# The Use of Spectrophotometry to Estimate Melanin Density in Caucasians[1]

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## Abstract

**The density of cutaneous melanin may be the property of the skin that protects it from damage by solar radiation, but there is not an accepted, noninvasive method of measuring it. To determine whether the density of cutaneous melanin can be estimated from reflectance of visible light by the skin, reflectance of 15-nm wavebands of light by the skin of the inner upper arm of each of 82 volunteers was measured at 20-nm intervals with a Minolta 508 spectrophotometer. A 3-mm skin biopsy was then taken from the same site, and four nonserial sections of it were stained with Masson Fontana for melanin. The melanin content of the basal area was calculated using the NIH Image analysis system. We show that cutaneous melanin in Caucasians can be estimated by the difference between two measurements of reflectance of visible light by the skin: those at wavelengths 400 and 420 nm. This new spectrophotometric measurement was more highly correlated ($r = 0.68$) with the histological measurements of cutaneous melanin than was skin reflectance of light of wavelength 680 nm ($r = 0.33$). Reflectances in the range of 650–700 nm have been used previously in skin cancer research. This relatively accurate measurement of melanin is quick and noninvasive and can be readily used in the field. It should provide improved discrimination of individual susceptibility to epidermal tumors in Caucasians and information about melanin's biological role in the causation of skin cancer.**

## Introduction

Researchers in several fields have been interested in estimating the concentration and type of melanin in tissues of epidermal origin, including the skin, hair, and iris. For anthropologists, the interest has been in tracing the geographic and cultural origins of individuals and populations (1). In medicine, this interest has principally stemmed from the desire to determine susceptibility to epidermal tumors (2).

The risk of melanoma is determined by sun exposure and by skin phenotype. The observation of differences in risk of melanoma between racial groups, with Caucasians having a much higher incidence of melanoma than Africans or Asians, strongly supports this inference about the role of skin phenotype. It also seems probable that, among Caucasians, risk of both melanoma and other skin tumors differs by skin phenotype.

Measurable properties that could reasonably be thought to be related to or to characterize skin phenotype include eye color, hair color, and skin color. Those properties have been used as indicators of skin phenotype because they appear to be clear markers of the type of biological difference that distinguishes Caucasians from lower-risk populations. Although the basis for the choice of measurements has not usually been made explicit, it is likely that they have been used as proxies for melanin density or melanin type in the skin.

There have been attempts to estimate melanin density in the skin using reflectometers. As early as 1939, Edwards and Duntley (3) demonstrated that the reflectance of light in the visible spectrum (400–700 nm) is influenced by the presence of melanin, as well as by hemoglobin and carotene. Melanin was found to absorb more light at shorter wavelengths and to exhibit steadily decreasing absorption up to 700 nm. Absorptions by hemoglobin and by carotene were more intermittent, with peaks at 418–429 nm and 542–576 nm (hemoglobin) and at 482 nm (carotene). The subsequent development of noninvasive skin reflectance measurements of melanin density focused on reflectance at wavelengths of 650–700 nm in the belief (4) that, although absorption by melanin at those wavelengths was relatively low, the total absorption of light could be attributed solely to the presence of melanin because there was no contribution by hemoglobin or carotene. The extent to which melanin is truly estimated by reflectance at those wavelengths or any other has not been previously assessed by direct comparison with actual melanin density in the skin.

Here, we compared the epidermal concentration of melanin, analyzed histologically in skin biopsy specimens, with skin reflectance, measured by a spectrophotometer, to find the best reflectance estimate of melanin density. The skin biopsy and the spectrophotometric measurement for each subject were obtained from a site on the inner upper arm, an area not usually exposed to the sun. This site was defined as the midpoint of a line drawn from the epicondyle of the humerus to the point where the axilla joins the upper chest. We also examined the correlation of the commonly used proxies for skin phenotype, eye, hair color, and self-perceived skin type with the direct measurement of cutaneous melanin density.

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## Subjects and Methods

To recruit participants, leaflets were placed in specialist clinics at the public hospital in Hobart, Tasmania, Australia. The 82 volunteer subjects were visitors and staff, not patients, of the hospital.

