Research Article doi: 10.47866/2615-9252/vjfc.4162

Study on the simultaneous determination of bixin and norbixin in foods by high-performance liquid chromatography

Vu Thi Trang[1*], Le Thi Thuy[1], Luu Thi Huyen Trang[1], Nguyen Nhu Thuong[1], Vu Thi Nhat Le[1], Do Doan Phuong Loan[2], Ngo Thuy Linh[2], Nguyen Thi Anh Huong[3], Le Thi Hong Hao[1,3]

[1]National Institute for Food Control, Hanoi, Vietnam

[2]Hanoi University of Pharmacy, Hanoi, Vietnam

[3]University of Science, Vietnam National University, Hanoi, Vietnam

(Received: 07/11/2023; Accepted: 11/12/2023)

Abstract

Bixin and norbixin are two types of apocarotenoids that can be extracted from the seeds of cashew nuts (*Bixa orellana*). They are the main components of annatto, a natural food coloring agent, which is widely used in the food industry to impart yellow and orange-red hues to various food products. The objective of this study was to develop a method for the simultaneous determination of bixin and norbixin in different food samples using high performance liquid chromatography (HPLC-PDA). The samples were extracted with ethanol by ultrasonic vibration at room temperature for 20 minutes. The extracted samples were then analyzed by HPLC-PDA using XBridge C18 chromatography column (150 mm x 4.6 mm; 5 µm), and isocratic elution with a mobile phase consisting of 0.5 % formic acid in water: MeOH (15:85, v:v) at 450 nm detection wavelength. The method showed good specificity, high correlation coefficients ($R^2 > 0.9999$) for the calibration curves, acceptable repeatability (1.9 - 4.2% for bixin and 4.1 - 5.9% for norbixin), satisfactory recovery (96.0 - 107% for bixin and 95.0 - 109% for norbixin), low limits of detection (LOD) (0.17 - 0.5 mg/kg for both bixin and norbixin), and low limits of quantification (LOQ) (0.56 - 1.31 mg/kg for both bixin and norbixin). The method was successfully applied to the simultaneous determination of bixin and norbixin in 35 food samples. The results showed: concentrations ranging from 736 mg/100g to 5911 mg/100g (bixin) and from 8.91 mg/100g to 51.9 mg/100g (norbixin) for raw material samples and from 5.85 mg/kg to 490 mg/kg (bixin) and 0.83 ng/kg to 8.02 mg/kg (norbixin) for food samples.

Keywords: *Bixin, norbixin, food, HPLC, PDA.*

1. INTRODUCTION

During food processing, to increase sensory value, color and appeal of food products, natural or synthetic coloring agents can be used. Currently, the trend of using natural

[*] *Corresponding author: Tel: (+84) 916149106 Email: trangvt@nifc.gov.vn*

colorants is increasingly concerning because of the safety for users. In particular, bixin is the main apocarotenoid ingredient extracted from cashew nuts to form annatto color - a natural colorant, containing about 5% pigment, of which 70 - 80% is bixin [1, 2]. The bixin compound is chemically unstable and is often converted to isomerization in the trans-bixin (β-bixin) form - a double bond isomer. Bixin is soluble in fats and alcohol but insoluble in water. When exposed to alkali, the methyl ester group is hydrolyzed to produce dicarboxylic acid called norbixin - a water-soluble derivative [1-3]. Both of these substances (Figure 1) are food colorants with INS codes INS 160b (i) and 160b (ii), respectively [4, 5]. Bixin is known as an antioxidant, capable of destroying free radicals [3, 6], and preventing the growth of cancer cells [7-9], the isomeric form of norbixin has antibacterial activity (especially gram-positive bacteria) [10, 11]. In the food industry, bixin and norbixin create colors ranging from yellow to orange-red which are usually used in various foods such as cheese, margarine, milk and dairy products, bread, and desserts. mouth, snacks [12-14].

