Certification of tartrazine, allura red and carmoisine content in powdered juice reference material

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Abstract
In this research work, the production of reference material of powdered juice certified for three of the water-soluble azo-dyes is described herein. The water-soluble azo-dyes under study are tartrazine, allura red, and carmoisine. Homogeneity and stability study of the prepared reference material were reported. Three independent analytical procedures were used for the certification. These procedures include two chromatographic methods and one spectrophotometric method. A statistical analysis of the measurement results was done, and the certified values of tartrazine, allura red, and carmoisine in powdered juice and their accompanied uncertainties were estimated as 2071.93±142.87, 34.66±1.67, and 5.043±0.476 mg/kg respectively. This reference material is a valuable tool for many food testing laboratories, especially in analytical methods' validation, confirmation and measurement results' quality assurance.

Keywords: Food additives; water-soluble azo dyes; certified reference materials; Homogeneity study; Certification of reference materials.

1. Introduction

Food colors play a crucial role in giving sufficient colors to food to attract consumers(Lin et al., 2018).Food colors include tartrazine, allura red, and carmoisine. They are water-soluble azo-dyes, which are approved in the European Union as color additives with E numbers of E102, E129, and E122 in that order. There is an approval for these azo-dyes in the United States by Fedral Food, Drug and Cosmetic Act (FD&C) as color additives and given the names of FD&C yellow 5 and FD&C Red 40 for tartrazine and allura red while carmoisine is banned. Also, there is an international approval for these azo dyes as color additives, and they are present in the list of Codex Alimentarius with International Numbering System (INS) number 102, 129, and 122(Bastaki, Farrell, Bhusari, Pant, & Kulkarni, 2017).

Tartrazine, allura red, and carmoisineare used in swiss rolls, jams, juice, yogurts, cheesecake mix, beverages, dietary supplements, pharmaceutical worldwide(Bastaki et al., 2017; Basu & Kumar, 2014).As mentioned earlier, food colors additives make food attractive to consumers by giving them the desirable color. There are different factors effects on the sensory acceptability of color on foodstuff for consumers. These factors comprise geography, local humanities, and social background. When the food color is used to identify flavor, it can be a strategic marketing communications purpose.

There are many studies on the effect of artificial food colors (AFC) on children for more than 35 years. In these studies, meta-analysis had shown that the hyperactivity and allergy were higher in children who consume AFC of an amount equal to or more than 50 mg than those who consume AFC < 50 mg. This amount is less than the acceptable daily intake (ADI) determined by US food and drug administration(Lin et al., 2018).There is a strong linkage between the effect of azo dyes and many diseases such as human bladder cancer, hepatocarcinmac, and splenic sacromas(Basu & Kumar, 2014).The Acceptable Daily Intake for tartrazine, allura red, and carmoisine were established recently to be 10, 7, and 4 mg/kg bw/day for the three water-soluble azo-dyes, respectively(Bastaki et al., 2017).

The analytical control of color additives, which are made by laboratories who care for the food industry has to verify that their test results are valid and reliable. That is why validation and quality control procedures must be usedto ensurethe results' validity. Appropriate matrix certified reference material should be used for such control. The assessment of laboratory

performance can be made using matrix certified reference materials (CRMs)(Ahn, Lee, Lee, &Kim, 2016; Chew et al., 2016; Linsinger, Pauwels, van der Veen, Schimmel, & Lamberty, 2001; Olivares, Souza, Nogueira, Toledo, & Marcki, 2018; Van Leeuwen et al., 2006). They play a crucial role in quality control(Chew et al., 2016), assure the measurement results' accuracy, perform the traceability of measurement results to the SI unit, and for measuring instrument calibration as well as estimation of uncertainty of the measurement results(Tahoun, Yamani, & Shehata, 2019).

Reference materials production compriseshomogeneity study, stability study,characterization, and value assignment. This present work described herein provides a matrix reference materialfrom powdered juice to be certified for tartrazine, allura red, and carmoisine**.**This CRM will be valuable in the food industry, especially in the food additives section(Shehata, Rizk, Farag, & Tahoun, 2015).

2. Materials and methods

2.1   Chemicals and reagents

High-performance liquid chromatography (HPLC) grade solvents methanol and acetonitrile, ammonium acetate (98%), sodium acetate trihydrate (99%) and hydrochloric acid (36.5-38%) were purchased from Sigma-Aldrich (Germany). Ammonia solution (33%) were purchased from Adwic. High purity water was obtained through a Milli-Q water purification system (Millipore, Bedford, MA, USA). Tartrazine and carmoisine powder for standard preparation were purchased from Alpha (India), while allura red powder was purchased from Loba (India).

2.2   The chromatographic system used for determination of azo-dyes

The chromatographic analysis was carried out using HPLCAgilent 1100 integrated system equipped with a G1313Aautomated injector, a G1311A pump, and a G1315B multiwavelength diode-array detector (Agilent, Santa Clara, CA, USA).The chromatographic separation of the compounds wasachieved with a reversed-phase column, Eclipse XDB-C18(4.6 $\times$150 mm, 5 µm) (M1), Zorbax NH$_2$ (9.4 $\times$ 250 mm, 5µm) from Agilent (M2). The chromatographic data were analyzed usingAgilent Chemstation Rev. B.02.01-SR1 (260). The

compoundsunder study were identified by their retention times and theirUV spectral characteristics.

2.3   *UV-visible spectrophotometer*

The purity measurement was carried out using UV-Vis spectrophotometer Analytik Jena specord 250 plus equipped with 15 samples tray as method 3 (M3). Measurements were carried out using a glass cell for the range covering 430, 504, and 516 nm. The UV spectrum containsabsorption bands; to get information about the wavelength of the individual bands of a complex absorption spectrum; the resolution of it into individual band is a mandatory by a deconvolution process. The deconvolution was performed using originLab 8 software by employing Gaussian symmetric peak fitting.

