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Validation and measurement uncertainty of HPLC method for simultaneous determination of 10 dyes in adulterated *Phellodendron*

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Abstract As global interest in herbal medicines has increased, the adulteration of herbal medicines has become a critical safety issue. Adulteration with dyes to improve the appearance of low-quality products is of particular concern. This study aimed to develop a high-performance liquid chromatography (HPLC) method to detect dyes added as adulterants to *Phellodendron*. Samples were analyzed on a C18 column using 50 mM ammonium acetate and acetonitrile as the mobile phase. All calibration curves showed good linearity (r^2 ≥0.9999) over the five-point concentration range (1-50 mg/kg). Limit of detection ranged from 0.04-0.35 mg/kg, and limit of quantification ranged from 0.11-1.07 mg/kg. The repeatability and reproducibility for these measurements were 94.2-103.3% and 96.6-103.8% for accuracy and 0.14-2.28 RSD (%) and 0.80-2.37 RSD (%) for precision. Moreover, the measurement uncertainty of the low, medium, and high concentrations for 10 dyes was considered. Thus, this HPLC method is suitable for detecting color adulteration of *Phellodendron*.

Keywords: Adulteration, Herbal medicine, Measurement uncertainty, Synthetic dye, Validation

Introduction

Herbal medicine has been used for its therapeutic effects for over 5,000 years in China. Due to the purported health benefits, few side effects, and relatively low cost, the interest in herbal medicines has increased worldwide (Lin et al., 2018). In 2020, the global herbal medicine market was valued at 138,350 million USD, and it is expected to reach 218,940 million USD by the end of 2026, growing at a compound average growth rate of 6.7% between 2021 and 2026 (MarketandResearch.biz, 2021). However, with this development, safety issues of herbal medicines continue to arise. The global spread of the herbal medicine industry is accelerating the destruction of species and habitats. Moreover, the distribution structure is so complex that there is a risk of chemical and physical exposure to various harmful substances (Bouzembrak et al., 2018).

One of the recent controversial issues in the distribution process of herbal medicines is the addition of various colors/dyes to enhance the appearance of herbal medicines (Guo et al., 2019). Tar dyes are synthesized from benzene or naphthalene contained in coal tar, which is a by-product of coal drying. These synthetic coloring agents enhance the appearance of products, enable a simple means of distinguishing products of similar appearance, sometimes make it easier for children to take medicines, and have useful functions, such as preserving color by preventing the decomposition of the contents. However, exposure to tar dyes may cause hepatotoxicity, asthma, hives, allergic reactions, and tumors.

Many dyes have been banned because of their adverse health effects. Adulterated herbal medicines are detected throughout the global market, posing a serious threat to consumers' safety and well-being (Ichim, 2019). For example, the herbal medicine *Phellodendron* (*Phellodendron amurense* Ruprecht) was recently found to be contaminated with auramine O dye (Xu et al., 2019), used to improve color retention so that the product could yield a higher retail price. *Phellodendron* is widely known as one of the 50 basic herbs of traditional herbal medicine and is commonly used around the world (Xu et al., 2018). *Phellodendron* releases toxins (Li et al., 2016), regulates immunity (Cai et al., 2018), and has effects, such as antibacterial (Liang and Wang, 2018; Lu et al., 2015), antitumor (Tsujii et al., 2020) and anti-inflammatory (Choi et al., 2014). However, auramine O is a carcinogenic dye that can cause liver and kidney toxicity. Therefore, these commonly used medicinal plant resources are at risk of adulteration, so careful conservation and resource management strategies are required.

In this study, we intend to establish a method to quickly detect color adulteration of Hwangbaek (*Phellodendron*), a commonly distributed herbal medicine. Recently, a HPLC-UV method to determine five dyes in *Typha orientalis* was developed and validated by Ministry Food and Drug Safety (MFDS) (Ko et al., 2021). Colors that may be incorporated illegally into *Phellodendron* without detection by the naked eye are yellows and reds, including tartrazine, amaranth, new coccine, sunset yellow, azorubine, erythrosin B, acid red 73, orange II, auramine O, and metanil yellow. Validation (linearity, accuracy, precision, limit of detection [LOD], and limit of

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quantification [LOQ]) for the simultaneous HPLC analysis of these 10 dyes in commercially available *Phellodendron* is conducted, and system suitability is evaluated. In addition, the applicability of the developed method is reviewed by monitoring whether the dyes are detected in commercial *Phellodendron*.

