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Degree in chemistry

FINAL DEGREE PROJECT

Determination of Allure Red, Brilliant Blue and Tartrazine in solid samples by digital image analysis

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Determination of three food colorants in solid samples

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ABSTRACT

This study aims to determine the colorants Allure Red, Brilliant Blue and Tartrazine in solid samples, such as Jellybeans. The objective is to determine those foods coloring by digital image analysis in order to obtain a fast and cheap method, which is easy to apply. It has been created univariate and multivariate calibration models with images taken by a Smartphone for two different standards, Jelly and Candy.

It also has been developed an image taking procedure in order to control factors that could interfere in the image quality, such as brightness or shadows. Once the images have been taken, they are processed and the data is checked in control charts to verify if they are suitable for generating calibrations or determining the previously mentioned dyes.

At the beginning, it was observed that there could be factors interfering in the intensity of the color of the image that were neither the lighting nor the shadows. In order to verify the existence of those factors, Student *t* and *Fischer* tests were carried out in different tests explained in Appendix I and Appendix II. These tests demonstrated that, finally, the depth of the standards used to develop the calibration curve directly affect the intensity of the color, so different calibration curves were obtained in which significant differences were. So as a conclusion, it could be said that this method follows the Lambert-Beer law.

Finally, it has been tried to validate the method with real samples, but it was not possible to do it since it could have not been validated with a parallel method.

LABURPENA

Ikerketa honen helburua Allura Gorria, Urdin Distiratsua eta Tartrazina determinatzea da lagin solidoetan, gomazko gozokietan esaterako. Helburua koloratzaile horien determinazioa egitea da irudi digitalen analisiaren bidez. Modu honetan, metodo azkar eta merkea lortzen da aplikatzeko erraza dena. Gainera, bi kalibraketa eredu desberdin eratu dira, bat aldagai bakarreakoa eta bestea aldagai anitzekoa. Modelo hauek Smartphone batekin harturiko argazkiekin osatu dira, bi patroi multzo desberdinekin, bata gelatinazkoa eta bestea gomazko gozokia izanik.

Argazkiaren hartzearen prozedura bat garatu da, argazkian bertan eragin dezaketen faktoreak kontrolatzeko asmoz eta modu honetan argazkiaren kalitatea kontrolatzeko. Faktore horiek iluminazioarekin zerikusia dute, itzalak eta argitasuna. Behin argazkiak harturik daudela, prozesatzen dira eta lorturiko datuak kontrol grafiko erabiliz egiaztatzen dira. Honen helburua, argazki hori determinazioetako eta kalibraziorako egokian diren jakitea da.

Hasiera batean, ikusi zen faktoreak argazkiaren kolorearen intentsitatean eragiten zutela. Faktore hauek ez ziren ez itzalak ezta argitasuna ere. Faktore horiek existitzen zirela konprobatzeko asmoz, *Student t* eta *Fischer* test batzuk garatu ziren berauengan diferentzia adierazgarririk zeuden egiaztatzeko. Test hauek aurrera eraman ziren saiakuntzak Appendix I eta Appendix II-an agertzen dira. Test hauek azkenik demostratu zuten azkenean patroi bakoitzaren sakontasunak zuzenki eragiten zuela patroien kolorean. Ondorioz Lambert-Beer legea jarraitzen dutela.

Azkenik, metodoaren balidazioa egiten saiatu gara lagin errealetara eramanez, hala ere, ezinezkoa izan da denbora falta eta kanpo-balidazio metodo baten faltagatik.

1. Introduction

1.1. Food colorants: Allure Red, Blue Brilliant and Tartrazine

Food additives are substances with no nutritional value. Nevertheless, they improve appearance in food and drinks making them more desirable for consumers. For that reason, food industry uses food additives (preservatives, colorings, antioxidants, etc.) in processed food or drink available in the market.

The use of additives ensures the appearance and eases the preparation, preservation, storage and transport of nourishments.[1] In addition, they improve the taste, flavor, color, texture of food preserving the homogeneity of the product. The additives used in processing are divided in two groups: [2]

- Naturally occurring compounds or additives isolated from natural sources. They have lower coloring strength, they are more sensitive and not stable under temperature, oxygen, light or different pH conditions.
- Synthetic chemicals.

However, considering their function as additive the classification changes into 15 different groups:[2]

- **Colorings:** They are used for changing the color of a product or intensifying it.
- **Preservatives:** They are applied to avoid biological changes in the product, such as, the appearance of mold, fermentation or getting spoiled.
- **Antioxidants:** To avoid the catalytic oxidation by heat, light and air.
- **Stabilizer:** They have the aim of maintaining the chemical equilibrium, inhibiting possible reactions.
- **Emulsifier:** They enable to mix two immiscible phases.
- **Thickening agents:** To develop viscosity.
- **Jellying agents:** they are used to obtain jellies.
- **Aromatic agents:** they contribute to reinforce flavor and smell.
- **Flavor enhancers:** They reinforce flavor.
- **Artificial sweeteners:** they use provide a higher sweet level.
- **Antifoaming agents:** not wanted foam is avoided by they use.
- **pH regulators:** their objective is to control, neutrality, acidity and alkalinity by adding, salts, acids or bases.

Furthermore, food additives are listed on the product labels.[3] In those lists, the function and the specific substance must be specified by its name or even the E code. If a product has the E number, guarantees the overcome of the European Union's security controls. By this method, we obtain a single form to denominate each additive in any country. The following table (see Table 1) explains this classification according to the additive function.[2]

Table 1. E code classification of food additives according to their function.

FUNCTION	E CODE
Colorings	E-100-E-199
Preservatives	E-200-E-299
Antioxidants & acidity regulators	E-300-E-399
Stabilizers	E-400-E-499
pH regulators & antilump agents	E-500-E-599
Flavor enhancer	E-600-E-699
Various	E-900-E-999

According to the E codes (see Table 1), the scale from E-100 to E-199 correspond to colorants. Nowadays, food colors are used for preservation and improvement of the color appearance in food products. In recent years however, it has been an important increase in the use of natural colorings due to the consumers' preference. Nevertheless, natural food colors are not as stable as the synthetic ones and production costs are higher.[3] In a similar way to synthetic food dyes, they could be classified into azo dyes, triphenilmethane dyes, thriarylmethane dyes, xanthene dyes, indigotine dyes and quinoline dyes. On the one hand, azo dyes, contain the azo group (-N=N-) which has the chromophore function in the molecular structure.[2] Allure Red and Tartrazine are located in this group. On the other hand, triarylmethane dyes, they have triphenylmethane backbones. It is an organic salt to which Blue Brilliant belongs to.[4]

As to food colorants, they also have their particular E code which would describe the color and substance (see Table 2).

Table 2. E code classification of food colorants by colors.

COLORS	E CODE
Yellow	E-100-E-109
Orange	E-110-E-119
Red	E-120-E-129
Blue and purple	E-130-E-139
Green	E-140-E-149
Brown and black	E-150-E-159
Variety of colors	E-160-E-199

As to Table 2 and taking into account the colorants to be described in this project Allure Red (E-129), Blue Brilliant (E-133) and Tartrazine (E-102) correspond properly to the previous classification.

1.1.1. Allure Red (E-129)

Allure Red is an azo dye, which could be easily found in different products, such as soft drinks, bakery products, candies and ice creams as it is one of the most used dye of food industry. Like E-133, E-129 it is also obtained from petroleum. Allure Red or Disodium 6-hidroxy-5-[(2-methoxy-5-methyl-4-sulfophenyl)azo]-2-naphthalenesulfonate according to its IUPAC name, it has the molecular structure provided in Figure 1. In Figure 1B, it is possible to appreciate the chromophore azo group (-N=N-). E-129 will have the maximum absorbance at 504nm in water solution.

Figure 1. Allure Red food coloring (E-129). (A) The physical appearance of Allure Red, a dark red solid powder. (B) The molecular structure of E-129.

As its chemical structure is $C_{18}H_{14}N_2Na_2O_8S_2$ the correspondent molecular weight will be 496.42 g/mol. According to Figure 1, Allure Red is a dark red solid dust.[2,5,6]

1.1.2. Brilliant Blue (E-133)

Brilliant Blue as mentioned before, it is a triarylmethane blue color synthetic dye. Its IUPAC name is disodium; 2-[[4-ethyl-[(3-sulfonatophenyl)methyl] amino]phenyl]-[4-4[ethyl- [(3-sulfonatophenyl) methyl] azaniumylidene] cyclohexa-2,5-dien-1-ylidene] methyl] benzenesulfonate. The chemical formula is $C_{37}H_{34}N_2Na_2O_9S_3$ and the molecular weight 792.85 g/mol. It is a sodium salt (see Figure 2) obtained from petroleum. In addition, this molecule will give its maximum absorbance at 630nm. [7]

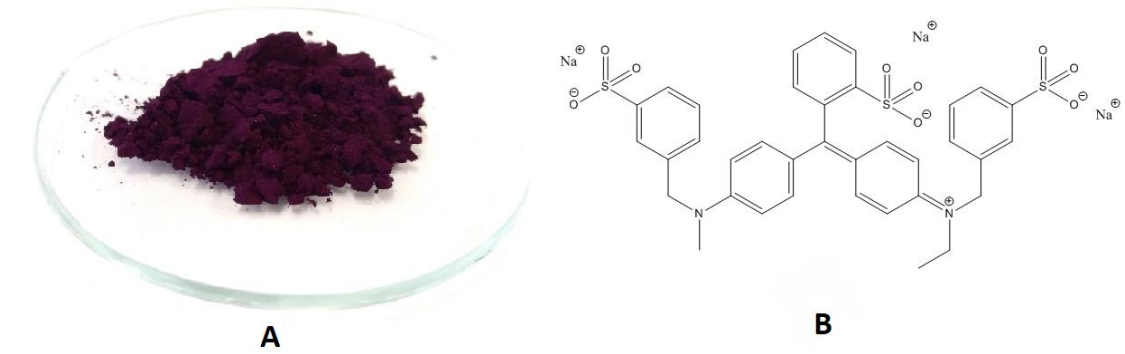

Figure 2. Blue Brilliant (E-133) food coloring. (A) Its physical appearance, a dark blue-purple solid powder. (B) The molecular structure of E-133.

According to the physical characteristics, it is a dark blue solid dust (see Figure 2). It is sensitive to light and oxidants.[7]

1.1.3. Tartrazine (E-102)

Tartrazine or Trisodium 5-hydroxy-1-(4-sulfonatophenyl)4-4[(E)-(4-sulfonatophenyl)diazenyl]-H pyrazole-3-carboxylate (IUPAC name) it is an azo dye as well as Allure Red. In Figure 3, the molecular structure of $C_{16}H_9N_4Na_3O_9S_2$ is provided. Its molecular weight is 534.36g/mol. In Figure 3B the azo group is possible to be seen, thank to this chromophore group Tartrazine give the maximum absorbance at 427 nm.

Figure 3. Tartrazine food coloring (E-102). (A) It is an orange solid powder. (B) The molecular structure of E-102.

This petroleum derivative substance appears in many candies, soft drinks, or sauces in the market, and daily use products. In addition, according to Figure 3 Tartrazine is an orange solid dust. [7,8]

1.1.4. Toxicity

Candies, jellies or soft drinks are consumed daily. Their flashy colors, make these foodstuff desirable and even more when talking about children. As these products contain synthetic colorants and their consumption is high, it is important to know the possible effect they could have on human beings.

Brilliant Blue, Allure Red and Tartrazine are synthetic colorants and some of the most used of the food industry. Synthetic food colors and other type of food additives have always been suggested to have an effect on children's behavior. These type of substances are also called AFCA (Artificial Food Colors and food Additives). Ben Feingold made the initial claims about the problem more than 30 years ago. He claimed that AFCA may produce overactive, impulsive and inattentive behavior. Unfortunately, children who show this behavioral pattern usually are diagnosed with hyperactivity disorder.[9]

Notwithstanding, these are not the only problems artificial food color could cause. Latest research has shown that Allure Red, could cause DNA damage in rats and mice, at different doses. Not only that, E-129 may cause allergic reactions such as urticaria or asthma, especially when mixing it with other colorants. In addition, Brilliant Blue, could create certain cytotoxicity and genotoxicity on human blood's lymphocyte cells and also ophthalmological issues. Nevertheless, azo dyes are what most concern the researchers, especially Tartrazine. Several studies suggest the cytotoxicity and genotoxicity of E-102, as it damages directly human lymphocytes. When its consumption carries out high doses, it exists the risk of carcinogenesis, this does not only take into account the consumption but also the accumulation of the colorant in human body. Finally, it may cause neurotoxicity and deficits in learning and memory. [10]

Considering the society and researchers' concern the EU Scientific Committee for food (SCF) and Joint FAO/WHO Expert committee on Food Additives (JECFA) decided to evaluate the Acceptable Daily Intake, ADI (see Table 3). [2]

Table 3. ADI values for Tartrazine, Brilliant Blue and Allure Red.

FOOD COLORING	ADI VALUES	
	mg/kg	mg/70kg
Tartrazine	7.5	525
Blue Brilliant	12.5	875
Allure Red	7	490

The ADI values indicate the dose a person could daily intake, without effects in the organism. ADI values are specified by mg colorant per kg of body mass (see Table 3).

In this project, the determination has been carried out in solid samples. Those solid samples are supposed to be jellybeans.

Table 4. Maximum colorant (mg/kg) levels of daily intake.

PRODUCT	mg/kg			
	Tartrazine	Allure Red	Brilliant Blue	Group III (Colors with combined maximum limit)
Unflavored fermented milk products, not heated after fermentation	-	-	-	150
Other creams	-	-	-	150
Unripen cheese.	-	-	-	150
Edible cheese rind	-	-	-	*Quantum Satum*
Cheese products	100	-	-	100
Edible ices	-	-	-	150
Dried fruits and vegetables	-	200*	200*	-
Fruit and vegetables in vinegar, oil or brine	-	200*	200*	-
Canned or bottled fruit and vegetables	-	200*	20, 200*	-
Chewing gum	-	-	-	300**
Decorations, coatings and fillings	-	-	-	30** (only fillings) 500** (decorations, coatings, ...)
Flavored drinks	-	-	-	150
Processed nuts	-	-	-	100

For that reason, taking into account the data provided in Table 4, it could be seen that for chewing gums, decoration, coatings, fillings and flavored drinks, the samples with more similarity with the objective only have the maximum colorant level of daily intake for a combination of colorants. This fact makes more difficult to know the maximum levels for each colorant independently and becomes easier to exceed the level applied.

1.2. Digital image analysis.

Colorimetry is the science that defines the methods of decomposition, analysis and description of visible light with the aim of quantifying color information in order to perform a wide variety of analysis. [11] Nowadays with the development of technology, there is a need of finding new

analysis methods, which are easy to apply, environmentally friendly, little use of reagents and samples and with reliable results. For that reason, the use of small technological devices is becoming little by little more important and so the digital image analysis.

In analytical chemistry, the most used optical instrumentation always has a light source, a device for the selection of the wavelength, a capture device or even a detector in order to recognize the signal and process it.[12] Nevertheless, to develop digital image analysis, an image-taking device is only needed. The device could be a scanner, a mobile phone, a digital camera or even a multispectral camera. Each device would have an illumination system.

Digital image analysis has been developed in different analytical areas from food analysis to covering industry. In this project, it has been used to determine three different food coloring in solid samples. A mobile phone has been used to capture the image. The Smartphone provides a wide range of advantages[12]:

- As to the importance of cameras nowadays, mobile phone manufacturers have introduced more developed cameras in cell phones. Therefore, they could provide a great picture sometimes equal (or even better) to digital cameras.
- They combine fast response and simplicity during the analysis.
- Real time communication with computers or other devices in order to improve the results with software.
- Integrated photodetector[13].
- The use of different color spaced by which it makes possible different responses.

However, it also has some drawback to be commented as cell phone cameras have limitations compared to digital cameras:[12]

- Fixed focus lenses
- Little optical zoom
- Smaller sensors that could produce quality loss in pictures.
- Impossibility to control every patter that interfere in the image taking procedure.

Furthermore, it is not only the image-taking device what is needed for the digital image analysis. The use of color spaces is an absolute need nowadays in order to determine the response. The RGB and HSV are the most common color space when capturing images. In this project, RGB system was used since this color space has various similarities with human vision system work. Color spaces are a way of describing one color by a combination of three primary colors. In this case channels. RGB model has three channels R (red), G (green) and B (blue). [12]

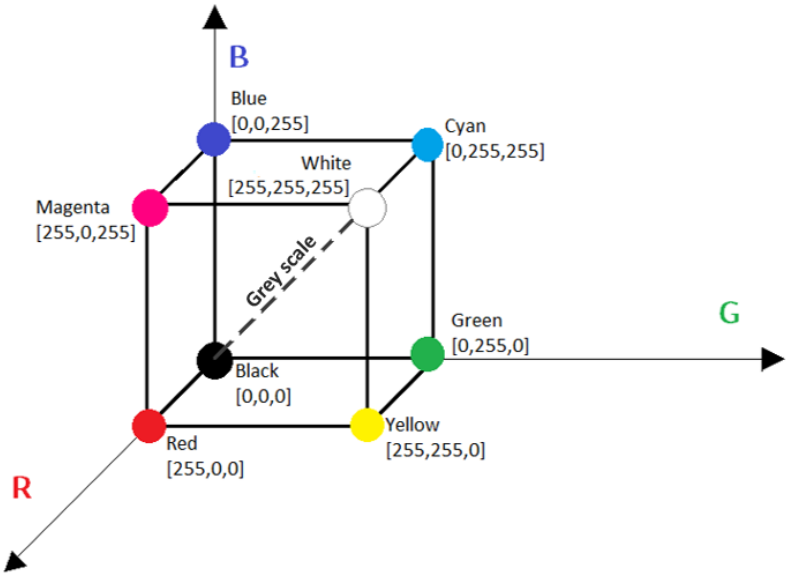

Figure 4. RGB space representation in a cube.

