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Discovery of Environmental Rhodamine B Contamination in Paprika during the Vegetation Process

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ABSTRACT: Recently, rhodamine B (RhB) in paprika and chilli has attracted much attention. Almost all the literature has deemed that the detectable RhB was attributed to malicious intents in the fabrication process. However, the occurrence of increasing cases with ultratrace levels of RhB was difficult to understand on the basis of that statement. Here, we report on the discovery of environmental RhB contamination in paprika during its vegetation process. Samples including paprika, soils, and stems collected from seven fields in the Xinjiang Region, China, were detected by ultraperformance liquid chromatography−tandem mass spectrometry. Far from any anthropogenic addition, the ultratrace RhB concentrations in all the paprika samples provided unambiguous evidence that environmental RhB contamination in paprika had really occurred over its growth period. Further illation suggests that the soil contaminated by RhB is one of the major contamination sources and that there may be a degradation of RhB in paprika during the late maturation stage. The discovery has significant implications for re-evaluating the origin of the RhB in paprika- and chilli-containing products.

KEYWORDS: *rhodamine B, paprika, UPLC−MS/MS, environmental contamination*

INTRODUCTION

Rhodamine B (RhB), an important xanthene dye with high water solubility, is widely used as a fluorescent dye in a variety of applications, ranging from a fluorescent reagent in the laboratory to a tracer dye in biotechnology application and a colorant in chemistry industries such as glass, fireworks, paper, textile, plastic, paint drawing, and dyed pesticides.[1−8] Because of its intense red-orange color and low price, RhB (Figure 1) has also been attractive as a food colorant, particularly in paprika- and chilli-containing foods. However, following a thorough safety evaluation, there are increasing reports evidencing that RhB dyes have developmental toxicity,[9] mutagenicity,[10] and carcinogenic activities.[11] Hence, the European Food Safety Authority (EFSA) has declared RhB to be potentially genotoxic and carcinogenic.[12] Consequently, most countries, including those of the European Union (EU) and China, have banned its use in foodstuffs.[13]

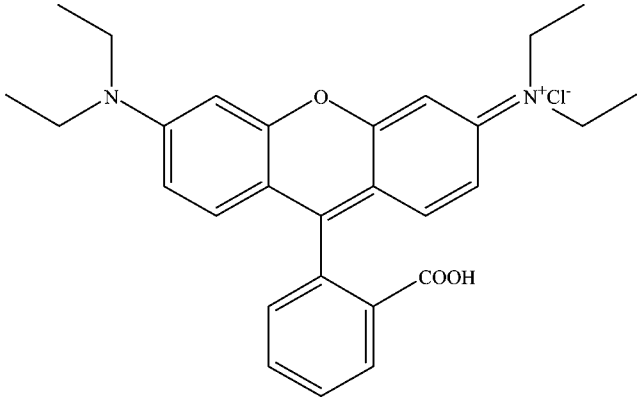

Figure 1. Molecular structure of RhB.

To monitor and prevent the abuse of RhB in foodstuffs or seasoning production, various analytical methods have been developed, including immunoassays,[14] capillary electrophoresis,[15] spectrophotometry,[16−19] high-performance liquid chromatography (HPLC),[20,21] high-performance liquid chromatography−mass spectrometry (HPLC−MS),[22] and even liquid chromatography−tandem mass spectrometry (LC−MS/MS).[23−25] Among them, LC−MS/MS is becoming the most popular method due to its outstanding performance both in qualitative and in quantitative analysis. Thus, in China, LC−MS/MS has been regulated as a public determination method referring to the official SN/T 2430-2010 standard method, with a limit of detection (LOD) of 5 μg/kg.[26] According to this standard method, RhB was not permitted to be found in foodstuffs, so the permissible detectable threshold was not more than the LOD of 5 μg/kg.

