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New LC-MS/MS Method for the Analysis of Allura Red Level in Takeaway Chinese Dishes and Urine of an Adult Chinese Population

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Supporting Information

ABSTRACT: Allura red is a widely used synthetic food dye. In this study, we developed and validated a LC-MS/MS method for the quantification of allura red in three popular takeaway Chinese dishes (braised pork, soy sauce chicken, sweet and sour pork) and human urine samples. High levels of allura red ranging from 2.85 to 8.38 mg/g wet weight were detected in the surveyed Chinese dishes. Of 113 participants who frequently consume the surveyed Chinese dishes (>once a week in the past 2 years), the median of their urinary allura red level was 22.29 nM/mM creatinine (95% CI = 19.48−25.03) . Risk assessment using Cox proportional hazard models showed that a 10-fold increase in urinary allura red was positively associated with high blood pressure (odds ratio of 1.75 (95% CI = 0.78−3.96)). Our findings provide new insights for the potential risk of hypertension for long-term allura red overconsumption.

KEYWORDS: *allura red, LC-MS/MS, Chinese dishes, urine, long-term exposure, high blood pressure*

INTRODUCTION

Allura red (Red No. 40 in U.S. FD&C and E129 in Europe) (Figure 1a) is one of the most widely used synthetic food additives in China and is present in many food products such as candies, beverages, and cakes due to its unique dark red color, low price, and excellent stability.[1] Takeaway meals are extremely popular in China, with many people eating once or twice a week from an increasing variety of catering outlets. Braised pork, soy sauce chicken, and sweet and sour pork are common in Chinese cuisine with the appearance of red color. The amount of allura red added in these dishes remains unclear, and no allura red limit has been set for Chinese cuisine by the food regulatory authorities.

Previous studies have indicated that azo dye overconsumption might cause hyperactivity in children[2] and induce DNA damage in the colon of mice.[3,4] In addition, in vitro analysis revealed the binding properties of allura red with human and bovine serum albumin.[5,6] Even though an acceptable daily intake (ADI) for allura red was established by the European Food Safety Authority (EFSA), the impact of allura red on human health has not been fully understood.

One of the limiting factors for the exposure assessment of allura red is the lack of the quantitative measurement of the dye in biological samples. Several methods have been reported for the determination of multiple color additives including allura red.[7−10] However, these methods were generally developed for food products with relatively simple food matrices.

It has been postulated that allura red might be metabolized to two compounds through azo reduction by the gut microbiome and excreted through feces.[7] Urine is the major route for the excretion of the parent form of synthetic dyes in humans and animals. Urine is a complex matrix containing high levels of urea, uric acid, creatinine, salts, and lipids, which might interfere with allura red quantification and cause ion suppression effect. The majority of current methods are not suitable for such complex biological fluids due to their low sensitivity and selectivity.[11] There is still a lack of a sensitive and accurate method for the quantification of allura red in urine samples.

In this work, we developed and validated a highly sensitive and reliable LC-MS/MS method to quantify allura red in three popular Chinese dishes and human urine using trypan blue as the internal standard. We measured the level of allura red in 103 urine samples from a Chinese population who had frequently consumed the surveyed Chinese dishes (>once a week) in the past 2 years. In addition, we examined the correlation between vital signs and urinary allura red level.

MATERIALS AND METHODS

Materials. Three popular Chinese dishes including sweet and sour pork, braised pork, and soy sauce chicken were purchased from five local restaurants in Yantai, China. A total of nine samples were collected for each kind of dish. The recipes are given in the Supporting Information. The purified standards of allura red (lot 071318), trypan blue (purity > 85%), and creatinine-(*methyl*-^{13}C) were bought from Sigma-Aldrich (Sigma-Aldrich, USA). LC-MS grade water, methanol, acetonitrile, and ammonium acetate (Ultra CHROMASOLV, Fluka, Switzerland) were also purchased from Sigma-Aldrich.

