Validated Method for the Determination of Phenylbutazone in Horse Meat with LC-MS/MS

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Method 63799

Key Words
TSQ Vantage, UltiMate 3000, TraceFinder 3.0, Horse Meat, In-house Validation, Phenylbutazone

1. Schematic of Method

Sampling and Internal Standardization

1. Weigh 2 g of homogenized horse meat to 15 mL centrifuge tube; add IS (20 ppb).

Enzymatic Hydrolysis

2. Add 4 mL acetate buffer (pH 4.5) and 50 µL β-glucuronidase.
3. Vortex sample and incubate for 1 hr at 37 °C.

Extraction

4. Cool sample and add 10 mL ACN; shake it vigorously.
5. Centrifuge sample at 4500 rpm for 5 min, collect supernatant.
6. Repeat extraction with 5 mL, centrifuge, and combine supernatants.

Defatting

7. Pass supernatant through Thermo Scientific™ HyperSep™ Silica (2 g) cartridge.

Clean up

8. Blow down extract to ~ 4 mL and apply onto C18 SPE cartridge.
9. Elute analyte with 4 mL ACN and evaporate to dryness.
10. Redissolve samples in 500 µL mobile phase, filter (0.2 µm PTFE).

LC-MS/MS

2. Introduction

Phenylbutazone (bute, PBT) is one of the most widely used (non-steroidal) anti-inflammatory (NSAiD) and painkiller drugs for dogs and horses. Bute was used as a treatment for rheumatoid arthritis and gout in humans as well, but its use was discontinued due to health concerns. It was found to cause different blood disorders (discrasia, anemia, leuko- and thrombocytopenia) and in some cases allergic or severe toxic reactions.

Horse meat is still widely used for human consumption in some European countries. For those animals marked for human consumption, a limited number of medicines can be administered. Any horse meat products that contain substances that are not on a prescribed list (like PBT) must be permanently excluded from the food chain. Despite this regulation, bute continues to be found in 2–5% of slaughtered horse samples, indicating non-compliance with existing meat tracking systems or that feed containing bute was eaten by a horse for which

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it not was intended. As a response to the possible human health threat from the discovery of horse meat in beef products in some European countries, the Standing Committee of the Food Chain and Animal Health (SCoFCAH) of the European Commission has required member states to test representative samples of beef meat products to see if they contain horse meat and to detect possible illegal residues like PBT.

A newly developed LC-MS/MS method for the determination of PBT in horse meat with LC-MS/MS is described here. The method was in-house validated and its applicability for routine testing according to the European legislation requirements is critically reviewed.[1]

3. Scope and Application

This method can be applied to horse and beef meat samples at a limit of quantification (LOQ) below 5 µg/kg, which is the action limit used by the European Commission for monitoring purposes. The method has been validated for the determination of phenylbutazone in horse meat.

4. Principle

This liquid-phase chromatographic method is based on offline sample preparation and triple quadrupole mass spectrometric detection. Due to the fact that a high amount (>90%) of PBT is bound to carrier proteins, samples were enzymatically hydrolyzed prior to extraction with acetonitrile.[2] The centrifuged extracts were collected and defatted on silica cartridge. The volume of the collected effluent was reduced prior to application onto C18 SPE cartridges for clean-up. The eluate was collected and evaporated under a nitrogen stream and, after reconstitution in initial mobile phase and filtration, the samples were directly injected into the LC-MS/MS system.

5. Reagent List

		Part Number
5.1	Purified Water	Obtained from Thermo Scientific™ Barnstead™ EASYpure™ II water system
5.2	Methanol, Fisher Chemical™ Optima™, LC-MS grade	10767665
5.3	Water, Optima, LC-MS grade	10505904
5.4	Acetonitrile, Optima, LC-MS grade	10001334
5.5	Formic acid, extra pure, >98%	10375990
5.6	Sodium acetate anhydrous	10103243
5.7	Acetic acid, HPLC grade	10060000
5.8	Ascorbic acid	Sigma-Aldrich®
5.9	β-glucuronidase from *Helix pomatia*	Sigma-Aldrich

6. Calibration Standards

6.1	Phenylbutazone	Sigma-Aldrich
6.2	Internal standard: Phenylbutazone – diphenyl $^{13}C_{12}$	Sigma-Aldrich

7. Standards Preparation

7.1 Stock Standard Solutions

Stock standard solutions (1000 µg/mL) were prepared individually. Phenylbutazone was prepared by weighing 25 mg of standard and dissolving in 25 mL of methanol. Phenylbutazone – diphenyl $^{13}C_{12}$ (IS) was prepared by weighing of 5 mg of standard and dissolving in 5 mL of methanol. Solutions were stored at -20 °C and were stable for 12 months.

