Rapid Method for Quantification of Seven Synthetic Pigments in Colored Chinese Steamed Buns Using UFLC-MS/MS without SPE

He-Gang Gao,* Wen-Jie Gong,** and Yong-Gang Zhao***†

*Shaoxing Center for Disease Control and Prevention, Shaoxing, Zhejiang 312071, P. R. China
**Cixi Municipal Center for Disease Control and Prevention, Ningbo, Zhejiang 315300, P. R. China
***Key Laboratory of Health Risk Appraisal for Trace Toxic Chemicals of Zhejiang Provincial, Ningbo Municipal Center for Disease Control and Prevention, Ningbo, Zhejiang 315010, P. R. China

Synthetic pigments are still used instead of natural pigments in many foods and their residues in food could be an important risk to human health. A simple and rapid analytical method combining the low-cost extraction protocol with ultra-fast liquid chromatography-tandem quadrupole mass spectrometry (UFLC-MS/MS) was developed for the simultaneous determination of seven synthetic pigments used in colored Chinese steamed buns. For the first time, ethanol/ammonia solution/water (7:2:1, v/v/v) was used as extraction solution for the synthetic pigments in colored Chinese steamed buns. The results showed that the property of the extraction solution used in this method was more effective than critic acid solution, which is used in the polyamide adsorption method. The limits of quantification for the seven synthetic pigments ranged from 0.15 to 0.50 μg/kg. The present method was successfully applied to samples of colored Chinese steamed buns for food-safety risk monitoring in Zhejiang Province, China. The results found sunset yellow pigment in six out of 300 colored Chinese steamed buns (from 0.50 to 32.6 μg/kg).

Keywords Synthetic pigments, ultra-fast liquid chromatography-tandem quadrupole mass spectrometry (UFLC-MS/MS), colored Chinese steamed buns

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laboratory batch tests, and it can be applied to the routine analyses for the determination of trace synthetic pigment residue in colored Chinese steamed bun samples.

**Experimental**

**Reagents and materials**

Tartrazine, amaranth, carmine, sunset yellow, allura red, brilliant blue and erythrosine were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetonitrile and ammonium acetate (AmAc) of HPLC grade were purchased from Merck Company (Darmstadt, Germany). Deionized water was purified using a Millipore water purification system (Millipore, Billerica, MA). Colored Chinese steamed bun samples were collected from 11 different markets in Zhejiang.

**Equipment**

UFLC-MS/MS analyses were performed using a Prominence UFLC XR system equipped with a DGU-20A3 degasser, a CTO-20AC column oven, an LC-20AD pump, an SIL-20AC autosampler (Shimadzu Corp., Tokyo, Japan) and an AB SCIEX TRIPLE QUAD TM 5500 mass spectrometer (Applied Biosystems, Foster City, CA). The UFLC-MS/MS system was controlled and data were analyzed on a computer equipped with Applied Biosystems/MDS Sciex Analyst 1.5.1 (Applied Biosystems).

**UFLC-QqQ-MS/MS analysis**

UFLC analysis was performed on a Shim-pack XR-ODS II (100 mm × 2.0 mm i.d., 2.2 μm). Analytes were separated by UFLC using 5.0 mmol/L AmAc in acetonitrile as eluent (A), and 5.0 mmol/L AmAc in water as eluent (B). The linear gradient was: 0 → 3.00 min, 2.00 → 10.0% A (98.0 → 90.0%}

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**Fig. 1** Structural formulas of the seven synthetic pigments.
B); 3.00 → 7.00 min, 10.0 → 80.0% A (90.0 → 20.0% B); 7.00 → 7.01 min, 80.0 → 2.00% A (20.0 → 98.0% B) and 7.01 → 9.00 min, 2.00% A (98.0% B). Chromatographic separation of the synthetic pigments was accomplished at a constant flow of 0.40 mL/min and the injection volume was 5.0 μL. The column was thermostated at 40°C to increase the retention time reproducibility. Mass spectrometry analysis was performed using an electrospray ionization source in negative mode. The operation conditions were as follows: ion spray voltage, −4500 V; curtain gas (CUR), 40 psi; cell exit potential (CEP), −10 V; collision gas (CXP), −10 V; collision cell exit potential (CXP), −10 V. Nitrogen was used in all cases. Multiple-reaction monitoring (MRM) mode was used for quantification. The results of the precursor ion, product ion, corresponding declustering potential (DP) and collision energy (CE) are shown in Table 1.

