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Research Paper

Quantification of the β_2-Adrenergic Feed Additives Ractopamine and Salbutamol by Reductive Amination–Assisted Modification

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ABSTRACT

β_2-Adrenergic compounds such as ractopamine and salbutamol can cause smooth muscle relaxation and are used for asthma and chronic obstructive pulmonary disease treatment. However, β_2-adrenergic compounds may also cause some adverse cardiovascular effects or behavioral changes in humans; therefore, it is important for analytical chemists to determine the amounts of β_2-adrenergic compounds in raw biological materials. Herein, novel analogs for standards and internal standard analogs were synthesized through reductive amination with isotopic H_2-formaldehyde, D_2-formaldehyde, and sodium cyanoborohydride. These analogs were used for quantifying β_2-adrenergic compounds such as ractopamine and salbutamol in ground pork samples under multiple reaction monitoring scanning modes by tandem mass spectrometry. H_2-Formaldehyde–modified ractopamine and H_2-formaldehyde–modified salbutamol acting as standards were used to prepare calibration curves, whereas D_2-formaldehyde was used to generate D_2-modified ractopamine and D_2-modified salbutamol analogs acting as internal standards. The H_2-modified ractopamine and H_2-modified salbutamol showed excellent correlation coefficients, limits of detection, and limits of quantification. In mass spectrometric detection, H_2-modified ractopamine showed 202% signal enhancement after modification and H_2-modified salbutamol showed 17% signal enhancement after modification.

HIGHLIGHTS

- A reductive amination–assisted method was used to synthesize standards and internal standards of ractopamine and salbutamol.
- Standard and internal standard analogs were fabricated by isotopic formaldehydes and sodium cyanoborohydride.
- A quantitative method of modified ractopamine and salbutamol was successfully validated.
- The reductive amination–assisted method enhances the signal for MS detection.

Key words: β_2-Adrenergic agonist; Ractopamine; Reductive amination; Salbutamol; Tandem mass spectrometry

β_2-Adrenergic agonists (β-agonists) interact with β_2-adrenergic receptors to cause smooth muscle relaxation; consequently, they are used for the treatment of asthma and chronic obstructive pulmonary disease *(7, 9, 13, 17, 19)*. In addition, β-agonists have been explored as agents to promote animal growth and lean muscle in poultry and domestic livestock, such as swine, cattle, and turkeys *(4, 8)*. Well-known β-agonists in animal feed are ractopamine (Paylean), colterol, zilpaterol, cimaterol, salbutamol, tulobuterol, terbutaline, and clenbuterol.

Some β-agonists are forbidden in livestock feed, and only ractopamine and zilpaterol are U.S. Food and Drug Administration (FDA) approved. According to FDA tolerances for ractopamine, residues are set at concentrations as low as 0.03, 0.05, and 0.1 ppm (mg/L) in the crude muscles of cattle, swine, and turkeys, respectively *(1)*. The ractopamine residue limits for the livers of these animals are set at 0.09, 0.15, and 0.45 ppm, respectively *(1, 4)*. In 2012, the Codex Alimentarius Commission announced that the maximum residue level for ractopamine is 0.01 ppm in muscle, 0.04 ppm in liver, and 0.09 ppm in kidney for swine and cattle, but turkeys were excluded *(18)*. For zilpaterol residues, the levels were set below 0.012 ppm in cattle liver and 0.01 ppm in cattle muscle *(2)*. Salbutamol has also been reported as a therapy for asthma, bronchospasm, and chronic obstructive pulmonary disease *(15)*. However, β-agonists are known to cause toxic effects upon overconsumption and may cause adverse effects such as irregular heartbeat, headache, anxiousness, dizziness, nausea, and

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TABLE 1. *Characteristics of ractopamine and salbutamol and method validation factors*

