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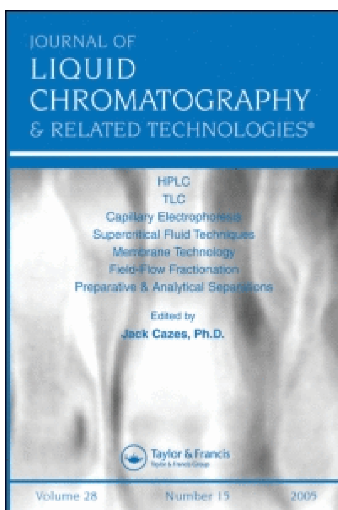

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Separation and Determination of β_2-Agonists in Swine Feed Using Field-Amplified On-Line Sample Stacking Method by Capillary Zone Electrophoresis

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Separation and Determination of β_2-Agonists in Swine Feed Using Field-Amplified On-Line Sample Stacking Method by Capillary Zone Electrophoresis

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Abstract: A rapid, simple, and reliable capillary zone electrophoresis method was developed for the separation and determination of β_2-agonists under the optimum buffer solution consisting of 50 mM H_3BO_3-$Na_2B_4O_7$ and 30 mM NaCl (pH 7.40). The field amplified sample stacking (FASS) technique was applied to the on-line concentration of β_2-agonists cimaterol, clenbuterol, and salbutamol. The data presented in this work indicate that the sensitivity could be improved significantly by using a short water plug in FASS. Under the optimum conditions, cimaterol, clenbuterol, and salbutamol were separated completely and determined with the correlation coefficient of $0.9996 \sim 0.9999$. The relative standard deviations (RSD) were less than 2.85% (run to run) and 3.10% (day to day) for peak area. The detection ranges ($S/N = 3$) were located from 0.01 to 1 μg/mL for each β_2-agonists. With this stacking measure, cimaterol, clenbuterol, and salbutamol in spiked swine feed were successfully determined with the recovery more than 95%.

Keywords: β_2-Agonists, Capillary zone electrophoresis, Cimaterol, Clenbuterol, Field amplified sample stacking, Salbutamol, Swine feed

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INTRODUCTION

There is a considerable interest in the determination and control of additives abused in animal feed, such as clenbuterol, cimaterol, and salbutamol, which are β_2-agonists originally used to treat the pulmonary diseases in veterinary and human asthma.[1–3] Since β_2-agonists may improve lean meat deposition and promote production efficiency, they are abused as feed additive for growth promotion of livestock in meat production and this can result in human food poisoning.[4,5] Thus, the determination of abused additive in livestock feed is quite important to the public health. The methods for routine determination of additive in livestock feed are required commonly to have be reliable, rapid, and convenient. The capillary electrophoresis technique can match the above requirements.

It is well known that capillary electrophoresis (CE) has many advantages such as highly efficient separation, simple operation, rapid detection, very low consumption of background electrolytes and samples. This is why the application of CE to the determination of β_2-agonists has made a long progress in the past decades.[6–11] However, the most commercial CE equipment equipped with a UV/vis detector has a drawback that limited the detection sensitivity due to a short detection light path. Generally, changing the component of the background electrolyte can promote the resolution and/or separation efficiency; using a sample concentrating technique can also improve the detection sensitivity. In order to broaden the CE-UV application in trace analysis, a number of practical techniques have been set up to concentrate analytes on-line. For example, Quirino1 et al. studied a concentration mechanism of sweeping and applied the method in high sensitivity analysis in capillary electrophoresis.[12] Smyth et al. detected drugs, dyes, and metal chelates by large volume sample stacking (LVSS).[13] Liu et al. finished an analysis of alkaloids in Sophora flavescens Ait by the field amplified sample stacking method.[14] Shi et al. used the field amplified sample injection technique to separate and determine the β_2-agonists in urine sample and to acquire a satisfactory result.[15] After surveying the literatures, it is worthwhile noticing that the sensitivity of the same method for determining β_2-agonists varies with a different sample. That is, for determining the β_2-agonists in swine feed the new conditions must be reselected again.