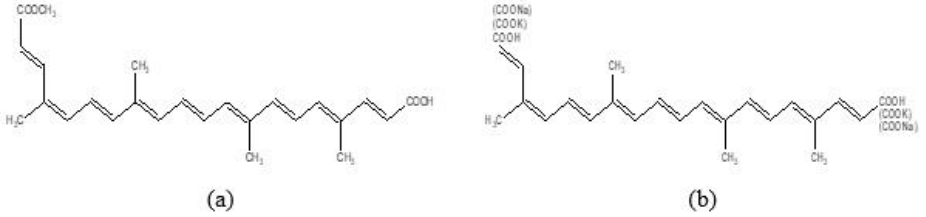

***Figure 1**. Structure of cis-bixin cis Bixin (a) và cis Norbixin (b)*

Currently, bixin and norbixin are analyzed using methods such as molecular absorption spectroscopy (UV-Vis) [15], thin-layer chromatography (TLC) [16], liquid chromatography-mass spectrometry (LC- APCI/MS) [17], high performance liquid chromatography (HPLC-PDA) [18-22]. In this study, the HPLC-PDA method was chosen to simultaneously determine bixin and norbixin in some food samples due to its high sensitivity and selectivity, along with its popularity in laboratories today.

2. MATERIAL AND METHOD

2.1. Research subjects

The research subjects were bixin and norbixin and the food samples were randomly purchased in the Hanoi markets including cashew flour, cashew oil, candy, fruit juice, milk and milk products, cheese, margarine, and canned food.

2.2. Standards and reagents

The chemicals used in the study all met analytical standards for liquid chromatography. The standard substances included bixin from HPC, CAS: 6983-79-5, batch number: 808355, purity 90.6%, norbixin from TRC, CAS: 542-40-5, batch number: 9-WBZ-34 -1, purity 89.41%. Other solvents and chemicals: acetic acid, formic acid, ethanol, and methanol (MeOH) from Merck.

2.3. Equipment

High performance liquid chromatography system HPLC (Alliance, Waters) equipped with PDA detector; XBridge C18 chromatography column (150 mm x 4.6 mm; 5 μm) and other laboratory instruments.

2.4. Method

2.4.1. Sample treatment

Samples were thoroughly homogenized before analysis. Based on studies [18-22], due to different bixin and norbixin contents for raw material and food samples, the amount of sample weighed was not the sample to thoroughly extract the analytes from the sample matrix. For samples (cashew powder, cashew oil), weigh accurately 0.1 - 0.5g; for food samples, accurately weigh 1 – 5 g into a 50 mL centrifuge tube and then add 20 mL of ethanol solvent, vortexed shake for about 2 - 3 minutes, then ultrasonic for 20 minutes at room temperature, centrifuge for 5 minutes at 6000 rpm. Then, the supernatant was decanted into a 50 mL volumetric flask. The residue was extracted again with 20 mL of ethanol, the extract was combined, then made up to the mark with extraction solvent, filtered through a 0.2 µm filter into a sample vial, and analyzed on the HPLC-PDA system.

Samples used for the optimization: cashew flour, cashew oil, cakes, margarine, and juice samples. The blank cake sample was used to do optimized operations. The blank sample was analyzed following the same procedure as the test sample.

2.4.2. HPLC -PDA analytical condition

According to studies [19-21], the HPLC conditions for simultaneous analysis of bixin and norbixin are as follows: PDA detector at a wavelength of 450 nm; XBridge C18 (150 mm x 4,6 mm; 5 µm). The mobile phase consisted of 0.5% formic acid and MeOH at a ratio of 15:85 (v:v) in isocratic mode, the flow rate was 1 mL/min, injection volume was 10 µL.

2.4.3. Method validation

The studied method was validated for specificity (evaluation of blank sample, standard sample, spiked blank sample), linear range, standard curve, limit of detection (LOD), limit of quantification (LOQ), precision is evaluated through the relative standard deviation (RSD%) when analyzing 6 times (n=6) on real samples under the same analytical conditions, recovery is evaluated by spiking standards at 3 concentration levels: low, medium, high within the working concentration range. The results were evaluated according to AOAC regulations [23] according to the corresponding concentration levels.

3. RESULTS AND DISCUSSION

3.1. Optimize the bixin và norbixin analyzed method

3.1.1. Optimze mobile phase

In chromatography, the mobile phase is one of the main factors that greatly affects separation efficiency. Through reference [21], the mobile phase was selected for investigation including 0.5% formic acid or 0.5% acetic acid combined with MeOH in a ratio of 15:85 (v:v). The results are shown in Figure 2.

