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Research on mammalian pigmentation has ranged from basic studies on biochemistry of melanin formation to more practical studies involving effects of nutrition, environment and genetics on hair color. In mink such studies have an added significance as fur color is of considerable economic importance; however, research in this area has been hampered by lack of a quantitative measure for pigmentation.

Historically, fur color in mink has been evaluated visually; this has the disadvantage of lacking repeatability, sensitivity and objectivity. One existing chemical method for measuring hair color employing spectrophotometric procedures is based on the knowledge that hair pigmentation is derived from microscopic melanin granules within the hair, the corollary being that hair color intensity is a function of melanin granule concentration. The studies reported

herein were undertaken to further improve this spectrophotometric method of quantitating the amount of melanin pigmentation in hair. In this regard, trials were conducted in the areas of spectrophotometry, ultrasonic homogenization and sample preparation. Other tests were made to gain further information on application of the method.

Spectrophotometry. Determining melanin granule concentrations involves establishment of the turbidity of suspensions of melanin granules. Hence, trials were conducted to determine effects of light source wave length, granule concentration and type of spectrophotometer employed on these turbidimetric measurements. These trials indicate that wave length must be specified, as although the absorption curve is linear, optical density values progressively decrease with longer wave lengths. It was further shown that optical density values of varying concentrations of melanin granules follow Beer's law within the tenfold concentration range studied. This strengthens the fundamental validity of the method since the data indicate optical densities of melanin granule suspensions are directly related to granule concentrations. Results further indicate that the technique can be adapted to any spectrophotometer; however, absorption curves vary as a result of differences in cuvette size and other differences inherent to the equipment giving relative but not absolute results. Establishment of standard curves for particular instruments would render values directly comparable.

Ultrasonic Homogenization. Investigations in this area indicate that homogenization of the digested hair by sound waves increases the optical density of melanin granule suspensions by separating groups of pigment granules into individual particles. One and one-half minutes of ultrasonic homogenization, at all four power intensity levels studied, resulted in maximum optical density values indicating complete granule separation. Homogenization of melanin granule suspensions increased the repeatability and consequently the sensitivity of the technique by maximally increasing the surface area of the suspended particles and by uniformly dispersing the particles.

Sample Preparation. Studies indicate that grinding the hair sample was not necessary to achieve complete granule liberation when followed by ultrasonic homogenization. This modification resulted in a more useable procedure since grinding of hair required considerable time and effort; however, more difficulty was encountered in obtaining a representative sample of guard hair and underfur, which slightly lowered the repeatability of the procedure.

Application of Method. Research indicates that the procedure of hair color analysis, as modified by spectrophotometry and ultrasonic homogenization techniques, is sensitive to small differences in hair pigmentation and can be applied to relatively large numbers of samples. These trials further indicate that hair pigmentation as expressed by "melanin value" is definitely correlated to visually

appraised intensities of hair pigmentation. The research also suggests that numerous applications of the technique exist.

The studies reported herein indicate that the hair color analysis, as modified, is sensitive and repeatable and can be fairly rapidly applied to relatively large numbers of samples.

AN IMPROVED CHEMICAL METHOD FOR MEASURING INTENSITY OF HAIR PIGMENTATION

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INTRODUCTION

Hair color in mammals has been and continues to be a subject of considerable interest. In humans, intensity of hair pigmentation seems to be associated with age. Most children have hair that is lighter in color than it will be in their adult years. In later years a phenomenon, not yet explained, results in a failure of normal pigment formation and greying occurs. Geneticists have found the study of coat color in the laboratory mouse especially interesting not only from the standpoint of coat color patterns but also because coat color is often a qualitatively inherited trait which can be used as a barometer for other heritable characteristics. Color and sheen of hair coat have long been recognized as indications of a healthy condition; conversely, a failure of normal pigment formation and dullness of hair indicates an unhealthy condition.

Coat color was the object of a study by Schleger (1962) who used a crude evaluation to establish a positive correlation between intensity of red pigment and average daily gain both in the Africander and British breeds of beef cattle. Schleger (1967) subsequently reported positive correlations between coat color type and shade of red color with milk yield.

Hair color is a prime consideration in the raising of mink, and standard dark mink are valued, along with other considerations, according to intensity of fur pigmentation, with pelts from darker animals receiving a premium when marketed. For this reason, mink ranchers have made continual attempts to produce darker mink both by genetic selection and by feeding practices. Some ranchers have been successful in developing blood lines of very dark mink. Many theories about effects of various feedstuffs upon fur color have also developed but are supported by little evidence. One reason for so little factual information in this area is a lack of an objective method for evaluating fur color.

At the Oregon State Agricultural Experiment Station results from tests involving more than 100 experimental diets fed to 1500 male mink indicate that level of several major feed nutrients is not strongly associated with either an increase or a decrease in fur pigmentation (Stout, Adair and Oldfield, 1966). However, the Oregon Station has accumulated research data which indicate that intensity of fur pigmentation is increased when mink are fed purified diets (Stout, Adair, Costley and Oldfield, 1967a).

It has long been recognized that many specific nutrients are important in pigment formation and that a deficiency of one or more of these nutrients results in a failure of normal fur pigmentation. Such nutrients include: copper, zinc, pantothenic acid, folic acid,

para-amino benzoic acid, lysine and biotin (Frost and Douglas, 1948), and recently iron has been added to this list (Stout, Bailey, Adair and Oldfield, 1968).

If specific nutrients are important in pigment formation it follows that feedstuffs containing these nutrients must also be important. In addition, evaluation of nutritional effects on fur color in the past may have been thwarted by lack of a quantitative method sensitive enough to distinguish slight variations in fur color.

The objective of this study was to further improve a quantitative determination of fur color based upon a method developed by Lea (1954) and later modified by Stout and Costley (1967). The improvements sought were in three general areas: (1) to gain increased repeatability and consequently sensitivity, (2) to develop standardization of technique and (3) to simplify the technique thus making it applicable to large numbers of determinations.

Such a technique, if accurate and reliable, could ultimately make possible a quantitative assessment of genetic, nutritional and environmental effects upon hair color for any species. Other possible uses for such a method with regard to mink might include establishment of fur color standards which could be used both in breeder selection and pelt grading. The method could also be used by researchers working with species other than mink for both hair color analysis and isolation of melanin granules from hair and other tissues.

LITERATURE REVIEW

Color

Color is a property of matter arising from the interaction between an object and a light source which results in a visual response (Hofert 1967). Burnham, Hanes and Bartleson (1963) list several factors which are related to visual color experience, including: the physical characteristics of radiant energy that stimulate the eye to perceive color, the chemical components of such things as pigments and dyes which render color to an object, the physiological activity of of nerves leading from the eye, the psychophysical relationships between specific amounts and kinds of color stimuli and a particular color response and the psychological response of an individual when the eye is stimulated by radiant energy.

The color of an object depends primarily on the spectral distribution of the incident light. For example, when a surface which appears white because it reflects the entire color spectrum is illuminated with a red light, only the red spectrum is reflected, and it appears red; if illuminated with a blue light, it appears blue. Color also depends upon the absorbent properties of the object. If a light is projected on a black surface, which will not reflect light, it appears black because the entire color spectrum is absorbed (Smith and

Cooper, 1957). When one sees an object, illuminated with white light, which appears green, the object is reflecting only the green portion of the spectrum and absorbing all other wave lengths.

Eaton (1966) maintains that the color one sees is dependent not only upon the reflectant and absorbent properties of the object but also upon the observer's mental attitude, past experience, surroundings, expectation and eye fatigue.

Hair Color

Melanin is the pigment responsible for color of hair and skin in mammals and feathers in birds. It is distributed throughout the pigmented tissue in microscopic particles called melanin granules. These were first isolated from feathers of chickens and examined under a light microscope by Frieling (1936), who characterized them as either dark (eumelanin) or light (pheomelanin). Hutt (1953) subsequently isolated these granules and found that granules of eumelanin were larger than pheomelanin granules and were only slightly soluble in sodium hydroxide. Pheomelanin granules were spherical and entirely soluble in sodium hydroxide.

Melanin granules are produced in specialized cells called melanocytes. Both eumelanin and pheomelanin can be synthesized within the melanocytes of one hair follicle (Wolfe and Douglas, 1966). Melanocytes originate from melanoblasts, which are produced in early stages of fetal development and derived embryologically from

the area of the neural crest and migrate to their location in the epidermis. Melanocytes selectively synthesize melanin pigments and secrete these as granules between and within other cells which become pigment carriers. This inoculation of pigment is accomplished through dendritic prolongations of the melanocyte (Rawles, 1953).

Active melanocytes giving rise to hair pigments are localized in the upper hair bulb along the border of a papilla cavity (Lyne, 1965) (Figure 1).

