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Simultaneous determination of Brilliant green and Crystal Violet dyes in fish and water samples with dispersive liquid-liquid micro-extraction using ionic liquid followed by zero crossing first derivative spectrophotometric analysis method

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Abstract

In this study, dispersive liquid-liquid micro-extraction using ionic liquid (IL-DLLME) combined with zero crossing first derivative spectrophotometric method was applied to quantitative determination of triphenylmethane dyes in binary mixtures. The 1-methyl-3-octylimidazolium hexafluorophosphate [OMIM][PF_6] ionic liquid was used to extract Brilliant Green (BG) and Crystal Violet(CV) dyes from aqueous solutions. The amplitude of the zero crossing first derivative spectra at 670 nm and 532 nm were selected for the determination of BG and CV, respectively. Significant factors influencing the extraction of BG and CV such as sample pH, kind of extraction solvent, amount of extractant, extraction and centrifuging times and ionic strength were investigated. Under the optimal conditions, the calibration curves for the simultaneous determination of both dyes were found to be linear in the range of 10-500 μg L^{-1} with detection limits (LODs) of 2.7 μg L^{-1} and 1.4 μg L^{-1} for BG and CV, respectively. The relative standard deviation (RSD%) for five replicate simultaneous determinations of BG and CV were 4.7% and 1.7%, respectively. Extraction efficiencies of the BG and CV dyes in the presence of interfering ions were also investigated. Sample preparation based on the quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction combined with the IL-DLLME method and zero crossing first derivative spectrophotometric detection was applied for the simultaneous analysis of BG and CV in fish and water samples with quantitative recoveries.

Keywords: Crystal Violet; Brilliant Green; Dispersive liquid-liquid microextraction; Derivative spectrophotometry; Food analysis

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1. Introduction

The triphenylmethane dyes such as Brilliant Green (BG) and Crystal Violet (CV) (Fig.1) are kinds of synthetic colorants which are widely used for coloring in the textile industry and biological stains [1]. BG is a member of the triphenylmethane dyes family that has antifungal properties [2]. In the paper production industry, about 0.8-1.0 Kg of BG per ton of paper is consumed. It is water soluble and toxic so that it has mutagenic and carcinogenic effects on aquatic biota and humans. It may also cause irritation with redness and pain when it comes in contact with the skin[3]. On the other hand, this dye has been used in the aquaculture industry for many decades due to its anti-microbial, anti-parasitic and anti-fungal properties for the treatment and prevention of certain fish diseases [4, 5]. Crystal violet (CV) is a well-known dye that is also applied as a dermatological agent, veterinary medicine, biological stain, additive to poultry feed to inhibit propagation of mold, fungus and intestinal parasites, paper printing, textile dying, and etc.

BG, CV and their metabolites may cause human carcinogenesis and mutagenesis, so that these dyes are classified as the third class of dyes (strong toxins) [6]. It is proven that such dyes can induce reproductive abnormalities in fish [7-9], and renal and hepatic tumors in mice [10]. The fish tissues can readily absorb CV from water and reduce it metabolically to the leuco moiety (leucocrystal violet, LCV). According to the European Union (EU), the developed analytical methods must meet the European minimum required performance limit (MRPL) at 2 ng/g for the sum of the parent drugs and their leuco forms. Meanwhile, in the US Food and Drug Administration (FDA), a minimum sensitivity of 1 ng/g is required for the regulatory testing [11].

Although, the EU and the FDA do not authorize BG and CV but BG and CV are often illegally used in the fish farming industry and treatment of certain fish disease owing to low cost, easy availability, high efficiency against fungus, bacteria and parasite. However, the use of BG and CV

causes environmental contamination and affects human health [12]. Therefore, monitoring of these residues in aquatic products is very important. The development of simple and sensitive methods for the determination of BG and CV is of great importance and interest among the chemical analysts [13].

Various techniques such as spectrophotometry [14], electrochemistry [15], electro-chemiluminescence (ECL) [16-18], enzyme linked immunosorbent assay (ELISA)[19], capillary electrophoresis [20], liquid chromatography with visible and/or fluorescence detection [21-24], liquid chromatography–mass spectrometry [25], and liquid chromatography-diode array detection [26] have been applied for quantitative analysis of triphenylmethane dyes and their leuco-metabolites in fish tissue. It is proven that the chromatography and electrophoresis techniques have high sensitivity for the determination of dyes in complex matrices but they have several potential drawbacks such as the need for time consuming sample pre-treatment, the usage of toxic solvents, and the resulting waste products[27, 28]. UV-vis. absorption spectrophotometry is more economical and simpler for analysis of dyes than chromatography and electrophoresis techniques, however, employment of this technique for the simultaneous analysis of dyes in multi-component dye systems often encountered problem owing to overlapping their zero-order absorption spectra. For resolving the spectral overlaps as well as removing the background of signals originated from the other components in samples, the conventional first-order derivative spectrophotometry has been used[28]. Derivative spectrophotometry has widely found applications in different fields of analysis, clinical and biochemical, pharmaceutical, as well as in inorganic or organic analysis [29, 30]. If the derivative peak height (amplitude) of analyte is measured at wavelength in which spectra of the other components pass through zero, the amplitude of the derivative spectrum is proportional only to the concentration of analyte. This approach of quantitative determination is called ″zero-crossing

technique″. It allows quantitative determination of one analyte in the presence of matrix or the simultaneous determination of two analytes in their binary mixtures. In addition, due to dependence of derivatization result on the shape of zero-order absorption spectra, broad signals led to flatting and finally zeroing, so that signals of analyte are amplified. Thus, the influence of the background is eliminated and compare to the classical determination, selectivity is increased [30].

Due to low concentrations of analytes and presence of matrix effects, often a suitable pretreatment step is required prior to their determinations. In the recent decade, dispersive liquid-liquid microextraction (DLLME) is becoming popular extraction treatment due to the simplicity of operation, rapidity of extraction, low cost, environmental friendliness, and high enrichment factor [31, 32]. In this method, a ternary solvent system consisting of an extraction solvent (water immiscible), a dispersing solvent (water and organic solvent miscible) and the aqueous sample solution is used, so that a cloudy solution consisting of fine droplets of the extractant is formed by the fast injection of the mixture of extraction and dispersing solvents to the aqueous sample. In this way, the contacting area between the extraction solvent and the aqueous sample is high so that the equilibrium state is achieved rapidly, and the analytes are enriched into the microdroplets of the extracting phase. The small volume of the used extracting phase can be separated by centrifugation, and collected easily by a microsyringe for the subsequent instrumental analysis [33, 34]. Some dispersive solvents such as ethanol, acetone, and acetonitrile have been used in DLLME. Ionic liquids (ILs) have unique characteristics such as lower vapor pressure, miscibility in aqueous phases, higher viscosity and are generally more environmentally friendly than some organic solvents. Incorporation of functional groups into cations or anions of ILs producing extractants with selective binding sites that improves selectivity of the ILs based microextraction methods [35]. In order to facilitate dispersion of the hydrophobic IL into the aqueous solution and ensure the

adequate formation of microdroplets, ultrasonic energy, microwaves energy, high temperature or vortex have also been used [36, 37].

In this study, we integrate the advantages of ionic liquid based DLLME (IL-DLLME) and first derivative spectrophotometric method by zero crossing technique for simultaneous determination of trace amount of BG and CV in their binary mixtures. Various experimental parameters that affect the extraction process have been carefully studied. To the best of our knowledge, there is no report on the use of IL-DLLME/zero crossing first derivative spectrophotometry for extraction and determination of the BG and CV dyes in aqueous solutions. The QuEChERS extraction approach combined with the developed method was designed for the simultaneous analysis of BG and CV in real samples.

2. Experimental

2.1 Instrumentation

A UV/Vis spectrophotometer Specord 210 Plus (Analytik Jena, Germany) equipped with 1 cm quartz microcells (0.7 mL of capacity) with a fixed slit width of 2 nm and a scan speed of 1200 nm min^{-1} was used for recording the zero order and first derivative absorption spectra. The WinASPECT software package was used for data acquisition and manipulation.

An analytical electrical balance model H30 (Gallenkamp, Germany) was employed to weigh the materials. The pH measurements were carried out by using BP3001 benchtop pH meter equipped with a combined glass electrode (Trans instruments, Singapour). In order to separate the phases, a centrifuge model EBA20 (Hettich, Germany) was used. An automatic blender (Fan azmagostar, Iran) was used to prepare the real samples. A Magnetic stirrer (Gallenkamp, Germany) and a R2062 rotary evaporator (Heidolph, Germany) were used in the synthesis of ionic liquid. Also, deionized water was prepared by using Aqua max system (Young – Lin, South Korea).

2.2 Chemicals and reagents

1-Methylimidazole, 1-bromobuane, 1-bromohexane, 1-bromooctane, and potassium hexafluorophosphate (KPF_6) were used to synthesize the 1-methyl-3-butylimidazolium hexafluorophosphate ([BMIM][PF_6]), 1-methyl-3-hexylimidazolium hexafluorophosphate ([HMIM][PF_6]) and 1-methyl-3-octylimidazolium hexafluorophosphate ([OMIM][PF_6]) ionic liquids and provided from Merck (Darmstadt, Germany). The purity of Brilliant green dye (BG, $C_{27}H_{33}N_2$-HO_4S, 482.64 g mol^{-1}) supplied by Merck was 95%. Crystal violet (CV, $C_{25}N_3H_{30}Cl$, 407.97 g mol^{-1}) was provided from Fluka (Neu-Ulm, Germany) with a purity exceeding 90% and used as received. Deionized water was used for preparing all solutions. The stock solutions of BG and CV (1000 µg L^{-1}) were prepared by dissolving appropriate amounts of BG or CV in deionized water. Working standard solutions were freshly prepared by successive dilutions of the stock solutions with the water. A 0.1 M phosphate buffer solutions were prepared with phosphoric acid and sodium hydroxide solutions. The pH of the solutions was adjusted by 0.1 M NaOH or phosphoric acid solutions using pH meter. Ethanol was used as a diluting solvent in microextraction.

