Talanta 129 (2014) 431–437

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Sulfonated polystyrene magnetic nanobeads coupled with immunochromatographic strip for clenbuterol determination in pork muscle

Kesheng Wu [a,b,1], Liang Guo [a,1], Wei Xu [a], Hengyi Xu [a], Zoraida P. Aguilar [c], Guomao Xu [b], Weihua Lai [a], Yonghua Xiong [a,*], Yiqun Wan [a]

[a] State Key Laboratory of Food Science and Technology, Nanchang University, 235 Nanjing East Road, Nanchang 330047, PR China
[b] Jiangxi Institute of Veterinary Drug and Feedstuff control, Nanchang 330047, PR China
[c] Zystein, LLC, Fayetteville AR72704, USA

ARTICLE INFO

Article history:
Received 2 April 2014
Received in revised form
30 May 2014
Accepted 8 June 2014
Available online 14 June 2014

Keywords:
Clenbuterol
Immunochromatographic strip
Magnetic solid phase extraction
Magnetic sulfonated polystyrene nanobeads

ABSTRACT

A magnetic solid-phase extraction method (MSPE) was developed to pre-concentrate and cleanup clenbuterol (CLE) from pork muscle. Novel sulfonated polystyrene magnetic nanobeads (spMNBs) were synthesized via a one-pot emulsion copolymerization method by using divinylbenzene, styrene, and sodium styrene sulfonate in the presence of oleic acid-modified and 10-undecylenic acid-modified magnetic ferrofluid. The resulting spMNBs exhibited high adsorption efficiency for CLE and for 10 other common beta-adrenergic agonists, namely, brombuterol, ractopamine, tulobuterol, bambuterol, cimbuterol, mabuterol, clorprenaline, penbutolol, salbutamol, and cimaterol. The adsorption behavior of the spMNBs for CLE was described by the Langmuir equation with a maximum adsorption capacity of 0.41 mg/g. Under the optimized parameters, the extraction of CLE from 0.5 g of pork muscle required 25 mg of the spMNBs at a shortened adsorption time (0.5 min). The proposed MSPE was coupled with colloidal gold nanoparticle-based immunochromatographic assay (MSPE–AuNPIA) for the quantitative detection of CLE residue in pork muscle. The limit of detection and limit of quantification for the pork muscle were 0.10 and 0.24 ng/g, respectively. The intra-day and inter-day assay recoveries at three CLE spiked concentrations ranged from 92.5% to 98.1%, with relative standard deviations ranging from 3.2% to 13.0%. The results of MSPE–AuNPIA were confirmed by liquid chromatography-tandem mass spectrometry (LC–MS/MS). The CLE values obtained with MSPE–AuNPIA agreed with those obtained with LC–MS/MS.

1. Introduction

Clenbuterol (CLE), a representative beta-adrenergic drug, has been misused as a nutrient repartitioning agent in the livestock industry in the past decades [1,2]. China, the United States, and most European countries have prohibited the use of CLE as a feed additive [3]. Various methods have been developed to detect CLE. These methods include liquid chromatography coupled with mass spectrometry (LC–MS) [4,5], gas chromatography coupled with MS [6], capillary electrophoresis with electrochemical detection [7], enzyme-linked immunosorbent assay (ELISA) [8], colloidal gold nanoparticle immunochromatographic assay (AuNPIA) [9,10], surface plasmon resonance [11], and surface-enhanced Raman scatter immunoassay [12]. Among these methods, AuNPIA is the most popular because of its simple operation, low cost, and rapidity (within 10 min); it has been successfully used to monitor CLE residue in swine urine. For the detection of tissue matrix via AuNPIA, sample pretreatments are necessary to remove matrix interference and increase CLE concentration. Classical methods for the pre-concentration and cleanup of CLE in tissue samples include extraction with perchloric acid solution, liquid–liquid extraction with isopropyl alcohol/ethyl acetate, rotary evaporator, and solid-phase extraction [4,7]. Currently, a few commercial solid-phase extraction cartridges with different surface functional groups have been widely used in CLE sample preparation. Examples of these cartridges include reversed-phase [13], strong cation-exchange [14], and mixed-mode cation-exchange [15] cartridges. Mixed-mode cartridges show higher sensitivity, better loading capacity, and better efficiencies than single-mode stationary-phase cartridges in CLE sample pretreatment because of the presence of interactions that contribute to analyte retention [16]. However, these traditional pretreatment methods cannot satisfy the demand of rapid screening analysis because of their

* Corresponding author. Tel.: +0086 791 833 4578; fax: +0086 791 833 3708.
E-mail address: yhxiongchen@163.com (Y. Xiong).
[1] Authors with equal contributions.

http://dx.doi.org/10.1016/j.talanta.2014.06.007
0039-9140/

complicated operation, large amounts of organic pollution, and time-consuming process [17–19].

Magnetic solid-phase extraction (MSPE) based on superparamagnetic submicro-materials exhibits numerous advantages, such as fast adsorption kinetics, high adsorption/extraction efficiency, low magnetic absorbents consumption, high enrichment factor, convenient withdrawal with magnetic separator, and suitability for rapid screening operation [20–24]. Various magnetic materials that contain different surface-functionalized chemical groups have been successfully used to absorb metal ions from polluted water [25–27], phenolic compounds from environmental samples [28,29], melamine from egg samples [30], sulfonamides and tetracyclines from milk samples [31], and trace amounts of leuco-malachite green from fish tissue [32]. However, MSPE has yet to be used to extract CLE from tissue samples.

In recent years, many portable reader-based quantitative strip assays have been developed for food safety monitoring of *Brucella* [33], deoxynivalenol [34,35], fumonisins [36], enrofloxacin [37,38], etc. The ratio of the optical density (OD) of the test line (A_T) to that of the control line (A_C) is used to normalize the effects of operation temperature, immunoreaction time, matrix interference, and strip-to-strip variability [36,39]. Considering these concepts, we have previously developed a quantitative strip for the rapid detection of CLE in swine urine [40]. In the present study, novel sulfonated polystyrene magnetic nanobeads (spMNBs) that contain sulfonic acid and benzene ring groups (synthesized using a one-pot mini-emulsion copolymerization method) were used to pre-concentrate and cleanup CLE from meat samples. This spMNB-based extraction/pre-concentration method for CLE was combined with AuNPIA to develop a convenient quantitative MSPE–AuNPIA strip test for the sensitive and rapid detection of CLE residue in pork muscle. The performance of MSPE–AuNPIA for the quantitative detection of CLE was optimized in terms of sensitivity, reproducibility, accuracy, reliability, and rapidity. The results obtained with MSPE–AuNPIA for CLE detection were compared with those obtained with LC–MS/MS to confirm the accuracy of this new method.

