Application Note

Food & Beverage Testing

Measuring Veterinary Drugs in Meat Using Matrix Calibration and Standard Additions

Applying Easy Injector Workflows for automated calibration

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Abstract

This application note demonstrates the use of the new Easy Injector workflows for automated calibrations in the Agilent MassHunter software, with external calibration in solvent, matrix-matched calibration, and standard addition. As an example, veterinary drug compounds will be measured in meat extract by matrix-matched calibration in comparison to a calibration in solvent and standard addition. The Easy Injector program provides different injector workflows that remove the need for programming, saving time, allowing higher ease-of-use, and leading to fewer errors.

Introduction

A widely known problem in quantitative LC/MS analysis of analytes dissolved in complex matrices like plant or meat extracts are so-called matrix effects. These effects are due to matrix compounds eluting from the analytical column at the same time as the analytes of interest. These matrix compounds can decrease or increase the response of the analyte compound by competing for ionization in the mass spectrometer's ion source or by producing masses identical to the analyte. There are commonly used strategies to avoid or minimize these matrix effects, such as creating external calibration curves in blank matrix, or standard addition.

This application note demonstrates the influence of a complex meat matrix on the analysis of veterinary drugs and the compensation of matrix effects by using matrix-matched calibration and standard addition. With the easy and fast setup of standard workflows like generation of calibration curves of dilutions, the Easy Injector workflow program significantly saves time and costs.[1] The Easy Injector workflow program capability is available in the Agilent MassHunter acquisition software in the user interface of the Agilent 1290 Infinity III Multisampler. The automated generation of a matrix-matched calibration and standard addition will be demonstrated using the Easy Injector workflow.

Experimental

Instrumentation

- Agilent 1290 Infinity III High-Speed Pump (G7120A)
- Agilent 1290 Infinity III Multisampler (G7167B)
- Agilent 1290 Infinity III Multicolumn Thermostat (G7116B)
- Agilent Ultivo triple quadrupole LC/MS with Agilent Jet Stream source

Software

- Agilent MassHunter acquisition software (v. 12.2)
- Agilent MassHunter Qualitative Analysis software (v. 12.0)
- Agilent MassHunter Quantitative Analysis software (v. 12.1)

Column

Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 150 mm, 1.9 µm (part number 699675-912)

UHPLC method parameters

Parameter	Value
Flow Rate	0.5 mL/min
Solvents	A) 5 mM ammonium formate + 0.1% formic acid in water B) 5 mM ammonium formate + 0.1% formic acid in methanol
Gradient	Time (min) %B 0.00 5 6.00 60 6.10 100 Stop time: 7 min Post time: 3 min
Column Temperature	40 °C
Needle Wash	3 s acetonitrile
Injection Volume	1 µL and 0.9 µL in case of standard addition

MS method parameters

Parameter	Value
Acquisition Mode	Dynamic MRM. All transitions, including details of molecular weights, fragments, voltages, and collision energies, are listed in appendix Table A1
Polarity	Positive
Capillary Voltage	3,600 V in positive mode
Drying Gas Flow	8 L/min
Drying Gas Temperature	300 °C
Nebulizer Pressure	40 psi
Sheath Gas Temperature	200 °C
Sheath Gas Flow	10 L/min
Nozzle Voltage	350 V
Q1 and Q2: Resolution	Unit (0.7 amu), optimized by autotune
Delta EMV	0 V

Chemicals

- 5 M ammonium formate solution (G1946-85021)
- Formic acid for LC/MS (G2453-85060)
- Dimethyl sulfoxide (DMSO)
- Ethylenediaminetetraacetic acid, disodium salt, dihydrate (Na-EDTA)

Solvents

- Agilent InfinityLab acetonitrile for LC/MS (part number 5191-5101-001)
- Agilent InfinityLab water for LC/MS (part number 5191-5121-001)
- Agilent InfinityLab methanol for LC/MS (part number 5191-5111-001)

Additional material

- Agilent Captiva EMR—Lipid cartridge, 6 mL, 600 mg (part number 5190-1004).
- Agilent vial insert, 400 µL, glass, flat bottom (part number 5181-3377).
- Agilent vial, screw top, amber, write-on spot, certified, 2 mL (part number 5182-0716).
- Agilent screw cap, bonded blue, PTFE/red silicone septa (part number 5190-7024).

