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Ion-Pair Based Dispersive Liquid-Liquid Microextraction for Spectrophotometric Analysis of Carmoisine in Food: A BAGI and AGREE Study

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Abstract

This paper discusses the identification of a new dispersive Liquid-liquid microextraction method for evaluating carmoisine dye using spectrophotometry. It has been established that Carmoisine dye is an anionic azo food dye used in the food industry in some countries such as Iraq. This method involves the extraction of carmoisine from aqueous solutions using chloroform as the extraction solvent in a DLLME procedure. Parameters influencing the DLLME efficiency, including pH, type and volume of extraction as well as dispersive solvents (chloroform and ethanol), under a centrifugation conditions, were thoroughly optimized to complete extraction conditions. Moreover, the method showed a linear calibration curve in the range of 1.0-10.0 mg/L. The low values of both the limit of quantification (0.297 mg/L) and the limit of detection (0.099 mg/L) indicate a more sensitive DLLME method. The applicability and environmental impact of the developed method were assessed using the Blue Applicability Grade Index (BAGI) and the Analytical Greenness metric (AGREE), yielding scores of 70.0 and 0.55, respectively, indicating a practical method with moderate adherence to green analytic chemistry principles. The optimized DLLME procedure was successfully applied to the determination of carmoisine dye in various food samples.

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1. Introduction

The advancement of instrumental and chemical methods for extracting, evaluating, and quantitatively analyzing synthetic food colors has become crucial for the food and beverage industry, as well as in academic and governmental institutions, in assessing the safety and quality of food products [1, 2]. Carmoisine is a synthetic dye that belongs to the azo dye class. It is also known as Azorubine or E122 in Europe (Figure 1) [3]., and used to add red color to food, medicine, cosmetics, and beverages, because they can provide food with a more stable color, synthetic dyes have a wide range of applications [4]. Currently, certain dyes are permitted for use as food coloring agents in various developed countries, while many others have been banned over the past two decades due to their toxicity and potential carcinogenic effects [5, 6]. One of these dyes is azorubine, which has the potential to be toxic when absorbed and metabolized in the human body. It can have adverse effects on and after biochemical markers in important organs including the liver and kidneys, at both higher and lower doses [7, 8]. Many methods had used for determination of Carmoisine dye include, electrochemical [9, 10], Differential Pulse Polarography [11], RP-HPLC-UVVis detection [12], HPLC-UV-DIODE array detection [13], LC-MS [14], capillary electrophoresis [15], zero-order spectrophotometric [16], single step of

spectrophotometric [17], digital image analysis [18, 19], and four derivative spectrophotometric [20]. Moreover, the various preconcentration and extraction methods that have been developed for determination dye such as Cloud point extraction using Triton X-114 [21]. Cloud point extraction using Triton X-100 [22, 23], Cloud point extraction using Brij 58 [24], magnetic solid phase extraction [25, 26], solid phase extraction [27, 28], Liquid-Liquid Extraction (LLE) [29], and Microextraction [30, 31]. In recent years, dispersive liquid-liquid microextraction (DLLME) has been utilized as a pre-concentration and determination method in analytical chemistry, due to its several benefits, including low cost, safety, low consumption of organic solvents, and speed. This reduced solvent consumption makes DLLME more environmentally friendly and cost-effective **[32, 33].**

Additionally, the DLLME technique is easy, with good recoveries and high pre-concentration factors. The present work aims to determine and extract carmoisine in different samples, such as jelly, soft drinks, and candy using the DLLME method. This method involves forming an ion pair complex between the carmoisine dye and 2,3,5-Triphenyltetrazolium chloride (TTC) reagent using the extraction solvent ($CHCl_3$) and disperser solvent (ethanol). The aim of using the TTC reagent is to increase the solubility of the carmoisine in the organic extraction solvent (chloroform) compared to the charged carmoisine ions. However this leads to a more successful transfer of carmoisine from the aqueous phase to the chloroform phase during the DLLME process. Finally the environmental impact of this study was evaluated the Analytical Greenness metric (AGREE) and the blue Applicability Grade Index (BAGI) to assess the practicality and suitability of the developed DLLME method.

2. Experimental Work

2.1. Instrumental

The Analytikjena Specord40 spectrophotometer (Germany) equipped with a 1.0 cm quartz cell was used to acquire UV-visible spectra. A WTW digital pH meter (pH 7110, Germany) with a combined glass electrode was used to measure pH values. Additionally, a centrifuge (DLAB, China) was used to expedite the phase extraction process.

