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Nota de aplicación

Determination of Beta-Adrenergic Receptor Agonists in Animal Tissues and Urine Using Liquid Chromatography-Tandem Quadrupole Mass Spectrometry

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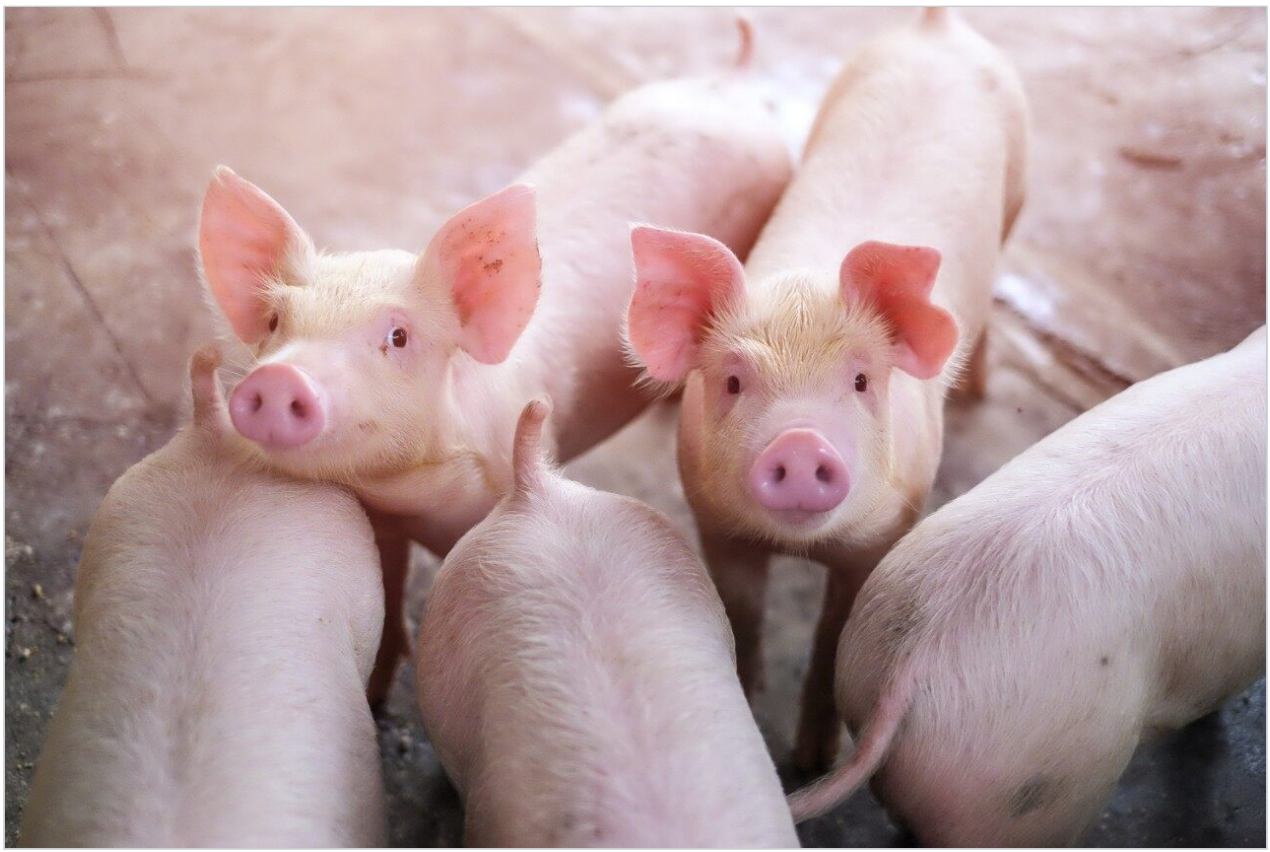

Abstract

Here we show a specific, targeted method that we developed in collaboration with Fera Science for β-agonists determination in a range of sample types that meets requirements for both official control and food business operators' due diligence testing.

Benefits

A specific, targeted method for β-agonists determination in a range of sample types that meets requirements for both official control and food business operators' due diligence testing.