**Sample preparation**

Batch tests were conducted to evaluate the excellent sensitivity and selectivity of the UFLC-MS/MS method. A sample of 1.0 g was weighed into a 10 mL polypropylene centrifuge tube, and then 5.0 mL of ethanol/ammonia solution/water (7:2:1, v/v/v) was added. The contents were homogenized for 1.0 min using an Ultra Turrax mixer. Subsequently, the mixture was centrifuged at 6800 rpm for 2.0 min. The residues were again extracted with 5.0 mL of ethanol/ammonia solution/water (7:2:1, v/v/v). The supernatant was concentrated to dryness with a nitrogen stream and was redissolved with 100.0 μL of initial mobile phase and filtered using a 0.22 μm polytetrafluoroethylene (PTFE) membrane prior to its injection into the UFLC-MS/MS system.

**Method validation**

Individual stock standard solutions were prepared at 1000 mg/L level by exact weighing and dissolution in water-methanol (1:1, v/v). The stock mixture standard solution (10.0 mg/L) was prepared by appropriate dilution of the stock solutions with water-methanol (1:1, v/v). Matrix-matched calibration standards were spiked with concentration in the range of 0.5 – 100 μg/kg for tartrazine, amaranth, carmine, brilliant blue and allura red, and 0.25 – 50 μg/kg for sunset yellow and erythrosine. The matrix-matched calibration curves made by peak area vs. concentration (μg/kg) were used to calibrate spike samples in the recovery experiments.

Spiked recoveries were performed at concentrations of 0.8, 8.0, and 80 μg/kg (equivalent to 8, 80, and 800 μg/L) for tartrazine, amaranth, carmine, brilliant blue, and allura red, and 0.4, 4.0 and 40 μg/kg (equivalent to 4, 40, and 400 μg/L) for sunset yellow and erythrosine used in colored Chinese steamed bun samples, respectively. For each spiked sample, a stock mixture solution of the standards was added to 1.0 g of sample, which was free from the target compounds. The spiked samples prepared were stored at 4°C for about 12 h to let the synthetic pigments permeate uniformly into the samples.

The method was evaluated by linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy. Matrix-matched calibration standards with concentrations ranging from 0.25 to 100 μg/kg were prepared for the calibration curves. Calibration curves of peak area of quantitative ion pairs (as shown in Table 1) against the analyte concentration were used to calibrate spike samples in recovery experiments. LOD and LOQ were determined based on a signal-to-noise ratio of 3 (S/N = 3) and 10 (S/N = 10), respectively. Both the method accuracy and precision were estimated by tartrazine, amaranth, carmine, brilliant blue and allura red spiked at concentrations of 0.8, 8.0, and 80 μg/kg (equivalent to 8, 80, and 800 μg/L) and sunset yellow and erythrosine spiked at 0.4, 4.0 and 40 μg/kg (equivalent to 4, 40, and 400 μg/L) in blank samples, respectively. The method accuracies were expressed as the recoveries, and the method precisions were expressed as the intra-day and inter-day relative standard deviations (RSDs). The intra-day RSDs were obtained by repeating the three levels of spiked samples six times within a day, and the inter-day RSDs were obtained by repeating the three levels of spiked samples in triplicate on six separate days within a 2-week period.

