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A New Assay for Quantifying Pentosidine Levels in Avian Species

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A NEW ASSAY FOR QUANTIFYING PENTOSIDINE LEVELS IN AVIAN SPECIES

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Abstract

In previous studies, a protein crosslink called pentosidine was found to increase linearly with age in bird species. The assay used was too costly and time consuming to be considered practical. The goal of our study was to develop a new assay to determine pentosidine concentration in avian species. Skin samples were harvested from loons of known and unknown age as well as from five roosters that were all five months of age. The assay developed utilized total protein extraction with a buffer to remove collagen from the skin samples, fluorometry, and Beer's Law to determine the concentration of pentosidine in collagen.

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Introduction

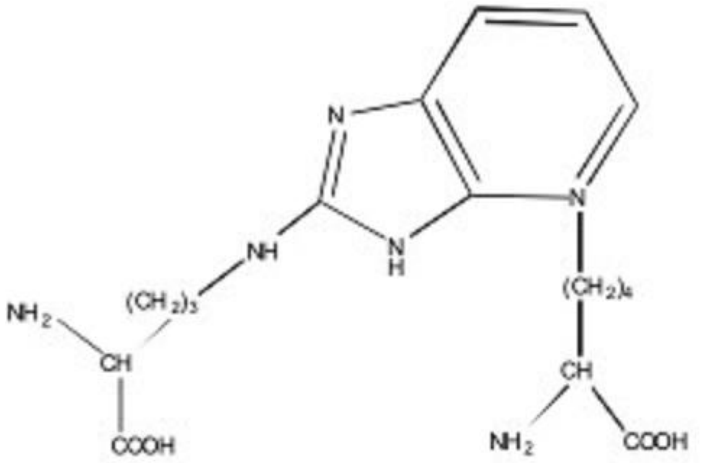
In mammals, it has always been possible to determine age by observing progressive changes in dentition over time. For birds, however, there are no outward physical attributes that can be assessed to accurately approximate age. Once full plumage has been achieved, it is virtually impossible to distinguish the age of one adult from another. When birds arrive at a rehabilitation center for treatment, it is important for researchers and veterinarians to know whether a patient is dying from old age or from some illness. It is imperative to have this information due to the limited resources available to wildlife clinics. Determining a patient's age can help rehabilitators decide how far to proceed with potentially expensive treatments. As such, it is necessary for researchers to have an assay at their disposal to determine the age of birds arriving at their clinic.

The goal of this project was to create a new assay for determining pentosidine concentration in collagen samples collected from avian species. Due to the fact that this compound has been found to increase linearly with age in birds (Iqbal et al. 1997), it was thought that pentosidine levels in the skin of test subjects could be used to determine their level of maturity. In previous studies, the assay used was both time-consuming and expensive due to the use of HPLC and hydrolysis. In this study, it was hypothesized that the length of time and expense of the assay could be reduced by using total protein extraction and fluorometry to completely bypass HPLC and hydrolysis.

Formation of Pentosidine

Pentosidine is a protein crosslink, formed by the non-enzymatic addition of glucose to protein, which is comprised of a lysine and an arginine residue that are linked by a pentose (see **Diagram 1**).

Diagram 1: Pentosidine Molecule (Hatfield et al. 2009)

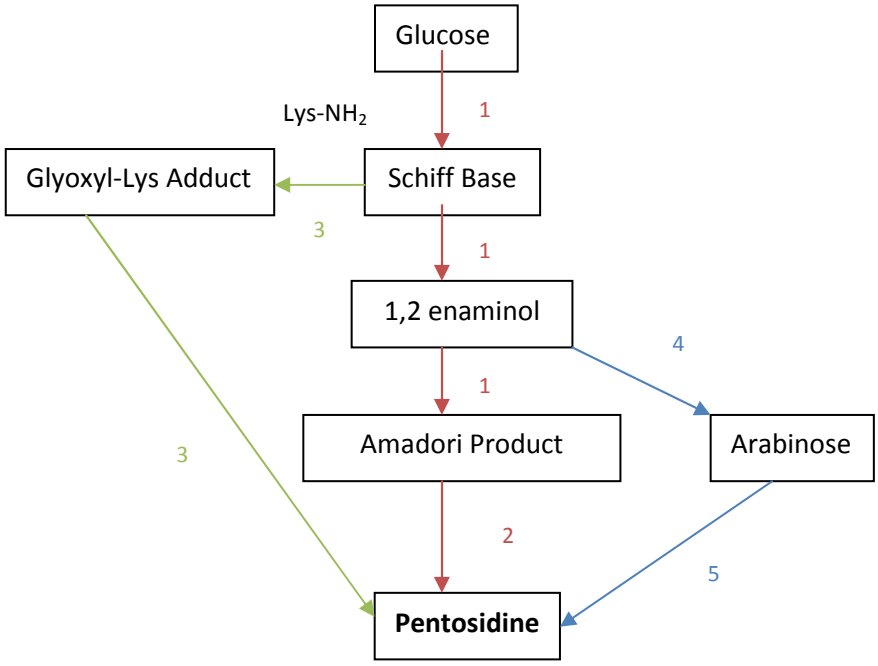


It has been found to form *in vitro* by the reaction of ribose with lysine and arginine (Grandhee et al. 1991). This crosslink has measurable fluorescent properties and is found only in collagen. The process of forming crosslinks marks progressive linear changes in the extracellular matrix during the aging process *in vivo* and these changes have been shown to cause decreased solubility and proteolytic digestibility, increased heat denaturation time, and accumulation of yellow and fluorescent material *in vitro* (Sell et al. 1989).

Pentosidine is a member of a select family of molecules known as advanced glycation endproducts (AGEs). The formation of AGEs, in which there is a chemical reaction between amino-containing compounds and the carbonyl group of reducing sugars, is known as the Maillard reaction. In recent years, the Maillard reaction has been increasingly studied *in vivo* and

has come to be known as glycation under *in vivo* conditions. As can be seen from **Diagram 2** below, Cho et al. (2007) have distinguished the formation of pentosidine in five stages during the process of glycation. These stages led to the discovery of three possible pathways for pentosidine synthesis. In the first pathway, pentosidine formation is initiated at stage (1) with the formation of a Schiff base. The Schiff base then undergoes Amadori rearrangement to form an Amadori product. In the second stage (2) of the first pathway a series of chemical reactions take place, which lead to the formation of several AGEs, including pentosidine. The second pathway begins with the third stage (3), which involves Schiff base degradation to produce glyoxyl and glyoxyl-protein adducts. These adducts can then be converted to pentosidine, completely bypassing Amadori rearrangement. The third pathway begins with stage four (4) and involves the formation of arabinose. The last stage (5) describes the formation of pentosidine from arabinose, which was generated in stage four (4) (Cho et al. 2007).

Diagram 2: Formation of Pentosidine and the Maillard Reaction (Cho et al. 2007)



Key: Red = Pathway 1; Green = Pathway 2; Blue = Pathway 3

Human Applications of Pentosidine

Glycation has been implicated in the development of several age and diabetes related pathologies that are the cause of nephropathy, neuropathy, retinopathy, and sclerosis (Cho et al. 2007). It has been determined that glycation leads to the formation and accumulation of AGEs. Due to crosslinking, AGE-modified matrix proteins behave abnormally with respect to other matrix components and cell surface receptors in that they cause greater permeability of the basement membrane to macromolecules (Cho et al. 2007). As a result, the interaction between AGE-modified plasma proteins and AGE-specific receptors on macrophages induces receptor-mediated production of reactive oxygen species. This then leads to increased oxidative stress in biological systems (Cho et al. 2007). In a study by Hartog et al. (2007) it was postulated that increased AGEs in dialysis patients might play a role in the development of a condition known as

diastolic dysfunction, which describes a problem in the way the heart fills with blood between beats. The data obtained indicated that, although plasma AGEs were not significantly associated with diastolic function, accumulation of tissue AGEs strongly correlated with the development of diastolic dysfunction.

