Analytical Methods

Development of molecular imprinted column-on line-two dimensional liquid chromatography for selective determination of clenbuterol residues in biological samples

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A B S T R A C T

A novel method coupling molecular imprinted monolithic column with two-dimensional liquid chromatography was developed and validated for the analysis of clenbuterol in pork liver and swine urine samples. The polymers were characterized by using Fourier transform infrared spectroscopy, nitrogen adsorption desorption analyses, frontal analysis and the adsorption of selectivity. The results indicated that the imprinted columns were well prepared and possessed high selectivity adsorption capacity. Subsequently, the MIMC-2D-LC (molecular imprinted monolithic column-two dimensional liquid chromatography) method was developed for the selective analysis of clenbuterol in practical samples. The accuracy ranged from 94.3% to 99.7% and from 93.7% to 99.6% for liver and urine, respectively. The relative standard deviation (RSD) of repeatability was lower than 8.6% for both analyses. The limit of detections was 16 ng mL⁻¹ for liver and 25 ng mL⁻¹ for urine, respectively. Compared with the reported methods, the disturbance of endogenous impurity could be avoided by the 2D-LC method.

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1. Introduction

β₂-Agonists which can bind to the bronchial smooth muscle β₂-adrenoceptor, were originally used as remedial medicine for asthma and other related diseases in humans (Wu et al., 2015). However, these compounds have also been misapplied as growth stimulants in stockbreeding, which can transform nutrients from fat tissues to the muscular tissue (Du, Zhao, et al., 2014). Accordingly, all β₂-agonists, as the feed additives for growth stimulation, are forbidden in livestock in both China and European Union (Gao et al., 2014). However, it still permits such drugs to be used as the feed additive for livestock both in United States and Canada (Du, Lei, et al., 2014). Therefore, it is essential to monitor the abuse of β₂-agonists in grazieri (Du et al., 2013).

Currently, lots of analytical methods have been developed for the determination of β₂-agonists in animal tissues, feeds and urine: enzyme-linked immune sorbent assay (ELISA), gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS) and high performance liquid chromatography (HPLC). ELISA is easy to operate, while it is easily affected by environmental factors which lead to a high rate of false positive results (Cheng et al., 2014). GC–MS and LC–MS have the advantages of high sensitivity, nevertheless, the instruments are expensive and need to be equipped with professional technical personnel (Guo et al., 2015). HPLC is the most common used method, whereas conventional one-dimension detection models is liable to be interfered by endogenous contaminants in complex biological samples (Thippani, Pothuraju, Ramisetti, & Shaik, 2013). In addition, the routine sample pretreatment process is time-consuming and low selectivity (Luo et al., 2014). Therefore, it is desired extremely to develop a rapid, effective and specificity method for the detection of β₂-agonists residues in biological samples.

Compared with routine chromatography, multi-dimensional liquid chromatography (MDLC) provides a better separation effectiveness (Sarrut, Crétier, & Heinisch, 2014). Therefore, multi-dimensional liquid chromatography, especially two-dimensional liquid chromatography (2D-LC), has been extensively applied in complex specimens’ analysis (Bailey & Rutan, 2011). Two-dimensional liquid chromatography is a technique in which two independent chromatography columns are involved to analysis

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the samples. According to the separation patterns, 2D-LC can also be classified as off-line 2D-LC and on-line 2D-LC. Compared with off-line 2D-LC, on-line 2D-LC has the advantage of automation and better reproducibility. On-line 2D-LC can be further classified as comprehensive 2D-LC and heart cutting 2D-LC, which depending on the first dimension sections are completely transferred into the second dimension or not (Wang et al., 2015). Therefore, on-line heart cutting 2D-LC is an ideal mode for the analysis of the specific categories compounds in complex matrices due to its advantages of rapid and reliability (Ginzburg, Macko, Dolle, & Brüll, 2011).

Furthermore, the β2-agonists are usually existed in biological matrices with trace amounts (Xiong, Gao, Li, Yang, & Shimo, 2016). The complex matrices can cause the decreasing of sensitivity and reduce the life cycle of HPLC columns. Accordingly, some sample pretreatments, such as solid phase extraction, is an essential procedure. Nevertheless, routine commercial SPE packing material suffers from the disadvantage of poor selectivity and low recovery, which lead to the sample pre-treatments is time-consuming and tedious. Consequently, specific recognition adsorbents for SPE use are in demand.

