A method for the determination of amantadine in chicken was established using Shimadzu Triple Quadrupole Mass Spectrometer LCMS-8040. After extraction and cleanup, the commercially bought chicken sample was separated by Ultra-High-Performance Liquid Chromatograph Nexera X2 and then quantified by Triple Quadrupole Mass Spectrometer LCMS-8040. The calibration curve of amantadine exhibited good linearity within the concentration range of 0.1-50 μg/L, and the correlation coefficient of calibration curve was 0.9996. Six consecutive analyses were conducted for low-, mid- and high-concentration standard working solutions (0.5, 1 and 5 μg/L) and the RSD of retention time and peak area were 0.04-0.91% and 0.48-2.77% respectively, indicating good instrument precision.

Keywords: Chicken; Amantadine; Triple quadrupole mass spectrometry

### Introduction
In November 2012, it was revealed by the media that a large broiler food company fed chicken and other poultry with feeds containing antiviral veterinary drugs such as amantadine. This resulted in the entire growth phase of chicken (from hatch/egg to adult (cooked food)) to shorten significantly to a mere 45 days. Media termed these chickens as fast-growing chickens. The company was a large supplier of KFC and McDonald’s, and this incident brought about a strong response in society when reported. As early as 2005, the Ministry of Agriculture of China have released "Emergency Notice on Checking Amantadine and Other Anti-Viral Drugs" and explicitly required the immediate cessation of production, operation and use of such drugs (e.g. amantadine and rimantadine).

As a banned veterinary drug, the detection and control of amantadine residues is particularly important. Currently, most detection methods employ HPLC-fluorescence method or GC method after pre-column derivatization. These methods consist of laborious multi-step procedures which are time-consuming and less sensitive. UHPLC tandem MS has been increasingly used for this application. In this application note, a high-sensitivity and high-selectivity LC-tandem MS method was developed for the determination of amantadine residues in chicken. It serves as a comprehensive reference for many applications and regulatory personnel.

### Chemical structure of amantadine

![Chemical structure of amantadine](image)

### Experimental

#### 1.1. Instruments
Shimadzu Nexera X2 UHPLC and Triple Quadrupole Mass Spectrometer LCMS-8040 was used. The specific configuration included LC-30AD×2 infusion pumps, DGU-20A5R Online Degasser, SIL-30AC Autosampler, CTO-30AC Column Oven, CBM-20A System Controller, Triple Quadrupole Mass Spectrometer LCMS-8040, and LabSolutions Ver. 5.60 Chromatography Workstation.

#### 1.2. Analytical Conditions
Liquid chromatography (LC) parameters
- Chromatographic column: Shimadzu Shim-pack XR-ODS III, 2.0 mm (I.D.) x 150 mm (L) x 2.2 μm
- Mobile phase A: solution containing 0.1% formic acid and 5 mM ammonium acetate
- Mobile phase B: Acetonitrile
- Elution mode: gradient elution; the initial concentration of phase B was 5%; Refer to Table 1 for the gradient program.
- Flow rate: 0.4 mL/min
- Column temperature: 30°C
- Injection volume: 2 μL
Table 1: Gradient Program

<table>
<thead>
<tr>
<th>Time(min)</th>
<th>Module</th>
<th>Command</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>Pumps</td>
<td>Pump B Cone.</td>
<td>22</td>
</tr>
<tr>
<td>2.00</td>
<td>Pumps</td>
<td>Pump B Cone.</td>
<td>22</td>
</tr>
<tr>
<td>2.40</td>
<td>Pumps</td>
<td>Pump B Cone.</td>
<td>95</td>
</tr>
<tr>
<td>3.10</td>
<td>Pumps</td>
<td>Pump B Cone.</td>
<td>95</td>
</tr>
<tr>
<td>3.11</td>
<td>Pumps</td>
<td>Pump B Cone.</td>
<td>5</td>
</tr>
<tr>
<td>5.00</td>
<td>Controller</td>
<td>Stop</td>
<td></td>
</tr>
</tbody>
</table>

Mass spectrometry (MS) parameters
- Ionization mode: ESI (+)
- Ion spray voltage: 2 kV
- Nebulizing gas: Nitrogen 2.0 L/min
- Drying gas: Nitrogen 12 L/min
- Collision gas: Argon
- DL temperature: 300°C
- Heating module temperature: 450°C
- Scan mode: Multiple Reaction Monitoring (MRM)
- Dwell time: 100 msec
- Pause time: 3 msec
- MRM parameters: Refer to Table 2

Table 2: MRM Parameters

<table>
<thead>
<tr>
<th>Compound name</th>
<th>CAS No.</th>
<th>Precursor ion</th>
<th>Product ion</th>
<th>Q1 Pre Bias (V)</th>
<th>CE (V)</th>
<th>Q3 Pre Bias (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amantadine</td>
<td>768-94-5</td>
<td>152.2</td>
<td>135.2*</td>
<td>-30</td>
<td>-22</td>
<td>-30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>93.2</td>
<td>-30</td>
<td>-28</td>
<td>-17</td>
</tr>
</tbody>
</table>

Note: * represents quantifier ion

1.3. Preparation of Standard Solutions and Sample Pretreatment

Preparation of standard working solutions:
Standard working solution at the concentration of 10 mg/L was prepared using methanol, and then sequentially diluted with 5% acetonitrile solution to obtain a series of standard working solutions at concentrations of 0.1, 0.5, 5, 10 and 50 μg/L.

