

# Analysis of 157 Veterinary Drugs with Agilent Intelligent Reflex

## Authors

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## Abstract

The analysis of multiple residues is required for the safe management of veterinary drug residues in livestock and fishery products. A 157-drug simultaneous multiresidue testing method has been implemented for this management. The testing method specifies quantitative limits for both livestock and fishery products, as well as their respective residues. For some residues, an exceptionally low quantitative limit of 0.0001 mg/kg (0.1 ppb) is applied. Since most standard substances are manufactured and supplied as grouped mixed standards, in accordance with the quantitative limits outlined in the testing method, it is common to generate a calibration curve for all components within the same concentration range (e.g., 0.1 to 20 ppb).

These identical trace concentration calibration curves lead to confusion when interpreting results for medium- and high-concentration residues, thereby reducing analytical efficiency due to an increased number of unnecessary reanalyses.

Agilent Korea has applied the newly developed intelligent reflex function to the 157-drug simultaneous multi-residue testing method to enhance analytical productivity and accuracy.

As a result, analysis time was reduced by up to 25% under standard sample analytical conditions. Automated reanalysis of high-concentration samples above the calibration curve range with intelligent reflex showed an average improvement of 41.6% in interquartile deviation, and an average reduction of 55% in the proportion of residues exceeding 70 to 130% of the injected concentration.

The instrument limit of detection (ILOD) for the 157-drug simultaneous multiresidue analysis with an Agilent 1290 Infinity II LC System and Agilent 6475 triple quadrupole mass spectrometer (LC/TQ) was 0.047 ppb (ng/mL) on average for group 1 and 0.033ppb (ng/mL) on average for group 2. The calibration curve linearity within the quantification range (0.1 to 20 ppb) was 0.994 for group 1 and 0.995 for group 2 on average.

## Introduction

Agilent has developed and launched the intelligent reflex function to enhance analytical efficiency.

Intelligent reflex includes the following three features:

1. Automatic analysis of blank samples for detection of carryover.
2. Automatic reanalysis with reduced injection volume for samples above the calibration curve range.
3. After rapid screening, only positive samples are sent for reanalysis under standard analytical conditions.

Agilent Korea applied the first and second features of intelligent reflex to the 157-drug simultaneous multi-residue analysis method for veterinary drugs and evaluated the improvement in analytical efficiency.

The Agilent 1290 Infinity II LC System and Agilent 6475 triple quadrupole LC/MS were used for the analysis, and the instrument limit of detection (ILOD) and calibration curve linearity were also verified before analysis.

To evaluate the concentration accuracy of residues exceeding the calibration curve range, four negative livestock and fishery samples (pig, chicken, shrimp, and salmon) were spiked with residues at 2.5 times the highest calibration standard concentration.

## Experimental

### Standards and reagents

All reagents used for analysis were of LC/MS grade, and the standards used were group-based mixed standards and individual standards manufactured by Kemidas Co., Ltd. in accordance with the 157-drug test method for veterinary drugs.

To evaluate the calibration curve and instrumental detection limit, the calibration curves for groups 1 and 2 were prepared by stepwise dilution with water/methanol (1:1, v/v) according to the test method, resulting in seven solutions (0.1, 0.2, 0.5, 1, 5, 10, and 20 ppb (ng/mL)) that were used for the analysis.

The four livestock and fishery product samples were processed according to Food Standards and Specifications / 8.3.1 Simultaneous Multi-Residue Test Method for Veterinary Drugs in Livestock and Fishery Products. Negative sample extracts (90%) were mixed with a 500 ppb standard (10%) to prepare a final sample concentration of 50 ppb.

**Table 1.** Standard information manufacturer: Kemidas Co., Ltd.

Group 1	Group 2
6 mixture group VDLF mix (1c, 2c, 3c, 4c, 5c) BEZ mix-18a	8 mixture group VDLF mix (7c, 8c, 9c, 10c, 11c, 12c) NIZ mix-8a, SFB mix-7a
3 single residues oxolinic acid, cefadroxil, cefalexin	4 single residues carbendazim, propoxur, N,N-Bis-(4-nitrophenyl)urea, isometamidium chloride
Excluded 2 residues desfuroylcefotiofur, fumagillin	Excluded 2 residues efrotomycin, pirlimycin

### Instrument and analysis conditions

For rapid and stable separation, the [Agilent 1290 Infinity II LC System](#) was used, and for high-sensitivity analysis of the separated compounds, the [Agilent 6475 triple quadrupole LC/MS](#) equipped with Agilent Jet Stream electrospray ionization (ESI) technology was used.

