Color of Cultures of *Staphylococcus epidermidis*
Determined by Spectral Reflectance
Colorimetry

RICHARD W. BROWN

National Animal Disease Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa

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ABSTRACT

BROWN, RICHARD W. (National Animal Disease Laboratory, Ames, Iowa). Color of cultures of *Staphylococcus epidermidis* determined by spectral reflectance colorimetry. J. Bacteriol. 91:911-918. 1966.—A colorimeter with a reflectance attachment was used to study pigment production by *Staphylococcus epidermidis* strains grown on a medium containing Trypticase Soy Agar (BBL) and cream. The color of each culture was first characterized by reflectance colorimetry for dominant wavelength, purity, and luminous reflectance \(Y\) and was then classified visually into 1 of 10 color grades. There was not complete agreement in grading colors by the two methods, inasmuch as cultures were considered more pigmented in relation to other cultures by the reflectance method were sometimes graded visually as less pigmented, and vice versa. Nevertheless, when the cultures were visually graded as being more pigmented, there was a concomitant increase in the average values of dominant wavelength and purity with a decrease in \(Y\) for the cultures in each higher grade. Thus, the nonpigmented cultures had the lowest dominant wavelength and purity values but the highest \(Y\) (brightness) values, whereas the most pigmented cultures had the highest dominant wavelength and purity values, but the lowest \(Y\) values. These results indicated that the cultures did not produce pigments of different hues (greenish-yellow, yellow, yellowish-orange) each with high, medium, and low degrees of purity and brightness. The value \((I - z)\), where the chromaticity coordinate \(z = Z/(X + Y + Z)\), was found to be proportional to the purity value. An inverse relationship between the tristimulus \(Z\) and purity values was also demonstrated. All cultures tested by the reflectance method were also classified according to the type of spectral absorption curve obtained with pigments extracted from the cultures with methanol. A comparison of these methods indicated that determining the type of spectral absorption curve would be better for differentiating strains of *S. epidermidis*, whereas the use of the reflectance method would be better for determining differences of pigment production within strains.

In a previous study, strains of *Staphylococcus epidermidis* isolated from bovine udders were differentiated on the basis of the absorption spectra of the pigments extracted with methanol (3). The method demonstrated qualitative differences in the pigments and, to some extent, quantitative differences in pigment production, but it did not provide information on the color of the colonies. Sompolinsky (4) used standard color cards to measure the shade and intensity of the color of colonies for differentiating strains of *S. aureus*. This method provides a means of defining the color of a colony better than the use of a few selected names. It is, nevertheless, still subjective, and the results are affected by lighting conditions and the observer's ability to make fine color distinctions or even distinctions between certain colors. In addition, any color-mixture system provides spaced scales of color which require estimations of those colors that do not match the color standards. Although an objective method of defining colors numerically by color-
metry has been available for many years, it has been used primarily in certain industries (i.e., textile, paint, food). Recent publications indicate the value of this method in studying the taxonomy of streptomyces (2) and birds (Zeiss Information No. 51, 1964).

This report describes a method for using spectral reflectance to define numerically the color of cultures of *S. epidermidis* grown on an agar medium. The value and limitations of the method for differentiating strains of *S. epidermidis* are also discussed.

**Materials and Methods**

*Cultures*. The cultures of *S. epidermidis* used in this study were isolated from milk samples obtained from an experimental herd of 20 cows sampled at weekly intervals. The methods of isolation, identification, and storage have been described (3). The organisms were also tested for pigment production by the method of analyzing spectrophotometrically the methanol-extracted pigments (3).

