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Cover Page Footnote

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ORIGINAL ARTICLE

Three-way analysis-based pH-UV-Vis spectroscopy for quantifying allura red in an energy drink and determining colorant's pKa

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Abstract

Three-way analysis-based pH-UV-Vis spectroscopy was proposed for quantifying allura red in an energy drink product without the need for chromatographic analysis, and determining the colorant's pKa without using any titration technique. In this study, UV-Vis spectroscopic data matrices were obtained from absorbance measurements at five different pH levels from pH 8 to pH 12 and arranged as a three-way array (wavelength x sample x pH). In the three-way analysis procedure, parallel factor analysis (PARAFAC) was implemented to decompose the three-way array into a set of trilinear components. Each set of three components relates to spectral, pH and relative concentration profiles of allura red and sample matrix in the energy drink. First, UV-Vis spectra of the colorant's acid-base pair and sample's matrix were characterized by using the estimated spectral profile. Then, from the pH profile the pKa value was found to be 11.28 for the related colorant. Finally, allura red in energy drink samples was determined using the estimated concentration curve in the relative concentration profile. In the quantitation procedure, the working concentration range was 0.8-19.2 μg/mL. PARAFAC approach was tested in terms of selectivity, precision, and accuracy of the method. Added recovery results obtained by applying the proposed method to spiked samples were between 101.5% and 103.5%. In the application of the method to the analysis of real samples, successful results were reported. For a comparison, an ultra-performance liquid chromatographic method was developed for the quantitation of the colorant. Compared to the chromatographic method, we observed that PARAFAC model was simple and less expensive without requiring separation.

Keywords: Allura red AC dye, Energy drinks, pKa estimation, Quantitation, Three-way analysis

1. Introduction

The analysis of various food products is usually accomplished by hyphenated chromatographic methods, such as ultra-performance liquid chromatography with photodiode array detector and mass spectrometry detector (UPLC-PDA and UPLC-MS), which are designed to eliminate matrix effects. However, these techniques suffer from major disadvantages, such as being high-cost, time consuming, and need for well-trained workforce [1, 2]. In contrast, UV-Vis spectroscopy provides low-cost and simple analysis but it suffers from poor selectivity and interference from food matrix [3]. The use of three-way analysis methods for UV-Vis spectroscopic data can overcome the interference problem while maintaining the advantages of spectroscopy. [4, 5]. Moreover, three-way analysis-based spectroscopy may be used to reveal multiple characteristics of the analyte. For example, both the quantity and the pKa of an analyte can be simultaneously determined by three-way analysis of pH-UV-Vis absorbance data. In previous works, a three-way analysis method was applied to pH-absorbance dataset for the simultaneous determination of the active pharmaceutical ingredients in syrup formulations

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and their pKa value in spite of the matrix effects [6, 7]. Various three-way analysis tools [8-13] have been used to solve complex problems in different fields of analytical chemistry [14-24]. However, three-way analysis methods have not been used for determining an analyte and its pKa value in food samples in literature. In this regard, we concluded that PARAFAC (parallel factor analysis) decomposition of spectroscopic data would provide a novel perspective for mathematical elution of analyte and interferents in food samples.

In food industry, several additives are used to increase the flavor, appearance and shelf life of products. Most of the food additives have weak acidic or basic character. The physicochemical phenomena in human body, such as absorption, permeability and distribution depend on the pH value of the biological media and the substance's acid-base dissociation constant [25]. Moreover, the acidity constant is very important to reveal physicochemical behaviors of additives in food products. Hence, the pKa value of any food additive is an important parameter for the production of food products [25, 26].

Allura red AC, which is a synthetic food colorant with the E number E129, is obtained from coal tar and petroleum products. Synthetic colorants are divided into five classes which are named as azo, triarylmethane, chinophthalon, indigo, and xanthenes. Allura red is a member of azo dyes' group. Allura red, which is named as disodium 6-hydroxy-5-(2 methoxy-5-methyl sulphophenylazo)-2-naphthalenesulphonate, contains two sulpho groups, one phenolic group (−OH) and one azo group (-N=N-). The molecular formula of allura red was given in Fig. 1. Allura red is widely used in food and pharmaceutical preparations as a coloring agent. Azo dyes have significant adverse effects on human health. For instance, consumption of allura red may cause asthma, DNA damage, allergic reactions and increased hyperactivity in children. Some countries have banned the use of allura red while the other countries have restricted it [27-34]. For this reason, the analysis of allura red is an important task in term of analytical chemistry and food industry.

