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A Step-by-step Implementation of the Instrumental Color Measurement in the Routine Pharmaceutical Quality Control

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Abstract: Research done in the last five decades showed the applicability and advantage of instrumental color measurement to many areas in the pharmaceutical field where the determination of appearance is relevant, including the development, production and quality control of API, excipients and finished pharmaceutical products. The development of instrumental color measurement applications in the pharmaceutical sector, in recent years led to a significant degree of harmonization through the major pharmacopoeias,United States Pharmacopoeia (USP), European Pharmacopoeia (Ph. Eur.) and Japanese Pharmacopoeia(JP). Still, instrumental color measurement has not become a routine method in the quality control of medicines, as is visual color determination. In the measurement of color, colorimetry takes advantage of spectroscopic measurements by integrating the spectroscopic results with human visual perception, using standard color spaces. Unlike qualitative color tests that rely on visual observation alone, quantitative tristimulus colorimetry provides precise measurements of color attributes. In this paper the step-by-step process of implementation of the instrumental color measurement described in pharmacopoeias is presented:performance of the actual measurements, entailing proper handling of the instrument in the required measurement geometry, and assuring its suitabilityfor color measurement, as well as appropriate application of the formulae and correct calculation of the color parameters. The method was appliedfor determination of the color of solution of three pharmaceutical excipients and three finished pharmaceutical products.The results from testing method performance highlighted some key aspects forthe proper implementation and interpretation of pharmacopoeial requirements for the instrumental color measurement,especially helpful for the first-time user.

Keywords:instrumental color measurement, manual calculation, pharmaceutical routine quality control, spectrophotometric color measurement, tristimulus colorimetry

I. INTRODUCTION

Instrumental color measurement is used in a variety of fields to ensure color accuracy and consistency, including industries such as food and beverage, textiles, and cosmetics [1]. Research done in the last five decades showed as well the applicability and advantage of the instrumental measurement of color to many areas in the pharmaceutical field where determination of appearance is relevant, including the development, production and quality control of active pharmaceutical ingredients, excipients and finished pharmaceutical products [2][3][4][5]. The development of instrumental color measurement applications in the pharmaceutical sector as well as the well documented drawbacks of the compendial visual method for color assessment [6], in recent years led to a significant degree of harmonization through the major pharmacopoeias,United States Pharmacopoeia (USP), European Pharmacopoeia (Ph. Eur.) and Japanese Pharmacopoeia (JP),regarding the instrumental method. [7][8][9][10].

To understand the process of implementation of the instrumental method, a brief overview of the underlying theory of color measurement is necessary.

Human perception of color consists of three components:a human observer,a white light source and an observed object. In order to quantify human color perception each component must be represented as numbers.Methods for expressing color numerically were developed by an international organization concerned

with light and color, the "Commission Internationale de l'Eclairage" (CIE), as early as in 1931[6][11][12][13].

Let`s explore the first component, the human observer. Red, green and blue are the three primary colors sensitive to the human eye, whichare detectedvia three types of receptors, called cones, located in the retina (a layer at the back of the eye).Light radiation inthe visible region of the electromagnetic spectrum (360 − 780 nm) selectively excites the respective cones,followed by neuro-processing in the brain of the number of cones that were activated and the strength of their signal, resulting in the perception of color[14][15]. Thus, all colors can be represented as a mixture of three radiant stimuli, appropriately chosen to excite all three receptors in the eye.Through extensive color-matching experiments with human subjects having normal color vision it was achieved to developand quantify the color ability of a standard or average, human observer. The experimentswere performed by projecting colors from acrossthe visible spectrum onto a screen.Looking through a hole that allowed subjectsa defined field of view (observer angle),multiple people matched each spectral color light usinga combination of red, green, and blue lights.The curves generated from this data resulted in $\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$ functions, referred to as color matching distributing coefficients giving the relative amount of stimulation of each receptor caused by light of thatwavelength. Standard Observer color matching functions have been published by the CIE for two different fields of view, the first for the 2° Standard Observer in 1931, and the second for the 10° Standard Observerin 1964[5][16][17]. In the calculation of color coordinates, the 1931 2° and 1964 10° Standard Observerfunctionsreflect the human response to the visiblespectrum.

The second component affecting color perception is the light source, which is defined as a standard illuminant by the CIE. Standard illuminants are designed to mimic common viewing situations (e.g., room lights, daylight) and representa theoretical source of visible light with a profile (standardized intensity spectrum describing the spectral illuminant power as a function of wavelength,S_λ) that is published. They are designated by a letter or by a letter-number combination. Illuminants A, B, and C, introduced in 1931, represent average incandescent light, direct sunlight, and average daylight [7][17][18].

