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Establishment of an Analytical Method for Azorubine, an Undesignated Food Colorant in Korea

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ABSTRACT - Azorubine is a synthetic tar color containing azo-bond in the molecular structure. This food colorant has been allowed to be used for beverages, cheese and dried fruits in the European Union and for some food in Australia. Even though it is applicable as a food color in many countries, this compound has not been permitted in Korea so far as a food additive. Thus, this study was performed to establish an analysis method for azorubine in Korea by comparison of three HPLC analysis methods for azorubine and other azo-compounds which are officially used in the European Food Safety Authority (EFSA, EU), the Food Standard Agency (FSA, England) and the National Institute of Food and Drug Safety Evaluation (NIFDS, Korea). The analysis method of the FSA for azorubine showed the best linearity ($r^2 = 0.999$), limit of detection (LOD, 0.07 μg/mL), limit of quantification (LOQ, 0.20 μg/mL), precision (0~0.5%) and accuracy (98.6~100.7%) among tested HPLC methods using a C-18 column and diode array detector (DAD) with ammonium acetate solution and acetonitrile as an eluent solution. Finally selected method of FSA was further verified by inter-day and intra-day experiments with linearity, LOD, LOQ, precision and accuracy. Recovery test showed the recover ratios of 97~103%, 95~101%, and 93~102% in beverages, breads/snacks and other foods, respectively. Inter-laboratory test represented the absolute value of z-score of less than 2 which means satisfactory levels in this test. Selected method of FSA showed reliable analytical results in application test using food samples collected in commercial markets in Europe.

Key words : food colorant, azorubine, E122, analysis, method validation

Azorubine (disodium 4-hydroxy-2-[(E)-(4-sulfonato-1-naphthyl) diazenyl] naphthalene-1-sulfonate)[1)] is a red synthetic dye from the azo dye group[2)] with molecular formula of $C_{20}H_{12}N_2Na_2O_7S_2$. It has a molecular weight of 502.44 g/mol with CAS registry number of 3567-69-9 or E number of 122 in EU. It has a number of synonyms including carmoisine, Food Red 3, azorubine S, Brillantcarmoisin O, Acid Red 14, and C.I. 14720[3)]. This colorant (Fig. 1) is soluble in water[4)] and slightly soluble in ethanol solution, but insoluble in vegetable oil[5)]. Due to general stability of azo dyes azorubine is pH and heat stable, and it don't fade away when exposed to light and oxygen[6)]. Therefore, it has been used in heat treated food such as blancmange, marzipan, and etc[7)].

Azorubine is allowed in the EU according to the code of European Parliament and Council Directive[8)], as food colorant in several foodstuffs such as beverages, dried fruits, confectionery, processed cheese, and sauces. The maximum guide level in foodstuffs is 50~500 mg/kg for each food. In the twenty-seventh report[9)] of the Joint FAO/WHO Expert Committee on Food Additives, an acceptable daily intake (ADI) for azorubine was determined as 0~4 mg/kg of body weight. According to this report it is not mutagenic,

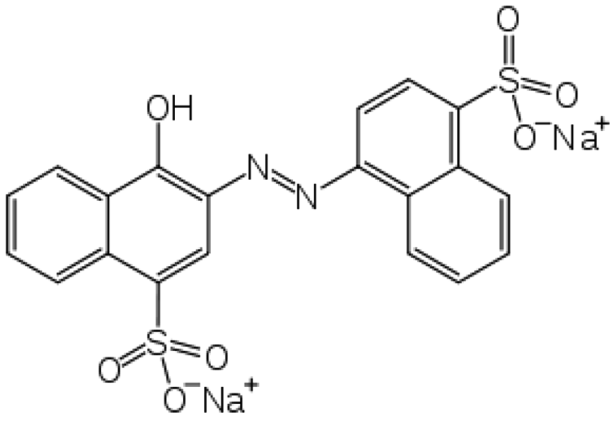

Fig. 1. Chemical structure of azorubine.