Each pixel has a value between 0 and 225 for each channel. RGB space could be represented as a cube, where each axis corresponds to a channel. In Figure 4, the cube could be seen. Depending on the value the pixel has in each axis (R, G or B) it would have one color or another. On the one hand, white color will correspond to [255,255,255]. On the other hand, black will correspond to [0,0,0]. Between both colors commented right now, a diagonal could be painted as could be seen in Figure 4, and it will correspond to the grey scale. [14]

After taking the image, a software will be needed. The software must have the possibility to treat images, and by the use of easy algorithm, the signals will be obtained.

1.3. Univariate analysis.

In this project, a univariate analysis was firstly used in order to achieve a regression. The regression analysis is used to explain certain variable, for example Y, depending on variable X, or depending on several variables X_1, X_2, ... X_k. For the first situation, it will be a univariate regression, and in the second case, a multivariate regression. In both cases, the model is linear and it is assumed that the dependency between Y and the variable X assumes the form 1 for the univariate analysis, and for 2 for the multivariate:[15]

$$(1)\quad Y = a + bX + error$$

$$(2)\quad Y = a + b_1X_1 + b_2X_2 + \ldots + b_kX_k + error$$

The error term is necessary to appear because every time an X is observed, not always Y value is going to be the same. For example, if X is the height of a person, and Y the weight, every time we

look at a stature, it will not always get the same weight in Y. The univariate model is useful for predicting linear relationships.[15]

Furthermore, in this project, by the use of Matlab, each image is itemized in three channels R, G and B. In addition, one channel is selected for each color (see part 4.1) and a signal transformation is carried out. Finally, it is developed a logarithmic relation between white signal (A_0) and standard signal (A) which will be represented in the Y axis.

$$(3) \quad log \frac{A_0}{A}$$

By representing Equation X in the Y axis in front of the concentration of each standard a linear regression is achieved. So finally, the linear regression for this method is the following:

$$(4) \quad log \frac{R_0}{R} = Cb + a$$

1.4. Multivariate analysis.

The multivariate analysis has its basis on the statistical principle of multivariate statistics, which takes into account more than one statistical variable at a time. In this type of analysis is taken into account the effects of every variable and offers the opportunity to observe the variable that most affects in the analysis.

In this project, it has been carried out a Partial Least Square-Regression (PLSR). This method is similar to PCR (Principal Component Regression) and it is possible to obtain the same prediction result. The difference is that it is based on smaller number of components. So instead of finding the maximum variance between the response and the independent variables its objective is to find a regression model. The regression is achieved by projecting the predicted variables and the observable ones into a new space. In other words, the aim is to find relations between two matrices X and Y. It will try to find in X space the multidimensional direction that will explain the maximum variance (again multidimensional) in the Y space direction.[16]

The difference is that it is based on smaller number of components. PLSR is being a successful technique not only in chemometrics but also in other fields. The PLSR models are similar to PCA (Principal Component Analysis) and PCR models. The difference is that PLSR is basically focused on Y and the most relevant Y information is expected to be in the first component. So that first component is going to give the most information possible of the model created.[17]

2. Objectives

The aim of this project is to develop a new method to determine Allure Red, Brilliant Blue and Tartrazine independently in solid samples by using digital image analysis. The mentioned image should be taken with a Smartphone in order to simplify the method. The purpose is to create a new fast method where inexpensive instrumentation is needed. Therefore, several aspects should be developed:

- Find out the best conditions for taking images and control them, such as brightness and shadows.
- Detect the factors that could interfere in the color intensity.
- Calculate statistical parameters in order to check the reliability of the method and the tests carried out.
- Obtain the figures of merit of the methods.
- Apply the method to real samples.

In this way, an attempt will be made to develop the mentioned method for the determination of food dyes in solid samples.

3. Experimental

3.1. Equipment and software.

For the digital image analysis, a mobile phone was used to obtain the images. The mobile phone is a Bq Aquaris X with a dual camera of 8MP for the frontal camera and 16MP for the one at the back.

Once the image was taken, they were sent to a computer, which was equipped with software for image treatment. For the treatment of the image, the computer was provided by Matlab program R2013b (The Mathworls, Inc.Natick, MA) with the Image Processing Toolbox tool™. It has to be said that before treating any image in Matlab, images have to be cut in this case with Microsoft Office Picture Manager. This way, the automatization with a Matlab code script was possible.

3.2. Standard preparation.

In this project, two different standard preparations have been carried out. On the one hand, jelly preparation with three different food coloring, Brilliant Blue, Allure Red and Tartrazine. On the other hand, candy preparation in order to get closer to possible real samples. As well as before, Brilliant Blue, Allure Red and Tartrazine were added.

3.2.1. Jelly standard preparation

To determine colorants in solid samples, firstly it was necessary to prepare 3 different stock solutions, one with Tartrazine, other with Brilliant Blue and a last one with Allure Red of 100 mg/L. Following with, standards from 1 mg/L to 12 mg/L would be prepared. In order to solidify standards, 8 g of Royal Jelly would be added for 250 mL of solution. Next, the mixture was heated and mixed up for 20 minutes. Finally, it was necessary to cool them down in an appropriate container during a whole night. As they are solid standards a change in concentration units must be done from mg/L to mg/kg.

3.2.1.1. Allure Red jelly standards

For Allure Red jelly standards, a 95 mg/L stock solution was done and 7 standards of different concentrations from 1 mg/L to 10 mg/L were prepared. Then, standards were solidified in the fridge overnight in each mold. It is important not to forget preparing a standard with no colorant

to use as blank. It has to be said, that the concentrations were changed to mg/kg since samples were solid. Finally, standards were prepared for the image taking procedure.

3.2.1.2. Brilliant Blue jelly standards:

Like for Allure Red, for Brilliant Blue other 7 standards of different concentrations were prepared from a stock solution of 100 mg/L. The standards were of 250 mL and have these concentrations from 1 mg/L to 11 mg/L. It is important not to forget to prepare the blank standards. After the solidification, the concentration units must be changed into mg/kg. Afterwards, the image taking procedure was done.

3.2.1.3. Tartrazine jelly standards

On the other hand, 7 Tartrazine jelly standards were prepared from a stock solution of 100 mg/L. Finally, 7 standards of 250 mL with concentrations from 1mg/L to 10mg/L were prepared from a stock solution. After adding 8 g of jelly, it is necessary to heat and mix the standards for 20 minutes, afterwards, they were cooled in order to solidify them. Therefore, the new concentrations were from 1 mg/kg to 11mg/kg. It is important not to forget the standard without colorant as the blank. Subsequently, the standards must be prepared for the image taking procedure.

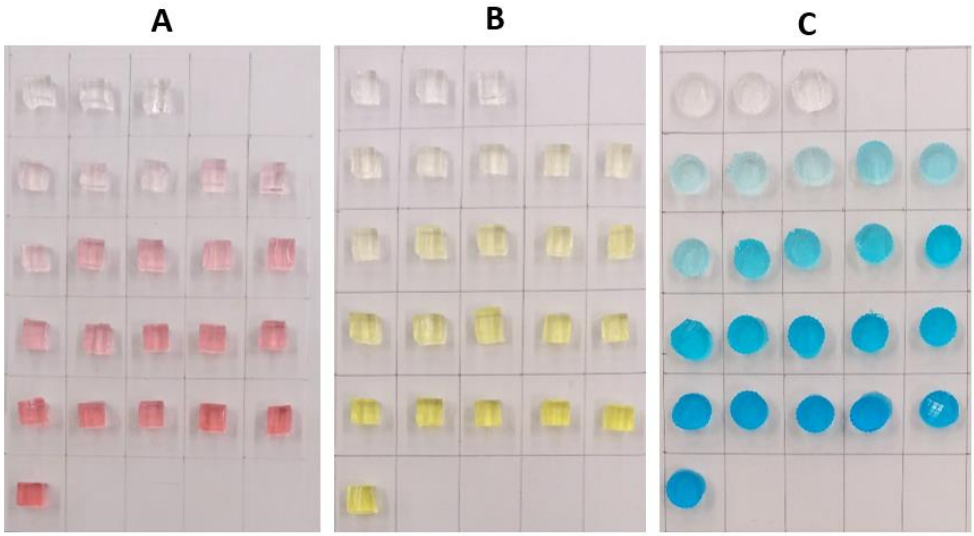

Figure 5. Jelly standards for each colorant. A Allure Red jelly standards. B correspond to Tartrazine jelly standards and C correspond to Brilliant Blue jellies.

Figure 5 represent each jelly standard with its correspondent colorant. Figure 5A corresponds to Allure Red jelly standards after carrying out the previously mentioned procedure. Figure 5B correspond to Tartrazine jelly standards and finally standards in 5C have Brilliant Blue.

3.2.2. Candy standard preparation

With the objective of getting closer to possible real samples, candy standards were prepared. Firstly, as in Jelly standard preparation stock solutions must be prepared as well as the standards. In this case, 2 different solutions (A and B) must be prepared for each standard. These are the content of each solution (see Table 5).

Table 5. A and B solutions' content for Candy standard preparation.

INGREDIENT	A	B
Standard solution	250 mL	-
Powder jelly	8 g	-
Sucrose	-	33g
Glucose syrup	-	31.5 g
Citric acid	-	1.5 g
Water	-	24.5 mL

As it can be seen in Table 5, A and B solutions were prepared. Firstly, A as well as in Jelly standard preparation. Secondly, for the B one, syrup, sucrose and water were mixed and heated. When everything in both solutions is dissolved, mixture must be mixed and heated up again. After 20 minutes, citric acid was added.[18]

Finally, after letting it cool in the fridge overnight, solid candy samples were obtained as it is possible to see in Figure 5.

Figure 6. Brilliant Blue candy standard after solidifying.

3.2.2.1. Allure Red candy standards

For the preparation of Allure Red candies, two different stock solutions were used, the first one of 95 mg/L and the second one of 10 mg/L. From here, 8 standards were done of 100 mL. Firstly, the A solution of Table 5 was prepared and mixed with the B one. After letting the standard cool for a night, they were prepared for the image taking procedure as could be seen in Figure 7A.

3.2.2.2. Brilliant Blue candy standards

Brilliant Blue candies were prepared using 115 mg/L stock solution for the preparation of 8 standards of 100 mL. After adding the ingredients of Table 5, and doing the necessary steps for the candy preparation and letting them cool in the fridge for a night, it is important to change the

standard concentrations from mg/L to mg/kg as now the standards are solid. The final concentrations of them are between 1 mg/kg to 9 mg/kg. (see Figure 6 and Figure 7C)

3.2.2.3. Tartrazine candy standards

For the Tartrazine standards, a stock solution of 240 mg/L was prepared and 8 different standards were done in 100 mL flasks. Then the 8 g of powder jelly was added and heated for 20 minutes as explained above in Table 5. At the same time, the other solution with sucrose, glucose syrup and citric acid was prepared. After mixing both and letting them cool in the fridge, 8 standards from 1 mg/kg to 8 mg/kg are prepared for taking the image. (see Figure 7B)

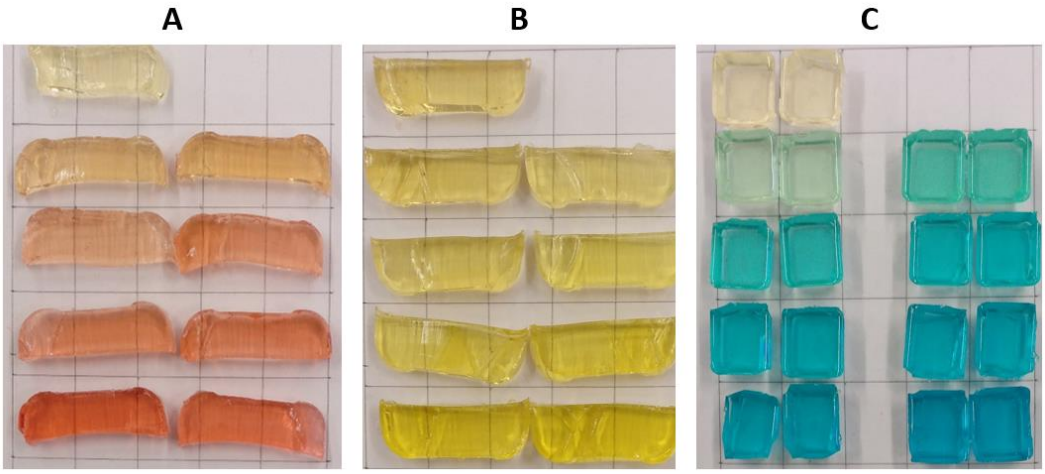

Figure 7. Candy standards. A correspond to Allure Red candy standards. B to Tartrazine candy standards and C to Brilliant blue candy standards.

According to Figure 7, three different groups of standards could be seen. This Figure represents the final result of the candy standards with its colorant. A represents the Allure Red candy standards. B correspond to Tartrazine candy standards and finally figure 7 C correspond to Brilliant Blue candy standards.

3.3. Image-taking procedure.

Illumination is a vital factor when taking digital images, as well as trying always to take the same photograph. Therefore, the Smartphone and the standards should always be in the same place in the laboratory. In this case, the standards were placed on a template, as can be seen in Figure 6, in order to have as much control as possible about random lighting changes around. However, according to Figure 6, brightness and shadows are visible in one of the standards principally. Unfortunately, in Figure 6 image imperfections can be better perceived. To solve this problem, different measurements are possible to take (some of them are explained in 3.3.1.).

Firstly, it is not advisable to take the photograph with the Smartphone's camera automatic settings. The ISO (the camera's capacity to capture light) has to be adjusted to 200, since in other previous studies good results have been obtained. [19]Secondly, it is advisable to take more than one image, in order to compensate possible errors that shadows and brightness can produce.

Figure 8. Standard positioning in the white template. Placed in groups of three marked in black, red, green, purple and yellow.

In addition, according to Figure 8, inside the marked black square, 3 blank standards are possible to be seen. These standards are useful to see the effect that light has on the ones with colorant, since afterwards its signal will be used in the calibration. Finally, adding a color pattern (see Figure 11) in each and every time a photo is taken, allows us to do an image control study before every determination.

3.3.1. Previous studies

As mentioned above, the lighting plays a crucial role in the determination of the dye. Therefore, controlling the factors that interfere with it was an important task. Several tests were made in order to get the best image with the lowest number of brightness and shadows and the highest possible quality. Firstly, with the aim of controlling the brightness, we used a box illuminated with indirect light. The box was white inside to reflect as much light as possible and not obtain very dark images. In the Figure 9 A, B and C, it is possible to see how the brightness and shadows disappeared completely compared to Figure 9D, E and F. In addition, the standards were cooled in different volume molds, in order to see the possible effects in the determination. Besides, differences could be perceived depending on whether the standard was inside or outside the mold and between them. (see Appendix I).

Therefore, a study of the possible interfering factors was done (see Appendix I). These are the possible factors to interfere in the color intensity determination:

- Box with indirect light.
- Different mold.
- Standard inside or outside the mold.
- Differences in standard volume.

To conclude and after seeing the results of Appendix I, as a conclusion, the images must be taken outside the box and molds, since better results are obtained and it is easier preparation is carried.

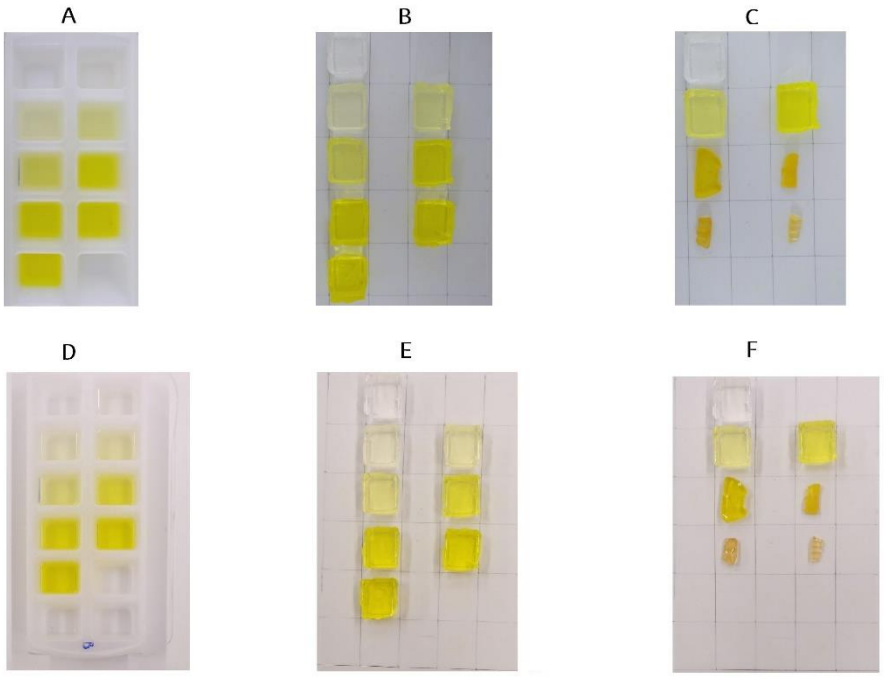

Figure 9. Differences in lighting, brightness and shadows when taking the images. A is an image taken with the standards inside the mold and box. In image B standards are outside the mold but inside the box. C, there are the standards outside the mold and 4 real samples, everything inside the box. Image D is taken outside the box but standards are inside of the mold. E, standards are outside the mold and outside the box. Like in E, F standards are outside the box and mold and some real samples appear.

