Development and validation of analytical method for determining the artificial dyes in breakfast cereals by means of high performance liquid chromatography

Desenvolvimento e validação de método analítico para determinação de corantes artificiais em cereais matinais utilizando cromatogafia líquida de alta eficiência

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ABSTRACT
Validation of analytical methodology is an important tool to ensure the applicability and scope of a technique for laboratory routine, establishing the limits of the quality parameters of instrumental measurements and the statistical reliability by estimating the procedure performance. There are several normative documents for establishing the figures of merit, namely, limit of detection (LOD), limit of quantification (LOQ), linearity, selectivity, repetitivity, intermediate precision and recovery. This work aimed at developing and validating an analytical methodology for quantifying the artificial dyes in cereal by means of high performance liquid chromatography (HPLC). All of the validation process of this study was performed according to the recommendations by Thompson et al, and following the guidance stated in the document INMETRO DOQ-CGCRE-08 and the harmonized Guidelines IUPAC. After completing all of the validation steps, the methodology showed to be precise, exact, linear over a wide concentration range and its analysis has not being influenced by the food matrix for HPLC determination. Moreover, the methodology has shown as an important contribution since no official methodologies have been available for determining artificial dyes in breakfast cereals.
Keyword. breakfast cereals, validation, artificial dyes.

RESUMO
A validação de metodologia analítica é uma importante ferramenta para assegurar a aplicabilidade e a abrangência de uma técnica na rotina laboratorial, estabelecendo-se os limites dos parâmetros de qualidade das medidas instrumentais e da confiabilidade estatística, por meio de estimativa das figuras de mérito. Existem vários documentos normativos que estabelecem essas figuras de mérito, sendo: limite de detecção (LD), limite de quantificação (LQ), linearidade, efeito matriz, repetitividade, precisão intermediária e recuperação. Neste trabalho foi feita a elaboração e validação de um método analítico para quantificar corantes artificiais em cereal matinal por cromatografia líquida de alta eficiência (CLAE). Todo o processo de validação desta pesquisa foi realizado de acordo com o preconizado por Thompson et al utilizando-se as recomendações contidas no documento do INMETRO DOQ-CGCRE-08 e no Guia harmonizado IUPAC. Após o cumprimento de todas as etapas, o método mostrou ser preciso, exato, linear em ampla faixa de concentração, e sua análise não foi influenciada pela matriz alimentar na determinação por CLAE. Além disso, o método mostrou-se como contribuição importante em vista de não existirem metodologias oficiais para realizar a determinação de corantes artificiais em cereais matinais.
Palavras-chave. cereal matinal, validação, corantes artificiais.

INTRODUCTION

The consumption of breakfast cereals is growing 15-20 % a year, as the population is seeking healthier foods, rich in fiber and nutrients. The breakfast cereals are excellent sources of carbohydrates and also have vitamins, fiber and minerals such as iron, associated with low fat, being recommended to people of all ages[3]. As it is an industrialized food, it is not free from the presence of artificial colors. In a recent study, Mattos et al[4], by applying the nutrition survey in relation to food consumption by children and adolescents, found that 13 % of respondents consume breakfast cereal / cereal bar once or more per day.

According to RDC Resolution No. 263 of September 22nd, 2005, the National Agency of Sanitary Surveillance (ANVISA)[5], which approves technical standards for products of cereals, starches, flours and bran, these are processed cereals products obtained from grain laminates, cylindered, rolled, puffed, flocked, extruded, pre-cooked and/or other technological processes considered safe for food production, and may contain other ingredients as far as they do not mischaracterize the products. They can also present diverse coverage, shape and texture.

Another component that is allowed to the production of breakfast cereals are artificial colors, which provide wide range of colors, offering all shades of colors in the visible spectrum. Moreover, most of this type of colors has high stability, uniformity in color conferred, high tinctorial power, exemption from microbiological contamination and relatively low production cost[6].

It is known that the use of chemical additives, including the artificial dyes, is one of the most controversial advances in food industry, due to all the toxicological aspects relating to these substances. With the increasing of the use of additives, countries began to establish laws to control their use, establishing specifications and criteria of use[7].