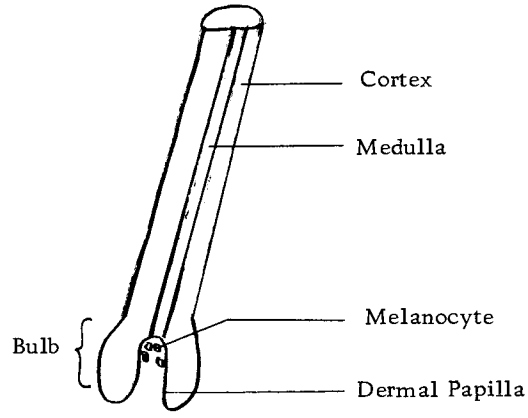


Figure 1. Location of active melanocytes in hair bulb.

Melanin synthesized by these melanocytes is deposited between and within epithelial cells as they move upward during hair growth to be incorporated in the developing hair shaft (Rawles, 1953). Lyne (1965) while studying the size of melanin granules found that they pass from hair bulb melanocytes mainly into the center, medulla, of the hair shaft; however, a few pass into the outer portion, cortex, of the hair. He noted melanin granules surround the hair at the base of the canals of active follicles and that accumulations of pigment granules may build

up in the hair canals before a new hair is formed. Lyne further noted discharged granules are at first randomly distributed throughout the cytoplasm of the hair cell; subsequently they become organized into layers and ultimately oriented so that the long axis of both granule and hair shaft are parallel.

Hair growth in mammals is characterized by three phases: anagen, the growing phase; catagen, the transitional phase; and telogen, the resting phase. Follicular melanocytes begin synthesizing and depositing melanin granules into the hair medullary cells of the growing hair shaft during the third substage of the anagen phase (Chase, Ranch and Smith, 1951). Pigment synthesis continues until the active hair cycle terminates with the onset of the catagen stage (Straile, Chase and Artsenault, 1961).

During the early stages of melanin granule development within the melanocyte, thin unit fibers aggregate within a membranous boundary to form compound fibers. As these compound fibers become cross-linked and oriented parallel to one another, the shape of the melanin granule develops. When this organization is complete melanin pigment is deposited in the matrix at predetermined sites along the fibers. As deposition continues the internal structure of the matrix is obscured by the electron dense pigment (Wolfe and Douglas, 1966).

Although it is generally accepted that melanin granules are responsible for hair pigmentation, there is some disagreement in the

literature regarding their importance in the final expression of hair color. Maximow and Bloom (1945) maintain that differences between dark and light hairs are primarily dependent upon the air space within the hair substance while the amount of melanin pigment is of less importance. Russell (1946) while studying different color types in mice found the concentration of melanin granules to be of primary importance in the intensity of hair pigmentation and that size, shape, clumping and arrangement of these granules within the medullary cells and along the hair shaft was of secondary importance. McGrath and Quevedo (1965), while studying the histology of various types of hair of the mouse, found the numbers of melanin granules to vary greatly within one hair shaft. The highest numbers were found in the hair tips with a progressive decrease toward the base. The extreme basal segment of many such hairs contained few if any pigment granules. McGrath further compared hair color of two strains of mice, a light strain and an intensely dark strain, and found that although large variations occurred within each strain there were consistently more melanin granules in the darker strain.

Measurement of Hair Color

Hair color in various species of animals is of considerable interest, and numerous research studies have been devoted to the effects of genetic, environmental and nutritional factors which alter

it. Further, hair color is of special importance in the production of fur bearing animals such as the mink. Consequently, several methods have been used in an attempt to quantitatively evaluate hair color. Basically these can be separated into either subjective or objective methods, which will be discussed separately.

Subjective Evaluation

Visual Appraisal. Traditionally, fur color in mink has been assessed by visual inspection both for breeder selection and for pelt sales. This visual evaluation of fur color has developed into an art, and men are trained commercially in detecting both small and large differences in coat color. These trained individuals include mink ranchers, auction company personnel, commercial fur buyers and fur matchers of the garment trade.

Travis and Schaible (1960) listed several considerations relative to the grading of pelts for color, including: (1) establishment of some type of reference point, (2) elimination of grading near undesirable backgrounds such as aluminum roofs or red buildings and (3) wearing neutral rather than brilliant colors while grading.

The advantages of visual appraisal are its simplicity and inexpensiveness. Its disadvantages are lack of repeatability due to lack of permanent standards for comparison, lack of standardized lighting and personal bias. A fundamental disadvantage of this method lies in

the inability of the human eye, even that of the most qualified grader, to distinguish slight variations in fur color, unless the comparison is made with furs side by side.

Color Comparison Charts. Color comparison charts have been used extensively in color determinations by artists and interior decorators but have not found extensive use in hair color evaluations. Schleger (1962), while studying the correlation between intensity of red hair color and average daily gain in beef cattle, was able to facilitate his evaluation by comparing the color of an individual animal to one of several color standards from a color chart.

Such charts have the advantages of being quick, relatively inexpensive and substantially more repeatable than strict visual appraisal. Their disadvantages lie in the facts that lighting and backgrounds are not necessarily standardized, that the evaluation can be influenced by personal bias and that, as with visual appraisal, it is impossible to distinguish slight variations in hair color.

Objective Evaluation

Spectrophotometric Methods. Light reflection and absorption have been used extensively in all areas of science. So extensively have they been employed that the field of colorimetry has itself developed into an active area of scientific investigation. However, hair color evaluation by spectrophometric methods has been slow to

develop. One such procedure has been employed by the United States Federal Trade Commission (Moos, 1967) to evaluate possible alterations in fur color which have resulted from dressing procedures applied to mink pelts. This technique involved the measurement of light reflectance from a 2.5 inch square of mink fur, which had been combed to straighten the hair evenly, mounted on cardboard and covered tightly with a transparent film.

Although this procedure was apparently repeatable, its utilization in detecting differences in fur color could be questioned. As described, the technique would primarily measure pigmentation differences occurring in the guard hair; however, variations in hair pigmentation usually occur in the underfur. Also, since the fur samples are taken from the back area, their removal would substantially decrease the sale value of the pelts. For these reasons this technique would be of questionable value for routine color evaluations of mink pelts.

Other reflectance techniques have been developed for evaluations of materials other than hair. Stewart, Zipsi and Watts (1965), for example, described a spectrophotometric method of evaluating pigmentation of meat. This technique was based on the ratio of absorbed and scattered light from a spectrophotometer. Such a technique possibly could be applied to the evaluation of hair pigmentation. Hofert (1967) presents a method of separating shades of colors by

reflectance techniques. His procedure separates color by photoelectric currents and is able to numerically evaluate color types. The technique does not appear to have direct application in evaluating hair pigmentation.

Optical Density Methods. An entirely new approach to the problem of hair color measurement was taken by Lea (1954) who developed a method for analyzing hair color based on measuring differences in concentration of melanin granules by determining the optical density of suspensions of melanin granules which had been freed from hair by a digestion procedure. This area was investigated in an attempt to establish a quantitative evaluation of differences in hair color so that genetic effects on hair pigmentation could be more accurately assessed.

Lea's technique was based on the fact that hair proteins, which are principally keratins, are soluble in liquid urea and that melanin pigment granules are insoluble in this solvent. Thus, digestion of hair samples in urea liberated the melanin granules forming a suspension which could be measured spectrophotometrically. Lea's method was developed utilizing the findings of Clark (1951), who studied the solvent properties of urea giving special attention to its action on relatively insoluble materials.

The hair color analysis method described by Lea involved the treatment of hair in a soxhlet apparatus for 1.5 hours to remove

exogenous lipids. The hair was dried and cut into lengths of approximately .5 cm. Ten mg of the cut hair were placed in 3 g of urea in a long thermometer pocket. A 150 ml flask containing commercial xylol was fitted with a two-way adapter; a reflux condenser was placed in one neck while a thermometer pocket was placed in the other. The apparatus kept the xylol at its boiling point of approximately 140° C. The hair-urea mixture was stirred continuously at a speed of 17 revolutions per minute. This digestion process continued for three hours, at which time the hair-urea suspension was cooled and diluted to 10 ml, and the optical density was determined using a Spekker absorptiometer. Relative color intensity was expressed in terms of optical density per 3 g of hair.

Lea applied this procedure to human, cat and rabbit hair with repeatable results; however, he was not able to use it on hair from very black animals since some of the hairs remained undissolved.

Schleger (1962) determined optical densities of melanin granule suspensions for hair of both British and Africander breeds of beef cattle using a technique modified slightly from Lea's original method. Schleger's modifications included not extracting the hair with ether, using approximately 200 mg of hair and discontinuous stirring throughout the three hour digestion period.

