

SPIRIT COLLECTIONS: ACCELERATED AGING STUDIES CONCERNING THE STABILITY OF KERATIN IN ETHANOL AND FORMALIN

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Abstract.—The keratins are a closely related family of chemically stable proteins composing mammalian hair, horn, hooves, and avian feathers. Sheep hair (wool) has been much studied chemically because of its economic value. To our knowledge, no studies have been conducted on the long-term stability of other keratins from a museum perspective. We present here differences in the stability of feathers and hair under simulated aging conditions. Feathers and hair were heated dry, in 70% ethanol, and in 70% ethanol plus 1% formalin at 180°C for periods of 1 and 2 days. Feather keratin was approximately 50% less stable in ethanol than was hair keratin, as evidenced by the amount of amino acids lost from the sample and appearing in the solution. Feathers and hair, heated for the same periods of time under dry conditions, exhibited the same pattern of stability. As a corollary, the amino acid patterns of fresh hair and feathers from our samples of different species were found to be distinct and indicative of their originating taxon.

The word “keratin” denotes a class of linear structural proteins that is characterized by disulfide bonds that link the sulfur atoms of two cysteine amino-acid residues. To a large degree, the stability of keratin is dependent on these bonds. Keratins are found primarily in the epidermal regions of vertebrate animals and function in a protecting capacity. This class of proteins encompasses the outer layer of skin, hair and fur, horn, nails (and hooves), feathers, and bird beaks (Yu et al. 1993).

Keratins are pervasive in natural science and anthropological collections and are stored either “wet” or dry. Mammalian pelts and bird skins are stored dry, as are many cultural objects made from keratin. Entire bodies of animals and animal parts are maintained for research and taxonomic purposes. These are stored both as dried specimens and preserved in fluids. Despite this widespread presence in natural history museums, a protocol for keratin storage has yet to be developed. The variety of “dry” conditions includes storage at a constant room temperature and moderate relative humidity, as well as refrigeration in cold storage vaults at various temperatures and relative humidities. Fluid storage involves putting keratinous material in mixtures of reactive chemicals such as ethanol and formalin and often involves “topping off” when the level of liquid in the specimen container decreases from evaporation. This addition of fluid has an unknown effect on the possible leaching of chemicals from the specimen (von Endt 1994).

For these reasons, and because the chemistry of keratin is relatively obscure, optimal storage conditions are unknown. The effects of such factors as temperature and water vapor must be researched and controlled to ensure the integrity of stored specimens and artifacts of scientific and historical significance. Before optimal storage conditions can be determined, the nature of keratin itself must be examined. It is important to understand the chemical reactions undergone by ker-

atin, as well as the mechanisms by which it deteriorates. Because its disulfide bonds characterize keratin, it should be possible to examine specific proteinaceous materials such as hair and feathers and apply the resulting data to the class of keratins as a whole. Because there are some structural similarities between keratin and collagen, another structural protein of great importance in natural science collections, some of the information obtained by studying keratin may be applicable to the properties of collagen as well.

The Nature of Keratin

Keratins appear in many forms in all vertebrate phyla (Menefee 1977). Keratins are separated into "soft" and "hard" categories. Soft keratins encompass the bulk of the relatively low-sulfur-containing outer layer of skin, whereas the hard keratins include the relatively high-sulfur-containing hair, hoof, nail, feather, and bird beak (Arai et al. 1993, Yu et al. 1993).

Keratins are significant for their insolubility (as are other structural proteins such as mature collagen). They have developed biologically as "a mechanically tough protective coat" (Menefee 1977). There are three primary methods by which fibrous keratin macromolecules are stabilized: (a) crystallization between polymer chains may occur biologically, (b) disulfide crosslinks between cysteine molecules may be formed to produce the amino acid cystine (and keratins characteristically contain extensive intermolecular cystine crosslinking) (Arai et al. 1993, Menefee 1977, Mercer 1961), and (c) covalent crosslinks between polypeptide chains may be introduced by chemical processes (such as tanning).