In order to solve this problem, molecular imprinting technology has attracted more attention (Luo et al., 2015). Since the recognition process of molecular imprinted polymers (MIPs) are comparable with the specificity identification of the antigen-antibody, it has a comprehensive application prospects (Kamra et al., 2016). In this study, we established a novel MIMC-2D-LC method, which coupled molecular imprinted column with C18 column for selectively recognizing clenbuterol in biological matrices. FT-IR, nitrogen gas adsorption and desorption analysis and frontal chromatographic was used to characterize the physicochemical properties and the recognition properties. And, we successfully analyzed the clenbuterol in liver and urine samples with the developed method.

2. Experimental

2.1. Reagents and chemicals

Clenbuterol (CLB, 99.0%) was purchased from Jinjie Pharmaceutical Co. Ractopamine and terbutaline sulfate were purchased from Ganzhang Pharmaceutical Co. (Wuhan, China). Ambroxol hydrochloride, noradrenaline Bitartrate and isoprenaline hydrochloride were purchased from Hefeng Pharmaceutical Co. (Shanghai, China). Dopamine was purchased from J&K China Chemical Ltd. (Beijing, China). The chemical structures of these compounds were illustrated in Fig. S1. Methacrylic acid (MAA) was purchased from Tianjin Chemical Reagent Plant (Tianjin, China). Ethylene glycol dimethacrylate (EGDMA) was obtained from J&K China Chemical Ltd. (Beijing, China). 2,2'-Azobisisobutyronitrile (AIBN) was purchased from Shanghai Shanpu Chemical Ltd. (Shanghai, China). Methanol and acetonitrile were of HPLC grade, purchased from Tianjin Kemio Chemical Reagent Co. (Tianjin, China). Water was purified with MilliQ 1805b (Shanghai, China). Ethylene glycol dimethacrylate (EGDMA) was purchased from Tianjin Chemical Reagent Plant (Tianjin, China).

2.2. HPLC conditions

An on-line two-dimensional liquid chromatography system, including two CMC I-PUMP chromatography pumps (Zhejiang FULLI analytical instruments Co. Ltd, China), an Agilent 1260 diode array detector (Agilent Technologies Inc., USA), a CMC I-UV UV detector (Zhejiang FULLI analytical instruments Co. Ltd, China) and a ten-port valve switching system. A LC-20A pump, a SPD-20AT spectrophotometer, a CBM communications bus module and a LC Solution work station (all from Shimadzu Co., Japan). LC-2010AT high performance liquid chromatograph (Shimadzu Co., Japan).

High-performance liquid chromatography was performed on an LC-20 HPLC system. The UV detector was set at 213 nm with a flow rate of 1.0 mL min⁻¹. The column void volume was measured by injecting 20 μL of acetone (0.1%, v/v), in a mobile phase of acetonitrile-buffer phosphate (20 mmol L⁻¹, pH = 4.0) (65:35, v/v). All of the mobile phases were filtered through 0.45 μm membranes before used.

The capacity factor (k') is calculated from the equation 
\[ k' = \frac{t_R - t_0}{t_0} \]
where \( t_R \) is the retention time of the eluted substances and \( t_0 \) is the retention time of the void marker. The imprinting factor (IF) is calculated from the equation 
\[ IF = \frac{k_{MIPs}}{k_{NIPs}} \]
where \( k_{MIPs} \) is the capacity factor of the target molecule eluted from the imprinted column and \( k_{NIPs} \) is the capacity factor of the target molecule eluted from the non-imprinted column. The separation factors are calculated as 
\[ \alpha = \frac{k_{C1}}{k_{C2}} \]
where \( k_{C1} \) and \( k_{C2} \) are the capacity factors of clenbuterol and other analogues on molecular imprinted column, respectively.

