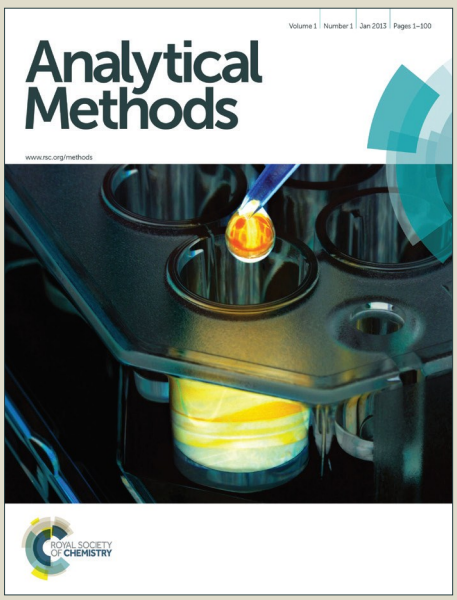
Analytical Methods

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

New eco-friendly methodology for determination of Amaranth dye in foodstuffs using diffuse reflectance spectroscopy

Eduardo Luiz Rossini[a], Maria Izabel Milani[a], Leonardo Pezza[a], Helena Redigolo Pezza[a,*]

[a] Instituto de Química, Universidade Estadual Paulista "Julio de Mesquita Filho", UNESP, R. Prof. Francisco Degni 55, P.O. Box 355, 14800-900, Araraquara, SP, Brazil

*** Corresponding author**

Fax: +55-16-33327932

E-mail address: hrpezza@iq.unesp.br (H.R. Pezza)

Abstract

A simple, fast, low cost, and environmentally friendly analytical methodology was developed for the determination of Amaranth dye in foodstuff samples, offering low consumption of reagents. Spot tests were performed using diffuse reflectance spectroscopy measurements at 530 nm of Amaranth dye on the surface of a qualitative filter paper as the solid support. Caramel coloring, an interferent in the analysis, was eluted from the spot test using aliquots of acid buffer. The calibration curve of absorbance (A_R) as a function of the square root of the dye concentration ($[Amaranth]^{1/2}$) was described by: $A_R = -0.0176 + 14.556\ [Amaranth]^{1/2}$ ($R^2 = 0.999$). The proposed method showed a linear range of 1.00×10^{-5} to 5.00×10^{-4} mol L^{-1} and detection and quantification limits of 1.13×10^{-6} and 1.25×10^{-5} mol L^{-1}, respectively. The technique was applied for the determination of Amaranth in foodstuff samples and the results were consistent with those obtained by a comparative method.

Keywords: Amaranth, diffuse reflectance spectroscopy, green analytical methodology, foodstuffs.

1. Introduction

Dyes are added to foods and beverages with the sole purpose of giving them color, making then look more attractive to the final consumer; however, they do not have any nutritive value. On the contrary, since Fischer demonstrated the carcinogenic properties of Scarlet Red dye, other dyes have been tested to evaluate their mutagenicity/carcinogenicity[1].

The ingestion of these food additives can cause adverse reactions such as hives, headaches, angioedema, and gastrointestinal disorders, in addition to their potential carcinogenic and/or mutagenic effects[2]. Azo dyes can be metabolized in the gastrointestinal system, producing genotoxic and mutagenic compounds[3].

Among the artificial dyes used in the food industry, Amaranth, also known as Bordeaux S, is widely employed due to its low cost and high stability[4]. Its chemical structure consists of two sulfonated naphthalene rings connected by an azo group (Figure 1), so the compound is classified as an acid or azo dye. The intake of this food additive has been associated with adverse health effects, which led the United States to ban its use in 1976[5]. The use of this substance is also prohibited by the European Union Food and Drink Confederation, in some cases[5]. The Brazilian Health Surveillance Agency (ANVISA) has set maximum limits for the dye in foods and beverages ranging from 0.005 g per 100 mL (currant syrup) to 0.01 g per 100 mL (liquid base for ice cream, and gelatin)[6].

Considering the importance of quality control of food products and the extensive use of Amaranth dye in industry, reliable methods are required for its detection and measurement. Several analytical methodologies have been reported for the determination of Amaranth in foods and beverages (Table 1) using techniques including fluorimetry[4], spectrophotometry[7], electrochemistry[8], electrophoresis[9], and high performance liquid chromatography with UV-Vis[10-14] or MS detection[15].

