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# Simultaneous determination of capsanthin and capsorubin in foods by high-performance liquid chromatography using PDA detector

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***Abstract***

A high-performance liquid chromatography method with a photodiode array detector (HPLC-PDA) was developed for the simultaneous determination of Capsanthin and Capsorubin in food. These are the two main carotenoids responsible for the red color found in *Capsicum annuum* and lily *(Lilium).* Analytes were extracted by ultrasonic vibration method with solvent of acetonitrile (ACN): ethyl acetate containing 0.1% butyl hydroxytoluene (BHT) (1:1, v/v) at a temperature of 40$^{o}$C for 20 minutes. The sample extract solution was determined by HPLC–PDA with the following conditions: InterSustain C18 column (250 mm x 4.6 mm; 5 µm), mobile phase consisting of a 5 mM ammonium acetate solution, ACN, MeOH, and water following with gradient program, detection wavelength at 450 nm. The method has good specificity, the calibration curve of Capsanthin and Capsorubin has $R^2 > 0.9997$ and the repeatability and recovery meet AOAC requirements. The limit of detection (LOD) and limit of quantification (LOQ) were 0.20 mg/kg and 0.67 mg/kg, respectively. The method was applied to simultaneously determine Capsanthin and Capsorubin in 35 different food products including chili powder, spices, soft drinks, canned products, confectionery, ice cream, and cheese. Capsanthin was detected with a content of 10.5 - 15.8 mg/kg in chili powder and 3.86 - 9.78 mg/kg in spices, capsorubin was detected with a content of 2.86 - 3.92 mg/kg in chili powder and 0.76 - 1.64 mg/kg in spices.

***Keywords****: Capsanthin, capsorubin, HPLC, PDA, food.*

## 1. INTRODUCTION

Currently, the use of synthetic colors is often abused due to their low price, high economic efficiency, and good color fastness. However, if these synthetic colorants are used in the wrong dosage or type, they will cause serious impacts on consumers' health [1]. Therefore, the trend of using naturally derived colorants is attracting attention from

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manufacturers and consumers. The benefit of using this colorant is to give the product a natural color, increase sensory value, ensure safety, and non-toxicity, and have anti-oxidant properties, eliminate free radicals, and anti-obesity [1-3, 5-7]. In particular, paprika extract and paprika oleoresin containing the main colorants capsanthin and capsorubin are increasingly being used to replace synthetic colorants [2-5, 7 ] with INS codes 160c (i) for paprika oleoresin and INS 160c (ii) for paprika extract, respectively [9, 10].

Capsanthin and capsorubin (Figure 1) give natural red, belonging to the xanthophyll group of carotenoids [4-7]. They are found in *Capsicum annuum* species including red bell peppers, New Mexico peppers, and cayenne peppers [4-8]. Capsanthin is also found in some kinds of lilies (Lilium) [8]. Besides their role as food colorants, capsanthin and capsorubin also have antioxidant activity that is beneficial for health [11, 12]. Among them, capsanthin is a substance with good antioxidant capacity due to the presence of 11 conjugated double bonds, a conjugated keto group, and a cyclopentane ring [13, 14]. In addition, capsanthin can also inhibit hydroperoxide formation, eliminate free radicals, prevent cancer [5, 15], and maintain intraocular pressure (anti-glaucoma) [16].