2.3. Preparation of the imprinted column

CLB-molecularly imprinted columns were synthesized according to the reported method (Luo et al., 2011). MAA was used as the functional monomer, EGDMA as the cross-linking reagent, AIBN as the initiator, toluene and dodecanol as the mixed porogenic solvents. In brief, 95.6 mg clenbuterol, 136.4 μL MAA, and 762 μL EGDMA were dissolved in 3.08 mL mixed solvent of toluene and dodecanol (18:82). After the addition of 6.6 mg AIBN, the mixture was sonicated for 10 min. After being purged with nitrogen for 15 min, the mixture was introduced into a stainless-steel column (100 mm × 4.6 mm, i.d.). Then the polymerization was proceeded at 50 °C in a 101-AB oven (Tianjin Taisite Instrument Co, China) for 20 h. After polymerization, the column was elutriated with a mixture of methanol and acetic acid (4:1, v/v) until the baseline smoothly. Meanwhile, the non-imprinted polymers (NIPs) were prepared by same processes in the absence of clenbuterol.

2.4. Physical and morphological characterization

Fourier transform infrared spectra were performed by an FTIR-8400S spectrometer (Shimadzu, Japan) with a scanning range from 400 to 4000 cm⁻¹. Nitrogen adsorption and desorption analyses were evaluated by an Autochem 2920 (Quantachrome, USA) with a bath temperature of 77 K.

2.5. Frontal chromatography

The binding performances of the imprinted and non-imprinted monolithic columns were investigated by consecutive frontal analysis at a flow rate of 1.0 mL min⁻¹. The detector was performed at 213 nm, and mobile phases containing 3.61–36.08 μmol (1–10 μg mL⁻¹) of clenbuterol was used. The columns were first equilibrated with the mobile phase acetonitrile-buffer phosphate. Then a low concentration of solute, 3.61 μmol of clenbuterol, was passed through the column until a plateau appeared. Thereafter, a second solution of higher concentration, 7.22 μmol of clenbuterol, was applied directly to the column, without any regeneration of the columns with the blank mobile phase. This process was repeated until the plateau for the 36.08 μmol solution was observed. Then, the chromatography conditions were adjusted and the breakthrough curves for other conditions were obtained by the same manners. The gradient delaying volume of the whole system was examined by using 0.1 mg mL⁻¹ of sodium nitrate to the columns.
under the same conditions. The retention time was obtained at half height of each breakthrough curve. The apparent binding constant \((K)\) and the number of active binding sites \((mL)\) of binding sites and drug are calculated by the following equation:

\[
1/m_{\text{app}} = 1/KmL[D] + 1/mL
\]

where \(m_{\text{app}}\) is the amount of drug which adsorbed at saturation; \([D]\) is the concentration of the solute applied to the analysis. Then the \(K\) and \(mL\) can be calculated from the regression line of \(1/m_{\text{app}}\) against \(1/[D]\) \((Lei et al., 2007)\).

2.6. Construction of two-dimensional chromatographic system

The molecular imprinted column was combined with a RP-HPLC column by an online 10-port column switcher. The molecular imprinted column-two dimensional liquid chromatography system is illustrated in Fig. S2. When the 10-port column switcher was set at the position A, the target compound or its structural analogues was separated from other interferences. Meanwhile, the second dimensional column was balanced by its mobile phase. When the switcher was transformed at the position B, any retention fraction that was recognized by the molecular imprinted columns was transferred into the ODS enrichment column. Then the retention fractions were directly eluted into the second dimensional HPLC system for a further separation when the switcher back to the position A.

2.7. Sample preparation

In order to evaluate the usefulness of the developed method, the samples of pork liver and urine were chosen as the biological matrix.

The pretreatment of pork liver was according to the reported method \((Du et al., 2013)\). Briefly, two grams of liver samples mixed with 1 mL of saline were homogenized. After spiked with IS solution \((the final concentration was ranged from 0.1 to 10 \(\mu\)g/mL\), the samples were extracted with 2 mL of acetonitrile and then vortex-mixed for 3 min. After a centrifugation at 4000 rpm for 5 min, the supernatant was collected and evaporated to dryness under nitrogen stream. Subsequently, the residual was dissolved by 4.0 mL of mobile phase. Finally, the re-suspended samples was filtered with a 0.45 \(\mu\)m of Millipore filter. The filtrate was injected into the two-dimensional chromatographic system directly.