[Insert Table 1]

Fluorescence spectroscopy techniques present low limits of detection and quantification, and are selective, although there have been no applications of the methodology using real samples[4]. Spectrophotometric techniques have the advantage of being simple and readily available in many laboratories, although a disadvantage is that spectral overlap of different substances can occur, requiring the application of mathematical steps that make the overall process of analyte determination much more laborious and complicated[7]. Electrochemical techniques for quantification of Amaranth dye have been successfully developed[8], although they have sometimes shown low reproducibility, which makes it more

difficult to use these methodologies. Electrophoresis is a technique that employs aqueous buffers for analyte separation[9]; however, it requires a specialized operator, and the instrumentation used has higher added costs, compared to the equipment needed in the new analytical method described here.

Procedures involving chromatographic separation are most widely used, due to their low limits of detection and quantification, possibility of automation, robustness, reproducibility, the ability to eliminate interferences, and possibility of simultaneous analysis of different dyes. However, this method uses large quantities of solvents[10-16] and necessitates laborious pretreatment steps for extraction of the dye from beverages, employing solid-phase extraction (SPE)[12-15], resulting in large quantities of waste after the analyses. Although SPE is one of the most widely used procedures, there are other ways to make an extraction even more eco-friendly, such as ultrasound-assisted solvent extraction[17], using organic solvents and centrifugation to extract both hydrophilic and hydrophobic pigments, and microwave-assisted microextraction using an ionic liquid[18], which avoids the use of volatile and toxic organic solvents. Due to the variety of food products containing azo dyes as additives, there is no generally accepted standard procedure for their extraction in laboratories, as described in a recent review about methods for the analysis of different azo dyes employed in food industry[19].

Hence, there is a need to develop new methodologies that are safer for the operator and the environment, and that comply with the principles of Green Chemistry[20], as well as being fast, simple, inexpensive, and reliable.

[Insert Figure 1]

In the search for alternatives that are environmentally more friendly, the use of diffuse reflectance spectroscopy has been described for the analysis of drugs[21,22], pesticides[23], and other contaminants[24]. The association of diffuse reflectance spectroscopy with the spot test is eco-friendly, because it consists of a simple, fast, inexpensive technique that generates small amounts of waste with low toxicity, minimizing or even eliminating risks to the operator or to the environment. Furthermore, diffuse reflectance spectroscopy measurements can be performed *in situ* using a very simple reflectance photometer[25] or a portable diffuse reflectance spectrophotometer. The use of diffuse reflectance spectroscopy has several advantages, but the presence of other dyes such as Ponceau 4R and Azorubine causes interference in the analysis of Amaranth dye due to spectral overlap (analogously to spectrophotometry), so separation procedures are necessary.

Filter paper is used as the spot test platform in clinical[26], environmental[27], forensic[28], drug[21], and food[29] analyses because it is white, which ensures a bright and high contrast background[29]. Paper-based analysis uses small quantities of reagents, generates negligible waste, and the paper is obtained from renewable sources with low added cost[21].

There is a lack of studies describing the determination of dyes in food and beverage samples using spot tests on a paper support associated with diffuse reflectance spectroscopy[30], and there have been no reports of the determination of Amaranth dye using this technique. Some of the works described in the literature for the analysis of dyes using a solid support have employed thin layer chromatography (TLC)[31-33], which is a technique that is difficult to reproduce and uses toxic organic solvents.

Here, a new method is described for the analysis of Amaranth dye in foodstuffs using spot test associated with diffuse reflectance spectroscopy. Unlike other methods, no sample clean-up is required, resulting in a technique that is faster, simpler, cheaper, and more eco-friendly than the conventional procedures.

2. Experimental

2.1. Reagents and standard solutions

Amaranth (analytical grade), anhydrous sodium acetate (analytical grade), and acetic acid (99% minimum) were purchased from Sigma-Aldrich. Sodium hydroxide (analytical grade, 98.2%) and acetonitrile (HPLC grade) were purchased from Mallinckrodt. Ammonium hydroxide (28-30%) was from Synth. Ammonium acetate (analytical grade, 98%) was from Merck, and methanol (HPLC grade) was from J. T. Baker.

A stock solution of 1.00 x 10^{-2} mol L^{-1} Amaranth was prepared in deionized water. Working solutions of Amaranth were freshly prepared by appropriate dilution of the stock solution with deionized water.

Acetate buffer solution pH 4.3 was prepared by mixing the appropriate volumes of acetic acid (0.1 mol L^{-1}) and sodium acetate (0.1 mol L^{-1}), and the exact pH was checked by a digital pH-meter.

Dilutions were made using deionized water (18.2 MΩ.cm) obtained from a Milli-Q system (Millipore, Brazil).

Whatman No. 1 filter paper was used as the solid support in the spot tests. The paper was cut into pieces sized 4.5 x 3.5 cm, with a 1.5 x 3.5 cm extension for ease of handling.

