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Development and Validation of an Analytical Method for Betanine and Isobetanine in Processed Food Products Labeled with Beet Red

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ABSTRACT - Red beet (*Beta vulgaris* L.) is a root vegetable and a popular functional food ingredient of dark red-purple appearance due largely to betacyanins, principally betanine (75-95%) and its isomer, isobetanine (15-45%). This study developed an analytical method for beet red in terms of betanine and isobetanine in processed food products labeled with beet red as a food additive. High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) was used with a C_{18} column. Linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision and uncertainty in measurement were calculated for method validation. Matrix-matched calibration was applied to the candy, ice cream, and cocoa product, respectively, and R^2 was ≥0.9998, showing a high level of linearity. The LOD and LOQ were 0.16 to 0.32 and 0.48 to 0.97 mg/L, respectively. As a result of repeated intra-day and inter-day experiments to validate the accuracy and precision of the analytical method, the recovery rates were 96.0-103.1% and 100.0-102.2%, respectively and the RSD% was 0.5-3.3% and 0.9-3.8%, respectively. Moreover, the measurement uncertainty was estimated to be 1.71-12.43% depending on the matrix and the measured concentration. In this study, betanine and isobetanine were quantified (8.4-3,823.4 mg/kg) by applying the developed analytical method to processed food products (n = 26; e.g., candy, ice cream, and other processed foods) labeled with beet red as a food additive.

Keywords: Beet red, Betanine, Isobetanine, Natural food additive, Quantitative Analysis

Red beet (*Beta vulgaris* L.) is a root vegetable containing abundant nutrients and bioactive compounds, particularly phenolics, flavonoids, and betalains (red-purple betacyanins and yellow betaxanthins), with anti-inflammatory, antioxidant, and anticancer properties[1-3]. The betacyanins—betanine (75-95%) and its isomer, isobetanine (15-45%)—account for 41% of beet red pigment and are the most abundant components[4-6]. The cyclic amine group and phenolic of betanine are excellent electron donors and are largely responsible for the potent antioxidant properties of beet red[7].

Betanine shows stability while maintaining color between pH 3.0 and 7.0, and it is suitable for use in slightly acidic foods[8]. Moreover, beet extract powder is permissible as a food colorant under Section 73.40 in Title 21 by the U.S. Food and Drug Administration (FDA) and approved as a natural food additive—Beetroot Red/Beet Red (162/E162)—for colorant purposes by the European Union (E.U.) and Korea[9-13]. The ADI is listed as 'Not specified' by the European Food Safety Authority (EFSA) due to insufficient toxicity data[11]. However, betanine has a long history as a dietary constituent. As the main colorant in E162, betanine exposure resulting from its use as a food additive lies within the same range as its exposure from the diet[12]. As a result, it was confirmed that it was safe as a food additive[11].

According to the Korean Ministry of Food and Drug Safety's (MFDS) regulations, beet red is prohibited in tea, coffee, red pepper powder, shredded red pepper, kimchi, red pepper paste, vinegar, and processed spice products[13]. However, the analysis method for beet red as a food additive in food has not been reported in Korea. Furthermore, although the Joint FAO/WHO Expert Committee on Food Additives (JECFA) notified the method for quantifying the red dye of beet red, it is difficult to find a study on the determination method in food[14].

Therefore, this study intends to develop an analytical determination method of two chemical markers betanine and

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isobetanine, as the key compounds in beet red. In order to obtain the reliability of the analytical method, the proposed method in this study is validated, and the content of betanine in various matrices of processed foods is applied to the quantification.

Materials and Methods

Chemicals and materials

Betanine was purchased as a red beet extract diluted with dextrin (B0397, Tokyo Chemical Industry, Tokyo, Japan). Sodium acetate anhydrous, acetic acid, and sodium phosphate were obtained from Sigma Aldrich Co. (St. Louis, MO, USA). HPLC-grade water, acetonitrile, and chloroform were from J.T. Baker (Phillipsburg, NJ, USA). The use of beet red in local distributed foods was investigated, and processed products labeled with beet red as a food additive ingredient were collected. To confirm the applicability of the developed analytical method, 26 foods, including various types of candy (n = 5), ice cream (n = 3), other processed foods (n = 9), processed sugar products (n = 7), and weight control formula (n = 2), were purchased and used as samples in 2019.

