Universidade de Aveiro Departamento de Química
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DANIELA FILIPA SANTOS DIAS

CARACTERIZAÇÃO FÍSICA DE PREPARAÇÕES LÍQUIDAS NÃO ESTÉREIS: APARÊNCIA

PHYSICAL CHARACTERIZATION OF NON-STERILE LIQUID PREPARATIONS: APPEARANCE

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Engenharia Química, realizada sob a orientação científica do Doutor Mário Manuel Quialheiro Simões, Professor Auxiliar do Departamento de Química da Universidade de Aveiro e co-orientação do Doutor António Lucas Nunes, Responsável do Setor de Investigação e Inovação Tecnológica da Bluepharma Indústria Farmacêutica SA.

Dedico este trabalho aos meus pais, à minha irmã e à minha madrinha. Por tudo.

"Not all those who wander are lost".

J.R.R Tolkien

o júri

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Palavras-chave

Medicamento, Aparência, Espaços de Cor, Avaliação Visual e Instrumental, Tolerância da Cor

Resumo

A presente dissertação tem como objetivo o desenvolvimento e implementação de um método de avaliação da cor de uma preparação líquida não estéril analisada em diferentes intervalos de tempo, após estar sujeita a diferentes condições de temperatura e humidade relativa no seu período de pré-estabilidade. A avaliação ocorreu através da comparação entre a formulação e diferentes escalas da cor reproduzidas com base na Farmacopeia Europeia 9.7.

Devido ao facto de a avaliação visual poder ser subjetiva, procedeu-se a uma avaliação instrumental da cor a partir de espaços de cor tridimensionais e bidimensionais, nomeadamente o espaço de cor CIELAB.

Dado que a representação bidimensional utilizada para a especificação instrumental da cor não considera todos os atributos associados à mesma (não inclui a luminosidade), determinou-se a Diferença CIELAB (ΔE^*) entre a cor das amostras de referência das escalas e a cor da formulação em análise. A Diferença CIELAB considera todos os parâmetros que influenciam a sensação da cor e permite determinar qual das amostras de referência tem a cor mais semelhante à formulação em análise. Por fim, recorrendo à amostra de referência com a cor mais intensa de uma das escalas, estabeleceu-se uma caixa retangular de tolerância para a cor da formulação em avaliação: todas as formulações com coordenadas fora do limite da caixa devem ser rejeitadas e investigadas. No entanto, a tolerância definida com as coordenadas da cor mais intensa de uma das escalas é meramente representativa: o objetivo é, através de histórico de coordenadas da cor da formulação ao longo do tempo, estabelecer o limite de tolerância com as coordenadas associadas a uma formulação que se encontre no limite das especificações associadas à mesma.

Keywords

Drug Product, Appearance, Color Spaces, Visual and Instrumental Evaluation, Color Tolerances

Abstract

The present dissertation consists in the development of a visual and instrumental method for the evaluation of the color of a non-sterile liquid formulation. The color of the formulation was analyzed at different time intervals, after being subjected to different conditions of temperature and relative humidity in its pre-stability period. The evaluation occurred by comparing the formulation with different color scales reproduced based on the European Pharmacopoeia 9.7.

Due to the fact that the visual assessment could be subjective, an instrumental color evaluation was performed from three- and two-dimensional color spaces, namely the CIELAB color space.

Since the two-dimensional representation used to the instrumental specification of color does not consider all the attributes associated with color (does not include brightness), the CIELAB Difference (ΔE^*) between the color of the standard solutions of the scales and the formulation analyzed under different conditions was determined. The CIELAB Difference between two colors considers all the parameters that influence the color sensation and allows determining which of the standard solution has the most similar color to the formulation under analysis. Finally, using the standard solution with the most intense color of one of the scales reproduced, a rectangular color tolerance box was established to limit the acceptable tolerance for the coordinates of the formulation under analysis: all the formulations with the color coordinates outside the tolerance limits should be potentially rejected and investigated. However, the tolerance defined with the most intense color of one of the scales is merely representative: the main objective is, over time, to obtain a history of the coordinates of the color of the formulation and establish as tolerance limit the coordinates of the color associated with a formulation that is at the limit of the specifications associated with it.

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NOMENCLATURE

CDMO	Contract Development and Manufacturing Organization
CIE	*Commission Internationale de l'Éclairage*
CMO	Contract Manufacturing Organization
CQA	Critical Quality Attributes
EMA	European Medicines Agency
FDA	Food and Drug Administration
GMP	Good Manufacturing Practices
ISO	International Organization for Standardization
LMS	Long, Medium and Short wavelength system
QbD	Quality by Design
QTTP	Quality Target Product Profile
RH	Relative Humidity (%)
USP	United States Pharmacopoeia
a^*	Red/Green color content of a color
b^*	Yellow/Blue color content of a color
L^*	Brightness of a color
Δa^*	Difference of Red/Green color content between two colors
Δb^*	Difference of Yellow/Blue color content between two colors
ΔL^*	Difference of brightness between two colors
ΔE^*	Numerical difference between two colors considering all parameters that influence the color sensation.

CHAPTER I

1.1. CONTEXTUALIZATION

The project consisted in the development of a method for visual and instrumental color evaluation of non-sterile liquid preparations, in particular a liquid preparation for application in the oral mucosa. Liquid preparations for application on the mucosa have increasingly been used as an alternative to solid pharmaceutical dosage forms, due to some advantages, such as the ease of administration and the availability for immediate absorption.

In Chapter I, it is possible to find an introduction in which the theoretical concepts used to support this dissertation are presented, including a brief description of pharmaceutical industry and the characteristics of non-sterile liquid preparations for the application in the mucosa. Since the main objective of the project is the implementation of a color evaluation method, also in Chapter I it is presented a theoretical background related to color, to all parameters that influence the color sensation and to the colorimetric models for color evaluation present in the bibliography.

Appearance is a critical quality attribute of the drug product, so its specification criteria must be met. The change in color may indicate some non-conformity in the formulation specifications. It is therefore important to carry out tests to assess the color of drug products during stability programs. The color evaluation should be performed visually and instrumentally to avoid any subjective questions associated with the visual evaluation of color.

The experimental description of the color evaluation method can be found in Chapter II. The visual color evaluation method was developed based on the European Pharmacopoeia 9.7.

Although European Pharmacopoeia does not require it, an instrumental method of color quantification based on United States Pharmacopoeia (2019) has also been developed. Subsequently, in Chapter III, it is possible to observe the experimental results obtained, as well as their analysis. The analysis of the results occurred in three stages: visual evaluation of the color, instrumental evaluation of the color and attempt to establish tolerances for the color of a specific non-sterile liquid preparation (composition not disclosed). The visual assessment consists of a comparison of the color between the formulations under analysis and the color scales prepared based on the European Pharmacopoeia 9.7. The instrumental evaluation consisted of measuring the color coordinates and representing them in color space models, namely in the CIELAB color space. The third stage consists in the attempt to establish tolerances for the color of the formulation.

Finally, the conclusions regarding the results obtained are presented in Chapter IV, as well as suggestions for future work.

1.2. BLUEPHARMA INDÚSTRIA FARMACÊUTICA, S.A.

This dissertation was developed at Bluepharma Indústria Farmacêutica, S.A. (headquarters of Bluepharma are presented in **Figure 1)**, in the department of Research and Innovation, in the framework of a curricular internship for the master thesis.

Figure 1 - Bluepharma Indústria Farmacêutica, S.A. [*Adapted from* [1]].

Bluepharma is a pharmaceutical company, based in Coimbra, founded in 2001, after the acquisition of an industrial unit belonging to the German multinational Bayer.[1]

Currently, Bluepharma employs more than 580 employees, distributed in an economic group of 17 companies. Even though it has a very extensive national activity, the main focus of Bluepharma is the export of its generic pharmaceutical products, mainly to the United States of America, Europe, Middle East, Africa and Asia.[1]

In 18 years of activity, Bluepharma has conquered several honorable milestones, among them the certification of quality (ISO 9001/2000), environmental (ISO 14001/1999) and occupational health and safety (OHSAS 18000) and the Innovation Award. It is also certified in Good Manufacturing Practices (GMP) by the European Medicines Agency, the U.S. Food and Drug Administration (FDA) and ANVISA (regulatory entity of Brazil).[1], [2]

Bluepharma is dedicated to different areas of activity, such as Research, Pharmaceutical Development, Business Development, Industrial Activities and Marketing Activities. Its activity includes the manufacture of its own drug products or for customers, research and production of new pharmaceutical drugs and the marketing of generic drugs.[1]

Bluepharma started its activity as a CMO (Contract Manufacturing Organization) and now is also a CDMO (Contract Development and Manufacturing Organization). During the years, Bluepharma focused its activity also on disruptive innovation, in particular on the creation of new products and services.[2]

1.3. PHARMACEUTICAL INDUSTRY

Pharmaceutical industry is one of the most important and innovative industries in the world today and, in a simplified way, aims at the development of drug products that improve the quality of human lives.[3],[4]

The pharmaceutical industry is based on research, development and innovation of drug products and is driven by technological and scientific developments that allow the expansion of an existing market or the creation of new markets.[5]

In **Figure 2** is represented a scheme of the life cycle of the drug product. It is possible to observe the stages through which a drug product passes, from its development to its consumption. Bluepharma covers almost the entire value chain of the drug products produced, from research and development to their marketing and quality control.[6]

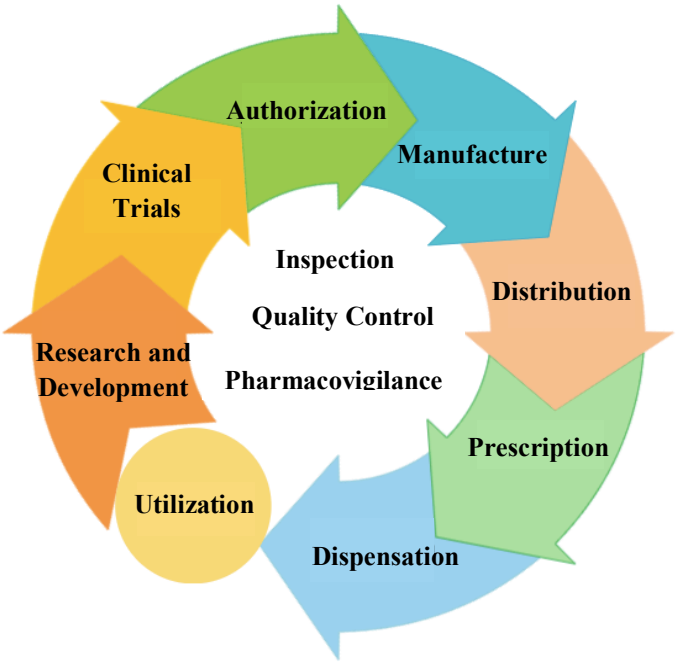


Figure 2 - Life cycle of a drug product [*Adapted from* [6]].

As highly regulated industry, all the activities associated with the pharmaceutical industry are subject to legislation and approval policies regarding the development, manufacture, quality control, marketing and sale of products.[3],[7]

Before introducing a drug product on the market, it is necessary to obtain approval from international regulatory authorities, such as the FDA in the United States of America or EMA in Europe, or regional regulatory authorities, such as INFARMED, in the case of Portugal. In order to obtain the approval from the regulatory authority, it is crucial that the drug product has high levels of quality, efficacy and safety associated with it.[3]

Drug products are released onto the market in a certain pharmaceutical form. According to the regulatory authorities, the classification of pharmaceutical forms depends on three criteria: physical form of the dosage, route of administration and type of release. **Figure 3** is a scheme where the different forms of pharmaceutical dosage are represented considering the route of administration and the physical form.[9],[10]

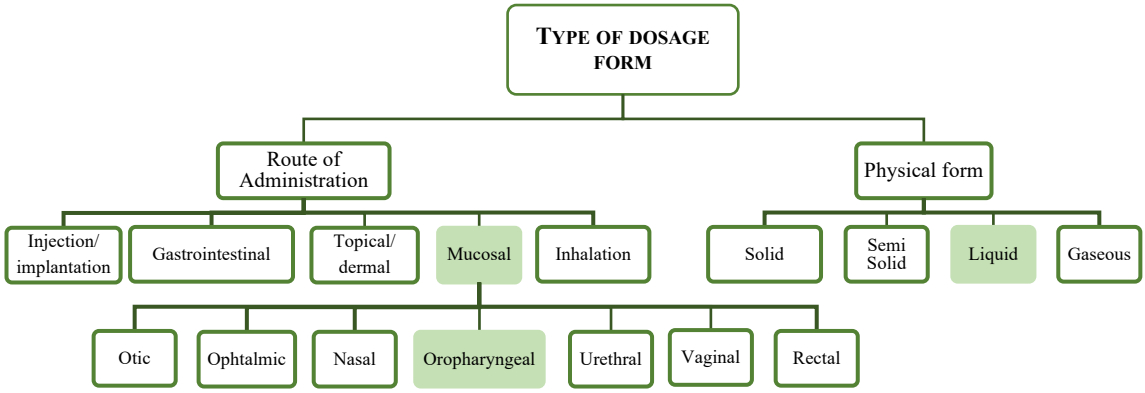


Figure 3 - Characterization of pharmaceutical dosage forms [*Adapted from* [10]].

Based on the United States Pharmacopoeia (USP), the release of the drug product can be immediate or modified. In the immediate release, drug products are formulated to release the active substance right after the administration. Modified release is the deliberate change in the active substance (speed and/or site of action change) of the formulation through specific procedures and can be Extended-Release or Delayed-Release. Extended-Release is the type of pharmaceutical dosage form in which the release of the active substance is extended compared to that observed in an immediate release pharmaceutical form administered via the same route. Delayed- release is the type of pharmaceutical dosage form deliberately modified to prolong the release of the drug substance for some period of time after the initial administration.[10]–[12]

The drug product under analysis can be classified regarding its physical form as liquid and regarding its route of administration as liquid for mucous administration (in particular oral mucosal administration). Its type of release can be adjusted to be immediate or extended, depending on the final composition.

A brief description of the liquid pharmaceutical dosage forms for the application in the oral mucosa are presented in the next sub-chapter.

1.3.1. PHARMACEUTICAL DOSAGE FORMS FOR ORAL MUCOSA ADMINISTRATION

Oral solid dosage forms are the most conventional way to administer pharmaceutical drug products. However, there are some challenges associated with this type of pharmaceutical dosage forms, in particular gastrointestinal degradation and first-pass liver metabolism. These occurrences potentially decrease the bioavailability of the drug product. In order to overtake some of these

limitations, alternative strategies have emerged, such as administration of pharmaceutical drug products directly into the oral mucosa.[11],[13]

Non-sterile liquid preparations have specific characteristics which are described in the following sub-chapter.

1.3.1.1. NON-STERILE LIQUID PREPARATIONS

Liquid preparations have increasingly been used as an alternative to tablets and capsules due to their ease of administration and availability for immediate absorption.[14]

Liquid preparations are generally very versatile; however, they have certain limitations, including the potential risk of instability. Nevertheless, liquid preparations for the administration in the mucosa present several advantages when compared to the other pharmaceutical dosage forms.[13] Due to the ease of administration and the absence of pain in the act of the administration, consumers can be autonomous in taking the drug product. Because it is a liquid preparation, administration to children, to elderly and to patients with reduced capacities is facilitated.[16] Moreover, the effect of the pharmaceutical product could be more accelerated, as the mucous membranes are highly vascularized and allow rapid permeation of the drug product into the systemic circulation.[4], [16]

In pharmaceutical industry, the development of drug products is based on a systematic approach recommended by authorities called Quality by Design (QbD). Very briefly, the application of this approach implies the identification of well-defined objectives and scientific knowledge about all the stages involved in the product development process. Combining the knowledge of the processes with quality risk management tools, it is possible to produce drug products with the quality, efficiency and safety parameters ensured. The pharmaceutical development begins with the establishment of the Product Quality Profile - QTTP. QTTP corresponds to the definition of ideal criteria associated with the quality of the product to be developed. In order to ensure the quality, safety and efficacy of the product, the Critical Quality Attributes (CQA) of the product under development are identified. These attributes, which may be of a chemical, physical, biological or microbiological nature, must be known and controlled during the drug development process, at the time of finish and during the period of stability.[19]–[22]

The study of the stability of a drug product is extremely important, because it is the basis to establish its life cycle, shelf life and behavior over time. The stability of a drug product refers to its ability to maintain its physical, chemical, microbiological, therapeutic and toxicological characteristics and properties throughout its storage and use period.[20]

From several CQAs identified by the Formulation team as critical to the characterization of non-sterile liquid preparations in a specific product (information not disclosed), this dissertation focused on the appearance of the formulation. In particular, the focus in this dissertation is the color

evaluation of a formulation, implementing an instrumental method to assess the change of color over time under specific temperature and relative humidity conditions.

The appearance of the drug product is a parameter that must comply with the respective specification of the product. During the development of this specific product, the specification was established as being from almost transparent to slight pale yellow when assessed under visual inspection. In order to perform the most accurate visual and instrumental evaluation of the color, it is necessary to know in detail the parameters that influence the color sensation and the colorimetric models of color quantification present in the bibliography. In the following sub-chapter, a theoretical background on the color phenomena is presented.

1.4. COLOR EVALUATION OF NON-STERILE LIQUID PREPARATIONS

1.4.1. COLORIMETRY AND CIE

The sensation of color produced by the human eye results from an interaction between three principal parameters: light, an object and an observer. Therefore, in order to perform an accurate color evaluation, it is important to understand in detail all the three principal elements involved in color perception. Colorimetry is the science that is responsible for the study of the color phenomena and of the parameters that influence the color sensation. In the following sub-chapters some theoretical concepts related to light, object and observer are presented.[23],[24]

1.4.1.1. LIGHT

Light is an energy source that propagates in the form of electromagnetic waves with different wavelengths and frequencies. The electromagnetic spectrum (represented in **Figure 4**) consists of a scale of electromagnetic radiation in which all the types of electromagnetic waves are represented: radio waves, microwaves, infrared, visible light, ultraviolet, x-rays and gamma rays. Visible light is located at wavelengths between 380 nm and 780 nm. Shorter wavelengths correspond to violet color and longer wavelengths are associated with red color.[25],[26]

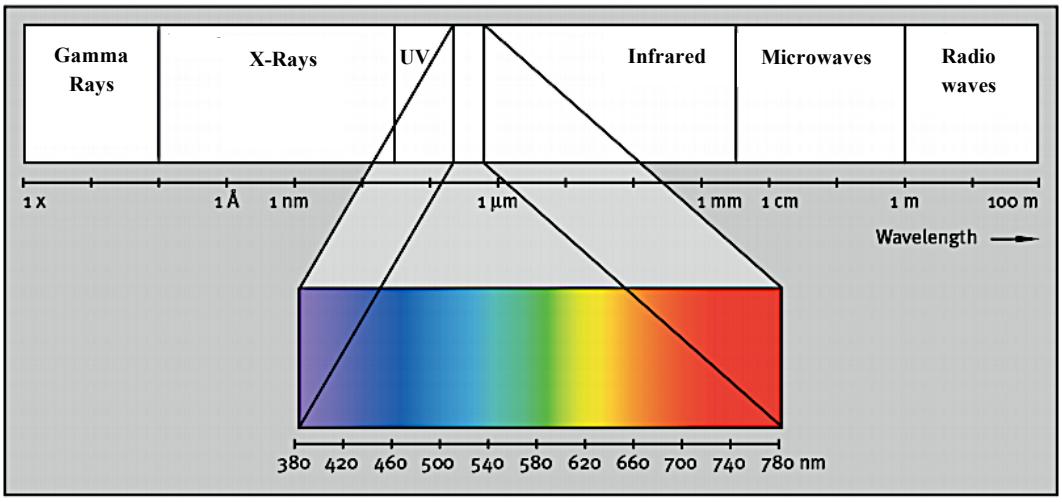


Figure 4 - Electromagnetic Spectrum [*Adapted from* [26]].

The appearance/color of an object/sample depends not only on its physical properties, but also on the nature of the light that falls on it. The incidence of different types of light sources on the same object results in different perceptions of color.[25], [27]

A light source is defined as the radiation that emits many photons in the visible region of the electromagnetic spectrum. There are several types of light sources, among them radiant black bodies, sunlight and tungsten incandescent lamps. A black body consists of a hypothetical object that absorbs light at all wavelengths. The entire light incident on the object is absorbed and results on a total black coloration of the object. However, when exposed to higher temperatures, it acquires brightness and can be considered as a light source.[28]

Sunlight contains the same amount of short, long and medium wavelength electromagnetic waves, reason why it is considered white light.[25]

Tungsten incandescent lamps, as the name indicates, are made up of tungsten filaments. These filaments are distributed in a mixture of inert gases supported by electric energy conducting wires and wrapped in a glass support.[25] The passage of electric current through one of the conducting wires causes a vibration in the molecules of the tungsten filament, making it incandescent, which leads to the heating and subsequent incandescence of the lamp.[28],[29]

The selection of light source is critical for the correct evaluation of the color.[25] As can be seen from the **Figure 5**, the spectral behavior of the sunlight/white light (represented in **Figure 5 A**) is different from the incandescent lamp (represented in **Figure 5 B**), leading to different perceptions on the evaluation of the color.[23],[26],[27],[28]

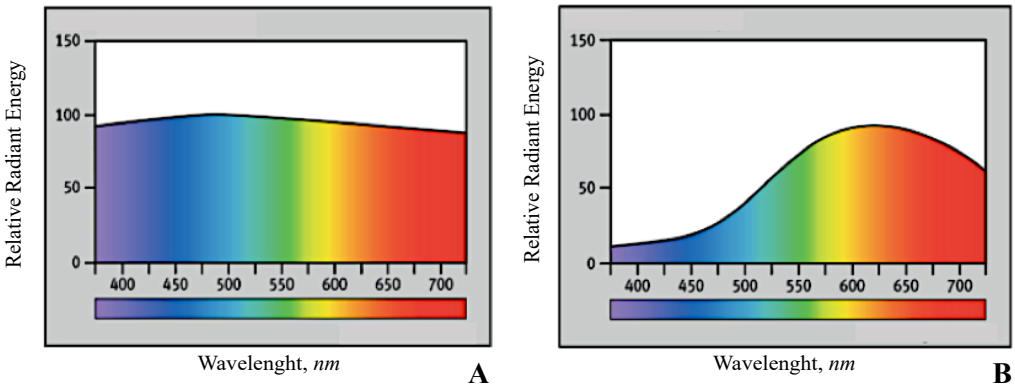


Figure 5 - Spectrum corresponding to sunlight/white light (A) and spectrum corresponding to the incandescent light of a lamp (B) [*Adapted from* [26]].