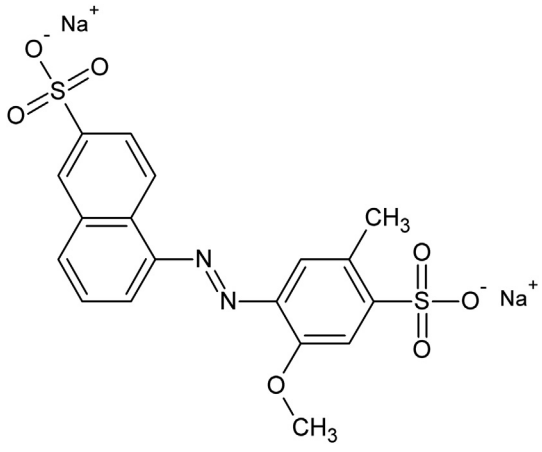


Fig. 1. Chemical structure of allura red AC (E129).

Some papers including high-performance liquid chromatography [28-31], spectrophotometry [32, 35-37], and capillary electrophoresis [38-40] were reported for the quantitative analysis of allura red in commercial energy drink samples. Several methods including potentiometric, spectrophotometric and electrophoretic techniques have been used for determining acidity constant of colorants in the literature [41-45]. In these studies, pKa determination required the use of conventional titration procedure or an extra software.

Literature survey has revealed that this is the first study to analyze allura red in energy drinks by three-way analysis of pH-UV-Vis-absorbance data. Moreover, this study is the first one in the literature to predict the quantity and pKa of allura red, simultaneously.

The focus of this study was the quantitation of allura red and its pKa determination by applying PARAFAC to the same pH-UV-Vis spectroscopic dataset. The amount of allura red in energy drink was analyzed from the second mode (concentration profile) and pKa value of allura red was determined from the third mode (pH profile) using the PARAFAC decomposition of spectral dataset. In this application, the quantitative analysis and pKa prediction was based on the mathematical extraction of individual spectral bands of allura red from the sample's spectral bands in food sample. The proposed method was validated in terms of accuracy, precision, and specificity for the quantitative analysis of the colorant's content by independent validation samples. Then, PARAFAC analysis was successfully applied to pH-UV-Vis-absorbance measurement data for the quantification of allura red and the determination of its pKa value.

In this investigation, a new UPLC method was developed as a reference method for the quantitative determination of allura red in energy drink sample. The determination results of allura red in energy drink provided from the PARAFAC application were statistically compared to those obtained by the UPLC method. The assay results provided by UPLC method were also compared to those obtained by classical UV-spectroscopic method.

We concluded that, three-way analysis methods based on the resolution of pH-UV-Vis spectroscopic dataset were new and useful methodological approaches over more laborious and expensive methods for the analysis of food products and the pKa determination.

2. Materials and methods

2.1. Apparatus and software

Absorbance spectra (Shimadzu UV-2550 with UVPC software, Shimadzu, Japan) were recorded and transferred to a spreadsheet (Microsoft Excel Software, Microsoft, USA) prior to processing by the N-way Toolbox [46] in Matlab (Mathworks Inc., USA). Regression, quantification and figure plots were performed using an in-house algorithm written in Matlab platform. Chromatographic analyses were performed using a Waters Acquity H-Class System (Waters, USA) equipped with a quaternary solvent manager, a sample manager, and a photodiode array (PDA) detector. Empower2 software (Waters, USA) was used to record UPLC data.

2.2. Chemicals and reagents

Allura Red AC was supplied by Sigma-Aldrich, USA. Reagent grade CH_3COOH, H_3PO_4, triethylamine and gradient grade acetonitrile were purchased from Sigma Aldrich, USA. NaOH and H_3BO_3 produced by Riedel-de-Haën, Germany were used. All solutions, which were used for spectrophotometric and chromatographic analysis, were prepared by ultrapure water, obtained by Milli-Q Gradient A10 Millipore Purification System (Merck Millipore, USA). Commercial energy drink sample (Burn Energy Drink) was produced by the Coca Cola Company and obtained from a local market in Ankara, Turkey. The energy drink sample was stored at room temperature.

2.3. Preparation of calibration and sample solutions

Britton Robinson (BR) buffer solutions at five different pH levels (pH 8- pH 12, $\Delta pH = 1$) were used for all spectrophotometric analysis. Buffer solutions were prepared by mixing fixed amounts of CH_3COOH (0.04 M), H_3BO_3 (0.04 M) and H_3PO_4 (0.04 M). The pH values of buffers were adjusted by 0.1 M NaOH (SevenCompact S220-Basic, Mettler Toledo, USA).

10 mg of allura red standard was weighed and dissolved in 100 mL buffer solution. The stock solution for each pH was prepared individually and freshly. All standard solutions were prepared from these stock solutions.

The calibration set was prepared in the range of 0.8-19.2 μg/mL by using the stock solution. In a similar manner, independent test samples (in the range of 0.8-14.4 μg/mL) were obtained from the stock solution of allura red. The inter-day and intra-day samples in three levels (3.2, 8.0 and 12.8 μg/mL) were prepared to get method validity. The standard addition samples were prepared by the addition of allura red's standard solution (0, 3.2, 8.0 and 11.2 μg/mL) to 0.8 mL of commercial energy drink. This procedure was repeated at each pH level for the preparation of calibration, validation and unknown sample sets.