2. 3 Synthesis of ionic liquids

The synthesis of [OMIM][PF_6] was performed according to the method previously reported by Obliosca et al. [38] and purified according to the literature [39, 40]. Briefly, appropriate amounts of 1-methylimidazole and an excess of 1-bromooctane was reacted in a two-necked round-bottom flask with refluxing at 80 ˚C for 48h to obtain1-methyl-3-octylimidazolium bromide ([OMIM][Br]). The resulting viscous liquid was cooled to room temperature and then was washed several times with ethyl acetate (3×20 mL) to remove unreacted starting materials. Finally the product was dried under vacuum at 80˚C. Afterwards, appropriate amount of [OMIM][Br] and KPF_6 aqueous solution

was stirred for 24h at room temperature. The upper phase was removed and the [OMIM][PF_6] ionic liquid was dissolved in dichloromethane. Then, the mixture was washed several times (3×20 mL) with deionized water and was dried at 80°C. Other ionic liquids [BMIM][PF_6] and [HMIM][PF_6] were synthesized in the same way except for using 1-bromobutane or 1-bromohexane for the synthesis of [BMIM][PF_6] and [HMIM][PF_6] ionic liquids, respectively.

2. 4 Sample preparation

The preparation procedure of a trout fish tissue was carried out as follow: A trout fish was caught from a local fishing pond (Abiz, Ghaen, Iran). The skin and bones were removed, and the muscles were minced homogenized in the blender and frozen before being analyzed [41]. Five grams of tissue samples were weighed in a 50-mL polypropylene tube and spiked with appropriate concentrations of BG and CV, mixed thoroughly and kept at room temperature for 30 min. The QuEChERS sample preparation method was used for the extraction of dyes residue from fish sample. The QuEChERS method was carried out by adding 5 mL of deionized water and 10 mL of acetonitrile solution containing formic acid (1%; v/v) to the spiked tissues and shaken for 1 h. Afterward, 1 g of anhydrous sodium acetate and 4 g of anhydrous sodium sulfate were added to the tube and the mixture was vigorously shaken for 1 min and then centrifuged at 5,000 rpm for 5 min. The upper phase was completely separated, filtered to remove impurities and heated to near dryness in a hot water bath. After cooling, the sample was prepared in a phosphate buffer and the pH was adjusted to 4.0. Finally, the prepared sample solution was treated with the recommended DLLME before the simultaneous determination of target dyes by zero-crossing first derivative spectrophotometry.

The river water samples were obtained from the Band-e-Dareh River (Birjand, Iran). Prior to analysis, the aqueous sample was passed through 0.45 μm membrane filter and kept in glass bottle

at 4°C in a refrigerator. The extraction of the target dyes from unspiked and spiked water samples was carried out according to the recommended IL-DLLME procedures.

2.5 DLLME procedure

An aliquot of standards or sample solution containing 100 μg L^{-1} of BG and/or 100 μg L^{-1} of CV was pipetted into 15 mL scaled-glass test tubes with conical bottom and diluted to the mark by addition of phosphate buffer solution (pH = 4). Then, 500 μL sodium chloride (10%, w/v) and 150 μL [OMIM][PF_6] were transferred to the tube and the mixture was shaken for 3 min. The turbid solutions were centrifuged at 4,000 rpm for 10 min. The upper aqueous phase was removed with a syringe, and the volume of the settled phase was diluted to 0.65 mL with ethanol and then entirely transferred into a quartz microcell and determined spectrophotometrically. The zero order UV-vis. spectra were recorded in the wavelength region of 500-700 nm with a resolution of 2 nm against the reagent blank which was treated on dye free solution by the IL-DLLME. For the simultaneous determination of BG and CV, the binary mixture solutions containing BG and CV dyes were prepared and after extraction of the target dyes by the recommended IL-DLLME method, the first order derivative absorption spectra were recorded for the evaluation of dye contents.

3. Results and discussion

3. 1 Optimization of DLLME procedure

To achieve maximum efficiency in the DLLME procedure, several individual experimental parameters including the sample pH, the type of buffer, the type and volume of ionic liquid extractant, the extraction time, the centrifuging time and salt concentration were optimized for each individual dye solution. In each experiment, zero order absorption spectra of the dyes were recorded in the wavelength region of 500-700 nm. BG and CV showed maximum absorbencies at 628 nm and 590 nm, respectively, in their single solutions. The first order derivative spectra were obtained

using the software of instrument with wavelength interval of 2 nm ($\Delta\lambda$=2 nm). To evaluate various effective parameters in the IL-DLLME, the amplitudes of zero crossing first order derivative spectra at 670 nm and 532 nm were selected for the determination of BG and CV, respectively.

3. 1. 1 Effect of sample pH

The pH is an important analytical parameter and plays a special role in the extraction of triphenylmethane dyes. These dyes can exist in the form of colored cationic form or as undissociated basic form as the carbinol [42]. The carbinol form is relatively insoluble in water and forms at high pH value. The formation of carbinol form is also depends on the concentration of the dye. In this study, the effect of pH on the extraction performance of BG and CV was investigated in the pH range of 2-7. BG has two pK_a values of 2.62 and 4.93 while pK_a value for CV is 0.8; consequently at pH<7, BG and CV are mostly dissociated into the cationic form. Thus, the target dyes would be well extracted at low pH values. The results are shown in Fig. 2A, indicating that the maximum extraction efficiency was occurred at pH = 5.0 for BG and pH = 3.0 for CV. At higher pH value than 7.0, OH^- as a nucleophile could be contribute to the structural changes occurred in the dye molecules, producing carbinol form that became easy to remove dyes from aqueous solutions [43]. Therefore, the pH of the sample solution was adjusted to 5.0 and 3.0 for the extraction of BG and CV, respectively, in the following experiments.

The effect of type of buffer on the extraction efficiency of the dyes was also investigated. In this study, acetate, phosphate and Robinson buffers (0.1 M) were prepared at the optimal pH values of 5.0 and 3.0 for microextraction of BG and CV, respectively. As seen in Fig. 2B, the highest extraction efficiency was achieved by using phosphate buffer for both dyes. Therefore, phosphate buffer was used in the preparation of dye samples.

3.1.2 Selection of the microextraction solvent

The extraction solvent is a very important parameter for DLLME processing. Initial conditions necessary for the extraction solvent such as larger density than water, low solubility in water, and high extraction efficiency for the target analytes were considered. In comparison with the organic solvents that used in DLLME methods, ILs are efficient alternatives with properties of less toxicity, less volatility, less contaminating effect and higher enrichment factor. Based on these criteria, three ionic liquids [BMIM][PF_6], [HMIM][PF_6], and [OMIM][PF_6] were examined as extraction solvent. The results are shown in Fig. 3A revealed that [OMIM][PF_6] provided the best extraction efficiencies among three investigated solvents most probably due to higher hydrophobicity. Therefore, [OMIM][PF_6] was selected as an appropriate extraction solvent for BG and CV dyes. The ionic liquid based DLLME process was performed without any dispersing solvent.

3.1.3 Effect of the volume of ionic liquid

To investigating the effect of extraction solvent volume, different volumes of [OMIM][PF_6] IL from 25 to 200 μL were tested. The results in Fig. 3B indicated that the extraction efficiency increased with increase the [OMIM][PF_6] IL volume from 25 μL to 150 μL, and then it remained constant over the volume range of 150-200 μL. When the IL volume was too large, the amount of sedimented IL increased, so that the concentration of target dyes in the IL phase decreased, resulted in decreasing of the extraction efficiencies. Consequently, 150 μL of [OMIM][PF_6] IL was used for the microextraction of target dyes.

3.1.4 Effect of extraction time

In the DLLME, the extraction time is defined as the time between injecting the extraction solvent and centrifugation [44]. In this experiment, the effect of extraction time was examined in the range of 0.5-10 min at room temperature. It is expected that increasing the extraction time facilitates the mass transfer of target dyes to the IL phase which lead to enhance the extraction efficiencies. It was

found that more than 95% of BG ad CV were extracted within 3 min at 100 μg L^{-1} and then remained relatively constant over the time range of 3-10 min. It seems the extraction of analytes into the extractant was achieved almost instantaneous. Therefore, the optimum extraction time for BG and CV was chosen 3 min (Fig. 4A).

3.1.5 Effect of centrifuging time

Centrifugation allows the quick and easy collection of the extractant phase at proper time. Centrifuging time affects the volume of the settled phase by extraction. Therefore, the right choice of centrifuging time has a great effect on extraction efficiency [45]. In this regard, the effect of this parameter on the extraction efficiency of dyes was studied in the range from 5 to 25 min at a centrifugation speed of 4,000 rpm. The results illustrated in Fig. 4B, showing that the maximum extraction efficiencies were obtained in 10 min. In short centrifuging times, the IL phase didn't completely separate and collect at the bottom of the test tube. At longer centrifuging times, extraction efficiency decreased could be attributed to combine the IL microdroplets again which led to more dissolving IL in the aqueous phase. Based on these observations, centrifuging the cloudy mixture for 10 min at 4,000 rpm was used for further experiments.

