# Determination of Auramine O in animal feedstuffs using ultra performance liquid chromatography tandem mass spectrometry

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## Abstract

A method based on ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UPLC/MS/MS) was developed for simple and rapid determination of the residues of Auramine O in animal feedstuffs. The samples were extracted by MeOH: H2O + HCOOH 0.1% and then analyzed in multiple reaction monitoring (MRM) mode. The mobile phase was ultrapure water with 0.1% formic acid. Under the optimized detection conditions, the linear range for Auramine O was 20 - 100µg/L and the linear correlation coefficients found more than 0.99. The limit of quantification of Auramine O was 0.34 mg/kg. The recoveries of Auramine O ranged from 64.71 - 94.12% with relative standard deviations (RSD) of 4.93 - 8.31% with the concentration range of 20 - 100 µg/L. This method is simple, effective, sensitive and is suitable for the determination and confirmation of Auramine O in animal feedstuffs.



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## 1 Introduction

Auramine O is a hazardous diarylmethane dye, and used as a fluorescent stain. It is very soluble in ethanol and water and used a coloring agent for industry. According to the International Cancer Research by WHO (IARC), Auramine O is the chemical ranked 5$^{th}$ in the 116 carcinogens worldwide. Harmful if swallowed, Auramine O may cause vomiting, diarrhea, liver and kidney damage. Skin contact with this chemical may produce toxic effects: swelling, blistering, pain or redness[1]. Due to the toxic effects of this substance on health, Auramine O is an unauthorized food additive in the United States, Japan and EU.

In Vietnam, The Ministry of Agriculture and Rural Development issued the Circular No. 42/2015/TT-BNNPTNT dated November 16, 2015 announcing that Auramine O is in the additional list of chemicals and antibiotics banned from import, manufacture, trade or use in feed for livestock and poultry. Auramine O has been used by private food makers and retailers for coloring sour bamboo shoots, feeds for fish, chicken, shrimp, etc. This chemical may have serious effects on consumers' health. In fact, there are many methods which have been developed for the determination of Auramine O in food [2,3,4,5]; however, to the best of our knowledge, no analytical method for the determination of Auramine O in animal feedstuffs has been established. In addition, there is limited literature on the determination of Auramine O by UPLC/MS/MS. In this study, we developed a simple and rapid method to detect Auramine O by ultra performance liquid chromatography-tandem mass spectrometry. The method is applicable to various animal feedstuffs.

## 2 Material and methods

### 2.1 Reagents and chemicals

Acetonitrile, methanol (MeOH) and water were purchased from Merck, Germany. Formic acid was analytical grade (Spain). Auramine O (85.5%) was purchased from Sigma-Aldrich Co. A stock standard solution (100µg/ml) was prepared in methanol based on the known purity and molecular weight. From stock solution, one working solution (500ng/ml) for MS/MS optimization was prepared by diluting stock solution. A calibration curve consisting of at least 4 points (20, 50, 100, 200, 500ng/ml) was prepared in methanol with 0.1% formic acid. All samples found to contain Auramine O were diluted into this range for

quantitation. Twenty animal feedstuffs samples were purchased from a local market in Hanoi, Vietnam.

2.2 Chromatography conditions

A Waters Acquity UPLC was used in this study. Separation was carried out on an Acquity BEH $C_{18}$ column (100mm x 2.1 x 1.7µm) maintained at 30ºC. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (methanol). The analysis was performed under gradient conditions as follows: Initial gradient conditions were set to 20% B and held for 1.35 min before incorporating a linear gradient increasing to 80% at 1.50 min. At 7.0 min the gradient was programmed to initial condition for column (total run time 10 min). The flow rate was 0.3 ml/min. The injection volume was 10 µl in full loop injection mode.

Multiple reaction monitoring mode was applied to detect Auramine O, and the detection parameters optimized by Masslynx 4.1 software. Detection was carried out by Waters Acquity TQD triple quadrupole MS fitted with electrospray probe operated in the positive ion mode. The precursor and product ions were determined by direct infusion (10µl/min) into the MS. The following parameters were optimal: capillary voltage, 0.5kV; in source temperature, 150ºC; desolvation gas flow rate, 600L/h. Argon was used as the collision gas, and the collision cell pressure was 3.8 mBar. Other parameters are shown in Table 1.