2.4. Chromatographic analysis

The chromatographic separation was successfully performed on a Waters BEH C_{18} analytical column (100 mm x 2.1 i.d., 1.7 μm) (Waters, USA) by using a mobile phase system containing a mixture of acetonitrile and 0.1 M CH_3COOH solution containing 0.2% triethylamine (18:82, *v/v*) with 0.2 mL/min flow rate. The stock solution of allura red was prepared by dissolving 10 mg of allura red powder in 100 mL ultrapure water. Then, the stock solution was filtered with a cellulose nitrate filter with a pore size of 0.2 μm. All standard solutions were prepared from this filtered stock solution. As in UV-Vis spectrophotometry, the calibration curve of allura red in the range of 0.8-19.2 μg/mL was used for the chromatographic analysis of the related colorant. Chromatographic detection of samples was performed at the maximum wavelength (240.0 nm).

2.5. Preparation of energy drink samples

The energy drink samples were degassed for 20 minutes. For the analysis, 0.8 mL of this solution containing allura red was transferred into a 10 mL calibrated flask and the volume was completed with the buffer solution. The same procedure was repeated ten times for each pH media.

For chromatographic studies, the commercial energy drink sample was sonicated and filtered with cellulose nitrate filter with a pore size 0.2 μm. Then 0.8 mL of commercial energy drink was transferred to 10 mL volumetric flask and the volume was completed using ultrapure water.

3. Results and discussion

Generally, the quantitative analysis of analytes in food products has been carried out by chromatographic methods, such as UPLC. In some cases, these chromatographic approaches may not provide desirable analysis results due to co-elution of active compounds, poor resolution, similar behavior of active compounds, and interference of sample's matrix on the analysis. Thus, a simple and efficient analytical method is necessary. With this aim, three-way analysis of pH-UV-Vis-absorbance dataset may be preferred because it is rapid, low cost and do not require preliminary separation procedures. In this respect, PARAFAC is one of the most popular and widely used techniques for the applications that require fast analysis of a large number of food samples to get safe and quality products.

The spectral characterization, pKa determination, and quantitation of allura red in the presence of interferences were performed with the estimated profiles extracted from the pH-UV-Vis-absorbance data array using PARAFAC modeling that did not require any separation step. The details about the PARAFAC application to pKa analysis and quantitative determination of allura red in commercial energy drink were explained below.

3.1. PARAFAC method

The first step in the application of PARAFAC model to pH-spectral datasets was to find an optimal pH range that exhibits variation in spectral bands. In addition, in order to be able to determine the pKa value, the chosen pH range should include the pKa value of the related analyte. For these aims, the UV-Vis absorption spectra of allura red and commercial energy drink were recorded in the wavelength range of 207-620 nm (every 0.2 nm) at different pH media. From the recorded spectra, the pH range from pH 8.0 to pH 12.0 was found to be the optimal one for the PARAFAC implementation. As it can be seen from Fig. 2a, the standard allura red gave different spectral bands at the selected pH range. In a similar manner, the change in the spectral bands of the energy drink product containing allura red at the same selected pH range was observed (see Fig. 2b).

Then, the UV-Vis spectra of calibration, validation and unknown samples for each pH were recorded between 207 and 620 nm (every 0.2 nm). From the recorded spectra of the samples in the specified wavelength range, for mathematical treatments, the absorbance data matrix of size 1033 × 52 was collected by reducing the size of the data with the interval of 2 for each sample and for each pH value.

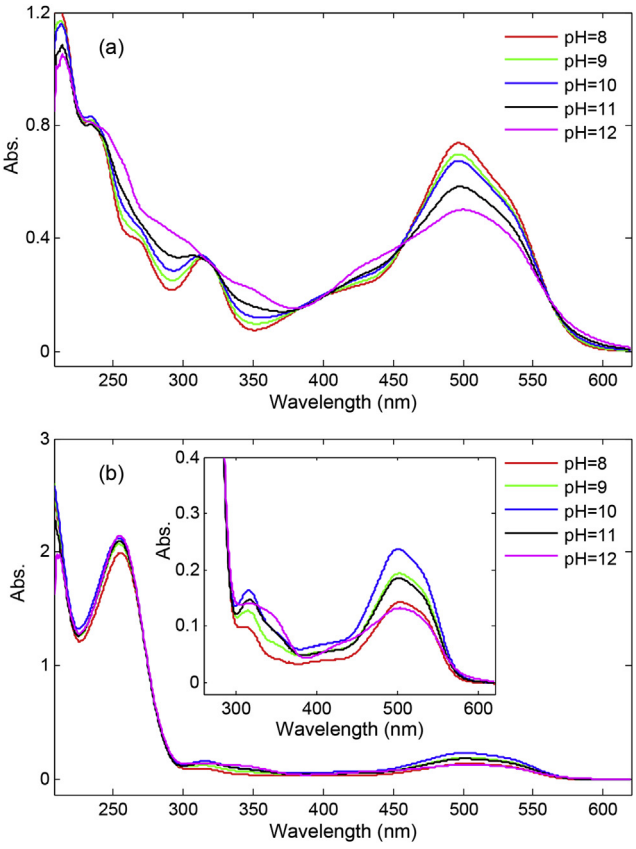


Fig. 2. UV/VIS spectra of a) allura red and b) real sample containing allura red at five different pH levels.

