CHAPTER IV

RESULTS AND DISCUSSION

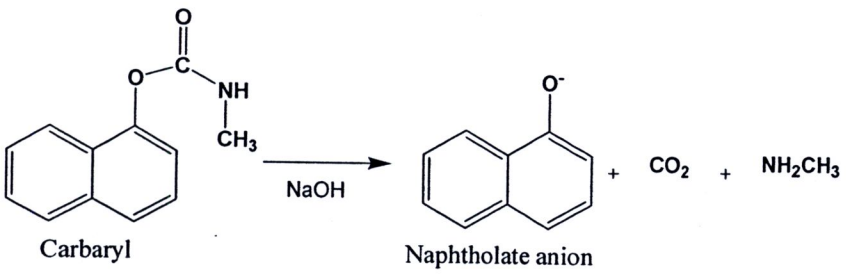
The results and discussion are divided into four parts. The first part (section 4.1) involves the spectrophotometry for determination of CBR using DMA as the derivatizing agent and simultaneous determination of CBR and CBF using AP as the derivatizing agent. In this part, the optimum conditions for the derivatization of CBR and CBF are reported. The second part (section 4.2) involves sample preparation, optimization for extraction and preconcentration of CBR and CBF by CPE in vegetable samples. The third part (sections 4.3 to 4.5) summarizes the results for recovery of CBR and CBF using simultaneous determination in spiked samples and validation of the proposed method. In addition, comparison of the proposed spectrophotometry with HPLC is discussed. The fourth part (section 4.6) demonstrates the application of the proposed method for determination of CBR and CBF in vegetable samples.

4.1 Spectrophotometry of CBR and CBF

Two derivatizing agents were used. The derivatization of CBR using DMA as the derivatizing agent is based on coupling reaction of the hydrolyzed phenolate product with diazonium ion in aqueous solution. While, the derivatization of CBR or CBF using AP as the derivatizing agent involves the reaction of the hydrolyzed phenolate product with derivatizing agent in the presence of an oxidizing agent.

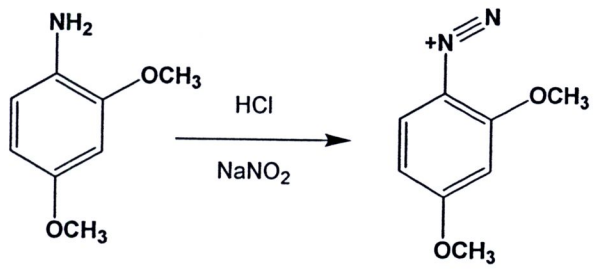
4.1.1 Derivatization of CBR using DMA

The derivatization of CBR is easily performed via diazotization reaction to form azo-dye compound. The reaction is generally two steps reaction namely hydrolysis and coupling, as shown in Figure 4.1.



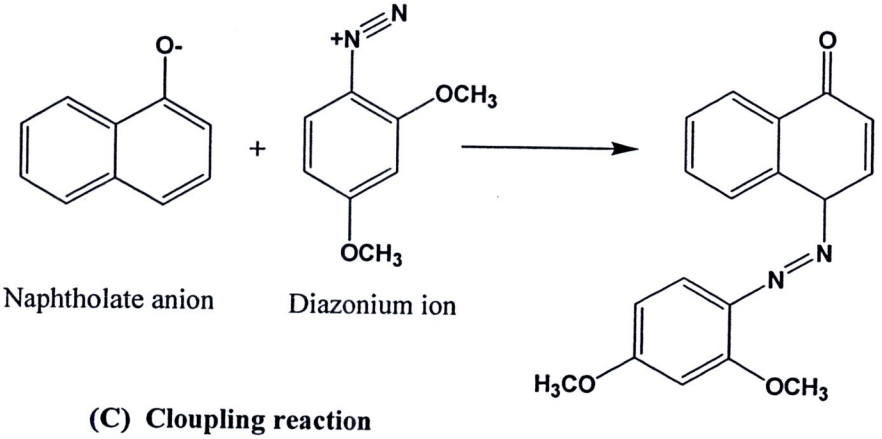
Carbaryl Naphtholate anion

(A) Hydrolysis of CBR



2,4-dimethoxyaniline Diazonium ion

(B) Diazotization of DMA



Naphtholate anion Diazonium ion

(C) Cloupling reaction

Azo dye (510 nm)

Figure 4.1 Derivatization reaction of CBR; DMA as the derivatizing agent

First step, CBR was hydrolyzed with NaOH to form naphtholate anion (Figure 4.1 (A)), while DMA was mixed with $NaNO_2$ and HCl to form diazonium ion (Figure 4.1 (B)). Second step, the naphtholate anion was mixed with diazonium ion to form azo dye (Figure 4.1 (C)). The orange-red color derivative with the maximum absorption at 510 nm was obtained.

To obtain high yield of derivatization, the effect of the concentrations of DMA, $NaNO_2$, HCl and NaOH on the derivatization of CBR (3.0 mg L^{-1}) was investigated. The studied concentrations of DMA, $NaNO_2$ and HCl were 0.2-1.0 mmol L^{-1}, 0.1-1.0 mmol L^{-1} and 2.0-10.0 mmol L^{-1}, respectively. While concentration of NaOH was studied according to pHs ranged from 2.0 to 14.0. The results are shown in Figure 4.2. The optimum concentrations for the formation of diazonium ion (see Figure 4.2 (A-C)) were 0.6 mmol L^{-1}, 0.2 mmol L^{-1} and 2.0 mmol L^{-1} for DMA, $NaNO_2$ and HCl, respectively. The optimum concentration of NaOH for hydrolysis of CBR was 10.0 mmol L^{-1}, which corresponds to pH higher than 12.0 (see Fig 4.2 (D)).

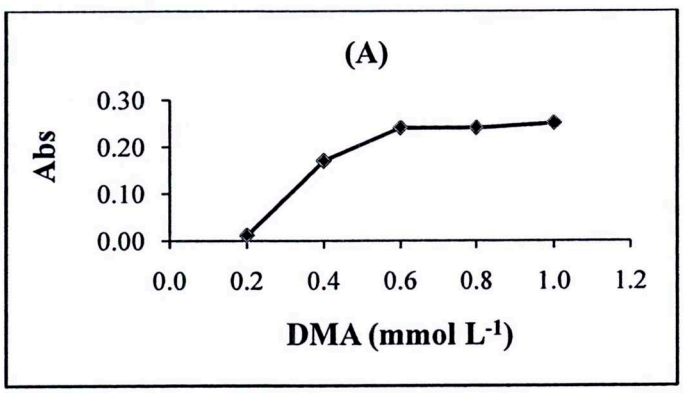


Figure 4.2 Effect of the concentrations of (A) DMA, (B) $NaNO_2$, (C) HCl and (D) NaOH on the absorbance of CBR derivative

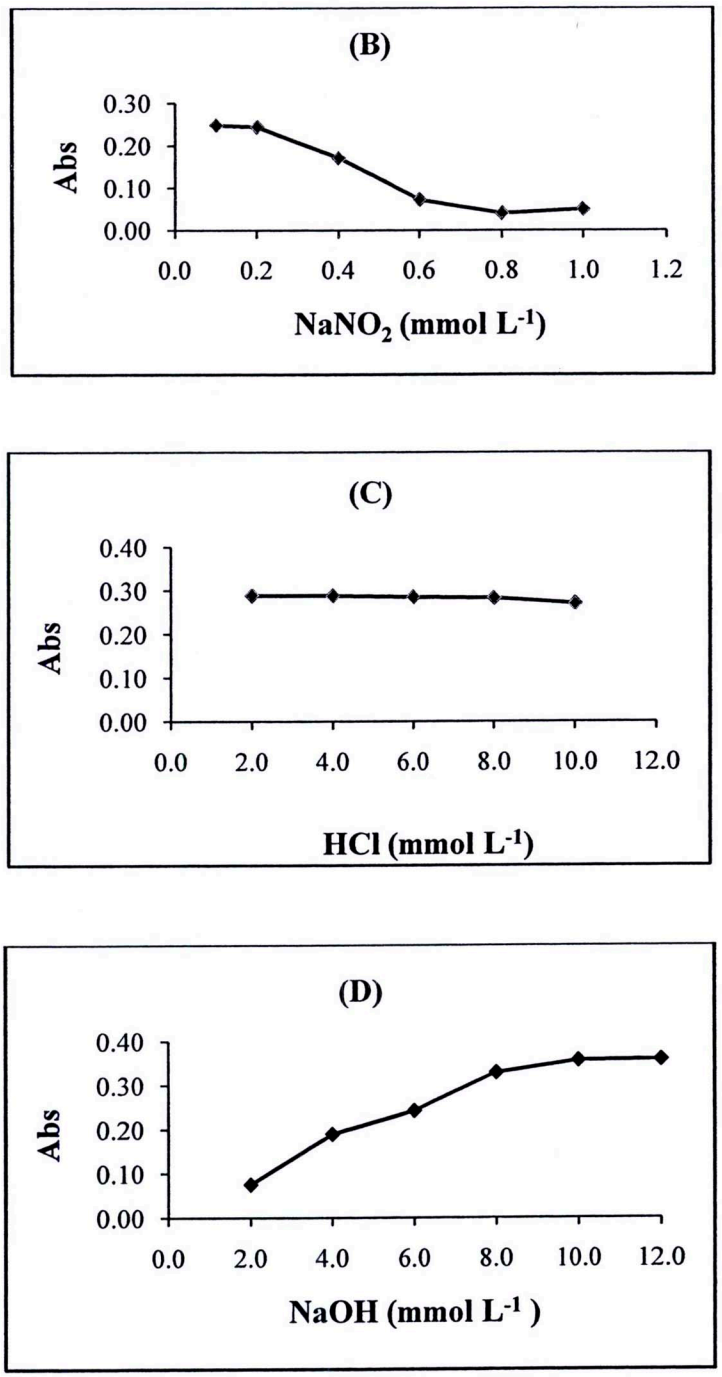


Figure 4.2 Effect of the concentrations of (A) DMA, (B) $NaNO_2$, (C) HCl and (D) NaOH on the absorbance of CBR derivative (Cont.)

The stability of the derivative was also studied by leaving the derivative to stand for over 2 hours and measured the absorbance at 510 nm every 20 min. The results are shown in Figure 4.3, indicated that the derivative was stable at least 2 hours.

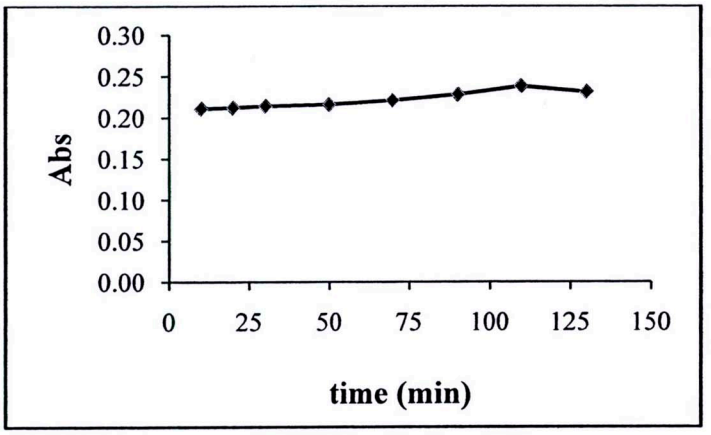


Figure 4.3 Stability of CBR derivative

The optimum condition for diazotization of CBR are summarized in Table 4.1.

Table 4.1 Optimum condition for the derivatization of CBR using DMA

Reagent/parameter	Concentration (mmol L^{-1})
2,4-dimethoxyaniline (DMA)	0.6
Sodium nitrite ($NaNO_2$)	0.2
Hydrochloric acid (HCl)	2.0
Sodium hydroxide (NaOH)	10.0

In addition, under the optimum condition only CBR gave colored derivative with high molar absorptivity ($\varepsilon = 1.71 \times 10^4$ L mol^{-1} cm^{-1}), while the other carbamates could not form derivative with DMA. Thus, it can be concluded that the spectrometric method using DMA as the derivatizing agent is selective for CBR.

4.1.2 Derivatization of CBR and CBF using AP

The derivatization of CBR and CBF using AP is based on the reaction of phenolate products, obtained from alkaline hydrolysis of CBR and CBF, with AP in the solution containing oxidizing agent. Oxidizing agent is required in this reaction to stabilizing the colored products (Venkateswarlu et al., 1995). In this study, potassium ferricyanide was used as the oxidizing agent. The derivatization reaction of CBF is shown in Figure 4.4 (Chen et al., 2009). The derivatization was carried out as follow: CBF (3.0 mg L^{-1}) was hydrolyzed with NaOH and stand for 10 min to complete hydrolysis, the pH of the solution is adjusted for stabilizing of the reaction by adding the borate buffer. AP was then added and followed by potassium ferricyanide, the solution was mixed well and adjusted the final volume to 10.00 mL with water. The absorbance was measured at 530 nm after 10 min.

The derivatization of CBR was carried out as the procedure described for CBF but the absorbance was measured at 510 nm.