7.2 Working Standard Solutions

The working standard solutions containing 10 µg/mL were prepared individually by dilution of individual stock standard solutions with methanol. Solutions were stored at -20 °C and were stable for 6 months.

7.3 Spiking Standard Solutions

Spiking standard solution (200 µg/L) of phenylbutazone was prepared by dilution of working standard solution with methanol. Spiking internal standard solution (1000 µg/L) was prepared by dilution of working standard solution with methanol. All standard spiking solutions were stored at -20 °C and were stable for 3 months.

8. Apparatus

		Part Number
8.1	Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC system	
8.2	Thermo Scientific™ TSQ Vantage™ triple quadrupole mass spectrometer	
8.3	Fisher precision balance	10145222
8.4	Sartorius analytical balance	1056-4833
8.5	EASYpure II water system	10010682
8.6	Vortex shaker	1013-2562
8.7	Vortex universal cap	1014-2902
8.8	BrandTech® accu-jet® pipettor	1047-5062
8.9	Centrifuge, Thermo Scientific™ Heraeus™ Multifuge™ X3R	10667815
8.10	Thermo Scientific 16 port SPE vacuum manifold	11390731
8.11	Heating Bath B-491, Büchi®	1069-5793
8.12	Thermo Scientific™ Orion™ 2 Star, pH meter	1015-5714
8.13	Horizontal shaker 501 digital, IKA® Werke	1053-6011
8.14	Waring® laboratory blender	10221962
8.15	Evaporator EVMT-130-32-16	1027-9991

9. Consumables

		Part Number
9.1	Thermo Scientific™ Accucore™ C18 (50 × 2.1 mm, 2.6 µm) HPLC column	17126-052130
9.2	Centrifuge tubes, PP disposable 50 mL	05-539-8
9.3	Glass tubes, 15 mL	FB12170
9.4	LC vials	3205111
9.5	LC caps	3151266
9.6	Thermo Scientific™ Finnpipette™ Pipette 100–1000 µL	3214535
9.7	Pipette Finnpipette 20–200 µL	3214534
9.8	Pipette Finnpipette 10–100 µL	3166472
9.9	Pipette Finnpipette 500–5000 µL	3166473
9.10	Pipette Finnpipette 1000–10000 µL	3214536
9.11	Pipette holder	651211
9.12	Pipette tips 0.5–250 µL, 500/box	3270399
9.13	Pipette tips 1–5 mL, 75/box	3270420
9.14	Pipette tips 100–1000 µL, 200/box	3270410
9.15	Pipette tips 20000–10000 µL, 40/box	3270425
9.16	Pipette Pasteur soda lime glass 150 mm	FB50251
9.17	Pipette suction device	3120891
9.18	PTFE syringe filter 0.2 µm	F2513-4
9.19	SPE cartridges, HyperSep Silica (2 g, 15 mL)	60108-710
9.20	SPE cartridges, HyperSep C18 (0.5 g, 6 mL)	60108-305
9.21	Spatula, 18/10 steel	3458179
9.22	Spatula, nylon	3047217
9.23	Tube rack (15 mL)	1034-3461
9.24	Tube rack (50 mL)	1024-1861
9.25	1 mL disposable syringes	1066-4161
9.26	Vial rack (2 mL)	12211001

Glassware

		Part Number
9.27	Beaker, 50 mL	10527211
9.28	Beaker, 100 mL	10769541
9.29	Beaker, 25 mL	10683771
9.30	Volumetric flask, 25 mL	10107901
9.31	Volumetric flask, 10 mL	10406681
9.32	Volumetric flask, 5 mL	10770803
9.33	Volumetric flask, 100 mL	10675731

10. Procedure

10.1 Sample Preparation[3]

10.1.1

50 g of horse meat was homogenized with laboratory blender.