A framework of spectral records and data measurements to obtain a three-way array of pH-absorbance datasets was illustrated in Fig. 3a–c. For the implementation of the PARAFAC algorithm, the spectra of 52 samples (consisting of 6 calibration samples, 6 test samples, 12 standard addition samples, 9 inter-day samples, 9 intra-day samples and 10 unknown samples) at pH 8 was obtained as an output in the form of UV-Vis-absorbance data matrix as shown in Fig. 3a. A similar procedure was repeated for each pH from pH 8 to pH 12. Thus, the pH-UV-Vis-absorbance data matrix of size 1033 × 52 were created for each pH value as displayed in Fig. 3b. In the next step, each matrix of UV-Vis-absorbance data was added to a three-way array. Since the number of pH media was 5, we obtained a three-way array with dimensions 1033 × 52 × 5 (wavelength x sample x pH) as explained and given in Fig. 3c.

In the implementation of three-way analysis, the PARAFAC model was used for the decomposition of the three-way array (pH-UV-Vis spectroscopic array in this study), $\underline{\mathbf{X}}$, illustrated in Fig. 3c, into trilinear

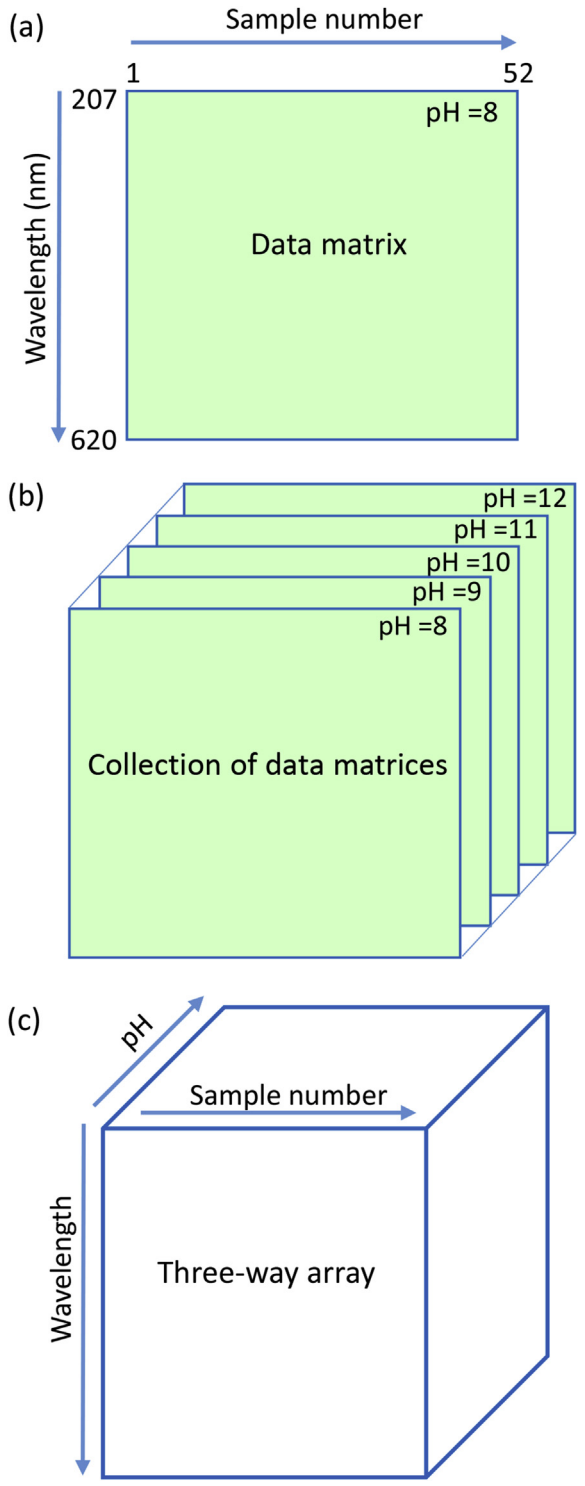


Fig. 3. Representative illustration of going from a matrix to a three-way array for UV-VIS spectra of samples at different pH media (pH8, pH9, pH 10, pH 11 and pH 12). Here we showed a) data matrix with dimensions 1033 × 52 (wavelength x sample), b) collection of data matrices and c) three-way array.

