Analytical Methods

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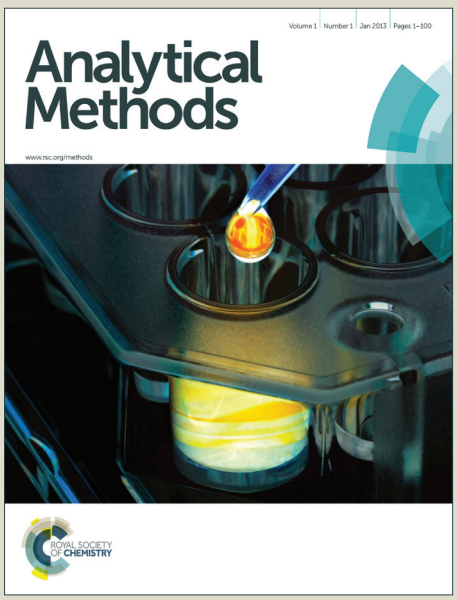

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PAPER

Chemiluminescence Diminishment on a Paper-Based Analytical Device: High Throughput Determination of β-agonists in Swine Hair

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β-agonists are a group of illegal but widely used feed additives in stockbreeding industry. In order to achieve simple-to-use, fast and high-throughput testing of this banned chemical, here we suggest a paper-based analytical device on which a chemiluminescence diminishment method was performed. In this approach, extracts from swine hair samples as well as luminescence reagents, such as luminol and potassium periodate solution, in a low volume were applied on our device. It was found that the light emission was diminished by the β-agonists extracted from in swine hair samples. The degree of diminishment is proportional to the concentration of the β-agonists from 1.0×10^{-5} to 1.0×10^{-8}mol/L. Also, the concentrations of solutions for chemiluminescence were optimized. The mechanism and reaction kinetics of chemiluminescence were discussed as well. The detection limit was obtained as 1.0×10^{-9}mol/L, and the recoveries were achieved from 96% to 110%, both of which suggested that our method will be favourable to the field application for swine hair samples.

Introduction

β-agonists, a class of sympathomimetic agents which act upon the β-adrenoceptors, was widely used as a bronchodilator for treatment of bronchial asthma, chronic bronchitis, and emphysema.[1] Researchers later found that the β-agonists could stimulate protein accretion and inhibit the adipose accumulation of animal.[2, 3] So the β-agonists as feed additives had been used to promote lean meat content of beef and pork. However, once livestock animals were fed with the β-agonists, the residues remain in their meat and liver for a long time and then enter into food cycle of human beings. Also, it is worth metion that it is difficult to eliminate them in the household cooking processes [4] and this can lead to a potential risk to human health. For the sake of public health and safety, many countries have forbidden the use of the β-agonists in stockbreeding industry, especially in European Union, Russia and China. Thus highly sensitive analytical methods for quantification and confirmation of trace residues of β-agonists in animal tissues have been reported, on such as gas chromatography,[5-7] high performance liquid chromatography,[8-10] electrochemistry,[11-13] colorimetry,[14, 15] surface-enhanced Raman spectroscopy,[16-18] capillary electrophoresis[19-21] and immunoassay.[22-24] However, most of these methods required complicated and expensive instruments, meanwhile these methods were time consuming and laborious, which did not meet the demand of real time analysis. Therefore it is necessary to develop easy-to-use, inexpensive, high throughput and portable method for detecting β-agonists at low concentrations with attractive precision and accuracy.

Paper-based analytical devices (PADs) have gained significant attention in the recent few years because they are fabricated with an extremely inexpensive and common material, paper. First paper is compatible with many chemicals and biochemicals; second, chemical can be transported using capillary force without assistance of external forces in paper matrix; third, paper are portable ,simple to use and easy to discard; also, pretreatment of crude samples and detection of analytes has been reported in high throughput mode.