There are three different parameters that are used to characterize a symmetrical spectral band. These parameters are: position (wavelength), intensity (absorbance), and width (Full width at half maximum [FWHM]). The deconvolution of the UV/Vis spectra is performed by the Gaussian function. When the independent variable λ is expressed in nm, the approximation of the spectral bands can be done using the Gaussian line shape. Equation 1 is used to describe the absorbance A at a wavelength λ:

$$A = \ A_{max} \ \exp\left[-(4\ln 2)\frac{(\lambda - \lambda_{max})^2}{\Delta\lambda^2}\right], \tag{1}$$

where A_{max} is the band maximum absorbance, λ_{max} is the wavelength at Amax and $\Delta\lambda$ is the band half width.

2.4   *Materials processing and packaging*

Powdered juice samples were obtained fromlocal markets (Cairo, Egypt). 200 grams of powdered juicewere first groundedthen filtered through 25µmmesh sieve for obtaining homogenized material and packed in 5g brown glass bottles to prevent light exposure and humidity uptake; the bottles were stored at 4°C.

2.5   *Sample preparation*

Azo-dyes understudy in powdered juice was extracted by taking 0.25 g of powdered juice in 5 ml water and shaking with vortexer for 1 minute. The sample was then heated in a water

bath at 100˚C for 10 minutes then filtered through a 0.45 µL nylon syringe filter into a 1 ml amber glass vial. The filtrate contains the compounds understudy.

2.6   Chromatographic separations

10 µLwas injected into the HPLC system. The separation wascarried out using Eclipse XDB C18 (4.6×150 mm, 5 µm) (M1) orZorbax NH_2 (9.4 × 250 mm, 5 µm) (M2) at flow rate 1, 1.5mL/minrespectively using step gradient from methanol and watermixture as shown in Table 1.

Table 1: The Elusion parameters for the two methods of HPLC

M1				M2			
Time (min)	0.1M Ammonium acetate solution (%)	Acetonitrile (%)	Flow rate (ml/min)	Time (min)	0.7 M Sodium acetate trihydrate (%)	Methanol (%)	Flow rate (ml/min)
0	95	5	1	0	90	10	1.5
20	40	60	1	8	80	20	1.5
21	95	5	1	12	70	30	1
				18	70	30	1
				25	60	40	1.5
				30	90	10	1.5