Consequently, the objectives of this study was to 1) apply the HPLC-UV method developed by MFDS for simultaneous detection of 10 synthetic dyes added as adulterants to *Phellodendron*, 2) validate the method, 3) estimate measurement uncertainty, and 4) apply the proposed method to commercial *Phellodendron*. By establishing a reliable method, it is intended to become a part of pre-emptive safety management for ensuring the quality of *Phellodendron*.

Materials and Methods

Materials

For quantification, standard compounds tartrazine (03322), amaranth (87612), new coccine (18137), sunset yellow (465224), azorubine (52245), erythrosin B (87613), acid red 73 (49823), orange II (69143), auramine O (861030), and metanil yellow (44426) were purchased from Sigma-Aldrich (St Louis, MO, USA). The chemical structures of the standard compounds used in the experiment are shown in Fig. 1. Water (4218-88) and acetonitrile (9017-88) was purchased from J.T. Baker Chemical Co. (Radnor, PA, USA) as an HPLC grade, respectively. Ammonium acetate (431311) was obtained from Sigma-Aldrich, and HCl was purchased from Samchun (H0424, Samchun, Seoul, Korea). *Phellodendron* was purchased from the Korean herbal medicine market in order to study the applicability of this method. Considering the size of the market and the domestic population, a total of 24 *Phellodendron* samples were collected from Gyeongdong oriental market (Seoul), Daegu oriental market (Gyeongsang-do), Jecheon oriental market (Chungcheong-do), and Iksan oriental market (Jeolla-do).

Sample preparation

Two grams of homogenized sample was placed in a centrifuge tube, followed by the addition of 20 mL of 70% methanol with 50 mM ammonium acetate, and the mixture was vortexed, sonicated for 20 min and then centrifuged at 3,200×g for 10 min. After centrifugation, the supernatant was transferred to another tube. Then, 20 mL of 70% methanol containing 100 mM HCl was added to the remaining precipitate. After that, sonication and centrifugation were performed repeatedly, following the method above. The supernatants were pooled and filtered through a 0.45-μm syringe filter (Sartorius Minisart®, Sartorius RC, Göttingen, Germany).

Analytical instruments

HPLC was carried out using an Agilent Technologies 1260 Infinity II HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a quaternary pump, autosampler, column, and diode array detector (DAD). The Osaka Soda C18 UG 120Å column (4.6 mm i.d.×250 mm, 5 μm) was applied for chromatographic separation and set to 30°C. A binary mobile phase of 50 mM ammonium acetate in water (solvent A) and acetonitrile (solvent B) with gradient elution was used. The gradient program was 0-40 min, 5-45% B; 40-45 min, 45% B; 45-46 min, 45-100% B; 46-50 min, 100% B; 50-51 min, 100-5% B. The flow rate was 1.0 mL/min. The DAD was set at a wavelength of 428 nm for tartrazine, auramine O, and metanil yellow and 500 nm for amaranth, new coccine, sunset yellow, azorubine, erythrosin B, acid red 73, and orange II. The UV-Visible spectrum was observed in the range of 200-600 nm.