1.4.1.2. OBJECT

During the interaction of light with an object, various phenomena can occur, including absorption, reflection and transmission, from which it is possible to observe the color of objects.[30] On one hand, the absorption of light occurs selectively at a given wavelength through pigmented particles (atoms) on the surface of the object. Briefly, the absorption of light leads to the formation of a colored object, whose color corresponds to the radiation associated with the wavelength not absorbed by the pigments.[23],[25] On the other hand, the reflection phenomena correspond to the fraction of incident light that is reflected by an object at certain wavelengths and the transmission corresponds to the fraction of the incident light that is transmitted by an object.[31]

Herein, the instrumental color evaluation performed was based on the transmittance phenomena, following the recommendation of United States Pharmacopoeia (USP). **Figure 6** shows a schematic representation of the transmittance phenomena.[31],[32]

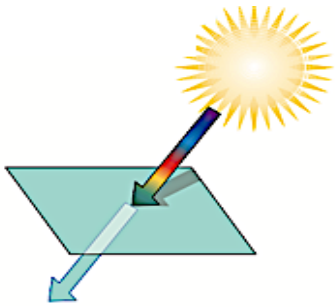


Figure 6 - Transmittance phenomena [*Adapted from* [31]].

1.4.1.3. OBSERVER

The perception of color by the human eye is the result of the incidence of a photon beam on specialized cells present in the retina of the observer. The retina captures light and transforms it into nervous impulses, which are transmitted to the brain via the optic nerve to be interpreted.[24],[25] The retina is composed of photosensitive cells: the rods and the cones. The rods are light-sensitive photoreceptors throughout the visible region of the spectrum; however, because they are not selective in light absorption, they cannot detect color. On the other hand, cones absorb at specific wavelengths and detect color in the presence of a high light intensity (daylight vision).[33]

Figure 7 shows a representation of the human eye and the photoreceptor cells. Each one of the photoreceptor cells (cones and rods) has a pigment that has more sensitivity to absorb at certain wavelengths.[25] There are three types of cones: one with sensitivity in the red zone, other with sensitivity in the green zone and another with sensitivity in the blue zone. One of the formal systems for designating cones is the LMS (long, medium, short) system. Red cones are associated with the perception of long wavelengths, green cones are associated with medium wavelengths and blue cones are associated with shorter wavelengths.[25], [34] More details about the LMS system are present in the sub-chapter 1.5.1.2.

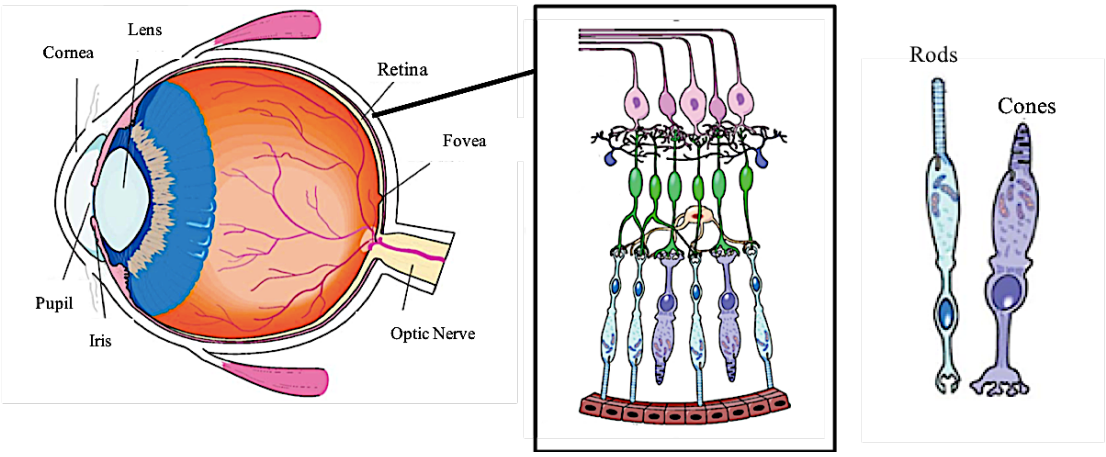


Figure 7 - Human eye representation and photoreceptors cells [*Adapted from* [34]].

In the sub-chapters above, the parameters that influence the sensation of color were briefly presented. Taking into consideration these parameters, colorimetric models were developed that allow the numerical specification of the color in space. These models are presented in the following sub-chapter.

1.5. COLORIMETRIC MODELS AND COLOR SPACES

Isaac Newton was the pioneer of the study of the nature of colors, demonstrating in 1671 that when sunlight (white light) falls on a glass prism, a band (spectrum) composed of different colors (red, orange, yellow, green, blue, indigo and violet) is originated.[26] This phenomena is represented in **Figure 8.**

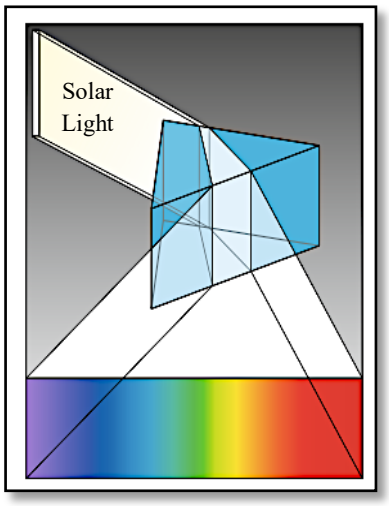
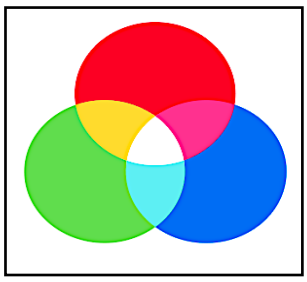


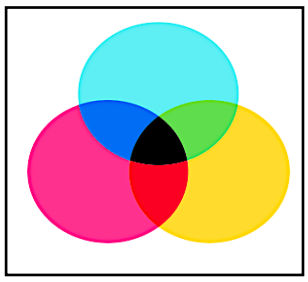
Figure 8 - Light dispersion through a prism [*Adapted from [26]*].

Associated with the phenomenon of color sensation are many theories that have been developed overtime. These theories are based on the premise that all colors could be obtained by mixing the light of three different wavelengths associated with the primary colors (red, green and blue) - Trichromatic Theory.[34],[35]

The various color sensations are derived from the mixture of the two or more primary colors, associated with two basic color models of the Trichromatic Theory: the additive model and the subtractive model (both models are present in **Figure 9**). The additive model consists of the sum of two or more primary colors (red, green and blue), which allows the secondary colors (magenta, cyan and yellow) to be obtained. The subtractive model consists of the mixture of several pigments that absorb in a certain wavelength range of white light. The primary colors of the subtractive model are magenta, yellow and cyan. When two or more colors are mixed, they subtract from white the primary colors of the additive model (red, green and blue) and reduce the reflected radiation in order to cancel it out. This phenomenon creates a black sensation.[25], [36]



Additive Model



Subtractive Model

Figure 9 - Additive and Subtractive Model [*Adapted from* [36]].

The visual assessment of color based on Trichromatic Theory is associated with a high level of subjectivity and abstraction. Therefore, several color classification models were established, involving fields of study less subjective, as can be seen in **Table 1**.[37]

Table 1 - Color Classification Models.[37]

Examples	Premises	Study Field
Trichromatic Theory	Color evaluation based on the three additive primary colors (red, green and blue) and on the spectral sensitivity of the cones present in the retina. Constitutes the base theory of the other models.	Psychology
Munsell Color Model	Evaluation of color based on visual perception by the human being and based on physical parameters (hue, chroma and brightness).	Psychophysics
CIEXYZ	Evaluation of the color based on the determination of the spectral distribution of incident light.	Colorimetry
MacAdam Color Space RGB Color Space CIELAB Color Space	Quantitative evaluation of color based on psychological, physical and mathematical parameters associated with color.	Psycometry

As mentioned before, the simplest color classification theory consists of the Trichromatic Theory, which is based only on psychological parameters and do not allow the reproduction of all the colors of the visible spectrum.

The Munsell Color Space Model, in addition to the psychological field, also uses physical parameters for color classification, which allows obtaining more colors.[28] The three main parameters associated with color are hue, chroma and brightness. The hue of a color corresponds to the initial perception of color that can be identified in a given object (blue, red, orange, pink, among others). Chroma indicates the sharpness or lack of luminosity of a color and represents how close the observed color is to its pure hue or grey color. The brightness corresponds to the relative lightness or darkness of the color of an object.[27], [28], [34]

The perception of these parameters still depends on the physiological characteristics of the human eye, making this model also subjective. Therefore, CIEXYZ model gained interest as it considers not only psychological, but also physical and mathematical parameters associated with the sensation of color.[25] CIEXYZ model constituted the basis for the establishment of color space models for color specification (MacAdam, RGB and CIELAB). These models are discussed in more detail in the following sub-chapters.[28], [38]

1.5.1. COMISSION INTERNATIONALE DE L'ÉCLAIRAGE (CIE)

There are several theories associated with color sensation, however, the search for a universal color classification system increased. Thus, *Commission Internationale de l'Éclairage* (*CIE*), an international entity whose objective is the study of light, lighting and color spaces was created.[39]

As already mentioned, the sensation of color results from the interaction between light, the object and the observer. In order to universalize the study of color, the CIE standardized parameters that influence the color sensation.[25], [28], [38]

1.5.1.1. COLOR EVALUATION PARAMETERS STANDARDIZED BY THE CIE

Since the sensitivity of the retina to color is different at the center and periphery, it was essential to standardize the angles of observation to measure the color of formulations. According to the CIE, two standard observers (2° and 10°) are mainly used. The standard observers represent the sensitivity of the human eye to the color. A 2° standard observer limits the observation area to the central part of the retina, in which only cones are present (responsible for detecting color in the presence of light). A 10º standard observer includes, in addition to the central part, another area of the retina that contains rods (responsible for detecting color in conditions of reduced light). Because the 10° standard observer allows a wider field of view, it is possible to obtain more realistic values regarding the quantitative evaluation of the color, reason why this angle is used more often in the industry. **Figure 10** shows a representation of the two types of standard observer placed at the same distance from the object and different fields of view.[25], [28], [34], [37]

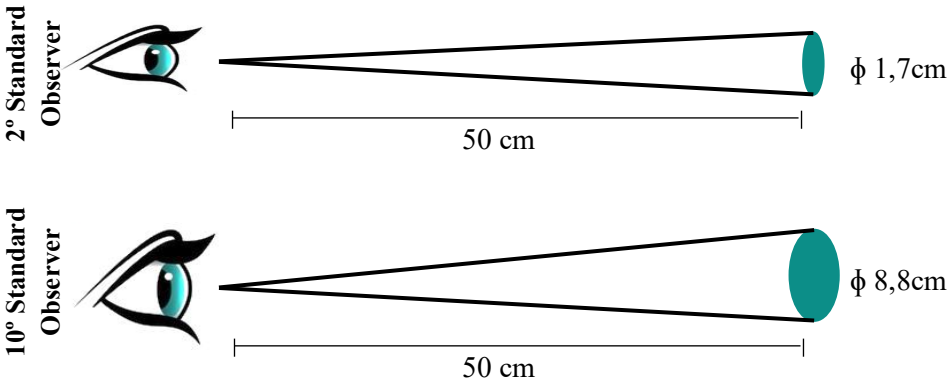


Figure 10 - Schematic representation of the different standard observers [*Adapted from [39]*].

Since color is a property of light, it was also necessary to standardize light sources in order to be able to perform color measurements under the effect of various types of lighting. CIE standardized a set of illuminants with specific characteristics, among them illuminant A, C and D65. An illuminant consists of a standard numerical representation of the spectral energy distribution curve of a given radiation that simulate different types of light sources. [28], [34], [37] Illuminant A has the same spectral distribution as a black body when exposed to a temperature of $2856\ K$. It represents an incandescent tungsten lamp.[25] Illuminant C simulates sunlight but does not contemplate the ultraviolet region of the electromagnetic spectrum and is therefore not the best illuminant to simulate sunlight.[25],[34] The most indicated illuminant to simulate sunlight is D65, with a color temperature of $6500\ K$. This illuminant represents the sunlight more rigorously. Its specification resulted from a study of the daylight in different geographic regions. **Figure 11** shows the spectral distribution of illuminants A and D65.[25], [34], [37]

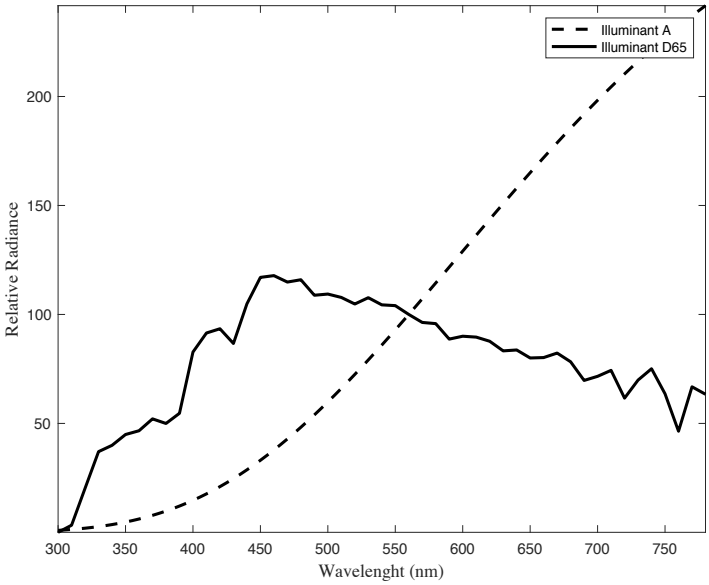


Figure 11 - Spectral distribution of illuminants A and D65 [*Adapted from [25]*].

1.5.1.2. COLOR QUANTIFICATION BASIC MODELS STANDARDIZED BY THE CIE

According to CIE, all colors result from the mixture of the three primary colors: red, blue and green (Trichromatic Theory). However, due to the limitations associated with this theory - such as the difficulty in representing all the colors of the visible spectrum -, the CIE implemented a mathematical model in which the three additive primary colors (red, green and blue) were represented by the variables X, Y and Z, also known as tristimulus, and to which are associated color-matching functions x, y and z. These functions represent proportions of each primary color necessary to reproduce almost all the colors in the visible spectrum.[25],[38] Through the conversion of the three main colors into mathematical functions, it was possible to verify that these assumed negative values, which mathematically does not make sense, because it is not possible to mix negative amounts of colors.

In order to overtake these limitations, the CIEXYZ model was implemented. This model is only based on positive values of the trichromatic components, as can be seen in **Figure 12**. In addition to these mathematical functions representing the three primary colors, they also represent the sensitivity that the cones present in the human retina have in absorbing light at certain wavelengths (LMS System referred to in subchapter 1.4.1.3). In this way, it makes sense to use these functions as a basis for the quantification of color, due to the fact that they consider the psychological and mathematical factors associated with colors.[25], [40],[41]

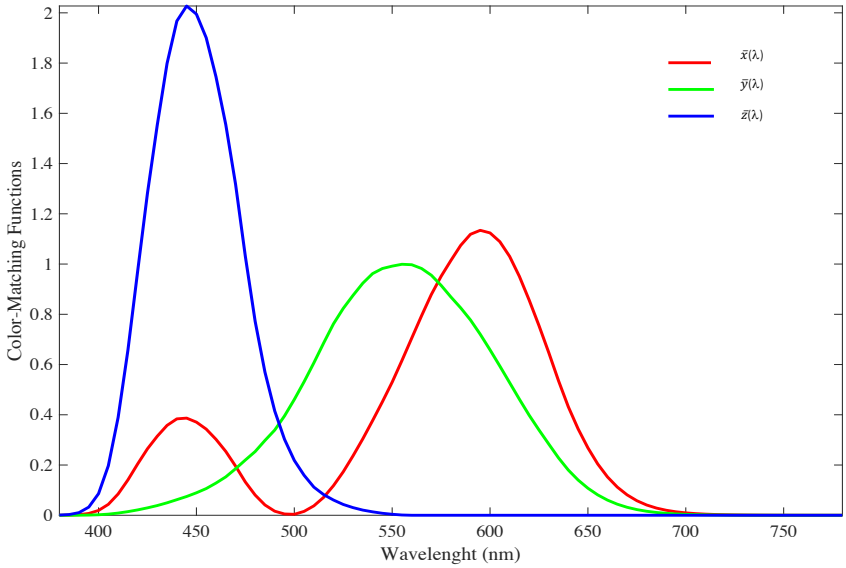


Figure 12 - Color-Matching Functions for a 10º standard observer [*Adapted from* [25]].

The CIEXYZ model is the psychological and mathematical basis of all the color spaces used to specify the color. It is the simplest model to numerically specify a color, through a xy Chromaticity Diagram.[25], [38],[42]

In **Figure 13** the coordinates of two different colors overlapping in the Chromaticity Diagram are represented. The line segment that connects both coordinates only indicates that the mixture between the two colors represented allows obtaining all the colors on which the segment is positioned in the diagram.[25],[38]

One of the main objectives of the present dissertation is to determine the relation between the numerical difference and the visual noticeable difference of the colors of the samples under analysis. The numerical difference between the color coordinates represented in the Chromaticity Diagram is not related to the color differences that can be visually perceived. Because of these reasons, it was not possible to use the CIE XYZ model to specify the color in the present dissertation.[25], [38],[43]

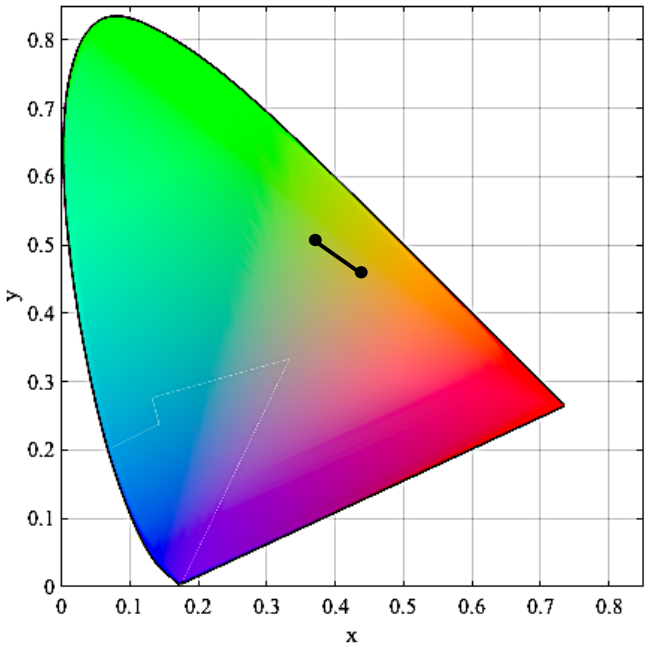


Figure 13 - Chromaticity Diagram [*Adapted from* [25], [42]].

In addition to the limitation described above, there is also the fact that the Chromaticity Diagram does not consider the attribute of brightness. This attribute is crucial to the color reproduction, because the alteration of brightness values causes perceptual changes in color.[30] Therefore, in order to overcome the limitations of CIEXYZ model and the Chromaticity Diagram, Color Space Models were implemented.[25], [38],[43]

1.5.2. COLOR SPACES

There are several Color Space Models presented in the literature, including Munsell, MacAdam, RGB and CIELAB Color Spaces Models.[28] The main objective of the study of the different color spaces was to understand which one of the models would be more appropriate for the color evaluation intended in this project. For the quantification of the color, it is intended to use a color space as uniform as possible that allows the clear determination of the difference between colors and relate the numerical difference between two colors to the noticeable perceptual difference.[25], [37], [38],[43]

1.5.2.1. MUNSELL COLOR SPACE

In the Munsell Color Space all the colors on which the three-dimensional models are based are represented, as can be seen in **Figure 14**.[28] This color space model associates different characteristics of hue, chroma and brightness parameters for all the primary and secondary colors, enabling the reproduction of all the colors present in the visible spectrum. For example, cyan

results from mixing blue and green primary colors. However, by changing the characteristics of hue, chroma and brightness, it is possible to obtain other colors through cyan.[37], [43]

This color space model is very important for the visual evaluation of the color performed in this dissertation, because it is from this that it is possible to specify the color range of the non-sterile liquid preparation under analysis between almost transparent and slight pale yellow. The pale yellow color consists of the yellow color with the characteristics of hue, chroma and brightness altered.[28]

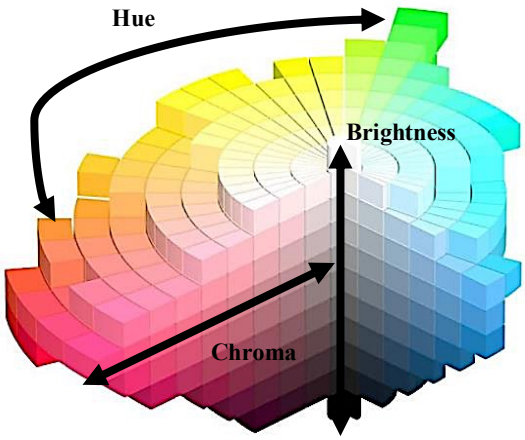


Figure 14 - The Munsell Color Space Model [*Adapted from* [25]].