3.1.6 Effect of ionic strength

The ionic strength of the sample solution is an important analytical parameter that affects the extraction efficiency. Generally, ionic strength is affected by two processes: electrostatic interactions and salting out [11]. The salting out effect increases the extraction efficiency, while electrostatic interactions between salt ions and the target analyte decrease the extraction efficiency.

In this work, the effect of ionic strength was studied with the addition of NaCl salt to the aqueous dye solutions ranged from 0.1 to 30% (w/v). It is evident from Fig.5 that the extraction efficiency of target analytes increased with increasing NaCl concentration from 0.1 to 0.5% (w/v) and then was

remained constant up to 20% (w/v) and after that eventually decreased. The solubility of IL in the water phase increased with the increase of high ionic strength above 20% (w/v), because of increasing the density and viscosity of the aqueous phase. Hence, the IL phase couldn't separate from aqueous phase and the sediment phase volume decreased. These results revealed that the recommended method has potential for analysis of the target dyes from saline solutions up to 20% (w/v).

3.2 The determination of BG and CV in their single solutions

Under the optimal conditions, the zero order absorption spectra of each dye were recorded in the wavelength range of 500-700 nm. The maximum absorption at wavelengths of 628 and 590 nm were used for the analysis of BG and CV at different concentrations in their single dye solutions, respectively. The calibration curves were linear in the range of 1–500 μg L^{-1} for BG and CV dyes, however, the correlation deviated at very high mixture concentrations.

3. 3 The simultaneous analysis of BG and CV in binary mixtures

According to the results obtained from the optimization process for target dyes, except for the pH, other optimal parameters were similar for the microextractions of BG and CV. Therefore, the simultaneous determination of BG and CV in binary mixtures was carried out under the optimal conditions at pH 4 where both dyes could extract with high extraction efficiencies. Although, a large amount of CV extracted at pH= 3, but significant amount of BG remained in the aqueous phase. At pH=5, the extraction efficiency of BG was higher than CV, however, both dyes could be extracted at pH=4 with high extraction efficiencies. Accordingly, for the simultaneous determination of BG and CV in binary solutions, pH= 4 was selected as the optimal pH value.

For the simultaneous determination of BG and CV, a binary mixture containing 100 μg L^{-1} of each dye was prepared and after microextraction of target dyes by the proposed method, the zero order

absorption spectra was recorded (Fig. 6A). Although BG and CV in single solution showed well defined zero order absorption spectra in the wavelength range of 500-700 nm with maximum absorbance at 628 nm and 590 nm, respectively, but, these spectra were overlapping in the binary mixture. Therefore, simultaneous determination of the two dyes was not possible by direct measurement of absorbance using zero-order absorption spectra. The treatment of derivative absorption spectra has been the basis of simultaneous analysis of BG and CV dyes in binary mixtures. Fig. 6B depicts the first order derivative absorption spectra of BG and CV in single and binary mixture solutions in the wavelength range of 500-700 nm. Simultaneous determination was performed by first derivative absorption spectra of dyes using zero-crossing method based on measurement of first derivative absorbance value of BG dye at 670 nm ($^1D_{670}$) in the presence of CV, where the derived absorbance of CV was zero (zero crossing point), and at 532 nm ($^1D_{532}$) for CV dye in the presence of BG, where the derived absorbance of BG dye was zero or near to zero value (zero crossing point). Thus, the both dyes were assayed in the concentration range of 10-500 μg L^{-1} by measurements of the peak amplitude of the first derivative spectra at 670 nm for BG and 532 nm for CV (Fig. 7).

The accuracy of the zero-crossing first order derivative spectrophotometry method for analyzing the BG and CV dyes concentrations in binary solutions was checked with the recovery studies [46]. Two set standard solutions containing BG and CV dyes at different concentrations were prepared. In the first set, the CV concentration was fixed at 200 μg L^{-1} while the BG concentration was changed from 10 to 500 μg L^{-1}(Fig. 7A). In the second set, the similar trend was used to get standard solutions of CV having concentrations from 10 to 500 μg L^{-1} in the presence of 200 μg L^{-1} BG (Fig. 7B). Finally, to prove the validity of the proposed method, synthetic mixtures of different concentrations of both dyes changing from 10 to 500 μg L^{-1} were assayed (Fig. 7C). The results

were computed against the previously constructed standard curves. The recoveries (R, %), errors (E, %) and average percentage errors (C, %) were calculated using Eqs. from (1) to (3), respectively, where C_t is the theoretical concentration and C_m is the measured concentration in μg L^{-1}. In Eq. (3), N is the number of measurements.

$$R\% = \frac{C_m(\mu g L^{-1})}{C_t(\mu g L^{-1})} \times 100 \quad (1)$$

$$E\% = \frac{C_m(\mu g L^{-1}) - C_t(\mu g L^{-1})}{C_t(\mu g L^{-1})} \times 100 \quad (2)$$

$$C\% = \frac{\sum_{i=1}^{N} \left| C_m - C_t / C_t \right|}{N} \times 100 \quad (3)$$

The results of analysis of the synthetic mixtures are summarized in Table 1. As could be seen from Table 1, the recovery values for BG and CV were in the range of 96 - 105% and the average percentage errors (E,%) were 3.2 and 3.5% for BG and CV, respectively. It was shown that the obtained concentrations agreed reasonably well with the theoretical concentrations of both dyes for reliable measurements. According to the recovery studies, the simultaneous determination of BG and CV contents in binary mixtures can be accurately performed by using the zero crossing first derivative spectrophotometric method.

3.4. Analytical performance

From the measurements made under the optimum conditions, calibration graphs for the determination of each dye in their single solutions and the simultaneous determination of BG and CV dyes in the binary mixtures were constructed based on the first derivative spectra of the BG and CV dye solutions and their mixtures. The linearity was obeyed in the concentration ranges of 1-500 μg L^{-1} and 10-500 μg L^{-1} for single and binary dye solutions, respectively. The limit of detection based on three times of standard deviation of the blank (S_b) divided by the slope of calibration curve

(m) in simultaneous determinations of BG and CV were 2.7 μg L^{-1} and 1.4 μg L^{-1}, respectively. Also, the relative standard deviations (RSD%) for five replicate simultaneous determinations of BG and CV at 50 μg L^{-1} of each dye were 4.7% and 1.7%, respectively. Because the amount of BG and CV in 10 mL sample solution are measured after preconcentration by DLLME in a final volume of 150 μL, the analytes were concentrated by a factor of 67 (Table 2).

3. 5 Interference study

The effect of different ions on the simultaneous determination of BG and CV by the IL-DLLME method was investigated by the measurement of the absorbencies of dyes using zero crossing point first derivative spectrophotometry. An ion was considered as interferent when it caused a variation in the absorbencies of the analytes greater than ± 5% [47]. For this study, various amounts of ionic species were added to the binary mixtures of dyes each at 50 μg L^{-1} and processed by the IL-DLLME. In the first test, a 5000-fold weight ratio of the interferent to the dyes was investigated. If interference occurred, this ratio was gradually reduced until the interference stopped. As the results show in Table 3, a large excess amount of common cations and anions did not interfere in the simultaneous determination of BG and CV.

3.6 Application to real samples

In order to evaluate the analytical applicability of the recommended method, it was applied to the simultaneous determination of BG and CV in river water and trout fish samples. Real samples were prepared according to section 2.4. In both samples, concentrations of BG and CV by standard addition method at 670 nm ($^1D_{670}$) and 532 nm ($^1D_{532}$), respectively, were calculated. The results of analysis are summarized in Table 4. The presented method was compared with the other liquid phase microextraction methods based on UV-Vis. spectrophotometric detection for determination of trace levels of BG and CV dyes [3, 5, 13, 48, 49] and the results are shown in Table 5. The

established IL- DLLME combined with zero crossing first derivative spectrophotometry showed advantages like relatively low detection limits, wide linear concentration range, cost-effectiveness and easily sample handling properties that makes the method a reliable tool in simultaneous monitoring BG and CV in aqueous phase.

4. Conclusion

The IL-DLLME system is an environmentally friendly and simple pre-concentration way prior to spectrophotometric determination of trace amounts of BG and CV. Although BG and CV in their single solutions showed well defined zero order spectra in the wavelength range of 500-700 nm with maximum absorption at 628 nm and 590 nm, respectively, but in the binary mixture, the zero order absorption spectra of BG and CV dyes overlapped. By the first derivation of zero order spectra, the broad shape of zero-order spectra could flat, so that signals of analytes were amplified. Using the first derivative absorption spectra and zero-crossing method, simultaneous determination of BG ad CV dyes was feasible. The present method showed notable points like simplicity, efficiently, rapidly and low cost which is a good option for determination of the BG and CV dyes even in low sample volumes. The obtained results demonstrated the capability of the method for simultaneous detection of BG and CV at trace level in fish and water samples.

