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Matrix Effects in Analysis of β-Agonists with LC-MS/MS: Influence of Analyte Concentration, Sample Source, and SPE Type

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Supporting Information

ABSTRACT: The synergistic influences of analyte concentration, sample source, and solid-phase extraction (SPE) type on matrix effects in the multiresidue analyses of eight β-agonists with LC-ESI-MS/MS were evaluated. Porcine muscle and liver extracts and urine from diverse sources were purified by strong or mixed-mode cation exchange and molecularly imprinted polymer SPE cartridges, respectively. Three spiked concentrations (2, 10, and 20 ng/mL) of eight β-agonists in the purified matrices and the different sample sources were analyzed. The results show that for most β-agonists there are significant differences in matrix effects between analyte concentrations or sample sources ($P < 0.05$), whereas there is no significant difference in matrix effects between different SPE cartridges ($P > 0.05$). Results from main effects testing indicated that analyte concentration was the main effector.

KEYWORDS: *β-agonists, liquid chromatography−tandem mass spectrometry, matrix effects, porcine tissues and urine*

INTRODUCTION

β-Agonists such as clenbuterol, salbutamol, and ractopamine are well-known to promote or improve feed efficiency and obtain greater muscle to fat ratio in farm animals. However, their residues in animal tissues can cause potential risk for human health because of their presence in foods produced from these animals. β-Agonists have been forbidden as growth-promoting agents in many countries and communities such as the European Union,[1] China, and Malaysia. However, some of them may be still illegally used in food-producing animals. Therefore, a multiresidue method for monitoring the illegal use of β-agonists is very necessary to ensure animal food safety.

With the introduction of atmospheric pressure ionization (API) sources, liquid chromatography−triple-quadrupole mass spectrometry (LC-MS/MS) has become a powerful analytical tool. With many recognized advantages in terms of sensitivity, selectivity, and specificity, which allow for the analysis of trace amounts of target analytes in complex matrices, this technique has become the method of choice for detecting β-agonists.[2−5] However, one limitation associated with LC-MS/MS analysis is the presence of matrix effects (MEs), which can either suppress or enhance the signal of the target analyte and thus diminish the precision and accuracy of subsequent measurements.[6−9] Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) suffer from MEs, but the former is more susceptible to it.[10−12] The mechanism of MEs is not fully understood, but it probably originates from the competition between the coeluting interferences and the analytes. The reactions taking place between nonvolatile materials and the analyte have been indicated as the most probable cause of MEs.[13] Owing to the above reason, much attention should be paid to overcome MEs in the development of a LC-MS/MS method.

In bioanalysis, many factors such as matrix type,[10] sample preparation technique,[10] sample source,[11] and physicochemical properties of analyte[14] have been proved to play a role in influencing MEs.[15] In the residue analysis of β-agonists, Fiori et al.[16] reported an evaluation of two different purification procedures to minimize matrix-induced ion suppression phenomena in ion-trap LC-MS^n multiresidue β-agonist analysis. The experimental results indicate that molecularly imprinted polymers (MIPs) cleanup of calf urine is effective in reducing ion suppression phenomena below 10%, which is not achievable with conventional SPE C_{18}. On the basis of MIP SPE and conventional Clean Screen Dau (CSD) SPE, Van Hoof et al.[17] developed an ion-trap LC-MS^n multiresidue method for the detection of β-agonists in calf urine. It was observed that there is less suppression of the signals when urine is pretreated with MIP cartridges. Moragues et al.[18] also developed an ion-trap LC-MS^n multiresidue method for determining seven β-agonists in animal liver and urine. This method efficiently decreased the matrix effect through a washing step with hexane in the SPE procedure and a liquid−liquid extraction with *tert*-butyl methyl ether after the elution step. To the best of our knowledge, however, little attention has been devoted to systematically evaluate the factors of MEs and find the main effector of matrix effects in multiresidue analysis of β-agonists with triple-quadrupole MS, which is more reproducible and repeatable in quantitation than ion-trap LC-MS^n. We believe that discovering the main effectors is very beneficial to overcoming matrix effects.