β_2-Adrenergic compound	Molecular formula	Molecular wt	MRM transition		LOD (ng/mL)[a]	LOQ (ng/mL)[a]	R^2
			Precursor ion	Product ions			
Ractopamine	$C_{18}H_{23}NO_3$	301.38	302	284, 164			
H_2-modified ractopamine	$C_{19}H_{25}NO_3$	315.41	316	298, 178	0.14	0.39	0.9994
D_2-modified ractopamine	$C_{19}H_{23}D_2NO_3$	317.42	318	300, 180			
Salbutamol	$C_{13}H_{21}NO_3$	239.31	240	166, 148			
H_2-modified salbutamol	$C_{14}H_{23}NO_3$	253.34	254	180, 162	0.15	0.40	0.9999
D_2-modified salbutamol	$C_{14}H_{21}D_2NO_3$	255.35	256	182, 164			

[a] Limits of detection (LODs) and limits of quantification (LOQs) values were ascertained following a statistical method. LOD, the value of the average of blank + 3SD of blank; LOQ, the value of the average of blank + 10SD

vomiting *(14, 16)*. Furthermore, the relationship between cancer patients and β-agonists has been studied by measuring ractopamine and zilpaterol in urine samples, and the results showed low correlation *(4)*.

Because of these unwanted effects, β-agonists are forbidden in livestock feeds in many countries and should not be present in crude muscle and ground viscera. Various analytical methods have been developed to characterize and quantify the β-agonists in human body fluids and animal tissues, including the use of an electrochemical electrode modified with polytaurinezirconia nanoparticles to detect ractopamine and salbutamol in pig muscle and human urinary samples *(16),* online stacking capillary electrophoresis to quantify ractopamine *(22),* and combined solid-phase extraction and high-performance liquid chromatography (HPLC) with UV and mass spectrometry (MS) detection to quantify ractopamine, clenbuterol, and salbutamol *(5, 6, 24)*. Furthermore, HPLC coupled with tandem mass spectrometry (MS/MS) *(11)* and gas chromatography coupled with MS/MS have also been reported *(12, 20)*.

To control and monitor the stability of analytical instruments, suitable internal standards matching the analytes are needed *(11)*. In this study, we have demonstrated a novel modification strategy called the reductive amination assistance method to synthesize internal standard ractopamine and salbutamol derivatives for quantification *(12, 20)*. The reductive amination strategy is based on that used for comparative quantitative proteomics techniques *(3, 10, 23)*. H_2-Formaldehyde and the reducing agent sodium cyanoborohydride ($NaBH_3CN$) can be used to modify ractopamine and salbutamol into H_2-modified analogs as standards for the preparation of calibration curves. Likewise, D_2-formaldehyde and $NaBH_3CN$ can transform ractopamine and salbutamol into their D_2-modified analogs for use as internal standards. We applied this method to MS detection by multiple reaction monitoring (MRM) and validated the method with reference to its dynamic range, correlation coefficient, limit of detection (LOD), limit of quantification (LOQ), and average recoveries at low, intermediate, and high concentrations. This work led to us to establish a quantitative method for the determination of the β-agonists ractopamine and salbutamol.

MATERIALS AND METHODS

Reagents and chemicals. Ground pork produced by the Taiwan Sugar Corporation was purchased from a supermarket. The following materials were all obtained from Sigma (St. Louis, MO): β-agonists ractopamine hydrochloride and salbutamol sulfate, $NaBH_3CN$, trifluoroacetic acid, a sodium acetate buffer, formic acid (>98%), and H_2-formaldehyde solution (36.5 to 38% in water). D_2-Formaldehyde (20% in water) was obtained from Isotec Corp. (Miamisburg, OH). Sodium hydroxide and hydrochloric acid, used to adjust buffer pH, were obtained from J.T. Baker (Phillipsburg, NJ). Liquid chromatography (LC)–MS grade solvents including methanol and acetonitrile were purchased from Merck (Seelze, Germany). Deionized water (18.3 MΩ cm) was produced using a Millipore system (Millipore, Bedford, MA). Polytetrafluoroethylene filters with a pore size of 0.22 μm were also obtained from Millipore.