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Table 1. Results of percentage recovery, error and average error values for BG and CV in binary solutions by first order derivative spectra method

Theoretical (μg L^{-1})		Measurement (μg L^{-1})		Recovery (%)		Error (%)	
C_{BG}	C_{CV}	C_{BG}	C_{CV}	BG	CV	BG	CV
200	10	194.1	10.4	97.0	104.4	-2.9	4.4
200	25	207.0	24.0	103.5	95.9	3.5	-4.1
200	50	207.7	52.2	103.8	104.4	3.8	4.4
200	100	203.7	103.2	101.8	103.2	1.8	3.2
200	200	197.7	210.0	98.8	105.0	-1.2	5.0
200	300	208.0	290.7	104.0	96.9	4.0	-3.1
200	400	191.0	401.7	95.5	100.4	-4.5	0.4
200	500	208.3	522.7	104.2	104.5	4.2	4.5
10	200	9.7	190.0	96.7	95.0	-3.3	-5.0
25	200	24.3	190.7	97.3	95.3	-2.7	-4.7
50	200	48.3	195.5	96.7	97.8	-3.3	-2.2
100	200	104.7	204.9	104.7	102.4	4.7	2.4
200	200	209.3	193.0	104.7	96.5	4.7	-3.5
300	200	310.7	206.9	103.5	103.5	3. 5	3.7
400	200	419.7	210.0	104.9	105.0	4.9	5.0
500	200	518.0	208.6	103.6	104.3	3.5	4.3
10	10	10.3	9.8	103.3	98.0	3.3	-2.0
25	25	24.7	24.3	98.7	97.1	-1.3	-2.9
50	50	47.7	47.6	95.3	95.2	-4.7	-4.8
100	100	98.0	104.5	98.0	104.5	-2.0	4.5
200	200	197.7	210.0	98.8	105.0	-1.2	5.0
300	300	288.7	313.6	96.2	104.5	-3.8	4.5
400	400	384.7	400.5	96.2	100.1	-3.8	0.1
500	500	488.3	501.9	97.7	100.4	-2.3	0.4
					%C	3.3	3.5

Table 2. Figures of merit of the proposed method

Parameters	Analytical feature	
	BG	CV
Linear range in single solution	1-500	1-500
Correlation coefficient in single solution (R^2)	0.9997	0.9996
Linear range in binary solution	10-500	10-500
Correlation coefficient in binary solution (R^2)	0.9974	0.9955
Limit of detection in binary solution(μg L^{-1})	2.7	1.4
%RSD (n = 5) in binary solution	4.7	1.7
Enrichment factor	67	67

Table 3. Effect of coexisting ions on the absorbance of 50 μg L^{-1} BG or CV in binary solutions

Foreign ions	Tolerance ratio[a]	
	BG	**CV**
Li^+, K^+,Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+},SO_4^{2-},SCN^-	5000	5000
$H_2PO_4^-$	4000	4000
F^-, I^-	3000	3000
PO_4^{3-}	2000	2000
CH_3COO^- ,Cu^{2+}	1000	1000
NO_2^-	500	500

[a] Amount of foreign ion/amount of BG or CV

Table 4. Analytical results for BG and CV determination in real samples by the recommended procedure

Sample	Added concentration ($\mu g\ L^{-1}$)		Found concentration ($\mu g\ L^{-1}$) ± SD[a]		Recovery (%)	
	BG	CV	BG	CV	BG	CV
Fish	0	0	0.7 ± 0.5	0.7 ± 0.4	-	-
	10	10	10.8 ± 0.4	9.6 ± 0.4	100.6±3.4	90.0 ± 3.7
	50	50	41.3 ± 0.3	48.8 ± 1.0	81.5±0.6	96.3 ± 2.1
	100	100	90.3 ± 1.7	97.1 ± 2.4	89.7±1.7	96.4 ± 2.4
Water	0	0	0.9 ± 0.4	0.8 ± 0.7	-	-
	10	10	10.9 ± 0.2	9.1 ± 0.6	100.0 ±1.7	83.9 ± 4.5
	50	50	41.8 ± 0.8	48.4 ± 0.8	82.1 ± 1.6	95.1 ± 1.6
	100	100	91.7 ± 1.2	94.8 ± 2.3	90.9 ± 1.2	94.0 ± 2.3

[a]Triplicate analyses(n=3)

Table 5. Comparison of the reported liquid phase extraction methods with the recommended method based on UV-Vis. detection

Analyte	Method	LOD ($\mu g\ L^{-1}$)	RSD (%)	Linear range ($\mu g\ L^{-1}$)	EF	Ref.
BG	[a] CPE	15	2.7	50-2000	-	3
CV	[b] TC-IL-DLLME /HPLC	0.03	7.6	0.25-20	276	5
CV	[c] IL-CIAME	1.16	2.3	3.6-15	152	13
CV	[a] CPE	4.8	-	16-1000	-	48
CV	[d] IL-MAE/HPLC	0.08	5.9	0.1-25	-	49
BG&CV	IL-DLLME- ZCDSP	BG; 2.7 CV; 1.4	BG; 4.7 CV; 1.7	10-500	67	This work

[a] Cloud point extraction

[b] Temperature-controlled ionic liquid dispersive liquid–liquid microextraction/High performance liquid chromatography

[c] Ionic liquid cold-induced aggregation microextraction

[d] Ionic liquid-based microwave-assisted extraction/High performance liquid chromatography

[e] Ionic liquid based dispersive liquid-liquid microextraction followed by Zero-crossing first derivative spectrophotometric method (ZCDSP)

Figure captions

Fig. 1 Chemical structures of **(A)** brilliant green (BG) and **(B)** crystal violet(CV)

Fig. 2 The effect of **(A)** pH and **(B)** type of buffer on the extraction efficiency of BG and CV after IL-DLLME(Conditions: Sample volume, 10 mL; dye concentration, 100 μg L^{-1}; acetate buffer, 0.1M; [OMIM][PF_6] IL, 100 μL; extraction time, 1 min; centrifugation time, 20 min).

Fig. 3 The effect of **(A)** type of ionic liquid and **(B)** volume of ionic liquid on the extraction efficiency of BG and CV after IL-DLLME (Conditions: Sample volume, 10 mL; pH=5 for BG, pH=3 for BG; dye concentration, 100 μg L^{-1}; phosphate buffer, 0.1M; [OMIM][PF_6] IL, 100 μL; extraction time, 1 min; centrifugation time, 20 min)

Fig. 4 The effect of **(A)** extraction time and **(B)** centrifuge time on the extraction efficiency of BG and CV after IL-DLLME(Conditions: Sample volume, 10 mL; pH=5 for BG, pH=3 for BG; dye concentration, 100 μg L^{-1}; phosphate buffer, 0.1M; [OMIM][PF_6] IL, 150 μL

Fig. 5 The effect of salt concentration on the extraction efficiency of BG and CV after IL-DLLME (Conditions: sample volume 10 ml, dye concentration 100 μg L^{-1} pH=5 for BG, pH=3 for BG; dye concentration,100 μg L^{-1}; phosphate buffer, 0.1M; [OMIM][PF_6] IL, 150 μL; extraction time, 3 min; centrifugation time, 20 min)

Fig. 6 (A) Zero order absorption spectra and **(B)** first order derivative absorption spectra of BG and CV in single and binary solutions of each dyes at 100 μg L^{-1}

Fig. 7 First order derivative spectra of BG and CV in binary solutions **(A)** at 200 μg L^{-1} constant concentration of CV with varying concentration of BG in the range of 10-500 μg L^{-1}, **(B)** at 200 μg L^{-1} constant concentration of BG with varying concentration of CV in the range of 10-500 μg L^{-1}, and **(C)** with varying concentrations of BG and CV in the range of 10-500 μg L^{-1}

