STRUCTURAL IDENTIFICATION AND QUANTITATION OF AURAMINE O IN FOODS

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Abstract

Auramine O is an industrial dye which was listed in the group 2B of carcinogenic agents. It had been illegally used in livestock and poultry feed, as well as in bamboo shoot in Vietnam. The confiscated chemical was also identified by IR, TLC, MS. An efficient method based on LC/MS/MS was developed for determination of the former. Recoveries were obtained from 73.5 to 95.4 % (with RSD_R 2.93 - 6.35 %) for chicken meat and from 66.2 to 88.7 % (with RSD_R 6.35-7.13 %) for bamboo shoot. Method Detection Limit (MDL) and Method Quantitation Limit (MQL) were respectively 3 and 10 µg/kg.

Key words: Liquid chromatography coupled to tandem mass systems, Auramine O, LC/MS/MS, QuEChERS.

1. Introduction

Auramine O (AO) is a diarylmethane dye used as a fluorescent stain as well as coloring agent for textile. The chemical structure was illustrated in Figure 1. AO has been listed in the group 2B of carcinogenic agents since 2007 in Japan and in Europe [1]. Recently, it was illegally added into some food and feed in Vietnam for coloring purpose. This was a serious problem for Vietnam socio economy. According to the Circular No. 42/2015/TT-BNNPTNT dated November 16, 2015 of the Ministry of Agriculture and Rural Development, AO had been banned to use in livestock and poultry feed in Vietnam [2].

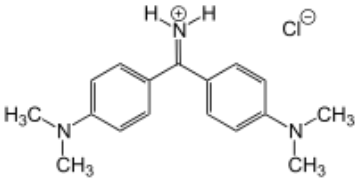

Figure 1. Chemical structures of AO.

There were various analytical methods for determination of basic colorants in foods, having some drawbacks such as time-consuming steps, available only for samples with high level of basic colors [3,4]. Besides, AO was also determinated by HPLC method with PDA detector, obtained a LOD at 0.05 µg/g [5].

In this study, liquid chromatography coupled to tandem mass systems (LC/MS/MS) was used to analyze AO in chicken meat and bamboo shoot. The QuEChERS extraction technology was applied for the cleanup step.

2. Experimental

2.1. Industrial AO chemical

The industrial AO chemical was confiscated from a company where AO had been mixed into livestock and poultry feed.

2.2. Reagents and chemicals

All solutions were prepared with ultrapure Milli-Q water (Milli-Q, Milford, MA), which was used for preparing aqueous mobile phase. Acetonitrile, $MgSO_4$ and NaCl were purchased from Merck. d-SPE kit (150 mg $MgSO_4$, 50 mg C18, 50 mg PSA) and EMR-lipid QuEChERS were purchased from Agilent .

Auramine O (purity 89 %), formic acid (HCOOH) was purchased from Sigma-Aldrich. A solution 0.1 % HCOOH was prepared by diluting 1 mL HCOOH in 1 L ultrapure water.

2.3. Preparation of standard solutions

Stock standard solutions of AO were prepared by dissolving AO standard in acetonitrile in a volumetric flask at a concentration of 100 mg/L. The stock standard solutions were further diluted with

acetonitrile to give standard solutions for the recovery. For the calibration curves, the stock solutions were diluted with acetonitrile to give seven working standard solutions for analysis (with concentrations of 1, 5, 10, 25, 50, 75 and 100 μg/L).

2.4. Preparation of sample solutions from food

In this study, we chose two kinds of samples (bamboo shoot and chicken meat) from market for research. All samples were finely cut and homogenized. 2 g sample was accurately weighted and dissolved in 10 mL of acetonitrile. The sample solution was then shaken for 30 min and a mixture (0.8 g MgSO4 and 0.2 NaCl) was subsequently added and mixed with a vortex mixer for 1 min. The solution was finally centrifuged at 6000 rpm for 10 min, and 5 mL of the supernatant was collected into a QuEChERS EMR dSPE kit after activation with 5ml water. Then, the kit was mixed for 2 min, centrifuged at 3000 rpm for 5 min. Next, 5 mL of supernatant was transferred to an EMR polish tube, mixed for 2 min, centrifuged at 3000 rpm for 5 min, and 1 mL of the supernatant was filtered through a 0.22 μm PTFE filter into HPLC vial. It is now ready for LC/MS/MS analysis (Fig. 2).