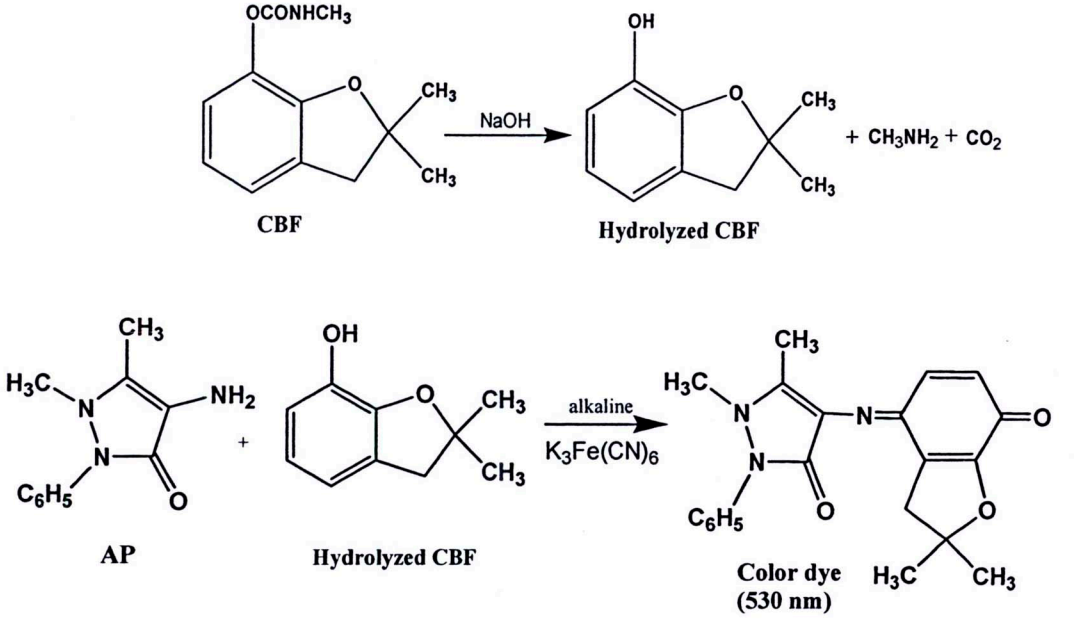


Figure 4.4 Derivatization reaction of CBF using AP as the derivatizing agent

In order to obtain the highest sensitivity, the effect of concentrations of AP, borate buffer, $K_3Fe(CN)_6$, and NaOH was investigated. The results are summarized below.

4.1.2.1 Effect of derivatizing agent concentration

The concentration of derivatizing agent (AP) was studied in the range 1.0-7.0 mmol L^{-1}. The other parameters including borate buffer, $K_3Fe(CN)_6$ and NaOH were 5.0 mmol L^{-1} borate buffer, 12.0 mmol L^{-1} $K_3Fe(CN)_6$ and 0.02 mol L^{-1} NaOH, while concentrations of CBR and CBF were 3.0 mg L^{-1}. The concentrations were calculated for a total volume of 10.00 mL. The effect of AP concentration on the absorbance of color dye is shown in Figure 4.5. For the determination of CBF, the absorbance increased with the increasing concentration of AP upto 5.0 mmol L^{-1} and afterwards, the absorbances were constant. While the determination of CBR, the absorbance increased until the concentration of AP was 4.0 mmol L^{-1}. To ensure the complete reaction, 5.0 mmol L^{-1} of AP was selected for further studies.

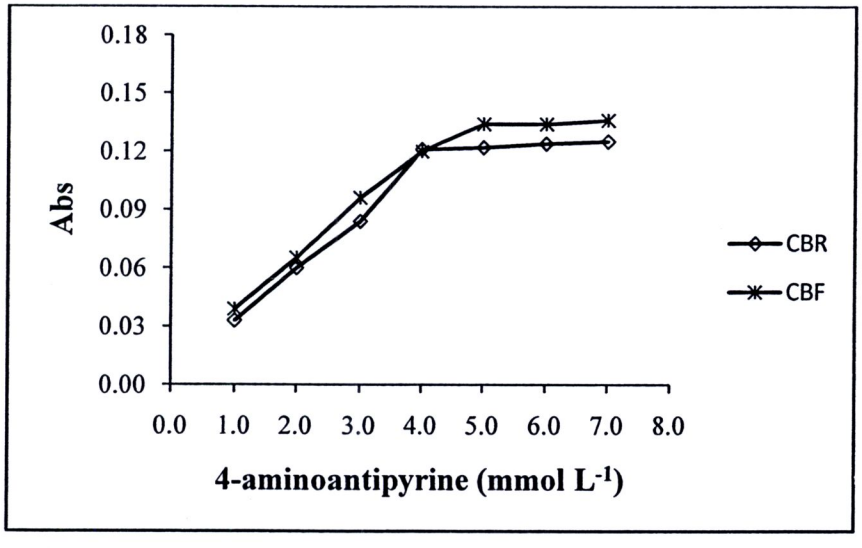


Figure 4.5 Effect of AP concentration on the absorbance of CBR and CBF derivatives

4.1.2.2 Effect of oxidizing agent

Potassium ferricyanide was chosen as oxidizing agent and its concentration was studied in the range 2.4-16.8 mmol L^{-1}. The results are shown in Figure 4.6.

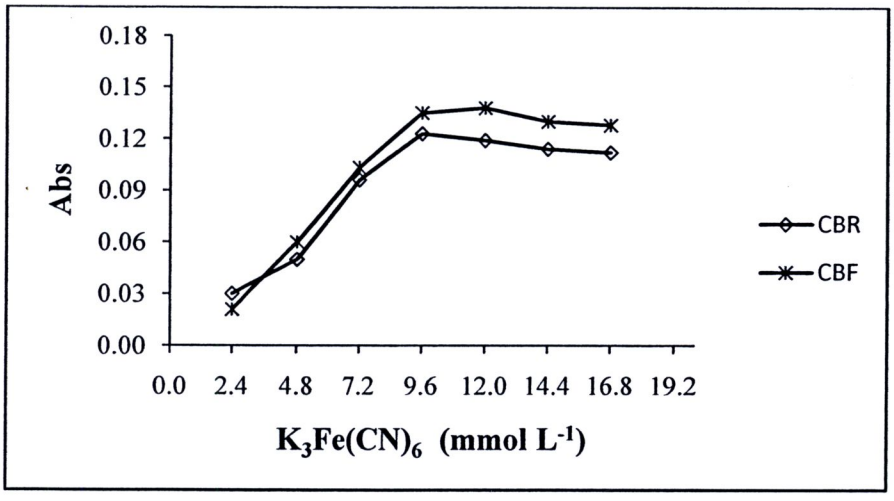


Figure 4.6 Effect of potassium ferricyanide concentration on the absorbance of CBR and CBF derivatives

The results (Figure 4.6) showed that in case of CBR, absorbance sharply increased with the increasing of potassium ferricyanide concentration and the maximum absorbance was obtained at 9.6 mmol L^{-1}. Afterward, the absorbances were slightly decreased. For CBF, the absorbance increased with the increasing of $K_3Fe(CN)_6$ concentration up to 12.0 mmol L^{-1}. 9.6 mmol L^{-1} $K_3Fe(CN)_6$ was chosen for further studies.

4.1.2.3 Effect of pH

The studied pH was 2.0-14.0, pH of the solution is related to concentration of NaOH that used in the hydrolysis step. Figure 4.7 shows the effect of pH on the absorbance of CBR and CBF derivatives.

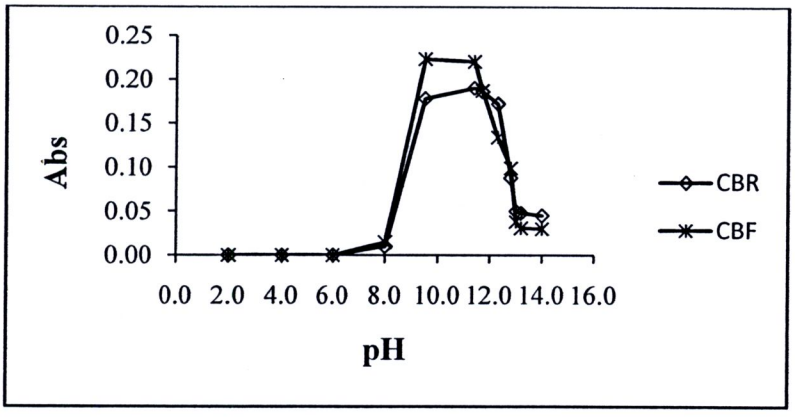


Figure 4.7 Effect of pH on absorbances of CBR and CBF derivatives

The results showed that no reaction occurred at pH 2-6, indicating from zero absorbance in acidic medium, i.e. CBR and CBF could not hydrolyze. When pH was higher than 8.0, the absorbance sharply increased and the maximum absorbance was obtained at pH 9.0. However, pH higher than 12.0, the absorbance was suddenly decreased. Thus, buffer solution is needed to control pH.

4.1.2.4 Effect of buffer concentration

According to effect of pH (4.1.2.3), pH 9.5 was chosen at the optimum pH. Borate buffer pH 9.5 was used and its concentration was studied as the results shown in Figure 4.8.

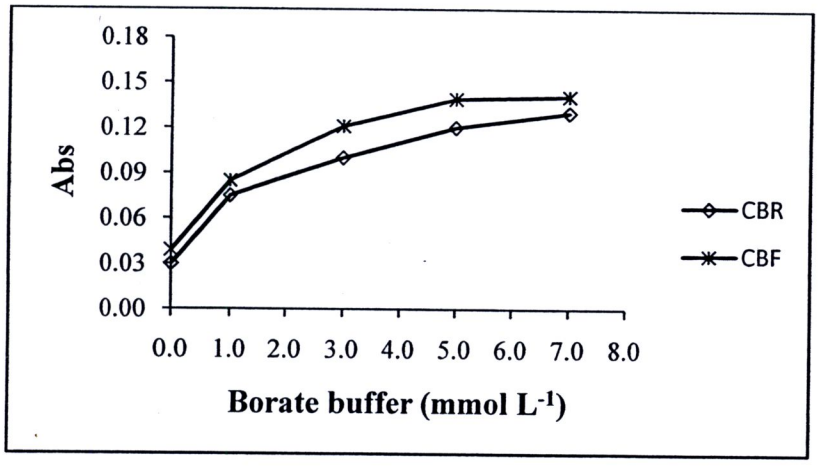


Figure 4.8 Effect of concentration of borate buffer on the absorbances of CBR and CBF derivatives

The results showed that absorbance increased with the increasing of borate buffer concentration. However, at 7.0 mmol L^{-1} borate buffer, the solution became cloudy and when buffer concentration higher than 7.0 mmol L^{-1}, precipitation was observed. Thus, 5.0 mmol L^{-1} borate buffer was used for further studies.

4.1.2.5 Stability of the derivatives

The stability of the derivative was also studied by leaving the derivative to stand for over 2 hours and measured the absorbance every 20 min at 510 nm for CBR derivative and 530 nm for CBF derivative. The results are shown in Figure 4.9, indicating that the derivatives were stable at least 2 hours.

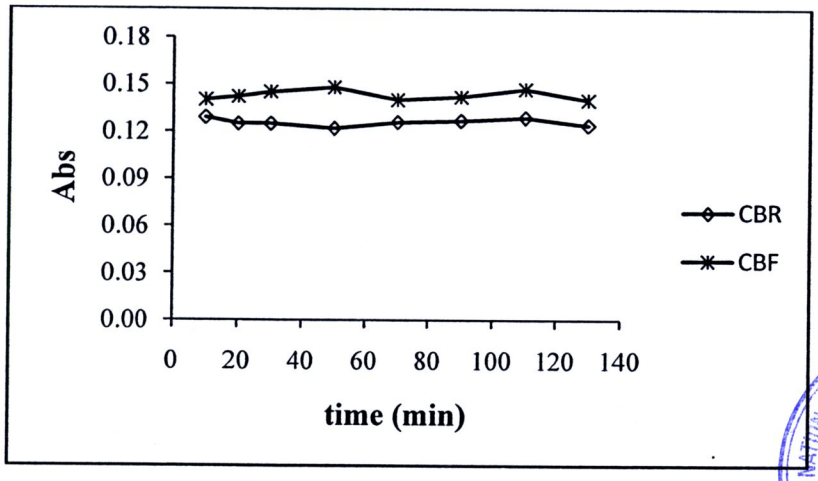


Figure 4.9 Stability of CBR and CBF derivatives of AP

The optimum conditions for derivatization of CBR and CBF are summarized in Table 4.2.

Table 4.2 Optimum condition for the derivatizations of CBR and CBF using AP

Reagent/paremeter	Concentration (mmol L^{-1})
4-aminoantipyrine (AP)	5.0
Potassium ferricyanide ($K_3Fe(CN)_6$)	9.6
pH	9.0-12.0
Borate buffer solution (pH= 9.5)	5.0

In addition, molar absorptivities of CBR and CBF derivatives with AP were 8.75×10^3 L mol^{-1} cm^{-1} and 1.38×10^4 L mol^{-1} cm^{-1}, respectively.

4.2 Sample preparation

4.2.1 Extraction by QuEChERS

QuEChER method was used for extraction of CBR and CBF in vegetable samples. The advantages of QuEChERS are extraction of analytes, removal of exceed water in sample and chlorophyll can be achieved in single step (Lee at al., 2008). The extraction efficiency is expressed as recovery. Cucumber and yard long bean were chosen as the representative samples for light color and dark color samples, respectively. The parameters affect the extraction efficiency of cucumber and yard long bean were investigated. The results are presented in the following topics (4.2.1.1 to 4.2.1.3).