In the present paper, a rapid, simple, and reliable on-line sample stacking method, field amplified sample stacking (FASS), was described for separation and determination of cimaterol, clenbuterol, and salbutamol in swine feed. A common running buffer consisting of borate-boric acid and sodium chloride was adopted as background electrolyte (BGE) to improve separation of three β_2-agonists. FASS was based on the conductivity difference between the sample solution and background electrolyte and it could obviously improve detector response. Compared

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with conventional CE,[16] the enhancement factors were found to be more than 10. The proposed method has been used for determination of β_2-agonists in spiked swine feed successfully.

EXPERIMENTAL

Apparatus

Separation was performed on a P/ACE 5510 apparatus (Beckman Instruments, USA) equipped with a UV detector and wavelength filters (190, 200, 214, 254, and 280 nm). A fused silica capillary (total length 57 cm, 75 μm i.d.; Beckman Coulter, USA) was used. The distance from the point of injection to the detection window was 50 cm. System Gold software was used for data acquisition. Absorbance was recorded at 214 nm. A CQ-250 ultrasonic cleaner (Shanghai, China) was employed for sonicating degas and cleaning. Samples were centrifuged on an Allegra-64 R centrifuge (Beckman, USA). Polyethylene membranes (0.45 μm) were used for filtering (Shanghai Xinya Purified Appliances Factory, China). In addition, a mode 211 pH-meter (Hanna, Italy) was used to adjust the pH of solutions.

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Reagents and Solutions

All reagents used were of analytical reagent grade. Clenbuterol and salbutamol were purchased from Sigma-Aldrich Co. (Augsburg, Germany); Cimaterol was obtained from Toric (Ellisvile, MO, USA); Acetone, sodium borate, and sodium chloride were purchased from Shanghai Chemical Reagents Co. (Shanghai, China); Boric acid was provided by Beijing Chemical Reagents Co. (Beijing, China). Swine feed was purchased from the local animal feed market.

Ultra-pure water was made from a Milli-Q water system (Millipore, Germany) and was used in the preparation of all solutions. Stock standard solutions (300 μg/mL) of cimaterol, clenbuterol, and salbutamol were prepared with ultra-pure water, placed in the icebox under +2°C. They were diluted with 1/100 running buffer solution, which served as BGE to the desired concentration before the use. The running buffer were prepared fresh daily.

Procedures

At the beginning and the end of each experimental step, the capillary was rinsed with 0.1 mol/L NaOH and ultra-pure water for 5 min separately,

followed by the BGE for 10 min. It was rinsed with ultra-pure water and BGE for 5 min, respectively, between runs, so as to improve the repeatability of the electroosmotic flow and migration time. All solutions of BGE and samples were filtered through a 0.45 μm polyethylene membrane filter and degassed by ultrasonication before determination. The separation temperature of 25°C and the applied separation voltage of 20 kV were adopted in this study, respectively. Electrokinetic injection was performed from the positive end of the capillary.

Samples

Swine feed of 5.00 g was accurately weighed and accurate amounts of standard cimaterol, clenbuterol, and salbutamol were separately added into the feed sample. The spiked sample was mixed with 50 mL of acetone with ultrasonic vibration for 30 min. It was taken out and shaken for 2 min. The extract was centrifuged for 10 min at 4000 rpm. Then 5.0 mL of the clear upper liquid was pipetted and evaporated to dryness under nitrogen and then dissolved with 2.0 mL of 1/100 running buffer solution. The solutions could be directly injected after filtering through a 0.45 μm membrane.

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RESULTS AND DISCUSSION

Effect of the Running Buffer

A series of buffer solution, including sodium dihydrogen phosphate-disodium hydrogen phosphate, sodium borate-boric acid, sodium citric acid-citric acid, and sodium acetic-acetic acid were tested for their effects on the separation of cimaterol, clenbuterol, and salbutamol in the pre-experimental. The results showed that the analytes could be separated completely only in buffer solution consisting of sodium borate-boric acid and sodium chloride. Therefore, H_3BO_3-NaCl-$Na_2B_4O_7$ buffer solution[17] was chosen as the running buffer.