Stout (1966) employed Lea's published method on fur samples from standard dark mink and found the same disadvantages reported

by Lea, i.e. incomplete digestion of dark hair samples, poor repeatability, lack of sensitivity and excessive time required. Because of these disadvantages, he began to investigate ways of making this technique more useable. Since melanin granule concentration was known to be a prime factor influencing intensity of hair pigmentation, the fundamental principles of Lea's method were deemed valid. Stout's work was originally directed toward establishing optimum time and temperature relationships for hair digestion. It was noted that if digestion proceeded too long at the high temperatures used the urea would polymerize forming biuret and the insoluble cyanuric acid. The xylene bath originally used was disbanded as maximum temperatures were limited. Subsequently, baths of glycerine and flowing sand were employed but discontinued due to the volatility of the former and lack of temperature control of the latter. Finally, a silicone oil bath was used which proved to be satisfactory for the high temperatures required to minimize digestion time. It was established, using this equipment, that optimum digestion of hair required 15 minutes at a temperature of 190° C. With time and temperature for digestion established, the procedure became more useable since complete digestion was achieved and considerably larger numbers of samples could be run in shorter periods of time. Repeatability, though greatly improved, remained variable, but this was partially overcome by employing more elaborate grinding procedures.

This research resulted in the establishment of an improved method for measuring hair color, but a greater degree of sensitivity, which comes with precise repeatability, was still sought and such was the object of this thesis.

EXPERIMENTAL

Objectives

The primary objective of this research was to improve an existing chemical method for measuring intensity of pigmentation in hair. Basic procedures were available but lacked complete standardization and were laborious and thus not feasible for large numbers of determinations. Improvement of the original method for assessing hair color was sought in the four general areas of repeatability, reliability, sensitivity and simplification. Numerous trials were conducted to improve different aspects of these four areas and include: (1) standardization of spectrophotometry, (2) homogenization of melanin granule suspensions by ultrasonic techniques, (3) simplification of sample preparation and (4) establishment of relationships between "melanin values" and intensity of hair pigmentation.

Methods

The original technique for measuring concentrations of melanin granules within hair was based on the knowlege that hair proteins (primarily keratins) were soluble in liquid urea at high temperatures, whereas melanin granules were not. These properties allow melanin granules to be released from hair and placed in aqueous suspension. Subsequently, their relative concentration can be measured

spectrophotometrically. The following general procedures used prior to modifications suggested by this research were presented at the fifty-ninth annual meeting of the American Society of Animal Science (Stout and Costley, 1967).

Sample Preparation

Small strips of dried skin with attached fur, no more than 1/2 inch wide, were cut bilaterally from the edges of a mink pelt in the area between the tail and hind leg. Fur from these strips was clipped as closely as possible with barber shears to get representative amounts of both guard and underfur fibers. This fur was placed in a Goldfisch ether extraction apparatus for two hours to remove lipids. After thoroughly dry, samples were ground in a micro-Wiley mill through a #60 mesh screen. Fineness of grind appeared to be especially important as poorly ground hair led to incomplete digestion and poor repeatability. The tendency of hair to float in the grinder was overcome by adding reagent sodium chloride which forced the hair through the screen. The sodium chloride was subsequently removed by dissolving in distilled water and filtering through a sintered glass filter. Common table salt was found to be unsatisfactory as it contains $CaCl_2$ which is relatively insoluble in water and can not be separated from the hair sample. Washed hair samples were placed in a drying oven for 24 hours and dessicated for 24 hours before weighing.

Melanin Granule Liberation

Approximately 30 mg of ground hair were weighed accurately into a micro-kjeldahl flask, and 4 g of urea were placed so as to cover the hair. The flask containing hair and urea was placed in a silicone oil bath previously heated to 190° C. ±0.5. (For a description of equipment used see Appendix 1.)

Exactly 15 minutes were allowed for digestion; the reaction was stopped by removing the flask from the bath and cooling with distilled water. The digested sample was transferred quantitatively to a 100 ml volumetric flask, diluted to approximately 98 ml with distilled water, allowed to equilibrate to room temperature for about one hour and then diluted exactly to 100 ml.

Optical Density Determination

Optical density of the resultant melanin granule suspension was determined using a Coleman spectrophotometer set at 485 millimicrons using matched cuvettes (13 mm i.d.). Optical density values were converted to a "melanin value" expressing optical density of melanin granule suspensions per unit weight of hair according to the following formula:

$$\text{Melanin Value} = \frac{\text{Optical Density X 10}}{\text{Weight of Hair (grams)}}$$

The research presented in this thesis involved modification of

various of these procedures to obtain more sensitive and reliable estimates of hair color intensity.

Results

Spectrophotometry

Measurement of hair color intensity by this technique was based on being able to determine differences in optical densities of melanin granule suspensions. Trials were conducted to investigate various aspects of the optical density determination, including establishing the effect of spectrophotometric wave length setting, assessing the effect of concentration of melanin granules on resultant optical densities and determining differences in optical density values obtained from different spectrophotometers.

Trial 1A. A representative sample of mink fur (control #7130) was digested and diluted to 100 ml as previously described. The optical density of this suspension was measured with a Zeiss spectrophotometer (Appendix 1) at wave lengths ranging from 380-640 millimicrons. Resultant values are given in Table 1 and depicted in Figure 2.

Table 1. Optical density values of a melanin granule suspension with respect to wave length--Trial 1A.

Wave Length (millimicrons)	Optical Density
380	.435
390	.426
400	.419
410	.412
420	.406
430	.403
440	.399
450	.395
460	.388
470	.385
480	.382
490	.380
500	.374
510	.370
520	.365
530	.360
540	.355
550	.352
560	.350
570	.344
580	.340
590	.335
600	.332
610	.330
620	.325
630	.320
640	.315

(Zeiss PMQ spectrophotometer slit width .01.)

These data indicate that changes in wave length of transmitted light result in changes in optical density values of melanin granule suspensions, which points to the need for specifying a precise wave length. The data further indicate that melanin granule suspensions lack an absorption curve, within the spectrum studied. The

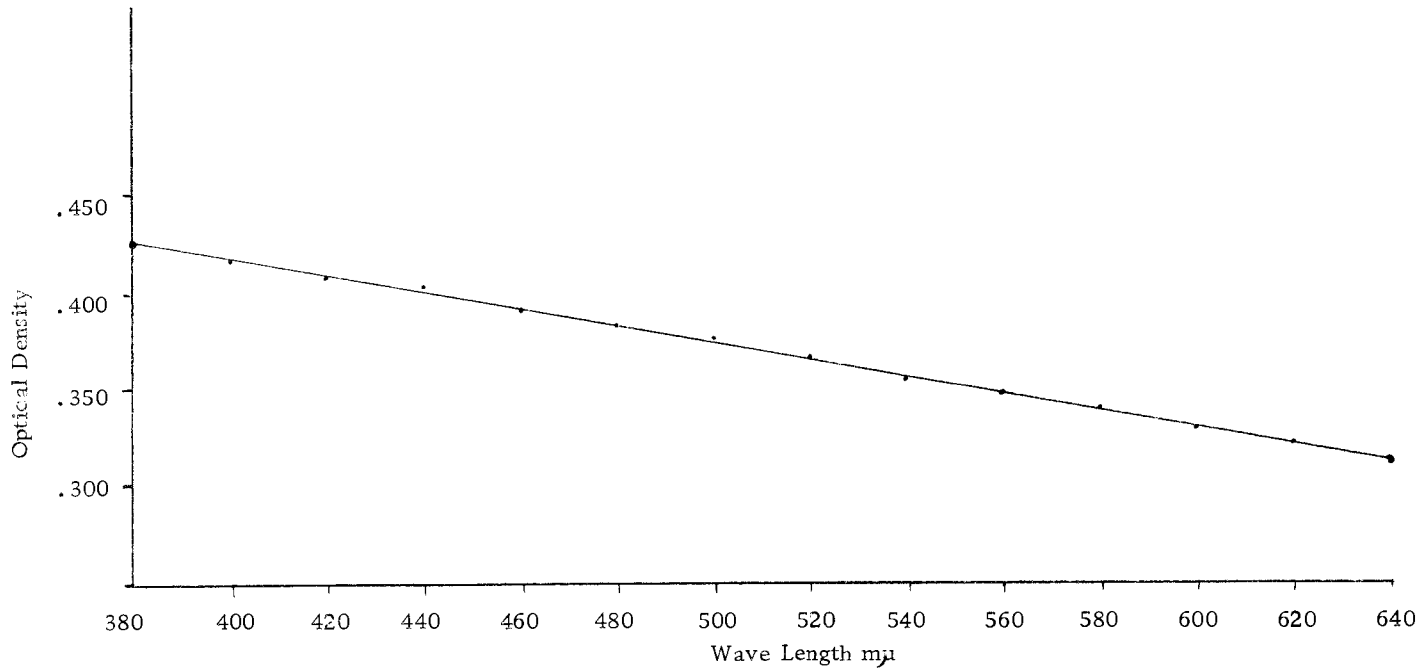


Figure 2. Optical density values of a melanin granule suspension as influenced by wave length setting - Trial 1A. (Zeiss PMQ2 Spectrophotometer)

relationship between wave length and optical density was found to be linear, progressing from higher to lower values with shorter to longer wave lengths. A logical reason for this is that shorter length light waves have greater opportunity to be deflected by the melanin particles, resulting in a lower percentage of light transmittance and a higher optical density. Because of the linear relationship, the choice of wave length is arbitrary. Originally 485 millimicrons was chosen, but subsequently difficulty was encountered in adjusting the Zeiss spectrophotometer to 100 percent transmittance using distilled water, since at this wave length the spectrophotometer was at the top of its transmittance scale. Consequently, 480 millimicrons was chosen as a suitable wave length.