*Media*. The medium used for pigment production consisted of Trypticase Soy Agar (BBL) with cream and was suggested by Nancy O. Sturgan, Department of Veterinary Science, Pennsylvania State University, University Park. Cream was obtained from raw milk (Holstein-Friesian) that was collected aseptically from an udder that was not infected, and was then placed in a 2-liter separatory funnel and held at 4°C overnight. The cream was dispensed in 50-ml volumes in 125-ml Erlenmeyer flasks and was sterilized by heating at 100°C for 15 min. The Trypticase Soy Agar was prepared by adding 10 g of medium to 200 ml of distilled water in a 500-ml Erlenmeyer flask and then autoclaving at 121°C for 15 min. The cream and agar were held in a water bath at 50°C before mixing and pouring into glass (100 mm in diameter) or plastic (60 mm in diameter) petri plates. The plates were inoculated at 37°C for 20 hr and were then inoculated or held at 4°C. The maximal period of storage before use was 3 weeks.

The surface of the cream-agar was scored at six places with a no. 7 stainless-steel cork borer (13 mm in diameter) which was sterilized by flaming before use on each plate. Approximately 0.01 ml of a 20-hr beef infusion broth culture was spread within a scored area with either a 1-ml or a Pasteur pipette. The former was preferred, because of greater ease in spreading the liquid. The inoculated plates were incubated at 30°C for 48 hr. The pigmented areas were separated from the surrounding medium by cutting through the agar with a no. 12 cork borer (21 mm in diameter); they were then removed with a stainless-steel spatula (14-mm blade diameter). Each agar wafer was inverted on a metal washer (outer diameter, 35 mm; inner diameter, 14 mm; thickness, 2.5 to 3 mm) with the pigmented area positioned over the hole. To permit easier handling of the agar wafers, media should be at least 3 mm thick.

*Color measurement*. A Bausch & Lomb Spectronic 20 colorimeter with a color-analyzer reflectance attachment was used according to the method described in the Bausch & Lomb instrument reference manual. The washer with the inverted agar wafer was placed over the measuring aperture and was centered by aligning a line marked on the washer with one marked on the machine. Outside light was excluded by placing a light-proofing cap (inner diameter, 35 mm; depth, 64 mm) over the washer. The small clearance between the washer and the cap was an additional aid in centering the sample.

A number of cultures (38) were tested at one time. Based on the results obtained, 24 cultures were selected from this group and were retested after 3 months of storage on Tryptose Agar (Difco) slants at 4°C. After the reflectance measurements were completed in both tests, each culture was graded visually by two persons for the amount of pigmentation, and was classified by mutual agreement into one of 10 color grades ranging from nonpigmented (grade 1) to the most pigmented (grade 10). After grading, the agar wafers were refrigerated overnight, and their colors were then classified with Munsell high-gloss color standard chips (1.7 x 2 cm; Munsell Color Co., Baltimore, Md.). Reflectance measurements were made of selected Munsell color chips.

**Definitions**. The three characteristics used to describe a color by reflectance spectrophotometry are dominant wavelength, purity, and luminous reflectance (*Y*), shown in Table 1. Dominant wavelength indicates what part of the spectrum has been mixed with some neutral standard to match a given color. Purity indicates the degree of approach of this color to the spectrum color designated by the dominant wavelength. Thus, a color designated by a dominant wavelength of 600 nm and a purity of 50% would indicate an orange hue of about half the saturation of spectrum orange. Luminous reflectance is the ratio of reflected to incident luminous flux and can be considered a measure of brightness or lightness. The tristimulus values (*X*, *Y*, and *Z*) are the amounts of the three reference stimuli (red, green, and blue) required to give, by additive combination, a match with the color considered, and were calculated from reflectance data by use of the Bausch & Lomb trichromatic computer program. The chromaticity coordinates (*x*, *y*, and *z*) are computed from the tristimulus values as fractions of their totals; e.g., *x* = *X*/(*X* + *Y* + *Z*). The preceding information was taken in part from Judd and Wyszecki (1). The dominant wavelength and purity values were computed by plotting *x* and *y* on a chromaticity diagram enlarged photographically 3.25 times the diagram illustrated in the Bausch & Lomb reference manual. The tristimulus *Y* value was used as a measure of luminous reflectance (brightness). Chromaticity diagrams (45 x 50 cm) for plotting *x* and *y* are available from the Munsell Color Co.

