A fast method for quantitative analysis of antibiotic residues

Consumption of meat on the global scale has doubled in the past 20 years and reached more than 320 million tons in 2019. The meat industry has been feeding growth-promoting antibiotics to food producing animals for years in order to improve efficiency. However, scientists have raised concerns that, in conjunction with the general overuse of antibiotics in humans, this use of so called 'sub-therapeutic' levels of antibiotics in livestock may lead to serious health risks for people.

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The LCMS-8060 NX triple quadrupole mass spectrometer (LC-MS/MS).

These antibiotics or veterinary drugs, also known as medicinal products for veterinary use, are substances or combinations of substances to treat, prevent or diagnose diseases in animals.

The EU works to support the development and authorisation of safe, effective and quality veterinary medicinal products for food production and companion animals, ensuring the availability of drugs and guaranteeing the highest levels of public health, animal health and a safe environment.

Legal framework

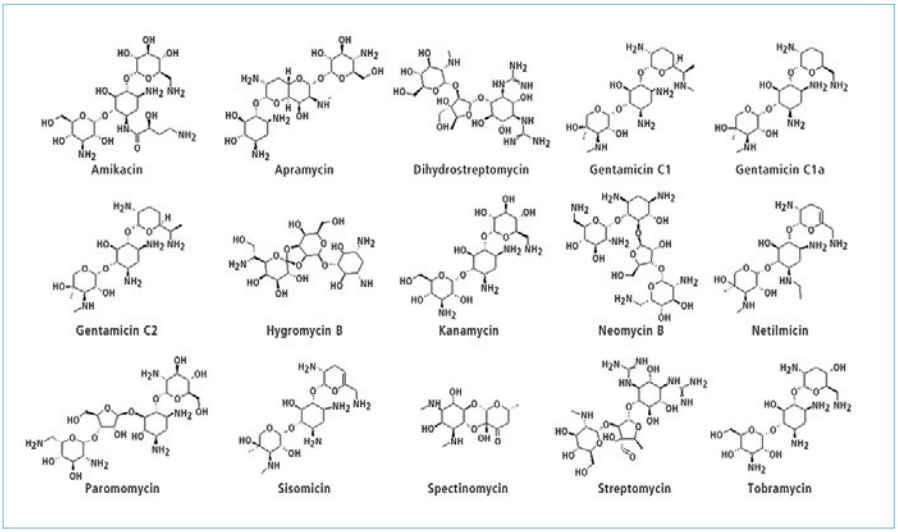
The law governing veterinary medicinal products in the EU sets standards to ensure adequate health protection with established Maximum Residue Limits (MRLs) for certain drugs in target tissues and animal species.

It has also identified pharmacologically active compounds that are prohibited and considered to be a hazard at any level (EU regulation EC 37/2010; Commission Decision 2003/181/EC; 21CFR Part 556 Tolerances for Residues of New Animal Drugs in Food).

The current legal framework for veterinary medicinal products and medicated feed has been replaced by Regulation (EU) 2019/6 on veterinary medicinal products and Regulation (EU) 2019/4 on medicated feed; the new regulations will apply from 28th January 2022.

For veterinary drugs testing to meet the new requirements, Shimadzu offers the full solution with its high sensitivity LCMS-8060 NX triple quadrupole mass spectrometer (LC-MS/MS).

Fig. 1. Targeted aminoglycosides.



Method package for meat samples and milk

Aminoglycosides (AGs) are an antibiotic family widely used for the treatment of bacterial infections in cattle, sheep, pigs and poultry.

They have a broad-spectrum activity and are used against Gram-positive and Gram-negative bacteria. AGs can cause severe side effects such as oto- and nephrotoxicity which has not however prevented the widespread use of AGs in veterinary applications due to their low costs. Because of their high affinity for tissues, they may occur in meat, milk or eggs if the withholding period has not been observed or if used improperly.

Consumption of food containing aminoglycosides can therefore be potentially hazardous to human health and MRLs for these compounds must be strictly controlled.

Aminoglycosides are very polar compounds poorly retained by reversed-phase liquid chromatography. Ion-pairing reagents are not desirable as they can easily contaminate the analytical system and interfere in other methods.

A Method Package has been developed to overcome these problems. It comprises a protocol to generate clean extracts in a variety of commodities and a rapid quantitative method using hydrophilic interaction liquid chromatography (HILiC) combined with triple quadrupole mass spectrometry detection.