AGE accumulation in tissue has also been implicated in the progression of chronic, age-related diseases such as atherosclerosis, chronic renal failure, Alzheimer's disease, and diabetes mellitus (Meerwaldt et al. 2004). A study by Meerwaldt et al. (2004) investigated the correlation between pentosidine levels and diabetes. It revealed that skin autofluorescence, due to the presence of pentosidine, was 25% higher in Type I diabetes patients than in people without the disease. The study also found that skin autofluorescence was higher in patients with Type II diabetes than in those with Type I, indicating greater oxidative stress, and thus AGE-accumulation, in Type II patients (Meerwaldt et al. 2004).

Pentosidine in Birds as a Marker for Age

As previously stated, pentosidine is the result of a process called glycation in which proteins are non-enzymatically modified by reducing sugars. The pentosidine molecule is irreversibly synthesized in the body and its formation is an unavoidable outcome of aging (Fallon et al. 2006). Due to the fact that pentosidine accumulates with age, it is a good biomarker of aging in mammals and, more recently, it has been utilized in determining age in avian species (Iqbal et al. 1997). In a study by Iqbal et al. (1997) collagen pentosidine levels in domestic chickens (*Gallus gallus)* were found to increase linearly with age. Following this study, other wild bird species (*Larus calfornicus, Coracias caudata, and Carduellis cucullata)* were found to have similar accumulation patterns, confirming that pentosidine is a reliable biomarker for aging (Chaney et al. 2003).

Because there is no outward anatomical attribute in avian species that can be used to reliably predict age, it is important to establish an assay for an age biomarker in wild birds to help provide more insight into their life cycles and population fluxes. According to Chaney et al., a "reliable estimation of a bird's age could play a critical role in species' survival plans and pairing of endangered species" (2003). Developing a method for measuring pentosidine in birds will benefit both conservation and rehabilitation. Wildlife clinics, such as the one at Cummings School of Veterinary Medicine at Tufts University, perform necropsies to determine the cause of death of many wild bird species. In these cases, it is important for researchers to know how old a particular specimen is in order to determine if illness, toxicity, or simply old age was the cause of death.

Pentosidine Stability and Localization in Tissues

Pentosidine has been found to be stable in both mammals and avian species. In a study by Versijl et al. (2000), it was discovered that, in humans, advanced glycation endproducts, such as pentosidine, have half-lives of 15 years in skin collagen. Fallon et al. (2006) proved that, for at least one year, pentosidine levels do not change in bird skins, and researchers concluded that pentosidine may remain stable in skin samples much longer than a year with proper preservation and storage techniques. This also suggests that pentosidine concentration can be accurately assessed at the time of death as long as a specimen is preserved fairly soon after dying.

In studies by Chaney et al. (2003) and Fallon et al. (2006), pentosodine levels were shown to fluctuate in certain areas of the same bird specimen. Higher levels of pentosidine were found in the wing and thigh than in the upper and lower back, abdomen, and breast (Fallon et al. 2006). It was also discovered that pentosidine levels in the webbing of the foot are lower than in the skin of the breast (Chaney et al. 2003). This fluctuation could be attributed to imperfections

in experimental technique but other reasons, such as differences in vascularity, relative temperature, amount of collagen deposition in different tissues, and extraction technique, may be the cause of this difference. Fallon et al. (2006) suggest that there could be a variation in the rates of collagen turnover or concentrations of tissue antioxidants due to these differences.

Experimental Technique

The purpose of this project was to develop a quick and inexpensive assay to determine pentosidine levels as an indication of age in birds, specifically loons, arriving at Cummings School of Veterinary Medicine at Tufts University for necropsy. The experimental protocol used for collagen extraction in previous studies was time-consuming and costly. The procedure involved multiple extractions from the skin by hydrolysis using either special Teflon-lined tubes or amperes that were left in a high concentration of hydrochloric acid for about 20 hours at a high temperature followed by HPLC. Because hydrolysis had to be performed twice, once to extract the collagen and once to determine how much collagen was in the sample, the entire process took about a week to complete.

The protocol in the original experiments took far too long and was far too expensive to be practical for any wildlife clinic run solely on public donations. With this in mind, new techniques were researched that could be used in developing the new procedure. In the end, an assay was developed that took only two days to perform and bypassed both hydrolysis and HPLC. Instead of hydrolysis, RIPA (Radio-Immunoprecipitation Assay) buffer was used to extract collagen. It worked by lysing the cells and breaking down tissue, thus allowing for the release and solubilization of all proteins, including collagen. RIPA buffer extracted collagen samples were read in a fluorometer and the values were used to determine pentosidine concentration in each sample. A modified Sircol^{TM} Soluble Collagen Assay was used to

establish how much collagen was in each sample. For this assay, Direct Red 80 dye was utilized and bound to a specific site on the collagen molecules. Once the dye had bound, an alkali reagent was added in order to bring the dye-bound molecules back into solution and the absorbance (540nm) was read. In order to validate the accuracy of this experimental protocol, skin was harvested from five young roosters of the same age and assayed. Three skin samples were excised and assayed from the same loon (loon #6) to prove that the assay results could be reproduced.

This study was expected to prove that the new procedure described above would be a viable assay for determining pentosidine concentration in avian species. If the assay was successful, it was thought that the five roosters of the same age would have similar pentosidine levels, and that the three trials run on loon #6 would have the same concentration in comparison to each other. From the results gathered, it was concluded that this assay might be a viable method for determining protein concentration in avian species, but that further testing must be done on a larger population of test samples to determine the true statistical significance of the assay.

Materials and Methods

Skin Harvesting

In this study, both loon and rooster skin was used. Between 10 and 15 grams of loon skin were harvested per bird from the upper abdomen during necropsy at Cummings School of Veterinary Medicine at Tufts University. In order to remove the skin, either a 10 blade scalpel or surgical scissors were used. The skin was then frozen in a -80^{o}C freezer under saline until used. After thawing, the feathers were removed by scraping with a 10 blade scalpel. Five roosters of the same age (five months old), which were previously slaughtered and plucked by scalding and

an automated plucking machine, were also used in this study. Between two to four grams of skin from the abdomen were taken from each rooster to be used in the experiment. Because the roosters were kept in a freezer in the lab at Worcester Polytechnic Institute, not having enough skin was not a concern. More skin was taken from the loons because the skin was collected at Tufts Veterinary School in Grafton, Massachusetts and the loons were disposed of after the necropsy. Therefore, ample amount of skin was collected to make sure that there would be enough during the experiment.

In addition to the scraping method of removing the feathers, a scalding and plucking method was attempted. Because simply scraping the skin and feathers with a scalpel leaves some of the feather shafts behind, another method of removing feathers was sought. Initially, plucking the feathers from the bird during necropsy was attempted. However, this proved to be an extremely arduous and time-consuming task. In order to pluck feathers from birds, the entire bird is generally scalded in very hot water (about 70-80^{0}C) for about 1 minute. While at Cummings School of Veterinary Medicine at Tufts University, it was not possible to put the entire loon into a large vat of hot water. Therefore, the pieces of skin were thawed to room temperature at Worcester Polytechnic Institute and placed in simmering hot water for about 10 seconds. Because it was still difficult to pluck the feathers, the same piece of skin was placed in boiling water for about 10 seconds. The feathers did not release any easier, but as many feathers as possible were plucked and that piece of skin still underwent the remaining procedure to fluorometry in duplicate. However, no peaks were obtained in the fluorometer and it was decided to continue with the manual removal of feathers with a scalpel.

