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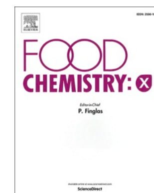

Improvement of analytical method for three azo dyes in processed milk and cheese using HPLC-PDA

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ABSTRACT

This study aims to develop and validate a method for simultaneously measuring three azo dyes (azorubine, brilliant black BN, lithol rubine BK) not designated in Korea. The HPLC-PDA analysis method was validated based on the ICH guidelines, and the color stability was evaluated. The milk and cheese samples were spiked with azo dyes, the correlation coefficient of calibration curve ranged from 0.999 to 1.000 and the recovery rates of azo dyes were 98.81 ~ 115.94%, with RSD of 0.08 ~ 3.71%. The LOD and the LOQ in milk and cheese ranged from 1.14 to 1.73 μg/mL and 3.46 to 5.25 μg/mL, respectively. In addition, the expanded uncertainties of the measurements ranged from 3.3421 to 3.8146%. The azo dyes appeared to be color stable for more than 14 days. The results indicate that this analytical method is suitable for extracting and analyzing azo dyes in milk and cheese samples, which are not permitted in Korea.

1. Introduction

Color is one of the most important attributes for evaluating food, as it is a parameter that determines product choice for consumers. Various food dyes of natural and synthetic origin are added to and consumed in food (Arrizabalaga-Larrañaga, Epigmenio-Chamú, Santos, & Moyano, 2021; Yamjala, Nainar, & Ramisetti, 2016). Synthetic dyes are widely used in food because they are easy to synthesize compared to natural dyes; can exhibit excellent coloration at a relatively low cost; are highly stable to light, temperature, pH, and redox agents; and are less prone to contamination by microorganisms (Oreopoulou et al., 2009). As a result, synthetic dyes are used more frequently than natural dyes in many processed foods (Choi, 2012). However, some synthetic dyes are toxic and can adversely affect the human body, causing allergies, asthma, DNA damage, and hyperactivity (Li et al., 2014). Some synthetic dyes are hyperactive and pose potential risks to human health, especially in children, when consumed in excess, some are considered genotoxic, neurotoxic, and carcinogenic and are also reported to cause thyroid tumors (Palianskikh et al., 2022; Sulaiman, Shah, & Khan, 2022). In many countries, the use of additives in the food industry to protect public health is subject to strict legal regulations based on the maximum permissible limit (MPL) by acceptable daily intake (ADI), and national guidelines such as the European Union (EU) and Codex Alimentarius (CODEX) (Cheibub, de Lyra, Alves, Donagemma, & Netto, 2020). Nevertheless, regulated or banned synthetic dyes are often abused in food and used illegally to obtain brighter colors and reduce costs (Liu et al., 2019). Thus, the development of sensitive and reliable analytical methods to analyze and monitor synthetic dyes in artificially colored foods is very important for food safety.

Among the synthetic dyes used in the food industry, azo dyes, which are synthetic organic dyes containing an azo group as part of their structure, are widely used and account for ~ 65% of the commercial dye market (Ahlström, Eskilsson, & Björklund, 2005). Azo dyes are used as a colorant in various food products such as jams, candies, confectionery, ice cream, jellies, alcoholic beverages, and soft drinks. However, despite their widespread use, they have been reported to have negative effects on human health, such as those arising from the production of aromatic amines (Beitollahi, Nejad, & Tajik, 2021). The ecological and toxicological association of dyes and organic pigment manufacturers (ETAD) has cataloged azo dyes that form aromatic amines. This list contains more than 500 azo dyes, of which at least 142 dyes are still available in the global market (Chavan, 2011). Some of these azo dyes that have an E

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number used as a food additive code in the European Food Safety Authority (EFSA) have been or are currently used as food additives (European Commission, 2012). We developed simultaneous analytical methods for azorubine, brilliant black BN, and lithol rubine BK, which are not designated in Korea among food additives currently approved in the EU or CODEX, and these standards are now available (Codex Alimentarius, 2021; European Commission, 2012). These three dyes have been designated overseas but are not designated in Korea. Thus, these colorants may be present in imported food and may be illegally distributed in Korea. Simultaneous analysis of the three azo dyes in flavored fluid milk drinks, flavored processed cheeses, and edible cheese rind, among the various food groups permitted by EU and CODEX, was performed.

Expensive and state-of-the-art equipment was utilized to detect trace amounts of colorants using Raman spectroscopy, ionic liquid-based extraction, membrane filtration, capillary electrophoresis, cloud point extraction, high-performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC–MS) (Rebane, Leito, Yurchenko, & Herodes, 2010; Wu et al., 2018). These methods involve high costs and require highly skilled experimenters and high-purity solvents and gases, thereby rendering their universal use difficult for monitoring various food products (Sulaiman et al., 2022). On the other hand, inexpensive methods or those that do not require high-purity solvents, such as ultraviolet–visible (UV/Vis) spectroscopy, has low sensitivity to trace food dyes, involve complex sample preparation methods, and require a pre-concentration step (Asensio-Ramos, Ravelo-Pérez, González-Curbelo, & Hernández-Borges, 2011). Also, although an analytical method has been reported for azorubine, there are no studies on the development and validation of an economical and efficient method for the simultaneous analysis of the three azo dyes under the same analytical conditions (Kim et al., 2016b). Although the limit of detection (LOD) and limit of quantification (LOQ) are commonly reported, the measurement uncertainty of these methods is often not reported (Harp, Miranda-Bermudez, & Barrows, 2013).

In this study, a sample pretreatment method for milk and cheese was developed to analyze the three azo dyes, the analytical method was validated using the high-performance liquid chromatography-photo diode array (HPLC-PDA) method, and measurement uncertainty was measured. The applicability of the validated analytical method was confirmed by monitoring the presence of illegal azo dyes in domestic and imported milk and cheeses samples distributed in Korea. In addition, by investigating the color stability and determining the recovery rate during storage in milk, it was evaluated whether the simultaneous analysis of the three azo dyes was effective during distribution.