Introduction

Beta-adrenergic receptor agonists (β-agonists) are synthetic compounds that mimic some of the effects of naturally-occurring compounds by binding to beta-receptors on the surface of cells within the muscle, fat, and other tissues of animals. Some β-agonists are used in human medicine for the treatment of conditions such as asthma, but they have also been used in livestock production to enhance growth and alter body composition.[1] The structures of some of the key β-agonists are shown in Figure 1.

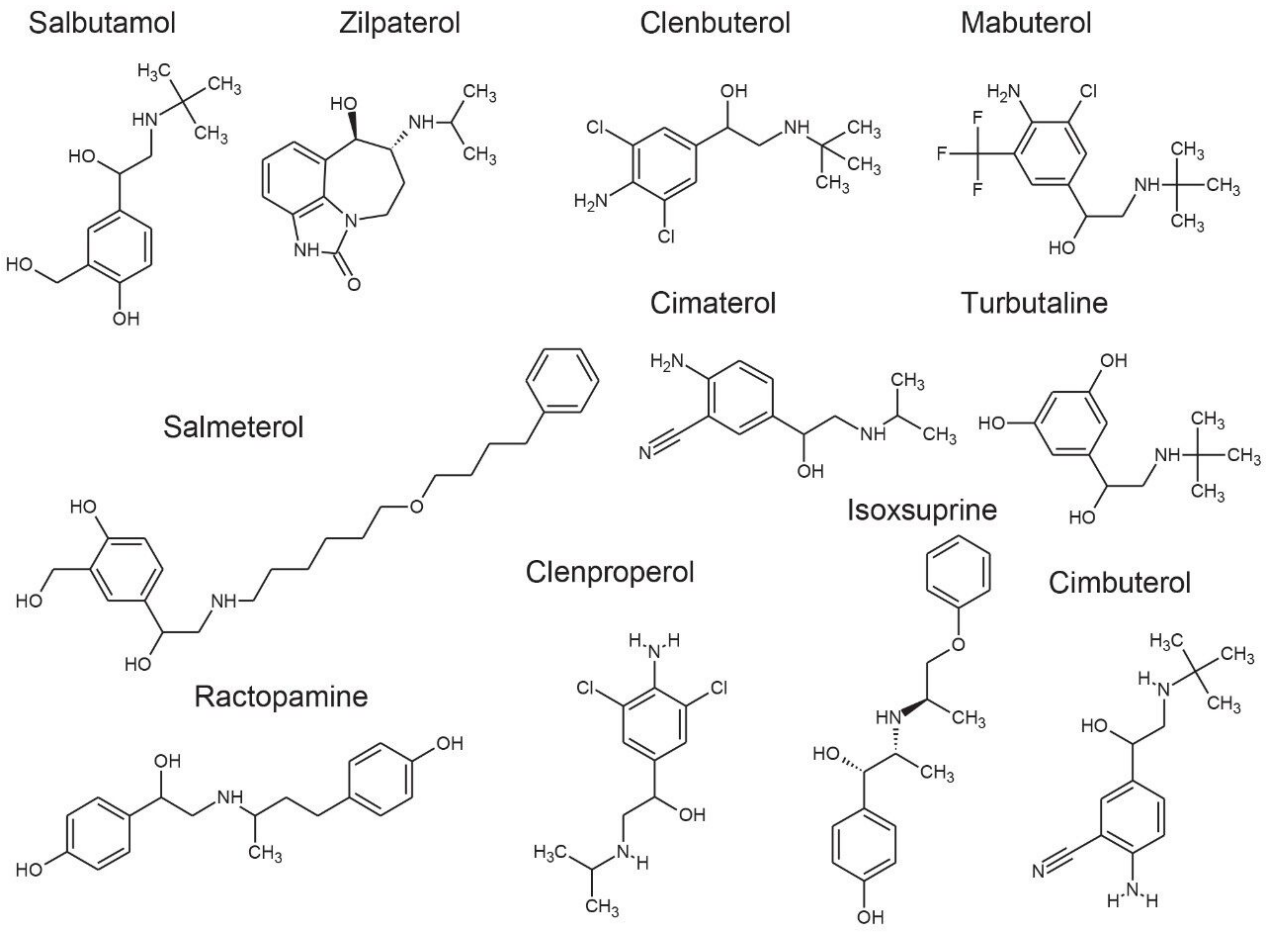

Figure 1. Structures of some of the key β-agonists.

