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Multi-Residue Analysis of 18 Dye Residues in Animal Products by Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT - This study aimed to develop an analytical method for determination of 18 dyes in livestock and fishery products by liquid chromatograph-tandem mass spectrometry (LC-MS/MS). The developed method was validated for linearity, accuracy, limit of quantifications (LOQ) and recovery based on the CODEX guideline (CAC/GL-71). Target matrices (beef, pork, chicken, egg, milk, flatfish, eel, and shrimp) were extracted using acetonitrile (containing 1% of acetic acid) and then, purified with C_{18} and primary secondary amine (PSA). Calibration linearity was obtained (r^2>0.98) and LOQs were 0.002 mg/kg in animal products. The recoveries of dyes were ranged from 63 to 112% and relative standard deviations (RSDs, %) were less than 15%. The residues of 18 dyes were investigated in real samples (n=124) collected from retail markets in South Korea. As a result, a total of seven samples showed positive results for target analytes in fish samples. However, there was no violation according to the maximum residue limits set by the Korean Food Code. The proposed method will be used for routine analysis of dye residues in livestock and fishery products.

Key words : Dye, Residue, Live stocks, Fishery products, LC-MS/MS

Triphenylmethane dyes including brilliant green (BG), crystal violet (CV), malachite green (MG), pararosaniline base (PB) and Victoria Blue are a group of organic dyes widely used in industry for dyeing wool, silk, nylon, paper and leather[1-4]. Such dyes are banned from fishery products around the world due to mutations and toxic effects. Nevertheless, due to it is low cost and high efficacy, it is widely used in aquaculture for the prevention and treatment of fungal and parasitic infections of fish[5-7]. Littlefield et al.[8] and Culp et al.[9] reported carcinogenicity was observed in rats fed MG for 2 years and in animals exposed to CV for extended periods. In particular, MG and leuco malachitegreen (LMG) can cause carcinogenesis, mutations, chromosomal fractures, group formation and toxicity in different animal species. CV can induce fish genital abnormalities and found to be associated with an increased risk of human bladder cancer[10,11]. Moerover, acridine dyes acriflacine (ACR) and proflavine (PRO), have used to disinfectants during since 1918[12]. The rhodamine B (RB) is used as dye laser material and herbicide sprays in water environment pollution studies[13,14]. Nile blue A (NB) is known to localize selectively from animal tumors[15]. Methylene blue (MB) is widely used in microbiology and medicine[16].

Aquaculture industry has continued to grow impressively for fishery products consumption since the late 1980s. The average per capita consumption grew about 1.5 percent annually from 9.0 kg in 1961 to 20.2 kg in 2015[17]. However, non-compliant samples in fishery products are increasing due to the unintended and over use of chemicals. In 2005, malachite green was found in imported eel produced by China. According to the Ministry of Health, Labor and Welfare (MHLW) in Japan, methylene blue was detected in 0.013 mg/kg in Chinese eel in 2017. There are trade issues associated with certain dye chemicals particularly with MG and its related congeners in fishery products. Thus, MRLs (Maximum Residue Limit) have been established for veterinary drug residues and unintended used compounds in fishery products by Ministry of Food and Drug Safety (MFDS) in Korea[18,19]. The minimum required performance levels for MG, CV, MB and its metabolites were set at 0.002 mg/kg in 2019. Other dye substances shall not be detected in food in accordance with the related food safety laws and regulations.

MG and CV are rapidly metabolized in several fish species and they are easily reduced to their leuco-form,

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such LMG and leuco crystal violet (LCV) metabolites. The colorless LMB is light sensitive and rapidly redevelop the blue color of MB. Thus, the metabolite of MB, Azure A, B, and C (AZ-A, AZ-B, AZ-C) can be marker residues in fish tissues, a multi-residue detection method is required[20]. In previous studies, multi-residue analysis of dyes has been difficult to develop the simultaneous analytical methods due to the properties of leuco-type of MG and CV. In this study, we develop the stable analytical method without the oxidation reaction, and optimize multi-residue method using QuEChERS procedure of 18 dyes in animal products. The proposed method was applied in fish samples collected from retail markets to determine residue analysis of dyes.

