*Angel Carracedo,*[1] *M.D.; José Manuel Prieto,*[1] *B.S.;*
*Luis Concheiro,*[1] *M.D.; and Javier Estefanía,*[1] *B.S.*

# Isoelectric Focusing Patterns of Some Mammalian Keratins

**REFERENCE:** Carracedo, A., Prieto, J. M., Concheiro, L., and Estefanía, J., **"Isoelectric Focusing Patterns of Some Mammalian Keratins,"** *Journal of Forensic Sciences,* JFSCA, Vol. 32, No. 1, Jan. 1987, pp. 93-99.

**ABSTRACT:** An isoelectric focusing method followed by silver staining has been used for the study of keratins from a total of 97 individuals belonging to 17 families and 39 species. The method distinguishes perfectly between two different animal species. In addition, there are often considerable differences between breeds and even occasionally slight individual differences which in no way impede the identification of a particular species as such.

**KEYWORDS:** criminalistics, keratins, hair, isoelectric focusing, hair identification, species identification

The examination of physical properties of hair has been routinely used in forensic science for species identification. Nevertheless, the value of this method is often questionable because an individual can produce a variety of hair types with different structures and the hair can be affected by environmental factors [*1*].

For this reason, electrophoretic procedures in the study of keratins have proved useful for determining the species of the hair specimen.

Although the heterogeneity of hair proteins from different species can be proved with moving boundary electrophoresis [*2*] or with conventional electrophoretic procedures (such as polyacrylamide or starch gel electrophoresis) [*3,4*], the best results are obtained when two-dimensional electrophoretic procedures are used. This involves complicated methodology including fluorography [*5,6*].

Isoelectric focusing of keratins, without carboxymethylation and followed by silver staining, has proved to be a useful method for identifying hair from some species [*7*], but a study with a longer number of individuals and species (including different breeds) was necessary to establish its general utility. With that aim, we undertook the present study.

## Material and Methods

A total of 97 individuals belonging to 17 families and 39 species were analyzed (Table 1). The samples were obtained from different corporal areas of the animals listed in Table 1 and washed with petroleum ether, ethanol, and water and dried and cut into small pieces.

Received for publication 13 Feb. 1986; accepted for publication 14 April 1986.

[1]Associate professor, associate investigator, professor and director, and associate investigator, respectively, Department of Legal Medicine, University of Santiago de Compostela, Galicia, Spain.

TABLE 1—*Ninety-seven individuals analyzed.*

| Order | Family | Species | Number |
|---|---|---|---|
| Primates | Hominidae | *Homo sapiens* | 10 |
| | Cercopithecidae | *Papio papio* | 1 |
| | | *Macacca irus* | 1 |
| | | *Macacca sylvanus* | 1 |
| | | *Erythrocebus patas* | 1 |
| | | *Cercocebus galeritus* | 1 |
| | | *Cercopithecus cephus* | 1 |
| | | *Cercopithecus mitis* | 2 |
| | Pongidae | *Pan troglodytes* | 1 |
| Carnivora | Canidae | *Canis familiaris* | 7 |
| | | *Canis lupus* | 3 |
| | | *Vulpes vulpes* | 1 |
| | Viverridae | *Genetta genetta* | 1 |
| | Felidae | *Felis catus* | 6 |
| | | *Felis concolor* | 1 |
| | | *Panthera leo* | 2 |
| | | *Panthera tigris* | 1 |
| | Mustelidae | *Martes foina* | 1 |
| | | *Meles meles* | 1 |
| | | *Mustela vison* | 1 |
| | | *Mustela furo* | 1 |
| | Ursidae | *Ursus americanus* | 1 |
| | Procyonidae | *Procyon lotor* | 1 |
| Artiodactyla | Suide | *Sus scrofa* | 7 |
| | Camelidae | *Camelus dromedarius* | 1 |
| | | *Lama glama* | 1 |
| | Cervidae | *Cervus elaphus* | 1 |
| | | *Cervus unicolor* | 1 |
| | | *Dama dama* | 2 |
| | Bovidae | *Bos taurus* | 10 |
| | | *Bos indicus* | 1 |
| | | *Bos africanus* | 1 |
| | | *Bison bonasus* | 1 |
| | Caprinae | *Capra hircus* | 3 |
| | | *Ovis aries* | 6 |
| Perissodactyla | Equidae | *Equus caballus* | 6 |
| | | *Equus asinus* | 4 |
| Lagomorpha | Leporidae | *Oryctolagus cuniculus* | 4 |
| Rodentia | Caviidae | *Cavia porcellus* | 2 |

Extraction of keratins from at least ten samples from each animal was carried out according to Marshall and Gillespie [*6*] but without carboxy methylation.