Although the administration of β-agonists as growth-promoting agents in food-producing animals is banned in many countries due to concerns over human health, there are exceptions.[2] Ractopamine and zilpaterol are authorized for the production of some animals (e.g. cattle and pigs) in a limited number of countries such as the U.S., Canada, and Brazil. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established Maximum Residue Limits (MRLs) for ractopamine in cattle and pig muscle (both 10 µg/kg), which have been adopted by the Codex Alimentarius Commission (Codex) and implemented in some countries (e.g. Canada).

Others have alternative limits for ractopamine, such as the tolerances set in the U.S. (30 and 50 µg/kg in cattle and pig muscle, respectively).

However, many countries disagree with the Codex standards and are restricting or banning meat products containing β-agonists. In the EU, MRLs only exist for clenbuterol (cattle and horse). Other β-agonists are prohibited for use in food producing animals. As no Minimum Required Performance Limits (MRPLs) were

set for these banned β-agonists,[3] the EU relies on Recommended Concentrations (RCs) to indicate required performance of associated analytical methods. In contrast to MRPLs, RCs have no legal standing and are used for guidance only.

Importing countries can set even lower limits for testing based upon trading decisions to provide better warranties to their customers and to gain commercial advantage. For example, Russia established an action level of 0.1 µg/kg for ractopamine in imported beef consignments; one hundred times lower than Codex MRL. However, as Russia has a zero tolerance policy, if a laboratory found any confirmed residues at lower concentrations, the consignment would be rejected. This drives down the analytical limits for those involved with pre-export testing.

Most countries have established strict surveillance programs for official control purposes to check compliance with regulatory limits, for both domestic and imported produce. Monitoring compliance within these limits requires the use of highly sensitive and selective analytical methodology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS). As the frequency of detection of residues is typically low, samples tend to be screened using a reduced but adequate level of quality control. This includes the use of stable isotope analogs as surrogates to monitor and mitigate the impact of any sample-to-sample variability in matrix effects. Any suspect positives are re-analyzed with the same method but with additional analytical quality control suitable for quantification (e.g. multi-level matrix-extracted calibration). This method had also been validated as suitable for confirmation4 with values for Decision Limit (CCα) as low as reasonably achievable.

Experimental

Sample preparation

Extracts were generated by Fera using a validated method based on methods developed by the EURL BVL in Germany.[5,6,7] Samples of meat and fish were extracted by blending with an aqueous buffer/methanol solution and subjected to enzyme hydrolysis to cleave any conjugated β-agonists, and to help solubilize any residues.

Solid-phase extraction (SPE) was carried out on a mixed mode-type cartridge prior to determination using UPLC-MS/MS. Samples of urine were extracted by SPE after enzyme hydrolysis. Stable isotope analogues were available for many of the analytes, and were added to all samples prior to extraction. Matrix-extracted standards were prepared at the Screening Target Concentration (STC) when screening and at multiple

levels, typically over the range 0.5x to 10x STC, for any confirmatory analysis. Typically, STC values are set at half the maximum limit, which for β-agonists varies (see Table 1). For example, the STC for clenbuterol in animal muscle was set at 0.05 µg/kg, whereas that for terbutaline was set at 5.0 µg/kg. Sample extracts were filtered prior to subsequent analysis by UPLC-MS/MS.

