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Improved HPLC-UV method for determination of five synthetic dyes in *Typha orientalis*

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Abstract: Synthetic azo dyes are used extensively in herbal medicines to render the medicines more visually attractive to consumers. This study developed and validated a rapid high-performance liquid chromatography (HPLC) method to determine whether synthetic colorants such as Tartrazine, Auramine O, Metanil yellow, Sunset yellow, and Orange II are used extensively in *Typha orientalis*. To increase the recovery of the synthetic dyes, this method employed containing 50 mM ammonium acetate in 70 % methanol at first extraction and 100 mM HCl in 70 % methanol at second extraction. Five synthetic pigments in *Typha orientalis* were separated by gradient elution with a mobile phase consisting of acetonitrile and 50 mM ammonium acetate in distilled water at ultra-violet (UV) detection 428 nm or 500 nm. Additionally, this study established the liquid chromatography tandem mass spectrometry (LC-MS/MS) method to confirm positive samples suspected by HPLC results. The HPLC-UV method had good linearity, indicating $r^2 > 0.999$. The recoveries of the samples spiked with three different concentration ranged from 73.8~91.5 %, and relative standard deviation values indicated 0.2~5.2 %. The established LC-MS/MS could successfully identify the synthetic pigments in herbal medicine samples. The study demonstrates that *Typha orientalis* adulterated by yellowish synthetic dyes can be successfully distinguished when using the HPLC-UV method.

Key words: herbal medicines, synthetic dyes, *Typha orientalis,* HPLC, LC-MS/MS

1. Introduction

To maintain the natural color and to create a desirable colored appearance during storage and distribution, synthetic azo dyes are employed in various products including food, cosmetics, and drugs because of their relatively facile and cheap production.[1] Therefore, certain synthetic colorants are abused in herbal medicines to make the medicines more visually attractive to consumers. Rapid Alert System for Food and Feed portal (RASFF) reports that several risk information related to adulteration of synthetic colorants in herbal medicines have been reported as follows: turmeric powders were adulterated using

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Orange II (2019. 9, Belgium) or Sudan I and Sudan II (2017.5, Finland). Another case of Orange II being used extensively was when it was artificially added to safflowers (2019, 4, Germany and the United Kingdom). According to the survey of adulterated traditional Chinese medicines in China from 2003 to 2017, the adulteration of synthetic dyes in herbal medicines have gradually increased.[2] As majority of the herbal medicines consumed in Korea are mainly imported from Asian countries, hence, there is an urgent requirement to establish an analysis method that can distinguish whether these synthetic colorants are indeed illegally added in these medicines. Therefore, we conducted preliminary experiments using thin-layer chromatography to determine whether synthetic dyes in potential herbal medicines; *Typha orientalis* was found to be one of the herbal medicines that contained synthetic colorants. This was the reason why *Typha orientalis* was targeted in the development of the test method to detect illegal dyes.

The presence of illegal dyes in herbal medicines can result in public health issues because it is unsafe to use synthetic colorants. Tartrazine is reported to have serious toxicological risks because of its mutagenicity and carcinogenicity,[1] and is also known to cause adverse health effects such as hyperactivity in children, allergies, and asthma.[3] In addition, a joint Food and Agriculture Organization (FAO)/World Health Organization Expert Committee on Food Additives and the European Union (EU) Scientific Committee for Food have standardized the acceptable daily intake for Tartrazine to be 7.5 mg kg^{-1} of body weight.[3] The addition of Sunset yellow to foods such as fresh meat is prohibited since it can cause allergies, intolerances, and other symptoms such as itching, rhinitis, purple skin, migraines, and blurred vision in sensitive people.[4] In addition, the ingestion of large amounts of Sunset yellow will result in kidney and liver damage. Thus, in the EU, concentration limit of Sunset yellow as a food additive is < 50 ppm.[5] For Metanil yellow, the (FAO) suggests an acceptable daily intake of 0 ~ 0.3 mg kg^{-1} of body weight for this dye.[6] According to recent reports, the safe threshold level of Metanil yellow is 0.25 %.[7] Orange II, which is also known as 2-naphthol Orange II and Acid Orange 7, is an acidic orange dye. It is soluble in water, and very slightly soluble in ethanol.[8]

Orange II is reportedly poses a severe threat to human health due to the intermediates formed after cleavage of the azo bond.[9] Although Orange II is not a skin sensitizer and does not exhibit *in vivo* genotoxic potential, it is considered to lead to skin, mucous membrane, eye, and upper respiratory tract irritation.[10] Auramine O is a cationic dye that has been categorized as a carcinogenic compound (Group 2B) by the International Agency for Research on Cancer.[11] Auramine O is also reported to cause DNA damage in the liver, kidney, and bone marrow of mice.[12] Because of its toxicity, it is an unauthorized food additive in Japan, the EU, and the United States.[11] However, despite such restrictions, Auramine O is reportedly illegally and extensively used in food products, pharmaceuticals, and medicinal herbs to improve their appearance.[13]

Unlike many studies related to illegal dyes in food products have been reported,[14-16] few methods have been published regarding the determination of illegal dyes in herbal medicines. Recently, it is reported to quantify the amounts of dyes added artificially to safflowers using near-infrared spectroscopy.[17] Guo *et al*.[18] developed and demonstrated a hybrid quadrupole-Orbitrap high-resolution mass spectrometry (HR-MS) method to detect 21 synthetic dyes in herbal medicines. In general, LC-MS/MS analysis method is known as more sensitive and selective procedure than HPLC methods, however, analysis costs would be high if advanced techniques were employed for determination of synthetic dyes.

