# Sensitive and Fast Measurement of Aminoglycoside Antibiotics in Milk, Meat or Eggs by HILIC-MS/MS and Identification using multi-MRM Spectrum Mode

Mikaël LEVI[1]; Ichiro HIRANO[1], Jun WATANABE[1] - [1] *Shimadzu Corporation, Kyoto, Japan*

## 1. Introduction

Aminoglycoside are an antibiotic family widely used for the treatment of bacterial infections in cattle, sheep, pigs and poultry. Due to their high affinity for tissues, the consumption of meat, milk or eggs containing aminoglycosides (AGs) can be potentially hazardous for human health. Regulatory agencies have set maximum residue limits (MRL) for these compounds with veterinary use. Depending on the countries, the animal species, the commodity or the AG, these MRL are different. For food safety laboratories testing large numbers of samples, a method capable to cover as many compounds, matrices and regulated range as possible would be of great help.

In addition, some AGs are strictly banned for some commodities (*e.g.* spectinomycin in eggs) or have low MRL. So in the case of a positive sample, a strict identification of the compound is necessary to confidently report potential fraud.

AGs are very polar compounds poorly retained by reversed-phase liquid chromatography and ion-pairing reagents are not desirable when users share several methods on a single system.

Here we present a method using hydrophilic interaction liquid chromatography (HILiC) with high sensitivity mass spectrometer to reach low limits of quantification, combined with muli-MRM Spectrum Mode for formal identification.

## 2. Materials and Method

### 2-1. Sample Preparation

Frozen meat samples were homogenized using a knife mill (Grindomix GM200, Retsch). 5 g of homogenized sample or mixed eggs or 10 mL of milk were placed in a polypropylene tube. After addition of 20µL of ISTD solution (Ribostamycin) and 20 mL of extraction solution (10 mM NH4OAc, 0.4mM EDTA, 0.5% NaCl, 2% trichloroacetic acid in water), sample were vortex mixed and shaked for 10 min. After centrifugation for 10 min at 4000 rpm, the supernatant was transferred to a clean PP tube. Extraction was repeated and supernatant combined. Extract pH was then adjusted to pH 6.5 +/- 0.25.

Further purification was then perfomed by Solid Phase Extraction (SPE) using mixed-mode sorbent (WCX Express 96-well plate 30 mg, Biotage) and Extrahera automate (Biotage). 0.5 mL of extract was loaded without prior conditioning. Then the sample was washed with 1 mL of ammonium acetate buffer 50mM pH7. After sorbent drying, target compounds were eluted with 250 µL of aqueous formic acid 10% (v/v). Purified extracts were then diluted 5 times with mobile phase A prior to transfer into a polypropylene vial and analysis.

### 2-2. Analytical Conditions

Two methods were used. First method for fast quantitative screening is using a fast gradient with two MRM by compound. The second method for positive sample confirmation, used same mobile phases and column but with a longer gradient and 15 MRM per compound. Parameters are described parameters in Table 1 and 2.

**Table 1:** HILiC Conditions

| | |
|---|---|
| System | : Nexera X2 |
| Column | : GL Sciences Inertsil Amide 3µm 100x2.1mm |
| Temperature | : 50°C |
| Mobile Phases | : A: Water + 250 mM ammonium formate + 1% formic acid<br>B: Acetonitrile |
| Flow Rate | : 800 µL/min |
| Injection Volume | : 5 µL |
| (Quant screening) | |
| Gradient | : 75 % B (0.1min) to 30%B in 1 min. 30%B (2 min). 30%B to 75%B in 0.1 min |
| Total Run Time | : 4.5 min |
| (ID Confirmation) | |
| Gradient | : 70 % B (0.1min) to 60%B in 6 min. 60%B to 50 (3min). 50%B to 70%B in 0.1 min |
| Total Run Time | : 13 min |