2. Materials and methods

2.1. Chemicals and reagents

Brilliant black BN (≥95%), lithol rubine BK (≥95%), sodium phosphate dibasic (≥99.0), citric acid (≥99.5%), and ammonium acetate (≥98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Azorubine (95.0%) was purchased from Tokyo Chemical Industry (TCI, Tokyo, Japan). Methanol (≥99.9), acetonitrile (≥99.9), hexane (95%), and acetone (≥99.3) were acquired from J. T. Baker (Philipsburg, NJ, USA). Dimethyl sulfoxide (≥99.0) was purchased from Junsei Chemical (Tokyo, Japan). Ethyl alcohol, anhydrous (≥99.9), and petroleum ether (90.0%) were purchased from Samchun (Seoul, Korea).

2.2. Food materials

Domestic and imported samples comprising 25 types of colored and flavored processed milk and 25 types of cheeses and rind were purchased from supermarkets in Korea. The shelf life of each sample was sufficient during the experiment, and all samples were refrigerated and used for experiments. White milk and cheese samples were used for validation of the analytical methods. After the method was validated, the applicability of the method for the detection of the three azo dyes, in 50 types of colored milk and cheese samples was examined.

2.3. Preparation of three azo dyes standard solution

Calibration and stock solutions used in the experiment were prepared as follows. First, 10 mg of the three azo dyes were dissolved in 10 mL of dimethyl sulfoxide (DMSO) each to prepare three stock solutions at a concentration of 1,000 μg/mL. Three milliliters of the three prepared dye solutions were mixed to yield a 9 mL solution containing the three dyes. Then, 1 mL of DMSO was added to yield the standard mixture containing 300 μg/mL of each dye. Following this, the mixture was diluted to two-thirds of its concentration using DMSO to prepare 7-point standard solutions containing 26.34–300 μg/mL of each dye.

2.4. Optimization of HPLC instrument conditions

We have established the optimal analysis conditions for the simultaneous identification of the three azo dyes using HPLC-PDA as they achieved the best results. First, three analytical methods were reproduced and compared to achieve optimal analysis conditions (Kim et al., 2016b; Kim, Park, Suh, & Lee, 2016c; Yoshioka & Ichihashi, 2008). For the three analysis conditions, ammonium acetate at different concentrations and an organic solvent (methanol or/and acetonitrile) were used as the mobile phase. The suitability of the analytical methods was evaluated based on the standard's peak shape, peak area, baseline, and correlation coefficient. Among the three analysis conditions, the Kim et al. (2016b) analytical method exhibited the best performance for the simultaneous discrimination of the three azo dyes. For more detailed optimization, an analytical method was developed to select instrument parameters such as column type and temperature. The Agilent 2695 series of analytical instruments used in this study was equipped with a column oven, quaternary pump, autosampler, and Waters 996 PDA detector to analyze materials over a wide range of wavelengths in the ultraviolet and visible region. SunFire C18 analytical columns with dimensions (diameter and length) of 4.6 × 150 mm and 4.6 × 250 mm and Shiseido Capcell Pak C18 UG120 columns with dimensions of 4.6 × 250 mm were used. All columns were filled with 5.0 μm particles. Among the three columns, the Shiseido Capcell Pak C18 UG120 (4.6 × 250 mm, 5.0 μm) column was selected for its superior resolution. To establish the optimal temperature conditions for the selected column, we set the column temperature to 25℃ after comparing the standards of the dyes at temperatures of 25, 35, and 45℃. The mobile phase solution was vacuum degassed by filtration through a 0.45 μm nylon membrane filter (Whatman, Amersham, UK). Mobile phases A and B were 0.02 M ammonium acetate solution and methanol, respectively, and linear gradient elution was conducted as follows for a run time of 30 min: 0 to 10 min, 95% to 10% A; 10 to 11 min, 10% to 30% A; 11 to 13 min, held at 30% A; 13 to 15 min, 30% to 26% A; 15 to 16 min, 26% to 0% A; 16 to 20 min, held at 0% A; 20 to 25 min, 0% to 95% A; 25 to 30 min, held at 95% A. The sample injection volume was set to 10 μL and the flow rate was set to 0.8 L/min. Azorubine and lithol rubine BK were detected at 520 nm while brilliant black BN was detected at 608 nm. Data were processed using the Empower software.

2.5. Optimization of the extraction method of three azo dyes

The optimal pretreatment method for extracting azo dyes from milk and cheese samples was established using the optimized HPLC-PDA analytical method. The sample pretreatment method was determined based on the recovery of the sample spiked with a known concentration of the standard. We compared the results with those of Kim et al. (2016c) who reported a pretreatment method for high-fat food, the method of Chun (2016), and the method specified for tar color analysis in the Korea Food Code (Fig. S1). Among these, we found that the sample preparation

method reported by Kim et al. (2016c) was optimal for the sample matrix used in this study. To extract the three azo dyes, 10 mL of milk and 10 g of cheese samples were accurately weighed in a beaker. In this study, cheese and rind were finely mixed with a mortar and pestle. 40 mL of Ethanol at 70% was added to the sample, and to remove milk fat, a 20 mL sample was shaken three times with hexane, following which the hexane layer was removed. The mixture was left in a water bath at 50℃ for 20 min. Next, this mixture was centrifuged at 10,000 rpm for 10 min to discard impurities, and the supernatant was collected. Before injecting the sample for HPLC analysis, the volume massed up to 100 mL using ammonium acetate at 0.02 M (mobile phase), and the resultant solution was filtered using a 0.45 μm syringe filter.