HPLC is commonly used for the analysis of dyes because of its sensitivity, precision, accuracy, time efficiency, low cost, and robust nature.[19] Although HPLC test methods often require long time-consuming detection processes (> 30 min for a single run) in addition to laborious and complex pretreatment methods (solid-phase extraction (SPE), rotary evaporation, etc.), chromatographic methods offer good selectivity and detection limits.[20] As such, the development of a simple and rapid sample extraction and preparation

process is essential, since the solvent and extraction conditions can significantly affect analyte recovery rates. Although SPE is a commonly used sample pretreatment method,[21] it is a laborious and time-consuming process, and requires the use of special sorbents.[22] Considering the economic and convenience aspects, the HPLC analysis is considered as a more reasonable procedure to quantify the pigments adulterated in herbal medicines, instead of LC-MS/MS analysis method because it is an expensive analysis method and requires advanced techniques.

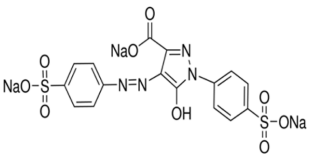
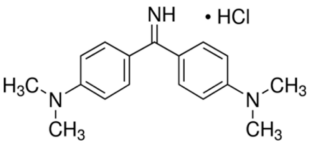
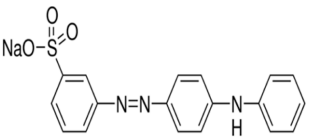
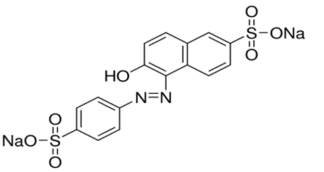
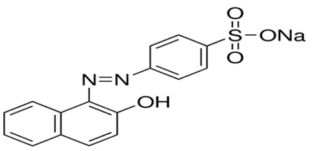
For developing a novel HPLC analysis method, we referred the HPLC-UV method coupled with solvent extraction using 70 % ethanol for determination of illegal dyes in herbal medicines.[23-24] Through preliminary test, the HPLC-UV method using 70 % ethanol was found to indicate very low recovery in Auramine O among five pigments. To overcome such a problem, this study was to develop an improved HPLC-UV method coupled with optimal solvent, which can successfully extract synthetic dyes from *Typha orientalis.* The improved HPLC-UV method was also validated to confirm its reliability to determine five yellowish colorants such as Tartrazine, Auramine O, Metanil yellow, Sunset yellow, and Orange II in *Typha orientalis.*

2. Experimental

2.1. Materials and reagents

The herbal drug materials (*Typha orientalis*) were purchased from an herbal medicinal store

Table 1. Permitted usages and physicochemical characteristics of the five synthetic colorants employed in the present study

Compound	MW (g mol^{-1})	Chemical structure	CAS No.	pKa	log P
Tartrazine	534.36		1934-21-0	9.40	-10.17
Auramine O	303.83		2456-27-2	9.80	3.33
Metanil yellow	375.38		587-98-4	-	5.90
Sunset yellow	452.37		2783-94-0	10.36	-1.18
Orange II	350.32		633-96-5	10.8	4.95

located in Daejeon, Korea, whereby most samples were imported from China. Prior to carrying out any further experiments, the identities of the *Typha orientalis* samples were confirmed through sensory tests performed by a specialist. Voucher specimens (19-279) were deposited at the National Institute of Food and Drug Safety Evaluation, in Osong. The surficial appearance of *Typha orentalis* was observed by using a scanning electron microscopy (SEM/SEC SNE-4500M, Korea); the analysis condition was set as follows: 5 mm (30 kV) of resolution, approximately 5 to 30 kV of acceleration voltage, electron gun of pre-centered tungsten filament cartridge, SE/BSE of detector and standard mode.

Tartrazine (≥99.0 %), Auramine O (≥85.0 %), Metanil yellow (≥98.0 %), Sunset yellow (≥95.0 %), and Orange II (≥99.0 %) (*Table* 1) used at this study were purchased from Sigma-Aldrich, Co. (MO, USA). HPLC grade acetonitrile, methanol, and ammonium acetate were purchased from Merck KGaA (Darmstadt, Germany). Distilled water was prepared using a Cascada^{TM} AN water purification system (Pall corporation, NY, USA) with a specific resistance of 18.0 MΩ.