In spite of the strict directives, there are still a great many cases each year of RhB dye discovery in some chilli-containing food products reported by the EU and China. In most cases, the concentration of RhB dyes exceeded the threshold of 5 μg/kg; thus, the sources of these cases were reasonably judged as illegal addition. However, with the detection ability being pushed below the microgram per kilogram level, increasing cases with the concentrations of RhB dyes much lower than 5 μg/kg have frequently been found. Due to the ultratrace amount in the range of 0.01−1 μg/kg, these cases cannot be absolutely explained by artificial addition. As such, some other potential sources of RhB dye contamination in chilli or paprika caused by environmental pollution during the plant growth,

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Table 1. Type and Amount (kg) of Samples Collected in Different Fields

field no.	paprika type	*Capsicum* species	soil	stem	paprika fruit/maturation stage: green (80 days)[a]	orange (86 days)[a]	red (95 days)[a]
1	urn	*C. frutescens*	2.2	1.5	2.3	2.5	2.5
2	urn	*C. frutescens*	2.1	1.7	2.8	2.7	2.3
3	sweet	*C. annuum*	3.0	–[b]	2.1	2.0	2.0
4	urn	*C. frutescens*	2.5	–	–	2.9	2.8
5	urn	*C. frutescens*	2.8	1.3	–	2.9	2.1
6	line	*C. frutescens*	–	1.9	–	2.1	2.0
7	urn	*C. frutescens*	–	1.7	2.0	2.3	2.4

[a]The maturation stage was expressed by the fruit color and number of days after sowing. [b]A dash means no such sample.

Table 2. Analysis Parameters of the UPLC–MS/MS Method for RhB

sample type	matrix-matched linear equation[a]	LR[b] (μg/kg)	R^2	LOD (μg/kg)	LOQ (μg/kg)	recovery (%)	RSD (%)
stem powder	$y = 2408.4x - 6.39$	0.05–20.0	0.9991	0.02	0.07	90.1–98.8	9.2
paprika powder	$y = 2562.8x + 40.64$	0.05–20.0	0.9993	0.03	0.09	86.9–93.5	7.8
soil	$y = 3107.1x + 53.91$	0.01–10.0	0.9990	0.02	0.06	78.8–99.6	8.6

[a]y and x represent the peak area and the concentration of the spiked working solution, respectively. [b]Linear range.

harvest, and storing processes should be explored. However, it is a pity that because almost all the researchers have generally deemed that RhB in paprika or chili products is added intentionally, most attention is paid to developing analytical methods for the determination of RhB residues in foodstuffs while less attention has been focused on environmental RhB contamination[27,28] and still less on the contamination source and mechanism.

The objective of this study is to identify the occurrence of environmental RhB contamination in paprika during its vegetation process, to assess the variation of RhB contamination levels in paprika samples among different growing fields, paprika types, and maturation stages, and to simply explore the environmental source of RhB contamination in paprika during its growth process. This study has significant implications for re-evaluating the origin of RhB in paprika- and chilli-containing products.

MATERIALS AND METHODS

Chemicals and Materials. RhB standard was purchased from Dr. Ehrenstorfer (Augsburg, Germany), and its individual standard stock solution was prepared in methanol with a concentration of 10 mg/kg. Methanol, acetonitrile, and 2-propanol of HPLC grade were purchased from Fisher Scientific (Waltham, MA), while HPLC grade formic acid was supplied by Anpu Scientific Instrument Co., Ltd. (Shanghai, China). All the water used was ultrapure water prepared and refreshed daily. All other reagents such as ethanol and acetone were of analytical grade.

Apparatus. Ultraperformance liquid chromatography (UPLC)–MS/MS analysis was performed on a Waters Acquity UPLC system integrated with an electrospray ionization triple-stage quadrupole mass spectrometry (ESI-TQ-MS) system (Waters Corp., Milford, MA) through an autosampler system (Waters Corp.). The analytical data from UPLC–MS/MS were collected and processed using the software MassLynx V4.1.

A Master-S UVF model laboratory water purification system (Hetai Instrument Co., Ltd., Shanghai, China) was used to prepare ultrapure water, and an FW135 universal grinder (Taisite Instrument Co., Ltd., Tianjin, China) was employed for the trituration of the samples. An XW-80A model vortex instrument (Jingke Co., Ltd., Shanghai, China) and a TH-300B model ultrasound cleaner (Tianhua Ultrasound Instrument Co., Ltd., Jining, China) set at 40 W were used to improve the extraction efficiency, and an H-2050R model freezing centrifuge (Changsha Xiangyi Centrifuge Equipment Co., Ltd., Changsha, China) was used to separate the phases after extraction.