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carcinogenic or teratogenic with no serious histopathological effects.

So far, this colorant has not been allowed in any kinds of foods in Korea, Japan and Canada. In addition, it was delisted from additives for drugs and cosmetics in FDA[10,11] and azorubine has been never approved for foods in USA due to the possible adverse effects on the human health. Azorubine could alter biochemical markers related to renal and hepatic function[12] and this colorant induced oxidative stress in vital organs by formation of free radicals. Furthermore, increased hyperactivity aggregate (GHA) was reported for 3-years and 8-9 years old children by exposure to drinks with artificial food color and additives (AFCA) containing azorubine[13].

For the analysis of this colorant, high performance liquid chromatography (HPLC) system was applied in analysis methods of official institution such as the European Food Safety Authority (EFSA) and the Food Standard Agency (FSA)[14,15]. Similar HPLC system using C8 column was also reported by Bonan, S. et al.[16] to analyze azorubine in food-stuffs and beverages with mobile phase of acetonitrile and sodium acetate solution using diode array detector (DAD). Simultaneous HPLC analysis method of azorubine with eight water-soluble food colorants was also established in soft drinks using LC-DAD with C18 column and mobile phase of tetrabutylammonium (TBA) phosphate buffer[17].

Even though azorubine is allowed as food additive in EU, this colorant is not permitted for foods in many countries. Therefore, there is a high possibility in misuse of azorubine in imported or exported foods. Due to the increase of imported food the establishment of analysis method for azorubine is necessary in Korea. In this present study, various analysis methods and pretreatment conditions were compared and optimized in order to develop an official method for azorubine in Korea. In addition, the optimized HPLC method was validated by linearity, LOD, LOQ, accuracy, precision, recovery and inter-laboratory test. As a final step, this verified method was applied to analyze azorubine in a total of 109 samples including imported foods and overseas foods.

Materials and Methods

Food samples

A total of 109 food samples were collected from markets in South Korea (76 samples) and from the EU countries including Portugal (3 samples), Bulgaria (14 samples), Spain (5 samples) and Czech (11 samples). Samples were classified into three different groups (beverages, breads/snacks and other foods) and stored for the analysis.

Chemicals and reagents

Azorubine with 98% purity was purchased from Sigma-Aldrich (Yongin, Korea). Water, methanol, acetonitrile, ethanol and hexane (HPLC grade) were obtained from Burdick & Jackson (Ulsan, Korea). Ammonium acetate (HPLC grade, 99.0%, Netherlands), tetrabutyl ammonium bromide (ion pair chromatography grade, 99.0%, India), and formic acid (mass spectrometry grade, 98%, St. Louis, USA) were supplied by Sigma-Aldrich (Yongin, Korea). Sodium hydroxide solution (extra pure grade, 10%), ammonia water (extra pure grade, 25%) and acetic acid (30%) were products of Duksan Pure Chemical Co. Ltd (Ansan, Korea). Hydrochloric acid (1 mol/L) from Samchun Chemicals (Pyeongtaek, Korea) was applied in all experiment.

Equipment

The HPLC system of Agilent Technologies 1200 series (San Jose, USA) including binary pump, degasser, automatic liquid sampler, thermostated column compartment and 1260 diode array detector with wavelengths of 516 nm and 520 nm was used to establish azorubine analysis method. Two different size of C18 columns were applied ; Agilent Eclipse XDB-C18 column (250 × 4.6 mm, 4.6 μm) and Agilent Eclipse XDB-C18 column (150 × 4.6 mm, 4.6 μm) for the official method of the EFSA, the FSA, and NIFDS, respectively.

An LTQ Velos Pro series LC-(ESI)-MS/MS system equipped with electrospray ionization and an Accela HPLC system of Thermo Fisher Scientific (San Jose, USA) using a Thermo Scientific Hypersil GOLD C18 column (100 × 2.1 mm, 1.9 μm, USA) were used to verify the azorubine compound.