4.2.1.1 Type of organic solvent

The solvents used were 1.0% acetic acid and 99.0% organic solvent. Two organic solvents including methanol and acetonitrile were studied. The volume of organic solvents was varied in the range 10.00-40.00 mL.

Figure 4.10 shows the effect of organic solvent on the recovery of CBR and CBF in spiked samples (cucumber and Yard long bean), which two derivatizing agents namely AP and DMA were used. The solution containing 1.0% acetic acid in 99.0% acetonitrile was selected for extraction of CBR and CBF because it gave higher recovery than 1.0% acetic acid in 99.0% methanol for all cases.

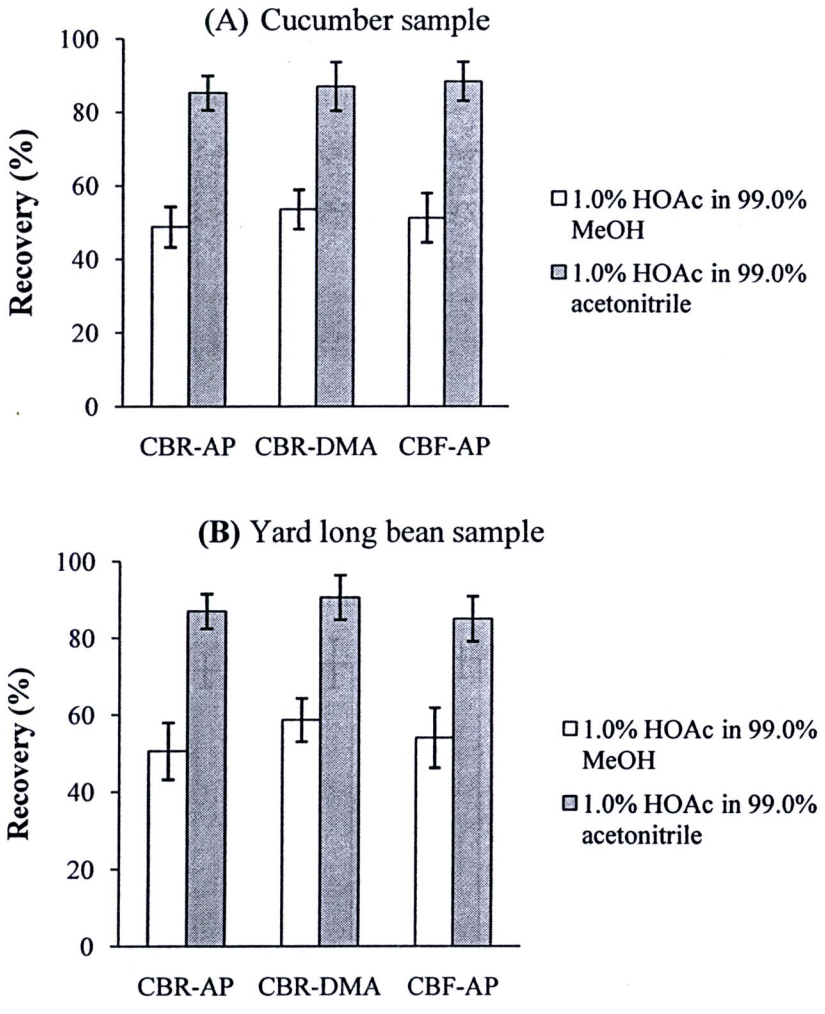


Figure 4.10 Effect of extraction solvent on the recoveries of CBR and CBF in cucumber and yard long bean. Extraction conditions: 15 g sample, 20 mL solvent, 5.0 g $MgSO_4$ and Na_2SO_4, 1.0 g CH_3COONa, 0.1 g Carbograph and 0.5 g PSA; *(n=3)*

4.2.1.2 Amount of chemicals for removing exceed water

Various chemicals were investigated for the removal of exceed water including Na_2SO_4, $MgSO_4$ and CH_3COONa, which the studied amounts were in the range 5.0-15.0 g, 1.0-10.0 g and 0.5-5.0 g, respectively.

For QuEChERS method, the exceed water needs to remove in order to ensure that the analyte disperses only in organic phase. The amounts of chemicals used for removing exceed water of the studied samples were different,

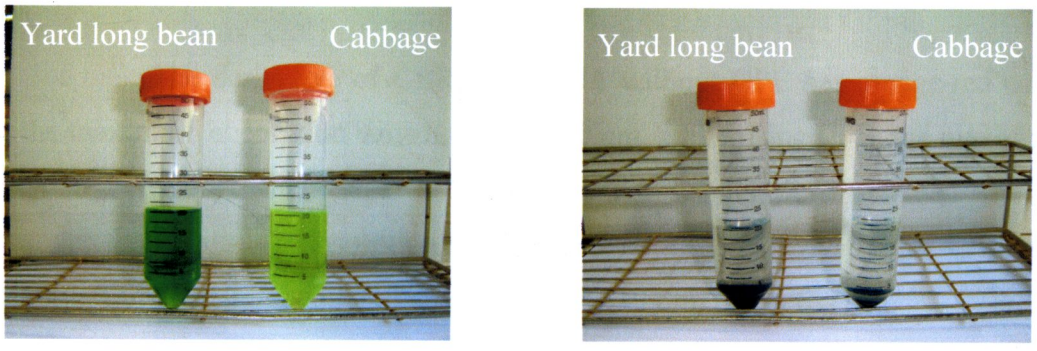
which depended on the moisture of samples. The sample with higher water content such as cucumber needed higher amounts of the chemical (Na_2SO_4). The suitable contents are summarized in Table 4.3.

Table 4.3 The optimum amount of chemicals used for removing of exceed water

Chemical	**Amount (g)**				
	Cucumber	Cabbage	Yard long bean	Kale	Mustard
Na_2SO_4	15.00	10.00	10.00	7.00	7.00
$MgSO_4$	4.00	4.00	4.00	4.00	4.00
CH_3COONa	1.00	1.00	1.00	1.00	1.00

4.2.1.3 Study of chemicals for removing chlorophyll and other compounds

Chlorophyll is a green pigment that composite in numerous vegetables. It can interfere the measurement of absorbance if it remains in sample. Carbograph and PSA have been reported to remove chlorophyll (Lee et al., 2008). Carbograph is non-porous graphitized carbon black (GCB), while PSA is a weak anion exchanger that has two amine groups. PSA was used to eliminate fatty acids, organic acid, organic pigments and sugars. The amounts of Carbograph and PSA were studied. Figure 4.11 shows effect of Carbograph and PSA for removing of chlorophyll in yard long bean and cabbage. It is clearly seen that the color of samples cloud remove by Carbograph and PSA.



(A) (B)

Figure 4.11 Effect of Carbograph and PSA for removing of chlorophyll.
(A) before the addition of Carbograph and PSA
(B) after the addition of Carbograph and PSA

Table 4.4 summarizes the results obtained from the using of Carbograph and PSA. It was found that, the amounts used depended on the dark and light of the color. Cucumber has light color, it used less amounts of Carbograph and PSA when compared to the others.

Table 4.4 The optimum amount of Carbograph and PSA for removing of chlorophyll

Chemical	**Optimum amount (g)**				
	Cucumber	Cabbage	Yard long bean	Kale	Mustard
Carbograph	0.05	0.10	0.40	0.70	0.70
PSA	0.20	0.20	0.50	0.50	0.50

4.2.2 Preconcentration by CPE

CPE uses surfactant to extract the target analytes from a large bulk aqueous solution. The analytes is extracted into a less volume of surfactant-rich phase (SRP) that resulting a higher preconcentration. The parameters influence CPE include concentration of surfactants (TX-114), types and concentration of salts, equilibration time, equilibration temperature and centrifugation time were investigated. The target analytes (CBR and CBF) in aqueous extract obtained from QuEChERS were studied.

The efficiency of CPE is evaluated using the optimum conditions (sections 4.1.1-4.1.2).

The derivative of CBR with DMA without CPE has orange-red color with the maximum absorption wavelength at 510 nm, while using CPE, the maximum absorption wavelength shifted to 505 nm. Similar results were obtained for AP derivative, the maximum absorption wavelengths of the AP derivative of CBR and CBF shifted from 510 nm and 530 nm to 480 and 510 nm, respectively.

4.2.2.1 Effect of concentration of TX-114

The effect of concentration of TX-114 on the extraction of CBR and CBF was studied because the preconcentration factor depends on the concentration of surfactant (Zhou et al., 2008). The effect of concentration of TX-114 was studied in the range 0.5 to 2.5 % w/v, while the other conditions were as follow: 1% w/v NaCl, room temperature equilibration for 10 min, followed by centrifugation for 20 min. As can be seen in Figure 4.12, the absorbance of both CBR and CBF increased with the increasing of TX-114 concentration and showed the highest absorbance at 1.5% w/v TX-114. Beyond this point, the absorbance decreased. Therefore, 1.5% w/v TX-114 was selected for further studies.

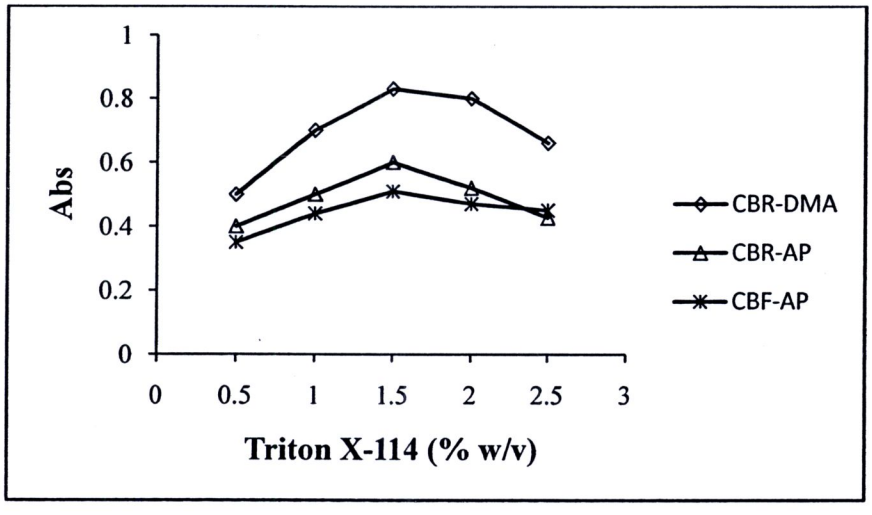


Figure 4.12 Effect of concentration of TX-114 (%w/v) on the absorbance of the standard CBR and CBF (3.0 mg L^{-1}) derivatives

4.2.2.2 Effect of type and concentration of salt

The addition of salts has been reported to facilitate the separation of two phases in CPE (Zhu et al., 2008). Different types of salts including NaCl, Na_2CO_3 and Na_2SO_4 were investigated in an equal mol (1.7 mmol). After centrifugation step, in the cases of Na_2CO_3 and Na_2SO_4, the SRP was suspended in the AQP. It was difficult to withdraw the AQP form SRP. While NaCl, the SRP was clearly separated from AQP. Thus, NaCl was selected and studied at concentration ranged from 0.2 to 5.0% w/v. Considering Figure 4.13, effect of NaCl concentration; it is clearly observed that increasing of NaCl concentrations resulted in the increasing of absorbance. The highest absorbance was obtained when the NaCl concentration was higher than 1.0% w/v for CBR-DMA and CBR-AP, while CBF-AP, the absorbance increased slightly as NaCl concentration was up to 1.5% w/v. After these points, the absorbance was remain constant. However, when the NaCl concentration was higher than 3% w/v, the SRP moved from the bottom of the solution to the middle zone. In this case, it was difficult to separate the SRP from bulk aqueous phase, resulted in poor accuracy and reproducibility. This observation was also reported (Zhou et al., 2008). To obtain high extraction efficiency, 2.0% w/v NaCl was selected for further studies.

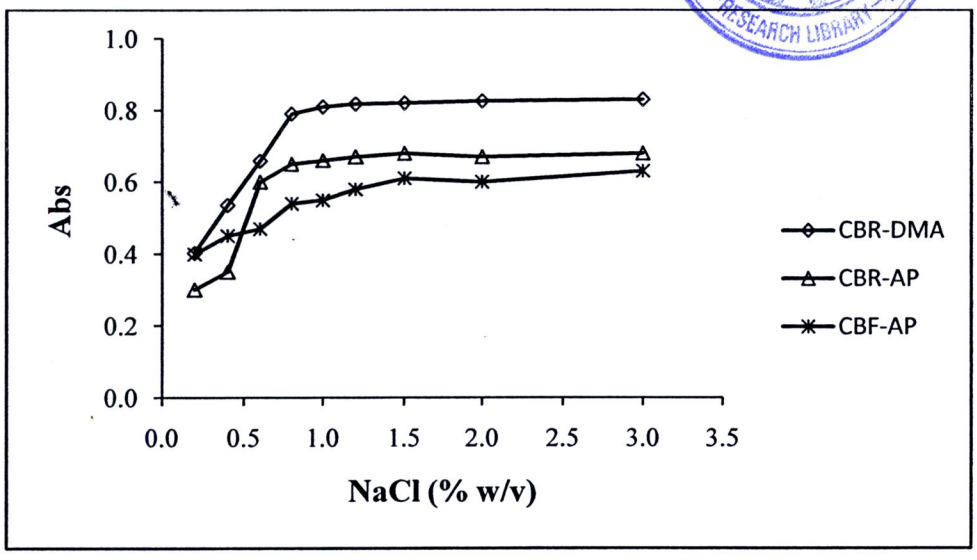


Figure 4.13 Effect of NaCl concentration for CPE.
Condition: 1.5 % w/v TX-114, equilibrated at 25 °C and centrifuged at 3,500 rpm for 10 min

4.2.2.3 Effect of equilibration temperature and equilibration time

Although CPE using non-ionic TX-114 surfactant can be performed at around 25 °C because its cloud-point temperature is 23 °C, (Carabias-Martinez et al., 2000), theoretically, the optimal equilibration temperature of the CPE occurs when the equilibration temperature is 15-20 °C higher than the cloud-point temperature of the surfactant (Liu et al., 2007). In order to achieve highest extraction efficiency, the effect of temperature in the range 25-55 °C was investigated. The results are shown in Figure 4.14, the absorbance increased slightly as the temperature was increased up to 55°C. Therefore, temperature at 55 °C was selected as equilibration temperature for further CPE experiments.