The third component is the observed object. An object absorbs part ofthe light from the light source and reflects/transmitsthe remaining light. This reflected/transmitted lightenters the human eye, and the resulting stimulation of the retina is recognized as the object's "color" by the brain. Each object absorbs and reflects/transmits light from different portions of the spectrum and indifferent amounts. These differences in absorption and reflection/transmission are what make the colors of different objects different. As the color of a material comes from the reflected/transmitted visible light, aUV-Visible spectrophotometer can be used to measure either thepercent of light transmitted ($\%T$) or reflected ($\%R$) across thevisible spectrum. As either of these measurementgeometries can be used, this analysis can be applied to both liquidand solid products[12][17].

Tristimulus colorimetry is an objective instrumental approach for color measurements where the critical parameters of color perception (observer,illuminant and object) are standardized and well controlled. Hence, for any color, the amount of stimulation of each receptor in the eye can be defined and calculated as a set of CIE tristimulus values (X, Y, Z) from the CIE Standard Observer functions, a selected CIE illuminant and the reflectance or transmission of the sample. At each wavelength, thecolor matching distributing coefficients $\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$, are multiplied by the CIE standard illuminant. Then that value is multiplied by the reflectance or transmission of the sample at each wavelength. The values for all the wavelengths are then summed and integrated to display CIE X, Y, Z color values[8][9][13].

The tristimulus values express the measured visible spectrum of a specimenas a single coordinate, but the coordinate space thus obtained is not uniform. It means that identical color differences in various places of color space do not correspond to identical geometrical distances. The color space chosen is visually uniform if the geometric distance between two colors in the color space is correlated with the perceived difference between the two colors. To create a three-dimensional visually uniform color space in which any color is located by its coordinates, the three attributes of color: hue, lightness and saturation may be used, where hue constitutes the outer rim of the solid with lightness as the center axes and saturation as the horizontal spokes. By creating scales for hue, lightness and saturation, color measure can be expressed numerically in much the same way as length or weight [5][7][12][13].

In an attempt to define a visually uniform color space different mathematical transformations of tristimulus values X, Y, Z are used. At present, CIE *Lab* color space(CIELAB),defined by the CIE in 1976, is an international standard widely used for color measurement, which is built on opposing color theory. The formulas necessary to transform tristimulus values$X$, Y, Z into this space are available in the literature. In a CIE *Lab* color space, the resulting L^*, a^*, b^*, C^*_{ab}, and h^*_{ab} values indicate the following:L^* is the vertical axis and represents measureof lightness, is always positive and ranges from 0 (black) to 100(white, colorless); a^* and b^* coordinates are found on a plane, representing a cross-section perpendicular to the lightness axis, are usually between -100 and +100 anddefine the degree of redness (positive a^*) or greenness(negative a^*), yellowness (positive b^*) or blueness (negativeb^*); C^*_{ab} is the chroma (intensity or saturation of the color) in the (a^*, b^*) plane, $C^*_{ab} = \sqrt{(a^*)^2 + (b^*)^2}$, and is determined by the geometric distance from the vertical axis;h^*_{ab} is the hue

angle about the lightness axis in the (a^*, b^*) plane, measured from the a^* axis increasing counterclockwise, and is reported as avalue from 0 to 360 degrees (Fig. 1). The coordinates a^* and b^* are zero for neutralcolors (white, grays and blacks). Red, yellow, green, blue, and intermediatehues are differentiated by various hue angles. Colors of the same hue angle become more intense (i.e., chromatic) as they movefurther from the vertical axis (larger chroma). The higher thevalues for a^* and b^*, the more saturated a color is [7][8][9][11][12].

The coordinates of a color in a visually uniform color space may be used to calculate the deviation of a color from a chosenreference point. The composite change, or difference in color between two points in the CIE *Lab* color space (ΔE^*), using the difference in the individual color components L^*, a^*, and b^*, can be calculated as a simple Euclidian distance in space [5][7][9][12][13].

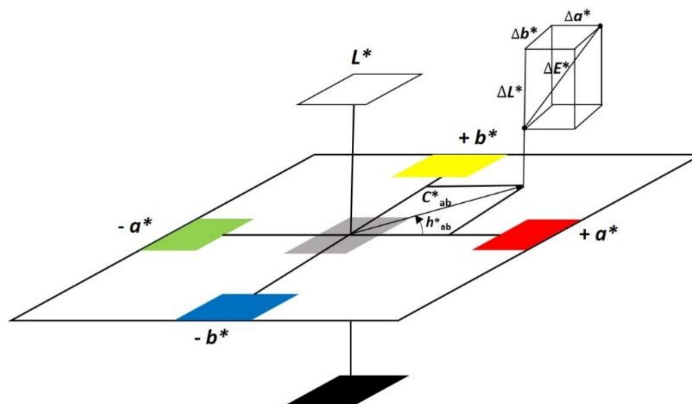

Fig. 1. CIE *Lab* color space (CIELAB).

Adapted from source: "Validation of an Instrumental Measuring Procedure According to the European Pharmacopoeia Chapter 2.2.2", 2016, Hach Lange GmbH, Validation report LICO 690 - Pharm. Eur V2016-02. Retrieved from Hach website:
https://info.hach.com/rs/463-MOY-364/images/Validation%20LICO%20690%20PhEur%20V02_E%20%281%29.pdf[19].