So far, four ways have been applied for the detection of analytes on PADs: chemiluminescence (CL),[25-27] electrochemistry(EC),[28-31] colorimetry[32-34] and electrochemiluminescence (ECL).[35, 36] Among these methods, CL is especially attractive for PAD due to its high sensitivity, wide linear range, simple and inexpensive instrumentation, very short analysis time and more importantly, it does not need any light source.[37]

The shelf time for detection of β-agonists in samples is also a very important parameter, especially for food safety investigation. Urine and plasma have been proposed as the sources of samples for detection of residues of β-agonists in animals,[17, 18, 38]. However, their major drawbacks of using them as the source is that β-agonists cannot be detected in urine with a shelf time longer than 11 days after sampling, and β-agonists in plasma is undetectable 4 days after the last application.[39, 40] The analysis of β-agonists in retinal has been shown to extend its detection period to about 20 weeks.[41] However, retinal can only be sampled at slaughterhouses. Recently, hair emerged as an important substrate for the detection of residues of illicitly administered β-agonists. This is not only because hair can be easily collected from tliving animals and stored until analysis owing to its biological stability and to its physical state, but also because hair analysis allows the possibility of revealing historical record of drugs exposure, dating

back over a period of months. With these considerations above, we collect swine hairs to analyze the β-agonists residues in this work.

In this paper, the reaction between luminol and KIO_4 was performed to generate CL, and we found that the CL intensity can be diminished by the β-agonists. On the basis of this phenomenon, a novel PAD-CL sensor, which was designed as "96-well plate", was developed for high throughput, fast, sensitive and convenient determination of β-agonists. And as a result, 48 samples could be detected on the same PAD. The validity of our method was demonstrated with detection limit and relative standard deviation for five replicate measurements of the β-agonists obtained. This proposed method was applied to the determination of the β-agonists in swine hair and the recoveries were satisfactory (range 96% - 110%).

Materials and methods

Design and fabrication of the paper device

As shown in Fig.1, the μPAD was designed (CorelDRAW x4, Corel Corporation) in a "96-well plate" form, on which there are blank solution detection areas (pink area) and sample solution detection areas (white area), respectively. The pattern was transferred to a filter paper with a wax colour printer (FUJI XEROX Phaser 8560DN). The printed paper was then placed in an oven at 150 ℃ for 3 minutes, and the wax melted and edistributed into the paper matrix and formed the hydrophobic wall on the paper. The patterned paper was ready for use after removing the paper from the drying oven and allowing it to cool to room temperature.

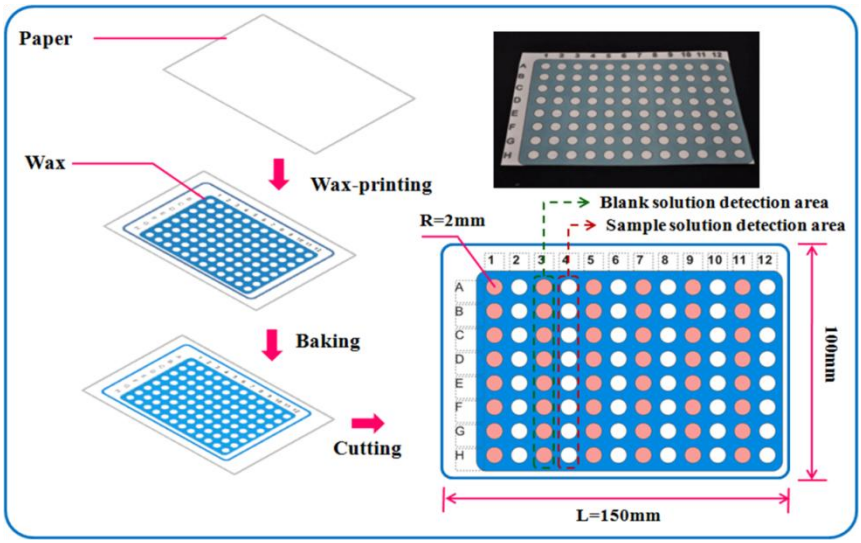

Fig.1. Schematic of the μPAD fabrication process by wax-printing method. The blue part represents wax in the μPAD. Four major steps were involved as follows for fabrication of the μPAD by wax-printing: a. the pattern of μPAD was designed with CorelDRAW software; b. the pattern was transferred to paper by wax-printing; c. the wax-printed paper was put into a drying oven to melt the wax (150 ℃, 3 minutes), d. the μPAD were cooled to room temperature.