The effect of pH on separation was investigated using 50 mM H_3BO_3-NaCl-$Na_2B_4O_7$ buffer solution from pH 6.40 to 8.00. As shown in Figure 1, the migration time of the analytes decreased with increasing pH from 6.40 to 7.00. In the range of pH 7.20 to 7.60, the migration time of the analytes were kept almost a constant and satisfactory separation was achieved. Then, the migration times of the analytes increased slightly. Hence, pH 7.40 of H_3BO_3-NaCl-$Na_2B_4O_7$ solution was considered as a running buffer.

The effect of the buffer concentration on the separation was examined in the range of 40–100 mM H_3BO_3-$Na_2B_4O_7$ at pH 7.40. It was

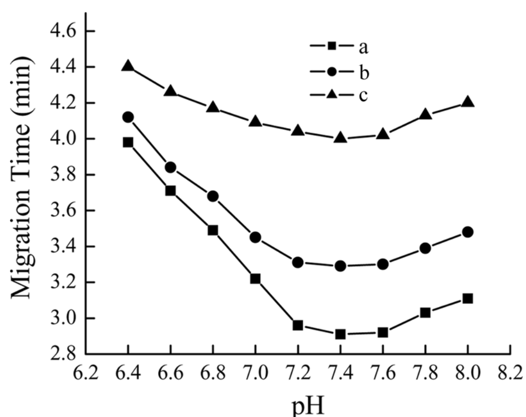

Figure 1. Effect of running buffer pH on the separation of analytes. CE conditions: Fused-silica capillary (75 μm I.D. × 57 cm); UV detection wavelength: 214 nm; concentration of running buffer: 50 mM H_3BO_3-$Na_2B_4O_7$ and 30 mM NaCl; separation voltage: 20 KV; electrokinetic injection with 10 KV for 13 s after preliminary pressure injection of water for 4 s; capillary temperate: 25°C. Peak identification and concentration of each analyte: (a) 0.2 μg/mL cimaterol; (b) 0.2 μg/mL clenbuterol; (c) 0.2 μg/mL salbutamol.

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found that the migration time of the analytes increased with the buffer increased concentration. As the ionic strength of the buffer solution increased with the concentration, the electroosmotic flow in the capillary decreased and a better resolution was observed at 50 mM of H_3BO_3-$Na_2B_4O_7$. Therefore, 50 mM of H_3BO_3-$Na_2B_4O_7$(pH 7.40) was selected.

Effect of Sodium Chloride

A variety of components in the background electrolyte can change the electroosmotic flow to improve separation selectivity; for example, Green et al.[18] reported a separation improvement of proteins by adding alkali metal salts to running buffer, due to a increase of ion strength and the adsorption of alkali on the wall of silica capillary. Figure 2 shows an investigation for the effect of NaCl on the separation of analytes. It could be seen that the separation of cimaterol and clenbuterol was improved by adding NaCl to the running buffer from 15 mM to 45 mM. The increased ion concentration decreases the electroosmotic flow and improves the separation of analytes. However, the large Joule heat would be produced and the separation time be prolonged if the ion concentration was too high, so 30 mM NaCl was used in the study.

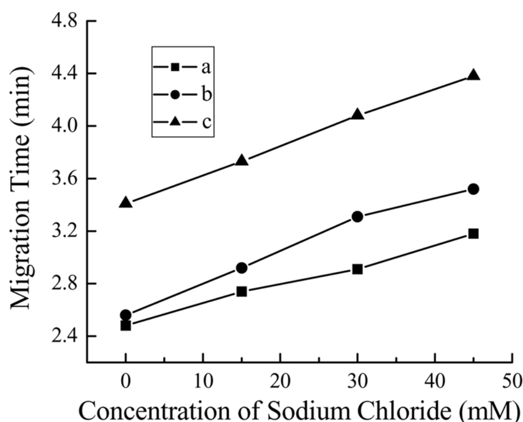

Figure 2. Effect of the sodium chloride on the separation of analytes. CE conditions: H_3BO_3-NaCl-$Na_2B_4O_7$ running buffer with pH 7.40; other conditions are the same as Figure 1. Peak identification: (a) cimaterol; (b) clenbuterol; (c) salbutamol.