Creating Powdered Skin Samples

The skin harvested from both loons and roosters was minced into smaller pieces with scissors and placed in a 50ml Waring blender. Liquid nitrogen was poured over the skin and the samples were blended until they became powder-like in consistency. One gram of each sample was then weighed out and put into individually labeled 15mL conical tubes. This was done in duplicate.

Prior to determining the final method for powdering the skin samples as described above, three other methods were attempted. Originally, the skin was "minced" with a razor blade. At room temperature, it was very difficult to obtain tiny pieces of skin. A combination of surgical scissors and a razor blade was tried, but still did not produce small enough pieces. According to Chaney et al. (2003), freezing skin samples under dry ice and mincing them with a razor blade produced small enough pieces to perform the assay. In accordance with this discovery, a skin sample was dipped in liquid nitrogen and mincing was attempted. However, this made mincing even harder because the razor blade was not able to penetrate the frozen piece of skin.

Two more methods were tried before the final powdering protocol was determined. First, the skin was placed in three layers of aluminum foil to form a packet and dipped in liquid nitrogen for several seconds. The packet was hit a few times with a rubber mallet hoping to crush the skin into a powder like consistency. Unfortunately, only flat pieces of skin were obtained. Next, a small amount of skin was placed in liquid nitrogen until thoroughly frozen. The skin was placed in frozen mortar and a cold pestle was used to try and crush the skin into a fine powder. The skin would not break up into smaller pieces or crush into a powder. It was decided that the best method to produce powdered skin samples was the use of liquid nitrogen and a Waring blender.

Delipidation

After blending, each sample was dilipidated in 4mL of chloroform/methanol (2:1) with mild shaking in a Mistral Multi-Mixer mechanical shaker for 18 to 22 hours at room temperature. It was discovered that, after 18 to 22 hours of exposure to chloroform methanol (2:1), the 15ml conical tubes containing the samples became brittle and ran the risk of breaking in the centrifuge. As a result, the samples were transferred to new 15mL conical tubes before centrifugation. These tubes were then spun for 10 minutes at 1453 x g and the supernatant was removed, leaving only the delipidated skin pellet.

This form of delipidation was used in all journal articles relating pentosidine to bird age (Chaney et al. 2003, Fallon et al. 2006, Iqbal et al. 1997) and it worked well from the beginning of the study. A few weeks into the project, it was discovered that Cliche et al. (2003) removed the fat from chicken skins by heating homogenized skin at 40^{o} C for 1 hour. Therefore, this method of delipidation was attempted. To observe the necessity of having a delipidation step, one sample of skin was simply powdered and not delipidated. These different attempts of delipidation were done in duplicate with the same loon skin sample. On the heated samples of skin, a small layer of fat was removed at the top of the pellet after spinning in the centrifuge.

After the total protein extraction step (described in the next section), the supernatants of each delipidation were different colors. The chloroform:methanol delipidation produced a clear supernatant, while the heat delipidation produced a yellow, cloudy supernatant. Additionally, no delipidation produced a dark brown, cloudy supernatant. All three supernatants were read in the fluorometer and only the chloroform:methanol delipidation produced peaks in the area of pentosidine. It was decided that this form of delipidation was the best method to use.

Total Protein Extraction

The total protein extraction in this study utilized RIPA (Radio-Immunoprecipitation Assay) buffer. The RIPA buffer used for this assay contained 1% Nonidet-P40, 0.2% of SDS, and 49.5mL of 10mM TRIS buffer at pH 7.4 and was stored at 4^0 C. Phosphatase and protease inhibitors were not needed in this assay because the goal was to break apart the skin and release as much collagen as possible into the supernatant. Degradation of proteins was not a concern. The first extraction required 4mL of RIPA buffer to be added to each delipidated skin sample. The samples were vortexed and left at room temperature for one hour and spun in a centrifuge at 1453 x g for 10 minutes. The supernatant was then transferred to a second conical tube containing the second gram of delipidated skin. The duplicate then underwent the same extraction as the first gram of skin. The supernatant from this sample was used in the remaining procedure as described in the following sections.

Before deciding on the above protocol for this study, one other attempt at extracting the collagen was made and a few changes in the RIPA buffer extraction were instituted. Using the method by Cliche et al. (2003), collagen extraction from the skin of chickens was attempted using acetic acid and pepsin. First, the skin was delipidated with chloroform:methanol as stated above. The skin pellet was homogenized with an Omni polytron in distilled water for about 1 minute. Unfortunately, the polytron did not work well and the skin could not be completely homogenized. The resulting homogenate was centrifuged at 1453 x g for 10 minutes. After removing the supernatant, 3ml of 0.5 M Acetic acid at pH 2.5 was mixed with the pellet for 24 hours at room temperature. A second extraction was done using 3ml of 0.5 M acetic acid at pH 2.5 and pepsin at 1mg/ml for 24 hours at room temperature and the supernatant was run in the

fluorometer. The results showed no peaks in the wavelength range of pentosodine and this method was abandoned.

In addition to the extraction techniques attempted above, the effect of leaving the samples at room temperature was tested against leaving them in an ice bath. Using the same sample of loon skin and testing it in duplicate for room temperature versus an ice bath, it was discovered that there was a slight increase in the amount of pentosidine found in each sample for the room temperature tubes. Therefore, it was determined that the RIPA buffer extraction should be performed at room temperature.

Fluorometry

After total protein extraction, the supernatant from each sample was transferred to a new set of 15mL conical tubes. A small amount of a dense, clear, liquid layer formed below the skin and was not included in the supernatant for analysis in the fluorometer. The supernatant was then transferred to fluorometer UV-Vis macro cuvettes and read in a PerkinElmer LS50B Luminescence Spectrophotometer with excitation at 325nm and emission ranging from 300-500nm. If there was no time for analysis in the fluorometer, the supernatant was frozen in a -80^{o}C freezer until it could be read. Both the wavelength and the height of the peak were recorded for each sample. After it was read in the fluorometer, the supernatant was either used right away in the collagen determination portion of the experiment or frozen in a -80^{o}C freezer until needed.

To establish a standard curve of pentosidine, 0.5µmol of pure pentosidine, obtained from Dr. Vincent Monnier of Case Western Reserve University**,** was reconstituted with 100µl of distilled water to yield a 1.89mg/ml solution of pentosidine (MW = 378.43g/mol) in water. A

1:3 serial dilution was then performed 10 times until the lowest concentration of pentosidine in RIPA buffer was 0.032 μg/ml. The concentrations of pentosidine were 630, 210, 70, 23.3, 7.78, 2.6, 0.864, 0.288, 0.096, and 0.032 μg/ml. This dilution series was then read in the fluorometer at the same excitation and emission wavelengths described above for the test samples. While the assay was being developed, it was discovered that the dilution series read virtually the same fluorescence units each time it was run after it was frozen at -80^{0}C and thawed. Therefore, the dilution series was placed in the freezer after the correct dilution series was established.

Collagen Determination

In order to determine how much collagen was in each sample, a slightly modified version of the "Sircol^{TM} Soluble Collagen Assay" (2007) from Biocolor Ltd. was used. Using the instruction manual, each solution needed for the assay was made. The collagen used for the standard curve was made from Type I collagen from rat tail (Sigma) and made into a concentration of 1mg of collagen per mL of 0.5 M acetic acid. The final concentrations in the standard curve for collagen were 200, 100, 40, 20 and 8 μg/ml. The dye reagent was prepared by using Direct Red 80 (Sigma) and by creating a 10mg concentration of Direct Red 80 per mL of distilled water (Lee et. al 1998). The alkali reagent used was 0.5 M NaOH.