3. Results and discussion

Three methods were used for the selected azo-dyes characterization in powdered juice. Two different HPLC methods, the columns are different and one spectroscopic method. In the case of the chromatographic methods, water was used for the extraction, while the separation was made by Eclipse C18 (M1) and Zorbax NH_2(M2) columns. The chromatograms of M1and M2 are shown in Figure 1 and 2. It is obvious fromthese figures thatthe azo-dyes understudy was separated in powdered juice.However method 3 (M3) revealed that the spectra of the sample hadundergone deconvolution using origin, giving results that are very close to the chromatographic methods, as shown in Figure 3.
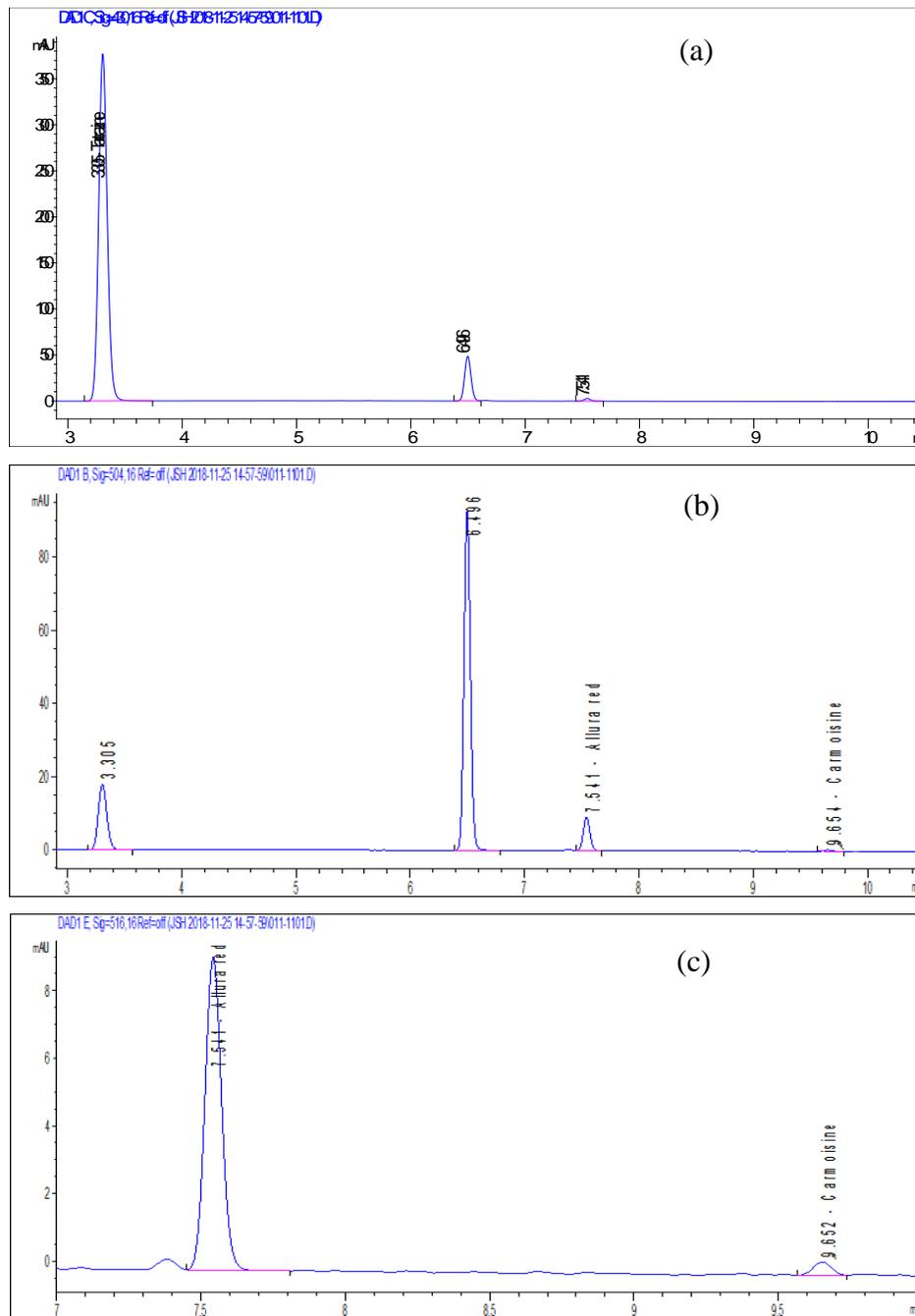
Figure 1 HPLC chromatogram M1 for powdered juice samples a) at 430nm b) at 504nm and c) at 516 nm
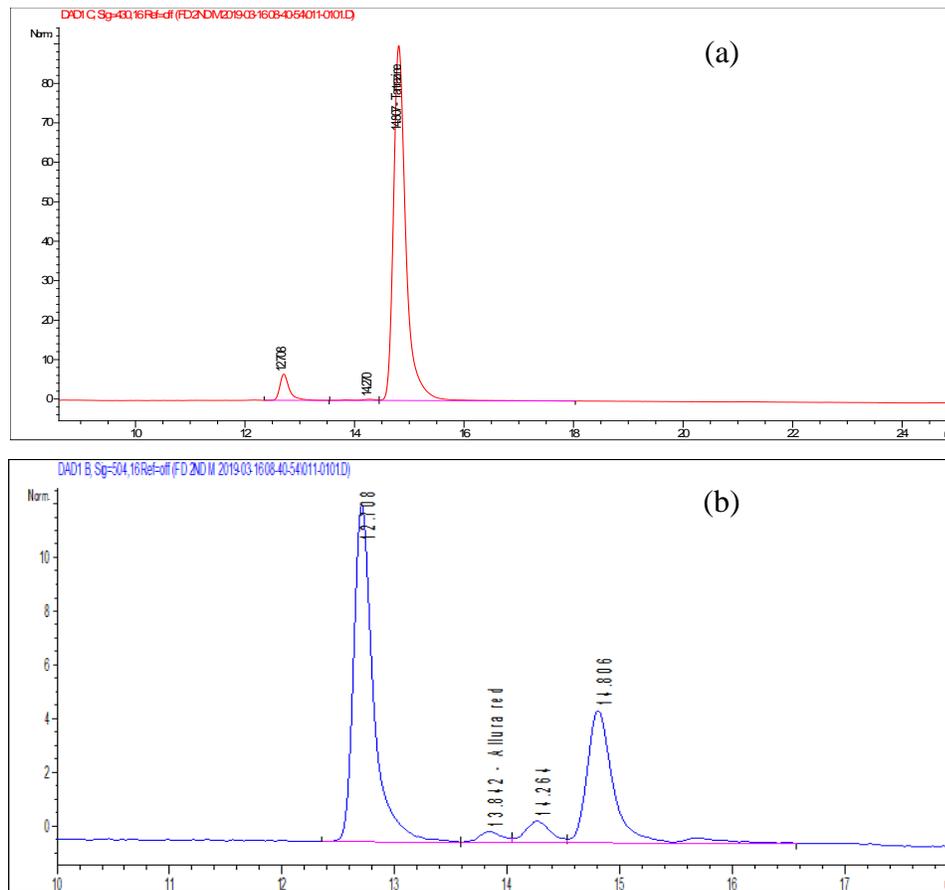
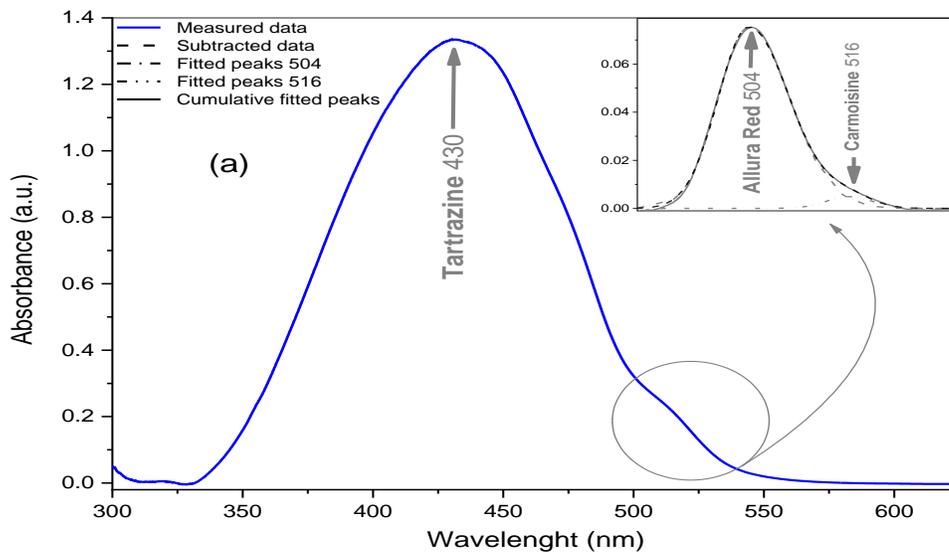**Figure 2 HPLC chromatogram M2 for powdered juice samples a) at 430nm and b) at 504nm**