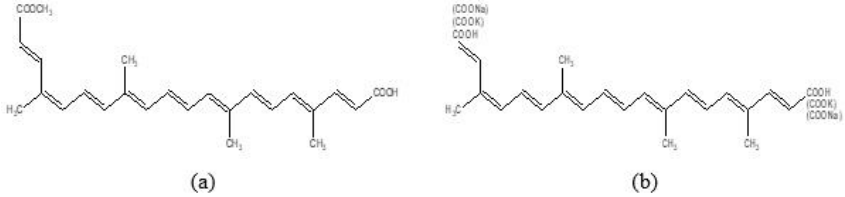

Figure 2. *Analytical chromatogram of bixin and norbixin using mobile phase formic acid 0.5%:MeOH (15:85, v:v)*

The results show that in the two investigated solvent systems, using 0.5% formic acid: MeOH (15:85, v:v) gives very good separation results of analytes with sharp and balanced peaks. Therefore, this solvent system was selected for use in further studies.

3.1.2. Investigation on mobile phase concentration

The concentration of formic acid in the mobile phase was investigated at levels of 0.2%, 0.5%, 1.0% (section 2.4.2). The results are presented in Table 1.

***Table 1**: Results of mobile phase concentration investigation*

Concentration of formic acid %	***Peak area***			
	Bixin		***Norbixin***	
0.2	243299	235177	166991	165765
0.5	243799	247861	173128	174698
1.0	230932	230293	168940	166627

The results that there is no statistically significant difference when using formic acid mobile phase solution at different concentrations of 0.2%; 0.5%; 1.0%. However, at a concentration of 0.5%, the chromatographic peak signal was higher than when using the other two concentrations. Therefore, a 0.5% formic acid concentration in the mobile phase was used for other investigations.

3.2. Optimize sample treatment

3.2.1. Optimize bixin và norbixin extraction solvent

Referring to reports [18-22], three solvents were selected for investigation including acetonitrile, methanol, and ethanol. The cashew powder samples were analyzed twice in this study. The results are shown in Figure 3.

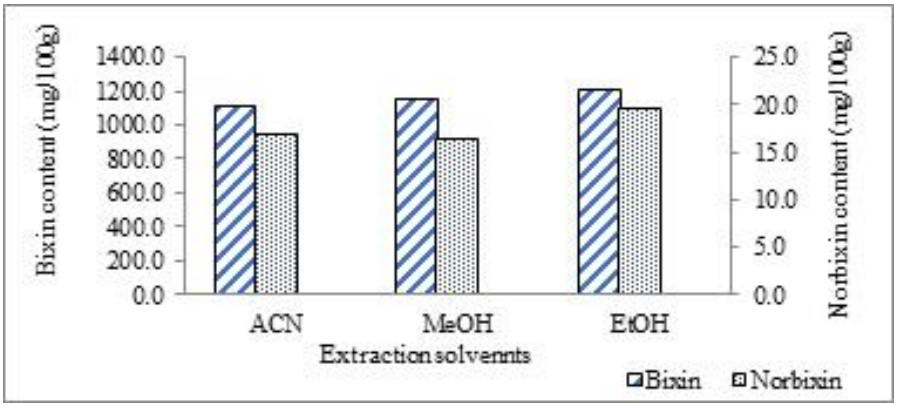

***Figure 3.** Result of extraction solvent study*

The results in Figure 3 show that all three extraction solvents gave relatively good recovery with bixin and norbixin contents in the range of 850 - 1250 mg/100g and 15 - 20

mg/100g, respectively. However, the best extraction efficiency was obtained when using ethanol solvent. Therefore, the solvent ethanol was chosen in this study.

3.2.2. Optimize extraction solvent ratio

Due to the different solubility of bixin and norbixin, bixin dissolves well in organic solvents, and norbixin dissolves well in water, so combining water and organic substances in the extraction solvent is necessary to increase extraction efficiency. According to papers [22], the ratio of ethanol (combined with H_2O) in the extraction solvent was investigated with values including 20%, 40%, 60%, 80%, 100%. The results of the study conducted with 4 different sample matrices (cashew flour, cashew oil, butter, cake) are presented in Figures 4 and Figure 5.