2.2. Sample preparation

The samples (four different brands of currant syrup: A-D; liquid base for ice cream: E; and gelatin: F) were purchased locally in the city of Araraquara (São Paulo, Brazil). Analysis was performed according to the proposed method, with liquid samples being diluted with acetic acid/acetate buffer and solid samples being solubilized and diluted with acetic acid/acetate buffer. For analysis using the comparative method, the samples were prepared at three-fold greater dilutions with deionized water.

2.3. Equipment

Diffuse reflectance measurements were made using a portable spectrophotometer (USB2000, Ocean Optics), with the aid of OOIBase32 software (Ocean Optics). The spectrophotometer was coupled to an integrating sphere using an optical fiber.

The comparative method employed a Shimadzu UFLC-20A HPLC system with DAD detector[11].

2.4. Methodology

In the proposed method, a 20 µL aliquot of the sample or standard solution was spotted onto the center of the filter paper using a micropipette fixed in a holder, according to the procedure described previously[21], and dried at ambient temperature, followed by addition of two 10 µL aliquots of acid buffer and further drying for about 10 min. Diffuse reflectance measurements of Amaranth were then performed at a wavelength of 530 nm.

2.5. Study of matrix interferences

Evaluation of matrix interferences was conducted using standard addition and recovery tests. The sample matrices were fortified with standard solutions at levels between 50% and 250%, followed by determination using diffuse reflectance spectroscopy.

2.6. Comparative methodology

The Amaranth dye was extracted from the samples using natural wool, with heating on a hotplate (Corning), as described in the literature[34]. There is no generally accepted standard procedure for the extraction of azo dyes from food products[19]. In the present study, natural wool was selected for dye extraction because it was readily available, inexpensive, and could be reused several times after washing. An aliquot of sample was transferred quantitatively to a 100 mL beaker, together with a 15 cm length of natural wool thread that had been pretreated previously[34]. Acetic acid solution (0.05 mol L^{-1}) was then added to give a final volume of 20 mL. The system was heated to boiling for 3 min. The wool was then removed and washed

with two portions of cold deionized water. The washed wool was transferred to another 100 mL beaker, to which was added about 15 mL of 10% ammonium hydroxide solution. The system was boiled for 3 min and the resulting solution was transferred to a 100 mL beaker. The wool was washed two more times with hot 10% NH_4OH solution, and the alkaline solutions were combined in another 100 mL beaker. This solution was carefully evaporated to 10 mL, then cooled and transferred to a 25 mL volumetric flask, and the final volume was made up with deionized water (at room temperature).

Analysis by the comparative HPLC method was performed as described in the literature[11]. The experimental conditions were: C_{18} column (250 mm x 4.6 mm x 5 μm) with an internal C_{18} guard column; 0.1 mol L^{-1} ammonium acetate (pH 6.9) as mobile phase A; a mixture of methanol and acetonitrile (90:10, v/v) as mobile phase B; isocratic elution with 8% of mobile phase A; run time of 6 min; flow rate of 1.0 mL min^{-1}; injection volume of 20 μL; DAD detector with a fixed wavelength of 520 nm; column temperature of 35 °C.

3. Results and Discussion

Reproducible quantitative analysis by reflectance measurements using a paper platform requires consideration of several factors that can influence the homogeneity and intensity of the spot test reaction.

According to Wendlant and Hecht[35], the color of the spot test should be uniform over the entire surface in order to ensure reproducible reflectance measurements. Here, consideration was made of important parameters described previously for spot test reactions[36,37], such as the rate of reagent addition, the quality of the filter paper, pH, and the volume of solution added. All these details are important for the uniformity of the color spot test. Investigations were carried out to establish the most favorable conditions for the spot test reaction on the filter paper, in order to achieve maximum color development at 530 nm.

The solutions were spotted onto the center of the filter paper using a micropipette fixed in a holder, according to the procedure described previously (section 2.4). The effects of pH and volume of acid buffer solution on the color intensity and uniformity of the spot test were optimized in univariate mode.

3.1. Evaluation of pH and volume

Tests were first performed to determine the most suitable pH for the measurements, because due to the presence of sulfonic acid groups in Amanranth dye, the spot shape and analytical response depend on pH. For analysis in acid solution, the selected buffer was acetic acid/sodium acetate with a measured pH of 4.3, while for analysis in alkaline solution the

buffer was ammonia/ammonium with a measured pH of 8.9. Analyses were also made using deionized water with a measured pH of 6.9.

The results (Table 2) showed that the highest absorbance was achieved at alkaline pH. This was expected, because for the analyte structure shown in Figure 1, the dye is completely deprotonated at alkaline pH, which maximizes the resonance between the electrons of the molecule, hence increasing the absorbance. Lower absorbance was expected in an acid solution, due to less effective resonance. However, the dye was more protonated and therefore less polar, and it was less efficiently eluted on the filter paper by the aqueous solution (a polar mobile phase). This resulted in the formation of a smaller stain that was more intensely concentrated in the center of the spot, so the absorbance was higher than expected in acid medium.