10.1.2

2 g of homogenized meat was weighed into 50 mL polypropylene centrifuge tube. 40 µL of IS spiking solution was added. Sample was mixed by vortex and let to stay for 10 min.

10.1.3

4 mL of acetate buffer (2.7 g of sodium acetate and 0.17 g of ascorbic acid were diluted in 100 mL of H_2O, the pH was adjusted to 4.5 with acetic acid) and 50 µL of β-glucuronidase were added. The sample was vortexed and incubated for 1 h at 37 °C in a water bath.

10.1.4

After cooling the sample, 10 mL of acetonitrile (ACN) was added and the sample was shaken by the horizontal shaker for 5 min and consequently centrifuged (5 °C, 5000 × g, 5 min). The supernatant was transferred into clean 50 mL centrifuge tube. A second extraction procedure was performed by addition of 5 mL of ACN, shaking for 5 min and centrifugation (5 °C, 5000 × g, 5 min).

10.1.5

The combined extracts (ca. 19 mL) were passed through the SPE HyperSep Silica (2 g, 15 mL) cartridge and collected entirely in a glass tube. The cartridge was washed with 4 mL of mixture ACN:H_2O (75:25, v/v) and the portion was added to the eluate.

10.1.6

The eluate was evaporated by nitrogen flow to approximately 4 mL at 50 °C.

10.1.7

The extract was loaded onto the C18 cartridge preconditioned with 2 mL of methanol and 2 mL of 0.02 M ascorbic acid. The glass tube was washed with 2 × 3 mL portions of 0.02 M ascorbic acid and both portions were loaded on the column. The cartridge was washed with 2 mL of 0.02 M ascorbic acid, 2 mL of H_2O and 2 mL of mixture ACN:H_2O (30:70, v/v). The analytes were eluted from the cartridge with 4 mL of ACN and evaporated to dryness by nitrogen stream at 50 °C.

10.1.8

The extract was reconstituted in 0.5 mL mixture of ACN: 0.1% formic acid (50:50, v/v) and filtered through the syringe filter (PTFE, 0.2 µm) directly to the vial.

10.2 LC Conditions

LC analysis was performed on an UltiMate 3000 UHPLC system equipped with Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System and Thermo Scientific™ TraceFinder™ software version 3.0 for system control, acquisition, and data evaluation.

The LC conditions were as follows:

Analytical column:	Accucore C18 (50 × 2.1 mm, 2.6 µm)
Total run time:	7.5 min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	Acetonitrile
Gradient:	Table 1
Injection volume:	10 µL
Column temperature:	Ambient

Table 1. Gradient program of the UHPLC method

Time [min]	A%	B%	Flow rate [µl/min]
0	50	50	350
5	0	100	350
5.5	0	100	350
5.6	50	50	350
7.5	50	50	350

10.2.1 Injector Settings

The injector settings were as follows:

Injector:	UltiMate 3000 autosampler
Injection mode:	Normal
Cleaning solvent:	MeOH
Draw speed [µL/s]:	2
Pre clean with solvent 2 [steps]:	3
Draw delay [ms]:	500
Dispense speed [µL/s]:	5
Dispense delay [ms]:	200
Dispense to waste speed [µL/s]:	5
Sample height [mm]:	0.2
Inject wash:	Both
Wash volume [µL]:	100
Wash speed [µL/s]:	5
Loop wash factor:	2

10.3 Mass Spectrometric Conditions

Mass spectrometric analysis was carried out using a TSQ Vantage triple quadrupole system. Data acquisition for quantification and confirmation was performed in the selected reaction monitoring mode (SRM). All SRM traces (parent, qualifier, and quantifier ion) were individually tuned for each target analyte by direct injection of the individual working standard solution (100 ng/mL). Data acquisition and processing was performed using TraceFinder 3.0 software.