**Table 2:** MS/MS conditions for fast quantitative screening

| | | | |
|---|---|---|---|
| System | : LCMS-8060 | | |
| Ionization | : Heated ESI | | |
| Probe Voltage | : +1.5 kV (positive ionization) | | |
| Temperature | : Interface: 400°C | | |
| | Desolvation Line: 150°C | | |
| | Heater Block: 300°C | | |
| Gas Flow | : Nebulizing Gas: 3 L/min | | |
| | Heating Gas: 20 L/Min | | |
| | Drying Gas: 3 L/min | | |
| Dwell Time / Pause time | : 6 ms / 1.5 ms | | |
| MRM | : Compound | MRM Quant | MRM Qual |
| | Spectinomycin | 351.1 > 207.0 | 351.1 > 98.2 |
| | Apramycin | 540.3 > 217.1 | 540.3 > 378.3 |
| | Dihydrostreptomycin | 584.3 > 263.2 | 584.3 > 246.0 |
| | Gentamicin C1a | 450.2 > 322.2 | 450.2 > 163.0 |
| | Gentamicin C1 | 478.3 > 322.3 | 478.3 > 157.1 |
| | Gentamicin C2 | 464.3 > 322.1 | 464.3 > 160.0 |
| | Hygromycin B | 528.3 > 177.1 | 528.3 > 352.1 |
| | Kanamycin | 485.3 > 163.0 | 485.3 > 324.2 |
| | Neomycin B | 615.3 > 161.1 | 615.3 > 163.1 |
| | Streptomycin | 582.3 > 263.2 | 582.3 > 246.12 |
| | Amikacin | 586.3 > 425.2 | 586.3 > 163.3 |
| | Netilmicin | 476.3 > 299.2 | 476.3 > 191.2 |
| | Paromomycin | 616.3 > 163.1 | 616.3 > 293.2 |
| | Sisomycin | 448.3 > 254.1 | 448.3 > 271.2 |
| | Tobramycin | 468.3 > 324.0 | 468.3 > 163.0 |

## 3. Results

### 3-1. Calibration

As the method should fit any type of sample, calibration standards were prepared in aqueous 1% formic acid. The calibration range was set up by combining all MRL from Europe, Japan and USA for all target compounds and all commodities. For each compound, the lowest MRL divided by 10, or the practical achievable concentration with S/N > 10 was considered as the target limit of quantification. The highest MRL +50% was used to define the highest calibration level.

When no MRL was defined, the Japan rule using 10 ng/g as default MRL was employed for the highest level. Finally, taking into account the sample preparation protocol, volumetric concentration were established. The table 3 summarize the calibration ranges used. Some typical chromatograms at LOQ are provided in figure 1.

**Table 3:** Calibration ranges

| | Calibration range (µg/kg) | |
|---|---|---|
| Compound | lowest (Low MRL/10) | highest (high MRL + 50%) |
| Amikacin | 1.3 | 10 |
| Apramycin | 4.0 | 2021 |
| Dihydrostreptomycin | 8.2 | 2015 |
| Gentamicin(*) | 3.4 | 5041 |
| Hygromycin | 1.4 | 10 |
| Kanamycin | 6.7 | 2542 |
| Neomycin (+) | 33.9 | 10245 |
| Netilmicin | 1.3 | 10 |
| Paromomycin | 33.4 | 1508 |
| Sisomycin | 1.3 | 10 |
| Spectinomycin | 6.8 | 4995 |
| Streptomycin | 20.1 | 2036 |
| Tobramycin | 1.4 | 10 |