[Insert Table 2]

From the results described above, it was expected that alkaline pH would be most suitable for the analyses. However, in the currant syrup samples, Amaranth was present together with Caramel IV natural coloring, which has a brown color that interfered in the determination of the red-colored artificial dye. It would therefore be necessary to perform a separation of the dyes, keeping Amaranth in the center of the spot and Caramel IV at the edges of the spot.

For these reasons, acid pH was chosen for the determinations, because due to the lower polarity of Amaranth in an acid solution, it was retained in the center of the spot, while the Caramel IV was more easily eluted into the solid support (paper).

In order to improve the separation of the two dyes, after application of the sample, two consecutive 10 µL aliquots of acid buffer were added so that the Caramel IV was eluted to the edges of the stain, while the Amaranth dye remained retained in the center of the spot. The difference between the samples with and without the buffer elution can be seen in Figure 2.

[Insert Figure 2]

3.2. Analytical curve, limit of detection (LOD), and limit of quantification (LOQ)

In reflectance analysis, the optical density for reflectance measurements is described by A_R = - log T_R, analogous to absorbance[38]. The analytical curve was constructed using Amaranth standard solutions in a concentration range from 1.00 x 10^{-5} to 5.00 x 10^{-4} mol L^{-1}. A linear relationship was observed between A_R and [Amaranth]$^{1/2}$ (Figure 3), described by A_R

= -0.0176 + 14.556 [Amaranth]$^{1/2}$, with a correlation coefficient (R^2) value of 0.999 indicating an excellent linear relation. The LOD and LOQ values, determined according to IUPAC recommendations[39], were 1.13 x 10^{-6} and 1.25 x 10^{-5} mol L^{-1}, respectively.

[Insert Figure 3]

3.3. Study of matrix interferences

Possible matrix interferences were investigated by standard addition and recovery tests, with fortification of the samples with Amaranth standards at levels ranging from 50% to 250%. The recoveries obtained were between 90.5 and 98.8% for currant syrup, 90.1 to 99.4% for ice cream liquid base, and 95.5 to 103% for gelatin. These data indicated that the compositions of the three commercial products did not significantly interfere in the analysis, so sample clean-up steps were not required.

3.4. Determination of Amaranth dye in foodstuff samples

The proposed method was applied using six commercial samples: four currant syrups (A-D), one ice cream liquid base (E), and one gelatin sample (F). The results were compared with those obtained by the comparative method[11]. Statistical analysis using the Student's t-test showed that there was no significant difference between the results obtained using the two methods (Table 3).

[Insert Table 3]

Among the samples analyzed, it was noted that currant syrup samples A and D showed Amaranth dye contents above the limit permitted by Brazilian law. The other samples presented Amaranth dye contents below the permitted limit.

3.5. Performance of the proposed method for Amaranth determination

Compared with other methodologies previously reported in the literature for Amaranth assays (Table 4), the proposed reflectometric method showed lower sensitivity. Nevertheless, considering the quantities of Amaranth present in commercial products sold in Brazil, the proposed method is adequately sensitive for the determination of this analyte in such samples (Table 3).

Regarding dye extraction, the proposed method does not require a sample clean-up step and the separation of Caramel IV colorant (an interferent present in some samples) is made at

the moment of application to the paper, only requiring elution with two 10 µL aliquots of acid buffer.

The main advantage of this new methodology is the absence of the use of organic solvents harmful to health and the environment, as well as the low cost of the equipment used in the determinations, compared to the operating costs of the equipment used in other methodologies.

[Insert Table 4]

4. Conclusions

This study demonstrates the feasibility of employing diffuse reflectance spectroscopy coupled to a spot test on a filter paper support for the assay of Amaranth dye in food samples. The proposed method was applied to different types of samples and showed good performance for the analysis of Amaranth contained in foods. Compared to the methodologies generally used, this new method is much faster, simpler, avoids the use of organic solvents, and is more environmentally friendly, with minimal reagent use and waste generation. The sensitivity of the method is sufficient to quantify the analyte in the types of samples tested. This new technique is performed in aqueous medium, and no sample pretreatment steps or clean-up steps are required (except dilution or solubilization), making it simpler and more practical than other methods.

Acknowledgements

The authors are grateful to CNPq, CAPES, and FAPESP (process n° 2013/09701-1) for financial support.

References

[1] L. M. G. Antunes and M. C. P. Araújo, *Rev. Nutr.*, 2000, **13**, 81-88.