Quantification of beet red

Standard betanine was naturally extracted from beet red, and it was calculated as the sum of betanine and isobetanine. The molar extinction coefficient of betanine was applied to calculate the betanine concentration by equation (1) according to Stintzing et al.[15]

$$\text{Betanine content (mg/L)} = [(A \times DF \times MW \times 1000) / (e \times l)] \quad (1)$$

where A = absorption at λ_{max} (535 nm), DF = the dilution factor, MW = molecular weight of betanine (550 g/mol), e = molar extinction coefficient of betanine (60,000 L/mol·cm in H_2O), l = the length of the cuvette (1 cm).

Therefore, the betanine standard stock solution prepared with 10% used as a standard chemical is 186.08 mg/L. This stock solution was serially diluted to concentrations of 9.30, 18.61, 37.22, 93.04, and 186.08 mg/L to prepare a calibration curve.

Sample preparation

Betanine and isobetanine, as marker compounds of beet red, were analyzed with reference to the preparation procedure reported by Gandía-Herrero et al.[16] Briefly, 5 g of the sample was precisely taken into a 50 mL centrifuge tube and diluted with 30 mL of 20 mM sodium phosphate buffer (pH 6.0), and the extraction was performed twice. Afterward, the supernatant was collected and adjusted to 50 mL. For a sample with fat, a fat-extraction procedure was performed in which after adjusting the extract to 50 mL, 10 mL chloroform was added to 20 mL of the extract. After filtration through a 0.45 μm membrane filter, the filtrate was used for HPLC analysis.

Analytical instrument

For HPLC-DAD analysis, the Agilent Technologies HPLC 1100 series was used. The column was a Phenomenex Luna C18 (4.6 × 250 mm, 5 μm), and the detection wavelength was set to 536 nm. For the mobile phase, (A) 0.1% trifluoroacetic acid in water and (B) 0.1% trifluoroacetic acid in 60% acetonitrile were used under gradient conditions such that (A) decreased from 100% to 5% over 20 min. Table 1 shows the details of the HPLC analytical conditions.

Method validation

Validation was carried out according to the type of matrix because the quantification was affected by the matrix. Betanine standard was added to the blank sample of each matrix (candy, ice cream, and cocoa product) to obtain concentrations of 9.30, 18.61, 37.22, 93.04, and 186.08 mg/L to confirm the linearity. Matrix-matched calibration was prepared from each concentration, the sum of the HPLC peak areas of betanine and isobetanine were obtained, and the coefficient of determination (R^2) of the calibration curve was calculated. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated using equation (2) and (3) with reference to the ICH Q2 (R1) Validation of Analytical Procedures: Text and Methodology [17].

$$\text{LOD} = \frac{3.3 \times \sigma}{S} \quad (2)$$

Table 1. HPLC-DAD conditions for the analysis of betanine and isobetanine

Instrument	HPLC 1100 series (Agilent)		
Column	Phenomenex Luna C_{18} (4.6×250 mm, 5 μm)		
Detector	DAD (536 nm)		
Mobile phase	A- 0.1% Trifluoroacetic acid/Water B- 0.1% Trifluoroacetic acid/60% Acetonitrile		
	Time (min)	A (%)	B (%)
	0	100	0
	20	5	95
	Post run	100	0
Flow rate	10 μL		
Injection volume	1.0 mL/min		
Column temp.	25°C		

$$LOQ = \frac{10 \times \sigma}{S} \quad (3)$$

where σ = the standard deviation of the response, S = the slope of the calibration curve.

In order to evaluate the precision and accuracy of the proposed method, concentrations of 18.61, 93.04, and 186.08 mg/L were added to the blank sample of each matrix (candy, ice cream, and cocoa product). The recovery rate for accuracy, and the relative standard deviations (RSDs) for precision, were measured by performing six repetitions (intra-day) per day and three repetitions (inter-day) for 3 days.

The Horwitz ratio (HorRat) is a performance parameter that reflects the acceptability of a chemical method of analysis with respect to precision. The $HorRat_r$ value is evaluated by calculating the method repeatability (intra-day precision and inter-day precision), as shown in equation (4).

$$HorRat_r = RSD_r / PRSD_{r,} \quad (4)$$

where RSD_r = the observed repeatability precision and $PRSD_{r,}$ = the predicted repeatability precision, computed by a simple exponential equation ($PRSD_r = 2C^{-0.15}$) from the mean concentration, C, found or added, expressed as a mass fraction[18].

Measurement uncertainty assessment

"A parameter, which is associated with the result of a measurement and characterizes the dispersion of the values that could reasonably be attributed to the measurand" is known as the uncertainty of a measurement[19]. The Guide to the Expression of Uncertainty in Measurement (GUM) and the EURACHEM method referred the measurement uncertainty budget for the methods[20,21]. The analysis of betanine and isobetanine were evaluated in association with the sources of measurement uncertainty, including the repeatability for the determination of betanine and isobetanine in the sample (*uRP*), sample preparation (*uSP*), standard stock solution (*uSSS*), and calibration curve (*uCal*). The expanded uncertainty (*Uc*) was calculated with a coverage factor (k) of 2 at the confidence level of 95%.