The extraction of clenbuterol from swine urine samples was performed by a general LLE procedure. Briefly, 4 mL of urine sample was spiked with IS solution \((the final concentration was ranged from 0.1 to 10 \(\mu\)g/mL\). Then, 160 \(\mu\)L of 5% \(NH_3\cdot H_2O\) and approximately 200 mg of NaCl were added and the solution was mixed on a vortex. Subsequently, LLE was performed with 1 mL of dichloromethane and a continuous shaking for 10 min. After a centrifugation at 4000 rpm for 5 min, the dichloromethane phase was separated and dried under nitrogen. The residue was re-suspended with 1 mL mobile phase. It was filtered with a 0.45 \(\mu\)m of Millipore filter and injected to the two-dimensional chromatographic system directly.

2.8. Method validation and practical sample analysis

The method validation was performed following the recommendations of the ICH, including specificity, linearity, range, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision. In order to evaluate the stability of the MIMC-2D-LC system, a month continual test was repeated \((about 200 injections)\) by the same molecular imprinted column. Finally, five pork live samples, obtained from local markets, were analyzed by the MIMC-2D-LC system.

3. Results and discussion

3.1. Preparation of imprinted monolith

In order to obtain a molecule imprinted column which can be applied to the two-dimensional chromatographic analysis system, the performance of packed columns and monolithic columns was studied. Since it can provide a better permeability and separating capacity, the monolithic columns was selected. Relevant literature shows that, porogenic solvent is an important factor which affects the molecular imprinted monolith’s properties \((Luo et al., 2011)\). Primarily, the porogenic solvent affects the strength of non-covalent interactions between functional monomers and templates. Secondly, the porogenic solvent can generate large pores to assure good flow-through properties. Therefore, a mixed solvent of toluene and dodecanol was chosen as porogenic solvent. In the mixed solvent system, the pore size of imprinted polymer was directly influenced by the content of toluene, and finally, 18% \((\nu/v)\) of toluene was selected. In addition, in order to obtain the best adsorption performance, the amounts of template and crosslinker were optimized. Ultimately, the molar ratio of template-monomer was chosen as 1:4 and the volume ratio of monomer-crosslinker was selected as 15:85.

3.2. The adsorption properties of the molecule imprinted monolithic columns

3.2.1. Frontal analysis

The representative breakthrough curve of frontal analysis is shown in Fig. S3. Following the increase of the analysis concentration, the equilibrium time became shorter and the slope of breakthrough curves became larger. The saturated adsorption of the drugs \((m_{\text{app}})\) were obtained from the breakthrough curves \((Lei et al., 2007)\). In accordance with Eq. (2), linear relationships between \(1/C\) and \(1/m_{\text{app}}\) were obtained, with correlation coefficients from 0.9838 to 0.9986 \((Fig. S4)\), suggesting that the adsorption of clenbuterol was attributed to the Langmuir isotherm, and clenbuterol was binding to a single type of site on the imprinted column \((Ma et al., 2015; Sobansky & Hage, 2012)\).

Fig. 1a and d showed that the amount of drug adsorbed at saturation and the binding constants between imprinted stationary phase and clenbuterol significant increased with the increase of pH. The possible reason was that, the surface charge of the drugs and the dissociation condition of the carboxyl group on the surface of binding sites were significant changed after pH increase. The results demonstrated that, the implanted materials were well prepared and the specific recognition process was induced by hydrogen bonds. Fig. 1b and e also showed that the hydrophobic force had an influence on the recognition process. When the content of acetonitrile in the mobile phase at a low level, non-specificity hydrophobic force occupied a dominant position in the adsorption process. With the increase of water in mobile phase, hydrogen bond force was strengthened gradually. The results shown in Fig. 1c and f indicated that, the column temperature also had an influence on the recognition, though the influence was slight \((Matsuda, Li, Zheng, & Hage, 2015)\). These results are consistent with the previous discovery \((Luo et al., 2011)\).