In1977, the use of artificial dyes was regulated by the Health Surveillance Secretariat of the Ministry of Health (SVS / MS), that it issued Resolution CNNPA No. 44, which established the general conditions of preparation, classification, presentation, description, composition and essential factors of quality of the colors used in food and beverages[8]. Brazil internalized national legislation on the basis of harmonized instruments in Mercosur related to food additives, of which we can mention the GMC Resolution No. 19/93 (General List Harmonized Additives - Mercosur), GMC Resolution No. 14/93 (General List Harmonized Dye - Mercosur), GMC Resolution No. 101/94 (List of Food Additives with their Functional Class) and GMC Resolution No. 38/01 (Incorporation of Additives in Schedules Harmonized)[9-12].

The SVS / MS approved in 1997 and Ordinance No. 540, which establishes Technical Regulations for food additives, with appropriate definitions, classification and employment[13]. Additives authorized for human use are divided into 23 groups, among them, the artificial colors .This ordinance allows the use of the following colors: Sunset Yellow (INS 110), Quinoline Yellow (INS 104); Carmoisine (INS 122), Brilliant Blue (INS 133), Patent Blue V(INS 131), Amaranth (INS 123); Erythrosine B (INS 127), Indigo Carmine (INS 132), Brown HT (INS 155); Bright Black (INS 151), New Coccine (INS 124),Tartrazine (INS 102), Allura Red (INS 129), Fast Green FCF (INS 143). The International Numbering System (INS) for Food Additives was developed by the Codex Committee on Food Additives and Contaminants in Foods to establish an international system of numerical identification of food additives in ingredient lists as an alternative to the statement of the specific name of the additive.

On August 5th, 1999, ANVISA, current agency of the Ministry of Health which operates in regulation, approved the use of food additives by establishing its functions and maximum limits for the different food groups, in this including the resolution No. 60 of 2007 (Cereals and products of or based on cereals), where it is allowed to use the following colors: Sunset Yellow, Allura Red and Brilliant Blue.[14]

The increasing of the food trade leads to a growing need to develop methods of analysis that are increasingly reliable, efficient and fast. For artificial color is not enough simply to detect the color, but each color, or mixture of these, must be detected and quantified individually, which has been hampered mainly by the lack of appropriate analytical methodologies[15]. Various separation techniques such as High performance liquid chromatography (HPLC), Gas Chromatography (GC) and Capillary Electrophoresis (CE) are being used to determine qualitatively and quantitatively the artificial color[16]. In Brazil, much still needs to be done to develop new analytical methods for the determination of artificial colors in food.

To ensure reliability of the analytical method, it must undergo an assessment also known as validation. According to ISO/IEC 17025[17] validation: "is the confirmation by means of tests and presentation of objective evidences that certain requirements are met for a given intended use."

The validation ensures applicability and scope of an analytical method for routine monitoring, establishing the limits of the quality parameters of instrumental measurements and statistical reliability by estimating the method performance[18]. A well defined and documented validation provides the regulatory agencies substantial evidence that the method and the systems fit the desired use[19].

In order to standardize these procedures, the regulatory agencies of the United States, Japan and the European Union began to organize, since the 90s, the International Conference on Harmonization (ICH), establishing standards in research procedures and drug development. Also in the 90's the International Union of Pure and Applied Chemistry (IUPAC) published a guide validation in analytical chemistry[20]. In this context ICH draws up a guide on method validation[21, 22]. Among the international bodies that also have technical documents that define guidelines for validation of analytical methods we can cite the IUPAC, AOAC (Association of Official Analytical Chemists), ISO (International Standard Organization), and US-FDA (United States Food and Drug Administration)[23, 24].

For laboratories to have technical capability to perform an analytical test they must get an accreditation from accrediting agencies, which can be either national or international bodies. In Brazil today, the National Institute of Metrology, Quality and Technology (INMETRO) is the institution responsible for this accreditation, which uses as a tool the guidance on validation of analytical methods DOQ-CGCRE-0082. ANVISA also has the Resolution No 899 of May 29th 2003 as a guide for the procedure of the validation of analytical methods[25].

The validation of analytical methodology can occur in two distinctive ways: the intralaboratory validation and the interlaboratory one. The intralaboratory validation is indicated for the purpose of validation of new methods have been developed in the laboratory or to verify the application of the analytical method adopted by other sources. In this type of validation all figures of merit are measured, except reproducibility1. On the other hand, interlaboratory validation evaluates all analytical performance parameters, obtained by collaborative study, which involves several laboratories[26].