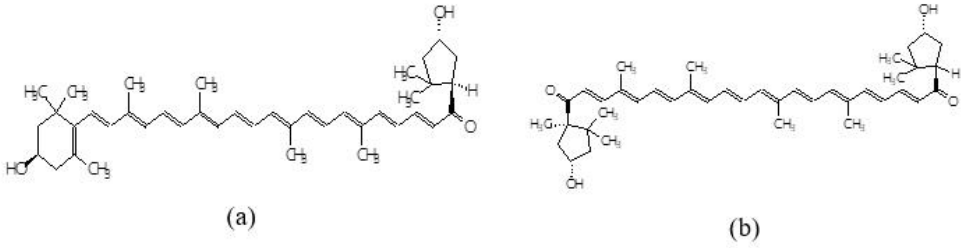


***Figure 1.*** *Structure of capsanthin (a) and capsorubin (b)*

Although natural dolor agents are considered safe and have many benefits for human health, they are expensive, less durable, and have lower color intensity than synthetic ones. Furthermore, some products on the market today are unclear in declaring the type of color in the label. This makes consumers feel concerned when using those products, as well as making it difficult for management agencies to control and post-inspect. In addition, the system of regulations and standards of natural colorants in our country is currently incomplete, so determining the type of colorants, especially colorants of natural origin is extremely important and necessary.

Currently, a number of studies have been published on the determination of capsanthin and capsorubin such as molecular absorption spectroscopy (UV-Vis) [17], thin-layer chromatography (TLC) [18], high performance liquid chromatography (HPLC-PDA) [19-24]. Because the structure of these natural colorants contains many double bonds which have absorbance at the ultraviolet-visible region, thus, high performance liquid chromatography combined with a diode chain detector (HPLC-PDA) is considered due to its popularity, high sensitivity, selectivity, and accuracy. Therefore, the HPLC-PDA method was chosen in this study to simultaneously determine capsanthin and capsorubin in foods.

## 2. MATERIAL AND METHOD

### 2.1. Subjective

The subjects analyzed were capsanthin and capsorubin. The food samples randomly purchased on the Hanoi markets include chili powder, spices, confectionery, juice, canned food, ice cream, cheese, and canned food.

### 2.2. Reagent and standard

The chemicals used in the study are of analytical purity used for liquid chromatography. The standard substances included capsanthin from Chemface CAS number: 465-42-9, lot number: CFS202201, purity 98%, capsorubin from Cayman CAS number: 470-38-2, lot number: 0624453-3; 95% purity. Other chemicals and solvents were methanol (MeOH), ethanol, acetonitrile (ACN), ethyl acetate, diethyl ether, dimethyl sulfoxide, butyl hydroxytoluene (BHT) from Merck, distilled water ($H_2O$) for laboratory use.

### 2.3. Apparatus

High performance liquid chromatography (HPLC) system (Shimadzu) equipped with PDA detector; Intersustain C18 chromatography column (250 mm x 4.6 mm; 5 µm) and corresponding pre-column (5 mm x 4.6 mm; 5 µm) and other common equipment and tools in the laboratory.

### 2.4. Method

*2.4.1. Sample treatment*

According to reference [19], the sample treatment is proposed as follows:

Accurately weigh 0.2 - 5.0 g of the homogenized sample into a 50 mL falcon tube, add 20 mL of ACN and Ethyl acetate containing 0.1% BHT solution at the ratio of 1:1 (v/v), and shake well. Ultrasonic vibration at 40$^o$C for 20 minutes. Decant the extract into a 100 mL vacuum rotary evaporation flask. The residue was extracted again once with the same solvent. Conduct vacuum evaporation at 35 ± 5$^o$C. Dissolve the residue with 10 mL of ACN. The solution was filtered through filter paper and a 0.2 µm filter membrane before injection into the HPLC system.

To determine the most optimal sample processing conditions, refer to documents [19-24], some factors were considered to investigated: sample extraction solvent composition (MeOH, acetone, ethyl acetate, 95% ethanol containing BHT 0.1% and DMSO, acetone:anhydrous ether (1:1, v/v), acetone:ethyl acetate (1:2, v/v), ethyl acetate:ACN (1:1, v/v) containing BHT 0.1%; concentration of BHT solution (0%, 0.1%, 0.2%, 0.5%); extraction temperature (30$^o$C, 40$^o$C, 50$^o$C, 60$^o$C); extraction time (10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 60 minutes) and number of sample extractions (1 time, 2 times, 3 times, 4 times).