2. Experimental

2.1. Materials and reagents

Oleic acid (OA), 10-undecylenic acid (UA), divinylbenzene (DVB), styrene, sodium p-styrenesulfonate (NaSS), potassium persulfate (KPS), CLE, ractopamine, mabuterol, salbutamol, cimbuterol, brombuterol, cimaterol, bambuterol, clorprenaline, penbutolol, and tulobuterol were purchased from Sigma-Aldrich (St. Louis, MO, USA). CLE–D_9 was purchased from Dr. Ehrenstorfer (Augsburg, Germany). The ELISA kit for CLE and CLE–bovine serum albumin conjugates (CLE–BSA; molar ratio of 15:1) were purchased from Wuxi Zodoboer Biotech Co., Ltd. (Wuxi, China). The BioDot XYZ platform combined with a motion controller, BioJet Quanti3000k dispenser, and AirJet Quanti3000k dispenser for solution dispensing were supplied by BioDot (Irvine, CA, USA). All other chemicals and reagents were analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ultrapure water (18 MΩ/cm) produced by using a Milli-Q system (Milford, MA, USA) was used in all experiments.

A CLE stock solution (1.0 mg/mL) was prepared by dissolving CLE in methanol and stored at −20 °C until use. Working standard solutions were prepared weekly using appropriate dilutions of the stock solution. Pork muscle samples that were confirmed CLE positive or CLE free by LC–MS/MS were provided by Wuxi Zodoboer Biotech. Fortified pork muscle samples were prepared as follows for the accuracy and precision analyses. Briefly, the stock solution was diluted in methanol to obtain the intermediate CLE standard solution (200 ng/mL). CLE-free pork muscle samples (1.0 g) were fortified by adding the intermediate standard solution to produce spiked samples containing 0.25, 0.5, and 1.0 μg/kg. The spiked samples were incubated in the dark for 30 min at an

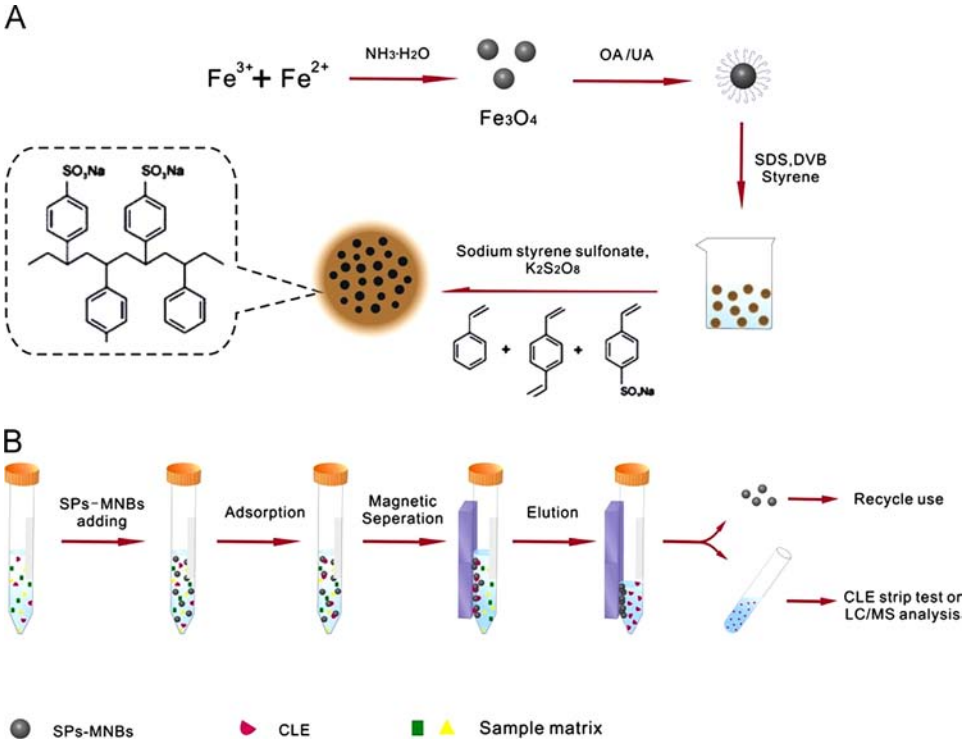

Fig. 1. Schematic for spMNB preparation and spMNB-based MSPE for CLE adsorption from pork muscle samples.

ambient temperature. All spiked samples were homogenized and stored at −20 °C and thawed at 4 °C before analysis.

2.2. Preparation and characterization of spMNBs

The schematic of spMNB preparation is shown in Fig. 1A. Magnetite (Fe_3O_4) nanoparticles (MNPs) were prepared via a modified chemical co-precipitation method [41]. Briefly, 16.0 mmol $FeSO_4 \cdot 7H_2O$ and 8.0 mmol $FeCl_3 \cdot 6H_2O$ were dissolved in 150 mL of water. The mixture was purged with N_2 for 15 min at 50 °C to remove O_2. Approximately 12.5 mL of ammonia water (25–28%) was added under vigorous stirring for 0.5 h. The MNPs were magnetically collected at the bottom of the reaction vessel. The MNPs were washed five times with water and ultrasonically dispersed in 100 mL of water. OA (1.2 mL) and UA (0.4 mL) were added, and the mixture was stirred for 3 h at 70 °C under N_2. The OA- and UA-modified MNPs precipitated at the bottom of the flask, were washed with ethanol for five times, and then dried with N_2. The modified MNPs (1.0 g) were mixed with 1.4 g of styrene and 0.28 g of DVB and then sonicated for 5 min. Water (90 mL) containing 0.207 g of sodium dodecyl sulfate was added, and the mixture was ultrasonicated for 20 min. The mixture was treated with an ultrasonic cell disruptor at 280 W for 20 min to produce a mini-emulsion, to which 0.48 g of NaSS and 0.06 g of KPS were added. The mixture reacted at 70 °C for 24 h under vigorous stirring. The resulting spMNBs were separated using a magnet and rinsed sequentially with 0.1 M HCl, 0.1 M NaOH, and ultrapure water.