Even though it is very complete and allows determining perceptually differences between colors, Munsell Color Space Model has some limitations, such as there are no known mathematical models capable of converting the color representation from the Munsell system into numerical values.[25] The Munsell Color Model is also subjective because depends on the physiological characteristics of each human being, the geometry/quantity of the objects/samples whose color is being compared and the lighting present at the time of color evaluation.[27]

All these limitations led to the non-use of the Munsell Color Space in the instrumental evaluation of color, although it was used to the visual color evaluation.

1.5.2.2. MACADAM COLOR SPACE

By representing the coordinates of one specific color in the chromaticity diagram, it is possible to establish a space that includes all colors that are indistinguishable from the color located in the center of the space. This space is called The MacAdam Color Space Model and demonstrates that the color difference can be measured using a metric in a Chromaticity Diagram.[25], [44]

Through the analysis of **Figure 15** (representation of The MacAdam Color Space) it is possible to verify that the spaces created are ellipses, which are not uniform color spaces. Due to this fact, the numerical difference between two colors does not correspond to the visual difference that can be perceptually detected (proving that the Chromaticity Diagram is not a perfect model of

human color perception), which is one of the main objectives of the present dissertation. In order to achieve this objective, it is crucial to represent colors in a practically uniform model, where the color spaces do not resemble ellipses, but rather circles.[43], [45]

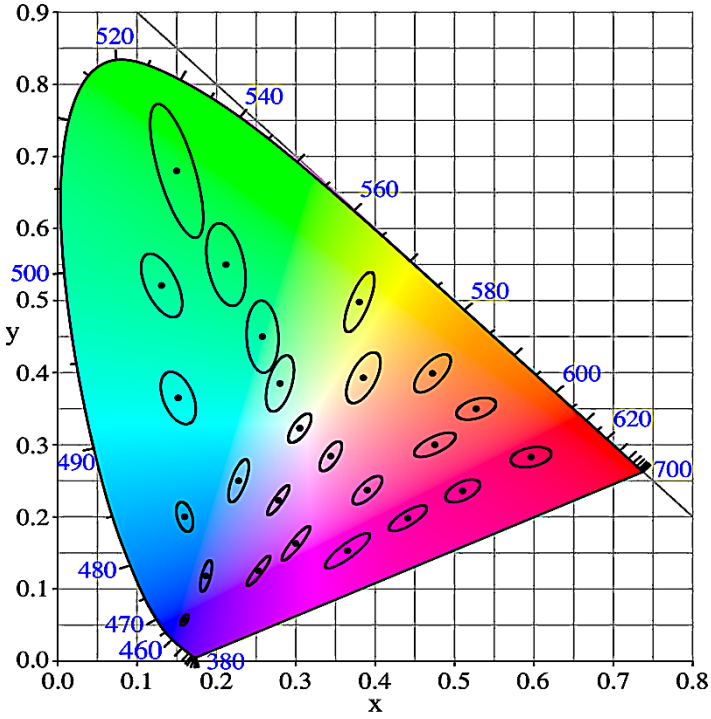


Figure 15 - The MacAdam Color Space Model [*Adapted from* [28]].

1.5.2.3. RGB COLOR SPACE

The RGB model is often represented by a three-dimensional cube, as shown in **Figure 16**, where it is possible to visualize all the colors that can be obtained from the mixture of the primary and secondary colors. The RGB cube present in **Figure 16** was developed using Matlab Software ® and the code is represented in **Attachment E**.[42]

The red, green and blue primary colors (RGB colors) are represented at the vertices of the cube, as well as the secondary colors and the black and white. The value of the coordinates of the colors varies between 0 and 255 in the three axis.[43], [45]

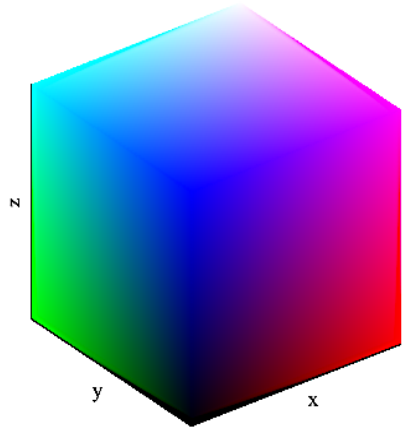


Figure 16 - RGB Color Space [*Adapted from* [42], [43]].

Although it is possible, through the RGB color space, to determine numerically the difference between two colors considering all the attributes associated with it, this model has limitations that concerns to human visual perception, because it is difficult to determine the visual differences between two colors in the three-dimensional models. [37], [43], [46]

Even though not used integrally, some concepts of the three-dimensional RGB model were applied for the instrumental evaluation of color in this dissertation. The instrumental assessment of color was performed based on a two-dimensional cartesian diagram whose axis correspond to the coordinates of the RGB colors and yellow, mathematically converted into the coordinates of the model in use. The following sub-chapter explains in more detail the model used for the instrumental evaluation of color.

1.5.2.4. CIELAB Color Space Model

In order to overtake the limitations associated with MacAdam, Munsell and RGB color spaces, many color spaces were developed, using as reference the colors of the Munsell Color Space Model.[28], [38] One of the most feasible systems created was CIELAB Color Space Model, which allows the conversion of the Munsell Color Space Model into a mathematical model. This color space suggests that the brain has antagonistic perceptions of color (perception of green/red, perception of blue/yellow and perception of white/black) and consists of the representation of color through three-dimensional coordinates: chromaticity coordinates (a^* and b^*) and brightness (L^*). [27], [28]

Thus, the CIELAB color space considers the field of psychology (perception of color by the human brain), physics (coordinates are related to physical parameters), and mathematics, since it specifies the color numerically.[47] **Figure 17** shows the three-dimensional CIELAB Color Space.

All the figures related to this color space model were obtained using the Matlab Software ® and the codes are present in **Attachment D**.[42],[48]

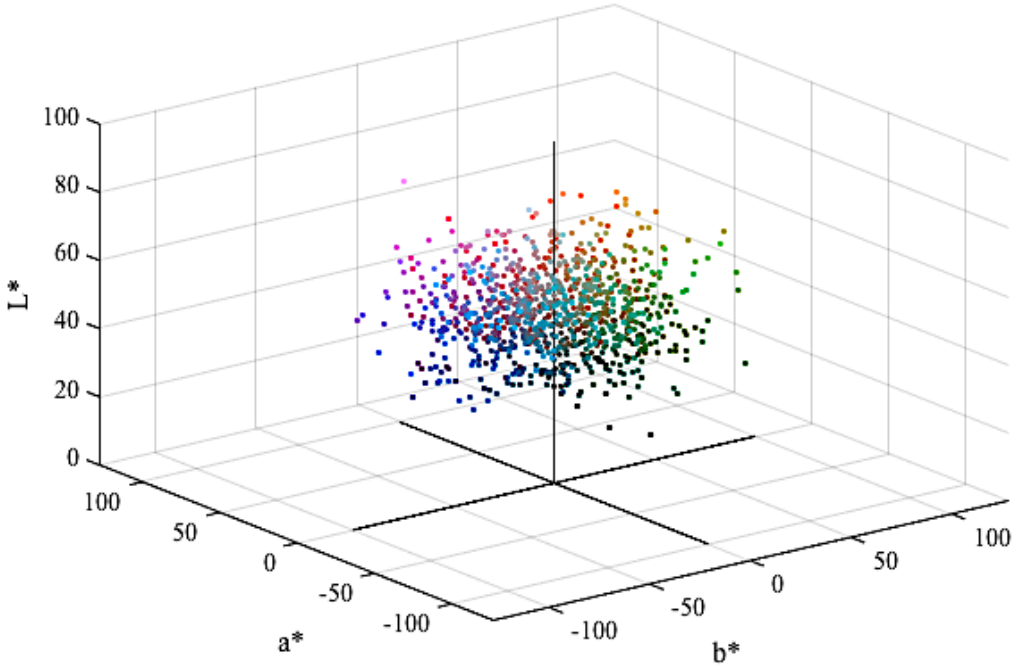


Figure 17 - Three-Dimensional CIELAB Color Space [*Adapted from* [48]].

The a^* coordinate represents the axis of antagonistic sensations red/green, the b^* is related to the axis of antagonistic sensations yellow/blue. The white color is obtained when the L^* coordinate takes the value of 100. If L^* takes a value of zero, the color obtained is black.[37], [43], [47]

In the sub-chapter about MacAdam color space model (subchapter 1.5.2.2), the importance of using a color space as uniform as possible to represent the color coordinates was demonstrated. CIELAB color space is approximately uniform for the perception of small color difference and allows to establish a linear color space in which the distance between the coordinates of each color is connected to the noticeable perceptually color difference between the colors.[25],[34],[43]

As mentioned in sub-chapter 1.5.2.3 (RGB color space model), it is complicated to detect perceptually differences between colors in three-dimensional models. By projecting the color coordinates of the CIELAB Color Space Model into the plane (Erro! A origem da referência não foi encontrada.), it is possible to obtain two-dimensional cartesian diagrams (present in **Figure 19**). Two-dimensional representation of colors facilitates the graphical detection of perceptual differences between two colors.[48], [49]

Herein, the instrumental evaluation of the color was performed using the two-dimensional diagram in which are represented the b^* coordinates as function of a^* coordinates (yellow/blue

content as function of red/green content), thus demonstrating the application of the basic concept of RGB color space in two-dimensional diagram format.[47], [48]

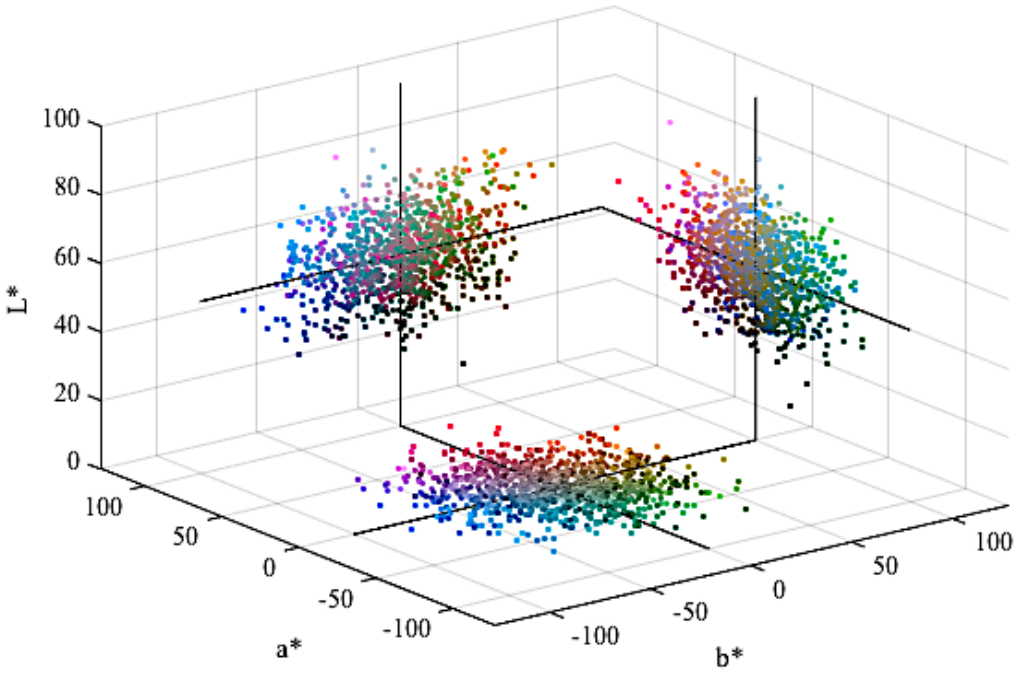


Figure 18 - Projection of the three-dimensional color space into two-dimensional planes [*Adapted from [48]*].

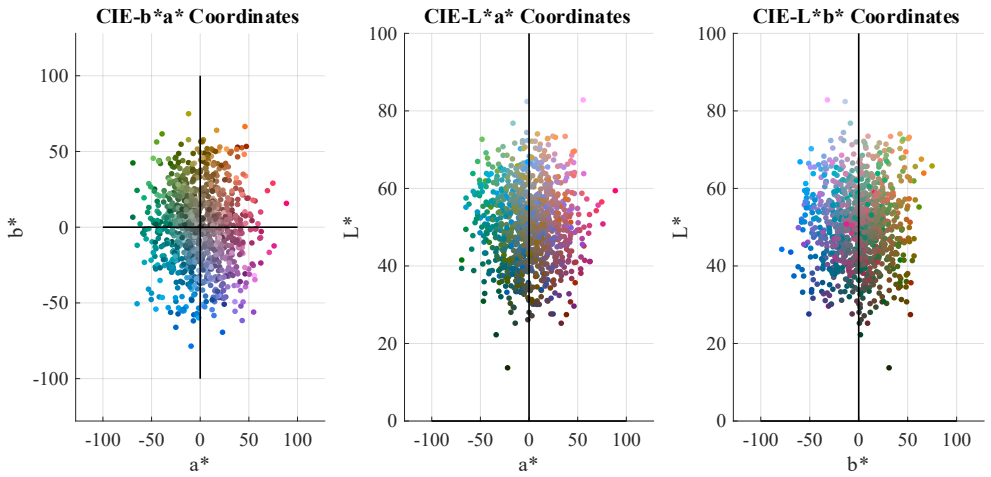


Figure 19 - Two-dimensional color spaces obtained through the projection of three-dimensional color coordinates [*Adapted from [48]*].

Through the CIELAB color space it is possible to quantify the differences between the colors taking into consideration all the parameters associated with the color (hue, chroma and brightness), thereby overcoming the limitations of the Chromaticity Diagram referred to in the sub-chapter 1.5.1.2.[50]

The CIELAB Difference between two colors (ΔE^*) corresponds to the metric distance between the two coordinates of the colors located in the three-dimensional CIELAB color space and can be calculated using Equation (1). In addition, it also represents the numerical response of the human eye to the sensation of color.[25], [37], [51] Thus, from the ΔE^* parameter it is possible to relate the numerical difference between the color coordinates and the visual perceptions that are possible to obtain, considering all the parameters related to color[25].

Table 2 shows the color differences that can be perceived according to the value of ΔE^*.[50]

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{\frac{1}{2}} \quad (1)$$

Table 2 - Color difference perception based on ΔE^*parameter.[50]

ΔE^*	Color Difference Perception
0 to 1	Not noticeable.
1 to 2	Noticeable to an experienced observer.
2 to 3, 5	Noticeable to an inexperienced observer.
3, 5 to 5	Difference between the two colors is evident.
$>$ 5	Distinction between two different colors.

The parameters Δa^*, Δb^* and ΔL^* correspond to the difference between the values of color coordinates of the formulation samples under analysis and the standard solutions of the scales, as described in Equations (2) to (4).[25], [37], [51]

$$\Delta a^* = a^* - a^*_{ref} \quad (2)$$

$$\Delta b^* = b^* - b^*_{ref} \quad (3)$$

$$\Delta L^* = L^* - L^*_{ref} \quad (4)$$

Δa^* parameter indicates whether the sample is more reddish ($\Delta a^* > 0$) or greener ($\Delta a^* < 0$) than the reference. When Δb^* is positive, it is possible to conclude that the sample is more yellowish than the reference and when it is negative it is concluded that it is more bluish. ΔL^* value indicates whether the sample analyzed is lighter ($\Delta L^* > 0$) or darker ($\Delta L^* < 0$) than the reference. **Table 3** shows the definition of the CIELAB parameters.[37], [47]

Table 3 - Definition of the CIELAB parameters.[37]

Parameter	Definition
$\Delta a^* > 0$	The sample is more reddish than the reference.
$\Delta a^* < 0$	The sample is greener than the reference.
$\Delta b^* > 0$	The sample is more yellowish than the reference.
$\Delta b^* < 0$	The sample is more bluish than the reference.
$\Delta L^* > 0$	The sample is lighter than the reference.
$\Delta L^* < 0$	The sample is darker than the reference.

Using the CIELAB color model, a tolerance for the color of a sample can be established using Equations (2) and (3). In this case, the parameters Δa^* and Δb^* correspond to the difference between the color coordinates of the sample under analysis and the desirable color coordinates for the sample.[37],[52]

The tolerance limit (that corresponds to the rectangular box that is possible to observe in **Figure 20)** is established by projecting the parameters Δa^*, Δb^*, $-\Delta a^*$ and $-\Delta b^*$ on the axes. The samples under analysis whose color coordinates are inside the box are accepted because they are within the tolerance limits. All samples whose color coordinates are outside the box are rejected. [52]

Color tolerance can only be established because the CIELAB color space is practically uniform, as can be seen in **Figure 20**, where a hypothetical rectangular color tolerance box is represented placed on a completely uniform representation of the two-dimensional diagram of b^* coordinates as a function of a^* coordinates. This representation was obtained using Matlab Software ® and the code is shown in **Attachment E**.[42],[49]

In the present project it was not possible to establish the tolerance for the color of the formulation under analysis, because for this it is necessary to obtain a history of the coordinates of the color of the formulation overtime. This subject will be explained in more detail in the Chapter III (sub-chapter 3.4).

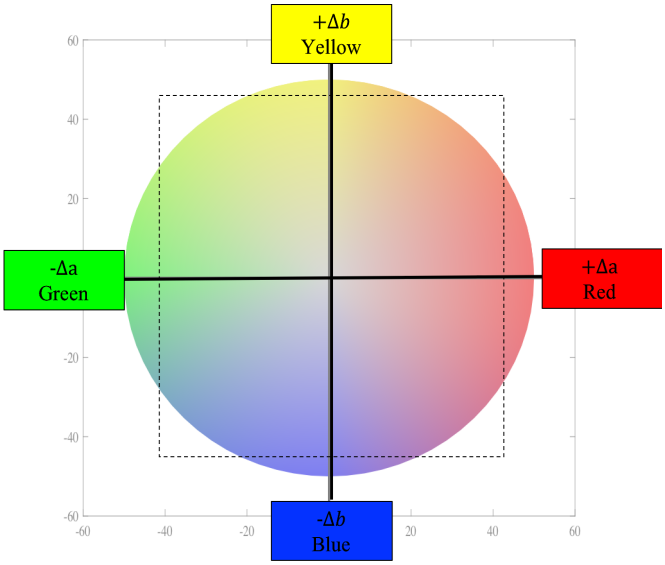


Figure 20 - Rectangular color tolerance box [*Adapted from* [42], [49]].

CHAPTER II - EXPERIMENTAL PART

2.1. OBJECTIVES

The aim of the present dissertation is the implementation of a color evaluation method for non-sterile liquid preparations. To this end, the evaluation of color is divided into three stages: visual evaluation of color, instrumental evaluation of color based on CIELAB Color Space Model and an attempt to establish a tolerance for the color of the formulation under analysis. **Figure 21** shows a representative scheme of the objectives of the color evaluation and the methodology used.

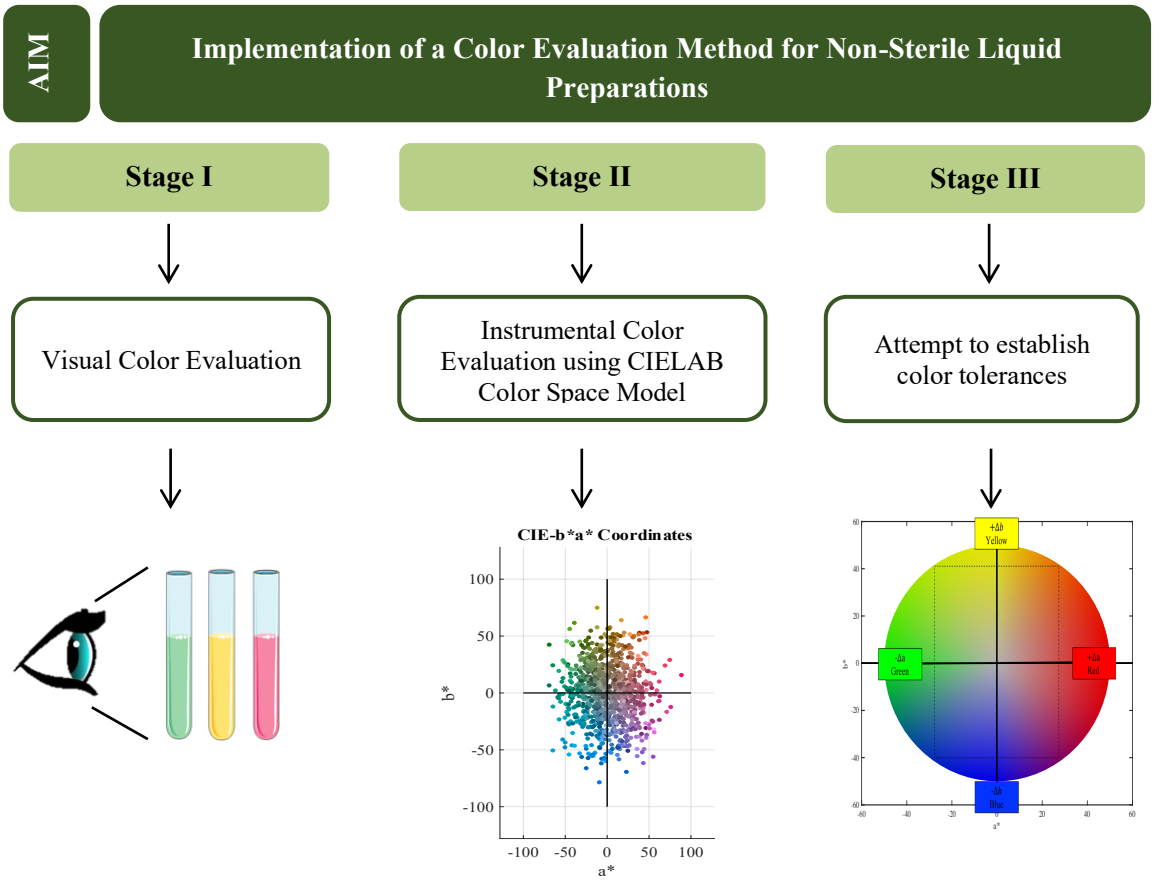


Figure 21 - Objectives and methodology for the analysis of the results.