[2] A. P. Hutchinson, B. Carrick, K. Miller and S. Nicklin, *Toxicology Letters*, 1992, **60**, 165-173.

[3] K. T. Chung and C. E. Cerniglia, *Mutation Research*, 1992, **277**, 201-220.

[4] H. Zhu, W. Huang and F. Wang, *Advanced Materials Research*, 2014, **1010-1012**, 835-838.

[5] EFSA Panel on Food Additives and Nutrient Sources added to Food, *EFSA Journal*, 2010, **7**, DOI: 10.2903/j.efsa.2010.1649.

[6] Brazilian Health Surveillance Agency - ANVISA. Resolution No. 5, January 15th, 2007.

[7] J. J. Berzas-Nevado, C. Guiberteau-Cabanillas, A. M. Contento-Salcedo and R. Martín-Villamuelas, *Analytical Letters*, 1999, **32**, 1879-1898.

[8] M. Wang, Y. Sun, X. Yang and J. Zhao, *Food Chemistry*, 2015, **179**, 318-324.

[9] A. V. Jager, F. G. Tonin and M. F. M. Tavares, *Journal of Separation Science*, 2005, **28**, 957-965.

[10] M. J. Culzoni, A. V. Schenone, N. E. Llamas, M. Garrido, M. S. D. Nezio, B. S. F. Band and H. C. Goicoechea, *Journal of Chromatography A*, 2009, **1216**, 7063-7070.

[11] K. Ma, Y. N. Yang, X. X. Jiang, M. Zhao and Y. Q. Cai, *Chinese Chemical Letters*, 2012, **23**, 492-495.

[12] F. I. de Andrade, M. I. F. Guedes, I. G. P. Vieira, F. N. P. Mendes, P. A. S. Rodrigues, C. S. C. Maia, M. M. M. Ávila and L. M. Ribeiro, *Food Chemistry*, 2014, **157**, 193-198.

[13] S. Bonan, G. Fedrizzi, S. Menotta and C Elisabetta, *Dyes and Pigments*, 2013, **99**, 36-40.

[14] X. Q. Li, Q. H. Zhang, K. Ma, H. M. Li and Z. Ghuo, *Food Chemistry*, 2015, **182**, 316-326.

[15] F. Martin, J. M. Oberson, M. Meschiari and C. Munari, *Food Chemistry*, 2016, **197**, 1249-1255.

[16] General Standard for the Labeling of Prepackaged Foods, National Standard of the People's Republic of China (GB 7718-2004), Standard Press of China, Beijing, 2004.

[17] Y. Shen, X. Zhang, W. Prinyawiwatkul and Z. Xu, *Food Chemistry*, 2014, **157**, 553-558.

[18] Y. Song, L. Wu, N. Li, M. Hu and Z. Wang, *Talanta*, 2015, **135**, 163-169.

[19] K. Yamjala, M. S. Nainar and N. R. Ramisetti, *Food Chemistry*, 2016, **192**, 813-824.

[20] P. T. Anastas and M. M. Kirchhoff, *Accounts of Chemical Research*, 2002, **35**, 686-694.

[21] V. H. M. Luiz, L. Pezza and H. R. Pezza, *Microchemical Journal*, 2013, **109**, 68-72.

[22] L. S. Lima, P. L. Weinert, S. C. Lemos, R. Sequinel, H. R. Pezza and L. Pezza, *Spectrochimica Acta Part A*, 2009, **71**, 1999-2004.

[23] Y. Liu, Y. Ning, W. Cai and X Shao, *Analyst*, 2013, **138**, 6617-6622.

[24] R. N. Okparanma, F. Coulon and A. M. Mouazen, *Environmental Pollution*, 2014, **18**, 298-305.

[25] S. Seng and M. Kita, *Journal of Chemical Education*, 2007, **84**, 1803-1805.

[26] A. Kumar, A. Hens, R. K. Arun, M, Chatterjee, K. Mahato, K. Layek and N. Chanda, *Analyst*, 2015, **140**, 1817-1821.

[27] R. S. J. Alkasir, A. Rossner and A. Andreescu, *Environmental Science and Technology*, 2015, **49**, 9889-9897.

[28] M. O. Salles, G. N. Meloni, W. R. de Araújo and T. R. L. C. Paixão, *Analytical Methods*, 2014, **6**, 2047-2052.

[29] V. H. M. Luiz, L. Pezza and H. R. Pezza, *Food Chemistry*, 2012, **134**, 2546-2551.

[30] M. H. Sorouraddin and M. Saadati, *Sensors and Actuators B: Chemical*, 2010, **145**, 367-372.