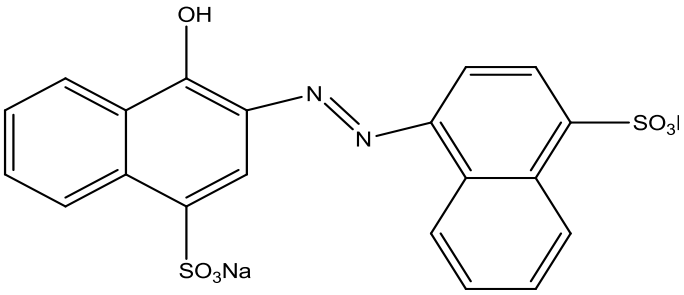


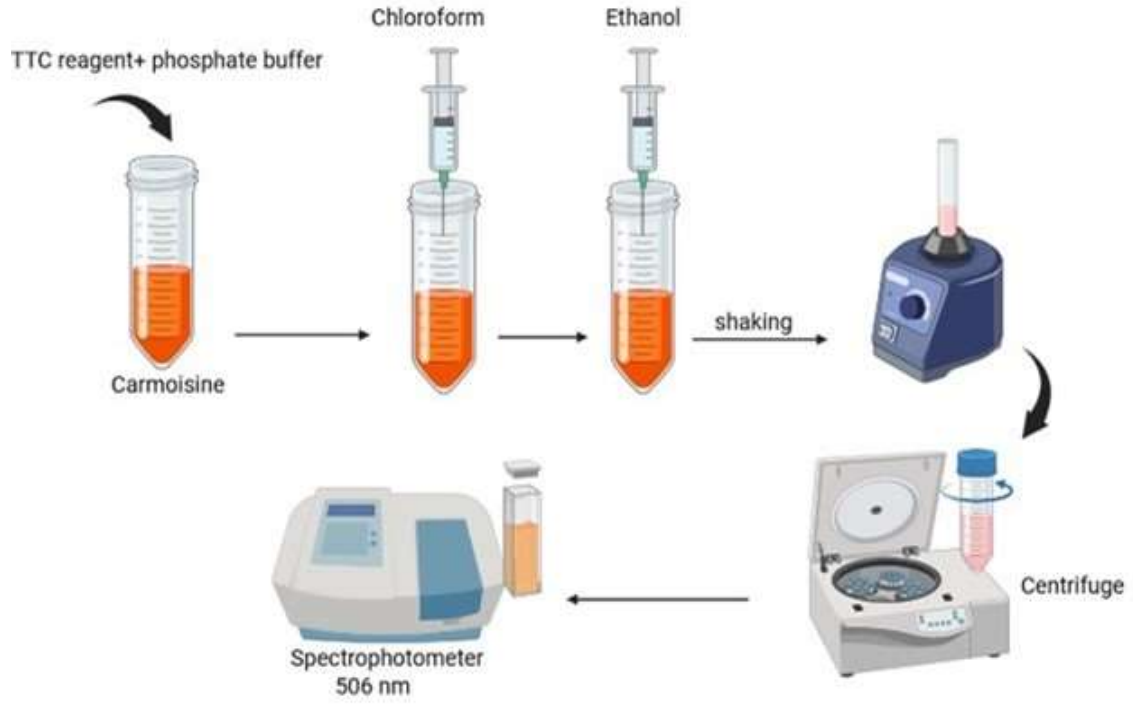
Figure1: Chemical structure of carmoisine dye

2.2. Reagents

All chemical substances were of analytical grade. A stock solution of carmoisine (BDH), 500 mg/L, was prepared by dissolving 50 mg of carmoisine (M.Wt= 502.43 g/mol) in distilled water and diluting to 100 mL in a volumetric flask. Also, a stock solution of 2,3,5-Triphenyltetrazolium chloride (TTC) reagent (Sigma-Aldrich), 500 mg/L was prepared by dissolving 50 mg of TTC in the distilled water and completing it to the mark in a volumetric flask (100 mL). All buffer solutions were prepared according to the reference [22].

2.3. DLLME procedure

For DLLME under perfect conditions, 1 mL of the solution including carmoisine with a concentration of 1.0 to10.0 mg/L in a glass test tube with a conical bottom. Added 1.5 mL of (1%) TTC reagent and 1.0 mL of phosphate buffer (pH=6), then completed it with distilled water to 10 mL. A total of 400 µL of chloroform, used as the extraction solvent, and 800 µL of ethanol, serving as the disperser solvent, were rapidly injected into the solution using a micro syringe, followed by gentle shaking. This resulted in the formation of a cloudy solution within the centrifuge tube. The mixture was then centrifuged for 6 minutes at 3000 rpm, while the sediment organic phase was separated, and 600 µL of ethanol was used for analysisat room temperature [34, 35]. The highest absorbance of the extracted carmoisine dye was determined at a wavelength of 506 nm, except for the carmoisine dye. The entire set of components was used to produce a blank solution, Scheme 1.



Scheme 1: Schematic of the DLLME procedure

2.4. Sample preparation

All food samples were sourced from local supermarkets, where some measured quantities of soft drinks, candy, and jelly were dissolved in deionized water and gently heated to ensure the complete dissolution of the jelly and candy. The resulting solutions were then filtered and diluted to a final volume of 10 mL in a volumetric flask. After treating, a sample of the solution using the DLLME method, carmoisine dye was simultaneously determined.