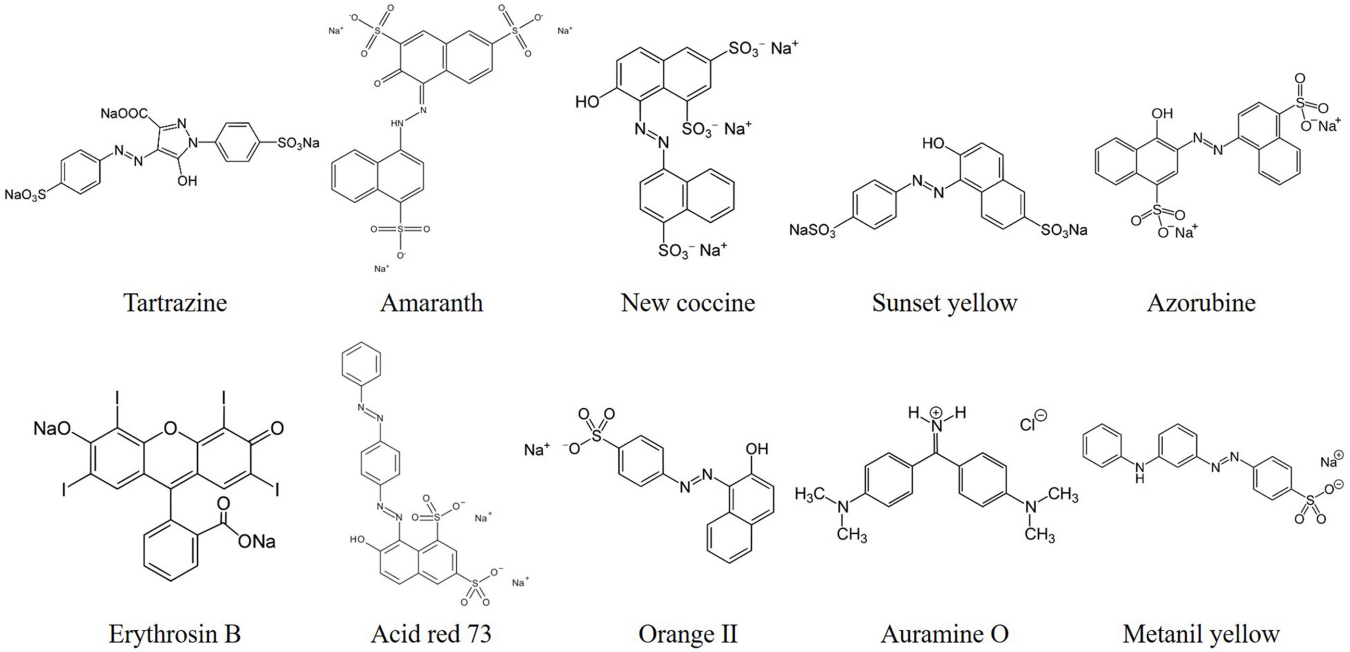

Fig. 1. Chemical structures of 10 dyes used in the present study.