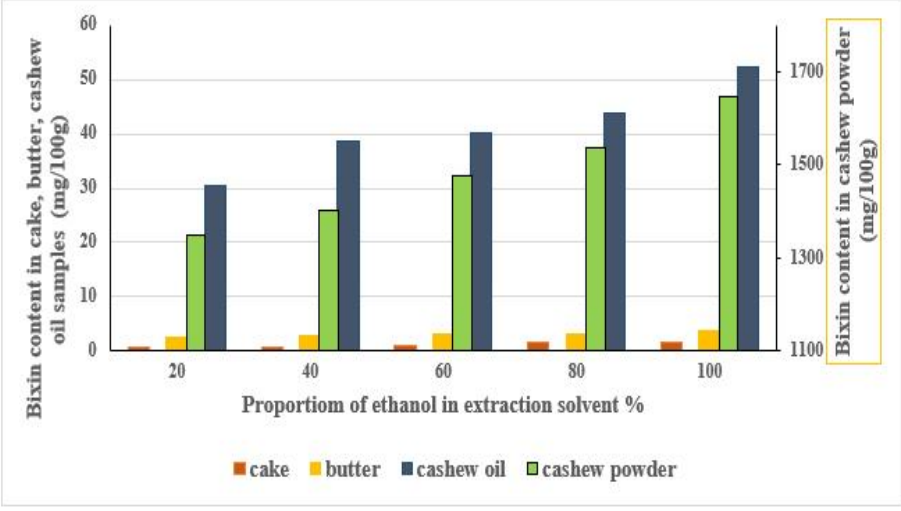

***Figure 4**. Graph of bixin extraction efficiency in different ethanol solvent ratio*

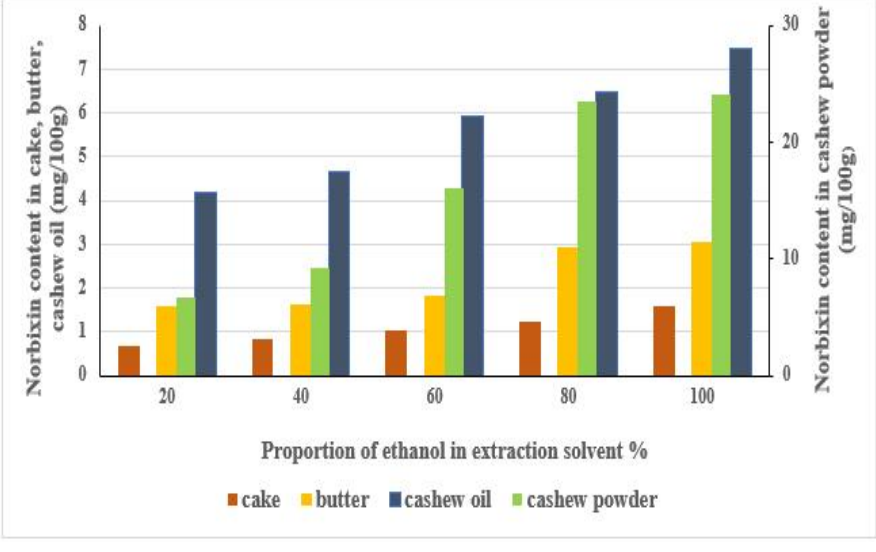

***Figure 5**. Graph of norbixin extraction efficiency in different ethanol solvent ratio*

The results in Figure 4 and Figure 5 show that the content of the two analytes increased when increasing the ethanol ratio from 20% to 100% and the highest extraction efficiency was achieved when using 100% ethanol for both bixin and norbixin. Therefore, 100% ethanol solvent was chosen as the solvent to simultaneously extract the two analytes in this study.

3.2.3. Optimize sample extraction time

Extraction time is also an important factor in obtaining the highest extraction efficiency. The extraction times selected for investigation were: 5 minutes, 10 minutes, 15 minutes, 20 minutes, 30 minutes on the cashew powder sample. The results are shown in Figure 6.

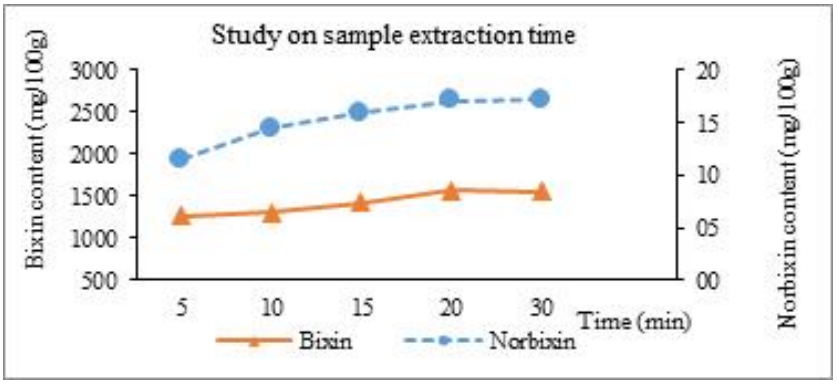

Figure 6. *Result of sample extraction time study*

The study results in Figure 6 show that, with an extraction time of 5 minutes, the analytes were not completely extracted, so the obtained analyte content was the lowest. The analyte content increased when increasing the extraction time from 10 minutes to 20 minutes and did not change significantly when increasing the extraction time to 30 minutes. Therefore, to save extraction time while still ensuring extraction efficiency, a time of 20 minutes was chosen for further studies.