2.2. CONTEXTUALIZATION OF COLOR EVALUATION METHODS BASED ON PHARMACOPOEIAS

Initially, a bibliographic research was carried out for the implementation of the color evaluation method of non-sterile liquid preparations. The bibliography analyzed for the color evaluation of the non-sterile liquid preparation was based on the methods present in the United States Pharmacopoeia - USP (*Chapter 631: Color and Chromaticity*), Brazilian Pharmacopoeia 5th edition (*Chapter 5.2.12: Liquid Color*) and European Pharmacopoeia 9.7 (*Chapter 2.2.2: Liquid Coloration Degree*). The description of the Brazilian Pharmacopoeia and USP methods are similar: the main objective is the visual comparison between the formulation samples under analysis and the color scales reproduced. The difference between Brazilian Pharmacopoeia and USP is that the last one advises the application of instrumental methods for the evaluation of color.[32],[53]–[55]

Although the basic principle is the same, the method described in the European Pharmacopoeia presents differences regarding the preparation of standard solutions of the color scales. The standard solutions are prepared, in all cases, from primary solutions of cobalt chloride (red stain), ferric chloride (yellow stain) and cupric sulfate (blue stain). However, while in the case of Brazilian Pharmacopoeia and USP only one color scale is prepared, in European Pharmacopoeia five scales with different color hues are prepared, using different concentrations of the primary solutions.[53]–[55]

The visual color evaluation should be performed under the same conditions: against a white background, under daylight, transversally (in the case of Brazilian Pharmacopoeia and USP) and both vertical and horizontally, for the case of European Pharmacopoeia. **Table 4** describes the objectives and conditions of evaluation for each of the methods.[53]–[55]

Although the visual evaluation methods for all Pharmacopoeias have been performed, the focus of this dissertation is the experimental procedure and results based on the European Pharmacopoeia, with the addition of the application of instrumental methods suggested by USP. It is important to note that European Pharmacopoeia does not advise the instrumental quantification of color.[32], [53]

Table 4 - Description of each of the color evaluation methods present in USP and in the Brazilian and European Pharmacopoeias.[32],[53]–[55]

Test	Reference	Objective	Conditions of Evaluation
Liquid Color	Brazilian Pharmacopoeia (Chapter 5.2.12)	Color comparison between the formulation samples and standard solutions of the color scale.	Transversely, against a white background and under daylight.
Color and Chromaticity	USP <631>	Color comparison between the formulation samples and standard solutions of the color scale. In addition to the visual evaluation, an instrumental method to quantify the color is proposed.	
Liquid Coloration Degree	European Pharmacopoeia (Chapter 2.2.2.)	Method I	Horizontally, against a white background and under daylight.
		Method II	Vertically, against a white background and under daylight.

2.3. EXPERIMENTAL PROCEDURE BASED ON EUROPEAN PHARMACOPOEIA

In the following chapter, the experimental procedure for the color evaluation will be presented, based on methods described in The European Pharmacopoeia (*Chapter 2.2.2: Liquid Coloration Degree*). The sub-chapter 2.3.1 refers to the visual evaluation of color and explains the experimental procedure for the preparation of the solutions for the different color scales. Chapter 2.3.2. describes the instrumental procedure for color measurement of the samples under analysis.

2.3.1. PROCEDURE FOR THE PREPARATION OF THE DIFFERENT COLOR SCALES

The experimental procedure of visual color evaluation based on European Pharmacopoeia 9.7 consists of the preparation of five distinct color scales, based on three principal solutions: yellow, red and blue. These solutions result from the dissolution of different salts in hydrochloric acid $2{,}5\ \%(V/V)$. The hydrochloric acid solution $2{,}5\ \%(V/V)$ is prepared from hydrochloric acid $37\ \%$. As it is an acid, additional laboratorial practices are required in its preparation. Before pipetting the acid slowly directly into the volumetric flask, a considerable amount of ultra-purified

water must be introduced into the flask. The meniscus of the volumetric flask is only adjusted after the solution has cooled down to room temperature, because the heat causes the liquid expansion.[56]

For the preparation of the yellow solution, ferric chloride is dissolved in hydrochloric acid 2,5 $\%(V/V)$. Then, the solution must be placed in an ambar bottle and protected from light, because it is photosensitive. The red solution is prepared through the dissolution of cobaltous chloride in hydrochloric acid 2,5 $\%(V/V)$ and the blue solution is prepared using cupric sulfate and hydrochloric acid 2,5 $\%(V/V)$. All primary solutions should be prepared in volumetric flasks and the dissolution of the salt should be done under magnetic stirring.[53],[56]

After the preparation of the initial main solutions (yellow, red and blue), the primary solutions are prepared, considering **Table 5**. Using the five primary solutions, the scales of the standard solutions are prepared, considering **Table 6**. The preparation of the standard solutions must be done immediately after the preparation of the primary solutions, due to the fact that the primary solutions are unstable and the color changes over time. As these solutions contain photosensitive compounds, they must be protected from light. The experimental procedure to prepare the color scales is presented on a scheme in **Figure 22**.[53]

Table 5 - Portions of the different principal solutions (yellow, red and blue) for the preparation of primary solutions.[53]

	Volume of the solutions (mL)				
Standard solution	**Yellow (Y)**	**Red (R)**	**Blue (B)**	**HCl 10 $g \cdot L^{-1}$**	**Total**
B (Brown)	15.0	15.0	12.0	8.00	50.0
BY (Yellow-Brownish)	12.0	5.00	2.00	31.0	50.0
Y (Yellow)	12.0	3.00	0.000	35.0	50.0
GY (Yellow-Greenish)	24.0	0.500	0.500	0.000	25.0
R (Red)	5.00	10.0	0.000	35.0	50.0

Table 6 - Portions of the different primary solutions for the preparation of standard solutions.[53]

		Volume (mL)	
	Color Scales	**Primary solution**	**HCl (10 g·L^{-1})**
B	**B1**	7.500	2.500
	B2	5.000	5.000
	B3	3.750	6.250
	B4	2.500	7.500
	B5	1.250	8.750
	B6	0.5000	9.500
	B7	0.5000	9.750
	B8	0.15000	9.850
	B9	0.1000	9.900
BY	**BY1**	10.00	0.000
	BY2	7.500	2.500
	BY3	5.000	5.000
	BY4	2.500	7.500
	BY5	1.250	8.750
	BY6	0.5000	9.500
	BY7	0.2500	9.750
Y	**Y1**	10.00	0.0000
	Y2	7.500	2.500
	Y3	5.000	5.000
	Y4	2.500	7.500
	Y5	1.250	8.750
	Y6	0.5000	9.500
	Y7	0.2500	9.750
GY	**GY1**	2.500	7.500
	GY2	1.500	8.500
	GY3	0.8500	9.150
	GY4	0.5000	9.500
	GY5	0.3000	9.700
	GY6	0.1500	9.850
	GY7	0.0750	9.925
R	**R1**	10.00	0,0000
	R2	7.500	2.500
	R3	5.000	5.000
	R4	3.750	6.250
	R5	2.500	7.500
	R6	1.250	8.750
	R7	0.5000	9.500

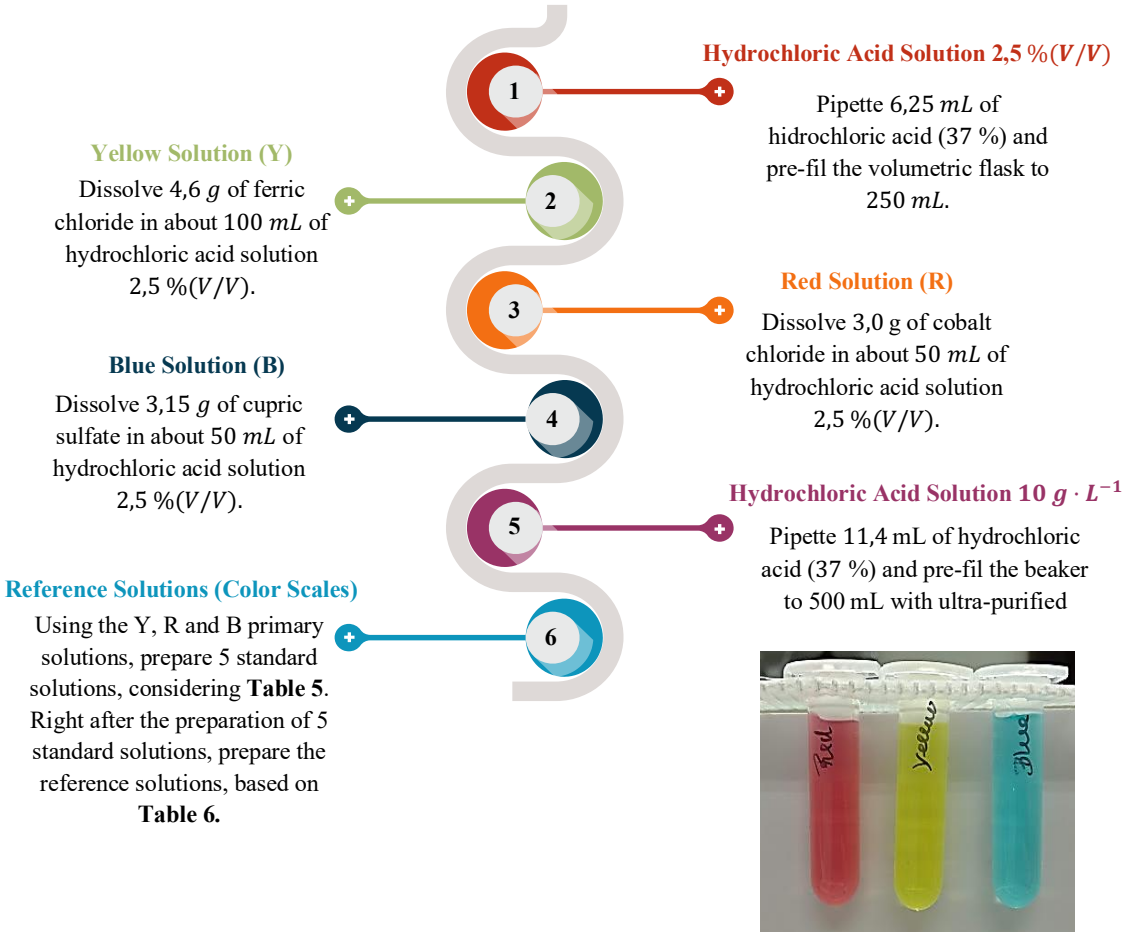


Figure 22 - Experimental procedure for the preparation of the color scales based on European Pharmacopoeia 9.7 and the primary solutions.

2.3.2. INSTRUMENTAL COLOR MEASUREMENT OF COLOR SCALES

As already mentioned, the visual evaluation of the color could be subjective. Therefore, an instrumental method to quantify the color was applied. In order to determine the color coordinates based on CIELAB color space model a spectrophotometer was used.

Spectrophotometry is the science that studies the interaction of light with the object, by comparing and quantifying the light that the object selectively absorbs or transmits. Important to note that the main components associated with the spectrophotometer are the light source (illuminant), the monochromator, the storage cells of the solutions, the radiation detectors (transducers) and the signal indicators. For these particular studies, the spectrophotometer used was a double beam spectrophotometer. **Figure 23** shows the operating mechanism of this type of spectrophotometer.[57]–[59]

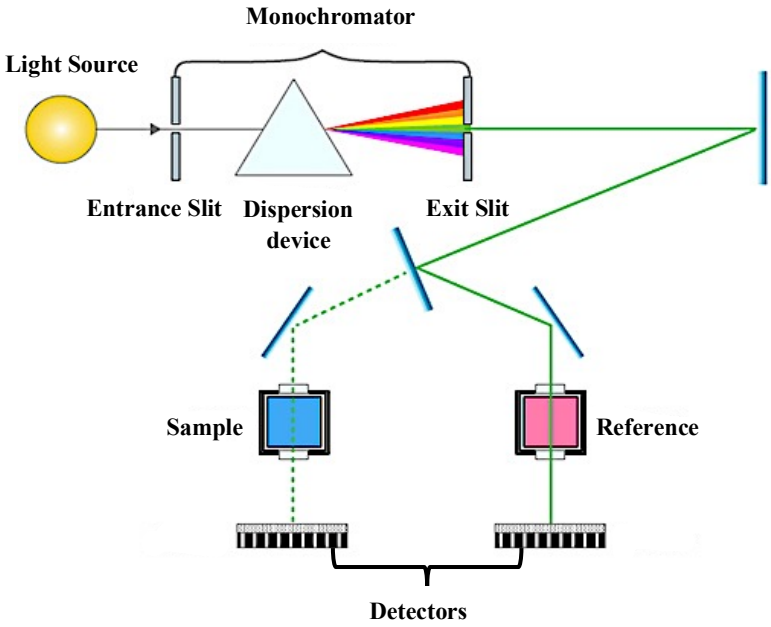


Figure 23 - Double beam spectrophotometer: operating mechanism [*Adapted from* [57]].

In this type of equipment, one light beam passes through the blank to the transducer, while at same time, another radiation beam passes through the sample under analysis until it reaches the second transducer. Through the determination of transmittance curve, it is possible to determine the color coordinates of the sample under analysis.[57]–[59]

Initially, it was necessary to configure a method for measuring the color coordinates in the spectrophotometer. **Table 7** and **Figure 24** describe the configuration of the method used in the color measurements. This configuration consisted of the specification of the three parameters responsible for the color sensation: the light source, the observer and the object. As for the light source, the illuminant D65 was selected, the European Pharmacopoeia 9.7 advises that the evaluation of the color should be performed under daylight and the illuminant D65 is the one with the most similar characteristics. As for the observer, a standard observer of 10° was selected, because from this angle it is possible to obtain a wider view of the sample. To determine the color coordinates, the transmittance values were read at the wavelengths of the visible range of the spectrum (380 nm to 770 nm), as it is advised in USP.[25], [32], [37], [53]

Table 7 - Configuration of the model of the spectrophotometer used in color measurement.[54], [58]

	Parameters	Configuration
Settings	Cycle Mode	None
	Display	Transmittance
	Correction	Reference
Device	Slit	1 nm
	Lamp change at	320º
	D2E automatically	X
	HL automatically	X
Mode	Measurement mode	Spectral Scan
	Range	380-770 nm
	Delta λ	1,0 nm
	Speed	50 $nm \cdot s^{-1}$
Accessories	Accessory	None

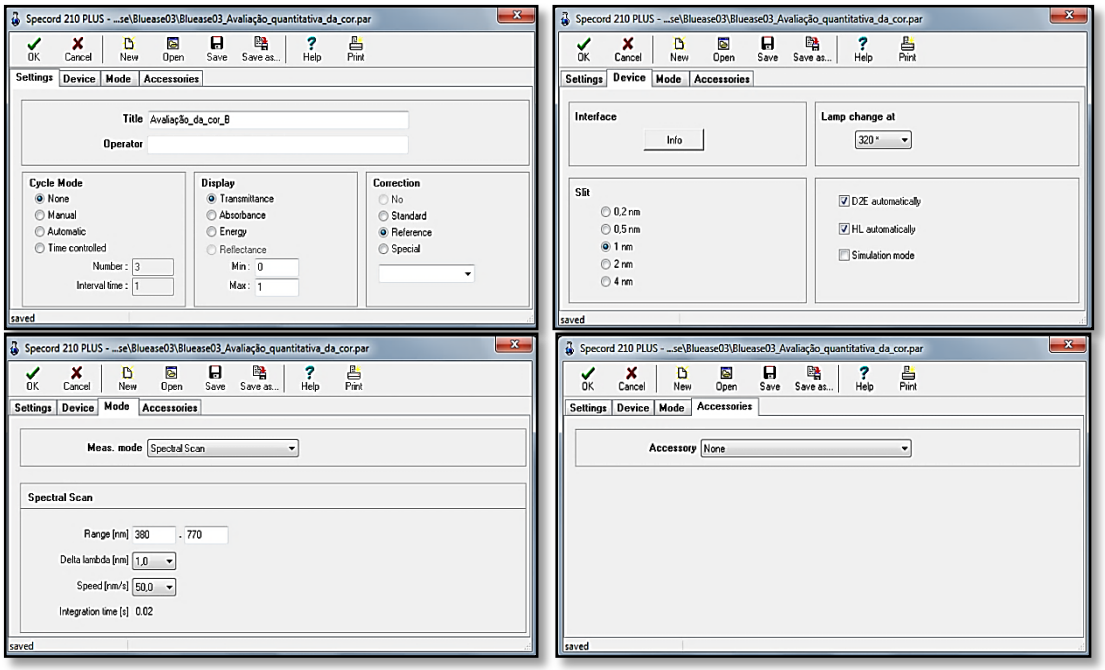


Figure 24 - Method for the measurement of the color coordinates in the spectrophotometer.

The procedure of the instrumental method is described schematically in **Figure 25**. Initially, after turning on the equipment and waiting for the lamps to warm up, it is necessary to configure the method for instrumental color evaluation. Then, the transmittance of the blank (reference) solution is read using quartz cells. Subsequently, and keeping the cell with the reference solution in the spectrophotometer, the standard solutions and formulation are measured, taking into consideration to always wash the cell with ethanol or other cleaning solution between each

measurement. Finally, the color coordinates are determined using a tool present in the software used.[32],[56], [58]

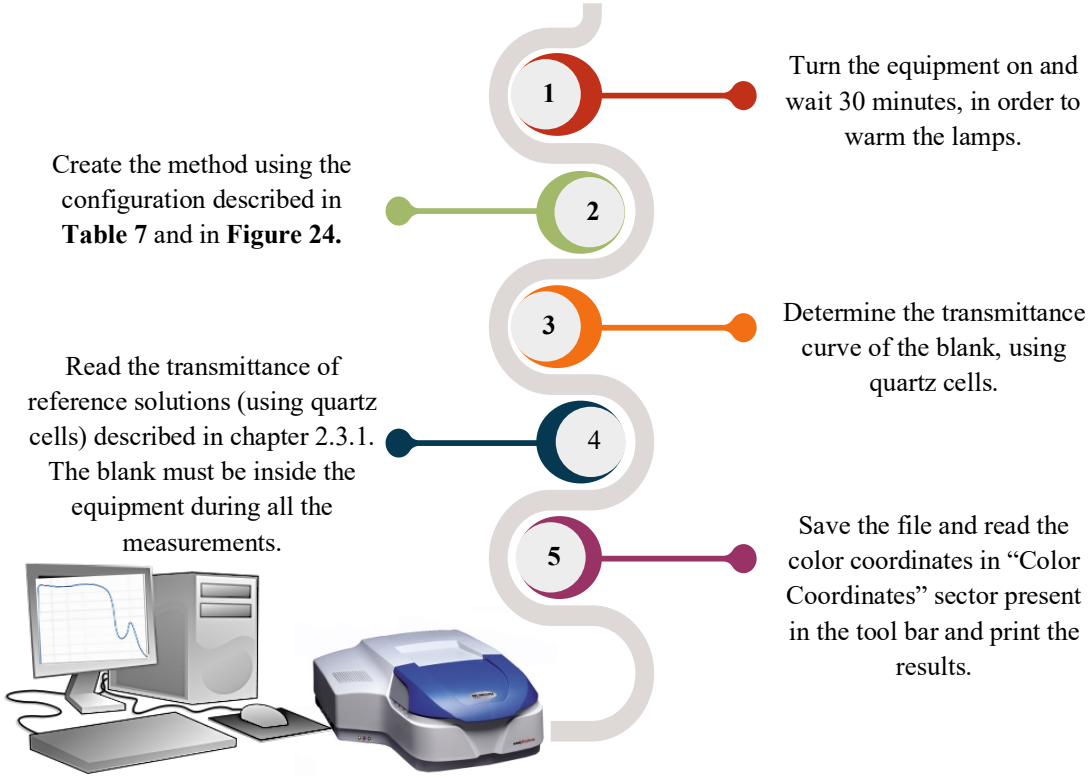


Figure 25 - Experimental procedure for the instrumental color evaluation.