Analysis methods and pretreatment conditions

Two analytical methods for azorubine from the EFSA[14] and the FSA[15] were compared with the method of National Institute of Food and Drug Safety Evaluation (NIFDS, Korea)[18] for food colorant. The conditions of the all tested HPLC methods were described in Table 1.

According to EFSA Journal[14], azorubine can be analyzed in water-soluble foods such as fruit flavored drinks, alcoholic drinks, jams and sweets after water extraction or dilution by using HPLC with diode array detecter[19]. Pretreatment conditions from the EFSA Journal were slightly modified to increase recovery ratio of azorubine. Samples were homogenized and diluted with water. The beverage samples were degassed by strong stirring, if necessary. Diluted samples were sonicated for 15 min to extract the azorubine and filtered through filter paper (whatman qualitative filter paper, Grade 4, 150 mm). In the final step, the solutions were filtered again through 0.45 μm pore size syringe filter before injection into HPLC.

Table 1. The HPLC conditions of all tested methods

Items	EFSA (EU)			FSA (UK)			NIFDS (Korea)		
Column	Agilent Eclipse XDB-C18, Analytical., 4.6 × 250 mm, 4.6 μm			Agilent Eclipse XDB-C18, Analytical. 4.6 × 150 mm, 4.6 μm			Agilent Eclipse XDB-C18, Analytical. 4.6 × 150 mm, 4.6 μm		
Detector	Agilent 1200 Diode array detector, 516 nm			Agilent 1200 Diode array detector, 520 nm			Agilent 1200 Diode array detector, 516 nm		
Column Temp.	25°C			45°C			25°C		
Mobile phase (Gradient)	A: Ammonium acetate solution 1% (m/v), pH 7.5 (addition of a sodium hydroxide solution 10% (m/v)) B: A mixture of methanol : acetonitrile (80:20, v/v)			A: 0.02M Ammonium acetate B: Acetonitrile			A: 0.025M Ammonium acetate (containing 0.01M TBA-Br):ACN:MeOH = 65:25:10 B: 0.025M Ammonium acetate (containing 0.01M TBA-Br):ACN:MeOH = 40:50:10		
	Time (min)	Mobile phase		Time (min)	Mobile phase		Time (min)	Mobile phase	
		A%	B%		A%	B%		A%	B%
	0	100	0	0	95	5	0	100	0
	2.0	100	0	20.0	50	50	2.0	100	0
	22.0	47.5	52.5	24.0	0	100	24.0	60	40
	37.6	0	100	28.0	0	100	26.0	0	100
	40.0	0	100	30.0	95	5	35.0	0	100
	41.0	100	0	35.0	95	5	36.0	100	0
	43.0	100	0				40.0	100	0
Flow rate	1.5 mL/min			1.0 mL/min			1.0 mL/min		
Run time	43 min			35 min			40 min		

The FSA reported the official HPLC analysis method for azorubine in food including jellies, gums, mallows and etc[15]. The samples were homogenized after dilution with water and 4% ammonia solution, and they were centrifuged at 10,000 rpm for 10 min. The supernatant was evaporated by a rotary evaporator (Eyela, Japan). The dried residue was redissolved in the mobile phase solution (0.02 M ammonium acetate).

The analysis method from the NIFDS using HPLC was also applied for the analysis of azorubine in foods such as soft drink, candy, jelly and snack[18,20]. After pretreatment according to the method of NIFDS, the extract was further purified through the C18 SPE cartridges. In order to improve the recovery ratio of azorubine in foods, the new pretreatment method was developed and the conditions were described in Fig. 2.

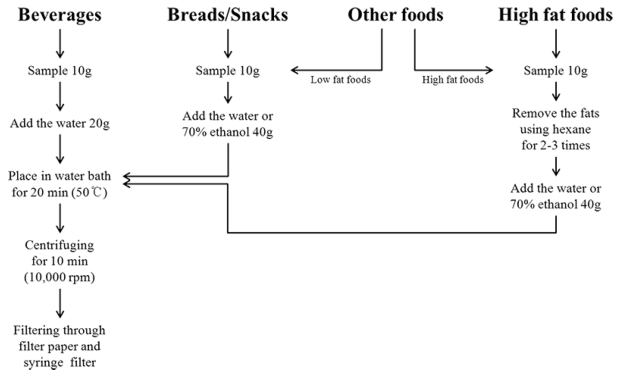

Fig. 2. Developed pretreatment conditions in foods.