**Results and Discussion**

**Optimization of UFLC-MS/MS conditions**

For optimization of the detection of seven synthetic pigments by MS, a standard solution of analytes (100 μg/L in water-methanol (1:1, v/v)) was infused directly into the MS. The objective of this set was to select representative ions (precursor and product ions) and to obtain values of DP, EP, CEP, CE and CXP for their detection. The confirmation procedures by the Commission Decision 2002/657/EC (Commission Decision 2002/657/EC 2002) for banned substances were established by using a minimum of four identification points. In this experiment, four identification points, one parent (1.0 point) and two transitions (each 1.5 points) were monitored. The final MS/MS conditions are detailed in Table 1.

In order to achieve an optimal chromatographic separation, the gradient elution and the effects of AmAc and ammonia concentration on the chromatographic separation were studied. Finally, a UFLC-MS/MS method was established to determine
The aim of the present study was to investigate the sample extraction procedure by using critic acid solution (pH 4.0) and ethanol/ammonia solution/water (7:2:1, v/v/v) as extraction solvents.\textsuperscript{13} And the effectiveness of the two different extraction solvents on extraction efficiency and analyte recovery were studied with blank samples spiked at a concentration of 0.8 μg/kg for tartrazine, amaranth, carmine, brilliant blue and allura red, and 0.4 μg/kg for sunset yellow and erythrosine. The average recoveries and RSDs of the studied analytes are shown in Fig. 2. It can be seen that some analytes, i.e., amaranth, carmine and erythrosine could not be extracted from the samples resulting in low recoveries when using critic acid solution as the extraction solvent (Fig. 2c). The recoveries of tartrazine, amaranth, carmine, sunset yellow, allura red, brilliant blue and erythrosine were 88.2, 57.3, 67.7, 98.2, 101.2, 92.2, and 20.5%, respectively. However, with the use of ethanol/ammonia solution/water (7:2:1, v/v/v) as the extraction solvent, the recoveries ranged from 89.2 to 102.2% (Fig. 2b). This reveals that the extraction property of ethanol/ammonia solution/water (7:2:1, v/v/v) is more effective than critic acid solution, which is used as the extraction solvent in the polyamide SPE adsorption method.\textsuperscript{13} In addition, the advantages of using the developed extraction procedure are obvious, namely a short sample preparation time without adsorption and desorption in the polyamide SPE procedure.

### Method linearity, accuracy, LOD and LOQ

The linearity of the calibration curves made by peak area vs. concentration (μg/kg) was studied using matrix-matched calibration standards in samples. The response function was found to be linear with a determination coefficient (r²) higher than 0.9990 in the tested range listed in Table 2 for the seven synthetic pigments.

The method accuracies were expressed as the recoveries, and the method precisions were expressed as the intra- and inter-day RSDs. The results are summarized in Table 3. It shows that the majority of mean recoveries were in the range of 82.6 – 109% with the intra-day RSDs ranging from 1.7 to 5.9% and inter-day RSDs ranging from 2.0 to 5.8%.

The LODs and LOQs for the analyzed synthetic pigments are shown in Table 2. The LODs and LOQs, which were calculated on the analysis of seven synthetic pigments spiked at 0.5 μg/kg for tartrazine, amaranth, carmine, brilliant blue and allura red, and 0.25 μg/kg for sunset yellow and erythrosine in blank samples that yielded an S/N ratio of 3 and 10, were in the range of 0.045 – 0.15 μg/kg and 0.15 – 0.50 μg/kg, respectively.