2.5. *Recovery tests and method validation*

Recovery were performed to evaluate the accuracy of the method. Seven samples at three concentrations of 60, 100 and 200 μg/kg were prepared for chicken meat and bamboo shoot. The samples were kept at room temperature for 30 min and then treated as described in the section "Preparation of sample solutions from food".

Calibration curves were prepared with AO standard solutions at concentrations of 1–100 μg/L to examine the linearity of the calibration curves. Intraday precision (RSD_r) and interday precision (RSD_R) were assessed by analyzing above samples during a day and on a different day, respectively. The limits of detection (LOD) and quantification (LOQ) were estimated by 3SD and 10SD with SD is the standard deviation of the minimum spiked sample.

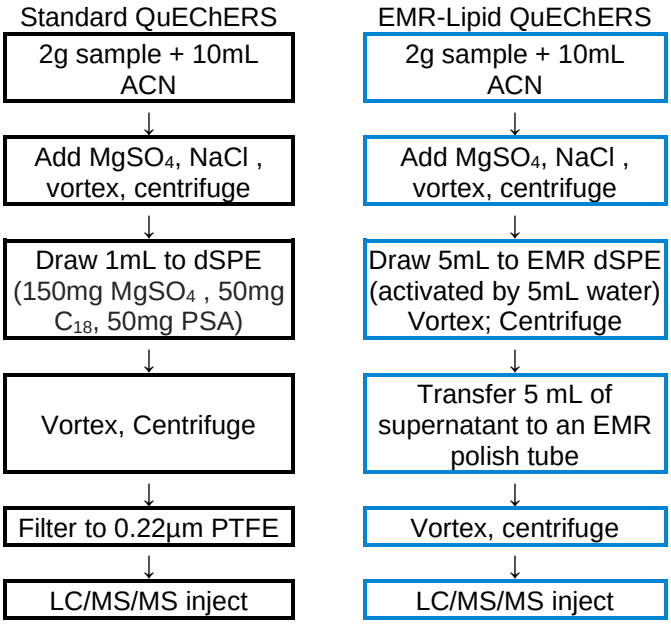

Figure 2. Extraction procedure.

2.6. Fourier Transform Infrared Spectroscopy combined with Attenuated Total Reflection (FTIR-ATR)

Infrared spectra were recorded at room temperature on a Bruker IFS 128 Fourier Transform Infrared spectrometer. Recordings were obtained with a resolution of 4 cm^{-1}, a spectral width between 400 and 4000 cm^{-1}. ATR has a diamond /ZnSe crystal (128 scans).

2.7. Thin layer chromatography (TLC)

TLC was performed on Silica gel 60 F_{254} thin plate which supplied by Merck. Develop solvent system was n-butanol:ethanol:water=2:1:1. Rf value was 8.5/10 in 20 minutes.

2.8.LC/MS/MS analysis

LC analysis was performed by using a HPLC-electrospray ionization-MS/MS (HPLC–ESI-MS/MS) instrument from Agilent (HPLC 1200; Mass spectrometry Agilent 6410). Chromatographic separation was performed on a reversed-phase InertSustain column ODS (i.d.: 4.6 mm x 50 mm; particle size: 5 μm). The mobile phase consisted of acetonitrile (mobile phase A) and HCOOH 0.1 % (mobile phase B). The isocratic condition for HPLC pump was set at ratio 1/1 (MP_A/ MP_B). The flow

rate was 0.4 mL/min and the injection volume was 1 μL; the column oven was at 40°C. The sample solution for HPLC was injected and analyzed in the ESI (+) mode with mass parameters in table 1.

Table 1. Mass detector parameters

Analyte	Q1>Q3 (m/z)	Dwell (ms)	Fragmentor (V)	Collision energy (eV)
Quantitation mass	268.2>147	200	100	30
Confirmation mass	268.2>252	200	100	30

3. Results and Discussion

3.1. *Identification of the industrial AO chemical*

The IR spectrum of industrial AO sample (Fig. 3) confirmed that the former was auramine O. Doublet band at 3382 and 3188 cm^{-1}, and broad band at 2965 cm^{-1} could be assigned to $=NH_2^+$. Band at 1371 cm^{-1} corresponded to CH_3 groups. Band at 1592 cm^{-1} came from C=N group. Multiplicity of bands at 821 cm^{-1} was characterized for para-substituted aromatic. Besides, TLC (Fig. 4) showed that the AO sample had the same Rf value in comparing with the AO standard.