Some veterinary drugs exhibited significant carryover, which posed challenges in analyzing low-concentration samples after high-concentration ones. The 1290 Infinity II LC System was equipped with the Agilent proprietary Multiwash feature to minimize carryover.

**Table 2.** Agilent LC analysis conditions.

Agilent 1290 Infinity II UHPLC System (Pump: Binary)				
Column	Poroshell 120EC-C18, 2.1 x 150 mm, 2.7- $\mu$ m (part number 693575-902)			
Column Temperature	40°C			
Injection Amount	2.5 $\mu$ L			
Mobile Phase	A: 0.1% Formic acid in DI water B: 0.1% Formic acid in ACN			
Gradient	Time (min)	A	B	Column flow rate
	0	90	10	0.35 mL/min
	1	90	10	0.35 mL/min
	6	60	40	0.35 mL/min
	10	5	95	0.35 mL/min
	10.5	5	95	0.45 mL/min
	13	5	95	0.45 mL/min
	13.3	90	10	0.35 mL/min
	15.0	90	10	0.35 mL/min
Multiwash Conditions				
Solvent	Step	Time (s)	Seat back flush	Needle wash
10% IPA in DI Water	1	7	On	On
70% MeOH in DI Water	2	7	On	On
10% ACN in DI Water	3	3	On	On

**Table 3.** Agilent MS analysis conditions.

Agilent 6475A LC/TQ	
Ionization Source	ESI, with Agilent Jet Stream
Acquisition Mode	dMRM
Gas Temperature	180 °C
Gas Flow Rate	12 L/min
Nebulizer	30 psi
Sheath Gas Temperature	370 °C
Sheath Gas Flow Rate	12 L/min
Capillary Voltage (V)	(+)3500, (-)3500
Nozzle Voltage (V)	(+)0, (-)0
Polarity	Positive / Negative

## Results and discussion

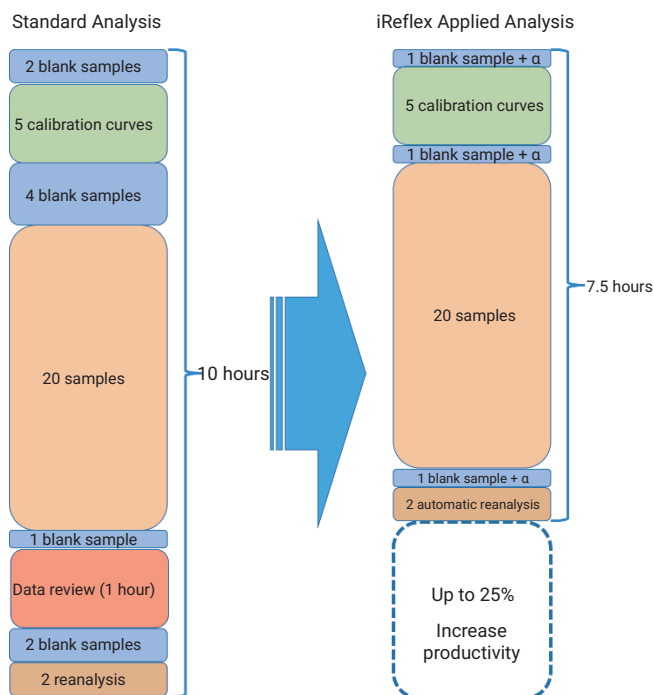
### Evaluation of productivity improvement with intelligent reflex

Agilent intelligent reflex supports two functions: detection of carryover, which triggers automatic analysis of blank samples as needed, and automatic reanalysis with reduced sample volume when concentrations above the calibration curve range are detected.