2.6. Method validation

The developed analytical method to simultaneously determine the three azo dyes was validated according to the International Conference on Harmonization (ICH) guidelines (Ich, 1996). Method validation was performed by evaluating specificity, linearity, LOD, LOQ, precision, and accuracy according to the ICH guidelines. Specificity requires that the peaks of a particular analyte are not overlapped with the peaks of other components and that the unique components can be selectively identified. Specificity was assessed by confirming that there was no interference between other analytes and matrices. Linearity is determined by the correlation coefficient of the matrix calibration curve. Calibration curves were generated at 7 calibration points in the range of 26.34–300 μg/mL by spiking a standard mixture containing the three azo dyes in milk and cheese samples without food coloring. The concentration range of the standard curve was set including the maximum level of the three azo dyes regulated in milk and cheese by EU and CODEX (Codex Alimentarius, 2021; European Commission, 2012). All samples spiked with the standard mixture were analyzed using the developed pretreatment procedure. The LOD and LOQ of three azo dyes were calculated using the following formula based on the slope and the standard deviation: LOD = $3.3(\sigma/S)$; LOQ = $10(\sigma/S)$, where σ is the mean standard deviation and S is the slope of the calibration curve (Ich, 1996). For the developed analytical method, precision, and accuracy were determined from the relative standard deviation (RSD) and recovery for three spiked levels, by spiking 50, 100, and 150 μg/mL. The blank samples for both matrices containing no food coloring were used to examine the recovery rates. Recovery rate was obtained using the following formula: recovery (%) = $[(C_f - C_u) / C_a] \times 100$, where C_f is the concentration of the spiked sample, C_u is the concentration of the sample, and C_a is the concentration of the standard. These performances were assessed for intra-day (repeated three times per day) and inter-day (repeated three times in three days) measurements according to the ICH guidelines (Ich, 1996).

2.7. Evaluation of color stability of milk products containing azo dyes during storage

2.7.1. Experimental conditions

The longer the storage period, the lower the pH of milk, which can be attributed to the growth of microorganisms or the separation of lactose (Weston, Kuchel, Ciftci, Boyer, & Chandrawati, 2020). Accordingly, the color stability of the azo dyes was examined to determine if real distributed products can be monitored smoothly using the validated analytical method over the storage period. The experiment was designed referring to the methods proposed by Bermúdez-Aguirre, Yáñez, Dunne, Davies, and Barbosa-Cánovas (2010) and Ma et al. (2020). The pH of the milk samples was arbitrarily adjusted using a buffer (citric acid-sodium phosphate dibasic buffer) solution. Milk experimental groups of pH 4.7, 5.46, and 6.42, corresponding to the 3 stages of milk deterioration, were prepared, and it was confirmed that the milk does not coagulate. The pH 6.7 of fresh milk was used as a control. According to Tejayadi (2004), the recommended minimum composition of colorants to produce aseptic flavored milk at a temperature of 2-80℃ is 0.01 wt%. Therefore, 10 mL of pasteurized whole milk and 5 mg/mL of azo dye (in DMSO) were mixed, the pH was adjusted with buffer, and the volume massed up to 50 mL using distilled water. The final concentration of the azo dye in the resultant solution is 0.01 wt%. Considering that the shelf life of pasteurized milk is 12–14 days, the storage period was set to a maximum of 14 days. The color was measured five times at a cycle of 3.5 days, including 0 days, to obtain the initial value, and the colorant content was analyzed three times by HPLC at a cycle of seven days to confirm the residual level. All experimental groups were refrigerated.

2.7.2. Measurement of color

Hunter color parameters (L, a, and b) were measured for milk samples during storage. Each color parameter was measured using a Konica Minolta CR-400 colorimeter (Konica Minolta, Osaka, Japan) in the reflection mode, and each milk sample was placed evenly in 20 mL aliquots on sterile plates (90 × 20 mm) (SPL, Gyeonggi, Korea). Before experiments, the colorimeter was calibrated using a white ceramic plate (L = 90.77, a = 0.01, b = 1.74) to standardize the instrument. All samples were measured in triplicate.

2.7.3. Measurement of residual level of azo dyes

The content of food colorant during the storage period of 14 days was confirmed using HPLC. Samples were collected for storage periods of 0, 7, and 14 days and analyzed after subjecting them to the pretreatment process. The residual level of colorant in each experimental group was expressed as a rate of change of colorant concentration over the storage period. The colorant content on day 0 was defined as 100% and subsequent values were determined based on this value.

2.8. Measurement uncertainty

The measurement uncertainty for azo dye in milk was estimated according to the Guide to the Expression of Uncertainty in Measurement (GUM) using HPLC-PDA concerning Jang et al. (2021) and Kim et al. (2016a). To measure uncertainty, a metrological approach was used based on in-laboratory data such as precision studies, data on analytical performance, and quantification of each dye (Ellison & Williams, 2012; NIST, 1993). Accordingly, we have considered and recorded the uncertainties arising from balances, reference materials, volumetric devices, calibration curves, sample preparation, and tool elements. The expanded uncertainty (U) was obtained using the following equation and it is obtained by multiplying the combined standard uncertainty by the coverage factor, k = 2, at a level of approximately 95% confidence.

$$\boldsymbol{u}_c^2(\boldsymbol{y}) = \sum_{i=1}^{N} \left(\frac{\partial f}{\partial x_i}\right)^2 \boldsymbol{u}^2(\boldsymbol{x}_i) \tag{1}$$

$$V_{eff} = \frac{\boldsymbol{u}_c^4(\boldsymbol{y})}{\sum_{i=1}^{N} \frac{[c_i u(x_i)^2]}{v_i}} \tag{2}$$

$$U(\boldsymbol{y}) = \mathbf{k}\boldsymbol{u}_c(\boldsymbol{y}) \tag{3}$$

U: Expanded uncertainty.
k: Coverage factor.
V_{eff}: Effective degree of freedom.
v_i: Degree of freedom.