Trial 1B. This trial was designed to determine the relationship between optical density and concentration of melanin granule suspensions. Also, differences in optical density values obtained from two different models of spectrophotometers were investigated. Two mink fur samples were prepared and digested as previously described and then pooled and diluted to 200 ml; from this suspension, serial dilutions were made so that resultant concentrations were 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 percent of the original pooled sample. The optical density of each diluted sample was determined on both Zeiss and Coleman spectrophotometers, and results

are shown in Table 2 and Figure 3.

Table 2. Optical density values of serial dilutions of melanin granule suspensions determined on Zeiss and Coleman spectrophotometers--Trial 1 B.

Sample Concentration (% of original)	Optical Density	
	Zeiss (480 mμ)	Coleman (480 mμ)
0	.000	.000
10	.040	.045
20	.075	.090
30	.114	.133
40	.151	.180
50	.190	.222
60	.226	.252
70	.265	.312
80	.304	.355
90	.342	.400
100	.375	.445

Data from this trial indicate that optical densities of serially diluted melanin granule suspensions are linear and directly dependent upon concentration and follow Beer's Law within the range of concentrations used. The observed linear relationship between melanin granule concentration and suspension optical density is further evidence of the validity of this method for measuring hair color, since concentration of melanin granules is a prime factor influencing the intensity of hair pigmentation (McGrath and Quevedo, 1965).

Measurement with Zeiss and Coleman spectrophotometers gave similar type curves, although slopes differed. Optical density values determined with the Coleman instrument were higher than those for

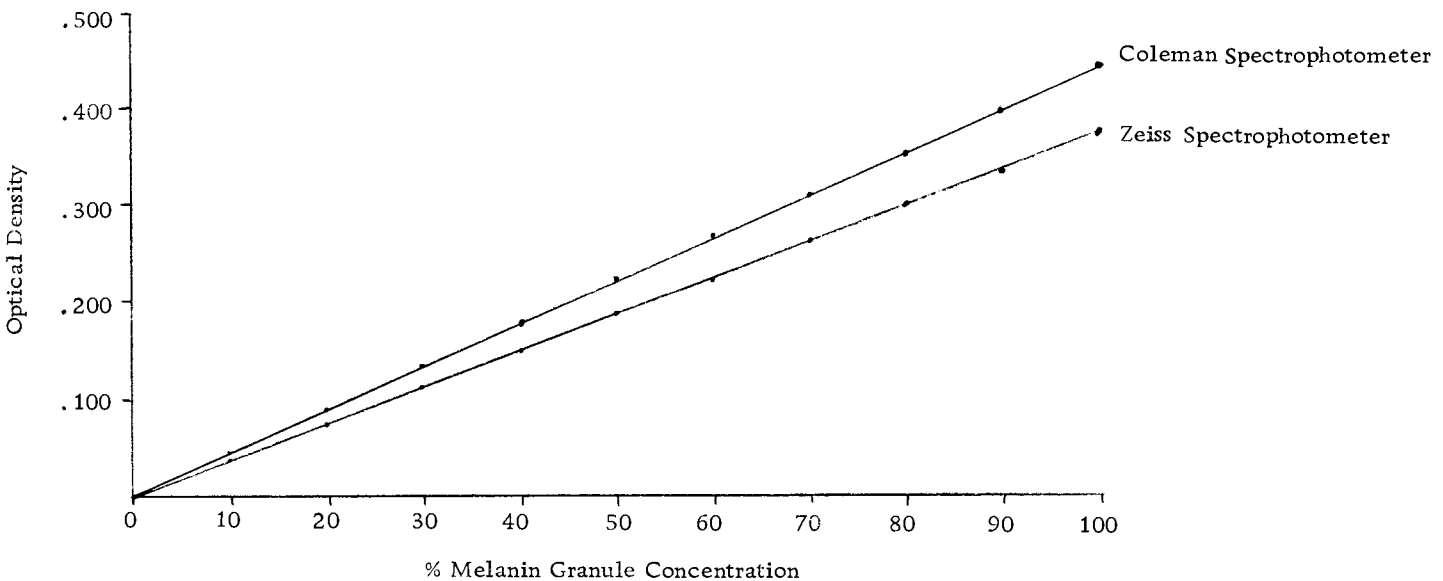


Figure 3. Optical density of a Melanin granule suspension at concentrations ranging from 0 to 100% as determined on two makes of spectrophotometers - Trial 1B.

the Zeiss at every concentration level studied (Figure 3). This is explainable in that the Coleman employs 13 mm cuvettes as opposed to 10 mm cuvettes for the Zeiss. With larger cuvettes, the suspension is deeper; consequently, less light is transmitted resulting in higher optical density values. Therefore, hair color measurements using this technique and expressed on an optical density basis are comparative for different instruments.

Ultrasonic Homogenization of Suspended Melanin Granules

Suspensions of melanin granules from digested hair contain particles which are visible to the naked eye. Since melanin granules from mink fur are usually less than one micron in size and consequently invisible to the eye, it was assumed that such suspensions actually contained groups of granules rather than individual particles. It also seemed possible that if these granule aggregates could be separated into individual particles without structural disruption the sensitivity and repeatability of the technique could be greatly improved. For these reasons, trials were run to determine the effects of ultrasonic homogenization on properties of granule suspensions.

Trial 2A. Objectives of this trial were to determine the value of ultrasonic homogenization as well as the optimum length and power intensity required for maximum separation of melanin granule suspensions without granule breakdown. Eight samples of mink fur (control

#7130) were weighed, digested and diluted to 100 ml each. From these, four suspensions were randomly pooled and diluted to 500 ml; the remaining four were pooled and diluted to 425 ml. From each pooled suspension, four 100 ml aliquants were taken. The resultant eight suspensions were homogenized ultrasonically at intensities of either 40, 55, 70 or 85 percent of full power (125 watts) at 15 second intervals with a Biosonik II ultrasonic homogenizer (Appendix 1). After each homogenization treatment optical densities were measured using a Zeiss spectrophotometer set at 480 millimicrons and a slit width of .01 millimicrons. Results are given in Table 3 and Figure 4.

Table 3. Effects of length and intensity of ultrasonic homogenization on optical density of melanin granule suspensions--Trial 2A.

Time minutes	Intensity (% of full power) 40	55	70	85
	Replica 1 (500 ml dilution)			
0.00	.187	.187	.187	.187
0.25	.248	.258	.262	.264
0.50	.264	.288	.291	.300
0.75	.285	.288	.301	.300
1.00	.289	.298	.302	.311
1.25	.301	.310	.308	.316
1.50	.311	.313	.316	.324
1.75	.308	.313	.310	.322
2.00	.308	.318	.312	.323
2.25	.309	.317	.319	.322
2.50	.309	.320	.314	.323
3.00	.313	.320	.312	.327
3.50	.311	.323	.311	.327
4.00	.313	.323	.312	.326
8.00	.314	.324	.312	.324

Table 3 continued

Time min.	Intensity (% of full power) 40	55	70	85
	Replica 2 (425 ml dilution)			
0.00	.217	.221	.217	.215
0.25	.289	.306	.312	.318
0.50	.320	.312	.342	.339
0.75	.334	.331	.353	.350
1.00	.341	.345	.358	.358
1.25	.351	.353	.359	.359
1.50	.354	.356	.355	.360
1.75	.357	.354	.359	.358
2.00	.357	.357	.359	.358
2.25	.357	.360	.359	.357
2.50	.356	.356	.358	.358
2.75	.357	.356	.359	.358
3.00	.357	.357	.358	.356
3.50	.358	.360	.359	.357
4.00	.357	.356	.359	.358
8.00	.357	.356	.359	.357

Results of ultrasonic homogenization indicate a marked increase in the optical density of melanin granule suspensions during the first minute of treatment at all intensity levels studied. Between 1 and 1.5 minutes there were only slight increases in optical densities, and after 1.5 minutes there were essentially no changes in optical densities.

