Application note

Color analysis for pharmaceutical products using UV-Visible absorption techniques

Introduction

The collection of reflected light by our eyes leads to the perception of an object's color, specifically light in the visible range of the electromagnetic spectrum (~400 nm – 700 nm). As our eyes are sensitive to variations in color and brightness,[1] small changes in the color of an object can be easily observed. In pharmaceutical manufacturing, the color of a drug product is important to analyze for QA/QC purposes. Not only is it necessary to minimize batch-to-batch variations for aesthetic purposes, but changes to the color of a product can have implications for the quality of the products. Specifically, variations from the anticipated color could indicate impurities are present in the product or that the material has degraded.[2–4] This is particularly important for materials which are easily decomposed, including light, moisture, and oxygen/air-sensitive substances.[5]

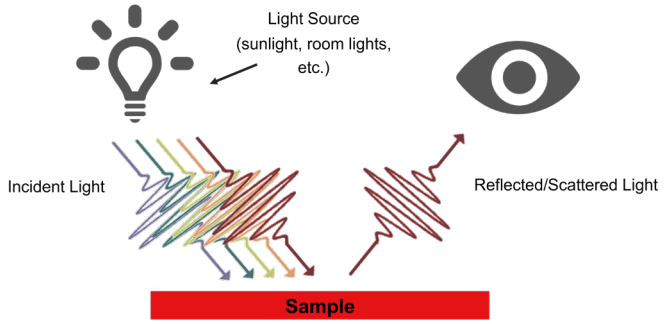


Figure 1: Diagram of how the color of an object is perceived.

Qualitatively, a comparison of the color of a finished drug product with an accepted standard can be used to ensure the material's color matches. However, inherently this methodology will introduce person-to-person variations.[6] Additionally, environmental effects, such as the light source or the presence of shadows, can influence the perceived color. As the color of a material comes from the reflected visible light, spectroscopic measurements of a material in the visible spectral range can be used to provide a more rigorous and quantitative method for assessing color. Consequently, a UV-Visible spectrophotometer can be used to measure either the percent of light transmitted (%T) or reflected (%R) across the visible spectrum for this purpose. As either of these measurement geometries can be used, this analysis can be applied to both liquid and solid products.

The American Society for Testing and Materials (ASTM),[7] as well as USP <1061>,[8] have detailed descriptions of the mathematics that can be used to assign the sample's color a coordinate in a graphical representation of color, also referred to as a color space. The tristimulus values, calculated through the equations 1 – 3,

$$X = \sum_{400}^{700} k * S(\lambda) * \bar{x}(\lambda) * R(\lambda)\Delta\lambda \quad (1)$$

$$Y = \sum_{400}^{700} k * S(\lambda) * \bar{y}(\lambda) * R(\lambda)\Delta\lambda \quad (2)$$

$$Z = \sum_{400}^{700} k * S(\lambda) * \bar{z}(\lambda) * R(\lambda)\Delta\lambda \quad (3)$$

are the basis of most other color spaces developed by the Comission Internationale de l'Eclairage (CIE).[9] These formulas include the measured reflectance ($R(\lambda)$), the spectral power of an illuminant ($S(\lambda)$), a color matching function ($\bar{x}(\lambda),\bar{y}(\lambda),\bar{z}(\lambda)$), and the normalization factor (k).

As described previously, the color of an object is highly dependent on environmental factors, such as light source and the field of view of the object. For example, the intensity of the light across the visible spectrum can be very different for various light sources and can lead to differences in how the color is observed. In the tristimulus equations, this factor is taken into account through the inclusion of the spectral power of the illuminant, $S(\lambda)$. A standardized intensity spectrum describing the spectral illuminant power as a function of wavelength was developed to describe a typical intensity spectrum for common illuminants (e.g., room lights, daylight), and is included in equations 1 – 3. Additionally, the observer angle, which defines the field of view of the material, can also alter the perceived color and is also accounted for in tristimulus equations through the color-matching functions.