2.3. Sample preparation

Weigh 2.0g of animal feedstuff (accurate to 0.01g) into a 50ml polypropylene centrifuge tube homogenized and add in 10ml MeOH:$H_2O$ (9:1) with 0.1% formic acid. The sample solution was then vortexed for 10 mins and placed into an ultrasonic bath for 30 mins. The solution was finally centrifuged at 5000rpm for 10mins at room temperature, and the supernatant was collected into a 20ml volumetric flask. The same procedure as described above was performed two times. The mixture was centrifuged at 5000rpm for 10 min at room temperature, and the supernatant was collected into the above volumetric flask and diluted to the volume with mobile phase, then 1ml of the solution was filtered with 0.22µm filter membrane, transferred to an autosampler vial, degassed and injected into UPLC-MS/MS.

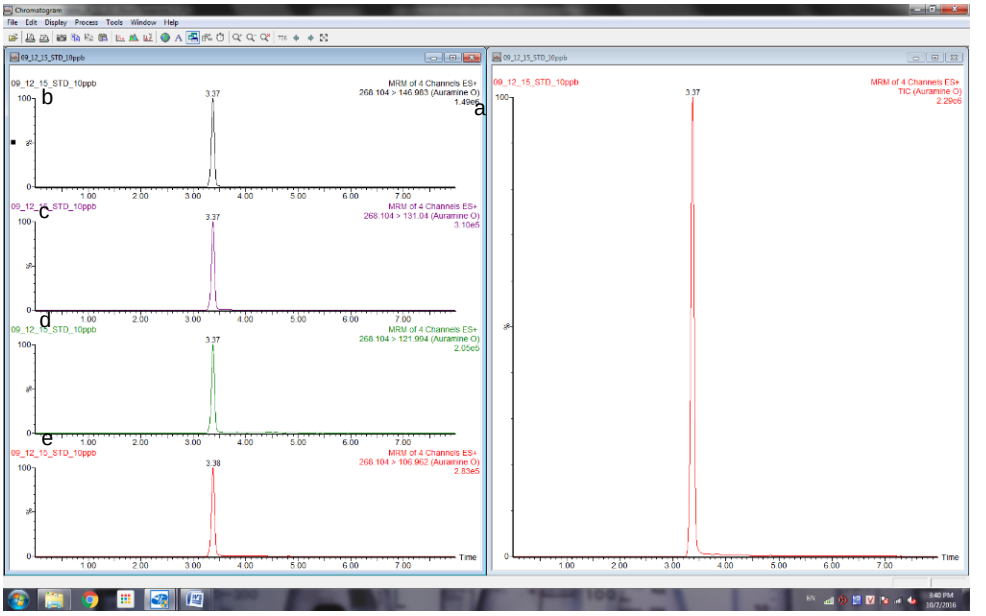


**Fig. 1** Chromatogram of Auramine O in standard solution (10 ng/ml).

*(a) Chromatogram Total Ion Chromatogram (TIC). (b) Chromatogram m/z = 146.98. (c) Chromatogram m/z = 131.08. (d) Chromatogram m/z = 121.99. (e) Chromatogram m/z = 106.96*

**Table 1** Multiple reaction monitoring (MRM) parameters for LC-MS/MS analysis of Auramine O

| Retention time (min) | Parent ion (m/z) | Product ions (m/z) | Dwell time (s) | Cone voltage (V) | Collision Energy (eV) |
|---|---|---|---|---|---|
| 3.37 | 268.10 | 106.96 | 0.078 | 48 | 42 |
| | | 121.99 | 0.078 | 48 | 32 |
| | | 131.04 | 0.078 | 48 | 54 |
| | | 146.98[a] | 0.078 | 48 | 32 |