Although the method was sometimes performed on a single hair (about 3 cm) extracted with 15 $\mu$L of extracting solution for 48 h at room temperature, usually proportionally higher amounts of hair and extracting solution were used.

The extracting solution was prepared by dissolving 0.09 g of Tris (Sigma) and 7.2 g of urea (Merck) in 9.6 mL of water, and then adding 120 mg of dithiothreitol (DTT) (BioRad) immediately before use.

The extracting solution was briefly centrifuged and 5 $\mu$L of 0.1 $M$ DTT were added to 25 $\mu$L of supernatant at least 10 min before typing.

The sample was then ready to be run by isoelectric focusing.

Isoelectric focusing was conducted using Pharmacia systems FBE 3000 and ECPS 2000/300 (Pharmacia Fine Chemicals, Uppsala, Sweden) and LKB systems Ultrophor, Multitemp, and Maxidrive 5000 (LKB, Bromma, Sweden).

Polyacrylamide gel isoelectric focusing was carried out in 0.5 mm of polyacrylamide gels at a gel concentration of ($T$ = acrylamide + bis/100) $T$ = 4.5% and cross-linking of ($C$ = bis/acrylamide + bis) $C$ = 2.8%. Sucrose (Merck) was added as a stabilizing agent at a total concentration of 12% (w/v). Ampholyte concentration was 2.8% (v/v).

Usually a mixture (1 : 1) of pharmalyte 2.5 to 5 and ampholine 5 to 7 was used to obtain an appropriate pH range. Other pH ranges (pH 2.5 to 5, 3.5 to 5, 4 to 6, 5 to 7, and 6.5 to 9) were also used.

Polymerization was carried out with 2% (v/v) riboflavin (BioRad) solution (20 mg/100 mL of distilled water) under ultraviolet light (360 nm).

Samples were applied to Whatman 3MM filter papers (1 by 1 cm) at a distance of 2 cm from the cathode. The electrode solutions were 1% (v/v) ethanolamine (Merck) for the cathode and 1 $M$ phosphoric acid (Merck) for the anode.

Focusing was carried out at 15-W constant power. A maximum voltage of 2500 V with unlimited current was used. Electrofocusing was carried out for 180 min at a cooling temperature of 8°C.

After isoelectric focusing, the gels were stained with the silver staining method of Carracedo et al. [*8*], with some modifications. Gels were stained as follows. First, they were prefixed in 12% trichloroacetic acid (Merck) for 15 min and washed three times for 20 min in 200 mL of 50% ethanol at 50°C in a shaking water bath to remove the carrier ampholytes. The gels were then washed in 5% methanol and 7% acetic acid (100 mL total amount) for 20 min and then fixed in 100 mL of 10% glutaraldehyde for 20 min. Next, the gels were washed four times for 15 min in 200 mL of distilled water. They were then soaked in 100 mL of 0.05% dithiothreitol (BioRad) for 20 min, and then treated for 30 min in 100 mL of 0.1% silver nitrate (Sigma). The gels were then given two rinses, first in 100 mL of distilled water and then with a small amount of developer. The gels were then soaked in the rest of the developer (75 $\mu$L of 37% formaldehyde in 150 mL of 3% sodium carbonate) until enough contrast in the bands was obtained.

The staining was stopped by adding 10 mL of 2 $M$ citric acid and shaking for 5 min. The gels were then rinsed with distilled water and wrapped in protective cellophane sheets.

The silver staining method of Sammons et al. [*9*] was also used.

## Results and Discussion

Figures 1 to 5 show the keratins patterns of different species in different pH ranges.

The patterns were typed from a total of at least ten samples from each animal and the results were always exactly the same.