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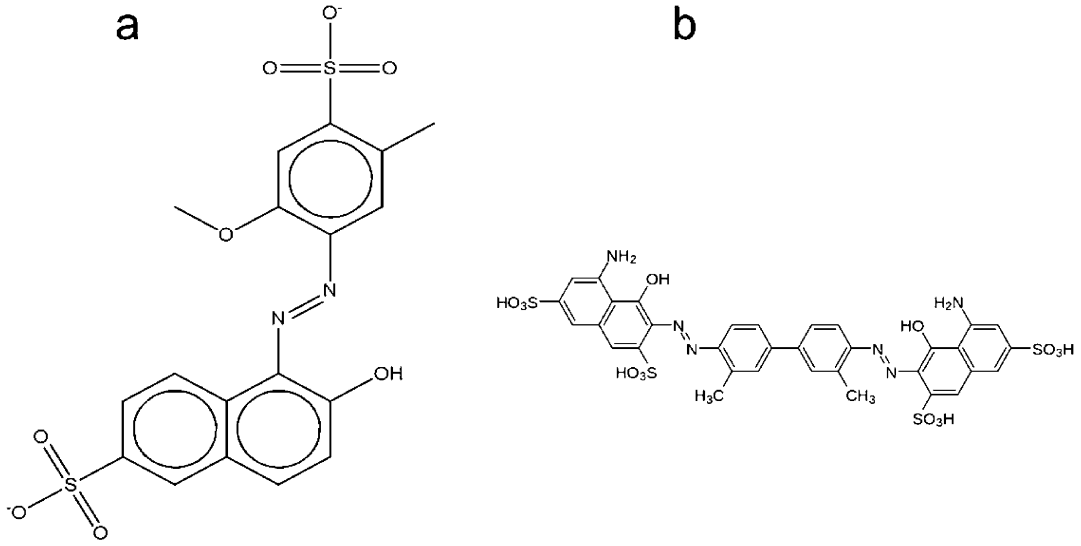

Figure 1. Chemical structures of (a) allura red and (b) trypan blue. Trypan blue was used as the internal standard for quantification of allura red.

Table 1. Parameters for Multiple Reaction Monitoring (MRM)[a]

compound	polarity	Q1	Q3	DP	CE	CXP
allura red	negative	225.1	207.0	−80	−22.1	−23
trypan blue (IS)	negative	435.2	185.0	−144.58	−57.8	−20.94

[a]DP, declustering potential (V); CE, collision energy (eV); CXP, collision cell exit potential (eV).

Questionnaires. The study is part of the nation-wide SUPERB Project (Study of Use of Products and Exposure-Related Behavior) involving 10 diverse regions in China. The frequency of consumption of three Chinese dishes (braised pork, soy sauce chicken, sweet and sour pork) in the past 2 years (between 2013 and 2014) was collected via questionnaire.

Clinic Visit. The study protocol was reviewed and approved by the ethics committees of Yantai Yuhuangding Hospital, Shandong province, China (Protocol YYH-2005-05-18-1). Written informed consent was obtained from each of the participants. Briefly, from January 2015 to May 2015, volunteers who consumed the above three Chinese dishes frequently (>one a week) were requested to visit our local clinics in Yantai Yuhuangding Hospital. A total of 44 men and 59 women were recruited. The volunteers' vital signs including body temperature, blood pressure, heart rate, and breathing rate were measured by the nurses. The body mass index (BMI) of each volunteer was also collected. Urine samples were collected and stored in 50 mL polypropylene cups at −80 °C until analysis. To check the reproducibility, a second spot urine sample was collected from each participant at a different time on his/her visiting day (at least 2 h apart within a single day).

Allura Red Extraction. For Chinese cuisine, the samples were mixed thoroughly and lyophilized in a lyophilizer (Labconoco, USA). About 50 mg of the powder was weighed and transferred to 2 mL tubes. Nine volumes of methanol/water (20:80, v/v) with 10 μM trypan blue was added and mixed thoroughly. The mixture was then sonicated for 10 min at room temperature and centrifuged at 16000*g* for 10 min at 4 °C. The supernatant was collected and stored at −80 °C until analysis.

For human urine, 90 μL of urine was mixed thoroughly with 10 μL of trypan blue (final concentration of 10 μM) and 400 μL of MeOH/ACN (50:50, v/v). The mixture was incubated on ice for 10 min and then centrifuged at 16000*g* for 10 min at 4 °C. The protein and large molecules were precipitated, and the supernatant was taken for LC-MS/MS analysis.