3.2.2. Thermodynamics study

For a single type of site binding system, the enthalpy change \(\Delta H\), entropy change \(\Delta S\) and Gibbs free energy \(\Delta G\) can be calculate in accordance with the equations as follows:

\[
\ln K = -\Delta H/(RT) + \Delta S/R
\]

\[
\Delta G = \Delta H - T\Delta S
\]
By applying Eqs. (2) and (3) to the data in Fig. 1f, $D_H$, $D_S$ and $D_G$ in the adsorption of the imprinted stationary phase were calculated to be $3.08 \text{ kJ mol}^{-1}$, $158.16 \text{ J mol}^{-1} \text{ K}^{-1}$ and $-44.86 \text{ kJ mol}^{-1}$ respectively, on $30^\circ \text{C}$. It was found that $D_H$ was greater than zero, which indicated the increase of entropy was an important process promoting the interactions between drugs and the recognition sites.

Fig. 1. The active binding sites number and the binding constants of different conditions ($n = 3$). (a) The active binding sites number of different pH; (b) The active binding sites number of different amount of organic phase; (c) The active binding sites number of different temperature; (d) The binding constants of different pH; (e) The binding constants of different amount of organic phase; (f) The binding constants of different temperature.

3.2.3. Selectivity experiments

The specific recognition properties were evaluated by the template molecule and seven structural analogues. The imprinted factors for clenbuterol, ractopamine, salbutamol, terbutaline sulfate, isoprenaline hydrochloride, noradrenaline bitartrate, ambroxol hydrochloride and dopamine were 33.20, 8.85, 1.79, 2.66, 3.40, 44.86 kJ mol$^{-1}$. It was found that $D_H$ was greater than zero, which indicated the increase of entropy was an important process promoting the interactions between drugs and the recognition sites.
0.75, 1.04 and 0.39, respectively (Fig. 2a). The results indicated that the obtained molecular imprinted column had a high selectivity for clenbuterol and a good cross-recognition for ractopamine. Meanwhile, the molecular imprinted column had a poorer affinity for other analogues. The possible reason is that, in the binding process, many specific recognition sites matched with the template are generated in the polymers. Although the ractopamine has a larger molecular dimension than others compounds, it possesses similar chemical groups with the template molecule. For other analogues, due to the slight differences in the chemical structure, the matching degree with the recognition site was not very well. Therefore, the imprinted factors of imprinted materials for the other six analogues were lower than those for clenbuterol and ractopamine. The results fully confirmed that the imprinted material has a great specificity.

3.3. The physical and chemical properties of the molecule imprinted monolithic columns

3.3.1. Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) spectra were used to characterize the functionality of the imprinted stationary phase. As shown in Fig. S5, the MIPs + Cl (curve 3) indicated the polymer which the template was not removed. The peaks at 3500 cm\(^{-1}\) and 1630 cm\(^{-1}\) on all the curves are characteristic of \(\text{OH}\) vibrations of hydroxyl groups. The absorption peak observed
at 1725 cm$^{-1}$, 1650 cm$^{-1}$ and 2950 cm$^{-1}$ were attributed to the stretching vibration of C=O, C=C and C–H, respectively. Compared with other polymers, the peak at 1395 cm$^{-1}$ and 3132 cm$^{-1}$ were newly found on the curve of MIPs + Cl, which were ascribed to the bending mode of $\equiv$C(CH$_3$)$\equiv$ bond and the stretching vibration of $\equiv$NH$_2$ of clenbuterol. In conclusion, we successfully prepared the MIPs using developed method.

3.3.2. Nitrogen gas adsorption and desorption analysis
Surface area and porosity were determined by nitrogen gas adsorption and desorption analysis using an Autochem 2920 analyzer (Micromeritics, USA). The BET surface area, the Langmuir surface area, the BJH adsorption and desorption areas, the external surface area, and the total pore volume were all calculated from the nitrogen gas sorption isotherms (Fig. S6a), and the result showed that these parameters of MIPs were higher than NIPs. The isotherms of MIPs and NIPs show some hysteresis, indicating that the monoliths were porous materials (Sun, Zhao, Wang, Huang, & Liu, 2014). The pore diameter distribution plots of the MIPs and NIPs in Fig. S6b showed a peak at a pore diameter of 16 nm and 17 nm, respectively. The parameters of the mesopore structure are shown in Table S1. Compared MIPs with NIPs, the parameters of MIPs were all higher than those of the NIPs, demonstrating that the MIPs had more mesopores than the NIPs. The differences in mesoporosity between them might be due to the variation of the polymerization in the presence the template (Kempe, Parareda Pujolràs, & Kempe, 2014).