Gels of 0.4 and 0.8 mm show similar results and the patterns remain stable through time, and even one-year-old hair shows the same pattern, although in old hairs the bands are fainter.

The most appropriate pH range for typing noncarboxymethylated keratins is obtained with a 1 : 1 mixture of pharmalyte pH 2.5 to 5 and ampholine 5 to 7, at a total concentration of 6% (v/v).

Patterns obtained from primate hairs can be seen in Fig. 1.

The patterns show a certain degree of similarity; nonetheless, differences between species (Fig. 1) are quite clear both in regard to the number and position of the bands, and to the color.

The closer two species are phylogenetically, the greater the similarity in their patterns, as is seen with the two Macacca studied, which showed almost no differences.

Occasionally, minimal differences between individuals belonging to the same species were observed (for example, the two samples of *Macacca irus,* or the human samples), but these differences did not hamper species identification.


FIG. 1—*Patterns of keratins from hairs of* (a) Papio papio, (b) Pan troglodytes, (c) Macacca sylvanus, (d) Macacca irus, (e) Erythrocebus patas, (f *and* g) Cercopithecus mitis, (h) Cercocebus galeritus, (i) Cercopithecus cephus, *and* (j *and* k) Homo sapiens.

In humans, individual differences are more striking in the range of pH 5 to 7, as previously demonstrated [7].

The patterns corresponding to Carnivora order are shown in Figs. 2 and 3.

Felidae and Canidae can be seen in Figs. 2 and 3. The keratin patterns seen in felid hair are characteristic for each species, although the similarity between tiger and puma patterns is striking: they differ only in one band at pI 4.7.

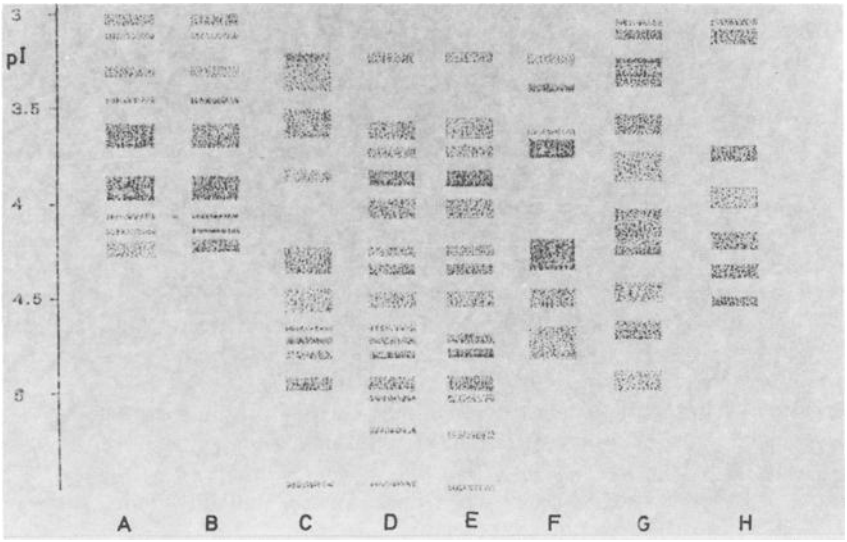


FIG. 2—*Keratin patterns from hairs of* (a) Felis catus *(common),* (b) Felis catus *(Siamese),* (c) Panthera leo, (d) Felis concolor, (e) Panthera tigris, (f) Ursus americanus, (g) Procyon lotor, *and* (h) Genetta genetta.