Materials and chemical reagents

The standard clenbuterol (CLB), ractopamine (RAC), salbutamol (SBL), terbutaline (TBL) and clencyclohexerol (CCL) solutions (1.0×10^{-3}mol/L) were respectively prepared by dissolving 0.0314g, 0.0338g, 0.0239g, 0.0549g, 0.0319g of CLB (NIFDC, China), RAC (IQSTAP, CAAS, China), SBL (NIFDC, China), TBL (Sigma, St. Louis, MO), CCL (WITEGA, Berlin, Germany) in 100mL deionized (DI) water. These stock solutions were stored in refrigerator at 4℃. Stock solution of luminol (1.0×10^{-3} mol/L) was prepared by dissolving 0.0177g of luminol (Aladdin Industrial Co., Shanghai, China) in 100mL of 0.1mol/L NaOH. The working solution of luminol was prepared by directly diluting this stock solution to appropriate concentrations with appropriate concentrations of NaOH. KIO_4 stock solution (1.0×10^{-2}mol/L) was prepared by dissolving 0.23g KIO_4 (Tianjin Guangfu Fine Chemical Research Institute, China) in 100mL DI water. All the other reagents used were of analytical reagent grade.

Apparatus

Wax color printer (FUJI XEROX Phaser 8560DN) was purchased from FUJI Xerox Co., Ltd. Whatman chromatography paper#1 (200mm×200mm) was obtained from GE Healthcare Worldwide (Pudong Shanghai, China). This type of filter paper was chosen because of its uniform composition and free from contamination of additives that may affect CL reaction. Reagent solutions were introduced by TJ-2A Micro Flow Rate Syringe Pump (Longer Precision pump Co. Ltd., Baoding, China).The CL signal was detected and recorded by MPI-B CL analyzer (Xi'an Remex Electronic High-Tech Ltd., China). LS-55 fluorescence spectrophotometer (Perkin-Elmer, USA) was used for CL spectroscopy.

Hair sample preparation

The swine hair samples were obtained from Institute of Quality Standard and Testing Technology, Chinese Academy of Agricultural Sciences (Beijing, China). The hairs were thoroughly washed with a solution of 1% SDS for 30 min. The sample was then rinsed in DI water as frequently as necessary until there was no SDS in the washing water. Hair samples were dried in an oven at 40℃, and subsequently cut into small pieces of 1-2 mm. A total of 100 mg of each washed swine hair sample

was weighed, and internal standard solution and 1mL of 0.1 mol/L hydrochloric acid were added. This mixture was then incubated in a 60 ℃ water bath for 18h, removed, cooled to room temperature, and centrifuged at 4000rpm for 5 min. The supernatant was kept for chemiluminescence assay.

Chemiluminescence assay procedure of this μPAD

The direct detection of β-agonists in complex hair matrices is a difficult task, and some sample clean-up treatment is mandatory before performing the analysis. In this work, molecular imprinting technology, which can offer tailor-made selectivity for the extraction of trace analytes in complex matrixes, was used to remove hair matrix interferences. Molecularly Imprinted Polymer (MIP) SPE Cartridges was bought from Sigma (St. Louis, MO). The MIP phase was conditioned and equilibrated sequentially with 1mL methanol and 1mL DI water. After 2.0 ml pre-treated hair sample solution was loaded to the conditioned MIP column, the column was washed sequentially with 2×1mL DI water (elution of salt and matrix interferences), 1mL acetonitrile (selective removal of hydrophobic interferences) and 1mL 60% acetonitrile/40% DI Water (selective removal of hydrophilic interferences). The tube was dried for 2 min under full vacuum after the every stage of the wash step. The β-agonists were eluted with 2×1mL 1% formic acid in acetonitrile. The eluted sample was evaporated under full vacuum (80℃) and reconstitute with 1mL DI water prior to CL analysis. At testing, as shown in Fig.2, firstly, 5μL luminol solution was added on the μPAD, followed by being dried in the air. Then the μPAD was put into CL analyzer and aligned exactly onto the photomultiplier of the CL analyzer. For a typical CL assay, firstly, 5μL of solution A (the mixture of the blank solution (DI water) and KIO_4) was dropped onto the blank solution detection area by a syringe pump, then after 90s, 5μL of solution B (the mixture of the sample solution and KIO_4) was dropped onto the sample solution detection area by another syringe pump. The data acquisition and treatment were performed with the MPI-B CL data processing software (Xi'an Remex Electronic High-Tech Ltd.). And the signal was recorded using a computer. The concentration of the β-agonists was quantified by ΔI ($\Delta I = I_0 - I$, where I and I_0 designate the CL signals in the presence and absence of the β-agonists, respectively).