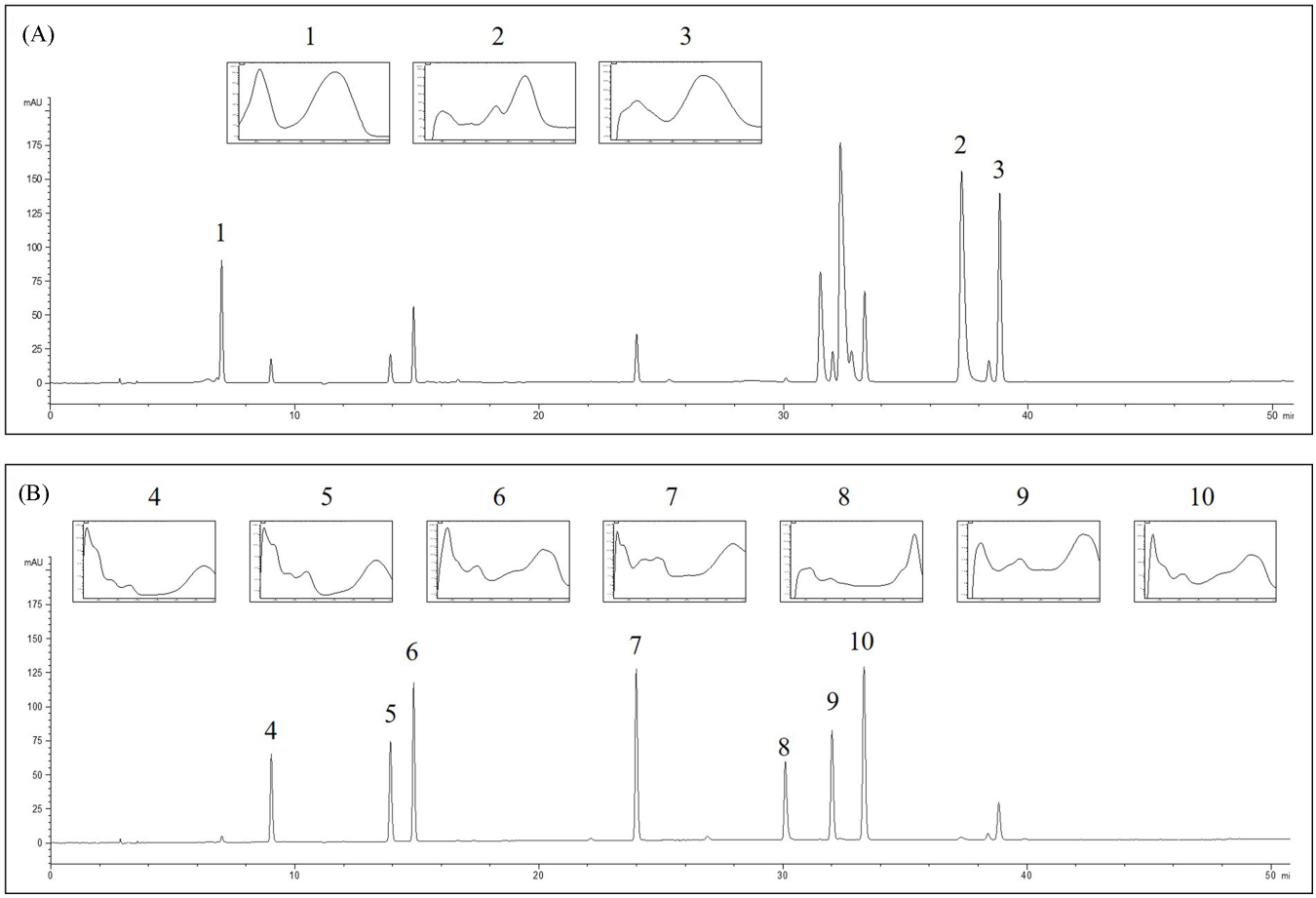

Fig. 2. HPLC chromatograms and spectra of 10 dyes in spiked *Phellodendron*. (A) chromatogram and UV-Visible spectrum of the standard solution 428 nm (1. Tartrazine, 2. Auramine O, and 3. Metanil yellow) and (B) chromatogram and UV-Visible spectrum of the standard solution 500 nm (4. Amaranth, 5. New coccine, 6. Sunset yellow, 7. Azorubine, 8. Erythrosin B, 9. Acid red 73, and 10. Orange II)

Column parameter

Four separate column parameters (retention factor, *k'*; separation factor, *a*; resolution factor, *Rs*; the number of theoretical plates, *N*) were assessed (Kim et al., 2013; Gliszczynska-Swiglo and Sikorska, 2004). The value of *k'* was calculated as $k'=(t_r-t_0)/t_0$, where t_0 is the retention time of the solvent, and t_r is the retention time of the standard. The optimum range of *k'* for chromatographic separation is $1 \le k' \le 10$. The α value is a measure of the time or distance between the maxima of two peaks, and the optimal range is $\alpha > 1$. R_s is a coefficient that determines how well two peaks are separated. It is assessed as $R_s=2\times(t_1-t_2)/(w_1+w_2)$, where t_1 and t_2 are the retention times of two adjacent peaks, and w_1 and w_2 are the widths of each peak. The optimal range is $R_s>1.5$. N is calculated as $N=5.54(t_r/w_{0.5})^2$, where $w_{0.5}$ is the width at half the peak height. Columns with a high N number are regarded as more efficient than columns with a lower N number. Moreover, a column with a high N number has a narrower peak shape at a given retention time than a column with a lower N number.

Validation

Validation was performed to ensure the reliability of the HPLC method for simultaneous determination of 10 dyes prohibited in *Phellodendron*. For validation, the specificity, linearity, LOD, LOQ, accuracy, and precision were evaluated with reference to the evaluation criteria of the Association of Official Analytical Chemists (AOAC) guideline (Latimer, 2016). The retention time of tartrazine tends to be delayed due to the difference in the 'degree of dissociation at the time of binding' between the column and the sample extraction solvent, so that it does not coincide with the peak of the ESTD standard solution, so a matrix-matched standards calibration method was applied. Specificity was observed for impurities or interference through HPLC analysis of the 50 mM ammonium acetate in 70% methanol as blank, standard-non-spiked *Phellodendron* sample, and standard-spiked *Phellodendron* sample (Fig. 2). The linearity, LOD, and LOQ were measured for seven replicates at five concentrations, and a matrix-matched standards calibration was used. The standard solution was added to *Phellodendron* that contained no added dyes to obtain final concentrations of the standard solution of 5, 10, and 25 mg/kg; the sample pretreatment was the same. It was measured for 3 days, and intra-day and inter-day accuracy and precision were measured using the average value of the recovery and the relative standard deviation result of each concentration. In addition, HorRat value was calculated in accordance with AOAC criterion (Latimer,

2016). HorRat(r), HorRat values for repeatability is calculated as RSDr/PRSD(R), HorRat(R), HorRat values for reproducibility is calculated as RSD_R/PRSD(R) ($PRSD(R)=2C^{-0.15}$, C=Connection values).