The average size and morphology of the spMNBs were determined using JEM-2100 transmission electron microscopy (JEOL, Japan). Fourier-transform infrared (FTIR) spectra were obtained over the range of 400–4000 cm^{-1} using a Nicolet 5700 FTIR spectrometer (Thermo Fisher Scientific, Inc., USA). The sulfonic group density on the surface of the spMNBs was determined as previously described with some modifications [42]. Briefly, the electrode of an FE30 conductivity meter (Mettler Toledo, Shanghai, China) was immersed in 40 mL of ultrapure water containing 10 mg of spMNBs. The pH was adjusted to 10.5 using 0.1 mol/L NaOH. HCl (0.2 M) was added dropwise under gentle stirring. The titration curve was plotted using the conductivity of the solution against the titrant volume of the HCl solution. The sulfonic group density was calculated as $Ds=[(V_2-V_1)\times C]/m$, where Ds (μmol/mg) is the density of the sulfonic group on the spMNBs, V_1 and V_2 are the HCl titrant volumes at the first and second inflection points of the titration curve, respectively, C is the concentration of the HCl solution, and m is the mass of the spMNBs.

2.3. Optimization of adsorption parameters with spMNBs

The adsorption experiments were performed by adding 1.0 mg of the spMNBs to 1.0 mL of an aqueous solution containing 5.0 ng/mL CLE. The mixture was shaken vigorously with a vortex mixer to form a homogeneous solution and then incubated with a thermostat oscillator. After reaching equilibrium adsorption, the spMNBs were separated with a magnetic separator (0.4 T) for 3 min. After washing with 1.0 mL of 0.01 M phosphate-buffered saline (PBS, pH 7.0), CLE was eluted with 100 μL of methanol containing 5% ammonia solution. The eluent was dried with nitrogen and re-dissolved with 200 μL of 0.01 M PBS. The adsorption efficiency was determined using a commercial CLE ELISA kit. The absorption process was optimized by adjusting several parameters, including solution pH (2.0–11.0), NaCl concentration (0–3.0 mol/L) at pH 7.0, environmental temperature (5.0–35 °C), and adsorption time (0.5–3 min).

The adsorption isotherm of CLE on the spMNBs was studied at pH 7.0 and 25 °C under varying CLE concentrations (5.0–500 ng/mL). The equilibrium adsorption capacity was calculated as $q_e=[(C_0-C_e)\times V]/M$, where q_e (mg/g) is the equilibrium adsorption capacity, C_0 and C_e (mg/L) are the initial and equilibrium concentrations of CLE in the supernatant, respectively, V (L) is the volume of the supernatant, and M (g) is the weight of the spMNBs.

2.4. MSPE-based strip assay for the quantitative detection of CLE

2.4.1. Extraction of CLE from pork muscle using spMNBs

The magnetic separation of CLE from pork muscle using the spMNBs is illustrated in Fig. 1B. Briefly, 1.0 g of homogenized muscle sample was mixed with 5.0 mL of 0.01 M HCl using an ultrasonicator and then centrifuged at 10,000 rpm for 5.0 min. The supernatant was collected, and the pH was adjusted to 7.0. The supernatant was centrifuged at 12,000 rpm for another 5.0 min to discard the precipitate. The spMNBs (25.0 mg) were added to 3.0 mL of the extract, which is equivalent to 0.5 g of the muscle sample, and then diluted with an equal volume of ultrapure water. The mixture was incubated at room temperature for 0.5 min. Subsequently, the CLE–spMNBs were separated using an external magnetic field and washed with 5.0 mL of PBS. The CLE from the CLE–spMNBs was eluted with 3.0 mL of methanol containing 5% ammonia solution. The eluent was dried with nitrogen and then reconstituted with 200 μL of 0.01 M PBS for use in the AuNPIA strip assay.

2.4.2. Quantitative detection of CLE from pork muscle sample

The CLE strip for the quantitative test was fabricated as previously described with some modifications [20]. Colloidal gold-labeled anti-CLE mAb (AuNP–mAb) was prepared by adding 1.0 mL of anti-CLE mAb (0.8 μg/mL) to 10 mL of colloidal gold (30 nm diameter) solution. After blocking and centrifugation, the AuNP–mAb was re-suspended in 1.0 mL of PBS containing 5% sucrose, 1% BSA, and 0.5% Tween 20, and then sprayed onto a treated conjugate pad at a density of 2.5 μL/cm. CLE–BSA conjugate (0.5 mg/mL) and goat anti-mouse IgG (1.0 mg/mL) were spotted on the nitrocellulose membrane at the test (T) and control (C) lines, respectively.

For the quantitative test, an 80 μL sample eluent was added to the sample well. The ratio of the OD of A_T to that of A_C was recorded using a commercial HG-8 strip reader from Shanghai Huguo Science Instrument Co., Ltd. (Shanghai, China). The CLE concentration in the sample eluent was automatically calculated based on the built-in linear calibration curve. The calibration curve was constructed by plotting the B/B_0 signal against the logarithm of the CLE concentration, where B_0 and B represent the A_T/A_C ratios of the negative sample and a series of CLE standard solutions, respectively. The standard CLE solutions were prepared by spiking a stock solution (1.0 mg/mL) with PBS to a final concentration of 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 2.0, or 2.5 ng/mL. The standard linear regression equation was obtained by using the software that was built into a device called the “watchdog”. The “watchdog” was interfaced with the reader to store, normalize, and analyze the raw data.