Unfortunately, brightness and shadows are not the only factors affecting the image taking procedure and of course the coloring determination. The depth of the standard affects directly in the determination of the correspondent color. According to Figure 10, you can see 7 standards with different concentrations of Allure Red (Figure 10 A, B and C) or Brilliant Blue (Figure 10 D, E and F). The three images of each color correspond to equal standards, but there is only one difference, the depth of them. The standards are cut in different depths, A and D 0.5 cm; B and E 1.0 cm and C and F 1.5 cm. The intensity of the color changes according to the depth of the sample, even having the same coloring concentration, as can be seen in Figure 7.

3.3.2. Study of depth optimization

The study of depth in the jellies could be explained with the Lambert-Beer law. The absorbance is related to the concentration of the substance, (c) by the law of Lambert-Beer, which is summarized with the equation: $A = \varepsilon\, b\, c$, where c is expressed in mol / L; b is the length of the optical path (width of the cell containing the solution of the substance) and is expressed in cm; and ε is the molar absorptivity, property characteristic of each substance corresponding to the amount of radiation absorbed at a given wavelength per unit of concentration, being its units L mol^{-1} cm^{-1} (note that the absorbance has no units). In this project, the absorbance would be the intensity of the color, and therefore the depth of the sample (b in the equation) to determine would directly affect the concentration according to Lambert-Beer law.

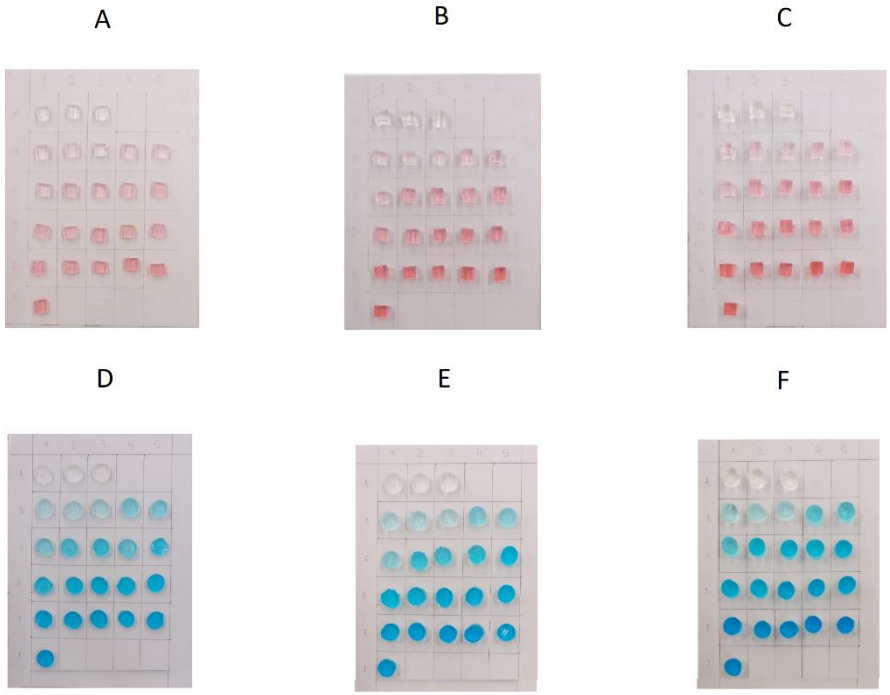

Figure 10. Standards with different depths. From A to C Allure Red standards of same concentrations. From D to F Brilliant Blue standards of same concentrations. It is possible to appreciate the difference in color intensity when the standard has higher depth. A and D 0.5 cm; B and E 1cm, C and F 1.5 cm.

Therefore, after having observed that the depth of the standards and samples were directly related to their concentration, it was decided to make a study to optimize the depth. In this study, the aim was to find a depth that would work to determine the three food colors and thus standardize the method. Three different depths were studied, 0.5, 1.0 and 1.5 cm, and differences in the calibration curves were examined (Appendix II).

3.3.2.1. Allure Red

In the study carried out for the Allure Red dye, it could be seen that the minimum depth of the standard had to be 1.0 cm and maximum 1.5 cm so that in the determination of the concentration there were no significant differences as can be seen in the Table 6. To reach to that conclusion, student *t* test and *Fischer* test were performed (see Appendix II).

Table 6. Allure Red's significance *t* tests in the depth for the determination of the coloring concentration.

Depth	Slope	Typical error of regression
0.5 cm-1.0 cm	Significant dif.	Significant dif.
1.0 cm-1.5 cm	NO significant dif.	NO significant dif.
0.5 cm-1.5 cm	Significant dif.	Significant dif.

Table 6 as previously mentioned, significant differences appear, what means that a calibration curve performed by standards of 0.5 cm for example could not be used for synthetic or real samples of 1 cm of depth.

3.3.2.2. Brilliant Blue

Although, applying the study to the Brilliant Blue dye, it was found that the depth margin was greater than in the Allure Red. According to Table 7, it is possible to be seen how the range would go from 0.5 cm to 1.5 cm. In Appendix II *student t* and *Fischer* test were carried out.

Table 7. Brilliant Blue's significance *t* tests in the depth for the determination of the coloring concentration.

Depth	Slope	Typical error of regression
0.5 cm-1.0 cm	NO significant dif.	NO significant dif.
1.0 cm-1.5 cm	NO significant dif.	NO significant dif.
0.5 cm-1.5 cm	Significant dif.	Significant dif.

Even so, it is not recommended to obtain a calibration curve with 0.5 cm-depth standards with those of 1.5 cm since there are significant differences between them and the calibration will probably be erroneous.

3.3.2.3. Tartrazine

Finally, for the Tartrazine dye, the measurements had to be modified, since significant differences were observed between the previous anterior depths. Therefore, the depths that were taken into account were the following (see Table 8); 0.5 cm, 0.80 cm, 1.0 cm and 1.50 cm. Considering this

information, it was seen that the minimum depth would be 0.80 cm and the maximum 1.0 cm (see Appendix II). Therefore, there were no significant differences in the calibration curve (see Table 8).

Table 8. Tartrazine's significance *t* test in the depth for the determination of the coloring concentration.

Depth	Slope	Typical error of regression
0.5 cm-0.80 cm	Significant dif.	Significant dif.
0.80 cm-1.0 cm	NO significant dif.	NO significant dif.
1.0 cm-1.5 cm	Significant dif.	Significant dif.

As a conclusion, it was decided that 1.0 cm would be the depth that the standards should have in order to take the digital image correctly as one centimeter depth would be valid for the three dyes analyzed in this project.

3.4. Image control.

In the determination of colorants or any other substance in digital image analysis, the image control becomes a vital factor. Furthermore, taking an incorrect photo will become our analysis useless, and it will necessarily be repeated. For that reason, there are some factors or actions that will provide the assurance of having obtained a correct photo, hence a correct analysis and determination. For that reason, two steps have to be followed: Color pattern and Quality control method.

3.4.1. Color pattern

The color pattern is a piece of Foamy (Ethylene Vinyl Acetate) of ten different colors as could be seen in Figure 11. This color pattern will provide a filter by which some images, the one which are not adequate for determination, will be discarded. The pattern will provide us a light control, by applying it into each and every image (see Figure 11).

The pattern must be applied to every digital image, due to even though the image is always taken in the same place, factors exist which could not be controlled, such as, laboratory illumination or even the effect the weather could have. Those factors could directly affect in the color intensity and for that reason the color pattern would definitely help to discard undesirable images.

Analyzing the color pattern an image can differ significantly from the rest. Since the lighting of a photo is different, this will be reflected in the measurements of the colors of the pattern. Some limits are established considering the mean and standard deviation of each color patch in the pattern (see Figure 11). An image outside the limits will be discarded.[19]

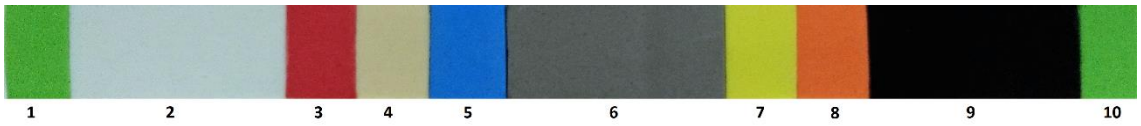

Figure 11. Control color pattern and number assignation to each one. 1: Green_A; 2: White; 3: Red; 4: Beige; 5: Blue; 6: Grey; 7: Yellow; 8: Orange; 9: Black; 10: Green_B

In this work, it has been carried out and image control study. As in any analytical control system, it is possible to develop different charts, in here, Shewhart charts are carried out and applied. 15 different images were chosen to develop the studies. Those images were taken to determine the three food colorants, in different days and seasons in order to obtain a graphic that englobes any possible lighting situation. Therefore, it will be necessary for each image to have the color pattern and that the standards meet the conditions mentioned in the previous paragraph. In this way, as can be seen in next figure, the pattern will be added to them, in order to verify that they are suitable for the determination.

Figure 12. Allure Red candy standards with the color pattern.

Finally, the result will be Figure 12. It is possible to observe 8 different candy standards with different Allure Red concentration. In addition, the blank standard is also there and of course the previously mentioned color pattern in order to control if the image is correct in order to develop the determination method.

3.4.2. Shewhart control chart

After having chosen the 15 photographs, 10 different Shewhart charts where done. One for each color patch of the control color pattern. In this way, the images used for the different determinations were previously tested in these charts in order to verify they were useful.

To plot the graphs, the RGB average of each color of the pattern are calculated. With the RGB average, the central line (5 equation) of the graphics is represented in a black discontinuous line in Figure 13. Once the average is calculated, the standard deviation (s) was calculated from the 15 images. The standard deviation is not only used to develop the warning line (6 equation) , represented in discontinuous green lines, but also, the action lines (7.equation) discontinuous red lines, as it is possible to see in next figure [20]

If an image is located between both warning lines, there will not be any risk with the illumination if that image is used for the determination. However, if the image is located between the warning and action lines, those images needed to be checked out. If the same happens in the other nine Shewhart control charts it is preferable not to use it as it possibly has a lightning problem. In addition, if the image is outside the action limits, that photograph will immediately be discarded and it will not be used for determination due to the evidential light problem.[20]

$$(5)\quad Central\ line\ (CL) = \frac{\sum_{i=1}^{15} RGB\ average_i}{15}$$

$$(6)\quad Warning\ lines = CL \pm 2s$$

$$(7)\quad Action\ lines = CL\ \pm 3s$$

For example, according to Figure 13 it is possible to see that there are some points located outside the action lines or between the warning and action lines. Those images were automatically discarded, as a possible illumination problem was evident according to the 10 different graphics. So, as explained above (section 3.3. image taking procedure), more than one image is recommended to take, so that new photograph will again be check out in Shewhart control charts and if the problem continues on, a new photograph must be taken with more control on the laboratory illumination.

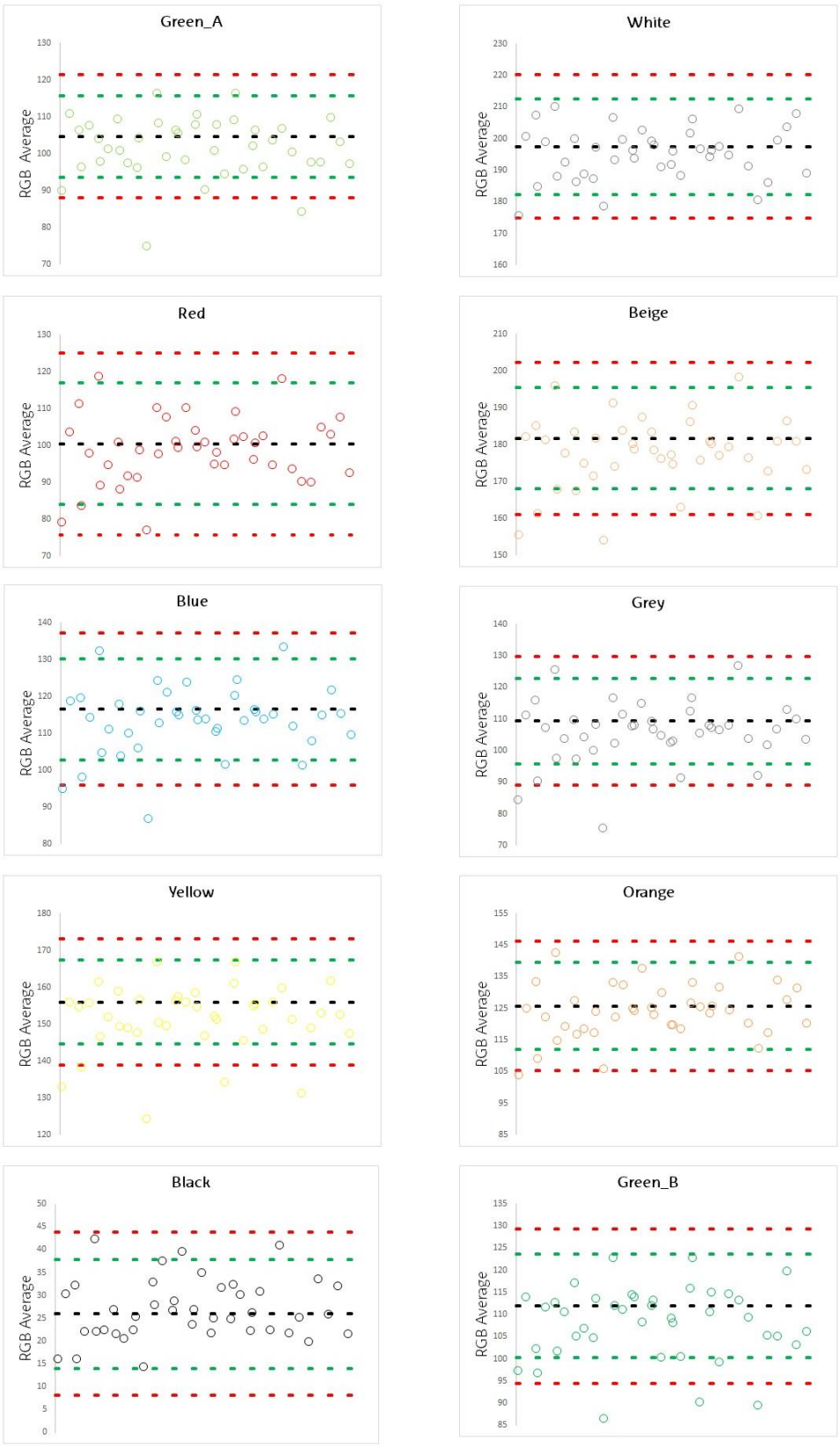

Figure 13. Shewhart control charts. Ten different control charts, one for each color of the color pattern.

4. Results and discussion

Before starting to explain the obtained results, it is important to remember that two different standards have been developed. The first standards are the jelly ones, which only have gelatin, water and colorant in the matrix. The second type of standards are called candy standards; these ones have a more complex matrix (see Table 5). In Figure 14 could be seen both standards, A correspond to Jelly Brilliant Blue standards and B correspond to Allure Red candy standards. Difference can be noticed in the blank standard in both pictures (the first line in both).

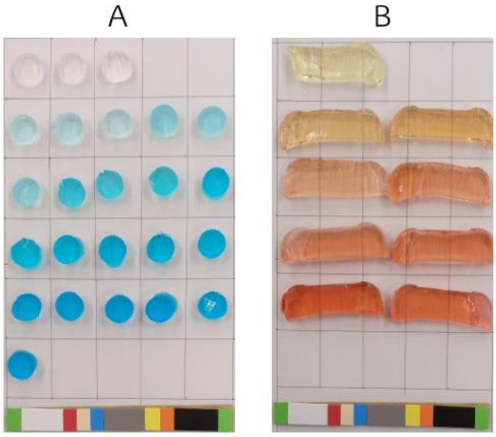

Figure 14. Representation of the two different standards. A correspond to Jelly standards in this case for Brilliant Blue. B correspond to candy standards in this case for Allure Red.

So two different calibration curves have been performed for each colorant, one for jelly standards and another one for candy standards.

4.1. Univariate analysis for the determination of Allure Red, Brilliant Blue and Tartrazine in both Jelly and Candy standards

For the determination of food coloring in a univariate analysis, the RGB system was used. As previously mentioned, RGB system is composed by three different channels, Red, Green and Blue. In each case, depending on the examined color, a different signal will be achieved in each channel and hence; the calibration curve will not always be achieved in the same one. For the calibration curve, the logarithm of each channel's signal is represented in front of the concentration (mg/kg).

4.1.1. Results of the determination of food coloring in Jelly based standards

4.1.1.1. Determination of Allure Red

For the determination on each food coloring, the same procedure was carried out when obtaining the calibration curve. Firstly, in order to obtain a proportional relation between signal and coloring concentration, it is important to relate two different signals in a logarithm. The logarithm would correlate the blank and standard signal continuously and it will be represented against concentration, so a proportional relation would be obtained.

According to Figure 15 three different regressions can be seen, in red color appear the Red channel of the RGB space, in green color G channel (green) and finally in blue the last channel of the RGB space the blue channel. Each one of them was carried out with Allure Red Jelly standards following the instructions of 3.2. section. Each calibration corresponds to a channel of the RGB model. To decide the most accurate for the determination, the most important factor is the R^2. In which R channel is discarded due to its R^2 value is worse compared to G or even B. For that reason, to determinate Allure Red Green channel is selected.