Measurement uncertainty assessment

Measurement uncertainty refers to "a parameter that characterizes the variance of values that are relevant to the measurement result and that can reasonably contribute to the measurement," as defined by the CODEX Alimentarius Commission (CODEX, 2008). Assessing the measurement uncertainty for this method was carried out by modifying the EURACHEM/CITAC guide and the Guide to the Expression of Uncertainty in Measurement (GUM) method (Ellison and Williams, 2012).

The sources of the measurement uncertainty of the analysis of the dyes were evaluated in the standard stock solutions (*uSSS*), sample preparation (*uSP*), repeatability for the determination of dyes in samples (*uRP*), and calibration curves (*uCal*). At a confidence level of 95%, the error of these components was calculated as an expanded uncertainty (*Uc*) using a coverage factor of 2 (*k*).

Results and Discussion

Column parameters

Ten dyes that can be illicitly incorporated into herbal medicines were successfully isolated within 60 min using the Osaka Soda C18 UG 120Å column. Column parameter values were calculated to obtain reliability for the degree of separation of the column (Table 1). The k' values were 1.01 to 9.98, and all of them were within the range of $1 \leq k' \leq 10$. All α-coefficients showed acceptable separations, from 1.05 to 1.90. In addition, *Rs* was 2.69 to 23.85, which is a suitable value. *N* values were also calculated (refer to the Methods section).

HPLC validation

The specificity was confirmed using the peak retention time and spectrum of 10 standard dyes (as solutions) added to *Phellodendron*. When comparing the *Phellodendron* sample without added dyes and the *Phellodendron* sample spiked with the standard solution of the dye, it was confirmed that there was no matrix effect of the interfering substance on the peak retention time (Fig. 2).

Table 1. Column performance results for 10 dyes in *Phellodendron*

Parameters	retention factor (k')	separation factor (A)	resolution factor (R_s)	efficiency (N)[1)]
Tartrazine	1.01	-	-	27750
Amaranth	1.55	1.53	7.68	46328
New coccine	2.93	1.90	16.64	96883
Sunset yellow	3.20	1.09	2.69	143826
Azorubine	5.79	1.81	23.85	281427
Erythrosin B	7.49	1.29	13.62	338496
Acid red 73	8.04	1.07	4.21	442383
Orange Ⅱ	8.41	1.05	3.07	404762
Auramine O	9.34	1.11	6.83	226933
Metanil yellow	9.98	1.07	4.84	510745

[1)]Number of theoretical plates.

Table 2. Calibration parameters of 10 dyes in *Phellodendron*

Parameters	range of calibration (mg/kg)	slope (±SD)	intercept (±SD)	regression coefficient (r^2)	LOD[1)] (mg/kg)	LOQ[2)] (mg/kg)
Tartrazine	1-50	11.3±4.0	2.9±0.8	0.9999±0.0001	0.22	0.67
Amaranth	1-50	8.0±2.8	-0.9±0.7	1.0000±0.0001	0.28	0.86
New coccine	1-50	10.1±3.6	-0.1±1.1	0.9999±0.0001	0.35	1.07
Sunset yellow	1-50	13.9±4.9	-0.4±1.4	1.0000±0.0000	0.34	1.02
Azorubine	1-50	17.4±6.2	-1.8±1.2	1.0000±0.0000	0.22	0.67
Erythrosin B	1-50	9.2±3.3	-1.3±0.5	1.0000±0.0000	0.19	0.57
Acid red 73	1-50	12.3±4.4	-1.2±1.0	1.0000±0.0000	0.28	0.85
Orange Ⅱ	1-50	20.2±7.2	-1.2±1.2	1.0000±0.0000	0.20	0.61
Auramine O	1-50	40.5±14.3	-5.1±0.4	1.0000±0.0000	0.04	0.11
Metanil yellow	1-50	23.3±8.2	-1.6±1.3	1.0000±0.0000	0.19	0.57

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

Table 3. Intra-day and inter-day accuracy and precision results of 10 dyes in *Phellodendron*

