairs cannot be made). These overlapping values represent only 14% of the total number of distinct α/β-pairs which were observed for the 62 species: 18 of the 133 different α/β-pair values. Thus, 86% of the observed α/β-pairs provided a positive identification of the individual's analyzed blood sample to the species level of determination. To the best of our knowledge, there is no other single protein system studied by a single analytical technique which can so effectively resolve species from such a wide range of taxa as can the hemoglobin system when analyzed by electrospray ionization mass spectrometry. The ability of the technique to resolve proteins of 16,000 amu at a level of ±3 amu (0.02% resolution capability) offers an analytical system able to identify a large number of α- and β-chain molecular weight markers precisely.

While electrospray ionization mass spectrometry cannot differentiate all species, the basis of the technique for resolving proteins according to their molecular weights does offer distinct advantages over tech-

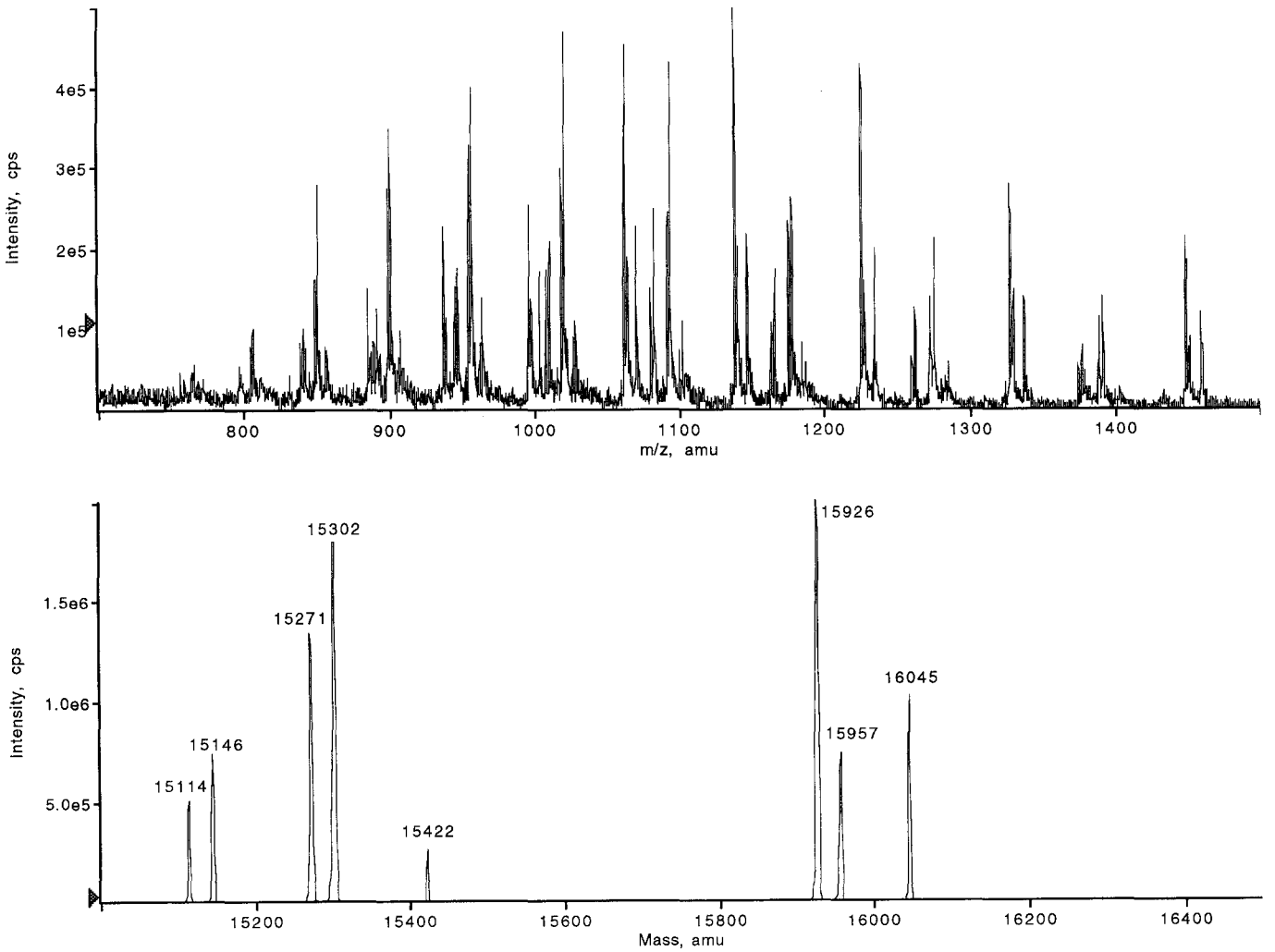


FIG. 2. Originally acquired (top) and devonvoluted (bottom) spectra of *Puma concolor.* Mass values derived from the devonvolution of the original spectrum are shown. Charge states of the α- and β-chains are not given in the originally acquired spectrum due to the complexity of the spectrum.