2.5. Confirmation of CLE in pork muscle samples by LC–MS/MS

The performance of the developed MSPE–AuNPIA strip test was further confirmed by using a LC–MS/MS system (Agilent Corporation, MA, USA) composed of a triple-quadrupole instrument (Agilent 6410) and an LC system (Agilent 1200 series). A field-minced pork muscle sample (5.0 g) that was spiked with 100 μL of the internal standard solution with 10 ng/mL of CLE–D_9 was used in the test. The sample extraction, cleanup, and LC–MS/MS operation were performed in accordance with the national standard GB/T 22286-2008 of China (the detailed description can be found in the Supplementary Material). The LC–MS/MS system was controlled using MassHunter

software (Agilent Corporation, MA, USA). Chromatographic separation was performed with an Agilent Zorbax XDB-C18 column (50 mm × 2.1 mm, 1.8 μm) maintained at 30 °C. The mobile phase consisted of solvent *A* (0.1% formic acid in water) and solvent *B* (0.1% formic acid in acetonitrile). The gradient was as follows: 2% solvent *B* at t=0–1.5 min; a linear gradient from 2% to 80% solvent *B* at t=1.5–3 min; and then a linear gradient from 80% to 90% solvent *B* at t=3.0–5.5 min. At 5.5 min, the gradient was programmed back to the initial conditions to re-equilibrate the column for 2.0 min. The flow rate was 0.20 mL/min, and the injection volume was 10 μL in full-loop injection mode. Ionization was achieved using electrospray ionization in positive-ion mode at an ionization voltage of 4000 V. Detection was performed in selected reaction monitoring (SRM) mode to study the adsorption of CLE on sp-MNBs and in multiple reaction monitoring mode to study the co-adsorption of common β-agonists on sp-MNBs. High-purity nitrogen gas (99.99%) was used for desolvation, as well as for cone and collisions. The recorded SRM transitions were *m/z* 277/203 (quantitation ion) and 277/259 (qualitative ions) for CLE and *m/z* 286/204 (quantitative ion) for CLE–D_9.

3. Results and discussion

3.1. Synthesis and characterization of spMNBs

The spMNBs were synthesized via a one-pot mini-emulsion copolymerization method. A representative transmission electron micrograph of the spMNBs is shown in Fig. 2A. The spMNBs exhibit regular spherical morphology with an average diameter of 130 ± 28 nm. To obtain a high sulfonic group density on the surface of the spMNBs, the amounts of NaSS were optimized by adding 0.24, 0.32, 0.48, 0.64, and 0.96 g of NaSS to 90 mL of the mini-emulsion for the modified spMNBs. The sulfonic group density on the resulting spMNBs was determined by potentiometric titration using a conductivity meter to measure signal changes (Fig. 2B). The maximum sulfonic group density of 4.8 μmol/mg was reached when the NaSS dosage was 0.48 g. The FTIR spectra (Fig. 2C) verified the successful copolymerization of DVB, styrene, and NaSS on the OA- and UA-modified MNPs. Four peaks at 2925.38, 2852.17, 1425.17, and 1400 cm^{-1} in the spMNB spectra indicate the presence of aromatic skeletal vibration and C–H stretching vibration in the benzene ring [43,44]. The peak at 585.24 cm^{-1} is a typical characteristic of Fe–O stretching vibration in Fe_3O_4 [45,46]. The characteristic signals at 1034.78 and 1125.18 cm^{-1} in the spMNB and NaSS spectra correspond to the symmetric and asymmetric stretching vibrations of S=O, indicating the formation of a sulfonic group on the surface of the spMNBs [44,47].

3.2. Optimization of the MSPE procedure

Several parameters, including pH and ionic strength in the solution, adsorption time, and environmental temperature, were optimized to achieve the maximum adsorption efficiency for CLE using the spMNBs. The pH of the solution may affect the ionization of spMNBs and CLE. Theoretically, protonated CLE (p*Ka*=9.6) benefits the formation of electrostatic interactions with the sulfonic group of the spMNBs under acidic and neutral conditions (pH ≤ 7.0). However, low pH can increase the concentration of hydrogen ions, which may compete with the protonated CLE at the sulfonic binding site. The effect of pH on adsorption efficiency is illustrated in Fig. 3A. The adsorption efficiency of the spMNBs for CLE increased from 17.6% to 40.6% at pH 2.0–5.0 and then peaked to 99.2% at pH 7.0. The adsorption efficiency for CLE slightly decreased but remained higher than 90% as the pH increased from 7.0 to 11. At pH > 7, the amount of CLE molecules existing in the neutral form increased. Thus, the hydrophobic and not the electrostatic interaction between CLE and the benzene group on the spMNBs dominated the CLE adsorption. The CLE adsorption efficiency for CLE remained higher than 90% at pH 6.0–11 because of the combined effect of hydrophobic and electrostatic interactions. Compared with single-mode stationary adsorbents, the novel spMNBs demonstrated a relatively higher extraction efficiency for CLE at a wider pH range [15]. Unless stated otherwise, all succeeding experiments were conducted at pH 7.0.

The adsorption efficiency significantly decreased from 97.8% to 40.6% as the NaCl concentration was increased from 0 M to 1.0 M (Fig. 3B). The adsorption efficiency for CLE on the spMNBs increased to 93.1% as the NaCl concentration was increased to 3.5 M. Thus, NaCl concentration at the range of 0–1.0 M interfered with the electrostatic interaction between the sulfonic group and the protonated CLE. However, high NaCl concentrations (≥ 3.5 M) enhanced the hydrophobic interaction between the CLE and benzene group on the spMNBs by decreasing the availability of water, resulting in increased adsorption efficiency. To simplify the operation, the adsorption was performed without additional salt in the subsequent experiments.

The CLE adsorption performance of the spMNBs was further evaluated under different incubation times and temperatures. As shown in Fig. 3C and D, the spMNBs exhibited > 94% adsorption efficiency after incubation at 5.0–35 °C for 0.5–15 min. These results indicated that CLE exhibited rapid adsorption kinetics on the spMNBs at the given temperature range. This phenomenon could benefit the development of a rapid sensor system.

3.3. Co-adsorption of common β-agonists on spMNBs

To investigate the adsorption capacity of the spMNBs for other common β-agonists, 0.5 mg of the spMNBs were added to a mixture containing 20 ng/mL CLE, salbutamol, cimbuterol, brombuterol, cimaterol, ractopamine, mabuterol, bambuterol, clorprenaline, penbutolol, and tulobuterol over the pH range of 2.0–11.0. The remaining β-agonists in the supernatant were analyzed using LC–MS/MS. The results indicated that the spMNBs exhibited > 95% extraction efficiency for CLE, brombuterol, ractopamine, tulobuterol, bambuterol, cimbuterol, mabuterol, clorprenaline, and penbutolol at pH 6.0–9.0. In addition, 75.24% adsorption efficiency for salbutamol at pH 6.0–7.0 and 82.69% for cimaterol at pH 7.0 were achieved.

