Food Chemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Food Chemistry

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

Short communication

Rapid detection of clenbuterol in milk using microfluidic paper-based ELISA

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ARTICLE INFO

Keywords:
Paper-based microfluidics
Food safety
Clenbuterol
Milk
ELISA
Immunoassay

ABSTRACT

In this study, a paper-based microfluidic enzyme-linked immunosorbent assay (ELISA) was developed as a screening system for rapid detection of clenbuterol, which is illegally used as a growth promoter for food-producing animals. The microfluidic paper-based analytical device (μPAD) was combined with ELISA and the intrinsic properties of paper allowed the entrapment of antibody through cellulosic fibres, validating to be an alternative to 96-well ELISA microplate for food safety monitoring. Detection of clenbuterol in milk was achieved by measuring the intensity of colour change that was proportional to the analyte concentration with a detection limit of 0.2 ppb. The μPAD effectively reduces the cost, volume of reagents, and time required to run ELISA for food sample testing.

1. Introduction

Clenbuterol is widely used as bronchodilator and decongestant in clinical management of both humans and animals (Tang et al., 2017). However, clenbuterol has also been misapplied as a growth promoter for food-producing animals, which decreases fat accumulation and increases muscular mass (Ramos et al., 2003). Clenbuterol has a long half-life in animal tissues (*e.g.,* meat, kidney and liver), milk and urine (Yuan et al., 2017). The accumulation of clenbuterol in animal bodies and milk can be transmitted to humans through the food chain and cause food poisonings, such as palpitations and muscular tremors (Barry & Graham, 2013). Thus, the administration of clenbuterol as a feed additive for food-producing animals has been strictly prohibited by many countries (Tang et al., 2017). The maximum residue limits set by the Food and Agriculture Organization (FAO) are 0.05 μg/l in milk, 0.2 μg/kg in muscle, and 0.6 μg/kg in liver and kidney.

Various analytical techniques have been employed to detect clenbuterol in food products, including high performance liquid chromatography (HPLC) (Guo et al., 2017), liquid chromatography-mass spectrometry (LC-MS) (M. Li et al., 2017) and gas chromatography-mass spectrometry (GC-MS) (Ramos et al., 2003). Although these techniques are accurate and sensitive, they require expensive and bulky equipment, complex sample preparation and highly trained personnel. Alternatively, enzyme-linked immunosorbent assay (ELISA) has been established to determine clenbuterol in polystyrene 96-well microtiter plate (Pleadin et al., 2013), which is high-throughput, sensitive and specific. However, the conventional ELISA is still considered relatively expensive to food industry, as it requires large volumes of analyte samples and reagents (especially antibodies) as well as costly plate readers ($CAD 20–30 K) for data collection. Therefore, the conventional ELISA is not well suited for rapid on-site detection of food chemical hazards, and there is an urgent need to develop an affordable, simple, easy-to-use point-of-care (POC) diagnosis technique for the detection of clenbuterol in foods.

The microfluidic fiber-based analytical device is an innovative study platform (Martinez, Phillips, Butte, & Whitesides, 2007; Nilghaz, Guan, Tan, & Shen, 2016), which integrates the function of analytical devices on textile (Nilghaz et al., 2016) and paper-based substrate to reduce reagent volume and shorten analysis time (Cate, Adkins, Mettakoonpitak, & Henry, 2015; Zhang et al., 2015). Paper-based ELISA has been widely applied to detect various molecules in complex biological matrices, such as HIV-1 antibodies (Cheng et al., 2010), human performance biomarkers (Murdock et al., 2013) and tumor biomarkers (Hsu et al., 2016; Wang et al., 2012) in human serum. ELISA can be performed on a paper-based multiwall plate, in which the samples and reagents are deposited onto the hydrophilic test zones confined by the hydrophobic barriers (*e.g.,* wax, polystynrene and alkyl ketene dimer) (Li, Ballerini, & Shen, 2012). Paper-based ELISA is low-cost, biodegradable, easy-to-modify, and convenient for transportation. Numerous fabrication approaches are available for μPADs that can customize various non-standard formats. The fabrication techniques include, but are not limited to, wax printing (Carrilho, Martinez, & Whitesides, 2009), wax screen-printing (Wang et al., 2012), photolithography (Carrilho, Phillips, Vella, Martinez, & Whitesides, 2009), inkjet etching