The MS conditions were as follows:

Ionization mode:	Heated Electrospray (HESI)
Scan type:	Selected reaction monitoring (SRM)
Polarity:	Negative ion mode
Spray voltage [V]:	2500
Ion sweep gas pressure [arb]:	0
Vaporizer temperature [°C]:	320
Sheath gas pressure [arb]:	20
Aux Gas Pressure [arb]:	10
Capillary temperature [°C]:	310
Collision gas pressure [mTorr]:	1.5
Cycle time [s]:	0.3
Peak width:	Q1/Q3 the full width of a peak at half its maximum height (FWHM) of 0.70 Da

The parameters for SRM analysis for target compound and internal standard are displayed in Table 2.

11. Calculations

11.1 Identification

Identification of phenylbutazone and phenylbutazone – diphenyl $^{13}C_{12}$ was confirmed by the presence of transition ions (quantifier and qualifier) at retention times (+/-2.5%) to the corresponding standards. In multiple reaction monitoring (MRM) mode the measured peak area ratios for qualifier to quantifier ion should be in close agreement (according to Commission Decision 2002/657/EC) with those of the standards as shown in Table 2. The quantifier and qualifier ion were selected among the product ions produced by the fragmentation of the selected parent ion on the basis of the intensity.

11.2 Quantification

For quantification internal standardization was used, measuring peak area ratios for standards in matched matrixes. Phenylbutazone – diphenyl $^{13}C_{12}$ was used as the internal standard for phenylbutazone. The calibration curve was plotted as the relative peak area (phenylbutazone versus the phenylbutazone – diphenyl $^{13}C_{12}$) as a function of the compound concentration. The phenylbutazone concentration in the samples was determined from the equation:

$$c_{PBT} = \left(\frac{A_{PBT}}{A_{IS}} - b \right)$$

c_{PBT} – phenylbutazone concentration in µg/kg

A_{PBT} – peak area of the phenylbutazone

A_{IS} – peak area of internal standard

b – y-intercept

a – slope of the calibration curve

12. Method Performance

The method was in-house validated according to the criteria specified in European Commission Decision 2002/675/EC for a quantitative method.[1] The validation parameters were determined by spiking phenylbutazone-free horse meat at levels of 2.5, 5, and 7.5 µg/kg. The measured parameters were specificity, linear range, repeatability, accuracy, limit of detection and quantification (LOD and LOQ), and limit of decision and capability (CCα and CCβ). Data were evaluated by TraceFinder 3.0 software. A screenshot of result overview is presented in Figure 1.

12.1 Samples and Quality Control Materials

For preparation of matrix-matched calibration samples and spiking of samples for validation, horse meat was obtained from a local butcher. The meat was analyzed by repeated measurements to confirm that it was free of phenylbutazone.

For determination of accuracy, a former proficiency test sample FAPAS – 02157 of bovine muscle test material from the Food and Environmental Research Agency (York, UK) was analyzed.

12.2 Specificity

Using SRM the specificity was confirmed based on the presence of the transition ions (quantifier and qualifier) at the correct retention times corresponding to those of the phenylbutazone and phenylbutazone – diphenyl $^{13}C_{12}$. The measured peak area ratios of qualifier/quantifier were in the range (defined in Commission Decision 2002/657/EC) comparing to the standards (Table 2).[1]

12.3 Linearity and Calibration Curve

The linearity of calibration curves was assessed over the range from 0–30 µg/kg for phenylbutazone. The correlation coefficient of linear function was 0.9941. The calibration curve was created from five matrix-matched calibration standards (0, 2.5, 5, 7.5, 10 µg/kg) which were injected in each batch in triplicate.

12.4 Precision

Precision (repeatability) of the method was determined using independently spiked blank horse meat at three different levels (2.5, 5, and 7.5 µg/kg) (within day repeatability) with % RSD of 5.2, 2.0, and 6.3 respectively. In one day the set of three levels with six repetitions was measured. For the determination of the repeatability (day-to day-repeatability) two other sets at one level (5 µg/kg) with six repetitions were measured over the next two days with a % RSD of 9.8. The results are summarized in Table 3 and Table 4. The LC-MS/MS chromatogram of phenylbutazone spiked and extracted in horse meat at 2.5 mg/kg is shown in Figure 2.