Sample Collection and Preparation. Paprika fruit samples as well as the corresponding stem and soil samples were collected randomly from seven different fields (30–50 km apart from each other) in Xinjiang Uygur Autonomous Region, China. Within each field, paprika samples at three different stages of ripening, including green, orange, and red fruits, were cut from the same plant respectively on Aug 9, 15, and 24, 2011, which were the 80th, 86th, and 95th days after seeding, respectively. At every collection time, each batch of samples in the same color was collected on three different plants at similar vegetable statuses. After the last harvest, the corresponding paprika stems and surface vegetative soil down to 20 cm were collected. The sample type and amount are listed in detail in Table 1.

After homogenization of the whole paprika or stem samples, an aliquot of approximately 200 g was selected as a subsample for sample preparation. The subsamples were powdered by a grinder after being dried at 80 °C in the air-dry oven. The soil samples were laid in the sun to dry completely and then pulverized and passed through an 80-mesh stainless steel sieve. All the powdered samples were packaged in sterile polyethylene bags and stored in a desiccator at room temperature until analysis.

Extraction Procedure. A 1.0 g weighed paprika or stem sample was placed in a 50 mL centrifuge tube and extracted with 10 mL of methanol on a vortex machine for 2 min, followed by extraction with the aid of ultrasound for 10 min. After freezing centrifugation at 10 000 rpm for 5 min, the supernatant was collected and filtered through a 0.22 μm membrane for injection into the UPLC–MS/MS system by an autosampler vial.

The soil samples were weighed at 10.0 g every time and then extracted according to the above procedure except that the time of ultrasound-assisted extraction was prolonged from 10 to 30 min.

UPLC Conditions. Chromatographic separation was carried out on a Waters Acquity BEH C_{18} column (2.1 × 50 mm, 1.7 μm) at 35 °C. The injection volume was 5 μL, and the total flow rate was 0.35 mL/min. The mobile phase consisted of (A) acetonitrile with 0.1% formic acid and (B) water with 0.1% formic acid. Gradient elution was conducted as follows: Solvent A was initialized at 50%, followed by a linear gradient to 70% A within 0.5 min and a linear gradient to 95% A in the subsequent 2.5 min. Finally, after maintenance for 1 min, the mobile phase was recovered to 50% A within 1 min. The total run time of the program was 5.0 min.

Mass Conditions. The identification and quantitation of RhB were achieved by the ESI-TQ-MS system using multiple reaction monitoring (MRM) in positive ion mode. The identification ion pairs of RhB were set as 443.4/399.3 and 443.4/355.2, the former of

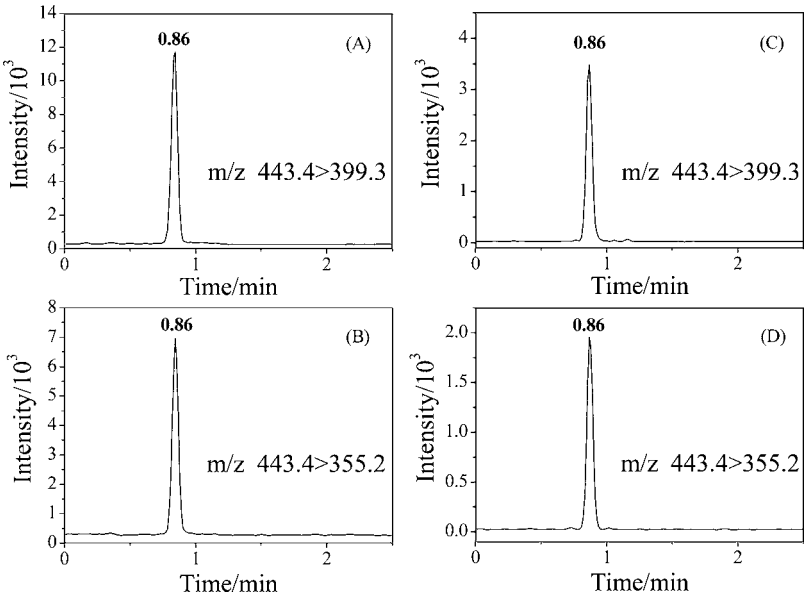

Figure 2. UPLC−MS/MS chromatograms of the RhB standard solution (0.5 μg/kg) (A, B) and paprika sample (0.2 μg/kg) (C, D).