Despite its everyday use in a variety of fields, the instrumental color measurement, to this day, has not become a routine method in the quality control of active pharmaceutical ingredients, excipients and finished pharmaceutical products [2] as the visual color determination. According to the available data, the main reason for this could be that, there are no individual monographs in the USP and Ph. Eur. of active pharmaceutical ingredients, excipients or finished pharmaceutical products that require appearance/color of solution to be determined instrumentally, except for certain packaging materials. Furthermore, mainly it is understood that calculation and interpretation of spectroscopic measurements will require the use of specialized software. Although specialized software for instrumental color measurement offers convenience of use, financial reasons may stand in the way of the actual implementation of the instrumental method for some routine quality control laboratories, thus still making the visual color assessment the more applicable method. Considering that for any endeavor dedication is needed, it is nevertheless not too laborious to conduct the calculations in commonly used statistical software and design the calculator spreadsheets for instrumental color determination for everyday laboratory use, making the instrumental method as easy and accessible for use as the visual method.

Pharmacopoeias doprovide guideline on the performance of the method, but the actual step-by-step implementation of the instrumental method for the first-time user requires an in-depth literature inquiry and understanding of the theory of color measurement, as well as settling some key issues regarding the proper handling of the instrument in the required measurement geometry and assuring its suitability of use for the concrete measurement of color, as well as proper application of the given formulae and correct calculation of the final results.

The aim of this paper was to facilitatethe implementation and interpretation of pharmacopoeial requirements of the instrumental color measurement in the quality control laboratory, especially for the first-time user. For that purpose, a lay out of the step-by-step process is presented, with focus on themethod performance and assurance of its suitabilityforquality control of pharmaceutical ingredients and finished pharmaceutical products.

II. MATERIALS AND METHODS

Chemicals and reagents:

Iron trichloride anhydrous (SLR grade), cobalt chloride hexahydrate (AR grade) and copper sulfate pentahydrate (AR grade) were provided by Thermo Fisher Scientific, UK. Hydrochloric acid, concentrated (37%, RPE – ISO – For analysis) was purchased from Carlo Erba, France. Ethanol 96% (Ph. Eur) was provided

by Alkaloid Skopje, North Macedonia. Purified water was obtained with an Aqua-Win® Pure Water System (AQUA-WIN WATER CORPORATION, Taiwan) and water for chromatography was obtained with a TKA-LAB Reinstwasser system (Niederelbert, Germany). For investigation of method applicability tested excipients were: sodium benzoate (≥99.5%, AnalaR NORMAPUR® analytical reagent) purchased from VWR Chemicals, citric acid monohydrate (RPE-ACS-ISO - Reag. Ph. Eur. - Reag. USP) and glycerol (RPE-ACS - Reag. USP) obtained by Carlo Erba, France. Tested finished pharmaceutical products were three different dosage forms: diazepam, 10 mg/2 mL solution for injection; topotecan, 4 mg/4 mL concentrate for solution for infusion and esomeprazole, 40 mg/vial powder for solution for injection or infusion, manufactured by three different manufacturers, purchased from local pharmacies.

European Pharmacopoeia (Ph. Eur.) color reference solutions:
According to Ph.Eur. 2.2.2., Degree ofColoration of Liquids [9], yellow, red and blue primary solutions were prepared. These three primary solutions and 10 g/L HCl were used to prepare five standard solutions:B (brown), BY (brownish-yellow),Y (yellow), GY (greenish-yellow) and R (red), by mixing the prescribed volumes of solutions.Finally, for each color family, the prescribed reference solutions for color comparison, 37 in total, were prepared from each of the standard solutions and 10 g/L HCl, mixing the appropriate volumes. Reference solutions thus obtained were: B_1–B_9, BY_1-BY_7, Y_1-Y_7, GY_1-GY_7 and R_1-R_7.

Sample solutions of excipients:
Sample solutions of tested excipients for determination of color of solution were prepared according to the prescribed in test for Appearance of solution in the respective Ph. Eur. monographs[20][21][22].
For sodium benzoate, the sample solution (Solution S) was prepared by dissolving 10.0 g of sodium benzoate in carbon dioxide-free water (prepared from purified water) and diluted to 100.0 mL with the same solvent. For citric acid monohydrate, the sample solution was prepared by dissolving 2.0 g of the examined substance in purified water, and diluted to 10.0 mL with the same solvent. For glycerol, solution S was prepared bydiluting 100.0 g of the substance to 200.0 mL with carbon dioxide-free water (prepared from purified water). The sample solution for color comparison, is prepared by further diluting 10.0 mL of solution S to 25.0 mL with purified water.