[31] S. J. Lyle and M. S. Tehrami, *Journal of Chromatography A*, 1979, **175**, 163-168.

[32] F. Soponar, A. C. Mot and C. Sârbu, *Journal of Chromatography A*, 2008, **1188**, 295-300.

[33] S. Dixit, S. K. Khanna and M. Das, *Journal of AOAC International*, 2008, **91**, 1387-1396.

[34] W. Pregnolatto and N. P. Pregnolatto, *Normas Analíticas do Instituto Adolfo Lutz*, Instituto Adolfo Lutz, São Paulo, 1985.

[35] W.W. Wendlant and H.G. Hecht, *Reflectance Spectroscopy*, Interscience Publishers, New York, 1996, p. 297.

[36] L. Pezza, M. Tubino, C. B. Melios and H. R. Pezza, *Analytical Sciences*, 2000, **16**, 313-315.

[37] M. A. Gotardo, J. O. Tognolli, H. R. Pezza and L. Pezza, *Spectrochimica Acta A*, 2008, **69**, 1103-1109.

[38] A. Ghauch, C. Turnar, C. Fachinger, J. Rima, A. Charef, J. Suptil and M. Martin-Bouyer, *Chemosphere*, 2000, **40**, 1327-1333.

[39] M. Thompson, S. L. R. Ellison and R. Wood, *Pure and Applied Chemistry*, 2002, **74**, 832-855.

FIGURE CAPTIONS

Figure 1 – Structural formula of Amaranth dye.

Figure 2 – Spot tests without elution (left) and with elution using two 10 µL aliquots of acid buffer (right).

Figure 3 – Analytical curve of Amaranth dye.

Table 1 - Analytical methodologies for the determination of Amaranth in foods and beverages.

Matrix	Extraction procedure	Analytical Method	Observations	Ref.
Fizzy drinks	No extraction procedure involved	Spectrophotometry	Uses multivariate calibration method, acid buffer, and ethanol	7
Fruit drink	No extraction procedure involved	Square wave stripping voltammetry	Uses modified electrodes with graphene oxide	8
Not applied to real samples	Not applied	Fluorescence spectrometry	Requires 45 min for measurements	4
Fruit juice powders, lollipops, and hard and soft chewable treats	Hard samples were triturated, heated at 40 °C, diluted, and filtered through 0.45 µm membranes. Soft samples were diluted, vortexed, and centrifuged	Capillary zone electrophoresis method with UV/Vis detector	Uses acetonitrile, tetraborate, and surfactant	9
Soft drinks	Samples were homogenized and filtered through 0.45 µm membranes	HPLC with UV/Vis detector	Uses methanol as mobile phase and second order algorithms to quantify the analytes	10
Beverages	Performed based on the modified Chinese official analytical method[16]	HPLC with UV/Vis detector	Uses acetonitrile as mobile phase	11
Carbonated soft drinks	Samples were diluted with water and degassed in an ultrasonic bath. Amaranth dye was extracted using C_{18} SPE with isopropyl alcohol that was evaporated and filtered through 0.45 µm cellulose membranes	Dye identification by thin layer chromatography and quantification by HPLC with UV/Vis detector	Uses isopropyl alcohol and methanol as mobile phase in chromatographic identification and quantification, and in the extraction procedure	12
Beverages and solid matrices such as meat, cakes, and fruit	Beverage samples were diluted (1:1) with deionized water and centrifuged. Water-ethanol solutions were added to solid samples that were then homogenized, shaken, and centrifuged. The dye was extracted by polyamide SPE. The final extract was made up with methanol/ammonia solution	HPLC with UV/Vis detector	Uses acetonitrile as mobile phase and ethanol and methanol in the extraction procedure	13
Beverages, syrup, and chewing gum	Beverage samples were degassed or heated. All samples were evaporated and adjusted to pH 3. Syrup and chewing gum samples were also centrifuged and filtered through 0.45 µm nylon membranes. Amaranth dye was then extracted in a polyamide column, evaporated, and filtered through a 0.22 µm filter	HPLC with UV/Vis detector	Uses methanol and acetonitrile as mobile phase and ethanol in the extraction procedure	14
Sugar and gummy confectionary, ice-cream, and chocolate sweets	Samples were homogenized and solubilized with water and acetonitrile, or extracted with aqueous/organic phases and centrifuged. Amaranth dye was then extracted using SPE with ethanol and ammonia	HPLC - electrospray ionization-tandem mass spectrometry	Uses dry ice, liquid nitrogen, acetonitrile, hexane, and methanol in sample preparation. Mobile phase consists of acetonitrile and aqueous solution	15

Table 2 - Absorbance measurements (530 nm) at different pHs.

pH	Absorbance (A_R)[a]
4.3	0.483±0.009
6.1	0.419±0.012
8.9	0.536±0.021

[a] Average of three determinations.