3.4. Adsorption isotherm

The equilibrium isotherm (Fig. 4) was obtained using different CLE initial concentrations (5.0–500 ng/mL) at 25 °C under the optimized adsorption conditions. The adsorption behavior could be described using the Langmuir equation: $C_e/q_e = 1/(K_L \times q_m) + C_e/q_m$, where q_m is the maximum adsorption capacity (mg/g), C_e is the equilibrium concentration of CLE (mg/L), q_e is the amount of CLE adsorbed per unit weight of spMNBs at equilibrium concentration (mg/g), and K_L is the Langmuir constant related to the affinity of binding sites (L/mg). A linear curve of $C_e/q_e = 2.426\ C_e + 0.0192$ was obtained by plotting C_e against C_e/q_e (R^2=0.993). The constants q_m and K_L were 0.41 mg/g and 148 L/mg, respectively. The fact that the Langmuir isotherm fits the experimental data very well may be due to homogeneous and monolayer adsorption.

3.5. MSPE for CLE in pork muscle samples

To simplify the sample pretreatment process, the CLE in the pork muscle extract was obtained using a modified Randox ELISA kit (Randox, Co. Antrim, U.K.). CLE is an aniline-type β-agonist; thus, 5 × volume of 0.01 M HCl solution was used to obtain the minced meat extract. The extract was diluted with an equal

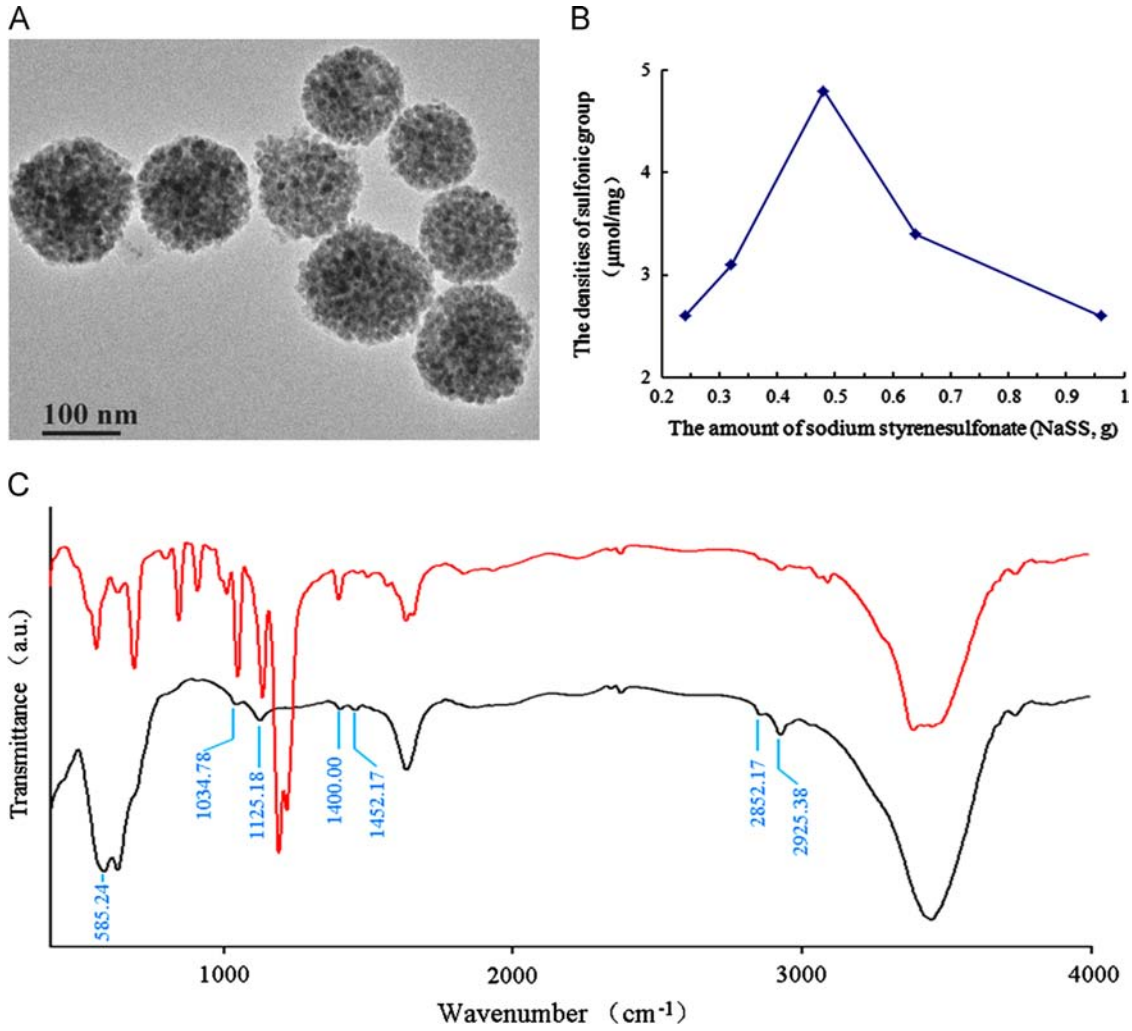

Fig. 2. Characterization of spMNBs: (A) transmission electron micrograph of spMNBs, (B) the effect of the dosage of sodium styrenesulfonate on the sulfonic group density on the spMNBs, and (C) FTIR spectrum of sodium styrenesulfonate (red) and spMNBs (black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

volume of ultrapure water to dilute polar substances, including free amino acid, polypeptides, and inorganic salt. In the MSPE of spMNBs (5–35 mg) from muscle samples (0.5 g), 25 mg of the spMNBs were necessary to ensure high adsorption efficiency ($>95\%$) in a 6.0 mL adsorption solution. The effect of five eluent solutions, including 50–100% methanol and methanol containing 5% ammonia solution, were also evaluated. Methanol was beneficial to CLE elution (see Fig. S1), whereas methanol containing 5% ammonia solution provided a better recovery (94%). This result could be attributed to the possibility that the ammonium ion could compete with the CLE on the sulfonic group, which could also improve the dispersion of magnetic adsorbents in the solution.