The primary structure of keratin consists of a molecular backbone formed by amino acids covalently linked by peptide bonds between the N-terminus, or amino end of one molecule, and the C-terminus, or carboxylic acid end of another molecule. The secondary structure of keratin results primarily from hydrogen bonding between amino-acid molecules and is an alpha helix. However, if the filament is stretched, it converts from an alpha helix to a beta-pleated sheet. In the case of keratin, a tertiary structure results from disulfide bonds linking cysteines located across alpha helices from one another. The most complex spatial arrangement of keratin, the quaternary structure, involves the interaction of multiple amino-acid chains. This quaternary conformation resembles a coiled-coil rope, often called a supercoil (James et al. 1995, Steinert et al. 1994, Tucker et al. 1989, Wilk et al. 1995).

Some of the chemistry of keratin reactivity is still somewhat unclear. However, not only do many old keratin artifacts exist in museums that indicate keratin stability, but under mild laboratory conditions the protein is quite insoluble because of its disulfide crosslinks, which implies that the potential for successful fluid storage also exists.

Previous Research

To our knowledge, no information is available concerning the long-term stability of keratin in natural history collections, especially of keratin stored in fluids. It appears to be unstable, however, because few, if any, existing mammalian skins in collections were collected prior to 1840 (C. Hawks pers. comm. 1994). To some degree, this observation about skins may also be the result of specimens deteriorating to the point where they are deemed no longer useful in the collection

(perhaps because of pest damage prior to the widespread use of pesticides) and are discarded.

The medical and law enforcement professions have studied hair, while the textile industry has advanced the study of wool. The Commonwealth Scientific and Industrial Research Organization (CSIRO) in Australia has been the primary source of wool research (Dowling and Sparrow 1991).

There have been four major directions in modern keratin research. Initial studies used light- and scanning-electron microscopy to describe the physical appearance of the hair and feathers themselves. These methods have been used by law enforcement agencies to catalog the microscopic and morphological features of hair from different species of animals (Hicks 1977) and to document the feather morphology of different species of birds (R. Laybourne pers. comm. 1994).

The molecular structure of keratin fibers has been examined using methods such as x-ray diffraction and infrared spectroscopy. Crewther et al. (1965) state that the "elucidation of the structure of alpha-keratin is a task of such enormous complexity that it has stimulated a great deal of research. . . ." The accepted structure for alpha keratin consists of multiple helices coiled around two central filaments, with the entire supercoil held together by disulfide bridges and hydrogen bonds (Parry et al. 1977). Although x-ray diffraction and infrared spectroscopy cannot provide structural models (Crewther et al. 1965), they can confirm or refute proposed models. The supercoil satisfies the data supplied by these analytical methods. For a detailed discussion of the structure of keratin, see the review articles by Crewther et al. (1965) and Bradbury (1973).

A third focus of keratin research pertains to the mechanical properties of wool fibers and feathers (Bonser and Purslow 1995, Wortman and Zahn 1994). The mechanical properties of wool have been studied by the textile, clothing, and hairdressing industries, which are concerned with the processes that occur as the fiber is stretched, set, and supercontracted. The physical alterations caused by the stress of stretching result in a conversion from the alpha-helix conformation to the beta-pleated sheet conformation, a change in the secondary structure of the protein. The supercontraction of a fiber is the shrinkage that takes place when it is stretched through setting, then steamed again and allowed to contract. This phenomenon differs from the contraction that occurs when a fiber is stretched and allowed to return to its original state without additional treatment. The effects on the measurement of these three mechanical properties when the fiber is soaked in water has been examined also (Bradbury 1973, Crewther et al. 1965).

The fourth series of studies involves protein sequencing and has been a primary focus of researchers at CSIRO. By 1991, 28 of the more than 100 proteins in wool fibers had been sequenced, encompassing about 5,000 amino-acid molecules. This represents an estimated 60-80% of the protein content of wool based on weight (Dowling and Sparrow 1991). With advances in genome mapping, however, "it is likely that any further wool protein sequences will be determined via DNA technology" (Dowling and Sparrow 1991) and will not involve sequencing the protein directly by the sequential removal of amino acid molecules.