Munsell notation of a color is written in the form hue value/chroma, such as 2.5 Y 8/6, which indicates a Munsell hue of 2.5 yellow, a Munsell value of 8/6, and a Munsell chroma of 8/6. Hue can be described in terms of the adjectives red, green, blue, etc., and is analogous to dominant wavelength. The value notation indicates the degree of lightness or darkness of a
TABLE 1. Growth characteristics and objective color descriptions of two strains of Staphylococcus epidermidis isolated four times at weekly intervals from bovine udders

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>Isolate</th>
<th>Type of growth*</th>
<th>Munsell color notation</th>
<th>Dominant wavelength†</th>
<th>Purity</th>
<th>Y</th>
<th>Type of spectral absorption curve‡</th>
</tr>
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<tr>
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<td></td>
<td>SM</td>
<td>2.5Y 8/10</td>
<td>578</td>
<td>.590</td>
<td>46.84</td>
<td>IV</td>
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<tr>
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<td></td>
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<td>—</td>
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<td>.650</td>
<td>44.08</td>
<td>IV</td>
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<td>—</td>
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<td>.622</td>
<td>45.87</td>
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</tr>
<tr>
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<td></td>
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<td>.616</td>
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<td></td>
</tr>
<tr>
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<td></td>
<td>.31</td>
<td>.027</td>
<td>1.27</td>
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<tr>
<td>5076 D</td>
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<td>rG</td>
<td>10YR 8/10</td>
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<td>.028</td>
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</table>

* Type of growth on cream-agar. Abbreviations: S = smooth surface; r = slightly rough surface; M = mat finish; G = glossy finish.
† Estimated to nearest 0.25 μm.
‡ Literature Cited (3).

color in relation to a neutral gray scale, which extends from a theoretically pure black (0/) to a theoretically pure white (10/), and is analogous to the tristimulus Y value. The chroma notation indicates the strength (saturation) or degree of departure of a particular hue from a neutral gray of the same value and is analogous to purity. The information on the Munsell system of color notation was taken in part from their bulletin on publications, materials, and equipment.

RESULTS

Growth and pigmentation characteristics. Fifty-one cultures grew with a smooth surface, whereas 7 cultures grew with a slightly rough or irregular surface. The surface finish of the growth was either glossy or mat, and classification of the 58 cultures showed 31 glossy, 20 mat, and 7 with a combination of both finishes. When 24 of these cultures were retested, 17 produced the same finish, 6 changed from mat to glossy, and 1 changed from glossy to mat.

Two forms of irregular pigmentation also were observed. One group of five cultures showed most of the growth area uniformly pigmented, except for a peripheral ring of darker pigment. These cultures were not used for comparative studies. Another group of nine cultures showed an interlacing of lighter and darker pigmented areas. In seven cultures, the light areas showed a mat finish, and the dark areas a glossy finish.

Comparative studies in grading. To determine whether the reflectance method was measuring differences in the pigmented cultures commensurate with the visual method of grading, the dominant wavelength and purity values of the cultures in each grade were averaged and plotted for each parameter (Fig. 1 and 2). Although there is considerable overlapping of values among cultures that showed similar pigmentation, the data presented indicate that the dominant wavelength and purity values increased as the pigmentation increased. In the first test, the average values were less for both parameters in grades 6 and 8 than the average values in the next lower grades of 5 and 7, respectively.

To determine whether this would occur again, the 19 cultures that comprised grades 4 through 8, and 1 culture from each of the other 5 grades, were retested. These results are shown as the second test averages in Fig. 1 and 2. In contrast to the results of the first test, a progressive increase in average values was found for both parameters in grades 5 through 8. The decrease in dominant wavelength values between grades 3 and 4 (Fig. 1) and purity values between grades 8 and 9 (Fig. 2) are not significant because of the small numbers involved, and only emphasize the lack of complete agreement between visual grading and the spectral reflectance method when classifying colors that show small differences. Possible sources of error that can cause
colors to be graded differently by the two methods are discussed later.