Figure 3 The UV Absorbance curve of tartrazine, allura red and carmoisine in powdered juice

3.1   Homogeneity study

The between-bottle variability and the within-bottle variability can bestudied in the homogeneity study. The quantitative determination of inhomogeneity of the materials was achieved by the analysis of five bottles that were randomly selected from the whole set of 25 bottles for thematrix, and five independent subsamples of each bottle. This scheme was repeated once, leading to a total offive sample preparations per unit. All samples were analyzedby HPLC/DAD with Eclipse-XDB-C18 column (M1)under repeatabilityconditions ensuring that all samples were quantified againstthe same calibration. The results of the homogeneity studiesare shown in Tables 3-5.

3.2   Stability study

Azo-dyes in powdered juice candidates for certification was submitted to stability studies(Jiménez, Ventura, Segura, & De la Torre, 2004; Lee et al., 2010).Assessment ofthe stability was performed through the analysis of samples at three-time points (0 months, two months, four monthsand six months)at 4˚C and 20˚C. Five samples were analyzed at each time pointat each temperature. Two replicates per sample were analyzed,and the average results are shown in Tables6-8.

3.3   Traceability of measurements

In this part of the work, metrological traceability of the measurement results was based on the purity of tartrazine, allura red, and carmoisine purchased from Alpha and Loba traceable mass and volume measurements and appropriate uncertainties. For calibration of HPLC/DAD system with Eclipse-XDB-C18 column, stock solutions of tartrazine, allura red, and carmoisine were prepared by weighing 0.02019 g, 0.02104 g and 0.01994 g in 50.015051g, 52.66753 and 49.94592 g ultrapure water respectively. Fourteen calibration solutions were prepared from each stock solution as follows: 4.4-177.3 mg/kg for tartrazine, 0.11-42.90 mg/kg for allura red, and 0.16-42.27 mg/kg for carmoisine.

The calibration solutions from each range were injected into the HPLC/DAD system with C18 Eclipse column. Meanwhile, for calibration of the HPLC/DAD system with Zorbax NH2 column, stock solutions of tartrazine and allura red and were prepared by weighing 0.0126g and 0.00986 g in 50.67204 g and 56.10394 g in ultrapure water respectively. Eight calibration

solutions were prepared from tartrazine stock solution as follows 50-130 mg/kg for tartrazine and five calibration solutions were prepared from allura red stock solution of mass fractions 0.5-3.5 mg/kg. These calibration solutions were injected into the HPLC/DAD system with Zorbax NH2 column. Examples for some of the obtained calibration curves are shown in, Figure 4, 5, and 6 for method 1, 2, and 3 respectively. The linearity of the calibration curves for each method was checked by calculation of the residuals and plotting them, as shown in Figure 7. It can be seen that the residuals in all calibration curves are randomly distributed on both sides of the zero-axis, which confirms the linearity of the curves.
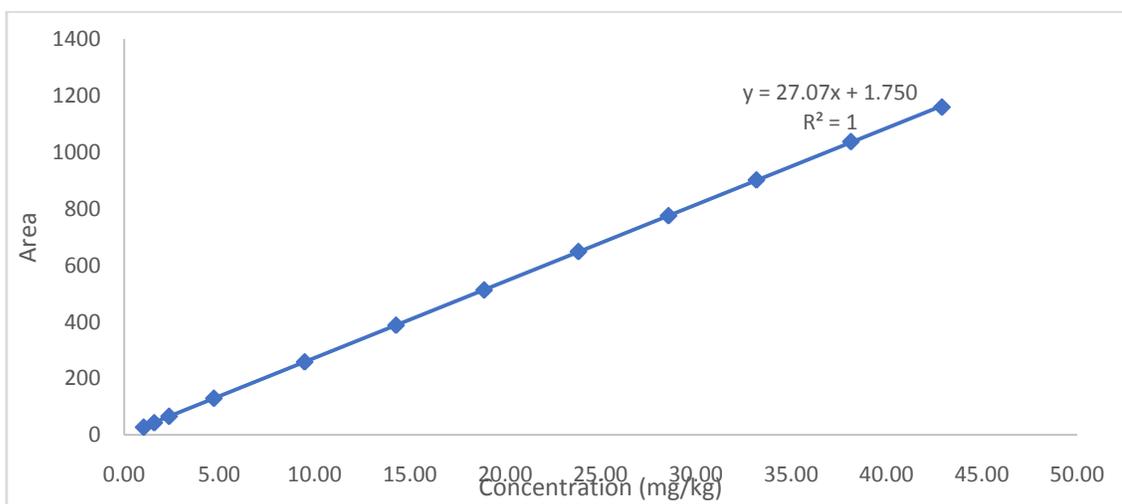