2.2. Preparation of the mixed standard solution

Each synthetic dye was individually dissolved in 70 % or 100 % methanol at a concentration of approximately 2500 μg/mL. The color standard solutions were prepared to a concentration of 250 μg/mL by mixing the synthetic dye solutions and diluting with 70 % methanol. The concentration range of the prepared calibration standards were from 1 to 40 μg/mL. Both the stock and sample solutions were filtered through a 0.45 μm syringe filter (Millipore, Bedford, USA) prior to injection into the HPLC system.

2.3. Selection of optimal extraction condition

To select an optimal solvent to extract the five colorants from *Typha orientalis,* the several candidate extraction solvents were used. The candidate extraction solvents were as follows: i) 70 % ETOH, ii) 70 % MeOH, and iii) 50 mM ammonium acetate in 70 % MeOH coupled with 100 mM HCl in 70 % MeOH. That is, after spiking the mixed standard solution to blank sample solution, the synthetic dyes were extracted by using the candidate solvents following sonication for 30 min in water bath. After extraction, the sample solutions were subjected to centrifugation at 3220 × g for 10 min, after which time the first and second supernatants were combined and filtered prior to HPLC analysis. Also, for increasing the recovery of Auramine O, this study added HCl solution into 70 % MeOH extraction solution and compared the recoveries obtained from when the HCl concentration in 70 % MeOH was adjusted to be 10, 25, 50, or 100 mM to choose the optimal HCl concentration added into 70 % MeOH.

2.4. Sample preparation and HPLC conditions

The *Typha orientalis* sample (2.0 g) was added to a 50 mL centrifuge tube and a 50 mM ammonium acetate in 70 % MeOH (20 mL). After vortexing quickly, the sample tubes were sonicated for 30 min to extract the dyes from the samples, and then subjected to centrifugation at 3220 × g for 10 min. The first supernatant was collected and a 100 mM HCl in 70 % methanol solution (20 mL) was added to the precipitate. After centrifugation under the same condition, the first and second supernatants were combined and prior to injection into the HPLC system the sample solution was filtered through a 0.45 μm syringe filter (Millipore, Bedford, USA).

The HPLC instrument employed herein consisted of a Waters e2695 separation module and a photodiode-array (PDA) detector (Waters 2998) running Empower3 (Waters, Milford, MA, USA). A Shiseido capcell-pak C18 UG120 column (250 × 4.6 mm, 5 μm, Tokyo, Japan) was used for HPLC analysis. The detection wavelength for Tartrazine, Auramine O, and Metanil yellow was set to 428 nm, whereas that for Orange II and Sunset yellow was set to 500 nm. The mobile phase comprised acetonitrile (solvent A) and distilled water containing 50 mM ammonium acetate (solvent B). A linear gradient was employed as follows: 0 min, 5 % solvent A; 15 min, 45 % solvent A; 40~45 min, 45 % solvent A; 46 min, 5 % solvent A. The flow rate

was 1.0 mL/min, and the injection volume was 10 μL.

2.5. LC-MS/MS conditions

LC-MS/MS was used to confirm the identities of the detected compounds. More specifically, ultra-fast liquid chromatography tandem mass spectrometry (UFLC-MS/MS) analyses were performed using a NexeraX2 system equipped with a DGU-20A degasser, a CTO-20AC column oven, an LC-30AD pump, an SIL-20AC autosampler, and an LCMS-8060 triple quadruple LC-MS/MS spectrometer (Shimadzu Corporation, Tokyo, Japan). Data acquisition and processing were performed using LCMS LabSolutions software version 5.82 (Shimadzu). UFLC analysis was carried out using a UK-C18 column (C18, 100 mm × 2.0 mm i.d., 3 μm). For LC-MS/MS analysis, the HPLC sample was diluted using the initial mobile phase of LC-MS/MS, then filtered using a 0.2 μm polytetrafluoroethylene (PTFE) membrane prior to injection into the UFLC-MS/MS system. UFLC-MS/MS separated the analytes with mobile phases as 5 mM ammonium acetate in acetonitrile containing 0.1 % acetic acid (solvent A) and 5 mM ammonium acetate in water containing 0.1 % acetic acid (solvent B). A linear gradient was employed as follows: 0-1 min, 0 % solvent A; 1-3 min, 0-30 % solvent A; 3~ 7 min, 30~80 % solvent A; 7~9 min, 80 % solvent A; 9.1 min, 0 % solvent A. Chromatographic separation of the synthetic dyes was accomplished at a constant flow of 0.3 mL/min and using an injection volume of 5 μL. The mixed standard and sample solutions were appropriately diluted with the mobile phase for LC-MS/MS analysis.

3. Results and Discussion

3.1. Appearance and microscopic observation of Typha orientalis

The pollen grains of *Typha orientalis* are classified based on their morphological characteristics, including their dispersal unit, aperture, shape, polarity, pollen wall, and ornamentation.[25] *Typha orientalis* is a perennial aquatic plant that is commonly found in marshes, lake edges, drainage ditches, and other wetlands.[26] In general, when the *Typha orientalis* are placed on water, an authentic *Typha orientalis* should float (*Fig.* 1(A)), whereas the counterfeits that foreign substances might have adulterated does not float (*Fig.* 1(B)). This study used the authentic *Typha orientalis,* which was a light, yellowish brown powder that behaved like a fluid and floated on water.