3. Results and discussion

3.1. Optimization of HPLC instrument conditions

HPLC conditions for the determination of three azo dyes were investigated and optimized. First, three different analytical conditions using HPLC were compared to establish the analytical conditions for azo dyes. For the three different analytical conditions, components were separated using a common C18 column, and different maximum

absorption wavelengths were set for detecting red colorants (azorubine and lithol rubine BK) at 520 nm and black colorant (brilliant black BN) at 608 nm using a photo diode array (PDA) detector. Azo dye peaks were selectively separated in all the methods. The method proposed by Kim et al. (2016b) showed excellent peak shapes for all analytes. Using the methods proposed by Kim et al. (2016c) and Yoshioka and Ichihashi (2008), sharp and clear peaks were obtained for azorubine and lithol rubine BK, whereas the peaks of brilliant black BN were split. In addition, the peak width obtained using the method proposed by Kim et al. (2016b) was about twice that obtained using other methods. Moreover, Kim et al. (2016b) analytical conditions had a clean baseline and were environmentally friendly as it required a relatively low concentration of mobile phase compared to other methods. In the method proposed by Kim et al. (2016b), the column type and column temperature were compared under three conditions. Three types of columns from different companies and with different lengths were used. A calibration curve according to the peak area was prepared for four concentrations of the standard mixture solution. One concentration included in the range of the calibration curve was analyzed, and the correlation coefficient and peak area were compared. In this case, excellent peak resolution was obtained using all three columns for the three azo dyes. In the SunFire C18 column (4.6 × 150 mm, 5 μm) and Shiseido C18 column (4.6 × 250 mm, 5 μm), the correlation coefficient of two of the three standard substances was 1.0000, and that of the other substance was 0.9999. In the other SunFire C18 column (4.6 × 250 mm, 5 μm), the correlation coefficient of one of the three standard substances was 1.0000 while those of the other two substances were 0.9995 and 0.9996. The peak area obtained using the Shiseido C18 column (4.6 × 250 mm, 5 μm) was the highest for all the standard substances. Accordingly, it was chosen as the optimal column. Finally, the column temperature was optimized by varying the column oven temperature of the HPLC system. Columns are heated to adjust the spacing between different peaks or control the pressure inside the HPLC device. The optimal column temperature for azo dye analysis was established by constructing a calibration curve using four concentrations of the standard mixture and comparing the correlation coefficients. At a column temperature of 25 °C, the correlation coefficients were 1.0000, 1.0000, and 0.9999, which were superior to the correlation coefficients at 35 and 45 °C. Therefore, the optimal column temperature was set at 25 °C.

3.2. Optimization of extraction method of three azo dyes

To extract the three azo dyes from milk and cheese, the sample pretreatment conditions were optimized. To this end, the correlation coefficient and recovery rate after pretreatment were compared by applying known concentrations of the standard mixture to milk and cheese samples. All the pretreatment methods compared for the extraction of the azo dyes involved removing milk fat, mixing the sample with an organic solvent, and applying temperature treatment. These steps remove impurities such as milk fat, lactose, and protein (casein), which are specific to dairy products, to the extent possible and eliminate substances that interfere with HPLC analysis. The method of Chun (2016) did not detect some dyes in cheese samples or the recovery rate was higher than 80–120%, and the MFDS (2021) method also had some dyes that were out of the range of the guidelines. Using the pretreatment method by Kim et al. (2016c), all azo dyes were well recovered from both matrices and an excellent correlation coefficient was obtained. Therefore, Kim et al. (2016c) method with the best recovery of three azo dyes from milk and cheese was determined to be the optimal extraction method for the analysis.

3.3. Method validation

Specificity, linearity, LOD, LOQ, precision, and accuracy were evaluated to validate the analytical method. To construct the calibration curve of each azo dye, a mixture of three azo dye standards was added to a sample without dyes, followed by preparation to a concentration of 26.34–300 μg/mL. Three known concentrations (low, medium, and high concentrations) of the standard were also measured by spiking into the dye-free sample of white milk and cheese. Method validation was performed using the developed and optimized analytical conditions and sample pretreatment method. The specificity of a chromatogram is a measure of how well an analyte can be determined without interference from other components (each analyte being analyzed simultaneously, with impurities in the sample, sample matrix, etc.). Fig. 1 shows the chromatograms of the three azo dye standards obtained using the developed HPLC-PDA analytical method. In milk and cheese samples, individual peaks with different retention times were sufficiently separated, without overlapping peaks, and no matrix interference was observed, demonstrating the specificity of this analytical method. Linearity was determined from the correlation coefficient of the calibration curve for each azo dye. Table 1 shows the validation results for milk and cheese matrices. All the correlation coefficients of milk and cheese samples were 0.9999 or higher, showing excellent linearity (Fig. S2). LODs were in the range of 1.28–1.61 μg/mL for milk samples and 1.14–1.73 μg/mL for cheese samples. LOQs were in the range of 3.89–4.87 μg/mL for milk samples and 3.46–5.25 μg/mL for cheese samples. The LODs and LOQs were less than 100 or 150 mg/kg, which is the acceptable level of flavored fluid milk drinks and flavored processed cheeses and edible cheese rind products according to the EU and CODEX for the three azo dyes (Codex Alimentarius, 2021; European Commission, 2012). Therefore, the sensitivity of the HPLC-PDA analytical method developed in this study was excellent, and the method was suitable for the quantitative detection of the three azo dyes. To ensure the reproducibility and reliability of the developed pretreatment method, the RSD and the recovery rate were evaluated by adding 50, 100, and 150 μg/mL of standard mixture solutions to the milk and cheese matrices performing the pretreatment process (Table 2). The accuracy of the analytical method was determined through a recovery experiment. The recovery rates for the three concentrations of the azo dyes in milk and cheese were 98.81–102.90% and 94.26–115.94%, respectively, indicating excellent recovery according to the ICH guidelines and demonstrating the accuracy of this assay (Ich, 1996). Precision was evaluated based on the RSD using the mean and standard deviation of a total of nine analyses conducted by measuring three times a day for three days. For all the azo dyes, the RSD in milk and cheese was up to 3.71%. Thus, the precision was less than 5%, indicating that the analytical method developed in this study showed excellent precision and accuracy in the range of 26.34–300 μg/mL in milk and cheese for three azo dyes that are not designated in Korea.