12.5 Accuracy

Method accuracy was determined using independently spiked blank samples at three different levels. Accuracy was evaluated by comparison of found values with standard addition in spikes. Recovery values ranged between 95.6–103.9% (Table 3). Additionally, accuracy was established by analyzing a former proficiency test sample FAPAS – 02157 of bovine muscle test material in duplicate. All measured concentrations of phenylbutazone were within the acceptable satisfactory range (Table 5). The LC-MS/MS chromatogram of phenylbutazone detected in reference material is presented in Figure 3.

12.6 Matrix Effect

The matrix effect was investigated by comparison of calibration results in solvent and in matrix. The Youden plot of both calibration series was applied. The slope of the fitted linear resulted in y = 0.9821x, which represents less than 20% deviation from the idealistic y = x value, indicating no matrix effect for the investigated matrix (Figure 4).

12.7 LOD and LOQ

Limits of detection and quantification were estimated following the IUPAC approach, which consisted of analyzing the blank sample to establish noise level and then estimating LOD and LOQ for signal/noise, 3 and 10 respectively. LOQ was found to be at 2.0 µg/kg and LOD at 0.8 µg/kg.

12.8 CCα and CCβ

Both CCα and CCβ were established by the calibration curve procedure according to ISO 118434 guideline.[4] The blank material fortified at and above the lowest possible level (for analytes without MRL) in equidistant steps was used. The calculated values were 1.0 µg/kg for CCα and 1.29 µg/kg for CCβ (Table 3).

12.9 Robustness

The robustness of the method was tested by the analysis of samples with another HPLC-MS/MS system and the sample preparation carried out by another chemist. All results were comparable to the validated data presented in this document.

13. Conclusion

The reported in-house validated method enables quantification of PBT residues from horse meat matrix. With the applied sample preparation method, effective deconjugation and prevention of early degradation of target compound with high recovery and good repeatability was achieved. The sample pretreatment procedure requires two clean-up steps, which ensures clean meat extracts with no matrix effect and enables high sensitive quantification. In-house validation of the method was carried out according to the current European legislation recommendations. Taking into account the current rapidly growing interests in phenylbutazone measurement we have proven that this method is fit for purpose and can be applied for routine testing analysis.

14. References

1. Commission Decision 2002/657/EC. Off. J. Eur. Commun. L221/8 (2002).
2. http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/191606.htm.
3. Jedziniak, P.; Szprengier-Juzkiewicz, T.; Olejnik, M.; Zmudzki, J. Determination of non-steroidal anti-inflammatory drugs residues in animal muscles by liquid chromatography-tandem mass spectrometry. *Anal. Chim. Acta*. **2010**, *672*, 85-92.
4. ISO 11843: Capability of detection (1997).

15. Annex

Tables and Figures

Table 2. MS/MS parameters for selected reaction monitoring of PBT and PBT-IS and ion ratios (Qual/Quant) in matrix and in standard mixture (the agreement between ion ratios should be in the permitted tolerance, which is defined in the Commission Decision 2002/657/EC)

Analyte	Rt Time (min)	Molecular Weight	Precursor Ion $[M-H]^+$	Quantifier Ion (CE)	Qualifier Ion (CE)	Tube Lens	Ion Ratio (Solvent)	Ion Ratio (Matrix)	Difference In Ion Ratio (%)
PBT	1.67	308.37	307.10	279.10 (21)	130.50 (24)	95	60.7	63.2	4.2
PBT-IS	1.67	320.29	319.17	291.30 (20)	136.82 (24)	95	59.6	61.3	2.9

CE = Collision energy (eV)

Table 3. Validation results: Method linearity, method recovery (%), and method repeatability expressed as RSD (%) for spiked samples of horse meat at three different spike levels with six replicates, limit of detection and quantification (LOD and LOQ), and limit of decision and capability (CCα and CCβ)

Parameter		Phenylbutazone
Linearity	Slope	0.0425
	Intercept	-0.0056
	R^2	0.9941
Spiking Levels (µg/kg)	I.	2.5
	II.	5.0
	III.	7.5
Recovery (%)	I.	103.9
	II.	97.5
	III.	95.6
Repeatability (%)	I.	5.2
	II.	2.0
	III.	6.3
LOD (µg/kg)		0.80
LOQ (µg/kg)		2.00
CCα (µg/kg)		1.00
CCβ (µg/kg)		1.29