The tristimulus values can condense the measured visible spectrum of a sample down to a single coordinate, however, the coordinate space is not uniform.[9] The lack of uniformity can lead to issues gauging the difference between the color of a sample and the color of a reference standard. In pharmaceutical applications, specifically in QA/QC functions, the ability to compare the sample to an accepted standard, as well as establish acceptance criteria, is critical. Consequently, a uniform color space must be used instead. CIE developed a set of mathematical functions which convert the calculated tristimulus coordinates into a uniform, cylindrical (CIE L*a*b*) or spherical (CIE L*C*h*) coordinate system (Figure 2), which is built on opposing color theory.

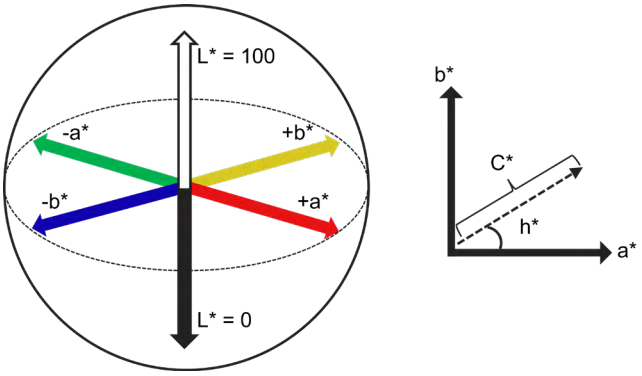


Figure 2: CIE L*a*b* and CIE L*C*h* coordinates

Coordinates for the more commonly used CIE L*a*b* color space are generated through the following mathematical functions,[7, 8]

$$L^* = 116\left(\frac{Y}{Y_n}\right)^{\frac{1}{3}} - 16 \quad (4)$$

$$a^* = 500\left[\left(\frac{X}{X_n}\right)^{\frac{1}{3}} - \left(\frac{Y}{Y_n}\right)^{\frac{1}{3}}\right] \quad (5)$$

$$b^* = 200\left[\left(\frac{Y}{Y_n}\right)^{\frac{1}{3}} - \left(\frac{Z}{Z_n}\right)^{\frac{1}{3}}\right] \quad (6)$$

where X, Y, and Z are the calculated tristimulus values and X_n, Y_n, and Z_n are the tristimulus values of a perfectly reflecting white diffuser. Here L^* describes how light (100) or dark (0) the materials are, a^* represents how red (positive) or green (negative) the sample is, and b^* demonstrates how yellow (positive) or blue (negative). As this transformation results in a more uniform color space, a better representation of the color difference (ΔE^*) between the sample and a standard can be developed. The color difference formula (eq 7) describes how a color difference is mathematically determined,

$$\Delta E^* = \sqrt{(L^*_{sam} - L^*_{std})^2 + (a^*_{sam} - a^*_{std})^2 + (b^*_{sam} - b^*_{std})^2} \quad (7)$$

where L^*_{sam}, a^*_{sam}, and b^*_{sam} represent the CIE L*a*b* values for the sample and L^*_{std}, a^*_{std}, and b^*_{std} represent the CIE L*a*b* values for the standard.[8] As a rule of thumb, two colors are considered to be indistinguishable from one another by eye if the color difference between the two substances is less than 3.

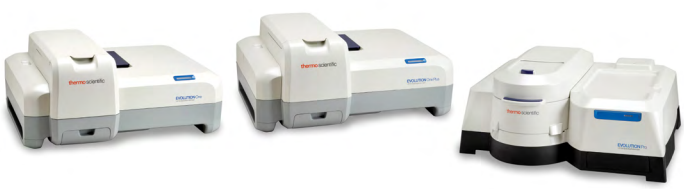
The CIE L*C*h* color space uses the same coordinate system as the CIE Lab system, except it reports the chroma (C_{ab}^*) and hue (h_{ab}^*) of the substance in place of a^* and b^*. Chroma is calculated through equation 8,

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}} \quad (8)$$

and describes how colorful a substance is wherein a small C_{ab}^* represents a more pale or muted color, while a large C_{ab}^* describes a substance with a very vibrant color. Hue describes the color of the object and is calculated through equation 9.

$$h^*_{ab} = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (9)$$

Color analysis can be a quick and useful tool for assessing the overall quality of a given product prior to further downstream processing. Through UV-Visible absorption spectroscopy, the analysis can be made more rigorous, allowing for a more accurate measurement of color. Herein, we describe how color analysis can be applied to both solid and liquid samples using the Thermo Scientific™ Evolution™ Spectrophotometers and Thermo Scientific™ Insight™ Pro Software. Furthermore, descriptions of the USP requirements for color analysis of samples are explained in relation to the instrumental analysis method.