3.4. Monitoring of three azo dyes in milk and cheese samples

The use of azorubine, brilliant black BN, and lithol rubine BK in domestic and imported milk and cheese products was monitored using the analytical method developed and validated in this study. We collected 25 types of milk samples and 25 types of cheese samples from supermarkets in Korea. Three azo dyes were not detected in all milk and cheese samples. Since the three azo dyes are not designated in the Korea Food Additives Code, it is reasonable to show non-detection in all 50 types of samples (MFDS, 2021).

3.5. Evaluation of color stability of milk products containing azo dyes during storage

3.5.1. Color stability of three azo dyes according to Hunter color parameters

Milk contains essential antibacterial factors including lactoperoxidase, immunoglobulins, lysozyme, and lactoferrin (Atasever, Ozdemir, Gulcin, & Kufrevioglu, 2013; Kalin et al., 2022; Sisecioglu, Uguz, Cankaya, Özdemir, & Gülçin, 2011), but when milk is stored for a prolonged period, the molecular environment changes because microorganisms

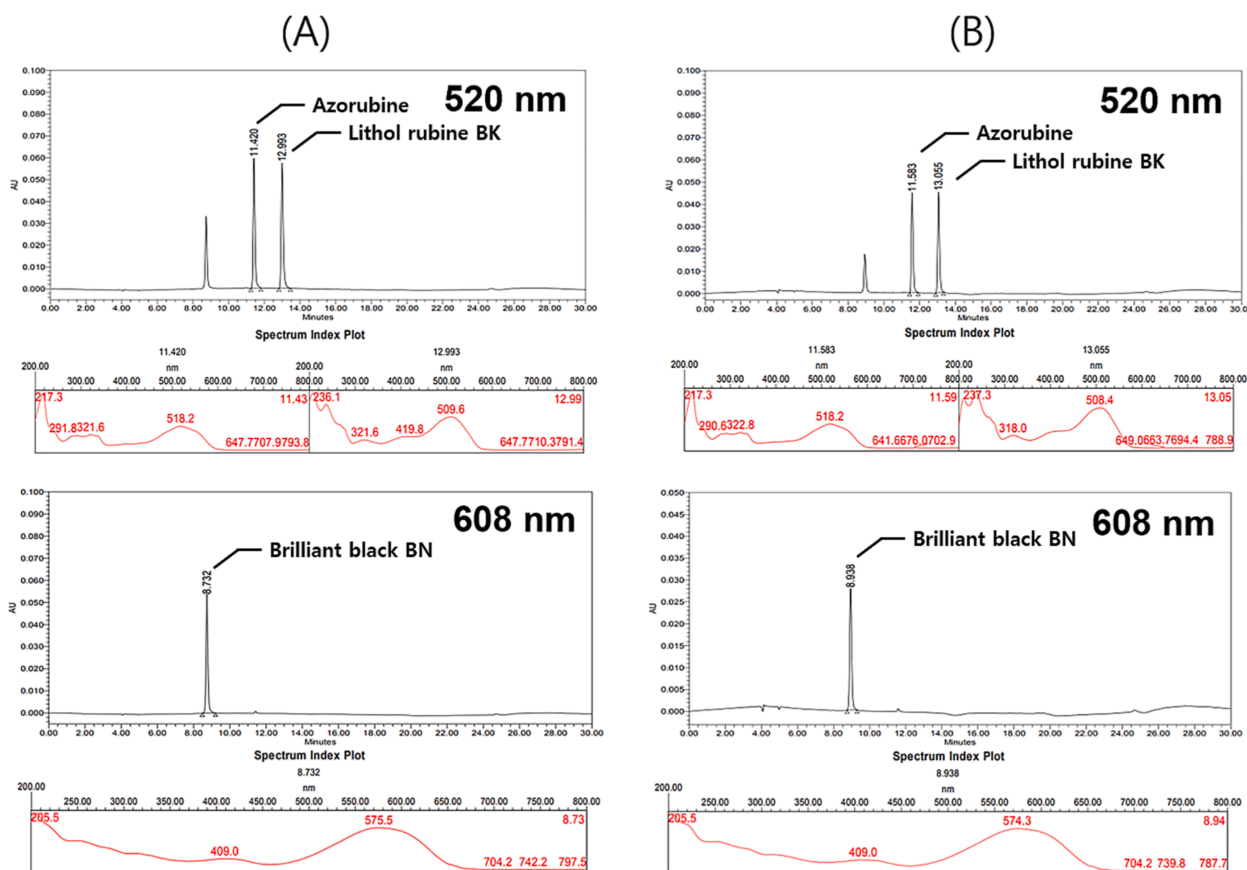

Fig. 1. Chromatograms and spectrums of three azo dye standards using the developed HPLC-PDA method. Signals were detected at 520 nm for azorubine and lithol rubine BK and 608 nm for brilliant black BN in spiked (A) milk and (B) cheese.

Table 1
Correlation coefficients of the calibration curves, LOD, and LOQ of azorubine, brilliant black BN, and lithol rubine BK for milk and cheese samples using HPLC.

