Quantitative Analysis of Ractopamine in Beef using Automated Online Sample Preparation with Liquid Chromatography-Tandem Mass Spectrometry

Yang Shi, Bill Yu, Catherine Lafontaine, François Espourteille
Thermo Fisher Scientific, Franklin, MA 02038, USA

Application Note 63554

Key Words
Transcend TLX-1, TurboFlow Technology, TSQ Quantum Access MAX, Ractopamine, Beef

Goal

To develop a rapid and sensitive automated online sample preparation LC-MS/MS method to determine ractopamine in beef.

Introduction

Ractopamine is a phenethanolamine member of the family of β2-adrenergic receptor agonists (β-agonists). It has been widely used as a veterinary additive drug in livestock production to promote leanness in meat, accelerate average daily weight gain and improve feed efficiency[1]. Recently, there have been growing concerns about the safety of meat containing ractopamine residues due to its potential health risks for humans[2]. Over 150 countries including China and the European Union (EU) have banned the use of ractopamine in animal feeds, but in other countries such as the US and Japan, ractopamine use is allowed. The maximum residue limit (MRL) of ractopamine has been set at 30 ppb for beef and 50 ppb for pork in the US and 10 ppb for beef muscle by Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA)[3]. The EU also proposed 10 ppb as the minimum required performance limit (MRPL)[4].

Ractopamine has been a specific trade concern since a number of shipments of US beef containing trace level contamination were rejected by the Taiwanese government as early as 2007. Therefore, it is essential to develop a sensitive, reliable and effective analytical method for quantitative measurement of ractopamine in samples of animal meat and organs. A number of methods have been reported to monitor ractopamine residue in meat-producing animals using a combination of chromatographic techniques with mass spectrometry, including gas chromatography/mass spectrometry (GC/MS)[5] and liquid chromatography/mass spectrometry (LC/MS)[4,6]. These approaches usually require complicated off-line sample clean-up procedures, primarily based on solid phase extraction (SPE), which can be very time-consuming, labor-intensive and are vulnerable to variability due to errors in manual preparation.

Thermo Scientific TurboFlow technology has been widely used as an automated online sample extraction technique in the food testing industry[7] and was recently applied to β-agonists in urine[8]. Sample extracts are directly injected onto a narrow diameter TurboFlow column, minimizing lengthy offline sample preparation steps. High linear velocities inside the column force large molecules to quickly flow through to waste while small molecules are retained. In this application note, we describe a simple and rapid method using TurboFlow™ technology and tandem MS for the quantitative analysis of ractopamine in beef.

Thermo SCIENTIFIC

Experimental

Reagents/Matrix

Ractopamine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA) and its isotope labeled internal standard (IS), ractopamine-d_6 hydrochloride, was obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). All other chemicals and solvents were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Ground beef was purchased from a local supermarket.

Sample Preparation

Four replicates of 5 grams each of homogenized ground beef were weighed into separate 50-mL conical polypropylene centrifuge tubes. Each 5 g sample was first extracted with 20 mL of extraction solvent consisting of 0.2 % formic acid in 80:20 methanol/H_2O by vortexing for 2 minutes followed by centrifugation at 5,700 RCF and 15 °C for 10 minutes. The resultant replicate supernatants were decanted and combined into a glass bottle. A second 10-mL solvent extraction was performed as above and the supernatants combined with the first extracts. The extract was then filtered through a 0.45 µm syringe filter and the resulting solution was used to make matrix calibrators and quality control (QC) samples. The overall sample preparation time is approximately 30 min. Twenty milliliters each of two solvents (ground beef extract and extraction solvent) were spiked with an IS stock solution of ractopamine-d_6 (1 µg/mL) to a final concentration of 5.0 ng/mL. The calibration concentration levels, were 0.00 (blank sample), 0.06, 0.12, 0.30, 0.60, 1.2, 3.0, 6.0, 30.0, 60.0 and 120.0 ng/g.

LC/MS conditions

LC/MS analysis was performed using a Thermo Scientific Transcend TLX-1 system powered by TurboFlow technology coupled to a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer. Heated electrospray ionization source or HESI was selected for its sensitivity and ruggedness. As required in Commission Decision 2002/657/EC[9], one quantifier and two confirmation ions were selected. The precursor and product ion fragments of ractopamine and its IS were detected using positive ionization selective reaction monitoring (+SRM) as listed in Table 1.