LC-MS/MS Analysis. The separation of allura red was performed on an Agilent UHPLC (UHPLC1260, Agilent, USA). The mobile phase consisted of a gradient of water (A) and methanol (B), both containing 20 mM ammonium acetate. Ten microliters of extract was injected onto a Nucleosil C_{18} column (150 × 2.1 mm, 5 μm, Macherey-Nagel, Germany) held at 30 °C for chromatographic separation. Elution was performed using the following gradient: 0−100% B at 0−15 min, 100% B at 15−18 min, 0% B at 18.1−23 min. The flow rate was 300 μL/min. All of the samples were kept in a Leap PAL CTC autosampler with the temperature controlled at 4 °C prior to injection during analysis.

A Qtrap 5500 hybrid triple-quadrupole mass spectrometer (SCIEX, USA) equipped with electrospray source operating in negative mode was used for detection in multiple reaction monitoring (MRM) mode. The MRM transitions and source conditions were optimized using purified standards. The optimal conditions for allura red and trypan blue are listed in Table 1. The parameters for electrospray ionization source were set as follows: source temperature, 500 °C; curtain gas, 30; ion source gas 1, 35; ion source gas 2, 35; spray voltage, −4500 V; and CAD gas, high.

Method Validation. The method was validated by determining the lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), linearity, reproducibility, specificity, and recovery (Table 2 and Figure 2). Briefly, different concentrations (25, 50, 100, 500, 1000, and 1500 μg/g) of allura red were spiked into three Chinese dishes and human urine and analyzed using the developed method. The linearity was evaluated using six-point standard curves. The linear range and regression coefficient (r^2) were calculated. The LLOQ (S/N

Table 2. Validation Data for LC-MS/MS Analysis of Allura Red in Chinese Cuisine and Human Urine[a]

matrix	LLOQ	ULOQ	recovery in samples (%)	CV_{inter} (%)
braised pork	25 μg/g	1500 μg/g	95.9	6.53
soy sauce chicken	25 μg/g	1500 μg/g	98.3	7.14
sweet and sour pork	50 μg/g	1500 μg/g	87.4	6.96
human urine	0.1 nM	3000 nM	97.6	5.98

[a]LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; CV_{inter}, interbatch coefficient variance.

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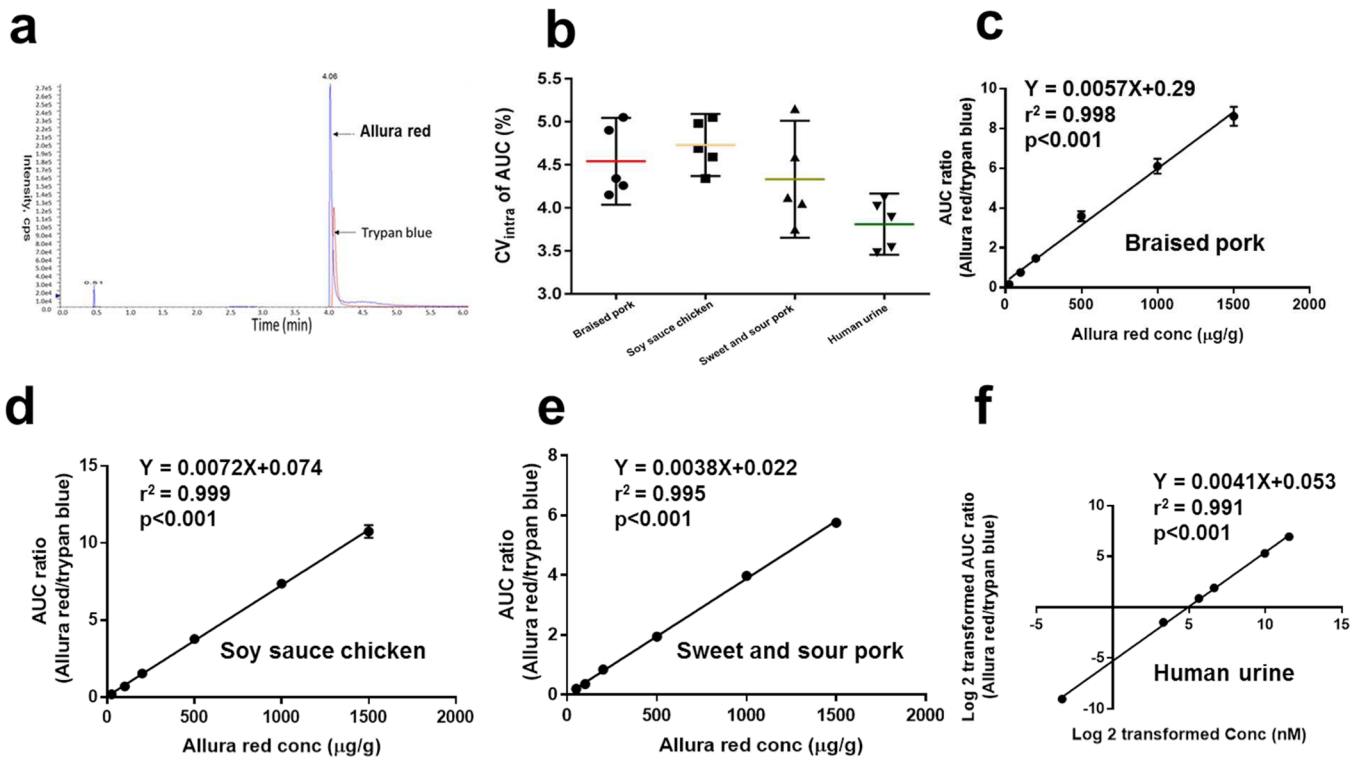