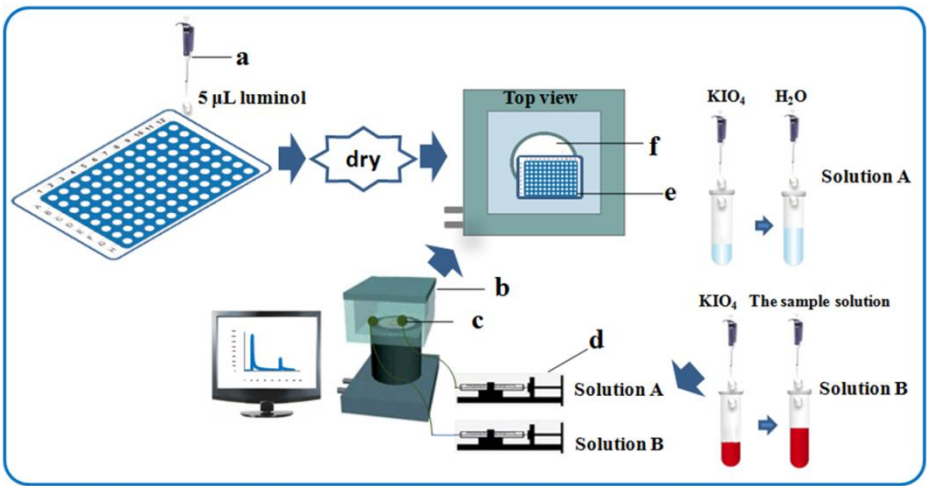

Fig. 2 Schematic of a typical CL assay procedure: a. pipette; b. CL analyzer; c. injection hole; d. syringe pump; e. PAD; f. photomultiplier.

Results and discussion

Chemiluminescence diminishment in the presence of β-agonists

The CL kinetic characteristics of the reactions were studied in Fig. 3. Curve 1, which shows I_0 of the blank solution, was the CL kinetic curve obtained when 5μL of the solution A (the mixture of the blank solution (DI water) and KIO_4) was injected into the blank solution detection area. 90s later, the CL reaction terminated and the CL signal declined to baseline. When the CL signal returned to baseline, 5μL of the solution B (the mixture of the sample solution and KIO_4) was then injected into the sample solution detection area, and c β-agonists signal (I),were obtained as curve 2 in Fig. 3. . It can be seen that the reaction between luminol and KIO_4 generated CL signals, and the emission of the CL were quenched by the β-agonists. In order to explore the mechanism of the CL reaction, the following experiments were performed, and their results are discussed below.

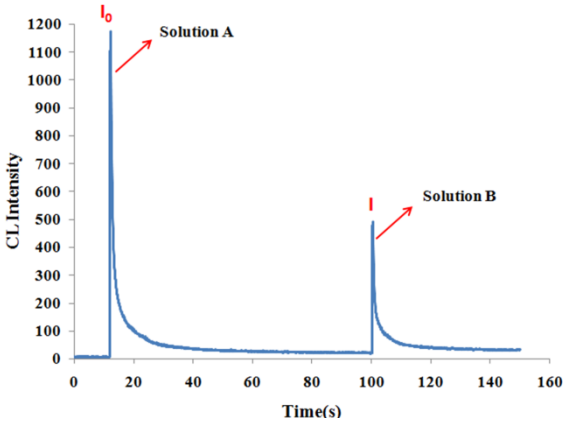

Fig. 3 Kinetic curves of CL. curve 1: the CL signal of solution A; curve 2: the CL signal of solution B. Operating conditions: luminol concentration, 1.0×10^{-4} mol/L in 0.1mol/L NaOH; KIO_4 concentration, 1.0×10^{-4}mol/L; β-agonists concentration,

1.0×10^{-5}mol/L.