Materials and Methods

Reagents and chemicals

The chemical characteristics were presented in Table 1. LMG, LCV and AZ-B standards were purchased from Dr.ehrenstofer (Augsburg, Germany), Wako Pure Chemical Industries Inc. (Osaka, Japan), USP (Rockville, MD, USA), respectively. The other standards were purchased from Sigma-Aldrich (Buchs, Switzerland). HPLC grade acetonitrile

Table 1. Molecular characteristics of 18 dye compounds

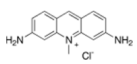
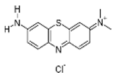
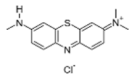
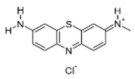
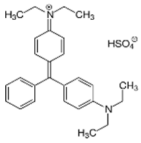
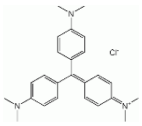
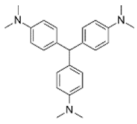
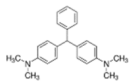
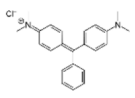
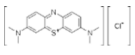
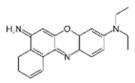
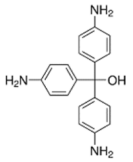
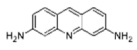
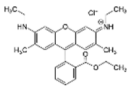
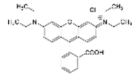
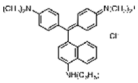
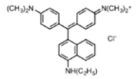
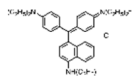
Compounds	IUPAC Name	CAS No.	Molecular formula	Molecular weight	Log P_{ow}	pka	Structure
Acriflavine (ACR)	3,6-diamino-10-methylacridin-10-ium chloride	65589-70-0	$C_{14}H_{14}ClN_3$	259.7	-	-	
Azure A (AZ-A)	N',N'-dimethylphenothiazin-5-ium-3,7-diamine chloride	531-53-3	$C_{14}H_{14}ClN_3S$	291.8	-	-	
Azure B (AZ-B)	dimethyl-[7-(methylamino)phenothiazin-3-ylidene]azanium chloride	531-55-5	$C_{15}H_{16}ClN_3S$	305.8	-	-	
Azure C (AZ-C)	7-methyliminophenothiazin-10-ium-3-amine chloride	531-57-7	$C_{13}H_{12}ClN_3S$	277.8	-	-	
Brilliant Green (BG)	[4-{[4-(diethylamino)phenyl]-phenylmethylidene}cyclohexa-2,5-dien-1-ylidene]-diethylazanium hydrogen sulfate	633-03-4	$C_{27}H_{34}N_2O_4S$	475.6	-	-	
Crystal Violet (CV)	Tris(4-(dimethylamino)phenyl)methylium chloride	548-62-9	$C_{25}H_{30}ClN_3$	408.0	3.18	9.40 (at 25°C)	
Leucocrystal Violet (LCV)	4-[bis[4-(dimethylamino)phenyl]methyl]-N,N-dimethylaniline	603-48-5	$C_{25}H_{31}N_3$	373.5	-	-	
Leucomalachite Green (LMG)	4-[[4-(dimethylamino)phenyl]-phenylmethyl]-N,N-dimethylaniline	129-73-7	$C_{23}H_{26}N$	330.5	-	5.46	
Malachite Green (MG)	4-{[4-(Dimethylamino)phenyl](phenyl)methylidene}-N,N-dimethylcyclohexa-2,5-dien-1-iminium chloride	569-64-2	$C_{23}H_{25}ClN_2$	364.9	0.62	10.3	
Methylene Blue (MB)	7-(dimethylamino)phenothiazin-3-ylidene]-dimethylazanium chloride	61-73-4	$C_{16}H_{18}ClN_3S$	319.9	0.75	3.14	
Nile Blue A (NB)	(5-aminobenzo[a]phenoxazin-9-ylidene)-diethylazanium sulfate	3625-57-8	$C_{20}H_{20}ClN_3O$	353.8	-	-	

Table 1. (Continued) Molecular characteristics of 18 dye compounds

Compounds	IUPAC Name	CAS No.	Molecular formula	Molecular weight	Log P_{ow}	pka	Structure
Pararosaniline Base (PB)	Tris(4-aminophenyl)methanol	467-62-9	$C_{19}H_{19}N_3O$	305.4	-	-	
Proflavine (PRO)	Acridine-3,6-diamine	92-62-6	$C_{13}H_{11}N_3$	209.3	1.83	8.06 (at 20°C)	
Rhodamine 6G (R6G)	Ethyl 2-[3-(ethylamino)-6-ethylimino-2,7-dimethylxanthen-9-yl]benzoate	989-38-8	$C_{28}H_{31}N_2O_3Cl$	479.0	-	-	
Rhodamine B (RB)	[9-(2-carboxyphenyl)-6-diethylamino-3-xanthenylidene]-diethylammonium chloride	81-88-9	$C_{28}H_{31}ClN_2O_3$	479.0	1.95	-	
Victoria Blue B (VBB)	[4-[bis[4-(dimethylamino)phenyl]methylidene]naphthalen-1-ylidene]-phenylazanium chloride	2580-56-5	$C_{33}H_{32}ClN_3$	506.1	-	-	
Victoria Blue R (VBR)	[4-[Bis[4-(dimethylamino)phenyl]methylidene]naphthalen-1-ylidene]-ethylazanium chloride	2185-86-6	$C_{29}H_{32}ClN$	458.0	-	-	
Victoria Pure Blue BO (VBO)	[4-[bis[4-(diethylamino)phenyl]methylidene]naphthalen-1-ylidene]-ethylazanium chloride	2390-60-5	$C_{33}H_{40}ClN_3$	514.1	-		