100μl of each sample was mixed in a microfuge tube with 1mL of the Direct Red 80. The tubes were placed on a Mistral Multi-mixer mechanical shaker for two hours. Next, the tubes were spun at 15,741 x g in a centrifuge for 15 minutes. The supernatant was carefully removed by first inverting the tubes and emptying out the bulk of the reagent. The dye that was not removed by simply inverting the tube was carefully drawn out with Kim-wipes. It was very important to have the pellet located and carefully hold the Kim-wipe just above it. If the Kim-

wipe touched the pellet, the pellet would detach from the surface and attach to the Kim-wipe, which meant the collagen extraction sample would have to be repeated.

With the excess dye removed, 1ml of alkali reagent was added and the solution was vortexed until the pellet went back into solution. Using a Jenway spectrophotometer, the absorbance of each sample was read in UV-Vis semi micro cuvettes at 540nm. The spectrophotometer was blanked using distilled water and the resulting A_{540} of both the test samples and the standard curve were recorded.

Results

Many different methods of sample preparation were explored in this study. The first of these was comparing two methods for removing feathers from samples (Table 1). The next was discovering the most efficient way to turn samples into a fine powder (Table 2). For the delipidation portion of the protocol, three different methods were attempted (Table 3). Lastly, two different types of collagen extraction were tried (Table 4). It was decided that feather removal by scalpel, powdering by Waring blender/liquid nitrogen, 2:1 chloroform/methanol delipidation, and collagen extraction by RIPA buffer was the best protocol for this study.

A standard curve was constructed for pentosidine using the fluorometer (Table 5, Figure 1) and used to determine pentosidine values for Loon #6 and five young roosters (Table 6 and 7). Similarly, a standard curve was constructed for collagen concentration, using a spectrophotometer set to A_{540} (Table 8, Figure 2), and used to determine the collagen concentrations for Loon #6 and five young roosters (Tables 9 and 10). Lastly, the ratio of pentosidine concentration/collagen concentration was determined for all assayed samples (Table 11).

Mechanics

Two methods for removing feathers from skin sample were explored. The outcomes of each trial are displayed in Table 1. It was determined that removal by scalpel was the best method for cleaning skin samples and was used for all skin samples assayed for the remainder of this study.

Table 1: Comparison of Two Methods for Removing Feathers

Method for Removing Feathers	**Observations**
Scalpel (#10 Blade)	Peaks were obtained on the fluorometer for these samples – this method was chosen as the best for this experiment
Scalding and Plucking	No peaks were obtained on the fluorometer for these samples – this method was not used for the experiment

In addition to feather removal, it was also important to determine the most efficient way to powder skin samples prior to collagen extraction. Several methods were attempted including mincing with scissors/razor blade, a mortar/pestle coupled with liquid nitrogen, aluminum foil/mallet coupled with liquid nitrogen, and a Waring blender coupled with liquid nitrogen. The results of these attempts are displayed in Table 2. It was determined that the use of a Waring blender and liquid nitrogen was the best method for powdering skin and was used on all samples going forward.

Table 2: Method for Powdering Skin

Method for Powdering Skin	Observations
Mincing with Scissors/Razor Blade	Small square sections of skin – not sufficient for extraction
Mortar and Pestle, Liquid Nitrogen	Large, frozen chunks, unable to form a powder – not sufficient for extraction
Aluminum Foil, Mallet, Liquid Nitrogen	Flat, frozen piece of skin – not sufficient for homogenization/extraction
Waring Blender, Liquid Nitrogen	Fine powder – Good for extraction – this method was chosen as the best for this experiment

Delipidation

Three different delipidation conditions (heat at 40^{o} Celsius, 2:1 Chloroform:methanol, and no delipidation) were attempted for skin samples as displayed in Table 3. It was found that the supernatant for each of these methods was different in color and that the only method that yielded peaks on the fluorometer was 2:1 chloroform:methanol delipidation. As a result, it was decided that this type of delipidation would be used on all samples used in the study.

Table 3: Delipidation Methods

Delipidation Method	Observations
Heat at 40^{o} Celsius	supernatant was yellow in color- No peaks were obtained on the fluorometer for these samples
2:1 Chloroform Methanol	supernatant was clear in color- Peaks were obtained on the fluorometer for these samples
None	supernatant was brown and cloudy- No peaks were obtained on the fluorometer for these samples

Collagen Extraction

Table 4 exhibits the two extraction methods explored in this study. The first of these was RIPA buffer extraction and the second was extraction by pepsin and acetic acid. No peaks were produced on the fluorometer for samples extracted with pepsin and acetic acid while peaks were

obtained for samples extracted with RIPA buffer. RIPA buffer extraction was determined to be the best method for this study.

Table 4: Extraction Methods

Extraction Method	Observations
RIPA Buffer	Peaks were obtained on the fluorometer for pentosidine– supernatant was clear in color
pepsin and acetic acid	No peaks were obtained on the fluorometer for pentosidine –supernatant was clear in color

Fluorometry

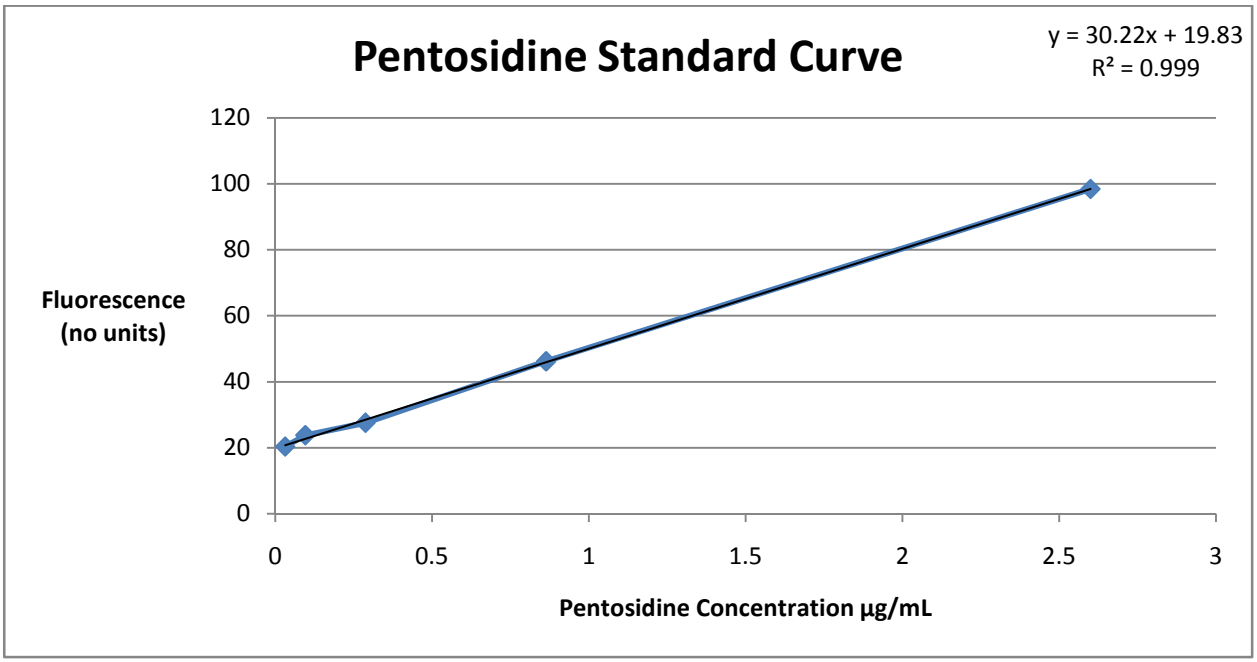
Table 5 shows the fluorescence values for each concentration of pentosidine used in the standard curve. Originally, 1:10 dilutions were made from a concentrated pentosidine sample, but it was discovered that they were not sufficient for creating a standard curve. 1:3 dilutions were then made and were found to produce an acceptable curve with an approximately linear distribution. Though, initially, this curve was repeated each time new samples were read, it was found that the standard pentosidine values were the same each time. As a result, it was decided that the curve could be run once and used for all samples.