Data presented in Table 3 indicate higher power intensities were more effective than lower intensities in separating melanin granule aggregates into individual particles during the first minute of treatment. However, past one minute of homogenization separation reached a peak and remained at that level regardless of intensity used.

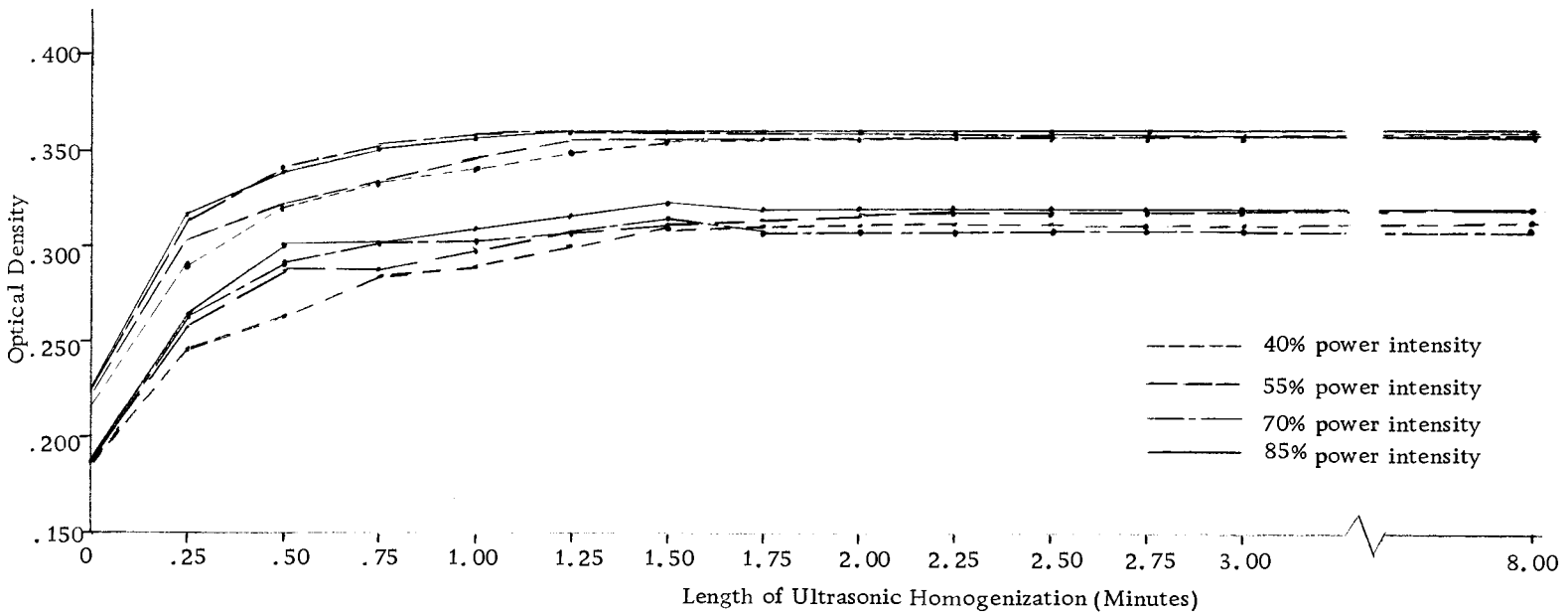


Figure 4. Effect of ultrasonic homogenization on optical density of two melanin granule suspensions of different concentrations run at power intensities ranging from 40 to 85 percent - Trial 2A.

In replica 1 the highest optical densities were achieved at the 85% intensity level and the lowest at the 70% level. Replica 2 showed final optical densities highest from the 70% homogenization intensity and the lowest from the 55% level although differences were slight. These data indicate that optical density differences noted after one minute of ultrasonic homogenization were a result of experimental error.

This trial indicates that ultrasonic homogenization increases the optical density of melanin granule suspensions by separating melanin granule aggregates into individual granules which effectively increases the reflective surface area of the suspension. The data further point out that from 1.25 to 1.50 minutes are required for maximum separation and that after this time intensity levels of 40, 55, 70 and 85 percent of full power are equally effective for separation.

Trial 2B. In view of results of trial 2A, this trial was designed to investigate the value of ultrasonic homogenization for improving the repeatability of the method of melanin measurement. It was known that ultrasonic homogenization would uniformly disperse melanin particles, and it was assumed that this should lead to increased repeatability of the optical density measurement and provide an increased sensitivity to the method. To verify this assumption, duplicate samples of finely ground mink fur (control #7130) weighing 15,

20, 25, 30 or 35 mg were prepared. Each sample was digested and diluted to 100 ml and the optical density determined both before and after ultrasonic homogenization. Variation in sample weight was used as a means to investigate effects of this treatment on suspensions differing in concentration. Results of this trial are given in Tables 4 and 5.

Table 4. Melanin values of mink fur (control #7130) before and after ultrasonic homogenization--Trial 2B.

Sample Weight (mg)	Before		After	
	Melanin Value	Variation* %	Melanin Value	Variation* %
15.22	71.0	2.02	123.5	2.32
15.33	72.4		120.7	
19.99	98.5	18.83	126.1	0.79
20.47	81.6		125.1	
25.32	74.1	1.26	122.3	0.00
25.32	75.0		122.3	
29.90	71.9	0.98	123.1	0.68
30.02	72.6		123.9	
34.74	67.9	3.38	120.6	0.37
35.29	70.3		120.2	

*Represents difference between duplicates divided by their mean.

Table 5. Effect of ultrasonic homogenization on repeatability of hair color analysis run on five hair samples in duplicate--Trial 2B.

Treatment	Mean Melanin Value	Mean Variation between Duplicates	Standard Deviation	Coefficient of Variation (%)
none	75.5	5.29	3.34	4.42
Ultrasonic Homogenization*	122.8	.84	1.86	1.51

* Carried out for 1.50 minutes at 85% full power.

These data indicate that melanin values of mink fur samples are not influenced by sample weight, within the weight range studied. Duplicate samples of 20 mg showed marked variation in melanin values before ultrasonic homogenization, but after treatment values were comparable (Table 4). The reason for this variability is not known; however, it seems possible that in the cooling and dilution processes the melanin granules may be grouping in a nonsimilar fashion. As a result, a sample with large aggregates of granules would have less surface area and consequently a lower optical density. Ultrasonic homogenization separates melanin granules resulting in a homogeneous suspension and less variation among duplicate samples; further, the treatment results in a uniform increase in "melanin values" by increasing the surface area of the suspension.

A linear increase in optical density was observed as sample weight increased, which is in agreement with results obtained in

trial 1B with serial dilutions. Data in Table 5 substantiate the hypothesis that ultrasonic homogenization of melanin granule suspensions increases the repeatability of the method. Both standard deviations and coefficients of variation bear this out.

Sample Preparation

With application of the findings of the spectrophotometry and ultrasonic homogenization experiments, repeatability and sensitivity of this method were improved; nevertheless, the technique remained laborious, which limited its application. For this reason, effort was directed towards simplifying sample preparation. Considerable time was involved in grinding hair samples and in removing sodium chloride by dissolution and filtration. Prior to application of ultrasonic homogenization, fineness of grind was definitely important in obtaining repeatable results; however, after ultrasonic homogenization it was found that fineness of grind did not affect repeatability of melanin value determinations (trial 2A). In view of these indications, trials were run to determine if grinding was necessary to achieve repeatability in the measurement of melanin granule concentrations.

Trial 3A. Melanin values of five samples from different locations on mink fur #7566 were determined both before and after grinding. Each sample was run in duplicate, and the percent variation was calculated. These results are given in Table 6.

Table 6. Melanin values of fur taken from different locations on standard dark mink pelt #7566 both before and after grinding--Trial 3A.

Sample Number	Unground Melanin Value	Unground Percent Variation*	Ground Melanin Value	Ground Percent Variation*
1A	126.3	1.12	126.1	.11
1B	127.7		126.2	
2A	155.4	2.05	154.3	.10
2B	152.2		154.1	
3A	114.7	1.45	114.1	.43
3B	113.0		113.6	
4A	131.1	0.35	131.6	.84
4B	130.7		132.8	
5A	128.4	0.85	129.2	.33
5B	129.5		128.8	

* Represents differences between duplicates divided by their mean.

These values indicate that the repeatability of these determinations was not seriously affected by elimination of grinding the hair when ultrasonic homogenization was employed. There was an increase in the overall mean variation between duplicates of unground samples (1.16%) when compared to ground samples (.36%). However, this variation was not regarded as excessive. This increased variation may be due to greater sampling error, since it is more difficult to get representative samples of guard and underfur from unground hair. These findings are not in agreement with results obtained prior to the use of ultrasonic homogenization where lack of grinding

resulted in incomplete hair digestion and consequently in poor repeatability. From this it was concluded that if ultrasonic homogenization is used, grinding of the fur samples is not required.