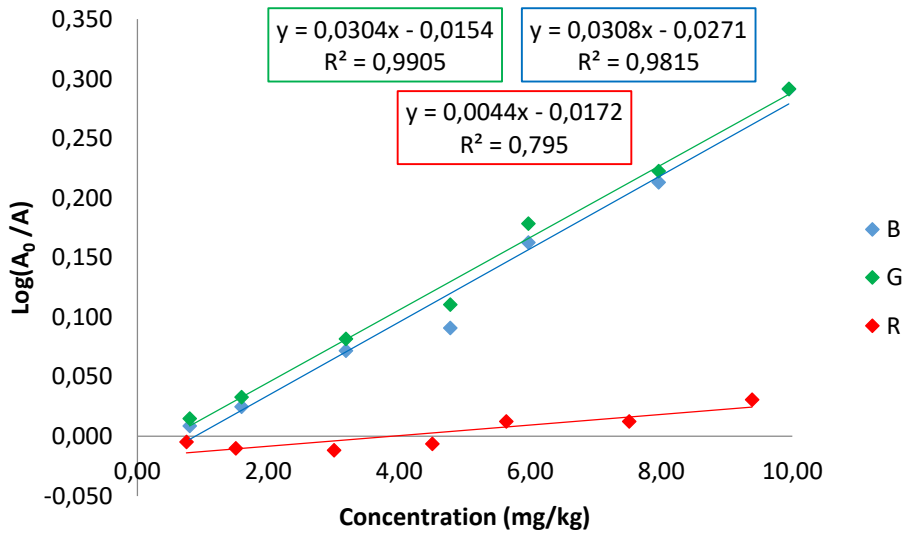

Figure 15. Representation of the three canals of RGB system for Allure Red

After considering the correlation coefficient, G channel is the one chosen for the determination as its R^2 is the best one, the one with less errors in the regression. Figure 16 represents the chosen calibration curve. It should be said that G_0 represents blank signal and that together with G (standard signal) are related in the logarithm represented in the Y axis. To finish with the calibration, the logarithm is represented against standard concentration in mg/kg in the X axis.

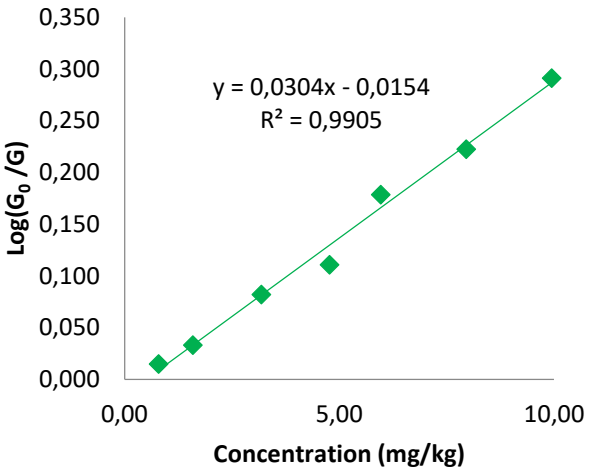

Figure 16. G channel's representation for Allure Red jelly standards. The logarithm correlates the white signal and the standard signal for G channel in front of different concentrations of jelly standards.

Taking into account the regression (see Figure 14) it could be said that linearity is favorable as the correlation coefficient is higher than 0.9. Nevertheless, the fourth point with a concentration of 5 mg/kg could have been deleted as it is far from the linearity, but finally it was decided to let it there. Taking into account the regression curve, limit of detection and quantification were calculated. Both limits were calculated by Equation 8 and 9 in where, $S_{y/x}$ corresponds to the typical error of the regression and b_1 to the slope of the regression. The detection and quantification limits are of 1.2 mg/kg and 3.5 mg/kg respectively.

$$(8)\quad LOD = \frac{3.3 \cdot S_{y/x}}{b_1}$$

$$(9)\quad LOQ = 3 \cdot LOD$$

For the measurement of the repeatability 6 images where chosen, all of them taken in the same day. It was verified that they were useful for determination by Shewhart charts. As it was difficult to perform, the method with real samples it was calculated the %RSD value for each standard and its logarithmic signal.

According to Table 9, standards are represented as S1, S2, S3, S4, S5, S6 and S7. In addition, each standard has its concentration and the RSD% of the chosen RGB channel signal's logarithmic relation. As could be seen in Table 9, when taking into account standards with low concentration of Allure Red, the %RSD value is quite high and leads to errors. In this case for standards 1,2 and 3 the values are higher than %10 showing a lack of accuracy in the method.

Table 9. LOD, LOQ and Repeatability values for Allure Red Jelly standards.

LOD mg/kg	LOQ mg/kg	Standards	S1	S2	S3	S4	S5	S6	S7
1.2	3.5	Concentration mg/kg	0.8	1.6	3.2	4.8	5.9	7.9	9.9
		Repeatability %RSD	35.2	27.4	12.5	9.1	3.2	5.6	7.1

In addition, the concentration is one factor that affects directly in the color intensity, nevertheless, shadows and brightness affect directly in the digital image and not only that, both are factors of high difficulty to control. Furthermore, standards with low concentration of food coloring will be more affected by brightness and shadows than the ones with higher concentrations. This could be possibly, why the %RSD values are higher for the first three standards.

4.1.1.2. Determination of Brilliant Blue

The same procedure was carried out to develop the calibration curves in Figure 17 for Brilliant Blue. As previously mentioned, the RGB model has three different canals, so three different calibration curves could be obtained from one image. Figure 17 demonstrated that the calibration curve with the highest intensity is not always the most adequate. Taking into account the R^2 value, for Brilliant Blue the regression with less linearity and with best proportionality is the one in G channel. However, there is only one possible to be used, G channel curve, as its R^2 value is the best one compared to the others, even though the high intensity of Red canal.

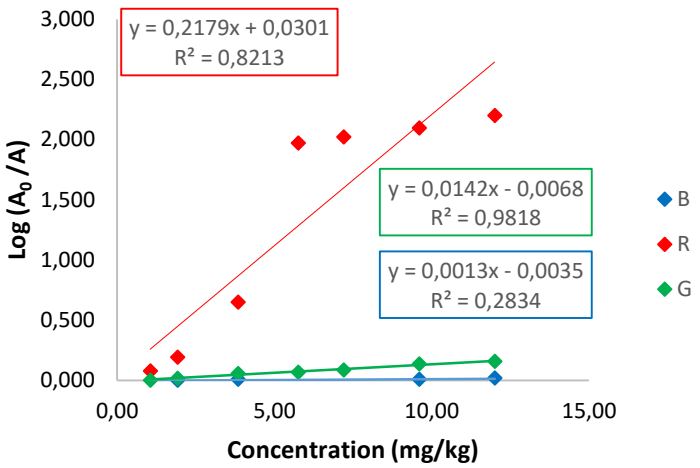

Figure 17. Representation of RGB system for Brilliant Blue Jelly standards according to its concentrations. There are three calibration curves, one for each channel. Each channel is represented with its color.

According to Figure 18 the linear relationship between the logarithmic relation and the concentration in mg/kg is possible to see. The logarithm relates white standards signal and the ones with food coloring; each logarithm is represented against each standard concentration in mg/kg. The obtained correlation coefficient is adequate, with a value of 0.9818, for digital image analysis. However, the values for detection and quantification limits (calculated by Equation 8 and 9) are of 2 mg/kg and 6 mg/kg respectively.

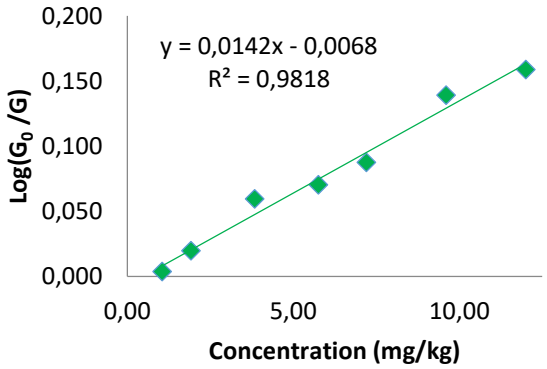

Figure 18. Calibration curve for Brilliant Blue Jelly standards in G channel of RGB system. Y axis correspond to the logarithmic relation of G channel's blank signal and the standard signal.

According to Table 10, standards are represented as S1, S2, S3, S4, S5, S6 and S7. In addition, each standard has its concentration and the RSD% of the chosen RGB channel signal's logarithmic relation. As done for Allure Red, repeatability was measured with every standard and taking into account 5 images taken in the same day. Results could be seen in Table 10. According to table, the %RSD values for repeatability in the logarithmic signal are quite high for standards 1 and 7 with values of %27.5 and %18.9 respectively.

Table 10. LOD, LOQ and Repeatability values for Brilliant Blue jelly standards.

LOD mg/kg	LOQ mg/kg	Standards	S1	S2	S3	S4	S5	S6	S7
2	6	Concentration mg/kg	1.1	1.9	3.8	5.8	7.2	9.6	12.0
		Repeat. %RSD	27.5	9.4	7.1	6.7	5.2	2.8	18.9

The value for the first standard could be possible as it has very low concentration not reaching of 1 mg/kg of Brilliant Blue, therefore the intensity of the color could have not been well detected in the image. However, the contrary happens for the 7th standard. It has the highest concentration so the signal is started to be saturated and the %RSD value is higher for that reason.

4.1.1.3. Determination of Tartrazine

For the determination of Tartrazine, as well as with Allure Red and Brilliant Blue, three different calibration curves are obtained (see Figure 19). As previously mentioned with Allure Red and Brilliant Blue each calibration curve corresponds to a channel of the RGB model. Taking into account Figure 19, in Red color appear the Red channel of the RGB space, in green color G channel (green) and finally in blue the last channel of the RGB space the blue channel. In this case, the calibration chosen for the determination is the B one. Its intensity and the good correlation coefficient enables the choice.

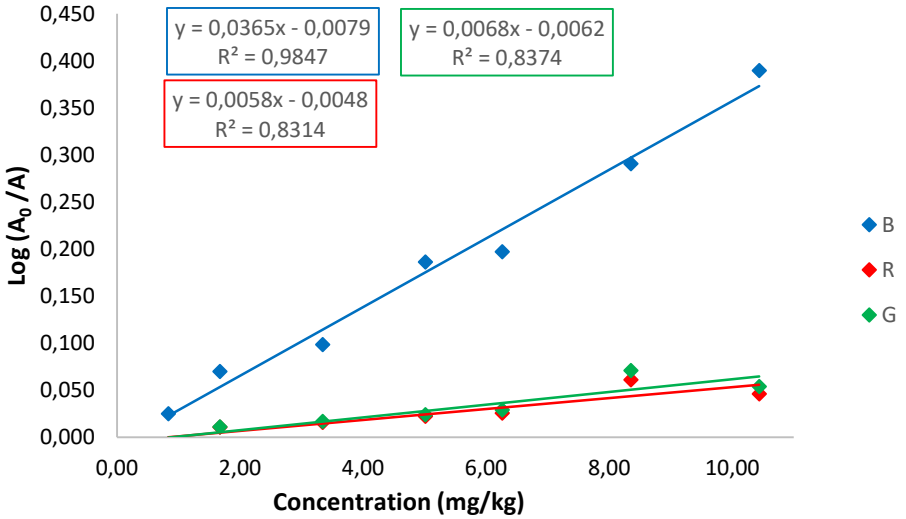

Figure 19. Representation of RGB system for Tartrazine Jelly standards according to its concentrations. Each channel R, G or B has its own calibration curve. For Tartrazine the best one is B due to its R^2 value and the signal intensity.

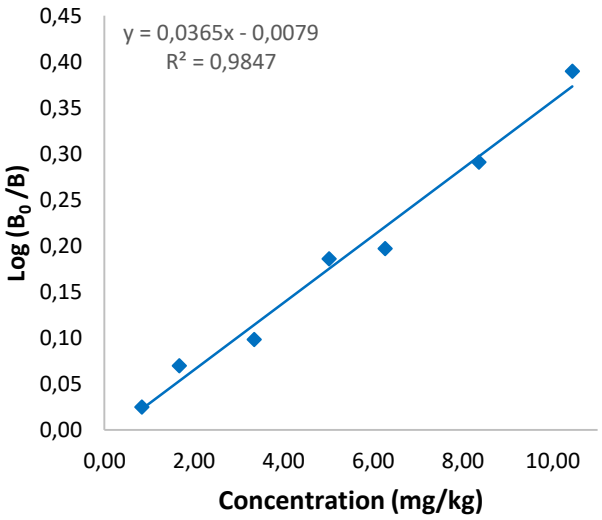

Figure 20. Calibration curve for Tartrazine Jelly standards in the B channel of RGB system. The logarithm relates white standards signal with the food coloring ones. Each signal is represented against its standards concentration in mg/kg.

In Figure 20, the chosen B regression can be seen. Taking into account its correlation coefficient of 0.9847 could be said that the linearity is adequate but it could be better as it is not higher than 0.99. In addition, this regression for Tartrazine has the limit of detection value of 1.6 mg/kg and the quantification limit value of 4.7 mg/kg. The reproducibility was measured with seven images taken in different moths of the year.

According to Table 11, standards are represented as S1, S2, S3, S4, S5, S6 and S7. In addition, each standard has its concentration and the RSD% of the chosen RGB channel signal's logarithmic relation. Nevertheless, as with the other food colorings, it was difficult to measure real samples, for that reason %RSD value was calculated for each standard. It was taken into account the logarithmic signal for its calculation. Furthermore, six images were taken into account to develop the repeatability as could be seen in Table 11.

Table 11. LOD, LOQ and Repeatability values for Tartrazine candy standards.

LOD mg/kg	LOQ mg/kg	Standards	S1	S2	S3	S4	S5	S6	S7
1.6	4.7	Concentration mg/kg	0.8	1.7	3.3	5.0	6.3	8.4	10.4
		Repeat. %RSD	9.3	8.4	1.6	14.2	5.2	1.9	3.4

In this case, compared to Brilliant Blue and Allure Red, the results are better. The highest value is %14.2, it could be due to shadows and brightness. As explained before with Allure Red, both factors interact directly in the image an affect in the color intensity.

4.1.2. Results of the determination of food coloring in Candy based standards

4.1.2.1. Determination of Allure Red

Taking into account Figure 21, in Red color appear the Red channel of the RGB space, in green color G channel (green) and finally in blue the last channel of the RGB space the blue channel. Even though B calibration curve has the highest intensity, G is the one to be used for the colorant determination due to its R^2 value. Nevertheless, it is obvious that R is not going to provide useful information. This happens because it is the same color as the one to be determined.

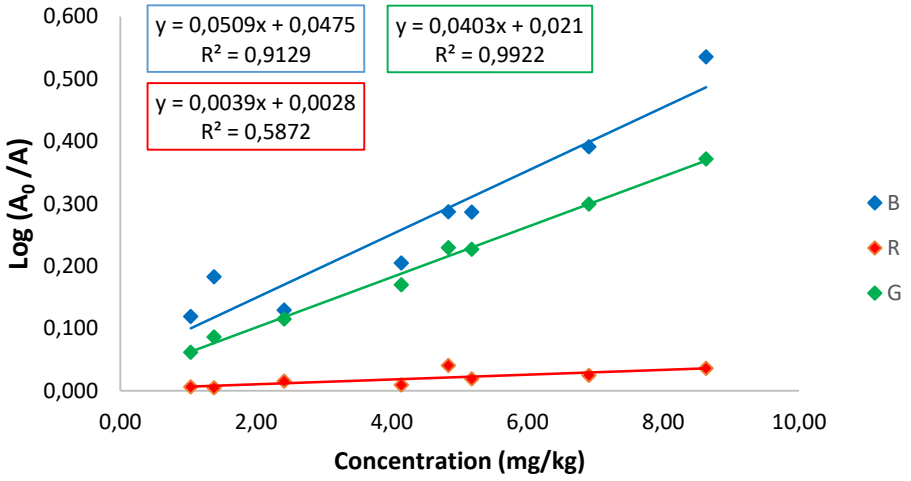

Figure 21. Representation of three calibration curves of Allure Red standards. All of them developed with the same standard image, but the signal is achieved in different canals of the RGB system.

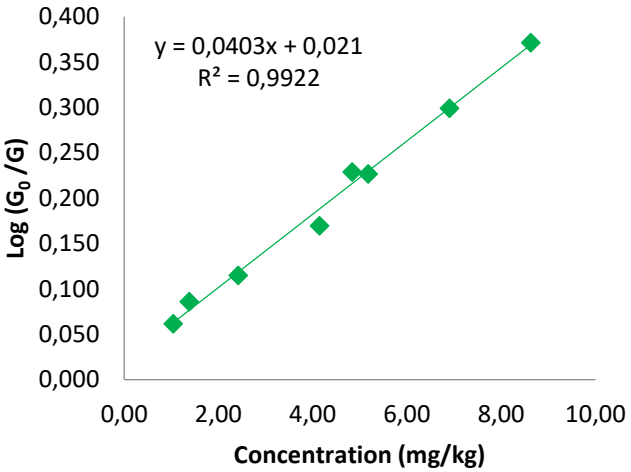

Figure 22. Representation of G channel of the RGB model for Allure Red Candy standards. G0 represents the white signal in G channel and G represents the standard signal in G channel. The logarithm that relates the blank and standard is represented against concentration in mg/kg.

Looking at Figure 22, it is possible to appreciate the linearity with the correlation coefficient of 0.9922. In this case, for candy Allure Red standards, it could be said that the linearity is adequate as the value is higher than 0.99. The limits of detection and quantification (calculated with Equation 8 and 9) in this case are 0.84 mg/kg and 2.5 mg/kg respectively (see Table 12).

For repeatability (see Table 12), it was calculated the %RSD value of the standards logarithmic signal used to develop the calibration as done with Jelly standards. For the calculation 7 images were taken into account all of them of the same day as done previously. In addition, if a comparison is done between Table 12 and Table 9 it must be said that results are quite better.

Table 12. LOD, LOQ and Repeatability values for Allure Red candy standards.

LOD mg/kg	LOQ mg/kg	Standards	S1	S2	S3	S4	S5	S6	S7	S8
0.84	2.5	Concentration mg/kg	1.2	1.7	2.9	4.9	5.8	6.2	8.3	10.4
		Repeat. %RSD	8.3	12.0	3.2	1.9	3.4	4.2	2.5	1.9

There is only one value that is higher than %10, 2nd standard exactly. The improvement could be the result of taking into account more points of the standard (Appendix III), in order to obtain a more homogeneous signal in the calibration curve.