Samplesolutions of finished pharmaceutical products:
Sample solutions of finished pharmaceutical products were prepared according to methods for color of solution prescribed for the respective finished pharmaceutical productthat were accessible to the laboratory.
For diazepam, 10 mg/2 mL solution for injection, and topotecan, 4 mg/4 mL concentrate for solution for infusion, sample solutions for color examination represent the products as such, without further dilution.
For esomeprazole, 40 mg/vial powder for solution for injection or infusion, sample solution represents the reconstituted product: the contents of 1 vial were dissolved with 10 mL of water for chromatography.

Visual examination:
Visual examination of the degree of coloration of the sample solutions for the excipients was carried out in accordance to the official method of their respective Ph.Eur. monograph[20][21][22]. For the finished pharmaceutical products visual examination was carried out in accordance to testing methods for color of solution prescribed for the respective finished pharmaceutical product, that were accessible to the laboratory. In all cases visual method II of Ph. Eur. 2.2.2.[9] was performed, by comparing each of the sample solutions to their respective reference solution (sodium benzoate was compared to Y_6; citric acid monohydrate to B_9; glycerol to B_9; diazepam, 10 mg/2 mL solution for injection to GY_6; topotecan, 4 mg/4 mL concentrate for solution for infusion to Y_3; esomeprazole, 40 mg/vial powder for solution for injection or infusion to B_5).The evaluation of the color was performed in diffuse daylight, using identical tubes of colorless, transparent, neutral glass with a flat base and an internal diameter of 18 mm (Glassco, India), the depth of the layer of the examined liquid being 40 mm, viewing vertically against a white background.

Spectrophotometric measurement:
Spectrophotometric measurements were conducted on an Agilent 8453 UV/Vis Spectrophotometer (Agilent Technologies, USA) equipped with a general-purpose Agilent ChemStation software for UV-visible spectroscopy. All measurements were recorded in transmission geometry and reported as % Transmission (%T), scanning over the visible range 400-700 nm, at 10 nm intervals, using a 1 nm spectral bandwidth and1 nm data interval.Forverification of the calibration in transmission mode, before measurement of the solutions, purified water was used as the blank solution to establish a 100% transmission baseline. All tested solutions were measured six times using a 1 cm path lengthquartzcuvette, at room temperature (20 °C to 25°C).

Calculation of CIE *Lab*values:

Color analysis of spectra was performed manually in Microsoft Excel (Microsoft Corporation). All calculations performed correspond to the descriptions outlined in Ph.Eur. 2.2.2., USP <631> and USP <1061>[7][8][9] for the instrumental method, based on the International Commission on Illumination (CIE)color models. Tristimulus color calculations of the tristimulus values (*X*, *Y* and *Z*), performed from 400 to 700 nm, with 10 nm spacing, for the CIE 2° Standard Observer and standard illuminant C (diffuse daylight) (C/2), were made by the following equations:

$$X = \sum_{400}^{700} T_\lambda \bar{x}_\lambda S_\lambda \Delta\lambda / Y` \quad (1)$$

$$Y = \sum_{400}^{700} T_\lambda \bar{y}_\lambda S_\lambda \Delta\lambda / Y` \quad (2)$$

$$Z = \sum_{400}^{700} T_\lambda \bar{z}_\lambda S_\lambda \Delta\lambda / Y` \quad (3)$$

$$Y` = \sum_{400}^{700} \bar{y}_\lambda S_\lambda \Delta\lambda \quad (4)$$

$Y`$ = normalising constant characterizing the stimulation of one receptor typeand the used illumination;
S_λ = relative spectral power distribution ofthe CIEstandard illuminant C;
$\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$ = color matching distributioncoefficients for CIE 2° StandardObserver;
T_λ= spectral transmittance of thematerial, in %;
λ = wavelength, in nanometers.

The products $\bar{x}_\lambda S_\lambda$, $\bar{y}_\lambda S_\lambda$, $\bar{z}_\lambda S_\lambda$ representing the weighting factors($Wx(\lambda)$, $Wy(\lambda)$,$Wz(\lambda)$) for the CIE *Lab*C/2 are referenced in USP <631>.

The tristimulus values were then used to calculate the CIE *Lab* color space co-ordinates: L*(lightness or brightness),a*(red-green) and b*(yellow-blue),defined by:

$$L^* = 116(Y/Y_0)^{1/3} - 16 \quad (5)$$

$$a^* = 500[(X/X_0)^{1/3} - (Y/Y_0)^{1/3}] \quad (6)$$

$$b^* = 200[(Y/Y_0)^{1/3} - (Z/Z_0)^{1/3}] \quad (7)$$

where Y/Y_0> 0.01 and X_0, Y_0, and Z_0 are the tristimulus values of water, in this case for CIE standard illuminant C, $Y_0$is set equal to 100.0, thus X_0 = 98.0 and Z_0 = 118.1.

The values of each color parameter represent the mean of six measurements on each of the tested solutions.