When compared with typical veterinary drug sample analysis conditions, the method using intelligent reflex along with the Multiwash feature showed a maximum improvement of 25% in analytical productivity.

#### Sample analysis conditions

- Instrument analysis time: 15 minutes/sample
- Number of standards for calibration curve: 5
- Number of analytical samples per batch: 20
- Time required for first review of results after analysis: 60 minutes
- Detection of concentrations above the calibration range for at least one residue in 10% of the total samples analyzed (2 samples, in the case of 20 samples analyzed)



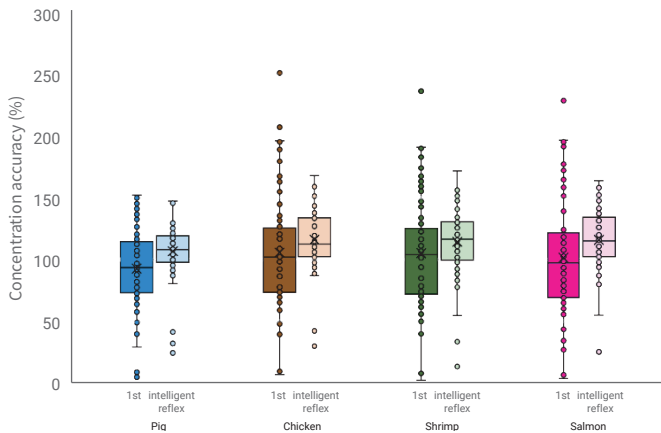
**Figure 1.** Comparison of productivity improvement between standard analysis and intelligent reflex-applied analysis.

### Evaluation of accuracy improvement for concentrations above calibration curve with intelligent reflex

Agilent intelligent reflex supports the feature of automatically reducing the sample volume and reanalyzing the sample when concentrations above the calibration curve range are detected.

The extracts of each matrix were spiked and prepared with residue concentrations 2.5 times higher than the highest calibration standard, then analyzed using the intelligent reflex feature. Following the analysis at concentrations above the calibration curve, the sample injection volume was automatically reduced, and reanalysis was performed within the calibration curve range.

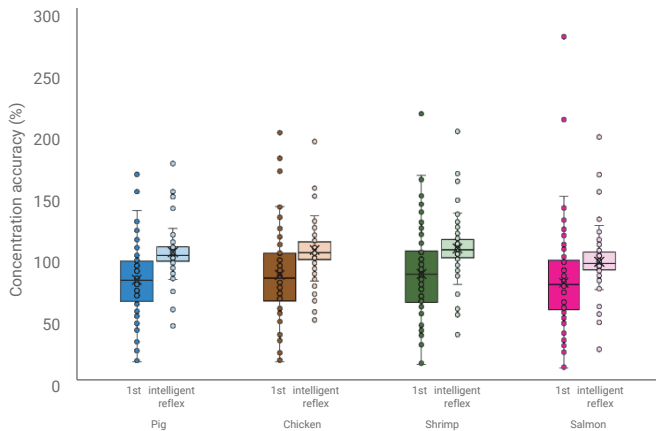
The initial analysis, where concentrations exceeded the calibration curve range, was compared with those from the reanalysis after reducing the sample injection volume using intelligent reflex.



**Figure 2.** Group 1 comparison of concentration accuracy with intelligent reflex analysis for residues exceeding the calibration curve.

In the first analysis for group 1, where concentrations exceeded the calibration curve range, the interquartile deviation was found to be an average of 24.9 (pig: 20.7, chicken: 26.2, shrimp: 26.7, salmon: 26.2). However, in the reanalysis within the calibration range using intelligent reflex, the interquartile deviation decreased to an average of 14.6 (pig: 10.8, chicken: 15.6, shrimp: 15.6, salmon: 16.2).

This reflects a 42% average decrease in deviation due to reanalysis within the calibration curve range using the automatic reinjection workflow. Additionally, the proportion of residues falling outside the 70 to 130% range of the injection concentration was found to improve by an average of 40% with automated reanalysis.