4.1.2.2. Determination of Brilliant Blue

Brilliant Blue candy standard determination was carried out in the same way. Firstly, three different calibrations were developed (see Figure 23) one for each channel. In this case as happens with Jelly standards (with Brilliant Blue food coloring) the calibration chosen is the one of G channel even though its low intensity the correlation coefficient is the best one and for that reason must be chosen.

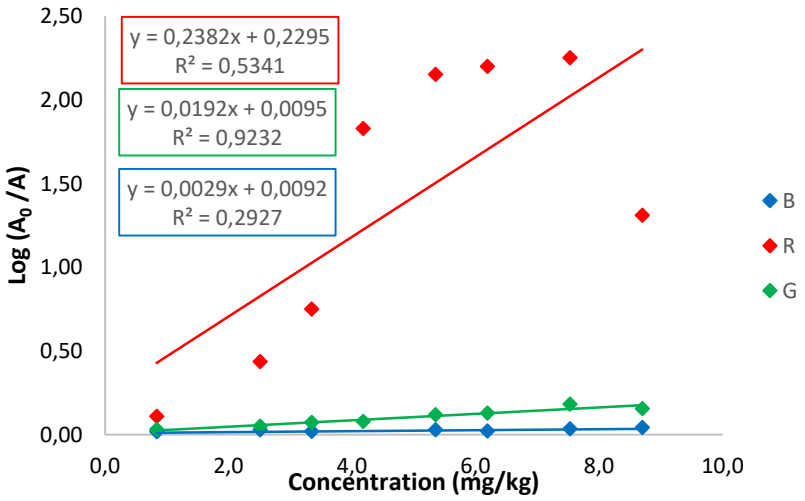

Figure 23. Different calibration curves performed with Brilliant Blue Candy standards. As there are three channels R, G and B three calibrations are obtained. However, for Brilliant Blue, G regression is the only one usefull due to its R^2 value. Each regression curve is represented in the color of the correspondent channel of the RGB space system.

Taking into account the proportionality in the regression curve (see Figure 24) some factors must be highlighted. First, its linearity, the correlation coefficient is 0.9232, lower than with Allure Red, but even it is below 0.99 it is quite adequate. The limit of detection is 1.8 mg/kg and the quantification limit, 5.3 mg/kg.

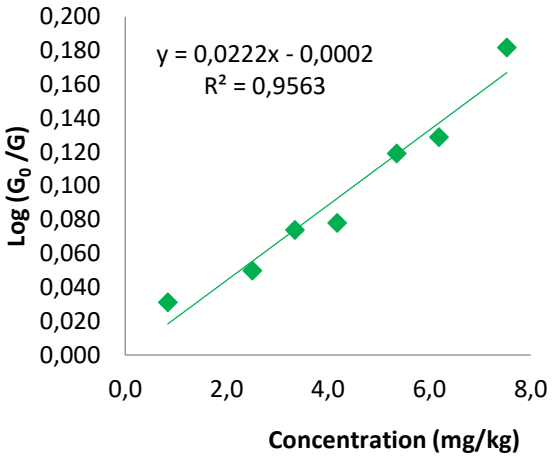

Figure 24. Representation of G channel of the RGB model for Brilliant Blue Candy standards. G0 represents the white signal in G canal and G represents the standard signal in G channel. The logarithm that relates the white with standards signal is represented against concentration in mg/kg.

According to Table 13 repeatability was measured by %RSD value. For the development of it 8 images were taken into account as previously said each one of them correspond to the same day. Surprisingly, for Brilliant Blue candy standards the results are very favorable. Each %RSD value is below the 4%.

Table 13. LOD, LOQ and Repeatability values for Brilliant Blue candy standards.

LOD mg/kg	LOQ mg/kg	Standards	S1	S2	S3	S4	S5	S6	S7	S8
1.8	5.3	Concentration mg/kg	0.8	2.5	3.3	4.2	5.3	6.1	7.5	8.7
		Repeat. %RSD	3.5	3.7	2.5	3.2	1.0	2.2	1.4	2.5

In this case, it does not exist evidence of saturation or even shadows or brightness. As a conclusion, quite good results were obtained, especially if they are compared to the ones in Table 10.

4.1.2.3. Determination of Tartrazine

For the determination of Tartrazine food coloring, the chosen calibration was the B channel one. Taking into account Figure 25, in Red color appear the Red channel of the RGB space, in green color G channel (green) and finally in blue the last channel of the RGB space the blue channel. As done with Allure Red and Brilliant Blue the correlation coefficient and the intensity are taken into account and the B channel has the best numbers (see Figure 25).

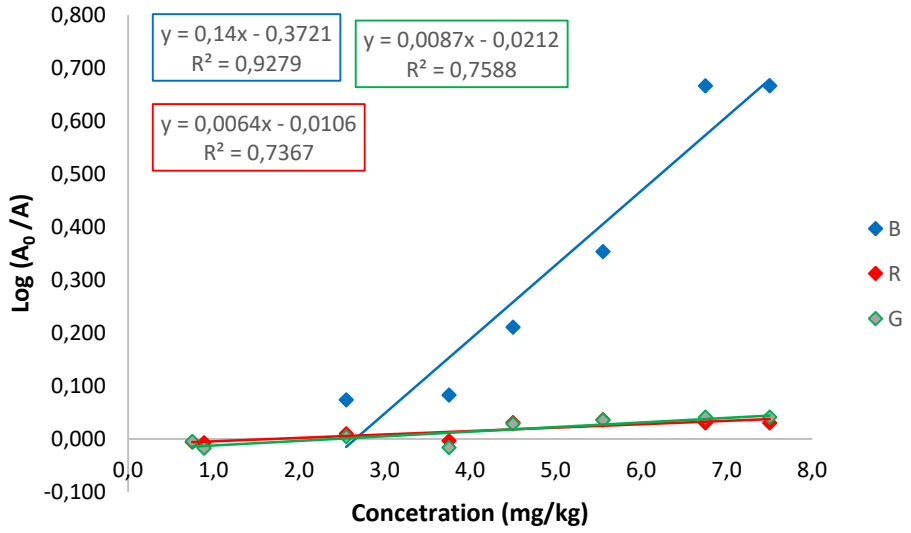

Figure 25. Three different calibration curves performed with candy standards of Tartrazine, each curve represents each canal of the RGB model. B regression will be the optimum one to determine Tartrazine due to its R^2 value and the intensity of the signal.

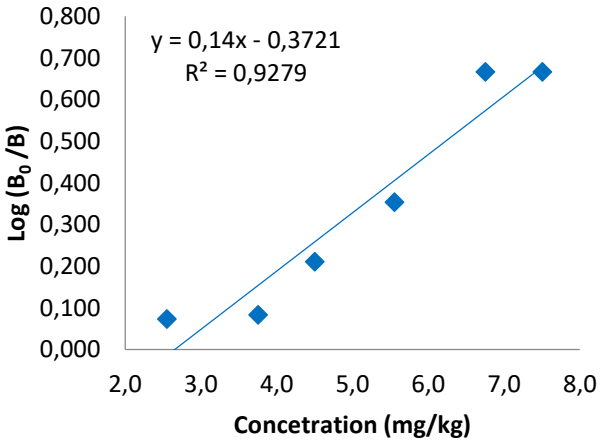

Figure 26. B canal's representation of the calibration curve developed for Tartrazine Candy standards. B_0 represents the White signal and B represents the standard signal. Both of them related in a logarithm are represented against each standard concentration in mg/kg.

According to Figure 26 the regression curve of channel B, the correlation coefficient is 0.9279; higher than 0.99 so it could be said that the linearity of it is correct. However, compared to the Tartrazine Jelly Standards B calibration curve, it is worse. The determination in candy standards was tougher due to the difficulty to differentiate the white standards to those with low concentration of Tartrazine. The ingredients in Table 5 give yellow color to the standards so the white ones are not transparent enough to be determined perfectly and may interfere in the determination compared to the Jelly standards.

The calibration (see Figure 26) has a limit of detection of 1.9 mg/kg and a limit of quantification of 5.8 mg/kg. The repeatability in Table 14 was carried out with 6 images taken in the same day. As previously mentioned, it was not possible to perform it with real samples so the %RSD value was done for each standard's logarithmic signal.

Table 14. LOD, LOQ and Repeatability values for Tartrazine candy standards.

LOD mg/kg	LOQ mg/kg	Standards	S1	S2	S3	S4	S5	S6	S7
1.9	5.8	Concentration mg/kg	0.9	2.6	3.7	4.5	5.5	6.8	7.5
		Repeatibility %RSD	5.1	15.7	18.2	12.2	8.7	3.9	5.3

According to Table 14 there are only two values higher than %10 the 2nd and 3rd Tartrazine candy standards. Nevertheless, compared to Tartrazine jelly candy repeatability (see Table 11) the result have improved enormously to a positive direction.

4.1.3. Discussion

The aim of performing also candy standards was to get closer to real samples and try to determine them. Nevertheless, Appendix III shows an Interference study. This study was developed as there was the aim of using either Jelly standard regression or Candy standard regression for the determination of food colorings in candies. Unfortunately, as commented in Appendix III, Interferences exist so it was inviable determining the three colorants with jelly standards.

However, further tests were developed in order to see how each variable in the regression affect to it. Multivariate analysis gave the opportunity to create different PLS models and obtain information of how the created model adjusts to the reference one. In order to see if the model is the correct one or in near to be, the slope of this new regression must be 1 or be close to it.

4.2. Multivariate analysis for the determination of Allure Red, Brilliant Blue and Tartrazine in both Jelly and Candy standards.

As previously mentioned, it was decided to perform an interference study (Appendix III) in order to prove there were significant differences between jelly and candy calibrations. In addition, another alternative was to perform multivariate analysis with the aim of getting a Partial least model (PLS) model in order to determine synthetic samples. As well as in univariate analysis firstly Jelly standard calibrations (in this case PLS model) was developed and after the candy ones. Furthermore, the PLS models were validated with standards which were not taken into account when developing the model and several data was achieved, such as; RMSEC (Root Mean Square Error of Calibration), RMSEP (Root Mean Square Error of Prediction) or RMESCV (Root Mean Square Error of Cross Validation), in order to compare them with the univariate study.

4.2.1. PLS results for Jelly standards

4.2.1.1. Allure Red

In order to develop the PLS model, there were not only taken into account the logarithmic signal of the channel with better R^2 value and intensity. In this case, the logarithmic relation of every channel; raw signals of R, B and G channel and the concentrations were considered. Seven variables in total. The objective was to obtain a robust model in were every variable was considerate.

Figure 27 is the representation of the achieved PLS model in where four different representations are shown. Above in the left side the representation of the score values is shown. According to that representation, it could be said that the standards are not classified in groups. On the other hand, taking into account the representation of the loading values, it could be seen that G and B channels are the ones with most effect in the model.

Furthermore, according to explained variance representation, in this occasion, Factor 2 demonstrated a better accuracy and by which 98.7% of the variance was explained. Finally, in predicted vs. reference representation is possible to observe two different calibrations. The one in red corresponds to the standards used for the calibrations and the one in blue the standards discarded from the calibration which were applied for validation (cross validation).

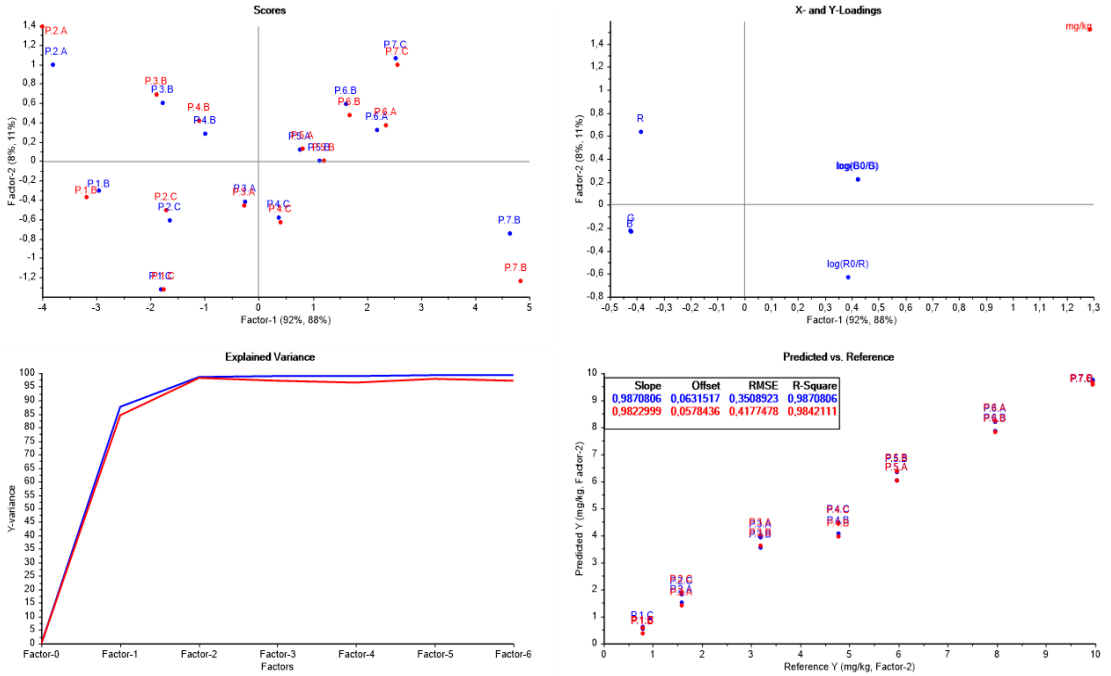

Figure 27. Different graphs obtained in the development of Allure Red jelly standards PLS model. Above in the left side, scores graph. Above in the right side, loadings graph. Below on the left side, the explained variance and in the right side Predicted vs reference representation. In red color the calibration curve is represented. In blue color, the standards discarded and recalculated for validation.

Nevertheless, if standard 4 is eliminated surely a better result would have been obtained. When developing this kind of graph, it is desired to obtain a slope value of 1 or near to it. In this case, the slope is 0.98 for both validated and calculated. Therefore, it could be said that favorable results have been obtained.

4.2.1.2. Brilliant Blue

Brilliant Blue PLS model is very similar to the one obtained for Allure Red (see Figure 27). The results achieved are shown in Table 16. However, according to Figure 28, the representation of predicted vs. reference; slope value is 0.94 near 1 but it is not as accurate as in Allure Red, due to standards 2, 3 and 7. As obtaining a good slope was difficult 3 factors were needed but they are not enough.

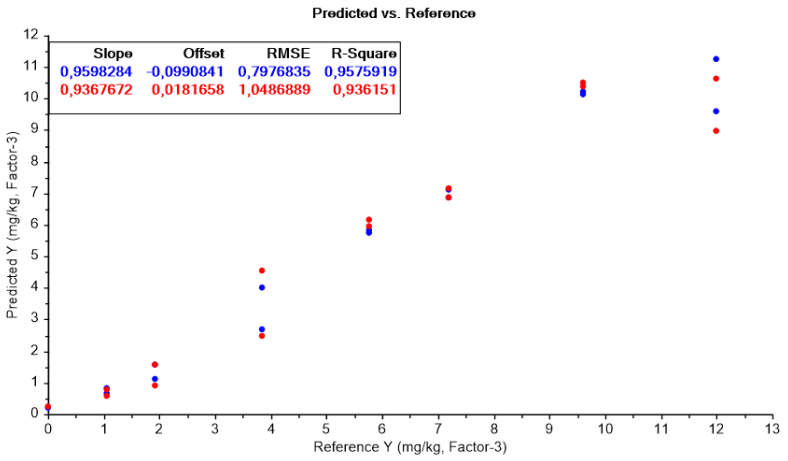

Figure 28. Predicted vs Reference representation of the model PLS developed for Brilliant Blue in Jelly standards.

Comparing it with Allure Red Jelly standard PLS it is obvious that results are quite worse. There are some standards to be possibly eliminated, such as the last ones as it affects directly. If Table 10 is taken into consideration and compared to Figure 28, the same standards with high %RSD value are the ones that do not overlap in here.

4.2.1.3. Tartrazine

However, for Tartrazine Jelly standards, the results are more desirable compared to the ones in Allure Red and Brilliant Blue (see Table16). The obtained slope value is very close to 1 so they are very positive because the value is 0.99.

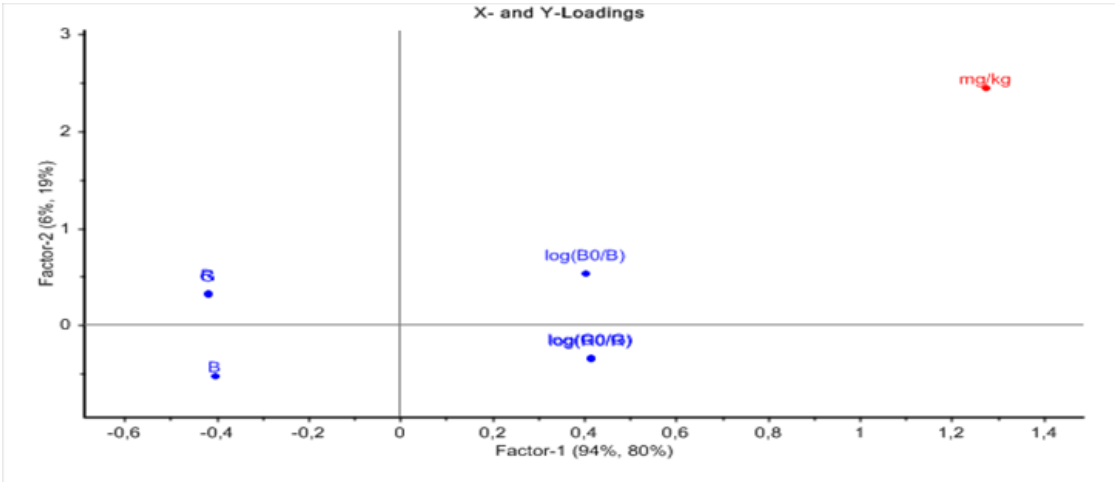

Figure 29. Loading value representation of the developed PLS model for Tartrazine in Jelly standards.