3.4. Two-dimensional chromatographic system

3.4.1. Optimization of the chromatographic conditions
In general, the separation mechanism of the columns which were used to construct the 2D-LC system, should be orthogonal. For the molecule-imprinted column, the separation mechanisms included hydrogen bonds, electrostatic attraction, hydrophobic interaction, dipole force, π-π interaction, etc. Reversed-phase chromatography is mainly based on hydrophobic to separate. Two chromatographic columns’ separation patterns should own certain orthogonality, which can be used to build a 2D-LC system. Column switching interface is an important factor influencing the effects of separation. When using sample injection loop as a column-switching interface, the 2D signal was extremely weak due to the limited amount of the sample, which was transferred into the second dimensional system. When using a large volume sample loop, the 2D chromatographic peak was distorted and broadened. Therefore, a C18 pre-column was needed as the enriching column.

Compatibility of 2D-LC mobile phase is an important factor which can influence the separation effects. Since the interactions between the imprinted columns and the drugs mainly includes the hydrogen bond, the electrostatic attraction and the ionic bond. The pH has a great influence on the capacity factor. The retention behavior of the target molecules on the reversed-phase HPLC is closely related to the amount of organic phase. Therefore, by adjusting the pH and the organic phase ratio in mobile phase, the adsorption and desorption of the target molecules on the enriching column can be realized. Finally, acetonitrile-0.5% formic acid (15:85, v/v) was chosen as the first dimensional chromatographic mobile phase; methanol-0.1% formic acid (60:40, v/v) was chosen as the second dimensional chromatographic mobile phase; the flow rate was set as 0.8 mL min$^{-1}$; the column oven was set to 30 °C and the detection was monitored at 213 nm for clenbuterol.

3.4.2. Method validation
For the method validation, the linearity, precision, accuracy, LOD and LOQ were investigated at three QC levels. Representative chromatograms of spiked pork liver and urine samples on 2D-LC and the routine HPLC are compared in Figs. 3 and 4. It can be seen that, the endogenous impurity showed a certain disturbance to the determination of clenbuterol when using C18 column alone. On the contrary, the disturbance of endogenous impurity could be avoided by using the 2D-LC system. The result indicated that the

![Fig. 3. 2-Dimensional chromatogram of clenbuterol in pork liver samples. (a) The first dimension chromatogram of clenbuterol; (b) The chromatogram using C18 column alone; (c) The second dimension chromatogram of clenbuterol; Peak identifications: Rf. No retention components on the molecular imprinted column, Rf. Retention components on the molecular imprinted column.](image-url)
established method could be used to detect clenbuterol in pork liver and urine samples.

The calibration curve was constructed by using the areas of the chromatographic peaks measured at ten spiked levels. The linear regression equation obtained in the range of 0.1–12.5 μg mL\(^{-1}\) was \(y = 1004.13x + 3651.04\), with a correlation coefficient of 0.9980 for the pork liver samples. Besides, the linear equation for swine urine was \(y = 3723.24x + 1240.86\), with a correlation coefficient of 0.9995, in the range of 0.075–10 μg mL\(^{-1}\). The LODs and LOQs based on S/N = 3:1 and S/N = 10:1 were 16 ng mL\(^{-1}\) and 50 ng mL\(^{-1}\) for pork liver samples, and were 25 ng mL\(^{-1}\) and 75 ng mL\(^{-1}\) for swine urine samples, respectively. Method accuracy and precision were established at three concentration levels of QC samples (0.1, 1 and 10 μg mL\(^{-1}\)). The intra-day precision was evaluated by six repeated injections of each spiked standard. Similarly, the inter-day precision was examined by performing the assays on six consecutive days. The estimated results are shown in Table 1. The constructed MIMC-2D-LC system was continuously used for a month to analysis the biological samples (about 200 injection), then, the separation efficiency and the back pressure of the whole system had no significant change, which provided a good durability for the MIMC-2D-LC system.