Standards

The used veterinary drug standard comprised 25 compounds (10 ppm, in acetonitrile/water) and was produced by Ehrenstorfer, Germany.

Calibration

The calibration curves were created using the Easy Injector workflow program in a fully automated sequence with the 1290 Infinity III Multisampler (Figure 1). The Multisampler created dilutions from a given stock solution (500 ppb) and diluent and put them into vials for later injection. The first run of the sequence comprised the Easy Injector workflow program with the Multisampler for generation of the dilutions together with a short cleaning method at high organic composition. The following injections in the sequence were using the dilutions prepared by the Easy Injector workflow program without manual interaction. Calibration points at 125, 31.25, 7.81,1.95, and 0.488 ppb were created with pure solvent and blank matrix by a dilution factor of 1:4.

Standard addition

For the quantitative measurement through standard addition, a separate calibration curve had to be created for each sample for comparison with the added standard and two calibration points (which represented different concentrations of the standard). These curves were produced with the Easy Injector workflow program (Figure 2). Two standard stock solutions at 10 and 100 ppb were used. A calibration concentration of 1 and 10 ppb was achieved by dilution of the 10 and 100 pbb standard by 1:10 (v:v) in sample. The samples were measured in the order: Sample > Calibration 1 ppb > Calibration 10 ppb. All necessary calculations were automatically performed in the MassHunter Quantitative Analysis software.

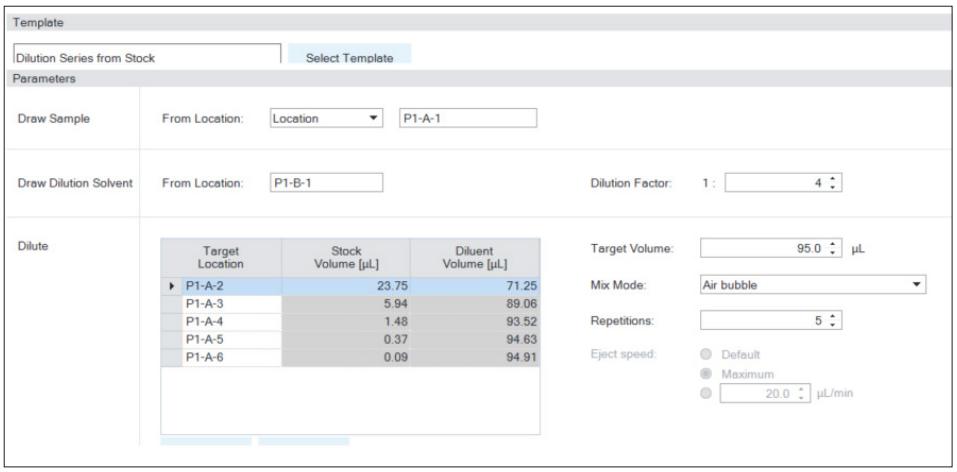

Figure 1. Easy Injector program for a dilution series from stock. The stock solution at 500 ppb (sample) and the diluent were put in defined positions. The dilution factor of 4 was selected, as well as the target volume and the mixing mode. The dilutions were ejected in screw capped vials with flat bottom inserts at defined positions for later injection in the sequence.