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
Synthetic pigment & Linear equation\textsuperscript{a} & r² & Linear range/μg kg\textsuperscript{-1} & LOD/μg kg\textsuperscript{-1} & LOQ/μg kg\textsuperscript{-1} & MRL\textsuperscript{b}/g kg\textsuperscript{-1} \\
\hline
Tartrazine & \(Y = 1.39 \times 10^4 X - 6.69 \times 10^3\) & 0.9996 & 0.5 – 100 & 0.13 & 0.43 & 0.08 \\
Amaranth & \(Y = 2.75 \times 10^4 X + 3.11 \times 10^4\) & 0.9991 & 0.5 – 100 & 0.15 & 0.50 & 0.25 \\
Carmine & \(Y = 2.06 \times 10^4 X + 7.31 \times 10^3\) & 0.9996 & 0.5 – 100 & 0.11 & 0.36 & 0.2 \\
Sunset yellow & \(Y = 5.44 \times 10^4 X + 7.5 \times 10^3\) & 0.9994 & 0.25 – 50 & 0.045 & 0.15 & 0.02 \\
Allura red & \(Y = 1.77 \times 10^4 X + 8.57 \times 10^3\) & 0.9992 & 0.5 – 100 & 0.10 & 0.33 & 0.07 \\
Brilliant blue & \(Y = 2.62 \times 10^4 X - 8.72 \times 10^4\) & 0.9998 & 0.5 – 100 & 0.072 & 0.22 & 0.015 \\
Erythrosine & \(Y = 3.55 \times 10^4 X - 2.85 \times 10^3\) & 0.9996 & 0.25 – 50 & 0.050 & 0.17 & 0.05 \\
\hline
\end{tabular}
\caption{Linear equations, determination coefficient (r²), linear ranges, limits of detection (LODs) limits of quantification (LOQs) and means maximum residue limit (MRL) of seven synthetic pigments}
\end{table}

\textsuperscript{a} Y: peak area; X: mass concentration, μg/kg. \textsuperscript{b} MRL for pigments tested in this work are referred to National Standard of the People’s Republic of China.\textsuperscript{17}
Synthetic pigments were illegally added to Chinese steamed buns by some illicit manufacturers in Zhejiang Province, China. The determination of synthetic pigments applied in food samples include fast analysis and a low-cost extraction procedure, the advantages of the technique described control the extraction efficiency. The obtained results showed that sunset yellow was found in six out of 300 colored Chinese steamed buns (0.50 to 32.6 μg/kg for sunset yellow and erythrosine) was used to control the extraction efficiency. The obtained results showed that sunset yellow was found in six out of 300 colored Chinese steamed buns (0.50 to 32.6 μg/kg), and other synthetic pigments were not detected in the analyzed samples with the proposed method. The typical UFLC-MS/MS chromatograms for one examined sample are shown in Fig. 3. It indicates that the synthetic pigments were illegally added to Chinese steamed buns by some illicit manufacturers in Zhejiang Province, China.

Furthermore, a comparison study among different methods for the determination of synthetic pigments applied in food samples was also performed, and the results are shown in Table 4. As is shown in the table, the proposed method gives a simpler and faster way for the extraction of synthetic pigments in solid food samples and provides a relatively lower LOD, higher recoveries of synthetic pigments and better precision.

**Table 3** Validation parameters obtained for the seven synthetic pigments at three concentration levels in colored Chinese steamed buns

| Synthetic pigment | Added/μg kg⁻¹ | Recovery, % | RSD, % |
|-------------------|---------------|-------------|--------|
|                   | Intra-dayᵃ | Inter-dayᵇ | Intra-dayᵃ | Inter-dayᵇ |
| Tartrazine        | 0.8       | 97.0        | 92.3     | 1.8     | 2.6 |
|                   | 8.0       | 92.6        | 88.2     | 2.4     | 3.9 |
|                   | 80.0      | 90.2        | 93.6     | 3.3     | 2.6 |
| Amaranth          | 0.8       | 100         | 112      | 2.3     | 2.9 |
|                   | 8.0       | 85.8        | 93.2     | 2.8     | 3.6 |
|                   | 80.0      | 96.0        | 88.6     | 4.8     | 5.8 |
| Carmine           | 0.8       | 91.2        | 92.4     | 5.9     | 3.5 |
|                   | 8.0       | 109         | 96.6     | 3.7     | 5.5 |
|                   | 80.0      | 92.6        | 96.4     | 1.7     | 5.5 |
| Sunset yellow     | 0.4       | 90.0        | 93.6     | 2.2     | 3.2 |
|                   | 4.0       | 92.5        | 100      | 2.6     | 2.6 |
|                   | 40.0      | 101         | 95.8     | 1.8     | 3.6 |
| Allura red        | 0.8       | 98.2        | 106      | 4.6     | 3.9 |
|                   | 8.0       | 86.2        | 99.2     | 2.0     | 3.7 |
|                   | 80.0      | 92.4        | 96.6     | 1.9     | 4.3 |
| Brilliant blue    | 0.8       | 102         | 100      | 3.2     | 3.6 |
|                   | 8.0       | 94.1        | 103      | 1.9     | 2.0 |
|                   | 80.0      | 96.2        | 98.7     | 3.3     | 3.2 |
| Erythrosine       | 0.4       | 82.6        | 92.8     | 3.2     | 5.2 |
|                   | 4.0       | 90.5        | 89.1     | 3.4     | 3.7 |
|                   | 40.0      | 90.9        | 92.3     | 5.2     | 5.6 |