Method validation

The final selected method for the azorubine analysis was verified by evaluation of linearity, LOD, LOQ, precision, accuracy and recovery according to the method of Sistla, R. et al.[21]. To estimate the linearity, 6 points of calibration ranges (5, 10, 20, 50, 100 and 300 μg/mL) were selected and the linearity was calculated by the correlation coefficient (r^2) of the calibration curve. LOD and LOQ were calculated based on the standard deviation of the response and the slope as suggested by ICH[22] using three low concentrations (5, 10 and 20 μg/mL) to make the calibration curve and the intermediate concentration (10 μg/mL) was measured seven times repetitively.

Inter-day and intra-day tests were conducted to measure accuracy and precision[22]. Inter-day test was performed by measurement at 6 different concentrations of azorubine (5, 10, 20, 50, 100 and 300 μg/mL) once in a day for three days. Intra-day test was performed by 5 times of repeated measurements at 30, 60, 120 and 240 μg of azorubine per mL in a day. The ICH recommends minimum three concentrations

and three replicates for these measurements.

Recovery was checked with three different food sample groups (beverages, breads/snacks and other foods) after addition of azorubine to the blank food samples to give final concentrations of 5, 10 and 50 μg/mL in three replicates.

The selected method were also verified in five different laboratories by inter-laboratory test, in which the laboratories were coded as A, B, C, D, and E. Calibration curves were prepared at 6 levels of azorubine (5, 10, 20, 50, 100, and 300 μg/mL) and the recoveries using this method were cross-checked in five laboratories with the beverages samples containing three levels of azorubine (5, 10 and 50 μg/mL). The results of inter-laboratory test were expressed as z-score[23].

Sample analysis

Azorubine levels were analyzed by HPLC in 109 collected food samples collected from foreign country. The measurement was repeated three times for each sample and the level of colorant was calculated and adjusted by recovery ratio.

Analysis by LC-(ESI)-MS/MS

Azorubine peak in HPLC chromatogram was further identified by determination of m/z value and mass fragment pattern using LC-(ESI)-MS/MS (an LTQ Velos Pro series LC-(ESI)-MS/MS system equipped with electrospray ionization). Primary separation was performed with a Thermo Scientific Hypersil GOLD C18 column (100 × 2.1 mm, 1.9 μm, USA) in Accela HPLC system. A mixture of water and acetonitrile containing 0.1% formic acid was used as mobile phase in this study. LC-(ESI)-MS/MS conditions for the analysis of azorubine were described in Table 2.

Table 2. The LC-(ESI)-MS/MS analysis conditions

Devices	Parameters	Conditions		
HPLC	Column	Thermo Scientific Hypersil GOLD C18, 2.1 × 100 mm, 1.9 μm		
	Mobile phase	A: 0.1% Formic acid in water B: 0.1% Formic acid in ACN		
		Time (min)	Mobile phase	
			A %	B %
		0	90	10
		8.0	20	80
		13.0	90	10
		15.0	90	10
	Flow rate	0.3 mL/min		
	DAD	λ. = 520 nm		
	Run time (min)	15 min		
MS/MS	Electron ionization mode	ESI (electro-spray ionization) Negative ion mode		
		Mode	ESI negative mode	
		Spray voltage	5.0 kV	
		Sheath gas	35	
		Aux gas	5	
		Capillary temp.	275°C	
		Collision energy	35 V	

Results and Discussion

HPLC and LC-(ESI)-MS/MS analysis

Three HPLC analytical methods for azorubine were compared in terms of retention time, LOD, LOQ, heigh/width ratio of peak, and running time as shown in Table 3. Chromatogram using analytical methods from the EFSA, the FSA and the NIFDS showed peaks of azorubine on 23, 10 and 24 min after injection, respectively (Fig. 3). Among them, the method of the FSA showed the symmetrical peak of azorubine with highest height/width ratio in shortest running time. LOD and LOQ of this method were 0.07 and 0.20 μg/mL, respectively.