Some studies of protein deterioration have been conducted that relate to keratin, but have not dealt with protein deterioration specifically, and therefore their relevance is unknown (Whitaker and Fujimaki 1980). Proteins can undergo hydrolysis (with water or water vapor), catalyzed by small amounts of either acid or

base. Amino acid amide groups and disulfide bonds are susceptible to attack under alkaline (higher pH) conditions. Alkaline conditions also can cause amino acid racemization, elimination reactions, the formation of new, destabilizing products such as lysinoalanine, and the formation of degradation products such as dehydroalanine. In addition, the disulfide bonds of cystine (an amino acid composed of two cysteine molecules bonded across their respective sulfur atoms) are susceptible to reduction, whereas both the S-methyl group of methionine and the thiol group of cysteine are susceptible to oxidation (Whitaker and Fujimaki 1980).

Keratin can be completely denatured and solubilized with urea and mercaptoethanol (Means and Feeney 1971), but this method may progress beyond dissolution, break peptide bonds, and destroy important chemical information. A more gentle method of solubilizing keratin while retaining more of its integrity involves breaking the disulfide bonds by either oxidation or reduction reactions. If left alone however, the disulfide bonds will regenerate themselves but in different ways. By converting the reactive sulfur atoms to thiols or sulfonyls, recreation of the disulfide bridges can be prevented. It is therefore common practice to oxidize or reduce the bond and then alkylate it. This solubilizes the protein, allowing its preparation for analysis, in addition to retaining as much chemical information as possible.

MATERIALS AND METHODS

Samples of feathers were collected primarily as molts from captive parrots and parakeets, supplied by a local pet owner. Samples of hair for the heating experiments were taken fresh from the tail of a horse quartered at a local stable, and from a dog, cat, guinea pig, and rabbit from a local veterinarian. Volunteers supplied human hair.

For the "wet" samples, approximately 1 mg of hair and feather was cut, weighed, and placed into 10 × 150 mm Pyrex® test tubes that contained 1 ml of solution. The "dry" samples were placed into empty tubes. Simulated storage solutions were formulated from 70% grain (ethyl) alcohol that was diluted from material purchased as a 95% solution from a local liquor store. Grain alcohol was chosen, because it contains no stabilizers, in contrast to laboratory alcohol. Solutions of ethanol and formalin were made by adding 70% ethanol to 1 ml of a 37% formaldehyde solution to a final volume of 100 ml of solution. Tubes containing the samples and solutions then were sealed in a flame and placed into a laboratory oven maintained at 180 ± 1°C. At specified time intervals, the tubes were removed from the oven, opened, and any remaining solid sample removed, dried, and weighed to determine weight loss during the experiment. The liquid was dried in the tube over silica gel under vacuum. The dried liquid and their respective hair or feather sample then were sealed separately in tubes containing 200 µl of 6 N hydrochloric acid, flushed three times with nitrogen to remove any oxygen and prevent sample oxidation, and then heated for 20 hr at 100°C to hydrolyze proteins and peptides to amino acids. The hydrolyzed samples then were dried over silica gel under vacuum and placed into a sample vial containing 0.25 M sodium citrate solution at pH 3.2, prior to introduction into the analyzer.

Amino-acid analysis was conducted on a specially constructed high-pressure liquid chromatograph, similar to the one described by Benson and Hare (1975) and by von Endt (1994) in its current modified form. Briefly stated, a 2-mm

internal diameter (i.d.) \times 150-mm long stainless-steel column filled with 3 μ m diameter cation exchange resin beads was connected to a series of buffer chambers via high-pressure tubing and a high-pressure piston pump. The chambers were maintained under 1.7×10^5 pascals (25 psi) helium pressure. The pump drew buffers from the chambers in a timed sequence as determined by a sample changer/controller. Four of the separating buffers consisted of 0.25 M sodium citrate of differing pH, each containing 1 g/L of ethylenediaminetetraacetic acid. The final buffer was a 0.2 M boric acid solution containing 1 g/L sodium chloride. Buffers entered the column in a sequence of ascending pH: 3.25, 4.5, 6.5, and 10. The column effluent then was mixed with a stream of *O*-phthalaldehyde (OPA) and 2-mercaptoethanol as the detecting reagent. *O*-Phthalaldehyde reacts with amino acids containing primary amines (most amino acids) to produce an OPA/mercaptoethanol/amino-acid product that fluoresces under UV light. The fluorescence is detected, acquired, and recorded by a detector and data system. Two common amino acids contain secondary amines in a ring structure (proline and hydroxyproline) but do not react under these conditions, and are not detected.