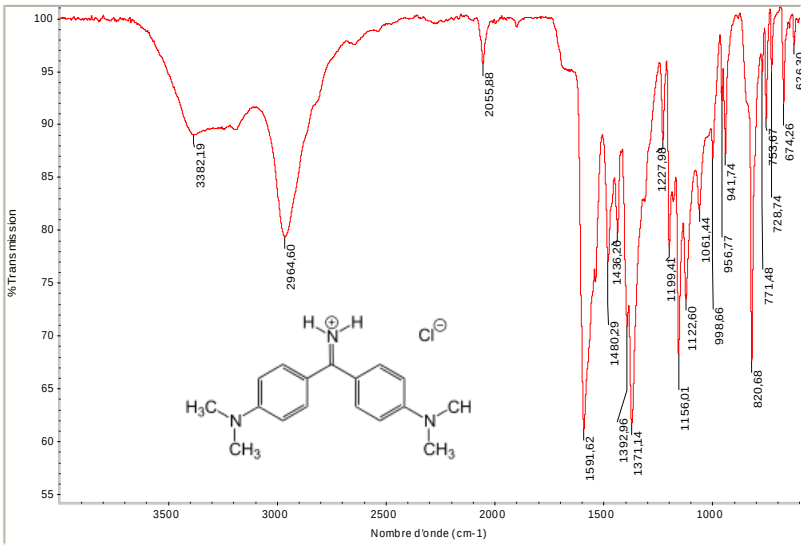

Figure 3. IR spectrum of the confiscated sample

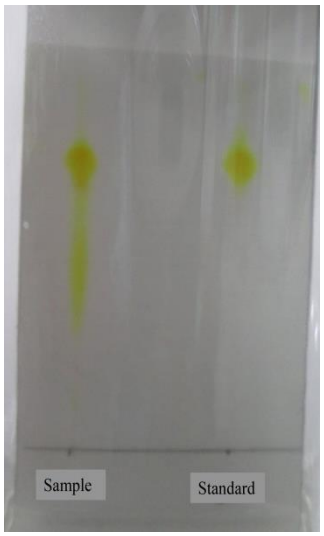

Figure 4. TLC of the confiscated sample and standard after 15 minutes.

The industrial AO sample was also determined by LC/MS/MS. The full scan (Fig. 5) showed a main peak which appeared at retention time 1.9 min. The mass spectrum also showed that peak at m/z = 268 corresponded to parent mass plus one proton [M+H]. Two main fragments was m/z = 147 and 252 which can be assigned to structures illustrated in Figure 5.

The analytical result permits to confirm that the confiscated sample was auramine O.

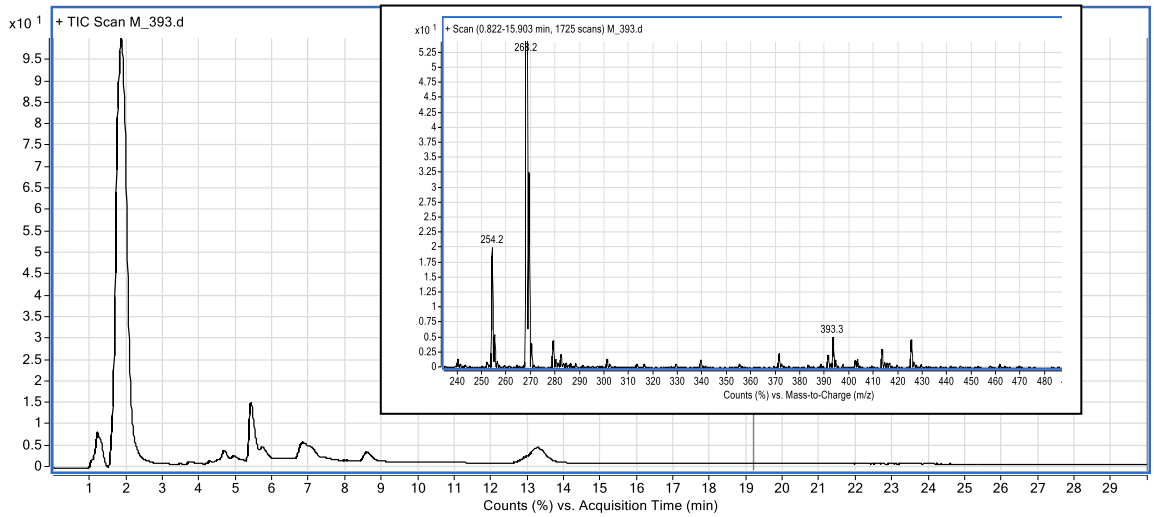

Figure 5. LC full scan and MS of the confiscated sample.