CHAPTER III - RESULTS AND DATA ANALYSIS

3.1. COLOR SCALES FOR COLOR EVALUATION

According to European Pharmacopoeia 9.7 (*Chapter 2.2.2: Liquid Coloration Degree*), color evaluation of samples to analyze and standard solutions should be carried out horizontally and vertically under white background.[53]

Figure 26 shows the color scales observed from a vertical and horizontal view under white background.[53]

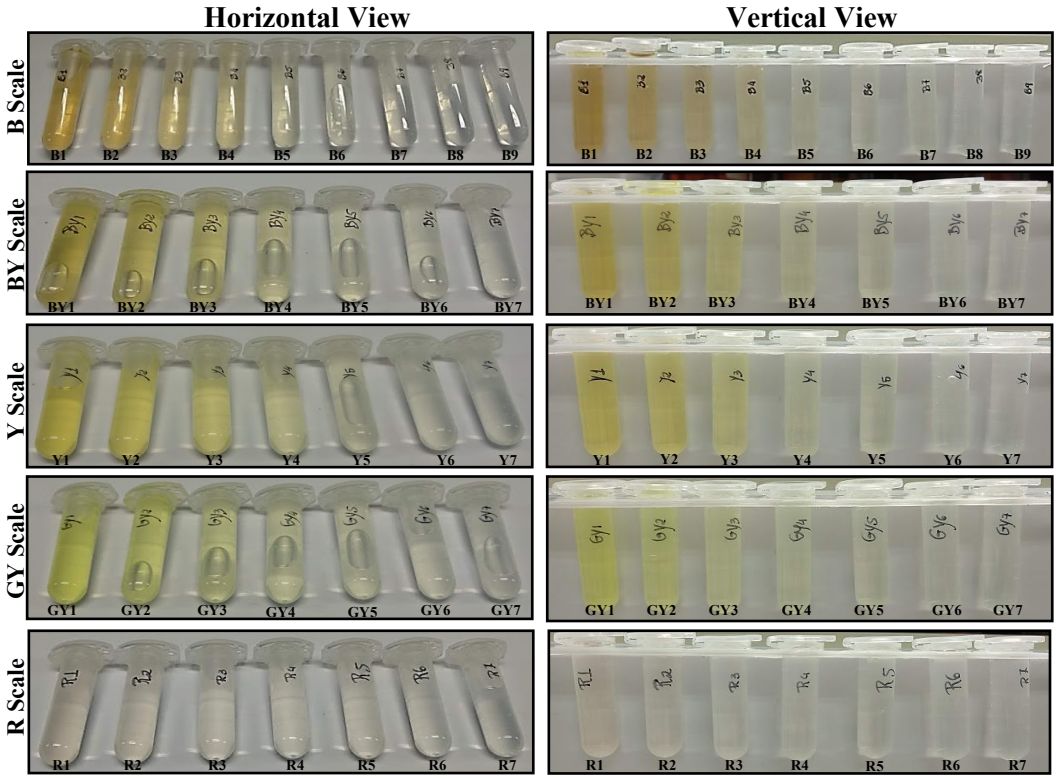


Figure 26 - Horizontal and vertical view of color scales reproduced based on European Pharmacopoeia 9.7.

Using the color scales proposed in the European Pharmacopoeia 9.7, several formulation samples were visual and instrumentally analyzed. The samples under analysis present similar composition, however undergone different pre-stability conditions and times.

The formulation A T0M was analyzed immediately after its production, and therefore the moment of its evaluation was T0 months. The formulation B was subject to conditions of normal temperature and relative humidity (25 °C/60 % RH) and to conditions of forced degradation (40 °C/75 % RH) and its color evaluation was performed at 3 months (T3M) and 6 months (T6M) of stability. In **Figure 27** an illustrative scheme of the formulations whose color will be analyzed visually and instrumentally is given.

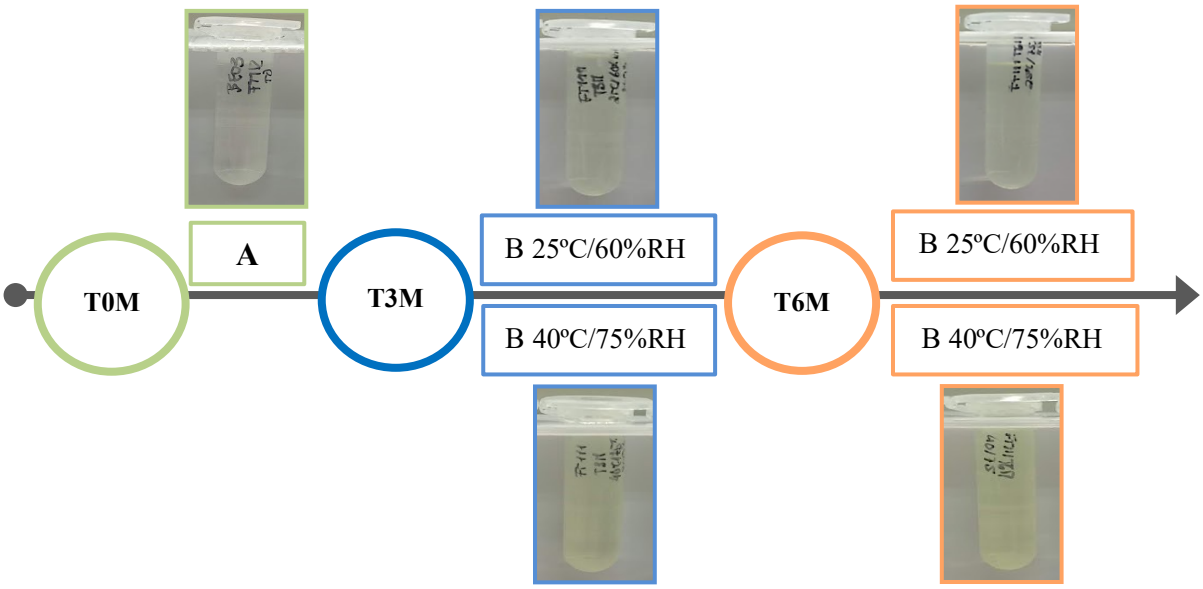


Figure 27 - Formulations under analysis and respective time points of color evaluation.

The analysis of the experimental results was divided into three stages:

- **Stage I** - Visual evaluation of the color of the samples to be analyzed, through comparison with the standard solution of all the scales reproduced based on European Pharmacopoeia 9.7.
- **Stage II** - Color quantification from instrumental methods, in order to verify if the visual evaluation is coherent with the instrumental one.
- **Stage III** - Attempt to determine rectangular boxes to define the color tolerance.

The following sub-chapters present the experimental results and the respective analysis.

3.2. Stage I – Visual Color Evaluation

The visual evaluation of the color consisted of the comparison between each of the formulation samples present in **Figure 27** and the standard solutions prepared based on European Pharmacopoeia (BY, Y and GY scales). The scales B and R were discarded because the color of the standard of each of these scales departs, visually and instrumentally, from the color observed in the formulations under analysis, as it is possible to observe in **Figure 28** and in the **Figure 30** (present in the sub-chapter 3.3). Thus, the focus of the visual analysis was on the BY, Y and GY scales, since they are those that best represent the color range of the formulation.[53]

Regarding the method of observation, the European Pharmacopoeia suggests the horizontal and vertical color evaluation of the samples. Both evaluations were carried out. The visual evaluation of the color according to a horizontal field of view corresponded to the evaluation from a vertical visual field. However, only the results obtained through the comparison with the vertical

scale are presented. The methodology applied to the visual evaluation consisted of a comparison between the color of each one of the samples under analysis and each one of the colors of the standard solutions. In order to carry out a visual evaluation as accurate as possible, the color perception of different people was considered.[53]

Visual color evaluation shall be carried out under the same conditions in order to avoid the metamerism effect. This effect consists of the inconsistency between two same colors when evaluated under different conditions. It can be caused by illumination, standard observer geometry and different sample sizes/quantities. It is also important to use an experienced observer to visually evaluate the samples under analysis. The potential subjectivity associated to the color, also called color blindness, derives from the excessive overlap between the color-matching functions (functions present in **Figure 12**). This overlap prevents the tristimulus (red, green and blue curves of **Figure 12**) from developing in their fullness, decreasing the ability to distinguish colors. Thus, in order to assess the feasibility of a color vision of a person, there are certain tests, for example The Ishihara Color Test and genetic tests for color blindness screening.[61]–[63] A description and example of Ishihara Color Test are given in **Attachment F.**

Herein, the analysis of the color scales and formulations was performed under white light, against a white background and in vertical and horizontal field of view, as suggested by the European Pharmacopoeia.[64] The results of the color evaluation for all the formulations compared to the BY, Y and GY scales are shown in **Figure 28**. **Figure 28 A** is related to the formulations under normal conditions of pre-stability (25 °C/60 % RH) and **Figure 28 B** is related to conditions of forced degradation (40 °C/75 % RH).

Analyzing the results of the visual evaluation over time, it is possible to verify a trend in the change of color of the formulations. At the time of preparation (formulation A T0M), the color of the formulation is almost transparent. Over time, it acquires a slight pale yellow color (formulations B T3M and B T6M). Under conditions of forced degradation (40 °C/75 % RH), the staining of the formulation is a more intense pale yellow than under conditions of degradation at lower temperatures and relative humidity. Forced degradation is a type of degradation of a drug product under severe conditions. Comparing the color evolution in both conditions of stability, it can be seen that the most noticeable color change occurs in the first three months of stability. In the remaining three months, the change in color is not so noticeable, so the comparison of the color of the formulations with the color scales is maintained. **Table 8** shows a summary of the results of the visual evaluation obtained for each of the formulation under analysis.

Table 8 - Results of visual color evaluation for BY, Y and GY scales.

	Results of Visual Color Evaluation		
Formulations	**BY**	**Y**	**GY**
A T0M	[BY6 - BY7]	[Y6 - Y7]	[GY6 - GY7]
A T3M (25ºC/60%RH)	[BY4 - BY5]	[Y4 - Y5]	[GY4 - GY5]
B T3M (40ºC/75%RH)	[BY4 - BY5]	[Y4 - Y5]	[GY4 – GY5]
B T6M (25ºC/60%RH)	[BY4 - BY5]	[Y4 - Y5]	[GY4 - GY5]
B T6M (40ºC/75%RH)	[BY3 – BY4]	[Y3 - Y4]	[GY3 - GY4]

Analyzing **Table 8**, it can be seen that at the time of preparation (Formulation A) the color of the formulation is in between the color of the standard solutions 6 and 7 of all scales. These standard solutions are associated with an almost transparent stain. After three months, the color of the formulations is located between the slight pale yellow colored standard solutions (between samples 4 and 5 in the case of stability conditions 25 °C/60 %RH and of force degradation conditions 40 °C/75 %RH). Thus, it is possible to verify that over time, the color of the formulation varies between transparent and slight pale yellow.

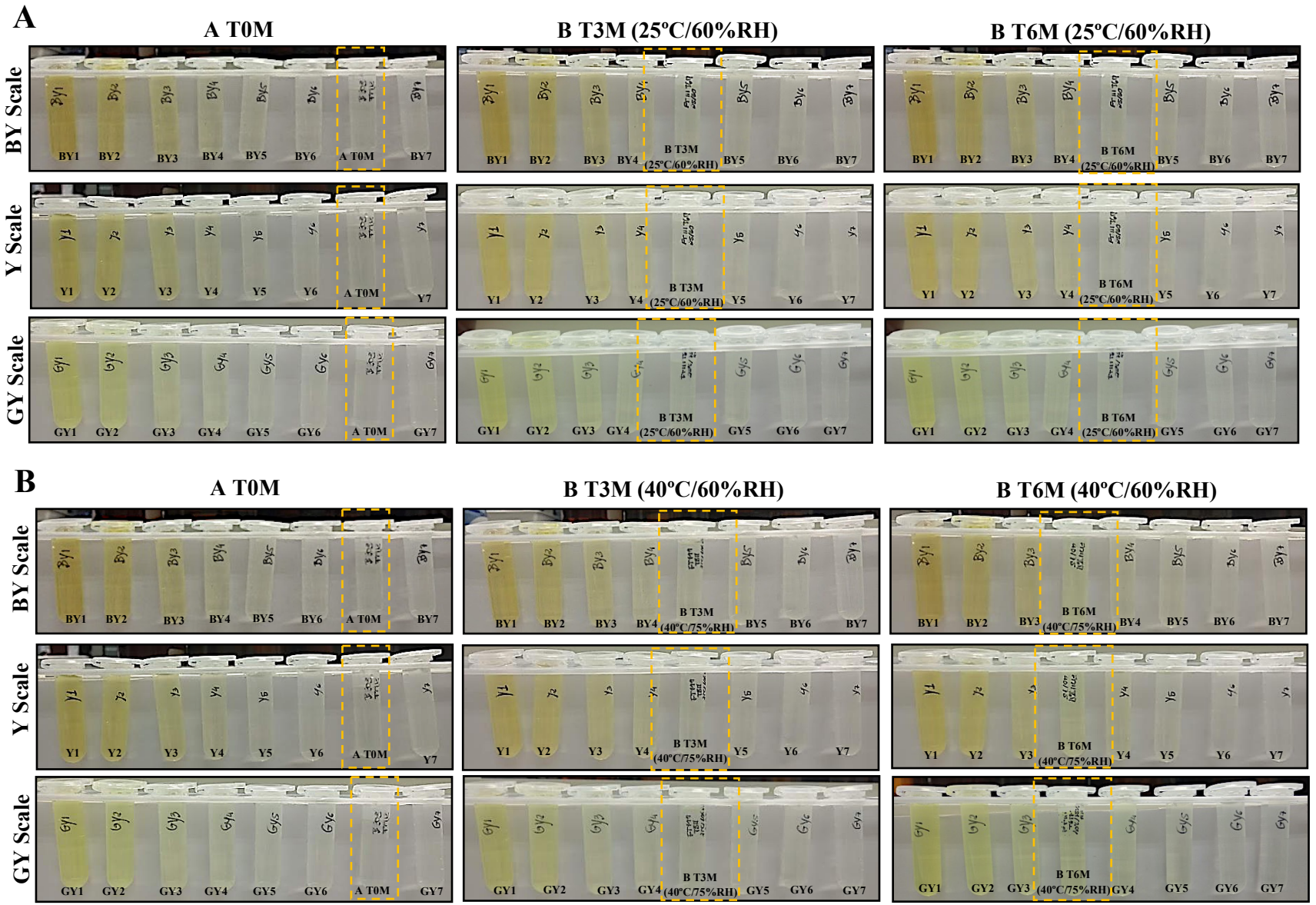


Figure 28 - Visual Color Evaluation for the formulations A and B. **Figure A** is related to the analysis of the formulation in normal pre-stability conditions and **Figure B** is related to conditions of forced degradation. The formulations are identified with dashed shapes.

3.3. INSTRUMENTAL METHOD OF COLOR EVALUATION

In order to overpass the potential subjectivity associated with the visual evaluation of each person, the intensity of color was numerically quantified in the samples under analysis together with quantification of the standard scale solutions pre-prepared (standard solutions).

The quantification and specification of the color was performed using CIELAB color space model, as mentioned in the sub-chapters above. This two-dimensional model is based on Δa^* e Δb^* parameters, which are calculated from the difference between the coordinates of the sample under analysis or standard solutions of the color scales and the coordinates of a transparent liquid. In this particular case, water was used as a standard for transparent liquid.[37]

In this study, one could detect the perceptual difference between colors in a two-dimensional diagram, however it is important to note that one of the parameters associated with color - brightness - is not considered during two-dimensional instrumental assessment.[37] In order to consider the brightness, the difference of two colors could be determined by calculating the ΔE^* parameter. ΔE^* will allow to match the color of a sample under analysis with a specific color of standard solutions from the pre-prepared color scales. Smaller values of ΔE^* indicate a greater proximity between the two colors.[37], [50]

In addition to configuring a method for measuring the color coordinates in the spectrophotometer, it is important to define which blank (reference) solution will be used to perform the instrumental color evaluation in the spectrophotometer. The appropriate blank solution to measure the color coordinates in the spectrophotometer was defined through the measurements performed with two different blank solutions: HCl $2{,}5\ \%(V/V)$ (solvent from the standard solutions of the scales) and ultra-purified water (standard transparent liquid). **Figure 29** shows the color coordinates a^* and b^* for the two tested blanks and color coordinates of the formulation A T0M for each one of the blanks. As can be seen in **Figure 29**, the coordinates of the blanks are similar, since both have a transparent coloration. The coordinates of the formulation are practically the same using any of the two different blanks, which indicates that as long as it is transparent (or close to transparent), the selection of $2{,}5\ \%(V/V)$ or ultra-purified water as blank in the spectrophotometer measurements has no impact on the quantification of color through the two-dimensional model a^* and b^*. Thus, ultra-purified water was used, since it is the transparent standard liquid.

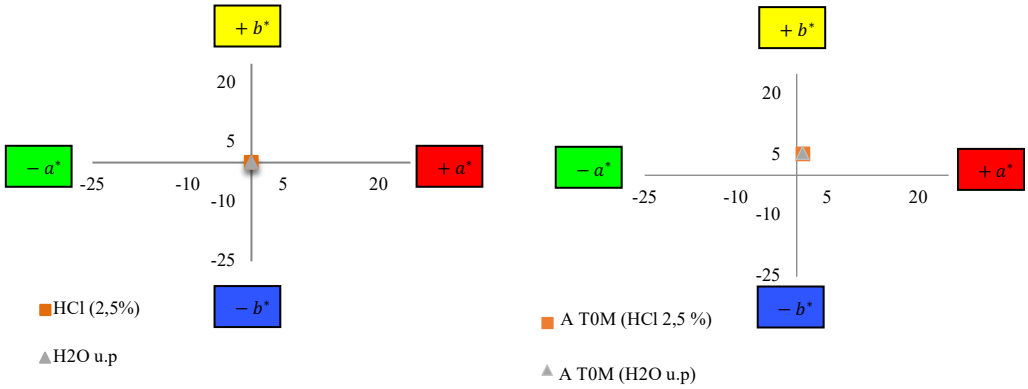


Figure 29 - Two-dimensional color coordinates of the blanks and of the formulation A T0M measured with the different blanks.

As mentioned in **Table 6**, the European Pharmacopoeia distinguishes five different color scales: brown (scale B), yellow brownish (scale BY), yellow (Scale Y), yellow greenish (scale GY) and red (Scale R). Initially, all the color scales were prepared. However, from the visual and instrumental analysis, it was possible to verify that not all of the scales adjust well the expected evolution of color coordinates of the samples under analysis. **Figure 30** shows the graphical representation of the two-dimensional color coordinates of the standard solutions of each scale and of the formulations under analysis, as well as a dashed arrow that indicates the trend of the color evolution of the formulation.[53]

The scale of the two-dimensional diagram used for the color representation varies between -128 and 128 (result of converting the coordinates of the RGB color space described in the sub-chapter 1.5.2.3 to the CIELAB color space by mathematical equations). However, even though the scales vary between brown and red, the colors of the standard solutions belonging to each scale are very little intense, as can be seen in **Figure A 1** in **Attachment A**. Thus, it is not justified to use a scale varying between -128 and 128 for the axes of the cartesian diagram, because in this way, all coordinates would be clustered very close to the origin of the cartesian diagram, making difficult to evaluate the color from the graphical representation. Thus, the scale of the axes was established between -25 and 25.[47]

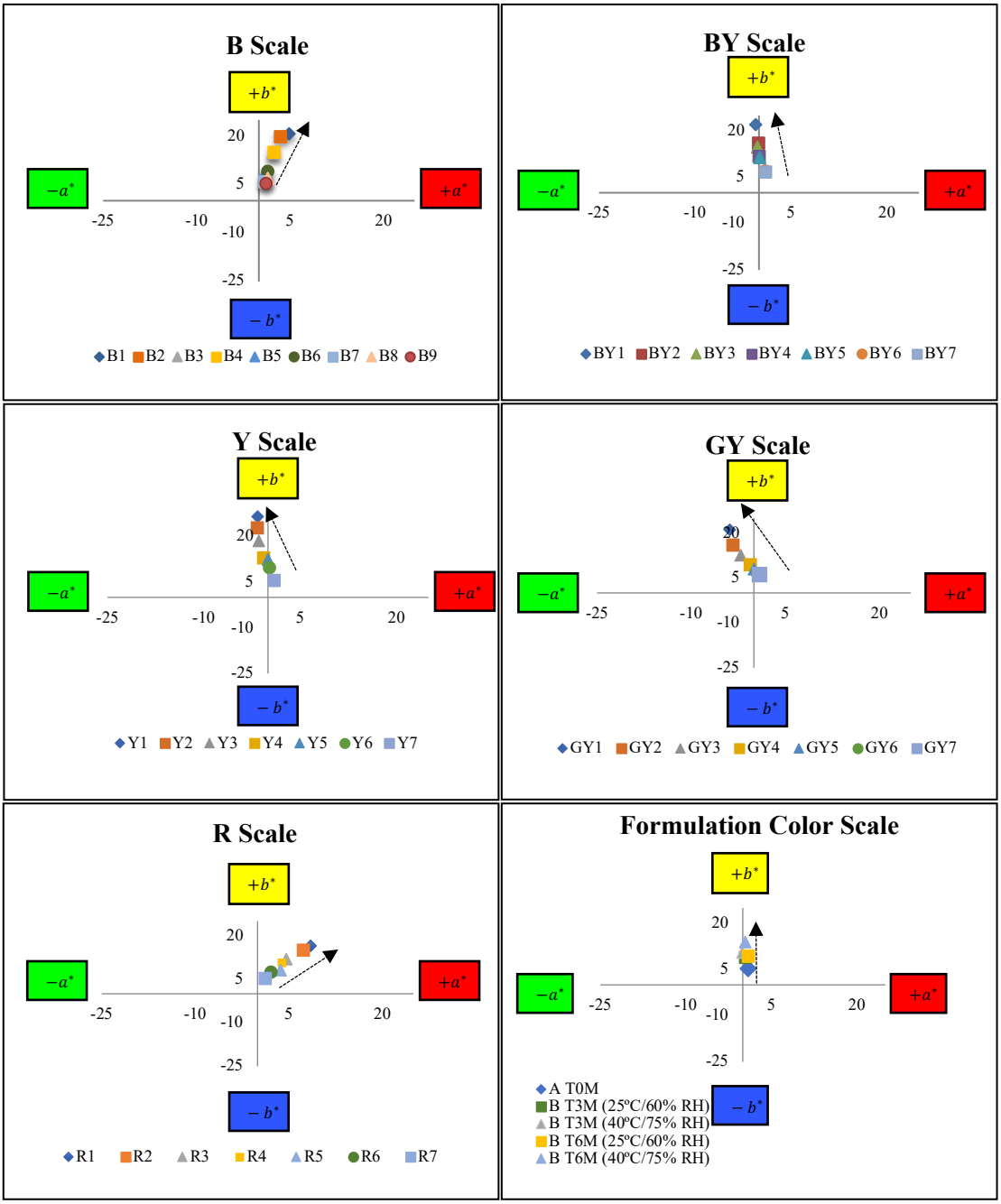


Figure 30 - Two-dimensional color coordinates of the standard solutions for each one of the scales. The dashed arrow represents the evolution of the color of the standard solutions over the color scale.