Thermo Scientific Evolution Spectrophotometers

Experimental

Materials

USP color-matching solutions were prepared based on descriptions in USP's chapter <631>,[10] which includes methods to analyze and report the color of solution phase samples. Briefly, three stock solutions were generated:

- 0.27 M $CoCl_2 \bullet 6H_2O$ (red solution)
- 0.17 M $FeCl_3 \bullet 5H_2O$ (yellow solution)
- 0.23 M $CuSO_4 \bullet 5H_2O$ (blue solution)

These solutions were mixed in different proportions to prepare the color-matching solutions A – T as defined in USP <631> (see Table 1).[10]

Table 1: Proportions of stock color solutions used to prepare color matching solutions A – T based on USP <631>.[10]

Color Matching Solution	Volume $CoCl_2 \bullet 6H_2O$ (mL)	Volume $FeCl_3 \bullet 5H_2O$ (mL)	Volume $CuSO_4 \bullet 5H_2O$ (mL)	Volume H_2O (mL)
A	0.1	0.4	0.1	4.4
B	0.3	0.9	0.3	3.5
C	0.1	0.6	0.1	4.2
D	0.3	0.6	0.4	3.7
E	0.4	1.2	0.3	3.1
F	0.	1.2	0.0	3.5
G	0.5	1.2	0.2	3.1
H	0.2	1.5	0.0	3.3
I	0.4	2.2	0.1	2.3
J	0.4	3.5	0.1	1.0
K	0.5	4.5	0.0	0.0
L	0.8	3.8	0.1	0.3
M	0.1	2.0	0.1	2.8
N	0.0	4.9	0.1	0.0
O	0.1	4.8	0.1	0.0
P	0.2	0.4	0.1	4.3
Q	0.2	0.3	0.1	4.4
R	0.3	0.4	0.2	4.1
S	0.2	0.1	0.0	4.7
T	0.5	0.5	0.4	3.6

For comparison against a more realistic example, two different cough syrups were analyzed. One sample was labeled "Daytime" and the other "Night-time." Additionally, a set of four antacid tablets of different colors were analyzed herein. The tablets were crushed into powders using a mortar and pestle.

Instrument parameters

UV-Visible measurements described herein were collected using an Evolution One Plus Spectrophotometer. For all samples, spectral measurements spanning 280 nm and 780 nm were collected using a 1.0 nm spectral bandwidth and 2 nm data interval.

The USP color-matching solutions were measured in transmission geometry and reported as % Transmission (%T), and the cough syrup samples were reported in absorption units. For both sample sets, deionized water was used to establish a 100% transmission baseline as the blank solution. All USP matching solutions were measured using a plastic 10 mm cuvette, while the cough syrup samples were measured in a 10 mm and 1 mm quartz cuvette.

The antacid samples were measured in reflection geometry using an integrating sphere accessory (ISA-220) with a powder cell holder. A white Spectrlon© disk was used to establish a 100% reflection baseline as the blank. The resulting data was reported as % Reflectance (%R).

Color analysis parameters

For all samples described herein, the CIE L*a*b* color values were calculated using Insight Pro Software. The D65 illuminant with a 10° observer angle was chosen to reflect the color of all samples. Color difference measurements were also performed through this software feature. All calculations performed correspond to the descriptions outlined in USP <1061>[8] and ASTM-E308.[7]

Results and discussion

Analysis of liquid samples—color matching solutions

According to USP <631>, color-matching solutions are to be used as a comparison point against the produced liquid product to ensure the product matches the expected color. As many liquid-based pharmaceutical products are yellow in hue, the USP monograph includes a procedure for making a set of standard solutions of varying yellow (Figure 3d).[10] EP has a different procedure outlined for making color standards and includes a wider range of colors, including brown, green and blue, among others.[11]

As shown in Figure 3d, some samples appear by eye to be similar and almost indistinguishable in color. However, as the purpose of these standards is to serve as different matching solutions, the variations in the color may be slight and difficult to compare without instrumental methods like UV-Visible color analysis. To demonstrate this concept, the percent transmittance of each matching solution was collected and are shown in Figures 3a – 3c.