Chicken sample pretreatment:
2 g (± 0.02 g) of chicken sample was accurately weighed in a 50 mL centrifuge tube. 10 mL of acetonitrile:1% trichloroacetic acid solution (1:1) was added to the chicken sample. The mixture was homogenized for 1 minute and centrifuged at 10,000 rpm for 20 min. Solid phase extraction was performed where 4 mL of the supernatant was transferred to WondaSep (InertSep) MPC column for cleanup. The detailed SPE cleanup procedure is shown below:

- **Activation:** 5mL of methanol; 5mL of water
- **Load sample**
- **Rinsing:** 3mL of 2% HCl; 3mL of methanol
- **Elution:** 5 mL of 5% ammonium hydroxide-methanol-isopropanol (5:80:15)
- **Dry the eluent under N₂ and re-dissolve it to 1mL, followed by filtering**
- **LC-MS/MS analysis**

Results and Discussion

2.1. Q1 Scan and Product Ion Scan Mass Spectra of the Amantadine Standard

![Figure 1](image1.png) [The Q1 scan mass spectrum of amantadine standard](image1.png)

![Figure 2](image2.png) [Product ion scan mass spectrum (CE value was -18V) of amantadine](image2.png)
2.2. MRM Chromatogram of the Amantadine Standard

![MRM Chromatogram of the Amantadine Standard Sample (1 μg/L)](image)

2.3. Calibration and Linearity
Calibration is performed using a 5-point curve at concentrations 0.1, 0.5, 5, 10 and 50 μg/L. Quantitation is conducted using the external standard method and the calibration curve obtained (Figure 4) had good linearity, with the linear equation $Y = (16661.2)X + (8416.12)$ and correlation coefficient $R$ is 0.99960.

![Calibration Curve of Amantadine](image)

2.4. Precision Experiment
The prepared standard working solutions at concentrations of 0.5, 1 and 5 μg/L were determined successively six times to examine instrument precision. The repeatability results of retention time and peak area are shown in Table 3. The RSD of retention time and peak area of standards (at low-, mid- and high-concentrations) were in the range of 0.04-0.91% and 0.48-2.01% respectively, indicating good instrument precision.

<table>
<thead>
<tr>
<th>Conc.(μg/L)</th>
<th>RSD% (R.T.)</th>
<th>RSD%(Area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.91</td>
<td>2.01</td>
</tr>
<tr>
<td>1</td>
<td>0.15</td>
<td>2.77</td>
</tr>
<tr>
<td>5</td>
<td>0.04</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Table 3: Repeatability Results of Retention Time and Peak Area of Amantadine (n=6)
2.5. Method Performance
In order to evaluate the sensitivity of the method, 7 replicates were prepared at the concentration of 0.01 μg/L and injected. The method detection limit (MDL) and lower limit of quantitation (LOQ) were calculated based on the standard deviations (S) determined from 7 injections where MDL = 3.14 x S, and LOQ = 4 x MDL. The results are shown in Table 4.

Table 4: Detection Limit and Lower Limit of Quantitation of Amantadine

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Standard deviation (S)</th>
<th>MDL (μg/L)</th>
<th>LOQ (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amantadine</td>
<td>0.0058</td>
<td>0.0181</td>
<td>0.0725</td>
</tr>
</tbody>
</table>

2.6. Matrix Spike and Recovery Experiment
A small amount of amantadine standard working solution was added to 2 g of blank chicken sample to achieve a matrix spike solution at a concentration of 1 μg/kg. The matrix spike sample was extracted, purified, and analyzed according to the conditions in 1.2. Figure 5 and 6 show the MRM chromatogram of blank chicken, and matrix spike sample (1 μg/kg). The recovery was determined to be 71%.

Figure 5: MRM Chromatogram of the Blank Chicken Sample

Figure 6: MRM Chromatogram of the 1 μg/kg Matrix Spike Sample
Conclusion
A method for the determination of amantadine residues in chicken using Shimadzu Triple Quadrupole Mass Spectrometer LCMS-8040 was developed. After extraction and cleanup, the chicken sample was separated by Nexera X2 UHPLC and then quantified by Triple Quadrupole Mass Spectrometer LCMS-8040. The calibration curve of amantadine exhibited good linearity within the range of 0.1-50μg/L. Six consecutive determinations were conducted for each of the standard working solutions at low-, mid- and high-concentration, and the RSD of retention time and peak area were below 0.91% and 2.77% respectively. This simple method demonstrates a fast and high-sensitivity detection of amantadine in chicken.
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