components (or loadings). The PARAFAC decomposition model for an element X_{ijk} of a three-way array $\underline{\mathbf{X}}$ can be given by the following mathematical equation:

$$X_{ijk} = \sum_{n=1}^{N} a_{in} b_{jn} c_{kn} + E_{ijk} \quad (1)$$

where N is the number of components in the fitting model, E_{ijk} is an element of residual error of array E. $a_{in,} b_{jn}$ and c_{kn} are an element of the column vector of $a_{in,} b_{jn}$ and c_{kn}, corresponding to spectral, pH and relative concentration profiles for each N components, respectively.

In the deconvolution of three-way data array, different PARAFAC approaches based on the use of different component numbers with and without constraints were investigated [47, 48]. The validation of the PARAFAC model has a very important role to get acceptable analysis results. For this purpose, core consistency diagnostic (CORCONDIA) [49] is a key parameter for estimating the appropriateness of the PARAFAC model for the explanation of fitting data. In the PARAFAC modeling, the number of components was three and non-negativity constraints were used in all of three modes. In the three-component PARAFAC modeling with non-negativity constraints, the values of CORCONDIA and explained variation were reported as 98.51% and 99.60% to obtain the correct profiles for spectral, pH and sample modes. In the PARAFAC deconvolution procedure of three-dimensional data, the estimated profiles for spectral, pH and sample loadings were displayed in Fig. 4a–f.

From Fig. 4a–c, the individual contributions of components can be seen for the acid-base species of allura red and the matrix of the sample. To interpret carefully the PARAFAC results, the matrix's signals were removed from the estimated profiles showing spectral, pH and sample modes (See Fig. 4d–f). In Fig. 4d, the spectral characterization of allura red was given as resolved spectral profile of colorant's acid-base species with the original UV-Vis spectrum of pure allura red (black dotted line). As can be seen in this figure, a good coincidence between the estimated spectrum (red line) and the original spectrum (black dotted line) of the analyzed colorant was reported. In order to avoid complexity, Fig. 4e showing the acid (red line) and base (blue line) forms in the resolved pH profile was obtained with the elimination of sample matrix (black dotted line) from Fig. 4b, presenting the pH profile obtained from spectral PARAFAC implementation. pKa value of allura red was estimated by using the pH profile of acid-base pair showing different behaviors with the change in different media (See Fig. 4b and 4e). As seen in Fig. 4b, the behavior of the sample matrix, shown with the black line, was constant in the pH profile. The relative concentration levels of allura red's acid-base components were extracted

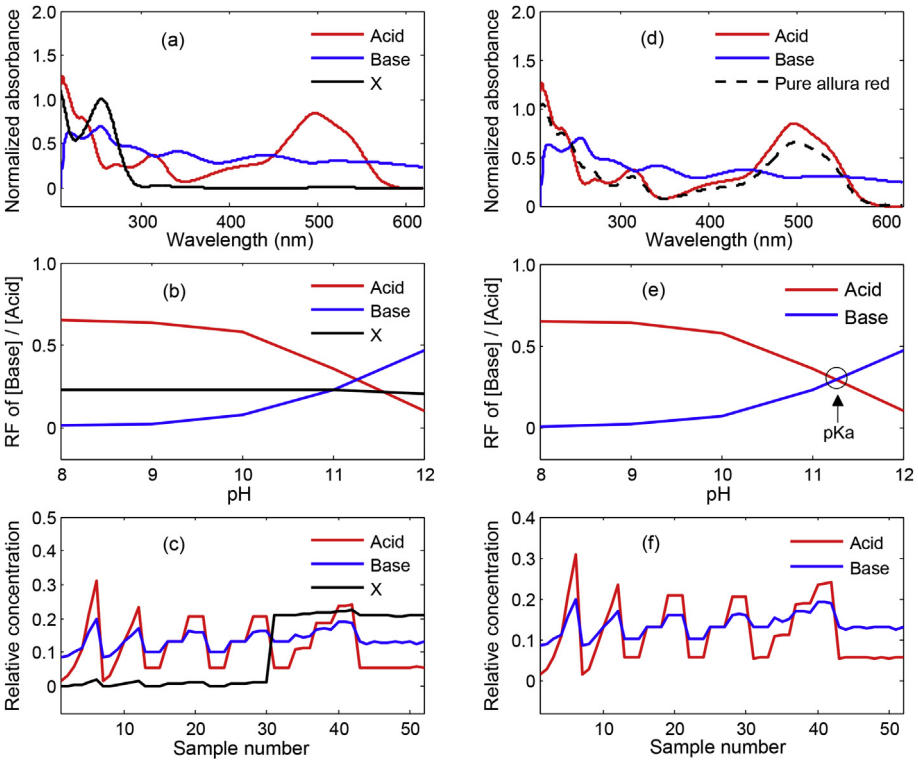


Fig. 4. Acid-base forms of allura red with (a-c) and without (d-f) the interference. (RF: relative fraction of acid-base species of allura red on the dissociation equilibrium).

from the pH profile containing the signals of acid-base forms and sample matrix, indicated in Fig. 4c, and then they were presented in Fig. 4f. The concentration profile of acid and base components of allura red, obtained from the sample mode using PARAFAC model, was used for the determination of the relative amount of the colorant in all samples containing calibration, validation and unknown samples in the presence of the interferences of the sample matrix.