The validation of an analytical method is a constant and dynamic process. The entire validation process must be well defined and documented, resulting in documents that provide objective and overwhelming evidence that the measurement methodology developed or adapted is appropriate for the intended use. Thus, the validation should be well planned in order to have a satisfactory performance. In this context, Feinberg[27] proposes the following scheme for what he calls the life cycle of an analytical method, according to Figure 1.

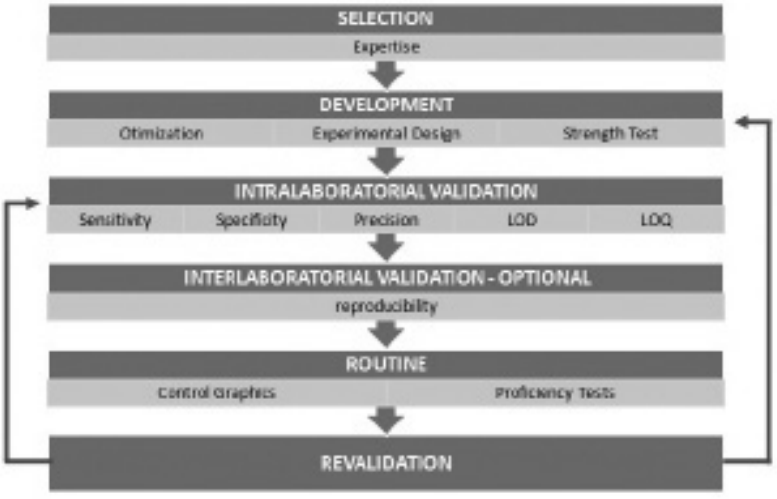

Figure 1. Life cicle of an analytical method

According to the document INMETRO DOQ-CGCRE-082, the parameters that must be determined in the validation process vary with the type of assay. For a quantitative analysis of minor elements and traces, it is necessary to calculate precision, selectivity, recovery, robustness, linearity, working range, the limit of detection (LOD) and the limit of quantification (LOQ).

This work aimed at the formulation and validation of an analytical method for quantification of artificial colors in cereal by High performance liquid chromatography (HPLC).

MATERIAL AND METHODS

All the validation process of this research was performed according to the recommendations by Thompson et al[1]. The following validation parameters were evaluated: detection limit,

quantification limit, linearity, selectivity (matrix effect), repeatability, intermediate precision and recovery.

Materials

The patterns of the artificial colors Sunset Yellow, Allura Red and Brilliant Blue were purchased from Sigma provider, and the purity of each dye is respectively, 90 %, 80 % and 65 %. The chromatographic grade methanol which was employed was purchased from Tedia and other reagents (acetic acid PA, ammonium acetate PA, isopropyl alcohol PA, ammonium hydroxide PA and ethanol 96 %) from Merck.

The equipment used was a liquid of high efficiency chromatograph with gradient mode of operation, brand Waters, detector coupled with UV/vis WATERS-2487 (variable and dual wavelength) and reverse phase column Symmetry® C18 column (dimensions: 150 mm X 3.9 mm - 4 μmDI). The chromatographic data were analyzed using Empower 2 software.

Analytical Method

Chromatographic parameters

The mobile phase consisted of a solution of water / methanol 70:30 (v/v) and the conditioning phase consisted of a solution of aqueous 0.08 M ammonium acetate/ methanol 70:30 (v/v). Both were filtered on hydrophilic membrane of 0.45 μm and degassed before use. Analyses were performed at a temperature of 25 ° C with a flow of 0.5 mL/min and injection volume of 20 μL. The duration of the analyzes were fixed in 20 minutes, using UV detection at 475 and 600 nm.

Preparation of Standard Stock Solutions

For each colors used in the experiment three stock solutions were prepared. In preparing the stock solutions of the color Sunset Yellow mass between 134.59 and 134.60 milligrams of standard were weighed. For the Allura Red color masses between 124.10 and 124.20 milligrams of the color pattern were weighed and for the Brilliant Blue masses between 166.17 and 166.19 milligrams were weighed. All solutions had their volume heightened in a 100 mL calibrated volumetric flask.