Figure4Calibration curve of HPLC/DAD with Eclipse RP column (M1) by allura red
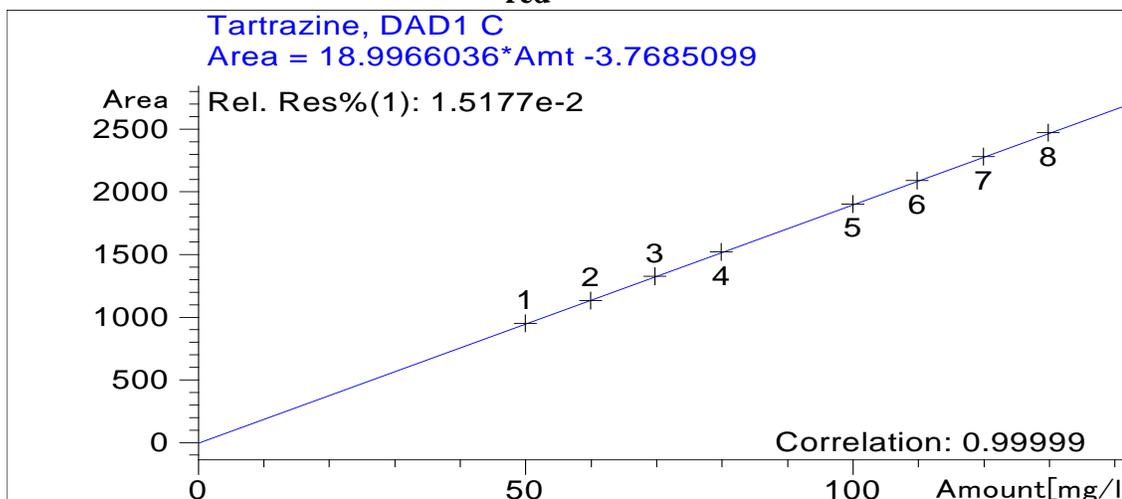

Figure5 Calibration curve of HPLC/DAD with Zorbax NH2 column (M2) by tartrazine
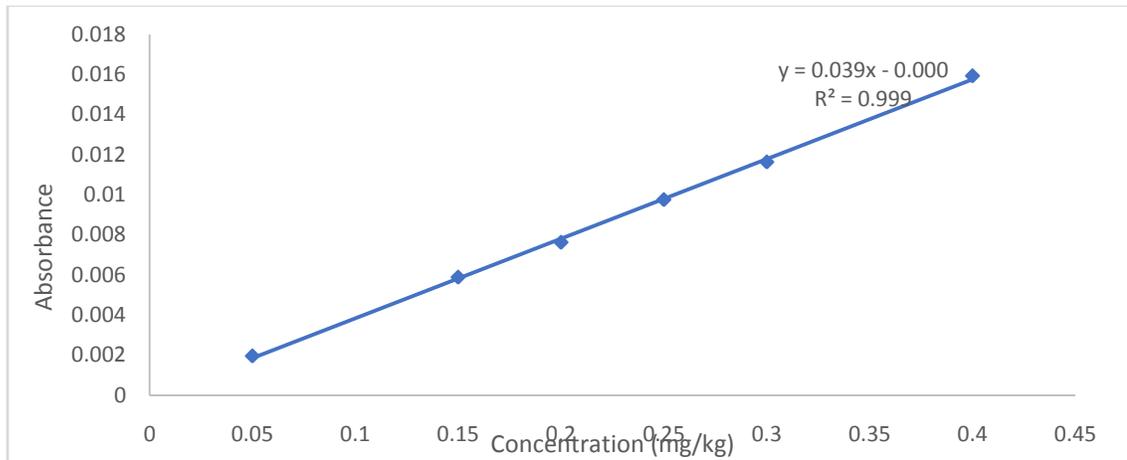
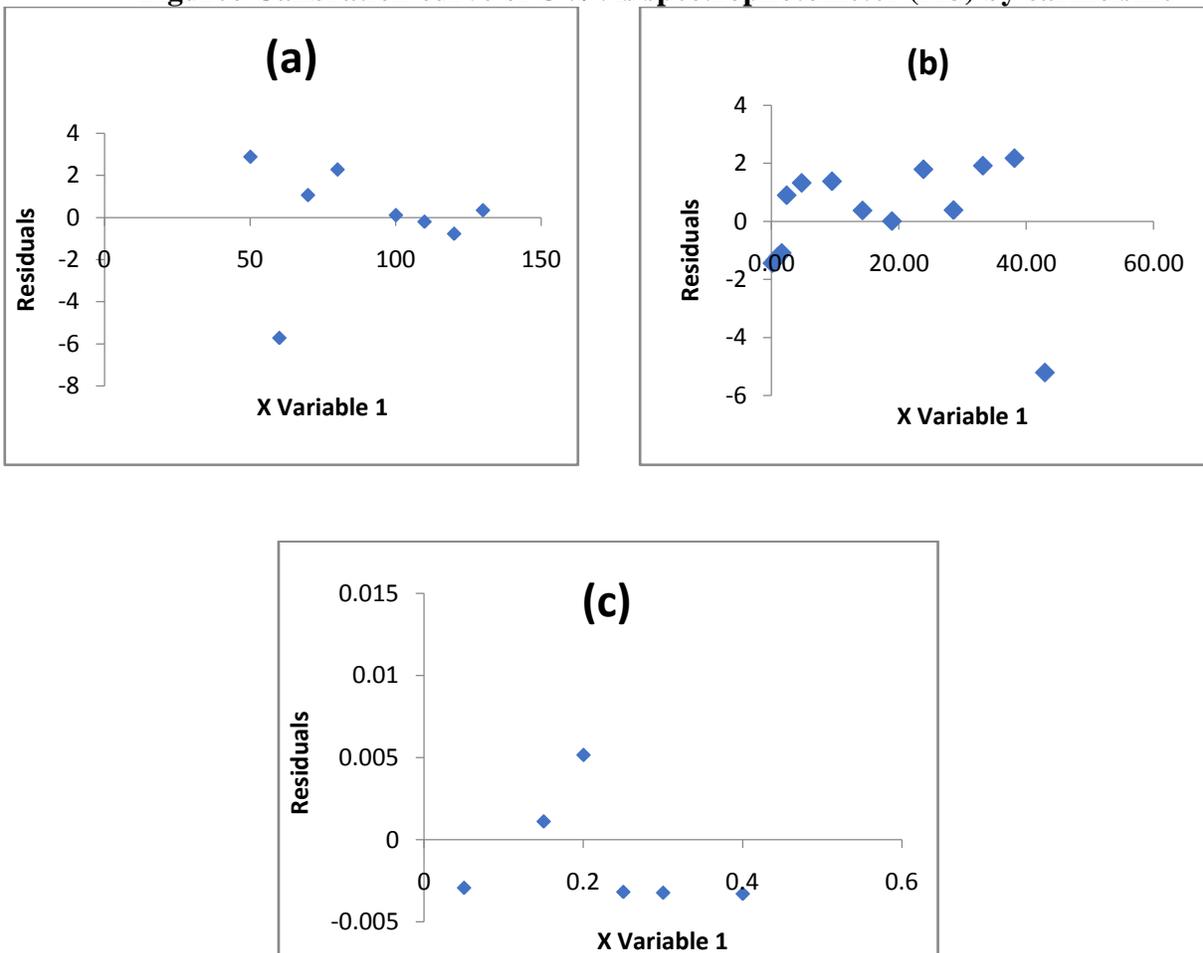**Figure6 Calibration curve of UV/Vis spectrophotometer (M3) by carmoisine**