Table 5: Standard Curve for Pentosidine

Concentration (µg/ml)	Fluorescence (no units)
630	>1000
210	>1000
70	>1000
23.3	742.4
7.78	276.9
2.60	98.4
0.864	46.2
0.288	27.6
0.096	23.83
0.032	20.41

Figure 1 illustrates the results displayed in Table 1 as a graph. The linear equation obtained for this chart was found to be y = 30223x + 19.83 and the R value was determined at 0.99, indicating a strong linear relationship. Only pentosidine concentrations that gave readings below 100 were used due to the fact that the samples assayed in this study were never higher than that value.

Figure 1: Pentosidine Standard Curve



The sample calculation below (sample calculation 1) illustrates the process for mathematically determining pentosidine concentration for Trial 1 of Loon #6 from Table 6 using the equation obtained from the standard curve in Figure 1.

Sample Calculation 1: Determining Pentosidine Concentration for Trial 1

$y = 30.22x + 19.83$

$56.9 = 30.22x + 19.83$

$56.9 – 19.83 = 30.22x$

$37.07/30.22 = x$

Protein Concentration = 1.22 µg/mL

Three skin samples were harvested from the same loon (Loon #6) and each was assayed using the finalized procedure (2:1 chloroform/methanol delipidation, RIPA buffer extraction, fluorometry) as described in the Materials and Methods. The results obtained from each of these attempts were similar, indicating that this assay is reproducible.

Table 6: Reproducibilty of the Pentosidine Assay

Trial	**Fluorescence (no units)**	**Pentosidine Concentration (µg/mL)**
1	56.9	1.22
2	53.2	1.10
3	45.6	0.85

Skin samples were harvested from five roosters of the same age and assayed using the finalized procedure described in the Materials and Methods. The results for each of two trials performed for the five roosters can be seen in Table 7. All of the roosters had similar fluorescence and pentosidine concentrations between the two trials except for roosters 2 and 3.

Table 7: Fluorescence and Pentosidine Concentration for Five Roosters Reproduced Two Times

Rooster	Fluorescence (no units) Trial 1	Pentosidine Concentration (µg/mL) Trial 1	Fluorescence (no units) Trial 2	Pentosidine Concentration (µg/mL) Trial 2
1	25.5	0.19	24.8	0.16
2	24.6	0.16	36.0	0.54
3	38.4	0.61	28.8	0.29
4	35.9	0.53	31.1	0.37
5	27.6	0.26	29.6	0.32

Collagen Determination

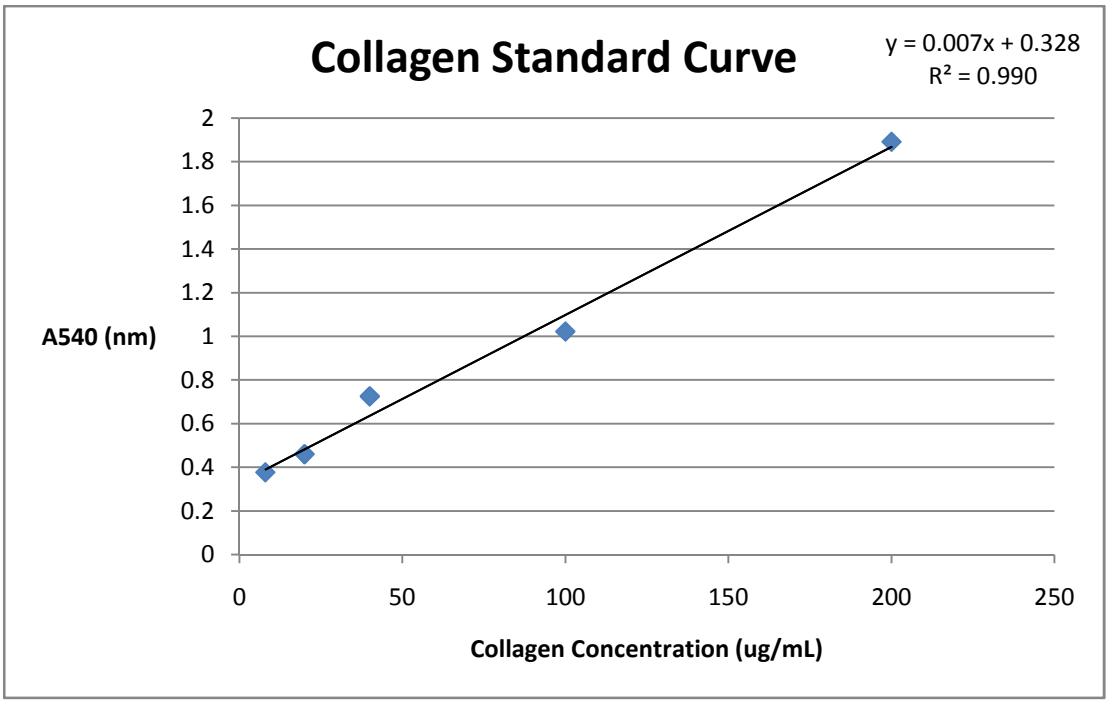
The absorbance (540 nm) for each concentration of collagen used in the standard curve can be seen in Table 8. This curve was attempted several times, however, only one attempt yielded an acceptable curve with an approximately linear distribution. As a result, this curve was the one used to determine collagen concentration.

Table 8: Collagen Standard Curve A_{540}

Concentration (ug/ml)	Absorbance at 540nm
200	1.891
100	1.022
40	0.725
20	0.460
8	0.377

Figure 2 illustrates the graph of the data from Table 8. The linear equation obtained was y = 0.007x + 0.328 and the R value obtained was 0.99, indicating a strong linear relationship.

Figure 2: Collagen Standard Curve



Sample Calculation 2 illustrates how to mathematically determine collagen concentration for Trial 2 of Loon #6 from Table 9 using the standard curve from Figure 2 and the linear equation obtained.

Sample Calculation 2: Determining Collagen Concentration for Trial 2

Y = 0.007x + 0.328

1.991 = 0.007x + 0.328

1.991 – 0.328 = 0.007x

1.663/0.007 = x

Collagen Concentration for Trial 2 = 237.57 ug/mL

The collagen determination results for Loon #6 are displayed in Table 9. Only Trials 2 and 3 were analyzed due to the loss of the sample for Trial 1.

Table 9: Collagen Results for Loon #6

Trial	Absorbance at 540nm	Collagen Concentration (µg/mL)
2	1.991	237.57
3	1.642	187.71

The collagen determination results for two trials of each of the five roosters can be seen in Table 10. The sample for rooster 3 had to be diluted 1:2 on the second trial due to the fact that its A540 was off the scale of the spectrophotometer when diluted. When determining this concentration, the diluted absorbance of 1.384 was entered into the equation and calculated to be 150.86. This was then multiplied by the dilution factor (2) to equal a final concentration of 301.71 µg/mL. A paired t-test was performed on the collagen concentration results for the five roosters and the P-value obtained was found to be 0.1144. Because a paired t-test is usually used to test whether differences in data points for a specific test subject (e.g. before and after a medical treatment) are statistically significant, it was expected that the p-value for this test would be higher than 0.05, indicating that there is no significant difference between each trial and that any variability between samples can be attributed to random chance.