Similar result was reported by Yoshioka, N. and Ichihashi, K.[24] which exhibited retention time of 10 min for azorubine in HPLC chromatogram using C18 column (50 mm × 4.6 mm, 1.8 μm) and mobile phase of ammonium acetate solution, methanol and acetonitrile with gradient elution at 50°C.

All peaks of azorubine in the selected HPLC analysis were further identified using mass analysis. The full mass scan spectrum in LC-(ESI)-MS/MS analysis showed m/z of 457

Table 3. The comparison of azorubine peaks in all tested HPLC methods

	EFSA (EU)	FSA (UK)	NIFDS (Korea)
Retention time (min)	23.3	10.5	24.7
LOD (μg/mL)	0.11	0.07	0.09
LOQ (μg/mL)	0.35	0.20	0.27
Height (mAU)	449.4	1012.4	342.5
Width (min)	0.1075	0.0729	0.2101
Height/Width ratio	69.7	231.5	27.2
Peak Area (mAU*s)	3138.8	4762.6	4700.9
Column	C18-25cm	C18-15cm	C18-15cm
Time (min)	43	35	40
Correlation of coefficient	0.99	0.99	0.99
Standard deviation (μg/mL)	1.35	1.20	1.56
RSD (%)	0.4	0.2	0.3

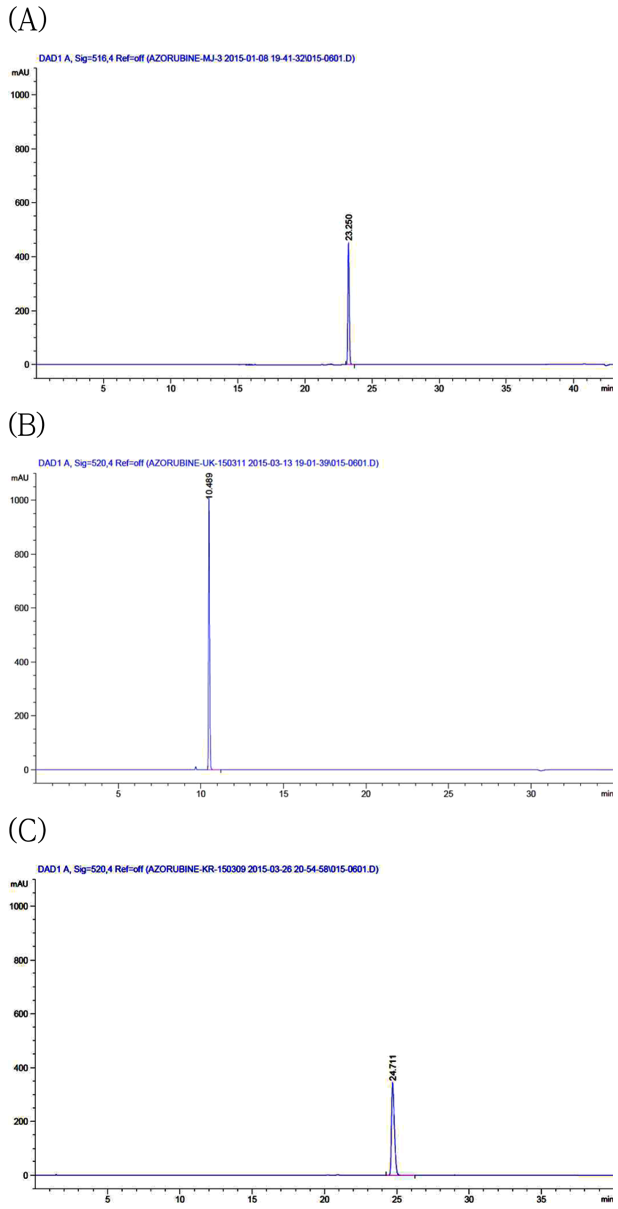

Fig. 3. Reproduced chromatograms using three HPLC methods from EFSA (A), FSA (B) and NIFDS (C).