Reflectance by the skin of wavebands of light that were 15 nm wide was measured at 20-nm intervals in the wavelength range of 400–700 nm with a Minolta 508 spectrophotometer. Measurements were taken with the arm set at heart height and with the head of the spectrophotometer resting on the skin so that there was a complete seal with the skin surface but no depression of the skin (to avoid surface blanching or flushing). The spectrophotometer output also included the $L^*$ value, the measurement of lightness of color across the visible light spectrum, used with the Commission International d'Eclairage system of color assessment (5).

A 3-mm punch biopsy was then taken at the site of reflectance measurement. Biopsies were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned at 4 $\mu$m. Four nonserial sections were taken at approximate 400-$\mu$m intervals and stained using the Masson Fontana method for melanin (6). To obtain an optimal specimen for use in image analysis, the Neutral red counterstain was omitted. For quantitative studies on the histological material, image acquisition and analysis were performed using a Macintosh Power 7600 computer connected to a Sony monochrome charge-coupled device camera on an Olympus BH2 microscope and using the public domain NIH Image program (available from http://rsb.info.nih.gov/nih-image). From each section, using the ×20 objective, 4–10 fields were captured as grayscale images. The thresholding function of NIH Image was used to select the stained melanin pigment. The area of pigment was measured in $\mu m^2$, and its density was calculated as a proportion of the total area of the deep layers epidermis (corresponding to the first two cell layers above the epidermal-dermal junction). All samples were quantified by the same operator, who was blind to other data on the subjects.

Self-reported information on eye color (brown, hazel or green, and blue), hair color (black, dark brown, midbrown, "mousy" brown, blond, and red), untanned skin color (dark, olive, olive/medium, medium/fair, and fair), skin reaction to 1st h of sun exposure (no burn at 24 h then good tan after 1 week, tender burn then moderate tan, painful burn then light tan, and painful burn then no tan), depth of tan at the end of summer (dark, medium, light, and practically none), and freckling (7-point scale from none to heavy) was gathered by questionnaire.

In data analysis, the reflectance readings were averaged across three repetitions, which allowed within-person variation to be estimated. The measurements of melanin and of the basal area were aggregated across sections, and the density of melanin was calculated by dividing the total melanin by the total basal area. Linear regression analysis was used to model the association of melanin density with the averaged reflectance readings. The extent of linear association was summarized by correlation coefficients and by the proportion of total variance in melanin density explained by the regression model. The ordered categories of self-reported phenotypic characteristics were assigned consecutive, ascending integer scores.

## Results

The characteristics of the 82 subjects recruited into the study are reported in Table 1. The distributions of eye color and hair color at age 5 years were similar to those we found in a random sample ($n$ = 410) of the 14–15-year-olds in this predominately Anglo-Celtic population (7), 90% of which traces its ancestry to the British Isles.

*Table 1* Characteristics of the study sample of 82 adults

| Characteristic | No. of subjects |
|---|---|
| Sex | |
| Female | 55 |
| Male | 27 |
| Age (yr) | |
| 21–39 | 31 |
| 40–49 | 27 |
| 50–69 | 24 |
| Eye color | |
| Brown | 11 |
| Hazel | 18 |
| Green | 8 |
| Blue | 45 |
| Hair color (at age 5) | |
| Dark | 19 |
| Mid brown | 10 |
| Light brown | 20 |
| Blond | 25 |
| Red | 8 |
| Hair color (at age 25) | |
| Dark | 27 |
| Midbrown | 21 |
| Light brown | 19 |
| Blond | 7 |
| Red | 8 |
| Self-assessed untanned skin color | |
| Olive | 5 |
| Olive/medium | 17 |
| Medium/fair | 38 |
| Fair | 11 |
| Skin reaction to 1st h of sun | |
| No burn/good tan | 8 |
| Tender burn/moderate tan | 42 |
| Painful burn/light tan | 17 |
| Painful burn/no tan | 14 |
| Depth of tan at end of summer | |
| Dark | 20 |
| Medium | 39 |
| Light | 10 |
| Practically none | 12 |
| Freckling of face (as a child) | |
| Grade I | 20 |
| Grade II | 28 |
| Grade III | 20 |
| Grade IV | 9 |
| Grade V | 3 |
| Grade VI (severe) | 1 |
| Freckling of back/shoulders (as an adult) | |
| Grade I | 5 |
| Grade II | 29 |
| Grade III | 15 |
| Grade IV | 22 |
| Grade V | 6 |
| Grade VI (severe) | 4 |