If necessary, a second method for formal peak identification using MRM spectrum mode can be applied without changing reagents.

This article focuses on the use of the method package to assess the safety level of several meat samples and milk.

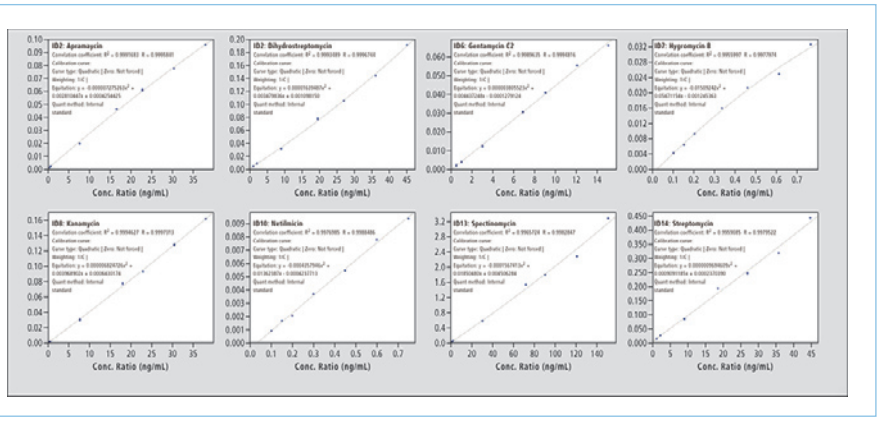


Fig. 3. Representative calibration curves of aminoglycosides.

Sample preparation

Meat samples such as the famous Kobe beef, chicken breast and liver, pork cutlet and cow milk were purchased from a local supermarket and used for the following determinations. After grinding, 5g of sample was treated as described in the Aminoglycoside Antibiotics Method Package.

Briefly, after addition of internal standard (ribostamycin), compounds were extracted twice with acidic buffer.

Extracts were then purified by weak-cation exchange and diluted by a factor of five before injection (5µg).

Each sample was also spiked at 0.5 times and 1.5 times the MRL.

All samples were prepared once, except the beef sample spiked at 0.5 × MRL, which was prepared in six replicates.

Table 1. Maximum residue limits for the selected samples and calibration ranges.

Substances	Maximum residue limits			Calibration ranges	
	low MRL JP (µg/kg)	low MRL EU (µg/kg)	high MRL JP (µg/kg)	LLOQ (µg/kg)	ULOQ (µg/kg)
Amikacin	No value	No value	Default 10	2	15
Apramycin	60	1,000	500	6	750
Dihydrostreptomycin	200	500	600	20	900
Gentamicin (sum)	100	50	200	10	300
Hygromycin	No MRL	No MRL	Default 10	2	15
Kanamycin	40	100	500	4	750
Meomycin	500	500	500	50	750
Netilmicin	No MRL	No MRL	Default 10	2	15
Paromomycin	No MRL	500	Default 10	2	15
Sisomicin	No MRL	No MRL	Default 10	2	15
Spectomycin	200	200	2,000	20	3,000
Streptomycin	200	200	600	20	900
Tobramycin	No MRL	No MRL	Default 10	2	15

LC-MS/MS analysis

Purified extracts were assayed applying LC-MS/MS conditions and ready-to-use methods included in the LC-MS/MS Method Package for Aminoglycoside Antibiotics. A calibration curve prepared in mobile phase was set for quantification of samples.

They were first assayed with a fast quantitative method employing HILiC conditions to elute compounds with a gradient of acetonitrile and a formate buffer. Cycle time for analysis was 4.5 minutes.

Detection was performed in Multiple Reaction Monitoring (MRM) mode with two transitions acquired per compound. For positive samples (over the MRL), a second injection of purified extracts was executed to assess peak identity. For this purpose, a second method with the same column and mobile phases but alternative gradient and 15 MRM per compound (except ISTD) was used.

The analytical system was a Nexera X2 UHPLC coupled with LCMS-8060 triple quadrupole mass spectrometer (Fig. 2). Data processing was achieved using LabSolutions Insight v.3.1 with screening option.

Results

Depending on the animal species and the target tissues, MRLs were different. Specified MRLs for a variety of aminoglycosides are listed in Table 1.