The influence of the equilibration time for the CPE was also investigated in the range 5-30 min. The absorbance increased sharply and was highest at 20 min (Figure 4.15). Afterwards, the absorbance remained constant, so 20 min was chosen to ensure the complete extraction.

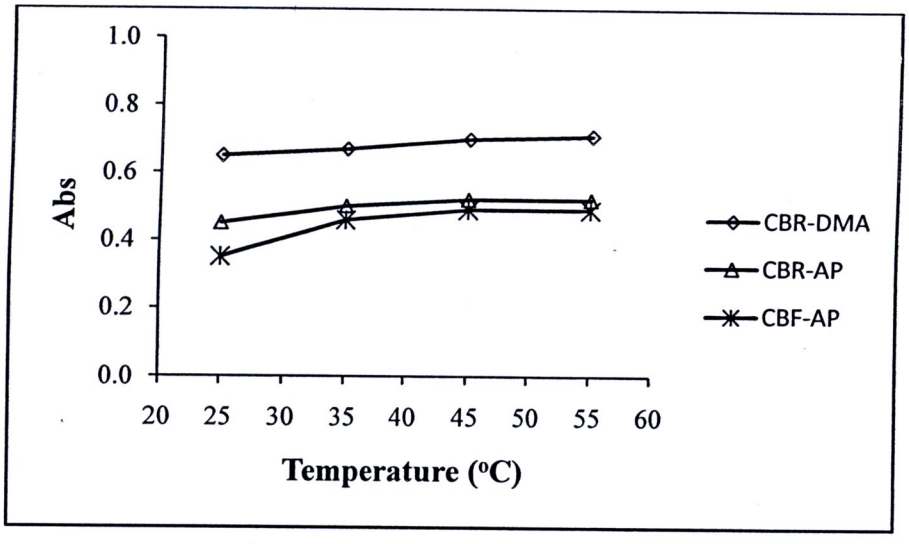


Figure 4.14 Effect of equilibration temperature.
CPE condition: 1.5% w/v TX-114, 2.0% w/v NaCl and the other parameters are described in Figure 4.13

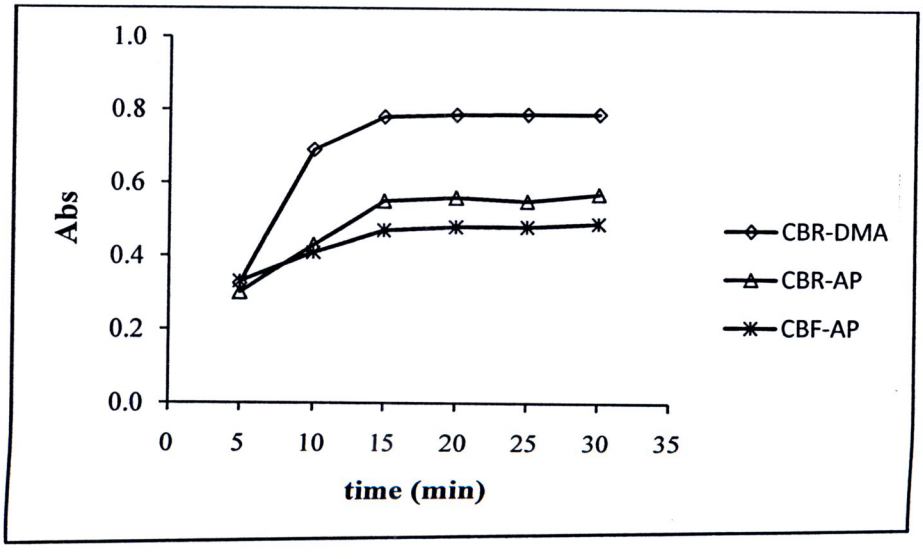


Figure 4.15 Effect of equilibration time.
CPE condition are described in Figure 4.14

4.2.2.4 Effect of centrifugation time

The effect of centrifugation time on the absorbance was also studied in the range of 5-30 min at 3,500 rpm. The results (Figure 4.16) show that the

centrifugation time had insignificantly influence on the extraction. However, to ensure the complete separation of two phases, centrifugation for 10 min was used throughout the experiments.

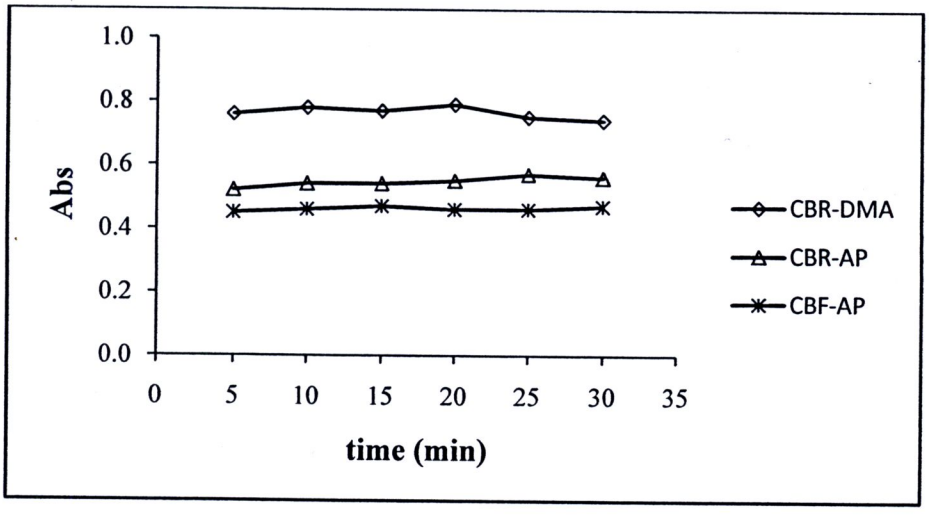


Figure 4.16 Effect of centrifugation time.
CPE conditions: 1.5% w/v TX-114, 2.0 % w/v NaCl and equilibrated at 20 min

In conclusion, the optimum condition for CBE of CBR and CBF are summarized in Table 4.5.

Table 4.5 Optimum condition for CPE of CBR and CBF derivatives

Reagent/parameter	Optimum value
Triton X-114	1.5% w/v
Type and concentration of salts	2.0% w/v NaCl
Equilibration temperature	55 °C
Equilibration time	20 min
Centrifugation time	10 min

4.3 Simultaneous determination of CBR and CBF

Since CBR and CBF can derivatize with AP, under the same condition CBR and CBF give different colors. The derivative of CBR has orange-red color with the maximum absorption wavelength at 480 nm, while CBF-derivative has orange color with the maximum absorption wavelength at 510 nm. Therefore, it is interesting to investigate the application of spectrophotometry for simultaneous determination of CBR and CBF. There are a number of publications on simultaneous analysis using spectrometry (Benamor et al., 2008 ; Elham et al., 2002), however most of them deal with metal ions. Various methods have been used for simultaneous analysis by spectrometry including partial least squares method (Khalaf et al., 1996), derivative spectrophotometry using a zero-crossing technique (Benamor et al., 2008 ; Elham et al., 2002) and zero-order spectrophotometry using simultaneous equations technique (Willard et al., 1974). In this study, simultaneous equations and zero-crossing techniques were explored.

4.3.1 Simultaneous equations technique

Figure 4.17 (A) and (B) shows the typical spectra of standard CBR and CBF derivatives and spiked cucumber sample (3.0 mg L^{-1}), respectively.

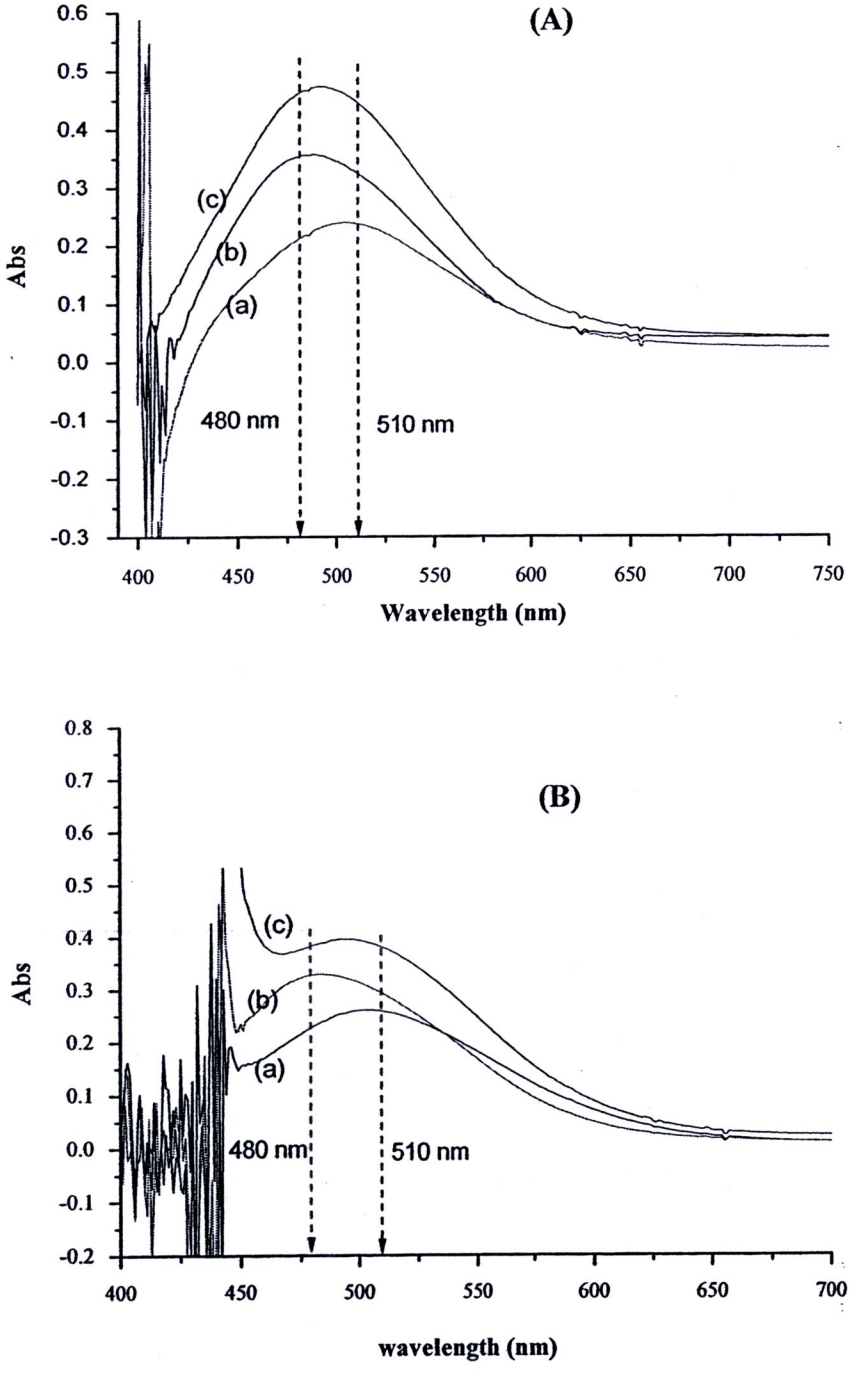


Figure 4.17 Absorption spectra of CBR and CBF derivatives: (A) standard solution and (B) spiked cucumber sample.