3.6. Analytical performance

Quantitative analysis was performed using the AuNPIA strip. The standard calibration curve was obtained by testing 11 standard solutions of CLE from 0 ng/mL to 2.5 ng/mL prepared in 0.01 M PBS. The A_T/A_C ratios between the negative control and real samples were designated B_0 and B, respectively. The logarithmic competitive inhibition curve (see Fig. S2) exhibited a linear range between 0.05 and 1.2 ng/mL CLE with a half maximal inhibitory concentration (IC_{50}) of 0.16 ± 0.02 ng/mL ($n=3$). The linear regression equation was $y=-0.221\log(x)+0.119$ ($R^2=0.995$), where y is B/B_0 and x is the CLE concentration.

The limit of detection (LOD) and limit of quantitation (LOQ) of the MSPE–AuNPIA strip assay were evaluated as previously described [48]. Twenty pork muscle sample extracts that were ascertained to be free of CLE by LC–MS/MS were prepared using spMNBs. The CLE signals from the extracts were determined with the AuNPIA strip, and then the LOD was calculated using the mean of the measured CLE response in the 20 blank samples plus threefold standard deviation. The calculated LOD value was 0.10 ng CLE/g pork muscle. The calculated LOQ value was 0.24 ng CLE/g pork muscle based on the mean plus tenfold standard deviation. The specificity of the assay was evaluated by running 10 other common structurally related β-agonists. The results showed ~1% cross-reactivity of the proposed MSPE–AuNPIA strip to mabuterol, $<0.5\%$ to salbutamol, and no cross-reaction ($<0.1\%$) to cimbuterol, brombuterol, cimaterol, ractopamine, bambuterol, clorprenaline, penbutolol, and tulobuterol.

Recovery studies were conducted to evaluate the accuracy and precision of the novel MSPE–AuNPIA strip assay using 0.25, 0.5, and 1.0 ng/mL CLE spiked in pork muscle samples (Table 1). The average intra-day assay recoveries ranged from 93.0% to 97.7%, with relative standard deviations (RSDs) ranging from 3.2% to 13.0%. The inter-day assay recoveries ranged from 92.5% to 98.1%, with RSDs ranging from 8.6% to 11.9%. To investigate the influence of different batches of spMNBs on the adsorption performance, three spMNB batches were synthesized and evaluated (Table 2).

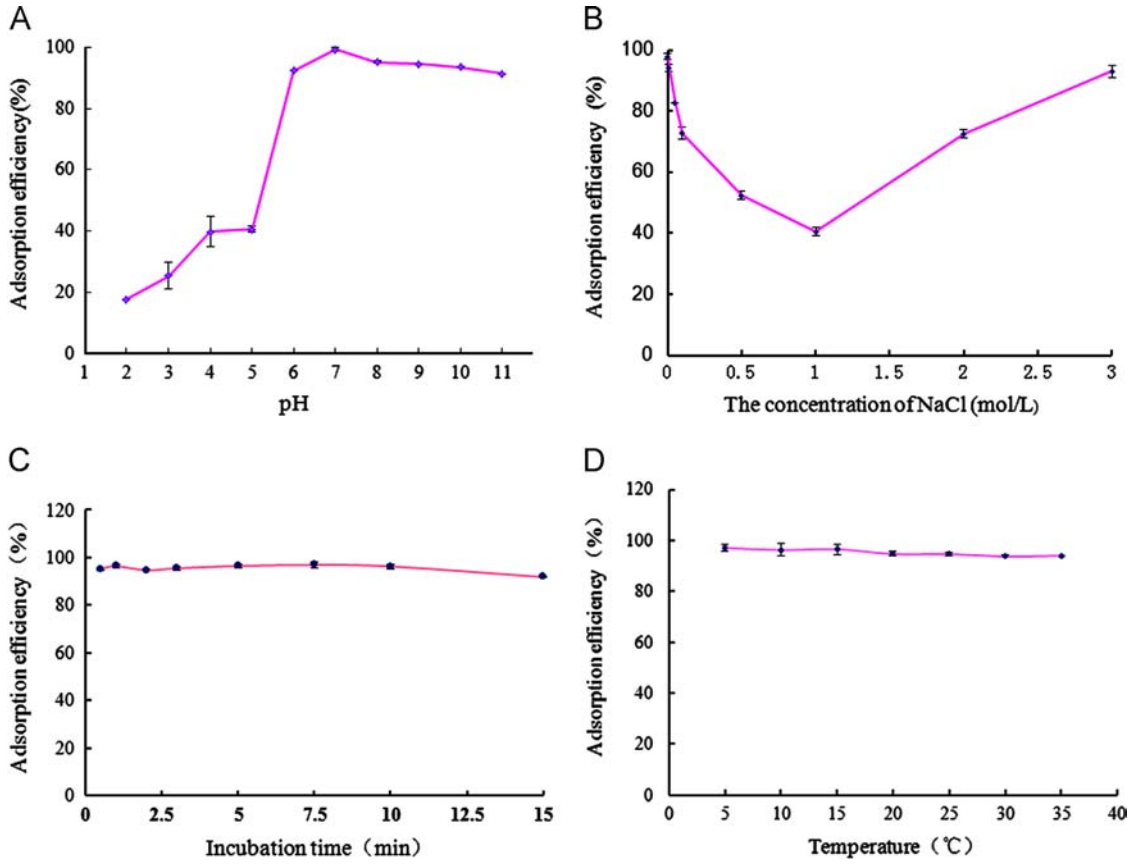

Fig. 3. Optimization of adsorption parameters of spMNBs for CLE. Effects of (A) pH; (B) ionic strength (pH 7.0); (C) incubation time; and (D) environmental temperature (pH 7.0). Error bars were based on three duplicate measurements.

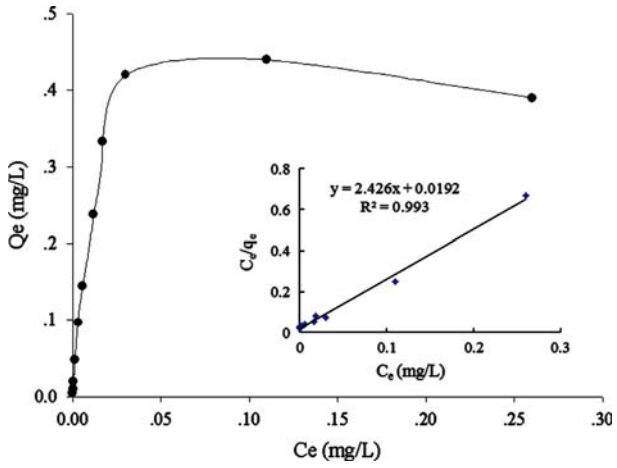

Fig. 4. Adsorption isotherm and Langmuir model simulation (inset) for the adsorption of CLE on spMNBs. (Inset) Aqueous solution (1 mL, pH 7.0) containing CLE concentration from 5.0 ng/mL to 500 ng/mL and 1 mg spMNBs.