RESULTS AND DISCUSSION

Modern hair from four mammalian species (human, dog, cat, and guinea pig) and feathers from two individuals of the same species of bird were examined for their relative amino-acid content using an amino-acid analyzer. The chromatograms of seven selected amino acids from each analysis, and a standard for comparison, are presented below in Figure 1A-F. The sample of feather keratin (Fig. 1B) differs from the group of mammalian hair samples, because it contains relatively greater proportions of aspartic acid (amino-acid peak 1), serine (peak 3), glycine (5), and valine (7). There are also noticeable differences in the amino-acid content of the mammalian hairs themselves. Human hair (Fig. 1E) is most closely related in amino-acid content to that from the dog (Fig. 1F) (this observation is not meant to imply a close phylogenetic linkage), but differs from it in that the amounts of serine (3) and glutamic acid (4) are more nearly equal to each other in the dog hair than in the human hair. Also, the dog hair contains slightly more glycine (5), relative to alanine (6), than does human hair, whereas the human hair contains slightly more serine (2), relative to aspartic acid (1), than does the dog. The guinea pig (Fig. 1C) and cat (1D) differ from each other especially in their relative amounts of serine (peak 3), glutamic acid (peak 4), and glycine (peak 5). Glutamic acid is present in greater amount than is serine in the guinea pig chromatogram; this chromatogram is unique in that regard. Although cat hair exhibits the same general serine/glutamic acid relationship, as do the mammals other than guinea pig, the relatively large amount of glycine (5) in cat hair helps set it apart from the other mammals. In summary, the mammalian hairs and feathers analyzed for this study, and the taxa they represent, exhibit amino-acid patterns that seem to allow each taxon sampled to be distinguished from the others.

In an early study, Darkus and Gillespie (1971) used electrophoretic data to distinguish among four genera of sheep and goats, but were not able to distinguish among individual breeds of sheep using this technique. Hrdy and Baden (1973) also indicated that there were no differences in the electrophoretic patterns of human hair they examined from six areas of the world, but they reported that

differences did exist at the family level for nonhuman primates. Amino-acid analysis also was unable to distinguish among the human hairs (no amino-acid data was presented for the nonhuman primates). Gillespie and Frenkel (1974) and Gillespie and Marshall (1977) noted, however, that the concentration of various amino acids did differ in echidna claw and quill, ox hair, horn, and hoof, and rabbit hair and claw, indicating that the potential does exist for using amino-acid analysis to distinguish among taxa.

The chromatographic results of each sample also can be adjusted to account for differences in detectability ("color yield") of the individual amino acids illustrated in the chromatogram of the standard (Fig. 1A). When this correction is made, a numerical value can be assigned to each amino acid in each sample, and the data can be compared in a quantitative manner. The results from treating the analyses in this manner are summarized below in Table 1. Here, each amino acid was normalized to glutamic acid (GLU), so that the values presented in the table are ratios with respect to GLU.