Table 3 - Determination of Amaranth dye in food samples.

Sample	Concentration of Amaranth[a] (proposed method)	Concentration of Amaranth[a] (comparative method[11])	Calculated t[b]
A[c]	0.0053±0.0003	0.0053±0.0001	0.323
B[c]	0.0017±0.0001	0.0015±0.0001	4.252
C[c]	0.0024±0.0001	0.0024±0.0001	0.043
D[c]	0.0061±0.0001	0.0062±0.0001	0.666
E[d]	0.0025±0.0001	0.0023±0.0001	2.790
F[e]	0.0043±0.0003	0.0045±0.0001	1.704

[a]Results expressed in g(dye)/100 mL of product ready for consumption.

[b]Tabulated Student's t-value equal to 4.303 for two degrees of freedom and a confidence interval of 95%.

[c] Currant syrups;

[d] ice cream liquid base;

[e] gelatin sample.

Table 4 – Comparison of analytical performances of the proposed method and previously reported methodologies for the determination of Amaranth dye in different matrices.

Analytical Method	Linear range	LOD	LOQ	Ref.
Spectrophotometry	$3.3 \times 10^{-6} – 5.3 \times 10^{-5}$ mol L^{-1}	-	-	7
Square wave stripping voltammetry	$5 \times 10^{-10} – 4 \times 10^{-6}$ mol L^{-1}	1.0×10^{-8} mol L^{-1}	-	8
Fluorescence spectrometry	$1 \times 10^{-7} – 1 \times 10^{-3}$ mol L^{-1}	3.27×10^{-7} mol L^{-1}	-	4
Capillary zone electrophoresis with UV/Vis detector	-	$7.78 \times 10^{-7} – 3.80 \times 10^{-6}$ mol L^{-1}	-	9
HPLC with UV/Vis detector	$8.27 \times 10^{-8} – 4.96 \times 10^{-6}$ mol L^{-1}	-	-	10
HPLC with UV/Vis detector	$1.65 \times 10^{-7} – 4.91 \times 10^{-4}$ mol L^{-1}	4.30×10^{-8} mol L^{-1}	1.42×10^{-7} mol L^{-1}	11
Dye identification by thin layer chromatography and quantification by HPLC with UV/Vis detector	$4.96 \times 10^{-7} – 5.96 \times 10^{-6}$ mol L^{-1}	6.62×10^{-8} mol L^{-1}	1.49×10^{-7} mol L^{-1}	12
HPLC with UV/Vis detector	$1.65 \times 10^{-6} – 1.65 \times 10^{-4}$ mol L^{-1}	-	-	13
HPLC with UV/Vis detector	$7.44 \times 10^{-8} – 2.98 \times 10^{-5}$ mol L^{-1}	1.49×10^{-8} mol L^{-1}	7.44×10^{-8} mol L^{-1}	14
HPLC - electrospray ionization-tandem mass spectrometry	$1.65 \times 10^{-5} – 1.65 \times 10^{-4}$ mol L^{-1}	-	-	15
Diffuse reflectance spectroscopy	$1.00 \times 10^{-5} – 5.00 \times 10^{-4}$ mol L^{-1}	1.13×10^{-6} mol L^{-1}	1.25×10^{-5} mol L^{-1}	This work