which being set as the quantitative ion pair. The source temperature and desolvation temperature were set at 120 and 420 °C, respectively. As the collision gas, argon had a flow rate of 0.24 mL/min, whereas the desired amount of N_2 was 700 L/h.

Analytical Parameters. The main analytical parameters of the selected analytical method are presented in Table 2. The matrix-matched calibration curves of the paprika powder, stem, and soil were built from six corresponding blank samples spiked with gradient concentrations. The LOD and the limit of quantitation (LOQ) were calculated on the basis of signal-to-noise ratios (S/N) of 3 and 10, respectively. Recovery experiments were carried out by spiking three different concentrations (5, 10, and 15 μg/kg) of standard solutions into a selected sample and carrying them through the entire extraction procedure as for the samples. Meanwhile, the precision of the analytical method was determined by repeatedly analyzing the spiked samples five times.

Data Analysis. The concentrations of the sample solutions (C_x, μg/kg) were obtained through the external standard method by the workstation automatically, and the concentrations of RhB (X, μg/kg) in paprika or related samples were calculated by multiplying C_x by the dilution factors (10 for paprika and stems and 1 for soils) according to the sample amounts.

Analytical Quality Assurance. Prior to analysis of a sample set, the UPLC−MS/MS system performance and calibration were verified for the analyte. A solvent blank (methanol) was injected to ensure that the system was free from contaminants or interfering peaks. Analytical quality assurance measures for RhB determination also involved inserting a procedural blank in each batch of 8 samples and a certified standard reference material after every 40 injections. Batches of samples were deemed acceptable if spiked samples indicated recovery rates above 85% and RSDs below 15%.

Each sample was extracted in duplicate, and each solution was analyzed twice; then the mean of four values was used for interpretation. If the difference of the analyses was greater than 15% of the mean value, then the analysis was repeated. Any sample (mainly soil samples) showing no response or a value less than the LOQ was condensed to some extent to recheck or else reported as not detected (ND).

RESULTS AND DISCUSSION

Identification of Environmental RhB Contamination in Paprika. Presently, a common viewpoint has been accepted that almost all the detectable RhB contaminations in paprika or chilli are attributed to illegal addition. However, we found a large number of cases with ultratrace RhB at levels of 0.01−1 μg/kg in paprika or chilli powder during the raw material testing process. Considering the negligible pigmentation effect of ultratrace RhB and the cost of the crime, these cases are difficult to understand with malicious intents. Consequently, a hypothesis arose that there may be an environmental RhB contamination occurring during the growing process of paprika and chilli.

To verify the hypothesis, the identification of RhB in raw paprika samples was conducted by using a UPLC−MS/MS system, which is well-known for its incomparable specificity for unknown compound identification as well as excellent analytical performance such as a short run time, a strong separating capacity, high sensitivity, and stability. The confirmation criteria are according to European Decision 2002/657/CE:[29] (a) the chromatographic retention time of the analyte must be the same as that obtained from the calibration solutions, with a tolerance of ±0.5%; (b) the two qualitative ions' intensities must be, at least, 3 times greater than the base noise of the MS detector; (c) the relative abundance between the qualitative ions in the samples should be approximately equal to that in the standard, with acceptable deviations described in the legislation.