3. Results and Discussion

3.1. Optimum Conditions of DLLME

All measurements for the optimum DLLME conditions were performed in triplicate (n=3), with the results shown in Figure 2.

3.2. TTC reagent as an ion-pair complexation reagent effect

The effect of (1%) TTC reagent volume on the extraction of carmoisine dye was investigated within a range of 0.2 to 3.0 mL, with the results presented in Figure 2A. A TTC reagent volume of 1.5 mL was determined to be optimal for the subsequent experiments. The role of 2, 3, 5-triphenyltetrazolium chloride (TTC) reagent in this procedure is to form an ion pair complex with carmoisine dye, which leads to improved sensitivity of the DLLME method, enhanced extraction efficiency, and gives a higher absorbance signal. This carmoisine dye-TTC ion pair complex exhibits increased solubility in the organic extraction solvent (chloroform) compared to the charged carmoisine ions alone. The complex is estimated at a wavelength of 506 nm, which leads to a more active transfer of carmoisine from the aqueous phase to the chloroform phase during the DLLME process [36].

3.3. Buffer type and pH effect

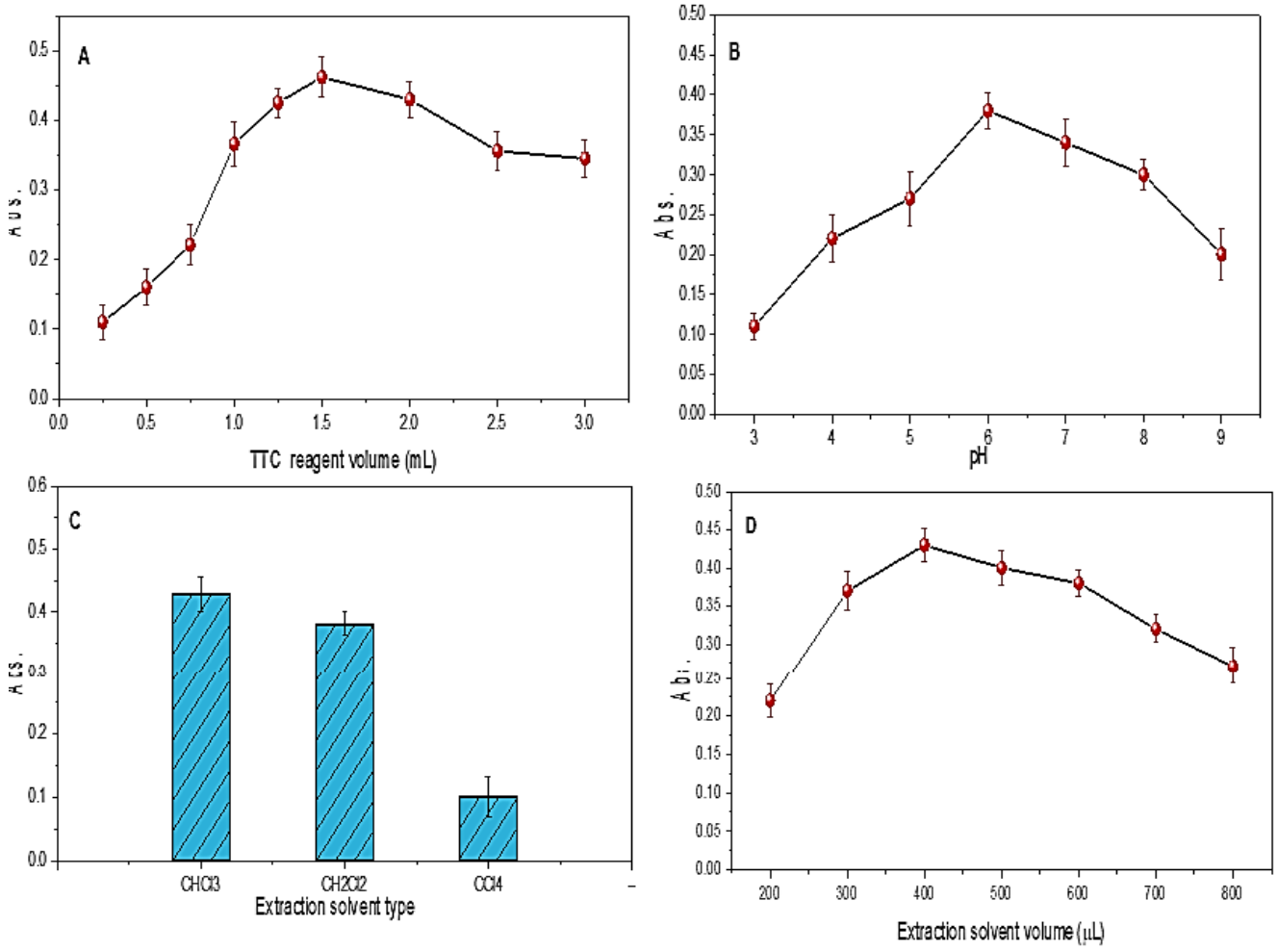
The stability of the pH depends on the buffer solution because, it affects the chemical properties of the carmoisine dye-TTC (analyte) and its distribution between the aqueous and organic phases (chloroform). The absorbance values for each buffer solution type are as follows: Acetate buffer - 0.325, Phosphate buffer - 0.426, and Dimethylammonium phosphate buffer - 0.191. Therefore, the phosphate buffer was found to be the most effective in the extracting of carmoisine dye-TTC, compared to the other buffers. Figure 2B illustrates the influence of pH value on the absorbanceof carmoisine dye-TTC at 506 nm (Figure 5). The results indicate that a phosphate buffer at pH 6 yields the highest absorbance and is therefore chosen as the optimal condition [37].