Figure 2. LC-MS/MS method development and validation for the analysis of allura red: (a) representative LC-MS/MS chromatogram of allura red (225.1−207.0) and trypan blue (435.2−185.0) monitored in MRM negative mode; (b) reproducibility illustrated by intrabatch coefficient variance (CV_{intra} or RSD) of allura red AUCs. A pooled sample from each kind of dish and human urine was analyzed five times a day for 5 days. The CVs of allura red AUC on each day and across 5 days were calculated. (c−f) Linearity evaluation. Allura red was spiked into the matrix at concentrations of 25, 50, 100, 500, 1000, and 1500 μg/g. The samples were extracted and analyzed using the developed method. A linear regression analysis was conducted.

> 10) and ULOQ were the two end points of the standard curves. The intrabatch and interbatch reproducibilities were assessed by injecting each kind of sample five times in 1 day for 5 days. The CV_{intra} (coefficient of variance) and CV_{inter} were calculated. The allura red negative samples were spiked with trypan blue internal standard, extracted, and analyzed for specificity. No significant interfering peaks were observed at the retention time of allura red.

Urinary Creatinine. The creatinine level in urine was measured according to Jaffe's colorimetric method on an automated plate reader using a creatinine assay kit (Sigma-Aldrich). Briefly, 10 μL of urine was mixed with 50 μL of reaction mix on a 96-well plate. The reaction was kept for 60 min in the dark, and the absorbance at 570 nm was recorded. Creatinine concentration was calculated using a standard curve run at the same time.

Data Analysis. Data analysis was conducted using Graphpad 6.0 (Graphpad Prism). Absolute concentration of allura red in Chinese cuisine was calculated using matrix-specific standard curves and the concentration of trypan blue and converted to wet weight (ww) basis (mg/g, ww). The concentration of allura red in human urine samples was normalized with urinary creatinine level.[12] The Mann−Whitney nonparametric test was used to determine the significant difference of urinary allura red between male and female participants. Spearman correlation was used to determine the correlation between age and urinary allura red level. Multivariate linear regression and correlation model were used to assess the associations between urinary allura red concentration and vital signs. Odds ratio and 95% confidence intervals (CIs) were calculated using logistic regression.

RESULTS AND DISCUSSION

Method Development. The MRM transitions and compound-specific parameters for allura red were optimized using the purified standard. A previous study had used 452.1 ($[M - H]^-$) as the parent ion for LC-MS/MS quantification.[7] In this study, we found that 225.1 ($[(M - 2H)/2]^{2-}$) in negative mode was much more abundant than 452.1 and was the dominant parent ion for allura red. The dominant daughter ion (fragment) was 207.0 (Figure SI-2). For quantitative LC-MS/MS methods, the use of internal standards is essential for the normalization of extraction variability, detection signal shift, and matrix effect. No internal standards have been used in the previous methods, which might cause erroneous concentration measurements of allura red.[13] Here, we selected trypan blue as the internal standard for quantification of allura red because it is also an azo dye and has a chemical structure similar to that of allura red (Figure 1b). In addition, trypan blue is not a food dye and not present in the matrix. The MRM transitions for trypan blue are 435.2 (Q1, parent ion) and 185.0 (daughter ion, Q3) (Figure SI-3). The compound-specific parameters for allura red and trypan blue are listed in Table 1. We then tested the analysis of allura red on different types of LC columns. Allura red was eluted completely from a Nucleosil C_{18} column, whereas it was not detected on the aminopropyl column (Figure SI-4). A representative chromatogram is shown in Figure 2a. Allura red and trypan blue were coeluted together at 4 min, which makes trypan blue suitable as an internal standard for the quantification of allura red.