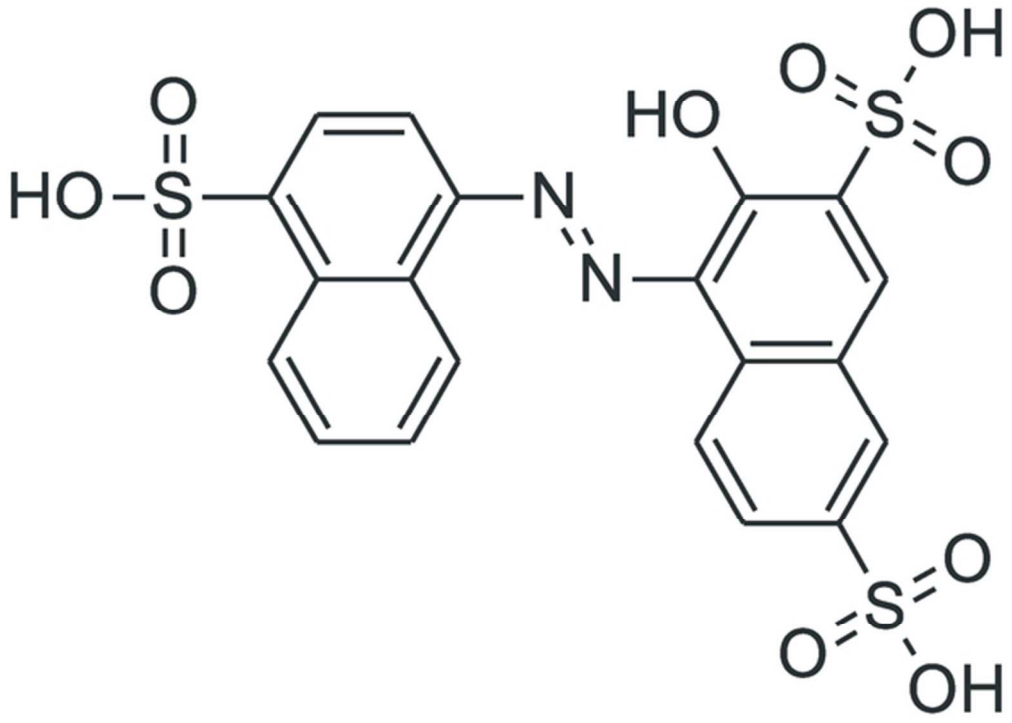


Figure 1 – Structural formula of Amaranth dye.
52x38mm (300 x 300 DPI)

Figure 2 – Spot tests without elution (left) and with elution using two 10 µL aliquots of acid buffer (right).
38x18mm (300 x 300 DPI)

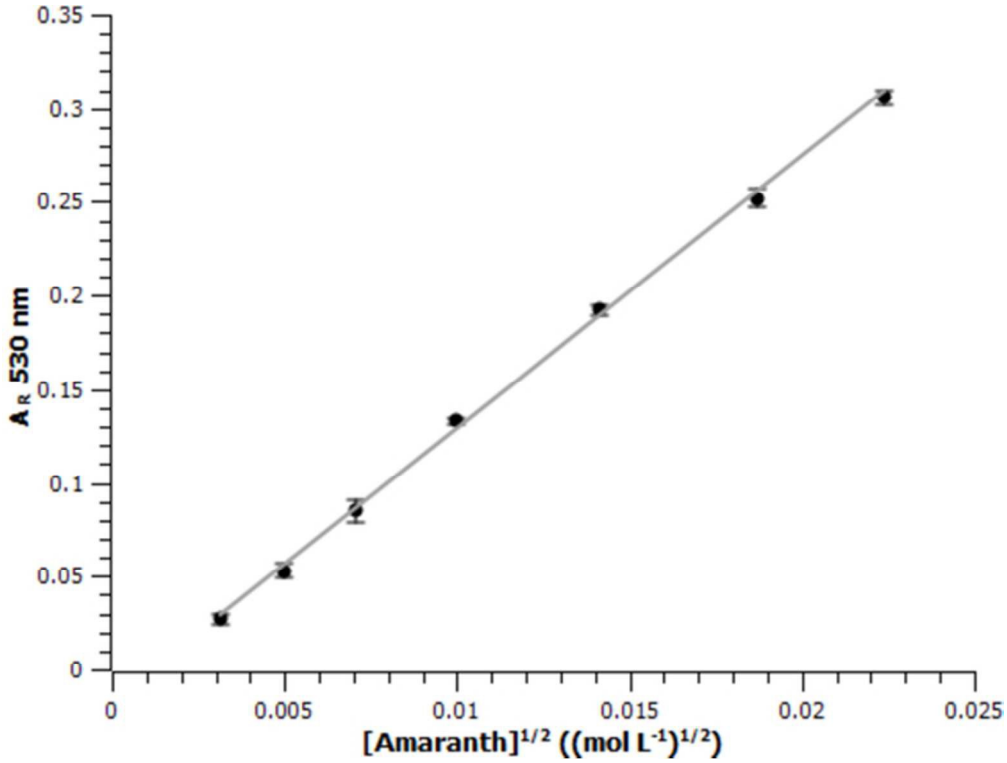
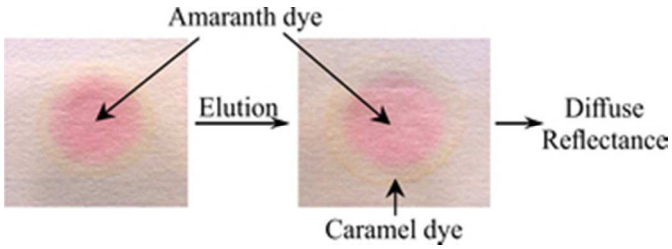


Figure 3 – Analytical curve of Amaranth dye.
54x40mm (300 x 300 DPI)



A green analytical methodology was developed employing diffuse reflectance spectroscopy for analysis of Amaranth in foodstuffs without clean-up steps.
28x9mm (300 x 300 DPI)