3.4. Extraction solvent effect

The influence of the type and volume of extraction solvent was investigated. In the chosen extraction solvent, the confirmed properties of the solvent that need to be considered are low solubility in water, the ability to extract analytes of interest (carmoisine dye-TTC), and higher density than water. Depending on these requirements, three extraction solvents containing chloroform, dichloromethane, and carbon tetrachloride were chosen. $CHCl_3$ has a higher efficiency of extraction than others (Figure 2C). A series of volumes of chloroform containing ethanol, ranging from 200 to 800 µL, was tested. As shown in Figure 2D, the absorbance of the organic phase decreased with increasing chloroform volume, due to the dilution effect, which reduced the concentration of carmoisine dye-TTC in the organic phase. 400 µL of chloroform was selected in the next steps [38].

3.5. Disperser solvent effect

The influence of the type and volume of the disperser solvent was studied. The disperser solvent's role is to help the extraction solvent (CHCl3) disperse into the aqueous sample (carmoisine dye-TTC) as very fine a droplet, which leadsto an increase in the contact area between both solvents and more efficient extraction of the analyte (carmoisine dye-TTC) [39]. The effect of disperser solvent type is shown in Figure 2E. Ethanol has a higher absorbance value and was an important factor in achieving good extraction performance; consequently, ethanol was utilized as a disperser solvent. The influence of the ethanol volume on the DLLME extraction of carmoisine was evaluated in the volume range of 500 to 1300 µL, and the findings are shown in Figure 2F. It can be seen that the absorbance of carmoisine dye-TTC appeared to reach its maximum at 800 µL. Therefore, 800 µL was chosen as the optimum volume of ethanol.



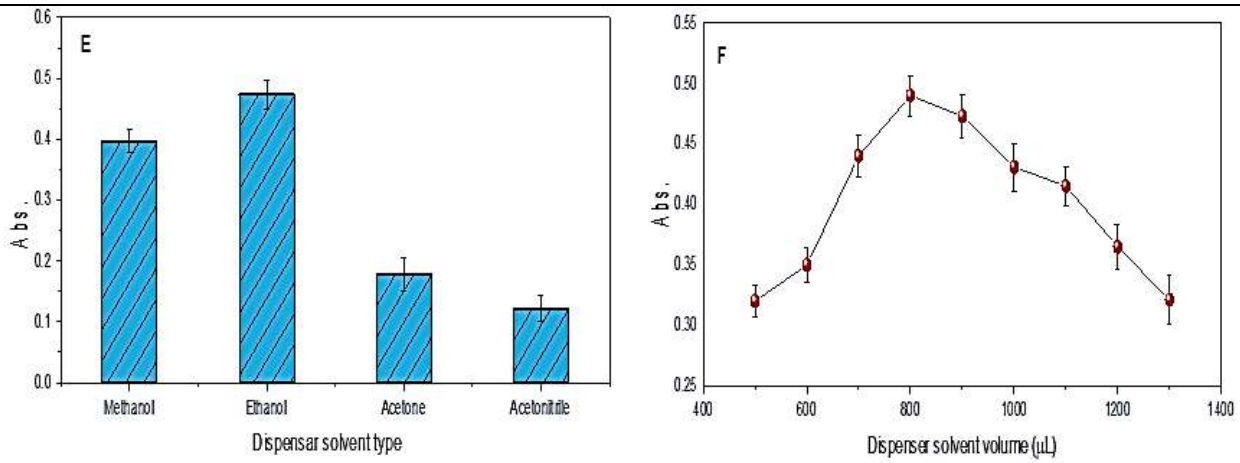


Figure 2: Effect of (A) TTC reagent complexation, (B) pH, (C) Extraction solvent type, (D) Extraction solvent volume, (E) Disperser solvent type, and (F) Disperser solvent volume on the absorbance of carmoisine dye