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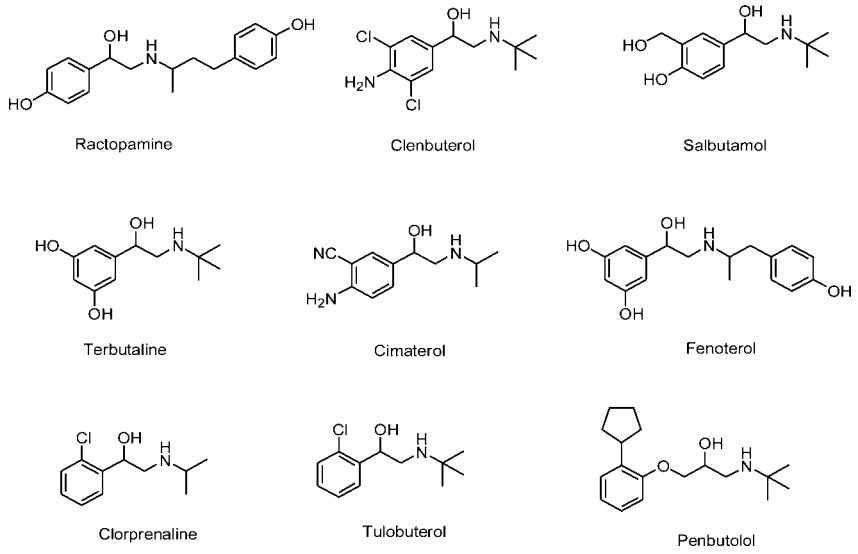

Figure 1. Chemical structures of nine β-agonists.

The purpose of this work was to assess the synergistic influence of analyte concentration, sample source, and SPE type on matrix effect in the multiresidue analyses of β-agonists by liquid chromatography−electrospray ionization quadrupole mass spectrometry using analysis of variance (ANOVA). Blank porcine muscle, liver, and urine samples spiked at three concentration levels from three different sources were cleaned up by three SPE cartridges, namely MIP, strong cation exchange (SCX), and mixed-mode cation exchange (MCX), and then analyzed using LC-ESI-MS/MS in multiple-reaction monitoring (MRM). Eight β-agonists including clenbuterol, salbutamol, terbutaline, cimaterol, fenoterol, clorprenaline, tulobuterol, and penbuterol were investigated. This experimental method and results obtained from this study would contribute to solve matrix effects and choose optimal analyte calibration concentration, matrix-matched sample source, and SPE cartridge type for scientific and accurate determination of β-agonists in a given matrix.

MATERIALS AND METHODS

Chemicals. Standards of ractopamine (RCT), clenbuterol (CLB), salbutamol (SAL), terbutaline (TER), cimaterol (CIM), fenoterol (FEN), clorprenaline (CLO), tulobuterol (TUL), and penbuterol (PEN) (Figure 1) were provided by Dr. Ehrenstorfer (Augsburg, Germany). Ethylene glycol dimethacrylate (EGDMA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Acrylamide (AM) and 2,2′-azobis(isobutyronitrile) (AIBN) were purchased from Kermel Chemical Reagents Development Center (Tianjin, China). Acetonitrile, methanol, and formic acid purchased from Fisher Scientific Co. (Pittsburgh, PA, USA) were of HPLC grade. Other reagents were of analytical grade. An Anpel MCX SPE cartridge (60 mg, 3 mL) was purchased from Anpel Co. (Shanghai, China), and an SCX SPE cartridge (500 mg, 3 mL) was purchased from Supelco Co. (Bellefonte, PA, USA).

Preparation of Standards. Stock solutions of RCT, CLB, SAL, TER, CIM, FEN, CLO, TUL, and PEN were prepared in methanol at a concentration of 1 mg/mL and stored at −20 °C. Working solutions were diluted from the stock solutions with 10% methanol in water containing 0.1% formic acid before use.

Preparation of MIP Cartridges. Synthesis of imprinted polymers and preparation of MIP cartridges were based on the methods described by Zhang et al.[19] MIP was synthesized by bulk polymerization in an acetonitrile−triethylamine system using ractopamine as template and acrylamide as monomer. A molecularly imprinted solid-phase extraction (MISPE) cartridge packed with 150 mg of MIP particles was applied to the following experiments.

Sample Collection. Porcine muscle, liver, and urine samples were collected from the cities of Liuan, Hefei, and Haikou in China. The tissue samples were homogenized and then stored at −20 °C with urine samples. A previous analysis was conducted to ensure that they contain none of the studied analytes.