An inverse relationship was found between the tristimulus Y values (brightness) and the amount of pigmentation. The highest average Y values occurred in grade 1 and decreased progressively with each grade to the lowest average in grade 10. The exceptions were grades 6 and 8 in the first test group and grade 9 in the second test group, wherein the average Y values were higher than those in the next lower grade. The inverse relationship between brightness and saturation of the pigments is shown by comparing Y and purity values in Fig. 3.

To determine the reproducibility of the method with strains isolated repeatedly from the same source of infection, two cows infected with S. epidermidis in one quarter of each udder were studied. Milk samples were taken from each infected quarter four times at weekly intervals and were inoculated on bovine blood agar plates. After incubation, a representative colony of each was picked and inoculated into a tube of Trypticase Soy Broth which was incubated and then used to inoculate the cream-agar. Spectral absorption curves of methanol-extracted pigments were also determined for each isolate.

The results of this study are presented in Table 1. The isolates of strain 5076D showed greater variation than the isolates of strain 4950B for all three characteristics; however, the standard deviations for both strains indicate that the method is quite reproducible when different isolates of the same strain are tested. Higher standard deviations were obtained when 24 different strains (Fig. 1 and 2) were each tested twice. The standard deviations of the differences between the first and second tests of the 24 strains were 0.99, 0.040, and 1.80 for dominant wavelength, purity, and Y, respectively. The variation was greater than that shown for the cultures in Table 1 and might be attributed to the fact that the 24 cultures were stored 3 months before retesting.

The visual classification of pigmentation of the two strains did not agree with that deduced from the reflectance measurements. On blood-agar, the colonies of culture 4950B were less pigmented than those of 5076D. The Munsell color chip selected as the best color match for each culture on the first test also indicated that 4950B was less pigmented on cream-agar. This was based on the fact that 2.5Y represents a yellow hue and
10YR a yellowish-orange hue. The difference in hues was not reflected in higher dominant wavelength values for 5076D. The fact that the same value/charoma notation (8/10) was used for both hues suggested that the Y and purity values of both cultures would be similar. This was true for the Y values; however, 4950B gave higher purity values in all tests, and consequently would be classified as more pigmented than 5076D by the spectral reflectance method.

Purity value substitutes. One drawback to the reflectance method for routine testing of pigmented cultures is the necessity of calculating the chromaticity coordinates and then plotting them on a chromaticity diagram to determine the dominant wavelength and purity values. According to Judd (personal communication), $(I - z)$ is proportional to purity for yellow-green to red colors, where the chromaticity coordinate $z = Z/(X + Y + Z)$. This relationship was valid for the cultures of S. epidermidis tested (Fig. 4), and indicated that $(I - z)$ can be used as a measure of purity. Substituting tristimulus Z values for $(I - z)$ indicated that Z also could be used as a measure of purity (Fig. 5). Since the relationship for Z is inverse, an increase in Z would indicate a decrease in the purity of a color; however, a direct proportion could be obtained by using $(100 - Z)$.

Similar curves with different slopes resulted when tristimulus Z vs. purity values were plotted for Munsell color standards 2.5Y 7/2 to /12, 8/2 to /16, and 8.5/2 to /12 (Fig. 6). Other Munsell color standards (5Y 8.5/1 to /14, 10YR 7/1 to /14 and 8/1 to /14, 7.5YR 8/2 to /8), regardless of the hue, produced curves that could be superimposed on those curves in Fig. 6 that had the same Munsell values (7/8, 7/1, and 8.5/1). This was not true when the $(I - z)$ values for the Munsell color standards listed above were plotted against the purity values. All $(I - z)$ values fell along the line presented in Fig. 4, regardless of the hue or Y values. Consequently, the use of Z values as a comparative measure of purity would be useful only if the colors had similar Y values, whereas $(I - z)$ values could be used as a measure of purity regardless of the Y values.