Table 10: Collagen Results for Roosters Attempted Twice

Rooster	A540nm 1st Trial	Collagen Concentration (µg/mL) 1st Trial	A540nm 2nd Trial	Collagen Concentration (µg/mL) 2nd Trial
1	0.793	66.43	0.985	93.86
2	0.708	54.29	1.547	174.14
3	0.595	38.14	1.384 (diluted 1:2)	301.71*
4	1.545	173.86	1.687	194.14
5	1.249	131.57	1.077	107.00

Pentosidine/Collagen Determination

The process for determining the ratio of µg pentosidine/µg collagen for Loon # 6 Trial 2 is illustrated in Sample Calculation 3.

Sample Calculation 3: Determining µg pentosidine/µg collagen for Loon # 6 Trial 2

µg pentosidine = 1.103 µg/mL

µg collagen = 237.57 µg/mL

µg pentosidine per µg collagen $= \frac{1.103 \mu g \text{ pentosidine}}{mL} \; x \; \frac{mL}{237.57 \text{ µg collagen}}$

Ratio = 4.64 x 10^{-3} µg pentosidine/µg collagen

Table 11 displays the ratio of pentosidine to collagen for trials two and three of Loon #6 as well as for each of the two trials performed for each of the five roosters. The ratio is very similar for each of the two loon trials and similar between all of the rooster trials except for rooster # 3. As for the collagen concentrations, a paired t-test was performed for the pentosidine/collagen values for the five roosters and the P-value was found to be 0.903. As explained above, a high value would be expected if the assay had good reproducibility.

Table 11: The ratio of µg pentosidine/µg collagen for Loon #6 and Five Roosters

Sample	Trial	µgPentosidine/ µgCollagen
Loon #6	2	4.64 x 10^{-3}
	3	4.54 x 10^{-3}
Rooster 1	1	2.81 x 10^{-3}
	2	1.75 x 10^{-3}
Rooster 2	1	2.91 x 10^{-3}
	2	3.07 x 10^{-3}
Rooster 3	1	1.61 x 10^{-2}
	2	9.84 x 10^{-4}
Rooster 4	1	3.06 x 10^{-3}
	2	1.92 x 10^{-3}
Rooster 5	1	1.95 x 10^{-3}
	2	3.02 x 10^{-3}

Discussion

Mechanics

In removing feathers from the skin samples, two methods were explored (Table 1). The first involved simply removing as many feathers as possible with a scalpel while the second involved scalding the skin in boiling water and then plucking the feathers. After reading the samples in the fluorometer, peaks were produced for the samples cleaned with a scalpel while none were seen for those cleaned by scalding. It was decided that scalding a small sample essentially cooks the skin, which may cause collagen to become denatured, rendering the protein insoluble. Another possibility is that heat may break down the pentosidine crosslink, resulting in no fluorescence. Therefore, scalding small amounts of skin should not be used to remove feathers from samples. As can be seen from Table 7 in the results section, pentosodine was still easily quantified in the rooster skins, even though they were plucked using a scalding method. It is possible that the process by which a professional slaughter house scalds chickens allows heat to be distributed evenly in such a way that does not denature the collagen. In the future, it may be worthwhile to determine a method for plucking the birds before removing the skin. However, this was attempted unsuccessfully multiple times and requires a great amount of strength to remove all the feathers. Another method that might be useful to try is utilizing a scalpel to remove feathers before excising a skin sample from a specimen of interest. Having the skin still attached to the bird might make the feathers easier to remove because the skin is still tight.

Several methods were explored for turning the harvested skin into a fine powder (Table 2). The first of these was mincing the skin using scissors and/or a razor blade. It was found that the samples could not be minced finely enough to produce a fully homogenized sample in later steps of the procedure. In the second method, a section of skin was placed into a mortar and

doused with liquid nitrogen. A pestle was then employed to crush the skin into a fine powder. Though other studies found this technique to be sufficient, in this experiment, the skin only seemed to shatter into larger pieces that were not conducive to the rest of the experiment. A third method explored involved wrapping skin sections in three layers of aluminum foil, dipping the packet in liquid nitrogen, and then hitting it with a mallet. This technique also did not produce the fine powder necessary for collagen extraction. Instead, it only seemed to flatten the skin. For the last and final method explored, skin samples were ground up in a Waring Blender with liquid nitrogen. This technique was, by far, the most successful and produced the fine powder necessary for the assay. As a result, this was the method used for the rest of the experiment. When using the Waring Blender technique, it was found that there had to be a good balance between the amount of liquid nitrogen and skin. If an excess of liquid nitrogen is used, the skin becomes too frozen and the blades of the blender are not able to rotate. If not enough liquid nitrogen is used, then the skin does not powder, but shreds and wraps around the blades of the blender. Therefore, it takes a process of trial and error to determine how much liquid nitrogen to use for each piece of skin. Furthermore, if a homogenizer is available, then grinding the skin into a fine powder may not be necessary. Merely cutting the skin into smaller pieces and homogenizing the sample with the delipidation solution may be sufficient.

Delipidation

Three methods were explored for delipidation of the samples (Table 3). These were: delipidation with 2:1 chloroform and methanol for 18-22 hours, delipidation with heat at 40^0C for 30 minutes, and no delipidation at all. Heat dilipidation was tested because, according to Cliche et al. (2003), it was possible to delipidate chicken skin with heat for 30 minutes and it was thought that loon skin might be delipidated in the same manner. It was also unclear why

delipidation was necessary in the first place. As a result, testing was performed to see if delipidation could be omitted all together. To test these different forms of delipidation, three assays using tissue from the same loon were run. No fluorescent emissions at or around 380nm with an excitation of 325nm were found for samples that were delipidated with heat or had no delipidation (Table 3). The supernatant for the samples delipidated with heat was yellow in color while those with no delipidation had a brown, cloudy supernatant. The reason why the supernatants were yellow or brown may have been because there was fat left behind, proving that heat delipidation is not sufficient for complete fat removal. Samples with no delipidation might have had the darkest supernatant because they contained the highest concentration of fat. For the samples delipidated with 2:1 chloroform methanol, the supernatant was clear and fluorescent emissions at or around 380nm with an excitation of 325nm were easily obtained. As a result, using chloroform methanol to delipidate the samples was used. It is important to note that when these different delipidations were tested, they all had the same collagen extraction with RIPA buffer. When Cliche et. al (2003) used heat to delipidate chicken skin, they were using a different extraction method with acetic acid and pepsin. It could be that, in conjunction with an acetic acid and pepsin extraction, delipidating with heat would work.

Collagen Extraction

When this study began, using a total protein extraction with RIPA buffer always worked and there was a fluorescent emission at 380nm with an excitation of 325nm. After about six weeks of using RIPA buffer, two journal articles by Nalinanan, et al. (2007 and 2008) were discovered which stated that a combination of pepsin and acetic acid could be used to extract collagen from the skin of fish. Further research proved that pepsin and acetic acid works well for extracting collagen from chicken skin (Cliche et al. 2003). Therefore, this method was tested

with loon skin in conjunction with the 2:1 chloroform/methanol delipidation. This method was tested with the hopes of establishing a better method for extracting as much collagen as possible. Unfortunately, no fluorescent emissions at or around 380nm with an excitation of 325nm were found using this protocol for collagen extraction. There were very wide emissions between 470nm and 500nm, which could not have been pentosidine because it fluoresces around 380nm with an excitation of 325. It could be that another compound, either in the pepsin/acetic acid or in the extract itself, was emitting fluorescence in the 470nm – 500nm range. In any case, what was fluorescing was not pentosidine, indicating that the pepsin/acetic acid method for extracting collagen was not an acceptable method for determining pentosidine concentration in skin.