LC-MS/MS instrumentation and software. Sample detection and analyses were processed by a Thermo Finnigan Acella 1250 autosampler and an ultrahigh-performance liquid chromatograph (UHPLC; Thermo Fisher Scientific, Waltham, MA) linked to a Thermo Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer. The interface between the UHPLC and MS systems was a micro-electrospray ionization ion source in positive ion mode at an operating spray voltage of 3,000 V. The vaporizing and capillary temperatures were set at 350 and 300°C, respectively, and the pressures of the sheath gas and auxiliary gas were maintained at 35 and 10 absolute units, respectively. For fragmentation, the nitrogen gas in the collision chamber was adjusted to 1.0 unit, and the collision energy was set at 25 V. An autosampler was used to deliver the sample into a 10-μL sample loop, and separation was performed on a Capcell Pak MG II C18 analytical column (inside diameter, 1.5 by 100 mm, 3 μm; Shiseido, Tokyo, Japan). The UHPLC flow rate was maintained at 140 μL/min, and the mobile phases were water (A) and 100% acetonitrile containing 0.1% formic acid (B). MS analysis was performed in MRM mode for quantification of the isotopic analogs of ractopamine and salbutamol. MRM processing procedures were applied by first determining the *m*/*z* values of the precursors and then establishing the *m*/*z* values of the product ions. The MRM transitions were set as listed in Table 1. Xcalibur 2.2 software (Thermo Finnigan Inc., San Jose, CA) was used to manage the autosampler, UHPLC, and MS/MS and also to acquire and process raw MS data. In the statistical analyses, Xcalibur LCquan 2.7 software (Thermo Finnigan Inc.) was used to process the raw MS data and prepare calibration curves to quantify the analogs of ractopamine and salbutamol.

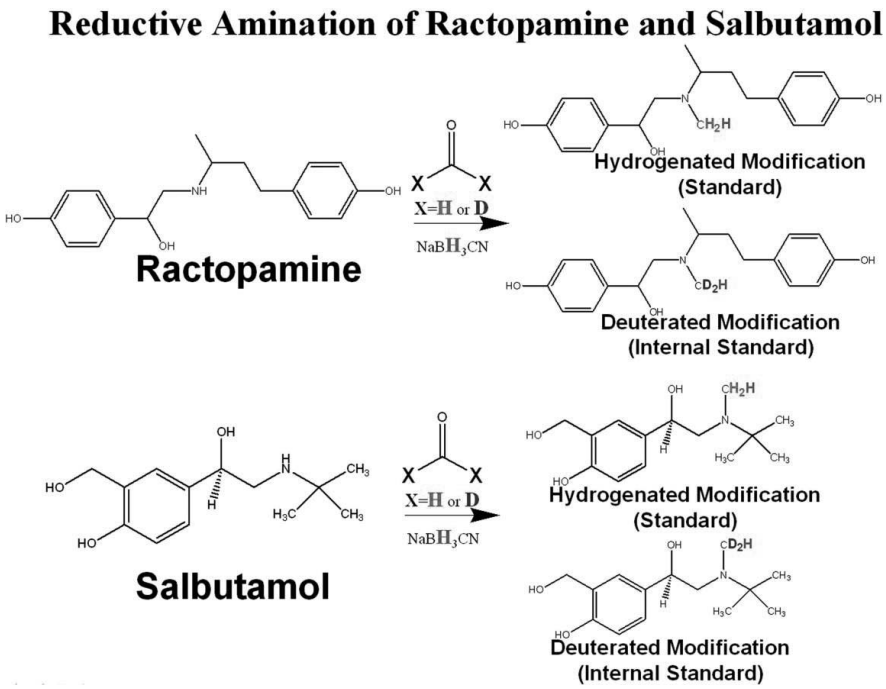

FIGURE 1. *Synthetic scheme for reductive amination of ractopamine and salbutamol compounds with isotopic formaldehydes and sodium cyanoborohydride ($NaBH_3CN$).*