Consequently, the selected sample processing conditions included: the sample was extracted twice with 20 mL of ethanol solvent for 20 minutes at room temperature, centrifuged, and the extract combined into a 50 mL volumetric flask; then filter the sample through a 0.2 µm membrane and analyze on the HPLC-PDA system using a C18 column (150 mm x 4.6 mm; 5 µm), 0.5% formic acid mobile phase: MeOH (85:15, v:v) at wavelength 450 nm, flow rate 1 mL/min, sample injection volume 10 µL.

3.3. Method validation

3.3.1. Specificity

Specificity was evaluated through analysis of blank samples (blank cake samples) standard samples and spiked blank samples (cake samples). The results are shown in Figure 7.

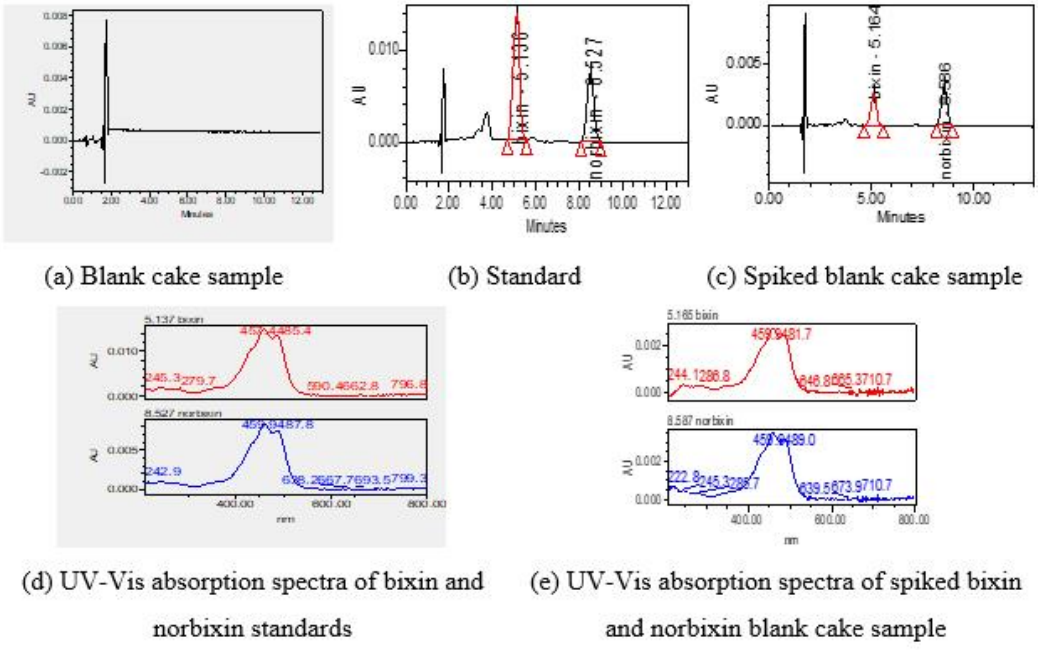

***Figure 7**. Result of specificity*

The analysis results in Figure 7 show that the blank sample does not give a signal of the analyte, the standard sample and the spiked blank sample give a signal with retention time that does not deviate by more than 0.1 minute. The UV-Vis absorption spectra of the standard and spiked cake samples are the same. Thus, the method has a specificity that meets the requirements of AOAC [23].

3.3.2. Determine standard curve, LOD, LOQ

Standard curves for determining bixin and norbixin were constructed in the concentration range of 0.05 mg/L to 10 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 a value of 4≤ R ≤10 [23]. The results are shown in Table 2.