(ACN), methanol (MeOH) and dimethylsulfoside (DMSO) were purchased from Merck Inc. (Darmstadt, Germany). Sodium sulfate anhydrous (Na_2SO_4) and acetic acid were purchased from Sigma-Aldrich octadecylsilane (C_{18}) and PSA (Primary secondary amine) were obtained from Waters (Milford, MA, USA), Agilent Technologies (Santa Clara, CA, USA), respectively. In addition, formic acid (≥95%) and ammonium acetate of guaranteed reagent grade from Sigma Aldrich. Syringe filter was acquired 0.2 μm PTFE (polytetrafluoroethylene, Barcelona, Spain). Livestock and fishery products were purchased from local markets in Korea. After homogenizing in high speed food blender, the edible tissue of animal samples were stored low -20°C. Each animal samples were confirmed to be free of targeted analytes.

Preparation of stock and standard solutions

To prepare the samples for analysis, each individual standard (10 mg) was accurately weighed and placed in a 100 mg/L volume flask. Standard solutions of CV were prepared by dissolving the standards in MeOH/DMSO (95:5, v/v). Other standard stock solutions were prepared for each compound in MeOH. All stock solutions stored at -20°C in amber glass bottles to prevent photolysis. Working solutions were prepared right before use by ACN/water (1:1, v/v).

Sample preparation

A portion (2 g) of homogenized sample was weighed and placed into 50-mL centrifuge tube. Add 1 mL of 1% acetic acid in water to each sample and strongly mixed for 1 min. After, add 10 mL 1% acetic acid in ACN and shaken. Na_2SO_4 (2 g) was added and mixed with sample for 5 minutes. The tube with the sample was centrifuged at 4,500×g for 10 min (4°C). The supernatant was transferred to 50 mL falcon tubes containing C_{18} (100 mg) and PSA (100 mg). Shake over 5 min and centrifuge at 4,500 g, 4°C for 10 minutes. And then, 1 mL supernatant filtered by 0.2 μm PTFE and placed in a vial (Fig. 1).

LC-MS/MS analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed on a Shimadzu (Kyoto, Japan) LC-MS/MS (LCMS-8060) with Cadenza CD-C_{18} (2.0 mm I.D.×150 mm, 3.0 μm). The temperature column was maintained at 40°C and the flow rate was 0.3 mL/min. The injection volume was 2 μL, and the analysis was performed with gradient elution using (A) 10 mM ammonium acetate and 0.1% formic acid in ACN, (B) 0.1% formic acid in water as the mobile. Triple quadrupole tandem mass analysis was performed under an electrospray ionization mode (ESI) source in positive mode (ESI+). Data collection carried out

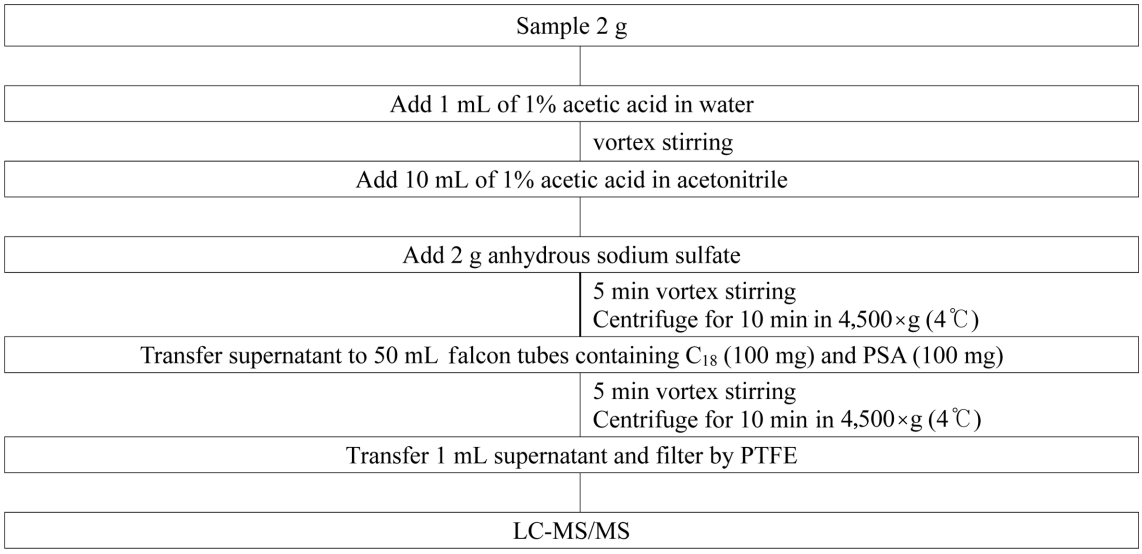


Fig. 1. Analytical procedure of 18 dyes in fishery product samples.