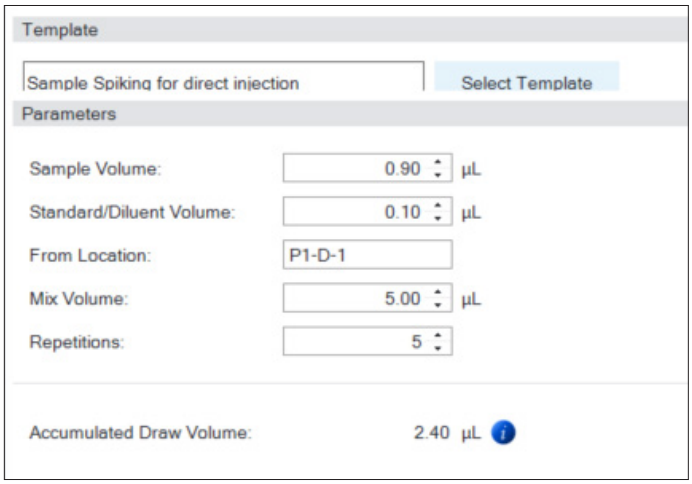

Figure 2. Easy Injector workflow for sample spiking for direct injection. The selected sample volume was mixed with the defined standard solution volume taken from a defined position, and injected. The sample position was defined in the sequence; the position in the Easy Injector workflow program corresponded to the position of the required stock solutions.

Sample (blank matrix) preparation[2]

1. Weigh 2 g of comminuted meat (pork) sample into a 50 mL centrifuge tube.
2. Add 2 mL of 0.1 M Na-EDTA solution and shake vigorously for 2 minutes.
3. Centrifuge at 5,000 rpm at 4 °C for 5 minutes. Transfer supernatant to tube 2.
4. Add 8 mL of ACN/2% formic acid/2% DMSO (V/V/V) to the sample residue in tube 1.
5. Shake for 5 minutes, and centrifuge at 5,000 rpm at 4 °C for 5 minutes.
6. Decant supernatant to tube 2.
7. Vortex the combined extract in tube 2 for 2 minutes, and centrifuge at 5,000 rpm at 4 °C for 5 minutes.
8. Transfer 5 mL of supernatant to a Captiva EMR—Lipid 6 mL cartridge and allow elution by gravity.
9. Add 1.25 mL of 80:20 ACN/water into the EMR—Lipid cartridge for secondary elution.
10. Gradually apply vacuum to drain the cartridge when there is no visible liquid left in cartridge.
11. Combine 0.5 mL of sample eluent and 0.3 mL of water in the sample vial, and vortex.
12. Blank matrix/samples are now ready for LC/MS/MS analysis.

The obtained blank matrix was post-spiked to generate the samples used in this study.

Results and discussion

The first calibration for quantitative determination of the 25 veterinary drugs in the used standard was performed in pure solvent with the Easy Injector program, as described in the Experimental section. The applied dilution was 1:4 starting from a stock solution at 500 ppb; the highest calibration point was at 125 ppb and the lowest at 0.488 ppb (Figure 3). The second calibration curve was acquired in the same manner, except that a blank meat matrix was used for dilution.

Typically, if a sample is extracted for measurement from a matrix, there are also compounds extracted which are not of interest but may influence the measurement. This influence can decrease or increase the measured concentration of the desired analyte. To demonstrate the effect on a meat extract sample which was spiked with 10 ppb of the veterinary drug mixture, the sample was measured and quantified by means of the calibration curves generated in pure solvent and blank matrix.