Analytical Validation. The method was analytically validated by determining the reproducibility, linearity, specificity, and recovery. We showed that the peak area (AUC) reproducibility was excellent. The average intrabatch coefficient of variance (CV_{intra}) for 5 days was <5% (Figure 2b). The 5-day interbatch CV (CV_{inter}) was between 5.98 and 7.14%. The

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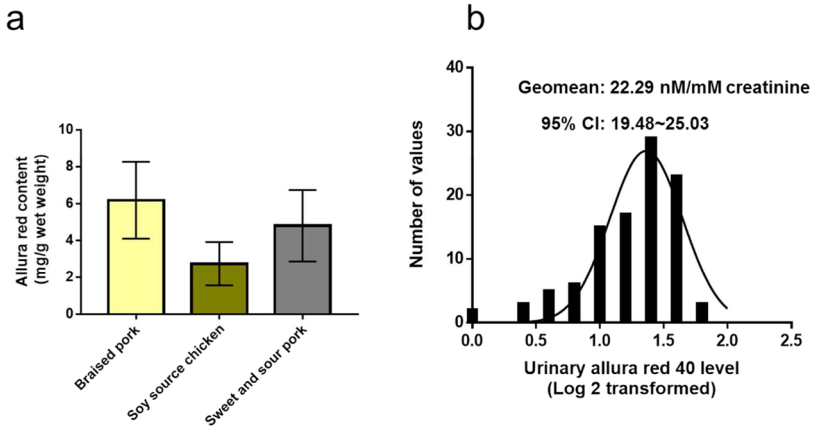

Figure 3. Analysis of allura red in Chinese cuisine and urine samples of a Chinese population: (a) content of allura red in three popular takeaway Chinese dishes (mean ± SD, n = 9); (b) distribution of urinary allura red in an adult Chinese population. Data were log 2 transformed before statistical analysis.

method also showed good linearity with regression coefficients >0.9 (Figure 2c–f). The LLOQ for allura red was 25 μg/g in braised pork and soy sauce chicken, 50 μg/g in sweet and sour pork, and 0.1 nM in human urine (Table 2). The recovery of allura red in three Chinese dishes and human urine was between 87 and 98%. Our method was about 40-fold more sensitive than the published methods.[7,14] In addition, no pretreatment is needed in our method, which was usually required for the previous methods.[15−17]

Concentration of Allura Red in Chinese Cuisine. We then analyzed the level of allura red in three popular Chinese dishes including sweet and sour pork, braised pork, and soy sauce chicken using the developed LC-MS/MS method. Nine samples were collected from three different catering centers for each dish. As shown in Figure 3a, allura red was detected in all three kinds of Chinese dishes we tested. The average allura red level in braised pork was 6.17 mg/g ww (range, 4.09−8.38 mg/g ww). The level of allura red in soy sauce chicken was between 1.55 and 3.91 mg/g ww. Of nine samples tested, sweet and sour pork had an average allura red level of 4.79 mg/g ws with a maximum value of 7.48 mg/g ww and a minimum value of 2.85 mg/g ww. The allura red in these Chinese dishes might be from the commercial chili pepper powder, chili oil, soy sauce, or the direct addition by chefs during cooking.[18] The current food regulations in China have not set a legal limit for the use of allura red in Chinese cuisine. EFSA has established an ADI of 0−7 mg/kg body weight/day.[19] If a 60 kg adult takes a full meal (500 g) of any of the above three collected Chinese dishes per day, the amount of allura red consumed (>23.8 mg/kg body weight/day) could be far beyond the ADI.