Trial 3B. Employing information gained from spectrophotometry and ultrasonic homogenization experiments, a trial was designed to determine the repeatability of the melanin determination procedure both before and after grinding. For this trial, approximately 20 g of fur were collected from mink pelt #7130; this fur was divided into two equal parts. One part was ground as before, and the other part was left unground. Fifteen melanin value determinations were run on each sample and means, standard deviations and coefficients of variation calculated (Table 7).

Table 7. Melanin values of mink fur samples (control #7130) with or without grinding--Trial 3B.

Ground	Unground
125.2	126.6
126.1	126.2
126.3	127.1
125.0	125.4
125.0	125.9
127.7	126.8
124.9	124.9
125.2	126.3
126.3	126.7
125.1	124.8
126.2	126.5
126.3	126.3
126.9	126.1
126.2	126.9
126.1	125.5

Table 7 continued

	Ground	Unground
Mean	125.8	125.9
Standard Deviation	.66	.78
Coefficient of Variation (%)	.52	.61

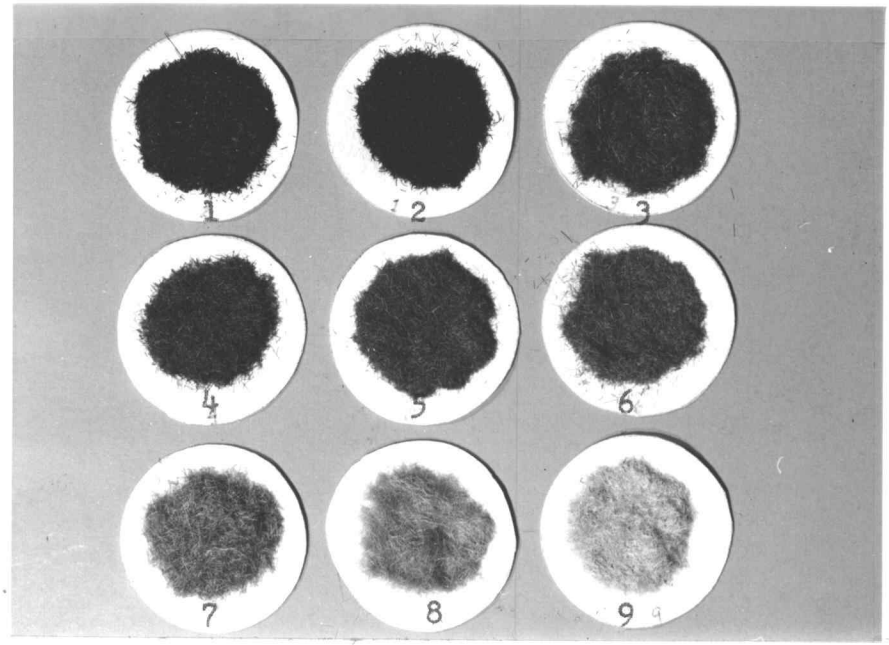
Results confirm those of trial 3A and indicate that measurement of melanin granule concentration on unground hair is as repeatable as measurement on ground hair when samples are homogenized ultrasonically.

Application

Results of the foregoing experiments support the underlying thesis that concentration of melanin granules in hair can be quantitatively analyzed. A necessary step in establishing an analysis for hair pigmentation intensity is to demonstrate that an increase in melanin granule concentration is actually related to increased hair pigmentation. Consequently, trials were conducted to determine the relationship between hair color and melanin granule concentration.

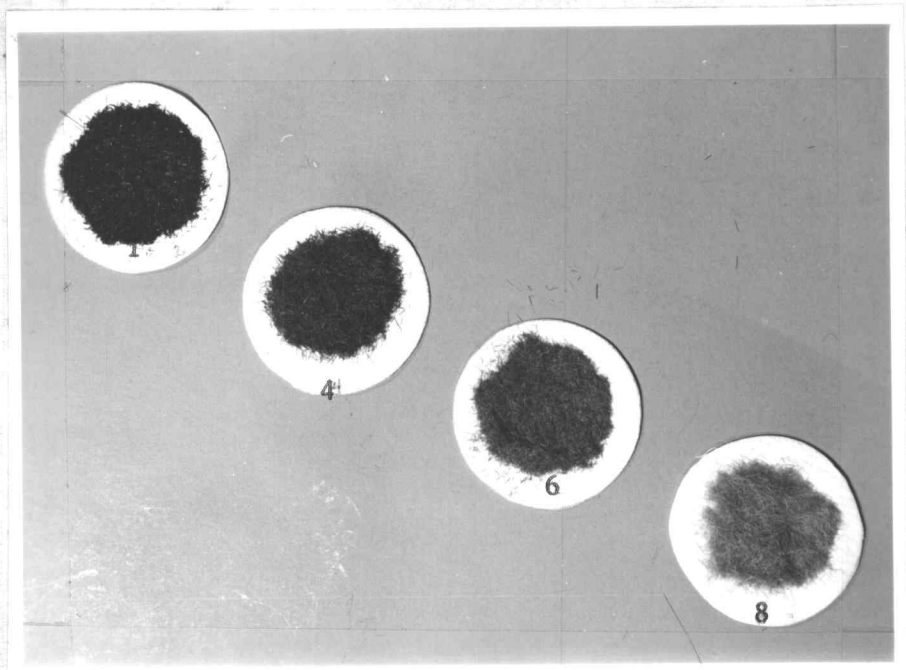
Trial 4A. In this trial, nine individual human hair samples ranging from light to dark in color were collected, defatted and arranged visually by color from darkest to lightest. A photograph of these ground hair samples is shown in Figure 5. Since only slight

variations were present in color intensity of samples three through seven, another photograph portrays the obvious color intensity differences of samples 1, 4, 6 and 8 (Figure 6). Melanin values by methods previously presented were determined for these samples and are given in Figures 5 and 6. Data in Figure 6 show that the decrease in melanin value corresponds to decreased hair pigmentation as determined visually.



Sample Number	Visual Ranking	Melanin Value
1	1	58.3
2	2	80.1
3	3	25.2
4	4	27.3
5	5	22.1
6	6	20.3
7	7	17.5
8	8	15.1
9	9	10.5

Figure 5. Melanin values of 9 ground human hair samples visually ranked in order of intensity of pigmentation--Trial 4A.



Sample	Melanin Value
1	58.3
4	27.3
6	20.3
8	15.0

Figure 6. Melanin values of ground human hair samples ranging from dark to light in color--Trial 4A.

Progressively decreasing melanin values were measured for hair samples one through nine (Figure 5). However, melanin values of samples one and two and of samples three and four were reversed as compared with visual rankings. Sample two was chosen as the darkest sample initially, but because sample one appeared more glossy it was subsequently placed above sample two. Analyzed melanin values show that sample two is considerably darker than sample one (Figure 5). This supports the hypothesis that the human

eye is unable to distinguish variations in color as it is influenced by other factors, such as glossiness.

A possible explanation for variation in results obtained on human hair is that it is often heterogeneous, with blond hair, for example, containing significant numbers of black hairs and black hair containing significant amounts of lighter pigmented hairs. These "off color" hairs alter the "melanin value" since they may contribute more or less to the optical density of a melanin granule suspension than to the hair color. Another possible explanation for variation lies in the fact that the samples may contain the red-yellow pigment, pheomelanin, which is soluble in weak bases (Rawles, 1944) and should not, theoretically, contribute to optical density of a melanin granule suspension but would render pigment to hair.

The melanin determination procedure was primarily designed for the purpose of evaluating differences in fur color in mink, and this trial is included only as evidence that there is a definite correlation between visual color differences of hair and melanin granule concentration.

Trial 4B. This trial was designed to further evaluate the relationship between analytical fur color measurement and visual color appraisal using standard dark mink pelts. Pelts were graded visually by a commercial fur grader using the following grading system: grade one for intensely dark pelts, grade two for medium dark pelts

and grade three for lighter pigmented pelts.

Ten dried mink pelts from each of the three color grades were randomly selected, and samples of fur from each were analyzed in duplicate to determine melanin concentrations. Resultant data were analyzed statistically by the Student t test (Steel and Torrie, 1960) to determine if there were significant differences in mean melanin values between each of the three grades. Results are tabulated in Table 8 and graphically presented in Figure 7.

Table 8. Melanin values of fur samples from standard dark mink pelts visually graded 1, 2 or 3--Trial 4B.

Fur Color Grade		
1	2	3
147.2	140.7	111.5
145.0	135.0	108.3
142.6	130.9	105.9
140.6	129.0	104.5
134.2	127.5	104.3
131.3	124.7	99.1
125.7	122.9	95.1
125.4	116.2	90.6
119.4	110.4	85.2
99.9	99.9	*
Mean Melanin Value 131.1	123.7	100.5
Standard Deviation 13.6	11.5	9.2

* One sample in grade 3 was not analyzed since insufficient hair was available.