3.2. *Quantitation of AO in chicken meat and bamboo shoot*

3.2.1. *Optimization of the clean-up process on QuEChERS method*

Sample extraction in food commodities is routine for many laboratories using the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method. Removal of lipid interferences from complicated matrices (like chicken meat) is especially important for techniques such as QuEChERS and protein precipitation, as these methods coextract large amounts of matrix with the target analytes [6]. We compared two QuEChERS kits from Agilent: d-SPE kit and EMR (Enhanced Matrix Removal) -lipid QuEChERS kit with the spiked sample at concentration 250 μg/kg as figure 2. The results and chromatograms are shown Table 2 and Figure 6.

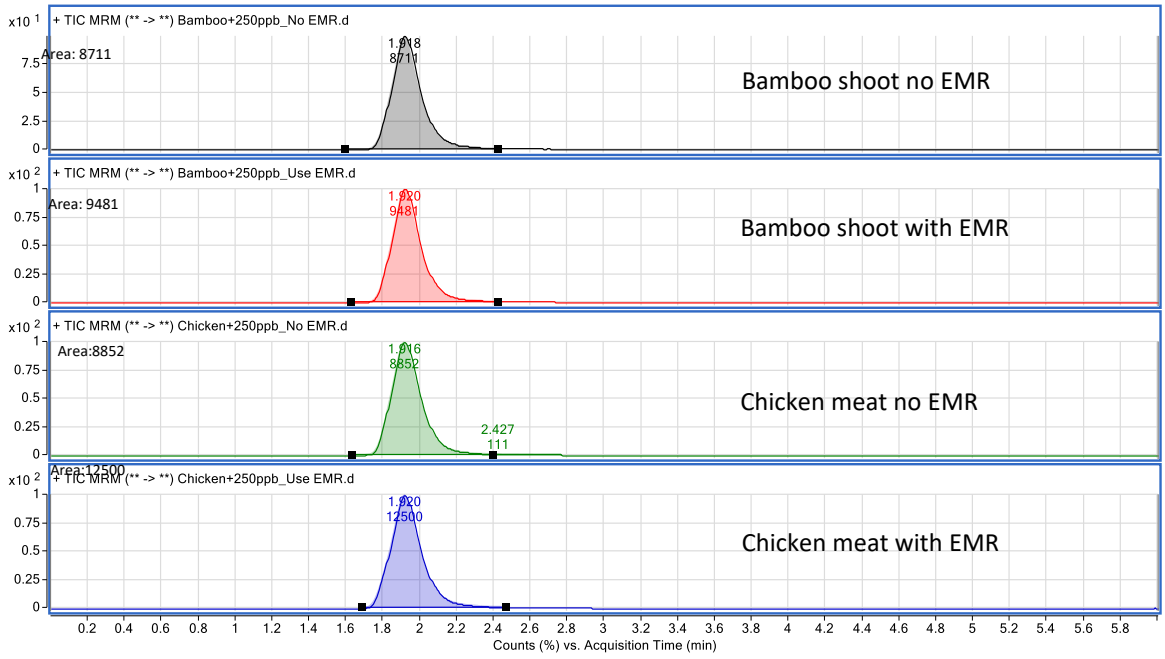

Figure 6. LC/MS/MS MRM chromatograms of samples spiked AO (250 μg/kg) – with and without EMR-lipid kit.

Table 2. Recovery compare of two method – with and without EMR-lipid kit

Sample	Spike level (μg/kg)		Recovery (%)
Chicken meat	EMR	250	90.2
	No EMR	250	69.6
Bamboo shoot	EMR	250	75.8
	No EMR	250	68.6

The results showed that there was no significant difference between the two kits for bamboo shoot. By contrast, against the high lipid sample as chicken meat, recovery efficiency was significantly improved from around 70 % to 90 %. So, we chose the ERM-lipid QuEChERS kit for sample handling procedure in food for AO's analysis because it can also be applied to extract chicken meat as well as various types of processing food.