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Effect of Water Plug

A short plug of water before electrokinetic injection of the sample provides proper electric field enhancement at the injection point and renders an empty region for concentration ions deeper into the column and away from the inlet end.[19] The effect of water plug injection time on the peak areas was investigated in this work. Results indicated that the peak area of the analytes increased with increasing the injection time from 1 s to 4 s, and than decreased from 5 s to 7 s. It was found that the determination degraded at 8 s. The reason was the analytes could not move out of the trap of a too-long water plug and resulted in a decrease in sensitivity.[19] Hence, 4 s was chosen as the optimal injection time of a water plug.

The Injection Voltage and Time

The amount of the analytes injected with FASS is dependent on injection voltage and time under the experimental conditions. To understand how these parameters affected the sensitivity, a constant length of water plug and injection time was kept and the injection voltage was varied from 5 KV to 10 KV. The peak areas of the analytes increased with the increase of injection voltage rapidly. However, the maximum voltage for injection is limited at 10 KV in the P/ACE 5510 capillary electrophoresis. Therefore, the injection voltage of 10 kV was used. The

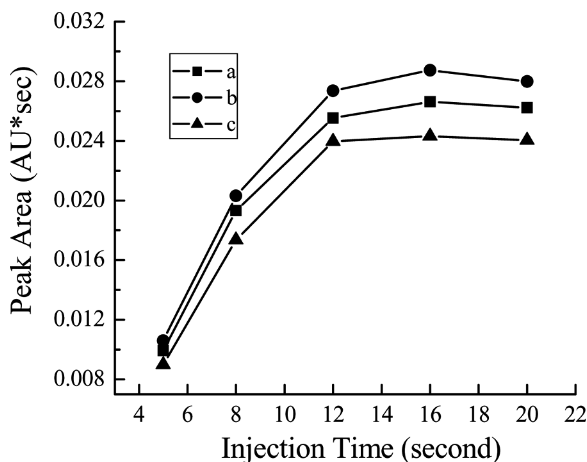

Figure 3. Effect of injection time on the peak areas of analytes. CE conditions: 50 mM H_3BO_3-$Na_2B_4O_7$ and 30 mM NaCl running buffer with pH 7.40; other conditions are the same as Figure 1. Peak identification: (a) cimaterol; (b) clenbuterol; (c) salbutamol.

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sensitivity was tested by changing the injection time from 5 s to 20 s under the condition of 10 KV injection voltage. As shown in Figure 3, the peak areas of the analytes increased rapidly with the sample injection time from 5 s to 12 s; with a further increase of injection time, the peak areas increased slightly in the range of 12–16 s. From 16 s to 20 s the peaks began to broaden. Therefore, 13 s was chosen as the injection time for subsequent experiments.

The Separation Voltage

It was found that with an increase of the separation voltage, migration time decreased and the peak shapes became sharper, while the electric current in the capillary obviously increased. Owing to the too high electric current in the capillary causing the peak to broaden, 20 kV was used as the separation voltage.

Under the selected optimum conditions, a complete baseline separation of standard mixture containing cimaterol, clenbuterol, and salbutamol was obtained within 5 min as shown in Figure 4.

Linear Range and Detection Limit

To determine the linearity between the peak areas and concentrations of clenbuterol, cimaterol, and salbutamol, a series of standard mixtures of

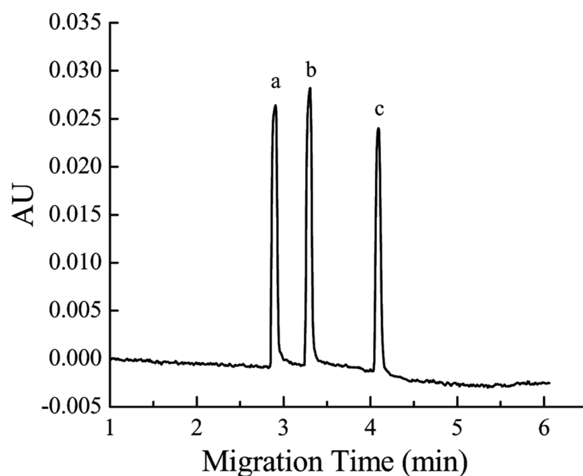

Figure 4. Electrophoregram of standard mixture of three β_2-agonists. CE conditions are the same as in Figure 3. Peak identification: (a) cimaterol; (b) clenbuterol; (c) salbutamol.