for precursor ion of azorubine in negative mode which explains the presence of azorubine with sodium ion ($[M^{-}2Na]^{-}$). This mother ion exhibited three major daughter ions of 377, 297 and 391 m/z in MS/MS analysis as shown in Fig. 4. Similar results were reported by Feng, F. et al.[25] which presented the precursor ion of azorubine as 457 m/z and the product ion of 206.8 m/z and 377.2 m/z in same analysis.

Validation of HPLC analysis

In general, HPLC analysis method can be verified by linearity of calibration curve, LOD, LOQ, accuracy, precision, and recovery. Furthermore, inter-laboratory test gives

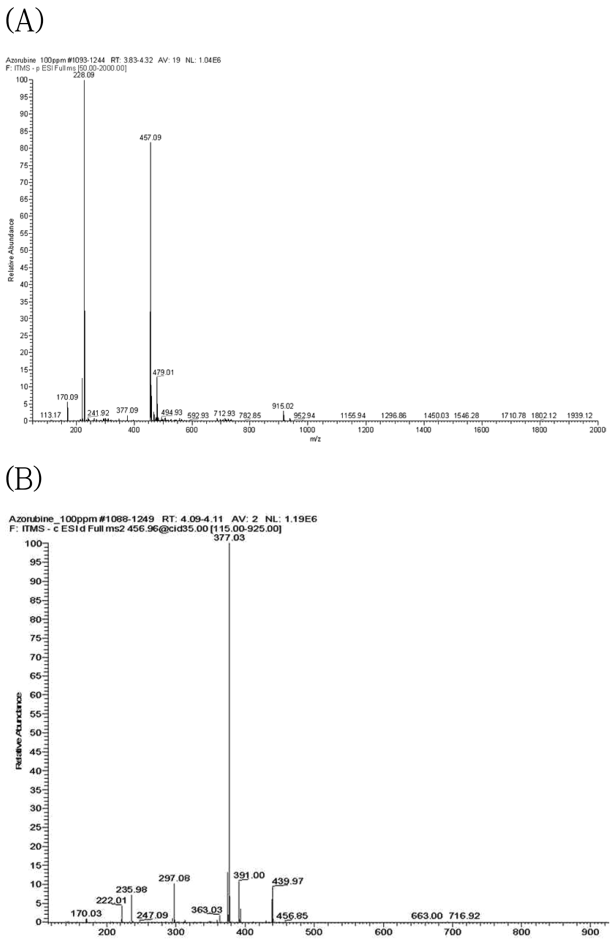

Fig. 4. Full mass scan in negative mode (A) of azorubine and MS/MS analysis (B) of mother peak (m/z = 377, 297, and 391) in MS analysis using LC-(ESI)-MS/MS.

reliability to the method. Linearity of calibration can be expressed by correlation coefficients. The accuracy and precision were estimated using intra-day and inter-day test to check systematic and unavoidable random errors[26].

As a first verification of tested HPLC method, the linearity was checked in calibration curve. All correlation coefficients of tested three HPLC methods were calculated as over 0.999. Similar data of linearity were reported in other study of Fortuna, T. et al.[27] and Bonan, S. et al.[16] exhibiting correlation coefficients of 0.9996 and 0.99, respectively.

All tested methods represented slightly different values in LODs and LOQs. As shown in Table 3, the analytical methods of the EFSA, the FSA and the NIFDS showed 0.11, 0.07 and 0.09 μg/mL of LODs and 0.35, 0.20 and 0.27 μg/mL of LOQs, respectively. (Table 3). Similar tendency of LOD and LOQ was reported by Fortuna, T. et al.[27] as 1.33 and 3.99 μg/mL. All of the checked LODs and LOQs in our experiment were lower than those of method performed by Fortuna, T. et al.[27].