3.5. Practical samples analysis

In order to verify the applicability of the method, mice livers were prepared and detected after gavage in with 20 times of the clinical dosage. Then, the clenbuterol was found at the concentration of 0.43 μg mL\(^{-1}\). However, due to a long half-time, the residues may more serious when illegal use clenbuterol as growth stimulants in stockbreeding. With the purpose of verifying the validity of the clenbuterol illegal used has been controlled, the MIMC-2D-LC method was applied to analyze five pork liver samples obtained from different local markets. The results showed that clenbuterol was not detected in all samples, which indicated that the abuse of clenbuterol was effectively controlled in local livestock.

<table>
<thead>
<tr>
<th></th>
<th>Pork liver</th>
<th></th>
<th>Swine urine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 μg mL(^{-1})</td>
<td>1 μg mL(^{-1})</td>
<td>10 μg mL(^{-1})</td>
<td>0.1 μg mL(^{-1})</td>
</tr>
<tr>
<td><strong>Accuracy (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-day</td>
<td>96.3</td>
<td>98.1</td>
<td>99.1</td>
<td>97.0</td>
</tr>
<tr>
<td>Inter-day</td>
<td>94.3</td>
<td>99.7</td>
<td>98.3</td>
<td>96.5</td>
</tr>
<tr>
<td><strong>Precision (RSD%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-day</td>
<td>6.6</td>
<td>4.5</td>
<td>4.9</td>
<td>7.5</td>
</tr>
<tr>
<td>Inter-day</td>
<td>5.6</td>
<td>5.2</td>
<td>4.6</td>
<td>8.6</td>
</tr>
</tbody>
</table>

Table 1
Results of accuracy and precision (n = 6).
3.6. Comparison study

The MIMC-2D-LC method was compared with the reported methods for the detections of clenbuterol. The results are presented in Table 2. The results demonstrated that the established MIMC-2D-LC method has either similar or better sensitivity for the detection of clenbuterol, and it also provides a new method for the determination of other β2-agonists in different complex samples.

4. Conclusions

In summary, the clenbuterol molecularly imprinted columns were synthesized by in-situ polymerization. Fourier transform infrared spectroscopy and nitrogen gas adsorption and desorption analysis were used to characterize the properties of the imprinted monolithic stationary phase. Besides, frontal chromatographic and thermodynamics study were involved to discuss the mechanism of the recognition. The results demonstrated that the imprinted columns had a high specificity. A novel and reliable MIMC-2D-LC method was developed for the determination of clenbuterol in pork liver and swine urine samples. Compared with routine detection methods, this method has better selectivity and specificity, which can efficiently avoid the disturbance of endogenous impurities. It also exhibits good recoveries and reproducibility, to meet the requirements for the analysis of trace amount of clenbuterol. Additionally, our established method is a promising approach for the analysis of other drug residues in biological matrix.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.09.021.

Table 2
Comparison of different methods for clenbuterol determination.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample</th>
<th>LOD (ng ml⁻¹)</th>
<th>Recovery (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPE-GC–MS</td>
<td>Feed</td>
<td>2.4</td>
<td>70.8–92.0</td>
<td>Zhao, Jin, Chen, Li and Yu (2011)</td>
</tr>
<tr>
<td>SPE-HPLC</td>
<td>Liver</td>
<td>10</td>
<td>91.1–113.1</td>
<td>Luo et al. (2011)</td>
</tr>
<tr>
<td>SPE-Quatum dots-UV</td>
<td>Chicken</td>
<td>12</td>
<td>92.0–99.1</td>
<td>Qiao and Du (2013)</td>
</tr>
<tr>
<td>SPE-HPLC</td>
<td>Milk</td>
<td>120</td>
<td>92.0–97.0</td>
<td>The Huy, Seo, Zhang, and Lee (2014)</td>
</tr>
<tr>
<td>SPE-HPLC</td>
<td>Pork</td>
<td>120</td>
<td>74.1–107.8</td>
<td>Tang et al. (2016)</td>
</tr>
<tr>
<td>MIMC-2D-LC</td>
<td>Pork liver</td>
<td>16</td>
<td>94.3–95.7</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>Swine urine</td>
<td>50</td>
<td>93.7–99.6</td>
<td></td>
</tr>
</tbody>
</table>

References


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