Matrix	Analyte	Range (μg/mL)	Slope	Intercept	Correlation coefficient (R^2)	LOD (μg/mL)	LOQ (μg/mL)
Liquid (Milk)	Azorubine	26.34–300	2954.3	1896.8	1.0000	1.61	4.87
	Brilliant black BN	26.34–300	2572.8	117.5	1.0000	1.28	3.89
	Lithol rubine BK	26.34–300	2984.1	−1360	1.0000	1.50	4.56
Solid (Cheese)	Azorubine	26.34–300	1769	−445.63	0.9999	1.14	3.46
	Brilliant black BN	26.34–300	345.94	−2402.1	0.9999	1.73	5.25
	Lithol rubine BK	26.34–300	1797.9	−6100	1.0000	1.30	3.94

begin to multiply, and lactose is broken down by lactic acid bacteria (Weston et al., 2020). The quality of milk deteriorates rapidly with an increasing number of lactic acid bacteria and other bacteria (Choudhary, Joshi, Pandey, & Joshi, 2019). Spoiled milk is characterized by a decrease in pH over pH values of 6.42, 5.46, and 4.77, corresponding to three stages of milk degradation (Ma et al., 2020). In this study, pH was adjusted for azorubine, brilliant black BN, and lithol rubine BK, which were arbitrarily added to pasteurized whole milk. The change in chromaticity according to the storage period was measured using the Hunter color system, as shown in Table 3. The Hunter color parameter 'L' indicates lightness, which may vary in the range of 0–100. The color tends to be whiter with increasing L values and blacker for lower L values. 'a' value indicates redness: positive values indicate red color and negative values indicate green color, 'b' value indicates yellowness: positive values indicate yellow color and negative values indicate blue color. The pH of pasteurized whole milk samples was adjusted using citric acid-sodium phosphate dibasic buffer, and the samples were refrigerated for 14 days, which corresponds to the shelf life of pasteurized milk. For all the azo dyes, the L value fluctuated within the maximum of ± 0.31. The a and b values fluctuated within the maximum of ± 0.39 and ± 0.30, respectively, but generally tended to be constant. Therefore, the azo dyes, a synthetic colorant, exhibit excellent color stability over the storage period of pasteurized whole milk.

3.5.2. Residual level of three azo dyes using the HPLC-PDA method

The stability of azorubine, brilliant black BN, and lithol rubine BK during the refrigeration of colored milk was evaluated by measuring the change in the residual levels of these dyes. The recovery rate was converted by setting storage day 0 as 100%, as shown in Table 3. Some azo dyes showed a slight decrease in the residual amount with a prolonged storage period; however, all the azo dyes showed recovery rates of 83.5% or more. Therefore, the analytical conditions validated in this study are useful for measuring the azo dye content during the storage of milk.

3.6. Measurement uncertainty

Method validation demonstrates the reliability of analytical results, but it is not sufficient to accurately interpret and compare results (Rozet, Marini, Ziemons, Boulanger, & Hubert, 2011). A measurement result is only an estimate of the probability of reaching a target value and a specific quantity of the measurand. Measurement uncertainty reflects

Table 2
RSD and recoveries of azorubine, brilliant black BN, and lithol rubine BK for milk and cheese samples spiked at three different concentrations using HPLC (n = 3).

Matrix	Analyte		Concentration (μg/mL)	Mean ± SD * (μg/mL)	RSD * (%)	Recovery (%)
Liquid (Milk)	Azorubine	Intra-day	50	50.75 ± 0.74	0.92	101.49
			100	100.57 ± 0.08	0.08	100.57
			150	148.79 ± 0.14	0.10	99.19
		Inter-day	50	50.15 ± 0.65	1.29	100.31
			100	99.56 ± 0.69	0.69	99.56
			150	148.21 ± 0.80	0.54	98.81
	Brilliant black BN	Intra-day	50	51.43 ± 0.23	0.44	102.86
			100	100.26 ± 0.77	0.77	100.26
			150	149.04 ± 1.21	0.81	99.36
		Inter-day	50	50.28 ± 0.82	1.64	100.57
			100	100.42 ± 0.47	0.46	100.42
			150	148.78 ± 0.61	0.41	99.19
	Lithol rubine BK	Intra-day	50	51.45 ± 0.22	0.43	102.90
			100	101.34 ± 0.32	0.31	101.34
			150	150.43 ± 0.49	0.33	100.29
		Inter-day	50	50.56 ± 0.29	0.57	101.12
			100	100.76 ± 0.35	0.35	100.76
			150	149.74 ± 0.35	0.23	99.82
Solid (Cheese)	Azorubine	Intra-day	50	57.92 ± 1.13	1.95	115.84
			100	113.09 ± 2.31	2.04	113.09
			150	170.27 ± 1.08	0.63	113.51
		Inter-day	50	57.97 ± 0.84	1.45	115.94
			100	107.21 ± 1.42	1.32	107.21
			150	163.65 ± 2.76	1.69	109.10
	Brilliant black BN	Intra-day	50	56.79 ± 3.55	3.13	113.58
			100	101.20 ± 4.34	1.43	101.20
			150	144.49 ± 3.02	1.05	96.32
		Inter-day	50	55.03 ± 2.57	1.17	110.05
			100	104.85 ± 4.56	1.45	104.85
			150	144.93 ± 3.71	1.28	96.62
	Lithol rubine BK	Intra-day	50	57.92 ± 1.37	2.37	115.83
			100	112.68 ± 1.98	1.76	112.68
			150	170.19 ± 1.53	0.90	113.46
		Inter-day	50	55.28 ± 2.05	3.71	110.55
			100	108.89 ± 1.07	0.98	108.89
			150	157.33 ± 0.40	0.25	104.89

* SD: Standard deviation.
* RSD: Relative standard deviation.