For those substances where no MRL has been officially defined, a 'default' MRL of 10µg/kg should be considered for any chemical tested. For apramycin, dihydrostreptomycin, gentamicin, kanamycin, neomycin, spectinomycin and streptomycin, the calibration range was then set to cover from 10% of the lowest MRL to 150% of the highest one. For other compounds without official MRL, the calibration range was set from 20% to 150% of 10µg/kg. Calibration values can be found in Table 1.

Seven calibration levels, regularly observed within the range were prepared. Calibration standards with an accuracy within 85-115% were selected.

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Table 2. Repeatability in beef sample at 0.5 × MRL

	AMI	APRA	DHSTP	GENT C1a	GENT C1	GENT C2/C2a	HYGRO	KANA
Mean conc. (µg/kg)	5.38	225.0	350.0	45.6	47.5	48	5.32	21.1
Recovery (%)	107.0	89.0	117.0	90.4	94.2	95.2	107.0	102.0
RSD (%)	19.9	7.7	10.0	10.8	10.2	6.9	7.1	12.0
	NEO	**NETIL**	**PARO**	**SISO**	**SPC**	**STP**	**TOB**	**–**
Mean conc. (µl/kg)	228.0	5.03	4.39	4.47	275.0	348	4.66	–
Recovery	91.4	101.0	88.1	88.4	110	114	91.5	–
RSD (%)	8.8	10.0	8.1	4.4	11.0	11.9	6.2	–

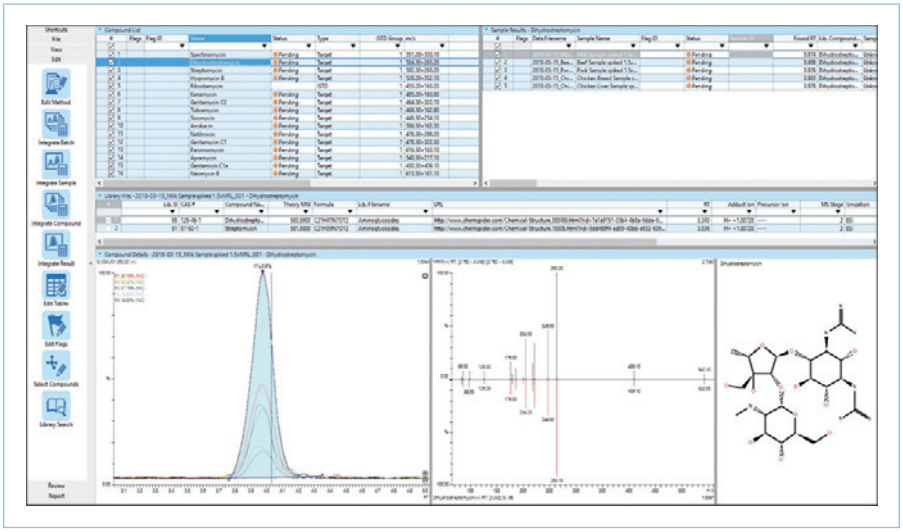

Fig. 4. Library search result of dihydrostreptomycin MRM spectrum (spiked at 1.5 × MRL).

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Representative calibration curves are shown in Fig. 3.

The samples without spiking were detected as being free of aminoglycoside residues.

Recovery was then calculated in spiked samples using the calculated concentrations. Recoveries were in the acceptable range of 70-120% for all compounds and all types of samples.

Repeatability has been assessed in beef sample spiked at 0.5 × MRL. Results are presented in Table 2. The % RSD was less than 20% which is suitable for such an application.

For increased confidence in identification of compounds exceeding the MRL, additional injection of the extracts can be done using a second method with elongated gradient time and acquisition of 15 MRM transitions per compound.

MRM signals are then merged to create a spectrum in which every fragment is acquired at optimum collision energy.

An example of search result by LabSolutions Insight software with screening option is illustrated in Fig. 4.

The samples can be processed and the library search can be done automatically in batch mode.

In this case, a high identification score can be obtained. Dihydrostreptomycin recorded a score of 95, while the second hit (streptomycin, a very close compound) achieved a score of only 51.

Conclusion

A newly developed Method Package was successfully applied to real meat and milk samples.

The quantitative method gave good recoveries and accuracies, even for non-regulated compounds at trace levels.

It can be applied to a variety of samples without using matrix-matched calibration curves. A complementary method gives increased confidence in identification for over-the-limit compounds employing MRM spectrum mode. ■

References are available from the authors on request