Comparative test of colors using CIE *Lab*values:

In order to apply the calculated CIE *Lab* valuesto determinecompliance of the sampleswith existing individual monographs, the CIE *Lab* valuesof respective sample and reference solutions were evaluated according to the approach outlined in USP <631>, Method IIA - Maximum Level of Color test[7]. First, the hue angle(h^*_{ab}) of each sample solution and its respective reference solution was calculated according to the formula

$$h^*_{ab} = \text{Arctan}\ (b^*/a^*) \quad (8)$$

and the result obtained in degrees. Since a*and b*can obtain both positive and negative values, the quadrant information depending on the sign (−/+) of the a*and b*values, has to be later accounted for,such that a full 360° hue angle potential is used (hue anglerepresents a value from 0° to 360°) (Fig. 2). To establish the value of the final hue angle, to the result obtained in degrees calculated by the above mentioned formula, the following transformation is applied:

- if both a* and b* have a positive value (quadrant I), no transformation is applied, and the result is taken as such;
- if both a* and b* have a negative value (quadrant III) or a* has a negative and b* has a positive value (quadrant II), 180 is added to the result;
- if$a$* has a positive and b* has a negative value (quadrant IV), 360 is added to the result[23].

The absolute value of the difference between the hue angles of the sample solution and its respective reference solution(Δh^*_{ab})should be less than 15.

Then, in order to compare the color difference of 2 solutions, or a deviation from a defined color, the difference between two points in the CIE *Lab* color space (ΔE*) is calculated by the equation:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (9)$$

where ΔL*, Δa*, and Δb* represent the differences in color coordinates of the compared solutions.

To establish the compliance of the samples to the maximum level of color represented by the respective reference solution, the color differencebetween the sample solution and purified water (ΔE_T*) and the color differencebetween the referencesolution and purified water ($\Delta E0$*), were calculated.Requirement is $\Delta E_T^* < \Delta E0^*$.

Mathematically, when a ΔE^* is calculated(Eq. (9)), a spherical distance from a center point is createdin the chosen color space. That is, ΔE^* does not possess a directionalvector component. The solutions would pass the ΔE^* requirement but maynot have the expected hue and may fail the visual appearancerequirement. The criterion for Δh^*_{ab}(vector component) limits the color space to acceptable hues for the sample solution as compared to the existing color reference solution[6].

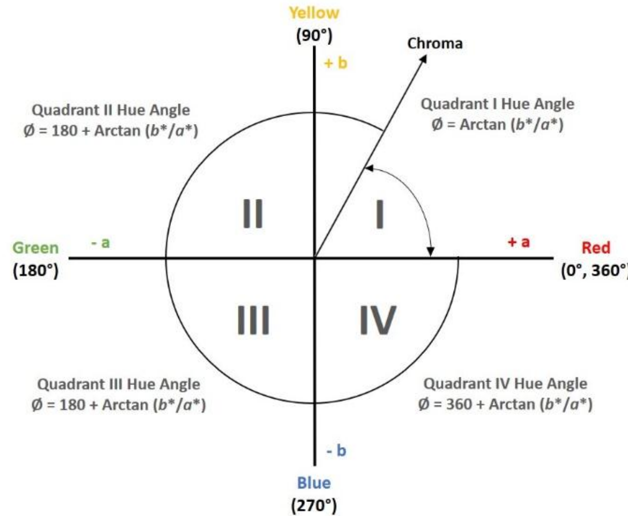

Fig. 2. System diagram of the (a*, b*) plane of the CIE *Lab* color space.To determine the final hue angle (h^*_{ab}) quadrant information depending on the sign (−/+) of the $a^*$and b^* values has to be accounted for.
Adapted from source:"Hue angle determinations and statistical analysis for multiquadrant Hunter L, a, b data", by M. R. McLellan, L. R. Lind, and R. W. Kime, 1995, Journal of Food Quality, 18, p. 235-240 [23].

III. RESULTS

The test for color of solution of the samples was performed at first visually, comparing each of the sample solutions to their respective reference solution. In all cases the sample solutions were not more intensely colored than the corresponding reference solutions.

The test was then repeated instrumentally using the same test and reference solutions.CIE *Lab* color space co-ordinates were calculated for all of the solutions (Table 1 and Table 2). Numerical values from the instrumental color comparison using the calculated CIE *Lab* values, obtained for all 6 samples, were in total compliance with the test requirements (Table 3), corroborating the results from the official visual test.

An example of the calculation of the tristimulus values X, Y and Z and the corresponding CIE *Lab* color space co-ordinates for Ph. Eur. reference solution $Y_7$is given in Table 4 and Table 5, respectively.

Table 1: CIE *Lab* (C/2) values determined for the Ph. Eur. 2.2.2 Color reference solutions.