Fortified concentration	Compound	Intra day			Inter day		
		Accuracy[1)]	Precision[2)]	Hor[3)]	Accuracy[1)]	Precision[2)]	HoR[4)]
5 mg/kg	Tartrazine	94.2±1.9	2.04	0.16	96.6±1.4	1.50	0.12
	Amaranth	101.4±2.3	2.28	0.18	102.9±2.0	1.98	0.16
	New coccine	96.7±1.1	1.15	0.09	97.3±1.9	1.97	0.16
	Sunset yellow	99.2±0.4	0.38	0.03	100.7±1.8	1.80	0.14
	Azorubine	99.6±0.8	0.81	0.07	100.7±1.6	1.54	0.12
	Erythrosin B	98.4±2.1	2.13	0.17	99.2±2.4	2.37	0.19
	Acid red 73	101.6±0.6	0.59	0.05	102.5±1.3	1.26	0.10
	Orange II	103.3±0.8	0.74	0.06	103.6±1.2	1.18	0.09
	Auramine O	102.7±0.4	0.44	0.04	103.8±1.8	1.70	0.14
	Metanil yellow	102.7±0.1	0.14	0.01	103.8±1.5	1.49	0.12
10 mg/kg	Tartrazine	101.9±1.3	1.27	0.11	101.9±0.8	0.80	0.07
	Amaranth	101.7±0.4	0.44	0.04	101.6±0.9	0.92	0.08
	New coccine	96.8±0.7	0.67	0.06	96.8±1.5	1.51	0.13
	Sunset yellow	99.1±0.5	0.50	0.04	100.6±1.2	1.23	0.11
	Azorubine	99.4±0.2	0.21	0.02	100.2±1.0	1.00	0.09
	Erythrosin B	97.6±0.8	0.80	0.07	99.0±1.6	1.65	0.15
	Acid red 73	100.1±0.8	0.77	0.07	100.2±1.6	1.56	0.14
	Orange II	100.0±0.6	0.61	0.05	101.0±0.9	0.85	0.08
	Auramine O	99.2±0.3	0.33	0.03	100.5±1.3	1.31	0.12
	Metanil yellow	100.3±0.4	0.37	0.03	101.2±0.8	0.83	0.07
25 mg/kg	Tartrazine	102.5±1.5	1.44	0.15	103.7±1.6	1.50	0.15
	Amaranth	102.9±1.0	0.99	0.10	103.3±1.2	1.15	0.12
	New coccine	99.9±0.7	0.67	0.07	100.1±1.2	1.18	0.12
	Sunset yellow	100.6±0.4	0.42	0.04	101.4±1.2	1.13	0.12
	Azorubine	100.3±0.6	0.58	0.06	100.9±1.2	1.17	0.12
	Erythrosin B	96.7±0.4	0.46	0.05	98.1±1.6	1.58	0.16
	Acid red 73	100.6±0.6	0.57	0.06	101.3±1.1	1.10	0.11
	Orange II	101.0±0.3	0.30	0.03	102.1±1.3	1.27	0.13
	Auramine O	101.6±0.3	0.27	0.03	102.5±1.2	1.17	0.12
	Metanil yellow	101.4±0.4	0.40	0.04	102.3±1.1	1.09	0.11

[1)]Mean±SD, %
[2)]RSD (%)
[3)]Horrat ratio for repeatability
[4)]HoRrat ratio for reproducibility

The linearity, LOD, and LOQ were confirmed by measuring five concentrations within the range of 1 to 50 mg/kg of the standard solution, seven times in total. The regression coefficient values (r^2) of all 10 dyes added to *Phellodendron* were 0.9999 or higher, and the linearity values met the criteria (≥0.995) specified in the "Methods, Method Verification, and Validation" protocol (Food and Drug Administration, 2014). LOD and LOQ were calculated by multiplying the standard deviation of the intercept by the average of the slope of the calibration curve and multiplied by 3.3 and 10, giving values of 0.04 to 0.35 mg/kg and 0.11 to 1.07 mg/kg, respectively. The calibration parameters, LOD, and LOQ values are shown in Table 2.

The intra-day and inter-day accuracy were 94.2-103.3 and 96.6-103.8%, respectively, and the intra-day and inter-day precision were 0.14-2.28 RSD (%) and 0.80-2.37 RSD (%), respectively. Excellent intra-day accuracy and precision data (n=6) and inter-day accuracy and precision data (n=9) for 10 dyes added to herbal medicines can be confirmed. The total values are shown in Table 3. These parameters comply with the AOAC test guideline criteria (≤5.3%). In addition, the HorRat values were 0.02 to 0.18 for repeatability and 0.07 to 0.19 for reproducibility (Table 3). These values fall within the acceptable HorRat ranges of 1.3 or less for repeatability and 2.0 or less for reproducibility, as defined by the AOAC standard (Latimer, 2016).