the quality of the analytical procedure and aids in the interpretation of results so that a measurement can be said to be complete when it is accompanied by measurement uncertainty (Dias, Camões, & Oliveira, 2008; Rozet et al., 2011). Therefore, the uncertainty of the quantification of three azo dyes contained in milk was estimated using the HPLC-PDA method, and the estimated calculation of uncertainty was standardized using a method based on the GUM and the draft Ellison and Williams (2012) proposed by the Dimensional Metrology approach. Five separate sources of uncertainty were considered in this study: U_{prep}, the uncertainty associated with sample preparation; U_{RM}, uncertainty related to reference material; U_{std}, uncertainty associated with standard stock solutions; U_{cal}, uncertainty associated with the calibration curve; and U_{rep}, the uncertainty associated with repeatability (Table 4). The corresponding calibration certificates and repeated tests at laboratory temperature ensured the uncertainties related to instrumental equipment. U_{prep} was measured using a chemical balance and 100 mL volumetric flask. The uncertainty of the chemical balance was measured by the certificate of calibration (0.0003 g), readability (0.000029 g), stability (0.000032 g), and the uncertainty of the volumetric flask was measured by the certificate of calibration (0.018 mL), the repeatability (0.00031 mL), and the change in volume with temperature (0.08 mL). The relative standard uncertainties for the chemical balance and 100 mL volumetric flask were 0.000003 and 0.000868, respectively. The combined uncertainty of the sample preparation was 0.0.000868. U_{RM} was determined based on the certificate of analysis for each colorant. The stock solution uncertainty, U_{std}, was measured using a chemical balance and a 10 mL volumetric flask. A 10 mL volumetric flask was measured with a certificate of calibration (0.008 mL), repeatability (0.00071 mL), and change in volume with temperature (0.008 mL). The relative standard uncertainties for the chemical balance and 10 mL volumetric flask were 0.015612 and 0.000939, respectively. The uncertainty of the calibration curve was obtained for each dye, which was measured in triplicate at seven concentrations. The repeatability uncertainty was evaluated to determine the average content of each dye in a sample of 10 mL. Fig. 2 provides an overview of the contribution of each uncertainty source. The major contribution to uncertainty comes from the reference material and the calibration curve. The combined and expanded uncertainty, considering a coverage factor of 2 and a confidence level of approximately 95%, were calculated for all three standards used for calibration.

4. Conclusion

In this study, using HPLC-PDA, we developed a method to simultaneously analyze three azo dyes (azorubine, brilliant black BN, and lithol rubine BK), which are undesignated in Korea, in milk and cheese products. The simultaneous analytical method was validated for specificity, linearity, LOD, LOQ, precision, and accuracy according to the ICH guidelines. Additionally, the measurement uncertainty of the analytical process was a measure for the HPLC-PDA method. The major contribution to uncertainty comes from the reference material and the calibration curve. The analytical method was suitable for the quantitative measurement of three azo dyes in milk and cheese products. The validated analytical method was applied to real food products distributed in Korea, and the presence of the three azo dyes was examined. None of the tested samples showed the presence of these unspecified azo dyes. These results suggest that the validated simultaneous analytical method is suitable for the identification and quantitative analysis of the three azo

Table 3
Effect of pH on color stability and residual levels during storage. All data of chromaticity were measured three times and expressed as mean ± SD (n = 3).

Azorubine

	pH	Day 0	3.5	7	10.5	14
L *	4.77	48.6 ±0.02	48.3 ±0.41	48.8 ±0.03	48.6 ±0.02	48.7 ±0.00
	5.46	47.4 ±0.01	48.3 ±0.40	47.6 ±0.00	47.6 ±0.02	47.9 ±0.00
	6.42	48.2 ±0.00	48.4 ±0.23	47.9 ±0.04	47.9 ±0.02	48.1 ±0.01
	6.70	48.2 ±0.01	48.1 ±0.27	48.1 ±0.04	48.2 ±0.01	48.2 ±0.01
a *	4.77	22.3 ±0.03	22.3 ±0.09	22.6 ±0.04	22.2 ±0.01	22.2 ±0.02
	5.46	22.4 ±0.02	22.3 ±0.15	22.6 ±0.03	22.4 ±0.05	22.8 ±0.01
	6.42	22.5 ±0.03	22.3 ±0.08	22.2 ±0.01	22.5 ±0.02	22.3 ±0.03
	6.70	22.1 ±0.04	22.3 ±0.15	22.1 ±0.05	22.3 ±0.02	22.3 ±0.05
b *	4.77	1.5 ±0.10	2.1 ±0.69	1.6 ±0.01	1.4 ±0.01	1.3 ±0.01
	5.46	3.0 ±0.02	2.4 ±0.77	3.1 ±0.01	3.1 ±0.01	2.9 ±0.01
	6.42	3.0 ±0.03	2.4 ±0.77	3.1 ±0.01	3.1 ±0.01	2.9 ±0.01
	6.70	3.0 ±0.00	2.8 ±0.25	3.2 ±0.02	3.1 ±0.01	2.9 ±0.01
R level*	4.77	100		92.0		93.4
	5.46	100		97.3		96.4
	6.42	100		101.7		99.1
	6.70	100		101.0		102.3