The average recoveries ranged from 81.5% to 105%, with RSDs ranging from 5.8% to 12.8%. These results confirmed that the spMNBs in the MSPE–AuNPIA assay exhibited good reproducibility for CLE detection/cleanup.

To verify the reliability of the new quantitative spMNBs for MSPE–AuNPIA analysis, eight muscle samples were tested and further confirmed by LC–MS/MS analysis. The values (Table 3) obtained from MSPE–AuNPIA and LC−MS/MS were evaluated by regression analysis, the results show that the two methods have a good correlation (R^2=0.98, see Fig. S3).

Table 1
Accuracy and precision of MSPE–AuNPIA in CLE-spiked pork muscle samples.

CLE-spiked pork muscle sample (ng/g)	Intra-day assay		Inter-day assay	
	Recovery (%, n=3)	RSD (%)	Recovery (%, n=3)	RSD (%)
0.25	97.7	13.0	92.5	10.3
0.5	93.0	8.4	98.1	12.1
1.0	95.9	3.2	93.1	9.2

Table 2
CLE recovery and RSD of CLE with three batches of spMNBs.

CLE-spiked pork muscle sample (ng/g)	Batch 1		Batch 2		Batch 3	
	Recovery (%, n=3)	RSD (%)	Recovery (%, n=3)	RSD (%)	Recovery (%, n=3)	RSD (%)
0.25	105	2.5	98.4	3.0	81.5	6.9
0.5	87.8	7.4	89.7	6.5	99.9	11.6
1.0	95.8	3.0	85.5	1.6	92.7	6.9

4. Conclusion

AuNPIA assay has been widely used in monitoring CLE residual in swine urine. However, this rapid screening method is unsuitable in determining CLE concentration in tissue samples because of its complex sample pretreatment. To simplify the process, novel spMNBs containing sulfonic acid and benzene ring groups on the surface were used for the rapid and convenient cleanup and pre-concentration of CLE from pork muscle. The spMNB-based MSPE

Table 3
Comparison of CLE residual in real pork muscle samples between MSPE–AuNPIA and LC–MS/MS ($n=3$).

Sample number	CLE concentration (ng/g, mean ± SD)	
	MSPE–AuNPIA	LC–MS/MS method
1	0.21 ± 0.011	0.27 ± 0.013
2	2.55 ± 0.36	2.91 ± 0.15
3	0.36 ± 0.026	0.41 ± 0.021
4	0.56 ± 0.040	0.67 ± 0.026
5	2.11 ± 0.31	2.24 ± 0.14
6	1.29 ± 0.13	1.83 ± 0.11
7	0.72 ± 0.061	0.91 ± 0.034
8	1.79 ± 0.22	2.25 ± 0.18

method exhibited fast adsorption kinetics for CLE, with a high maximum adsorption capacity of 0.41 mg CLE/g spMNB. Compared with the CLE traditional pretreatment method, the novel MSPE–AuNPIA method simplified the sample preparation and reduced the extraction time from 1.5 h to 30 min. In the MSPE–AuNPIA method, approximately 40 min is needed for one pork muscle sample to obtain results with satisfactory trueness, repeatability, and intermediate precision. Therefore, the spMNB-based AuNPIA is a promising rapid and convenient screening technique for CLE detection in pork muscle samples.

Acknowledgments

This study was supported by a grant from the "Twelfth Five-Year Plan" for National Science and Technology Support Program (2012BAK17B02 and 2013BAD19B02), the National Natural Science Foundation of China (No. 31360385), the Ground Plan of Science and Technology in Jiangxi Province Department of Education (KJLD13011) and the Innovation Fund Designated for Graduate Students of Jiangxi Province (YC2013-B022).

Appendix A. Supplementary Material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.06.007.