Figure 7 Residual errors of the calibration curves of (a)HPLC-RP column by tartrazine, (b) HPLC-NH$_2$ column by standard allura red and (c)UV/Vis spectrophotometer by standard carmoisine

3.4   Statistical treatment of data

3.4.1 Normality (Kolmogorov-Smirnov test)

Normality tests were used to examine the normal distribution of the data obtained from three different analytical methods. The probability value (P-value) is an indication of the normality of the data. When it is more significant than 0.05, this means that the measurement results undergo the normal distribution model as shown in Table 2. Figure 8 is an example for the normal probability plot in which the concentration results (mg/kg) of allura red have been plotted against the percent cumulative frequency and the linear distribution is evidentfor the normality of the data. The percent cumulative frequency can be calculated from equation 2:

$$\% \; cumulative frequency = \frac{100 \times cumulative frequency of each measurement}{(n+1)} \quad (2)$$

A normality test was performed using Minitab 16.

Table 2: The p-value obtained from the normal probability plot

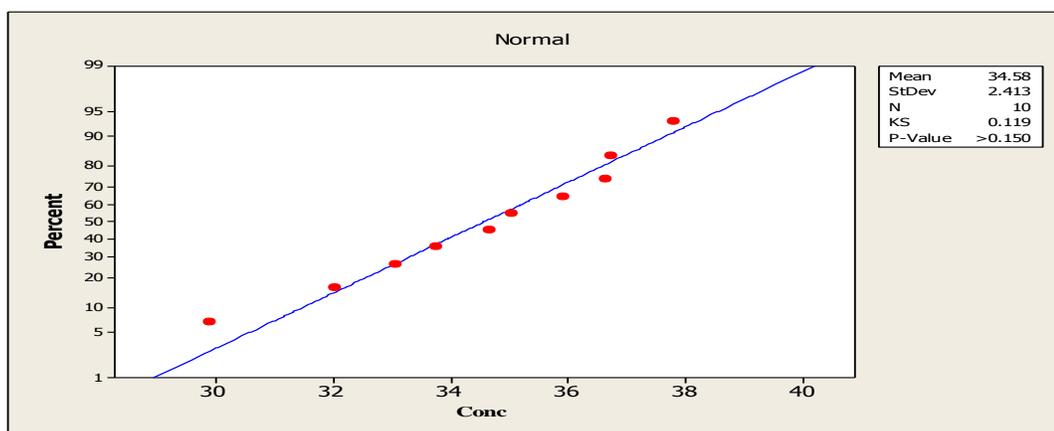| Analyte	Method	p-Value
Tartrazine	M1	>0.150
	M2	0.086
	M3	>0.150
Allura red	M1	>0.150
	M2	>0.150
	M3	>0.150
Carmoisine	M1	>0.150
	M3	>0.150

Figure 8Normal probability plot for allura red in powdered juice RM by M2

3.4.2 Dixon's tests for outlier

The Dixon's test (Q-test) is performed for identification and rejection of outliers for univariate data of sample size up to 30. The data was verified to be normally distributed before applying this test. This test can be performed for both high and low outliers by the following procedure:

1- Arrange the n results in ascending order to give the values x_1 to x_n
2- Calculate the statistic Q from equation 3

$$Q = \frac{Suspected value - Nearest value}{Largest value - Smallest value} \tag{3}$$

3- Compare the statistic Q with the critical values.
4- If the critical value is larger than statistic Q this means that the relevant observation is not outlier.

Costat statistical software was used for outlier detection. Outliers were detected and eliminated according to the test recommendation(Ellison, Barwick, & Farrant, 2009). According to this information, the outliers were eliminated. The P-value after outliers elimination was > 0.05.