The associations of melanin density and reflectance measurements were first examined on subjects recruited before June 1996. For these 32 subjects, the strongest association with a single reflectance explained only 31% of the variance in the melanin density measurements, but a regression model containing a term for reflectance at 400 nm and a term for reflectance at 420 nm explained 43% of the variance. Including further reflectance measurements at other wavelengths in the regression model produced only minor improvements in fit. The best

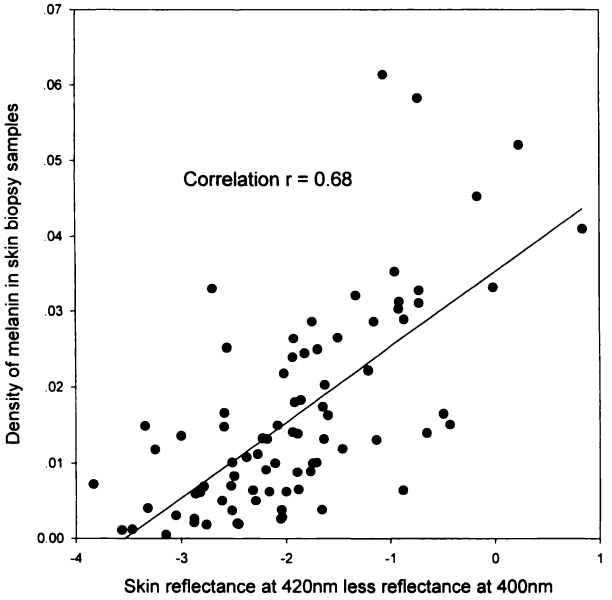


*Fig. 1.* Cutaneous melanin density and difference of skin reflectances at 400 and 420 nm for 82 healthy volunteer subjects. Melanin density was calculated as the proportion of the total area of the deep layers of the skin sections that were stained as melanin.

three-predictor model (reflectances at 400, 420, and 500 nm) explained 46% of variance, and the best four-predictor model (reflectances at 400, 420, 460, and 480 nm) explained 47% of the variance. In all models, the coefficient of reflectance at 400 nm had the expected negative sign. When included in a model with reflectance at 400 nm, the coefficient of reflectance at 420 nm became positive. The sum of these coefficients was almost zero, and imposing a linear restriction so that they summed to zero produced little change in the fit of the regression model ($P = 0.52$). Their difference (reflectance at 420 nm minus reflectance at 400 nm) was chosen as the most parsimonious predictor of melanin. It explained a similar proportion of variance as the separate terms for reflectances at 400 and 420 nm.

These results were confirmed in the 50 subjects recruited from June 1996 to November 1996. For this group, the best single predictor of melanin was reflectance at 400 nm. It explained 39% of the variance. The best two-variable model again contained terms for reflectance at 400 and 420 nm and explained 57% of the variance. Including further terms produced only minor improvements in fit, the sum of the coefficients was zero ($P = 0.87$), and the difference term provided a good fit.

For all 82 subjects, the linear regression model, containing a term for reflectance at 400 nm and a term for reflectance at 420 nm as the only predictors, explained 48% of the variance. In stepwise regression, with reflectance measurements and the other indicators of phenotype as candidates for inclusion, the first two variables entered into the model were terms for reflectance at 400 nm and reflectance at 420 nm. Hair color at age 5 years was added to form the best three-term model (50% of variance explained), and hair color at age 25 was added to form the best four-term model (51% of variance explained).

Fig. 1 displays the association between the estimated melanin density of skin biopsy samples and the difference of reflectances at 400 and 420 nm for all 82 subjects. The correlation coefficient for linear association was $r = 0.68$. A square root transformation of melanin density would have produced

*Table 2* Correlation coefficients for the linear association of histologically determined melanin density in skin biopsy samples with measurements of skin reflectance at the same site

| Measurement of reflectance | All 82 subjects |
|---|---|
| Previously used reflectance measurements | |
| Reflectance at 680 nm | −0.33[a] |
| $L^*$ for 400–700-nm spectrum | −0.46[b] |
| Measurement suggested by our analysis | |
| Reflectance at 420 nm minus reflectance at 400 nm | −0.68[b] |

[a] $P < 0.01$.
[b] $P < 0.01$.