Table 4. Expanded uncertainty of 10 dyes in spiked *Phellodendron* and contributions of the individual uncertainty sources

Concentration	Compound	Measurement (mg/kg)	Combined standard uncertainty (mg/kg)	Expanded uncertainty (mg/kg)	Relative uncertainty (%)
5 mg/kg	Tartrazine	4.71	0.23	0.46	9.66
	Amaranth	5.03	0.09	0.18	3.49
	New coccine	4.85	0.12	0.25	5.10
	Sunset yellow	4.86	0.08	0.16	3.38
	Azorubine	4.94	0.05	0.10	2.11
	Erythrosin B	5.06	0.08	0.16	3.23
	Acid red 73	5.05	0.07	0.13	2.63
	Orange Ⅱ	5.05	0.05	0.10	2.07
	Auramine O	5.18	0.07	0.15	2.81
	Metanil yellow	5.08	0.05	0.11	2.12
10 mg/kg	Tartrazine	9.90	0.23	0.46	4.69
	Amaranth	10.04	0.10	0.19	1.91
	New coccine	9.63	0.13	0.27	2.79
	Sunset yellow	9.73	0.10	0.20	2.07
	Azorubine	9.86	0.08	0.15	1.56
	Erythrosin B	10.07	0.10	0.19	1.91
	Acid red 73	9.95	0.09	0.18	1.86
	Orange Ⅱ	9.89	0.08	0.16	1.62
	Auramine O	9.98	0.09	0.19	1.86
	Metanil yellow	9.95	0.08	0.16	1.64
25 mg/kg	Tartrazine	24.51	0.31	0.62	2.51
	Amaranth	25.33	0.21	0.42	1.67
	New coccine	24.70	0.21	0.43	1.74
	Sunset yellow	24.72	0.19	0.38	1.53
	Azorubine	24.86	0.18	0.37	1.48
	Erythrosin B	24.99	0.19	0.37	1.49
	Acid red 73	24.98	0.19	0.38	1.51
	Orange Ⅱ	25.13	0.18	0.36	1.42
	Auramine O	25.53	0.19	0.37	1.47
	Metanil yellow	25.07	0.18	0.36	1.42

Uncertainty of measurement

In this study, the measurement uncertainty was determined for each of 10 dyes at 5, 10, and 25 mg/kg. The evaluation was conducted considering the factors *uSSS*, *uSP*, *uCal*, and *uRP*. All measurement uncertainty values are shown in Table 4. In the *Phellodendron* sample containing 10 dyes, the relative uncertainty multiplied by the coverage factor (k=2) was 1.42-9.66% at the 95% confidence level, confirming that it was suitable according to the CODEX standard (≤32%)(CODEX, 2008). In addition, it was seen that the higher the dye concentration, the smaller the value of the relative measurement uncertainty, decreasing in the order of 2.07-9.66, 1.56-4.69, and 1.42-2.51 mg/kg at the concentrations of 5, 10, and 25 mg/kg, respectively.

The percentage of each factor for the sum of the relative measurement uncertainties was also determined. At the concentration of 5 mg/kg, the uncertainty was mainly found in the calibration curves (*uCal*), and the value was 0.85-7.32%. At the concentration of 10 mg/kg, the calibration curves (*uCal*) and standard stock solutions (*uSSS*) were the main factors that contributed to the uncertainty, with values of 0.43-3.01 and 0.81-0.94%, respectively. At the concentration of 25 mg/kg, the uncertainty was mainly associated with the standard stock solutions (*uSSS*), and the value was 0.79-1.01%. The overall results are shown in Fig. 3.

Application of the HPLC method to commercial *Phellodendron*

In order to apply the proposed HPLC method to *Phellodendron*, 24 *Phellodendron* products were purchased and considered for their applicability. Considering the size of the market and the population in Korea, the products were purchased from Seoul,