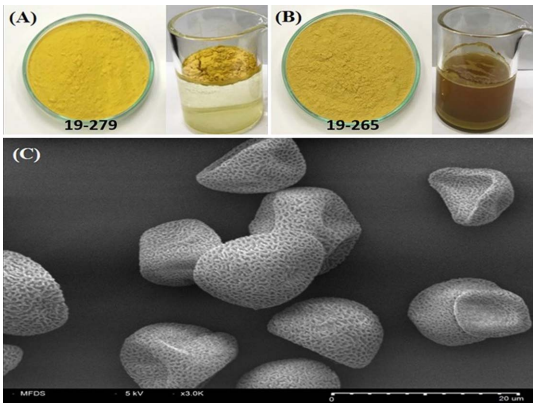


Fig. 1. Photographic and scanning electron microscopy images of *Typha orientalis.* A: A genuine product of *Typha orientalis*; B: A fraudulent product of *Typha orientalis*; C: *Typha orientalis* observed by scanning electron microscopy (pore formation).

In addition, the pollen of Typha is divided into two types, namely tetrads, in which the pollen grains form clusters of four, and monads, in which pollen grains are present individually.[26] The surface structure of the *Typha orientalis* sample was analyzed by SEM, which confirmed that the pollen surfaces of *T. orientalis* were consistent with their intrinsic characteristics as reported in a previous study.[27] SEM imaging also indicated the presence of apertures on the particle surfaces, as shown in *Fig.* 1(C). The presence of pores on the surface of *T. orientalis* seemed to hold certain synthetic dyes. Especially, Auramine O known as a cation dye was not completely extracted from *T. orientalis*, therefore, this study introduced the extraction solution contained acid solution to aid the extraction of Auramine O.

3.2. Optimization of the extraction and HPLC analysis

In China, an HPLC method has been used previously

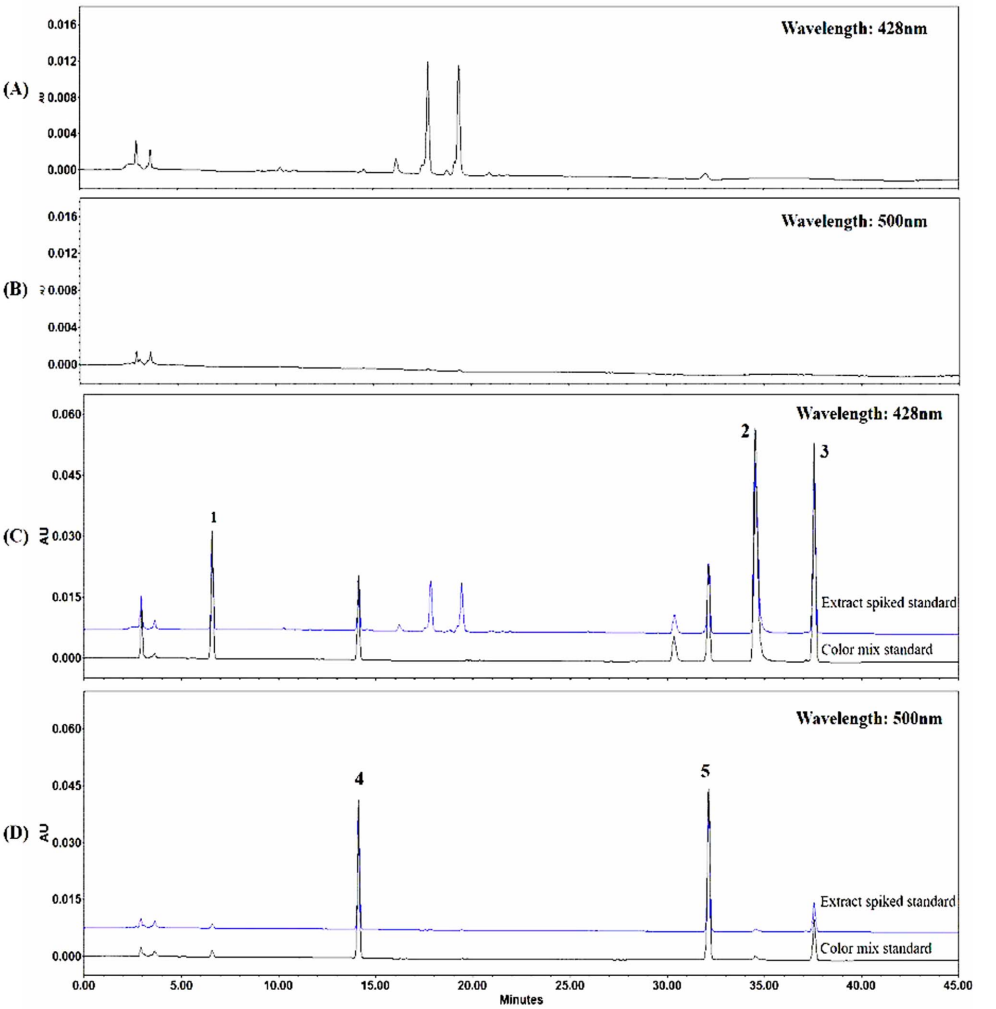


Fig. 2. HPLC Chromatograms of five synthetic dyes in standard solution and the *Typha orientalis* extract. A: Extract of the blank sample at 428 nm; B: Extract of the blank sample at 500 nm; C: The standard solution and the extract of the spiked sample at 428 nm; D: The standard solution and the extract of the spiked sample at 500 nm. 1: Tartrazine, 2: Auramine O, 3: Metanil yellow, 4: Sunset yellow, 5: Orange II.