Samples used to do optimization: samples of chili powder, cakes and spice sauces. Cake samples that do not contain analytes (capsanthin and capsorubin) were used as blank samples. The blank sample was analyzed following the same procedure as the test sample.

*2.4.2. HPLC - PDA condition*

Based on research [19], HPLC conditions for simultaneous analysis of capsanthin and capsorubin are as follows: PDA detector, Intersustain C18 chromatography column (250 mm

x 4.6 mm; 5 µm) and corresponding pre-column, column chamber temperature: 30°C, flow rate: 1.0 mL/min, injection volume: 10 µL.

To obtain the best chromatographic separation efficiency, factors were investigated including mobile phase composition (ammonium acetate, ACN, MeOH, $H_2O$), ammonium acetate buffer concentration (5 mM, 10 mM, 20 mM, 50mM), and gradient elution program.

*2.4.3. Method validation*

The analytical method is validated for specificity (evaluation of blank sample, standard sample, spiked blank sample), standard curve, limit of detection (LOD), limit of quantification (LOQ), precision is evaluated through relative standard deviation (RSD%) when analyzing 6 times (n=6) on real samples under the same analytical conditions, recovery is evaluated by adding standards at 3 concentration levels: low, medium, high within the working concentration range. The results were evaluated according to AOAC regulations [25] corresponding concentration levels.

## 3. RESULT AND DISCUSSION

### 3.1. Optimization of HPLC-PDA conditions

*3.1.1. Select detection wavelength*

To select the wavelength for simultaneous analysis of capsanthin and capsorubin using a PDA detector, the UV-Vis spectrum was scanned in the range of 190 - 800 nm to obtain the absorption maximum of each substance (Figures 2, 3).

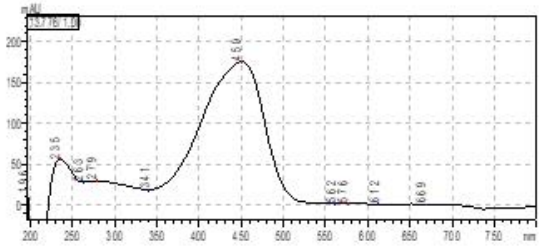

***Figure 2**. Absorbance spectrum of capsanthin at wavelength 450 nm*

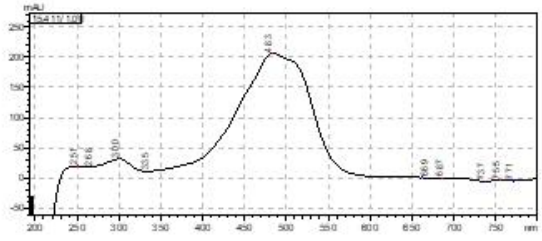

***Figure 3**. Absorbance spectrum of capsanthin at wavelength 483 nm*

From the results in Figures 2 and Figure 3, Capsanthin and capsorubin have maximum absorption spectra at wavelengths of 450 nm and 483 nm, respectively. To reduce the influence of solvents and impurities but still ensure the sensitivity of the method and facilitate the result processing, a wavelength of 450 nm was chosen to simultaneously determine these two substances.

*3.1.2. Optimization of mobile phase composition and gradient program*

In chromatography, the mobile phase is one of the factors that greatly affects separation efficiency. According to the previous study [19], mobile phase solvents are surveyed in section 2.4.2 with the gradient program. The results show that optimal conditions were obtained with a solvent mixture of ammonium acetate, ACN, MeOH, and $H_2O$ according to the gradient program given in Table 1 and the chromatogram in Figure 4.