Ph. Eur. 2.2.2 Color reference solutions			
Reference solution	***L****	***a****	***b****
B1	87.92	0.22	27.61
B2	91.75	-0.51	19.43
B3	93.76	-0.72	14.99
B4	95.86	-0.81	10.34
B5	97.81	-0.70	5.87
B6	99.16	-0.39	2.66
B7	99.43	-0.21	1.45
B8	99.65	-0.14	0.99
B9	99.72	-0.10	0.60
BY1	94.24	-6.35	30.33
BY2	95.78	-5.85	24.10
BY3	96.99	-4.78	17.12
BY4	98.33	-3.03	9.46
BY5	99.10	-1.81	5.41
BY6	99.56	-0.80	2.43
BY7	99.59	-0.42	1.45
Y1	96.51	-9.17	31.51
Y2	97.61	-8.06	24.80

Y3	98.46	-6.31	17.49
Y4	99.17	-3.81	9.57
Y5	99.58	-2.06	4.91
Y6	99.94	-0.92	2.08
Y7	99.96	-0.46	1.03
GY1	98.84	-13.62	29.55
GY2	99.29	-9.55	19.23
GY3	99.53	-6.43	12.37
GY4	99.64	-4.07	7.74
GY5	99.83	-2.65	4.89
GY6	99.74	-1.40	2.60
GY7	99.95	-0.73	1.33
R1	91.31	8.77	19.30
R2	93.32	6.63	14.21
R3	95.48	4.46	9.38
R4	96.65	3.35	6.95
R5	97.82	2.17	4.48
R6	99.06	0.97	1.99
R7	99.46	0.44	1.05

Table 2: CIE *Lab* (C/2) values determined for the sample solutions.

Sample solution	***L****	***a****	***b****
Sodium benzoate	100.08	-0.13	0.43
Citric acid monohydrate	99.65	-0.06	0.46
Glycerol	99.87	-0.01	0.11
Diazepam, 10 mg/2 mL solution for injection	100.32	-0.81	1.62
Topotecan, 4 mg/4 mL concentrate for solution for infusion	99.70	-7.31	13.44
Esomeprazole, 40 mg/vial powder for solution for injection or infusion	99.66	-0.31	0.89

Table 3: Instrumental color comparison using the calculated CIE *Lab* values of the tested excipients and finished pharmaceutical productsand their respective reference solutions.

Sample solution	$h_T{}^*_{ab}$	$\Delta E_T{}^*$	**Reference solution for maximum level of color**	$h0^*_{ab}$	Δh^*_{ab}	$\Delta E0^*$
Sodium benzoate	106.49	0.46	**Y6**	113.75	7.26	2.28
Citric acid monohydrate	97.03	0.58	**B9**	99.08	2.06	0.66
Glycerol	95.31	0.17	**B9**	99.08	3.78	0.66
Diazepam, 10 mg/2 mL solution for injection	116.56	1.84	**GY6**	118.27	1.71	2.95
Topotecan, 4 mg/4 mL concentrate for solution for infusion	118.53	15.29	**Y3**	109.84	8.70	18.65
Esomeprazole, 40 mg/vial powder for solution for injection or infusion	108.94	0.99	**B5**	96.82	12.11	6.30

Table 4: Example of the calculation of the tristimulus values *X*, *Y* and *Z*for Ph. Eur. reference solution Y_7 (performed from 400 to 700 nm, with 10 nm spacing, for CIE 2° Standard Observer and standard illuminant C).

λ (nm)	$Wx(\lambda) = \bar{x}_\lambda S_\lambda$	$Wy(\lambda) = \bar{y}_\lambda S_\lambda$	$Wz(\lambda) = \bar{z}_\lambda S_\lambda$	%*T*	$T\lambda Wx(\lambda)\Delta\lambda/Y$\`	$T\lambda Wy(\lambda)\Delta\lambda/Y$\`	$T\lambda Wz(\lambda)\Delta\lambda/Y$\`	$Wy(\lambda)\Delta\lambda$
400	0.099	0.003	0.463	80.06367	0.0793	0.0024	0.3707	0.030
410	0.325	0.009	1.547	88.61567	0.2880	0.0080	1.3709	0.090
420	1.292	0.038	6.207	93.77017	1.2115	0.0356	5.8203	0.380
430	2.968	0.123	14.496	96.69800	2.8700	0.1189	14.0173	1.230
440	3.959	0.261	19.860	98.18733	3.8872	0.2563	19.5000	2.610
450	3.931	0.443	20.728	99.00733	3.8920	0.4386	20.5222	4.430
460	3.360	0.692	19.286	99.38717	3.3394	0.6878	19.1678	6.920
470	2.283	1.061	15.022	99.56850	2.2731	1.0564	14.9572	10.610
480	1.116	1.612	9.479	99.64900	1.1121	1.6063	9.4457	16.120
490	0.363	2.358	5.286	99.72500	0.3620	2.3515	5.2715	23.580
500	0.048	3.414	2.868	99.70483	0.0479	3.4039	2.8595	34.140
510	0.092	4.842	1.512	99.69950	0.0917	4.8274	1.5075	48.420
520	0.578	6.449	0.720	99.73417	0.5765	6.4319	0.7181	64.490