Mechenism of chemiluminescence diminishment

The CL spectra of the β-agonists-KIO_4-luminol and KIO_4-luminol reaction systems were recorded and the results are shown in Fig. 4. The KIO_4 solution was injected into the luminol solution and the CL spectrum of the mixture was measured (Fig. 4a). In parallel, the mixture solution of the β-agonists and KIO_4 was injected into a luminol solution and CL spectrum was obtained (Fig. 4b). Both the spectra had same peak emission wavelength at 425nm, indicating that the luminant of both the reactions was the excited state of 3-aminophthalate ion (3-AP*).[42]

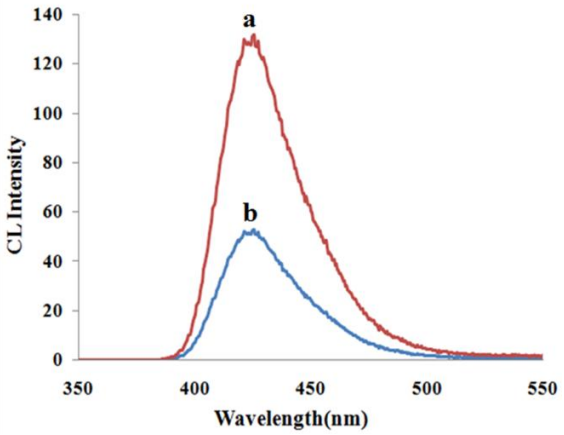

Fig.4. CL emission spectra, operating conditions: luminol concentration, 1.0×10^{-4} mol/L in 0.1mol/L NaOH; KIO_4 concentration, 1.0×10^{-4}mol/L; β-agonists concentration, 1.0×10^{-5}mol/L.

The fluorescence spectra of β-agonists alone, and β-agonists-KIO_4 mixture were displayed respectively in Fig. 5. It can be seen that the wavelength of peak emission for the β-agonists was 310 nm and interestingly this peak of β-agonists at 310nm disappeared when KIO_4 was intoduced. This is attributed to the fact that β-agonists were oxidized by KIO_4. And then 5μL β-agonists (1.0×10^{-4}mol/L), 5μL KIO_4 (1.0×10^{-4}mol/L) and 5μL KIO_3 (1.0×10^{-4}mol/L, the reaction product of KIO_4 with the β-agonists) were respectively dropped on the μPAD by syringe pumps. As shown in Fig. 6, only the KIO_4 solution gave a strong CL signal, but the CL signals of the KIO_3 and β-agonists were both very weak.

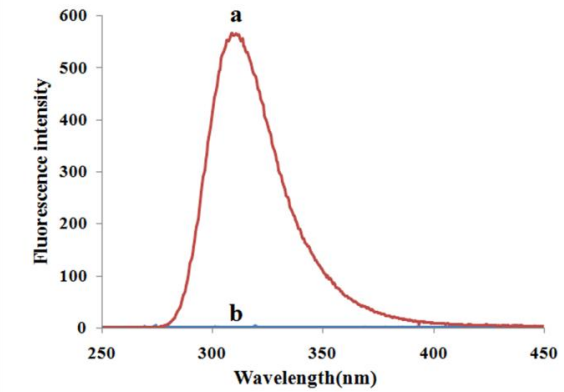

Fig.5 The fluorescence spectra of β-agonists and β-agonists-KIO_4, a: 5.0×10^{-4}mol/L β-agonists, b: the mixture of 5.0×10^{-4}mol/L β-agonists and 5.0×10^{-3}mol/L KIO_4.

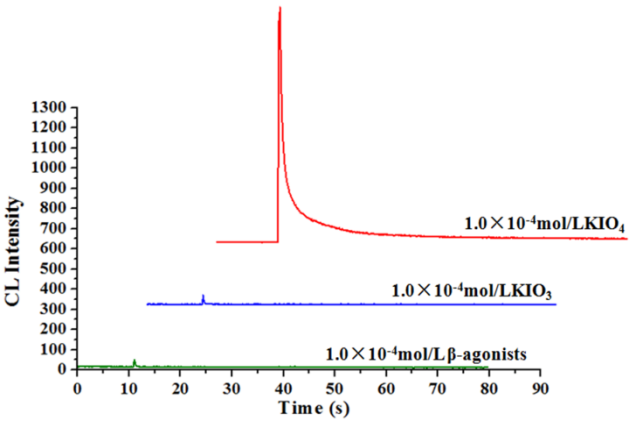

Fig.6. Temporal curve of CL of β-agonists, KIO_4 and KIO_3. Operating conditions: luminol concentration, 1.0×10^{-4} mol/L in 0.1mol/L NaOH.

Based on the above experiments, here we propose the mechanism of diminishment effect of the β-agonists on the luminal-KIO_4 CL system as follows: in alkaline medium, KIO_4 oxidizes luminol to produce 3-aminophthalate (AP) ion in an excited state, and then the excited state of 3-AP back to the ground state and produces CL. When the β-agonists was added to the KIO_4 solution, KIO_4 (the oxidant of luminol–KIO_4 CL system) was consumed, and that led to the diminishment of the CL intensity.