Results and Discussion

Validation

In order to check specificity, linearity, LOD, and LOQ, the results of preparing a calibration curve by adding betanine standard to the solution extracted from candy, ice cream, and cocoa product are shown in Table 2. With no other interfering substances in the retention time (RT) of betanine (RT = 8.3 min) and isobetanine (RT = 8.7 min) in each matrix, it was confirmed that the developed test method had high resolution and selectivity through HPLC analysis of the a sample spiked with standard and the samples[22,23] (Fig. 1). The American Association of Analytical Chemists (AOAC) guideline defines high linearity as $R^2 > 0.99$[24]. The R^2 in each matrix was ≥0.9998, confirming the linearity. LOD and LOQ values of 0.16 and 0.48 mg/L in candy, 0.32 and 0.97 mg/L in ice cream, and 0.29 and 0.84 mg/L in cocoa product, respectively, were obtained. To achieve more accurate and precise analysis results, the current study performed validation using a matrix-matched calibration curve.

As shown in Table 3, three concentrations of betanine standard (18.61, 93.04, and 186.08 mg/L) were added to blank samples of candy, ice cream, and cocoa product, respectively, for sample preparation. Accuracy and precision were evaluated by conducting six repetitions per day and three repetitions on 3 days. As a result, the intra-day accuracy was 96.0-100.4% for candy, 102.8-103.1% for ice cream, and 99.5-101.1% for cocoa product. Furthermore, the inter-day accuracy was 100.0-102.2% for candy, 101.0-101.4% for ice cream, and 100.6-101.0% for cocoa product. The intra-day RSD% was 0.5-3.3% for candy, 1.4-1.8% for ice cream, and 1.1-1.5% for cocoa product, and the inter-day RSD% was 0.9-3.8% for candy, 1.0-1.9% for ice cream, and 1.7-2.4% for cocoa product. This confirmed the reliability of the analytical method fulfilled the requirements of the AOAC guideline[24]. The results show that $HorRat_r$ values were 0.12-0.64 for intra-day and 0.25-0.74 for inter-day, which are suitable for repeatability (<1.3) in compliance with the HorRat values of the AOAC guideline[25].

Table 2. Calibration parameters of beet red (sum of betanine and isobetanine)

Parameters	Candy	Ice cream	Cocoa product
Regression coefficient (R^2) (Mean±SD)	0.9998±0.0002	1.0000±0.0001	1.0000±0.0001
Slope (Mean±SD)	4.1904±0.0757	4.1765±0.01623	4.3019±0.0414
Intercept (Mean±SD)	1.0493±0.4766	-0.5826±0.8246	-0.9301±0.0526
LOD (mg/kg)[1)]	0.16	0.32	0.29
LOQ (mg/kg)[2)]	0.48	0.97	0.84

[1)] Limit of detection, [2)] Limit of quantification.