Sample Extract and Cleanup. A 10 mL aliquot of acetonitrile was added to 2.0 g of blank porcine muscle or liver; after homogenization, the samples were shaken (300 mot/min, 10 min) and centrifuged (8000 rpm, 4 °C 10 min), and then 1 mL of the supernatant was removed, dried, and reconstituted in 5 mL of 0.02 mmol/L ammonium acetate as the loading solution, the pH of which was adjusted to 6.6 for MIP cartridges and 5.2 for MCX and SCX cartridges. Urine was directly 5-fold diluted with 5 mL of 0.02 mmol/L ammonium acetate at pH 6.6 and 5.2, respectively.

On the basis of the previous study,[19] the MIP SPE procedure was as follows: conditioning with methanol and water, then loading 5 mL of sample, washing with water and methanol, and finally eluting with 5 mL of methanol containing 4% ammonia in water.

The MCX cartridge was conditioned with methanol, water, and 2% formic acid in water successively. Thereafter, 5 mL of sample was loaded onto the cartridge, which was then washed with 2% formic acid in water and methanol and eluted with 5 mL of methanol containing 4% ammonia in water.

The SCX cartridge was conditioned with methanol, water, and 30 mmol/L hydrochloric acid, and then 5 mL of sample was loaded onto the cartridges, followed by a washing step with water and methanol. Finally, the cartridges were eluted with 5 mL of methanol containing 4% ammonia in water.

All of the eluates were collected and evaporated to dryness at 45 °C, and the residues were reconstituted in 2 mL of 10% methanol in water containing 0.1% formic acid, respectively. Then nine β-agonists at concentrations of 2, 10, and 20 ng/mL in blank sample extracts and neat solution were prepared before LC-MS/MS analysis. Samples from three different sources were analyzed in triplicate.

Instrument Conditions. An Agilent 1200 HPLC system (Palo Alto, CA, USA) coupled to an Applied Biosystems API 4000 triple-quadrupole mass spectrometer (Foster City, CA, USA) equipped with an ESI source was employed. Chromatographic separation was performed using a Luna C_{18} column (150 mm × 2 mm, 5 μm) purchased from Phenomenex (Torrance, CA, USA). Solvents A and B were 0.1% formic acid in water and acetonitrile, respectively. The flow rate was at 0.25 mL/min. The linear elution gradient profile consisted of 0−2.0 min, 0−45% B; 2.0−6.0 min, 45% B; 6.0−7.0 min, 45−0% B; 7.0−13.0 min, 0% B. The injection volume was 5 μL. Mass analyses

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were performed using an electrospray ion source in positive ionization mode. Multiple-reaction monitoring (unit mass resolution) experiments were carried out. The operation conditions were as follows: ion spray voltage, 5.0 kV; source temperature, 600 °C; curtain gas, 20 psi; ion source gas 1 and gas 2, 55 and 50 psi, respectively. Dwell time was 150 ms for all nine β-agonists, and Table 1 shows the optimized parameter values and the MRM transitions of the analytes.

Table 1. Multiple-Reaction Monitoring Settings for MS/MS Analysis of Nine β-Agonists

analyte	precursor ion $[M + H]^+$, m/z	product ions, m/z	DP,[a] V	CE,[b] eV
ractopamine	302.2	284.2, 164.1[c]	54	20, 23
clenbuterol	277.1	258.9, 203.0[c]	48	19, 23
salbutamol	240.0	222.0, 148.0[c]	60	19, 24
terbutaline	226.1	169.8, 152.1[c]	54	23, 24
cimaterol	220.0	143.2, 202.2[c]	55	21, 16
fenoterol	304.2	134.8, 107.0[c]	54	14, 15
clorprenaline	214.2	196.1, 154.1[c]	56	17, 18
tulobuterol	228.1	171.8, 154.1[c]	54	20, 24
penbuterol	292.2	201.2, 236.2[c]	54	30, 25

[a]DP, declustering potential. [b]CE, collision energy. [c]Quantification ions.