*Table 3* Correlation coefficients for the linear association of histologically determined melanin density and the difference of reflectance measurement with common indicators of phenotype

| Indicator of phenotype | Melanin density | Reflectance at 420 nm minus reflectance at 400 nm |
|---|---|---|
| Eye color | −0.01 | −0.09 |
| Hair color | 0.05 | −0.11 |
| Self-assessed untanned skin color | −0.38[a] | −0.50[b] |
| Skin reaction to 1st h of sun exposure | −0.42[b] | −0.53[b] |
| Depth of tan at end of summer | −0.39[b] | −0.46[b] |
| Freckling | | |
| Face (as a child) | −0.25[c] | −0.23[c] |
| Back/shoulders (as an adult) | −0.20[a] | −0.12 |

[a] $P < 0.01$.
[b] $P < 0.001$.
[c] $P < 0.05$.

normally distributed residuals and a marginally higher correlation coefficient ($r = 0.69$). The correlations were similar in females ($r = 0.64$) and males ($r = 0.80$). Within-subject variation accounted for 1.37% of the total variation in the three measurements of the difference of reflectance measure, and the mean, median, and minimum and maximum values of the SDs of the repeated measurements on each subject were 0.082, 0.062, 0.012, and 0.412 respectively.

This correlation is compared with those for the other reflectance measurements that have been used to estimate skin phenotype in Table 2. For measurements of skin reflectance at wavelengths in the 650–700-nm range, the strongest association with melanin density was for reflectance at 680 nm. Both it and the summary measurement of skin lightness, the $L^*$ value, were associated with melanin density, but neither was as strongly associated as was the difference of reflectances at 400 and 420 nm.

Table 3 summarizes the associations of other measurements that have been used to indicate skin phenotype with melanin density measured directly and by the difference of reflectances at 400 and 420 nm. The melanin density measurements and the difference of reflectance measurement were more closely associated with self-reported skin color and skin type (skin reaction to 1st h of sun and depth of tan at the end of summer) than they were with eye or hair color. Freckling is not purely an indicator of phenotype, being influenced by sun exposure as well (8). Nonetheless, associations with it were not as strong. The associations of skin color and skin type with the previously used reflectance measurements (data not shown)

were weaker than those for melanin density and the difference of reflectance measurement.

## Discussion

This direct comparison with histological measurement of epidermal melanin showed that it is possible to estimate cutaneous melanin using reflectance by skin of light within the 400–700-nm range. Reflectance in the waveband 650–700 nm was found to be associated with melanin density. The $L^*$ value, which integrates reflectance across the visible light spectrum (400–700 nm), also provided a reasonable estimate of melanin density. The measurement of choice, though, was that which used information from two wavebands from the lower end of the visible light spectrum. This measurement, the difference of reflectance at 400 and 420 nm, explained around half of the variance in melanin density.

This is probably an underestimate of the true association because of measurement error in the histological determination of epidermal melanin. A review of the imaged biopsy samples of three subjects produced recalculations of melanin density that, on average, differed by 24%, whereas an analysis of the spectrophotometer readings indicated that the measurement error in the difference of reflectance measurement was only about 1% of the total variance in this measure.

The importance of the 400- and 420-nm wavelengths in predicting melanin density can best be explained by referring to what is known about the absorption of visible light by the skin. Absorption at those wavelengths is principally due to the presence of cutaneous melanin and hemoglobin. There is a lesser contribution by carotene. Melanin absorbs more light at 400 nm than it does at 420 nm, but absorption by hemoglobin is high at either wavelength. We assume that by subtracting the spectrophotometric estimate of reflectance at 420 nm from that at 400 nm, we remove the contribution by hemoglobin, which is similar at both wavelengths. The difference is the reflectance, which is principally determined by the absorption by the melanin in the skin. If this is so, the confounding effect of absorption by hemoglobin at either 400 or 420 nm has been substantially eliminated by taking the difference of reflectances at the two wavelengths.

If melanin is the relevant property of the skin that protects it from damage by solar radiation, the difference of reflectance measurement should provide improved discrimination of individual susceptibility to epidermal tumors, at least in populations of northern European ancestry. The next step is to determine whether this measurement of melanin density, which can be readily applied in the field, predicts risk of melanoma and nonmelanoma skin cancer.

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