Table 2. Multiple reaction monitoring (MRM) parameters of dyes

Com-pounds	Retention time	Molecular weight	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
ACR	4.02	259.7	224.1	181.1	45
				182.1	35
				309.1	30
AZ- A	4.25	291.8	256.1	199.1	42
				214.1	34
				241.1	27
AZ- B	4.40	305.8	269.9	212.1	52
				228.1	34
				254.1	33
AZ- C	4.06	277.8	241.9	173.1	46
				200.1	34
				277.1	27
BG	6.31	475.6	385.1	397.1	53
				341.2	39
				355.2	36
CV	6.01	408.0	372.1	235.1	55
				340.2	55
				356.2	39
LCV	7.94	373.5	372.1	238.2	28
				239.2	35
				358.3	32
LMG	7.93	330.5	331.0	223.1	55
				239.2	31
				316.2	21
MG	5.51	364.9	329.1	165.2	55
				208.2	34
				313.2	36

Table 2. (Continued) Multiple reaction monitoring (MRM) parameters of dyes

Com-pounds	Retention time	Molecular weight	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
ME	4.56	319.9	284.0	240.1	33
				252.1	52
				268.1	34
NB	5.27	353.8	318.0	246.1	51
				260.1	44
				274.1	35
PB	4.41	305.4	288.1	151.1	49
				167.1	49
				195.1	30
PRO	4.02	209.3	210.1	166.1	32
				192.1	36
				193.1	28
R6G	5.85	479.0	433.1	341.2	49
				386.2	41
				415.2	34
RB	5.67	479.0	433.1	355.1	55
				385.2	47
				399.2	43
VBB	6.30	506.1	470.1	333.1	53
				349.2	38
				454.2	44
VBR	6.19	458.0	422.1	301.2	35
				393.2	38
				406.2	40
VBO	6.94	514.1	478.2	390.2	55
				405.2	50
				434.2	43

*The underlined bold letters expressed as quantification ion.

using the multiple reaction monitoring (MRM). The optimized parameters are listed in Table 2.

Method validation

The method was validated according to the procedures described in the Codex guideline (CAC/GL-71)[21]. The measured parameters were the linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and recovery. The intra-day and inter-lab precision are expressed as relative standard deviations (RSDs, %) and were evaluated by spiking blank samples (n=5). The inter-lab validation was performed at three target concentrations of 1×LOQ (0.002 mg/L), 2×LOQ (0.004 mg/L), and 10×LOQ (0.02 mg/L) in all animal product samples. The LOD was calculated at a signal to noise ratio (S/N) of 3, and the LOQ value was calculated using a S/N ratio of 10. The LOQ of the examined analytes were 0.002 mg/kg. Matrix-matched calibration standards were prepared by spiking blank matrix samples at 6 concentration levels (0.001, 0.002, 0.004, 0.01, 0.02, 0.03 mg/L). Recovery was assessed at spiking concentrations of 1×LOQ, 2×LOQ, and 10×LOQ.

Results and Discussion

Optimization of LC-MS/MS conditions

Several multi-residue assays were performed by ionization using LC-MS/MS because of high sensitivity, quantitation and qualitative analysis[7,20]. Moreover, the QuEChERS (quick, easy, cheap, effective, rugged and safe) method is very simple and efficient analysis for time reduction. The mobile phase was established (A: 0.1% formic acid in water/ 10 mM ammonium acetate and B: 0.1% formic acid in ACN). For mobile phases A, 10 mM ammonium acetate, 10 mM ammonium formate, and 0.1% formic acid in water/ 10 mM ammonium acetate were compared. As a result, 10 mM ammonium formate showed peak tailing in dyes analysis. Using 10 mM ammonium acetate and adding formic acid has increased the sensitivity and highest peak area[22]. To optimize mobile phases B, ACN and 0.1% formic acid in ACN was compared. The result showed that only ACN with formic acid has good peak shape. C_{18} column was used to separate target compounds in animal products. Mass spectrum was confirmed in full scan mode to generate precursor ions and product ions, and the product ions with the best sensitivity were set as quantitative ions. Of all product ions, only two product ions with high sensitivity were established by qualitative ions (Table 2).