Evaluation of Matrix Effects. Postextraction addition (PEA) experiments were carried out to quantify ME according to the strategy applied by Matuszewki et al.[11] The percentage of ME is calculated as

$$\text{ME}\ (\%) = B/A \times 100$$

where A and B represent the peak area of the standard solution and the standard spiked after extraction, respectively. A ME value of 100% indicates that no matrix effect is present. If the value is <100%, there is signal suppression, and if the value is >100%, there is signal enhancement.

Postcolumn infusion (PCI) experiments were also conducted to qualitatively assess ME in correspondence with the technique developed by Bonfiglio et al.[14]

Statistical Analyses. ANOVA involving multiple factors was performed using SPSS 17.0 software to elucidate any effect of analyte concentration, sample source, and SPE type on MEs. The main effects of three factors were tested to find out the main effector of MEs. For these analyses, Duncan's test (p = 0.05) was performed to evaluate whether MEs varied significantly among analyte concentrations, sample sources, and SPE types, respectively.

RESULTS AND DISCUSSION

Analyte Concentration Dependence. Because ractopamine was used as template molecule in the synthesis of imprinted polymers, it was wrapped in the polymers and could not be removed completely in the washing step.[19] Therefore, when the MIP was used as packing material with the SPE cartridge, the leakage of the residual template from the MIP was inevitable. Finally, this leakage would influence the accurate determination of ractopamine in trace level. Therefore, subsequent analyses were performed only for the other eight analytes (CLB, SAL, TER, CIM, FEN, CLO, TUL, and PEN). The PEA results show that MEs of the eight β-agonists in muscle samples spiked at three levels are 68.7−103.7% (Table 2), whereas those in liver and urine samples are 67.0−103.6 and 63.8−106.2%, respectively (Supporting Information, Tables S1 and S2). The results of ANOVA indicated that MEs were significantly different for all analytes in three matrices among the three analyte concentrations ($P < 0.05$). MEs reduced with the increase of the analyte concentration when the matrix concentration was unchanged. An analogous result was

Table 2. ME of Eight β-Agonists at Different Concentrations in Muscle Samples

	ME,[a] %		
analyte	2.0 ng/mL	10 ng/mL	20 ng/mL
clenbuterol	78.3 ± 3.7 a	95.7 ± 5.2 ab	103.7 ± 5.5 b
salbutamol	70.2 ± 3.7 a	87.7 ± 3.3 b	98.9 ± 4.4 b
terbutaline	71.1 ± 3.2 a	84.2 ± 3.3 b	94.6 ± 4.5 b
cimaterol	68.7 ± 3.3 a	86.9 ± 3.5 b	100.1 ± 4.7 b
fenoterol	74.3 ± 3.5 a	77.0 ± 4.3 a	94.8 ± 5.2 b
clorprenaline	75.6 ± 3.3 a	89.9 ± 3.5 b	99.2 ± 4.6 b
tulobuterol	79.3 ± 3.7 a	86.3 ± 3.8 a	104.8 ± 5.2 b
penbuterol	75.7 ± 3.9 a	91.3 ± 4.0 b	96.5 ± 6.0 b

[a]Each value represents the mean ± SD (n = 81). Values in the same row with different letters are significantly different ($P < 0.05$).

obtained by Van Hout et al.[20] in the analysis of clenbuterol residue in urine using LC-APCI−ion-trap mass spectrometry. Hence, the concentration of analyte chosen for validating MEs should be as close as possible to that expected in the real samples. In addition, when using an internal standard, its concentration should be similar to that of the analyte to ensure that it suffers from similar MEs; otherwise, it could not calibrate effectively the results of analysis.

Sample Source Dependence. The PEA results show that MEs of eight analytes in muscle samples from different regions including Liuan, Hefei, and Haikou are 73.1−110.8% (Table 3),

Table 3. ME of Eight β-Agonists in Muscle Samples from Different Sources

	ME,[a] %		
analyte	Liuan	Haikou	Hefei
clenbuterol	110.8 ± 8.1 a	88.0 ± 3.9 ab	78.9 ± 3.3 b
salbutamol	95.5 ± 5.6 a	88.1 ± 3.6 b	73.1 ± 2.9 b
terbutaline	96.6 ± 4.8 a	79.7 ± 3.5 b	73.5 ± 3.0 b
cimaterol	91.0 ± 5.0	88.0 ± 3.6	76.8 ± 3.1
fenoterol	85.2 ± 5.3	85.1 ± 3.9	75.8 ± 3.8
clorprenaline	92.2 ± 4.9 ab	94.9 ± 3.6 a	77.6 ± 3.0 b
tulobuterol	93.5 ± 5.5 ab	96.8 ± 4.0 a	80.0 ± 3.4 b
penbuterol	97.3 ± 5.8 ab	84.5 ± 4.3 a	81.7 ± 3.7 b