(a). Ion for quantification

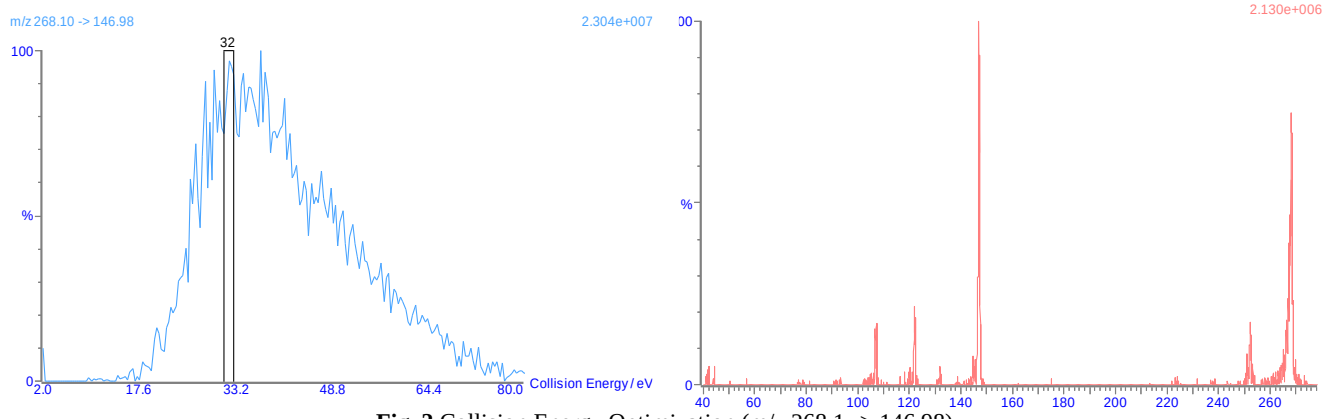


**Fig. 2** Collision Energy Optimization (m/z 268,1 -> 146,98)

## 3 Result and discussion

### 3.1. The selectivity and specificity of the technique

The specificity was evaluated by analyzing blank sample. Additionally, according to the European Commission Decision 2002/657/EC[5], a minimum of four points was required. In this experiment, for four identification points, one parent ion (1 point) and two transitions (each 1.5 points) were monitored. Ion $[M+H]^+$ was chosen as the precursor ion because the ion was the most abundant peak in the mass spectra when mobile phase consisted of methanol and water with 0.1% formic acid. The selected transitions for Auramine O and the optimal MS/MS conditions were shown in Table 1. Collision energy optimization m/z 268,1 > 146.98 was shown in figure 1. No evident matrix effect was observed in this method. The results in Table 2 showed that the peak areas of each ion in the standard solution and the analyzed sample matrix solution were similar and the ion intensities in all sample solutions were within the permitted range, consistent with the European Commission Decision 2002/657/EC[6].

**Table 2** The results of the sample matrix effect and ion ratio in the analysis process

| **Concentration (ng/ml)** | | **20** | **50** | **100** | **Ion ratio intensities** |
|---|---|---|---|---|---|
| Standard solution peak area | A147<br>A131 | 336328<br>69554 | 900189<br>184574 | 1680739<br>348948 | 0.14 – 0.26 |
| Spiked sample peak area | A147<br>A131 | 332469<br>57545 | 834628<br>189331 | 1680372<br>362215 | 0.17 – 0.22 |

### 3.2. Linearity, LOD and LOQ

For analysis, the relationship between peak area and concentration was found to be linear from 20 to 500ng/g, the correlation coefficients of the calibration curve were above 0.99. Using this curve, recoveries can be calculated at each fortification level.