Optimization of extraction and purification

In our previous method of Korean Food Code, leuco type compounds (LMG and LCV) need to convert parent compounds (MG and CV) through oxidation process by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). For this reason, both all parent compounds and its metabolites should be analyzed simultaneously. Due to oxidation process is performed in the dyes analysis the material is decomposed and thus it is difficult to analyze properly[23]. In addition, the MB metabolite, LMB, is very unstable to light and is free to oxidize to methylene blue, making it difficult to quantify and there is no standard[24,25]. Therefore, the method was developed by analyzing the metabolite and the raw material simultaneously except the DDQ oxidation process, which is a general dyes analysis method. Xu et al.[26] reported, MB defined AZ-A and C as analytical, including the main metabolite AZ-B. First, the shrimp tissue was not well dispersed in the organic solvent, it was occurred a large deviation in the recovery rate. To improve the protein tissues were dispersed and mixed strongly after adding 1% acetic acid in water before extraction to increase the extraction efficiency. 1% acetic acid in ACN was used as the extraction solvent, and 1% acetic acid was much better than 0.5% acetic acid for extracting in recovery[27]. In addition, since the aqueous solution was used before extraction, sodium sulfate was used to remove the water in the sample. C_{18} and PSA were used to effectively remove interfering substance and fats. C_{18} is hydrocarbon chains to eliminate fats and nonpolar interfering substance. Further, PSA is a weak anion exchange sorbent that retains carboxylic acids and fatty acids in the sample because it contains two amino group[28]. Filtrations with PTFE, PVDF, and nylon cartridges were compared with standard solution for filtration loss. As a result, nearly 100% recovery was retained by PTFE in the process of filtration. However, PVDF and nylon filters were lower recovery than PTFE filter. In this study, the dye compounds of were stabilized metabolites without DDQ oxidation. In addition, the proposed analytical method showed high accuracy, acceptable sensitivity and recovery according to CODEX guideline.

Method validation

A specificity was showed through the analysis of blank sample (beef, pork, chicken, egg, milk, flatfish, eel, and shrimp) and not interfering substance was observed (n=5). The chromatograms of target compounds were shown in Fig. 2. LOQ was 0.002 mg/kg from S/N ratio of 10. LOQ including calibration standards were prepared by spiking blank matrix samples at 6 concentration levels. All compound calibration linearity was obtained (r^2>0.98) and spiking concentrations of 1×LOQ, 2×LOQ, and 10×LOQ, (n=5). As a result, precision (repeatability and reproducibility) at intra-day of dyes were ranged from 63 to 112% with RSD less than 15% (Table 3). In egg samples, the recoveries of LCV and LMG were ranged 62.7-69.6%.

