Survey of canthaxanthin content in commercial chicken eggs sampled from Hunan province, China

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Abstract. Canthaxanthin (Cx) is a feed additive widely used for pigmenting egg yolk. In this study, a ultra performance liquid chromatography (UPLC) method was used to detect Cx in commercial chicken eggs sampled from Hunan province, China. Results showed that 43.75% of samples (14 out of 32) possessed detectable amount of Cx, ranging from 0.8 ± 0.2 µg/g to 152.8 ± 10.4 µg/g, and 25.0% of samples (8 out of 32) carried residues of Cx at levels higher than the approved maximum residue limit (MRL = 30.0 µg/g). Interestingly, higher detection rate and above-MRL rate were reported in samples that claimed to be free-range eggs and were collected from traditional farmers’ markets. This survey illustrates that Cx might occasionally be used in excess for breeding hens, in the surveyed area, especially in those that are not branded. Therefore, more strict regulatory measures are urgently required to be taken up by the Chinese government to retain the safety of chicken eggs and to ensure that consumers are not deceived by the color of yolks pigmented with Cx.

1. Introduction

Chicken egg, one of the most important foods in human diet worldwide, is not only highly nutritious, but also a rich source of diverse bioactive components [1]. Consumers usually judge the nutritional quality of eggs by yolk color, and eggs with richer yolk color are considered to be richer in nutrients [2]. Free-range eggs [3, 4], which are produced by chickens that graze naturally on grass, bugs, and seeds, commonly have bright orange yolks and are considered to contain higher percentage of nutrients and healthy fats. On the contrary, caged eggs [4], produced by chickens that live in tightly packed cages and primarily feed on grain, tend to have pale-yellow yolks with nutritional content lower than in the bright-orange counterparts. Hence, free-range eggs are more attractive to consumers and priced higher in the market [5, 6]. In reality, the color of yolk is actually determined by the hen’s diet, and farmers can easily regulate it based on their feed [7, 8].

There are many related studies on the influence of factors such as breeding environment and feed addition on egg quality [9-11]. Generally, higher the percentage of carotenoids in a hen’s diet, richer is the color of the egg-yolk [5]. Hence, natural or synthetic carotenoids are widely utilized as feed
additives for egg-yolk pigmentation, thereby making the caged egg-yolks appear bright orange, similar to free-range egg-yolks. Fraudulent issues of using carotenoids to pigment the yolk of caged eggs, and selling at higher price, like that of the free-range eggs, have been reported in many countries. On the International Day for Protecting Consumers’ Rights in 2019, an event titled “made-up free-range eggs” was shown by China Central Television (CCTV), where Canthaxanthin (Cx) was used to pigment the yolk of eggs. Cx is a naturally occurring red carotenoid, whose synthesized formulations have been widely used as feed additives for pigmenting egg yolks and aquatic products [12]. Cx is not associated with any concern of genotoxicity, and even has some beneficial effects to human health [13]; however, a side effect of its extreme over-dosage results in the deposition of minute crystals in the eye [14]. Therefore, the maximum residue limit (MRL) for Cx in egg yolks has been proposed to be 30.0 μg/g by the European Food Safety Authority (EFSA) [15].

Therefore, an over-dosage of Cx in hen’s diet might be considered as both fraudulent issue and food safety issue to consumers. However, residue-level of Cx in egg yolks had not been a wide concern till recently. Therefore, this study aimed to determine the residues of Cx in commercial chicken eggs sampled from Hunan Province, China.

2. Materials and methods

2.1. Instruments and chemicals

An Acquity H-Class Ultra Performance Liquid Chromatography (UPLC)® system coupled with ultraviolet detector from Waters Inc. was used for Cx analysis. Separation was performed using an Acquity UPLC® BEH C18 column (50 mm × 2.1 mm × 1.7 μm) from Waters Inc. Low temperature centrifuge (Thermo Fisher Scientific Inc.), AUW120D electronic balance (Shimadzu Inc., Japan) and Lab Dancer mini vortex mixer (IKA Inc., Germany) were the other key instruments used in the current study.

Chromatographic-grade acetonitrile and n-hexane were purchased from Merck Inc. (Germany). Standard Cx was purchased from Dr. Ehrenstorfer GmbH (Germany). All other chemicals, such as anhydrous sodium sulfate, were of analytical grade and purchased from a local company. The water used was of ultra-pure grade, prepared using a Labinstru VDUPF-20 ultrapure water system (Beijing, China).

2.2. Samples

The survey was conducted in 2018 to analyze Cx content in commercial chicken eggs sampled from Hunan province, China. In total, 32 samples were collected from supermarkets or traditional farmers’ markets, and 30 eggs, randomly picked from a batch, were included per sample. Subsequently, the samples were transported to our laboratory and maintained at low temperature, prior to Cx extraction and analysis.