LC conditions

UPLC system:	ACQUITY UPLC I-Class with FTN Autosampler
Column:	CORTECS UPLC C_{18}+ 1.6 µm, 2.1 × 100 mm
Mobile phase A:	0.1% formic acid (aq.)
Mobile phase B:	0.1% formic acid in acetonitrile
Flow rate:	0.6 mL/min
Injection volume:	5 µL
Column temp.:	40 °C
Sample temp.:	10 °C
Run time:	11 min

Gradient:

Time (min)	%A	%B	Curve
0.0	100	0	–
0.2	100	0	6
5.0	50	50	6

Time (min)	%A	%B	Curve
5.1	0	100	6
8.0	0	100	6
8.1	100	0	6

MS conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI +
Capillary voltage:	1.0 kV
Desolvation temp.:	600 °C
Desolvation gas flow:	1000 L/Hr
Source temp.:	140 °C
Cone gas flow:	0 L/Hr

The two MRM transitions that showed the best selectivity were used for each of the β-agonists and one for each stable isotope analogue. The data were acquired using MassLynx 4.1 Software and processed using TargetLynx XS Application Manager. Table 2 summarizes conditions for all MRM transitions and retention times. Cone voltage was set to 40 V throughout, with the exception of the in-source fragment at *m/z* 202.1, which was selected as a precursor ion for cimaterol (75 V). The optimum dwell time was set automatically using the Autodwell function based upon 4s wide peaks and 12 data points per peak.

Compound	Animal muscle	Animal offal	Poultry offal	Urine
Terbutaline (TER)	5.00	5.00	5.00	1.50
Cimaterol (CIM)	0.25	0.25	0.25	0.25
Salbutamol (SAL)	2.50	2.50	2.50	0.50
Zilpaterol (ZIL)	2.50	2.50	2.50	0.50
Cimbuterol (CIMB)	0.25	0.25	0.25	0.25
Hydroxymethyl Clenbuterol (HMC)	0.05	0.10	0.10	0.10
Clenproperol (CLE)	0.25	0.25	0.25	0.25
Ractopamine (RAC)	0.50	0.50	0.50	0.50
Clenbuterol (CLEN)	0.05	0.10	0.10	0.10
Tulobuterol (TUL)	0.05	0.10	0.10	0.10
Brombuterol (BRO)	0.05	0.10	0.10	0.10
Isoxsuprine (ISO)	0.25	0.25	0.25	0.25
Mabuterol (MAB)	0.05	0.10	0.10	0.10
Clenpenterol (CLENP)	0.25	0.25	0.25	0.25
Mapenterol (MAP)	0.05	0.10	0.10	0.10
Salmeterol (SALM)	2.50	2.50	2.50	0.50

Table 1. STC values (µg/kg) for β-agonists in difficult sample types.

Compound	Retention time (min)	MRM	CE (eV)
Terbutaline	1.57	**226.1>107.0** 226.1>125.0	30 25
Terbutaline-d9	1.57	235.1>153.0	15
Cimaterol	1.57	202.1>116.0 **220.1>143.0**	30 25
Cimaterol-d7	1.57	227.1>161.0	20
Salbutamol	1.59	240.2>121.0 **240.2>148.0**	30 20
Salbutamol-d3	1.59	243.2>151.0	20
Zilpaterol	1.59	**262.2>185.1** 262.2>202.1	25 20
Zilpaterol-d7	1.59	269.1>251.1	10
Cimbuterol	1.79	**234.2>160.0** 234.2>143.0	15 25
Cimbuterol-d9	1.77	243.2>161.0	15
Hydroxymethyl Clenbuterol	2.15	**293.1>203.0** 295.1>205.0	15 15
Clenproperol	2.21	263.1>132.0 **265.1>132.0**	25 25
Clenproperol-d7	2.21	270.1>169.0	25
Ractopamine	2.30	302.2>107.0 **302.2>164.0**	30 15
Ractopamine-d6	2.30	308.2>168.0	15
Clenbuterol	2.49	**277.1>203.1** 279.1>205.1	15 15
Clenbuterol-d9	2.48	286.1>204.0	15
Tulobuterol	2.48	**228.1>154.0** 228.1>172.0	15 10
Tulobuterol-d9	2.47	237.2>155.0	15
Brombuterol	2.72	367.1>214.0 **367.1>293.1**	30 15
Isoxsuprine	2.81	**302.2>107.0** 302.2>150.0	30 20
Mabuterol	2.84	311.1>217.1 **311.1>237.1**	25 15
Mabuterol-d9	2.83	320.1>238.1	15
Clenpenterol	2.83	**291.1>203.0** 293.1>205.0	15 15
Mapenterol	3.18	325.1>217.1	25

agonists and stable isotope analogues (quantatitive transitions in bold).