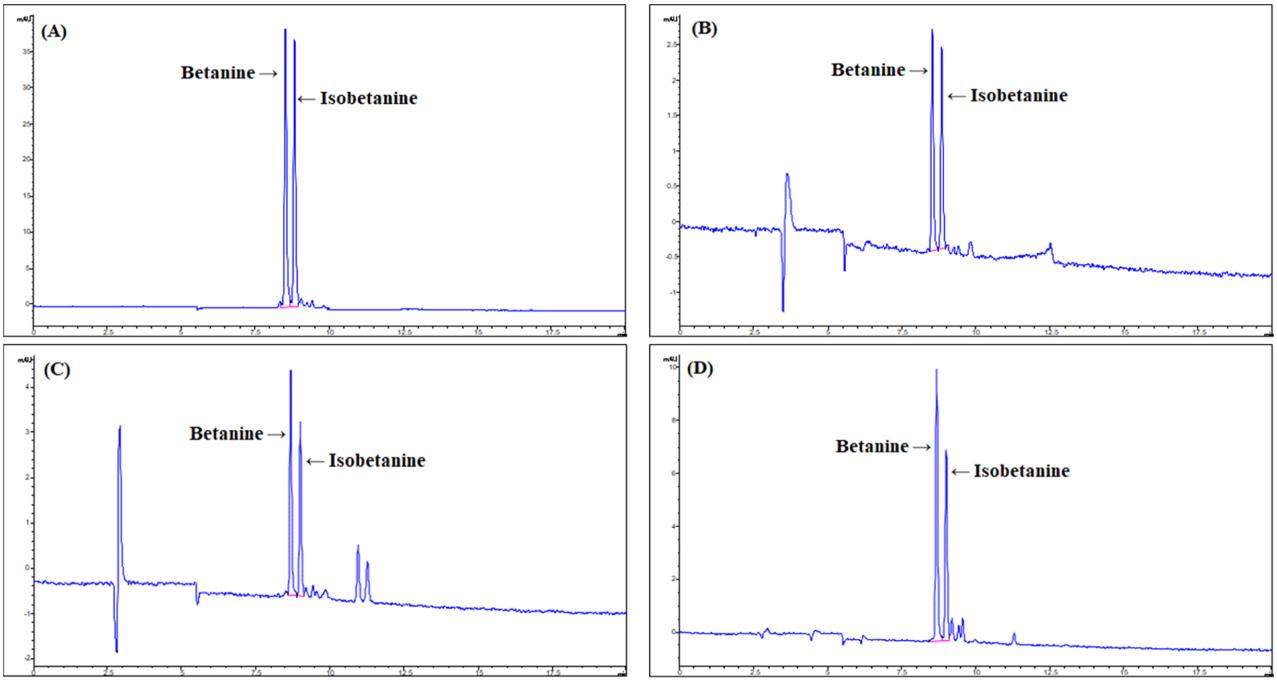

Fig. 1. HPLC chromatograms of a candy spiked with beet red standard (A), a candy sample (B), an ice cream sample (C), and a cocoa product sample (D).

Table 3. Accuracy, precision, and relative measurement uncertainty results of beet red (sum of betanine and isobetanine) using HPLC

Samples	Added standards (mg/kg)	Intra-day[1)]			Inter-day[2)]			Relative uncertainty (%)
		Accuracy (%)	Precision (%RSD)	$HorRat_r$[3)]	Accuracy (%)	Precision (%RSD)	$HorRat_r$[4)]	
Candy	18.61	96.0±3.1	3.3	0.64	100.0±3.8	3.8	0.74	12.43
	93.04	99.5±0.5	0.5	0.12	101.2±1.7	1.7	0.42	2.52
	186.08	100.4±1.1	1.1	0.30	102.2±0.9	0.9	0.25	2.09
Ice cream	18.61	103.1±1.9	1.8	0.35	101.0±1.9	1.9	0.37	3.36
	93.04	102.8±1.6	1.6	0.40	101.4±1.2	1.2	0.30	1.92
	186.08	103.0±1.5	1.4	0.39	101.0±1.1	1.0	0.28	1.80
Cocoa product	18.61	99.5±1.1	1.1	0.21	100.6±2.5	2.4	0.47	6.04
	93.04	101.1±1.5	1.5	0.37	100.7±1.8	1.8	0.45	2.12
	186.08	100.0±1.1	1.1	0.30	101.0±1.7	1.7	0.47	1.71

[1)] Analysis was conducted six times/day.
[2)] Analysis was conducted three times on three days.
[3)] $HorRat_r$ ratio for intra-day repeatability.
[4)] $HorRat_r$ ratio for inter-day repeatability.

Table 4. Concentration (mg/kg) and the range of beet red (sum of betanine and isobetanine) in various foods

Food category	Number of sample	Range (mg/kg)		Average (mg/kg)
		Min.	Max.	
Candy	5	8.4	138.6	43.5
Ice cream	3	34.3	49.2	42.8
Other processed product	9	26.1	2,422.4	594.9
Processed sugar product	7	36.7	3,823.4	701.2
Weight control formula	2	218.0	907.6	562.8