niques currently being used for species identification. The 0.02% resolution capacity of ESIMS for proteins the size of hemoglobin proteins clearly makes this technique much more discriminating than any electrophoretic technique currently being employed to differentiate protein markers for species identification. Two examples may be used to illustrate this point from the data shown in Table 1. The first involve the elk samples (*Cervus elaphus*) which were analyzed. Some of the 53 samples analyzed comprised the subspecies red deer; the red deer samples were obtained from New Zealand, while the elk samples came from Colorado, Washington, Oregon, and the Canadian provinces of Alberta and Saskatchewan. Previous electrophoretic work used to differentiate between red deer and North American elk distinguished these two subspecies as a result of their resolved hemoglobin markers (5). The conclusion drawn from the work is that two distinct types of hemoglobin exist: one for red deer and one for elk, with the F_1 hybrids displaying both hemoglobin types. The ESIMS data shown in Table 1, however, indicate that at least five separate hemoglobins are found within *C. elaphus,* albeit two of the five hemoglobins do predominate in the species according to the samples analyzed. The discrepancy between the ESIMS and electrophoretic results regarding the number of hemoglobins exhibited by *C. elaphus* may be attributed to the manner in which each technique resolves proteins. Amino acid substitutions which differentiate proteins structurally, yet cause only subtle pI changes between the proteins, may go undetected in electrophoresis as a result of the molecular pH dependence which governs electrophoretic separations. ESIMS, on the other hand, resolves proteins based

solely upon their molecular weight differences. Consequently, amino acid substitutions which alter the molecular weight values between proteins can be resolved by mass spectrometry regardless of the pI changes occurring between the molecules. Thus, resolution of proteins based upon molecular weight differences offers a unique advantage in unraveling information concerning the amount of polymorphism displayed by a particular genetic locus. In the case of *C. elaphus,* the mass spectral data in Table 1 show the presence of two distinct α-chains and three distinct β-chains combining to form at least five hemoglobin genotypes within the species based upon the major hemoglobin protein signals displayed in the samples analyzed.

Insight into the number of unique hemoglobin types derived from mass spectral data was also displayed in white-tailed deer (*Odocoileus virginianus*) when the data collected were compared to those found in the literature. We observed 16 types of hemoglobins in the 62 white-tailed deer samples evaluated. These 16 hemoglobins were assembled from 4 types of α-chains and 9 different types of β-chains. The total number of α-chains observed in the mass spectral analysis conforms to that observed through electrophoresis and protein sequencing (2b), while, at most, only five distinct β-chains have been reported for white-tailed deer (2b, 2c). Partial sequencing data reported for two of the α-chains of white-tailed deer showed a single amino acid difference at position 24 (Tyr-24 $\rightarrow$ Phe-24) (2a). These two α-chains were not separable through electrophoresis or other chromatographic techniques and yet would likely be resolved through ESIMS since the difference between the two proteins is 16 amu; coincidently, a molecular weight difference which we observed between the two α-chain molecular weights of 15113 and 15129.

The above examples point to situations where electrospray ionization mass spectrometry may discriminate polymorphic forms of hemoglobin that can go undetected through conventional electrophoresis. It should also be pointed out that a technique which so readily provides the molecular weights of proteins is a valuable aide in corroborating sequencing data. While we found many of the reported sequences to agree with the data appearing in Table 1, in several instances the values we observed were not the same as those reported. As an example, we observed three distinct β-chain types for *Felis catus* (15946, 15973, and 16002) and yet none of the five sequences reported for the β-chains of hemoglobin found in the domestic cat correspond to the molecular weights that we observed (6, 7). It is quite possible that the molecular weights of 15946, 15973, and 16002 for the β-chains observed by ESIMS simply reflect molecular weight values for β-chains which have not yet been sequenced. On the other hand, either the molecular weights we observed are somehow in error or the reported sequences are not correct. Considering the differences between obtaining sequencing data versus molecular weight data for a given protein, we believe that there is a greater likelihood of error in the reported sequences of the *F. catus* β-chain data.