Optimization of Reaction Conditions

The influence of KIO_4 concentration on the CL intensity was examined in the range between 4.0×10^{-5} and 4.0×10^{-4} mol/L. The results showed that ΔI reached its peak value and then turned to drop after when the concentration of KIO_4 was at 1.0×10^{-4} mol/L, ΔI then decreased. Thus, the concentration of 1.0×10^{-4} mol/L KIO_4 was chosen in the following experiment.

Also, the effect of luminol concentration was examined from 6.0×10^{-5} to 6.0×10^{-4}mol/L. The result showed that the maximum ΔI was obtained when the concentration of luminol was 1.0×10^{-4}mol/L. So the 1.0×10^{-4}mol/L luminol was applied for the subsequent experiment.

The concentration of NaOH was also an important factor for the detection of the β-agonists. By varying NaOH concentration in luminol solution in 0.06–0.6 mol/L range, it could be observed that at a low NaOH concentration, the ΔI increases when the concentration of NaOH goes up; when the NaOH concentration is greater than 0.1mol/L, the ΔI starts to decay. Therefore, the NaOH concentration was optimized as 0.1mol/L.

Effect of β-agonists type on CL

Under the optimal experimental condition, the CL behaviors of most-commonly used β-agonists, TBL, RAC, SBL, CLB and CCL single standard solutions at the concentration of 1.0×10^{-6}mol/L were tudied respectively on our PADs. As shown in Fig.7, their CL intensities demonstrated negligible deviations between each other. This indicates that β-agonists quantity as an entire value can be measured by this CL method.

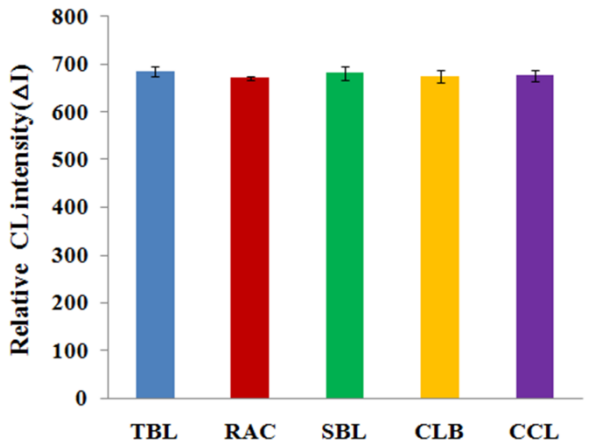

Fig.7. The relative CL value of different types of β-agonists.

Performance of the method for β-agonists measurements

Under the optimal conditions described above, the plot of ΔI versus the β-agonists concentration showed good linearity from 1.0×10^{-5}mol/L to 1.0×10^{-8}mol/L, and the detection limit was 1.0×10^{-9} mol/L (the control intensity minus two times standard deviation). The regression equation can be expressed as $\Delta I = 1492 + 166.1 \log C$ (C being the β-agonists concentration (mol/L) with a correlation coefficient of 0.990 (n =6)), as seen in Fig.8. The relative standard deviation was 2.7% for the determination of a 1.0×10^{-6} mol/L the β-agonists standard solution. (n =5)