3.6. Rotation speed and time effect

To attain efficient pre-concentration and facilitate easy phase extraction, the rotation speed and duration were optimized. The rotation speed helps to create a fine dispersion of the extraction solvent ($CHCl_3$) in the aqueous sample (carmoisine dye-TTC), which leads to an increase in the contact area between them. The rotation time needs to be sufficient to allow the analyte (carmoisine dye-TTC) to transfer effectively from the aqueous phase to the extraction solvent ($CHCl_3$). The impact of rotation speed was examined within the range of 1000 to 5000 rpm. As shown in Figure 3A, a rotation speed of 3000 rpm yielded the highest absorbance, making it suitable for achieving quantitative extraction in this system. To ensure satisfactory extraction, the rotation time was extended from 1.0 to 10.0 minutes, as depicted in Figure 3B. Consequently, a rotation time of 6.0 minutes was selected for the subsequent experiments. The continuous variation technique (Job's method) was used to accurately determine the ideal molar ratio between the carmoisine dye and TTC Reagent. The Job's method constant. It was demonstrated that the ratio for the procedure was 1:2 (carmoisine dye + TTC reagent) (see Figure 4).

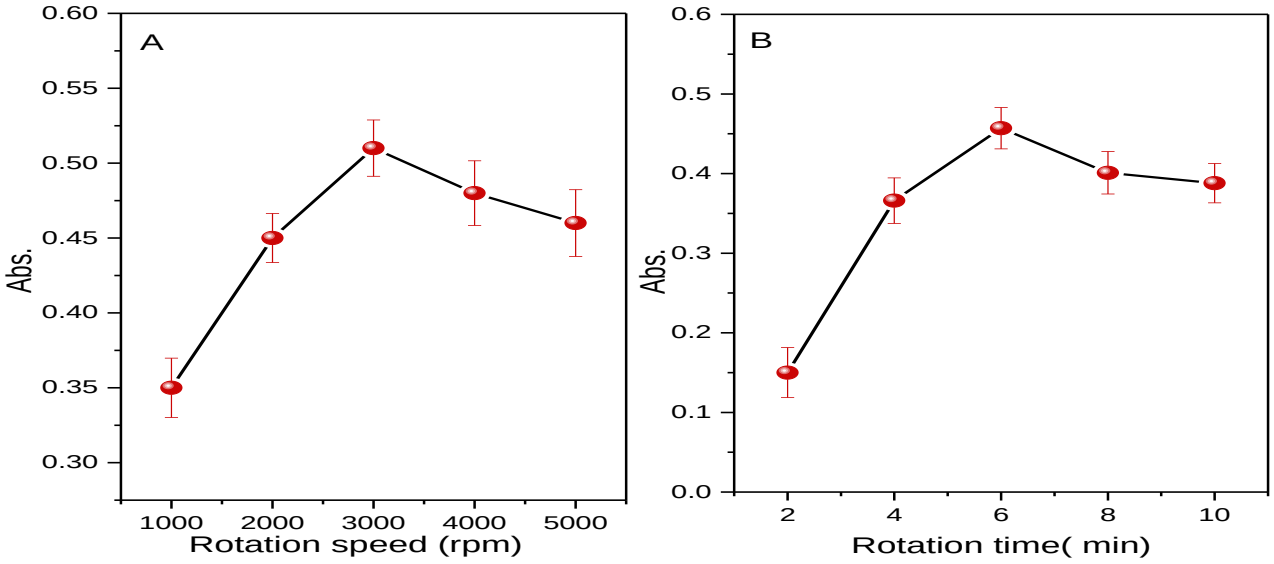


Figure 3: Effect of (A) rotation speed and (B) rotation time on the absorbance of carmoisine dye-TTC Determining the molar ratio between carmoisine dye and TTC Reagent (Job's method)