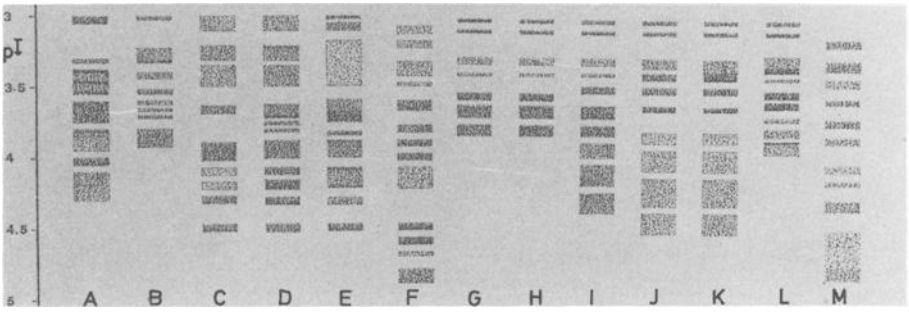


FIG. 3—*Keratin patterns from hairs of* (a) Canis familiaris *(Great Dane),* (b) Canis familiaris *(poodle),* (c) Mustela viso, (d) Martes foina, (e) Mustelo furo, (f) Meles meles, (g *and* h) Canis familiaris *(German shepherd),* (i, j, *and* k) Canis familiaris *(mixed breed),* (l) Canis lupus, *and* (m) Vulpes vulpes.

Two different breeds (common and Siamese) of cats display quite similar patterns, differing only in intensity and width of some of the bands.

Clear differences are evident in patterns of different breeds of dogs (Fig. 3). These patterns are constant within the same breed and in fact, hybrids show patterns common to each parent breed.

Patterns from other Carnivorae can be seen in Fig. 2. The salient feature here is the presence of minimal individual differences in the badger, which, again, did not hinder species identification.

Figures 4 and 5 show the patterns of the orders Perissodactyla and Artiodactyla. Of the former, *Equus caballus* and *Equus asinus* were studied, revealing predominantly cathodic banding with no sexual or breed differences. Different breeds of *Capra hircus* and *Ovis aries* showed different patterns, in contrast to various breeds of *Bos taurus,* which had similar patterns. Finally, Caviidae and Leporidae exhibit different patterns with no individual or breed differences apparent in the samples analyzed (Fig. 5).

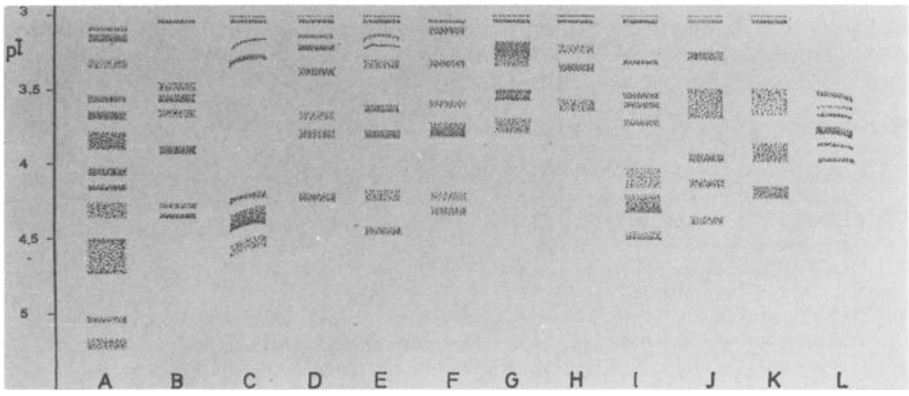


FIG. 4—*Keratin patterns from hairs of* (a) Bison bonasus, (b) Camelus dromedarius, (c) Lama glama, (d) Bos inducus, (e) Bos africanus, (f) Cervus unicolor, (g) Cervus elaphus, (h) Dama dama *(white variety),* (i) Dama dama *(brown variety),* (j) Ovis aries *(Valaquia sheep),* (k) Ovis aries *(Camerun sheep), and* (l) Ovis aries *(Merino breed).*