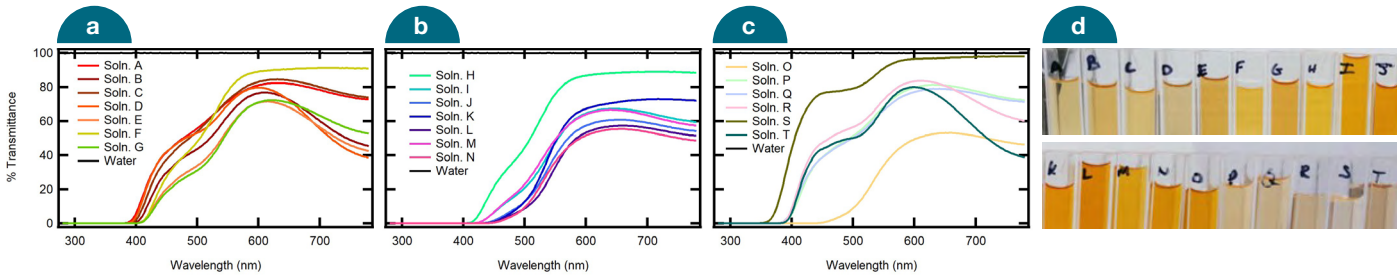


Figure 3: Absorption spectra of USP color matching solutions (a) A – G, (b) H – N, (c) O – T. (d) An image of the USP color matching solutions.

From these spectra, it is clear there are small differences in the transmittance, and consequently absorption, of each matching solution; however, color difference calculations were needed to rigorously compare the colors. As described previously, the CIE L*a*b* values were calculated using the Insight Pro Software. A select set of color-matching standards were chosen for comparison and are included in Table 2 as these standards (Soln. A and B, Soln. J and K, and Soln. Q and R) appear similar enough to each other in color that they are difficult to tell apart.

Table 2: CIE Lab and color difference values for select USP color matching solutions (A, B, J, K, Q, R). Color difference calculations were carried out for samples which appear similar by eye.

Solution	*L**	*a**	*b**	*ΔE**
A	87.5	0.5	28.5	**9.7**
B	83.3	2.4	37.0	
J	69.1	12.0	80.0	**12.5**
K	73.9	12.5	91.5	
Q	85.1	2.6	28.3	**5.2**
R	88.1	2.5	24.0	

The color difference values calculated between matching solutions A and B, J and K, and Q and R are relatively low; however, a numerical limit is required to put these difference values into context. In the pharmaceutical industry, different formulations may require different methods of comparison against a color-matching standard. For example, one product may need to have no discernable color (achromatic), while another must meet a minimum color value. Consequently, USP has developed a set of criteria which can be used to set acceptable limits for the calculated color difference from a standard (Table 3).

There are four main test limits which can be used depending on the color expectations for the analyzed product. Each test defines a limit to an acceptable color difference between the material and a given standard. For a sample which should have no color, the first test in Table 3 (colorless/achromatic) defines the necessary color difference limit as $\Delta E^* < 1$, where the color-matching standard is purified water.

For samples where the sample has an expected color, there are a few different options for analysis. If the color must match a given standard color exactly, the second test in Table 3 (Indiscernible from Standard) is required. Here, the color difference between the product and the color matching standard is used and must be less than 3. As mentioned previously, this defines the color difference that is discernable by the human eye.[10] The last two analyses define maximum and minimum color limits. Here, a sample can either be more or less colorful than a given standard. USP defines $\Delta h_{ab}{}^*$, the difference in hue between the sample and matching standard chosen must be less than 15. When setting the maximum or minimum color limit, instead of comparing the color difference against a number, two different analyses are required: one where the color of the standard is compared to the color of pure water ($\Delta E_{std}{}^*$) and one where the color of the product is compared against pure water (ΔE^*).

Table 3: Passing criteria for color difference tests from USP <631>.[10] For the maximum and minimum color difference measurements, $\Delta E_{std}{}^*$ refers to the color difference between a matching standard and purified water while ΔE^* refers to the color difference of the sample against purified water.