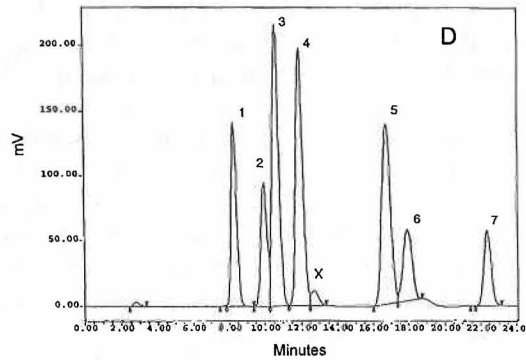
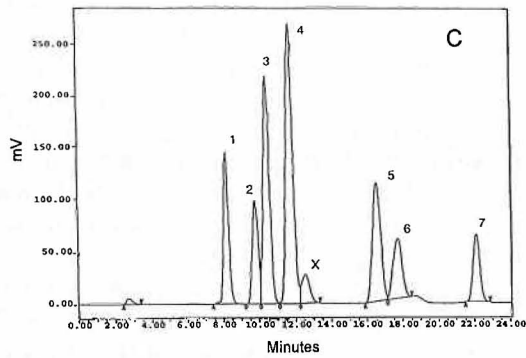
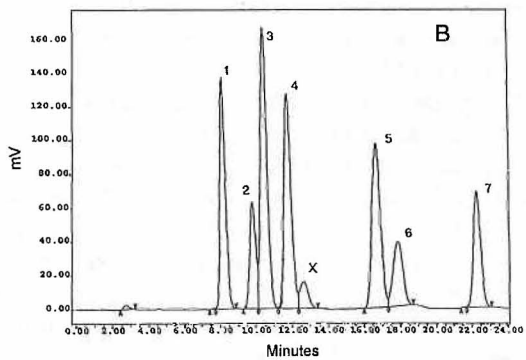
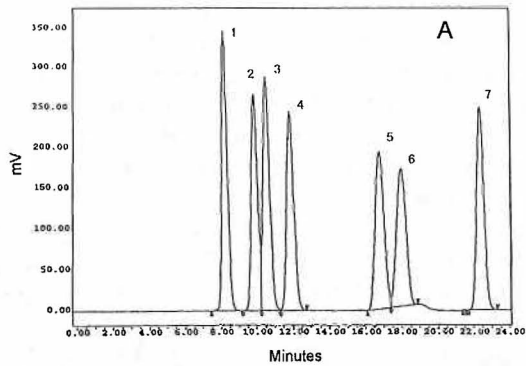
Examination of the amino-acid content of the hair and feather samples summarized in Table 1 reveals differences in the relative proportions of amino acids, as seen visually in Figure 1B–F. In addition, the contrasting relative amounts of the amino acids imply the presence of different keratin proteins in the feathers and hair and suggest that the chemical reactivity and hence decomposition may also be dissimilar and require different conditions for optimum storage.

Aging experiments simulating fluid storage, and using a single temperature, indicate that dramatic differences can be found in the stability of keratin heated in different fluids, and that an additional series of aging experiments using different temperatures and storage conditions are warranted to further explore the stability of keratin.

Figures 2–4, below, illustrate the stability differences of keratin heated in simulated storage fluids. In each of the figures, the upper chromatogram is an analysis of a sample of modern keratin (hair or feather). The lower part of each figure is a chromatogram of a portion of the modern sample artificially aged at 180°C and 24 hr either in 70% ethanol, or in 70% ethanol + 1% formalin. Further, each illustrated analysis represents the same original weight of sample, so that the results may be compared directly to one another.

Figure 2 illustrates the difference in amino-acid content between untreated hair (the upper portion of the figure) and hair heated at 180°C in 70% ethanol for 1 day (the lower portion of Fig. 2). First, notice that the millivolt scale on the left side of the figure indicates that the "treated" hair has lost about half of its amino acids. (Peak height [millivolt] readings can be used for comparison in this case because the samples are being compared in a more descriptive, semi-quantitative manner, as well as having been prepared using equal protein concentrations. Normally, quantification involves a comparison of the integrated area of each peak.) Figure 2 also indicates that the amino acids labeled A and B have disappeared in the heated sample, a new amino acid labeled C has appeared, and the proportions of all the amino acids have changed. Finally, the amount of ammonia (labeled D) in the "treated" hair has increased, indicating increased deterioration. Other changes can be seen in this area of the chromatogram, but represent other, unknown deterioration products.

The results of heating hair in the presence of a small amount (1%) of formalin



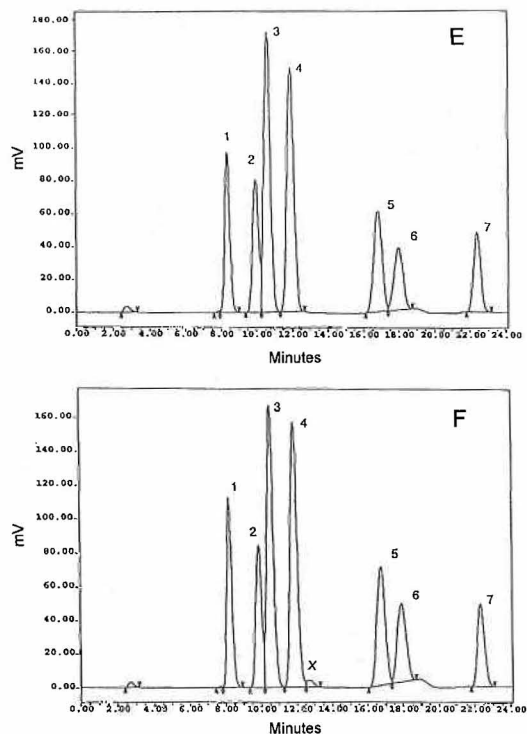


Figure 1. Continued.