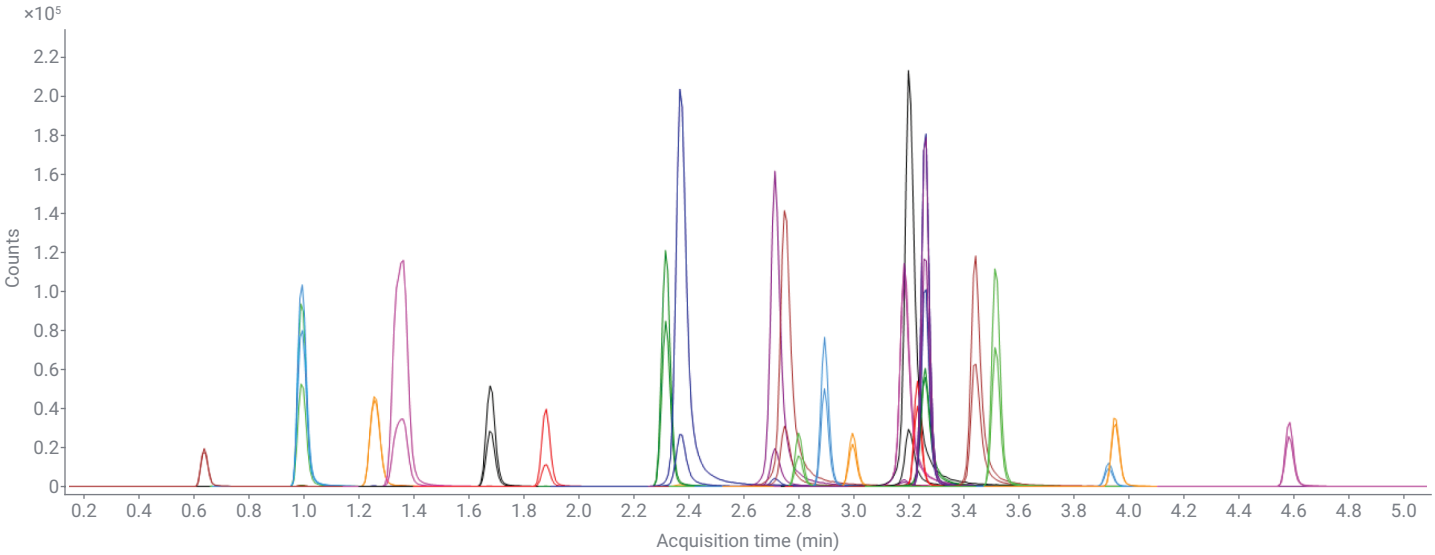

Figure 3. MRM quantifier and qualifier overlay of 25 veterinary drugs at 125 ppb each.

In the results obtained for the quantification with the calibration in pure solvent, a suppression of the compound sulfanilamide down to only 20% of the expected concentration could be seen (Figure 4A). The responses of some other compounds were increased, leading to an increase of 20% for their measured concentrations. Using a matrix-matched calibration curve, these effects can be accounted for. This step enables quantification close to the expected values of analytes suffering from ion suppression, such as sulfanilamide, or ion enhancement when using solvent calibration curves. The typical recoveries obtained for the 10 ppb spiked meat sample quantified by the matrix-generated calibration curve were between 95% and 105% (Figure 4B).