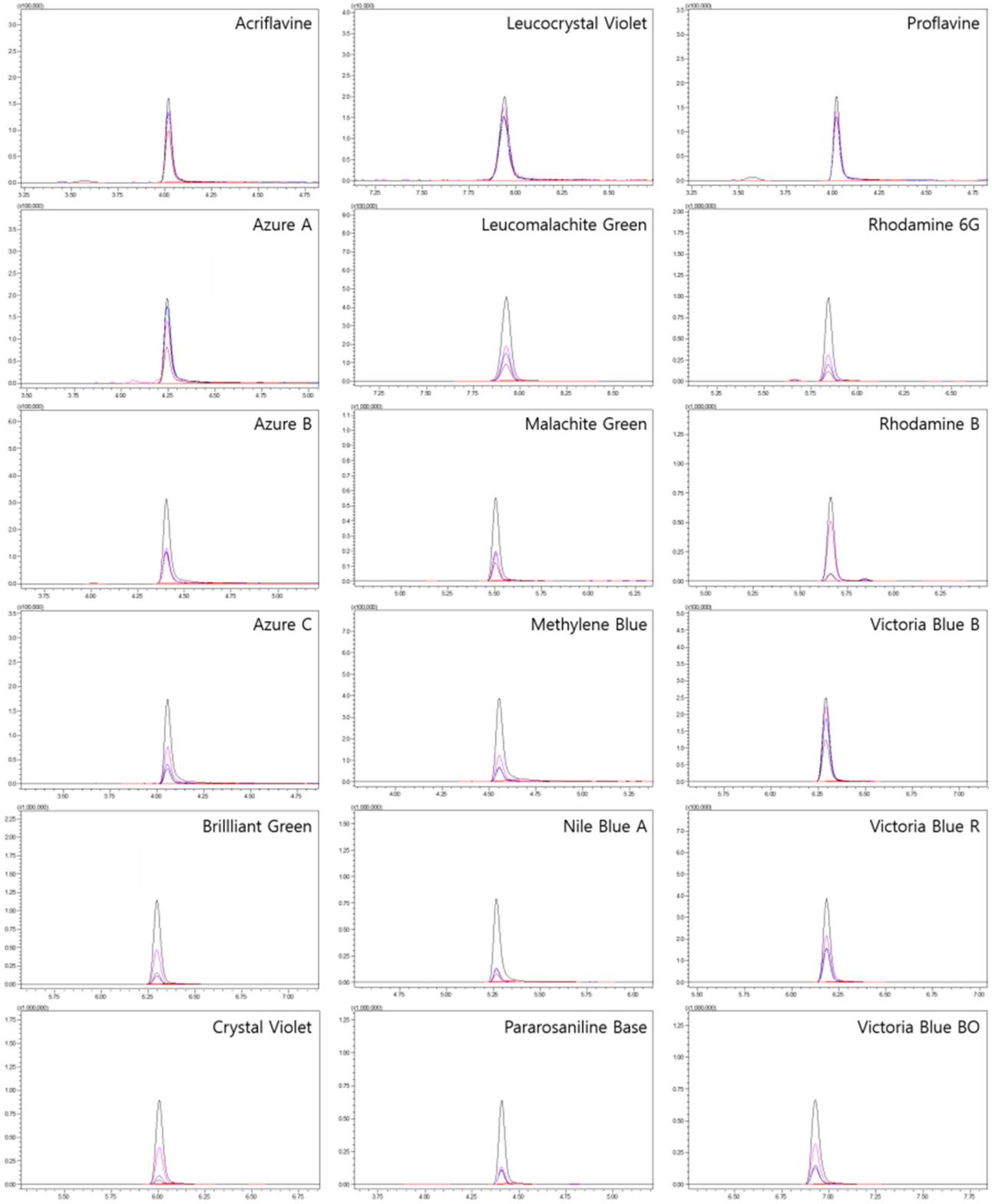


Fig. 2. Total ion chromatograms of 18 dyes by LC-MS/MS (0.002 mg/kg).

These results may be related to high protein and lipid content in egg matrix. The lipoproteins may have affected the sufficient extraction and forms emulsions with the extraction solvents[28]. The inter-lab test was conducted to evaluated the ruggedness of which results was ranged from 70 to 120% with RSD less than 25%. Compared to previous

Table 3. Recovery and CV (coefficient variation) at target testing levels for all matrices