Table 4. Method precision expressed as RSD (%) – at one level – with three sets with six replicates

Parameter		PBT
Spiking Level (µg/kg)	II.	5.0
Identification (t_r)	Repeatability (%)	0.5
	Intermediate precision (%)	0.9
Quantification (peak area)	Within day repeatability (%)	2.0
	Day to day repeatability (%)	9.8

Table 5. Results of certified reference material – Fapas 02157 (bovine muscle) – phenylbutazone – assigned value 19.3 ± 8.4 µg/kg

Sample	Found Concentration (µg/kg)
CRM 1	14.6
CRM 2	15.8

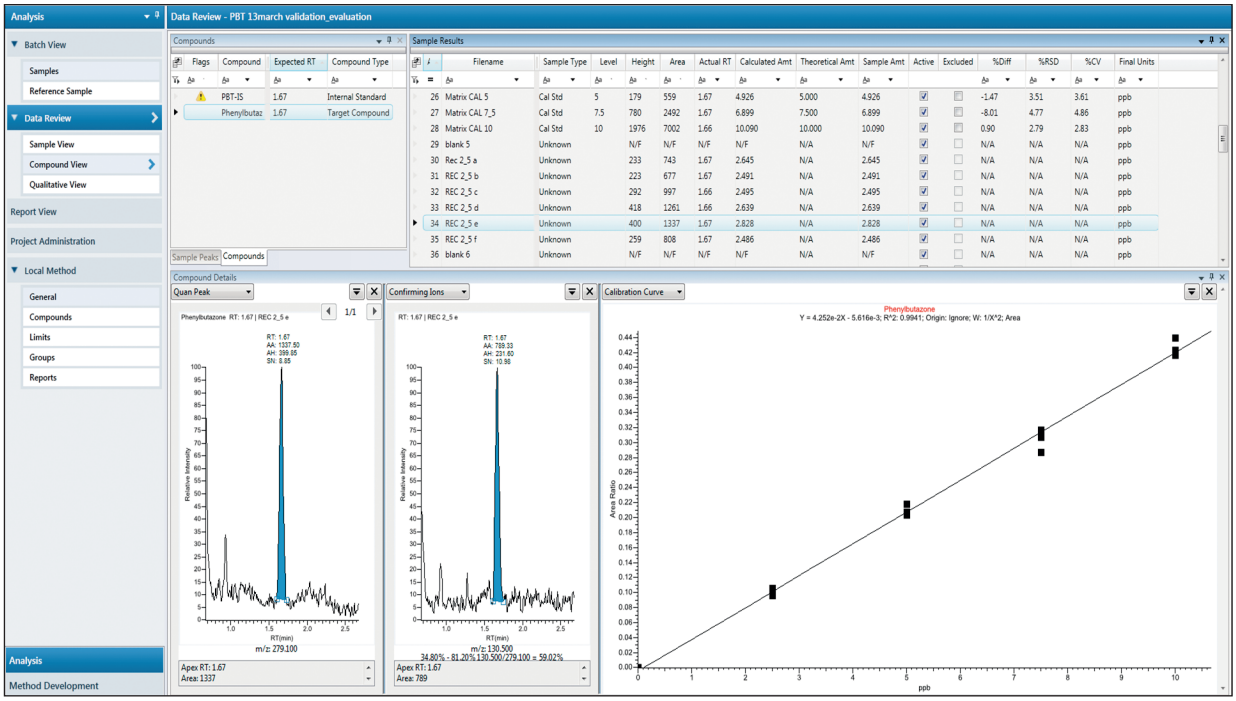

Figure 1. Screenshot data evaluation overview with TraceFinder 3.0 software

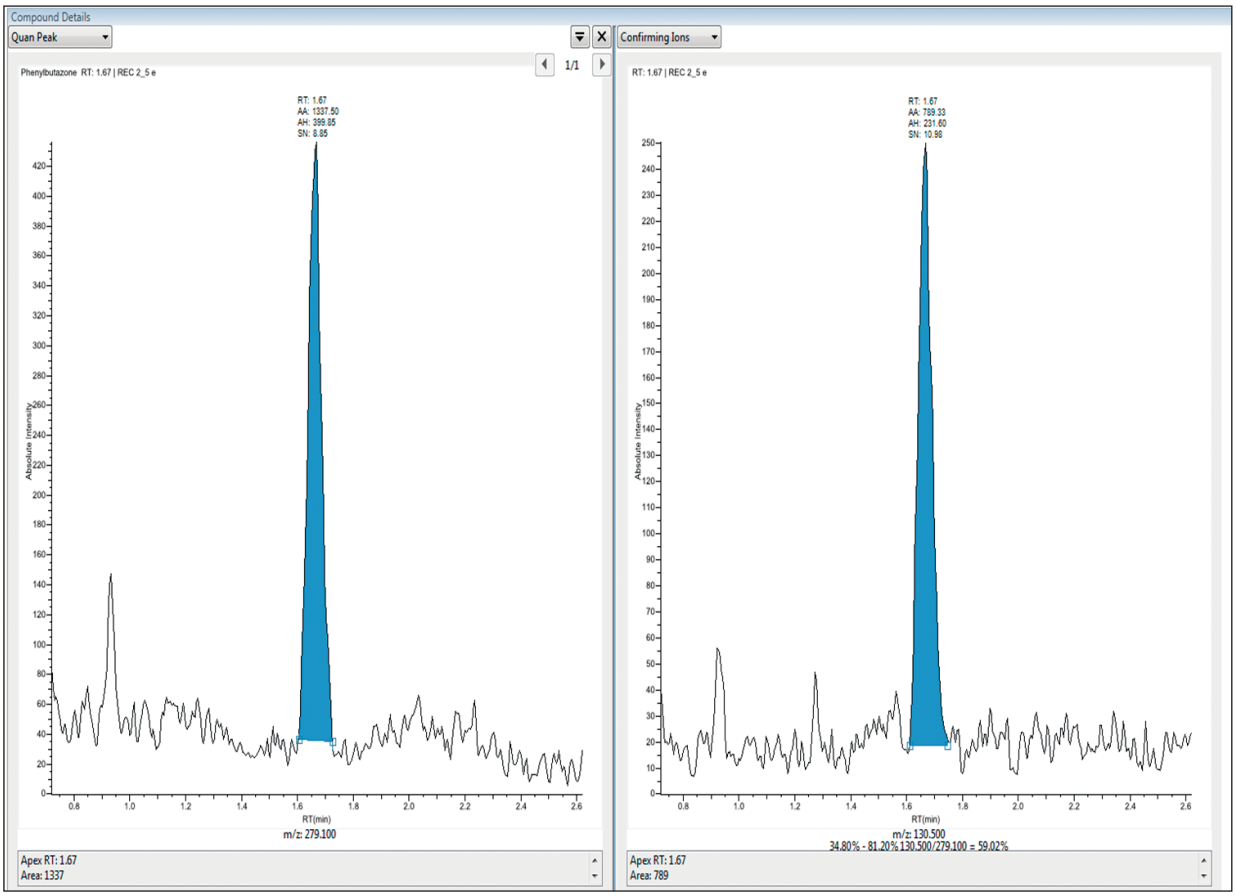

Figure 2. LC-MS/MS chromatogram of phenylbutazone in horse meat at 2.5 µg/kg. Left: quantifier ion *m/z* 279.1, right: qualifier ion *m/z* 130.5 at 1.67 min.