***Table 2:** Standard curve, LOD, and LOD of the method*

Analyte	*Calibration curve*	*R^2*	*LOD (mg/kg)*	*LOQ (mg/kg)*	*Coefficient (R)*
Bixin	y= 84303x - 920.27	0.9999	0.4 - 0.5	1.3 -1.7	4.3 - 7.0
Norbixin	y = 108335x - 3158.9	0.9999	0.17 - 0.4	0.56 - 1.3	5.3 - 6.9

The results in Table 2 show that the coefficient correlation R^2 = 0.9999, demonstrating a good linear relationship between signal and concentration within the standard curve. The LOD and LOQ values of bixin are 0.4 - 0.5 mg/kg and 1.3 - 1.7 mg/kg, respectively, and of norbixin are 0.17 - 0.4 mg/kg, respectively. and 0.56 - 6.9 mg/kg, with a satisfactory R-value compared to AOAC regulations [23].

3.3.3. Precision and accuracy

The precision of the method is evaluated through repeatability (relative standard deviation RSD %, n = 6) and accuracy is evaluated through recovery R % by adding standards at 3 different concentration levels corresponding to the start point, end point, and middle of the standard curve. The precision and accuracy were conducted on 5 different sample matrices: cashew powder, cashew oil, margarine, cake, and juice. The results are presented in Table 3.

Table 3: *Result of repeatability (RSD%) and recovery of the method*

Sample matrix	***RSD%***		***Recovery (R%)***	
	Bixin	***Norbixin***	***Bixin***	***Norbixin***
Cashew powder	2.6	4.5	97.6-103	96.3-105
Cashew oil	0.96	4.2	96.0-105	95.0-107
Margarine	3.4	5.9	97.1-102	95.9-107
Cake	1.9	4.1	96.1-107	96.3-104
Juice	4.2	4.4	97.5-105	97.9-109

The results shown in Table 3 demonstrate that the repeatability and recovery of bixin and norbixin in the corresponding sample matrices meet the requirements according to AOAC regulations [23]. The method is suitable for these compounds (bixin and norbixin) in food samples.

3.3. Application in real sample

The results of the simultaneous analysis of bixin and norbixin content in 35 food samples collected in the Hanoi market are summarized in Figure 8.

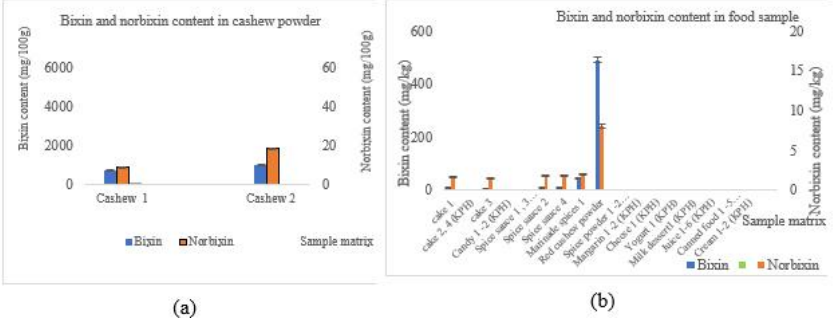

Figure 8. *Result of bixin and norbixin in cashew powder (a) and food sample (b)*

By using the developed method, the contents of bixin and norbixin were evaluated in 35 different food samples. The result is that bixin and norbixin were detected in 9/35 samples (accounting for 25.7%) including cashew powder, cashew oil, spices, and cakes and were not detected in other samples. In particular, 100% of the cashew powder samples contained these two colorants with bixin and norbixin contents ranging from 736-5911 mg/100g and 8.91-51.9 mg/100g, respectively. Cashew oil samples contain bixin and norbixin with concentrations of 49.0 mg/100g and 0.8 mg/100g, respectively. The spice samples found that 3/7 samples (accounting for 42.9%) contained bixin and norbixin with concentrations ranging from 8.76-46.6 mg/kg and 0.83 -1.90 mg/kg, respectively. The cake samples found that 2/6 samples contained bixin and norbixin at levels of 5.85-10.5 mg/kg and 1.21-1.54 mg/kg, respectively. Thus, bixin and norbixin participate in creating color, increasing the sensory properties of food products. Compared with the researched results [18-22], the applied objects of the method are more diverse, including raw material samples and food samples with a large concentration range.