Sample Preparation

The determination of artificial colors was performed by the extraction of the color present in the food with an alcoholic solution of ammonia 10 % (v/v) to boiling repeatedly, then centrifuging this color solution obtained by subsequent filtration in hydrophilic membrane and insulation through elution of aqueous isopropyl alcohol 60 % (v/v) in cartridges C18 SEP-PACK®. After complete evaporation of the isopropyl alcohol and the eluate dilution in water the sample is analyzed by HPLC, as follows:

Extraction of the colors contained in the sample

Weigh 30 g of sample, equivalent to a portion of cereal, separated by color, grinding to a thin powder. Weigh 5 g of the homogenized sample, add 20 mL of deionized water and mix with the aid of a glass rod. Add 40 mL of alcoholic solution of ammonium hydroxide 10 % and lead to a water bath at boiling point for 10 minutes. Remove from water bath and let stand until separating the precipitate, the supernatant is passed slowly to another beaker. Wash the precipitate several times in a water bath, in order to complete the volume to 90 mL or until filtrate leaves no more color in the supernatant. Centrifuge supernatant at 5,000 rpm for 20 minutes to separate the phases and filter the liquid portion in to hydrophilic membrane filter 0.45 μm. Complete the filtrate to 100 mL with deionized water in a volumetric flask calibrated. Take 20 mL in volumetric calibrated pipette, and put into the graduated test tube and evaporate to dryness, completing to 5 mL with deionized water. Bring to ultrasound for 10 minutes at 27 °C to ensure complete solubilization of the dye in water.

Treatment of the cartridge SEP-PAK ®C18

Treat the cartridge SEP-PAK ® C18 with 2 mL of isopropyl alcohol PA and then with 5 mL of aqueous solution of 1% acetic acid (v/v).

Isolation of color

Pass 5 mL of the solution of the extraction of the color sample in the cartridge SEP-PACK® C18 treated and subsequently extract the color that was retained with 10 mL of aqueous isopropyl alcohol 60 %. Evaporate all the alcohol and make up to volume of 10 mL with deionized water and bring to ultrasound for 10 minutes at 27 °C to ensure complete solubilization of the color in water. Finally, filter in a cartridge containing hydrophilic membrane filter of 0.45 μm (Millex®) and analyze in the chromatograph.

Validation of Analytical Method

Assays for the validation of the proposed method were performed according to the recommendations

contained in the document INMETRO DOQ-CGCRE-08[2] and the Guidelines harmonized IUPAC (International Union of Pure and Applied Chemistry)[1].

The premises of validation are that the method should be applicable to specific analyte, the specific matrix in the maximum or minimum specific levels. Thus, the applicable range of the method depends on the minimum specified level (LM) to be evaluated, and may be expressed in terms of standard deviation for reproducibility (SR) calculated by the equation Horwitz[28] and Thompson et al[1] or in terms of limit of detection (LOD) and limit of quantification (LOQ)[29]. For the validation of the analytical method in question the minimum applicable range was calculated, from the LM of each color in analytical aliquot, following the recommendation of ANVISA described in Resolution 60/07[14], from 0.03 g/100 g product to Sunset Yellow and 0.02 g/100 g product to Allura Red color and Brilliant Blue, taking into consideration the proposed analytical procedure and rate of analysis in question. Thus, the applicable minimum range was determined by the following formula:

$$LM \pm 0{,}06LM \times (LM)^{-0{,}1505}$$

Where: LM = minimum specific level

The choice of this formula occurred because the final concentration of the analyte in question is above 0.1 mg/kg.

The next stage of the experimental design was to determine the LOD and LOQ. It is understood by LOD the lowest concentration of analyte present in a sample being analyzed which can be detected, but not necessarily quantified under the experimental conditions established[2]. But the limit of LOQ is the smallest amount of analyte in a sample that can be determined with precision and trueness under the experimental conditions established. To determine these two values were used the following equations:

$$LOD \leq \frac{1}{10} \times LM$$

$$LOQ \leq \frac{1}{5} \times LM$$

Where: LOD = limit of detection
LOQ = limit of quantification
LM = minimum specific level