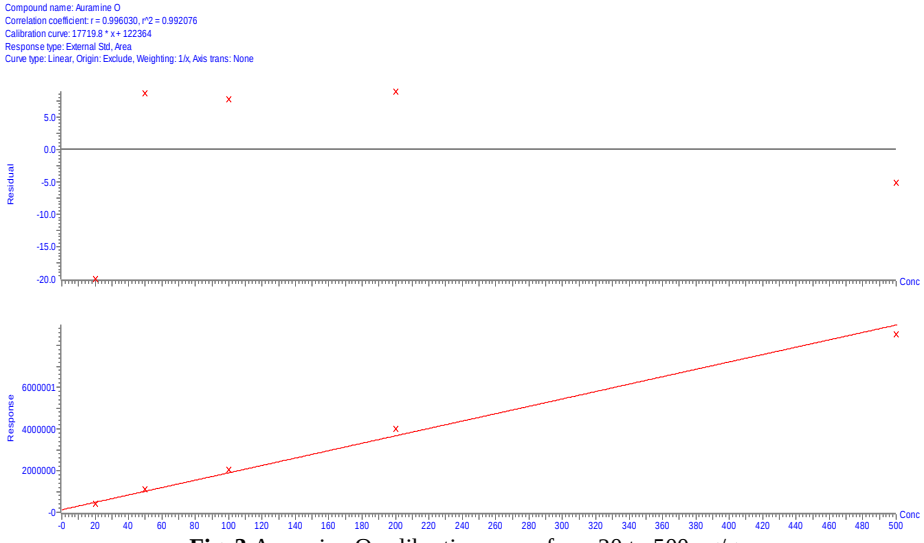


**Fig. 3** Auramine O calibration curve from 20 to 500 ng/g

For the purpose of method validation, concentration of 0.5mg/kg (equivalent to the final concentration of 50ng/g in extract) was chosen to determine the limit of detection (LOD). 10 blank standard addition samples were prepared at the above concentration, extracted and analyzed according to the optimal conditions. Auramine O was confirmed in all LOD spikes with retention time and ion ratio requirements met. The results were given in Table 3 and showed the R calculated values of 4.08 that was in the range $4 < R_{calculated} < 10$, so the measured concentration of the fortifies samples is accordant, the estimated limit of detection (LOD) is reliability[7]. The limit of quantitation (LOQ) was chosen to be 3 times greater than LOD (1.5mg/kg). At this concentration, both ions produced signals well above 10 times noise and no interferences were observed.

**Table 3** Results of the limit of detection and quantitation of the method

| Spike level | Results (mg/kg) |
|---|---|
| SPK 0.5-1 | 0.44 |
| SPK 0.5-2 | 0.52 |
| SPK 0.5-3 | 0.40 |
| SPK 0.5-4 | 0.45 |
| SPK 0.5-5 | 0.43 |
| SPK 0.5-6 | 0.42 |
| SPK 0.5-7 | 0.41 |
| SPK 0.5-8 | 0.43 |
| SPK 0.5-9 | 0.41 |
| SPK 0.5-10 | 0.45 |
| $X_{TB}$ | 0.44 |
| SD | 0.03 |
| LOD | 0.11 |
| LOQ | 0.34 |
| R calculated | 4.08 |

3.3. The recovery

The accuracy and precision of the method were evaluated by recovery tests. Four animal feedstuffs were spiked at LOQ (0.34 ppm), 2 x LOQ (0.68 ppm) and 10 x LOQ (3.4 ppm). The spikes samples were extracted and analyzed in accordance with the already described method. Table 4 - 6 showed the recoveries and relative standard deviations (RSD) obtained by the developed analytical method. The recoveries of Auramine O range from 64.71- 94.12% with relative standard deviations (RSD) of 4.93 – 8.31% respectively.

**Table 4** Results of the recovery (H%) and the relative standard deviation (RSD%) at concentration $C_o$ = 0.34 mg/kg

| Run | Concentrations (mg/kg) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | H% | Day 2 | H% | Day 3 | H% | Day 4 | H% |
| 1st | 0.25 | 73.53 | 0.27 | 79.41 | 0.25 | 73.53 | 0.23 | 67.65 |
| 2nd | 0.24 | 70.59 | 0.25 | 73.53 | 0.26 | 76.47 | 0.25 | 73.53 |
| 3rd | 0.25 | 73.53 | 0.26 | 76.47 | 0.25 | 73.53 | 0.24 | 70.59 |
| 4th | 0.23 | 67.65 | 0.22 | 64.71 | 0.24 | 70.59 | 0.26 | 76.47 |
| 5th | 0.26 | 76.47 | 0.23 | 67.65 | 0.27 | 79.41 | 0.22 | 64.71 |
| 6th | 0.23 | 67.65 | 0.22 | 64.71 | 0.22 | 64.71 | 0.25 | 73.53 |
| Average | 0.243 | 71.47 | 0.242 | 71.18 | 0.248 | 72.94 | 0.242 | 71.18 |
| SD | 0.01 | | 0.02 | | 0.01 | | 0.01 | |
| RSD% | 4.94 | | 8.67 | | 6.85 | | 6.12 | |

**Table 5** The results of the recovery ( H%) and the relative standard deviation (RSD%) at concentration $C_o$ = 0.68 mg/kg