[a]Each value represents the mean ± SD (n = 81). Values in the same row with different letters are significantly different ($P < 0.05$).

whereas those in liver and urine samples are 71.2−116.4 and 74.1−114.7%, respectively (Supporting Information, Tables S3 and S4). The results of ANOVA indicated that the MEs were significantly different for CLB, SAL, TER, CLO, TUL, and PEN in muscle and liver samples and for CLB, CLO, and TUL in urine samples among different sample sources ($P < 0.05$). In the routine analyses of abused veterinary drugs, a large number of different porcine subjects across the country are involved, and the matrix composition of their tissues and biofluids might be widely different. Researchers suggested that samples originating from different sources should be used when an LC-MS/MS method in bioanalysis is validated.[11,21] It seems that the same is applicable for residue analysis in animal tissues.

SPE Type Dependence. Several strategies have been suggested to compensate for or eliminate matrix effects. These strategies include improvements of sample preparation procedures, modifications of chromatographic and mass spectrometric conditions, and applications of calibration methods. Extensive sample cleanup can reduce the presence

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of interfering components in the final extract, thus effectively eliminating matrix effects. Compared to other sample cleanup procedures, SPE represents the most efficient way to overcome MEs. SPE based on MIPs involving a molecular recognition mechanism was proved to achieve higher selectivity than conventional SPE.[22,23] Because SCX, MCX, and MIP SPE procedures are used frequently for the routine analysis of β-agonists,[19,24−26] we aim to compare their ability to reduce matrix effects through ANOVA when multiple factors are involved. The PEA results show that MEs of eight analytes in muscle samples purified by SCX, MCX, and MIP cartridges are 76.6−97.5% (Table 4), and those in liver and urine samples are

Table 4. ME of Eight β-Agonists in Muscle Samples Cleaned up by Different SPE Cartridges

	ME,[a] %		
analyte	SCX	MCX	MIP
clenbuterol	91.7 ± 5.1	97.5 ± 4.9	88.5 ± 4.2 a
salbutamol	77.9 ± 3.6	91.5 ± 3.6	87.3 ± 4.3
terbutaline	80.4 ± 3.7	85.7 ± 3.7	83.7 ± 3.6
cimaterol	76.6 ± 3.8 a	90.1 ± 3.9 ab	89.0 ± 3.8 b
fenoterol	80.7 ± 4.3	83.8 ± 4.4	81.6 ± 3.9
clorprenaline	86.1 ± 3.8	92.8 ± 3.8	85.7 ± 3.7
tulobuterol	82.1 ± 4.2 a	93.0 ± 4.3 ab	95.2 ± 4.1 b
penbuterol	87.5 ± 4.6	93.2 ± 4.5	82.8 ± 4.4

[a]Each value represents the mean ± SD (n = 81). Values in the same row with different letters are significantly different (P < 0.05).

75.0−95.6 and 70.1−95.9%, respectively (Supporting Information, Tables S5 and S6). Results of ANOVA indicated that MEs were significantly differenf only for CIM and TUL in muscle sample, CIM and PEN in liver sample, and SAL, TER, and FEN in urine sample among the three SPE procedures (P < 0.05). Namely, due to the diversity of SPE type, MEs were not significantly different for most analytes in muscle, liver, and urine matrices (P > 0.05), indicating that SCX, MCX, and MIP SPE cartridges might be able to remove the matrix-interfering components from muscle, liver, and urine and thus diminish MEs of most analytes effectively. Consequently, only other performance criteria such as recovery of analyte, price of cartridge, and SPE time need to be considered in the choice of SPE cartridge.