Compounds	Target testing level (mg/kg)	Beef (n=5)		Pork (n=5)		Chicken (n=5)		Egg (n=5)		Milk (n=5)		Flatfish (n=5)		Eel (n=5)		Shrimp (n=5)	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
	0.002	88.6	4.64	92.5	3.52	93.4	4.12	90.8	6.05	105	0.94	96.9	2.84	99.6	2.32	106	1.96
ACR	0.004	98.0	1.43	105	4.10	103	2.69	102	2.13	101	2.41	98.5	2.18	102	1.69	104	1.13
	0.02	93.5	0.97	102	2.93	98.9	1.84	97.0	1.18	99.1	1.44	102	0.91	99.4	1.26	100	1.98
	0.002	90.1	3.51	106	3.37	88.5	5.44	102	2.11	98.7	3.29	99.2	4.27	97.6	8.04	106	2.54
AZ- A	0.004	93.6	1.91	105	4.62	98.8	4.03	96.0	3.59	95.2	3.98	98.6	2.48	97.4	1.11	102	3.20
	0.02	94.9	1.19	97.3	2.99	96.3	1.37	98.3	3.28	93.9	0.88	104	0.80	103	1.35	102	1.62
	0.002	106	3.70	105	3.80	105	1.88	101	2.99	104	3.94	99.3	3.89	105	4.33	108	4.40
AZ- B	0.004	98.0	2.62	98.6	5.81	102	4.62	96.2	1.61	97.1	3.54	94.6	1.05	99.8	4.69	102	2.72
	0.02	97.6	0.58	96.4	1.22	97.5	1.07	97.4	0.76	96.1	1.38	105	0.84	104	0.69	101	0.88
	0.002	89.3	3.58	97.9	4.04	101	3.33	97.2	5.13	95.9	3.47	103.0	2.45	106	1.85	107	2.88
AZ- C	0.004	97.1	4.71	102	2.43	102	3.76	102	2.78	98.8	3.31	98.9	1.95	104	3.39	98.3	1.61
	0.02	95.7	3.22	100	2.07	99.9	2.75	98.0	0.70	97.3	1.56	102	0.97	101	1.81	101	2.35
	0.002	96.5	3.50	88.0	3.12	93.3	2.24	97.5	2.88	99.9	2.48	107	1.17	101	2.54	112	6.33
BG	0.004	96.4	2.09	92.9	4.08	97.5	2.15	94.0	1.43	97.4	1.70	102	1.26	103	3.64	106	2.04
	0.02	95.7	2.24	101	1.72	97.4	1.85	91.6	3.16	100	1.50	99.2	0.81	96.8	1.43	93.0	1.27
	0.002	86.7	0.92	90.1	2.51	92.1	2.07	94.2	0.96	94.1	0.56	101	1.15	106	1.84	94.6	2.78
CV	0.004	93.5	1.97	93.6	5.15	97.8	2.16	96.0	1.57	95.2	2.02	96.4	0.97	98.6	1.16	98.0	1.23
	0.02	103	1.63	100	1.94	99.7	0.67	101	1.32	103	1.11	99.7	0.94	96.5	1.21	100	0.59
	0.002	97.0	1.61	80.4	3.77	96.3	1.46	62.7	11.8	105	2.65	104	4.84	100	2.45	108	5.81
LCV	0.004	92.5	2.06	87.4	7.32	94.4	1.04	63.8	13.9	94.9	1.62	105	4.66	90.7	7.74	109	2.34
	0.02	82.1	3.12	80.4	2.81	89.9	1.44	63.2	12.7	92.1	1.51	105	2.45	83.7	9.06	108	1.07
	0.002	103	2.20	84.4	3.73	96.5	1.37	69.6	8.42	106	2.15	104	4.98	99.8	1.53	107	5.38
LMG	0.004	92.9	2.44	86.3	6.48	93.6	1.35	67.0	12.2	95.1	1.14	101	3.97	89.3	6.38	107	2.26
	0.02	83.0	3.70	79.2	3.12	92.7	1.44	66.5	11.5	93.5	1.63	105	1.79	84.0	7.42	107	1.11
	0.002	102	4.80	112	3.60	99.9	1.68	101	4.60	106	1.37	97.3	1.09	96.5	3.63	101	11.2
MG	0.004	104	4.41	98.8	5.17	103	3.64	101	2.33	99.1	1.30	95.1	2.58	101	6.30	100	2.45
	0.02	102	2.99	99.5	2.93	101	4.80	96.6	3.57	99.1	1.50	108	1.69	103	2.82	106	3.52
	0.002	107	4.18	110	4.17	104	2.74	108	4.12	98.3	4.07	103	4.48	101	1.57	102	5.31
ME	0.004	97.4	1.92	97.7	3.45	99.3	3.61	96.7	2.34	92.9	2.43	96.8	1.80	99.3	2.69	96.7	3.61
	0.02	95.6	1.10	96.6	1.17	96.1	0.44	95.3	1.14	96.1	1.74	101	1.07	102	1.35	101	0.81
	0.002	93.5	2.29	96.3	2.98	101	2.61	101	2.68	94.6	1.19	91.8	2.33	100	1.15	104	2.65
NB	0.004	93.1	0.77	96.4	3.13	99.7	1.07	97.5	0.69	94.5	1.71	93.9	0.90	98.1	0.64	101	1.45
	0.02	97.9	1.93	98.9	1.17	99.4	0.64	98.0	1.70	98.9	0.87	104	1.06	101	1.44	101	0.38
	0.002	105	2.16	102	2.44	106	3.42	102	1.28	95.4	1.42	97.5	1.90	95.3	1.09	97.1	3.53
PB	0.004	104	2.75	100	3.49	103	0.70	102	1.03	96.6	2.07	95.1	0.83	96.6	1.01	96.2	1.38
	0.02	100	1.35	98.7	1.46	97.5	1.62	100	1.31	100	1.28	101	1.10	102	1.28	103	1.20
	0.002	86.2	3.01	88.3	4.69	102	1.21	88.1	2.83	98.5	3.77	92.4	2.81	90.2	2.54	99.6	2.29
PRO	0.004	101	1.57	101	1.02	107	2.63	104	2.17	103	2.40	96.6	1.78	99.2	1.01	101	1.87
	0.02	99.5	2.41	98.8	1.31	96.8	2.71	99.7	1.02	98.3	2.43	99.1	1.69	102	1.50	102	2.02

Table 3. (Continued) Recovery and CV (coefficient variation) at target testing levels for all matrices