Assignment: (a) CBF derivative, (b) CBR derivative and (c) mixture of CBR and CBF derivative

The concentration of CBR and CBF can be determined using the simultaneous equations (Willard et al., 1974) as follow:

$$A_{\lambda 1} = C_1(\varepsilon_1)_{\lambda 1} + C_2(\varepsilon_2)_{\lambda 1} \quad (4.1)$$

$$A_{\lambda 2} = C_1(\varepsilon_1)_{\lambda 2} + C_2(\varepsilon_2)_{\lambda 2} \quad (4.2)$$

From eqns. 4.1 and 4.2, the concentration of each analyte can be calculated as follow:

$$C_1 = \frac{(\varepsilon_2)_{\lambda 2} A_{\lambda 1} - (\varepsilon_2)_{\lambda 1} A_{\lambda 2}}{(\varepsilon_1)_{\lambda 1} (\varepsilon_2)_{\lambda 2} - (\varepsilon_2)_{\lambda 1} (\varepsilon_1)_{\lambda 2}} \quad (4.3)$$

$$C_2 = \frac{(\varepsilon_1)_{\lambda 1} A_{\lambda 2} - (\varepsilon_1)_{\lambda 2} A_{\lambda 1}}{(\varepsilon_1)_{\lambda 1} (\varepsilon_2)_{\lambda 2} - (\varepsilon_2)_{\lambda 1} (\varepsilon_1)_{\lambda 2}} \quad (4.4)$$

Where, $A_{\lambda 1}$ and $A_{\lambda 2}$ are the absorbances of CBR or CBF derivative at wavelengths 480 nm and 510 nm, C_1 and C_2 are the concentrations of CBR and CBF derivative, ε_1 and ε_2 are molar absorptivities of CBR and CBF derivatives, respectively.

After sample or spiked sample solutions derivatized, the absorbances were measured at 480 nm (the maximum absorption wavelength of CBR derivative) and 510 nm (the maximum absorption wavelength of CBF derivative), thus terms $A_{\lambda 1}$ and $A_{\lambda 2}$ in eqns. 4.1 and 4.2 were obtained. Terms $(\varepsilon_1)_{\lambda 1}$ and $(\varepsilon_1)_{\lambda 2}$ were calculated from the absorbance of known concentration of CBR derivative at 480 nm and 510 nm, respectively. While $(\varepsilon_2)_{\lambda 1}$ and $(\varepsilon_2)_{\lambda 2}$ were obtained from the calculation of absorbance of known concentration of CBF derivative at 480 nm and 510 nm, respectively. Table 4.6 lists the simultaneous equations for the determination of CBR and CBF in spiked vegetable samples (0.5 mg kg^{-1}).

Table 4.6 Simultaneous equations for the study of recovery of CBR and CBF (at 0.5 mg kg^{-1})

Sample	Simultaneous equation
Cucumber	$0.054 = 1.24\times10^4\ C_{CBR} + 0.90\times10^4\ C_{CBF}$ $0.055 = 1.11\times10^4\ C_{CBR} + 1.07\times10^4\ C_{CBF}$
Cabbage	$0.064 = 1.49\times10^4\ C_{CBR} + 0.99\times10^4\ C_{CBF}$ $0.064 = 1.37\times10^4\ C_{CBR} + 1.13\times10^4\ C_{CBF}$
Kale	$0.050 = 1.21\times10^4\ C_{CBR} + 0.92\times10^4\ C_{CBF}$ $0.051 = 1.09\times10^4\ C_{CBR} + 1.07\times10^4\ C_{CBF}$
Yard long bean	$0.065 = 1.23\times10^4\ C_{CBR} + 0.89\times10^4\ C_{CBF}$ $0.064 = 1.09\times10^4\ C_{CBR} + 1.04\times10^4\ C_{CBF}$
Mustard	$0.053 = 1.22\times10^4\ C_{CBR} + 0.90\times10^4\ C_{CBF}$ $0.054 = 1.11\times10^4\ C_{CBR} + 1.08\times10^4\ C_{CBF}$

Where, C_{CBR} and C_{CBF} are concentrations (mol L^{-1}) of CBR and CBF, respectively.

The obtained recoveries of CBR and CBF using AP as the derivatizing agent are summarized in Topic 4.4.3 (Table 4.10).

4.3.2 Zero-crossing technique

The first-derivative spectrophotometry using a zero-crossing technique was also applied for the simultaneous analysis of CBR and CBF. In this study, AP was used as the derivatizing agent. First-derivative spectrophotometry can enhance the detection selectivity by recording derivatives spectra which closely overlapped absorption peaks can be resolved. Zero-crossing technique was used to select suitable wavelengths for obtaining linear calibration graphs. The method is based on the measurements of the absolute value of the derivative spectrum of the mixture at an abscissa value (wavelength) where the sensitivity of one component of the mixture goes to zero (zero-crossing point). At this wavelength, the intensity is directly proportional to the other component (Benamor et al., 2008).

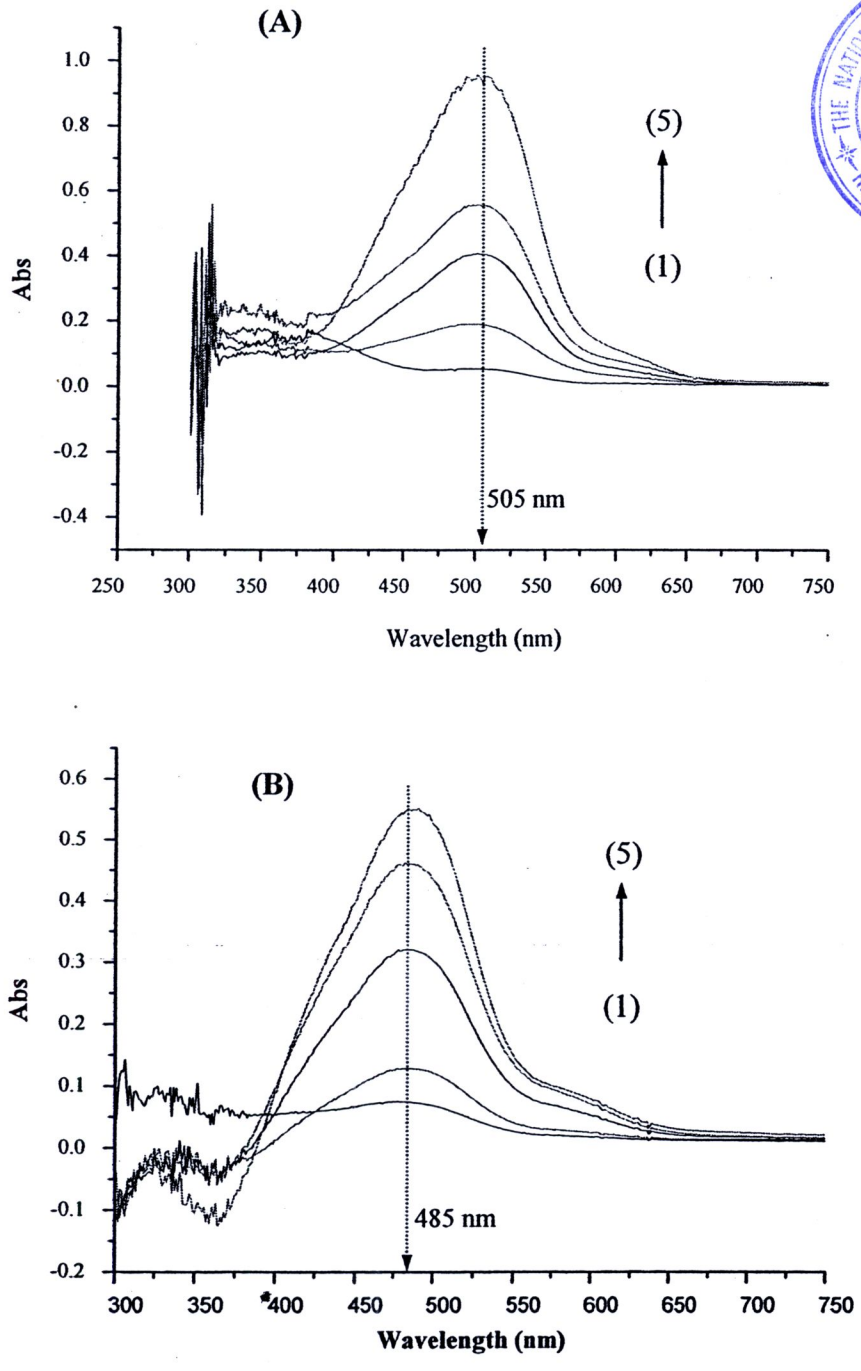


Figure 4.18 Absorption spectra of AP derivatives of (A) CBF and (B) CBR. Assigment: (1), (2), (3), (4) and (5) are the concentrations of CBF or CBR at 0.5, 1.0, 3.0, 5.0 and 7.0 mg L^{-1}, respectively

Figure 4.18 shows the absorption spectra (zero-order mode) of the derivatives, the maximum absorption wavelength of CBF derivative and CBR derivative are 505 nm and 485 nm, respectively. The first-derivative spectra of CBF and CBR derivatives are shown in Figure 4.19.

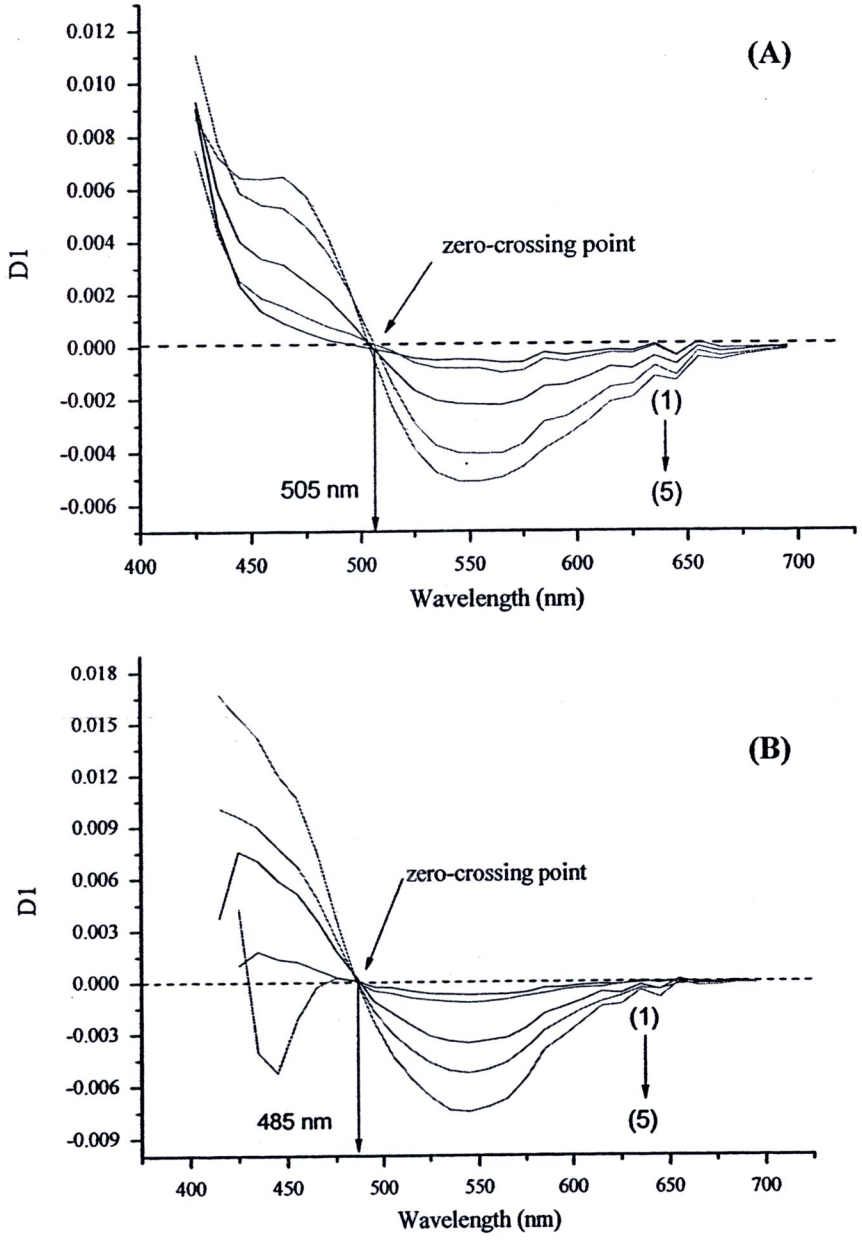


Figure 4.19 First-derivative spectra of (A) CBF and (B) CBR.
Assigment: (1), (2), (3), (4) and (5) are concentrations of CBR or CBF at 0.5, 1.0, 3.0, 5.0 and 7.0 mg L^{-1}, respectively

From Figure 4.19, zero-crossing wavelengths of CBF and CBR derivatives are 505 nm and 485 nm, respectively. The first-derivative spectra of mixtures containing increasing amounts of CBF or CBR at the fixed concentration of CBR or CBF are depicted in Figure 4.20 (A) and (B), respectively.