in ethanol (to simulate the amount that might be found in fixed specimens) are illustrated in the lower portion of Figure 3 and can be compared to the analysis of the untreated sample above it. In Figure 3, virtually all amino acids have disappeared in the treated specimen, and the millivolt scales indicate that only about 10% of the hair remains. Figure 4 indicates that feather also is not stable when stored in ethanol. The millivolt scales show that feather heated in 70% ethanol alone has lost well over 50% of its amino acids (compared to hair in Fig. 1 that lost about 50%).

CONCLUSIONS AND FUTURE DIRECTIONS

On the basis of these data, a series of conclusions can be drawn. First, note that the keratins are unstable under artificial aging conditions using high temperatures. What is of interest here is the ability to compare the stability of keratin from different sources within a reasonable length of time. If one assumes that the reactions seen at these higher temperatures are the same general type that can be

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Figure 1. All parts of this figure are chromatograms of seven amino acids generated by an amino acid analyzer. Peak 1 = aspartic acid, 2 = threonine, 3 = serine, 4 = glutamic acid, 5 = glycine, 6 = alanine, 7 = valine, and X = an unknown. Part A of the figure represents a standard mix of amino acids used for identification and quantification. Part B represents a parrot feather, C is guinea pig hair, D is cat hair, E is human hair, and part F, dog hair.

Table 1. Selected amino acids in keratin from different sources normalized to glutamic acid.

Source	Amino acids							
	ASP	THR	SER	GLU	GLY	ALA	ILE	LEU
Feather A	1.44	0.62	1.81	1.00	0.37	0.29	0.12	0.46
Feather B	1.09	0.50	1.31	1.00	0.78	0.31	0.19	0.31
Carmen	0.68	0.55	1.07	1.00	0.36	0.26	0.09	0.26
Wendy	0.64	0.54	1.14	1.00	0.42	0.26	0.12	0.25
Horse	0.81	0.41	1.02	1.00	0.37	0.29	0.12	0.46
Cat	0.70	0.47	1.08	1.00	0.70	0.30	0.09	0.23
Guinea pig	0.54	0.36	0.80	1.00	0.43	0.25	0.08	0.21

ASP, aspartic acid; THR, threonine; SER, serine; GLU, glutamic acid; GLY, glycine; ALA, alanine; ILE, isoleucine; LEU, leucine.

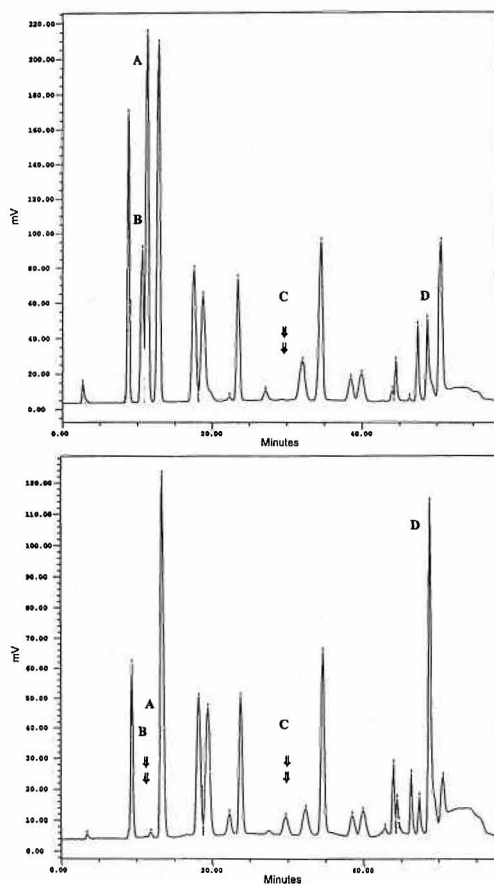


Figure 2. An amino-acid analysis of modern hair (upper part of the figure). The lower portion is hair that has been heated in 70% ethanol at 180°C for 24 hr.