Parts A and B of Figure 2 show the UPLC−MS/MS chromatograms of the RhB standard solution (0.5 μg/kg). As can be seen, the retention time of the RhB standard was at 0.86 min, and the abundance ratio of the qualitative ion pairs m/z 443.4/355.2 to m/z 443.4/399.3 was calculated to be 0.59. For comparison, the chromatograms of a random raw red paprika sample are displayed in Figure 2C,D. As expected, the retention times of the two qualitative ion pairs were also at 0.86 min, completely consistent with that of the standard. The abundance ratio of ion pair m/z 443.4/355.2 to ion pair m/z 443.4/399.3 was calculated to be 0.56, slightly lower than the value of the standard (0.59) by 5.1%, which was within the accepted range of 10%. Therefore, according to the criterion, it can be confirmed that the analyte in the sample was just the target compound RhB. In other words, the paprika sample was indeed contaminated by RhB.

As described in the Materials and Methods, all paprika samples were directly picked by researchers themselves from

the paprika planting base, and neither addition activities nor contact with RhB-containing materials took place during the sample preparation and analysis processes. Therefore, it can be ensured that RhB had already existed in the paprika before sample collection. This evidence confirmed that, far from any anthropogenic activity, the paprika had really been contaminated by the RhB in the plant growth environment over its growth period.

Levels of Environmental RhB Contamination in Paprika Samples. The levels of environmental RhB contamination in red paprika from seven different fields in Xinjiang (China) were investigated. The mean values together with RSDs of the RhB concentrations of all seven red paprika samples are presented in Table 3. All the paprika samples were found to be contaminated by RhB to different degrees. The concentrations of RhB ranged from 0.13 to 0.35 μg/kg, which were far below the permissible detectable threshold of 5 μg/kg.[26] This result indicated that all the tested samples suffered from environmental contamination of RhB during the vegetation period, suggesting that the occurrence of environmental RhB contamination in paprika during the vegetation period may be a ubiquitous phenomenon in the surveyed fields.

Table 3. Concentrations of RhB in Red Paprika from Different Fields

field no.	paprika type	RhB concn (μg/kg)	RSD (%) (n = 3)
1	urn	0.20	5.0
2	urn	0.20	10.4
3	sweet	0.21	8.2
4	urn	0.18	8.5
5	urn	0.13	8.9
6	line	0.35	10.3
7	urn	0.19	10.4

The effect of the growing field on the level of RhB contamination was evaluated by comparing the RhB concentrations of five urn paprika samples from five different fields. As seen from Table 3, only one urn sample in field 5 had a relatively low value of 0.13 μg/kg, whereas four other samples had close levels around 0.2 μg/kg. This result suggested that although in theory the level of RhB contamination in paprika must be closely related to the growing field, no obvious correlation was found between the RhB contamination level of paprika and the growing field in this study. This may be ascribed to an important matter that the same planting method was popularized and adopted in these surveyed fields since Xinjiang was regarded as a large-scale paprika planting base in China.

Moreover, the difference among different paprika types was simply evaluated. The highest concentration of 0.35 μg/kg was observed in line paprika, while the sweet paprika and urn paprika had close values near 0.2 μg/kg. These data seemed to imply that the line paprika had a higher contamination level than urn and sweet paprika, but a conclusion could not be easily drawn because of the limited amount of samples in the present study. Further sampling and analysis should be specially emphasized on this subject in the future.

To assess the evolution of the RhB contamination level over the maturation stage, green, orange, and red paprika samples were collected from four fields for RhB determination. As expressed in Figure 3, a clear variation tendency in different colors was obtained with respect to the concentration level of RhB in the following order: orange paprika > green paprika > red paprika. Because the three colors of green, orange, and red represent three maturity levels of general maturity, moderate maturity, and full maturity, respectively, the tendency reflected an unambiguous evolution process: the RhB concentration in paprika increased with the paprika maturity level from general maturity to moderate maturity and then decreased from moderate maturity to full maturity. The evolution of the RhB concentration with maturity levels revealed there may be a degradation process of RhB in paprika fruit in the late maturation stage.