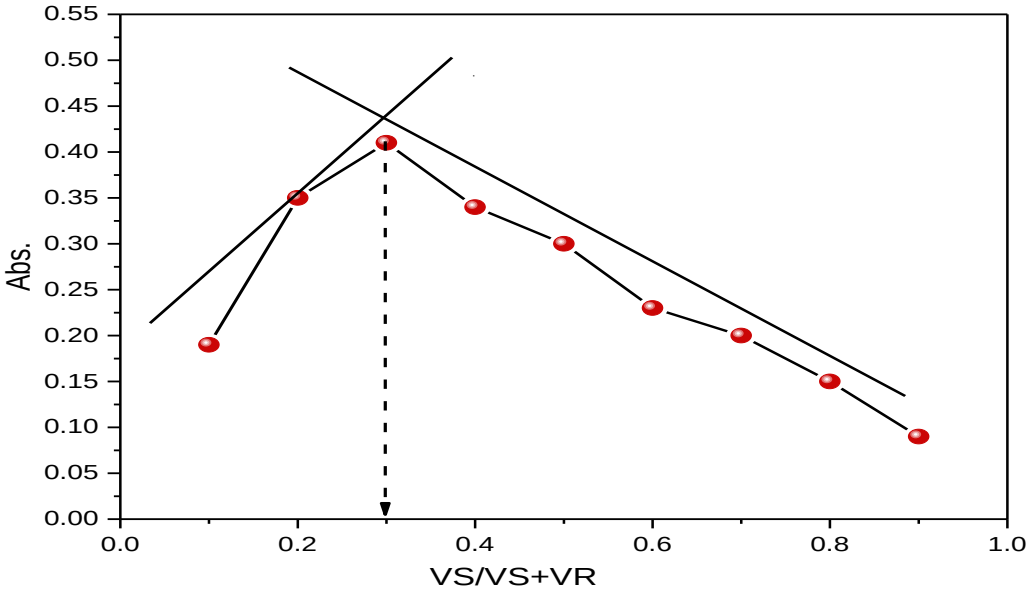


Figure 4: The Job method

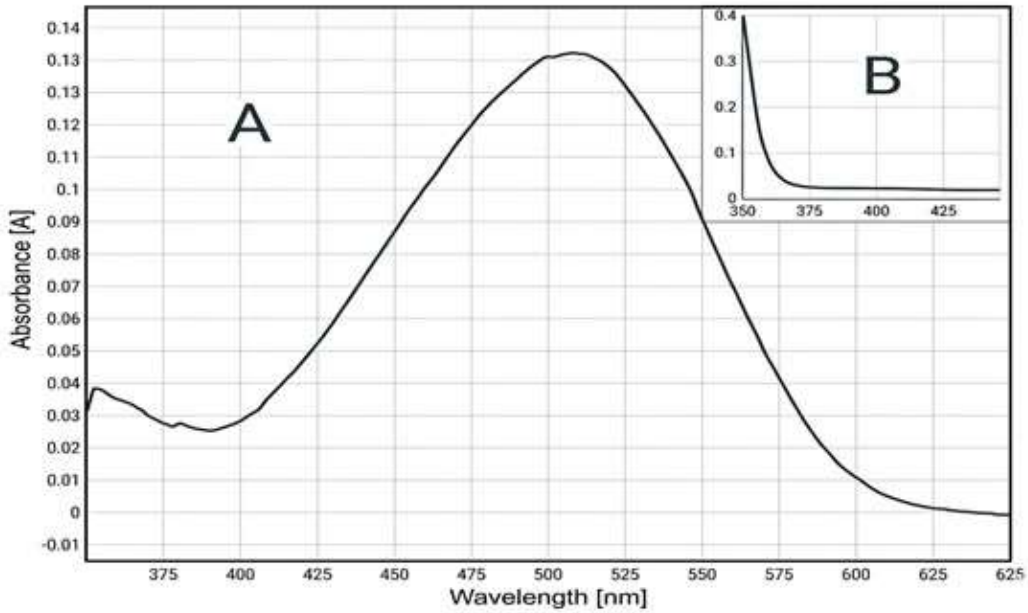


Figure 5: (A) The absorption spectra of the carmoisine dye-TTC ion-pair complex and (B) the blank.

3.7. Analytical Figures of Merit

Following the optimization of all parameters, the quantitative characteristics of the proposed method were evaluated. The linear dynamic range (1.0-10.0 mg/L) and correlation coefficient (R^2 = 0.9976) were established to assess the method's performance. A low Sandell's sensitivity (9.1 × 10^{-3} mg/cm^2) indicates a more sensitive method. The low values of both the limit of quantification (LOD=0.297 mg/L) and the limit of detection (LOQ= 0.099 mg/L) indicate a more sensitive DLLME method, capable of detecting minimal amounts of the carmoisine dye-TTC complex. Confidence limits (C.L.) were calculated for three different concentrations of the carmoisine dye-TTC complex (0.5 mg/L, 1.5 mg/L, and 7.5 mg/L). The C.L. values are relatively narrow, indicating acceptable accuracy and precision within the working range of the DLLME procedure (Table 1) (see Figure 6).