| Run | Concentrations (mg/kg) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | H% | Day 2 | H% | Day 3 | H% | Day 4 | H% |
| 1st | 0.48 | 70.59 | 0.52 | 76.47 | 0.5 | 73.53 | 0.47 | 69.12 |
| 2nd | 0.55 | 80.88 | 0.55 | 80.88 | 0.54 | 79.41 | 0.5 | 73.53 |
| 3rd | 0.56 | 82.35 | 0.51 | 75.00 | 0.46 | 67.65 | 0.55 | 80.88 |
| 4th | 0.45 | 66.18 | 0.45 | 66.18 | 0.53 | 77.94 | 0.56 | 82.35 |
| 5th | 0.53 | 77.94 | 0.51 | 75.00 | 0.55 | 80.88 | 0.51 | 75.00 |
| 6th | 0.52 | 76.47 | 0.55 | 80.88 | 0.52 | 76.47 | 0.52 | 76.47 |
| Average | 0.515 | 75.74 | 0.515 | 75.74 | 0.517 | 76.03 | 0.518 | 76.18 |
| SD | 0.042 | | 0.037 | | 0.033 | | 0.033 | |
| RSD% | 8.155 | | 7.184 | | 6.383 | | 6.371 | |

**Table 6** The examination results of the recovery (H%) and the relative standard deviation (RSD%) at concentration $C_o$ = 3.4 mg/kg

| Run | Concentrations (mg/kg) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | H% | Day 2 | H% | Day 3 | H% | Day 4 | H% |
| 1st | 2.65 | 77.94 | 3.02 | 88.82 | 2.55 | 75.00 | 2.58 | 75.88 |
| 2nd | 3.05 | 89.71 | 2.67 | 78.53 | 3.2 | 94.12 | 2.94 | 86.47 |
| 3rd | 2.55 | 75.00 | 3.1 | 91.18 | 2.51 | 73.82 | 2.54 | 74.71 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 4th | 2.73 | 80.29 | 2.85 | 83.82 | 2.56 | 75.29 | 2.87 | 84.41 |
| 5th | 2.86 | 84.12 | 2.78 | 81.76 | 3.06 | 90.00 | 3.15 | 92.65 |
| 6th | 3.12 | 91.76 | 3.05 | 89.71 | 2.53 | 74.41 | 2.96 | 87.06 |
| Average | 2.827 | 83.15 | 2.912 | 85.65 | 2.735 | 80.44 | 2.84 | 83.53 |
| SD | 0.225 | | 0.171 | | 0.310 | | 0.236 | |
| RSD% | 7.965 | | 5.872 | | 11.320 | | 8.310 | |

3.4. Applications of the method to analyze the Auramine O in real samples

Forty samples commercially available from the local market were taken and analyzed for Auramine O using the above method. Fortunately, no Auramine O was found in these samples (LOD = 0.11mg/kg).

## 4 Conclusions

In the present study, a rapid and sensitive method for the determination of Auramine O in the animal feedstuff has been developed by using ultra performance liquid chromatography with tandem mass spectrometry. This method was validated with fortified animal feedstuff samples and good recoveries with excellent RSD were obtained. LOD and LOQ were found to be sufficiently low to determine the residues of Auramine O. The simple sample preparation combined with the short run time means any sample can be analyzed within one day. The method is very useful for monitoring unauthorized colorants with reasonable cost.

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## Xác định Auramine O trong thức ăn chăn nuôi bằng kĩ thuật sắc kí lỏng siêu hiệu năng hai lần khối phổ

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**Tóm tắt** Trong nghiên cứu này, qui trình phân tích sử dụng thiết bị sắc kí lỏng siêu hiệu năng khối phổ hai lần (UPLC/MS/MS) được phát triển và áp dụng nhằm xác định hàm lượng Auramine O trong thức ăn chăn nuôi. Mẫu được chiết bằng hỗn hợp dung dịch MeOH:$H_2O$ (9:1) + HCOOH 0,1%, và định lượng trên UPLC/MS/MS. Pha động sử dụng nước siêu tinh khiết với 0,1% axit formic. Ở điều kiện tối ưu, khoảng tuyến tính của Auramine O từ 20 đến 100µg/L và hệ số tương quan tuyến tính đạt trên 0,99. Giới hạn định lượng Auramine O là 0,34mg/kg. Trong khoảng nồng độ nghiên cứu (20 - 100µg/L), tỉ lệ thu hồi của Auramine O trong khoảng 64,71 – 94,12% với độ lệch chuẩn (RSD) là 4,93 – 8,31%. Phương pháp đạt độ nhạy và độ chọn lọc cao, qui trình chiết mẫu đơn giản, thời gian phân tích nhanh, phù hợp trong phân tích hàm lượng Auramine O trong thức ăn chăn nuôi và các mẫu thực phẩm khác.

**Từ khóa** Auramine O; Thức ăn chăn nuôi; UPLC/MS/MS.