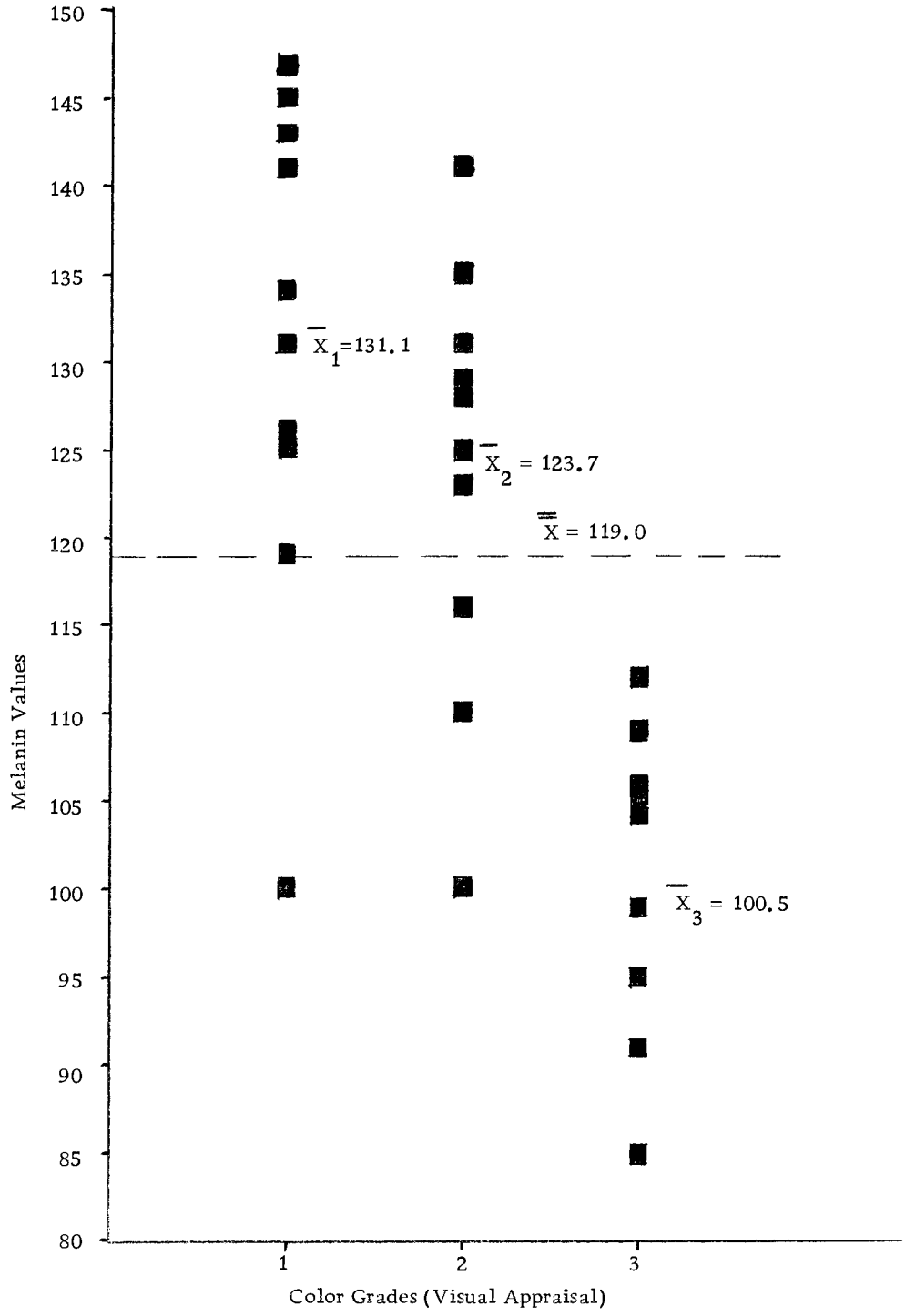
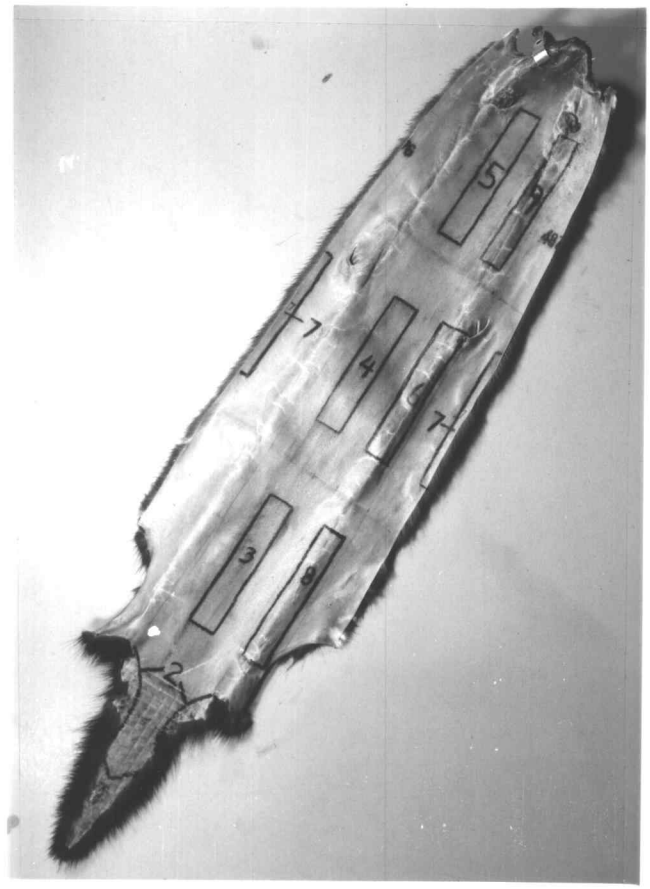


Figure 7. Melanin values of fur samples from standard dark mink pelts graded 1, 2 or 3 - Trial 4B.

Data in Table 9 indicate mink graded as number one generally have a higher melanin value than those graded as number two, although the difference was not significant statistically ($P < 0.15$). Color grades one and two were significantly higher than grade three ($P < 0.01$). Figure 7 illustrates the ranking of melanin values in relation to one another within each assigned color grade. The positive correlation between visual assessment of color and measured melanin values is evident, but considerable overlapping between grades occurs. The melanin value of one sample in grade one was very low and contributed especially to the lack of statistical significance between grades one and two. The reason for this extremely low value was not known.

These data suggest that chemical analysis of melanin granule concentration is useful in evaluating fur color quantitatively and is more sensitive than visual appraisal methods.

Trial 4C. In order to determine the extent of variation in fur color in relation to body location and in an attempt to determine a sampling site for future melanin analysis, a mink pelt was sectioned as show in Figure 8. Melanin values on unground fur samples from these sections were determined and are presented in Figure 8.



Sample Site	Melanin Value
1	153.8
2	113.8
3	130.9
4	129.0
5	133.5
6	105.3
7	90.5
8	101.3
9	97.8

Figure 8. Melanin values of mink fur (#7566) sampled at various locations on the pelt--Trial 4C.

The data indicate gross differences in melanin granule concentration are found in fur taken from various locations on a standard

dark mink pelt. The dorsal strip along the midline of the back was extensively pigmented while other locations were pigmented to a lesser degree (Figure 8). This extensively pigmented dorsal strip is present in most mammalian species although not always apparent to the untrained eye.

These results necessitate the establishment of a sampling site for hair color determinations; however, the data does not provide good evidence for the selection of a sampling site, since only one pelt was used and vast pigmentation differences were obtained. Further, the choice of a sampling area is limited to sites one or two (Figure 8), since sampling from other sites would result in damage to the pelts. Neither of these sites represent an area that is pigmented to approximately the same extent as the area observed during pelt evaluation, the dorsal strip. Additional research with more mink pelts is needed to map the pigmentation patterns of standard dark mink and to establish the optimum sampling site.

DISCUSSION

Color is a property of matter which seemingly can only be ascertained visually. Nevertheless, there is a definite physical basis for color, and consequently the problem of objective evaluation of color is amenable to chemical and physical evaluation. This thesis describes research designed to improve the repeatability and consequently the value of a technique designed to measure concentrations of melanin granules in hair, as indicative of the extent of pigment in hair. Data, as herein reported, indicate that original objectives have been accomplished and that the procedure may be extremely useful in measuring the intensity of hair pigmentation.