Concentration of Allura Red in the Urine of a Chinese Population. The characteristics of 103 participants in this study are listed in Table 3. There were about equal numbers of males and females. The age range was 19−65 years old with about 51% of participants at 19−34 years old, 30% at 35−50 years old, and 20% at 51−65 years old. These volunteers have frequently consumed the above three Chinese dishes in the past 2 years. The allura red in the collected urine samples was analyzed and adjusted by creatinine concentration using the developed LC-MS/MS method. The reproducibility of unadjusted allura red in repeated urine samples was excellent (interclass correlation coefficient > 0.75). The level of allura red was log-normal distributed (Figure 3b). The median of allura red in the urine of the tested Chinese population was 22.29 nM/mM creatinine. The 95% confidence interval (CI) was between 19.48 and 25.03 nM/mM creatinine (Figure 3b). Spearman's rank correlation coefficient results showed a slightly positive correlation between the urinary allura red and age in both male and female participants (Table 4). Animal studies reported that allura red is either directly excreted by urine or converted to other compounds in the intestine by gut microbiota and excreted by feces.[20] It is likely that the composition of gut microbiota changes with age, which results in less conversion of allura red and more direct excretion through urine.[21] The median of allura red in the women's urine was slightly higher than that for the men without statistical significance (p = 0.12, Figure SI-5). It was reported that about

Table 3. Characteristics of the Participants[a]

category	N (%) or mean ± SD
gender	
men	44 (42.3%)
women	59 (57.3%)
age (years)	
19−34	52 (50.5%)
35−50	31 (30.1%)
51−65	20 (19.4%)
BMI, kg/m^2	19.2 ± 4.1
race	
Han	103
vital signs	
SBP	129.4 ± 27.4
DBP	83.1 ± 18.2
heart rate	94.8 ± 17.6
breathing rate	26.7 ± 5.9

[a]BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 4. Spearman's Correlation between Age and Urinary Allura Red Level

	urinary allura red level	
age	Spearman r	p value
male	0.579	<0.001
female	0.663	<0.001
all participants	0.613	<0.01

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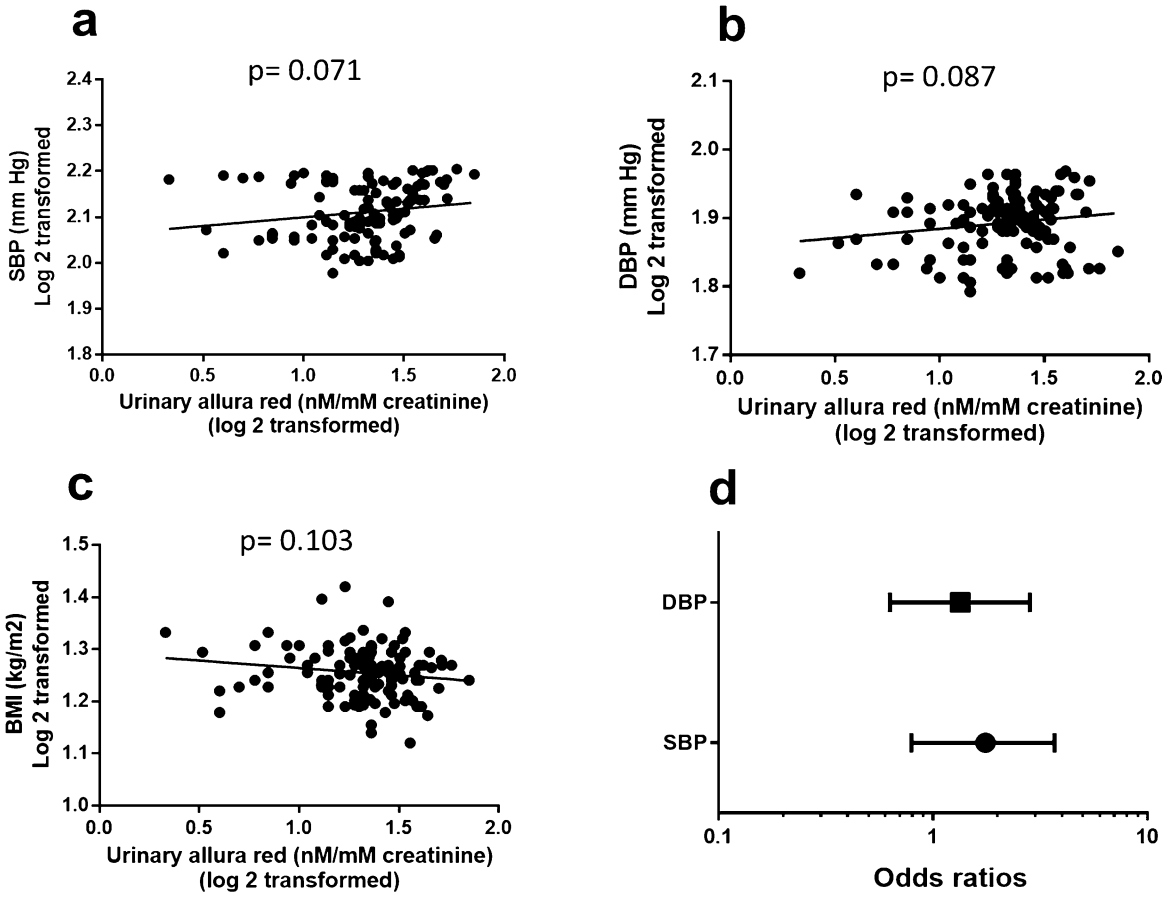