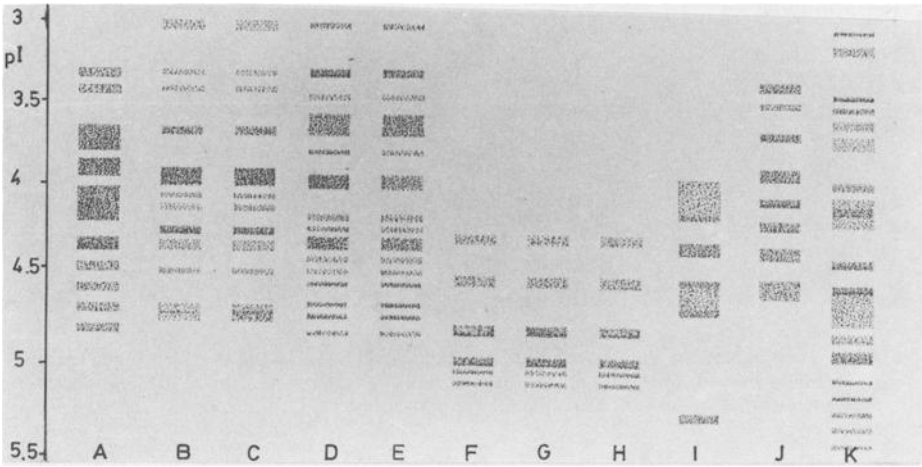


FIG. 5—*Keratin patterns from hairs of* (a) Capra hircus, (b *and* c) Bos taurus, (d) Sus scrofa *(common pig),* (e) Sus scrofa *(wild boar),* (f *and* g) Equus caballus, (h) Equus caballus *(mare),* (i) Equus asinus, (j) Oryctolagus cuniculus, *and* (k) Cavia procellus.

In short, then, this method distinguishes perfectly between two different animal species. In addition, there are often considerable differences between breeds and even occasionally slight individual differences which in no way impede the identification of a particular species as such. Obviously, however, the smaller the phylogenetic proximity of the species, the greater the differences in the patterns.

We believe that this method, which can be applied to a single hair, should be used as a method for species identification in forensic science laboratories. Also, the possibilities that it presumably offers for individual diagnosis of human hair samples should be investigated.

*Acknowledgments*

This work has been supported by Grant 2654/83 from the "Comisión Asesora de Investigación Científica y Técnica (Ministerio de Educación y Ciencia)."

The authors wish to thank Esther Rodríguez and Jesús Carracedo for preparing figures and photographs.

## References

[*1*] Gillespie, J. M. and Marshall, R. C., "Proteins of the Hard Keratins of Echidna, Hedgehog, Rabbit, Ox and Man," *Australian Journal of Biological Science,* Vol. 30, 1977, pp. 401-409.

[*2*] Gillespie, J. M. and Inglis, A. S., "A Comparative Study of High-Sulfur Proteins from α-Keratins," *Comparative Biochemistry Physiology,* Vol. 15, 1965, pp. 175-185.

[*3*] Day, T. H., "Interspecific Variation in the Hair Proteins," *Comparative Biochemistry Physiology,* Vol. 43, 1972, pp. 361-367.

[*4*] Hrdy, D. and Baden, H. P., "Biochemical Variation of Hair Keratins in Man and Non-Human Primates," *American Journal of Physical Anthropology,* Vol. 39, 1973, pp. 19-24.

[*5*] Marshall, R. C., Gillespie, J. M., and Klement, V., "Methods and Future Prospects for Forensic Identification of Hairs by Electrophoresis," *Journal of the Forensic Science Society,* Vol. 25, No. 1, Jan. 1985, pp. 57-66.

[*6*] Marshall, R. C. and Gillespie, J. M., "Comparison of Samples of Human Hair by Two-Dimensional Electrophoresis," *Journal of the Forensic Science Society,* Vol. 22, No. 4, Oct. 1982, pp. 377-385.

[*7*] Carracedo, A., Concheiro, L., and Requena, I., "The Isoelectric Focusing of Keratins in Hair Followed by Silver Staining," *Forensic Science International,* Vol. 29, 1985, pp. 83-89.

[8] Carracedo, A., Concheiro, L., Requena, I., and López-Rivadulla, M., "A Silver Staining Method for the Detection of Polymorphic Proteins in Minute Bloodstains after Isoelectric Focusing," *Forensic Science International,* Vol. 23, 1983, pp. 241-248.

[9] Sammons, D. W., Adams, L. D., and Nishizava, E. E., "Ultrasensitive Silver-Based Color Staining of Polypeptides in Polyacrylamide Gels," *Electrophoresis,* Vol. 2, No. 3, July 1981, pp. 304-307.

Address requests for reprints or additional information to
Dr. A. Carracedo
Department of Legal Medicine
Faculty of Medicine
Santiago de Compostela (Galicia) Spain