The three-dimensional color coordinates (considering the brightness, content in red/green and content in yellow/blue of the standard solutions of the scales and of the formulation are present in **Attachment A**, in **Figure A 1** and **Figure A 2**. As in the visual color evaluation, the instrumental analysis focused on the BY, Y and GY scales. The color coordinates of the reference samples on the BY, Y and GY scales are located in the region between the first and second quadrant of the two-dimensional cartesian representation, very close to the origin and the b* axis, which

corresponds to the range of colors between which the color of the formulation differs: almost transparent to slight pale yellow.

After the visual and instrumental selection of the appropriate color scales, the instrumental evaluation of the non-sterile liquid preparation under analysis was performed.

3.3.1. INSTRUMENTAL EVALUATION OF THE FORMULATIONS

The results of the instrumental evaluation are presented in the following sub-chapters, in which it is possible to observe the representation of the coordinates of each formulation in the different scales, as well as a summary table with the instrumental results obtained. In the summary table is also possible to find the results of the difference between the colors of the formulations and of each of the standard solutions of the three scales. This difference confirms the visual examination in which was identified the standard solutions that was more similarity to each sample under analysis.

3.3.1.1. FORMULATION A T0M

The results of the instrumental color evaluation of the formulation A T0M are shown in **Figure 31.** The visual evaluation was a challenge as the sample presented a markedly transparent feature. Nevertheless, the visual observations positioned the sample in between solutions 6 and 7 of all the scales (BY, Y and GY scales). The instrumental analysis (that does not consider brightness) partially confirms the visual evaluations as it indicates that the color of the formulation A T0M is close to standard solutions 7 (the more transparent of each set of scales).

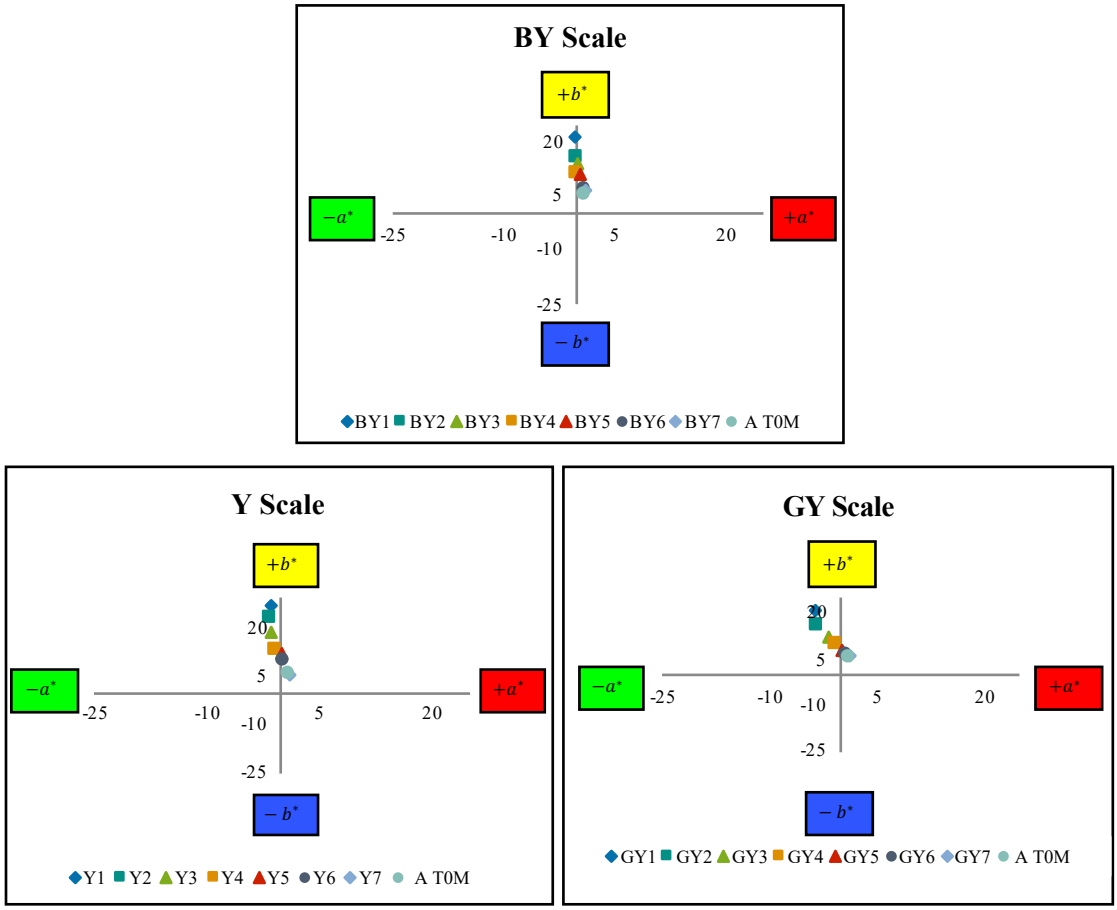


Figure 31 - Two-dimensional instrumental color evaluation of formulation A T0M.

Through the analysis of ΔE^* parameter (depicted in **Table B 1 – Attachment B)** it is possible to conclude that formulation A T0M is closest to the standard solution Y7, as showed the smaller value of ΔE^*. Note that the value of ΔE^* (greater than 5) indicates, based on **Table 2**, that the two colors under comparison are likely distinguishable by an experienced observer. Nevertheless, in this particular case of "transparent" samples (incolor), such was not visually confirmed, as this evaluation was difficult to assess only by visual examination. In **Table 9** is present a summary of the visual and instrumental results obtained for the formulation A T0M. As mentioned, the qualitative results correspond partially to the quantitative results.

Table 9 - Summary of visual and instrumental results for formulation A T0M.

	BY Scale	Y Scale	GY Scale
Visual Evaluation	[BY6 - BY7]	[Y6 - Y7]	[GY6 - GY7]
Instrumental Evaluation (Two-dimensional)	Colorless than BY7	Colorless than Y7	Colorless than GY7
Evaluation based on ΔE^* parameter (Three-dimensional)	_____	Y7	_____

3.3.1.2. FORMULATION B T3M (25ºC/60% RH)

Figure 32 shows the results of the instrumental color evaluation of the formulation B T3M (25°C/60 %RH). The visual color examination placed the samples under analysis between the colors of standard solutions 4 and 5. In reality, the results of instrumental analysis indicate that the color of the samples locates in more transparent ranges (for BY Scale is located between BY5 and BY6, for Y Scale is located between Y6 and Y7 and for GY Scale is located between GY6 and GY7). Importantly, this potential mismatch could be understandable as the visual differences of the standard solutions 4, 5 and 6 in all the color scales studied are almost unnoticeable for an inexperienced observer.

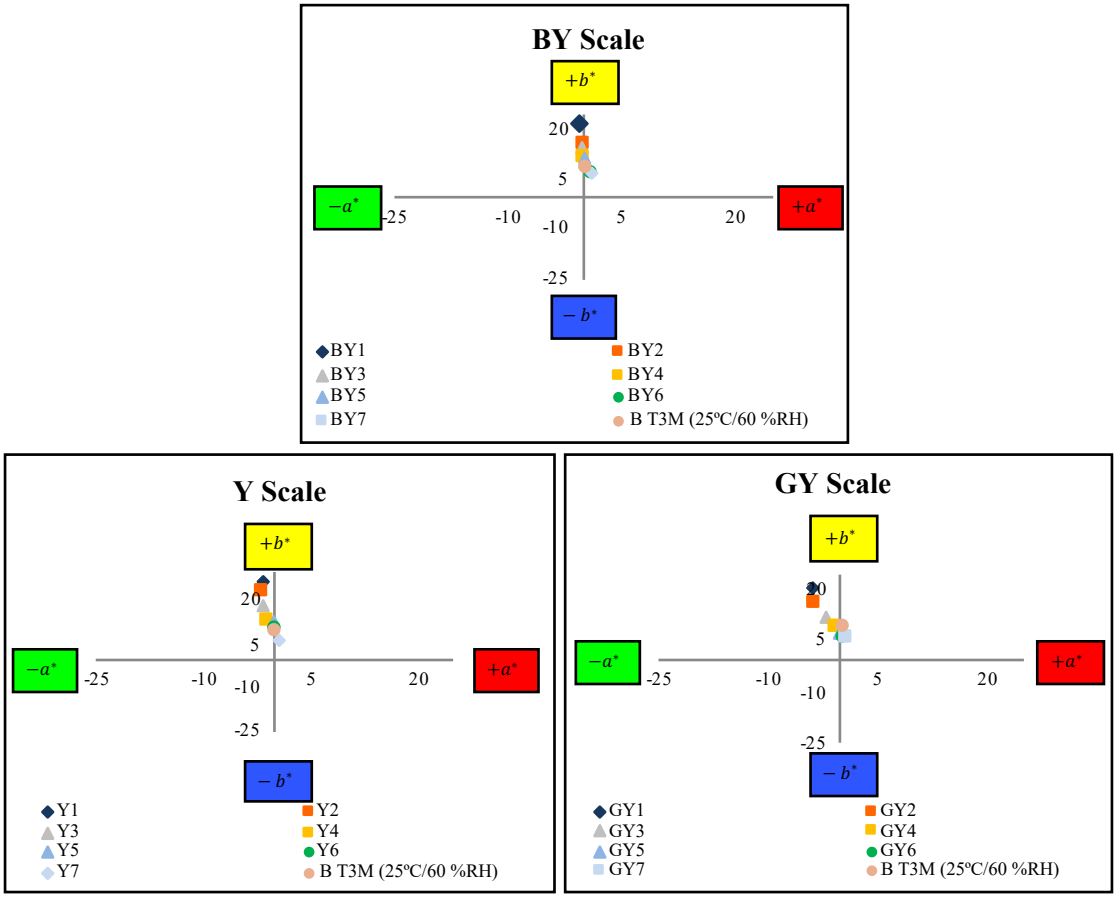


Figure 32 - Two-dimensional instrumental color evaluation of formulation B T3M (25°C/60 %RH).

Analyzing **Table B 2 (Attachment B)** it is possible to conclude that the standard solution that has the closest color to the sample under analysis is Y6. The value of ΔE^* (lower than 2) indicates that the color difference may be only perceived by an experienced observer, potentially because the differences between colors are too small for the human eye to detect. In **Table 10** is a summary of the visual and instrumental results obtained.

Table 10 - Summary of visual and instrumental results for formulation B T3M (25ºC/60% RH).

	BY Scale	**Y Scale**	**GY Scale**
Visual Evaluation	[BY4 – BY5]	[Y4 – Y5]	[GY4 – GY5]
Instrumental Evaluation (Two-dimensional)	[BY5 – BY6]	[Y6 – Y7]	[GY6 – GY7]
Evaluation based on ΔE^* parameter (Three-dimensional)	_____	Y6	_____

3.3.1.3. FORMULATION B T3M (40ºC/75% RH)

Figure 33 presents the results of the instrumental color evaluation of the formulation B T3M (40°C/75 %RH). The visual color examination placed the samples under analysis between the colors of standard solutions 4 and 5 for all color scales. The results of instrumental analysis indicate that the color of the samples is located between BY4 and BY5 for BY Scale, for Y Scale is located between Y5 and Y6 and for GY Scale is located between GY4 and GY5.

The visual results obtained correspond to the instrumental results on the BY and GY scales. In the Y scale, the mismatch between the visual examination and the instrumental one, are likely related to the fact that the visual color difference between standard solutions 4 and 5 could not be completely noticeable by the human eye; the difference between the colors of these two standard solutions may be too small for the human eye to detect easily.

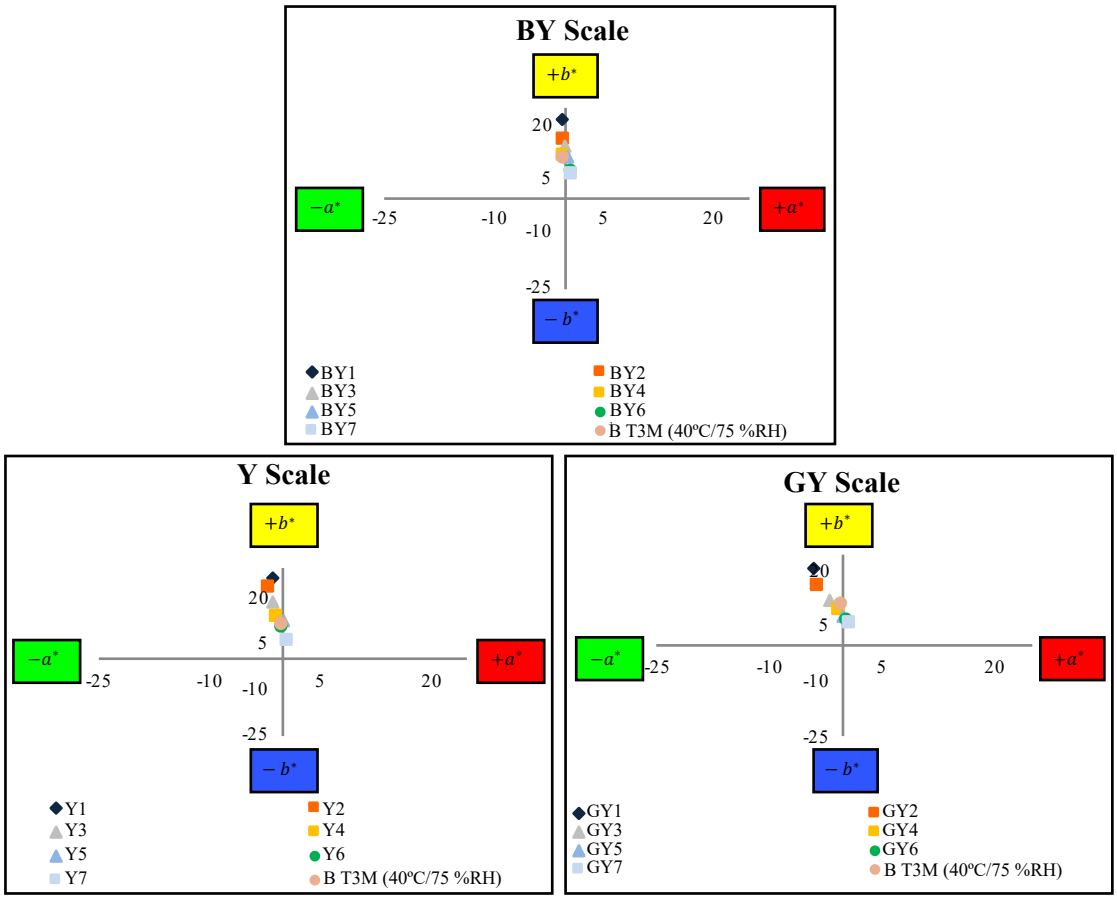


Figure 33 - Two-dimensional instrumental color evaluation of formulation B T3M (40°C/75 %RH).

The standard solution whose color is closest to the color of the formulation under analysis is GY4. Given that ΔE^* is between 1 and 2, it is possible to conclude that the two colors are really closer to each other in the color space and the difference only may be detected by an experienced observer. The values of ΔE^* are present in **Table B 3 (Attachment B).** In **Table 11** is a summary of the visual and instrumental results obtained.

Table 11 - Summary of visual and instrumental results for formulation B T3M (40ºC/75% RH).

	BY Scale	Y Scale	GY Scale
Visual Evaluation	[BY4 – BY5]	[Y4 – Y5]	[GY4 – GY5]
Instrumental Evaluation (Two-dimensional)	[BY4 – BY5]	[Y5 – Y6]	[GY4 – GY5]
Evaluation based on ΔE^* parameter (Three-dimensional)	_____	_____	GY4

3.3.1.4. FORMULATION B T6M (25ºC/60% RH)

The results of the instrumental color evaluation of the formulation B T6M (25°C/ 60 %RH) are shown in **Figure 34**. The visual examination located the formulation between standard solutions 4 and 5 of all the scales. For the BY and Y scales, the formulation is located between the standard solutions 5 and 6 and for the GY case, its location is in between the standard solutions 4 and 5. The visual and instrumental results are not completely accurate in the cases of the BY and Y scales. The differences in the results may be justified due to the visual difference between the color of the standard solutions 4 and 5 not being fully noticeable for the human eye perception.

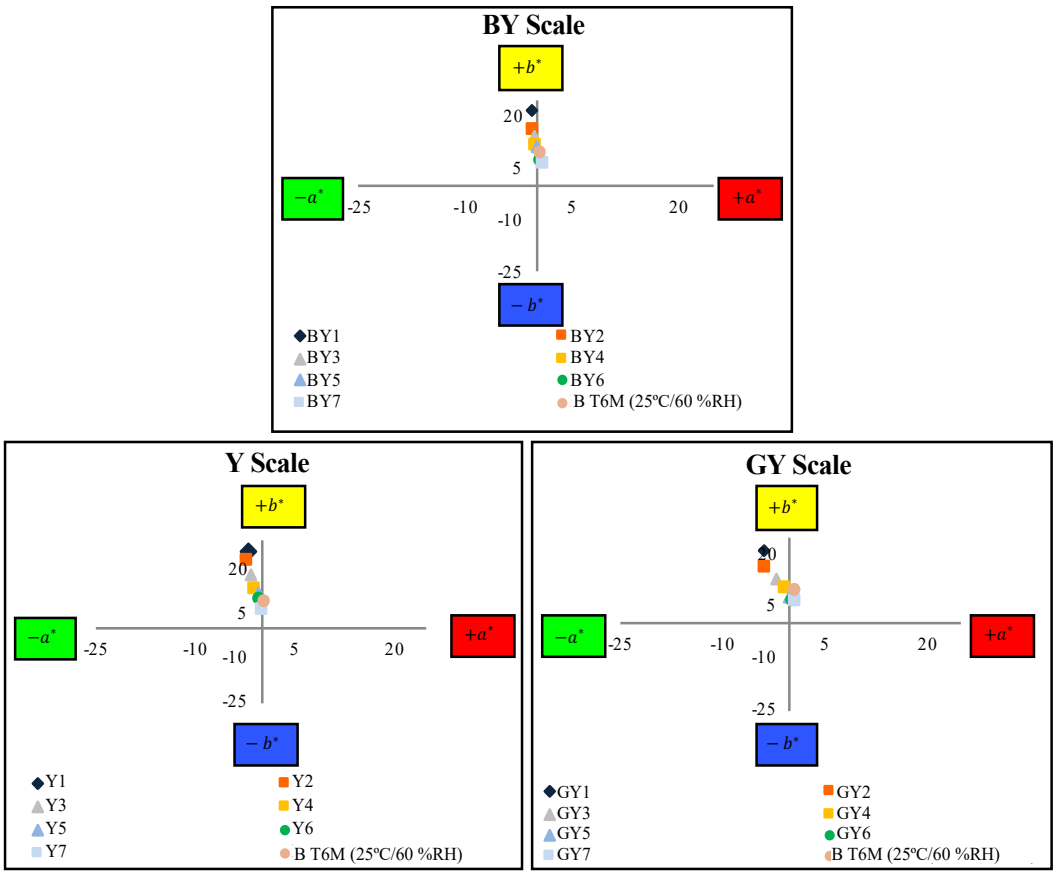


Figure 34 - Two-dimensional instrumental color evaluation of formulation B T6M (25°C/60 %RH).

Table B 4 (Attachment B) allows concluding that the standard solution that has the closest color to the sample under analysis is GY5. The value of ΔE^* (lower than 2) indicates that the color difference may be only perceived by an experienced observer. **Table 12** shows the results obtained for the visual and instrumental evaluation.

Table 12 - Summary of visual and instrumental results for formulation B T6M (25ºC/60% RH).

	BY Scale	Y Scale	GY Scale
Visual Evaluation	[BY4 – BY5]	[Y4 – Y5]	[GY4 – GY5]
Instrumental Evaluation (Two-dimensional)	[BY5 – BY6]	[Y5 – Y6]	[GY4 – GY5]
Evaluation based on ΔE^* parameter (Three-dimensional)	_____	_____	GY5

3.3.1.5. FORMULATION B T6M (40ºC/75% RH)

The instrumental color evaluation of formulation B T6M (40°C/75 %RH) can be seen in **Figure 35**. The visual color examination allowed placing the samples under analysis between the colors of standard solutions 3 and 4 (range of slight pale yellow standard solutions). The results of instrumental analysis indicate that the color of the samples locates in slight pale yellow ranges. For BY Scale is located between BY3 and BY4, for Y Scale is located between Y5 and Y6 and for GY Scale is located between GY3 and GY4.