3.4.3 Homogeneity and stability measurements

3.4.3.1 Powdered juice matrix reference material homogeneity

The results in Tables 3-5were analyzed by ANOVA, and the uncertainty of the material variability was calculated. The judgement of the material homogeneity can by evaluated through this kind of uncertainty(Linsinger et al., 2001; Olivares et al., 2018; Van Leeuwen et al., 2006). From the ANOVA,we have found that F-calculated is smaller than F-critical, which means that the powdered juice reference material was homogeneous. Uncertainty of the material homogeneity σ_h was calculated according to equation 4(Jiménez et al., 2004; Lee et al., 2010; Pauwels, Lamberty, & Schimmel, 1998)and was found 23.32 for tartrazine, 0.598 for allura red, and 0.033 for carmoisine as reported in Tables 3-5.

$$\sigma_h(u_{bb}) = \sqrt{MS_{within}/n} \cdot \sqrt[4]{2/\upsilon MS_{within}} \tag{4}$$

Table 3: Mass fraction (mg/kg) of tartrazine in powdered juice RM

G1	G2	G3	G4	G5	σ_h
1958.79	1992.91	1874.84	1904.76	2116.2	
1941.26	2020.1	2018.29	2024.35	2047.84	
1905.92	2186.65	2194.93	2024.95	2047.86	23.32
2028.95	2213.76	1892.72	2034.14	1970.96	
2049.28	2204.78	1898.07	2203.97	2096.35	

Table 4: Mass fraction (mg/kg) of allura red in powdered juice RM

G1	G2	G3	G4	G5	σ_h
32.53	35.45	33.79	36.82	32.06	
32.50	29.76	36.75	38.28	37.20	
37.96	33.07	34.02	36.67	29.66	0.598
31.03	29.93	36.28	37.81	32.55	
38.62	35.93	34.69	35.06	33.25	

Table 5: Mass fraction (mg/kg) of carmoisine in powdered juice RM

G1	G2	G3	G4	G5	σ_h
4.87	5.13	5.02	4.84	5.08	
4.84	5.15	5.05	5.31	5.00	0.033
4.95	5.18	5.02	4.95	5.13	
4.96	5.01	4.79	4.94	4.97	

Note: G refers to group

3.4.3.2 Stability of the powdered juice matrix reference material

The stability study measures a sample as a function of time. It can be seen from the results that are shown in Tables6-8 at storage temperatures of 4°C, and 20 °C that statistically significant trends were not observed along the six month storage period, which indicates the material stability. The uncertainty of the material stability (S) was measured from equation 5(van der Veen, Linsinger, Lamberty, & Pauwels, 2001):

$$S = \sum_{i=1}^{n} \varepsilon_i^2 = \sum_{i=1}^{n} (Y_i - \beta_0 - \beta_i X_i^2) , \qquad (5)$$

where ε_i is the random error, β_0 is the solpe, β_i is the intercept, Y_i is the concentration of azo dyes under study and X_i is the time.

Table 6: Mass fraction (mg/kg) of tartrazine in powdered juice RM

Time(0-6M) Temperature	0 M	2 M	4 M	6 M	Slope	S
20 ∘C	2090	2066	2072	2093	0.7263	0.52751
4 ∘C	2094	2073	2129	2072	0.3740	0.13988

Table 7: Mass fraction (mg/kg) of allura red in powdered juice RM

Time(0-6M) Temperature	0 M	2 M	4 M	6 M	Slope	S
20 ∘C	34.62	35.43	33.33	35.20	-0.0185	0.00034
4 ∘C	34.30	33.30	34.55	33.9	0.0022	0.000005

Table 8: Mass fraction (mg/kg) of carmoisine in powdered juice RM

Time(0-6M) Temperature	0 M	2 M	4 M	6 M	Slope	S
20 ∘C	5.045	5.065	5.14	5.085	0.0097	0.00009
4 ∘C	5.050	4.990	4.980	5.070	0.0025	0.000006

3.4.4 Uncertainty of the measurement results

Uncertainty is defined as 'a non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used'(De Bièvre, 2012). Measurement uncertainty comprises, in general, many components.In the present certification work, sources of uncertainty come from the calibration of measuring instruments, repeatability of measurements, material variability (homogeneity and stability), and the bias allowance. The contribution of each of these sources will be described, and the combined standard uncertainty will be calculated based on ISO GUM (De Bièvre, 2012).

3.4.4.1 Uncertainty of the calibration process

Uncertainty of the calibration of HPLC/DAD-Eclipse XDB-C18, HPLC/DAD- Zorbax NH_2 and the UV-Vis spectrophotometer come from purity of the tartrazine, allura red and carmoisine provided by Alpha and Loba (0.196, 0.146, 0.172%), gravimetric dilution of tartrazine, allura red and carmoisine and the slope and intercept of the calibration curve. The combined standard uncertainty can be described from equation 6:

$$Combined standard duncertainty = \sqrt{(TypeA Standard uncertainty)^2 + u_{cal}^2} \quad (6)$$

Type A standard uncertainty can be expressed from the following equation:

$$TypeA = \sqrt{Variance(\bar{y})} \quad (7)$$

$$\text{Variance}(\bar{y}) = \frac{MS_{rows} + MS_{column} - MS_{error}}{Total number of measurement}, \quad (8)$$

where MS_{Rows} is the average mean square of the rows, MS_{error} is the average mean square of the error, MS_{column} is the average mean square of columns, n is the total number of measurement in each method.The uncertainty of the calibration process, type A standard uncertainty and the mean of each method are reported in Table 9.