***Table 1.*** *Gradient program*

| *Time (min)* | *Amoni acetate (%)* | *ACN (%)* | *MeOH (%)* | *$H_2O$ (%)* |
|---|---|---|---|---|
| 0 | 95 | 0 | 5 | 0 |
| 2 | 95 | 0 | 5 | 0 |
| 4 | 75 | 0 | 25 | 0 |
| 8 | 0 | 5 | 90 | 5 |
| 15 | 0 | 5 | 90 | 5 |
| 18 | 95 | 0 | 5 | 0 |
| 20 | 95 | 0 | 5 | 0 |

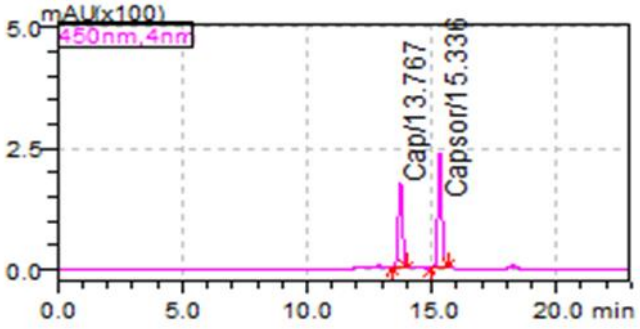


***Figure 4****. Standard chromatogram of capsanthin and capsorubin according to gradient program*

From the results obtained in Table 1 and Figure 4, it can be seen that the analytes have very good resolution (Rs = 3.2), and sharp and well-balanced peaks, suitable for the simultaneous determination of these two colors in food.

*3.1.2. Optimization of mobile phase concentration*

After selecting the mobile phase composition, the mobile phase concentration was also investigated with the levels in section 2.4.2. The analysis results are obtained in Table 2.

***Table 2****. Result of mobile phase concentration optimization*

| ***Concentration (mM)*** | ***Peak area (mAU)*** | | | |
|---|---|---|---|---|
| | ***Capsanthin*** | | ***Capsorubin*** | |
| | ***Run 1*** | ***Run 2*** | ***Run 1*** | ***Run 2*** |
| 5 | 700514 | 701254 | 1016944 | 1025610 |
| 10 | 700705 | 700215 | 1028698 | 1021001 |
| 20 | 704464 | 701240 | 1052782 | 1024201 |
| 50 | 707017 | 707215 | 1016944 | 1021025 |

The results in Table 2 showed that there is no statistically significant difference when using different concentrations of ammonium acetate. Furthermore, the use of high concentrations of ammonium acetate can cause contamination of the analytical column and

system, increasing analysis costs. Therefore, in this study, ammonium acetate concentration of 5 mM was chosen for subsequent investigations.

### 3.2. Optimization of sample treatment

*3.2.1. Optimization of sample extraction solvent*

Capsanthin and capsorubin both dissolve well in organic solvents. Based on studies [19-23], the solvents were selected for investigation according to section 2.4.1. The results of the analysis are shown in Figure 5.

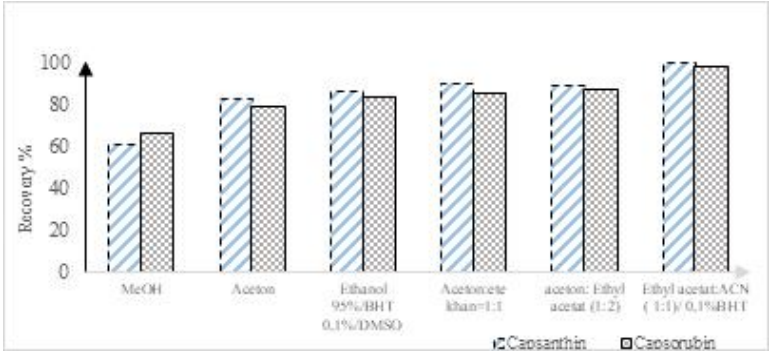


***Figure 5.*** *Result of sample extraction solvent optimization*

The results shown in Figure 5 show that, when using the solvent ACN:ethyl acetate containing 0.1% BHT solution (1:1, v/v), the recovery of capsanthin and capsorubin was the highest (reaching > 90 %). Therefore, this solvent was chosen to perform further investigations.