to quantify dyes such as Tartrazine, Auramine O, and Metanil yellow in *Typha orientalis* became an important reference method in development of a HPLC test method within short time. The HPLC analysis method employs a 70 % ETOH solution to extract three synthetic colorants from the herbal medicines.[23-24] This study added two yellowish synthetic dyes and adjusted the column temperature to 30 ºC for increasing the separation capacity. Additionally, we improved the peak shapes in HPLC analysis by changing the concentration of ammonium acetate in mobile phase solvent B from 20 to 50 mM. As shown in *Fig.* 2, we confirmed that the chromatogram obtained using these HPLC conditions indicated a great sensitivity and there were no interference peaks in the chromatogram obtained by HPLC analysis. The HPLC-UV method was used to measure the content of synthetic dyes at two wavelengths, i.e., 428 nm for Tartrazine, Auramine O, and Metanil yellow, and 500 nm for Sunset yellow and Orange II, thereby resulting in enough sensitivity for all five colorants.

Also, for increasing the recovery of Auramine O,

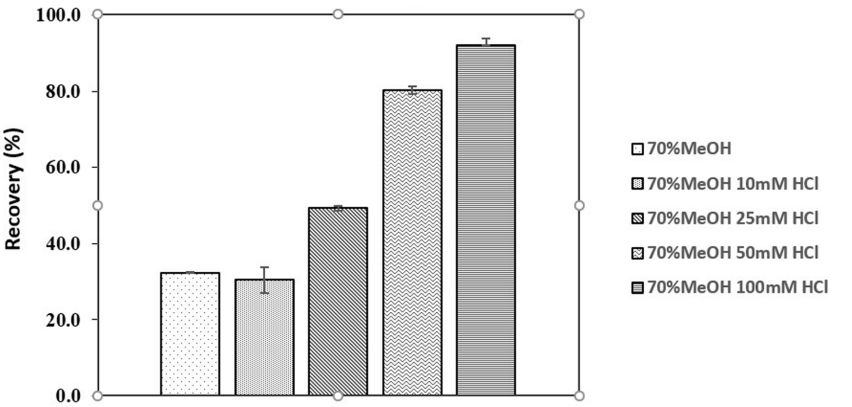


Fig. 3. Changes in the recovery rates of the Auramine O colorant upon increasing the acid concentration in the 70 % methanol extraction solvent.

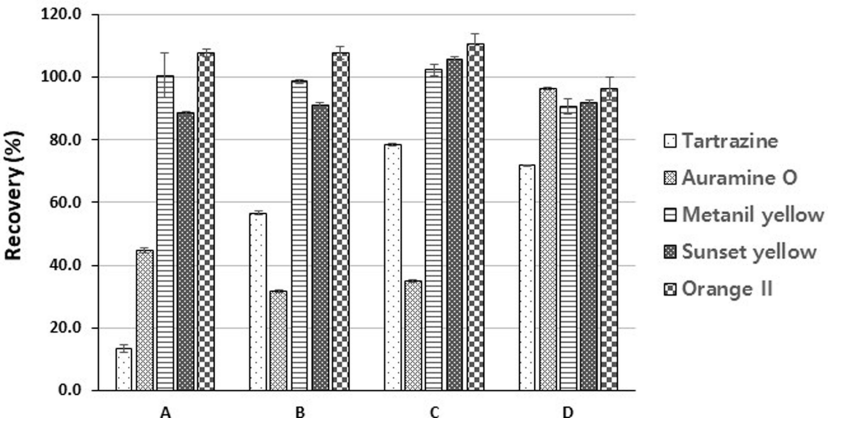


Fig. 4. Recovery rates of five colorants obtained after extraction using different solvents for the *Typha orientalis*-spiked colorants. A : extraction using only 70 % EtOH (x 2); B: extractions using only 70 % MeOH (x 2); C: extraction using 50 mM ammonium acetate in 70 % MeOH (x 2); D: extraction using 50 mM ammonium acetate in 70 % MeOH(1^{st}) + 100 mM HCl in 70 % MeOH (2^{nd})