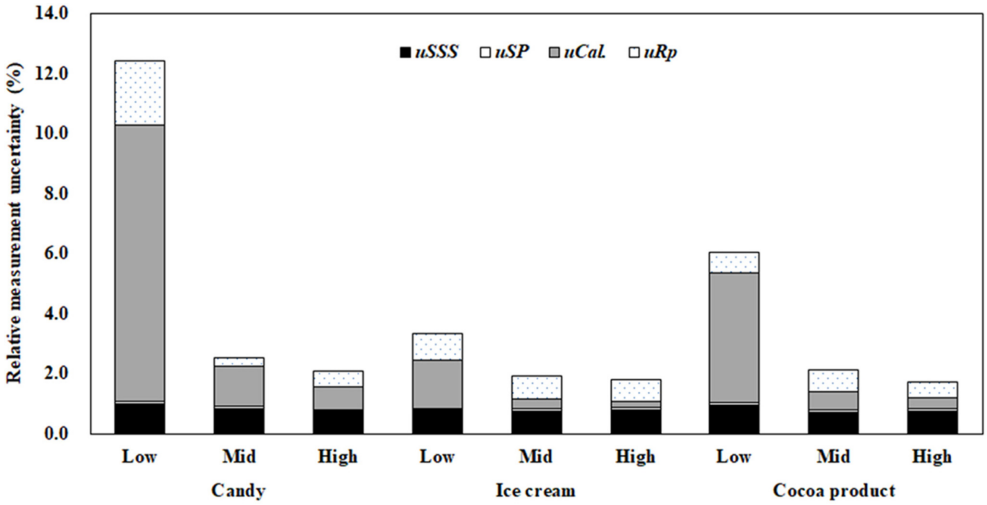

Fig. 2. Measurement uncertainty contribution to the expanded uncertainty in the determination of betanine and isobetanine in candy, ice cream, and cocoa product by the proposed HPLC-DAD method (low: 18.61 mg/kg, medium: 93.04 mg/kg, and high: 186.08 mg/kg).

Assessing measurement uncertainty

This study used HPLC-DAD to determine the measurement uncertainty for betanine and isobetanine in processed food products. This assessment was performed by considering measurement uncertainty factors related to the betanine and isobetanine analysis, such as sample weight, final volume, standard weight, purity, the standard solution, the calibration curve, and repeatability. The sum of the spiked betanine and isobetanine content in candy, ice cream, and cocoa product was calculated with 95% confidence interval, hence, compared to the analysis results, the relative *Uc* was found to be 1.71-12.43%. These results are considered acceptable as they satisfy the CODEX criteria (<16%)[19]. Furthermore, Table 3 show the influence of each uncertainty factor on the overall uncertainty, expressed as the percentage (%) of each uncertainty factor with respect to the sum of the relative standard uncertainties. Moreover, the effect of each uncertainty factor on the overall uncertainty was expressed as the percentage (%) of each uncertainty factor with respect to the sum of the relative standard uncertainties shown in Fig. 2. For candy, ice cream, and cocoa product spiked with 18.61, 93.04, and 186.08 mg/kg, uncertainty associated with the calibration curve (*uCal*) was the highest in association with the sources of measurement uncertainty. Thus, to obtain more accurate and precise result, more careful experiments are required in these steps to reduce measurement uncertainties of analysis.

Application

The developed analytical method was applied to quantify betanine and isobetanine as marker compounds for the detection of beet red in processed food products containing this color additive. As a result of the quantitative analysis of various types of candy (n = 5), ice cream (n = 3), other processed foods (n = 9), processed sugar products (n = 7), and weight control formula (n = 2), betanine and isobetanine were detected in all 26 processed food products (Table 4). Especially, processed sugar products showed the highest betanine content, at an average concentration of 701.2 mg/kg, followed by other processed products at 594.9 mg/kg and weight control formulas at 562.8 mg/kg. It was confirmed that the proposed analytical method was applicable to various matrices of food products containing beet red.

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국문요약

Red beet roots (*Beta vulgaris* L.)는 천연색소로 붉은 색계열의 betacyanins은 75-95%의 betanine와 이성질체인 isobetanine 15-45%으로 존재한다. 본 연구는 비트레드를 사용한 식품에 대해 HPLC-DAD를 이용하여 지표성분인 betanine 및 isobetanine에 대해 분석법을 확립하였으며, 유효성 검증을 위해 직선성, 검출한계, 정량한계, 정확성, 정밀성, 측정불확도를 측정하였다. 캔디류, 빙과류, 코코아 가공품의 matrix에 적용하여 matrix matched calibration법을 사용하였으며 R^2이 0.9998 이상으로 높은 직선성을 보였다. 검출한계와 정량한계는 각각 0.16-0.32 mg/L, 0.48-0.97 mg/L으로 확인되었다. 분석법의 정확성 및 정밀성을 검증하기 위해 intra-day 및 inter-day 반복 실험 결과, 회수율은 96.0-103.1 %, 100.0-102.2 %이였으며, RSD는 0.5-

3.3 %, 0.9-3.8 %로 산출되었다. 측정불확도는 매트릭스 및 측정 농도에 따라 1.71-12.43%로 평가되었다. 또한, 확립된 분석법의 적용성 검토를 위해서 비트레드 색소를 사용한 가공식품 26종을 분석한 결과, betanine과 isobetanine을 정량 할 수 있었다 (8.4-3,823.4 mg/kg).

Conflict of interests

The authors declare no potential conflict of interest.

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