A comparison of the curve in Fig. 5 with those in Fig. 6 indicated that the cultures of S. epidermidis produced pigments with Y values similar to Munsell color standards of 7/ value. However, the Munsell color standards selected as providing the best visual match for the cultures had 8/, 8.5/, and 9/ values. No explanation can be given for this discrepancy.

Spectral absorption curve comparisons. A number of cultures (56) tested for pigment production on cream-agar were also classified according to

FIG. 3. Relationship of the tristimulus Y values (brightness) and purity values (saturation) of pigments produced by cultures of Staphylococcus epidermidis grown on Trypticase Soy Agar-cream.

FIG. 4. Relationship of $(I - z)$ and purity values of pigments produced by cultures of Staphylococcus epidermidis grown on Trypticase Soy cream agar, where the chromaticity coordinate $z = Z/(X + Y + Z)$. 
FIG. 5. Relationship of the tristimulus Z and purity values of pigments produced by cultures of Staphylococcus epidermidis grown on Trypticase Soy Agar-cream.

FIG. 6. Relationship of the tristimulus Z and purity values of Munsell Color standards of the same hue (2.5 Y) but different values (7/, 8/, 8.5/) and chromas (/2 to /16).

the type of spectral absorption curve given by the methanol-extracted pigments. The range and average purity values for the organisms giving each type of curve are presented in Fig. 7. The nonpigmented cultures gave a type I curve. All methanol extracts from the cultures that gave types II and VI curves had to be concentrated three times to provide satisfactory curves. This indicated poor pigment production, which was also shown by most of these cultures on the cream-agar. The cultures that gave types IV, V, and S. aureus curves showed comparable ranges of pigment production on the cream-agar. The eight cultures classified by both visual and reflectance methods as the most pigmented on cream-agar gave type III curves.

FIG. 7. Comparison of the purity values of pigments produced by cultures of Staphylococcus epidermidis grown on Trypticase Soy Agar-cream and classified according to the type of spectral absorption curve obtained with pigments extracted from cultures with methanol (3).

DISCUSSION

There was not complete agreement when the colors of pigmented growth were graded visually and by spectral reflectance. According to Judd and Wyszeck (1), "The measurement of a uniform, mat, opaque, nonpolarizing, and non-
fluorescent specimen usually does not present a problem in modern spectrophotometry." They discussed, however, the problems involved when a specimen exhibits nonuniformity of color, glossiness, translucence, polarization, and fluorescence. Consequently, any deviation in one of these factors could result in different measurements with colors that are judged to be identical.

The nonuniformity of pigmentation might account for the discrepancy in the second test group of cultures in grade 9 (Fig. 2) giving lower purity values than the cultures in grade 8 and, likewise, strain 5076D giving lower purity values than 4950B (Table 1). In both instances, the cultures that appeared more pigmented showed a few white foci or plaques dispersed throughout the pigmented area of growth. Therefore, if a plaque fell in the area of illumination, it could have caused a lower purity value.

Mat and glossy surfaces were exhibited by the cultures of S. epidermidis grown on cream-agar. The limited data obtained do not permit any conclusion as to the influence either surface has on the visual grading or reflectance measurements. The only apparent advantage of noting the type of surface formed would be for differentiating cultures with comparable reflectance values. The results with cultures 4950B and 5076D (Table 1) indicate that the type of growth is probably characteristic and constant for a strain if the isolates are tested immediately after isolation.

In one instance, the discrepancy between the reflectance measurements and the visual grade may have been due to an error in visual grading. In the first tests (Fig. 1 and 2), the Munsell color standards selected as the best matches were 2.5Y 8/6 for the five cultures in grade 5, and 2.5Y 8.5/6 and 8.5/8 for the two cultures in grade 6. The Munsell power of a color is defined as the product of Munsell value by Munsell chroma and correlates well with its power to attract attention (1). The 8.5/8 value indicates a lighter color than 8/6; consequently, the higher Munsell power of the cultures in grade 6 may have influenced their classification in the higher grade, because the pigments were lighter rather than more saturated.