this study tried to use 70 % MeOH solution which contained HCl as a second extraction solution because Auramine O is a cation dye. Comparing the recoveries obtained from 70 % MeOH solution when the HCl concentration in it was adjusted to be 10, 25, 50, or 100 mM, based on the results, the optimal HCl concentration was decided. As shown in *Fig.* 3, an HCl concentration of 100 mM in 70 % MeOH gave the highest recovery in Auramine O. The improved HPLC-UV method employed an initial extraction using 50 mM ammonium acetate in 70 % MeOH and a subsequent extraction using 100 mM HCl in 70 % MeOH. Through the preliminary tests, the recovery of Auramine O indicated approximately 21 % when using the reference HPLC method in *Typha orientalis.* To improve its recovery, this study tried to employ several candidate extraction solvents. As shown in *Fig.* 4, Auramine O showed very low recovery despite extraction was done twice using 70 % ETOH, 70 % MeOH, or 50 mM ammonium acetate in 70 % MeOH. Tartrazine also indicated less than 50 % recovery at 70 % ETOH and 70 % MeOH solution. However, the combination, which comprised 50 mM ammonium acetate in 70 % MeOH used at first extraction and 100 mM HCl in 70 % MeOH employed at second extraction, exhibited entirely satisfactory

recoveries in five synthetic dyes, indicating the range of 72.0~96.4 % recovery (*Fig.* 4).

3.3. Method validation

The improved HPLC method was validated in terms of its linearity, LOD, and inter- and intra-day precisions and accuracies. The regression equations, correlation coefficients, LOD, LOQ, and retention times of the analytes are listed in *Table* 2. As indicated, the correlation coefficients (r^2) showed great linearity (0.999) for all five synthetic colorants. In addition, LOD and LOQ values in the range of 1.2~5.8 and 4.0~17.6 μg/g were obtained, respectively (*Tabl*e 2). Even though Tartrazaine and Auramine O indicated to be comparatively higher LOQ, this study demonstrates that the adulteration of synthetic dyes in herbal medicines can be sufficiently distinguished under the LOQ concentration.

As shown on *Fig.* 2, the *Typha orientalis* blank sample itself had several peaks, however, there were no inference peaks against the analytes at the HPLC analysis environment; therefore, its selectivity was successfully achieved. In recovery evaluation, the intra-day and inter-day recoveries of four synthetic dyes except Tartrazine indicated satisfactory values (83.8~91.6 %). Tartrazine samples spiked with low concentration indicated recoveries ranged from 3.8~75.0 %, however, those spiked with middle and high concentration revealed comparatively satisfactory recoveries (*Table* 3). The intra-day precision (repeatability) of the proposed method was in the range of 0.1~5.2 % (*Table* 3), while the inter-day precision

Table 2. LOD, LOQ, and linearity values for the method developed in the present study

Colorant	Wave length (nm)	Retention time (min)	Range (μg/mL)	LOD (μg/g)	LOQ (μg/g)	Equation of calibration plot	Linearity (r^2)
Tartrazine	428	6.5	1–40	5.8	17.6	y = 20762x − 139	0.999
Auramine O	428	34.6	1–40	5.6	17.0	y = 76527x − 21531	0.999
Metanil yellow	428	37.7	1–40	1.2	4.0	y = 41910x − 6030	0.999
Sunset yellow	500	14.2	1–40	2.8	8.4	y = 28207x − 1183	0.999
Orange II	500	32.2	1–40	3.4	10.0	y = 32879x − 5155	0.999

Table 3. Intra- and inter-day recovery rates and precisions for the measurement of colorants (n = 3)

Colorant	Spiked conc. (μg/mL)*	Intra-day		Inter-day	
		Recovery %)	RSD (%)	Recovery %)	RSD (%)
Tartrazine	5	73.8 ± 1.4	1.9	75.0 ± 0.7	1.0
	10	80.6 ± 1.8	2.2	78.8 ± 1.5	1.9
	25	82.2 ± 4.3	5.2	83.9 ± 1.2	1.4
Auramine O	5	91.6 ± 1.5	1.6	91.1 ± 2.3	2.5
	10	90.6 ± 0.7	0.7	90.9 ± 0.1	0.1
	25	91.5 ± 0.1	0.1	91.2 ± 0.2	0.2
Metanil yellow	5	87.8 ± 1.2	1.4	88.4 ± 1.7	1.9
	10	86.6 ± 1.0	1.1	86.6 ± 0.2	0.3
	25	87.8 ± 0.2	0.2	87.4 ± 0.3	0.3
Sunset yellow	5	83.8 ± 1.1	1.4	84.8 ± 0.7	0.8
	10	85.2 ± 0.3	0.4	85.0 ± 0.8	0.9
	25	85.5 ± 0.5	0.6	85.4 ± 0.2	0.2
Orange II	5	89.8 ± 1.3	1.5	90.3 ± 1.4	1.5
	10	88.9 ± 1.0	1.1	89.4 ± 0.4	0.5
	25	90.2 ± 0.4	0.4	90.0 ± 0.1	0.1

*The concentration refers to the concentration of the final sample solution prepared for HPLC analysis.

(intermediate precision) was 0.1~2.5 %. These precision values were within the values suggested in the association of official analytical chemists (AOAC) validation guidelines.[28] As the proposed HPLC-UV method indicated satisfactory recovery and precision values, it was considered as a simple and economic procedure that can detect synthetic dyes in *Typha orientalis*.