Corroboration of hemoglobin sequencing data and the assessment of intraspecies hemoglobin polymorphism are certainly a part of the versatility associated with utilizing electrospray ionization mass spectrometry for the analysis of blood. The most remarkable results, however, obtained from the electrospray ionization mass spectrometric analysis of blood are a consequence of the accommodating nature of the hemoglobin molecule itself to be so species specific. This fact, coupled with the ease with which sample preparation, analysis, and interpretation is performed, makes electrospray ionization mass spectrometry an attractive analytical tool for species determination from blood. The capability of ESIMS to differentiate proteins of 16,000 amu to a level of ± 3 amu provides an analytical technique that can readily unravel the wide array of hemoglobin polymorphism expressed by many species and allows viewing these multiple hemoglobin types as a group of hemoglobin unique to a given species. Certainly the 16 different hemoglobin types expressed in white-tailed deer, all of which seem to be species specific, exemplifies the extent to which ESIMS may detect the many varied forms of hemoglobin within a given species and the subsequent capacity to resolve all these forms from those found in other species.

The database of 980 samples evaluated for this paper displayed 133 distinct hemoglobin α/β-pairs, of which 86% were diagnostic to the species level of determination. While some may consider the database shown in Table 1 to be insufficiently large to assess the technique of ESIMS for species identification, access to large, curated protein databases such as SWISS-PROT (7) and the Protein Information Resource (8) provide a substantial amount of information on hemoglobins found in various species, and thus lend further insight into judging the potential of ESIMS to accurately perform species identifications. We have extracted from these databases the reported values for the molecular weights of hemoglobin α- and β-chains. This information coupled with our own results from Table 1, as well as additional blood samples we have examined, has provided us with an in-house database of hemoglobin molecular weights encompassing 326 species: 7 species of amphibians, 9 species of reptiles, 12 species of fish, 101 species of birds, and 197 species of mammals. These 326 species are defined by 380 distinct α/β-pairs, of which 53 were found to be overlapping. The entire database of 326 species displays an 86% diagnostic success rate

for identifying species when considering the 53 overlapping α/β-pairs from the 380 distinct hemoglobins in the database. As in Table 1, hemoglobin types were considered overlapping when the α- and β-chain molecular weight markers were coincidental at value of ± 4 amu.

Analyzing the α/β-pairs in Table 1 against the in-house database of 326 species affords an additional 9 overlapping α/β-pairs apart from the existing 18 α/β-pairs already highlighted in Table 1. The newly disclosed α/β-pairs of 15037/16038, 15115/16136, 15129/16068, 15139/15967, 15167/16179, 15230/15940, 15264/16308, 15292/16293, and 15532/16236 for Cretian goat, mourning dove, axis deer, mourning dove, green-winged macaw, sika deer, hooded merganser, Canada goose, and harpy eagle, respectively, were found to overlap with other species. Taking these additional 9 overlapping hemoglobin makers into account affords 27 total overlapping α/β-pairs from the 133 displayed in Table 1, or a decrease in efficiency of the technique for identifying the species in Table 1 from 86 to 80%. While our in-house database is not compiled strictly from ESIMS data, and has a considerable contribution from outside sources of hemoglobin values represented by single individuals for a given species, it is reassuring that there is not a significant difference in the calculated reliability of ESIMS to differentiate species from the data compiled within Table 1 to the reliability observed when the Table 1 data are examined against a much larger database of hemoglobin molecular weights.

A final point should be made concerning overlapping α/β-pair molecular weight values. In some instances the primary structures found for the α- and β-chains which overlap may be different and their molecular weights just happen to be coincidental. As an example, ESIMS fails to differentiate most of the members representing the family *Ursus* from a significant portion of the elk population examined (*Cervus elaphus*), and from one of the analyzed horses (*Equus caballus*). The α/β-pair centered at 15112/16009 is the coincidental α/β-pair marker for all of these animals. However, HPLC analysis of blood samples from bear, elk, and horse would indicate a difference between the primary structures of the genera's α-chains and those of the genera's β-chains based upon differing retention times (9). Consequently, HPLC analysis of blood samples from overlapping bear, elk, or horse samples prior to the mass spectrometric analysis should differentiate these species. Thus, some of the known 27 overlapping α/β-pair molecular weight markers uncovered from those listed in Table 1 should be differentiable by implementing HPLC separation of hemoglobin samples prior to mass spectrometric analysis. Consequently, the ability of the technique to differentiate species could be significantly increased by applying a straightforward adaptation to the instrumental setup. We continue to examine the rich resource of blood for information on species identifications.

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