Preparation of isotopic standards of ractopamine and salbutamol. Ractopamine and salbutamol were dissolved in methanol at 10 mg/mL (10,000 ppm), and then the concentration was adjusted with deionized water to 4 μg/mL. Aliquots (20 μL) of ractopamine and salbutamol were taken, and the pH was adjusted to 5.6 using sodium acetate buffer (160 μL). The ractopamine and salbutamol samples were reacted separately with both 10 μL of 4% H_2-formaldehyde and 4% D_2-formaldehyde for 5 min, and the four resulting mixtures were reduced with 0.6 M $NaBH_3CN$ for 1 h. A schematic of the reductive amination with isotopic formaldehyde and $NaBH_3CN$ for the preparation of standards and internal standards is illustrated in Figure 1.

MRM transitions of isotopic ractopamine and salbutamol analogs. Isotopic formaldehyde-modified ractopamine and formaldehyde-modified salbutamol were diluted with 30% acetonitrile in 0.1% formic acid and introduced to the MS directly by syringe to determine the *m/z* of the precursor and product ions for establishing MRM transitions. The fragmentation spectra of the modified ractopamine and salbutamol are shown in Figure 2, along with the spectra of their unmodified analogs. The following MRM transitions were established: 302 > 284 and 302 > 164 for unmodified ractopamine (Fig. 2A), 316 > 298 and 316 > 178 for H_2-formaldehyde–modified ractopamine (Fig. 2B), 318 > 300 and 318 > 180 for D_2-formaldehyde–modified ractopamine (Fig. 2C), 240 > 166 and 240 > 148 for unmodified salbutamol (Fig. 2D), 254 > 180 and 254 > 162 H_2-formaldehyde–modified salbutamol (Fig. 2E), and 256 > 182 and 256 > 164 for D_2-formaldehyde–modified salbutamol (Fig. 2F). These MRM transitions are listed in Table 1.

Method validation. Calibration curves were plotted to estimate the LODs and LOQs, and the recoveries for real samples were demonstrated. To prepare calibration curves for modified ractopamine and salbutamol, ractopamine and salbutamol stock solutions were modified with H_2-formaldehyde and diluted to 1, 2, 5, 10, 20, 50, 100, 200, 500, and 1,000 ng/mL. D_2-Formaldehyde–modified ractopamine and D_2-formaldehyde–modified salbutamol analogs were added to the corresponding samples as internal standards.

To assess average recovery, stock solutions of unmodified ractopamine and salbutamol were diluted with deionized water to three concentrations: low (20 ng/mL), intermediate (100 ng/mL), and high (500 ng/mL). Individual samples were mixed with 0.5 g of ground pork and blended with the corresponding D_2-formaldehyde–modified analogs as internal standards. The ground pork and β-agonist mixture was treated with acetonitrile (1,400 μL) for protein precipitation and removal of solid substances. After vortexing for 5 min and centrifugation at 13,400 × *g* for 20 min at 4°C, the supernatants were removed and dried using nitrogen gas at 37°C. These samples were then redissolved in sodium acetate buffer (180 μL) and 10 μL of 4% H_2-formaldehyde. After shaking the samples for 5 min, 0.6 M $NaBH_3CN$ was added to complete the reductive amination. Before analysis, all samples mixtures, including H_2-formaldehyde–modified ractopamine and H_2-formaldehyde–modified salbutamol blended with D_2-formaldehyde–modified ractopamine and D_2-formaldehyde–modified salbutamol, were filtered through 0.22-μm-pore-size polytetrafluoroethylene filters, individually.

RESULTS AND DISCUSSION

Reductive amination. We generated novel standards and internal standards of ractopamine and salbutamol and developed an MS detection platform for ractopamine and salbutamol modified by reductive amination with isotopic formaldehyde. The H_2-formaldehyde–modified ractopamine and H_2-formaldehyde–modified salbutamol were used to prepare calibration curves, and the D_2-formaldehyde–modified ractopamine and D_2-formaldehyde–modified salbutamol were used as internal standards to facilitate ractopamine and salbutamol quantification. A schematic for the reductive amination of ractopamine and salbutamol with isotopic formaldehyde is shown in Figure 1.