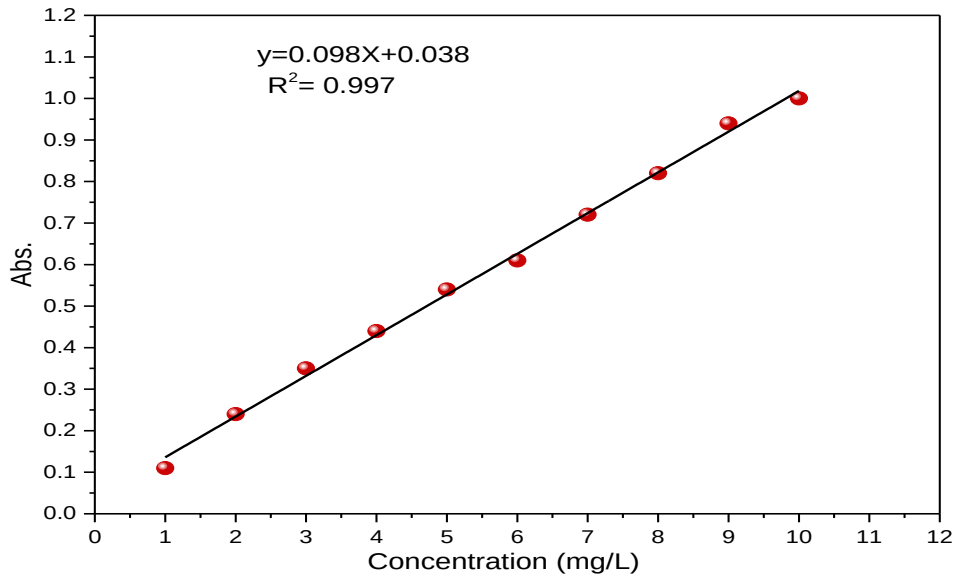


Figure 6: Calibration curve of carmoisine dye-TTC using DLLME

Table 1: Statistical parameters of carmoisine dye-TTC using the DLLME method

Parameter	DLLME
λ max(nm) and color	506 and Red
linearity range (mg/L)	1.0-10.0
Sandell's sensitivity (mg/cm^2)	9.1×10^{-3}
Correlation coefficient (R^2)	0.9976
Regression equation	Y=0.098X+0.038
Slope(m)	0.098
Intercept(a)	0.038
Limit of detection (LOD) (mg /L)	0.099
Limit of quantification (LOQ) (mg /L)	0.297
*C.L. for the 0.5 (mg/L) at 95%	0.52± 0.022
*C.L. for the 1.0 (mg/L) at 95%	1.23± 0.170
*C.L. for the 1.5 (mg/L) at 95%	1.42± 1.221
Sandell's Sensitivity= M.W of carmoisine dye /1000×ε, LOD = 3.3 ×S_{blank}/m, LOQ= 10×S_{blank}/m, S_{blank}=standard deviation of blank, which was calculated from three replicate measurements of the blank solution (n=3),C.L. =Confidence Limits.	

4. Application

The proposed procedures have been utilized for the determination of carmoisine dye in a variety of food samples (spiked standards experiments). As indicated in Table 2, the smaller relative error (R.E. %) indicates the accuracy of the DLLME method. A high recovery (Rec. %) value indicates high efficiency, and a lower relative standard deviation (RSD %) signifies better precision. These results demonstrate the applicability of the DLLME method for carmoisine dye in food samples such as jelly, soft drinks, and candy.

Table 2: Determination of carmoisine dye in real samples

sample	Concentration of carmoisinedye mg/mL		R.E. % (n=3)	Rec.% (n=3)	RSD% (n=3)
	Add	Found			
Jelly	----	0.98	-----	----	2.33
	1.0	2.13	7.5	107.5	1.75
	2.0	2.89	-3.02	96.9	1.31
Candy	----	0.76	----	----	0.93
	1.0	1.66	-5.6	94.3	2.51
	2.0	2.72	-1.4	98.5	4.01
Soft drink	----	1.93	----	----	1.41
	1.0	2.85	-2.7	97.2	3.36
	2.0	3.90	-0.76	99.2	0.99

5. Comparison with reported methods

In the proposed method, organic solvent (Chloroform) as extraction solvent and (ethanol) as disperser solvent) were applied for the DLLME of Carmoisine dye, achieving a low LOD and a good Linearityrange. Several methods have been used for the determination of Carmoisine dye. Compared to these methods, the proposed method simplifies and can be used for the determination of Carmoisine dye in food samples such as jelly, soft drinks, and candy. While this method's LOD of 0.099 mg/L is higher compared to some other methods that offer lower detection limits, the current DLLME method offers a simpler and more cost-effective method (such as UV-Vis spectrophotometer and low consumption of organic solvents).

Table 3: A comparison of the suggested methods with previous studies

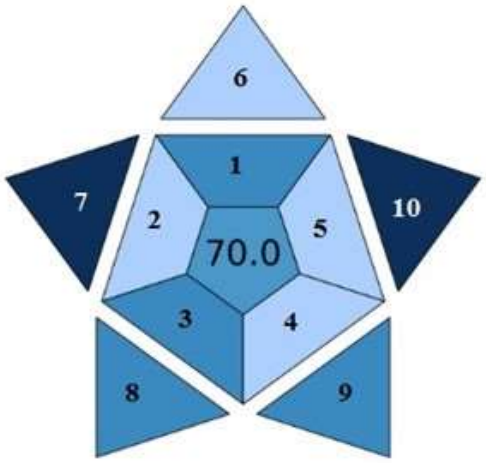
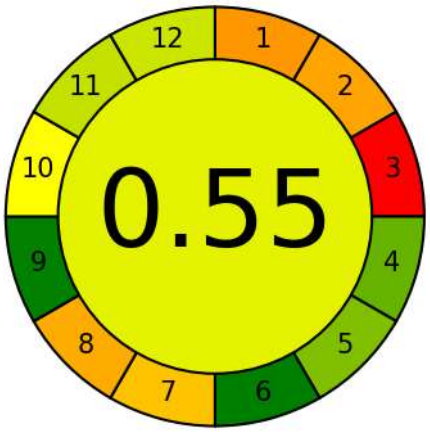
Method	Linearity, mg/L	LOD, mg/L	Ref.
Cloud point extraction	0.05-1.5	0.015	[40]
Dispersive liquid-liquid microextraction	0.01-1.2	0.003	[41]
HPLC	2–10	0.086	[42]
HPLC	5 - 50	0.2	[43]
Aqueous Two-Phase System and Spectrophotometric Detection	0.0001-0.12	1.4×10^{-5}	[44]
Digital image analysis	20-250	4.82 and 8.05	[22]
Cloud point extraction	0.05 -5.0	7.2	[45]
Quantitative SERS Analysis	0.5-500	----	[46]
DLLME	1.0-10.0	0.099	Present work