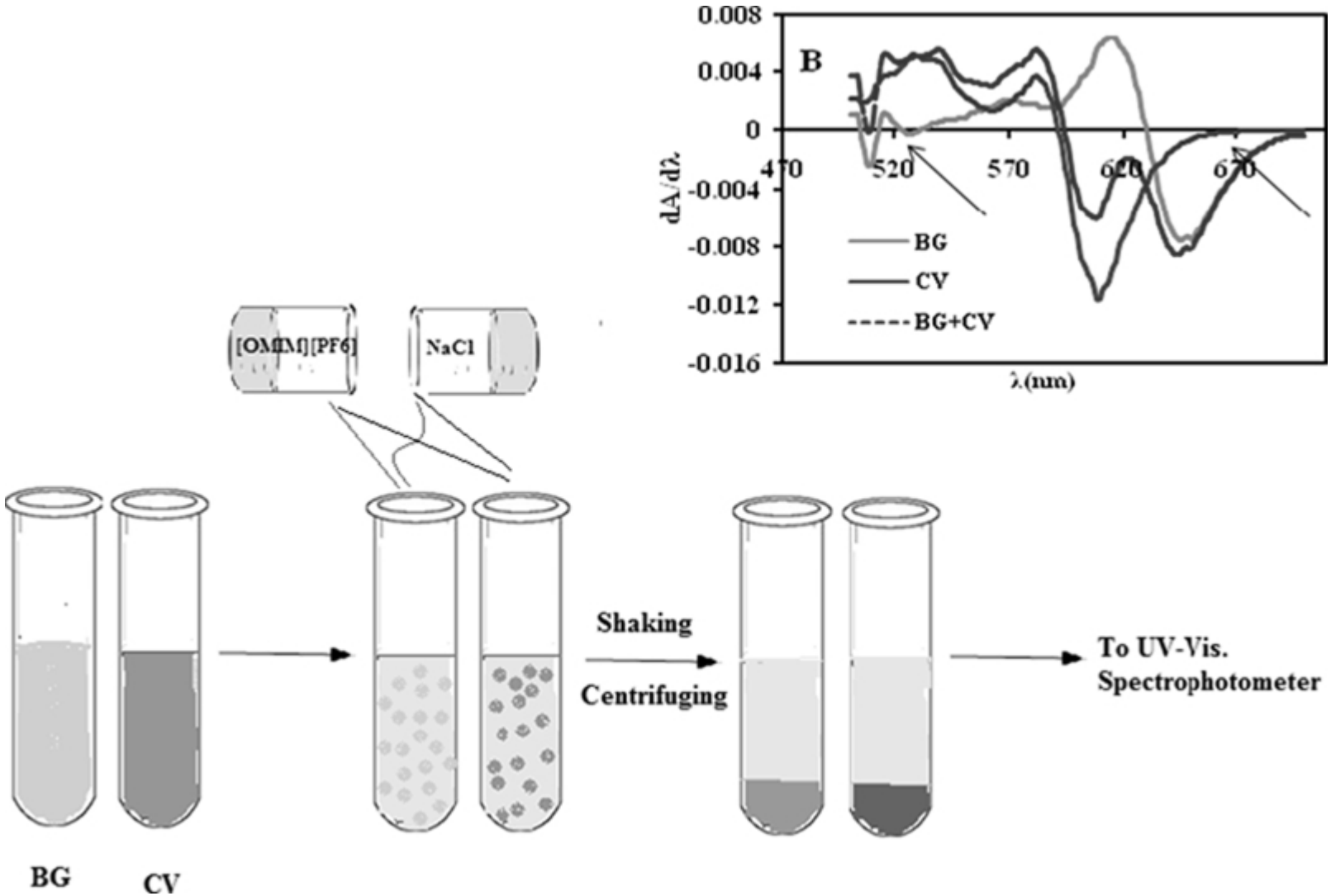
Highlights

► Simultaneous determination of CV and BG dyes that zero order spectrophotometric sepectra are overlapped

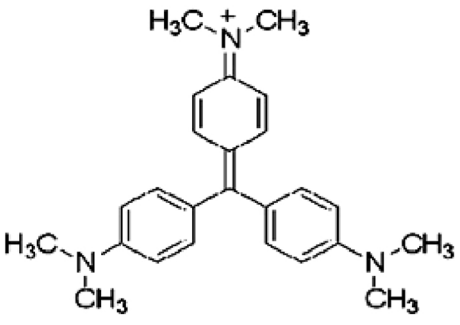
► The use of environmental friendliness ionic liquid extractant [OMIM][PF_6]

► Simple, efficient, rapid and cost effectiveness method for determination of the BG and CV dyes

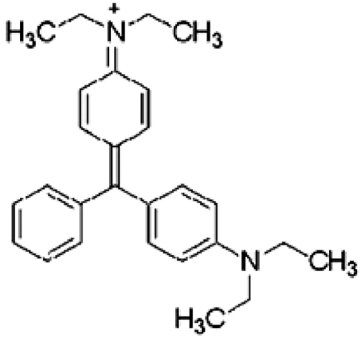
► Successful applicable in determination of BG and CV dyes in fish and water samples