Table 9: Methods arithmetic mean and type A standard uncertainty

Analyte	Method	Mean	Type A Standard Uncertainty	u_{cal} Calibration	Combined Standard Uncertainty
Tartrazine	M1	2032.03	16.226	0.338	16.230
	M2	2069.29	14.702	0.197	14.703
	M3	2114.52	16.157	0.308	16.160
Allura red	M1	34.58	0.554	0.154	0.575
	M2	34.56	0.266	0.197	0.331
	M3	34.85	0.203	0.382	0.433
Carmoisine	M1	5.021	0.117	0.107	0.192
	M2	4.959	0.127	0.004	0.172

3.4.4.2 The between method variance and method weights

The weight of each method is inversely proportional to its variance. Using method weights gives an unbiased estimate of the true value with minimum variance.(Epstein, 1991; Susannah B Schiller, 1996). Using an algorithm for the between method variance and the square of its combined standard uncertainty estimation is described in the Paule-Mandel weighing scheme. The method weights are defined as described in equation 9, 10 and their values were expressed in Table 10.

$$W_i = [1/(S_i^2 + \sigma_b^2)], \quad (9)$$

$$\text{where } \sigma_{b=} \sqrt{\frac{MS_{between} - MS_{within}}{number of runs}}$$

The weighing factor is

$$w_i = {W_i}\Big/{(\sum_1^M W_i)} \tag{10}$$

Table 10: The between method variance, method weights and weighing factor for each method

Analyte	Method	Between method variance	Method weight (Wi)	Weighing factor(wi)
Tartrazine	M1	7.52	0.0031	0.314
	M2		0.0037	0.369
	M3		0.0031	0.317
Allura red	M1	0.255	2.55	0.208
	M2		5.73	0.468
	M3		3.96	0.323
Carmoisine	M1	0.034	0.040	0.500
	M2		-	-
	M3		0.040	0.500

3.4.4.4 Certified value (weighted mean) and its uncertainty

The certified value can be obtained from the weighted mean of the method means which was computed according to the weighting algorithm of Paule and Mandel and is often implemented for combining data from independent chemical analysis methods (Epstein, 1991; Hässelbarth, Bremser, & Pradel, 1998; May et al., 2000; Nalobin & Osintseva, 2007; S B Schiller & Eberhardt, 1991; Susannah B Schiller, 1996). The weight for each method is inversely proportional to the sum of the variance of its mean and the between-method variance. The weighted average of the X_i^-.

$$\tilde{X} = \sum_1^M w_i \bar{X}_i \tag{11}$$

The combined standard uncertainty S for the weighted mean is calculated as:

$$S = \sqrt{\sum_1^M w_i^2 S_i^2} \tag{12}$$

Using this weighting scheme, the method weights, the weighted means, the weighted mean, and the weighted uncertainty have been calculated, and the results are tabulated in Table 11.

Table 11: Weighted mean and weighted uncertainty (S) for tartrazine, allura red and carmoisine in powdered juice

Analyte	Method	Weighted mean	Assigned value	Weighted uncertainty (S)
Tartrazine	M1	638.95	2071.89	15.64
	M2	763.31		
	M3	669.63		
Allura red	M1	7.21	34.66	0.414
	M2	16.18		
	M3	11.27		
Carmoisine	M1	2.50	5.05	0.203
	M2			
	M3	2.55		

3.4.4.5 Uncertainty of the bias allowance

Bias allowance is a systematic error due to the difference in methods. It is taken as the maximum absolute deviation of any method mean from the weighted mean, as expressed in equation 13(Epstein, 1991; JCGM, 2008; S B Schiller & Eberhardt, 1991). It has been calculated and reported in Table12.

$$Bias allowance = max_i |\bar{X}_i - \tilde{X}| \tag{13}$$

3.4.4.6 The certified uncertainty

The degrees of freedom were calculated according to equation 14based on the data obtained from method weights and weighted uncertainty and were found 31.31, 31.64 and 19.67 for tartrazine, allura red, and carmoisine respectively

$$df(effective) = \frac{\left(\sum_1^M w_i^2 S_i^2 + \hat{\sigma}_h^2\right)^2}{\left(\sum_1^M \frac{(w_i^2 S_i^2)^2}{n_i - 1} + \frac{\hat{\sigma}_h^4}{df_h}\right)}, \tag{14}$$

where w_i is the weighing factor, S_i is the weighted uncertainty, σ_h is the material variability, n_i is the total number of measurement for each method and df_h is number of samples measured for material variability estimation minus one.

The weighted uncertainty was calculated according to the scheme explained above and is given in Table 12. The certified uncertainty was calculated according to equation 15, including the estimate of the bias allowance and reported in Table 12.

$$U = t_{1-\alpha/2}\sqrt{S^2(\tilde{X}) + \sigma_h^2 + u_{stability}} + Bias.\,allowance, \tag{15}$$

where S_x^2 is the weighted combined standard uncertainty and σ_h^2 is the uncertainty of the material in-homogeneity.

Table 12: Certified value and expanded uncertainty for each compound

Analyte	Assigned Value(mg/kg)	Bias allowance	σ_h	$u_{stability}$	U_{exp} (%)	Type
Tartrazine	2071.89	42.62	23.32	0.817	6.97	Certified value
Alllura red	34.66	0.437	0.598	0.019	2.58	Certified Value
Carmoisine	5.049	0.084	0.033	0.010	9.82	Certified value

4. Conclusion

A matrix reference material of powdered juice has been developed for the content of tartrazine, allura red, and carmoisine by three independent analytical methods in full compliance with ISO Guide 30-35. The methods were based on either using different instruments or separation using different columns. Statistical treatment of the measurement results had been accomplished; the assignment of the certified values for tartrazine, allura red, and carmoisine in powdered juice was achieved, and their expanded uncertainties were estimated as 2071 (mg/kg) $\pm6.97\%$, 34.66 (mg/kg) $\pm2.58\%$, and 5.049 (mg/kg) $\pm9.82\%$ respectively. It has been found from the certified values and their associated uncertainties that the level of uncertainties is acceptable, and this is because of the good homogeneity and stability of powdered juice. This reference material affords the foundation for more development in quality control and quality assurance of food color analysis in powdered juice. It is not only valuable in proficiency testing

schemes and inter-laboratory comparisons but also for the validation of a method. Thus, it can be concluded that the prepared reference material seems to be very promising for color additives.

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