Underlying Principles and Assumptions

The analytical method for estimating hair pigment content as presented in this thesis assumes that intensity of hair pigmentation is primarily dependent upon melanin granule concentration and that factors apart from this have only lesser modifying effects. This assumption is supported experimentally by establishment of positive correlations between intensities of pigmentation in human hair and "melanin values" determined analytically (trial 4A). Further, serial dilutions of a melanin granule suspension as well as granule

suspensions prepared from a series of hair samples with decreasing weights resulted in linear decreases in melanin values (trials 1B and 2B). Collectively, these three trials supply good evidence that the assumption made concerning granule concentration and extent of pigmentation is well founded. These experimental findings agree with those of Russell (1946) and of McGrath and Quevedo (1965) but are not in accord with the findings of Maximow and Bloom (1945), who maintain that concentration of melanin granules has only a secondary effect on hair pigmentation and that other factors, such as diameter of the hair shaft and air space within the hair fiber, are of more importance.

Application

Lea (1954) originally utilized the basic principles of this method, i.e., the solubility of keratins in liquid urea and the concentrations of melanin granules found in variously pigmented hair samples, to quantitatively evaluate hair color in genetic studies. This procedure was later slightly modified and employed by Schleger (1962) to determine the extent of correlations between hair color and average daily gain in beef cattle. Subsequently, extensive modifications were made at Oregon State University (Stout and Costley, 1967), and the modified method has been used to determine effects of various feedstuffs on fur color in standard dark mink (Stout, Adair, Costley

and Oldfield, 1967a) and to evaluate changes that occur in the color of mink pelts resulting from dressing procedures applied to the pelts (Stout, Adair, Costley and Oldfield, 1967b).

Potentially, a useable method for measuring hair color could have wide application in assessing the effects of nutrition, environment or genetics on fur color in mink. Availability of such a tool would also make possible the determination of estimates of heritability of fur color intensity in mink as well as other mammalian species. Further, the procedure could be used to establish fur color standards for pelts of standard dark and conceivably other color types of mink. These standards could be beneficial in the marketing of pelts, since intensity of pigmentation in dark mink is an important economic consideration and heretofore has been evaluated subjectively.

Aside from measuring hair pigmentation, the technique might find application in the area of isolating melanin granules from hair or other tissue. Such a procedure has not been perfected, but it seems likely that it could be with additional research. If developed, it could be beneficial to those engaged in pigmentation research, especially in the areas of basic studies on melanin biosynthesis and abnormal pigment formation occurring as a result of certain metabolic abnormalities.

Advantages

Measurement of melanin granule concentration by the procedure reported herein is an objective estimation of pigmentation and therefore should be more sensitive and repeatable than visual appraisal. This conclusion is supported by experimental results which indicate that a general positive correlation exists between the analytical method and color grades determined visually; however, extensive overlapping of melanin values between color grades occurs. This points out that visual appraisal is able to distinguish definite pigmentation differences, as would be expected, but has difficulty in distinguishing slight differences, especially when factors of time and space are involved (trial 4B). Additional experimental evidence indicates that both extensive and slight pigmentation differences are found in the hair taken from various locations of the same pelt (trial 4C) and that this analytical procedure can distinguish these pigmentation differences which are not necessarily apparent to the eye.

Standardization of spectrophotometry and use of ultrasonic homogenization have contributed significantly to the potentially high repeatability and sensitivity achievable with this procedure. Further, utilization of ultrasonic homogenization made possible the reduction of sample preparation time by eliminating the necessity of grinding hair in order to obtain complete digestion. Experimental data on

this point indicate that elimination of grinding slightly increased the variability of results obtained, but this increase was not considered excessive (trials 3A and 3B).

This method for evaluating hair color is considered superior in several ways to other spectrophotometric methods of hair color analysis previously employed. It is better suited to large numbers of determinations than those methods described by Lea (1954) and Moos (1967). Further, because the procedure could conceivably be applied to either guard and underfur fibers together or separately, it appears to be more reliable, sensitive and useable than other spectrophotometric evaluations of hair color.

Disadvantages

A fundamental limitation of the procedure is that the "melanin values" derived are relative but not absolute; i. e., the technique is based on spectrophotometry rather than gravimetry. Therefore, the "melanin values" obtained will vary from one laboratory to another unless exactly the same spectrophotometric equipment is employed. It is possible, however, that standard curves could be established which would allow direct comparisons of "melanin values" from one laboratory to another.

In trials not reported, an attempt was made to establish standard curves based on weight of melanin granules per ml of suspension.

This would permit expression of the pigmentation on a weight basis. In these trials fur samples were digested, as described, and the liberated melanin granules were dialyzed for 24 hours to remove urea and other low molecular weight materials. The melanin granules were subsequently lyophilized and weighed. Results were not considered successful since high molecular weight, nondialyzable materials other than melanin granules, possibly residual keratins, were present in the dried samples. These residue substances appeared to be soluble in water and thus would not be expected to contribute to the optical density in the turbidimetric measurement of melanin granule suspensions.

Another possible error in the procedure arises from difficulty in obtaining representative samples of hair. This is especially true when analyzing small amounts of unground fur containing both guard and underfur fibers. This procedure, like other sensitive chemical procedures, is only as accurate as the sampling methods employed, and sampling error increases as smaller amounts of materials are used. Further, choice of sampling site is of extreme importance in fur color evaluations since definite pigmentation differences are known to occur on various locations of the same mink pelt (trial 4C). In this regard, additional research with more pelts is needed to determine a suitable sampling site for future determinations and also to determine the correlation between sample site and

pelt pigment.

The assumption that pigmentation of standard dark mink fur is entirely eumelanin may be a further source of error. It is entirely possible that pheomelanin, which is soluble in even weak bases, may be present and influence the visible intensity of fur pigmentation without contributing to the optical density of eumelanin granule suspensions.

With these possible errors being recognized, the research reported herein indicates that the procedure is more accurate and useable than other existing methods of hair color evaluation. Conceivably, it could be applied to numerous research problems as well as being employed for practical purposes in the area of fur production. However, additional research in the areas of establishing standard curves, determining concentrations of melanin granules by direct counts from variously pigmented hair samples, selecting an appropriate sampling site and perfecting melanin granule isolation procedures would substantially increase usefulness of the technique.

SUMMARY

Research in the areas of spectrophotometry, ultrasonic homogenization, sample preparation and method application was conducted to improve an existing method for measuring hair color. The following points summarize this research.

Spectrophotometry

The light absorption curve of an aqueous suspension of melanin granules isolated from standard dark mink fur is linear between 360 and 480 millimicrons and exhibits a decreasing optical density with shorter wave lengths. Consequently, the choice of wave length to be used for spectrophotometric measurement is arbitrary but must be specified. Within the tenfold concentration range studied, optical densities of melanin granule suspensions follow Beer's law. Further, the weight of a given hair sample, so long as it is known, is not important in determining "melanin values" since a linear relationship exists between sample weight and optical density values.

Ultrasonic Homogenization

Ultrasonic homogenization of melanin granule suspensions effectively separates clusters of melanin granules into uniformly suspended individual particles, which has the effect of increasing

granule surface area and consequently optical density of the suspension. This leads to increased repeatability and therefore increased sensitivity of the method. Homogenization for 1.5 minutes, at all intensity levels studied, resulted in maximum granule separation; treatment beyond 1.5 minutes had no further effect upon optical density values.

Sample Preparation

Research indicated that grinding of hair samples was unnecessary to achieve optimum digestion and granule liberation when ultrasonic homogenization was employed. When grinding was not employed, however, it was more difficult to obtain representative samples of fur, and variability of the procedure was increased slightly.

Method Application

The value of the analytical procedure for measuring melanin is that it is objective and has sufficient sensitivity to distinguish slight as well as pronounced differences in hair pigmentation. In relation to other methods currently being employed, it is more sensitive, repeatable and useable. The analytical melanin determination procedure, as described in this thesis, has both research and

practical application possibilities and has been found to be reliable, sensitive and highly repeatable when applied to representative samples of hair containing eumelanin as the primary pigment.

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APPENDIX

APPENDIX

I. Ether Extraction

1. Goldfisch Extractor 1164
   Laboratory Construction Company
   Kansas City, Missouri.

II. Digestion

1. Silicone Oil
   Silicone Products Department
   General Electric Co.
   Waterford, New York .

2. Precision Electronic Relay Control Box P-10
   Precision Scientific Company
   Chicago, Illinois.

3. Micro-Set Thermoregulator V-7
   Precision Scientific Company
   Chicago, Illinois.

4. Precision Electric Heaters Cat. 33890 and 33891
   Precision Scientific Company
   Chicago, Illinois.

5. Powerstat 116
   The Superior Electric Co.
   Bristol, Connecticut.

III. Ultrasonic Homogenization

1. Bronwill Biosonik II
   High Intensity Ultrasonic Probe
   Bronwill Scientific
   Rochester, New York.

IV. Spectrophotometry

1. Carl Zeiss Spectrophotometer PMQ2
   Carl Zeiss, Inc.
   New York, New York.