3.2. Validation of PARAFAC method

From the perspective of analytical chemistry, validation is a requirement for the confirmation of a new method that will be used for quality control of samples. In our research article, the validation procedure and related details for the PARAFAC analysis were described below.

The calibration curve was calculated from the independent and dependent variables. The statistical results for the regression analysis and related parameters were summarized in Table 1. As seen in this table, it was observed that LOD (limit of detection) and LOQ (limit of quantitation) were 0.20 μg/mL and 0.66 μg/mL, respectively.

In the method validation procedure test sample set was prepared and the PARAFAC model was applied to determine the content of allura red in independent test samples. The obtained results were illustrated in Table 2 to show the ability of the PARAFAC approach for the quantification of the colorant in samples of the test set. This experiment

Table 1. Statistical results of least square regression analysis and its related parameters.

Parameter	PARAFAC model	Classical UV-VIS spectroscopy	Classical UPLC
m	1.60×10^{-2}	5.35×10^{-2}	2.98×10^{-1}
n	3.62×10^{-3}	1.04×10^{-2}	4.68×10^{-2}
r	0.9999	0.9997	0.9998
SE(m)	1.07×10^{-4}	6.25×10^{-4}	2.90×10^{-3}
SE(n)	1.06×10^{-3}	6.18×10^{-3}	2.87×10^{-2}
SE(r)	3.34×10^{-3}	1.02×10^{-2}	4.87×10^{-3}
LOD	0.20	0.35	0.29
LOQ	0.66	1.16	0.96

m: Slope of regression equation.
n: Intercept of regression equation.
r: Correlation coefficient.
SE (m): Standard error of slope.
SE (n): Standard error of intercept.
SE (r): Standard error of correlation coefficient.
LOD: Limit of detection (μg/mL).
LOQ: Limit of quantitation (μg/mL).

Table 2. Recovery data obtained by applying PARAFAC model.

Sample number	Test sample (μg/mL)	Predicted amount (μg/mL)	Recovery (%)
1	0.8	0.81	101.7
2	1.6	1.57	97.9
3	4.8	4.91	102.3
4	8.0	8.06	100.8
5	11.2	11.18	99.9
6	14.4	14.46	100.4
Average			100.5
Standard deviation			1.53
Relative standard deviation			1.52

and its results showed that the method indicated a good capability to get precise and accurate results. In the assessment of the PARAFAC model's precision and accuracy, intra- and inter-day samples were prepared and analyzed. Their results for the quantitation of allura red in inter-day and intra-day samples containing colorant at three different concentration levels (3.2, 8.0 and 12.8 μg/mL) were shown in Table 3. In this test, the PARAFAC application gave a good agreement between actual and predicted concentrations.

In the method validation studies, the analysis of standard addition samples has a very important role to reveal the presence or absence of the effect of sample matrix on the quantitation of the related compound. For this aim, standard addition samples (at three concentration levels (low: 3.2 μg/mL, medium: 8.0 μg/mL and high: 11.2 μg/mL) with a constant amount of commercial energy drink product) were prepared as described in the section "2.3. Preparation of Calibration and Sample Solutions". PARAFAC analysis was subjected to standard addition samples and the added recovery results and relative standard deviations were computed and presented in Table 4. Although a signal for sample matrix interferences was observed in all profiles, the signal of sample's matrix was eliminated due to second-order advantage of PARAFAC model, then relative concentration profile was used for the quantitation. Thus, this application showed that PARAFAC implementation in the analysis of the food samples gave us more selective, precise

Table 3. Analysis results of intra-day and inter-day studies by PARAFAC model.

	Added (μg/mL)	Found (μg/mL)	Recovery (%)	RSD	RSE
Intra-day	3.2	3.3	101.9	0.21	1.85
	8.0	8.0	100.4	0.03	0.41
	12.8	12.7	99.4	0.12	−0.60
Inter-day	3.2	3.3	101.9	0.14	1.88
	8.0	8.0	100.4	0.19	0.38
	12.8	12.7	99.1	0.09	−0.95

RSD: relative standard deviation.
RSE: relative standard error.

Table 4. Analysis results obtained from standard addition samples by PARAFAC model.