6. Blueness and Greenness Assessment

Table 4 presents the blue applicability grade index (BAGI) evaluation of the dispersive liquid-liquid microextraction (DLLME) method for the carmoisine-TTC dye ion pair complex, utilising chloroform as the extraction solvent and ethanol as the dispersing solvent, with UV-Vis spectroscopy for detection. The BAGI score of 70.0 indicates a practical and blue method, supported by the use of simple instrumentation (UV-Vis), a miniaturised extraction technique (DLLME), common reagents (ethanol, chloroform, TTC, and phosphate buffer solution), and a semi-automated procedure. The use of 800 µL of ethanol provided maximum extraction efficiency by effectively dispersing the chloroform into the aqueous sample, increasing the contact area for better analyte transfer—an important aspect of the DLLME procedure that contributes to the overall performance of the extraction process and, consequently, the BAGI score. Table 4 also shows the Analytical GREEnness metric (AGREE) score for the dispersive liquid-liquid microextraction (DLLME) method, which received a score of 0.55, indicating moderate adherence to green analytical chemistry principles. The extraction solvent (chloroform) and dispersion solvent (ethanol) in this DLLME procedure were optimized for the extraction of the carmoisine-TTC dye ion pair complex. Chloroform was selected as the extraction solvent based on its low water solubility, analyte extraction capacity, and higher density than water, with 400 µL being optimal to avoid dilution effects. Ethanol was selected as the dispersion solvent due to its high extraction efficiency, with 800 µL being the optimal volume for effectively dispersing chloroform into the aqueous sample, thus maximizing contact

area and improving carmoisine-TTC dye extraction—factors that influence the overall "greenness" score in the AGREE diagram [47-49].

Table 4: Blueness and Greenness assessment of carmoisine dye-TTC using the DLLME method

BAGI	
1. Type of analysis (Quantitative) 2. Multi- or single-element analysis (2-5 compounds) 3. Analytical technique (Simple instrumentation available in labs -UV-Vis) 4. Simultaneous sample preparation (2-10) 5. Sample preparation (Minimalized extraction sample preparation- DLLME) 6. Samples per h (2-4) 7. Reagents and materials (Common commercially available reagents (ethanol, Chloroform, TTC , and phosphate buffer) 8. Preconcentration (Required, one-step preconcentration). 9. Degree of automation (Semi-automated with common devices - UV-Vis spectrophotometer) 10. Amount of sample (10 mL food/environmental)	
AGREE	
1. Sample treatment (external and batch analysis-reduced number of steps) 2. Sample amount (10 mL of the tube contains Carmoisine dye sample and reagents) 3. Devise positioning (off-line- UV-Vis spectrophotometer) 4. Sample preparation stages (4 steps: added reagents, shaking with vetrox, centrifuge, and analysis with UV-Visspectrophotometer) 5. Automation, miniaturization (semi-automatic and miniaturized) 6. Derivatisation(none) 7. Waste (10 mL) 8. Analysis throughput (1 analytein a single run using a UV-Vis spectrophotometer and 5 samples/hour) 9. Energy consumption (UV-Vis spectrophotometer, low energy 0.1 kWh) 10. Source of reagents (some bio-based reagents) 11. Toxicity (Yes, 400µl of chloroform) 12. Operator's safety (ethanol- highly flammable solvent and chloroform-toxic to aquatic life)	

7. Conclusion

In the present work, the DLLME method coupled with micro volume UV-Vis spectrophotometry has been successfully used for the sensitive assessment of carmoisine dye, showing the potential for a greener analytical approach through the miniaturization of toxic organic solvent usage. In addition to its ease of operation, the proposed methods offers several advantages, including speed, low sample volume requirements, and cost-effectiveness. 2,3,5-Triphenyl tetrazolium chloride (TTC) was employed as a reagent for producing an ion-pair complex with carmoisine dye. Furthermore, the method's practicality and moderate environmental impact were supported by BAGI and AGREE scores of 70.0 and 0.55, respectively, suggesting its suitability for real sample applications. The good accuracy, low detection limits, and excellent precision achieved, indicate

that this DLLME-UV-Vis method holds considerable promise for the reliable determination of carmoisine dye in various food samples.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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