*3.2.2. Investigate the effect of BHT solution concentration*

The capsanthin and capsorubin are easily decomposed by factors such as temperature, light, sample extraction and storage conditions [4, 6]. To reduce the influence of these factors, the antioxidant BHT was added to the sample treatment with the investigated concentrations: 0%, 0.1%, 0.2%, 0.5%. The analysis results are obtained in Figure 6.

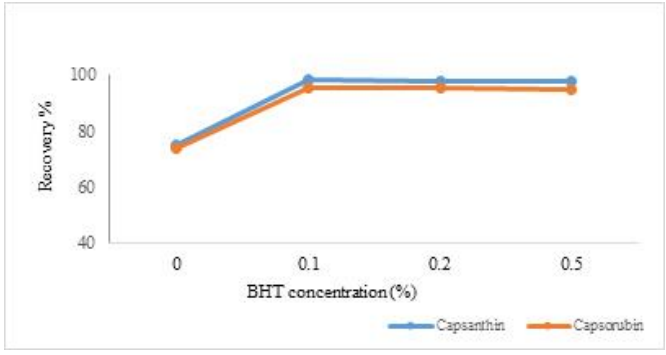


***Figure 6.*** *Result of BHT concentration*

The results in Figure 6 show that the recovery of analytes was low when the antioxidant BHT was not used and increased when BHT was added to the sample extraction process at a concentration of 0.1% and did not change significantly when continuing to increase the concentration by 0.2%, 0.5%. Therefore, to control the influence of environmental

conditions on the sample extraction process and ensure extraction efficiency, a BHT concentration of 0.1% was chosen for further study.

*3.2.3. Optimization of extraction temperature*

Temperature affects the solubility and extraction ability of the sample matrix. The capsanthin and capsorubin are easily decomposed by high temperatures [4, 6]. Therefore, in the sample extraction process, it is necessary to control this factor to ensure the best extraction efficiency. According to the previous reports [19, 21], different temperature levels were investigated on chili powder samples according to section 2.4.1. The results obtained are shown in Figure 7.

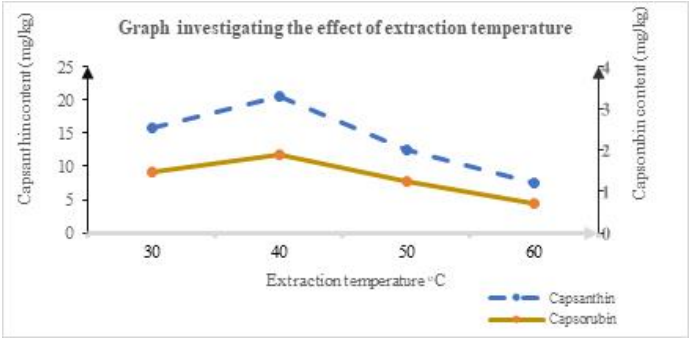


***Figure 7**. Results of the extraction temperature study*

Figure 7 shows that the analyte content increases when the temperature increases from 30 - 40$^{o}$C and then gradually decreases when the temperature continues to increase from 50$^{o}$C and 60$^{o}$C. Because these natural colors are greatly affected by temperature, as the temperature increases, the solubility of the substances increases, so the analyte content obtained increases accordingly. However, these substances decompose when the temperature is high, so the analyte content decreases. Therefore, to ensure extraction efficiency, a temperature of 40$^{o}$C was chosen for this study.

*3.2.4 Optimization of extraction time*

The extraction times selected for investigation to obtain the most optimal extraction efficiency of analytes were 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 60 minutes. The survey was carried out on chili powder samples. The results are shown in Figure 8.