4. CONCLUSION

The method was successful in simultaneously determining the two main colorants of Annatto food coloring, bixin, and norbixin, in food using high performance liquid chromatography (HPLC-PDA). The analytical procedure has been optimized for a number of food sample matrices. The method achieved high sensitivity and has been validated to meet AOAC requirements. The method was applied to simultaneously analyze the content of bixin and norbixin in 35 different food samples in which bixin and norbixin were detected in cashew powder, cashew oil, spices, and cake samples. Research will continue to be expanded to simultaneously determine bixin and norbixin in different types of food samples.

ACKNOWLEDGEMENT

This research is sponsored by the National Institute for Food Control, under project code NIFC.ĐTCS.22.10.

REFERENCES

[1]. G.J. Lauro, "A primer on natural colours", *Cereal Food World*, vol. 36, pp. 949–953, 1991.

[2]. H.D. Preston, M.D.Rickard, "Extraction and chemistry of annatto", *Food Chemistry*, vol. 5, no. 1, pp. 47–56, 1980.

[3]. Shahid-ul-Islam, Luqman J. Rather, Faqeer Mohammad, "Phytochemistry, biological activities and potential of annatto in natural colorant production for industrial applications – A review", *Journal of Advanced Research*, vol. 7, pp. 499–514, 2016.

[4]. Annatto extracts (Aqueous-processed bixin). Jecfa FNP 52 Add 11 (2003) and FAO JECFA Monographs 1 (2005).

[5]. Annatto extracts (Aqueous-processed norbixin). Jecfa FNP 52 Add 11 (2003) and FAO JECFA Monographs 1 (2005).

[6]. C.R. Silva, L.M.G. Antunes, M.L.P. Bianchi, "Antioxidant action of bixin against cisplatin-induced chromosome aberrations and lipid peroxidation in rats", *Pharmaceutical Research*, vol. 43, pp. 561–566, 2001.

[7]. M.K. Reddy, R.L. Alexander-Lindo, M.G. Nair, "Relative inhibition of lipid peroxidation, cyclooxygenase enzymes, and human tumor cell proliferation by natural food colors", *Journal of Agricultural and Food Chemistry*, vol. 53, pp. 9268–9273, 2005.

[8]. L.M.G. Antunes, L.M. Pascoal, M.L.P. Bianchi, F.L. Dias, "Evaluation of the clastogenicity and anticlastogenicity of the carotenoid bixin in human lymphocyte cultures", *Mutation Research*, vol. 585, pp. 113–119, 2005.

[9]. J.D. Tibodeau, C.R. Isham, K.C. Bible, "Annatto constituent Cis Bixin has selective antimyeloma effects mediated by oxidative stress and associated with inhibition of Thioredoxin and Thioredoxin reductase", *Antioxid Redox Signal*, vol. 13, pp. 987–997, 2010.

[10]. V. Galindo-Cuspinera, D.C. Westhoff, S.A. Rankin, "Antimicrobial properties of commercial annatto extracts against selected pathogenic, lactic acid, and spoilage microorganisms", *Journal of food protection*, vol. 66, no. 6, pp. 1074–1078, 2003.

[11]. V. Alindo-Cuspinera, S.A. Rankin, "Bioautography and Chemical Characterization of Antimicrobial Compound(s) in Commercial Water-Soluble Annatto Extracts", *Journal of Agricultural and Food Chemistry*, vol. 53, no. 7, pp. 2524–2529, 2005.

[12]. E.J. Kang, R.E. Campbell, E. Bastian, M.A. Drake, "Annatto usage and bleaching in dairy foods", *Journal of Dairy Science*, vol. 93, pp. 3891–3901, 2010.

[13]. A. Venugopalan, P. Giridhar, G.A. Ravishankar, "Food, ethanobotanical and diversified applications of Bixa Orellana L.: a scope for its improvement through biotechnological mediation", *Indian Journal of Fundamental and Appled Life Science*, vol. 1, no. 4, pp. 9–31, 2011.

[14]. M. Scotter, "The chemistry and analysis of annatto food colouring: a review", *Food Additives & Contaminants*, vol. 26, pp. 1123–1145, 2009.

[15]. W. Rahmalia, J. F. Fabre, Z. Mouloungui, "Effects of cyclohexane/acetone ratio on bixin extraction yield by accelerated solvent extraction method", *Procedia Chemistry*, vol. 14, pp. 455-464, 2015.

[16]. M.L. Wegdan, A.H. Rasmia, A.T. Wafaa, A.H. Amira, "Determination of natural colors by thin layer chromatography", *Journal of Applied Sciences Research*, vol. 4, no. 12, pp. 2013-2017, 2008.