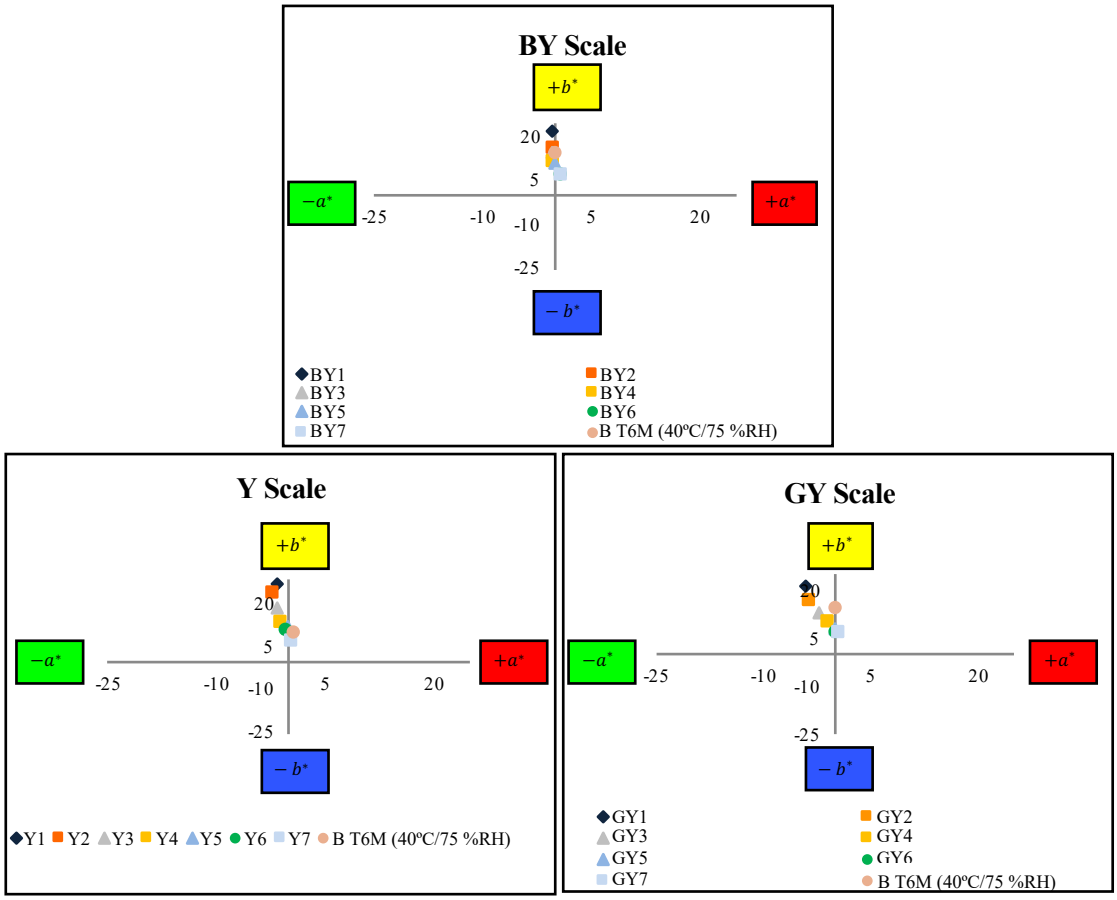


Figure 35 - Two-dimensional instrumental color evaluation of formulation B T6M (40°C/75 %RH).

Table B 5 (Attachment B) allows concluding that the standard solution that has the closest color to the sample under analysis is BY3. The value of ΔE^* (lower than 2) indicates that the color difference may be only perceived by an experienced observer. **Table 13** shows a summary of the results obtained for the visual and instrumental evaluation of the formulation color.

Table 13 - Summary of visual and instrumental results for formulation B T6M (40ºC/75% RH).

	BY Scale	Y Scale	GY Scale
Visual Evaluation	[BY3 – BY4]	[Y3 – Y4]	[GY3 – GY4]
Instrumental Evaluation (Two-dimensional)	[BY3 – BY4]	[Y5 – Y6]	[GY3 – GY4]
Evaluation based on ΔE^* parameter (Three-dimensional)	BY3	_____	_____

Analyzing the coordinates of the color of the formulations under analysis (in the two-dimensional color space), it is possible to conclude that they all present a yellowish hue, derived

from the positive value of the parameter Δb^*. Through the analysis of **Figure A 2** (present in **Attachment A**), it is possible to verify that the coordinates of the formulations (exposed to different conditions and analyzed in different time points) are very close to each other, proving that the difference between them is not extremely significant. Analyzing also the brightness parameter (L^*) of the formulations, it is possible to verify that it has a relatively high value, which justifies the fact that the samples are all of a low intensity color. Comparing the results obtained between the two-dimensional instrumental analysis and the ΔE^* parameter (three-dimensional evaluation), it can be verified that the results are coincident with each other. Thus, it is possible to conclude that the brightness parameter does not affect the quantification of color in the case of formulations and standard solutions under analysis, due to the fact that they all have similar brightness intensities (as can be seen in **Figure A 1** and **Figure A 2** in **Attachment A**). Therefore, two-dimensional instrumental analysis quantifies color accurately (for the samples in study in the present dissertation), even if it does not consider the three parameters associated with color (hue, chroma and brightness).

The color scales reproduced based on the European Pharmacopoeia 9.7 (namely BY, Y and GY) are suitable for the color evaluation of the formulations under analysis. This conclusion can be obtained both visually (it is possible to establish the comparison between the colors of the standard reference solutions and the colors of the formulation) and instrumentally, from the analysis of the **Figure A1** and **Figure A2** present in **Attachment A**. The color coordinates of the standard solutions and the formulation are in the same region of the three-dimensional CIELAB Color Space.

The visual and quantitative evaluation of the color allowed verifying that during the stability time, the liquid formulation color evolved from almost transparent to a slight pale yellow coloration. This color evolution of the formulation is more evident in forced degradation conditions (high temperature and high percentage of relative humidity). It was also noted that the change in color occurs preferably during the initial three months of stability. Overall, the color change of the formulations appear to be subtle, being mainly detected using instrumental tecnhiques. The simple visual examination of these samples revealed to be a challenge and with some bias associated. Despite the slightly changes in color during the period of time tested, the CQAs of the formulation (including color) fully comply with the specifications proposed for this product, revealing that this minor color change has no influence on the safety, efficacy and performance of the drug product.

In the following sub-chapter, an attempt to establish a color tolerance for the non-sterile liquid preparation under study is presented.

3.4. COLOR TOLERANCES

Through the CIELAB color space, it is possible to determine the tolerance for a specific color, as represented in **Figure 36.** The tolerance limit was determined using the color coordinates of the standard solutions with the most intense color of the GY Scale (that correspond to a pale yellow color). The values of Δa^*, $-\Delta a^*$, Δb^* and $-\Delta b^*$ correspond to the difference between the color coordinates of the formulation/standard solutions under analysis and of the ultra-purified water (standard transparent liquid).[52] These limits are merely examples, because the objective in the future is to establish the tolerance based on the color coordinates of a formulation whose CQAs (analytically studied) are at the limit of the specifications. For these, it is necessary to gain more data of this composition; in particular, it is important to correlate the intensity of yellowish of the composition with the loss on analytical stability and product quality of the formulation or performance of the product over time. The change in color is expected to indicate some non-conformity in the formulation specifications, thus, by studying the coordinates of the color of the formulations, it is possible to assess their conformity.

The objective in the future is that while the color coordinates are located inside the space delimited by the tolerance, the composition can be accepted and comply with specification. If the color coordinates are located outside the tolerance limits defined for a specific product, the formulation should be rejected (as it does not comply with the specification) and the cause of the color change beyond the defined limits should be investigated.

It is not possible, for now, to obtain the tolerance limits for the color of the formulation due to lack of stability history of the formulation.

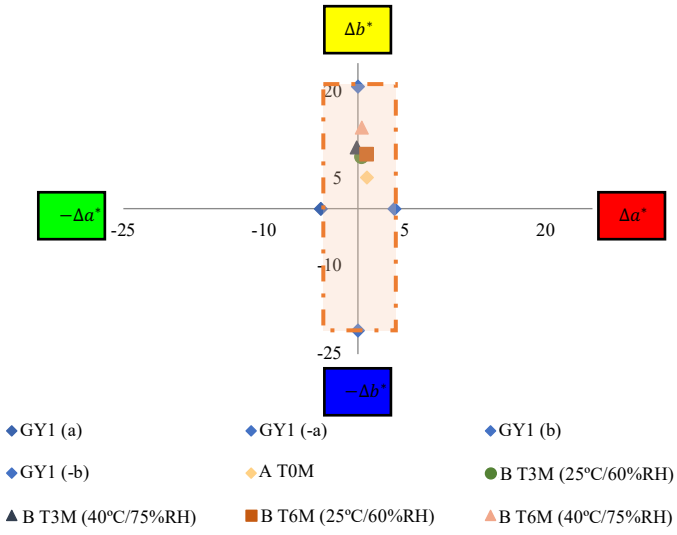


Figure 36 - Color tolerance box using the most intense color of the GY Scale.

CHAPTER IV - CONCLUSIONS AND FUTURE WORK

The objective of this dissertation was the implementation of a method for the evaluation of the color of non-sterile liquid preparations. The development of the method was based on the European Pharmacopoeia 9.7 and consisted on the visual and instrumental evaluation of the color using five color scales and a non-sterile liquid formulation subject to different pre-stability conditions (25 °C/60 %RH and 40 °C/75 %RH), analyzed at different time points (at the time of preparation and after three and six months in the stability chambers).

The colors of the standard solutions and formulations were quantified based on the CIELAB color space model. The representation of the color coordinates of the standard solutions and the formulation on a two-dimensional cartesian diagram based on CIELAB color space allowed the location of the color of the formulation within the scales. Using the CIELAB model, it is possible to quantify the difference between two colors and associate this numerical difference with the difference that an observer could detect. In this way, it is possible to determine which color of the standard solutions of the scales is closer to the color of the formulation under analysis, allowing in a potential more precise way to specify the real color of a liquid preparation. **Table C 1 (**present in **Attachment C)** shows a summary of the results of the visual and instrumental evaluation obtained for the formulation under analysis, at the different time points and in the different pre-stability conditions. The color scales used for the visual and instrumental evaluation of the color are constituted by standard solutions that vary between the 1 (sample with more intense color of the scale) and the 7 (sample with less intense color of the scale).

The formulation A T0M (analyzed immediately after preparation) has a transparent staining, being close to the standard solutions 7 of all scales (almost transparent ranges), both visually and instrumentally. After three months in stability, the formulation acquires a slight yellowish hue. Comparing the color of the formulations under normal pre-stability conditions (25 °C/60 %RH) and under forced degradation conditions (40 °C/75 %RH) , it can be seen, visually and instrumentally, that the slight pale yellow color is more intense under forced degradation conditions. In this way, the color of the formulation is closer to the more intense colors of the scales (3, 4 and 5). It is also possible to conclude that the color change of the formulation between three and six months of stability is not very significant and is only detectable under conditions of forced degradation. From **Table C 1** (present in **Attachment C**), it is also possible to verify which one of the standard solutions has the most similar color to that of the formulation. The results of the numerical difference between the colors (which take into account the brightness) coincide with the instrumental results represented in the two-dimensional model. Thus, it can be concluded that, due to the fact that all samples have a similar degree of brightness, this does not affect the instrumental measurement considering only the hue and chroma parameters associated with the color (the three

dimensional color coordinates – including the brightness parameter - are present in **Figure A 1** and **Figure A 2**, represented in **Attachment A)**. Thus, it is concluded that the two-dimensional CIELAB model used is accurate for the instrumental evaluation of the color for the samples analyzed in this dissertation. The CIELAB model, because it is practically uniform, also allowed the establishment of a rectangular box with tolerances for color. As an example, one of the standard solution of one of the scales was used for the construction of the tolerance limits of the box. However, the objective in the future is to define as tolerance the coordinates of the color of a formulation that is at the limits of its specifications (CQAs attributed to the formulation, such as impurities, assay, pH, viscosity, among others).

The analysis of the results of this dissertation is divided into three stages: visual evaluation according to the European Pharmacopoeia 9.7, instrumental evaluation and a tentative to establish the definition of tolerances for the color of the formulation. During the curricular internship, it was possible to conclude on the first two stages (the results are presented in **Table C 1 – Attachment C**). The visual and instrumental analysis allows concluding that the color scales reproduced based on the European Pharmacopoeia (BY, Y and GY) are suitable for evaluation of the color of the formulation. For the definition of the tolerance box for the formulation color, it is necessary to obtain a history of the color coordinates of the formulation, which was not possible due to the temporal limitation of the internship. Thus, as a suggestion for future work, it is advisable to analyze the CQAs of formulations over time and on different stability conditions (mainly under conditions of forced degradation, as it is the worst case study), in order to be able to find a formulation that is at the limit of the specifications of the CQAs. It is important to note that instrumental color evaluation performed in this dissertation is not required by the European Pharmacopoeia 9.7, it is only advised by USP. The preparation of the color scales differs form European Pharmacopoeia and USP, which may have an impact on the overall results obtained. Briefly, in this work it was implemented the quantification method described in USP not with USP color standars but rather with European color standards. However, the compositon is very similar, the slight differences could have an impact on the evaluations performed. For future work, the implementation of a visual and instrumental color evaluation method according to USP and the Brazilian Pharmacopoeia will be performed. The reason behind the decision of perform the color evaluation based on European Pharmacopoeia is the fact that this product will be develop for European market. Thus only qualificative assessment is required. The inclusion of a quantification is proposed as an add on to the project. Overall, the implementation of the color evaluation method described in this dissertation will allow, in the future, to establish the color specifications of liquid pharmaceutical dosage forms. Through the color quantification, it will be possible to universalize the color specifications, which will potentially facilitate the description of this important CQA.

CHAPTER V - REFERENCES

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CHAPTER VI - ATTACHMENTS

A. THREE-DIMENSIONAL COLOR COORDINATES – RESULTS

Figure A 1 and **Figure A 2** show the results of the color coordinates of the scales reproduced by the European Pharmacopoeia 9.7 and of the formulations under analysis, taking into consideration all the parameters of the CIELAB model: red/green content (a^*), yellow/blue content (b^*) and brightness (L^*). The brightness parameter is not considered in the two-dimensional CIELAB model. As can be seen from **Figure A 1**, the brightness parameter (L^*) of the standard solutions of the scales has a high value, which justifies the fact that the samples are all of a low intensity color. The color coordinates of the reference samples and the formulation are in the same region of the CIELAB color space, which allows concluding that the color scales reproduced (BY, Y and GY) are suitable for the color evaluation of the formulations under analysis .

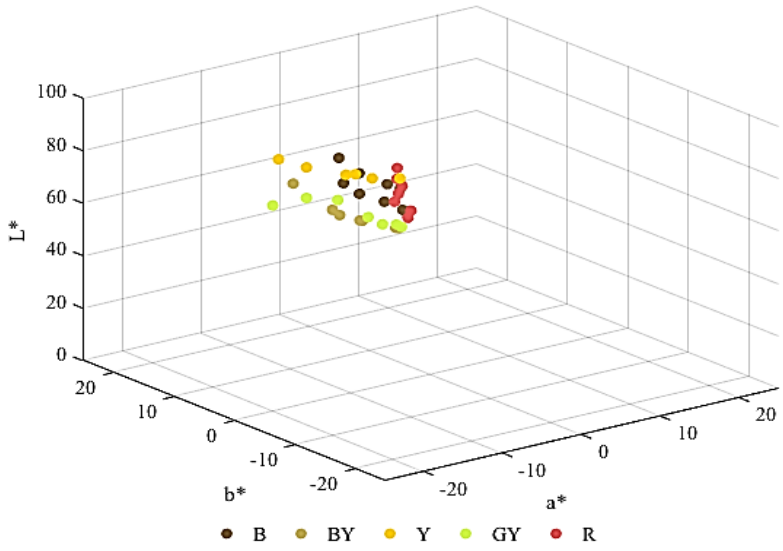

Figure A 1 - Three-dimensional representation of the color coordinates of the color scales.

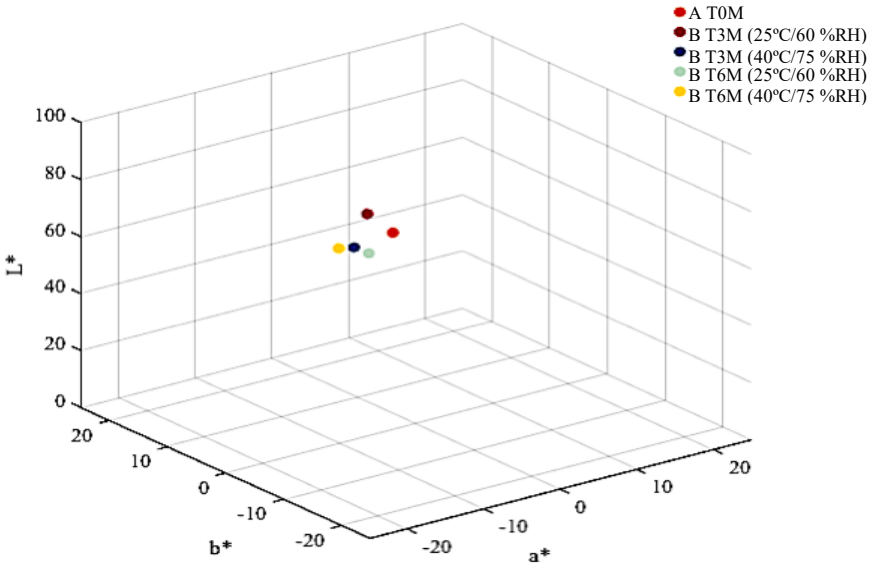

Figure A 2 - Three-dimensional representation of the color coordinates of the formulations under analysis.

B. COLOR DIFFERENCE (ΔE^* PARAMETER) – RESULTS

In the **Table B1** to **Table B5** are the results obtained for the color difference parameter ΔE^*, calculated from Equation (1).

Table B 1 - Color difference between formulation A T0M and standard solutions of each scale.

Standard Solutions	ΔE^*
BY1	18.8535
BY2	17.1933
BY3	16.8668
BY4	14.9195
BY5	14.7602
BY6	12.8214
BY7	12.5627
Y1	21.4098
Y2	17.7957
Y3	13.5601
Y4	8.5467
Y5	7.9585
Y6	6.2040
Y7	**6.1674**
GY1	21.2092
GY2	13.2901
GY3	9.3739
GY4	10.6638
GY5	11.7444
GY6	10.4759
GY7	11.2366

Table B 2 - Color difference between formulation B T3M (25ºC/60% RH) and standard solution of each scale.

Standard Solutions	ΔE*
BY1	17.8696
BY2	18.3817
BY3	18.4715
BY4	17.2527
BY5	17.1679
BY6	16.4138
BY7	16.2512
Y1	18.0858
Y2	14.3669
Y3	10.0011
Y4	4.26157
Y5	3.42579
Y6	**1.08548**
Y7	4.8331
GY1	21.2826
GY2	12.8834
GY3	9.68075
GY4	13.3174
GY5	15.0356
GY6	14.2403
GY7	15.1117

Table B 3 - Color difference between formulation B T3M (40ºC/75% RH) and standard solutions of each scale.

Standard Solutions	ΔE*
BY1	11.5309
BY2	6.88873
BY3	6.29981
BY4	4.35122
BY5	4.25359
BY6	5.07207
BY7	5.21845
Y1	18.5463
Y2	15.9781
Y3	13.4981
Y4	12.7248
Y5	12.9535
Y6	13.4497
Y7	17.2524
GY1	11.8444
GY2	6.98723
GY3	5.08147
GY4	**1.27824**
GY5	3.42542
GY6	4.42899
GY7	5.12380

Table B 4 - Color difference between formulation B T6M (25ºC/60% RH) and standard solutions of each scale.

Standard Solutions	ΔE*
BY1	12.9489
BY2	7.11802
BY3	6.12598
BY4	3.42639
BY5	3.19057
BY6	2.94091
BY7	3.15250
Y1	20.5356
Y2	18.1066
Y3	15.7247
Y4	14.7990
Y5	14.9384
Y6	15.2439
Y7	18.7256
GY1	12.7388
GY2	9.15189
GY3	7.43833
GY4	2.08801
GY5	**1.85126**
GY6	3.18436
GY7	3.50874

Table B 5 - Color difference between formulation B T6M (40ºC/75% RH) and standard solution of each scale.

Standard Solutions	ΔE*
BY1	9.38008
BY2	2.24557
BY3	**1.33011**
BY4	2.27087
BY5	2.52401
BY6	6.93859
BY7	7.33095
Y1	18.3175
Y2	16.6662
Y3	15.4678
Y4	16.3724
Y5	16.7539
Y6	17.7532
Y7	21.9523
GY1	8.27801
GY2	7.94447
GY3	8.54554
GY4	5.70159
GY5	6.26362
GY6	8.03956
GY7	8.21483

C. SUMMARY TABLE OF THE OBTAINED RESULTS

Table C 1 shows the results obtained for the visual and instrumental evaluation of the color. In addition, it also shows the standard reference solution with the color closest to the formulation under analysis (evaluation based on the ΔE^* parameter).

Table C 1 - Summary table of the obtained results for visual and instrumental color evaluation.