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https://doi.org/10.1016/j.foodchem.2017.12.022
Received 31 July 2017; Received in revised form 5 December 2017; Accepted 7 December 2017

(Abe, Suzuki, & Citterio, 2008), plasma treatment (X. Li, Tian, Nguyen, & Shen, 2008), stamping (Curto et al., 2013) and paper cutting (Fenton, Mascarenas, López, & Sibbett, 2009). Each approach has advantages and limitations from the perspectives of cost, speed, simplicity of fabrication, and safety (Carrilho, Martinez, & Whitesides, 2009; He, Wu, Fu, & Wu, 2015). Wax printing and wax screen-printing show more merits in simple and rapid fabrication (printing/patterning and baking; < 5 min), low-cost materials (wax and paper) and no safety issue (no use of organic solvents or toxic photoresist agents) (Carrilho, Martinez, & Whitesides, 2009).

In this study, paper-based colorimetric ELISA was established to detect clenbuterol in milk. Wax printing and wax screen-printing were conducted to prototype paper-based devices. The device fabrication and antibody immobilization were investigated to optimize assay performance. Further, the limit of detection (LOD) of this paper-based clenbuterol assay was evaluated. This study demonstrates the development of a portable, low-cost, rapid and sensitive technique for the detection of clenbuterol in milk for the first time.

2. Materials and methods

2.1. Chemicals and materials

The detailed materials information can be found in Supplementary Information.

2.2. Fabrication and surface functionalization of μPADs

The fabrication of μPADs as the substrate for ELISA was divided into three steps: a) device design; b) fabrication of the device using wax-printing/patterning on paper; and c) surface modification of the paper. The layout of μPADs is similar to that of the conventional 96-well plate with minimized size, which is in an array of 8-row by 12-column and has a microzone diameter of 4 mm and a centre-to-centre distance of 6 mm. To inhibit the migration of sample fluids among microzones, hydrophobic barriers were designed in black on a white background using AutoCAD (Autodesk Inc., San Rafael, CA, the United States). Subsequently, microzone plate patterns were printed on a Whatman® cellulose chromatography paper using a wax printer Xerox Phaser 8560 N (Xerox Canada, Toronto, ON, Canada) (Carrilho, Martinez, & Whitesides, 2009). Since the wax printing method requires a relatively expensive wax printer, we also used a woven mesh as an alternative technique to support an ink-blocking stencil as a wax patterning technique, known as “screen patterning”. The screen mesh was designed as a multi-well plate (polyester, pore size: 75 μm) and fabricated by a local company in Vancouver, Canada. Chromatography paper was pasted on the back of the screen mesh. A piece of paraffin wax with the melting point of ≥65 °C was rubbed through the screen mesh for 2 min. The chromatography paper was then separated from the mesh. The printed/screen-patterned paper was subsequently placed in an oven set at different temperatures (*i.e.,* 70, 90 and 110 °C) for different time periods (*i.e.,* 30, 60, 120 and 180 s) to establish an optimal heating condition for the fabrication of the device. The optimal condition was employed to uniformly melt the wax on the paper to form hydrophobic barriers among the microzones. The fabrication techniques were evaluated and the best candidate was selected for device fabrication.