After discovering that pepsin and acetic acid was not a good method for extracting collagen from bird skin, it was necessary to begin work on perfecting the RIPA buffer extraction. As previously stated, using RIPA buffer in conjunction with chloroform/methanol delipidation always provided a fluorescent emission at 380nm with an excitation of 325nm. Unfortunately, the height of the peak at 380nm was at the lower end of the standard curve for all samples (see tables 2A and 3A in the Appendix). This meant that a way to extract a greater amount of collagen/pentosodine from the samples had to be discovered. A double extraction with RIPA buffer was then attempted and reproduced three times with the same loon (Table 6). As can be seen from Table 6, the double extraction does increase the height of the fluorescent emission at 380nm. In fact, heights increased from about 35 fluorescence units for the single extraction to 55 fluorescence units with the double extraction. Hence, the double extraction using RIPA buffer was the preferred method for extracting collagen.

Fluorometry

After obtaining the pentosidine standard curve, a linear equation of y = 30.22x + 19.83 was established which allowed for the calculation of pentosidine concentration (μg/mL) in samples from their fluorescence (Table 5, Figure 1). In constructing the standard curve, only the standards with values of 98.4 fluorescence units and lower were used due to the fact that none of the samples had fluorescence readings higher than 56.9 fluorescence units. It was discovered that the pentosidine concentrations for the three Loon #6 trials (Table 6) were similar in value while those for the five roosters varied greatly (Table 7). The attempts for roosters 1 and 5 contained about the same amount of pentosidine. However, roosters 2-4 all contained at least a difference of about 0.16μg/ml of pentosidine with the largest difference being 0.38μg/ml of pentosidine. This was not considered a problem because collagen determination was yet to be performed. It was predicted that roosters with higher pentosidine concentrations most likely had samples higher in collagen.

Collagen Determination

As with the fluorometry, a standard curve for collagen was constructed and the resulting linear equation (y = 0.007x + 0.328) was used to find the collagen concentrations (μg/mL) for each of the samples (Table 8, Figure 2). This linear curve was originally intended to be used only for all the loon attempts as well as for the first attempt for the 5 roosters. All of these samples were run on the same day and, ideally, a new linear curve should be created each day that a new sample is read to account for changes in the machine, cuvettes, or slight variations in how the samples are measured. However, a standard curve could not be constructed for the second attempt of the five roosters because the absorbencies did not decrease in a linear fashion. For

instance, the 100µg and 20µg samples had higher absorbencies than the 200µg and 40µg. The standard curve was attempted twice for the second rooster attempt and, in both cases, the absorbencies would not decrease in a linear fashion. This may have happened due to the pellet being disturbed in the dye extraction using the Kim-wipes, or it is possible that not enough Direct Red 80 dye was removed. In the future, the mechanics of this part of the assay might need to be adjusted. If the linear equation used in this study turned out to be incorrect, then this does pose a problem to the results of this assay. It means that the results obtained were not accurate and using the "Sircol™ Soluble Collagen Assay" (2007) from Biocolor Ltd. is not the appropriate assay to determine how much collagen is in skin samples.

A paired t-test was performed on the data obtained for trials 1 and 2 of the five roosters (Table 10). Usually, this type of statistical analysis is used in analyzing the effects of a treatment on test subjects. For instance, it compares the values obtained from patients before treatment to those taken after. In a case such as this one, where researchers hope to see a difference in values as a result of the treatment being given, a p-value of 0.05 or less indicates that the value differences are not likely to be due to random chance. Conversely, a high p-value indicates that any differences seen between the before and after values are not statistically significant. In this study, the two trials for each of the five roosters were compared using the paired t-test. Because it was hoped that the assay would be found to be reproducible, it was expected that the p-values obtained would be very high, thus indicating that any variability observed between the two trials was due to random chance. The p-value obtained was much greater than 0.05 (0.1144) and this high value further confirms that this assay can be reliably reproduced.

It should be noted that, when the samples of Loon #6 were read, only trials two and three were analyzed (Table 9). This was due to the fact that the sample from trial one was lost. Table

10 shows the results of the collagen determination for the five roosters. As previously mentioned, the collagen determination of the second attempt of the five roosters should not have used the first linear equation (Figure 2). However, being unsuccessful in creating a second linear equation for the second attempt of the five roosters, all of the collagen determination was done using the original linear equation. In the second trial, rooster #3's absorbance at 540nm was too high to be read by the spectrophotometer and so the sample had to be diluted 1:2. This dilution factor was taken into consideration when calculating collagen concentration (Sample Calculation 3).

In creating the standard collagen curve, originally five 1:5 dilutions were made to result in concentrations between 0.32µg and 200µg. However, it was discovered that the assay was only sensitive down to 8µg. As a result, a 1:2 dilution was made of both the 200µg and 40µg samples, thus creating a standard curve of 200µg, 100µg, 40µg, 20µg, and 8µg. In the future, a 1:3 dilution might be best.

It is also important to note that, when creating the Direct Red 80 solution (10mg/ml), the directions of the Sircol™ Collagen Determination Assay (2007) specify that it be mixed with picric acid. However, research by Lee et. al (1998) indicates that picric acid is not necessary for the binding of the dye with collagen. Therefore, distilled water was used in this study instead of the expensive and potentially dangerous acid. The Sircol™ Collagen Determination Assay Manual also specifies that the dye only needs to sit with the collagen for 30 minutes. After trying to read a number of samples that were left in the dye for only 30 minutes, it was discovered that a large enough pellet could not be obtained to get a sufficient reading in the spectrophotometer. It was decided that samples should be left in the dye for two hours before centrifugation. This made a substantial difference in the size of the pellet obtained. When the

solution was left to sit for only 30 minutes, the pellet was either so small it couldn't be seen, or there was no pellet. However, after 2 hours, the pellet was easily visible for all samples. It is possible that if the dye was mixed with picric acid only 30 minutes would be necessary.

Pentosidine Concentration/Collagen Concentration

The values of the ratios of (pentosidine μg/mL)/(collagen μg/mL) were the most important in this study as they allowed for comparison between the samples. This is crucial to this experiment because, if this step is not done, then the only value being compared is the amount of pentosidine in each sample. Knowing the amount of pentosodine is not enough to establish a connection between the amount of pentosidine and age. Because pentosidine is found in collagen, more collagen in a sample will mean that there is more pentosidine. Therefore, if a bird's concentration of pentosidine is measured and there is no amount of collagen to compare it to, a high pentosidine reading may lead to the mistaken conclusion that the bird is old. However, the sample may also have a large amount of collagen, which explains why there is a large amount of pentosidine but does not indicate that specific subject is old.

If the experiment worked, it was expected that the two trials for Loon#6 would be the same or very similar, that the two trials for each individual roosters would be the same or similar, and that the values would be similar among all five roosters. This was found to be largely true (Table 11). The two Loon#6 attempts were almost exactly the same in value indicating that the assay is reproducible. Roosters one, two, four, and five all had similar values amongst each other as well. However, rooster three showed great variability both between its two trials and in comparison to the other roosters. This was thought to be because, in the second collagen trial, not enough of the dye reagent was removed from the tube before assaying, resulting in an

absorbance that could not be read by the spectrophotometer. Diluting the sample in order to be read may also have contributed to this discrepancy in the data. A paired t-test was performed for the pentosidine/collagen values of the five roosters and the p-value was found to be 0.903. As discussed above, a high p-value confirms the reliability and reproducibility of this assay.