TurboFlow Method Parameters	
System:	Transcend TLX-1 system
Column:	TurboFlow Cyclone-P, 0.5 x 50 mm
Injection Volume:	25 µL
Solvent A:	0.1 % Formic Acid in Water
Solvent B:	0.1 % Formic Acid in Methanol
Solvent C:	0.01M Ammonium Hydroxide in Water
Solvent D:	1:1:1 Acetonitrile (ACN): Isopropanol: Acetone (v:v:v)

HPLC Method Parameters	
Analytical Column:	Accucore C18, 3 x 50 mm, 2.6 µm
Solvent A:	0.1 % Formic Acid in Water
Solvent B:	0.1 % Formic Acid in ACN
Solvent C:	100 % Isopropanol

Mass Spectrometer Parameters	
Mass spectrometer:	TSQ Quantum Access MAX
Ionization Source:	Heated electrospray ionization II (HESI II)
Ion Polarity:	Positive ion mode
Spray Voltage:	2.5 KV
Sheath Gas Pressure (N_2):	60 arbitrary units
Auxiliary Gas Pressure (N_2):	30 arbitrary units
Vaporizer Temperature:	350 °C
Capillary Temperature:	300 °C
Tube Lens Voltage:	110 V
Collision Gas Pressure (Ar):	1.5 mTorr
Q1 Resolution:	*m/z* 0.7 (full width at half maximum)
Q3 Resolution:	*m/z* 0.7

Figure 1 illustrates the schematic diagram of a typical TurboFlow online sample extraction system. The LC method schematic view in Thermo Scientific Aria OS software is shown in Figure 2. The entire experiment was controlled by Aria OS version 1.6.3.

Table 1. The +SRM transitions for ractopamine and its internal standard as run on the mass spectrometer operating with a HESI source

Analyte	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (CE)	Tube Lens
Ractopamine	302.141	164.074 (Q)	15	83
		107.122	29	
		121.089	23	
Ractopamine-d_6	308.170	168.094 (Q)	15	85
		107.137	30	
		121.090	22	