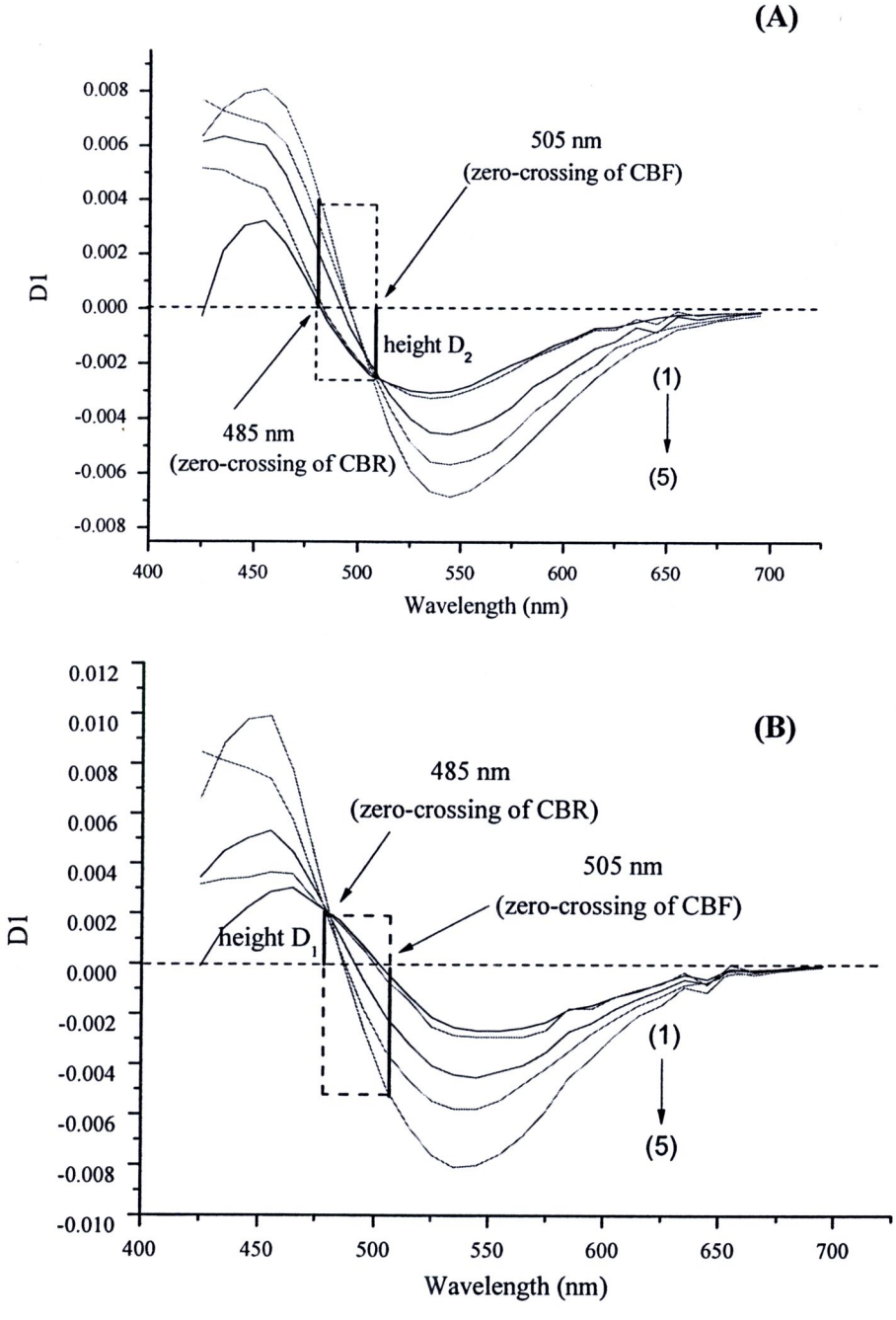


Figure 4.20 First-derivative spectra of CBF and CBR mixture.

(A): a mixture of CBR and an increasing concentrations of CBF, CBR= 3.0 mg L^{-1}; CBF = (1) 0.5, (2) 1.0, (3) 3.0, (4) 5.0, (5) 7.0 mg L^{-1}.

(B): a mixture of CBF and an increasing concentrations of CBR, CBF= 3.0 mg L^{-1}; CBR = (1) 0.5, (2) 1.0, (3) 3.0, (4) 5.0, (5) 7.0 mg L^{-1}

The first-derivative value (1^{st}-value) is $\Delta Abs/\Delta\lambda$ can be deduced from the first-derivative spectra using zero-crossing technique. It is the vertical distance from peak to through. The calibration graphs were prepared by plotting the first-derivative value versus concentrations of CBR and CBF derivatives. The heights D_1, D_2 in the first-derivative spectra of mixture (see Figure 4.20) are the values taken at wavelengths 485 nm (zero-crossing wavelength of CBR derivative) and 505 nm (zero-crossing wavelength of CBF derivative), respectively. D_1 and D_2 are proportional to the concentrations of CBF and CBR, respectively. Taking Figure 4.20 as an example, D_1, D_2 values and linear equations for CBR and CBF are summarized in Table 4.7.

Table 4.7 First-derivative values and linear equations for the determination of CBR and CBF (spiked 3.0 mg L^{-1}); n = 3

Determination	485 nm		505 nm		Linear equation
	Concentration (mg L^{-1})	1^{st}-value	Concentration (mg L^{-1})	1^{st}-value	
CBR	0.5 1.0 3.0 5.0 7.0	0.00039 0.00071 0.00225 0.00343 0.00463	0.5 1.0 3.0 5.0 7.0	0.00210 (D_2)	$D_2=6.56\times10^{-4}X+1.16\times10^{-4}$ $R^2 = 0.997$
CBF	0.5 1.0 3.0 5.0 7.0	0.00260 (D_1)	0.5 1.0 3.0 5.0 7.0	0.00082 0.00108 0.00270 0.00399 0.00576	$D_1=7.55\times10^{-4}X + 3.78\times10^{-4}$ $R^2 = 0.997$
Concentration of CBR = 3.02 ± 0.06 mg L^{-1} Concentration of CBF = 2.95 ± 0.08 mg L^{-1} (spiked concentration of CBR and CBF = 3.0 mg L^{-1})					Error: CBR = 0.67% CBF = 1.67%
					Recovery : CBR = 101% ±2.15 *%RSD = 2.1* CBF = 98% ± 2.85 *%RSD = 2.9*

From Table 4.7, the deduced regression equations for the determination of CBR and CBF are shown in equations 4.5 and 4.6, respectively.

$$D_2 = 6.56x10^{-4} C_{CBR} + 1.16x10^{-4} \quad (4.5)$$

$$D_1 = 7.55x10^{-4} C_{CBF} + 3.78x10^{-4} \quad (4.6)$$

Where, C_{CBR} and C_{CBF} are the concentrations (mg L^{-1}) of CBR and CBF, respectively.

For the determination of CBR, the linear equation at wavelength of CBF derivative (Eqn. 4.5) and D_2 value of 0.00210 (height D_2 at 505 nm) were used. The obtained concentration of CBR in mixture was 3.02 mg L^{-1}. The concentration of CBF in the mixture of CBF and CBR can be calculated as described for CBR. Using Eqn. 4.6 ($D_1 = 7.55x10^{-4} C_{CBF} + 3.78x10^{-4}$) and D_1 (height at 485 nm) of 0.00260, the obtained concentration of CBF was 2.95 mg L^{-1}. The obtained results revealed that the zero-crossing technique could use for the determination of CBR and CBF mixture with high accuracy (98-101% recovery).

Zero-crossing technique was also investigated for the determination of trace concentration level. A mixture 1.0 mg L^{-1} of CBR and CBF was studied. The first-derivative spectra are shown in Figure 4.21 and calculated recoveries are presented in Table 4.8.

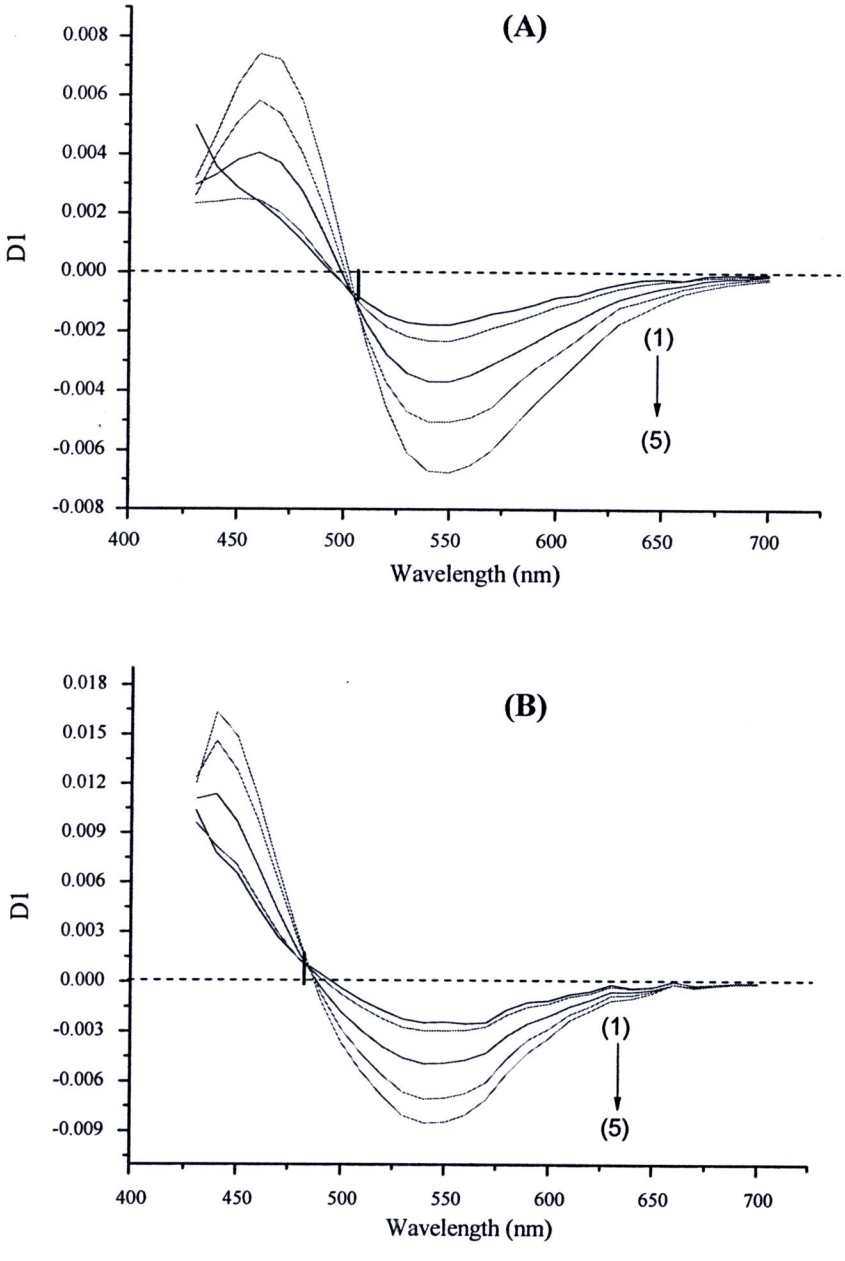


Figure 4.21 First-derivative spectra of CBR and CBF mixture.

(A): a mixture of CBR and an increasing concentrations of CBF, CBR= 1.0 mg L^{-1}; CBF = (1) 0.5, (2) 1.0, (3) 3.0, (4) 5.0, (5) 7.0 mg L^{-1}.

(B): a mixture of CBF and an increasing concentrations of CBR, CBF= 1.0 mg L^{-1}; CBR = (1) 0.5, (2) 1.0, (3) 3.0, (4) 5.0, (5) 7.0 mg L^{-1}

Table 4.8 First-derivative value and linear equations for the determination of CBR and CBF (spiked 1.0 mg L^{-1}); n = 3

Determination	485 nm		505 nm		Linear equation
	Concentration (mg L^{-1})	1^{st}-value	Concentration (mg L^{-1})	1^{st}-value	
CBR	0.5 1.0 3.0 5.0 7.0	0.00068 0.00089 0.00201 0.00315 0.00465	0.5 1.0 3.0 5.0 7.0	0.00082 (D_2)	$D_2=6.04x10^{-4}X+2.82x10^{-4}$ $R^2 = 0.995$
CBF	0.5 1.0 3.0 5.0 7.0	0.00105 (D_1)	0.5 1.0 3.0 5.0 7.0	0.00074 0.00115 0.00238 0.00359 0.00454	$D_1 =5.86x10^{-4}X + 5.45x10^{-4}$ $R^2 = 0.996$
Concentration of CBR = 0.89 ± 0.08 mg L^{-1} Concentration of CBF = 0.86 ± 0.07 mg L^{-1} (spiked concentration of CBR and CBF = 1.0 mg L^{-1})					Error: CBR = 11% CBF = 14%
					Recovery : CBR = 89% ±7.93 *%RSD = 8.9* CBF = 86 % ±7.94 *%RSD =9.2*

It is clearly seen (from Tables 4.7 and 4.8) that zero-crossing technique provided less error which apply to high concentration than the detection at trace concentration level (recovery < 90%).

4.4 Validation of CPE coupled to spectrophotometry

Using the CPE conditions (1.5% w/v TX-114, 2.0% w/v NaCl, 20 min equilibrated at 55 °C and 10 min centrifugation), the preconcentration factor obtained was about 10, calculated after the addition of methanol to SRP. The SRP (containing of analytes) after the addition of methanol was hydrolyzed and derivatized with derivatizing agent (DMA or AP) and then measured the absorbance. The validated parameters were linearity, LOD, LOQ, precision, accuracy and interference study.

4.4.1 Linearity

In order to reduce the matrix effect in samples, the matrix match calibration was applied. Under the optimum conditions for both CPE and spectrophotometry, the calibration graphs were constructed by detecting the analyte derivative at different concentrations using matrix match calibration plot. Five concentration levels of analytes ranging from 0.5 to 7.0 mg L^{-1} were prepared in the sample matrices. The typical absorption spectra of CBR derivatives and the calibration plots are shown in Figure 4.22. The linear equations for determination of CBR using DMA as the derivatizing agent are summarized in Table 4.9.