Comparing Tartrazine jelly standards PLS model's loading values representation (see Figure 29) to the one obtained for Allure Red jelly standards PLS model's it could be seen that are similar. Taking into account Figure 29, R and G channels have the same effect in the model but in this case B has an independent model. According to the univariate study, the study for Tartrazine B channel was chosen and this representation could explain the election done.

It has to be said that this model reinforce the results obtained in the univariate regression for Tartrazine Jelly standards and adjust perfectly well to data in Table 11. As the results of the obtained %RSD values are quite low and the best one compared with the other colorants and in Table 16 the obtained slope value again is the best one compared with Allure Red and Brilliant Blue.

4.2.2. PLS results for Candy standards

4.2.2.1. Allure Red

According to candy standards, a multivariate study was carried out as previously done for jelly standards. The objective was again to obtain a model in which more variables were taken into account in order to obtain a more robust model of calibration. In Figure 30, the scores value representation could be seen, as well as the loadings value, explained variance and predicted vs. reference representation.

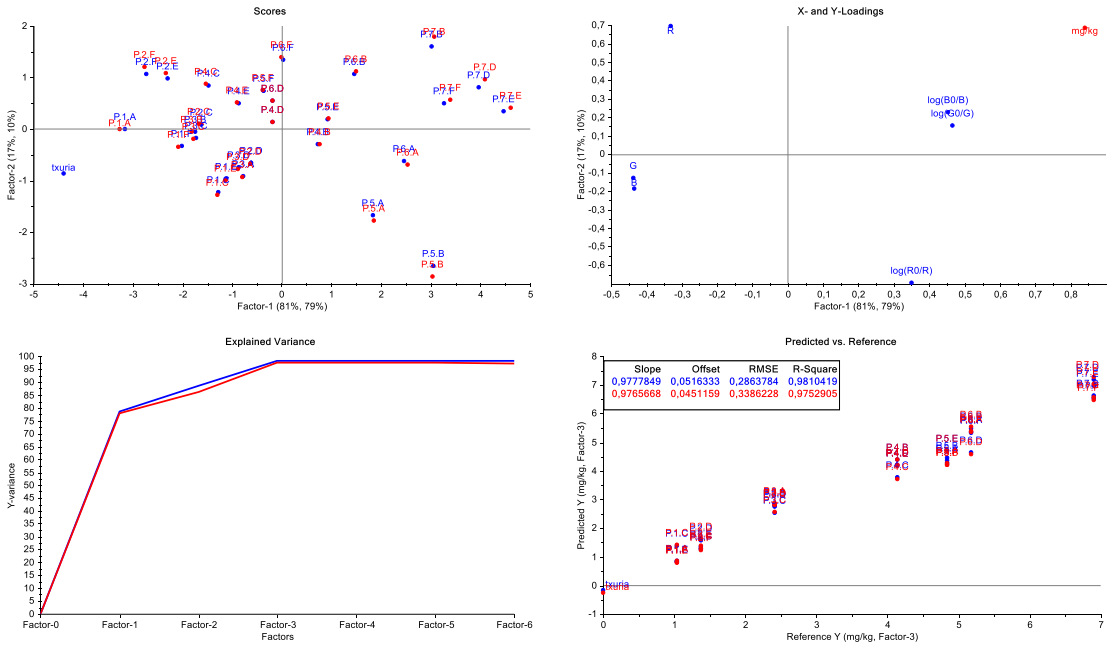

Figure 30. Different graphs obtained in the development of Allure Red jelly standards PLS model. Above in the left side, scores graph. Above in the right side, loadings graph. Below on the left side, the explained variance and in the right side Predicted vs reference representation. In red color the calibration curve is represented. In blue color, the standards discarded and recalculated for validation.

Furthermore, scores representation in Figure 30 show, no groups so at the beginning could be said that there are no differences between the standards used to develop the calibration.

According to the representation of Predicted vs. References the pursue of this representation is to obtain a slope value of 1 or a number which is very close to it. In this case the value corresponds to 0.97, which is favorable for digital image analysis. However, in order to obtain those values, some standards were absolutely discarded as they used to give problems. For that reason, the model was developed with only seven standards.

Nevertheless, according to the representation of explained variance, it could be seen why three factors were used to develop the model. The explained variance representation, provides information of how many factors provide the mayor information, in this case three which represents 98.15% of the information of the model.

4.2.2.2. Brilliant Blue

According to Brilliant Blue candy standards, generally the results obtained are quite worse if they are compared to the ones obtained for Allure Red candy standards. Taking into account Figure 31, no groups are observable, so it could be said that there are no differences between the standards. .

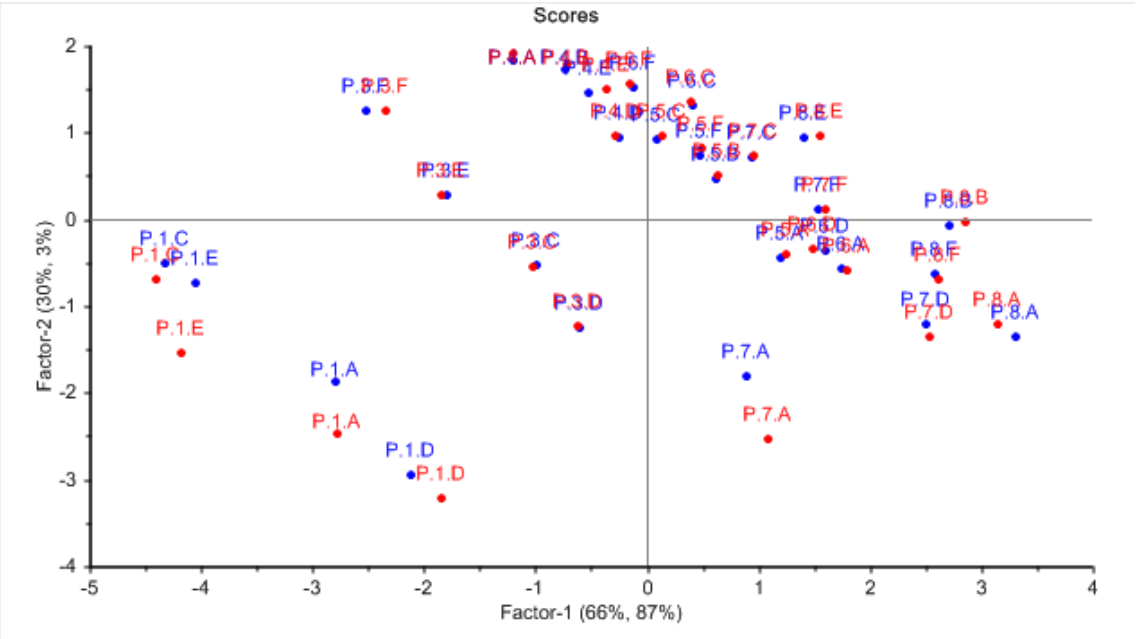

Figure 31. Predicted vs Reference representation of the developed model of PLS for Brilliant Blue candy standards.

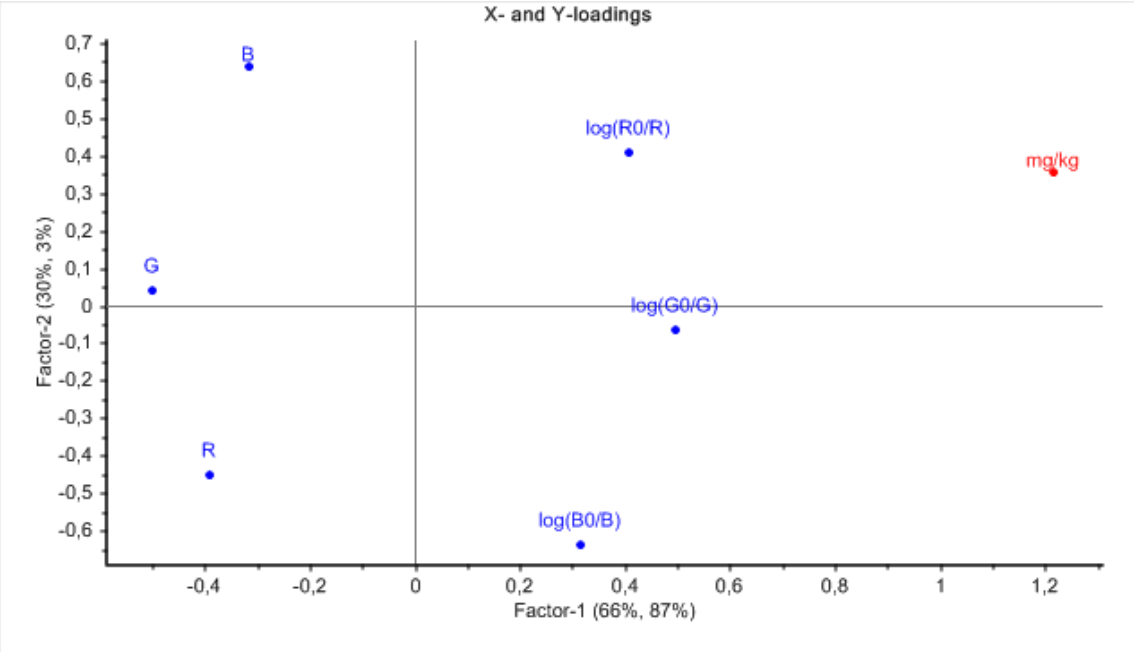

Figure 32. Representation of the loading value of the Brilliant Blue candy standards PLS model.

However, taking into account which variables affect most in the model, (see Figure 31). According to the figure, the Loading value representation helps to know which variables affect the most in the model wanted to be developed. It could be seen that channel G and the logarithmic relation again from channel G affect the most or maybe that the channel which has more changes is the Green one. Therefore, the decision taken for the univariate analysis of taking into account G channel for the calibration may be the correct one. Compared this figure to Figure 29 for example which corresponds to Tartrazine colorant, differences exist. In this case, each channel has different effects in the model.

4.2.2.3. Tartrazine

In addition, according to Tartrazine candy standards, it could be seen that the obtained slope in Figure 33 is a bit worse compared to the PLS model developed for Jelly standards in Figure 29. In this case, the value corresponds to 0.97, which continues to be favorable for digital image analysis as it is higher than 0.9.

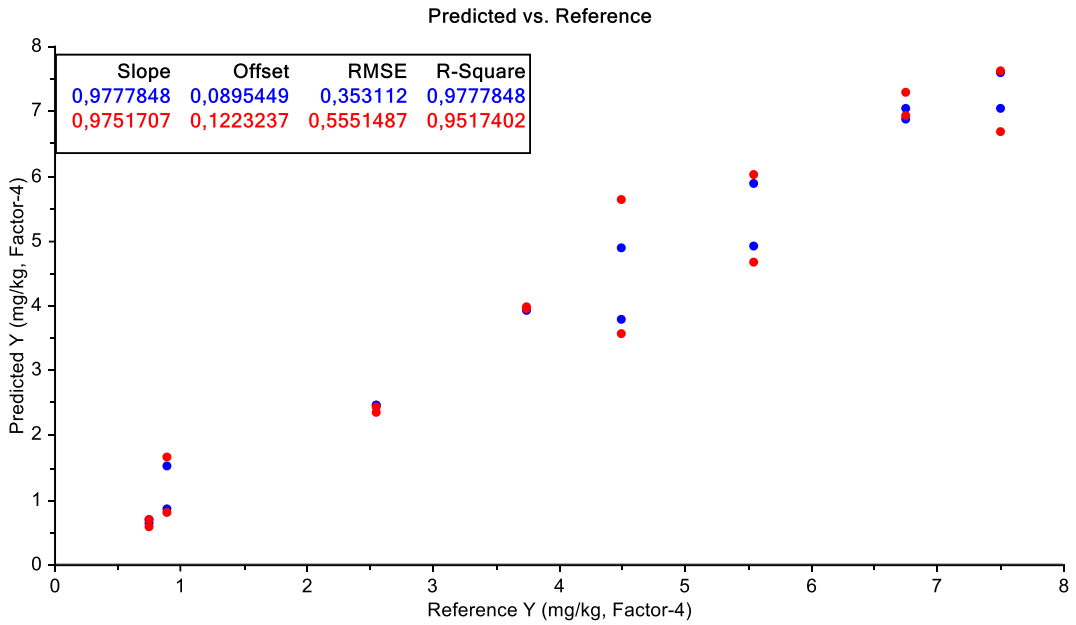

Figure 33. Predicted vs Reference representation of the PLS model developed for Tartrazine Candy standard

However, in this case there were needed 4 factors in order to explain the most information possible for this model, 93.68% So compared to the other candy standards and also the jelly standards PLS models this data in a bit higher, which becomes it worse. Not only that, in Figure 33, some represented standards appear with a bit of dispersion what indicates that the model is nor the best one.

4.3. Discussion

To conclude, it has to be said that as Table 15 shows, the achieved results are very similar in both cases, Jelly standards and candy standards when univariate calibration is used. The channel chosen for each colorant is maintained and the linearity is very similar. However, it is important to underline that in general, better results about linearity are obtained in jelly standards. Nevertheless, as the analysis was developed with digital image analysis having a correlation coefficient higher than 0.9 is always adequate.

Table 15. Summary table which represents the results achieved in univariate analysis.

	Univariate Analysis					
	Jelly standards			Candy standards		
	Allure Red	Brilliant Blue	Tartrazine	Allure Red	Brilliant Blue	Tartrazine
RGB Channel	G	G	B	G	G	B
Linearity	0.9905	0.9818	0.9847	0.9922	0.9232	0.9279
Repeatability %RSD*	3.2	5.2	5.2	3.4	1.0	8.7
LOD	1.2	2.0	1.6	0.8	1.8	1.9
LOQ	3.5	6.0	4.7	2.5	5.3	5.8

*For the Repeatability, in this table the value that appears correspond to the 5th standard of each colorant and standard type.

In addition, taking into account the %RSD values of repeatability, in this table appears the %RSD value correspondent to the 5th standard from Table 9 to Table 14 as it has a medium concentration in each regression. Having said that, candy standard's values for Brilliant Blue colorant show a huge improvement. However, for Allure Red and Tartrazine worse results are obtained. Finally, similar results are obtained for LOD and LOQ values in both types of standards.

On the other hand, according to the multivariate analysis several PLS models were carried out. In addition, after building the model, they were validated with standards which were not taken into account when developing the PLS model. In Table 16, a summary table with the results obtained from the multivariate analysis is shown. For both jelly standards and candy standards in each colorant, the slope value of the reference vs predicted plot, RMSEC (Root Mean Square Error of calibration), REcal (Relative Error of the calibration), RMSEP (Root Mean Square Error of Prediction), REval (Relative Error of validation) and RMSECV (Root Mean Square Error of Cross Validation) were calculated.

According to Table 16, relative error of calibrations (REcal) obtained were between 1.92 and 7.59 % for jelly standards and 3.44 and 6.34% for candy standards. In both cases the lowest value was

obtained for Allure Red and the highest for Tartrazine. Nevertheless, all values were below 10% showing good accuracy for calibration in the model.

In addition, the relative error of validations (REval) obtained were 3.52% for Allure Red, 3.56% Brilliant Blue and 10.94% for Tartrazine jelly standards and 9.49, 2.72 and 9.34% for candy standards respectively. The error is higher again for Allure Red. However, it continues to be below 10% which demonstrated good accuracy for the developed PLS model. Nevertheless, as explained with REcal, the relative errors for candy standards are higher as some ingredients as the syrup has a direct effect in the color.

According to the RMSEC value, it could be said that better results are obtained for Brilliant Blue in candy standards, however, taking into account Allure Red and Tartrazine better results are achieved for jelly standards. Furthermore, according to the relative error of the calibration, better results are obtained generally for both cases (jelly and candy standards) in multivariate study compared to the univariate one. Taking into account Table 15, could be seen that in univariate study, only taking into account the logarithmic signal, the %RSD values are good. In addition, when developing the PLS model and trying to obtain a robust model in where the logarithmic relations of each channel and the signal of every single channel with no changes are taken into account, better results are obtained. Firstly, for jelly standards, an improvement was obtained for Allure Red and Brilliant Blue. Secondly, for candy standards, the improvement happens only for Tartrazine standards.

Table 16. Summary table of the results obtained in multivariate analysis. RMSEC, RMSEO, RMSECV, REcal (Relative error of the calibration) and REval (Relative error of the validation).

	Multivariate Analysis					
	Jelly standards			Candy standards		
	Allure Red	Brilliant Blue	Tartrazine	Allure Red	Brilliant Blue	Tartrazine
Slope value	0.98	0.94	0.99	0.97	0.94	0.97
RMSEC	0.35	0.82	1.46	0.70	0.52	2.09
REcal	1.92	3.73	7.59	3.44	2.56	6.34
RMSEP	0.46	0.56	1.48	1.64	0.54	1.51
REval	3.52	3.56	10.94	9.49	2.72	9.34
RMSECV	0.42	1.05	0.43	0.34	0.64	0.56

Following with the RMSEP and REval values, which explains the validation error and the relative error of it, desirable data was obtained. It has to be said that only in Allure Red candy standards are obtained worse results compared to Allure Red jelly standards. To conclude with, similar data was obtained generally, due to the similarity in the models as they take into account the same

variables. In addition, the multivariate model provides a more robust model in where is preferable to determine these three colorants compared to the univariate model.

Multivariate analysis has advantages compared to the univariate model. In this case, the multivariate model takes into consideration more variables, which are not taken into account in the univariate model. So it as previously mentioned, a more robust model could be obtained.