Q: Quantifier

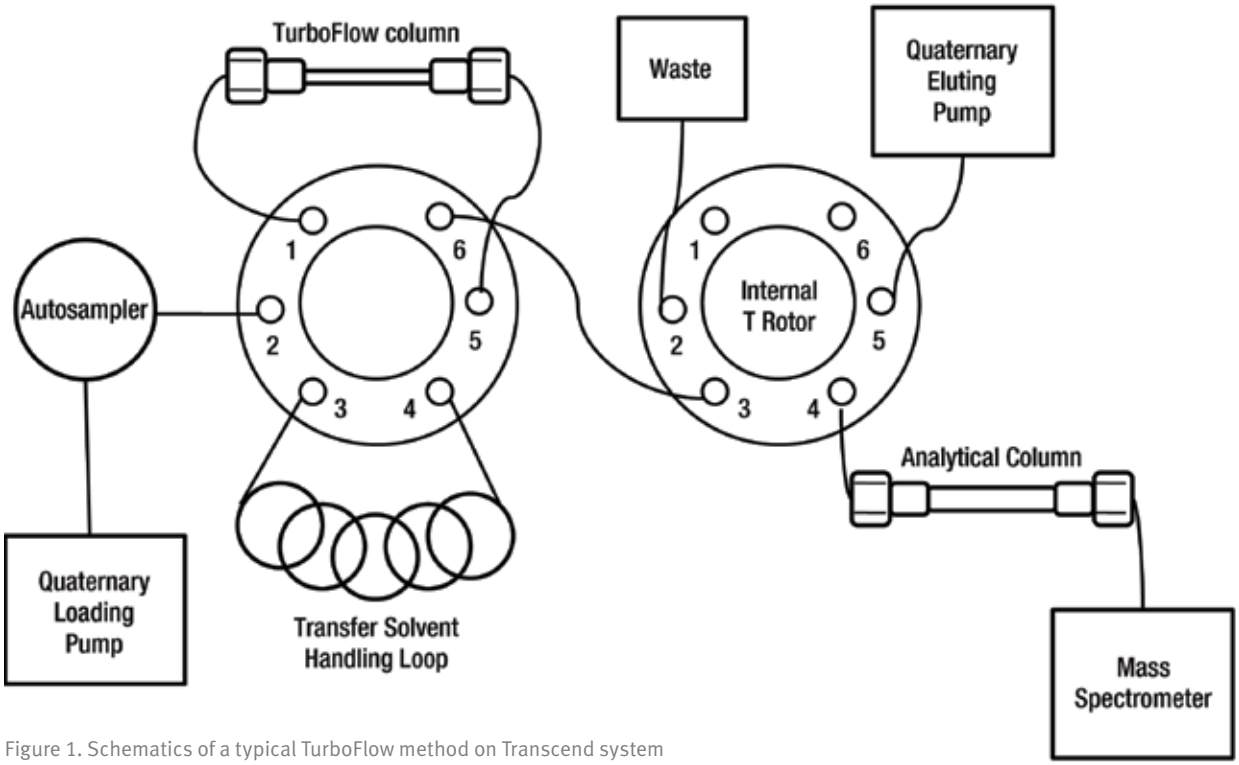

Figure 1. Schematics of a typical TurboFlow method on Transcend system

Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B	%C	%D
1	0.00	30	1.50	Step	100.0	-	-	-	====	out	0.70	Step	100.0	-	-	-
2	0.50	10	0.20	Step	100.0	-	-	-	====	out	0.70	Step	100.0	-	-	-
3	0.67	60	0.20	Step	100.0	-	-	-	T	in	1.30	Step	100.0	-	-	-
4	1.67	30	1.50	Step	-	-	-	100.0	====	in	0.70	Step	40.0	60.0	-	-
5	2.17	30	1.50	Step	-	-	100.0	-	====	out	0.70	Ramp	-	100.0	-	-
6	2.67	10	1.50	Step	-	-	-	100.0	====	in	0.70	Step	-	100.0	-	-
7	2.83	30	1.50	Step	-	-	-	100.0	====	out	1.00	Step	-	-	100.0	-
8	3.33	60	1.50	Step	90.0	10.0	-	-	====	in	0.70	Step	-	100.0	-	-

Start Data Window 0.02 min Data Window Length 5.05 min

Figure 2. TurboFlow method schematic diagram as viewed in Aria OS software

Results and discussion

Limit of detection and quantitation

In the present study, in addition to a double blank sample (processed matrix sample without IS) and a zero sample (processed matrix sample with IS), ten calibration concentration levels ranging from 0.06 to 120 ng/g were used. Good linearity was observed over the entire tested range for 25 µL injections except at the two lowest concentration levels. As shown in Figure 3, the correlation coefficient obtained using weighted (1/x) linear regression analysis of standard curves was greater than 0.99 for beef. For the concentration range studied, limits of quantitation (LOQ) was estimated from triplicate injections (coefficient of variation CV < 20%) of standard solutions at a concentration level corresponding to a minimum signal-to-noise (S/N) ratio of 10. The limit of quantification of the current protocol was 0.30 ng/g in beef. Figure 4 shows the extracted ion chromatogram of ractopamine at LOQ in beef.

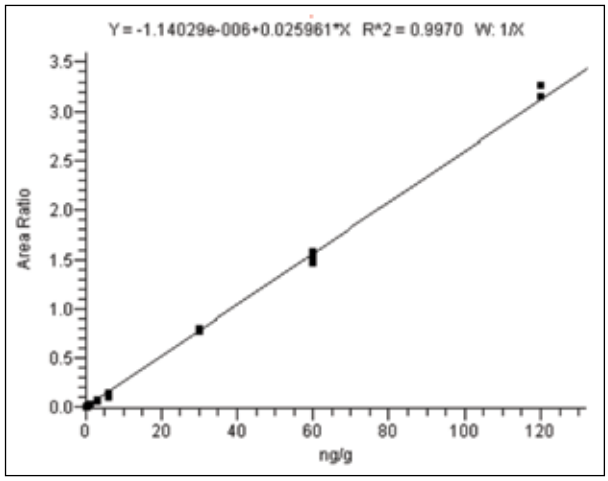

Figure 3. Linear regression curve of ractopamine standards based on area ratio with internal standard ractopamine-d_6