The method for collagen concentration determination will have to be refined in further studies to make it a more reliable technique. Furthermore, to determine the age of unknown birds, a standard curve of known age birds for each species would have to be obtained for pentosidine/collagen. With the standard curve, it would be possible to enter in the values for the unknown bird ages into the linear equation obtained and determine exactly how old each specimen is.

This new assay for quantifying pentosidine concentration in avian species is promising in its potential uses. In the past, the assay for determining pentosidine concentration was too costly and time consuming to be practical for field researchers and wildlife centers to use. This new assay, however, bypasses hydrolysis and HPLC completely, both cutting costs and reducing time. In further studies, it will be necessary to create a standard age curve for loons so that age can be approximated based solely on pentosidine per collagen concentration. Additionally, further research is necessary on a much larger population size to further confirm the reliability of this assay.

In the future, this technique will be useful for wildlife researchers in that it gives them a basic idea of how old a bird is upon necropsy. It can then be decided whether age has an effect on accumulation of heavy metals as well as whether or not a specimen died of illness or simply old age. Though currently this technique can only be used on deceased birds, the ultimate goal is

that this assay can be refined enough to be used in living birds arriving at wildlife clinics for treatment. This assay will give veterinarians and rehabilitators the insight they need to determine what types of treatment options to pursue for their patients and ultimately has the potential to revolutionize the avian research field.

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Appendix

Table 1A: Original Standard Curve for Pentosidine

Concentration of Pentosidine (mg/mL)	Fluorescence (nm)
1.892×10^{-3}	1001.57
1.892×10^{-4}	121.9
1.892×10^{-5}	31.5
1.892×10^{-6}	27.0
1.892×10^{-7}	19.0
1.892×10^{-8}	21.2
1.892×10^{-9}	17.9

Table 1A shows the original standards used for the pentosidine curve. This curve had to be further diluted because once the concentration reached 1.892×10^{-5}, the fluorescence no longer changed significantly enough and did not go to a low enough value to be useful for our assay.

Figure 1A: Original Pentosidine Standard Curve

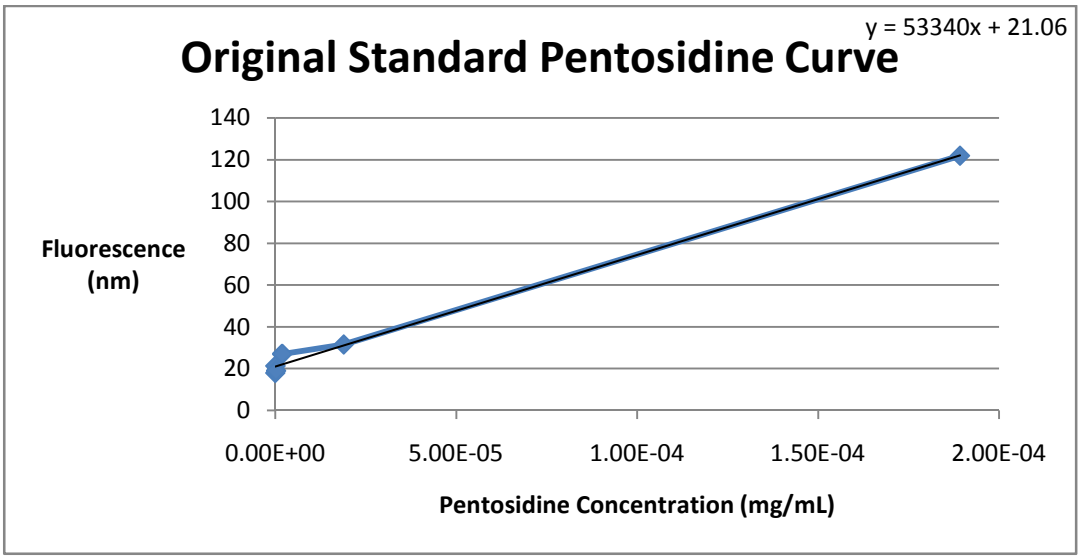

Figure 1A shows the original standard pentosidine curve that could not be used due to insufficient dilutions.

Table 2A: Pentosidine Data for Known Age Loons

Identification	Fluorescence (nm)
"Known 6"	26.6
TV 08- 230	51.1
TV 08- 294	13.2

Table 2A shows the fluorescence data for the known age loons. Pentosidine concentration could not be determined because the fluorescence values were too low for the standard curve constructed in Figure 1 of the main report. If two or more extractions had been done, it would have been possible to get high enough fluorescence values to find pentosidine concentration.

Table 3A: Pentosidine Data for Unknown Age Loons

Identification	Fluorescence (nm)
TV 07- 446 (youngish)	13.85
TV 08- 282	29.4
TV 08- 259	24.0
ME 07-117	22.8

Table 3A shows the fluorescence data for the unknown age loons. Pentosidine concentration could not be determined because the fluorescence values were too low for the standard curve constructed in Figure 1 of the main report. If two or more extractions had been done, it would be possible to get high enough fluorescence values to find pentosidine concentration.

Table 4A: Original Collagen Standard Curve

Concentration of Collagen (ug/mL)	A540 (nm)
200ug	2.238
100ug	1.746
40ug	0.523
20ug	0.332
8ug	0.153

Table 4A shows the original collagen standard curve data. This could not be used because the A540 values did not go low enough for the samples we read.

Figure 2A: Original Standard Curve

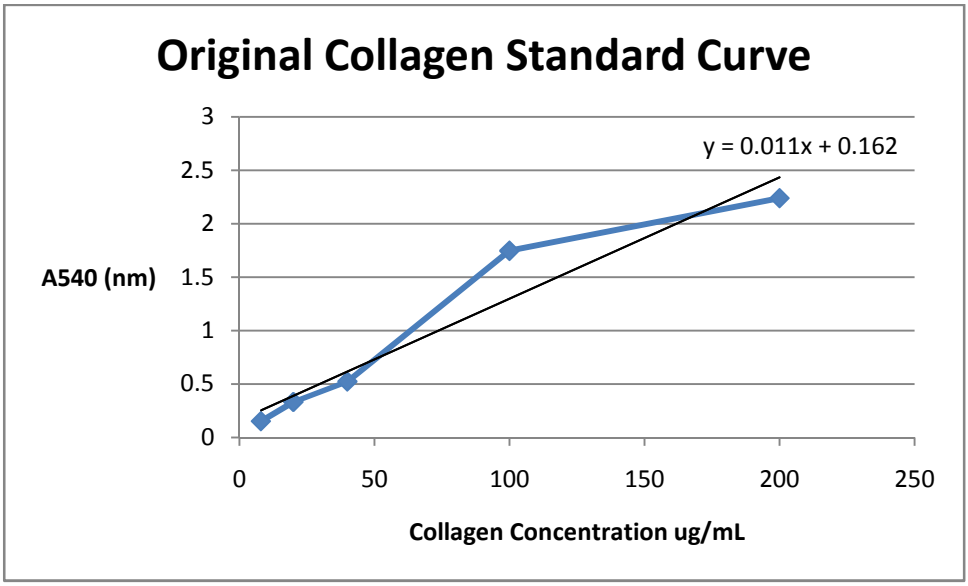

Figure 2A shows the original collagen standard curve with a linear equation of y = 0.011x + 0.162.

Table 5A: A540 for Known Age Loons

Identification	A540 (nm)
"Known 6"	0.367
TV 08- 230	0.028
TV 08- 294	0.205

Table 5A shows the A540 found for each known age loons.

Table 6A: A540 for Unknown Age Loons

Identification	A540 (nm)
TV 07- 446 (youngish)	1.508
TV 08- 282	0.858
TV 08- 259	1.984
ME 07-117	0.193

Table 6A shows the A540 for each of the unknown age loons.