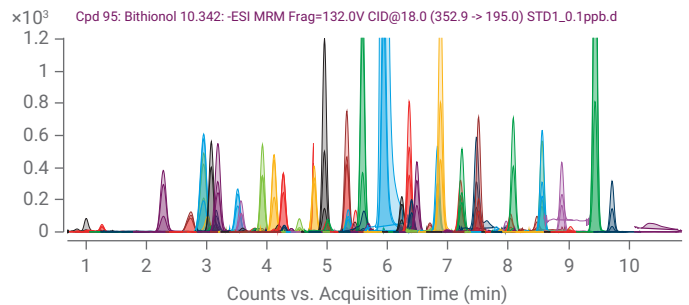
**Figure 3.** Group 2 comparison of concentration accuracy with intelligent reflex analysis for residues exceeding the calibration curve.

In the first analysis for group 2, the interquartile deviation was found to be an average of 19.3 (pig: 16.5, chicken: 19.6, shrimp: 21.0, salmon: 20.0). However, in the intelligent reflex analysis, the interquartile deviation decreased to an average of 7.0 (pig: 5.9, chicken: 7.2, shrimp: 7.4, salmon: 7.4).

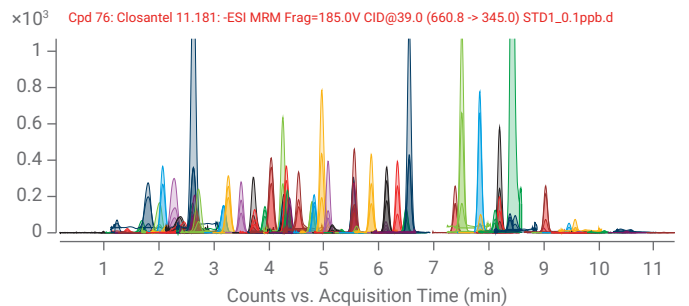
This indicates a 64% average decrease in deviation with automated reinjection analysis compared to analysis exceeding the calibration curve range. Additionally, the proportion of residues falling outside the 70 to 130% range of the injection concentration showed a 69% reduction, demonstrating improved results.

### Calibration curve lowest concentration dMRM chromatogram

The group-specific dynamic multiple reaction monitoring (dMRM) chromatograms at the lowest concentration used in the calibration curve, 0.1 ppb (ng/mL), are shown in Figure 4 and Figure 5.



**Figure 4.** Group 1 calibration curve lowest concentration dMRM chromatogram.



**Figure 5.** Group 2 calibration curve lowest concentration dMRM chromatogram.

### Instrumental detection limit

Agilent MassHunter Quantitative Analysis software supports automatic detection limit calculation with results from continuous analysis. Using this feature, the instrumental detection limit was calculated after performing six consecutive analyses of the mixed standard at the lowest concentration of the calibration curve, 0.1 ppb (ng/mL).

The instrumental detection limit was 0.047 ppb on average for group 1, and 0.033 ppb on average for group 2. For group 1, more than 85% of the components met the detection limit of less than 0.1 ppb, and for group 2, more than 93% of the components met the detection limit of less than 0.1 ppb.

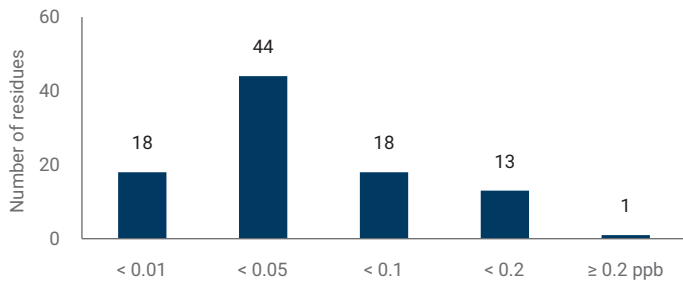


Figure 6. Instrumental detection limits for group 1 residues.

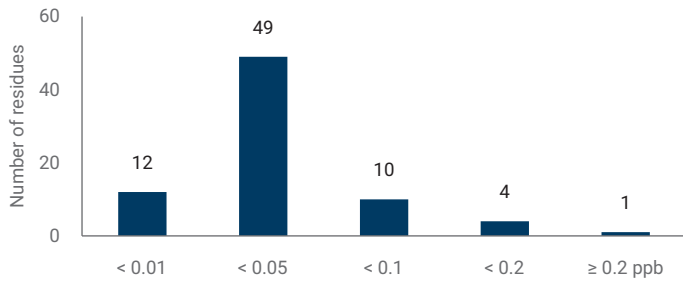


Figure 7. Instrumental detection limits for group 2 residues.