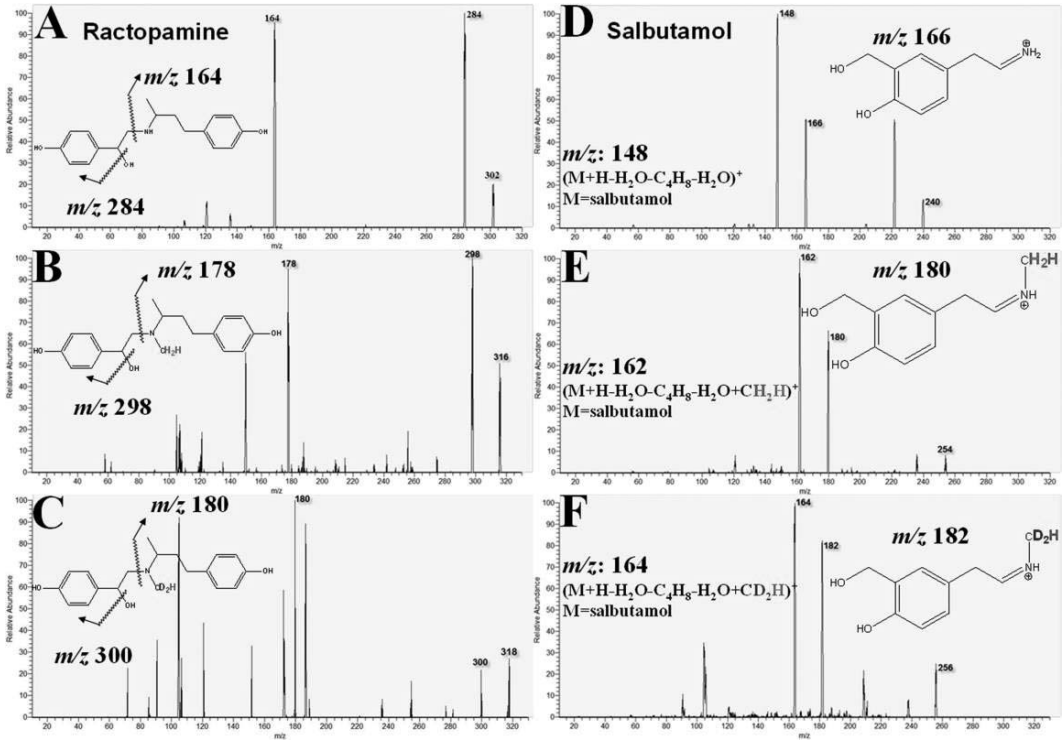

FIGURE 2. *MRM transitions. (A) Unmodified ractopamine (*m/z *302) and its product ions at* m/z *284 and 164. (B) H_2-Formaldehyde–modified ractopamine (*m/z *316) and its product ions at* m/z *298 and 178. (C) D_2-Formaldehyde–modified ractopamine (*m/z *318) and its product ions at* m/z *300 and 180. (D) Unmodified salbutamol (*m/z *240) and its product ions at* m/z *166 and 148. (E) H_2-Formaldehyde–modified salbutamol (*m/z *254) and its product ions at* m/z *180 and 162. (F) D_2-Formaldehyde–modified salbutamol (*m/z *256) and its product ions at* m/z *182 and 164.*