5. Conclusions

First, as a general conclusion, it has been possible to perform calibration curves that are sufficiently linear to determine the dyes, whether they are solid samples of gelatin or jellybeans. Even so, it has been seen that depending on the sample we want to determine the calibration standards should be consistent with the sample since it is not possible to use a regression made with gelatin standards to determine dye in candy since there are proven interferences.

Second, taking into account the image control procedure, it has to be said that many interfering factors were controlled. From the illumination to the depth or thickness. The optimum depth was found, 1 cm.

Not only that, to prove the integrity or reliability of the method, statistical procedures in order to find significant differences were carried out. Firstly, to detect and control the factors that could interfere in the image taking procedure. Secondly, to prove the interferences when using a Jelly calibration curve or candy calibration curve in order to determine dyes.

Unfortunately, it was not possible to perform the method with real samples, the method was validated, however, due to the lack of time it was not possible to develop the most important factor in the validation of a method, accuracy. For example, no recovery studies neither alternative method was developed. However, it has to be said that even the method was proved with real samples it would be impossible to know with absolute certainty the real concentration of samples.

5. Ondorioak

Lehendabizi, ondorio nagusitzat, lan honetan azkenik linealtasun bat mantentzen duten kalibrazioak lortu dira koloratzaileak determinatzeko, bai gelatina patroiekin eta gomazko gozokiekin. Gainera, determinatu nahi den laginaren arabera, ondorioztatu da kalibrazio kurba bat edo beste erabili beharko dela. Hau da, gelatina laginentzako gelatina patroiekin burututako kalibrazioa eta gomazko gozokien kasurako gomazko gozokien patroiekin garatutakoa.

Bigarrenez, argazkiaren kontrol prozedura kontutan hartuz gero, esan beharra dago bertan interferitzen zuten faktore askoren kontrola lortu dela. Iluminaziotik hasita patroiaren sakontasunera. Gainera, sakontasun optimoa lortu da; hau da, 1 cm.

Ez hori bakarrik, metodoaren fidagarritasuna konprobatzeko helburuarekin hainbat froga estatistiko erabili dira abiarazitako prozeduren artean diferentzia adierazgarriak zeuden ikusteko. Lehendabizi, argazkia hartzerako momentuan interferitu zezaketen faktoreak detektatzeko eta kontrolatzeko. Bigarren, interferentzien azterketa egiteko gelatina kalibrazioetan eta gomazko gozokia kalibrazioetan koloratzailea determinatzeko momentuan.

Zoritxarrez, ezinezkoa izan da metodoa lagin errealetara eramatea. Hala ere, metodoa balidatu da, baina denbora falta dela eta ez da balidazioaren atal garrantzitsu bat jorratu; zehaztasuna. Ez dira berreskurapen testik egin ezta balidazio metodo alternatibo bat martxan jarri ere. Hala ere, lagin errealekin lorturiko kontzentrazio horiek ezingo lirateke esan berez laginak dituenak diren. Metodo honek ezin baitu hori ongi ziurtatu.

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Appendix I: Study of factors that interfere in image taking procedure.

Volume study

It was decided to make a study on the impact of the volume of the standards since different molds were available (whose volumes were different). These molds were used to solidify the standards. In this way, enable the analysis of the possible impact on the slope and R^2 of the different regressions. Therefore, compare the concentrations achieved from real samples.

In this study, it is intended to determine Tartrazine in solid samples. Therefore, solutions with different concentrations of Tartrazine will be prepared and they will turn into gelatin as to be solid. Therefore, this study is carried out with jelly standards. In the solidification, 3 different molds (called A, B and C) will be used. Each one with a specific volume as it is possible to see in Table 17.

Table 17. Molds for the solidification of standards and its correspondent volumes.

MOLD	VOLUME (mL)
A	3.5
B	22.0
C	12.0

Then it will be developed a calibration curve for each mold. Therefore, there will be a calibration for mold A, another for B and finally another for C. In the development of these calibrations, other factors were taken into account, such as the place where the photo was taken (inside or outside of a box, which had indirect light) or if the standards were inside or outside the corresponding mold. In Figure 34 the applied molds could be seen.

- Pattern positioning:
    - Inside the box (indirect light).
    - Outside the box.
- Mold:
    - A
        - Inside the mold.
        - Outside the mold.
    - B
        - Inside the mold.

- Outside the mold.

- C
  - Inside the mold.
  - Outside the mold.

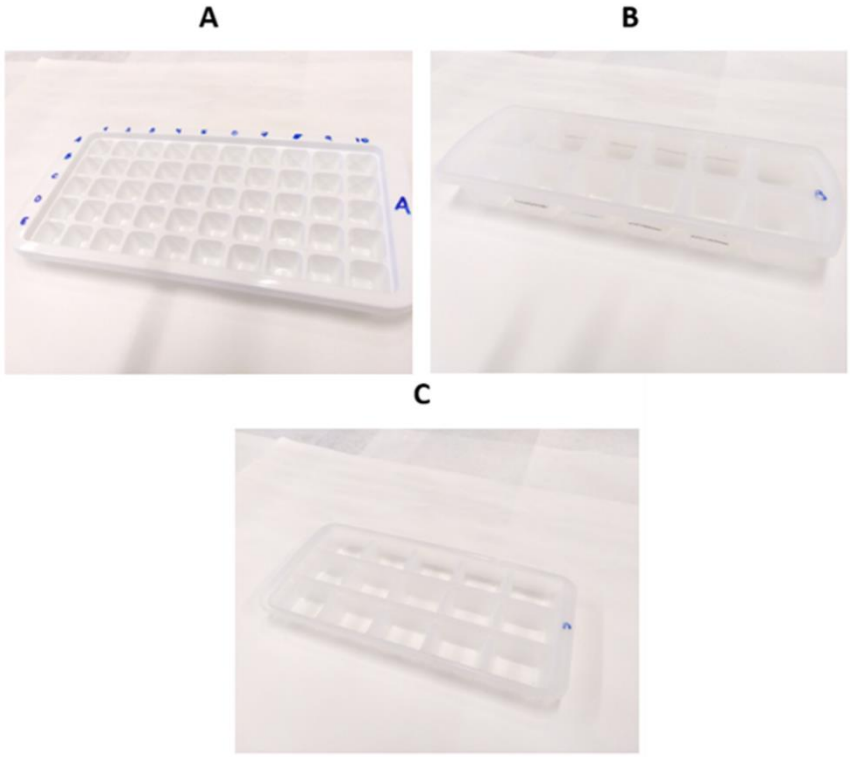

Figure 34. Three different molds applied in the study of factors that interfere in the image taking procedure. A correspond to A mold, B to mold B and finally C to mold C.

It must be taken into account that the concentration of real samples is obtained only from a calibration type, which is the ones obtained from standards that are outside its molds. The other calibration curves will be used to compare them with these ones mentioned in order to ensure they are correct.

Table 18. Comparison of slope and R^2 values outside and inside the box.

Mold		INSIDE THE BOX		OUTSIDE THE BOX	
		Slope	R^2	Slope	R^2
A	Inside	0.1092	0.9528	-	-
	Outside	0.0757	0.9932	-	-
B	Inside	0.1134	0.7843	0.1070	0.9932
	Outside	0.1144	0.9071	0.1311	0.9987
C	Inside	0.1137	0.9956	0.0824	0.9975
	Outside	0.1005	0.9970	0.0442	0.9790

As for Table 18, it is possible to compare and contrast de differences between slopes and R^2 values obtained from the different calibration curves. Foremost, even if the data of the mold A is missing, the regression data obtained outside the box are clearly better than the ones obtained inside it. In addition, taking into account the R^2 values of B mold, the improvement is obvious. For example, calibration inside the box and inside mold B has 0.7843 R^2 value, but if the image is taken outside the box, the error minimizes to 0.9932 for R^2. Taking into account the analyzed images, these data do not agree with them, since the images inside the box lacked shadows and brightness. Therefore, it is believed that two reasons can explain this difference.

First of all, there is a lack of light inside the box. Although at first the image seemed better, it is true that the box is only composed of an indirect focus. Therefore, when Matlab program analyses the image, the intensity of the selected pixels is not the same as without light. So it affects the calibration curve. Secondly, taking into account the values of the calibrations when the standards were inside the mold and inside the box, they are clearly worse (see Table 18). This may be due to the fact that the same mold does not allow the light to pass through it completely and in this way the selected areas are darker to analyze. On the other hand, it would not only affect a standard, but also the standards contiguous to it.

However, according to the slopes (see Table 18), the only significant difference is in mold C as the value was down from 0.1137 to 0.0824 inside the mold and from 0.1005 to 0.0442 outside it. As for the others, their values are quite similar.

Consequently, there are two main conclusions. Images inside the box are worse than the others taking into account the achieved data due to the lack of light inside it and the direct interference it has in the color intensity. Furthermore, there is a significant interference of the mold, as the images taken outside it are better.

Table 19. Comparison in the determination of Tartrazine in real samples when different calibrations are applied.

		INSIDE THE BOX			OUTSIDE THE BOX		
Mold		A	B	C	A	B	C
		mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Samples	1	19.3	12.1	15.0	-	11.5	34.2
	2	19.0	10.2	14.3	-	7.8	21.8
	3	10.8	5.4	8.2	-	4.5	11.8
	4	6.7	3.6	6.8	-	4.1	8.8
	5	5.0	1.2	2.9	-	2.2	4.5
Mold volumes		mL	mL	mL	mL	mL	mL
		3.5	22	12	3.5	22	12

All these differences in the calibration curves are showed in Table 19. It could be believed that the concentrations in all the proposed situations should be the same, although unfortunately this is not the case. The differences between all of them are remarkable. If we take into account the molds individually, the differences are not so striking but if they are compared together, the opposite happens. In this case no statistical test has been applied. According to the B mold data, it does not exist a significant difference between the concentrations inside or outside the box. However, in C mold happens the opposite. In addition, it is clear, that the more volume the standards used for the calibration have; the smaller concentration will be determined for real samples.

For that reason, a comparison of different volume standards is done.

Study of different volume standards

After having seen significant differences in the previous test, it was decided to make several comparisons. On the one hand, a volume study was carried out to see if the standards volume provided significant differences. To develop the study, two different molds (B and C) were taken into account. From each mold two different calibration curves were developed. B mold had a calibration curve with standards of 22 mL and another one with 10 mL standards. On the other hand, for mold C, the first calibration was made with 12 mL standards and the other with 10 mL standards.

In addition, as in the previous study, the box was reused with the aim of ensuring that images taken outside of it gave better results.

B mold

For the study of B mold, 22 mL and 10 mL standards were prepared and images were taken inside and outside the box. Table 20 shows all the data used for the development of the *F* and student *t* tests, in order to see significant differences between both volumes. In Table 20, slopes are represented as b_{11} and b_{12} respectively, the total number of data points (n_1 and n_2) in this case is 4 inside and outside the box. There are also taken into account the standard deviation of slopes (s_{b11} and s_{b12}) and regressions (s_{e1} and s_{e2}).[21]

Table 20. Different data achieved from calibration curves used for the volume study. Inside and outside the box with different volume standards

INSIDE THE BOX				OUTSIDE THE BOX			
Volume of 22mL		Volume of10mL		Volume of22 mL		Volume of10mL	
S_{b11}	0.03	S_{b12}	0.01	S_{b11}	0.003	S_{b12}	0,002
S^2_{b11}	0.0007	S^2_{b12}	0.00007	S^2_{b11}	0.00001	S^2_{b12}	0,000003
b_{11}	0.11	b_{12}	0.11	b_{11}	0.13	b_{12}	0,05
S_{e1}	0.22	S_{e1}	0.07	S_{e1}	0.03	S_{e1}	0,02
S^2_{e1}	0.05	S^2_{e1}	0.01	S^2_{e1}	0.0007	S^2_{e1}	0,0003
n_1	4	n_2	4	n_1	4	n_2	4

As to Table 21, according to the data inside the box, the *F* test of both slope and regression comparison indicates that the variances of the slopes and regressions are homogeneous as $F_{tab} \rangle F_{cal}$ happens. In addition, both student t tests demonstrate that there are no significant differences between the volumes inside the box.

Table 21. B mold's volume study, with slope and regression comparisons inside and outside the box. F_{tab} represents the tabulated F value; F_{cal} the calculated F value; t_{tab} represents the tabulated t value and t_{cal} the calculated t value.

INSIDE THE BOX							
SLOPE COMPARISON				REGRESSION COMPARISON			
F Test		*t* Test		*F* Test		*t* Test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
39.0	9.5	2.50	0.13	39.0	9.1	2.77	0.13
$F_{tab} \rangle F_{cal}$ HOMOGENEOUS		$t_{tab} \rangle t_{cal}$ No significant difference		$F_{tab} \rangle F_{cal}$ HOMOGENEOUS		$t_{tab} \rangle t_{cal}$ No significant difference	
OUTSIDE THE BOX							
SLOPE COMPARISON				REGRESSION COMPARISON			
F Test		*t* T est		*F* Test		*t* Test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
39.0	3.2	2.45	21.50	39.0	3.0	2.77	21.60
$F_{tab} \rangle F_{cal}$		$t_{tab} \langle t_{cal}$		$F_{tab} \rangle F_{cal}$		$t_{tab} \langle t_{cal}$	

HOMOGENEOUS	SIGNIFICANT DIFFERENCE	HOMOGENEOUS	SIGNIFICANT DIFFERENCE

However, unfortunately, outside the box does not occur the same. *F* test also shows that the variances between the slopes and regressions respectively are homogeneous, but there are significant differences in the t tests not only in the slope but also in the regression as $t_{tab}<t_{cal}$, which indicates a significant difference. This difference could be that the image is inadequate for the analysis, as this study was done before Shewhart charts were developed. However, when the image was checked out, it was near the warning limits in many colors of the color pattern, so the image may have been discarded.

C mold

As previously mentioned, two different molds have been used for this volume study. In Table 22 C mold's data is available. According to it, the data is obtained from images of standards inside and outside the box with indirect light with different standard volumes. In this case, the volume were 12 mL standards and 10 mL standards. As done in B mold volume study, *F* and *t* tests were carried out in order to see the possible significant differences between the volumes in the same mold. In Table 22, slopes are represented as b_{11} and b_{12} respectively, the total number of data points (n_1 and n_2) in this case is 4 inside and outside the box. There are also taken into account the standard deviation of slopes (s_{b11} and s_{b12}) and regressions (s_{e1} and s_{e2})

Table 22. C mold's volume study's data for F and t tests. Taking into account the different calibration curves obtained inside and outside the box, with different mL standards.

INSIDE THE BOX				OUTSIDE THE BOX			
12mL		10mL		12mL		10mL	
S_{b11}	0,004	S_{b12}	0,006	S_{b11}	0,003	S_{b12}	0,0009
S^2_{b11}	1,50E-05	S^2_{b12}	3,50E-05	S^2_{b11}	8,70E-06	S^2_{b12}	8,03E-07
b_{11}	0,10	b_{12}	0,11	b_{11}	0,04	b_{12}	0,05
S_{e1}	0,03	S_{e1}	0,05	S_{e1}	0,02	S_{e1}	0,008
S^2_{e1}	0,001	S^2_{e1}	0,003	S^2_{e1}	0,0005	S^2_{e1}	6,07E-05
n_1	4	n_2	4	n_1	3	n_2	4

According to the results (see Table 23) obtained from F test for the slope inside the box, as $F_{tab} > F_{cal}$ the variances are homogeneous (F_{tab} is the tabulated value of F; F_{cal} is the calculated value of F). The same happens for the regression comparison. In addition, according to the t test of both comparisons, as $t_{tab} > t_{cal}$ happens, there are no significant differences inside the box.

Furthermore, the F tests for images and calibrations outside the box show again the same, the variance of both slope and regression is homogeneous. According to the t test for the slope, it also demonstrates that there are no significant differences as $t_{tab} > t_{cal}$ meets. In addition, the same happens for the t test for regression.

Table 23. C mold's volume study, with slope and regression comparisons inside and outside the box. F_{tab} represents the tabulated F value; F_{cal} the calculated F value; t_{tab} represents the tabulated t value and t_{cal} the calculated t value.

INSIDE THE BOX							
SLOPE COMPARISON				REGRESSION COMPARISON			
F Test		*Student t* Test		*F* Test		*Student t* Test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
39.0	2.3	2.45	1.67	39.0	2.4	2.77	1.66
$F_{tab} > F_{cal}$ HOMOGENEOUS		$t_{tab} > t_{cal}$ No significant difference		$F_{tab} > F_{cal}$ HOMOGENEOUS		$t_{tab} > t_{cal}$ No significant difference	
OUTSIDE THE BOX							
SLOPE COMPARISON				REGRESSION COMPARISON			
F Test		*t* Test		*F* Test		*t* Test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
38.5	10.8	2.45	2.36	38.5	9.0	3.18	2.86
$F_{tab} > F_{cal}$ HOMOGENEOUS		$t_{tab} > t_{cal}$ No significant difference		$F_{tab} > F_{cal}$ HOMOGENEOUS		$t_{tab} > t_{cal}$ No significant difference	

To conclude, it has to be said that there are no significant differences, between the volumes, so there must be another interferential factor affecting the determination of food colorant according to the results in Table 18. For that reason, a mold study was done as it was believed that possibly the measurements of each one could affect the determination.

Mold study

For the development of the mold study, 10 mL of standards were poured and solidified in both molds B and C. In addition, these were the dimensions of each mold with 10 mL of standard (see Table 24:

Table 24. Dimensions of mold B and C.

	B	C
DEPTH	1.5 cm	1.5 cm
LENGTH	3.5 cm	4.0 cm
WIDTH	4.0 cm	3.0 cm

As to the comparison of mold B and mold C, is has been carried out in two ways. Again one of it inside the box and another one outside of it. It is important to say that this study is based on the Lambert-beer law. There is a suspicion that the depth, length or width of the standard could affect directly in the analysis. That is the reason why 10 mL were poured down the molds and the data in Table 25 was achieved.