530	1.519	7.936	0.381	99.78867	1.5158	7.9192	0.3802	79.360
540	2.786	9.145	0.195	99.86500	2.7822	9.1327	0.1947	91.450
550	4.285	9.831	0.086	99.92233	4.2817	9.8234	0.0859	98.310
560	5.877	9.834	0.038	99.97783	5.8757	9.8318	0.0380	98.340
570	7.323	9.148	0.020	100.01400	7.3240	9.1493	0.0200	91.480
580	8.414	7.990	0.015	100.02117	8.4158	7.9917	0.0150	79.900
590	8.985	6.629	0.010	100.04500	8.9890	6.6320	0.0100	66.290
600	8.958	5.321	0.007	100.04833	8.9623	5.3236	0.0070	53.210
610	8.324	4.177	0.003	100.03667	8.3271	4.1785	0.0030	41.770
620	7.055	3.146	0.001	100.05000	7.0585	3.1476	0.0010	31.460
630	5.327	2.196	0.000	100.03333	5.3288	2.1967	0.0000	21.960
640	3.692	1.442	0.000	100.05000	3.6938	1.4427	0.0000	14.420
650	2.352	0.887	0.000	100.03333	2.3528	0.8873	0.0000	8.870
660	1.360	0.503	0.000	100.04333	1.3606	0.5032	0.0000	5.030
670	0.713	0.261	0.000	100.03667	0.7133	0.2611	0.0000	2.610
680	0.364	0.132	0.000	100.04000	0.3641	0.1321	0.0000	1.320
690	0.172	0.062	0.000	100.03333	0.1721	0.0620	0.0000	0.620
700	0.154	0.055	0.000	100.02833	0.1540	0.0550	0.0000	0.550
Sum	98.074	100.000	118.230		***X***	***Y***	***Z***	***Y`***
White point[a]	98.074	100.000	118.232		97.7023	99.8949	116.2836	1000.000
$\Delta\lambda$ = 10 nm; Y/Y_0 = 0.999; X_0 = 98.074; Y_0 = 100.000; Z_0 = 118.232								

[a]The white point value is the exact value for the colorless/white standard.

Table 5: Example of the calculation of the CIE *Lab* (C/2) color space co-ordinates for Ph. Eur. reference solution Y_7.

CIE *Lab* Values	**Calculation with C/2**
	Y7
L*	99.96
a*	-0.46
b*	1.03

IV. DISCUSSION

Our primary intention was to implementthe prescribed Ph.Eur. 2.2.2. method for instrumental color measurement of liquids in the laboratory to enhance the quality of the results for the parameter appearance. Having in mind the availability and everyday use of a UV/Vis spectrophotometer, our initial notion was that implementing the method would be straightforward following the monograph text. An initial hurdle was acquiring a specialized software for calculation, which wasn`t the simplest choice due to financial reasons. For the unambiguous and correct execution of the method, also further clarification from extensive literature inquiry was needed.

Hence, from our experience with the implementation of the instrumental color measurement of the degree of coloration of liquids, the key takeaways are as follows:

- Instrument required for measurement in transmittance is a regular UV/Vis spectrophotometer, that has a bandwidth of 10 nm or less, well-calibrated as per manufacturer`s instructions or general pharmacopeia chapters for UV/Vis spectroscopy, in the visual range (at least in the operating range of 400 – 700 nm). A1 cm path length cuvette is adequate for performing measurements. The spectrophotometer measures the spectral transmittance of the object at each wavelength or ineach narrow wavelength range.The measured spectral data are independent of the instrument conditions (light source or filters), sincethe only input from the instrument for the calculation of the tristimulus values *XYZ*is the obtained transmittance data. From the transmittance data the tristimulus values *XYZ*can be calculated for any chosen combination of illuminant and observer.In addition to numerical data in various color spaces, the graph of the color′s spectral transmittance provides more detailed information about the nature of the color.Another advantage ofUV/Vis spectrophotometers for color measurement is that the sample is positioned reproducibly with respect to the illuminant and detector [6][12]. Measurement can be performed for opaque solids, as well, in reflectance geometry, but some additional reflectance accessories for the instrument are required depending on the nature of the samples. The calibration of the instrument is performed with calibrated ceramic color tiles, as recommended in the instrument manual (white ceramic tile is used to determine the top of the scale where all wavelengths are reflected 100%, black ceramic tile to determine the bottom of scale where all wavelengths are absorbed)[6].