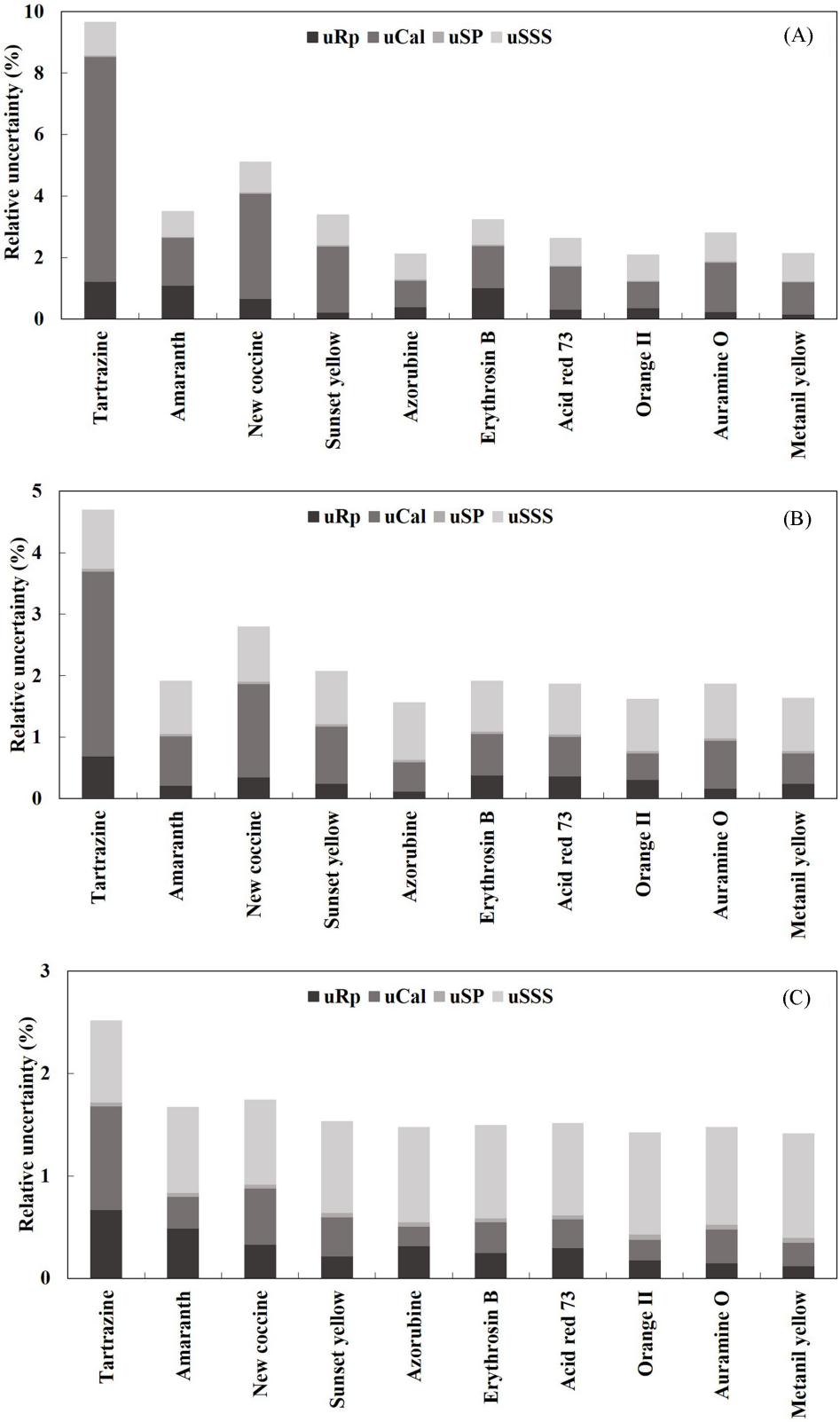

Fig. 3. Measurement uncertainty contributions (uRp: uncertainty of repeatability, uCal: uncertainty of the calibration of standard, uSP: uncertainty of sample preparation, and uSSS: uncertainty of standard stock solution) to the expanded uncertainty of 10 dyes in spiked *Phellodendron*. (A) 5 mg/kg spiking, (B) 10 mg/kg spiking, and (C) 25 mg/kg spiking

Gyeongsang-do, Chungcheong-do, and Jeolla-do. The analysis confirmed no overlapping peaks among the 10 dyes, and none of the dyes were detected in any of the *Phellodendron* products.

Conclusion

In this work, validation for specificity, linearity, precision, and accuracy was conducted on the HPLC-DAD method for quantifying 10 dyes that can be illicitly mixed into *Phellodendron*. Furthermore, this method was applied to commercial *Phellodendron* products, confirming its applicability. This study confirmed the factors that have the greatest influence on the uncertainty by calculating the measurement uncertainty, which improved the reliability of the analysis results. The method is suitable for the quantification of 10 dyes that can be found in adulterated *Phellodendron*.

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Conflict of Interest

The authors declare no conflict of interest.

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