Graphics Abstract

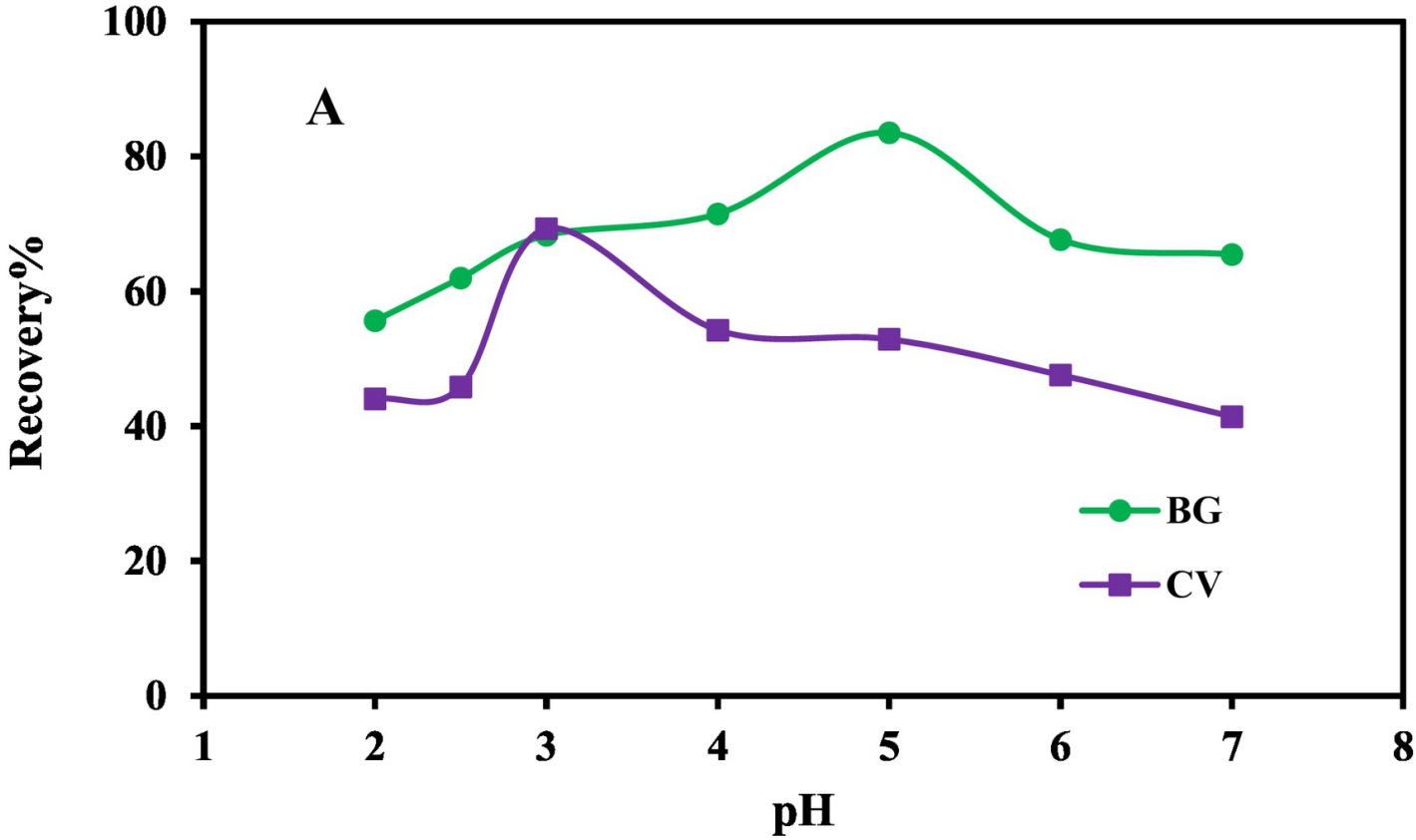


B



A

Figure 1


A
Recovery%
100
80
60
40
20
0
1
2
3
4
5
6
7
8
pH
BG
CV

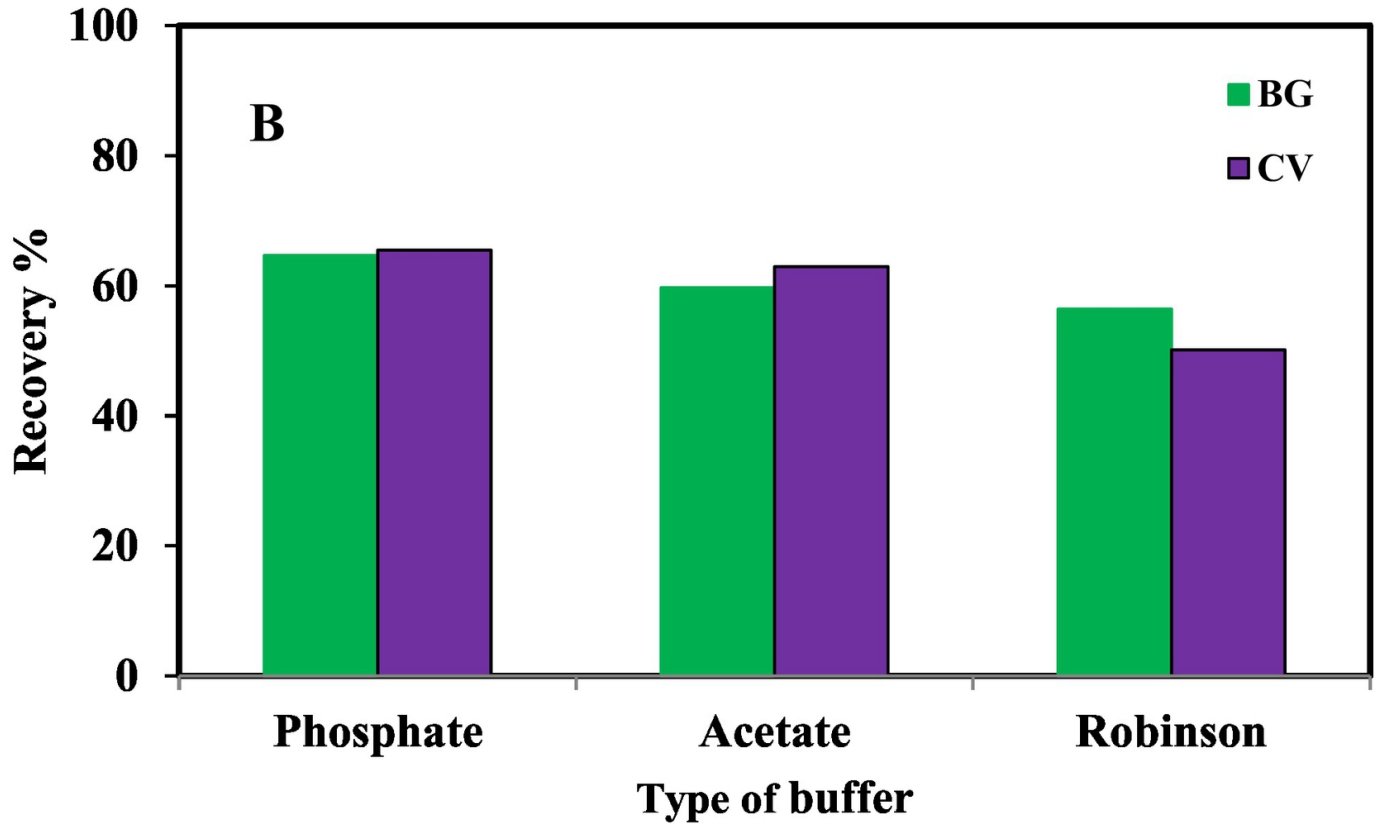
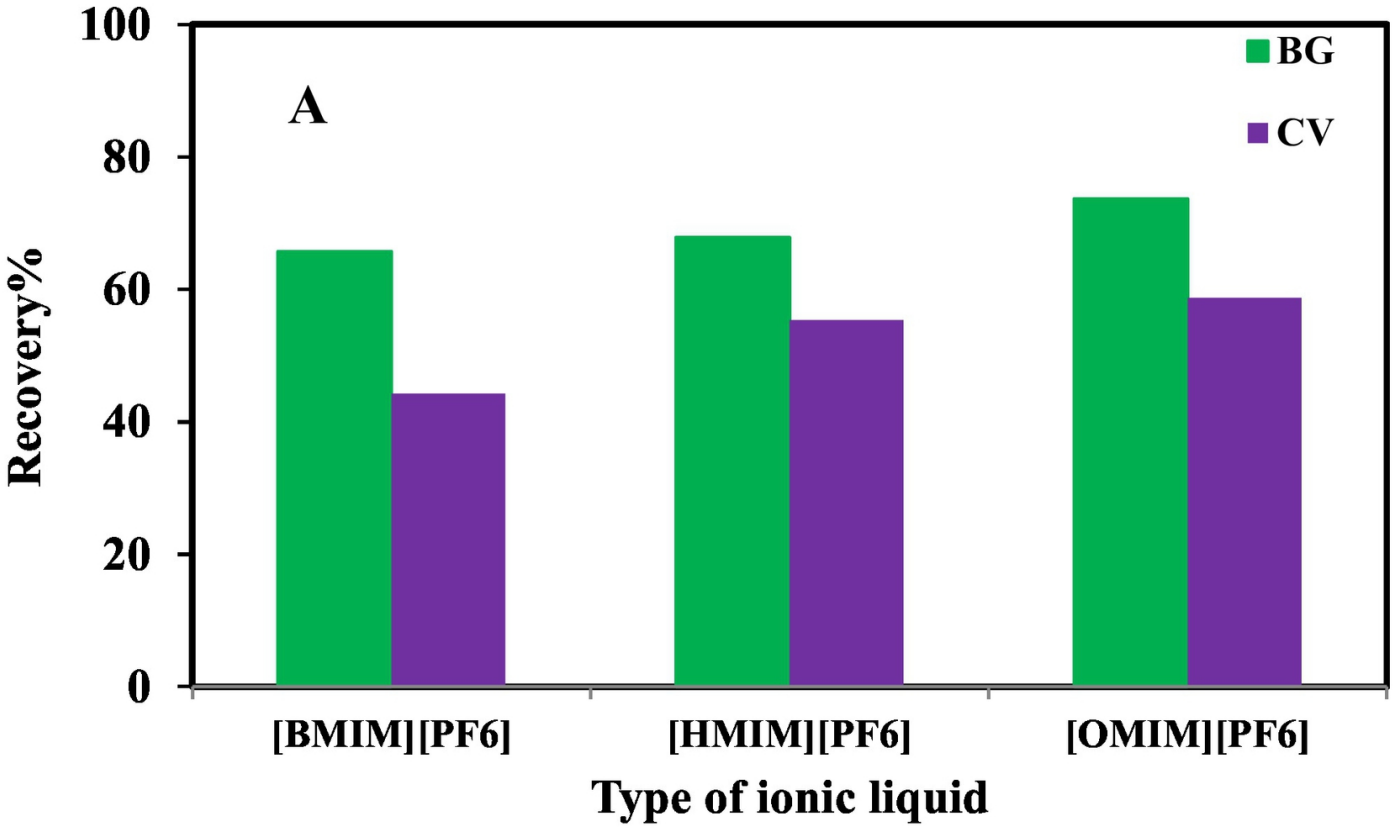

B
Recovery %
100
80
60
40
20
0
Phosphate
Acetate
Robinson
Type of buffer
BG
CV

Figure 2


A
BG
CV
Recovery%
100
80
60
40
20
0
[BMIM][PF6]
[HMIM][PF6]
[OMIM][PF6]
Type of ionic liquid

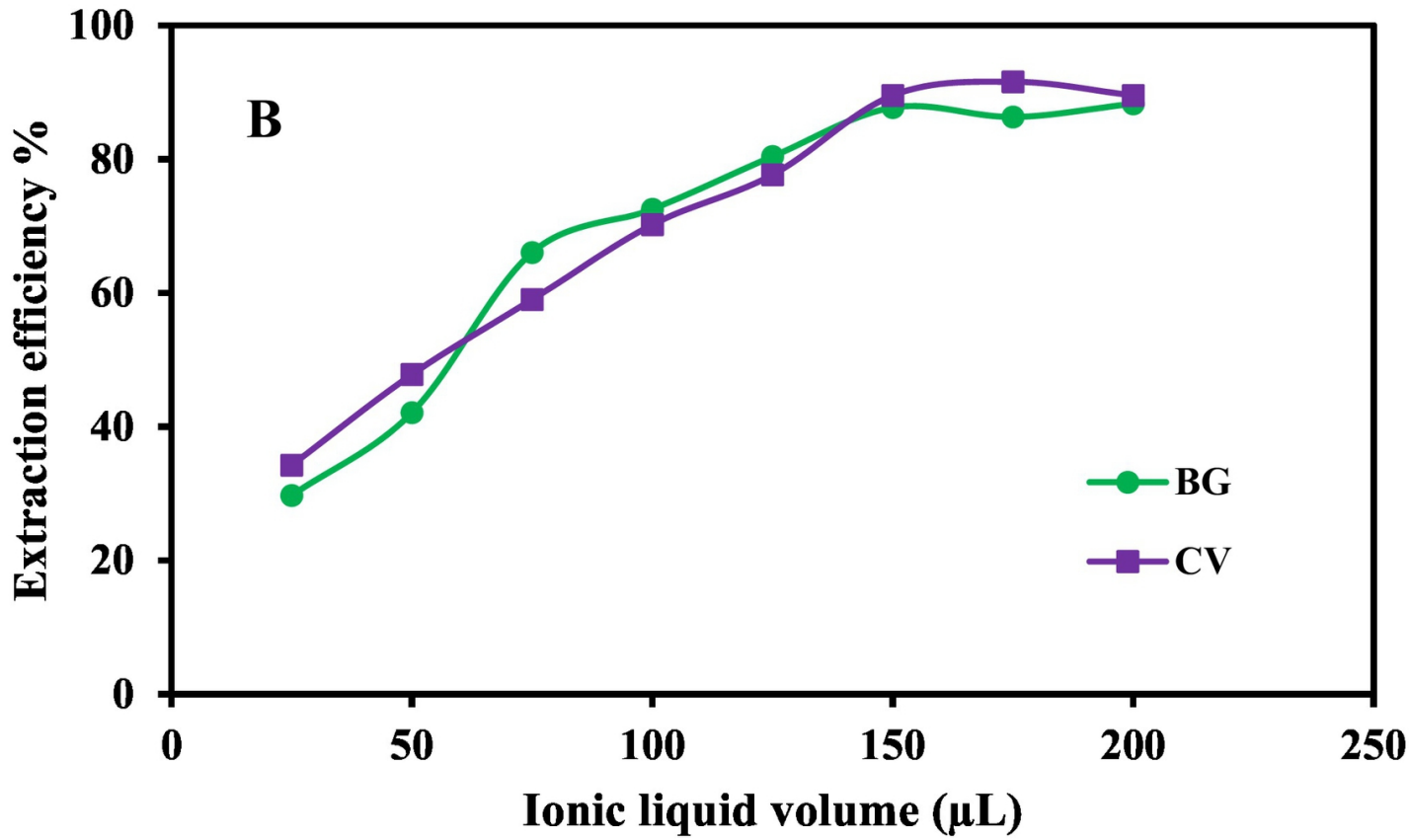
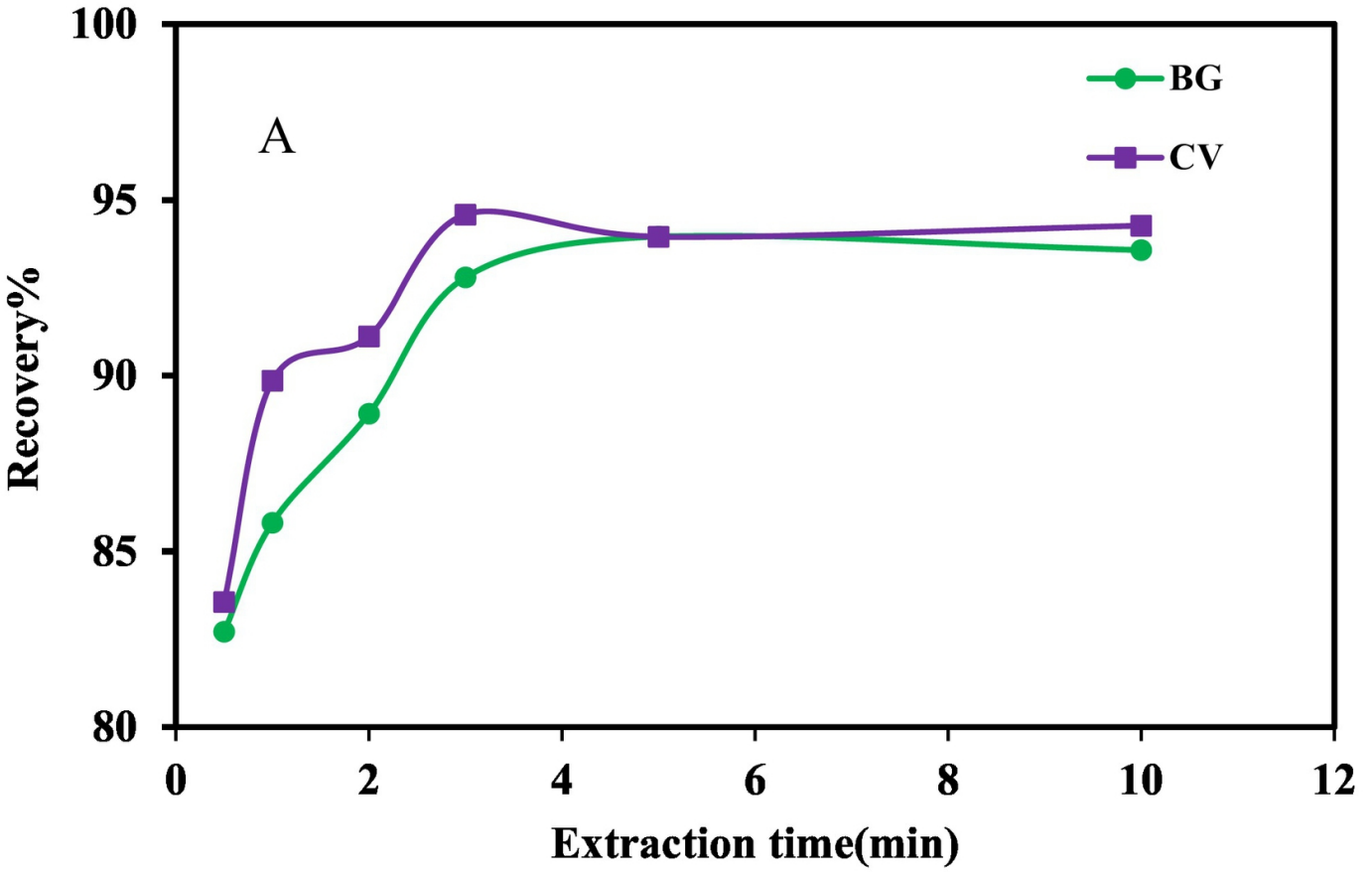