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these three analytes were tested from 0.001 to 1.0 μg/mL. The results indicated that peak area and analyte concentration had a good linear relation in the range of 0.01 to 1.0 μg/mL. The linear correlation was found to be $Y = 586.6 + 9255X$ ($r = 0.9997$) for cimaterol, $Y = 781.4 + 10195X$ ($r = 0.9999$) for clenbuterol, and $Y = 570.0 + 8356X$ ($r = 0.9996$) for salbutamol, respectively, and the detection limits were down to 1.9 ng/mL, 1.7 ng/mL, and 2.1 ng/mL, for cimaterol, clenbuterol, and salbutamol at a 3:1 signal-to-noise ratio.

Precision

The repeatability of the proposed method was evaluated by determinations of three β_2-agonists standard solutions containing

Table 1. Relative standard deviation (RSD %) for peak area

	Peak area RSD (%)	
β_2-agonists	Run to run (n = 6)	Day to day (n = 6)
Cimaterol	2.08	2.16
Clenbuterol	1.73	2.10
Salbutamol	2.85	3.10

Table 2. Results of the spiked samples by FASS method

β_2-agonists	Added concentration (μg/mL)	Found concentration (μg/mL)	Recovery (%)	RSD (%) (n = 6)
Cimaterol	0.1	0.0978	97.8	2.26
	0.5	0.485	97.0	1.13
	1.0	0.963	96.3	0.95
Clenbuterol	0.1	0.0982	98.2	2.26
	0.5	0.486	97.2	1.03
	1.0	0.965	96.5	0.94
Salbutamol	0.1	0.0968	96.8	3.10
	0.5	0.481	96.1	1.31
	1.0	0.952	95.2	0.95

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0.1 μg/mL of each (Table 1). Satisfactory results with RSD $\leq$ 2.85% for run to run and RSD $\leq$ 3.10% for day to day was obtained.

Real Application

Under the optimum conditions, the determination of cimaterol, clenbuterol, and salbutamol in spiked swine feed was carried out by the proposed method. The feed samples were treated with the procedure

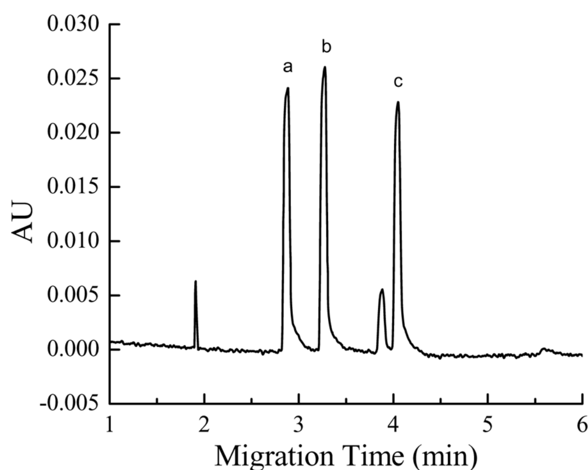

Figure 5. Electrophoregram of three β_2-agonists in spiked swine feed. CE conditions are the same as in Figure 3. Peak identification: (a) cimaterol; (b) clenbuterol; (c) salbutamol.

described in samples section. The typical electropherogram of the spiked samples is shown in Figure 5. The recovery and reproducibility were investigated by adding 0.4, 2, and 4 mg of the analytes to per kilogram swine feed, respectively. The results were listed in Table 2. The proposed method could satisfy the need of routine analysis for three β_2-agonists in swine feed.

CONCLUSIONS

This work presented a simple, rapid, and reliable method for the determination of β_2-agonists in swine feed. The proposed method remarkably improved the sensitivity and can be applied in routine analysis of livestock feed.

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