Main Effector of MEs. Results of main effect testing showed that analyte concentration played the most important role in influencing MEs for all analytes. PCI experiments allowed us to assess the differences of MEs among analyte concentrations. A standard solution of eight analytes (2 or 10 ng/mL) was constantly infused at 10 μL/min into the mass spectrometer using a T-piece while 5 μL of a blank sample extract (muscle, liver, or urine) was injected into the chromatographic column under the required chromatographic conditions. The typical MRM chromatograms of penbuterol are shown in Figure 2. Penbuterol had a retention time of 7.8 min (Figure 2A), and around this zone, PCI signals of penbuterol at 2 ng/mL (Figure 2B) experienced more severe suppression than that at 20 ng/mL (Figure 2C), agreeing with the results obtained from PEA experiments. Across the entire chromatographic run, ion suppression was also found in the solvent front (2 min) and during the last step of the elution gradient (7 min). Through modifying the chromatographic conditions, we can adjust the retention time of the analytes away from these time windows where there are obvious matrix effects.

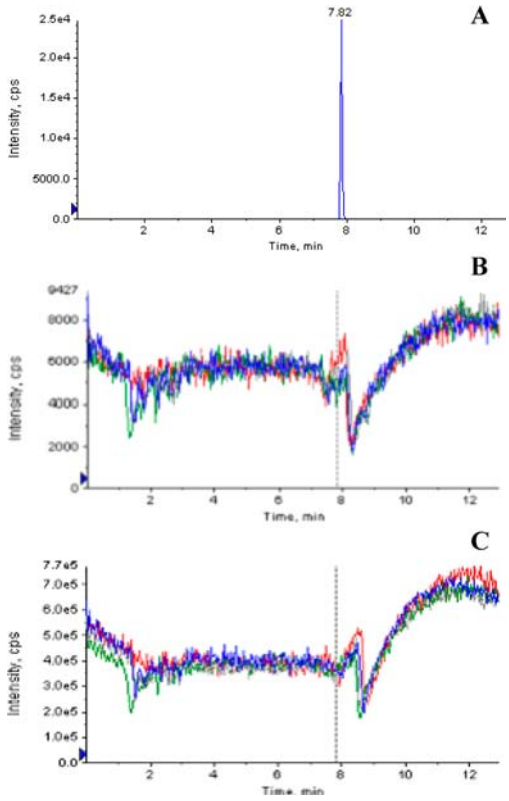

Figure 2. MRM chromatogram of penbuterol standard at a concentration of 2 ng/mL (A) and postcolumn infusion MRM chromatograms of sample extracts purified by MIP at the spiked concentrations of 2 ng/mL (B) and 20 ng/mL (C): muscle (blue line), liver (green line), urine (gray line), and 10% methanol in water containing 0.1% formic acid (red line). The dotted vertical lines represent the retention time of penbuterol.

In conclusion, eight β-agonists including clenbuterol, salbutamol, terbutaline, cimaterol, fenoterol, clorprenaline, tulobuterol, and penbuterol were determined in porcine muscle, liver, and urine samples by liquid chromatography−electrospray ionization tandem mass spectrometry, and the main factors affecting matrix effects were systemically assessed. The results show that analyte concentration and sample source have significant impact on matrix effects in the multiresidue analysis of β-agonists with LC-ESI-MS/MS, whereas SPE cartridge type has a minor influence on matrix effects. The present study may be helpful in the choice of optimal analyte calibration concentration and matrix-matched sample source for scientific and accurate quantitation of β-agonists.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

More details on matrix effects of eight β-agonists in liver and urine samples spiked at three levels (Tables S1−S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AIBN, 2,2′-azobis(isobutyronitrile); AM, acrylamide; ANOVA, analysis of variance; APCI, atmospheric pressure chemical ionization; API, atmospheric pressure ionization; CIM, cimaterol; CLB, clenbuterol; CLO, clorprenaline; CSD, clean screen dau; EGDMA, ethylene glycol dimethacrylate; ESI, electrospray ionization; FEN, fenoterol; LC-MS/MS, liquid chromatography−triple-quadrupole mass spectrometry; LC-MS^n, liquid chromatography−ion-trap mass spectrometry; MCX, mixed-mode cation exchange; ME, matrix effect; MIP, molecularly imprinted polymers; MISPE, molecularly imprinted solid-phase extraction; MRM, multiple-reaction monitoring; PCI, postcolumn infusion; PEA, postextraction addition; PEN, penbuterol; RCT, ractopamine; SAL, salbutamol; SCX, strong cation exchange; SPE, solid phase extraction; TER, terbutaline; TUL, tulobuterol.

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