3.2.2. *Validation of the method*

+Calibration curve

The calibration curve (Fig. 7) and data (Tab. 3) for AO exhibits linearity at the concentration of 1–100 μg/L. The regression coefficient were greater than 0.999.

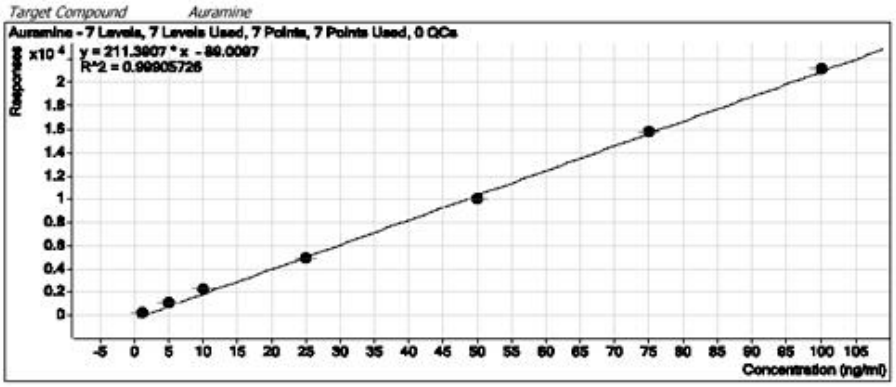

Figure 7. Calibration curve

Table 3. Data of AO standard for calibration curve

Calibration STD	Level	Response	RF	Exp Concentration (μg/L)
1ppb.d	1	202	201.9914	1
5ppb.d	2	1060	212.0328	5
10ppb.d	3	2279	227.8567	10
25ppb.d	4	4928	197.1046	25
50ppb.d	5	10062	201.2368	50
75ppb.d	6	15828	211.0424	75
100ppb.d	7	21248	212.4850	100

+Accuracy and precision

The accuracy and precision of the method were evaluated by recovery tests. Table 3 shows the recoveries, RSD_r and RSD_R obtained by the developed analytical method. The recoveries for chicken meat and bamboo shoot ranged from 73.5 to 95.4%, and from 66.2 to 88.7%, respectively. The reproducibility of the results was assessed by determining both the RSD_r and the RSD_R of the recovery tests. The RSD_r and RSD_R value ranged 2.23 – 5.03% and 2.93 – 6.35% for chicken meat. The corresponding figure for bamboo shoot ranged 5.0 – 6.67% and 6.35 – 7.13%, respectively.

Table 4. Accuracy and precision results

Sample	Spiked level (µg/kg)	Recovery %, n=14	RSD_r %, n=7	RSD_R %, n=14
Chicken meat	50	73.5	5.03	6.26
	100	85.7	2.23	2.93
	200	95.4	4.92	6.35
Bamboo shoot	50	66.2	6.47	6.58
	100	70.6	6.67	7.13
	200	88.7	5.00	6.35

+MDL and MQL

The MDL and MQL were determined by seven spiked samples at concentration 10 µg/kg for chicken meat and bamboo shoot. These samples were analysis and calculated SD of the final result. The MDL based on three times SD were 2.38 µg/kg for chicken meat, 3.01 µg/kg for bamboo shoot.The MQL based on ten times the SD were 7.94 µg/kg for chicken meat, 10.05 µg/kg for bamboo shoot. Hence, we reported the MDL and MQL at a concentration of 3 µg/kg and 10 µg/kg. Because no MRL regulation for Auramine O in food, but usually with a banned substance, the MQL at 10 µg/kg is suitable for the food safety control. The result was summerised in the table 5.

Table 5. MDL and MQL results

Sample	Spike (µg/kg)	SD (µg/kg)	MDL (µg/kg)	MQL (µg/kg)
Chicken meat	10	0.7937	2.38	7.94
Bamboo shoot	10	1.0048	3.01	10.05

4. Conclusions

The confiscated chemical was identified by many reliable techniques. It was auramine O -a carcinogenic agent. Besides, LC/MS/MS method with high performance was also developed for determination of Auramine O in processed foods. The achieved recoveries were from 73.5 to 95.4 % (RSD_R 2.93 - 6.35 %) for chicken meat, and from 66.2 to 88.7 % (RSD_R 6.35 - 7.13 %) for bamboo shoot. The modified method with EMR-Lipid QuEChERS kit showed a good recovery for samples in high lipid content. This is a simple, reliable technique to determine Auramine O like carcinogenic colorants in processed foods.

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