(*) sum of C1, C1a and C2 congeners
(+) using Neomycin B as marker

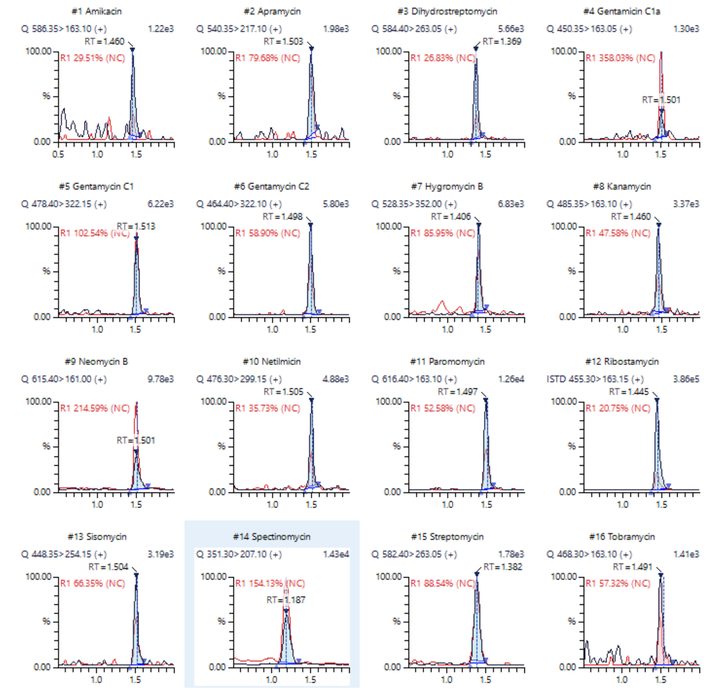


**Figure 1:** Chromatograms at the Limit of Quantification

### 3-2. Recovery

Several meat samples, eggs and milk samples were purchased from the local supermarket. All samples were processed as described in 2.1. Blank samples and samples spiked at 100 ng/g before extraction were analyzed. No compound were found in blank samples. Peak areas were compared to an aqueous standard at the same concentration.

The mean recoveries for each compound were superior to 90% and moreover, were homogenous within the type of samples tested. This illustrates the good extraction recoveries and low matrix effect obtained.

### 3-3. Identification using MRM Spectrum Mode

In MRM Spectrum Mode, 15 MRM transitions were acquired per compound. Signals were merged by the software (Labsolutions Insight Library Screening, Shimadzu Corp., Kyoto, Japan) to create a spectrum with optimized sensitivity for each fragment. By comparing this spectrum to a predefined library, identification becomes unambiguous. Thanks to ultrafast MRM features of the mass spectrometer used, there is no significant difference in sensitivity when acquiring 2 or 15 MRM per compound. Figure 2 shows library search display.

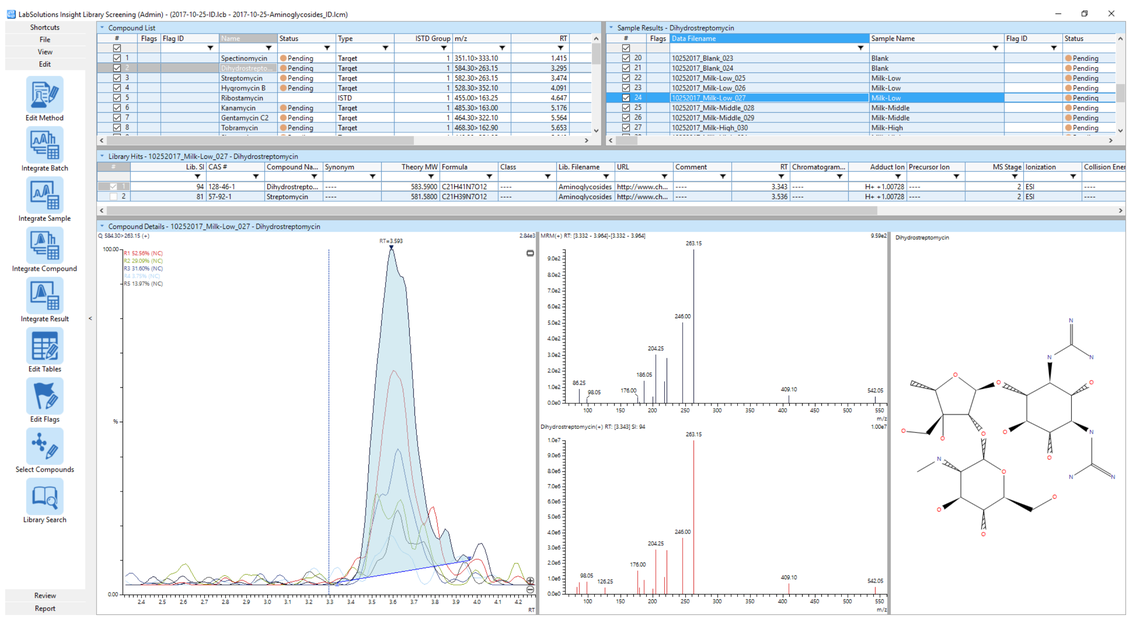


**Figure 2:** Multi-MRM library search results view in Labsolutions Insight Library Screening

## 4. Conclusions

A very sensitive HILiC-MS/MS method was developed to detect a large panel of aminoglycoside antibiotics without ion-pairing.

One method can be used for all kind of animal species or commodities, covering major food safety regulations.

The complete workflow, including sample preparation has been optimized to provide high-throughput. The good recoveries obtained across the tested matrices eliminate the use of matrix-matched calibration standards.

In addition to the fast quantitative screening method, a ID confirmation method using MRM Spectrum Mode can be performed with same mobile phases and column.