Figure 4. Correlations between urinary allura red and vital signs: (a) correlation with systolic blood pressure (SBP); (b) correlation with diastolic blood pressure (DBP); (c) correlation with body mass index (BMI); (d) odds ratios (95% CI) for blood pressure associated with a 10-fold increase of urinary allura red. Data were log 2 transformed prior to statistical analysis. Multiple variate linear regression, Pearson's correlation, and odds ratio analysis were performed.

5.7−19.8% of allura red were excreted via urine in rats.[20] Our study provided the first excretion data of allura red in adult human urine.

Correlations between Urinary Allura Red and Vital Signs. We next investigated the potential correlations between urinary allura red and vital signs in adults including systolic blood pressure (SBP), diastolic blood pressure (DBP), and body mass index (BMI). We found a positive association between urinary allura red and SBP in log 2 space (correlation p = 0.071) (Figure 4a). A 10-fold increase in urinary allura red concentration was associated with an odds ratio of 1.75 (95% CI = 0.78−3.96) for hypertension (SBP > 120 mmHg) (Figure 4d). The accumulation of urinary allura red was also positively correlated with the increase of DBP (correlation p = 0.087) (Figure 4b). The odds ratio was 1.33 for the association between a 10-fold increase in urinary allura red and prehypertension and hypertension of DBP (>80 mmHg) (Figure 4d). Although the association was not statistically significant, BMI tended to decrease with the increase of allura red level in urine (correlation p = 0.103) (Figure 4c).

Artificial colors have been reported to cause attention deficit hyperactivity disorder (ADHD) in children.[2] No previous studies have reported an association between allura red exposure and high blood pressure in adults. It is likely that long-term exposure of excess allura red induced oxidative stress and the increased oxidative stress led to hypertension.[22,23]

Due to the relatively small sample size, the link between long-term allura red consumption and high blood pressure needs to be further evaluated in a larger population. The fat and sugar contents could also be factors that might lead to high blood pressure in that population who consumed the above Chinese dishes frequently.

In conclusion, a high level of allura red was detected in the surveyed samples of three popular Chinese dishes including braised pork, soy sauce chicken, and sweet and sour pork. The amount of allura red taken in a standard meal was found to exceed the recommended ADI. Among the study populations, the geomean of allura red in the urine samples was 22.29 nM/mM creatinine with a 95% CI between 19.48 and 25.03 nM/mM creatinine. Our findings also suggested that overdose of allura red might be associated with hypertension in adults. Additional large-scale prospective population studies are needed to confirm our findings.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b05310.

MS/MS and LC spectra for allura red and trypan blue; comparison of male and female urinary allura red levels (PDF)

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Author Contributions

‖K.L. and Y.X. contributed equally and both recruited volunteers, developed the method, and wrote part of the manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; ADI, acceptable daily intake; BMI, body mass index; EFSA, European Food Safety Authority; MRM, multiple reaction monitoring; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; CV, coefficient of variance; SBP, systolic blood pressure; DBP, diastolic blood pressure; ADHD, attention deficit hyperactivity disorder

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