The reflectance attachment used in this study measured a rectangular surface area approximately 2 × 8 mm, whereas the area available for visual observation was approximately seven times larger. Consequently, some of the discrepancies between the two methods of grading might be minimized if comparable areas are measured. An instrument is available (Bausch & Lomb, Inc.) that measures an area approximately 12 × 12 mm, which might be preferable for pigment studies as described in this paper.

Besides the fact that pigment production is better on media containing cream, the cream also provides an opaque background. In reflectance measurements, the background is important, because with a translucent specimen there is the loss of radiant flux due to scattering on the surface of the specimen and also within the specimen (1). Even with the most pigmented cultures of S. epidermidis, growth on media without cream is translucent and results in lower reflectance measurements.

No information is available to determine whether polarization or fluorescence has any effect on the reflectance measurements of cultures of S. epidermidis grown on cream-agar.

From the data presented in Fig. 1, 2, and 3, the following conclusions are made concerning the color of the pigments produced by the cultures of S. epidermidis studied. As the cultures were visually graded as being more pigmented, there was a concomitant increase in the average values of dominant wavelength and purity with a decrease in Y for the cultures in each higher grade. Thus, the nonpigmented cultures have the lowest dominant wavelength and purity values, but the highest Y or brightness values, whereas the most pigmented cultures have the highest dominant wavelength and purity values, but the lowest Y values. These findings indicate that the cultures are not producing pigments of different hues (greenish-yellow, yellow, yellowish-orange) each with high, medium, and low degrees of purity and brightness; otherwise, high purity values (0.7 to 0.8) would also have been associated with the lower dominant wavelengths (570 mµ) and low purity values with the higher dominant wavelengths (580 mµ). Consequently, the dominant wavelength values would not be essential for comparing the pigments of the cultures of S. epidermidis studied. This fact, plus the fact that (I - z) or Z values can be substituted for purity values, would make it unnecessary to calculate and then plot the x and y chromaticity coordinates on a chromaticity diagram. Thus, the calculations for defining the color of the pigments could be simplified by using the tristimulus Y and Z or (I - z) values. However, unless sufficient data are obtained to determine the variability of pigment production within strains, any values obtained from a single set of reflectance measurements are not sufficient to make a valid differentiation of cultures. For example, the differentiation of strains 4950B and 5076D (Table 1) would depend on the purity values, since the average dominant wavelength and Y values for each were the same. If we assume that the mean plus or minus twice the standard deviation in-
includes 95% of all possible values that could be produced by each strain, then the upper limit of most possible purity values for strain 5076D would be .605, and the lower limit of most possible purity values for strain 4950B would be .562. Isolates 1 and 3 of 4950B and isolates 2 and 3 of 5076D gave purity values that fell within these limits of values, so that any one of the values could have been produced by either strain. Classifying the two strains as different, based on the purity values of either of the two isolates of each strain, would be invalid.

The data presented in Fig. 7 indicate that, if only the reflectance method is used, cultures with comparable purity values would be considered the same, even though a difference is indicated by the spectral absorption curves of the methanol-extracted pigments. Consequently, the use of the spectral reflectance method is better for determining differences of pigment production within strains of _S. epidermidis_ after the cultures are first differentiated by the type of spectral absorption curve.

The use of the spectral reflectance method with a cream-agar medium provides an objective method for studying quantitative differences in pigment production by _S. epidermidis_ under standard conditions and might be useful for studying the effect of light, incubation time, and temperature. If an inert material could be found that would disperse evenly in solid media and give a white background, chemically defined media could be used, and the effect of various chemical compounds on pigment production could be studied with this method.

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**LITERATURE CITED**