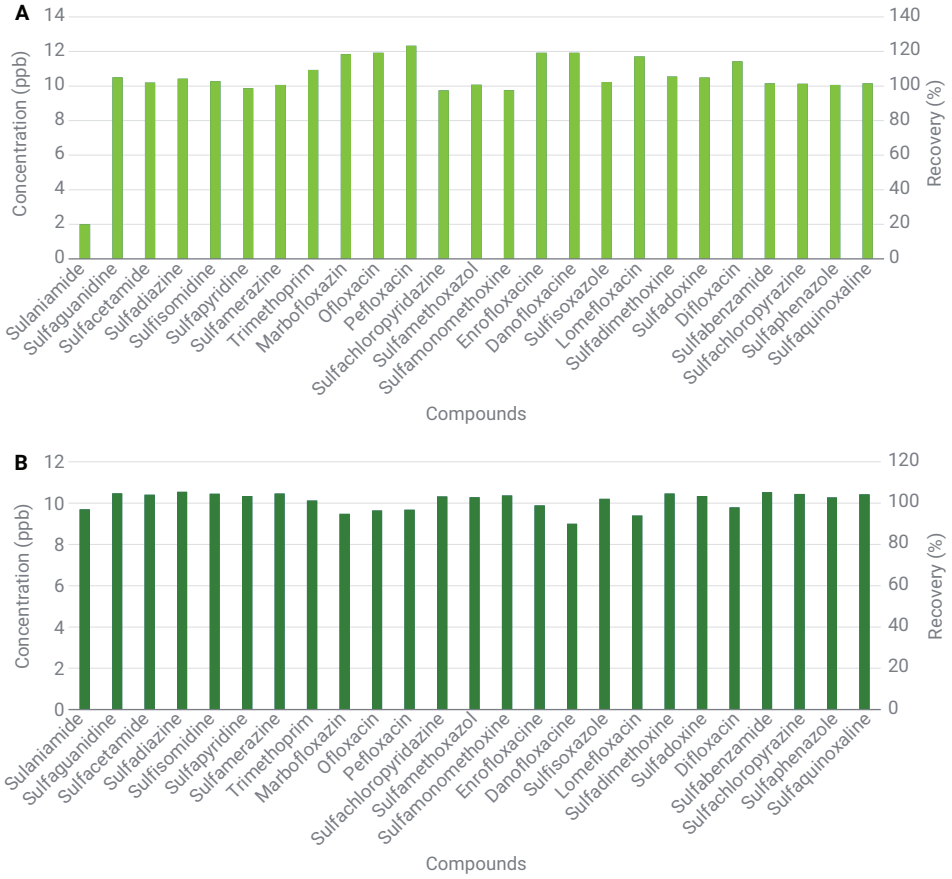

Figure 4. (A) Quantified concentration and recovery percentage of a 10 ppb spiked meat sample using pure solvent calibration. (B) Quantified concentration and recovery percentage of the 10 ppb sample measured with the matrix-matched calibration.

Then, 1 ppb spiked meat sample was measured and quantified using both calibrations. At this lower concentration level, the matrix compounds increased their influence on the quantification result. For instance, sulfanilamide could not be detected with the solvent calibration and was largely above the expected value quantified using matrix-matched calibration (Figures 5A and 5B). The signals for other compounds like trimethoprim, marbofloxacin, and ofloxacin show about 1.7 ppb in solvent calibration and about 1.2 ppb in matrix-matched calibration, as an example of matrix effect reduction compared to solvent calibration.

To improve the accuracy of the results obtained by quantifying lower concentrations in the meat extract sample, a standard addition experiment was done. For the generation of the calibration, the spiked sample was mixed with two concentrations of the standard solution at 10 and 100 ppb to achieve an additional concentration of 1 and 10 ppb, respectively. These dilutions were generated by the Easy Injector workflow directly in the Multisampler's injection needle and measured in a small sequence together with the original spiked sample.

The quantification results achieved are shown in Figure 6 (left axis). Concentrations range between 0.9 and 1.1 ppb. The recovery percentages are shown in Figure 6 (right axis). The recovery percentage results obtained by the automated standard addition are typically between 90% and 110%.

Figure 5. (A) Quantified concentration and recovery percentage of a 1 ppb spiked meat sample using pure solvent calibration. (B) Quantified concentration and recovery percentage of the 1 ppb sample measured with the matrix-matched calibration.