References

[1] H.H. Meyer, L.M. Rinke, J. Anim. Sci. 69 (1991) 4538–4544.
[2] G. Re, P. Badino, A. Novelli, C. Girardi, Vet. J. 153 (1997) 63–70.
[3] G.P. Zhang, X.N. Wang, J.F. Yang, Y.Y. Yang, G.X. Xing, Q.M. Li, D. Zhao, S.J. Chai, J.Q. Guo, J. Immunol. Methods 312 (2006) 27–33.
[4] M. Thevis, T. Schebalkin, A. Thomas, W. Schänzer, Chromatographia 62 (2005) 435–439.
[5] J. Pleadin, I. Bratoš, A. Vulić, N. Perši, J. Đugum, Rev. Anal. Chem. 30 (2011) 5–9.
[6] C.A. Fente, B.I. Vázquez, C. Franco, A. Cepeda, P.G. Gigosos, J. Chromatogr. B 726 (1999) 133–139.
[7] Y. Bao, F. Yang, X.R. Yang, Electroanalysis 24 (2012) 1597–1603.
[8] S.L. Zheng, S.Q. Song, H. Lan, G.R. Qu, R.X. Li, A.B. Wu, D.B. Zhang, Anal. Lett. 42 (2009) 600–614.
[9] G.P. Zhang, X.N. Wang, J.F. Yang, Y.Y. Yang, G.X. Xing, Q.M. Li, D. Zhao, S.J. Chai, J.Q. Guo, J. Immunol. Methods 312 (2006) 27–33.
[10] M.Z. Zhang, M.Z. Wang, Z.L. Chen, J.H. Fang, M.M. Fang, J. Liu, X.P. Yu, Anal. Bioanal. Chem. 395 (2009) 2591–2599.
[11] I.M. Traynor, S.R.H. Crooks, J. Bowers, C.T. Elliott, Anal. Chim. Acta 483 (2003) 187–191.
[12] G.C. Zhu, Y.J. Hu, J. Gao, L. Zhong, Anal. Chim. Acta 697 (2011) 61–66.
[13] Y. Gaillard, A. Balland, F. Doucet, G. Pepin, J. Chromatogr. B 703 (1997) 85–95.
[14] P.A. Guy, M.C. Savoy, R.H. Stadler, J. Chromatogr. B 736 (1999) 209–219.
[15] C.H.P. Bruins, C.M. Jeronimus-Stratingh, K. Ensing, W.D. van Dongen, G.J. de Jong, J. Chromatogr. A 863 (1999) 115–122.
[16] Y. Yang, X.D. Geng, J. Chromatogr. A 1218 (2011) 8813–8825.
[17] I.S. Ibarra, J.A. Rodriguez, J.M. Miranda, M. Vega, E. Barrado, J. Chromatogr. A 1218 (2011) 2196–2202.
[18] Q. Gao, D. Luo, J. Ding, Y.Q. Feng, J. Chromatogr. A 1217 (2010) 5602–5609.
[19] L. Sun, X. Sun, X.B. Du, Y.S. Yue, L.G. Chen, H.Y. Xu, Q.L. Zeng, H. Wang, L. Ding, Anal. Chim. Acta 665 (2010) 185–192.
[20] M. Safarikova, I. Safarik, Eur. Cell Mater. 3 (2002) 192–195.
[21] Y. Liu, H.F. Li, J.M. Lin, Talanta 77 (2009) 1037–1042.
[22] Q.L. Li, M.H.W. Lam, R.S.S. Wu, B.W. Jiang, J. Chromatogr. A 1217 (2010) 1219–1226.
[23] S.X. Zhang, H.Y. Niu, Z.J. Hua, Y.Q. Cai, Y.L. Shi, J. Chromatogr. A 1217 (2010) 4757–4764.
[24] A. Mehdinia, F. Roohi, A. Jabbari, J. Chromatogr. A 1218 (2011) 4269–4274.
[25] T. Tuutijärvi, J. Lu, M. Sillanpää, G. Chen, J. Hazard. Mater. 166 (2009) 1415–1420.
[26] M. Faraji, Y. Yamini, A. Saleh, M. Rezaee, M. Ghambarian, R. Hassani, Anal. Chim. Acta 659 (2010) 172–177.
[27] M.H. Mashhadizadeh, Z. Karami, J. Hazard. Mater. 190 (2011) 1023–1029.
[28] Y.F. Sha, C.H. Deng, B.Z. Liu, J. Chromatogr. A 1198–1199 (2008) 27–33.
[29] P. Dou, L. Liang, J.G. He, Z. Liu, H.Y. Chen, J. Chromatogr. A 1216 (2009) 7558–7563.
[30] Y. Xu, L.G. Chen, H. Wang, X.P. Zhang, Q.L. Zeng, H.Y. Xu, L. Sun, Q. Zhao, L. Ding, Anal. Chim. Acta 661 (2010) 35–41.
[31] J.A. Rodriguez, J. Espinosa, K. Aguilar-Arteaga, I.S. Ibarra, J.M. Miranda, Microchim. Acta 171 (2010) 407–413.
[32] L. Guo, J.W. Zhang, H. Wei, W.H. Lai, Z.P. Aguilar, H.Y. Xu, Y.H. Xiong, Talanta 97 (2012) 336–342.
[33] Q. Qu, Z.W. Zhu, Y.F. Wang, Z.J. Zhong, J. Zhao, F. Qiao, X.Y. Du, Z.J. Wang, R. F. Yang, L.Y. Huang, Y.Q. Yu, L. Zhou, Z.L. Chen, J. Microbiol. Methods 79 (2009) 121–123.
[34] J. Liu, S. Zanardi, S. Powers, M. Suman, Food Control 26 (2012) 88–91.
[35] H.U. Aamot, I.S. Hofgaard, G. Brodal, O. Elen, M. Jestoi, S.S. Klemsdal, World Mycotoxin J. 5 (2012) 339–350.
[36] L. Anfossi, M. Calderara, C. Baggiani, C. Giovannoli, E. Arletti, G. Giraudi, Anal. Chim. Acta 682 (2010) 104–109.
[37] Y.L. Zhao, G.P. Zhang, Q.T. Liu, M. Teng, J.F. Yang, J.H. Wang, J. Agric. Food Chem. 56 (2008) 12138–12142.
[38] X.L. Huang, Z.P. Aguilar, H.M. Li, W.H. Lai, H. Wei, H.Y. Xu, Y.H. Xiong, Anal. Chem. 85 (2013) 5120–5128.
[39] Q.H. Yang, X.Q. Gong, T. Song, J.M. Yang, S.J. Zhu, Y.H. Li, Y. Cui, Y.X. Li, B. B. Zhang, J. Chang, Biosens. Bioelectron. 30 (2011) 145–150.
[40] C.H. Li, W. Luo, H.Y. Xu, Q. Zhang, H. Xu, Z.P. Aguilar, W.H. Lai, H. Wei, Y. H. Xiong, Food Control 34 (2013) 725–732.
[41] F. Yan, J. Li, R. Fu, Z.Y. Lu, W.S. Yang, J. Nanosci. Nanotechnol. 9 (2009) 5874–5879.
[42] B.L.A. Prabhavathi Devi, K.N. Gangadhar, K.L.N. Siva Kumar, K. Shiva Shanker, R.B.N. Prasad, P.S. Sai Prasad, J. Mol. Catal. A:Chem. 345 (2011) 96–100.
[43] A.A. Bhuttoa, D. Veselyb, B.J. Gabrys, Polymer 44 (2003) 6627–6631.
[44] J.C. Yang, M.J. Jablonsky, J.W. Mays, Polymer 43 (2002) 5125–5132.
[45] I.J. Bruce, T. Sen, Langmuir 21 (2005) 7029–7035.
[46] S.X. Zhang, H.Y. Niu, Z.J. Hua, Y.Q. Cai, Y.L. Shi, J. Chromatogr. A 1217 (2010) 4757–4764.
[47] S.B. Brijmohan, S. Swier, R.A. Weiss, M.T. Shaw, Ind. Eng. Chem. Res. 44 (2005) 8039–8045.
[48] J.Z. Shen, Z. Zhang, Y. Yao, W.M. Shi, Y.B. Liu, S.X. Zhang, Anal. Chim. Acta 575 (2006) 262–266.