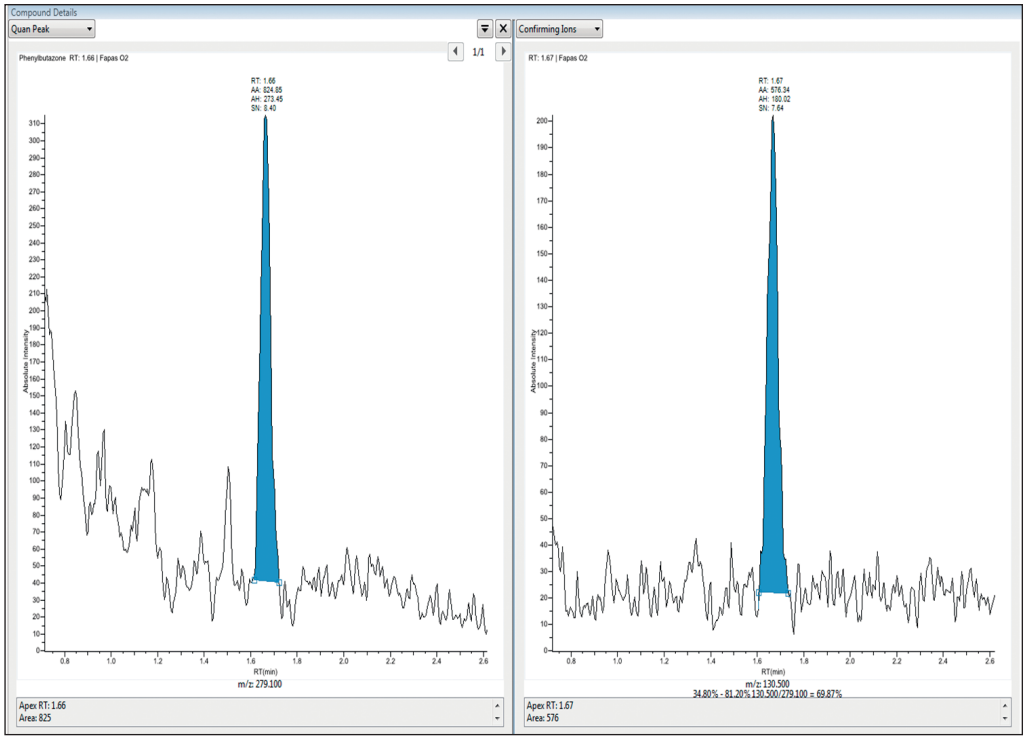

Figure 3. LC-MS/MS chromatogram of phenylbutazone in beef meat quality control reference material found concentration 15.8 µg/kg (assigned value 19.3 µg/Kg). Left: quantifier ion *m/z* 279.1, right: qualifier ion *m/z* 130.5 at 1.67 min.

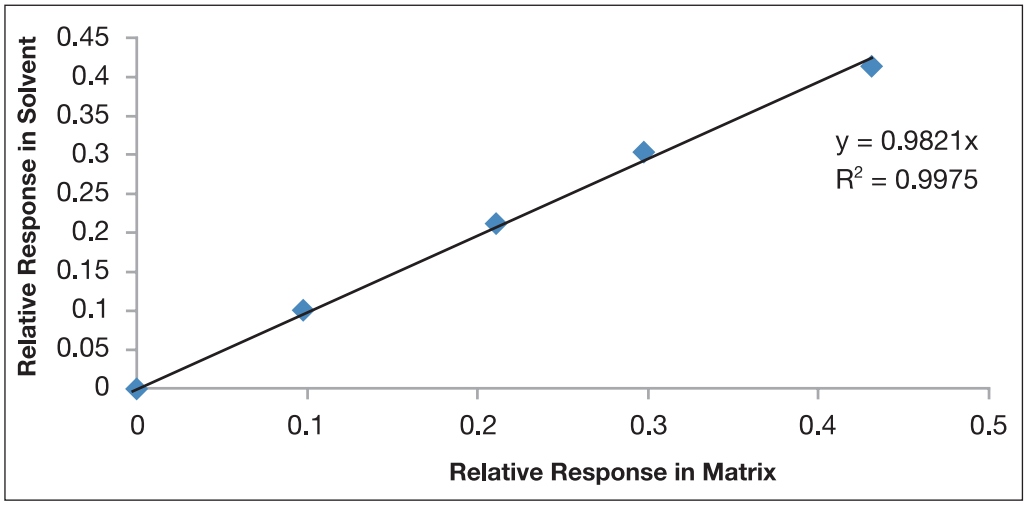

Figure 4. Matrix effect study. Plot of relative responses of calibration levels in solvent versus in horse meat.

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