As shown in Table 4, the accuracy was determined as

Table 4. Accuracy and precision data of selected HPLC method (FSA)

Inter-day test	Conc. (μg/mL, n = 3)					
	5	10	20	50	100	300
Mean	5.0	10.1	20.0	49.8	100.2	298.5
Precision (%)	0	0	0.1	0	0.3	0.5
Accuracy (%)	100.1	100.7	99.8	99.6	100.2	99.5
Intra-day test	Conc. (μg/mL, n = 5)					
	30		60	120	240	
Mean	29.6		59.9	119.5	237.2	
Precision (%)	0.1		0.2	0.3	0.3	
Accuracy (%)	98.6		99.9	99.6	98.8	

99.5~100.7% with the precision of 0~0.5%. Intra-day experiment and inter-day test also exhibited high accuracy of 98.6~99.9% with the precision of 0.1~0.3% (Table 4). The accuracy close to 100% means that the analysis had a high level of accuracy and the results are reliable. The precision level of less than 1% means that this analytical method is reliable. Vlase, L. et al.[28] performed similar intra-day and inter-day test at three concentrations (0.05, 0.40 and 6.07 μg/mL) of azorubine and this experiment showed the precision of 0.21~5.44% and 0.23~3.75% for intra-day test and inter day test, respectively.

The recoveries calculated from the methods of the EFSA, the FSA and the NIFDS were 13.4~93.0%, 78.5~86.4% and 0~42.6%, respectively (Table 5). The pretreatment method of the EFSA showed the good recoveries exhibiting more than 92% in beverages group, but poor recoveries of about 13.4~16.1% were observed in breads/snacks and in other food groups. The FSA method presented the highest recoveries among tested three methods, but it needed long time for concentration of sample solutions using rotary evaporator during pretreatment. In general, the recovery ratio considered good and acceptable values for method validation is between 80 and 120%[29]. Interestingly, recovery of between 91.6 and 103.4% could be achieved in this experiment as shown in Table 5, when the pretreatment condition of the NIFDS was modified. This modified method showed the recoveries of more than 90% in all sample groups at three concentrations (5, 10, and 50 μg/mL) expressing good value for recovery as explained by Brittain, H.[29]. Other researcher also studied recovery of azorubine in foods for method validation using HPLC[16] and it presented the mean of recovery of 78.2% in solid food matrices and 93.9% in beverage.

Inter-laboratory test was performed to calculate the recovery and z-score. All correlation coefficients from the results of study performed in five different laboratories were more than 0.999. The recoveries obtained were 94.7~112.0%, 93.7~100.7%, 94.4~103.1% for 5, 10 and 50 μg/mL, respectively (Table 6). In addition, z-scores were calculated as 0.26, −1.29 and −0.68 for 5, 10 and 50 μg/mL, respectively. The z-score is the main parameter for the standard deviation of the results and its absolute value less than 2 means that the performance of the laboratory is considered satisfactory[30]. Also, other study reported that the z-score of the absolute value of less than 2 means satisfactory results[23].

Sample analysis

Sample analysis was conducted using the selected HPLC method of FSA with the developed pretreatment process. Azorubine could be detected in 9 samples out of 109 collected samples. The results were shown in Table 7.