### Linearity of calibration curve

To evaluate the calibration curve linearity, mixed standards for each group were prepared via seven-step dilution to produce concentrations of 0.1, 0.2, 0.5, 1, 5, 10, and 20 ppb (ng/mL), and then analyzed.

The calibration curve  $R^2$  value of group 1 was 0.994 on average, and the calibration curve  $R^2$  value of group 2 was found to be 0.995 on average.

Then 95% of group 1 residues and 96% of group 2 residues showed stable linearity with calibration curve  $R^2$  values exceeding 0.98.

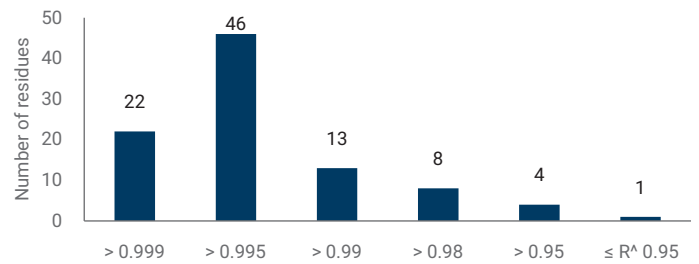


Figure 8. Linearity of the calibration curve of group 1 components.

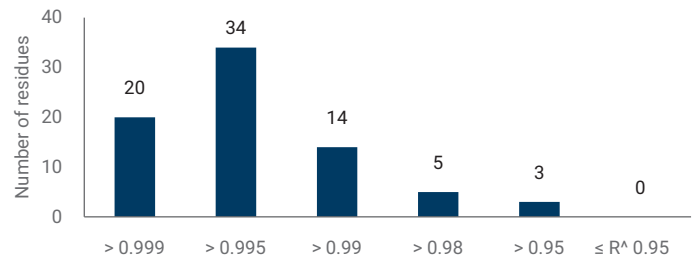


Figure 9. Linearity of calibration curve of group 2 components.

## Conclusion

Agilent applied the newly developed intelligent reflex feature to the 157-drug simultaneous multi-residue analysis method for veterinary drugs and evaluated its impact on analytical productivity and accuracy.

Results show that, compared to conventional analytical methods, analysis using intelligent reflex demonstrated up to a 25% increase in productivity.

In the evaluation of analytical accuracy, the application of intelligent reflex resulted in an average improvement of 55% compared to the conventional method, based on the number of residues exceeding the 70 to 130% threshold for samples with concentrations above the calibration curve.

The performance of the 157-drug simultaneous multiresidue analysis for veterinary drugs using the Agilent 1290 Infinity II LC system and Agilent 6475 triple quadrupole LC/MS was evaluated.

The instrument detection limit was found to be an average of 0.047 ppb (ng/mL) for group 1, and 0.033 ppb (ng/mL) for group 2. In terms of calibration curve linearity, group 1 exhibited an average of 0.994, while group 2 showed an average of 0.995, indicating stable results for performing the relevant testing methods.

LC/MS grade reagents, solvents, and validated standards were used in this experiment. Results may vary depending on the grade of reagents and solvents used and the condition of the standards. The four types of livestock and fishery products used in the experiment were tested after undergoing extraction and purification processes according to the official test method. However, since these four samples may not fully represent the entire scope of livestock and fishery products, differences in results based on the sample matrix may occur.

## References

1. Food Standards and Specifications, Article 8. General Test Method 8.3.1 Simultaneous Multi-Residue Test Method for Veterinary Drugs in Livestock and Fishery Products, Ministry of Food and Drug Safety, 2023.
2. Active & iterative data-dependent reinjection logic for maintaining throughput, uptime, and consistency in triple quadrupole LC/MS analysis, *Agilent Technologies*, Poster number MP326, ASMS 2022

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