B
Extraction efficiency %
100
80
60
40
20
0
BG
CV
0
50
100
150
200
250
Ionic liquid volume (µL)

Figure 3


A
BG
CV
Recovery%
100
95
90
85
80
0
2
4
6
8
10
12
Extraction time(min)

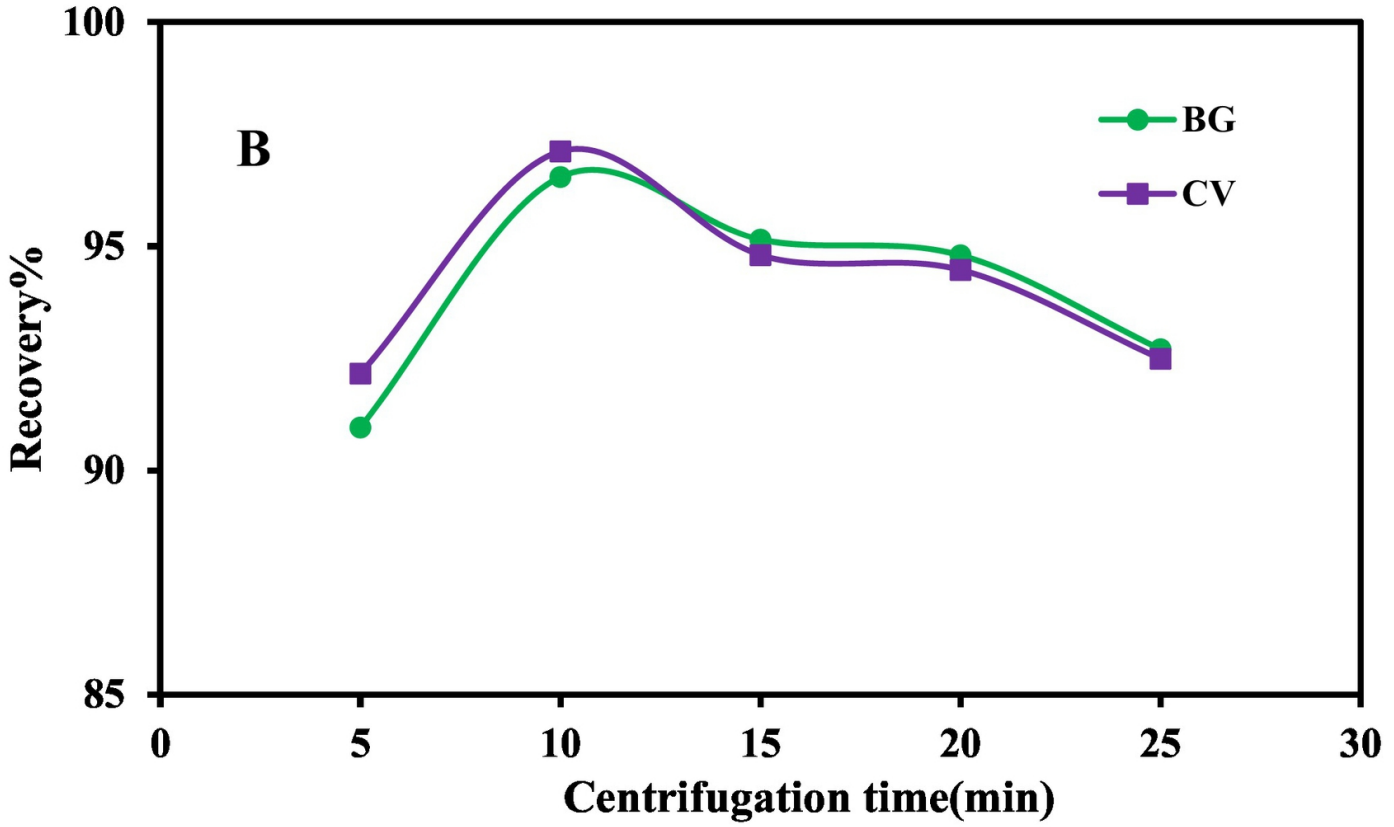

B
BG
CV
Recovery%
100
95
90
85
0
5
10
15
20
25
30
Centrifugation time(min)