2.3. Cx Extraction and analysis

The procedure used to determine Cx concentration was based on a standard, approved by the China Entry-Exit Inspection and Quarantine Bureau [16], and the methods developed by Furusawa [17], with some modifications. Ten eggs were randomly picked from each sample and treated in boiling water for 10 min; the yolks were separated from the albumen, crushed, and mixed. A 1.00-g sample was placed in a 50-mL centrifuge tube and 10 mL acetonitrile and 0.75 g anhydrous sodium sulfate were added. The samples were homogenized and extracted with ultrasonic-assist for 10 min. The samples were then centrifuged at 5000 × g at 4 °C for 5 min, and supernatant was collected. The precipitates were extracted again, as mentioned before, and the extracting solutions were combined. The extracting solutions were transferred to a 125-mL separating funnel, to which 20 mL n-hexane was added and vigorously shaken for defatting. After stratifying, the acetonitrile phase was collected and volume adjusted to 25 mL. The defatted extracting solution was filtered (through 0.22-μm) prior to UPLC analysis. Three replicates were prepared for each sample, and analyzed separately.
A mixture of pure water and acetonitrile (8:92, v/v) was used as a mobile phase for Cx analysis. Flow rate of the mobile phase was 0.4 mL/min and the column temperature was 35 °C. To determine the identity of substances, 0.4 μL of the samples were injected into the UPLC system, and a wavelength of 470 nm was used in the ultraviolet detector.

A series of standard sample solutions with concentrations ranging from 0.1 μg/mL to 10 μg/mL were prepared and analyzed, as described previously. Standard curve of Cx was thereafter prepared to calculate the amount of Cx in each chicken egg sample.

2.4. Quality control
A blank sample and a spike-in sample were analyzed as per the procedures mentioned above, together with the actual samples. If the analytical result of the blank sample was undetectable and recovery of the spike-in sample was higher than 80%, the corresponding test results of the actual samples were reported. To eliminate the effect of migration on retention time, a standard sample was analyzed after every 5 actual samples. Each sample was measured thrice.

3. Results and discussion

3.1. Performance of the proposed Cx detection method
Cx was efficiently separated under the chromatographic operating conditions described above, and its retention time was about 3 min, with only 5 min required for the analysis of each sample. The standard curve of Cx is listed in Table 1, with a correlation index ($R^2$) equal to 0.9999, which indicated a strong linear relationship between the Cx concentrations and its corresponding chromatographic response within the selected range.

| Indices                        | Performance          |
|-------------------------------|----------------------|
| Standard curve                 | $Y = 2.61X + 131.00$ |
| Linear range (μg/mL)           | 0.1–10               |
| Determination coefficient ($R^2$) | 0.9999              |
| Sensitivity                    | LOD (μg/g)           |
|                               | 0.1                  |
|                               | LOQ (μg/g)           |
|                               | 0.4                  |
| Accuracy/Recovery rate (%)     | low spiked level     |
|                               | 92.59                |
|                               | middle spiked level  |
|                               | 98.75                |
|                               | high spiked level    |
|                               | 102.29               |
| Reproducibility/Relative standard deviations (%) | intra-batch |
|                               | 4.97                 |
|                               | inter-batch          |
|                               | 7.86                 |

Matrix-matched standard sample of Cx was diluted in a 10-fold series and analyzed using UPLC to estimate the sensitivity of this detection method, where concentrations corresponding to S/N = 3 and 10 were defined as the limits of detection (LOD) and limits of quantification (LOQ), respectively. The LOD and LOQ of Cx detection were 0.1 μg/g and 0.4 μg/g, respectively.

Accuracy of the detection method was estimated by spiking blank samples in the recovery experiments. Results indicated the recoveries of Cx to range from 92.59% to 102.29%. The intra- and inter-batch reproducibility of this detection method was also evaluated by injecting three replicates of the same spike-in sample, on the same day and on three different days. The relative standard deviations (RSD) in intra-batch and inter-batch experiments were 4.94% and 7.86%, respectively. Performance of the Cx analysis method is summarized in Table 1; it indicates the effectiveness and reproducibility of this method.

3.2. Survey of Cx in commercial chicken eggs
The sample information and survey results of Cx in commercial chicken eggs sampled from Hunan province are summarized in Table 2 and Fig. 1. The results indicated that 43.75% of samples (14 out of 32) possessed detectable amounts of Cx ranging from 0.8 ± 0.2 to 152.8 ± 10.4 μg/g, and 25.0% of
samples (8 out of 32) contained residues of Cx higher than 30.0 µg/g, which is the MRL value approved for Cx in egg yolk. Twenty-two samples were claimed to be free-range eggs, although half of them carried detectable amounts of Cx and 27.27% of them (6 out of 22) contained residues of Cx higher than the corresponding MRL (30 µg/g). On the contrary, 10 samples were not claimed to be free-range eggs, and three of them contained detectable Cx while two of them had residues of Cx higher than 30 µg/g.