	Test	Color Standard	Passing Criteria
1	Colorless (Achromatic)	Purified Water	$\Delta E^* < 1$
2	Indiscernible from Standard	Color Matching Solution	$\Delta E^* < 3$
3	Maximum Color	Purified Water	$\Delta E^* < \Delta E_{std}{}^*$
4	Minimum Color	Purified Water	$\Delta E^* > \Delta E_{std}{}^*$

As the color difference values shown in Table 2 are intended to determine how similar the color of the two solutions are to one another, this analysis would follow the "Indiscernible from Standard" test. The passing criteria would require a calculated color difference of less than 3. For each set of standards, the color difference exceeds this limit, indicating they fail this test and are distinguishable from one another. This result highlights how small differences in color can be analyzed through the instrumental method, where it is difficult to perceive visually.

Analysis of liquid samples—cough syrup

The color-matching standards are ideal solutions with optimized component concentrations to produce a measurable spectrum in a standard 10 mm cuvette. Real samples may not be manufactured to produce UV-Visible absorption spectra that can be easily measured under these conditions. For example, Figure 4a includes the absorption spectra of a "Daytime" and "Night-time" cough syrup measured in a 10 mm cuvette. By eye, the "Daytime" syrup appears orange while the "Night-time" syrup appears red/purple.

As shown, both samples absorb greatly at wavelengths shorter than 550 nm (A > 3). In UV-Visible absorption measurements, it is good practice not to use highly absorptive samples for calculations or quantification, as very little light is allowed to pass through the sample and be detected by the system. For example, an absorption of 3 indicates 99.9% of the incident light is absorbed by the sample, leaving 0.1% of the light collected by the detector. Consequently, the absorption spectra in Figure 4a are not ideal for color analysis and result in the values described in Table 4.

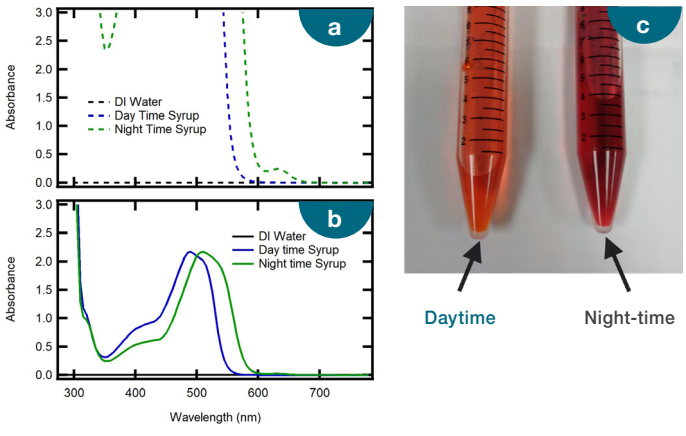


Figure 4: Absorption spectra of "Daytime" and "Night-time" cough syrup collected using a (a) 10 mm and (b) 1 mm quartz cuvette. (c) An image of the "Daytime" and "Night-time" cought syrup).

Table 4: CIE L*a*b* values for "Daytime" and "Night-time" cough syrup samples. Spectra were measured using a 10 mm and 1 mm path length.

	*L**		*a**		*b**	
Sample	10 mm cuvette	1 mm cuvette	10 mm cuvette	1 mm cuvette	10 mm cuvette	1 mm cuvette
Daytime	67.5	79.7	62.0	40.3	116.2	86.0
Night-time	40.2	62.3	68.5	72.2	69.2	27.6

To avoid issues for highly absorptive samples, instead a short pathlength cuvette can be used as absorption is directly proportional to pathlength according to Beer's law (eq. 10),

$$A = cl\varepsilon \quad (10)$$

where A is the collected absorbance, *c* is the concentration of the analyte, *l* is the path length, and ε is the molar absorptivity of the analyte. Changing the path length also circumvents the need to dilute the sample, avoiding some waste of the material.