Compounds	Target testing level (mg/kg)	Beef (n=5)		Pork (n=5)		Chicken (n=5)		Egg (n=5)		Milk (n=5)		Flatfish (n=5)		Eel (n=5)		Shrimp (n=5)	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
R6G	0.002	103	1.87	94.3	2.10	101	0.76	99.2	1.40	105	0.94	104	2.45	100	0.92	106	1.62
	0.004	102	1.19	94.9	3.77	98.5	1.28	96.4	1.11	101	2.41	100	0.82	101	1.02	105	1.33
	0.02	101	2.12	97.5	2.56	95.7	1.22	97.6	0.71	99.1	1.44	92.7	0.58	93.6	1.13	93.4	0.72
RB	0.002	104	2.66	97.2	1.60	99.7	1.03	105	1.92	103	1.45	91.8	1.98	101	1.80	102	1.92
	0.004	102	1.45	95.3	4.24	99.5	1.22	98.8	1.53	98.2	1.53	92.7	2.47	97.3	1.04	99.4	1.53
	0.02	99.7	2.56	96.7	1.43	98.4	1.77	98.0	0.64	101	1.64	100	1.01	99.2	0.72	103	1.65
VBB	0.002	98.1	2.01	95.5	0.99	95.5	2.17	94.0	0.86	103	1.61	101	1.51	96.5	4.63	106	1.59
	0.004	95.1	1.83	95.5	4.76	98.2	2.00	93.9	2.99	97.3	1.52	97.2	1.17	97.9	1.65	102	1.55
	0.02	90.5	1.67	95.2	1.43	96.0	1.29	95.0	1.24	99.0	1.36	102	5.46	103	2.25	104	1.65
VBR	0.002	98.8	2.19	103	2.32	99.9	1.94	103	0.99	101	2.09	98.1	2.42	96.9	1.31	103	1.27
	0.004	95.8	2.23	97.3	3.81	96.9	2.00	96.2	0.85	96.5	1.76	96.1	1.73	95.4	1.66	100	1.45
	0.02	92.6	1.73	95.4	1.63	96.4	1.36	94.3	1.35	98.5	1.42	99.9	1.16	100	1.32	104	1.57
VBO	0.002	104	2.95	93.7	2.35	94.5	2.42	93.0	1.42	105	0.83	103	1.46	99.2	1.15	108	1.70
	0.004	100	1.30	94.4	5.89	97.6	1.30	91.2	1.44	100	1.98	98.6	1.30	97.4	4.13	103	0.84
	0.02	97.6	1.61	94.5	1.06	99.1	0.51	91.4	1.63	102	0.98	104	1.47	100	1.88	104	1.11

studies, the proposed method is simple and simultaneous analysis of dye substances in food matrices with fast analysis, repeatability high sensitivity[20,23,29]. Therefore, dyes multi-residue method is expected to be used for future monitoring studies.

Application and real sample monitoring

The real sample monitoring was conducted to identify the applicability of the multi-residue determination for dye residues in animal products. Based on the previous detection history, fishery product samples (flatfish, eel, shrimp) were collected from domestic market (n=124). As a result, a total of seven cases were detected in fishery products. CV (LCV), MG, R6G were detected each sample. VBO was detected in four samples. However, the detected concentrations were very low concentrations (0.0001-0.0014 mg/kg). There was no violation result exceeding the Korean MRL (0.002 mg/kg for dye residues). Therefore, monitoring results shows that domestic fishery products are safe level of residues. Further studies are needed to control unintended contamination for chemical residues in animal products.

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국문요약

본 연구는 불법적으로 수산물에 사용될 수 있는 염료 18 종에 대한 안전관리 강화를 위해 정량 및 정성 분석이 가능한 LC-MS/MS 를 적용하여 검증하기 위해 수행되었다 . 확립된 시험법은 CODEX CAC/GL-71 가이드라인에 따라 직선성 , 정밀성 , 정량한계 및 회수율 등을 통해 유효성을 확인하였다 . 대상시료에 1% 아세트산을 함유한 아세토니트릴로 추출 후 C_{18} 과 PSA 로 정제하였다 . 본 실험에서 정량한계는 0.002 mg/kg 수준으로 정량한계를 포함한 농도에 따라 검량선을 작성하였고 모두 0.98 이상의 직선성을 확인하였다 . 또한 정확성은 63%-112% 이고 , 정밀도는 15% 이하로 재현성이 우수하였다 . 국내 유통 중인 수산물 124 건을 수거하여 개발된 분석법의 적용성 검증과 안전성을 확인하고자 잔류실태조사를 실시 하였고 그 결과 7 건이 미량으로 검출 되었고 부적합은 없었다 . 확립된 시험법은 수산물 안전관리에 활용할 수 있을 것으로 사료되는 바이다 .

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