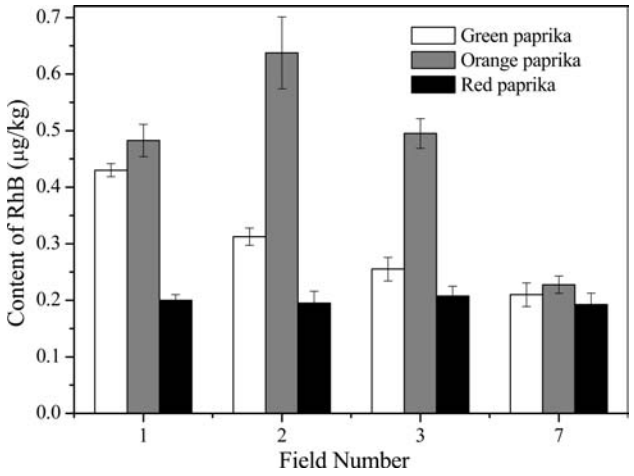

Figure 3. Comparison of RhB concentrations in paprika at different growth periods.

Source of Environmental RhB Contamination in Paprika. Like those of most land plants, the growth and quality of paprika rely mainly on three environmental conditions, including soil, water, and atmosphere. Soil plays a significant role in nutrients and water supplies for paprika, and the atmosphere acts as a reservoir for the prerequisite carbon dioxide for the photosynthesis process of plants. Because the selected fields in the present study are far away from the industrial district, it is impossible that the soil, water, and atmosphere environments of paprika growth are polluted directly through industrial emission. Therefore, the occurrence of environmental RhB contamination in paprika should originate from the use of some RhB-containing agronomic materials during the agricultural activities. RhB has been extensively used as a coloring agent in chemical industries to enhance the luster of glass, plastic, porcelain, rubber, paper, and textile products because of its strong and long-lasting fluorescence.[5,6] These products have been used as the basic raw or auxiliary materials in the production of some agricultural materials such as mulching film and dyed pesticide.[7,8] Once these RhB-containing agrochemical materials have been used in the agricultural activities, it is inevitable that the soil will be contaminated by RhB. Therefore, we speculated that the most potential source of environmental RhB contamination in paprika is be the soil contaminated by RhB.

To verify this assumption, RhB was determined in five soil samples corresponding to the paprika samples. As shown in Table 4, RhB was found in all five soil samples in the concentration range of 0.009−0.059 μg/kg. This indicated that although the exact contamination origin was still unknown, the soils were indeed contaminated by RhB. This evidence supported the hypothesis that the RhB-contaminated soil was

Table 4. Concentrations of RhB in Soils from Different Fields

field no.	RhB concn (μg/kg)	RSD (%) (n = 3)
1	0.040	1.1
2	0.059	4.3
3	0.012	4.3
4	0.017	5.9
5	0.009	9.8

one of the major sources of environmental RhB contamination in paprika during its vegetation process. A special survey on the origin of RhB-contaminated soil should be thoroughly carried out in the future, e.g., by interviewing the farmers or their advisors or suppliers and producers of agronomic materials. Such a study would provide some scientific foundation and feasible advice for how to alleviate the environmental RhB contamination in paprika during the growing process.

To further clarify the migration mechanism of RhB in the paprika growth process, the RhB concentrations in paprika stems were also determined and compared with the levels of soils and green, orange, and red paprika samples. As shown in Figure 4, a clear level order was observed as follows: orange

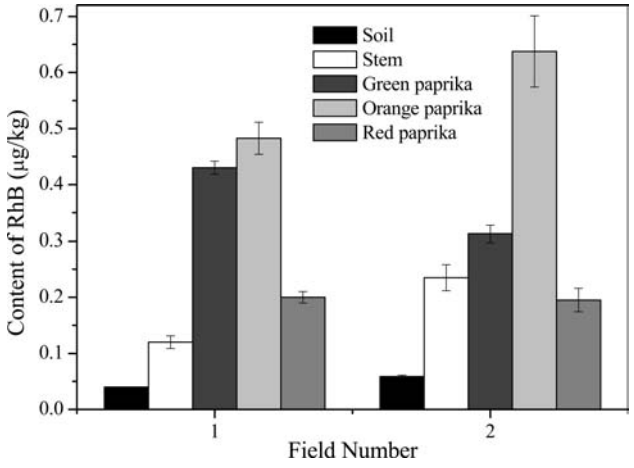

Figure 4. Comparison of RhB concentrations in the soil, stem, and paprika fruits.