Results and Discussion

The objective of this work was to evaluate the performance of the Xevo TQ-S micro instrument for the determination of 16 β-agonists in appropriate matrices rather than development and validation of a new method. Analysis was restricted to batches of various animal target tissues (e.g. liver), edible products (e.g. muscle), and urine collected from animals on the farm. Performance was evaluated based on its sensitivity for analytes in context with international regulations, the absence of isobaric interference, precision of UPLC-MS/MS measurements, accuracy and precision through the analysis of spiked samples and proficiency test materials, as well as compliance with identification and quantification criteria.

The LC conditions were modified from those reported in an earlier Waters application note.[8] The CORTECS C_{18}+ Column is a charged surface, high efficiency C_{18} column, based on a solid-core particle that delivers excellent peak shape for these basic analytes at low pH, while providing sufficient retention of the most polar β-agonists, both at a lower operating pressure than columns packed with fully porous sub-2 µm particles. The gradient was adjusted to provide sufficient chromatographic resolution of all 16 β-agonists from isobaric interference on both transitions. The selection of MRM transitions and optimization of critical parameters was performed by infusion of individual solutions of all the analytes and evaluation of the data by IntelliStart Software to automatically create acquisition and processing methods.

Excellent sensitivity and selectivity was demonstrated by the response for each analyte peaks detected from the analysis of matrix-extracted standards prepared at 1 x STC in a range of different sample types: bovine and poultry liver, salmon, and bovine urine. See Figure 2 and Figure 3 for examples.

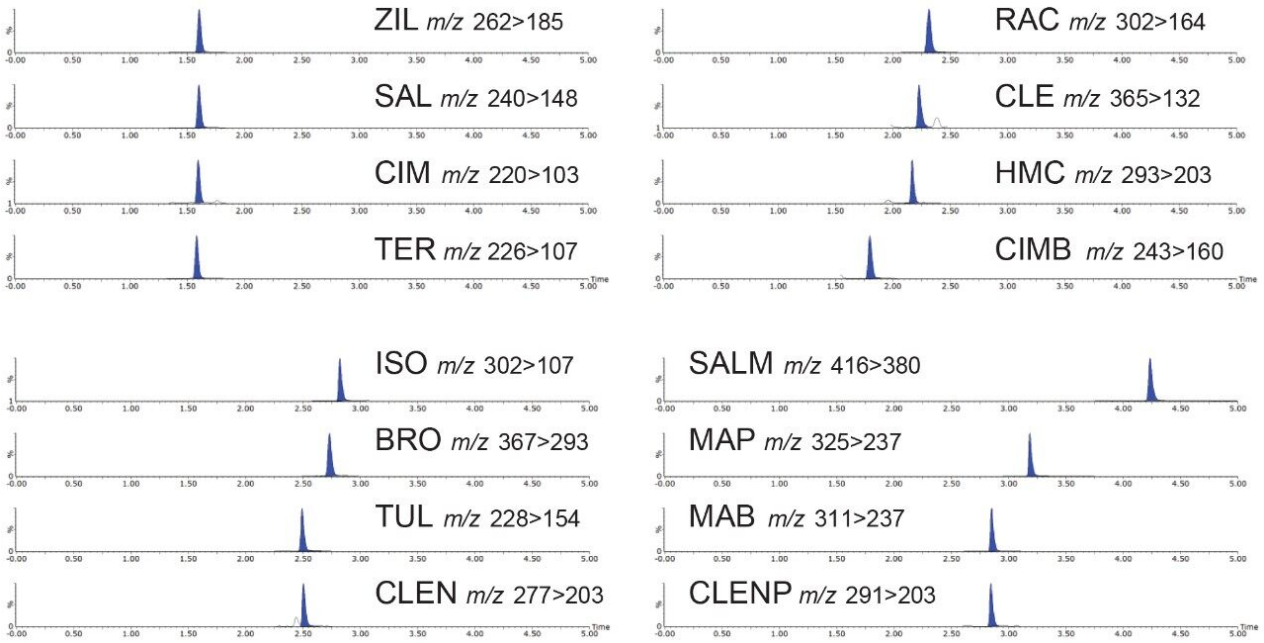

Figure 2. Chromatograms showing β-agonists from analysis of matrix-extracted standards of bovine liver at the STC.