MS fragmentation spectra for ractopamine and salbutamol analogs. Ractopamine, salbutamol, and their modified analogs including H_2-formaldehyde–modified ractopamine and H_2-formaldehyde–modified salbutamol and D_2-formaldehyde–modified ractopamine and D_2-formaldehyde–modified salbutamol were injected into the tandem MS system and analyzed to establish the MRM transitions. As shown in Figure 2A, unmodified ractopamine has an *m*/*z* value of 302 (precursor ion) and generates product ions of *m*/*z* 284 and 164 through fragmentation, as published previously *(21)*. H_2-Formaldehyde–modified ractopamine presents a peak at *m*/*z* 316 (precursor ion) and product ions at *m*/*z* 298 and 178 after fragmentation (Fig. 2B). D_2-Formaldehyde–modified ractopamine presents a precursor ion peak at *m*/*z* 318 and product ions at *m*/*z* 300 and 180 (Fig. 2C). Unmodified salbutamol (precursor ion at *m*/*z* 240) generates fragments at 166 and 148 *(24)*. Salbutamol and its modified analogs, including H_2-formaldehyde–modified salbutamol and D_2-formaldehyde–modified salbutamol, exhibit MRM transitions of 240 > 166 and 240 > 148 for salbutamol, 254 > 180 and 254 > 162 for H_2-formaldehyde–modified salbutamol, and 256 > 182 and 256 > 164 for D_2-formaldehyde–modified salbutamol (Fig. 2D through 2F). These MRM transitions are summarized in Table 1, along with the molecular formulas, molecular weights, LODs, LOQs, and correlation coefficients (R^2).

Signal enhancement of ractopamine and salbutamol. The extracted MS MRM peaks for ractopamine and salbutamol clearly show that the signals are enhanced by reductive amination. As shown in Figure 3, an identical amount of ractopamine (extracted *m*/*z,* 302 > 284; retention time [rt], 9.0 min; Fig. 3A) and H_2-modified ractopamine (extracted *m*/*z,* 316 > 298; rt, 9.2 min; Fig. 3B) were monitored simultaneously, and ractopamine shows an absolute count of 5.0×10^6, whereas H_2-modified ractopamine shows an absolute count of 1.5×10^7. Similarly, comparing salbutamol (extracted *m*/*z,* 240 > 166; rt, 7.0 min; Fig. 3C) with H_2-modified salbutamol (extracted *m*/*z,* 254 > 162; rt, 6.9 min; Fig. 3D), the absolute counts show

TABLE 2. *Signal enhancements with comparison of the integrated areas between modified and unmodified ractopamine and salbutamol MRM transitions*

	MRM/avg peak area ($n = 6$)				
	Unmodified compound	RSD (%)	H_2-modified compound	RSD (%)	Increasing percentage
Ractopamine	38,652,088	9.0	116,882,904	3.9	202
Salbutamol	70,795,898	3.5	83,056,238	4.6	17