Table 25. Data used in the mold study for F and t test. Obtained inside and outside the box with standards of 10 mL.

INSIDE THE BOX				OUTSIDE THE BOX			
B 10mL		C 10mL		B 10mL		C 10mL	
S_{b11}	0,008	S_{b12}	0,006	S_{b11}	0,002	S_{b12}	0,0009
S^2_{b11}	7,1E-05	S^2_{b12}	3,5E-05	S^2_{b11}	3,5E-06	S^2_{b12}	8,03E-07
b_{11}	0,11	b_{12}	0,11	b_{11}	0,05	b_{12}	0,05
S_{e1}	0,07	S_{e1}	0,05	S_{e1}	0,02	S_{e1}	0,008
S^2_{e1}	0,005	S^2_{e1}	0,003	S^2_{e1}	0,0003	S^2_{e1}	6,07E-05
n_1	4	n_2	4	n_1	4	n_2	4

According to Table 25, it is possible to see the obtained data for both molds outside or inside the box. As in previous studies, b_{11} and b_{12} represent the slopes respectively; standard deviation of

the slope (s_{b11} and s_{b12}) and regression (s_{e1} and s_{e2}) are not only represented in the table, but also the total number of data as n_1 and n_2.

The data in Table 25 provided the development of *F* and *t* tests in order to see if there were significant differences between the molds used in the determination. According to Table 26, it is possible to appreciate the results of both tests. Those tests were applied to the slope and regression (inside and outside the box), in order to obtain a more general result. Taking into account the slope comparison from inside the box, $F_{tab} \rangle F_{cal}$ (F_{tab} tabulated *F* value and F_{cal} calculated F value) what means the variance is homogeneous. Furthermore, according to the results of *t* test, as $T_{tab} \rangle T_{cal}$ it could be said that there are no significant differences between both molds B and C.

Table 26. F and t tests results for the mold study. It has been done a comparison for both slope and regression with the data from Table 25.

INSIDE THE BOX							
SLOPE COMPARISON				REGRESSION COMPARISON			
F Test		*t* Test		*F* Test		*t* Test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
39.0	2.0	2.45	0.14	39.0	2.0	2.77	0.14
$F_{tab} \rangle F_{cal}$ HOMOGENEOUS		$t_{tab} \rangle t_{cal}$ No significant difference		$F_{tab} \rangle F_{cal}$ HOMOGENEOUS		$t_{tab} \rangle t_{cal}$ No significant difference	
OUTSIDE THE BOX							
SLOPE COMPARISON				REGRESSION COMPARISON			
F Test		*t* Test		*F* Test		*t* Test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{ca8l}
39.0	4.3	2.45	0.08	39.0	4.35	2.77	0.08
$F_{tab} \rangle F_{cal}$ HOMOGENEOUS		$t_{tab} \rangle t_{cal}$ No significant difference		$F_{tab} \rangle F_{cal}$ HOMOGENEOUS		$t_{tab} \rangle t_{cal}$ No significant difference	

Additionally, according to the *t* test and *F* test outside the box, the results are the same (see Table 26). Furthermore, it could be said that there does not exist a significant difference between both molds. It is believed that those results are due to depth. Taking into account Table 26, 1.5 cm is the depth value, constant in both molds. According to Lambert Beer, the length of the cuvette has a direct impact in the determination of concentration. The same happens in digital analysis. In this case, image is taken from above, so the depth would directly interfere in the coloring determination.

Appendix II: Depth study

A depth study was carried out after considering depth as the factor that most influenced the regression. For this reason, it was decided to look for the optimum depth to mechanize the process and use it continuously. These were the conditions to obtain the optimal depth of the standards:

- Search the depth in the three colors individually.
- Perform three different measurements (depths of 0.5 cm, 1 cm and 1.5 cm) and verify if there are significant differences in regression and slopes.

Allure Red

For Allure Red three different regressions were obtained, one for 0.50 cm, another one for 1.0 cm and the last one for 1.5 cm. Table 27 shows the data obtained for each depth. As in previous studies, b_{11}, b_{12} and b_{12} represent the slopes respectively; standard deviation of the slope (s_{b11}, s_{b12} and s_{b13}) and regression (s_{e1} s_{e2} and s_{e3}) are not only represented in the table, but also the total number of data as n_1, n_2 and n_3.

Table 27. G channel's data for three different depths, 0.50 cm; 1.0 cm and 1.5 cm.

Blue Channel					
Depth of 0.5 cm		Depth of 1.0 cm		Depth of 1.5 cm	
S_{b11}	0,001	S_{b12}	0,002	S_{b13}	0,003
S^2_{b11}	8,25E-07	S^2_{b12}	3,56E-06	S^2_{b13}	7,45E-06
b_{11}	0,015	b_{12}	0,030	b_{13}	0,034
S_{e1}	0,007	S_{e2}	0,015	S_{e3}	0,022
S^2_{e1}	5,5E-05	S^2_{e2}	0,0002	S^2_{e3}	0,0005
n_1	7	n_2	7	n_3	7

As in previous studies, Table 27's data was used to perform both *F* and student *t* tests (see Table 28) in order to compare slopes and regressions. The aim is to see if 1.0 cm is the optimum one as happens with Lambert-beer law. Firstly, 0.5 cm and 1.0 cm of depth were compared. Both comparisons, slope and regression, demonstrate a significant difference as the calculated t value

is higher than the tabulated one. The same happens when comparing 0.5 cm and 1.5 cm. Therefore, a last comparison was performed between 1.0 cm and 1.5 cm of depth. On the contrary, to previous results, there was no significant difference between them. For that reason, it was decided to take 1.0 cm of depth as the optimum one for Allure Red. So every standard in following tests must have 1.0 cm of depth for Allure Red.

Table 28. F and T tests between different depth in order to compare slopes and regressions and see if there are significant differences.

Depth of jellies 0.50 cm-1.0 cm							
SLOPE COMPARISON				REGRESSION COMPARISON			
F Test		*t* Test		*F* Test		*t* Test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
7.15	0.23	2.18	7,37	7.15	0.23	2.23	6,99
$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}<t_{cal}$ SIGNIFICANT DIFFERENCE		$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}<t_{cal}$ SIGNIFICANT DIFFERENCE	
Depth of jellies 1.0 cm - 1.5 cm							
SLOPE COMPARISON				REGRESSION COMPARISON			
F Test		t *test*		*F* Test		*t* test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
7.15	0.48	2.18	0.89	7.15	0.48	2.23	0.85
$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}>t_{cal}$ No significant difference		$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}>t_{cal}$ No significant difference	
Depth of jellies 0.50 cm – 1.5 cm							
SLOPE COMPARISON				REGRESSION COMPARISON			
F Test		T test		F Test		T test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
7.15	0.11	2.18	6.40	7.15	0.11	2.23	6.06
$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}<t_{cal}$ SIGNIFICANT DIFFERENCE		$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}<t_{cal}$ SIGNIFICANT DIFFERENCE	

Brilliant Blue

For Brilliant Blue as with Allure Red, three different depths were compared, 0.50 cm; 1.0cm and 1.5 cm. In Table 29 the obtained data in G channel could be seen for each depth. Three different regressions were performed for each depth and the G channel was selected as in previous studies for Brilliant Blue.

Table 29. G channel's data in three different depths, 0.5 cm; 1.0 cm and 1.5 cm.

Green CHANNEL					
0.50 cm		1.0 cm		1.5 cm	
S_{b11}	0,001	S_{b12}	0,0008	S_{b11}	0,0015
S^2_{b11}	1,02E-06	S^2_{b12}	6,62E-07	S^2_{b11}	2,2E-06
b_{11}	0,011	b_{12}	0,013	b_{11}	0,015
S_{e1}	0,01	S_{e1}	0,009	S_{e1}	0,015
S^2_{e1}	0,0001	S^2_{e1}	7,2E-05	S^2_{e1}	0,0002
n_1	7	n_2	7	n_1	7

With the provided data of Table 29 *F* and *t* tests were put into practice to compare the slopes and regressions. According to Table 30, it could be seen that the comparison of 0.5 cm and 1.0 cm shows no significant difference for slope and regression ($T_{tab} > T_{cal}$) so using either of two depths the result practically will not change as there are no significant differences. However, before taking 1.0 cm as the optimum depth two more comparisons were done. The first one between 0.5 cm and 1.5 cm. This one proved significant difference for both slope and regression so no regressions could be performed with standards of 0.5 cm and 1.5 cm. Nevertheless, the last comparison between regression done with standards of 1.0 cm and another one with standards of 1.5 cm of depth, demonstrated no significant difference as could be seen in Table 30.

To conclude as previously done for Allure Red, 1.0 cm of depth was chosen again for Brilliant Blue regressions to be the optimum one for the standards. From now on, every regression prepared for any test with Brilliant Blue must have 1.0 cm of depth.

Table 30. *F* and *t* tests between different depth in order to compare slopes and regressions and see if there are significant differences.

0.50 cm-1.0 cm							
SLOPE COMPARISON				REGRESSION COMPARISON			
F test		*t* test		*F* test		*t* test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
7.15	1.54	2.18	1.97	7.15	1.54	2.23	1.87
$F_{tab} > F_{cal}$ HOMOGENEOUS		$T_{tab} > T_{cal}$ No significant difference		$F_{tab} > F_{cal}$ HOMOGENEOUS		$T_{tab} > T_{cal}$ No significant difference	
1.0 cm - 1.5 cm							
SLOPE COMPARISON				REGRESSION COMPARISON			
F Test		*t* test		*F* test		*t* test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
7.15	0.31	2.18	0.85	7.15	0.31	2.23	0.81
$F_{tab} > F_{cal}$ HOMOGENEOUS		$t_{tab} > t_{cal}$ No significant difference		$F_{tab} > F_{cal}$ HOMOGENEOUS		$t_{tab} > t_{cal}$ No significant difference	
0.50 cm – 1.5 cm							
SLOPE COMPARISON				REGRESSION COMPARISON			
F Test		T test		F Test		T test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
7.15	0.47	2.18	2.24	7.15	0.47	2.23	2.12
$F_{tab} > F_{cal}$ HOMOGENEOUS		$t_{tab} < t_{cal}$ SIGNIFICANT DIFFERENCE		$F_{tab} > F_{cal}$ HOMOGENEOUS		$t_{tab} > t_{cal}$ No significant difference	

Tartrazine

Finally, the same tests were performed for Tartrazine. In this case, 4 different depths were compared since comparisons between 0.50 cm, 1.0 cm and 1.5 cm prove significant difference continuously. Knowing that, it was decided to measure a new depth, 0.80 cm in this case, to compare and see if there were significant differences when comparing with 1.0 cm.

Table 31 show the data obtained from each depth regression in G channel. As previous studies, enable de development of T and F tests to compare slope and regression.

Table 31. G channel's data in three different depths, 0.50 cm; 0.80 cm; 1.0 cm and 1.5 cm.

G CHANNEL							
0.50 cm		0.80 cm		1.0 cm		1.5 cm	
S_{b11}	0,0011	S_{b14}	0,0012	S_{b12}	0,0020	S_{b13}	0,0022
S^2_{b11}	1,32E-06	S^2_{b14}	1,48E-06	S^2_{b12}	4,14E-06	S^2_{b13}	4,94E-06
b_{11}	0,021	b_{14}	0,037	b_{12}	0,036	b_{13}	0,049
S_{e1}	0,009	S_{e4}	0,010	S_{e2}	0,017	S_{e3}	0,020
S^2_{e1}	8,6E-05	S^2_{e4}	9,7E-05	S^2_{e2}	0,0003	S^2_{e3}	0,0004
n_1	7	n_4	7	n_2	7	n_3	7

In these last comparisons, there is only one that proves no significant difference, the one between 0.80 cm and 1.0 cm. As commented above, *t* tests for slope and regression between 0.5 cm and 1.0 cm prove significant difference. The same happens between 1.0 cm and 1.5cm. There for as it was impossible to determinate 1.0 cm as the optimum depth it was decided to carry out a last comparison between 0.80 cm and 1.0 cm.

Fortunately, it proved no significant difference and 1.0 cm was decided to be the optimum one. So from now on 1.0 cm of depth would be the one used in the standards in order to perform regressions (see Table 32).

Table 32. *F* and *t* tests between different depth in order to compare slopes and regressions and see if there are significant differences.

0.50 cm - 0.80 cm							
SLOPE COMPARISON				REGRESSION COMPARISON			
F test		*t* test		*F* test		*t* test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
7.15	0.89	2.18	9.13	7.15	0.89	2.23	9.17
$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}<t_{cal}$ SIGNIFICANT DIFFERENCE		$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}<t_{cal}$ SIGNIFICANT DIFFERENCE	
0.80 cm - 1.0 cm							
SLOPE COMPARISON				REGRESSION COMPARISON			
F test		*t* test		*F* test		*t* test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
7.15	0.32	2.18	0.79	7.15	0.84	2.23	0.75
$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}>t_{cal}$ No significant difference		$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}>t_{cal}$ No significant difference	
1.0 cm – 1.5 cm							
SLOPE COMPARISON				REGRESSION COMPARISON			
F test		*t* test		*F* test		*t* test	
F_{tab}	F_{cal}	t_{tab}	t_{cal}	F_{tab}	F_{cal}	t_{tab}	t_{cal}
7.15	0.84	2.18	4.07	7.15	0.84	2.23	3.86
$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}<t_{cal}$ SIGNIFICANT DIFFERENCE		$F_{tab}>F_{cal}$ HOMOGENEOUS		$t_{tab}<t_{tal}$ SIGNIFICANT DIFFERENCE	

Appendix III: Interference study

An interference study was developed with the aim of using candy or gelatin standards for the determination of food coloring. Previous tests were carried out with only Jelly and food coloring standards. However, our objective was to get closer to real samples and simulate them in order to develop the method and see if both linear regressions could be used in the coloring determination.

Firstly, it was decided to perform both calibration curves in the same graph. Orange calibration curve represent Jelly standards and the blue curve candy standards (Figure 35). Blue curve was developed with images in where candy standards were very small so signal only could be taken from the center of it. Figure 35 A shows a tendency in where both regressions would cross each other proving a possible interference. For that reason, it was decided to develop a second calibration curve for candy standards supposing that the interferences come from there.

In this occasion, bigger standards of 1 cm of depth were prepared and signals were taken not only from the center of it but also from everywhere, 6 signals in total. In this way, the obtaining a homogeneous curve was more possible. Unfortunately, the result was a total evidence of interferences due to the crossing of both curves (Figure 35 B).

Furthermore, *student t* and *F* tests were carried out to prove significant differences in the regressions and slopes of candy and gelatin calibration curves in Allure Red, Tartrazine and Brilliant Blue. Therefore, a confirmation of the interferences (see Table 33).

Table 33. *t* and *F* test results for slope and regression comparison between Jelly and Candy standards for each food coloring being examined.

	Slope	**Regression**
Allure Red	Significant Difference	Significant Difference
Tartrazine	Significant Difference	Significant Difference
Brilliant Blue	Significant Difference	Significant Difference

.

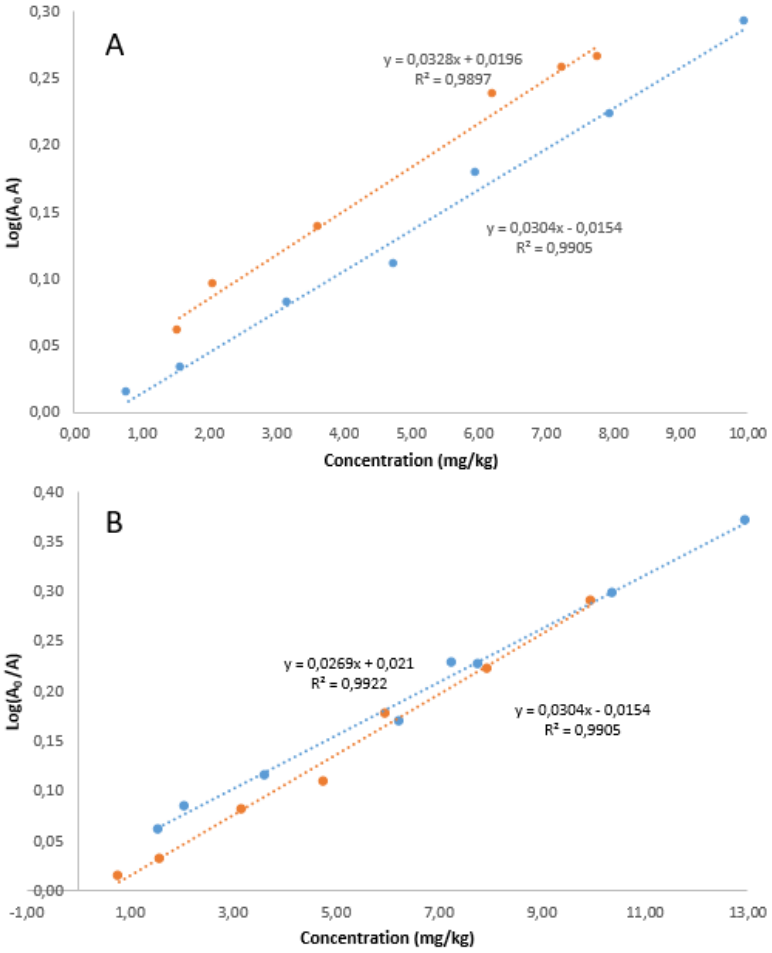

Figure 35. Comparison of two different regressions, orange done by jelly standards and blue with candy standards. A performed by three points of signal. B performed by 6 points of signal in order to obtain a more homogeneous regression.

To conclude, this interference study demonstrated that there is not possible to use both regressions for the same objective as interferences exist. So if the aim is to determine food coloring in candies, is preferable to use candy standards and ignore the jelly ones.