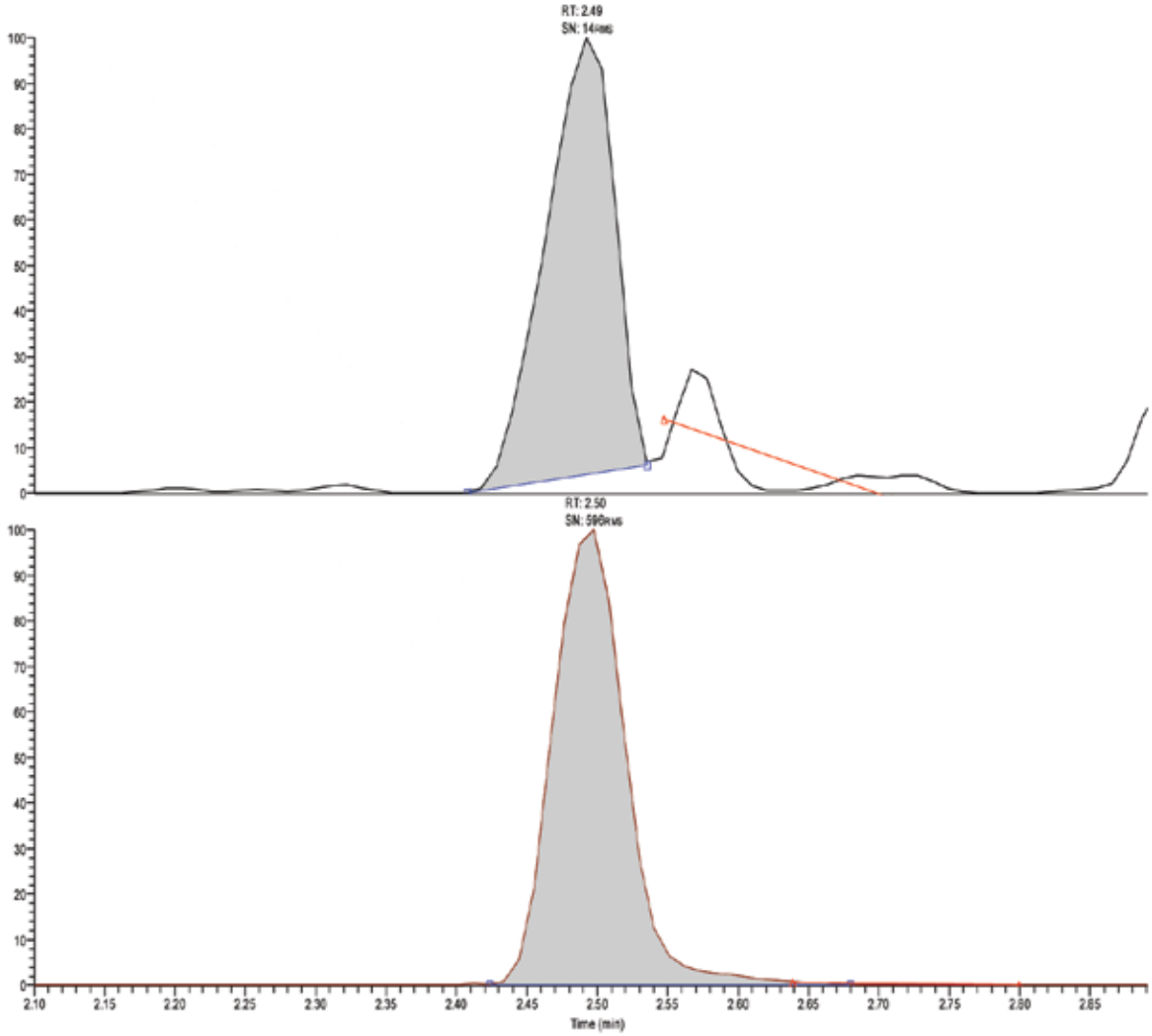

Figure 4. Extracted ion chromatography of ractopamine SRM *m/z* 164 transition at LOQ (upper trace) and ractopamine-d_6 at 5.0 ng/mL in matrix (lower trace)

Accuracy and precision

Method accuracy and precision were assessed using blank matrix spiked at four concentration levels injected in six replicates. Samples were spiked at 0.6, 3.0, 6.0 and 60.0 ng/g concentration levels. Between-run accuracy and precision was expressed as mean of the overall accuracy and precision data from four individual runs over three consecutive days. The accuracy is reported as percent of the nominal spiked known concentration value and the precision is expressed as the relative standard deviation (%RSD). Table 2 summarizes the data obtained from the method validation study. The within-run and between-run accuracy varied from 84.0 to 99.6 and 82.8 to 99.5 % of nominal concentrations, respectively, while within-run and between-run precision ranged from 2.6 to 11.2 %RSD and 2.9 to 14.0 %RSD, respectively. These values are all within the requirements (15% for both accuracy and precision) determined by various regulatory agencies[9,10].

Table 2. Results of the method validation

Ractopamine Spike level (ng/g)	Within-run Accuracy (Run 1) (%)	Between-run Accuracy (%)	Within-run Precision (Run 1) (%RSD)	Between-run Precision (%RSD)	Mean Recovery (%) (n=6)
0.6	94.9	97.3	11.2	14.0	86.3
3.0	89.9	85.7	8.1	8.9	91.7
6.0	84.0	82.8	7.2	7.7	85.5
60.0	99.6	99.5	2.6	2.9	103.8
Internal Standard			3.2	5.1	

Recovery

The recovery study was performed on beef matrix fortified with ractopamine at 0.6, 3.0, 6.0, and 60.0 ng/g. The recovery was assessed by comparing the detector response of a post-extracted spiked sample with that determined from a spiked neat standard sample at the same concentration. Addition of isotope labeled IS compensated for matrix interference during LC/MS analysis. Recovery values of 70–125% are deemed acceptable. As shown in Table 2, recoveries ranged from 85 to 104% in beef. Method performance data show the feasibility and reliability of using this approach for the determination of ractopamine in meat matrices.