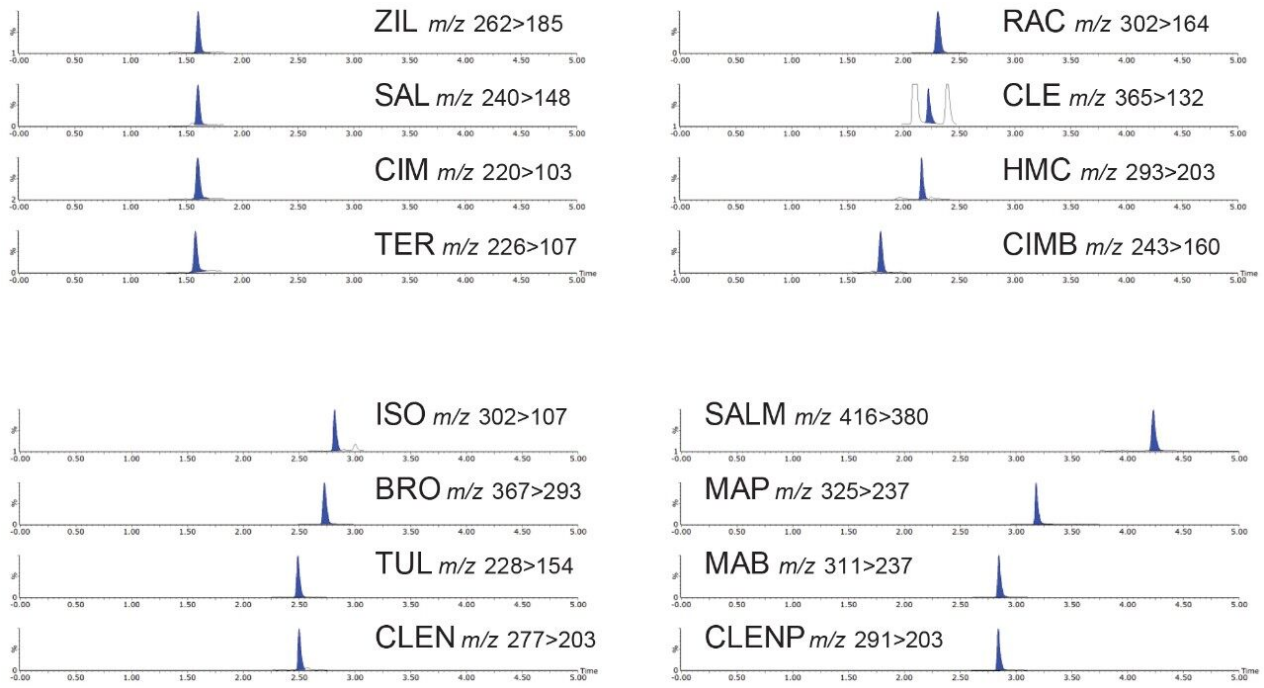

Figure 3. Chromatograms showing β-agonists from analysis of matrix-extracted standards of bovine urine at the STC.

Figure 4 shows the detection of ractopamine spiked into pork meat at 0.05 µg/kg. This demonstrates the suitability of the method for checking compliance with the Russian action level for imported meat of 0.1 µg/kg ractopamine. No interfering compounds were detected at the retention times of the β-agonists or stable isotope analogues in all of the tested blank samples, with the exception of the second transition for clenproperol in liver samples.