The linearity is the ability of the analytical method, within a given range, to obtain results which are proportional to the concentration of analyte in the sample, either directly or through mathematical calculations[30]. Thus, the linearity of the analytical method developed was checked from the drafting of three curves, with seven levels of concentration for each color, according to the methodology proposed by Souza and Junqueira[31]. From the values found for the applicable minimum track and taking into account LOQ and LOD were established the seven concentration points of the analytical curve, so that they were equispaced. Thus, each stock solution was diluted in deionized water to give concentrations of approximately 15, 20, 25, 30, 35, 40, 45 mg/L for Sunset Yellow color and approximate concentrations of 5, 10, 15, , 20, 25, 30 and 35 mg/L for the colors Allura Red and Brilliant Blue. All the final solutions were filtered through 0.45 μm hydrophilic membrane before injection. The results were statistically analyzed by linear regression by the least squares method[31].

According to Bratinova, Raffael and Simoneau[40], the selectivity of analytical method is its capacity of quantifying the analyte of interest in presence of other analytes, matrixes and other interferents. In a validation study of a chromatographic method, the selectivity is evaluated through blank sample analyses, following defined analytical procedures. The determination of the analyte would be guaranteed in the final solution of the essay even in the presence of possible interferents in the sample matrix. The absence of chromatographic peak in the same retention time of the colors indicates the suitable selectivity of method.

The selectivity evaluation of method three blank samples were analysed and the chrogratogram was visually inspected referring to the presence of chromatographic peak in the retention time corresponding to colors.

The validation, in general, is accomplished in simple solutions of the analyte. However, the sample is subject to possible interference from substances present in the matrix of the sample material. Due to this reason, a very useful tool for the validation to test these interferences is to determine the matrix effect. To verify the occurrence or not of the matrix effect, it is necessary to prepare standard curves in the array at the same concentrations used in determining the analytical curve prepared in the solvent in an extraction rate from the food treating a sample with composition equal or much like the object of study and that has no artificial colors in their composition[32]. After this procedure an analytical

curve is prepared on the same matrix and it is compared with the analytical curve obtained in aqueous solution by means of t Student Test to verify the equivalence of curves and finally determine whether the matrix interferes with the determination of the analyte[33].

The precision of an analytical method assesses the degree of agreement among individual results, when the method is applied repeatedly to multiple samplings of a homogeneous product in analytical conditions established[21, 25]. In this study, the precision was evaluated based on two method performances: the repeatability through six determinations genuine and the intermediate precision with the variation of the analyst. The repeatability was calculated according to what it is recommended by Albert and Horwitz[34], which evaluates the ratio Horwitz (HorRat), which can be seen in the following equation:

$$HorRat_r = \frac{CV\%_r \leq 2}{½\ CV\%_{HR}}$$

Where: CV%r = coefficient of variation of repeatability
CV%HR = coefficient of variation of Horwitz

In the case of this experimental drawing, the CV% HR was obtained by the value $2C^{(-0,1505)}$[28], where C is the concentration found or added, expressed as a mass fraction.

Intermediary precision (was calculated by what is determined by inmetro[2], according to the following equation:

$$Si_{(j,k)} = \sqrt{\frac{1}{2t} \text{x} \sum_{j=1}^{t} (y_{j1} - y_{j2})^2}$$

Where: $Si_{(j,k)}$= intermediate precision;
t= total number of samples tested;
y_{j1}=first result obtained for the sample j;
y_{j2}=second result obtained for the sample j.

Percentage recovery was estimated by analysis of samples fortified with a known concentration of artificial color. Samples were fortified at three different concentration levels related to the linear range studied: the lowest, the center and the highest. The values found were compared by the recovery established by FAO/ WHO in accordance with the LM analyte in question, which in this experimental design should be 80 to 110 % recovery[29]. Data for validation of the analytical methodology proposed were analyzed following the proposal advocated by Thompson et al[1], and for determining the linearity and limits of detection and quantification spread sheet developed by Bazilio et al[35] was used.

RESULTS AND DISCUSSION

The use of HPLC methodology is widely used in the determination of various substances in food including artificial colors. We used a validated methodology for determining intralaboratorial and artificial colors in candy by Pinheiro and Abrantes[36] as an initial guide, especially for the chromatographic conditions. But successive tests were conducted for the extraction of colors in the food matrix, which in this case was the breakfast cereal, as the aqueous extraction was inefficient due to the complex composition of the food.