Brilliant black BN

	pH	Day 0	3.5	7	10.5	14
L	4.77	43.9 ±0.01	44.1 ±0.01	44.0 ±0.00	43.9 ±0.00	43.9 ±0.01
	5.46	42.2 ±0.00	42.3 ±0.01	42.5 ±0.00	42.3 ±0.01	42.4 ±0.01
	6.42	42.7 ±0.00	42.5 ±0.00	42.9 ±0.00	42.8 ±0.01	42.8 ±0.00
	6.70	42.9 ±0.00	43.0 ±0.01	43.0 ±0.00	42.8 ±0.00	42.9 ±0.00
a	4.77	4.7 ±0.00	4.9 ±0.01	4.6 ±0.01	4.7 ±0.01	4.5 ±0.04
	5.46	6.0 ±0.01	6.2 ±0.01	5.7 ±0.02	5.8 ±0.01	5.5 ±0.02
	6.42	6.8 ±0.00	6.9 ±0.00	6.4 ±0.02	6.5 ±0.02	6.1 ±0.01
	6.70	6.7 ±0.02	7.0 ±0.01	6.1 ±0.01	6.4 ±0.01	5.9 ±0.02
b	4.77	−13.2 ±0.00	−13.7 ±0.01	−13.2 ±0.00	−13.3 ±0.02	−13.1 ±0.03
	5.46	−13.5 ±0.00	−13.9 ±0.00	−13.5 ±0.01	−13.4 ±0.01	−13.3 ±0.02
	6.42	−13.9 ±0.00	−14.0 ±0.00	−13.9 ±0.01	−14.0 ±0.02	−13.7 ±0.01
	6.70	−14.1 ±0.00	−14.4 ±0.00	−13.9 ±0.00	−14.0 ±0.01	−13.7 ±0.02
R level	4.77	100		83.4		83.0
	5.46	100		84.6		83.5
	6.42	100		92.3		89.9
	6.70	100		85.7		84.9

Lithol rubine BK

	pH	Day 0	3.5	7	10.5	14
L	4.77	54.6 ±0.01	54.6 ±0.02	54.5 ±0.02	55.0 ±0.00	54.5 ±0.04
	5.46	54.5 ±0.00	54.5 ±0.01	54.6 ±0.02	54.9 ±0.01	54.4 ±0.02
	6.42	54.4 ±0.00	54.3 ±0.05	54.4 ±0.02	54.6 ±0.03	54.0 ±0.03
	6.70	54.7 ±0.02	54.5 ±0.00	54.7 ±0.02	54.5 ±0.02	54.1 ±0.01
a	4.77	16.6 ±0.01	15.9 ±0.02	16.7 ±0.01	16.5 ±0.02	16.8 ±0.03
	5.46	16.3 ±0.00	16.0 ±0.05	17.0 ±0.06	16.3 ±0.03	16.7 ±0.09
	6.42	16.1 ±0.05	15.6 ±0.08	16.4 ±0.05	16.4 ±0.06	16.6 ±0.07
	6.70	16.0 ±0.02	15.7 ±0.04	16.6 ±0.04	16.2 ±0.03	16.6 ±0.04
b	4.77	13.7 ±0.01	13.3 ±0.02	13.3 ±0.01	13.4 ±0.01	12.9 ±0.02
	5.46	13.2 ±0.02	13.1 ±0.02	13.0 ±0.03	13.0 ±0.04	12.7 ±0.03
	6.42	14.2 ±0.02	14.2 ±0.03	14.1 ±0.02	14.4 ±0.03	13.8 ±0.03
	6.70	14.5 ±0.01	14.5 ±0.01	14.4 ±0.02	14.4 ±0.02	14.0 ±0.01
R level	4.77	100		90.7		90.5
	5.46	100		91.6		93.8
	6.42	100		99.0		102.8
	6.70	100		101.0		104.4

* L: Lightness.
* a: Redness (red to green).
* b: Yellowness (yellow to blue).
* R level: Residual level.

Table 4
Individual uncertainties of the sample preparation (U_{prep}), reference material (U_{RM}), standard stock solution (U_{std}), calibration curve (U_{cal}), repeatability (U_{rep}), and expanded uncertainty (U) according to the Eurachem Guide.

Analytes	U_{prep}	U_{RM}	U_{std}	U_{cal}	U_{rep}	U
Azorubine	0.0009	0.0304	0.0156	0.1152	0.0102	3.8146
Brilliant black BN	0.0009	0.0304	0.0156	0.1140	0.0106	3.3421
Lithol rubine BK	0.0009	0.0304	0.0156	0.1148	0.0061	3.5517

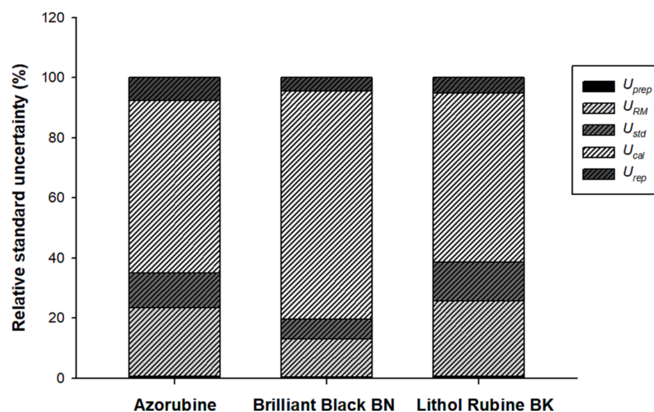

Fig. 2. Contribution of each source of uncertainty for food dyes analysis. U_{prep}, sample preparation; U_{RM}, reference material; U_{std}, standard stock solution; U_{cal}, calibration curve; and U_{rep}, repeatability.

dyes that are not designated in Korea. The method can be used to establish the safety of azo dyes in milk and cheese products. As some international organizations allow the use of azo dyes in various food matrices, a follow-up study on the development of a method that can analyze azo dyes in matrices other than milk and cheese should be conducted.

Data availability

The data that has been used is confidential.

CRediT authorship contribution statement

Se-Jeong Lee: Conceptualization, Methodology, Validation, Writing – original draft. **Xionggao Han:** Methodology, Formal analysis, Validation, Investigation. **Xiao Men:** Software, Data curation, Visualization, Project administration. **Geon Oh:** Software, Investigation, Resources, Visualization. **Sun-Il Choi:** Conceptualization, Validation, Data curation, Writing – review & editing, Supervision. **Ok-Hwan Lee:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

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