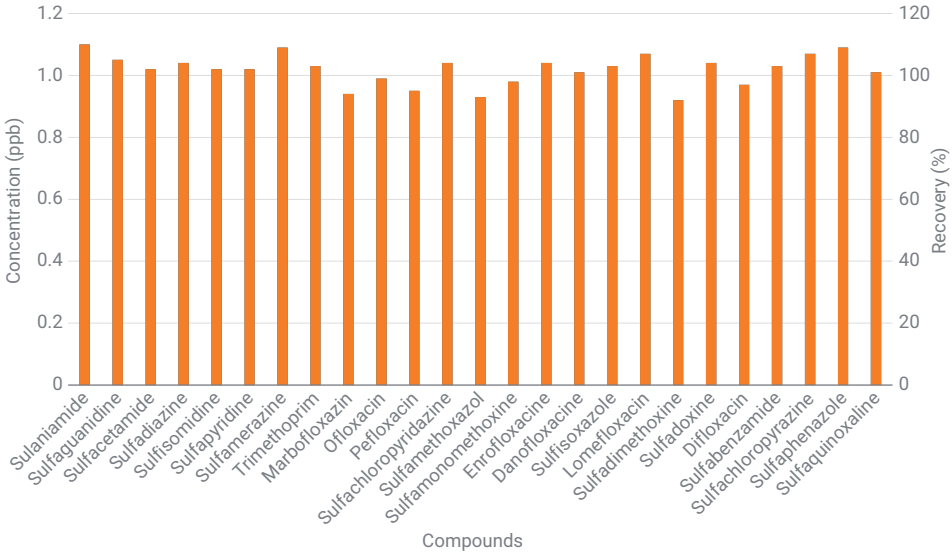

Figure 6. (Left axis) Quantitative results obtained for a 1 ppb sample using the standard addition technique. (Right axis) Recovery percentage of the quantitative obtained results for all spiked compounds at 1 ppb level.

Conclusion

This application note demonstrates the use of the Easy Injector program of the Agilent 1290 Infinity III Multisampler in Agilent MassHunter acquisition software for automated calibration and standard addition experiments. This functionality enables a fully automated workflow starting with an automated generation of a calibration curve for quantification. This step can be followed by an automated standard addition experiment for samples with concentrations at the lower quantification range.

References

1. Cakar, A. Easy and Fast Automation of Sample Preparation. Agilent Technologies application note, publication number 5994-7703EN, **2025**.
2. Zhao, L.; Lucas, D. Multiclass Multiresidue Veterinary Drug Analysis in Beef Using Agilent Captiva EMR—Lipid Cartridge Cleanup and LC/MS/MS. *Agilent Technologies application note*, publication number 5991-8598EN, **2017**.

Appendix

Table A1. MRM settings for used standard compounds.

Compound Name	RT (min)	Precursor (*m/z*)	Fragmentor (V)	Product 1 (*m/z*)	CE 1 (V)	Product 2 (*m/z*)	CE 2 (V)	Polarity
Sulfanilamide	0.64	173.0	80	155.8	1	91.9	16	Positive
Sulfaguanidine	1.00	215.1	80	155.9	9	108.0	20	Positive
Sulfacetamide	1.01	215.0	80	155.9	3	91.9	20	Positive
Sulfadiazine	1.27	251.1	90	156.0	15	92.0	20	Positive
Sulfisomidine	1.37	279.3	125	185.9	17	124.0	25	Positive
Sulfapyridine	1.69	250.1	97	183.9	17	156.0	10	Positive
Sulfamerazine	1.89	265.1	97	172.0	13	108.0	12	Positive
Trimethoprim	2.34	291.1	130	230.0	20	122.9	20	Positive
Marbofloxacin	2.39	363.1	97	345.1	20	72.0	20	Positive
Ofloxacin	2.74	362.1	103	344.1	20	318.1	20	Positive
Pefloxacin	2.77	334.1	113	316.1	20	290.1	19	Positive
Sulfachloropyridazine	2.82	285.0	100	156.0	10	108.0	22	Positive
Sulfamethoxazole	2.91	254.1	100	156.0	10	92.0	26	Positive
Sulfamonomethoxine	3.01	281.1	120	155.9	15	92.0	20	Positive
Enrofloxacin	3.20	360.0	130	342.1	18	316.2	18	Positive
Danofloxacin	3.23	358.2	140	340.1	22	255.0	46	Positive
Sulfisoxazole	3.24	268.1	100	156.0	10	113.0	10	Positive
Sulfadimethoxine	3.27	311.1	123	156.0	17	108.0	26	Positive
Sulfadoxine	3.27	311.1	120	156.0	14	91.9	30	Positive
Lomefloxacin	3.28	352.1	127	334.1	20	308.1	15	Positive
Difloxacin	3.47	400.1	120	382.1	20	356.1	20	Positive
Sulfabenzamide	3.53	277.1	90	155.9	10	92.0	20	Positive
Sulfachloropyrazine	3.94	285.0	106	156.0	9	92.1	29	Positive
Sulfaphenazole	3.96	315.4	130	92.0	43	65.0	78	Positive
Sulfaquinoxaline	4.59	301.1	110	155.9	11	108.0	29	Positive

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