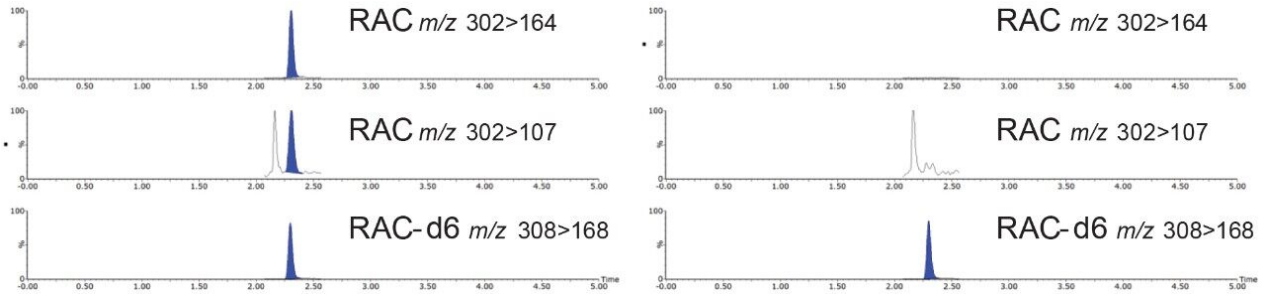

Figure 4. Chromatograms showing ractopamine and stable isotope analogue from analysis of matrix-extracted standard of pork meat spiked at 0.05 µg/kg and associated blank.

Carryover was observed to be typically very low, with the exception of the late eluting salmeterol. A maximum of 1.3% carryover was observed for salmeterol from measurements in solvent blanks after injection of standards, but this proved to be a consistent background in the system equivalent to ca. 1% of the STC level.

Measurement precision, as determined from the peak areas of replicate (n=25) injections of β-agonists matrix extracted standards (1x STC), was good for the majority of compounds (<5% RSD). Precision for salmeterol and brombuterol was worse in some matrices, having lower area counts due to lower recovery. When concentration was estimated using appropriate stable isotope analogues as internal standards, precision of the measurements was observed to improve for all matrices tested (RSD <4%). Retention times of the analytes from these replicate injections were very stable showing a maximum shift of ±0.01 minutes. Ion ratios were found to be within tolerance with the exception of clenproperol in liver samples, where there was an isobaric interference on the second transition, so an alternative MRM transition would be needed for confirmatory analyses.

Compound	Bovine liver	Porcine liver	Salmon	Bovine urine
Terbutaline (TER)	1.3	1.7	1.9	3.4
Cimaterol (CIM)	1.9	1.7	3.6	2.1
Salbutamol (SAL)	1.0	1.5	2.3	3.5
Zilpaterol (ZIL)	1.5	2.2	2.5	1.4
Cimbuterol (CIMB)	1.3	1.7	2.1	1.5
Hydroxymethyl clenbuterol (HMC)	1.7	2.0	3.2	2.1
Clenproperol (CLE)	1.4	2.2	3.4	2.7
Ractopamine (RAC)	2.2	1.4	2.6	2.3
Clenbuterol (CLEN)	1.2	2.3	3.2	1.9
Tulobuterol (TUL)	1.4	2.1	2.9	1.6
Brombuterol (BRO)	1.7	2.8	6.6	2.3
Isoxsuprine (ISO)	1.1	2.2	2.6	2.7
Mabuterol (MAB)	0.9	2.2	3.5	2.2
Clenpenterol (CLENP)	0.9	2.0	2.9	2.1
Mapenterol (MAP)	0.8	2.1	4.0	2.2
Salmeterol (SALM)	5.3	0.7	15	5.8

Table 3. Precision data (%RSDs) for measurement of β-agonists in the different matrices.

To assess performance of the method for confirmatory purposes, accuracy, precision, and identification criteria were evaluated through the analysis of incurred proficiency test materials and spiked blank samples. The linearity of response for two of the β-agonists in bovine liver matrix was evaluated using a bracketed calibration over a suitable concentration range of: 0.25 to 5.0 µg/kg for ractopamine, and 0.05 to 1.0 µg/kg for clenbuterol. The coefficients of determination were satisfactory (r^2 >0.99), and the residuals <4%.