Exp No.		Added (μg/mL)	Found (μg/mL)	Recovery	RSD
1	Sample +	3.2	3.2	103.5	1.15
2	Sample +	8.0	8.3	103.2	0.20
3	Sample +	11.2	11.4	101.5	0.58

RSD: Relative standard deviation.

and accurate results for recoveries and corresponding relative standard deviations for standard addition samples.

3.3. pKa determination of allura red

The pH profile obtained from three-way PARAFAC analysis of the tensor of pH-UV-Vis-absorbance datasets was used for the estimation of acidity constant (or pKa) of allura red. This pH profile for acid and base species of the analyzed colorant can be seen from Fig. 4e. In Fig. 4e, the red line and blue line correspond to acid and base species, respectively. The intersection of red (decreasing) and blue (increasing) lines in the pH profile were used to predict the pKa value of allura red in the analyzed energy drink. The numerical value of pKa for allura red was found to be 11.28 with a relative standard deviation of 1.197. This result was obtained from the average of three different measurements of the intersection point of acid and base lines in the related pH profile. In previously published studies, two different references on the colorant's acidity constant were reported [26, 50]. When these results were compared with the PARAFAC model, good coincidence was observed.

3.4. Quantitative analysis of energy drink product

In order to show the applicability of the investigated three-way resolution method, the PARAFAC model was applied to the three-way array of pH-UV-Vis-absorbance dataset and then the quantitation of allura red in commercial energy drink samples was performed. The relative concentration profile given in Fig. 4f was used for the calibration, prediction of validation samples and quantitative estimation of unknown concentration of allura red in commercial energy drink product.

The preparation of calibration samples was described in Section "Preparation of Calibration and Sample Solutions". In the preliminary studies, the concentration range of calibration samples was identified between 0.8–19.2 μg/mL. A linear regression analysis was applied to the independent variables (actual concentration of allura red in

calibration sample set) and dependent variables (estimated concentration levels of acidic component (red line)) in relative concentration profile of allura red's acid-base components illustrated in Fig. 4f. The statistical results obtained from the regression analysis and its related parameters were listed in Table 1. The computed linear regression function was used to estimate the amount of allura red in commercial energy drink samples. The obtained experimental results for the amount of allura red in analyzed commercial samples were presented in Table 5. In spite of the presence of interferences of sample matrix on the analysis of the related colorant, three-way resolution approach based on the PARAFAC application to pH-UV-Vis-absorbance dataset gave successful results for the analysis of a commercial energy product containing allura red and sample matrix without preliminary separation or extraction step.

3.5. Classical UPLC analysis

A new UPLC-PDA method was developed to determine the amount of allura red in the energy drink product. The UPLC analysis results for the related colorant were used to compare with those obtained by applying the PARAFAC tool to pH-UV-Vis-absorbance dataset.

In the development of the new classical UPLC method, Waters BEH C_{18} (100 mm x 2.1 i.d., 1.7 μm) column was chosen as a stationary phase for the chromatographic analysis of allura red in the analyzed energy drink. A mobile phase consisting of acetonitrile and 0.1 M CH_3COOH containing 0.2% triethylamine (18:82, *v/v*) with the flow rate of 0.2 μL/min and the sample injection volume of 1.0 μL were found to be appropriate chromatographic conditions providing acceptable elution of the colorant and sample matrix in a commercial food product. Chromatographic detection was done at the wavelength of 240.0 nm and at room temperature. The chromatograms of the calibration set of allura red in the concentration range of 0.8-19.2 μg/mL were recorded. Fig. 5a shows the representative chromatogram of allura red standard. The retention time for the subjected colorant was reported to be 2.544 min as indicated in Fig. 5a. In the calibration procedure, the linear regression equation and the related statistical results were given in Table 1. In the newly developed UPLC application to samples, the recorded UPLC chromatogram was illustrated in Fig. 5b. From this figure, we can see a good separation between the colorant and sample matrix in marketed energy drink product. Quantitative analysis results of allura red in commercial samples were given in Table 5. In the implementation of the newly developed UPLC method for commercial food samples, all the samples were prepared in water and filtered by cellulose nitrate filter with a pore size of 0.2 μm. The obtained UPLC results of allura red were used for the comparison of PARAFAC analysis.

Table 5. Quantitative analysis results of allura red in energy drink sample.

Exp. No	mg/250 mL		
	PARAFAC model	Classical UV-VIS spectroscopy	Classical UPLC
1	10.13	12.28	9.75
2	10.26	12.29	10.09
3	10.25	12.31	9.95
4	10.27	12.19	9.91
5	10.26	12.5	9.91
6	9.92	12.23	9.94
7	10.26	13.13	10.13
8	10.05	12.32	10.48
9	10.1	12.3	10.31
10	10.23	12.29	10.06
Average	10.21	12.38	10.05
SD	0.14	0.27	0.22
RSD	1.41	2.20	2.14
t-cal	1.42 ($p = 0.19$)	22.76 ($p = 2.9 \times 10^{-9}$)	t-crit: 2.26 ($\alpha = 0.05$)
F-cal	1.64 ($p = 0.24$)	3.23 ($p = 0.048$)	F-crit: 3.18 ($\alpha = 0.05$)

SD: Standard deviation.
RDS: Relative standard deviation.