Herein, both cough syrup samples were measured using a 1 mm cuvette, resulting in the absorption spectra in Figure 4b. Compared to the spectra shown in Figure 4c, the spectra collected show much more clearly the absorption features present in the sample. Included in Table 4 are the resulting color values based on the spectra collected with a shorter path length. These reported values are very different from the values calculated using the spectra collected with a longer path length. It is important to note that changing the path length not only changed the perceived lightness/darkness of the sample (*L**), but also how red/green (*a**) and how blue/yellow (*b**) the samples appear. This observation further illustrates the importance of measuring highly absorptive samples in a shorter path length to avoid significant deviations in the calculated color values. As good practice, quantification should only be performed when the highest peak absorption in the spectral region of interest is 1 A or lower. Given the calculated color values will be sensitive to the chosen path length, it is important any standard used for color difference calculations be measured using the same path length.

Analysis of solid samples

USP <631> specifically refers to color analysis procedures for liquids; however, color analysis can be performed using solid samples as well, according to USP <1061>.[8,11] For pharmaceutical analysis, the color of a solid drug product can also have implications on the quality of the material,[3–6] as described previously; however, it can also be used to indicate the dosage of a given product as well as comply with a company's branding or marketing needs.[6] For solid materials, measurements in reflection geometry are appropriate as it is difficult to pass light through a solid material without scattering effects. As described in equations 1 – 3, the tristimulus values, and therefore the CIE L*a*b* values, can be calculated using reflectance data, allowing for color analysis of solid samples.

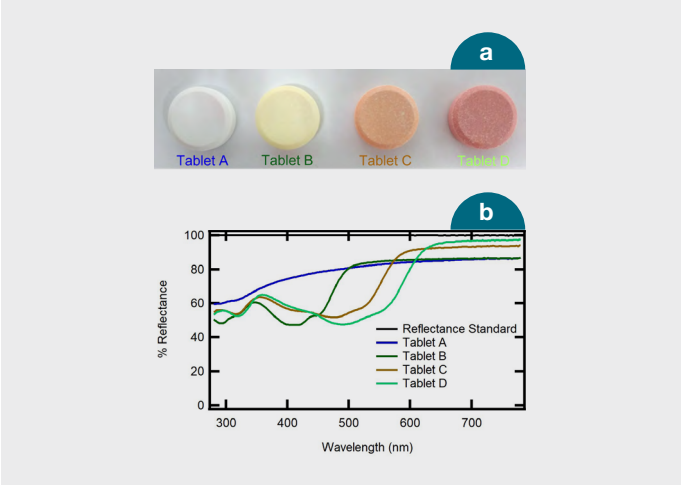


Figure 5 – (a) An image of the four antacid tablets measured. (b) Reflectance spectra of four antacid tablets (blue—Tablet A, dark green—Tablet B, brown—Tablet C, and light green—Tablet D) and a white reflectance standard (Spectralon).

Figure 5b includes the percent reflectance spectra (%R) of four antacid tablets (Fig. 3a) of varying colors. By eye, Tablets A – D appear white, yellow, orange, and red, respectively. The calculated CIE L*a*b* values for each sample are included in Table 5, along with the color values for a white Spectralon® reference material (99% reflectance). Color difference calculations were then performed to determine how different each antacid tablet was from the white reference material. Tablets B – D resulted in very high color differences (between 23 and 27) with respect to the reference standard, as anticipated as these samples are visually very different from the white standard. Tablet A, which appears white by eye, is closer in color to the reference, with a color difference of 8.7 compared with the color difference of the other three tablets, however as the calculated color difference is greater than 3, it is distinguishable from the reference standard and would fail a color matching test.

Table 5: Calculated CIE L*a*b* color values and color difference values for antacid tablets. Color Difference Calculations were carried out using the color values for the Spectralon® reference as the standard.

Sample	*L**	*a**	*b**	*ΔE**
Spectralon® Reference	100.0	0.0	0.0	—
Tablet A	92.8	0.3	3.4	7.92
Tablet B	92.8	-5.8	21.7	23.6
Tablet C	88.1	13.7	17.0	24.9
Tablet D	82.5	19.3	8.7	27.5

Conclusion

Color analysis can be an effective and quick method for QA/QC in pharmaceutical manufacturing. As shown in the experiments described herein, color analysis can be performed using the Evolution UV-Visible Spectrophotometers to carefully determine a material's color without person-to-person variations, allowing for a quantitative analysis of a produced pharmaceutical. Additionally, these measurements demonstrate the ability to analyze both liquid and solid samples following USP color analysis procedures.

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