Figure 4

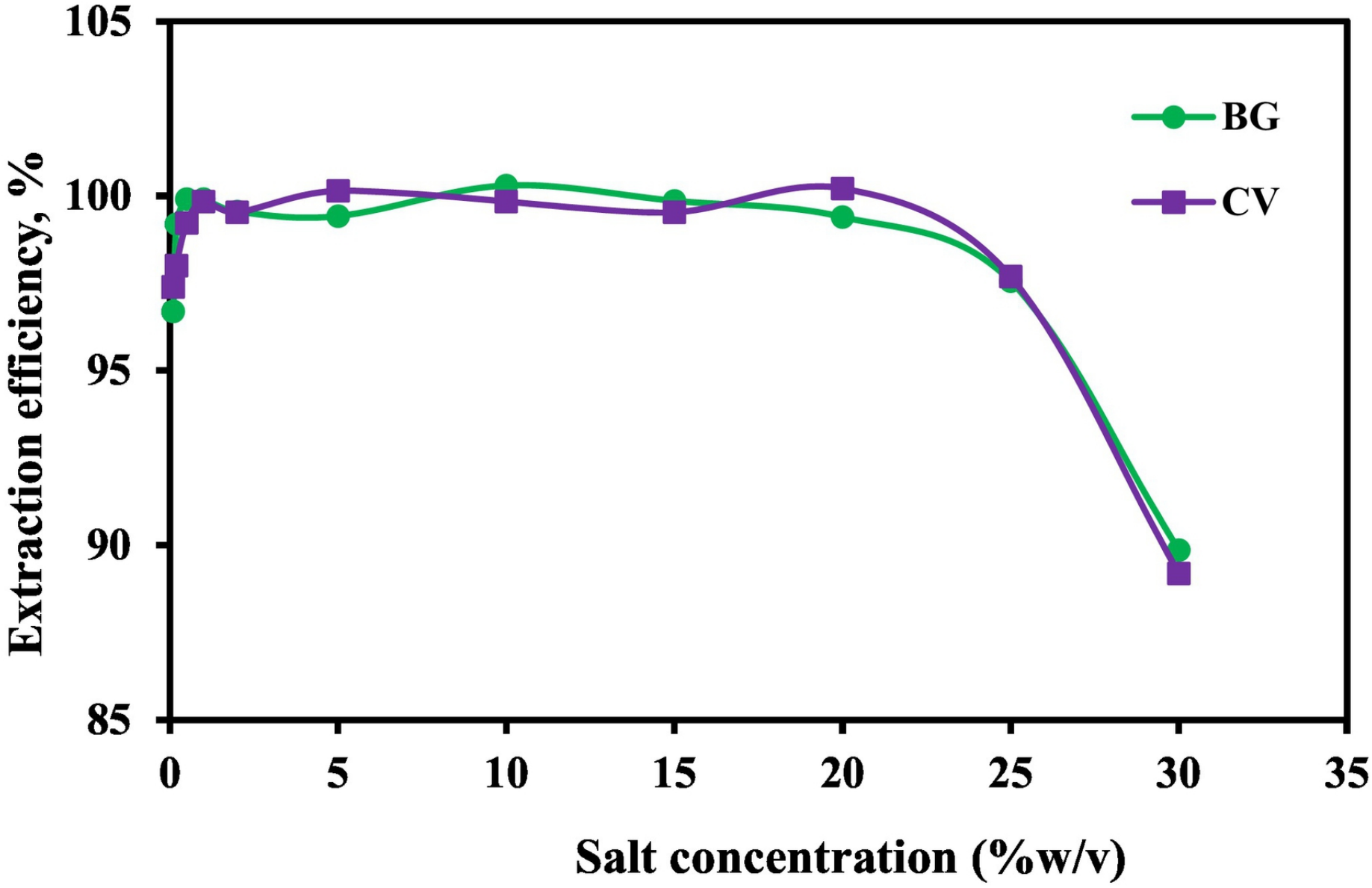

105
100
95
90
85
Extraction efficiency, %
0
5
10
15
20
25
30
35
Salt concentration (%w/v)
BG
CV

Figure 5

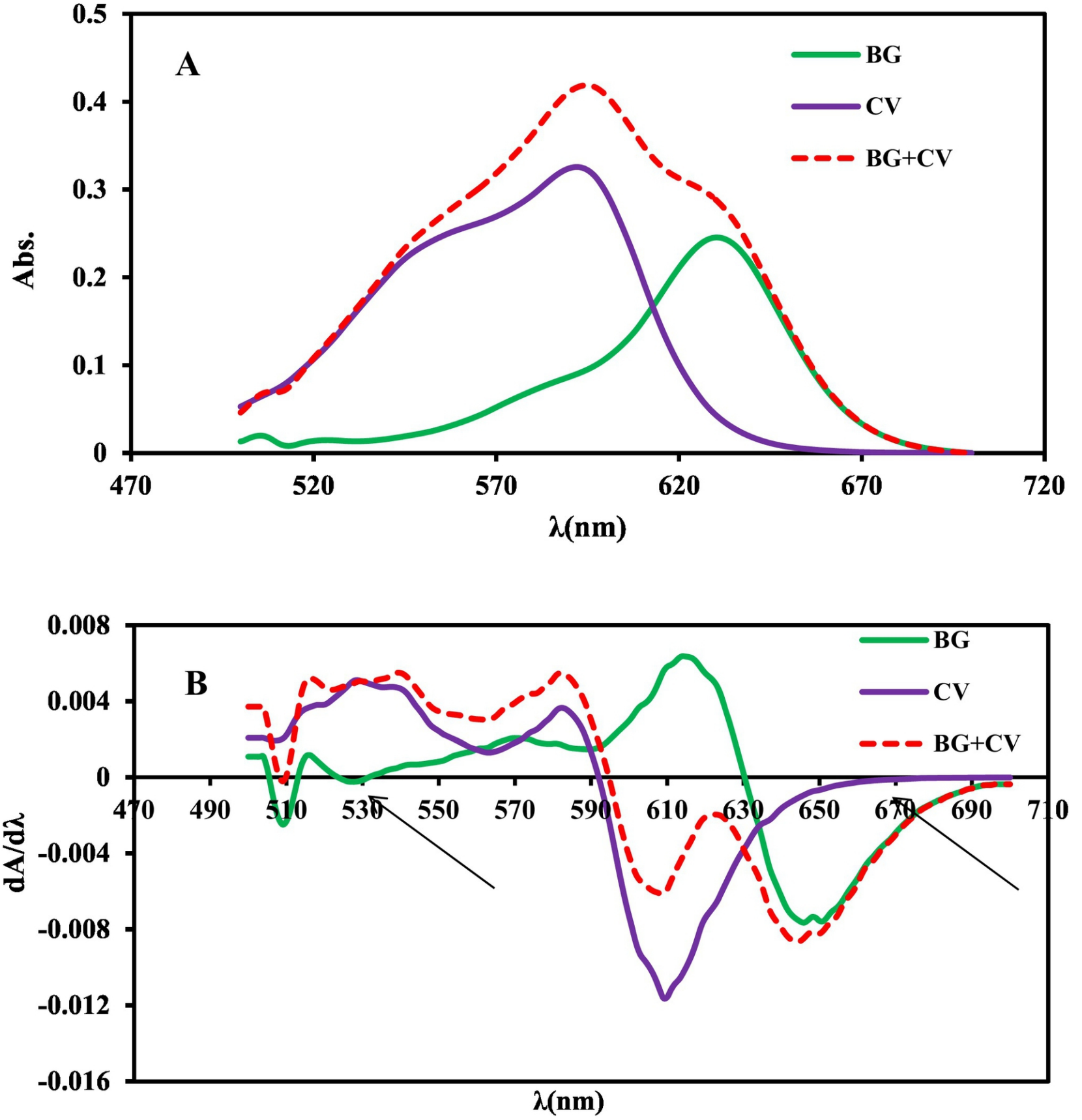

A
BG
CV
BG+CV
Abs.
0.5
0.4
0.3
0.2
0.1
0
470
520
570
620
670
720
λ(nm)
B
BG
CV
BG+CV
dA/dλ
0.008
0.004
0
-0.004
-0.008
-0.012
-0.016
470
490
510
530
550
570
590
610
630
650
670
690
710
λ(nm)

Figure 6

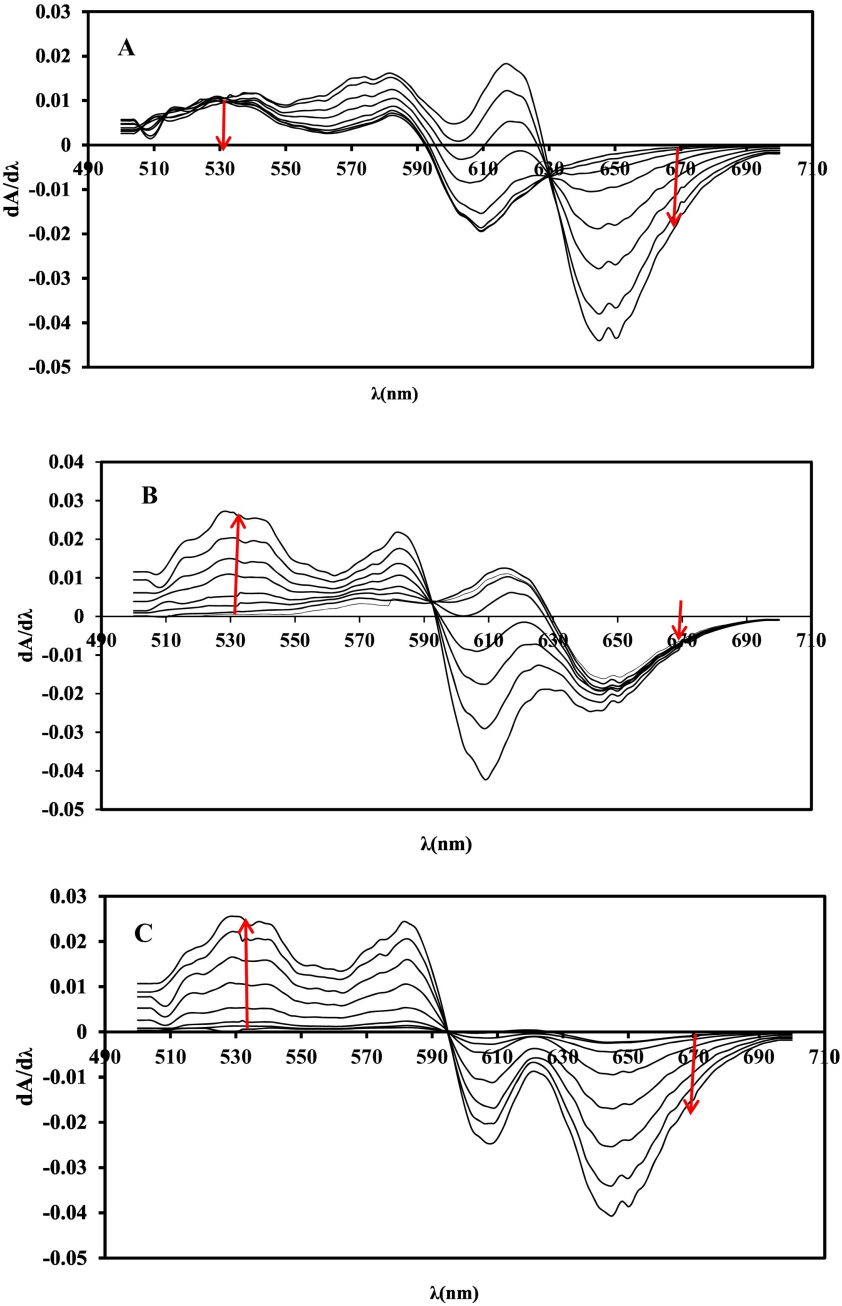

A
B
C
dA/dλ
λ(nm)
0.03
0.02
0.01
0
-0.01
-0.02
-0.03
-0.04
-0.05
0.04
490
510
530
550
570
590
610
630
650
670
690
710

Figure 7