Thus, based on research done in specific bibliography, we started with the principle of extraction in alcoholic solution of ammonia to artificial colors, showing that this extraction was more effective when subjected to heating[37]. Following the experimental tests, it was possible to extract in ethanol with 10 % ammonium hydroxide in heating at 100 °C for 20 minutes, with successive washes as described in the methodology.

As the use of three artificial colors in breakfast cereal is allowed in Brazil, methodology has been validated for each of these colors. The calibration curve for each color was constructed from seven concentration points, prepared on the same day the analyzes were performed. In Table 1 it is possible to observe the straight line equation obtained by the least squares method and correlation coefficient of each color studied.

Table 1. Information about the line equation and correlation coefficient for each dye analyzed

Artificial Dye	Line Equation	Correlation Coefficient
Sunset Yellow	Y = 2,65E+06 + 1,16E+05X	R2 = 0,9950
Allura Red	Y = 1,60E+06 + 1,05E+05X	R2 = 0,9982
Brilliant Blue	Y = 2,36E+04 + 1,33E+05X	R2 = 0,9482

The values of LOD and LOQ obtained are shown in

Table 2. These results demonstrate that the method is enough to detect and quantify the concentration levels of the three colors present in samples of cereal.

Table 2. LOD and LOQ of artificial colors analyzed

Artificial Dye	LOD (mg/L)	LOQ (mg/L)
Sunset Yellow	3	6
Allura Red	2	4
Brilliant Blue	2	4

The recovery efficiency of the method shows the insulation ability of the analyte of interest expressed as the percentage of known quantity of this analyte[38]. The recovery percentage was obtained by the ratio value between the concentration determined experimentally and the corresponding theoretical concentration after addition of the colors, at extreme levels (low and high) and central multiplied by 100. In the analytical method in question, the range was found to recover 82 to 97 % for Sunset Yellow color, 82 to 87 % for Allura Red and 81 to 86 % for Brilliant Blue color, with all three within the expected specifications for complex matrices[29,39].

In the essay of blank samples chromatographic peaks of interferents have not been observed in the same retention time referring to colors, showing adequate selectivity of the method.

The effect of matrix was determined and calculated from the data on the analytical curve of the solvent and analytical curve obtained with the matrix, calculating the effect matrix, from the F-Test Snedecor and t Student test with $\alpha = 0.05$, where t critical value of 2.02 was found and tb and ta value, respectively, of 0.62 and 0.50 for Sunset Yellow; tb and ta value, respectively, of 1.62 and 1.67, for Allura Red; and tb and ta value, respectively, of 0.06 and 0.33 for Brilliant Blue. According to these statistical results it is permitted to say that the method in question has no matrix effect, since statistically the analytical curves of the three colors in solvent and matrix are equivalent.

The precision of the method is also known as the extent of random error and represents the closeness of the results obtained from independent measurements of multiple samplings of a homogeneous sample[38] and it was evaluated considering the method performances repeatability and intermediate precision. To determine the repeatable extractions were performed six genuine under the same chromatographic conditions and by the same analyst. The results were evaluated according to the criterion of Albert and Horwitz[34]. The intermediate precision was calculated from the variation of the results obtained by two different analysts, being performed in triplicate. The results were also evaluated against acceptance criteria Horwitz and Albert[34] and can be seen in Table 3, together with the values of repeatability.

Table 3. Precision values of analytical method

Artificial Dye	Repetitivity (mg/L)	Intermediate Precision (mg/L)	Acceptance Criteria of Horwitz
Sunset Yellow	0,455	0,393	≤ 2,0
Allura Red	0,375	0,090	
Brilliant Blue	0,542	0,040	

CONCLUSION

The analysis method of artificial colors in breakfast cereals proposed constitutes an important tool in archiving the sanitary surveillance, as it contemplates all method performance proposed for the validation of analytical methods. The method showed to be precise, accurate, linear in wide concentration range and also showed that its determination is not influenced by the food matrix for determination by HPLC. Moreover, the methodology is useful since there are no official methods for the determination of artificial colors in breakfast cereals.

Yet, it is important to emphasize that validation is an ongoing process that seeks above all reliability and quality of results, thus being indicated to undergo constant reviews and if deemed necessary, revalidate the proposed method.

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