[17]. D. E. Breithaupt, "Simultaneous HPLC determination of carotenoids used as food coloring additives: applicability of accelerated solvent extraction", *Food chemistry*, vol. 86, no. 3, pp. 449-456, 2004.

[18]. F.E. Lancaster, J.F. Lawrence, "Determination of annatto in high-fat dairy products, margarine and hard candy by solvent extraction followed by high-performance liquid chromatography", *Food Additives & Contaminants*, vol. 12, no. 1, pp. 9-19, 1995.

[19]. H. Noppe et al., "Determination of bixin and norbixin in meat using liquid chromatography and photodiode array detection", *Food Additives and Contaminants*, vol. 26, no. 1, pp. 17-24, 2009.

[20]. M. J. Scotter et al, "Method development and analysis of retail foods for annatto food colouring material", *Food Additives & Contaminants*, vol. 19, no.3, pp. 205-222, 2002.

[21]. V. Cardeñosa, M. Lunar, S. Rubio, "Generalized and rapid supramolecular solvent-based sample treatment for the determination of annatto in food", *Journal of Chromatography A*, vol. 1218, no. 50, pp. 8996-9002, 2011.

[22]. T. Taham, F. A. Cabral, M.A.S. Barrozo, "Extraction of bixin from annatto seeds using combined technologies", *The Journal of Supercritical Fluids*, vol. 100, pp. 175-183, 2015.

[23]. AOAC International, Appendix F, *AOAC International Method committee Guidelines for standard method performance requirement*, 2016.

Nghiên cứu xác định đồng thời bixin và norbixin trong thực phẩm bằng sắc ký lỏng hiệu năng cao kết nối detector PDA

Vũ Thị Trang[1*], Lê Thị Thúy[1,3], Lưu Thị Huyền Trang[1], Nguyễn Như Thượng[1], Vũ Thị Nhật Lệ[1], Đỗ Đoàn Phương Loan[2], Ngô Thùy Linh[2], Nguyễn Thị Ánh Hường[3], Lê Thị Hồng Hảo[1,3]

[1]*Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia, Hà Nội, Việt Nam*

[2]*Trường Đại học Dược Hà Nội, Hà Nội, Việt Nam*

[3]*Trường Đại học Khoa học tự nhiên, Đại học quốc gia Hà Nội, Việt Nam*

Tóm tắt

Bixin và norbixin là hai hợp chất apocarotenoid được tìm thấy trong hạt của cây điều *(Bixa orellana)*, được chiết xuất để tạo thành màu annatto – một loại phẩm màu tự nhiên, được sử dụng phổ biến trong công nghiệp thực phẩm tạo màu vàng và màu đỏ cam. Nghiên cứu này nhằm xây dựng phương pháp xác định đồng thời bixin và norbixin trong một số thực phẩm bằng kỹ thuật sắc ký lỏng hiệu năng cao (HPLC-PDA). Mẫu được chiết siêu âm với dung môi ethanol ở nhiệt độ phòng trong thời gian 20 phút. Dịch chiết mẫu được phân tích trên hệ thống HPLC với điều kiện như sau: detector PDA được cài đặt tại bước sóng 450 nm, cột sắc ký XBridge C18 (150 mm x 4,6 mm; 5 µm), pha động chứa acid formic 0,5%: MeOH (15:85, v: v) theo chế độ đẳng dòng. Phương pháp có độ đặc hiệu tốt, đường chuẩn có hệ số xác định $R^2 \geq 0,9999$. Độ lặp lại trong khoảng 0,96 - 4,2% đối với bixin và 4,1 - 5,9% đối với norbixin; độ thu hồi của phương pháp với bixin và norbixin tương ứng là 96,0 - 107% và 95,0 - 109%, giới hạn phát hiện (LOD) của bixin và norbixin là 0,17 - 0,5 mg/kg và giới hạn định lượng (LOQ) là 0,56-1,31 mg/kg. Phương pháp đã được áp dụng để phân tích bixin và norbixin trong 35 mẫu khác nhau với hàm lượng tương ứng trong các mẫu nguyên liệu là 736 - 5911 mg/100g (bixin) và 8,91 - 51,9 mg/100g (norbixin) và trong thực phẩm là 5,85 - 490 mg/kg (bixin) và 0,83 - 8,02 mg/kg (norbixin).

Từ khóa: *Bixin, Norbixin, thực phẩm, HPLC, PDA.*