ᵃ. Intra-day, n = 6 replicates. ᵇ. Inter-day, n = 3 replicates × 6 days within a 2-week period.

**Sample analysis**

To further validate the feasibility of the proposed method, it was used for the analysis of 300 colored Chinese steamed buns. A blank extract spiked at the low calibration level (0.5 μg/kg for tartrazine, amaranth, carmine, brilliant blue and allura red, and 0.25 μg/kg for sunset yellow and erythrosine) was used to control the extraction efficiency. The obtained results showed that sunset yellow was found in six out of 300 colored Chinese steamed buns (0.50 to 32.6 μg/kg), and other synthetic pigments were not detected in the analyzed samples with the proposed method. The typical UFLC-MS/MS chromatograms for one examined sample are shown in Fig. 3. It indicates that the synthetic pigments were illegally added to Chinese steamed buns by some illicit manufacturers in Zhejiang Province, China.

Furthermore, a comparison study among different methods for the determination of synthetic pigments applied in food samples was also performed, and the results are shown in Table 4. As is shown in the table, the proposed method gives a simpler and faster way for the extraction of synthetic pigments in solid food samples and provides a relatively lower LOD, higher recoveries of synthetic pigments and better precision.

**Conclusions**

In this study, a simple extraction procedure followed by UFLC-MS/MS has been successfully applied for the determination of synthetic pigments used for coloring Chinese steamed buns. Also, compared to the traditional polyamide adsorption method, the advantages of the technique described include fast analysis and a low-cost extraction procedure. Furthermore, the results demonstrate that the accuracy and precision of the proposed UFLC-MS/MS method are satisfactory for analysis of trace synthetic pigments in a wide variety of food with artificial color. This work was extended to the application of the proposed approach to a total of 300 samples collected from 11 different markets in Zhejiang.

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Table 4  Comparison of the analytical features of current LC-based methodologies for the determination of synthetic pigments in solid food samples

| Sample                          | Sample preparation (main steps)   | Detection | Recovery, % | RSD, % | Method LOD/μg kg⁻¹ | Ref.  |
|---------------------------------|-----------------------------------|-----------|-------------|--------|---------------------|-------|
| Fish products                   | SPE                               | LC-DAD    | 52 – 93     | 4 – 10 | 700 (Sunset yellow) 700 (Amaranth) 700 (Allura red) 700 (Erythrosine) | 16    |
| Lemon bread                     | Ultrasound-assisted solvent       | LC-DAD    | 80.5 – 97.2 | —      | 50 (Allura red) 10 (Tartrazine) 20 (Amaranth) 10 (Sunset yellow) | 18    |
| Colored Chinese steamed buns    | Solvent extraction                | LC-MS/MS  | 82.6 – 109  | 1.7 – 5.9 | 0.13 (Tartrazine) 0.15 (Amaranth) 0.11 μg/kg (Carmine) 0.045 (Sunset yellow) 0.10 (Allura red) 0.072 (Brilliant blue) 0.050 (Erythrosine) | This work |

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