- Before actual measurement, for verification of the calibration in transmission mode, instrument should bechecked with purified water as a white standard and assigned a transmittance of 1.000 at all wavelengths. Then the tristimulus values of the nominally white or colorless standard, X_0, Y_0, and Z_0, are usually equal to the tristimulus values of the standard illuminant (if standard illuminant C is used, $X_0 = 98.0$, $Y_0 = 100.0$ and $Z_0 = 118.1$). Additionally, a black card (or block of the light path) can be used to determine the zero end of the lightness scale. If considered necessary by the lab, further assurance of system suitability before a sample set is generated, can include determination of wave length accuracy, or measurement of a chosen reference material. For reflectance measurements, standardization is accomplished by testing a white traceable ceramic tile. Furthermore, a common practice forin-use instrument check, is to utilize a green tile to confirm the tolerances in *X*, *Y*, *Z*. If desired, multiple color tiles can be incorporated into a color measurement method to further ensure system suitability. However, one tile, typically green, is suitable as it confirms the entire reflected spectrum across the instrument range and visible spectrum[6][7].
- Pharmacopoeia color reference solutions should be freshly prepared and measured and *L*a*b** values calculated for each of the solutions. Since ours was a preliminary study in a trial to implement the method, color reference solutions were prepared in one replicate and measured six times each. In order to implement a quantitative method that does not require preparation of the Ph. Eur. reference color solutions, and lock determined *L*a*b** as reference values for future comparison with sample solutions,better quality of the results should be obtained by preparing the reference color solutions in a sufficient number of replicates and measuring each replicate several times, and mean values are to be taken[12]. Robust statistics for evaluation of the validity of the data should be applied. A possible approach for this is detailed in [15]. From the work of these authors several other points can be taken in the preparation of the reference color solutions, besides preparing the solutions in multiple replicates: increased accuracy and reduced preparation-to-preparation variability can be obtained by preparing the solutions gravimetrically (weighing the prescribed volumes after determining the density); actual concentration of the 10 g/L HCl solution and temperature variation between 15 °C and 30 °C can affect the measured color of the reference solutions (in particular, yellow (iron) primary color solution is sensitive to HClconcentration and temperature variation), so authors suggest purchasing certified 10 g/L HCl solution and performing the measurements at room temperature; in the gravimetric preparation of reference solutions, the GY series showed the most variability (attributable to GY containing the highest percent of yellow primary solution), so increased number of replicates should be preparedfor the GY series in order to achieve statistical validity of results comparable to the other color series.
- The instrumental color measurement as described here is intended for the assessment of color of uniform clear liquid samples and opaque solids, and is not applicable to hazy liquids or translucent solids. The method may be applied to the liquid phase of dispersed systems in cases where the dispersed phase can be removed prior to color measurements.If the sample is turbid or hazy, it should be filtered or centrifuged before measurement. If the solution to be measured contains air bubbles or foam, these should be removed by centrifugation before evaluating the color[7][9].
- Detailed explanation and necessary data for the calculation of the CIE *Lab* valuesare given in USP <631>. Also this chapter includes a detailed example that provides the data and results that should be checked to verify/validate that the correct calculation method has been used.Furthermore, if possible, a statistical comparison of the manually calculated CIE *Lab* values with those from specialized software can be performed to validate the effectiveness of the manual method.Some examples of published nominal CIE *Lab*values for color reference solutions used in commercial software can be found in literature [19].
- An approach to use instrumental colorimetry to ensure compliance with existing monographs is outlined in USP <631>, describing the different metrics that may be used to compare obtained CIE *Lab*values of a sample to the CIE *Lab*values of a reference color solution. This is the approach that has been used in our examples in order to demonstrate the applicability of the instrumental method to existing monographs. In Ph. Eur. 2.2.2., it is referenced that correspondence to pharmacopoeial reference solutions(such as ‘test solution equals reference solution XY’, ‘test solution close to reference solution XY’ or ‘test solution between reference solutions XY and XZ’) can be obtained using appropriate algorithms, directing to the use of commercial software for evaluation of the obtained CIE *Lab* values.Furthermore, USP <631> provides information on how the CIE*Lab* values can be used directly as a quantitative measure of the color attributes of a sample, directed to specification setting for the color attributesin new monographs under development, which do not already have a visual color determination method.

The results of the experimental implementation of the instrumental color measurementin our laboratory contribute to the feasibility of the method in the routine quality control environment.

V. CONCLUSION

The exercise of implementation of the instrumental color measurementdescribed in pharmacopoeias for determination of color of solution of the chosen pharmaceutical excipients and finished pharmaceutical products, demonstrated the feasibility of the instrumental method for routine use, even in laboratories that do not have at their disposal commercial instruments for color measurement and where the method is applied for the first time. The lay out of the step-by-step process presented and the derived key pointscan serve to facilitate the implementation and interpretation of pharmacopoeial requirements of the instrumental color measurement in the quality control laboratory, especially for the first-time user.

Making the instrumental method a common method in the pharmaceutical field, as frequently used, especially in the routine quality control environment, as the visual color determination, should be a future goal in providing better quality data where color is an important aspect of the control strategy. This shift would be greatly facilitated by the further harmonization of the monographs concerned with determination of color between the pharmacopoeias, providing information which would make the instrumental color measurement applicable in any environment, making its use as equally straightforward as the currently widely used visual method. Furthermore, including the instrumental method in new monographs on active pharmaceutical ingredients, excipients or finished pharmaceutical products would also underscore the inevitability of turning color measurement into the default method, replacing the outdated and comparatively substantially less reliable, visual method.

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