paprika > green paprika > red paprika and stem > soil. Combining the present trend and the growth rhythm of paprika, a visual migration mechanism of RhB from soil to paprika fruit can be depicted: Soils were initially contaminated by RhB due to the use of some RhB-containing agrochemical materials in the agricultural activities. When paprika was planted in the RhB-contaminated soil, RhB migrated from the soil to the plant through the absorption of the root and then moved to all parts of the plant body such as the stem, leaf, and flower through plentiful transfusion tissues of the plant.[30] As the plant began to bear fruits, a portion of RhB was then diverted into the fruits and continually accumulated in them as time went on. With the color changing from green to orange, paprika fruit entered its moderate maturation stage and the RhB concentration in paprika reached its highest value. Nevertheless, when the fruit gradually grew old with the color from orange to red, the enrichment rate of RhB slowed. Moreover, approximately half of the RhB in the fruits might be degraded at this stage. As a result, the RhB level in red fruits was close to that in the stems, as expressed by Figure 4.

Correlation between RhB Contamination and Paprika Properties. The major ingredients of paprika and chilli are capsanthin and capsaicin, whose amounts are quantitatively represented by the color value and pungency degree, respectively. To study the correlation between RhB contamination and paprika properties, the color value and pungency degree of paprika samples were determined using UV−vis spectrophotometry[31] and the HPLC−UV method,[32] respectively. As shown in Figure 5, the color value of paprika displayed a variation tendency similar to that of the RhB level in paprika as field 6 > field 4 > field 5, suggesting an approximately positive correlation between them. Thus, the presence of paprika pigment was likely to be beneficial for the accumulation of RhB in paprika fruit.

In contrast, the variation trend of the pungency degree of the paprika was ranked to be field 6 > field 5 > field 4, which was not similar to that of the RhB level. This indicated that there was no correlation between pungency degree and RhB concentration. Hence, the existence of the capsaicin in paprika had no effect on the RhB contamination.

To sum up, this study clearly showed that environmental RhB contamination had really occurred in paprika over its growth period. The discovery has significant implications for re-evaluating the origin of the RhB in paprika- and chilli-containing products. Although the detectable RhB in paprika in most cases was undoubtedly attributed to illegal addition, the presence of RhB in many other cases appeared to come from an environmental source. This provides a more reasonable explanation for the occurrence of increasing cases with ultratrace concentrations of RhB in paprika or chilli. In the future, more extensive research and in-depth exploration should

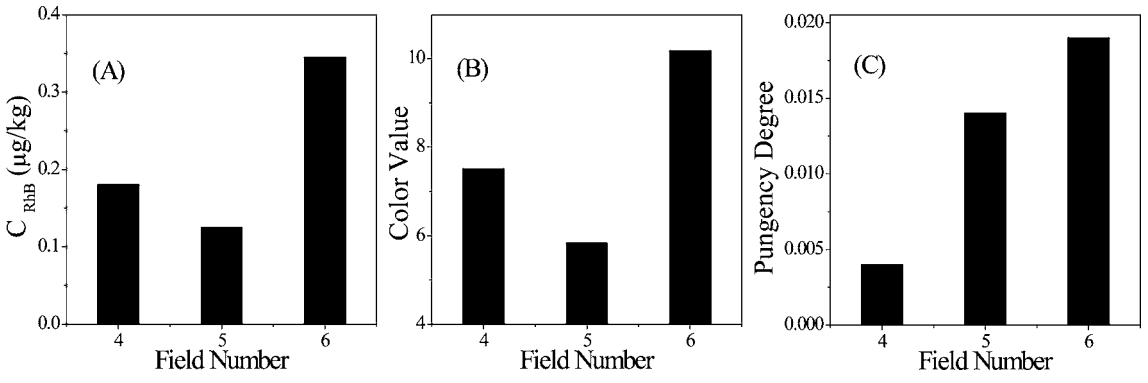

Figure 5. Correlation between RhB level (A) and color value (B) or pungency degree (C) of paprika.

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be conducted to confirm the universality of environmental RhB contamination in different regions and to investigate the contamination source and contamination mechanisms.

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Notes

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