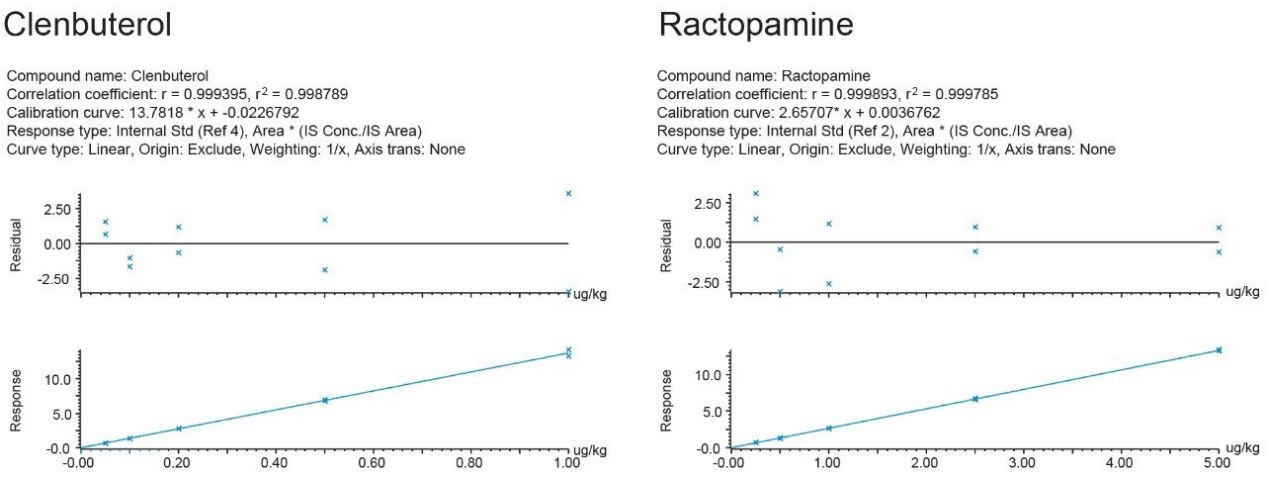

Figure 5. Calibration graphs for clenbuterol and ractapamine prepared in bovine liver matrix.

Three bovine liver proficiency test materials (BVL, Germany), contaminated with β-agonists, were prepared in duplicate and the extracts were analyzed by UPLC-MS/MS in triplicate (n=6). Sample A was found to contain both ractopamine (1.01 µg/kg, 2.6% RSD) and clenbuterol (0.27 µg/kg, 5.8% RSD); Sample B just ractopamine (3.69 µg/kg, 3.7% RSD); and Sample C contained clenbuterol only (0.18 µg/kg, 3.4% RSD). The mean measured concentrations of ractopamine and clenbuterol compared well with the assigned values in all three samples. In addition two samples of bovine liver, previously shown to be blank, were spiked with ractopamine (1.25 µg/kg) and clenbuterol (0.2 µg/kg) and analyzed in triplicate. Mean recovery and precision was 100% (1.9% RSD) and 101% (2.1% RSD), respectively, after correction for losses using the appropriate stable isotope analogue as an internal standard.

Requirements given in EU Commission Decision 2002/65/7/EC for identification were met (European Commission, 2002). The range of calculated ion ratios for ractopamine (0.95 to 1.00) and clenbuterol (0.62 to 0.64), from the replicate measurements of the incurred samples, were well within the tolerance of ±20% of the reference values for ractopamine (0.80 to 1.21) and clenbuterol (0.51 to 0.71), respectively. The retention times for ractopamine (2.30 to 2.31 min) and clenbuterol (2.49 min) showed little variation and were well within acceptance tolerance of ±2.5% (2.25 to 2.37 min and 2.43 to 2.55 min).

Conclusion

The ACQUITY UPLC I-Class System combined with the Xevo TQ-S micro provides excellent sensitivity for the

detection, identification, and quantification of β-agonists in a range of products. This method can be used for both screening and confirmation for official control purposes with a high degree of confidence, but also to meet the challenging requirements of pre-export testing, which often demands lower limits of quantification. Scientists must validate the method in their own laboratories and demonstrate that their calculated Detection Capability (CCβ) and Decision Limit (CCα) values are fit for purpose (i.e. as low as reasonably achievable).

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