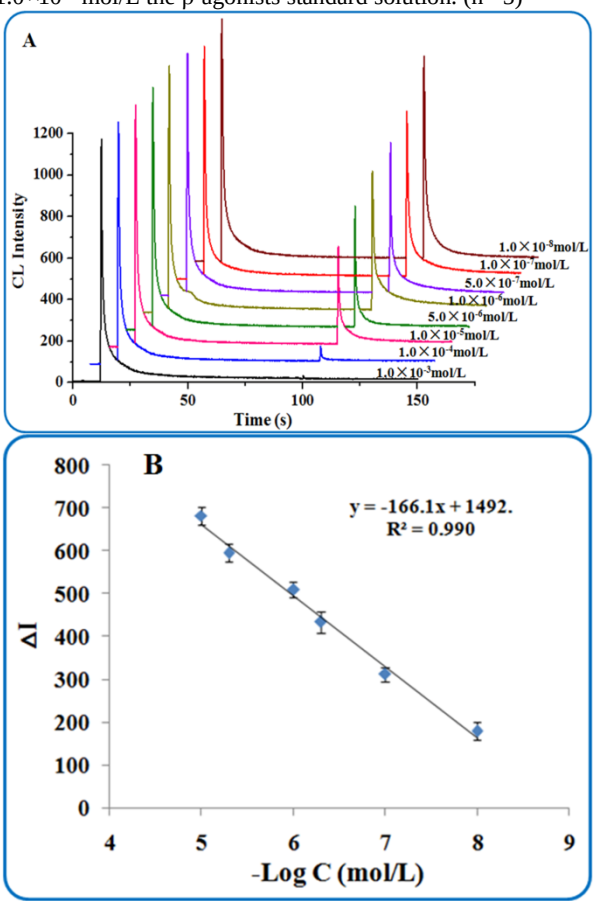

Fig 8.A: CL signals for eight different concentrations of the β-agonists standard solution under the optimal conditions, B: Calibration curve for standard solutions of the β-agonists.

Interference studies

In order to apply the method to the analysis of abused β-agonists in the swine hair, the interference of some inorganic and organic components, which exist in swine hair, was investigated by analyzing a standard solution of 1.0×10^{-7} mol/L the β-agonists. A foreign species is not regarded as an interfering substance if it causes a relative error <5%. Listed in Table 1, these results showed that MIP phase can be used as a sample clean-up treatment in this work and improve the selectivity of the method. And the proposed method was then used to determine the β-agonists in real field samples, such as swine hair.

Table 1 The tolerable relative concentration ratios of interfering substances against β-agonists without and with MIP cleaning step

Substances	Without MIP	With MIP
Na^+, K^+, Cl^-,NO_3^-,SO_4^{2-}, Glucose, Starch	500	1000
Alanine, Phenylalanine, Glycine, Cystine	100	500
Ca^{2+}, Mg^{2+}, Zn^{2+},CO_3^{2}	50	100
Tryptophan, Methioninc, Urea	10	100
Antondine, Amoxicillin, Lincomycin hydrochloride	5	100
Chloramphenicol	1	100
Fe^{3+},Fe^{2+},Mn^{2+}, Cu^{2+}	0.1	10

Analytical application of the method

Application of our proposed method was applied to determination of the β-agonists in swine hair samples. The swine hair samples were prepared by the procedure described previously. The results are shown in Table 2. The recoveries for different concentration levels of the β-agonists were between 96% to 110%.

Table 2 Determination results of β-agonists in swine hair samples

Sample	Added (mol/L)	Found (mol/L)	Recovery (%)	RSD (%,n=6)
The black swine hair	5.00×10^{-8}	5.22×10^{-8}	104	3.6
	1.00×10^{-7}	0.98×10^{-7}	98	2.7
	5.00×10^{-7}	4.86×10^{-7}	97	4.1
	1.00×10^{-6}	1.10×10^{-6}	110	2.7
	5.00×10^{-6}	5.14×10^{-6}	103	3.1
The white swine hair	5.00×10^{-8}	4.95×10^{-8}	99	2.1
	1.00×10^{-7}	1.06×10^{-7}	106	2.8
	5.00×10^{-7}	4.81×10^{-7}	96	3.7
	1.00×10^{-6}	1.00×10^{-6}	100	3.3
	5.00×10^{-6}	5.08×10^{-6}	102	3.3

Conclusion

In this study, we found that the β-agonists diminished the chemiluminescence emission and that was caused by the reaction between luminol and KIO_4 on the PAD. Utilizing this, a novel

PAD chemiluminescence sensor, which was designed in the form of a "96-well plate", was developed for high throughput, fast, sensitive and convenient determination of β-agonists. And 48 samples could be simultaneously detected on one piece of PAD. Also, this sensor was applied to measure the total β-agonists in the swine hair. The detection limit of this method was as low as 1.0×10^{-9}mol/L. Satisfactory recovery values between 96% to 110% were achieved. These figures, as well as the fact that samples and reagents were consumed in microlitres gave support to the validity of our PADs method. Further, our PADs methodology was straightforward fast, high throughput, accurate and reduced the reagents consumption and are suitable for developing miniaturization instrument for on-site analysis, such as point-of-care health diagnostics, food quality control and on-site environmental monitoring.

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Notes

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