3.4. Confirmation by LC-MS/MS analysis

To identify the five synthetic dyes, this study established the multiple-reaction monitoring (MRM) condition for LC-MS/MS analysis method (*Table* 4). Ionization was performed in both positive and negative electrospray-ionization (ESI) modes, and all compounds were unambiguously identified by their precursor peaks as reported in a previous study.[18,29-30] Auramine O was detected at 6.3 min using the positive ESI mode, giving precursor and product ions at *m/z* 268.0 and 147.1, respectively (*Fig.* 5). The other synthetic dyes were detected in negative ion mode: Tartrazine, 3.0 min, precursor ion *m/z* 467.1, and product ion *m/z* 198.0; Metanil yellow, 6.0 min, precursor ion *m/z* 352.2, and product ion *m/z* 156.0; Sunset yellow, 3.8 min, precursor ion *m/z* 203.4, and product ion *m/z* 171.0; and Orange II, 4.5 min, precursor ion *m/z* 327.0, and product ion *m/z* 170.9. The mixed standard solution of the five colorants in MeOH solution was analyzed using the established MRM conditions in LC-MS/MS analysis. This study confirmed that the five synthetic dyes were well separated and could be identified as shown on *Fig.* 5. The established LC-MS/MS method is likely to be applied for quantitative analysis of synthetic dyes in some samples suspected to be adulterated with be below LOQ concentration in HPLC analysis.

3.5. Cross-validation results

To confirm the validity of this HPLC method for the detection of the five synthetic dyes, inter-laboratory tests were performed on *Typha orientalis* samples fortified at the same concentrations at three different laboratories. We note that the r^2 values of the analytical calibration curves for five pigments were > 0.999 in all cases. The results obtained at the three laboratories are summarized in *Table* 5. For Tartrazine, recoveries were in the range of 76.5~83.0 % at Lab 2 and 75.1~80.60 % at Lab 3 when sample concentrations were 5, 10, and 25 μg/mL of the final sample solution. Although Metanil yellow showed low recovery as the range of 72.8~78.1 % at Lab 3, the other two laboratory indicated satisfactory recoveries. Therefore,

Table 4. MRM conditions of LC-MS/MS for identification of the synthetic dyes in herbal medicines

Colorants	Ion	Precursor ion	Product ion	Polarity	Collision Energy	References
Tartrazine	Target ion	467.1	198.0	-	18	Feng *et al*., 2011, Guo *et al*., 2019
	Ref. ion 1		171.9	-	21	
	Ref. ion 2		422.9	-	12	
Auramine O	Target ion	268.0	147.1	+	-29	Guo *et al*., 2019
	Ref. ion 1		131.0	+	-50	
	Ref. ion 2		252.1	+	-34	
Metanil yellow	Target ion	352.2	156.0	-	30	Liu *et al*., 2011
	Ref. ion 1		79.9	-	45	
	Ref. ion 2		287.9	-	20	
Sunset yellow	Target ion	203.4	171.0	-	15	Feng *et al*., 2011 Guo *et al*., 2019
	Ref. ion 1		181.1	-	21	
	Ref. ion 2		114.0	-	30	
Orange II	Target ion	327.0	170.9	-	23	Liu *et al*., 2011 Feng *et al*., 2011
	Ref. ion 1		156.0	-	31	
	Ref. ion 2		107.1	-	40	