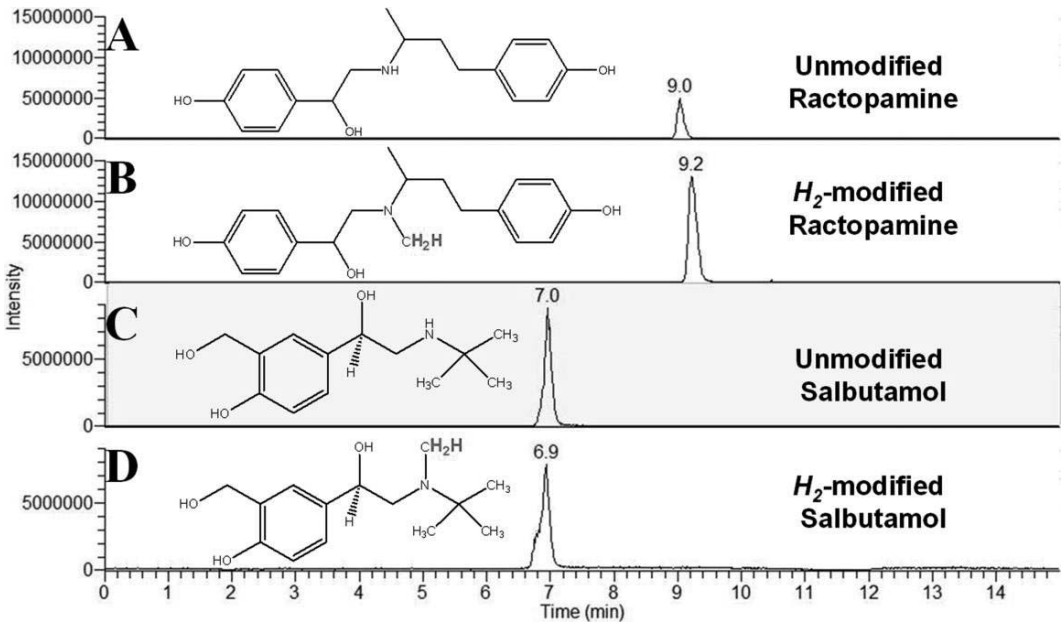

FIGURE 3. *Signal enhancement for H_2-formaldehyde–modified ractopamine and H_2-formaldehyde–modified salbutamol compared with the unmodified analogs. Extracted MRM spectra of (A) unmodified ractopamine (*m/z *302; rt, 8.3 min), (B) H_2-modified ractopamine (*m/z *316; rt, 9.2 min), (C) unmodified salbutamol (*m/z *240; rt, 7.0 min), and (D) H_2-modified salbutamol (*m/z *254; rt, 6.9 min).*

similar values of around 8.0×10^6. However, we compared the average integrated areas for the unmodified and H_2-modified ractopamine and salbutamol, and the ractopamine and salbutamol data sets show 202 and 17% enhancement, respectively, upon modification. The statistical results, including average area, relative standard deviations (RSDs) and signal percentage increases are shown in Table 2.

Method validation. Ractopamine and salbutamol were modified with H_2-formaldehyde to prepare calibration curves. H_2-Modified samples as calibration standards were prepared at 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100, 200, 500, and 1,000 ng/mL, and identical volumes of D_2-modified ractopamine and D_2-modified salbutamol were added as internal standards. The calibration curves, including H_2-modified ractopamine and H_2-modified salbutamol, were extracted from the peak areas of the MRM MS spectra of H_2-modified ractopamine and H_2-modified salbutamol. The calibration curves show an R^2 value of 0.9994 for H_2-modified ractopamine and 0.9999 for H_2-modified salbutamol. The statistical data are listed in Table 1. The LODs and LOQs for modified ractopamine and salbutamol are 0.14 and 0.39 ng/mL for ractopamine and 0.15 and 0.40 ng/mL for salbutamol, respectively.

We also used ractopamine and salbutamol analogs spiked at low (20 ng/mL), intermediate (100 ng/mL), and high (500 ng/mL) concentrations to assess the average recoveries of ractopamine and salbutamol in ground pork samples ($n = 3$). Through sample pretreatment and MS detection, the statistical average recoveries for H_2-modified ractopamine and H_2-modified salbutamol at low, intermediate, and high concentrations are, respectively, 108.0, 100.0, and 100.2 for H_2-modified ractopamine and 84.1, 100.0, and 103.7 for H_2-modified salbutamol (statistical data are shown in Table 3). Figure 4 shows an example of intermediate concentration (100 ng/mL) MS spectra. The H_2-modified ractopamine extracted MRM spectrum is shown in Figure 4A (*m*/*z* 316; rt, 8.3 min) with the internal standard D_2-modified ractopamine extracted MRM spectrum shown in Figure 4B (*m*/*z* 318; rt, 8.3 min). The H_2-modified salbutamol extracted MRM spectrum is shown in Figure 4C (*m*/*z* 254; rt, 6.9 min) with its internal standard D_2-modified salbutamol extracted MRM spectrum shown in Figure 4D (*m*/*z* 256; rt, 6.9 min).