Table 2. Sample information and survey results of canthaxanthin (Cx) in commercial chicken eggs sampled from Hunan province, China.

| No. | Sample site | Claimed to be free-range egg | Cx content (mean ± SD)(μg/g) |
|-----|-------------|-------------------------------|-----------------------------|
| 1   | SM<sup>a</sup> | Y                             | ND<sup>b</sup>              |
| 2   | SM          | Y                             | 7.4 ± 0.5                   |
| 3   | SM          | Y                             | ND                          |
| 4   | SM          | Y                             | 129.9 ± 26.9                |
| 5   | SM          | Y                             | ND                          |
| 6   | SM          | N                             | ND                          |
| 7   | SM          | N                             | 1.8 ± 0.9                   |
| 8   | SM          | Y                             | ND                          |
| 9   | SM          | Y                             | ND                          |
| 10  | SM          | N                             | ND                          |
| 11  | SM          | Y                             | 152.8 ± 10.4                |
| 12  | SM          | Y                             | ND                          |
| 13  | SM          | N                             | ND                          |
| 14  | SM          | Y                             | ND                          |
| 15  | SM          | Y                             | ND                          |
| 16  | FM<sup>b</sup> | Y                             | 4.6 ± 2.2                   |
| 17  | FM          | N                             | 33.8 ± 7.4                  |
| 18  | FM          | Y                             | 0.8 ± 0.2                   |
| 19  | FM          | Y                             | 84.5 ± 12.0                 |
| 20  | FM          | N                             | 39.1 ± 4.5                  |
| 21  | FM          | Y                             | 26.3 ± 16.3                 |
| 22  | FM          | Y                             | ND                          |
| 23  | FM          | N                             | ND                          |
| 24  | FM          | Y                             | 36.4 ± 16.2                 |
| 25  | FM          | N                             | ND                          |
| 26  | FM          | Y                             | 28.5 ± 1.7                  |
| 27  | FM          | N                             | ND                          |
| 28  | FM          | Y                             | 31.8 ± 4.4                  |
| 29  | FM          | Y                             | 123.2 ± 26.0                |
| 30  | FM          | Y                             | ND                          |
| 31  | FM          | N                             | ND                          |
| 32  | FM          | Y                             | ND                          |

<sup>a</sup> supermarket; <sup>b</sup> traditional farmers’ market; <sup>c</sup> checking the package or asking the salesman; <sup>d</sup> undetectable
Figure 1. The canthaxanthin (Cx) residue profile of chicken eggs sampled from Hunan province, China: A. comparison between eggs to check if they were indeed free-range eggs, as claimed; B. comparison between eggs sampled from supermarkets and traditional farmers’ markets.

In addition, when 15 samples from supermarkets and 17 samples from traditional farmers’ markets were compared, the Cx residue profiles of chicken eggs from both were obviously different. Over half (73.33%) of the samples from supermarkets contained undetectable amount of Cx while 58.82% of samples from traditional farmers’ markets contained detectable amount of Cx and 35.29% (6 out of 17) carried residues of Cx higher than 30 µg/g. Generally, eggs from the traditional farmers’ markets are not branded and hard to trace, hence prompting the producers of these eggs to intentionally use higher dose of Cx to pigment the yolk of eggs. However, most interesting were two samples from the supermarket, which were claimed to be free-range eggs, yet contained large amounts of Cx (129.9 ± 26.9 µg/g and 152.8 ± 10.4 µg/g, respectively). It, therefore, illustrated that extensive use of Cx as feed additives might be true in some farm hens as well, which supply branded eggs to supermarkets.

A similar work, which surveyed the Cx residues in commercial chicken eggs sampled from Yunnan province, was conducted by Luo et al. [19]. Results from this work indicated that 35.94% of the samples had detectable content of Cx and the content ranged from 7.37 µg/g to 91.3 µg/g. In addition, Schlatterer and Breithaupt [16] had investigated the Cx content of egg yolks obtained from local German supermarkets, and found Cx residues in all but ecological eggs; however, the Cx contents only ranged from 3.7 µg/g to 11.6 µg/g. The current results were comparable to those reported by Luo et al. [19], whereas the residue profile of Cx in eggs obtained from China’s markets are more serious than in eggs from German markets.

4. Conclusion
In conclusion, more strict regulatory measures are urgently required to be taken up by the Chinese government to retain the safety of chicken eggs and to ensure that consumers are not deceived by the color of yolks pigmented with Cx.

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