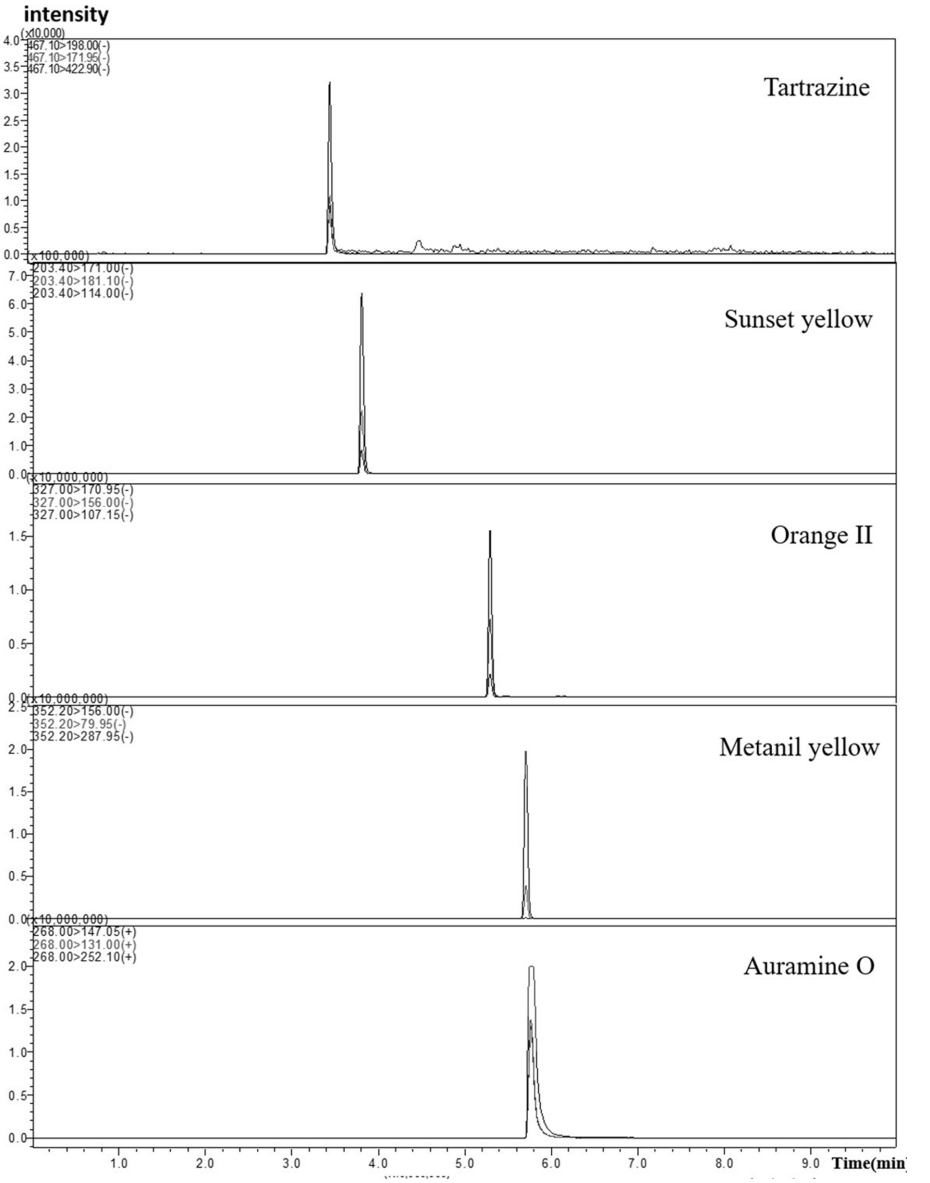


Fig. 5. LC-MS/MS chromatograms of the five yellowish synthetic dyes extracted by using the MRM condition established in the present study.

cross-validation demonstrates that the recoveries and precisions of the samples satisfied the values recommended in the AOAC guidelines.

4. Conclusions

This study developed and validated a simple and rapid HPLC method for determination of five yellow colorants in *Typha orientalis*. The analysis method used a simple two-step extraction method using 50 mM ammonium acetate in 70 % methanol and 100 mM HCl in 70% methanol to improve recoveries of the illegal dyes used in *Typha orientalis*. The validation results demonstrated that the proposed HPLC method exhibits satisfactory recoveries in the range of 73.8~91.5 %, with relative standard deviations < 20 %.

Table 5. Results of the cross-validation studies conducted at two other institutes (n = 3)

Colorant	Spiked conc. (μg/ mL)*	Lab1*		Lab2		Lab3	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Tartrazine	5	73.8 ± 1.4	1.9	83.0 ± 0.5	0.6	78.6 ± 2.1	2.7
	10	80.6 ± 1.8	2.2	80.7 ± 0.2	0.3	80.6 ± 1.4	1.7
	25	82.2 ± 4.3	5.2	76.5 ± 0.5	0.6	75.1 ± 0.6	0.9
Auramine O	5	91.6 ±1.5	1.6	90.2 ± 0.4	0.4	102.0 ± 2.1	2.1
	10	90.6 ± 0.7	0.7	86.8 ± 0.1	0.1	95.6 ± 2.0	2.1
	25	91.5 ± 0.1	0.1	87.2 ± 0.3	0.3	87.4 ± 2.4	2.7
Metanil yellow	5	87.8 ±1.2	1.4	87.1 ± 0.9	1	77.2 ± 1.5	1.9
	10	86.6 ± 1.0	1.1	83.2 ± 0.2	0.2	78.1 ± 1.7	2.2
	25	87.8 ± 0.2	0.2	82.3 ± 0.3	0.4	72.8 ± 0.5	0.6
Sunset yellow	5	83.8 ± 1.1	1.4	85.3 ± 0.7	0.8	102.5 ± 2.7	2.6
	10	85.2 ± 0.3	0.4	82.5 ± 0.4	0.5	98.2 ± 5.0	5.0
	25	85.5 ± 0.5	0.6	79.1 ± 0.4	0.5	94.6 ± 0.5	0.5
Orange II	5	89.8 ± 1.3	1.5	87.0 ± 0.6	0.7	98.1 ± 3.7	3.7
	10	88.9 ± 1.0	1.1	83.3 ± 0.3	0.4	93.2 ± 5.2	5.5
	25	90.2 ± 0.4	0.4	92.3 ± 0.3	0.4	91.1 ±0.7	0.8

Lab 1: National institute of Food and Drug Safety Lab 2: Chung-Ang University
Lab 3: Chungbuk National University

Considering these results, the proposed HPLC method could be an efficient and economic method to determine yellowish synthetic dyes in *Typha orientalis*.

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