To determine the required volume of working reagents on μPAD, different volumes (1–20 μl) of crystal violet dye solution [0.025% (w/v) in water] were added to the microzones before drying the device at 22 °C and the results were evaluated by the naked eyes. The device was then functionalized using chitosan and glutaraldehyde to enhance the wet strength of the paper and increase the immobilization of antibodies on the surface of the paper, similar to that proposed by Wang and co-authors (Wang et al., 2012). Briefly, a total of 10 μl of chitosan solution was deposited onto the detection zones of the paper and dried at ambient temperature. An aliquot of 5 μl of glutaraldehyde [2.5% (w/v) in 1 × PBS] solution was then added and incubated on the detection zone, followed by washing three times using 10 μl of 1 × PBS to remove the excess chitosan and glutaraldehyde. Between each wash, the washing buffer was adsorbed by a piece of blotting paper from the bottom of the device. After drying, the surface-modified μPADs were ready to use as the substrate for performing ELISA.

2.3. Stability of μPADs in food matrices with different pH

To evaluate the stability of μPADs with different food matrices, the devices were immersed in solutions with different pH values for 24 h. The acidic [pH = 1.7, 20% (v/v)] and basic solutions (pH = 9.5, 0.2 M) were prepared by diluting acetic acid and sodium carbonate-bicarbonate buffer in water. PBS was used as a neutral solution (pH = 7.4, 10 mM). The devices were then rinsed with distilled water before evaluation. The integrity of the hydrophobic barriers was evaluated by adding blue dye (5 μl) to each microzone.

2.4. Characterization of the immobilization capacity of μPADs

To characterize the antibody immobilization capacity of μPADs, a total of 3 μl of HRP-conjugated antibody and 5 μl of TMB obtained from a commercial ELISA kit were successively added to the microzones fabricated from untreated, chitosan-, glutaraldehyde- and chitosan/glutaraldehyde-functionalized papers. Upon the reaction of HRP-conjugated antibody with TMB, a colour change occurred. The images of microzones were qualified by the naked eyes and semi-quantified by capturing and analyzing the photos of the microzones using Adobe Photoshop before comparing the mean intensities.

2.5. Paper-based competitive ELISA

A competitive ELISA was employed to determine the concentration of clenbuterol in spiked water and milk. Briefly, a total of 3 μl of mouse monoclonal clenbuterol antibody (25 μg/ml) was spotted on each microzone and completely dried at room temperature. The excess antibodies were thoroughly washed off using 10 μl of washing buffer three times. An aliquot of 10 μl of blocking buffer was then added to each microzone in order to prevent the non-specific binding of the targeted analytes. Skim milk was obtained from a local grocery store in Vancouver and kept at 4 °C. The milk samples were confirmed to be free of clenbuterol using HPLC coupled with photodiode array detector (HPLC-DAD; Agilent Technologies, Santa Clara, CA, The United States). Water and milk samples were spiked with certain amounts of clenbuterol, making the final concentrations to be 0, 1, 10, 100 and 1000 μg/l. To minimize the negative impact of complex food matrices, the resultant spiked milk samples were 5-fold diluted using 1 × PBS before performing ELISA (Wu, Zhang, & Zhou, 2010). Then, sample solution (3 μl) was deposited onto each microzone and incubated at 22 °C for 2 min. The same amount (3 μl) of HRP-labeled clenbuterol (dilution factor: 1/100) was then added to each microzone. Following a 5-min incubation, the excess unbounded HRP-labeled clenbuterol was washed off using 10 μl of washing buffer for three times. Finally, a total of 5 μl of TMB was added to each microzone and left to react with HRP-labeled clenbuterol for 2 min. The images of microzones were obtained using a mobile phone camera (iPhone 6).

2.6. Image and data analysis

The images captured by the mobile phone camera were transferred to a computer for further analysis using image processing software Adobe Photoshop (Adobe Systems Inc., San Jose, CA, United States of America). An average blue intensity in CMYK-scale and black intensity in gray-scale value were calculated by selecting the whole area of each microzone. Statistical significance was determined using one-way the ANOVA Dunnett test (SPSS; IBM Canada Ltd., Markham, ON, Canada)