The all detected azorubine in samples using HPLC analysis were further identified LC-(ESI)-MS/MS chromatography. The precursor of 457 m/z ion was found in MS spectrum

Table 5. Recoveries of all tested pretreatment methods

	Sample groups	Spiked conc. (μg/mL)	Mean	SD	RSD (%)	Recovery (%)
EFSA	Beverages	100	92.99	0	0	93.0
	Breads/Snacks	100	13.40	0	0	13.4
	Other foods	100	16.05	0.07	0.44	16.1
FSA	Beverages	100	86.41	0.28	0.33	86.4
	Breads/Snacks	100	78.50	0	0	78.5
	Other foods	100	79.88	0.07	0.09	79.9
NIFDS	Beverages	100	42.64	0	0.07	42.6
	Breads/Snacks	100	-	-	-	0
	Other foods	100	1.00	0.07	0.44	1.0
Developed method	Beverages	100	99.25	0.49	0.50	99.3
	Breads/Snacks	100	91.57	0.07	0.08	91.6
	Other foods	100	103.43	0.07	0.07	103.4

Table 6. Inter-laboratory test of selected HPLC method (FSA)

	ug/mL	A	B	C	D	E	RSD (%)	z-score
Recovery (%)	5	95.8	98.5	94.7	109.4	112.0	0.08	0.26
	10	94.7	95.3	93.7	97.5	100.7	0.03	−1.29
	50	103.1	98.4	96.9	96.0	94.4	0.03	−0.68
$R^{2a)}$		0.999	0.999	0.999	0.999	0.999		

[a)]R^2 : correlation of coefficient

Table 7. The level of azorubine in positive food samples collected from foreign countries

Country	Sample type	Detected conc. (μg/mL)
Bulgaria	Beverages	8.36
Bulgaria	Beverages	11.87
Bulgaria	Beverages	41.68
Bulgaria	Beverages	91.51
Bulgaria	Beverages	10.37
Bulgaria	Beverages	162.02
Spain	Other foods	5.74
Spain	Other foods	6.79
Spain	Beverages	2.94

and the product of 377 m/z ions was detected, followed by 297 and 391 in MS/MS analysis as shown in MS/MS chromatogram of standard azorubine.

Conclusion

This study was performed to establish the analytical method for azorubine in Korea by comparing three HPLC analysis methods of the EFSA, the FSA and the NIFDS and by developing pretreatment process for Korean foods. The method of the FSA presented good results in LOD and, LOQ with highest accuracy and precision among tested methods. Newly developed pretreatment method in our experiment showed the high recovery ratio of more than 90% in all food sample groups. On the basis of these results, the HPLC method of the FSA was selected as the final method for azorubine analysis with newly developed pretreatment process exhibiting good recovery. When this final analysis method was further applied to detect azorubine in food samples produced from EU and other countries, azorubine could be analyzed successively in all positive food samples. So far, azorubine has not been permitted as a food colorant in Korea, even though it has approved in Europe. Therefore, there may be conflicts in food trades in the future, if foods containing azorubine, undesignated food colorant are imported into Korea without notice. The established analytical method in this study may play a good role in the future for the regulation of azorubine in foods in Korea.

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국문요약

국내 미지정 색소인 azorubine에 대한 우리나라의 공인 분석법을 설립하기 위해 제외국의 공인분석법들(유럽연합의 EFSA, 영국의 FSA, 우리나라의 식품의약품안전평가원)이 비교되었다. 재현된 HPLC 방법들 중 FSA의 분석법이 가장 우수한 분석 결과를 나타내어 azorubine분석법으로 최종 선정되었다. Azorubine 분석을 위한 위 세 기관의 전처리 방법은 모두 낮은 회수율을 나타내었다. 따라서 식품의약품안전평가원의 전처리법을 개선한 새로운 전처리 방법이 개발되었다. 최종 선정된 HPLC 분석법과 본 연구를 통해 개발된 새로운 식품 전처리법에 대한 분석법 밸리데이션을 실시하였다. 검증 실험에서 음료류에서 97~103%, 빵 및 과자류에서 95~101%, 기타 식품에서 93~102%의 회수율을 나타내었고, 실험실간 교차검증에서 −1.29~0.26의 z-score를 보여 신뢰할 수 있는 것으로 판단되었다. 이 결과로부터 본 연구에서 확립된 전처리법 및 분석법은 미지정 색소 azorubine이 사용된 식품검사에 활용될 수 있는 분석법으로 확인되었다.

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