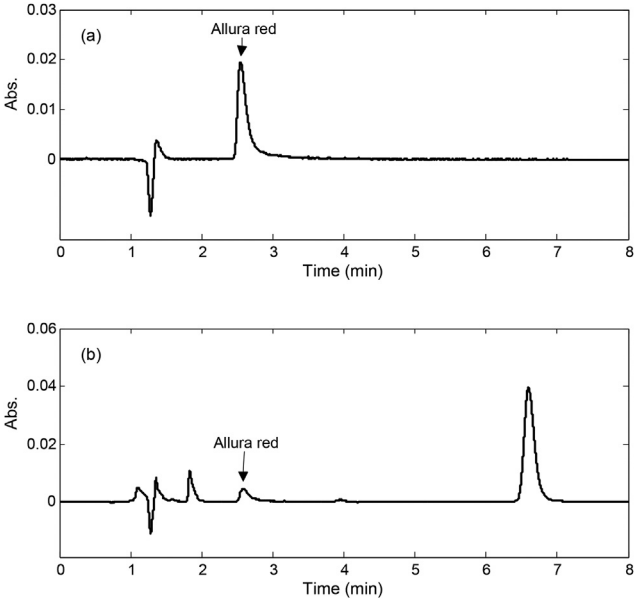


*Fig. 5. UPLC chromatogram of **(a)** standard solution of allura red and **(b)** commercial energy drink sample.*

3.6. Classical UV-Vis-spectroscopic analysis

In order to observe the performance of the PARAFAC model for the resolution of complex food samples, a classical UV-Vis-spectroscopic method was used for the determination of allura red in energy drink samples. This classical UV-Vis spectroscopy is based on direct absorbance measurements at 495 nm, which corresponds to the maximum wavelength for the absorption spectra of allura red at pH 10. Least square regression analysis and related statistical results for the method were listed in Table 1. Determination results of allura red obtained by the application of the classical spectroscopic approach to the commercial energy drink samples were indicated in Table 5. As can be seen from this table, the assay result obtained by classical UV-Vis-spectroscopy showed a significant deviation from the results obtained by PARAFAC and UPLC methods. As a result, classical direct UV-Vis spectroscopic measurements were not suitable for the quantification of colorant due to the presence of sample's matrix in energy drink product.

3.7. Statistical comparison of determination results

In this paper, statistical tests (t-test and F-test) were applied to compare the analysis results of allura red obtained by the application of three methods to commercial energy drink samples. In these statistical processes, t-test was used to compare the means for the analysis results of PARAFAC-UPLC pair and classical UV-Vis spectroscopy-UPLC pair, whereas F-test was used to compare the variances for the analysis results of PARAFAC-UPLC and classical UV-Vis spectroscopy-UPLC.

In the statistical tests, the critical values for t ($p = 0.05$ with number of degree of freedom of 9) and F ($p = 0.05$ with degrees of freedom $n_1 = 9$ and $n_2 = 9$) found in tables are 2.26 and 3.18, respectively. At the 95% confidence level, the difference between the experimental results for the commercial energy drink samples using PARAFAC and UPLC is not significant because t- and F-calculated values were less than those of t- and F-critical values (See Table 5). However, a significant difference between classical UV-Vis spectroscopic and UPLC results was reported due to the effect of sample matrix on the analysis of allura red in commercial energy drink samples.

4. Conclusions

In this article, the application of three-way PARAFAC model to the pH-UV-Vis absorbance dataset gave successful results for the quantification of allura red and its pKa determination in the presence of interferences of sample's matrix in a commercial energy drink product. The proposed three-way analysis method provided us with the simultaneous determination of the colorant's amount and its pKa value using the deconvolution of the same dataset and without using a classical titration procedure. The simultaneous determination of these two characteristics would not be possible by classical UV-Vis spectroscopic and chromatographic approaches. This is an advantage of PARAFAC model over classical UV-Vis spectroscopy and UPLC methods. For a comparison of PARAFAC results, a newly developed UPLC method and a classical UV-Vis spectroscopic method were applied to the quantitation of allura red in samples. In spite of the complexity of food sample and the effect of the sample's matrix, we observed that the proposed PARAFAC application was faster, simpler, more accurate, precise and reliable than the classical methods, without requiring a sample preparation procedure. PARAFAC application required neither a sophisticated equipment, nor technical expertise, nor a sample preparation procedure.

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