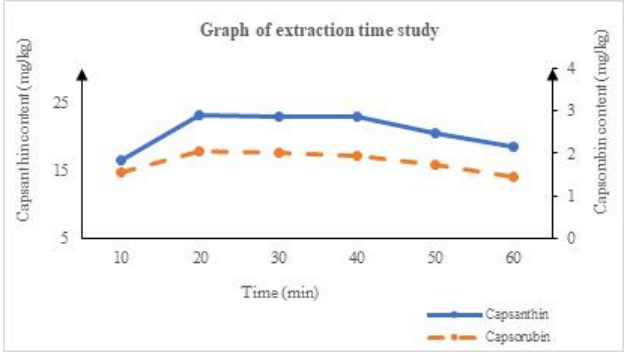


***Figure 8.** Results of the extraction time study*

Results from Figure 8 show that, with an extraction time of 10 minutes, the analytes have not been completely dissolved from the sample matrix, so the concentration obtained is low. When the time is increased to 20 minutes, the substances are completely dissolved and the amount of analyte obtained increases accordingly. However, these colors are easily decomposed by environmental factors such as light and temperature. Therefore, when extending the extraction time, these substances will change, leading to a gradual decrease in the obtained content when increasing the time from 30 minutes to 60 minutes. Therefore, to save time and ensure extraction efficiency, a time of 20 minutes was chosen.

*3.2.5. Optimization of repeated extractions*

To ensure that the analyte is thoroughly extracted from the sample matrix, repeated extraction is necessary. The number of repeated extractions surveyed were: 1 time, 2 times, 3 times, 4 times. The results are presented in Figure 9.

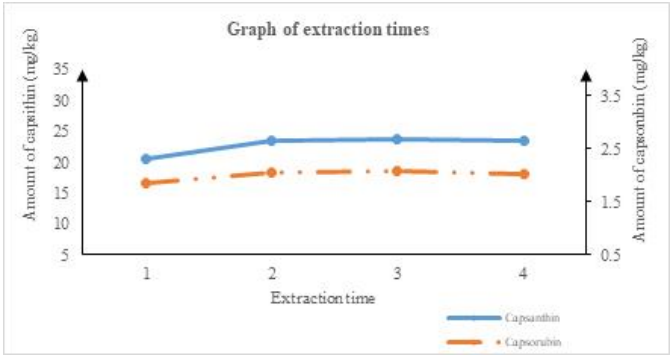


***Figure 9**. Results of the extraction time study*

According to Figure 9, when increasing the number of sample extractions from 1 to 2 times, the amount of analytes obtained increased and did not change significantly when continuing to increase the number of sample extractions 3 times and 4 times. Therefore, double extraction was chosen for this study.

**3.3. Method validation**

*3.3.1. Specificity*

Specificity was evaluated through analysis of blanks, standards, and spiked blanks. The results are shown in Figure 10.

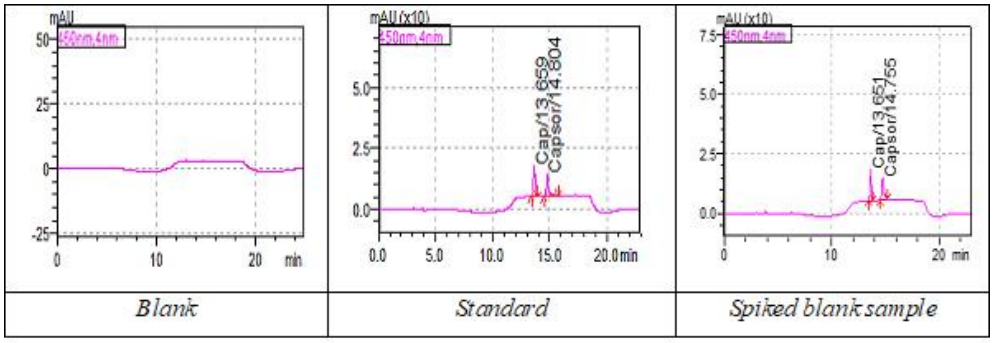


***Figure 10.** Result of specificity*

In Figure 10, the blank sample does not give a signal of the analyte, the standard sample and the spiked blank sample give a signal that does not deviate by more than 0.1 minute. Thus, the method has the required specificity [25].