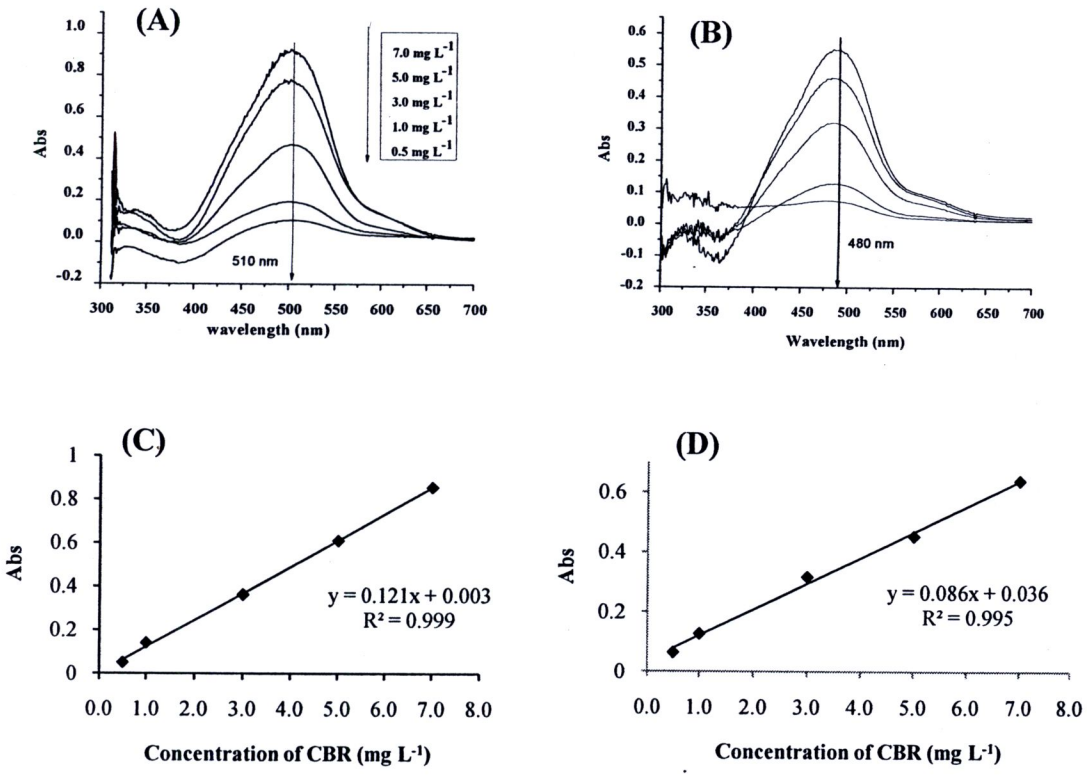


Figure 4.22 Absorption spectra and calibration plots of cucumber spiking with different concentrations of CBR.

(A) absorption spectra obtained from DMA derivative

(B) absorption spectra obtained from AP derivative

(C) calibration plot obtained from DMA derivative

(D) calibration plot obtained from AP derivative

Table 4.9 Linear equations of CBR using DMA as the derivatizing agent

Sample	Linear equation	R^2
Cucumber	Y = 0.121X + 0.003	0.999
Cabbage	Y = 0.111X + 0.011	0.998
Kale	Y = 0.096X + 0.013	0.995
Yard long bean	Y = 0.082X + 0.032	0.998
Mustard	Y = 0.096X + 0.013	0.995

This study used simultaneous equations technique for the simultaneous determination of CBR and CBF. In simultaneous equations technique, only molar absorptivity of known concentration of analyte at two wavelengths (the maximum absorption wavelength of each analyte) is required. Therefore, linear equations of CBR and CBF using AP as the derivatizing agent were not required. However, derivatization with AP gave good linearity as well as the DMA derivative (Figure 4.22 (B) and (D)), the photographs of CBR derivative with DMA and AP are shown in Figure 4.23.

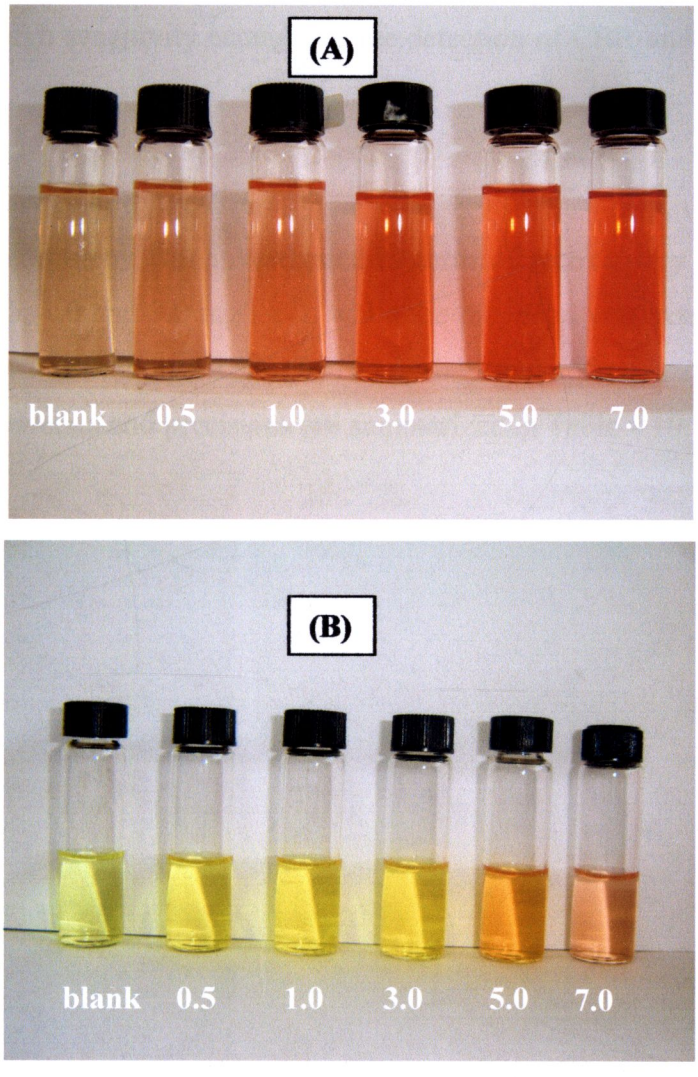


Figure 4.23 Photographs of CBR derivative with (A) DMA and (B) AP.
Assignment: blank, 0.5, 1.0, 3.0, 5.0 and 7.0 mg L^{-1} CBR and CBF, respectively

4.4.2 Limit of detections and limit of quantitations

LOD and LOQ obtained for CBR using DMA as the derivatizing agent and CPE were 0.10 mg L^{-1} and 0.5 mg L^{-1}, respectively. While, the spectrophotometric method without CPE gave the LOD and LOQ of 1.00 mg L^{-1} and 5.00 mg L^{-1}, respectively. In case of simultaneous determination of CBR and CBF using AP as the derivatizing agent, LOD and LOQ were determined by spiking standard mixture of CBR and CBF. The fortified samples were extracted by CPE, derivatized with AP and then measured the absorbance. The LOD and LOQ were 0.2 mg L^{-1} and 0.5 mg L^{-1}, respectively. It is clearly seen that CPE-spectrophotometric method provided high sensitivity enough for the detection of CBR and CBF residues at MRLs level.

4.4.3 Accuracy and precision

The accuracy was investigated in term of recovery by analyzing the spiked samples (0.5, 1.0 and 3.0 mg kg^{-1}), while the precision was examined in term of relative standard deviation (RSD) by analyzing the spiked samples in five replicates. The recoveries and precisions are summarized in Table 4.10.

Table 4.10 Recoveries and precisions for the determination of CBR and CBF in vegetable samples obtained by CPE-spectrophotometry

Sample	**Recovery (%), mean ± SD (*%RSD*)**		
Derivative of DMA	*Spike* (mg kg^{-1})		
	0.50	1.00	3.00
CBR derivative:			
Cucumber	88.00 ± 4.54 (*5.1*)	88.25 ± 6.04(*6.8*)	90.33 ± 8.16(*9.0*)
Cabbage	87.30 ± 3.03(*3.5*)	87.14 ± 8.64(*9.9*)	86.81 ± 6.35(*7.3*)
Kale	79.33 ± 4.52(*5.7*)	80.08 ±7.43(*9.3*)	82.43 ± 3.97(*4.8*)
Yard long bean	79.58 ± 8.91(*11.2*)	80.12 ± 9.67(*12.1*)	85.53 ± 7.28(*8.5*)
Mustard	78.23 ± 5.33(*6.8*)	79.55 ± 2.24(*2.8*)	80.29 ± 9.95(*12.4*)
Derivative of AP			
CBR derivative:			
Cucumber	98.06 ± 4.27(*4.4*)	86.34 ± 1.96(*2.3*)	85.83 ± 4.09(*4.8*)
Cabbage	87.49 ± 4.05(4.6)	82.26 ± 6.43(*7.8*)	83.26 ± 5.45(*6.5*)
Kale	80.56 ± 4.43(*5.5*)	83.65 ± 7.55(*9.1*)	101.39 ± 6.61(*6.5*)
Yard long bean	81.54 ± 7.43(*9.1*)	82.84 ± 4.56(*5.5*)	83.61 ± 6.27(*7.5*)
Mustard	78.56 ± 8.86(*11.3*)	80.44 ± 9.31(*11.5*)	81.25 ± 5.72(6.5)
CBF derivative:			
Cucumber	83.33 ± 3.94(*4.7*)	87.34 ± 3.06(*3.5*)	101.04 ± 5.20(*5.1*)
Cabbage	82.22 ± 4.30(*5.2*)	85.84 ± 3.35(*3.9*)	99.48 ± 6.39(*6.4*)
Kale	78.83 ± 4.05(*5.1*)	77.98 ± 8.76(*11.2*)	80.48 ± 1.44(*1.8*)
Yard long bean	72.23 ± 8.75(*12.1*)	82.34 ± 6.76(*8.2*)	75.48 ± 7.95(*10.5*)
Mustard	79.43 ± 9.97(*12.5*)	75.87 ± 6.45(*8.5*)	100.89 ± 3.03(*3.0*)

The recoveries for the detection of CBR using DMA as the derivatizing agent were 78.23-90.33%, while using AP, the recoveries were 78.56-101.39%. For the determination of CBF using AP as the derivatizing agent, the recoveries were in the range 72.23-101.04%. The results indicated that the CPE-spectrophotometric method provide acceptable recovery. In addition, the proposed method showed good precision with RSD less than 13%.

4.4.4 Interference study

The selectivity of the proposed spectrophotometric method was determined by adding different amounts of potential interfering species to sample solutions. For the determination of CBR using DMA as the derivatizing agent, the sample solution containing 1.0 mg L^{-1} CBR and then preconcentrated by CPE. The tolerance limit was taken as the concentration of the interfering species giving an error of absorbance lower than ±5% (Sadeghi et al., 2009). Selectivity for simultaneous determination of CBR and CBF was also studied in the same manner with the above mentioned criterion by using the solution containing 3.0 mg L^{-1} CBR and 1.0 mg L^{-1} CBF. The obtained results are summarized Table 4.11. As can be seen from the data, for the determination of CBR using DMA as the derivatizing agent, all of carbamate pesticides did not interfere even at high concentrations because under the optimum condition only CBR gave colored derivative with high molar absorptivity ($\varepsilon = 1.71\times10^{4}$ L mol^{-1} cm^{-1}) , while the other carbamates cloud not form derivative with DMA. Only organophosphate pesticide (MCP) could interference but the tolerance limit was high ($\geq$ 5.0 mg L^{-1}). For the simultaneous determination of CBR and CBF using AP as the derivatizing agent, the tolerance limits for foreign species including, BDC, MTM, MTC and PTO were higher than 5.0 mg L^{-1}, but for PPX, PMC and MCP were lower than 0.5 mg L^{-1}, and IPC interfered seriously ($\leq$ 0.1 mg L^{-1}).

Table 4.11 Effect of foreign pesticides on the determination of CBR and CBF

Foreign pesticide	Tolerance ($mg\ L^{-1}$)
Determination of CBR using DMA as the derivatizing agent:*	
PPX, IPC, CBF, PMC, BDC, MTM, MTC, PTO	not interfere
MCP	≥ 5.0
Simultaneous determination of CBR and CBF using AP as the derivatizing agent:**	
BDC, MTM, MTC, PTO	≥ 5.0
PPX, PMC	≤ 5.0
MCP	≤ 0.5
IPC	≤ 0.1

Experimental condition: * CBR ($1.0\ mg\ L^{-1}$)

**CBR ($3.0\ mg\ L^{-1}$) and CBF ($1.0\ mg\ L^{-1}$)

4.5 Comparison of spectrophotometry and HPLC for the determination of CBR and CBF

HPLC was used for comparison in this study. The optimum condition for the analysis of CBR and CBF by HPLC was investigated. Mobile phase composition was studied by varying methanol to water ratios ranged from 80/20 to 30/70 (v/v). Increasing of methanol resulted in the decreasing of retention time for both CBR and CBF as the results shown in Figure 4.24. The mobile phase of 50/50 v/v ($MeOH/H_2O$) was chosen, which CBF and CBR was completely separated with the resolution of 1.0 within 5 min. The obtained chromatogram is shown in Figure 4.25.