	Experimental Results of the Color Evaluation Method						
	Stage I			**Stage II**			
	Visual Evaluation			**Instrumental Evaluation**			**Closest standard solution based on ΔE^***
Color Scales / Formulations	**BY**	**Y**	**GY**	**BY**	**Y**	**GY**	
A T0M	[BY6 - BY7]	[Y6 - Y7]	[GY6 - GY7]	Colorless than BY7	Colorless than Y7	Colorless than GY7	Y7
B T3M (25ºC/60%RH)	[BY4 - BY5]	[Y4 - Y5]	[GY4 - GY5]	[BY5 - BY6]	[Y6 - Y7]	[GY6 - GY7]	Y6
B T6M (25ºC/60%RH)	[BY4 - BY5]	[Y4 - Y5]	[GY4 - GY5]	[BY5 - BY6]	[Y5 - Y6]	[GY4 - GY5]	GY5
B T3M (40ºC/75%RH)	[BY4 - BY5]	[Y4 - Y5]	[GY4 – GY5]	[BY4 - BY5]	[Y5 - Y6]	[GY4 - GY5]	GY4
B T6M (40ºC/75%RH)	[BY3 – BY4]	[Y3 - Y4]	[GY3 - GY4]	[BY3 - BY4]	[Y5 - Y6]	[GY3 - GY4]	BY3

D. CIELAB COLOR SPACE MODEL

In the present dissertation, the CIELAB coordinates were obtained directly from the spectrophotometer. However, if this were not possible, these coordinates could be obtained mathematically from the value of the tristimulus.

The determination of the CIELAB system coordinates requires, in a first instance, the conversion of the tristimulus X, Y and Z coordinates into X^*, $Y^*$and Z^*value functions through the Equations (A1) to (A3). Th e parameters X_n, Y_n (for standardize illuminants, Y_n takes the value of 100) and Z_n are the standard tristimulus for the illuminant that is being used to the color observation.[25], [65],[59]

Values of the tristimulus X, Y and Z for A, C and D65 illuminants are present in **Table D 1**.[25]

$$X^* = \sqrt[3]{\frac{X}{X_n}} \quad \text{(A1)}$$

$$Y^* = \sqrt[3]{\frac{Y}{Y_n}} \quad \text{(A2)}$$

$$Z^* = \sqrt[3]{\frac{Z}{Z_n}} \quad \text{(A3)}$$

Table D 1 - Tristimulus values for three types of illuminants for the two standard observers.

	2º Standard Observer		10º Standard Observer	
Illuminant	X_n	Z_n	X_n	Z_n
A	109.83	35.55	111.16	35.19
C	98.04	118.11	97.30	116.14
D65	95.02	108.82	94.83	107.38

When the ratio between $\frac{X}{X_n}$, $\frac{Y}{100}$ and $\frac{Z}{Z_n}$ is inferior or equal to the value of $0{,}008856$, the value functions X^*, $Y^*$and Z^*must be determined using the Equations (A4) to (A6). [25]

$$X^* = 7{,}787 \times \frac{X}{X_n} + 0{,}138 \quad \text{(A4)}$$

$$Y^* = 7{,}787 \times \frac{Y}{100} + 0{,}138 \quad \text{(A5)}$$

$$Z^* = 7{,}787 \times \frac{Z}{Z_n} + 0{,}138 \quad \text{(A6)}$$

From the values of X^*, $Y^*$and Z^*, it is possible to obtain the spatial coordinates L^*, $a^*$and b^*, by subtracting the value function of the tristimulus X* and the tristimulus Y*, as well as subtracting the value of the tristimulus Z* from the value of tristimulus Y*. This conversion is performed using the Equations (A7) to (A9).[28], [59], [66]

$$a^* = 500 \times (X^* - Y^*) \quad \text{(A7)}$$

$$b^* = 200 \times (Y^* - Z^*) \quad \text{(A8)}$$

$$L^* = 116 \times Y^* - 16 \quad \text{(A9)}$$

E. MATLAB CODES

E1. CHROMATICITY DIAGRAM

Figure E 1 shows the representation of the Chromaticity Diagram. The code used to obtain the Chromaticity Diagram is described below.[25], [42]

```
%Matlab Function for the Chromaticity Diagram

plotChromaticity()
```

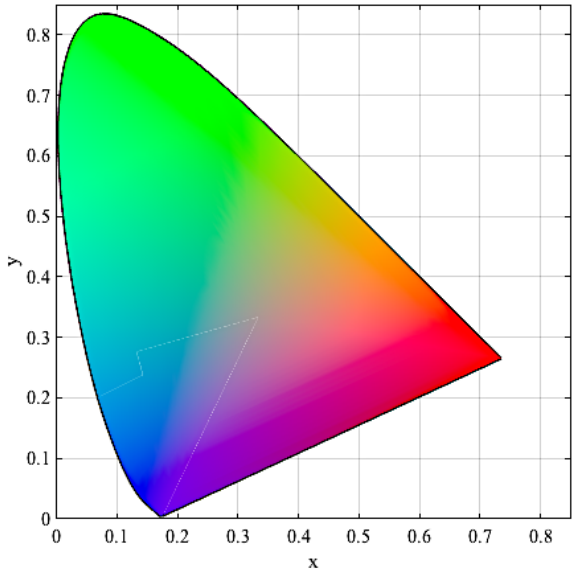

Figure E 1 - Representation of Chromaticity Diagram [*Adapted from* [25], [42]].

E2. RGB CUBE

In **Figure E 2** is represented the RGB Cube, obtained using the code below. [42], [67]

```
%Matlab Function for the RGB Cube

x=[NaN 0 1 NaN; 0 0 1 1;0 0 1 1;NaN 0 1 NaN;NaN 0 1 NaN;NaN NaN NaN NaN];
y=[NaN 0 0 NaN;0 0 0 0;1 1 1 1;NaN 1 1 NaN;NaN 0 0 NaN;NaN NaN NaN NaN];
z=[NaN 0 0 NaN;0 1 1 0;0 1 1 0;NaN 0 0 NaN;NaN 0 0 NaN;NaN NaN NaN NaN];
    cc=zeros(8,3); % Matrix involving 8 colors distributed in the 3 axis.
    cc(1,:)=[0 0 0]; % Black
    cc(2,:)=[1 0 0]; % Red
    cc(3,:)=[0 1 0]; % Green
    cc(4,:)=[0 0 1]; % Blue
    cc(5,:)=[1 0 1]; % Magenta
    cc(6,:)=[0 1 1]; % Cyan
    cc(7,:)=[1 1 0]; % Yellow
    cc(8,:)=[1 1 1]; % White
    cs=size(x);
    c=repmat(zeros(cs),[1 1 3]);
for i=1:size(cc,1)
    ix=find(x==cc(i,1) &...
          y==cc(i,2) &...
          z==cc(i,3));
    [ir,ic]=ind2sub(cs,ix);
     for k=1:3
          for m=1:length(ir)
          c(ir(m),ic(m),k)=cc(i,k);
          end
     end
end
    s=surf(x,y,z,c);
    shading interp; % Color blur
    xlabel('x');
    ylabel('y');
    zlabel('z');
    title('Cubo RGB')
    axis equal;
    axis on;
rotate3d on
```

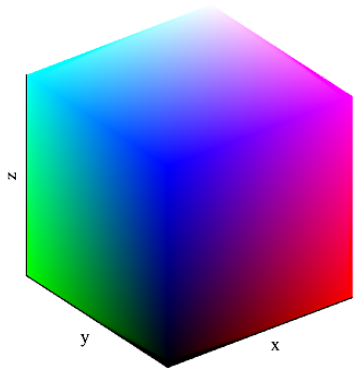

Figure E 2 -Representation of RGB Cube [*Adapted from* [42], [43]].

E3. CIELAB Color Space

In **Figure 17** is represented the three-dimensional CIELAB color space and in Erro! A origem da referência não foi encontrada. are the projections of the three-dimensional color space. Through the projections of the Erro! A origem da referência não foi encontrada. two-dimensional planes of the CIELAB color space are obtained (**Figure 19**). The code used to obtain all the three figures are present below.[42], [67]

```
%Matlab Function for the CIELAB Color Space

function f =plot_Lab(mode,Lab,createnewfig,markercolor,markersize,storeme)
% Imput
Mode          [scalar 2,4 and 6 to obtain the 3D plot, the projections and the two-dimensional plots]
Lab           [scalar 0 to obtain all the colors]
Createnewfig [scalar 0 to create different figures]
Markercolor  [scalar 0 to obtain all colors]
Markersize    [size of the point, for example, 10]
Storeme       [scalar 0 to export figure]

linewidth = 1; %Width of the axis
if mode==0
   demo();
   return;
end
%Graphical Representation
if createnewfig
   f = figure;
else
   f = gcf;
end
set(f,'PaperUnits','centimeters');
set(f,'name','plot_Lab - colorimg@ugr.es');
set(0,'units','pixels') ;
screen = get(0,'screensize');
set(f,'Position',[200,screen(4)-screen(4)/3-200,screen(3)/2,screen(4)/3]);
% Color of the points
if mode==2 || mode==4 || mode==6
   cform = makecform('lab2srgb','AdaptedWhitePoint',whitepoint('D65'));
   RGB = applycform(Lab',cform);
else
   RGB = markercolor;
end
%Axis Limits: L[-100,100]; a and b[-128,128]
min_Lab = min(Lab,[],2);
max_Lab = max(Lab,[],2);
if min_Lab(1) > 0
   min_Lab(1) = 0;
end
```

```
if min_Lab(2) > -128
   min_Lab(2) = -128;
end
if min_Lab(3) > -128
   min_Lab(3) = -128;
end
if max_Lab(1) < 100
   max_Lab(1) = 100;
end
if max_Lab(2) < 128
   max_Lab(2) = 128;
end
if max_Lab(3) < 128
   max_Lab(3) = 128;
end
switch mode
   case {1,2} %Two-dimensional subplots
      subplot(1,3,1);scatter(Lab(2,:),Lab(3,:),markersize,RGB,'fill');
      xlabel('a*'),ylabel('b*');
      title('CIE-b*a* coordinates');
      axis([min_Lab(3) max_Lab(3) min_Lab(2) max_Lab(2)]);
      grid on;
      hold onM
      subplot(1,3,2);scatter(Lab(2,:),Lab(1,:),markersize,RGB,'fill');
      xlabel('a*'),ylabel('L*');title('CIE-L*a* coordinates');
      axis([min_Lab(2) max_Lab(2) min_Lab(1) max_Lab(1)]);
      grid on;
      hold on;
      subplot(1,3,3);scatter(Lab(3,:),Lab(1,:),markersize,RGB,'fill');
      xlabel('b*'),ylabel('L*');title('CIE-L*b* coordinates');
      axis([min_Lab(3) max_Lab(3) min_Lab(1) max_Lab(1)]);
      grid on;
      hold on;
      % Black Axis
      subplot(1,3,1)
      line([min_Lab(3) max_Lab(3)],[0 0],'color',[0 0 0],'lineWidth',linewidth)
      line([0 0],[min_Lab(2) max_Lab(2)],'color',[0 0 0],'lineWidth',linewidth)
      subplot(1,3,2)
      line([min_Lab(2) max_Lab(2)],[0 0],'color',[0 0 0],'lineWidth',linewidth)
      line([0 0],[min_Lab(1) max_Lab(1)],'color',[0 0 0],'lineWidth',linewidth)
      subplot(1,3,3)
      line([min_Lab(3) max_Lab(3)],[0 0],'color',[0 0 0],'lineWidth',linewidth)
      line([0 0],[min_Lab(1) max_Lab(1)],'color',[0 0 0],'lineWidth',linewidth)
      %Export the plot
      if ischar(storeme)
         plot2svg(['Lab_coordinates_3D_',storeme,'.svg'],f,'png');
      end
   case {3,4} %Three-dimensional plot
      scatter3(Lab(3,:),Lab(2,:),Lab(1,:),markersize,RGB,'fill');
      xlabel('b*'),ylabel('a*'),zlabel('L*');
```

```
    title('CIE-L*a*b* coordinates');
    axis([min_Lab(3) max_Lab(3) min_Lab(2) max_Lab(2) min_Lab(1) max_Lab(1)]);
    grid on;
    hold on;
    % Black Axis
    line([min_Lab(3) max_Lab(3)],[0 0],[0 0],'color',[0 0 0],'lineWidth',linewidth);
    line([0 0],[min_Lab(2) max_Lab(2)],[0 0],'color',[0 0 0],'lineWidth',linewidth);
    line([0 0],[0 0],[min_Lab(1) max_Lab(1)],'color',[0 0 0],'lineWidth',linewidth);
    %Export the plot
    if ischar(storeme)
       plot2svg(['Lab_coordinates_3D_',storeme,'.svg'],f,'png');
    end
  case {5,6} %Three-dimensional projection plot
    scatter3(Lab(3,:),Lab(2,:),zeros(size(Lab,2),1),markersize,RGB,'fill');
    hold on;
    scatter3(Lab(3,:),100*ones(size(Lab,2),1),Lab(1,:),markersize,RGB,'fill');
    scatter3(100*ones(size(Lab,2),1),Lab(2,:),Lab(1,:),markersize,RGB,'fill');
    xlabel('b*'),ylabel('a*'),zlabel('L*');
    title('CIE-L*a*b* coordinates');
    axis([min_Lab(3) max_Lab(3) min_Lab(2) max_Lab(2) min_Lab(1) max_Lab(1)]);
    grid on;
    hold on;
    % Black Axis
    line([min_Lab(3) max_Lab(3)],[0 0],[0 0],'color',[0 0 0],'lineWidth',linewidth);
    line([0 0],[min_Lab(2) max_Lab(2)],[0 0],'color',[0 0 0],'lineWidth',linewidth);
    line([0 0],[max_Lab(2) max_Lab(2)],[min_Lab(1) max_Lab(1)],'color',[0 0 0],'lineWidth',linewidth);
    line([max_Lab(3) max_Lab(3)],[0 0],[min_Lab(1) max_Lab(1)],'color',[0 0 0],'lineWidth',linewidth);
    line([max_Lab(3) max_Lab(3)],[min_Lab(2) max_Lab(2)],[50 50],'color',[0 0 0],'lineWidth',linewidth);
    line([min_Lab(3) max_Lab(3)],[max_Lab(2) max_Lab(2)],[50 50],'color',[0 0 0],'lineWidth',linewidth);
    %Export the plot
    if ischar(storeme)
      plot2svg(['Lab_coordinates_3D_projected_',storeme,'.svg'],f,'png');
    end
```

E4. TWO-DIMENSIONAL UNIFORM CIELAB COLOR SPACE

Figure E 3 shows the uniform two-dimensional CIELAB color space used in the instrumental evaluation in the present dissertation. Below, is described the code used to obtain the uniform graphical representation.[42], [49]

```
plot([0 0],[-60 60],'k','LineWidth',2)
hold on
plot([-60 60],[0 0],'k','LineWidth',2)
axis([-60 60 -60 60])
gr = [0.7 0.7 0.7];
r = [.9 0 0];
g = [0 .9 0];
y = [.9 .9 0];
bl = [0 0 .9];
index = 0;
```

```
% First quadrant of the color space
index = index+1;
a = 50;
b = 0;
ab(index,:) = [a b];
col(index,:) = r;
    for i = 5:5:85
        index = index+1;
        a = cos(i*pi/180)*50;
        b = sin(i*pi/180)*50;
        ab(index,:) = [a b];
        c = (a*r+(50-a)*y)/50;
        col(index,:) = c;
    end
index = index+1;
a = 0;
b = 50;
ab(index,:) = [a b];
col(index,:) = y;
% Grey color
index = index+1;
a = 0;
b = 0;
ab(index,:) = [a b];
col(index,:) = gr;
patch('Vertices',ab, 'Faces',[1:size(ab,1)],'EdgeColor','none','FaceVertexCData',col,'FaceColor','interp')
clear ab;
index = 0;
% Second quadrant of the color space
index = index+1;
a = 0;
b = 50;
ab(index,:) = [a b];
col(index,:) = y;
    for i=95:5:175
        index = index+1;
        a = cos(i*pi/180)*50;
        b = sin(i*pi/180)*50;
        ab(index,:) = [a b];
        c=(b*y+(50-b)*g)/50;
        col(index,:) = c;
    end
index = index+1;
a = -50;
b = 0;
ab(index,:) = [a b];
col(index,:) = g;
% Grey color
index = index+1;
a = 0;
```

```
b = 0;
ab(index,:) = [a b];
col(index,:) = gr;
patch('Vertices',ab, 'Faces',[1:size(ab,1)],'EdgeColor','none','FaceVertexCData',col,'FaceColor','interp')
clear ab;
index=0;
% Third quadrant of the color space
index = index+1;
a = -50;
b = 0;
ab(index,:) = [a b];
col(index,:) = g;
    for i=185:5:265
        index = index+1;
        a = cos(i*pi/180)*50;
        b = sin(i*pi/180)*50;
        ab(index,:) = [a b];
        c=(-b*bl+(50+b)*g)/50;
        col(index,:) = c;
    end
index = index+1;
a = 0;
b = -50;
ab(index,:) = [a b];
col(index,:) = bl;
% Grey color
index = index+1;
a = 0;
b = 0;
ab(index,:) = [a b];
col(index,:) = gr;
patch('Vertices',ab, 'Faces',[1:size(ab,1)],'EdgeColor','none','FaceVertexCData',col,'FaceColor','interp')
clear ab;
index = 0;
% Fourth quadrant of the color space
index = index+1;
a = 0;
b = -50;
ab(index,:) = [a b];
col(index,:) = bl;
    for i=275:5:355
        index = index+1;
        a = cos(i*pi/180)*50;
        b = sin(i*pi/180)*50;
        ab(index,:) = [a b];
        c = (a*r+(50-a)*bl)/50;
        col(index,:) = c;
    end
index = index+1;
a = 50;
```

```
b = 0;
ab(index,:) = [a b];
col(index,:) = r;
% Grey color
index = index+1;
a = 0;
b = 0;
ab(index,:) = [a b];
col(index,:) = gr;
patch('Vertices',ab, 'Faces',[1:size(ab,1)],'EdgeColor','none','FaceVertexCData',col, 'FaceColor','interp')
clear ab;
plot([0 0],[-60 60],'k','LineWidth',2)
plot([-60 60],[0 0],'k','LineWidth',2)
```

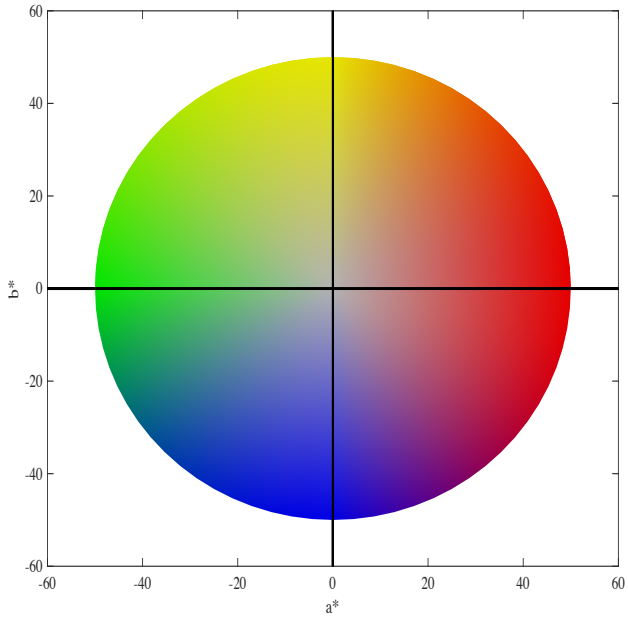

Figure E 3 - Uniform CIELAB two-dimensional color space [*Adapted from* [42], [49]].

F. COLOR BLINDNESS TEST

Due to the subjectivity that can be associated with visual color evaluation, it is important to perform this test using people who do not present color vision deficiencies. One of the tests used for the detection of visual color deficiencies (acute and moderate deficiencies) is the Ishihara color test. This test consists of the presentation of a set of colored plates to the person being analyzed. These colored plates contain a pattern consisting of color circles with similar colors and in the middle of the pattern, there are circles of a different color, grouped together that form a number or a shape only visible to people without color blindness. There are different Ishihara plates, each with a different level of difficulty of distinguishing colors. An example of an Ishihara plate used for the

color blindness test is shown in **Figure F 1**. People who can distinguish colors correctly can see the number 74 represented in the plate. People with some difficulty in distinguishing colors see either the number 21 (in cases of moderate color vision deficiency) or they cannot see any number (acute deficiency).

However, in order to detect minor deficiencies in relation to color distinction, genetic testing is advised. In the present dissertation, genetic tests may not be required because the difference between colors is possible to detect by a experienced observer (considering ΔE^* parameter).

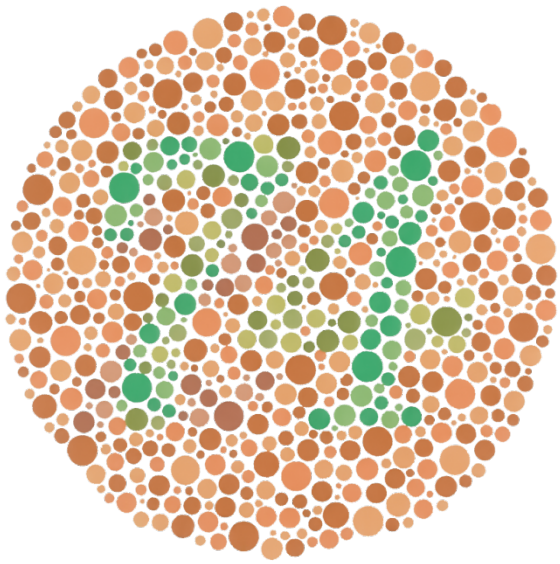

Figure F 1 - Ishihara plate [*Adapted from* [61]].