*3.3.2. Standard curve, the limit of detection (LOD) and limit of quantification (LOQ)*

Standard curves for the determination of capsanthin and capsorubin were constructed in the concentration range of 0.25 mg/L to 50 mg/L. The limit of detection (LOD) and limit of quantification (LOQ) were determined by analyzing real samples with low concentrations (about 5 - 7 times the estimated LOD) with values of $4 \leq R \leq 10$ [25]. The results are shown in Table 3.

***Table 3.*** *Result of calibration curve, LOD, and LOQ of capsanthin and capsorubin*

| ***Compound*** | ***Standard curve*** | ***$R^2$*** | ***LOD (mg/kg)*** | ***LOQ (mg/kg)*** | ***R*** |
|---|---|---|---|---|---|
| Capsanthin | y= 71635x - 1400.1 | 0.9997 | 0.20 | 0.67 | 8.2 |
| Capsorubin | y = 104395x - 6739.4 | 1.0000 | | | 6.3 |

The results in Table 3 show that the standard curve was built in the range from 0.25 mg/L to 50 mg/L with the coefficient correlation of capsanthin and capsorubin being $R^2$ = 0.9997 and $R^2$ = 1, respectively, demonstrating a good linear relationship between the obtained signal and the analyte concentration. The LOD and LOQ values of the method are 0.20 mg/kg and 0.67 mg/kg for both substances with R values meeting the requirements compared to regulations [25].

*3.3.3. Precision and accuracy*

The precision of the method is evaluated through the repeatability (relative standard deviation RSD %, n = 6) and the accuracy is evaluated through the recovery R % by adding standards at 3 concentration levels 3 concentration levels: low, medium, and high within the working concentration range. 3 different sample matrices including chili powder, cake, and spice sauce were used in this study. The results are shown in Table 4.

***Table 4****. Result of precision and accuracy*

| ***Matrix*** | ***Precision (RSD%)*** | | ***Accuracy (R %)*** | |
|---|---|---|---|---|
| | ***Capsanthin*** | ***Capsorubin*** | ***Capsanthin*** | ***Capsorubin*** |
| Chilli powder | 4.3 | 3.8 | 82.2-98.4 | 87.1-94.9 |
| Cake | 4.8 | 3.3 | 92.5-98.3 | 80.7-93.6 |
| Spice sauce | 5.2 | 6.2 | 87.6-98.2 | 80.8-91.2 |
| *AOAC regulation* | *7.3-11* | | *80-110* | |

The results in Table 4 show that the method has satisfactory precision and accuracy compared to AOAC [25] at the content thresholds. The method is suitable for analyzing natural colorants on food sample matrices.

### 3.4. Simultaneous analysis of capsanthin and capsorubin content in foods

The method, after validation to meet AOAC requirements, was applied to simultaneously analyze capsanthin and capsorubin content in 35 food samples including chili powder, canned food, spices, ice cream, juice, candy, margarine, cheese, and whey. Each sample was analyzed twice and the average value was taken. The results are shown in Figure 11.