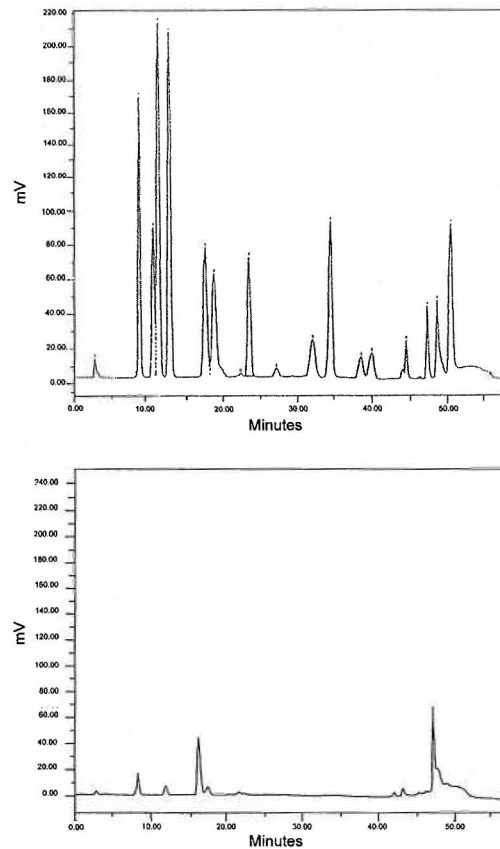


Figure 3. An amino-acid analysis of modern hair (upper part of the figure) and hair heated at 180°C in 70% ethanol and 1% formalin for 24 hr.

observed at lower (room) temperature, then reasonable inferences can be drawn from these aging experiments and projected to museum conditions. One also can note that feather is less stable than hair.

When keratins are "stored dry" they last longest. They last less long in 70% ethanol, and least long in 70% ethanol + 1% formalin. These observations imply that even small amounts of formalin promote deterioration reactions in the keratins, seen especially dramatically under the experimental conditions described above. Further, under these experimental conditions, the difference between "longest" and "least long," as seen in the chromatographic data is several orders of magnitude. The chromatographic and tabular data also indicate that keratins are compositionally distinguishable among taxa.

By comparing the rate of change in amino acids at several temperatures under several "storage conditions," such as dry, 70% ethanol, and 70% ethanol plus 1% formalin, it should be possible to assign a numerical value to a rate of change that can be related to storage temperature as well as storage medium. This information then can be used to predict an expected "lifetime" of keratin under specific conditions of storage at a specific temperature. These data then can be com-

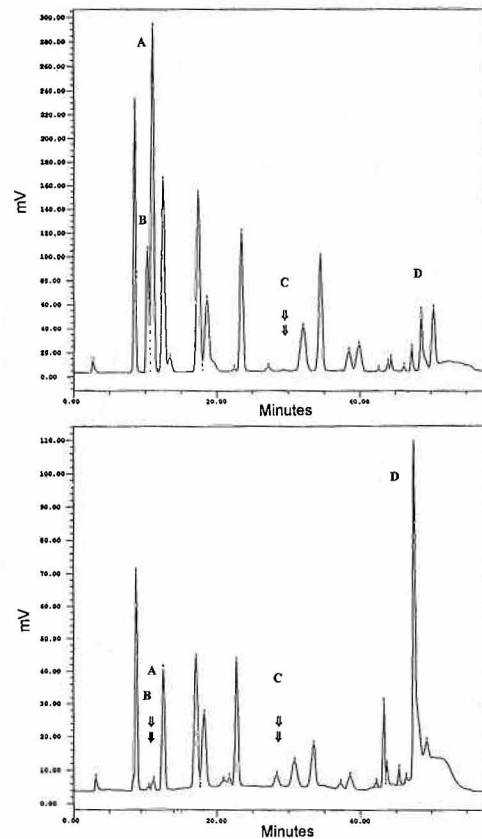


Figure 4. An amino acid analysis of modern feather (upper part of the figure) and feather heated in 70% ethanol at 180°C for 24 hr.

pared to that from actual museum specimens, and lead us not only to a better understanding of the stability of keratin in natural history museums, but also the data can be used to adjust museum storage conditions to ensure maximum specimen lifetime and utility.

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