Carryover

The level of carryover was determined by evaluating the analyte peak area in a matrix double blank injected immediately after the highest calibration matrix standard. Peak area should not be greater than 20% of the LOQ. To minimize autosampler cross-contamination, the injector and syringe were washed with 0.1% formic acid in 2% ACN and 0.1% formic acid in ACN three times each between injections. There is relatively low affinity between ractopamine and hydrophobic alkyl phases. Carryover associated with the TurboFlow column was inhibited by washing the column with over 150 column volume of 1:1:1 ACN isopropanol acetone (v:v:v) after the analytes were transferred onto the analytical column. The analytical column was washed with 1.5 column volume isopropanol after the gradient completion. A blank sample was also injected after each standard or sample to minimize the possible impact of carryover on accuracy and precision. As a result, no measurable carryover peak was detected.

Conclusion

A quick, rugged, sensitive and automated online LC/MS method was developed to measure ractopamine in animal meat matrices. The TurboFlow method eliminates the need for time-consuming sample preparation procedures such as offline SPE, allowing for higher throughput and better reproducibility. The method quantitation limit has been determined to be 0.30 ng/g, which is significantly lower than the limits set by the US government and MRPL proposed by the EU. Owing to the large volume and/or multiple injection capability of Transcend TLX system[11], the quantitation limit could be further lowered if necessary.

The relevant data window of this method represents only a part of total run time, thus sample throughput can be doubled or even quadrupled by multiplexing across a two or four channel Transcend TLX system. As a result, up to 40 samples per hour can be analyzed. There is also a potential to add other β-agonists, such as clenbuterol, into the same method.

Acknowledgements

The authors would like to thank Matthew Berube of Thermo Fisher Scientific for instrument setup.

References

1 Apple, J. K.; Rincker, P. J.; McKeith, F. K.; Carr, S. N.; Armstrong, T. A.; PAS; Matzat, P. D. *Prof. Anim. Sci.*, **2007**, *23*, 179–196.

2 European Food Safety Authority, *EFSA J.*, **2009**, *1041*, 1-52.

3 The sixty-second meeting of the Joint FAO/WHO Expert Committee on Food Additives, FAO Food and Nutrition Paper 41/16, **2004**.

4 De Brabander, H. F.; Le Bizec, B.; Pinel, G.; Antignac, J.-P.; Verheyden, K.; Mortier, V.; Courtheyn, D.; Noppe, H. *J. Mass Spectrom.*, **2007**, *42*, 983–998.

5 Wang, J.P.; Li, X.W.; Zhang, W.; Shen, J.Z. *Chromatographia*, **2006**, *64*, 613-617.

6 Sakai, T.; Hitomi, T.; Sugaya, K.; Kai, S.; Murayama, M.; Maitani, T. *J. Food Hyg. Soc. Jpn.*, **2007**, *48*, 144-147.

7 Shi, Y.; Lafontaine, C.; Fink, J. A.; Espourteille, F. A. *Food Quality*, **2009**, **Aug/Sept.**

8 Bernsmanna, T.; Furst, P.; Godula, M. *Food Additi. Contam.*, **2011**, *28*, 1352-1363.

9 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, **2002**.

10 Food and Drug Administration, U.S. Department of Health and Human Services, Guidance for Industry, Bioanalytical Method Validation, **2001**.

11 Herman, J.L. *Rapid Commun. Mass Spectrom.*, **2005**, *19*, 696-700.

ISO 9001 REGISTERED COMPANY Thermo Fisher Scientific, San Jose, CA USA is ISO Certified.

www.thermoscientific.com

Africa-Other +27 11 570 1840
Australia +61 2 8844 9500
Austria +43 1 333 50 34 0
Belgium +32 53 73 42 41
Canada +1 800 530 8447
China +86 10 8419 3588
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland /Norway/Sweden +46 8 556 468 00
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9434
Italy +39 02 950 591
Japan +81 45 453 9100
Latin America +1 608 276 5659
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
South Africa +27 11 570 1840
Spain +34 914 845 965
Switzerland +41 61 716 77 00
UK +44 1442 233555
USA +1 800 532 4752

Thermo SCIENTIFIC
Part of Thermo Fisher Scientific

AN63554_FS119_E 05/12S