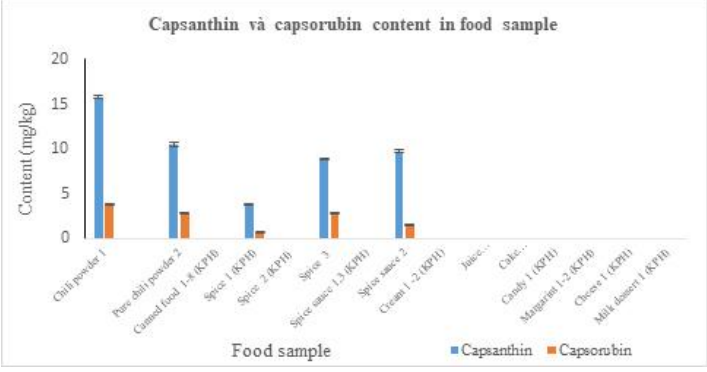


***Figure 11.*** *Results of capsanthin and capsorubin content in some food samples*

*Note: "KPH" is not detected*

The analysis results in Figure 11 show that 5/35 samples detected capsanthin and capsorubin, including 02/02 chili powder samples and 03/06 spice samples. In particular, chili powder samples contain capsanthin with a content of 10.5 - 15.8 mg/kg and capsorubin with a content of 2.86-3.92 mg/kg, spice samples contain capsanthin with a content of 3.86 - 9.78 mg/kg and capsorubin from 0.76 - 1.64 mg/kg. The appearance of capsanthin and capsorubin in spice samples may come from the chili ingredient in the spice or the spice samples supplemented with food additives INS 160c (i) (paprika oleoresin) and INS 160c (ii) (paprika extract) contains capsanthin and capsorubin.

## 4. CONCLUSION

The research was successful in developing a method to simultaneously analyze capsanthin and capsorubin in foods using the HPLC-PDA method. The method has been validated for standard curve, specificity, accuracy, precision, LOD, and LOQ meeting AOAC requirements. The analytical procedure was applied to simultaneously analyze the above two colorants in 35 food samples. The results detected these colorants in some samples of chili powder and spices. The analytical results show that the method can be applied for the simultaneous determination of capsanthin and capsorubin in food samples.

## ACKNOWLEDGEMENT

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# Xác định đồng thời capsanthin và capsorubin trong thực phẩm bằng phương pháp sắc ký lỏng hiệu năng cao sử dụng detector PDA

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***Tóm tắt***

Phương pháp sắc ký lỏng hiệu năng cao (HPLC-PDA) được sử dụng để xác định đồng thời capsanthin và capsorubin trong thực phẩm. Đây là hai hợp chất carotenoid chính tạo màu đỏ được tìm thấy trong loài ớt chuông (*Capsicum annuum)* và loa kèn *(Lilium).* Chất phân tích được chiết siêu âm bằng hỗn hợp acetonitril (ACN): Ethyl acetat tỷ lệ 1:1 chứa dung dịch butyl hydroxytoluen (BHT) 0,1% tại nhiệt độ 40$^{o}$C trong 20 phút. Dịch chiết sau đó được xác định bằng sắc ký lỏng hiệu năng cao HPLC–PDA với các điều kiện: Cột InterSustain C18 (250 mm x 4,6 mm; 5 µm) và tiền cột tương ứng (5 mm x 3,9 mm; 5 µm), pha động gồm hỗn hợp các dung môi amoni acetat 5mM, ACN, MeOH và $H_2O$ theo chương trình gradient, bước sóng phát hiện 450 nm. Phương pháp có độ đặc hiệu tốt, đường chuẩn của capsanthin và capsorubin có hệ số xác định $R^2 > 0,9997$ và độ lặp lại, độ thu hồi đạt yêu cầu của AOAC. Giới hạn phát hiện (LOD) và giới hạn định lượng (LOQ) của hai chất này là 0,20 mg/kg và 0,67 mg/kg. Phương pháp đã được áp dụng để xác định đồng thời capsanthin và capsorubin trong 35 sản phẩm thực phẩm khác nhau gồm bột ớt, gia vị, nước giải khát, đồ hộp và bánh kẹo, kem, pho mai. Kết quả phân tích đã phát hiện hàm lượng capsanthin là 10,5 - 15,8 mg/kg trong ớt bột và 3,86 - 9,78 mg/kg trong gia vị, hàm lượng capsorubin là 2,86 - 3,92 mg/kg trong ớt bột và 0,76 - 1,64 mg/kg trong gia vị.

***Từ khóa:*** *Capsanthin, capsorubin, sắc ký lỏng hiệu năng cao, HPLC, thực phẩm.*