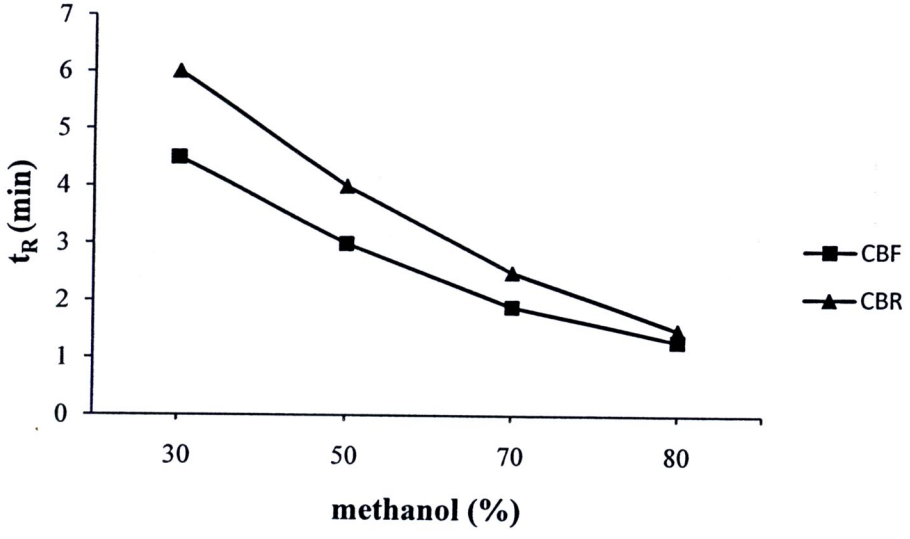


Figure 4.24 Effect of the methanol on the retention of CBF and CBR

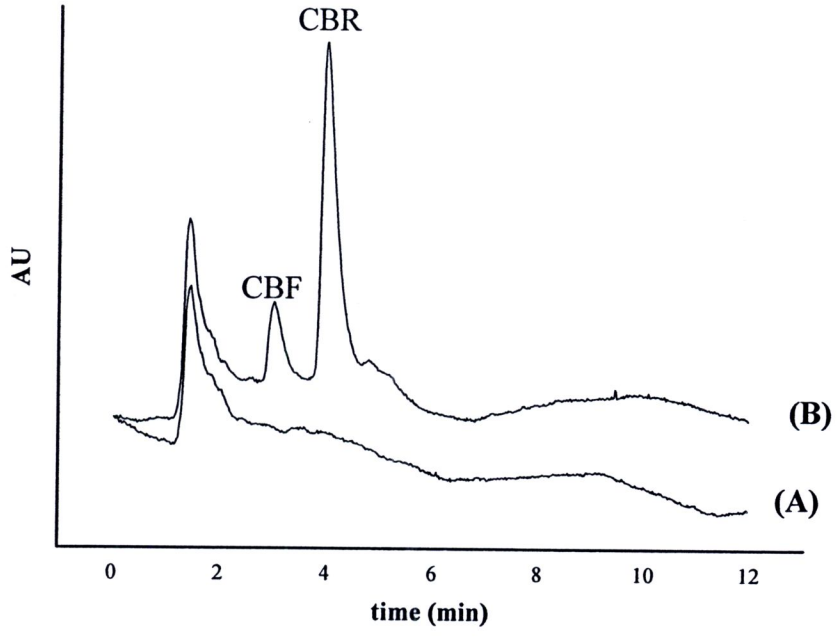


Figure 4.25 Chromatograms of CBR and CBF.

(A) cucumber (blank) and (B) spiked cucumber (1.0 mg kg^{-1}), HPLC conditions: monolithic column, mobile phase: MeOH/H_2O (50/50 v/v), flow rate: 0.5 mL min^{-1}, injection volume: 20 µL, detection wavelength: 270 nm

In order to ensure about accuracy and precision of the proposed spectrophotometric method, HPLC was used to determine CBR and CBF in spiked vegetable samples (1.0 mg kg^{-1} of CBR and CBF) after their preconcentration by CPE. The recoveries of CBR and CBR using HPLC technique are summarized in Table 4.12.

Table 4.12 Recoveries and precisions for determination of CBR and CBF in vegetable samples obtained by CPE-HPLC*

Sample	**Recovery (%), mean ± SD (*%RSD*) (n=5)**	
	CBR	CBF
Cucumber	88.13 ± 3.13 (*3.6*)	88.89 ± 6.81 (*7.7*)
Cabbage	86.11 ± 3.36 (*3.9*)	89.99 ± 5.71 (*6.3*)
Kale	83.54 ± 3.91 (*4.6*)	81.95 ± 8.63 (*10.5*)
Yard long bean	78.47 ± 6.95 (*8.9*)	85.45 ± 9.01 (*10.5*)
Mustard	78.33 ± 5.36 (*6.8*)	79.02 ± 5.14 (*6.5*)

*CPE condition: as described in Table 4.5, HPLC condition: as described in Figure 4.25, except detection wavelength; 220 nm for CBR and 270 nm for CBF.

The recoveries of spiked samples (1.0 mg kg^{-1}) using the spectrophotometry (Table 4.10) were compared with the recoveries obtained from HPLC (Table 4.12). Figure 4.26 shows bar charts for the comparison. It is clearly seen that the recoveries obtained from the spectrophotometry are comparable to those obtained from HPLC. In addition, *t*-test (at 95% confidence level) was applied to test for the difference of the methods. The results revealed that there were not significantly different between the proposed spectrophotometric method and HPLC, $t_{calculated} < t_{table}$, as the typical results are shown in Tables 4.13 and 4.14.

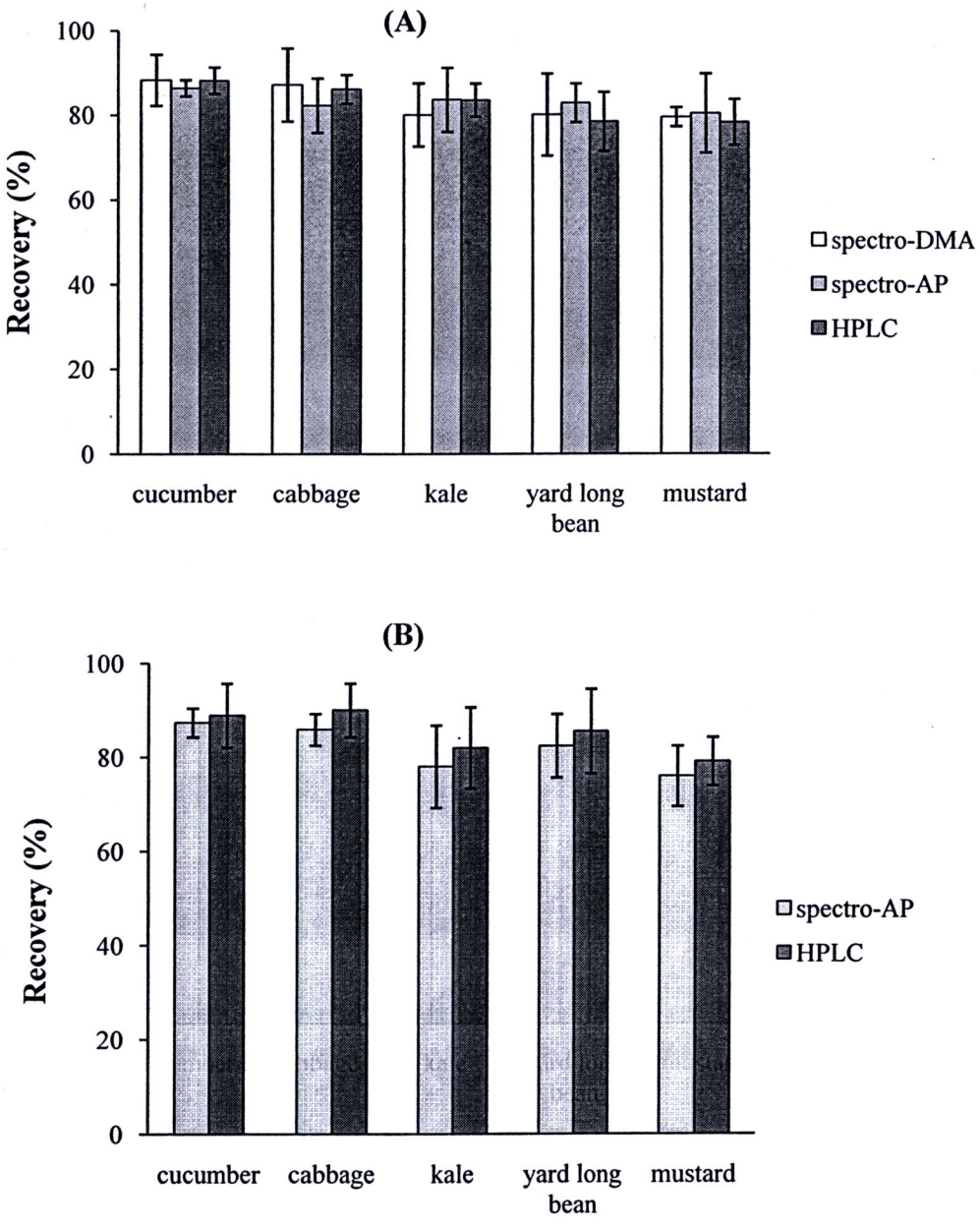


Figure 4.26 Comparison of the recoveries obtained from the proposed method and HPLC, where (A) CBR and (B) CBF; n = 5

Table 4.13 Comparison of the recoveries obtained from the proposed method and HPLC (cucumber spiked at 1.0 mg kg^{-1})

Derivatizing agent	Carbaryl			Carbofuran		
	Spectophoto-metry	HPLC	$t_{calculated}$	Spectrophoto-metry	HPLC	$t_{calculated}$
DMA	88.89 92.06 95.86 81.67 82.77 $\bar{x}$ =88.25 SD=6.04	86.39 91.67 91.39 85.83 85.37 $\bar{x}$= 88.13 SD=3.13	0.08	-	-	-
AP	83.56 87.75 85.23 88.41 86.75 $\bar{x}$=86.34 SD=1.96	86.39 91.67 91.39 85.83 85.37 $\bar{x}$=88.13 SD=3.13	1.09	87.56 89.45 90.32 82.44 86.93 $\bar{x}$= 87.34 SD=3.06	90.21 97.45 92.65 83.32 80.82 $\bar{x}$=88.89 SD=6.81	0.685

Comparison for other samples at 95% confidence level are summarized in Table 4.14.

Table 4.14 Comparison of the recoveries obtained from the proposed method and HPLC (spiked samples at 1.0 mg kg^{-1})

Sample: Derivatizing agent	Carbaryl			Carbofuran		
	Spectrophoto-metry	HPLC	$t_{calculated}$	Spectrophoto-metry	HPLC	$t_{calculated}$
Cabbage:						
DMA	$\bar{x}$=87.14 SD=8.64	$\bar{x}$=86.11 SD=3.36	0.35	- -	- -	-
AP	$\bar{x}$=82.26 SD=6.43	$\bar{x}$=86.11 SD=3.36	1.67	$\bar{x}$=85.84 SD=3.35	$\bar{x}$=89.99 SD=5.71	1.98
Kale:						
DMA	$\bar{x}$=80.08 SD=7.43	$\bar{x}$=83.54 SD=3.91	1.30	- -	- -	-
AP	$\bar{x}$=83.65 SD=7.55	$\bar{x}$=83.54 SD=3.91	0.04	$\bar{x}$=77.98 SD=8.76	$\bar{x}$=81.95 SD=8.63	1.02
Yard long bean:						
DMA	$\bar{x}$=80.12 SD=9.67	$\bar{x}$=78.47 SD=6.95	0.44	- -	- -	-
AP	$\bar{x}$=82.84 SD=4.56	$\bar{x}$=78.47 SD=6.95	1.66	$\bar{x}$=82.34 SD=6.76	$\bar{x}$=85.45 SD=9.01	0.87

Table 4.14 Comparison of the recoveries obtained from the proposed method and HPLC (spiked sample at 1.0 mg kg^{-1}) (Cont.)

Sample: Derivatizing agent	Carbaryl			Carbofuran		
	Spectrophoto-metry	HPLC	$t_{calculated}$	Spectrophoto-metry	HPLC	$t_{calculated}$
Mustard:						
DMA	$\bar{x}$=79.55 SD=2.24	$\bar{x}$=78.33 SD=5.36	0.66	- -	- -	-
AP	$\bar{x}$=80.44 SD=9.31	$\bar{x}$=78.33 SD=5.36	0.62	$\bar{x}$=75.87 SD=6.45	$\bar{x}$=79.02 SD=5.14	1.21

At 95% confidence level, t_{table} = 2.78 for n – 1 = 4 degrees of freedom (see Table 2B in appendix B).

4.6 Determination of CBR and CBF in vegetable samples

Five samples of five vegetables including, cucumber, cabbage, kale, yard long bean, and mustard were studied. The samples were extracted using QuEChERS method and then preconcentrated by CPE. Each sample solution was divided into three portions, two portions were analyzed by spectrophotometry using DMA and AP as derivatizing agents and the third portion was analyzed by HPLC. The results indicated that no contamination of CBR and CBF was detected in all the studied samples.