In summary, a sensitive method with signal enhancement by a convenient modification with reductive amination was developed to quantify β-agonists. This modification requires inexpensive agents including formaldehyde and $NaBH_3CN$ to synthesize internal standards. Initially, we

TABLE 3. *Statistical average recoveries of ractopamine and salbutamol analogs spiked in ground pork samples (*n = *3)*

Spike quantity (ng/mL)	Ractopamine			Salbutamol		
	Determined quantity (ng/mL)	RSD (%)	Recovery (%)	Determined quantity (ng/mL)	RSD (%)	Recovery (%)
500	501.0	2.6	100.2	518.5	7.9	103.7
100	100.0	6.7	100.0	100.0	8.1	100.0
20	21.6	4.1	108.0	16.8	4.7	84.1

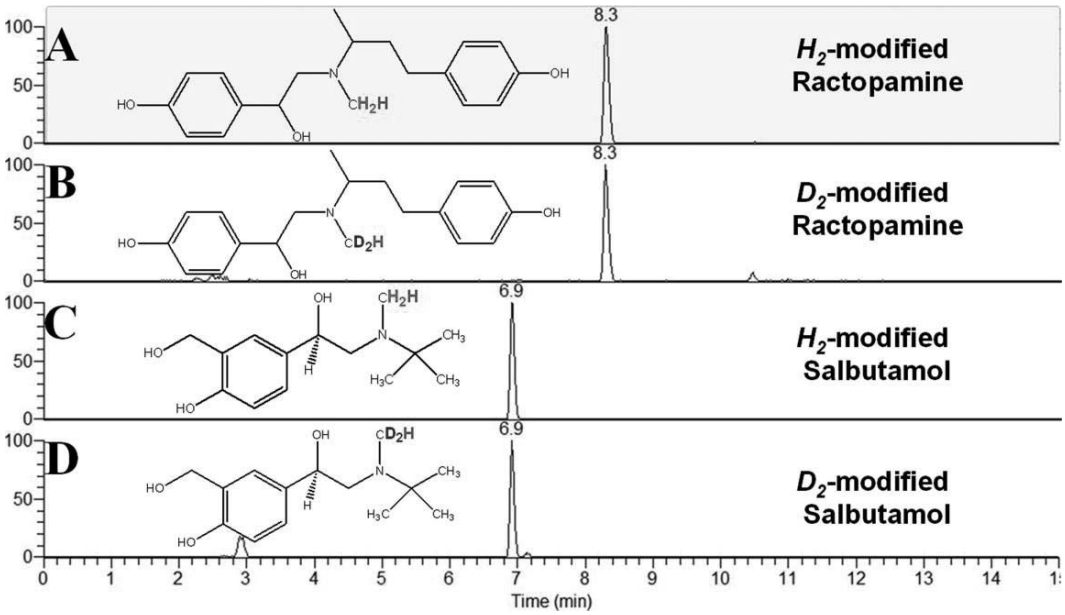

FIGURE 4. *At intermediate concentration (100 ng/mL) in ground pork samples, the extracted MRM MS peaks from raw data for the spectra of H_2-modified ractopamine, D_2-modified ractopamine, H_2-modified salbutamol, and D_2-modified salbutamol. (A) H_2-Modified ractopamine with MRM transitions of 316 > 298 and 316 > 178. (B) D_2-Modified ractopamine with MRM transitions of 318 > 300 and 318 > 180. (C) H_2-Modified salbutamol with MRM transitions of 254 > 180 and 254 > 162. (D) D_2-Modified salbutamol with MRM transitions of 256 > 182 and 256 > 164.*

used ractopamine and salbutamol as an example to demonstrate the practicality of reductive amination in β-agonist quantification. Through reductive amination, eventually, the analogs of ractopamine and salbutamol showed beneficial qualities for MS detection, with signal enhancements of 202 and 17%, respectively. In the method validation, modified ractopamine and salbutamol showed dynamic linear ranges from 1 to 1,000 ng/mL, with an R^2 value of 0.9994 and 0.9999, respectively. Furthermore, the average recoveries for ractopamine and salbutamol ranged from 84.1 to 108.0%.

We intend to extend this reductive amination modification to other β-agonists and develop quantitative methods using tandem MS for all β-agonists. We believe that using reductive amination to synthesize standards and internal standards to enhance MS signal intensities will benefit MS detection and promote the development of food safety and foodomics.

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